

**NATIONAL FOREST
GENETICS LABORATORY (NFGEL)**

**Standard Operating Procedures for
Starch Gel Electrophoresis**



United States
Department of
Agriculture

Forest Service

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INTRODUCTION

The National Forest Genetic Electrophoresis Laboratory (NFGEL) was established in 1988 as a unique link between Forest Service Research and the National Forest System. NFGEL is a national facility located at the Institute of Forest Genetics, Placerville, California, and administered by the Pacific Southwest Research Station. The lab provides an opportunity for forest managers to:

- (1) assess genetic structure and relative amounts of genetic diversity,
- (2) evaluate genetic improvement activities,
- (3) identify species, hybrids, clones, their seed, and their pollen, and
- (4) describe the genetic structure and unique genetic characteristics of threatened, endangered, and sensitive plants in an effort to maintain their genetic integrity.

We work with coniferous species from all regions of the United States and a variety of herbaceous and woody angiosperms native to our North American Forests. Our conclusions facilitate the implementation of appropriate management strategies for our forests and their species.

The electrophoretic technique of identifying different forms of enzymes is an invaluable tool for conducting genetic analysis of forest plants. Enzymes are separated by movement through an electric field based on differences in their charge, size, and shape. Isozymes are different forms of the same enzyme and are phenotypic expressions of alleles.

This manual describes the standard operating procedures (SOPs) used in isozyme electrophoresis and daily laboratory work. It does not include isoelectric focusing of enzymes or DNA extraction and characterization, other procedures used in this laboratory.

The protocols in this manual were obtained from several different laboratories and other sources where starch gel electrophoresis is used for genetic analysis. We acknowledge the contributions of Tom Adams, Oregon State University; A Manual for Starch Gel Electrophoresis: New Microwave Edition, by T. L. Marty, D. M. O'Malley, and R. P. Guries, University of Wisconsin-Madison; Tom Conkle, Paul Hodgskiss, Connie Millar, and Diane Delany, Pacific Southwest Research Station, USDA Forest Service; Jim Hamrick, University of Georgia-Athens; Eric Knapp and Kevin Rice, University of California Davis; and Nicholas Wheeler, Weyerhaeuser Company.

MATERIALS HANDLING

COLLECTION

Proper collection procedures are essential to ensure the quality of electrophoretic analysis. The Project Submission Collection Guides can be found in Appendix A.

Often genetic variation is partitioned by geography. Therefore, samples should be accompanied by details about the place and date of collection and the name and contact information for the collector. Material from seed or clone banks should be accompanied by as much detail about the source as is available. Collection location should include township/range/section or latitude/longitude or UTM, in addition to a written explanation of the description. Remember to include the state, county, and National Forest in which the collection was made.

Seed

Gymnosperms

For tree genotyping and most other purposes, we ask for 25 clean and viable seeds per tree. For other purposes (e.g. seed lot identification, pollen contamination, and mating system studies), sampling strategies are determined by lab staff and the customer. Seed packets are labeled with tree number, ramet number, and/or clone ID.

Angiosperms

Lab staff and the customer determine sampling strategies for specific projects. Seed packets are labeled with identification name or number.

Vegetative Material

Gymnosperms

Approximately two, 6" long branchlets per tree are needed for needle analysis. An average of five dormant vegetative buds, including up to 6" of stem per branchlet, are needed for bud analyses. However, as many as fifteen buds per tree may be needed, as in Douglas-fir. It is critical that buds be dormant and vegetative.

Only healthy looking buds with green, moist meristems are used. Larger buds are usually desirable. Needles should (1) be healthy-looking, preferably second-year or older tissue, (2) have little or no evidence of disease or insect damage, and (3) be relatively dry. Place tissue into Ziploc plastic bags containing damp but not wet paper towels. Bud and needle tissue should be kept cold and moist during collection, similar to scion material. Do not wrap tissue in damp towels. Place bags in an ice chest. Keep bags out of the sun. Do not leave collection bags inside vehicles in the summer heat, and always store in the shade of a tree or vehicle. Blue ice packs should be used inside the ice chest to keep plant materials cool. (Remember to freeze blue ice packs before use.) Insulate sample bags from blue ice using newspaper. Store bagged tissue in a refrigerator until they are ready to ship.

Angiosperms

Collect healthy-looking, disease-free leaves. Leaves are best collected in the morning on cool days. There is evidence of decreased enzyme activity in some species through the summer months, so springtime collections are desirable. Collect and store leaves as described above for needles and buds. The amount of tissue required depends on project objectives.

SHIPPING

Both seeds and vegetative material may suffer from poor shipping and storage conditions. To ensure proper processing of material, customers are asked to (1) alert NFGEL when materials are sent, (2) ship by a quick delivery service for Monday through Thursday arrival, and (3) enclose in the package a Packing List (see Appendix A). Package should be well labeled as to contents ("seed" or "vegetative material"), and to "refrigerate immediately" if the material is vegetative.

Upon receipt of material, NFGEL staff records information regarding the shipment (date received, species, project number, accession numbers, and contact person). Each seed lot or individual collection of buds or leaves receives a lab accession number.

LABORATORY STORAGE

Seed

Seeds to be analyzed within 4 weeks are placed in labeled Ziplock bags and stored in the refrigerator at 5°C. For longer storage, low moisture (under 9% moisture) seeds contained in labeled Ziploc bags are stored in a walk-in freezer (-20°C). If frozen seed packets are opened, excess moisture is introduced and can be absorbed by the seed. In order to maintain seed quality, sealed packets must be allowed to reach room temperature before they are opened and the desired quantity of seed is removed. The remaining seed is refrozen. If all seed in a packet are to be used, the packet does not need to come up to room temperature.

Vegetative Material

Immediate preparation of tissue upon arrival in the lab is optimum. However, depending on the species (especially conifers), tissue may be refrigerated for up to several weeks in the laboratory before preparation. Condition of tissue should be checked every week and paper towels dampened as needed.

If longer-term storage is required, tissue should be prepared for analysis in microtiter plates and frozen (see Sample Preparation) at -70°C. We do not recommend freezing whole tissue before dissection into extraction buffer, as repeated thawings affect sample quality.

LABORATORY PROCEDURES

SEED STRATIFICATION AND GERMINATION

General Comments

Conifer seed offer some advantages over other tissue and plant systems in regard to genetic analysis. The megagametophyte of conifer seed is haploid. Therefore, an individual genotype may be determined by analyzing multiple megagametophytes. (We analyze ten per individual.) The genotype of the pollen gamete can be deduced by analysis of the diploid embryo and subtracting the contribution of the female megagametophyte.

Most seed, gymnosperm or angiosperm, is germinated before isozyme analysis. We germinate only a portion of a seed lot at a time to guard against loss due to pathogen attacks or error. Germination protocols are listed by species. As new species come in, lab personnel consult with available resources (seed laboratories, land grant colleges, the internet, and published sources) for species-specific germination instructions. Two good general sources are: (1) Young, A. J., and C.G. Young, 1986, *Collecting, Processing, and Germinating Seeds of Wildland Plants*. (2) Agriculture Handbook No. 450: 1974, *Seeds of Woody plants of the United States*.

Response to germination conditions is species-specific. Some seed are killed by chemical residues left over in the production process of specific brands of germination paper or Kimpacks. In these species minimize the seed's contact with the bleach and hydrogen peroxide (H₂O₂) solutions, and use the lowest concentration that kills pathogens.

Due to pathogen problems, some species, or some collections within a species, require extra measures to effect successful germination. Those measures may include prolonged agitation in bleach (sodium hypochlorite) solutions of various concentrations, or soaking in various concentrations of hydrogen peroxide (H₂O₂). See Pathogens, Section 4, below.

Gymnosperms

General Conifer Germination Instructions

Label one plastic petri plate top and bottom for each NFGEL accession number. Spread seed on petri plates lined with Kimpack (No. 402 from Seedburo Equipment) paper soaked in 1% H₂O₂ solution or water (soak Kimpack to the point JUST before liquid pools in the bottom of petri plate; over-soaking Kimpack enhances mold growth). Allow seed to imbibe water (or 1% H₂O₂) at room temperature for 48 hours before starting stratification and germination. If time is available, cold stratify seed at approximately 4°C for the appropriate time before beginning. For some species, longer stratification periods are needed. Place an individual seed lot into a mesh bag that is identified by NFGEL number (unique identifier) and secure (close) the opening. Place the mesh bag(s) into a container of oxygenated water (19 – 24 °C) large enough to allow for circulation. A fish tank bubbler works well to oxygenate the water. One method to maintain H₂O temperature is to place the container under a running water faucet in a sink and adjust hot/cold valves. Start oxygenation and soak seed for 48 hours. Remove mesh bags and drain. Remove seed, place in a secured plastic bag (6.0 mil) that is identified by NFGEL number (unique identifier) and store at ± 4 °C for recommended cold stratification period.

Germinate seed at specified photoperiod and temperature regimes (Table 1). Monitor seed daily and keep Kimpack wet with 1% H₂O₂ solution or with water, as needed. If pathogens becomes a problem, rinse with Na(OCl) (sodium hypochlorite; see below). Expect germination at the specified time, counting from when the seeds in petri plates are placed in the appropriate light and temperature regime to promote germination. Seed is considered germinated when the embryo reaches the appropriate size. However, when analyzing only megagametophytes, it is sufficient for the radical to have just emerged from the seed coat before dissecting the tissue. Refrigerate germinated seeds in petri dish lined with moist Kimpack until tissue preparation, to retard mold growth. If pathogens seriously interfere with germination, see the Pathogens section below.

Species-specific comments (Table 1)

Fraser Fir: Store seed at low seed moisture and temperature. Under ordinary storage conditions, this seed retains little or no viability after one year. Stratify in petri plates after soaking in H₂O₂. Place 1 MM Whatman filter paper on top of the Kimpack to prevent seedlings from sticking.

Sugar Pine: Locate the pointed end of the seed. Using a sharp scalpel, nick the seed (cut off a piece of the outer seed coat so that only inner coat is exposed). Only a small cut is needed. Note: Germination is not required for analysis of sugar pine megagametophyte tissue. Simply soak seed in 1% H₂O₂ for 48 hours, rinse with distilled water, and store for up to several days in the refrigerator until preparation.

Western White Pine: Treat as Sugar Pine.

Angiosperms

Dicots

Bitterbrush: soak seed for 48 hours in 1% H₂O₂. Pour off the majority of the solution, place seed and some H₂O₂ in zip-lock bags, seal, and refrigerate for 48-60 days. Place seed on petri plates containing 1% H₂O₂ soaked filter paper and germinate using 16 hr photoperiods (86F light, 68F dark).

Grasses (Figure 1)

General grass germination instructions

Fold a 20.3 cm X 28 cm sheet of germination paper (Anchor Paper Co.) lengthwise 2 cm off center and divide it into five equal sections, each assigned a NFGEL accession number noted at the outer edge of the wide section and again at the inner fold. Draw a pencil line lengthwise 4.5 cm from the fold on the wide section of the folded sheet and then moisten the germination sheet with distilled water. Place three seeds from each of five designated individuals (by the appropriate NFGEL number) along the drawn line, and fold the narrow section of the paper to the wide section, covering the seed. Roll folded sheets into tubes, then place them in groups of 4 to 6 in 250 ml glass beakers covered with plastic wrap and rubber banded to retain moisture. Set beakers under strong light source (12 hour photoperiod) at room temperature. Keep paper moist with distilled water. Seed germinates in 10-16 days.

Species-specific comments.

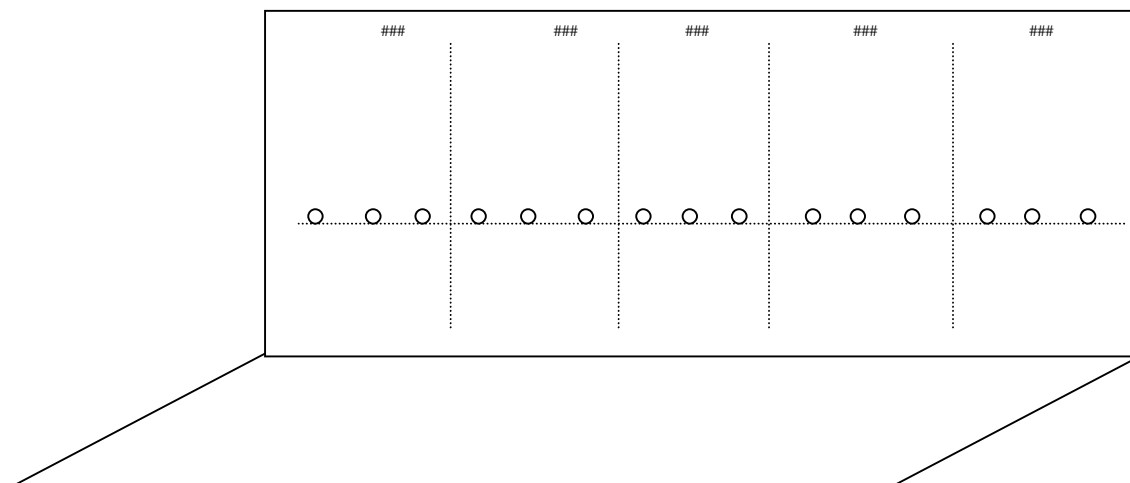
Bromus: Place beaker with tubes under 24-hour fluorescent light at approximately 74 °F for the duration of germination. Germination occurs in approximately 6-14 days, and growth of seedling to standard preparation size in 12-28 days.

Achnatherum hymenoides: Mechanically scarify seed. Make a solution of 0.2% potassium nitrate and 0.01% gibberellic acid (add 1g potassium nitrate and 50 mg gibberellic acid to 500 ml distilled water. Keep cold and limit light). Place scarified seed in petri dish lined with solution moist kimpack. Place in cold stratification for 4 weeks (35°F) with out light. Germinate using 60°F/8 hour light and 40°F/16 hour dark. Germination occurs in 14 to 28 days.

Table 1. Species-specific details of germination procedures for conifers.

Species	Hours in H ₂ O ₂	Cold Stratification (35°F) (days)	Day length (hours)	Temperature regime (light/dark)	Expect germination	Desired embryo length	Comments
PINES							
Loblolly Pine	48	30-60	8+	86°F/68°F	7 – 28 days	1 cm	Pathogens are frequent problems. Dies in 5 min. of bleach
Longleaf Pine	0	30	12	70°F/68°F	3 – 14 days		Water Kimpack with 3% H ₂ O ₂
Ponderosa Pine	48	30-60	8	86°F/68°F	7 – 10 days		
Shortleaf Pine	48	15-60	18	82°F/72°F	14-28 days	2-5 mm	
Sugar Pine	48-72	60-90	8	86°F/68°F	7-28 days		See text. For megs, see text.
Western White Pine	48-72	30-120	8	86°F/68°F	14-28 days		For megs, see text.
OTHERS							
Douglas-Fir	48	30	8	86°F/68°F	7 – 10 days	½ – 1 cm	
Fraser Fir	6	30	8	86°F/68°F	7 – 28 days		See text.
Western Larch	48	0-40	8	86°F/68°F	6 – 8 days	1 cm	

Figure 1. Creased germination paper with three grass seeds per individual.



Pathogens

If pathogens such as mold prevent germination, extra measures may be necessary. As a first step in preparing a seed for germination, agitate the seed in 1% sodium hypochlorite (bleach). Many angiosperms respond best to a quick bleach rinse followed by distilled water rinse. Agitate conifer seeds in 1% bleach from 30 seconds to five minutes. Experiment to determine what exposure to bleach maximizes germination while minimizing mold. For some species, it is possible to increase the concentration of H_2O_2 to 3% and/or hydrate Kimpack with H_2O_2 rather than with distilled water.

Minimize exposure to bleach and to H_2O_2 . They kill pathogens, but they also kill seeds if they penetrate the seed coat. The appropriate exposure time varies. Seed response to these chemicals is species-specific, and the need for bleach treatment is population specific.

Note: Store-bought bleach is 5% sodium hypochlorite. For a 1% bleach solution, mix 1 part “store bought” bleach with 4 parts water.

SAMPLE PREPARATION

Testing New Tissues

When preparing tissue that has not been previously assayed, we test for optimum tissue amount, the type and amount of extraction buffer, and extraction method. We use as guidelines our previous experience with similar tissues (Appendix B) and the relevant literature. Seed of most plant species are prepared in 0.2 M phosphate buffer (pH 7.5) without the use of liquid nitrogen. Vegetative tissue is usually prepared in either Melody/Neale or Gottlieb’s buffer (Appendix B), with recalcitrant tissue needing a grinding step in liquid nitrogen. A factorial test is set up to decide the final preparation method for the tissue, using as variables extraction buffer, amount of tissue, liquid nitrogen (use or not), and storage method (freeze the slurry, absorb slurry onto wicks and freeze the wicks, or freeze tissue that has been dissected into extraction buffer). Different extraction buffers or other procedures may be tested, depending on the species.

The effectiveness of these preparation methods are determined by using the samples for electrophoresis and staining for enzymes that resolve reliably in nearly all taxa. In most cases, effectiveness is judged by running an LB gel stained for ACO, LAP, PGI, PGM, and an SB gel stained for GOT, 6PGD, TPI, and UGPP. (See Appendix C for gel abbreviations.)

Paper wicks are made from Whatman 3MM Chromatography paper. This paper is cut into wicks that may be 2 mm wide (for megagametophytes), 2.5 mm wide (for conifer embryos) or 3 mm wide (for

most tissues). Do not confuse wick widths with the designation for the chromatography paper. For consistency, the lab sends uncut paper to Northfork Products (Dan Klaybor, P.O. Box 621, Syracuse, Indiana 46567) to be cut into wicks.

Gymnosperms

Seeds

Megagametophytes

Label 96-well microtiter plates and place at -20°C for 15 minutes prior to tissue preparation. Remove chilled plates from freezer and place on blue ice packs covered with damp cloths. To each well, add the appropriate extraction buffer. Use 100 ul for saturating three 2.0 mm wicks, or 150 ul for four to six 2.0 mm wicks. If running two megagametophytes in a single well, add an extra 25 ul of buffer.

Remove germinated seed from the refrigerator. Work quickly and keep tissue cold at all times to maintain sample quality and prevent enzyme degradation. Peel off seed coat and tissue surrounding the gametophyte. Seed tissue should appear healthy and white in color. Avoid dark-colored, yellowed, or soft seed. Discolored seeds produce inconsistent results, and should only be used if necessary.

Sugar pine and western white pine megagametophytes do not require germination before analysis. After the seeds have imbibed water, the seeds are rinsed twice with distilled water and approximately one fourth of the sugar pine megagametophyte (the quarter closest to the radicle) is dissected into a microtiter plate well. Entire western white pine megagametophytes are used after the seeds imbibe water.

Remove all embryo pieces and discard. Place megagametophyte or part of the megagametophyte in a microtiter plate well containing extraction buffer and macerate completely with an acrylic grinding rod or other grinding tool. Rinse and dry grinding tips thoroughly between samples. Place the pre-determined appropriate number of wicks into each well. The number of wicks is dependent on the number of gels that will be run and how many wicks are to be archived as backups in the ultralow freezer.

After wicks become saturated, load those to be used that day according to position onto prepared gels, wiping excess tissue from each wick. Place the remaining wicks in pre-chilled and labeled microtiter plates. Use one plate for each set of wicks (i.e. if there are 4 wicks/grinding block well, separate wicks into 4 sets between the 4 microtiter plates). Keep microtiter plates on blue ice blocks and work quickly. The entire process should take less than two hours to prevent enzymatic loss. Store plates at negative 70°C.

The lid of the microtiter plate is labeled with the project number, species, and set number. The plate itself is labeled with the project, set number, species, and date.

Embryos

When analyzing embryos, germinated seed is necessary to initiate enzyme activity. Seed is prepared as for megagametophyte sampling. Remove and place all parts (including root and shoot) of a green, healthy embryo in a microtiter plate well. Embryo length is species dependent and may range from ~0.5-2.0 cm, as measured by the exposed part of the embryo. Embryos may have to be dissected with a scalpel into smaller pieces for tissue to stay submerged in extraction buffer. Freeze submerged embryo pieces until the day of the run. Thaw microtiter plate at room temperature, place on blue ice and grind as for megagametophytes. Wider wicks (2.5-3 mm) are usually needed for embryos as compared to megagametophytes due to decreased resolution of diploid tissue.

Vegetative Material

Buds

Dormant vegetative buds should be used for isozyme analysis; avoid reproductive buds (those that will produce flowers). Buds that have begun to elongate do not work well.

Douglas-fir and true firs (*Pseudotsuga* and *Abies*): Cut bud in half longitudinally and excise the primordium. The primordium should be moist and green. Place primordium in a cold microtiter plate well containing bud extraction buffer (see Appendix B). Keep plate on ice block. The amount of buffer and bud tissue needed is dependent on the number of buffer systems and enzyme stains, which will be used. Usually, two to six primordia per individual are added to 100µl of buffer in a single well. Store plate at negative 70°C. The morning of the electrophoresis run, thaw samples at room temperature, grind completely, and place the appropriate number of wicks in each well. Load wicks onto gels after wicks become saturated. Buds may be ground, wicked, and frozen prior to the day of the run. However, resolution decreases if this method is used because of repeated sample thawings.

Pines (*Pinus*): Cut vegetative bud in half longitudinally. Remove bud scales. Use a piece approximately 2 mm on a side ($= 8 \text{ mm}^3 = 1/4$ the size of pencil eraser) per sample well. Prepare as for fir buds.

Needles

Because of high levels of phenolic and secondary compounds contained in gymnosperm needle tissue, a rigorous extraction procedure is usually needed for sample preparation. Grind needle tissue in mortars using liquid nitrogen, and extract enzymes into the appropriate extraction buffer (see Appendix B3). Place slurry samples in microtiter plate wells and freeze plates at -70°C . The morning of the electrophoresis run, thaw samples at room temperature and place the appropriate number of wicks into each well. Wick width should be 3mm or greater. Load onto gels after wicks become saturated.

Some conifer species do not require a liquid nitrogen preparation for needles. *Cupressus* and *Chamaecyparis* needles may be dissected directly into extraction buffer. Place 8 mm^3 of needle tissue into a microtiter plate well containing 150 μl of extraction buffer. Freeze the plate at -70°C until the morning of electrophoresis. (See Appendix B.)

Angiosperms

Seeds

Stratify and germinate seed as specified under Seed Stratification and Germination. In general, the seedling is considered ready for processing when $\sim 2.5\text{mm}$ of root emerges, but optimum size is species-specific. Excise seedling (including cotyledons), from seed coat. For most species, the entire seedling should be processed, including root. Cut seedling into small pieces with sharp scalpel. Place seedling tissue into microtiter plate wells submerged in 150 μl of buffer, and freeze plates at -70°C . Thaw samples at room temperature the morning of the run, grind thoroughly, saturate samples onto 3mm wicks, and load gels.

Vegetative Material.

Refrigerate leaf tissue until preparation. For some species, use a hole punch to remove a disc 7 mm in diameter from a leaf. Place 1/2 to 3 hole punches (depending on species and number of wicks needed) into a cold microtiter plate well containing the appropriate extraction buffer. Freeze samples at -70°C , and grind the morning of the run. For more recalcitrant tissue (i.e. Bitterbrush), grind leaf tissue to a fine powder using liquid nitrogen (see Appendix B2) and extract enzymes into the appropriate extraction buffer. Slurry samples are saturated onto 3-4mm wide wicks on the morning of the run.

Red Pine Controls

Red pine (*Pinus resinosa*) megagametophytes are used as controls on all gels because they show no intraspecific genetic variation in isozymes (except for a very rare occurrence in IDH and GOT). A single wick is placed on each side of all sample groupings. An extra red pine wick is placed between the first two groups on the left side of every gel to ensure proper gel orientation. The use of controls allow for short and long-term consistency, both within and between gels.

Preparation of red pine control wicks begins by soaking red pine seeds in tap water, at room temperature, for 24 hours. Then drain and rinse the seeds and either process them immediately or store them in the refrigerator. The seeds can be kept refrigerated for several weeks.

Sort 2 mm wide wicks, made from Whatman 3MM chromatography paper, into groups of ten. Prelabel cryovials "Red Pine."

Keep cryovials, buffer, mortar, pestle, and microtiter plate on ice packs. Place approximately 50 seeds in a cold mortar and add 1.2 ml of 0.2 M phosphate buffer, pH 7.5. Do not grind the seeds; place the cold pestle on them, push down, and rock the pestle back and forth. This breaks up the seeds and produces a milky slurry. Grinding the seeds produces a grit that clings to wicks and gels. It is important that the slurry does not freeze at this step! Using a spatula, scrape the seed fragments up onto the side of the mortar and press them to release the slurry. Pick up the slurry with a pipette. Avoid picking up seed fragments. Transfer 150 μl of slurry to each microtiter plate well. Place a group of ten wicks in each well and allow the slurry to soak into the wicks. Then place each group of ten wicks into a separate prelabeled cryovial. Put cryovials in a plastic bag with the date, and place in -70°C freezer. Cryovials may be stored temporarily in the -20°C freezer in the lab during preparation. Use the oldest wicks first.

GEL PREPARATION

Comments

NFGEL uses two different thicknesses of gel frames. Thick frames yield seven slices per gel; thin frames yield up to five slices.

Mold Assembly

Assemble gel frames by piecing together the four sidebars. Label the underside of a glass plate with the appropriate buffer abbreviation LB, SB, MC6, MC8, H7, H8, and gel number. (See Appendix C for buffer abbreviations). Frames should be assembled so that long pieces are placed over the shorter sidepieces. Place gel frames on the glass plate. Some gels are likely to stick to the glass. If this is a problem, spray the glass with Pledge furniture polish. (Pledge forms a non-stick barrier that the lab has found not to affect the gel. Other wax barriers may work as well.)

Starch Preparation

Testing Starch Quality

The quality of starch varies from lot to lot, even when supplied by major chemical companies such as Sigma. It is critical to verify that protein migration and resulting band resolution are still optimum when receiving a new lot of starch. Even when holding constant the percent starch, band separation can be very poor when using some starch lots. Also, some starches create slurries that are difficult to pour and which set into gels that are difficult to slice.

When ordering a new lot of starch, we request a small initial starch shipment for testing. We test the material by pouring gels made with two or three buffer systems and running samples on those gels. Side by side comparisons are made using the current starch lot. NFGEL uses an average of 30 kg of starch a year.

Making Gels

Assemble and label all required equipment, make any necessary buffers, and bring the starch to room temperature. Assemble the vacuum pump.

Weigh the required amount of starch and place in a side-arm flask. Measure the appropriate amount of buffer for the side-arm flask using a graduated cylinder and pour it into the side-arm flask with the starch (see Appendix C). Place the side-arm flask on the shaker at a sufficient speed to keep the starch suspended in the buffer. Measure the appropriate volume of buffer for the boiling (volumetric) flask using a graduated cylinder, and pour it into the flask. Place the boiling flask into a microwave oven. Heat just until the buffer boils.

When the buffer in the microwave begins to boil, remove and gently swirl to de-gas. As you swirl the starch slurry, add the boiling buffer. (This prevents lumping in the starch slurry.) Mix well, return to the microwave oven, and cook for 4 minutes, or until the entire volume boils gently, swirling once. Cooking time may vary by starch lot, starch supplier, volume of starch, and microwave.

Remove from oven, swirl, and de-gas under vacuum until large bubbles rise from the starch solution and no small bubbles are left on the flask bottom. Pour solution into the assembled frame(s). While the gel solution is still hot and liquid, use a small spatula to remove any air bubbles or debris. **GELS THAT CONTAIN LARGE AMOUNTS OF UNDISSOLVED STARCH OR NUMEROUS AIR BUBBLES SHOULD BE DISCARDED.** Always wear face shield, heat resistant gloves, and apron when handling hot starch solutions. Immediately fill empty side-arm flask with hot tap water to aid in cleaning.

Allow gels to cool until they become firm in texture and opaque. Cover cooled gel with plastic wrap, label appropriately, and place in refrigerator. Gels can be poured up to 24 hours in advance of the run.

ELECTROPHORESIS

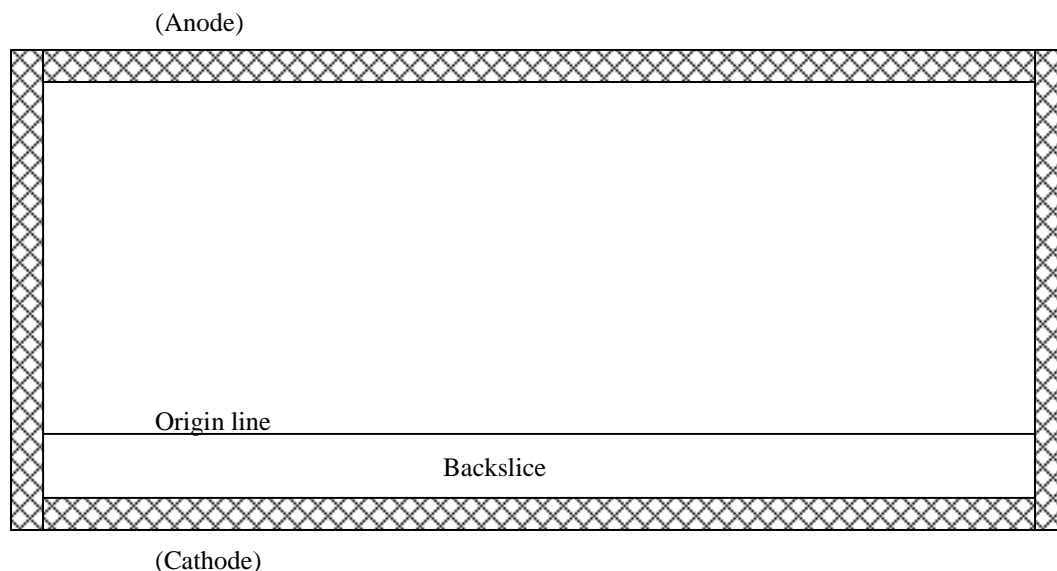
Loading Samples

On the morning of the run, remove microtiter plates containing either unground tissue samples in extraction buffer, tissue slurry, or prepared wicks from the freezer and thaw them at room temperature. Then either absorb tissue slurry onto wicks made from Whatman 3MM Chromatography paper, or macerate

unground tissue completely with a rod or a dremel tool, and absorb the resulting slurry onto wicks.

Trim away excess gel that has flowed on top of the gel frame. Orient the gel so that the width is greater than the height. Using a scalpel, make a horizontal cut 2.2 cm (1.0 inch) in from the bottom of the frame, creating a one-inch wide strip the length of the gel (Figure 2). Wicks will be placed and held between these two gel sections. This will also serve as the origin line.

Figure 2. Gel in frame, showing origin line one inch from frame and backslice (cathodal piece).



Load wicks into the gel's origin line, using an appropriate spacing template. Keep plates containing wicks on ice packs and work quickly so everything remains cold. Place wicks saturated with diluted red food coloring (4:1, H₂O to coloring) on the insertion line at each edge of the gel, near the frame, as dye markers to track the migrating front.

Running Gels

After the wicks are loaded, place the gel (still on its glass plate) on the gel tray that is filled with the appropriate tray buffer. Buffer should be cold. Cover gel with plastic wrap. Use "Handiwipes" (also referred to as towels or sponges) to conduct the charged buffer through the gel. Handiwipes must be soaked in the appropriate buffer prior to use. (When first used, cloths should be rinsed several times in distilled H₂O, soaked 48 hours in the appropriate buffer, and placed on the anode side of the tray). Place two cloths together and fold into 8 thicknesses. Place in buffer chamber. Repeat for other buffer chamber. Place one edge of each cloth on top of the exposed gel. Approximately 1 cm (1/2 inch) of the gel should be in contact with the Handiwipes on both anode and cathode ends. Do not expose the insertion line and wicks on the cathode end; wicks should remain under the plastic wrap. There must be continuous contact between the gel and the cloth. Fold plastic wrap over the Handiwipes on each side (Figure 3). Place trays in refrigerator and cover gel with a plastic bag filled with chilled H₂O.

Before placing run trays in the refrigerator check that all power supplies are "off" or in the "on/standby" position. Our gel running refrigerator is equipped with a safety shut-off device which activates when doors are opened, but in case of malfunction, it is best to check before entering the refrigerator. As another precaution, be sure you are standing on a rubber mat while manipulating electrical equipment. Connect tray terminals to power supply. Samples are run from the cathode to the anode ("run to red"). The current used depends on the buffer system and on the thickness of the gels (Table 2).

After running for 15 minutes, remove wicks from gels. To remove wicks: (1) remove gel/tray from refrigerator, (2) fold back plastic wrap and cloth and remove front frame piece, (3) separate front slice of gel from main body of gel, (4) remove wicks, (5) push gel pieces back together and replace frame piece,

(6) insert 1 to 3 glass rods between gel and tray frame along cathode side of gel, (7) remove any air bubbles trapped between the gel and glass plate, (8) replace cloth, and (9) return to refrigerator and reconnect to power supply. Place water bag back on gel and place frozen blue ice block on top of water bag. Turn power units back on and adjust to appropriate settings.

Figure 3. Gel tray with gel. To simplify the diagram, the gel frame is not shown, and a cloth is shown only on the anodal end. When the gel is being run, the two gel pieces are pushed together. The gel pieces are temporarily separated to remove wicks 15 minutes after the run begins.

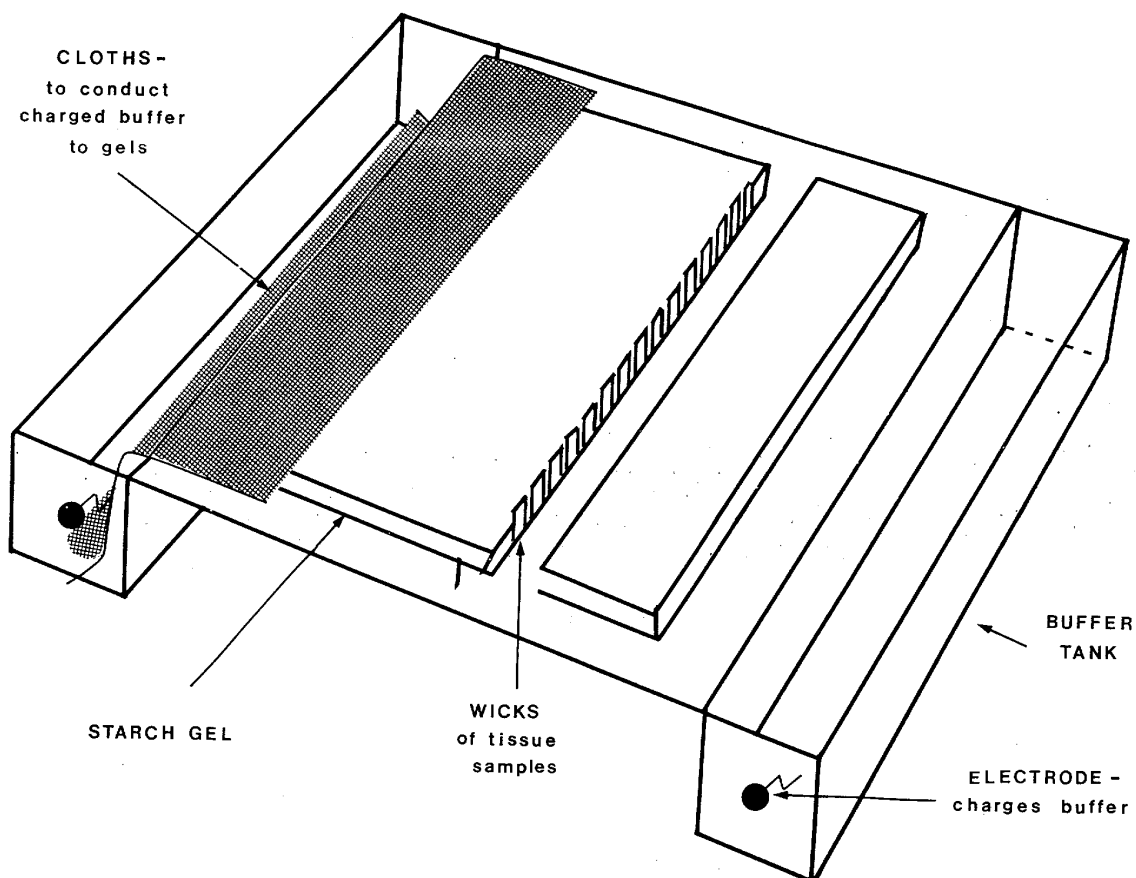


Table 2. Currents (in milliamps) for electrophoresis.

Buffer	Thin (start)	Thin (after dewicking)	Thick (start)	Thick (after dewicking)
LB	75	75	100	110
SB	70	70	80	85
MC	50	55	60	65
H7	45	45	50	55

During the course of the run, voltages should never exceed 320V. Voltages will generally increase over time at constant mA. Check progress of the dye markers periodically. Adjust mA if needed. Voltage readings should be approximately 250V for gels with MC buffer, 320V for LB, 180V for SB, and 60V for H.

Samples are run until the dye marker has moved a distance of 8 cm from the origin in LB, SB, and H gels, and 9 cm in MC gels. (However, if bands are very close together, resolution may be improved by running markers further; determine during the testing phase). Enzyme migration speeds up greatly on LB buffers near the end of the run. Dye markers do not work as well on H gels and fronts must be checked visually. H gels are run until the visual front runs against the far frame.

If a gel is nearing the end and cannot be sliced immediately, turn the power pack down to 5 mA to slow its progress. Gels should not sit without current, because the enzymes will diffuse, making bands broader and fuzzy.

At the end of the run, the current dial on the power pack must be turned down to zero current before the gel is detached. This prevents a subsequent power surge from damaging the unit when the pack is turned back on.

SLICING

When the samples have migrated the correct distance through the gel, the power sources are turned off, the leads disconnected, and the gels removed from the refrigerator. The gel trays are emptied and rinsed with tap water and the cloths are squeezed out and placed in distilled water to soak.

Gels are usually trimmed to a width of 6 cm measured from the origin line. With SB and LB gels, some of the slices will need to be wider due to the proteins migrating farther than 6 cm. The wider slices require a wide staining pan and a double stain recipe. Use a pipette to notch the gel with successive holes representing gel number. Final notches will appear on the left side of the anodal edge when the origin is oriented horizontally along the bottom. A corner cut represents five, and a hole cut represents one. Make sure holes are clearly cut through the entire gel.

Remove gels from glass plates and place them on paper towels to remove excess moisture. Center the gel on the slicing plate, and place one-millimeter thick plastic spacer strips adjacent to each side of the gel to create the thickness of each slice. Our gel slicer is made from a coping saw with the blade replaced by a 0.01 gauge wire (a plain, not wrapped, metal guitar E string). It is used to slice gels horizontally. To slice the gel, apply sufficient downward pressure on the slicer to keep the wire taut across the plastic strips, and pull the wire toward you in a steady motion. Additional plastic strips are added to raise the cutter to produce each consecutive slice. To obtain two different width slices from one gel, simply trim the gel to an 8 cm width, slice the gel, then trim the slices back to 6 cm when necessary.

After slicing, flip the gel over (the slice that was against the glass plate is now on top), return it to its original plate, and remove and save the top slice. The next slice is referred to as slice #1, 2, 3, 4, 5, etc. Cover the gel with plastic wrap and place it in the refrigerator until ready for staining. (With some leaf tissue, the top slice can be stained for RBC).

For some species, include the cathodal piece (backslice) of the gel in the pan when staining for ACP, GOT, or PGI. In this case, slice the cathodal section along with the other portion of the gel, and be sure and notch the cathodal piece with the pipette for gel number to keep its orientation correct.

STAINING

See Appendix E for stain recipes. Weigh and measure dry and liquid stain components while gels are running. Wear gloves, lab coat, dust-mask style respirator and safety goggles when weighing dry chemicals. For a given stain recipe, carefully weigh out each dry chemical and place them into an Erlenmeyer flask. If necessary, use an anti-static gun to prevent the powder from clinging to the flask surface. Use a clean spatula and fresh weigh paper for each chemical. Set used weigh paper and spatula in a beaker of water immediately after use. Gloves can be disposed of in the regular garbage. Close the flasks with rubber stoppers to prevent absorption of moisture from the air. Amber flasks should be used for those stains that contain light-sensitive chemicals (i.e., GOT, LAP, and CAT). When all the dry components are weighed, thoroughly clean the balance, and wipe the lab bench with moist paper towels. Return the chemicals to their proper storage location. In a second flask, for a given stain, combine non-enzymatic

liquid components. Use rubber stoppers to prevent evaporation. Place those that need to be warmed into a 37°C incubator.

When the gels are sliced and ready for staining, add the liquid components in one flask to the dry components in the second flask for a given stain. Add additional components last (i.e. MTT, PMS, enzymes), as listed in our recipes as "during staining". Pour the stain into the pan, and add the gel slices(s). Place the pan in the incubator, unless the reaction requires refrigeration (e.g. CAT), or darkness at room temperature (e.g. AAT); check stain recipe for instructions. Continue staining until all the slices are stained and are developing.

The FEST stain (approximately 1 ml) is evenly applied with a disposable pipette to a gel slice placed on the UV illuminator in the digital camera hood. Adjust the settings for the camera for UV/EtBr photography (see FILMING, below) prior to staining. Monitor the development of the stain and photograph the gel as bands appear. Multiple photos may be necessary to capture the greatest intensity or multiple loci.

As the patterns become sufficiently visible on incubating stains, remove the pans from the incubator. Some gel stains may have to be removed before over-staining occurs, resulting in blurred stain patterns (i.e. UGPP, PGI, PGM, and CAT in some species). For the few gels requiring overnight incubation, turn off the heat switch and leave the pan in the incubator. Collect the stain waste in a beaker and dispose of it in the bottle labeled "Isozyme Waste" stored in the fume hood. For ALD and TPI gel stains, rinse water and fixer are stored in bottles for special disposal. Rinse slices with distilled H₂O. Some gels may require a second rinse to eliminate stain solution that leaches out of the gels. After rinsing, gels are ready to be photographed. Some patterns must be photographed before they fade or the background becomes dark (e.g. CAT). Do not allow slices to dry out; cover them with distilled water if gels are to be scored that day.

SCORING

The enzyme stain and buffer system combinations to be used on a species are determined in a testing phase at the beginning of a project. We also test whether stains resolve better near the top or bottom of a gel. We base our decisions on genetically interpretable patterns, good band resolution, and sufficient migration between bands (alleles).

After the genetic interpretations of the gel isozyme patterns have been determined, the scoring becomes routine. The tools that are needed to score include: metric ruler, calipers, light box, calculator, scoring sheet, and allele chart (specific for each species and tissue). If the patterns are faint, place gels on the light box if scoring from fresh gels, or adjust the contrast on the computer image to better identify bands.

Select a gel and check the migration distances of the controls (wicks made from Red Pine tissue). (The control genotypes are listed on the allele chart and standardize samples within and among gels.) Identify the position of each sample and measure the alleles present. Determine the relative distances and convert to the appropriate allele designation from the allele chart. The most common allele is usually designated as allele #1. A diploid sample with a single band at the 1 position is considered homozygous for 1, and receives the score of 11. A heterozygous sample will usually have the common allele and one less common allele. An example of this score might appear as 12. If samples are unscorable, a 0 is placed as the score. Proceed through the gel, scoring all the samples.

If controls show no activity for a particular enzyme, allele designations may be determined by allele frequency. Most loci have an allele that is usually observed in higher frequency than the other alleles. The common allele is used as a standard for identifying other alleles.

For consistency and accuracy, two independent scores are taken. Discrepancies are checked and a final score is assigned.

FILMING

Gel images are preserved using a digital camera and a 35mm camera, both over a light box. The gels are best filmed after blotting to prevent reflection. Gels are photographed fresh, immediately after bands develop and before overstaining occurs. Some stains (e.g., UGPP, 6PGD and others) may need to be photographed more than once in order to capture the best resolution for multiple loci. This decision will be made on a species-by-species basis.

Place the gel (or gels; up to 2 may be captured on the same image) to be photographed on a glass plate. All images must be labeled with the Project number, species, date, stain, buffer system, and set number(s) (written in Sharpie directly on the glass plate for 35mm images; incorporated into an overlay in a computer image).

To capture an image using the 35mm camera, set the glass plate on the illuminated light box. Turn on the camera. The exposure and aperture have been pre-set and do not need adjusting. Center the gel(s), and set the zoom and focus. Use the notches in the gel, not the bands, as the standard for focusing the camera. Press the exposure button to capture the picture.

Always wear gloves and a lab coat when working at the digital camera/EtBr station. To capture images using the digital camera, first set the camera and computer system for the appropriate illumination (UV or white box). For UV images (i.e., FEST), flip up the white box to expose the UV illuminator. Then turn the filter rotor to 2, turn the top ring on the camera lens full clockwise, and turn the bottom ring to the black mark between 3 and 5. For white light images (e.g. other stains), flip the white box back down and turn the filter rotor to 1. Then turn the top ring on the camera lens back counter-clockwise until it is just past 16. Turn the bottom ring to the black mark between 2 and 3. Use both the reflective and trans-illumination options when capturing UV and white light images.

FIXING AND STORING

If the gels are to be stored after photographing, drain water off the gels and add just enough fixative (4:5 parts ethanol:water) to cover the slices. Leave the gels overnight in fixative in covered pans in the refrigerator. Do not use fixer on AK, CAT, DIA, or FEST. The next day, blot gel slices on paper towels to remove excess fixative before wrapping. Wrap each gel individually in plastic wrap, keeping the notches in the upper left hand corner. Label each gel with the buffer system, stain, date, set number, and project number, writing on the plastic wrap using a permanent marker.

Store gels from a single run day in a large Ziploc bag labeled with the species, run date and set numbers. Include an extra paper towel in the bag to absorb excess moisture during storage. Gels are stored by project in large plastic boxes at 4°C in the refrigerator until the analysis and report are completed.

DATA ENTRY

Allele scores are entered into the computer. Sample identifications, geographical information, allele scores, set number, and sample positions are entered into a spreadsheet or database.

CHEMICAL INVENTORY PROCEDURES

A complete inventory of all chemicals purchased by NFGEL is maintained in an MS-Access database (k://gene/nfgel/Chemical Inventory/Chemical Tracking.mdb). Eight details are logged into the database for every order placed: the chemical ordered, the catalog number and vendor, the date the order was placed, the quantity in each unit, the number of units ordered, the extended price and the initials of the person placing the order. When the order arrives, each unit is checked in, comparing the original requisition with the packing list, and checking catalog numbers to insure the proper chemical formulation was sent. The date the shipment arrived is written in black ink on each unit, and the chemicals are stored in the appropriate locations. After processing the new chemicals, four additional details are tracked in the Chemical Tracking database: the date the chemical arrived, the lot number of the unit, the date the unit was opened and the date it was emptied. Additionally, any comments pertaining to the shipment, storage, or ordering procedure are noted in the database.

When a unit of a chemical is emptied, the container is washed thoroughly and placed in the cardboard box labeled “NFGEL Empty Chemicals” in the chemical weigh room. At this time, additional units of the chemical should be available in the appropriate storage area. Always use the unit with the oldest black arrival date first. When a new unit is opened, the date is written in red below the black arrival date on the chemical’s label. It is the responsibility of the person starting a new bottle of a chemical to check the lot numbers of the empty and newly opened units in the Chemical Tracking database, and enter the dates each unit was emptied/opened accordingly.

Once a week, or more frequently if necessary, the lab manager records the empty chemical bottles, checks the remaining supplies of each chemical, and, if necessary, places orders for new stocks. Chemical orders are routinely placed using government VISA purchasing. Laboratory personnel are alerted when an order arrives so that the chemical is promptly checked-in and stored following the procedure described above.

QUALITY ASSURANCE PROGRAM

SHIPPING MATERIAL

Detailed instructions are given to collectors for treatment of material. Material is not shipped if it will arrive on a weekend. Material is placed in a refrigerator upon arrival at NFGEL.

RECEIVING MATERIAL

Material is assigned a project number upon arrival. This is noted in the Plant Materials Received notebook, on the packing list, and on the project board. When material is checked in, the packing list is initialed and dated, and a computer message is sent to the customer verifying receipt. Discrepancies and damage are noted. All paperwork for a project is placed in its assigned project binder.

STORAGE OF PLANT MATERIALS

NFGEL staff regularly monitor both fresh and frozen tissue stored in the laboratory. Vegetative material stored in Ziploc bags in the refrigerator are checked each week, and paper towels moistened or changed as needed.

Samples stored for long periods of time are placed in the ultralow freezers. These freezers preserve samples at -80°C, and are monitored by an automatic backup power system. When power to the ultralow freezers is interrupted, a generator automatically starts and provides power to the two units. The generator is maintained weekly, and is powered by the facility propane supply.

QUALITY ASSURANCE BLIND SAMPLES

We require an additional ten percent random samples be created when genotyping individuals. For example, a project in which 200 individuals are to be genotyped would have 220 samples assayed, 20 of which would duplicate seed or vegetative tissue samples in the initial 200. These samples are compared after the project is completed and prior to data analysis. This enables us to monitor the entire laboratory process. If the customer does not include these QA samples, NFGEL staff randomly creates the additional samples. If sufficient material is not available, it is necessary to contact the customer for additional samples.

In other cases, such as when analyzing single megagametophytes, embryos, or seedlings, back-up wicks (replicate wicks for a given sample) are created, electrophoretically run after the project is completed, and compared to the data obtained from the original wicks. This serves as a check of the electrophoretic and scoring quality.

For lengthy projects (several months in duration), back-up wicks for at least two sets are created in quantities large enough to run several times throughout the project. Example: a project lasting six months using four buffer systems. Two sets (30-60 samples per set) are prepared using 12 extra wicks per sample (if tissue allows), or replicate plates for vegetative tissue. These sets are re-analyzed using the back-up

wicks at two month intervals throughout the project. This checks the scoring precision and accuracy throughout the project.

QUALITY ASSURANCE SHEETS

For a given run day any factors that may affect gel quality are entered on the quality assurance (QA) sheet. QA sheets are filled out prior to each gel run in order to track the day's progress. Information noted on a project development QA sheet includes slice order, stains and buffers, set identities, wide or narrow slicing, and any other special notation. Project development QA sheets are kept in project binders for quick reference. Daily QA sheets record project number, gel identities, species, stains and comments. Completed daily QA sheets are filed in the QA sheet binder by date.

POWER SUPPLIES

Power Packs

The use of constant current power supplies enables us to tightly control the voltage and current through the gels without needing to check them frequently. The Hoeffler power supplies have connections for running two gels at a time. We use this feature only when necessary and never connect two gels of different buffer systems, or two thick-framed gels to the same power pack.

Calibrating Power Packs

Check power packs to make sure the power supply is off. Check that the power pack leads are free and not connected. Turn power back on. Turn amp dial counter clockwise to stop, and then one fourth turn clockwise to activate. Turn volt dial until power pack reads 320 volts. Power pack is now calibrated for a maximum of 320 volts. Turn power pack off.

SCORING

For consistency and accuracy, two independent scores are taken. Discrepancies are checked and a final score is assigned.

STAINING

Some solutions (such as malic acid, albumin, CAT buffer) are made fresh weekly to improve staining clarity. This eliminates poor resolution associated with solutions that are sensitive to aging.

BUFFER SOLUTIONS

The date each buffer was prepared is recorded on the buffer solution carboys. Temperature sensitive solutions (MC6, MC8, and H) are refrigerated. The pH of each buffer is monitored weekly before pouring gels.

CHEMICAL INVENTORY PROCEDURES

Optimal enzyme resolution ultimately depends upon maintaining a fresh supply of chemicals. To insure a reliable supply, many sensitive chemicals are ordered in small quantities, and all chemicals are stored under the appropriate conditions.

NFGEL tracks details of every chemical purchased and used in an MS-Access database. The date each chemical was ordered, arrived, was opened, and was emptied, in addition to the lot number of each unit, is tracked on a continual basis. Maintaining a database of current chemical inventory allows NFGEL to track the storage and rate of use of sensitive chemicals, monitor any changes in data quality due to chemical activity or age, and take the appropriate measures to remedy the problem.

WICKS AND WICK TEMPLATES

Templates are used for placing sample wicks on gels. The use of templates makes the wick locations very consistent among gels and eases scoring. Greater consistency is achieved using pre-cut wicks purchased from Northfork Products. (Dan Klaybor, P.O. Box 621, Syracuse, Indiana 46567, 219-457-4551). We provide Whatman 3MM chromatography paper and wicks are cut to specified sizes.

CONTROLS

Red pine controls are placed frequently along the gel, normally at the beginning and end of each group of ten samples. One red pine sample is used between the first two groups on each gel for further visual recognition of gel orientation. The use of a standard control marker on all projects provides consistent reference bands during scoring, potentially increasing the consistency of data.

STANDARD SCREENING

New species are screened with our standard operating procedures for staining while incorporating specific stains that are described in the literature.

GEL DOCUMENTATION

Photographs of gels containing representative alleles and allelic combinations are taken using a 35mm camera with a close-up lens, a Polaroid camera, a digital camera, thermal camera (AlphaImager 3300; AlphaInnotech), or computer scanner with thermal printer. Using multiple gels, all alleles at a given locus are photographed and the photos filed by tissue-type within species. Digital images of each gel are preserved in two back-up locations.

LABORATORY MAINTENANCE

To ensure the high quality standards of our data, rigorous preventative maintenance procedures are followed. Because NFGEL is located in a mountainous region, we must protect our equipment and samples against numerous power outages throughout the year.

FREEZERS AND REFRIGERATORS

Lab Freezer

The lab freezer should be defrosted every 6-12 months, or as needed. The freezer is a Whirlpool, model EV190F. The freezer is not frost-free (A frost-free freezer would be inappropriate for this lab because it could cause excessive freezer burn and its occasional heating cycles might destroy tissue and harm sensitive chemicals). To defrost:

- (1) Turn the freezer off by turning the dial located in the upper right-hand corner to the off position.
- (2) Transfer desiccators and seed bags to the refrigerators for temporary storage. Check carefully to make sure all the prepared tissue, whether in cryovials or in microtiter trays, are transferred to the -70°C ultralow freezer.
- (3) Remove the ice packs and place in the sink, remove the wire rack from the bottom of the freezer.
- (4) Place plastic dishpans on the shelves to catch ice and water, place paper towels around the drain hole at the bottom left to catch water, and place additional towels on the floor under the drain.
- (5) Allow 3 hours for the ice to melt. (A hair dryer may speed the process.) Do not chip ice off the coils as they may be damaged. Keep the floor as free of ice and water as possible because it is a safety hazard.
- (6) Periodically dump water and ice that collects in the pans.

(7) When all the ice is out of the freezer, wipe out any water, turn the temperature to the coldest setting, replace the wire rack and ice packs, and allow the freezer to cool for about half an hour before putting the desiccators back.

Lab Refrigerators

Condensers in the two refrigerators are inspected every 6 months. Remove the two screws on the bottom panel of the refrigerator. Remove the panel and blow out the dust using air hose and compressed air. Always wipe out the condenser pan.

Ultralow Freezer

The ultralow freezer filters should be inspected every 6 months to check for clogging. The Reuco, chest ultralow freezer has a foam filter that is removed, washed, dried, and replaced when needed. The Solow, upright freezer does not have a filter.

Installation and Repairs for the ultralow freezers:

Oscar Kerndt, ATR Mobile Shops, P.O. Box 629,
West Sacramento CA 95691, (916) 372-6070, 1-800-344-2871 (24-hr
pager).

Freezer Insulation Panels--All Temp Insulation, Sacramento CA. Ask for 3/4" blue styrofoam for a -70°C freezer. Sometimes replacements can be ordered from freezer manufacturer.

POWER OUTAGES

Ultralow Freezers

The two ultralow freezers are connected to an automatic electricity generator that provides power to the units in the case of a power outage. The automatic response of the generator can be tested by switching breakers 4 and 6 to the Off position in the gray panel box to the left of the transistor panel. The generator should automatically start when power is cut to the freezers. When power is restored to the freezers (by switching the breakers back to the On position), the transfer box will delay 5 minutes before returning the freezers to PG&E power. The generator will automatically shut off another 5 minutes after the freezers are returned to PG&E power.

If the ultralow compressors do not work when the backup generator starts after the breakers have been switched to Off, check the breakers located in the right side of the transfer case (the blue box near the gray panel box). If these circuits have been broken, switch the breakers back to the On position.

Generator

The generator must be maintained on a weekly basis in order to keep the internal parts lubricated, insuring it will start if power is interrupted. The weekly maintenance consists of cleaning the generator shed of any leaves and debris, starting the generator manually, and allowing it to run for approximately 10 minutes. In order to start the generator automatically, first change the small toggle switch on the left of the transfer case (the blue box near the panel box) from Automatic to Handcrank. Then check the level of oil on the dipstick located in the center of the machine. To manually start the generator, hold the toggle switch on the generator in the Start position until the machine has completely started (about 5-10 sec.). The generator starter motor is designed to run constantly until the generator comes up to speed. Releasing the toggle switch before the generator is completely started will cause greater stress on the unit than holding it too long. The generator will become loud, vibrate, and then calm, indicating that it has started completely. Allow the generator to run for around 10 minutes.

To turn off the generator, hold the toggle switch in the Stop position until the generator is completely quiet (around 5 sec.). Failure to hold the switch long enough will cause the generator to start again and put unnecessary stress on the motor. **ALWAYS RETURN THE TOGGLE SWITCH IN THE TRANSFER CASE BACK TO THE AUTOMATIC POSITION OR THE SYSTEM WILL NOT FUNCTION PROPERLY IN THE EVENT OF A POWER OUTAGE.** The generator's oil and oil filter should be changed every 200 hours or yearly.

Laboratory Freezer and Refrigerator

In case of power outages of more than 12 hours, transfer the seed and chemicals found in the

laboratory freezer. The upright freezer does not cool as efficiently when powered by the generator, and should not be opened unless absolutely necessary. Transfer the desiccators in large ice chests, and the seed in boxes. Try to enter the ultralow freezer only once to maintain the internal temperature.

The desiccator and seed in the lab refrigerator should be stored in an ice chest with the ice packs from the lab freezer.

VACUUM PUMP/AIR COMPRESSOR

Welch vacuum pump model number 8917A; serial number ED081752. In order to insure consistent and reliable activity, the vacuum pump used to degas gels must undergo regular maintenance. If it is not to be used for extended periods, the motor should be turned on once a week and allowed to run 10 minutes in order to keep the working parts lubricated. The oil should be changed every two weeks, or more often if necessary. Used oil recovered from the pump motor must be disposed of consistent with county regulations.

The oil and equipment used to maintain the vacuum pump is ordered from Lab Safety Supply under the following catalog numbers:

Motor Oil, 1 gallon, #8995P-15

Filler plugs, #61-6020

Plug O-ring, #66-0212

LAB EQUIPMENT AND PROCEDURES

pH Meter (Corning 240)

Always unplug the fill hole prior to using the electrode. Make sure the solution in the electrode is not more than one-inch below the fill hole, this can cause variations in pH readings. Fill the electrode with Orion filling solution (Ag/AgCl) until solution is within one inch of fill hole.

Calibrate every day before using. First rinse electrodes with distilled water from a squirt bottle. Press the "MODE" button until the pH light is on. Lower the electrode into the pH7 buffer and gently swirl the jar. While still swirling, press "CAL", when reading stabilizes press "READ". Rinse electrodes with distilled H₂O. Next, place electrodes in the pH 10.0 or pH 4.0 calibration buffer. Gently swirl the jar and press "CAL" again. When meter stabilizes press "READ". Most of our solutions are in the higher pH range so it is advisable to use the 2-points of pH 7 and 10. Always use the pH 7.0 buffer first followed by other buffers for calibration.

To measure the pH of a solution, begin by rinsing the electrode and patting dry. Place electrode in sample and swirl vessel gently. Press "READ" to start, when stabilization occurs, press "READ" again to freeze action. The "AUTO" control can be used in all modes. The meter automatically detects a stable endpoint and freezes the display. This is convenient during calibration but not very useful during titration. For accurate pH measurements of Tris buffers, we use a Tris-compatible electrode. Freeze display by pressing, "READ".

When not in use, always store electrode in test tube of electrode storage solution and secure the plug in the hole with a rubber band. If meter is showing slow responses, continuous drifting, or erratic readings, drain and refill with fresh Orion filling solution. If cleaning and maintenance fail to rejuvenate the electrode, replace it.

On the last Friday of each month, during the monthly lab cleaning, drain the electrode of filling solution. If there are crystals in the tip of the electrode, dissolve and remove with warm water. Replace with fresh solution. Also replace pH4, 7 and 10 calibration solutions with fresh solutions mixed from the premixed powder capsules. Initial the maintenance sheet located on the lab wall.

Fume Hood

The fume hood Face Velocity Calibration Kit is located in the cabinet under the fume hood. Inside there is a Vaneometer and a squeeze bottle of Flowchecker powder. Put the Vaneometer in the fume hood about three inches from the edge. Make sure it is level by checking the built-in level on its top. Turn the fan on. Move the sash to the velocity sticker marking one of the vertical sides of the hood. Compare the reading on the Vaneometer with the suggested velocity reading on the sticker. You may check airflow by squeezing the Flowchecker twice. Watch the powder to see if it is drawn up in the exhaust vent or just hangs in the air indicating a lack of proper airflow.

Buffer Monitoring and Preparation

Buffers are the cornerstones of enzyme electrophoresis. Changes in buffer pH often indicate that the buffer is aging or is contaminated. To monitor the pH, take a small sample (25ml) from each buffer every Monday morning. Allow the sample to come to room temperature. After the pH meter is calibrated, measure the pH of each sample. If any of the samples indicate that the pH of the buffer is more than 0.25 pH points different from the desired pH, discard the buffer and replace with fresh buffer.

Buffers are made in small quantities (usually 10 liters) to ensure freshness, and kept in carboys with spigots. Any buffers needing to be kept cool (e.g. MC and H buffers) are placed in the refrigerator. Whenever a new batch of buffers or any other solution is prepared, the date and pH are recorded on the container label. The recipes for all gel buffers are in Appendix C.

Washing Procedures

Large sidearm flasks that have been used for pouring gels are filled with warm water and set to soak. A small brush may be needed in the side arm. It is important to clear this hole to allow the vacuum to operate properly.

Small staining flasks should be washed in soapy water and rinsed with tap water.

Buffer cloths are soaked prior to use to remove the sizing agent present. For proper "breaking-in", use new cloths on the anodal side of the tray. After a days run, they are soaked in distilled water, squeezed to remove liquid, folded, placed in Ziploc bags by buffer, and stored damp in the refrigerator.

After a run day, dump the tray buffers down the sink and rinse trays with water. Turn trays upside down to dry.

After grinding blocks have been used for sample prep, they are rinsed and any remaining seed is removed with a scalpel or other fine point instrument. "Q-Tips" may also be helpful to clean writing from blocks with ethanol. Air dry in the rack. Grinding tools are cleaned immediately after use to prevent problems arising from dried-on tissue.

Staining pans can be washed in soapy water and rinsed. Bottoms of pans stain easily and are hard to clean. Steel wool soap pads will remove excessive stain residue. It becomes necessary to buy new pans when they are too stained to give a clear view of the gels.

Dirty glass pipettes should be placed tips-up in a pipette washer that has been filled to cover tips with soapy water. One soap tablet is added to pipette holding column. Tablets (Alcotabs) are located above sink area. Turn water on a low input rate (2L/min) and allow the pipettes to wash for approximately 15 minutes or until suds are absent from drain water. Then turn up water (12L/min) for 20 minutes to rinse the pipettes. After rinsing, catch the wash cycle after it has filled and started to drain. Turn off the water and allow washer to drain. Remove the pipette holder and place on dish rack to air dry the pipettes. When dry, return the pipettes to the appropriate drawer divider and replace the pipette holder back into the washer. Fill washer with soapy water to cover pipettes.

All other equipment and tools can be washed in soapy water, rinsed well and air dried in the rack. For safety reasons, do not combine scalpels or other metal items with regular dish washing. Do not soak scalpels or other metal items, to prevent rusting.

Eye Wash and Emergency Shower

Shower should be tested every month and the eyewash should be tested every week to check for proper operation and to clean rust or other debris from pipes. To test the shower, put a 5-gallon bucket up to the showerhead while another person pulls down the handle. After the water runs clear or the bucket becomes heavy, push handle back up to shut water off. To test the eyewash, turn handle and allow it to run until water becomes clear. Date and initial the posted preventative maintenance schedule.

HAZARDOUS WASTE DISPOSAL

Isozyme Hazardous Waste

Two stain recipes, TPI and ALD, and the Yew extraction buffer contain hazardous chemicals and must be stored and disposed of separately from non-hazardous waste. Store TPI and ALD stains, their rinse water, and their fixers in a separate plastic bottle from the non-hazardous stain wastes. Yew extraction buffer is stored separately from stain waste. For short-term storage, hazardous liquid waste is stored in the fume hood until the collection bottles are full. Full bottles are stored in the chemical shed until disposal.

Contacts

Hazardous waste disposal must be arranged with a private contractor, who will pick up and dispose of material according to local, state, and federal guidelines. These events may be coordinated with the Station Safety Officer, IFG, and/or for NFGEL alone.

NON-HAZARDOUS WASTE DISPOSAL

Isozyme Liquid Waste

Tray buffers, extraction buffers, and all water left from washing glassware, etc., can be disposed of down the drain.

Non-hazardous stains (all except TPI and ALD), their rinse water, and used fixer have passed analysis and bench tests, but is collected in the laboratory and disposed of at the water treatment plant outside of Placerville. This waste should not go down the sink only because IFG is on a septic system. All non-hazardous waste is collected in plastic bottles, labeled appropriately, and stored in the flammable cabinet located in the basement until a trip is made to the water treatment plant. Non-hazardous waste should be disposed of at least twice a year, or more often if necessary.

Isozyme Solid Waste

All solid wastes created by isozyme analysis, including starch gels after staining and/or fixing and any agar stains other than TPI and ALD, can be disposed of in the laboratory trash.

Contacts

To schedule a time to deposit non-hazardous isozyme waste at the water treatment plant, call Dave Johnston at (530) 621-5896 (voice). His fax number is (530) 626-7130. The phone for Union Mine (the location of the water treatment plant) is (530) 295-0429.

SAFETY PROCEDURES

NFGEL has a written Health and Safety Plan that includes laboratory and office safety guidelines and rules, evacuation procedures, and Job Hazard Analyses. This plan was developed to achieve and maintain a safe and healthy working environment.

The written Chemical Hygiene Plan outlines proper use, storage, and handling of chemical substances. These plans comply with all Forest Service and OSHA regulations.

All employees must be familiar with the NFGEL Health and Safety Plan and with the Forest Service Health and Safety Code Handbook, in particular Chapter 60 on Hazardous Materials.

Each NFGEL employee has the responsibility to be aware of unsafe and unhealthy situations. Employees are responsible for reporting to their supervisor unsafe practices and/or conditions when they lack the ability or authority to correct these situations. All employees are responsible for seeking clarification on any problem or subject that might pose a question.

APPENDICES

APPENDIX A: PROJECT SUBMISSION

Appendix A1: General Collection Guide for Seeds

Appendix A2: General Collection Guide for Vegetative Materials

Appendix A3: Packing List

General Collection Guide For Seeds

National Forest Genetic Electrophoresis Lab (NFGEL)

IMPORTANT! Sample collectors are required to make contact with NFGEL staff **BEFORE** collections begin. Call (530) 622-1609 or 1225 for orientation to collection procedures, Quality Assurance (QA) sampling, and equipment. Questions will gladly be answered. We want to help you make your project a success, and that means collections must be optimal!

COLLECTION AND PACKING OF ALL SEED:

Only viable cleaned seed is acceptable for testing. Please let us know if it is not possible for your seed to be cleaned before arriving at NFGEL, we will be glad to discuss your options. Pay attention to collection dates to ensure seed is still viable.

If there is any question about the sampling strategy for your project, call or e-mail the lab staff. Include QA samples if required for your project, as determined by NFGEL staff.

Label seed packets with the identification information (such as ramet, clone, or individual ID). Send seed in well labeled manila envelopes or plastic ziplock bags. Amounts of seed required, number of individuals and populations needed for your project will be determined when your project is developed with NFGEL staff. Let us know if you need remaining seed returned upon completion of your project.

Cardboard boxes are fine for shipping seed. Do not use damp towels or blue ice. Include and accurate packing list using seed identifications.

For collection guidelines specific to your project, an additional page of instructions may be enclosed.

SHIPPING:

It is OK for seed to arrive at the lab on a Friday, or the day before a holiday.

1. **SEND** via traceable delivery service to:

NFGEL, USDA Forest Service
2480 Carson Road
Placerville, CA 95667

2. **MARK** the contents on the outside of the package, "PACKAGE CONTAINS SEED".

3. **ENCLOSE** the packing list (following page) and e-mail a copy to NFGEL at vhipkins@fs.fed.us We will reply when your seed is received.

4. **CALL** NFGEL at (530) 622-1609 or (530) 622-1225

General Collection Guide For Vegetative Materials

National Forest Genetic Electrophoresis Lab (NFGEL)

IMPORTANT! Sample collectors are required to make contact with NFGEL staff **BEFORE** collections begin. Call (530) 622-1609 or 1225 for orientation to collection procedures, Quality Assurance (QA) sampling, and equipment. Questions will gladly be answered. We want to help you make your project a success, and that means collections must be optimal!

COLLECTION AND PACKING:

Samples must be free from all visible disease and/or insect damage.

Samples should be placed in ziplock plastic bags large enough to prevent tissue from being folded, crushed, or damaged. If samples are very wet, pat with paper towels until tissue is just damp to prevent mold. Place a damp (not wet) paper towel into each sample bag to help prevent tissue dessication. Each sample should be well identified with labels in permanent ink. Write the identification on the outside of each ziplock bag, and also insert a matching label inside the bag. Place bags into an ice chest with frozen blue ice to keep samples cool in the field. **KEEP BAGS AND ICE CHESTS OUT OF THE SUN.** Do not leave collection bags inside vehicles in summer heat. Include QA samples if required for your project, as determined by NFGEL staff.

Store bagged tissue in a refrigerator until shipping. So not allow bag to touch the refrigerator walls as this often causes the samples to freeze. Ship samples in a cooler with well frozen blue ice packs to keep samples cold in transit. **NEVER** use wet ice. Insulate samples from direct contact with blue ice using crumpled newspaper or other packing material. Use enough blue ice to cover the bottom of the cooler. Hot summer months may require more blue ice for protection. Don't pack samples together so tightly that samples are crushed and bruised when they arrive; use another cooler if needed. If you ship in a Styrofoam ice chest (they are inexpensive), please place the ice chest in a cardboard box to prevent the cooler from breaking in transit.

SHIPPING:

Ship vegetative materials as soon as possible; within one day of collection is best. It is important to coordinate shipping of materials with NFGEL so that samples may be processed quickly upon arrival. **NEVER MAIL THE PACKAGE SO THAT IT ARRIVES AT THE LAB ON A FRIDAY OR THE DAY BEFORE A HOLIDAY.**

1. **SEND** by a quick and traceable delivery service. (Federal Express has the best delivery time for us, but UPS or U.S. Postal Overnight Service are fine.) Send to:
NFGEL, USDA Forest Service
2480 Carson Road
Placerville, CA 95667.
2. **MARK** on the outside of the package: "VEGETATIVE MATERIAL – REFRIGERATE IMMEDIATELY UPON ARRIVAL."
3. **ENCLOSE** a packing list (see a following page) and E-MAIL a copy to NFGEL at vhipkins@fs.fed.us. We will reply when materials are received.
4. **CALL** NFGEL at (530) 622-1609 or 1225 when material is shipped.

Packing List For Submitting Sample Material

(revised 2/03)

<i>Please complete items # 1 – 8</i>	
(1) Name of contact person:	
(2) Address:	
(3) Telephone number:	
(4) E-mail address:	
(5) Date collected (vegetative material only):	
(6) List of enclosed species, individual or sample identification (any necessary identification information), and approximate amount of material per sample (number of seeds/collection, buds/individual, leaves/plant, etc.)	
(7) QA samples. REMINDER: Be sure to include an extra sample from 10% of your individuals for our Quality Assurance Program if NFGEL advises you this is applicable to your project.	
(8) Comments:	
<i>To be completed by NFGEL</i>	
Date received:	Received by:
NFGEL #s:	E-mail reply sent:
QA samples enclosed:	
<u>SEND TO:</u> USDA Forest Service – NFGEL, 2480 Carson Road, Placerville, California 95667 e-mail: vhipkins@fs.fed.us phone: 530-622-1225; fax: 530-622-2633	

APPENDIX B: SAMPLE PREPARATION

Appendix B1: Extraction Buffers

Appendix B2: Instructions for Leaf or Needle Prep with Liquid Nitrogen

Appendix B3: Sample Preparation Instructions by Species

- a. Pines**
- b. Other Conifers**
- c. Woody Angiosperms**
- d. Herbaceous Plants**

Appendix B1: Extraction Buffers

GERMANIUM DIOXIDE EXTRACTION BUFFER	
CHEMICAL COMPONENTS	
STEP 1 - germanium dioxide distilled water Heat on hot plate with stir bar until dissolved. Cool to room temperature	0.075 g 75 ml
STEP 2 - diethyldithiocarbamic acid borax (sodium tetraborate) sodium bisulfite PVP 40 L-ascorbic acid Mix together in a beaker to break up lumps. Combine the chemicals from steps 1 and 2. While stirring, add 8.0 ml 0.16 M potassium phosphate buffer.	0.275 g 1.1 g 0.3 g 4.0 g 4.0 g
STEP 3 - DMSO 2-phenoxyethanol mercaptoethanol Stir together and keep chilled in an ice bath.	8.0 ml 0.5 ml 0.16 ml
COMMENTS: Must be prepped and used in the fume hood. Keep adding ice as needed to ice bath. Keep solution covered until ready to use. Keep all pipettes used in the extraction buffer inside the fume hood, throw away at the end of the day in a closed bag.	
CITATION: Mitton et al., 1979. J. Heredity 79: 86-89.	

GOTTLIEB EXTRACTION BUFFER		
CHEMICAL COMPONENTS	100 ml recipe	50 ml recipe
LIQUIDS: distilled water 1 M Tris-HCl, pH 7.5 10% MgCl ₂ (10 mM) 1 M KCl (10 mM) *2-mercaptoethanol, 14 mM*	70 ml 10 ml 2 ml 1 ml 100 µl	35 ml 5 ml 1 ml 0.5 ml 50 µl
DRY: EDTA (final concentration = 1 mM) PVP-40 (final concentration = 5%)	0.037 g 5 g	0.019 g 2.5 g
DIRECTIONS: Store in refrigerator up to 1 week.		
COMMENTS: *Using the fume hood, add mercaptoethanol after the solution has been brought to volume.*		
CITATION: Gottlieb 1981.		

MELODY / NEALE EXTRACTION BUFFER	
CHEMICAL COMPONENTS	100 ml recipe
LIQUIDS: 1 M Tris-HCl, pH 8.0 distilled water	10 ml 70 ml
DRY: PVP - 40 sucrose EDTA, Na ₂ salt dithiotreitol (DTT) ascorbic acid bovine albumin NAD NADP pyrodoxal-5- phosphate	10 g 10 g 0.17 g 0.15 g 0.02 g 0.10 g 0.05 g 0.035 g 0.005 g
DIRECTIONS: Mix until dissolved. Bring to volume using distilled water. Store in refrigerator.	
COMMENTS: Refrigerate for up to one week. Good for conifer buds and plant leaves.	
CITATION: Pitel, J. A. and W. M. Cheliak. 1984. Effects of extraction buffers...	

NEALE'S DOUGLAS FIR PRIMORDIA BUFFER	
CHEMICAL COMPONENTS	100 ml recipe
LIQUIDS: distilled water	100 ml
DRY: PVP - 40 PVP -360 sucrose EDTA, Na ₂ salt dithiotreitol (DTT) ascorbic acid bovine albumin NAD NADP pyrodoxal-5- phosphate	7 g 1 g 10 g 0.17 g 0.015 g 0.02 g 0.10 g 0.05 g 0.035 g 0.005 g
DIRECTIONS: Titrate to pH 6.7 with 1M Tris-HCl, pH 8.0. Add 200 µl 2-Mercaptoethanol.	
COMMENTS: Prepare in fume hood and use in fume hood. Refrigerate for up to one week.	
CITATION:	

PHOSPHATE BUFFER, 0.2 M, PH 7.5		
CHEMICAL COMPONENTS	1000 ml recipe	
LIQUIDS: distilled water	900 ml	
DRY: Sodium phosphate, monobasic Sodium phosphate, dibasic	3.56 g 23.86 g	
DIRECTIONS: Mix and bring volume up to 1000 ml.		
COMMENTS: This buffer is used as a wick buffer for sample preparation, and for the GOT stain.		
CITATION: Modified from Conkle et al. 1982		

Appendix B2: Leaf or Needle Extraction Using Liquid Nitrogen

In a fume hood assemble: empty, labeled microtiter plates on ice packs
beaker containing disposable glass pipettes
beaker containing small metal spatulas

Have on hand: liquid nitrogen
liquid nitrogen dipper
tissue that is to be prepared
dishpan full of soap and cold water
pen and paper, scissors, and paper towels
approximately 24 porcelain mortars and pestles that have been stored at least
one hour at 0C. (Coo's size 60313, 65 ml).
100 ml extraction buffer

Obtain 1 mortar and pestle from the freezer. Choose your individual, record pertinent information. Use scissors to cut healthy, green, disease-free tissue into 1-4cm square pieces. Place the tissue in a cold mortar until there are 2-3 tissue layers (about the size and thickness of a quarter). Wipe the scissors clean with a paper towel.

Cover the tissue with liquid nitrogen. When the liquid nitrogen has almost evaporated, use the pestle to crush the frozen needles to a fine powder. Add more liquid nitrogen and crush again if necessary. **KEEP TISSUE COLD.** Place the used pestle into the dishpan of cold soapy water. Add 1-2 ml of extraction buffer to the needle powder in the mortar and mix well using a small metal spatula. When the cold powder is saturated, not dripping, scrape the mixture up onto the sides of the mortar where it will freeze. Allow the extraction buffer/powder mixture to thaw. As it thaws, a liquid buffer/enzyme solution will drain to the bottom of the mortar. Add more extraction buffer if necessary.

Collect the buffer/enzyme solution that has drained into the bottom of the mortar with a clean, disposable, glass pipette. Transfer the solution to the appropriate microtiter plate well. Place used glass pipettes into a sealable plastic bag for later disposal. Leave all dirty pipettes and spatulas in the hood so the odor is contained while you work. Place the used mortars into the dishpan. Wash, dry, and return mortars to the freezer. Cold water in the dishpan helps eliminate the mercaptoethanol odor.

We use 7 drops of buffer/enzyme solution in each well. We usually have 4-5 mortars that contain the powder/buffer mixture thawing in the fume hood at one time. It takes two people 3 hours to prepare 30 individuals, three replicates of each individual. One person is responsible for crushing and labeling; the second person is responsible for adding extraction buffer to the powder and transferring the buffer/enzyme solution to microtiter plates. Of the three replicates, one is for primary use and the other two are used as back-up plates.

Appendix B3. Sample Preparation Instructions by Species

Table 1. Pines

(Note: A cube 2 mm on a side = $8 \text{ mm}^3 = 1/4$ the size of pencil eraser.) When putting two megs on one wick, add one extra drop of buffer. Liquid nitrogen is not used for pine tissues. In general, 1 drop = $50 \mu\text{l}$, although this varies depending on the pipette used. Method 1 = Submerge tissue in buffer in microtiter plate wells. Freeze at -70°C . On the morning of the run, thaw at room temperature, grind the tissue, and absorb slurry onto wicks. Method 2 = Grind tissue in microtiter plate wells with buffer. Soak wicks. Freeze wicks at -70°C . On the morning of the run thaw at room temperature.

Species	Tissue	Tissue quantity	Buffer	Buffer quantity	Gel format	Wicks	Sample Preparation
Jeffrey Pine; <i>Pinus jeffreyi</i>	buds	8 mm^3	Melody/Neale	$150 \mu\text{l}$	3 groups of 10 wicks	3 mm	Method 1. (4 to six set replicates prepared if buds are large.)
Limber Pine; <i>Pinus flexilis</i>	buds	8 mm^3	Melody/Neale	$150 \mu\text{l}$	3 groups of 10 wicks	3 mm	Method 1. Store backup wicks frozen. Make backups when buds are too small for replicates.
Loblolly Pine; <i>Pinus taeda</i>	buds	8 mm^3	Melody/Neale	$150 \mu\text{l}$	3 groups of 10 wicks	3 mm	Method 1.
Loblolly Pine; <i>Pinus taeda</i>	megs	whole	0.2 M phosphate buffer, pH 7.5	$150 \mu\text{l}$	Megs for genotyping: 6 groups of 10 megs (using two megs/ wick in the last two positions of each group). Meg for reading individual samples: 4 groups of 10 wicks.	2 mm 2.5 mm	Method 2.
Loblolly Pine; <i>Pinus taeda</i>	meg/ embryo pairs	whole megs, embryos 10 mm long	0.2 M phosphate buffer, pH 7.5	$100 \mu\text{l}$	3 groups of 14 wicks each. 7 meg/embryo pairs/group	2 and 2.5 mm	Megs: Method 1; use 2 mm wicks. Embryos: Method 1, 2.5 mm wicks. Meg and embryo go in separate microtiterplate wells.
Longleaf Pine; <i>Pinus palustris</i>	megs	whole	0.2 M phosphate buffer, pH 7.5		10 megs/ individual; 8 sample wicks per individual (last two wicks are double megs); 6 individuals/set	2 mm	Method 2.
Ponderosa Pine; <i>Pinus ponderosa</i>	buds	8 mm^3	Melody/Neale	$150 \mu\text{l}$	3 groups of 10 wicks	3 mm	Method 1. (4 to six set replicates prepared.)
Ponderosa Pine; <i>Pinus ponderosa</i>	megs	whole	0.2 M phosphate buffer	$100 \mu\text{l}$	See Loblolly Pine Megs	2 mm	Method 2.
Sand Pine; <i>Pinus clausa</i>	megs	whole	phosphate buffer	$100 \mu\text{l}$	See Loblolly Pine Megs	2 mm	Method 2.

Shortleaf Pine; <i>Pinus echinata</i>	meg/embryo pairs	whole	0.2 M phosphate buffer	100 µl	Set = 3 groups of 7 seeds (42 wicks total)	2 and 2.5 mm	See Loblolly Pine meg/embryo pairs.
Slash Pine; <i>Pinus elliotii</i>	buds	8 mm ³	Melody/Neale	150 µl	3 groups of 10 wicks	3 mm	Method 1. (Store backup wicks frozen.)
Slash Pine; <i>Pinus elliotii</i>	needles	the small amount which fits onto the end of a small metal spatula	germanium dioxide	1 ml		3 mm	Grind tissue in small, cold mortar with cold pestle. Transfer slurry to microtiter plate wells. Freeze at –70°C. On the morning of the run, thaw, then soak wicks in slurry.
Sugar Pine; <i>Pinus lambertiana</i>	buds	8 mm ³	Melody/Neale	150 µl	3 groups of 10 wicks	3 mm	Method 1.
Sugar Pine, <i>Pinus lambertiana</i>	megs	the ¼ of the meg closest to the embryo	0.2 M phosphate buffer, pH 7.5	100 µl	See Loblolly Pine megs	2 mm	Method 2.
Virginia Pine; <i>Pinus virginiana</i>	buds	8 mm ³	Melody/Neale	100 µl	3 groups of 10 wicks	3 mm	Method 1.
Western White Pine; <i>Pinus monticola</i>	megs	whole meg	Melody/Neale	150 µl	3 groups of 10 wicks	2 mm	Method 2.
<i>Pinus massoniana</i>	buds	8 mm ³	Melody/Neale	150 µl	3 groups of 10 wicks	3 mm	Method 1.

Table 2. Other Conifers

Sample preparation instructions: N₂ = liquid nitrogen. Method 1 = Submerge tissue in buffer in microtiter plate wells. Freeze at -70°C. On the morning of the run, thaw at room temperature, grind the tissue, and absorb slurry onto wicks. Method 2 = Grind tissue in microtiter plate wells with buffer. Soak wicks. Freeze wicks at -70°C. On the morning of the run thaw at room temperature.

Species	Tissue	Tissue quantity	Buffer	Buffer quantity	N ₂	Gel format	Wick	Sample preparation
Cedar, Alaska Yellow; <i>Cupressus nootkatensis</i>	needles	0.15 g	Gottlieb (Tris HCl, pH 7.5)	600 µl	yes		3 mm	Grind needles in buffer in chilled acrylic plates, transfer slurry to microtiter plate wells, treat as in Method 1.
Monterrey Cypress; <i>Cupressus macrocarpa</i>	needles	0.15 g	Gottlieb (Tris HCl, pH 7.5)	600 µl	yes	2 groups of 10, 1 group with 5 wicks, 4 QA'a	3 mm	Grind needles in buffer in chilled acrylic plates, transfer slurry to microtiter plate wells, treat as in Method 1.
Cedar, Port Orford; <i>Cupressus lawsoniana</i>	needles	0.15 g	Gottlieb (Tris HCl, pH 7.5)	600 µl	yes		3 mm	Grind needles in buffer in chilled acrylic plates, transfer slurry to microtiter plate wells, treat as in Method 1.
Cedar, Port Orford; <i>Cupressus lawsoniana</i>	needles	3 mm ³	Gottlieb (Tris HCl, pH 7.5)	150 µl	no		3 mm	Method 1. Absorb extract onto 5 wicks, saving 2 backup wicks at -70°C.
Douglas-Fir; <i>Pseudotsuga menziesii</i>	buds	2 to 6 buds/tree (depending on size)	Melody/Neale	150 µl	no	3 groups of 10 wicks	3 mm	Method 1. 6 wicks/samples.
Douglas-Fir; <i>Pseudotsuga menziesii</i>	megs		0.2 M phosphate buffer, pH 7.5	100 µl	no		2.5 mm	Method 2.
Fir, Fraser; <i>Abies fraseri</i>	meg/embryo pairs		0.2 M phosphate buffer, pH 7.5	100 µl	no	3 groups of 14 wicks (7 meg/emb. pairs)	2 and 2.5 mm	Method 1: Megs: use 2 mm wicks, embryos: 2.5 mm wicks. Meg and embryo go in separate microtiter plate wells.
Larch, Western	eggs		0.2 M phosphate buffer, pH 7.5	100 µl	no		.5 mm	Not worthwhile; megagametophytes were too small to provide enough enzyme to assay.
Yew, Pacific; <i>Taxus brevifolia</i>	needles		Mitton et al. 1979		yes			Grind in chilled mortar and pestle, using liquid N ₂ . Place buffer into the mortar. Transfer slurry to microtiter plates and freeze at -70°C. On morning of run, thaw at room temperature and absorb onto wicks.

Table 3. Woody Angiosperms

Sample preparation instructions: N₂ = liquid nitrogen. Method 1 = Submerge tissue in buffer in microtiter plate wells. Freeze at -70°C. On the morning of the run, thaw at room temperature, grind the tissue, and absorb slurry onto wicks.

Species	Tissue	Tissue quantity	Buffer	Buffer quantity	Liquid N2	Gel format	Wicks	Sample preparation
<i>Populus angustifolia</i> ; Narrowleaf Cottonwood	leaves	Two 7 mm discs (paper hole punch)	Gottlieb (Tris HCl, pH 7.5)	150 µl	No	3 groups of 10 wicks	3 mm	Method 1.
<i>Populus deltoides</i> ; Plains and Eastern Cottonwood	leaves	Two 7 mm discs (paper hole punch)	Gottlieb (Tris HCl, pH 7.5)	150 µl	No	3 groups of 10 wicks	3 mm	Method 1.
<i>Populus fremontii</i> ; Fremont Cottonwood	leaves	Two 7 mm discs (paper hole punch)	Gottlieb (Tris HCl, pH 7.5)	15 µl	No	3 groups of 10 wicks	3 mm	Method 1.
<i>Populus tremuloides</i> ; Trembling Aspen	leaves	Two 7 mm discs (paper hole punch)	Gottlieb (Tris HCl, pH 7.5)	150 µl	No	3 groups of 10 wicks	3 mm	Method 1.
<i>Populus trichocarpa</i> ; Black Cottonwood	leaves	Two 7 mm discs (paper hole punch)	Gottlieb (Tris HCl, pH 7.5)	150 µl	No	3 groups of 10 wicks	3 mm	Method 1.
<i>Purshia tridentata</i> ; Bitterbrush	leaves	8mm ³	Gottlieb (Tris HCl, pH 7.5)	150 µl	Yes	3 groups of 10 wicks	3 mm	Method 1.

Table 4. Herbaceous Plants

Sample preparation instructions: N₂ = liquid nitrogen. Method 1 = Submerge tissue in buffer in microtiter plate wells. Freeze at -70°C. On the morning of the run, thaw at room temperature, grind the tissue, and absorb slurry onto wicks. Method 2 = Grind tissue in microtiter plate wells with buffer. Soak wicks. Freeze wicks at -70°C. On the morning of the run thaw at room temperature.

Species	Tissue	Tissue Quantity	Buffer	Buffer Quantity	N ₂	Gel format	Wicks	Sample preparation
<i>Achnatherum hymenoides</i> ; Indian Ricegrass;	seedling	entire seedling with root	Melody/Neale	450 µl	No	3 groups of 10 seedlings each	3 mm	Method 2, transferring 3 drops of slurry into each of three microtiter plate wells.
<i>Achnatherum occidentale</i> s; Western Needlegrass;	seedling	entire seedling with root	Melody/Neale	450 µl	No	3 groups of 10 seedlings each	3 mm	Method 2, transferring 3 drops of slurry into each of three microtiter plate wells.
<i>Achnatherum speciosum</i> ;	seedling	entire seedling with root	Melody/Neale	450 µl	No	3 groups of 10 seedlings each	3 mm	Method 2, transferring 3 drops of slurry into each of three microtiter plate wells.
<i>Achnatherum thurberiana</i> ;	seedling	entire seedling with root	Melody/Neale	450 µl	No	3 groups of 10 seedlings each	3 mm	Method 2, transferring 3 drops of slurry into each of three microtiter plate wells.
<i>Bromus carinatus</i> ;	seedling	entire seedling with root	Melody/Neale	400 µl	No	3 groups of 10 seedlings each	3 mm	Grind entire seedling; Method 2.
<i>Collomia rawsoniana</i> ;	leaves	2 medium sized leaves, ca. 3 cm long	Gottlieb (Tris HCl, pH 7.5)	400 µl	Yes	3 groups of 10 seedlings each	3 mm	Grind in mortar & pestle with liquid N ₂ . Method 2.
<i>Elymus elymoides</i> ;	seedling	entire seedling with root	Melody/Neale	450 µl	No	3 groups of 10 seedlings each	3 mm	Method 2, transferring 3 drops of slurry into each of three microtiter plate wells.
<i>Elymus glaucus</i> ;	seedling	entire seedling, with root	Melody/Neale	400 µl	No	3 groups of 10 seedlings each	3 mm	Use seedlings 8-11 mm long. (Length excludes root, and roots were removed from samples in error.) Method 2, transferring 3 drops of slurry into each of two microtiter plate wells.
<i>Festuca idahoensis</i> ;	leaves	3 3" long leaf blades	Gottlieb (Tris HCl, pH 7.5)	400 µl	No	1 wick/ individual, 10 wicks/ group, 3 groups	3 mm	Method 2, transferring 3 drops of slurry into each of two microtiter plate wells.
<i>Frasera fastigiata</i>	leaves	3 discs 7 mm in diameter	Gottlieb (Tris HCl, pH 7.5)	200 µl		3 groups of 10 individuals each.	3 mm	Method 2. Soak 6 wicks. Backup set: 2 discs of leaf tissue/sample in microtiter plate well with 4 drops buffer. Freeze.
<i>Frasera umpquaensis</i>	leaves	3 discs 7 mm in diameter	Gottlieb (Tris HCl, pH 7.5)	200 µl		3 groups of 10 individuals each.	3 mm	Method 2. Soak 6 wicks. Backup set: 2 discs of leaf tissue/sample in microtiter plate well with 4 drops buffer. Freeze.
<i>Hackelia diffusa</i>	leaves	10 mm ²	Gottlieb (Tris HCl, pH 7.5)	400 – 500 µl	Yes	3 groups of 10 individuals each.	3 mm	Grind in cold mortar with liquid N ₂ . Place buffer in mortar to make slurry. Transfer 2-3 drops slurry to well of microtiter plate. (Make original + 2 replicate plates.) Freeze at -70°C. On morning of run, thaw at room temperature and soak onto 4 wicks.
<i>Hackelia venusta</i>	leaves	10 mm ²	Gottlieb (Tris HCl, pH 7.5)	400 - 500 µl	Yes	3 groups of 10 individuals each.	3 mm	See <i>Hackelia diffusa</i> .

Species	Tissue	Tissue Quantity	Buffer	Buffer Quantity	N ₂	Gel format	Wicks	Sample preparation
<i>Lewisia cantelovii</i>	leaves	½ of 7 mm diameter disc	Melody/Neale	150 µl	No	3 groups of 10 seedlings each	3 mm	Method 1. Soak 6 wicks.
<i>Lewisia congonii</i>	leaves	½ of 7 mm diameter disc	Melody/Neale	150 µl	No	3 groups of 10 seedlings each	3 mm	Method 1. Soak 6 wicks.
<i>Lewisia serrata</i>	leaves	½ of 7 mm diameter disc	Melody/Neale	150 µl	No	3 groups of 10 seedlings each	3 mm	Method 1. Soak 6 wicks.
<i>Periderdia erythrorhiza</i>	seedlings	entire (shoot, bulbil and root)	Melody/Neale	500 µl	Yes	3 groups of 10 seedlings each	3 mm	Grind in cold mortar with liquid N ₂ . Place buffer in mortar to make slurry. Transfer 3 drops slurry to well of microtiter plate. Freeze at –70°C. On morning of run, thaw at room temperature and soak onto wicks.
<i>Periderdia oregona</i>	seedlings	entire (shoot, bulbil, and root)	Melody/Neale	500 µl	Yes	3 groups of 10 seedlings each	3 mm	See <i>Periderdia erythrorhiza</i> .
<i>Rorippa subumbellata</i> ; Tahoe Yellow Cress	leafy shoot		Gottlieb (Tris HCl, pH 7.5)	500 µl	No	3 groups of 10 seedlings each	3 mm	Submerge shoot in buffer in chilled well. Grind. Soak wicks. Method 2.
<i>Saxifraga bryophora</i>	leaves	2 discs, 7 mm diameter	Gottlieb (Tris HCl, pH 7.5)	100 µl		3 groups of 10 seedlings each	3 mm	Method 1. Soak 6 wicks.
<i>Sisyrinchium sarmentosum</i> ; Pale Blue-eyed Grass	leaves	8-11 cm long	Gottlieb (Tris HCl, pH 7.5)	400 µl		3 groups of 10 individuals each.	3 mm	Grind tissue in cold mortar with buffer. Transfer 2 or 3 drops of slurry to well of cold microtiter plate. One replicate plate per set. Freeze at –70°C. On morning of run, thaw and soak 3 wicks.

APPENDIX C: GELS AND THEIR BUFFERS

Appendix C1: Gel and Tray Buffers

Solutions are made in 10 L quantities and stored in carboys. Morpholine Citrate, Histidine gel buffer, and System 8 Parts A & B are stored in the refrigerator to prolong shelf life. All other buffers are stored at room temperature.

GENERAL INSTRUCTIONS :

Fill a labeled carboy with 5 L of distilled H₂O. Place 4 L distilled H₂O and a large stir bar into a 4 L NALGENE beaker. Using a stir plate, gradually add dry chemicals to churning water. Once in solution, add the contents of the beaker into the carboy, pH, and bring up to 10 L total volume. Measure the buffer pH and record it on the carboy. If pH is more than 0.10 off the optimum it is best to start the recipe over to insure the quality of the buffer. Small adjustments in pH can be made by the addition of small amounts of the recipe's ingredients. Check pH weekly to insure buffers are still in the optimum range (+/- 0.10 pH)

GEL BUFFER RECIPES

LITHIUM BORATE GEL BUFFER (pH~8.3)		
CHEMICAL COMPONENTS		
Tris (Sigma 7-9 or Trizma Base)		62 g
Citric acid, monohydrate		16 g
DIRECTIONS: Bring almost to volume of 10 liters. Adjust pH to 8.3 and bring to final volume.		
CITATION:		
SODIUM BORATE GEL BUFFER (pH~8.8)		
CHEMICAL COMPONENTS		
Tris (Sigma 7-9 or Trizma Base)		121.1 g
Citric acid, monohydrate		14.5 g
DIRECTIONS: Bring almost to volume of 10 liters. Adjust pH to 8.8 and bring to final volume .		
CITATION:		
MORPHOLINE CITRATE BUFFER (pH~6.1 or 8.0)		
CHEMICAL COMPONENTS	pH 6.1	pH 8.0
Citric acid, anhydrous	76.85 g	76.85 g
After citric acid is dissolved, add: N-(3-aminopropyl)-morpholine	approx. 95 ml	approx 175 ml
DIRECTIONS: Bring almost to volume of 10 liters. Adjust pH and bring to final volume		
COMMENTS: Store in refrigerator.		
CITATION: Wendel and Weeden 1989, referencing Clayton and Tretiak 1972. Modified.		

TRAY BUFFER RECIPES

LITHIUM BORATE TRAY BUFFER (pH~8.3)	
CHEMICAL COMPONENTS	
Boric Acid	118.9 g
Lithium hydroxide	18.0 g
DIRECTIONS: Bring almost to volume of 10 liters. Adjust pH to 8.3 and bring to final volume	
CITATION: Wendel and Weeden 1989, referencing Ashton and Braden 1961.	

SODIUM BORATE TRAY BUFFER (pH~8.0)	
CHEMICAL COMPONENTS	
Boric Acid	185.5 g
Sodium hydroxide	20.0 g
DIRECTIONS: Bring almost to volume of 10 liters. Adjust pH to 8.0 and bring to final volume.	
CITATION: Wendel and Weeden 1989, referencing Poulik 1957.	

MORPHOLINE CITRATE TRAY BUFFERS are the same as the gel buffers.

Appendix C2: Gel Recipes

Gels are 11% starch.

Approximate cooking time under current lab conditions are:

2 thick gels=3'30"

2 thin gels=2'40"

1 thick gel=1'50"

1 thin gel=1'20"

LITHIUM BORATE; Thick Frames (210mm x 124mm x 12mm)			
INGREDIENTS	2 gels (1000 ml)	1 gel (500 ml)	
Starch	110 g	55 g	in a 2-liter vacuum flask
LB tray buffer	100 ml	50 ml	in a graduated cylinder
+ LB gel buffer	220 ml	110 ml	in a graduated cylinder
LB gel buffer	680 ml	340 ml	in a 1-liter boiling flask
COMMENTS: Total 320 ml liquid in graduated cylinder for 2 gels, 160 ml for 1 gel.			

LITHIUM BORATE; Thin Frames (210mm x 124mm x 8mm)			
INGREDIENTS	2 gels (784 ml)	1 gel (392 ml)	
Starch	86.25 g	43.13 g	in a 2-liter vacuum flask
LB tray buffer	78 ml	39 ml	in a graduated cylinder
+ LB gel buffer	172 ml	86 ml	in a graduated cylinder
LB gel buffer	534 ml	267 ml	in a 500 ml boiling flask
COMMENTS: Total 250 ml liquid in graduated cylinder for 2 gels, 125 ml for 1 gel.			

SODIUM BORATE BUFFER; Thick Frames (210mm x 124mm x 12mm)			
INGREDIENTS	2 gels (1000 ml)	1 gel (500 ml)	
Starch	110 g	55 g	in a 2-liter vacuum flask
SB gel buffer	320 ml	160 ml	in a graduated cylinder
SB gel buffer	680 ml	340 ml	in a 1-liter boiling flask

SODIUM BORATE BUFFER; Thin Frames (210mm x 124mm x 8mm)			
INGREDIENTS	2 gels (784 ml)	1 gel (392 ml)	
Starch	86.25 g	43.13 g	in a 2-liter vacuum flask
SB gel buffer	250 ml	125 ml	in a graduated cylinder
SB gel buffer	534 ml	267 ml	in a 500 ml boiling flask

MORPHOLINE CITRATE pH 8 or 6.1; Thick Frames (210mm x 124mm x 12mm)			
INGREDIENTS	2 gels (1000 ml)	1 gel (500 ml)	
Starch	110 g	55 g	in a 2-liter vacuum flask
MC buffer	50 ml	25 ml	in a graduated cylinder
+ distilled H ₂ O	270 ml	135 ml	in a graduated cylinder
distilled H ₂ O	680 ml	340 ml	in a 1-liter boiling flask
COMMENTS: Total 320 ml liquid in graduated cylinder for 2 gels, 160 ml for 1 gel.			

MORPHOLINE CITRATE pH 8 or 6.1; Thin Frames (210mm x 124mm x 8mm)			
INGREDIENTS	2 gels (784 ml)	1 gel (392 ml)	
Starch	86.25 g	43.13 g	in a 2-liter vacuum flask
MC buffer	40 ml	20 ml	in a graduated cylinder
+ distilled H ₂ O	210 ml	105 ml	in a graduated cylinder
distilled H ₂ O	534 ml	267 ml	in a 500 ml boiling flask
COMMENTS: Total 250 ml liquid in graduated cylinder for 2 gels, 125 ml for 1 gel.			

APPENDIX D: STOCK SOLUTIONS

AAT Solution, pH 7.4		
CHEMICAL COMPONENTS	800 ml recipe	1000 ml recipe
LIQUIDS: distilled water	800 ml	1000 ml
DRY: alpha-Ketoglutaric acid L-Aspartic acid PVP-40 (polyvinylpyrrolidone) EDTA, Na ₂ salt Sodium phosphate, dibasic	0.292 g 1.07 g 4.00 g 0.40g 11.36 g	.365 g 1.338 g 5.0 g .500 g 14.20 g
COMMENTS: The PVP goes into solution slowly. Store in the refrigerator. Keep it for up to three weeks.		
CITATION: Wendel and Weeden, 1989.		

cis-Aconitic Acid, 5%		
CHEMICAL COMPONENTS	20 ml recipe	
LIQUIDS: distilled water	20 ml	
DRY: cis-Aconitic Acid	1 g	
COMMENTS: Store in the refrigerator.		
CITATION:		

ACP Buffer		
CHEMICAL COMPONENTS	500 ml recipe	
LIQUIDS: distilled water glacial Acetic Acid 10% MgCl ₂	400 ml 2.35 ml 5.1 ml	
DRY: Sodium acetate, trihydrate	1.2 g	
DIRECTIONS: pH to 4.0 with NaOH, and bring total volume to 500 ml with distilled water.		
CITATION: Conkle et al. 1982, referencing Scandalious 1969.		

Agar, 1.3% , liquid		
CHEMICAL COMPONENTS	10 ml recipe	
LIQUIDS: distilled water	10 ml	
DRY: Bacto-agar	0.13 g	
DIRECTIONS: Mix in a beaker using a magnetic stir bar on a stirring hot plate. Make sure agar is completely dissolved. Do not pour over gel slices until beaker is cool enough to touch to your bare skin without burning.		
COMMENTS: 10 ml recipe covers one slice; adjust recipe for number of slices required. Used with AK and HK stains. We use Bacto-agar. If substituting agarose, make a 1% solution.		
CITATION:		

L-Aspartic Acid, 0.2 M, pH 7.5		
CHEMICAL COMPONENTS	1000 ml recipe	
LIQUIDS: distilled water 4N NaOH	900 ml as needed	
DRY: L-Aspartic Acid	26.62 g	
DIRECTIONS: Slowly adjust pH to 7.5 with 4N NaOH, approximately 50 ml. Bring to a final volume of 1 liter with distilled water. Store in refrigerator.		
COMMENTS: The chemical does not completely dissolve until the NaOH is added.		
CITATION:		

Bovine Albumin, 3%		
CHEMICAL COMPONENTS	10 ml recipe	
LIQUIDS: distilled water	10 ml	
DRY: Bovine Albumin	0.30 g	
COMMENTS: Store in the refrigerator for up to one week.		
CITATION:		

Catalase Buffer		
CHEMICAL COMPONENTS	200 ml recipe	500 ml recipe
LIQUIDS: distilled water	200 ml	500 ml
DRY: Sodium phosphate, monobasic Sodium phosphate, dibasic	3.70 g 3.58 g	9.25 g 8.95 g
COMMENTS: Mix until dissolved. Store in the refrigerator. Make fresh weekly.		
CITATION: Conkle et al. 1982, referencing Scandalious 1962.		

Citric Acid, 0.41 M		
CHEMICAL COMPONENTS	500 ml recipe	
LIQUIDS: distilled water	500 ml	
DRY: Citric Acid, anhydrous	39.39 g	
COMMENTS: Store in refrigerator for up to one month. This solution is used to adjust the pH of the Histidine Citrate tray buffer.		
CITATION:		

Dihydroxyacetone Phosphate Solution		
CHEMICAL COMPONENTS	1 ml recipe	
LIQUIDS: distilled water	1 ml	
DRY: 1 bottle of Dihydroxyacetone phosphate =	25 mg	
COMMENTS: Store in refrigerator.		
CITATION:		

Esterase Buffer, pH 6.4		
CHEMICAL COMPONENTS	1000 ml recipe	
LIQUIDS: distilled water	to 1000 ml	
DRY: Sodium phosphate, monobasic (NaH ₂ PO ₄) Sodium phosphate, dibasic (Na ₂ HPO ₄)	13.9 g 5.36 g	
CITATION: Conkle et al. 1982		

Fructose-6-phosphate (F-6-P)		
CHEMICAL COMPONENTS	50 ml recipe	
LIQUIDS: distilled water	50 ml	
DRY: Fructose-6-phosphate	0.274 g	
COMMENTS: Store in the refrigerator for approximately up to one month.		
CITATION:		

Glucose-1,6-diphosphate, 0.05% (G-1,6-DP)		
CHEMICAL COMPONENTS	50 ml recipe	
LIQUIDS: distilled water	50 ml	
DRY: Glucose-1,6-diphosphate	0.025 g	
COMMENTS: Store in the refrigerator.		
CITATION:		

Glucose-6-phosphate dehydrogenase solution (G-6-PDH solution), 50 units/ml		
CHEMICAL COMPONENTS	200 ml recipe	20 ml
LIQUIDS: distilled water	160 ml	16 ml
DRY: Glycine Bovine Albumin	0.74 g 0.2 g	0.074 g 0.02 g
STEP 1: Mix distilled water, glycine, and bovine albumin. When well mixed, raise pH to 8.0 with NaOH (using 1-2 drops).		
LIQUID: 10% MgCl ₂ 1N NaOH	2 ml as needed	0.2 ml as needed
DRY: NAD Glucose-6-phosphate dehydrogenase (Sigma #G-5760; not dry)	0.04 g 10,000 units	0.004 g 5,000 units
STEP 2: Add these these chemicals to the solution make in Step 1. When well mixed, check pH again, raise to pH 8.0 with NaOH (2-4 drops).		
STEP 3: Bring total volume to 200 ml. Place 2 ml or 5 ml amounts into cryovials. Store in the ultralow freezer.		
COMMENTS: Thaw only the amount you need to use that day; it does not work the next day. Do not overheat when thawing. Do not substitute Sigma #G-5760 formulation.		
CITATION: Weyerhaeuser Corporation.		

Gel Fixative (40% ethanol)		
CHEMICAL COMPONENTS		
LIQUIDS: distilled water 95% ethyl alcohol (reagent alcohol)	5 parts 4 parts	
COMMENTS: Store in airtight bottle.		
CITATION:		

Glyceraldehyde-3-phosphate dehydrogenase solution, 500 units/slice		
CHEMICAL COMPONENTS	1 ml recipe	0.2 ml recipe
LIQUIDS: distilled water	1 ml	0.2 ml
DRY: Glyceraldehyde-3-phosphate dehydrogenase (1 bottle of Sigma # G-9263)	5,000 units	1,000 units
COMMENTS: Refrigerate for up to one week, or freeze to increase shelf life.		
CITATION:		

Hexokinase Buffer		
CHEMICAL COMPONENTS	100 ml recipe	50 ml recipe
LIQUIDS: distilled water	100 ml	50 ml
DRY: Hexokinase (Sigma# H- 4502)	1,000 units	500 units
COMMENTS: Mix well. Store in the refrigerator for up to two weeks.		
CITATION:		

Hydrogen peroxide	(1%)	(3%)
CHEMICAL COMPONENTS	1000 ml recipe	1000 ml recipe
LIQUIDS: distilled water Hydrogen Peroxide, 30%	966.7 ml 33.3 ml	900 ml 100 ml
COMMENTS: Store in the refrigerator in an airtight container.		
CITATION:		

Ketoglutarate, 0.1M		
CHEMICAL COMPONENTS	100 ml recipe	
LIQUIDS: 0.2 M Phosphate Buffer, pH 7.5	100 ml	
DRY: Ketoglutaric Acid	1.461 g	
COMMENTS: Store in the refrigerator.		
CITATION:		

Magnesium chloride, 10% (MgCl₂)		
CHEMICAL COMPONENTS	500 ml recipe	
LIQUIDS: distilled water	500 ml	
DRY: Magnesium chloride	50 g	
CITATION:		

DL-Malic Acid, 0.5 M, pH 7.0			
CHEMICAL COMPONENTS	1000 ml recipe	500 ml recipe	250 ml recipe
LIQUIDS: distilled water 4N NaOH approximately.....	600 ml (250 ml)	300 ml (125 ml)	150 ml (62.5 ml)
DRY: dl-Malic Acid	67.05 g	33.52 g	16.76 g
DIRECTIONS: Mix water and malic acid. Adjust pH to 7.0 with NaOH. Bring recipe to total volume with distilled water. Store in the refrigerator.			
COMMENTS: Make fresh weekly. Does not work well if kept to the next week. The 250 ml volume is difficult to pH. When it reaches pH 6, start using very small amounts of NaOH. If pH exceeds 7, throw away and start over again.			
CITATION:			

MTT solution (5 mg/ml)		
CHEMICAL COMPONENTS	100 ml recipe	
LIQUIDS: distilled water	100 ml	
DRY: MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenoltetrazolium bromide)	0.5 g	
COMMENTS: **CAUTION** Use goggles and respirator while mixing. Mix and store in opaque bottle. Limit light exposure. Store in refrigerator.		
CITATION:		

NAD Combination Solution		
CHEMICAL COMPONENTS	25 ml recipe	50 ml recipe
LIQUIDS: distilled water	25 ml	50 ml
DRY: NAD (B-nicotinamide adenine dinucleotide) NBT (nitro blue tetrazolium) PMS (phenazine methosulfate)	.1 g .1 g .05 g	0.2 g 0.2 g 0.1 g
COMMENTS: Store in refrigerator in opaque bottle for up to two weeks.		
CITATION:		

NADP Combination Solution		
CHEMICAL COMPONENTS	25 ml recipe	50 ml recipe
LIQUIDS: distilled water	25 ml	50 ml
DRY: NADP (B-nicotinamide adenine dinucleotide phosphate) NBT (nitro blue tetrazolium) PMS (phenazine methosulfate)	.1 g .1 g .05 g	0.2 g 0.2 g 0.1 g
COMMENTS: Store in refrigerator in opaque bottle for up to two weeks.		
CITATION:		

Phosphate Buffer, 0.2 M, pH 7.5		
CHEMICAL COMPONENTS	1000 ml recipe	
LIQUIDS: distilled water	900 ml	
DRY: Sodium phosphate, monobasic Sodium phosphate, dibasic	3.56 g 23.86 g	
DIRECTIONS: Mix and bring volume up to 1000 ml.		
COMMENTS: This buffer is used as a extraction buffer for sample preparation, and for the GOT stain.		
CITATION:		

PMS Solution (3mg/ml)		
CHEMICAL COMPONENTS	100 ml recipe	
LIQUIDS: distilled water	100 ml	
DRY: PMS (phenazine methosulfate)	0.3 g	
COMMENTS: **CAUTION** Use goggles and respirator while preparing. Store in opaque bottle in the refrigerator.		
CITATION:		

Potassium Chloride, 1M (KCl)		
CHEMICAL COMPONENTS	500 ml recipe	
LIQUIDS: distilled water	450 ml	
DRY: Potassium chloride	37.27 g	
DIRECTIONS: Bring to total volume of 500 ml with distilled water.		
CITATION:		

Potassium Phosphate Buffer, 1M		
CHEMICAL COMPONENTS	1000 ml recipe	
LIQUIDS: distilled water	1000 ml	
DRY: Potassium phosphate, monobasic, anhydrous	136 g	
DIRECTIONS: Mix well. Store in refrigerator.		
CITATION:		

Potassium Phosphate Buffer, 0.16M		
CHEMICAL COMPONENTS	1000 ml recipe	
LIQUIDS: 1M potassium phosphate buffer (monobasic) 1N NaOH	100 ml 59 ml	
DIRECTIONS: Mix liquids together and bring to final volume of 1000 ml with distilled water.		
COMMENTS: Store in the refrigerator.		

Pyridoxal-5'-phosphate, 0.5%		
CHEMICAL COMPONENTS	50 ml recipe	
LIQUIDS: distilled water	50 ml	
DRY: Pyridoxal-5'-phosphate	0.25 g	
COMMENTS: Store in the refrigerator.		
CITATION:		

RBC solution		
CHEMICAL COMPONENTS	500 ml recipe	
LIQUIDS: distilled water methanol (can be the cheapest grade, used to clean floors) glacial acetic acid	250 ml 250 ml 50 ml	
COMMENT ON RBC SOLUTION: Store in the refrigerator for several weeks.		
CITATION:		

Sodium Acetate Buffer, 0.07M, pH 4.0		
CHEMICAL COMPONENTS	500 ml recipe	
LIQUIDS: distilled water Acetic Acid	450 ml as needed	
DRY: sodium acetate	4.763 g	
DIRECTIONS: Mix water and sodium acetate. Adjust pH to 4.0 with acetic acid (approximately 1 ml). Bring to a total volume of 500 ml with distilled water.		
CITATION:		

Sodium Hydroxide (NaOH)		
CHEMICAL COMPONENTS	500 ml recipe	500 ml recipe
LIQUIDS: distilled water	1 N = 1 M	4 N
DRY: Sodium hydroxide (NaOH) pellets	400 ml	350 ml
	20 g	80 g
COMMENTS: Bring to final volume after the dry NaOH is dissolved.		
CITATION:		

Tris-HCl	0.2 M, pH 7.0	0.5 M, pH 8.0	1 M, pH 8.0 or pH 7.5
CHEMICAL COMPONENTS	500 ml recipe	500 ml recipe	1000 ml recipe
LIQUIDS: distilled water concentrated hydrochloric acid (HCl)	450 ml as needed	450 ml as needed	700 ml as needed
DRY: Tris (Sigma 7-9)	12.11 g	30.38 g	121.1 g
DIRECTIONS: Mix water and Tris. Bring to appropriate pH using concentrated hydrochloric acid. Bring to final volume by adding distilled water.			
CITATION:			

Tris-Maleate Buffer, 0.2 M, pH 3.8		
CHEMICAL COMPONENTS	1000 ml recipe	
LIQUIDS: distilled water 4N NaOH	900 ml as needed	
DRY: Maleic Acid Tris (Sigma 7-9)	23.2 g 24.2 g	
DIRECTIONS: Adjust pH if needed with 4N NaOH. Add distilled water to a total volume of 1000 ml. Store in the refrigerator.		
COMMENTS: Maleic acid is NOT Malic acid!		
CITATION:		

APPENDIX E: ENZYME STAINS

Appendix E1: Enzyme Stain Recipes

Appendix E2: Stain/Buffer Combinations

- a. Pines**
- b. Other Conifers**
- c. Woody Angiosperms**
- d. Herbaceous Plants**

Appendix E1: Enzyme Stain Recipes

STAIN: Aspartate Aminotransferase (AAT)	Amount/Number of Slices			
CHEMICAL COMPONENTS	1	2	3	4
BUFFER: AAT Solution	50 ml	100 ml	150 ml	200 ml
DRY: Fast Blue BB Salt	0.05 g	0.1 g	0.15 g	0.2 g
DIRECTIONS: Incubate at room temperature in the dark.				
COMMENTS: Fast Garnet GBC salt can replace the Fast Blue BB salt, but gives a pattern or dark orange on an orange background. This enzyme is also known as Glutamic Oxaloacetate Transaminase GOT; see alternate recipe under than heading. Stain the backslice.				
CITATION: Wendel and Weeden 1989.				

STAIN: Aconitase (ACO)	Amount/Number of Slices	
CHEMICAL COMPONENTS	1	2
BUFFER: (warm) 1 M Tris-HCl, pH 8.0 10% MgCl ₂ distilled H ₂ O to . . .	5 ml 0.25 ml 25 ml	10 ml 0.5 ml 50 ml
DURING STAINING: NADP combination solution 5% cis-Aconitic Acid solution Isocitrate dehydrogenase (IDH) (Sigma #I-2002; 20 units/slice) 3% Bovine Albumin solution	1.25 ml 1 ml 260 ul 1 ml	2.5 ml 2 ml 520 ul 2 ml
DIRECTIONS: Incubate of 37° C for a while.		
COMMENTS: If nothing appears on gel within 20 minutes, add more IDH. After 20 minutes, don't bother. This recipe is preferred. If lot number I-2002 of IDH is not available, substitute *I-2516 at 0.003 g/slice and leave in incubator overnight (no heat). This recipe is not for grasses. Concentration of IDH varies by lot number. Adjust volume needed accordingly.		
CITATION: Conkle et al. 1982, who reference Yeh and O'Malley 1980. Modified.		

STAIN: Aconitase (ACO) for grasses	Amount/Number of Slices	
CHEMICAL COMPONENTS	2	
BUFFER: (warm) 1 M Tris-HCl, pH 8.0 10% MgCl ₂ distilled H ₂ O to . . .	15 ml 0.1 ml 70 ml	
DRY: NBT NADP	0.01 g 0.01 g	
DURING STAINING: PMS solution Isocitrate dehydrogenase (IDH; 20 units/slice) 5% cis-Aconitic Acid	0.8 ml 1.4 ml 3 ml	
DIRECTIONS: Incubate at 37° C		
COMMENTS: If nothing appears on gel within 20 minutes, add more IDH. After 20 minutes, don't bother. This recipe for <i>Elymus glaucus</i> (grass).		
CITATION: Restoration Ecology paper by Knapp and Rice		

STAIN: Alcohol Dehydrogenase (ADH)	Amount/Number of Slices	
CHEMICAL COMPONENTS	1	2
BUFFER: 1 M Tris-HCl, pH 8.0 distilled H ₂ O to	2.5 ml 25 ml	5 ml 50 ml
DURING STAINING: Grain alcohol (76%) OR Grain alcohol (95%) NAD combination solution	0.9 ml 0.7 ml 1.25 ml	1.8 ml 1.3 ml 2.5 ml
DIRECTIONS: Incubate at 37°C in covered container.		
COMMENTS: (1) You can use 95% ethanol (0.5 ml for one slice or 1 ml for two). However, the other 5% may include toxic chemicals that inhibit ADH function. You may substitute vodka or tequila for 95% ethanol. Adjust the amounts of alcohol and dH ₂ O to provide the correct amount of ethanol. Remember that the "proof" = twice the percent alcohol. (2) ADH is an inducible enzyme, and tissues that have been growing in different environments may differ in ADH activity.		
CITATION: Conkle et al. 1982, who reference Scandalious 1969. Modified.		

STAIN: Cinnamyl Alcohol Dehydrogenase (CADH)	Amount/Number of Slices	
CHEMICAL COMPONENTS	1	2
BUFFER: (warm) 1 M Tris-HCl, pH 8.0 10% MgCl ₂		45 ml 0.2 ml
DRY: NADP		0.01 g
DURING STAINING: Cinnamyl Alcohol Acetone * (add to Cinnamyl Alcohol to dissolve) MTT PMS		0.02 g 5 ml 2 ml 0.8 ml
DIRECTIONS: Incubate at 37°C in covered container.		
COMMENTS: (1) * Dissolve Cinnamyl Alcohol in Acetone. Mix buffer and dry. (3) Add Cinnamyl Alcohol/Acetone, MTT, and PMS to the combined buffer/dry flask.		
CITATION: Tropical. Modified.		

STAIN: Catalase (CAT)	Amount/Number of Slices	
CHEMICAL COMPONENTS	1	2
STEP 1: (cold) Catalase buffer (cold), pH 6.5 Cover the slice and soak for 30 minutes in the refrigerator. Slice must be well submerged. Drain thoroughly.	sufficient to submerge the slice	sufficient to submerge the slices
STEP 2: (room temperature) 2% Potassium iodide (KI) solution, made fresh just before staining. Soak slices for 2 minutes at room temperature, then rinse in distilled water	1 g KI in 50 ml H ₂ O	2 g KI in 100 ml dH ₂ O
STEP 3: (room temperature) 0.03% Hydrogen peroxide (H ₂ O ₂) solution, made fresh weekly. Soak gel in this solution at room temperature until it develops, agitating by hand or mechanically. Do not overstain; about ten minutes is long enough. Rinse in distilled water.	1.5 ml 1% H ₂ O ₂ in 50 ml H ₂ O	3 ml 1% H ₂ O ₂ in 100 ml H ₂ O
COMMENTS: (1) Gels may become very dark. You may not be able to stop the over staining, so read or photograph as soon as you can. (2) Wrap gels in plastic wrap for storage. (3) These slices show fingerprints easily, even with gloves. Handle gels as carefully and as little as possible. (4) Do not use fixer on CAT slices.		
CITATION: Conkle et al. 1982, who reference Scandalious 1969.		

STAIN: Diaphorase (DIA)	Amount/Number of Slices		
CHEMICAL COMPONENTS	1 or 2	3	4
BUFFER: (warm) 1 M Tris-HCl, pH 8.0 distilled H ₂ O to . . .	10 ml 50 ml	15 ml 75 ml	20 ml 100 ml
DRY: Bacto-agar 2,6-dichlorophenol-indophenol b-NADH	0.5 g 0.002 g 0.030 g	0.75 g 0.003 g 0.045 g	1 g 0.0045 g 0.06 g
DURING STAINING: MTT 2,6 – Dichlorophenol-indophenol	2 ml 0.75 ml	3 ml 1.1ml	4 ml 1.5 ml
DIRECTIONS: STEP 1 (agar solution): Dissolve Bacto-agar in about $\frac{3}{4}$ (about 38 ml) of the buffer solution. Dissolve agar by heating with microwave or with hot plate. Cool agar to 45° C			
DIRECTIONS: STEP 2 (other chemicals): Combine dry chemicals with remaining buffer (about 12 ml). Dissolve well. Then add the MTT solution.			
DIRECTIONS: Step 3: Combine the two solutions and pour over gel slices. Incubate at 37°C.			
COMMENTS: This stain may identify the same enzyme as MNR. Do not use fixer.			
CITATION: Adams, with NADH modified.			

STAIN: Esterase (EST)	Amount/Number of Slices	
CHEMICAL COMPONENTS	1	2
BUFFER: Esterase buffer	25 ml	50 ml
DRY: alpha-Naphthyl acetate beta-Naphthyl acetate	0.025 g 0.025 g	0.05 g 0.05 g
DURING STAINING: acetone Fast Blue RR Salt	2.5 ml 0.05 g	5 ml 0.1 g
DIRECTIONS: 1. Dissolve dry chemicals in acetone. 2. Add the buffer. 3. Add the Fast Blue RR Salt.		
COMMENTS: Can use Fast Garnet GBC Salt.		
CITATION: Wendel and Weeden 1989. Adams.		

STAIN: Fluorescent Esterase (FEST)	Amount/Number of Slices	
CHEMICAL COMPONENTS	1	2
DRY: 4-Methylumbelliferyl acetate (leave in freezer before measuring, and replace immediately)	0.001 g*	0.001 g*
DURING STAINING: acetone 0.2 M Tris-HCl pH 7.0 or 8.0 (dissolve dry chemical in acetone, then add Tris-HCl)	3 ml 10 ml	3 ml 10 ml
DIRECTIONS: Dissolve frozen 4-Methylumbelliferyl acetate in acetone before adding Tris-HCl. *Can't accurately measure 0.001 g. Use a tiny amount on the tip of a spatula. Place slice on glass plate. Using a pipette, cover slices uniformly with liquid; use a generous amount. Cover with unwrinkled plastic wrap. Read under UV light.		
COMMENTS: When using LB buffer, read at approximately 10 minutes. With MC buffer, read at 20 minutes. If doing FEST-8, use 2 ml 1M Tris-HCl, pH 8, and 8 ml of dH ₂ O.		
CITATION: Wendel and Weeden 1989. Modified.		

STAIN: Fructose-1,6-diphosphatase (FDP)	Amount/Number of Slices			
CHEMICAL COMPONENTS	1	2	3	4
BUFFER: (warm) 1 M Tris-HCl, pH 8.0 10% MgCl ₂ solution distilled H ₂ O to . . .	5 ml 0.1 ml 25 ml	10 ml 0.2 ml 50 ml	15 ml 0.3 ml 75 ml	20 ml 0.4 ml 100 ml
DRY: Fructose-1,6-diphosphate Phosphoglucose isomerase	0.1 g 0.01 g	0.2 g 0.02 g	0.3 g 0.03 g	0.4 g 0.04 g
DURING STAINING: distilled H ₂ O, added to above dry ingredients: After dissolving, add: 3% Bovine Albumin solution NADP combination solution G6PDH (frozen, 50 units/ml)	5 ml 0.5 ml 2.5 ml 0.4 ml	10 ml 1 ml 5 ml 0.8 ml	15 ml 1.5 ml 7.5 ml 1.2 ml	20 ml 2 ml 10 ml 1.6 ml
DIRECTIONS: During staining, dissolve dry chemicals in distilled water. Then add the other "during staining" chemicals. Incubate at 37°C.				
COMMENTS:				
CITATION: Wendel and Weeden 1989. Modified! They use NAD and an NAD-dependent G6PDH, and they omit Bovine Albumin.				

STAIN: Fumerase (FUM)	Amount/Number of Slices	
CHEMICAL COMPONENTS	1	2
BUFFER: 0.2 M Tris HCl, pH 8.0	25 ml	50 ml
DRY: Fumaric Acid	0.3 g	0.6 g
DURING STAINING: MDH enzyme (malic dehydrogenase) NAD combination solution	2 drops = 80 μ l 2.5 ml	4 drops = 160 μ l 5.0 ml
DIRECTIONS: Incubate at 37°C.		
COMMENTS:		
CITATION:		

STAIN: Glucose-6-phosphate dehydrogenase (G6PD)	Amount/Number of Slices			
CHEMICAL COMPONENTS	1	2	3	4
BUFFER: (warm) 1 M Tris-HCl, pH 8.0 10% MgCl ₂ distilled H ₂ O to . . .	5 ml 0.25 ml 25 ml	10 ml 0.5 ml 50 ml	15 ml 0.75 ml 75 ml	20 ml 1.0 ml 100 ml
DRY: NADP Glucose-6-phosphate, disodium salt	0.004 g 0.02 g	0.007 g 0.04 g	0.01 g 0.06 g	0.014 g 0.08 g
DURING STAINING: MTT solution PMS solution	0.75 ml 0.5 ml	1.5 ml 1 ml	2.25 ml 1.5 ml	3 ml 2 ml
DIRECTIONS: Incubate at 37°C.				
COMMENTS: The addition of Bovine Albumin solution at 30 mg/slice can be used to improve resolution in some species. Douglas-fir benefits from albumin.				
CITATION: Conkle et al. 1982, who reference Shaw and Prasad 1970. Modified.				

STAIN: Glutamic Acid Dehydrogenase (GDH)	Amount/Number of Slices	
CHEMICAL COMPONENTS	1 or 2	3 or 4
BUFFER: (warm) 1 M Tris-HCl, pH 8.0 distilled H ₂ O to . . .	5.6 ml 50 ml	11.2 ml 100 ml
DRY: l-Glutamic Acid (mono Na salt)	2 g	4 g
DURING STAINING: NAD combination solution	2.5 ml	5 ml
DIRECTIONS: Incubate at 37°C.		
COMMENTS:		
CITATION: Conkle et al. 1982, who reference Shaw and Koehn 1968.		

STAIN: Glutamate oxaloacetate transaminase (GOT)	Amount/Number of Slices			
CHEMICAL COMPONENTS	1	2	3	4
BUFFER: 0.2 M Phosphate Buffer, pH 7.5 0.2 M L-aspartic Acid	20 ml 4.6 ml	40 ml 9.1 ml	60 ml 13.6 ml	80 ml 18.2 ml
DRY: Fast Blue BB Salt	0.08 g	0.1 g	0.24 g	0.2 g
DURING STAINING: distilled H ₂ O 0.5% Pyridoxal-5-phosphate solution 3% Bovine Albumin solution 0.1 M Ketoglutaric Acid solution	6 ml 0.6 ml 1.1 ml 1.4 ml	11 ml 1.1 ml 2.1 ml 2.7 ml	16 ml 1.6 ml 3.2 ml 4 ml	21 ml 2.1 ml 4.2 ml 5.4 ml
DIRECTIONS: 1. Mix buffers and “during staining” chemicals. 2. Mix Fast Blue BB Salt in the distilled H ₂ O. 3. Mix the two solutions together. 4. Incubate at room temperature in the dark.				
COMMENTS: Fast Garnet GBC salt can replace the Fast Blue BB salt, but gives a pattern or dark orange on an orange background.				
CITATION: Conkle et al. 1982, who reference Brewbaker et al. 1968. Modified.				

STAIN: Glycerate-2-Dehydrogenase (GLYDH)	Amount/Number of Slices			
CHEMICAL COMPONENTS	1	2	3	4
BUFFER: (warm) 1 M Tris-HCl, pH 8.0 1 N NaOH dH ₂ O to . . .	2.5 ml 0.7 ml 25 ml	5 ml 1.4 ml 50 ml	7.5 ml 2 ml 75 ml	10 ml 2.7 ml 100 ml
DRY: DL-Glyceric Acid, hemi-CA Salt NAD	0.2 g 0.02 g	0.4 g 0.04 g	0.6 g 0.06 g	0.8 g 0.08 g
DURING STAINING: MTT solution PMS solution	1 ml 0.75 ml	2 ml 1.5 ml	3 ml 2.25 ml	4 ml 3 ml
COMMENTS: Incubate at 37°C in dark.				
CITATION:				

STAIN: Isocitrate dehydrogenase (IDH)	Amount/Number of Slices			
CHEMICAL COMPONENTS	1	2	3	4
BUFFER: (warm) 1 M Tris-HCl, pH 8.0 10% MgCl ₂ distilled H ₂ O to . . .	2.5 ml 0.2 ml 25 ml	5 ml 0.3 ml 50 ml	7.5 ml 0.4 ml 75 ml	10 ml 0.5 ml 100 ml
DRY: Isocitric acid NADP	0.005 g 0.003 g	0.01 g 0.005 g	0.015 g 0.008 g	0.02 g 0.01 g
DURING STAINING: MTT solution PMS solution	0.75 ml 0.5 ml	1.5 ml 0.75 ml	2.25 ml 1 ml	3 ml 1.25 ml
DIRECTIONS: Incubate at 37°C.				
COMMENTS:				
CITATION: Conkle et al. 1982, who reference Nichols and Ruddle 1973. Modified.				

STAIN: Leucine Aminopeptidase (LAP)	Amount/Number of Slices			
CHEMICAL COMPONENTS	1	2	3	4
BUFFER: (warm) 0.2 M Tris-Maleate Buffer, pH 3.8 1 N NaOH distilled H ₂ O to . . .	17 ml 1.7 ml 25 ml	33 ml 3.3 ml 50 ml	50 ml 5 ml 75 ml	65 ml 6.5 ml 100 ml
DRY: **1-leucine B-naphtylamide** Fast Black K Salt	0.007 g 0.007 g	0.013 g 0.013 g	0.02 g 0.02 g	0.035 g 0.025 g
DURING STAINING: distilled H ₂ O	8 ml	17 ml	25 ml	33 ml
DIRECTIONS: Mix water with dry chemicals before adding buffer ingredients. Incubate at 37°C.				
COMMENTS: ** Carcinogen**				
CITATION: Conkle et al. 1982, who reference Scandalious 1969. Modified.				

STAIN: Malate Dehydrogenase (MDH)	Amount/Number of Slices			
CHEMICAL COMPONENTS	1	2	3	4
BUFFER: (warm) 1 M Tris-HCl, pH 8.0 0.5 M DL-Malic Acid, pH 7.0 distilled H ₂ O to . . .	2.5 ml 12.5 ml 25 ml	5 ml 25 ml 50 ml	7.5 ml 37.5 ml 75. ml	10 ml 50 ml 100 ml
DURING STAINING: NAD combination solution	1.25 ml	2.5 ml	3.75 ml	5 m.
DIRECTIONS: Incubate at 37°C.				
COMMENTS:				
CITATION: Conkle et al. 1982, who reference Nichols and Ruddle 1973. Modified.				

STAIN: Malic Enzyme (ME)	Amount/Number of Slices			
CHEMICAL COMPONENTS	1	2	3	4
BUFFER: (warm) Morpholine-Citrate buffer, pH 6.1 0.5 M DL-Malic Acid, pH 7.0 10% MgCl ₂	25 ml 20 ml 1 ml	50 ml 40 ml 2 ml	75 ml 60 ml 3 ml	100 ml 80 ml 4 ml
DRY: NADP	0.01 g	0.02 g	0.03 g	0.04 g
DURING STAINING: MTT solution PMS solution	1 ml 1 ml	2 ml 2 ml	3 ml 3 ml	4 ml 4 ml
DIRECTIONS: Incubate at 37°C in the dark. .				
COMMENTS: Stains slowly (fuzzy). May get IDH staining. Fresh DL-Malic acid very important. Turns out better on Loblolly Pine than Douglas-Fir. For a few species (such as <i>Taxus</i>), ME activity is revealed more clearly if the malic acid solution is brought to pH 8.0.				
CITATION:				

STAIN: 6-Phosphogluconate Dehydrogenase (6PGD)	Amount/Number of Slices			
CHEMICAL COMPONENTS	1	2	3	4
BUFFER: 1 M Tris-HCl, pH 8.0 10% MgCl ₂ distilled H ₂ O to . . .	2.5 ml 0.2 ml 25 ml	5 ml 0.3 ml 50 ml	7.5 ml 0.4 ml 75 ml	10 ml 0.5 ml 100 ml
DRY: 6-Phosphogluconic acid, trisodium NADP	0.006 g 0.003 g	0.012 g 0.005 g	0.018 g 0.008 g	0.024 g 0.010 g
DURING STAINING: MTT solution PMS solution	0.75 ml 0.5 ml	1.5 ml 0.75 ml	2.25 ml 1 ml	3 ml 1.25 ml
DIRECTIONS: Incubate at 37°C.				
COMMENTS:				
CITATION: Wendel and Weeden 1989. Modified.				

STAIN: Phosphoglucose Isomerase (PGI)	Amount/Number of Slices			
CHEMICAL COMPONENTS	1	2	3	4
BUFFER: 1 M Tris-HCl, pH 8.0 10% MgCl ₂ distilled H ₂ O to . . .	2.5 ml 0.25 ml 25 ml	5 ml 0.5 ml 50 ml	7.5 ml 0.75 ml 75 ml	10 ml 1 ml 100 ml
DRY: NADP	0.0035 g	0.007 g	0.01 g	0.014 g
DURING STAINING: MTT solution PMS solution Glucose-6-phosphate dehydrogenase (frozen, 50 units/ml) Fructose-6-phosphate solution	0.75 ml 0.25 ml 0.25 ml 1.75 ml	1.5 ml 0.5 ml 0.5 ml 3.25 ml	2.25 ml 0.75 ml 0.75 ml 5 ml	3 ml 1 ml 1 ml 6.5 ml
DIRECTIONS: Incubate at 37°C.				
COMMENTS: Sometimes the backslice is stained (Collomia).				
CITATION: Conkle et al. 1982, who reference Brewer 1970.				

STAIN: Phosphoglucomutase (PGM)	Amount/Number of Slices			
CHEMICAL COMPONENTS	1	2	3	4
BUFFER: 1 M Tris-HCl, pH 8.0 10% MgCl ₂ distilled H ₂ O to . . .	2.5 ml 0.5 ml 25 ml	5 ml 1 ml 50 ml	7.5 ml 1.5 ml 75 ml	10 ml 2 ml 100 ml
DRY: Glucose-1-phosphate NADP	0.025 g 0.005 g	0.05 g 0.01 g	0.075 g 0.015 g	0.1 g 0.02 g
DURING STAINING: MTT solution PMS solution G-6-PDH (frozen, 50 units/ml) Glucose-1,6-diphosphate solution	1.25 ml 1 ml 0.5 ml 1 ml	2.5 ml 2 ml 1 ml 2 ml	3.75 ml 2.75 ml 1.5 ml 2.75 ml	5 ml 3.5 ml 2 ml 3.5 ml
DIRECTIONS: Incubate at 37°C.				
COMMENTS:				
CITATION: Wendel and Weeden 1989. Modified; they recommend NAD and NAD-dependent G6PDH.				

STAIN: Shikimic Acid Dehydrogenase (SKD)	Amount/Number of Slices		
CHEMICAL COMPONENTS	1 or 2	3	4
BUFFER: (warm) 1 M Tris-HCl, pH 8.0 10% MgCl ₂ distilled H ₂ O to . . .	10 ml 0.5 ml 50 ml	15 ml 0.75 ml 75 ml	20 ml 1 ml 100 ml
DRY: Shikimic acid NADP	0.2 g 0.01 g	0.3 g 0.015 g	0.4 g 0.02 g
DURING STAINING: MTT solution PMS solution	2 ml 1 ml	3 ml 1.5 ml	4 ml 2 ml
DIRECTIONS: Incubate at 37°C.			
COMMENTS:			
CITATION:			

STAIN: Triosephosphate Isomerase (TPI)	Amount/Number of Slices			
CHEMICAL COMPONENTS	1	2	3	4
BUFFER: 1 M Tris-HCl, pH 8.0 distilled H ₂ O to . . .	5 ml 25 ml	10 ml 50 ml	15 ml 75 ml	20 ml 100 ml
DRY: NAD EDTA Arsenic acid	0.01 g 0.01 g 0.15 g	0.015 g 0.015 g 0.22 g	0.025 g 0.025 g 0.37 g	0.03 g 0.03 g 0.44 g
DURING STAINING: Glyceraldehyde-3-phosphate dehydrogenase (5,000 units/ml for 500 units/slice) 1% dihydroxyacetone phosphate MTT solution PMS solution	100 µl 50 µl 1 ml 1.5 ml	200 µl 100 µl 2 ml 3 ml	300 µl 150 µl 3 ml 4.5 ml	400 µl 200 µl 4 ml 6 ml
DIRECTIONS: Incubate at 37°C.				
COMMENTS: DISPOSE OF STAIN IN SEPERATE HAZARDOUS WASTE CONTAINER. This recipe only works with the dry version of G-3-PDH, Sigma #G9263. Use 500 units/slice of Glyceraldehyde-3-phosphate dehydrogenase; check concentration of stock solution.				
CITATION:				

STAIN: Uridine diphosphoglucose pyrophosphorylase (UGPP)	Amount/Number of Slices			
	1	2	3	4
CHEMICAL COMPONENTS				
BUFFER: (warm) 1 M Tris-HCl, pH 8.0 10% MgCl ₂ distilled H ₂ O to . . .	1.5 ml 0.1 ml 30 ml	3 ml 0.2 ml 60 ml	4.5 ml 0.3 ml 90 ml	6 ml 0.4 ml 120 ml
DRY: uridine-5'-diphosphoglucose disodium pyrophosphate NAD	0.025 0.02 g 0.01 g	0.05 0.04 g 0.02 g	0.075 0.06 g 0.03 g	0.1 0.08 g 0.04 g
DURING STAINING: MTT solution PMS solution Glucose-1,6-diphosphate solution G-6-PDH (frozen, 50 units/ml) Phosphoglucomutase (1.67 units/slice)	1 ml 1 ml 1 ml 1 ml 75 µl	2 ml 2 ml 2 ml 2 ml 150 µl	3 ml 3 ml 3 ml 3 ml 225 µl	4 ml 4 ml 4 ml 4 ml 300 µl
DIRECTIONS: Incubate at 37°C.				
COMMENTS: Do not use tetrasodium pyrophosphate. Accurate measurement of disodium pyrophosphate effects results.				
CITATION: Weyerhaeuser recipe standardized to NFGEL procedures.				

Appendix E2. Stain/Buffer Combinations Used for Different Species.

Table 1. Pines

In the list under each buffer, stains are in order by preferred slice from the gel; with the bottom slice of gel first on the list. LB = a lithium borate electrode buffer (pH 8.3) used with a Tris citrate gel buffer (pH 8.3). SB = a sodium borate electrode buffer (pH 8.0) used with a Tris citrate gel buffer (pH 8.8). MC6 = a morpholine citrate electrode and gel buffer (pH 6.1). MC8 = a morpholine citrate electrode and gel buffer (pH 8.0). See Appendix C for buffer recipes and Appendix E1 for stain recipes. A normal gel slice is six cm wide; a wide slice is eight cm wide. A backslice is one from the portion of the gel on the cathode side of the line where wicks were inserted. ME7 stain recipe uses a malic acid solution brought to pH 7.0; for ME8, the solution is brought to pH 8.0.

Species	LB	SB	MC6	MC8	Comments, and additional buffers
Caribbean Pine; <i>Pinus caribaea</i> ; buds	PGM1,2; ACO; SKD; LAP1,2; DIA	CAT1,2; UGPP; TPI1,2; GOT1,2,3; G6PD		FEST; PGI1,2; MDH1,2,4; IDH; 6PGD1,2	Cut FEST and the SB stains wide. Score on day of run: SKD1; PGM2; ACO; TPI1; 6PGD1; and IDH. Use backslice on GOT. Can leave ACO in incubator overnight; may darken.
Jack Pine; <i>Pinus banksiana</i> , meg/embryos pairs	b-GAL; FEST; LAP1,2; ME7; PGM; ACO	GDH; GOT2,3; SOD; UGPP1,2	FDP1,2,3; MDH2,4; IDH; 6PGD1,2	AK1; HK1; PGI1,2; MDH2,4; IDH	Read SOD on GDH (SB), SOD shows in megs only. On MC8, HK and AK may be the same. Stains listed here are in the correct buffer system from their order on the map, but unable to verify actual stain order used. Test first.
Jeffrey Pine; <i>Pinus jefferyi</i> ; buds	PGM1,2; ACO; ME7; PGI1,2; ADH; LAP1,2; FEST	CAT; UGPP; G6PD; GOT 1,2,3; SOD	MDH1,2,3; SKD1; IDH1; 6PGD; DIA1,2; FEST		Notes for Ponderosa Pine buds also apply for Jeffrey Pine. Jeffrey and Ponderosa Pine can be distinguished by six stains: ADH, 6PGD, PGI2, PGM2, SKD, and UGPP1.
Limber Pine; <i>Pinus flexilis</i> ; buds;	PGM1,2; PGI2; ACO1; ADH1; LAP1,2; FEST2	CAT1,2; GOT1,2,3; UGPP1; TPI1	IDH1; MDH1,2,3; FDP1; 6PGD1,2; DIA1		Cut UGPP, TPI and FEST wide. Read FEST after 3-5 minutes. Read PGI and 6PGD early to avoid background. Use Wendel and Weeden recipe for GOT.
Loblolly Pine; <i>Pinus taeda</i> ; buds	PGM1,2; ACO; SKD; LAP1,2; DIA	CAT1,2; UGPP; TPI1,2; GOT1,2,3; G6PD		FEST; PGI1,2; MDH1,2,4; IDH; 6PGD1,2	Cut FEST and the SB stains wide. Score on day of run: SKD1; PGM2; ACO; TPI1; 6PGD1; and IDH. Use Backslide on GOT. Can leave ACO in incubator overnight; may darken.
Loblolly Pine; <i>Pinus taeda</i> ; megagametophytes	PGM1; LAP1,2; ACO; ME7; FEST2; PGI1,2	GOT2,3; 6PGD1; UGPP1	SKD1,2; MDH1,2; FEST2; 6PGD1,2	SKD1,2; MDH2,4; IDH1; 6PGD1,2	Use backslice for GOT. Read FEST as soon as you stain. Cut FEST and all SB stains wide.
Loblolly Pine; <i>Pinus taeda</i> ; embryos	PGM1; LAP1,2; ACO; ME7; FEST2; PGI1,2	GOT3; UGPP1; 6PGD1	SKD1,2; MDH1,2; 6PGD1,2	SKD1,2; MDH2,4; IDH1; 6PGD1,2	Use backslice for GOT. Read FEST as soon as you stain. Cut FEST and all SB stains wide.
Loblolly Pine; <i>Pinus taeda</i> ; meg/embryo pairs	ACO1; LAP1,2; PGM1,2; FEST1,2; PGI1,2; ME7	GOT1,3; 6PGD1; UGPP1; TPI1,2; GDH1	MDH1,2; SKD1,2; DIA1; 6PGD2; IDH1		Read FEST after 8 minutes. Cut FEST and all SB stains wide. Use backslice for GOT.

Species	LB	SB	MC6	MC8	Comments, and additional buffers
Longleaf Pine; <i>Pinus palustris</i> ; buds	PGM1,2; ACO; SKD; LAP1,2; DIA	CAT1,2; UGPP; TPI1,2; GOT1,2,3; G6PD		FEST; PGI1,2; MDH1,2,4; IDH; 6PGD1,2	Cut FEST and the SB stains wide. Score on day of run: SKD1; PGM2; ACO; TPI1; 6PGD1; and IDH. Use Backslide on GOT. Can leave ACO in incubator overnight; may darken.
Longleaf Pine; <i>Pinus palustris</i> ; megagametophytes	PGM1,2; LAP1,2; ACO; ME; FEST1; PGI2; ADH	GOT2,3; 6PGD1; G6PDH1,2; GLYDH; TPI2		MDH1,2,3,4; 6PGD2; IDH1; AK1,2	Read FEST immediately. Cut LAP wide, and also TPI if you want to score TPI1. No backslide needed for GOT. Stains tested and dropped: PGI1 & TPI1
Monterey Pine, <i>Pinus radiata</i> ; megs	ADH; PGM1,2; PGI1,2; ME7; LAP1,2	UGPP1,2; TPI1,2; CAT; AAT1,3; G6PD2	SKD; IDH1,2; FEST2; MDH1,2,3; DIA1; FDP; 6PGD1,2		LB: ADH can be left in incubator overnight and may darken up. PGI and LAP must be photographed right away, as bands get blurry.
<i>Pinus masoniana</i>	PGM1,2; ACO; SKD; LAP1,2; DIA	CAT1,2; TPI1,2; GOT1,2,3; G6PD		FEST; PGI1,2; MDH1,2,4; IDH; 6PGD1,2	Use backslide on GOT. Score day of run: ACO, IDH, SKD1, 6PGD1, PGM2, TPI1, and UGPP1
Ponderosa Pine; <i>Pinus ponderosa</i> ; buds	PGM1,2; ACO; ME7; PGI1,2; ADH; LAP1,2; FEST	CAT; UGPP; G6PD; GOT 1,2,3; SOD	MDH1,2,3; SKD1; IDH1; 6PGD; DIA; FEST	MDH; IDH; DIA2; FDP	Score SKD1,2; CAT; DIA1,2; and PGM2 fresh. Include albumin in ACO and G6PD stains. Photograph CAT. Use backslide for GOT. Cut UGPP and FEST wide. FDP may be added only on MC8 (use a later slice number, i.e. top of the gel). MDH1, IDH2, and DIA4 may also be run on MC8. DIA and CAT alleles cannot be compared between tissues because they migrate differently in bud and megagametophyte tissues. Read FEST after 10 minutes; read as combination between LB and MC6 buffers.
Ponderosa Pine; <i>Pinus ponderosa</i> ; megagametophytes	PGI1,2; PGM1,2; LAP1,2; ME7; ACO; FEST2; ADH	GOT1,2,3; 6PGD1,2; UGPP1,2; CAT1,2; G6PD	DIA1; SKD1; MDH1,2,3; FEST2	IDH1,2; DIA2; SKD2; FDP2; 6PGD1,2	Cut FEST and UGPP wide. Score SKD1,2, CAT, DIA fresh. Read FEST2 after 20 minutes; read as combination between LB and MC6. Use backslide for GOT. Include albumin in ACO and G6PD stains.
Sand Pine; <i>Pinus clausa</i> ; megagametophytes	PGM1,2; LAP1,2; FEST1; ACO1; ME7; PGI1,2; ADH	GOT2,3; UGPP1,2; 6PGD1; G6PD1,2; PGI3; CAT1	SKD1,2; MDH1,2; ACP1,2; DIA1; 6PGD2	IDH1; MDH3,4; 6PGD2; FDP1,2; AK1,2	Cut UGPP and G6PD wide. Read 6PGD2 off MC6; use MC8 as a check. Use backslide for GOT. Can read UGPP3 on SB (lies between UGPP1 and UGPP2 in distance on that system. Read FEST immediately.

Species	LB	SB	MC6	MC8	Comments, and additional buffers
Shortleaf Pine; <i>Pinus echinata</i> ; meg/embryo pairs	PGM1,2; ACO1; PGI2; ME7; ADH; LAP1,2; FEST2	6PGD1; TPI1,2; UGPP1,2; G6PD2; GOT2,3; GLYDH	MDH1,2; FDP2; DIA2	MDH4; 6PGD2; IDH1	Read FEST, GLYDH, FDP, and DIA immediately. Cut UGPP, TPI, and FEST wide. Use backslice of GOT. UGPP1 overstains quickly; read quickly and continue staining for UGPP2 (or rinse quickly and don't score UGPP2). DIA2, MDH1, MDH2, and FDP2 come up on MC6; difficult to score in embryos. MDH4 must be run on MC8.
Slash Pine; <i>Pinus elliottii</i> ; buds	PGM1,2; SKD1; LAP1,2; DIA2	CAT1,2; TPI1,2; GOT1,2,3		FEST2; PGI1,2; MDH1,2,4; IDH; 6PGD1,2	Cut FEST and the SB stains wide. Score on day of run: SKD1; PGM2; TPI1; UGPP; 6PGD1; IDH. Use Backslide on GOT. MC8 stains can be run on MC6 for confirmation. LB; ACO can be added in second slice position and left in incubator overnight (it may darken). ME7 may be added to LB. SB; can add G6pd and UGPP at end. MC8; AK can be added.
Sugar Pine; <i>Pinus lambertiana</i> ; buds	FEST; PGM1,2; ACO; PGI2; ADH1,2; LAP1,2	CAT; UGPP; G6PD; GOT1,2,3; SOD	MDH1,3; SKD1,2; IDH; 6PGD2; DIA		Read FEST after 5 minutes. Cut FEST and UGPP wide. Read fresh: CAT, UGPP, SKD, and DIA.
Sugar Pine; <i>Pinus lambertiana</i> ; megagametophytes	FEST; PGM1,2; ACO; ME7-1,2; PGI2; ADH1,2; LAP1,2	CAT; UGPP; G6PD; GOT1,2,3; SOD	FEST; MDH2,4; SKD1,2; IDH; 6PGD1,2; DIA		Read FEST after 10 minutes. No backslice for GOT. Cut wide: UGPP, GOT, TPI, and FEST. FDP can be run on SB. MNR and FUM can be run on MC6.
Sugar Pine; <i>Pinus lambertiana</i> ; embryos	PGM; LAP; PGI; ACO; ADH	UGPP; GOT; TPI	(toss the first slice); SKD; IDH; MDH; 6PGD		Pour all gels thin. Cut SB gels wide for all stains. Watch UGPP for overstaining. Toss out bottom slice on MC6 gel.
Virginia Pine, <i>Pinus virginiana</i> ; buds	PGM1,2; PGI1,2; ACO; ADH; LAP1,2; FEST	CAT; GOT1,2,3; UGPP; TPI		IDH; MDH1,2,4; FDP; 6PGD1,2; DIA	Cut FEST, UGPP, and TPI wide.
Western White Pine, <i>Pinus monticola</i> , seed	LAP1,2; ACO; PGM1,2; PGI1,2	GOT1,3		IDH; 6PGD1,2; FEST; MDH2,4; MNR1,2	DIA, UGPP, & TPI recipes should be tried. MC6 and MC8 buffers should be tested, along with slice order trials on all buffers.

Table 2. Other Conifers (not pines)

Species	LB	SB	MC6	MC8	Comments, and additional buffers
Cedar, Alaska Yellow; <i>Chamaecyparis nootkatensis</i> ; needles	FEST1; PGM1; ME7; PGI1,2	UGPP1; TPI1; GOT1,2; ACP; G6PD; GDH; SOD1,2		MDH1,2; 6PGD1,2; IDH; SKD2; MNR; FDP	SB: Cut TPI wide. MC8: FEST1,2 can be added to the last position. There is no needle activity in ACO, LAP, DIA and CAT. GOT does not require backslide. Dropped because of poor resolution: EST and ADH.
Cedar, Atlantic White, <i>Chamaecyparis thyoides</i> ; needles	FEST1; PGM1; ME7; PGI1,2	UGPP1; TPI1; GOT1,2; ACP; G6PD; GDH; SOD1,2		MDH1,2; 6PGD1,2; IDH; SKD2; MNR; FDP	Cut TPI wide. There is no needle activity in ACO, LAP, DIA and CAT. GOT does not require backslide.
Cedar, Hinoki, <i>Chamaecyparis obtusata</i> ; needles	FEST1; PGM1; ME7; PGI1,2	UGPP1; TPI1; GOT1,2; ACP; G6PD; GDH; SOD1,2		MDH1,2; 6PGD1,2; IDH; SKD2; MNR; FDP	Cut TPI wide. There is no needle activity in ACO, LAP, DIA and CAT. GOT does not require backslide.
Cedar, Port Orford; <i>Chamaecyparis lawsoniana</i> ; needles	FEST1; PGM1; ME7; PGI1,2	UGPP1; TPI1; GOT1,2; ACP; G6PD; GDH; SOD1,2		MDH1,2; 6PGD1,2; IDH; SKD2; MNR; FDP	Cut TPI wide. There is no needle activity in ACO, LAP, DIA and CAT. GOT does not require backslide.
Cypress, Chinese, <i>Cupressus tortulosa</i> ; needles	FEST1; PGM1; ME7; PGI1,2	UGPP1; TPI1; GOT1,2; ACP; G6PD; GDH; SOD1,2		MDH1,2; 6PGD1,2; IDH; SKD2; MNR; FDP	Cut TPI wide. There is no needle activity in ACO, LAP, DIA and CAT. GOT does not require backslide.
Cypress, Leyland, <i>Cupressocyparis leylandii</i> ; needles	FEST1; PGM1; ME7; PGI1,2	UGPP1; TPI1; GOT1,2; ACP; G6PD; GDH; SOD1,2		MDH1,2; 6PGD1,2; IDH; SKD2; MNR; FDP	Cut TPI wide. There is no needle activity in ACO, LAP, DIA and CAT. GOT does not require backslide.
Cypress, Monterey; <i>Cupressus macrocarpa</i> ; needles	FEST1,2; PGM1; ME7; PGI1,2	UGPP1; TPI1; GOT1,2; ACP; G6PD; GDH; SOD1,2		MDH1,2; 6PGD1,2; IDH; SKD2; MNR; FDP	Cut TPI wide. There is no needle activity in ACO, LAP, DIA and CAT. GOT does not require backslide.
Douglas-fir; <i>Pseudotsuga menziesii</i> ; megagametophytes or buds	LAP1,2; PGI2; PGM1,2; ACO 1,2; FEST1,2	GLYDH; UGPP1,2,3; G6PD; GOT1,2,3; SOD; GDH; CAT	DIA; MDH1,3; 6PGD1; SKD1; IDH1		Cut FEST, GOT, PGM, LAP and UGPP wide. Stain FEST for 3 minutes. No albumin in G6PD stain. No backslide for GOT. Read PGM1, UGPP, CAT, DIA, and SKD fresh. Read UGPP after approximately 10 minutes. Use CAT for megs only.
Fir, Fraser; <i>Abies fraseri</i> ; meg/embryo pairs	PGM1; ACO; ME7; LAP1	UGPP1,3; TPI2; GOT3; GDH; CAT; PGI2	IDH2; 6PGD2; MNR1,2; SKD; FEST; MDH2		
Larch, Western; <i>Larix occidentalis</i> ; megagametophytes	GOT; ACO; LAP; PGM; PGI		G6PD; MDH; 6PGD; IDH; GLYDH	HISTIDINE BUFFER:G6P D; MDH;6PGD; IDH; PGM	Use backslide for GOT, and cut wide G6PD, PGM, LAP, and GOT.
Yew, Western; <i>Taxus brevifolia</i> ; needles	ADH; ME8; PGM	G6PD; GOT1,2; UGPP	SKD; PGI1,2; FDP2; MDH2; IDH	SKD; MDH; 6PGD2	Cut all SB stains wide.

Table 3. Woody Angiosperms

Species	LB	SB	MC6	MC8	Comments, and additional buffers
<i>Populus angustifolia</i> ; Narrowleaf Cottonwood; leaves from suckers	FEST2; ME7; PGM1; LAP1,2; PGI1,2	UGPP1,2; TPI1,2; GOT1,2		IDH1; 6PGD1,2; MDH1,2; DIA1	Toss bottom slice on MC8. Cut all SB slices wide. Read FEST in 5 – 10 minutes. ACO could have been used, but was not due to a bad lot of IDH chemical.
<i>Populus deltoides</i> ; Eastern and Plains Cottonwood; leaves from suckers	FEST2; ME7; PGM1; LAP1,2; PGI1,2	UGPP1,2; TPI1,2; GOT1,2		IDH1; 6PGD1,2; MDH1,2; DIA1	Toss bottom slice on MC8. Cut all SB slices wide. Read FEST in 5 – 10 minutes. ACO could have been used, but was not due to a bad lot of IDH chemical. UGPP2, TPI1, and TIP2 failed to resolve in samples from the Louisiana and nearby areas.
<i>Populus fremontii</i> ; Fremont Cottonwood; leaves from suckers	FEST2; ME7; PGM1; LAP1,2; PGI1,2	UGPP1,2; TPI1,2; GOT1,2		IDH1; 6PGD1,2; MDH1,2; DIA1	Toss bottom slice on MC8. Cut all SB slices wide. Read FEST in 5 – 10 minutes. ACO could have been used, but was not due to a bad lot of IDH chemical.
<i>Populus tremuloides</i> ; Trembling Aspen; leaves from suckers	LAP1,2; PGM1,2; ME7; PGI1,2;	UGPP1,2; CAT; 6PGD1; GOT1		SKD; MDH1,2,3; 6PGD2; IDH1; DIA1	Cut UGPP wide. Read CAT quickly; it will fade fast. Do not let PGI overstain.
<i>Populus trichocarpa</i> ; Black Cottonwood; leaves from suckers	FEST2; ME7; PGM1; LAP1,2; PGI1,2	UGPP1,2; TPI1,2; GOT1,2		IDH1; 6PGD1,2; MDH1,2; DIA1	Toss bottom slice on MC8. Cut all SB slices wide. Read FEST in 5 – 10 minutes. ACO could have been used, but was not due to a bad lot of IDH chemical.
<i>Purshia tridentata</i> ; Bitterbrush; leaves	FEST1; LAP1; PGM2; PGI1,2	UGPP3; GOT1; CAT1; TPI1,2; 6PGD1		FDP1; MDH1,2; DIA1; IDH1	Cut TPI wide. No back slice needed for GOT. Read Fest immediately. Read fresh; UGPP1, CAT1, TPI1, TPI2, FDP1, and DIA1. Stains dropped: SKD & ME7.
<i>Quercus rubra</i> ; Northern Red Oak; leaves	PGI1,2; PGM1; LAP1	GOT1,2; TPI1,2; SOD1,2; G6PD; UGPP	6PGD1,2; SKD; IDH; MDH1,2; DIA1,2		Acorns were run successfully on IEF gels, #5080: MDH, EST, DIA, PHI, ACP, PGM. PER and Silver stain were poor.

Table 4. Herbaceous Plants

Species	LB	SB	MC6	MC8	Comments, and additional buffers
<i>Achnatherum hymenoides</i> ; Indian Ricegrass; seedlings	LAP1; PGM1,2; ME7-1,2; IDH1	UGPP1; TIP1,2; GOT1,2,3,4; PGI4; 6PGD1,2	MDH1,2,4,5; SKD1,2,3; MDH		Cut SB slices wide except 6PGD. Double score GOT, 6PGD, PGI, ME7 and TPI and same day because they fade. No backslice needed on GOT. MDH: stain in 2 positions and score the best resulting gel. Low to no activity stains: ACO, DIA, G6PD, and GLYDH.
<i>Achnatherum occidentale</i> ; Western Needlegrass; seedlings	LAP1; PGM1,2; ME7-1,2; IDH1	UGPP1; TIP1,2; GOT1,2,3,4; PGI4; 6PGD1,2	MDH1,2,4,5; SKD1,2,3; MDH		Cut SB slices wide except 6PGD. Double score GOT, 6PGD, PGI, ME7 and TPI and same day because they fade. No backslice needed on GOT. MDH: stain in 2 positions and score the best resulting gel. Low to no activity stains: ACO, DIA, G6PD, and GLYDH.
<i>Achnatherum speciosum</i> ; seedlings	LAP1; PGM1,2; ME7-1,2; IDH1	UGPP1; TIP1,2; GOT1,2,3,4; PGI4; 6PGD1,2	MDH1,2,4,5; SKD1,2,3; MDH		Cut SB slices wide except 6PGD. Double score GOT, 6PGD, PGI, ME7 and TPI and same day because they fade. No backslice needed on GOT. MDH: stain in 2 positions and score the best resulting gel. Low to no activity stains: ACO, DIA, G6PD, and GLYDH.
<i>Achnatherum thurberiana</i> ; seedlings	LAP1; PGM1,2; ME7-1,2; IDH1	UGPP1; TIP1,2; GOT1,2,3,4; PGI4; 6PGD1,2	MDH1,2,4,5; SKD1,2,3; MDH		Cut SB slices wide except 6PGD. Double score GOT, 6PGD, PGI, ME7 and TPI and same day because they fade. No backslice needed on GOT. MDH: stain in 2 positions and score the best resulting gel. Low to no activity stains: ACO, DIA, G6PD, and GLYDH.
<i>Bromus carinatus</i> ; whole seedlings; phenotypic analysis	FEST; PGM; ME7; IDH; DIA	UGPP; TPI; GOT1,2; PGI1,2; 6PGD; G6PD		MDH1,2; SKD	Cut MDH, and all SB slices wide. Read UGPP early before blurring. Read FEST immediately. Read TPI before background gets too dark. MDH: leave in incubator (heat off) overnight for MDH2 to improve. Throw away first slice of LB gel before staining FEST.

Species	LB	SB	MC6	MC8	Comments, and additional buffers
<i>Collomia rawsoniana</i> ; Rawson's Flaming Trumpet; leaves	ADH; ME7; PGM1,2; ACO; PGI1,2	UGPP1; TPI1,2; G6PDH; GOT1,2; CAT		FEST-3; DIA1,2; SKD; MDH1,2,3,4; 6PGD1,2; IDH	Read FEST between 3 and 5 minutes. ACO, ADH, G6PD take a while to get dark. ACO and ADH can be left in incubator overnight, no heat. CAT and PGI have to be read right away. Stain backslice of PGI. ADH was dropped from analysis.
<i>Elymus elymoides</i> ; Squirreltail; seedlings	LAP1; PGM1,2; ME7- 1,2; IDH-1	UGPP1; TPI1,2; GOT1,2,3,4; PGI4; 6PGD1,2	MDH1,2,4,5; SKD1,2,3; MDH		Cut SB slices wide except 6PGD. Double score GOT, 6PGD, PGI, ME7 and TPI and same day because they fade. Stain MDH twice, score only in the clearest resulting position. GOT: no backslice needed. Poor to no activity: ACO, DIA, G6PD & GLYDH.
<i>Elymus glaucus</i> ; Blue Wild Rye; seedlings	LAP1,2; ACO1,2,3,4; PGM1,2; ME7-1,2; IDH1,2; DIA1,2	UGPP1,2; TPI1,2; GOT1,2,3,4; PGI1,2,3,4; 6PGD1,2; G6PD1,2	MDH1,2,3,4,5 ,6		Cut UGPP, TPI, and GOT wide. Use grass recipe for ACO. Use Wendel and Weeden recipe for GOT. ACO will not appear if roots are not included in seedling sample prep.
<i>Festuca idahoensis</i> ; Idaho Fescue; leaves; phenotypic analysis	ME7; PGM; PGI	UGPP; TPI; GOT1,2; 6PGD; GLYDH; CAT		SKD; DIA	Cut UGPP, TPI, GOT, and FEST wide. Read FEST immediately. Stains tested and dropped: ADH, ACO, FEST, IDH & MDH.
<i>Frasera fastigiata</i> ; leaves	ADH; PGM; LAP; PGI1,2	UGPP; GOT1,2; TPI1,2; CAT		MDH1,2; DIA1,2; IDH; FEST	System 5 Buffer: ME, 6PGD. Run System 5 at 50 mA to the second line. Cut SB stains wide.
<i>Frasera umpquaensis</i> ; leaves	ADH; PGM; LAP; PGI1,2	UGPP; GOT1,2; TPI1,2; CAT		MDH1,2; DIA1,2; IDH; FEST	System 5 Buffer: ME, 6PGD. Run System 5 at 50 mA to the second line. Cut SB stains wide.
<i>Hackelia diffusa</i> ; leaves; phenotypic analysis	PGM; ME7; PGI1,2; LAP	GLYDH; G6PD; UGPP1,2; GOT1,2; TPI1,2		MDH; SKD; IDH; 6PGD	No albumin on G6PD. UGPP and PGI tend to overstain. On LAP, bad tissue can look like an up.
<i>Hackelia venusta</i> ; leaves; phenotypic analysis	PGM; ME7; PGI1,2; LAP	GLYDH; G6PD; UGPP1,2; GOT1,2; TPI1,2		MDH; SKD; IDH; 6PGD	No albumin on G6PD. UGPP and PGI tend to overstain quickly. On LAP, bad tissue can look like an up.

Species	LB	SB	MC6	MC8	Comments, and additional buffers
<i>Lewisia cantelovii</i> ; leaves; scoring sheets	ADH; PGII,2; LAP1,2; PGM1,2; ME7; FEST2	UGPP1; GOT1,2; TPI1,2,3,4; G6PD	IDH1; 6PGD1; MDH1,2	SKD; IDH2; 6PGD2; EST; FDP	Read fresh: UGPP, TPI, DIA, IDH2, SKD, EST. Remove albumin from G6PD stain. Cut UGPP, FEST, GOT, TPI, and DIA wide. Read FEST: try immediately, some may take up to 10 minutes. EST fades within one half hour. No backslice for GOT. Agar method: AK in top third, DIA in top third. Stains with no activity: CAT, HK, MPI, GLUDH, MNR, SOD, GDH & FUM. Photograph night of run, gels tend to fade or add extra bands overnight. Stains dropped because of poor activity: ACO, GLYDH, DIA, ALD, & AK.
<i>Lewisia condonii</i> ; leaves	ADH; PGII,2; LAP1,2; PGM1,2; ME7; FEST2	UGPP1; GOT1,2; TPI1,2,3,4; G6PD	IDH1; 6PGD1; MDH1,2	SKD; IDH2; 6PGD2; EST; FDP	Read fresh: UGPP, TPI, DIA, IDH2, SKD, EST. Remove albumin from G6PD stain. Cut UGPP, FEST, GOT, TPI, and DIA wide. Read FEST: try immediately, some may take up to 10 minutes. EST fades within one half hour. No backslice for GOT. Agar method: AK in top third, DIA in top third. Stains with no activity: CAT, HK, MPI, GLUDH, MNR, SOD, GDH, & FUM. Photograph night of run, gels tend to fade or add extra band overnight. Stains dropped because of poor activity: ACO, GLYDH, DIA, ALD, & AK.
<i>Lewisia serrata</i> ; leaves	ADH; PGII,2; LAP1,2; PGM1,2; ME7; FEST2	UGPP1; GOT1,2; TPI1,2,3,4; G6PD	IDH1; 6PGD1; MDH1,2	SKD; IDH2; 6PGD2; EST; FDP	Read fresh: UGPP, TPI, DIA, IDH2, SKD, EST. Remove albumin from G6PD stain. Cut UGPP, FEST, GOT, TPI, and DIA wide. Read FEST: try immediately, or some may take up to 10 minutes. EST fades within one half hour. No backslice for GOT. Agar method: AK in top third, DIA in top third. Stains with no activity: CAT, HK, MPI, GLUDH, MNR, SOD, GDH & FUM. Photograph night of the run, gels tend to fade or add extra bands overnight. Stains dropped because of poor activity: ACO, GLYDH, DIA, ALD, & AK.

Species	LB	SB	MC6	MC8	Comments, and additional buffers
<i>Lupinus latifolius</i> ; Lupine; seeds	FEST1,3; LAP; ACO; PGI1,2; ME7; PGM; ADH	TPI1,2; GOT1,2; GLYDH; CAT	SKD; DIA; 6PGD; ACP1,2; IDH		Cut FEST, TPI, and GOT wide. Use backslime for ACP2. Read CAT and FEST immediately (they comes up instantly). Read ME, and PGI as soon as they come in; they begin to spread and blur quickly. Double score the night of the run: FEST, CAT, PGI, LAP, PGM, ME, PGI, SKD, GLYDH, IDH, and 6PGD. Leave ACO in incubator (heat off) overnight.
<i>Perideridia erythrorhiza</i> ; seedlings	ADH; PGI2; PGM1,2; ME7	TPI1,2; GOT; UGPP; GDH		FEST1; 6PGD2; MDH1,2,3,4	Cut wide: FEST, TPI, GOT & UGPP. Check FEST right away. Seedlings prepped without a root bulb will probably not stain for ADH, PGM-1, GDH, FEST & MDH-4. FEST2 is inconsistant. No backslime is needed for GOT. Stains tested and dropped: G6PDH, 6PGD1, DIA, SKD, CAT and IDH.
<i>Perideridia oregona</i> ; seedlings	ADH; PGI2; PGM1,2; ME7	TPI1,2; GOT; UGPP; GDH		FEST1; 6PGD2; MDH1,2,3,4	Cut wide: FEST, TPI, GOT & UGPP. Check FEST right away. Seedlings prepped without a root bulb will proably not stain for ADH, PGM-1, GDH, FEST & MDH-4. FEST-2 is inconsistant. No backslime is needed for GOT. Stains tested and dropped: G6PDH, 6PGD1, DIA, SKD, CAT, and IDH.
<i>Rorippa subumbellata</i> ; Tahoe Yellow Cress; whole shoots from adult plants	FEST1,2,3,4; LAP1; PGI1,2; ACO1; PGM1,2; ME7	UGPP1; GOT1; TPI1,2; GLYDH; CAT	MDH1; DIA1; IDH1; 6PGD1,2; SKD1		Cut wide: FEST, GOT, TPI, and UGPP. Read immediately: FEST, UGPP. ACO, LAP, and PGM come up slowly and faintly. No activity: G6PD on SB.
<i>Saxifraga bryophora</i> var. <i>tobiasiae</i> and var. <i>bryophora</i> ; Tobias' Saxifrage; leaves	ME7; LAP; PGM; PGI	UGPP; GOT; TPI; CAT		MDH; DIA; IDH; SKD; 6PGD; FEST	Cut SB stains wide.
<i>Sisyrinchium sarmentosum</i> ; Pale Blue-eyed Grass; leaves phenotypic analysis	FEST; LAP; ADH; PGM; PGI; ME7	G6PD; TPI; UGPP; GOT	IDH; MDH; DIA; 6PGD		Cut wide: FEST, TPI, UGPP. No backslime for GOT. Buffers tested and dropped: Histidine pH7, Histidine pH8, and MC8. Stains tested and dropped: GDH, CAT, SKD, GLYDH, AK, FDP, ACO & RBC.

APPENDIX F: RARELY USED RECIPES**Appendix F1: Gel and Tray Buffers****Appendix F2: Gel Recipes****Appendix F3: Stock Solutions****Appendix F4: Stain Recipes**

Appendix F1: Rarely Used Gel And Tray Buffer Recipes

RARELY USED GEL BUFFERS

HISTIDINE-CITRIC ACID GEL BUFFER (pH~7.0 or 8.0)	
CHEMICAL COMPONENTS	1 liter
Histidine (final concentration = 0.005 M)	9.58 g
DIRECTIONS: Bring almost to final volume with distilled water. Adjust pH to 7.0 (or 8.0) with 4N NaOH (approx. 10-11 mls) Bring to final volume.	
COMMENTS: Store in refrigerator.	
CITATION: Adams recipe. Wendel and Weeden 1989, referencing Fildes and Harris 1966.	

SYSTEM 5 GEL BUFFER: Dilute 140 l of System 5 tray buffer to 4 liters.

SYSTEM 8-, PARTS A & B			
CHEMICAL COMPONENTS			
Part A distilled water Lithium hydroxide (LiOH) Boric acid pH is 7.6; adjust to pH 8.0 with additional dry LiOH. Store in refrigerator.			4 liters 9.6 g 95.6 g
Part B distilled water Tris (Sigma 7-9 or Trizma Base) Citric acid, anhydrous Refrigerate.			4 liters 49.44 g 13.44 g
COMMENTS: Refrigerate parts A and B in separate containers.			
Produce finished gel buffer by combining parts A and B as follows:			
	for 2 liters	for 3 liters	for 4 liters
Part A	153.6 ml	230.4 ml	307.2 ml
Part B	652.8 ml	979.2 ml	1305.6 ml
DIRECTIONS: Bring almost to final volume with distilled water. pH is 8.0, bring to pH 7.6 with dilute HCl. Bring to final volume.			
CITATION: (Hamrick's)			

SYSTEM 11 GEL BUFFER, pH 7.0		
CHEMICAL COMPONENTS	for 2 liters	for 4 liters
L-Histidine-HCl, monohydrate	3.775 g	7.55 g
DIRECTIONS: Bring almost to volume with distilled water. Adjust pH to 7.0 using dilute NaOH, and bring to final volume.		
COMMENTS: This equals 0.009 M L-Histidine, HCl monohydrate.		
CITATION: (Hamrick's)		

RARELY USED TRAY BUFFER RECIPES

HISTIDINE-CITRIC ACID TRAY BUFFER (pH~7.0 or pH 8.0)	
CHEMICAL COMPONENTS	2 liters
Citric Acid, trisodium salt (Sodium Citrate) distilled water	211.64 g little less than 2000 ml
DIRECTIONS: Bring almost to volume. Adjust to pH with 0.41 M Citric acid (approximately 15-16 ml), then bring to volume of 2 liters.	
CITATION: Adams recipes. Wendel and Weeden 1989, referencing Fildes and Harris 1966.	

SYSTEM 5 TRAY BUFFER (pH~7.2)	
CHEMICAL COMPONENTS	
Tris (Sigma 7-9) Citric acid, monohydrate	108 g 53.32 g
DIRECTIONS: Bring almost to volume. Titrate to pH 7.2 with concentrated HCl, using a glass pipette. Bring to final volume of 10 liters.	
CITATION: Soltis et al. 1983	

SYSTEM 8- TRAY BUFFER					
CHEMICAL COMPONENTS					
Produce finished tray buffer by combining part A and water as follows:					
	for 2 liters	for 3 liters	for 4.5 liters	for 6 liters	for 10.5 liters
distilled water	621.3 ml	932 ml	1398 ml	1864 ml	3262 ml
Part A*	1330 ml	2000 ml	3000 ml	4000 ml	7000 ml
DIRECTIONS: (* - see gel buffer recipe.) Adjust pH to 8. Bring to final volume.					
COMMENTS: This equals 0.388 M LiOH, 0.263 M Boric Acid					
CITATION: Hamrick's recipe, a modification of Soltis et al., 1983					

SYSTEM 11 TRAY BUFFER		
CHEMICAL COMPONENTS	for 2 liters	for 4 liters
Citric Acid, trisodium salt (Sodium Citrate)	235.28 g	470.56 g
COMMENTS: Bring almost to volume. Adjust pH to 7.0 with dilute HCl, then bring to volume.		
CITATION: Hamrick's recipe		

Appendix F2: Rarely Used Gel Recipes

HISTIDINE CITRATE pH 7 or 8; Thin Frames (210mm x 124mm x 8mm)			
INGREDIENTS	2 gels (784 ml)	1 gel (392 ml)	
Starch	86.25 g (90.2 if Sigma)	43.13 g (45.08 if Sigma)	in a 2-liter vacuum flask
HC buffer	79 ml	39 ml	in a graduated cylinder
+ distilled H ₂ O	171 ml	86 ml	
distilled H ₂ O	534 ml	267 ml	in a 500 ml boiling flask
COMMENTS: Total 250 ml liquid in graduated cylinder for 2 gels, 125 ml for 1 gel.			
CITATION:			

SYSTEM 5, 8-, or 11; Thick Frames (210mm x 124mm x 12mm)			
INGREDIENTS	2 gels (1000 ml)	1 gel (500 ml)	
Starch		55 g (57 if Sigma)	in a 2-liter vacuum flask
appropriate gel buffer		159 ml	in a graduated cylinder
appropriate gel buffer		341 ml	in a 1-liter boiling flask
CITATION:			

SYSTEM 5, 8-, or 11; Thin Frames (210mm x 124mm x 8mm)			
INGREDIENTS	2 gels (784 ml)	1 gel (392 ml)	
Starch	86.25 g (90.2 if Sigma)	43.13 g (45.08 if Sigma)	in a 2-liter vacuum flask
appropriate gel buffer	250 ml	125 ml	in a graduated cylinder
appropriate gel buffer	534 ml	267 ml	in a 500 ml boiling flask
CITATION:			

SYSTEM 3 BUFFER; Thin Frames (210mm x 124mm x 8mm)			
INGREDIENTS	2 gels (784 ml)	1 gel (392 ml)	
Starch	86.25 g (90.2 if Sigma)	43.13 g (45.08 if Sigma)	in a 2-liter vacuum flask
System 3 tray buffer	50 ml	50 ml	in a graduated cylinder
+ distilled H ₂ O	200 ml	100 ml	
distilled H ₂ O	534 ml	267 ml	in a 1-liter boiling flask
COMMENTS: Total 250 ml liquid in graduated cylinder for 2 gels, 125 ml for 1 gel.			
CITATION:			

Appendix F3: Rarely Used Stock Solutions

Fumaric Acid Buffer (for Sugar Pine FUM stain)		
CHEMICAL COMPONENTS	400 ml recipe	
LIQUIDS: distilled water 4N NaOH	400 ml as needed	
DRY: Fumaric Acid	13.8 g	
DIRECTIONS: Mix dry ingredient into final volume of 400 ml. Use heat. Fumaric acid will not dissolve until pH reaches 8.0. Raise pH slowly with NaOH.		
COMMENTS: Mix fresh weekly.		
CITATION:		

Fumaric Acid Buffer (NOT for Sugar Pine)		
CHEMICAL COMPONENTS	400 ml recipe	
LIQUIDS: distilled water 4N NaOH	400 ml as needed	
DRY: Fumaric Acid	14.5 g	
DIRECTIONS: Mix dry ingredient into final volume of 400 ml. Use heat. Fumaric acid will not dissolve until pH reaches 8.0. Raise pH slowly with NaOH.		
COMMENTS: Mix fresh weekly.		
CITATION:		

STAIN: Acid Phosphatase (ACP)	Amount/Number of Slices	
CHEMICAL COMPONENTS	1	2
BUFFER: ACP buffer	30 ml	60 ml
DRY: **Fast Garnet GBC Salt** Na-a-Naphthyl acid phosphate	0.05 g 0.05 g	0.1 g 0.1 g
DIRECTIONS: Incubate at 37° C.		
COMMENTS: **CARCINOGEN** Stain the backslice. (Enzyme may migrate to the cathode.)		
CITATION: Conkle et al. 1982, who reference Scandalious 1969. Modified. Wendel and Weeden 1989.		

Appendix F4: Rarely Used Enzyme Stain Recipes

STAIN: Adenylate Kinase (AK), agar method	Amount/Number of Slices			
	1	2	3	4
CHEMICAL COMPONENTS				
BUFFER: (warm) 0.2 M M Tris-HCl, pH 7.0 10% MgCl ₂ solution	4.5 ml 0.05 ml	9 ml 0.1 ml	13.5 ml 0.15 ml	18 ml 0.2 ml
DRY: Adenosine-5-diphosphate (ADP) NADP Glucose	0.006 g 0.006 g 0.225 g	0.012 g 0.012 g 0.45 g	0.018 g 0.018 g 0.675 g	0.024 g 0.024 g 0.9 g
DURING STAINING: Hexokinase buffer G6PDH (frozen, 50 units/ml) PMS solution MTT solution	1.5 ml 0.25 ml 0.1 ml 1 ml	3 ml 0.5 ml 0.2 ml 2 ml	4.5 ml 0.75 ml 0.3 ml 3 ml	6 ml 1 ml 0.4 ml 4 ml
AGAR SOLUTION: Bacto-agar distilled H ₂ O	0.13 g 10 ml	0.26 g 20 ml	0.39 g 30 ml	0.52 g 40 ml
DIRECTIONS: STEP 1: Combine all ingredients except agar and water. STEP 2: In separate beaker, combine agar and water. Heat to dissolve agar thoroughly. Cool to 60° C. STEP 3: Combine buffer solution and agar solution. Pour over slices. Incubate at 37° C for a while. Leave overnight at room temperature.				
COMMENTS: Do not put it in fixer. Peel off agar and wrap gel in plastic wrap the day after the run.				
CITATION: Wendel and Weeden 1989. Modified.				

STAIN: Aldolase (ALD)	Amount/Number of Slices			
	1	2	3	4
CHEMICAL COMPONENTS				
BUFFER: 1 M Tris-HCl, pH 8 distilled H ₂ O to	5 ml 25 ml	10 ml 50 ml	15 ml 75 ml	20 ml 100 ml
DRY: Fructose-1,6-diphosphate Arsenic acid NAD	0.125 g 0.038 g 0.01 g	0.25 g 0.076 g 0.02 g	0.375 g 0.114 g 0.03 g	0.5 g 0.152 g 0.04 g
DURING STAINING: Glyceraldehyde-3-phosphate dehydrogenase MTT solution PMS solution	15 µl 1 ml 1.5 ml	30 µl 2 ml 3 ml	45 µl 3 ml 4.5 ml	60 µl 4 ml 6 ml
DIRECTIONS: Incubate at 37°C.				
COMMENTS: Dispose of stain in HAZARDOUS WASTE container.				
CITATION: Conkle et al. 1982, who reference Yeh and O'Malley 1980.				

STAIN: Alanine Amino Peptidase (ALAP)	Amount/Number of Slices	
CHEMICAL COMPONENTS	2	
BUFFER: 0.2 M Tris-Maleate Buffer, pH 3.8	60 ml	
DRY: Alanine-b-naphthylamide Fast Black K Salt	0.05 g in 2 ml DMSO 0.05 g	
DIRECTIONS: Combine all ingredients. Dissolve well. Incubate at 37° C in the dark.		
CITATION:		

STAIN: Beta-Galactosidase (b-GAL)	Amount/Number of Slices			
CHEMICAL COMPONENTS	1	2	3	4
BUFFER: Sodium Acetate Buffer, pH 4.0	5 ml	10 ml	15 ml	20 ml
DRY: 4-methylumbelliferyl-b-D-galactoside (leave in freezer until ready to stain; replace immediately)	0.005 g	0.01 g	0.015 ml	0.02 ml
DURING STAINING: Concentrated ammonium hydroxide	as needed	as needed	as needed	as needed
DIRECTIONS: Combine the ingredients. Drip over gel until covered. Cover gel with plastic wrap for 35 to 45 minutes. Coat with concentrated ammonium hydroxide (except for gels run on Histidine buffer pH 7). Read under a UV light.				
CITATION:				

STAIN: Beta-Glucosidase (b-GLU)	Amount/Number of Slices			
CHEMICAL COMPONENTS	1	2	3	4
BUFFER: Sodium Acetate Buffer, pH 4.0	5 ml	10 ml	15 ml	20 ml
DRY: 4-methylumbelliferyl-b-D-glucoside (leave in freezer until ready to stain; replace immediately)	0.01 g	0.02 g	0.03 g	0.03 g
DURING STAINING: Concentrated ammonium hydroxide	as needed	as needed	as needed	as needed
DIRECTIONS: Combine the ingredients. Drip over slice with pipet. Cover gel with plastic wrap for 35 to 45 minutes. Rinse with tap water. Sprinkle with concentrated ammonium hydroxide (except for gels run on Histidine buffer pH 7). Read under a UV light.				
COMMENTS: Histidine buffer, pH 7: read after 30 minutes. Morpholine buffers: view after 45 minutes.				
CITATION:				

STAIN: (GLUDH)	Amount/Number of Slices	
CHEMICAL COMPONENTS	1	2
BUFFER: 0.2 M Tris-HCl, pH 8	25 ml	50 ml
DRY: Glucose NAD	0.25 g 0.015 g	0.5 g 0.03 g
DURING STAINING: MTT solution PMS solution	1 ml 1.5 ml	2 ml 3 ml
CITATION:		

STAIN: Hexokinase (HK)	Amount/Number of Slices	
CHEMICAL COMPONENTS	2 or 4	
BUFFER: 1 M Tris-HCl, pH 8.0 10% MgCl ₂ distilled H ₂ O to . . .	7.5 ml 0.75 ml 75 ml	
DRY: Adenosine-5'-triphosphate (ATP) Glucose	0.020 g 0.070 g	
DURING STAINING: Glucose-6-phosphate dehydrogenase (frozen, 50 units/ml) NADP combination solution	1.6 ml 4.0 ml	
DIRECTIONS: Incubate at 37°C.		
COMMENTS: Best results when used on the Morpholine-Citrate pH 8 buffer system.		
CITATION: Modified Adams		

STAIN: Menadione Reductase (MNR)	Amount/Number of Slices	
CHEMICAL COMPONENTS	1 or 2	
BUFFER: (warm) 0.1 M Tris-HCl, pH 7.0	60 ml	
DRY: Menadione sodium bisulfite NADH NBT	0.01 g 0.025 g 0.01 g	
DIRECTIONS: Incubate at 37°C.		
COMMENTS: See also DIA. These stains may reveal the same enzyme.		
CITATION: This recipe from IFG. Conkle et al. 1982. Modified.		

STAIN: Phosphomannose isomerase (PMI or MPI)	Amount/Number of Slices			
CHEMICAL COMPONENTS	1	2	3	4
BUFFER: 1 M Tris-HCl, pH 8.0 distilled H ₂ O to . . .	1 ml 25 ml	2 ml 50 ml	3 ml 75 ml	10 ml 100 ml
DRY: d-Mannose-6-phosphate Phosphoglucose isomerase (PGI)	0.013 g 0.005 g	0.025 g 0.01 g	0.038 g 0.015 g	0.05 g 0.02 g
DURING STAINING: NADP combination solution G-6-PDH (frozen, 50 units/ml)	1.25 ml 0.4 ml	2.5 ml 0.8 ml	3.75 ml 1.2 ml	5 ml 1.6 ml
DIRECTIONS: Incubate at 37°C.				
CITATION:				

STAIN: Ribulose biphosphate carboxylase (RBC)	Amount/Number of Slices	
CHEMICAL COMPONENTS	1	2
BUFFER: RBC solution	ca. 600 ml	ca. 1200 ml
DRY: Coomassie Brilliant Blue, or Amido Black, or Ponceau S, or other general protein stain	0.025 g	
DIRECTIONS: Dissolve the dye in about half the RBC solution. Allow to stand, then carefully decant solution into remaining buffer, leaving undissolved bits behind. Cover the slice with stained RBC solution. Incubate at room temperature. Later, pour off, then rinse gel with RBC solution repeatedly until color contrast is maximized. Then rinse thoroughly with water.		
COMMENTS: Use only on leaf tissue! (RBC is involved in photosynthesis.) The methanol in the RBC solution fixes the gel; no further fixative needed.		
CITATION: Wendel and Weeden 1989.		

STAIN: Sorbitol dehydrogenase (SRDH)	Amount/Number of Slices	
CHEMICAL COMPONENTS	2	
BUFFER: (warm) 0.2 M Tris-HCl, pH 8.0	50 ml	
DRY: sorbitol	7 g	
DURING STAINING: NAD combination solution	2.5 ml	
DIRECTIONS: Incubate at 37°C in the dark.		
CITATION: Adams.		

STAIN: Superoxide Dismutase (SOD)	Amount/Number of Slices	
CHEMICAL COMPONENTS	1 or 2	3 or 4
BUFFER: 1 M Tris-HCl, pH 8.0 1 M NaOH distilled H ₂ O to . . .	3.75 ml 1.5 ml 75 ml	7.5 ml 3 ml 150 ml
DURING STAINING: NAD combination solution	2.5 ml	5 ml
DIRECTIONS: Incubate at 37°C.		
CITATION:		

APPENDIX G: NFGEL FORMS

- a. Allele Map Form**
- b. Chemical Inventory Form**
- c. Data Check Form**
- d. NFGEL Project Summary**
- e. Prep Sheet**
- f. QA Sheet**

NFGEL Project Summary

Project #

Contact Person

Name:

Phone #:

Email Address:

Species

Project Objectives

Date Submitted

Material Submitted

Material Preparation

Prep. Dates:

Gel Format

Buffers and Stains

Run Dates

Quality Assurance Plan

Analysis and Final Product

Additional Notes

Sample Prep Sheet

Project #: _____ Species: _____

SET #: _____

Plant Mat'l: _____

Extraction Buffer: _____ Date: _____ By: _____

Prep Type: _____

Wick Size: _____ Vol/Well: _____

Prep Date: _____ By: _____

Run Date: _____

Buffers: _____

Total # Plates (original+reps) _____

1. **RP** _____ 14. **RP** _____ 26. **RP** _____

2. _____ 15. _____ 27. _____

3. _____ 16. _____ 28. _____

4. _____ 17. _____ 29. _____

5. _____ 18. _____ 30. _____

6. _____ 19. _____ 31. _____

7. _____ 20. _____ 32. _____

8. _____ 21. _____ 33. _____

9. _____ 22. _____ 34. _____

10. _____ 23. _____ 35. _____

11. _____ 24. _____ 36. _____

12. **RP** _____ 25. **RP** _____ 37. **RP** _____13. **RP** _____

SET #: _____

Plant Mat'l: _____

Extraction Buffer: _____ Date: _____ By: _____

Prep Type: _____

Wick Size: _____ Vol/Well: _____

Prep Date: _____ By: _____

Run Date: _____

Buffers: _____

Total # Plates (original+reps) _____

1. **RP** _____ 14. **RP** _____ 26. **RP** _____

2. _____ 15. _____ 27. _____

3. _____ 16. _____ 28. _____

4. _____ 17. _____ 29. _____

5. _____ 18. _____ 30. _____

6. _____ 19. _____ 31. _____

7. _____ 20. _____ 32. _____

8. _____ 21. _____ 33. _____

9. _____ 22. _____ 34. _____

10. _____ 23. _____ 35. _____

11. _____ 24. _____ 36. _____

12. **RP** _____ 25. **RP** _____ 37. **RP** _____

(QA) Sheet

Run Date:

Gel #	Buffer	Set#	Project #	Species	Stains

Comments:

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