Methodologies for Allozyme Analysis Using Cellulose Acetate Electrophoresis

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1 Introduction

Although cellulose acetate has been employed as an electrophoresis medium for several decades (Chin 1970), its use was, until recently, largely restricted to diagnostic applications in clinical settings. The success of early membranes was limited by their extreme fragility and their lack of resolution as compared to competing media such as acrylamide and starch. In the mid 1970's, a new generation of cellulose acetate membranes was developed which employed a mylar backing to produce a more robust product. These membranes also possessed superior resolution to earlier membranes and have rapidly gained wide acceptance in the medical sector.

Diffusion of the technique into other disciplines has been remarkably slow. For example, most papers in population genetics reporting data collected using this system have been published since 1985. As a group, these papers have established that cellulose acetate electrophoresis is not only simpler and more rapid, but that it is also more sensitive and provides superior resolution to starch or acrylamide electrophoresis (Easteal and Boussy 1987). Because of its simplicity, cellulose acetate electrophoresis is particularly attractive for systematists and ecologists who employ allozyme analysis as a tool rather than as a primary area of research. The technique is also likely to be useful in undergraduate laboratory courses in genetics and evolution because of the short time required to obtain results.

Our experience with cellulose acetate electrophoresis has confirmed that it generally provides resolution equal or superior to that obtained using either starch or acrylamide gels. Of course, no system is perfect and cellulose acetate is, on occasion, inferior to conventional methodologies for specific allozyme loci. However, there are a number of advantages which ordinarily make cellulose acetate the preferred medium. Gel run times with cellulose acetate can be as short as 10 minutes, and therefore, large sample sizes can be processed rapidly. For example, in a survey of fish enzymes, 2700 individuals were screened for variation at 1 enzyme in 7 days and 5 species were surveyed for 22 enzymes in a single day (Gauldie and Smith 1978). Aside from the short run times, cellulose acetate gels are ready to use just 20 minutes after being placed in the gel buffer. Not only is the labor of gel preparation avoided, but there is tremendous flexibility in research plans. There is no need, for example, to decide how many gels will be run a day in advance. Although the gels themselves are relatively expensive, the savings on labor, stain reagents and electrode buffers are considerable.

The sensitivity of cellulose acetate electrophoresis is a major advantage to anyone interested in surveying levels of genetic variation in natural populations of small organisms. In our experience, enzyme activity can be obtained from 0.5-2 µl of extract, meaning that single individuals of species with body lengths of 0.5 mm or less can be analyzed. There is a limit to its sensitivity; don't try single protozoans!

There are two thorough electrophoresis handbooks available, one focussing on the use of acrylamide and starch media (Harris and Hopkinson 1976) and one detailing the use of cellulose acetate as the gel matrix (Richardson et al. 1986). Both are excellent references, filled with information on an assortment of topics related to electrophoresis (ie. population genetics, sampling strategies and data analysis). Other texts contain one to several chapters dealing with enzyme electrophoresis and related topics (Whitmore 1990, Hillis and Moritz 1990). The major drawback of these books is their format and bulk; few first time users are interested in a technique which requires 300 pages of explanation. Furthermore, the staining protocols, while sufficiently detailed for those experienced in electrophoretic methods, often ignore some of the basic information which novitiates require to obtain results.

The methods described in this handbook are those which we routinely use in our analyses of genetic variation in freshwater zooplankton populations. However, these same methods have been successfully used with only minor modifications to analyze patterns of allozyme variation in populations of fish, flatworms, molluscs, insects, terrestrial plants and marine algae.

The handbook is not intended to detail the theory of electrophoresis, but rather to succinctly present the cellulose acetate electrophoresis protocol. If at times, the manual presents portions of the methods in a "black box" fashion, it is done for simplification. Those intrigued by the technique will find more complete explanations of the mechanics of the methods in other references (Harris and Hopkinson 1976; Richardson et al. 1986).

The handbook has been divided into three sections which aim to provide even the inexperienced electrophoresis user with sufficient information to process samples successfully. The preliminary section includes information on equipment requirements, setting up buffer tanks and loading and running gels. This section of this book is written for Helena Laboratories' Titan® III cellulose acetate plates. Alternate brands of cellulose acetate gel plates are available and protocols for their use may deviate from those which we propose. The second section of this manual is a "cookbook" which details stain and buffer recipes and information on the enzyme systems such as the number of zones of activity, the quaternary structure of the enzyme and the optimal buffer system. The final section provides information on gel interpretation, troubleshooting and taxa successfully studied using this system. Finally, it also includes references pertaining to the use of this method in studying genetic variation in natural populations, as well as catalog numbers for purchasing equipment and chemicals.

2 General Methodology

2.1 Equipment Requirements

The following equipment is necessary for electrophoresis: a refrigerator with a freezer unit for chemical storage, gel electrophoresis tanks, a D.C. power supply, cellulose acetate gel plates and an applicator kit. The kit contains two sample well plates, one applicator and one aligning base (Fig. 1). In addition, a microwave oven is useful for preparing agar for the overlays as well as a drying oven for holding melted agar, incubating plates during staining and drying stained plates. Additional useful apparatus include a small light box for scoring gels, plexiglass sheets (13 cm x 13 cm) to support gels while they stain, 15 ml scintillation vials to hold stain mixtures, 60 ml amber glass dropper bottles for stock solutions, 800 ml beakers for soaking gels, plastic trays for soaking stained gels, and micro-pipettes for measuring volumes of the linking enzymes used in stain mixtures. Most of this equipment (except the refrigerator, microwave and drying oven) may be easily packed into a single ice chest and the entire system transported for use in field localities.

2.2 Gel Plates

We use the Super Z-12 applicator kit and Titan® III cellulose acetate plates. The dull matte upperside of each gel is made of acetate whereas the smooth shiny back is mylar. The gel plates used in this laboratory are 76 mm x 76 mm. With plates of this size, wells 1 and 12 are occasionally difficult to score because of irregular running along the sides of the plate. The use of larger plates (eg. 94 mm x 76 mm) avoids this problem, but is more costly.

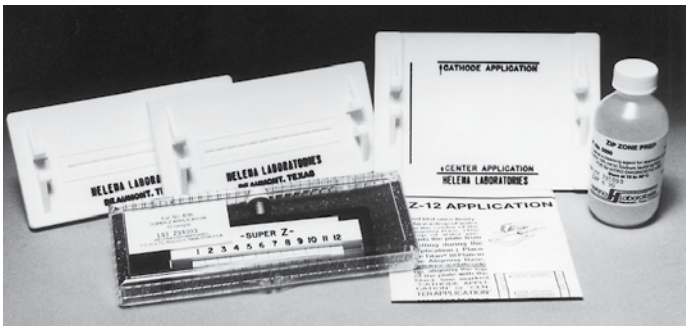


Figure 1: Helena Super Z-12 Applicator kit.

2.3 Tank Setup

Both sides of the electrophoresis tank are filled with the appropriate electrode buffer until the platinum electrodes, which run along the bottom across the width of the tank, are completely submerged in the buffer (Fig. 2, 3). Support rails should be positioned in the tank on either side of the partition (approximately 70 mm apart for the 76 mm x 76 mm size gel plates). A wick is placed on either side of the partition to draw the buffer from the reservoir. During a run, the gel plates rest on the wicks and are supported by the rails. The wicks may be cut from either blotting paper or Whatman filter paper and should be the width of the tank and long enough to reach the bottom of the tank from the rail (approximately 24 cm x 9 cm).

The wicks should be turned over every day to ensure that the paper along the support rail is moist. Replace wicks when the buffer is changed or when wick is damaged. The tank buffer needs to be changed only when a reduction in resolution is observed. For Tris Glycine buffer, more than 100 runs may be performed before replacement is warranted. Other buffers, such as CAAPM, may require replacement after 20 runs.

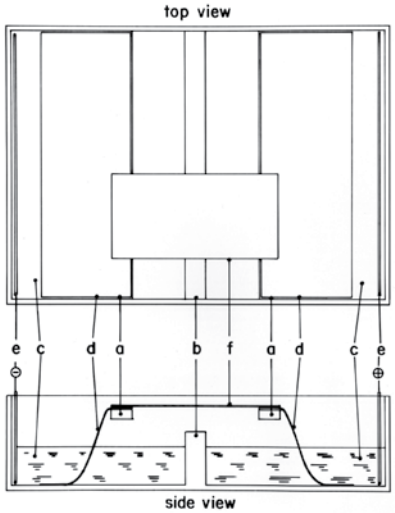


Figure 2: Top and side view of electrophoresis tank. Tank dimensions: 30 cm (L) x 26 cm (W) x 5 cm (D).

a = supporting rail
b = partition
c = buffer
d = wick
e = electrode with platinum electrode along bottom of tank
f = cellulose acetate plate

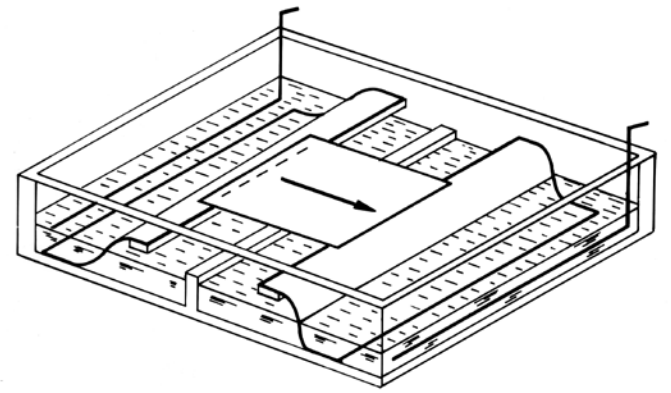


Figure 3: Operational electrophoresis tank. Three cellulose acetate plates will fit in one tank simultaneously.

Gel tanks should be stored in a refrigerator when not in use to reduce evaporation of and fungal growth in the electrode buffer. Refrigeration during electrophoresis, however, is not necessary because of short run times and low current flow. Therefore, gels can be run at room temperature without reduced resolution due to enzyme degradation.

2.4 Gel Soaking

Cellulose acetate plates are soaked in the same buffer as the electrode buffer. This is often referred to as a continuous buffer system. Multiple gels can be simultaneously soaked in an 800 ml beaker with individual gel plates separated by glass rods to ensure complete soaking of every plate. Care must be taken, however, to prevent the formation of bubbles on the gel plate as it is immersed. This is accomplished by submerging the plates at a slow, constant rate into the gel buffer. Plates should be soaked for at least 20 minutes, but can be left refrigerated in the buffer for several days without harm. The gel buffer should be replaced when it becomes cloudy with cellulose acetate particles.

2.5 Sample Preparation

For samples collected but not immediately run, care must be taken to ensure that enzyme degradation does not occur. The tissues should be stored in liquid nitrogen or in an ultrafreezer at -70°C until processed.

2.5.1 Animals

Small (0.5-5 mm) animals are placed directly into Helena sample wells and 5-10 µl of either distilled water or a grinding solution (ie. Tris HCl, pH=8.0) is added. To stabilize certain enzymes (eg. G6PDH), either NAD or NADP (in a concentration of 10 mg cofactor/ml solution) may be used. A metal spatula can be employed to crush the animals once they are in the wells. Care must be taken not to exchange fluid between wells during the homogenization process. Grinding continues until a smooth extract is obtained (approximately 10-30 sec/animal, depending on their size). Poor homogenization of the extracts may clog the applicator. A clogged applicator will leave "blobs" instead of thin lines when the samples are applied to the plate.

Larger animals (ie. > 5 mm) are prepared by grinding tissues in Eppendorf tubes or in a grinding block. The block, usually made of plexiglass, has a series of depressions drilled out where tissues may be placed. In either case, tissues are ground on ice in 20-500 µl (depending on amount of tissue) of grinding solution or distilled water using a plexiglass rod. In some cases, centrifugation of large tissue samples may be desirable to obtain an aliquot free of particulate matter. This is most readily done by centrifuging 1 ml extracts in Eppendorf tubes in a microfuge for 90 seconds. Once homogenized (and centrifuged if necessary), a 10 µl aliquot of extract is added to a well of the sample plate with a micro-pipette. The balance of each extract may be frozen in Eppendorf tubes for later analysis. In many cases, when the animal is sufficiently large to permit dissection, extracts are prepared separately from a variety of tissue types – liver, muscle and eye are most commonly surveyed in vertebrates.

2.5.2 Plants

Soft or young plant tissues may be ground in either a grinding block or Eppendorf tubes. Homogenizing tougher tissues may be accomplished in a mortar with a pestle using an abrasive such as fine, clean sand. Most plant tissues must be homogenized in a grinding buffer (Section 3.3) in order to obtain good resolution of banding patterns. In some cases, addition of polyvinylpolypyrrolidone (PVPP) or a few grains of instant coffee to the sample during grinding improves enzyme stability by removing phenolic compounds. The use of young tissues (eg. seedlings, leaf buds) is desirable because they contain lower concentrations of secondary compounds which often act to reduce or destroy enzyme activity.

2.6 Sample Loading

When extracts have been prepared or added to the wells of the sample plate, a gel is removed from the soaking buffer. The cellulose acetate plate is blotted dry between sheets of filter paper. It is criti-

cal that the gel be blotted dry – if excess moisture remains on the gel surface, the loading zone will be broad resulting in impaired resolution. The plate is placed mylar side down on the aligning base. Care must be taken to ensure that the cellulose acetate plate lies flat and does not shift when the extracts are loaded. To prevent movement, the aligning plate can be moistened with a drop of gel buffer before the cellulose acetate plate is set on it. The plate must also be centered on the aligning base to ensure that all samples are applied. The Super Z aligning base may either be milled in the centre (approximately 2 mm deep) or plate positions marked with a pen to facilitate the positioning of the gel plates during extract application.

Using the applicator, extracts are applied one or more times to the same position on the plate. The optimal number of applications to each load zone will vary depending on the amount of enzyme activity. For high activity enzymes (eg. GPI), one application at each load zone is sufficient. Enzymes with lower levels of activity (eg. TPI), may require 3 or 4 applications at each load zone. One or two load zones may be applied to the same plate, with the first load zone near one end and the other load zone centrally positioned on the gel. The use of two load zones permits 24 individuals to be analyzed on each plate.

If extracts from one set of wells are to be applied to several plates, the plates can be loaded sequentially. Once loaded, plates are rested on the wicks in the tank (without current being applied) while subsequent plates are loaded. The applicator must be cleaned by blotting its teeth on filter paper before applications are made with other extracts. Failure to properly clean the teeth may result in the staining of residual extracts and confusing zymograms. The use of two applicators (1 for each set of 12 wells) is beneficial, especially when analyzing small animals to eliminate the potential for contamination and to avoid wastage of extract during blotting of the applicator.

2.7 Gel Running

The plates are placed acetate side down on the wicks in the electrophoresis tank. The load zone located near the end of the gel should not come into contact with the wicks. Since the current runs from the cathodal to the anodal electrode (negative to positive), the load zones on the plate should be positioned at the cathodal end of the tank for the majority of the enzyme systems which migrate anodally. For those systems which migrate cathodally, extracts should be loaded only near the center of the gel.

There should be no air between the wicks and the cellulose acetate plates (ie. the plates must lie flat). A small aliquot of buffer can be applied to the zone of contact between the plates and wicks using a pipette to displace air trapped during placement of the gel plate. As excessive buffer addition can cause variable migration, it is usually preferable to simply run a finger along the length of the gel at both ends to ensure complete contact and displacement of air. If the plates are warped, microscope slides may be used as weights to ensure complete contact.

Electrophoresis is ordinarily carried out at room temperature at 200 volts for 15 minutes. Both voltage and run time may be adjusted to optimize separation and resolution. Current flow varies with buffer – 8 mA/plate with CAAPM and approximately 2 mA/plate with Tris Glycine (TG). It may also be necessary to vary the pH of the buffer or to change the buffer system completely to improve resolution. The position at which the sample is applied on the cellulose acetate plate can also affect the resolution. The best resolution is ordinarily obtained from samples loaded near the center of the plate.

2.8 Gel Staining

Stocks of the chemicals are usually made up in 10-20 ml batches and placed in amber dropper bottles. Larger quantities can be made of the frequently used chemicals (eg. 50-100 ml of NAD, MTT, and PMS). When kept at 4°C, chemical stocks are ordinarily stable for at least 1 month. Sodium azide (1 µl sodium azide stock/ml chemical stock) is added to the stock solutions of many chemicals to prevent bacterial degradation. For more complex stocks (e.g. AAT solution #1, TPI solution), large batches can be prepared and then frozen in small quantities. Chemicals which are not going to used for an extended period of time should be frozen. Detailed stain recipes are listed on pages 11-20.

Fifteen ml glass scintillation vials make useful containers for preparing individual stain mixtures. Each stain consists of approximately 2 ml of a mixture of chemical stocks and 2 ml of agar. Most of the stain components may be combined while the gel is running and the resultant mixture will be stable. Certain chemicals (ie. PMS, dye salts and linking enzymes) should not be added until immediately before use to avoid deterioration of the stain. Many stain mixtures

(exclusive of the labile and photosensitive chemicals) can be frozen in scintillation vials. Nicknamed allozymsicles, the frozen mixtures allow the preparation of many similar mixtures at one time. This saves time, prevents substrate degradation, and leads to more reliable assembly of the stain mixture. When required for use, one or more vials are thawed in a water bath or a microwave. Allozymsicles are ideal when undertaking large scale population surveys. When the gel run is complete, the final stain ingredients are added and the plates removed from the tank and placed mylar side down on a plexiglass sheet. Again, care must be taken to ensure that the cellulose acetate plate lies flat. Once plates have been removed from the tank, they should be stained immediately before they dry out. Melted agar (ideally held in a drying oven at 60°C) is added to the stain last and the mixture is poured over the plate. After the stain has been poured, the plate should not be moved until the agar has set (approximately 1 minute). Incubation of the plates in the dark at temperatures above 25°C often accelerates the staining process – a drying oven can be used for this purpose. If prolonged heating is required, however, the gel plate should be placed in a covered dish to prevent dessication. If several plates have been run together, it is best to turn off the current and remove and stain the plates one at a time to prevent drying of the plates.

For tissue specific studies or work on extremely small organisms, the amount of tissue often limits the number of plates which can be loaded. In these cases, several techniques may be used to maximize the number of loci stained. Firstly, the sequential loading of low, followed by medium and then loci with high staining intensity can greatly increase the number of loci which can be scored. Secondly, a single gel plate can be stained for 2 or more enzymes.

There are two methods to stain a single plate for several enzymes. In some cases there is sufficient similarity in stain ingredients that one can produce a mixed stain that includes all reagents needed for two or more enzymes. For example, to stain for both LDH and PGM, just add 1-2 drops of lactic acid to a normal PGM stain. To stain for LDH, PGM and AO simply add one drop of benzaldehyde to the previous mixture. Obviously, before constructing a complicated reaction mixture such as the one just suggested, enzymes should be stained individually to ensure that the bands for each enzyme do not overlap. This method, however, can be of tremendous value when one is trying to ascertain multilocus phenotypes of very small animals.

Where the stain reagents can be mixed, the method just described is the best to use. However, some reaction mixtures are incompatible (eg. AAT and GPI, AMY and any dehydrogenase). Multiple enzymes may still be stained in tandem if the enzyme bands are sufficiently separated on the plate. A plastic barrier may be used to prevent the first stain from covering the entire gel surface. For example, a GPI/AO stain may be poured over the portion of the gel containing these enzymes and the agar allowed to solidify. The barrier is then removed and AMY substrate is poured over the remainder of the gel.

As an example of the effectiveness of these techniques, 16 loci have been scored from individuals with a body length 0.5 mm (Taylor, unpublished) by loading plates in the following order and employing some multiple stain combinations.

Plate	Enzyme	#loci
1	PEP (PP)	1
2	PEP (LG)	2
3	AO/LDH	2
4	AAT	2
5	PGM	1
6	FUM	1
7	GPI	1
8	APK/ME	2
9	IDH	2
10	MDH	2

2.9 Gel Scoring and Storage

Once the plate has stained sufficiently to resolve the enzyme, the agar/stain overlay can be removed by holding the plate under cold running water or by peeling the overlay off and rinsing the plate with water. Plates should be soaked in a tray of water until scored. The scoring is most easily accomplished on a light box. For details on gel interpretation see Section 4.3 (page 24). If two load zones were applied to a plate, it is typical that their separation will vary.

To keep plates for extended periods of time, they should be first soaked in cold water for 20 minutes (to allow diffusion of residual stain reagents which darken the background), then blotted dry and placed in an oven at approximately 60°C for 10 minutes. The bands fade on drying, but will regain much of

peat all this information, but a few comments may be helpful. Firstly, there is a simple relationship between quaternary structure (ie. the number of subunits in the functional enzyme) and allozyme phenotypes. The two most common quaternary structures are monomers (the functional enzyme is composed of a single enzyme subunit) and dimers (two subunits make up the functional enzyme). Tetramers, in which the functioning enzyme is composed of four subunits, are less common. At monomeric loci heterozygotes typically have a double banded phenotype, and homozygotes a single banded phenotype (Fig. 4a). Many studies have shown that subunit assembly in multimeric enzymes is random. Hence, in an individual heterozygous at a dimeric enzyme for slow (S) and fast (F) alleles, three enzyme configurations will be seen (SS, SF, FF). Because subunits randomly associate, the heterodimer (SF) is twice as abundant as the two homodimers (Fig. 4b). Thus, an individual heterozygous at a dimeric enzyme shows a three banded phenotype with the central band staining twice as intensely as the peripheral bands. Similarly, heterozygotes for tetrameric enzymes are five banded, with band intensities of 1/16, 4/16, 6/16, 4/16 and 1/16 respectively (Fig. 4c). Often only 3 zones of activity can be discerned in individuals heterozygous at tetrameric enzymes because the 3 central bands stain so much more intensely than the peripheral bands. In these cases, the central bands do not align with either of the homozygotes, but are located in the region between them.

The extent and nature of phenotypic diversity at a specific allozyme locus depends not only upon the quaternary structure of the enzyme, but also upon the number of alleles present at the locus. When 2 alleles are present, 3 phenotypes are expected, with 3 alleles, 6 phenotypes and with 4 alleles, 10 phenotypes. In most cases, the number of alleles at polymorphic loci ranges from 2-3. In a few exceptional cases, more than 10 alleles have been detected making gel scoring a nasty task.

Figures 4a-4c: Diallelic allozyme polymorphisms at monomeric, dimeric, and tetrameric loci. At each locus 11 represents the fast homozygote, 22 the slow homozygote and 12 the heterozygote.

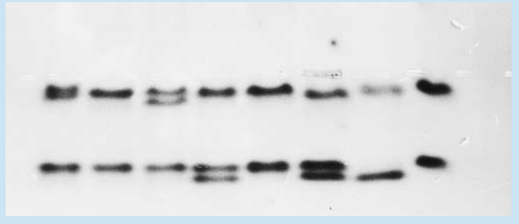


Figure 4a: Leucine aminopeptidase polymorphism in *Dreissena polymorpha* (Mollusca). Sixteen different individuals were analyzed. Phenotypes in the lower zone are scored (from left to right) as follows: 11, 11, 11, 12, 11, 12, 22, 11.

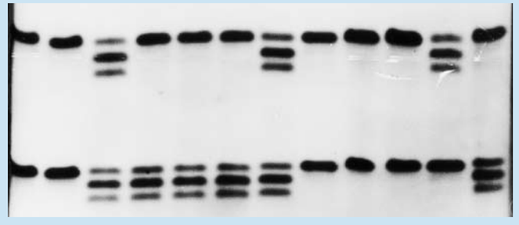


Figure 4b: Glucose-6-phosphate isomerase polymorphism in *Notodromas monacha* (Ostracoda). Twenty-four different individuals were analyzed. Phenotypes in the lower zone are scored (from left to right) as follows: 11, 11, 12, 12, 12, 12, 12, 11, 11, 11, 11, 12.

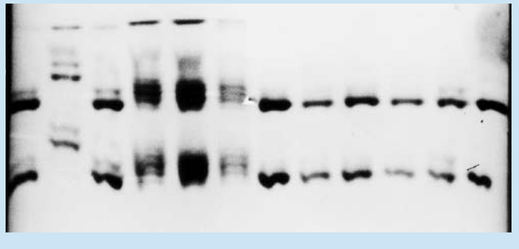


Figure 4c: Lactate dehydrogenase polymorphism in *Daphnia pulex* (Cladocera). Twelve individuals were scored with two duplicate zones of application. Phenotypes in each zone are scored (from left to right) as follows: 22, 11, 22, 12, 12, 12, 22, 22, 22, 22, 22, 22.

4.2 Equipment Requirements

1. **Gel tanks**
   May be purchased or manufactured in any workshop. Note that the electrode wire should be made of platinum.

2. **Power supply**
   The E.C. minicell power supply available for either 110 or 220 volt input is an excellent unit. The supply will run two tanks at one time. It is produced by:
   E.-C. Apparatus Corp.
   3831 Tyrone Blvd. N.
   St. Petersburg, Fla.,U.S.A. 33709
   U.S. Toll Free- 1-800-624-2232
   Outside U.S.- Telex 51-4736 HALA.

3. **Applicator kit and cellulose acetate plates**
   Kit includes 2 sample well plates, an applicator, and an aligning base available from:
   Helena Laboratories
   P.O. Box 752
   Beaumont, Texas
   U.S.A., 77704-0752
   U.S. Toll Free 800-231-5663
   Outside U.S.A., 409-842-3714

We use the Helena Super Z-12 applicator kit (Catalog # 4093), and 76x76 mm Titan® III Cellulose Acetate plates (Catalog # 3033).

Additional useful items

1. **Refrigerator** – refrigerator section for buffer and chemical stock solution storage
   – freezer section for chemical storage
2. **Microwave oven** – for melting agar
3. **Drying oven** – for agar storage, gel incubation and gel drying
4. **Ultrafreezer/cryogenic storage system** – for sample storage at -70°C
5. **Light box** – for scoring gels
6. **Pipettors (fixed volumes)** – 5, 10, 50, 100 µl

4.3 Gel Interpretation

With the development of cellulose acetate electrophoresis, the acquisition of allozyme data has been tremendously simplified. This is dangerous – the interpretation of these data still requires considerable experience, and most individuals need at least 6 months before they are either fully confident or competent in gel scoring. The interpretational difficulties are greatest in the analysis of enzymes that exist as multiple isozymes. For example, many species have 4-6 different gene loci which produce enzymes with esterase activity. When a gel is stained for esterases, the products of all these loci costain. Similarly if one stains for general proteins, the gel often has 20-30 zones of staining. The interpretation of such variation is something that should be attempted only after one gains considerable experience.

It is best for a novice to work with a diploid, bisexual taxon in which one can verify the validity of gel scoring efforts by comparing the inferred genotypic frequency distribution to that expected at Hardy-Weinberg (H.W.) equilibrium. Past work has shown that genotypic frequencies at enzyme loci are almost always in H.W. equilibrium in sexual taxa. This restraint imposes no limits on the taxa available for study when working with homeotherms – there aren't any asexual birds or mammals. However, in other groups one must be careful – asexual and/or polyploid taxa are common. The study of patterns of variation in asexual groups is complicated by the fact that genotypic frequencies often deviate from H.W. expectations. Moreover, asexuals are often polyploid and show either tri- or tetrasomic inheritance patterns. Problems arising from polyploidy are not restricted to asexuals, but are also frequently encountered in plants and some animal groups (eg. salmonid fish are all polyploid). Work on such groups is no place for the neophyte!

There are three books available which provide a useful introduction to the nuances of gel scoring. Manwell and Baker (1970) provide a summary of allozyme variation in a broad range of taxa. By contrast, the treatise by Harris and Hopkinson (1976) focuses solely on patterns of allozyme variation in the human species. Finally, a recent book by Richardson et al (1986) provides a good coverage of allozyme phenotypes in a variety of taxa.

The present handbook does not attempt to re-

their original intensity when rehydrated. Notes may be made on the mylar side of the gel plate using a water insoluble marker. We retain all of our gels until a study is complete to permit a re-examination of gel scoring in light of, for example, the detection of a new allele in later gels.

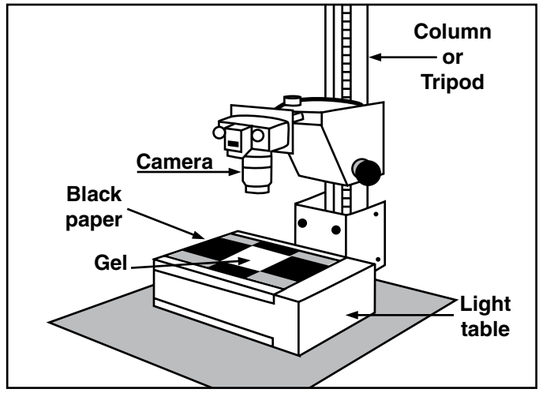
2.10 Photography

2.10.1 Equipment Needed

1) Light table, daylight balanced (3400 kelven) If other than daylight balanced, exposure time may need to be lengthened or shortened according to the strength of the light.
2) 50mm macro lens, for close-up, full frame photographs
   A normal 50mm lens may be used, but the gel will appear smaller within the frame of the film.
3) Photography column or vertical tripod for mounting the camera

2.10.2 Photography Set Up

Mount the camera on the photography column about 8 to 10 inches above the light table. (A normal 50mm lens will need to be further from the light table and thus will require a longer exposure time to compensate for the added distance the light must travel to reach the film.) Set the gel on the light table and adjust the height of the camera to frame the gel as fully as possible. Place black paper around the gel in box frame fashion. This way, only light that comes through the gel reaches the camera. This enhances the appearance of the electrophoretic pattern.



2.10.3 Suggested Aperture and Shutter Speed Settings

The following F/stops and shutter speed settings are recommended as a good starting point for a test run. Shutter speed (not F/stop) settings may need to be lengthened or shortened depending on the type of camera lens, light table, density of the gel, etc.

Plus-X Pan ASA/ISO 125 Black-and-White Film
F/5.6 @ 1/125 Sec.
F/5.6 @ 1/60 Sec.
F/5.6 @ 1/30 Sec.
F/5.6 @ 1/15 Sec.
F/5.6 @ 1/4 Sec.
F/5.6 @ 1/2 Sec.
F/5.6 @ 1 Sec.

EPP Ektachrome Plus ASA/ISO 100 Color Slide Film
F/5.6 @ 1/125 Sec.
F/5.6 @ 1/60 Sec.
F/5.6 @ 1/30 Sec.
F/5.6 @ 1/15 Sec.
F/5.6 @ 1/8 Sec.
F/5.6 @ 1/4 Sec.
F/5.6 @ 1/2 Sec.
F/5.6 @ 1 Sec.
F/5.6 @ 2 Sec.

Kodacolor ASA/ISO 200 Color Print Film
F/5.6 @ 1/125 Sec.
F/5.6 @ 1/60 Sec.
F/5.6 @ 1/30 Sec.
F/5.6 @ 1/15 Sec.
F/5.6 @ 1/8 Sec.
F/5.6 @ 1/2 Sec.
F/5.6 @ 1 Sec.

3 Gel Cookbook

3.1 Stain Recipes

The following section of the handbook includes recipes for 30 different enzymes. The recommended enzyme name and its Enzyme Commission (EC) number is provided (International Union of Biochemistry Nomenclature Committee 1984) with its usual abbreviation. Most stain mixtures consist of 5-10 ingredients. The recipes indicate the amount of each reagent required, and information on reagent concentrations is provided in Section 4.1 (p. 22). Ingredients marked with an asterisk (*) are optional, in the sense that staining activity is usually adequate in their absence. Optional ingredients are either stain buffers or metallic cofactors. If staining fails or is weak in their absence, their addition may help. Ingredients marked with a double asterisk (**) are either photosensitive (eg. dye salts, PMS) or labile (eg. linking enzymes). They should be added to the stain mixture immediately before its use.

Before using the recipes, two points should be noted. Firstly, care should be taken when handling some of the chemicals due to their known or suspected carcinogenic or mutagenic properties. Caution labels appearing on bottles should be noted and the proper precautions taken. Secondly, many of the recipes include G6PDH as an ingredient. We invariably use G6PDH isolated from Leuconostoc. This enzyme employs NAD rather than NADP as a cofactor. As NAD costs less (only 10% as much as NADP), use of G6PDH from Leuconostoc is desirable from an economic standpoint. Other forms of G6PDH require NADP as a cofactor, and if used NAD should be replaced by NADP.

Our recipe list is far from complete. It does include most of the enzymes which are "good performers"; that is those which usually stain intensely and are not prone to degradation. Our list has been developed primarily as a result of work with invertebrates, and individuals working on vertebrates or plants will undoubtedly identify gaps in our repertoire. Your pet enzyme can usually be adapted from a starch gel recipe by miniaturizing stain volumes and increasing reagent concentrations.

Following each enzyme recipe, four annotated comments are presented. The first of these annotations provides information on the quaternary structure of the enzyme. The quaternary structure of most enzymes is a conserved trait (Ward 1977), but some show a flexible structure. This is particularly true when several isozymes are present. In this case, one isozyme can have a monomeric structure and the other a multimeric structure. A brief discussion of the impact of quaternary structure on allozyme phenotypes is provided in Section 4.3 (p. 24). The second annotation provides information on the number of isozymes – ie. different gene loci which produce an enzyme with this substrate specificity. The number of isozymes is fairly stable among taxa for most enzymes, but certain peripheral enzymes (eg. esterases, phosphatases) exhibit considerable variation in the number of isozymes. In the case of this annotation, the information provided is based on our experience with invertebrate taxa. The third annotation provides information on the need for specific grinding buffers and specific run times. The final annotation indicates buffer systems which can be employed. When there is a choice of buffers, the optimal system will be indicated.

Maleic acid		
L-Malic acid		M-9138
D-Mannose-6-phosphate†	20	M-6876
β-Mercaptoethanol		
MTT	10	M-2128
NAD†	2	N-7381
NADP†	2	N-0505
α-Napthyl acetate		N-6875
α-napthyl acid phosphate		N-7000
Na_2HAsO_4	10	A-6756
L-Phenylalanyl-L-leucine (dipeptide)	10	P-3876
L-Phenylalanyl-L proline (dipeptide)	10	P-6258
6-Phosphogluconic acid†	20	P-7877
Phospho-L-arginine	20	P-5139
PMS	2	P-9625
Polyvinylpolypyrrolidone		P-6755
Polyvinylpyrrolidone		PVP-40
Pyridoxal-5-phosphate		P-9255
Pyruvic acid		P-2256
Sodium azide	250	S-2002
Starch (soluble potato)		S-2630
D-(+)Trehalose	10	T-5251
Trizma base		T-1503

Enzymes*	**units/ml**	**Catalog #**
Aldolase	200	A-1893
L-amino oxidase	10	A-5174
G3PDH	2650	G-0763
G6PDH * (from Leuconostoc, uses NAD as cofactor)	300	G-5885
* Many other types of G6PDH are available, but they use NADP as a cofactor. If such enzymes are used, NAD should be replaced with NADP in all recipes which include G6PDH.		
α-Glycerophosphate dehydrogenase	80	G-6751
Hexokinase	250	H-5500
IDH	50	I-1877
L-LDH	2750	L-2500
MDH	200	M-7383
Peroxidase	1000	P-8125
GPI	600	P-5381

† Indicates solutions which should be fixed with 1 µl sodium azide stock/ml solution for storage.
*Many of the enzymes are only available as concentrated solutions or as solids. Dilute to the concentrations shown in this table before use.

4 Background Information

4.1 Chemical List

The concentrations and manufacturer listed for the chemicals should be considered suggestions only. If an enzyme stains too intensely, concentrations of some of the components may be reduced. Obviously, the expensive ones should be reduced first. Conversely, concentrations of some solutions may need to be increased if staining intensity is weak.

Chemical	Conc mg/ml	Sigma Catalog #
cis-Aconitic acid	10	A-3412
ADP	10	A-2754
4-(3-Aminopropyl) morphine (Aldrich Catalog #12, 309-9)		
Aspartic acid		A-9006
ATP	10	A-5394
Benzaldehyde		B-6259
Benzidine DiHCl		B-3125
Citric acid		C-0759
o-Diansidine (di-HCl)	4	D-3252
Fast Blue BB salt		F-0250
Fast Red TR salt		F-1500
Fast Black K salt		F-7253
D-Fructose-1,6-diphosphate†	100	F-0377
Fructose-6-phosphate†	20	F-3627
Fumaric acid†	100	F-1506
D-glucose	315	G-5000
Glucose-1-phosphate† (grade III)	50	G-7000
(grade VI)	50	G-1259
D-Glucose-6-phosphate†	20	G-7879
DL-α-Glycerophosphate†	40	G-2138
Glycine		G-7126
Hypoxanthine†	10	H-9377
DL-Isocitric acid†	100	I-1252
α-Ketoglutaric acid		K-1875
DL-Lactic acid		L-1375
L-Leucine-b β-napthylamide HCl	0.5	L-0376
$MgCl_2$	20	

† Indicates solutions which should be fixed with 1 µl sodium azide stock/ml solution for storage.

1. Aconitate Hydratase (ACON) EC 4.2.1.3

16 drops cis-Aconitic acid (pH adjusted to 8.0)
1.5 ml NADP
6 drops $MgCl_2$*
5 drops MTT
5 drops PMS**
10 µl IDH**
2 ml agar

Quaternary structure – monomer

Number of isozymes – two (two loci), slower zone probably is mitochondrial form of the enzyme

Notes – grind tissues in NADP

Buffer system – CAAPM

2. Adenylate Kinase (AK) EC 2.7.4.3

0.6 ml Tris HCl, pH=7.0*
1.5 ml NAD
1.5 ml ADP solution
6 drops $MgCl_2$
5 drops MTT
5 drops PMS**
15 µl Hexokinase**
5 µl G6PDH**
2 ml agar

ADP Solution
0.10 g ADP
3.15 g D-glucose
10.0 ml water

Quaternary structure – monomer

Number of isozymes – two or more zones, the genetic control is uncertain

Buffer system – TG

3. Alcohol Dehydrogenase (ADH) EC 1.1.1.1

0.6 ml Tris HCl, pH=7.0*
1.5 ml NAD
5 drops MTT
3 drops ethanol or isopropanol
5 drops PMS**
2 ml agar

Quaternary structure – dimer

Number of isozymes – 1 or 2

Buffer system – TG

*Optional ingredient

**Labile or photosensitive, add immediately before use

4. Aldehyde Oxidase (AO) EC 1.2.3.1

0.6 ml Tris HCl, pH=8.0*
1 drop Benzaldehyde
5 drops MTT
5 drops PMS**
2 ml agar

Quaternary structure – dimer

Number of isozymes – 1 to 3

Notes – With the addition of 1.5 ml NAD, this reaction mixture will stain for aldehyde dehydrogenase (AD).

Buffer system – TG

5. Alkaline Phosphatase (ALP) EC 3.1.3.1

2 ml α-Napthyl acid phosphate solution
5 drops $MgCl_2$
5 drops saturated solution of Fast Blue BB salt solution**

α-Napthyl acid phosphate solution
50 ml Tris HCl, pH=9.0
200 mg NaCl
100 mg polyvinylpyrrolidone*
10 mg α-Napthyl acid phosphate

Quaternary structure – monomer, dimer

Number of isozymes – 2

Notes – α-napthyl acid phosphate solution is not stable. Small batches should be made and frozen.

Buffer system – CAAPM

6. α-Amylase (AMY) EC 3.2.1.1

2 ml starch solution
2 ml agar

Incubate for 15 minutes. Remove the agar overlay by carefully peeling it back. Place plate in a dish of iodine solution until staining occurs. Remove the plate from the iodine solution and rinse with water thoroughly.

Starch solution
33 ml 1M K_2HPO_4
66 ml 1M KH_2PO_4
600 mg NaCl
10 gm soluble starch
900 ml water

Iodine solution
16.6 gm KI
25.4 gm I_2
2 l water
Dilute 1:2 for use

Quaternary structure – monomer

Number of isozymes – 3; the lowest migrating zone stains most intensely

Notes – negatively staining system; gel remains white where enzyme activity is present

Buffer system – TG

*Optional ingredient

**Labile or photosensitive, add immediately before use

3.2 Gel and Electrode Buffer Recipes

We have aimed to keep buffer diversity to a minimum and have obtained excellent resolution for each of the 29 enzymes listed in Section 3.1 using just two buffers (TG and CAAPM). The primary difference between these two buffers is their pH. TG has a pH of 8.5, whereas CAAPM has a pH of 7.0. In general, high pH buffers such as TG provide better separation of variants than do low pH buffers. However, many enzymes are more stable at a lower pH and one must occasionally use the latter buffer. If you fail to obtain activity for a specific enzyme or if resolution is poor, you may wish to experiment with other buffers. There is a host of possibilities (Harris and Hopkinson 1976), but we suggest that you restrict your efforts to continuous buffer systems (ie. those in which the gel soaking buffer and tank buffer are identical).

CAAPM

42.0 g Citric acid (anhydrous)

50.0 ml 4-(3-aminopropyl) morpholine

Make up to 1 liter. Dilute 1:4 CAAPM buffer:water for use.

Tris Glycine (TG)

30 g Trizma base

144 g Glycine

Make up to 1 liter. Dilute 1:9 TG:water for general use.

3.3 Stain Buffer Recipes

0.1 M Tris Maleate Buffer, pH=5.3

1.2 g Trizma base

1.2 g maleic acid

2.4 ml 1M NaOH

Dilute to 100 ml with water

0.1 M Sodium Phosphate Buffer

30.5 ml 0.2M Na_2HPO_4

19.5 ml 0.2M NA H_2PO_4

Dilute to 100 ml with water. Adjust pH to 7.0.

0.09M Tris HCl pH=7.0

44.4 g Trizma Base

350 ml 1M HCl

Make up to 4 liters. Check and adjust pH as necessary.

0.09M Tris HCl pH=8.0

44.4 g Trizma Base

248 ml 1M HCl

Make up to 4 liters. Check and adjust pH as necessary.

0.20M Tris HCl pH=9.0

98.6 g Trizma Base

120 ml 1M HCl

Make up to 4 liters. Check and adjust pH as necessary.

3.4 Agar Overlay

4.0 g Bacterial grade agar

250 ml water

Heat the mixture until it boils vigorously. This is most easily accomplished by heating for 2-3 minutes in a microwave. Store covered at 60°C between use.

Grinding Buffer for Plants

10 ml 0.1 M Tris HCl, pH=8.0

0.5 g β-mercaptoethanol

50-500 mg Polyvinylpolypyrrolidone (optional), may be added as a solid at the time of grinding. The amount will vary with the quantity of tissue being homogenized and the volume of grinding buffer added.

29. Triose-Phosphate Isomerase (TPI) EC 5.3.1.1

15 drops TPI substrate
1.5 ml NAD
5 drops Na_2HAsO_4
5 drops MTT
5 drops PMS**
15 µl G3PDH**
2 ml agar

TPI Substrate

20 ml 0.02M Tris HCl, pH=8.0*
650 mg DL-α-glycerophosphate
220 mg Pyruvic acid
20 mg NAD
20 µl Glycerophosphate dehydrogenase
20 µl Lactate dehydrogenase

Incubate at 37°C for 2 hours. The reaction is then stopped by dropwise addition of concentrated HCl until pH=2.0. The pH should then be readjusted to 8.0 immediately.

Quaternary structure – dimer

Number of isozymes – 1

Notes – The TPI substrate is time consuming to make, but once generated should be stable for approximately one month. As an alternative, 200 µl of dihydroxyacetone phosphate can be employed as a substrate but it is expensive.

Buffer system – TG (preferred), CAAPM

30. Xanthine Dehydrogenase (XDH) EC 1.1.1.204

1.0 ml Tris HCl, pH=8.0*
1.5 ml NAD
20 drops Hypoxanthine
5 drops MTT
5 drops PMS**
2 ml agar

Quaternary structure – dimer

Number of isozymes – 1

Notes – hypoxanthine does not easily dissolve in water. It can be dissolved in acetone or alternatively solubilized by heating before adding to stain mixture.

Buffer system – TG (preferred), CAAPM

*Optional ingredient **Labile or photosensitive, add immediately before use

7. Arginine Kinase (ARK) EC 2.7.3.3

0.5 ml Tris HCl, pH=8.0*
1.5 ml NADP or NAD
5 drops $MgCl_2$
5 drops Phospho-L-arginine
5 drops ADP + D-glucose solution
5 drops MTT
5 drops PMS**
10 µl Hexokinase**
10 µl G6PDH**
2 ml agar

Quaternary structure – monomer

Number of isozymes – 1 or more loci, varies among taxa

Buffer system – TG

8. Asportate Amiro Transferase (AAT) EC 2.6.1.1

3 ml Solution #1
10 drops Fast Blue BB salt (saturated solution)**
2 ml agar

Solution #1

200 ml 0.1M Sodium Phosphate, pH=7.0
10 mg Pyridoxal-5-phosphate
460 mg L-Aspartic acid
260 mg α-Ketoglutaric acid
Adjust to pH=7.4. This step is critical!

Quaternary structure – dimer

Number of isozymes – 2; the anodal zone corresponds to the supernatant form, the weakly cathodal zone to the mitochondrial form

Notes – Solution #1 degrades in a week. Batches should be subdivided and frozen until required.

Buffer system – TG

9. Carbonate Dehydratase (CD) EC 4.2.1.1

Soak gel for 5 minutes in 0.1% bromothymol blue solution. Blot the gel plate and then pass a stream of CO_2 over its surface.

Bromothymol blue solution

0.1 g bromothymol blue
100 ml 0.1 M Tris HCL buffer (pH = 9.0)

Quaternary Structure – Monomer

Number of isozymes – 2

Notes – Activity appears rapidly then fades
– Dry ice in a container with the gel plate can be used to produce the CO_2

Buffer system – TG

*Optional ingredient **Labile or photosensitive, add immediately before use

10. Carboxylesterase (EST) EC 3.1.1.1

2.0 ml 0.1M Tris maleate, pH=5.3
200 µl α-naphthyl acetate solution
10 drops saturated fast red TR salt**
2.0 ml agar

α-naphthyl acetate solution
10 ml water
10 ml acetone
0.1g α-naphthyl acetate

Quaternary structure – monomer, dimer

Number of isozymes – at least 4

Notes – run in CAAPM at 130 volts for a minimum of 20 minutes

Buffer system – CAAPM

11. Fumarate Hydratase (FUM) EC 4.2.1.2

1.0 ml Tris HCl, pH=7.0*
1.5 ml NAD
5 drops Fumaric acid (adjust to pH=8.0)
5 drops MTT
5 drops PMS**
50 µl MDH**
2 ml agar

Quaternary structure – tetramer

Number of isozymes – 1

Buffer system – TG

12. Glucose-6-Phosphate Dehydrogenase (G6PDH) EC 1.1.1.49

0.6 ml Tris HCl, pH=8.0*
1.5 ml NADP
12 drops D-Glucose-6-phosphate
6 drops $MgCl_2$
5 drops MTT
5 drops PMS**
2 ml agar

Quaternary structure – tetramer

Number of isozymes – 1

Notes – enzyme is labile and degrades if tissues are not homogenized in NADP

Buffer system – TG

*Optional ingredient **Labile or photosensitive, add immediately before use

26. 6-Phosphogluconate Dehydrogenase (6PGDH) EC 1.1.1.44

0.6 ml Tris HCl, pH=8.0*
1.5 ml NADP
6 drops 6-Phosphogluconic acid
6 drops $MgCl_2$
5 drops MTT
5 drops PMS**
2 ml agar

Quaternary structure – dimer

Number of isozymes – 1

Buffer system – TG (preferred), CAAPM

27. Superoxide Dismutase (SOD) EC 1.15.1.1

1.0 ml Tris HCl, pH=8.0
5 drops MTT
5 drops PMS**
2 ml agar

Quaternary structure – dimer

Number of isozymes – 1 or 2

Notes – expose gel to light while incubating
– this enzyme produces white bands on diffuse tetrazolium background
– this enzyme often does not stain as well on cellulose acetate as it does in acrylamide or starch gels

Buffer system – TG

28. α,α-Trehalase EC 3.2.1.28

1.0 ml Tris HCl, pH=8.0*
5 drops Trehalose
4 drops ATP
1.5 ml NADP
5 drops $MgCl_2$
5 drops MTT
5 drop PMS**
5 µl Hexokinase**
5 µl G6PDH**
2 ml agar

Quaternary structure – monomer

Number of isozymes – 1

Notes – migrates cathodally in some taxa

Buffer system – TG

*Optional ingredient **Labile or photosensitive, add immediately before use

23. Mannose-6-Phosphate Isomerase (MPI) EC 5.3.1.8

1.0 ml Tris HCl, pH=8.0*
1.5 ml NAD
5 drops D-Mannose-6-phosphate
5 drops MTT
5 drops PMS**
5 µl PGI**
20 µl G6PDH**
2 ml agar

Quaternary structure – monomer

Number of isozymes – 1

Buffer system – TG

24. Peptidase (PEP) EC 3.4.11 or 3.4.13

2.0 ml 0.02M NA_2HPO_4 (adjusted to pH=7.5)
4 drops Peroxidase
8 drops o-Dianisidine (diHCl salt)
2 drops $MnCl_2$
8 drops Peptide[2] (use desired dipeptide)
4 drops L-amino acid oxidase**
2 ml agar

Quaternary structure – some are dimers; others are monomers

Number of isozymes – 2-3 for most dipeptide substrates

Notes – Dipeptides commonly used include: leucyl alanine, phenylalanine proline, and leucyl glycine

Buffer system – TG

25. Phosphoglucomutase (PGM) EC 5.4.2.2

1.0 ml Tris HCl, pH=8.0*
1.5 ml NAD
5 drops $MgCl_2$
5 drops Glucose-1-phosphate solution
5 drops MTT
5 drops PMS**
20 µl G6PDH**
2 ml agar

Quaternary structure – monomer

Number of isozymes – 1

Buffer system – TG

Glucose-1-Phosphate solution

250 mg glucose-1-phosphate, Grade III
250 mg glucose-1-phosphate, Grade VI
5.0 ml water

*Optional ingredient

**Labile or photosensitive, add immediately before use

13. Glucose-6-Phosphate Isomerase (GPI) EC 5.3.1.9

1.0 ml Tris HCl, pH=8.0*
1.5 ml NAD
5 drops Fructose-6-phosphate
5 drops MTT
5 drops PMS**
10 µl G6PDH**
2 ml agar

Quaternary structure – dimer

Number of isozymes – 1

Buffer system – TG

14. Glyceraldehyde-3-Phosphate Dehydrogenase (G3PDH) EC 1.2.1.12

1.5 ml NAD
1 ml D-Fructose-1,6-diphosphate solution
5 drops Na_2HAsO_4
5 drops MTT
5 drops PMS**
2 ml agar

Quaternary structure – tetramer

Number of isozymes – 1

Buffer system – TG (preferred), CAAPM

Fructose-1, 6-diphosphate solution

100 mg fructose-1, 6-diphosphate
50 µl Aldolose
4.0 ml water
Incubate at 37°C for 1 hour
Freeze in 1 ml aliquots

15. Glycerol-3-Phosphate Dehydrogenase (GPDH) EC 1.1.1.8

0.6 ml Tris HCl, pH=8.0*
1.5 ml NAD
22 drops DL-α-Glycerophosphate
5 drops MTT
5 drops PMS**
2 ml agar

Quaternary structure – dimer

Number of isozymes – 1

Buffer system – CAAPM

16. Hemoglobin (HEM)

Saturated benzidine in absolute ethanol
Make 1:5 solution of glacial acetic acid:benzidine
Incubate plate for 5 minutes
Remove and incubate plate in 5%H_2O_2

Quaternary structure – dimer in many taxa

Number of isozymes – 2 or more loci

Buffer system – TG

*Optional ingredient

**Labile or photosensitive, add immediately before use

17. Hexokinase (HEX) EC 2.7.1.1

0.6 ml Tris HCl, pH=7.0*
1.5 ml NAD (2.5 mg/ml)
16 drops ATP solution
5 drops $MgCl_2$
5 drops MTT
5 drops PMS**
10 µl G6PDH**
2 ml agar

ADP Solution
0.25 g ATP
5.0 g D-glucose
10.0 ml water

Assumed quaternary structure – monomer

Number of isozymes – 1

Buffer system – TG (preferred), CAAPM

18. Isocitrate Dehydrogenase (IDH) EC 1.1.1.42

1.0 ml Tris HCl, pH=7.0*
1.5 ml NADP
15 drops DL-Isocitric acid
8 drops $MgCl_2$
5 drops MTT
5 drops PMS**
2 ml agar

Quaternary structure – dimer usually, monomer in some taxa

Number of isozymes – 2. The faster zone (supernatant form) is subject to breakdown. The slower zone (mitochondrial form) stains intensely.

Notes – grind in NADP

Buffer system – TG (preferred), CAAPM

19. Lactate Dehydrogenase (LDH) EC 1.1.1.27

1.0 ml Tris HCl, pH=7.0
1.5 ml NAD
4 drops DL-Lactic acid
5 drops MTT
5 drops PMS**
2 ml agar

Quaternary structure – tetramer

Number of isozymes – 2-3 isozymes in plants and vertebrates, but just 1 in many invertebrates

Notes – this enzyme commonly stains as "nothing" dehydrogenase

Buffer system – TG

*Optional ingredient

**Labile or photosensitive, add immediately before use

20. Leucine Aminopeptidase (LAP) EC 3.4.11.1

2.0 ml L-Leucine-β-napthylamide solution
1 drops saturated fast black K salt**
2.0 ml agar

L-Leucine-β-naphthylamide solution
20 ml 0.1M Tris maleate, pH=5.3
10 mg L-Leucine-β-naphthylamide HCl

Quaternary structure – monomer

Number of isozymes – 1

Notes – run in CAAPM at 130 volts for a minimum of 20 minutes

Buffer system – CAAPM

21. Malate Dehydrogenase (MDH) EC 1.1.1.37

1.0 ml Tris HCl, pH=8.0*
1.5 ml NAD
13 drops Malic substrate
5 drops MTT
5 drops PMS**
2 ml agar

Malic substrate
180 ml water
20 ml Tris HCl, pH=9.0
3.68 gm L-Malic acid
Adjust to pH=8.0.

Quaternary structure – dimer

Number of isozymes – 2; cathodal band is mitochondrial form, anodal band is supernatant form

Buffer system – for MDH-1 – TG (preferred), CAAPM; for MDH-2 – CAAPM

22. Malate Dehydrogenase $NADP^+$(ME) EC 1.1.1.40

0.6 ml Tris HCl, pH=8.0*
1.5 ml NADP
12 drops Malic substrate
2 drops $MgCl_2$
5 drops MTT
5 drops PMS**
2 ml agar

Assumed quaternary structure – tetramer

Number of isozymes – 1

Buffer system – TG

*Optional ingredient

**Labile or photosensitive, add immediately before use

4.4 Taxa Studied

The following is a list of taxa in which levels of allozyme variation have been investigated using cellulose acetate electrophoresis. The list is not complete, but should be considered as a sample of the prior studies published.

PLANTS

Solidago sempervirens
Innes and Hermenutz 1988
Betula glandulosa
Hermanutz et al. 1988
Polytrichum juniperinum
Innes 1990

INVERTEBRATES

Protozoa

Trypanosoma cruzi
Lanham et al. 1981

Insecta

Tetranychus urticai
Easteal and Boussy 1987
Drosophila spp.
Easteal and Boussy 1987
Aphids
Hebert et al. 1991
Collembolans
Boileau et al. 1991
Aedes aegypti
Easteal and Boussy 1987

Crustacea

Cladocera
Daphnia spp.
Hebert 1985
Hebert et al. 1988; 1989a
Innes 1989
Innes et al. 1986
Weider and Hebert 1987a, b
Weider et al. 1987
Daphniopsis ephemeralis
Schwartz and Hebert 1987
Simocephalus spp.
Boileau et al. 1991
Polyphemus pediculus
Weider 1989
Anostraca
Artemiopsis steffansoni
Boileau et al. 1991
Branchinecta paludosa
Boileau et al. 1991
Notostraca
Lepidurus arcticus
Beaton and Hebert 1988
Copepoda
Heterocope septentrionalis
Boileau and Hebert 1988a
Leptodiaptomus spp.
Boileau and Hebert 1988b
Ostracoda
Cypridosis vidua
Havel and Hebert 1989
18 species
Havel et al. 1990

Mollusca

Dreissena polymorpha
Hebert et al. 1989b

Turbellaria

Mesostoma lingua
Hebert and Payne 1985
Mesostoma ehrenbergii
Hebert and Beaton 1990

Onychophora

Peripatus jamaicensis
Hebert et al. 1991

VERTEBRATES

Pisces

Stizostedium vitreum
Billington et al. 1990
22 marine species
Gauldie and Smith 1978

Mammalia

Pistrellus spp.
Adams et al. 1987b
Eptesicus spp.
Adams et al. 1987a

4.5 Troubleshooting

The beginner should not expect perfect gels on the first attempt. Don't give up! Often the problem is minor and the solution simple. Here are suggestions to overcome troubles encountered most often.

Symptom	**Check points**
No staining	Were all chemicals added to stain mixture? Were all chemicals fresh?
No migration of bands	Was the power supply turned on or plugged in? Were the electrodes submerged in the buffer?
Enzyme migrates in reverse	Was gel properly oriented in tank? Does the enzyme migrate cathodally? Were electrode connections reversed?
Enzyme stains weakly	Were all chemicals fresh? Were the extracts too dilute, or were too few applications made per load zone?
Enzyme stains too intensely	Were substrate concentrations and/or linking enzymes too high? Were there too many applications/load zone?
Wobbly bands	Did plates lie flat during gel run? Were wicks recently turned?
Wide fuzzy bands	Was plate sufficiently blotted? Was extract applied as thin lines? Was the applicator cleaned?
Extra unaccountable bands	Was the applicator cleaned?

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6 References

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