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P. G. Benson
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Inquiries to:
The Editor,
Fisheries Research Centre,
P.O. Box 297,
Wellington,
New Zealand.

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Contents

The first draft of this manual was produced in 1985 and submitted for publication in October 1986. Publication was delayed partly because P. G. Benson left New Zealand in 1985 and P. J. Smith was overseas for all of 1987. In the interim, some modifications were made to the electrophoretic techniques used at Fisheries Research Centre, and these have been incorporated in this manual.

Abstract

Benson, P. G. and Smith, P. J. 1988: A manual of techniques for electrophoretic analysis of fish and shellfish tissues. *N.Z. Fisheries Technical Report No. 13*. 32 p.

The gel electrophoresis methods used at Fisheries Research Centre are described. Methods for the collection, storage, and preparation of samples are outlined as are details of the starch gel, cellulose acetate gel, and iso-electric focusing techniques used. Staining methods to detect protein and enzymatic products and the interpretation of stained gels are discussed. Details of gel buffers and staining recipes are given.

Introduction

This manual describes the procedures developed and adapted at Fisheries Research Centre (FRC) for the electrophoretic analysis of fish and shellfish tissues. It has been produced in response to the many requests which have been received for technical details of our electrophoretic methods. Although electrophoresis is a standard laboratory procedure, the techniques have been modified at FRC for the routine running and testing of many fish or shellfish tissue samples with simplified equipment. The work has concentrated on tissue enzymes run in starch or cellulose acetate gels for population studies of teleosts, elasmobranchs, crustaceans, and molluscs. Iso-electric focusing (IEF) is used to analyse general proteins as biochemical fingerprints for species and fillet identification.

The theory of electrophoresis and its biological application

Electrophoresis is the term used to describe the movement of ions in solution under the influence of an electric field. If a direct current is transmitted through a medium containing two ionic species A^+ and B^-, the cationic (A^+) ions will move towards the cathode (the negative pole, usually coloured black), and the anionic (B^-) ions will move towards the anode (the positive pole, usually coloured red). The rate of movement of A^+ and B^- will be determined by the force to which they are subjected, i.e., $Q \times N$, where Q is the field strength and N the net charge of the ion. This force is opposed by a frictional force as the ion moves through the supporting medium. The frictional force is determined by the size and shape of the ion and the viscosity of the medium. Therefore, the migration rate of an ion depends on the applied current, the shape and size of the ion, and the net charge carried by the ion. Thus, ions which differ from each other in charge, shape, or size will also differ in migration rate and in theory can be separated by electrophoresis. However, two ions which show the same migration rates are not necessarily identical.

In zone electrophoresis, the separated ions remain as discrete areas or zones on the supporting medium and can be detected by histochemical staining. Zone electrophoresis was first used by König in 1937 to separate proteins in snake venom, but it was not until the development of starch gel electrophoresis and specific histochemical staining techniques in the late 1950s that the technique was used by geneticists. There was a rapid expansion in the use of electrophoretic techniques when it was realised that many enzymes and proteins show genetic variation. Genetic variation is exhibited as electromorphs, or protein bands, which migrate at different rates through the gel and are detected with specific enzyme, or protein, stains.

Sample collection and storage

When collecting and storing animal tissues for electrophoresis it is most important to prevent or minimise protein denaturation. This is best achieved by:

1. sampling freshly killed fish or shellfish, or sampling as soon as practicable after death for trawl-damaged specimens;
2. immediately and rapidly freezing the sampled tissues (dissected tissues should not be kept on deck for long periods);
3. transporting the frozen samples to the laboratory without any rise in temperature.

Liquid nitrogen vivostats, with a wide neck for the easy removal of samples, are the preferred equipment for freezing and storing samples at sea. The vivostats are light portable freezers that can be taken on any vessel, e.g., a liquid nitrogen vivostat with a capacity of 40 l can store up to 1500 tissue samples for 3 weeks. Additional supplies of liquid nitrogen may be needed to top up vivostats for longer sea voyages. The only drawback to the use of liquid nitrogen vivostats is that large cylinders are not permitted on small aircraft.

The fish tissues collected include whole blood, sera, eyes, livers, hearts, and white skeletal muscle. Eyes and hearts are removed whole, whereas livers and white skeletal muscle are sampled as half thumb-size pieces, the muscle being a small fillet from behind the vent. The tissues are put into pre-labelled minigrip plastic bags which are clip-sealed and dropped into liquid nitrogen. The temperature of liquid nitrogen (−195 °C) ensures that the samples are snap-frozen. Air must be squeezed out of the minigrip bag when it is sealed, to prevent the bag from exploding when it is dropped into the liquid nitrogen. The bags are not folded when they are put in the liquid nitrogen because the plastic becomes brittle at low temperatures and cracks on fold lines.

There are several sizes of minigrip bags, the most useful being about 90 × 50 mm, with a white label on one side for labelling with an oil-based marker pen. Plastic or borosilicate glass tubes can be used, but the frozen tissue samples are more difficult to remove from these without partially thawing the sample. If the tissue samples are small, they can be put into tubes and homogenised directly in these in the laboratory. Biological data (e.g., length and sex) of specimens can be recorded on the bag. Where more detailed information is collected, separate sheets are used to record the data, and the tissue bags or tubes are identified by a code number.

Blood samples require additional processing at sea, and for routine electrophoretic studies other tissues are preferred. Whole blood is collected from the gill or heart by puncture with a vacutainer or syringe needle. The size of the vacutainer or syringe is determined by the size of the fish to be sampled: a minimum draw of 2 ml is required, even for small fish, whereas up to 10 ml can be used for large fish such as tuna. Whole blood samples require an equal volume of preservative (6 parts 5% trisodium citrate in distilled water and 4 parts glycerol) to be added to prevent rupture of red cells during freezing. If vacutainers are used, the preservative can be added directly to the tube after blood collection; if syringes are used, a separate glass or plastic tube is required. The tube must be shaken to ensure mixing of blood and preservative, and a small air gap must be left between the blood-preservative mixture and bung to allow for expansion during freezing. The labelled tubes are stored at −20 °C or lower.

When serum is required, the blood sample is centrifuged at about 1000 rpm for 5 min and the clear top layer of serum is pipetted off into a clean labelled tube. Red-coloured serum samples should be discarded because they are contaminated with haemoglobin. Serum samples should be stored at −20 °C or lower.

Solid tissue samples remain in liquid nitrogen until the vivostat is returned to the laboratory, where they are removed and stored at −70 °C.

Other suitable but less preferred methods of storage collection in the field are:

1. Dry ice (−78 °C) in an insulated container. The disadvantage of dry ice is its short life (1–2 days).
2. Ship's freezer (often only −20 °C). The disadvantage is that the samples are not snap frozen, because of the higher temperature, but freeze gradually, and there is difficulty in trans-shipping samples from the vessel to the laboratory without thawing.

Sampling can occasionally be done in fish processing and packing factories or on trawlers in port. However, such fish may have been dead for several days, stored on ice. These fish are suitable for human consumption, but some of the more sensitive enzymes will have denatured, thus reducing the range of enzymes that can be successfully screened for in the samples. This method is not recommended for general sampling, but can be used in projects such as biochemical species identification based on stable enzymes (e.g., lactate dehydrogenase) or general proteins.

Marine invertebrates, such as squid and large crustaceans, can be dissected at sea and their tissues stored like fish. Smaller gastropods and bivalves can be sent live to the laboratory, packed in sacks dampened with sea water, inside an insulated container. Many species remain alive for several days with this method. Where sample sites are far from a rapid method of transport, shellfish can be shucked on site and the soft tissue frozen in minigrip bags. The dried and labelled shells can be stored separately if required. Alternatively, the adductor (foot muscle) and a piece of digestive gland can be removed and placed in separate labelled tubes. These are frozen in liquid nitrogen as outlined above.

Sample preparation

All stages of tissue preparation must be kept at 4–5 °C. If a walk-in cold room is not available, ice is essential. Tubes and buffers can be pre-chilled in the refrigerator, but must be held in an ice-water slurry on the bench. Tubes are numbered or put in numbered racks to avoid confusing the samples. The centrifuge tubes must be medium-walled rimless borosilicate tubes or disposable polypropylene tubes which withstand high speed centrifugation. The dimensions are selected to suit the centrifuge heads available and the size of the tissue samples. For small pieces of tissue, 10 mm (external diameter) × 75 mm tubes are used.

Tissue samples are ground up in an equal volume of cold distilled water for starch and cellulose acetate electrophoresis, whereas 4–5 volumes of distilled water are used per volume of tissue for IEF. A round bottom glass rod with a slightly narrower diameter than the internal diameter of the tube is used to macerate the sample in cooled distilled water or homogenising buffer. (Several homogenising buffers have been tested, but cooled distilled water has produced as good a resolution on the final stained gels.) During homogenisation the tube is held in an ice-water slurry. The glass rod is rinsed in distilled water and dried between samples. For samples collected from processing plants, an equal volume of 1% mercaptoethanol in 0.2 M tris-HCl pH 8.0 can be used as a homogenising buffer to help stabilise gel phenotypes. Mercaptoethanol is a sulphydryl reducing agent and reduces changes caused by interactions of the sulphydryl groups in the enzymes during storage.

Homogenised samples are allowed to stand at 4 °C for 15–30 min before centrifugation in a high-speed refrigerated centrifuge at 4 °C. The centrifuge and head are pre-cooled to 4 °C before the samples are added. The samples are centrifuged for about 10 min at 10 000–30 000 g. After centrifuging, the samples have separated into three layers: an upper oil or fat layer, varying in thickness depending on the tissue, species, and season at time of sampling; a clear supernatant containing the soluble enzymes and proteins; and a lower layer of tissue and cell debris. The sample for electrophoresis is removed from the clear middle layer and pipetted on to paper inserts for starch gels or into sample wells for loading on to cellulose acetate plates. Excess supernatant is pipetted into labelled minitubes (0.5 ml or less) and frozen at −70 °C for repeat tests. Enzymes in solution must be stored at −70 °C to retain activity; those stored at −20 °C rapidly lose activity. Blood samples require little laboratory preparation. They can be partially thawed and a subsample removed with a pipette for loading on to paper inserts or into sample wells.

In practice, many enzymes resolve well on gels without centrifuging the tissue sample. Where centrifuging is not required, a multiple grinding block can be used for homogenisation instead of individual tubes. This allows many samples to be ground at once. The block is pre-cooled in a refrigerator, and the tissue samples are added to each well, followed by an equal volume of cold distilled water. Blocks can be made up according to the size and number of tissue samples that need to be processed. For larvae and small tissue samples, a block with 48 holes (5 mm diameter × 5 mm deep) is used and the specimens are ground with a glass rod as described above. For larger tissue samples, such as a piece of liver or skeletal muscle, a 12 hole (15 mm diameter × 15 mm deep) block with matching 12 rod (10 mm diameter × 20 mm deep) block is used. The rods are about 5 mm less in diameter than the wells to allow adequate movement for grinding. For small tissue samples and larvae, staining intensity can be increased if homogenised samples are held at 4 °C, and covered to reduce evaporation, for 15–30 min before being loaded into gels. This probably allows soluble enzymes to diffuse out of the homogenised tissue. For starch gels, filter paper inserts are dipped directly into the wells, whereas for cellulose acetate gels, the samples are pipetted from the block into the cellulose acetate loading wells.

Starch gel electrophoresis

Introduction

Starch gel electrophoresis is used in many laboratories for population-genetic surveys in preference to other gel media, such as acrylamide or cellulose acetate, because it is easier to use and is cheaper. Starch gels give good resolution of most enzymes. Each gel can provide a lot of data if many samples are put in the gel and it is multi-sliced to stain for several enzymes.

Equipment

Stabilised power supply (300 V, 300 mA)
Gel mould and base plate
Gel slicing rods and 0.01–0.05 mm wire
Electrode buffer tanks and sponge wicks
Twinflex wire (to connect electrode tanks to power supply) and platinum electrode wire
Electric heating mantle and matching round bottom flasks
Electric stirrer and rod
Water flow, or electric, vacuum pump
Staining trays
Staining oven (37 °C)
Micropipette

Power supply. There are several power packs suitable for electrophoresis. Some are designed for a specific electrophoretic kit, whereas others are multi-purpose models. The power source should be capable of delivering either a constant voltage, or a constant current, though a constant voltage is mostly used in electrophoresis. For general starch electrophoresis, a power pack with a voltage output capability of at least 300 V (preferably 400 V) and a current output of at least 150 mA is required. Some power packs contain two independent power supplies which can be set at different voltages. Some also have two or more outlets from each supply so they can run two or more gels.

Gel mould. One mould is required for each gel. The gel mould consists of a base and four edging strips. A base made of glass plate measuring 210 × 180 × 2 mm is preferable to perspex or plastiplex because it does not warp. The edges of the glass plate should be bevelled for ease of handling. The edging strips determine the size of the gel mould: two of these (210 × 15 × 6 mm) are put on top of the glass plate on the long sides and the other two (150 × 15 × 6 mm) are put on top of the glass plate across each end. The four strips form a rectangular frame on the glass base and are held on by two rubber bands (Figure 1). The effective inside dimensions of the gel mould are 180 × 150 × 6 mm, which gives a total gel volume of 162 ml.

Electrode tanks. Two electrode tanks are required for each gel. Rectangular plastic boxes (available from most supermarket or hardware stores) measuring about 220–230 × 85–110 × 90–95 mm are suitable. The cathodal tank carries the cathode, a 1 mm thick stainless steel wire about 250 mm long; and the anodal tank carries the anode, a 0.2–0.5 mm thick platinum wire about 250 mm long. Platinum wire is essential at the anode because stainless steel rapidly oxidises when subjected to a current. One wick is required in each electrode tank to connect the buffer to the gel. Plain household sponges which are not soap impregnated (available from most hardware stores), or three layers of surgical gauze (available from pharmacies), are used as wicks. They should be 150 × 220–300 × 3–5 mm when wet, and they should be rinsed in electrode buffer before use.

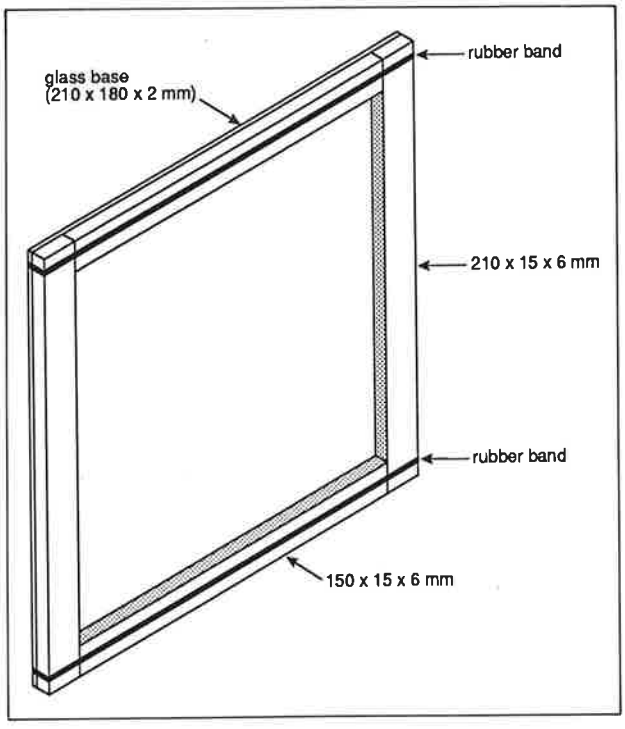


Figure 1: A starch gel mould consisting of a glass plate base and plastic edging strips held together by two rubber bands.

The electrode tanks are prepared by pouring the appropriate electrode buffer into both pre-washed tanks. The buffer should be freshly made, or less than 2 weeks old if stored in a refrigerator. The tanks are filled to a depth of about 3 cm, sufficient to cover the wick and electrode.

Preparation of starch gels

Starch gels are made up at 12% weight per volume. The properties of starch gels depend on the brand and batch of starch used. The weighed starch is put in a round-bottom borosilicate boiling flask. The appropriate volume of gel buffer is added (Figure 2a), each 30 g of starch requires 250 ml of buffer, and shaken vigorously to ensure that no lumps of starch are stuck to the inside edge of the flask and that all

of the starch is in suspension. The flask is put in an electric heating mantle and the contents are stirred by an electric laboratory stirrer (Figure 2b). This heating mantle should be able to accommodate boiling flasks of 1–4 l.

The gel is heated and stirred. Stirring is essential to maintain an even dispersion of the starch and prevent lumps forming in the gel. As the gel is heated, the starch suspension becomes viscous and the colour changes from a cloudy white to a clear grey. When the temperature has reached about 90 °C the gel is ready for pouring. **The gel must not be overheated or boiled.** It should be checked with a thermometer, but with experience the correct temperature can be judged by the colour and viscosity of the starch and by touch. The flask is taken from the heating mantle by use of a heat protective glove, and the gel is de-gassed with a water-flow vacuum pump or an electric vacuum pump (Figure 2c). This step is essential to remove the air beaten into the starch suspension by the stirrer. After de-gassing, the gel is poured into clean moulds on a level bench (Figure 2d). Each mould is over-poured to a reverse miniscus above the surface of the mould. The gels are cooled at room temperature for 30–60 min and then put in a refrigerator (4 °C) for at least 15 min before use. Once the gels have cooled, the top layer (miniscus) is sliced off with a fine wire (e.g., 0.007 mm 80 : 20 nickel-chromium alloy wire) (Figure 2e). This produces an even 6 mm thick starch gel ready for the sample inserts. Gels are made up fresh each day because they do not store well.

Insertion of homogenates into the starch gel

Filter paper inserts are prepared by cutting strips from sheets of filter paper. The inserts are 6 mm deep, to fit in the gel, and 2–4 mm wide. The width depends on the final resolution of the stained protein or enzyme bands and the number of samples to be fitted on the gel. A Whatman No. 1 filter paper is usually sufficient, but thicker papers can be used if more protein or enzyme is required in the gel. Thicker papers are essential for transferrins in serum samples. Thick inserts tend to cause the gel to split at the insert line during electrophoresis, but this can be overcome by removing the inserts after the first 30–40 min of the electrophoretic run and closing up the gel. Inserts should be cooled between two sheets of glass in a refrigerator before use. Samples are pipetted on to the cooled inserts, on a sheet of glass held in an ice tray to keep it cool during bench use. The pipette must be rinsed and blotted dry on tissue paper between samples.

An insert line is cut in the gel with a sharp scalpel, about 60 mm from one end. The two halves of the gel are gently pulled apart at this line, and the soaked inserts are put in the gel from left to right about 2 mm apart (Figure 2f). The soaked inserts are picked up with fine forceps and excess supernatant is blotted off. The inserts must be blotted or excess supernatant can run between the inserts in the gel and cross-contaminate samples. Up to 40 inserts can be placed across a standard starch gel. The gel is closed up by gently pushing the short gel mould plastic strips, and it is then placed across the two electrode tanks with the insert line at the cathodal end. For enzymes which migrate a short distance along the gel, two insert lines can be cut in the gel, thereby doubling the number of samples run per gel. Often the samples in the insert line closest to the cathodal end of the gel run farther than those in the more anodal insert line, so the position of the two insert lines has to be determined by trial and error.

Electrophoresis

The gel and wicks are covered by a sheet of thin clear plastic to reduce evaporation during the run. A sheet of glass (100 × 180 × 2 mm) is put on top of the plastic layer between the wicks, supported by the gel moulding. A cold freezer pack at 4 °C, or tray of ice slurry, is put on the glass plate to absorb heat from the gel during electrophoresis (Figure 3). Gels are run in a refrigerator to maintain buffers and gels at about 4 °C. For short runs, of about 3 h or less, gels can be run on a laboratory bench at room temperature with an ice pack on top of the gel.

The electrodes are connected to the power pack, the voltage is set for the particular buffer system (Appendix 1), the current is set at maximum, the power is switched on, and the gel is run for the recommended time (3–6 h, depending on the buffer system). After the specified run time, the power is switched off and the gel is prepared for staining.

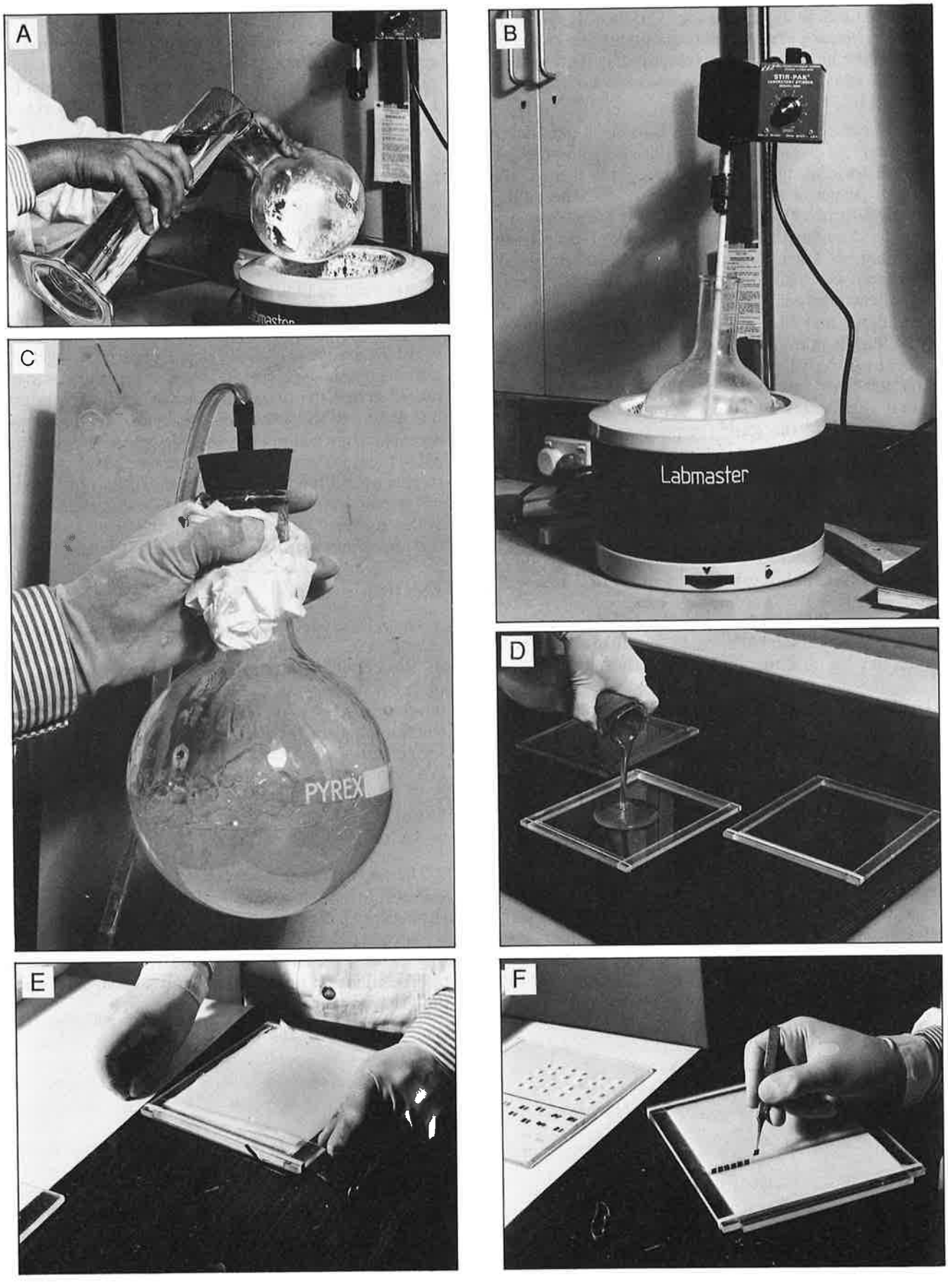


Figure 2: Preparation of starch gels. A, adding gel buffer to the starch; B, heating and stirring the gel in an electric heating mantle; C, de-gassing the hot gel; D, pouring the hot gel into the moulds; E, slicing off the top miniscus of the cooled gel; F, placing inserts into the gel.

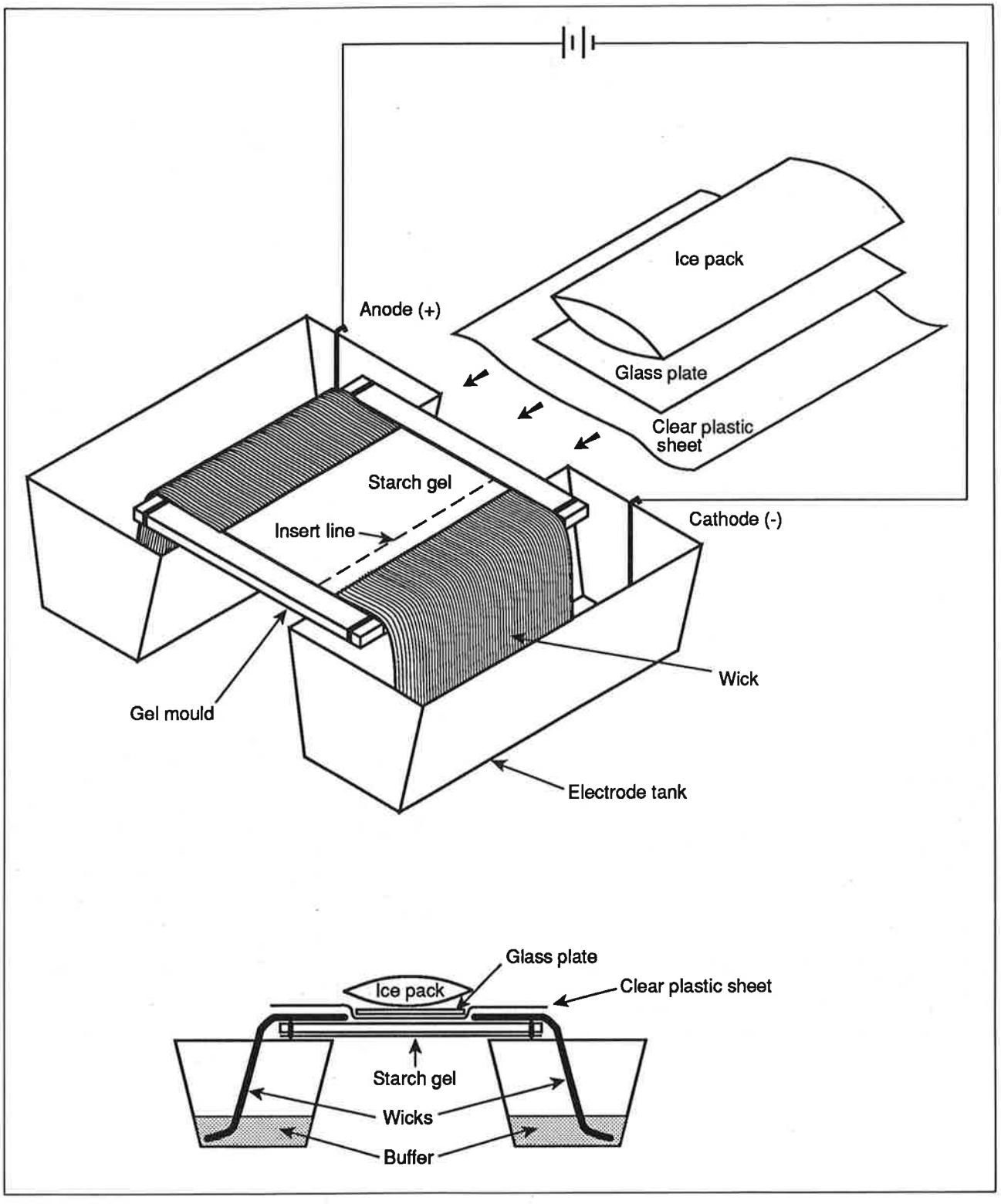


Figure 3: Arrangement of electrophoretic tanks and starch gel.

Cellulose acetate electrophoresis

Introduction

Most cellulose acetate gels are run with a kitset apparatus and preweighed chemicals. The Helena Laboratories system has been used at FRC because it is readily available and easy to use, though other kits have recently come on to the market.

The main advantages in using cellulose acetate rather than starch gels are the shorter running times (about 30–60 min, compared with 3–6 h for starch gels) and the much smaller volumes of staining solution required for a given number of samples (i.e., less than 5 ml of staining solution for a run of 24 samples, compared with 20 ml of staining solution for 24–40 samples on a starch slab). This can be a major saving when expensive enzymes are used in the staining solution. However, for several enzymes, better resolution is obtained in starch than in cellulose acetate gels, so both are used in initial screening programmes.

Equipment

Stabilised power supply (200 V, 150 mA)
Electrode tank
Gel loading base and sample applicator
Micropipette
Staining chamber

A power supply similar to that used for starch electrophoresis is suitable for cellulose acetate electrophoresis, but the maximum current used for cellulose acetate gels is generally lower, and a maximum output of 200 V is sufficient for most purposes.

Procedure

The appropriate buffer (Appendix 1) is poured into the anodal and cathodal chambers to a depth of 3–5 mm. A double layer of disposable paper wicks, supplied with the kit, is placed along the inside wall of each electrode chamber, which allows the wicks to soak up buffer (Figure 4a). The wick ends must dip beneath the surface of the buffer to make contact between gel and buffer. Wicks are not needed in other cellulose acetate kits because the gel is folded into the buffer chamber.

Cellulose acetate gel plates are purchased ready-made. They are soaked in the appropriate buffer for at least 10 min, before use, so they are adjusted to the correct pH. Care must be taken to lower the plates slowly into the buffer solution to prevent air pockets forming between the gel and the plastic base (Figure 4b). Gel plates can also be soaked by pouring buffer into a header tank with a drip feed to a lower chamber (Figure 4c).

Each well of the loading base has 10 μl of sample pipetted into it (Figure 4d). The gel is taken from the buffer solution and dried with soft tissue to remove excess buffer (hard laboratory tissues will scratch the gel). The gel is put on the application plate, and the samples in the loading base are printed on the gel, about 2 cm from one end, with the applicator (Figure 4e). The gel is put in the electrophoretic tank with the printed face down and with the insert line closest to the cathodal end of the tank (Figure 4f). Two plastic strips (from the starch gel moulds) are put on top of the gel plates, over the tank support bar, to ensure even contact between the wick and the gel. When there are three gel plates in the tank, the lid is closed and the electrodes are connected to the power supply pack. (Voltages and conditions are given in Appendix 1.) For enzymes which resolve with a short run of 2–3 cm, it is possible to print 2 or 3 separate starting lines and run 16 or 24 samples per gel. If two enzymes have very different migration rates, both can be stained on the same gel. Cellulose acetate gels can be run in a refrigerator at about 4 °C, though for many enzymes equal resolution is obtained by running the gels at room temperature. A small volume of ice is added to the middle chamber of the electrode tank, or a tray of ice-water slurry is put on the tank lid, to provide cooling during a room temperature run.

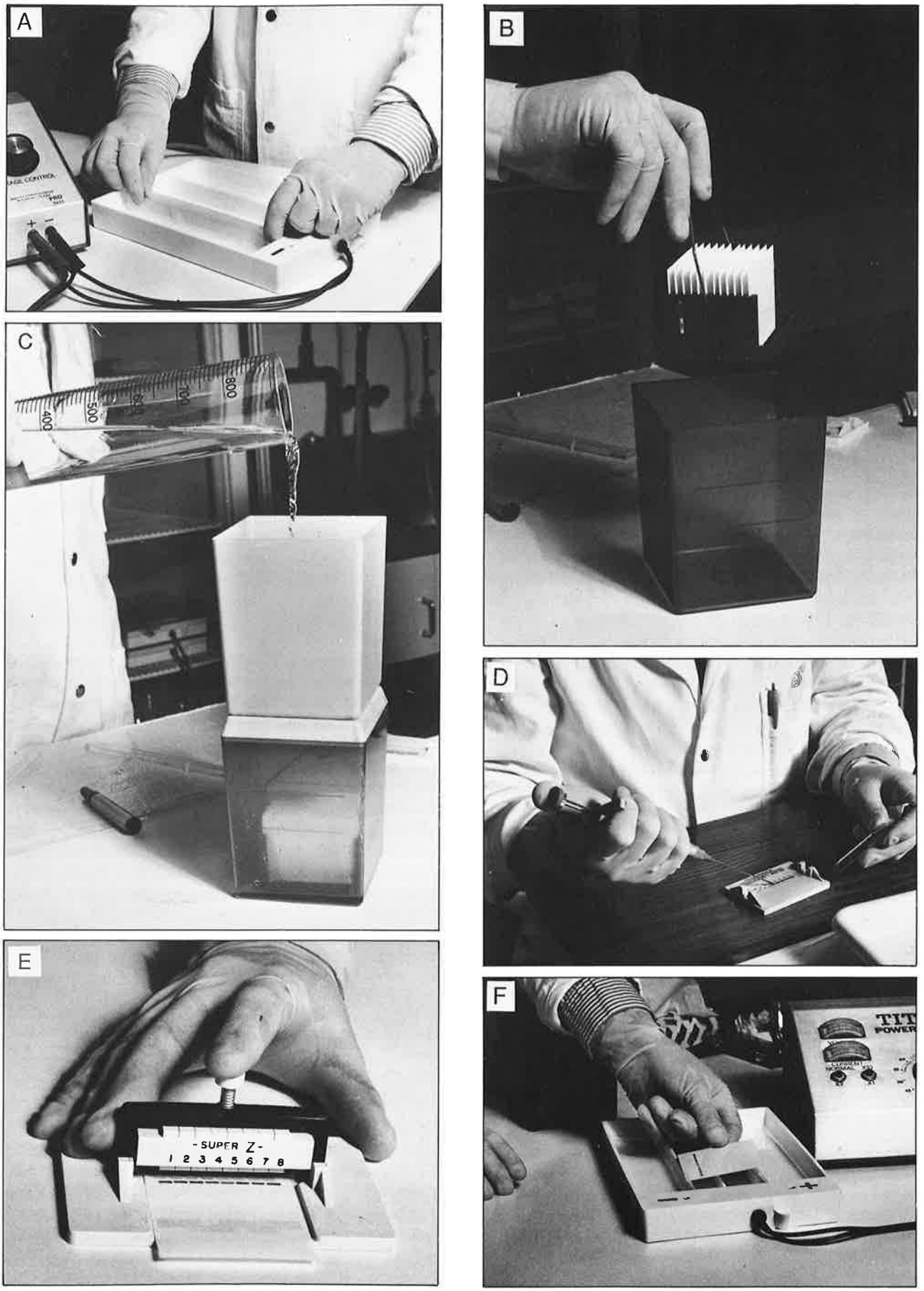


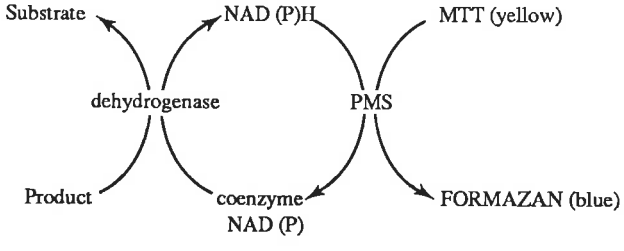
Figure 4: Preparation of cellulose acetate gels for electrophoresis. A, placing electrode wicks into the electrode tank; B, adding the plates to the gel buffer; C, pouring the gel buffer into the header tank to soak the plates in the lower chamber; D, adding supernatant to the loading base; E, printing samples on the gel; F, putting the gel plates into the electrophoretic tank.

Staining and interpretation of starch and cellulose acetate gels

Introduction

The position of enzyme or protein bands in the gel is detected by adding a specific enzyme or protein staining solution to the gel. Most enzyme-substrate products are colourless, so the reaction is linked to a colour producing reagent. When it is not possible to link the enzyme-substrate reaction directly to a colour producing reagent, the product is coupled to another enzyme-substrate reaction which is linked to a coloured reaction, e.g., the detection of phosphoglucose isomerase or phosphoglucomutase depends on them being coupled to the glucose-6-phosphate dehydrogenase-substrate reaction. The development of this direct enzyme staining technique has meant gel electrophoresis can be used to measure genetic variation over a wide range of enzymatic loci in almost any animal or plant species.

The electron transfer dye methyl thiazolyl tetrazolium (MTT) is used widely in staining reactions as an electron acceptor. In dehydrogenase reactions, it is reduced from a yellow soluble compound to a dark blue insoluble formazan. It requires phenazine methosulphate (PMS) as a catalyst, and the enzyme requires the coenzyme nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP). The general reaction for a dehydrogenase is shown below:



For oxidases and peroxidases, 3-amino-9-ethyl carbazole is used as an electron acceptor and is reduced to a brown amino ethyl carbazole precipitate. Enzyme reactions using napthol as a substrate (e.g., esterases) can be identified directly with diazonium salts, which produce coloured diazo compounds. Other chemical methods for seeing enzyme reactions depend on a negative reaction in which the gel is coloured and the enzyme produces clear bands at the site of activity (e.g., catalase). Some enzymes (e.g., esterases) can be identified with a fluorescent stain in which the enzyme-substrate reaction generates a fluorescent product, which has to be viewed under ultraviolet light.

Many of the reagents used in enzyme staining are not stable at room temperature and must be stored desiccated at 4 °C, or below 0 °C. The manufacturer's instructions provided with the chemical should be followed. The staining solutions are mixed just before use (details are given in Appendix 2). To speed up stain preparation, standard reagents such as MTT, PMS, NAD, and NADP can be made up as stock solutions and 1–2 ml are added to the substrate solution. These stock solutions must be kept at 4 °C in dark bottles. If acidic substrates are used, the pH must be buffered to between 7 and 8 before the other stain ingredients are added. Our staining recipes are adapted from the work by Allendorf *et al.* (1978), Harris and Hopkinson (1976), Selander *et al.* (1971), and Shaw and Prasad (1970).

Several of the reagents, such as MTT, PMS, diazonium salts, and 3-amino-9-ethyl carbazole, are possibly carcinogens and must be handled with care. Some brief safety guidelines are given on page 22.

Staining starch gels

At the end of each electrophoretic run the voltage supply is switched off and the power pack is disconnected before the gel is taken from the electrode tanks. It is a good idea to work with one hand in a pocket at this stage to reduce the severity of an accidental shock. The two long gel mould strips are removed, and the top and bottom 3 cm of gel, which have been under the buffer wicks, are sliced off. The inserts are removed, and the top right hand corner of the gel is nicked to identify left and right in the gel slices. The gel is sliced with a 0.007 mm nickel-chromium alloy wire supported on plastic guides along the edge of the gel (Figure 5a). A series of guides (10 × 300 mm and 1–2 mm thick) are put on top of each other to prepare up to six 1 mm slices. The gel is sliced into the required number of sections, and each slice is peeled off on a plastic sheet (Figure 5b). The gel sticks to the plastic, which means the delicate slices are easy to handle. The gel slice, on its plastic sheet, is put in a white staining tray of slightly larger dimensions. This technique uses the minimum stain to soak the gel. Shallow plastic trays, available from hardware stores, make suitable staining trays.

The freshly made stain (about 20 ml per gel slice) is poured on to the cut gel surface and incubated by use of the methods recommended for each stain, usually at 37 °C in the dark (Appendix 2). The gels are checked periodically to score the results and interpret them, as outlined below.

It is possible to stain the cut gel surface for two enzymes by use of an agarose overlay. The staining solution is made up in 10 ml of buffer, and 10 ml of boiled and cooled 1% agarose in stain buffer are added to the stain. This is swirled gently and immediately poured on to the cut gel surface where it sets as a thin gel. Once the enzyme bands have stained and have been scored, the agar overlay gel can be removed, taking most of the stain reagents with it, and the cut gel surface restained for another enzyme.

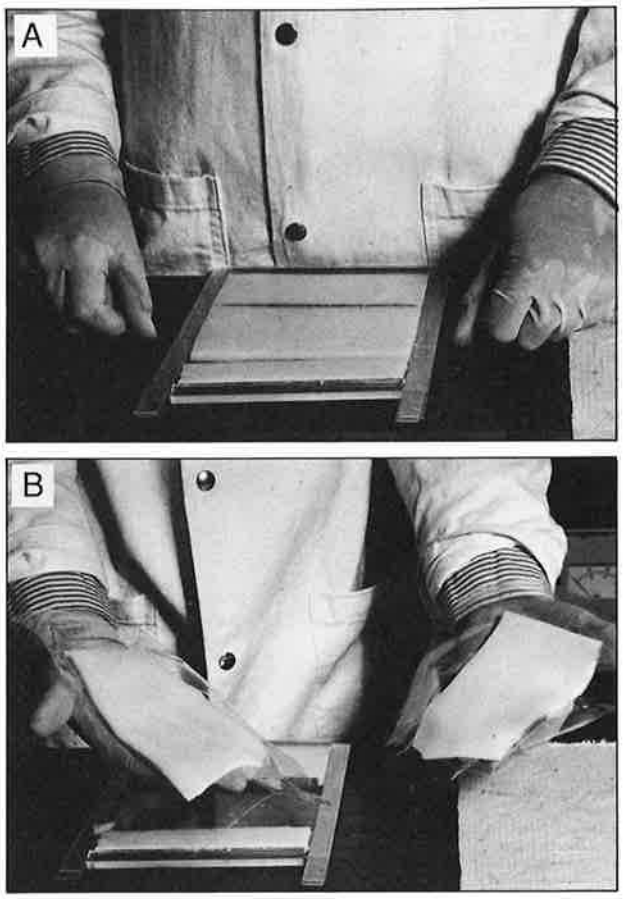


Figure 5: A, slicing a starch gel; B, two halves of a sliced starch gel on plastic backing sheets.

Destaining, fixing, and preservation of starch gels

It is often desirable to prevent the gel from "overstaining" for preservation or for photographic recording. When the bands are at the optimum scoring level, the excess stain solution is washed off with distilled water and the gel is soaked in destain (5 parts methanol, 5 parts water, and 1 part glacial acetic acid). Some starch gels can be stored in destain for several years. The gel shrinks and becomes more opaque, which enhances the bands. This method works well for general proteins and esterases, but for enzymes with MTT and PMS in the stain, the destain solution slowly removes the bands. For short-term preservation, starch gels are rinsed in distilled water and sandwiched between two sheets of thin clear plastic; care must be taken to ensure that there are no air bubbles and that there is an overlap of 5–10 cm of plastic around the gel. Gels last for several weeks before the bands fade if they are stored dry and in the dark. They can be photocopied to provide a more permanent record.

An alternative procedure for long-term gel preservation is to stain the starch gel slice as above and then fix it in 7% acetic acid solution for 15–30 min. The gel slice is washed in distilled water twice to remove excess acid and soaked in 5% glycerol for 15–30 min. It is then put on a glass plate, which has been covered by a sheet of plastic, and covered with another sheet of plastic. The plastic sheets must overlap by 5–10 cm around the gel and must have been pre-soaked in 5% glycerol solution. Air bubbles between the layers of plastic are removed by gentle squeezing. Glycerol solution poured on the gel reduces the formation of air bubbles when the gel is covered. The overlapping edges of the plastic sheets are folded over the back of the glass plate, and the excess glycerol is removed by blotting with filter paper. The plates are dried at room temperature in the dark, to avoid any fading of the bands. Alternatively, they can be dried in an oven at 60–80 °C. When the gel and plastic are dry, the sheets are cut with a scalpel about 1 cm from the border of the dried gel. The gel in its protective plastic layer is stripped off the glass and can be photographed before storage.

Staining cellulose acetate gels

After the cellulose acetate gels have run for the required time, the voltage supply is switched off, the cellulose acetate plates are taken from the electrophoresis tank and put in a suitable staining chamber. A plastic box such as the starch gel electrode box (measuring about 225 × 100 × 90 mm) containing warm water (about 2–3 cm deep) creates a suitable humidity chamber for staining. Three 100 ml beakers, each containing 2–3 cm of warm water, are put in the box and an acetate plate is put on top of each beaker, printed side up. The stain is applied to the gel with a small paintbrush. A much smaller volume of stain is required for cellulose acetate plates than for starch slices; 5 ml of stain will cover several plates. A cover may be put on top of the box to reduce light intensity and maintain a warm humid environment. Cellulose acetate gels stain more rapidly than starch gels with the equivalent stain, and they can "overstain" quickly, so they must be checked frequently. The staining reaction can be stopped by washing off the excess stain with distilled water. The gel plate is then photographed, or photocopied, immediately.

An alternative method is to put the gel plate on a sheet of plastic, paint on the stain, and put another sheet of plastic on top of the plate to prevent the stained gel from drying out. The staining reaction can be stopped by putting the plate in a freezer. Some plates can be stored in this manner for more than a year without losing the stained bands.

Photographic records of gels

Black and white film (such as 80 ASA Ilford FP4) is used to photograph the gels. The gel is illuminated from below with a commercial photographic light box or two 25 cm fluorescent tubes in a ventilated box with a 0.5 cm translucent plastic top. The area around the gel is masked out with black card. The operation should be carried out in a darkened room, or the gel area should be shrouded from excessive surface illumination. Freshly stained gels are blotted dry with soft tissue to reduce reflective glare.

Cellulose acetate gel plates and thin starch gel slices with strongly staining bands can be photocopied to provide a permanent record.

Interpretation of stained gels

In the absence of breeding studies, strict criteria must be applied to the genetic interpretation of stained gels. Gel patterns can be easily interpreted if the tissues have been well preserved and the appropriate buffer was used. Poorly resolved gels with thick or diffuse bands should be discarded, and the enzyme should be tested in another buffer system or tissue. If clear resolution cannot be obtained, the enzyme must be omitted from the survey.

Inheritance studies have been carried out in a wide range of organisms, from *Drosophila* to humans, and including several teleosts (Appendix 3), so it is possible to work out the expected gel patterns (number of loci, tissue specificity, and quarternary structure) for many enzymes before running a gel. A locus appears as a band, or zone of bands, staining with regular intensity and set apart from other loci. Overlapping loci are found, but interpretation can be difficult without a breeding study.

Heteropolymer zones can form between two loci such that there are three zones of bands in the stained gels. An example is the enzyme phosphoglucose isomerase, which controls two loci in teleosts higher than Clupeiformes and shows a heteropolymer zone between the two loci. The zone has an additive pattern: with a homozygote at both loci, the heteropolymer zone has one band; with a heterozygote at one locus, the heteropolymer zone has two bands; and with a heterozygote at both loci, the heteropolymer zone has four bands, though in many species the two middle bands overlap to produce a thicker middle band (Figure 6).

At a specific locus an individual can show a single or multiband phenotype. Homozygotes resolve as a single band and heterozygotes as multiband phenotypes for most enzymes in most species. Occasionally, two-band homozygotes are reported with a corresponding increase in the multiband heterozygote. The number of bands in the heterozygote depends upon the quarternary structure of the enzyme. The expected gel phenotypes for homozygotes and heterozygotes of monomeric, dimeric, trimeric, and tetrameric enzymes are shown in Figure 7, and specific examples are shown in Figure 8. The quarternary structure of commonly used enzymes in electrophoretic studies is given in Appendix 2. If the gel patterns do not agree with these expected patterns, then a genetic interpretation of the gel phenotypes is questionable. An exception is malic enzyme, which has a quarternary structure in higher vertebrates, whereas two-band heterozygotes have been reported in teleosts and crustaceans.

The collection of tissue samples at sea, sometimes an hour or more after the death of the fish, can result in poor gel resolution for some enzymes. The effects include:

1. Poor resolution of heterozygotes. For some enzyme loci the heterozygotes resolve as a thick band instead

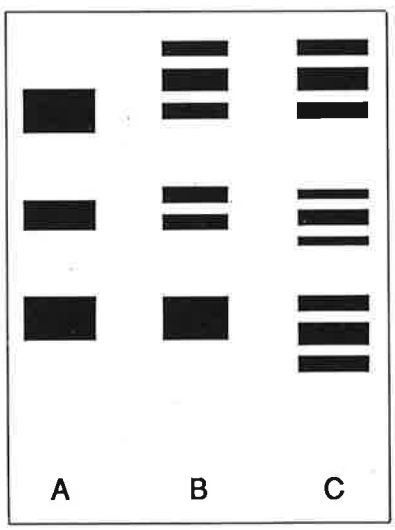


Figure 6: Gel phenotypes for an enzyme with two loci and a heteropolymer zone. A, two homozygotes; B, one heterozygote and one homozygote; C, two heterozygotes.

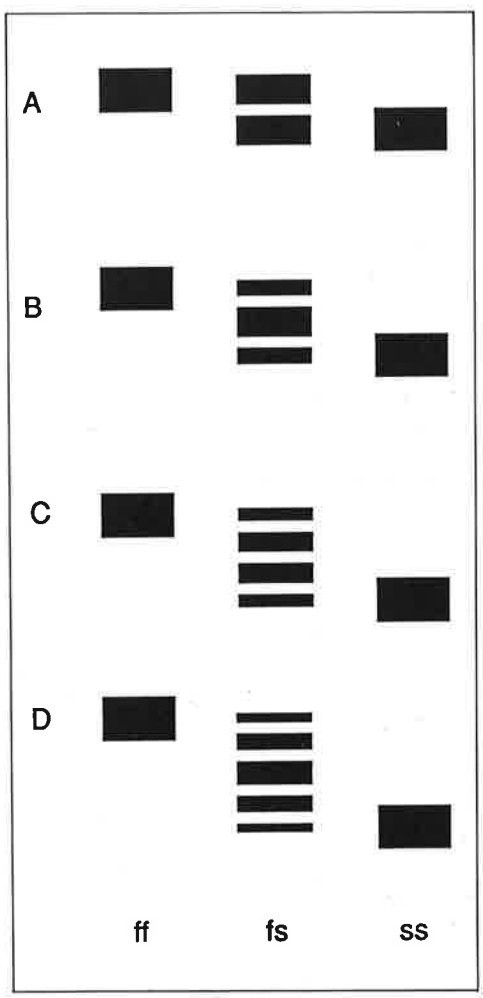


Figure 7: Expected gel phenotypes. A, monomeric; B, dimeric; C, trimeric; D, tetrameric enzymes (ff, fast homozygote; fs, heterozygote; ss, slow homozygote).

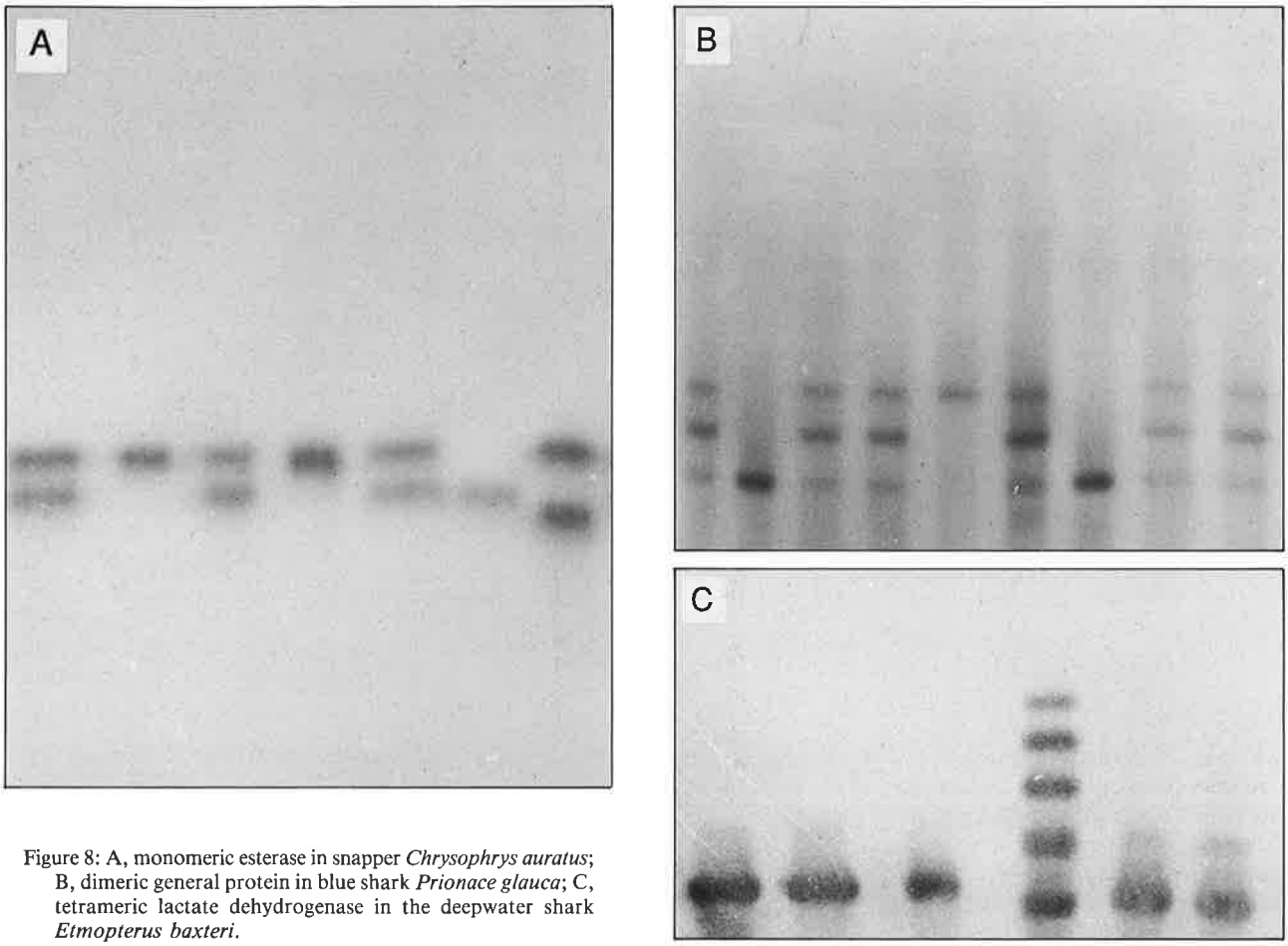


Figure 8: A, monomeric esterase in snapper *Chrysophrys auratus*; B, dimeric general protein in blue shark *Prionace glauca*; C, tetrameric lactate dehydrogenase in the deepwater shark *Etmopterus baxteri*.

of three (dimeric) or five (tetrameric) bands. Where both homozygous classes are present and resolve as clear thin bands at either end of the thick band phenotypes, they may be interpreted as heterozygotes. However, if only a few thick band specimens are found, these individuals cannot be scored with confidence as heterozygotes in the absence of the rare second homozygote.

2. Loss of bands in heterozygotes. In some dimeric and tetrameric heterozygotes the outer bands stain less intensely than the middle bands, and with prolonged storage times these outer bands lose all staining activity. For a dimeric enzyme (such as isocitrate dehydrogenase) this produces a single-band heterozygote, but of different mobility to the two homozygotes (Figure 9a). For these enzymes it would not be possible to interpret the phenotypes without access to gels scored before the storage loss occurred. A similar storage loss for a tetrameric enzyme (such as sorbitol dehydrogenase) produces a three-band heterozygote which can be distinguished from the homozygotes (Figure 9b). Occasionally with alcohol dehydrogenase in frozen teleost liver samples, only homozygous phenotype classes are found, which suggests storage loss of the heterozygote bands, but without access to fresh material it is not possible to interpret such gels.
3. Gain of storage bands. Some enzymes appear more sensitive to storage conditions than others and produce storage bands after tissues have been kept in a freezer for several weeks. With care it is possible to interpret some of these gel phenotypes. Storage artefacts can be checked by holding the supernatant overnight at 4 °C, or room temperature, and rerunning the gel. The storage bands should show enhanced staining activity against freshly prepared samples. Some typical storage band artefacts are shown in Figure 10, and specific examples are shown in Figure 11. Occasionally at FRC the dimeric phosphoglucose isomerase locus in liver samples has resolved as two-band homozygotes and four-band heterozygotes; this is most noticeable in samples stored at −20 °C for several months. A more extreme example is where a homozygote might have two storage bands and resemble a dimeric heterozygote. However, with care these storage artefacts can be distinguished from heterozygotes by the different staining intensities of the bands: the storage bands show decreasing staining intensity in one direction, whereas the heterozygote shows strong staining in the middle band and weaker, but equal, staining intensity in the other two bands (Figure 10b).

Data recording

Loci are labelled alphabetically, or numerically, starting at the most anodal locus (e.g., *Mdh*-A,

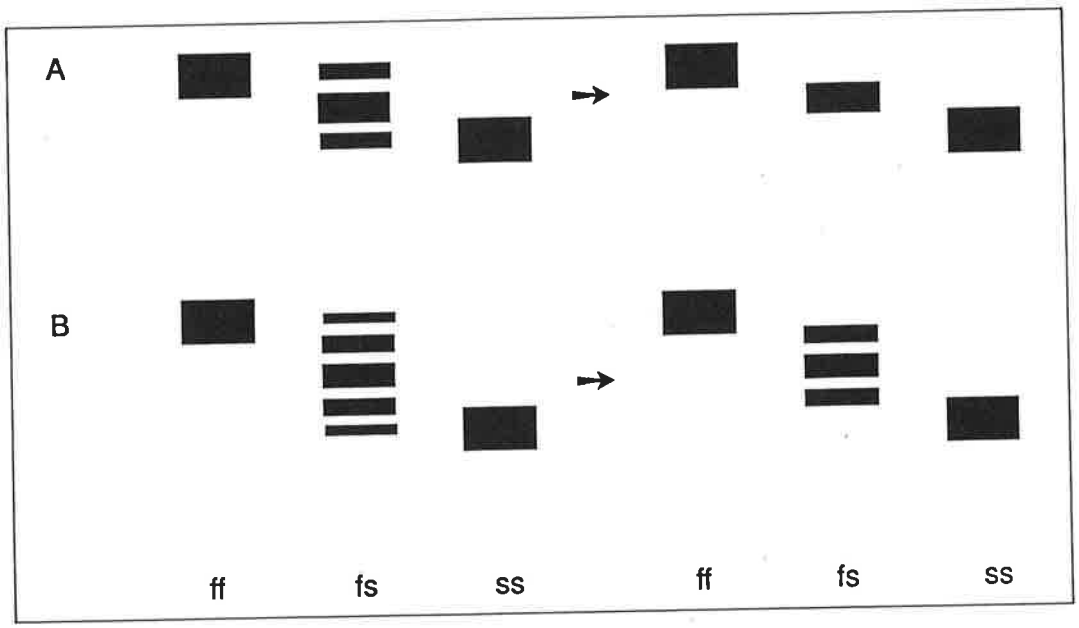


Figure 9: Loss of bands. A, dimeric; B, tetrameric enzymes with prolonged storage (ff, fast homozygote; fs, heterozygote; ss, slow homozygote).

Mdh-1). In some laboratories loci are labelled from the insert line towards the anode, and the cathodally migrating loci are given a negative value. Electromorphs are labelled in descending order of anodic mobility (e.g., *Mdh*-1^{a} or *Mdh*-1^{b}). Some laboratories label the common electromorph as 100

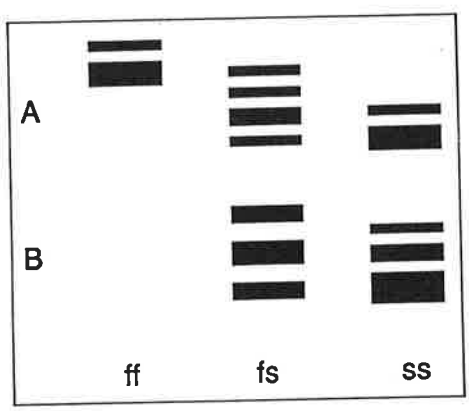


Figure 10: Storage artefacts. A, a dimeric enzyme showing two-band homozygotes and four-band heterozygotes; B, relative staining intensities in a homozygote with two storage bands compared with a dimeric heterozygote (ff, fast homozygote; fs, heterozygote; ss, slow homozygote).

and all other electromorphs as a relative measurement of this electromorph (e.g., *Gpi*-A^{92}). At FRC electromorphs are referred to as fast, medium, or slow according to migration rate (other rare electromorphs are referred to as extra fast or extra slow). This provides an easy scoring system for phenotype classes (e.g., ff, fm, mm, ms, fs, ss) when running many specimens. Each gel must be run with a control sample, usually a heterozygote of the common electromorphs, to ensure continuity of scoring throughout a project. With three, or more, electromorphs in a population, two, or more, controls might be required for each gel. Specimens showing rare phenotypes can be rerun adjacent to each other to check relative migration rates.

Data are recorded on a gel-run sheet (Appendix 4). This sheet is used for each gel to record the species, tissues, sample details, electrophoretic conditions, and result. Photographs can be attached or sketches of gels made on the back of the sheet. If a computer terminal is available, data can be put directly into a file as the gels are scored. Without this facility, gel data are stored on gel-run sheets and later transferred to a computer file, or tabulated into the number of specimens in each phenotype class for analysis with a hand calculator.

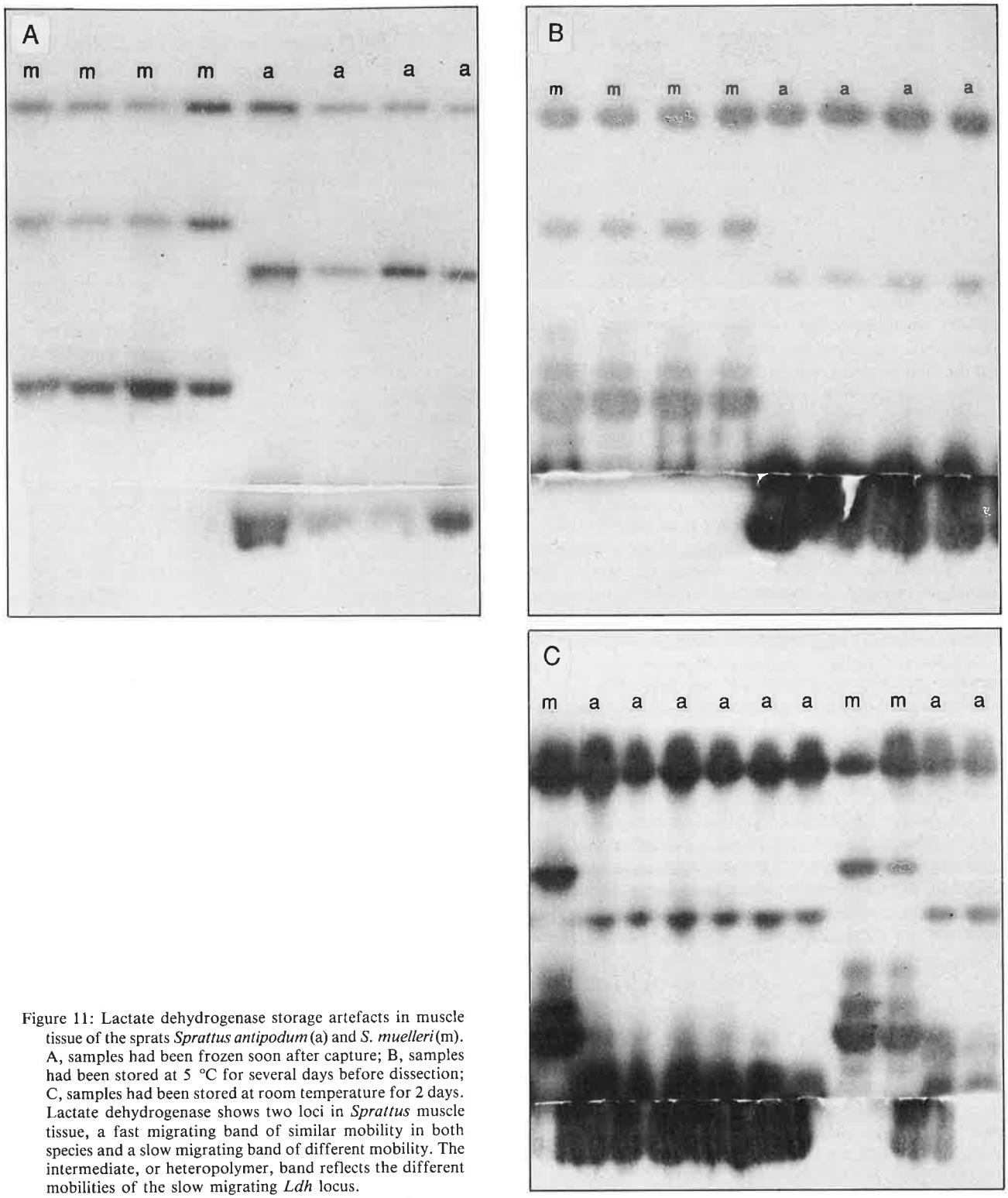


Figure 11: Lactate dehydrogenase storage artefacts in muscle tissue of the sprats *Sprattus antipodum* (a) and *S. muelleri* (m). A, samples had been frozen soon after capture; B, samples had been stored at 5 °C for several days before dissection; C, samples had been stored at room temperature for 2 days. Lactate dehydrogenase shows two loci in *Sprattus* muscle tissue, a fast migrating band of similar mobility in both species and a slow migrating band of different mobility. The intermediate, or heteropolymer, band reflects the different mobilities of the slow migrating *Ldh* locus.

Iso-electric focusing

Introduction

Iso-electric focusing is used to achieve a higher resolution than standard electrophoresis by separation in a pH gradient. This is done by adding a complex mixture of aliphatic amino carboxylic acids (generally known under the trade name ampholines) to the gel and by making the anode a strong acid and the cathode a strong alkali. The ampholines contain compounds with a wide variety of iso-electric points and have a high buffering capacity. When a protein sample is put in the IEF gradient, the individual proteins migrate to and concentrate at a position where they are electrically neutral (their iso-electric point). Iso-electric focusing is a powerful technique, and it is possible to separate molecules which differ in iso-electric points by as little as 0.02 pH units.

Polyacrylamide is the usual gel-medium for IEF; it can be made to exact specifications, is less expensive, and gives high resolution. However, acrylamide has the disadvantage of being a nerve toxin, and the gels do not always fully polymerise. Iso-electric focusing grade agaroses have recently become available as an alternative to polyacrylamide; they are non-toxic and give a resolution similar to polyacrylamide gels. (Ordinary agaroses result in unstable pH gradients because of their residual charges, a phenomenon known as gradient drift.) Iso-electric focusing grade agarose gels are run on a horizontal or flat bed system with a cooling plate beneath the gel to reduce heat build-up during the run. There are several systems available which can be supplied as complete kits with stabilised power supply, a flat bed, gel sheets and moulds, electrode wicks, inserts, and templates. The Pharmacia system was used at FRC because it was available in New Zealand.

Iso-electric focusing is used as a forensic tool to identify unknown fillets or specimens by their protein fingerprints. The unknown specimen is run against a series of controls from known species. In practice, a bank of frozen muscle samples and protein fingerprints is built up from the common teleosts; because of the high resolution and excellent reproductibility of IEF, the preliminary identification can be made by a comparison with protein fingerprints in preserved gels or photographs. Examples are shown in Figure 12.

The high resolution obtained with IEF means it can be used as a rapid method to distinguish closely related and phenotypically similar species, thereby avoiding the need to run a wide range of specific enzymes. Additional genetic variation can be detected by IEF: starch-gel electrophoresis of phosphoglucomutase in humans reveals two electromorphs, whereas IEF reveals four. Additional genetic variation has not yet been found in teleosts, and the greater expense and time required to run specific enzymes in IEF have precluded its use in routine population surveys.

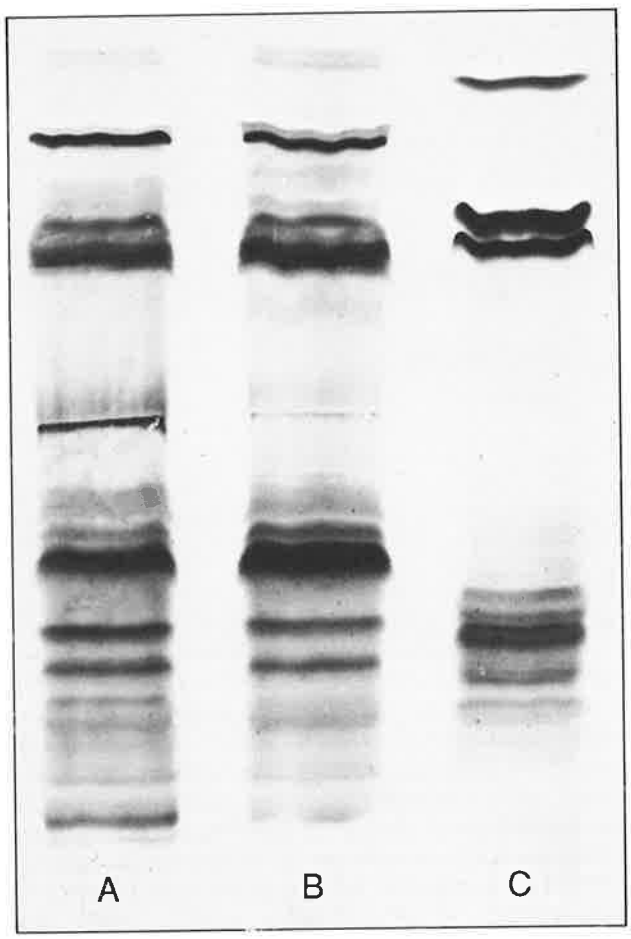


Figure 12: A dried agarose gel stained with Coomassie Blue R-250. A, ling, or kingclip, *Genypterus blacodes*; B, fillet marketed as roughy; C, orange roughy, *Hoplostethus atlanticus*.

Equipment

Stabilised power supply (3000 V, 300 mA)
Flat bed cooling base and lid
Supply of cooled water at 10–15 °C
Gel backing sheets and gel moulds
Glass plate and clips
Levelling table
Micropipette

A power pack of higher capacity is required for IEF. Some models have built-in timers so they can be set to automatically switch off after the electrophoretic run. Others allow for an external electronic timer or a volt-hour integrater (for use with a constant current and variable voltage) to be connected. This latter facility is essential for IEF.

Preparation of agarose gels

The gel backing sheet (a hydrophilic polyester sheet) is put on a glass plate on a levelling table. Pharmacia "gel bond", which has hydrophilic and hydrophobic

surfaces, is used as the backing sheet at FRC. The gel is allowed to set on the hydrophilic side, which is recognised by putting a drop of distilled water on the gel bond (it will form droplets on the hydrophobic side, but will spread on the hydrophilic side). The gel sheet must make even contact with the glass plate. Air bubbles are gently squeezed out by rolling the gel sheet with a tube. The gel sheet and surrounding glass plate are dried with soft tissue, then the gel mould is placed on the gel sheet and clamped down with spring clips. The sheet and mould are warmed in an oven to above 50 °C to ensure that the gel does not set too quickly when poured.

To prepare the gel, 3.6 g of sorbitol and 0.3 g of IEF grade agarose are dissolved in 27.0 ml of distilled water by gentle heating and stirring. The solution is boiled gently until clear. The mixture is cooled to 75 °C and 1.9 ml of ampholine solution (Phamacia "pharmalyte") are added. Wide range ampholines (pH 3–10) are used in most analyses of fish fillets. Narrow range ampholines (e.g., pH 5–8 or pH 6.5–9) may be used for specific enzymes or proteins (focusing over a restricted range) after initial tests in wide range ampholines. The gel is swirled gently and poured into the warm gel mould. It must be poured quickly and evenly by running the flask around the edge of the mould. The lip of the flask can touch the gel to assist in spreading. Air bubbles should be pricked immediately with a needle because the gel sets rapidly.

The gel is allowed to set for 10–15 min before the mould is removed. The gel must harden for about 1 h at 4 °C, or overnight at room temperature. For overnight storage, the gel is put in a humidity chamber made from a plastic tray, with moist paper towelling and another tray stacked on top.

Running the gel

About 2 ml of distilled water are put on to the cooling plate of the electrophoretic unit, and the gel sheet is gently pulled across the cooling plate, so the water spreads to a thin film under the gel with no air bubbles. It is essential to avoid air bubbles because they insulate the gel from the cooling effect of the plate. Excess water around the edge of the gel is mopped up with soft tissue.

The two electrode strips, supplied with the IEF accessory kit, are soaked in the appropriate electrode solutions, 1 M NaOH for the cathode and 0.05 M H_2SO_4 for the anode (for narrow range ampholines 0.2 M histidine is used for the anode). The electrode strips are dried on filter paper and placed on the gel. Up to 25 μl of sample are pipetted on to each filter paper wick and these are put on to the gel surface (Figure 13). Smaller volumes (3–5 μl) can be put directly on to the gel surface by use of a stencil template.

The electrical conductivity of the gel changes during the course of the run, particularly as the pH gradient is established. To avoid overheating or excessively long run times, the gel is run on constant power. As the gel resistance changes the voltage increases to a maximum preset level and the current decreases, from a maximum preset level.

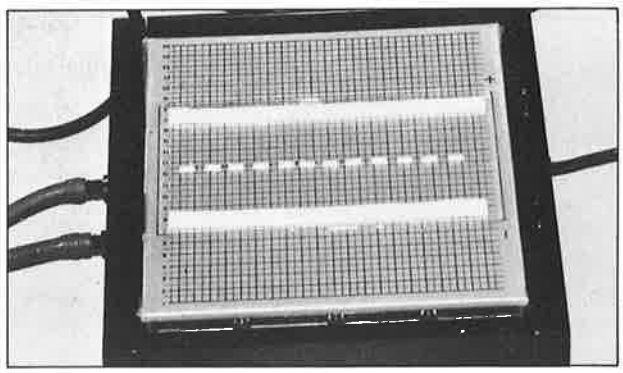


Figure 13: Electrode wicks and sample inserts on an agarose gel on the cooling base.

The power supply is set to deliver a maximum of 1500 V, 150 mA, and 10 W. The gel is run for 15 min, then the electrode strips are removed, dried, and replaced. The electrode strips absorb moisture during the run and it is essential to dry them. After 45 min the inserts are removed and the electrode strips are dried again. The gel is run for a maximum of 90 min to give a total focusing time of 1500–2000 volt hours. Cooled water, at 10 °C, is circulated through the flat bed during the run. Lower temperatures produce condensation inside the chamber and can cause arcing across the gel surface. The pH gradient can be measured with a surface electrode at the end of the run.

Fixing and staining the gel

After electrophoresis the gel is fixed for 30 min in a solution of 5% sulphosalicylic acid and 10% trichloroacetic acid in distilled water. The gel is washed twice in destaining solution (35% ethanol, 10% acetic acid, and 55% distilled water) for 15 min each time. It should be agitated several times in the destain solution to remove excess fixative. The gel is then dried by putting it under three layers of filter paper and a weight of about 1 kg for 15 min. This produces a thin opaque gel which is further dried with a hairdryer until it is transparent (Figure 14). The dried gel is stained

Figure 14: Drying an agarose gel (on a gel backing sheet) with a hairdryer.

for 5–10 min in 0.2% Page Blue 83 (or 0.2% Coomassie Blue R-250) dissolved in destaining solution. It is essential to use fresh stain because old stain tends to precipitate and leave crystals on the gel sheet. The gel is destained for about 30 min until the background is clear; it should be agitated several times during destaining. Finally, the gel is dried with a hair dryer and scanned under visible light. The dried gel can be stored for several years in a dry, dark environment.

Laboratory safety procedures

The usual laboratory safety procedures should be followed in electrophoretic laboratories. In addition, two areas of potential danger (the high voltage used in electrophoretic runs and the toxic chemicals in enzyme stains) must be considered. General guidelines taught to staff using the FRC laboratory are:

1. A laboratory coat is worn and changed once a week.
2. Long hair is tied back.
3. Low-heeled shoes are worn and open-toed shoes are avoided.
4. The power supply is switched off and the electrode tanks are disconnected before and after an electrophoretic run and when setting up or removing a gel. The "one hand in a pocket" rule is used to reduce the severity of an accidental shock.
5. Disposable gloves are worn when handling the enzyme staining reagents. A fume cupboard or restricted part of the laboratory is used for handling and weighing these chemicals.
6. Stained gels, chemicals, and gloves are disposed of by sealing them in a strong plastic bag which is put directly into a waste disposal bin. They are not disposed of with general paper waste in the laboratory.
7. Excess staining solutions are poured down the sink and the tap is left running for about 5 min.

Acknowledgments

The methods described in this report owe much to the contacts we have had with other "gel-jockeys", both through visiting their laboratories and having them work with us. In particular, we thank Dr Alan Jamieson (Lowestoft), Dr Bob Gauldie (FRC), and Dr Peter Baverstock (Adelaide) for freely showing us their techniques.

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Appendix 1

Electrophoretic buffers used at Fisheries Research Centre

Buffers used in cellulose acetate gel electrophoresis

Buffer (reference)	Buffer recipe	pH	Recommended voltage (V)
1. Barbitone-acetate (Sargent and George 1975)	6.5 g sodium acetate 8.9 g barbitone sodium 1.1 g barbitone 1.0 l distilled water	8.6	130
2. Barbitone (Sargent and George 1975)	10.3 g barbitone sodium 1.8 g barbitone 1.0 l distilled water	8.6	140
3. Tris-EDTA-borate (TEB) (Sargent and George 1975)	78.6 g tris 7.8 g EDTA 6.0 g boric acid 1.0 l distilled water	9.0	150
4. TEB-barbitone discontinuous buffer (Smith 1968)	Anode buffer 25.2 g tris 2.5 g EDTA 1.9 g boric acid 1.0 l distilled water	9.0	170
	Cathode buffer 5.2 g barbitone sodium 0.9 g barbitone 1.0 l distilled water	8.6	
	(Presoak cellulose acetate plates in a 50 : 50 mix of anode and cathode buffers)		
5. HR tris-barbital-sodium barbital (Helena Cat. No. 5805)	Dissolve contents of sachet in 1 l distilled water (Ionic strength 0.05)	8.8	180
6. Supra-heme TEB (Helena Cat. No. 5802)	Dissolve contents of sachet in 1 l distilled water (Ionic strength 0.02)	8.2–8.6	140
7. Citrate buffer (Sodium citrate/citric acid) (Helena Cat. No. 5121)	Dissolve contents of sachet in 1 l distilled water (Ionic strength 0.30)	6.0–6.3	130
8. Barbital/sodium barbital (Helena Cat. No. 5016)	Dissolve contents of sachet in 1 l distilled water (Ionic strength 0.05)	8.6	140
9. Barbital/sodium barbital (Helena Cat. No. 5017)	Dissolve contents of sachet in 1 l distilled water (Ionic strength 0.075)	8.6	140

Buffers used in starch gel electrophoresis

Buffer system (reference)	Buffer pH		Buffer recipe		Recommended voltage (V)	Running time (h)
	Gel	Electrode	Gel buffer	Electrode buffer		
1. Histidine-citric acid (Shaw and Prasad 1970)	7.0	7.0	Dissolve 0.8 g histidine in 1 l distilled water Adjust to pH 7.0 with NaOH	Dissolve 78.8 g anhydrous citric acid in 1 l distilled water Adjust to pH 7.0 with NaOH	100	4
2. Phosphate citrate (Selander *et al.* 1971)	7.0	6.7	Dissolve 1.1 g anhydrous K_2HPO_4 and 0.3 g anhydrous citric acid in 1 l distilled water	Dissolve 29.1 g anhydrous K_2HPO_4 and 5.7 g anhydrous citric acid in 1 l distilled water	100	4–6
3. Sodium hydroxide-boric acid buffer (Shaw and Prasad 1970)	8.5	8.0	Dissolve 1.9 g boric acid and 0.5 g NaOH in 1 l distilled water	Dissolve 18.6 g boric acid and 2.0 g NaOH in 1 l distilled water	160	3
4. Tris-citrate (Selander *et al.* 1971)	6.7	6.3	Dissolve 1.0 g tris and 0.6 g anhydrous citric acid in 1 l distilled water Adjust to pH 6.7 with 1 M NaOH	Dissolve 27.0 g tris and 18.1 g anhydrous citric acid in 1 l distilled water Adjust to pH 6.3 with 1 M NaOH	160	3
5. Tris-citrate (Selander *et al.* 1971)	8.0	8.0	Dissolve 2.8 g tris and 1.0 g anhydrous citric acid in 1 l distilled water	Dissolve 83.2 g tris and 27.4 g anhydrous citric acid in 1 l distilled water	100	4
6. Tris-citrate (Fujio 1977)	7.0	7.0	1 : 9 dilution of electrode buffer with distilled water	Dissolve 18.8 g tris and 8.6 g anhydrous citric acid in 1 l distilled water	200	4
7. Tris-citrate (Syner and Goodman 1966)	7.0	6.0	Dissolve 1.5 g tris and 0.8 g anhydrous citric acid in 1 l distilled water	Dissolve 45.8 g tris and 29.6 g anhydrous citric acid in 1 l distilled water	200	4

Appendix 1—continued

Buffer system (reference)	Buffer pH		Buffer recipe		Recommended voltage (V)	Running time (h)
	Gel	Electrode	Gel buffer	Electrode buffer		
8. Tris-citrate/lithium hydroxide-boric acid (Ridgway *et al.* 1970)	8.0	8.0	Dissolve 3.6 g tris and 1.0 g anhydrous citric acid in 1 l distilled water Make up gels with 99% gel buffer and 1% electrode buffer, or make up gels with 96% gel buffer and 4% electrode buffer	Dissolve 2.5 g lithium hydroxide and 18.5 g boric acid in 1 l distilled water	230	3–4
9. Tris-citrate/lithium hydroxide-boric acid (modified after Ridgway *et al.* 1970)	8.0	8.0	Dissolve 1.8 g tris, 1.0 g citric acid, 2.6 g boric acid, and 0.5 g lithium hydroxide in 1 l distilled water	As above	230	3–4
10. Tris-citrate/lithium hydroxide-boric acid (Selander *et al.* 1971)	8.2	8.1	Dissolve 6.2 g tris and 1.6 g anhydrous citric acid in 1 l distilled water Make up gels with 90% gel buffer and 10% electrode buffer (this gives a pH of 8.2)	Dissolve 1.2 g lithium hydroxide and 11.9 g boric acid in 1 l distilled water	250	3–4
11. Tris-citrate/sodium hydroxide-boric acid (Poulik 1957)	8.7	8.2	Dissolve 9.2 g tris and 1.0 g anhydrous citric acid in 1 l distilled water	Dissolve 18.5 g boric acid and 2.4 g NaOH in 1 l distilled water	250	3
12. Tris-HCl/sodium hydroxide-boric acid (Selander *et al.* 1971)	8.5	8.2	Dissolve 1.2 g tris in 1 l distilled water Adjust to pH 8.5 with conc. HCl	Dissolve 18.5 g boric acid in 1 l distilled water Adjust to pH 8.2 with NaOH	250	1.5
13. Tris-EDTA-borate (Selander *et al.* 1971)	8.0	8.0	1 : 9 dilution of electrode buffer in distilled water (i.e., 10% electrode buffer)	Dissolve 60.6 g tris, 40.0 g boric acid, and 6.0 g EDTA (disodium salt) in 1 l distilled water	200	4
14. Tris-EDTA-borate (Dando 1970)	8.7	8.7	gel + anode Dissolve 5.5 g tris, 1.6 g boric acid, and 0.04 g EDTA (disodium salt) in 1 l distilled water	Cathode Dissolve 15.2 g tris, 4.3 g boric acid, 0.1 g EDTA (disodium salt) in 1 l distilled water	200	6
15. Tris-EDTA-citrate (Buroker *et al.* 1975)	7.0	7.0	1 : 19 dilution of electrode buffer in distilled water	Dissolve 16.4 g tris, 8.6 g anhydrous citric acid, and 0.5 g EDTA (disodium salt) in 1 l distilled water Adjust to pH 7.0	150	4–6
16. Tris-EDTA-maleate (Selander *et al.* 1971)	7.4	7.4	1 : 9 dilution of electrode buffer in distilled water	Dissolve 2.0 g magnesium chloride (hexahydrate), 3.7 g EDTA (disodium salt), 11.6 g maleic acid, and 12.1 g tris in 1 l distilled water Adjust to pH 7.4 with 0.1 M NaOH	100	5

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Appendix 2

Enzyme and general protein stains used at Fisheries Research Centre for frozen fish and shellfish tissue samples

Enzyme (abbreviation) E.C. No.*	Quarternary structure†	Stain ingredients: PMS (mg)	MTT (mg)	NAD (mg)	NADP (mg)	Substrate		Other chemicals and instructions		Suitable buffer systems‡
Acid phosphatase (ACP) 3.1.3.2	m or d	-	-	-	-	α-napthyl phosphate	25 mg	$MgSO_4$ Fast Blue BB 0.05 M citric acid (pH 4.5) Incubate gel at 37 °C (dark)	50 mg 10 mg 20 ml	S2
Aldolase (ALD) 4.1.2.13	t	3	5	5	-	fructose 1,6-diphosphate	100 mg	Sodium arsenate glyceraldehyde-3-phosphate dehydrogenase 0.1 M tris-HCl, pH 8.0	10 mg 20 µl 20 ml	S5, S13
Alkaline phosphatase (AKP) 3.1.3.1	m or d	-	-	-	-	α-napthyl phosphate	25 mg	$MgSO_4$ Fast Blue BB 0.06 M boric acid (pH 9.7) Incubate gel at 37 °C (dark)	50 mg 10 mg 20 ml	CA6, S8
Aconitate hydratase (ACON) 4.2.1.3	m	3	5	-	5	Cis-aconitic acid (75 mg cis-aconitic acid in 25 ml 0.4 M tris-HCl, pH to 8.0)	20 ml	Iso-citrate dehydrogenase $MgCl_2$ Incubate gel at 37 °C (dark) (ACON sometimes produces identical gel phenotypes to IDH)	5 µl 5 mg	S5
Adenosine deaminase (ADA) 3.5.4.4	m	3	5	-	-	Adenosine	20 mg	Xanthine oxidase Nucleoside phosphorylase 0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark)	5 µl 5 µl 25 ml	CA1, S5
Adenylate kinase (AK) 2.7.4.3	m	3	5	-	5	Adenosine diphosphate	10 mg	Hexokinase Glucose-6-phosphate dehydrogenase $MgCl_2$ Glucose 0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark)	5 µl 1 µl 5 mg 40 mg 30 ml	CA1, CA2, S3, S12, S15
Alcohol dehydrogenase (ADH) 1.1.1.1	d	3	5	5	-	Ethanol Iso-propanol	5 ml 5 ml	0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark)	20 ml	CA6, S2, S8
Aspartate aminotransferase (AAT) or Glutamate-oxaloacetate-transaminase (GOT) 2.6.1.1	d	-	-	-	-	(a) Pyridoxal-5-phosphate α-ketoglutaric acid DL-aspartic acid 0.2 M tris-HCl (pH 8.0) (Ensure pH is 8.0)	5 mg 50 mg 200 mg 100 ml	AAT substrate solution Fast Blue BB Incubate gel at 37 °C (dark)	25 ml 5 mg	S5, S8 S13
		-	-	-	-	(b) α-ketoglutaric acid L-aspartic acid $Na_2H_2PO_4$ Polyvinyl pyrrolidone (PVP) Distilled water	40 mg 100 mg 1 g 0.5 g 50 ml	Add 0.5 g Fast Garnet GBC to substrate solution and pour on to sliced gel Leave for 1 min then pour off		
Catalase (CAT) 1.11.1.6	m or t	-	-	-	-	Solution A KI Acetic acid Distilled water	 2 g 2 ml 98 ml	1. Soak the sliced gel in gel buffer with the pH adjusted to 6.5 (by HCl) at 4 °C for 30 min 2. Wash the buffer off and soak in solution A for 30 s		S8
and Peroxidase (PER) 1.11.1.7	m					Solution B 0.03% H_2O_2 N.B., if making stock solutions A and B, store in dark glass bottles in a refrigerator		3. Wash off solution A with running water, rinse three times 4. Soak in solution B until peroxidase bands appear as dark blue bands and catalase bands appear as white 5. Wash and fix with 50% glycerol		
Creatine phosphokinase (CPK) 2.7.3.2	d	3	5	-	5	Phosphocreatine Adenosine diphosphate	15 mg 10 mg	Hexokinase Glucose 6-phosphate dehydrogenase $MgCl_2$ Glucose 0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark)	5 µl 1 µl 20 mg 40 mg 25 ml	S6
Diaphorase (DIA) 1.6.2.2	m	-	5	-	-	2,6-dichlorophenol-indophenol	5 mg	NADH 0.2 M tris-HCl (pH 8.0)	10 mg 20 ml	S14

Appendix 2—continued

Enzyme (abbreviation) E.C. No.*	Quarternary structure†	Stain ingredients PMS (mg)	MTT (mg)	NAD (mg)	NADP (mg)	Substrate		Other chemicals and instructions		Suitable buffer systems‡
Esterase (EST) 3.1.1.1	m or d	-	-	-	-	(a) α- or β-naphthyl salt (1% in acetone)		Add a few drops of 1% substrate (in acetone) to sliced gel surface Stain with 20 ml Fast Blue BB solution (approximately 10 mg per 20 ml 0.2 M tris-HCl pH 8.0)		S8–11
		-	-	-	-	(b) 4-methylumbelliferyl acetate (1% in acetone)		1. Add several drops of 1% substrate (in acetone) to sliced gel surface 2. Stand the soaked gel under a u.v. lamp (the resulting esterase bands show up as luminescent white bands)		S9
Fructose 1,6-diphosphatase 3.1.3.11	t	3	5	-	5	fructose 1,6-diphospate	20 mg	$MgSO_4$ Glucose-6-phosphate dehydrogenase Glucosephosphate isomerase	10 mg 1 µl 1 mg	
Fumaratehydratase (FUM) 4.2.1.2	t	3	5	5	-	Fumaric acid solution (60 mg fumaric acid per 50 ml 0.2 M tris-HCl (pH 8.0), pH adjusted to 8.0 by NaOH)	30 ml	Malic dehydrogenase Incubate gel at 37 °C (dark) (FUM sometimes produces the same gel phenotypes as MDH)	5 µl	CA1, S5
General protein (PROT)	m or d	-	-	-	-			1. Soak the sliced gel in 1% Coomassie Blue R-250 or 2% Amido Black (in destain) for 30–60 min 2. Rinse in destain 3. Soak gel in destain solution (1 part acetic acid, 5 parts distilled water, 5 parts methanol) to remove background stain		CA1, S8
Glucose dehydrogenase (GDH) 1.1.1.47	d	3	5	5	-	D-glucose	5 g	Phosphate buffer (pH 7.5) Incubate gel at 37 °C (dark)	25 ml	S5
Glucose-6-phosphate dehydrogenase (G6PDH) 1.1.1.49	d	3	5	-	5	Glucose-6-phosphate	25 mg	0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark)	20 ml	CA7
Glutamate dehydrogenase (GlutDH) 1.4.1.3	-	3	5	5	-	Glutamic acid	15 mg	0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark)	25 ml	S8, S9
Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) 1.2.1.12	t	3	5	10	-	Na_4 fructose 1,6-diphosophate-$6H_2O$ Andolase 0.2 M tris-HCl (pH 8.0) Incubate at 37 °C for 30 min before staining	545 mg 0.6 ml 10 ml	Add 25 ml 0.2 M tris-HCl (pH 8.0) to the PMS, MTT, and NAD Pour in the substrate solution (pre-incubated at 37 °C) Pour over the sliced gel Incubate at 37 °C (dark)		S5
Glycerol-3-phosphate dehydrogenase (GPDH) 1.1.1.8	d	3	5	5	-	DL-α-glycerophosphate	30 mg	0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark)	20 ml	S2
Guanine deaminase (GDA) 3.5.4.3	d	3	5	-	-	Dissolve 50 mg guanine in warm NaOH solution Add 50 ml distilled water	10 ml	Xanthine oxidase Substrate mixture Phosphate buffer Incubate gel at 37 °C (dark)	5 µl 3 ml 25 ml	S5
Haemoglobin (Hb)	m	-	-	-	-			Use general protein stain on whole blood or red cell tissues		S8

Appendix 2—continued

Enzyme (abbreviation) E.C. No.*	Quarternary structure†	Stain ingredients PMS (mg)	MTT (mg)	NAD (mg)	NADP (mg)	Substrate		Other chemicals and instructions		Suitable buffer systems‡
Hexokinase (HK) 2.7.1.1	m	3	5	–	5	Adenosine triphosphate	40 mg	Glucose-6-phosphate dehydrogenase $MgCl_2$ Glucose 0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark)	5 µl 20 mg 900 mg 25 ml	S3, S12
D-2-hydroxyacid dehydrogenase (HADH) 1.1.99.6	d	3	5	10	–	1. Dissolve 2 g D-gluconic acid lactone in 25 ml distilled water 2. Raise pH to 12.5 with NaOH 3. Incubate at ambient temperature for 30 min (occasionally stirring) 4. Alter pH to less than 8.0 with conc. HCl. 5. Add 25 ml 0.2 M tris-HCl (pH 8.0)		Add 25 ml of fresh substrate to PMS, MTT, and NAD Incubate gel at 37 °C (dark)		S2
β-hydroxybutyrate dehydrogenase (HBDH) 1.1.1.30	d	3	5	5	–	D(-) hydroxybutyric acid	20 mg	0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark)	20 ml	S4, S5
Iso-citrate dehydrogenase (IDH) 1.1.1.42	d	3	5	–	5	DL-isocitric acid	20 mg	$MnCl_2$ 0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark)	5 mg 20 ml	S2
Leucine amino peptidase (LAP) 3.4.1.1	m	–	–	–	–	L-leucine β-napthylamide HCL	20 mg	1. Dissolve 20 mg of substrate in 5 ml dimethyl formamide		CA1, S8
						or L-leucine 4-methoxy-β-napthylamide	20 mg	2. Add 95 ml phosphate buffer (pH 7.0) 3. Incubate gel for 15 min (starch) or 2 min (cellulose acetate) 4. Pour off and add Fast Black K (SIGMA) 50 mg Fast Black K in 100 ml 0.1 M phosphate buffer		CA7, S8
Lactate dehydrogenase (LDH) 1.1.1.27	t	3	5	10	–	DL-lactic acid (lithium salt)	25 mg	0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark)	20 ml	CA6, S1, S5, S7, S8, S15
Malate dehydrogenase (MDH) 1.1.1.37	d	3	5	10	–	Malic acid solution 0.35 g DL-malic acid per 0.2 M tris-HCl (pH 8.0) Solution adjusted to pH 8.0 with NaOH	5 ml 25 ml	0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark)	25 ml	CA6, S4, S5, S14–16
Malic enzyme (ME) 1.1.1.40	m or t	3	5	–	5	Malic acid solution (As for MDH)	5 ml	0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark)	25 ml	S4, S5
Mannose phosphate isomerase (MPI) 5.3.1.8	m	3	5	–	5	Mannose 6-phosphate	15 mg	Glucosephosphate isomerase Glucose-6-phosphate dehydrogenase 0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark)	1 mg 1 µl 20 ml	CA8, CA9 S8–11
Nucleoside phosphorylase (NP) 2.4.2.1	tr	3	5	–	–	Inosine	10 mg	Xanthine oxidase Phosphate buffer (pH 7.0) Incubate gel at 37 °C (dark)	5 µl 20 ml	S4
Octopine dehydrogenase (Oct DH) 1.5.1.11	m	3	5	–	10	Octopine	50 mg	0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark)	25 ml	S5, S14
Peptidase (PEP) 3.4.1.1	m or d	–	–	–	–	Peptide (e.g., one of: leucine-glycine-glycine; valine-leucine	20 mg	Horseradish peroxidase *Bathrops atrox* (snake) venom 3-amino-9-ethyl carbazole (dissolve 3-amino-9-ethylcarbazole in 5 ml acetone before adding to stain) $MnCl_2$ Na_2HPO_4 buffer (pH 7.5) Incubate gel at 37 °C (dark)	1 mg 1 mg 10 mg 5 mg 35 ml	

Appendix 2—continued

Enzyme (abbreviation) E.C. No.*	Quarternary structure†	Stain ingredients: PMS (mg)	MTT (mg)	NAD (mg)	NADP (mg)	Substrate		Other chemicals and instructions		Suitable buffer systems‡
Peroxidase (PER) 1.11.1.7	m	-	-	-	-	3% H_2O_2	0.5 ml	Dissolve 50 mg 3-amino-9-ethyl carbazole in 5 ml dimethyl formamide and 0.5 ml H_2O_2 and add: 0.1 M calcium chloride 0.5 M sodium acetate (pH 5.0) Incubate gel in the above for 30-60 min at 4 °C After brown-red bands appear, rinse the gel and fix in 50% glycerol in water	 2 ml 92.5 ml	S8
						or refer to CAT — this recipe has tended to give better results				
Phosphoglucose isomerase (PGI or GPI) 5.3.1.9	d	3	5	-	5	Fructose 6-phosphate	15 mg	Glucose-6-phosphate dehydrogenase 0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark) (N.B. PGI bands tend to appear very quickly. Wash and fix (destain) after bands appear)	1 µl 20 ml	CA4, CA5, S9
Phosphoglucomutase (PGM) 2.7.5.1	m	3	5	-	5	Glucose-1-phosphate	25 mg	Glucose-6-phosphate dehydrogenase 0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark)	1 µl 20 ml	CA5, S5, S9
6-phosphogluconate dehydrogenase (PGDH) 1.1.1.44	d	3	5	-	5	6-phosphogluconic acid	20 mg	0.2 M tris-HCl (pH 8.0)	20 ml	CA1, CA3 S5, S13, S16
Pyruvate kinase (PK) 2.7.1.40	t	-	-	-	-	Phosphoenolpyruvate	20 mg	ADP NADH Magnesium sulphate Potassium chloride Fructose 1,6-diphosphate Lactate dehydrogenase 0.1 M tris HCl (pH 8.0) View under u.v. light	30 mg 10 mg 10 mg 10 mg 10 mg 10 µl 20 ml	
Superoxide dismutase (SOD) 1.15.1.1	d	3	5	10	-			0.2 M tris-HCl (pH 8.0) Stain in dark overnight, then expose to light	20 ml	S13
Sorbitol dehydrogenase (SDH) (L-iditol dehydrogenase) 1.1.1.14	t	3	5	10	-	Sorbitol	25 mg	0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (in dark)	20 ml	
Transferrin (Tf)	m	-	-	-	-			1. Add an equal volume of 4.5% 6,9-diamino-2-ethoxyacridine lactate (rivanol) in gel buffer to serum sample before electrophoresis, shake sample, and centrifuge at low speed for 5 min to precipitate non-transferrin proteins Use supernatant for electrophoresis Rivanol runs as a bright yellow cathodal band 2. After electrophoresis stain for 2 min in 1% amido black (in fixing solution) 3. Wash in fixing solution to remove background stain		S8
Xanthine dehydrogenase (XDH) 1.2.1.37	d	3	5	10	-	Hypoxanthine	10 mg	0.2 M tris-HCl (pH 8.0) Incubate gel at 37 °C (dark) (XDH activity might represent xanthine oxidase)	25 ml	S13

* Enzyme Commission number.
† m, monomer; d, dimer; t, tetramer; tr, trimer.
‡ s, starch; CA, cellulose acetate. Numbers refer to Appendix 1.

Appendix 3

Inheritance of enzyme and protein phenotypes in marine and freshwater teleosts

Protein	Species	Quarternary structure*	Reference
Adenosine deaminase	*Zoarces viviparus* (mother-offspring)	m	Simonsen and Christiansen 1981
	Oncorhynchus tshawytscha	m	Kobayashi *et al.* 1984
Alcohol dehydrogenase	*Salvelinus* hybrids	d	May *et al.* 1979a
Aspartate aminotransferase	*Clupea harengus*	d	King 1983
	Salvelinus hybrids	d	May *et al.* 1979a
	Oncorhynchus keta	d	May *et al.* 1975
	Salvelinus fontinalis	d	Wright *et al.* 1980
Creatine kinase	*Salmo gairdnerii*	d	Utter *et al.* 1979
Diaphorase	*Salmo trutta*	m	May *et al.* 1979b
Esterase	*Poeciliopsis monacha* (mother-offspring)	m	Leslie and Vrijenhoek 1977
	Xiphophorus hybrids	m	Morizot and Siciliano 1979 Siciliano and Wright 1976
	Pomoxis hybrids	m	Metcalf *et al.* 1972
	Zoarces viviparus (mother-offspring)	m	Simonsen and Frydenberg 1972
	Salmo hybrids	m	Nyman 1970
Glyceraldehyde-phosphate dehydrogenase	*Xiphophorus* hybrids	t	Siciliano and Wright 1976 Morizot and Siciliano 1982a
	Xiphophorus maculatus	t	Morizot *et al.* 1982
Glycerol-3-phosphate dehydrogenase	*Pleuronectes platessa*	d	Purdom *et al.* 1976
	Oncorhynchus gorbuscha	d	Aspinwall 1973
	Coregonus clupeiformis	d	Clayton *et al.* 1973
	Salvelinus hybrids	d	May *et al.* 1979a
Isocitrate dehydrogenase	*Fundulus heteroclitus*	d	Van Beneden *et al.* 1981
	Clupea harengus	d	Kornfield *et al.* 1981 King 1983
	Salmo gairdnerii	d	Allendorf and Utter 1973 Ropers *et al.* 1973 Reinitz 1977
	Poeciliopsis monacha (mother-offspring)	d	Leslie and Vrijenhoek 1977
	Xiphophorus hybrids	d	Morizot and Siciliano 1982b Siciliano and Wright 1973, 1976
	Oncorhynchus keta	d	May *et al.* 1975
	Salvelinus hybrids	d	May *et al.* 1979a
Lactate dehydrogenase	*Fundulus heteroclitus*	t	Place and Powers 1978
	Clupea harengus	t	Kornfield *et al.* 1981 King 1983
	Salmo gairdnerii	t	Utter *et al.* 1973
	Poeciliopsis monacha (mother-offspring)	t	Leslie and Vrijenhoek 1977
	Cyprinus carpio	t	Valenta *et al.* 1977
	Salvelinus fontinalis	t	Wright and Atherton 1970
	Salvelinus hybrids	t	May *et al.* 1979a
	Salmo hybrids	t	Morrison 1970 Morrison and Wright 1966
	Xiphophorus hybrids	t	Morizot and Siciliano 1979
Malate dehydrogenase	*Fundulus heteroclitus*	d	Place and Powers 1978
	Pleuronectes platessa	d	Purdom *et al.* 1976
	Salmo gairdnerii	d	Utter *et al.* 1973
	Stizostedion vitreum vitreum	d	Clayton *et al.* 1971
	Oncorhynchus gorbuscha	d	Aspinwall 1974
	Oncorhynchus tshawytscha	d	Bailey *et al.* 1970 Kristiansson and MacIntyre 1976
	Micropterus hybrids	d	Wheat *et al.* 1971
	Salvelinus hybrids	d	May *et al.* 1979a
	Salmo trutta	d	May *et al.* 1979b
Malic enzyme	*Clupea harengus*	t	Kornfield *et al.* 1981
	Salvelinus hybrids	t	Stoneking *et al.* 1979
Mannosephosphate isomerase	*Xiphophorus* hybrids	m	Morizot and Siciliano 1979
	Salvelinus hybrids	m	May *et al.* 1979a
Peptidase	*Poeciliopsis monacha* (mother-offspring)	d	Leslie and Vrijenhoek 1977

Appendix 3—continued

Protein	Species	Quarternary structure*	Reference
Phosphoglucomutase	*Fundulus heteroclitus*	m	Place and Powers 1978
	Clupea harengus	m	Kornfield *et al.* 1981
	Zoarces viviparus (mother-offspring)	m	Hjorth 1971
	Pleuronectes platessa	m	Purdom *et al.* 1976
	Oncorhynchus nerka	m	Utter *et al.* 1973
	Salmo gairdnerii	m	Diebig *et al.* 1979
Phosphogluconate dehydrogenase	*Fundulus heteroclitus*	d	Van Beneden *et al.* 1981
	Poeciliopsis monacha (mother-offspring)	d	Leslie and Vrijenhoek 1977
	Xiphophorus hybrids	d	Scholl and Anders 1973
Phosphoglucose isomerase	*Fundulus heteroclitus*	d	Place and Powers 1978
	Clupea harengus	d	Kornfield *et al.* 1981 King 1983
	Pleuronectes platessa	d	Dando 1974 Purdom *et al.* 1976
	Platichthys flesus	d	Dando 1974
	Zoarces viviparus (mother-offspring)	d	Yndgaard 1972
	Xiphophorus hybrids	d	Morizot and Siciliano 1982b Siciliano and Wright 1976
	Salvelinus hybrids	d	May *et al.* 1979a
Protein	*Poeciliopsis monacha* (mother-offspring)	d	Leslie and Vrijenhoek 1977
	Xiphophorus hybrids	d	Scholl and Anders 1973
	Poeciliopsis monacha (mother-offspring)	m	Leslie and Vrijenhoek 1977
	Salvelinus fontinalis	presence/absence	Eckroat and Wright 1969
Sorbitol dehydrogenase	*Salvelinus* hybrids	t	May *et al.* 1979a
Superoxide dismutase	*Salmo gairdnerii*	d	Utter *et al.* 1973
	Carp hybrids	d	Valenta *et al.* 1977
	Oncorhynchus tshawytscha	d	Kristiansson and MacIntyre 1970
	Salvelinus hybrids	d	May *et al.* 1979a
Transferrin	*Salmo gairdnerii*	m	Utter *et al.* 1973
	Oncorhynchus kisutch	m	Utter *et al.* 1973
	Carp hybrids	m	Beck *et al.* 1983

* m, monomer, d, dimer, t, tetramer.

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Appendix 4

Example of a gel-run sheet for recording electrophoretic data

ELECTROPHORESIS RESULTS SHEET

SPECIES ... P. subtmangulata ... LOCATION ... Nelson ...

TISSUE ... Digestive gland ... VESSEL/DATE ... 9 1 86 ...

Prep. | Hand | | Centrifuge | ✓ | Controls ... D'ville 41 & 68 ...

Gel | Starch | ✓ | CA | ✓ | IEF | | Date tested ... 6 6 86 ...

Buffers ... TCI TCS Rid Ac CA Cit ...

Gel pos.	Sample code no.	GENOTYPES									
		Mdh-1	Mdh-2	αGpd	Ldh	Gpi	bGpd	Aat	Idh	Est-1	Est-2
1	6	F	F	FS	F	F	FS	M	F	F	F
2	70	↑	↑	F	↑	S	F	M	F	↑	↑
3	29			FS		FS	FS	M	F		
4	31			F		FS	F	M	F		
5	52			F		FS	F	MS	F		
6	54			FS		F	F	M	F		
7	55			F		F	S	M	F		
8	61			F		FS	F	MS	F		
9	Control 41			F		FS	F	MS	F		
10	62			F		FS	FS	M	*F F		
11	63			F		FS	F	M	F		
12	64			F		FS	F	M	F		
13	65			FS		F	F	M	F		
14	69			FS		FS	F	M	F		
15	71			F		FS	FS	M	F		
16	72			F		S	FS	M	F		
17	77			FS		FS	FS	M	F		
18	Control 68			FS		FS	S	M	F		
19	79			S		*FS	FS	S	F		
20	81			F		FS	FS	M	F		
21	86			F		FS	FS	M	F		
22	87			S		FS	F	M	F		
23	88			FS		FS	F	M	F		
24	95			F		F	S	M	F		
25	99			FS		FS	S	M	F		
26	100	↓	↓	F	↓	FS	FS	M	F	↓	↓
27											
28											
29											
30											

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