Bio-Phoresis® Horizontal Electrophoresis Cell

Instruction Manual

Catalog Number
170-2900

For Technical Service
Call Your Local Bio-Rad Office or
in the U.S. Call **1-800-4BIORAD**
(1-800-424-6723)

BIO-RAD

Note

To insure best performance from the Bio-Phoresis horizontal electrophoresis cell, become fully acquainted with these operating instructions before using the cell to separate samples. Bio-Rad recommends that you first read these instructions carefully. Then assemble and disassemble the cell completely without casting a gel. After these preliminary steps, you should be ready to cast and run a gel.

Bio-Rad also recommends that all Bio-Phoresis horizontal electrophoresis cell components and accessories be cleaned with a suitable laboratory cleaner, such as Bio-Rad Cleaning Concentrate, catalog number 161-0722, and rinsed thoroughly with distilled water, before use.

Model _____

Catalog No. _____

Date of Delivery _____

Warranty Period _____

Serial No. _____

Invoice No. _____

Purchase Order No. _____

Warranty

Bio-Rad Laboratories warrants the Bio-Phoresis horizontal electrophoresis cell against defects in materials and workmanship for 1 year. If any defects occur in the instrument during this warranty period, Bio-Rad Laboratories will repair or replace the defective parts free. The following defects, however, are specifically excluded:

1. Defects caused by improper operation.
2. Repair or modification done by anyone other than Bio-Rad Laboratories or an authorized agent.
3. Use of fittings or other spare parts supplied by anyone other than Bio-Rad Laboratories.
4. Damage caused by accident or misuse.
5. Damage caused by disaster.
6. Corrosion due to use of improper solvent or sample.

This warranty does not apply to parts listed below:

1. Platinum wire, glass plates.

For any inquiry or request for repair service, contact Bio-Rad Laboratories after confirming the model and serial number of your instrument.

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Section 1
Getting Started

1.1 General Description

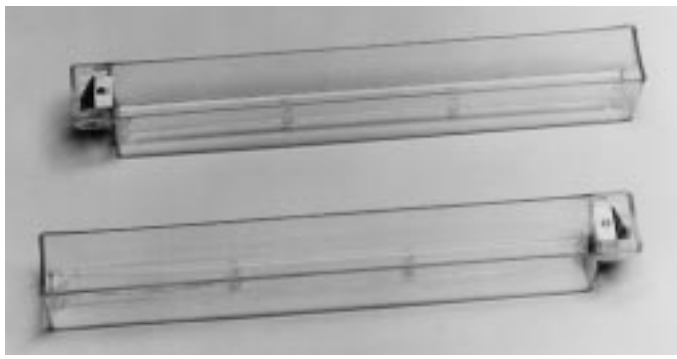
The versatile Bio-Phoresis horizontal electrophoresis cell accommodates interchangeable accessories which allow you to perform immunoelectrophoresis, electrophoresis, and electrofocusing in the same cell. Only the addition of the appropriate electrodes, a power supply, and an applications kit for the specific technique is required.

The Bio-Phoresis cell features:

- Large epoxy coated aluminum cooling platform (125 x 360 mm) surface for increased capacity with high cooling efficiency.
- Location grid on the cooling platform surface for easy location of protein bands and determination of pH gradients.
- Built-in condensation control to eliminate fogging on the lid and to prevent condensed liquid from dripping on the gel.
- Unique electrofocusing electrode holder that exerts uniform electrode pressure on the gel for better resolution.
- Direct connect electrofocusing electrodes and electrode/buffer chambers that eliminate connection wires.
- Molded construction for strength and durability.
- Platinum wire electrodes.
- Leveling feet to prevent skewing of gels.
- Safety interlock to prevent cell operation with the lid open.

1.2 Cell Components

The Bio-Phoresis cell has three major components: the cell body with safety interlock, condensation coil, and electrode contacts; the cell lid; and the ceramic cooling platform with the location grid. The addition of optional electrofocusing electrodes and an electrofocusing electrode holder, or electrophoresis electrode/buffer chambers, is all that is needed to complete the unit.



1.3 Specifications

Construction

Cell body	Injected molded Noryl® plastic
Cell lid	Thermal formed acrylic plastic
Electrode/buffer chambers	Thermal formed acrylic plastic
Electrofocusing electrodes	Extruded acrylic plastic
Electrode holder	Acrylic plastic
Condensation coil	Stainless steel
Cooling platform	Epoxy coated aluminum

Electrical

Maximum input voltage	3,000 volts DC

Shipping weight

Basic unit	6 Kg

Size	
Basic unit (with lid down)	469 mm (L) x 290 mm (W) x 67.5 mm (H)
Working surface	
Cooling platform	125 mm (W) x 360 mm (L)
Buffer capacities	
Both electrode/buffer chambers in total	725 ml

1.4 Safety

Power to the Bio-Phoresis cell is to be supplied by an external DC voltage power supply. **This power supply must employ a safety isolation transformer to isolate the DC voltage output with respect to ground.** All of Bio-Rad's power supplies meet this important safety requirement. Regardless of which power supply is used, the maximum specified operation parameters for the cell are:

3,000 VDC ⎓	maximum voltage limit
25 watts	maximum power limit
50 ˚C	maximum ambient temperature limit

Current to the cell, provided from the external power supply, enters the unit through the lid assembly, providing a safety interlock to the user. Current to the cell is broken when the lid is removed. Do not attempt to circumvent this safety interlock and always turn the power supply off before removing the lid, or when working with the cell in any way.

1.5 Equipment

Catalog Number	Equipment
170-2900	**Bio-Phoresis Horizontal Electrophoresis Cell**
Accessories	
170-2903	**Electrofocusing Electrodes**, 360 mm, 2
170-2902	**Electrofocusing Electrodes**, 120 mm, 2
170-2904	**Posi-Force® Electrofocusing Electrode Holder**
170-2901	**Electrode/Buffer Chambers for Immunoelectrophoresis**

1.6 General Instructions

The Bio-Phoresis horizontal electrophoresis cell is shipped assembled, without electrodes. Electrode/buffer chambers and electrofocusing electrodes and holders are optional. Refer to the Bio-Rad catalog for a complete description and ordering information.

Unpack the electrophoresis cell carefully, and inspect the unit for damage. Complete the warranty card and mail it to Bio-Rad.

Caution: When operating the unit, do not use the safety interlock on the lid as a power switch. Turn off the power supply before opening the lid.

1.7 Analytical Isoelectric Focusing

1. Connect 1/4" tubing from the inlet port of the cell to the outlet port of a refrigerated circulating water bath set to 4 ˚C. To keep the temperature in the condensation coil slightly lower than that in the cooling stage, water will circulate through the condensation coil first.

2. Connect 1/4" tubing to the outlet port of the cell and direct it to the inlet port of the refrigerated circulating water bath.

   Note: If the coolant pressure entering the cell is too high, the adhesive seal on the cooling platform will burst. To prevent leaks from occurring always monitor the flow of coolant entering the cell prior to starting a run, and use ≥ 1/4 inch diameter tubing throughout the system to reduce the pressure inside the cooling platform.

   Recirculating water baths typically have flow rates of 1 liter/min. The internal pumping system of the water bath will provide regulated flow rates that are suitable for the Bio-Phoresis cell. Because of this compatibility, it is recommended that a recirculating water bath be used to cool the Bio-Phoresis cell.

   Tap water can be used to cool the Bio-Phoresis cell, but precautions must be made to regulate its flow. Sudden changes in pressure (i.e., when the tap is accidentally opened to full flow) can burst the seal of the cooling platform. Before hooking tap water to the Bio-Phoresis cell, check that all tubing is ≥ 1/4 inch in diameter and that the flow entering the cell is ≤ 1-2 liter/min. Once the flow is properly set do not adjust it while the hose is connected to the cell. The Bio-Phoresis cell does have an internal pressure relief valve that will actuate at 8 psi to compensate for fluctuations in water pressure at the 1-2 liter/min flow rate. This valve cannot compensate for extreme fluctuations in volumes and pressures.

3. Level the cooling platform and turn on the refrigerated water bath. Place a bubble level on the cooling platform, and adjust the leveling feet on the bottom of the cell until the bubble is centered. Check to be sure the cell does not wobble. Allow the temperature to equilibrate before beginning electrofocusing.

4. With the cooling water at 4 ˚C, place a previously prepared gel or granulated bed on a thin film of water or 1% glycerol on the cooling stage. Eliminate bubbles by placing additional liquid at the edge, or by relaying the plate. Remove excess liquid from the stage.

5. Attach the long or short electrodes to the electrode holder. Position the electrode as near the edge of the holder as possible.

6. Position electrolyte soaked paper strips on the gel or granulated bed. See IEF instruction sections for the specific technique and details of electrolyte selection.

7. Place the electrode holder with attached electrodes over the cooling platform. Place the holder in the slots in the cell to position the electrode over the gel or granulated bed. Adjust the electrodes so that they will contact the electrolyte soaked paper strips when lowered into the cell. Tighten the screws on the electrodes to prevent them from moving. Make sure the electrodes are positioned so that the colored dot (red or black) on the electrode holder corresponds with the colored dot (red or black) on the cell.

8. Apply sample or samples. The electrode holder is open to allow easy sample application. Make a final check of electrode contact and spacing.

9. Close the lid and start electrophoresis. Always close the lid before switching the power on. Always switch the power off before opening the lid. Power will switch off automatically when the lid is opened; however, to prevent premature failure of the safety interlock switch, the lid should not be used as an on-off switch.

1.8 Set-up for Immunoelectrophoresis

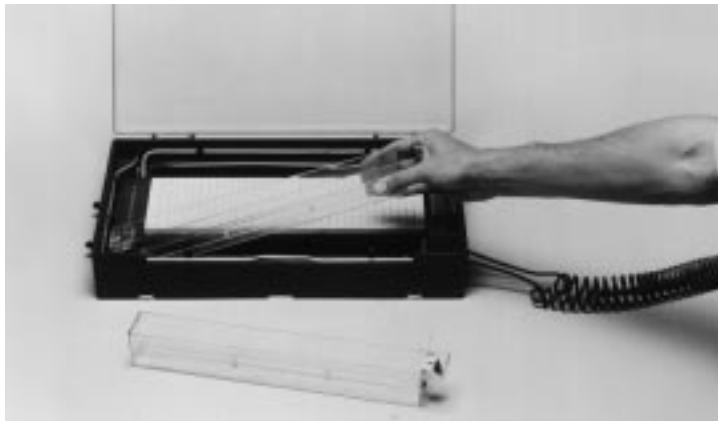
Together, the ceramic cooling stage and condensation coil eliminate artifacts due to condensation on the gel surface and inside the lid (see below).

1. Connect 1/4" tubing from the water source (tap water line or the outlet port of a refrigerated water bath) to the inlet port of the cell.
2. Connect 1/4" tubing to the outlet port of the cell and direct it to the drain (or to the inlet port of the refrigerated water bath).

   Note: If the coolant pressure entering the cell is too high, the adhesive seal on the cooling platform will burst. To prevent leaks from occurring, always monitor the flow of coolant entering the cell prior to starting a run, and use ≥ 1/4 inch diameter tubing throughout the system to reduce the pressure inside the cooling platform.

   Recirculating water baths typically have flow rates of 1 liter/min. The internal pumping system of the water bath will provide regulated flow rates that are suitable for the Bio-Phoresis cell. Because of this compatibility, it is recommended that a recirculating water bath be used to cool the Bio-Phoresis cell.

   Tap water can be used to cool the Bio-Phoresis cell, but precautions must be made to regulate its flow. Sudden changes in pressure (i.e., when the tap is accidentally opened to full flow) can burst the seal of the cooling platform. Before hooking tap water to the Bio-Phoresis cell, check that all tubing is ≥ 1/4 inch in diameter and that the flow entering the cell is ≤ 1-2 liter/min. Once the flow is properly set, do not adjust it while the hose is connected to the cell. The Bio-Phoresis cell does have an internal pressure relief valve that will actuate at 8 psi to compensate for fluctuations in water pressure at the 1-2 liter/min flow rate. This valve cannot compensate for extreme fluctuations in volumes and pressures.
3. Level the cooling platform and turn on the cooling water. Place the bubble level on the cooling platform, and adjust the leveling feet on the bottom of the cell until the bubble is centered. Check to be sure the cell does not wobble.
4. Allow the temperature of the cell to equilibrate before beginning electrophoresis.



5. Install the electrode/buffer chambers. Place the electrode/buffer chambers in the cell alongside the cooling platform. Locate the chamber with the colored dot (red or black) which corresponds with the colored dot (red or black) on the cell. This will insure that the anode and cathode connections are correct. The electrical connections are at opposite ends to equalize the electrical field.

6. Pour equal amounts of buffer into each chamber. Chambers should be nearly full to insure adequate buffer volume and to shorten the distance from the buffer to the cooling platform. Each chamber will hold a maximum volume of 362 ml.

7. Place a small amount of water or 1% glycerol on the cooling platform. Then place one edge of the previously prepared gel plate in the liquid and lay it down slowly so that a thin film of liquid forms between the plate and the cooling platform. The liquid maximizes heat transfer to the cooling platform. Any bubbles will cause irregularities in sample migration. Make sure that any excess liquid extruded from between the gel plate and the surface of the cooling platform is removed. Be sure that the plate is aligned parallel to the buffer chambers. Multiple gel plates must be aligned evenly with each other as well.

8. Place wicks and apply sample. Connect the gel to the buffer using Ultra-Wicks soaked with buffer. Overlap the edge of the gel by 1/2 to 1 cm. Optional agarose gel bridges are available. Place the other end of the wicks into the inside side of the electrode/buffer chamber. This separates the wicks from the platinum electrodes and prevents them from being in contact with the more concentrated electrolysis products, which could decrease conductivity and cause problems associated with pH changes. Apply sample as required for the particular separation technique being performed. (Refer to the section about the technique you are using.)

9. Close the lid and start electrophoresis.

   Caution: Always close the lid before switching the power on. Always switch the power off before opening the lid.

   Connect the cables from the cell to the power supply, red to red and black to black. Turn on the power supply and set to the desired power setting to start electrophoresis.

1.9 Maintenance

Wash the Bio-Phoresis cell with mild soap and water, scrubbing the electrode contacts with a soft brush. Do not immerse the unit, or allow the electrical outlets or cables to get wet. Exercise caution when cleaning the electrodes and buffer chambers; platinum wire will break if excessive force is used in scrubbing. Never use hot water, abrasive powders, or non-aqueous solvents on the surface of the cell.

Always wipe the cell dry with absorbent paper towels after an electrophoresis run. Examine the platinum wire after use for bends which may affect the performance of the electrode. Replace the platinum wire if necessary; bends will cause unusual spikes and ridges in the gels.

Section 2
Analytical Polyacrylamide Gel Isoelectric Focusing (IEF-PAGE)

2.1 Historical Perspective

Isoelectric focusing, or electrofocusing, is the result of a series of methodological and theoretical advances from as early as 1912. Early workers described the separation of amphoteric material in membrane-divided, multi-compartment electrolysis chambers. Tiselius (1941), and later Kolin (1954), developed the idea of stationary electrolyte concentration distributions in an electrical field, and were able to perform separations using “artificial” pH gradients generated by buffering electrolytes.[1] Modern electrofocusing was born out of the

theoretical work of Swensson (now called Rilbe) on the generation of “natural” pH gradients with carrier ampholytes,[2-4] and the synthetic work of Vesterberg, who produced the first synthetic carrier ampholytes that met Swensson's criteria.[5]

2.2 The Electrofocusing Principle - How Does it Work?

Conventional electrophoresis separates proteins and other charged molecules by electrophoretically-driven migration through a sieving matrix that is buffered at a constant pH. Each component of the mixture assumes its own characteristic velocity based on molecular size and surface charge. This velocity is constant throughout the electrophoresis experiment and is counteracted by diffusion, which tends to broaden the bands. There is no tendency toward equilibrium in conventional electrophoresis, and the protein bands will run off the gel if the electrical field is not interrupted.

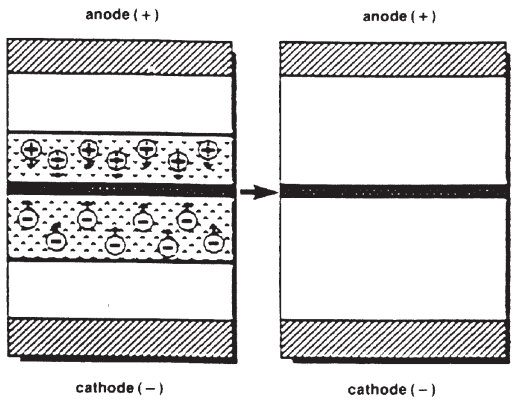


Fig. 2.1. Diagrammatic view of electrofocusing. Approach to focused state on left; focused state on right.

On the other hand, electrofocusing separates proteins on the basis of surface charge alone as a function of pH. The separation is done in a non-sieving medium (sucrose density gradient, agarose, or polyacrylamide gel) in the presence of carrier ampholytes, which establish a pH gradient increasing from the anode to the cathode. Since a protein contains both positive (amines) and negative (carboxyl) charge-bearing groups, the new charge of the protein will vary as a function of pH.

A pH gradient is established in the gel by preliminary electrophoresis, or concomitant with protein separation. As the protein migrates into an acidic region of the gel, it will gain positive charge via protonation of the carboxylic and amino groups. At some point. the overall positive charge will cause the protein to migrate from the anode (+) to a more basic region of the gel. As the protein enters a more basic environment, it will lose positive charge and gain negative charge, via ammonium and carboxylic acid group deprotonation, and consequently, will migrate away from the cathode (-). Eventually, the protein reaches a position in the pH gradient where its net charge is zero (defined as its p*I* or isoelectric point). At that point, the electrophoretic mobility is zero. Migration will cease, and a concentration equilibrium of the focused protein is established. A pictorial summary of this phenomenon is given in Figure 2.1.

Unlike conventional electrophoresis, electrofocusing is, as its name implies, a focusing phenomenon. Should the protein move slightly away from its p*I* on the gradient, it will acquire a net charge corresponding to its position on the gradient, and be drawn back to its pI, producing a tightly focused protein zone. Consequently, electrofocusing is a high resolution technique, capable of separating closely related proteins into very narrow bands. The empirical

limit of resolution for electrofocusing has been reported to be as low as 0.005 pH units;[6] however, a good practical limit to the resolving power is about 0.02 pH units.[7]

The resolving power of electrofocusing actually depends on a number of parameters. The mathematical equation describing resolution is given by:[8]

$$\Delta \text{ pI} = 3 \times \sqrt{\frac{D \, d(\text{pH}) / dx}{E \, (-du / d\text{pH})}}$$

where:

D = protein diffusion coefficient

dpH/dx = the slope of the pH gradient

du/dpH = the mobility slope of the protein (a constant near the p*I*)

E = local field strength

Therefore, the highest resolution for a given protein is obtained with shallow (i.e. narrow range) gradients and high field strength. An excellent summary and presentation of electrofocusing theory can be found in reference 1.

2.3 Carrier Ampholytes

Carrier ampholytes are complex mixtures of amphoteric buffers that form smooth pH gradients in an applied electrical field. During electrofocusing, these buffers stack according to their individual p*I*s across the gel, producing a step gradient. In order for the gradient to appear smooth and continuous, a large number of these buffering components must be present. This is also a requirement for separating a complex mixture of proteins.

Bio-Lyte ampholytes are derivatized low-molecular weight amines that are electrophoretically separated and reblended to give smooth and reproducible gradients. Narrow range Bio-Lyte ampholytes are produced and tested so that, under normal circumstances, no additional blending or fortification will be necessary to achieve the desired shallow gradient.

For preparative applications, proteins often must be separated from the carrier ampholytes in the focusing zone. This may be done by dialysis,[7] ultrafiltration,[9] or ion exchange chromatography.[10,11]

2.4 Choice of Support Matrix

The electrofocusing process must be stabilized against convection and, to a lesser extent, diffusion, by a support matrix. This can be anything from a liquid column stabilized by a sucrose density gradient to a gel cast from agarose or polyacrylamide. The principal criteria for a good support matrix are that it should be relatively non-sieving so that molecular size is not a factor in protein mobility, and that it must be free of charged groups which would give rise to internal fluid flow and distortion of the pH gradient. Only those supports suitable for horizontal electrofocusing in the Bio-Phoresis cell will be discussed. A complete discussion of electrofocusing matrices is given in reference 1.

For preparative work, a granulated bed of Bio-Lyte electrofocusing gel (catalog number 163-1194) provides a suitable matrix for purifying up to 1 gram of protein in a focused band.[12] The gel is formed of cross-linked polyacrylamide and has been specially processed to minimize carboxylic acid content. A detailed discussion of preparative electrofocusing with the Bio-Phoresis cell is given in Section 6.

For analytical work, both agarose and polyacrylamide gels provide good supports for electrofocusing. Agarose has the advantage of very large pore structures (as large as 500 nm), making it an ideal non-sieving medium; however, it suffers from varying degrees of residual negative charge from sulfate groups. For this reason, only agarose proven for electrofocusing applications should be used (Zero -m_r Agarose, catalog number 162-0022). Agarose concentrations may vary between 0.5 and 1.25%. Proteins as large as 50 x 10^6 daltons have been successfully electrofocused in agarose.[13]

2.5 Review of Applications

Several reviews are available for various applications of electrofocusing. A complete manual for electrofocusing, including methodology and applications, has been published[1] and an earlier version is still available.[14] Other reviews in the field are: general methodology and applications, including clinical;[15] clinical applications;[16] membrane proteins;[17] new methodology;[18,19] plant and food proteins;[20] methodology applied to two-dimensional electrophoresis.[21]

In addition, three extensive listings of protein isoelectric points have been published.[22-24] These will prove useful in correlating data in various protein systems.

2.6 References for IEF-PAGE

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21. Dunn, M. J. and Burghes, A. H. M., *Electrophoresis*, **4**, 97 (1983).
22. Righetti, P. G. and Caravaggio, T., *J. Chrom.*, **127**, 1 (1976).
23. Malamud, D. and Drysdale, J. W., *Anal. Biochem.*, **86**, 620 (1978).
24. Righetti, P. G., Tudor, G. and Ek, K., *J. Chrom.*, **220**, 115 (1981).

2.7 Equipment for Analytical Isoelectric Focusing

Catalog Number	Product Description
170-2900	**Bio-Phoresis Horizontal Electrophoresis Cell (basic cell, electrodes not included)**

Electrofocusing Electrodes

170-2902	**Electrofocusing Electrodes,** 120 mm, 2
170-2903	**Electrofocusing Electrodes,** 360 mm, 2
170-2904	**Posi-Force Electrode Holder (required)**

Power Supply

165-5056	**PowerPac 3000 Power Supply,** 100/120 VAC
165-5057	**PowerPac 3000 Power Supply,** 220/240 VAC

Kits

170-4253	**Analytical Electrofocusing Kit,** 120 V. Includes: CTL Casting Tray; 110 V Photopolymerization Light; Electrofocusing Wicks (100); Glass Plates 45 x 125 mm (30), 100 x 125 mm (50); Sample Application Pieces (200)
170-4258	**Analytical Electrofocusing Kit,** 220 V. Same as 110 V kit except light is 220 V
170-4260	**Ultra-Thin Layer Electrofocusing Kit,** 120 V. Includes; Ultra–Thin Layer Casting Tray; Photo–polymerization Light (110 V); Gel Support Film, 100 x 125 x 0.2 mm (100); Glass Plates, 100 x 125 x 1.5 mm (50); Electrofocusing Wicks, 0.7 x 33 cm (100); Sample Application Pieces(200)
170-4261	**Ultra-Thin Layer Electrofocusing Kit,** 220 V. Same as 110 V except light is 220 V

Accessories and Reagents
Analytical IEF Accessories

170-4259	**UTL Casting Tray,** 0.2 and 0.4 mm gels
170-4219	**CTL Casting Tray,** 0.8 and 1.6 mm gels
170-2981	**Mini Casting Tray**
170-4262	**Gel Support Film for Polyacrylamide,** 100 x 125 x 0.2 mm, 100
170-4204	**Electrofocusing Strips,** 0.7 x 33 cm, 100
170-2985	**Sample Templates** (5)
170-4104	**Glass Plates,** 125 x 100 x 1.5 mm, 50
170-4230	**Glass Plates,** 125 x 205 x 1.5 mm, 15
170-4046	**Leveling Table,** 20 x 30 cm
170-4237	**Surface pH Electrode**
170-4240	**Photopolymerization Light,** 110 VAC

Catalog Number	Product Description
170-4242	**Photopolymerization Light,** 220 VAC

Polyacrylamide Gel Reagents

161-0100	**Acrylamide, 99.9%,**[A] 100 g
161-0101	**Acrylamide, 99.9%,**[A] 500 g
161-0200	**Bis,**[A] 5 g
161-0201	**Bis,**[A] 50 g
161-0500	**Riboflavin,**[B] 25 g
161-0501	**Riboflavin–5'–Phosphate,**[B] 10 g
161-0700	**Ammonium Persulfate,**[B] 10 g
161-0800	**TEMED,**[B] 5 ml
161-5100	**PAGE Reagent Starter Kit,** includes: Acrylamide, 100 g; Bis, 5 g; TEMED, 5 ml; Ammonium Persulfate, 10 g
161-0310	**IEF Standards,**[E] p*I* 4.6–9.6
163-1112	**Bio-Lyte 3/10 Ampholyte, 40%,**[D] 10 ml
163-1113	**Bio-Lyte 3/10 Ampholyte, 40%,**[D] 25 ml
163-1132	**Bio-Lyte 3/ 5 Ampholyte, 20%,**[D] 10 ml
163-1142	**Bio-Lyte 4/6 Ampholyte, 40%,**[D] 10 ml
163-1143	**Bio-Lyte 4/6 Ampholyte, 40%,**[D] 25 ml
163-1152	**Bio-Lyte 5/7 Ampholyte 40%,**[D] 10 ml
163-1153	**Bio-Lyte 5/7 Ampholyte 40%,**[D] 25 ml
163-1192	**Bio-Lyte 5/8 Ampholyte, 40%,**[D] 10 ml
163-1193	**Bio-Lyte 5/8 Ampholyte, 40%,**[D] 25 ml
163-1162	**Bio-Lyte 6/8 Ampholyte, 40%,**[D] 10 ml
163-1163	**Bio-Lyte 6/8, Ampholyte, 40%,**[D] 25 ml
163-1172	**Bio-Lyte 7/9Ampholyte, 40%,**[D] 10 ml
163-1182	**Bio-Lyte 8/10 Ampholyte, 20%,**[D] 10 ml
161-0400	**Coomassie Brilliant Blue R-250,**[A] 10 g
161-0406	**Coomassie Brilliant Blue G-250,**[A] 10 g
161-0417	**Crocein Scarlet,**[A] 10 g
161-0443	**Silver Stain Kit,**[D] includes: oxidizer concentrate, silver reagent concentrate, and developer. Enough to stain approximately 24 gels.

All electrophoresis purity reagents listed in this section , except the DNA Size Standards, are shipped at room temperature. For longer shelf life, follow these storage instructions:

A. Store at room temperature.
B. Store desiccated at room temperature.
C. Store desiccated at -20 ˚C.
D. Store at 4 ˚C.
E. Store at -20 ˚C.
F. Store desiccated at 4 ˚C.

Section 3
Preparing Thin Layer and Ultra-Thin Layer Polyacrylamide Gels for Analytical IEF

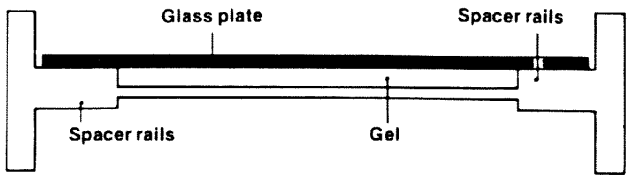


Fig. 3.1. Reversible casting tray.

3.1 Capillary Thin Layer Caster (CTL)

The capillary thin layer caster (CTL) provides the fastest and easiest method to cast electrofocusing gels. The double sided trays are available in two sizes: thin layer (catalog number 170-4219) for 0.8 and 1.6 mm thick gels, and ultra thin layer (catalog number 170-4259) for 0.2 and 0.4 mm gels. The trays consist of an acrylic plate with precisely defined spacer rails as shown in Figure 3.1. The acrylic surface imparts a slight inhibitory effect on acrylamide polymerization, eliminating sticking and tearing of the gel.

Important Considerations in Matrix Preparation

Because they are prepared from monomers, polyacrylamide gels can be tailored to meet particular separations requirements. The most common gel composition for horizontal electrofocusing is T = 5%, C = 3%, where:

$$\%\ T = \frac{\text{g acrylamide + g crosslinker}}{\text{Total volume of monomer solution}} \times 100$$

$$\%\ C = \frac{\text{g crosslinker}}{\text{g acrylamide + g crosslinker}} \times 100$$

This formulation will give a suitably non-sieving gel for proteins up to 10^6 daltons, that is still rigid enough to handle conveniently. A slightly stronger gel of T = 5%, C = 4% may be used for protein samples under 200,000 daltons.[1]

The choice of a catalyst system is extremely important in electrofocusing, since any residual ions will affect the final attainable voltage, and can lead to overheating and gross distortions in the gel. For this reason a three-phase catalyst system of ammonium persulfate, riboflavin-5'-phosphate, and TEMED is recommended. This system, catalyzed by light, will give reproducible polymerization with a minimum of ionic contamination.

The formation of polyacrylamide gels has been extensively studied,[2-4] and a detailed discussion of practical considerations is available in bulletin 1156.

Thin or Ultra-Thin Layer?

Thin layer electrofocusing in polyacrylamide gels has been a popular technique for over a decade. Although the earliest gels were 1.5 to 3.0 mm thick, a large number of recent publications cite the use of 1 mm thick gels for routine work. The capillary thin layer caster (CTL) will produce gels 0.8 and 1.6 mm thick. These thicknesses are appropriate to most routine work, with sample loads of up to 10 µg per band.

Recently, very thin gels (0.4 mm or less) have been described in electrofocusing applications.[5,6] These ultra-thin gels provide superior heat dissipation, and allow the use of substantially increased field strengths to achieve improved resolution in a short run time. In addition, staining and destaining procedures are significantly faster with ultra-thin gels, resulting in a valuable time savings.

The chief drawbacks of ultra-thin electrofocusing gels have been the extreme fragility of the gel, and the difficulty of casting a uniform thin layer. The former has been overcome by the use of a treated polyester support film that covalently binds to acrylamide;[6] the latter, by Bio-Rad's patented ultra-thin layer casting tray, which will easily produce 0.4 and 0.2 mm gels.

When selecting a gel thickness, several factors must be considered. Ultra-thin gels offer high resolution, fast run times (usually 60 minutes), very fast staining and destaining (1 to 2 hours, total), and economy of reagents, especially carrier ampholytes. On the other hand, ultra-thin gels are more sensitive to salt contamination in the sample and have a limited loading capacity (maximum = 1-5 µg per band). In addition, the gel support film for polyacrylamide is not compatible with electrophoretic or capillary blotting, and the gel must be dried directly on the polyester backing.

Detailed instructions for casting thin (0.8 and 1.6 mm) gels are in Section 3.4; instructions for ultra-thin (0.2 and 0.4 mm) gels are in Section 3.5.

3.2 Stock Solutions for Ultra-Thin Layer and Thin Layer Polyacrylamide IEF

Always use high quality distilled deionized water to prepare stock solutions for electrofocusing.

1. Monomer Concentrate

24.25% (w/v) acrylamide

0.75% (w/v) Bis (N, N' methylene-bis-acrylamide)

Dissolve 24.25 g acrylamide and 0.75 g Bis in water, bring to a final volume of 100 ml, and filter through a 0.45 µ filter. Store protected from light at 4 ˚C. This solution may be stored up to 1 month.

2. 0.1% (w/v) riboflavin-5'-phosphate (FMN)

50 mg riboflavin-5' -phosphate

50 ml water

This solution may be stored up to 1 month at 4 ˚C protected from light.

Note: Riboflavin can be substituted for riboflavin-5'-phosphate, however FMN takes minutes to solubilize while riboflavin will take 4-6 hours.

3. 10% (w/v) ammonium persulfate

100 mg ammonium persulfate

1 ml water

Prepare fresh daily. Make sure that the ammonium persulfate is completely dissolved before using.

4. 25% glycerol (w/v)

Add 25 g glycerol to 50 ml H_2O. Dilute to 100 ml with H_2O.

5. TEMED (N, N'-tetramethylene-ethylenediamine)

Use TEMED neat from the bottle. Use only pure, distilled TEMED. Store cool, dry, and protected from light.

3.3 Reagents for Polyacrylamide Electrofocusing Gels

The following volumes will product sufficient reagent for two 45 x 125 x 0.8 mm gels, or two 100 x 125 x 0.2 mm gels:

Monomer Solution

H_2O	5.5 ml
Monomer concentrate (25% T, 3.0%C)	2.0 ml
25% (w/v) glycerol	2.0 ml
Ampholyte	0.5 ml

Catalyst Solution

10% (w/v) ammonium persulfate	15 µl
0.1% (w/v) FMN	50 µl
TEMED (neat)	3 µl

Note: The selection of appropriate ampholyte is determined by the pH range required for a particular separation. Bio-Lyte ampholytes are specifically blended to produce a linear gradient within the stated range and no further blending is generally needed. Particular separations, however may require a combination of two or more ampholytes to achieve a desired result.

3.4 Casting Thin Layer Gels

Important: The glass plates and the casting tray must be clean and dry. Do not use organic solvents, abrasive cleaners, or hot water on the casting tray. Clean the glass plates with soap and water and rinse with deionized water followed by ethanol. Wipe the plates dry with lint-free paper.

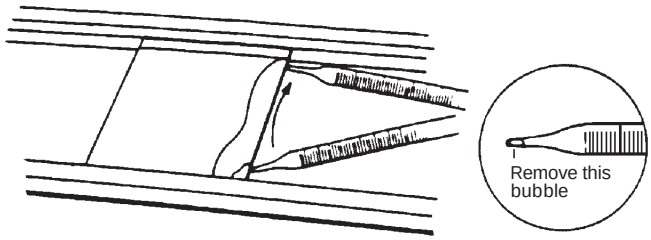
1. Place the glass plate(s) on the casting tray.
2. Prepare the monomer-ampholyte solution (see Section 3.3).
3. Degas the solution for 5 minutes with gentle agitation under aspirator vacuum.
4. Prepare the catalyst solution.

Note: Use freshly prepared persulfate solutions.

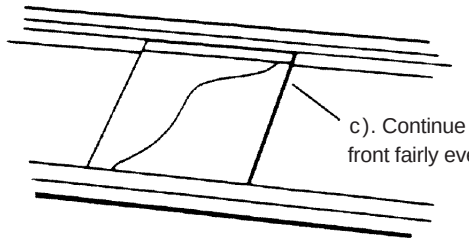
5. Mix the required catalyst solutions with the degassed monomer and swirl gently.

   Caution: Do not mouth pipet acrylamide solutions. Wear gloves. Acrylamide is a neurotoxin.

6. Pipet the solution between the glass plate and the casting tray as shown in Figure 3.2.
   a. Hold the pipet at a 45˚ angle and clear the air bubble from the tip.
   b. Start the monomer flow at one end of the glass plate and slowly move the pipet to the other spacer.



a). Clear bubble from end of pipet. b). Start solution at one corner of plate and move it along the edge to form a front. Be sure the solution contacts both the plate and tray. Avoid trapping air bubbles by releasing the solution slowly.



c). Continue to inject solution, keeping the front fairly even to avoid forming bubbles.

Fig. 3.2. Injecting monomer/ampholyte solution in CTL casting system.

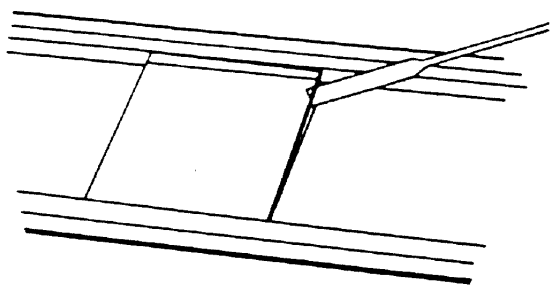


Fig. 3.3. Loosening the plate.

c. When a liquid front is established across the plate, slowly add the remaining monomer from the midpoint of the plate.

d. Control the flow rate to prevent air bubbles. If a bubble is trapped, remove it by sliding the plate sideways until the bubble is at the edge.

e. Keep the gel from protruding beyond the plate by underfilling the tray. The gel will shrink somewhat as it polymerizes.

7. Position the photopolymerization light over the tray.

8. Irradiate the solution for approximately 1 hour.

9. To lift the gel from the casting tray:

   a. Lift one corner with a flat spatula inserted between the gel and the casting stand (see Figure 3.3).

   b. When air appears under the gel, gently lift the plate free from the casting tray.

   A good, detailed discussion, with references of the practical aspects of casting polyacrylamide gels is found in Bio-Rad bulletin 1156.

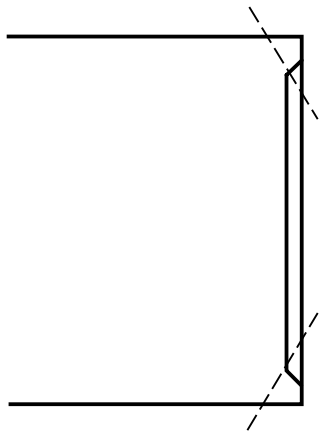


Fig. 3.4. Removing the corners.

   c. Flip the plate glass side down onto the casting tray and further irradiate for 15 minutes to eliminate unmpolymerized monomer on the gel surface.

   d. Remove the corners of the gel and any portion that reaches the edge of the plate (see Figure 3.4). This is done to prevent electrical shorting along the edges.

10. The gel may be used immediately, or it may be stored at 4 ˚C for several days. To store the gel, wrap it in Parafilm and place it in a Zip-Lock™ bag to prevent dehydration.

3.5 Casting Ultra-Thin Layer Gels

Using Gel Support Film

1. Cut the gel support film to the dimensions of the glass plate.

2. Test for the hydrophobic side by placing a drop of water on the film. The water beads on the hydrophobic side, and spreads on the hydrophilic side.

3. Pipet a few drops of water onto the clean glass plate.

4. Place the hydrophobic side of the gel support film against the plate.
5. Roll the gel support film flat with a test tube or similar object to force out excess water and air bubbles.
6. Carefully wipe or blot off any excess liquid at the edges. The gel support film is now ready for use, and should be used as soon as possible.

   Note: Basic ampholytes (pH >8.0) have been found to interfere with gel adhesion to the support film for polyacrylamide. Increasing the concentration of ammonium persulfate to 0.7 mg/ml in the final acrylamide gel solution (containing less than 2.5% ampholyte and 1 µl TEMED/ml) should alleviate the problem. In this case, prefocusing is strongly recommended to remove excess ammonium persulfate. Adhesion may also be affected by prolonged soaking in acid solutions, such as in staining and destaining. Do not soak longer than absolutely necessary to achieve the desired result.

   Important: The glass plates and the ultra-thin layer casting tray must be clean and dry. Do not use organic solvents, abrasive cleaners, or hot water on the casting tray. Clean the glass plates with soap and water and rinse with deionized water followed by ethanol. Wipe the plates dry with lint-free paper.

   Warning: Do not mouth pipet acrylamide solutions. Wear gloves. Acrylamide is a neurotoxin.

Casting

1. Prepare the monomer/ampholyte solution.
2. Prepare the catalyst solutions.
3. Prepare the gel support film for polyacrylamide as described above (mandatory for 0.2 mm gels).
4. When casting 0.4 mm gels, it is not absolutely necessary to use the gel support film. The gel may be cast directly on the glass plate using the procedure given in Section 3.4.
5. To cast a gel on the gel support film, place one edge of the glass plate bearing the film against the spacer rails of the casting tray with the film side down.
6. Pipet 5 ml (for a 100 x125 mm plate) of the monomer ampholyte solution near the edge of the glass plate. Slide the plate as you would a microscope slide and gently lower the plate to the tray. Apply slight pressure to the glass plate directly over the rails and wipe any excess monomer solution from the edges. Remove any trapped air bubbles by gently sliding the glass plate on the tray just far enough to place the bubbles at the edge.
7. An alternative method is to lay the plate/film flat on the casting tray and inject the monomer solution with a narrow gauge needle. Care must be taken not to inject the solution between the glass and the film.
8. Position the polymerization light over the tray and irradiate for 0.5 to 1 hour. The edge of the gel should shrink somewhat from the edge of the plate.
9. After polymerization, to lift the gel from the casting tray:
   a. Lift one corner with a flat spatula inserted between the casting stand and the gel support film.
   b. Twist gently. When air appears under the gel, lift the plate free from the casting stand.
   c. Should the gel support film separate from the glass plate, simply peel the supported gel from the casting stand. The slight inhibitory property of the casting tray insures easy removal.

d. Irradiate the surface of the gel for 10 minutes to eliminate unpolymerized monomer. Use gels immediately.

Section 4
Sample Preparation and Application for IEF-PAGE

4.1 Sample Preparation

Protein samples for isoelectric focusing must be free of precipitates, and substantially salt-free. Small samples (1 to 10 µl) in typical biochemical buffers are usually tolerated, though better results can be obtained with solutions in deionized water, 2% ampholytes or 1% glycine. Suitable protein solutions may be prepared by dialysis, or gel filtration with Bio-Gel® P-6DG gel.

A convenient technique for preparing small samples is to load a small amount of hydrated Bio-Gel P gel into a 0.5 ml plastic microcentrifuge tube with a small hole in the bottom. Load the sample on top of the gel and place this tube into a 1.5 ml plastic microcentrifuge tube. Spin for 5 seconds in a microcentrifuge. The sample will be adequately desalted for isoelectric focusing, and can be isolated from the bottom of the larger tube.

Many samples will require the use of urea, ethylene glycol, non-ionic detergents (e.g. Triton® X-100, NP-40, Lubrol WX, or octylglucopyranoside), or zwitterionic detergents (CHAPS, CHAPSO).

Even in the presence of detergents, some samples may resist solubility due to salt requirements. Only if high salt is an absolute requirement should it be present in a sample, and even then, substantial distortions and anomalies can be expected, especially in high voltage focusing experiments.

4.2 Sample Application

There are many suitable methods for applying samples to thin-layer polyacrylamide gels. The most common methods are paper strips, surface addition, and indentations cast into the gel. Samples up to 25 µl may be conveniently applied with 1 cm squares of rectangular filter paper strips. This method suffers in that some proteins may not be properly eluted from the paper strip. An interesting variation is to use triangular pieces, which will give focused zones of increasing protein concentration, providing sample loading information.[1]

Protein solutions may be dropped or streaked directly onto the gel surface, or a sample template mask (catalog number 170-2985) can be used to contain the sample. Sample wells can be formed in the surface of the gel with the CTL casting tray. Attach the appropriate size piece of Dymo tape to the stand in the proper location and cast the gel. The taped areas will leave imprints in the surface that can be used as sample application wells. When using sample wells, the sample solution should contain 2% ampholyte to prevent field discontinuity at the point of application.

Another method which has been reported involves forming droplets of the sample in low melting temperature agarose and applying these sample “beads” to the surface of the gel.[9]

Choice of sample application technique is governed largely by the protein to be focused.

4.3 Position of Application

There are no fixed rules regarding the positioning of the sample on the gel. Samples should not, in general, be applied to areas where protein bands are expected to focus. To protect the proteins from extreme pH exposure, the samples should not be applied closer than 1 cm from either electrode. The best strategy for a new protein is to make three points of application; one at each end and one near the middle of the gel, and observe the resulting focusing pattern.

4.4 Setting Up for Running the Gel (IEF-PAGE)

Before starting, connect the Bio-Phoresis cell to a refrigerated circulator capable of maintaining 2˚ to 4 ˚C.

1. Wet the ceramic side of the platform with a drop of water, 0.1% Triton X-100, or 0.5% glycerol to insure good contact with the glass plate or the gel support film, and to facilitate heat transfer.
2. Place the gel plate on the platform. Do not trap air bubbles between the gel and the ceramic. Wipe any excess liquid from the platform.
3. Cut the electrofocusing strips (35 x 0.7cm) to 4 mm less than the width of the gel. Do not allow the wicks to extend beyond the sides of the gel.
4. Soak the electrode strips in the appropriate buffer solution (see Table 4.1). Place on a glass plate and blot excess buffer.

Table 4.1 Recommended Electrode Buffers

	Buffer 1	Buffer 2
Cathode Buffer (-)	1 N NaOH	20 mM lysine, free base
		20 mM arginine, free base
		2 M ethylenediamine or
		2 M ethanolamine
Anode Buffer (+)	1 N H_3PO4	20 mM aspartic acid
		20 mM glutamic acid

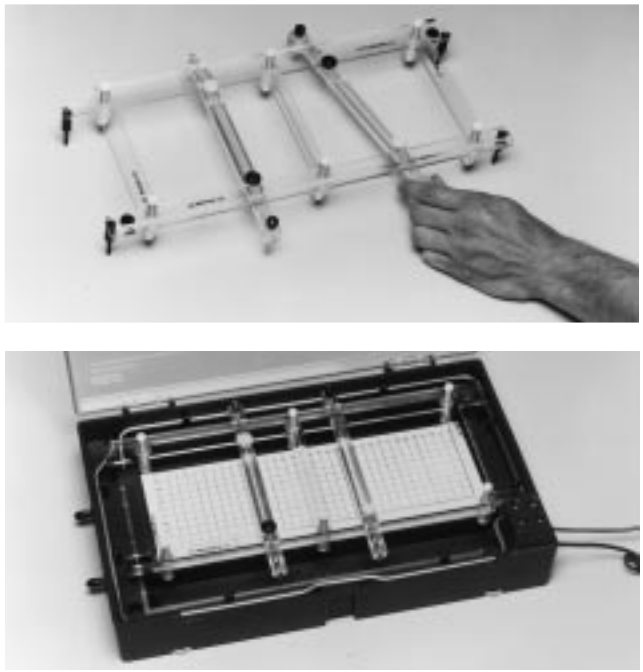
We recommend the use of Buffer 2 when using high voltages, as in ultra-thin electrofocusing.

5. Carefully position the electrode strips on the correct ends of the gel and gently press them against the gel to insure good contact [acidic electrolyte = (+) electrode (red), basic electrolyte = (-) electrode (black)].
6. Adjust the electrodes in the electrode holder so that the wires run through the center of each strip. Tighten the clamp screws to secure the electrodes in place and position the holder over the gel.

   Note: The sample may be loaded at this point, or after prefocusing the gel. Prefocusing is recommended if high catalyst concentrations have been used to form the gel. A suitable prefocusing condition is 400 volts for 15 minutes.
7. Load the sample as outlined in Section 4.2.

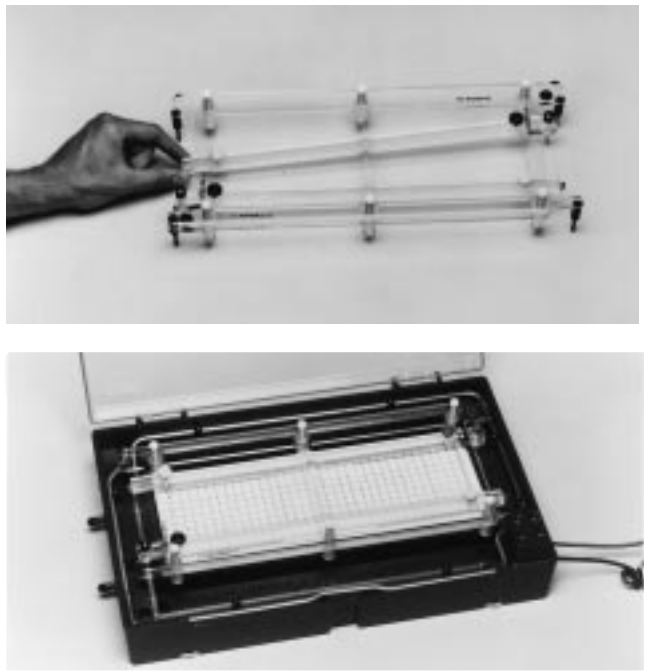
Assembly of Electrodes for Analytical Isoelectric Focusing

A.



A. Posiforce electrode holder assembled with 120 mm electrofocusing electrodes.

B.



B. Posiforce electrode holder assembled with 360 mm electrodes.

4.5 Run Conditions

1. Run conditions vary with sample, sample preparation, and choice of ampholytes. The conditions listed below are appropriate for Bio-Lyte ampholytes with protein samples dissolved in deionized water.

2. Notice that the run parameters require constant power conditions from the start of the run until the maximum voltage is reached. This is to prevent overheating and subsequent dehydration of the gel.

3. When the maximum voltage is reached, switch to constant voltage regulation until focusing is complete.

4. A good way to monitor the progress of an isoelectric focusing experiment is to observe the migration of visible marker proteins of known p*I* to their isoelectric points. The IEF protein market kit (catalog number 161-0310) provides eight natural proteins, including four visible proteins that are clearly discernable during an IEF run.

Table 4.2 Running Conditions for Isoelectric Focusing

		pH 3/10	pH 2/5	pH 3/5	pH 4/6	pH 5/7	pH 6/8	pH 7/9	pH 8/10
Initial power[1] (watts)	45 x 125 mm	3-5	3-5	3-5	3-5	3-5	3-5	3-5	3-5
	100 x 125 mm	6-10	6-10	6-10	6-10	6-10	6-10	6-10	6-10
	205 x 125 mm	12-20	12-20	12-20	12-20	12-20	12-20	12-20	12-20
Final voltage[2] (approximate)	0.2 mm thick	3,000 V	3,000 V	3,000 V	3,000 V	3,000 V	3,000 V	3,000 V	3,000 V
	0.4 mm thick	2,000 V	2,000 V	2,000 V	2,000 V	2,000 V	2,000 V	2,000 V	2,000 V
	0.8 mm thick	1,500 V	1,500 V	1,500 V	1,500 V	1,500 V	1,500 V	1,500 V	1,500 V
	1.6 mm thick	1,200 V	1,200 V	1,200 V	1,200 V	1,200 V	1,200 V	1,200 V	1,200 V
Total time[3] (minutes)	0.2 mm thick	30-60	30-60	30-60	30-60	30-60	30-60	30-60	30-60
	0.4 mm thick	45-90	45-90	45-90	45-90	45-90	45-90	45-90	45-90
	0.8 mm thick	90-120	90-120	90-120	90-120	90-120	90-120	90-120	90-120
	1.6 mm thick	180	180	180	180	180	180	180	180

1. Regulate at constant power until maximum voltage is reached; power conditions shown apply to all gel thicknesses.
2. Regulate at constant voltage after reading maximum voltage.
3. These are approximate running times. Time will vary with mobility of the sample and migration distance. pH gradients are usually developed after 60 minutes.

4.6 Band Detection

In general, proteins are detected by fixing and staining. If the proteins are not fixed immediately, the high resolving power of electrofocusing can be lost to diffusion. Small proteins and proteins with basic pIs are particularly difficult to fix. Autoradiography, biological activity, group specific staining, and immunological methods are a few examples of alternative detection techniques currently being used.

Section 4.7 gives two recommended staining procedures for electrofocusing. Either method produces acceptable results, however, Method B is significantly easier to use and, in some cases, will produce a sharper pattern.

The cupric sulfate in the staining and destaining solutions effectively eliminates any background staining due to the presence of ampholytes.

4.7 Fixing and Staining

Method A	**Method B**
Fixative:	**Fixative:**
4% Sulfosalicylic acid 12.5% Trichchloracetic acid 30% Methanol	Not necessary
Immerse gels in this solution for 30 minutes.	
Stain:	**Stain:**
27% Isopropanol or ethanol 10% Acetic acid 0.04 % Coomassie Blue R-250 0.5% $CuSO_4$	Same as Method A, but with 0.05%crocein scarlet
(0.05% Crocein scarlet optional)	
Dissolve the $CuSO_4$ in water before adding the alcohol. Either dissolve or add it to the solution at the end.	Crocein scarlet, a highly soluble dye which rapidly binds to protein, is included to assure rapid fixation of the bands. This procedure is adequate if Coomassie Blue R-250 is used alone.
Immerse the gel in the stain for at least 2 hours.	

4.8 Destaining

First destaining solution:

12% isopropanol or ethanol

7% acetic acid

0.5% $CuSO_4$

Dissolve the cupric sulfate in water before adding the alcohol. Immerse the gel in two or three 500 ml changes of this solution until the background is nearly clear. Gentle agitation and slight heating will speed the destaining process.

Second destaining solution:

12% isopropanol or ethanol

7% acetic acid

Immerse the gel in this solution to remove the last traces of stain and $CuSO_4$.

Note: Gels supported on gel support film for polyacrylamide cannot withstand prolonged soaking in acidic solutions. Total staining and destaining time for 0.2 mm ultrathin gels should not exceed 2 hours. Longer soaking periods will cause the gel to separate from the backing, making further handling extremely difficult.

4.9 Other Detection Methods

1. Coomassie Blue G-250 "Quick Stain"

   This technique is nearly as sensitive as Coomassie R-250, but requires no destaining and will not stain ampholytes. It cannot be used in the presence of detergents, except urea.

   3.5% perchloric acid
   0.025% Coomassie G-250

   Immerse gels in this solution for 1 hour. Place in 7% (v/v) acetic acid for intensification and preservation.

2. Electrophoretic Transfer

   Resolved proteins can be transferred electrophoretically from electrofocusing gels onto nitrocellulose using the Trans-Blot® cell (catalog number 170-3905). This sheet can then be stained with 10% amido black or probed with labeled antisera. Gels supported on gel support film cannot be transferred electrophoretically.

3. Ultrasensitive Silver Stain

   Bio-Rad's Silver Stain Kit (catalog number 161-0443) is 10 to 50 times more sensitive than Coomassie blue (see Bulletin 1089) and is compatible with both supported and unsupported gels.

4.10 Gel Drying and Preservation

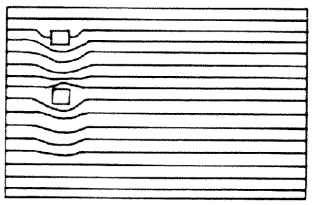
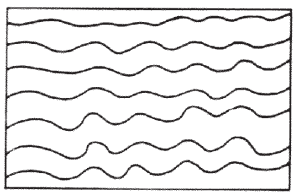
To dry ultra-thin gels on gel support film:

Place the destained gel in a dust free area with good ventilation (fume hood is excellent for this purpose) and allow the gel to dry overnight at room temperature. Alternatively, the gel can be carefully dried with a heat gun on a low heat setting. Dried gels can be stored in plastic photograph holders or taped directly into notebooks (gel side down).

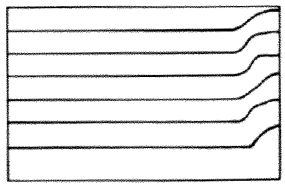
To dry unsupported thin layer gels:

Soak the gel in 7% acetic acid, 5% (v/v) glycerol for 1 hour. Place it on a thick filter paper which has been thoroughly wetted, and smooth out any air bubbles. Dry the gel, covered with Saran-Wrap®, in a gel dryer (catalog number 165-1961) according to the dryer instructions.

4.11 Troubleshooting Polyacrylamide Electrofocusing Gels

Problem	Cause	Solution
1. Distortion in gradient where sample was applied. 	a. Too much salt in sample.	a. Dialyze against ampholyte, 1% glycine, or water, or desalt with Bio-Gel P-2 or P-6 gel.
	b. Sample not in same percentage and range of ampholyte as rest of gel.	b. Sample should have nearly the same percentage and range of ampholytes as the gel.
	c. Sample applied too near the anode.	c. Apply elsewhere on plate. Remove paper within 15 to 30 minutes.
	d. Sample load excessive.	d. Dilute sample.
	e. Sample precipitation.	e. Spin to remove insolubles or use detergents.
2. Distortion across gel not associated with sample application. 	a. Uneven cooling.	a. Put water, mineral oil, 1% Triton X-100, or other non-ionic detergent between the glass plate and the platform. Eliminate air bubbles.
	b. Uneven or excess electrolyte on wicks.	b. Blot wicks until just moist. Don't exert excessive pressure on wicks when placing electrodes. Prevent acid or base from entering gel between electrode strips.
	c. Excessive catalyst.	c. Cut down amount of APS or prefocus at low voltage.
	d. Poor electrode contact.	d. Clean electrodes and check for bends or kinks in the wires.

4.11 Troubleshooting Polyacrylamide Electrofocusing Gels (continued)

Problem	Cause	Solution
3. Large distortion of gradient at gel edge. 	a. Electrode wick too long.	a. Gel should extend 2 to 3 mm beyond wick.
	b. Electrode wick too wet.	b. Blot wick until just moist.
	c. Condensation on platform and plate edge comes in contact with electrolyte and gel.	c. Hook cover cooling coil to cold water before platform. If humid, adjust cover coil 5° cooler than platform.
4. Condensation inside lid.	a. Inadequate cooling.	a. Check cooling water - 4 to 10 °C at 10 L/hour.
	b. Power setting too high.	b. See Table 4.2.
	c. Salt concentration too high.	c. See Solution 1a.
	d. Wrong polarity.	d. Basic electrolyte at cathode; acidic electrolyte at anode.
5. Sample streaking.	a. Particles in sample.	a. Centrifuge sample before application.
	b. Sample adsorbed onto applicator.	b. Change application method. (See Section 4.2.)
	c. Precipitation at point of application.	c. Try a different position. Use additives (1% glycine, urea,non-ionic detergents, amphoteric detergents).
6. Current increases instead of decreasing.	a. Inadequate cooling.	a. See Solution 4a.
	b. Wrong polarity.	b. See Solution 4d.
	a. Cover not completely closed.	a. Close cell cover completely to engage magnetic interlock.
	b. Open circuit.	b. Check electrical connections to power supply and electrode jacks. Check fuses. Check connections to house power.
	c. Poor contact between electrodes and electrode strips.	c. The electrode should lie flat against the whole length of the electrode strip.

4.11 Troubleshooting Polyacrylamide Electrofocusing Gels (continued)

Problem	Cause	Solution
7. Low or no starting current.	a. Cover not completely closed.	a. Close cell cover completely to engage magnetic interlock.
	b. Open circuit.	b. Check electrical connections to power supply and electrode jacks. Check fuses. Check connections to house power.
	c. Poor contact between electrodes and electrode strips.	c. The electrode should lie flat against the whole length of the electrode strip.
8. Sparking at gel edge.	a. Gel too close to edge of plate.	a. Trim gel to leave 1 to 2 mm glass along entire plate edge.
9. Sparking next to electrodes.	a. Liquid from electrode strip causing short circuit under plate.	a. Blot to remove excess liquid. Use mineral oil or a non-conducting liquid instead of water to achieve good contact between platform and plate.
10. pH gradient does not cover expected range.	a. Focused too long or use of excessive power.	a. At recommended constant power, proteins should be focused within 3 hours.
	b. Basic gels stored too long.	b. Use basic gels (pH>7) immediately to prevent hydrolysis of acrylamide to acrylic acid, which causes electroendosmosis.
	c. Poor quality acrylamide and Bis.	c. Use highest quality acrylamide and Bis to avoid polymerizing acrylic acid into the gel.
	d. Old acrylamide and Bis stock solutions.	d. Prolonged storage of acrylamide and Bis leads to acrylic acid formation.
	e. Ampholyte contamination or deterioration.	e. Check ampholyte for bacterial contamination. Solution will look cloudy. Inspect under a microscope. Check date of receipt and storage conditions.

4.11 Troubleshooting Polyacrylamide Electrofocusing Gels (continued)

Problem	Cause	Solution
10. (Continued.)	f. Inefficient cooling.	f. Cool to 4-10 °C.
11. Gels distort, crack, or melt.	a. Ampholyte contamination.	a. See 10e.
	b. Excessive power.	b. See Table 4.2.
	c. Voltage drop too high due to excessive conductance gap between electrolyte and narrow range ampholyte.	c. See Table 4.1.
	d. Sample load excessive or sample too high in salt concentration.	d. Reduce sample load. See Solution 1 a.

4.12 Casting Troubleshooting

Problem	Cause	Solution
1. Gel does not stick to plate; gel feels sticky and soft, or is still liquid.	a. Incomplete polymerization.	a. Use only high purity acrylamide and Bis. Inferior monomers are difficult to polymerize. Store solutions in amber bottles at 4 °C for no more than 2 weeks. Make fresh catalyst solutions daily. Replace APS and TEMED. Follow proper storage procedures for these reagents. Use higher catalyst levels with ampholytes other than Bio-Lyte ampholytes. Degas longer if gels polymerized with APS and Riboflavin-5'-Phosphate. See Section 3.3.

4.12 Casting Troubleshooting (continued)

Problem	Cause	Solution
2. Gel does not stick to plate; gel feels firm and is not sticky.	a. Polymerization time is too long, causing gel to dry onto plastic at the edges of the plate. Especially true with gels polymerized with APS.	a. Optimize polymerization time by placing excess solution in a 12 x 75 mm glass test tube and putting the test tube under the polymerization light. Remove plate 15 minutes after test tube gels.
	b. Incorrect procedure used to lift plate from CTL casting tray.	b. Do not slide plate sideways before removing it. Do not pry the plate up with fingers. Twist the spatula to raise the plate edge nearest the spacer. This allows air to penetrate between the gel and the plastic without forming a vacuum.
	c. Dirty glass plates.	c. Wash plates with Bio-Rad Cleaning Concentrate, and rinse them thoroughly with deionized or distilled water.

4.13 References (IEF-PAGE)

1. Righetti, P. G., Isoelectric Focusing: Theory, Methodology, and Applications, Elsevier Biomedical Press, Amsterdam (1983).
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Section 5
Agarose Gel Electrofocusing

5.1 Introduction

Agarose isoelectric focusing separates large proteins and antibodies that cannot be readily characterized on polyacrylamide IEF gels due to polyacrylamide's smaller pore sizes. Molecules greater than 200,000 daltons can easily be separated on a 1% agarose gel.

The agarose gels are formed within minutes by simply boiling the agarose mixture and pouring it onto a glass plate in a procedure similar to that for immunoelectrophoresis in agarose gels. Sorbitol and glycerol are incorporated into the agarose gel to increase gel viscosity and to counteract electroendosmosis (EEO), a major cause of sample smearing. EEO is the cathodic flow of water in the neutral and alkaline parts of the gel caused by the low concentration of fixed charge carboxyl groups on the gel matrix. These carboxyl groups are not charged at pH 3.5 or lower, but acquire their full charges at pH 5.5 and higher. As a consequence, gel shrinkage occurs at the pK of the carboxyl groups, resulting in "flooding" of water and solutes at the alkaline portion of the gel. EEO decreases when gel viscosity increases.[1]

These sorbitol and glycerol additives also allow higher voltages (>1,000 V) toward the end of the IEF run.

The procedure for agarose isoelectric focusing is simple, consisting of the following seven steps. Each of these steps is described in detail in Sections 5.3 through 5.6.

1. Pour the agarose gel.
2. Dehydrate the gel to remove excess liquid.
3. Focus the gel for 1.5 to 3 hours.
4. Immerse the gel in fixative solution.
5. Immerse the gel in ethanol to remove background.
6. Immerse the gel in stain.
7. Destain the gel.

5.2 Equipment for Agarose Isoelectric Focusing

Catalog Number	Product Description
Bio-Phoresis Cell	
170-2900	**Bio-Phoresis Cell (basic cell, electrodes not included)**
Electrofocusing Electrodes	
170-2902	**Electrofocusing Electrodes,** 120 mm, 2
170-2903	**Electrofocusing Electrodes,** 360 mm, 2
170-2904	**Posi-Force Electrode Holder (required)**

Catalog Number	Product Description

Power Supply

165-5056 **PowerPac 3000 Power Supply,** 100/120 VAC
165-5057 **PowerPac 3000 Power Supply,** 220/240 VAC

Application Kit

170-4250 **Agarose Electrofocusing Kit**, includes Gel Support Film for Agarose, 100 x 125 mm (100); Glass Plates, 100 x 125 (50); Electrofocusing Wicks, 0.7 x 33 cm (100); Blotting Filter Paper (200) Sample Application Pieces (200); and instructions.

Accessories and Reagents Analytical IEF Accessories

170-2981 **Mini Casting Tray**
170-4251 **Gel Support Film for Agarose,** 100 x 125 mm, 100
170-4204 **Electrofocusing Strips,** 0.7 x 33 mm, 100
170-4257 **Sample Application Pieces,** 200
170-4252 **Blotting Filter Paper,** 150 x 175 mm, 200
170-4104 **Glass Plates,** 125 x 100 x 1.5 mm, 50
170-4230 **Glass Plates,** 125 x 205 x 1.5 mm, 15
170-4046 **Leveling Table,** 20 x 30 cm
170-4237 **Surface pH Electrode**
170-2985 **Sample Templates,** 5

Reagents

162-0022 **Zero -m_r Agarose,**[A] 10 g
163-1112 **Bio-Lyte 3/10 Ampholyte,** 40%,[D] 10 ml

All electrophoresis purity reagents listed in this section , except the DNA Size Standards, are shipped at room temperature. For longer shelf life, follow these storage instructions:

A. Store at room temperature.
B. Store desiccated at room temperature.
C. Store desiccated at -20 ˚C.
D. Store at 4 ˚C.
E. Store at -20 ˚C.
F. Store desiccated at 4 ˚C.

Catalog Number	Product Description
163-1113	**Bio-Lyte 3/10 Ampholyte,** 40%,[D] 25 ml
163-1132	**Bio-Lyte 3/5 Ampholyte,** 20%,[D] 10 ml
163-1142	**Bio-Lyte 4/6 Ampholyte,** 40%,[D] 10 ml
163-1142	**Bio-Lyte 4/6 Ampholyte,** 40%,[D] 25 ml
163-1152	**Bio-Lyte 5/7 Ampholyte,** 40%,[D] 10 ml
163-1192	**Bio-Lyte 5/7 Ampholyte,** 40%,[D] 25 ml
163-1192	**Bio-Lyte 5/8 Ampholyte,** 40%,[D] 10 ml
163-1193	**Bio-Lyte 5/8 Ampholyte,** 40%,[D] 25 ml
163-1162	**Bio-Lyte 6/8 Ampholyte,** 40%,[D] 10 ml
163-1163	**Bio-Lyte 6/8 Ampholyte,** 40%,[D] 25 ml
163-1172	**Bio-Lyte 7/9 Ampholyte,** 40%,[D] 10 ml
163-1182	**Bio-Lyte 8/10 Ampholyte,** 20%,[D] 10 ml
161-0310	**IEF Standards,** p*I* 4.6-9.6
161-0400	**Coomassie Brilliant Blue R-250,**[A] 10 g
161-0417	**Crocein Scarlet,**[A] 10 g

5.3 Preparing the Plates for Agarose

Agarose IEF gel contains:

1% Agarose

2% Ampholytes

5% Sorbitol

20% Glycerol

1. Add 1 gram zero -m_r agarose and 5 grams sorbitol to 40 ml of 25% glycerol and 20 ml distilled water. Place a stir bar into the flask and immerse the flask in a beaker of water. Heat the water to boiling (100 ˚C) and stir to dissolve components (30 minutes).
2. Turn off heat and add ampholytes while stirring the agarose mixture (5 ml of 40% ampholytes or 10 ml of 20% ampholytes). Add more hot (100 ˚C) distilled water for a final volume of 100 ml.

To improve adhesion between the plate and agarose, precoat the plate with a thin film of agarose (0.2%) and dry it before casting the gel. Plastic support films (i.e. gel support film for agarose) may also be used.

Note: Casting plates with gel support film for agarose

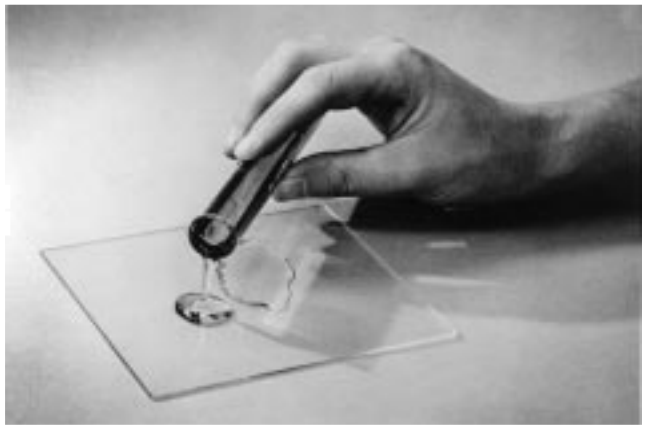
To eliminate precoating the glass plates with agarose, and to allow the plates to be reused, polyester gel support film for agarose may be applied to the plates. Gel support film for agarose is specially treated to provide a permanent record of the procedure. It facilitates staining/destaining, and can be stored easily in laboratory notebooks.

To apply gel support film for agarose:

1. Cut the gel support film for agarose to the dimensions of the glass plate.
2. Test for hydrophobic side by placing a drop of water on the film. The water beads on the hydrophobic side, and spreads on the hydrophilic side.
3. Pipet a few drops of water onto the glass plate.

4. Place the hydrophobic side of the gel support film for agarose against the plate.
5. Roll the gel support film for agarose flat with a test tube of similar object to force out excess water and air bubble.

3. Heat a pipet in the hot water and pipet 20 ml of agarose solution into warm test tubes. Pour one test tube onto a 100 x 125 mm glass plate.



The agarose gel will be 1.2 mm thick. Use the lip of the test tube to pull the hot agarose to the corners of the plate. The gel will solidify within 10-15 minutes.

4. After the gel has solidified, cover the gel with Parafilm® plastic film and let the gel "age" in a humid chamber at 4 ˚C for a minimum of 4 hours, preferably overnight.[2-4]

5.4 Applying the Sample

1. After "aging," place the agarose gel on a level surface and overlay it with blotting filter paper for 5 to 10 minutes to remove excess liquid. After this time, place the gel on the cooled (10 ˚C) electrophoresis stage.

   Note: It is advantageous to place water or a dilute (0.1%) detergent solution between the glass plate and the cooling stage to increase heat transfer.

2. Cut electrofocusing wicks to 9.5 cm long. Apply appropriate electrolytes in excess. After 5 minutes, gently blot the excess electrolytes from the wicks and place the wicks on the agarose gel.

pH range	Typical Electrolytes	
Ampholyte	**Anode**	**Cathode**
3-5	0.5 M acetic acid	0.25 M HEPES
4-6	0.5 M acetic acid	0.5 M ethanolamine
5-7	0.1 M glutamic acid	0.5 M ethanolamine
6-8	0.1 M glutamic acid	0.5 M NaOH
7-9	0.25 M MES	0.5 M NaOH
8-10	0.25 M MES	0.5 M NaOH
3-10	0.5 M acetic acid	0.5 M NaOH

3. There are several ways to apply the sample.
   a. Apply the sample as 1-3 µl droplets or streak out 4-15 µl on the surface of the gel.
   b. Place filter paper squares on the surface of the gel and pipet the samples onto them.

Filter Paper (6 x 6 mm square)	Sample Volume
Bio-Rad Sample Application Pieces (catalog number 170-4257)	**10 µl**
Bio-Rad Agarose Blotting Paper (catalog number 170-4252)	**15 µl**

To prevent edge effects, place the samples 1 cm from each edge and 0.5 cm from the electrode wick. Bio-Rad recommends application near the basic (-) electrode. Initially it is good practice to try different locations on the gel.

Note: The sample should be free of precipitate, preferably free of salt and in 2% ampholytes. If the sample is labile, apply it after the pH gradient is formed to minimize focusing time.

5.5 Running the Agarose Gel

1. Place electrodes directly on the electrode wicks or use Ultra Wicks to connect the electrode wicks to the buffer chambers.
2. Run the gel at a constant 7.5 watts per 100 x 125 mm plate for 1 1/2 to 3 hours at 8-10 ˚C. The voltage will increase in that period from approximately 400 V to 1,200 V.
3. Use a surface pH electrode to measure the gradient directly. Leave the gel on the cooling stage to measure the pH at about the same temperature the gel was focused. After pH measurements are taken, the gel may be refocused for 15 minutes to sharpen up the bands.
4. After refocusing the gel, remove the electrode wicks and place the gel in fixative solution[5] for 15 minutes.

   Fixative Solution

   30% Methanol

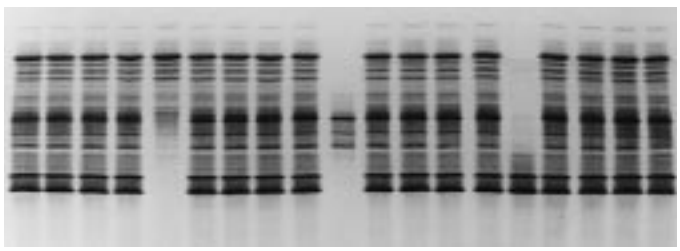
   5% Trichloroacetic acid (TCA)

   3.5% Sulfosalicylic acid (SSA)

5. To insure a clear gel background, take the agarose gel directly from the fixative solution to a 95% ethanol bath.[2-4] Immerse the gel in ethanol for 30 minutes with occasional swirling.
6. After the ethanol wash, remove the sample paper squares and place the gels on a level surface. Soak one piece of filter paper in ethanol and place it on top of the gel. Then place additional filter paper (8-10 sheets) or folded paper towels on top of the ethanol-soaked paper. Press the gel with a 1 kg weight for 30 minutes.
7. After pressing, dry the gel completely with an air blow dryer or under a laboratory hood's fan.

5.6 Detection in Agarose

8. Autoradiography, biological activity, group specific staining, and immunological techniques are just a few examples or many detection techniques currently used. The typical IEF stain is Coomassie brilliant blue R-250.



Stain

0.3% Coomassie brilliant blue R-250

28% isopropanol or ethanol

14% acetic acid

Filter the stain before use. The gel should be stained a minimum of 30 minutes at room temperature.

9. Destaining the gels should take only 30 minutes at room temperature

   Destain Solution

   28% isopropanol or ethanol

   15% acetic acid

10. Air dry the gel plate

5.7 Troubleshooting (Agarose IEF)

Problem	Cause	Solution
1. Excessive pooling of water at the cathode (-).	a. Poor quality agarose.	a. Use zero - m_r agarose only.
	b. Gel has not been sufficiently blotted dry prior to run.	b. Blot all excess liquid from gel prior to run.
2. Distortion in gradient where sample is applied.	a. Too much salt in sample.	a. Dialyze against ampholyte, 1% glycine or water, or desalt with Bio-Gel P-2 or P-6 gel.
	b. Sample applied too near the anode.	b. Apply elsewhere on plate minimum of 1 cm from anode.
	c. Sample load excessive.	c. Dilute sample.
	d. Sample precipitation.	d. Spin to remove insolubles or use detergents.
3. Distortion across gel not associated with sample application.	a. Uneven cooling.	a. Put water, mineral oil, or other nonionic detergent between the glass plate and the cooling platform. Eliminate air bubbles.
		b. Blot wicks until just moist. Don't exert excessive pressure on wicks when placing electrodes. Prevent acid or base from entering gel between electrode strips.
4. Large distortion of gradient at gel edge.	a. Electrode wick too long.	a. Gel should extend 2-3 mm beyond wick.
	b. Electrode wick too wet.	b. Blot wick until just moist.

5.7 Troubleshooting (Agarose IEF) (continued)

Problem	Cause	Solution
5. Condensation inside cell lid.	a. Inadequate cooling.	a. Check cooling water - 4 °C to 10 °C at 10L/hour.
	b. Power setting too high.	b. See section 13.2.
	c. Salt concentration too high.	c. See Solution 2a.
	d. Wrong polarity.	d. Basic electrolyte at cathode, acidic electrolyte at anode.
6. Sample streaking.	a. Particles in sample.	a. Centrifuge sample before application.
	b. Sample absorbed onto applicator.	b. Change application method.
	c. Precipitation at point of application.	c. Try a different position. Use additives (1% glycine, urea, non-ionic detergents, amphoretic detergents).
7. Current increases instead of decreasing.	a. Inadequate cooling	a. See Solution 5a.
	b. Wrong polarity.	b. See Solution 5d.
8. Low or no starting current.	a. Cover not completely closed.	a. Close cell cover completely to engage magnetic interlock.
	b. Open circuit	b. Check electrical connections to power supply and electrode jacks. Check fuses. Check connections to house power. Check magnet alignment with trip switch.
	c. Poor contact between electrodeds and electrode strips.	c. The electrode should lie flat against the whole length of the electrode strip.
9. Sparking at gel edge.	a. Gel too close to edge of plate.	a. Trim gel to leave 1 to 2 mm glass along entire plate edge.
10. Sparking next to electrode.	a. Liquid from electrode strip causing short circuit under plate.	a. Blot to remove excess liquid. Use mineral oil or a non-conducting liquid instead of water to achieve good contract between platform and plate.

5.7 Troubleshooting (Agarose IEF) (continued)

Problem	Cause	Solution
11. pH gradient does not cover expected range.	a. Focused too long or use of excessive power.	a. At recommended constant power, proteins should be focused within 3 hours.
	b. Basic gels stored too long.	b. Use basic gels (pH>7) immediately to prevent hydrolysis of acrylamide to acrylic acid, which causes electroendosmosis.
	c. Poor quality acrylamide and Bis.	c. Use highest quality acrylamide and Bis to avoid polymerizing acrylic acid into the gel.
	d. Old acrylamide and Bis stock solutions.	d. Prolonged storage of acrylamide and Bis leads to acrylic acid formation.
	e. Ampholyte contamination or deterioration.	e. Check ampholyte for bacterial contamination. Solution will look cloudy. Inspect under a microscope. Check date of receipt and storage conditions.
	f. Inefficient cooling.	f. Cool to 4-10 °C.

5.8 References for Agarose-IEF

1. Rilbe, H., **Electrofocusing and Isotachophoresis,** (Radola, B. J. and Graesslin, D., eds.) Walter de Gruyter and Co., publishers, 43 (1977).
2. Rosen, A., **Electrophoresis '79,** (Radola, B. J., ed.) Walter de Gruyter and Co., publishers, 105 (1980).
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Section 6
Preparative Granulated Bed Isoelectric Focusing

6.1 Introduction

Isoelectric focusing of proteins with synthetic ampholytes fractionates the proteins into narrow concentrated bands at their isoelectric points. The use of narrow range ampholytes in a granulated bed matrix allows high resolution separation of relatively large quantities of sample as well as easy recovery of the individual bands after focusing. The horizontal bed medium is composed of beaded hydrophilic gel material with low residual charge providing an excellent anticonvection medium, which provides the high resolution indicative of isoelectric focusing. The problem of isoelectric precipitation, common in density gradient IEF, is eliminated.

The granulated bed procedure produces stable pH gradients of high resolving power. It simplifies the preparation of granulate beds for IEF by introducing a convenient "wicking" procedure for controlled dehydration of the bed before focusing.

6.2 Equipment for Granulated Bed Isoelectric Focusing

A circulating water bath cooling to 4 ˚C is recommended.

Catalog Number	Product Description
Bio-Phoresis Cell	
170-2900	**Bio-Phoresis Cell (basic cell, electrodes not included)**
170-2902	**Electrofocusing Electrodes,** 120 mm, 2
170-2904	**Posi-Force Electrode Holder (required)**
Power Supply	
165-5056	**PowerPac 3000 Power Supply,** 100/120 VAC
165-5057	**PowerPac 3000 Power Supply,** 220/240 VAC
Accessory Kit	
170-4254	**Preparative Electrofocusing Kit,** includes: **Gel Tray,** 200 x 110 x 8 mm **Gel Divider,** 200 x 110 mm **Electrofocusing Gel,** 50 g **Sample Applicator** **Electrofocusing Strips** (100) **Filter Paper** (500)
Optional Accessories	
163-1194	**Bio-Lyte Electrofocusing Gel**
170-4124	**Filter Paper**
170-4046	**Leveling Table**
731-1550	**Polypropylene Econo-Column® Chromatography Column**
170-4237	**Surface pH Electrode**
For 200 mm Beds:	
170-4297	**Glass Gel Tray,** 200 mm
170-4208	**Gel Divider and Sample Applicator,** 200 mm

Catalog Number	Product Description

Chemicals

Bio-Lyte Carrier Ampholytes

163-1112	**Bio-Lyte 3/10 Ampholyte,** 40%
163-1132	**Bio-Lyte 3/5 Ampholytye,** 20%
163-1142	**Bio-Lyte 4/6 Ampholyte,** 40%
163-1152	**Bio-Lyte 5/7 Ampholyte,** 40%
163-1162	**Bio-Lyte 6/8 Ampholyte,** 40%
163-1172	**Bio-Lyte 7/9 Ampholyte,** 40%
163-1182	**Bio-Lyte 8/10 Ampholyte,** 20%

6.3 Preparation of Gel and Ampholytes

Note: It is essential to wash Bio-Lyte electrofocusing gel with good quality distilled water before use. Residual water soluble ionic material must be removed to prevent interference in the formation of the pH gradient. A conductance of less than 1.0 µmho is recommended.

6.4 Procedure for Preparation of Bio-Lyte Electrofocusing Gel

1. Swell the entire contents (50 g) of the gel in approximately 1,800 ml distilled water for at least 2 hours. The dry gel is most conveniently measured by weight.
2. Wash the hydrated gel in a large Buchner funnel with high quality distilled water. A volume of 8 liters is usually sufficient to remove all residual ionic material. If a conductivity meter is unavailable for checking the wash water, a 10 liter wash volume is recommended.
3. After washing, transfer the slightly compacted gel bed to a liter glass beaker, rinsing it out with distilled water. To dilute the gel, add enough distilled water to take the 50 g up to a final volume of 930 ml. Because of its fine particle size and high water regain, Bio-Lyte electrofocusing gel will form an approximate 100% slurry.

   The addition of ampholytes and/or sample will provide enough excess liquid for proper formation of the gel bed. The slurry should pour easily and should be free of lumps. Store at 4 ˚C.

Swell 50 g gel in 1800 ml distilled water.

Wash hydrated gel.

Re-hydrate to total volume of 930 ml.

Table 6.1. Recommended Electrolyte and Bio-Lyte Mixtures for Specific pH Gradients.

pH Range	3/10	3/5	4/6	5/7	6/8	7/9	8/10	Anode	Cathode
2.8-5.2		**1**						0.1-1 N H_3PO_4	2% 4/6 Bio-Lyte ampholyte
3.0-5.6		**2**	**1**					0.1-1 N H_3PO_4	0.1-1 N MES
3.0-6.0	**2**	**3**						0.1-1 N H_3PO_4	2% 4/6 Bio-Lyte ampholyte
3.5-5.5		**1**	**1**					0.1-1 N H_3PO_4	0.1-1 N NaOH
4.0-5.5		**1**	**2**					0.1-1 N H_3PO_4	2% 4/6 Bio-Lyte ampholyte
4.0-6.0			**1**					0.1-1 N H_3PO_4	2% 5/7 Bio-Lyte ampholyte
4.5-7.5	**2**			**3**				0.1-1 N H_3PO_4	0.1-1 N NaOH
5.0-6.5			**1**	**2**				2% 5/7 Bio-Lyte ampholyte	2% 5/7 Bio-Lyte ampholyte
5.5-8.5				**1**	**1**	**1**		0.1-1 N H_3PO_4	0.1-1 N NaOH
5.5-9.0		**1**			**3**	**1**		0.1-1 N H_3Po_4	0.1-1 N NaOH
6.0-8.0					**1**			2% 6/8 Bio-Lyte ampholyte	2% 6/8 Bio-Lyte ampholyte
7.0-9.5						**3**	**1**	0.1-1 N H_3PO_4	0.1-1 N NaOH
7.5-10.0						**1**	**3**	0.1-1 N H_3PO_4	0.1-1 N NaOH
8.0-10.0							**1**	0.1-1 N MES	0.1-1 N NaOH
3.5-9.5	**1**							0.1-1 N H_3PO_4	0.1-1 N NaOH

6.5 Selection of Ampholytes

Since preparative IEF procedures usually seek to isolate a single component in a mixture, it is most common to employ a relatively narrow pH gradient encompassing the isoelectric point of the component of interest. Table 6.1 shows a series of overlapping pH ranges which can be formed using Bio-Lyte ampholytes and ampholyte blends as indicated. The graphs represent typical pH gradients found in granulated bed IEF.

Typically 0.1-1 N NaOH at the cathode (-) and 0.1-1 N H_2SO_4 or H_3PO_4 at the anode (+) end of the tray are used. However with some ampholyte ranges, other electrolytes must be used to avoid conductivity gaps and pH extremes. Table 6.1 provides recommended electrolytes as well as ampholyte blends for various pH ranges. Electrolytes closer to the end pH of the ampholyte range may be advantageous.

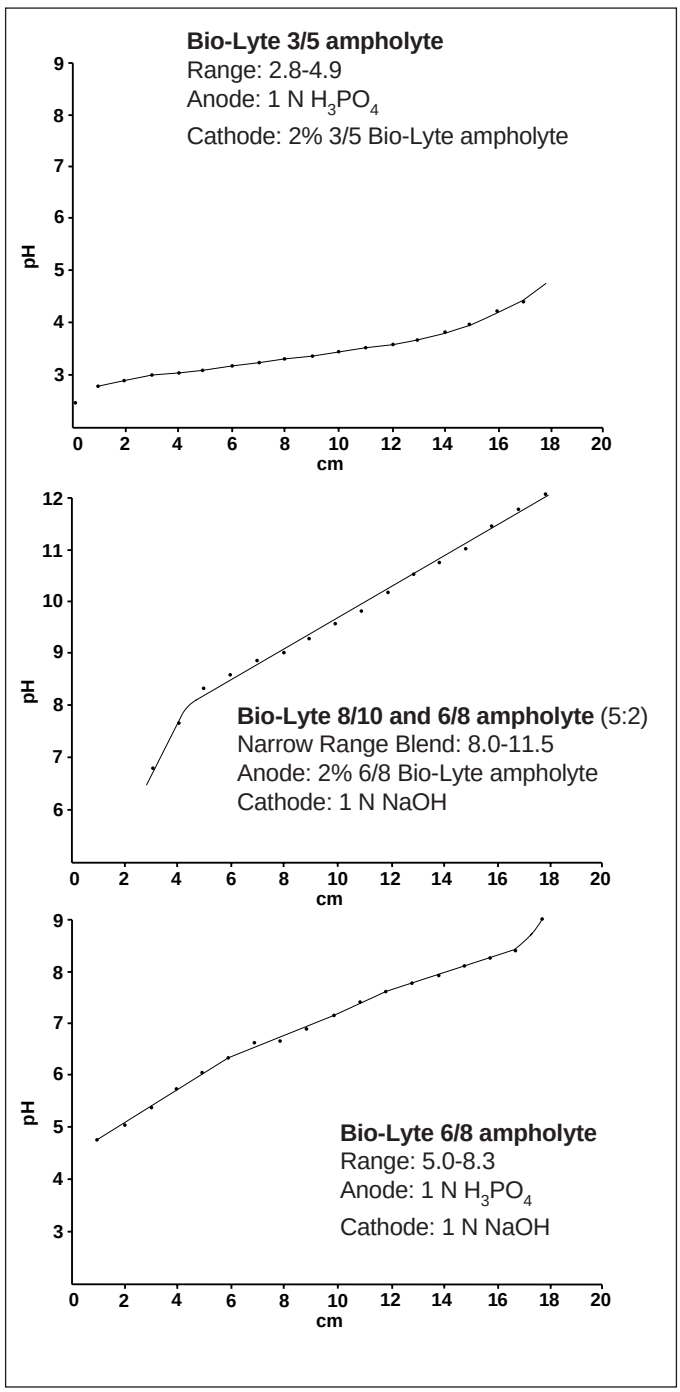
6.6 Narrow pH Ranges

It is often necessary to separate protein subunits or individual proteins which have p*I*s very close together. The narrow ampholyte range of 2 pH units may prove unsatisfactory at times. The following procedure is recommended for generating a pH range of 0.5, 1.0, or 1.5 pH units.

1. To the maximum amount of gel slurry, add narrow range ampholytes for a final concentration of 6-8%.
2. Dehydrate the gel bed completely. Do not add sample.
3. Focus the gel bed as usual to set up the pH gradient.
4. Measure the pH gradient and cut out that portion of gel which corresponds to the pH range of interest.

5. Re-slurry the gel in a minimum amount of water. **Do not add additional ampholytes.** It may be advantageous to add some plain gel slowly to increase gel bed volume.
6. Pour the thin gel bed and proceed with dehydration of the gel matrix and sample application.
7. The focused gel will now have a pH gradient of much narrower range, therefore further separating and resolving the bands of interest.

   The following graphs represent typical pH gradients found in granulated bed IEF.



6.7 Sample Preparation

Granulated bed IEF is capable of providing high resolution at high sample levels. Concentrated samples facilitate loading of several hundred milligrams of protein.

Lyophilization, vacuum dialysis, ammonium sulfate precipitation, or alternative concentrating procedures may be desirable in order to fully use the capacity of the technique.

Introducing ionic buffer components into the bed along with the sample must be avoided. While 1-2 cc of a protein solution in a dilute buffer (E.G. 0.01 M Tris, pH 8) may not seriously affect resolution, the sample is best prepared as a 2% ampholyte solution. The best method of sample preparation may be investigated on a small aliquot of the material of interest. A suggested protocol is given below.

1. Dialyze an aliquot of material against distilled water. If soluble, bring to 2% ampholyte concentration. (For selection of ampholytes see next section.) With crude mixtures which exhibit precipitation, screen for solubility of the component of interest (E.G. enzyme activity, immunological screening of soluble and insoluble phase, etc.). If the species of interest is soluble, centrifuge to remove insoluble material and bring the supernatant to 2% ampholyte concentration.
2. In cases of precipitation upon dialysis against distilled water, determine if an aliquot of the suspension becomes soluble upon addition of 2% 3/10 Bio-Lyte ampholyte or 1% glycine. Dialysis against one of these is then advised.
3. Investigate the solubility of the component of interest at a pH away from its p*I*. Use narrow range ampholytes to investigate the solubility at an acidic or basic pH (e.g. add Bio-Lyte 3/5 or Bio-Lyte 8/10 ampholyte to the protein suspension in distilled water).
4. Determine the solubility in urea or in a non-ionic detergent such as Triton X-100. Such reagents should be checked to assure they do not contain ionic impurities.

   Note: The sample must be as free from ionic species as possible as well as in a 2-4% ampholyte solution before application to the gel bed.

6.8 Preparing the Granulated Bed

The washed gel must be mixed with the appropriate ampholytes, distributed evenly in the gel tray, and dehydrated to the proper running consistency.

Using paper wicks to draw off excess moisture from each end of the bed is the simplest way to dehydrate the bed to the proper, slightly firm consistency, and entails no risk of over dehydration or of denaturation of sample proteins. It is recommended in all cases where the sample is sufficiently concentrated (10-100 mg/ml) and/or limited in volume (1-25 ml) that the sample applicator be used. Air drying is an alternative method for high volume samples.

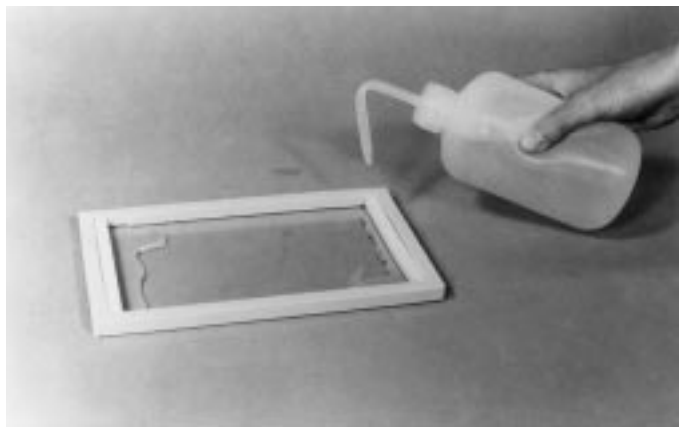
Precise measurement of the degree of dehydration is less important with the Bio-Phoresis cell than with other apparatus. Instead of the bed dilution which generally occurs, the bed actually loses about 0.5% of its moisture when run in the Bio-Phoresis cell with the cooled condensation coil.

6.9 Wicking Technique: Procedure

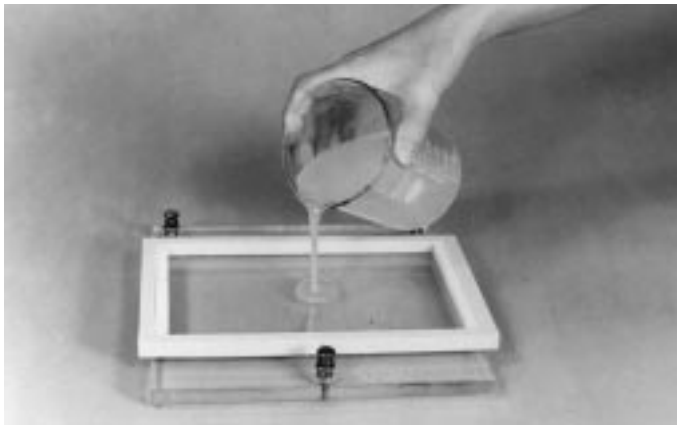
1. Measure out the quantity of ampholytes needed to produce a final concentration of 2-4%. A 2% ampholyte solution is adequate for most work, but increased concentrations may be beneficial with high sample loads. Remember that commercial ampholytes are supplied either as 20% or 40% solutions.

2. Mix the ampholytes into the required amount of gel (see Table 6.2). The gel/ampholyte solution may be degassed under vacuum for approximately 10 minutes.
3. Clean the gel tray (Bio-Rad cleaning concentrate, catalog number 161-0722, is recommended), rinse, and dry.
4. Cut eight to ten thicknesses of electrofocusing wick to size and place at each end of the tray.
5. Wet the wick thoroughly with distilled water and remove excess water from the tray.
6. Place the gel tray on a smooth, level surface. Pour the gel ampholyte slurry evenly throughout the tray. With a gentle sliding motion, distribute the slurry evenly throughout the tray.

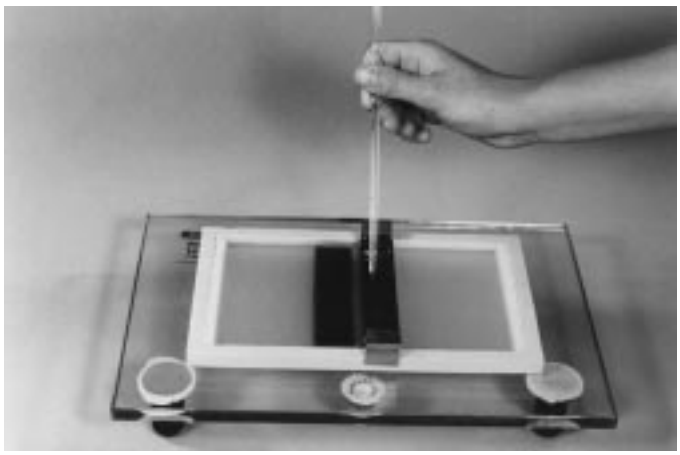
Add ampholytes for 2-4% final concentration.



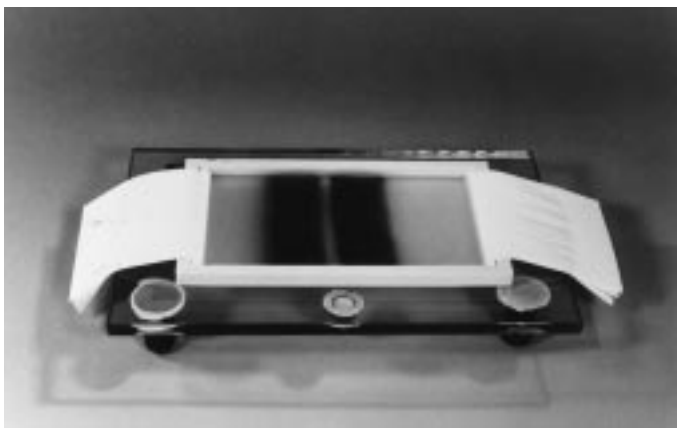
Wet electrofocusing wicks.



Pour gel-ampholyte slurry.



Wick off excess moisture from all but center portion. Add sample to center portion with applicator.



Wick off moisture from center.

7. Place 5-10 filter paper squares per side (10 x 10 mm, catalog number 170-4124) with their edges on the electrofocusing wicks. A relatively dry zone with a dull, non-reflective appearance will appear near the two ends, and should not exceed 1/3 the bed length on either side. Change the wicks as the rate of wicking slows, and stop wicking when a wet area large enough for sample application remains in the center of the gel.

8. Add 1 to 5 ml (depending on the depth of the gel bed) of sample to the gel inside the applicator. Mix the gel and sample **very well** while holding the applicator firmly in place. Remove the applicator.

   Applicator volumes should not exceed 5 ml, so more than one application per bed may be necessary. Up to 3 applications may be made in the 20 cm tray and up to 5 in 30 cm tray. Table 6.2 suggests maximum sample loads for various gel bed volumes.

Table 6.2. Sample Application

Gel Tray	IEF Gel Slurry Before dehydration	Suggested Maximum Sample Load
20 cm	50 ml	100 mg
20 cm	100 ml	300 mg
20 cm	150 ml	500 mg
30 cm	75 ml	250 mg
30 cm	150 ml	500 mg
30 cm	200 ml	750 mg
30 cm	250 ml	1,000 mg

9. Remove the remaining excess moisture by using the filter paper wicking technique previously described. The sample will diffuse toward the electrode wicks, but will remain in the gel matrix. The dehydrated gel matrix should not move when the tray is inclined to 45°.

6.10 Air Drying Technique

1. Mix the sample, which has been equilibrated with the ampholyte solution to be used, with the degassed gel/ ampholyte slurry. Samples lyophilized from distilled water or 2% ampholyte solution may be added to the slurry as solid and dissolved prior to pouring the gel bed.

2. Clean the tray, position and wet the electrofocusing wicks, and dry the tray as described in Section 2.1, steps 3-5. Pour and distribute the slurry as described in step 6.

3. Dry the gel by evaporation, using a fan or hair dryer to pass air over the bed. The air flow must not be strong enough to visibly disturb the surface of the gel slurry. The gel should be dried until it appears non-reflective or until it is dehydrated to about 68% of its original weight. Monitor the drying procedure carefully, as it is possible to over dehydrate the gels with this technique leading to cracking of the bed. When properly dehydrated the bed should not move when inclined to 45°.

6.11 Focusing the Granulated Bed

1. Place the tray in the Bio-Phoresis cell precooled with chilled water (4-10 °C) passed through the cooling coil and then through the cooling stage. Be sure to wet the stage with water (or a 1% nonionic detergent solution). A uniform film of water between the tray and the stage is necessary for maximum heat transfer from the tray to the stage.

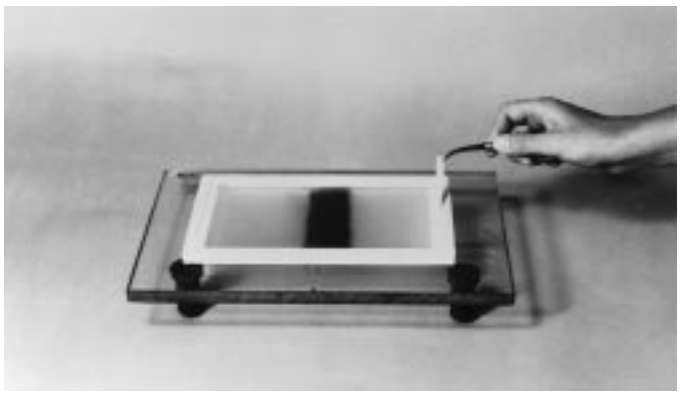
2. Dry out the cell. Carefully remove electrofocusing wicks which are above the level of the dehydrated gel bed (about 1-3 at each end). Cut to size and wet a single dry wick, with the terminating electrolyte for each end of the gel (see Table 6.1), blot excess moisture (Kim Wipes® are recommended), and place on top of the other wicks.

3. Position electrodes on the wicks, close the lid, and apply power to the bed. A constant power supply, such as the PowerPac 3000 power supply, is recommended. With such a power supply, overnight runs will generally focus at 1,400-1,600 V after 12-16 hours. Constant voltage power supplies can also be used by manually increasing the voltage over the first few hours to maintain a power level of ca. 4-6 watts (20 cm) or 7-10 watts (30 cm). Overnight focusing at ~700 V will usually be adequate to focus the sample.

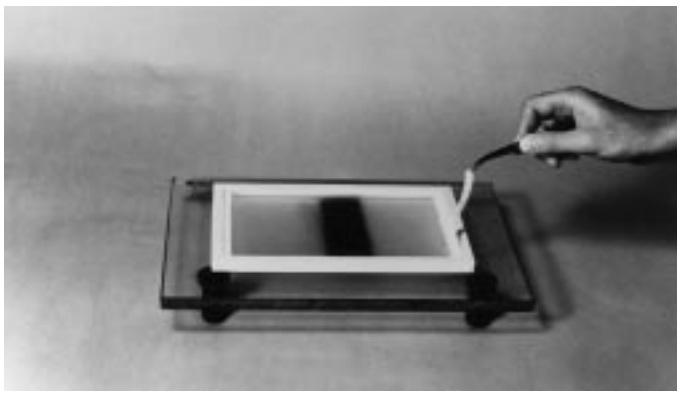
Table 6.3 provides guidelines to operating conditions.

Table 6.3 Granulated bed IEF Operating Conditions

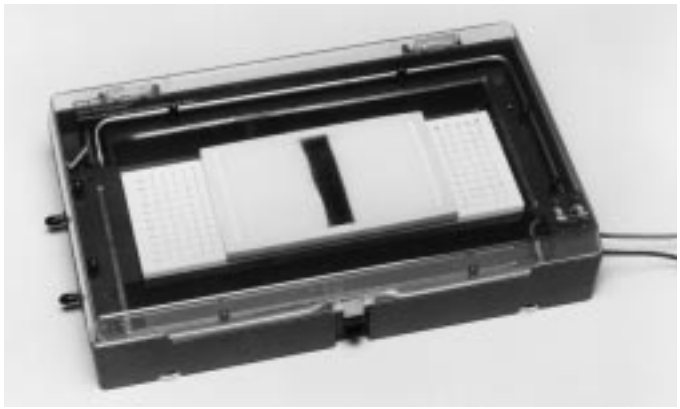
Power Supply	Tray Size	Initial Setting	Final Setting	IEF Time
Constant Power	20 cm	4-7 watts	4-7 watts	12-16 hr
Constant Power	20 cm	11-14 watts	11-14 watts	6-8 hr
Constant Power	30 cm	6-12 watts	6-12 watts	12-16 hr
Constant Power	30 cm	18-22 watts	18-22 watts	6-8 hr
Constant Voltage				
0-1,000 V DC	20 cm	250 V	500-700 V	18 hr
	30 cm	300 V	700-1000 V	18 hr
0-500 V DC	20 cm	250 V	500 V	18-24 hr
	30 cm	Not recommended		



Remove excess wicks.



Add wicks with terminating electrolytes.



Place on cooled, wet stage and focus.

6.12 Harvesting and Analysis of Results

The paper print technique offers a method for locating protein before harvesting, so only those sections of the gel with appreciable protein content need be harvested. The paper print technique results in a permanent record of the entire gel surface. Protein bands can be seen after staining and destaining, however this is limited to detection of protein on the gel surface. For an alternative method of detection, refer to reference 4 at the end of this section.

6.13 Procedure for Making Paper Prints

1. Cut Whatman 31ET filter paper to the size of the gel bed (19 x 11 cm or 29 x 11 cm).
2. After focusing is complete, remove the top electrode strip from the cathode and the anode.
3. Carefully roll the paper onto the surface of the bed without trapping any air bubbles.
4. After 1 to 2 minutes remove the paper and dry with a heat gun or in a 110-120 ˚C oven.
5. Place the top electrode strips in position and continue to focus while processing the paper print. This will prevent any diffusion of protein while analyzing the paper print.

   At this point any staining technique which does not stain ampholytes may be used. The following is one such method. Two other techniques are described using either light green SF or Coomassie violet R-150 dyes.

6. Wash the print for 15 minutes in 10% (w/v) trichloroacetic acid. Repeat the wash two times.

7. Stain the print for 15 minutes with 0.1% Coomassie brilliant blue R-250 in methanol/water/acetic acid in the proportions 50/50/10.

8. Destain in methanol/water/acetic acid (50/50/10), until protein bands are visible.

9. Use the paper print as a guide to orient the harvesting grid in Section 6.15.

6.14 Harvesting Techniques

The harvesting grid divides the gel into 0.7 cm wide sections. These can be scooped out and placed in small columns for elution of the protein, or the protein can be eluted by centrifuging the fractions in microcentrifuge tubes and pooling the supernatants.

The grid limits band diffusion during the harvesting procedure, and it provides a template for determination of the pH gradient using a surface micro-electrode. The pH gradient can also be determined with a standard combination electrode if the gel is first harvested into test tubes and diluted with water or saline.

The protein content of the fractions can be assessed by measuring the OD_{280} or using the Bio-Rad Protein Assay. Biological (e.g. enzyme activity) or immunological (double diffusion against an antisera specific for the component of interest) assays or stains can assist in locating specific components. Analytical IEF of the fractions can provide a profile of the separation.

As an alternative harvesting method, visible protein bands can be cut directly from the gel after the pH gradient is established without the use of the grid. Any band containing more than 10 mg protein will generally be visible as a clear area in the gel. This method gives complete recovery with maximum purity and minimum dilution.

6.15 Procedure for Harvesting with Fractionating Grid

1. If no bands are visible, illuminate the gel with long wave UV light to make sure that bands run straight across the gel, making it suitable for harvesting with the grid. Ampholyte bands, visible under long wave UV light, will follow the same contours as the protein bands.

2. Tare test tubes to accept the gel bed fractions. Place a disposable polypropylene column (catalog number 731-1110), packed with a small layer of larger mesh gel filtration beads (Bio-Gel® P-2 gel, 100-200 mesh, catalog number 150-0140) in each tube.

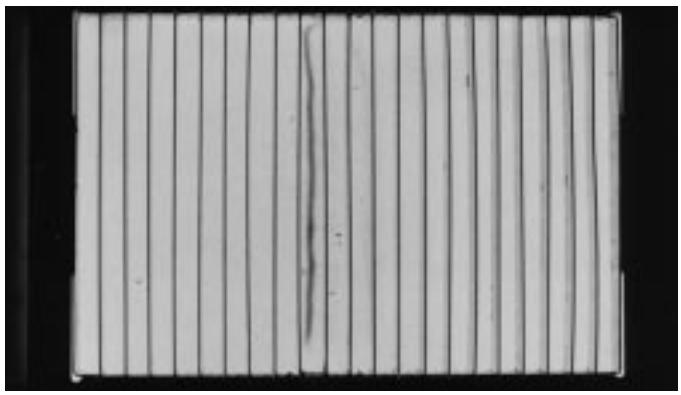
3. Remove the electrofocusing wicks from the gel tray. Make a print of the bed, if desired, as outlined in the previous section. Press the grid through the gel bed. The bed is left on the stage to maintain the temperature during direct pH measurement.

4. a. Measure the pH of every second section using a surface pH electrode (catalog number 170-4238).

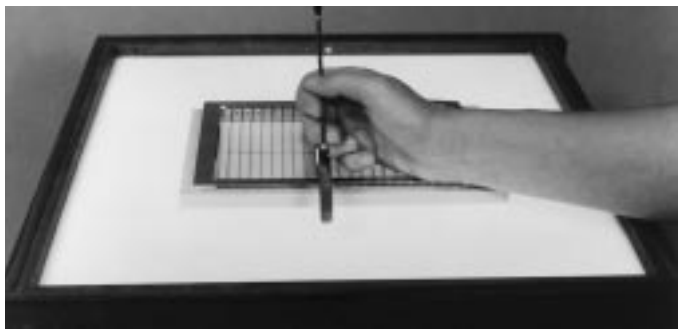
   b. For determination of the gradient with a standard combination electrode, carefully remove the fractions with a spatula and place in clean 16 x 100 mm test tube. Add 1.50 ml of distilled water or 0.2 M NaCl to liquefy the fraction.

   Measure the pH of the tube mixtures (alternate tubes may be adequate), then transfer the solution to the columns for complete elution with appropriate buffer, water, or saline.

The O.D.$_{280}$ or the protein content of the supernatant as measured with the Bio-Rad Protein Assay can be determined prior to elution. Generally, only those tubes with appropriate protein contents are eluted.



Place grid in gel bed.



Harvest gel and place in columns.

5. Harvest the gel from the sections and transfer to the columns. Use a minimum amount of buffer, water, or saline to wash the gel into the column. Break off the tip and collect the sample. When all the buffer has entered the gel, carefully add 3.0 to 4.0 ml buffer to complete elution. The fine particle size of the electrofocusing gel, which provides excellent resolution, elutes relatively slowly. Larger mesh beads must be placed in the end of the column to prevent small amounts of this fine material from passing through (step 2).
6. Determine the weight of the fractions. This provides a better estimate of volume than visual adjustment of tubes to "equal" volume. Determine the O.D.$_{280}$ or protein content with the Bio-Rad Protein Assay. Bio-Lyte ampholytes do not interfere with the Bio-Rad Protein Assay, and they generally do not interfere with enzymatic or immunological screening of fractions to locate components of interest.

6.16 Procedure for Harvesting Without Grid ("Snowplow" Method)

1. Press a razor (catalog number 170-4120) or thin spatula with a flat end, against the bottom of the gel tray next to the band and turn to a 45˚ angle away from the band.
2. Pass the blade along the edge of the band so that the gel is pushed aside, similar to the action of a snowplow. This leaves the band where it is but with a cleared space running along one side. (The gel that was pushed aside may be collected as a side fraction.)

3. Now repeat steps 1 and 2 to push the band of interest away from the rest of the intact bed. The band may now be removed with a spatula.
4. The gel on the other side of the band can now be removed as a side fraction or other bands of interest may be removed.

6.17 Sample Separation

Ampholytes have been removed from protein solutions by variety of methods including:

Dialysis

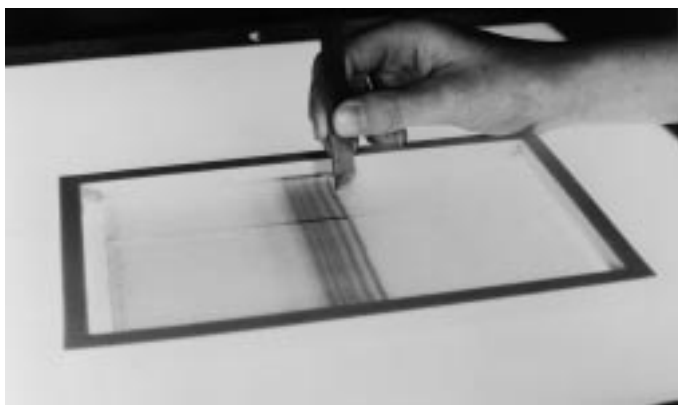
Gel filtration

Ion exchange chromatography

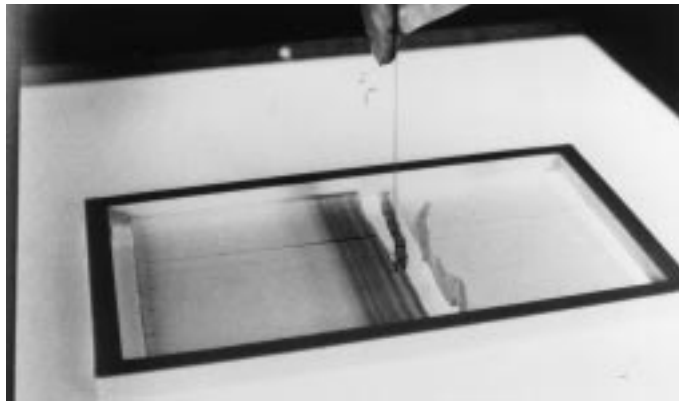
Ammonium sulfate precipitation

Hydroxylapatite chromatography

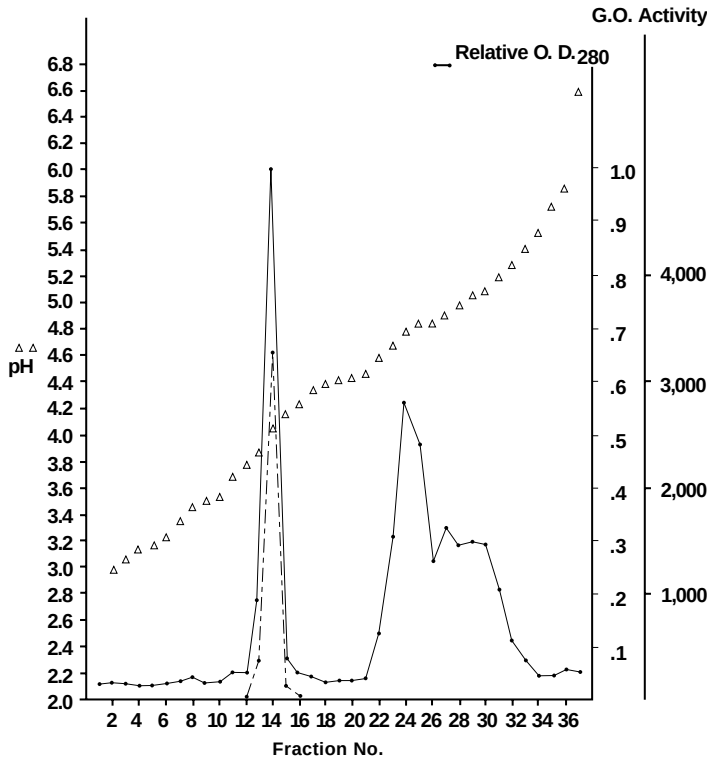
Elute protein in small column.



Press razor blade into gel and scrape away portion beside bands.



Scrape away and harvest bands.



Preparative Electrofocusing of Glucose Oxidase. 50 mg glucose oxidase in a volume of 12.38 ml was obtained from 930 mg of a crude enzyme preparation in 25 ml volume by preparative electrofocusing. A 1:1 mixture of 3/5 and 4/6 Bio-Lyte ampholytes was added to a bed formed with 25 g Bio-Lyte electrofocusing gel. After focusing for 14 hours at 9.5 watts constant power, the gel was sectioned into thirty-seven 0.7 cm wide fractions and harvested. Fraction 14 had 86% of the recovered activity with ten times the specific activity of the original sample. The percent yield of glucose oxidase in fraction 14 was 52.5%.

6.18 Troubleshooting Guide -Granulated Bed IEF

Problem	Cause	Solution
Bubbles near one or both ends of the gel bed, sometimes associated with localized condensation on the lid.	1. Too much salt in sample.	1. Dialyze against 1% glycine, ampholyte, or water. Otherwise, desalt with Bio-Gel P-2 gel, P-6 gel, or other comparable gels.
	2. Wrong polarity.	2. The more basic electrolyte should be at the - electrode (cathode) and the more acidic at the + electrode (anode).
	3. Heat produced by conductance gaps due to improper terminating electrolytes.	3. See Table 6.1 for suggested terminators.
Protein bands are skewed or have wiggles.	1. Bed not sufficiently dehydrated.	1. Gel beds prepared as per instructions should be dehydrated to approximately 68% of their original weight. If more water is used greater weight loss will be necessary.
	2. Gel bed regained moisture during run due to condensation.	2. With Bio-Phoresis cell this will not happen if the chilled water (4-10 °C) is first passed through the cover coil and then through the cooling stage.
	3. Gel and ampholyte slurry not homogeneous.	3. The slurry must be completely mixed to prevent localized differences in ampholyte concentrations.
	4. Electrode wicks too wet resulting in electrolyte spilling into the gel bed.	4. The electrode wicks should be placed on a glass plate and then thoroughly soaked with electrolyte. Then the wick should be blotted until it is just moist.

6.18 Troubleshooting Guide -Granulated Bed IEF (continued)

Problem	Cause	Solution
	5. Pressing electrode unevenly when placing on wicks resulting in localized spill-over of electrolyte.	5. Gently lay the pre-measured electrodes on top of the electrode wicks, avoiding direct pressure via thumb or fingers onto the electrode.
Current increases with time.	1. Wrong polarity.	1. The more basic electrolyte should be at the - electrode (cathode) and the more acidic at the + electrode (anode).
	2. Inadequate cooling.	2. Check that the cooling water is 4-10 °C and flowing at least 10 liters per hour.
	3. Contamination of anode with cathode electrolyte or vice versa.	3. Keep electrolytes physically separated during wick preparation.
Condensation above gel tray not localized at the ends.	1. Inadequate cooling.	1. Check that the cooling water is 4-10 °C and flowing at least 10 liters per hour.
	2. Too much power applied.	2. See Table 6.3.
	3. Bed not sufficiently dehydrated.	3. Gel beds prepared as per instructions should be dehydrated to approximately 68% of their original weight. If more water is used greater weight loss will be necessary.
Too low or no starting current.	1. Cover not completely closed.	1. The cover of the cell must be completely closed to engage the magnetic interlock.
	2. Open circuit.	2. (a) Check connections to power supply and electrode jacks. (b) Check fuses and connections to line voltage.
		3. The electrode should lie flat against the whole length of the electrode strip.

6.19 References (IEF)

1. Deincee, H. and Radola, B., *Anal. Biochem.*, **48**, 536 (1972).
2. Of the several papers screened, the 31 ET clearly performs the best in this application.
3. Radola, B., *Biochem. Biophys. Acta*, **295**, 412 (1973).
4. Frey, M. and Radola, B., *Electrophoresis*, **3**, 216 (1982).

Section 7
Immunoelectrophoresis

7.1 Background

Immunoelectrophoresis combines the principles of electrophoresis with traditional Ouchterlony (double gel diffusion) techniques. Grabar-Williams first introduced the method in 1953. Serum proteins were first separated based on electrophoretic migration (zone electrophoresis), then allowed to diffuse through agar containing antiserum. Distinct precipitate lines subsequently formed between each antibody/antigen interaction. Since the positioning and areas enclosed by the precipitate is proportional to the amounts of antibody and antigen present, this procedure provided the first means of immunological quantitation.

Several modifications of the Grabar-Williams procedure have evolved. Immunoelectrophoresis has become a complex, but useful, analytical and clinical tool. The most common methods are presented in this section. For more complete discussions the following references are recommended:

Handbook of Immunoprecipitation-in-Gel Techniques, Edited by N. H. Axelsen, *Scand. J. Immunol., Vol. 17, Suppl. 17*, (1983).

A Manual of Quantitative Immunoelectrophoresis, Edited by N. H. Axelsen, J. Kroll, and B. Weeke, *Scand. J. Immunol., Vol. 2, Suppl. 1*, (1973).

Quantitative Immunoelectrophoresis, New Developments and Applications, Edited by N.H . Axelsen, *Scand. J. Immunol., Suppl.*2, 1975.

Electrophoretic and Electro-Immunochemical Analysis of Proteins, Edited by C-B Laurell, *Scand. J. Clin. Lab. Invest., Vol. 29, Suppl. 124*, 1972.

Quantitative Immunoelectrophoretic Methods: A Literature Survey, Ronald Verbruggen, *Clin. Chem.*, **21/1**, 5-43, 1975.

Immunodiffusion Analyses Useful in Clinical Chemistry, Alfred J. Crowle, *Advan. in Clin. Chem.*, **20**, 181-224, 1978.

Refer to Section 1 “Getting Started” for Bio-Phoresis horizontal cell set-up and positioning of buffer chambers.

7.2 Techniques

A. Zone Electrophoresis

Zone electrophoresis separates proteins according to charge. The gel forms a porous network which acts as a stabilizing medium for the buffer, but which has pores large enough to allow even the largest protein molecules to pass unimpeded. The pH of the buffer is adjusted so the proteins of interest bear the same electrical charge, but have different charge densities. When an electric field is applied, the proteins migrate toward the oppositely charged electrode. The higher their charge-to-mass ratio, the faster they move, so the sample separates into a series of distinct zones in order of charge densities. These zones are then identified by staining. The proteins may be recovered by cutting out and solubilizing portions of the gel.

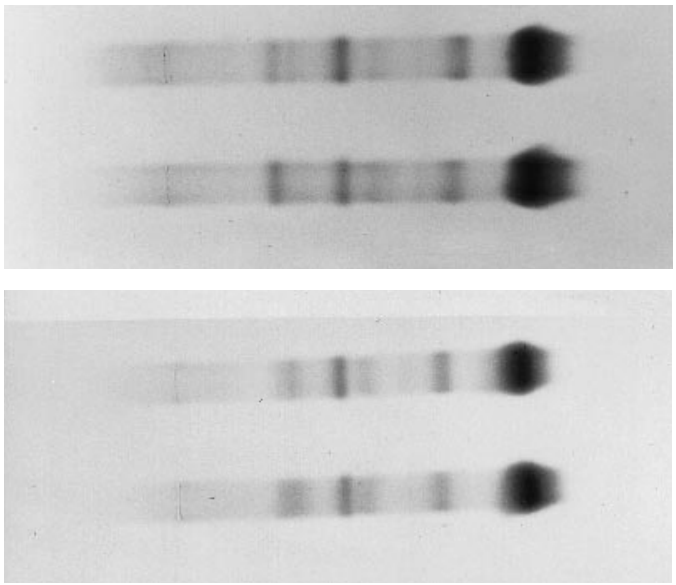


Fig. 7.1. Zone electrophoresis used for qualitative analysis of rabbit and goat sera.

B. Rocket and Fused Rocket

Rocket immunoelectrophoresis is a technique for the quantitative analysis of a single antigen in a complex mixture of proteins. The protein sample is placed in a well and moved by electrophoresis into a gel containing evenly distributed antibodies. The ratio of antibody to antigen increases as the antigen is diluted during migration. The highly concentrated tip of the antigen moves through the gel, while the slight quantities left at the sides form precipitin lines. Migration continues until the antigen is dilute enough to form the tip of the rocket shaped precipitin peak. The peak height is proportional to the concentration of antigen.[1,2]

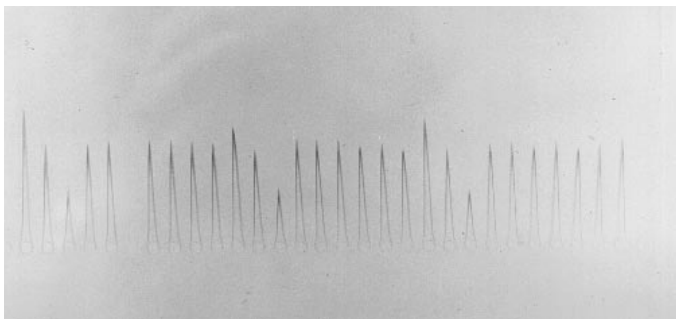


Fig. 7.2. Quantitation of albumin in different human serum samples by rocket immunoelectrophoresis of whole serum into gel containing rabbit antihuman albumin. Quantity of albumin in each sample is proportional to peak height.

The fused rocket technique measures the protein distribution among fractions collected in separation experiments. Samples of each fraction are placed into evenly spaced wells and allowed to diffuse. This produces a broad sample band, and therefore a continuous line precipitate pattern. The samples are electrophoresed into agarose gel containing antiserum to the sample under investigation. The resulting antigen-antibody precipitates reveal the elution profiles for individual proteins.

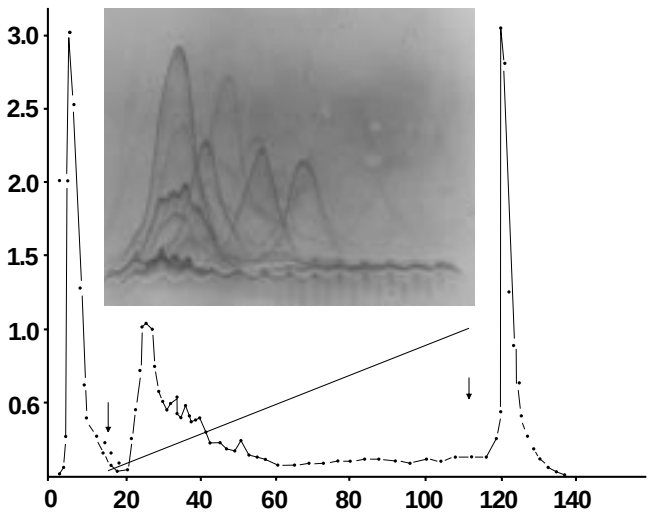


Fig. 7.3. Fused rocket immunoelectrophoresis used to show distribution of components in fractions obtained by gradient elution of serum proteins from DEAE Affi-Gel® blue columns. Five microliter aliquots of fractions 15-20 were applied to the wells. Absorbance pattern of entire separation is shown in surrounding chromatogram.

C. Grabar-Williams

Grabar-Williams immunoelectrophoresis is a two dimensional electroimmunodiffusion technique used for qualitative analysis of the components in a sample.

The samples are first separated by zone electrophoresis. For the second dimension, antisera is placed in a trough cut in the gel parallel to the direction of the electrophoretic separation As the samples and antiserum diffuse toward each other, each protein meets an increasing concentration of antiserum, including its own specific antibody. When the ratio of antiserum to antigen is high enough, they combine to form an arc shaped precipitate. The arc patterns from each sample are stained and compared.

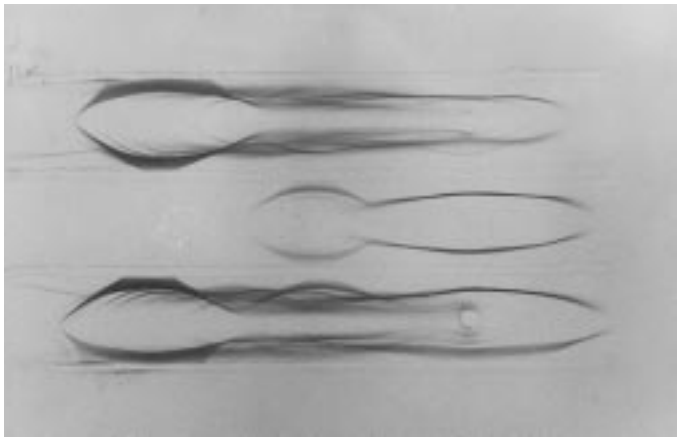


Fig.7.4. Grabar-Williams immunoelectrophoresis used for qualitative comparison of three different human sera preparations. Electrophoretically separated sera samples were allowed to diffuse against rabbit anti-whole human serum.

D. Two-Dimensional (Crossed)

Two-Dimensional (crossed) immunoelectrophoresis is a powerful method for the identification and estimation of complex antigen-antibody systems. This procedure has two stages:

1. Electrophoresis of antigens in agarose gel, then
2. Further electrophoresis at right angles to the first separation into an antibody-containing gel.

The area of the peaks formed by antigen-antibody precipitates is proportional to the antigen concentration.

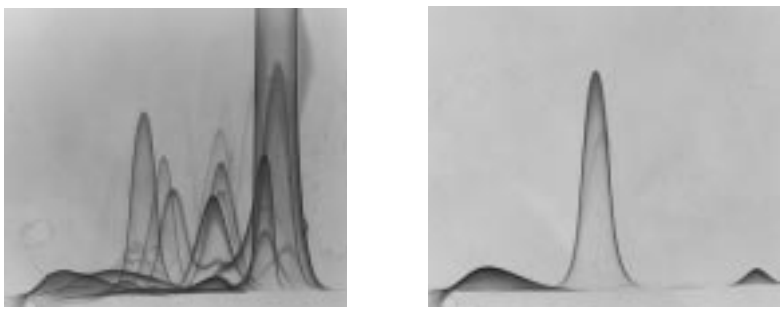


Fig. 7.5. 2-D immunoelectrophoresis used to monitor chromatographic purification of IgG. Plate 1 shows 2-D immunoelectrophoresis of the starting serum; Plate 2 shows 2-D immunoelectrophoresis of the IgG fraction of the serum following purification.[3]

7.3 Equipment and Chemicals for Immunoelectrophoresis

Equipment

Catalog Number	Product Description
170-2900	**Bio-Phoresis Cell**
170-2901	**Electrode/Buffer Chamber,** pair

Power Supply

165-4710	**Model 1000/500 Power Supply,** 100/120 V
165-4711	**Model 100/500 Power Supply,** 220/240 V
165-5056	**PowerPac 3000 Power Supply,** 100/120 VAC
165-5057	**PowerPac 3000 Power Supply,** 220/240 VAC
170-4016	**Leveling Table**

Glass Plates

170-4100	**50 x 50 x 1 mm,** 100
170-4101	**70 x 100 x 1.5 mm,** 50
170-4103	**100 x 100 x 1.5 mm,** 50
170-4104	**100 x 125 1.5 mm,** 50
170-4105	**100 x 200 x 1.5 mm,** 50
170-4107	**100 x 150 x 1.5 mm,** 50
170-4110	**70.7 x 70.7 x 1.2 mm,** 50
170-4127	**Ultra Wicks**
170-4120	**Cutting Blades**
170-4040	**Plate Holder**
170-4044	**Stain/Washing Bath**
170-4124	**Filter Paper**
170-4048	**Brass Bar**

U-Frame

170-4049	**100 x 100 x 1.0 mm**
170-4052	**205 x 110 1.0 mm**

Gel Puncher

170-4038	**1.5 mm**
170-4026	**2.0 mm**
170-4027	**2.5 mm**
170-4028	**3.0 mm**
170-4029	**4.0 mm**

Catalog Number	Product Description

Templates

170-4031	**Multiwell,** 100 mm
170-4024	**Multiwell,** 210 mm
170-4099	**Grabar-Williams**
170-4033	**Introductory Puncher and Template**
170-4097	**Rocket Peak Height and Area Estimator**
170-4098	**Radial Immunodiffusion Reader**

Immunoelectrophoresis Kits

170-4243	**Rocket and Two-Dimensional Immunoelectrophoresis Kit**
170-4245	**Radial Immunodiffusion Kit**
170-4246	**Immunoelectrophoresis Staining Kit**
170-4247	**Universal Immunoelectrophoresis Kit**

Additional Chemicals

Stains and Tracking Dyes

161-0404	**Bromophenol Blue**
161-0400	**Commassie Brilliant Blue R-250**
161-0402	**Amido Black 10B**
161-0417	**Crocein Scarlet**

Agarose Gel forming Reagents and Buffers

162-0100	**Agarose Standard Low -m_r**
162-0001	**Agarose High -m_r**
170-3002	**Agarose Immunodiffusion Tablets**
170-3003	**Agarose Immunoelectrophoresis Tablets**
170-3001	**IEP Buffer,** contains: sodium diethylbarbiturate 20.6 g; diethylbarbituric acid, 4 g; sodium azide, 1g. Makes 1 L 0.1 M solution.
170-3020	**IEP Buffer(Laurel),** contains: sodium diethylbarbiturate, 13.14 g; diethylbarbituric acid, 2.07 g; calcium lactate, 0.40 g. Makes 1 L 0.12 M solution.
170-3005	**IEP Buffer (Monthony),** contains: tricine, 17.2 g; tris, 39.2 g; calcium lactate, 0.424 g; sodium azide, 0.8 g. Makes 1 L 0.096 M solution.

7.4 Buffer Preparation

Tris-Tricine Buffer (IEP Buffer IV)

Although the literature recommends several barbital buffers for preparing the agarose gel and for using in the electrophoresis cell, Bio-Rad suggest a Tricine buffer specifically suited for zone electrophoretic and immunoelectrophoretic separations. Using the Tricine buffer eliminates stocking and working with the controlled substance sodium barbital. Compared with barbital buffer, the Tricine formulation produces higher buffering capacity and causes only slight variance in the relative position of proteins after electrophoresis.[4]

To prepare Tris-Tricine buffer (catalog number 170-3005):

1. Dissolve one package of buffer in deionized water, and make up to one liter.

2. Dilute one part buffer with three parts water before use.

The final buffer is Tris buffered 0.025 M Tricine, pH 8.6.

Agarose Immunoelectrophoresis Tablets

Agarose Immunoelectrophoresis Tablets, which include buffer, are a convenient way to prepare agarose gels for electrophoresis. The tablet form eliminates the need to weigh ingredients, and requires less melting time than premelted gel aliquots.

Each table yields a 1% agarose gel in Tris buffered 0.025 M Tricine, pH 8.6.

7.5 Casting Plates

Gel Support Film for Agarose

To eliminate precoating the glass plates with agarose, and to allow the plates to be reused, polyester gel support film for agarose may be applied to the plates. Gel support film for agarose is specially treated to provide a permanent record of the procedure. It facilitates staining/destaining, and can be stored easily in laboratory notebooks.

To apply gel support film for agarose:

1. Cut the gel support film for agarose to the dimensions of the glass plate.
2. Test for hydrophobic side by placing a drop of water on the film. The water beads on the hydrophobic side, and spreads on the hydrophilic side.
3. Pipet a few drops of water onto the glass plate.
4. Place the hydrophobic side of the gel support film for agarose against the plate.
5. Roll the gel support film for agarose flat with a test tube or similar object to force out excess water and air bubbles.

To Cast Plates:

Note: 15 ml of agarose makes enough gel for a 1.5 mm thick coating on a 100 x 100 mm plate. If the gel will contain antibody, adjust the amount of water or buffer added to the agarose to compensate for the amount of antibody solution added. Prepared agarose can be stored in small, sealed containers at 4 ˚C for several weeks.

1. Prepare a 1% agarose solution.
   a. Dissolve an agarose immunoelectrophoresis tablet in 5 ml hot distilled water, vortex or invert to mix, and place the tube in boiling water for 10 minutes to insure that the tablet melts.

or

b. Add 1 gram of agarose powder to 100 ml of buffer, immerse the tube in boiling water for 10 minutes, and stir frequently.

2. Apply gel support film for agarose to the plate, or moisten a paper wipe with agarose solution and coat the plate with a thin film. This improves adhesion between the agarose and the plate. Dry the plate with warm air.

3. Allow the agarose solution to cool to 55 ˚C.

4. For immunoelectrophoretic procedures, add antibody and mix.

5. Place the glass plate on a level surface.

6. Cast an even layer of gel on the plate.

   a. Pour the agarose solution directly from the test tube. Hold the test tube in the right hand within a centimeter or two of the plate, and start pouring to the right of plate center. Use the lip of the test tube to draw the agarose solution into any voids remaining on the plate.

   or

   b. Use a prewarmed pipet to place the agarose solution on the plate.

   or

   c. Clamp a U-frame between the coated plate and a second plate. With a prewarmed pipet, introduce the agarose solution into the opening between the plates. When the gel has solidified, slide the uncoated plate off.

   For best results, store the gel overnight in a closed container. However the plates may be used immediately after the gel has solidified.

7.6 Preparing and Using Tracking Dye

Bromophenol blue is the most common tracking dye for monitoring the migration of samples.

To use bromophenol blue:

1. Dissolve the dye in electrophoresis buffer to give a 0.01% concentration.

2. Dilute samples 1:1 with bromophenol blue/buffer solution.

3. Place samples in gel wells.

In serum samples, albumin binds to the dye and migrates as a second blue band behind the excess tracking dye.

7.7 Loading the Buffer Chamber

The buffer chambers should be loaded while inside the electrophoresis cell.

7.8 Running Recommendations

Sections 8-12 describe, in detail, the working procedures for zone electrophoresis and rocket, fused rocket, Grabar-Williams, and two-dimensional immunoelectrophoresis.

The following recommendations apply to all methods:

a. Always adjust the feet of the cell to level the platform before placing the glass plate on the surface. This prevents skewing of the sample.

b. For proper heat conduction, place water between the platform and the glass plate. This is done most easily by putting water at the edge of the plate with a pipet and allowing capillary action to draw it through.

c. For high visibility of the precipitates in immunoelectrophoretic separations power conditions of 5 V/cm are advised. At these conditions tap water can be used as a coolant. Use the table below to determine cooling requirements.

5 V/cm or below:	Approximately 17 ˚C. Tap water may be used if it is at least 5 ˚C below ambient temperature.
6-10 V/cm	12-15 ˚C.
10-20 V/cm:	94-10 ˚C.

d. Other quantitative immunoelectrophoretic techniques, such as line and intermediate gel, use similar equipment and chemicals in related procedures.

Section 8
Agarose Zone Electrophoresis

8.1 Applications

With zone electrophoresis, up to twenty protein samples can be separated on a single 110 x 205 mm glass plate. Zone electrophoresis is especially useful for screening purposes, such as routine clinical analysis of proteins in biological fluids. The number, position, and intensity of the resulting protein bands can be used to identify pathological samples. The method can also be adapted for preparative work on a 5 to 100 g scale.

Zone electrophoresis is also useful as a guide in protein fractionation work. It identifies proteins by their mobility compared to a reference. The intensity of the zone suggests relative concentrations.

8.2 Zone Electrophoresis Procedure

1. Set up the well forming apparatus (use combs and comb holders from submarine gel electrophoresis systems).

   a. Place the comb holder over a 100 x 100 mm plate.

   b. Adjust the well forming comb so the teeth are about 0.2 mm above the glass. (A piece of paper should pass between the teeth and the glass.) This adjustment insures that a thin layer of agarose remains at the bottom of the wells. (Section 8.3, Template 1.)

c. Coat the comb teeth with a thin film of silicone grease or petroleum jelly to keep the agarose from adhering to the teeth.

2. Prepare the plate (see Section 7.5).

   a. Make a 1% agarose solution from agarose tablets or powder. Place the plate on a level surface.2.

   b. Cast 15 ml of agarose on a 100 x 100 mm plate.

3. Make wells in the agarose.

   a. Place the comb holder over the agarose plate immediately.

   b. After the gel has solidified, lift the comb and comb holder vertically to remove it. A steady, gentle lifting will keep the agarose from tearing.

   c. To remove excess fluid from the wells, touch the well surfaces carefully with filter paper.

4. Put the plate in the Bio-Phoresis cell.

   a. Place the plate on the Bio-Phoresis cell cooling platform.

   b. Place a 0.6 x10 cm strip of paper wick at both edges of the gel.

   c. Place the Ultra Wicks or agarose gel wicks on top of the paper wicks to connect the gel to the buffer.

   d. For maximum cooling efficiency, put water between the plate and the cooing stage.

5. Add the sample to the wells.

   a. Dilute the protein samples 1:1 with 0.01% bromophenol blue.

   b. Without disturbing the wells or well edges, place 5 µl of sample into each well. (Sample volume may be increased for wells larger than 0.75 x 7 mm.)

   c. For best protein band resolution, begin electrophoresis right after adding the samples.

   Note: In zone electrophoresis it is essential to use perfectly straight edged wicks to connect the gel to the buffer.

6. Begin electrophoresis.

   a. Electrophorese at 20 V/cm with cooling at 4 to 10 ˚C for 1 hour, or until the sample has migrated approximately 6 cm from the well. If shorter separation time is required, higher voltages may be used.

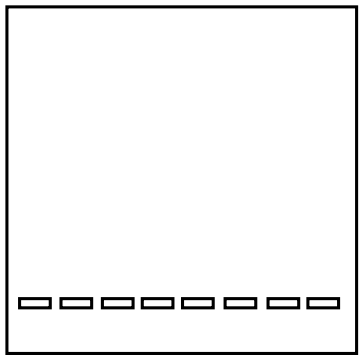
   b. To obtain sharp, even zones, refill the wells with buffer if they become dry during electrophoresis.

7. Fix the protein and stain (see Section 13.1).

   Resolved proteins can be electrophoretically transferred to nitrocellulose paper for further immunological assay work.[29]

8.3 Zone Electrophoresis Gel Cutting Template

Cast 15 ml agarose on the plate. Place the well former on the plate immediately and remove after the gel has completely solidified.



Template 1. Zone electrophoresis.

Section 9
Rocket Immunoelectrophoresis

9.1 Applications

Rocket immunoelectrophoresis is ideal for screening sera for individual proteins. For example, α-2-macroglobulin has been analyzed to compare variations with age, sex,[5] and pregnancy;[6] variations in relation to pulmonary disease,[7]diabetes mellitus,[8] and angioneurotic edema;[9] variations in cerebrospinal fluid;[10] and in studies of capillary permeability.[11] IgG from normal human serum has been analyzed by rocket immunoelectrophoresis at pH 5.0 using carbamylated antibodies which did not migrate at that pH.[12]

Rocket immunoelectrophoresis can be used in reverse to screen sera for antibodies, or to determine their concentration.[13] The buffer must run at a pH equal to the pI of the antigen in the gel to prevent the antigen from migrating. For example, human albumin is incorporated into the agarose gel, while rabbit anti-human albumin is applied into the sample wells. Electrophoresis at pH 4.85 produces rocket shaped precipitates whose heights are proportional to the antibody concentration.[1]

9.2 Rocket Immunoelectrophoresis Procedure

1. Prepare the plates (see Section 7.6).
    a. Make up a 1% agarose solution using agarose tablets or powder. (For each 100 x 100 mm plate, place 15 ml of the 1% agarose solution in a test tube to make a 1.5 mm thick gel.)
    b. For reproducible quantitative results, use antibody and sample concentrations that will result in peak heights from 20 to 40 mm.
    c. When the agarose tube has cooled to 55 ˚C, add the antibody. Mix gently to prevent air bubble formation.
    d. Place the glass plate on a level surface and cast an even layer of gel.

2. Punch wells in the gel.

    a. Attach a gel puncher to a vacuum source and punch 2.5 mm sample wells, using a punching template as a guide. Space the wells 5 mm apart, 10 to 20 mm from the edge of the plate (see Section 9.3, Template 2).

3. Place the plate in the Bio-Phoresis cell.

    a. Connect the gel to the buffer using a single thickness of Ultra Wick or filter paper (5 to 10 layers), or agarose gel wicks, moistened with buffer and overlapping the edge of the plate by 1/2 to 1 cm.

    b. For maximum cooling efficiency, place water between the plate and the platform.

4. Add samples to the wells.

    Note: Do not use a blow-out pipet, because the sample may splatter outside the wells.

    a. Place a 5 µl sample into each well.

    b. Apply the samples as rapidly as possible to minimize diffusion and consequent precipitate distortion.

5. Electrophorese.

    a. Switch on the current.

    b. Adjust the voltage to 1.5 to 2.0 volts per cm for overnight electrophoresis, or with proper cooling (4 to 10 ˚C) 20 volts per cm for a 2 to 3 hour run.

6. Stain the rocket patterns with Coomassie brilliant blue R-250 (see Section 13.2).

7. Measure the peak height to compare reference and unknown sample.

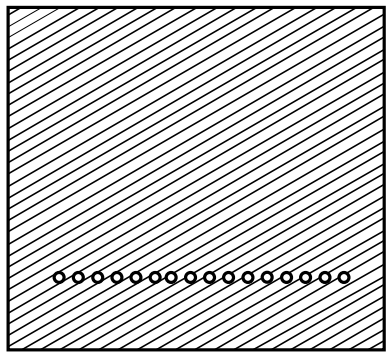
    a. Using a rocket peak height estimator, superimpose the base line on the base of the peak and the peak height line on the tip of the rocket. The multiplying effect permits reading of peak height to 0.2 mm.

    Depending on the volume of sample applied and the peak shape, up to 20 samples can be applied to a 100 mm plate. Individual proteins for which monospecific antibodies are not available may often be identified by radiolabeling, fluorescence, or specific staining techniques.

9.3 Rocket Gel Cutting Template

To prepare a 1.5 mm thick gel add the appropriate amount of antiserum to the agarose to produce the total volume given below:

Plate Size, mm	Agarose + Antibody, ml
50 x 50	7.5
70 x 100	10
100 x 100	15
205 x 110	34



Template 2. Rocket immunoelectrophoresis 15 ml agarose + antiserum.

Section 10
Fused Rocket Immunoelectrophoresis

10.1 Applications

Some examples of fused rocket analysis applications include:

a. Crude extracts of cells infected with herpes simplex virus were separated by ion exchange chromatography on DEAE cellulose columns. The fraction collected were analyzed by fused rocket immunoelectrophoresis to determine the distribution of virus antigens.[14]

b. Commercially available phytohemagglutinin, which was to be used for estimating lymphocyte activity, was purified by gel filtration. The fused rocket technique was used to identify the fractions.[15]

c. The trypsin degradation of human serum proteins was followed by fused rocket immunoelectrophoresis. Aliquots of the incubation mixture were taken at various intervals, inhibitor was added, and the samples were analyzed against whole antiserum.[16]

10.2 Fused Rocket Immunoelectrophoresis Procedure

1. Prepare the plates (see Section 7.5).

   a. Make up a 1% agarose solution using agarose tablets or powder.

b. Place a 100 x 100 mm glass plate on a level surface.

c. Place a brass bar 27 mm from the edge of the plate.

d. Pour 4.2 ml agarose solution.

e. Cut the agarose free from the bar, leaving a strip 25 mm wide.

f. Mix 1.0 ml antiserum with 10.5 ml agarose and pour onto the rest of the plate (see Section 10.3).

Note: The volume of antisera can be adjusted to give higher or lower peak heights.

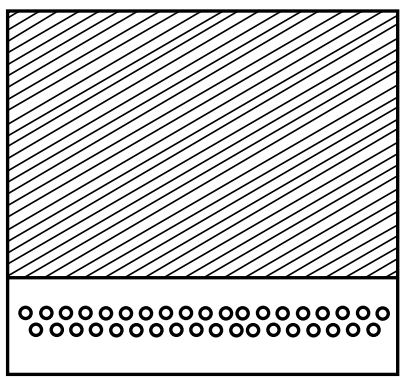
g. Punch out a series of 2.5 mm diameter sample wells following the pattern of template 3.

2. Pipet 5 µl aliquots of each desired separation fraction in sequence into the wells.
3. Let the samples diffuse into the gel for 30 to 60 minutes.
4. Place the plate in the Bio-Phoresis cell.
5. Connect the gel to the buffer with wicks.
6. Electrophorese at 1.5 to 2.0 V/cm for 16 to 20 hours.
7. Press and stain (see Section 13.2).

On a 100 x 100 cm plate, up to 40 fractions can be examined. Using antiserum raised against the particular sample will provide a complete elution profile, including minor impurities. Fused rocked immunoelectrophoresis using mono or polyspecific antibodies show the distribution of individual antigens. The distance between the precipitate line and the sample well is proportional to the concentration of the antigen in the fractions.

10.3 Fused Rocket Gel Cutting Template

Place a brass bar 27 mm from the edge of the glass plate and pour a 4.2 ml agarose solution. Cut the agarose free from the bar, leaving a strip 25 mm wide. Mix 1.0 ml antiserum with 10.5 ml agarose and pour onto the rest of the plate.



Template 3. Fused rocket immunoelectrophoresis 10.5 ml agarose +1.0 ml antiserum.

Section 11
Grabar-Williams Immunoelectrophoresis

11.1 Applications

Grabar-Williams immunoelectrophoresis is used clinically for qualitative analysis of serum proteins, particularly in analysis of immunoglobulin abnormalities. The position and shape of the precipitin arcs is used in diagnosing abnormalities in the clonal synthesis of immunoglobulins.

11.2 Graber Williams Electrophoresis Procedure

Grabar-Williams immunoelectrophoresis is also a valuable tool for protein comparisons in purification work. For example, the purity of an antigen solution can be determined using an antisera to the initial crude preparation. Fractionation work can be analyzed, even using crude polyspecific antisera, if known standards are available for comparison.

1. Prepare the plates (see Section 7.5).
   a. Make up a 1% agarose solution from agarose powder or tablets.
   b. Cast 15 ml of agarose on a 100 x 100 mm plate.
   c. Use the Grabar-Williams immunoelectrophoresis punching template to make 2.5 mm wells and troughs in the gel. Place the template bridge over the gel as a guide (see Section 11.3).
   d. Cut the troughs with a gel knife.

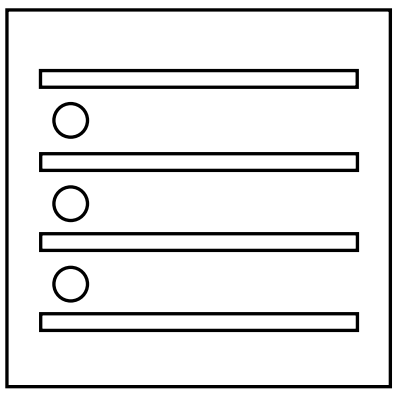
   Note: Do no remove the gel from the troughs until after electrophoresis.
2. Place the plates in the Bio-Phoresis cell.
   a. Use wicks to connect the gel to the buffer.
   b. For maximum cooling efficiency, place water between the plate and the platform.
3. Add the sample to the wells.
   a. Place 5 µl sample into each well.
   b. Dilute serum samples 1:1 with bromophenol blue to follow the position of the albumin visually.
4. Start electrophoresis.
   a. Electrophorese at 8 to 10 Vcm for 1 hour.
   b. For non-serum proteins, the electrophoresis time can be adjusted to obtain best separation.
5. Remove the gel from the troughs
   a. Lift the plate form the cell and remove the gel from the troughs using the slot removing tool. First slice the gel at each end of the trough with the 1mm edge of the tool. Then carefully slide this edge under the gel from the beginning to the end of the trough. The gel will slide onto the tool as it moves along.
6. Fill the trough with antisera.
   a. Use a minimum volume of 100 µl.

7. Prepare the plate for incubation.
    a. Place the plate in a closed container to prevent the gel from drying during incubation.
    b. The plate may be placed in the plate holder and incubated in the closed staining bath.
    c. Add a moistened paper towel to the bottom of the container to retain humidity.
8. Incubate at room temperature. Precipitin arcs start to become visible after 6 to 8 hours, and the reaction is complete after 24 hours.
9. Press, wash, dry, and stain (see Section 13.2).

    Sample components in a complex mixture can be identified by reaction with their monospecific antisera. Standard antigens can be run simultaneously with a sample preparation to identify unknown individual arcs by comparison of the position of their precipitin arcs.

11.3 Grabar-Williams Gel Cutting Template

Cast 15 ml agarose on the plate. Do not remove the troughs until electrophoresis is over.



Template 4. Grabar-Williams immunoelectrophoresis 15 ml agarose.

Section 12
Two-Dimensional (Crossed) Immunoelectrophoresis

12.1 Applications

Two-dimensional immunoelectrophoresis is valuable for comparing sera and other biological fluids. A rapid screening technique has been developed for routine clinical use. The first and second dimension gels are incorporated onto the same plate, eliminating the gel transfer step.[17]

Purification steps such as precipitation or ion exchange chromatography can be monitored qualitatively by two-dimensional immunoelectrophoresis. The rapid purification of protease-free IgG by DEAE Affi-Gel blue gel has been analyzed in this manner.[3]

Two-dimensional immunoelectrophoresis has also been used to provide immunogen for production of monospecific antisera,[18,19] bypassing tedious biomechanical methods. This technique appears to be widely applicable to protein purification.

Running the samples in tandem is an effective technique for comparing two related samples such as plasma and serum.[20] Apply the samples to the plate in a manner that results in double fused peaks for antigens which occur in both samples, and single peaks for those antigens present in only one of the samples.

When only small amounts of sample are available, the sample can be added to a reference, and their tandem patterns can be compared.[21]

Two-dimensional immunoelectrophoresis can also be performed using other media or techniques in the first dimension.

First dimension can consist of isoelectric focusing [22,23] or SDS-PAGE[24] separations followed by immunoelectrophoresis into antibody-containing agarose gel.

Monoclonal antibody specificity can also be examined in crossed immunoelectrophoresis.[25] Antigen mixtures are first separated electrophoretically in one dimension and precipitated by electrophoresis into an antibody containing gel in the second dimension.

12.2 Two-Dimensional Immunoelectrophoresis Procedure

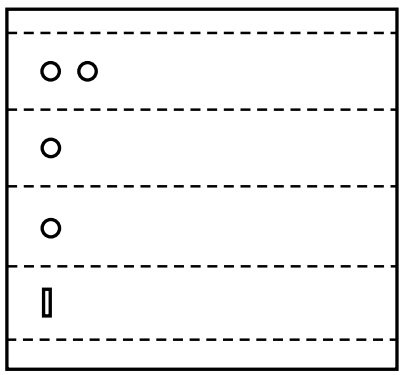
1. Prepare the plates (see Section 7.5).
   a. Make up a 1% agarose solution and cast 15 ml of agarose on a 100 x 100 mm plate.

   Note: Do not use gel support film for agarose or precoat plates at this step.

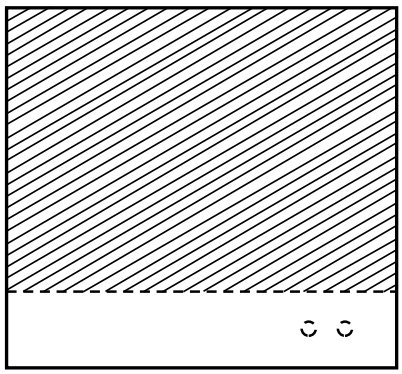
   b. Punch out 2.5 mm sample wells, using two-dimensional template 5 (Section 12.3).
2. Place the plate in the Bio-Phoresis cell.
   a. Use Ultra Wicks to connect the gel to the buffer.
   b. For maximum cooling efficiency, place water between the plate and the platform.
3. Place 5 µl of sample into each well.
   a. Dilute whole serum samples 1:1 with bromophenol blue and follow the position of the albumin proteins visually.
   b. For non-serum protein samples, duplicates should be run and one gel strip stained to observe its mobility under these conditions.(Samples should be allowed to migrate only 6 to 7 cm.)
4. Electrophorese at 8 to 10 V/cm for 1 hour. (For non-serum protein samples, electrophoresis time may be adjusted.)
5. Cut the gel into strips.
   a. Remove the plate from the cell and cut the gel slab into four strips, using 2-D Template 5 as a guide (Section 12.3).
   b. Transfer each strip to a separate clean plate (2-D Template 6).
   c. Cast a mixture of 11 ml agarose and 1 ml antiserum on the rest of the plate. (The volume of antiserum may be adjusted to give higher or lower peak heights.)
   d. Gel support film for agarose or precoated plates may be used.
6. Place the plates in the Bio-Phoresis cell. There must be a temperature difference of at least 5 ˚C between the cell coolant and the lowest room temperature, to prevent condensation on the gel.
7. Electrophorese at right angles to the first dimension at 1.5 to 2.0 V/cm overnight.

8. Press and stain (see Section 14.2).
9. Estimate the approximate concentration of each antigen in the sample by comparing the area under each peak with the corresponding area of a reference sample.
    a. Use the peak height and area estimator to quantitate peak areas. Superimpose the base line on the base of the peak and the peak height line on the tip of the peak. A half-height scale permits measurement of peak width for area estimation.
10. To identify individual peaks, compare their position with patterns formed by reference samples under identical conditions. Identify individual antigens by using monospecific antisera, specific staining methods, or by intermediate crossed-line immunoelectrophoresis.[26]

12.3 Two-Dimensional Electrophoresis Gel Cutting Templates



Template 5. First dimension 2-D immunoelectrophoresis. Cast 15 ml agarose on a 100 x 100 mm plate to produce a 1.5 mm thick gel.



Template 6. Second dimension 2-D immunoelectrophoresis 11.0 ml agarose + 1.0 ml antiserum.

Section 13
Stains and Staining Procedures for Immunoelectrophoresis

Various stains are used to make proteins and antigen-antibody precipitates more visible.

13.1 Zone Electrophoresis Stains and Staining Procedures

For zone electrophoresis, Amido Black 10B gives a distinct protein pattern and produces a colorless background.

Picric Acid Fixing Solution

Fix the protein before drying and staining. Bio-Rad recommends a picric acid fixing solution.

To prepare picric acid fixing solution:

Ingredients:

Picric acid	**14 g**
Deionized water	**1,000 ml**
Glacial acetic acid	**200 ml**

1. Add the picric acid to the deionized water.
2. Heat the mixture to approximately 60 °C to dissolve the acid.
3. Filter the saturated picric acid.
4. Add the acetic acid to the solution.

Amido Black 10B Staining Solution

To prepare amido black 10B staining solution:

1. Dissolve the stain in the acid, ethanol, and water solution.
2. Leave the solution at room temperature for 12 to 16 hours.
3. Filter the solution.

Zone Electrophoresis Fixing-Staining Procedure

1. Place the plate in picric acid solution for 15 minutes to fix the protein.
2. Wash in 95% (w/v) ethanol for 3 minutes.
3. Press and dry the gel.
4. Stain with Amido Black 10B staining solution for 5 minutes.
5. Destain until background is clear, usually about 15 minutes.

13.2 Immunoelectrophoresis Stains and Staining Procedures

For immunoelectrophoresis, Coomassie brilliant blue R-250 is the most sensitive stain and is the recommended stain for immunoprecipitates.

If whole antisera or large volumes of antibody (more than 20 µl/ml agarose) are used, remove the non-precipitated proteins by alternately pressing and washing the gel. Inadequate washing causes background staining because excess proteins remain in the gel.

If purified immunoglobulin or small volumes of antisera are used, the multiple pressing and washing is unnecessary.

Immunoelectrophoresis Destaining Solution

This solution is used to remove the excess staining solution from the gel. To regenerate the staining solution after use, pass it through a carbon filter.

To prepare destaining solution:

Mix:

Ethanol 95%	**450 ml**
Acetic acid	**100 ml**
Deionized water	**450 ml**

Immunoelectrophoresis Staining Procedure

1. Turn off power to the Bio-Phoresis cell and remove the plates.
2. Fill the sample wells with water.
3. Soak one piece of filter paper with deionized water and place it on the plate.
4. Place 8 to 10 sheets of dry filter paper on the plate.
5. Cover with a second glass plate.
6. Apply at least 10 g/cm^2 pressure (1 kg for 100 x 100 mm gel) for 10 to 15 minutes. (Use a heavy glass plate or similar object.)
7. Leaving the filter paper adjacent to the gel on the plate, remove the top pieces of filter paper.
8. Soak the piece of filter paper adjacent to the gel with water and remove it carefully.

   Note: When using whole antisera or over 20 µl antibody/ml agarose, place the plates in a plate holder and immerse in 0.1 M NaCl or physiological buffered saline for 10 minutes. Rinse in deionized water for 10 minutes. Then repeat Steps 2 through 8.
9. Dry the gel with warm air.
10. Place the plates in the plate holder and immerse in stain for 20 to 30 minutes.
11. Immerse the plates in three changes of destaining solution, 10 minutes each time.
12. Let the plates dry.

Silver Staining

Immunoprecipitates in agarose gels can be silver stained using an adaptation of the conventional protein-potassium ferrocyanide complex and silver nitrate. This technique is reported to be more sensitive than amido black methods.[28]

Section 14
Troubleshooting Guide for Immunoelectrophoresis

14.1 General Problems

Problem	Possible Cause
1. No precipitate in gel	a) no antibody in gel
	b) wrong antibody in gel
	c) imbalance in antigen/antibody proportion - concentration of antibody in gel is too high. All antigen precipitates at wells or interface of antibody containing gel
	d) no current (diffusion rings)
	e) anode and cathode reversed - cathodic precipitates
	f) marked pH changes in buffer and/or gel
	g) add antibody when agarose is too hot, therefore, precipitate out and cannot bind antigen
2. More than one precipitate	a) antibody is not monospecific
	b) antigen is not pure
	c) there is partial immunochemical identity and/or electrophoretic heterogeneity of the proteins
	d) current is interrupted before the end of electrophoresis
3. Oblique or "skewed" peaks	a) poor contact between wicks and gel
	b) crooked wicks or plates not placed straight on stage
	c) unequal thickness of gel
	d) cut in the gel causing current to run in a skewed pattern
	e) electrophoresis platform is not horizontal
4. Double contoured peaks	a) temperature differences between bottom and surface of the gel - gel is too thick
	b) inhomogeneity of gel due to unbalance cooling causes water condensation of the gel (wells fill with water)
	c) evaporation from the gel causes higher concentration of gel in the surface layer
	d) wicks are placed too close or overlap the wells

14.1 General Problems (continued)

Problem	Possible Cause
5. Diffused or "washed	a) water condensation on gel - too much cooling
out" peaks or arcs	b) water on lid drips onto gel - check that cooling is set up properly with electrophoresis apparatus
	c) electrophoresis not run long enough
6. Jagged incomplete peaks	a) fluid in the wells has emptied too fast and the antibody concentration is too high
	b) both antigen and antibody concentration were chosen too high so that partial blocking of the gel pores has developed
	c) electrophoresis not run long enough
7. Blunt, hazy, or "balloon" peaks	a) remaining antigen excess in the top area of the peak when the current was stopped too soon
	b) overfill of the wells leaving exess antigen unbound
	c) round tips and lower heights from running too slow
	d) electrophoresis not run long enough
8. Poor reproducibility or non-linearity in rocket QIEP	a) rockets may be too high; linearity is lost if peaks are above 40 mm high
	b) pipet calibration is off
	c) tip of pipet not wiped of excess sample on outside
	d) age and pH of buffer has not been checked
9. "Valley" precipitation arc running into first dimension gel in 2-D or fused rocket QIEP	a) due to overflow of the antibody-containing agarose onto the non-antibody gel slide
10. Drying out or "warping" of the gel	a) excessive heating of the gel - too much current
	b) cooling water is not running at correct temperature for current applied
11. Dark spots seen all over gel after staining/ destaining	a) agarose not completely dissolved (not boiled enough) - pieces of agarose retain the stain
	b) stained artifacts (bacteria growth, etc.) due to no azide in agarose and/or buffer, especially seen in Grabar-Williams and immunodiffusion plates

14.1 General Problems (continued)

Problem	Possible Cause
	c) stain is not filtered to remove insolubles
	d) drops of sample on gel
12. Dark background after staining/destaining	a) precipitated out antibody in gel - due to adding antibody when agarose is too hot (greater than 60 °C, not yet equilibrated)
	b) gel not washed with saline solution prior to staining
	c) cuts in the gel near wells cause stain to run underneath gel through the cut
13. Dark staining around wells	a) too high antibody concentration; therefore antigen precipitates at the well
	b) too low antigen concentration applied
	c) cuts in the gel near wells cause stain to run underneath gel through the cut
14. Precipitin lines disappear after destaining	a) destained too long
	b) concentration of acetic acid in destaining solution to high
	c) changed destaining solutions too quickly; therefore dissolved precipitation area

14.2 Specific QIEP Troubleshooting

Problem	Possible Cause
Barbital-sodium barbital buffer	a) overnight electrophoresis may change pH from 8.3 to between 10 and 11. This could cause a conductivity gap to set up in the middle of the gel, hence, this section can dry out. Antibody and antigen-antibody precipitate will "wash out" in this area. This effect is not seen with either Tris-barbital or Tris-tricine buffer
2-D (crossed) Immunoelectrophoresis	a) no current in the second dimension will cause first dimension gel to end up looking like Graber-Williams immunodiffusion
	b) reverse polarity in second dimension run will show only cathodic proteins running into the antibody gel - all others will run out of gel into cathode buffer

Section 15
References for Immunoelectrophoresis

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