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Model 111 Mini IEF Cell

Instruction Manual

Catalog Numbers 170-2975 and 170-2976

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Note

To insure best performance from the Model 111 Mini IEF 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 Model 111 Mini IEF 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 _____

Serial No. _____

Invoice No. _____

Purchase Order No. _____

Warranty

Bio-Rad Laboratories warrants the Model 111 Mini IEF 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
General Information

1.1 Introduction

Bio-Rad's Model 111 Mini IEF Cell introduces a simple and innovative "inverted" gel format for analytical isoelectric focusing applications. The gel is run upside-down, directly contacting the electrodes, to eliminate the need for electrode buffers and wicks. There is no need for active cooling during the focusing process. Condensation within the cell is not a problem because the gel is run upside-down, and therefore, the condensate cannot fall onto the gel.

The compact Model 111 Mini IEF Cell contains two graphite electrodes with an inter-electrode distance of 5 cm. The graphite electrodes are removable for easy cleaning. A maximum applied voltage of 450 volts is sufficient to yield tight bands, with excellent separation, within 90 minutes. The Model 111 Mini IEF Cell makes use of ultra-thin 0.4 mm gels, which provide better heat dissipation than thicker gels. Casting these gels is simple with the casting tray and gel support film provided with the cell. Acrylamide and agarose gels are both cast using the same casting tray.

1.2 Specifications

Construction

Outer chamber	Fabricated acrylic
Cell lid	Polycarbonate
Casting tray	Fabricated acrylic
Electrodes	High purity graphite, 0.95 cm diameter
Sample templates	Polyvinyl chloride

Shipping weight	**1.8 kg**
Overall size	24.6 (l) x 11.4 (w) x 4.8 cm (h)
Casting tray	20.3 (l) x 13.9 (w) x 2.3 cm (h)
Gel size	125 (w) x 65 (l) x 0.4 mm (t)
Glass plate size	125 x 65 mm
Voltage/power limit	500 VDC / 5 W
Casting tray temperature limit	60 °C

Note: The Model 111 Mini IEF Cell is not compatible with acetone, chlorinated hydrocarbons (i.e., chloroform), or aromatic hydrocarbons (i.e., toluene, benzene).

To clean the cell components use a mild detergent, such as Bio-Rad's Cleaning Concentrate (catalog number 161-0722) or ethanol.

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1.3 Safety



Power to the Model 111 Mini IEF Cell is to be supplied by an external DC voltage power supply. This power supply must be ground isolated in such a way that the DC voltage output floats 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 operating parameters for the cell are:

500VDC ⎓	maximum voltage limit
5 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 anyway.

IMPORTANT:

This Bio-Rad instrument is designed and certified to meet IEC1010-1* safety standards. Certified products are safe to use when operated in accordance with the instruction manual. This instrument should not be modified or altered in any way. Alteration of this instrument will:

- Void the manufacturer's warranty
- Void the IEC1010-1 safety certification
- Create a potential safety hazard

Bio-Rad is not responsible for any injury or damage caused by the use of this instrument for purposes other than for which it is intended or by modifications of the instrument not performed by Bio-Rad or an authorized agent.

*IEC 1010-1 is an internationally accepted electrical safety standard for laboratory instruments.

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1.4 Model 111 Mini IEF Cell Components

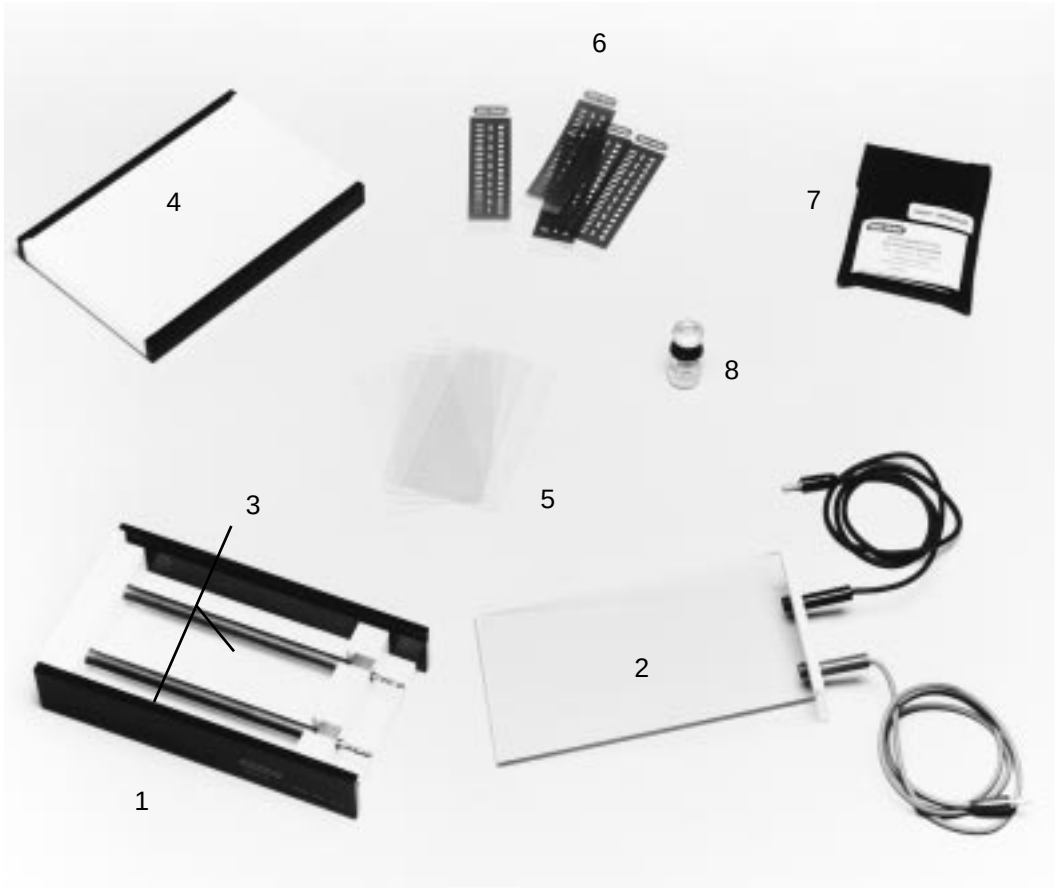


Fig. 1.1. Model 111 Mini IEF Cell. Outer chamber (1), sliding, interlocking lid with power cables (2), graphite electrodes (3), casting tray (4), glass plates (5), sample templates (6), gel support film (7), and 5 ml Bio-Lyte 3/10 ampholyte (8).

1.5 Capillary Thin Layer Gel Casting Tray

The Capillary Thin Layer Gel Casting Tray provides the fastest and easiest method for casting electrofocusing gels.

The casting tray consists of an acrylic plate with precisely defined spacers of 0.4 mm thickness as shown in Figure 1.2. The acrylic surface imparts a slight inhibitory effect on acrylamide polymerization, eliminating sticking and tearing of the gel.

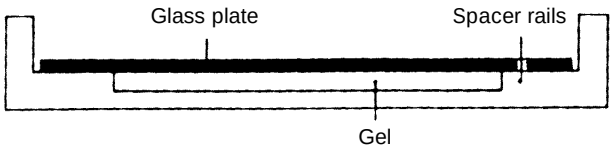


Fig. 1.2. Casting tray.

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Section 2
Introduction to Isoelectric Focusing

2.1 The Electrofocusing Principle

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.

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 net charge of the protein will vary as a function of pH.

A pH gradient is established concomitantly 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 away 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 pI or isoelectric point). At that point, the electrophoretic mobility is zero. Migration will cease, and a concentration equilibrium of the focused protein is established.

2.2 Carrier Ampholytes

Carrier ampholytes are complex mixtures of amphoteric buffers that form a smooth pH gradient in an applied electrical field. During electrofocusing, these buffers stack according to their individual pIs across the gel, producing a linear 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.

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2.3 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. A complete discussion of electrofocusing matrices is given in Reference 1.

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 (Bio-Rad's 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.

Section 3
Polyacrylamide Gel Isoelectric Focusing

3.1 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:

$$\%T = \frac{\text{g acrylamide + g crosslinker}}{\text{total solution volume in ml}} \times 100$$

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

This formulation will give a suitable 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.

The choice of a catalyst 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, and a detailed discussion of practical considerations is available in Bio-Rad's bulletin 1156.

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3.2 Stock Solutions for Polyacrylamide IEF Gels

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

1. Monomer concentrate (T=25%, C=3%)

   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 µm 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.

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_20.

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 produce sufficient reagent for two 125 x 65 x 0.4 mm gels:

Monomer-ampholyte solution

H_2O	5.5 ml
Monomer concentrate (25% T, 3% C)	2.0 ml
25% (w/v) glycerol	2.0 ml
*Ampholyte	0.5 ml

Catalyst solutions

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

*Volume required for 40% ampholyte solutions (Bio-Lyte 3/10, 4/6, 5/7, 6/8, 7/9 ampholytes). For 20% ampholyte (Bio-Lyte 3/5, 8/10 ampholytes) add 1 ml ampholyte and reduce H_2O volume to 5.0 ml.

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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 Use of Gel Support Film for Polyacrylamide

1. Remove a sheet(s) of Gel Support Film for Polyacrylamide (catalog number 170-2983) from the package, and then reseal the package. Polyacrylamide gel support film is sensitive to light over prolonged periods and must be kept sealed in the package until use. After the gel has adhered to the support film, the film is no longer light sensitive.
2. Polyacrylamide gel support film has two surfaces, a treated (hydrophilic) side which the acrylamide adheres to, and a hydrophobic surface. The sheets are packed with printed interleaf paper which protects the treated surface. All sheets are packed treated side up. A drop of water will bead on the hydrophobic surface and spread on the hydrophilic surface.
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 polyacrylamide support film. Increasing the concentration of ammonium persulfate to 0.7 mg/ml in the final acrylamide gel solution (add 70 µl of 10% ammonium persulfate per 10 ml solution) should alleviate the problem. 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.

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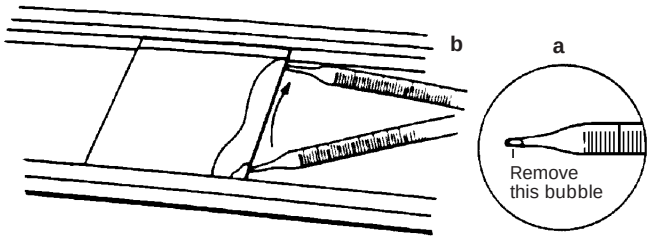
3.5 Casting Polyacrylamide Gels

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.

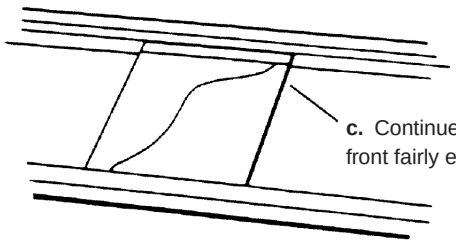
1. With the gel support film facing down, place the Glass Plate(s) on the casting tray so that it rests on the spacer bars.
2. Prepare the monomer-ampholyte solution (see Section 3.3). Degas the solution for 5 minutes under vacuum. Do not degas longer, as a slight amount of O_2 is required to catalyze riboflavin-5-phosphate.
4. Prepare the catalyst solutions.

   Note: Always use freshly prepared persulfate solutions
5. Add the catalyst solutions to 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 3.1.
   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.
   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.



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. 3.1. Injecting monomer/ampholyte solution in casting tray.

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7. Position a photopolymerization light (such as Bio-Rad's catalog number 170-4220 or 170-4242. See Section 9.2) over the tray. Any fluorescent desk lamp is a suitable alternative.
8. Irradiate the solution for 45 minutes.
9. To lift the gel from the casting tray:
    a. Lift one corner with a flat spatula inserted between the gel and the casting tray (see Figure 3.2).
    b. When air appears under the gel, gently lift the plate free from the casting tray.
10. Flip the plate, glass side down, onto the casting tray and further irradiate for 15 minutes to eliminate unpolymerized monomer on the gel surface.

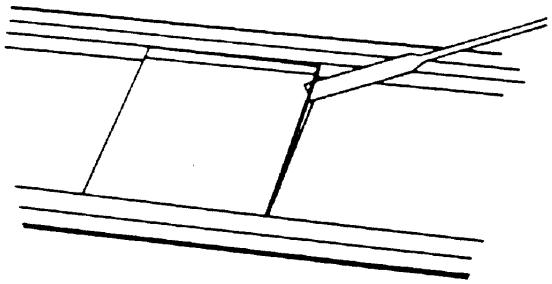


Fig. 3.2. Lifting the gel from the casting tray.

3.6 Sample Preparation

Protein samples for isoelectric focusing must be free of precipitates. Substantially salt-free samples 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 Spin® 6 chromatography columns (catalog number 732-6000 (10) or 732-6002 (25)) or with Bio-Gel® P-6DG desalting gel (catalog number 150-0738).

Many samples will require the use of urea, ethylene glycol, non-ionic detergents (i.e. Triton® X-100 detergent, NP-40, Lubrol® WX detergent, 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.

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3.7 Sample Application

Sample application is most conveniently accomplished using the included Sample Templates.

1. Place the template on top of the polymerized gel. The colored portion of the template should coincide with the longer dimension of the gel. The application position for the sample varies (see Section 3.8) but allow 1 cm at both the top and bottom of the gel for the gel to contact the electrodes.
2. Apply samples using a pipettor capable of delivering between 0.5 µl and 2 µl. Volumes above 2 µl are not generally recommended for use with the sample template.
3. Allow samples to diffuse into the gel for 5 minutes.
4. Carefully remove the template from the gel.

   Note: For larger samples, one may custom-form application strips from filter paper or use Bio-Rad's Sample Application Pieces (catalog number 170-4257). Apply the sample to the filter paper and then place the filter paper at the point of application on the gel. Allow the sample to diffuse into the gel for 5 minutes. The disadvantage of this method is that some proteins may not be completely eluted from the strip to the gel.

3.8 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.

Section 4
Running the Gel

4.1 Set Up Procedure

1. Slide the lid of the Model 111 Mini IEF Cell toward the electrode plugs to remove it.
2. Remove the Graphite Electrodes from the cell and rinse them with distilled water to remove traces of acrylamide from the previous run. The electrodes may be gently wiped with a laboratory tissue if necessary. Place the electrodes back into the cell.
3. Lightly moisten the graphite electrodes with water. Turn the gel with the adsorbed samples upside-down and place it directly on top of the electrodes. Position the gel carefully the first time, as re-positioning may damage the gel surface. Do not remove the glass plate from the gel/gel support backing because its weight insures good contact between the gel and the electrodes.
4. Carefully slide the lid back onto the Model 111 Mini IEF Cell. Plug the power cables of the cell into a power supply able to generate 500 V constant voltage. The cell is now ready to separate samples.

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4.2 Run Conditions

1. Focusing is carried out under constant voltage conditions in a stepped fashion. Begin focusing at 100 V for 15 minutes.
2. Increase voltage to 200 V for 15 minutes.
3. Finally, increase the voltage to 450 V for an additional 60 minutes.

   Note: Step increases of voltage are necessary to prevent overheating and subsequent dehydration of the gel. Failure to follow this procedure will result in poor resolution. One advantage of using Bio-Rad's PowerPac 3000 or Model 1000/500 power supply is that either power supply can be programmed to carry out this stepped increase in voltage automatically.
4. Typical running conditions for various ranges of Bio-Lyte ampholytes in the Model 111 Mini IEF Cell are shown in Table 4.1. Any gross variation from these values could indicate problems associated with polymerization, buffer preparation, etc.

Table 4.1 Typical Running Conditions

Bio-Lyte pH Range	Current/Power at 100 V	Current/Power at 200 V	Current/Power at 450 V
3-10	5-6 mA/0.5-0.6 W	5-6 mA/1 W	4 mA/2 W
4-6	3 mA/0.3 W	3-4 mA/0.6-0.8 W	4-5 mA/2 W
5-7	3 mA/0.3 W	3-4 mA/0.6-0.8 W	4-5 mA/2 W
6-8	3 mA/0.3 W	3-4 mA/0.6-0.8 W	4-5 mA/2 W
7-9	3 mA/0.3 W	3-4 mA/0.6-0.8 W	4-5 mA/2 W
8-10	10 mA/1 W	20 mA/4 W	4 mA/2 W

5. A good way to monitor the progress of an isoelectric focusing experiment is to observe the migration of visible marker proteins of known pI to their isoelectric points. The IEF Standards (catalog number 161-0310) provide eight natural proteins, including four visible proteins that are clearly discernable during an IEF run. These markers are useful with all non-denaturing IEF buffer systems.
6. As the focusing nears completion, the current will decrease from the values listed in the 450 V column. This is a general indication that the focusing is near completion.

   Note: The Model 1000/500 Power Supply measures current to the microampere range. This expanded monitoring of the changes in current allows better estimation of the time the current stabilizes and the experiment is finished.

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Section 5
Sample Detection and Gel Storage

5.1 Removing the Gel

1. After electrofocusing is complete, turn off the power supply and unplug the power cables.
2. Slide the protective lid from the cell and remove the gel from the electrodes. At this point separate the gel/gel support film from the glass plate.
3. Remove the Graphite Electrodes and clean with water and a tissue. Rinse the chamber with distilled water. Put the electrodes back into the cell to prevent damage during storage. Cleaning the electrodes immediately after the run will increase their life span.

5.2 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.

This section gives two recommended staining procedures for electrofocusing using Coomassie blue R-250, which has a detection limit for most proteins in µg quantities. Either method produces acceptable results, however, Method B is significantly easier to use and, in some cases, will produce a sharper pattern. For more sensitive detection down to ng levels of protein, the Bio-Rad Silver Stain (catalog number 161-0443) is recommended.

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

Fixing and Staining

Method A	**Method B**
Fixative: 4% sulfosalicylic acid 12.5% trichloroacetic acid 30% methanol Immerse gels in this solution for 30 minutes.	**Fixative:** Not necessary
Method A	**Method B**
Stain: 27% isopropanol or ethanol 10% acetic acid 0.04% Coomassie brilliant blue R-250 0.5% $CuSO_4$ (0.05% crocein scarlet optional) Dissolve the $CuSO_4$ in water before adding the alcohol. Either dissolve the dye in alcohol or add it to the solution at the end. Immerse the gel in the stain for approximately 1-2 hours.	**Stain:** Same as Method A, but with 0.05% crocein scarlet Crocein scarlet, a highly soluble dye which rapidly binds to protein, is included to assure rapid fixation of the bands. This procedure is inadequate if Coomassie brilliant blue R-250 is used alone.

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5.3 Destaining (For both Method A and Method B)

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:
25% isopropanol or ethanol
7% acetic acid
Immerse the gel in this solution to remove the last traces of stain and $CuS0_4$.

Note: Prolonged soaking of gels with gel support film backings in acidic solutions may cause the gel to separate from the backing. Staining and destaining steps should be no longer than 3-4 hours.

5.4 Other Detection Methods

1. Coomassie Blue G-250 “Quick Stain”

   This technique is nearly as sensitive as Coomassie blue R-250 staining, 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 blue G-250

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

2. Ultrasensitive Silver Stain

   Bio-Rad’s Silver Stain (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.

5.5 Gel Drying and Preservation

Place the destained gel in a dust free area with good ventilation (a 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).

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Section 6
Agarose Gel Electrofocusing

6.1 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 heating the agarose mixture and pouring it into the casting tray much the same way as casting acrylamide 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.

The procedure for agarose isoelectric focusing is simple, consisting of the following seven steps. Each of these steps is described in detail in Sections 6.3 through 6.6.

1. Cast the agarose gel.
2. Dehydrate the gel to remove excess liquid.
3. Focus the gel for 90 minutes.
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.

6.2 Preparing Agarose Gels

Agarose IEF gel contains:

1% agarose, zero-M_r

2% ampholytes

5% sorbitol

10% glycerol

1. Add 0.5 grams of Bio-Rad's Zero-M_r Agarose and 2.5 grams of sorbitol to 20 ml of 25% glycerol and 10 ml of 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 the components (30 minutes).
2. Place the casting tray into a prewarmed 55 °C oven and allow it to equilibrate. The tray should remain in the oven until just prior to pouring the gels.
3. Turn off heat and add ampholytes while stirring the agarose mixture (2.5 ml of 40% ampholytes or 5 ml of 20% ampholytes). Add more hot (100 °C) distilled water for a final volume of 50 ml.
4. Allow solution to cool to about 55 °C before pouring agarose. A hot water bath set to 55 °C is optimal.

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Note: Do not heat the casting tray to more than 55 °C. Extended heating above this temperature may cause the spacers to separate from the casting tray and may cause the tray to warp.

5. Remove a sheet of Gel Support Film for Agarose (catalog number 170-2984) from the package.
6. 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.
7. Pipet a few drops of water onto the glass plate.
8. Place the hydrophobic side of the Gel Support Film for Agarose against the plate.
9. Roll the Gel Support Film for Agarose flat with a test tube or similar object to force out excess water and air bubbles.
10. Remove the casting tray from the oven and place it on a level surface.
11. Heat a glass pipet with hot water and pipet 4 ml of the warm agarose solution in a bead across the width of the casting tray.
12. Carefully lower the Glass Plate (with the gel support film side down) onto the agarose. Allow the agarose to spread completely underneath the glass plate. Some agarose may extend outside the glass plate.
13. If there are any areas under the Glass Plate not filled with agarose, simply tilt the casting tray and the agarose should fill in these areas quickly.
14. If there are any air bubbles trapped, tapping on the Glass Plate should remove them.
15. The gel will solidify within 10-15 minutes. At this point, let the gel “age” at 4 °C (refrigerate) for a minimum of 4 hours, preferably overnight. The gels may be left in the casting tray. Simply cover the tray with plastic wrap to prevent dehydration of the gel.
16. To lift the gel from the casting tray:
    a. Lift one corner with a flat spatula inserted between the gel and the casting tray (Figure 6.1).

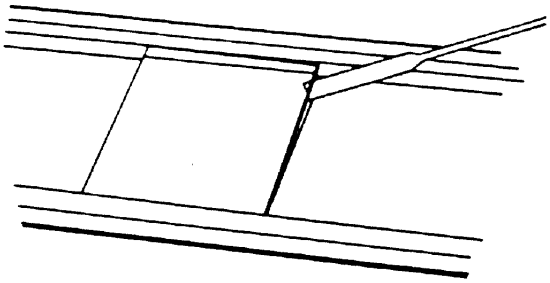


Fig. 6.1. Loosening the plate.

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6.3 Sample Preparation

Protein samples for isoelectric focusing must be free of precipitates. Substantially salt-free samples 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 desalting gel (catalog number 150-0738).

A convenient technique for preparing small samples is to load a small amount of hydrated Bio-Gel P-6DG 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 (i.e. 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.

6.4 Sample Application

Sample application is most conveniently accomplished using the included Sample Templates.

1. Place the template on top of the polymerized gel. The colored portion of the template should coincide with the longer dimension of the gel. The application position for the sample varies (see Section 6.5) but one should allow 1 cm at both the top and bottom of the gel where the gel will contact the electrodes.
2. Apply samples using a pipettor capable of delivering between 0.5 µl and 2 µl. Volumes above 2 µl are not generally recommended when using the sample template.
3. Allow samples to diffuse into the gel for 5 minutes.
4. Carefully remove the template from the gel.

   Note: For larger amounts and sizes of samples, one may custom-form application strips from filter paper, or use Bio-Rad's Sample Application Pieces (catalog number 170-4257). Apply the sample to the filter paper and then place the filter paper at the point of application on the gel. Allow the sample to diffuse into the gel for 5 minutes. The disadvantage of this method is that some proteins may not be completely eluted from the strip to the gel.

6.5 Position of Application

There are no fixed rules regarding the positioning of the sample on the gel. Samples should not, in general, be applied in 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.

6.6 Set Up Procedure

1. Slide the lid of the Model 111 Mini IEF Cell toward the electrode plugs to remove it.
2. Remove the Graphite Electrodes from the cell and rinse them with distilled water to remove traces of agarose from the previous run. The electrodes may be gently wiped with a laboratory tissue if necessary. Place the electrodes back into the cell.

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3. Lightly moisten the Graphite Electrodes with water. Turn the gel with the adsorbed samples upside down and place it directly on top of the electrodes. Position the gel carefully the first time as re-positioning may damage the gel surface. Do not remove the glass plate from the gel/gel support backing because its weight insures good contact between the gel and the electrodes.

4. Carefully slide the lid back onto the Model 111 Mini IEF Cell. Plug the power cab cell into a power supply able to generate 500 V constant voltage. The cell is now ready to separate samples.

6.7 Run Conditions

1. Focusing is carried out under constant voltage conditions in a stepped fashion. Begin focusing at 100 V for 15 minutes.

2. Increase voltage to 200 V for 15 minutes.

3. Finally, increase the voltage to 450 V for an additional 60 minutes.

   Note: Step increases of voltage are necessary to prevent overheating and subsequent dehydration of the gel. Failure to follow this procedure will result in poor resolution.

4. A good way to monitor the progress of an isoelectric focusing experiment is to observe the migration of visible marker proteins of known pI to their isoelectric points. The IEF Standards (catalog number 161-0310) provide eight natural proteins, including four visible proteins that are clearly discernable during an IEF run. These markers are useful with all non-denaturing IEF buffer systems.

5. As the focusing nears completion, the current will decrease substantially. This is a general indication that the focusing is near completion.

6.8 Sample Detection

1. After the focusing is complete, turn off the power supply and disconnect the power cables from it.

2. Slide the lid off the Model 111 Mini IEF Cell and carefully remove the gel from the Graphite Electrodes. Rinse the electrodes with water and wipe them gently with a tissue. Replace the electrodes in the cell.

3. Place the gel/gel support film (Glass Plate is removed at this point) into fixative solution for 15 minutes.

   Fixative Solution
   30% methanol
   5% trichloroacetic acid (TCA)
   3.5% sulfosalicylic acid (SSA)

4. To insure a clear gel background, take the agarose gel directly from the fixative solution to a 95% ethanol bath. Immerse the gel in ethanol for 30 minutes with occasional swirling.

5. After the ethanol wash, 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 dry 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.

6. After pressing, dry the gel completely with an air blow dryer or under a laboratory hood's fan.

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

   Stain
   0.2% 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.

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

   Destain Solution
   28% isopropanol or ethanol
   14% acetic acid

9. Air dry the gel plate.

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Section 7
Troubleshooting

7.1 General Troubleshooting

Problem	Cause	Solution
1. Excessive pooling of water at the cathode(-) Agarose only.	a. Poor quality agarose.	a. Use Bio-Rad's Zero - M_r Agrose only
	b. Gel has not been sufficiently blotted dry 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 ampholyte1% glycine or water, or desalt with Bio-Gel P-2 or P-6DG gel.
	b. Sample is 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. 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,amphoteric detergents).
4. pH gradient does not cover expected range.	a. Focused too long or use of excessive voltage.	a. At recommended constant voltage, proteins should be focused within 1.5 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 into the gel.
	d. Old acrylamide and Bis stock solutions.	d. Prolonged storage of acrylamide and Bis leads to acrylic acid formation.

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Problem	Cause	Solution
	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.
5. No current/power at recommended voltage.	a. Poor gel/electrode contact.	a. Lightly moisten Graphite Electrode before applying gel.
	b. Electrodes dirty.	b. Remove residue with tissue and water. Always clean electrodes after run.
	c. Poor electrical contact in unit.	c. Make sure lid is pushed on completely and electrodes are properly seated.

7.2 Casting Troubleshooting: Polyacrylamide Gels

Problem	Cause	Solution
1. Gel does not adhere to backing; sticks to casting tray.	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 4 weeks. Make fresh catalyst solutions daily. Use higher catalyst levels with ampholytes other than Bio-Lyte ampholytes. Degas longer if gels are polymerized exclusively with APS or TEMED. Degas for shorter time if riboflavin is one of the catalysts. Primary amine ampholytes, especially basic species, contribute to short chain polymer formation like the polymerization seen when high levels of TEMED are used. Reduce ampholyte concentration to 1%, do not degas or add polymerization inhibitor. Only use TEMED when combined with APS and riboflavin-5'-PO_4.

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Problem	Cause	Solution
	b. Polymerization time is too long, causing gel to dry onto plastic at the edges of the plate. Especially true with gels polymerized with APS.	b. 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 solution gels.
	c. Incorrect procedure used to lift plate from casting tray.	c. 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.
	d. Dirty glass plates and casting tray.	d. Wash plates and casting tray with Bio-Rad's Cleaning Concentrate, and rinse them thoroughly with deionized or distilled water.
	e. Use of wrong side of gel support film.	e. Gels must be cast on the treated (hydrophilic) side of gel support film.
	f. Use of Bio-Lyte 8/10 ampholyte.	f. Adhesion is decreased when using ampholytes with a pH ≥ 8.0. Increase ammonium persulfate to 0.7 mg/ml (7 µl of 10% APS stock per ml solution) and TEMED at 1 µl/ml.
	g. Gel support film exposed to light for extended periods.	g. Always keep gel support film sealed in its light-proof package. Store at room temperature.

7.3 Casting Troubleshooting: Agarose Gels

Problem	Cause	Solution
1. Gel does not adhere to gel support film; sticks to casting tray.	a. Use of incorrect side of gel support film.	a Always cast gels on treated (hydrophilic) surface of gel support film.
	b. Dirty glass plates and casting tray.	b. Wash plates and casting tray with Bio-Rad's Cleaning Concentrate, then rinse thoroughly with deionized or distilled water.
	c. Casting tray not equilibrated to 55 °C.	c. Allow 15-30 minutes for casting tray to equilibrate to 55 °C in oven.
	d. Agarose solution too hot or too cold.	d. For maximum adhesion, cast agarose solutions at 55 °C.

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Section 8
References

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2. Righetti, P.G. and Drysdale, J.W., Isoelectric Focusing, North-Holland Publishing Company, Amsterdam (1976).
3. Radola, B.J. Modern Methods in Protein Chemistry, 21, Walter de Gruyter & Co., New York (1983).

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Section 9
Equipment and Accessories

9.1 Equipment

Catalog Number	Description
170-2975	**Model 111 Mini IEF Cell,** includes outer chamber and interlocking lid; 2 Graphite Electrodes; gel casting tray; 5 Glass Plates; Gel Support Film for Acrylamide, 50 sheets; 5 Sample Templates; 5 ml of Bio-Lyte 3/10 ampholyte; and instructions
170-2976	**Model 111 Mini IEF Cell,** same as above, without casting tray

9.2 Accessories

Catalog Number	Description
170-2980	**Graphite Electrodes,** 2
170-2981	**Capillary Thin Layer Gel Casting Tray**
170-2982	**Glass Plates, 125 x 65 x 1.5 mm,** 5
170-2985	**Sample Templates,** 5
170-4220	**Photopolymerization Light,** 110 V
170-4242	**Photopolymerization Light,** 220 V
170-4257	**Sample Application Filter Paper,** 200 pieces

9.3 Gel Support Film

Catalog Number	Description
170-2983	**Gel Support Film for Acrylamide,** 125 x 65 mm, 50 sheets
170-2984	**Gel Support Film for Agarose,** 125 x 65 mm, 50 sheets

9.4 Isoelectric Focusing Chemicals and Reagents

Catalog Number	Description
161-0310	**IEF Standards,** pI 4.6-9.6
161-0100	**Acrylamide,** 99.9%, 100 g
161-0101	**Acrylamide,** 99.9%, 500 g
161-0107	**Acrylamide,** 99.9%, 1 kg
161-0103	**Acrylamide,** 99.9%, 2 kg
161-0108	**Acrylamide,** 99.9%, 5 kg
161-0200	**Bis (N,N'-Methylene-bis-acrylamide),** 5 g
161-0201	**Bis (N,N'-Methylene-bis-acrylamide),** 50 g
161-0501	**Riboflavin-5'-Phosphate,** 10 g
161-0700	**Ammonium Persulfate,** 10 g
161-0800	**TEMED,** 5 ml
161-0801	**TEMED,** 50 ml
162-0022	**Zero -m_r Agarose,** 10 g

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9.5 Bio-Lyte Ampholytes

Catalog Number	Description
163-1112	**Bio-Lyte 3/10 Ampholyte, 40%,** 10 ml
163-1132	**Bio-Lyte 3/5 Ampholyte, 20%,** 10 ml
163-1142	**Bio-Lyte 4/6 Ampholyte, 40%,** 10 ml
163-1152	**Bio-Lyte 5/7 Ampholyte, 40%,** 10 ml
163-1192	**Bio-Lyte 5/8 Ampholyte, 40%,** 10 ml
163-1162	**Bio-Lyte 6/8 Ampholyte, 40%,** 10 ml
163-1172	**Bio-Lyte 7/9 Ampholyte, 40%,** 10 ml
163-1182	**Bio-Lyte 8/10 Ampholyte, 20%,** 10 ml
163-1113	**Bio-Lyte 3/10 Ampholyte, 40%,** 25 ml
163-1143	**Bio-Lyte 4/6 Ampholyte, 40%,** 25 ml
163-1153	**Bio-Lyte 5/7 Ampholyte, 40%,** 25 ml
163-1193	**Bio-Lyte 5/8 Ampholyte, 40%,** 25 ml
163-1163	**Bio-Lyte 6/8 Ampholyte, 40%,** 25 ml

9.6 Stains

Catalog Number	Description
161-0400	**Coomassie Blue R-250,** 10 g
161-0406	**Coomassie Blue G-250,** 10 g
161-0417	**Crocein Scarlet,** 10 g
161-0443	**Silver Stain Kit,** includes: oxidizer concentrate, silver reagent concentrate, and developer, enough to stain approximately 48 mini IEF gels
161- 0449	**Silver Stain Plus Kit,** includes: fixative enhanced concentrate, silver complex solution, reduction moderator sloution, image development reagent, development accelerator reagent, enough to stain approximately 40 mini IEF gels

9.7 Sample Preperation

Catalog Number	Description
732-6000	**Bio Spin 6 Chromatography Column,** 10
732-6002	**Bio-Spin 6 Chromatography Column,** 25

9.8 Power Supplies

Catalog Number	Description
165-5056	**PowerPac Power Supply,** 100 VAC
165-5057	**PowerPac Power Supply,** 220 VAC
165-4710	**Model 1000/500 Programmable Power Supply,** 100/120 VAC, 50/60 Hz
165-4711	**Model 1000/500 Programmable Power Supply,** 220/240 VAC, 50/60 Hz

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