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## Section XII: Isoelectric Focusing of Proteins on Agarose Gels

### Introduction

Separation of proteins in complex mixtures for analytical resolution can be achieved by isoelectric focusing (IEF), in which proteins are separated based on their net charge (isoelectric point, or pI) in the presence of a pH gradient. Analytical focusing is carried out either in polyacrylamide gels—most recently prepared with immobilized pH gradients—or in agarose gels prepared with mobile carrier ampholytes, which form a pH gradient when subjected to electrophoresis. Separation in agarose gels is more rapid, since the pores of the agarose gel are larger than those of polyacrylamide gels.

#### Advantages

- No toxic monomer solutions are required
- Separation of proteins larger than 2,000 kDa
- Shorter staining times
- Nontacky and compressible (blottable)
- No catalysts to interfere with separation

#### Applications

- Immunofixation directly in the gel
- Crossed immunoelectrofocusing
- Direct tissue isoelectric focusing
- Preparative isoelectric focusing

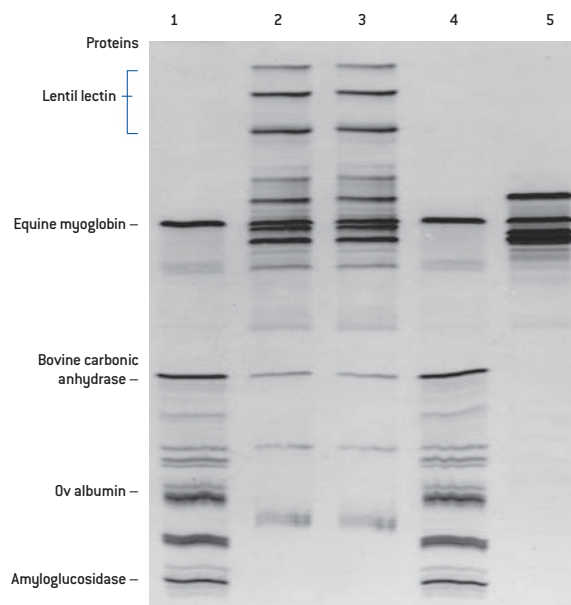
### Compatible agaroses

Isoelectric focusing on agarose gels requires the use of an agarose that has no measurable electroendosmosis (EEO). Lonza has developed two products that can be used for this application, specifically IsoGel® Agarose and IsoGel® Agarose IEF Plates.

- IsoGel® Agarose is a highly purified agarose that is easy to prepare and produces a less restrictive gel than polyacrylamide, allowing rapid focusing of high molecular weight proteins (>2,000 kDa).
- IsoGel® Agarose IEF Plates are ready-to-use 125 mm x 100 mm precast gels that eliminate gel preparation time and provide easy handling throughout IEF processing.

The photograph below demonstrates the separation performance of proteins focused on an IsoGel® Agarose IEF Plate.

Alternatively, Lonza offers IsoGel® Agarose IEF Precast Plates.



Separation of proteins in a IsoGel® Agarose IEF Plate, pH 3-10. Lanes 1 & 4: in-house pI Marker. Lanes 2 & 3: Broad Range pI 4.45-9.6 marker (BioRad). Lane 5: Hemoglobin, HB Type AFSC (PE Wallac). 2.5 µl of each sample were loaded on the gel and prefocused at 1 watt for 10 minutes and focused at 2000 volts (max), 25 mA (max), 25 W (max) for 60 minutes on a GE Healthcare MultiPhor® II chamber at 10°C. The gel was stained with Crowle's Stain.

## Section XII: Isoelectric Focusing of Proteins on Agarose Gels

### Preparation of Agarose Isoelectric Focusing Gels

#### Suggested analytes and catholytes

When selecting analytes and catholytes for any pH gradient, it is important to closely bracket the ends of the pH range of the ampholytes. Avoid creating pH discontinuities between the ends of the ampholyte range and the bracketing electrolytes.

**NOTE:** pH is dependent on temperature. 25°C pH values are provided for selection of electrolytes. Under running conditions, the pH will be slightly higher.

	Analyte	
	Concentration	pH (25°C)
Phosphoric acid	1 M	1.0
Sulfuric acid	0.2 M	1.6
Acetic acid	0.5 M	2.6
L-glutamic acid	40 mM	3.2
Indole acetic acid	3 mM	3.8
L-tyrosine	4 mM	4.5

	Catholyte	
	Concentration	pH (25°C)
Threonine	50 mM	5.8
Glycine	50 mM	6.15
Hepes	0.4 M	7.3
L-Histidine (free-base)*	40 mM	7.35
Bicine	0.1 M	8.0
Sodium hydroxide	1 M	13.0

\*Do not substitute Histidine HCl for free-base.

#### Preparation of the gel casting assembly

1. Spread a few drops of distilled water or 0.1% nonionic detergent on one glass plate.
2. Lay a sheet of GelBond® Film, cut slightly smaller than the glass plate, onto the plate with the hydrophilic side up.

**NOTE:** Water droplets spread on the hydrophilic side but bead up on the hydrophobic side of the film.

3. Cover the GelBond® Film with a sheet of blotting paper or the interleaving paper supplied with the film.
4. Firmly roll with a rubber roller or wipe with tissues to squeeze out any air bubbles and excess fluid from behind the GelBond® Film.
5. Carefully wipe off any excess liquid at the edges.
6. Place the U-frame spacer on top of the GelBond® Film. If a U-frame spacer is unavailable, place two spacers on the GelBond® Film along either side and one spacer across the bottom edge.
7. Place the second glass plate over the spacer(s).
8. Clamp the assembly with the stationery clamps, using 2 clamps per side and bottom.
9. Warm the cassette in a 55°C forced hot air oven for 15 minutes.

**NOTE:** GelBond® Film may warp if the cassette is heated too long or above 75°C.

#### Materials

- GelBond® Film (110 mm x 125 mm, Cat. No. 53745)
- Blotting paper or interleaving paper supplied with Gelbond® Film
- Two thick glass plates (110 mm x 125 mm)
- A plastic 0.8 mm-thick U-frame spacer or 3 single spacers, 0.8 mm-thick
- Six stationery binder clamps
- Blotting paper, rubber roller or tissues
- Forced hot air oven set to 55°C

#### Reagents

- Distilled water or 0.1% nonionic detergent

## Section XII: Isoelectric Focusing of Proteins on Agarose Gels

### Preparation of Agarose Isoelectric Focusing Gels — continued

The following procedure is to prepare a 10 ml IsoGel® Agarose gel.

#### Solution preparation

1. Choose a 50 ml beaker or Erlenmeyer flask.
2. Add 8 ml distilled water and a stir bar to the flask or beaker.
3. Premeasure 0.1 g IsoGel® Agarose.
4. Premeasure 1.0 g d-sorbitol.
5. Sprinkle in the premeasured agarose powder, while the solution is rapidly stirring.
6. Using a spatula, break up and disperse any agarose clumps and scrape down any powder adhered to the walls of the flask.
7. Add the d-sorbitol while the solution is rapidly stirring.
8. Remove the stir bar.
9. Follow the procedures on page 83 for dissolving agarose.
10. Cool the solution to approximately 60°C.
11. Add 0.63 ml of ampholyte solution with a 1-cc syringe.
12. Stir the solution well to mix.
13. Maintain the agarose solution at 60°C - 65°C until casting.
14. Correct for evaporation by adding warm distilled water immediately before gel casting.

#### Materials

- Erlenmeyer flask or beaker (50 ml)
- Microwave
- Boiling water bath or hot plate
- Magnetic stir plate
- Magnetic stir bar
- 1-cc tuberculin syringe
- Water bath set to 60°C
- 20-cc syringe
- Prewarmed cassette assembly
- Parafilm® or tape
- Spatula

#### Reagents

- IsoGel® Agarose (see page 56)
- Distilled water
- Ampholytes
- D-sorbitol
- Boiling distilled water
- 60°C distilled water

## Section XII: Isoelectric Focusing of Proteins on Agarose Gels

### Preparation of Agarose Isoelectric Focusing Gels — continued

#### Gel casting instructions

1. Flush a 20 cc syringe with boiling water to thoroughly heat it.
2. Expel all water from the barrel and needle.
3. Immediately fill the syringe with the warmed agarose solution.
4. Slowly inject the agarose solution into the pre-warmed cassette.

**NOTE:** Try to avoid introducing air bubbles into the cassette by injecting the solution in a slow-steady stream.

5. Fill the cassette assembly to the top with agarose solution.
6. Seal the top of the cassette with Parafilm® or tape to prevent evaporation.
7. Allow the casting assembly to cool at room temperature.
8. Place the gel at 4°C for one hour.

#### Disassembly of casting cassette

1. Remove the tape and clamps.
2. With the cassette lying flat, insert a flat spatula between the glass plates.
3. Twist gently to break the seal.
4. Carefully remove the top plate, leaving the gel and the GelBond® Film attached to the back plate.
5. Remove the spacers.
6. Lift the GelBond® Film from the back plate by inserting a flat spatula under the film.
7. Gently lift film and gel away from glass plate.

#### Sample Preparation

Successful isoelectric focusing, in part, depends upon the condition of the sample. Situations such as insolubility or high salt content, particularly in the case of high sample loading, should be addressed before the sample is loaded onto the gel. Listed below are general guidelines for sample treatment.

**High Salt Content:** Dialyze the sample against distilled water, 1% glycine, or 0.05 M - 0.1 M ammonium bicarbonate solution.

**Dissociation of protein aggregates, subunit assemblies or to unfold peptide chains:** Add urea to a final concentration of 4 M - 9 M to both the sample and the gel.

**Samples that are hydrophobic or poorly soluble at or near their pI point:**

Add either nonionic or zwitterionic detergents to the sample and the gel at a final concentration of 0.05% - 1.0%. Detergents should be added to the agarose solution once the agarose has been dissolved and cooled to 60°C.

Nonionic detergents	Zwitterionic detergents
Triton® X-100	CHAPS (available from Sigma Chemical Co., St. Louis, MO)
Nonidet® (NP-40)	Zwittergent® 3-14 (available from Calbiochem/Behring, LaJolla, CA)
Tween® 80	

## Section XII: Isoelectric Focusing of Proteins on Agarose Gels

### Running Agarose IEF Gels

**Caution:** Make certain the power supply is turned off before proceeding.

#### Procedure for gel placement

1. Set the refrigerated circulator bath to 10°C - 15°C.

**NOTE:** To prevent condensation on the gel and platen, do not circulate the coolant to the IEF chamber just prior to focusing.

2. Spread 0.2 ml - 0.3 ml of distilled water on the cooling platen of the IEF chamber.
3. Lower the gel (film-side down) onto the wetted area. Avoid trapping air under the GelBond® Film.
4. Wipe excess fluid from the edges of the film.
5. Blot the surface of the gel briefly with a sheet of fine-grained blotting paper.
6. If necessary, trim the edges of the gel parallel to the direction of focusing with a razor blade to ensure the edges are even and free of cracks or small tears.

#### Procedure for electrode wick application

1. Cut two electrode wicks to the exact width of the gel or slightly shorter.
2. Completely immerse one wick in catholyte solution.
3. Place the wick on blotting paper to remove excess fluid.
4. Place on the negative electrode contact of the gel.
5. Completely immerse a second wick in anolyte solution.
6. Place the wick on blotting paper to remove excess fluid.
7. Place on the positive electrode contact of the gel.

**NOTE:** The wicks must lie parallel to each other on the ends of the gel, evenly touching the surface.

8. Place a glass plate on top of the gel and wicks for approximately one minute.

**NOTE:** This ensures uniformity of contact between wicks and gel and serves to smooth the wick surface in preparation for electrode contact.

#### Materials

- Horizontal IEF chamber
- Fine-grained blotting paper
- Razor blade
- Refrigerated circulator bath set to 10°C - 15°C
- Kimwipe® Tissues or equivalent
- Electrode wicks
- Scissors
- Forceps
- Glass plate slightly larger than the gel
- Sample applicator mask
- Power supply

#### Reagents

- Distilled water
- Catholyte solution
- Anolyte solution

#### Procedure for sample application

1. Place the sample applicator mask across the gel at least 1 cm from either wick (e.g., 3 cm from cathode).
2. Load sample and pl markers into the slots (2 µl - 5 µl maximum; 2 - 10 mg/ml concentration).

**NOTE:** In direct tissue isofocusing, tissue samples may be placed directly onto the applicator slots.

3. Ensure electrodes and electrical contacts are clean and there are no breaks in the wire or ribbon.
4. Place the electrodes on the wicks (not the gel surface), aligning them so they lie in parallel upon the wicks.
5. Set the power supply at 1 W constant power.
6. Apply power for 10 minutes.
7. Turn power off.
8. Remove the sample applicator mask.
9. Gently remove any precipitated sample from the gel surface with blotting paper.

## Section XII: Isoelectric Focusing of Proteins on Agarose Gels

Running Agarose IEF Gels — continued

### IEF power settings and focusing time

1. Start circulation of the coolant to the IEF chamber.
2. Set the voltage, current and power according to the appropriate running conditions listed in the table below.
3. Once focusing is complete, turn off the power.
4. Discard the wicks.
5. Place the gel in fixative solution.

**NOTE:** The separation progress can be monitored by observing the visible proteins in the pI markers coming into focus and noting the decreasing rate of current flow on the power supply's milliampere indicator. Focusing is attained when the visible pI markers are sharply resolved and the current has stopped decreasing significantly (less than 1 mA in 10 minutes).

### Running Conditions

Ampholyte	Voltage pH Range	Current (limiting)	Power	Anolyte (upper limiting)	Catholyte	Focusing Time (minutes)
2.5 - 4.5	250 V	MAX	10 W	*1 M H <sub>3</sub> PO <sub>4</sub>	0.05 M Threonine	90
3.5 - 9.5	500 V	MAX	10 W	0.5 M HOAc	1 M NaOH	90
3.5 - 9.5	1000 V	MAX	25 W	0.5 M HOAc	1 M NaOH	40
3.5 - 9.5	1500 V	MAX	25 W	0.5 M HOAc	1 M NaOH	30
4 - 6.5	500 V	MAX	10 W	2% solution of pH 2.5 - 4.5 Ampholyte	0.04 M L- Histidine (free-base)	90
5 - 8	500 V	MAX	10 W	2% solution of pH 2.5 - 4.5 Ampholyte	0.1 M NaOH or 0.1 M Bicine	90

\*1M H<sub>3</sub>PO<sub>4</sub> can be replaced by 0.5 M acetic acid

## Section XII: Isoelectric Focusing of Proteins on Agarose Gels

### Staining Proteins in Agarose IEF Gels

#### Introduction

Either Coomassie® Blue or Crowle's Double Stain can be used to stain IEF gels. Coomassie® stain is used when increased sensitivity is desired, and Crowle's stain produces gels with clear background and sharp resolution.

#### Staining proteins with Coomassie® Blue Stain or Crowle's Double Stain

##### Preparation of staining solutions

Fixative solution	
180 ml	Methanol
30.0 g	Trichloroacetic acid
18.0 g	Sulfosalicylic acid
Adjust volume to 500 ml with distilled water	
Coomassie® Stain	
1.0 g	Coomassie® Brilliant Blue R-250
250 ml	Ethanol
90 ml	Glacial acetic acid
Adjust volume to 1 liter with distilled water	
Coomassie® Destaining Solution	
250 ml	Ethanol
90 ml	Glacial acetic acid
Adjust volume to 1 liter with distilled water	
Crowle's Double Stain	
2.5 g	Crocein scarlet (C.I. 26905)
150.0 mg	Coomassie® Brilliant Blue R-250
50 ml	Glacial acetic acid
30.0 g	Trichloroacetic acid
Adjust volume to 1 liter with distilled water	
Crowle's Destaining Solution	
3 ml	Glacial acetic acid
Adjust volume to 1 liter with distilled water	

#### Materials

- Flask
- Stir bar
- Magnetic stir plate
- Forceps
- Paper towel
- Whatman® 3MM chromatography paper
- 1 kg-2 kg weight
- Glass plate
- Forced hot air oven set to 50°C-55°C
- Staining vessel
- Clamps

#### Reagents

- Methanol
- Trichloroacetic acid
- Sulfosalicylic acid
- Distilled water
- Coomassie® Brilliant Blue R-250
- Ethanol
- Glacial acetic acid
- Crocein scarlet (C.I. 26905)



## Section XII: Isoelectric Focusing of Proteins on Agarose Gels

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### Staining Proteins in Agarose IEF Gels — Continued

Follow the steps below to stain the gel after electrophoresis using either Coomassie® Blue or Crowle's Double Dtain.

1. Place the gel in Fixative solution for 10 minutes.
2. Remove the gel and rinse the surface with distilled water.
3. Drain excess water.
4. Place on a paper towel, gel side up.
5. Pre-wet a piece of Whatman® 3MM chromatography paper with distilled water.
6. Place on gel surface.
7. Overlay the blotting paper with four to six layers of absorbent paper toweling.
8. Place a glass plate on top of the paper toweling.
9. Place a 1 kg - 2 kg weight on top of the toweling for 10 minutes.
10. Remove the weight, the glass plate, and the paper toweling.
11. Rewet the blotting paper thoroughly with distilled water.
12. Gently lift the blotting paper off gel surface.
13. Wash the gel in distilled water for 5 minutes to remove residual fixative and ampholytes.
14. Dry the gel completely in a forced hot air oven (50°C - 55°C).

**NOTE:** Drying usually takes less than 30 minutes.

15. Stain with Coomassie® or Crowle's double stain solution for 15 - 30 minutes without agitation.

**NOTE:** Float gel-face down into the stain, so precipitated stain will not settle on the gel surface.

16. Remove the gel and rinse with distilled water.
17. Place the gel in Destaining solution for 3 minutes.
18. Briefly rinse again in distilled water.
19. Clamp (gel side out), onto a glass plate to prevent curling during drying.
20. Dry the gel in a forced hot air oven (50°C - 55°C) for approximately 15 minutes or dry at room temperature overnight.

**NOTE:** Gel may crack if over dried by heating.

## Section XII: Isoelectric Focusing of Proteins on Agarose Gels

## Staining Proteins in Agarose IEF Gels — continued

**Staining Proteins in Agarose IEF Gels with Silver Stain**

A modified silver stain procedure has been developed for use with agarose gels cast on GelBond® Film. After electrophoresis, the gels are fixed, press blotted, and completely dried before staining. Perform all fixing and staining steps in acid-cleaned (50% HNO<sub>3</sub>) glassware. All washes are done with constant agitation in a volume of at least 250 ml (gel volume:reagent volume = 1:22). Coomassie® Brilliant Blue stained gels may be silver stained after drying. In this case, proceed directly to step 14.

**Preparation of staining solutions**

Fixative solution	
180 ml	Methanol
30.0 g	Trichloroacetic acid
18.0 g	Sulfosalicylic acid
Adjust volume to 500 ml with distilled water	
Silver Stain Solution A	
50.0 g	Sodium carbonate, anhydrous in 1 liter distilled water (Stable for 2 - 3 weeks at room temperature)
Silver Stain Solution B	
In the order given dissolve the following reagents in 1 liter of distilled water, while mixing rapidly.	
2.0 g	Ammonium nitrate
2.0 g	Silver nitrate
10.0 g	Dodeca-tungstosilicic acid
6.7 ml	37% formaldehyde (Stable 1 week at room temperature stored in the dark)
Stop solution	
1%	Acetic acid

**Materials**

- Paper towel
- Whatman® 3MM chromatography paper
- 1 kg-2 kg weight
- Glass plate
- Forceps
- Clamps
- Forced hot air oven set to 50°C-55°C
- Staining containers
- Beakers
- Magnetic stir plate
- Magnetic stir bar
- Acid-cleaned dish
- Orbital or rocking platform shaker

**Reagents**

- Methanol
- Trichloroacetic acid
- Sulfosalicylic acid
- Distilled water
- Glutaraldehyde
- Dithiothreitol (DTT)
- Anhydrous sodium carbonate
- Ammonium nitrate
- Silver nitrate
- Dodeca-tungstosilicic acid
  - (Gallard-Schlesinger Cat. No. 305453)
- 37% formaldehyde
- Acetic acid

**Caution:** Materials and methods shown here present hazards to the user and the environment. Refer to the safety information on page 210 before beginning these procedures.

**Caution:** Acetic acid causes burns and respiratory irritation. Take precautions to prevent exposure.

**Caution:** Glutaraldehyde is toxic and must be handled in a fume hood.

## Section XII: Isoelectric Focusing of Proteins on Agarose Gels

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### Staining Proteins in Agarose IEF Gels — continued

Follow the steps below to stain the gel after electrophoresis using silver stain

1. Place the gel in Fixative solution for 10 minutes. **If gel is prestained with Coomassie® Blue and dried, proceed to step 14.**
2. Place the gel on a paper towel, gel side up.
3. Pre-wet a sheet of Whatman® 3MM chromatography paper with distilled water.
4. Place on gel surface.
5. Overlay the blotting paper with four to six layers of absorbent paper toweling.
6. Place a glass plate on top of the paper toweling.
7. Place a 1 kg - 2 kg weight on top of the toweling for 10 minutes.
8. Remove the weight, the glass plate, and the paper toweling.
9. Rewet the blotting paper thoroughly with distilled water.
10. Gently lift the blotting paper off the gel surface.
11. Wash the gel in distilled water for 5 minutes to remove residual fixative and ampholytes.
12. Clamp (gel side out), onto a glass plate to prevent curling during drying.
13. Dry the gel in a forced hot air oven (50°C - 55°C) for approximately 15 minutes or until dry.
22. Rinse the gel in distilled water.
23. Wipe any silver deposits from the back of the film.
24. Allow to air dry.

**NOTE:** Gel may crack if over dried by heating.

14. Soak the dried gel in 2% glutaraldehyde for 10 minutes.
15. Wash in distilled water for 10 minutes using mild agitation.
16. Soak the gel for 10 minutes in 0.01% (DTT) dithiothreitol.
17. Wash in distilled water for 10 minutes using mild agitation.
18. Pour an equal volume of Silver stain solution B into vigorously stirring Silver stain solution A (75 ml B and 75 ml A for each gel to be stained).
19. Transfer the solution to an acid-cleaned glass dish containing one gel.
20. Stain the gel for 10 minutes with gentle agitation.

**NOTE:** There will be some background.

21. Transfer the gel to a Stop solution and gently agitate for 5 minutes.

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### Press Blot Transfer of Proteins from Agarose IEF Gels

Press blot transfer is a quick method of removing proteins from agarose gels. The procedure involves overlaying the gel with a buffer-soaked nitrocellulose membrane covered by a thick filter pad and several layers of dry paper toweling. The assembly is then covered by a glass plate. After just 1½ minutes of press blot, approximately 20% of the proteins are transferred from the gel to the membrane. Up to 85% transfer can be achieved with a 35 - 40 minute blotting time. Transferred proteins can be detected on the membrane and on the gel by standard methods.

#### Procedure

1. Prepare Tris-saline buffer, pH 7.5.
2. Cut one piece of nitrocellulose membrane and thick filter paper to the same dimension as the gel.

**NOTE:** Wear gloves to prevent contamination by extraneous proteins.

3. Evenly wet the nitrocellulose membrane in the Tris-saline buffer by holding one end of the membrane with smooth-tipped forceps and lowering the other end into the buffer container, dropping the membrane flat on the buffer surface.

**NOTE:** The membrane must be completely saturated with buffer.

4. Remove the gel from the focusing chamber.
5. Place on a flat surface, gel side up.
6. Place the buffer-soaked nitrocellulose membrane onto the gel surface.

**NOTE:** Avoid trapping air bubbles between the gel and the membrane.

7. Place one piece of buffer soaked filter paper on top of the membrane.
8. Place three layers of dry paper toweling on top of the filter paper.
9. Cover with a glass plate slightly larger than the gel surface. No other weight is required.
10. Press blot for 1½ minutes or longer, as desired.
11. Remove glass plate and discard the paper toweling and filter paper.

#### Materials

- Nitrocellulose membrane
- Thick filter paper
- Scissors
- Smooth-tipped forceps
- Container
- Paper towels
- Glass plate

#### Reagents

- Tris-saline buffer, pH 7.5
  - (50.0 g Tris-HCl, 0.94 g Tris-Base, 58.48 g NaCl adjust to 2 liters with distilled water)

## Section XII: Isoelectric Focusing of Proteins on Agarose Gels

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### Preparative Isoelectric Focusing

Separation of relatively large amounts of biologically active macromolecules is possible by isoelectric focusing in agarose. Typical high-yield recoveries of applied proteins are obtainable with retention of biological activity. This preparative isoelectric focusing procedure is based on the work of Cantarow, *et al.* As much as 120.0 mg of protein can be focused in 9.5 ml (0.75 x 10 x 11.5 cm) of 1% IsoGel® Agarose containing 2.5% ampholytes.

#### Procedure

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1. Follow steps 1 - 14 for Gel Preparation on pages 179-181. Also see Sample Preparation on page 181.
2. Add the protein sample to the agarose solution once cooled to 60°C.
3. Stir the solution well to mix.
4. Cast the gel and disassemble the casting cassette following the steps previously described on page 179.
5. Place the gel on the 10° C cooling platen for 10 minutes (if gel has not already been chilled after casting).
6. Focus following the steps previously described on pages 182-183.

#### Procedure for recovering proteins from preparative gel

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1. Excise the gel slice containing the protein of interest by using a spatula to strip the agarose from the GelBond® Film in 5 mm-wide slices.
2. Place the agarose strip in a 5-cc plastic syringe fitted with an 18-gauge needle.
3. Macerate the gel slice by expelling it into a clean tube.
4. Add 4 ml of phosphate-buffered saline (PBS) to the macerated gel.
5. Cover the tube securely with Parafilm®
6. Place on a test tube rocker for 16 hours at 4°C.
7. Centrifuge the tube for one minute at 100 rpm.
8. Separate the supernatant from the gel using a serum separator.

#### ■ Materials

- Spatula
- 5-cc plastic syringe with an 18-gauge needle
- Clean centrifuge tube
- Test tube rocker at 4°C
- Centrifuge
- Serum separator
- Parafilm®

#### ■ Reagent

- Phosphate-buffered saline (PBS)

## Section XII: Isoelectric Focusing of Proteins on Agarose Gels

### Immunofixation/Immunoperoxidase or Autoradiography

Detection of separate species can be accomplished by protein stains or by overlaying gels with specific antibody solutions coupled to enzymes that will eventually produce a visible end product. The antibody-peroxidase conjugate system or autoradiography with <sup>125</sup>I- labeled antibody are frequently used for this purpose. Immunoperoxidase labeling of focused proteins is performed according to Saravis, *et al.* After focusing, fixing, and drying the gel, treat the gel as described below.

#### Procedure for Immunoperoxidase Staining/Avidin-Biotin Modification

1. Soak the gel in 3% hydrogen peroxide for 10 minutes.
2. Rinse with distilled water.
3. Soak the gel in 2.28% periodic acid for 5 minutes.
4. Rinse with distilled water.
5. Soak the gel in 0.02% sodium borohydride for 2 minutes.
6. Rinse with distilled water.
7. Place the gel in 0.05 M Tris-saline, pH 7.6 for 10 minutes.
8. Incubate the gel with 1:5 normal serum for 10 minutes (same species as secondary antibody).
9. Incubate the gel for 2 - 4 hours at room temperature with Anti-serum (primary antibody).
10. Rinse the gel with 0.15 M PBS, pH 7.4 for 1 hour at room temperature.
11. Incubate the gel with Secondary\* antibody (e.g., goat anti-mouse IgG) for 2 - 4 hours at room temperature.
12. Rinse the gel with 0.15 M PBS for 1 hour at room temperature.
13. Incubate the gel with avidin solution (40 mg/gel) in PBS for 1 hour at room temperature (stock solution of avidin is usually 20 mg/ml).
14. Rinse the gel with 0.15 M PBS for 1 hour at room temperature.
15. Incubate the gel with horseradish peroxidase\* (33 mg/gel) for 2 hours at room temperature.
16. Rinse the gel with 0.15 M PBS for 1 hour at room temperature.
17. Incubate the gel with diaminobenzidine (0.15 mg/ml) and hydrogen peroxide (0.03%) in 0.01 M Tris 0.15 M NaCl for 1 - 17 hours at room temperature.
18. Rinse the gel in PBS and dry.

#### ■ Materials

- Staining containers

#### ■ Reagents

- Hydrogen peroxide
- Distilled water
- Periodic acid
- Sodium borohydride
- 0.05 M Tris-saline (pH 7.6), normal serum (same species as secondary antibody)
- Anti-serum (primary antibody)
- 0.15 M PBS (pH 7.4)
- Secondary antibody
- 20 mg/ml avidin solution
- Horseradish-peroxidase
- 0.15 mg/ml diaminobenzidine
- 0.01 M Tris/0.15 M NaCl

**NOTE:** If the avidin/biotin modification is used, proceed with steps 12 and 13 using biotinylated reagents (marked with \* in steps 11 and 15).

## Section XII: Isoelectric Focusing of Proteins on Agarose Gels

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### Direct Tissue Isoelectric Focusing (DTIF)

This method employs the application of tissue or cell pellets directly onto the surface of the IEF gel without concentration of samples, dialysis to remove salts, or the salt extraction of soluble proteins from tissue. DTIF allows more soluble protein per milligram of tissue to enter the gel than is recoverable by extraction procedures and minimizes denaturation of biologically active proteins that can be damaged by extraction.

1. Prepare solutions and cast gels as previously described on pages 179-181.
2. Prepare for focusing as previously described on page 182.
3. Place the sample applicator mask across the gel at least 1 cm from either wick (e.g., 3 cm from cathode).
4. Drape a tissue slice over the open slot of the applicator mask.
5. Ensure the electrodes and the electrical contacts are clean and there are no breaks in the wire or ribbon.
6. Place the electrodes on the wicks (not the gel surface), aligning them so they are in parallel upon the wicks.
7. Set power supply at 1 W (constant power).
8. Prefocus for 10 - 15 minutes to allow sample uptake.
9. Turn power supply off.
10. Remove the tissue slice and the applicator mask.
11. Focusing is continued using standard conditions.
12. After focusing is complete, the gel is fixed, stained and dried following standard procedures.

## Section XII: Isoelectric Focusing of Proteins on Agarose Gels






### Resolution Reference Guide

Phenomenon	IEF Band Appearance	Possible Causes	Remedy
Streaks or gaps perpendicular to bands		<ul style="list-style-type: none"> <li>• Particulates in sample</li> <li>• Old or denatured sample</li> <li>• Isoelectric precipitation of sample applied too close to pI point</li> <li>• Poor soluble sample, which precipitates</li> </ul>	<ul style="list-style-type: none"> <li>• Filter or centrifuge sample before applying.</li> <li>• Replace sample.</li> <li>• Do not refocus gel before applying sample.</li> <li>• Apply sample in different location.</li> <li>• Use solubilizing additive, i.e., non-ionic, Zwitterionic</li> <li>• Try different application location.</li> </ul>
Fuzzy bands		<ul style="list-style-type: none"> <li>• Run incomplete</li> <li>• Focusing time too long</li> <li>• Insufficient fixation</li> </ul>	<ul style="list-style-type: none"> <li>• Use pI marker to monitor run or measure pH gradient before removing gel from platen.</li> <li>• Decrease focusing time or power/voltage.</li> <li>• Increase fixation time. Be sure to place gel in fixative immediately after IEF run.</li> </ul>
Skewed bands		<ul style="list-style-type: none"> <li>• Electrode not clean</li> <li>• Uneven electrode contact</li> <li>• Sample placed too close to edge of gel</li> <li>• Electrode wicks too short</li> <li>• Old or incorrect electrolyte solution</li> <li>• Overloading of sample</li> <li>• Excessive salt in sample</li> </ul>	<ul style="list-style-type: none"> <li>• Rinse electrode with distilled water, then dry.</li> <li>• Wicks should be evenly wetted and placed on gel parallel to each other.</li> <li>• Place electrodes firmly on wicks but not so fluid is squeezed from wicks.</li> <li>• Apply <math>\geq 5</math> mm from edge of gel.</li> <li>• Wicks should extend to edge of gel.</li> <li>• Make fresh solution.</li> <li>• Decrease protein load.</li> <li>• Reduce salt concentration by gel filtration or dialyze against 1% glycine.</li> </ul>
Missing or faint bands		<ul style="list-style-type: none"> <li>• Protein not denatured by fixative</li> <li>• Sample unstable at pH of site of application</li> </ul>	<ul style="list-style-type: none"> <li>• Establish appropriate denaturing conditions.</li> <li>• Use alternative application site.</li> </ul>
Wavy bands		<ul style="list-style-type: none"> <li>• Excessive salt in sample</li> <li>• Overloading with sample, distorting pH gradient</li> <li>• Improper analyte used</li> <li>• Old or incorrect electrolyte solutions</li> <li>• Dirty electrodes</li> </ul>	<ul style="list-style-type: none"> <li>• Reduce salt concentration by gel filtration or dialyze against 1% glycine.</li> <li>• Applying smaller sample volume.</li> <li>• Replace analyte.</li> <li>• Make fresh solutions.</li> <li>• Rinse electrode with distilled water.</li> </ul>
Arc-shaped bands		<ul style="list-style-type: none"> <li>• Thin spot in gel</li> </ul>	<ul style="list-style-type: none"> <li>• Reduce evaporation by keeping gel sealed until ready for use.</li> <li>• Keep chamber lid closed except when handling gel.</li> </ul>







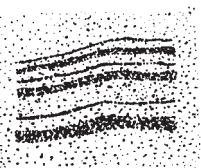
## Section XII: Isoelectric Focusing of Proteins on Agarose Gels

Resolution Reference Guide — continued

Phenomenon	IEF Band Appearance	Possible Causes	Remedy
Burning or sparking		<ul style="list-style-type: none"> <li>• Electrode wicks too dry</li> <li>• Focusing too long</li> <li>• Thin spot in gel</li> <li>• Using incorrect electrolyte solutions</li> <li>• Excessive power input</li> </ul>	<ul style="list-style-type: none"> <li>• Anodal wick should be wet not dripping wet; cathodal wick should be damp.</li> <li>• Reduce focusing time to minimum required to obtain linear pH gradient.</li> <li>• Keep gel sealed until ready for use.</li> <li>• Always use recommended solutions.</li> <li>• Check power supply settings.</li> </ul>
Sparking along edge of gel onto cooling plate		<ul style="list-style-type: none"> <li>• Excess moisture on the gel, or under gel</li> <li>• Electrode strips overhanging ends of gel</li> <li>• Liquid expelled at sides of electrode due to EEO flow of water to cathode</li> <li>• Excess electrode pressure when placing electrodes on gel</li> </ul>	<ul style="list-style-type: none"> <li>• Remove excess moisture by gently blotting gel.</li> <li>• Cut electrode stripes to size of gel.</li> <li>• Occasional blotting may be necessary.</li> <li>• Use firm but light pressure.</li> </ul>
Formation of 'ditch' ultimately causing gel to burn out		<ul style="list-style-type: none"> <li>• Gradient drift</li> <li>• High current causing excessive heating early in run</li> <li>• Uneven gel thickness due to gel drying out</li> <li>• Overblotting of gel, causing gel to dry out</li> </ul>	<ul style="list-style-type: none"> <li>• Check purity or reagents.</li> <li>• Use a low power for first 10-15 minutes of run during sample uptake.</li> <li>• Open foil package containing gel just before use.</li> <li>• Initially blot only until blotting paper picks up moisture.</li> </ul>
Sample smearing or precipitation		<ul style="list-style-type: none"> <li>• Sample overload</li> <li>• Sample aggregation</li> <li>• Sample applied too near pI or at a pH where it is insoluble</li> </ul>	<ul style="list-style-type: none"> <li>• Decrease sample concentration.</li> <li>• Solubilize sample with neutral surfactant or 1% glycine.</li> <li>• Change application site. Solubilizing additives or detergents may be necessary.</li> </ul>
Incomplete Sample Uptake		<ul style="list-style-type: none"> <li>• Sample application mask not left on long enough</li> <li>• Insolubles or aggregates in sample</li> <li>• Get too wet when sample applied</li> </ul>	<ul style="list-style-type: none"> <li>• Allow at least 10 minutes.</li> <li>• Filter or centrifuge sample.</li> <li>• Blot gel before sample application.</li> </ul>
Sample retained in sample application well		<ul style="list-style-type: none"> <li>• Sample applied too near pI</li> </ul>	<ul style="list-style-type: none"> <li>• Change application site.</li> </ul>
Fluid expression		<ul style="list-style-type: none"> <li>• At Cathode</li> <li>• Localized elsewhere</li> </ul>	<ul style="list-style-type: none"> <li>• Blot cathode wick with filter paper until damp before application.</li> <li>• Monitor run with pI markers.</li> <li>• Detach sample before electrofocusing.</li> <li>• Decrease sample concentration.</li> <li>• Check location of electrodes.</li> <li>• Remove excess moisture. Wicks must be blotted before use.</li> </ul>
• General (over entire gel)		<ul style="list-style-type: none"> <li>• Unblotted wick</li> <li>• Overfocusing</li> <li>• Salt in sample</li> <li>• Sample overload</li> <li>• Uneven electrode contact to wicks</li> <li>• Electrode wicks too wet</li> <li>• Local hot spot from spillage of electrode solution onto gel</li> <li>• Insufficient/excessive cooling</li> <li>• Excessive power applied to gel</li> <li>• Gel not adequately blotted</li> <li>• High ambient humidity</li> <li>• Cathodal drift (pooling of fluid near cathode—over saturating wick)</li> </ul>	<ul style="list-style-type: none"> <li>• Avoid this area for sample application.</li> <li>• Check coolant flow and temperature.</li> <li>• Check power supply settings.</li> <li>• Use blotting paper.</li> <li>• Use tray with desiccant in chamber.</li> <li>• Blot pooled liquid if necessary.</li> </ul>
• Excessive amount along electrode wicks. Persists for duration of run		<ul style="list-style-type: none"> <li>• Reversed polarity of electrode wicks (lower pH at cathode; higher at anode)</li> <li>• Localized hot spots caused by bubbles between gel film and chamber platen</li> </ul>	<ul style="list-style-type: none"> <li>• Check pH of wicks and polarity of plugs into power supply. Reverse polarity if incorrect.</li> <li>• Check for air bubbles—remove if present.</li> </ul>
• Spotted over gel			

## Section XII: Isoelectric Focusing of Proteins on Agarose Gels

Resolution Reference Guide — continued

Phenomenon	IEF Band Appearance	Possible Causes	Remedy
Condensation inside chamber		<ul style="list-style-type: none"> <li>Inefficient cooling</li> <li>Voltage/power setting too high</li> </ul>	<ul style="list-style-type: none"> <li>Adjust coolant temp. to 10° C.</li> <li>Adjust to 1W for 1st 10 min. 25W limiting thereafter (pH 3-10 e.g.).</li> </ul>
Get thinning <ul style="list-style-type: none"> <li>at electrode</li> <li>near of point of application</li> <li>generalized</li> </ul>		<ul style="list-style-type: none"> <li>Improper electrolyte solution</li> <li>Sample overload</li> <li>Get left out of cassette too long under low humidity conditions</li> </ul>	<ul style="list-style-type: none"> <li>Check electrolyte solution.</li> <li>Decrease sample concentration.</li> <li>Leave gel in cassette until ready to use or place in humidity chamber.</li> </ul>
Uneven pH gradient/pH gradient different than stated for ampholyte used		<ul style="list-style-type: none"> <li>Incorrect calibration of pH electrode</li> <li>Get overfocused—excessive gradient drift</li> <li>Impure distilled water in sample or electrolyte solutions</li> <li>Incorrect electrolyte solution</li> </ul>	<ul style="list-style-type: none"> <li>Check pH electrode.</li> <li>Do not exceed time required to form a linear pH gradient.</li> <li>Check water for heavy metals or ionic contaminants.</li> <li>Check solution.</li> </ul>
pH gradient is not linear		<ul style="list-style-type: none"> <li>Insufficient run time</li> </ul>	<ul style="list-style-type: none"> <li>Use IsoGel® pl markers to check progress. Measure pH gradient before removing gel.</li> </ul>
pH gradient not attaining upper limits		<ul style="list-style-type: none"> <li>Overfocusing</li> <li>pH measurements may be wrong</li> </ul>	<ul style="list-style-type: none"> <li>Decrease runtime.</li> <li>Use surface pH electrode.</li> </ul>
pH range shifted toward cathode		<ul style="list-style-type: none"> <li>Cathodal drift (entire gradient shifts towards cathode)</li> </ul>	<ul style="list-style-type: none"> <li>Reduce run time (as much as possible) to attain desired gradient profile.</li> </ul>
Current increases with time		<ul style="list-style-type: none"> <li>Electrodes applied at the wrong ends of the gel</li> </ul>	<ul style="list-style-type: none"> <li>Use recommended anolyte/catholyte solution.</li> <li>Apply (+) electrode to anode. (-) electrode to cathode.</li> <li>Reverse polarity</li> </ul>
Diagonal band migration		<ul style="list-style-type: none"> <li>Uneven electrode contact</li> <li>Too much pressure on electrodes</li> <li>Uneven electrode placement</li> </ul>	<ul style="list-style-type: none"> <li>Apply electrodes evenly on wicks.</li> <li>Adjust for light but firm contact.</li> <li>Ensure electrodes are parallel and perpendicular to the sample migration.</li> </ul>
Lateral band migration		<ul style="list-style-type: none"> <li>Air pocket (s) trapped beneath mask</li> <li>Too much sample applied to applicator mask</li> <li>Sample mask left on too long/ not long enough</li> </ul>	<ul style="list-style-type: none"> <li>Press gently to expel before sample applied.</li> <li>Reduce sample volume, increase sample concentration.</li> <li>Remove mask after 10 minutes at 1 watt.</li> </ul>
Sample "focuses" at wrong position		<ul style="list-style-type: none"> <li>Run incomplete</li> <li>Sample unstable at pH of application</li> <li>Removal of ligands during focusing</li> <li>Formation of complex aggregate</li> </ul>	<ul style="list-style-type: none"> <li>Allow longer focusing time.</li> <li>Use alternative application site.</li> <li>Check protein for this possible characteristic.</li> <li>Use urea to treat sample.</li> </ul>
A supposedly "pure" sample focuses as multiple bands		<ul style="list-style-type: none"> <li>Sample exists in various states of oxidation</li> <li>Sample disassociates into subunits</li> <li>Sample has become denatured</li> </ul>	<ul style="list-style-type: none"> <li>Pretreat sample accordingly.</li> <li>Natural phenomenon.</li> <li>Check procedures used to prepare sample.</li> </ul>
High background stain		<ul style="list-style-type: none"> <li>Residual ampholytes in gel</li> <li>Concentration of stain too high</li> <li>Stain is old</li> <li>Gel allowed to settle in bottom of staining dish</li> </ul>	<ul style="list-style-type: none"> <li>Follow recommended press blot procedure.</li> <li>Extend time of fixing, rinsing and destaining.</li> <li>Reduce concentration.</li> <li>Make fresh stain and be sure all dye is in solution.</li> <li>Float gel side down in stain for 15-30 minutes.</li> </ul>
Delamination (peeling) of gel from film support on staining after drying		<ul style="list-style-type: none"> <li>Residual fixative in dried gel</li> </ul>	<ul style="list-style-type: none"> <li>Follow recommended press blot/rinsing procedure.</li> </ul>

### References

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