SIGMA ELECTROPHORESIS™

ISOELECTRIC FOCUSING MARKER KITS
(Stock Nos. IEF-M1A & IEF-M2)

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INTRODUCTION

Isoelectric Focusing (IEF) is a highly sensitive electrophoretic technique which separates proteins according to their Isoelectric Points (pI). IEF provides a degree of resolution not attainable by most other charge separation procedures (e.g. electrophoresis, ion exchange chromatography). This high degree of resolution may also be attained on a preparative scale.

In order to fully utilize IEF, a method of pI calibration is necessary. Most calibration techniques are tedious (e.g. gel sectioning) or expensive (e.g. use of a pH surface electrode), and often are not reproducible. However, by using a series of markers with well-defined pIs, a standard curve can be easily obtained.

Sigma Isoelectric Focusing Kits (Stock Nos. IEF-M1A and IEF-M2) consist of a series of vials, each containing a purified protein with a well-defined isoelectric point; a vial containing a mixture of the proteins; and a separate vial containing a marker dye with a well-defined pI. The protein mixture forms evenly-spaced, distinct bands over the designated pH range. To ensure stability, the markers are lyophilized and stored below 0^{0}C.

Sigma's Isoelectric Focusing Kits are satisfactory for use on a flat bed apparatus with two commonly used brands of ampholytes-Ampholine® (Righetti, 1975) and Pharmalytes® (Soderberg et al., 1971). The markers are not suitable for use in gels containing urea. Preliminary trials may be necessary for use with other systems.

KIT FOR ISOELECTRIC FOCUSING RANGE 3.6-9.3 (Stock No. IEF-M1A)

Kit contains one vial each of the eight proteins below, one vial of Methyl Red pI marker 3.8 and one vial of IEF Mix 3.6-9.3. Store below 0°C.

	pI(20-24°C)	Product No.
Amyloglucosidase from *Aspergillus niger*	3.6	A 2910
Trypsin Inhibitor from Soybean	4.6	T 1021
ß-Lactoglobulin A from Bovine Milk	5.1	L 5137
Carbonic Anhydrase II from Bovine Erythrocytes	5.9	C 6403
Carbonic Anhydrase I from Human Erythrocytes	6.6	C 6653
Myoglobin from Horse Heart	6.8	M 9267
	7.2	
Lectin from *Lens culinaris*	8.2	L 1277
	8.6	
	8.8	
Trypsinogen from Bovine Pancreas	9.3	T 1146
Methyl Red (Marker Dye)	3.8	M 5768
IEF Mix 3.6-9.3	3.6-9.3	I 3018

*NOTE: A second trypsinogen band (pI approximately 9.1) may appear on focusing.

KIT FOR ISOELECTRIC FOCUSING RANGE 3.6-6.6 (Stock No. IEF-M2)

Kit contains one vial each of the seven proteins below, one vial of Methyl Red pI marker 3.8 and one vial of IEF Mix 3.6-6.6. Store below 0°C.

	pI(20-24°C)	Product No
Amyloglucosidase from *Aspergillus niger*	3.6	A 2910
Glucose Oxidase from *Aspergillus niger*	4.2	G 7146
Trypsin Inhibitor from Soybean	4.6	T 1021
ß-Lactoglobulin A from Bovine Milk	5.1	L 5137
Carbonic Anhydrase II from Bovine Erythrocytes	5.4	C 3666
Carbonic Anhydrase II from Bovine Erythrocytes	5.9	C 6403
Carbonic Anhydrase I from Human Erythrocytes	6.6	C 6653
Methyl Red (Marker Dye)	3.8	M 5768
IEF Mix 3.6-6.6	3.6-6.6	I 8012

REAGENTS REQUIRED BUT NOT PROVIDED:

Item	Product No.
Fixing Solution	F 7264
Brilliant Blue G Concentrate	B 8522
Methanol	M 3641
Acetic Acid, Glacial	A 6283
Glycerol	G 8773
Deionized Water	

PROCEDURE FOR USE OF ISOELECTRIC FOCUSING MARKERS

A. PREPARATION OF SOLUTIONS

1. **Fixing Solution (Trichloroacetic Acid/Sulfosalicylic Acid)**
   Use Fixing Solution (Product No. F 7264) OR
   dissolve and dilute to 1 L with deionized water:

   115.0 g Trichloroacetic Acid (Product No. T 4885)
   34.6 g 5-Sulfosalicylic Acid (Product No. S 2130)

2. **Staining Solution**

   Use Brilliant Blue G Concentrate (Product No. B 8522) OR dissolve 1 g Coomassie Brilliant Blue G (Product No. B 0770) in 1 L of Destaining Solution (prepared in step A.3.).

3. **Destaining Solution**

   Combine:
   750 ml Methanol (Product No. M 3641)
   150 ml Glacial Acetic Acid (Product No. A 6283)
   2100 ml deionized water
   NOTE: Always add acid to water.

4. **Preserving Solution**
   Add 90 ml Glycerol (Product No. G 8773), with stirring, to 900 ml Destaining Solution.

B. PREPARATION OF MARKER MIXTURES AND PROTEIN SAMPLE SOLUTIONS

1. **Marker Mixtures**

   IEF Mix 3.6-9.3 (Product No. I 3018)
   IEF Mix 3.6-6.6 (Product No. I 8012)
   Dissolve the appropriate IEF Mix in 0.25 ml of deionized water. Allow 5 minutes for the proteins to completely dissolve. The mixture may be stored frozen for up to 6 months after reconstitution. A slight haze which has no effect on protein focusing may be seen after reconstitution.

2. **Individual Markers**

   Dissolve each vial of individual markers, Product Nos. L 1277 and C 3666, in 0.25 ml of deionized water. Dissolve each vial of the remaining individual markers in 0.5 ml of deionized water. This will provide stock solutions of 4 mg/ml. These solutions may be diluted for use as individual markers or combined to prepare a marker mixture best suited for the pH range being studied. In either case, the recommended final protein concentration should be in the range of 0.4-0.6 mg protein/ml except for L 1277 which is 0.8-2.0 mg/ml. Individual protein markers should be used in conjunction with at least 2 or 3 other markers as part of a standard curve. Lectin from *Lens culinaris* and ß-Lactoglobulin A will be hazy upon reconstitution. This in no way affects the electrofocusing and results in an insignificant loss of protein.

   The marker dye included in each kit is 1.5-2.0 mg of Methyl Red HCl. Dissolve the dye in 1.0 ml of deionized water to obtain a solution which may be applied directly to the gel without further dilution.

The marker dye should be run in a separate lane, if possible, since some proteins may bind the dye and alter its banding pattern. Methyl Red will be light yellow on the cathodic side (the suggested area of application) and will turn pink as it focuses in the acid range.

3. **Sample Protein Solutions**

   Protein samples to be tested should be dissolved in, or dialyzed against, deionized water. The final protein concentration should be in the range of 0.4-1 mg/ml. If salt is required to dissolve the protein, a 1% glycine solution or a (1:30) dilution of Pharmalyte® or Ampholine® may be used.

   Some salts which are not zwitterions interfere with the pH gradient and may distort banding patterns.

C. ELECTROFOCUSING OF SIGMA MARKERS

1. Run gels at 1 W/cm of gel length. Prefocusing should not be required for most purposes. If paper applicators are used, they should be removed 1/2 hour into the run.
2. The power guidelines specified perform well with Sigma Markers and a wide variety of other proteins. Some proteins may require longer or shorter focusing periods. In such cases, trials may be performed to determine optimal focusing times.

   The progress of the electrofocusing may be followed by using Methyl Red pI Marker Dye (Product No. M 5768), Myoglobin (Product No. M 9267) and/or Glucose Oxidase (Product No. G 7146) at 4 mg/ml. The marker dye when applied cathodically will change color from light yellow to dark pink as it focuses in the acid range. The marker dye may reach its pI sooner than some proteins, so focusing time may need to be extended.

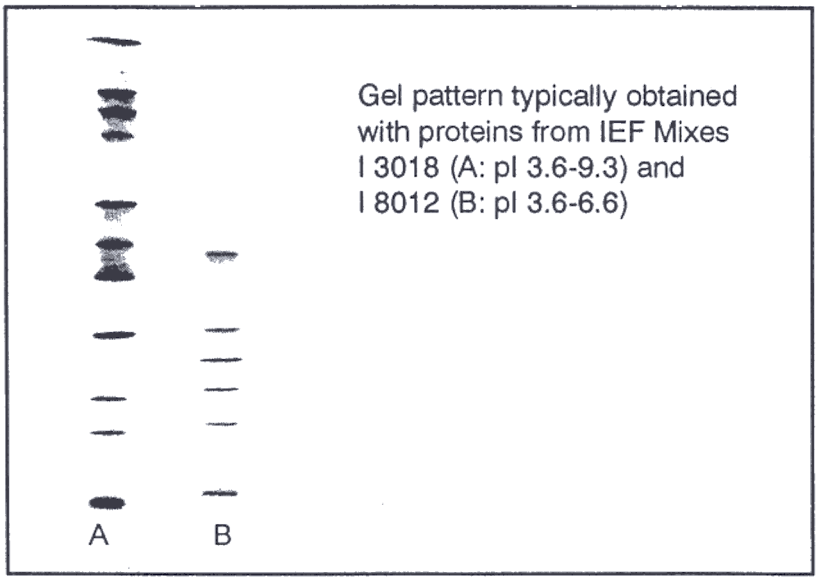
D. FIXING, STAINING AND DESTAINING

1. After electrofocusing is complete, remove the wicks from the slab and place the gel in Fixing Solution for 1 hour.
2. Rinse the gel several times in deionized water.
3. Incubate in Staining Solution for 1 hour at room temperature.
4. Place in Destaining Solution until the gel is clear, changing the destaining solution frequently at first to remove the bulk of the stain. If any dye precipitates on the gel, gently wipe with a clean cotton ball or a lab wipe soaked in Destaining Solution.

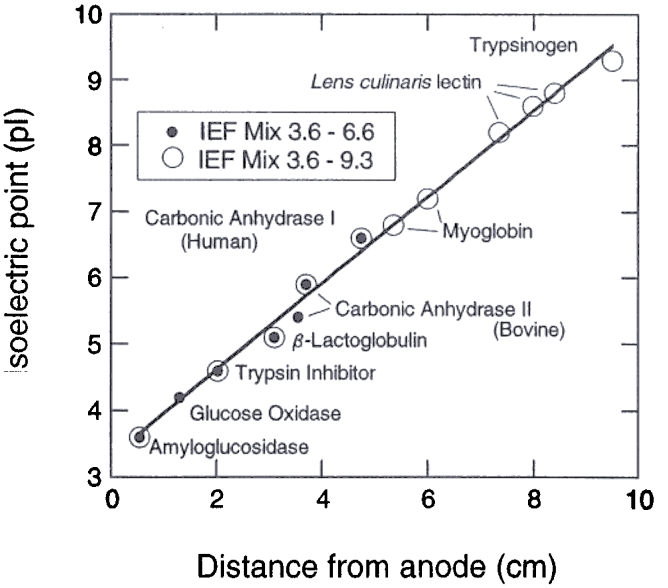
E. GEL PRESERVATION

After the gel is destained, soak it in Preserving Solution for 1 hour. The gel may then be processed as follows:

Place a glass plate having the same dimensions as the gel in a tray partially filled with Preserving Solution. Lay the gel with gel bond support on the glass plate (gel side up). Soak a dialysis membrane sheet in Preserving Solution and lay it on top of the gel. Wrap the ends of the membrane sheet around the glass plate and press out any trapped air bubbles. Set aside at room temperature. After 2 days, the gel "sandwich" consisting of the electrophoresis film, the gel, and the dialysis membrane may be removed from the glass plate.



Calibration Curve typically obtained with proteins from **IEF** Mixes 3.6-6.6 and 3.6-9.3.



NOTE: This typical curve cannot be used to derive laboratory test results. Each laboratory must prepare its own calibration curve.

F. CALCULATIONS FOR WIDE RANGE ELECTROFOCUSING

1. Using graph paper or a rule, determine the distance of migration of each band from the anode.
2. Plot the pI values of the standards vs. migration distance to obtain a standard curve.
3. The pI value of the sample protein can be determined from its migratory distance and the standard curve.

References

Laas, T., *et al.*, Prot. Biol. Fluids **27,** 683-686 (1971).

Nguyen, N.Y. and Chrambach, A., Anal. Biochem. **82,** 226-235 (1977).

Radola, B., Electrophoresis, **1,** 43-56 (1980).

Righetti, P.G., Laboratory Techniques in Biochemistry and Molecular Biology, Vol. II: Isoelectric Focusing: Theory, Methodology and Applications. Work, T.S. and Work, E., gen. eds., Elsevier Biomedical, Amsterdam. (1983).

Righetti, P.G., Laboratory Techniques in Biochemistry and Molecular Biology, Vol. V, Part II: Isoelectric Focusing. Work, T.S. and Work, E., gen. eds., North Holland Publishing Co., Amsterdam. (1976).

Soderberg, L., *et al.,* Prot. Biol. Fluids **27,** 687-691 (1979).

TROUBLESHOOTING GUIDE

PROBLEM	POSSIBLE CAUSES	SOLUTION
Missing or faint bands	1. Not enough sample loaded	1. Load more sample
	2. Protein not denatured by fixative	2. Try different fixatives
	3 Staining solution not sensitive to protein	3. Try activity stain or another stain
	4. Sample unstable at the application site	4. Change application site
Fuzzy Bands	1. Insufficient time	1. Increase run time
	2. Sample overfocused	2. Decrease power, voltage, or focusing time
	3. Fixation incomplete	3. Increase fixation time. Immediately fix after focusing.
Skewed Bands	1. The electrode is not clean	1. Wash electrode with distilled water and dry
	2. Uneven electrode contact	2. Ensure even contact along the entire electrode
	3. Sample applied too near edge of gel	3. Apply sample 0.5-1 cm from gel edge

	4. Electrode wicks too short	4. Leave no part of gel edge uncovered by wick
	5. Sample overloaded	5. Reduce sample volume or dilute sample
	6. Excessive salt in sample	6. Reduce salt concentration by gel filtration or dialysis against 0.2 M glycine, pH 6-8
	7. Outdated or incorrect electrolyte solution	7. Prepare fresh solutions
Wavy Bands	1. Excessive salt in sample	1. Reduce salt concentration by gel filtration or dialysis against 0.2 M glycine, pH 6-8
	2. Sample overloaded	2. Reduce sample volume or dilute sample
	3. Outdated or incorrect electrolyte solution	3. Prepare fresh solutions
	4. Anolyte not optimal for separation	4. Try different anolyte
	5. Electrode is not clean	5. Wash electrode with deionized water and dry
Bowed Bands	1. Excessive salt in sample	1. Lower salt concentration by gel filtration or dialysis against 0.2 M glycine, pH 6-8
	2. Thin spot in gel	2. Avoid gel drying by keeping sealed in plastic wrap until used, or keep chamber cooled and covered to prevent drying
Chamber Condensation	1. Improper cooling	1. Lower coolant temperature
	2. Wattage too high	2. Lower wattage
	3. Excessive salt in sample	3. Lower salt concentration by gel filtration or dialysis against 0.2 M glycine, pH 6-8

	4.	Humidity too high	4.	Flush chamber with dry air
Thinning of gel at electrodes or over the entire gel	1	Incorrect electrolyte solution	1	Check electrolyte
	2.	Gel dehydrated	2.	Leave gel in plastic wrap until ready to use
pH gradient different than stated ampholyte used	1	Incorrect calibration of pH electrode	1	Recalibrate electrode
	2.	Gradient drift toward cathode	2	Decrease running time
Nonlinear pH gradient	1.	Insufficient run time	1a. b.	Increase run time Measure pH before removing gel (if possible)
Current increases with time	1.	Electrode attached on the wrong side of gel	1.	Apply positive electrode to anode and negative electrode to cathode. Check for proper catholyte or anolyte solution
Angled band migration	1.	Uneven electrode	1.	Ensure even contact along the entire electrode
	2.	Too much pressure on electrode	2.	Adjust for light but firm contact
	3.	Electrodes are not parallel	3.	Make sure electrodes are parallel
Sample doesn't focus where expected	1.	Insufficient run time	1.	Increase run time
	2.	Sample overfocused	2.	Decrease power, voltage, or focusing time
	3.	Sample unstable at pH of application site or precipitates near pI	3.	Change application site
	4.	Removal of cofactor or other ligands	4.	Check characteristics of proteins
Observation of multiple bands on apparently "pure sample"	1.	Sample exists in various oxidation states	1.	Try dithiothreitol, 2-mercaptoethanol or other non-charged stabilizer
	2.	Protein dissociates into subunits	2.	Natural occurrence

	3.	Sample has become denatured	3.	Glycerol, glycine or other suitable substance may help stability
	4.	Protein focuses as isomers	4.	Natural occurrence
High background stain	1	Incorrect stain used	1	Brilliant Blue G gives fewer background problems than Brilliant Blue R.
	2.	Residual ampholytes	2.	Fix in TCA/SSA to remove ampholytes
	3.	Outdated staining solution	3.	Make sure all dye is in solution or prepare fresh staining solution
	4.	Gel allowed to sit too long in staining solution	4.	Float gel side down to prevent solution stain from precipitating on gel surface
Peeling of gel from film	1	Not using electrophoresis film	1.	Use electrophoresis film or glass plate treated with methacryloxypropyl trimethoxysilane
Sparking or burning of gel	1	Electrode wick too dry	1.	Anodal wick should be wet but not dripping wet; cathodal wick should be damp
	2.	Sample overfocused	2.	Reduce focusing time
	3.	Thin spot in gel	3.	Avoid dehydrating gel
	4.	Excessive salt in sample	4.	Lower salt concentration by gel filtration or dialysis against 0.2 M glycine, pH 6-8
	5.	Incorrect electrolyte solution	5.	Use the recommended electrolytes
	6.	Excessive power input	6.	Check power setting
Edge of gel sparking onto cooling plate	1.	Excess moisture on or under gel	1.	Blot away excess moisture
	2.	Electrode wick overhanging end of gel	2.	Make wicks flush with gel ends

Sample smearing or precipitating	1. Sample aggregated	1. Dissociate sample with neutral surfactant or 0.2 M Glycine, pH 6-8
	2. Sample applied too near pI or at pH where it's insoluble	2. Change application site or add nonionic surfactants (e.g. Triton X-100 or Nonidet P-40)
Sample does not move from application site	1. Sample applied too near pI	1. Change application site
Sample does not focus	1. CO_2 interferes with gradient	1. Flush chamber with nitrogen

SIGMA REAGENTS FOR ISOELECTRIC FOCUSING

A 3553	Acrylamide, Electrophoresis Reagent
A 4058	Acrylamide, 40% Solution
A 7802	Acrylamide/Bis, 40% Solution (29:1 ratio)
A 7168	Acrylamide/Bis, 40% Solution (37.5:1 ratio)
A 9926	Acrylamide/Bis, 40% Solution (19:1 ratio)
A 4804	Agarose, Electrophoresis Reagent, EEO 0.02
A 3678	Ammonium Persulfate, Electrophoresis Reagent
B 5133	Brilliant Blue G
B 4523	Brilliant Blue G-TCA Solution (Rapid Stain)
B 8522	Brilliant Blue G Concentrate
E 9890	Ethylenediamine, Electrophoresis Reagent
E 9508	Ethanolamine, Free Base
F 7264	Fixing Solution, 5X Concentrate
G 1126	DL-Glutamic Acid
G 8898	Glycine, Electrophoresis Reagent
G 8773	Glycerol, Electrophoresis Reagent
H 3375	HEPES, Free Acid
H 8000	L-Histidine, Free Base
I 5629	Iminodiacetic Acid, Free Acid
M 6514	γ-Methacryloxypropyltrimethoxysilane
M 7279	N,N'-Methylene-bis-acrylamide, Electrophoresis Reagent

Pharmalytes®

P 1522	pH 3.0-10.0
P 1647	pH 2.5-5.0
P 0783	pH 4.2-4.9
P 0658	pH 4.5-5.4
P 1772	pH 4.0-6.5
P 0533	pH 5.0-6.0
P 1897	pH 5.0-8.0
P 0408	pH 6.7-7.7
P 2147	pH 8.0-10.5

	Ampholine®	
A 5174		pH 3.5-10.0
A 5424		pH 3.5-5.0
A 5549		pH 4.0-6.0
A 5674		pH 5.0-7.0
A 5799		pH 5.0-8.0
A 5924		pH 6.0-8.0
A 6049		pH 7.0-9.0
A 4549		

	Ampholine® Preblended	
A 4549		pH 3.5-9.5
A 4799		pH 4.0-6.5
A 4924		pH 5.0-6.5
A 5049		pH 5.0-8.0

I 1506	Sephadex® IEF
S 8526	Sodium Hydroxide, 1.0 M Solution, Electrophoresis Reagent
S 5881	Sodium Hydroxide, Anhydrous Pellets
P 0180	Phosphoric Acid, 0.1 M Solution
S 3147	5-Sulfosalicylic Acid, Dihydrate, Electrophoresis Reagent
T 9281	TEMED, Electrophoresis Reagent
E 0389	Electrophoresis Film for Polyacrylamide Gels, 7 mil
E 1380	Electrophoresis Film for Agarose Gels, 7 mil