Isoelectric focusing

Adapted from Chapter 7, Gel Electrophoresis of Proteins, by David E. Garfin, Pages 197-268, in Essential Cell Biology, Volume 1: Cell Structure, A Practical Approach, Edited by John Davey and Mike Lord, Oxford University Press, Oxford UK (2003). Used by permission of Oxford University Press.

Isoelectric focusing is an electrophoretic method in which proteins are separated on the basis of their p*I*s (1-12). It makes use of the property of proteins that their net charges are determined by the pH of their local environments. Proteins carry positive, negative, or zero net electrical charge, depending on the pH of their surroundings.

The net charge of any particular protein is the (signed) sum of all of its positive and negative charges. These are determined by the ionizable acidic and basic side chains of the constituent amino acids and prosthetic groups of the protein. If the number of acidic groups in a protein exceeds the number of basic groups, the p*I* of that protein will be at a low pH value and the protein is classified as being *acidic*. When the basic groups outnumber the acidic groups in a protein, the p*I* will be high with the protein classified as *basic*. Proteins show considerable variation in isoelectric points, but p*I* values usually fall in the range of pH 3-12 with a great many having p*I*s between pH 4 and pH 7 (13, 14).

Proteins are positively charged in solutions at pH values below their p*I* and negatively charged above their isoelectric points. Thus, at pH values below the p*I* of a particular protein, it will migrate toward the cathode during electrophoresis. At pH values above its p*I*, a protein will move toward the anode. A protein at its isoelectric point will not move in an electric field.

When a protein is placed in a medium with a linear pH gradient and subjected to an electric field, it will initially move toward the electrode with the opposite charge (*Figure 1*). During migration through the pH gradient, the protein will either pick up or lose protons. As it does, its net charge and mobility will decrease and the protein will slow down. Eventually, the protein will arrive at the point in the pH gradient equaling its p*I*. There, being uncharged, it will stop migrating. If a protein at its p*I* should happen to diffuse to a region of lower pH, it will become protonated and be forced toward the cathode by the electric field. If, on the other hand, it diffuses into a pH higher than its p*I*, the protein will become negatively charged and will be driven toward the anode. In this way, proteins condense, or focus, into sharp bands in the pH gradient at their individual, characteristic p*I* values.

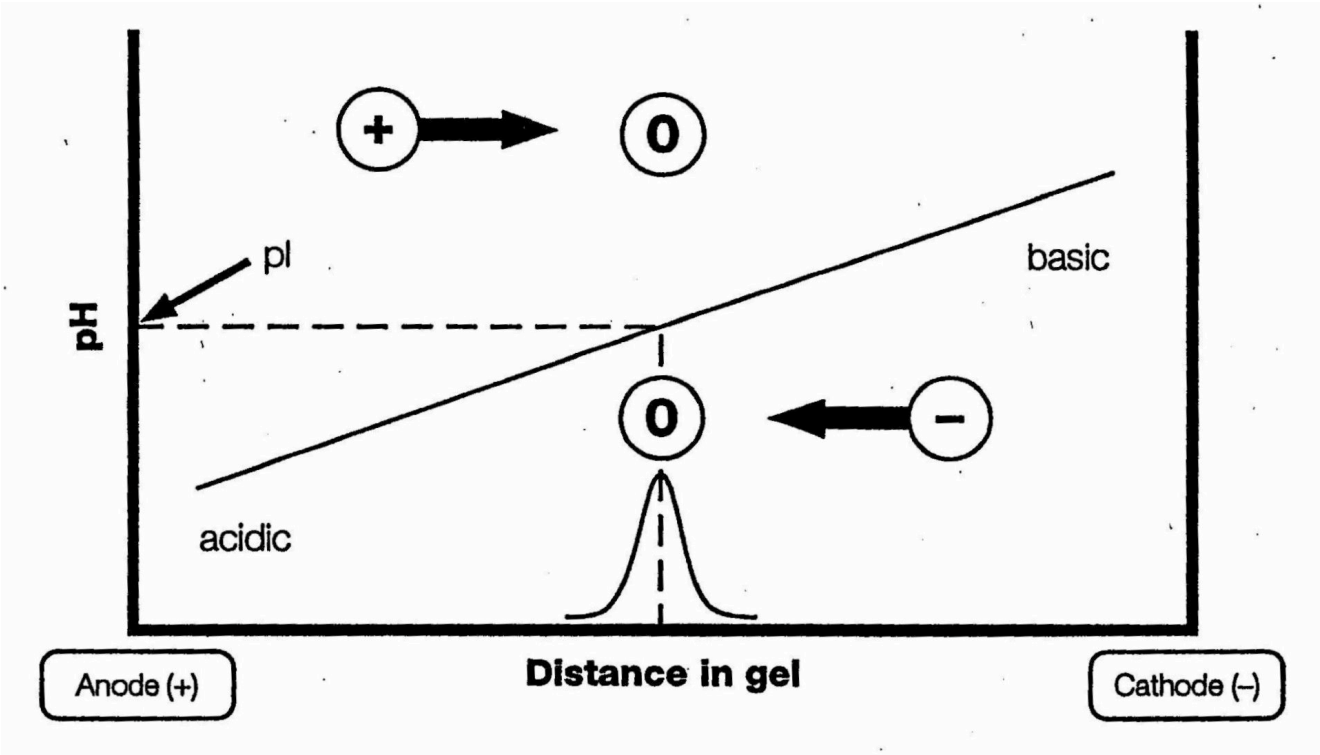

Figure 1. Isoelectric focusing. The motion of a protein undergoing isoelectric focusing is depicted (circles). The protein is shown near its p*I* in a pH gradient. Both the pH gradient and the motion of the protein are controlled by an electric field. At pH values lower than the p*I*, the protein is positively charged (+) and it is driven toward the cathode (arrow). Above the p*I*, the protein is negatively charged (-) and it moves toward the anode. There is no net electrical force on the protein at its p*I* (0). The protein focuses in a gaussian distribution centered at the p*I*.

Focusing is a steady-state mechanism with regard to pH. Proteins approach their respective p*I* values at differing rates but remain relatively fixed at those pH values for extended periods. This type of motion is in contrast to conventional electrophoresis in which proteins continue to move through the medium until the electric field is removed. Moreover, in IEF, proteins migrate to their steady-state positions from anywhere in the system. Thus, the sample application point is arbitrary. In fact, the sample can be initially distributed throughout the entire separation system.

1. Establishing pH gradients

Stable, linear pH gradients are the keys to successful IEF. Establishment of such gradients is accomplished in two ways with two different types of molecules, carrier ampholytes and acrylamido buffers.

Carrier ampholytes (*ampho*teric electro*lytes*) are mixtures of molecules containing multiple aliphatic amino and carboxylate groups. They are small (about 300-1000 Da in size) multi-charged organic buffer molecules with closely spaced p*I* values and high conductivity. Ampholytes are included directly in IEF gels. In electric fields, carrier ampholytes partition into smooth pH gradients that increase linearly from the anode to the cathode. The slope of a pH gradient is determined by the pH interval covered by the carrier ampholyte mixture and the distance between the electrodes. The use of carrier ampholytes is the most common and simplest means for forming pH gradients.

Acrylamido buffers are derivatives of acrylamide containing both reactive double bonds and buffering groups. Their general structure is CH_2 = CH-CO-NH-R, where R contains either a carboxyl [-COOH] or a tertiary amino group [*e.g.*, -$N(CH_3)_2$]. They are covalently incorporated into polyacrylamide gels at the time of casting. The key acrylamido buffers have pK values at pH 1, 3.6, 4.6, 6.2, 7.0, 8.5, 9.3, 10.3, and >12. They can be used to cast just about any conceivable pH gradients. In any given gradient, some of the acrylamido compounds act as buffers while others serve as titrants. Published formulations and methods are available for casting the most common gradients (48, 50). Because the buffering compounds are fixed in place in the separation medium, the gels are called "immobilized pH gradients", or IPGs. IPGs offer the advantage of gradient stability over extended runs. They are, however, more cumbersome and expensive to cast than carrier ampholyte gels. IPGs are commercially available in sheet form in a few pH ranges. A greater variety of pH ranges are available in IPGs that have been cut into strips for the IEF first dimension of 2-D PAGE.

IEF is a high-resolution technique that can routinely resolve proteins differing in p*I* by less than 0.05 pH unit. Antibodies, antigens, and enzymes usually retain their activities during IEF. The proper choice of ampholyte or IPG range is very important to the success of a fractionation. Ideally, the pH range covered by an IEF gel should be centered on the pI of the proteins of interest. This ensures that the proteins of interest focus in the linear part of the gradient with many extraneous proteins excluded from the separation zone.

With carrier ampholytes, concentrations of about 2% (w/v) are best. Ampholyte concentrations below 1% (w/v) often result in unstable pH gradients. At concentrations above 3% (w/v) ampholytes are difficult to remove from gels and can interfere with protein staining. When casting IPGs, follow published recipes and use buffering powers of about 3 meq throughout the gradient (method not presented).

2. Gels for isoelectric focusing

As an analytical tool, IEF is carried out in large-pore polyacrylamide gels (5%T, 3%C) which serve mainly as anticonvective matrices. Polyacrylamide IEF gels are polymerized with an initiator system including riboflavin for photopolymerization. Photochemical initiation of polymerization with a combination of the three compounds riboflavin, ammonium persulfate, and TEMED, results in more complete polymerization of IEF gels than does chemical polymerization in gels containing low-pH ampholytes (15). Suitable initiator concentrations are 0.015% ammonium persulfate, 0.05% TEMED, and 5 µg/ml riboflavin-5'-phosphate. Photochemical polymerization is allowed to continue for 2 hr, with the second hour under direct lighting from a nearby fluorescent lamp.

The most common configuration for analytical IEF is the horizontal polyacrylamide slab gel. Gels are cast with one exposed face on glass plates or specially treated plastic sheets. They are placed on cooling platforms and run with the exposed face upward. Electrolyte strips, saturated with 0.1-1 *M* phosphoric acid at the anode and 0.1-1 *M* sodium hydroxide at the cathode, are placed directly on the exposed surface of the IEF gel. Electrodes of platinum wire maintain contact between the electrical power supply and the electrolyte strips. In another possible configuration, the gel and its backing plate are inverted and suspended between two carbon rod electrodes without the use of

electrolyte strips. IPG strips for 2-D PAGE are often run with the gel facing down in dedicated IEF cells.

Protocol 1. Casting gels for isoelectric focusing

Equipment and reagents

- Gel casting apparatus for a horizontal electrophoresis cell; *e.g.*, the GE MultiPhor
- Fluorescent lamp
- 30%T, 3%C acrylamide stock solution[a]
- 50% glycerol[b]
- 0.1% riboflavin-5'-phosphate (FMN)[c]
- Carrier ampholytes with a pH range spanning the p*I*s of the proteins of interest[d]
- 10% APS[e] and TEMED[f].

Method

The formulation given here is for 12 ml of gel solution containing 5% T (3%C) acrylamide, 2% carrier ampholytes, 5% glycerol. The volume needed depends on the casting apparatus that is used; adjust volumes accordingly. This recipe is sufficient for casting one gel of 100 x 125 x 0.8 mm (10 ml) or four 100 x 125 x 0.2 mm (10 ml total). 8 M urea can be substituted for the glycerol if desired (see ref. 16).

1. Assemble the casting apparatus according to the manufacturer's instructions. The use of gel support film for polyacrylamide is highly recommended.

2. Combine

30%T, 3%C acrylamide stock	2.0 ml
Carrier ampholytes (40%)	0.6 ml
50% glycerol	1.2 ml
Water	8.2 ml
0.1% FMN	60 μl
10% APS	18 μl
TEMED	4 μl

Swirl the solution gently to mix the components.

3. Transfer the gel solution to the casting apparatus with a pipette and bulb.

4. Position a fluorescent lamp about 3-4 cm from the gel solution and illuminate the solution for about one hour.

5. Open the gel cassette or lift the gel from the casting tray to expose the face of the gel. Place the gel with the open face upward and illuminate it with the fluorescent lamp for an additional 30 min.

6. The gel may be used immediately or it can be covered with plastic wrap and stored at 4°C for several days. Best results are sometimes obtained when IEF gels are left overnight at 4°C before use.

[a] To prepare 30%T, 3% C acrylamide solution, dissolve 29.1 g of acrylamide and 0.9 g of bisacrylamide in 72.5 ml of water. The final volume will be 100 ml. See the *Caution* note regarding acrylamide in the Application Focus on Gel Electrophoresis of Proteins on this website.

[b]To prepare 50% glycerol, mix 50 g of glycerol with 63 g of water (100 ml final volume).
[c]To prepare 0.1% FMN, dissolve 50 mg of FMN in 50 ml of water.
[d]Carrier ampholytes are usually supplied at 40% (w/v) concentration, but some are at 20% (w/v).
[e]To prepare 10% APS, dissolve 100 mg of APS in 1 ml of water. APS solutions should be made fresh daily, but may perform satisfactorily for up to a week. Store at room temperature.
[f]TEMED is used undiluted as supplied.

Ultrathin gels (<0.5 mm) allow the highest field strengths and, therefore, the highest resolution of the analytical methods. Electrofocusing can also be done in tubes, and this configuration once constituted the first dimension of 2-D PAGE (17). Because of difficulties in handling and reproducibility with tube gels, IPG strips have largely replaced them.

Good visualization of individual bands generally requires a minimum of 0.5 µg each with dye staining or 50 ng each with silver staining (see the Application Focus about the Detection of Proteins in Gels on this website). One of the simplest methods for applying samples to thin polyacrylamide gels is to place filter paper strips impregnated with sample directly on the gel surface. Up to 25 µl of sample solution can be conveniently applied after absorption into 1-cm squares of filter paper. A convenient size for applicator papers is 0.2 x 1 cm, holding 5 µl of sample solution. Alternatively, 1- to 2-µl samples can be placed directly on the surface of the gel. In most cases, IPG strips (which are provided in dehydrated form) are rehydrated in sample-containing solution prior to electrophoresis (18). Rehydration loading allows higher protein loads to be applied to gels than do other methods. It is particularly popular because of its simplicity.

There are no fixed rules regarding the positioning of the sample on the gel. In general, samples should not be applied to areas where they are expected to focus. To protect the proteins from exposure to extreme pH, the samples should not be applied closer than 1 cm from either electrode. Forming the pH gradient before sample application also limits the exposure of proteins to pH extremes.

Precast IEF mini gels (6 cm long by 8 cm wide and 1 mm thick) are available for carrying out carrier-ampholyte electrofocusing. A selection of IPG sheets is also available for horizontal IEF. Vertical IEF gels have the advantage that the electrophoresis equipment for running them is available in most laboratories and they can hold relatively large sample volumes. Because vertical electrophoresis cells cannot tolerate very high voltages, this orientation is not capable of the ultrahigh resolution of horizontal cells. To protect the materials of the electrophoresis cells (mainly the gaskets) from caustic electrolytes alternative catholyte and anolyte solutions are substituted in vertical IEF runs. As catholyte, 20 m*M* arginine, 20 m*M* lysine is recommended in vertical slab systems (0.34 g arginine free base and 0.36 lysine free base in 100 ml of water). The recommended anolyte is 70 m*M* H_3PO_4, but it can be substituted with 20 m*M* aspartic acid, 20 m*M* glutamic acid (0.26 g aspartic acid and 0.29 g glutamic acid in 100 ml of water).

3. Power conditions and resolution in isoelectric focusing

The pH gradient and the applied electric field determine the resolution of an IEF run. According to both theory and experiment (1, 8, 12), the difference in p*I* between two resolved adjacent protein IEF bands (ΔpI) is directly proportional to the square root of the pH gradient and inversely proportional to the square root of the voltage gradient (field strength) at the position of the bands:

$$\Delta pI \propto [(\text{pH gradient})/(\text{voltage gradient})]^{1/2}.$$

Thus, narrow pH ranges and high applied-voltages give high resolution (small Δp*I*) in IEF.

In addition to the effect on resolution, high electric fields also result in shortened run times. However, high voltages in electrophoresis are accompanied by large amounts of generated heat (see the discussion of Joule heating, in the Electrical Considerations section of the Application Focus on Gel Electrophoresis of Proteins on this website). Thus, there are limitations on the magnitudes of the electric fields that can be applied and the ionic strengths of the solutions used in IEF. Because of their higher surface-to-volume ratio, thin gels are better able to dissipate heat than thick ones and are therefore capable of higher resolution (high voltage). Electric fields used in IEF are generally of the order of 100 *V*/cm. At focusing, currents drop to nearly zero since the current carriers have stopped moving by then.

4. Protein solubilization for isoelectric focusing

A fundamental problem with IEF is that some proteins tend to precipitate at their p*I* values. Carrier ampholytes sometimes help overcome pI precipitation and they are usually included in the sample solutions for IPG strips. In addition, nonionic detergents or urea are often included in IEF runs to minimize protein precipitation.

Urea is a common solubilizing agent in gel electrophoresis. It is particularly useful in IEF, especially for those proteins that tend to aggregate at their p*I*s. Urea disrupts hydrogen bonds and is used in situations in which hydrogen bonding can cause unwanted aggregation or formation of secondary structures that affect mobilities. Dissociation of hydrogen bonds requires high urea concentrations (7-8 *M*). If complete denaturation of proteins is sought, samples must be treated with a thiol-reducing agent to break disulfide bridges (protein solutions in urea should not be heated above 30°C to avoid carbamylation).

High concentrations of urea make gels behave as if they had reduced pore sizes. This is because of either viscosity effects or reductions in the effective size of water channels (pores). Urea must be present in the gels during electrophoresis, but, unlike SDS, urea does not affect the intrinsic charge of the sample polypeptides. Urea solutions should be used soon after they are made or treated with a mixed-bed ion-exchange resin to avoid protein carbamylation by cyanate in old urea.

Some proteins, especially membrane proteins, require detergent solubilization during isolation. Ionic detergents, such as SDS, are not compatible with IEF, although nonionic detergents, such as octylglucoside, and zwiterionic detergents, such as 2-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) and its hydroxyl analog CHAPSO, can be used. NP-40 and Triton X-100 sometimes perform satisfactorily, but some preparations may contain charged contaminants.

Concentrations of CHAPS and CHAPSO or of octylglucoside of 1-2% in the gel are recommended. Some proteins may require as high as 4% detergent for solubility. Even in the presence of detergents, some samples may have stringent salt requirements. Salt should be present in a sample only if it is an absolute requirement. Carrier ampholytes contribute to the ionic strength of the solution and can help to counteract a lack of salts in a sample. Small samples (1 to 10 µl) in typical biochemical buffers are

usually tolerated, but better results can be obtained with solutions in deionized water, 2% ampholytes, or 1% glycine. Suitable samples can be prepared by dialysis or gel filtration.

Protocol 2. Isoelectric focusing

Equipment and reagents

- Flat-bed electrophoresis cell; *e.g.*, the GE Multiphor
- Power supply capable of delivering 2-3000 *V* and 6 *W* at high voltage.
- Refrigerated water circulator if required
- 1 *N* NaOH[a] catholyte if required
- 1 *N* H_3PO_4[b] anolyte if required
- Electrode strips[c] if required
- Sample application strips[d]

Method

1. Set up the IEF cell as recommended by the manufacturer. This includes connecting a water circulator, if used, and preparing the cooling platform and electrode strips, if necessary.

2. Place sample application strips on a glass plate and pipette 5 μl of a protein sample to each strip. Place the application strips 1 cm from the anode end of the gel.

3. Position the gel in the IEF cell and make electrode contact as specified for the particular cell.

4. Close the the electrophoresis cell and connect the leads to the power supply; the red lead is the anode and the black lead is the cathode.

5. Set the running conditions as recommended by the manufacturer of the electrophoresis cell.

[a]To prepare 1 *N* NaOH, dissolve 4 g of NaOH in 100 ml of water.
[b]To prepare 1 *N* H_3PO_4, dissolve 2.3 ml of 85% H_3PO_4 (14.6 *M*, 44 *N*) in 97.7 ml of water.
[c]For electrode strips, cut thick filter paper about 7 mm wide and about 4 mm shorter than the gel.
[d]For sample application strips, cut thin filter paper to 0.2 x 1 cm, one strip per sample.

References

1. Andrews, A.T. (1986). *Electrophoresis: theory, techniques, and biochemical and clinical applications* (2nd edn). Oxford University Press, Oxford.
2. Låås, T. (1989). In *Protein purification: principles, high resolution methods and applications* (ed. J.-C. Janson and L. Ryden), p.376. VCH Press, Weinheim.
3. Dunn, M.J. (1993). *Gel electrophoresis: proteins*. BIOS Scientific Publishers, Oxford.
4. Allen, R.C. and Budowle, B. (1994)). *Gel electrophoresis of proteins and nucleic acids*. De Gruyter, Berlin.
5. Garfin, D.E. (1995). In *Introduction to biophysical methods for protein and nucleic acid research* (ed. J.A. Glasel and M.P. Deutscher), p. 53. Academic Press, San Diego.

6. Westermeier, R. (1997). *Electrophoresis in practice: a guide to methods and applications of DNA and protein separations* (2nd edn). VCH Press, Weinheim.
7. Låås, T. (1989). In *Protein purification: principals, high resolution methods, and applications* (ed. J.-C. Janson and L. Ryden), p.376. VCH Press, Weinheim.
8. Righetti, P.G. (1983). *Isoelectric focusing: theory, methodology, and applications*. Elsevier, Amsterdam.
9. Righetti, P.G. (1990). *Immobilized pH gradients: theory and methodology*. Elsevier, Amsterdam.
10. Garfin, D.E. (1990). In *Methods in enzymology* (ed. M.P. Deutscher). Vol. 182, p. 459. Academic Press, San Diego.
11. Righetti, P.G., Bossi, A., and Gelfi, C. (1998). In *Gel electrophoresis of proteins: a practical approach* (3rd edn) (ed. B.D. Hames), p. 127. Oxfore University Press, Oxford.
12. Garfin, D.E. (2000). In *Handbook of bioseparations* (ed. S. Ahuja), p. 263. Academic Press, San Diego.
13. Wilkins, M.R. and Gooley, A.A. (1997). In *Proteome research: new frontiers in functional genomics* (ed. M.R. Wilkins, K.L. Williams, R.D. Appel, and D.F. Hochstrasser), p. 35. Springer, Berlin.
14. Langen, H., Röder, D., Juranville, J.-F., and Fountoulakis, M. (1997). *Electrophoresis*, **18**, 2085.
15. Caglio, S. and Righetti, P.G. (1993). *Electrophoresis* **14**, 554.
16. Righetti, P.G., Bossi, A., and Gelfi, C. (1998). In *Gel electrophoresis of proteins: a practical approach* (3rd edn) (ed. B.D. Hames), p. 127. Oxfore University Press, Oxford.
17. Harrington, M.G., Gudeman, D., Zewert, T., Yun, M., and Hood, L. (1991). In *Methods: a companion to Methods in enzymology* (ed. M.G. Harrington). Vol. 3, No. 2, p. 98. Academic Press, San Diego.
18. Sanchez, J.-C., Rouge, V., Pisteur, M., Ravier, F., Tonella, L., Moosmayer, M. Wilkins, M.R., and Hochstrasser, D.F. (1997). *Electrophoresis*, **18**, 324.