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Immobilized pH Gradients: Analytical and Preparative Use

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The use of immobilized pH gradients for two-dimensional electrophoresis overcomes several of the limitations of carrier ampholyte-based isoelectric focusing. Procedures followed in the authors' laboratory for the analytical or preparative use of immobilized pH gradients are presented. © 1991 Academic Press, Inc.

High-resolution two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) has provided the means for detailed analysis of polypeptide constituents of whole cells or subcellular fractions from a wide range of sources. To date, for the most part 2-D PAGE has been utilized for analytical separations, following the method originally described by O'Farrell (1). Urea, reducing agent, and a nonionic detergent are used to solubilize and dissociate proteins into polypeptide subunits prior to the first-dimension isoelectric separation, using carrier ampholytes (CA). SDS–PAGE is used for the second-dimension separation based on molecular weight. 2-D PAGE has also been used successfully as the final purification step for the structural characterization of a relatively small number of polypeptides among the several hundred that can be observed for any given cell type, using analytical 2-D PAGE. For most polypeptides, structural studies are hampered in part because of the loss of resolution at higher protein loading in CA-based 2-D separations.

As an alternative to the use of carrier ampholytes for 2-D PAGE, we have utilized immobilized pH gradients (IPG) for the first-dimension separation (2, 3). Derivatives of acrylamide having carboxyl or tertiary amino groups with specific pK values are used for the preparation of gels in which the pH gradient is an integral part of the polyacrylamide matrix. Precise pH ranges that have narrow or broad pH gradients may be selected (Figs. 1 and 2). The problem of cathodic drift encountered with CA is eliminated, allowing reproducible focusing of polypeptides with isoelectric points up to 10. We have previously described the analytical and more recently the preparative use of IPG gels in 2-D separations (4). Preparative loads of 1 mg of protein may be applied to individual IPG strips without loss of resolution, thus substantially increasing the number of polypeptides that may be obtained from a small number of 2-D gels, in sufficient amount for structural studies. The approach we have utilized for analytical as well as preparative IPG-based 2-D gels is presented below.

METHODS

Equipment

Recommendations for sources of equipment and reagents are not exclusive. Substitutions can be made on the basis of demonstration of equivalence. Isoelectric focusing in IPG gels is performed on a horizontal electrophoresis apparatus with a temperature control block regulated at 20°C with coolant from an external cooling bath. It is important to avoid temperature fluctuation within and between electrophoretic runs because of temperature-related variability in polypeptide spot position (5). The first-dimension isoelectric focusing apparatus and accessories from Pharmacia–LKB, including a Multiphor horizontal flat bed electrophoresis apparatus, tray and template for holding IPG strips, platinum electrodes, sample application cups, and holder, are suitable for this application. Electrolyte reservoirs 5 mm wide and of appropriate length are prepared from 3-mm-thick filter paper (MN 440, Macherey-Nagel, Germany) or five layers of chromatography paper (3MM, Whatman). A 5-kV (Macrodrive5, Pharmacia–LKB) or a 10-kV (Glassman High Voltage, Inc., Whitehouse Station, NJ) power supply is used for focusing. Other related equipment includes a microgradient former, gel casting cassettes (260 × 200 × 0.5 mm), and rehydration cassettes (120 × 200 × 0.5 mm), all from Pharmacia–LKB.

For the second-dimension SDS–gel electrophoresis, the slab gel apparatus we use is the Dalt System (Hoefer),

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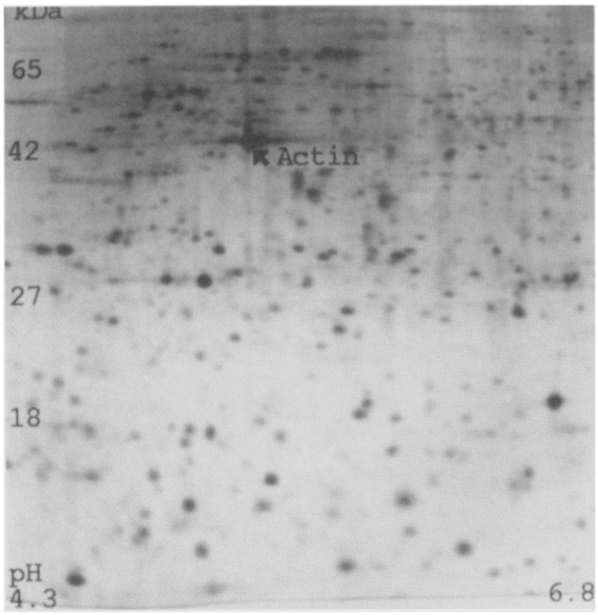

FIG. 1. Silver-stained two-dimensional gel of normal human peripheral blood lymphocytes. The first-dimension isoelectric focusing was performed for 110,000 V-h in an 18-cm-long pH 4–7 IPG gel. After cutting of the sample applicator and wick area from the anode end and the wick area from the cathode end of the IPG strips, the effective separation range is pH 4.3 (left) to pH 6.8 (right). The second-dimension SDS gel was an 11.4% (top) to 14% (bottom) acrylamide gradient.

which includes a gradient former and casting box for casting 20 (16.5 cm × 16.5 cm × 1.5 mm) gels simultaneously, and electrophoresis tanks that hold 10 gels each. Electrophoresis buffer is circulated by a pump to coils immersed in an external cooling bath regulated at 10°C. Gel cassettes are made of $\frac{1}{8}$-in. double-strength glass measuring 17.5 × 17.5 cm. Spacers are made from $\frac{1}{16}$-in. polycarbonate and measure 5 mm × 17.5 cm. For convenience spacers are glued to one of the glass plates with silicone sealant (Dow Corning). The power supply (0–600 V, 0–1.5 A) is from Hewlett–Packard. SDS-based separations on a flat bed using precast gels can be an alternative. The major drawback is the limited number of gels that can be utilized simultaneously.

Materials

A limited variety of IPG gels having narrow- and wide-range pH gradients which have a 10-cm separation distance are commercially available (Immobiline Dry Plate, LKB). For other separation distances and for customized pH gradients, gels are cast in the user's laboratory. We prefer larger size gels with a separation distance of 17 cm, for greater resolution and for the convenience of matching the size of the second-dimension plates that we utilize. "Electrophoresis Grade" reagents that we utilize are available from the following suppliers: acrylamide (for the first dimension), bisacrylamide, ammonium persulfate, urea, 2-mercaptoethanol, and agarose (Bio-Rad); acrylamide (for the second dimension) and sodium dodecyl sulfate (Serva); Nonidet P-40 (Hoefer/BDH); Temed (Bethesda Research Labs); Immobilines, Repel-Silane, GelBond PAG film, and Ampholines 3.5–10 (Pharmacia–LKB); Tris, glycine, and dithioerythritol (Sigma). Silicone oil (polydimethylsiloxane, trimethylsiloxy terminated, ≤100 cP) is from Petrarch Systems (Bristol, PA). All other chemicals are reagent grade or better. MilliQ water (Millipore) is used for all solutions.

Stock solutions for immobilized pH gradient gels.

A Immobiline stock solutions (0.2 M) are prepared by adding 25 g deionized water to each vial of Immobilines. Once reconstituted, Immobiline solutions are stored in aliquots of 3–4 ml at −20°C under a nitrogen atmosphere. Once thawed, an aliquot is kept at 4°C and is used within 2 weeks.

B A solution of 29.1% (w/v) acrylamide and 0.9% (w/v) bisacrylamide is stored at 4°C for up to 2 weeks.

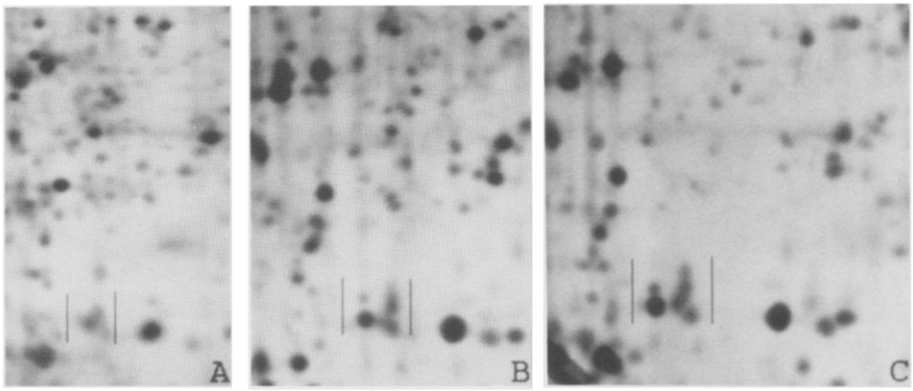

FIG. 2. Enhanced protein resolution with IPG-based separations. Close-up section covering a 0.5-pH-unit span of a standard CA-based gel with an effective separation range of 3.5 pH units (A) and corresponding sections of IPG gels with separation ranges of 3 (B) and 2 (C) pH units are shown. A cluster of spots between bars provides an example of enhanced resolution achievable with IPG [reprinted with permission of author (4)].

C Glycerol solution: 87% glycerol (w/w) is stored at room temperature.
D Ammonium persulfate: 10% (w/v) is stored at 4°C and is prepared weekly.
E Rehydration solution: 8 M urea, 0.5% NP-40, 10 mM DTE, and 0.4 mM orange G. Orange G serves as a tracking dye to monitor the uniformity of electrophoretic conditions within individual IPG strips during subsequent isoelectric focusing.
F Lysis solution: Solution A: 8 M urea, 2% (v/v) Nonidet P-40, 0.8% (w/v) Ampholine, pH 3.5 to 10, 2% (v/v) 2-mercaptoethanol. Store in 1-ml aliquots at −80°C. Solution B: Phenylmethylsulfonyl fluoride (PMSF), 15.5 mg/ml in 95% ethanol. Store at 4°C for 1 month. Immediately prior to sample solubilization, add 10 μl solution B to 1 ml solution A. NaF (25 mM) and Na vanadate (0.1 mM) may be added as phosphatase inhibitors.

Sample Preparation

For a number of 2-D studies the investigator does not know the subcellular localization of polypeptides that might be of interest. Consequently, analysis of total cellular proteins is desired. Furthermore, cell fractionation prior to analytical separations is undertaken, particularly for polypeptides that are below the detection threshold when total cellular proteins are analyzed. Cells are thoroughly washed with phosphate-buffered saline (PBS) and pelleted. Care is exercised to remove all traces of excess wash solution. It is desirable to prepare cell pellets in 1.5-ml microfuge tubes, with each pellet containing sufficient cells for the desired number of gels to be prepared simultaneously for that sample. Cell pellets are stored frozen at −80°C and are solubilized immediately before electrophoresis using a solubilization cocktail (lysis solution F). Because isoelectric focusing is sensitive to charge modification, it is important to minimize protein alterations (e.g., proteolysis, deamidation of glutamine and asparagine, oxidation of cystine to cystic acid, carbamylation) that can result from improper sample preparation. Once solubilized, samples may be stored frozen at −80°C for short periods (<1 month) without significant protein modification. It is important to standardize as best as possible the amount of protein loaded on first-dimension gels. For solid tissue, use wet weight and for cells in suspension, we prefer to use cell number as the quantitative variable in determining the amount of material to load on gels that will be subsequently silver-stained for several reasons:

(i) Sample size is frequently limiting, with little that can be spared for nongel use.

(ii) Accurate protein determination in the presence of reducing agents, detergent, and urea is time consuming and frequently unreliable with low amounts of protein.

(iii) Cell counts are easy to perform and are usually routinely available. Once the solubilized protein per cell equivalent for a particular cell type has been empirically determined, cell counts are sufficiently accurate to ensure reproducible loadings that yield overall spot intensities after silver-staining within ±20% among gels.

First-Dimension IEF with IPG

IPG gels are prepared using derivatives of acrylamide having carboxyl or tertiary amino groups with specific pK values. A linear pH gradient is prepared from a dense, acidic solution and a light, basic solution using a two-chamber microgradient former. The pH gradient is stabilized during polymerization of the Immobiline–acrylamide–bisacrylamide matrix by a co-linear gradient of glycerol. Formulations of buffering Immobiline mixtures with titrating Immobiline for the pH limit solutions for narrow pH gradients (1 pH unit) or for broad pH gradients (>1 pH unit, up to 6 pH units) have been published (6, 7). Details for only two broad pH gradients of 3 pH units each—pH 4 to 7 and pH 7 to 10—will be presented.

Gels are cast on a backing of GelBond PAG film. IPG gels are cast as slab gels (240 × 180 × 0.5 mm) with a pH gradient separation distance of 180 mm. Gels are extensively washed with deionized water to remove unpolymerized acrylamide and Immobiline monomers and catalysts. Gels are subsequently dried, wrapped in plastic film, and may be stored at −20°C for several months. Dried slab gels are cut into 3.5-mm-wide first-dimension strips, yielding 70 strips per slab. Protein denaturants and a reducing agent are added to the gel matrix during subsequent rehydration of IPG gels strips. CA may also be included in the rehydration solution.

Casting the IPG gel. The mold consists of two glass plates (260 × 200 mm), a 0.5-mm-thick U-shaped gasket cut from silicon rubber sheeting, and sufficient clamps to go completely around three sides of the mold. For a slightly thicker gel, a gasket made of neoprene with a nominal thickness of $\frac{1}{32}$ in. will yield a gel of 0.7-mm average thickness. Place one glass plate on an absorbent towel. Apply 1 ml water to the plate and cover with a sheet of GelBond PAG film (hydrophobic side down). Cover the film with a protective sheet of paper and remove air bubbles with a print block roller to ensure complete contact of the film with the plate. Remove the protective paper from the film and place the U-shaped gasket on the film. Siliconize a second glass plate with RepelSilane and allow to air-dry in a fume hood. Place the second plate, siliconized surface down, on the U-gasket and clamp on three sides. Chill the assembled mold in a cold box for 10 min prior to casting the gel.

For forming immobilized pH gradients a two-chamber gradient former is used. The gradient former consists of a mixing chamber with an outlet tube and clamp and is connected to a reservoir chamber of identical dimensions by a valve. Each chamber has a capacity of 15 ml. The position of the gradient former on the magnetic stirrer, the stirrer speed, and the height of the gradient former above the mold are critical for ensuring reproducible and

linear gradient formation. Make a note of these parameters for consistent results. Prepare the acidic (dense) solution and the basic (light) Immobiline mixtures as shown in Table 1. The measured pH values of the two limit solutions define the pH range that will be formed. For greatest reproducibility, prepare a large batch of the acidic and basic Immobiline mixtures and cast several gels in one session. When adjusted to neutral pH, these mixtures may be stored for several months at −20°C. Continue preparing the complete mixture as described in Table 1. Once the catalysts are added, work quickly. Place 11 ml of acidic solution in the mixing chamber. Momentarily open and close the valve between the chambers to fill the connecting channel with solution. Place 11 ml of basic solution in the reservoir. Open the outlet of the mixing chamber momentarily to fill the outlet tubing and close again. The magnetic stirrer speed should give adequate mixing without a vortex. Wait several minutes for the stirrer to warm up and attain a constant speed. Open the outlet and immediately open the valve between the mixing chamber and the reservoir. The liquid level in both chambers should drop at the same rate. When the gradient has been completely formed, allow the mold to remain undisturbed for 10 min to permit the gradient to stabilize. Transfer the mold to an oven at 50°C for 1 h to polymerize the gel. Allow the mold to cool to room temperature before disassembly. Wash the gel six times with 300 ml MilliQ water for 15 min each, and finally for 30 min with 2% glycerol. Air-dry the gel in a dust-free environment. Dry only long enough to achieve a uniform appearance to the gel surface. Wrap the gel in plastic film, date, and label. Be sure to label anode and cathode ends. Dried gels can be stored at −20°C for several months.

TABLE 1

Recipes for Preparing Immobilized pH Gradient Gels[a]

Stock solution	pH 4–7 Dense acidic	pH 4–7 Light basic	pH 7–10 Dense acidic	pH 7–10 Light basic
A Immobilines				
p*K* 3.6	846 µl	444 µl	794 µl	132 µl
p*K* 4.6	160 µl	1082 µl	—	—
p*K* 6.2	660 µl	220 µl	—	—
p*K* 7.0	—	396 µl	554 µl	274 µl
p*K* 8.5	—	—	514 µl	512 µl
p*K* 9.3	—	1286 µl	—	412 µl
Water	9.32 ml	7.58 ml	9.12 ml	9.46 ml
Measure pH at room temperature				
Reference pH	4.01 + 0.05	7.02 + 0.14	6.98 + 0.07	9.88 + 0.05
Adjust to pH 7 with 1 M NaOH or 1 M acetic acid.[b] All remaining reagent volumes are the same for each pH range gradient.				
B Acrylamide/bis	3.68 ml	3.68 ml		
C Glycerol	6.16 ml	—		
Water	1.16 ml	7.32 ml		
Add catalysts and immediately begin casting the gradient gel.				
Temed	15 µl	15 µl		
D Ammonium persulfate	80 µl	80 µl		

[a] Volumes are for casting two IPG gels.

[b] At this step stock mixtures of Immobilines may be stored at −20°C under N_2 for a week.

Rehydration and electrofocusing. With the gel still wrapped in plastic film, cut 3.5-mm-wide strips with a sharp blade or paper cutter. Engrave gel numbers on the GelBond PAG film near one end of the gel strips. Assemble a rehydration mold using two glass plates and the U-gasket originally used when casting the gel. Remove the protective plastic film from the gel strips and insert the strips into the mold. Fill the mold with rehydration solution (8 M urea, 0.5% NP-40, 10 mM DTE, and 0.4 mM orange G). Allow a minimum of 6 h for rehydration. Overnight rehydration yields similar results. When disassembling the rehydration cassette, keep the exposed surface of the IPG strips in contact with the glass plate (i.e., GelBond PAG film side up) to prevent the strips from drying until placed in the electrophoresis chamber.

Place the IPG gel strips 3–4 mm apart inside a tray placed on the cooling plate (20°C) of the electrophoresis apparatus. A channeled template in the tray serves to keep individual gel strips parallel to each other and appropriately spaced to accept specially designed sample cups having 100-µl capacity. IPG strips are placed with the acidic end at the anode. Electrode wicks consist of a layer of five strips (5 mm wide) of Whatman 3MM chromatography paper, or 3-mm-thick filter paper, soaked with 8 M urea and lightly blotted. Electrode wicks connect the gel strips at the ends. Install the electrodes and sample cups. Place a few microliters of lysis solution containing bromphenol blue in the sample cups to check for leakage. Cover gel strips with a layer of low-viscosity silicone oil. Alternatively, after 2 h of focusing allowing for sample entry into the gel, remove the sample applicators and cover the IPG strips with thin plastic film.

Sample solubilization. For analytical 2-D PAGE of total cell lysates, add 10 µl solubilization solution for each 1×10^6 frozen cells pelleted in a microfuge tube (~3 µg protein/µl). For preparative loadings, three times this protein concentration is used. Vortex five to six times for 4 to 5 s each over a 1-h period at room temperature to solubilize proteins. Do not cause excessive foaming while vortexing. Centrifuge for 3 min at 15,000*g* in a microfuge immediately prior to loading onto the gel. Avoid salt concentrations in the sample >50 mM. Salts are conveniently removed by diluting the sample to <50 mM salt and concentrating the sample with a Centricon ultrafiltration unit (Amicon) of appropriate molecular weight cutoff.

It should be pointed out that with certain samples, we have obtained 2-D patterns in which the cathodic end of the gel is blank over a margin of variable width, while the

remainder of the pattern appears intact and well focused. This phenomenon seems to be related to some unknown characteristic of the solubilized sample, since in repeat electrophoretic separations involving multiple samples, the same sample(s) displays this pattern consistently. This problem can be remedied by first precipitating proteins in the sample and subsequently solubilizing the precipitate using the standard solubilization cocktail.

Electrophoretic conditions. A 5-kV power supply is required. Initially, the voltage is 400 V. The voltage is doubled every 30 min to a maximum of 5 kV. Focusing is for a minimum of 50 kV-h. Alternatively, for preparative IPG isoelectric focusing we have used a 10-kV power supply. After reaching 5 kV, the voltage is increased to 6 kV for 1 h and 7 kV for a minimum of 6 h.

Second-Dimension SDS–Acrylamide Gel Electrophoresis

The second dimension separates proteins on the basis of molecular weight in an SDS gel. An 11.5 to 14% T (2.6% cross-linking) acrylamide gradient provides effective separation of proteins of mass from 15,000 to 100,000 Da. Proteins outside this range are less well resolved. Proteins with molecular weight less than 10,000 Da electrophorese close to the dye front and are not resolved. We do not use a stacking gel. A stacking gel results in more compact spot shape in the molecular weight dimension with spot elongation in the focusing dimension due to lateral band spreading of the protein as it forms a sharp zone in the stacking gel.

Second-dimension SDS slab gel stock solutions.

A Acrylamide, 30% (w/v) bisacrylamide, 0.8% (w/v). Filter and store at 4°C for 1 week.
B Gel buffer: 1.5 M Tris–HCl, pH 8.5 to 8.6. Store at 4°C.
C Glycerol: 87% (w/w). Store at room temperature.
D SDS: 10% (w/v). Store at room temperature.
E Temed, 40% (v/v). Store at 4°C.
F Ammonium persulfate: 10% (w/v). Store at 4°C for 1 week.
G SDS equilibration buffer: 0.125 M Tris–HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 65 mM dithioerythritol, 6 M urea, and 0.0005% bromphenol blue. Store at 4°C.
H Agarose: 0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS, 0.5% (w/v) agarose. Microwave or boil (>90 min) to dissolve agarose. Store at −20°C.
I Running buffer: 0.025 M Tris, 0.192 M glycine, 0.1% w/v SDS.

Casting polyacrylamide gradient SDS gels. Procedures are described for casting 20 polyacrylamide gradient slab gels simultaneously using the Dalt apparatus sold by Hoefer Scientific. Individual gel cassettes are assembled and stacked in the casting box. The dense (14% T) and light (11.5% T) acrylamide solutions are described in Table 2. Detailed procedures for the use of the Dalt gradient former are available from the manufacturer. Once the acrylamide gradient is cast, unique gel numbers typed with carbon ribbon on Whatman 1MM chromatography paper are inserted at one end of each cassette. The gel solution is overlaid with 1 to 2 ml of water-saturated sec-butanol. Allow the gel to polymerize for 1 h at room temperature. Carefully separate individual gel cassettes, remove polyacrylamide from the outside of the cassettes with a razor blade, and rinse the surface of the gel briefly with water. Place the gel upside down in a vertical position to drain the surface of excess water.

Support the slab gel in a vertical position to facilitate the application of the first-dimension gel. Place six to eight IPG gel strips in a tray with 50 ml SDS equilibration buffer (solution G) and equilibrate for 30 min with agitation. Drain excess equilibration solution from the IPG strips by placing them on their long edge on a lintless absorbent towel. Handle the gel strips by one end and remove about 5 mm of gel that was in contact with the electrode strips. An additional portion of gel in contact with the sample applicator is cut off if necessary so that the IPG strip will fit on the second-dimension gel. Note the orientation, anode and cathode, after trimming the gel strip. Place 1 ml of agarose (solution H) at 90°C onto the top surface of the slab gel and immediately immerse the IPG gel strip in the agarose solution. Carefully press the IPG strip with a spatula onto the surface of the slab gel to achieve complete contact. Allow the agarose to solidify for 5 min and then place the slab gel in the electrophoresis apparatus.

Electrophoresis conditions. Electrophoresis is performed at 10°C. The slab gel should be completely immersed in cold electrophoresis running buffer. Initially, electrophorese at 100 V until the bromphenol blue tracking dye has entered the separating gel about 1 cm (~30 min) and then increase the voltage to 400 V until the tracking dye reaches the bottom of the slab gel (~3 h). Alternatively, electrophorese at 80 V overnight. The current is initially 120 mA per slab gel and decreases to 60 mA at the end of electrophoresis.

TABLE 2

Recipe for Preparing Acrylamide Gradient SDS Gels[a]

Stock solution	Light solution	Dense solution
A Acrylamide/bis	150 ml	182 ml
B Gel buffer	100 ml	100 ml
C Glycerol	—	112 ml
D SDS	4 ml	4 ml
E Temed	100 µl	60 µl
F Ammonium persulfate	3 ml	2 ml
Water	142 ml	—
Final volume	400 ml	400 ml

[a] Volumes are for casting 20 gels 16.5 cm × 16.5 cm × 1.5 mm. The separating gel is an 11.4 to 14% T (2.6% C) acrylamide gradient, containing 0.375 M Tris–HCl, pH 8.5, and 0.1% (w/v) SDS.

CONCLUDING REMARKS

The procedures presented are for the most part identical for analytical and preparative 2-D gels. The major aspect of preparative IPG that requires consideration is the optimization of sample entry into the gel. Attention should be paid to avoid urea crystallization, which could occur in a dry atmosphere. High salt content in the sample should be reduced, and electrophoretic conditions should be modified (usually by reducing initial voltage) to limit protein precipitation in concentrated samples. This is particularly the case for structural proteins with a large molecular weight.

An important issue encountered with the initial use of IPG is the ability to relate the IPG-based pattern to previously obtained patterns, for the same type of samples, using CA-based separations. We have observed that in broad pH gradients such as 4–7, there exists substantial similarity in 2-D patterns between IPG- and CA-based gels. Constellations that include major spots are easily recognizable between patterns, allowing alignment of IPG- and CA-based patterns. Important circumstances in which to consider the use of IPG would be the need for high resolution, made possible by narrow gradients, the need for reproducible focusing of basic polypeptides, and the interest in isolating sufficient amount of a polypeptide for structural studies or for generating antibodies.

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