Review

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The current state of two-dimensional electrophoresis with immobilized pH gradients

The original protocol of two-dimensional electrophoresis with immobilized pH gradient (IPG-Dalt; Görg *et al.*, *Electrophoresis* 1988, *9*, 531–546) is updated. Merits and limits of different methods for sample solubilization, sample application (by cup-loading or in-gel rehydration) with respect to the pH interval used for IPG-isoelectric focusing are critically discussed. Guidelines for running conditions of analytical and micropreparative IPG-Dalt, using wide IPGs up to pH 12 for overview patterns, or narrow IPGs for zoom-in gels for optimum resolution and detection of minor components, are stated. Results with extended separation distances as well as automated procedures are demonstrated, and a comparison between protein detection by silver staining and fluorescent dyes is given. A brief trouble shooting guide is also included.

Keywords: Immobilized pH gradient / Two-dimensional polyacrylamide gel electrophoresis / Narrow gradients / Wide gradients / Alkaline proteins / Automation / Review EL 3871

Contents

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Abbreviations: DMAA, *N,N*-dimethylacrylamide; **IPG-Dalt**, two-dimensional gel electrophoresis with immobilized pH gradient

1 Introduction

More than ten years ago, a basic protocol of 2-D electrophoresis with immobilized pH gradients was described [1], summarizing the critical parameters inherent in isoelectric focusing with IPGs and a number of experimental conditions which were not part of the classic 2-D electro-

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phoresis repertoire with carrier ampholytes [2, 3]. Meanwhile, this procedure has become the standard procedure of IPG-Dalt for proteome research [4–8], permitting higher resolution [1, 9], improved reproducibility for interlaboratory comparisons [10, 11], higher loading capacity [12, 13] for micropreparative 2-D electrophoresis with subsequent spot identification by mass spectrometry [14, 15], Edman degradation and amino acid composition analysis [16], as well as the separation of basic proteins under equilibrium conditions [17–21].

The protocol described in 1988 [1] is still valid today: IEF is performed in individual IPG gel strips, and after equilibration with SDS buffer in the presence of urea, glycerol, and iodoacetamide, they are applied to a horizontal or vertical SDS gel. However, in order to visualize the global changes in protein expression for proteome analysis, 2-D electrophoresis should be able to resolve and separate the potentially hundreds or thousands of protein forms (including post-translational variants) present in a particular cell or tissue. For this highly demanding effort, several requirements must be met which allow (i) the extraction of all protein components, including hydrophobic membrane proteins, proteins with extreme p*I* (below pH 3 and above pH 10), as well as low copy number proteins in the presence of most abundant proteins, and (ii) a high resolution separation for the detection of a maximum of spot numbers, avoiding the presence of multiple proteins per spot and/or cross contamination of spots in 2-D gels. The latter can be achieved by using multiple overlapping, narrow IPGs in the first dimension (zoom-in gels) [1, 22] or by extending the separation distance [21], whilst a single wide pH gradient, *e.g.*, IPG 3–12 [21], offers an overview of the state of the proteome. The only technique currently available with sufficient resolving power for proteome analysis is 2-D electrophoresis. Owing to its still increasing resolving power, reproducibility, relative and increasing simplicity, IPG-Dalt has become the core technology of proteome research. The actual protocol of IPG-Dalt, as well as recent modifications with respect to sample application, running conditions, equilibration and the use of different pH gradients up to pH 12, extended separation distances, and automated procedures will be critically discussed in this paper.

2 The current protocol

2.1 Apparatus and chemicals

All equipment for IEF and SDS electrophoresis (IPGphor, Multiphor II horizontal electrophoresis unit, Hoefer DALT multiple vertical electrophoresis unit, EPS 3500 XL power supply, Multitemp II thermostatic circulator, Immobiline DryStrip Kit, gradient mixer, reswelling cassette and reswelling tray), Immobiline II, Pharmalyte 3–10, IPG buffers, Immobiline DryStrips, ExcelGel SDS, GelBond PAG-film, Repel Silane, TEMED and ammonium persulfate were from Amersham Pharmacia Biotech (Uppsala, Sweden). Acrylamido buffer solutions, p*K* 1.0, 10.3 and p*K* > 13, were a gift from Bengt Bjellqvist (Amersham Pharmacia Biotech). The Molecular Imager™ FX (Phosphor- and Fluoro-Imager) was purchased from Bio-Rad (Richmond, CA, USA), and the MD 300 laser densitometer from Molecular Dynamics (Sunnyvale, CA, USA). Acrylamide (2 × cryst.), *N,N*-methylenebisacrylamide and SDS were from Serva (Heidelberg, Germany). Thiourea, silicone oil, and *N,N*-dimethylacrylamide (DMAA) were obtained from Fluka (Buchs, Switzerland). DTT, iodoacetamide, Trizma base and agarose (Type I-a) were from Sigma (St. Louis, MO, USA). Argon was from Messer-Griesheim (München, Germany). CHAPS was from Boehringer Ingelheim (Ingelheim, Germany). SyproRuby was from Molecular Probes (Eugene, OR, USA). Urea, glycerol, Pefabloc® and all other chemicals (analytical grade) for electrophoresis and staining were obtained from Merck (Darmstadt, Germany).

2.2 Sample preparation

In general, deep-frozen cells or tissue (*e.g.*, mouse liver, myeloblast cells, yeast, plant seeds) were disrupted by different techniques such as grinding in a liquid nitrogen-cooled mortar, sonication, shearing-based methods, or homogenization. Proteins were then solubilized with sonication either (i) in lysis buffer [2] (9 M urea, 1% w/v DTT, 2–4% w/v CHAPS, 2% v/v carrier ampholytes, pH 3–10, and 10 mM Pefabloc® proteinase inhibitor); (ii) in thiourea lysis buffer [23] (2 M thiourea, 7 M urea, 2–4% w/v CHAPS, 1% w/v DTT and 2% v/v carrier ampholytes, pH 3–10, and 10 mM Pefabloc® proteinase inhibitor, or (iii) in hot SDS sample buffer (1% w/v SDS, 100 mM Tris-HCl, pH 7.0) and then diluted with an at least threefold exess of lysis buffer or thiourea lysis buffer [24]. Plant leaf proteins were solubilized according to [25]. Preparation of total ribosomal proteins (Tp802) from rat liver and HeLa cells was according to [26], preparation of histones from chicken erythrocytes according to [27]. TCA/acetone extract of mouse liver was prepared according to [18]. Briefly, mouse liver was ground in a liquid nitrogen-cooled mortar, and the obtained powder was immediately suspended in 20% TCA in acetone (–18°C) containing 0.2% DTT and kept at –18°C overnight in order to ensure complete protein precipitation. Following centrifugation, the supernatant was discarded and the pellet resuspended in acetone containing 0.2% DTT. The sample was spun again, the supernatant discarded and the pellet was dried under vacuum and then solubilized in lysis buffer and centrifuged (40 000 × *g*, 60 min, 15°C). The clear supernatants were stored in aliquots at –78°C until analyzed.

2.3 First dimension: IEF with IPG

The first dimension of IPG-Dalt, isoelectric focusing (IEF), was performed in individual 3 mm wide IPG gel strips cast on GelBond PAGfilm (either ready-made Immobiline DryStrips or laboratory-made). Samples were applied by cup-loading or by in-gel rehydration. IPG-IEF can be simplified by use of an integrated system, the IPGphor [21, 28, 29] where rehydration with sample solution and IEF are performed in a one-step procedure (see Section 2.3.4). The protocol of IPG-Dalt described in 1988 [1] with some modifications is valid for wide gradients in the pH range between 3–12 (*e.g.*, IPG 4–7, 4–9, 6–10, 3–10, as well as for IPG 3–12, 4–12, or 6–12), and for narrow IPGs in the acidic and neutral range ("zoom-in gels", *e.g.*, IPG 5–6). For narrow pH gradients above pH 9 (*e.g.*, IPG 9–12 or 10–12), a different protocol, minimizing the reverse electroendosmotic flow, was described [18, 20].

2.3.1 IPG gel casting

IPG gels (4%T, 3%C, 0.5 mm thick, 40, 110, 180 mm or 240 mm long) on GelBond PAGfilm were cast, washed, dried, and cut into individual 3 mm wide IPG gel strips by the procedure described previously [1, 30, 31]. To cast IPG gels pH 9–12 and pH 10–12, acrylamide was substituted by DMAA. When DMAA was used as monomer, %T was increased to 6% (instead of 4% acrylamide as usual) [18]. IPG gels with gradients 3–12, 4–12, 6–12, 8–12, and 9–12 [18–21], as well as narrow (1.5 pH units) IPGs in the acidic and neutral range between pH 3.5 and 7.5, were calculated (using the computer programs of Altland [32] and Giaffedra *et al.* [33]), tested, and optimized. A large number of recipes for a variety of IPGs have also been calculated by Righetti and co-workers [34, 35]. For IPG gel casting, a mold consisting of two glass plates, one covered with GelBond PAGfilm, the other bearing a 0.5 mm thick U-frame (Fig. 1A), was loaded from the top with the help of a gradient mixer according to the casting procedure of Görg *et al.* [36, 37] for ultrathin pore gradient gels (Fig. 1B). Prior to gel casting, the glass plate which bears the U-frame had to be made hydrophobic by treatment with Repel Silane in order to prevent the gel from sticking to the glass plate. Two Immobiline starter solutions (an acidic one and a basic one) were prepared as reported [1, 30, 31]. For better polymerization, the acidic and basic solutions had to be adjusted to pH 7 with sodium hydroxide and acetic acid, respectively. The acidic, dense solution was pipetted into the mixing chamber and the basic, light solution into the reservoir of the gradient mixer (Fig. 1B). When a pH plateau (2 cm wide) for the sample application area was desired, an extra portion of the dense solution was prepared and pipetted into the mold prior to pouring the gradient. After pouring the gradient into the precooled mold (refrigerator), the mold was kept at room temperature for 15 min to allow adequate leveling of the density gradient prior to polymerization for 1 h at 50°C. After polymerization, the IPG gel was removed from the mold and extensively washed with deionized water (6 × 10 min), immersed in 2% glycerol (30 min), and dried at room temperature in a dust-free cabinet, covered with a plastic film and, if not used immediately, stored at –20°C. The dried gels were stored frozen for up to one year. Prior to use, the IPG gel was cut into individual, 3 mm wide strips with the help of a paper cutter (Fig. 1C) [1]. Instead of laboratory-made gels, ready-made gels (Immobiline DryPlate or Immobiline DryStrip) were used, too.

2.3.2 Rehydration of IPG strips

IPG dry strips (ready-made or laboratory-made) are rehydrated with reswelling solution for cup-loading or directly with sample solution. (i) For sample in-gel rehydration [38, 39], the total cell/tissue lysate (0.5–5 mg of protein) was directly solubilized in 500 μL of a solution containing 8 M urea (or, alternatively, 2 M thiourea and 6 M urea), 0.5–4% CHAPS, 0.4% DTT and 0.5% v/v Pharmalyte 3–10. For 180 mm long and 3 mm wide IPG dry strips, 350 μL of this solution was pipetted into the grooves of the reswelling tray (Fig. 1E) or into the IPGphor strip holder (see Section 2.3.4 and Fig. 1F). For longer or shorter IPG strips, the rehydration volume was adjusted accordingly. The IPG strips were then inserted (gel side down) into the grooves without trapping air bubbles covered with silicone oil, and rehydrated overnight. (ii) For cup-loading, the IPG dry strips were rehydrated overnight to their original gel thickness (0.5 mm) in a reswelling solution containing 8 M urea (or, alternatively, 2 M thiourea and 6 M urea). 0.5–4% CHAPS, 0.4% DTT, and 0.5% v/v Pharmalyte 3–10. Rehydration of individual IPG strips was either performed in a reswelling cassette [1] (Fig. 1D) or in the reswelling tray, however, without sample (Fig. 1E). For IEF in very basic, narrow-range IPGs such as 9–12 or 10–12, the rehydration solution was modified as follows. For ribosomal proteins, reswelling solution was composed of 8 M urea, 10% v/v 2-propanol, 10% v/v glycerol, 1% w/v CHAPS, 20 mM DTT, 0.5% v/v Pharmalyte 3–10, and 0.2% w/v methylcellulose (or, alternatively, 16% v/v 2-propanol, 10% glycerol, 1% w/v CHAPS, 20 mM DTT and 0.5% v/v Pharmalyte 3–10). For IEF of histones the reswelling solution contained 10% v/v 2-propanol, 10% v/v glycerol, 20 m). For IEF of histones the reswelling solution containd 10% v/v 2-propanol, 10% v/v glycerol, 20 mM, DTT, and 0.5% v/v Pharmalyte 3–10, but no CHAPS [18, 20].

2.3.3 IEF on the Multiphor

Prior to IEF, the rehydrated IPG gel strips were rinsed with distilled water for 1 s and then blotted between two sheets of moist filter paper to remove excess reswelling

solution; this was done to avoid urea crystallization on the gel surface, which is held responsible for prolonged IEF and "empty" vertical lanes in the stained 2-D pattern. (i) For sample cup-loading, up to 40 rehydrated IPG strips were placed, side-by-side and 1–2 mm apart, onto the kerosene-wetted flat-bed cooling plate of the electrofocusing chamber (Fig. 1G), with the acidic end of the IPG gel strips facing the anode. Electrode paper strips (cut from 1 mm thick filter paper, *e.g.*, MN 440, Machery & Nagel, Düren, Germany) were soaked with deionized water, blotted against filter paper to remove excess liquid, and placed on top of the aligned IPG gel strips near the cathodic and anodic ends. When running basic IPGs 6–10, an extra paper strip soaked with 0.4% DTT was applied onto the IPG gel surface near the cathodic electrode strip [40]. Samples (20 μL; protein concentration 5–10 mg/mL) were pipetted into silicone rubber frames (size: 2 x 5mm) which had been placed on the gel surface, 5 mm from the anode or cathode. Alternatively, the Immobiline DryStrip kit was used (Fig. 1H). A 100 μL sample volume can be applied at a time; it is possible to apply a total of up to 200 μL onto a single IPG gel strip (for details see Immobiline DryStrip Kit Instruction Manual). For analytical purposes, typically 50–100 μg of protein were loaded onto a single, 180 mm long IPG gel strip. For micropreparative purposes up to several mg of protein were applied. When very basic pH gradients exceeding pH 10 (*e.g.*, IPG 3–12, 4–12, 6–12, 9–12, 10–12) or narrow-range ("zoom-in" gels) pH gradients (1 or 1.5 pH units) with extended focusing time were used, the IPG strips were covered by a layer of silicone oil. During IEF, electrode paper strips were replaced by fresh ones every 6 h. In case of IEF with very alkaline narrow-range IPGs, such as IPG 10–12, this procedure was performed once per hour. Alternatively, the cathodic electrode paper strip was wetted with water hourly to prevent it from drying out. (ii) The IPG strips rehydrated with sample solution (see Section 2.3.2) were put directly on the kerosene-covered cooling plate. When IEF was performed under a layer of silicone oil, the rehydrated IPG strips were placed in the grooves of the strip aligner of the Immobiline DryStrip kit and covered with silicone oil that had been degassed and flushed with argon prior to use. Electrode paper strips, soaked with deionized water and blotted with filter paper, were placed on the surface of the IPG strips. For running conditions see Table 1. For optimum 2-D patterns, initial voltage was limited to 50–150 V and then increased stepwise to 3500 V. IEF was continued at 3500 V to the steady state. Current and power settings were limited to 0.05 mA and 0.2 W per IPG gel strip, respectively. Optimum focusing temperature was 20°C [41]. Running conditions depended on parameters such as the pH interval used, separation distance, additives to the rehydration solution, sample amount, and temperature [17–21, 30, 31, 42]. Typical values for orientation are given in Table 1. After IEF, those IPG gel strips that were not used immediately for the second dimension, or which were kept for further reference, could be stored between two sheets of plastic film at –78°C for several months (Fig. 1K).

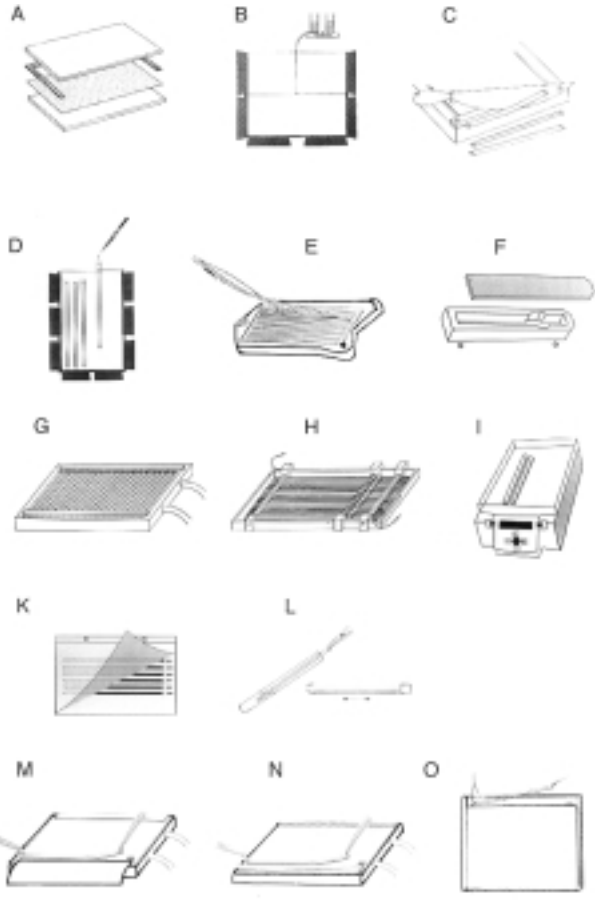

Figure 1. Procedure of IPG-Dalt [1, 31]. (A) Assembly of the polymerization cassette prior to casting IPG and SDS gels on plastic backing (glass plates, GelBond PAGfilm, 0.5 mm thick U-frame). (B) Casting of IPG and/or SDS pore gradient gels. (C) Cutting of washed and dried IPG slab gels (or Immobiline DryPlates) into individual IPG strips. Rehydration of individual IPG gel strips (D) in a vertical rehydration cassette, (E) in the reswelling tray, and (F) in the IPGphor strip holder. IEF in individual IPG gel strips (G) placed directly on the cooling plate of the IEF unit, (H) in the DryStrip kit, and (I) on the IPGphor. (K) Storage of the IPG strips after IEF. (L) Equilibration of IPG gel strips prior to SDS-PAGE. Transfer of the equilibrated IPG gel strip onto the surface (M) of laboratory-made horizontal SDS gel alongside the cathodic electrode wick or (N) onto a ready-made horizontal SDS gel along the cathodic buffer strip. (O) Loading of the equilibrated IGP gel strip onto a vertical SDS gel.

2.3.4 IEF with IPGphor

The total cell/tissue lysate (0.5 – 5 mg of protein) was solubilized in 500 μL of a solution containing 8 M urea (or, alternatively, 2 M thiourea and 6 M urea), 0.5–4% CHAPS, 0.4% DTT and 0.5% v/v Pharmalyte 3–10. The required number of strip holders (up to 12) was put onto the cooling plate's electrode contact area of the IPGphor (Fig. 1I), and 350 μL of sample-containing rehydration solution (for 180 mm long IPG strips) was evenly pipetted in the strip holder channel, 1–2 cm from the electrodes. The IPG strips were lowered, gel-side-down, onto the rehydration solution without trapping air bubbles, and overlayered with 1 mL of silicone oil before the plastic cover was applied. Once the safety lid was closed, rehydration and IEF were carried out automatically according to the programmed settings (Table 2), preferably overnight. As indicated in Table 2, low voltage (20–50 V) was already applied during the rehydration step for improved sample entry of high M_r proteins into the polyacrylamide gel [21]. IEF was then started with low voltage (200 V, followed by 500 V and 1000 V). For optimum results in case of samples with high salt concentrations, or when narrow pH intervals are used, it is beneficial to insert moist filter papers (size: 4×4 mm^2) between the electrodes and the IPG strip prior to raising the voltage to 8000 V. After terminating IEF, the IPG strips were stored as described in Section 2.3.3.

2.4 Equilibration of IPG strips

Prior to the second dimension (SDS-PAGE on horizontal or vertical systems), the IPG strips were equilibrated for 2 $\times$ 15 min with gentle shaking (Fig. 1L) in 10 mL of a solution containing Tris-HCl buffer (50 mM, pH 8.8), 6 M urea, 30% w/v glycerol, 2% w/v SDS, and a trace of bromophenol blue. DTT (1% w/v) was added to the first, and iodoacetamide (4% w/v) to the second equilibration step [1, 43]. After equilibration, the IPG strips were aligned on filter paper along one edge for 1 min to remove excess liquid before they were applied to horizontal or vertical SDS gels [1, 30, 31].

2.5 Second dimension: SDS-PAGE

SDS-PAGE was either performed on horizontal or on vertical systems. Horizontal systems are ideally suited for the use of ready-made gels (*e.g.,* ExcelGel SDS^{TM}), whereas vertical systems (*e.g.*, the Hoefer DALT multiple slab gel unit [44, 45] are preferred for multiple runs in parallel.

2.5.1 SDS-PAGE on horizontal systems

Horizontal SDS electrophoresis was performed on ready-made (ExcelGel) or laboratory-made slab gels cast onto GelBond PAGfilm by the casting procedure described by Görg *et at.* [1, 36, 37] for ultrathin pore gradient gels. Gel size was $250 \times 195 \times 0.5$ mm^3. Typically, the laboratory-made SDS gels were composed of a 6%T stacking gel (minimum length, 40 mm) and a 12–15%T linear gradient resolving gel. On several occasions, 13%T homogeneous resolving gels were used as well. In both stacking and resolving gel, cross-linker concentration was 3%C. The buffer system was 375 mM Tris-HCl, pH 8.8, and 0.1% SDS in both stacking and resolving gel. Paper wicks (*e.g.*, Ultra Wicks; Bio-Rad), soaked in electrode buffer (192 mM glycine, 25 mM Tris, 0.1% w/v SDS) [46] were placed on the gel surface so that they overlapped the gel ends by 10 mm while the other end was immersed into the electrode buffer reservoir. Paper wicks were substituted by electrode buffer strips made of polyacrylamide when ready-made gels (ExcelGel SDS, Tris-Tricine buffer system, linear acrylamide gradient from 12 to 14%T) were used. The equilibrated and blotted IPG strips were transferred onto the SDS gel by simply placing one long (240 mm or 180 mm) or several shorter (*e.g.,* 110 mm or 70 mm) IPG strips, gel side down, onto the surface of the stacking gel alongside the cathodic electrode paper wick (Fig. 1M) or buffer strip (Fig. 1N). The optimum distance between the IPG strip and the buffer strip (or electrode wick) is 2–3 mm. Electrophoresis was performed with a maximum voltage of 150 V for about 60 min until the bromophenol blue dye had migrated off the IPG strip(s) by 3–4 mm. Then the IPG strips were removed from the surface of the SDS gel and the cathodic electrode wick (or buffer strip, respectively) was moved forward by 5 mm so that it overlapped with the former application area of the IPG strip. In case of a rather wet IPG strip application area, excess liquid was removed from the SDS gel surface by blotting the IPG strip application area with a moist filter paper. Electrophoresis was continued at maximum settings of 600 V (30 mA, 30 W) when using laboratory-made gels, and 800 V (40 mA, 30 W max.) in the case of ExcelGel SDS. The temperature of the cooling plate was set to 20°C. Total running time was approximately 4–5 h.

2.5.2 Mulitple SDS-PAGE on vertical systems

Multiple SDS slab gel casting and vertical SDS electrophoresis were performed as described [44, 45]. Gel thickness was 1 mm, total acrylamide concentration typically 13%T homogeneous, and cross-linker concentration 2.7%C. Deviating from the horizontal procedure no stacking gel was required. To load the equilibrated IPG strip, the gel cassettes were placed in an upright position and filled with 2–3 mL of hot (75°C) agarose solution containing 0.5% agarose in electrode buffer (25 mM Tris, 192 mM glycine, 0.1% w/v SDS and 0.03% w/v bromophenol blue). Then the IPG strip was inserted between the glass plates with a spatula and brought in close contact with the upper edge of the SDS gel (Fig. 10). After the

agarose had set, ten gels were simultaneously run overnight in the Dalt tank (settings: 200 V, 150 mA, 20°C).

2.6 Visualization of proteins

When the bromophenol blue front had completely migrated out of the SDS gel, the resolved polypeptides were fixed in ethanol/acetic acid/water (4/1/5) for at least 1 h (but normally overnight). Analytical gels were usually stained with silver nitrate [47–50], whereas micropreparative gels were preferably stained with Coomassie Brilliant Blue (0.1% in ethanol/acid/ water 4/1/5) for 1–3 h. Then the gel was destained, however not completely, with ethanol/acetic acid/water (3/1/6) and then immersed in distilled water overnight to intensify the spot color. Postelectrophoretic fluorescent labeling was carried out with SYPRO Ruby. After fixing in acetic acid/methanol/water (7/10/83) for 30 min, up to four gels were simultaneously stained overnight in 500 mL of staining solution. Proteins which had been radiolabeled with [^{35}S]methionine were visualized by phosphor imaging as follows: After SDS-PAGE, the 2-D gels were directly dried onto a sheet of filter paper with the help of a vaccum gel dryer [51]. The dried gels were then exposed to phosphor imaging plates (Kodak, Rochester, NY, USA) for 24–96h. SYPRO Ruby and [^{35}S]methionine-labeled proteins were visualized using the Molecular Imager FX.

2.7 Pattern evaluation

Silver and Coomassie blue-stained gels were digitized at 88 μm resolution using the MD 300 laser densitometer, whereas SYPRO Ruby and [^{35}S]methionine-labeled gels or phosphor imaging plates were scanned at 100 μm resolution using the Molecular Imager FX. Computerized 2-D gel analysis (spot detection, spot editing, pattern matching, database construction) was performed with the help of the Image Master II software package.

3 Sample handling and entry

3.1 Sample preparation and prefractionation

The main problem in the visualization of total cell or tissue extract proteins is the highly dynamic range of expression of proteins and the chemical diversity of proteins over a wide range of molecular weights, isoelectric points, and solubilities. Although a one-step procedure for protein extraction would be highly desirable with respect to simplicity and reproducibility, there is no single solution for all protein categories. The most popular IEF sample solubilization buffer is still based on O'Farrell's [2] lysis buffer (9 M urea, 4% NP-40 and 50–100 mM DTT). However, this standard IEF sample solution is not ideal for the solubilization of all protein classes, in particular for membrane or other hydrophobic proteins. Merits and limits of new detergents, chaotropes, and reducing agents have been thoroughly discussed by Rabilloud *et al.* [23, 52–55] and Herbert *et al.* [56–58] .

The current protein solubilization procedures used in our laboratory are solubilization with (i) modified O'Farrell's lysis buffer (9 M urea, 2–4% CHAPS, 1% DTT, 2% v/v carrier ampholytes), (ii) thiourea/urea lysis buffer (2 M thiourea, 7 M urea, 2–4% v/v CHAPS, 1% DTT, 2% carrier ampholytes) [23], (iii) boiling with SDS sample buffer, followed by dilution with excess urea or thiourea/urea lysis buffer [59, 24], and (iv) TCA/acetone precipitation of proteins and resolubilization in urea (or thiourea/urea) lysis buffer [18]. Solubilization of protein in boiling SDS buffer followed by dilution with lysis buffer is a useful procedure in order to increase the solubilization of the majority of proteins and in order to inhibit protease activity during sample preparation. However, horizontal streaks in the 2-D pattern are observed when the samples initially solubilized in 1% SDS are not diluted with an at least threefold excess of urea (or thiourea/urea) lysis buffer to displace the SDS from the proteins and to replace it with a nonionic or zwitterionic detergent. Additionally, obtaining sufficient dilution can become a problem when micropreparative protein loads are required. TCA/acetone precipitation has been found useful (i) for the inactivation of proteases to minimize protein degradation, (ii) removal of interfering compounds, and, especially, (iii) for the enrichment of very alkaline proteins such as ribosomal proteins from total cell lysates [18]. However, attention has to be paid to protein losses due to incomplete precipitation and/or resolubilization of proteins.

Due to the high dynamic range and diversity of expressed proteins in eucaryotic tissues it is sometimes advisable to carry out a prefractionation step to reduce the complexity of the sample, and/or to enrich certain proteins (*e.g.,* low-copy number proteins or alkaline proteins). Prefractionation of proteins can be achieved either (i) by isolation of cell compartments and/or organelles (*e.g.*, ribosomes, nuclei, mitochondria, or plasma membrane fractions [60] by high-speed centrifugation, (ii) by sequential extraction procedures with increasingly powerful solubilizing buffer (usually aqueous buffers, organic solvents such as ethanol or chloroform/methanol, and detergent-based extraction solutions) [58, 61, 62], (iii) by precipitation (*e.g.*, with TCA/acetone) [18], or (iv) by separation methods such as free-flow electrophoresis [63], chromatography, and/or affinity purification of protein complexes.

3.2 Geometry of the IPG gel strip

The gel thickness as well as the width of the strip should be kept at a minimum, as described in 1988 [1], in order to minimize the electroendosmotic effects caused by the

immobilized buffering groups in the IPG gel matrix with concomitant gel gluing and hindered protein transfer from first to second dimension. The addition of glycerol and urea to the SDS equilibration buffer [1] is the only remedy to date. In contrast to gel width or thickness, the separation distance of the IPG strip can be easily increased from 4 cm [1] to 11, 18 [1], or 24 cm [21], or even more. Limitations are only given by the equipment used (power supply, size of the IEF chamber).

3.3 Sample application procedures: merits and limits

3.3.1 Sample application

Samples can be applied to the IPG strip either by cup-loading [1] or by in-gel rehydration [38, 39]. In case of cup-loading, samples are applied either at the anode or cathode. However, in our experience, sample application near the anode proved to be superior to cathodic application, with some exceptions. For example, human cardiac proteins were preferably applied at the cathode [10], whereas not only most kinds of plant proteins but also mouse liver, yeast cell proteins, *etc.*, yielded best results when applied at the anode. When using basic pH gradients such as IPGs 6–10, 7–10, or 6–12, anodic application was preferred for all the different samples investigated [1, 17–21, 31, 40].

3.3.2 Sample amount and sample volume

The optimum amount of protein to be loaded onto a single IPG gel strip for optimum resolution, maximum spot numbers, and minimum streaking/background smear depends on parameters such as pH gradient (wide or narrow), separation distance, and protein complexity of the sample. (i) For analytical 2-D electrophoresis followed by silver staining, 50–100 μg of protein of a total cell lysate per IPG strip being 180 mm long and 3–6 pH units wide, proved to be the optimum for the majority of samples. Samples can be applied by cup-loading or in-gel rehydration. The optimum sample volume for cup loading is 20–100 μL. Volumes less than 20 μL are not recommended because of the increased risk of protein precipitation at the application point. (ii) For micropreparative 2-D electrophoresis, in-gel rehydration is preferred (Fig. 2), although up to 1 mg of protein (sample volume: 100 μL) was successfully applied by cup-loading, using an IPG 4–7 [12]. By using narrow pH gradients (1 pH unit), up to 10 mg of protein were loaded onto a single IPG gel strip, either by (repeated) sample cup application [13], or by in-gel rehydration [38, 39]. High protein load, which may lead to overloaded and distorted patterns is usually more problem in the second dimension (SDS-PAGE) than in the first-dimensional run. Nevertheless, protein concentration in the sample solution should not exceed 10 mg/mL. Otherwise, loss of protein and horizontal or vertical streaking due to protein aggregation and precipitation may occur. In case of in-gel rehydration, IPG dry strips have to be reswollen with a defined sample volume (see Section 3.3.3). Optimum sample volume is 350 μL for an 18 cm long and 3 mm wide IPG strip (see below). For longer or shorter strips, the sample volume has to be calculated correspondingly.

3.3.3 Sample entry

In general, for proper sample entry, voltage has to be limited during the initial stage of IEF, depending on the protein amount, sample volume, and conductivity of the sample solution (*e.g.*, salt, carrier ampholytes, *etc.*). For example, for analytical runs in combination with cup-loading (sample volume: 20 μL), voltage has to be restricted to 150 V during the first 60 min and to 300 V during the next 60 min (Table 1). When more than 20 μL are applied, IEF at 300 V is prolonged for 30 min each per additional 10 μL. Critical samples with high salt concentrations can be desalted directly in the IPG gel by restricting the voltage to 50–100 V during the first 4–5 h [40]. In case of large sample volumes (micropreparative runs and/or narrow IPGs), voltage should be limited to 50 V overnight for improved sample entry (Table 1).

In case of in-gel rehydration, protein losses are observed when sample volumes for rehydration of the dry IPG strip significantly exceed the calculated amount of the IPG gel strip volume (*e.g.*, 0.5 × 3 × 180 mm) because proteins preferably remain in the surplus solution instead of entering the IPG gel matrix. Although in-gel rehydration works with the majority of samples, it needs to be confirmed that alkaline, hydrophobic and/or high M_r proteins have entered the IPG gel properly. By applying low voltage (30 V) during reswelling using the IPGphor, protein entry, especially of high M_r proteins, is improved [21]. In-gel rehydration is successfully applied for wide IPGs, *e.g.*, IPG 4–7, 4–9, or 4–10, and narrow IPGs with one pH unit in the pH range between 3.5–7.5 [22], whereas for very alkaline IPGs, such as 9–12 and 10–12 [18], cup-loading at the anode is still required.

4 Optimization of running conditions

IEF has to be performed to the steady state in order to obtain optimum reproducibility and pattern quality [1, 17]. The total number of Vh to reach the steady state depends on sample load, pH interval used, separation distance, *etc.*, as described in the following sections.

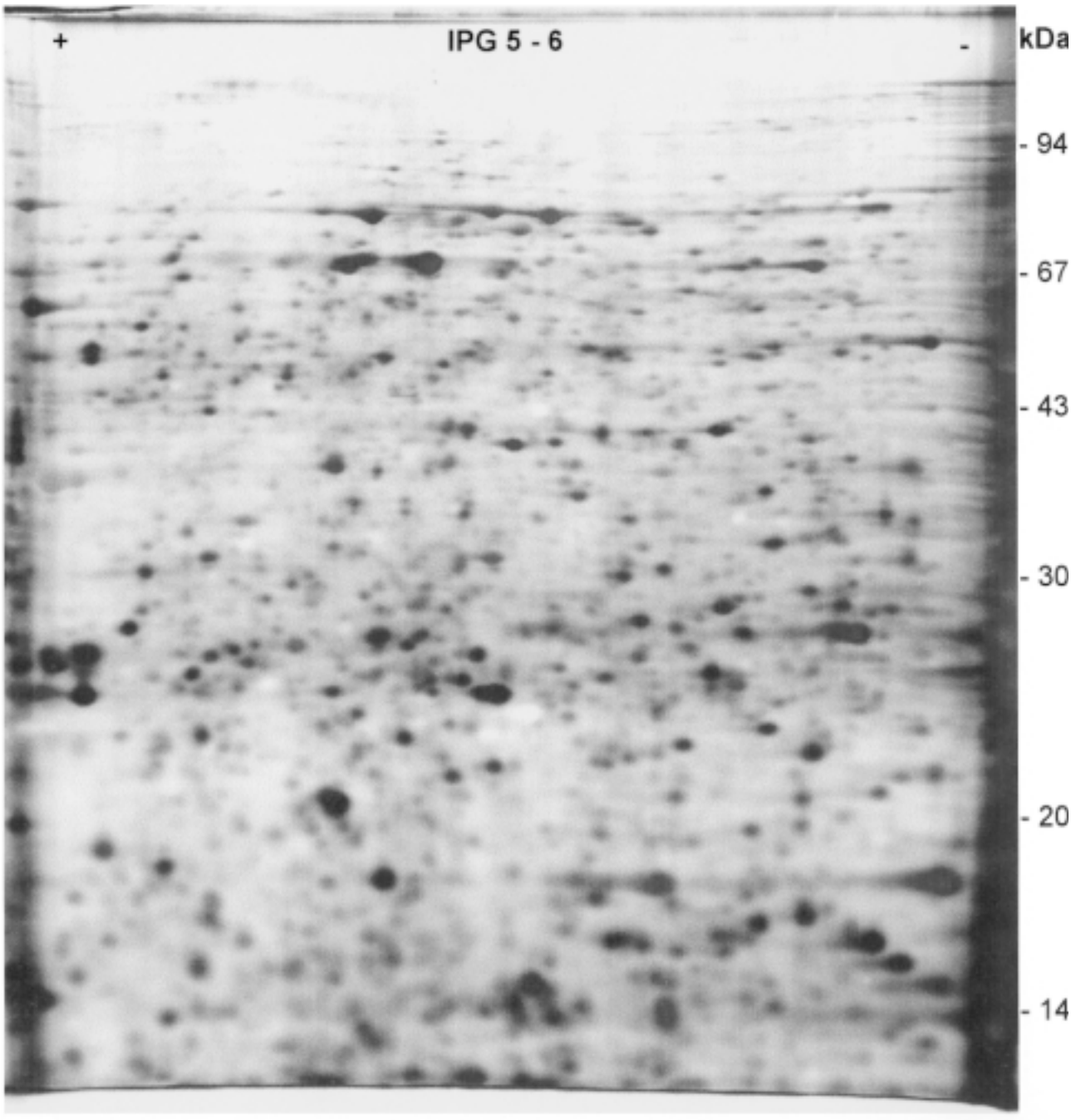

Figure 2. Micropreparative IPG-Dalt of mouse liver proteins. First dimension, IPG 5–6 (ready-made); separation distance, 180 mm; sample application by in-gel rehydration (500 μg protein); IEF was performed on the IPGphor; running conditions: 12 h at 8000 V max. (for details see Table 1). Second dimension, vertical SDS-PAGE (13%T constant); silver stain.

4.1 Immobilized pH gradients

4.1.1 Wide pH intervals with 3–7 pH units up to pH 10

Wide IPGs up to pH 10, such as IPG 4–7, 4–9, or 3–10 are still run according to the protocol described in 1988 [1], with minor modifications. These gradients are compatible with cup-loading as well as with in-gel rehydration, and they work well on the Multiphor and on the IPGphor. Wide IPGs up to pH 10 are ideally suited for analytical (sample load, 50–100 μg) and – at least to a certain extent – for micropreparative runs (sample load: up to 1 mg). For typical running conditions, see Tables 1 and 2.

Table 1. Running conditions using the Multiphor

Gel length	180 mm	
Temperature	20°C	
Current max.	0.05 mA per IPG strip	
Power max.	0.2 W per IPG strip	
Voltage max.	3500 V	
I Analytical IEF		
Initial IEF:		
Cup loading (20–50 μL)	In-gel rehydration (350 μL)	
150 V, 1 h	150 V, 1 h	
300 V, 1–3 h	300 V, 1–3 h	
600 V, 1 h		
IEF to the steady state at 3500 V:		
1–1.5 pH units	4 pH units	7 pH units
e.g. IPG 5–6 .... 24 h	IPG 4–8 ..... 10 h	IPG 3–10 L ..... 6 h
e.g. IPG 4–5.5.. 20 h	IPG 6–10 ..... 10 h	IPG 3–10 NL .. 6 h
3 pH units	5–6 pH units	8–9 pH units
IPG 4–7 ..... 12 h	IPG 4–9 ..... 8 h	IPG 3–12 ..... 6 h
IPG 6–9 ..... 12 h	IPG 6–12 ..... 8 h	IPG 4–12 ..... 8 h
II Extended separation distances (240 mm)		
IEF to the steady state at 3500 V:		
IPG 3–12 ..... 8 h		
IPG 4–12 ..... 12 h		
IPG 5–6 ..... 40 h		
III Micropreparative IEF		
Initial IEF:		
Cup loading (100 μL)	In-gel rehydration (350 μL)	
50 V, 12–16 h	50 V, 12–16 h	
300 V, 1 h	300 V, 1 h	
IEF to the steady state at 3500 V:		
Focusing time of analytical IEF plus approximately 50%		

4.1.2 Narrow IPGs with 1–1.5 pH units

With complex samples such as eukaryotic cell extracts, 2-D electrophoresis on a single wide-range pH gradient reveals only a small percentage of the whole proteome because of insufficient spatial resolution and the difficulty to reveal low copy number proteins in presence of the most abundant proteins. Besides the prefractionation procedures discussed in Section 3.1, one remedy is the use of multiple overlapping narrow IPGs in the first dimension (zoom-in gels) [1, 22] and/or extended separation distances [21] (i) to achieve an optimum resolution in order to avoid multiple proteins in a single spot for unambiguous protein identification, and (ii) to facilitate the application of higher protein amounts for the detection of minor components.

Zoom-in gels in the pH range between pH 4 and 7 (IPG 4–5, IPG 4.5–5.5, IPG 5–6, *etc.*) work with both in-gel rehydration and cup-loading, and may be run either on the Multiphor apparatus or on the IPGphor (Fig. 2). These gels are typically used for micropreparative purpose with sample loads up to several milligrams. In order to avoid protein precipitation and horizontal streaking, low voltage (30–50 V) is applied during the initial stage of IEF (Tables 1 and 2). Because of the long focusing time (usually > 24 h), the surface on the IPG strips has to be protected by a layer of silicone oil to prevent them from drying out. For optimum results, it is advisable to remove the electrode paper strips after several hours and replace them by fresh ones. This is of particular importance when the sample contains high amounts of salt.

4.1.3 Narrow IPGs at the basic extreme up to pH 12

Strongly alkaline proteins such as ribosomal and nuclear proteins with closely related p*I*s between 10.5 and 11.8 were focused to the steady state by using narrow IPGs 10–12 and 9–12 (Fig. 3) [18]. In order to obtain highly

Table 2. Running conditions using the IPGphor

Gel length	180 mm		
Temperature	20°C		
Current max.	0.05 mA per IPG strip		
Voltage max.	8000 V		
I Analytical IEF			
Reswelling	30 V, 12–16 h		
Initial IEF	200 V, 1 h; 500 V, 1 h; 1000 V, 1 h		
IEF to the steady state	Gradient from 1000 V to 8000 V within 30 min; 8000 V to the steady state, depending on the pH interval used		
1–1.5 pH units	4 pH units	7 pH units	
e.g. IPG 5–6 ..... 8 h	IPG 4–8 ..... 4 h	IPG 3–10 L ..... 3 h	
e.g. IPG 4–5.5 .. 8 h		IPG 3–10 NL .... 3 h	
3 pH units	5–6 pH units	8–9 pH units	
IPG 4–7 ..... 4 h	IPG 4–9 ..... 4 h	IPG 3–12 ..... 3 h	
		IPG 4–12 ..... 3 h	
II Micropreparative IEF			
Reswelling	30 V, 12–16 h		
IEF to the steady state	Focusing time of analytical IEF + additional 50% (approx.)		

reproducible 2-D patterns, different optimization steps with respect to pH engineering and gel composition, such as the substitution of DMAA for acrylamide and the addition of isopropanol to the IPG rehydration solution, were necessary in order to suppress the reverse electroendosmotic flow which gives rise to highly streaky 2-D patterns [18]. Sample cup loading at the anode and IEF under silicone oil are required.

4.1.4 Wide IPGs up to pH 12

Theoretical 2-D patterns calculated from sequenced genomes [64, 65] not only indicate that the majority of proteins of a total cell lysate possess p*I*s between pH 4 and 9, but also that a considerable protein quantity with p*I*s up to pH 12 can be expected. With classical 2-D electrophoresis using carrier ampholyte-generated pH gradients, basic proteins can only be separated by NEPHGE [66], with poor resolution and reproducibility. In contrast to carrier ampholyte IEF, the IPG technology also provides steady-state IEF of even very alkaline proteins, which was demonstrated by using narrow IPGs 9–12 and 10–12 for the separation of ribosomal and nuclear proteins [18]. However, in order to obtain an overview of the actual state of the proteome of a cell or tissue, wide IPGs 3–12 and 4–12 were generated [19, 21]. Excellent 2-D patterns of cell/tissue extracts and/or TCA/acetone precipitated proteins for the visualization of basic proteins exceeding pL 10, which are usually not included in lysis buffer extracts [19, 21], as well as the Triton X-100 insoluble cell fraction of *Mycoplasma pneumoniae* (Hermann *et al.*, submitted) were obtained. Moreover, IPG 4–12, which is flattened between pH 9–12, proved to be a most useful gradient for the separation of very alkaline proteins such as ribosomal proteins [19]. Most fortunately, because the strong water transport to the anode (reverse electroendosmotic flow), inherent to narrow IPGs exceeding pH 10 (see Section 4.1.3), is negligible, the wide gradients 3–12, 4–12, and 6–12 [19, 21] can be run under standard conditions without isopropanol. Moreover, by using IPGs 3–12 and 4–12, entry and focusing of high M_r proteins is significantly improved (Fig. 4).

4.2 Extended separation distances

Extended separation distances for maximum resolution of complex protein patterns have been described [67]. Whilst size stability and handling of fragile tube gels might become a problem, handling of longer IPG gel strips cast on plastic backings does not require special precautions. For example, 24 cm long IPG gel strips with pH 3–12 (Fig. 4) and 4–12 have been successfully applied [21]. Moreover, by using 24 cm long IPG strips with narrow pH gradients such as IPG 5–6, highly resolved 2-D patterns were obtained (Fig. 5).

4.3 Optimum focusing time

In principle, optimum focusing time for optimum 2-D pattern quality and reproducibility is the time needed for the IEF pattern to reach the steady state [1, 17]. Insufficient

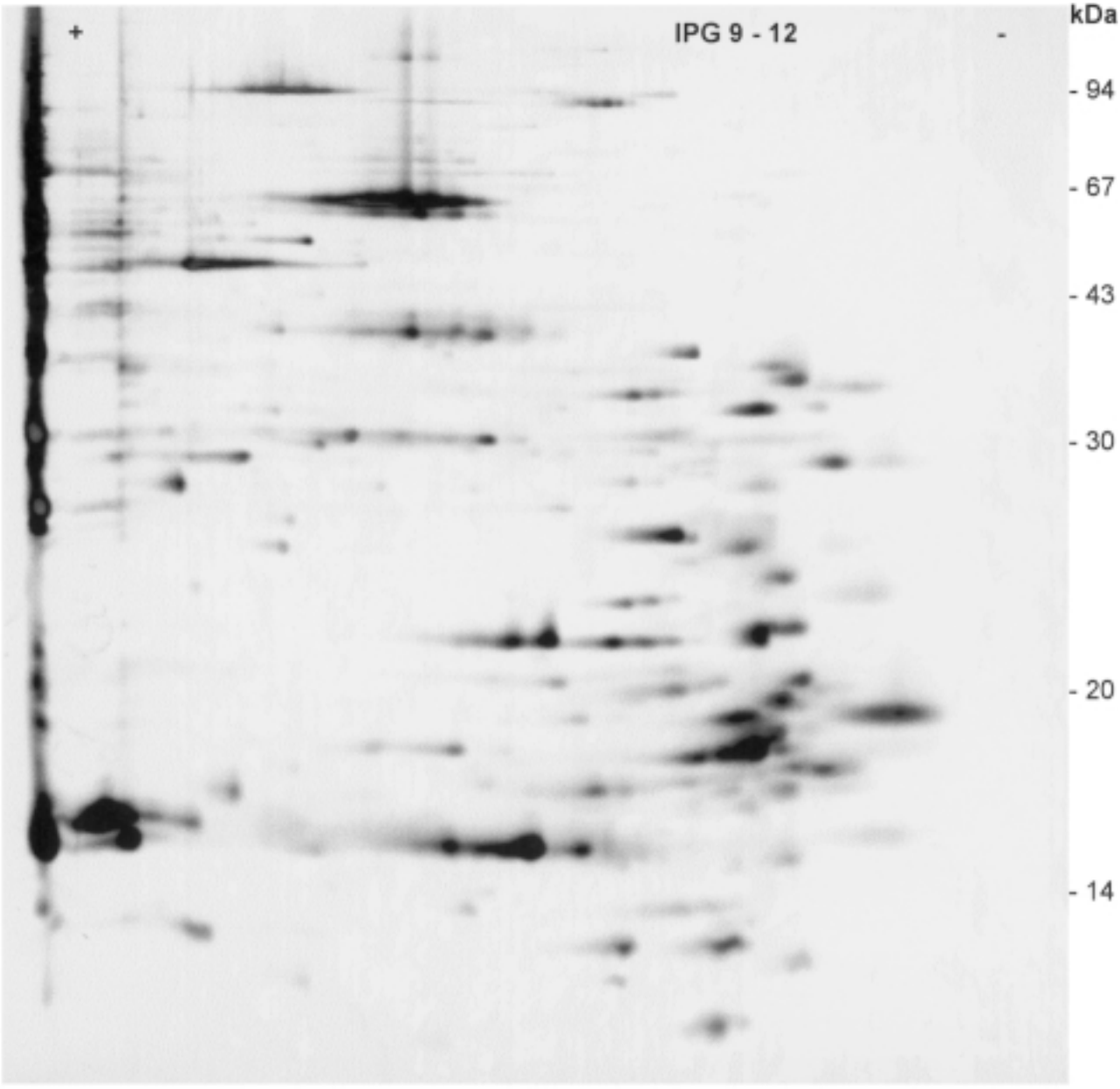

Figure 3. Analytical IPG-Dalt of very alkaline mouse liver proteins (TCA/acetone precipitation) [18]. First dimension, IPG 9–12 (8 h at 5000 V max.); separation distance, 180 mm; sample application by cup-loading near the anode. Second dimension, vertical SDS-PAGE (13%T constant); silver stain

focusing time will cause horizontal and vertical streaks. On the other hand, overfocusing should also be avoided. Although it does not result in a shift of proteins towards the cathode because of the stability of the IPG, increasing water exudation at the gel surface due to active water transport leads to distorted protein patterns, horizontal streaks at the basic gel end and loss of proteins. It should also be kept in mind that focusing time is prolonged in case the IEF strips have been rehydrated to 0.3 mm only instead of 0.5 mm. As a guideline, optimum focusing times determined for a number of different wide and narrow IPGs are given in Tables 1 and 2.

4.4 IEF under a layer of silicone oil or without?

Comparisons of the 2-D polypeptide patterns obtained by IEF in pH 4–7, pH 4–9, as well as pH 6–10 gradients, under, and without, a layer of silicone oil did not show significant differences up to the basic end [40]. Protecting the gel surface with a layer of silicone oil neither prevented the formation of urea crystals nor water exudation

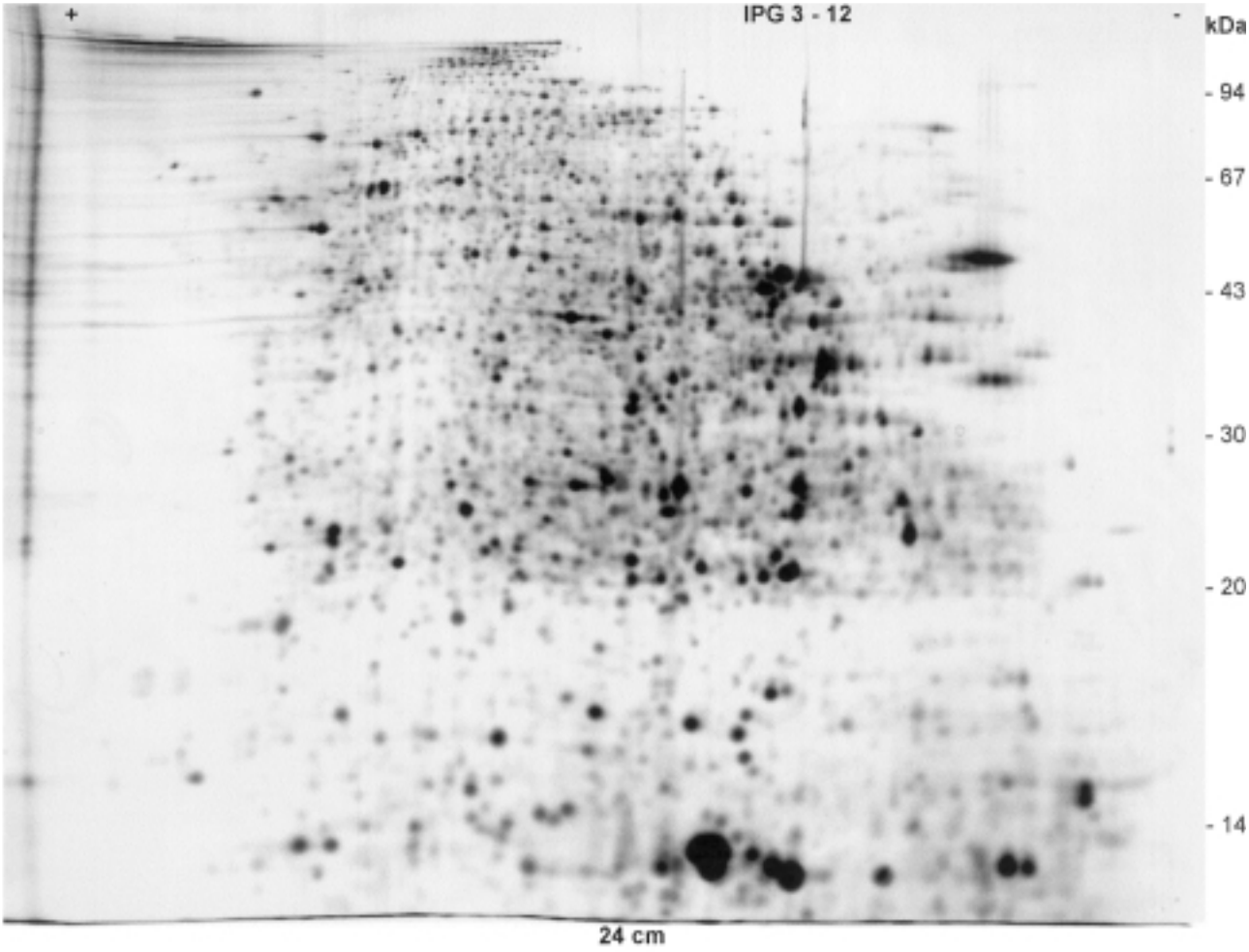

Figure 4. Wide IPG extended separation distance [21]. Analytical IPG-Dalt of mouse liver proteins (lysis buffer extract). First dimension, IPG 3–12; separation distance, 240 mm; sample application, cup-loading near the anode; running conditions, 6 h at 3500 V max. (see Table 1). Second dimension, vertical SDS-PAGE (13%T constant); silver stain.

(due to active water transport) on the gel surface during IEF, so that in these cases the "under-oil procedure" seemed to be of no advantage. Since the procedure without silicone oil is easier to perform, we prefer focusing without oil for analytical IEF using IPGs 4–7, 4–9, and 3–10. However, there are important exceptions: IEF under oil is strongly recommended (i) for micropreparative runs with narrow pH gradients and/or extensively long focusing times, and (ii) when very alkaline IPGs exceeding pH 10 (*e.g.*, IPG 3–12 or 10–12) are used [18–21]. However, silicone oil should be degassed and flushed with argon prior to use in order to remove oxygen.

4.5 Temperature

The effect of the temperature at which IEF with IPG is performed, on spot positions and pattern quality, has been studied in detail [41]. Since IEF in the presence of 8 M urea at low temperatures is not suitable due to the formation of urea crystals, the optimum focusing temperature was found to be 20°C rather than 10°C. Elevated temperatures allow more rapid focusing due to lower viscosities and increased mobilities of proteins. Increased temperature also results in improved sample entry, clearer background, less streaking, and well-defined spots up to the basic end of the gel. However, the focusing temperature not only affects pattern quality, but also has an influence on the spot positions of the 2-D polypeptide patterns. Shifts in spot positions were primarily found at the extremes of the pH gradient, whereas in the pH region between 6–7, position shifts were less marked [41]. Nevertheless, temperature control is essential in order to allow meaningful comparison of 2-D patterns.

5 Equilibration

Because the focused proteins bind stronger to the IPG gel matrix (with fixed charged groups) than to carrier ampholyte gels, prolonged equilibration time is important

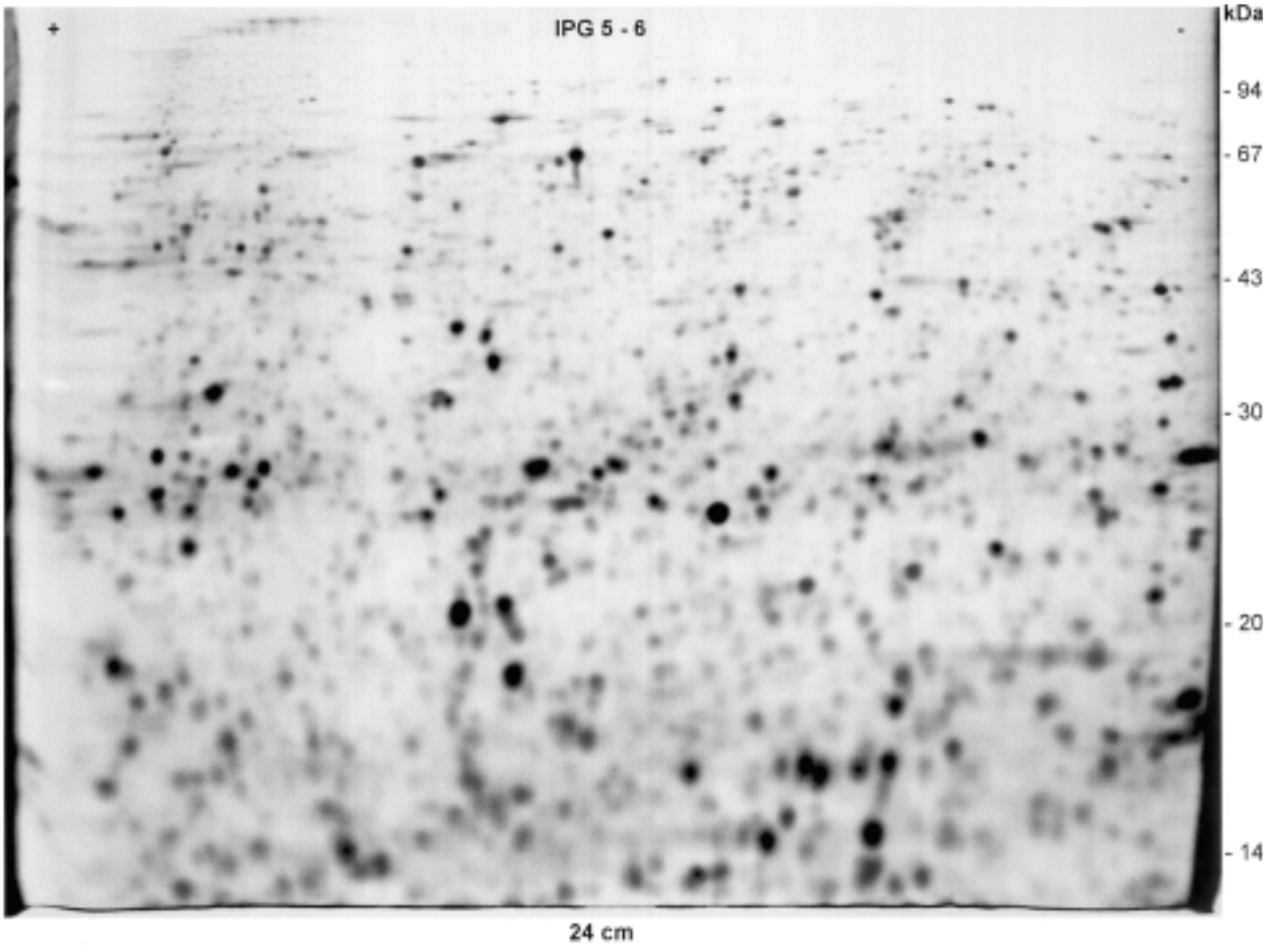

Figure 5. Narrow IPG with extended separation distance. Analytical IPG-Dalt of mouse liver proteins (lysis buffer extract). First dimension, IPG 5–6 (laboratory-made); separation distance, 240 mm; sample application by cup-loading at the anode; running conditions, 40 h at 3500 V max. (see Table 1). Second dimension, vertical SDS-PAGE (13%T constant); silver stain.

to completely load all proteins with SDS for improved protein transfer from the first to the second dimension. In order to achieve sufficient loading with SDS, at least 2 × 10 min equilibration with gentle shaking at room temperature is required. Increased temperature (heating) of the IPG strips during the equilibration step did not yield better results. However, protein transfer is considerably improved when urea and glycerol are incorporated in the equilibration buffer [1, 68, 69], whereas thiourea is not recommended because it causes streaks in the 2-D pattern (unpublished results). The IPG gel strips are typically equilibrated for 2 × 15 min with gentle shaking in 2 × 10 mL of equilibration solution, which is composed of 50 mM Tris buffer (pH 6.8 or 8.8, see below), 2% SDS, 6 M urea, and 30% w/v glycerol. Additionally, DTT is incorporated in the first equilibration step for reduction of the disulfide bonds and complete unfolding of proteins, and iodoacetamide in the second step to remove excess DTT held responsible for "point streaking" in silver-stained gels [43]. By using pH 8.8 instead of pH 6.8, proteins are alkylated with iodoacetamide, which prevents reoxidation of sulfhydryl groups of proteins during SDS-PAGE, and which is preferred for spot identification by mass spectrometry. Alternative procedures using tributylphosphine instead of DTT and iodoacetamide have been successfully applied for hydrophobic proteins (*e.g.*, wool filament proteins) [57, 58].

Loss of proteins during the equilibration step and subsequent transfer from the first to the second dimension are (i) due to proteins which remain in the IPG strip because of adsorption to the IPG gel matrix due to insufficient equilibration times, and (ii) due to wash-off effects. Experiments with radioactive labeled proteins have shown that up to 20% of the proteins may get lost during equilibration. However, time courses have also revealed that the

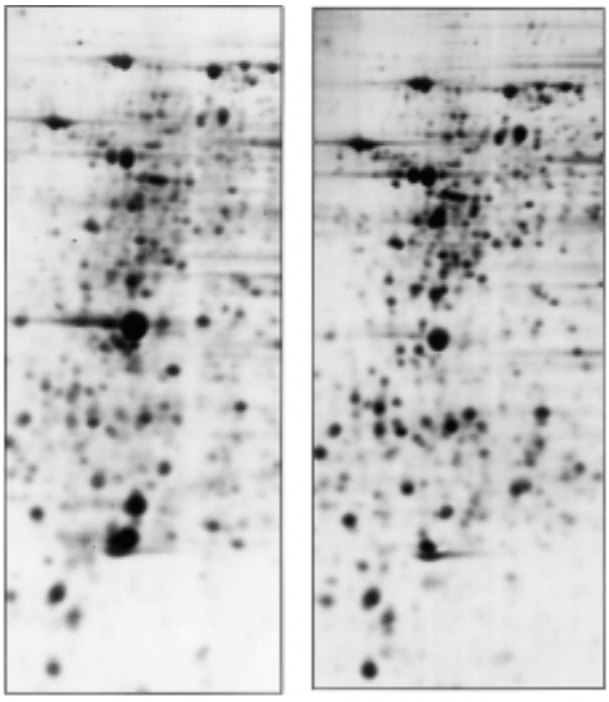

Figure 6. Comparison of protein detection with (A) SYPRO Ruby and (B) silver staining. IPG-Dalt of mouse liver proteins. First dimension, IPG 4–9 (section). Second dimension, vertical SDS-PAGE (13%T constant). The 2-D gel was stained with SYPRO Ruby, destained, and then stained with silver nitrate.

majority of proteins is lost during the very first minutes of equilibration, whereas protein losses in the second equilibration step are only marginal (Harder *et al.*, unpublished results). Obviously, the proteins lost had been primarily located on the surface of the IPG strip, which might have caused background smear in the second dimension anyway. These studies have also shown that the loss of proteins during equilibration is reproducible for a given sample.

6 Horizontal *versus* vertical SDS gels

After equilibration, the IPG strips are applied to a horizontal or vertical SDS gel. Horizontal SDS gels are covalently attached to a plastic support, which has the advantage of preventing alterations in gel size during the staining procedure (due to shrinkage in organic solvents and expansion upon rehydration in aqueous solvents). Spot sharpness is increased in comparison to vertical gels because of the decreased gel thickness (0.5 mm instead of 1 mm) which allows higher voltages to be applied, resulting in less protein diffusion due to the shorter running times [40]. Despite these advantages, multiple runs and computer-driven spot cutting are often required for proteome analysis. In this case, multiple vertical second-dimensional electrophoresis (Dalt system) is the method of choice.

7 Fluorescent dyes *versus* silver staining

Apart from radioactive labeling, highly sensitive detection of 2-D electrophoretically separated proteins can be achieved by (i) silver staining or (ii) using fluorescent compounds (for review see [70]). Both fluorescent and silver staining are compatible with subsequent protein analysis by mass spectrometry. Due to their high sensitivity, silver staining methods are ideal for the (i) detection of trace components within a protein sample and (ii) analysis of protein samples available in only limited quantity. The detection limit is as low as 0.1 ng of protein per spot. Despite these advantages, silver staining has several drawbacks, such as (i) the poor reproducibility of several stains, (ii) limited dynamic range, and (iii) the fact that certain proteins stain poorly, negatively, or not at all. Moreover, silver staining procedures are quite labor-intensive. In contrast, fluorescent staining is slightly less sensitive, but more reproducible and easier to perform. Three approaches are possible: (i) preelectrophoretic derivatization of proteins where proteins are coupled with a fluorescent dye prior to the IEF step of 2-D electrophoresis, (ii) labeling of proteins after the first dimension (IEF), and (iii) postelectrophoretic labeling after SDS-PAGE. Examples of preelectrophoretic stains are monobromobimane and 2-methoxy-2,4-diphenyl-3(2H)-furanone (MDPF). The main disadvantage of MDPF is that it reacts with the primary amino groups and thus introduces protein charge modifications which may result in altered protein mobility during IEF. Protein charge modification is less of a problem with monobromobimane, which reacts with the sulfhydryl groups of proteins.

Altered protein mobility during IEF due to protein charge modifications can be overcome (i) either by labeling proteins with MDPF after IEF, prior to the second dimension, or (ii) by labeling the proteins with fluorescent dye molecules after the electrophoretic separation has been completed. Examples of these postelectrophoretic dyes are SYPRO Orange, SYPRO Red and SYPRO Ruby. Staining with these dyes is noncovalent and can be accomplished in a simple one-step procedure. Since these fluorescent compounds apparently bind to the SDS surrounding the proteins, staining shows little protein-to-protein variability. Staining is usually completed after 2–3 h (although better results are obtained after overnight staining) with little or no destaining required. This approach is expected to be easily adapted for use with

automated instrumentation (see Section 8). Detection limit of these fluorescent dyes is in the range of 1–2 ng of protein per spot, and linear range is over three orders of magnitude. Disadvantages are (i) that photography or electronic image acquisition is required to document results and (ii) that GelBond PAGfilm adversely affects staining. In our hands, best results were obtained with SYPRO Ruby. The patterns obtained with silver staining and SYPRO Ruby staining were similar, but not identical (Fig. 6).

8 Automation

Despite improvements such as the possibility to run ten (or even 20) second-dimensional SDS gels parallel (Dalt apparatus) [44], 2-D electrophoresis has long been a labor-intensive and poorly automated procedure [71]. Only recently things have changed considerably: (i) by the availability of ready-made gels (IPG DryStrips, ExcelGel SDS) on stable plastic supports [72], (ii) by the introduction of the IPGphor [28, 29], (iii) by automated silver staining devices, and (iv) by fluorescent protein labeling, which is much less labor-intensive than silver staining procedures. In particular the IPGphor, in combination with ready-made IPG strips, where sample in-gel rehydration and IEF are performed overnight in unattended operation, is an important step towards automation of the 2-D procedure.

9 "Streak business"

Streaks are the most harmful experience for beginners, but also for 2-D experts, and the most time-consuming trouble-shooting business. The many different reasons for this have been extensively described [31] and therefore only some major causes and remedies are listed below. For details see http://www.weihenstephan.de/blm/deg.

9.1 Protein solubility

One of the major reasons for streaky 2-D patterns and/or background smear is poor protein solubilization or insuffient solubility of the proteins during IEF. Therefore, special attention has to be paid to cell lysis conditions, inactivation of protease activities, choice of adequate detergents, chaotropes, and/or amount of reducing agents.

9.2 Horizontal streaks

Horizontal streaks localized in different areas of the 2-D gel are primarily due to insufficient solubility of particular proteins (*e.g.*, caused by protein overloading, protein interactions with contaminants, diminished solubility near the p*I*) during IEF. Horizontal streaking, particularly at the basic end, might be due to different causes, such as significant overfocusing times (see Section 4.3), or depletion of DTT during IEF. This reducing agent is charged at alkaline pH, and thus migrates towards the anode, depleting the cathodic gel end with DTT. The result is reoxidation of sulfhydryl groups and a concomitant loss of solubility for certain proteins, especially those which interact *via* disulfide bonds such as prolamins or keratins, leading to horizontal streaks at the cathodic gel end. By increasing the amount of DTT in the reswelling solution of the IPG strips from 0.2 to 0.4%, the streaking was diminished, whereas higher amounts of DTT exceeding 0.4% resulted in distorted protein patterns. However, the shortage of reducing agent can be avoided by placing an extra paper strip soaked with DTT near the cathode [40]. Additionally, by using IPG 6–12 [21] instead of 6–10, diminished horizontal streaks at the basic end were observed.

9.3 Vertical streaks

Vertical streaks in the 2-D gel pattern can be caused by salt fronts, protein aggregates and/or incomplete focusing in the first dimension. Vertical "empty" lanes are often due to urea crystals formed during IEF on the surface of the IPG strip. Replacement of the electrode papers during IEF and slow sample entry help to avoid salt fronts whereas formation of urea crystals can be inhibited by rinsing the rehydrated IPG strip with water prior to IEF (see Section 2.3.3). Point-streaking due to dust and DTT is remedied by the addition of iodoacetamide [43] (see Section 5).

9.4 Detergent/chaotrope smear

Traditionally, detergents such as Triton X-100 and Nonidet P-40 have been used. However, because of repeatedly encountered quality problems giving rise to background smear in the silver-stained 2-D patterns, the sulfobetaine CHAPS is preferably used. Background smear caused by thiourea is avoided by using high-quality products.

10 Outlook

The reproducible high-resolution separation of complex protein mixtures is crucial for successful proteomics, and to date there is no alternative to 2-D electrophoresis whose intrinsic strength as opposed to other separation methods is that it can reveal thousands of proteins at a time. Whereas conventional 2-D technology with carrier ampholyte-generated pH gradients is increasingly exhausted due to its limitations with regard to resolution and reproducibility, IPG-Dalt [1] has proved amazingly flexible with respect to the requirements of proteome

analysis. The needs for successful proteomics are (i) increased resolving power, (ii) the ability to analyze very alkaline, hydrophobic, membrane and/or high M_r proteins under steady-state conditions, (iii) the ability to detect minor components in the presence of large quantities of housekeeping proteins, (iv) simplification and automation of the laborious 2-D electrophoresis procedure, (v) the ability to perform high-throughput analysis, and (vi) methods for protein quantitation that are sensitive, rapid, simple, reliable, and inexpensive. During the past ten years, IPG-Dalt has constantly been refined to accomplish at least several of these goals, *e.g.*, by the development of basic IPGs up to pH 12 for the analysis of very alkaline proteins, or the introduction of overlapping narrow IPGs to stretch the first dimension for higher resolution and the analysis of minor components, or the development of ready-made IPG strips and automated procedures. Limitations remain in the field of the analysis of hydrophobic and/or membrane proteins, as well as the lack of sensitive and reliable techniques for protein quantitation, although the launch of sensitive fluorescent dyes has considerably improved the latter situation. Steps have also been taken to develop nonradioactive dual labeling techniques for the visualization of differentially expressed proteins. In conclusion, although not being perfect, a method of 2-D electrophoresis with IPGs has been established that fufills the basic requirements of proteome research and that is being continuously improved to meet the demands of the future.

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