

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

186,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



High Speed Isoelectric Focusing of Proteins Enabling Rapid Two-Dimensional Gel Electrophoresis

Gary B. Smejkal and Darren J. Bauer

*University of New Hampshire,
Hubbard Center for Genome Studies, Durham, NH,
USA*

1. Introduction

1.1 Theoretical resolution of two-dimensional electrophoresis

While two-dimensional electrophoresis (2-DE) of proteins, as we know it, was first described nearly four decades ago (O'Farrell, 1975), it continues to be a critical component of comprehensive proteomics analysis today. Continuing refinements of 2-DE, particularly the development of immobilized pH gradients (Righetti, 1990), new chaotropes (Vecchio et al. 1984, Rabilloud 1998) and detergents (Seddon 2004, Rabilloud et al. 1999) that increase and maintain the solubility of hydrophobic proteins, and sample fractionation strategies (Smejkal and Lazarev 2005, Di Girolamo et al. 2011, Boschetti et al. 2007) that lessen the complexity of proteomes, have further increased the utility of the method.

Immobilized pH gradients (IPGs) are stable and highly reproducible pH gradients for the isoelectric focusing (IEF) of proteins. IPGs are capable of separating protein charge isoforms differing by 0.001 pI units, a resolution an order of magnitude higher than labile carrier ampholyte generated pH gradients, which can resolve differences of only 0.01 pI units (Hamdan and Righetti, 2005). While carrier ampholytes may be comprised of as many as 686 chemical entities with 3899 charge species, the polydispersity and focusing properties of these compounds curtails dramatically at alkaline pH intervals (Righetti et al. 2007.)

IPGs spanning 0.1 pH units have been described for the separation of proteins differing by a single amino acid substitution (Cossu and Righetti 1987). Hence, thousands of proteins can hypothetically be separated over the physiological pH range and this number of theoretical proteins is potentially squared when coupled with an orthogonal separation such as sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Richardson et al. (2008) elicited a total of 5,525 proteins from 2D gels of *Escherichia coli* lysate from multiple IPGs covering very narrow overlapping pH ranges.

IPGs can discriminate single post-translational modifications such as phosphorylation, in which the addition of a single phosphate group influences net charge of the protein, but changes its molecular mass by less than 78 Da. Only rarely can a single phosphorylation event be discriminated by a measureable mobility shift in SDS PAGE, and only if a

significant conformation change is induced which is preserved following SDS denaturation (Kovacs et al. 2003). For example, the phosphorylation of a single tyrosine in the 609 amino acid sequence of human serum albumin shifts the molecular mass from 69,367 to 69,289 Da, a mere 0.1% change in total protein mass.

SDS PAGE may be limited in its capacity to resolve such small mass differences, since it is not exclusively based on size separation. Proteins are separated on the basis of their capacity to bind SDS and it is generally assumed that all of the proteins in an amalgam will have very similar charge:mass ratios. On average, proteins bind 1.4 g SDS per gram of protein, such that the protein constitutes only about 42% of the total mass of the nascent SDS-protein complex. However, glycoproteins depart from this “rule” since the hydrophilic glycan moiety reduces the hydrophobic interactions between the protein and SDS. To the contrary, hydrophobic membrane proteins have been shown to bind up to 4.5 g SDS per gram of protein (Rath et al. 2009), in which case the protein represents only 19% of the total mass of the SDS-protein complex. The transmembrane insulin receptor at 460 kDa binds enough Triton X-100 to inflate this mass to over 1,000 kDa (Hjelmeland and Chrambach, 1981).

Further, the binding of different detergents will yield very different protein masses. For example, the molecular mass of cytochrome P-450 is 100 kDa using CHAPS as detergent, but over 300 kDa when using sodium cholate (Hjelmeland et. al 1983). Interestingly, adenylate cyclase exhibits twice its molecular size in 0.01% Lubrol than it does in 0.1% Lubrol (Chrambach 1985).

The separation efficiency of PAGE, defined as the number of theoretical plates, is increased in the presence of SDS which lowers the diffusion coefficient of proteins (Lunney et al. 1971, Chen and Chrambach 1979) and as many as 400,000 theoretical plates/meter have been predicted for a microfabricated SDS PAGE system (Herr and Singh, 2004). Moreover, electrophoresis provides an effective means of concentrating “rare” proteins. A 10,000 fold concentration of the cardiac biomarker cTnI has been described (Bottenus et. al 2011).

1.2 High speed isoelectric focusing of proteins

Recently, an IEF apparatus operating at an unprecedented 12,000V maximum voltage was described, enabling IEF to be completed within three hours (Smejkal and Bauer 2010). The IEF-100 is a microprocessor controlled IEF apparatus in which voltage, current, power, and temperature are controlled, monitored, and can be outputted by computer interface. In its standard configuration, the IEF-100 can run up to six IPG strips and features adjustable electrodes that can be moved along the length of the tray to accommodate different IPG strip lengths.

This chapter describes the development of protocols enabling rapid IEF of proteins prior to 2-DE. Important considerations critical to the expediency of IEF include (i) meticulous sample preparation and presentation of samples of low initial conductivity to IEF since contaminating ions increase sample conductivity and slows the rate at which maximum voltage is reached, (ii) single or dual electrode configurations, (iii) the placement of wicks and electrodes, and (iv) the addition of carrier ampholytes. The convention of adding carrier ampholytes is challenged, since increased conductivity slows the progression of IEF. Similarly, electrode placement and wick geometry may also affect the rate of IEF. The use of

oversized wicks provides a reservoir for contaminating ions to accumulate off gel. The rapid focusing of contaminating ions such as phosphate, HEPES, and even Tris can result in a localized increase of conductivity resulting in a precipitous voltage drop in that vicinity which hinders the proper focusing of proteins. For properly prepared samples of extremely low conductivity, maximum voltage of 12,000V could be reached in less than two hours with IEF being completed within three hours.

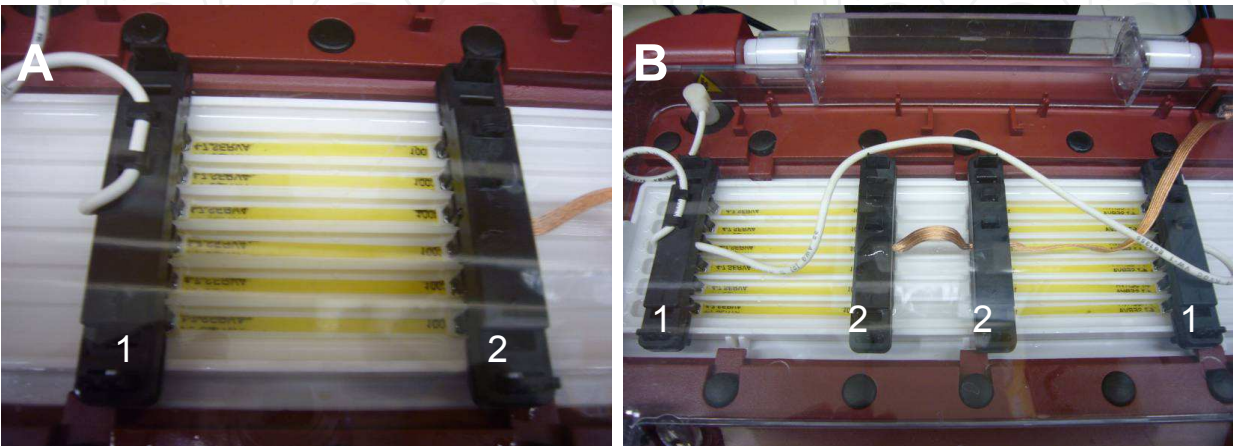


Fig. 1. IEF with (A) single or (B) dual electrode assembly. Note the positions of the anode (1) and cathode (2) in the dual electrode assembly. (From Smejkal and Bauer, 2011. Reproduced with permission from American Biotechnology Laboratory.)

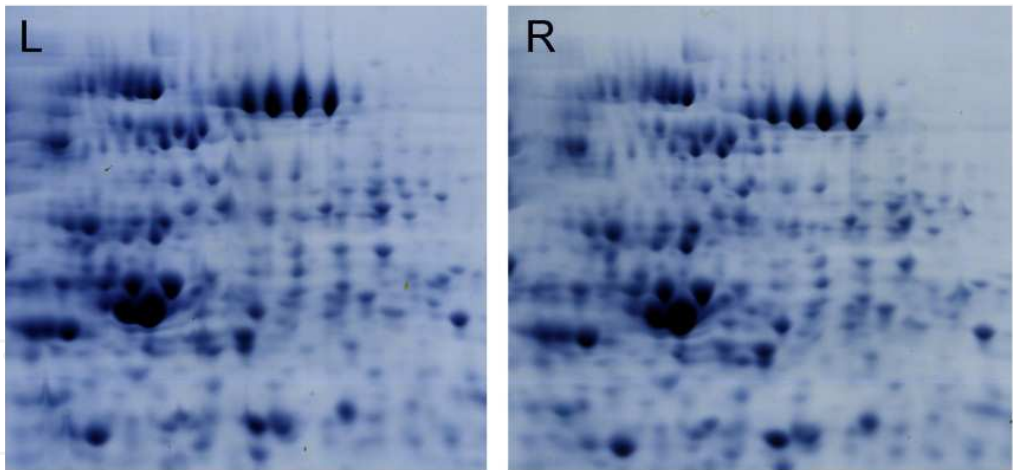


Fig. 2. Identical regions enlarged from 2D gels showing reproducibility of duplicate IPGs run simultaneously with left (L) or right (R) electrode pairs wired in parallel. IPGs were pH 3-10 non-linear. SDS PAGE was performed on 11% polyacrylamide gels. (From Smejkal and Bauer, 2011. Reproduced with permission from American Biotechnology Laboratory.)

Finally, using an extra wide vertical PAGE format, two IPGs can be run simultaneously on the same second dimension gel, improving reproducibility and doubling throughput while halving the number of SDS PAGE gels required. Recipes are provided for casting easy, highly reproducible gels with broad linear separation range similar to polyacrylamide gradients (Table 2).

2. Materials and methods

2.1 Materials

The IEF-100 and SE-640 dual vertical gel electrophoresis unit, and the Protein Determination Reagent were from Hoefer Scientific (Holliston, MA, USA). IPG BlueStrip IPGs and Servalyte pH 3-10 carrier ampholytes were from Serva Electrophoresis GmbH (Heidelberg, Germany). AG501-X8 ion-exchange resin was from BioRad (Hercules, CA, USA). Tributylphosphine (TBP), 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), Orange G, and protease inhibitors were from Sigma-Aldrich Chemicals (St. Louis, MO, USA). *m*-Cresol purple was from US Biochemicals (Cleveland, OH). Conductivity meter B-163 was from Horiba (Kyoto, Japan). The Sonicator 450 probe sonicator was from Branson Ultrasonics (Danbury, CT, USA).

2.2 Preparation of bacterial lysates

Proteins were prepared from log phase cultures of *Escherichia coli* strain OP50. One hundred milligrams of packed cells (approximately 10^{11} cells) were recovered from 100 mL cultures by centrifugation at 4,000 RCF for 20 minutes at 4° C. The cell pellets were washed once by resuspension in water containing protease inhibitors (2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1 mM EDTA, 130 uM bestatin, 14 uM *trans*-epoxysuccinyl-l-leucylamido-(4-guanidino)butane, 1 uM leupeptin, and 0.3 uM aprotinin) followed by centrifugation at 4,000 RCF for 10 minutes at 20° C. The cells were resuspended in 4.5 mL of 7M urea, 2M thiourea, 5% CHAPS supplemented with 5 mM TBP, 0.01% *m*-cresol purple, and protease inhibitors. All subsequent steps were performed at 18°-20° C to prevent the precipitation of urea or CHAPS. Cells were disrupted using a probe sonicator at 70% power and 30% duty cycle for 40X 1 second cycles with cooling to room temperature following each 10 cycles. Cellular debris was removed by centrifugation at 14,000 RCF for 10 minutes. Conductivity of the sample was 350 us/cm.

2.3 Inactivation of proteases

The prompt addition of protease inhibitors is required, since contrary to popular belief, residual enzyme activity may persist in the presence of urea and CHAPS (Olivieri et al. 2001, Smejkal et al. 2007) and even SDS (Arikan 2008). For example, proteolysis occurs so rapidly and extensively in the moulting microcrustacean *Daphnia pulex* that protease inhibitors must be infused into the live organisms prior to protein extraction in order to obtain reliable and reproducible 2-DE (Bauer et al. 2009). Alternatively, thermal denaturation has been shown to reliably curtail protease activity (Robinson et al. 2009, Grassl et al. 2009) and phosphatase and kinase activity (Smejkal et al. 2011) as evidenced by 2-DE and mass spectrometry.

2.4 Simultaneous reduction and alkylation of protein disulfides

The reduction of protein disulfides must be followed by alkylation to prevent the spurious formation of mixed disulfides during IEF, as they may lead to the formation of unnatural adducts and artifactual spots in 2-DE (Herbert et al. 2001). Alkylation also prevents the desulfuration of cysteines that can generate labile dehydro alanine residues that are susceptible to cleavage at the peptide bond (Herbert et al. 2003).

reagent	formulation	comments
Sample Solubilization Reagent	7M urea, 2M thiourea, 5% CHAPS	Dissolve solids completely. Add 1 g of AG501-X8 resin to each 50 mL of solution and mix by gentle inversion until conductivity is less than 10 uS/cm. Add pH indicator AFTER ion-exchange. Filter through 0.45 micron filter and store frozen aliquots for up to six months. Add Reducing Reagent fresh before each use.
Reducing Reagent	200 mM TBP	Simultaneous reduction and alkylation is possible when the non-thiol TBP is substituted for DTT. Provided in single use glass ampules. Rapidly oxidizes in air. Use within 30 minutes of opening ampule. Add 1/40 volume of TBP to the sample and 1/100th volume of alkylating reagent.
Alkylating Reagent	1M acrylamide	For more complete and more specific alkylation of Cys residues than iodoacetamide (IAA). Ion-exchange with AG501-X8 resin, filter and store at room temperature for three months. Do not freeze.
pH Indicator	1% <i>m</i> -cresol purple	Useful pH indicator during sample preparation. If sample turns yellow, add concentrated Tris until color transitions to purple. Filter and store at room temperature for six months. Add 1/100th volume to each sample.
IEF Tracking Dye	1% Orange G	Useful for monitoring the progression of IEF. Filter and store at room temperature for six months. Add 1/100th volume to each sample.
Tris Concentrate	1M Trizma base	Add 1/1000th volume when sample pH is below 7.6 as indicated by color shift of <i>m</i> -cresol purple to yellow. Repeat as necessary until color has shifted to purple. Filter and store refrigerated for three months.
Quenching Reagent	2M DTT	Add 1/40th volume to quench the alkylation reaction. Add 1/40th volume to diluted samples prior to IEF. Prepare fresh and store frozen for two months.
IPG Equilibration Reagent	375 mM Tris-HCl pH 8.8, 3M urea, 3% SDS, 50 mM DTT, 0.005% BPB, 0.005% phenol red, 0.005% bromophenol blue	Filter and store frozen for three months.
Acrylamide-Bis	29.2% acrylamide, 0.8% methylene bisacrylamide	The solids are completely dissolved, then incubated with AG501-X8 resin until conductivity is less than 20 uS/cm. Filter and store refrigerated for three months in amber glass bottles that are half-filled. Do not store in plastic containers. Do not freeze.
Gel Buffer Concentrate	1.5 mM Tris-HCl pH 8.8	Buffer component of second dimension PAGE gels. Filter and store refrigerated for three months.

APS Concentrate	10% ammonium persulfate	Add 1 mL of water to 1 g of ammonium persulfate and immediately listen for “snap, crackle, pop”. (If no effervescence occurs upon the addition of water, seek fresh source of ammonium persulfate.) Adjust volume to 10 mL and use within two hours. Frozen aliquots are stable for at least three months. Do not freeze thaw.
Tris-glycine-SDS Running Buffer	25 mM Tris, 144 mM glycine, 0.1% SDS	Do not adjust pH with HCl. Can be prepared as a 10X concentrate, filtered, and stored for three months at room temperature. Do not store refrigerated.

Table 1. Reagent preparation for IEF and 2-DE.

stock solution	final concentration	volume (mL)
30% acrylamide-Bis	11%	18.3
1.5M Tris-HCl pH 8.8	375 mM	12.5
H ₂ O	-	18.9
10% ammonium persulfate	0.04%	0.2
98% TEMED	0.12%	0.06
total volume		50

Table 2. Casting second dimensional polyacrylamide gels.

Degassing is unnecessary. Admix acrylamide-Bis, Tris buffer, and water in a 50 mL tube and mix by gentle inversion taking care not to introduce bubbles. This solution is stable at room temperature for several hours. Add TEMED and mix by gentle inversion. Add ammonium persulfate, mix, and pour gels within one minute. Carefully overlay each gel surface with 25% isopropanol within one minute of pouring. Polymerization time should be 15-20 minutes.

Once polymerized, rinse the top of the gels with water. The polymerized gels can be stored at room temperature for 24 hours with a layer of water on top to prevent drying. Do not store the gel with running buffer on top. Do not store the gels refrigerated.

Rinse the top of the gel with running buffer immediately before placing the IPG strip. Do not use an agarose overlay.

Using the non-thiol reducing agent TBP, the alkylation of protein cyteines can proceed immediately by adding a molar excess of acrylamide or dimethlyacrylamide. The reactivity of acrylamide with cysteine sulfhydryls has been shown to be at least two orders of magnitude higher than with lysine NH₂ when the reaction is terminated within two hours (Hamden et al. 2001, Bordini et al. 2000). Other non-thiols such as tris(carboxyethyl) phosphine (TCEP) can be substituted for TBP. However, it should be noted that TCEP is provided as TCEP-HCl which acidifies the sample and can result in the precipitative loss of proteins unless properly buffered.

Iodoacetamide (IAA) is relatively inefficient as an alkylating agent. IAA may nonspecifically alkylate lysine and methionine residues in addition to cysteines, and may propagate apocryphal charge trains in two-dimensional gels. Further, IAA is rapidly consumed in the

presence of thiourea (Galvani et al. 2001a) and its activity is inhibited by both SDS and CHAPS (Galvani et al. 2001b). Therefore, IAA cannot be used to alkylate proteins during the SDS equilibration of IPGs that precedes second dimension PAGE.

In these experiments, bacterial lysates were alkylated by the addition of 10 mM acrylamide. *m*-Cresol purple was added as pH indicator which transitions from yellow to purple at pH 7.6. Samples exhibiting a shift to yellow color indicates pH too low for effective alkylation. When necessary, the pH is adjusted by adding concentrated Tris until the sample transitions to purple. The alkylation reaction was terminated after two hours by the addition of 50 mM DTT.

2.5 Acetone precipitation of proteins

Proteins were then precipitated by the addition of six volumes of 100% acetone to give a final concentration of 86%, followed by incubation for one hour at room temperature with intermittent vortexing. Precipitation is carried out at room temperature since urea and CHAPS will precipitate at lower temperatures (Smejkal et al. 2007). Protein recoveries from *E. coli* cell lysates by acetone precipitation were typically 90-95% when the initial protein concentration was at least 3 mg/mL. Acetone precipitation is not recommended when the initial protein concentration is less than 1 mg/mL. Moreover, acetone precipitation cannot be used for samples containing PBS or HEPES, since these buffers precipitate and concentrate with the proteins and their carryover interferes with IEF (Smejkal et al. 2005).

The protein flocculent was pelleted by centrifugation at 14,000 RCF for 10 min and redissolved in 2 mL of 7M urea, 2M thiourea, 4% CHAPS that was ion-exchanged with the AG501-X8 resin. Conductivity of the reagent was less than 10 uS/cm after 30-60 minutes of incubation with the ion-exchange resin. The reagent was filtered to remove the resin and used immediately. Protein concentration of the reconstituted proteins was 6.4 mg/mL, as determined by Bradford assay using the Protein Determination Reagent. Conductivity was 180 uS/cm.

For IEF, the proteins were diluted to 1 mg/mL in the ion-exchanged reagent and supplemented with 50 mM DTT and 0.01% Orange G tracking dye. The final sample conductivity was less than 100 uS/cm.

2.6 IEF comparing single or dual electrodes

IPGs pH 3-10 nonlinear, 7 cm length, were rehydrated overnight with 140 µL of bacterial lysate in the provided rehydration trays. To prevent drying, the trays were sealed in zippered plastic storage bags with a dampened paper towel to maintain a humid environment. Unless specified otherwise, hydrated strips were adhered gel side facing upward to the IEF-100 running trays with a few drops of mineral oil. Standard 15 X 6 X 1 mm wicks dampened with deionized H₂O were blotted nearly dry and placed with 2-3 mm overlapping each end of the IPG strip. Electrodes were positioned and the running tray was flooded with mineral oil.

IEF of 2 X 12 IPGs were programmed to run in a single step for three hours at 12,000V, 50 uA, 0.6W in two separate IEF-100 units configured with dual electrodes. Current limiting at 50 uA resulted in the formation of a roughly linear voltage gradient that reached 12,000V

maximum voltage in 133 minutes. Temperature remained constant at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$. IEF was continued at 12,000V until 12 total kiloVolthours (kVh) had elapsed.

Six additional IPGs were run on the IEF-100 using the standard electrode pair. Run was programmed as a single step limited at 12,000V, 25 μA , and 0.3W. Current limiting at 25 μA resulted in the formation of a roughly linear voltage gradient that reached 12,000V maximum voltage in 124 minutes. IEF was continued at 12,000V until 12 total kVh had elapsed.

2.7 Comparing IEF with or without carrier ampholytes

IEF was performed with 0% or 0.25% Servalyte pH 3-10 carrier ampholytes. Initial sample conductivity of 180 $\mu\text{S}/\text{cm}$ was increased to 240 μS following the addition of Servalyte.

2.8 The use of oversized wicks in IEF

Standard 15 X 6 X 1 mm wicks provided with the IEF-100 were compared to 30 X 6 X 2 mm wicks. Large wicks act as reservoirs in which extraneous ions could run off of the gel at each end of the pH gradient, as evidenced by the accumulation of anionic tracking dye in the anodal wick, and were standard in the Proteome Systems IsoelectrIQ IEF system. (Sydney, NSW, Australia). However, IEF run times of 8-10 hours at 10,000V maximum voltage have been reported (Smejkal and Lazarev 2005). In contrast to the standard configuration, IPGs were run gel side facing down in the tray. The tray was first flooded with mineral, since the strips should not contact the plastic tray directly. Wicks were saturated with distilled water, then blotted nearly dry and placed in the plastic tray. IPGs were placed with the gel side facing downward with at least 3 mm overlap at each end of the strip. Electrodes were placed in contact with the wick approximately 10 mm from the edge of the IPG. Effectively the

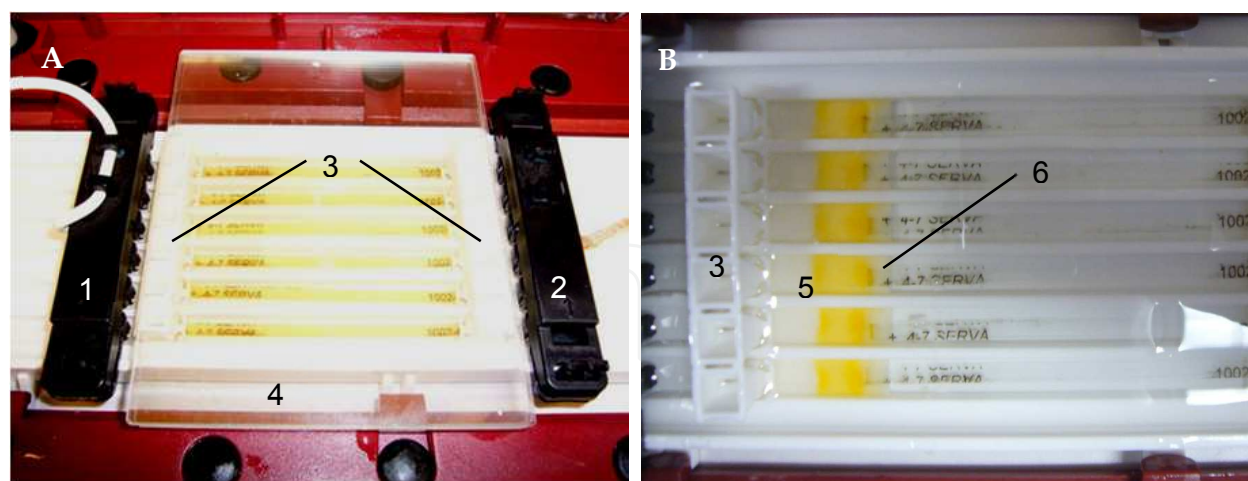


Fig. 3. IPGs loaded gel side facing downward in contact with oversized 30 X 6 X 2 mm wicks. (A) Empty sample loading cups (3) were used to ensure connectivity between IPGs and the large wicks and a stack of glass plates (4) was placed on top the assembly to apply uniform pressure. The anode (1) and cathode (2) were positioned with the wick acting as bridge between the IPG and electrode. (B) Exploded view following IEF showing the accumulation of the Orange G tracking dye in the wick (5) rather than at the acidic end of the IPG (6).

large wick acted as a bridge between the IPG and the electrode. The IPGs were held in uniform contact with the wick using cup loading strips supplied with the IEF-100. The cups were filled with mineral oil and a stack of glass plates was placed on top of the assembly (**Figure 3**).

2.9 Second-dimension PAGE

11% polyacrylamide gels were cast in 180 X 80 X 1 mm glass cassettes following the recipe in Table 2. When prepared as described in Table 1, acrylamide-bisacrylamide solutions do not require degassing provided they are stored in glass bottles, since plastics contain dissolved oxygen which inhibits polymerization. Likewise, stock solutions should be equilibrated to room temperature, since cold solutions contains more dissolved oxygen. When necessary, the ammonium persulfate concentration was adjusted to allow polymerization in 15–20 minutes. Gels taking longer than 30 minutes to polymerize were discarded. This rapid polymerization favors more numerous, but shorter polymers and higher conversion of acrylamide monomer than in most commercial precast gels. By comparison, precast gels formed at slower polymerization rates have fewer, but longer polymer chains and may contain higher amounts of acrylamide monomer. (Unpolymerized acrylamide monomer in the gel can react with protein cysteines during electrophoresis, if not alkylated beforehand.) Exactly 12.5 mL of acrylamide solution was added to each gel cassette within one minute of the addition of the catalysts. A sharp interface was produced by overlaying the top of the unpolymerized gel immediately with 25% isopropanol. Following polymerization, the gel surface was washed copiously with distilled water.

Immediately following IEF, the IPG strips were each equilibrated 2X 10 minutes in the SDS Equilibration Buffer described in Table 1. The strips were placed on the polyacrylamide gels without an agarose overlay and PAGE was performed immediately at 100V for 80 minutes. Gels were fixed a minimum of two hours in 25% ethanol, 10% acetic acid and stained overnight with colloidal Coomassie Brilliant Blue (CBB) G-250 (Wijte et al. 2006, Smejkal 2004).

3. Results and discussion

IEF throughput can be doubled using a modified electrode module in which two pairs of electrodes are wired in parallel (Smejkal and Bauer 2011). The acidic ends of the gradients are positioned proximally and the basic ends of the gradients are at opposing ends of the running tray (**Figure 1**). 2D gels produced with either single or dual electrodes were super imposable (**Figure 2**). Compared to the standard single electrode pair, voltage gradients were nearly superimposable for paired IPGs run at twice the current and IEF is completed in less than three hours (**Figure 4**). No significant increase in operating temperature was observed. From time course IEF, it was judged that the focusing of bacterial proteins was completed in 12 kWh (**Figure 5**). In current limited IEF, maximum voltage reached 12,000V in less than two hours. This eliminates the need to program multiple steps. A single step in which maximum voltage is set to 12,000V results in the formation of a “natural” voltage gradient where voltage climbs at a rate limited by the programmed current limit. (In turn, the risk of generating heat during IEF is minimized when conservative current limits are

used.) Once maximum voltage is reached, 3 kVh is accumulated every 15 min at 12,000 V such that IEF in the IEF-100 is at least three times faster than previously described protocols.

The rapid focusing is attributed to the preparation of samples of initially low conductivity, since initially high conductivity and delays IEF slowing the nascent voltage gradient.

Sample preparation is critical to the success of IEF since contaminating ions raise the conductivity of the hydrated IPG. Moreover, residual buffer such as PBS must be removed prior to IEF and the failure to do so may have disastrous consequences. During IEF, PO_4^{-3} and HPO_4^{-2} ions accumulate at the anodic end of the IPG. This gross negative charge drives water, predominately as H_3O^+ ions, towards the cathode resulting in the collapse of the IPG gel in that region. When this happens, IEF ceases.

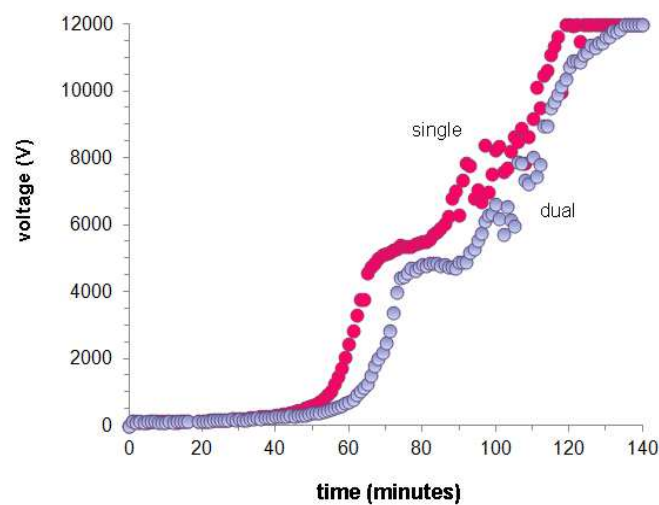


Fig. 4. Voltage gradients formed during IEF with single (red) or double (blue) electrodes current limited at 25 and 50 uA, respectively. Both runs were completed at 12 kVh.

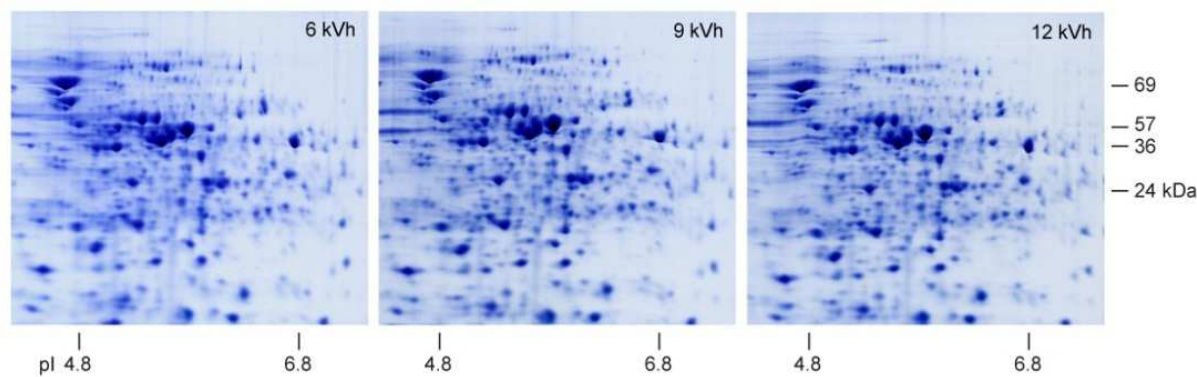


Fig. 5. IEF at 12,000V maximum voltage in the IEF-100. *E. coli* cell lysates were focused on IPGs pH 4-7 for 6, 9, or 12 kVh without carrier ampholytes. IEF for 12 kVh was completed in less than three hours. Second dimension PAGE was performed on 11% polyacrylamide gels prepared as described in Table 2. Gels were stained with colloidal CBB. (Figure adapted from Smejkal and Bauer, 2010. Reproduced with permission from American Biotechnology Laboratory.)

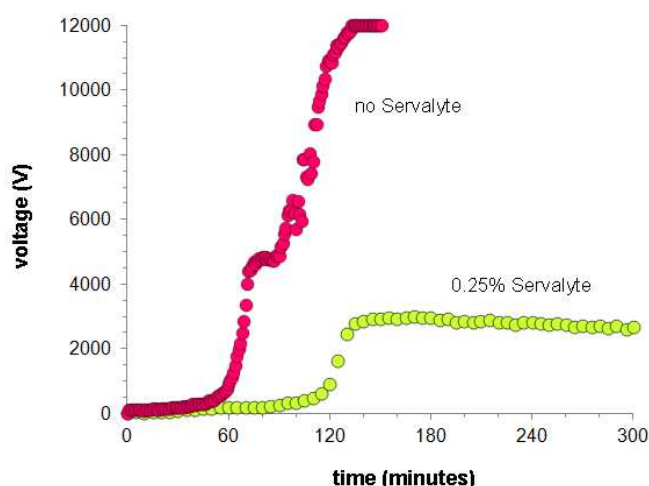


Fig. 6. Comparison of voltage gradients formed during IEF current limited at 25 μ A with or without 0.25% Servalyte pH 3-10 carrier ampholytes. IEF without carrier ampholytes (red) reached 12,000V maximum voltage in less than two hours. IEF with 0.25% Servalyte (green) did not exceed 2,500V and reached only 10 kWh in 5.5 hours.

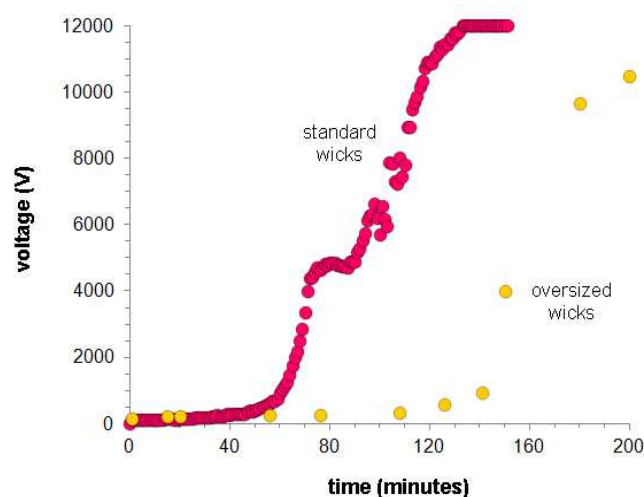


Fig. 7. The effect of wick size on voltage during IEF. Identical samples were run using standard 15 X 6 X 1 mm wicks (red) or 30 X 6 X 2 mm wicks (yellow). Using the oversized wick, voltage reached less than 1000V after two hours and IEF failed to reach maximum voltage of 12,000V after three hours. Only 6.8 kWh were accumulated.

The addition of 0.25% Servalyte carrier ampholytes also delayed IEF. When 0.25% carrier ampholytes were added to the samples, the voltage did not exceed 3,500V after three hours of IEF (**Figure 6**). This is because carrier ampholytes focus in the IPG where resulting in a minimum of conductivity gaps. Hence, conductivity remains constant. In samples lacking carrier ampholytes, conductivity gaps are rapidly created and voltage must increase to maintain constant current.

Similarly, IEF was prolonged when using oversized wicks (**Figure 7**). Voltage was less than 1000V after two hours and IEF failed to reach maximum voltage of 12,000V after three hours. The accumulation of ions in the enlarged wicks results in a precipitous voltage in that region which bridges the IPG to the electrode.

4. References

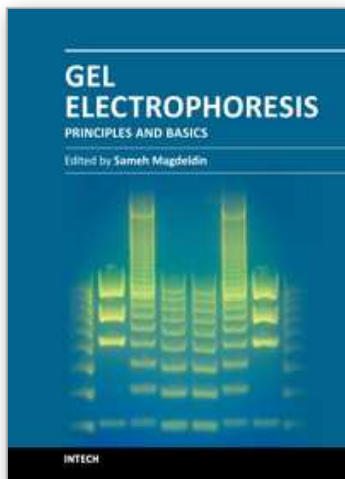
- Arikan, B. (2008). Highly thermostable, thermophilic, alkaline, SDS and chelator resistant amylase from a thermophilic *Bacillus* sp. isolate A3-15. *Bioresource Technol.*, 99, 3071-3096
- Bauer, D. J., Smejkal, G. B., Thomas, W. K., Robinson, M. H., Kuo, W. P. (2009). *HUPO 8th World Congress*, Toronto, Canada, September 14-20, 2009.
- Bordini, E., Hamdan, M., Righetti, P. G. (2000). *Rapid Comm. Mass Spectrom.*, 13, 840-848.
- Boschetti E., Monsarrat, B., Righetti, P.G. (2007). The "Invisible Proteome": How to capture the low-abundance proteins via combinatorial ligand libraries. *Current Proteomics*, 4, 198-208.
- Bottenus, D., Jubery, T.Z., Ouyang, Y., Dong, W.J., Dutta, P., Ivory, CF. (2011). A 10,000 fold concentration increase of the biomarker cardiac troponin I in a reducing union microfluidic chip using cationic isotachopheresis. *Lab Chip*, 11, 890-898.
- Chen, B., Chrambach, A., (1980). The effect of SDS on protein zone dispersion in polyacrylamide gel electrophoresis. *Anal. Biochem.*, 102, 409-418.
- Chrambach, A. (1985). *The Practice of Quantitative Gel Electrophoresis*. VCH: Weinheim, pp 1-195.
- Cossu, G., Righetti, P.G. (1987). Resolution of G gamma and A gamma foetal haemoglobin tetramers in immobilized pH gradients. *J. Chromatogr.*, 398, 211-216.
- Di Girolamo F., Bala K., Maxey C., Chung M., Righetti, P.G. (2011). "Proteomineering" serum biomarkers. A Study in Scarlet. *Electrophoresis*, 32, 976-980
- Galvani, M., Hamdan, M., Herbert, B., Righetti, P. G. (2001b). *Electrophoresis*, 22, 2058-2065.
- Galvani, M., Rovatti, L., Hamden, M., Herbert, B., Righetti, P. G. (2001a). *Electrophoresis*, 22, 2066-2074.
- Grassl, J., Westbrook, J. A., Robinson, A., Boren, M., Dunn, M. J., Clyne, R. K. (2009). *Proteomics*, 9, 4616-4626.
- Hamdan M., Righetti, P.G. (2005). Proteomics today: Protein assessment and biomarkers using mass spectrometry, 2D electrophoresis, and microarray technology. John Wiley & Sons: Hoboken, pp 219-265.
- Hamden, M., Bordini, E., Galvani, M., Righetti, P. G. (2001). *Electrophoresis*, 22, 1633-1644.
- Herbert, B., Galvani, M., Hamden, M., Olivieri, E., McCarthy, J., Pedersen, S., Righetti, P. G. (2001). *Electrophoresis*, 22, 2046-2057.
- Herbert, B., Hopwood, F., Oxley, D., McCarthy, J., Laver, M., Grinyer, J., Goodall, A., Williams, K., Castagna, A., Righetti, P.G. (2003). Beta-elimination: An unexpected artefact in proteome analysis. *Proteomics*, 3, 826-831.
- Herr, A.E., Singh, A.K. (2004). Photopolymerized Cross-Linked Polyacrylamide Gels for On-Chip Protein Sizing. *Anal. Chem.* 76, 4727-4733.
- Hjelmeland, L., Nebert, D., Osborne, J. (1983). Sulfobetaine Derivatives of Bile Acids: Nondenaturing Surfactants for Membrane Biochemistry. *Anal. Biochem.*, 130, 72-82.
- Hjelmeland, L.M., Chrambach, A. (1981). Electrophoresis and electrofocusing in detergent containing media: A discussion of basic concepts. *Electrophoresis* 2, 1-11.
- Kovacs, K. A., Steinmann, M., Magistretti, P. J., Halfon, O., Cardinaux, J. R. (2003). *J. Biol. Chem.*, 278, 36959-36965.
- Lunney, J., Chrambach, A., Rodbard, D. (1971). Factors affecting resolution, band width, number of theoretical plates, and apparent diffusion coefficients in polyacrylamide gel electrophoresis. *Anal Biochem.* 40, 158-73.

- O'Farrell, P.H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.*, 250, 4007-21.
- Olivieri, E., Herbert, B., Righetti, P. G. (2001). *Electrophoresis*, 22, 560-565.
- Rabilloud T. (1998). Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis. *Electrophoresis*, 19, 758-760.
- Rabilloud T. (2002). Two-dimensional gel electrophoresis in proteomics: Old, old fashioned, but it still climbs up the mountains. *Proteomics*, 2, 3-10.
- Rabilloud, T., Blisnick, T., Heller, M., Luche S., Aebersold, R., Lunardi, J., Braun-Breton, C. (1999). *Electrophoresis*, 20, 3603-10.
- Rath, A., Glibowicka, M., Nadeau, V.G., Chen, G., Deber, C.M. (2009). Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *PNAS*, 106, 1760-1765.
- Richardson M.R., Liu S., Ringham H.N., Chan V., Witzmann F.A. (2008). Sample complexity reduction for two-dimensional electrophoresis using solution isoelectric focusing prefractionation. *Electrophoresis*, 29, 2637-2644.
- Righetti P.G., Simó C., Sebastiano R., Citterio A. (2007). Carrier ampholytes for IEF, on their fortieth anniversary (1967-2007), brought to trial in court: the verdict. *Electrophoresis*. 2007 28, 3799-810.
- Righetti, P.G. (1990). Immobilized pH Gradients: Theory and Methodology. Elsevier: Amsterdam, pp 1-397.
- Righetti, P.G., Stoyanov, A.V., Zhukov, M.Y. (2001). *The Proteome Revisited: Theory and Practice of All Relevant Electrophoretic Steps*. Elsevier: Amsterdam, pp 295-7.
- Robinson, A. A., Westbrook, J. A., English, J. A., Boren, M., Dunn, M. J. (2009). *Proteomics*, 9, 4433-4444.
- Seddon, A.M., Curnow, P., Booth, P.J. (2004). Membrane proteins, lipids and detergents: Not just a soap opera. *Biochimica et Biophysica Acta*, 1666, 105-117.
- Smejkal G.B., Bauer D.J. (2010). 2D in a Day: A Two-Dimensional Gel Electrophoresis Workflow Completed in Under Five Hours. *American Biotechnology Laboratory*, 28, 24-27.
- Smejkal G.B., Bauer D.J. (2011). Increased IEF Throughput Using a Dual-Electrode Assembly. *American Laboratory*, 42, 28.
- Smejkal GB, Li C, Robinson MH, Lazarev A, Lawrence N, Chernokalskaya E. (2006). Simultaneous Reduction and Alkylation of Protein Disulfides in a Centrifugal Ultrafiltration Device Prior to Two-Dimensional Gel Electrophoresis. *J. Proteomic Res.*, 5, 983- 987.
- Smejkal GB, Rivas-Morello C, Chang JH, Alexander J, Trachtenberg AJ, Lazarev A, Ivanov A (2011). Thermal stabilization of tissues and the preservation of protein phosphorylation states for two-dimensional gel electrophoresis. *Electrophoresis*, 32, 1-10.
- Smejkal, G. B., Witzmann, F. A., Ringham, H., Small, D., Chase, S. F., Behnke, J., Ting, E. (2007). *Anal. Biochem.*, 363, 309-311.
- Smejkal, G.B. (2004). The Coomassie Chronicles: Past Present, and Future Perspectives in Polyacrylamide Gel Staining. *Expert Rev. Proteomics*, 1, 381-387.
- Smejkal, G.B., Lazarev, A. (2005). Solution phase isoelectric fractionation in the multi-compartment electrolyzer: a divide and conquer strategy for the analysis of complex proteomes. *Briefings in Functional Genomics and Proteomics*, 4, 76-81.

- Vecchio, G., Righetti, P.G., Zanoni, M., Artoni, G., Gianazza, E. (1984). Fractionation techniques in a hydro-organic environment: I. Sulfolane as a solvent for hydrophobic proteins. *Anal. Biochem.*, 137, 410-419.
- Wijte, D., De Jong, A., Mol, M., Van Baar, B., Heck, A. (2006). ProteomIQ Blue, a potent post-stain for the visualization and subsequent mass spectrometry based identification of fluorescent stained proteins on 2D-gels. *J. Proteome Res.*, 5, 2033-8.

IntechOpen

IntechOpen



Gel Electrophoresis - Principles and Basics

Edited by Dr. Sameh Magdeldin

ISBN 978-953-51-0458-2

Hard cover, 346 pages

Publisher InTech

Published online 04, April, 2012

Published in print edition April, 2012

Most will agree that gel electrophoresis is one of the basic pillars of molecular biology. This coined terminology covers a myriad of gel-based separation approaches that rely mainly on fractionating biomolecules under electrophoretic current based mainly on the molecular weight. In this book, the authors try to present simplified fundamentals of gel-based separation together with exemplarily applications of this versatile technique. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Gary B. Smejkal and Darren J. Bauer Part (2012). High Speed Isoelectric Focusing of Proteins Enabling Rapid Two-Dimensional Gel Electrophoresis, Gel Electrophoresis - Principles and Basics, Dr. Sameh Magdeldin (Ed.), ISBN: 978-953-51-0458-2, InTech, Available from: <http://www.intechopen.com/books/gel-electrophoresis-principles-and-basics/high-speed-ief-and-increased-two-dimensional-gel-electrophoresis-throughput>

INTECH
open science | open minds

InTech Europe

University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

© 2012 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the [Creative Commons Attribution 3.0 License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

IntechOpen

IntechOpen