2-D in a Day: A Two-Dimensional Gel Electrophoresis Work Flow Completed in Under Five Hours

While O’Farrell¹ first described two-dimensional electrophoresis (2DE) of proteins 35 years ago, it remains one of the most widely used tools in proteomics today. Continuing refinements of 2DE, particularly the development of immobilized pH gradients,² new surfactants that increase and maintain protein solubility,³ and sample fractionation,⁴ have further increased the utility of the method.

Consider that immobilized pH gradient (IPG) strips are presently capable of separating proteins differing in charge by 0.002 isoelectric point (pI) units such that thousands of proteins can theoretically be resolved over the pH 3–10 range. This hypothetical number of separated proteins is nearly squared when a second dimension of electrophoresis is introduced, so that the number of proteins that can be arrayed by 2DE extends into the tens of thousands.⁵ Despite these benefits, 2DE is generally regarded as procedurally complex and often irreproducible, even considered a “black art” by some, and frequently includes lengthy electrophoresis times culminating over two days. It may be necessary to perform isoelectric focusing (IEF) overnight, further protracting the procedure. This paper describes a microprocessor-controlled IEF unit from Hoefer, Inc. (Holliston, MA) in which highly reproducible IEF of proteins is completed in less than 3 hr, enabling both dimensions of 2DE to be completed in approximately 5 hr.

Materials and methods

Materials

IPG BlueStrips were from Serva Electrophoresis GmbH (Heidelberg, Germany). Lyophilized Escherichia coli strain K12, broad-range protease inhibitor cocktail (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride E-64, bestatin, leupeptin, aprotinin, ethylenediaminetetraacetic acid [EDTA]), and tributylphosphine (TBP) were from Sigma-Aldrich (St. Louis, MO). The B-163 conductivity meter was from Horiba (Kyoto, Japan). AG501-X8 ion-exchange resin was from Bio-Rad (Hercules, CA). Cresol purple was from US Biochemicals (Cleveland, OH). All other reagents used were from Hoefer, Inc.

Sample preparation

Three hundred milligrams of lyophilized E. coli was suspended in 10 mL of sample solubilization reagent (prepared as described in Table 1) followed by the addition of 200 µL of 50× broad-range protease inhibitor cocktail and 250 µL of 200 mM TBP. The suspension was sonicated for 40× 1-sec cycles using a Sonicator 450 (Branson Ultrasound, Danbury, CT) with microtip probe at 70% maximum power. The proteins were alkylated for 2 hr following the addition of 10 mM acrylamide. The alkylation reaction was quenched by the addition of concentrated dithiothreitol (DTT) to a final concentration of 50 mM.⁶

Table 1: Reagent preparation for IEF and 2-DE

<table>
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<tr>
<th>Reagent</th>
<th>Formulation</th>
<th>Comments</th>
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<tr>
<td>Sample Solubilization Solution</td>
<td>7M urea, 2M thiourea, 4% CHAPS</td>
<td>Dissolve solids completely, then mix with AG501-X8 ion-exchange resin until conductivity is less than 10 uS/cm. Filter and store in frozen aliquots.</td>
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<tr>
<td>Alkylation Reagent</td>
<td>1M acrylamide</td>
<td>For more complete and more specific alkylation of Cys residues than iodoacetamide (IAA). Simultaneous reduction and alkylation is possible when the non-thiol tributylphosphine (TBP) is substituted for DTT. Add 1/100 volume to the sample and alkylate for two hours. Prepare fresh, store at room temperature, and use within one month. Do not freeze.</td>
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<tr>
<td>IEF Tracking Dye</td>
<td>1% Bromophenol Blue</td>
<td>Provides a visual monitor of the progress of IEF. Useful pH indicator during sample preparation.</td>
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<tr>
<td>Tris Concentrate</td>
<td>1M Trizma base</td>
<td>Add 10 mM Tris when sample pH is below 8.0 as indicated by color shift of bromophenol Blue to yellow. Repeat as necessary until color has shifted to blue. Filter and store refrigerated.</td>
</tr>
<tr>
<td>Reducing Reagent</td>
<td>2M DTT</td>
<td>Store in frozen aliquots.</td>
</tr>
<tr>
<td>IPG Equilibration Buffer</td>
<td>375 mM Tris-HCl pH 8.8, 3M urea, 3% SDS, 50 mM DTT, 0.005% BPB, 0.01% m cresol purple</td>
<td>Filter and store in frozen aliquots.</td>
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<tr>
<td>Acrylamide-Bisacrylamide Concentrate</td>
<td>29.2% acrylamide, 0.8% methylene bisacrylamide</td>
<td>The solids are completely dissolved, then incubated with an ion-exchange resin until conductivity is less than 10 uS/cm. Filter and store refrigerated in amber glass bottles that are half-filled. Do not store in plastic containers. Do not freeze.</td>
</tr>
<tr>
<td>4X Resolving Gel Buffer</td>
<td>1.5 mM Tris-HCl pH 8.8</td>
<td>Buffer component of second dimension PAGE gels. Filter and store refrigerated.</td>
</tr>
<tr>
<td>APS Concentrate</td>
<td>10% ammonium persulfate</td>
<td>Prepare fresh and immediately filter and freeze at -80°C. Frozen aliquots are stable for at least three months.</td>
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Proteins were precipitated by the addition of 86% acetone, followed by incubation at room temperature for 2 hr with intermittent vortexing. The flocculent was pelleted by centrifugation at 10,000 RCF for 10 min. The pelleted proteins were resuspended in 10 mL of fresh sample solubilization reagent that was ion-exchanged with AG501-X8 ion-exchange resin until conductivity was ≤ 10 µS/cm. The sample solubilization reagent was filtered to remove the resin, and 50 mM DTT was added immediately prior to use. Protein concentration was estimated to be 10 mg/mL using the Hoefer Protein Determination Reagent. Samples were diluted to 0.65 or 1.3 mg/mL in the ion-exchanged reagent.

**Isoelectric focusing in the Hoefer IEF100 unit**

IPG BlueStrips, 7 cm, pH 4–7 and 3–10 NL, were rehydrated overnight with 140 µL of each test solution (either 0.65 or 1.3 mg/mL) in the provided rehydration trays. To prevent drying, the trays were sealed in zipper storage bags with a dampened paper towel to maintain a humid environment. (Alternatively, the hydrating strips can be protected from drying by overlaying with a small amount of mineral oil.)

Strips were adhered gel side up to the IEF100 running trays with a drop of mineral oil. Wicks dampened with Milli-Q H₂O (Millipore Corp., Billerica, MA) 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 was performed using 0–6000 V or 0–12,000 V voltage gradients over 1 hr, followed by 12,000 V constant voltage. Current was limited at 25 µA per strip. Power was limited at 0.5 W per strip. Temperature was 20° ± 0.5 °C.

**Staining of IPG strips**

IPG strips were removed during the time course and fixed in 35% methanol, 10% acetic acid. IPG strips were stained with Coomassie brilliant blue (CBB) as described in Table 2.

**Second-dimension polyacrylamide gel electrophoresis (PAGE)**

Polyacrylamide gels were cast in the Hoefer SE235 Four Gel Multicaster as described in Table 3. A sharp interface was produced by overlaying the unpolymerized gels with 25% isopropanol. Catalyst concentrations were adjusted to allow polymerization in 15–20 min, after which the gel surface was rinsed with distilled water. Immediately following IEF, the IPG strips were each equilibrated 2× for 10 min in IPG equilibration buffer (Table 1). The strips were placed on the polyacrylamide gels without an agarose overlay. Gels were electrophoresed on the Hoefer SE260 Mighty Small Vertical Electrophoresis Unit at 120 V constant voltage for 60 min.

**Results and discussion**

From time course studies, it was judged that IEF was completed in 6–8 kVh. At low protein loads (0.6 mg/mL) many bands were focused within the first hour of IEF, suggesting that many samples might be focused in less time (Figure 1). The heavier protein loads (1.3 mg/mL) required only slightly longer run times (Figure 2), which could be completed in the IEF100 in approximately 2 hr. The rapid focusing is attributed to the careful attention given to preparing the sample with low conductivity, since the failure to remove salts from the sample results in initially high strip conductivity and delays IEF by causing a lag in the voltage gradient.

**Conclusion**

A work flow is described that enables 2DE to be completed within 5 hr (Figure 3). Using the Hoefer IEF100 unit, rapid focus-
ing of proteins is possible at voltages up to 12,000 V with short IEF times reported for 7-cm IPG strips. When attention is given to sample preparation, low initial conductivity of the IPG strips enables maximum voltage to be reached in 1.5–2 hr. When performed in the Hoefer SE260 Mighty Small Vertical Electrophoresis Unit, second-dimension PAGE is completed within 90 min. The prescribed “2-D in a day” can be easily expanded to include overlapping IEF and PAGE runs to further increase throughput, all completed within a regular work day.

Most manufacturers recommend that IEF of 7-cm IPG strips in their instruments be performed at voltages not exceeding 3500 V and 4000 V, respectively, unlike the IEF100, which runs at 12,000 V. In the IEF100, optimized protocols eliminate the need to program multiple steps. A single step in which maximum voltage is set to 12,000 V results in a natural voltage gradient where voltage climbs at a rate limited by the programmed current limit. Once maximum voltage is reached, 3 kVh are accumulated every 15 min at 12,000 V such that IEF in the IEF100 is at least three times faster than previously described protocols. Depending on the initial sample conductivity, the voltage ramp to 12,000 V is generally reached in less than 2 hr, roughly the same time required for competing devices to reach much lower voltage maximums. As exemplified in Tables 4 and 5, 6 kVh were accumulated in less than 2 hr during the ramp to 12,000 V, with 9 and 12 total kVh being reached 15 and 30 min later.

The utility of the 12,000-V capacity of the Hoefer IEF100 will be further realized when running 18- and 24-cm IPG strips, which often require run times in excess of 100 kVh.

References


The authors are with the Hubbard Center for Genome Studies, University of New Hampshire, Durham, NH 03824, U.S.A.; tel.: 603-204-4947; e-mail: smejkal.gary@gmail.com.

Hoefer, Inc.
84 October Hill Road, Holliston, MA 01746
Tel +1 508.893.8999, US Toll Free +1 800.227.4750, Fax +1 508.893.0176
Email: support@hoeferinc.com, www.hoeferinc.com