High-Performance 2-D Gel Electrophoresis Using Narrow pH-Range ReadyStrip™ IPG Strips

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Introduction

Proteomics is now generally accepted as an unbiased method to analyze protein expression; e.g., to elucidate cellular processes at the molecular level or to identify surrogate markers in treatment models. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is the heart of proteome technologies, as it is the only method currently capable of simultaneously separating thousands of proteins (Rabilloud 2000). The technique involves the separation of proteins by isoelectric focusing (IEF) in the first dimension. Proteins are separated in a pH gradient until they reach a stationary position where their net charge is zero. The pH at which a protein has zero net charge is called its isoelectric point (pI). In the second dimension, proteins are separated according to their relative molecular weight by SDS-PAGE (Figure 1). The 2-D PAGE technique that is used today originated from the work of O'Farrell (1975), who used denaturing IEF in the first dimension.

The main drawbacks of the original procedure for 2-D PAGE methodology were poor reproducibility due to instability of the carrier ampholyte pH gradient in the first dimension and the lack of sufficient sample loading capacity to visualize low-abundance proteins, which are probably more interesting. The introduction of immobilized pH gradients (IPGs) largely overcame these problems (Bjellqvist et al. 1982). The IPGs are formed by copolymerization of the pH gradient and the polyacrylamide support. Since the IPGs are commercially available (as ReadyStrip pH 3–6, pH 5–8, and pH 7–10 IPG strips from Bio-Rad), this has dramatically improved the reproducibility of 2-D PAGE and boosted its application in proteomics studies. It has become the method of choice for the separation of complex mixtures of proteins from tissues and cells because of its enormous high resolution and the fact that parameters affecting separation are controlled independently in the two dimensions.

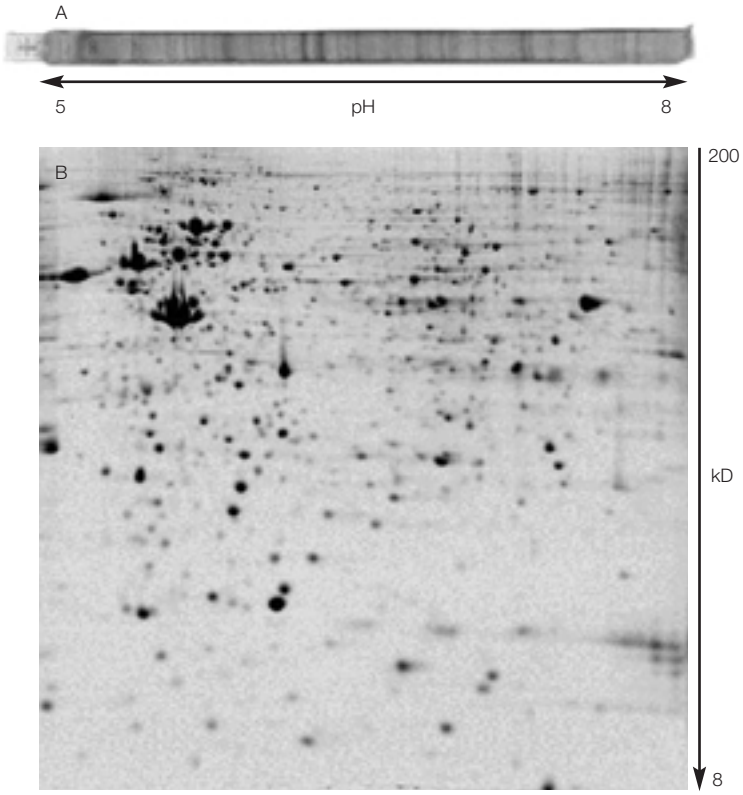

Fig. 1. Principle of 2-D electrophoresis. A, pre-B lymphoma cell extract (1 mg) was separated by IEF on a ReadyStrip pH 5–8 IPG strip, and stained with Bio-Safe™ Coomassie stain. B, Equilibrated strip was run in the second dimension by SDS-PAGE (12% acrylamide). The gel was stained with Coomassie Blue.

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Detailed Protocol for 2-D PAGE

Two-dimensional electrophoresis was essentially performed according to standard protocols (Gorg et al. 1988) with minor modifications as described here. Protein samples were prepared from the 697 pre-B lymphoma cell line, of which 10^7 cells corresponded to about 1 mg protein. Cells were directly dissolved in the sample buffer described by Rabilloud (1998). The sample buffer, which contains 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% Pharmalyte carrier ampholytes pH 3–10 (Pharmacia) for IEF, 0.01% Bromophenol Blue and one tablet of a cocktail of protease inhibitor (complete; Roche Bioscience), was aliquoted, stored at -80°C, and thawed only once. Before use, DNase and RNase were added. Protein samples were incubated for 15 min at room temperature and centrifuged for 10 min at 15,000 x g before applying them to the strips.

ReadyStrip pH 3–6 and pH 5–8 IPG strips (17 cm; Bio-Rad) were rehydrated overnight in 300 ml buffer containing 1–2 mg protein (Sanchez et al. 1997). Passive rehydration is preferred when loading semi-preparative amounts. After rehydration, paper wicks were added to absorb salts. Focusing conditions on the Bio-Rad PROTEAN® IEF cell were: 2 hr at 300 V to remove excess salts, voltage ramping for 4 hr to 10,000 V (current limit per strip was set at 50 µA), followed by focusing for 5.5 hr at 10,000 V (11.5 hr; 76 kV-hr). The focusing temperature was set at 15°C.

It was necessary to rehydrate the ReadyStrip pH 7–10 IPG strip in buffer without the protein. After rehydration, protein samples were applied through sample cups. In this case a conventional flatbed IEF system was used (Gorg et al. 1988). On this system, focusing conditions are adapted to ensure that the sample enters the gel with minimal precipitation at the interface with the cup. The following focusing conditions were

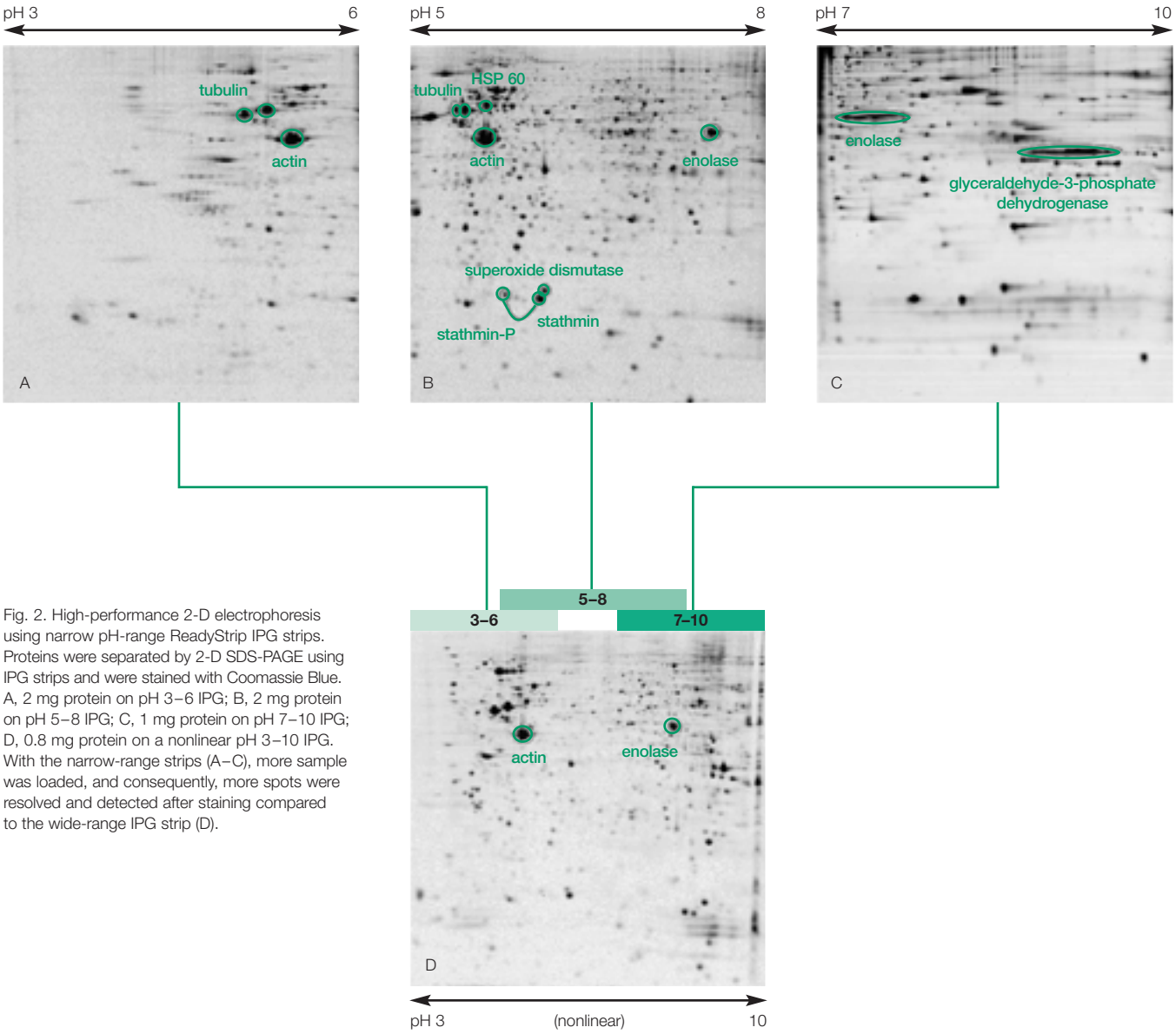

Fig. 2. High-performance 2-D electrophoresis using narrow pH-range ReadyStrip IPG strips. Proteins were separated by 2-D SDS-PAGE using IPG strips and were stained with Coomassie Blue. A, 2 mg protein on pH 3–6 IPG; B, 2 mg protein on pH 5–8 IPG; C, 1 mg protein on pH 7–10 IPG; D, 0.8 mg protein on a nonlinear pH 3–10 IPG. With the narrow-range strips (A–C), more sample was loaded, and consequently, more spots were resolved and detected after staining compared to the wide-range IPG strip (D).

therefore used: 3 hr at 300 V to ensure all proteins entered the strip, voltage ramping for 5 hr to 3,500 V, followed by focusing for 20 hr at 3,500 V (28 hr; 81 kV-hr). The focusing temperature was set at 20°C.

After focusing, the strips were equilibrated in a solution containing 6 M urea, 50 mM Tris-HCl, pH 8.5, 30% glycerol, 2% SDS for 12 min at room temperature with 2% DTT and for 6 min with 5% iodoacetamide. In the second dimension, strips were applied to 12%T, 2.6%C polyacrylamide gels. Gels were overlaid with 0.5% agarose solution to ensure good contact between the strip and the gel.

Gels were stained with colloidal Coomassie Blue (Anderson 1991) or with silver (Blum et al. 1987). The gels were scanned in a laser densitometer and image analysis was performed using Melanie software (Appel et al. 1997a, b).

Results and Discussion

On a standard Coomassie-stained 2-D gel with a wide pH 3–10 nonlinear IPG as the first dimension, about 1,500 spots can be detected with a protein load of about 1 mg (Figure 2D). However, to improve resolution and sensitivity, narrow-range IPG strips should be used. Such strips have higher protein loading capacity than standard wide-range IPGs (Righetti 1990), and consequently, an increased number of protein spots can be visualized at the same level of detection sensitivity.

Figure 2 shows the pH range of 3–10 expanded over three partially overlapping narrow pH-range ReadyStrip IPGs. The ReadyStrip pH 3–6 and pH 5–8 IPG strips were loaded with 2 mg of protein, whereas the ReadyStrip pH 7–10 strip was loaded with only 1 mg protein (by cup loading at the anodic site). Depending on the pH range, much higher amounts of protein can be loaded, although resolution might be diminished, especially at the more alkaline pH range. Our image analysis software detected 1,000 spots (for the IPG 3–6), 2,000 spots (IPG 5–8), and 900 spots (IPG 7–10) on these gels. While the overlapping pH ranges by definition have data repeated between gels, in total, more spots are easily resolved and detected on the narrow pH-range ReadyStrip IPGs.

Obviously, in these experiments the possibilities of sensitive protein detection have not been exhausted. With silver staining the detection limit can be lowered, albeit at the cost of simplicity and reproducibility. In preliminary experiments, the post-separation fluorescent dye SYPRO Ruby (available from Bio-Rad) seems to have the optimal combination of ease of use and sensitivity to make it the proteomics dye of the near future.

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