Leila H. Choe
Kelvin H. Lee

Chemical Engineering,
Cornell University,
Ithaca, NY, USA

A comparison of three commercially available isoelectric focusing units for proteome analysis: The Multiphor, the IPGphor and the Protean IEF cell

We tested and compared three different commercially available instruments for isoelectric focusing for proteome analysis by two-dimensional protein electrophoresis. These instruments, the Multiphor, the IPGphor, and the Protean IEF cell, were used with 18 cm immobilized pH gradient strips and run under various conditions. The total number of spots and features was obtained by Melanie software (Bio-Rad Laboratories) and separately by visual inspection. The Multiphor consistently resulted in the highest number of spots detected per gel independent of sample type, immobilized pH gradient (IPG) and method to calculate the number of spots. The Protean IEF cell had the next highest number of spots detected per gel. In the experiments performed, the IPGphor afforded good reproducibility in the total number of Melanie-detected spots from gel to gel while the Protean IEF cell offered better reproducibility in the total number of manually detected spots from gel to gel. Among gels run with the different instruments, differences in the quality of the ammoniacal silver stain were also observed. A measure of quantitative reproducibility suggests that the Protean IEF cell, which was the easiest instrument to use, performs better than the other instruments, although all three instruments had demonstrated good quantitative reproducibility in the experiments performed.

Keywords: Isoelectric focusing / Immobilized pH gradients / Proteome analysis / Two-dimensional protein electrophoresis EL 3803

1 Introduction

The need and trend for high throughput analysis of gene expression has motivated the development of new technology for mRNA analysis (microarrays) and proteome analysis (2-D gels, mass spectrometry, *etc.*). Many of these technologies have recently been reviewed [1, 2] and have been shown, both experimentally [3] and mathematically [4], to be necessary to understand the interaction among genes and gene products. One important step in the analysis of proteome expression using current technology is isoelectric focusing. Although some earlier work had been done, modern isoelectric focusing technology is often attributed to Tiselius' pioneering experimental work [5] and Svensson's theoretical studies [6]. Although this technology had been improved by the development of better carrier ampholyte production [7], acrylamide polymers for isoelectric focusing [8], and instrumentation, perhaps the single most important advance in isoelectric focusing for proteome analysis was the development of immobilized pH gradient (IPG) technology [9]. Among the key advantages of IPG technology *versus* conventional carrier ampholyte isoelectric focusing are: improved reproducibility in the charge separation [10], particularly when strips from a single lot are used; the ability to load greater quantities of protein either with specialized sample cups or by in-gel rehydration [11]; the ability to focus for longer times with less concern about cathodic drift and electroendosmosis; and the ability to fine-tune pH gradients for a particular charge range of proteins [12].

Given the requirement for proteome analysis [3, 4] in understanding gene networks, and motivated by the need for high throughput monitoring of gene expression, there has recently been a desire to develop new instrumentation for IEF, based on the advantages of IPG technology, which can reduce the variability introduced by human error and which can shorten the time required to perform IEF. Three currently available instruments are from Amersham-Pharmacia Biotech (Piscataway, NJ) and Bio-Rad Laboratories (Hercules, CA). These are the Multiphor (AP Biotech), the IPGphor (AP Biotech), and the Protean IEF cell (Bio-Rad). All three have inherent advantages and disadvantages and unique traits which make them the ideal instrument depending on the application and on the procedures used. The Multiphor has existed as a flat-bed electrophoresis system for more than two decades and

Correspondence: Dr. Kelvin H. Lee, Chemical Engineering, 120 Olin Hall, Cornell University, Ithaca NY 14853-5201, USA
E-mail: khlee@cheme.cornell.edu
Fax: +1-607-255-9166

Abbreviation: GFP, green fluorescent protein

Proteomics and 2-DE

© WILEY-VCH Verlag GmbH, 69451 Weinheim, 2000
0173-0835/00/0505-0993 $17.50+.50/0

has been adapted for use with IPG technology. More recently, the IPGphor and the Protean IEF cell have been introduced as instruments capable of higher voltages, longer focusing times (Vh), more careful temperature control, and simple operation in terms of sample handling and programming. Here we test, compare, and describe our observations using all three instruments run in parallel for use in proteome analysis.

2 Materials and methods

2.1 Reagents

Acrylamide (Cat. No. 161-0107), ammonium persulfate (161-0700), Bio-Lyte 3–10 (136-1112), Bio-Lyte 5–7 (163-1152), bromophenol blue (161-0404), DTT (161-0611), glycine (161-0718), piperazine diacrylamide (161-0202), SDS (161-0302), TEMED (161-0800), Tris (161-0719), and urea (161-0731) were purchased from Bio-Rad. CHAPS (C9426), citric acid monohydrate (C1909), 50% glutaraldehyde (G6403), Igepal Ca-630 (I7771), 85% phosphoric acid (P5811), and sec-butanol (B1888) were purchased from Sigma (St. Louis, MO). Glacial acetic acid (V194), 37% formaldehyde (5016), glycerol (509204), and 50% sodium hydroxide (770504) were obtained from Mallinckrodt (Paris, KY). 2-Mercaptoethanol (6010) and 28% ammonium hydroxide (AX130313) were purchased from EM Science (Gibbstown, NJ). 18 cm long Immobiline DryStrips, pH range 3–10 nonlinear (17-1235-01) and pH range 4–7 linear (17-1233-01) were purchased from Amersham Pharmacia Biotech. Light mineral oil (01211) was purchased from Fisher (Fair Lawn, NJ) and iodoacetamide (57670) was from Fluka (Buchs, Switzerland). Silver nitrate (378) was obtained from GFS Chemicals (Columbus, OH) and low odor kerosene (P3390) was from JT Baker (Phillipsburg, NJ). Tris hydrochloride (IB70162) was purchased from Kodak (Rochester, NY) and 100% ethanol (111000200CSGL) was obtained from Pharmco (Brookfield, CT).

2.2 Isoelectric focusing

We present data on three distinct runs using these instruments for proteome analysis by 2-DE. In all cases, non-isoelectric focusing steps were done in parallel. For simplicity, we designate the three conditions as A, B or C; the programmed voltages are summarized in Table 1.

2.2.1 Condition A

Normal human plasma was prepared as described online at Swiss-2D Page [13]. The plasma was separated on 18 cm pH 3–10 nonlinear strips available from AP Biotech and 60 μg of protein per lane was loaded by in-gel rehydration [11] during an overnight period in their respective rehydration trays. For the Protean IEF cell and the IPG-phor, strip rehydration was performed in 8 M urea, 2% CHAPS, 0.5% carrier ampholytes (pH 3–10 : pH 5–7, 2:1), 15 mM DTT and a trace of bromophenol blue at 50 V. The Multiphor solution was identical except for the use of

Table 1. Programmed voltage parameters

		Phase 1	Phase 2	Phase 3	Phase 4
Multiphor	A	500–3500 V 5 h	3500 V 17.5 h	–	–
IPGphor	A	500–3500 V 5 h	3500 V 17.5 h	–	–
Protean IEF cell	A	500–3500 V 5 h	3500 V 17.5 h	–	–
Multiphor	B	500–3500 V 5 h	3500 V 17.5 h	–	–
IPGphor	B	500 V 1 h	500–1000 V 2 h	1000–8000 V 15 h - linear	–
Protean IEF cell	B	500 V 1 h	500–1000 V 2 h	1000–9000 V 15 h - slow	–
Multiphor	C	500–3500 V 5 h	3500 V 19 h	–	–
IPGphor	C	500 V 1 h	500–1000 V 2 h	1000–8000 V 1 h - linear	8000 V 16.5 h
Protean IEF cell	C	500 V 1 h	500–1000 V 2 h	1000–9000 V 1 h - linear	9000 V 13 h

Details of run conditions A, B and C are provided in Sections 2.2.1–2.2.3. All gradients in voltage were programmed as linear gradients.

2% carrier ampholytes (pH 3–10 : pH 5–7, 2:1). We used 0.5% carrier ampholytes with the Protean IEF cell and the IPGphor because the use of 2% carrier ampholytes resulted in samples of relatively high conductivity that did not permit the Protean IEF cell and IPGphor power supplies to achieve their programmed voltages, whereas 0.5% carrier ampholytes, the amount recommended in both sets of manufacturer's instructions, did permit appropriate running conditions. Paper electrode wicks were dipped in 6 mM phosphoric acid or 0.5 mM sodium hydroxide and applied after rehydration but before isoelectric focusing. The samples were focused for 64 250 Vh [13, 14].

2.2.2 Condition B

Total cell lysate from *Escherichia coli* JM105 expressing intracellular green fluorescent protein (GFP) was prepared as previously described [15]. Again, 18 cm pH 3–10 nonlinear strips were used from AP Biotech and 25 µg of protein per lane was loaded by in-gel rehydration during an overnight period in their respective trays. Strips were rehydrated in buffers as suggested by the manufacturer of the instrument in the accompanying directions. A 50 V active reswell was done for the IPGphor and the Protean IEF cell. For the Protean IEF cell (which does not give a specific recipe for rehydration solution) we used 8 M urea, 2% CHAPS, 0.5% carrier ampholytes (pH 3–10 : pH 5–7, 2:1), 18 mM DTT and a trace of bromophenol blue – concentrations which fall within the manufacturer's recommended concentrations. Paper electrode wicks were dipped in deionized water and applied just prior to isoelectric focusing. Strips were focused for an average of 58 598 Vh.

2.2.3 Condition C

Total cell lysate from *Escherichia coli* JM105 expressing intracellular green fluorescent protein was prepared as in condition B. Here, 18 cm pH 4–7 linear strips from AP Biotech were used and 25 µg of protein per lane was loaded by in-gel rehydration (pH 3–10: pH 5–7, 1:1) during an overnight period in their respective rehydration trays. Strips were otherwise rehydrated as in condition B above and were focused for 132 910 Vh for the Protean IEF cell, 121 882 Vh for the IPGphor and 76 500 Vh for the Multiphor. These longer focusing times [13, 14] were designed to test the performance limits of these instruments: 3500 V upper limit for the Multiphor, 8000 V upper limit for the IPGphor, and 9000 V for the Protean IEF cell. A 50 V active reswell was performed for the Protean IEF and the IPGphor instruments.

2.3 Other steps

All samples were prepared in parallel and split for rehydration. Barnstead Nanopure water was used throughout. Equilibration was performed as previously described [15] and SDS-PAGE was performed in 1.5 mm thick 12%T, 2.6%C slab gels in a Protean IIxi Multi-Cell electrophoresis tank (Bio-Rad) at 40 mA per gel as previously described [15]. Six gels were cast at a time in Bio-Rad casting stands (Cat. No. 165-1911) and all gels were run in parallel for an individual experiment. Gels were stained in parallel using an ammoniacal silver stain as described previously [16]. Gels were scanned on a Molecular Dynamics personal densitometer attached to a 333 MHz Pentium II computer with 128 MB RAM running Windows NT workstation 4.0. Gel image files were directly imported

Table 2. Number of features and spots detected on each of three instruments run under three different conditions

		Melanie-detected features	SD/ average no. spots	% Multiphor	Manually detected features	SD/ average no. spots	% Multiphor
Multiphor	A	1167	3.3%	100	1222	3.0%	100
IPGphor	A	951	7.0%	81	958	8.1%	78
Protean IEF cell	A	1255	1.1%	108	1162	9.9%	95
Multiphor	B	1878	6.6%	100	2443	8.2%	100
IPGphor	B	1580	4.8%	84	1894	1.2%	78
Protean IEF cell	B	1836	1.9%	98	2117	4.1%	87
Multiphor	C	1292	10.8%	100	1543	5.8%	100
IPGphor	C	1172	1.5%	91	1364	10.0%	88
Protean IEF cell	C	1232	11.4%	95	1432	1.2%	93

Data are averaged over duplicate runs. The standard deviation in the number of spots detected per gel for each case is normalized against the average number of spots detected for that case and is presented as a percentage. Details of the run conditions A, B and C are provided in Sections 2.2.1–2.2.3.

Table 3. Quantitative measurements of %volume over 15 spots as depicted in Figs. 1–3.

		SD of samples/ average % volume
Multiphor	A	10.5
IPGphor	A	12.6
Protean IEF cell	A	7.5
Multiphor	B	10.3
IPGphor	B	15.2
Protean IEF cell	B	10.0
Multiphor	C	17.7
IPGphor	C	13.2
Protean IEF cell	C	13.8

Data are averaged over duplicate runs and the standard deviation of the data is determined. The deviation is normalized against the average %volume measurements. Details of run conditions A, B and C are provided in Section 2.2.1–2.2.3.

into the Melanie II software (Bio-Rad) and features were detected using default parameters for silver-stained gels as described in the Melanie manual. The total number of features was obtained using Melanie. Separately, gel images were subject to visual inspection. The total number of individual spots that could be discerned (Table 2) was counted by a single person (a hand tally counter was used to aid in the process) and the results were recorded. This process of manual spot counting provides the same data as spot counts obtained using automated Melanie spot detection corrected by visual inspection. Manual corrections to Melanie-detected spots are typically performed in a 2-DE-based proteome analysis and the decision to add, remove, or edit spot files are based exclusively on visual inspection. In addition to Melanie-detected and manually detected feature counting, two other quantitative assessments of instrument performance were made. First, the reproducibility in the number of observed spots was estimated by calculating the standard deviation in the spot count data and normalizing this value by the average number of observed features for Melanie-detected and manually detected features (Table 2). Second, quantitative reproducibility was estimated by selecting 15 spots for each condition (see Fig. 1–3) that covered a range of isoelectric points and molecular weights. The Melanie-determined %volume was obtained for each spot. The average %volume for a particular spot and the standard deviation for these measurements were calculated. The standard deviation was normalized against the average and these values were subsequently averaged over all 15 spots in a gel to provide a more quantitative assessment of reproducibility using the different instruments. These data are presented as percentages in Table 3. Finally, gels were studied for subjective staining characteristics.

3 Results

We ran six gels of human plasma as described in condition A and twelve gels of *Escherichia coli* JM105 expressing intracellular green fluorescent protein [15] as described in conditions B and C. Representative gel images from B are shown in Figs. 1–3. The total average number of spots for each of three runs (Sections 2.2.1–2.2.3) on each instrument is given in Table 2. We also present the same data in terms of the number of features detected on a particular instrument as a percentage of the number of features detected on the Multiphor. In nearly all cases we observe that 2-D gels generated with the Multiphor yield a greater number of spots than either the IPGphor or the Protean IEF cell. This observation holds whether we rely on computer-detected spots or visual inspection. The one exception is that, under condition A, more features were detected by Melanie with the Protean IEF than with the Multiphor. However, because Melanie detection is typically followed by a visual correction (and none was performed in generating this Melanie data) and because the manually detected data for these same gels suggests that the Multiphor resulted in a higher number of spots, we do not believe that this one data point suggests that the Protean IEF cell will generally result in more spots than the Multiphor. Furthermore, the Protean IEF cell consistently resulted in a greater number of spots than the IPGphor, as measured either by Melanie or by visual inspection.

The assessment of reproducibility in the total number of spots indicates that the Protean IEF cell performs somewhat better than either the IPGphor or the Multiphor. One can average the results in Table 2 for the reproducibility of Melanie-detected features separately for the Multiphor, IPGphor, and Protean IEF cell. This calculation yields an average percent deviation of 6.9% for Multiphor, 4.4% for IPGphor, and 4.8% for the Protean IEF cell for Melanie-detected features and 5.7% for Multiphor, 6.4% for IPGphor and 5.1% for the Protean IEF cell for manually detected features.

Qualitatively, we observe that the gels generated with the Protean IEF cell stain far less intensely (using ammoniacal silver stain) than the other two instruments. In particular, silver-stained spots on these gels have a reddish tint to their silver stain. Gels run with the IPGphor also demonstrate a significantly decreased staining intensity relative to the Multiphor gels and also have a reddish tint, although this tint is not as pronounced as on the Protean IEF cell gels. The IPGphor gels had increased streaking in the vertical direction as compared to either the Protean IEF cell or the Multiphor gels. This vertical streaking, which was most pronounced in the basic end of the gels, is probably due to increased protein precipitation, perhaps

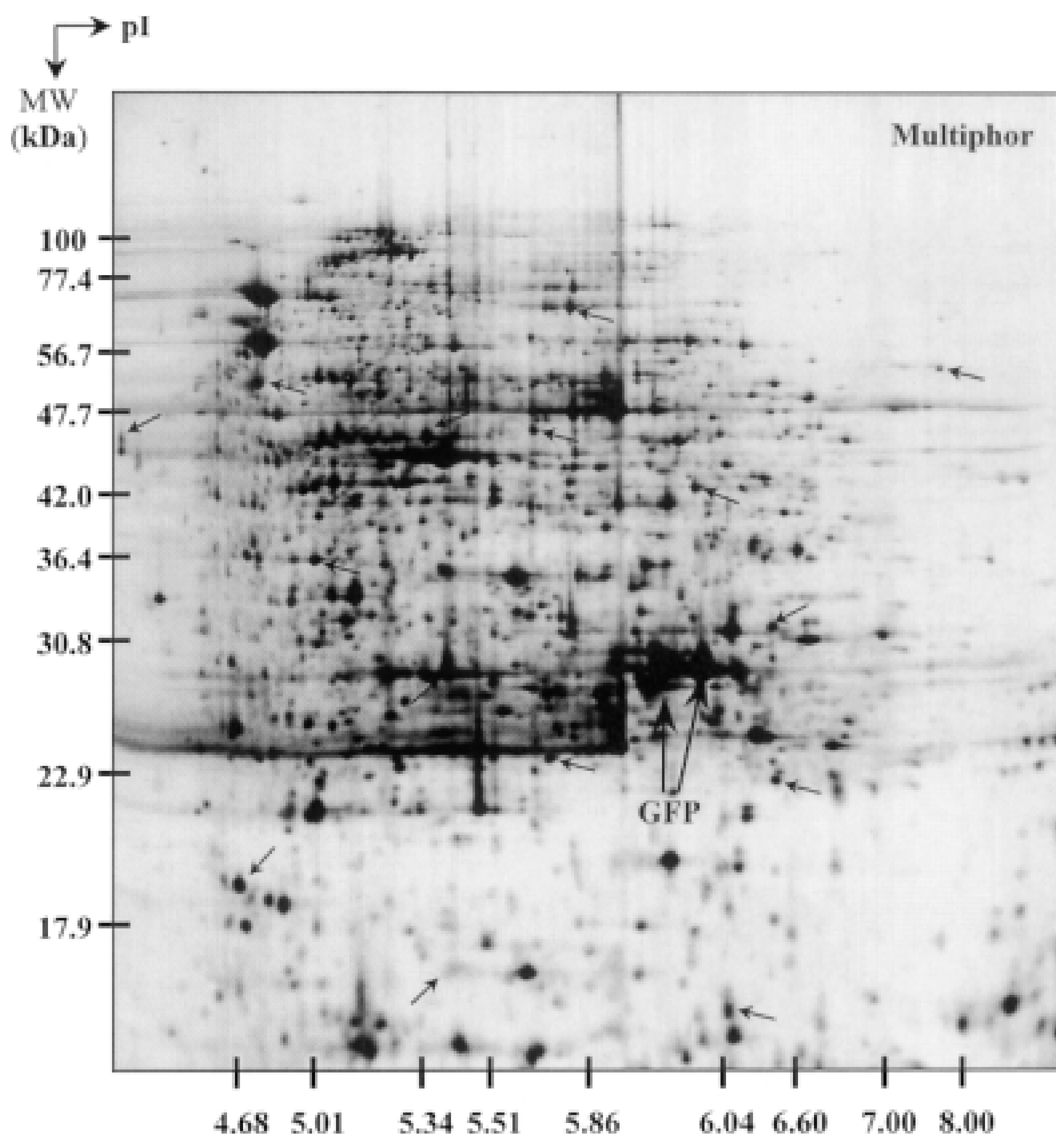

Figure 1. A representative gel from condition B run on the Multiphor. Total *E. coli* cell lysate from GFP-expressing cells was prepared and run on a pH 3–10 nonlinear gradient. The location of GFP is identified on this gel as a reference point. The location of the 15 spots used to generate the data in Table 3 are also given.

caused by longer focusing times. The streaking is likely enhanced by the use of the recombinant *E. coli* strain which expresses 15% of total protein as GFP which tends to result in increased streaking at the isoelectric points of GFP. However, in condition C, the focusing time for the Protean IEF cell was longer than that of the IPGphor and the streaking was still more pronounced on the IPGphor gels. In general, however, the Multiphor yielded the best resolution of proteins on the basic ends of the gels (see Figs. 1–3).

In conditions B and C we ran the instruments according to the manufacturer's directions as provided with the instruments with the goal of assessing the instruments closer to their respective voltage limits. Here again, the Multiphor yielded the greatest number of spots and features. Thus, we further find that the observations above appear to be somewhat sample-independent (human plasma and *E. coli*) as well as pH gradient-independent (pH 3–10 nonlinear and pH 4–7). The subjective qualitative differences identified above in staining intensity and quality also apply for these cases, suggesting that these differences in stain quality are also independent of pH gradient and rehydration buffer. We do not fully understand why a particular instrument may lead to such a qualitative difference in staining intensity and quality. We also find that the

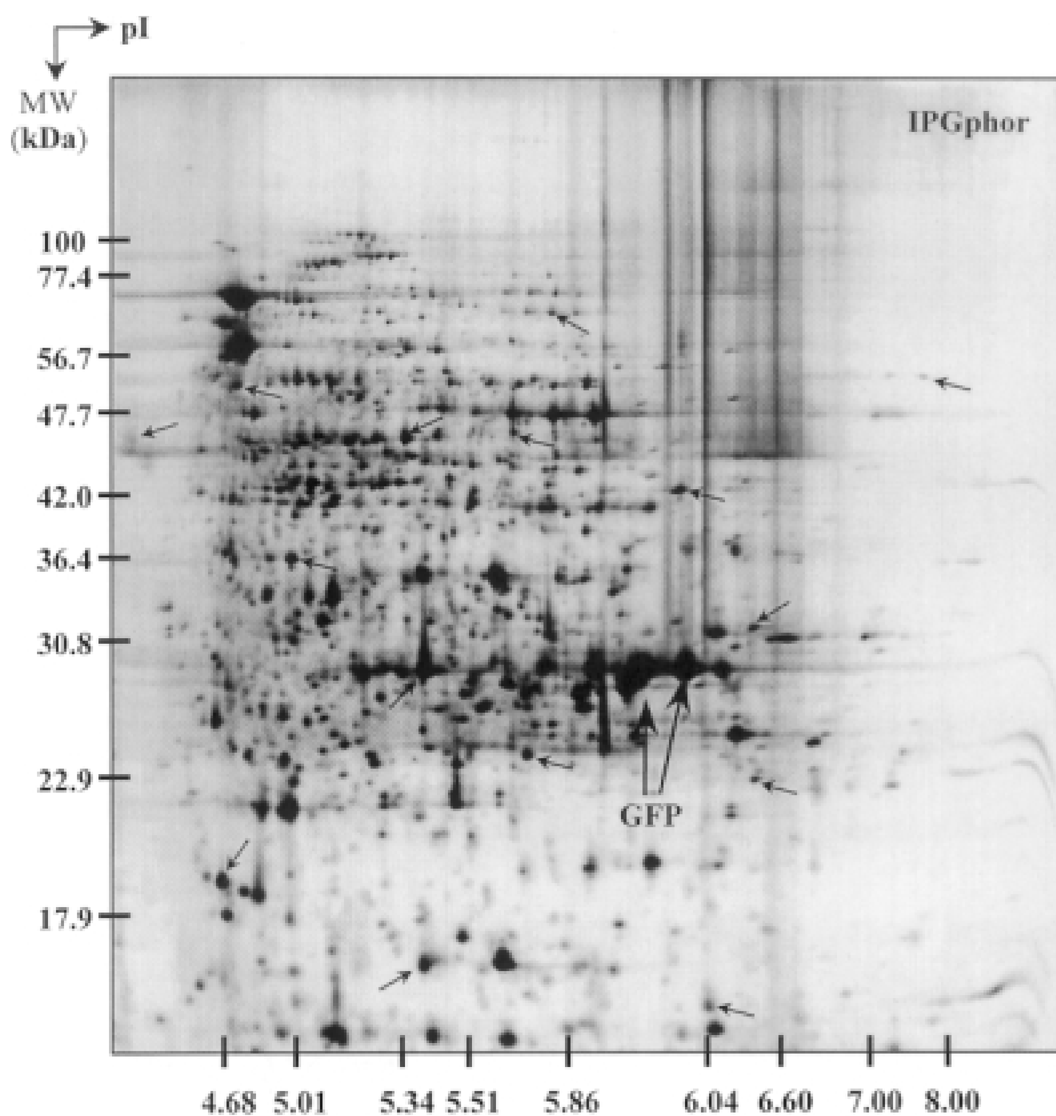

Figure 2. A representative gel from condition B run on the IPGphor. Sample, location of GFP, and location of the 15 relevant spots as in Fig. 1.

improved focusing in the basic end on the Multiphor is not pH gradient nor sample-dependent. We further observe major differences in the focusing patterns depending on which instrument is used – some prominent spots consistently appear or disappear depending on the instrument used. We can not offer a reasonable explanation for this observation at this time.

For a quantitative analysis we selected 15 spots and measured the reproducibility over individual samples and instruments. The data are presented in Table 3 as standard deviation normalized against average %volume for each of 15 spots. The data for condition A suggest that the Protean IEF cell had a variability of approximately 7.5% in the %volume measurements as compared to 10.5% for the Multiphor and 12.6% for the IPGphor. The Bio-Rad instrument performed better than the other two instruments in condition B while the IPGphor demonstrated the best performance for condition C. It is striking that none of these instruments shows significant quantitative variability for these measurements as measured by a Molecular Dynamics laser densitometer. The Multiphor under condition C demonstrates the greatest variability at less than 18%.

4 Discussion

We believe that the collective experience of the proteome field has shown that it is difficult, if not impossible, to generalize results from one specific experiment to experi-

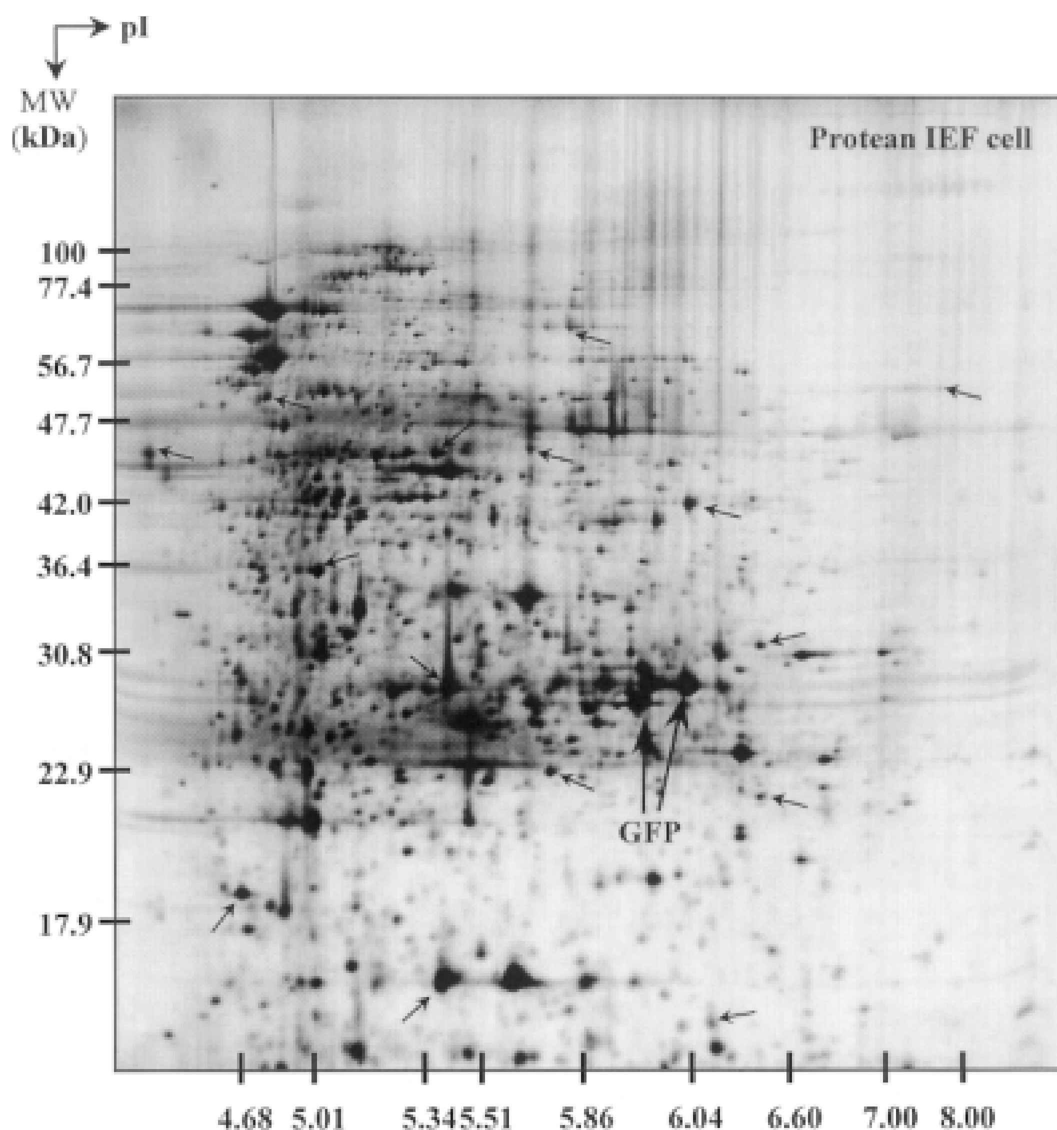

Figure 3. A representative gel from condition B run on the Protean IEF cell. Sample, location of GFP, and location of the 15 relevant spots as in Fig. 1.

ments with other samples and from samples run in different laboratories using different sources of chemicals, water, *etc.* Accordingly, we note that we have the most experience (>8 years) using the AP Biotech Multiphor which may have artificially led to biased results – although we tried to reduce this possibility by following the manufacturer's directions in some experiments. Optimization, over many months or years, with the IPGphor and the Protean IEF cell may lead to data that are as good or better than those obtained with the Multiphor; however, such an extended optimization period for each particular sample and each instrument is inconsistent with the concept of “out-of-the-box” isoelectric focusing for high throughput proteome analysis – a feature which is required before proteome analytical techniques can become ubiquitous and routine. Certainly, new users of IPG IEF technology demand an instrument capable of excellent resolution and quantitative reproducibility with little protocol optimization.

There are other extensions to this type of analysis which we have started to perform and will report later. For example, we did not use Bio-Rad ReadyStrip IPG strips on the Protean IEF cell for consistency in data interpretation. However, a combination of the Protean IEF cell with the Bio-Rad IPG strips may be optimal for that instrument, and because Bio-Rad IPG strips can be used on the Multiphor it is relatively straightforward to compare strips manufactured by either Bio-Rad or AP Biotech. However, given the cost of strips *versus* that of the instruments, one

might reasonably expect each laboratory to compare appropriate strips from different companies for each specific experiment.

Our analysis was subject to an optimization in the number of spots detected and, separately, in the quantitative reproducibility of resulting spots. However, depending on the particular application, such an optimization may not be appropriate. For example, one might wish to use IEF to focus micropreparative amounts of only one particular protein for chemical analysis (amino acid sequencing or MS analysis). In this case, the total number of spots seen over a large pH range is less important. However, given concerns [17] about solubilizing the largest number of proteins possible for effective proteome analysis, we chose to report data on the number of individual spots detected, with the Multiphor appearing to provide the best data. Of course, the reproducibility of such data is also an important factor in selecting an instrument; here the Protean IEF cell performed well. We further observed that the Bio-Rad Protean IEF cell is the simplest instrument to use among the three we tested. Finally, we note that our goals here are not to promote the use of any one instrument over others but to communicate our observations and experiences based on our sample preparation techniques performed by our hands. We have neither financial nor commercial interest in any of these instruments nor their manufacturers.

The work was supported, in part, by the National Science Foundation (BES-9874938), The New York State Science and Technology Foundation and Industrial Partners, DuPont, and Intel (98-238). The authors would also like to thank Bio-Rad Laboratories for the use of the Protean IEF cell and associated IPG strips and AP Biotech for use of the IPGphor.

Received August 30, 1999

5 References

[1] Service, R. F., *Science* 1998, *282*, 396–399.

[2] Anderson, N. L., Anderson, N. G., *Electrophoresis* 1998, *19*, 1853–1861.

[3] Gygi, S. P., Rochon, Y., Franza, B. R., Aebersold, R., *Mol. Cell. Biol.* 1999, *19*, 1720–1730.

[4] Hatzimanikatis, V., Lee, K. H., *Metab. Engineer.* 1999, *1*, 275–281.

[5] Tiselius, A., *Svensk Kem. Tidskr.* 1941, *58*, 305–310.

[6] Svensson, H., *Acta Chem. Scand.* 1961, *15*, 325–341.

[7] Vesterberg, O., *Acta Chem. Scand.* 1969, *23*, 2653–2666.

[8] Awdeh, Z. L., Williamsson, A. R., Askonas, B. A., *Nature* 1968, *219*, 66–67.

[9] Bjellqvist, B., Ek, K., Righetti, P. G., Gianazza, E., Görg, A., Postel, W., Westermeier, R., *J. Biochem. Biophys. Methods* 1982, *6*, 317–339.

[10] Corbett, J. M., Dunn, M. J., Posch, A., Görg, A., *Electrophoresis* 1994, *15*, 1205–1211.

[11] Sanchez, J.-C., Rouge, V., Pisteur, M., Ravier, F., Tonella, L., Moosmayer, M., Wilkins, M. R., Hochstrasser, D. F., *Electrophoresis* 1997, *18*, 324–327.

[12] Görg, A., Postel, W., Günther, S., *Electrophoresis* 1988, *9*, 531–546.

[13] Swiss-2D Page at http://www.expasy.ch/ch2d/ last accessed on 14 November 1999.

[14] Bjellqvist, B., Pasquali, C., Ravier, F., Sanchez, J.-C., Hochstrasser, D. F., *Electrophoresis* 1993, *14*, 1357–1365.

[15] Hatzimanikatis, V., Choe, L. H., Lee, K. H., *Biotechnol. Progr.* 1999, *15*, 312–318.

[16] Harrington, M. G., Gudeman, D., Zawert, T., Yun, M., Hood, L., *Methods Enzymol.* 1991, *3*, 98–108.

[17] Chevallet, M., Santoni, V., Poinas, A., Rouquié, D., Fuchs, A., Kieffer, S., Rossignol, M., Lunardi, J., Garin, J., Rabilloud, T., *Electrophoresis* 1998, *19*, 1901–1909.