

## TECHNICAL BRIEF

# Improved protocol for chromatofocusing on the ProteomeLab PF2D

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Beckman-Coulter has recently introduced the ProteomeLab PF2D™ for 2-D liquid separation of protein samples. The system features separation in the first dimension by chromatofocusing, followed by RP chromatography in the second dimension, allowing the analysis of complex proteomics samples. When used by the standard protocol, reproducibility and column life times are limited, making the use of the instrument very costly. We here present an improved protocol for chromatofocusing, which enhances column life by at least fivefold.

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Standard proteomics approaches rely on the 2-D separation of complex protein mixtures by gel electrophoresis [1]. However, 2-DE suffers from a number of shortcomings, such as limited loading capacity, inability to separate proteins with extreme *pI* values, or the difficulty of resolving proteins of small molecular weight. This has spurred the development of alternative protein separation and analysis tools such as SELDI-TOF [2] or liquid 2-D separation of proteins by column chromatography [3]. Beckman-Coulter has developed a corresponding system, the ProteomeLab PF2D™, which features separation by chromatofocusing in the first step, followed by RP chromatography in the second dimension [4–6]. The system is automated and allows separation of complex protein mixtures into several hundred fractions. Separation is followed by UV detection, allowing direct comparison of samples to detect changes in the proteome. Relevant fractions can then be subjected to MS to identify the proteins of interest.

A major shortcoming of the use of the ProteomeLab PF2D chromatographic system, if used according to the protocols recommended by the manufacturer, is the very limited

column life of the chromatofocusing column. Typical column life is four to six runs, after which the column back-pressure becomes too high for operation and the column has to be replaced. This makes the use of the instrument very costly and prompted us to seek improvements of the protocols for the use of this system. In an attempt to extend the life of the chromatofocusing column, we investigated factors that lead to clogging of the column. We identified three factors which limit column life. First, as no precolumns are used on the instrument, particulate or colloidal matter in the samples can clog the column. Second, the column wash procedure recommended by the manufacturer appears to cause protein precipitation in the column. Third, the recommended washing steps fail to remove all proteins from the column. We here describe an improved chromatofocusing protocol which improves column life by at least fivefold. It allows great savings in cost and enhances reproducibility when using the ProteomeLab PF2D.

The recommended washing steps for column regeneration involve 1 M NaCl, water, and 10–100% isopropanol. We found that high salt and isopropanol can cause protein precipitation in the column. Extensive experimentation revealed the most effective measures to prevent column clogging to be (i) filtration of all samples and buffers with 0.2 µm membrane filters, (ii) elimination of isopropanol washing steps, and (iii) the inclusion of 0.2% *n*-octyl-β-D-glucopyranoside (OG, Axon Labs) in all the eluents. These measures extended column life at least fivefold and thus significantly reduced

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**Abbreviation:** OG, *n*-octyl-β-D-glucopyranoside

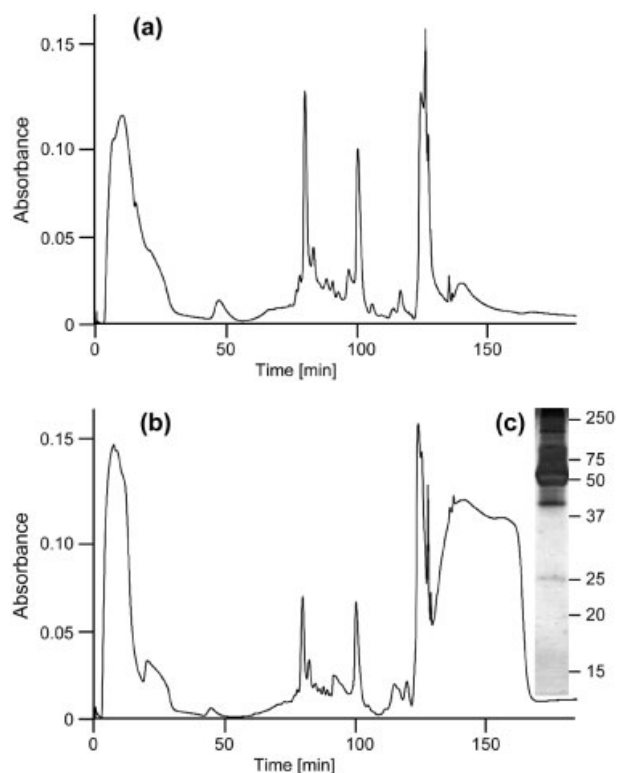
the operating cost of the ProteomeLab PF2D. A further gain in cost-effectiveness was achieved by the use of homemade buffers for chromatofocusing, as described below.

The improved protocol was tested, among others, with serum samples. Human blood was collected in the realm of a study that was approved by the Ethics Committee of the University of Berne. Blood (12 mL) was collected from healthy control patients with Sarstedt Monovette™ syringes and left overnight at 4°C to allow coagulation. Serum was then collected by centrifugation at 3000 × *g* for 15 min and stored at −80°C. For proteomics analysis, 100 μL of serum was centrifuged at 4°C for 20 min at 16 000 × *g* and the protein concentration was determined in the supernatant using the BioRad protein assay reagent. Samples were then diluted with start buffer to obtain a final protein concentration of 0.45–5 mg/mL, filtered through 0.22 μm Millipore Express PES Membrane filters, and 2 mL loaded on the chromatofocusing column.

Chromatofocusing was performed with the ProteomeLab PF2D (Beckman-Coulter, Fullerton, CA, USA) with an HPCF-1D column (250 mm × 2.1 mm, Eprogen, Darien, IL, USA). The pH gradients (internal and isocratic) were formed using two buffers, prepared essentially as described in [7]: “start” buffer (6 M urea, 25 mM Bis-Tris, 0.2% OG), pH 8.5 with 1 M ammonium hydroxide, and “elution” buffer (6 M urea, 10% v/v Polybuffer™ 74 (GE Healthcare), 0.2% OG, pH 4 with iminodiacetic acid). All buffers were filtered through 0.2 μm nylon membrane filters (NALGENE) and all flow rates were 0.2 mL/min. Column temperature was 20°C, and elution profiles were monitored at 280 nm.

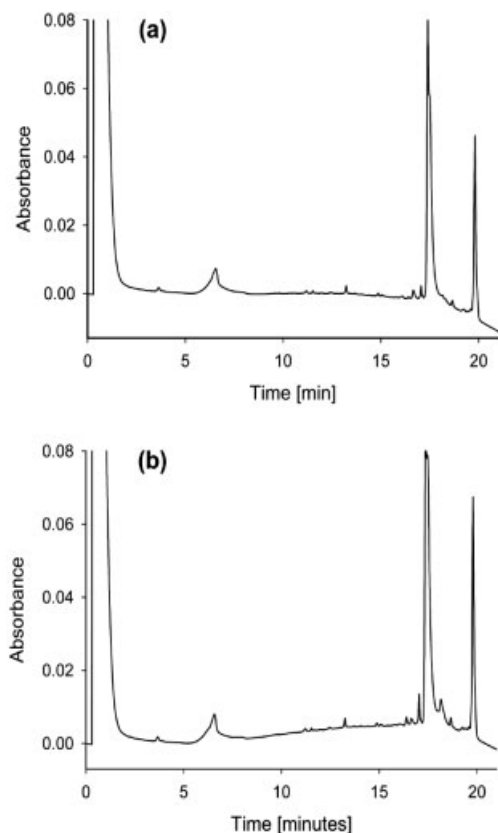
The chromatofocusing column was equilibrated with start buffer for 210 min, and the pH of the effluent was monitored in a flow-cell with a dead volume of 150 μL. Column equilibration was considered complete when the effluent had a stable pH of 8.5 ± 0.1. After sample application, the baseline was again allowed to stabilize for 20 min before elution with a pH gradient was ensued. Fractions were collected every 0.3 pH unit using a model SC 100 fraction collector. Figure 1a shows the elution profile of human serum proteins from a new chromatofocusing column. Elution with a pH gradient was followed by washing with 1 M NaCl for 40 min. Figure 1b shows essentially the same experiment, except that the 1 M NaCl wash solution also contained 0.2% OG. The figure shows that a large amount of UV-absorbing material was eluted from the column in the presence of detergent. This material could not be recovered by isopropanol or high-salt washing steps without detergent. The peak which was eluted with 1 M NaCl in the presence of OG was analyzed by sodium dodecylsulfate gel electrophoresis (Fig. 1c). The gel shows that the fraction contains mainly high-molecular weight proteins, with a major band probably representing albumin.

Using the wash procedure described above, there was no detectable degeneration of the column over 25 runs with a range of protein loading from 0.9 to 5 mg. The reproducibility of the runs was illustrated by separating fractions from



**Figure 1.** Elution profiles of the chromatofocusing column (first dimension), loaded with 1.5 mg of serum protein. (a) After loading, the column was eluted as described in the text, followed by washing with 1 M NaCl, which was started at 125 min. (b) Same experiment, but including 0.2% OG in the 1 M NaCl wash solution. Absorbance was measured at 280 nm. (c) Separation of the wash-peak (130–170 min) on a 12% sodium dodecylsulfate gel, stained with silver. The molecular weights are indicated in kDa.

the chromatofocusing column also in the second dimension on an RP column (33 mm × 4.6 mm, 1.5 μm nonporous ODS-IIIE C18 silica beads, Eprogen). A fraction (200 μL) in the pH range 5.71–6.05, from the chromatofocusing column was injected and eluted at a flow rate of 0.75 mL/min with a 0–100% linear gradient of solvent A (0.1% v/v TFA) and solvent B (0.08% v/v TFA in ACN) for 35 min. The column temperature was maintained at 50°C and the absorption of the effluent was monitored at 214 nm. Figure 2 shows a comparison of two such second-dimension separations of a chromatofocusing fraction obtained in run 1 with the corresponding fraction obtained in run 18 on the same chromatofocusing column. Clearly, the compositions of these two different fractions were not significantly different. This high level of reproducibility was not limited to a few fractions but was observed throughout the chromatofocusing profiles, showing that the chromatofocusing column had not suffered any detectable deterioration over the 18 runs. Additional second-dimension chromatograms of flow-throughs, pH fractions 5.56–5.26, and column salt washes can be viewed as Supplementary Material. Reproducibility of the runs was



**Figure 2.** RP separation of pH 5.71–6.05 fractions from chromatofocusing runs 1 (a) and run 18 (b) of human serum samples, corresponding to the region from 90 to 91.5 min in Fig. 1. The elution profiles were monitored at 214 nm. See text for other details.

independent of column loading in the range of 0.9–5 mg. Depending on the amount of protein loaded on the chromatofocusing column, the pressure during the run ranged from

1500 to 2500 psi, but even after 25 runs it returned to 500 psi following regeneration.

Sheng *et al.* [8] reported that the inclusion of 20% isopropanol in the IEF buffer increased the number of proteins they could identify in the serum. They also demonstrated improved recovery of protein from the column, but using purified BSA rather than complete serum. So, it remains unclear if the recovery of *all* proteins is improved and whether column life is increased by this procedure. Taken together, our results show that the use of OG throughout all separation and washing steps and the avoidance of isopropanol for regeneration greatly increases the life of the chromatofocusing column.

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