

Accelerated Articles

Multiplexed Proteomic Reactor for the Processing of Proteomic Samples

Weimin Hou, Martin Ethier, Jeffrey C. Smith, Yinglun Sheng, and Daniel Figeys*

The Ottawa Institute of Systems Biology, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, K1H 8M5 Canada

We report the development of a 96-well plate proteomic reactor for gel-free processing of minute amounts of complex proteomic samples. The device performs multiplexed trapping, enrichment, and biochemical processing of proteins, resulting in concentrated peptide solutions ready for mass spectrometric analysis. Individual wells on the reactor can process up to 2 μg of protein. We also report the coupling of the plate proteomic reactor with protein fractionation using size-exclusion chromatography for large-scale identification of proteins. To illustrate the potential of this approach, we separated 400 μg of MCF7 cell lysate using size-exclusion chromatography and processed 35 protein fractions on the reactor plate. Using stringent criteria when searching the data, a total of 875 unique proteins were identified. More relaxed searching conditions associated with a 1% false positive rate led to the identification of 2683 unique proteins, meaning that one protein was identified per 3–10 ng of total protein lysate loaded on the reactor plate.

The characterization of proteins by mass spectrometry has become the method of choice for many researchers in proteomics.^{1–4} Unfortunately, limitations such as the dynamic ranges of mass spectrometers and separation techniques, as well as the sensitivity of mass spectrometers, prevent the proteome from being exhaustively characterized. Therefore, the systematic identification and quantification of lower-abundance proteins, often with more biological significance, still remains elusive. Fortunately, we have clear evidence that lower-abundance proteins can be observed by mass spectrometry when sufficient starting material

is available and sample complexity is reduced. For example, we routinely observe lower-abundance proteins when doing immunopurification of protein complexes coupled to mass spectrometry.⁵ This indicates that with appropriate fractionation, based on either affinity purification or chromatographic means, lower-abundance proteins can be detected.^{6–8}

Current MS-based proteomic approaches are primarily integrations of peptide or protein separations and mass spectrometry technologies. For the past 30 years, gel electrophoresis has been used for protein prefractionation (1D and 2D),^{9,10} yet there are many intrinsic limitations associated with gel sample processing techniques, notably a limited dynamic range.⁹ Recently, gel-free alternatives for the fractionation of proteins and peptides have been proposed. Multidimensional fractionation of peptides (MudPIT) can identify thousands of proteins by separating a mixture of peptides through two successive and orthogonal liquid chromatographies (SCX and RP-C18);^{11–13} however, this approach also increases the complexity of the sample as each protein is enzymatically cleaved into multiple peptides that are then fractionated. Other approaches have focused on gel-free prefractionation of intact proteins using C18, which effectively increases the total number of proteins identified. This increase is due to the reduction of the complexity of the mixture content in each individual fraction.^{14,15}

* To whom correspondence should be addressed. Phone: 613-562-5800, ext 8674. Fax: 613-562-5655. E-mail: dfigeys@uottawa.ca.

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We have described the proteomic reactor technique, which processes minute amounts of protein samples on SCX resins.¹⁶ Although the reactor can process minute levels of protein, its format of a serial nature limits the number of samples that can be processed. Here, we report the development of a 96-well plate version of the proteomic reactor that allows multiplexed processing of minute amounts of protein sample. We have demonstrated the performance of this 96-well plate reactor using protein standards. Furthermore, the 96-well plate reactor was coupled with size-exclusion chromatography fractionation to obtain a broader coverage of the proteome. The performance of the plate reactor with SEC fractionation is demonstrated using MCF7 whole-cell lysate.

EXPERIMENTAL SECTION

Materials. Prototypical SCX Plates (reactor plate) were graciously provided by Millipore (Billerica, MA). The MultiScreen HTS Vacuum Manifold was obtained from Millipore (Billerica, MA), and 5 μm YMC ODS-A C18 beads came from Waters (Milford, MA). Sequencing grade trypsin was from Promega (Madison, WI). HPLC grade water with 0.1% (v/v) formic acid and acetonitrile with 0.1% (v/v) formic acid were from J. T. Baker (Phillipsburg, NJ).

Cell Culture and Harvest. P19 cells were cultured at 37 °C in 5% (v/v) CO₂ in a MEM medium supplemented with 7.5% (v/v) newborn calf serum and 2.5% (v/v) fetal bovine serum. Cells were harvested, washed twice with phosphate-buffered saline (PBS), and resuspended in modified RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, 0.25% (w/w) sodium deoxycholate, 1 mM EDTA) containing a cocktail of protease inhibitors (Roche Diagnostics, Laval, QC). Cell lysates were centrifuged for 10 min at 14 000 rpm to pellet cell debris. Supernatants were collected, and the protein concentration was assessed using a Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard (Sigma, St. Louis, MO). Human MCF-7 breast epithelial cancer cells were cultured at 37 °C in 5% CO₂ in a DMEM medium (GIBCOBRL, Burlington, ON) supplemented with 10% (v/v) fetal bovine serum. The cells were harvested following treatment with trypsin, washed with phosphate-buffered saline (PBS), and resuspended in a 2 mL of hypotonic buffer (50 mM Tris pH 7.5, 1 mM DTT, 1 mM EDTA, 5 mM CaCl₂, 5 mM MgCl₂, protease inhibitor cocktail) on ice for 10 min. Crude cell homogenate was then centrifuged at 3000g for 15 min. Supernatants were collected, and aliquots were stored at -80 °C. The protein concentration was assessed using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard (Sigma, St. Louis, MO).

Protein Prefractionation. The separation of 400 μg of MCF7 whole-cell lysate was carried out using a 300 \times 7.80 mm (5 μm) BioSep-SEC-S 3000 analytical column (Phenomenex, Torrance, CA). Samples were directly injected into the analytical column using a manual injection valve (Valco, Houston, TX) equipped with a 100- μL sample loop. The isocratic mobile phase (50 mM NaH₂-

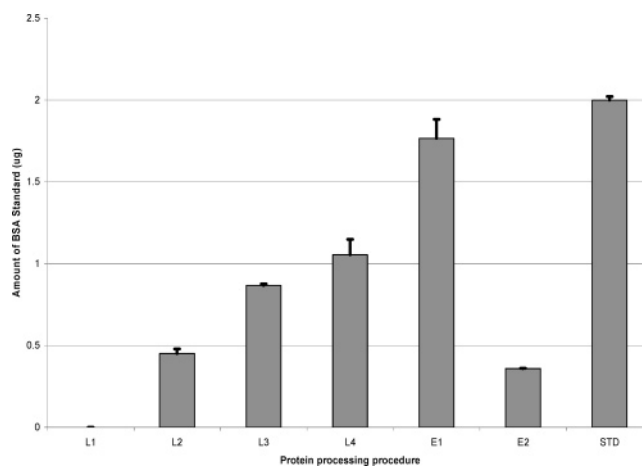


Figure 1. Characterization of the 96-well plate reactor. Lanes L1 to L4 represent experiments in which 1 μg of acidified BSA standard was introduced into the individual well sequentially for up to 4 μg of acidified BSA per well. The flow-through during every load (L1–L4) was collected. Two subsequent elutions were performed per well (E1 and E2) using aliquots of 25 μL elution buffer (200 mM ammonium bicarbonate). The flow-through was collected for each elution (E1 and E2). All the samples (L1–L4 and E1–E2), as well as 2 μg of BSA standard, were separated by SDS-PAGE, visualized by silver staining, and quantified using the ImageJ software. The increase in signal from L1 to L4 indicates that the binding on the reactor well was saturated and proteins flowed through the column. The decrease in signal from E1 to E2 indicates that the elution was efficiently performed.

PO₄) was delivered at 1 mL/min using an Agilent 1100 series microbore HPLC system (Agilent Technologies, Palo Alto, CA). The protein elution chromatography was monitored at 220 nm using a variable wavelength UV detector, and 40 fractions were collected on the basis of the elution time. About 200 μL of eluent was collected for each fraction, and 100 μL of eluent from each of the 35 selected fractions was introduced into a well on the plate reactor and processed as described below.

Sample Processing on the Plate Reactor. The wells on the 96-well plate were first conditioned by flowing 200 μL of wash buffer through them (8 mM potassium phosphate buffer, 20% (v/v) acetonitrile). Once the wells were conditioned, 10–200 μL of the protein samples at pH \sim 2.0 (acidified by 5% (v/v) of 1 M H₃PO₄) was mixed with \sim 0.5 μg of trypsin (inactive at pH 2.0). The samples were flown through the wells and were retained by the SCX resin at the bottom of each well. The retained protein samples were then washed with 30 μL of 10 mM potassium phosphate buffer (pH 3.0) and 30 μL of deionized water successively. The wells were then dried. Cysteine reduction occurred in the wells by adding 5 μL of 100 mM dithiothreitol and 10 mM ammonium bicarbonate (reducing buffer) for 30 min, after which the wells were washed with 5 μL of 10 mM potassium phosphate buffer (pH 3.0) and dried. The digestion of the protein was achieved by introducing 5 μL of digestion/alkylation solution (10 mM iodoacetamide, 100 mM Tris-HCl, pH 8) in each well. The digestion was allowed to proceed for 2 h, after which the resulting peptides were eluted into a collection plate using a volume of \sim 25 to 30 μL of 200 mM ammonium bicarbonate (elution buffer). All the above processes were carried out using a MultiScreen HTS vacuum manifold.

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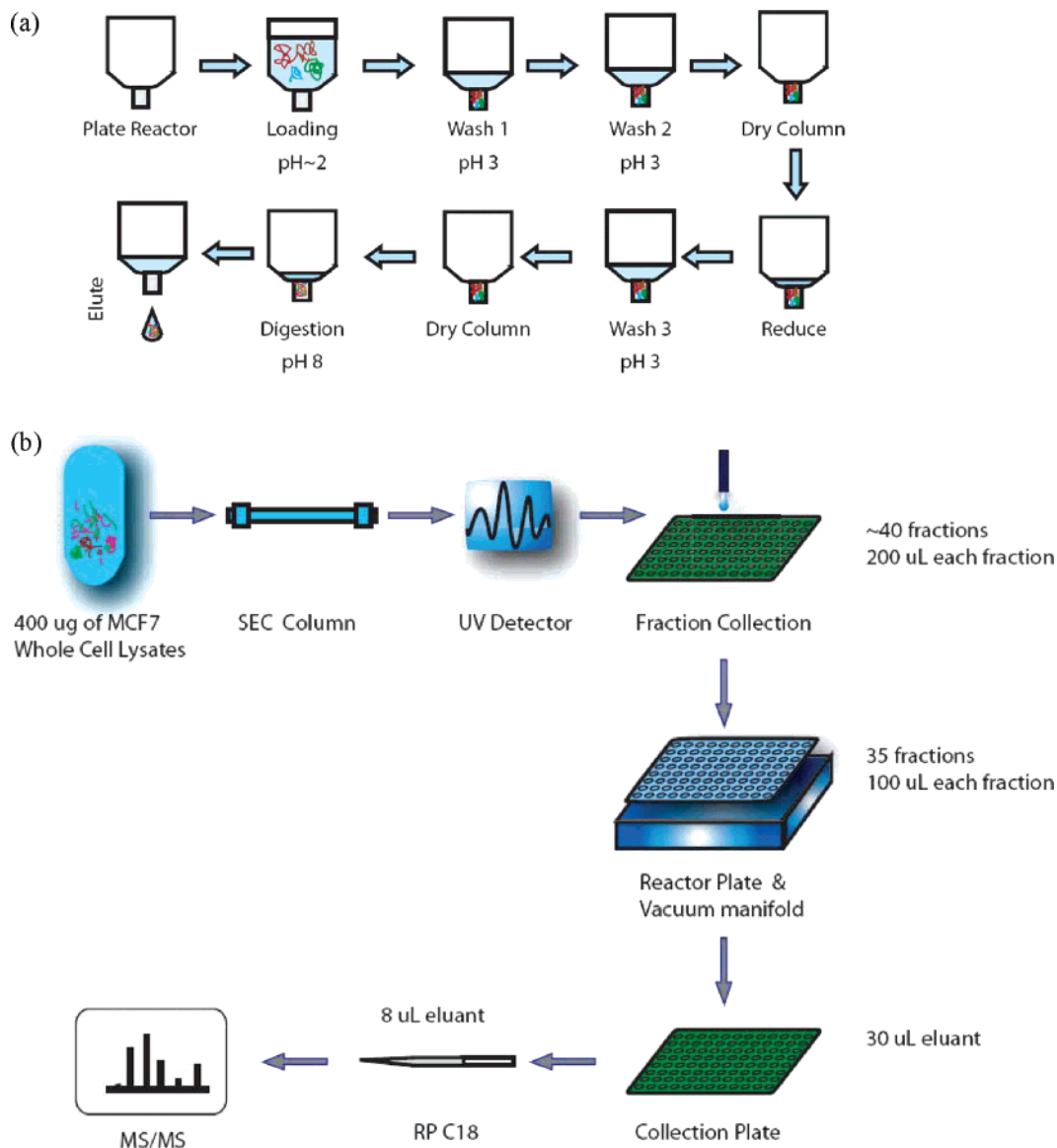


Figure 2. (a) The work flow of sample processing through the plate reactor. Details are provided in the Experimental Section. (b) The coupling of the reactor plate with size-exclusion chromatographic fractionation of proteins. Four hundred micrograms of MCF7 whole cell lysate was separated through size-exclusion chromatography. Thirty-five fractions were collected, and one-half of each fraction was introduced into a well on the reactor plate and processed as described in the Experimental Section. The resulting peptides were eluted in a volume of 30 μL , from which 8 μL was introduced into a LC/ESI-MS/MS system.

LC/ESI-MS/MS Analysis. The peptides obtained from P19 cell lysate derived from the reactor plate were analyzed using a QSTAR Pulsar quadrupole-TOF mass spectrometer (ABI/MDS Sciex, Concord, ON). Eight microliters out of 25 μL was loaded onto a 75 $\mu\text{m} \times 50 \text{ mm}$ precolumn (5 μm YMC ODS-A C18 beads, Waters, Milford, MA) at 1.5–2 $\mu\text{L}/\text{min}$ using an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA). Following a 12 min desalting step, the flow was split, and peptides were eluted through a second 75 $\mu\text{m} \times 50 \text{ mm}$ column (5 μm YMC ODS-A C18 beads) at $\sim 200 \text{ nL}/\text{min}$ using a 5–80% (v/v) gradient of acetonitrile with 0.1% (v/v) formic acid over 1.5 h. The LC effluent was interfaced with the QSTAR Pulsar quadrupole-TOF mass spectrometer via a 10- μm picotip emitter (New Objective, Woburn, MA) by electrospray ionization. Data-dependent MS scans were performed; the four most intense ions in each MS spectrum were subjected to MS/MS analysis using collision-

induced dissociation. The peptides from the MCF7 fractions were analyzed using a LCQ Deca XP mass spectrometer (ThermoFinnigan, San Jose, CA). Eight microliters of the total 30 μL was loaded onto a LC setup as described.¹⁶ Again, data-dependent MS scans were performed, and the three most intense ions in each MS spectrum were subjected to MS/MS analysis using collision-induced dissociation. The experiments were performed in dynamic exclusion mode. A peak could be sequenced a maximum of three times before being excluded for 3 min.

Protein Identification. The recorded MS/MS spectra were processed by the Mascot Distiller. For the P19 cell analysis, the resulting data files were searched against the mouse taxonomy in the NCBI nr database using Mascot; data files from all 35 MCF7 fractions were searched against the human taxonomy in the NCBI nr database using Mascot. The Mascot peptide and MS/MS tolerances for the experiment performed on a QSTAR mass

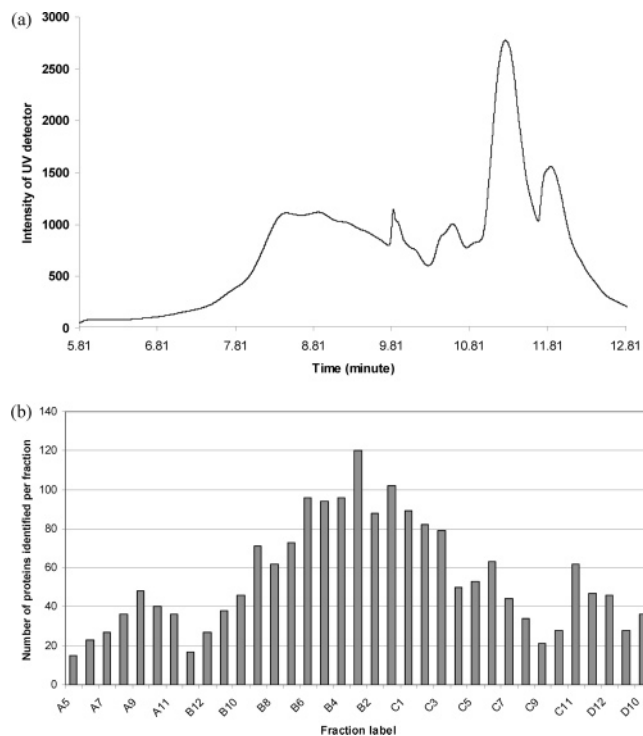


Figure 3. (a) The size-exclusion chromatographic separation of MCF7 whole cell lysate recorded at 220 nm using a variable wavelength detector. For each fraction, $\sim 200 \mu\text{L}$ was collected for a time interval of 12 s (0.20 min). (b) The corresponding total number of proteins identified in each of these 35 fractions. From fractions B6 to C2, an average of 94 proteins per fraction were identified.

spectrometer were set to ± 100 ppm and 0.2 Da, respectively. Similarly, the Mascot peptide and MS/MS tolerances were set for the LCQ Deca XP experiments at 2 and 0.8 Da, respectively. For all experiments, one missed cleavage was allowed, and the ion score threshold was set at 20. In addition, the protein cutoff score was set at 39 unless specified otherwise.

RESULTS AND DISCUSSION

We have demonstrated a very efficient device, termed the proteomic reactor, for processing minute amounts of proteins absorbed on SCX material.¹⁶ Briefly, the reactor extracts proteins from biological samples, exchanges their buffer, and allows chemical and enzymatic treatment of proteins in a volume of ~ 50 nL. The concentration effects achieved through this device have allowed us to identify proteins from several nanograms of total lysate, which has not been feasible in solution previously.¹⁶ Hence, we adapted the proteomic reactor protocol to a 96-well plate format. All of the protein processing abilities of the proteomic reactor may also be carried out in the 96-well reactor plate. Liquid flow is controlled by a MultiScreen HTS vacuum manifold; 10–200 μL of protein solution can be readily processed in each well. The protein mixtures retained in each well can then be desalted, washed, and digested; digestion is complete in ~ 2 h. Afterward, the resulting peptides are eluted from the reactor plate, collected, and analyzed by HPLC/ESI-MS/MS. Full processing of the proteins is routinely performed on the reactor plate in < 3 h, including 2 h for the enzymatic digestion.

Characterization of 96-Well Plate Reactor. The 96-well plate reactor contains ~ 300 nL of immobilized SCX media bound within

each well, as well as an $\sim 1 \text{ mm} \times 1 \text{ mm}$ (o.d.) exit capillary at the bottom of each well. The protein binding capacity and reproducibility of each well were measured using wells located at different positions across a 96-well reactor plate. The binding capacity of each well was measured to be 2 μg by sequentially introducing a known amount of protein standard (BSA) and monitoring the eluent by SDS-PAGE. Figure 1 demonstrates that the protein binding capacity was consistent from well to well and that 85% of protein samples can be recovered using 25 μL of elution buffer. While we processed the 35 fractions of MCF7 lysate, we used 30 μL of elution buffer to further improve the recovery rate of protein samples.

Processing of Proteins. The work flow of sample processing through the plate reactor is shown in Figure 2a; the details of the protocol are provided in the Experimental Section. We used P19 cell lysate to test the analytical performance of the 96-well reactor for identifying proteins. Different amounts of protein extracts from P19 whole cell lysate (0.2, 0.5, 1.0, 1.5, and 2.0 μg) were processed on the reactor plate as described above, and the resulting peptides were eluted in 25 μL of elution buffer. Eight microliters was analyzed by HPLC/ESI-MS/MS, and the resulting MS/MS were searched against the mouse taxonomy in the NCBI nr database using Mascot. As a result, 7, 11, 19, 35, and 68 proteins, respectively, were successfully identified from the different levels of the P19 cell lysate. It is worth noting that more than 60 unique proteins were identified with high confidence from $< 2 \mu\text{g}$ of untreated P19 whole cell lysate. In other words, by using the reactor plate, one protein was identified per 10 ng of total protein lysate introduced on the reactor well. This clearly demonstrates that the reactor plate efficiently processes complex protein mixtures from as little as 200 ng of total protein lysate.

Coupling of the Reactor Plate with SEC. We then coupled the reactor plate with size-exclusion chromatography fractionation of protein mixtures to create a completely gel-free technique for sample processing based on protein fractionation and protein processing (Figure 2b). To illustrate the potential of this approach, we fractionated MCF7 lysate and then used the reactor plate to process the fractions simultaneously. Briefly, 400 μg of MCF7 whole cell lysate was separated using size-exclusion chromatography with UV detection, and 40 fractions were collected using an Agilent 1100 fraction collector. The chromatographic traces and the regions in which fractions were collected are illustrated in Figure 3a. Thirty-five of the collected fractions were processed using the reactor plate as described above, resulting in 30 μL of peptide solution per reactor well. Eight microliters of peptide solution (27% of the sample) was analyzed by HPLC/ESI-MS/MS using an LCQ Deca XP mass spectrometer. During the course of 35 LC/MS runs, multiple columns and precolumns were used to avoid issues associated with column aging.

The distribution of the number of proteins identified per fraction is illustrated in Figure 3b. One thousand nine hundred and seventeen proteins were identified across 35 fractions, with an average of 55 proteins per fraction. From fractions B6 to C3, an average of 94 proteins per fraction were identified. Once redundancy was removed, we obtained 875 unique proteins based on a Mascot peptide threshold score of 20 and a protein score of 39. These results clearly demonstrate that protein fractionation coupled to the reactor plate greatly enhances the total number of

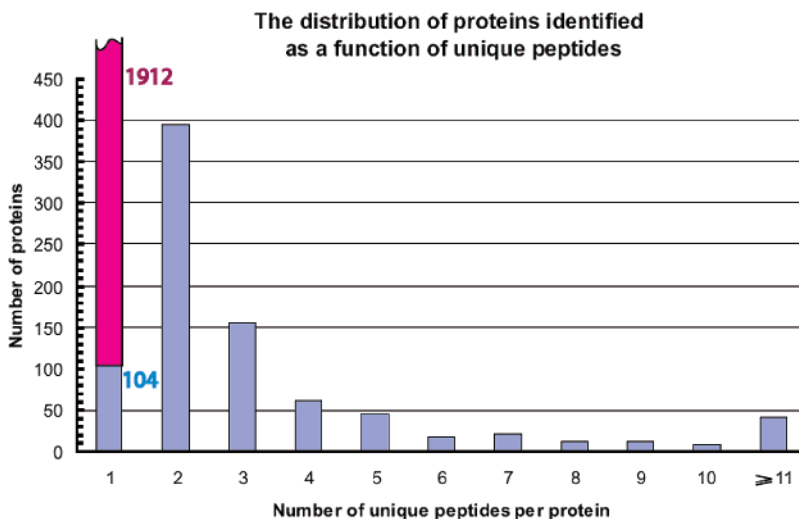


Figure 4. The distribution of proteins identified as a function of unique peptides. Eight hundred and seventy-five unique proteins were identified; 104 proteins were identified with a single peptide, and 771 proteins were identified with at least two unique peptides when the Mascot protein and peptide score thresholds were 39 and 20, respectively. When the protein score threshold was removed, 2683 unique proteins were identified; 1912 of these were identified with a single peptide.

proteins identified. Furthermore, as little as 10 ng of total protein lysate processed in the reactor was needed for each protein identification, which represents an exquisite level of sensitivity.

Higher-abundance proteins (i.e., proteins that have a higher Mascot score) tended to spread over multiple fractions, whereas, as expected, proteins with a lesser number of hits were found in a narrower range of fractions. Interestingly, of the 875 unique proteins identified from the combined data file, nearly 300 proteins were identified only through the combination of peptides identified in multiple fractions. This is probably related to the limited duty cycle of the HPLC/ESI-MS/MS over a 2-h total experiment, preventing a significant number of peptides from being fragmented in each run.

High Confidence in Protein Identification. The thresholds that were used for protein identification were stringent (an ion score cutoff of 20 for peptides and a protein score cutoff of 39 for proteins), which led to the identification of 875 unique proteins. As a result of the stringent searching criteria, 771 proteins were identified by at least two distinct peptides (with peptide ion scores ≥ 20), as shown in Figure 4. For those 104 proteins identified by a single peptide, the lowest peptide score was 39. This stringent filtering is likely leading to a high false negative level. A recent bioinformatic study of search engine ion score cutoffs reported that a Mascot peptide score threshold of 20 provides a false positive rate below 1%.¹⁷ Under these criteria (i.e., no protein score cutoff), we identified a total of 2683 nonredundant proteins, the majority of which were single peptide identifications (1912) (Figure 4).

Factors Limiting the Performance of the Plate Reactor. The overall performance of the plate reactor can be further improved. First, the binding capacity of each well on the plate is 2 μg or less of protein. Even though 400 μg of MCF7 protein lysate was separated by size-exclusion chromatography, no more than 70 μg of the protein samples was loaded on the reactor plate for

the 35 fractions. Hence, increasing the well capacity or the number of fractions would increase the total sample load. Second, to avoid saturating the precolumn, only 8 μL out of the 30 μL resulting peptides from each reactor plate well was injected onto the HPLC/ESI-MS/MS for protein identification. Increasing the HPLC capacity or performing multiple runs from the same sample would further enhance the performance.^{18,19} Third, rapid 2-h gradients were performed on the HPLC; increasing the duration of the gradient will increase the number of MS/MS scans generated. Finally, more sensitive mass spectrometers with faster duty cycles should also improve the performance of this approach.²⁰ We recently demonstrated that changing the mass spectrometer from an LCQ to an LTQ ion trap mass spectrometer can double the number of proteins identified from one fraction, all other processing methods being the same (data not shown).

CONCLUSIONS

In summary, we established a completely gel-free approach for large-scale protein identification using a 96-well plate reactor to process 35 fractions of a size-exclusion chromatographic separation of MCF7 lysate. Under stringent criteria, a total of 875 unique proteins were identified. A more relaxed ion score cutoff level associated with a 1% false positive rate resulted in the identification of 2683 unique proteins. This novel approach proves to be very efficient and sensitive in processing minute amounts of protein samples, demonstrated by one protein being identified per 3–10 ng of total protein lysate loaded on the reactor plate.

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SUPPORTING INFORMATION AVAILABLE

Spreadsheets for proteins, including gi, MW, Mascot score, no of peptides, and protein name. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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