Using the Empore™ C18 StageTips for High pH Peptide Fractionation in Global Proteomics

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Application Note
Life Science

Abstract
This application note demonstrates how the Empore™ C18 StageTips could be applied to high pH peptide fractionation in global proteomics to significantly increased peptide and protein identification.

Introduction
Extensive off-line peptide fractionation is used to reduce the complexity of the proteome and is critical to in-depth global proteome profiling. However, the fractionation of protein samples in the sub-microgram range can be limited due to sample loss during the fractionation process. Thus, it is essential to develop efficient microscale fractionation strategies. High-pH peptide fractionation is one of the methods among these efforts. Previous methods have either used a pump-based approach (e.g., using off-line HPLC), which is sophisticated and expensive, or use beads-based approaches, which are unreliable (e.g., need to weigh certain amount of bead material and do packing every time). Here, a novel high-pH peptide fractionation method using Empore C18 StageTips is demonstrated. The results show the method is simple, reliable, and robust.[1-3]

Experiment Setup
HeLa Whole Cell Lysate Preparation and Suspension Trapping Digestion

HeLa cells were maintained according to the ATCC protocol. Harvested cells were counted and briefly washed with PBS, and cell pellets were stored in -80 °C prior to use. Proteins from 1 × 105 cells were used for each digestion, and each experiment was performed in four replicates. Cells were resuspended in SDS lysis buffer (1% SDS, 25 mM DTT, 10 mM EDTA, 0.05% Tween-20, 100 mM Tris-HCl, pH 8.0) and sonicated in Misonix Sonicator 3000 Ultrasonic Cell Disruptor for 3 min (pulse: 20 s on, 20 s off). After sonication, cells were incubated at 95 °C for 10 min. Clear lysate was obtained after centrifugation at 16,000 g for 10 min. The iodoacetamide (25 mM) was added to the lysate, and proteins were alkylated in the dark for 20 min.
Protein digestion was performed using STrap protocol and in-house packed filter device with Whatman GF/F glass fiber membrane. Briefly, the alkylated proteins were first acidified by adding phosphoric acid to a final concentration of 1.2% and then mixed with 6 volumes of binding buffer (90% methanol, 100 mM triethylammonium bicarbonate, TEAB, pH 7.1). The sample was vortexed gently to ensure thorough mixing and then loaded onto the filter. Following a quick centrifugation at 400g for 1–2 min, the filter was washed 2–3 times with 200 µL binding buffer, and proteins were subjected to overnight digestion with 1 µg of MS-grade trypsin (in 150 µL of 50 mM TEAB) at 37 °C. The digested peptides were recovered by stepwise elution with 200 µL of 50 mM TEAB, 200 µL of 0.2% formic acid/water, and 200 µL of 0.2% formic acid/50% acetonitrile/50% water. Peptide elutions were pooled and dried in SpeedVac, followed by C18-based StageTips desalting. The peptides were subjected to direct LC-MS/MS analysis or further fractionation.

C18 StageTips Desalting Sample Preparation

Around 1 µg peptides were used for each experiment. For Empore C18 StageTips pre-treatment, the C18 tip and adapter were placed in a 2-mL microtube. In a first activation step 200 µL methanol was loaded on the C18 tip and spun at 4000 rpm for 1~2 min. In a second activation step, 200 µL of 80% ACN/0.5% HAc and spun at 4000 rpm for 1~2 min. All the liquid was discarded from the collection tube, and the C18 tip was equilibrated by loading 200 µL of H_2O/0.5% HAc onto the tip and spinning at 4000 rpm for 1~2 min. Again, all the liquid was discarded from the collection tube. 10 µL of sample (about 1 µg of peptides) was loaded onto the C18 tip and spun at 4000 rpm for 30 s. The flow through was collected and reloaded onto the tip and spun again. This step was repeated a total of three times. The C18 tip was then washed by loading 200 µL of H_2O/0.5% HAc onto the tip and spinning at 4000 rpm for 2~3min. This step may be repeated up to 2~3 times. Also, depending on the salt amount, the spin time may vary (2~4 min). The tips were then transferred to new collection tubes. The first elution step was performed by loading 200 µL of 60% ACN/0.5% HAc/40% H_2O onto the tip and spun at 4000 rpm for ~2 min. In the second elution step, 200 µL of 80% ACN/0.5% HAc/20% H_2O was loaded onto the tip and spun at 4000 rpm for ~2 min. This was repeated one more time and then samples were tried in a SpeedVac.

C18 StageTips Peptide Fractionation

High pH fractionation was performed using Empore C18 membrane tips (CDS Analytical, Oxford, PA). In brief, the peptides were resuspended into 200 µL of 10 mM ammonium formate, pH 10. Ten step-wise elution was carried out using resuspension buffer that contains 5%, 10%, 15%, 20% 25%, 30%, 35%, 40%, 50%, and 80%, respectively. Then, the ten fractions were combined into five fractions in a noncontiguous manner (e.g., f1+f6, f2+f7, etc.). The five fractions were dried in a speed vacuum and stored at -80 °C until LC-MS/MS analysis.

LC-MS/MS Analysis

The LC-MS/MS analysis was performed on an Ultimate 3000 nano-LC and Q-Exactive mass spectrometer system (Thermo Scientific) following a protocol published previously. Briefly, dried peptides were first resuspended into 20 µL of LC buffer A (0.1% formic acid in water) and then loaded onto a trap column (2 cm × 300 µm, PepMap C18, Thermo Scientific), followed by separation over a 150 min gradient (120 min to 35%, 10 min to 80%) in buffer B (0.1% formic acid in acetonitrile) at a flow rate of 200 nL/min in an in-house packed column (75 µm × 19 cm, 3.0 µm ReproSil-Pur C18-AQ media). The MS survey scans were acquired at a resolution of 70,000 over a mass range of

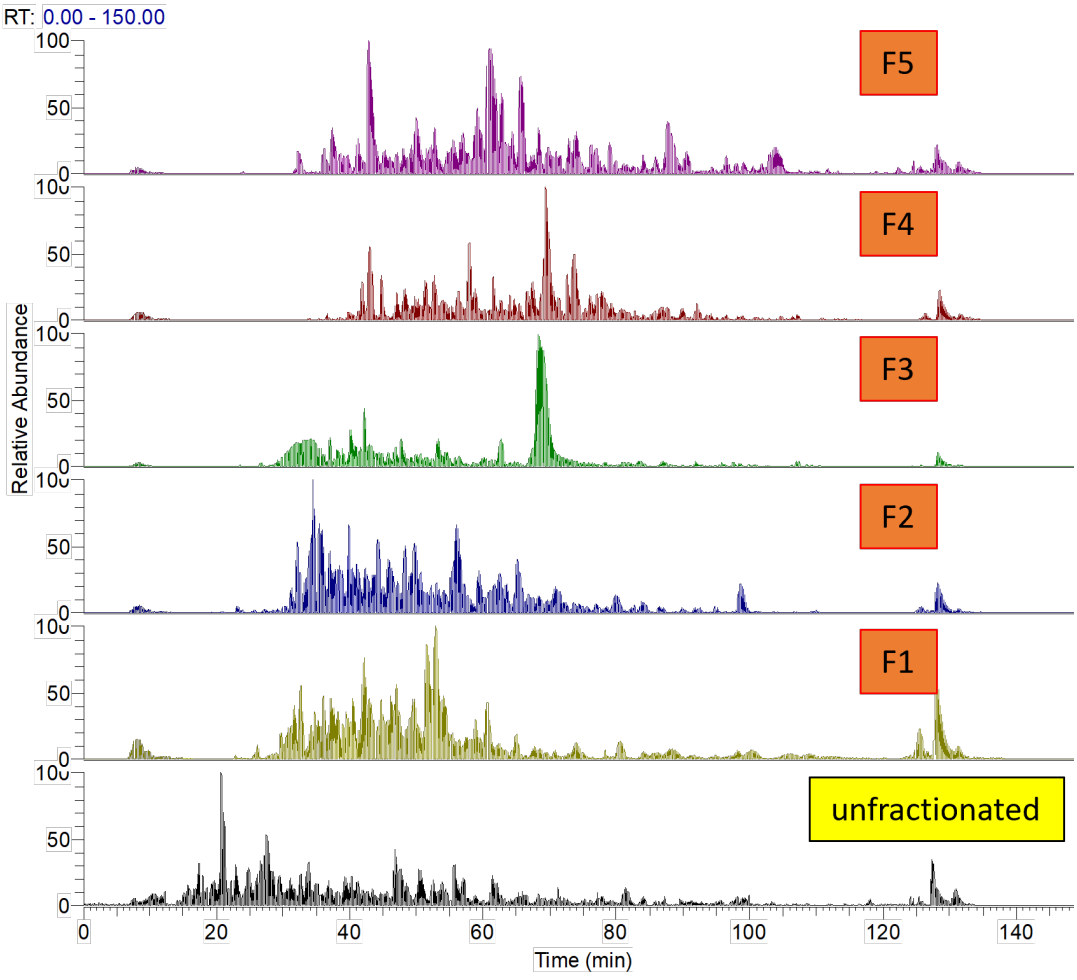

Figure 1. Chromatograms collected for the 5 fractions and for 1 unfractioned sample.

m/z 350–1800. In each cycle, the ten most intense ions were subjected to high-energy collisional dissociation (HCD), applying a normalized collision energy of 27%. The MS/MS scans were performed at a resolution of 17,500. Dynamic exclusion was set to 20 s. Unassigned ions or ions with charges four or more were rejected for HCD fragmentation. The LCMS raw data were processed with the Proteome Discoverer software (version 2.2, Thermo Scientific) using Sequest HT algorithm. The UniProt human database (20,195 sequences, reviewed only; version 2015_06) was used for protein search. Peptide should rank 1 and have a minimum length of seven amino acids. MS and MS/MS ion tolerances were set to 10 ppm and 0.02 Da, respectively. Only the peptide and protein identifications with false discovery rate (FDR) 1% or less were accepted in the final data set. Only one master protein was reported in the case that two or more proteins shared the same set of identified peptides.

Results and Discussion

In total, 10 fractions were collected and recombined in a non-contiguous manner to make 5 fractions. The resulting chromatograms from the 5 fractions are shown in Figure 1 where they are compared to the unfractioned protein in black. Figure 2a shows the number of peptides that were detected when each of the 5 fractions were eluted from the StageTips. The blue bar on the bottom of Figure 2a indicates that number of peptides that were uniquely identified from each fraction while peptides that were identified from two adjacent fractions are shown in the Venn diagram. On average, 57% of the peptides identified were unique to that fraction. The total proteins and peptides identified before and fractionation are summarized in Figure 2b. After fractionation, these numbers represent the sum of all proteins and peptides identified from each fraction, which includes both proteins and peptides that are unique and repeated. As a result of carrying out this high pH fractionation protocol, 50% more proteins and peptides were identified after fractionation. Figure 3 shows the percent of peptides (orange) and protein (blue) in the total sequence that were identified from fraction. In this figure peptides are only counted once they are first detected and any repeats detected in subsequent fractions are not considered.

Figure 4 shows key chemical properties, such as the isoelectric point (pI), molecular weight (MW), and the GRAVY index, were compared before and after fractionation. After fractionation, the mass shifts towards lower masses in Figure 4b indicating the separation of peptides during fractionation. Also, the GRAVY, or grand average hydropathicity, index is shown in Figure 4c. The GRAVY index is a measure of relative peptide hydrophilicity and hydrophobicity. In Figure 4c, the trend is becoming more negative, which means a shift to more hydrophilic peptides. This indicates that the protocol is effectively separating hydrophilic from hydrophobic peptides to help identify more hydrophilic peptides after fractionation.

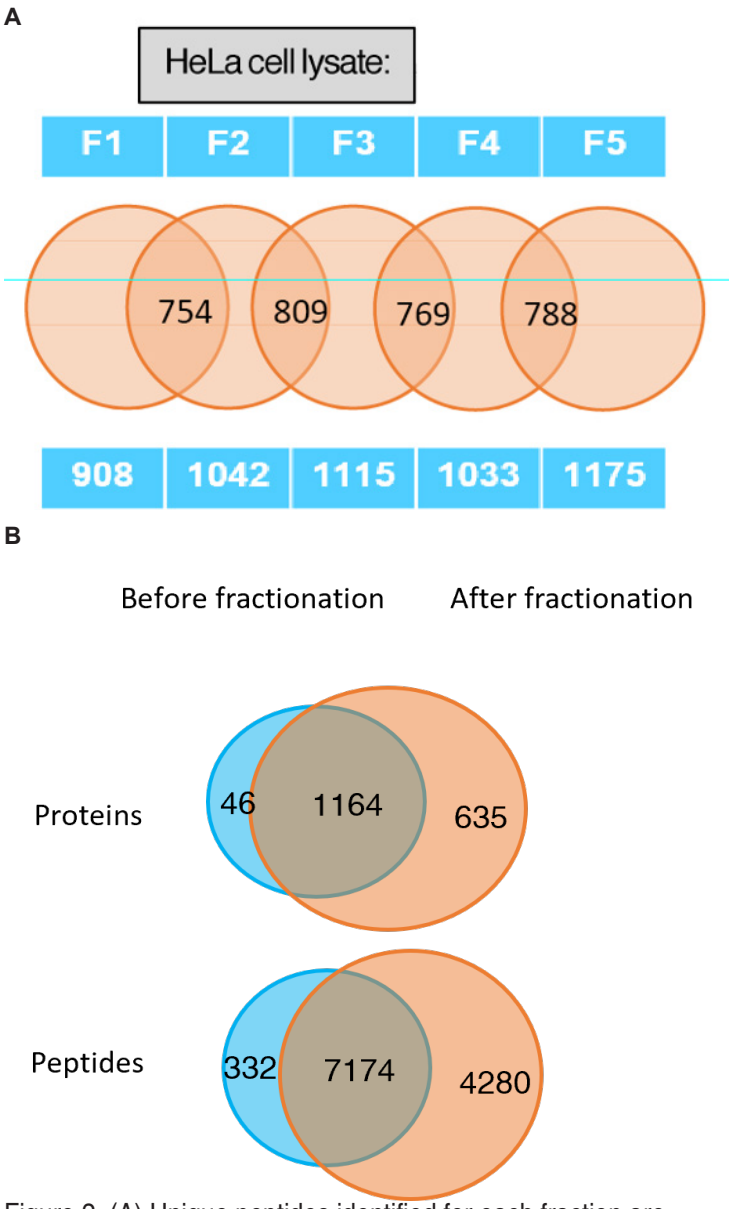

Figure 2. (A) Unique peptides identified for each fraction are in blue. Identified peptides that were repeats between two fractions are shown in the orange Venn diagram. (B) Total proteins and peptides identified before and after fractionation.

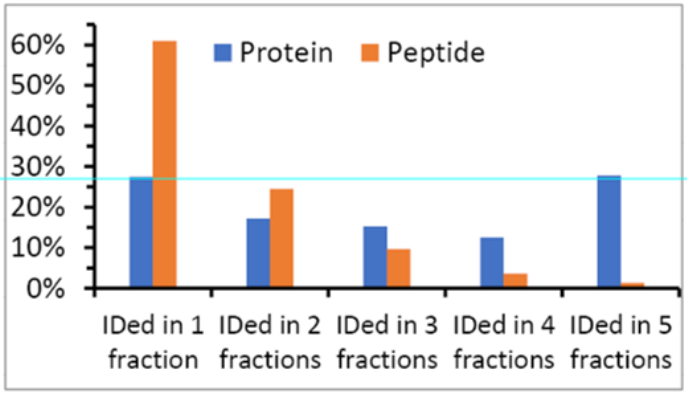

Figure 3. Percent of total proteins (blue) and peptides (orange) that were identified for each fraction.

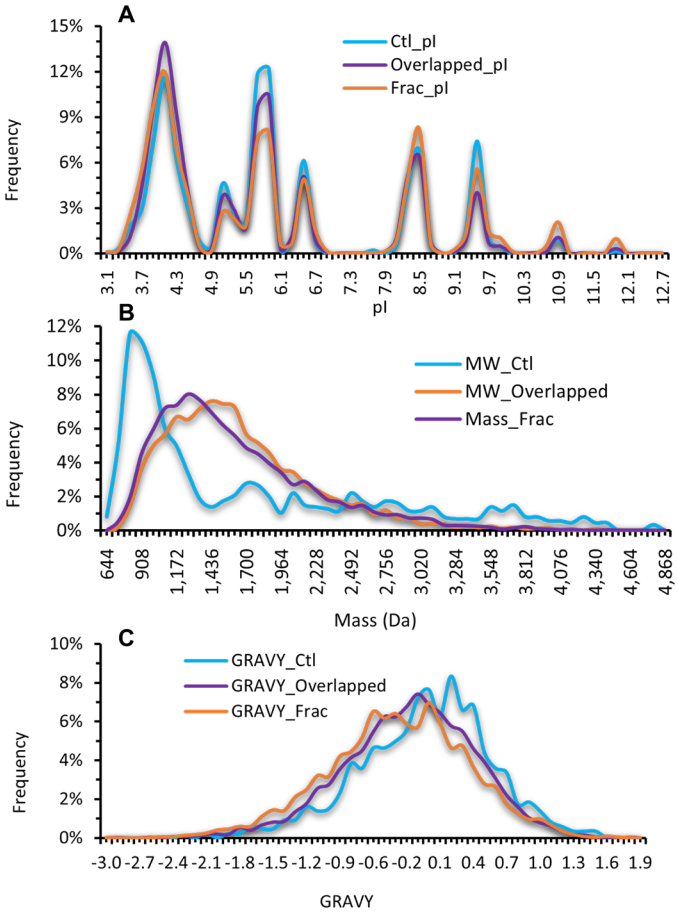

Figure 4. Percent frequency of peptides possessing a given (A) isoelectric point (pI), (B) mass in Da, and (C) GRAVY index value. The control (Ctl) is the unfractioned sample. This is compared the fractioned sample labeled as "Frac". "Overlapped" refers to the combination of both fractioned and unfractioned samples.

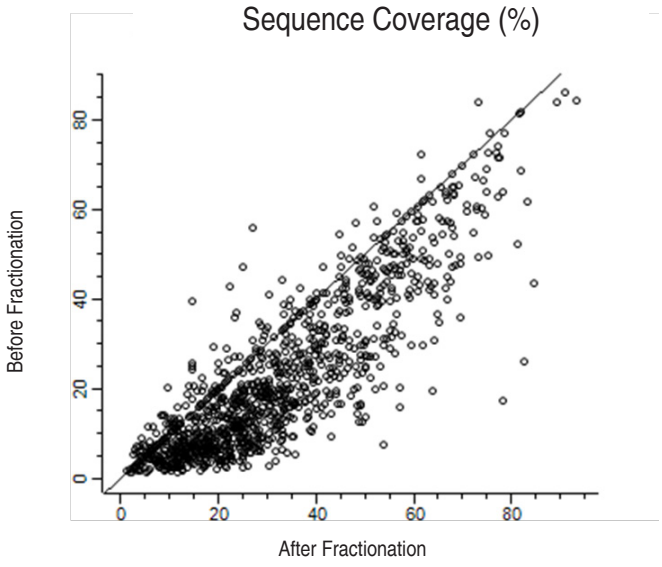

Figure 5. Percent peptide sequence coverage before and after fractionation.

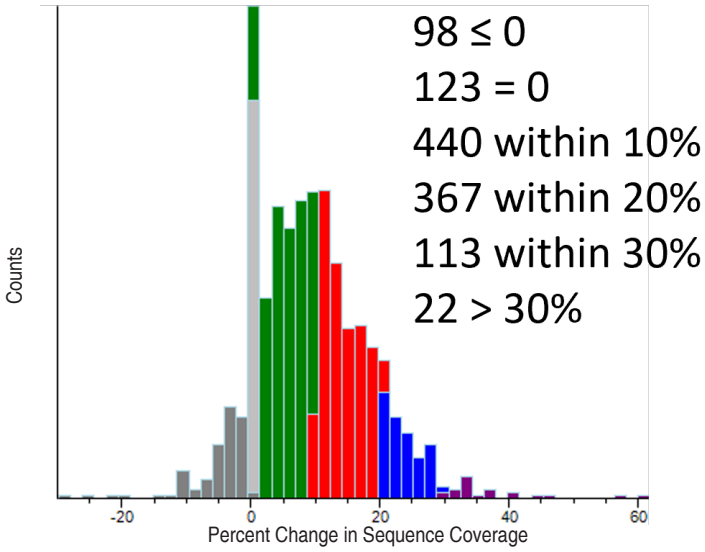

Figure 6. Number of peptides, after fractionation, whose with percent sequence coverage changed by ≤0% (drak gray), = 0% (light gray), within 10% (green), within 20% (red), within 30% (blue), and >30% (purple).

Figure 5 shows the percent of protein sequence coverage before and after fractionation. Sequence coverage is further broken in Figure 6. The numbers in the legend indicate numbers of proteins and their percent increase in sequence coverage following fractionation. For example, 98 proteins had a ≤0% increase in sequence coverage after fractionation. Following fractionation, the average sequence coverage increased by 10%.

Conclusions

This application has demonstrated a high pH fractionation protocol using the Empore C18 StageTips. The total number of proteins and peptides identified after performing the fractionation protocol were compared to an unfractionated sample. By using this fractionation protocol, the number of proteins and peptides that were identified increased by 50%. Additionally, this fractionation procedure greatly increased the total sequence coverage. On average, the sequence coverage increased by 10%. Compared to pump-based and bead-based methods, the method presented here is simple, reliable, and cost-effective.

References

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C18 StageTips Order Information

	Part Number
CDS Analytical	6091; 70-2019-1001-3
Fisher Sci.	13110-055
VWR	76449-262