CDS Empore™

Evaluation of Empore™ C18 Spin Column Ability to Increase Proteome Coverage via High pH Fractionation

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Application Note
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Abstract
Sample preparation is paramount to produce high quality LC-MS proteomic data. Large, bottom-up proteomic coverage from a single sample can be difficult to achieve due to the depth and complexity of a peptide population. Instrument systems for sample fractionation are expensive, require time, and regular maintenance for reliable results. Here we present a quick and affordable option for larger sample frationation, the Empore™ C18 spin column. We showcase improved proteome coverage of *E. coli* peptides sample by preparation via desalting combined with high pH reverse phase fractionation.

Introduction
Solid Phase Extraction (SPE) spin columns are now available prepacked with the same C18 Empore™ membrane as those found in our popular StageTips. For over 20 years the Empore™ membrane has been the SPE of choice for small volume peptide desalting and fractionation prior to LC-MS analysis. The addition of spin column format offers the same performance researchers expect but with a larger capacity, allowing researchers to process larger samples without having to split them between multiple StageTips and risk loss of sample (App Note #278). In this application note we detail a complete protocol from lysis to LC-MS analysis. Using *E. coli* as our sample, we first lysed the cells via SPEED[1] method. For digestion we utilized our new innovative membrane technology for cell and protein retention and on-filter digestion, the Empore™ E3technology™,[2] (App Note #279) spin columns. Finally, desalting and fractionation utilized our Empore™ C18 spin columns in preparation for LC-MS analysis. Furthermore, we share the results we obtained from this protocol showing an increase in proteomic coverage for the fractionated preparation.

Experimental Setup
1. Materials and Equipment

(1) Empore™ Spin Column - C18 (CDS Analytical, #70-2019-2001-0).
(2) Empore™ Spin Column - E3 (CDS Analytical, #70-2019-3101-0).
(3) Microtubes and Pipette tips - Low binding or maximum recovery microcentrifuge tubes (1.5 mL and/or 2.0 mL), Low binding tips (20 µL, 200 µL, and 1 mL).
(4) Sample for analysis - *E. coli* cells or sample of choice.
(5) Benchtop heater/shaker - Multi-Therm by Benchmark Scientific or equivalent.
(6) Benchtop centrifuge - accuSpin by Fisherbrand or equivalent.
(7) Vacuum centrifuge - SpeedVac system by ThermoFisher Scientific, Vacufuge system by Eppendorf, or similar system.
(8) UV-Vis Spectrophotometer - BlueStar A by Lab Tech or similar instrument.
(9) Quartz cuvette - 100 µL cuvette #SLB100 by Cuvet,co or similar.
(10) Trypsin Enzyme - Sequencing grade trypsin V5111 Promega or similar.
(11) Lysis and digestion solutions - Follow SPEED method[1] for lysis followed by modified Empore™ E3 User Guide for digestion
   a. Lysis solution: 100% trifluoracetic acid (TFA)
   b. Neutralization buffer: 1 M Tris buffer pH 12
   c. Precipitation solution: 100% acetonitrile (ACN)

d. Wash solution: 80% ACN in water
e. 10x Reduction/Alkylation solution: 100 mM tris(2-carboxyethyl)phosphine (TCEP), 400 mM 2-chloroacetamide (CAA)
f. Digestion buffer: 50 mM TEAB pH 8.5
g. 0.2% formic acid (FA) in water
h. 50% ACN, 0.1% FA in water

(12) Desalting & fractionation solutions
a. Activation solution I: 100% methanol (MeOH)
b. Activation solution II and elution soluion: 80% ACN, 0.5% acetic acid (HAc), 19.5% water
c. Equilibration and wash solution: 0.5% TFA in water
d. Wash solution II: water
e. Wash solution III: 0.5% acetic acid (HAc) in water
f. Fractionation solutions:

Table 1: Fractionation solutions and combinations for analysis

No.	Percent of ACN	Percent of 0.1% TEA	Fractions Combined
1	2%	98%	1 + 7
2	4%	96%	2 + 8
3	6%	94%	3 + 9
4	8%	92%	4 + 10
5	10%	90%	5 + 11
6	12%	88%	6 + 12
7	14%	86%	1 + 7
8	16%	84%	2 + 8
9	18%	82%	3 + 9
10	20%	80%	4 + 10
11	40%	60%	5 + 11
12	80%	20%	6 + 12

(13) LC-MS/MS System
a. Vanquish Horizon system, Thermo Scientific
b. Acclaim™ PepMap™ 100 C18 RSLC 1 mm ID x 15 cm, catalog # 164711
c. Q Exactive Plus Orbitrap, Thermo Scientific
d. HESI source

2. Procedure

E. coli cells were grown, harvested, and stored until cell lysis via SPEED method[1], followed by E3 on filter digestion using modified protocol from Empore™ E3 User Guide. Digestion procedure is as follows:

1. Lysis - 10 µL of pelleted E. coli pipetted into 10x volume of 100% TFA (100 µL)
   a. Pipetted to mix and break up pellet until solution is clear
   b. Let sit for 10 minutes to lyse cells
2. Neutralization - Add 10x volume of neutralization solution (1 mL) mixing solution to neutralize. Check pH to ensure falls between 8-9.
3. Reduction & alkylation - Add 1/10 volume of 10x reduction/alkylation solution (110 µL) to reach a 1x final concentration.
   a. Mix well
   b. Incubate 95 °C shaking at 1000 rpm for 5 minutes in a heater/shaker
4. Estimate protein concentration by measuring solution turbidity
   a. Transfer solution to cuvette with or without dilution to achieve absorption ≤ 1 AU
   b. Measure absorbance at 360 nm using UV-Vis spectrophotometer
   c. Estimate concentration by converting AU to µg/µL using conversion factor 1 AU = 0.79 µg/µL
   d. Proceed forward with desired amount of protein
5. Add 4x volume of ACN to reach final concentration of 80% ACN to precipitate protein prior to loading on E3 filter. Mix well.
6. Next followed steps from Empore™ E3 User Guide. Pipette 10-100 µg of precipitated protein into E3 spin column and spin through at 3,000 rpm (~900 x g) for 2 minutes or until solution completely passed. Discard flow through.
7. Wash E3 filter with the addition 200 µL of wash solution (80% ACN in water), spinning the solution through at 3,000 rpm for 1-2 minutes. Discard flow through and repeated step two additional times.
8. Skipped the reduction and alkylation step because protein has already been reduced and alkylated. Also skipped post reduction and alkylation wash steps.
9. Place E3 spin column into new low retention or low binding tube.
10. Added 200 µL of 50 mM TEAB buffer to the E3 filter with 1:50-1:100 ratio of trypsin enzyme relative to protein to digest. Place spin column and new tube into a heater/shaker at 37 °C and 200 rpm to digest overnight 16-18 hours.
11. Post digestion spin down digestion solution at 3,000 rpm for 2 minutes.
12. Elute with an additional 200 µL of 0.2% formic acid in water, spinning 3,000 rpm for 2 minutes.
13. Perform final elution with 200 µL of 50% ACN, 0.1% FA in water, spinning 3,000 rpm for 2 minutes.
14. Combined eluents and dry peptides via vacuum centrifugation.

E. coli peptides were next desalted and fractionated via Empore™ C18 spin columns in preparation for LC-MS analysis. Reccomended loading capcity is ≤ 300 µg (See App Note #278). Procedure for desalting in combination with fractionation is as follows:

1. Peptide sample was resuspended in 0.5% TFA solution to a volume of 300 µL at roughly estimated concentration of 1 µg/µL.

a. After addition of solution the tube was shaken at 1,000 rpm in the heater/shaker at ambient temperature for 15 minutes.
b. The sample was then sonicated in water bath for 10 minutes.
c. The sample was shaken again at 1,000 rpm for 15 minutes in the heater/shaker.
d. Lastly sample was spun down at max rpm for 5-10 minutes to pellet any precipitate prior to desalting and fractionation.

2. Empore™ C18 spin column was preconditioned prior to sample loading while the sample was being resuspended. For the desalting and fractionation steps the spin column was centrifuged at 500 x g.
   a. Pipette 400 µL of activation solution I (100% MeOH) and spin through for 30 seconds. Discard flow through.
      Note: Do not spin to dryness. Drying may negatively affect peptides samples and retention.
   b. Pipette 400 µL of activation solution II (80% ACN, 0.5% HAc, 19.5% water). Discard flow through.
      Note: Do not spin to dryness. Drying may negatively affect peptides samples and retention.
   c. Pipette 400 µL of 0.5% TFA in water and spin through for 30 seconds to equilibrate. Discard flow through.
      Note: Do not spin to dryness. Drying may negatively affect peptides samples and retention.
3. Place spin column into new low retention or low binding tube. Transfer peptide sample into Empore™ C18 spin column and spin through via centrifugation for about 4 minutes. Reload the flowthrough into the spin column and spin through an additional time.
4. Transfer Empore™ C18 spin column to a new tube. Desalt the bound peptide washing with 400 µL of wash solution I (0.5% TFA). Spin through the wash solution for 4 minutes, discard flow through and repeat the wash one to two additional times.
5. Pipette 400 µL of wash solution II (water) and spin through one time for 4 minutes discarding the flow through.
6. Transfer Empore™ C18 spin column to new low binding or low retention tube. Pipette 300 µL of the first (no. 1) fractionation solution (2% ACN, 0.1% TEA in water). Spin through completely for 2-4 minutes. Label tube and set aside as fraction 1.
7. Continue through the fractionation process by repeating step six using the next fractionation solutions one at a time until all 12 have passed through the spin column.
8. Combine fractions 1 + 7; 2 + 8; 3 + 9; 4 + 10; 5 + 11; 6 + 12; as shown in table 1.
9. Vacuum dry the now six fractions.
10. Resuspend each of the six fractions in 23 µL of 1% ACN, 0.1% FA in water following the same procedure in step 1 above. The sample is now ready for LC-MS injection and data acquisition.

E. coli sample for an un-fractionated comparison was desalted via Empore™ C18 spin columns in preparation for LC-MS analysis. Reccomended loading capcity is ≤ 300 µg (See App Note #278). Procedure for desalting is as follows:

1. Peptide sample was resuspended in 0.5% TFA solution to a volume of 300 µL at roughly estimated concentration of 1 µg/µL.
   a. After addition of solution the tube was shaken at 1,000 rpm in the heater/shaker at ambient temperature for 15 minutes.
   b. The sample was then sonicated in water bath for 10 minutes.
   c. The sample was shaken again at 1,000 rpm for 15 minutes in the heater/shaker.
   d. Lastly sample was spun down at max rpm for 5-10 minutes to pellet any precipitate prior to desalting and fractionation.
2. Empore™ C18 spin column was preconditioned prior to sample loading while the sample was being resuspended. For the desalting and fractionation steps the spin column was centrifuged at 500 x g.
   a. Pipette 400 µL of activation solution I (100% MeOH) and spin through for 30 seconds. Discard flow through.
      Note: Do not spin to dryness. Drying may negatively affect peptides samples and retention.
   b. Pipette 400 µL of activation solution II (80% ACN, 0.5% HAc, 19.5% water). Discard flow through.
      Note: Do not spin to dryness. Drying may negatively affect peptides samples and retention.
   c. Pipette 400 µL of 0.5% TFA in water and spin through for 30 seconds to equilibrate. Discard flow through.
      Note: Do not spin to dryness. Drying may negatively affect peptides samples and retention.
3. Place spin column into new low retention or low binding tube. Transfer peptide sample into Empore™ C18 spin column and spin through via centrifugation for about 4 minutes. Reload the flowthrough into the spin column and spin through an additional time.
4. Transfer Empore™ C18 spin column to a new tube. Desalt the bound peptide washing with 400 µL of wash solution I (0.5% TFA). Spin through the wash solution for 4 minutes, discard flow through and repeat the wash one to two additional times.
5. Perform final wash with 400 µL of wash solution III (0.5% HAc). Spin through the wash solution for 4 minutes, discard flow through.
6. Transfer Empore™ C18 spin column to new low binding or low retention tube. Pipette 100 µL of elution solution (80% ACN, 0.5% HAc, 19.5% water). Spin through completely for 1-3 minutes and keep eluent.
7. Repeat step 6 two more times and combine eluents. Volume of the eluted peptides should total 300 µL.
8. Vacuum dry the eluent solution.
9. Resuspend the dried peptide in 23 µL of 1% ACN, 0.1% FA in water following the same procedure in step 1. The sample is now ready for LC-MS injection and data acquisition.

3. LC-MS Conditions

Analysis of fractionated and unfractionated E. coli peptides was performed by a Vanquish Horizon LC system and Q Exactive Plus Orbitrap MS system utilizing a HESI source (all components Thermo Scientific). LC separation was performed on Acclaim™ PepMap™ 100 C18 1 mm ID x 15 cm. Mobile phase A: 0.1% formic acid (FA) in water, B: 0.1% FA in acetonitrile. Gradient: 120 minutes 3% B – 28% B. Post gradient wash and equilibration: 20 minutes 28% B – 80% B, 10 minutes 80% B, 5 minutes 80% B – 0% B, 10 minutes 0% B. Constant flow rate of 0.06 mL/min.

Data was collected in data independent acquisition (DIA) mode from minute 0 – 140. MS survey scans acquired from 400 – 900 m/z at a resolution of 70,000 with an AGC of 3e6 and maximum injection time of 76 ms. Each fragmentation cycle consisted of 40 scans of 12.5 m/z isolation windows. Ions were subjected to high-energy collisional dissociation (HCD) with a normalized collision energy of 30% for MS/MS scans. Scans were set to a resolution of 35,000 with a 2e5 AGC, maximum injection time of 52 ms. Raw data was analyzed by DIA-NN software[3] available on github. Search utilized a DIA-NN generated library using UniProt's E. coli proteome (UP000284592) downloaded September 9, 2023. Search utilized default DIA-NN settings with double-pass set for Neural network classifier. Fractionated and unfractionated runs were analyzed separately.

Results and Discussion

In this work, we utilized the Empore™ E3 spin columns (App Note #279) and C18 spin columns (App Note #278) to prepare *E. coli* proteomic samples from intact cells. Our aim was to compare the results of a single *E. coli* sample fractionated versus unfractionated. Fractionated and unfractionated were from the same *E. coli* sample split at the E3 digestion step. For this comparison, we analyzed LC chromatograms as well as results obtained from DIA-NN analysis of both preparations run separately.

LC-MS chromatograms for the different preparations are shown in Figure 1. The fractionated sample chromatogram shows a difference in signal profiles between different fractions (Figure 1A). This is indicative of the Empore™ C18 spin column's ability to gradually elute peptides off the membrane. Additionally, when compared to the unfractionated sample (Figure 1B), fractions 1 through 3 have the most signal early to mid on the chromatogram and fractions 4 through 6 contain the most signal mid to late chromatogram.

Analysis of the resulting raw files via DIA-NN gives further insight into data quality and the effect different preparation had on the results. Assessing the average missed cleavage by trypsin demonstrates the ability of Empore™ E3 spin column to perform efficient digestion (Figure 2A). In this case the unfractionated sample preparation showed the lowest missed cleavage at 19%,

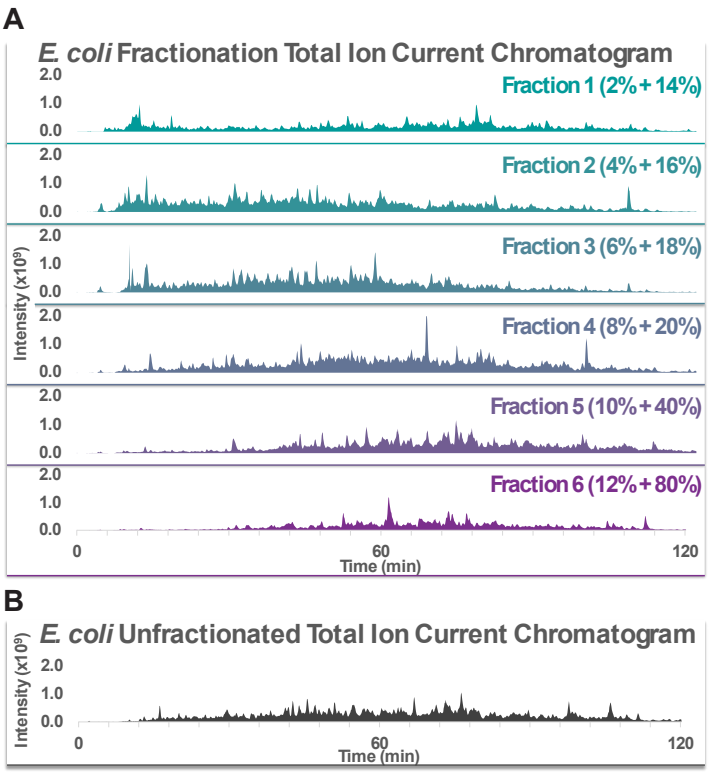
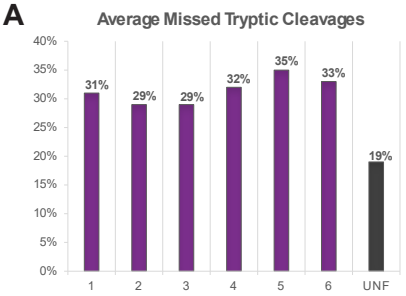
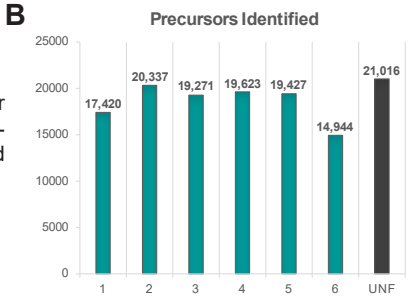


Figure 1. Chromatograms showing the total ion current (TIC) acquired via DIA on Q Exactive Plus over a 3% – 28% B gradient. **(A)** Chromatograms of the fractionated preparation with each combined fraction denoted. **(B)** Chromatogram of the unfractionated preparation.

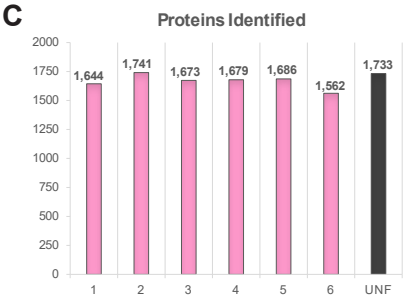
Figure 2. Bar graphs showing resulting data from DIA-NN analysis. X-axis denotes each fraction by the corresponding number and unfractionated as UNF.



(A) Bar graph showing missed cleavages comparing fractions of fractionated preparation to the unfractionated preparation.



(B) Bar graph showing number of precursors identified comparing fractions of fractionated preparation to the unfractionated preparation.



(C) Bar graph showing number of proteins identified comparing fractions of fractionated preparation to the unfractionated preparation.

whereas the fractionated samples averaged 31.5% for all fractions, falling within acceptable rates for proteomic preparations.

Comparing identified precursors, we show unfractionated samples had the highest number identified for a single run with 21,016, followed by fraction 2 of the fractionated preparation with 20,337 (Figure 2B). Fraction 6 contained the least with 14,944 identified. The fractionated sample contained on average 18,503 precursors identified with a range of 14,944 to 20,337, and total of 111,022 precursors identified.

Comparing the identified proteins, results similarly show close identification numbers between fraction 2 and the unfractionated preparation (Figure 2C). This time fraction 2 of the fractionation prep had the higher number with 1,741 identified proteins versus the 1,733 identified proteins in the unfractionated preparation. Again fraction 6 was found to have the least number of proteins identified at 1,562.

The combined fractionated results showed total unique proteins identified and precursors identified at 2,009 and 23,682 respectively (Figure 3A). This result shows a 15.9% increase in the protein and 12.6% increase in precursor identifications compared to the unfractionated preparation. Digging into the data, roughly 69% of the proteins were shared across all fractions, 28% of proteins were shared with at least one other fraction, and 2% were unique to each fraction. Therefore, fractionation could potentially boost protein identification anywhere from 2% to 30% based on this data. Further improvement of fractionation conditions could increase identification even more.

These resulting raw numbers are limited by the power of the LC-MS instrumentation. Another fractionated preparation following the same procedure and using the same *E. coli* sample was kindly run on a nanoLC coupled to a Thermo Scientfic's Oribitrap Eclipse Tribid Mass spectrophotometer by a friend. Their analysis resulted in 3,484 proteins identified (Figure 3B). Their method ran all six fractions independently, each using a two-hour gradient via C18 reverse phase. As of writing (2024), this result covers an estimated 65% of the *E. coli* proteome and is among the highest number in reported protein identifications by a single sample showing the high performance of Empore™ technology in combination with powerful LC-MS instrumentation.

Conclusions

We showcased a procedure for sample preparation making use of E3technology™ on-filter digestion followed by offline high pH fractionation via Empore™ C18 spin column leading to a 16% improvement in proteome identification and subsequently one of the highest number of proteins identified in single *E. coli* sample reported to date. Empore™ C18 spin columns are the perfect choice for large scale peptide desalting and fractionation with excellent reliable quantitative results without the use of large and expensive fractionation systems. This process utilizes simple and common laboratory equipment to produce quality data for any proteomic experiment.

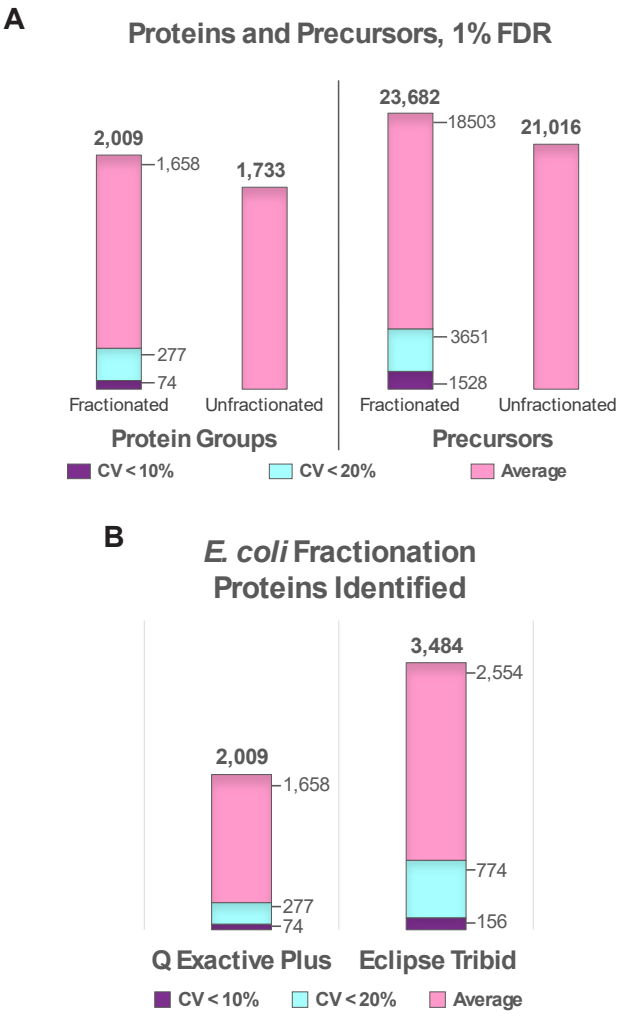


Figure 3. Combined analysis of fractionated results. **(A)** Number of identified proteins and precursors utilizing this procedure and equipment. **(B)** Comparison of the number of identified proteins from the same *E. coli* sample and procedure but different preparations and LC-MS systems for analysis.

Empore™ E3technology™ Order Information

Product Name	Part Number
E3tips, 10 µL	70-2019-3002-3
E3tips, 200 µL	70-2019-3001-1
E3filter, 0.5 mL	70-2019-3101-0
E3plate, 1.2 mL	70-2019-3201-9

Empore™ C18 Order Information

Product Name	Part Number
C18 StageTips, 10 µL	70-2019-1019-0
C18 StageTips, 200 µL	70-2019-1001-3
C18 Spin Columns, 0.5 mL	70-2019-2001-0

References

1. J. Doellinger et al., 2020, Molecular & Cell Proteomics *19*, 209-222
2. K. R. Martin et al., 2024, Cell Reports Methods *4*, 100796
3. V. Demichev et al., 2020, Nat Methods *17*, 41-44