High pH Reversed-Phase Peptide Fractionation in a Convenient Spin-Column Format

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Overview

Purpose: To demonstrate the applications of the new Thermo Scientific™ Pierce™ High pH Reversed Phase Peptide Fractionation Kit, together with Thermo Scientific™ Pierce™ Quantitative Colormetric and Fluorometric Peptide Assay kits in mass spectrometry-based large-scale proteomic experiments to increase protein/peptide identification numbers and protein sequence coverage with high analytical reproducibility.

Methods: HeLa lysate digests, both native and Thermo Scientific™ Tandem Mass Tag™ (TMT) reagents, were fractionated off-line in a high pH reversed-phase mode using spin-columns packed with polymer-based hydrophobic resin and bench top microcentrifuge, and individual fractions were analyzed by LC-MS. Absolute peptide quantities were determined prior to fractionation and sample injection using the quantitative peptide assays.

Results: Protein IDs increased by ~1.8 times and ~3 times for native and TMT-labeled samples, respectively, with fractionation of the complex digest samples.

Introduction

Many biologically relevant changes in the proteome occur at the mid-to-low range of the protein abundance scale. Off-line fractionation of complex peptide mixtures from sample digests enables deeper proteome sequencing through increased protein identifications and sequence coverage. Similar to strong cation exchange (SCX) peptide fractionation methods, high pH reversed-phase fractionation enables orthogonal peptide fractionation to low pH reversed-phased separation. In contrast to SCX fractionation, samples fractionated by high pH reversed-phase fractionation do not require desalting before LC-MS analysis. In this study, we assessed peptide/protein identification numbers, fractional resolution, and reproducibility of high pH reversed-phase fractionation in a convenient, spin column format.

Methods

Sample Preparation

Protein extracts from HeLa lysates were digested sequentially with Lys-C and trypsin. Initial protein concentrations were determined using a BCA assay. Subsequent peptide quantitation was performed using a Pierce Colorimetric Peptide Quantitation Assay (Product# 23275) and/or Pierce Fluorometric Peptide Quantitation Assay (Product# 23290). Portions of the digested samples were labeled with Tandem Mass Tag (TMT) reagents. Pierce High pH Reversed-Phase Fractionation Kits (Product# 84868) were used to fractionate both native and TMT-labeled digest samples into eight fractions by an increasing acetonitrile step-gradient elution. Fractions were dried in a vacuum centrifuge and re-suspended in 0.1% formic acid prior to LC-MS analysis.

Liquid Chromatography

Liquid chromatography was performed using Thermo Scientific™ Dionex™ Ultimate™ 3000 Nano LC system, utilizing a 50 cm C_{18} Thermo Scientific™ EASY-Spray™ column heated at 60°C. Two- or three-hour gradients were used in all experiments.

Mass Spectrometry

All samples were analyzed on a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer.

For native (unlabeled) samples, MS-level scans were performed with Orbitrap resolution set to 60,000; AGC Target 2.0e5; maximum injection time 50 ms; intensity threshold 5.0e3; dynamic exclusion 45 sec. Data dependent MS^2 selection was performed in Top Speed mode with HCD collision energy set to 28% and ion trap detection (AGC target 1.0e4, maximum injection time 35 ms).

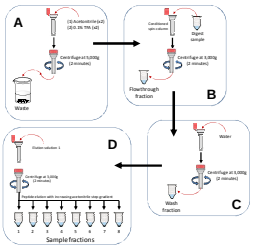
For TMT-labeled samples, MS-level scans were performed with Orbitrap resolution set to 120,000; AGC target 4.0e5; maximum injection time 50 ms; intensity threshold 5.0e3; dynamic exclusion 60 sec. Data-dependent MS^2 selection was performed in Top Speed mode with CID collision energy set to 35%. Data-dependent MS^3 was performed in Top N mode with Synchronous Precursor Selection set to 10 precursors and HCD collision energy set to 65% and Orbitrap resolution set to 60,000.

Data Analysis

All .raw files were processed using Thermo Scientific™ Proteome Discoverer™ 1.4 software. Data was searched against a custom human/yeast database using Sequest® HT search engine.

FIGURE 1. Off-line spin-column high pH reversed-phase fractionation workflow.

The column is first conditioned by successive washes with acetonitrile and aqueous 0.1% TFA by spinning at 5,000 g (A); a sample solution is then loaded onto the column and the flowthrough is collected by centrifugation at 3,000 g (B); bound peptides are further washed to remove any salts and/or weakly bound chemicals (C); bound sample peptides are then fractionated by stepwise elution with increasing acetonitrile concentration solutions at high pH (D).



Results

High pH reversed-phase fractionation orthogonality

To demonstrate orthogonality of low and high pH reversed phase separation modes, we fractionated 100 µg of HeLa digest according to the workflow in Figure 1 using low pH (0.1% trifluoroacetic acid, TFA) and high pH (0.1% triethylamine, TEA) elution solutions at 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, and 50% acetonitrile steps. The fractions were then analyzed individually by LC-MS (with 0.1% formic acid, FA, additive in the mobile phases). As shown in Figure 2, peptides in the high pH fractions cover more chromatographic space due to changes in hydrophobic retention properties during fractionation at high pH.

Reproducibility and fractional resolution

Analysis of the replicate sets of fractions revealed excellent reproducibility of the spin-column fractionation format. Fractional base peak chromatogram profiles shared high similarity across different replicate fractions (Figure 3). Reproducibility of the elution profiles, in terms of unique peptides identified and the numbers of unique peptides identified, was also good among the replicates for both the native (Figure 4) and TMT-labeled samples.

FIGURE 2. Orthogonality of low and high pH reversed-phase fractionation.

TIC chromatograms of fractions eluted with 7.5% acetonitrile solution at high pH (High-low) and low pH (Low-low) are compared to that of unfractionated digest sample.

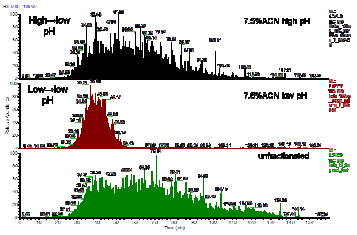
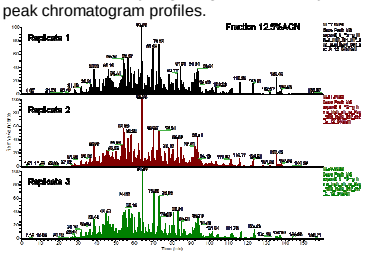


FIGURE 3. Fractionation reproducibility with spin-columns.

Base peak chromatograms of three replicate fractions are compared. Good reproducibility among replicate fraction sets is evidenced by high degree of similarity in the base peak chromatogram profiles.



Improvement in protein identification numbers

Due to the overall decrease in chromatographic density in each high pH reversed phase fraction, we are able to sample more peptides and acquire more MS^2 spectra. This leads to more spectral matches, more unique peptide identifications, and more protein identifications with higher sequence coverage. Upon fractionation of native HeLa digests and analysis of the individual fractions, nearly 1.8 times more protein groups were identified (Figure 3).

Similar analysis of the TMT-labeled HeLa sample fractions revealed nearly 3 times more protein groups compared to the unfractionated samples.

FIGURE 4. Reproducibility of fractional profiles and fractional resolution of native digest samples.

Numbers of unique peptides (A) and protein groups (B) identified in a given fraction are similar across the triplicate set. Over 70% of all peptides were identified in only one fraction, indicating excellent fractional resolution of the spin-column format (C). Nearly 2.5 times more unique peptides and 1.8 times more protein groups were identified upon the analysis of fractionated sample compared to unfractionated samples (black bars).

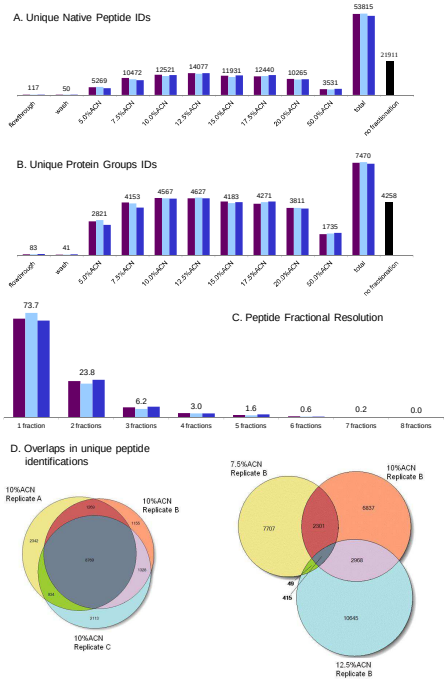
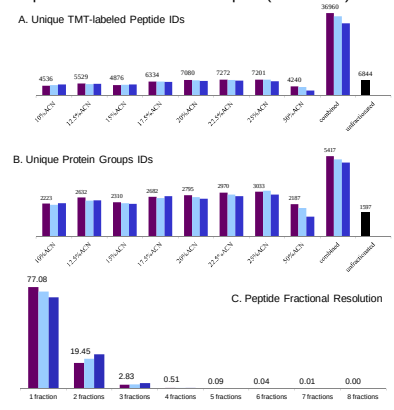


FIGURE 5. Reproducibility of fractional profiles and fractional resolution of TMT-labeled digest samples.

Numbers of unique peptides (A) and protein groups (B) identified in a given fraction are similar across the triplicate set. Over 75% of all peptides were identified in only one fraction, indicating excellent fractional resolution of the spin-column format (C). Over 5 times more unique peptides and nearly 3 times more protein groups were identified upon the analysis of fractionated sample compared to unfractionated samples (black bars).



Conclusions

- High pH reversed-phase fractionation of complex sample digests leads to higher numbers of unique peptide and protein identifications.
- Fractionation in a spin-column format offers excellent fractional resolution and reproducibility.
- Pierce High pH Reversed-Phase Fractionation Kit enables more comprehensive proteomic analysis of samples in a convenient, economical, and user-friendly format.

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