Use of high pH reversed-phase peptide fractionation to analyze proteins of medium to low abundance in complex mixtures

Decrease sample complexity and increase positive identification of proteins

Many of the biologically relevant changes in the proteome occur at the mid-to-low range of the protein-abundance scale. Traditional sample preparation and direct analysis by liquid chromatography/mass spectrometry (LC/MS) are often not sufficient to probe this part of the proteome, and it is often necessary to reduce the sample complexity by fractionation in an orthogonal dimension, prior to LC/MS analysis. Off-line fractionation of complex peptide mixtures from sample digests enables deeper proteome sequencing through increased protein identification and sequence coverage. Similar to peptide fractionation by strong cation exchange (SCX) chromatography, high pH reversed-phase fractionation enables peptide fractionation orthogonal to low pH reversed-phased separation. In contrast to SCX chromatography, 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.

Here we describe a simple, highly reproducible protocol to enable deep proteome sequencing (Figure 1). The Thermo Scientific™ Pierce™ High pH Reversed-Phase Peptide Fractionation Kit utilizes reversed-phase chromatography at high pH to separate peptides by hydrophobicity and provides excellent orthogonality to low pH reversed-phase LC/MS gradients. In this procedure, the following protocol is followed for peptide fractionation: (A) the column is first conditioned by successive washes with acetonitrile (ACN) and aqueous 0.1% trifluoroacetic acid (TFA) by spinning at 5,000 x *g*; (B) the sample solution is loaded onto the column, and the flow-through is collected by centrifugation at 3,000 x *g*; (C) the bound peptides are further washed to remove any salts and weakly bound chemicals; (D) the bound sample peptides are fractionated by stepwise elution with solutions containing increasing acetonitrile concentrations at high pH.

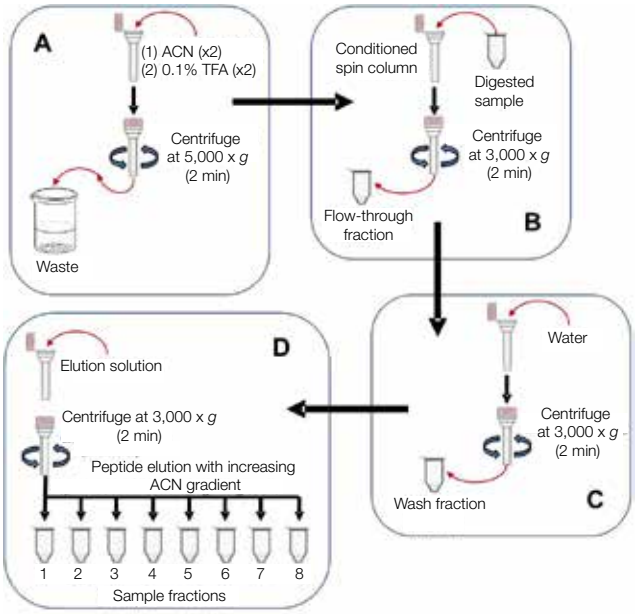

Figure 1. Procedure summary. Peptides are bound to the hydrophobic resin under aqueous conditions and desalted by washing the column with water by low-speed centrifugation. A step gradient of increasing acetonitrile concentrations in a volatile, high pH elution buffer is then applied to the columns to elute bound peptides into 8 fractions by centrifugation. Each fraction is then dried in a vacuum centrifuge and stored until analysis by mass spectrometry.

ThermoFisher SCIENTIFIC

Results

High pH reversed-phase fractionation orthogonality

To demonstrate orthogonality of low pH and high pH reversed-phase separation modes, we fractionated 100 µg of HeLa cell digest according to the workflow in Figure 1 using low pH (0.1% TFA) and high pH (0.1% triethylamine (TEA)) elution solutions at 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, and 50% ACN. The fractions were then analyzed individually on a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer (with 0.1% formic acid (FA), the additive in the mobile phase). Peptides in the high pH fractions cover more chromatographic space due to changes in hydrophobicities affecting the retention properties during fractionation (Figure 2).

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. Reproducibility of the elution profiles, in terms of unique peptides and protein groups identified, was also good among the replicates for both the native digests (Figure 3) and samples labeled with tandem mass tag (TMT) reagents (Figure 4). Overall, greater than 70% of all peptides were identified in only one fraction, indicating excellent fractional resolution of the spin-column format.

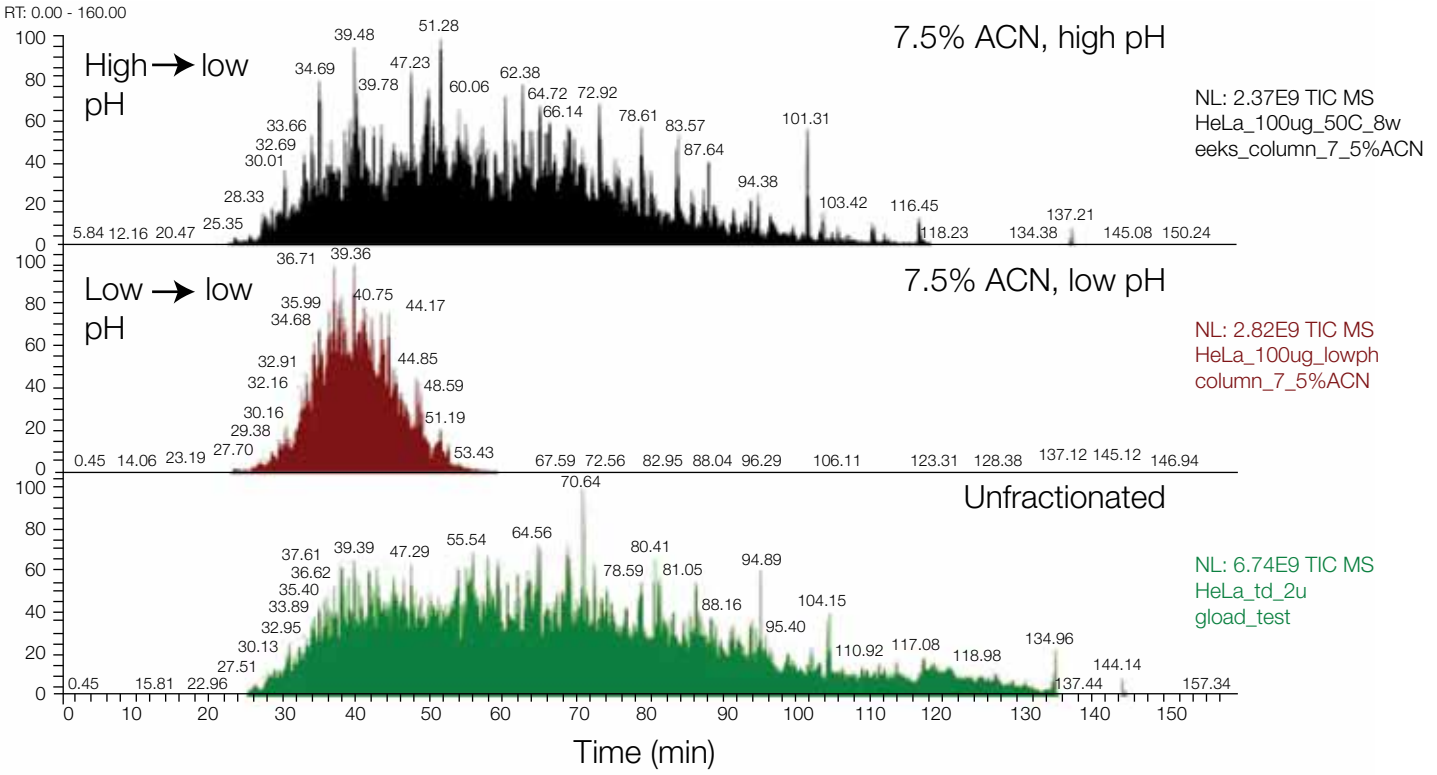

Figure 2. Orthogonality of low and high pH reversed-phase fractionation. The total ion chromatogram (TIC) of fractions eluted with 7.5% acetonitrile (ACN) solution at high pH (High → low) and low pH (Low → low) compared to an unfractionated digest sample.

Improvement in protein identification numbers

By decreasing the chromatographic density through the use of the high pH reversed-phase columns, we are able to sample a greater number of peptides and acquire a larger number of MS^2 spectra. This leads to more spectral matches, more unique peptide identifications, and more protein group identifications with higher sequence coverage. Upon fractionation of native HeLa cell digests and analysis of the individual fractions, nearly 1.7 times more protein groups were identified (Figure 3). Similar analysis of the HeLa cell digest fractions labeled with TMT reagents showed nearly 2.8 times more protein groups identified, compared to the unfractionated samples (Figure 4).

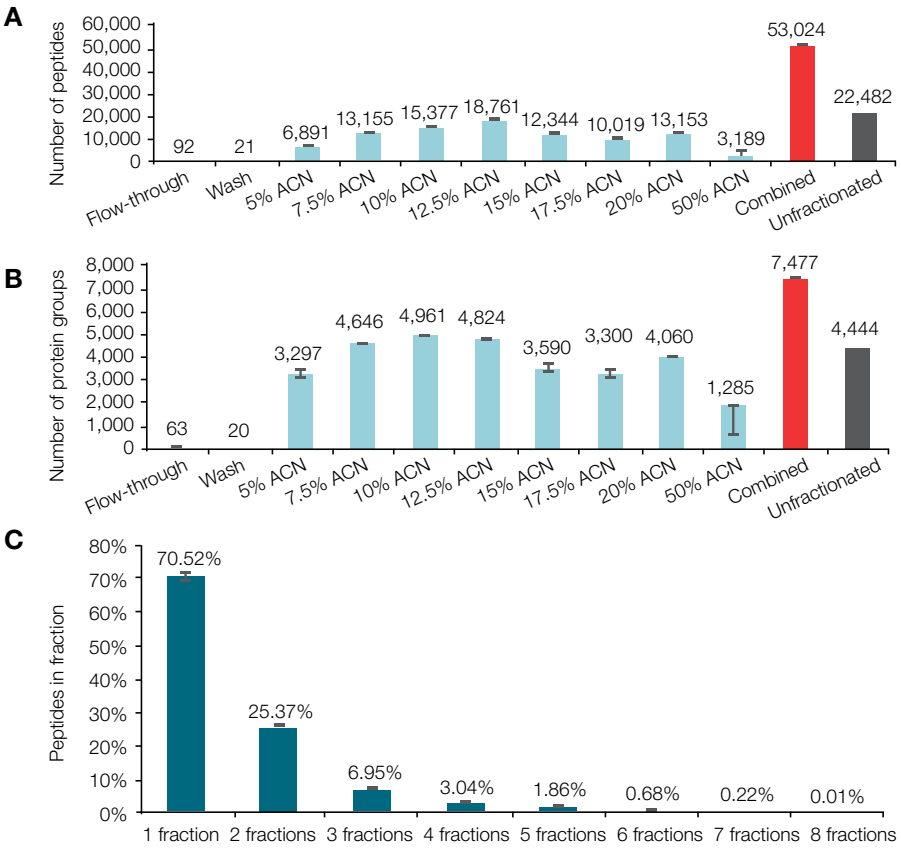

Figure 3. Reproducibility of fractional profiles, and fractional resolution of native digest samples. Number of **(A)** unique peptides and **(B)** protein groups identified for each fraction of native HeLa cell digest sample. **(C)** Fractional resolution is represented by the percent of unique peptides found in individual fractions.

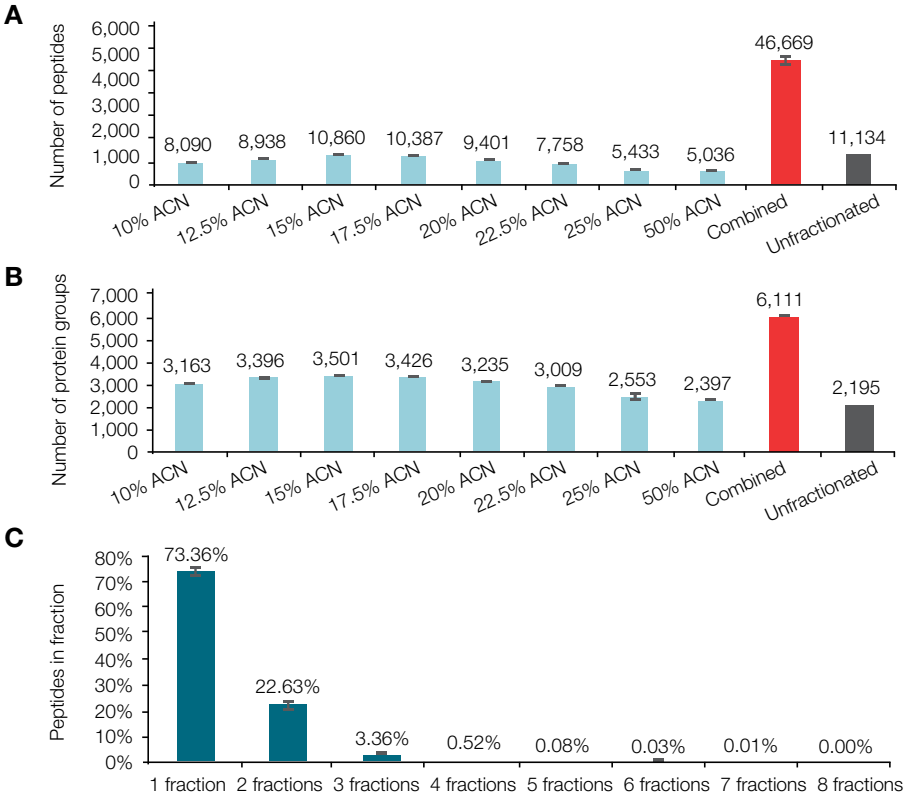

Figure 4. Reproducibility of fractional profiles, and fractional resolution of cell digest samples labeled with TMT reagents. Number of **(A)** unique peptides and **(B)** protein groups identified for each fraction of HeLa cell digest sample labeled with TMT reagents. **(C)** Fractional resolution is represented by the percent of unique peptides found in individual fractions.

Fractional profiles correlate with peptide quantities

The concentrations of peptides in each column fraction were quantified using the Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay (Cat. No. 23275) and/or Quantitative Fluorometric Peptide Assay (Cat. No. 23290). Overall, there was very good correlation between the quantitative peptide assay signal and TIC area counts for all fraction replicates (Figure 5A). Using the quantitative peptide assay, normalization was achieved for the column load for LC/MS analysis. For complex samples, increasing sample on-column load generally leads to a proportional increase in peptide detections, translating to a higher number of protein identifications.

Excellent fractional resolution is further demonstrated in Figure 5B, where three consecutive fractions are compared for three replicate fractions of the same elution step. Venn diagrams of unique peptide identifications show very high overlap of peptides from the same fraction, but low overlap when compared to neighboring fractions.

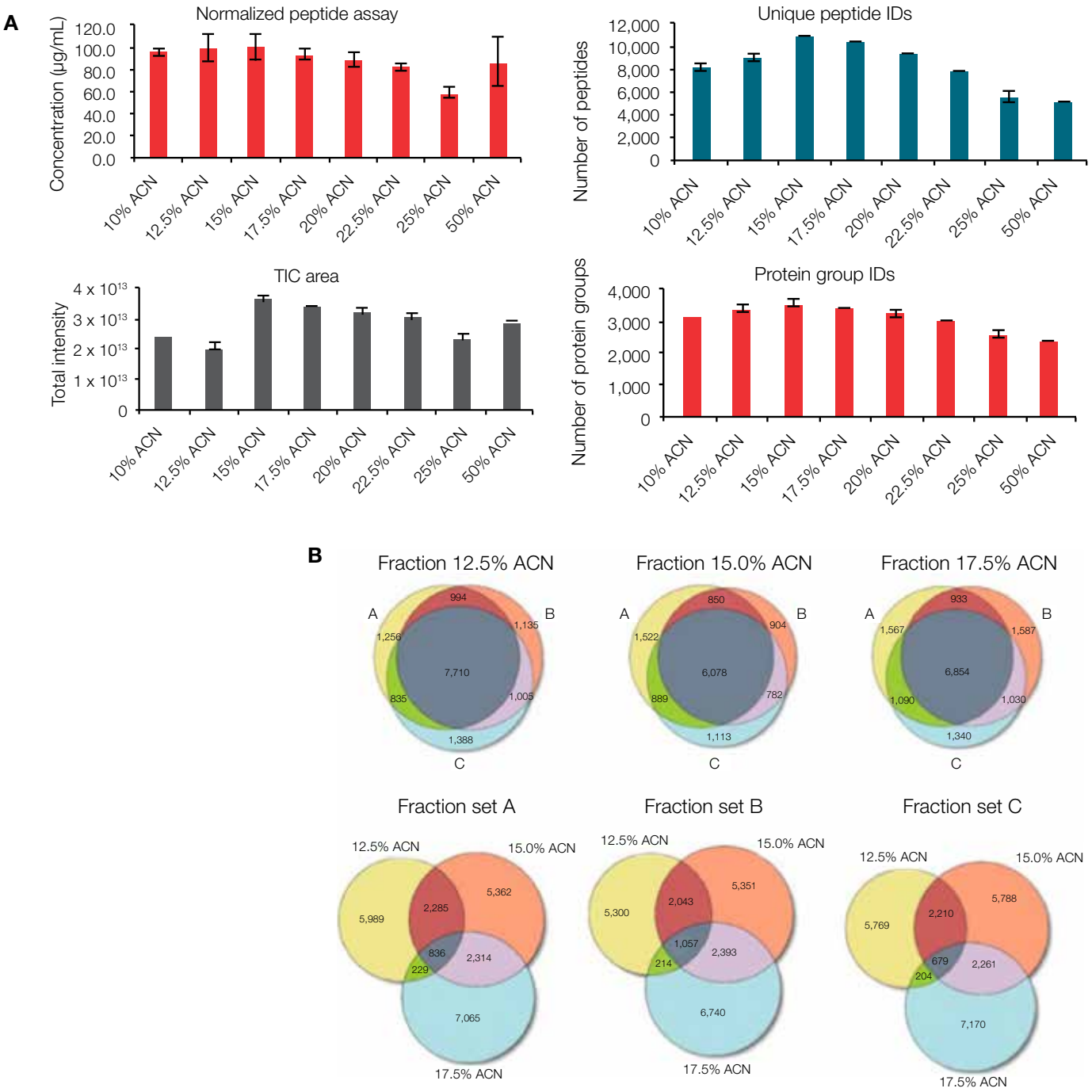

Figure 5. Fractional profiles of HeLa cell digest samples labeled with TMT reagents. **(A)** Comparison of normalized peptide assay signal, unique peptide IDs, TIC area counts, and protein group IDs for fraction sets of HeLa digests labeled with TMT reagents in triplicate and eluted with different concentrations of acetonitrile. **(B)** Overlap of unique peptide IDs in replicate fractions (top row) and in three consecutive fractions within a set.

thermo scientific

Conclusions

We have successfully developed a robust and reproducible protocol that utilizes off-line fractionation of peptide digests to decrease sample complexity, thereby increasing positive identification of proteins. The use of high pH reversed-phase fractionation of complex sample digests results in 1.7- to 2.8-fold increases in the number of unique peptide and protein group identifications. The high pH reversed-phase columns are efficient in fractionating both native protein cell digests and samples labeled with TMT reagents, allowing for a range of downstream target analysis techniques. Fractionation in a spin-column format offers excellent fractional resolution and reproducibility, enabling deep proteome sequencing.

Methods

Sample preparation

Protein extracts from HeLa cell lysates were digested sequentially with Lys-C and trypsin. Initial protein concentrations were determined using the Thermo Scientific™ Pierce™ BCA Protein Assay (Cat. No. 23224). Subsequent peptide quantification was performed using the Pierce Quantitative Colorimetric Peptide Assay (Cat. No. 23275) and/or the Pierce Quantitative Fluorometric Peptide Assay (Cat. No. 23290). Portions of the digested samples were labeled with Thermo Scientific™ TMTsixplex™ reagents (Cat. No. 90062). The Pierce High pH Reversed-Phase Peptide Fractionation Kit (Cat. No. 84868) was used to fractionate cell digest samples, both native and labeled with TMT reagents, into 8 fractions by eluting with an increasing acetonitrile step gradient. Fractions were dried in a vacuum centrifuge and resuspended in 0.1% formic acid prior to LC/MS analysis.

Liquid chromatography

Liquid chromatography was performed using the Thermo Scientific™ Dionex™ UltiMate™ 3000 Nano LC system, utilizing a 50 cm Thermo Scientific™ EASY-Spray™ C18 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; automatic gain control (AGC) target 2×10^5; maximum injection time 50 ms; intensity threshold 5×10^3; dynamic exclusion 45 sec. Data-dependent MS^2 selection was performed in top-speed mode with high-energy collision dissociation (HCD) energy set to 28% and ion trap detection (AGC target 1×10^4, maximum injection time 35 ms). For samples labeled with TMT reagents, MS-level scans were performed with Orbitrap mass spectrometer resolution set to 120,000; AGC target 4×10^5; maximum injection time 50 ms; intensity threshold 5×10^5; dynamic exclusion 60 sec. Data-dependent MS^2 selection was performed in top-speed mode with collision-induced dissociation (CID) energy set to 35%. Data-dependent MS^3 was performed in top-speed mode with synchronous precursor selection set to 10 precursors, HCD energy set to 65%, and Orbitrap mass spectrometer resolution set to 60,000.

Data analysis

All raw files were processed using Thermo Scientific™ Proteome Discoverer™ 1.4 software. Data were searched against a custom database of human/yeast gene sequences using the Sequest™ HT search engine and Percolator algorithm with 1% false discovery rate (FDR).

Find out more at **thermofisher.com/peptidefractionation**

ThermoFisher SCIENTIFIC