Peptide enrichment and fractionation

Successful analysis of low-abundance proteins and/or the identification of posttranslationally modified peptides often require several steps: enrichment, fractionation, and/or clean-up. Phosphorylation is arguably the most intensively studied PTM. Phosphoproteins are integral to global cellular signaling in disease and key to understanding biological regulation.

Unfortunately, many phosphopeptides are present at very low levels in a typical cell lysate. Initial approaches focused on enrichment with immobilized metal ion affinity chromatography (IMAC). However, enrichment and recovery of phosphopeptides using an IMAC system strongly depends on the type of metal ion and column material, and is often hampered by the nonselective enrichment of acidic residues. An alternative strategy is to carry out metal-oxide affinity chromatography using aluminum, titanium, zirconium, and other metal oxides; this was successfully applied for selective enrichment of phosphopeptides.

Many biologically relevant changes in the proteome occur at the mid-to-low range of the protein abundance scale. The fractionation of complex peptide mixtures from sample digests enables deeper proteome sequencing through increased protein identifications and sequence coverage. Numerous strategies are available, including strong cation exchange (SCX), peptide isoelectric focusing (pIEF), and SDS-PAGE. Similar to SCX peptide fractionation methods, high-pH reversed-phase fractionation enables peptide fractionation orthogonal 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.

Table 40. Peptide enrichment and fractionation kit selection guide.

	Fe-NTA Phosphopeptide Enrichment Kit	TiO_2 Phosphopeptide Enrichment Kit	Magnetic TiO_2 Phosphopeptide Enrichment Kit	High pH Reversed-Phase Peptide Fractionation Kit
				
Target	Phosphopeptides	Phosphopeptides	Phosphopeptides	All peptides
Binding/labeling mechanism	Metal-chelate affinity to phosphate groups	Metal-oxide affinity to phosphate groups	Metal-oxide affinity to phosphate groups	Hydrophobic interaction
Loading capacity/rxn*	0.5–5 mg	0.5–3 mg	100 µg	10–100 µg
Base support	IMAC-Agarose resin	Spherical porous TiO_2 bead	TiO_2 coated magnetic particles	Hydrophobic polymer based resin
Format	Spin column	Tip	Magnetic bead	Spin column
Processing time	45–60 min	45–60 min	15 min	30–60 min

* Based on a standard HeLa protein digest sample

Phosphopeptide Enrichment Kits

Phosphopeptides have high hydrophilicity and are low in abundance, resulting in poor chromatography, ionization, detection, and fragmentation. Phosphopeptide enrichment is therefore essential to successful MS analysis. We offer a variety of ligand and formats for the enrichment of phosphopeptides, including titanium dioxide (TiO_2) and Fe-NTA immobilized metal affinity chromatography (IMAC) resins. Because of unique binding characteristics of each ligand, Fe-NTA IMAC and TiO_2 phosphopeptide enrichment kits bind a complementary set of phosphopeptides from complex samples.

Choosing between the two ligands depends on the researchers' goals. Although these two ligands similar numbers of phosphopeptides per sample, there is only a 50% overlap between the identified phosphopeptides (Figure 50). Although there is a slight bias using TiO_2 enrichment toward multiply phosphorylated (i.e., two or more) peptides (Figure 51), each ligand type clearly has affinity for different phosphopeptide sequences. In contrast to our TiO_2 tip or magnetic supports, Fe-NTA spin columns have a much higher binding capacity and are recommended if additional fractionation steps will be utilized postenrichment for deeper proteome coverage and the detection of low-abundance phosphopeptides (Figure 52).

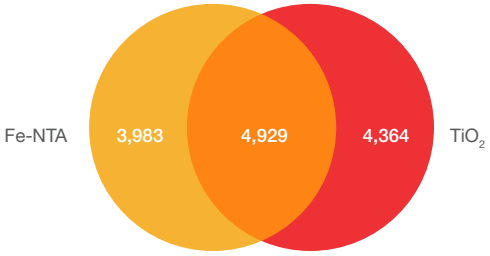


Figure 50. Fe-NTA and TiO_2 resins enrich a complementary set of phosphopeptides. The Venn diagram shows the number of phosphopeptides identified from 1.0 mg of peptides prepared from nocodazole-treated HeLa cells. Phosphopeptides were enriched with the Thermo Scientific™ High-Select™ Fe-NTA Phosphopeptide Enrichment Kit and the High-Select™ TiO_2 Phosphopeptide Enrichment Kit. Eluted peptides were analyzed with Trap column and Thermo Scientific™ Acclaim™ PepMap™ RSLC C18 (2 µm, 100Å, 75 µm x 50 cm) on a Orbitrap Fusion Tribrid Mass Spectrometer.

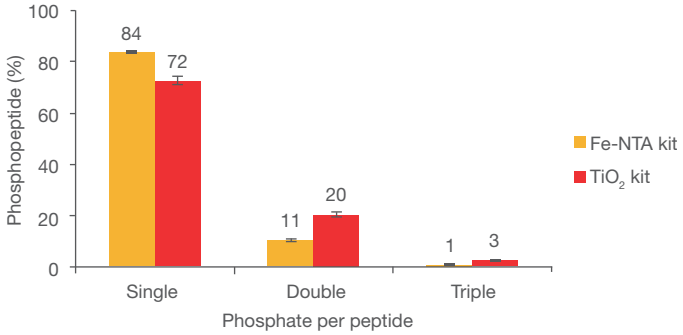


Figure 51. The multiple phosphopeptides profile. Both the Fe-NTA kit and TiO_2 kit effectively capture peptides with multiple phosphates. TiO_2 enrichment had a slight bias toward multiple phosphopeptides.

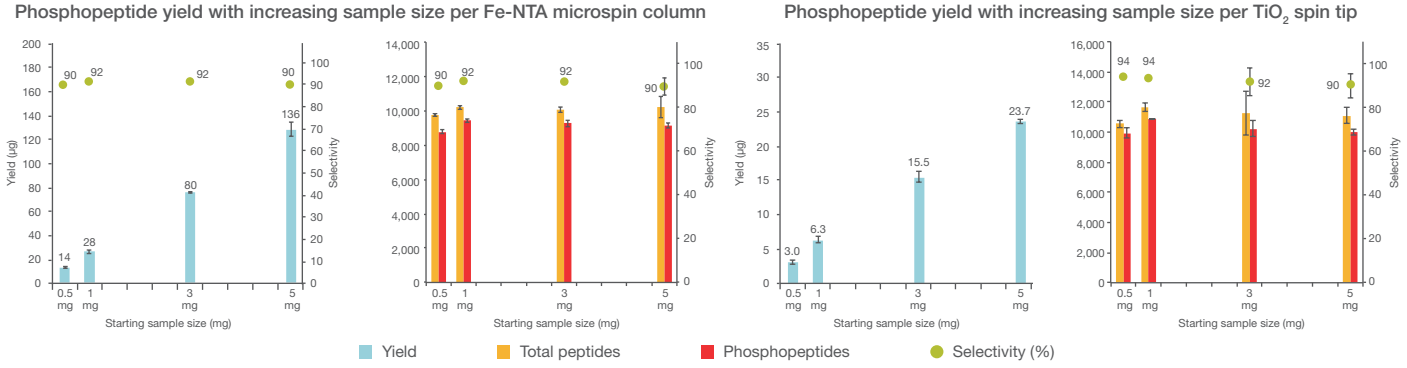


Figure 52. Enrichment and yield obtained from increasing sample sizes. Each Fe-NTA column or TiO_2 spin tip can enrich phosphopeptides from 0.5–5 mg or from 0.5-3 mg of a total protein digest in the starting samples, respectively. Phosphopeptide yield, determined by the Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay (Cat. No. 23275), was proportional to sample size with consistent ≥90% selectivity. The Fe-NTA kit can enrich five times more phosphopeptides than TiO_2 kits; it is recommended for users who have >2 mg of complex biological sample and need to get >50 µg phosphopeptide yield for further process such as fractionation.

High-Select Fe-NTA Phosphopeptide Enrichment Kit

Fe-NTA format optimized for high-binding capacity of phosphopeptides

Highlights:

- **Complete**—kit includes all columns and buffers for optimized phosphopeptide enrichment
- **Convenient**—prefilled spin-columns and ready-to-use buffers enable easy sample processing
- **High binding capacity**—each column enriches up to 150 µg of phosphopeptides from 5 mg of protein digest
- **High specificity**—recover phosphopeptides with >90% selectivity
- **Excellent recovery**—enriches more total and unique phosphopeptides than other commercially available resins
- **Complementary**—enriches a unique set of phosphopeptides that complements our TiO_2 kit

The Thermo Scientific™ High-Select™ Fe-NTA Phosphopeptide Enrichment Kit enables fast and efficient enrichment of phosphorylated peptides with greater than 90% specificity. This new and improved kit contains preformulated buffers and ready-to-use spin columns that provide a simplified and more rapid (45–60 min) procedure to enrich phosphopeptides from protein digests or peptide fractions for mass spec analysis. Each prefilled spin column contains a phosphopeptide-specific resin that offers excellent binding and recovery properties for enriching up to 150 µg of phosphopeptides. Each column has a loading capacity of 0.5–5 mg of a total protein digest and phosphopeptide yields are typically 2–4% of the starting sample. This kit fully complements our lysis, reduction, alkylation, and digestion reagents, along with C18, graphite spin, and high pH reversed-phase fractionation columns to provide a complete workflow for phosphopeptide enrichment.

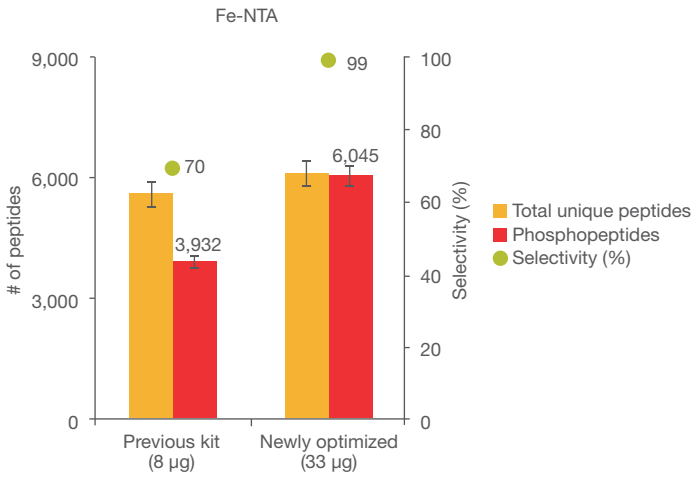


Figure 53. New High-Select Fe-NTA kit with significantly improved selectivity and yield. The average selectivity is 95% ± 2% per 1 mg of HeLa protein digest used for the enrichment. Phosphopeptide yield is improved 4-fold with the new Fe-NTA kit.

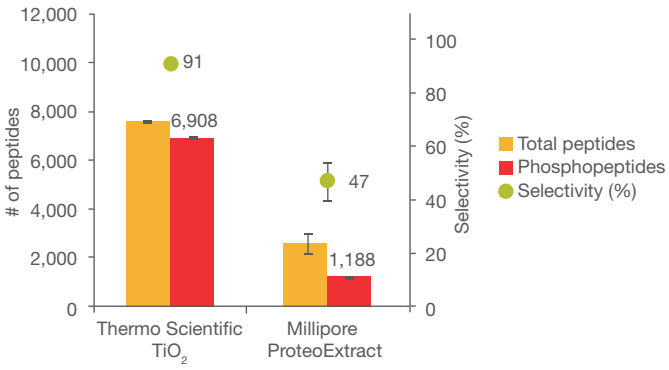
High-Select TiO_2 Phosphopeptide Enrichment Kit

TiO_2 spin tips selective for phosphopeptides

The Thermo Scientific™ High-Select™ TiO_2 Phosphopeptide Enrichment Kit enables efficient isolation of phosphorylated peptides from complex and fractionated protein digests for analysis by MS. This new and improved kit has eliminated a toxic component and provides a simplified and more rapid (45–60 min) procedure to enrich phosphopeptides from protein digests or peptide fractions for mass spec analysis. The spherical, porous TiO_2 resin spin tips and optimized buffers provide enhanced identification and enrichment of phosphopeptides with greater than 90% specificity. Each tip has a loading capacity of 0.5–3 mg of total protein digest and phosphopeptide yields are typically 1–3% of starting sample tip load. The easy-to-use protocol produces a high yield of clean phosphopeptide samples ready for MS analysis. This kit fully complements our lysis, reduction, alkylation, digestion, and high-pH reversed-phase fractionation columns to provide a complete workflow for phosphopeptide enrichment.

Highlights:

- **Complete**—kit includes all columns and buffers for optimized phosphopeptide enrichment
- **Convenient**—spin-tip format enables parallel processing of multiple samples
- **Highly specific**—recovers phosphopeptides with >85% selectivity
- **Complementary**—TiO_2 enriches a unique set of phosphopeptides that complements our Fe-NTA IMAC kit



	Thermo Scientific	EMD Millipore
Selectivity	91%	47%
Phosphopeptides	6,908	1,188
Yield	9.4 µg	70 µg*
Binding capacity	3 µg/mg dry resin	15 µg/mg dry resin

* Interference in Pierce peptide

Figure 54. Effective enrichment of phosphopeptides by the High-Select TiO_2 Kit. The High-Select TiO_2 Phosphopeptide Enrichment Kit was used to enrich phosphopeptides from 1 mg of protein digest from HeLa cell extract. The selectivity and yield of the kit was benchmarked against the EMD Millipore ProteoExtract™ Phosphopeptide Enrichment TiO_2 Kit. The same amount (1 µg) of eluted phosphopeptide determined by the Pierce™ Quantitative Colorimetric Peptide Assay (Cat. No. 23275) was analyzed on an Orbitrap Fusion Tribrid Mass Spectrometer.

Pierce Magnetic TiO_2 Phosphopeptide Enrichment Kit

TiO_2 magnetic particles for high-throughput phosphopeptide isolation

The Thermo Scientific™ Pierce™ Magnetic TiO_2 Phosphopeptide Enrichment Kit is used for isolating phosphopeptides from complex biological samples using titanium dioxide (TiO_2)-coated magnetic beads. The TiO_2 ligand selectively binds peptides containing phosphorylated serine (Ser), tyrosine (Tyr), or threonine (Thr), enabling phosphopeptide enrichment from protease-digested samples. The isolated phosphopeptides are compatible for analysis downstream by MS (Table 41).

The high-performance superparamagnetic TiO_2 particles are validated and optimized for use with high-throughput magnetic platforms such as the KingFisher Flex instruments. The beads also enable premium performance for simple benchtop applications using an appropriate magnetic stand.

Highlights:

- **Complete MS-compatible kits**—include ready-to-use binding, wash, and elution buffers that are optimized for phosphopeptide enrichment and downstream analysis by MALDI and ESI MS
- **Optimized for high-throughput screening**—procedure validated for processing 1–96 samples at a time; complete entire assay in about 15 min using a KingFisher Flex Instrument
- **Stable affinity ligand**—TiO_2 is specially coated as a film on the magnetic particles
- **Selective**—affinity system is selective for phosphorylated Ser, Tyr, and Thr; exhibits minimal nonspecific binding to acidic residues
- **Sensitive**—affinity format provides more than 1,000x greater sensitivity than traditional IMAC technologies; enables enrichment and MS measurement of less than 100 fmol of phosphoprotein

Table 41. Phosphopeptide enrichment improves MS identification of phosphoproteins. Two milligrams of a tryptic digest prepared from peripheral blood mononuclear cells (lymphocytes) with and without phosphopeptide enrichment were analyzed by MS. Enrichment was performed with the Pierce TiO_2 Phosphopeptide Enrichment Kit using the KingFisher 96 Instrument. Samples were analyzed on a LTQ Orbitrap Mass Spectrometer.

	Enriched	Unenriched
Total number of proteins identified	185	247
Total number of phosphoproteins identified	160	1
Total number of peptides identified	2,347	2,457
Total number of phosphopeptides identified	2,009	7
Total number of unique phosphopeptides identified	177	1
Relative enrichment for phosphopeptides (%)	86	0.3

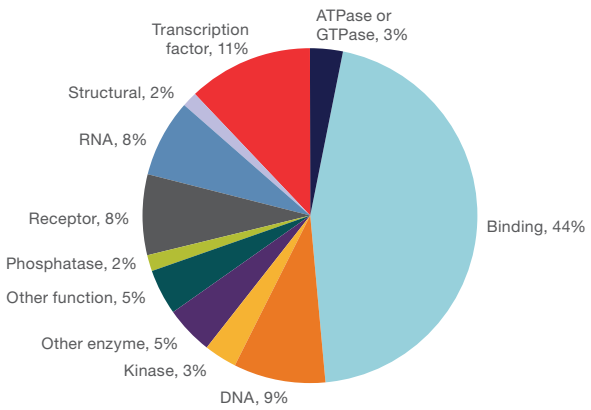


Figure 55. Major protein functions identified in a phosphoprotein- and phosphopeptide-enriched MS data set using the Pierce Magnetic TiO_2 Phosphopeptide Enrichment Kit.

Learn more at **thermofisher.com/peptidekits**

Pierce High pH Reversed-Phase Peptide Fractionation Kit

Easy-to-use peptide fractionation kit that reduces sample complexity and increases protein identification

The Thermo Scientific™ Pierce™ High pH Reversed-Phase Peptide Fractionation Kit increases protein identification from LC-MS analysis through orthogonal peptide fractionation of complex peptide samples.

Highlights:

- **Easy to use**—resin provided in single-use spin column format
- **Improved protein identifications**—protein identifications increased by ≥50% when compared to unfractionated samples
- **Reproducible**—elution profiles and fractional resolution vary by less than 20%
- **Optimized**—robust procedure for maximal protein identification and peptide recovery while minimizing fractional overlap
- **Compatible**—reagents have been validated with a variety of complex samples, including peptides labeled with Thermo Scientific™ Tandem Mass Tag™ (TMT™) reagents

To enable deep proteome sequencing, it is often necessary to reduce the sample complexity by fractionation in an orthogonal dimension prior to LC-MS analysis. The Pierce High pH Reversed-Phase Peptide Fractionation Kit uses high-pH reversed-phase chromatography to separate peptides by hydrophobicity and provides excellent orthogonality to low-pH reversed-phase LC-MS gradients. The kit is designed to improve protein identification through the use of a proprietary reversed-phase resin in an easy-to-use spin column format with a high-pH fractionation protocol. In contrast to strong cation exchange (SCX) fractionation, the high-pH reversed-phase fractions do not require an additional desalting step before LC-MS analysis.

The Pierce High pH Reversed-Phase Peptide Fractionation Kit includes a high-pH solution (0.1% triethylamine) and 12 spin columns containing pH-resistant reversed-phase resin. Each reversed-phase fractionation spin column enables fractionation of 10–100 µg of peptide sample using a microcentrifuge. Native phosphorylated samples labeled with TMT reagents and other complex peptide mixture samples can be fractionated using the kit. Combining the search results generated by the individual fractions improves protein sequence coverage and increases number of identified proteins relative to unfractionated samples

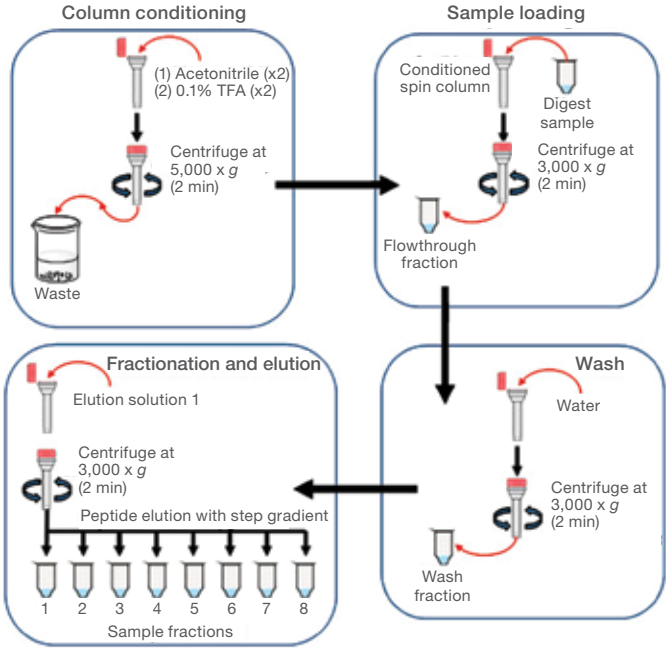


Figure 56. Procedure summary. Peptides are bound to the hydrophobic resin under aqueous conditions and desalted by washing the column with water by low-speed centrifugation. A step gradient of increasing acetonitrile concentrations in a volatile high-pH elution buffer is then applied to the columns to elute bound peptides into 8 different fractions collected by centrifugation. Each fraction is then dried in a vacuum centrifuge and stored until analysis by MS. During LC-MS analysis, peptides in each high-pH fraction are further separated using a low-pH gradient, thus reducing the overall sample complexity and improving the ability to identify low-abundance peptides.

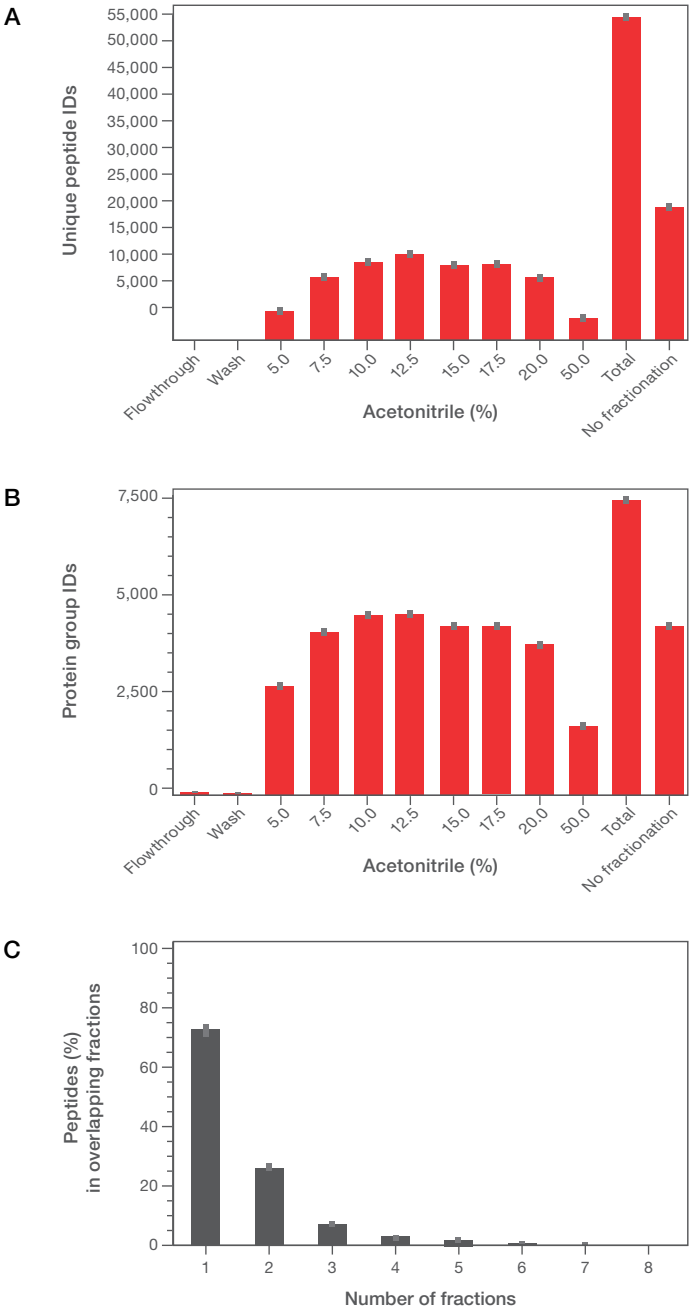


Figure 57. High-pH reversed-phase fractionation profile of 100 µg HeLa cell lysate tryptic digest. (A) Unique peptides and **(B)** protein groups identified in each elution fraction compared to the total number of identifications from a combined search of all elution fractions and a single injection of unfractionated sample. Over 100% more unique peptides and over 50% more protein groups are identified in the sample upon high-pH reversed-phase fractionation compared to analysis of a no-fractionation sample. **(C)** Excellent fractional resolution is attained, with only ~30% fractional overlap. The analysis, performed using triplicate sample sets, shows exceptional reproducibility, as indicated by the very narrow error bars.

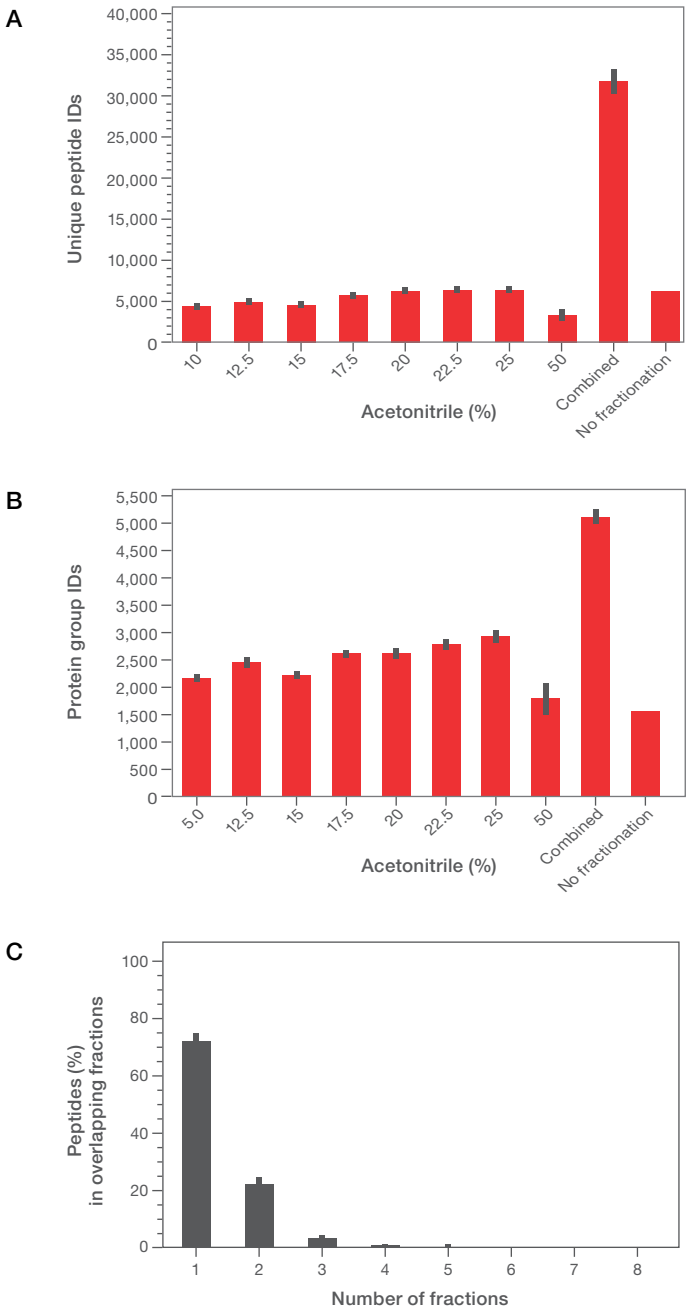


Figure 58. High-pH reversed-phase fractionation profile of 100 µg HeLa cell lysate tryptic digest labeled with TMT reagent. (A) Unique peptides and **(B)** protein groups identified in each elution fraction compared to the total number of identifications from a combined search of all elution fractions and a single injection of unfractionated sample. Over 100% more unique peptides and over 50% more protein groups are identified in the sample upon high-pH reversed-phase fractionation compared to analysis of a no-fractionation sample. **(C)** Excellent fractional resolution is attained, with only ~30% fractionation overlap. The analysis, performed using triplicate sample sets, shows exceptional reproducibility, as indicated by the very narrow error bars.

Product	Quantity	Cat. No.
Stains		
Pierce Silver Stain for Mass Spectrometry	1 L kit	24600
Imperial Protein Stain	1 L	24615
To view additional pack sizes and products, go to **thermofisher.com/proteinstains**		
Protein digestion		
Pierce Trypsin Protease, MS Grade, Frozen	100 µg	90305
Pierce Trypsin Protease, MS Grade	5 x 20 µg	90057
Pierce Trypsin Protease, MS Grade	5 x 100 µg	90058
Pierce Trypsin Protease, MS Grade	1 mg	90059
Lys-C Protease, MS Grade	20 µg	90051
Pierce LysN Protease, MS Grade	20 µg	90300
Pierce LysN Protease, MS Grade	5 x 20 µg	90301
Asp-N Protease, MS Grade	2 µg	90053
Glu-C Protease, MS Grade	5 x 10 µg	90054
Chymotrypsin Protease, MS Grade	4 x 25 µg	90056
In-Gel Tryptic Digestion Kit	Kit	89871
In-Solution Tryptic Digestion and Guanidination Kit	Kit	89895
Bond-Breaker TCEP Solution, Neutral pH	5 mL	77720
Pierce DTT (Dithiothreitol), No-Weigh Format	48 x 7.7 mg	20291
Pierce Iodoacetic Acid	500 mg	35603
Pierce Iodoacetamide, Single-Use	24 x 9.3 mg	90034
Pierce MMTS	200 mg	23011
Pierce N-Ethylmalemide (NEM)	25 g	23030
To view additional pack sizes and products, go to **thermofisher.com/msdigestion**		
Peptide enrichment and fractionation		
High-Select Fe-NTA Phosphopeptide Enrichment Kit	Kit	A32992
High-Select TiO_2 Phosphopeptide Enrichment Kit	Kit	A32993
Pierce TiO_2 Phosphopeptide Enrichment Spin Tips	96 tips	88303
Pierce Magnetic TiO_2 Phosphopeptide Enrichment Kit	96-rxn kit	88811
Pierce Magnetic TiO_2 Phosphopeptide Enrichment Kit, Trial Size	24-rxn kit	88812
Pierce High pH Reversed-Phase Peptide Fractionation Kit	12 columns	84868
Low Protein Binding Microcentrifuge Tubes, 2.0 mL	250 tubes	88379
Low Protein Binding Microcentrifuge Tubes, 2.0 mL	10 x 250 tubes	88380
To view additional pack sizes and products, go to **thermofisher.com/peptidekits**		

Product	Quantity	Cat. No.
Peptide clean-up		
Pierce C18 Spin Tips	96 tips	84850
Pierce C18 Tips, 10 µL bed	8 tips	87781
Pierce C18 Tips, 10 µL bed	96 tips	87782
Pierce C18 Tips, 100 µL bed	8 tips	87783
Pierce C18 Tips, 100 µL bed	96 tips	87784
Pierce C18 Spin Columns	25 columns	89870
Pierce C18 Spin Columns	50 columns	89873
Pierce Graphite Spin Columns	30 columns	88302
Pierce 96-Well Detergent Removal Spin Plates	2 plates	88304
Pierce Detergent Removal Spin Column, 125 µL	25 columns	87776
Pierce Detergent Removal Spin Column, 0.5 mL	25 columns	87777
Pierce Detergent Removal Spin Column, 2 mL	5 columns	87778
Pierce Detergent Removal Spin Column, 4 mL	5 columns	87779
Pierce Detergent Removal Resin	10 mL	87780
HiPPR Detergent Removal Spin Column	54 columns	88305
HiPPR Detergent Removal Spin Columns, 0.1 mL	24 columns	88306
HiPPR Detergent Removal 96-Well Spin Plates, 0.1 mL	2 plates	88307
To view additional pack sizes and products, go to **thermofisher.com/peptidecleanup**		
Peptide quantitation assays		
Pierce Quantitative Colorimetric Peptide Assay	Kit	23275
Peptide Digest Assay Standard	1.5 mL	23295
Pierce Quantitative Fluorometric Peptide Assay	500 assays	23290
96-Well Black Plate	25 pack	88378
To view additional pack sizes and products, go to **thermofisher.com/peptideassays**		