Application Note: 459

Efficient Enrichment and Identification of Cell Lysate Phosphopeptides Using an Automated, Magnetic Bead-Based System Coupled to High-Resolution LC-MS/MS and SRM

Bryan Krastins, David Sarracino, J. Rogers, P. Haney, T. Rezai, Amol Prakash, Mary F. Lopez, Thermo Fisher Scientific, Waltham, MA, USA; Michael Athanas, VAST Scientific, Cambridge, MA, USA; J. Degnore, Tufts University, Boston, MA, USA

Key Words

- KingFisher
- LTQ FT Ultra FTICR Mass Spectrometer
- Magnetic Beads
- Mass Spectrometry
- Phosphopeptide Enrichment
- Post-translational Modifications
- Selective Reaction Monitoring

Introduction

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. An efficient, automated and robust enrichment strategy in a proteomics workflow can facilitate the identification and quantification of these lower-abundance species. Previous approaches have focused on enrichment with immobilized metal ion affinity chromatography (IMAC).[1] 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 non-selective enrichment of acidic peptides.[2,3] Recently, metal oxide affinity chromatography using aluminum, titanium, zirconium and other metal oxides was successfully applied for selective enrichment of phosphopeptides.[4] We describe here an integrated, automated, high-throughput workflow using high-resolution LC-MS/MS for the selective enrichment and identification of phosphopeptides from cell lysates. Once the phosphopeptides are discovered and identified, selective reaction monitoring (SRM) assays using a triple quadrupole mass spectrometer can be developed to efficiently monitor and quantify the target peptides.[5]

Goal

The goal of this study was to simplify and automate the enrichment of phosphopeptides from cell lysates to facilitate identification and quantification by mass spectrometry.

Experimental

Cell Lysate Sample Preparation

Lymphocyte and monocyte cells (peripheral blood mononuclear cells – PBMC) were collected, prepared and isolated in cell preparation tubes (Becton Dickinson) following the product insert. Immediately after rinsing the PBMC pellet with PBS, the cells were lysed by adding 8 M GuHCl/150 mM Tris HCl/10 mM DTT pH 8.5. A probe sonicator was used briefly to break up DNA strands. Proteins were denatured by heating at 90 °C for 20 min and then incubated at 37 °C for 60 min. Iodoacetic acid was added to a final concentration of 40 mM and the lysate was incubated at room temperature in the dark for 60 min to alkylate the proteins. Subsequently, 2 M DTT was added to quench the reaction and then the PBMC sample was dialyzed against water with a Thermo Scientific 3500 MWCO Slide-a-Lyzer Dialysis Cassette. Following dialysis of reduction/alkylation solutions, the protein lysate was dialyzed against 50 mM Tris HCl/2 mM MgCl2 pH 8.0 plus 10 units of Benzonase® (EMD) (Benzonase buffer). The sample was dialyzed against the Benzonase buffer for 12-14 hours. Finally, the sample was again dialyzed against water, with at least one change for another 2-4 hours. The sample was then aliquotted into pre-weighed 1.5 mL microcentrifuge tubes and lyophilized to dryness. The microcentrifuge tubes plus dried pellets were re-weighed to calculate the dry protein weights.

Proteolytic Enzyme Digestion

Proteolytic enzyme digestion was initiated by re-suspending a 2 mg protein pellet in 500 µL of 8 M GuHCl/150 mM Tris/10 mM DTT pH 8.5. The samples were diluted to 10 mL with 50 mM Tris/5 mM CaCl2, pH 8.0 and then adding 20 µg of sequencing grade proteolytic enzyme (Pierce catalog #90055) resulting in a protein to proteolytic enzyme ratio of 1:100. The digests were incubated at 37 °C for 48 hours with shaking on an orbital shaker. The digestion reaction was quenched with the addition of 100 mL of TFA to a final concentration of 1%.

Sample Desalting Employing C18 Reverse Phase Resin

Thermo Scientific HyperSep C18 solid phase extraction media was used to desalt the digested samples prepared in the above step. A 500 mg packing volume SPE device (P/N 60108-304) was adapted for single use by centrifugation (500 xg for 3-5 min) or in a 96-well format using a plate adaptor (P/N 60300-302) and a HyperSep™ universal vacuum manifold (P/N 60104-230). In both cases the centrifugation and vacuum conditions need to be adjusted to achieve a processing time of 0.5-1 min residence time in the media bed. Long centrifugation times/high speeds or prolonged vacuum processing should be avoided to ensure that the reverse phase resin does not dry out, requiring re-wetting with organic solvent. The HyperSep C18 resin was conditioned before use; a) Fill the SPE device with acetonitrile and pass though C18 media slowly (1-2 min). Repeat two times. b) Follow with 0.25% (V/V in water) TFA to rinse the whole SPE device. Repeat two times. Load entire sample prepared as described above and process by centrifugation or vacuum to flow through the SPE media for a residence time of 0.5-1 min. Wash four times with 0.25% (V/V) TFA as described above. Elute the desalted sample with 1 mL of 80% acetonitrile/0.1% formic acid by centrifugation (single devices) or vacuum filtration.

Thermo SCIENTIFIC

Phosphopeptide Enrichment by Processing on the KingFisher Flex Purification System Using a Pierce Magnetic TiO_2 Phosphopeptide Enrichment Kit

Preparation of the KingFisher™ purification system

The Phosphopeptide Enrichment PCR Plate protocol, available for download from the Thermo Scientific KingFisher web site (www.thermo.com/kingfisher), a KingFisher Flex purification system with a PCR magnet head (Product No. 5400610) and a KingFisher Flex 96 tip comb for PCR magnets (Product No. 97002514), were used for this application.

Preparation of buffers, TiO_2 beads and enrichment on the KingFisher purification system

Binding buffer and washing buffer were prepared according to the Thermo Scientific Pierce Magnetic TiO_2 Phosphopeptide Enrichment Kit instructions (Product No. 88811). All plastic 96-well plates were rinsed three times with acetonitrile to minimize contamination from polymers leaching from the plastics.

The 20X TiO_2 magnetic beads were placed into a 15 mL polypropylene conical tube and mixed thoroughly by repeated inversion. Prepared binding buffer was added to the bead suspension (140 µL binding buffer per 10 µL of resin). Samples were then processed according to the protocol outlined in the kit and KingFisher method. After the samples were processed, the plates were removed and samples were transferred to clean 0.2 mL Thermo Scientific PCR Plate (Product No. AB1300) and lyophilized to dryness. Before LC-MS/MS analysis, samples were rehydrated in 5% (v/v) acetonitrile/1% (v/v) formic acid in water and injected directly into the mass spectrometer.

Mass Spectrometry Analysis with Ultra-High Resolution using the LTQ FT Ultra FTICR-MS

Samples in 5% (v/v) acetonitrile 1% (v/v) formic acid were injected with a Thermo Scientific Micro Autosampler onto a 75 µm x 25 cm fused silica capillary column packed with Thermo Scientific Hypersil GOLD aQ 5 µm media, in a 250 µL/min (1000:1 split to column) gradient of 5% (v/v) acetonitrile, 0.1% (v/v) formic acid to 30% (v/v) acetonitrile, 0.1% (v/v) formic acid over the course of 180 minutes with a total run length of 240 minutes. The Thermo Scientific LTQ FT Ultra hybrid mass spectrometer was run in a top 10 configuration at 200K resolution for a full scan, with monoisotopic precursor selection enabled, and +1, and unassigned charge state rejected. The analysis on the LTQ FT Ultra™ instrument was carried out with CID fragmentation.

Quantitative SRM Assay on the TSQ Quantum Ultra triple Quadrupole in Microspray Mode

The Thermo Scientific TSQ Quantum Ultra triple quadrupole mass spectrometer was equipped with a Thermo Scientific Surveyor MS pump, and Micro Autosampler (flow split pre-autosampler as used in the discovery instrument format). The source was the Thermo Scientific Ion Max source equipped with a low-flow metal needle, a 50 mm x 1 mm Hypersil GOLD aQ™ column (1.9 µm particle size) (part number 25302-051030). Solvent A was Fisher Optima LC-MS grade water with 0.2% (v/v) formic acid, and solvent B was Fisher Optima LC-MS grade acetonitrile with 0.2% (v/v) formic acid. Total separation time was 15 min.

Results and Discussion

Phosphopeptides were enriched from a cell lysate using an integrated, pre-analytical sample prep workflow. An outline of the workflow that incorporates automated phosphopeptide capture with magnetic beads is shown in Figure 1. Using the described workflow on a lysate of peripheral blood mononuclear cells (PBMC's), the result was a significant enrichment (86%) of the targeted peptides.

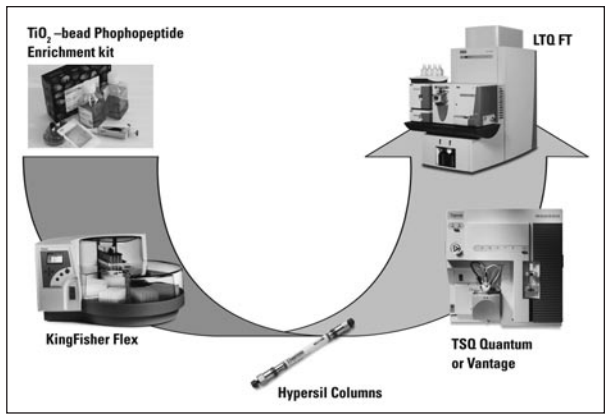


Figure 1: Integrated workflow for automated phosphopeptide enrichment and identification followed by SRM targeted assay development

A summary of the data acquired on the LTQ FT Ultra mass spectrometer is shown in Table 1. Figure 2A shows an example of a base peak chromatogram acquired at a resolution of 200 K with a mass accuracy of <2 ppm. Figure 2B is a zoom of the retention time 64.50 min. Examples of MS/MS spectra from two separate phosphopeptide precursor ions are shown in Figure 3A and B. In summary, a total of 181 unique phosphopeptides in 160 unique phosphoproteins were identified in the enriched fraction versus 1 unique phoshopeptide in the un-enriched fraction (Table 1).

Result	Enriched	Not Enriched
Total number of proteins identified	185	247
Total number of phosphoproteins identified	160	1
Total number of peptides identified	2347	2457
Total number of phosphopeptides identified	2009	7
Total number of unique phosphopeptides	181	1
Relative enrichment for phosphopeptides (%)	86	0.3

Table 1: Automated enrichment of phosphopeptides from blood lymphocytes using Pierce TiO_2 phosphopeptide enrichment kit and KingFisher purification system. Data acquired on an LTQ FT Ultra hybrid mass spectrometer. Starting material was 2 mg of total lymphocyte proteolytic enzyme peptide digest.

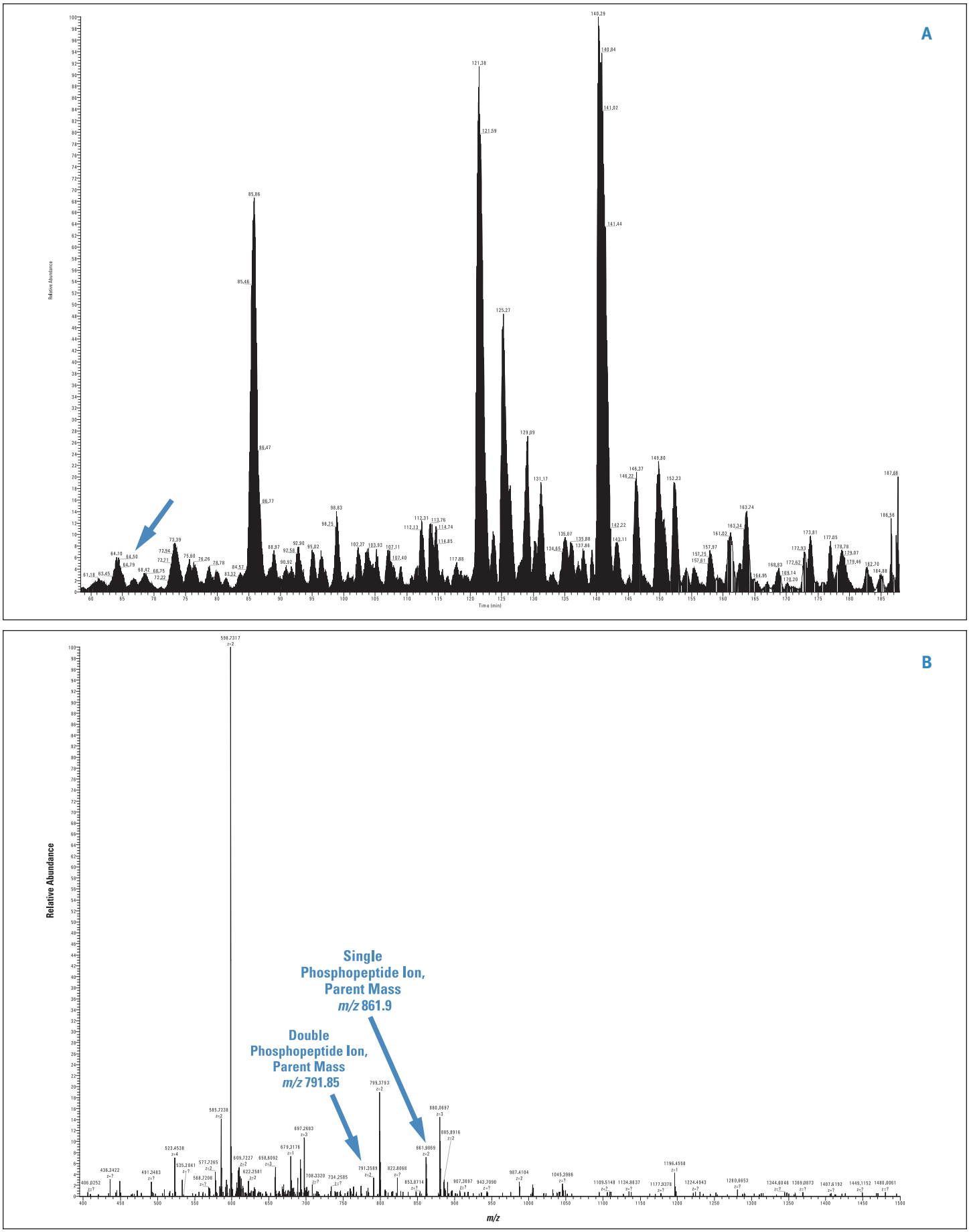
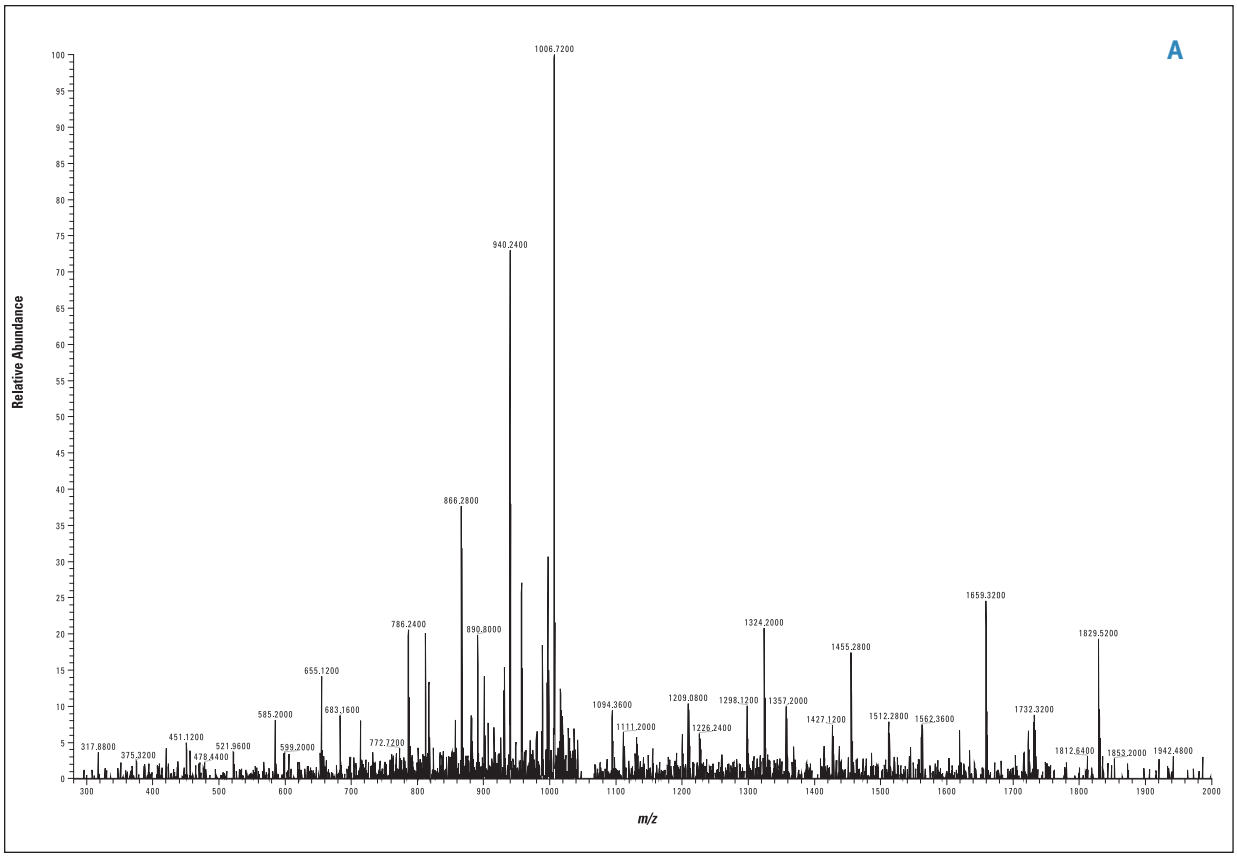


Figure 2: High-resolution LC-MS/MS data of phosphopeptides enriched from a cell lysate using the TiO_2 magnetic beads on the KingFisher Flex automated workflow platform. Panel A: Base peak chromatogram on the LTQ FT Ultra hybrid mass spectrometer. Panel B: Zoom of the full scan mass spectrum in (A) at retention time 64.50 min.



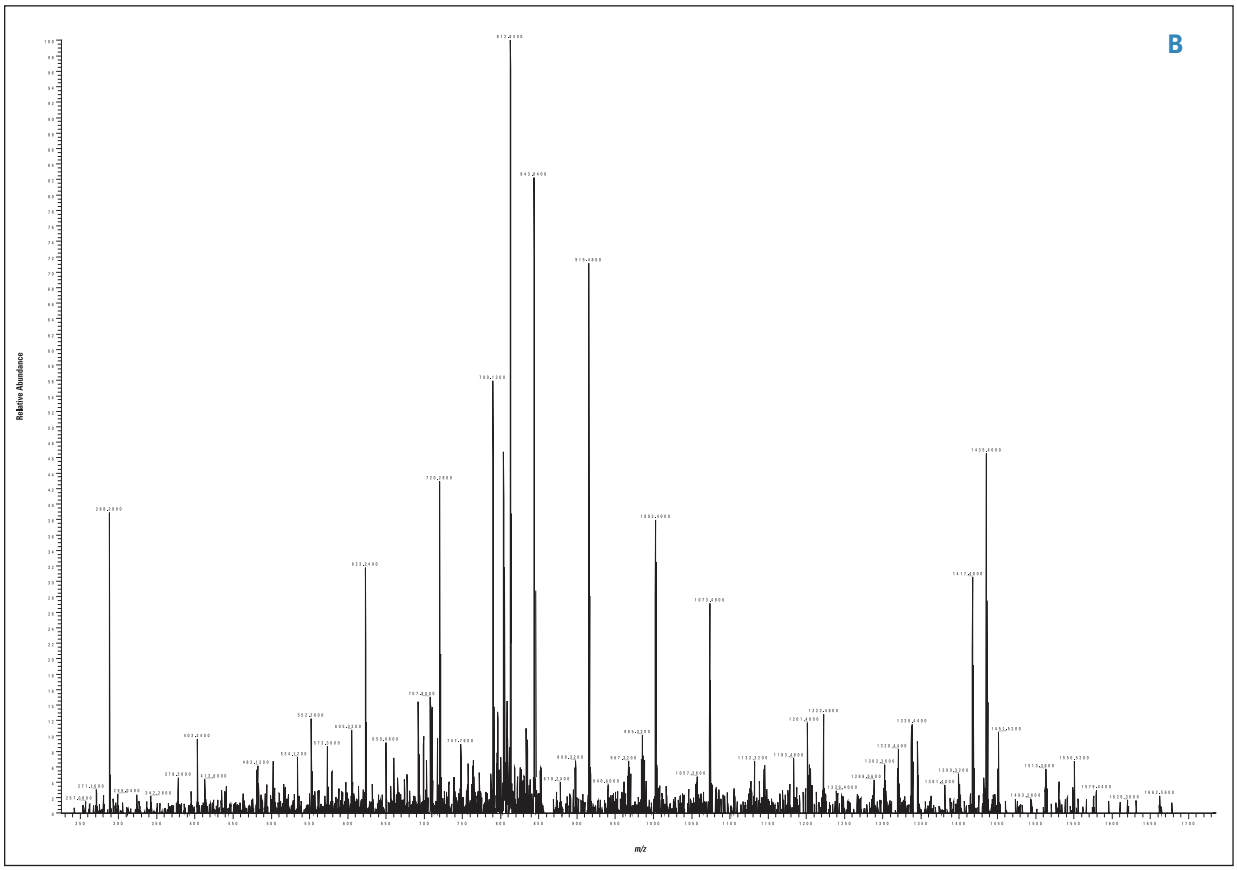


Figure 3: MS/MS fragmentation spectrum of phosphopeptide ions. Panel A: Double phosphopeptide ion, parent mass *m/z* 791.85.
Panel B: Single phosphopeptide ion, parent mass *m/z* 861.9.

Recently, emphasis has been placed on advancing proteomics studies from qualitative discovery experiments to quantitative assays that may be useful in a clinical research environment. Targeted selected-reaction monitoring (SRM) assays using triple quadrupole mass spectrometers provide a vehicle for cost effective, high-throughput quantification and monitoring of specific targeted disease biomarkers.

The phosphopeptide enrichment strategy outlined in this report, is useful for the identification of putative phosphorylated biomarkers. Subsequent to biomarker identification, targeted SRM assays can be developed for specific target peptides. An example of this strategy is shown in Figure 4. An SRM assay was developed to monitor synthetic phosphopeptide FLpSQDAPTVK with *m/z* 593.282 spiked into a background of PBMC cell lysate matrix (Figure 5). Six transitions (fragment ions) were monitored to ensure selectivity for the correct peptide sequence. In a neat background (no matrix), the level of detection (LOD) for the target peptide was 100 attomoles on column and the accurate level of quantification (LOQ) was 250 attomole on column. The SRM assay provided a rapid (15 min) quantitative, sensitive and selective method to monitor the target phosphopeptide.

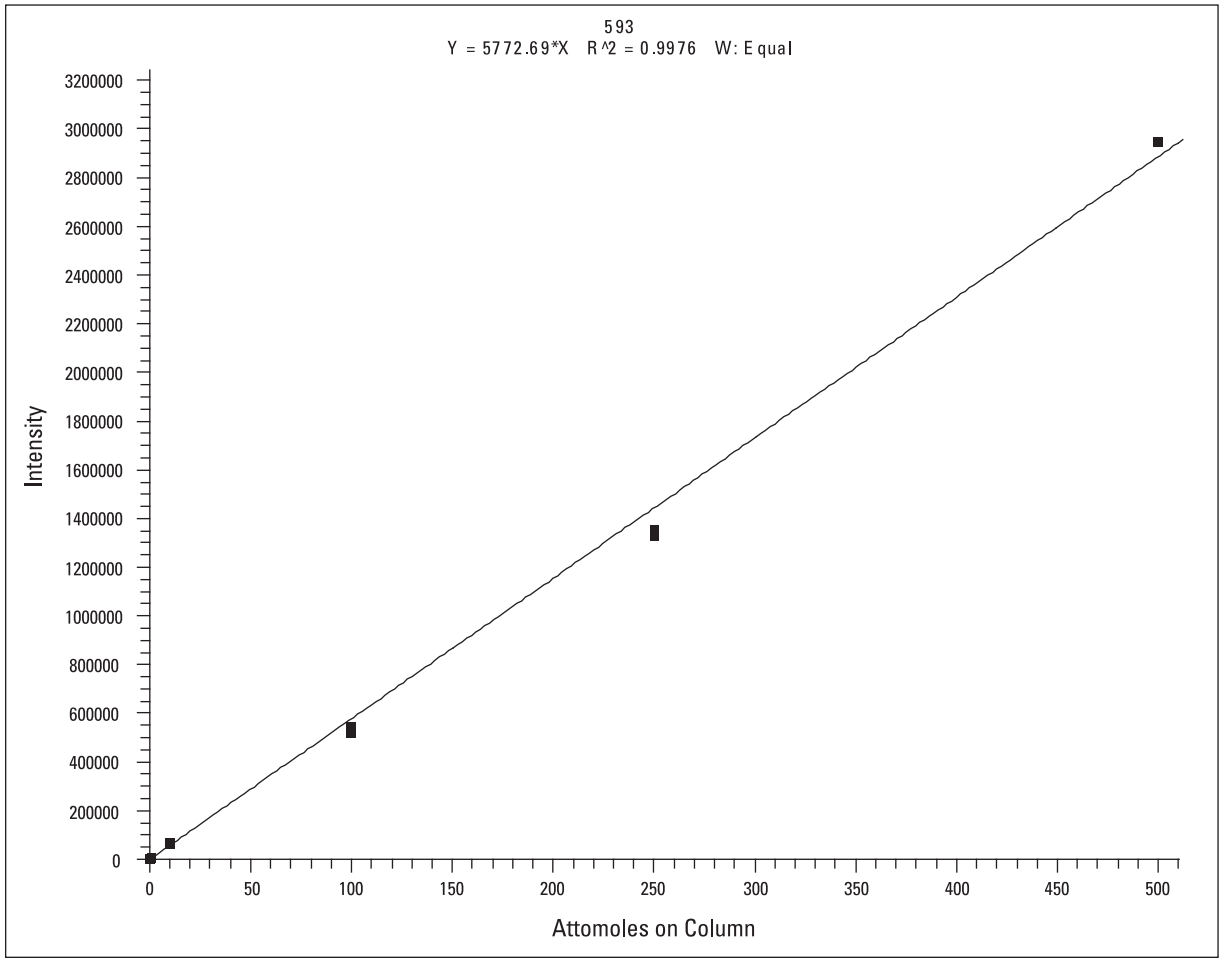


Figure 4: Neat calibration curve of synthetic phosphopeptide FLpSQDAPTVK. Detection was linear over the range 1 picomole – 100 attomoles on column with a correlation factor of 0.9976. LOD was 100 amol and LOQ was 250 amol on column.

Conclusion

An automated, high-throughput workflow for the enrichment of phosphopeptides from cell lysates was developed. Accurate measurement of phosphorylated proteins and peptides is crucially important due to their central role in cell signaling pathways. Unfortunately, phosphopeptides are typically present at very low levels in cell lysates. An efficient and robust pre-analytical enrichment step for phosphopeptides facilitates the understanding of these fundamental biological pathways. The enrichment of phosphopeptides described in this report using magnetic TiO_2 beads was comprehensive and selective. The described workflow enabled the discovery and identification of phosphopeptides in complex samples with the subsequent development of targeted SRM-based assays that may be useful for clinical research applications.

References and Acknowledgements

1. J. Porath, J. Carlsson, J. Olsson, and G. Belfrage. Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature* 258: 598 (**1975**).
2. Claire E. Haydon, Patrick A. Eyers, Lauren D. Aveline-Wolf, Katheryn A. Resing, James L. Maller and Natalie G. Ahn. Identification of novel phosphorylation sites on Xenopus laevis Aurora A and analysis of phosphopeptide enrichment by immobilized metal-affinity chromatography. *Mol. Cell. Proteomics* 2: 1055 (**2003**).
3. Cirulli C, Chiappetta G, Marino G, Mauri P, and Amoresano A. Identification of free phosphopeptides in different biological fluids by a mass spectrometry approach. *Anal Bioanal. Chem.* 392(1-2):147-59 (**2008**).
4. M. R. Larsen, T. E. Thingholm, O. N. Jensen, P. Roepstorff, and T. J. D. Jørgensen. Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide micro-columns. *Mol. Cell. Proteomics* 4: 873 (**2005**).
5. Hasmik Keshishian, Terri Addona, Michael Burgess, Eric Kuhn, and Steven A. Carr. Quantitative, Multiplexed Assays for Low Abundance Proteins in Plasma by Targeted Mass Spectrometry and Stable Isotope Dilution. *Molecular & Cellular Proteomics* 6.12:2213-2229 (**2007**).

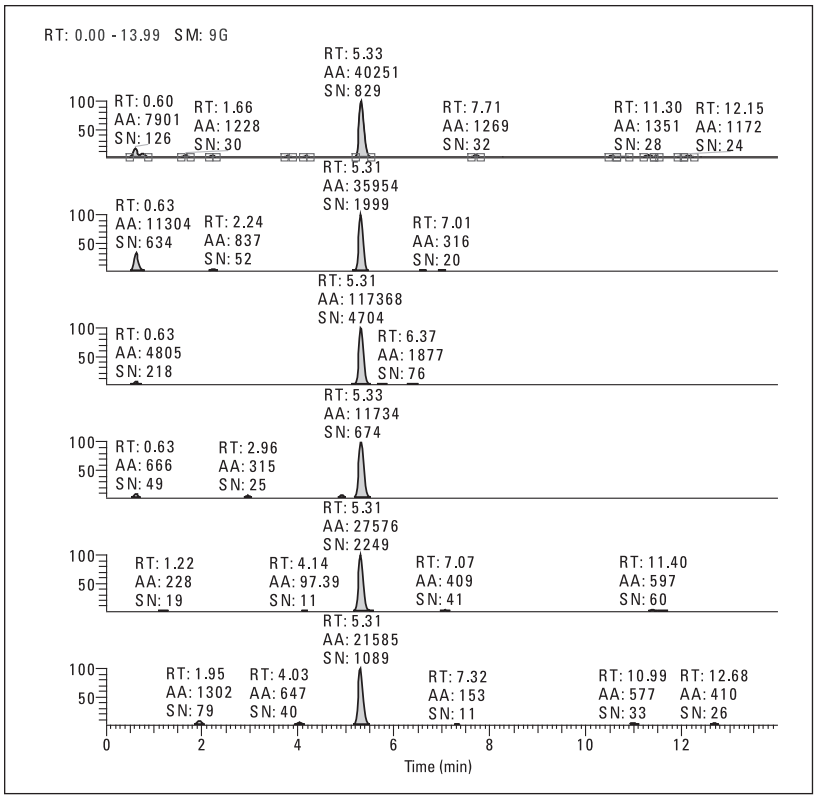


Figure 5: SRM of phosphopeptide FLS#QDAPTVK. Precursor ion *m/z* 593.282, Six transitions were monitored. Data were obtained on Quantum Ultra triple quadrupole in microspray mode, 50 mm × 1 mm Hypersil GOLD aQ column, flow rate 5 mL/min, 15 min total run time.

***View additional Thermo Scientific LC/MS application notes at:* www.thermo.com/appnotes**

In addition to these offices, Thermo Fisher Scientific maintains a network of representative organizations throughout the world.

Africa-Other
+27 11 570 1840
Australia
+61 2 8844 9500
Austria
+43 1 333 50 34 0
Belgium
+32 2 482 30 30
Canada
+1 800 530 8447
China
+86 10 8419 3588
Denmark
+45 70 23 62 60
Europe-Other
+43 1 333 50 34 0
Finland/Norway/ Sweden
+46 8 556 468 00
France
+33 1 60 92 48 00
Germany
+49 6103 408 1014
India
+91 22 6742 9434
Italy
+39 02 950 591
Japan
+81 45 453 9100
Latin America
+1 608 276 5659
Middle East
+43 1 333 50 34 0
Netherlands
+31 76 579 55 55
South Africa
+27 11 570 1840
Spain
+34 914 845 965
Switzerland
+41 61 716 77 00
UK
+44 1442 233555
USA
+1 800 532 4752

www.thermo.com

Thermo Fisher Scientific, San Jose, CA USA is ISO Certified.

AN63050_E 06/09M