Facilitating Phosphopeptide Analysis Using the Agilent HPLC Phosphochip

Application Note

Proteomics

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Abstract

The analysis of phosphopeptides can be particularly challenging since they are mostly present at very low concentrations in a complex proteolytic sample. Selective enrichment strategies have been introduced to capture phosphopeptides from the sample prior to MS analysis, which can enhance detection capabilities. One of the more successful phosphopeptide enrichment techniques is based on the selective capture of phosphopeptides using titanium dioxide (TiO_2) packing material. Although successful, these enrichment strategies still face challenges with regards to automation, robustness, reliability and reproducibility. The commercially available Agilent HPLC-Chip/MS provides a platform that can integrate an enrichment column, analytical column, associated connection capillaries, and a nanospray emitter directly on a single, small, reusable microfluidic chip. It also provides an easy to use, robust and reliable nanospray LC/MS platform when compared to conventional nanocolumn LC/MS technology. Integration of enrichment columns with different stationary phase materials on the chip can be used to selectively capture phosphopeptides during the sample loading process. In this Application Note, we will describe the design and performance of an HPLC-Chip with a hybrid TiO_2 and C18 enrichment column for selective phosphopeptide enrichment and analysis.

Agilent Technologies

Introduction

Protein phosphorylation is one of the most important post-translational modification (PTM) mechanisms for regulating protein function in cells. Myriad biological processes, including cell proliferation, migration, and apoptosis involve phosphorylation steps.[1–3] One of the major efforts in proteomics is devoted to the identification and understanding of phosphoproteomes in cells. Nevertheless, comprehensive identification of sites of protein phosphorylation remains a challenge, best left to experienced proteomics experts. Multidimensional separation techniques coupling reversed phase (RP) with strong cation exchange (SCX) or a strong anion exchange (SAX) step prior to liquid chromatography mass spectrometry (LC/MS) analysis is necessary to achieve more sensitive results for complex mixtures. Furthermore, selective enrichment of phosphorylated proteins and peptides is necessary to get better phosphoproteome coverage. Commonly used phosphopeptide enrichment technologies are immobilized metal affinity chromatography (IMAC), metal oxides of titanium, aluminum, or zirconium, and anti-phosphotyrosine antibodies. Although not completely overlapping, the titanium dioxide based approach is a particularly robust and automatable method with a high affinity for phosphopeptides.[4,5] However, multiple valve and micro-capillary connections create challenges in reliability, robustness, and reproducibility. Automation of the enrichment step and analytical separation is the best way to achieve run-to-run and lab-to-lab reproducibility.

Recent advances in HPLC-Chip/MS technology have allowed the automation of such a workflow, increasing ease of use and confidence of analysis. The new microfluidic chip, Phosphochip, is a unique, re-usable HPLC-Chip specifically designed for phosphopeptide enrichment and analysis.[6] The chip is a multilayer polyimide laminate that contains an enrichment section with titanium dioxide (TiO_2) beads flanked on both sides with C18 reversed phase material. (Figure 1) The three sections in the sandwich enrichment column are separated from each other by micro-fabricated frits. This enrichment section is connected to a reversed phase separation column ending in an integrated electro-spray tip. Through the chip cube, the HPLC-Chip/MS interface, a micro valve is connected directly with the chip surface. This creates a zero dead volume high pressure seal and the ultimate automation for sample loading, desalting, elution from trap column and gradient separation on the analytical column.

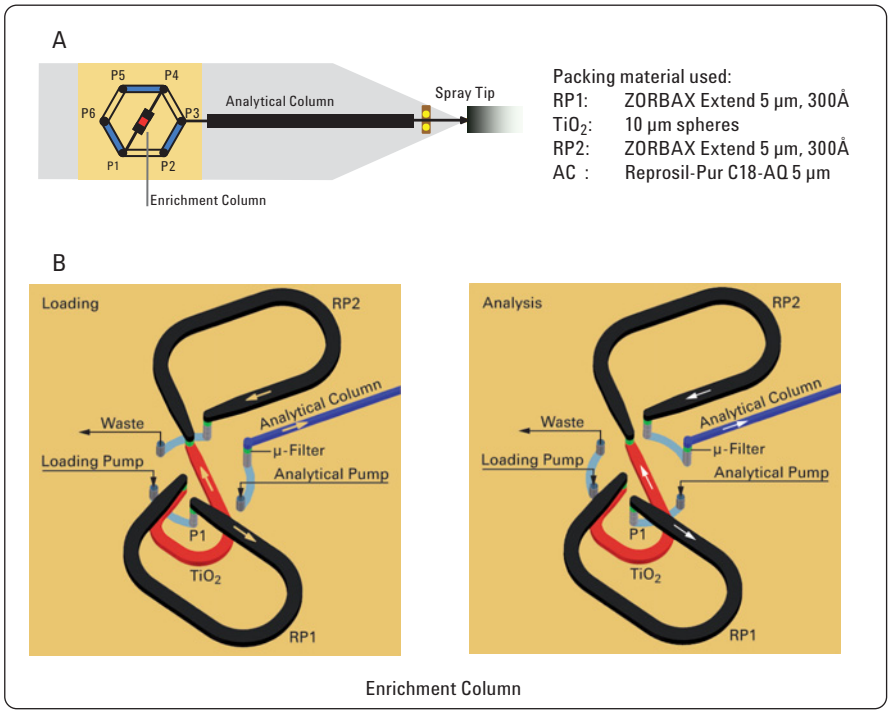

Figure 1
The unique design for Phosphochip. The Phosphochip (A) is a multi-layer microfluidic HPLC-Chip that features a sandwiched RP-TiO_2-RP trapping column. (B) In the enrichment column, RP is illustrated in black; TiO_2 is illustrated in red; the figure on the left illustrates the loading position for peptide enrichment and the figure on the right shows the separation of peptides through analytical column (please see next page on operation related to phosphopeptide enrichment and separation).

Experimental

Out-of-box Solution

The Phosphochip kit (G4240-62020) is a complete solution for phosphopeptide analysis on any Agilent HPLC-Chip/MS setup. This could be a Chip/Ion Trap, Chip/QQQ or Chip/QTOF. Conditioning of the HPLC system is required in order to saturate all potential phosphopeptide binding sites in the system. A conditioning and standard mix is provided in the reagent pack for this purpose. It is also recommended to replace capillaries to and from the inline filter with polyaryletheretherketone (PEEK) tubing from the kit for operation at high pH. The unique sandwiched enrichment column with RP1-TiO_2-RP2 packings, allows the trapping and analysis of both phosphorylated and non-phosphorylated peptides in one experiment. The micro valve connection on the chip allows the flow paths to be switched. The chip can be operated in two different modes for phosphopeptide enrichment. In the first mode, for phosphopeptide analysis only, most peptides will be trapped on RP1. The elution of peptides with an organic plug pushing all through and onto the TiO_2 phase, allows the enrichment of phosphopeptides, with unbounded peptides going to waste. Phosphopeptides are then desorbed with a special elution buffer and trapped on RP2. Finally, the gradient elution of phosphopeptides occurs on the analytical column. In dual mode analysis, a gradient elution of non-phosphorylated peptides is carried out instead of an organic plug and flow is directed through the analytical column. For more out-of-box operation of the Phosphochip, please refer to the User's Guide (G4240-90005).

Materials and Methods

Reference protein mixture
Three proteins, bovine serum albumin and bovine alpha and beta casein, were digested and combined to generate a protein reference mixture. Each was dissolved briefly in 1 M urea and 50 mM ammonium bicarbonate. The proteins were reduced in 1 mM dithiothreitol (DTT) and alkylated in 2 mM iodoacetamide, followed by digestion with trypsin overnight at a protein/protease ratio of 50:1. The final mixture was prepared for analysis by diluting the protein digests in 10% formic acid to a final concentration of 20 fmoL/µL.

Human cell lysate
Human U2OS osteosarcoma cells were resuspended in lysis buffer with phosphatase inhibitors (8 M urea, 50 mM NH_4HCO_3, 1 mM KF, 5 mM NaH_2PO_4, 1 mM Na_2VO_4), sonicated 2 times for 20 s each, then centrifuged at 13000 g and 4 ºC for 10 minutes to remove insoluble material. Lysate proteins were reduced with 1,4-dithiothreitol (10 mM) and alkylated with iodoacetamide reagent (20 mM). The sample was then diluted to 2 M of urea with lysis buffer lacking the urea, and 10 µg of trypsin was added. The sample was incubated for 4 hours at 37 ºC. Next, the sample was further diluted to 1 M of urea, another 10 ug of trypsin was added and the sample was incubated overnight at 37 ºC. The resulting peptides were separated by SCX chromatography and the main singly charged peptide fraction, known to also contain phosphopeptides, was further analyzed by LC/MS/MS.

SCX fractionation
SCX was performed using an Agilent 1200 Series LC system with two C18 Opti-Lynx (Optimized Technologies, Oregon City, OR) guard columns and a polysulfoethyl A SCX column (PolyLC, Columbia, MD; 200 mm × 2.1 mm inner diameter, 5 µm, 200Å). The digested cell lysate was dissolved in 0.05% formic acid and loaded onto the guard column at 100 µL/min and subsequently eluted onto the SCX column with 80% acetonitrile (ACN) and 0.05% formic acid. Peptides were fractionated using a multistage gradient of buffer A (5 mM KH_2PO_4, 30% ACN and 0.05% FA, pH 2.7) and buffer B (350 mM KCl, 5 mM KH_2PO_4, 30% ACN and 0.05% FA, pH 2.7), and 1-minute fractions were collected.

HPLC-Chip/MS
All LC/MS/MS experiments were performed using an Agilent 1200 Series HPLC-Chip/MS system connected to an Agilent 6520 Q-TOF mass spectrometer according to the Phosphochip User's Guide. Samples were analyzed using either a "regular" C18 HPLC-Chip (15 cm, 75 µm Reprosil C18 analytical column) or a Phosphochip, operated in dual mode, using 2-hour multi-step gradients of buffer A (0.6% HAc, 0.1% FA) and buffer B (0.6% HAc, 0.1% FA, 80% ACN). Per selected precursor (minimum intensity 2500), two MS2 spectra were generated with an acquisition time of 1000 ms, with a 60 second dynamic exclusion.

Data analysis
Resulting MS2 spectra were searched using Spectrum Mill against the IPI human database (v3.37). MS2 spectra from the same precursor mass were merged within a range of 1.4 *m/z* units and a retention time of 15 seconds. Variable modifications were the oxidation of methionine and the phosphorylation of serine, threonine and tyrosine. Precursor and product mass tolerance were both set at 50 ppm. Trypsin was chosen as the enzyme, with a maximum of 3 missed cleavages.

Results and discussion

Validation of phosphopeptide enrichment
According to the aforementioned protocol, 50 fmol of a trypsin digested mixture of the bovine proteins serum albumin (BSA), alpha-casein, and beta-

casein were applied, to validate the phosphopeptide enrichment of the Phosphochip. The majority of the peptides from the mixture were retained in the flowthrough fraction of the Phosphochip, while only a small number of peptides were detected in the Phosphochip elution. This is clear from the base peak chromatograms of the Phosphochip flowthrough and elution fractions (Figure 2). When examining specific peptides, all the casein derived phosphopeptides that were formed were detected exclusively in the elution fraction and not in the flowthrough (Figure 2). Comparison with a "regular" HPLC-Chip suggested near complete binding and recovery of the phosphopeptides with the TiO_2 based chip.[5] The majority of the non-phosphorylated peptides were observed only in the flowthrough of the Phosphochip and not in the elution (Figure 2).

Comparison between a regular HPLC-Chip and the Phosphochip

Since biological samples are much more complex in composition and can contain a large variety of different phosphopeptides, prefractionation of the peptides is required before the samples can be efficiently analyzed by LC/MS/MS. One common approach for prefractionation is strong cation exchange (SCX), which can separate peptides based on their net charge.[5-8] Tryptic peptides most often have two basic sites, the N-terminus of the peptide and the C-terminal lysine or arginine residue required for tryptic cleavage, resulting in a 2+ net charge. Phosphopeptides, in contrast, will mainly have a single net charge as the phosphorylated residue, which is negatively charged compensates for one positive charge.

Although this strategy enriches phosphopeptides into a few SCX fractions, the same fractions can also contain other peptides with a net single charge.

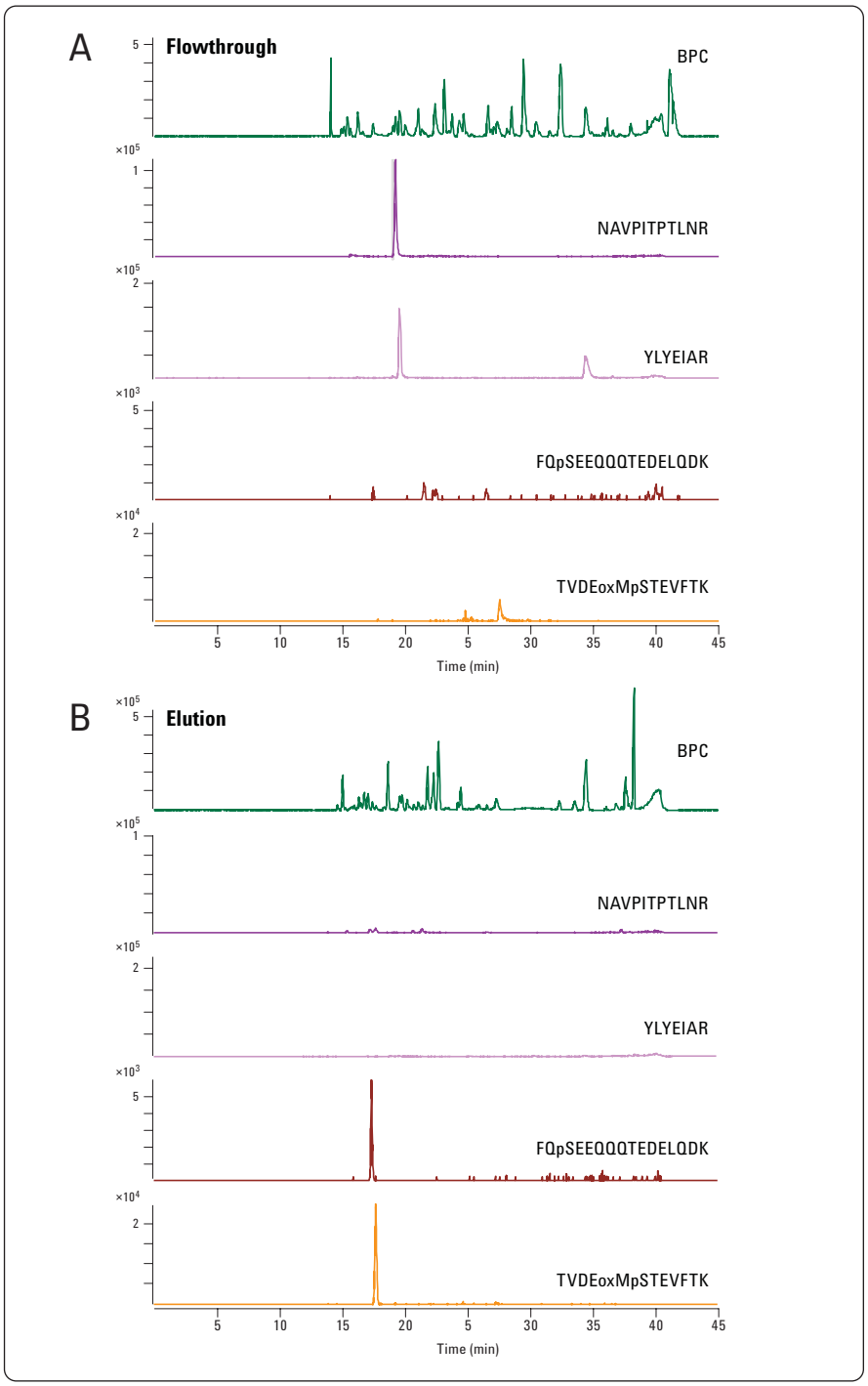

Figure 2

Enrichment of phosphopeptides from a standard protein mixture. (A) Base peak chromatograms (BPC) of the Phosphochip flowthrough and extracted ion chromatograms for two non-phosphorylated peptides (NAVPITPTLNR and YLYEIAR) and two phosphorylated peptides (FQpSEEQQQTEDELQDK and TVDEoxMpSTEVFTK) following analysis of a protein reference mixture. (B) identical chromatograms (note; equal y-axis) for the Phosphochip elution, revealing that the phosphopeptides are specifically recovered in the elution analysis.

This includes acetylated peptides and peptides derived from protein C-termini, as well as the many other cellular compounds that can carry a single charge, all of which often are much more abundant than the phosphorylated peptides of interest. Protein derived from a human osteosarcoma cell line (U2OS) was digested with trypsin to show the enrichment improvement that can be achieved on such samples using the Phosphochip. The resulting peptides were separated by SCX. A single SCX fraction expected to contain the majority of peptides with a net single charge was analyzed both with a regular HPLC Chip and with the Phosphochip (200 µg each).

As can be seen in Figure 3, the vast majority of the sample was again retained in the flowthrough of the Phosphochip. It is noteworthy that a large amount of the total sample (over 90% of the total combined signal) is observed in the flowthrough analysis on the TiO_2 chip. The subsequent analysis of the LC/MS/MS experiment using the Spectrum Mill search engine on the human IPI database allowed the identification of 109 unique phosphopeptides in the elution fraction of the Phosphochip with a Spectrum Mill score of at least 9. In contrast, only 11 phosphopeptides were identified when

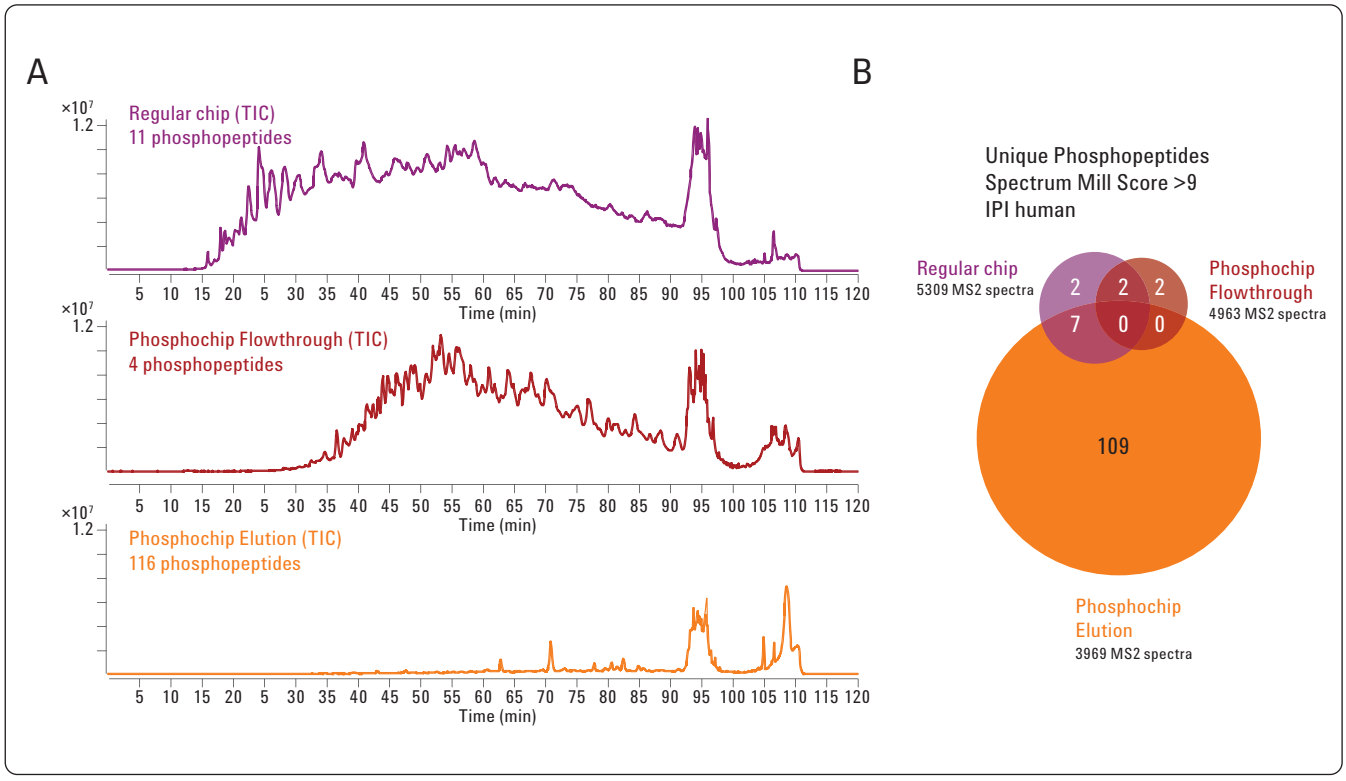

Figure 3

Comparison of phosphopeptide analysis on a regular HPLC-Chip and a Phosphochip. (A) Total ion current chromatograms of the U2OS singly charged peptide SCX fraction analyzed on a regular HPLC-Chip (top, purple) and on a Phosphochip (flowthrough in red, elution in orange). (B) The number of unique phosphopeptides identified with Spectrum Mill in the LC/MS/MS runs on the regular HPLC-Chip and in both the flowthrough and elution of the Phosphochip, as shown in panel A. Indicated are the total number of MS2 spectra searched and the scoring and database used.

the same sample was analyzed with a regular C18 HPLC-Chip. As expected, the major non-phosphopeptide components hindered phosphopeptide identification for the sample without the enrichment step.

Large scale identification of phosphopeptides from human cells

A common use of TiO_2 enrichment and SCX is global phosphoproteome characterizations. Often such experiments are performed using milligrams of material in order to gain access to phosphopeptides present at low levels.[7, 10, 11] Since it was clear that significantly more material could be analyzed by the TiO_2 based HPLC-Chip, the sample amount was increased by 5 times for easier identification of phosphopeptides. One milligram of protein was digested and the resulting peptides were separated by SCX chromatography. The full enriched singly charged fraction was brought on to a Phosphochip, allowing 5 times more material to be analyzed. From the TIC chromatograms shown in Figure 4, it is clear that significantly more material was observed in the Phosphochip elution. Equally apparent is the massive signal observed in the flowthrough analysis. Analysis of the LC/MS/MS results using Spectrum Mill allowed the identification of over 500 distinct phosphopeptides in the elution fraction using conservative parameters, while only 12 phosphopeptides were identified in the flowthrough analysis.[12] This scale of phosphopeptide identifications is quite similar to most recent global phosphoproteome analyses.[7, 9]

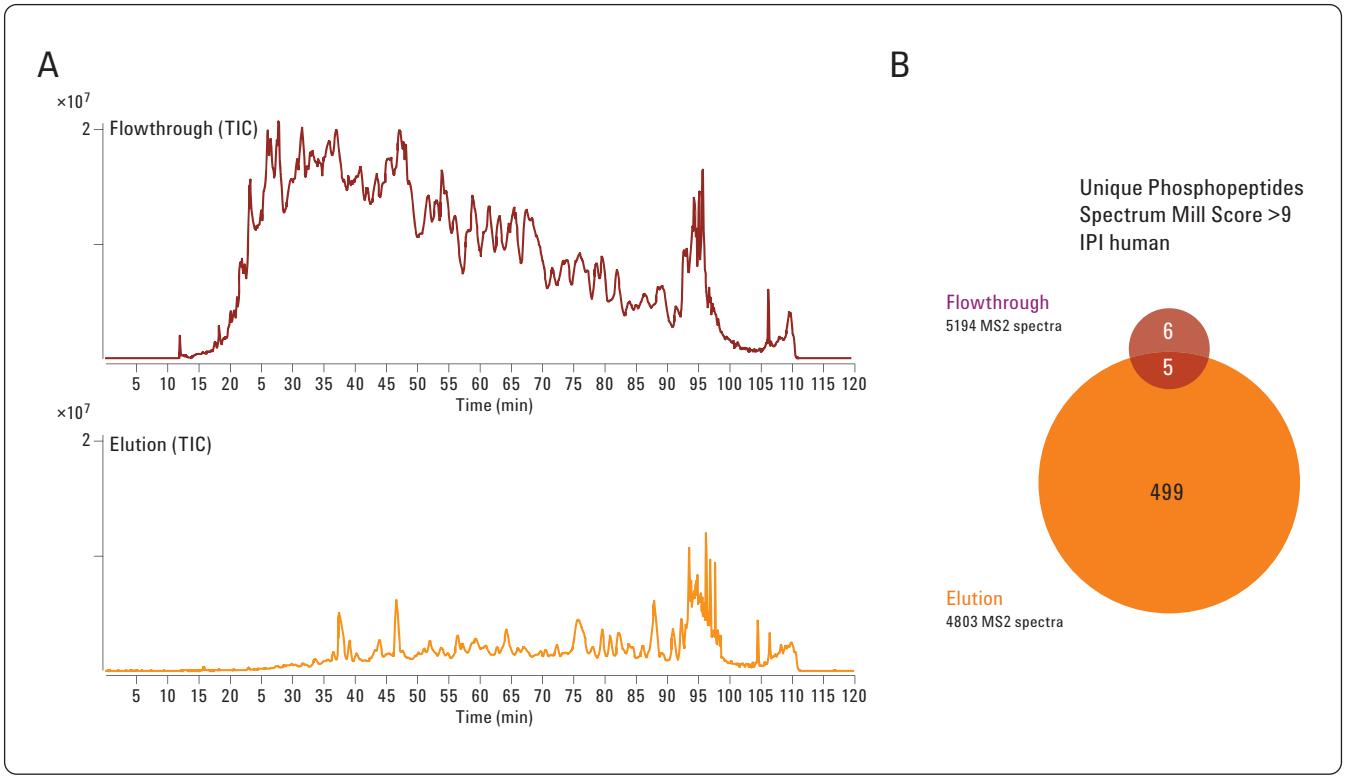

Figure 4

Large scale phosphopeptide enrichment from human cells. (A) Base peak chromatograms of the flowthrough (red) and elution (orange) from the large scale analysis of the U2OS singly charged peptide SCX fraction on the Phosphochip. (B) The number of unique phosphopeptides identified with Spectrum Mill from both the flowthrough and elution LC/MS/MS runs, shown in the panel A. Also indicated are the total number of MS2 spectra searched and the scoring and database used.

Phosphopeptide fragmentation and identification from Phosphochip elution fractions

The combination of phosphopeptide enrichment with a Q-TOF mass spectrometer has the advantage of high mass accuracy in fragmentation spectra, allowing the reliable identification of phosphopeptides, particularly when the fragmentation of the peptides is suboptimal. Phosphopeptide fragmentation spectra are generally difficult to interpret as the loss of the phosphate group is a common event during fragmentation. This leads to low intensity of the product ions deriving backbone fragmentation, which are required for identification. Figure 5 shows various phosphopeptide fragmentation spectra with their unambiguous assignment to phosphorylated human peptides, as obtained for peptides originating from the U2OS osteosarcoma cell line.

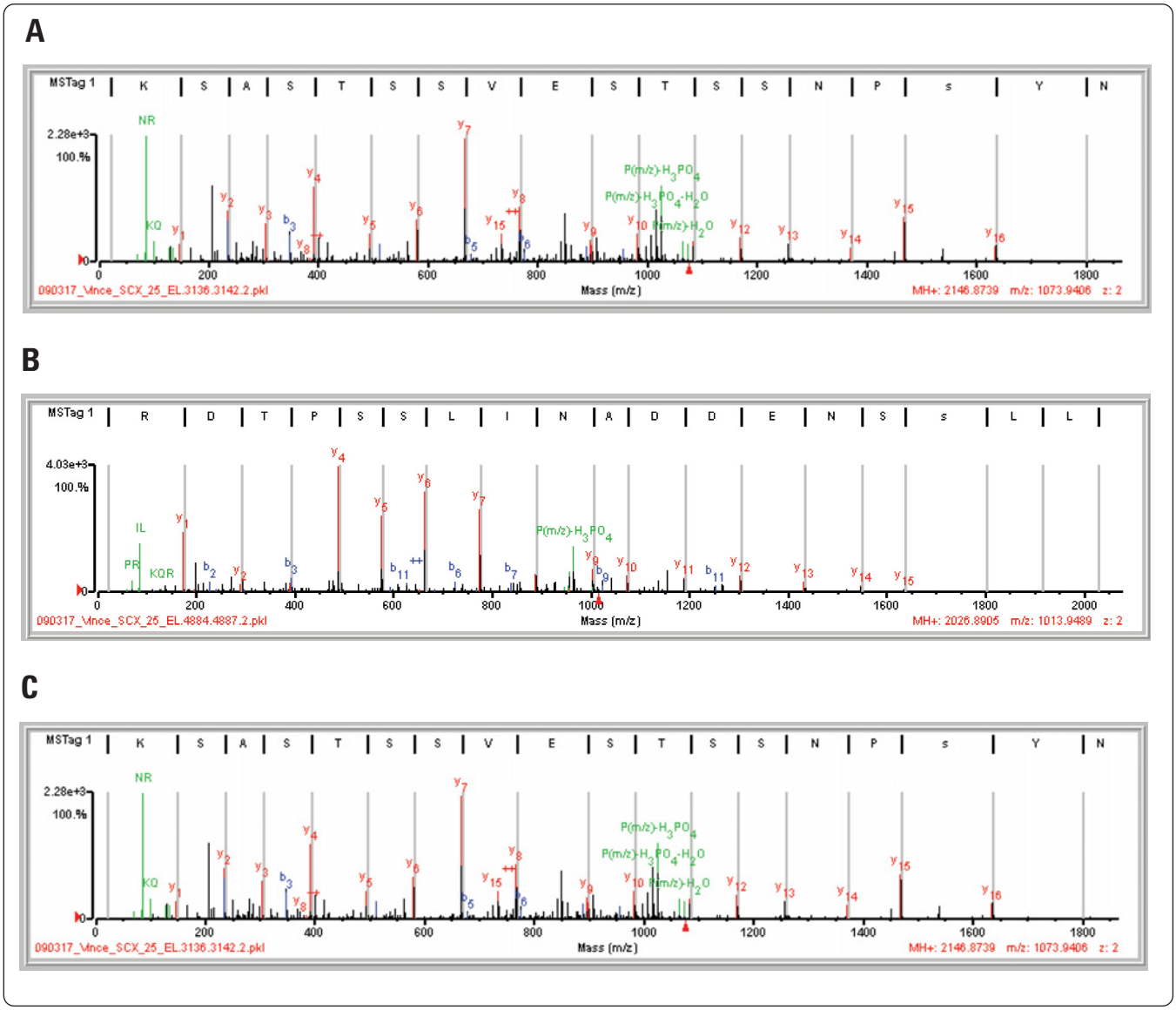

Figure 5

Fragmentation and identification of phosphopeptides from a human osteosarcoma cell line. Shown are the annotated fragmentation spectra of four phosphopeptides from the elution fraction of the Phosphochip analysis of net singly charged peptides from the human osteosarcoma cell line U2OS.

Conclusion

These experiments demonstrated the efficiency of phosphopeptide enrichment and analysis on a microfluidic HPLC-Chip, the Phosphochip. The Phosphochip workflow combines Agilent 1200 Nano and Capillary LC, HPLC-Chip/MS interface, and any Agilent 6000 Series mass spectrometer. This ultimate ease-of-use tool will help researchers studying phosphorylation achieve an increase in unique phosphopeptide identification from complex mixtures.

Ordering Information

	Product number
Phosphochip kit Includes Phosphochip, Reagent Pack (400-600, with ready-to-use Elution Buffer and Regeneration Mix and lyophilized Conditioning/Standard Mix)	G4240-62020
Phosphochip only (Orderable beginning of 2010)	G4240-62021
Reagent Pack only	400-600
PEEK sample transfer capillary (25 µm x 100 cm)	G4240-87309
PEEK capillary for use with micro inline filter (25 µm x 10 cm)	G4240-87310
Chip care and use sheet	G4240-90001
Phosphochip User's Guide	G4240-90005

References

1.
O. N. Jensen, "Interpreting the Protein Language Using Proteomics," *Nat. Rev. Mol. Cell Biol*. 2006, 7 (6), 391-403.

2.
G. Manning, et al. "The Protein Kinase Complement of the Human Genome," Science 2002, 298 (5600), 1912.

3.
M. Mann, O. N. Jensen, "Proteomic Analysis of Post-Translational Modifications," *Nat. Biotechnol*. 2003, 21 (3), 255-261.

4.
M. W. Pinkse, et al., "Selective Isolation at the Femtomole Level of Phosphopeptides from Proteolytic Digests Using 2D-NanoLC-ESI-MS/MS and Titanium Oxide Precolumns," *Anal Chem*, 2004. 76(14): 3935-43.

5.
M. W. Pinkse, et al., "Highly Robust, Automated, and Sensitive Online TiO_2-Based Phosphoproteomics Applied to Study Endogenous Phosphorylation in Drosophila Melanogaster," *J Proteome Res*, 2008. 7(2): 687-97.

6.
S. Mohammed, et al., "Chip-Based Enrichment and NanoLC/MS/MS Analysis of Phosphopeptides from Whole Lysates," *J Proteome Res*, 2008. 7(4): 1565-71.

7.
S. Lemeer, et al., "Comparative Phosphoproteomics of Zebrafish Fyn/Yes Morpholino Knockdown Embryos," *Mol Cell Proteomics*, 2008. 7(11): 2176-87.

8.
J. Peng, J. E. Elias, C. C. Thoreen, L. J. Licklider, and S. P. Gygi, "Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: the yeast proteome" *J Proteome Res*, 2003. 2(1): 43–50.

9.
J. Villen, and S. P. Gygi, "The SCX/IMAC Enrichment Approach for Global Phosphorylation Analysis by Mass Spectrometry." *Nat Protoc*, 2008. 3(10): 1630-8.

10.
J. V. Olsen, et al., "Global, In Vivo, and Site-Specific Phosphorylation Dynamics in Signaling Networks," Cell, 2006. 127(3): 635-48.

11.
J. Villen, et al., "Large-Scale Phosphorylation Analysis of Mouse Liver," *Proc Natl Acad Sci U S A*, 2007. 104(5): 1488-93.

12.
Raijmakers et al., "Exploring the human leukocyte phosphoproteome using a microfluidic RP-TiO_2-RP HPLC Phosphochip coupled to a Q-ToF mass spectrometer" submitted 2009.

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June 10, 2009
Publication Number 5990-4098EN

Agilent Technologies