

# Targeted SRM Assays Of Cell Lysate Phosphopeptides Enriched By An Automated, Magnetic, Bead-Based System

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**Overview** A workflow for automated enrichment, LC-MS/MS identification and SRM targeted assay of phosphopeptides from cell lysates was developed.

**Purpose:** Simplify and expedite sample enrichment of phosphopeptides from cell lysates for mass spectrometry analysis

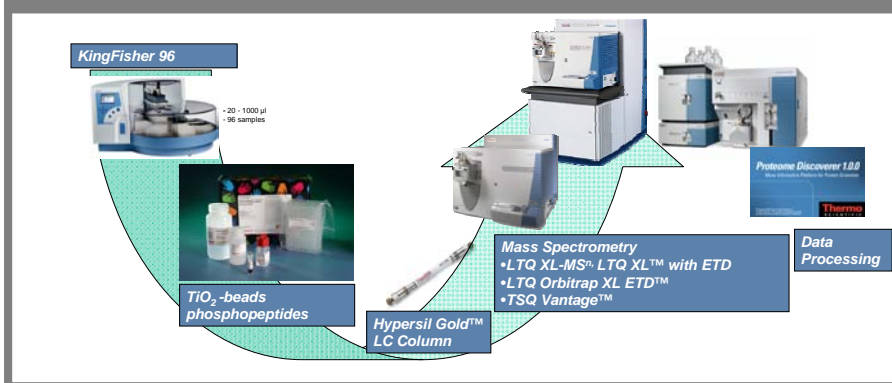
**Methods:** A Kingfisher automated system and TiO<sub>2</sub> magnetic beads were used to enrich phosphopeptides from cell lysates. Selective reaction monitoring (SRM) assays for targeted phosphopeptides were carried out on a Quantum Ultra triple quadrupole mass spectrometer. Proteotypic peptides and transitions for the targeted SRM assays were selected using a novel software algorithm automatically incorporating the most selective and sensitive predictions for SRM transitions

**Results:** The TiO<sub>2</sub> magnetic bead-based method resulted in the enrichment of numerous phosphopeptides. The automated phosphopeptide enrichment process facilitated the development of robust, quantitative targeted assays for phosphoproteins

## Introduction

Targeted SRM assays are increasingly being used for the quantification and monitoring of specific disease biomarkers (1). Unfortunately, many biomarkers, especially phosphopeptides involved in cell signaling, are typically present at very low levels in cell lysates. An efficient, automated and robust enrichment facilitates the detection and routine clinical assay of these lower abundance species. Previous approaches have focused on enrichment with IMAC (2). 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 (3,4). Recently, metal oxide affinity chromatography was successfully applied for selective enrichment of phosphopeptides using aluminum, titanium, zirconium and other metal oxides (5). We describe here an integrated, automated high-throughput workflow for the selective enrichment and identification of phosphopeptides from cell lysates using high resolution LC-MS/MS. This workflow includes the development of SRM targeted assay (on a triple quadrupole mass spectrometer) for selected phosphopeptides identified in the discovery experiments.

FIGURE 1. Integrated workflow for automated phosphopeptide capture, identification and SRM targeted assay development



## Methods

### Cell lysate sample preparation:

Lymphocyte and monocyte cells (peripheral blood mononuclear cells, PBMC) were collected, prepared and isolated in Cell Preparation Tubes (Beckton Dickinson) following the product insert. Immediately after rinsing the PBMC pellet with PBS, the cells were lysed by adding 8M GuHCl/ 150mM Tris HCl/10mM 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 40mM and the lysate was incubated at room temperature in the dark for 60 min to alkylate the proteins. Subsequently, 2M DTT was added to quench the reaction and then the PBMC sample was dialyzed against water with a 3500 MWCO Slide-a-Lyzer™ cassette (Thermo Fisher Scientific). Following dialysis of reduction/alkylation solutions, the protein lysate was dialyzed against 50mM Tris HCl/2mM MgCl<sub>2</sub> pH 8.0 plus 10 units of Benzamide (EMD) (Benzamide buffer). The sample was dialyzed against water with at least one change for another 2-4 hours. The sample was then aliquotted into pre-weighed 1.5mL microfuge tubes and lyophilized to dryness. The microfuge tubes plus dried pellets were re-weighed to calculate the dry protein weights.

**Enzymatic digestion:** Enzymatic digestion was initiated by resuspending a 2 mg protein pellet in 500mL of 8M GuHCl/150mM Tris/10mM DTT pH 8.5. The samples were diluted to 10mL with 50mM Tris/5mM CaCl<sub>2</sub>, pH 8.0 and then adding 20mg of Sequencing Grade trypsin (Promega) resulting in a protein to trypsin 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 100mL of TFA to a final concentration of 1%.

### Sample de-salting and solid phase extraction:

Samples were desalted using a 500mg C18 HyperSep disposable cartridge (Thermo Fisher Scientific). The cartridge was equilibrated with acetonitrile followed by 0.25% TFA. The sample was then added at a flow rate of 1mL per minute. The cartridge was then washed with 0.25% TFA and the peptides were eluted with 1ml of 80% acetonitrile/0.2% formic acid. Samples were brought to a final concentration of 2% formic acid immediately before using the Magnetic TiO<sub>2</sub> Phosphopeptide Enrichment Kit (Thermo Fisher Scientific).

### Phosphopeptide enrichment:

Phosphopeptides were enriched on the KingFisher™ Robot with a commercially available kit, Magnetic TiO<sub>2</sub> Phosphopeptide Enrichment Kit (Thermo Fisher Scientific).

FIGURE 2. Automated enrichment of phosphopeptides from blood lymphocytes using the Magnetic Titanium Dioxide Phosphopeptide Enrichment Kit and Thermo Scientific KingFisher. The results table shows the comparison between analysis conducted with enrichment or without enrichment. MS data were acquired on an LTQ FT™ MS ultra high resolution mass spectrometer. Starting material was 2 mg of total lymphocyte tryptic peptide digest.

Result	With Enrichment	Without Enrichment
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

FIGURE 3. High resolution LC-MS/MS data obtained from peripheral blood mononuclear cell (white blood cell) protein digest enriched with Pierce Magnetic TiO<sub>2</sub> Phosphopeptide Enrichment Kit processed on the KingFisher 96.

A. Full scan chromatogram obtained on LTQ FT Ultra high resolution mass spectrometer, resolution 200K, mass accuracy <2ppm.

B. Zoom of one full scan, retention time 64.50 minutes.

C. MS2 fragmentation spectrum of doubly phosphorylated peptide ion, parent mass 1582.70. Peptide sequence: SS@PFKVS@PLTFGR. Protein identified as Serum Deprivation Response Protein.

D. MS2 fragmentation spectrum of singly phosphorylated peptide ion, parent mass 1722.80. Peptide sequence LPS@GSGAASPTGSAVDIR. Protein identified as AHNAK Nucleoprotein

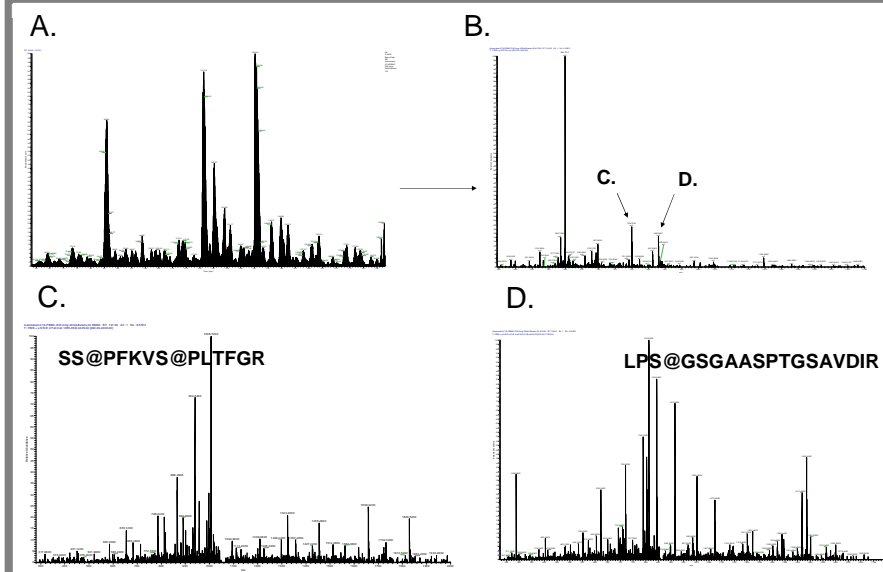
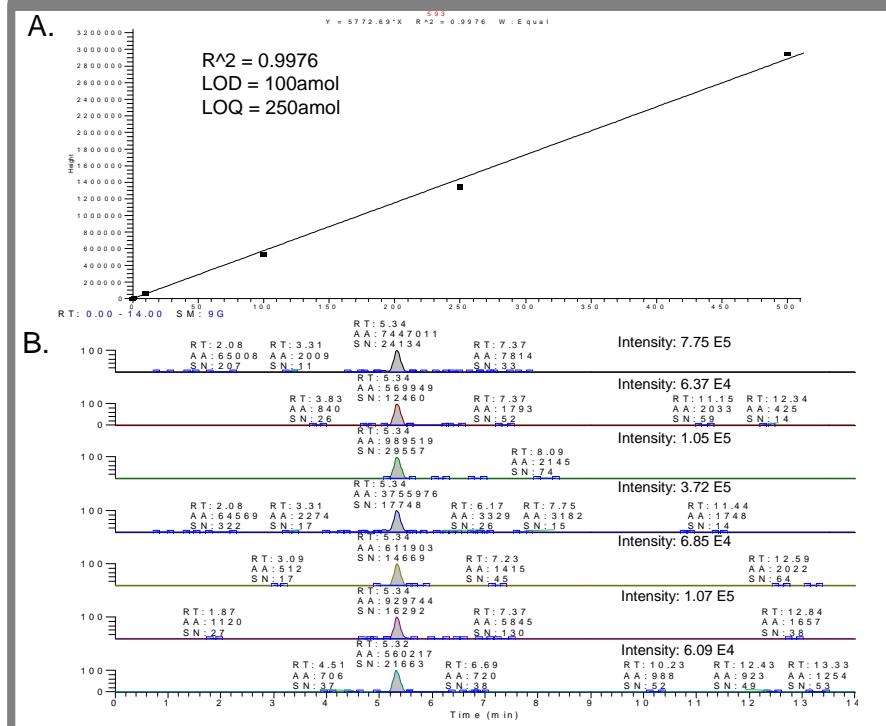


FIGURE 4a. Calibration curve of phosphopeptide FLS#QDAPTVK. Parent ion m/z 593.282, 6 transitions monitored. No internal standard used. Data obtained on Quantum Ultra in microspray mode, 50mM X 1mm Hypersil Gold Column, Flow rate 5µl/min 15 min total run time.

4b. 100fmol of phosphopeptide in background of 500ug of PBMC digest, phosphopeptides enriched on the KingFisher robot with a commercially available kit.



## Conclusions

1. An automated, high-throughput workflow for the enrichment of phosphopeptides from cell lysates was developed.
2. The enrichment of phosphopeptides using the Magnetic TiO<sub>2</sub> Phosphopeptide Enrichment Kit was comprehensive and selective.
3. The described workflow enables the discovery and identification of phosphopeptides and the subsequent development of targeted SRM-based assays that may be useful for clinical application.

## References

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