

# Isolation and Identification of Tyrosine Phosphorylated Peptides from Primary B-cells Using Phos-trap and Mass Spectrometry

## Introduction

Phosphorylation is a reversible protein modification involved in regulating a myriad of cellular events including receptor signaling, protein association and compartmentalization, activation and inhibition of protein function, and cell survival (1, 2). Antigen binding to B-cell receptor (BCR) results in a signal cascade involving multiple proteins and phosphorylation events. We are interested in characterizing changes in tyrosine phosphorylation (pY) on a global scale in response to BCR-ligand binding (3). However, the initial objective was to establish a general workflow in order to isolate pY peptides and identify as many pY sites as possible from resting and stimulated B-cells. In order to accomplish this, primary B-cells were stimulated with anti-kappa, a BCR crosslinking reagent, and pY peptides were isolated with an anti-pY antibody (4). Recovered peptides were further enriched for phosphopeptides using the Phos-trap™ Phosphopeptide Enrichment Kit or simply desalted using μZipTips® (Millipore Corp., Bedford, MA). Samples were then analyzed by nano-LC/MS on an LTQ®-Orbitrap®.

## General methods

Primary B-cells were isolated from mice and stimulated for 0 or 2 min with anti-kappa. Cells were then lysed with sonication in a urea lysis buffer containing phosphatase inhibitors. Protein concentration in the lysates was measured using the BCA protein assay kit (Pierce, Rockford, IL). An equivalent amount of protein from each sample was reduced, alkylated at the cysteine residues, and digested overnight at room temperature with trypsin.

Digested samples were acidified with TFA and 100 fmol of a standard phosphotyrosine peptide was added to each sample. The samples were then desalted by reverse phase chromatography and lyophilized.

Lyophilized peptides were dissolved in buffer (50 mM MOPS, pH 7.2, 10 mM sodium phosphate, 50 mM NaCl) and transferred to a microfuge tube containing a slurry of P-Tyr-100 antibody beads (Cell Signaling Technology, Danvers, MA). The samples were incubated with rotation overnight at 4 °C. The beads were washed and peptides were recovered with 0.15% TFA. Half of the recovered

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sample was concentrated by  $\mu$ ZipTip<sup>®</sup>, redissolved in 0.1% TFA, and manually loaded onto a C18 precolumn for nano-LC/MS. The remaining half of the recovered samples was enriched for phosphopeptides using the Phos-trap Phosphopeptide Enrichment kit (PerkinElmer) and manually loaded onto a C18 precolumn for nano-LC/MS.

Nano-LC/MS was performed on an LTQ<sup>®</sup>-Orbitrap<sup>®</sup>. The precolumn was placed inline with a 360 x 75  $\mu$ m column with integrated electrospray tip and equilibrated with 100% A. Peptides were eluted with a linear gradient of 0% B to 25% B in 120 min (Solvent A, 0.1M HOAc, in water; Solvent B, 0.1M HOAc in ACN). MS scans were as follows: 1 FTMS scan, 1 ITMS scan, 5 MS/MS scans of the most intense ions from the ITMS scan.

MS/MS data was searched using SEQUEST<sup>®</sup> with the following parameters: Variable Modifications of M – 16, STY – 80; Static Modification of C – 57; Enzyme as trypsin with 2 missed cleavages. The data was searched against a human, mouse, and rat “slice” of the NCBI nr database. Search results were filtered for Xcorr vs m/z of +1/2.0, +2/2.5, +3/3.0. pY peptides meeting this criteria from the 0 and 2 min samples were combined into one list and evaluated as below.

## Results

The distribution of peptide identifications from the two workflows, with and without Phos-trap enrichment (Figure 1), was compared by filtering the search results for minimum Xcorr vs m/z values and sorting the MS/MS scans into 3 groups: pY containing peptides, pS or pT containing peptides, and non-phosphopeptides (Figure 2). When no enrichment was employed, 27% of MS/MS scans

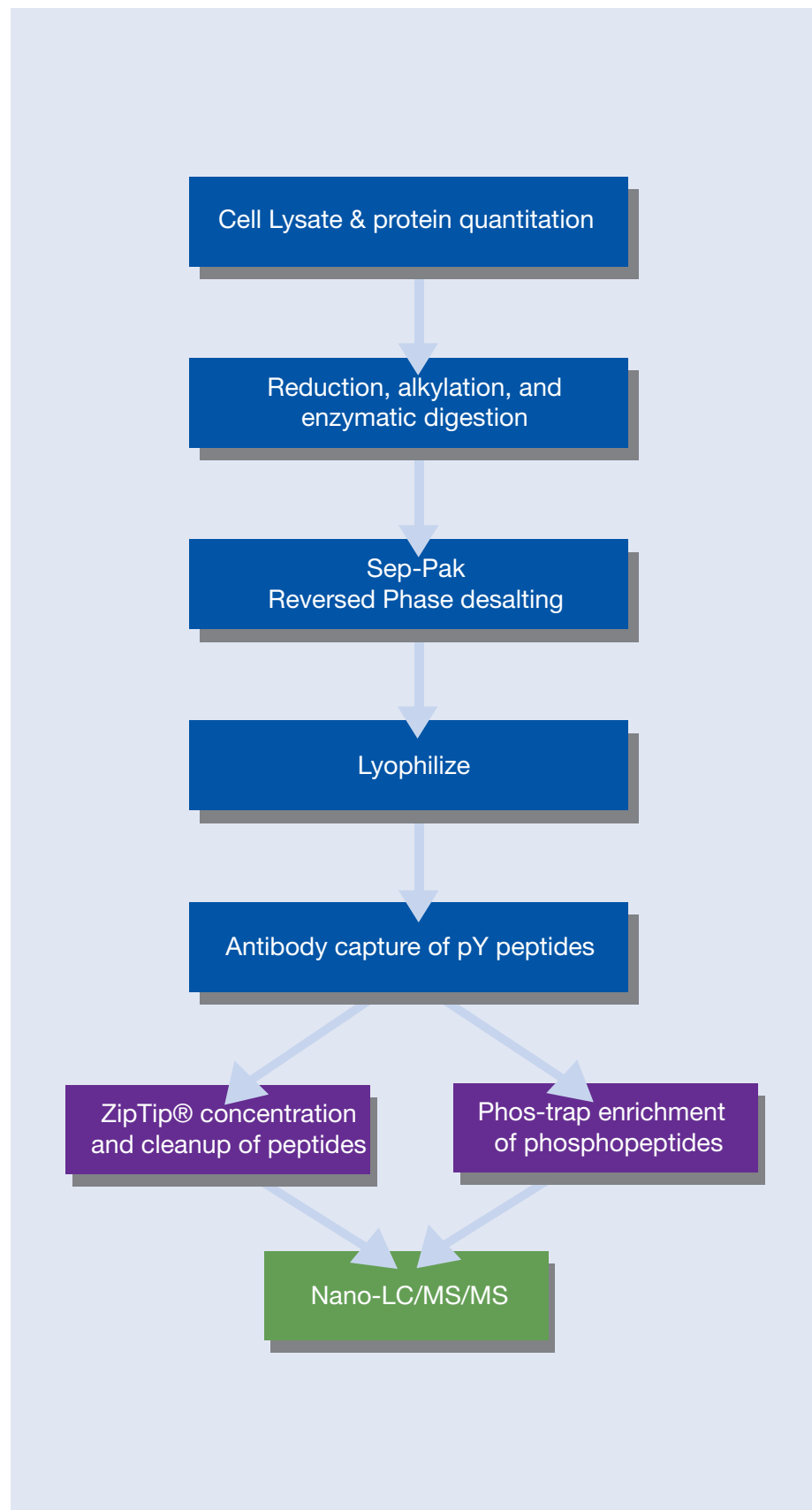


Figure 1. General workflow for experiments

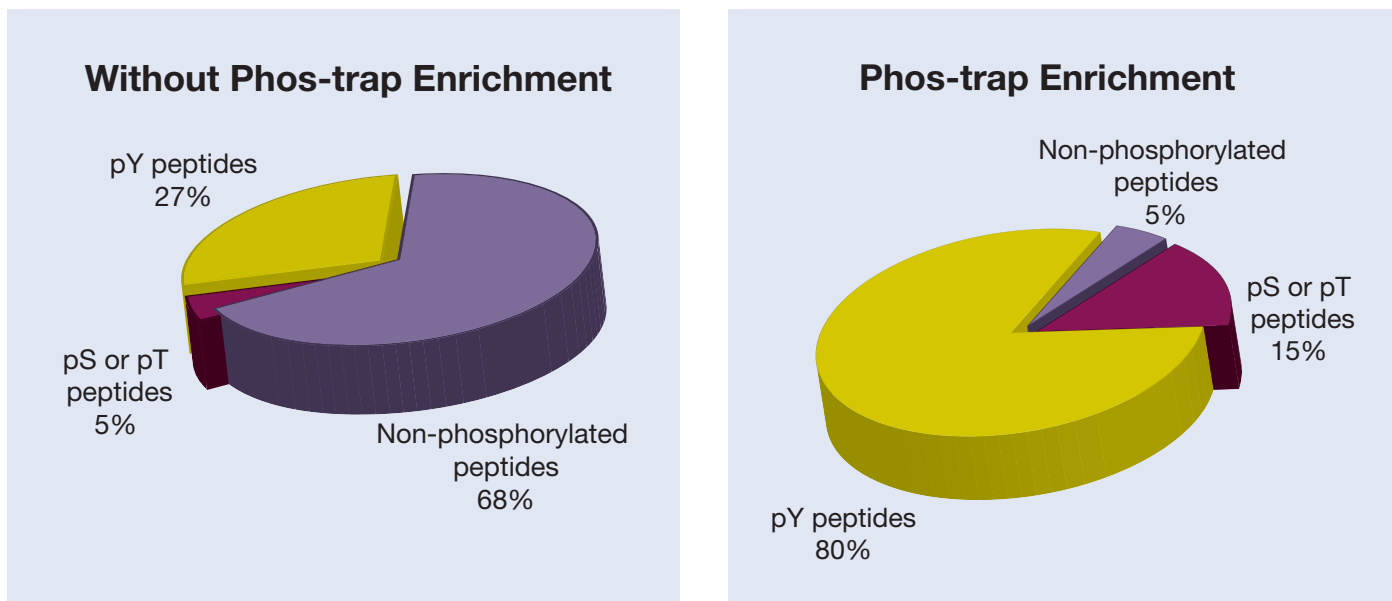


Figure 2. Distribution of peptides identified with Phos-trap and without Phos-trap enrichment. MS/MS scans were sorted into 3 groups based on search results, pY peptides, pS or pT peptides, or non-phosphorylated peptides. The number of MS/MS scans meeting the minimum criteria with Phos-trap and without Phos-trap enrichment were 1766 and 2940, respectively.

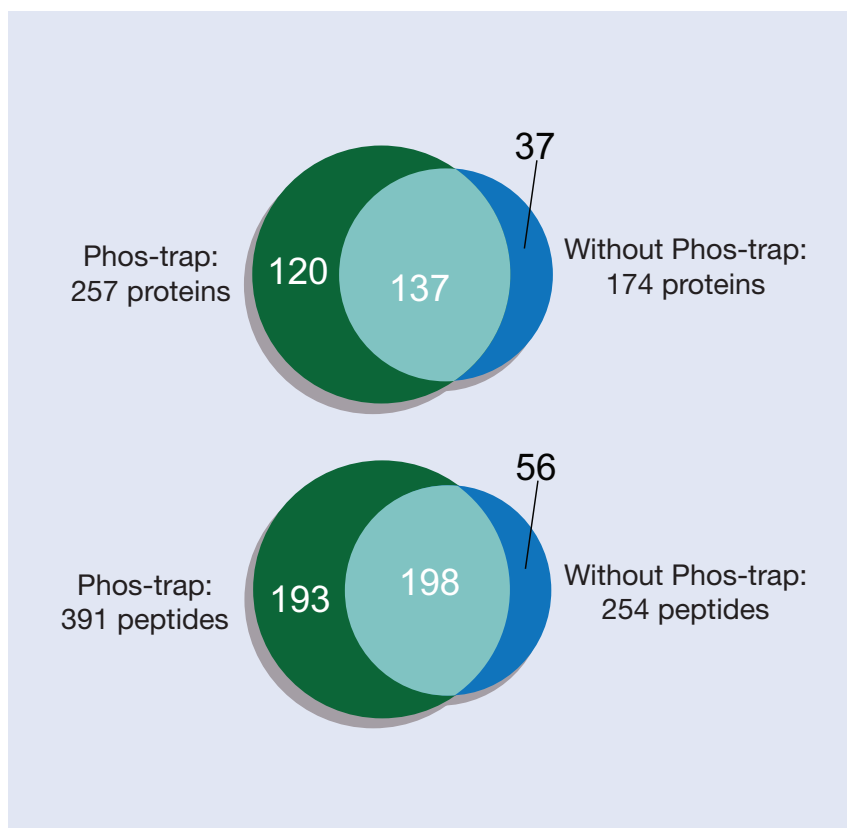


Figure 3. Degeneracy of peptide and protein identifications with Phos-trap and without Phos-trap enrichment. Venn diagrams showing non-redundant protein and peptide identifications meeting the minimum criteria from preparation with and without Phos-trap.

were matched to pY peptides while 68% of the scans were matched to peptides that did not have a phosphorylation site. In contrast, 80% of the scans following Phos-trap enrichment were matched to pY peptides with only 5% matching to non-phosphopeptides.

The degeneracy of pY peptide and protein identifications between the two workflows was also evaluated (Figure 3). 391 pY peptides from 257 proteins were identified from the Phos-trap enriched sample whereas 254 pY peptides from 174 proteins were identified when no enrichment was performed. 198 peptides from 137 proteins were common to both preparations. However, enrichment with Phos-trap enabled identification of 193 additional pY peptides from 120 proteins greatly increasing the number of pY sites. Additional pY peptides were also identified without Phos-trap enrichment (56 peptides from 37 proteins).

The total number of MS/MS scans meeting the filter criteria was greater when no enrichment was employed (2940 vs 1766). Given this, fewer pY peptides were identified from this sample set (254 vs 391). This suggests that the limited cycle time of the instrument during an LC analysis is spent collecting spectra of non-phosphorylated peptides at the expense of phosphopeptides and removal of non-phosphorylated peptides increases the number of identified phosphopeptides.

## References

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4. Rush J, Moritz A, Lee KA, Guo A, Goss VL, Spek EJ, Zhang H, Zha XM, Polakiewicz RD, Comb MJ. Immuno-affinity profiling of tyrosine phosphorylation in cancer cells. *Nat Biotechnol.* Jan;23(1):94-101 (2005).

## Ordering information

Product	Description	Product No.
Phos-trap Phosphopeptide Enrichment Kit	96 Sample Enrichments Phos-trap Kit contents: Phos-trap Magnetic Beads, binding buffer, washing buffer, elution buffer, 96-well V-Bottom polypropylene microplate and cover (2), manual.	PRT301001KT
Phos-trap 24 Enrichment Kit	24 Sample Enrichments Contents: Phos-trap Magnetic Beads, binding buffer, washing buffer, elution buffer, manual. Includes separations magnet.	PRT302001KT

Related Product	Description	Product No.
96-Well Magnet	Toroid design deep well plate magnet for use with magnetic bead applications	5083175

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