Enriching Posttranslationally-Modified Proteins

Efficient, specific enrichment of phosphoproteins, phosphopeptides, and glycoproteins

- **Quick, easy enrichment over a wide concentration range**
- **Specific and efficient for phosphorylation or glycosylation**
- **Speed up and improve results in downstream applications**

Posttranslational protein modification is one of three key mechanisms for regulating protein function and activity, together with translation and proteolysis. In many cases, posttranslational modifications are the first regulatory response to changes in the environment surrounding cells, organs, and the organism as a whole. Two very important posttranslational modifications are protein phosphorylation and glycosylation.

Protein Phosphorylation

Protein phosphorylation, a highly important mechanism for signal transduction in eukaryotic cells, is also observed in prokaryotic organisms (1–5). Signal transduction, transcriptional regulation, and cell division are just three examples of the many metabolic processes regulated via protein phosphorylation and dephosphorylation by kinases and phosphatases. Although only a relatively small percentage of proteins are phosphorylated in any given cell (6, 7) understanding the regulation of protein activity by phosphorylation is vital to understanding many cellular pathways. Enriching for phosphoproteins prior to analysis by 2-D PAGE or mass spectroscopy can increase the sensitivity of your assay and enable you to detect lower-abundance proteins.

Currently, the only available method for enriching the entire phosphoproteome is Immobilized Metal Affinity Chromatography (IMAC) with hard metal ions. Clontech has utilized this technology to develop efficient and convenient tools for specific phosphoprotein and phosphopeptide enrichment using both gravity/batch column and magnetic bead-based methods (Figure 1).

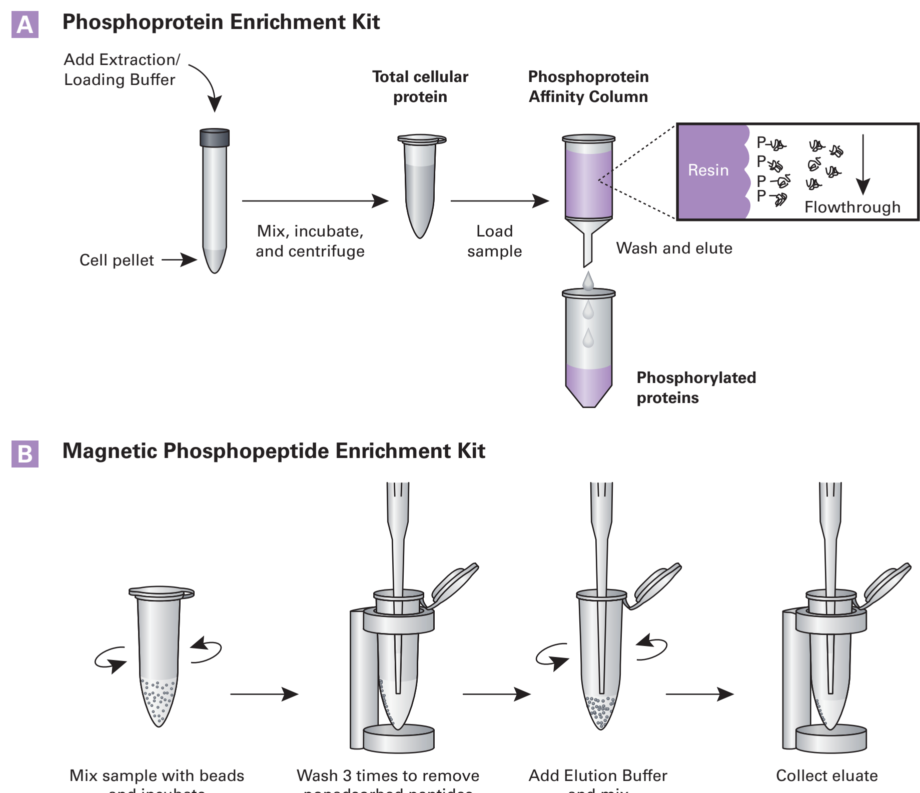

Figure 1. Examples of phosphopurification using column and magnetic bead-based methods from Clontech. The Phosphoprotein Enrichment Kit protocol (**Panel A**) utilizes a Phosphoprotein Affinity Column containing Phosphate Metal Affinity Chromatography (PMAC) resin to yield enriched phosphoproteins in less than 2 hr. The Magnetic Phosphopeptide Enrichment Kit protocol (**Panel B**) is carried out in a single microfuge tube with Phospho Magnetic Beads, using a magnetic separator, in a simple 30 min protocol. The **TALON® PMAC Magnetic Phospho Enrichment Kit** uses a similar magnetic bead-based protocol to enrich phosphoproteins.

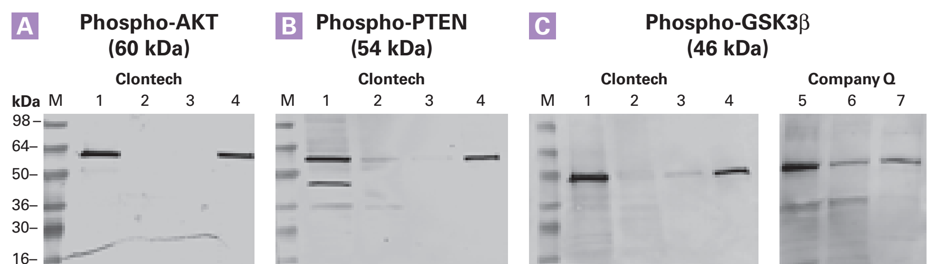

Figure 2. Highly selective enrichment of phosphorylated proteins using Clontech's Phosphoprotein Enrichment Kit. A Phosphoprotein Affinity Column was loaded with ~3 mg of total protein from HEK 293 cells. The extract (Lanes 1), flowthrough (Lanes 2), wash (Lanes 3), and eluate (Lanes 4) were then analyzed by Western blotting using antibodies specific for phosphorylated AKT (**Panel A**), PTEN (**Panel B**), and GSK3β (**Panel C**) proteins. The proteins were clearly detected in the eluate fraction. Compare these results to those in Lanes 5–7 (Panel C), which were loaded with the extract (Lane 5), flowthrough (Lane 6), and eluate (Lane 7) fractions obtained from the same cell line using Company Q's phosphoprotein purification system, and analyzed by Western blotting using antibodies specific for phosphorylated GSK3β protein.

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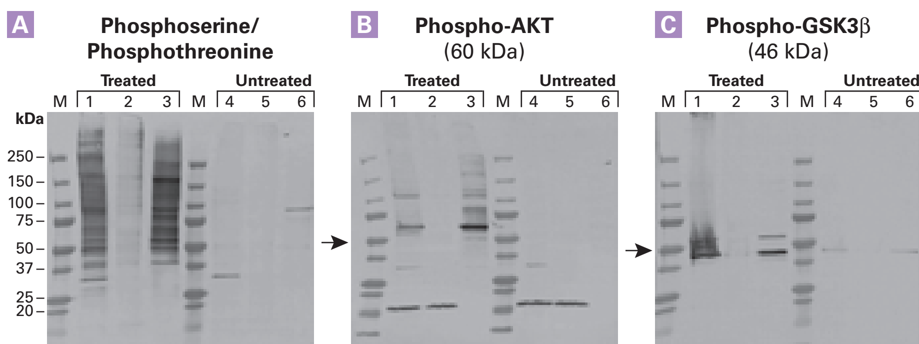

Figure 3. Calyculin A treatment enables specific enrichment of low-level phosphoproteins containing phosphoserine and phosphothreonine with the Phosphoprotein Enrichment Kit. Serine/threonine phosphorylation was induced above basal levels in HeLa cells by a 30 min treatment with 50 nM calyculin A (a serine/threonine phosphatase inhibitor which inhibits the activity of protein phosphatases PP1 and PP2A). Individual cell pellets, untreated or treated with calyculin A, were collected, lysed, and run on respective Phosphoprotein Affinity Columns. All fractions were analyzed by Western blotting using antibodies specific for phosphoserine and phosphothreonine (**Panel A**), phospho-AKT (**Panel B**), and phospho-GSK3β (**Panel C**). Lane M: molecular weight marker. Lanes 1 & 4: protein loaded. Lanes 2 & 5: flowthrough. Lane 3: eluate (phosphorylated ovalbumin). Lane 6: eluate (dephosphorylated ovalbumin). Phosphorylated protein levels were generally higher in all the treated samples and specifically enriched in Lanes 3 (the column eluate).

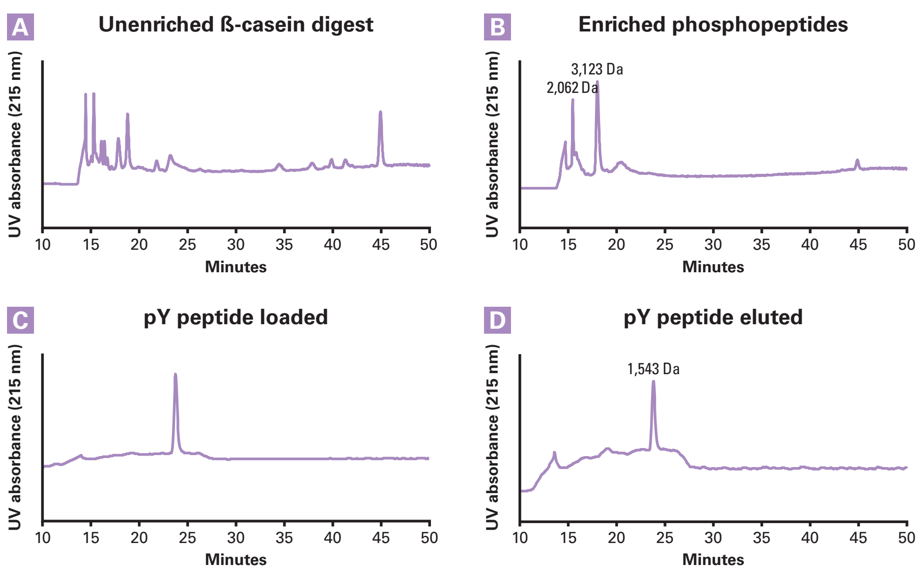

Figure 4. Highly specific phosphopeptide purification with the Magnetic Phosphopeptide Enrichment Kit. ß-casein protein digested with immobilized trypsin was diluted with Binding/Wash Buffer and mixed with Phospho Magnetic Beads at room temperature for 10 min. After washing, the enriched fraction was eluted with Elution Buffer. Reverse phase (RP)-HPLC analysis was performed on a Waters Breeze™ HPLC, XTerra® RP18 column (5 m, 4.6 x 150 mm) with UV detection at 215 nm (Solvent A: 0.1% TFA in water [v/v], Solvent B: 0.1% TFA in acetonitrile [v/v]). Reverse phase HPLC (RP-HPLC) data are shown for the crude protein digest in **Panel A** and for the eluate (enriched phosphopeptides) in **Panel B.** Synthetic pY peptide (pp60c-src Fragment 521–533) dissolved in Binding/Wash Buffer (**Panel C**) was mixed with Phospho Magnetic Beads. After washing, bound phosphopeptide was eluted with Elution Buffer (**Panel D**). The amino acid sequence of pY peptide is Thr-Ser-Thr-Glu-Pro-Gln-pTyr-Gln-Pro-Gly-Glu-Asn-Leu.

Phosphoprotein Enrichment

Our **Phosphoprotein Enrichment Kit** is based on our proprietary Phosphate Metal Affinity Chromatography (PMAC) resin, which binds proteins containing phosphorylated serine, threonine, and tyrosine residues, providing highly specific phosphoprotein enrichment (Figure 2). A straightforward protocol, using the Phosphoprotein Affinity Columns (which contain this resin) and buffers supplied in the kit, allows you to enrich up to 4 mg of total phosphoproteins from mammalian cell lines or tissue samples in less than two hours (Figure 1, Panel A). The PMAC resin may be used to enrich phosphoproteins that are normally present at undetectable basal levels, after blocking serine/threonine dephosphorylation with a phosphatase inhibitor such as calyculin A (Figure 3). We also offer the magnetic bead-based **TALON PMAC Magnetic Phospho Enrichment Kit,** for isolating microgram quantities of phosphoproteins from cell lines and tissues using Phospho Magnetic Beads and a magnetic separator (Figure 1, Panel B).

Phosphopeptide Enrichment

Additional downstream enrichment can be carried out at the peptide level with our quick and convenient phosphopeptide enrichment products, which are available in both a spin column format (the **Phosphopeptide Enrichment Spin Columns** and **Phosphopeptide Enrichment Buffer Kit**) and a magnetic bead-based format (the Magnetic Phosphopeptide Enrichment Kit). These kits utilize our PMAC resin to selectively bind peptides that contain phosphorylated serine, threonine, or tyrosine residues. They can be used to enrich phosphopeptides from trypsin-digested mammalian cell and tissue extracts, as well as tryptic digests of purified proteins. The specific binding of phosphoserine peptides from a protein digest, as well as a synthetic phosphotyrosine peptide, is shown in Figure 4. The Phosphopeptide

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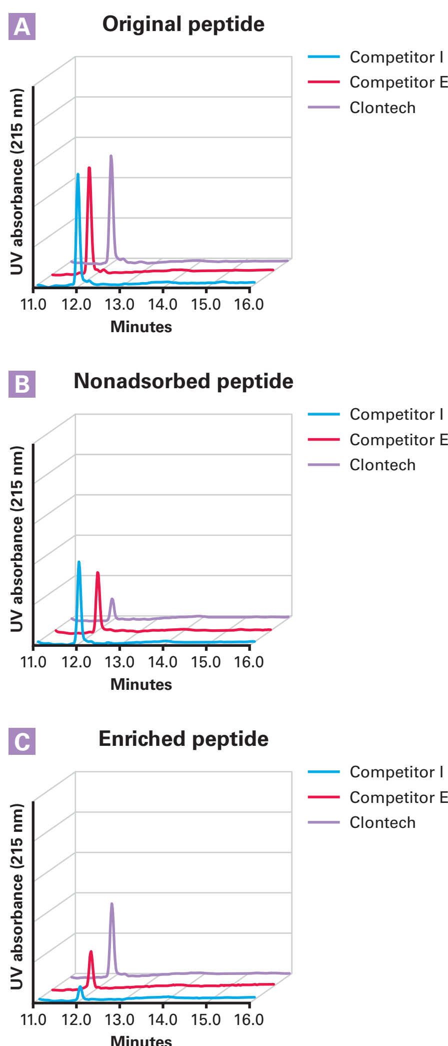

Figure 5. Clontech's Magnetic Phosphopeptide Enrichment Kit binds phosphopeptides more effectively than competing products. The binding capacity of Phospho Magnetic Beads from Clontech's Magnetic Phosphopeptide Enrichment Kit was compared to that of equal volumes of magnetic bead suspensions from two competitor kits. In this experiment, 20 µg of monophosphorylated peptide T1P (Sigma, Cat. No. P9615) was enriched using 100 µl aliquots of each bead suspension and the buffers provided in each respective kit. Samples were analyzed by reverse phase-HPLC (RP-HPLC) on a C18 column. **Panel A.** Original peptide before enrichment. **Panel B.** Nonadsorbed peptide after enrichment with each kit. **Panel C.** Enriched peptide eluted with each kit. Our kit provided higher yields than both competitors—and our Phospho Magnetic Beads displayed 10X the binding capacity of Competitor I's magnetic bead suspension.

Enrichment Spin Columns have the capacity to bind up to 250 µg of phosphopeptide and accommodate up to an 850 µl sample volume, while the Magnetic Phosphopeptide Enrichment Kit is designed for nanoscale purification in elution volumes as small as 20 µl. The kit utilizes Phospho Magnetic Beads, with a binding capacity of approximately 1–2 pmol of phosphate per µg of beads, which have been shown to bind phosphopeptides more effectively than competing products (Figure 5). These phosphopeptide enrichment products cover a wide range of purification scales to meet your research needs.

Protein Glycosylation

Posttranslational glycosylation plays numerous functional roles both inside and outside cells and affects over half of all secretory and cellular proteins (8). During glycoprotein synthesis, the glycans present in glycoproteins help regulate protein folding, oligomerization, quality control, sorting, and transport. They serve as "tags" that allow specific lectins and modifying enzymes to establish order among the diversity of maturing glycoproteins via a mechanism that is universal to eukaryotic systems (9). There is considerable evidence that attachment of a specific monosaccharide to core glycans or branches changes glycoprotein function, resulting in changes to cellular phenotypes. These changes may be involved in various biological or pathological processes such as cancer, infection, and reproduction (10).

Glycoprotein Enrichment & Detection

The ability to quickly enrich the entire glycoproteome before performing downstream analyses is vital to determining the function of this very important group of proteins. Clontech's new **Glycoprotein Enrichment Resin** and **Glycoprotein Western Detection Kit** offer powerful tools for glycoprotein enrichment and analysis. The resin provides efficient and specific enrichment of glycoproteins using

Product	Size	Cat. No.
Glycoprotein Enrichment Resin (NEW!)	10 ml	635647
Glycoprotein Western Detection Kit (NEW!)	20 rxns	635648
Phosphoprotein Enrichment Kit	6 preps	635624
Phosphoprotein Kit—Buffer A	500 ml	635626
TALON PMAC Magnetic Phospho Enrichment Kit	each	635641
Phosphopeptide Enrichment Spin Columns	25 columns	635634
Phosphopeptide Enrichment Buffer Kit	each	635635
Magnetic Phosphopeptide Enrichment Kit	each	635643

Related Products

- Mag-Trypsin (Cat. No. 635646)
- TALON® Metal Affinity Resin (Cat. Nos. 635501, 635502, 635503 & 635504)
- TALON® Magnetic Beads (Cat. Nos. 635636 & 635637)
- Thiophilic-Superflow™ Resin (Cat. Nos. 635616 & 635617)

either simple gravity flow columns or medium pressure FPLC columns, while the detection kit allows specific detection of glycoproteins on Western blots (11; see pages 8〜10 of this issue).

References

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