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Product Information

PhosphoProfile™ II Phosphopeptide Enrichment Kit

Catalog Number **PP0420**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product Description

Protein phosphorylation is a dynamic post-translational modification involved in the regulation of a variety of cellular events including signal transduction, gene expression, and apoptosis.[1] Reversible phosphorylation is controlled by the action of kinases and phosphatases for the addition and removal, respectively, of phosphate groups. The most common phosphorylation events in eukaryotes occur on serine, threonine, and tyrosine residues. As many as six other amino acids hold the potential for modification, adding additional complexity to the study of protein phosphorylation and further emphasizing the importance of identification of phosphorylation sites for characterization of signaling events.[2–4]

Mass spectrometry (MS) of phosphopeptides obtained from tryptic protein digests has become a powerful tool for the characterization and identification of phosphorylation sites. Such analysis is challenging due to several factors including a low stoichiometry of phosphorylation relative to non-phosphorylated analogs, heterogeneity of the phosphorylation state of a single phosphoprotein, and low abundance of certain phosphoproteins within cells. These factors contribute to the low concentration of phosphopeptides following trypsin digestion, resulting in weak signal intensities when analyzed by Matrix Assisted Laser Desorption/ Ionization Time of Flight (MALDI-TOF) or Electrospray Ionization (ESI) mass spectrometry. A combination of low intrinsic abundance, inefficient ionization, and/or signal suppression of most phosphopeptides may limit or even prevent detection, unless the phosphopeptide content is significantly enriched prior to analysis.[3–5]

Immobilized metal affinity chromatography (IMAC) has been commonly used for purification of phosphorylated compounds. The highest affinity and selectivity have been demonstrated with chelated gallium(III) and iron(III) ions.[3,6] The binding involves coordination of phosphate oxygen atoms with the metal ion.

The PhosphoProfile™ II Phosphopeptide Enrichment Kit is a convenient collection of all materials necessary to perform phosphopeptide enrichments from crude samples following tryptic digestion. The supplied phosphopeptide capture matrix is a novel Ga^{3+} chelate silica based on a proprietary nitriloacetic acid (NTA) analog. The silica beads are ~20 microns in diameter with a pore size of 1,000 Angstroms. The matrix is packed into spin columns for easy, microscale affinity capture of phosphopeptides.

Since the enrichment method described for this kit requires acidic conditions to promote specificity of interaction, it is best suited for enrichment from soluble tryptic peptides. Enrichment of phosphoproteins by this method is generally not recommended due to their poor solubility. The affinity isolation process significantly enriches phosphopeptide content; however, positive identification of phosphopeptides requires additional methods. Typical mass spectrometric based techniques include analysis by MALDI-TOF MS and ESI MS using Post-Source Decay (PSD), Collision Induced Dissociation (CID), ion trap (MS^n), and alkaline phosphatase treatments. All of these methods rely on identifying signature peptide mass loss of phosphate forms (see Table 1).[1,7]

Table 1.
Signature Mass Losses

Techniques for generation of signature mass loss	Phosphoserine/ threonine signature peptide mass loss (m/z)	Phosphotyrosine signature peptide mass loss (m/z)
Positive Ion PSD/CID	98 Da ($H_3PO_4^-$) preferential or 80 Da (HPO_3^-)	80 Da (HPO_3^-) preferential
Negative Ion PSD/CID	79 Da (PO_3^-) or 63 Da (PO_2^-)	79 Da (PO_3^-) or 63 Da (PO_2^-)
Alkaline Phosphatase Treatment	80 Da (HPO_3^-)	80 Da (HPO_3^-)

Components
The PhosphoProfile II Phosphopeptide Enrichment Kit can purify 24 samples containing up to 25 nmoles of phosphopeptides. The PHOS-Select Gallium Silica Spin Columns (Catalog Number P2873) are for a single use application.

- PHOS-Select™ Gallium Silica Spin Columns (Catalog Number P2873) — 24 each
  Pre-packed spin columns containing 50 µl (25 mg) of phosphopeptide capture matrix.
- Bind/Wash Solution — 10 ml
  (Catalog Number B8935)
  250 mM acetic acid in 30% acetonitrile
- Elution Solution (Catalog Number E0906) — 5 ml
  10% phosphoric acid solution optimized to enhance specific recovery of phosphopeptides, while maintaining MALDI-TOF MS compatibility.
- Proteomics Grade Trypsin — 3 × 20 µg
  (Catalog Number T6567)
- Trypsin Solubilization Reagent — 1 ml
  (Catalog Number T2073)
  1 mM HCl
- Enzyme Reaction Buffer — 25 ml
  (Catalog Number E0530)
- Collection Tubes — 2 × 50 each
  (Catalog Number T5449)

Equipment and Reagents Required but Not Provided
- Ultrapure water (18 MΩ·cm or equivalent)
- 37 °C incubator
- Bench-top centrifuge (microcentrifuge)

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
The listed reagents require preparation prior to use. Ultrapure water (18 MΩ·cm or equivalent) is recommended for use when reconstituting reagents.

- Proteomics Grade Trypsin Solution – Reconstitute 1 vial with 20 µl of Trypsin Solubilization Reagent to prepare a 1 mg/ml solution.
- Enzyme Reaction Buffer – Add 25 ml of water to the bottle. The resulting solution contains 100 mM ammonium bicarbonate.

Storage/Stability
For optimal performance, store the kit at 2–8 °C. Under these storage conditions, the product is stable for at least 2 years. The Enzyme Reaction Buffer is stable for up to one month after reconstitution when stored at 2–8 °C. The reconstituted Trypsin Solution should be aliquoted and stored at –20 °C. The Trypsin Solution is stable for at least 3 freeze-thaw cycles.

Procedure
Binding of phosphorylated compounds is highly pH dependent. The suggested working pH range is 2.5–3.0; however, binding will occur over the pH range of 2.0–5.5. Optimal binding is obtained using 250 mM acetic acid as a sample solvent. To obtain satisfactory results the sample should have a low ionic strength. This may require a buffer exchange or desalting of the sample prior to binding to the affinity gel. In addition, the desalting of a crude mixture may remove other interfering contaminants and improve enrichment.[8] The Bind/Wash Solution is formulated to minimize non-specific interactions and maximize capture of phosphopeptides.

Some researchers have attempted complete methyl esterification of peptide carboxyl groups to improve specificity with reported success.[9] Although promising, this step has not been incorporated into this procedure as there are difficulties with interpretation of incomplete esterification and other potential degradation products. The recommended procedure is optimized to minimize binding of acidic peptides.

The following procedure describes a typical enrichment method for use of the PHOS-Select Gallium Silica Spin Columns with the supplied Bind/Wash and Elution solutions following a tryptic digestion. The procedure should be used as a guideline and optimal conditions determined empirically for varying sample types.

1. Tryptic Digestion of Protein Samples - Tryptic digestion in solution should be performed according to the Technical Bulletin for the Proteomics Grade Trypsin (Catalog Number T6567) using the reagents supplied in this kit. For in-gel digestion, use of the Trypsin Profile IGD Kit (Catalog Number PP0100) is recommended.

   Reduction and alkylation of protein samples prior to tryptic digestion is also recommended. The ProteoPrep® Reduction and Alkylation Kit (Catalog Number PROTRA) contains tributylphosphine (TBP) and iodoacetamide (IAA) for reduction and alkylation, respectively, of protein disulfide bonds. Refer to the ProteoPrep Reduction and Alkylation Kit Technical Bulletin for suggested protocols.

   a. Solution Digestion - Dissolve the protein sample in 10 mM ammonium bicarbonate using a 10-fold dilution of the Enzyme Reaction Buffer (Catalog Number E0530). The prepared Enzyme Reaction Buffer is a 100 mM ammonium bicarbonate solution and a portion can be further diluted to a final concentration of 10 mM. For protein samples prepared in other buffers, exchange the buffer with 10 mM ammonium bicarbonate. Add the prepared Proteomics Grade Trypsin Solution to the protein solution employing the desired ratio of trypsin to protein. For the protein digestion, a 1:50 (w/w, enzyme to protein) ratio is recommended for use with this procedure; however, a ratio of between 1:100 and 1:20 (w/w) of enzyme to substrate may be suitable. The recommended incubation time is 5–18 hours at 37 °C, depending on the enzyme to protein ratio. Refer to the Proteomics Grade Trypsin Technical Bulletin for more detailed instructions and additional data.

   b. In-gel Digestion - An in-gel trypsin digestion may be achieved using the Trypsin Profile IGD Kit (Catalog Number PP0100). The Trypsin Profile IGD Kit contains reagents required for destaining gel slices, as well as a specific Trypsin Reaction Buffer to digest samples contained within gel pieces and a Peptide Extraction Solution.

2. Preparation of Samples Obtained from a Protein Tryptic Digest - Lyophilize or vacuum-dry the sample, then reconstitute in the supplied Bind/Wash Solution (Catalog Number B8935) to a final volume of 25–50 µl. Alternatively, for a sample digested in 10–100 mM ammonium bicarbonate (a sample prepared using the Trypsin Profile IGD Kit), add glacial acetic acid to obtain a final concentration of 250 mM acetic acid.

   A small sample (5 µg obtained from an in-gel digestion) may be added to 25–50 µl of Bind/Wash Solution. A sample load of up to 500 µg of digested protein is also suitable, but the sample volume should not exceed 50 µl per spin column.

3. Wash/Equilibration of PHOS-Select Gallium Silica Spin Column - Add 50 µl of Bind/Wash Solution to the PHOS-Select Gallium Silica Spin Column. Place the spin column into one of the provided 2 ml collection tubes (Catalog Number T5449) and centrifuge in a microcentrifuge for 30 seconds at 500 × *g* (e.g., 2,400 rpm in an Eppendorf® 5415C microcentrifuge). Discard any flow-through liquid. Repeat this step once. Save the collection tube for step 5.

4. Sample Loading - Place the spin column in a new 2 ml collection tube. Add 25–50 µl of the sample solution to the equilibrated spin column. Centrifuge in a microcentrifuge for 30 seconds at 500 × *g* to load the spin column. Incubate for 15 minutes at room temperature. The flow-through liquid in the collection tube may contain unbound peptides, which if desired may be pooled with the washes from step 5 and further analyzed.

5. Wash Spin Column with Bind/Wash Solution - Place the column in the collection tube saved from step 3. Add 50 µl of Bind/Wash Solution to the spin column and centrifuge in a microcentrifuge for 30 seconds at 500 × *g*. The flow-through liquid in the collection tube contains unbound peptides, which may be discarded or pooled with the flow-through liquid from step 4 and further analyzed. Repeat this step twice.

6. Wash Spin Column with Water - Wash the PHOS-Select Gallium Silica Spin Column once with 50 µl of water (18 MΩ·cm or equivalent) to remove any residual Bind/Wash Solution prior to elution.

7. Sample Elution - Place the PHOS-Select Gallium Silica Spin Column in a new collection tube. Add 25 µl of Elution Solution (Catalog Number E0906). Centrifuge in a microcentrifuge for 30 seconds at 500 × *g* and retain the flow-through liquid for phosphopeptide analysis. The initial spin will contain minimal peptides. Repeat this step, as the majority of phosphopeptides will elute in this second flow-through. Subsequent "pulsed elutions" may be needed for optimal peptide recovery. The Elution Solution is compatible with LC-MS and MALDI-TOF MS analysis. It is recommended to use 2,5-dihydroxybenzoic acid (DHB or gentisic acid) as the MALDI-TOF MS matrix, as it has been demonstrated that phosphoric acid in combination with DHB enhances phosphopeptide signals in MALDI-TOF mass spectra.[10] Alternatively, 0.4 M ammonium hydroxide may be used as a comparably efficient elution solution (not provided).

Related Products	Catalog Number
ProteoPrep Kits	
Total Extraction Sample	PROTTOT
Membrane Protein Extraction	PROTMEM
Universal Extraction	PROTTWO
ProteoPrep Reduction and Alkylation Kit	PROTRA
ProteoMass™ MALDI-MS Calibration Kits	
Protein and Peptide	MSCAL1
Peptide	MSCAL2
Protein	MSCAL3
2,5-Dihydroxybenzoic acid (DHB)	149357
Phosphatase Inhibitor Cocktail 1	P2850
Phosphatase Inhibitor Cocktail 2	P5726
Trypsin Profile IGD Kit	PP0100

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