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Product Information

PHOS-Select™ Iron Affinity Gel

Catalog Number **P9740**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Phosphorylation represents an important post-translational modification of proteins, playing a critical role in a multitude of cellular regulatory events. The identification and characterization of phosphorylation sites are important milestones in increasing the understanding of signaling processes. Mass spectrometry (MS) of phosphopeptides obtained from tryptic protein digests has become a powerful tool for characterization.[1] However, there is a general need to significantly enrich the phosphopeptide content to compensate for low abundance, poor ionization, and suppression effects.[2]

Immobilized metal affinity chromatography (IMAC) has been commonly used for purification of phosphorylated compounds. Highest affinity and selectivity have been demonstrated with chelated iron(III) and gallium(III) ions.[2] The binding involves coordination of the phosphate oxygen with the metal ion. It has been shown that iron(III)-nitriloacetic acid (NTA) type chelates have greater specificity for phosphorylated peptides than iron(III)-iminodiacetic acid (IDA) type chelates.[3] This is most likely due to the lower overall positive charge of an iron(III) NTA type complex (+1) compared to an IDA complex (+2). In addition, iron(III)-NTA type chelates demonstrate stronger bonding to phosphopeptides compared to gallium chelates.[2]

The PHOS-Select™ Iron Affinity Gel is a novel iron [Fe(III)] chelate matrix based on our proprietary NTA analog chelate ligand. This matrix provides high capacity affinity binding of molecules containing phosphate groups. Saturation binding assays with a model phosphorylated compound have determined the binding capacity to be ≥3 μmoles of phosphate per mL of gel.

The affinity isolation process significantly enriches phosphopeptide content. However, positive identification of phosphopeptides requires additional methods.

Typical mass spectrometry based techniques include analysis by matrix assisted laser desorption/ionization time of flight (MALDI-TOF) and electrospray ionization (ESI) using post-source decay (PSD), ion trap, and alkaline phosphatase treatments. All of these methods rely on identifying signature loss of phosphate forms (see Table 1).[4,5]

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^-)

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

The PHOS-Select Iron Affinity Gel may not be efficiently regenerated. Therefore, it is best utilized in a single-use application. It is recommended to read the entire technical bulletin prior to starting the procedure.

Storage/Stability

The resin is supplied as a ~50% suspension in 50% glycerol buffered at pH 5.0. For optimal performance, the product as supplied should be stored at –20 °C. Under these storage conditions, the product is stable for at least two years. Once the stabilizing buffer containing glycerol is removed by washing of an aliquot, it is suggested the portion be used within 4 hours.

Procedure

Binding of phosphocompounds is highly pH-dependent. The suggested working pH range is 2.5–3.0. However, binding can occur over the pH range of 2.0–5.5. Optimally, the sample should have a low ionic strength (final concentration of 250 mM acetic acid) to obtain the best results. 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 performance.[3] A 250 mM acetic acid solution containing 30% acetonitrile is recommended to minimize non-specific interactions.

Some researchers have attempted complete methyl esterification of peptide carboxyl groups to improve specificity, with reported success.[6] Although promising, this step has not been incorporated into this procedure, as there are difficulties with interpretation of incomplete esterification and other potential degradation processes. The recommended procedure is optimized to minimize binding of acidic peptides.

The PHOS-Select Iron Affinity Gel can be utilized either in batchwise or column formats. The procedure describes a batchwise format using the SigmaPrep™ Spin Columns (Catalog Number SC1000). This kit contains 25 SigmaPrep Spin Columns with frits packaged in collection tubes (Catalog Number H6787). There are 50 additional collection tubes (Catalog Number T7813) and 25 end caps (Catalog Number V2014). Several publications have cited use of P9740.[7-12]

Binding and Elution of phosphopeptides or other phosphocompounds

1. Preparation of samples obtained from a protein tryptic digest.
- Prepare a sample solution with up to 250 mM acetic acid with 30% acetonitrile.
- If necessary, adjust pH to 2.5–3.0 with 1 M HCl solution.
- A sample of up to 1 μmole of phosphopeptide per mL of packed gel is suitable.

2. Wash/equilibration of affinity gel:
- Carefully mix the PHOS-Select Iron Affinity Gel beads until they are completely and uniformly suspended.
- Immediately add 80 μL of the 50% slurry (40 μL of gel) into a clean SigmaPrep Spin Column that has been placed in a collection tube (Catalog Number H6787).
- To dispense beads, use a wide orifice pipette tip or cut ~1 mm off the end of a regular pipette tip to allow unrestricted flow of the bead suspension.
- Add 500 μL of wash/equilibration solution (250 mM acetic acid with 30% acetonitrile) to the tube.
- Vortex, and centrifuge in a microcentrifuge for 30 seconds at 8,200 × *g* (10,000 rpm in an Eppendorf® 5415C microcentrifuge).
- Discard the flow-through liquid.
- Repeat step 2 twice.
- Save the collection tube for steps 4 and 5.

3. Sample Loading:
- Place an end cap (Catalog Number V2014) onto the column outlet and place the column in a new collection tube (Catalog Number T7813).
- Add up to 500 μL of the sample solution to the equilibrated gel.
- Incubate 15–30 minutes with mixing (end-over-end rotation recommended).
- After incubation, remove the end cap and centrifuge as described in step 2. The flow-through liquid in the collection tube contains unbound peptides, which may be pooled with the washes from step 4 and analyzed further.

4. Affinity Gel Wash: Wash Equilibration Solution
- Place the column in the collection tube saved from step 2.
- Add 500 μL of wash/equilibration solution (250 mM acetic acid with 30% acetonitrile) to the tube.
- Vortex, and centrifuge in a microcentrifuge for 30 seconds at 8,200 × *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 3 and analyzed further.

5. Affinity Gel Wash: Deionized Water
Wash the gel once with 500 μL of water to remove any residual wash/equilibration solution prior to elution.

6. Sample Elution:

- Place an end cap onto the column outlet and place the column in a new collection tube.
- Add 100–500 μL of elution solution (400 mM ammonium hydroxide). See Table 2 for alternative elution solutions.
- Incubate for ~5 minutes with mixing (end-over-end rotation recommended).
- After incubation, remove the end cap and centrifuge as described in step 2. Retain the flow through liquid for phosphopeptide analysis.
- Optimal elution conditions have to be determined empirically.

Table 2.
Alternative Elution Conditions

Elution Solutions	Comments
200 mM sodium phosphate, pH 8.4	Provides competitive displacement. Requires salt removal for MALDI-TOF-MS.[3]
150–400 mM ammonium hydroxide	Provides significant recovery of phosphocompounds. Compatible with MALDI-TOF-MS.[3]
150 mM ammonium hydroxide with 25% acetonitrile	Provides significant recovery of phosphocompounds. Compatible with MALDI-TOF-MS.

Resin Viability

Resin viability may be tested with a phosphopeptide positive control of the researcher's choice. Follow the procedure for binding and elution of the phosphopeptides. Elution may be accomplished with 400 mM ammonium hydroxide. Capacity and recovery may be assessed using HPLC-MS.

Optimization of Results

Selectivity of binding, binding capacity, and recovery of molecules of interest can be optimized using the following recommendations:

1. Nonspecific Binding

Factors that may contribute to nonspecific binding are ionic and hydrophobic interactions. The metal chelate ligand has a positive charge (+1). To avoid binding of acidic peptides, adjust the pH of the sample to 2.5–3.0, which will result in the protonation of the majority of glutamic and aspartic acid residues. Load and wash at the recommended (250 mM) acetic acid concentration. To minimize other nonspecific interactions, acetonitrile in the sample and wash solution is recommended at up to 30%.

2. Gel Binding

Binding of phosphopeptides to the resin is time and concentration dependent. An incubation of 15 minutes may be sufficient. However, the incubation time may be increased up to 2 hours for incubations at 18–30 °C to ensure efficient binding of phosphopeptides from complex mixtures.

If the binding capacity of the gel is exceeded, there may be preferential binding of multiple phosphorylated compounds at the exclusion of monophosphorylated compounds. It is recommended to add the sample at varying concentrations to optimize capture of all phosphorylated compounds.

3. Recovery of Captured Molecules

Efficient recovery of phosphorylated molecules may be enhanced by optimization of the elution parameters. Maximal recovery is attained if the solution is allowed to incubate while mixing with the gel for 5 minutes. Longer incubation in basic solution may actually decrease recovery.

Elution with 400 mM ammonium hydroxide provides efficient recovery of both monophosphorylated and multiple phosphorylated molecules.

For direct analysis by MALDI-TOF-MS, compounds eluted with high (>150 mM) ammonium hydroxide may be neutralized with formic acid.

References

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Related Products	Catalog Number
SigmaPrep Spin Columns	SC1000
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ProteoPrep Reduction and Alkylation Kit	PROTRA
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Ammonium Bicarbonate	A6141
ProteoMass™ MALDI-MS Calibration Kits	
Protein and Peptide	MSCAL1
Peptide	MSCAL2
Protein	MSCAL3
Phosphatase Inhibitor Cocktail I	P2850
Phosphatase Inhibitor Cocktail II	P5726
Formic acid	F0507

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