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## ProteoExtract™ Phosphopeptide Capture Kit

**Cat. No. 525250**

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### 1. Introduction

In eukaryotic cells, post-translational modifications of proteins such as phosphorylation and dephosphorylation are involved in numerous metabolic pathways and the transmission of signals that control proliferation, differentiation and apoptosis. However, if the tightly controlled balance between phosphorylation and dephosphorylation becomes deregulated, it may lead to serious pathological conditions. Therefore, phosphoproteome analysis is important for elucidating the underlying mechanisms.

Mass spectrometry of phosphopeptides has become a powerful tool for phosphorylation site identification. However, there is a general need for more specific and efficient enrichment strategies for phosphorylated peptides in order to compensate for the low abundance of phosphopeptides, poor ionization and ion suppression effects. Immobilized metal ion affinity chromatography (IMAC) can be used to enrich for phosphorylated polypeptides without any bias towards specific phosphorylated amino acids.

The ProteoExtract™ Phosphopeptide Capture Kit is a dedicated tool to meet those requirements. The kit enables for selective capture of phosphorylated peptides derived from enzymatic digests of protein samples with high yield. Taking advantage of a novel chelate ligand, phosphopeptides are captured through specific interaction of phosphate groups with immobilized Zirconium (IV) ions on the surface of magnetic particles. The optimized buffers allow for high yield of isolated phosphopeptides. The elution buffer is directly compatible with downstream analysis by MALDI- and ESI mass spectrometry.

The magnetic particle format of the ProteoExtract™ Phosphopeptide Capture Kit allows for a convenient and fast process that yields phosphopeptides in 30 minutes ready for mass spectrometry analysis. A detailed protocol for phosphopeptide isolation is provided.

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**Kit Components (Sufficient for up to 100 phosphopeptide isolations each with a capacity of 2.5 nmol phosphopeptide/50 µl MagPrep<sup>®</sup> PhosphoBind beads)**

- 1) **Phospho Binding buffer** (Cat. No. KP31490)  
1 vial: 25 ml buffer
- 2) **Wash buffer 1** (Cat. No. KP31491)  
1 vial: 28 ml buffer
- 3) **Wash buffer 2** (Cat. No. KP31492)  
1 vial: 28 ml buffer containing Acetonitrile
- 4) **Elution buffer** (Cat. No. KP31493)  
1 vial: 14 ml buffer
- 5) **MagPrep<sup>®</sup> PhosphoBind** (Cat. No. KP31494)  
1 vial: 6ml of black particle suspension

## 2. Storage Conditions

For long term storage the test kit should be stored at 4° - 8°C. Refrigerated all kit components are stable for at least 12 months.

Do not freeze the MagPrep<sup>®</sup> PhosphoBind particles. Freezing-thawing cycles will destroy the particles' shape which results in loss of function.

For convenience all buffers provided with the kit may be stored at room temperature. Buffers are stable for at least 12 months.

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### 3. Technical Hints and Preparation Instruction

**3.1** All buffers provided in the kit are ready to use. All buffers should be equilibrated to room temperature prior to use.

**3.2** The vial designated MagPrep<sup>®</sup> PhosphoBind contains a particle suspension in storage buffer. For pipetting or aliquoting magnetic particles from the vial, gently mix the MagPrep<sup>®</sup> particles by inversion until completely suspended. The suspension can be considered homogeneous when no black sediment can be detected at the bottom of the vial.

**3.3** MagPrep<sup>®</sup> PhosphoBind particles can be separated using magnetic force or gravity. Any commercially available magnetic stand or separator that accommodates 1.5 ml microcentrifuges can be used (e.g. Magnetight stands by Novagen, Cat.No.69964-3 or Cat.No.70747-3).

Magnetizing is carried out by placing the test tube in the magnetic stand for 1-2 minutes. While pipetting solutions away from magnetic pellets the test tube stays inserted in the stand.

Demagnetizing is carried out by removing the test tube from the magnetic stand.

If no magnetic separator is available, a benchtop centrifuge may be used alternatively to separate the particles. Centrifuge the test tube for 1 min at 1500 – 2000x g. Do not centrifuge at higher g-values during the washing steps as this will lead to aggregation of the particles and incomplete resuspension.

**3.4** MagPrep<sup>®</sup> PhosphoBind particles are preactivated with Zirconium (IV) –ions and are stable when stored at +4 °C.

### 4. Reagents and Equipment not provided

- Micropipettes and tips, 10 µl, 200 µl and 1 ml size
- Microcentrifuge and rotor for >10,000x g and 1.5 ml tube size
- Magnetic stand or separator (optional)

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## 5. Protocol: Isolation of Phosphopeptides

### Before you start:

- Dilute 10-20µl of the sample with 100-200µl of **Phospho Binding Buffer**

(Samples that have been tryptic digested usually have a basic pH. To adjust pH for optimal binding conditions, samples must be diluted 10-fold in Phosphopeptide Binding Buffer prior to capture)

- All reagents should be warmed to room temperature.

1. Mix **MagPrep<sup>®</sup> PhosphoBind** particles by inversion until completely suspended.
2. Transfer 50 µl of the homogeneous particle suspension to a microcentrifuge tube and place the tube in a magnetic separator (or a centrifuge, see 3.3). Remove the supernatant carefully.
3. Add the diluted sample to the particles, demagnetize and mix by gentle vortexing.
4. Incubate with gentle agitation for 10 min. The use of a mixer at 1000 rpm is recommended. Alternatively, vortex the tube briefly several times during the incubation time.
5. Magnetize for 1-2 min. Remove supernatant (the supernatant can be discarded or alternatively saved for further analysis of unbound peptides).
6. Demagnetize and resuspend the particles in 100µl **Wash Buffer 1** by gentle vortexing.
7. Magnetize for 1-2 min. Remove the supernatant and discard.
8. Repeat steps 6 and 7.
9. Demagnetize and resuspend the beads in 100µl **Wash Buffer 2** by gentle vortexing.
10. Magnetize for 1-2 min. Remove the supernatant and discard.
11. Repeat steps 9 and 10.
12. In order to reduce the volume, centrifuge the tube for 1 min at 1000 – 2000x g. Remove the supernatant completely and discard.
13. Add 25µl of **Elution Buffer** and resuspend the particles by gentle vortexing.
14. Incubate with gentle agitation for 10 min. The use of a mixer at 1000 rpm is recommended. Alternatively, vortex the tube briefly several times during the incubation time.
15. Centrifuge the tube for 5 min at 10.000x g. Carefully transfer the supernatant to a new tube and save it for further analysis of the captured and purified phosphopeptides. Placing the tube into a magnetic separator after centrifugation will help to avoid pipetting of the MagPrep<sup>®</sup> PhosphoBind particles.

The protocol can easily be scaled-up or down. For samples with low phosphopeptide levels the volume of the particle suspension can be reduced to a minimum of 20 µl. For high phosphopeptide levels (more than 2.5 nanomoles) the volume of bead suspension must be increased accordingly. Decrease or increase the amount of elution buffer according to the changes in the particle suspension volume in step 2.

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## 6. Technical Appendix

### 6.1 Sample preparation for MALDI mass spectrometry

For MALDI mass spectrometry prepare the solution containing isolated phosphopeptides for analysis as follows:

- Matrix solution: 10 mg alpha-cyano-4-hydroxycinnamic acid in 1ml 70 % (v/v) acetonitrile in water; 0.1 % (v/v) TFA
- Mix 1 µl sample with 10 µl matrix solution
- Transfer 1 µl of sample/matrix mixture onto MALDI target plate
- After complete drying of the sample spot, carefully add a droplet of 1 µl high quality water to the sample spot and let sit on the spot for 2 sec.
- Carefully remove the water droplet using a pipette.
- The sample is now ready for MALDI mass spectrometry analysis.

MALDI mass spectrometry was successfully performed using different instrumentations e.g. Bruker Reflex and Ultraflex instruments, according to manufacturer's recommendation.

### 6.2 Sample preparation for ESI-LC/MS mass spectrometry

ESI-LC/MS mass spectrometry was performed as follows:

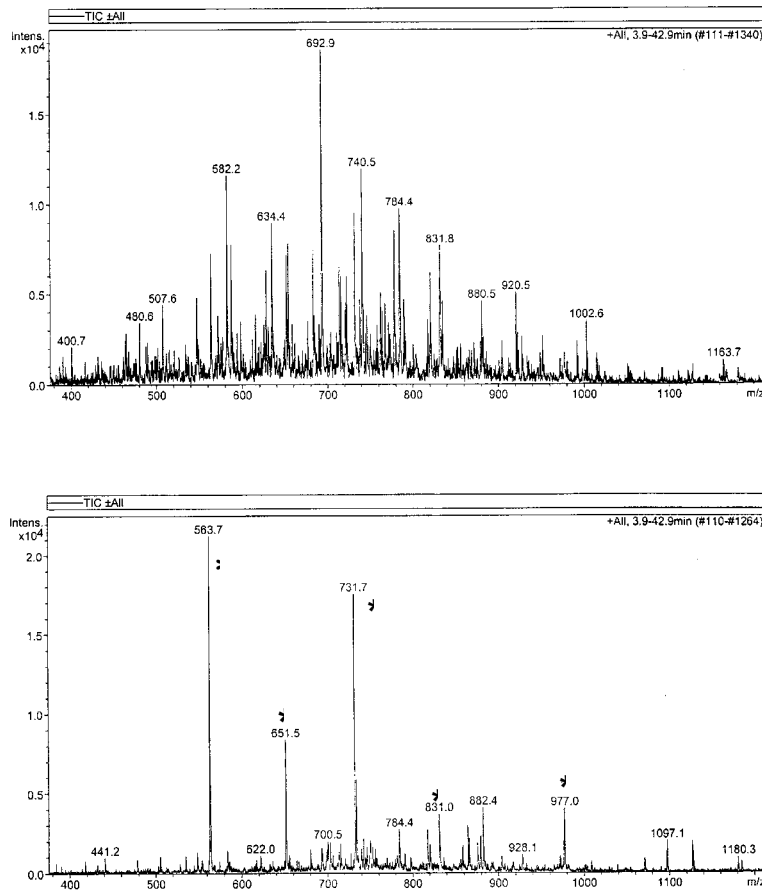
1-5µl sample volumes (picomol to low-femtomol range) were separated in gradient mode on silica-based 100 µm I.D. Chromolith™CapRod™ RP18e biocompatible monolithic capillaries (150 x 0,1mm, Merck KGaA, Darmstadt, Germany) using capillary/nano LC equipment. The mobile phases consisted of 0.1% formic acid in water/acetonitrile (98%:2%, v:v) and 0,08% formic acid in water/acetonitrile (20%:80%, v:v). For MS/MS the separated peptides were analyzed on-line by an ESI ion trap mass spectrometer (Esquire 3000plus, Bruker Daltonics, Bremen, Germany). The mass spectrometer was operated in the positive ion mode at a scan speed of 13,000 u/s with a peak width of less than 0.6 u (allowing clear resolution of singly and doubly charged ion species) in an m/z range of 100 - 3000. The peptides were fragmented using auto-MS/MS, active exclusion and two precursor ions.

### 6.3 Application example

Figure 1 demonstrates the efficiency and specificity of phosphopeptide capturing and analysis by ESI-LC/MS analysis. A mixture containing equimolar amounts of bovine albumin, bovine histone IIB1 and bovine alpha-casein was digested with trypsin using the ProteoExtract™ All-in-One Trypsin Digestion Kit (Calbiochem Cat. No. 650212). The resulting peptide mixture was subsequently spiked with two synthetic phosphopeptides, one phosphorylated at Serine (RII: DLDVPIGRFDRRVpSVAAE) and the other phosphorylated at Tyrosine (Angiotensin: DRVPpYIHPF). Following phosphopeptide capture, five predominant phosphopeptide ions could be clearly identified and assigned (see table 1). The

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observed background binding of non-phosphorylated peptides was particularly low with most non-phosphorylated peptides being completely removed.



**Figure 1: The ProteoExtract™ Phosphopeptide Capture Kit allows for efficient and selective phosphopeptide capture from complex mixtures.** A complex peptide mixture derived from a tryptic digest of bovine albumin, histone IIB1 and alpha-casein was spiked with two synthetic phosphopeptides and subsequently processed using the ProteoExtract™ Phosphopeptide Capture Kit. Mass spectrometry analysis was performed as described using an ESI-LC/MS equipment operated in positive mode. Only monophosphorylated phosphopeptides were detectable under these conditions. The predominant signals derive from phosphopeptide ions and are marked with asterisks.

**Table 1. Assignment of phosphopeptide ions to amino acid sequences**

No.	m/z	Sequence	Ion	Protein / Peptid
1	563,7	DRV <sub>p</sub> YIHFP	[M+2H] <sup>+2</sup>	[Tyr(PO <sub>3</sub> H <sub>2</sub> ) <sup>4</sup> ]-Angiotensin II
2	651,5	YKVPQLEIVN <sub>p</sub> SAEER	[M+3H] <sup>+3</sup>	α- S1- Casein
3	731,7	DLDVPIPIGRFDRRV <sub>p</sub> SVAAE	[M+3H] <sup>+3</sup>	RII-peptid
4	831.0	VPQLEIVN <sub>p</sub> SAEER	[M+2H] <sup>+2</sup>	α- S1- Casein
5	977.0	YKVPQLEIVN <sub>p</sub> SAEER	[M+2H] <sup>+2</sup>	α- S1- Casein

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