PerkinElmer Life and Analytical Sciences, Inc.

PHOS-TRAP™ 24
PHOSPHOPEPTIDE ENRICHMENT KIT

CATALOG NUMBER PRT302001KT

For Laboratory Use
CAUTION: Research Chemicals for Research Purposes Only

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I. PRODUCT NAME

Phos-trap™ 24 Enrichment Kit

(Cat. # PRT302001KT)

II. INTENDED USE

The Phos-trap™ Phosphopeptide Enrichment Kit is a simple and reliable tool for enrichment of phosphopeptides from biological samples, such as human serum and protein digests. The enriched samples are compatible and ready for downstream analysis of peptides by mass spectrometry. A magnet is included for serial processing of samples in microcentrifuge tubes. The flexible kit format is also adaptable for enrichment of phosphopeptides in 8-well strips or other suitable lab ware.

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III. INTRODUCTION

Protein phosphorylation is a reversible post-translational modification that forms the basis of cell signaling networks. Many cellular processes are regulated by the reversible phosphorylation of proteins and upwards of 30% of the proteins comprising the eukaryotic proteome are likely to be phosphorylated at some point during their existence[1]. The determination of the phosphorylation state of proteins is important with respect to defining protein kinase substrates, as well as revealing the activation state of signal transduction pathways.

These in turn have important implications with respect to the understanding of pathophysiological processes, such as cancer and other diseases.

Studying phosphorylated peptides in complex biological samples presents significant challenges due to their low abundance in the total proteome as well as poor ionization and ion suppression effects during mass spectrometric analyzes. Therefore, there is a need for robust and selective phosphopeptide enrichment tools. An ideal affinity support for phosphopeptide capture would likely be antibody-independent, able to measure phosphorylation of any type of substrate, and exhibit desirable product attributes, such as easy manufacturability, room-temperature storage, and prolonged shelf-life stability.

Immobilized metal ion affinity chromatography (IMAC)[2, 3] is based upon preferential ionic interactions between phosphomonoester groups on phosphopeptides and chelated divalent or trivalent transition metal ions, like Zn (II), Ga (III) and Fe (III)[4, 5], immobilized on a stationary phase. However, enrichment and recovery of phosphopeptides using an IMAC system strongly depends on the type of metal ion and column material, and is often hampered by the non-selective enrichment of acidic peptides[6]. Chemical modifications of peptide samples by methyl esterification are often required to improve the selectivity of enrichment[7]. Additionally, IMAC systems are at a disadvantage being based upon labile metal ion-organic chelator interactions, often resulting in leaching of metal ions during fractionation or storage.

Recently, metal oxide affinity chromatography (MOAC) was successfully applied for selective enrichment of phosphopeptides using aluminum, titanium, zirconium and other metal oxides[8-12]. MOAC circumvents many of the problems inherent to the IMAC systems and is simpler to use, maintain and does not require chelation of metal ions.

The Phos-trap™ 24 Enrichment Kit is based on robust enrichment of phosphopeptides using titanium dioxide coated magnetic beads. Fully optimized buffers and assay protocols allow efficient fractionation of complex biological samples, such as serum or protein digests containing complex mixtures of peptides, in less than 10 minutes. The resulting samples are ready to be analyzed by mass spectrometry or other detection tool of choice. The magnetic bead format offers additional advantages of sample fractionation without centrifugation or complex filtering equipment and is easily adaptable for automation on standard liquid handling equipment for hands-off high-throughput applications.

IV. KIT COMPONENTS AND STORAGE

Reagents supplied are intended FOR LABORATORY USE ONLY.

Table 1. Phos-trap™ Phosphopeptide Enrichment Kit components

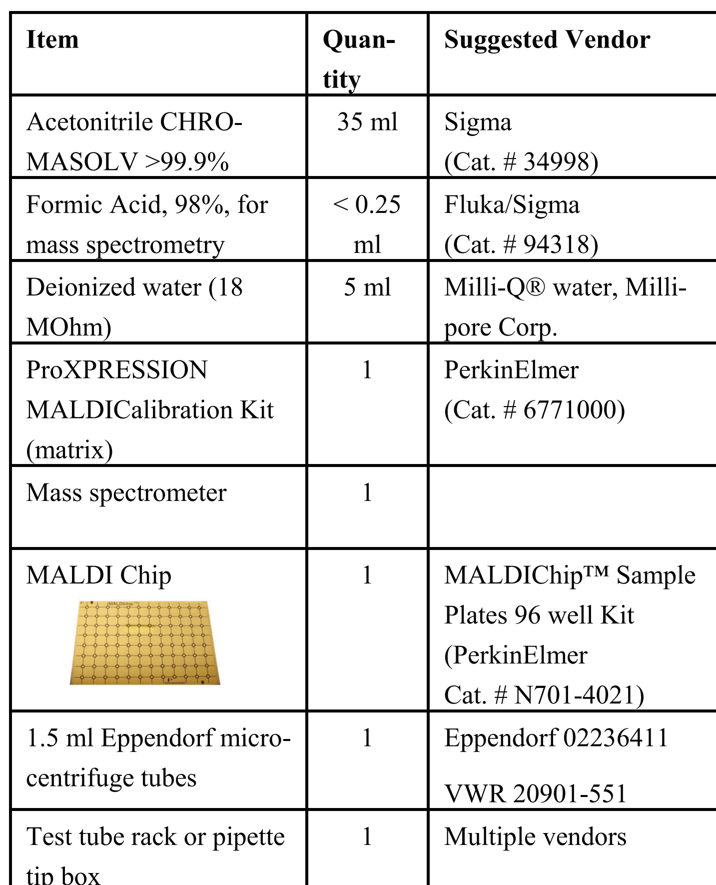
Kit Component	Quantity
20x Phos-trap™ Magnetic Beads	**1 vial: 0.25 ml**
Binding Buffer	**1 bottle: 100 ml**
Washing Buffer	**1 bottle: 25 ml**
Elution Buffer	**1 bottle: 3 ml**
Ring Magnets	**2**
Manual	**1**

Storage and Stability

All Phos-trap™ Phosphopeptide Enrichment Kit components should be stored at 4º - 8ºC. Kits are shipped at ambient temperature. Reagent expiration dates are listed on the product box label.

Do not freeze Phos-trap™ Magnetic Beads. Freeze-thawing may result in reduced performance or loss of function.

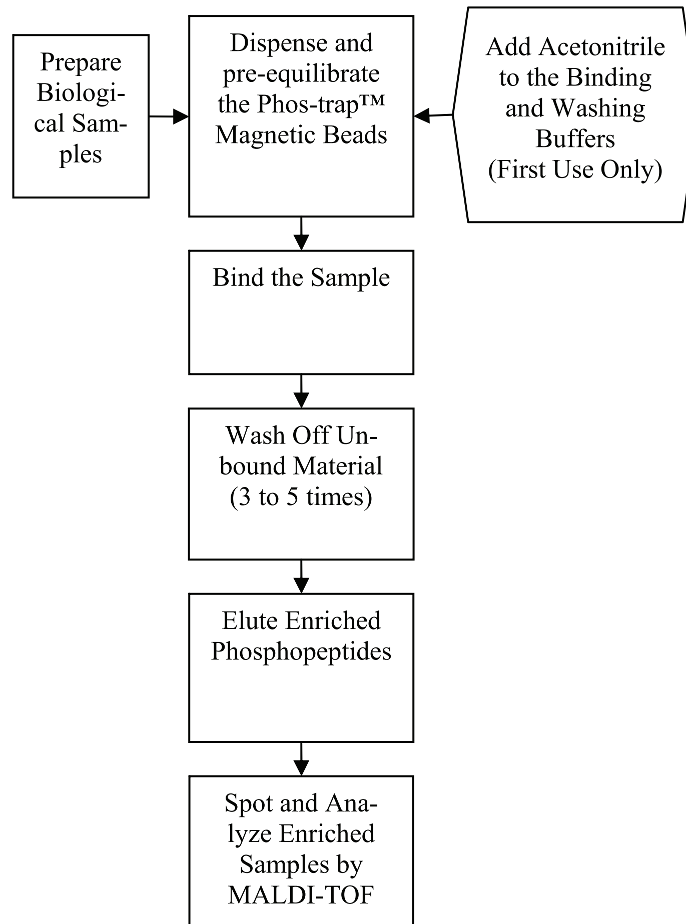
Table 2. User-provided materials



Item	Quan-tity	Suggested Vendor
Acetonitrile CHRO-MASOLV >99.9%	35 ml	Sigma (Cat. # 34998)
Formic Acid, 98%, for mass spectrometry	< 0.25 ml	Fluka/Sigma (Cat. # 94318)
Deionized water (18 MOhm)	5 ml	Milli-Q® water, Milli-pore Corp.
ProXPRESSION MALDICalibration Kit (matrix)	1	PerkinElmer (Cat. # 6771000)
Mass spectrometer	1	
MALDI Chip	1	MALDIChip™ Sample Plates 96 well Kit (PerkinElmer Cat. # N701-4021)
1.5 ml Eppendorf micro-centrifuge tubes	1	Eppendorf 02236411 VWR 20901-551
Test tube rack or pipette tip box	1	Multiple vendors

VI. REAGENT PREPARATION AND ASSAY PROTOCOL

A. Assay workflow at a glance and general recommendations



General recommendations:

1. The 1.5 ml Eppendorf microcentrifuge tube with beads should always be taken off the ring magnet assembly when adding the buffers or the sample to the tube to allow better suspension of beads.

2. Although not critical, excessive drying of beads should be avoided when possible. Drying of beads in between the liquid handling steps typically had no effect on fractionation results for multiple model protein digests and serum samples tested.

3. It is recommended to leave 5 to 10 µl of supernatant in the microcentrifuge tubes when aspirating the solutions from the beads to minimize accidental aspiration of beads. However, the Washing Buffer should be completely removed from the beads before eluting the samples especially when low volume of Elution Buffer is used.

4. The eluted sample can be directly spotted on a MALDI target and analyzed using a mass spectrometer. Drying down of the eluted samples may or may not increase the detected peak intensities depending on the sample type and composition. However, it is recommended to dry the eluted samples for storage or if the sample volume is changing significantly between the samples due to the elution buffer evaporation during prolonged processing of large set of samples.

5. The sample fractions not bound to the beads can also be analyzed by mass spectrometry the same way as the eluted samples. However, the unbound fraction of serum samples is typically not suitable for direct analysis and requires further sample clean up, such as reverse phase desalting.

6. The following MALDI matrices were successfully used with this kit: (1) 5 mg/ml to 10 mg/ml CHCA matrix in aqueous solution of 50 % acetonitrile containing 1% formic acid; (2) 20 mg/ml DHB matrix in aqueous solution of 50% acetonitrile containing 1% formic acid and 1% phosphoric acid. Other MALDI matrixes can also be used.

For LC-MS analysis, samples should be dried to about 20% of the elution volume, resuspended in 1% formic acid and desalted. Please see chapter XI for more information.

B. Sample Preparation

The Phos-trap™ 24 Enrichment Kit can be used for enrichment of phosphopeptides from at least two types of samples: (1) peptide mixtures derived from the digests of pre-fractionated proteins and (2) serum or plasma samples. Typically, 100 fmol to 1 nmol of the purified protein digest or 5 to 50 µl of human serum can be used for successful enrichment of phosphopeptides.

Prepare serum samples by high speed centrifugation to remove insoluble material followed by aspiration of the lipid layer to remove interfering phospholipids. No additional pre-fractionation of serum samples is typically required.

Phosphate buffers should be avoided for reconstituting peptide samples because phosphate anions may interfere with the selective enrichment of phosphopeptides.

High concentrations of acidic peptides may reduce the efficiency of phosphopeptide enrichment and detection results due to minor interaction of carboxyl groups with the TiO2 surface. Complex samples like cell lysate digests typically include high levels of acidic peptides and often require methylation of carboxyl groups prior to enrichment by Phos-trap. See chapter IX for a detailed procedure

C. Preparation of buffers (first use only)

Note: Skip this section if acetonitrile was already added to the vials of the Binding and Washing buffers in earlier experiments.

1. Add 100 ml of mass spectrometry grade acetonitrile to the vial of Binding Buffer. Mix the resulting contents of the vial. The container should be closed tightly to prevent evaporation of acetonitrile.
2. Add 25 ml of mass spectrometry grade acetonitrile to the vial of Washing Buffer. Mix the resulting contents of the vial. The container should be closed tightly to prevent evaporation of acetonitrile.

D. Dispensing and pre-equilibration of the Phos-trap™ Magnetic Beads

1. Estimate the number of fractionations to be performed and calculate the required volume of Phos-trap™ Magnetic Beads suspension as (number of fractionations + 1) x 10 µl = µl. The additional fractionation is added to compensate for any minor pipetting errors. Only the required amount of beads should be diluted as the diluted beads may not be stable for a prolonged storage period and thus should be used the same day.
2. Dispense (number of fractionations +1) x 190 µl = µl of deionized water into a 1.5 ml centrifuge tube (Table 1).

3. Mix the contents of the 20 x Phos-trap™ Magnetic Bead vial well, ensuring that all beads are uniformly dispersed. Repeated inversion or gentle vortexing of the vial should be sufficient for efficient dispersion of the beads.

4. Align the two ring magnets, one next to the other, as shown in Figure 1. Note that the magnets are brittle and should not be dropped or allowed to accelerate into one another uncontrollably. Also, care must be taken not to get your fingers caught between the magnets, as injury may result.

Figure 1. Proper alignment of ring magnets.

5. Place the stacked ring magnets onto the top of a pipette tip box, or test tube rack and insert the 1.5 ml microcentrifuge tube into the orifice of the stacked magnets, as shown in Figure 2. Smaller microcentrifuge tubes will not fit properly in the magnet

Figure 2. Proper placement of tube in ring magnet assembly.

6. Pipette the dispersed 20 x Phos-trap™ Magnetic Bead suspension into the 1.5 ml microcentrifuge tube with water prepared in step 2 of this section. It is recommended to cut off the narrow end of the pipette tip if smaller tips are used to ensure accurate pipetting of beads.
7. Mix the diluted beads well by closing the 1.5 ml microcentrifuge tube and repeatedly inverting it.
8. Dispense 200 µl of the diluted bead suspension per tube. Beads tend to sediment over time and require periodic mixing in between the bead dispensing steps.
9. Place the 1.5 ml microcentrifuge tube with beads into the orifice of the stacked ring magnets (Table 2) for 1 min. The beads will concentrate on the wall of the tube that is in closest contact with the magnet.
10. Aspirate the supernatant in the center of each tube, near the bottom.
11. Remove the tube with beads from the stacked ring magnet assembly and pre-equilibrate the beads with the previously prepared Binding Buffer (Section C, Step 1) by dispensing 200 µl of this buffer per tube. Fast dispensing of beads is typically sufficient to adequately re-suspend the beads in the tube.
12. Repeat steps 9 through 11 in this section two more times to rinse the beads thoroughly.

E. Enrichment of Phosphopeptides

1. Prepare MALDI matrix as suggested in General Recommendations, Step 8. (if needed).
2. Dilute the peptide mix or serum sample 1:10 with Binding Buffer containing acetonitrile. Typically, 10 µl to 100 µl of the sample, diluted in Binding Buffer, is applied to the beads.
3. Remove the tube with the pre-equilibrated beads from the magnet, add the peptide sample, diluted in Binding Buffer, to the beads, and mix the contents of the tube by pipetting the sample up and down.

4. Place the tube in the orifice of the stacked ring magnet assembly for 1 min and aspirate the supernatant containing the peptides not bound to the Phos-trap™ magnetic beads.

5. Remove the tube from the stacked magnet assembly and wash the beads with 200 µl of Binding Buffer. Aspirate the buffer as described in step 4 of this section.

6. Wash the beads as described in steps 4 and 5 three more times for relatively clean protein digests. Up to five washes of beads may be required for more complex biological samples such as human serum.

7. Wash the beads with 200 µl of Washing Buffer and discard the supernatant as described in step 4 of this section. For best performance, the Wash Buffer should be completely removed before proceeding to the sample elution step.

8. Remove the tube from the stacked ring magnet assembly. To elute the samples bound to the beads, add 5-10 µl of Elution Buffer to each tube. Smaller than 10 µl elution volumes can be used to increase the sensitivity levels. Caution should be taken to ensure good wetting of beads and avoiding contamination of the eluted sample with the beads.

9. Place the tube in the orifice of the stacked ring magnets for 1 min. and carefully aspirate the supernatant containing the eluted samples. Care should be taken to avoid aspirating the beads. Excessive amount of beads may interfere with peptide analysis by mass spectrometry.

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10. Mix equal volumes (0.5µl to 3µl) of an eluted sample and the 10 mg/ml CHCA MALDI matrix directly on a MALDI chip, allow the samples to crystallize, and analyze them using a MALDI-TOF mass spectrometer. Alternatively, the eluted samples can be dried down in the tube, resuspended in 10 µl of the 5 mg/ml CHCA MALDI matrix solution and spotted on a MALDI chip for analysis.

    For LC-MS analysis using reverse phase chromatography (e.g. C18), samples should be dried to about 20% of the elution volume, re-suspended in 5-10 µl of 1% formic acid and desalted. Please see chapter XI for more details.

VII. REPRESENTATIVE RESULTS

A. Enrichment of phosphopeptides from the tryptic digests of bovine β-casein and ovalbumin.

A peptide mix containing about 10 pmol of each of the β-casein tryptic peptides was fractionated using the Phos-trap™ Phosphopeptide Enrichment Kit. Total tryptic digest as well as the peptide fraction not bound to the Phos-trap™ magnetic beads and the enriched phosphopeptide fraction were analyzed using the prOTOF™ 2000 MALDI O-TOF mass spectrometer. The resulting spectra are depicted in Figure 1.

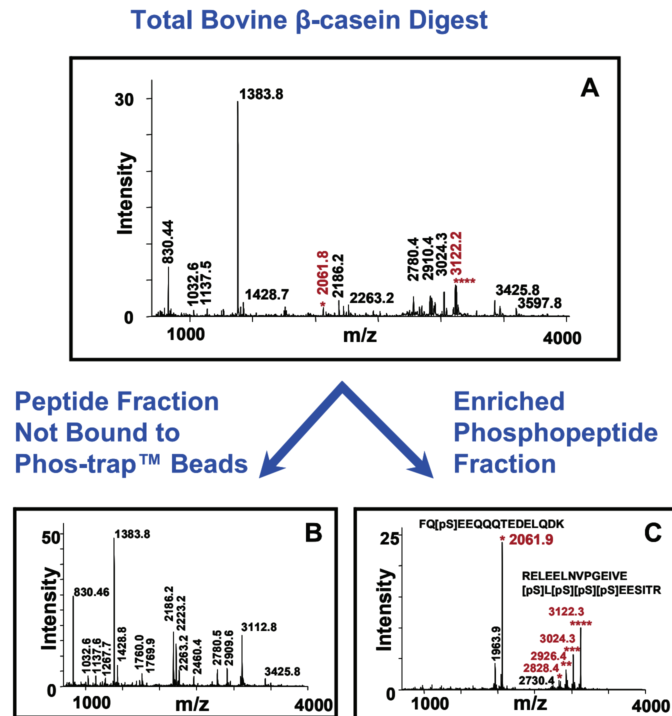

Figure 1. Enrichment of mono- and tetra-phosphorylated bovine β-casein peptides using the Phos-trap™ Phosphopeptide Enrichment Kit. MS spectra corresponding to the total β-casein digest, the peptide fraction not bound to Phos-trap™beads, and the enriched phosphopeptide fraction are depicted in Panels A, B, and C respectively. The stars above the peaks indicate the number of phosphate groups for the corresponding peptide. The enriched phosphopeptide sequences are shown in Panel C. The phosphoserine residues are abbreviated to pS.

Both mono-phosphorylated and tetra-phosphorylated peptides were selectively enriched while non-phosphorylated peptides were recovered in the fraction not bound to the beads.

Similarly, the phosphorylated peptides were selectively enriched from the ovalbumin tryptic digest using the Phos-trap™ Phosphopeptide Enrichment Kit (Figure 2).

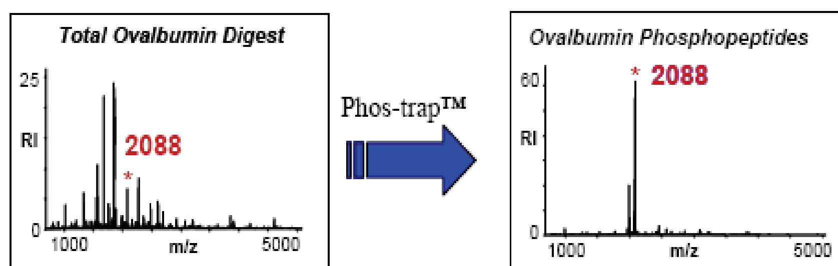

Figure 2. Enrichment of the major phosphorylated ovalbumin peptide (*) using the Phos-trap™ Phosphopeptide Enrichment Kit. RI = Relative Intensity.

B. Enrichment of phosphopeptides from human serum samples.

Ten microliters of normal human serum sample were diluted 1:10 with Binding Buffer and were directly fractionated using the Phos-trap™ Phosphopeptide Enrichment Kit. The resulting MALDI-TOF spectrum is depicted in Figure 3. Only the spectrum of the enriched fraction is shown as no significant peaks could be detected for the unfractionated total serum sample. The paired peaks with mass difference of 80 Da or 98 Da likely represent the phosphoric acid-related mass losses during the MALDI acquisition. These peaks were sequenced on an ABI QSTAR® MS/MS instrument, confirming that the enriched peptides were indeed phosphorylated (data not shown).

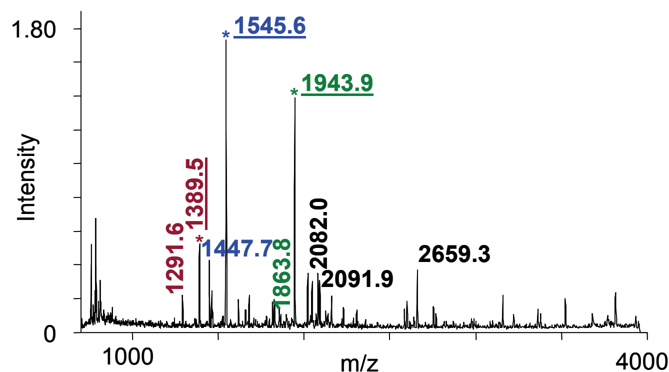

Figure 3. MALDI-TOF spectrum representing an enriched fraction of peptides from10 µl of normal human serum using the Phos-trap™ Phosphopeptide Enrichment Kit. The putative phosphorylated peptides identified by the presence of the corresponding de-phosphorylated during a MALDI acquisition peptide peaks (at -80 Da or -98 Da from the main peak) are marked with an asterisk.

VIII. TROUBLESHOOTING GUIDE

Possible Problem	Possible reason	Suggestions/ Solutions
No peaks could be detected by MALDI-TOF MS when fractionating a peptide mix derived from a digest of proteins	Low quality of acetonitrile or other user-supplied reagents	Use the reagents recommended in the manual
	Proteins insufficiently digested; the peptide sample is degraded.	Validate the protein digest by mass spectrometric analysis of a total digest
	Sample contains endogenous phosphate	Dialyze or desalt the protein samples before tryptic digestion
	The amount of analytes in the sample is below the detection limit	Increase the amount of fractionated sample
	Presence of beads in the eluted sample or loss of beads occurred during the fractionation.	Avoid aspirating the beads when eluting the samples. Use recommended magnet.

Possible Problem	Possible reason	Suggestions/ Solutions
No peaks could be detected by MALDI-TOF MS when fractionating a serum sample	Low quality of acetonitrile or other user-supplied reagents	Use the reagents recommended in the manual
	High content of phospholipids or other sources of endogenous phosphates.	Centrifuge the serum samples and aspirate the top lipid layer
	Contaminant level is too high	Decrease the amount of serum fractionated or increase the amount of beads used for fractionation
	The amount of analytes in the sample is below the detection limit.	Increase the amount of fractionated sample
	Presence of beads in the eluted sample or loss of beads occurred during the fractionation.	Avoid aspirating the beads when eluting the samples. Use recommended magnet
Peak intensities between the replicates vary significantly	Inconsistent dispensing of beads between tubes	Follow the recommendations in the protocol
	Loss of beads occurs while performing an assay	Use the recommended magnets, plates and liquid handling procedures
	Inconsistent liquid handling procedures	Follow the optimized protocol in the manual
	Presence of beads in the eluted sample	Avoid aspirating the beads when eluting the samples

Possible Problem	Possible reason	Suggestions/ Solutions
Significant non-selective enrichment of non-phosphorylated peptides	No acetonitrile was added to the Binding and Washing buffers	Add the amounts of acetonitrile recommended in the manual to the Binding and Washing buffers
	Acetonitrile concentration in the buffers is low	Close the buffer containers tightly to prevent evaporation of acetonitrile. Use the acetonitrile of the recommended in the manual quality in the properly stored containers
	Some highly acidic peptides may bind to the TiO_2 surfaces.	Increase the number of washes with Washing Buffer Methylate peptide carboxyl groups before enrichment on TiO_2
Low MS peak intensities	Interference from polymers leaching from plastic labware	Pre-wash tubes with acetonitrile
	Sample is degraded	Include phosphatases and protease inhibitors in sample preparation. Validate total peptide mix by MS analysis before fractionation using Phostrap™ Phosphopeptide
	Presence of beads in eluted sample	Avoid aspirating beads when eluting sample

IX. PROCEDURE FOR METHYLATION OF CARBOXYL GROUPS IN COMPLEX SAMPLES

The presence of significant amounts of highly acidic peptides may obscure the phosphopeptide enrichment and detection results due to minor interaction of carboxyl groups with the TiO_2 surface. It is recommended that carboxyl groups are methylated in complex peptide samples (e.g., cell lysate digests) before phosphopeptide enrichment using Phos-trap™ Magnetic Beads.

The following methylation protocol was adopted from the published procedure by Xu et al[13].

1. Prepare fresh the 2M methanolic HCl solution by drop wise addition of 160 µl of acetyl chloride (Fluka Cat # 00990) to 1 ml of anhydrous methanol (Sigma Cat # 322415).
2. Dry down or lyophilize a peptide sample and re-dissolve it in 50 µl of freshly prepared 2 M methanolic HCl. The volume of 2M methanolic HCl should be increased to 200 µl for samples exceeding 250 pmol of peptides.
3. Incubate the methylation reaction for 2 to 3 hours at room temperature.
4. Dry down or lyophilize the sample to remove the solvent.
5. Re-dissolve the peptides in 10 µl to 100 µl of Binding Buffer and proceed to enrichment of phosphopeptides using Phos-trap™ Magnetic Beads.

XI. Sample concentration and desalting using C18 ZipTip® and similar products

Sample concentration and desalting is not typically required, but may be beneficial when analyzing very low level phosphopeptides. The presence of organics in the elution buffer requires evaporation of the elution buffer before C18 chromatography.

1. After elution of peptides from Phos-Trap dry down the peptides completely or alternatively to 20% of the elution volume.

2. Re-suspend the sample in 18 M ohm deionized water containing 1% formic acid or 0.1% TFA

3. Your samples are now ready to apply to C18 ZipTips for concentration and desalting, please follow the manufacturer's recommended protocol. For best results avoid addition of chaotropic salts.

X. SAFETY CONSIDERATIONS

Wear appropriate gloves, protective clothing and eyewear and follow safe laboratory practices. Dispose of any materials in accordance with local, state and federal guidelines.

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XII. LICENSING

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XIII. NAME AND PLACE OF MANUFACTURE

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