

PerkinElmer Life and Analytical Sciences, Inc.



**PHOS-TRAP™ PHOSHOPEPTIDE
ENRICHMENT KIT**

CATALOG NUMBER PRT301001KT

**For Laboratory Use
CAUTION: Research Chemicals for Research Purposes Only**

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I. PRODUCT NAME

Phos-trap™ Phosphopeptide Enrichment Kit

(Cat. # PRT301001KT)

II. INTENDED USE

The Phos-trap™ Phosphopeptide Enrichment Kit is a simple and reliable tool for enrichment of phosphopeptides from up to 96 complex biological samples, such as human serum and protein digests. The enriched samples are compatible and ready for downstream analysis of peptides by mass spectrometry. The magnetic bead-based separation using the 96-well format is fast and can be performed manually or easily automated using standard liquid handling equipment. The flexible kit format is also adaptable for enrichment of phosphopeptides in spin tubes, 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 existence¹. 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 peptides⁶. Chemical modifications of peptide samples by methyl esterification are often required to improve the selectivity of enrichment⁷. 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 oxides⁸⁻¹². 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™ Phosphopeptide 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: 1 ml
Binding Buffer	1 bottle: 100 ml
Washing Buffer	1 bottle: 25 ml
Elution Buffer	1 bottle: 3 ml
96-well V-Bottom Polypropylene Plate	2
96-well Plate Cover	2
Manual	1

Storage and Stability

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

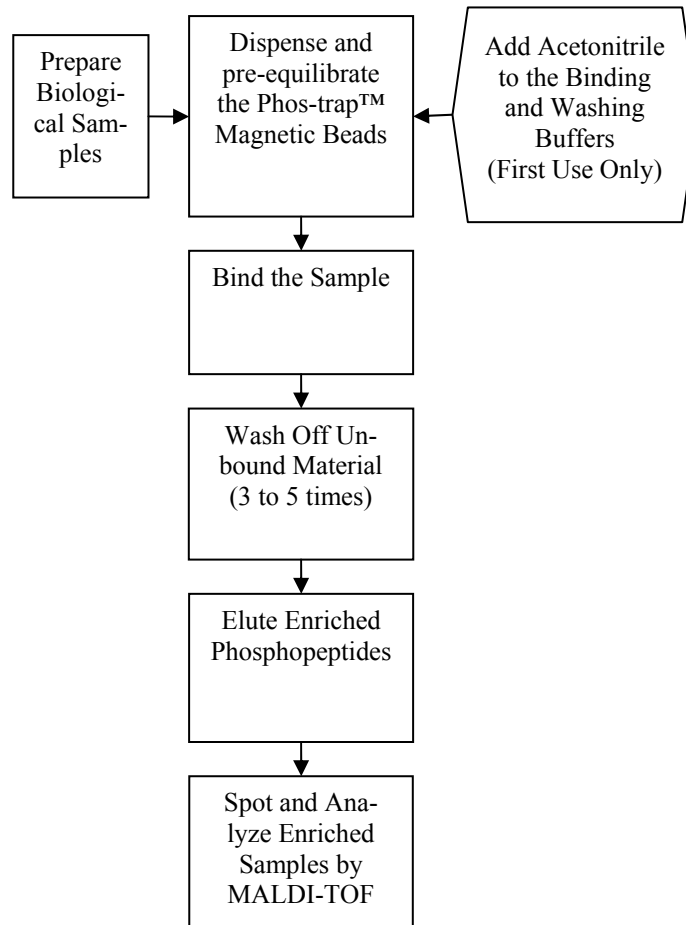
Do not freeze the Phos-trap™ Magnetic Beads. Freeze-thawing may result in reduced performance or loss of function.

Table 1. User-provided materials

Item	Quantity	Suggested Vendor
Acetonitrile CHROMASOLV >99.9%	130 ml	Sigma (Cat. # 34998)
Formic Acid, 98%, for mass spectrometry	< 1 ml	Fluka/Sigma (Cat. # 94318)
Deionized water (18 MΩm)	20 ml	Milli-Q® water, Millipore Corp.
ProXPRESSION MALDI Calibration Kit (matrix)	1	PerkinElmer (Cat. # 6771000)
Separation Magnet 	1	PerkinElmer (Cat. # 5083175)
Mass spectrometer 	1	prOTOF™ 2000 MALDI O-TOF Mass Spectrometer (PerkinElmer)
MALDI Chip 	1	MALDICHip™ Sample Plates 96 wells Kit (PerkinElmer Cat. # N701-4021)
50 ml polypropylene conical centrifuge tubes 	1	BD Falcon (Cat. # 352070)
Non-Sterile Solution Basins, 55 mL 	4	Labor Products, Inc. (Cat. # 730-001)

VI. REAGENT PREPARATION AND ASSAY PROTOCOL

A. Assay workflow at a glance and general recommendations



General recommendations:

1. It is recommended to use the provided V-bottom polypropylene plates and an O-ring- type 96-well magnet (Table 2) for efficient sample handling during fractionation. The O-ring magnets pull the beads along the perimeter near the bottom of each well enabling convenient aspiration of solutions from the middle of a V-shaped well without drawing the beads into a pipette tip. Other types of 96-well plates and magnets can be used as well, but caution should be taken not to draw the beads when aspirating the buffers.
2. Pre-washing the V-bottom polypropylene plates 3 times using 300 μ l of 100% acetonitrile per well before assay typically helps to minimize interference of polymers occasionally leaching from the plates.
3. The plate with beads should always be taken off the magnet when adding the buffers to the wells to allow better suspension of beads. Fast dispensing of solutions into the wells is typically sufficient to adequately re-suspend and wash the beads in the wells.
4. 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.
5. It is recommended to leave 5 to 10 μ l of supernatant in the wells 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 volumes of the Elution Buffer are used

6. The eluted sample can be directly spotted on a MALDI target and analyzed using suitable mass spectrometer. For analysis by LC-ESI-MS we recommend drying down the samples and reconstituting them in 1% formic acid.

7. 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 serum fraction not bound to the beads is typically not suitable for direct analysis and requires further sample clean up, such as sample dry down and reverse phase desalting.

8. 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.

B. Sample Preparation

The Phos-trap™ Phosphopeptide 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 μ l 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 Bead suspension as $(\text{number of fractionations} + 1) \times 10 \mu\text{l} = \mu\text{l}$. 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 $(\text{number of fractionations} + 1) \times 190 \mu\text{l} = \mu\text{l}$ of deionized water into a 50 ml polypropylene screw-cap centrifuge vial (Table 1).
3. Mix the contents of the 20X 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. Pipette the dispersed 20X Phos-trap™ Magnetic Bead suspension into the 50 ml vial with water prepared in step 2 of this section. It is recommended to use 1 ml pipette tips to dispense the beads or to cut off the narrow end of the pipette tip if smaller tips are used to ensure accurate pipetting of beads.
5. Mix the diluted beads well by closing the 50 ml vial with a screw cap and repeatedly inverting it.
6. Dispense 200 μl of the diluted bead suspension per well of the provided V-bottom 96-well plate using a 1 ml pipettor. Beads tend to sediment over time and require periodic mixing in between the bead dispensing steps.
7. Place the 96-well plate with beads on a magnet (Table 2) for 1 min. The beads will concentrate in the O-ring near the bottom of each well.

8. Aspirate the supernatant in the center of each well, near the bottom.
9. Remove the 96-well plate with beads from the magnet and pre-equilibrate the beads with the previously prepared Binding Buffer (Section C, Step 1) by dispensing 200 μ l of this buffer per well. Fast dispensing of buffer is typically sufficient to adequately re-suspend and wash the beads in the wells.
10. Repeat steps 7 through 9 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 μ l to 100 μ l of the sample, diluted in Binding Buffer, is applied to the beads.
3. Remove the plate 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 well by pipetting the sample up and down.
4. Place the plate on a magnet for 1 min and aspirate the supernatant containing the unbound peptides away from the Phos-trap™ magnetic beads
5. Remove the plate from the magnet and wash the beads with 200 μ l 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 the beads may be required for more complex biological samples such as human serum.

7. Wash the beads with 200 μ l of Washing Buffer and discard the supernatant as described in step 4 of this section. For best performance, the Washing Buffer should be completely removed before proceeding to the sample elution step.
8. Remove the plate from the magnet. To elute the samples bound to the beads, dispense 10 μ l of Elution Buffer to each well and pipette the contents of the well up and down a few times to ensure complete wetting of the beads. Smaller than 10 μ l elution volumes can be used to increase the sensitivity levels. Caution should be taken to ensure good wetting of the beads and avoiding contamination of the eluted sample with beads.
9. Place the plate on the magnet for 1 min and carefully aspirate the supernatant containing eluted samples. Care should be taken to avoid aspirating the beads. Excessive amounts of beads may interfere with peptide analysis by mass spectrometry.
10. Mix equal volumes (0.5 μ l to 3 μ l) 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 V-bottom 96-well plate, resuspended in 10 μ l 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 and resuspended in 10 μ l of 1% formic acid.

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.

Total Bovine β -casein Digest

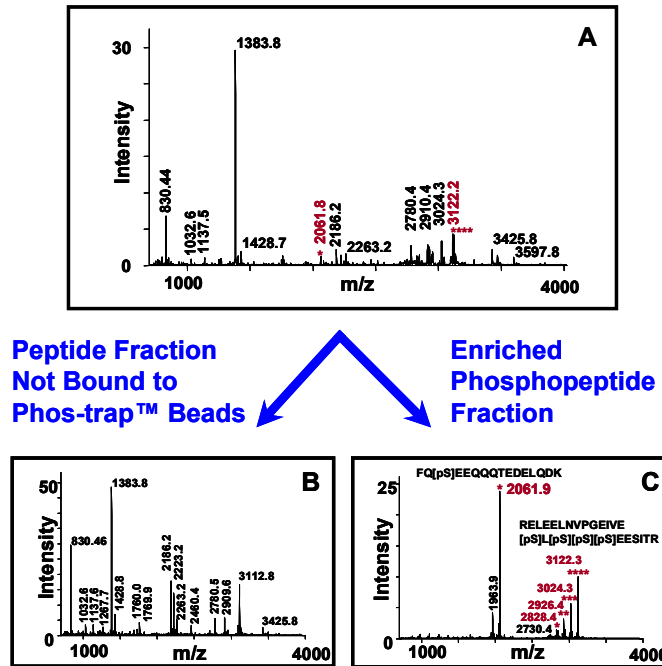


Figure 1. Enrichment of mono- and tetra-phosphorylated bovine β -casein peptides using the Phos-trap™ Phosphopeptide Enrichment Kit. MS spectra corresponding to the total β -casein digest, the peptide fraction not bound to Phos-trap™ beads, and the enriched phosphopeptide fraction are depicted in Panels 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).

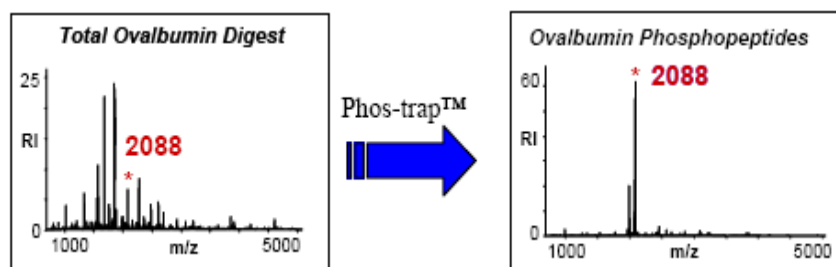


Figure 2. Enrichment of the major phosphorylated ovalbumin peptide (*) using the Phos-trap™ Phosphopeptide Enrichment Kit. RI = Relative Intensity.

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).

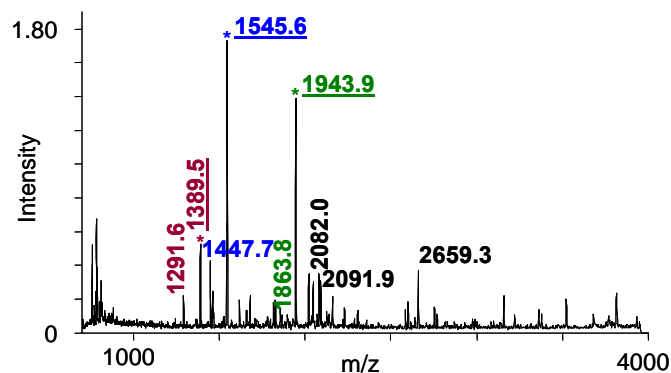


Figure 3. MALDI-TOF spectrum representing an enriched fraction of peptides from 10 μ l of normal human serum using the Phos-trap™ Phosphopeptide Enrichment Kit. The putative phosphorylated peptides identified by the presence of the corresponding de-phosphorylated during a MALDI acquisition peptide peaks (at -80 Da or -98 Da from the main peak) are marked with an asterisk.

VIII. TROUBLESHOOTING GUIDE

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

Possible Problem	Possible reason	Suggestions/ Solutions
No peaks could be detected by MALDI-TOF MS when fractionating a serum sample	<p data-bbox="641 449 925 506">Low quality of acetonitrile or other user-supplied reagents</p> <p data-bbox="641 562 917 646">High content of phospholipids or other sources of endogenous phosphates.</p> <p data-bbox="641 703 878 760">Contaminant level is too high</p> <p data-bbox="641 837 906 921">The amount of analytes in the sample is below the detection limit.</p> <p data-bbox="641 978 894 1077">Presence of beads in the eluted sample or loss of beads occurred during the fractionation.</p>	<p data-bbox="954 449 1192 506">Use the reagents recommended in the manual</p> <p data-bbox="954 562 1214 646">Centrifuge the serum samples and aspirate the top lipid layer</p> <p data-bbox="954 703 1219 814">Decrease the amount of serum fractionated or increase the amount of beads used for fractionation</p> <p data-bbox="954 837 1227 894">Increase the amount of fractionated sample</p> <p data-bbox="954 968 1214 1052">Avoid aspirating the beads when eluting the samples. Use recommended magnet</p>
Peak intensities between the replicates vary significantly	<p data-bbox="641 1100 919 1157">Inconsistent dispensing of beads between the wells</p> <p data-bbox="641 1213 927 1270">Loss of beads occurs while performing an assay</p> <p data-bbox="641 1327 927 1383">Inconsistent liquid handling procedures</p> <p data-bbox="641 1440 883 1497">Presence of beads in the eluted sample</p>	<p data-bbox="954 1100 1198 1157">Follow the recommendations in the protocol</p> <p data-bbox="954 1213 1227 1297">Use the recommended magnets, plates and liquid handling procedures</p> <p data-bbox="954 1327 1227 1383">Follow the optimized protocol in the manual</p> <p data-bbox="954 1440 1214 1497">Avoid aspirating the beads when eluting the samples</p>

Possible Problem	Possible reason	Suggestions/ Solutions
Significant non-selective enrichment of non-phosphorylated peptides	<p>No acetonitrile was added to the Binding and Washing buffers</p> <p>Acetonitrile concentration in the buffers is low</p> <p>Some highly acidic peptides may bind to the TiO₂ surfaces.</p>	<p>Add the amounts of acetonitrile recommended in the manual to the Binding and Washing buffers</p> <p>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</p> <p>Increase the number of washes with Washing Buffer</p> <p>Methylate peptide carboxyl groups before enrichment on TiO₂</p>
Low MS peak intensities	<p>Interference from polymers leaching from plastic labware</p> <p>Sample is degraded</p> <p>Presence of beads in eluted sample</p>	<p>Pre-wash the V-bottom plates with acetonitrile as recommended in the manual</p> <p>Include phosphatases and protease inhibitors in sample preparation. Validate total peptide mix by MS analysis before fractionation using Phos-trap™ Phosphopeptide</p> <p>Avoid aspirating beads when eluting sample</p>

IX. PROCEDURE FOR METHYLATION OF CARBOXYL GROUPS IN COMPLEX SAMPLES

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 TiO₂ 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 al¹³.

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.

XI. REFERENCES

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XII. LICENSING

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XIII. NAME AND PLACE OF MANUFACTURE

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