Preparation and Enrichment of Phosphopeptides from Phosphotyrosine Proteins Using IMAC and LC-MS/MS
AfCS Procedure Protocol PP00000193
Version 1, 11/03/03

The following procedure is specifically for the further enrichment of phosphopeptides from a phosphotyrosine pull-down. This is the final step for the preparation and enrichment of phosphopeptides using immobilized metal affinity chromatography (IMAC) for the identification of the phosphopeptides by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Procedure
1. Prepare and enrich phosphotyrosine proteins according to AfCS Procedure Protocol PP00000187, *Stimulation and Enrichment of Phosphotyrosine Proteins in RAW 264.7 Cells*.
2. Filter the triethylamine (TEA) eluate to remove detergent by using a Millipore Ultrafree spin column with a 10,000 kDa molecular weight cut off. Dilute the eluate tenfold with ammonium bicarbonate solution, 100 mM/methanol, 50% (100 mM NH$_4$HCO$_3$/50% MeOH), and repeat the dilution and ultrafiltration three times.
3. Add trypsin (20 µg/mg protein) to the filtered solution and mix well.
4. Incubate solution for 24 hr at 37 °C.
5. Lyophilize and redissolve the peptides in methanolic hydrochloride, 2 M (2 M methanolic HCl; 320 µl/mg of peptides).
6. Let the redissolved peptides sit for 2 hr at room temperature.
7. Lyophilize again and redissolve the peptides in acetonitrile, methanol, and water (equal volumes) solution (ACN/MeOH/H$_2$O [1:1:1]; 200 µl/mg of peptides).
8. Adjust pH to 3.5 with 10% acetic acid (HAc) before loading IMAC column.
9. Activate the micro-tip IMAC column (prepared according to AfCS Procedure Protocol PP00000163, *Construction of Micro-Tip for Use in IMAC*) by applying 200 µl of 100 mM gallium (III) chloride (GaCl$_3$) or 100 mM ferric chloride (FeCl$_3$) using a 200-µl pipette.
10. Rinse and equilibrate the micro-tip IMAC column with 100 µl of 0.2% HAc.
11. Load the sample eluted from the C18 cartridge (~1 mg of protein) onto the micro-tip IMAC column.
12. Wash the micro-tip IMAC column with 100 µl of 0.2% HAc.
13. Wash the micro-tip IMAC column with 200 µl of IMAC wash solution 1.
14. Wash the micro-tip IMAC column with 200 µl of IMAC wash solution 2.
15. Wash the micro-tip IMAC column with 100 µl of 0.2% HAc.
16. Elute the phosphopeptides with 20 µl of 200 mM sodium phosphate dibasic (Na$_2$HPO$_4$) into a 0.5-ml Eppendorf tube.
17. Equilibrate the capillary C18 high performance liquid chromatography (HPLC) column with HPLC mobile phase A3 for 20 min at a flow rate of approximately 0.5 µl/min.
18. Apply the sample eluted from the micro-tip IMAC column onto the C18 HPLC column using a 15-µl loop.
19. Desalt by washing with HPLC mobile phase A3 for 30 min.
20. Connect the HPLC column in-line with an ion trap mass spectrometer.
21. Elute the peptides with a gradient consisting of 0% to 50% HPLC mobile phase B3 for 80 min, then 50% to 80% HPLC mobile phase B3 for 20 min.
22. Set the mass spectrometer in a data-dependent mode and program to cycle continually through one mass spectrometry (MS) scan followed by tandem mass spectrometry (MS/MS) scans of the five most abundant ions in the MS scan.
23. Search the acquired MS/MS spectra against the mouse NCBI nonredundant database by using SEQUEST software. Include the search parameters to detect a differential modification of 80 Da on serine, threonine, and tyrosine.
24. Manually confirm the assignments of phosphopeptide sequences by comparing the acquired MS/MS spectra to the theoretical fragmentation pattern carefully.

Reagents and Materials
Ultrafree spin column: Millipore; catalog no. UFV5BGC25

Ammonium bicarbonate solution, 100 mM/Methanol, 50% (100 mM NH₄HCO₃/50% MeOH): AfCS Solution Protocol ID PS00000594

Trypsin, sequence grade: Promega; catalog no. V5111

Methanolic hydrochloride, 2 M (2 M Methanolic HCl): AfCS Solution Protocol ID PS00000595

Acetonitrile, methanol, and water (equal volumes) (ACN/MeOH/H₂O [1:1:1]): AfCS Solution Protocol ID PS00000593

Acetic acid, 10% (10% HAc): AfCS Solution Protocol ID PS00000491

pH Indicator strips: EMD Chemicals Inc.; catalog no. 9582

Gallium (III) chloride, 100 mM (100 mM GaCl₃): AfCS Solution Protocol ID PS00000525

Ferric chloride, 100 mM (100 mM FeCl₃): AfCS Solution Protocol ID PS00000524

Acetic acid, 0.2% (0.2% HAc): AfCS Solution Protocol ID PS00000521

IMAC wash solution 1: AfCS Solution Protocol ID PS00000526

IMAC wash solution 2: AfCS Solution Protocol ID PS00000527
Sodium phosphate dibasic, 200 mM (200 mM Na₂HPO₄): AfCS Solution Protocol ID PS00000530

HPLC mobile phase A3: AfCS Solution Protocol ID PS00000528

LCQ Deca ion trap mass spectrometer: Thermo Finnigan; catalog no. LCQ Deca XP Plus

HPLC mobile phase B3: AfCS Solution Protocol ID PS00000529

SEQUEST software: Thermo Finnigan; catalog no. SEQUEST Cluster

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