

STANDARD OPERATING PROCEDURE

Title: Enrichment of phosphopeptides by magnetic IMAC beads

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Purpose

The purpose of this document is to describe automated enrichment of phosphorylated peptides out of digested cell lysate using magnetic IMAC agarose beads.

Scope

This procedure may be used to enrich phosphorylated peptides out of cell lysate digests.

Responsibilities

It is the responsibility of person(s) performing this procedure to be familiar with laboratory safety procedures. The interpretation of results must be done by a person trained in the procedure and familiar with such interpretation.

Equipment

- KingFisher magnetic particle processor (Thermo Fisher, Waltham, MA).
- Titer plate shaker (Lab Line Instruments).
- Magnet (Thermo Fisher, A13346).

Materials

- Ni-NTA-magnetic beads, (Qiagen, 36113)
- EDTA, 500mM, (Sigma, E7889)
- FeCl₃, (Sigma, 451649)
- Water (H₂O), HPLC grade (Fisher, W5-1)
- Acetonitrile (ACN), HPLC grade (Fisher, A998-1)

- Methanol (MeOH), HPLC grade (Fisher, A412-1)
- Acetic Acid (HOAc), (Sigma, 242853)
- Trifluoroacetic Acid (TFA), (Thermo, 28901)
- Formic Acid (FA), (EDM, 11670-1)
- Ammonium Hydroxide [Fluka 1336-21-6]
- Flat bottom UV transparent 96-well plate (Corning, 3635)

Solutions

- 100mM EDTA:
 - 1 part 500mM EDTA stock
 - 4 parts HPLC water
- 10mM FeCl₃ aqueous solution. Must be made fresh daily:
Note: FeCl₃ should be store in a desiccator to prevent oxidation.
 - Prepare 100mM FeCl₃ solution by dissolving 16.22mg FeCl₃ in 1mL H₂O
 - Mix well
 - Dilute this stock 10:1 with H₂O to get 10mM FeCl₃
 - Mix well
- Bead Resuspension Solution – 1:1:1 ACN:MeOH:0.01% HOAc
 - 1 part ACN
 - 1 part MeOH
 - 1 part 0.01% HOAc in H₂O
- PO4 Load and wash Solution – 80% ACN, 0.1% TFA:
 - 1 part H₂O
 - 4 parts ACN
 - Add 0.1% TFA
 - Note: pH should be between 2.0 – 3.3
- PO4 Elute Solution – 1:1 acetonitrile/1:20 ammonia water
 - 1 part Acetonitrile
 - 1 part 1:20 Ammonia water

Procedure

Preparation of Ni-NTA Superflow Agarose and magnetic Beads

Note: this protocol is for a total slurry volume of less than 500uL.

1. Aliquot 100 ul of 5% slurry of beads per 200 ug sample.
2. Place beads on magnet and remove supernatant.
3. Rinse beads by repeating these steps 3x:
 - a. Add 1.0mL of H₂O to beads.
 - b. Vortex beads.
 - c. Place beads on magnet and remove supernatant.

4. Add 1.0mL of 100mM EDTA to beads, vortex and mix at ~1400rpm at room temperature for 30 minutes.
5. Rinse beads by repeating these steps 3x:
 - a. Add 1.0mL of H₂O to beads.
 - b. Vortex beads.
 - c. Place on magnet and remove supernatant.
6. Add 1.0 mL of 10mM FeCl₃ aqueous solution to beads, vortex and mix at ~1400rpm at room temperature for 30 minutes.
7. Rinse beads by repeating these steps 3x:
 - a. Add 1.0mL of H₂O to beads.
 - b. Vortex beads.
 - c. Place beads on magnet and remove supernatant.
8. Resuspend beads to a 5% slurry (100 ul) with 1:1:1: ACN:MeOH:0.01% HOAc.

Preparation of Peptide Sample

Note: these volumes are for 200ug peptide samples.

Note: final peptide concentration will be 1.0ug/uL.

1. Add 200uL of 80% ACN, 0.1% TFA to each sample in 96-well plates.
2. Spin down samples at 1000g for 30s.

Phosphopeptide Enrichment

1. Add 200uL of 80% ACN, 0.1% TFA to each sample in 96-well plates loaded with 100 μ L magnetic beads from 5% bead suspension..
2. Mix on the titer plate shaker at speed 4 for 30 minutes at room temperature.
3. The following steps were performed on the KingFisher magnetic particle processor with a PCR head:
 - a. Mix for 5 minutes.
 - b. Transfer for three washes (1 minute each in 0.1% TFA in 80% ACN, 200 μ L).
 - c. Elute in 200 μ L of 1:1 acetonitrile/1:20 ammonia:water for 5 minutes.
5. Lyophilize samples overnight to dryness.
6. Samples can be stored lyophilized at -80°C until ready for MRM analysis.

Reconstituting Samples (To be performed just prior to executing LC-MRM)

1. Reconstitute dried and desalted digests with 14 μ L of 3% acetonitrile, 0.1% formic acid to each sample.
2. Vortex sample, spin down, and transfer ~7 μ L to two 96-well.
3. LC-MRM analysis is performed according to SOP LC-03 and SOP MS-03.

Referenced Documents

SOP LC-03 Liquid chromatography trap elute.pdf

SOP MS-03 MRM mass spectrometry.pdf