Phosphopeptides purification

Boisvert Lab, may 2023

Protein digestion

1. Solubilize proteins directly in a solution of 8 M urea, 1 M NH_4HCO_3, 20 mM HEPES pH 8.0 (lysis buffer) (in MS-grade water). For a total cell extract, resuspend directly the cell pellet coming from a 100mm petri dish of confluent cells (~2 mg of total protein) in 500 µl of lysis buffer. Sonicate on ice (12 cycles of 5 sec PULSE /5 sec OFF, intensity of 20-25%), centrifuge 16,000 x g for 10 min at 4ºC and transfer the supernatant to a new lobind tube. Quantify your cell extract using BCA kit (compatible with 3M urea, so do not forget to dilute your sample). Generaly speaking, transfer 250 µl of cell extract containing up to 1 mg of total proteins to a new lobind tube and proceed to the next step.

2. Add DTT (5 mM final concentration). Boil at 95ºC for 2 minutes, then incubate at RT for 30 min.

3. Add chloroacetamide (7.5 mM final concentration). Incubate at RT for 20 min in the dark.

4. Add 750 µl of 1M NH_4HCO_3 (in MS-grade water) to the mixture so that the final concentration of urea is reduced to 2 M.

5. Add 10 µg trypsin and incubate at 30ºC overnight.

6. Acidify the sample to a final concentration of 0.2% TFA.

HYPERSEP cleaning

We use HYPERSEP C18, 100 mg /1 ml column (Thermo Scientific, cat: 60108-302). NB: you can increase the flow of the liquid by pushing air slowly with your P1000 pipet and a filter-tip.

1. Load 3 times 1 ml of 100% acetonitrile (wetting).

2. Load 3 times 1 ml of 0.1% TFA (equilibration).

3. Load your sample in the column with a flow of 0.5-1 ml/min, collect and reload the effluent twice.

4. Load 3 times 1 ml of 0.1% TFA (wash).

5. Elute 3 times with 500 µl of elution buffer (50% acetonitrile /1% formic acid) with a flow of 0.5-1 ml/min, collect the eluate in a new lobind tube.

6. Evaporate your sample using speedVac.

Phosphopeptides enrichment

We use Pierce High-Select TiO_2, 3 mg/200 ul tips (cat: A32993).

Materials required

• Collection tubes: lobind microcentrifuge tubes, 2 ml
• Water, MS-grade
• pH paper

Suspend peptide sample

1. Completely resuspend your peptides (1 mg) in 150 µl of Binding/Equilibration buffer (included in the kit). Use vortex mixer with tube stand if necessary. **Note**: For optimal results, lyophilized peptide samples must be entirely dissolved in Binding /Equilibration Buffer.
2. Optional: Verify pH of resuspended sample is <3 using pH paper.

Prepare column

1. Place a Centrifuge Column Adaptor in a 2 mL collection tube and insert a TiO2 Spin Tip into the adaptor.
2. Add 20 µl of Wash Buffer and centrifuge at 3000 × g for 2 minutes.
3. Add 20 µl of Binding/Equilibration Buffer and centrifuge at 3000 × g for 2 minutes.
4. Discard the flowthrough. Save the microcentrifuge tube for later "Wash column" step 1.

Bind phosphopeptide

1. Transfer the equilibrated TiO2 Spin Tip and adaptor into a new 2 ml microcentrifuge tube.
2. Apply 150 µl of suspended peptide sample to the spin tip. Centrifuge at 1000 × g for 5 minutes.
3. Reapply sample in the microcentrifuge tube to the spin tip. Centrifuge at 1000 × g for 5 minutes. If desired, retain the flowthrough for analysis.

Note: Applying sample twice to the spin tip results in an ~11% additional phosphopeptide yield**.**

Wash column

1. Transfer the TiO2 Spin Tip and adaptor into the collection tube saved from "Prepare column" step 4.
2. Wash column by adding 20 µl of Binding/Equilibration Buffer. Centrifuge at 3000 × g for 2 minutes.
3. Wash column by adding 20 µl of Wash Buffer. Centrifuge at 3000 × g for 2 minutes.
4. Repeat steps 2 and 3.

Note: Changing the order of wash column steps results in significantly higher nonspecific peptide binding.

5. Wash column by adding 20 µl of LC-MS grade water. Centrifuge at 3000 × g for 2 minutes.

Elute column

1. Remove excess liquid by blotting bottom of the spin tip on a clean lab tissue.
2. Place the spin tip and adaptor in a new collection tube and add 50 µl of Phosphopeptide Elution Buffer. Centrifuge at 1000 × g for 5 minutes. Repeat step once.
3. Dry the eluate immediately in a speed vacuum concentrator to remove Phosphopeptide Elution Buffer.

Note: Eluates cannot be stored in Phosphopeptide Elution Buffer because high pH will lead to loss of phosphates on phosphopeptides.

4. Suspend the eluate with 50 µl 1% formic acid. Concentration of the peptides can be estimated by Nanodrop absorbance at 205 nm.

Note: For <1 mg starting peptide sample amounts, suspend dried elute using 25 µl of 1% formic acid

Notes

1. Remember to wear gloves at all times during the procedure. Don't scratch your head, armpits, genital, or any part of your body during the procedure. Keratin contamination is less an issue after peptide digestion, as whole keratin proteins will not interfere with MS analysis.
2. When you make any stock solutions, avoid using any spatula that have not been cleaned thoroughly by water and ethanol. In fact, we routinely forgot using spatula at all, and tip the solid chemical into clean eppendorf tubes.
3. Peptides tend to stick to plastic surfaces on storage. We use 1.5 ml lobind tubes that are designed for storing peptides at low concentration.

Adapted from Pierce High-Select TiO_2 kit protocol.