Phosphopeptides purification

Boisvert Lab, may 2021

Protein digestion

1. Solubilize proteins directly in a solution of 8 M urea, 10 mM HEPES pH 8.0 (lysis buffer). How much sample to use depends on the amount of proteins, and the purpose of your experiment. 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 400 µg 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 50 mM NH_4HCO_3 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.

ZipTip 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.

7. Resuspend your peptides in 100 µl of 2% TFA (concentration of about 4 µg/µl).

Phosphopeptides enrichment

We use the titansphere TiO Kit (buffer and adaptor), 3 mg/200 ul tips (Canadian life science, cat: GL5010-21311;GL5010-21514).

1. Connect a centrifugal adaptor to a Waste Fluid tube, and insert the Spin Tip into the tube.

2. Add 20 µl of Buffer A, then centrifuge (3,000 x g for 2 min, RT) (conditioning).

3. Add 20 µl of Buffer B, then centrifuge (3,000 x g for 2 min, RT) (equilibration). Remove the 40 µl of effluent from the Waste Fluid tube.

4. Add 50 µl of your sample (200 µg) and 100 µl of Buffer B into the Spin Tip. To mix the sample with Buffer B, carefully perform up/down cycles (3 times of 150 µl volume) into the Spin Tip.

5. Centrifuge (1,000 x g for 10 min, RT) (adsorption). Collect and reload the effluent and centrifuge again (1,000 x g for 10 min, RT). Remove the 150 µl of effluent from the Waste Fluid tube.

5. Add 20 µl of Buffer B, then centrifuge (3,000 x g for 2 min, RT) (rinsing 1).

6. Add 20 µl of Buffer A, then centrifuge (3,000 x g for 2 min, RT) (rinsing 2). Repeat twice.

7. Put the Spin Tip into the recovery tube, add 50 µl of 5% ammonium hydroxide solution and centrifuge (1,000 x g for 5 min, RT).

8. Add 50 µl of 5% pyrrolidine solution and centrifuge (1,000 x g for 5 min, RT).

9. Evaporate your sample using speedVac.

10. Resuspend your phosphopeptides in 30 µl of 1% formic acid and quantify with nanodrop at 205 nm.

Buffer A: mix 125 µl of 2% TFA solution with 500 µl of 100% acetonitrile.

Buffer B: mix 50 µl of solution B (provided with the kit) with 150 µl of Buffer A.

Ammonium hydroxide 5%: add 5 µl of ammonium hydroxide 100% to 95 µl of MS-grade water.

Pyrrolidine 5%: add 5 µl of pyrrolidine 100% to 95 µl of MS-grade water.

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 titansphere TiO Kit protocol.