SCX -TiO_2 Phospho- peptide Separation Protocol

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Phosphatase inhibitors HIGHLY recommended. Do not dry phospho-petide eluent as these peptides will stick to sample tube. Note pH of samples ~pH2 is optimal, as well as a long incubation time at room temperature for successful binding to TiO_2.

Protein Digestion

- 100mM Iodoacetamide in 25mM NH_4HCO_3
- 45mM DTT in 25mM NH_4HCO_3
- 1µg/µl Trypsin Gold (50mM acetic acid reconstituted)

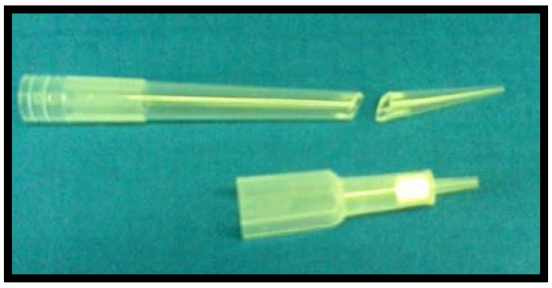
Firstly ensure your protein mixture is very near to pH 8, the optimal operating pH of Trypsin. Adjust as necessary.

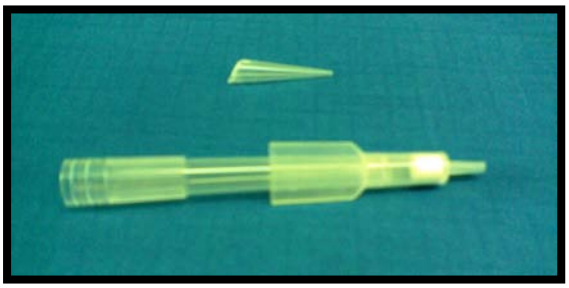
1. Take an aliquot and combine with running buffer (SDS PAGE running buffer) if required for digestion monitoring.
2. Add 45mM DTT (to a final concentration of 10mM), vortex and incubate at 50°C for 15mins.
3. Allow to cool slightly, then add 100mM Iodoacetamide (to a final concentration of 20mM), incubate at room temperature, in the dark, for 15mins.
4. Add Trypsin (1:100 (µg to µg) enzyme to substrate ratio, if Trypsin gold) and incubate over night at 37°C.
5. Take an aliquot and run with initial aliquot to test digestion efficacy if required.
6. Clean sample over Empore universal resin or Ziptip equivalent (C18 material)

Cleaning protocol with Thermo Scientific Hypersep C18 resin

(60300-422, 25mg/1ml)

1. Prime column with 2x 1ml MeOH.
2. Wash column with 2x 1ml 0.1% TFA.
3. Add 1ml of 0.1% TFA as well as your sample and another 1ml of 0.1%TFA (use to wash out remainder of sample form eppendorf). Elute slowly through the column.
4. Wash with 2x 1ml 0.1% TFA.
5. Elute sample with 2x 500µl 70% ACN/0.1% TFA.
6. Dry down samples to a small volume (≤200µl). Retain a aliquot of this sample- for the whole protein picture (sample 1- all proteins)





SCX Poly-Phospho-peptide Enrichment, Thermo Scientific Hypersep SCX (benzosulfonic acid, 25mg/1ml)

As TiO2 tends to bind phospho-peptides very strongly, and tends to not elute poly- phosphorylated peptides, it has proven more effective to perform an SCX Isolation first- to isolate poly-phosphos and then the remaining mono-phosphos can then be captured with the TiO2, and be successfully eluted.

- SCX Buffer A (loading and washing buffer): 10mM KH_2PO_4, 25% ACN, pH 3.
- SCX Buffer B (priming and elution buffer): 10mM KH_2PO_4, 25% CAN, 350mM KCl, pH3.

Reconstitute sample in Buffer A (500µl-1ml), and test the pH- making sure it is pH2-3.

1. Wash Column with 2x 1ml of MilliQ.
2. Prime the Column with 2x 500µl SCX Buffer B.
3. Wash the Column with 2x 1 ml SCX Buffer A.
4. Load sample, push through column slowly, **retaining the flow-through** as this will be applied to the TiO_2. This sample should be speed-vac'd down to a volume of approximately 100µl- and then used in step one of the TiO2 separation protocol.
5. Wash the sample with 2x 500µl SCX buffer A.
6. Elute sample with 500µl of SCX Buffer B (sample 2- Poly-phospho peptides).

NB: This sample should be cleaned (C18) as it contains a large amount of salt, which will cause problems with the LC-MS analysis. Remember to NEVER dry a phospho sample completely- as the peptides will stick to the tubes.

TiO_2 Separation.

This protocol is performed using 500µl eppendorfs instead of tips to allow for longer binding time and instead of elution from a tip, a spinning and supernatant approach is used.

- Loading buffer: 80% ACN/2% TFA and 200mg DHB, pH 2.
- Washing buffer: 80% ACN/ 2%TFA, pH 2.
- Elution buffer: 400mM NH_4OH, pH 11.

1. Re-suspend reduced sample (from step 4 above) in 80% ACN, 2% TFA and 200mg/ml DHB. Check the pH is 2.
2. To a micro spatula full of TiO2 beads in a 500µl eppendorf add 100µl 80% ACN, 2% TFA and ~200mg/ml DHB, and vortex for 10 seconds. Spin at 1000g. Remove supernatant and repeat.
3. Add sample to beads, seal well and rotate at room temperature for 1 hour.
4. Spin down at 1000g, remove the non-phosphorylated peptides. Retain if necessary (although this sample contains large amounts of DHB- which is good for MALDI- not so good for Electrospray MS, and is quite difficult to remove).
5. Wash 3 x 100µl with 80% ACN, 2% TFA and 200mg/ml DHB.
6. Wash 3x 100µl with 80% ACN, 2% TFA (note the yellow color should dissipate).
7. Elute phospho-peptides with 2x 30µl 400mM NH_4OH pH 11.

8. Add 5µl of 100% formic acid to adjust pH and load directly to MS plate or clean over C18 and ESI-MS/MS (sample 3- mono phospho peptides).