

ICAT labeling of affinity purified proteins and preparation of peptides for μ LC-MS/MS

Affinity purified protein samples are concentrated in Microcon 10 devices (Amicon) and buffer is exchanged by diluting the samples 10-fold with TNE (20 mM Tris-HCl (pH 8.3), 50 mM NaCl, 1 mM EDTA). After concentrating the samples to ~50 μ l, SDS is added to 0.3% and the samples are heated at 100 °C for 5 min. If a volatile solution was used for elution of the complex, the sample can be dried down and resuspended in TNE + 0.3% SDS.

Important considerations

- 1) Be aware of the salt concentration in the eluted sample to ensure efficient binding of the peptides to the cation exchange resin later in the protocol. The conductivity of the sample solution should be below that of a 5 mM KCl solution before loading on the cation exchange column.
- 2) If a peptide was used for elution of an immunoprecipitate, it is important to reduce the quantity of the peptide to the low pmol range by concentration and dilution in the concentrator device.
- 3) Reduce detergent concentrations to below the critical micelle concentration of the detergent before concentration.

Proteins are reduced by addition of TBP to 5 mM at 37 °C for 30 min, and then diluted by addition of 5X volume of 20 mM Tris-HCl (pH 8.3), 1 mM EDTA, 7.2 M urea. The goal of the dilution is to reduce SDS to 0.05% and bring urea to 6M. (TCEP can be used in place of TBP. If using TCEP increase Tris concentration to 50mM). Isotopically heavy or normal ICAT reagent is added to 1.5-1.75 mM and samples are incubated for 90 min at 22 °C. Reactions are quenched by addition of 10 mM DTT for 20 min at 37 °C, samples are combined, and proteins are digested by addition of 4 µg endoproteinase Lys-C (Beohringer Mannheim) at 37 °C for 3 h. SDS and urea concentrations are reduced to 0.01% and 1.2 M respectively, by addition of 20 mM Tris-HCl (pH 8.3), 1 mM EDTA, and samples are digested with trypsin (Promega, sequencing grade modified, 1:25 w/w) overnight at 37 °C.

The sample is diluted with an equal volume of Buffer A [5 mM KH₂PO₄ (pH 3) 25% CH₃CN] and the pH is adjusted to 3 with 10% trifluoroacetic acid (TFA). Peptides are fractionated by SCX chromatography. If the sample contains ~100ug protein, fractionation by HPLC (2.1 x 200 mm PolySULFOETHYL A, PolyLC, Inc.) is recommended. For smaller quantities of protein, fractionation on an SCX cartridge (ABI) or a homemade microcolumn is recommended. This step removes SDS and trypsin, and permits reduction of sample complexity. A typical HPLC gradient profile is: 0-15% Buffer B [5 mM KH₂PO₄ (pH 3) 600 mM KCl, 25% CH₃CN] over 30 min, 15-60% Buffer B in 20 min, and 60-100% Buffer B in 15 min at 0.2 ml/min. 0.4 ml fractions are collected. If using a cartridge, salt steps of 40, 100, 200, 350 and 600 mM can be used.

After reducing the acetonitrile concentration by partially drying the samples under reduced pressure, the samples are diluted 2-fold with 2X PBS and the pH is adjusted to ~7 with 1M NH_4HCO_3 . Labeled peptides are purified over monomeric avidin cartridges (ABI, Inc.), and washed with 2X PBS (pH 7.2), 1X PBS (pH 7.2), and 50 mM. NH_4HCO_3 (pH 8.3), 20% CH_3OH as described in the isotope labeling protocol. Peptides are eluted with 0.4% TFA in 30% CH_3CN , dried under reduced pressure, and resuspended in 10% CH_3CN , 0.1% TFA. For additional protein coverage the peptides from avidin flowthrough fractions can be captured on C18 cartridges (Vydac), washed with 0.5% CH_3CN , 0.1% TFA, and eluted with 60% CH_3CN , 0.1% TFA. Samples are dried and resuspended in 0.5% CH_3CN , 0.1% TFA.