Quick Start Guide

Protocol for biotinylation of proteins/peptides through cysteine residues

1) The protein mixture may be prepared by a variety of methods depending on the nature of the experiment. Protein sample preparation compatible with this method may be accomplished using the following:
   - ProteoPrep™ Universal Extraction Kit (Sigma-Aldrich PROT-TWO) for isolation of separate fractions of soluble and membrane proteins
   - ProteoPrep Membrane Extraction kit (Sigma-Aldrich PROT-MEM) for isolation of integral membrane proteins
   - ProteoPrep Sample Extraction Kit (Sigma-Aldrich PROT-TOT) for testing or optimizing protein extraction conditions.

   In each of these kits, reagents are provided for solubilization/denaturation and reduction of proteins.

   A denaturation/reduction protocol using individual reagents could be performed as follows:

   1. The protein solution should be at approx. 2 mg protein/ml in denaturing, buffered conditions (e.g. 6 M Guanidine HCl with 0.05 M HEPES pH 7.5).
   2. Add Tributylphosphine (TBP, Sigma-Aldrich T7567) to a final concentration of 5 mM from the 200 mM stock solution. React 1 hour at RT or 37°C.
2) Biotinylate through reduced sulfhydryls: Add Biotin-Iodoacetamide reagent to a final concentration of 2 -5 mM. Stir 1 hour at RT or 37°C. Protect reaction from light.
3) Dialyze to remove unreacted biotinylation reagent and denaturation agents. The dialysis may be performed against running water overnight or against a buffer such as 50 mM ammonium bicarbonate with multiple changes of dialysate. The protein may precipitate out of solution.
4) Digest the protein mixture by adding proteomics grade trypsin (Sigma-Aldrich T6567) to 1/50 wt/wt of total protein, i.e add 0.05 mg trypsin for 2.5 mg total protein. The trypsin should be predissolved in 1 mM HCl at 0.8 mg/ml prior to addition. If the protein solution was dialyzed against water, ammonium bicarbonate should be added to 50mM prior to addition of trypsin. Stir overnight. With digestion, the precipitated protein should completely redissolve.
5) The solution may be lyophilized or evaporated using a Speed vac to remove volatile buffers and to concentrate.
6) The peptides should be reconstituted in a low ionic strength buffer with acetonitrile to prepare for ion-exchange chromatography. When using SCX (Strong cation exchange) supports, a recommended buffer is 10 mM ammonium phosphate pH 3 containing 25 % acetonitrile.
7) Fractionate peptides on ion exchange chromatography. An example using cation exchange chromatography would require a 2.1 mm X 2 cm LC-SCX column (Supelco) with 25% acetonitrile, 10 mM ammonium phosphate pH 3 buffer and a non linear gradient of 0 - 0.5 M NH_4Cl over 30 minutes at 0.2 ml/minute. Up to 25 µg protein in 20 µl of water could be injected onto the column.

8) Collect fractions in a Streptavidin HC plate using a Foxy Jr. fraction collector directly from LC effluent. Collect 0.2 ml per well, i.e. 1 minute fractions.
9) After a 2 hour incubation at room temperature, transfer the Streptavidin plate to the platform of the Biomek 2000 at position B3 (with labware holder)
10) Set up the remaining requirements on the platform:
    - Tool Rack at A1 with WASH8, MP20,MP200
    - Reservoir module with quarter single reservoir at A2
    - Tip rack holder and P250 tips at A3 and A4
    - Tip rack holder and P20 tips at B1 and B2
    - Disposal container on left side of platform
    - MALDI target in modified labware holder* at B4
    - Bulk buffer 1: 18 megaohm water
    - Bulk buffer 2: 50 mM ammonium phosphate pH 7
11) Volumes of reagents required:
    - 15 ml Elution solution: 10 mg/ml α-cyano-4-hydroxycinnamic acid in 70% acetonitrile, 0.1% formic acid in quarter single reservoir at A2
    - 500 ml 18 megaohm water in bulk buffer 1
    - 250 ml 50 mM ammonium phosphate pH 7 in bulk buffer 2
12) Wash the streptavidin plate with 3 X 250 µl per well 50 mM ammonium phosphate pH 7 followed by with 3 X 250 µl per well water.
13) Elute peptides with 100 µl/well of 10 mg/ml α-cyano-4-hydroxycinnamic acid (ACCA) matrix in 70% acetonitrile, 0.1% formic acid. This solution may be supplemented to 1 mM biotin which may offer better elution.
14) Following 1 hour elution, mix the wells, three times per well.
15) Spot the elution fractions onto MALDI target. Use 1.5 µl per fraction spotted onto a 96 well Axima-CFR MALDI target.
16) Allow spots to dry for approximately 10 minutes at room temperature
17) Transfer MALDI target to Axima-CFR Mass spectrometer for data acquisition.(Shimazdu Biotech).

* The platform plate holder was modified to hold the Axima MALDI target as follows. Stainless steel pins were welded to a labware platform holder (Beckman Coulter p/n 609120). One 2.5 mm diameter pin, 7.0 mm from left side, 50 mm from top, 54 mm from the bottom of the holder. Two 1.5 mm diameter pins, 4.6 mm from right side, top pin 20 mm from top, bottom pin 25.5 mm from bottom of the holder. A 2 mm thick rubber mat was used under the MALDI target to adjust the height for pipetting.