Wear nitrile gloves and make sure area is free of dust and lint by wiping down area with ethanol using lint-free wipes.

1. Add 11.4 μl of 6 M urea, 100 mM Tris-HCl, pH 8.5 to every 2 μl of protein. There must be at least 200 ng (200 ng/2 μl) of a specific protein for detection by the mass spec.
2. Dilute 0.5 M TCEP 1:10 with HPLC grade water to make a 50 mM TCEP solution . Add 1.4 μl of the 50 mM TCEP to the protein solution prepared in step #1. This gives a final concentration of 5 mM TCEP. Mix the tube on an orbital shaker for 20 min at 24°C. Spin down briefly in microfuge to bring liquid to bottom of tube.
3. Dilute 500 mM IAA 10-1 using HPLC grade water to make 50 mM IAA. Add 3 μl 50 mM IAA so that protein has 10 mM final conc of IAA. Mix and spin down briefly in microfuge to bring liquid to bottom of tube. Incubate in the dark for 30 min.
4. Total volume is now 17.8 μl. Add formic acid and use pH paper to check for pH. Add formic acid until pH is less than pH 4.0.
5. The sample must be purified to remove urea. Use a Zip Tip C4 pipet tip (Millipore Corp) and a 10 μl pipet and follow the purification steps below:
6. Wet the zip tip with 10 μl acetonitrile (ACN). Discard the ACN. Repeat.
7. Wet the zip tip with 10 μl 0.1% formic acid. Discard the formic acid. Repeat.
8. Pipet the sample (from step 4 after pH adjustment to < pH 4.0) up and down at least 20 times.
9. Wash the zip tip with 10 μl 0.1% formic acid. Discard the formic acid.
10. Repeat the washing of the zip tip with 10 μl 0.1% formic acid. Discard the formic acid.
11. Wash the zip tip with 10 μl 5% methanol, 0.1% formic acid. Discard the methanol/formic acid.
12. Elute the sample into a 1.5 ml tubes using 10 μl 0.1% formic acid/50% ACN.
13. Repeat the elution into the same 1.5 ml tube.
14. Elute the sample into a 1.5 ml tubes using 10 μl 0.1% formic acid/99% ACN.
15. Dry liquid in 1.5 ml tube using speed vac.
16. Obtain Trypsin Gold stock at 1 μg/μl. Add 49 μl of W1 and 1 μl of Trypsin Gold stock at 1 μg/μl.
17. Add 30 – 50 μl of the diluted trypsin solution to the dried protein in the 1.5 ml tube. Mix and spin down briefly in microfuge to bring liquid to bottom of tube.
18. Digest overnight at 37°C.
19. Dry sample using a speed vac.
20. Add 4 μl of 0.1% formic acid in ACN (Buffer B). Add 16 μl of 0.1% formic acid in water (Buufer A) to the dried peptides. Mix to suspend peptides and transfer liquid to a vial insert.
21. Place insert in 1.5 ml tube and dry sample in vial insert using a speed vac.
22. Resuspend the peptides in 6 μl of 0.1% formic acid containing 0.7:2.8 ACN:water ratio (mass spec loading buffer) pipeting sample up and down on sides of vial insert. Spin down briefly using microfuge to settle liquid to bottom of vial insert.
23. Place vial insert in vial and place in appropriate position in mass spec autosampler.
24. Run on mass spec.

**Solutions**

**6 M Urea, 100 mM Tris-HCl, pH 8.5 – Make up fresh**

Dissolve 1.576 g Tris HCl in ~80 ml water. Adjust pH to pH 8.5. Bring volume up to 100 ml. Store for up to 6 months.

Dissolve 0.37 g Urea in 700 μl 100 mM Tris-HCl, pH 8.5. Make up fresh each time.

**Reducing agent**

0.5 M TCEP in Trypsin digestion kit, Rm 2-27, 4°C; dilute 10-1 with HPLC-grade water for 50 mM conc.

**Alkylating agent -** 500 mM Iodoacetamide (IAA)

Dissolve 0.092 g in 1 ml HPLC water. Make up fresh in amber 1.5 ml tube.

**Trypsin** – 1 μg/μl Trypsin gold stock in -80°C freezer

**Digestion Buffer (W1)** – 10 mg of ammonium bicarbonate + 5 ml ultrapure water (final conc 2 mg/ml = 25 mM). Store at 4°C for up to 3 months but always **check pH with pH paper before use to make sure it is at pH 8**. Trypsin digestion will not work in acidic solution.

**Buffer A** – 0.1% formic acid in HPLC-grade water (vol/vol)

**Buffer B** – 0.1% formic acid in acetonitrile (ACN)