1. Add membrane piece to 100 μl 8 M urea, 100 mM Tris-HCl, pH 8.5.
2. Add 1 μl 0.5 M TCEP (5 mM final conc) and shake for 20 min at 24°C.
3. Add 2 μl 500 mM IAA (10 mM final conc) and shake in the dark for 30 min.
4. Total volume is now 102 μl.
5. Spin down sample briefly to collect membrane at bottom of 1.5 ml tube. Discard fluid.
6. Add 100 μl W1 to membrane. Mix for 5 min at 24°C. Discard W1.
7. Repeat step 6 two more times to remove urea, Tris, TCEP, and IAA.
8. Make up a solution of Trypsin Gold to a concentration of 2 μg / 100 μl with W1. Add enough of the solution to cover the membrane. Incubate at 37°C overnight.
9. Collect the solution from the membrane after thoroughly vortexing and add to 1.5 ml tube.
10. Add 100 μl Buffer A (0.1% formic acid in water). Incubate at 24°C with mixing for 5 min. Collect the solution and add it to same 1.5 ml tube.
11. Add 75 μl Buffer A (0.1% formic acid in water) and 25 μl Buffer B (0.1% formic acid in ACN). Incubate at 24°C with mixing for 5 min. Collect the solution and add it to same 1.5 ml tube.
12. Speed vac solution in 1.5 ml tube to dryness.
13. Proceed to zip tip procedure.

**Zip Tip procedure**

1. Reconstitute the dried sample in 12 μl 0.1% formic acid.
2. Wet the zip tip with 10 μl acetonitrile (ACN). Discard the ACN.
3. Wet the zip tip with 10 μl 0.1% formic acid. Discard the formic acid.
4. Pipet the sample up and down at least 10 times.
5. Wash the zip tip with 10 μl 0.1% formic acid. Discard the formic acid.
6. Repeat the washing of the zip tip with 10 μl 0.1% formic acid. Discard the formic acid.
7. Elute the sample into a sample vial insert using 10 μl 0.1% formic acid/50% ACN.
8. Repeat the elution into the sample vial insert.
9. Elute the sample into a sample vial insert using 0.1% formic acid/99.9% ACN.
10. Repeat the elution into another tube, if desired, using 10 μl 0.1% formic acid/99.9% ACN into the sample vial insert.
11. Dry liquid in vial insert using speed vac.
12. Resuspend sample in vial insert with 6 μl of 0.1% formic acid containing 0.7:2.8 ACN:water ratio. Sample is ready for mass spec.

**Solutions**

**8 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 0.489 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

**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