# 8 M Urea In-solution Digest Protocol

## Material

Note: All reagents, solutions and vessels should be of high purity and keratin free to obtain optimal results.

- Urea lysis buffer
  8 M Urea (7.21 g for 15 ml)
  100 mM NaCl
  50 mM TEAB, pH 8.5

- Protease inhibitor tablets
  Complete Mini EDTA-free Protease Inhibitor tablets (Roche, 1 tablet for 10 ml lysis buffer)

- PMSF stock
  100 mM in DMSO (store aliquots at -20°C)

- Optional: Phosphatase Inhibitors
  PhosSTOP Phosphatase Inhibitor tablets (Roche, 1 tablet for 10 ml lysis buffer)

- DTT stock solution
  0.5 M in $H_2O$
- IAA (iodoacetamide) stock solution (always prepare fresh)
  0.5 M in $H_2O$
- Urea dilution solution
  50 mM TEAB pH 8.5
- Lys-C stock solution
  200 ng/µl solution (in 0.01% TFA), store at -20°C
- Trypsin stock solution
  200 ng/µl solution (in 0.01% TFA), store at -20°C
- TFA stock
  25% Trifluoroacetic acid in $H_2O$

## Procedure

### Cell lysis

1. Prepare lysis buffer by adding protease inhibitors, PMSF (1 mM final ➔ 1:100) and optionally phosphatase inhibitors.

2. Add appropriate amount of lysis buffer to the cell pellet, resuspend by pipetting and incubate on ice for 10 min.
3. Sonicate at 40% output 3x 10 s at 4°C with 30 s rest in between (0.5 s on 0.5 s off setting, put sample in ice-water bath).
4. Centrifuge 30 min at 4°C at 2500 g to remove cell debris. ➔ Transfer SN to fresh tube.
5. Determine protein concentration using a colorimetric protein assay (Bradford, Lowry etc.).

### Reduction & Alkylation

1. Add DTT to 10 mM final concentration and incubate for 1 h at 27°C to reduce disulfide bridges. Avoid temperatures over 30°C to reduce the amount of unwanted protein carbamylation.
2. Allow sample to cool down to RT. Add IAA to 30 mM final conc. Incubate for 30 min at RT in the dark.
3. Quench unreacted IAA by adding DTT to 10 mM final conc. and incubating 15 min at RT in the dark.

### Two-step digestion

1. Confirm pH to be at ~8.5.
2. Add proteinase Lys-C at 1: 100 enzyme:protein ratio, mix and incubate for 3 - 4h at 37°C.
3. Dilute mixture 1:5 with 50 mM TEAB pH 8.5, thereby reducing the urea conc. to 1.6 M.
4. Add trypsin at a 1:50 - 1:100 enzyme:protein ratio and incubate over night at 37°C.
5. Allow the digest to cool to RT and stop the digestion by adding TFA to 0.2% (vol/vol.) final conc. Verify that pH is <2.
6. Centrifuge at 2500 g for 10 min at RT and transfer supernatant containing the peptides to a fresh microtube
7. Proceed with peptide desalting using C18-StageTips (small peptide amounts) or SepPak solid phase extraction cartridges (large peptide amounts).