Systems Virology
of Emerging Respiratory Viruses

**P001.0P - General Protein Digestion Protocol (Tom Metz, 5-28-10)**

1) All samples are denatured in 50 mM ammonium bicarbonate containing 8 M urea, then shipped frozen on dry ice to PNNL.

OPTIONAL: Addition of Mini-Proteome

A. Perform BCA Protein Assay to determine protein concentration in samples. Determine the amount of protein to digest, take aliquots of samples to achieve the desired amount of protein.
B. Add 50 mM ammonium bicarbonate to each aliquoted sample for an equal final volume for all samples.
C. Add Mini-Proteome to each sample as per Mini-Proteome calculator.

**Mini Proteome Composition**

| Protein Name | Protein Description | Concentration (mg/mL) | Concentration (µM) | Monoisotopic Mass |
|---|---|---|---|---|
| P005\|G3P_RABIT | Glyceraldehyde 3-PDH | 0.21 | 5.9 | 35555 |
| P008\|MYG_HORSE | Apomyoglobin | 1 | 59 | 16941 |
| P010\|CYC_BOVIN | Cytochrome C | 0.363 | 31 | 11565 |
| | Total protein mass in Mini-Proteome: | 1.573 | | |
| | Mini-Proteome to sample protein ratio: | 1:100 | | |

Reduction

2) Prepare fresh 100 mM dithiothreitol (DTT) stock solution by weighing 15.4 mg of DTT and adding it to 1.0 mL of Nanopure water. Vortex until all DTT is dissolved.
3) Add appropriate amount of 100 mM DTT stock solution to each sample to reach a final concentration of 10 mM DTT in all samples. Vortex all samples briefly.
4) Incubate all samples for 1 h at 37ºC in a Thermomixer with constant shaking at 800 rpm.

Alkylation

5) Prepare fresh 400 mM stock solution of iodoacetamide by weighing 74.0 mg of iodoacetamide and adding it to 1.0 mL of Nanopure water. Vortex until all iodoacetamide is dissolved. Keep the iodoacetamide solution in the dark.
6) Add appropriate amount of 400 mM iodoacetamide stock solution to each sample to reach a final concentration of 40 mM iodoacetamide. Vortex all samples briefly.
7) Incubate all samples at 37ºC for 1 h in the dark in a Thermomixer with constant shaking at 800 rpm.

Digestion

8) In a separate tube (assuming that all samples are at the same protein concentration),
a. Add appropriate amount of 50 mM ammonium bicarbonate, pH 8.0, to dilute samples 10 fold
b. Add appropriate amount of 1 M $CaCl_2$ stock solution to samples to obtain a final concentration of 1 mM $CaCl_2$.
c. Vortex sample briefly.
9) To activate trypsin, reconstitute 20.0 µg (one vial) of trypsin with 20 µL of 50 mM ammonium bicarbonate, pH 8.0, and incubate for 10 min at 37ºC. Add appropriate amount of reconstituted trypsin to all samples to make a 1:50 (w/w) trypsin-to-protein ratio.
10) Incubate all samples for 3 h at 37ºC for tryptic digestion (if sample volumes are below 2 mL, then incubate in Thermomixer with constant shaking at 800 rpm; if sample volumes are above 2 mL, then incubate in an oven).
11) Snap freeze samples in liquid nitrogen after digestion.

SPE clean up

12) Clean up samples using 1 mL C18 SPE columns (a 1 mL/50 mg SPE column is sufficient for up to 2 mg of peptides, and a 1 mL/100 mg SPE column is sufficient for up to 5 mg of peptides). The following procedure is applicable to both sizes. Note: Columns can withstand going to dryness between steps as long as the dry period is extended for no longer than 30 seconds. However, the columns cannot be allowed to go dry between the methanol and 0.1% TFA steps.
13) Condition columns with 3 mL of methanol.
14) Rinse columns with 2 mL of acidified water (0.1% TFA)
15) Slowly add sample solutions to the columns (no faster than 1 mL/minute).
16) Wash columns containing samples with 4 mL of 95:5 $H_2O$:ACN, 0.1% TFA.
17) Allow columns to go to dryness and blot “needles” below columns dry.
18) Put collection tubes under columns, and, while SPE tubes are closed off from vacuum, add 1 mL of 80:20 ACN:$H_2O$, 0.1% TFA. Allow elution buffer to flow slowly through columns until columns are dry. Remove samples from SPE vacuum chamber.
19) Concentrate samples in speed-vac to a volume of ~50-100 µL. If necessary, add sufficient amount of 25 mM $NH_4HCO_3$ to dissolve any particulate matter on tube sides, and perform BCA protein assay on sample.
20) Perform BCA Protein Assay following concentration of cleaned sample for peptide concentration.
21) Snap freeze samples in liquid nitrogen and store at -80ºC until needed for analysis.

Note:

- ✓ Sequencing grade modified trypsin was purchased from Promega
- ✓ Ammonium bicarbonate and acetonitrile were purchased from Fisher Scientific
- ✓ Bicinchoninic acid (BCA) assay reagents and standards were purchased from Pierce
- ✓ Water was purified using a Barnstead Nanopure Infinity water purification system
- ✓ All other reagents were purchased from Sigma-Aldrich