2. In-solution digestion in membrane protein

Sample type: Cell sample	Method: Filter aided sample preparation (FASP)

2015. 8.

K-Bio 신약개발지원센터 진종화

1. Samples are prepared in 4% sodium dodecyl sulfate (SDS) and diluted in 8 M urea to dissociate SDS from the proteins.

2. Mix up to 30 µl of a protein extract with 200 µl of Urea 8.5 in the filter unit and centrifuge at 14,000 x g for 15-40 min.

3. Add 200 µl of Urea 8.5 to the filter unit and centrifuge at 14,000 x g for 15-40 min.

4. Discard the flow-through form the collection tube.

5. Note if your lysis step did not include a reduction step using DTT you should add 100 µl of 10 mM DTT in Urea 8.5, vortex for 1 min and incubate at 50 °C for 1 5 min and centrifuge at 14,000 x g for 15-40 min.

6. Discard the flow-through form the collection tube.

7. Add 100 µl IAA solution and vortex for 1 min and incubate without mixing for 20 min in the dark.

8. Centrifuge at 14,000 x g for 15-40 min.

9. Add 100 µl of Urea Sample Solution to the Spin Filter and 8. (Centrifuge at 14,000 x g for 15 min) Repeat this step twice.

10. The protein concentration in the vitreous and depleted plasma was measured using BCA methods according to the protocol provided by the manufacturer.

11. Aliquots of 100 µg protein were reduced, alkylated, and digested according to the protocol.

12. Briefly, 60 µl 9 M urea (5.4054 g) and 30 mM dithiothreitol (DTT; 0.04628 g) in 10 ml 100 mM Tris-base (pH 8.0) were added to each sample and incubated for 30 min at 37 °C.

13. Each sample was allowed to cool at room temperature before 9 µl 500 mM iodoacetamide

(IAA; 0.0925 g/ml) was added.

14. The solution was incubated for 20 min at room temperature.

15. To dilute the urea from 6 M to 0.6 M, 771 µl 100 mM Tris buffer (pH 8.0) was added to each sample.

16. Each sample was then digested with trypsin at a protein to enzyme ratio of 50:1 at 37 °C overnight.

17. To quench the digestion reaction, 50 µl 0.1% TFA was added.

2. In-solution digestion in membrane protein	
Sample type: Tissue sample	**Method: Filter aided sample preparation (FASP)**

2015. 8.

K-Bio 신약개발지원센터 진종화

1. Put the suitable amount of tissue into liquid nitrogen.
2. Slice the tissue using the scalpel.
3. Grind the sliced tissue in a pastel.
4. Put the lysis buffer (300 µl) and homogenize the sample.
5. Wash the pastel and add the washed buffer to sample.
6. Sonicate the sample (Pulse 1/1sec, 6 times, 4 cycle).
7. Centrifuge the sample at 13,500 rpm for 30 hr.
8. Transfer the supernatant to a new 1.5 ml tube.
9. Measure the protein concentration using BCA assay.
10. Samples are prepared in 4% sodium dodecyl sulfate (SDS) and diluted in 8 M urea to dissociate SDS from the proteins.
11. Mix up to 30 µl of a protein extract with 200 µl of Urea 8.5 in the filter unit and centrifuge at 14,000 x g for 15-40 min.
12. Add 200 µl of Urea 8.5 to the filter unit and centrifuge at 14,000 x g for 15-40 min.
13. Discard the flow-through form the collection tube.
14. Note if your lysis step did not include a reduction step using DTT you should add 100 µl of 10 mM DTT in Urea 8.5, vortex for 1min and incubate at 50 °C for 15 min and centrifuge at 14,000 x g for 15-40 min.
15. Discard the flow-through form the collection tube.

16. Add 100 µl IAA solution and vortex for 1min and incubate without mixing for 20 min in the dark.

17. Centrifuge at 14,000 x g for 15-40 min.

18. Add 100 µl of Urea Sample Solution to the Spin Filter and 15 (centrifuge at 14,000 x g for 15 min) Repeat this step twice.

19. The protein concentration in the vitreous and depleted plasma was measured using BCA methods according to the protocol provided by the manufacturer.

20. Aliquots of 100 µg protein were reduced, alkylated, and digested according to the protocol.

21. Briefly, 60 µl 9 M urea (5.4054 g) and 30 mM dithiothreitol (DTT; 0.04628 g) in 10 ml 100 mM Tris-base (pH 8.0) were added to each sample and incubated for 30 min at 37 °C.

22. Each sample was allowed to cool at room temperature before 9 µl 500 mM iodoacetamide (IAA; 0.0925 g/ml) was added.

23. The solution was incubated for 20 min at room temperature.

24. To dilute the urea from 6 M to 0.6 M, 771 µl 100 mM Tris buffer (pH 8.0) was added to each sample.

25. Each sample was then digested with trypsin at a protein to enzyme ratio of 50:1 at 37 °C overnight.

26. To quench the digestion reaction, 50 µl 0.1% TFA was added.

2. In-solution digestion in membrane protein	
Sample type: Plasma (RBC Membranes)	**Method: Detergent digestion**

2015. 8.

K-Bio 신약개발지원센터 진종화

1. Resuspended RBC membranes (10 µl; 80 µg protein) with 1 mL 100 mM Na_2CO_3 (pH 11).
2. Pass 5 times through a 25-gauge needle, mix by rotation (30 min, 4 °C), and centrifuge (90min, 245,000 x g), and remove the supernatant.
3. Repeat the process of suspension, rotation, pelleting, and washing (Twice more).
4. Add a 1:1 or 1:2 volume ratio of either 8 M urea, 6 M urea/2 M thiourea, or 8 M guanidine-HCl (pH 1.5, 10 min, RT) to Membrane samples and centrifuged (10 min, 9,300 x g).
5. Remove the supernatant and check its pH (Approximately 8.0).
6. Add 1 µg DTT to sample (per 50 µg protein) and incubate at RT for 30 min
7. Alkylate the reduced sample by supplementing with 5 µg iodoacetamide per 50 µg protein (20 min, RT).
8. Add Lys C (endoproteinase Lys C from Lysobacter enzymogenes; Sigma-Aldrich) to sample (1 µg/50 µg protein), incubate (3 hr, RT) and dilute (4 volumes 50 mM ammonium carbonate).
9. Add 1 µg trypsin/50 µg protein and incubate (15 hr, RT).

Reference

1. J. Jin, et. al., Detection of differential proteomes of human β-cells during islet-like differentiation using iTRAQ labeling. Journal of Proteome Research (2009).

2. J. Jin, et. al., Retinal proteome analysis in a mouse model of oxygen-induced retinopathy, Journal of Proteome Research (2013).

3. Erica M. Pasini, et. al., In-depth analysis of the membrane and cytosolic proteome of red blood cells. Blood (2006).