In-solution digestion in total proteins

Sample type: Cell sample	Method: Freeze-thaw lysis method

2015. 8.

K-Bio 신약개발지원센터 진종화

Whole cells are lysed using a modified freeze-thaw lysis method.

1. Harvest and wash the whole cell with ice-cold PBS three times
2. Resuspend with 500 µl ice-cold PBS containing a protease inhibitor cocktail, and frozen at -70 °C.
3. Repeat freeze-thaw cycles 3 times by pipeting up and down.
4. Sonicate lysed cell suspensions with a 25% pulse for 20sec three times.
5. Centrifuge Samples at 10,000 x g g for 30 min at 4 °C.
6. Remove the supernatants and measure the concentration using the BCA assay (Sigma-Aldrich, St Louis, MO).
7. Dry the each sample (a total of 100 or 200 µg) in a speed vacuum dryer for 3 hr, and resuspended with 20 µl of dissolution buffer (0.5 M triethylammonium bicarbonate and 0.05% SDS).
8. Reduce the disulfide bonds of the proteins with tris (2-carboxyethyl)phosphine (TCEP) (MW 286.65) at a final concentration of 12.5 mM at 37 °C for 1 hr.
9. Alkylate, the produced free thiols, with 40 mM iodoacetamide (MW 184.96) at room temperature in dark for 1 hr.
10. Dilute the solution with 20 mM TrisHCl (pH 8.3) to a final concentration of 1.0 M urea.
11. Digest with sequencing-grade modified trypsin at 20 µg per mg (20 ng/µg) of protein for overnight at 37 °C.

In-solution digestion in total proteins

Sample type: Cell sample	Method: Urea digestion method

2015. 8.

K-Bio 신약개발지원센터 진종화

1. Add 350-400 µl Lysis Buffer containing protease & phosphatase inhibitor and scrub the cells with a scrubber carefully and collect in tube.
2. Incubate the cells on ice for 10 min.
3. Lyse the cells by sonication for 1 min. Ampl: 21 and pulse: 0.7/1 sec.
4. Remove cell debris and nuclei by centrifugation for 10min 40 °C using 12,000 rpm.
5. Transfer the supernatant to a new 1.5 ml tube.
6. Measure the protein concentration using BCA assay.
7. Aliquots of 100 µg protein were reduced, alkylated, and digested according to the protocol.
8. 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.
9. Each sample was allowed to cool at room temperature before 9 µl 500 mM iodoacetamide (IAA; 0.0925 g/ml) was added.
10. The solution was incubated for 20 min at room temperature.
11. 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.
12. Each sample was then digested with trypsin at a protein to enzyme ratio of 50:1 at 37◦C overnight.
13. To quench the digestion reaction, 50 µl 0.1% TFA was added.

In-solution digestion in total proteins

Sample type: Tissue sample	Method: Urea digestion method

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 1/1 sec, 6 times, 4 cycle).
7. Centrifuge the sample at 13,500 rpm for 30hr.
8. Transfer the supernatant to a new 1.5 ml tube.
9. Measure the protein concentration using BCA assay.
10. Aliquots of 100 µg protein were reduced, alkylated, and digested according to the protocol.
11. Briefly, 60 µl 9M 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.
12. Each sample was allowed to cool at room temperature before 9 µl 500mM iodoacetamide (IAA; 0.0925 g/ml) was added.
13. The solution was incubated for 20 min at room temperature.
14. 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.
15. Each sample was then digested with trypsin at a protein to enzyme ratio of 50:1 at 37 °C overnight.
16. To quench the digestion reaction, 50 µl 0.1% TFA was added.

In-solution digestion in total proteins

Sample type: Plasma sample	Method: MARS depletion & Urea digestion

2015. 8.

K-Bio 신약개발지원센터 진종화

※ Depletion of high abundant proteins

1. To deplete plasma using a MARS column, plasma was diluted 5-fold with MARS buffer A (1:4).
2. Filter with a 0.22 μm Ultra free-MC Durapore centrifugalfilter.
3. Each plasma sample was applied to a MARS column on an LC-10AT HPLC system.
4. The sample loop volume of the HPLC was set to 200 μl, and 200 μl of 5-fold diluted plasma was injected into the MARS column.
5. The total LC run time of 37 min involved the following: 100% MARS buffer A at a flow rate of 0.7 ml/min for the first 11 min, sample injection, wash for 11 min, 100% MARS buffer B at a flow rate of 1.0 ml/min for 5 min, and 100% MARS buffer A at a flow rate of 0.7 ml/min for 10 min.
6. The UV detector was set to 280 nm for plasma injection, and the eluted fractions were collected in 250 μl aliquots.
7. The flow-through and bound fractions were eluted in 10 fractions (total, 2.5 ml), and the eluted fractions were pooled for MRM.

※ Digestion of Plasma sample.

1. The protein concentration in the vitreous and depleted plasma was measured using BCA methods according to the protocol provided by the manufacturer.
2. Aliquots of 100 μg protein were reduced, alkylated, and digested according to the protocol.
3. Briefly, 60 μl 9 M urea (5.4054 g) and 30mM 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.
4. Each sample was allowed to cool at room temperature before 9 μl 500 mM iodoacetamide (IAA;

0.0925 g/ml) was added.

5. The solution was incubated for 20 min at room temperature.
6. 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.
7. Each sample was then digested with trypsin at a protein to enzyme ratio of 50:1 at 37 °C overnight.
8. To quench the digestion reaction, 50 µl 0.1% TFA was added.