SOP: SP065

In-Solution Double Digestion and Depletion of Abundant Proteins

Materials and Reagents:

1. Protein of interest
2. 0.65 ml Deplasticized Eppendorf tubes (note 1)
3. Denaturing solution (6 M GuanHCl in 0.2M Tris-HCl, pH 8.6)
4. Vortexer
5. Reducing solution (0.4 M DTT, prepared in denaturing solution) (Note 2)
6. Alkylating Solution (18 mg iodoacetamide in 500 µL 10mM AMBIC) (Note 2)
7. 37˚C incubator
8. 10 mM ammonium bicarbonate
9. Slide-A-Lyzer MINI Dialysis Unit (Thermo #69552)
10. Dialysis tank
11. Savant Speed-Vac
12. Modified trypsin sequencing grade (Roache Molecular Biochemicals, catalog # 1 418 025) (note 2)
13. Acetonitrile (MS grade)
14. Burdick and Jackson Water (note 3)
15. Buffer A: 96.9% water, 3% ACN and 0.1% formic acid
16. Autosampler vials with lids

Protocol:

Day 1:

1._____ Savant Speed-Vac 100 µg of the protein of interest to minimal volume (2-5 ul) in a deplasticized Eppendorf tube. (Note 3 & 4)

2. _____ Resuspend protein in 100 µL of denaturing solution.

3._____ Add 1 µL of reducing solution, vortex briefly and incubate at room temperature for 3 hours.

4. _____ Add 10µL of alylating solution and incubate in the dark at 37˚C for 30 minutes.

5. _____ Transfer samples to Slide-A-Lyzer unit and dialyze samples using 10 mM ammonium bicarbonate. Incubate overnight at 4 ˚C.

Day 2:

6. _____ Remove samples from dialysis units and rinse membrane with additional 10 mM ambic, adding total sample to a new deplasticized Eppendorf tube.

7. _____ Dry sample in Savant Speed-Vac down to minimal volume (2-5 ul).

8. _____ Dissolve 25 µg of trypsin in 300 µl of 0.2 M ammonium bicarbonate (notes 5, 6 & 7). Add trypsin solution to the sample at a ratio of 50:1 (sample:trypsin), for 100 µg of protein add 2 µg (24 µL) of trypsin

9. _____ Add acetonitrile to a final concentration of 10%

10. _____ Incubate the digest reaction overnight at 37˚C.

Day 3:

11. _____ Terminate the reaction by adding 1/10th the digest volume of 10% TFA.

12. _____ Savant Speed-Vac and dry (note 8).

13. _____ Add buffer A to the sample and mix well. Sample should be at final concentration of 500 ng/μL - 1μg/μL for the LTQ (note 9).

14. _____ Centrifuge the sample for 5 minutes.

15. _____ Pipet the sample out of the tube, being sure not to touch the bottom of the tube with the pipet tip (there will be a small volume left in the tube).

16. _____ Pipet sample into an autosampler vial, being careful not to introduce any bubbles into the vial.

17. _____ Place the cap on the autosampler vial and label the vial.

18. _____ Store the samples at –20°C until analysis by LC-MS-MS.

Notes:

1. Eppendorf tubes are deplasticized by filling with 60% acetonitrile-0.1% TFA, followed by mixing, incubation at room temperature for 1 hour and decanting of the solution. This process is repeated two times for each tube and the tubes are dried in the Savant Speed-Vac.
2. These solutions should be made fresh and discarded after use.
3. This procedure can be used with less than 100 μg of protein, adjust reagents accordingly.
4. See SOP SP005 for use of the savant.
5. This procedure can be used with proteases other than trypsin, however the buffer for the digestions may differ for other proteases.
6. All solutions should be made using burdick and jackson water.
7. When preparing a new vial of trypsin, be sure to date and initial it. The reconstituted trypsin should be stored at 4˚C and can be used for up to two weeks.
8. Do not allow to dry completely. Leave 1-2 μl of liquid in the bottom of the tube.
9. Ideal final concentration varies greatly for the TQ-S depending on sample purity and purpose.

References:

Hellman, U., C. Wernstedt, J. Gonez, and C. H. Heldin. 1995. Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. Anal Biochem 224:451-5.

Rosenfeld, J. *et al*. 1992. In gel digestion of proteins for internal sequence analysis after 1 or 2-D gel electrophoresis. Anal Biochem 202: 173-179.