In-gel preparation for mass spectrometry analysis

Materials

25 mM ammonium bicarbonate (NH4HCO3; 1.98 mg/mL)/50% (v/v) acetonitrile

10 mM dithiothreitol (DTT, 1.54 mg/mL) in 25 mM NH4HCO3

55 mM iodoacetamide (10.2 mg/mL) in 25 mM NH4HCO3

25 mM NH4HCO3, pH 8

0.1 mg/mL trypsin (TPCK treated, AB Sciex) in 25 mM NH4HCO3, pH 8

50% Acetonitrile/50% 0.1% trifluoroacetic acid (TFA); (v/v)

Protocol

1. Excise protein bands from a stained polyacrylamide gel. Cut each gel piece into small particles (~1 mm2), and place into a 1.5 mL tube.

2. Add ~100 µL of 25 mM NH4HCO3/50% acetonitrile (or enough to immerse the gel particles) and vortex for 35-40 min on a low setting (more like shaking). Remove the solution and discard. Repeat this wash/dehydration step up to ~3 times.

3. Dehydrate gels with acetonitrile (100 µL). At this point the gel pieces should shrink and become an opaque-white color. If they do not, remove the acetonitrile and replace with fresh.

4. Remove acetonitrile and SpeedVac for 3-5 minutes.

5. Add 30 µL of the 10 mM DTT solution to cover the gel pieces, and reduce for 30-45 min at room temperature.

6. Replace the DTT solution with roughly the same volume of 55 mM iodoacetamide (30 µL). Incubate for 45 min at room temperature in the dark.

7. Remove the iodoacetamide solution and wash gel pieces with ~100 µL of 25 mM NH4HCO3 pH 8, for 10 min while vortexing.

8. Remove wash and dehydrate with ~100 µl acetonitrile. The gel pieces should shrink and become an opaque-white color. If they do not, remove the acetonitrile and repeat the washing-dehydration cycle until they do.

9. Remove the acetonitrile and dry the gel pieces in a vacuum centrifuge for 3-5 minutes.

10. Rehydrate gel particles in 25 µL trypsin solution and place on ice for 10-15 minutes.

11. Remove excess trypsin solution and overlay the rehydrated gel particles with 30 µL of 25 mM NH4HCO3 to keep them immersed throughout digestion.

12. Incubate 12 to 16 h at 37˚C. Recover peptides using a Zip-Tip

*LC-MS/MS*

Desalted samples were rehydrated in 0.1% formic acid (FA) and were analyzed by LC-MS using a nanoLC Eksigent 400 system (Eksigent, AB Sciex), coupled online to an TripleTOF6600 mass spectrometer (AB Sciex). Peptide separation was performed using liquid chromatography with a trap and elution conﬁguration using a nano trap column (350 μm × 0.5 mm, 3 μm, 120 Å, AB Sciex) and a nano ChromXP C18 reverse phase column (75 μm × 15 cm, 3 μm, 120 Å, AB Sciex) at 300 nl/min with a 90 min linear gradient of 8-30% acetonitrile in 0.1% FA, and then, with a 10 min linear gradient of 30% to 40% acetonitrile in 0.1% FA. The mass spectrometer was operated in information-dependent acquisition (IDA) mode, scanning full spectra (400–1500 *m/z*) for 250 ms, followed by up to 30 MS/MS scans (100–1800 *m/z* for 50 ms each), for a cycle time of 1.8 s. Candidate ions with a charge state between +2 and + 5 and counts above a minimum threshold of 125 counts per second were isolated for fragmentation, and one MS/MS spectrum was collected before adding those ions to the exclusion list for 12 s. Rolling collision energy was used with a collision energy spread of 15. The mass spectrometer was operated using the Analyst TF 1.7.1 software program (AB Sciex).

*Data Analysis*

Acquired spectra were searched against the UniProt reviewed database using the Paragon algorithm embedded in the ProteinPilot 5.0.1 software program (AB Sciex), with the following search parameters: (i) sample type: identiﬁcation, (ii) Cys alkylation: iodoacetamide, (iii) digestion: trypsin, (iv) instrument: TripleTOF 6600, (v) species: *Mus musculus*, (vi) ID focus: biological modiﬁcations, (vii) detected protein threshold: > 0.05 (10% conﬁdence). The detected protein threshold was set to the minimum level to enhance the number of wrong answers to enable the curve ﬁtting by an independent FDR analysis [W.H. Tang, I.V. Shilov, S.L. Seymour, Nonlinear ﬁtting method for determining local false discovery rates from decoy database searches, J. Proteome Res. 7 (2008) 3661–3667]. This was carried out by the target-decoy approach provided with the ProteinPilot software program, which was used to assess the quality of the identiﬁcations. Positive identiﬁcations were considered when identiﬁed proteins and peptides reached a 1% local FDR [L. Sennels, J.C. Bukowski-Wills, J. Rappsilber, Improved results in proteomics by use of local and peptide-class speciﬁc false discovery rates, BMC Bioinf. 10 (2009) 179].