Protocol: 1

Ingel Digestion Protocol with Incubation Overnight

Reference:

In-gel digestion for mass spectrometric characterization of proteins and proteomes. Shevchenko et al. Nature protocols. 2006.

Reagents:

All solvents and reagents used are of LC-MS quality.

A. Trypsin preparation can be of 2 types depending on sample numbers.

Prepartion1 (for bulk use): Prepare a solution of 13ng/µl Trypsin in 10mM ammonium bicarbonate containing 10% (vol/vol) Acetonitrile. Dissolve the content of 20µg vial in 1.5ml of the buffer. Make shortly before use; discard unused volume.

Preparation 2(for less use): If only a small volume of Trypsin buffer is required, the 20µg lyophilized enzyme can be re-dissolved in 1.5ml 1mM HCl (13ng/µl Trypsin prepared) and 100µl aliquots stored at -20°C. Note that after thawing frozen aliquots, pH should be adjusted by adding 15µl of 50mM ammonium bicarbonate shortly before use.

B. 100mM ammonium bicarbonate in water. Make ammonium bicarbonate buffer freshly in large (50–100 ml) volumes and discard after use.
C. Destaining solution: 100mM ammonium bicarbonate/Acetonitrile (1:1 vol/vol)
D. Extraction solution: 1:2(vol/vol) 5% formic acid/Acetonitrile.

Procedure:

1. Rinse the entire slab of a one or two-dimensional gel with water for a few hours, and excise bands (spots) of interest with a clean scalpel.
2. Cut excised bands (spots) into cubes (1mm). Note that smaller pieces could clog pipette tips.
3. Transfer gel pieces into a microcentrifuge tube and spin them down on a bench-top microcentrifuge.
4. Add 100µl of destaining solution and incubate with occasional vortexing for 30 min, depending on the staining intensity.
5. Spin the contents at 5000rpm for 1 minute. Carefully decant the supernatant.
6. Add 500µl of neat Acetonitrile and incubate at room temperature with occasional vortexing, until gel pieces become white and shrink and then remove acetonitrile. Although the bulk of Coomassie staining should be removed, it is not necessary to destain the gel pieces completely.
7. Dry all the gel pieces in vacuum centrifuge.
8. Samples are now ready for digestion. Alternatively, they can be stored at -20°C for a few weeks.
9. Add enough trypsin buffer to cover the dry gel pieces (typically, 50µl or more, depending on the volume of a gel matrix) and leave it in an ice bucket or a fridge.
10. After 30 min, check if all solution was absorbed and add more trypsin buffer, if necessary. Gel pieces should be completely covered with trypsin buffer.
11. Leave gel pieces for another 90 min to saturate them with trypsin and then add 10–20µl of ammonium bicarbonate buffer to cover the gel pieces and keep them wet during enzymatic cleavage.
12. Although after 30min dried gel pieces do not absorb any more buffer, the yield of tryptic peptides increases substantially while extending the incubation time, presumably because of slow diffusion of the enzyme into a polyacrylamide matrix.
13. Place tubes with gel pieces into thermostat and incubate samples at 37°C for overnight. CRITICAL STEP: It is important to avoid a temperature gradient between the bottom and the lid of the tube to prevent condensation of water at the inner surface of the lid and, consequently, premature dehydration of the gel pieces.
14. Chill tubes to room temperature, spin down gel pieces (10000rpm) using a microcentrifuge for a minute and withdraw the supernatant directly from the digest. As a typical volume of the digestion buffer is approximately 50µl, this leaves ample peptide material for the subsequent MS/MS analysis, if required.
15. Non-extracted digests can be stored at -20°C for a few months until it is decided if further LC MS/MS analysis.

16. Add 100µl of extraction buffer to each tube and incubate for 15 min at 37^{o}C in a shaker. For samples with much larger (or smaller) volume of gel matrix, add the extraction buffer such that the approximate ratio of 1:2 between volumes of the digest and extraction is achieved.
17. Spin down the gel pieces and carefully withdraw the supernatant. Utmost care must be taken not to take the gel pieces with the supernatant, if taken gel pieces may clog the auto-sampler or column.
18. Pool the respective supernatant and dry the contents in a vacuum centrifuge.
19. Dried extracts can be safely stored at -20^{o}C for a few months. For further LC MS/MS analysis, add 10–20µl of 0.1% (vol/vol) trifluoroacetic acid into the tube, vortex and incubate the tube for 2–5 min in the sonication bath and centrifuge for 15 min at 6.7g (10000 rpm) at the bench-top centrifuge and withdraw the appropriate aliquot for further MS analysis.

Protocol: 2

Accelerated Ingel Digestion Protocol

References:

1. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Shevchenko et al. Nature protocols. 2006.

2. Fast-Response Proteomics by Accelerated In-Gel Digestion of Proteins. Jan Havlis et al. Anal. Chem. 2003.

Reagents:

All solvents and reagents used are of LC-MS quality.

A. Trypsin preparation can be of 2 types depending on sample numbers.

Prepartion1 (for bulk use): Prepare a solution of 13ng/µl Trypsin in 10mM ammonium bicarbonate containing 10% (vol/vol) Acetonitrile. Dissolve the content of 20µg vial in 1.5ml of the buffer. Make shortly before use; discard unused volume.

Preparation 2(for less use): If only a small volume of Trypsin buffer is required, the 20µg lyophilized enzyme can be re-dissolved in 1.5ml 1mM HCl (13ng/µl Trypsin prepared) and 100µl aliquots stored at -20°C. Note that after thawing frozen aliquots, pH should be adjusted by adding 15µl of 50mM ammonium bicarbonate shortly before use.

B. 100mM ammonium bicarbonate in water. Make ammonium bicarbonate buffer freshly in large (50–100 ml) volumes and discard after use.
C. Destaining solution: 100mM ammonium bicarbonate/Acetonitrile (1:1 vol/vol)

Procedure:

1. Rinse the entire slab of a one or two-dimensional gel with water for a few hours, and excise bands (spots) of interest with a clean scalpel.
2. Cut excised bands (spots) into cubes (1mm). Note that smaller pieces could clog pipette tips.
3. Transfer gel pieces into a microcentrifuge tube and spin them down on a bench-top microcentrifuge.
4. Add 100µl of destaining solution and incubate with occasional vortexing for 30 min, depending on the staining intensity.
5. Spin the contents at 5000rpm for 1 minute. Decant the supernatant.
6. Add 500µl of neat Acetonitrile and incubate at room temperature with occasional vortexing, until gel pieces become white and shrink and then remove Acetonitrile. Although the bulk of Coomassie staining should be removed, it is not necessary to destain the gel pieces completely.
7. Dry all the gel pieces in vacuum centrifuge.
8. Samples are now ready for digestion. Alternatively, they can be stored at -20°C for a few weeks.
9. Add enough trypsin buffer to cover the dry gel pieces (typically, 50µl or more, depending on the volume of a gel matrix) and leave it in an ice bucket or a fridge.
10. After 30 min, check if all solution was absorbed and add more trypsin buffer, if necessary. Gel pieces should be completely covered with trypsin buffer.
11. Leave gel pieces for another 90 min to saturate them with trypsin and then add 10–20µl of ammonium bicarbonate buffer to cover the gel pieces and keep them wet during enzymatic cleavage.
12. Although after 30min dried gel pieces do not absorb any more buffer, the yield of tryptic peptides increases substantially while extending the incubation time, presumably because of slow diffusion of the enzyme into a polyacrylamide matrix.
13. Place tubes with gel pieces into thermostat and incubate samples at 56°C for 2 hours. CRITICAL STEP: It is important to avoid a temperature gradient between the bottom and the lid of the tube to prevent condensation of water at the inner surface of the lid and, consequently, premature dehydration of the gel pieces.
14. Chill tubes to room temperature, spin down gel pieces (10000rpm) using a microcentrifuge for a minute and withdraw the supernatant directly from the digest without further extracting the gel pieces. As a typical volume of the digestion buffer is approximately 50µl, this leaves ample peptide material for the subsequent MS/MS analysis, if required.

Protocol: 3

In-solution Digestion Protocol

Reference:

1. Proteomics Characterization of Mouse Kidney Peroxisomes by Tandem Mass Spectrometry and Protein Correlation Profiling. Sebastian Wiese et al. Mol Cel Proteomics. 2007.

Reagents:

All solvents and reagents used are of LC-MS quality.

A. Trypsin preparation can be of 2 types depending on sample numbers.

Prepartion1 (for bulk use): Prepare a solution of 13ng/µl Trypsin in 10mM ammonium bicarbonate containing 10% (vol/vol) Acetonitrile. Dissolve the content of 20µg vial in 1.5ml of the buffer. Make shortly before use; discard unused volume.

Preparation 2(for less use): If only a small volume of Trypsin buffer is required, the 20µg lyophilized enzyme can be re-dissolved in 1.5ml 1mM HCl (13ng/µl Trypsin prepared) and 100µl aliquots stored at -20°C. Note that after thawing frozen aliquots, pH should be adjusted by adding 15µl of 50mM ammonium bicarbonate shortly before use.

B. 100mM ammonium bicarbonate in water. Make ammonium bicarbonate buffer freshly in large (50–100 ml) volumes and discard after use.
C. 5% Formic acid (vol/vol) in water.

Procedure:

1. Adjust the pH of the protein sample by adding 50mM ammonium bicarbonate to ~8.5.
2. Vortex mix the sample and give a short spin.
3. Add 13ng/µl Trypsin Trypsin in 1:30 ratio. Mix well.
4. Place tubes with sample into thermostat and incubate samples at 55°C for 2hours/37°C for overnight.
5. Chill the tubes to room temperature provide a short spin. Add 5% formic acid till the pH reaches nearly 3.
6. Mix well. This can be subjected to MS analysis.