

An In-Gel Digestion Protocol

This protocol describes the digestion of a protein present in an SDS-PAGE gel band with trypsin. The band can be taken from either a 1D or 2D electrophoresis gel.

Reagents All reagents are prepared immediately prior to use. The water used in all components of the procedure is 17 megaohm/cm Nanopure® - or MilliQ® -type water. The acetonitrile and methanol are HPLC grade. All other reagents are the highest possible commercial grade available. The trypsin used is a sequencing-grade enzyme that has been modified to inhibit autolysis and to minimize non-tryptic protease activities. The reagents are prepared in either 20-mL glass vials with Teflon lined caps or 1.5-mL plastic microcentrifuge tubes.

1. Wash solution: Add 10 mL of methanol to 5 mL of water. Add 1 mL of acetic acid and adjust the total volume to 20 mL with water. The final concentrations are 50% (v/v) methanol and 5% (v/v) acetic acid.
2. 100 mM ammonium bicarbonate: Dissolve 0.2 g of ammonium bicarbonate in 20 mL of water.
3. 50 mM ammonium bicarbonate: Mix 2 mL of the 100 mM ammonium bicarbonate with 2 mL of water.
4. 10 mM DTT: Place 1.5 mg of dithiothreitol in a 1.5-mL plastic centrifuge tube. Add 1.0 mL of 100 mM ammonium bicarbonate and dissolve the dithiothreitol.
5. 100 mM iodoacetamide: Place 18 mg of iodoacetamide in a 1.5-mL plastic centrifuge tube. Add 1.0 mL of 100 mM ammonium bicarbonate and dissolve the iodoacetamide.
6. Trypsin solution: Add 1.0 mL of ice cold 50 mM ammonium bicarbonate to 20 μ g of sequencing-grade modified trypsin. Dissolve the trypsin by drawing the solution into and out of the pipette. Keep the trypsin solution on ice until use. The final concentration is 20 ng/ μ L trypsin.
7. Extraction buffer: Add 10 mL of acetonitrile to 5 mL of water. Add 1 mL of formic acid and adjust the total volume to 20 mL with water. The final concentrations must be 50% (v/v) acetonitrile and 5% (v/v) formic acid.

Centrifuge Tubes The 0.5- or 1.5-mL plastic microcentrifuge tubes that are used are purchased as low-binding, siliconized tubes. All tubes are rinsed, in USP-grade ethanol and air-dried prior to use.

The Digestion Procedure The volumes noted in this procedure are for protein bands cut from 2D gels. These bands, or spots, are on the order of 2 mm to 4 mm in diameter, with a gel thickness of 1 mm, and have total volumes less than ~20 μ L. The volumes given in this procedure would also be appropriate for 1D gel bands that are 1-mm to 2-mm wide in 1-cm lanes in a 1-mm-thick gel. Larger-volume gel bands would require a proportional increase in the volumes of each reagent.

Day one

1. Cut the protein bands from the gel as closely as possible with a sharp scalpel, and divide them into smaller pieces that are approximately 1mm³ to 2 mm³. Crushing or pulverizing the gel pieces is not necessary and may create fine particles that will block the capillary liquid chromatography column.
2. Place the gel pieces in a 1.5-mL plastic microcentrifuge tube.
3. Add 200 μ L of the wash solution and rinse the gel pieces overnight at room temperature. If desired, this washing step can be carried out over the weekend or, alternatively, for 4 h.

Day two

4. Carefully remove the wash solution from the sample with a plastic pipette and discard.
5. Add 200 μ L of the wash solution and rinse the gel pieces for an additional 2 to 3 h at room temperature.
6. Carefully remove the wash solution from the sample with a plastic pipette and discard.
7. Add 200 μ L of acetonitrile and dehydrate the gel pieces for ~5 min at room temperature. When dehydrated, the gel pieces will have an opaque white color and will be significantly smaller in size.
8. Carefully remove the acetonitrile from the sample with a plastic pipette and discard.
9. Completely dry the gel pieces at ambient temperature in a vacuum centrifuge for 2 to 3 min.
10. Add 30 μ L of 10 mM DTT and reduce the protein for 0.5h at room temperature.
11. Carefully remove the DTT solution from the sample with a plastic pipette and discard.
12. Add 30 μ L of 100 mM iodoacetamide and alkylate the protein at room temperature for 0.5 h.
13. Carefully remove the iodoacetamide solution from the sample with a plastic pipette and discard.
14. Add 200 μ L of acetonitrile and dehydrate the gel pieces for ~5 min at room temperature. When dehydrated, the gel pieces will have an opaque white color and will be significantly smaller in size.
15. Carefully remove the acetonitrile from the sample with a plastic pipette and discard.

16. Rehydrate the gel pieces in 200 μL of 100 mM ammonium bicarbonate, incubating the samples for 10 min at room temperature.
17. Carefully remove the ammonium bicarbonate from the sample with a plastic pipette and discard.
18. Add 200 μL of acetonitrile and dehydrate the gel pieces for ~5 min at room temperature. When dehydrated, the gel pieces will have an opaque white color and will be significantly smaller in size.
19. Carefully remove the acetonitrile from the sample with a plastic pipette and discard.
20. Completely dry the gel pieces at ambient temperature in a vacuum centrifuge for 2 to 3 min.
21. Prepare the trypsin reagent by adding 1000 μL of ice-cold 50 mM ammonium bicarbonate to 20 μg of trypsin. The concentration of trypsin in this solution is 20 ng/ μL . The solution is kept on ice until use.
22. Add 30 μL of the trypsin solution to the sample and allow the gel pieces to rehydrate on ice for 10 min with occasional vortex mixing. Watch that the gel pieces appear to have been rehydrated by the trypsin solution.
23. Drive the gel pieces to the bottom of the tube by centrifuging the sample for 30 sec. Carefully remove the excess trypsin solution from the sample with a plastic pipette and discard.
24. Add 5 μL of 50 mM ammonium bicarbonate to the sample. Vortex mix the sample. Drive the sample to the bottom of the tube by centrifuging for 30 sec, and carry out the digestion overnight at 37 °C.

Day Three

25. Extract the peptides produced by the digestion in three steps.
 - a. Add 30 μL of 50 mM ammonium bicarbonate to the digest and incubate the sample for 10 min with occasional gentle vortex mixing. Drive the digest to the bottom of the tube by centrifuging the sample for 30 sec. Carefully collect the supernate with a plastic pipette and transfer the sample to a 0.5-mL plastic microcentrifuge tube.
 - b. Add 30 μL of the extraction buffer to the tube containing the gel pieces, and incubate the sample for 10 min with occasional gentle vortex mixing. Drive the extract to the bottom of the tube by centrifuging the sample for 30 sec. Carefully collect the supernate with a plastic pipette and combine the extract in the 0.5-mL plastic microcentrifuge tube.
 - c. Add a second 30- μL aliquot of the extraction buffer to the tube containing the gel pieces, and incubate the sample for 10 min with occasional gentle vortex mixing. Drive the extract to the bottom of the tube by centrifuging the sample for 30 sec. Carefully collect the supernate with a plastic pipette and combine the extract in the 0.5-mL plastic microcentrifuge tube.

26. Reduce the volume of the extract to $<20 \mu\text{L}$ by evaporation in a vacuum centrifuge at ambient temperature. Do not allow the extract to dry completely.
27. Adjust the volume of the digest to $\sim 20 \mu\text{L}$, as needed, with 1% acetic acid. At this point, the sample is ready for analysis.

An In-Solution Digestion Protocol

Reagents All of the reagents are prepared immediately prior to use. The water used in all components of the procedure is 17 megaohm/cm Nanopure® - or MilliQ® -type water. The urea, Tris, ammonium bicarbonate, and acetic acid are the highest grade available. The trypsin used is a sequencing-grade enzyme that has been modified to inhibit autolysis and to minimize non-tryptic protease activities. The reagents are prepared in 20-mL glass vials with Teflon-lined caps of 1.5-mL plastic microfuge tubes.

1. Tris stock: Dissolve 12.1g of Tris base in 200 mL of water. Adjust the pH of the solution to pH 7.8 with 6 M HCl. Add water to give a final volume to 250 mL. The final Tris concentration is 0.4 M. This solution can be stored at 4 °C for 30 days.
2. 6 M urea, 100 mM Tris buffer: Place 2.0 g of urea in a 15-mL centrifuge tube. Add 1.25 mL of the Tris stock. Adjust the total volume to 5 mL with water. The final concentrations are 6 M urea and 100 mM Tris.
3. Reducing agent: Dissolve 30 mg of DTT in 750 μL of water. Add 250 μL of Tris stock and mix by gentle vortex. The final concentrations are 200 mM DTT and 100 mM Tris.
4. Alkylating reagent: Dissolve 36 mg of iodoacetamide in 750 μL of water. Add 250 μL of the Tris stock and mix by gentle vortex. The final concentration is 200 mM iodoacetamide and 100 mM Tris.
5. Trypsin solution: Add 25 μL of ice-cold Tris stock and 75 μL of ice-cold water to 20 μg of sequencing-grade modified trypsin. Dissolve the trypsin by drawing the solution into and out of the pipette. The final concentration of trypsin is 200 ng/ μL . The solution is kept on ice until use.

Sample Tubes The sample tubes are 1.5-mL plastic microcentrifuge tubes. Siliconized tubes can be used but, due to the high protein content of the sample, are not specifically necessary. All tubes are rinsed, first in USP-grade ethanol and second in water, and air-dried prior to use.

The Digestion Procedure The protein sample is evaporated and resuspended in the 6 M urea, 100 mM Tris buffer at 10 mg/mL. Resuspension may require careful mixing by drawing the sample into and out of a plastic pipette. Assuming, for the purpose of estimation, that the mixture is composed of 500 major, approximately equal abundance of proteins, then the 1 mg of total protein that is digested represents ~ 40 pmol of each protein (assuming an average molecular weight of 50 kDa). The final peptide concentration, assuming 100% yield, will be the 50 fmol/ μL range.

Day one

1. Place a 100- μL aliquot of the protein sample in the 6 M urea, 100 mM tris buffer, containing 1 mg of total protein, in a 1.5-mL plastic microcentrifuge tube.
2. Add 5 μL of the reducing reagent and mix the sample by gentle vortex.
3. Reduce the protein mixture for 1 h at room temperature.
4. Add 20 μL of the alkylating reagent and mix the sample by gentle vortex.
5. Alkylate the protein mixture for 1 h at room temperature.
6. Add 20 μL of the reducing agent to consume any unreacted iodoacetamide. Mix the sample by gentle vortex and allow the reaction to stand at room temperature for 1 h.
7. Reduce the urea concentration by diluting the reaction mixture with 775 μL of water. Mix the solution by gentle vortex. This dilution reduces the urea concentration to ~ 0.6 M, a concentration at which the trypsin retains its activity.
8. Add the 100- μL trypsin solution containing 20 μg of trypsin. Mix the sample by gentle vortex and carry out the digestion overnight at 37 °C. This amount of trypsin gives a protease-to-substrate ration 1-to-50.

Day Two

9. Stop the reaction and adjust the pH of the solution to <6 by adding concentrated acetic acid as needed. Test the pH by placing 1- μL aliquots of the sample onto an appropriated pH paper. The digest may be analyzed directly or concentrated by evaporation.

A Protocol for Sample Desalting by Solid-Phase Extraction Prior to Analysis

Reagents and Apparatus All reagents are prepared immediately prior to use.

1. ZipTip™: A C18 ZipTip™ from Millipore Corporation, Bedford, Massachusetts.
2. A 10- μL pipettor.
3. 1% trifluoroacetic acid: Add 10 μL of trifluoroacetic acid to 990 μL of water and vortex mix.
4. 0.1% trifluoroacetic acid: Add 100 μL of 1% trifluoroacetic acid to 900 μL of water and vortex mix.
5. 50% acetonitrile: Add 500 μL of acetonitrile to 500 μL of water and mix by vortexing.

Procedure

1. Prepare the sample.
 - a. Transfer a 10 μL aliquot of the digest to a 0.5-mL plastic microcentrifuge tube.
 - b. Add 1 μL of 1% trifluoroacetic acid to the digest and mix by drawing the sample into and out of the pipette tip.
2. Prepare the ZipTip™.
 - a. Wet the tip by drawing 10 μL of 50% acetonitrile into the tip. Expel the acetonitrile solution into a waste container. Repeat this process with another 10 μL aliquot of 50% acetonitrile.
 - b. Equilibrate the tip by drawing 10 μL of 0.1% trifluoroacetic acid into the tip. Expel the trifluoroacetic acid into the waste. Repeat this process with another aliquot of 0.1% trifluoroacetic acid.
3. Bind the peptides in the acidified digest to the ZipTip™. Repeatedly draw the acidified digest into the tip and expel it out of the tip, back into the microcentrifuge tube. Ten cycles are recommended. Carry out each movement slowly, to give good contact time between the sample and the column packing, and carefully, to avoid passing a large amount of air through the tip.
4. Wash the ZipTip™ by drawing 10 μL of 0.1% trifluoroacetic acid into the tip. Expel the trifluoroacetic acid solution into a waste container. Repeat this process with another 10- μL aliquot of 0.1% trifluoroacetic acid.
5. Elute the peptides from the ZipTip™.
 - a. Place 5 μL of 50% acetonitrile in a 0.5-mL plastic microcentrifuge tube. Note that this microcentrifuge tube will ultimately contain the eluted peptides.
 - b. Elute the peptides from the ZipTip™ by repeatedly drawing the 50% acetonitrile into the tip and expelling it out of the tip, remembering to recollect the eluant with each cycle. Five cycles are recommended. Carry out each movement slowly, to give good contact time between the solvent and the column packing, and carefully, to avoid passing air through the tip. At the end of this process the peptides from the digest are present in a 50% acetonitrile solution.

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by Michael Kinter and Nicholas E. Sherman.

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