SOP: In-Gel Digestion Protocol using ProteaseMAX™ Surfactant

This Protease Max protocol will work for trypsin, chymotrypsin, GluC, AspN, and LysC. Just use the same concentrations as for trypsin.

ProteaseMAX™ Surfactant 1% stock solution

- Add 100µl of freshly prepared 50mM ammonium bicarbonate (AmBic) to a vial of ProteaseMAX™ Surfactant (1 mg) to give a 1% solution
- Aliquot into 2-5 µl
- Snap-freezing on dry ice or nitrogen prior to storage at -70^{o}C

Regular Trypsin stock solution 1.0 µg/µl

- Add 100 µl of HPLC H_2O to 100 µg trypsin lyophilized powder (Trypsin Gold Cat.# V5280 from Promega)
- Aliquot into 2-5 µl
- Snap-freezing on dry ice or nitrogen prior to storage at -70^{o}C

Trypsin stock solution 0.1 µg/µl for low abundance protein

- Add 1 ml of HPLC H_2O to 100 µg trypsin lyophilized powder (Trypsin Gold Cat.# V5280 from Promega)
- Aliquot into 2-5 µl
- Snap-freezing on dry ice or nitrogen prior to storage at -70^{o}C

Reagents required

- 1 M Ammonium bicarbonate (AmBic or NH_4HCO_3)
    - Weigh 0.079 g AmBic powder and add HPLC H_2O until 1 ml
- 50 mM AmBic (1 ml x **4** tubes) -> *enough for ~4 samples*
    - 50 µl of 1 M AmBic
    - 950 µl of HPLC H_2O
- 50% ACN in 50 mM AmBic -> *enough for ~2.5 reactions (200 µl /sample twice)*
    - 500 µl of 50 mM AmBic
    - 500 µl of ACN
- 25 mM DTT in 50 mM AmBic (1 ml) -> *enough for ~4 reactions (200 µl /samples)*
    - 0.0039 g of DTT
    - 1000 µl of 50 mM AmBic
- 55 mM IAA in 50 mM AmBic (1 ml) -> *enough for ~4 reactions (200 µl /sample)*
    - 0.010 g of IAA
    - 1000 µl of 50 mM AmBic

In-Gel Digestion Protocol

Protease Max protocol will work for trypsin, GluC, AspN, and LysC and chymotrypsin. Just use the same concentrations as for trypsin.

1. Excise the protein band or spot of interest as tightly as possible to avoid having excess gel around the band/spot. For large bands, cut so the bands so they are no larger than 2 mm x 5 mm x 1 mm. Transfer bands into separate 1.5 ml microcentrifuge tubes.
2. Wash with 200µl of HPLC water by vortex for 30 seconds. Spin down in microcentrifuge and discard the water.
3. Destain for 10 minutes in 200µl of ACN/50mM AmBic (1:1 v/v) at **60°C**. Spin down in microcentrifuge. Discard supernatant. **Repeat once** (if necessary, until destained)
4. Dehydrate gel pieces by adding 200µl of 100% ACN, vortex gently for 5 minutes. Spin down in microcentrifuge. Discard the supernatant.
5. Dry for 5 minutes or until sample is dry. Cover samples with foil or a pipette box lid to keep dust out.
6. Rehydrate in 200µl of freshly prepared 25mM DTT in 50mM AmBic (reduction of disulfide bonds), and incubate for 20 minutes **at 60°C.**
7. Spin down in microcentrifuge. Discard supernatant. Add 200µl of freshly prepared 55mM iodoacetamide in 50mM AmBic, and incubate in the **dark** for 20 minutes **at room temperature**.
8. Spin down in microcentrifuge. Discard supernatant, and wash with 400µl of HPLC water by vortex briefly. Discard supernatant. **Repeat once**.
9. Dehydrate for 5 minutes in 200µl of ACN:50mM AmBic (1:1 v/v) with vortex gently at room temperature. Spin down in microcentrifuge. Discard supernatant.
10. Add 200µl of 100% ACN, vortex gently for 5 minutes. Spin down in microcentrifuge. Discard the supernatant.
11. Dry for approximately 5 minutes or until sample is dry. Cover samples with foil or a pipette box lid to keep dust out.

For high-abundant proteins

12. Thaw ProteaseMAX™ Surfactant 1% stock solution on ice, make a 0.01% working solution by adding 247.5 µl of 50 mM AmBic to 2.5 µl of ProteaseMAX™ Surfactant stock solution -> *enough for ~4 samples*.
13. Make a trypsin working solution of 12 ng/µl by mixing 1.2 µl of **1.0 µg/µl** trypsin with 98.8 µl of 0.01% ProteaseMAX™ Surfactant working solution (from step 13) -> *enough for ~4 samples*.
14. Rehydrate gel in 20µl of 12ng/µl trypsin in 0.01% ProteaseMAX™ Surfactant (from step 14) for 10 minutes.
15. Overlay with 30µl of 0.01% ProteaseMAX™ Surfactant (from step 13) and gently mix for several seconds.
16. Incubate for **1 hour at 50°C**. The digestion can also be performed at 37°C for 2 hr .

    ** We do not recommend continuing digestion for more than 1 hour at 50°C or 4 hours at 37°C. Incubating overnight might cause some decrease in peptide recovery.)

17. Collect the condensate from tube walls by spinning briefly, mix for a few seconds by vortexing at high speed, centrifuging at 12,000–16,000 × *g* for 10 min. This will help to pellet out the protease max.
18. Transfer the digestion reaction with extracted peptides into a new 1.5 ml Eppendorf tube.
19. Add 2.5 µl of 10% TFA to extracted peptides (final concentration of 0.5% to inactivate trypsin)
20. Add 25 µl of 0.1% TFA solution into gel pieces, vortex for 1-5 min, and centrifuging at 12,000 - 16,000 × *g* for 2 minutes. Combine the extract with the digest (step 18 + step 20). Discard gel pieces.
21. Dry in a Speed Vac® vacuum centrifuge until sample is dry. Resuspend sample in 3% ACN, 0.1% formic acid and bath sonicate for 5-10 minutes.
22. For MALDI-TOF/TOF MS analysis, the peptides can be desalted with a 10µl C18 ZipTip® Millipore. (Avoid micro-C18 (0.2µl bed volume) tips for peptide cleanup. The use of micro-C18 tips can result in a decrease in peptide recovery due to competition of the degraded surfactant and peptides for binding sites.)

For low abundant proteins

*****Follow the exact same steps 1-11 as protocol above

12. Thaw ProteaseMAX™ Surfactant 1% stock solution on ice, make a 0.01% working solution by adding 247.5 µl of 50 mM AmBic to 2.5 µl of ProteaseMAX™ Surfactant stock solution -> *enough for ~6 samples.*
13. Make a trypsin working solution of 2 ng/µl by mixing 2 µl of **0.1 µg/µl** trypsin with 98 µl of 0.01% ProteaseMAX™ Surfactant working solution (from step 13) -> *enough for ~ 4 samples*.
14. Rehydrate gel pieces in 20µl of 2ng/µl trypsin in 0.01% ProteaseMAX™ Surfactant (from step 14) for 10 minutes.
15. Overlay with 10 µl of 0.01% ProteaseMAX™ Surfactant (step 13) (enough to cover gel pieces) and gently vortex for several seconds.
16. Incubate **for 3 hours at 37°C**. Incubating overnight might cause some decrease in peptide recovery.

    ****** Note:** Protein digestion is complete and ProteaseMAX™ Surfactant degrades over the course of the digestion reaction.
17. Collect the condensate from tube walls by spinning briefly, mix for a few seconds by vortexing at high speed and centrifuging at 12,000–16,000 × *g* for 5 min.
18. Transfer the digestion reaction with extracted peptides into a new tube.
19. Add 20µl of 2.5% TFA to gel pieces and vortex for 15 minutes.
20. Combine the extract with the digest. Discard gel pieces.
21. Centrifuge the combined solution for 10 minutes at 12,000–16,000 × *g.*
22. Dry in a Speed Vac® vacuum centrifuge until sample is dry. Resuspend sample in 3% ACN, 0.1% formic acid and bath sonicate for 5-10 minutes.
23. For MALDI-TOF/TOF MS analysis, the peptides can be desalted with a 10µl C18 ZipTip® Millipore. (Avoid micro-C18 (0.2µl bed volume) tips for peptide cleanup. The use of micro-C18 tips can result in a decrease in peptide recovery due to competition of the degraded surfactant and peptides for binding sites.)