

**SOP: SP021.2**

Modified 3/8/2016 by MCL

**In-gel Digestion of Proteins****Materials and Reagents:**

1. Coomassie or SimplyBlue stained gel containing protein of interest
2. Razor blade
3. Glass plate
4. 0.65 ml Deplasticized Eppendorf tubes (note 1)
5. 37°C incubator
6. Vortexer
7. Savant Speed-Vac
8. Destain solution (60% acetonitrile in 0.2 M ammonium bicarbonate)
9. Extraction solution (60% acetonitrile in water, 0.1% TFA)
10. Modified trypsin sequencing grade (Roche Molecular Biochemicals, catalog # 1 418 025) (note 2)
11. Ultrapure water (note 3)
12. 0.2 M ammonium bicarbonate (ambic)
13. MS Buffer A: 96.9% water, 3% ACN and 0.1% formic acid
14. Autosampler vials with lids
15. 70% Ethanol

**Protocol:**

1. \_\_\_\_\_ Thoroughly clean the workspace, glass plate, and razor blade with 70% ethanol.
2. \_\_\_\_\_ Place a KimWipe or other white background under the glass plate to provide good visibility, then place the Coomassie or SimplyBlue stained gel on the glass plate.
3. \_\_\_\_\_ Excise protein spots of interest from the gel using a razor blade.
4. \_\_\_\_\_ Cut each spot into small pieces (~1 mm by 1 mm) and place the pieces in a deplasticized Eppendorf tube.
5. \_\_\_\_\_ Completely cover the gel pieces with destain solution (100-200 µl), vortex, and incubate at 37°C for at least 30 minutes, up to overnight.
6. \_\_\_\_\_ Pipet the destain solution into a waste container, taking care not to discard any gel pieces (note 4).
7. \_\_\_\_\_ Repeat steps 5-6 up to three times until the gel is completely destained (note 5).
8. \_\_\_\_\_ Dry the gel pieces in the Savant Speed-Vac (SOP SP005).
9. \_\_\_\_\_ Dissolve 25 µg of trypsin in 300 µl of 0.2 M ammonium bicarbonate (note 6).
10. \_\_\_\_\_ To the dried gel slices, add 0.6 µl trypsin solution for every 1 µg of protein to achieve a 1:20 trypsin:protein ratio (note 7).
11. \_\_\_\_\_ Incubate at room temperature until the trypsin solution is completely absorbed by the gel slices (~15 min).
12. \_\_\_\_\_ Add 10-15 µl of 0.2 M ammonium bicarbonate and gently break apart the pelleted gel pieces with the pipet tip.
13. \_\_\_\_\_ Continue to add 0.2 M ammonium bicarbonate in 10-15 µl increments until the final volume just covers the tops of the completely swelled gel pieces (note 8).

- 14.\_\_\_\_\_ Incubate the gel slices for 16 hours at 37°C. Do not exceed 18 hours of trypsin incubation.
- 15.\_\_\_\_\_ Add 100 µl of extraction solution to the gel slices and vortex.
- 16.\_\_\_\_\_ Incubate the extraction solution and gel slices at 37°C for 40 min (note 9).
- 17.\_\_\_\_\_ Briefly centrifuge the extract to collect condensation from the lid.
- 18.\_\_\_\_\_ Transfer the supernatant to a new deplasticized tube, taking care not to transfer any gel pieces.
- 19.\_\_\_\_\_ Repeat extraction of the gel pieces as in steps 15 to 18, pooling the supernatant with the extract collected in step 18.
- 20.\_\_\_\_\_ Place the combined extract in the Savant Speed-Vac and dry (note 10).
- 21.\_\_\_\_\_ Add MS Buffer A to the sample and mix well (note 11):
  - 15 µl for single protein spots
  - 1 µl/µg protein for known amounts of complex protein samples (whole gel lane)
- 22.\_\_\_\_\_ Centrifuge the sample for 5 minutes.
- 23.\_\_\_\_\_ 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).
- 24.\_\_\_\_\_ Pipet sample into an autosampler vial, being careful not to introduce any bubbles into the vial.
- 25.\_\_\_\_\_ Place the cap on the autosampler vial and label the vial (note 12).
- 26.\_\_\_\_\_ Store the samples at -20°C until analysis by LC-MS-MS.

**Notes:**

1. Deplasticized tubes:
  - Fill eppendorf tubes with Extraction Solution (60% acetonitrile-0.1% TFA)
  - Loosely cover with foil and incubate at room temperature for 1 hour
  - Decant the solution into a tray. Dispose of extraction solution as hazardous waste, or allow to evaporate in the hood
  - Repeat two more times (for a total of three rounds)
  - Loosely cover tubes with foil to prevent dust, and leave in the hood until completely dry (at least overnight), or dry tubes on the Savant Speed-Vac (SOP SP005)
  - Close the tube lids before storage
2. This procedure can be used with proteases other than trypsin, however the buffer for the digestions may differ for other proteases.
3. All solutions should be made using ultrapure water such as Burdick and Jackson HPLC Grade water, or equivalent (i.e. water from a MilliQ system equipped with a suitable polishing filter).
4. Destain solution should be disposed of as hazardous waste, or small amounts may be left to evaporate in the chemical fume hood.
5. Gel pieces will begin to dehydrate during this step, which can sometimes interfere with destaining. After the second round of destain, if the gel still contains a significant amount visible stain, or if the gel pieces start to turn white (indicating dehydration), rehydrate as follows:
  - Add 100 µl 0.2 M ambic
  - Allow gel pieces to re-swell for 15 minutes
  - Remove excess ambic
  - Add destain and proceed with the last incubation

6. When preparing a new vial of trypsin, be sure to date and initial it, and indicate "For In-Gel" (so as not to be confused with vials reconstituted at a different concentration for in-solution digests). The reconstituted trypsin should be stored at 4°C and can be used for up to two weeks.
7. If the protein amount is unknown, 3-5 µl of trypsin solution can be used for single protein spots.
8. Allow 10-15 minutes for swelling of the gel pieces between additions of ambic. Large gel spots may take as much as 100 µl to fully rehydrate.
9. After addition of the extract solution, vortex the tubes and put them in the incubator for 20 minutes, vortex again, then back in the incubator for 20 more minutes.
10. Dried samples can be stored at -20°C until ready to proceed with the next steps.
11. Targeted analyses will require additional steps, which may include adding isotopic standards for samples being analyzed by TQS.
12. Use orange caps for samples to be analyzed by Orbitrap. Use blue caps for samples to be analyzed by TQS.

**References:**

Hellman, U., C. Wernstedt, J. Gopez, 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.