# In-gel digestion of proteins

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## Trypsin in-gel digestion of proteins

### Materials

All solvents should be HPLC grade, NEVER use pipette tips when transferring acids >2% in concentration!

- Dithiothreitol DTT (Fisher, part # PI-20291); Stock solution: 1 M in $H_2O$
- Iodoacetamide IAA (Fisher, part # AC12227-0050); Stock solution: 100 mM in $H_2O$ (0.0185g/ml; always prepare fresh, light sensitive)
- Urea (Fisher, part # AC14075-0010 )
- Water (Fisher, part # W6-4 optima LCMS grade)
- Ammonium bicarbonate (Fisher, part # A643-500) Stock solution: 500 mM in $H_2O$ ($NH_4HCO_3$ (79.1g/mol): 3.955g/100ml)
- Acetonitrile (Fisher, part # A955-4 optima LCMS grade)
- 1 µg/µl Trypsin in 0.01% acetic acid (modified, sequencing grade, Promega, part # V5111, 5 x 20ug)

**Avoid Keratin contamination:**

When handling the gel and gel band(s) use extreme caution to avoid keratin contamination. Wear gloves at all times and use clean equipment (wipe all surfaces and tools with methanol/water and if possible use new unused razor blades). Keep the gel at a reasonable distance from your body, don't talk, sneeze etc over the gel…

**Avoid contamination from plasticizers:**

To avoid contamination from plasticizers, use all fresh solvents. Never use any plastic pipettes to transfer solvents from the original bottles, instead poor the solvent into a beaker. Repeated exposure of solvents to plastics will contaminate the stock solvents with plasticizers.
**Never** use plastic pipettes when handling concentrated (>2%) acids, use glass pipettes or Hamilton syringes (rinse the syringe with water after using it for concentrated acids to avoid corrosion of the metal needle).
If you don't know whether your stock solvents already are contaminated, buy new solvents and make sure no one is contaminating them!
Keep your solvents in glass bottles, preferably with Teflon lined lids. Never use bottle lids that have foil backed cardboard liners!

The excised band can be stored in de-ionized water for a few days at 4°C if necessary. Protein amounts should be in the hundreds of femtomole to 1 pmol range. A band that can be visualized by Coomassie blue stain, usually contains sufficient protein for identification.

The first (UWPR preferred) method takes a bit longer and involves reduction and alkylation of cysteine-containing peptides, this is useful for low level protein <1pmol and typically results in higher sequence coverage of the protein.

The other method is shorter and is used for protein identification purposes.

### *Trypsin digestion – UWPR recommended (for low level protein and improved sequence coverage)*

**(Includes reduction and alkylation of the protein )**

1. Excise protein spot/band, cut/dice into small pieces (~1 $mm^3$) and dehydrate in acetonitrile for approx. 10 min. repeat this step twice. Remove acetonitrile and SpeedVac untill dry. **Note** use gel loading pipette tips to remove solvents, and be careful not to lose the gel pieces.
2. Add 50-100 µl 5-**10mM DTT** in $H_2O$ (or 25mM ammonium bicarbonate) just enough to cover the gel pieces, vortex and spin down
3. Incubate at 56 $^oC$ for 45 min – 1hr.
4. Spin down, and pull off supernatant, cool down shortly.
5. Alkylate with iodoacetamide (184.96g/mol: 18.5mg/ml)) 55-**100mM** in 25mM ammonium bicarbonate (5 to 10 fold excess over DTT), incubate for 30min in the dark at room temperature.
6. Spind down, pull off supernatant, wash with **$H_2O$** (or 25mM ammonium bicarbonate) and pull off wash.
7. Dehydrate in acetonitrile for approx. 10min. Remove acetonitrile and SpeedVac untill dry.
8. Rehydrate gel pieces at 4 $^oC$ for 45 min in buffer containing trypsin and 50 mM ammonium bicarbonate. (Approx. 5 µl/$mm^2$ gel). The gel pieces should just be covered:
   Suggested amount of trypsin is 12.5 ng/uL of buffer for proteins that have been silver stained.
   **(1µg/µl trypsin solution → 1µl/80µl 50 mM ammonium bicarbonate)**
   Don't use more than 1 µg trypsin per sample for MS analysis.
9. Cover gel pieces with 50 mM ammonium bicarbonate. Digest overnight at 37 $^oC$ (or at least for 3 h).
10. Centrifuge gel pieces (4min) and collect (keep) supernatant. Use gel loading pipette tips to remove solvents, and be careful not to transfer the gel pieces as they could interfere with downstream MS analysis.
11. Further extract peptides by one change of $H_2O$ and three changes of 5% formic acid in 50% acetonitrile incubate 20 min for each of the changes, centrifuge then collect at room temp.
12. Reduce sample volume in speedvac until desired volume (5-10µl) has been reached, don't let the sample dry completely.

### *Trypsin digestion - short method (ID purposes only)*

(adapted from Shevchenko et al. Anal. Chem. 68, pp. 850-858, 1996)

1. Excise protein spot/band, cut/dice into small pieces and dehydrate in acetonitrile for approx. 10 min. repeat this step twice. Remove acetonitrile and SpeedVac until dry. Note use gel loading pipette tips to remove solvents, and be careful not to lose the gel pieces.
2. Add ~50µl $H_2O$, let it rehydrate (10-15 min). Dehydrate in acetonitrile for approx. 10min. Remove acetonitrile and SpeedVac until dry.
3. Rehydrate gel pieces at 4 $^oC$ for 45 min in buffer containing trypsin and 25-50 mM $NH_4HCO_3$. (Approx. 5 µl/$mm^2$ gel). The gel pieces should just be covered. Suggested amount of Trypsin is 12.5 ng/uL of buffer for proteins that have been silver stained.
   **1µg/µl Trypsin stock solution → 1µl/80µl 25-50 mM $NH_4$-$HCO_3$**
   Don't use more than 1 µg trypsin per sample for MS analysis.
4. Cover gel pieces with 25-50 mM ammonium bicarbonate.
5. Digest overnight at 37 $^oC$ (or at least for 4 h).
6. Centrifuge gel pieces (4min) and collect (keep) supernatant. Further extract peptides by one change of $H_2O$ and three changes of 5% formic acid in 50% $CH_3CN$, incubate 20 min for each of the changes, centrifuge then collect at room temp.
7. Reduce sample volume in speedvac until desired volume (5-10µl) has been reached, don't let the sample dry completely.
8. Note NEVER use more than 1 µg Trypsin per sample for MS analysis.

## On-Blot, In Situ Digest

*This was procedure adapted from the following article: Wong, S. C., Grimley, C., Padua, A., Bourell, J. H., and Henzel, W. J. (1993) Peptide Mapping of 2-D Gel Proteins by Capillary HPLC. Tech. In Prot. Chem. IV, 371-378*

### Materials and Solutions:

All solvents should be HPLC grade, NEVER use pipette tips when transferring acids >2% in concentration!

- Dithiothreitol DTT (Fisher, part # PI-20291); Stock solution: 1 M in $H_2O$ keep frozen at -20 °C
- Iodoacetamide IAA (Fisher, part # AC12227-0050); Stock solution: 200 mM in 0.5M NaOH (always prepare fresh, light sensitive)
- Ammonium bicarbonate (Fisher, part # A643-500) Stock solution: 500 mM in $H_2O$ ($NH_4HCO_3$ (79.1g/mol): 3.955g/100ml)
- Water (Fisher, part # W6-4 optima LCMS grade)
- Acetonitrile (Fisher, part # A955-4 optima LCMS grade): 10% Acetonitrile
- Methanol (Fisher, part # A456-4 optima LCMS grade)
- HCl
- Trypsin (modified, sequencing grade, Promega, part # V5111, 5 x 20ug): 0.2 µg/µl in 100 mM Tris-Base, pH 8.0, 10 % acetonitrile
- Polyvinylpyrrolidone PVP (Fisher, part # AC22747-0250): 0.25% PVP in 0.1% acetic acid
- Tris Base (Fisher, part # BP154-1)
- 0.5 M Tris-Base / 6M guanidine-HCl / 5 mM EDTA / 10% acetonitrile / 7 mM DTT / pH 8.5 with HCL (note: pH with HCl before adding the acetonitrile, starts out around pH 10. Add DTT fresh to a portion of this solution right before reduction)
- 100 mM Tris-Base, pH 8.0, 10 % acetonitrile

### Procedure:

1. Cut out the band or spot as narrowly as possible, avoid touching the blot, wear gloves.
2. Wet excised blot with 1-3 µL of methanol in a small tube. (the membrane is very hydrophobic and must be wetted with methanol. Do not let the membrane dry out during the procedure.)
3. Reduction. Reduce with 100 µL of 0.5 M Tris-Base / 6M guanidine-HCl / 5 mM EDTA / 10% acetonitrile / 7 mM DTT / pH 8.5 at 45 °C for 60 min.
4. Cool and add 10 µL of 200 mM IAA in 0.5 M NaOH, let react for 20 min. in the dark.
5. Wash the blot 3X with 500 µL 10% Acetonitrile.
6. To block the blot incubate in 200 µL of 0.25% PVP in 0.1% acetic acid for 20 minutes on shaker at room temp.
7. Wash the blot 3X with 500 µL 10% Acetonitrile.
8. Transfer blot to new tube.
9. Wash the blot 3X with 500 µL 10% Acetonitrile. (this extensive wash and transfer is necessary to remove excess PVP 360, which will give UV and mass spectral interference if not removed.)
10. Digest with 0.2 µg Promega modified trypsin in 100 µL of 100 mM Tris-Base, pH 8.0, 10 % acetonitrile for 24 hours at 37 °C.
11. Concentrate on a SpeedVac to 20 µl, inject 5 µL onto a capillary column for LC/MS analysis.

## Gel staining recommendations

We recommend gels be stained with either Commassie Blue or Sypro Ruby. These produce the best results. Stains with colloidal coomassie G-250 generally produce "better stains", most common commassie blue stains are compatible with mass spectrometry. Silver stained gels generally produce less robust results because they modify the proteins and crosslink the peptides to the gels. If you need to use silver stains, they must be mass spec compatible. Most commercial suppliers will indicate this on their product insert.

Here are a couple of examples:

### Pierce Stain Kit for Mass Spectrometry

Link to pierce

| Thermo Scientific Gel Stains for detection of total protein. | | | | | |
|---|---|---|---|---|---|
| Product Name | # Components[1] (# Steps [2]) | Time Required [3] | Detection Method | Limit of Detection | Mass Spec. Compatible |
| Krypton Fluorescent Protein Stain | 1 (7) | 2 hr 40 min | fluorescent | 0.25ng | Yes |
| Krypton Infrared Protein Stain | 1 (7) | 2 hr 40 min | fluorescent | 0.25ng | Yes |
| GelCode Blue Safe Protein Stain | 2 (5) | 2 hr 15 min | colorimetric | 9ng | Yes |
| GelCode Blue Stain Reagent | 1 (5) | 2 hr 15 min | colorimetric | 8ng | Yes |
| Imperial Protein Stain | 1 (5) | 2 hr 15 min | colorimetric | 3ng | Yes |
| Pierce Silver Stain for Mass Spec | 6 (17) | 1 hr 13 min | colorimetric | 0.25ng | Yes |
| Pierce Silver Stain Kit | 4 (15) | 2 hr 25 min | colorimetric | 0.25ng | Yes |
| Pierce Color Silver Stain Kit | 4 (9) | 4 hr 5 min | colorimetric | 0.1ng | Yes |
| Pierce Zinc Reversible Stain Kit | 3 (4) | 14 min | colorimetric | 0.25ng | Yes |

Notes:
1. Some products require additional reagents to be supplied by user
2. Number of steps includes multiple gel washes, solution changes, destaining steps, etc.
3. Time includes gel wash steps and all steps required for best sensitivity in a mini-gel. When less sensitivity is required, shorter staining and destaining times may be used with some protocols.

### Invitrogen SilverQuest Protocol

Visualize down to 3 ng of protein. Protocol listed below is for one mini-gel, 1.0 mm thick, for large gels double all solution volumes while keeping the same incubation time. Please do not use the "fast" protocol listed in the SilverQuest manual or the destainer. Development times between 5 and 6 minutes yield the best mass spectrometry results. Longer development time will result in "burning" of the protein bands, decreasing peptide recovery which decreases the opportunity for positive protein identification.

| **Step** | **Reagent** | **Basic Protocol** |
|---|---|---|
| Fix | Ethanol 40 ml<br>Acetic Acid 10 ml<br>Water to 100 ml | 100 ml, 20 min |
| Wash | Ethanol 30 ml<br>Water to 100 ml | 100 ml, 10 min |
| Sensitize | Ethanol 30ml<br>Sensitizer 10 ml<br>Water to 100 ml | 100 ml, 10 min |
| First Wash | Ethanol 30 ml<br>Water to 100 ml | 100 ml, 10min |
| Second Wash | Water 100 ml | 100 ml, 10 min |
| Stain | Stainer 1 ml<br>Water to 100 ml | 100 ml, 15 min |
| Wash | Water 100 ml | 100 ml, 1 min |
| Develop | Developer 10 ml<br>Developer Enhancer 1 drop<br>Water to 100 ml | 100 ml, 4-8 min |
| Stop | Stopper 10 ml<br>Add directly to Developing solution | 10 ml, 10 min |
| Wash | Water 100 ml | 100 ml, 10 min |