

Nature In-gel Digestion Protocol

Ref: Nature Protocols, Vol 1, No.6, 2856-2860, 2007, Andrej Shevchenko, Henrik Tomas, Jan V Olsen, & Matthias Mann. [“In-gel digestion for mass spectrometric characterization of proteins and proteomes”](#)

Materials

Reagents

- 1) Ammonium Bicarbonate (Fluka, Ctlg 09830-500G, MW 79.06)
- 2) Dithiothreitol (DTT;Pierce,Ctlg 20290, MW 154.25)
- 3) Iodoacetamide (Calbiochem, Ctlg 407710, MW185)
- 4) Trypsin (Porcine, Sequencing grade, modified; Promega Corp, 5 x 20ug, Ctlg V5111)
- 5) Water (Macron Chemicals, ChromAR, Ctlg 6795-10)
- 6) Acetonitrile (Fisher Scientific, HPLC grade, Ctlg A998-4)
- 7) Formic acid (Fisher Scientific, Optima LC/MS grade, Ctlg A117-50)
- 8) Trifluoroacetic acid (Thermo Scientific, Ctlg 28904)

Equipment

Laminar Flow Hood

Bench-top centrifuge (Eppendorf, 5415 R)

Shaker at 37C.

0.5 ml Polypropylene tubes

0.45m filters (ULTRAFREE-MC, Durapore-PVDF 0.45um, Millipore, Ctlg UFC30HV00)

Vacuum centrifuge (Speedvac)

Reagent Preparation

100mM Ammonium Bicarbonate in water: Dissolve 0.791g of Ammonium bicarbonate in 100ml of HPLC grade water. Make buffer daily in large (50-100ml) volumes and discard after use.

Critical Make shortly before use

10mM Ammonium Bicarbonate w 10% (vol/vol) Acetonitrile: 2ml of 100mM Ammonium Bicarbonate + 16ml of HPLC grade water + 2ml of Acetonitrile.20ml.

10mM DTT in 100mM Ammonium Bicarbonate: Dissolve 0.0154g in 10ml of 100mM Ammonium bicarbonate. This is good for 85 gel bands, 50ul/band. Make shortly before use. Make less for less number of gel bands.

Critical Make shortly before use

55mM Iodoacetamide in 100mM Ammonium Bicarbonate: Dissolve 0.1018g in 10ml of 100mM Ammonium bicarbonate. This is good for 85 gel bands, 50ul/band. Make shortly before use. Make less for less number of gel bands.

Critical Make shortly before use

5% Formic acid in water (vol/vol): Dissolve 1ml of Formic acid in 20ml of HPLC grade water. Make less for less number of gel bands.

100mM Ammonium bicarbonate/acetonitrile (1:1, vol/vol): Mix 10ml of 100mM Ammonium bicarbonate with 10ml of Acetonitrile. Total volume 20ml.

5% Formic acid/Acetonitrile (1:2, vol/vol): Mix 10ml of 5% Formic acid with 20ml of Acetonitrile. Total volume 30ml. Make less for less number of gel bands.

Trypsin, 13ng/ul: Dissolve the content of 20ug vial in 1.5ml of 10mM ammonium bicarbonate containing 10% (vol/vol) acetonitrile.

Critical Make shortly before use; discard unused volume. ***If only a small volume of trypsin buffer is required, the lyophilized enzyme can be dissolved in 200 ul of 1mM HCl and 10ul aliquots stored at -20C before use. Note that after thawing frozen aliquots, they can be dissolved in 75 ul of 10 mm ammonium bicarbonate containing 10% (vol/vol) acetonitrile.***

Precautions for reducing Keratin and Chemical Background

Wear gloves at all times and rinse them occasionally as they readily accumulate static charge and attract dust and pieces of hair and wool

If possible, perform all operations in a laminar hood and use dedicated set of pipettes, tips, tubes that should be stored in the hood in a dust-free environment

Do not use polymeric detergents (TWEEN, Triton, etc.) for cleaning flasks and glass plates for electrophoresis

Always visually check flasks, tubes and pipette tips for contaminating particles

Processing of bands from 1-D gels or spots from 2-D gels

Upon electrophoresis, proteins can be stained with Coomassie without negative effect. Imperial Coomassie stain works well.

Procedure

Excise protein bands (spots) **Timing ~ 5min per band per spot**

- 1) Rinse the entire slab of a one- or two-dimensional gel with water for a few hours, transfer the gel onto a glass plate and excise bands (spots) of interest with a clean razor blade or scalpel.
- 2) Cut excised bands (spots) into cubes (ca. 1 x 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.

In-gel reduction, alkylation and destaining of proteins **Timing 60min**

- 4) A reduction/alkylation step can be performed if this step is not performed prior to SDS-PAGE separation (such as is common in 2D gel protocols). If rapid identification of Coomassie-stained bands (spots) is intended, and recovery of cysteine containing peptides is not important, skip reduction/alkylation and proceed directly with the steps described in option B.

(A) Processing with reduction/alkylation

- (i) Add 500ul of neat acetonitrile and incubate tubes for 10min until gel pieces shrink (they become opaque and stick together).
- (ii) Spin gel pieces down, remove all liquid.
- (iii) Add 50ul of the DTT solution to completely cover gel pieces. Incubate 30min at 56C in a circulating air incubator or PCR heating block.
- (iv) Chill down the tubes to room temperature (ca. 22C), add 500ul of acetonitrile, incubate for 10min and then remove all liquid.
- (v) Add 50ul of Iodoacetamide solution to completely cover the gel pieces and incubate for 20min at room temperature in the dark.
- (vi) Add 500ul of neat acetonitrile, incubate tubes for 10min until gel pieces shrink, and then remove all liquid.

(B) Destain gel pieces excised from Coomassie-stained gels

Timing 30min

- (i) Add ca. 500ul of 100mM Ammonium bicarbonate/acetonitrile (1:1, vol/vol) and incubate with occasional vortexing for 30min.

Note: Skip this step if gel bands are already destained during reduction and alkylation.

- (ii) Add 500ul of neat acetonitrile, incubate tubes for 10 min at room temp with occasional vortexing, until gel pieces become white and shrink and then remove acetonitrile. Although the bulk of the Coomassie staining should be removed, it is not necessary to destain the gel pieces completely.

Note: Skip this step if gel bands are already destained during reduction and alkylolation.

PAUSE POINT Samples are now ready for in-gel digestion. Alternatively, they can be stored at -20C for a few weeks.

Saturate gel pieces with trypsin **Timing 120min**

- 5) Add 50-100ul of trypsin containing solution to cover the dry gel pieces and leave it in an ice bucket or a fridge.
- 6) After a ca. 30min, check if all solution was absorbed and add more trypsin buffer, if necessary. Gel pieces should be completely covered with trypsin buffer.
- 7) Leave the gel pieces for another 90min to saturate them with trypsin and then add 10-20ul of 10 mM ammonium bicarbonate, 10% ACN buffer to cover the gel pieces and keep them wet during enzymatic cleavage.

Critical Step Although after ca. 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 the polyacrylamide matrix.

Digestion

- 8) Place tubes with gel pieces into an air circulation incubator and incubate samples overnight at 37C for analyses performed at the limit of instrument sensitivity, which requires maximal peptide recovery.

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.

Extract peptide digestion products **Timing 15min**

- 9) Add 100ul of extraction buffer (1:2 (vol/vol) 5% Formic acid/acetonitrile) to each tube, vortex and incubate for 15min at 37C 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.***

Critical Step To withdraw the supernatant, use a pipette with a fine gel loader tip to prevent clogging the needle of the autosampler injector or nanoLC MS/MS column.

Note: Do not discard extracted gel pieces. If for any reason, the digestion failed, it can be repeated with the same gel pieces using the same enzyme (trypsin) or using another enzyme. Note that, in the later case, a strong peptide background may be encountered because of the digestion of residual intact trypsin.

Dry down extracted peptides in a speed vac Timing 15-45 min

10) Dry down the extracts in a vacuum centrifuge (speedvac). Larger volumes will take longer.

PAUSE POINT Dried extracts can be safely stored at -20C for a few months.

Redissolve tryptic peptides and filter before LC/MS analysis Timing 20min

- 11) For LC/MS/MS analysis, add 42ul of 5% formic acid into the tube, shake at 37C for 15 min.
- 12) Place the supernatant into individual Millipore Ultrafree-MC filter units (0.45um) and centrifuge in a table top centrifuge at 10,400 rpm (10,000xg) for 5min.
- 13) Transfer the sample with a pipette to an Agilent autosampler vial with plastic insert and proceed with LC/MS/MS analysis.

TIMING

Excise protein bands (spots): ~ 5min per band or spot

In-gel reduction and alkylation of proteins: 60min

Destain gel pieces and excised from Coomassie-stained gels: 30min

Saturate gel pieces with trypsin: 120min

Digestion: Overnight

Extract peptide digestion products: 15min

Dry down extracted peptides: 15-45 min

Redissolve and filter tryptic peptides for LC/MS/MS analysis: 20min