

Mann Protocol for In-Gel Digestion

1) Washing

- a) Before excising bands wash gels in ddH₂O for 15 min.
- b) Excise bands, cut as close to the band as possible to minimize excess gel material and cut band into 1mm cubes[†]. Place cubes in clean Eppendorf tube. The Proteomics Core has done research regarding digesting whole bands, bands cut into halves, and bands cut into cubes. The Sequest data shows the best coverage with whole bands, followed by halving the bands, then cutting into cubes. **Additional volume will need to be added when using the whole band procedure.**
 - i) * If bands are silver stained refer to the Destaining Protocol for Silver Stained Gel Bands
- c) Add 100uL ddH₂O to each band and incubate for 15 min.
- d) Pull off ddH₂O and add 40uL of 50/50 acetonitrile (ACN)/ddH₂O and incubate for 15 min.
- e) Pull off solution and add 40uL of ACN, incubate until gel pieces are white and sticky.
- f) Pull off solution and add 40uL of 100mM ammonium bicarbonate (Ambic) incubate 5 min.
- g) Add 40 ul of ACN to make 1:1 solution, incubate 15 min.
- h) Pull off sol'n and dry samples in speed-vac for approx. 15 min. (Must be very dry!)

2) Alkylation

- a) Remove samples from speed-vac and let cool.
- b) Add 40uL of 10mM DTT/100mM Ambic and incubate in water bath at 56°C for 45 min.
- c) Remove samples from bath and cool.
- d) Pull off solution and immediately add 40uL of 55mM iodoacetamide/100mM Ambic and incubate at room temperature for 30 min. in the dark.
 - i) ** If alkylating solution samples do not remove any solutions, skip the following steps, e, f and g. See solution digest protocol for Trypsin concentrations.
- e) Pull off sol'n and wash with 40uL of 100mM Ambic, incubate 5 min.
- f) Add 40uL of ACN to make 1:1 sol'n and incubate for 15 min.
- g) Pull of sol'n and dry gel pieces in speed-vac for 15 min. or until very dry.

3) Digestion

- a) Add 40uL (enough to cover pieces) of Trypsin digestion sol'n and incubate 45 min. on ice. Add more sol'n if pieces absorb all of sol'n.
- b) Pull off sol'n and discard, add 40-60uL of digestion buffer without Trypsin (enough to keep gel pieces covered overnight) and incubate overnight at 37°C.
- c) Acidify the digestion using 10ul of 2% TFA in Water, wait 1-2 minutes, remove the supernatant and save in clean microcentrifuge tube.
- d) Cover the gel slice (50 to 70uL for a single gel slice) with 0.1% TFA in water – place in a floating rack in a sonicating water bath and extract for 30 minutes with sonication. Combine supernatant with previous solution(s).
- e) Repeat step 3.d with 30% (ACN, 0.1% TFA) + 70%(H₂O, 0.1% TFA).
- f) Repeat step 3.d with 60% (ACN, 0.1% TFA) + 40%(H₂O, 0.1 % TFA).
- g) Speed-vac pooled supernatants to remove TFA/ACN (reduce solution to approx. 10 µl), vortex, and store at -20°C until analysis.

All solutions are made in microcentrifuge tubes. Stock solutions are pipetted direct from bottles into microcentrifuge tubes for further use.

Solutions:

50% ACN ddH₂O

250uL of ACN + 250uL ddH₂O

100mM Ambic

100uL of 1M Ambic in 900uL ddH₂O

10mM DTT/100mM Ambic (mix just prior to use)

5uL 2M DTT + 895uL ddH₂O + 100uL 1M Ambic

55mM IAA in 100mM Ambic (mix just prior to use)

10mg Iodoacetamide + 100uL 1M Ambic + 900uL H₂O

Digestion buffer without Trypsin:

5uL 1M CaCl₂ + 50uL 1M Ambic + 945uL ddH₂O

Trypsin digestion buffer:

Take 100ul of digestion sol'n add 12.5ul of 0.1ug/uL Trypsin

TFA/ ACN

300uL ACN, 0.1% TFA + 700uL H₂O, 0.1% TFA

600uL ACN, 0.1% TFA + 400uL H₂O, 0.1% TFA

Stock Solutions:

1) Make fresh weekly in plastic bottles.

a) 2M DTT
154.3mg/500ul H₂O

b) 1M CaCl₂
14.70g/100ml H₂O

c) 1M Ammonia Bicarbonate
7.906g/100ml H₂O

2) Make fresh monthly in glass bottles.

a) 2% TFA in water
0.5mL TFA in 25mL H₂O

b) 0.1% TFA in H₂O
0.5mL TFA in 500mL H₂O

Trypsin Solutions: (mix in shipping vial 1 hour prior to use, keep on ice)

Promega: use buffer supplied and dilute to 0.1ug/ul

Boehringer Mannheim: use 1mM HCL dilute to 0.1ug/ul

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