In Gel Digestion of Proteins for MALDI-MS

Buffers and Solutions for In Gel Digests:

500 mM Ammonium Bicarbonate [$(NH_4)HCO_3$]

* $(NH_4)HCO_3$-1.6 g
* Millipore water-to 40 mL final volume
* Filter through high protein binding filter [Nitrocellulose or PTFE; 0.45 μm pore size)

25 mM $(NH_4)HCO_3$

* 500 mM $(NH_4)HCO_3$-2 mL
* Millipore water-to 40 mL final volume
* Check pH (should be between 8.0-8.5)

25 mM $(NH_4)HCO_3$/50% Acetonitrile [ACN]

* 500 mM $(NH_4)HCO_3$-2 mL
* ACN (HPLC grade)-20 mL
* Millipore water-18 mL
* Make sure pH stays at 8.0-8.5

500 mM Ditiothreitol [DTT] stock

* DTT-77.1 mg
* 25 mM $(NH_4)HCO_3$-to 1 mL final volume
* Store at –20 ˚C in 50 μL aliquots

550 mM Iodoacetamide [IAA] stock

* IAA-101.7 mg
* 25 mM $(NH_4)HCO_3$-to 1 mL final volume
* Store at –20 ˚C in 50 μL aliquots

30 mM Potassium Ferricyanide (III) [$K_3Fe(CN)_6$]

* $K_3Fe(CN)_6$-494 mg
* Millipore water-to 50 mL final volume
* Wrap in aluminum foil and store at 4 ˚C up to three months.

100 mM Sodium Thiosulfate [$Na_2S_2O_3$]

* $Na_2S_2O_3$-237 mg
* Millipore water-to 50 mL final volume
* Store at 4 ˚C up to three months

Trypsin stock

* Trypsin [Promega Modified Sequence Grade]-20 μg
* 25mM (NH4)HCO3-1 mL (resuspend gently at room temperature)
* Store at –20 ˚C in 100 μL aliquots

10% N-octyl Glucoside

* N-octyl Glucoside [SIGMA]-250 mg
* Millipore water-to 2.5 mL final volume
* Store at room temperature

Gel fragment preparation

* Excise protein bands. Cut each into 1 mm pieces (not so small that they clog the pipet tip). Place into a low-binding Eppendorf tube (siliconized FISHER brand). Also cut out a gel piece from a protein-free region as control.

* Wash gel pieces with >10 volumes of Millipore water [~200 μL] for 10 minutes with intermittent vortexing, to wash out acetic acid.

Destaining

* For Coomassie Blue / Colloidal staining, destain **two times** (or until the color disappears) for 15 minutes with intermittent vortexing (low setting) with 250 μL of 50% Methanol in 100 mM $(NH_4)HCO_3$. Remove the solutions and discard. Dehydrate **twice** for 2-5 minutes with intermittent vortexing (low setting) with 200 μL neat Acetonitrile [ACN]. Remove the solutions and discard. The gel slices shrink and become white.

* For Non-Destructive Silver staining, destain **twice** for 15 minutes with intermittent vortexing (low setting) with 200ul of freshly prepared 1:1 solution of 100mM Sodium Thiosulfate $[Na_2S_2O_3]$ and 30 mM Potassium Ferricyanide $[K_3Fe(CN)_6]$. Discard the supernatants. Stop the reaction and wash out silver ions **twice** for 5 minutes with intermittent vortexing (low setting) with 250 μL of Millipore water. Equilibrate slices for 10 minutes with intermittent vortexing in 200 μL 25mM $(NH_4)HCO_3$ then dehydrate twice for 10 minutes vortexing with 200 μL 25mM $(NH_4)HCO_3$/50% ACN. Discard supernatants. The gel slices shrink and become white.

* For Sypro Ruby staining, no destaining necessary, dehydrate **twice** for 10 minutes with intermittent vortexing (low setting) with 200 μL 25 mM $(NH_4)HCO_3$/50% ACN. Remove the solutions and discard. The gel slices shrink and become white.

* Dry gel particles for 10 minutes in a vacuum centrifuge.

Reduction and Alkylation

* Rehydrate gel slices in 40-50 μL of freshly prepared 25 mM DTT [in 25 mM $(NH_4)HCO_3$]. Reduce the proteins for 30 minutes at 56 ˚C.

* Cool the samples to room temperature, pipet off any residual liquid and add 40-50 μL (same volume as previous step) of freshly prepared 55 mM Iodoacetamide [in 25 mM $(NH_4)HCO_3$]. Alkylate the proteins for 30 minutes at room temperature in the dark.

* Wash gel slices with 250 μL of Millipore water for 15 minutes, and discard supernatent. Resuspend gel in 200 μL of 25 mM $(NH_4)HCO_3$ for 10 minutes with intermittent vortexing and discard supernatent. Dehydrate gel pieces **twice** for 2-5 minutes each time with 200 μL of neat ACN. Discard the supernatants.

* Dry gel particles 10 minutes in a vacuum centrifuge.

Trypsin Digestion

* Rehydrate gel slices for 15 minutes at 4˚C in 20-30 μL [20 ng/μL] Trypsin (Promega Sequence Grade Modified) in 25 mM $(NH_4)HCO_3$ [make sure pH = 8.0-8.5].

* Overlay the rehydrated gel particles with a minimum amount of 25 mM $(NH_4)HCO_3$ to keep them immersed throughout the digestion. Incubate 16-24 hours at 37 ˚C.

Peptide Recovery

* Transfer the digest solution to a new low binding microcentrifuge tube.
* Extract digested peptides with one volume (40-60 μL) Millipore water/0.1% TFA by vortexing 20 minutes (high setting) at room temperature. Transfer solution to the low binding microcentrifuge tube.
* Perform an additional extraction with 60-70 μL of 5% TFA/70% ACN by vortexing 20 minutes (high setting).
* Pool extracted peptides and dry completely in a vacuum centrifuge (approx. 1 hour).

References

* Jimenez, C.R. Current Protocols in Protein Science. 1998; 16.4.1-16.4.5
* Gharahdaghi, F. *et al*. Electrophoresis.1999; 20: 601-605.
* Hirouki, K. *et al*. Rapid Commun. Mass Spectrom.