

EDMAN DEGRADATION SAMPLE PREPARATION PROTOCOLS

Distributed at ABRF 2002, Tutorial Session #2, N-terminal Edman Sequence Sample Preparation

1- Protocol for Chloroform Methanol Precipitation (For Removal of salt and detergents)

1. To sample of starting volume 100 ul
2. Add 400 ul methanol
3. Vortex well
4. Add 100 ul chloroform
5. Vortex
6. Add 300 ul H₂O
7. Vortex
8. Spin 1 minute @ 14,000 g
9. Remove top aqueous layer (protein is between layers)
10. Add 400 ul methanol
11. Vortex
12. Spin 2 minutes @ 14,000 g
13. Remove as much MeOH as possible without disturbing pellet
14. Speed-Vac to dryness
15. Bring up in 2X sample buffer for PAGE

Reference: Wessel, D. and Flugge, U. I. Anal. Biochem. (1984) 138, 141-143

2- Protocol for Concentrating Proteins Prior to SDS-PAGE

Protein samples containing Guanidine-HCl (up to 4 µl of 8 M Guanidine-HCl):

1. Add 5% N-Lauroyl sarcosine (Sigma L-5125 C₁₅ H₂₈ NO₃ Na, FW 293.4) to prevent guanidine from precipitating to 2x SDS Sample Buffer containing 10 mM DTT. Reduce and alkylate sample.

Microconcentration of proteins to remove salts or unwanted contaminants:

1. Choose microconcentrator tube with a protein cutoff smaller than molecular weight of the protein in sample. Amicon Microcon-10 MW Cutoff 10000, Amicon Centricon-30 MW Cutoff 30000
2. Add 1 μ l of 20% w/v SDS to dry microcon tube (if sample does not already contain SDS).
3. Slowly add sample (a few microliters at a time) to membrane until membrane is completely wet. Centrifuge to near (but not complete) dryness.
4. If intention is to desalt sample or buffer exchange: Add \sim 50 μ l water to microcon and spin until nearly dry. Repeat buffer exchange. Sample will remain on membrane.
5. Use new collection tube. Invert membrane and spin at low speed (1000x g) to elute protein from membrane. Add 2x SDS-Sample Buffer containing 10 mM DTT to membrane: vortex, invert and spin. Final volume should be \sim 20 μ l. Reduce and alkylate sample according to standard protocol.

Removing resin or beads from sample:

1. Add 20 μ l of 2x SDS-Sample Buffer containing 10 mM DTT to resin/beads. Incubate at 90°C for 2 minutes.
2. Transfer sample, including resin, to a microfilter and spin high speed (13000 x g).

3- Protocol for Protein Alkylation in Gel Sample Buffer with NIPIA

1. Lyophilize sample $<5 \mu$ g.
2. Reduce: Add 10 μ l of 10mM DTT in 2X Tris-glycine SDS sample buffer (1.54mg DTT in 1 ml) to protein sample.
3. Alkylate: Add 1 μ l of 200mM isopropyl iodoacetamide (NIPA) (4.5 mg NIPIA in 100 μ l methanol), and incubate 20 minutes at room temperature.
4. Add 1-2 μ l of BME or 5 μ l of 1M DTT to quench the reaction.
5. Load onto Tris-glycine gel using normal protocol.

Alternatively, or samples in high volume low salt:

1. Use a 20 μ l aliquot of sample. Samples with larger volume may require concentration using microcon cartridges.
2. Reduce: Add equal volume, 20 μ l, of 20 mM DTT (1.54mg DTT in 0.5ml) in 2X Tris-glycine SDS sample buffer to the sample. Incubate for 5 minutes at 85°C.
3. Alkylate: Add 4 μ l of 200 mM NIPA (4 mg NIPIA in 100 μ l methanol), or 1:10 with sample volume (ie. 1 μ l of NIPA per 10 μ l of total volume). Incubate 20 minutes at room temperature.
4. Add 1-2 μ l of BME or 5 μ l of 1M DTT to quench the reaction.
5. Load onto BioRad Tris-glycine 10 well/50 μ l comb-size gel, and run using normal protocol.

** 2X BioRad Tris-HCl Sample Buffer (non-reduced)

0.5M Tris-HCl, pH 8.3	2.0 ml
Glycerol	1.6 ml
10% (w/v) SDS	3.2 ml
0.5% Bromophenol Blue	0.8 ml
Deionized water	0.4 ml

Note: Bromophenol Blue should be made up in DI water, mixed for 1 hour, and filtered through a 0.5 μ m filter to remove particulates.

Reference: Henzel, W. J., Tropea, J., and Dupont, D. Anal. Biochem. (1999) 267, 148-160.

**REAGENTS:

N-isopropyl iodoacetamide (NIPIA) FW 227.04

Note: for mass spectrometry calculations add 99 to reduced mass of cysteine

Dithiothreitol (DTT) $C_4H_{10}O_2S_2$ FW 154.25

2-Mercaptoethanol (BME) $C_2H_6O_3$ FW 78.13 d=1.114 g/mL

4- Protocol for PVDF Electroblotting

1. Make 1L 10mM (3[cyclohexylamino] 1-propane sulfonic acid) (CAPS) pH 11 in appropriate methanol solution with 10mM Thioglycolic acid (1.14grams/ Liter) For proteins < 30 KDa use 20% methanol, 30-70 KDa use 10% methanol, >70 KDa use 5% methanol.
2. Applied Biosystems Problott PVDF sequencing membrane to gel size and wet with methanol.
3. Soak gel and membranes in CAPS buffer (see. 1).
4. Wet blotting paper with buffer and place a piece on both sides of a blotting clamp (see 1).
5. Place the gel on the cathode (Neg,black) side of the clamp, and place the membrane on the gel (facing the Pos, red,anode).
6. Wet membrane, remove the air bubbles from between the gel and membrane, and close the clamp.
7. Place the closed clamp in the slots of the blotting apparatus with the black (Neg.) and the red (Pos.) sides matched and check to make sure the electrodes are properly connected.
8. Run at 250mA contact current for 45min.
9. Stain blot with Coomassie Blue.

Reference: Matsudaira, P., J. Biol. Chem. (1987) 262, 10035-10038.

5- Sample Clean-Up Methods by PVDF Attachment Prior to Sequencing

1. Cut a small piece of PVDF membrane and wet thoroughly with methanol, followed by a water rinse.
2. Wet blot paper with water and place membrane on top of blot paper (to keep the membrane moist).
3. Pipet a small volume (~2 µl) of concentrated, pure sample onto membrane.
4. With a dry blot paper, hold against sides of membrane (as absorbent material) until the protein spot disappears.
5. Place membrane in a container and cover with a small amount of Coomassie R-250 stain for blots. Stain for ~60 seconds.
6. Discard stain and destain blot for 2-5 minutes, with destain changes, until spot appears and background is light.
7. Rinse with water and dry membrane. Excise spot with a razor blade and load onto sequencer.

6- Protocol for CNBr Cleavage

I. On Membrane.

1. Alkylate proteins prior to electroblotting. If proteins are already electroblotted, alkylate proteins on membranes (see protocols under 'Protein Alkylation: On PVDF membrane').
2. Place membrane slices containing reduced and alkylated protein in a 0.5 ml Eppendorf and wet with 1-2 μ l methanol.
3. Cleavage: Cover membrane with 30-50 μ l 0.1N HCl. Add a small CNBr crystal under the fume hood. Incubate at 45°C for 3 hours or room temperature overnight.
4. Remove acid and dry the membrane under a SpeedVac.

II. In Solution.

***Proteins should be salt free prior to this step!**

1. Lyophilize proteins in Eppendorf tube.
2. Resolubilize proteins in 50-100 μ l 70% formic acid or 0.1N HCl.
3. Cleavage: Add a small CNBr crystal under the fume hood. Incubate at 45°C for 3 hours with 0.1N HCl, or at room temperature for 17 hours with 0.1N HCl or 70% formic acid.
4. Remove acid under a SpeedVac.

Reference: Zalusky, C., Henzel, W. J., and Harris, W. H. J. Biochem. Biophys. Methods (1980) 3, 11-30.

7- Protocol for Deblocking Proteins with pfu Pyroglutamate Aminopeptidase (PGAP)

I. On Membrane.

1. Place PVDF sample in 0.5 ml Eppendorf tube. Wet membrane with 1 μ l methanol.
2. BLOCK. Add 200 μ l 0.5% polyvinyl pyrrolidone (PVP-360) in 0.1% Acetic acid. Shake for 20 minute at room temperature. Discard supernatant.
3. Rinse. Wash 3 times with 200 μ l milli-Q water. Transfer PVDF to a 0.2 ml PCR reaction tube.

4. DIGEST. Add 30 μ l of freshly made 1X PGAP buffer consisting of 50 mM Sodium Phosphate, 10 mM DTT, 1 mM EDTA, pH 7.0, recipe to follow:

50mM phosphate buffer:

1.08 g Sodium phosphate monobasic ($\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$)

3.27 g Sodium phosphate dibasic ($\text{Na}_2\text{HPO}_4 \cdot 7 \text{ H}_2\text{O}$)

q.s. to 200mL with milli-Q water

pH should be 7.0

1X PGAP buffer:

25 mL Phosphate buffer pH 7.0

39 mg DTT

9.3 mg EDTA

Dissolve lyophilized enzyme in 100 μ l fresh 1X PGAP buffer.

Add 1 mU *Pyrococcus furiosus* Pyroglutamate Aminopeptidase (TaKaRa)*. 10 μ l equals 1 mU.

Freeze the remaining enzyme in 10 μ l aliquots at -80°C . Incubate sample for 1 hours at 90°C .

Note: Watch reaction to be sure PVDF membrane does not dry out.

1. Discard enzyme solution. Rinse 3 times with 200 μ l MQ water.

2. Transfer PVDF to new 0.5 ml eppendorf tube.

3. Dry in speedvac, and store in refrigerator @ 5°C .

II. In Solution.

1. Lyophilize a few μ g blocked protein. Use neurotensin or other known PyroGlu-containing protein as a control.

2. REDUCE. Add 100 μ l 6M Guan-HCl, 0.2M Tris buffer titrated with glacial acetic acid to pH 8.0, 0.01M DTT (1.54 mg DTT in 1 ml of 6M Guan-HCl 0.2M Tris stock solution). Incubate at 45°C for 1 hour.

3. DESALT. Add protein solution to a Microcon-10 cartridge and spin at 14,000 g for 8 minutes.

4. WASH. Add 100 μ l Milli-Q water and spin at 14,000 g until 10 μ l remains, approximately 8 minutes. Invert and spin 2 minutes at 1000 g.

5. DIGEST. Add 40 μ l of freshly made 1X PGAP buffer consisting of 50 mM Phosphate, 10 mM DTT, 1 mM EDTA, pH 7.0, recipe to follow.

50mM phosphate buffer:

1.08 g Sodium phosphate monobasic ($\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$)

3.27 g Sodium phosphate dibasic ($\text{Na}_2\text{HPO}_4 \cdot 7 \text{H}_2\text{O}$)

q.s. to 200mL with milli-Q water

pH should be 7.0

1X PGAP buffer:

25 mL Phosphate buffer pH 7.0

39 mg DTT

9.3 mg EDTA

Dissolve lyophilized enzyme in 100 μ l fresh 1X PGAP buffer. Add 1 mU enzyme (TaKaRa)*. 10 μ l equals 1 mU. Freeze the remaining enzyme in 10 μ l aliquots at -80°C . Incubate sample for 2 hours at 75°C .

6. DESALT. Repeat steps 3 & 4 until 10 μ l remain, add 10 μ l 2X Tris-Gly reduced sample buffer, invert and spin 2 minutes at 1000 g.

7. Load samples to a 4-20% TG gel, electroblot, and sequence.

Pyrococcus furiosus Pyroglutamate Aminopeptidase (TaKaRa TAK7334)

*Note: Stability of the enzyme:

Lyophilized: room temp for 1 year 100% active

Dissolved: -20°C for 1 month 100% active, 5°C for 1 month 70% active

REAGENTS:

Polyvinyl pyrrolidone (PVP-360) FW 360,000

Tris (hydroxymethyl)aminomethane FW 121.14

Ethylenediaminetetraacetic acid tetra sodium salt (EDTA)

$\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}_8\text{Na}_4 \cdot 4 \text{H}_2\text{O}$ FW 452.2

Dithiothreitol (DTT) $\text{C}_4\text{H}_{10}\text{O}_2\text{S}_2$ FW 154.25

Sodium phosphate monobasic $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ FW 120.0

Sodium phosphate dibasic $\text{Na}_2\text{HPO}_4 \cdot 7 \text{H}_2\text{O}$ FW 142.0