Edman Degradation Sample Preparation Protocols

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

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 H_2O
7. Vortex
8. Spin 1 minute @ 14,0000 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

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 C_{15} H_{28} NO_3 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 µl 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 ~50 µl 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 ~20 µl. Reduce and alkylate sample according to standard protocol.

Removing resin or beads from sample:

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

Protocol for Protein Alkylation in Gel Sample Buffer with NIPIA

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

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Alternatively, or samples in high volume low salt:
1. Use a 20 µl aliquot of sample. Samples with larger volume may require concentration using microcon cartridges.
2. Reduce: Add equal volume, 20 µl, 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 85ºC.
3. Alkylate: Add 4 µl of 200 mM NIPA (4 mg NIPIA in 100 µl methanol), or 1:10 with sample volume (ie. 1 µl of NIPA per 10 µl of total volume). Incubate 20 minutes at room temperature.
4. Add 1-2 µl of BME or 5 µl of 1M DTT to quench the reaction.
5. Load onto BioRad Tris-glycine 10 well/50 µl 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 µm 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_2 H_6O_3 FW 78.13 d=1.114 g/mL

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 or Silver.

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

Sample Clean-Up Methods by PVDF Attachment Prior to Sequencing

I. Direct Spotting onto PVDF Membrane:
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.

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II. Concentrating with ProSorb Sample Preparation Cartridges (Perkin Elmer Part # 401950, pk. of 10)

1. ProSorb Cartridges are used for concentrating dilute samples onto a PVDF membrane and for sample clean up of unwanted salts or buffers. The ProSorb cartridge consists of a tube containing a PVDF membrane as the bottom. Sample is transferred through the cartridge by contact of the PVDF with the adsorbent filter directly underneath it. If sample is less than 100 µl, dilute sample into 100 µl of 0.1% TFA.
2. Wet PVDF membrane in ProSorb tube with 10 µl of methanol.
3. Add sample to reservoir. (Reservoir can hold up to 400 µl of liquid.)
4. Insure that the sample has contact with the absorbent filter and that fluid transfer has begun.
5. Add additional sample aliquots, if necessary (change absorbent after each 750 µl).
6. Remove sample reservoir and dry PVDF membrane. Punch out PVDF membrane or carefully cut the membrane out with a razor blade.
7. Sample is now ready to be placed in the sequencer.

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 µl methanol.
3. Cleavage: Cover membrane with 30-50 µl 0.1N HCl. Add a small CNBr crystal under the fume hood. Incubate at 45ºC 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 µl 70% formic acid or 0.1N HCl.
3. Cleavage: Add a small CNBr crystal under the fume hood. Incubate at 45ºC 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 Zalut, C., Henzel, W. J., and Harris, W. H. J. Biochem. Biophys. Methods (1980) 3, 11-30.

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 µl methanol.
2. BLOCK. Add 200 µl 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 µl milli-Q water. Transfer PVDF to a 0.2 ml PCR reaction tube.
4. DIGEST. Add 30 µl 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 ($Na_2HPO_4*H_2O$)
3.27 g Sodium phosphate dibasic ($Na_2HPO_4*7\ H_2O$)
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

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Dissolve lyophilized enzyme in 100 µl fresh 1X PGAP buffer.
Add 1 mU Pyrococcus furiosus Pyroglumate Aminopeptidase (TaKaRa)*. 10 µl equals 1 mU. Freeze the remaining enzyme in 10 µl aliquots at -80ºC. Incubate sample for 1 hours at 90ºC.
Note: Watch reaction to be sure PVDF membrane does not dry out.
1. Discard enzyme solution. Rinse 3 times with 200 µl MQ water.
2. Transfer PVDF to new 0.5 ml eppendorf tube.
3. Dry in speedvac, and store in refrigerator @ 5ºC.

II. In Solution.
1. Lyophilize a few µg blocked protein. Use neurotensin or other known PyroGlu-containing protein as a control.
2. REDUCE. Add 100 µl 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 45ºC 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 µl Milli-Q water and spin at 14,000 g until 10 µl remains, approximately 8 minutes. Invert and spin 2 minutes at 1000 g.
5. DIGEST. Add 40 µl 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 (Na_2HPO_4* H_2O)
3.27 g Sodium phosphate dibasic (Na_2HPO_4*7 H_2O)
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 µl fresh 1X PGAP buffer. Add 1 mU enzyme (TaKaRa)*. 10 µl equals 1 mU. Freeze the remaining enzyme in 10 µl aliquots at -80ºC. Incubate sample for 2 hours at 75ºC.
6. DESALT. Repeat steps 3 & 4 until 10 µl remain, add 10 µl 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: @ -20ºC for 1 month 100% active @ 5ºC for 1 month
70% active

REAGENTS:
Polyvinyl pyrrolidine (PVP-360) FW 360,000
Tris (hydroxymethyl)aminomethane FW 121.14
Ethylenediaminetetraacetic acid tetra sodium salt (EDTA)
$C_{10}H_{12}N_2O_8Na_4$*4 H_2O FW 452.2
Dithiothreitol (DTT) $C_4H_{10}O_2S_2$ FW 154.25
Sodium phosphate monobasic Na_2HPO_4* H_2O FW 120.0
Sodium phosphate dibasic Na_2HPO_4*7 H_2O FW 142.0