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Sample Preparation Guides for N-Terminal Edman Sequencing

WHAT PVDF MEMBRANE SHOULD I CHOOSE

We recommend the small pore (0.1 um) pure PVDF membranes from ABI (ProBlott), Millipore (Immobilon PSQ) or Bio-Rad (Trans-Blot). These membranes have a higher binding capacity than large pore (0.45 um) membranes, reducing the risk of protein loss by passage through the membrane and increasing the efficiency of transfer which can approach 100%.

ELECTROBLOTTING

Once a protein of interest has been separated from contaminating species by SDS-PAGE of 2D-GE, the pure protein can be transferred to a PVDF membrane for direct N-terminal sequence analysis. This “micro” purification protocol of SDS-PAGE followed by electroblotting onto a membrane has become the method of choice for structural analysis of proteins of low abundance or proteins difficult to purify by conventional column chromatography or HPLC. Electroblotting onto a PVDF membrane is fast, simple, and very efficient. The resulting “blots” are stable at -20C for several months.

The lab has a detailed protocol for SDS-PAGE/electroblotting derived from published procedures and personal experience. A good general reference is Chapter 3 of P. Matsudaira’s book, “A Practical Guide to Protein and Peptide Purification for Microsequencing” (1993). Factors affecting the efficiency of transfer and quality of subsequent sequence analysis and limitations of PVDF “blots” follow:

1. **Choice of Membrane**

We recommend the small pore (0.1 um) pure PVDF membranes from ABI (ProBlott), Millipore (Immobilon PSQ) or Bio-Rad (Trans-Blot). These membranes have a higher binding capacity than large pore (0.45 um) membranes, reducing the risk of protein loss by passage through the membrane and increasing the efficiency of transfer which can approach 100%.

2. Choice of Transfer Buffer

Blotting at high pH in CAPS buffer is recommended to avoid contamination with Tris and Glycine. While some people still prefer the conventional Tris-glycine buffer system, the blotting membrane must be extensively washed with ultra-pure water after electroblotting to remove these contaminants. Tris interferes with the Edman chemistry and glycine interferes with the interpretation of sequence data.

3. Visualization of PVDF-bound Proteins

There are many ways to visualize the transferred protein. Coomassie blue R-250 staining is the most popular (and sensitive) method followed by Ponceau S and Amido Black. Although metal (silver or gold) staining in the gel is very sensitive (1pmole), it should be avoided as no useful sequence information can be obtained from the transferred proteins.

4. Optimization of Transfer Conditions

Electroblotting is the most critical step in obtaining adequate amounts of "sequence-able" protein on the membrane. Careful optimization of the amperage and time required for transfer is important for the maximum recovery of your protein. Typically, 20kDa proteins will completely transfer from a mini gel at 0.5 A in 10 min. Large proteins (>100kDa) may require 45-60 min. Electroblotting at lower currents generally results in more efficient transfers but requires longer times. In most cases, proteins of similar size behave in a similar manner during transfer. But, occasionally, a protein's transfer characteristics will be unusually dependent upon pH, methanol concentration or transfer time.

5. Limitation of PVDF membranes in Sequence Analysis

It is not uncommon to carefully isolate, purify, and transfer enough protein for sequencing but not obtain any sequence information at all. When this happens, it is very important to ask two questions which 1st BASE can help answer. Was there enough protein on the blot? Was the protein "artificially" blocked? If the answers to these questions indicate you have isolated a protein which is N-terminally blocked by post-translational modification (over 80% of all eucaryotic proteins are), one must use a different approach (MS analysis) to obtain any sequence information.

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PREPARING PROTEIN SAMPLE(S) ON PVDF MEMBRANE

A. General Comments

1. To reduce the possibility of amino-terminal blockage of the protein:
    a. use the highest purity gel reagents possible
    b. make fresh stock solutions for important samples
    c. age gel prior to running protein
    d. reducing agents should be present during electrophoresis
2. Wear gloves. Handle gels and blots only at the edges to minimize background contamination.
3. The concentration of acrylamide in the lower gel (see B. below) can be from 7-18% but 12.5% is the most useful for 10-70Kd proteins.
4. Use a mini-gel system to maximize protein-to-gel ratio and load as concentrated a protein solution as possible without losing resolution. One wants to load the sequencer with as much protein and as little membrane as possible for best results.
5. Run a set of MW markers on each gel to serve as controls in case the unknown protein is blocked. One can sequence the first few residues of a marker band (ie. lysozyme at 14.3 kD) to make sure the gel reagents didn't chemically block the protein.
6. If identification of "cys" residues is desired, consider reduction and alkylation of the sample in the gel loading buffer.

B. Tris-Glycine Gels

1. Pour lower gel, cover with H2O sat'd butanol to break any bubbles and let sit overnight.
2. Pour off butanol, flush with water several times and dry completely with wipe.
3. Pour upper gel, let sit 30 min then flush wells by filling with water and shaking sharply; repeat two more times.
4. Dissolve protein in reducing sample loading buffer (r-S.L.B.) at 1-2 µg / 10 ul and let reduce for 10 min at 60 C.
5. Load the entire sample and run at 25 mA (constant current) for about 1 hour until the dye marker is near the bottom of gel.
6. If the sample is very dilute, pour an extra-long stacker. Completely fill the wells with the sample and run until the dye has just entered the stacker. Flush the wells with running buffer and load another aliquot. If needed, a third aliquot can usually be loaded without compromising resolution.

Recipe for 12.5 % gels:

	Lower gel	Upper gel
30% acrylamide/0.8% bis	4.20 ml	0.75 ml
4X U.G.B.		1.25 ml
4X L.G.B.	2.50 ml	
water	3.10 ml	2.90 ml
10% NH4 persulfate (fresh)	0.20 ml	0.10 ml
TEMED	10 µl	5 µl
TOTAL	10.00 ml	5.00 ml

C. Electroblotting onto a PVDF Membrane

1. Carefully remove the upper gel from the lower gel and briefly place the lower gel in a tray of blotting buffer.
2. Wet precut pieces of membrane with 100% methanol for a few seconds then transfer to blotting buffer in a second tray. Make sure the membrane is completely wetted.
3. Dip the sponges and precut filter paper pieces in blotting buffer in a third tray.
4. Assemble the blotting sandwich as follows:
   sponge // filter paper // gel // PVDF // filter paper // sponge
   a. make sure there are no trapped air bubbles between any of the layers
   b. assemble the sandwich on its holder so that the gel is toward the neg. (usually black) electrode and the membrane is toward the positive (usually red) electrode.
   c. make sure the sponges are in good condition; if the blotting cassette is easy to close, the sponges are no good.
5. Fill the blotting apparatus with blotting buffer and apply a constant voltage (ca. 70V) for 30 to 40 min.
   a. one can also transfer overnight at a low constant current (ca. 15 mA) in a cold room.

D. Staining the PVDF Membrane

1. Remove the membrane from the sandwich, briefly rinse with water then soak it in 100% methanol for a few seconds.
2. Stain the membrane in dye (Coomassie) solution with constant shaking for no more than 60 sec.
   a. Ponceau red and amino black are acceptable alternatives to Coomassie but are generally less sensitive.
3. Immediately de-stain in 50% methanol with constant shaking, changing the solution every couple of minutes until the solution remains colorless.
4. Rinse the membrane thoroughly with water and let dry 30-60 min. to increase the contrast of the bands.

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E. Stock Solutions

4X U.G.B.	6.05 gm Tris pH=6.8 (ca. 7 ml 6N HCl) 0.40 gm SDS to 100. ml with water
4X L.G.B.	18.17 gm Tris pH=8.8 (ca. 4 ml 6N HCl) 0.40 gm SDS to 100. ml with water
2X S.L.B.	12.5 ml 4X U.G.B. 20.0 ml 50% glycerol 3.0 gm SDS to 45. ml with water
Reducing S.L.B.:	0.45 ml 2X S.L.B. 0.45 ml water 0.05 ml 2-mercaptoethanol 0.05 ml 0.1% BPB dye solution
Tank buffer:	30.3 gm Tris pH=8.0 (10X stock) 144.1 gm glycine 10.0 gm SDS to 1000. ml with water
Blotting Buffer:	100. ml 10X CAPS buffer 100. ml methanol 800. ml water
10X CAPS:	22.13 gm CAPS pH=11 (2N NaOH) (store at 4 C.) to 1000. ml with water
Dye Solution:	1. dissolve 1.0 gm Coomassie blue in 400 ml methanol and stir for 1 hour. 2. add 10 ml acetic acid and 590 ml water. 3. stir for 30 min and filter thru a 0.45 µm filter.