Sequi-Blot PVDF Membrane for Protein Sequencing

Instruction Manual

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LIT240 Rev C

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Section 1
Introduction

The hydrophobicity of PVDF (polyvinylidene difluoride) membrane makes it an ideal support for binding proteins in electrophoretic and dot blotting applications. Proteins are tightly bound, and are quantitatively retained during exposure to acidic, basic, or organic solvents.[1,2] Resistance to acidic and organic solvents which would dissolve nitrocellulose or nylon membranes makes PVDF membrane an excellent support for amino-terminal protein sequencing.[1,3] The unique manufacturing process used for Bio-Rad's Sequi-Blot PVDF membrane insures higher protein binding capacity than other commercially available PVDF products.[4] Higher capacity increases the likelihood of sequencing proteins of interest, which makes Sequi-Blot PVDF membrane the best choice for

sequence analysis of blotted proteins and peptides.

For use of PVDF membrane as an immunoblotting support for western blot detection, Bio-Rad offers Immun-Blot® PVDF membrane which features lower signal to background results in this application than Sequi-Blot membrane. However, it does not exhibit the same high protein binding capacity, so Sequi-Blot PVDF membrane is the best one to use for protein sequencing.

Section 2
Membrane Wetting

Sequi-Blot PVDF membrane can be used as a direct replacement for the membrane currently being used in your sequencing protocol. No changes are required in the procedure, but the special steps given below are required to prepare the membrane for blotting. The hydrophobicity of the PVDF membrane makes it impossible to wet the membrane with aqueous solutions. Methanol or an alternative organic solvent is required to pre-wet the membrane prior to equilibration in transfer buffer. After equilibration, the membrane can be used in a semi-dry, tank, or capillary blotting system with any acidic or basic blotting buffer.

Note: Always handle membranes using gloves or forceps to prevent contamination.

1. Immerse the membrane in 100% methanol for a few seconds, until the entire membrane is translucent. In methanol, it wets immediately. (Solutions containing 50% methanol concentration can be used to prewet the membrane.)
2. Transfer the wetted membrane to a vessel containing transfer buffer or water. Incubate in buffer until it is equilibrated (² 2-3 minutes). The membrane will float on the surface of the buffer until completely equilibrated. After it is equilibrated it can be easily submerged into the aqueous solution. At this point, the membrane is ready to bind proteins in any blotting application.
3. After the membrane has been wetted with buffer, do not allow it to dry (white spots will form where the membrane is dry). Protein will not bind to the dried membrane, and dry spots will not rewet in aqueous solutions. If the membrane becomes dry prior to blotting, repeat steps 1 and 2 to rewet it.

Section 3
Dot Blotting

Dot blotting requires special precautions to insure that protein is bound to the PVDF membrane before it dries. When the membrane is dry, protein molecules will not bind tightly and will be washed off in subsequent analysis steps. For protein sequencing, the amount of protein bound is critical. For this application it might be better to try the direct adsorption method for attachment of proteins.[3] Direct adsorption consistently provides 90% binding of small amounts of protein. In this method, up to 300 pmol of protein is suspended in 200 µl of buffer and incubated with a small piece (5 x 9 mm) of wetted PVDF membrane for 1 hour

with agitation (rotary shaker) or 24 hours without. The incubation is carried out at 4 °C in a siliconized glass tube. As the amount of protein increases above 300 pmol, the percent of protein bound will decrease.

Note: The high binding capacity of Sequi-Blot PVDF membrane makes it diffi-cult to block in immunoassays. Use 0.5% casein, non-fat dry milk, or BSA, or Immun-Blot PVDF membrane for protein immunoblotting applications for best results.

Section 4
Electrophoretic Blotting for Protein Sequencing

This protocol is based on the techniques practiced by Dr. David Speicher in the Protein Micro-chemistry Lab at The Wistar Institute.[3] Each of the precautions recommended below reduces the potential of amino terminus blocking during the gel purification and blotting steps. Proteins and peptides larger than 10,000 daltons bind so strongly to PVDF that a Polybrene coated glass fiber filter is not required for optimal sequence analysis. Elimination of the Polybrene coated filters saves the time normally required for precycling, and reduces Polybrene associated background seen in the initial sequencing cycles. Alternative protocols for electrophoresis and blotting of proteins for sequence analysis, including options for recovering peptides from

proteins with a blocked amino terminus, are reviewed in bulletin 2212.

Follow your standard procedure for Laemmli SDS-PAGE, observing the changes outlined below (solution recipes are included after the blotting section):

1. Use reagents and solvents of the highest purity. Use of Bio-Rad's electrophoresis reagents without further purification is recommended.
2. Filter gel solutions, except running buffer, with a 0.45 micron filter and store at 4 °C. Store SDS stock solution at RT.
3. Solubilize samples with 2x or 5x solubilizing buffer with sucrose. Do not use urea in the solubilizing buffer.
4. Heat samples with solubilizing buffer at 37 °C for 10-15 minutes prior to loading onto the gel. Do not heat samples at 100 °C.

5. Allow the gel, including stacker, to polymerize completely. Let the cast gel stand for 24-72 hours at room temperature prior to use.
6. Add 11.4 mg/l (0.1 mM) thioglycolate to the upper running buffer prior to electrophoresis to scavenge reactive compounds left in the gel which cause N-terminal blocking.
7. Include 5 µg of a sequence standard (i.e. myoglobin or ß-lactoglobulin A) as a control in one lane.

Transfer Protocol

These instructions are for use with a tank blot apparatus such as the Trans-Blot® cell. Blotting in a Trans-Blot cell is preferable to semi-dry transfers for protein sequencing because tank blotting is more quantitative with higher binding yields. Follow the instructions provided with your blotting cell for assembly.

1. For most proteins, use a Towbin buffer[5] with methanol (MeOH).
2. PVDF membrane should be clean and free of wrinkles.
3. Wet the membrane following the protocol in the membrane wetting section.
4. Make sure there are no bubbles between the membrane and the gel.
5. After transfer, rinse the membrane three times (5 minutes each) with distilled water.
6. Stain for 5 minutes with the PVDF CBB R-250 membrane stain. **Do not use the standard CBB gel stain.** Destain for 10-15 minutes, or until background is light blue, with PVDF destain solution. **Avoid acetic acid in the stain and destain, as it might cause blockage of the amino terminus.**

Solutions

Stock sample buffer solution (5x without urea):

0.5 M sucrose	42.78 g
15% SDS	37.5 g
312.5 mM Tris	9.5 g
10 mM Na_2EDTA	0.925 g

Make to a volume of 225 ml with distilled water. Heat gently to get into solution and adjust to pH 6.9 with 1 N HCl. Adjust to a final volume of 250 ml. Store at 4 °C in 10 ml aliquots.

5x working sample buffer solution:

Add 100 µl of ß-mercaptoethanol and 100 µl of bromophenol blue (BPB) solution (0.05% w/v) to 2 ml of 5x stock sample buffer solution.

2x working sample buffer solution:

Add 1.5 ml of distilled water, 50 µl of ß-mercaptoethanol, and 50 µl of BPB solution (0.05% w/v) to 1 ml of 5x stock sample buffer solution.

Towbin buffer:

25 mM Tris	3.03 g
192 M glycine	14.4 g
20% methanol	200 ml

Adjust volume to 1 liter with dd H_2O. Prechill the buffer before use.

Note: Do not add acid or base to adjust pH. The buffer will range from pH 8.1 to 8.5, depending on the quality of Tris, glycine, dd H_2O, and methanol. Methanol should be analytical reagent grade, as metallic contaminants in low grade methanol will plate on the electrodes.

Sequi-Blot PVDF membrane stain:

0.025% Coomassie® Blue R-250 dissolved in a 40% MeOH solution

Sequi-Blot PVDF membrane destain:

50% MeOH solution

Section 5
Amino Acid Analysis by Hydrolysis of Membrane Bound Proteins

Amino acid analysis requires homogeneous proteins. SDS-PAGE electrophoresis provides a convenient way to purify proteins. Blotting to Sequi-Blot PVDF membrane provides a simple

way to isolate proteins separated in a gel. Protein samples which have been immobilized onto PVDF by either spotting, direct adsorption, or electrophoretic blotting can be hydrolyzed and subjected to amino acid analysis.[6,7] Amino acid analysis of blotted proteins using a post-column amino acid analyzer offers a way to quantitate the sample present prior to sequencing, as well as a way to determine the A.A. composition of the proteins of interest. This data can also be used to determine the efficiencies of the gel electrophoresis and blotting systems being used for sequencing, whether the protein of interest is present in enough quantity to provide useful sequence data, or whether the quantity of protein is not a problem and amino terminal blockage might be the cause of sequencing problems. For amino acid analysis:

1. Place the PVDF membrane piece containing the sample in a hydrolysis tube and add 200 µl of 6 N HCl containing 4% thioglycolic acid.
2. Seal the tube *in vacuo* and hydrolyze for 16-24 hours at 110 °C.
3. Extract the liquid containing the amino acids from the tube, and rinse the PVDF membrane with an additional 50-100 µl of 6 N HCl.
4. Combine the rinse wash with the extraction sample and evaporate to dryness.
5. Analyze samples with an amino acid analyzer.

Note: As a blank control to account for background, cut a piece of membrane the same size as the band of interest from a corner of the membrane and subject it to hydrolysis.

Section 6
Protein Detection

Staining

Proteins for sequencing can be detected with Coomassie brilliant blue (CBB) R-250 or with Bio-Rad's Colloidal Gold Total Protein Stain. CBB R-250 is the stain most commonly used for detection of proteins prior to protein sequence analysis. It is rapid (≥ 15–20 minutes), sensitive, and does not interfere or react with the reagents used in Edman degradation chemistry. See page 13 for the CBB R-250 stain preparation without acetic acid. If a protein can not be detected by CBB staining, there will not be enough present to sequence. See the Blotting for Protein Sequencing section for a protocol for CBB R-250 staining.

Section 7
For Technical Assistance

In the U.S., technical service is available by calling 1-800-4BIORAD (1-800-424-6723). Our Technical Service representatives are available to answer your questions from 8 AM to 5 PM (PST). Technical representatives can be contacted through your local Bio-Rad office. For more information about the use of PVDF membrane, request bulletin 2212.

Section 8
References

1. Matsudaira, P., *J. Biol. Chem.*, **262,** 10035-10038 (1987).
2. Hildebrandt, E. and Fried, V., *Anal. Biochem.*, **177,** 407-412 (1989)
3. Speicher, D. W., in **Techniques in Protein Chemistry** (T. Hugli, ed.), Academic Press, 24–35 (1989).
4. Speicher, D. W., Mozdzanowski, J., Beam, K. and Chem, D., *J. Protein Chemistry*, (1990).
5. Towbin, H., Staehelin, T. and Gordon, J., *Proc. Natl. Acad. Sci. USA*, **76,** 4350-4354 (1979).
6. Hulmes, J., Miedel, M. and Pan, Y., in **Techniques in Protein Chemistry** (T. Hugli, ed.), Academic Press, 7-16 (1989).
7. Nakagawa, S. and Fukuda, T., *Anal. Biochem.*, **181,** 75-78 (1989).

Section 9
Product Information

Catalog Number	Product Description
Sequi-Blot PVDF Membrane	
162-0180	**Sequi-Blot PVDF Membrane,** 10 x 15 cm, 10 sheets
162-0181	**Sequi-Blot PVDF Membrane,** 15 x 15 cm, 10 sheets
162-0182	**Sequi-Blot PVDF Membrane,** 20 x 20 cm, 10 sheets
162-0183	**Sequi-Blot PVDF Membrane,** 30 cm x 3 m, 1 roll
162-0185	**Sequi-Blot PVDF Membrane,** 20 x 20 cm, 3 sheets
162-0186	**Sequi-Blot PVDF Membrane,** 7 x 8.4 cm, 10 sheets

Catalog Number	Product Description
	Blotting Equipment
170-3910	**Trans-Blot Electrophoretic Transfer Cell**
170-3946	**Trans-Blot Cell with Plate Electrodes**
170-3930	**Mini Trans-Blot® Cell**
	Electrophoresis and Blotting Reagents
161-0400	**Coomassie Brilliant Blue R-250,** 10 g
170-6527	**Colloidal Gold Total Protein Stain,** 500 ml
161-0305	**Prestained SDS-PAGE Standards,** low range, 500 µl
161-0309	**Prestained SDS-PAGE Standards,** high range, 500 µl
161-0318	**Prestained SDS-PAGE Standards,** broad range, 500 µl
161-0326	**Polypeptide SDS-PAGE Standards,** 200 µl

Catalog Number	Product Description
161-0304	**SDS-PAGE Standards,** low range, 200 µl
161-0303	**SDS-PAGE Standards,** high range, 200 µl
161-0317	**SDS-PAGE Standards,** broad range, 200 µl