3.1.3 FluoroTrans® and BioTrace™ PVDF Membranes

Western blot transfer from an SDS-PAGE gel is a method that can be used to detect individual proteins in a given complex sample. The denatured proteins are resolved by electrophoresis then transferred out of the PAGE gel and onto a microporous membrane, where they are located by stains or by "probing" with specific detection reagents, such as antibodies to the analyte of interest.

The two most common membrane types used for western blots are nitrocellulose and polyvinylidene fluoride (PVDF). Nitrocellulose was one of the first membrane types used for western blotting. Its advantages are that it is easily wetted with an alchohol solution and it produces good immunoblotting results. PVDF membranes were developed in 1985 and exhibit improved protein retention under harsh conditions (i.e. in the presence of organic solvents or under acidic or basic conditions). The greater mechanical strength of PVDF membranes is an asset when handling the membrane compared to nitrocellulose. In addition to its chemical stability, PVDF offers advantages when stripping and reprobing in immunodetection applications.

Three different PVDF membranes with characteristics tailored to specific uses are available and are summarized in Table 3.3.

Table 3.3
Western Blot Transfer Membrane Application Guide

Product	BioTrace NT Membrane	BioTrace PVDF Membrane	FluoroTrans® PVDF Membrane	FluoroTrans W PVDF Membrane
Description	Pure Nitrocellulose	Polyvinylidene Fluoride	Polyvinylidene Fluoride	Polyvinylidene Fluoride
Application	Protein transfers Nucleic acid detection ELISA	Protein transfers Protein dot or slot blot	N terminal protein sequencing Protein dot or slot blot	Protein transfers Protein dot or slot blot, Detection with immunostaining
Advantages	High sensitivity Low background	Highest sensitivity Low background Chemical resistance High tensile strength	High binding capacity Lowest burn-through Chemical resistance High tensile strength Low fluorescence background	Highest sensitivity Low background Low burn-through High binding capacity Chemical resistance High tensile strength
Binding Interaction	Hydrophobic and electrostatic	Hydrophobic	Hydrophobic	Hydrophobic
Method of Immobilization	Western transfer Dot or slot blot Bake and UV crosslink (for nucleic acids)	Western transfer Dot or slot blot	Western transfer Dot or slot blot	Western transfer Dot or slot blot

Table 3.3 (continued)
Western Blot Transfer Membrane Application Guide

Product	BioTrace™ NT Membrane	BioTrace PVDF Membrane	FluoroTrans® PVDF Membrane	FluoroTrans W PVDF Membrane
Detection Methods	Radiolabeled probes Direct stains Fluorescence Enzyme-antibody conjugates	Direct stain Enzyme-antibody conjugates Cemiluminescence Chromogenic	Direct stain Enzyme-antibody conjugates Cemiluminescence Chromogenic Fluorescence	Direct stain Enzyme-antibody conjugates Cemiluminescence Chromogenic

FluoroTrans membrane is a PVDF microporous membrane with a rated pore size of 0.2 µm. It offers higher protein adsorption capacity and retention than any other commercially available membrane. Protein immobilized on FluoroTrans membrane is not easily removed, even with strong chaotropic agents. The high adsorption capacity, coupled with the high protein retention and resistance to chemical solvents, make this membrane the "gold standard" for use with N-terminal sequencing using the Edman chemistry.

Tip: *Some high background staining may be experienced when using total protein stains. This is presumably a consequence of the high internal surface area and adsorption potential afforded by such a material.*

FluoroTrans W membrane is a membrane that has been optimized for use in Western transfer applications. Sacrificing little in terms of binding capacity and retention, this membrane shows very low levels of protein “burn-through” during transfer. FluoroTrans W membrane yields very high sensitivity, excellent resolution, and very low background levels with all detection systems including total protein stains.

BioTrace PVDF membrane with a rated pore size of 0.45 µm performs especially well with chemiluminescent and colorimetric detection systems. It is highly resistant to organic solvents and aggressive aqueous solutions. BioTrace PVDF membrane is recommended for all protein transfers.

3.1 – Section 3.1.3

Protocol for FluoroTrans® and BioTrace™ PVDF Membranes

A. Materials Required

1. FluoroTrans, FluoroTrans W, or BioTrace PVDF blotting membranes
2. Protein blocking buffers
   a. 0.5% Casein in phosphate buffered saline (PBS)
   b. 1% non-fat, dried milk in PBS

Tip: *The best method for blocking membranes is 0.5% Hammersten grade casein (BDH 44020 or equivalent) diluted in buffer. Add casein to the buffer and heat on a stir plate to 80 °C or until dissolved; do not boil the solution. Cool to 25-40 °C before use.*

3. Blocking solutions may also contain 0.05% Tween 20 or other non-ionic surfactant which may enhance blocking and will also aid in rehydrating the membrane if the spotted membrane is stored in a dry state.
4. Goat Anti-Rabbit IgG (e.g., Sigma Chemicals PN R-3128)
5. Rabbit Anti-Mouse IgG (e.g., Sigma Chemicals PN M-9637)
6. Goat Anti-Rabbit IgG conjugated to Alkaline Phosphatase (e.g., Sigma Chemicals PN A-7650)
7. Nitro blue tetrazolium (NBT) (e.g., Sigma Chemicals PN N-6876) 75 mg/mL in 70% (v/v) dimethylformamide
8. 5-Bromo-4-chloro-3-indolyl Phosphate (BCIP) (e.g., Sigma Chemicals, PN B-8503) 50 mg/mL in di-methyl formamide
9. Buffer: 0.1 M Tris-HCL (pH 7.5), 0.1 M NaCl, 50 mM $MgCl_2$
10. Working substrate solution is prepared as follows: 33 µL NBT, 25 µL BCIP in 7.5 mL buffer. For optimal results, use 0.25 mL substrate/cm^2 membrane. Solution should be prepared fresh.

B. Handling and Pre-Wetting PVDF Membranes

1. Remove the roll of membrane from the package. Cut to desired dimensions of the SDS-PAGE gel.

Tip: *It is important to number the membranes with a pencil to ensure they can be identified during testing. Ink will run when the membranes are pre-wet in alcohol.*

2. Slowly lower the membrane into 80-100% (v/v) methanol or ethanol and agitate briefly. The membrane will become translucent as it wets.
3. Rinse the membrane with high purity water and equilibrate in transfer buffer for 5 minutes prior to use. Do not allow the membrane to dry out during processing. (If membrane does become partially dry, allow to dry fully, re-wet with < 20% alcohol, exchange to buffer, and proceed. This extra alcohol step does not usually interfere with detection.)

C. Western Transfer

Proteins should be immobilized on the membrane via electro-transfer or dot/slot blotting with a suitable manifold. The optimum amount of protein for detection usually varies between 1 and 10 µg per band. After transfer, membranes can be rinsed in transfer buffer to remove excess gel fragments.

1. If the membranes and absorbent pads are not precut to size, cut them to the size of the gel. Always wear gloves, handle the membrane with blunt-ended forceps (PN 51147), and cut the membrane while it is between sheets of the interleaving material.
2. Wet the membrane according to the procedure.
3. Equilibrate both the gel and membrane in transfer buffer.
4. Saturate six new absorbent pads (cut to size if needed) in transfer buffer (or use the number of pads recommended by the apparatus manufacturer). Place three pads on the anode (+) plate.
5. Carefully place the membrane on the saturated pads. Roll a clean glass pipette slowly and gently over the membrane in one direction to eliminate air bubbles that may exist between the pads and the membrane.
6. Place the gel on top of the membrane, rolling a glass pipette slowly and gently over the gel in one direction to eliminate air bubbles that may exist between the gel and membrane.
7. Place three absorbent pads on top of the gel, then place the cathode side (-) of the apparatus on top of the stack.
8. Insert the stack in the tank and add transfer buffer per the manufacturer's instructions.
9. Connect the tank to the power supply and start the transfer. Follow the manufacturer's recommendations for current. Transfers are generally complete in 15-90 minutes.
10. For PVDF membranes (FluoroTrans®, FluoroTrans W, and BioTrace™ PVDF): Do not allow the membrane to dry out at any point during the detection. If the membrane becomes partially dry, allow the membrane to dry fully, then re-wet with methanol and exchange to buffer before continuing.

D. Staining Methods

1. Coomassie* Blue protocol for total protein staining
   - **a.** Rinse the membrane blots in high purity water.
   - **b.** Place membranes in Coomassie Blue stain (0.2% Coomassie Blue (w/v), 40% methanol, 10% acetic acid) for 10 minutes.
   - **c.** Destain for 3 minutes with Coomassie Blue Destain I (80% methanol; 10% acetic acid).
   - **d.** Destain with Coomassie Blue Destain II (45% methanol; 10% acetic acid) for 1 hour. Replace with fresh Destain II solution and soak overnight.

2. Ponceau S Reversible Staining
   a. Place membrane in 0.01% (w/v) Ponceau S, 3% (w/v) Trichloroacetic acid solution at room temperature for 5 minutes.
   b. Wash in water for 2 minutes to destain the membrane.
   c. Wash an additional 10 minutes to remove all the stain.

E. Immunodetection

1. Block non-specific binding by using either a commercial blocking agent or one of the following blocking solutions.
   a. 2% non-fat, dry milk 10 mM Tris-HCl pH 7.5, 150 mM NaCl
   b. 1-5% BSA, 10 mM Tris-HCl pH 7.5, 150 mM NaCl
   c. 0.5% casein 10 mM Tris-HCl pH 7.5, 150 mM NaCl

Tip: *BSA (1-5%) may be used as a blocking agent for nitrocellulose membranes, but is not effective when using PVDF membranes. Casein provides superior blocking performance with all membrane types.*

2. Each of the following steps is performed in a suitably-sized container to allow 10 mL of solution to ensure adequate coverage of a 10 x 10 cm membrane area. Plastic bags, boxes, or tubes on a roller mixer may also be used to perform incubation steps. Make sure all NT blots are completely immersed beneath the surface of the liquid. Gently agitate samples during all incubation steps on an orbital shaker.
3. Place the membrane blots in a suitably sized container with suitable blocking solution.
4. Place the container on an orbital shaker and gently agitate for 30 minutes.

Tip: *At this point, the membrane blots may be dried at 37 °C for 10 minutes or air-dried at 20-30 °C for > 30 minutes and stored.*

5. Place the membrane blots into a clean, suitable container with primary detection antibody diluted in PBS to 1 µg/mL. Use 2 mL per 10 x 10 cm membrane area. Gently agitate the membrane blots on an orbital shaker for 30 minutes.
6. Replace primary antibody solution in the container with 10 mL wash solution. Wash membrane blots in 2x changes of wash solution, 5 minutes per wash. Gently agitate membrane blots on orbital shaker during wash steps.
7. Replace wash solution with 10 mL of PBS. Gently agitate on orbital shaker for 1 minute.
8. Replace the PBS solution with 2 mL (per 10 x 10 cm area) of the secondary detection conjugate diluted 1/1000 in Blocking Solution.
9. Repeat wash procedure listed above.
10. Replace wash solution with two changes of high purity water. Gently agitate on orbital shaker, 1 minute per change of rinse solution. (Substrate buffer may also be used for this rinse step.)

F. Detection Methods

1. Detection with Alkaline Phosphatase conjugate and BCIP/NBT substrate
   a. Equilibrate the membrane in reaction buffer I (10 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) at room temperature for 5 minutes.
   b. Remove the buffer and add Bromochloroiodyl phosphate/Nitrobule tetrazolium (BCIP/NBT) substrate. (Dissolve 82.5 mg BCIP, 42.5 mg NBT in 1 mL dimethylformamide. While stirring, add to 250 mL reaction buffer. Protect from light). Observe the reaction as color develops.
   c. When the reaction is complete, rinse the membrane twice with reaction buffer. Rinse the membrane twice with distilled water.
2. Detection with Horseradish Peroxidase and 3,3-Diaminobenzidine (HCl) substrate (DAB)

CAUTION: *DAB is a carcinogen and should be handled with appropriate safeguards. Carefully review the supplier's MSDS before proceeding.*

   a. Equilibrate the membrane in reaction buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween 20) at room temperature for 5 minutes.
   b. Add 0.003 mL of 30% H_2O_2 to 100 mL of DAB substrate (0.05% DAB in reaction buffer made fresh prior to use).
   c. Remove the membrane from the reaction buffer and add the above DAB solution.
   d. Gently agitate at room temperature and observe the reaction as color develops.
   e. Rinse the membrane twice with distilled water.
3. Detection with Horseradish Peroxidase and Chloronaphthol (CN)
   a. Equilibrate the membrane in reaction buffer (10 mM Tris-HCL pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 5 minutes at room temperature.
   b. Add 0.05 mL of CN substrate to 2 mL methanol. Add 8 mL TBS and mix. Add 0.035 mL of 30% H_2O_2 and mix again.
   c. Remove the membrane from the reaction buffer, blot briefly on blotting paper, and immerse it in the above solution.
   d. Gently agitate at room temperature and observe the reaction as color develops.
   e. When the desired intensity is achieved, stop the reaction by rinsing the membrane several times in distilled water.

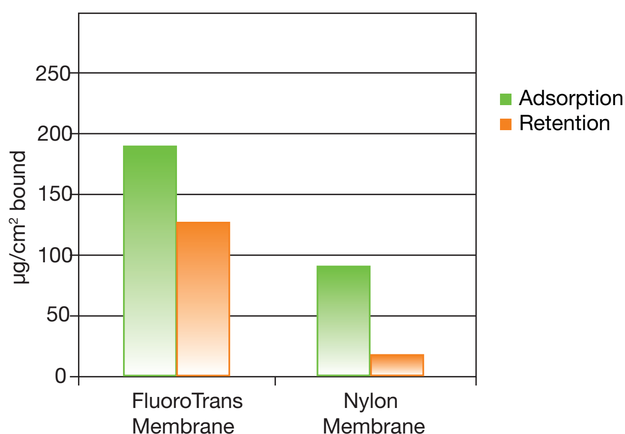
Application Data for FluoroTrans® and BioTrace™ PVDF Membranes

High Protein Binding and Retention on FluoroTrans Membrane

High internal surface area 0.2 µm pore size PVDF membranes, such as FluoroTrans membrane, exhibit high protein binding and retention under aggressive solvent conditions, such as detergents like SDS and chaotropic agents like urea. The mechanism of retention is complex and is thought to be related to the strong affinity of hydrophobic regions within a proteins structure for the PVDF surface and the strong dipole moment within the $-[CH_2 - CF_2]_{n-}$ chemical structure. An example of comparing protein retention on PVDF versus a charged nylon surface is shown in Figure 3.3. The results clearly show the initial higher passive adsorption of IgG to PVDF and its subsequent retention after challenge with SDS and urea.

Figure 3.3

Adsorption and Retention of Proteins by PVDF and Nylon Surfaces



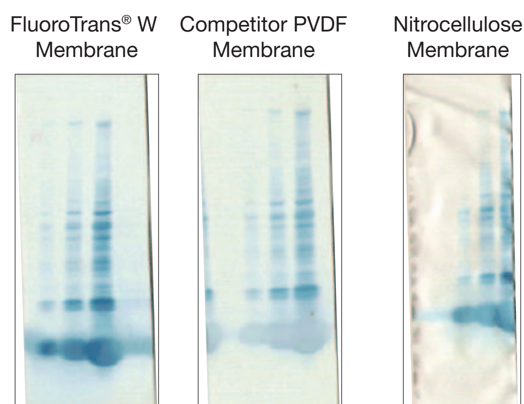
13 mm discs of FluoroTrans membrane and Biodyne® A nylon membrane were soaked in 2 mL per disc of phosphate buffered saline (PBS) containing 200 µg/mL goat IgG and 100,000 cpm/mL ^{125}I-labeled goat IgG. After soaking for 1 hour with agitation, discs were washed 3x with PBS. Binding (passive adsorption) was determined from cpm detected on the membranes. Membranes were then washed in 2 mL of 1% SBS, 2 M urea to remove protein not bound by covalent or strong non-covalent forces. Membranes were rinsed in water and counted again to measure the extent of protein retention by these membrane surfaces.

Western Transfer and Total Protein Stain

After Western blotting transfer, visualization of the protein pattern on the membrane and SDS-PAGE gels are important to assess the efficiency of electro-transfer. An example of post-transfer staining with Amido black is shown in Figure 3.4. The results show that PVDF membranes exhibit no change in dimension and do not shrink under these staining conditions, where nitrocellulose is not stable. The PVDF membranes show a high signal and low background staining with Amido black.

Figure 3.4

Amido Black Post-Transfer Staining of Western Blotting Membranes



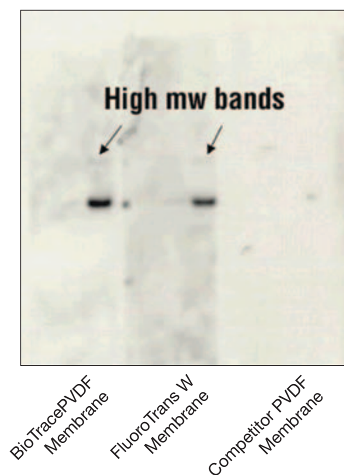
Dilutions of rabbit reticulocyte lysate were transferred to membranes and stained for 4 minutes in 0.1% Amido black, 45% methanol, 2% acetic acid. Membranes were de-stained in 90% methanol, 2% acetic acid.

High Sensitivity Immunodetection on BioTrace™ PVDF and FluoroTrans® W Membranes

Western blotted proteins on PVDF membranes can be detected by immunostaining coupled with high sensitivity detection reagents. Linking the alkaline phosphatase conjugated secondary antibody to degradation of a CDP Star* (Perkin Elmer, Boston, MA) chemiluminescent substrate yielding light output; or degradation of Attophos* (Promega Corp, Madison, WI) substrate which is detected by illumination at 488 nm excitation in the Storm* imager (GE Healthcare). Examples of chemiluminescent detection with CDP Star are shown in Figure 3.5 and chemifluorescence with Attophos in Figure 3.6. Both detection systems show strong signal to noise on BioTrace and FluoroTrans PVDF membranes.

Figure 3.5

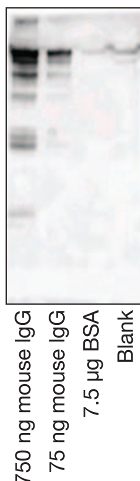
Chemiluminescent Detection on Western Blotted BioTrace and FluoroTrans PVDF Membranes



Three series of 0.01, 0.1 and 1 µg human serum albumin (HSA) per lane were transferred to different PVDF membranes. Membranes were blocked with casein solution and were detected with goat anti-HSA primary antibody and rabbit anti-goat IgG alkaline phosphatase conjugate. CDP Star chemiluminescent substrate was used. Membranes plus substrate were exposed to Amersham Hyperfilm MP for 10 minutes.*

Figure 3.6

Chemiluminescent Detection on Western Blotted FluoroTrans W PVDF Membranes



Mouse IgG and BSA control were separated by SDS-PAGE and transferred to FluoroTrans W membranes. Membranes were incubated with rabbit anti-mouse IgG samples followed by alkaline phosphatase conjugated goat anti-rabbit IgG. After washing, Attophos chemifluorescent substrate was added for three minutes before scanning in a Storm Imager (GE Healthcare) at 488 nm excitation.*

Troubleshooting for FluoroTrans® and BioTrace™ PVDF Membranes

Problem	Likely Cause(s)	Possible Remedies
Low sensitivity or absent signal	Low antibody activity or titer	Aliquot and store antibody solutions at -20 °C and avoid multiple freeze/thaw cycles. Use a higher concentration of primary antibody.
	Incomplete protein transfer (confirm by staining gel after transfer)	Increase transfer time. Decrease concentration of methanol in transfer buffer. Addition of 0.1% SDS can aid transfer of large proteins.
	Protein not binding to membrane	Assure that there are no air bubbles between the gel and membrane. Use a transfer buffer without SDS. If using BioTrace NT membrane, add methanol to the transfer buffer.
	Low conjugate activity	Store conjugates at 4 °C. Increase conjugate concentration or incubation time.
	Blocking agent interferes with antibody binding	Use a different blocking agent.
High background throughout membrane	Color development reaction too long	Stop reaction immediately when desired intensity is achieved.
	Poor quality antibody enzyme conjugate	Use affinity-purified secondary antibody.
	Incomplete blocking	Increase incubation time. Use a different blocking agent.
	Phosphatase or peroxidase activity present in blocking agent	Use a different or commercial blocking agent.
Background spots	Particulate present in buffers	Filter reagents prior to use.
Spurious bands or spots	Cross-reactivity of primary antibody	Further purify on or pre-adsorb membrane antibody. Use a monoclonal (if available). Decrease primary antibody concentration.
	Phosphatase or peroxidase activity in sample (confirm by omitting primary and secondary antibodies during detection)	Inactivate activity by heating blot at 80 °C for 20 minutes prior to blocking.

3.1 – Section 3.1.3

Ordering Information for FluoroTrans® and BioTrace™ PVDF Membranes

FluoroTrans Membrane, 0.2 µm, PVDF

Part Number	Description	Pkg
PVM020C-160	7 x 8.4 cm sheets	10/pkg
PVM020C-195	8.5 x 9 cm sheets	20/pkg
PVM020C-1015	10 x 15 cm sheets	10/pkg
PVM020C-2020	20 x 20 cm sheets	10/pkg
PVM020C-099	26 cm x 3.3 m roll	1/pkg

FluoroTrans W Membrane, 0.2 µm, PVDF

Part Number	Description	Pkg
BSP0158	7 x 9 cm sheets	10/pkg
BSP0157	10 x 15 cm sheets	10/pkg
BSP0159	20 x 20 cm sheets	10/pkg
BSP0161	26 cm x 3.3 m roll	1/pkg

BioTrace Membrane, 0.45 µm, PVDF

Part Number	Description	Pkg
66594	7 x 8.5 cm sheets	10/pkg
66542	20 x 20 cm sheets	10/pkg
66547	20 cm x 1 m roll	1/pkg
66543	30 cm x 3 m roll	1/pkg