3.1.1 Introduction

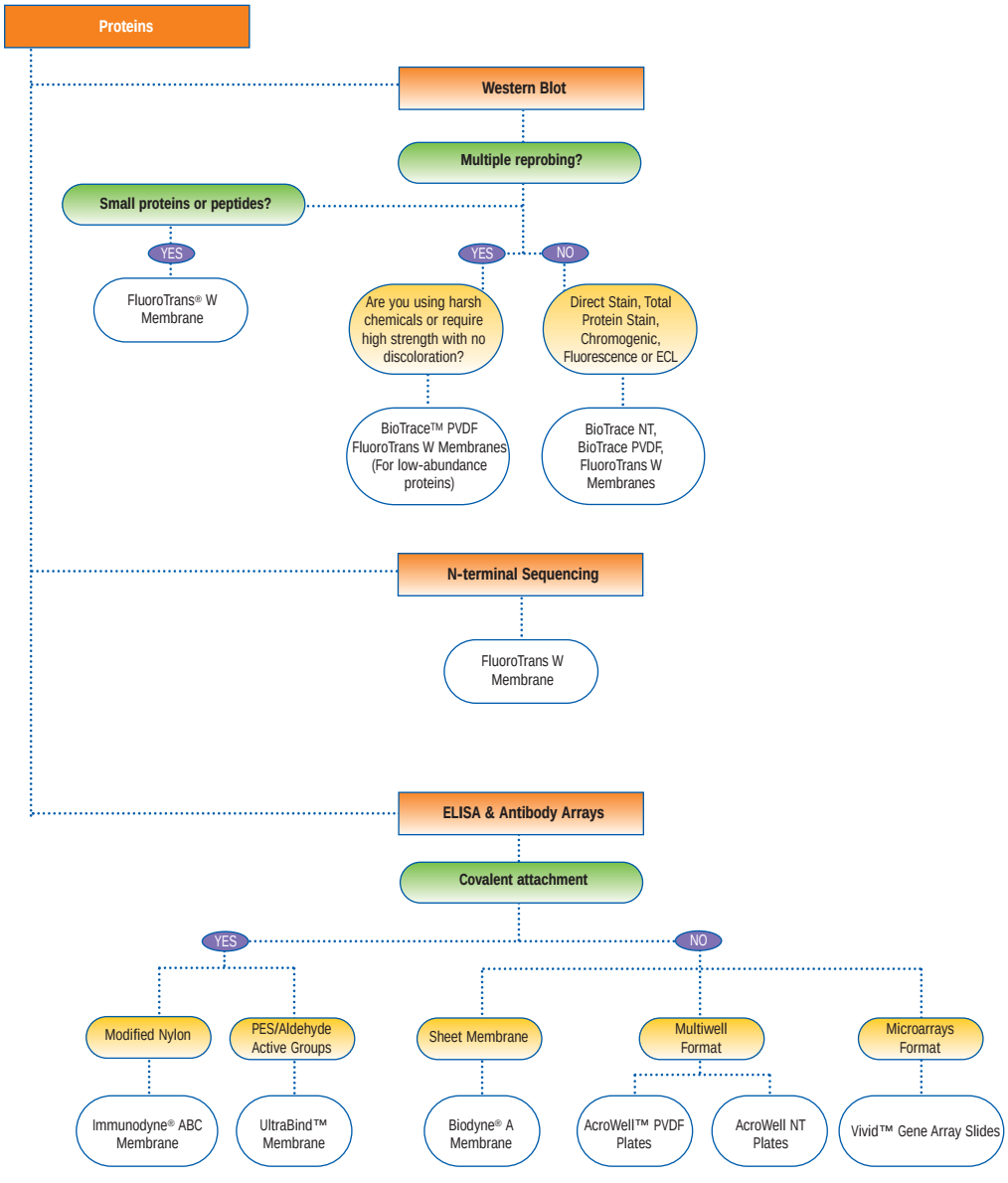
Membranes have been standard tools in molecular biology laboratories since the 1970s, when it was discovered that biomolecules could be spotted directly onto membranes (spot ELISA or DNA dot blots) or transferred from gels (Southerns, Northerns, Westerns).

The first membranes used for molecular detection were made from nitrocellulose, which has high affinity for biomolecules and low background with many detection techniques. PVDF membranes were found to have higher affinity for proteins than nitrocellulose and are now favored in Western transfers, although nitrocellulose remains in widespread use.

Pall Corporation manufactures membranes made from nitrocellulose, nylon, and PVDF for molecular detection applications.

Figure 3.1
Nitrocellulose, Nylon, and PVDF Membranes

Membrane Quick Selection Guide



Membrane Quick Selection Guide

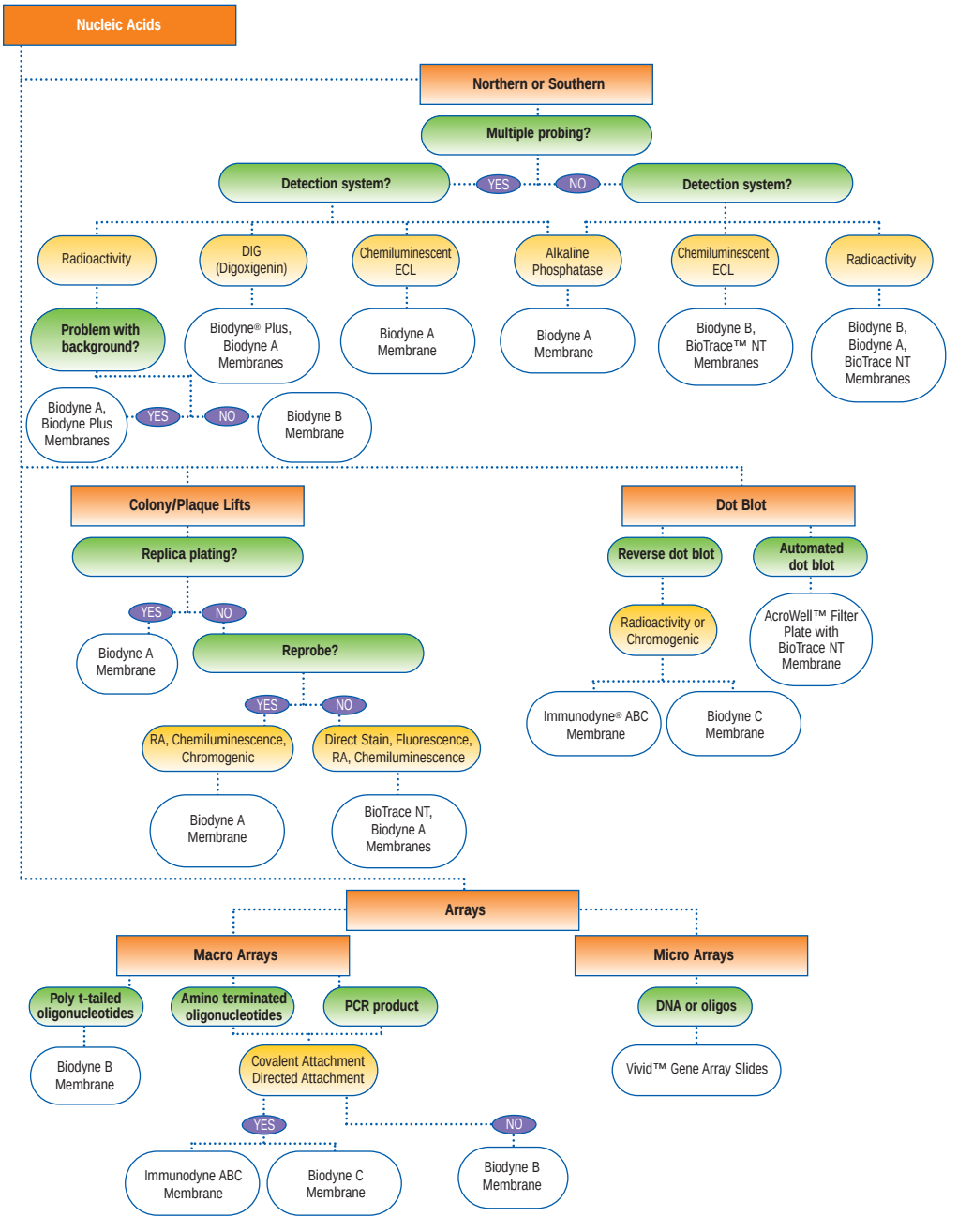


Table 3.1
Membranes and Devices for Use in Transfer and Immobilization Procedures

Product	Description	Works Best for:	Also Suited for:
Biodyne® A Membrane	Amphoteric Nylon 6,6	Colony/Plaque Lifts, DNA and RNA Transfers	Gene Probe Assays, DNA Fingerprinting, Nucleic Acid Dot/Slot Blots, Replica Plating, ELISAs
Biodyne B Membrane Biodyne Plus Membrane	Positively-charged Nylon 6,6	DNA and RNA Transfers, Multiple Reprobings, Replica Plating	DNA Fingerprinting, Nucleic Acid Dot/Slot Blots, Colony/Plaque Lifts (Biodyne B membrane)
Biodyne C Membrane	Negatively-charged Nylon 6,6	Reverse Dot Blots	Protein Immobilization, Affinity Purification, ELISAs
BioTrace™ NT Membrane	100% Pure Nitrocellulose	Colony/Plaque Lifts	Nucleic Acid and Protein Transfers, Protein Dot/Slot Blots
BioTrace PVDF Membrane	Polyvinylidene Fluoride	Protein Transfers	Nucleic Acid Transfers, Protein Dot/Slot Blots
FluoroTrans® W Membrane	Polyvinylidene Fluoride	FluoroTrans W: Western Transfers FluoroTrans: N-terminal Protein Sequencing	Southern Transfers
UltraBind™ Membrane	Modified Polyethersulfone	Solid-phase ELISAs	Affinity Chromatography, Hybridoma Screening
Immunodyne® ABC Membrane	Modified Nylon 6,6	Immuno Assays Diagnostic Applications Reverse Dot Blots	Enzyme Biosensor Applications, ELISAs
Vivid™ Gene Array Slides	Modified Nylon 6,6	Oligo and DNA Arrays	Antibody Arrays
AcroWell™ Filter Plates with BioTrace PVDF Membrane	Polyvinylidene Fluoride	Protein Detection ELISAs	Dot Blots Protein:Protein Interactions
AcroWell Filter Plates with BioTrace NT Membrane	100% Pure Nitrocellulose	Dot Blots ELISAs	Protein Dot Blots

Advantages	Binding Interaction	Method of Immobilization	Detection Methods
• High sensitivity • Low background • Net charge can be controlled by changing pH	Hydrophobic & Electrostatic Baking	UV Crosslink Chemiluminescent	• Radiolabeled Probes • Enzyme-antibody Conjugates • Chromogenic
• Positive charge over broad pH range • Highest sensitivity for nucleic acid applications (Biodyne B membrane) • No need to bake or UV crosslink	Electrostatic	Can be baked or UV crosslinked although not required	• Radiolabeled Probes • Enzyme-antibody Conjugates • Chemiluminescent • Chromogenic
• Negative charge over broad pH range • Surface carboxyl groups can be derivatized	Electrostatic	Derivatization	• Radiolabeled Probes • Enzyme-antibody Conjugates • Chromogenic
• Excellent strength • No support fabric • No detergents added • 100% pure nitrocellulose	Hydrophobic & Electrostatic	UV Crosslink Baking	• Radiolabeled Probes • Direct Stain, Fluorescence • Enzyme-antibody Conjugates • Chemiluminescent • Chromogenic
• Chemical resistance • No discoloration • Nonflammable • High strength	Hydrophobic	UV Crosslink Baking Alkaline Fixation	• Direct Stain • Enzyme-antibody Conjugates • Chemiluminescent • Chromogenic
• Strong protein binding • Sensitive detection • Very low burn-through • Good chemical compatibility	Hydrophobic	UV Crosslink Baking Alkaline Fixation	• Direct Stain • Enzyme-antibody Conjugates • Chemiluminescent • Chromogenic
• Permanent binding • No preactivation required • High protein-binding capacity	Covalent to Amine Groups	Air Drying	• Radiolabeled Probes • Enzyme-antibody Conjugates • Chromogenic
• Activated surface • No preactivation required • Intrinsically hydrophilic	Covalent to Amine and Carboxyl Groups	Air Drying	• Chemiluminescent • Chromogenic • Radiolabeled Probes • Fluorescent
• High signal:noise ratio • Low background • Microarray format • Serial barcode	Hydrophobic & Electrostatic	UV Crosslink Baking	• Fluorescent • Chemiluminescent • Chromogenic • Radiolabeled Probes
• High binding capacity • Chemical resistance • 96 well SBS-format • Choice of housing colors	Hydrophobic	Incubation and Vacuum Filtration	• Chemiluminescent • Chromogenic • Fluorescent
• High binding capacity • 96 well SBS-format • Choice of housing colors	Hydrophobic & Electrostatic	Incubation and Vacuum Filtration	• Chemiluminescent • Chromogenic • Radiolabeled Probes • Fluorescent

3.1.2 BioTrace™ NT Nitrocellulose Transfer Membrane

Techniques for Western transfer and protein detection were originally designed for nitrocellulose membranes. BioTrace NT membrane offers compatibility with protein staining and specific detection procedures that result in high background on other membrane types. BioTrace NT membrane is made from pure nitrocellulose. The membrane has a smooth surface finish and a bright, white color. BioTrace NT is a 0.2 µm pore size membrane with high adsorption capacity for proteins and nucleic acids, and is compatible with a wide range of detection protocols. Non-specific binding can be blocked with BSA, gelatin, caesin, or other surface passivating agents that are not effective on nylon or PVDF membranes. In addition, the membrane has lower "burn through" in Western blot electro-transfer than competitive nitrocellulose membranes, allowing longer transfer times and more complete transfer from the SDS-PAGE gel, thereby improving sensitivity.

A selection chart is provided in Table 3.2 to guide in choosing the appropriate product for an application between Pall nitrocellulose and polyvinylidene fluoride (PVDF) membranes.

Table 3.2
Western Blot Transfer Membrane Application Guide

FluoroTransW Product	BioTrace NT Membrane	BioTrace PVDF Membrane	FluoroTrans® PVDF Membrane	PVDF Membrane
Description	Pure Nitrocellulose	Polyvinylidene Fluoride	Polyvinylidene Fluoride	Polyvinylidene Fluoride
Application	Protein transfers Nucleic acid detection ELISA	Protein transfers Protein dot or slot blots	N terminal protein sequencing Protein dot or slot blotting	Protein transfers Protein dot or slot blots 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 3.2 (continued)
Western Blot Transfer Membrane Application Guide

FluroTransW Product	BioTrace™ NT Membrane	BioTrace PVDF Membrane	FluoroTrans® PV F Membrane	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

Protocol for BioTrace NT Nitrocellulose Transfer Membrane

Pall nitrocellulose membranes are recommended for protein transfer and detection. The procedures outlined below are intended as general guidelines. The transfer apparatus manufacturer's instructions should be followed when assembling the transfer tank. These membranes will provide excellent results with commercially available, non-radioactive detection kits using the manufacturer's published procedures.

A. Materials Required

1. BioTrace NT membrane
2. Phosphate buffered saline (PBS)
3. Non-amine containing buffers
4. Protein blocking buffers
   a. 0.5% Casein in 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.*

5. Blocking solutions may also contain 0.05% Tween 20 or other non-ionic surfactant which may enhance blocking and will also aid in re-hydrating the membrane if the spotted membrane is stored in a dry state.

B. Handling of the Membrane

1. BioTrace NT membrane should be handled carefully and with gloves. Nitrocellulose membranes can be brittle and may crack or tear, especially when dry.
2. Remove the sheet or roll of membrane from 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 in the Western blot transfer procedure.*

3. Slowly lower the membrane into 20% (v/v) methanol or ethanol and agitate briefly. The membrane will become translucent as it wets.

4. 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 BioTrace™ NT membrane: after the transfer is complete, allow the membrane to air dry at room temperature. This helps the proteins to bind more strongly to the membrane, preventing loss during subsequent washes and detection steps.

D. Ponceau S Staining Protocol for Reversible Total Protein Detection

1. Soak membranes in 0.01% (w/v) Ponceau S, 3% (w/v) Trichloroacetic acid for 1 minute.
2. Rinse several times in distilled water.
3. Photograph membrane and remove remaining stain with three changes of Tris

buffered saline (20mM Tris, 150mM NaCl, pH7).

4. Proceed to blocking step for immunodetection of specific bands.

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, dried 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
   - **d.** 0.05% Tween 20 (for BioTrace™ NT membrane only)

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 BCIP/NBT* substrate. (Dissolve 82.5 mg BCIP, 42.5 mg NBT in 1 mL dimethylformamide. Add, while stirring, 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.

d. Gently agitate at room temperature and observe the reaction as color develops.

e. Rinse the membrane twice with distilled water.

2. Detection with Horseradish Peroxidase and 3,3-Diaminobenzidine HCl (DAB)

CA *nd should be handled with appropriate safeguards.*
Car *OS 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 0.05 mL of 30% H_2O_2 to 100 mL of DAB substrate (0.05% DAB in reaction buffer, prepare 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)

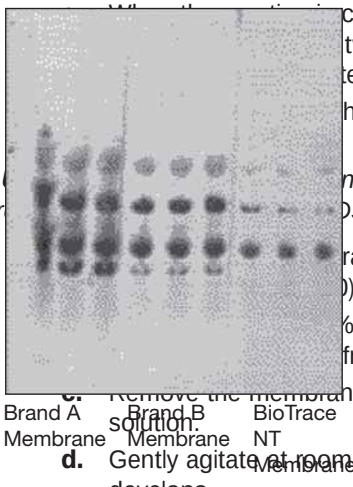
a. Equilibrate the membrane in reaction buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 5 minutes at room temperature.

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.



Pre-stained proteins were separated on an SDS-PAGE gel, electroblotted onto the indicated nitrocellulose membranes. A double layer of membrane was used, one directly against the gel, and followed by the second layer. Signal intensity on the second layer is indicative of "burn through," which can lead to loss of signal.

Application Data for BioTrace™ NT Nitrocellulose Transfer Membrane

An example of a Western blot electro-transfer experiment is shown in Figure 3.2. The resulting pre-stained proteins visible on the back up second membrane layer clearly showed reduced "burn through" with the BioTrace NT membrane.

Figure 3.2
Less "Burn-Through" with BioTrace NT Nitrocellulose Membranes in Western Blotting Electro-Transfer

Troubleshooting for BioTrace™ NT Nitrocellulose Transfer Membrane

Problem	Likely Cause(s)	Possible Remedies
Low sensitivity or absent signal	Low antibody activity or titer	Aliquot and store an ɔody solutions at -20 C and avoid multiple freeze/tl ıw cycles Use a higher concer ation 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 membrane antibody	Cross-reactivity of primary antibody	Further purify on or pre-adsorb 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

3.1.3 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.

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.

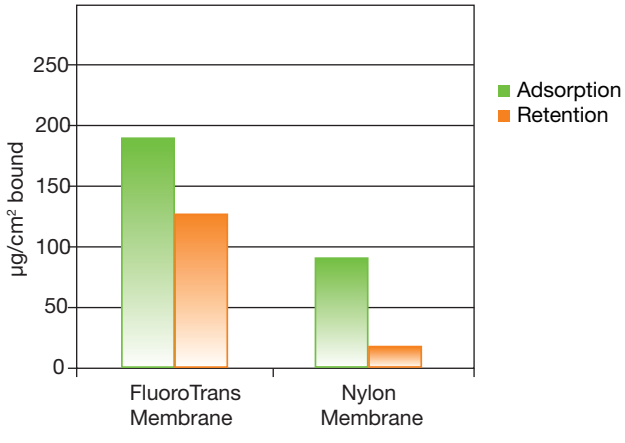
Application Data for FluoroTrans® and BioTrace™ PVDF Membranes

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



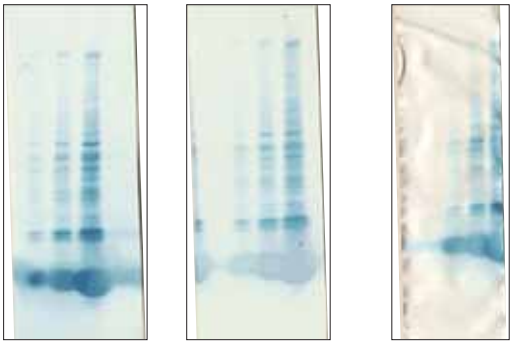
13 mm discs of FluoroTrans membrane and Biodyne® A nylon membrane were soaked in 2 mL per disc of phosphate buffered saline (PBS) containing 200 µg/mL goat IgG and 100,000 cpm/mL ^{125}I-labeled goat IgG. After soaking for 1 hour with agitation, discs were washed 3x with PBS. Binding (passive adsorption) 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



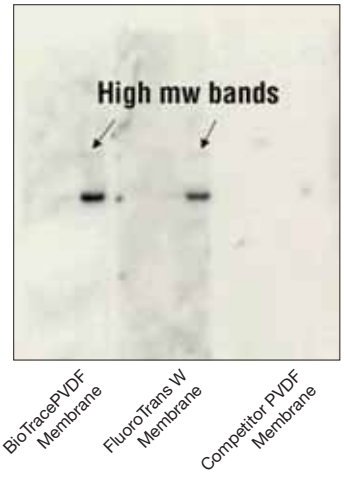
Dilutions of rabbit reticulocyte lysate were transferred to membranes and stained for 4 minutes in 0.1% Amido black, 45% methanol, 2% acetic acid. Membranes were de-stained in 90% methanol, 2% acetic acid.

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.

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

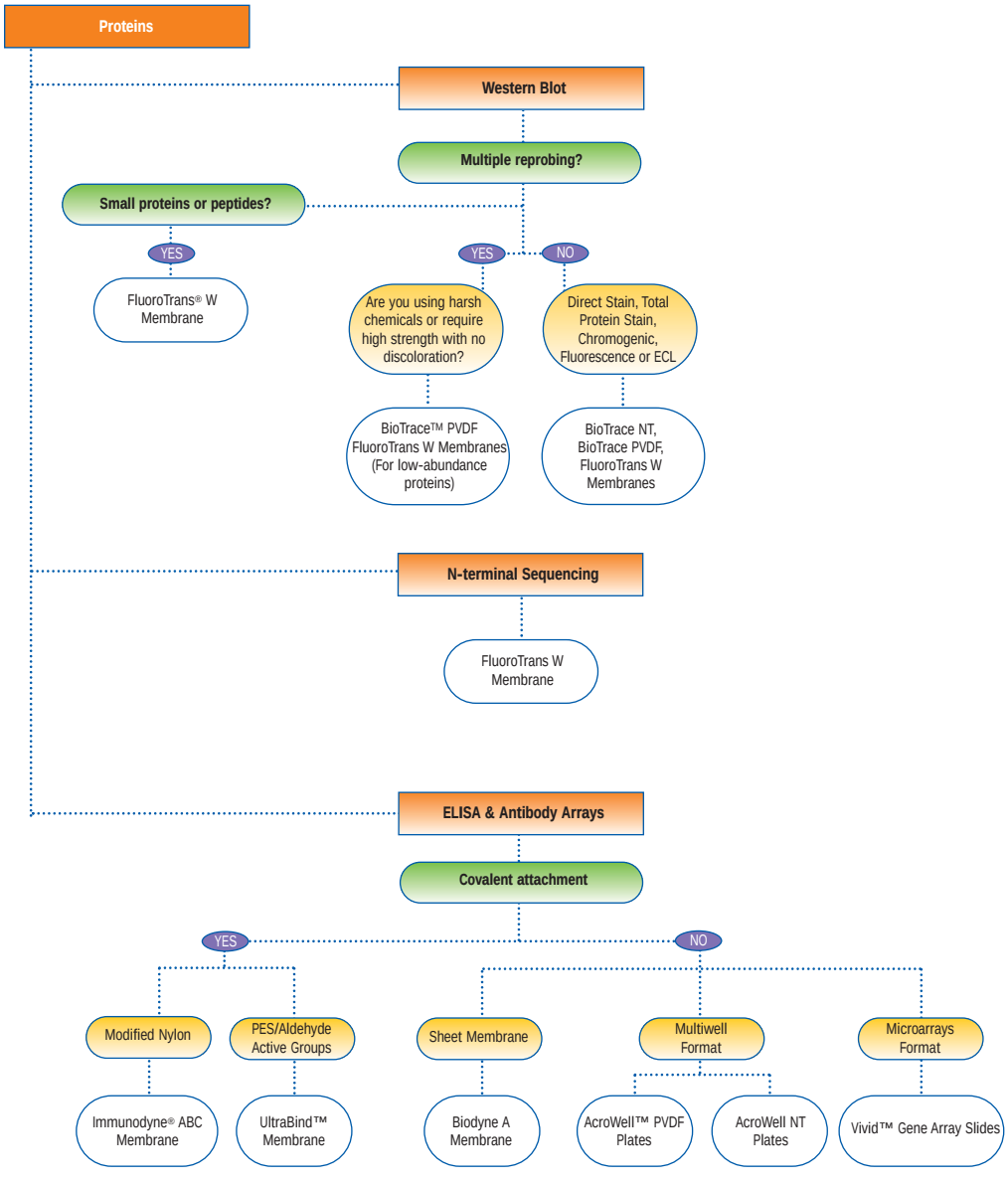
Part Number	Description	Pkg
66594	7 x 8.5 cm sheets	10/pkg
66542	20 x 20 cm sheets	10/pkg
66547	20 cm x 1 m roll	1/pkg
66543	30 cm x 3 m roll	1/pkg

3.2.1 Introduction

Membranes have been standard tools in molecular biology laboratories since the 1970s, when it was discovered that biomolecules could be spotted directly onto membranes (spot ELISA or DNA dot blots) or transferred from gels (Southerns, Northerns, Westerns).

Pall Corporation manufactures membranes made from nitrocellulose, nylon, and PVDF for molecular detection applications. Pall offers several activated membranes for covalent protein binding. Ultrabind modified polyethersulfone membrane has a high ratio of covalent to non-covalent protein binding. Immunodyne® ABC membrane offers the sharp spot geometry of nylon with a proprietary covalent attachment chemistry.

Membrane Quick Selection Guide



Membrane Quick Selection Guide

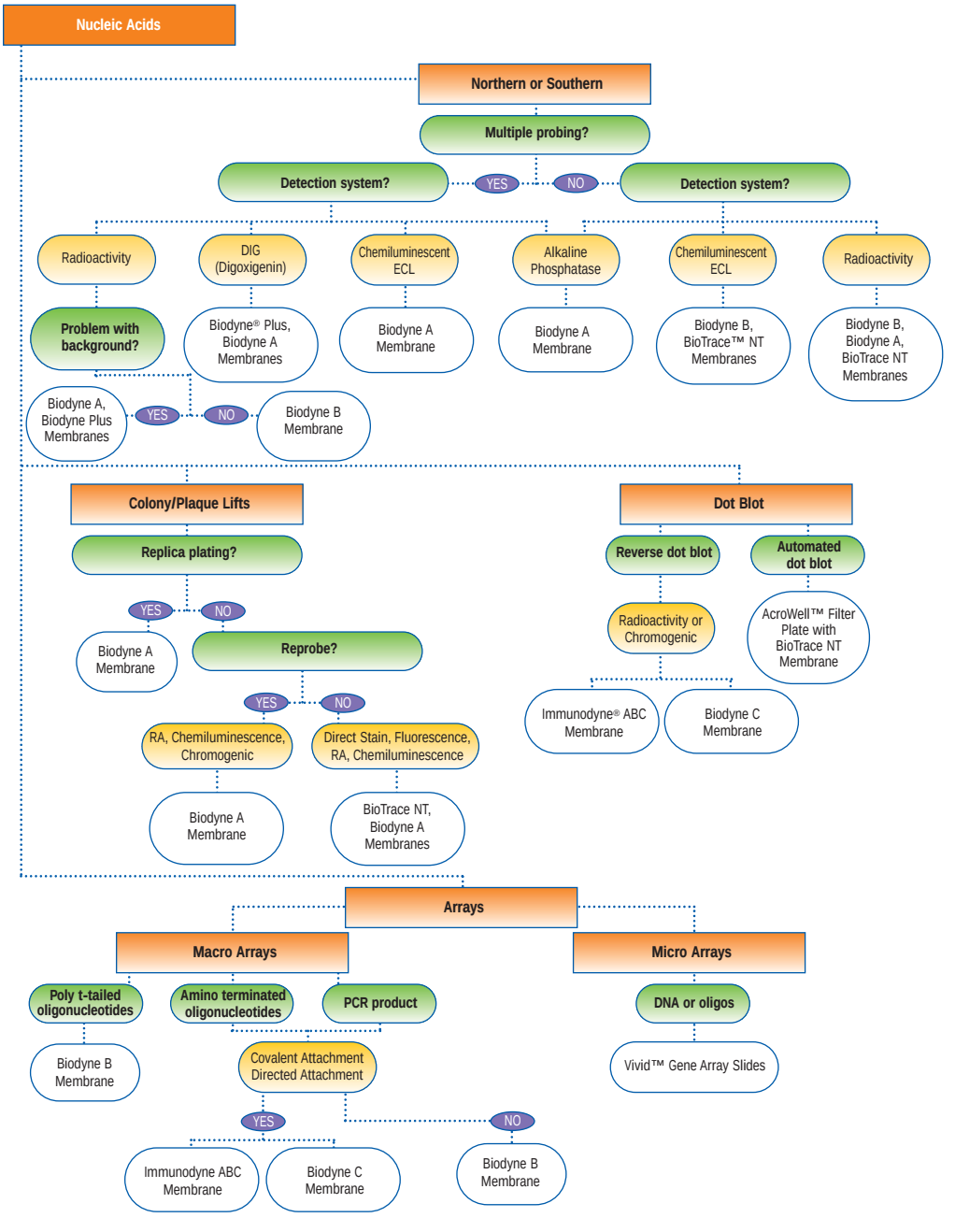


Table 3.4
Membranes and Devices for Use in Transfer and Immobilization Procedures

Product	Description	Works best for:	Also suited for:
Biodyne® A Membrane	Amphoteric Nylon 6,6	Colony/Plaque Lifts, DNA and RNA Transfers	Gene Probe Assays, DNA Fingerprinting, Nucleic Acid Dot/Slot Blots, Replica Plating, ELISAs
Biodyne B Membrane Biodyne Plus Membrane	Positively-charged Nylon 6,6	DNA and RNA Transfers, Multiple Reprobings Replica Plating	DNA Fingerprinting, Nucleic Acid Dot/Slot Blots, Colony/Plaque Lifts (Biodyne B membrane)
Biodyne C Membrane	Negatively-charged Nylon 6,6	Reverse Dot Blots	Protein Immobilization, Affinity Purification, ELISAs
BioTrace™ NT Membrane	100% Pure Nitrocellulose	Colony/Plaque Lifts	Nucleic Acid and Protein Transfers, Protein Dot/Slot Blots
BioTrace PVDF Membrane	Polyvinylidene Fluoride	Protein Transfers	Nucleic Acid Transfers, Protein Dot/Slot Blots
FluoroTrans W Membrane	Polyvinylidene Fluoride	FluoroTrans W: Western Transfers FluoroTrans: N-terminal Protein Sequencing	Southern Transfers
UltraBind™ Membrane	Modified Polyethersulfone	Solid-phase ELISAs	Affinity Chromatography, Hybridoma Screening
Immunodyne® ABC Membrane	Modified Nylon 6,6	Immuno Assays Diagnostic Applications Reverse Dot Blots	Enzyme Biosensor Applications, ELISAs
Vivid™ Gene Array Slides	Modified Nylon 6,6	Oligo and DNA Arrays	Antibody Arrays
AcroWell™ Filter Plates with BioTrace PVDF Membrane	Polyvinylidene Fluoride	Protein Detection ELISAs	Dot Blots Protein:Protein Interactions
AcroWell Filter Plates with BioTrace NT Membrane	100% Pure Nitrocellulose	Dot Blots ELISAs	Protein Dot Blots

Advantages	Binding Interaction	Method of Immobilization	Detection Methods
• High sensitivity • Low background • Net charge can be controlled by changing pH	Hydrophobic & Electrostatic Baking	UV Crosslink	• Radiolabeled Probes • Enzyme-antibody Conjugates • Chemiluminescent • Chromogenic
• Positive charge over broad pH range • Highest sensitivity for nucleic acid applications (Biodyne B membrane) • No need to bake or UV crosslink	Electrostatic	Can be baked or UV crosslinked, although not required	• Radiolabeled Probes • Enzyme-antibody Conjugates • Chemiluminescent • Chromogenic
• Negative charge over broad pH range • Surface carboxyl groups can be derivatized	Electrostatic	Derivatization	• Radiolabeled Probes • Enzyme-antibody Conjugates • Chromogenic
• Excellent strength • No support fabric • No detergents added • 100% pure nitrocellulose	Hydrophobic & Electrostatic	UV Crosslink Baking	• Radiolabeled Probes • Direct Stain, Fluorescence • Enzyme-antibody Conjugates • Chemiluminescent • Chromogenic
• Chemical resistance • No discoloration • Nonflammable • High strength	Hydrophobic	UV Crosslink Baking Alkaline Fixation	• Direct Stain • Enzyme-antibody Conjugates • Chemiluminescent • Chromogenic
• Strong protein binding • Sensitive detection • Very low burn-through • Good chemical compatibility	Hydrophobic	UV Crosslink Baking Alkaline Fixation	• Direct Stain • Enzyme-antibody Conjugates • Chemiluminescent • Chromogenic
• Permanent binding • No preactivation required • High protein-binding capacity	Covalent to Amine Groups	Air Drying	• Radiolabeled Probes • Enzyme-antibody Conjugates • Chromogenic
• Activated surface • No preactivation required • Intrinsically hydrophilic	Covalent to Amine and Carboxyl Groups	Air Drying	• Chemiluminescent • Chromogenic • Radiolabeled Probes • Fluorescent
• High signal:noise ratio • Low background • Microarray format • Serial barcode	Hydrophobic & Electrostatic	UV Crosslink Baking	• Fluorescent • Chemiluminescent • Chromogenic • Radiolabeled Probes
• High binding capacity • Chemical resistance • 96 well SBS-format • Choice of housing colors	Hydrophobic	Incubation and Vacuum Filtration	• Chemiluminescent • Chromogenic • Fluorescent
• High binding capacity • 96 well SBS-format • Choice of housing colors	Hydrophobic & Electrostatic	Incubation and Vacuum Filtration	• Chemiluminescent • Chromogenic • Radiolabeled Probes • Fluorescent

3.2.2 UltraBind™ Affinity Membrane

UltraBind is a modified polyethersulfone (PES) membrane that offers rapid covalent protein immobilization via an aldehyde chemistry. Proteins can be efficiently attached to this surface without prior chemical derivatization. The membrane has a pore size of 0.45 µm and a thickness of 152 µm typical and can immobilize immunoglobulin to a density of 135 µg/cm^2.

Protocol for UltraBind Affinity Membrane

A. Materials Required

1. UltraBind affinity membrane
2. Phosphate buffered saline (PBS)
3. Non-amine containing buffers (e.g., sodium carbonate)
4. Protein blocking buffers
   - **a.** 0.5% Casein in 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.*

5. 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.

B. Immobilization by Spot Wetting on a Dry Membrane

1. Prepare protein solution to be immobilized in PBS to a concentration of 1 mg/mL or higher. Filter with a Nanosep® 0.45 µm GHP membrane centrifugal device (volumes < 1.0 mL) or an Acrodisc® MF syringe filter with a 0.45 µm GHP membrane.

Tip: *The loading of protein onto an affinity activated surface is influenced by the concentration in the applied sample. High initial protein concentrations expose the activated surface to available primary amine groups and can lead to rapid high protein immobilization. The concentration of protein ligand should be optimized in preliminary experiments, where ligand loading and the functionality of the immobilized protein should be measured.*

2. Remove membrane from its protective storage pouch and place on a clean, dry surface that is non-adsorbent, such as a clean glass plate.

Tip: *Some activated membranes can be water- and light-sensitive depending on the activation chemistry. The aldehyde chemistry on UltraBind membrane is quite stable and the least affected by moisture.*

3. Sample (1-5 µL) is then taken up into a micro-volume dispensing pipette and a volume of 1-2 µL is dispensed as a pendant drop and brought into contact with the dry membrane. The liquid will rapidly spread and the spot diameter can be controlled by changing the volume and delivery rate. Avoid dispensing volumes > 2 µL as this will produce a very large and irregular spot. If larger volumes need to be spotted due to low ligand concentration, the following should be considered:
   a. Further concentrate the sample with a Nanosep® device with an Omega™ 10K ultrafiltration membrane.
   b. Spot multiple small volumes < 2 µL allowing each drop to dry in between applications.
4. Allow the drops to dry and react with the UltraBind™ chemistry by incubation at RT or 37 ºC for 30-60 minutes.
5. Cap the unreacted sites on the membrane by soaking in protein blocking buffer at RT or 37 ºC for 30-60 minutes.
6. Thoroughly wash the membrane in 2-3 changes of PBS to remove any unreacted sample and excess blocking buffer.
7. Store wet at 4 ºC in a suitable moisture retaining container for 1-2 days. If the membranes need to be stored longer, the following options should be considered:
   a. Add a bacteriostatic agent, such as 0.02% sodium azide or 0.01% thimersol to the storage buffer
   b. Consider drying the membrane in the presence of a wetting agent to aid in re-wetting after storage. Suitable agents are 0.02% (v/v) Tween-20 in PBS or 0.1% (w/v) trehalose in PBS.

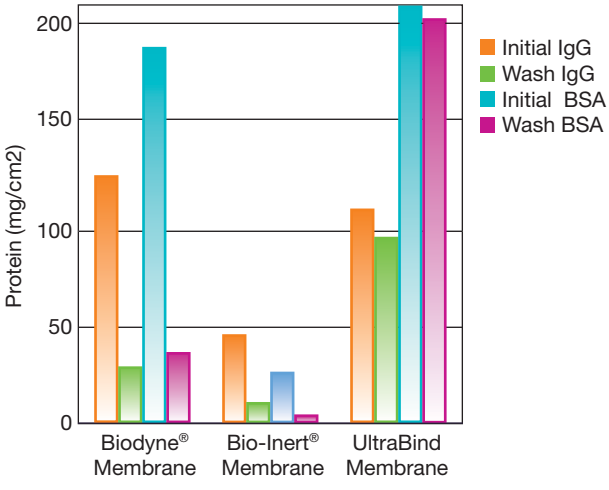
Application Data for UltraBind Affinity Membrane

Protein Binding Data

UltraBind activated affinity membrane can rapidly bind protein to yield a high covalent load. An example of a study to demonstrate immobilization of IgG and BSA is summarized in Figure 3.7. Biodyne® B membrane (charged nylon transfer membrane) and Bio-Inert® membrane (modified Nylon 6,6 membrane) were used as high and low binding capacity controls respectively. UltraBind membrane efficiently bound protein and retained it after the SDS/urea wash.

Figure 3.7

Protein Binding to UltraBind™ Aldehyde Activated Affinity Membrane



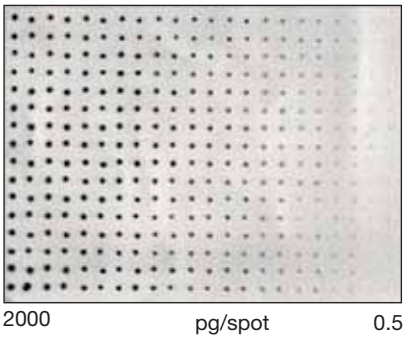
Membrane discs (13 mm) were soaked in a protein solution and washed to determine the capacity and strength of protein binding. Discs were soaked in radioactively labeled IgG and BSA (200 µg unlabeled protein with 100,000 cpm of ^{125}I-labeled tracer) for 60 minutes with agitation, rinsed, and either read in a scintillation counter or stripped using a 1% SDS/2 M urea wash.

Solid Phase Elisa for Human Serum Albumin (HSA) on Ultrabind Affinity Membrane

One key application of activated affinity membranes is high sensitivity solid phase ELISA detection of analytes in samples. An example of such an assay format with a spotted analyte dilution curve is shown in Figure 3.8. Signal was generated with a specific anti-HSA antibody followed by reaction with a secondary antibody detection reagent and BCIP/NBT substrate allowing detection of as little as 0.5 pg HSA spotted onto the UltraBind membrane substrate.

Figure 3.8

Antigen Detection (Dot Blot ELISA) with UltraBind Affinity Membrane



Dilutions of HSA ranging 2000 to 0.5 pg/spot were applied to an UltraBind membrane using a 96-pin transfer tool on a Matrix PlateMate Liquid Handling Station. The membrane was then blocked with 0.5% Hammersten-grade casein in PBS. HSA was detected with rabbit anti-HSA antibody followed with alkaline phosphatase conjugated goat anti-rabbit IgG.

Ordering Information for UltraBind™ Affinity Membrane

UltraBind Affinity Membrane

Part Number	Description	Pkg
66544	20 x 20 cm sheets	10/pk
66545	30 cm x 3 m roll	1/pkg

3.2.3 ELISA Assays Using Immunodyne® ABC Membranes

ELISA (enzyme-linked immunosorbent assay) is one of the most widely used quantitative tools for sensitive and reproducible analyte assays. These assays are rapid, simple to perform, and easily automated. The technique combines the high specificity of an antibody-antigen interaction with an enzyme-linked signal detection system. This powerful combination provides a unique quantitative assay format that can be readily adapted to Immunodydne ABC membranes. Success in ELISA assays can be influenced by the substrate used for the immobilization of the capture antibody employed in the common "sandwich" assay format.[1,2]

This protocol describes the detection of protein antigens using a "sandwich" ELISA technique in which an antigen is sandwiched between two different antibodies. The principle by which this ELISA technique operates is as follows:

- Immobilization of capture antibody on a suitable substrate
- Binding of antigen to immobilized antibody
- Binding of second antibody, linked to an enzyme, to bound antigen (formation of immune complex)
- Detection of immune complex using appropriate enzyme substrate

The protocol example described below uses a Goat Anti-Rabbit IgG (GAR) as a "capture" antibody, Rabbit Anti-Mouse IgG (RAM) as antigen, and Goat Anti-Rabbit Horseradish Peroxidase conjugate (GAR-POD) or Goat Anti-Rabbit Alkaline Phosphatase conjugate (GAR-AP) in an immune "sandwich" assay. Concentrations of immune reagents listed in these methods are specific for this example of a membrane-based ELISA. Immune reagents from other suppliers can be substituted, but will require some optimization of antibody concentrations, blocking conditions, immune incubation times, wash conditions, and choice of enzyme/substrate combination to obtain the desired result. In many cases alternative suppliers will provide guidance on using their reagents with substrates like Immunodyne ABC membrane.

Protocol for ELISA Assays Using Immunodyne ABC Membranes

A. Materials Required

1. Immunodyne ABC membrane (PN NBCH13R, 0.45 µm or PN NNCH13R, 1.2 µm)

Tip: *The two pore size membranes will exhibit different liquid flow rates, assuming the membrane thickness and sample viscosity are the same. This offers some degree of control over the exposure times of reagents with samples which can influence the kinetics of analyte capture or development of the ELISA assay signal. Residence time can be increased by using a membrane having a lower pore size rating. Assay sensitivity and background can also be impacted by membrane pore size. It is recommended to start with the 0.45 µm pore size membrane and consider the more open membrane if a shorter residence time is required.*

2. Phosphate buffered saline (PBS)

3. Non-amine containing buffers, such as sodium carbonate or acetate

4. Protein blocking buffers
   a. 0.5% Casein in 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.*

5. Blocking solutions may also contain 0.05% Tween 20 or other non-ionic surfactant, which may enhance blocking and will also aid in re-hydrating the membrane if the spotted membrane is stored in a dry state.
6. Wash solution: 0.1% Triton* X-100 (w/v) in PBS
7. Goat Anti-Rabbit IgG (e.g., Sigma Chemicals PN R-3128)
8. Rabbit Anti-Mouse IgG (e.g., Sigma Chemicals PN M-9637)
9. Goat Anti-Rabbit IgG conjugated to alkaline phosphatase (e.g., Sigma Chemicals PN A-7650)
10. Nitro blue tetrazolium (NBT) (e.g., Sigma Chemicals PN N-6876) 75 mg/mL in 70% (v/v) dimethyl formamide
11. 5-Bromo-4-chloro-3-indolyl Phosphate (BCIP) (e.g., Sigma Chemicals PN B-8503) 50 mg/mL in di-methyl formamide buffer: 0.1 M
12. Buffer 25 mM Tris-HCL (pH 7.5), 0.1 M NaCl, 50 mM $MgCl_2$
13. Working substrate solution is prepared as follows: 0.033 mL NBT, 0.025 mL BCIP in 7.5 mL above buffer buffer. For optimal results, use 0.25 mL substrate/cm^2 membrane. Solution should be prepared fresh.

Tip: *The above set of reagents have been found to give good ELISA assay performance. Other vendor reagents are available but will require some optimization to obtain the desired ELISA assay performance.*

B. Handling of the Membrane

1. Remove the roll of membrane from package. Cut to desired dimensions. (In this assay 1 cm wide strips are used.) The unused portion of the roll should be replaced in the mylar bag containing the supplied desiccant. Remove excess air from the bag prior to sealing (e.g. by tape or heat seal). Storage at room temperature or placement in vacuum desiccator is recommended.

Tip: *It is important to number the test strips with a pencil to ensure they can be identified during testing. Numbers should be applied to area not covered by spot.*

2. Dilute capture antibody in PBS. Buffers containing amino groups such as Tris should not be used for immobilization on Immunodyne® ABC membranes.

Tip: *Place the membrane strips on a non-absorbent surface.*

3. Spot load 1 µL spots containing 100 pg, 1 ng, 10 ng, 100 ng, and 1 µg of capture antibody onto squares of the membrane strip.
4. Air-dry the membrane for 5 minutes.

Tip: *Two to five minutes are sufficient for complete binding. Longer times may be used. In certain systems, highest sensitivities have been achieved when the membrane was blocked immediately after spot loading.*

C. Immunodetection

1. Each of the following steps is performed in a 100 mm petri dish containing 10 mL of solution to ensure adequate interaction between membrane strips and reagents. Plastic bags, boxes, or tubes on a roller mixer may also be used to perform incubation steps. Up to 5 membrane strips, 1 x 5 cm each, may be incubated in one petri dish. Make sure all strips are completely immersed beneath the surface of the liquid. Gently agitate samples during all incubation steps on an orbital shaker.
2. Place the membrane blots in a petri dish with 0.5% casein/PBS blocking solution.
3. Place the petri dish 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.*

4. Place the membrane blots into two clean petri dishes with RAM antigen diluted in PBS to 1 µg/mL. Use 10 mL of diluted antigen per petri dish. Gently agitate the membrane blots on an orbital shaker for 30 minutes.
5. Replace antigen solution in petri dishes with 10 mL wash solution per dish. Wash membrane blots in 2x changes of wash solution, 5 minutes per wash. Gently agitate membrane blots on orbital shaker during wash steps.
6. Replace wash solution in petri dishes with 10 mL of PBS. Gently agitate membrane strips on orbital shaker for 1 minute.
7. Replace the PBS solution in the petri dishes with 10 mL of Goat Anti-Rabbit IgG-alkaline phosphatase (GAR-AP) diluted 1/1000 in blocking solution.
8. Repeat wash procedure listed above.
9. Replace wash solution in petri dishes 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.)

D. Detection Methods

1. Prepare substrate solution immediately prior to use.
2. After performing previous steps, replace water in petri dishes with 7.5 mL per dish of substrate solution. Gently agitate membrane blots on orbital shaker for 5 minutes.
3. The reaction may be stopped by removing color development solution and adding high purity water.
4. Color intensity of the spots may be measured with a reflectometer. If a permanent record is required, the blots should be photographed or scanned shortly after development. Color intensity may fade with time.
5. As an alternative, peroxidase conjugates may also be used with appropriate substrates, i.e. 4CN, DAB or TMB. These may result in different sensitivities and would therefore require re-optimization.

Ordering Information for ELISA Assays Using Immunodyne® ABC Membranes

Immunodyne ABC Membrane

Part Number	Description	Pkg
NBCH13R	0.45 µm, 30 cm x 3 m roll	1/pkg
NNCH13R	1.2 µm, 30 cm x 3 m roll	1/pkg

References for ELISA Assays Using Immunodyne ABC Membranes

1. Goldsby, R.A., Kindt, T.J., Osborne, B.A., & Kuby, J. (2003). Enzyme-linked immunosorbent assay. *In Immunology*, 5th ed. (1926), 148–150.
2. Wikipedia, the free on-line encyclopedia (see http://en.wikipedia.org/wiki/ELISA).

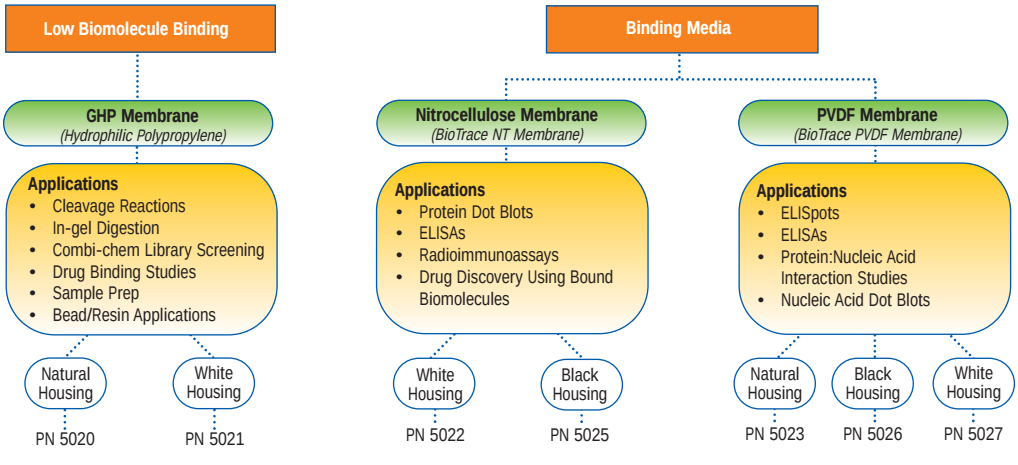
3.2.4 ELISA and Immunoassay Using AcroWell™ 96 Multi-Well Filter Plates with BioTrace™ PVDF and NT Membranes

The AcroWell 96 filter plates have been optimized for retentate and hybridization-based binding applications. The plates consist of a chemically resistant/biologically inert polypropylene filter plate assembly with two membrane layers sealed to the bottom of the plate using a patented sealing process that minimizes crosstalk. The bottom membrane support layer is hydrophobic PTFE that protects the upstream functional membrane. The PTFE acts as a barrier to passive flow, allowing lengthy or high temperature incubations with the wells filled with hybridization and immunodetection solutions. Small holes in the center of each well allow the fluid to pass under applied vacuum or centrifugation. Minimal hold-up allows for greater detection accuracy. A serialized barcode label allows for the use of automated tracking systems and identifies the membrane type. AcroWell 96 filter plates are available in clear, white, and black housing colors, which facilitates greater detection accuracy. Choose clear plates for time-resolved fluorescence, white plates for chemiluminescence/radioactivity, and black plates for fluorescein detection assays.

Tip: *The AcroWell 96 filter plates do not incorporate filtrate flow directors and should not be used when the filtrate needs to be collected for downstream analysis. For applications that require recovery of the filtrate, see our AcroPrep™ line of filter plates.*

A plate selection guide is shown in Figure 3.9 for the range of membranes available in this format. A summary of properties of the AcroWell 96 filter plates is presented in Table 3.5.

Figure 3.9
AcroWell Filter Plate Selection Guide



Detection results can be further optimized with proper selection of plate color. Use the following recommendations as a guideline for easy selection.

- *Natural (semi-opaque) – fluorescence, time-resolved fluorescence*
- *White (opaque) – radioactivity, chemiluminescence*
- *Black (opaque) – lowest background for fluorescence, time-resolved fluorescence*

Table 3.5
Properties of the AcroWell™ 96 Multi-Well Filter Plates

Specification	Parameter
Materials of Construction	
Membrane	GHP (hydrophilic polypropylene), BioTrace™ PVDF (hydrophobic), BioTrace Nitrocellulose
Media Support	Emflon membrane (PTFE) backed with non-woven polypropylene
Device	Polypropylene (lid polystyrene)
Effective Membrane Area	0.30 cm^2
Dimensions	
Length	12.78 cm (5.03 in.)
Width	8.51 cm (3.35 in.)
Height (without Lid)	1.44 cm (0.656 in.)
Height (with Lid)	1.70 cm (0.7 in.)
Tip Length	0.53 cm (0.21 in.)
Capacities	
Maximum Well Volume	300 µL
Recommended Volume	250 µL
Hold-up Volume (Membrane/Support)	< 10 µL
Maximum Centrifugal Force	3,000 x g
Centrifuge	Swinging bucket rotors
Operating Vacuum	25.4 cm Hg (10 in. Hg)
Typical Sample Processing Times for 0.25 mL Volume at 25.4 cm Hg (10 in. Hg)	GHP Membrane: 40 seconds BioTrace PVDF Membrane: 10 seconds BioTrace NT Membrane: 50 seconds
Fluorescence Detection (GHP Plates)*	Cy5 fluor < 25,000 (natural) < 40,000 (white) Fluorescein < 2,000 (natural) < 40,000 (white) Time-resolved fluorescence (DELFIA) < 2,000 < 3,000
Light Emitting Detection (GHP Plates)**	Signal to Noise 5:1 (natural) Signal to Noise 50:1 (white)

**Fluorescence was detected using the VICTOR* multi-label counter (PerkinElmer, Wallac) using standard filters and settings for each AcroWell 96 filter plate with GHP membrane.*

***Luminometry, 20 pg of an alkaline phosphatase-labeled antibody was placed in the AcroWell 96 filter plate with GHP membrane and assayed using LumiGLO* reagent. Adjacent wells were counted for light crosstalk and background.*

Protocol for ELISA and Immunoassay Using AcroWell™ 96 Multi-Well Filter Plates with BioTrace™ PVDF and NT Membranes

A. Materials Required

1. AcroWell 96 filter plates with BioTrace PVDF membrane and BioTrace NT membrane. For specifications, see Table 3.5.
2. High purity water or buffer, such as phosphate buffered saline (PBS)
3. Source of vacuum [25.4 cm Hg (10 in. Hg), (Pall vacuum manifold, PN 5017)]
4. Liquid handling source, such as the MultiPROBE* II HT EX workstation with Gripper* Integration Platform and Fusion* Universal Analyzer. The vacuum filtration option was also included.

B. Vacuum Manifold Filtration

1. During use, hold the filter plate so the membrane on the bottom is not touched or scratched. (Avoid contact between the membrane and other surfaces during incubation to prevent liquid flow due to contact wicking).
2. The AcroWell 96 filter plate with BioTrace PVDF membrane requires pre-wetting with 100 µL pure methanol, isopropanol, or 70% ethanol.

Tip: *Do not let the membrane dry or pre-wetting will need to be repeated. After wetting, apply vacuum and rinse with 100 µL water or buffer.*

3. Place the filter plate on the vacuum manifold. Rinse with 100 µL water or buffer.
4. Apply vacuum to manifold to initiate liquid flow. Recommended vacuum is 25.4 cm Hg (10 in. Hg).

Tip: *DO NOT exceed 38.1 cm Hg (15 in. Hg).*

5. Add the sample to be filtered to the plate. Incubate if required.
6. Apply vacuum.
7. Gently tap the plate to remove any hanging droplets.
8. Release vacuum from the manifold.

Tip: *Do not release the vacuum by pulling the corner of the plate. The manifold gasket will degrade.*

9. If needed, add wash solutions and filter.

C. Centrifugation in a Swinging Bucket Rotor

1. Place the AcroWell 96 filter plate on top of a receiver plate.
2. Insert the plates into a standard multi-well plate swinging bucket rotor assembly.
3. Place a duplicate pair of plates matching the weight of the test plate (add water to the receiver plate and match weight of the test plate).

Tip: *An imbalance can result with a single test plate if no counter balancing plate is used. If different volumes of sample are used in multiple plates, they will need to be balanced in pairs by addition of water to empty wells.*

4. Centrifuge at 500-3,000 x g for 1-2 minutes.

Tip: *The centrifugal force and time parameters can be varied to optimize the filtration rate of fluids in contact in the well of the plate.*

5. If needed, add wash solution(s) and repeat. Ensure that plates are in balance before centrifugation.

D. Immunodetection Protocol

1. Add the sample containing proteins to be bound to the AcroWell™ 96 filter plate with BioTrace™ NT membrane (PN 5022, 5025) or the pre-wetted AcroWell 96 filter plate with BioTrace PVDF membrane (PN 5023, 5026, 5027).
2. Incubate for 5-30 minutes and filter as directed.
3. A 5-10 dilution of a standard casein blocking solution has been shown to completely prevent further binding of biomolecules while avoiding filter fouling. Blocking may have to be individually optimized depending on which blocking solution is used.
4. To avoid sample loss or dehydration during high-temperature incubations, it is recommended that the AcroWell 96 filter plate be placed on a receiver plate and sealed into a bag containing a moist paper towel. Long incubations at high temperature may result in a small amount of weeping, usually less than 5% of the volume (20-40 µL).
5. Add wash solutions and filter as needed. Repeat 2-4 times.
6. Add detection solutions and place the AcroWell 96 filter plate directly into the detector.

Application Data for ELISA and Immunoassay Using AcroWell 96 Multi-Well Filter Plates with BioTrace PVDF and NT Membranes

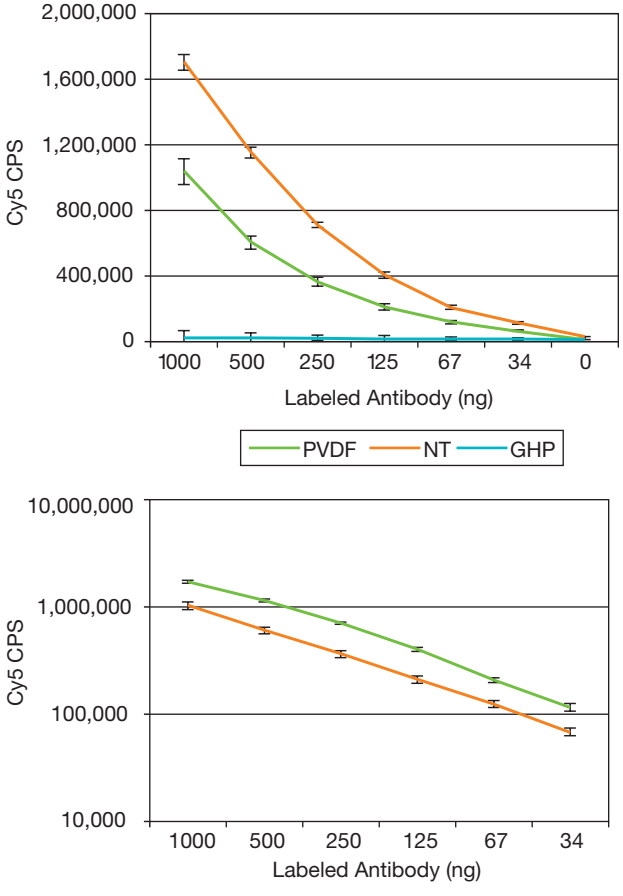
In order to achieve the highest sensitivities, radioactive ligands have been widely used for receptor binding assays. In a comparison, an Eu3+ labeled protein ligand was measured by time-resolved fluorescence (DELFIA) in an assay carried out in an AcroWell GHP membrane filter plate.

Protein Binding to BioTrace™ NT and BioTrace PVDF Membranes Effectively Bind Large Amounts of Protein and DNA

Experiments with these membranes in the AcroWell™ 96 filter plate configuration indicate that the PVDF and NT plates easily bind 1,000 ng/well of DNA or protein (see Figure 3.10).

Figure 3.10

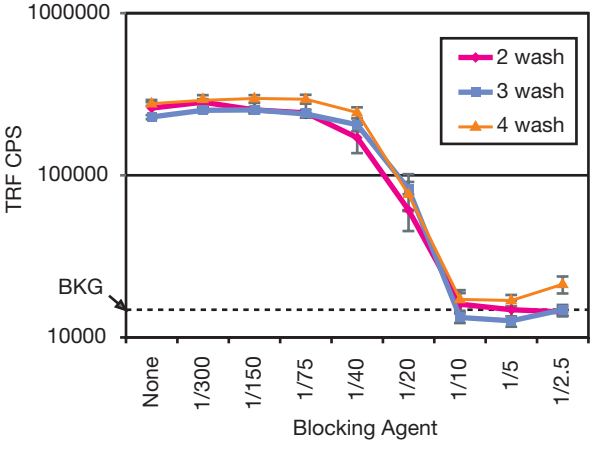
AcroWell 96 Filter Plates Use High Binding Capacity PVDF or NT Membranes



AcroWell™ 96 filter plates with BioTrace NT and BioTrace PVDF membranes reliably bind 1,000 ng protein per well. Saturation binding kinetics was seen at concentrations greater than 1,000 ng labeled antibody (not shown). The binding was resistant to washing and showed a linear correlation. The AcroWell 96 filter plates with GHP membrane, which were designed to be low in binding, bound very little labeled antibody even at high concentrations. Error bars indicate standard deviation, n = 8. Inset graphs show the logarithm plots for the BioTrace NT and BioTrace PVDF membrane-containing plates. CPS = Counts Per Second.

Once the desired proteins are bound, the addition of bovine serum albumin (BSA) or other standard protein-blot blocking solutions can further block non-specific binding. The interaction of each blocking solution may be different and should be optimized for blocking. A five-fold dilution of blotto (milk) buffer or 0.5% buffered BSA are adequate to prevent non-specific binding (see Figure 3.11).

Figure 3.11
Strong Binding Leads to Target Signal Retention with Repeated Washings



Complete binding occurs when blocking agents are absent or when inadequate concentrations of blocking agent are used. In contrast, increasing blocking agent completely blocks antibody binding, resulting in signals near plate background. Increasing the number of washes from 2 to 4 does not significantly alter signal strength at high or low antibody levels. Error bars indicate standard deviation, n = 8.
TRF = Time-resolved Fluorescence
CPS = Counts Per Second
BKG = Plate Background

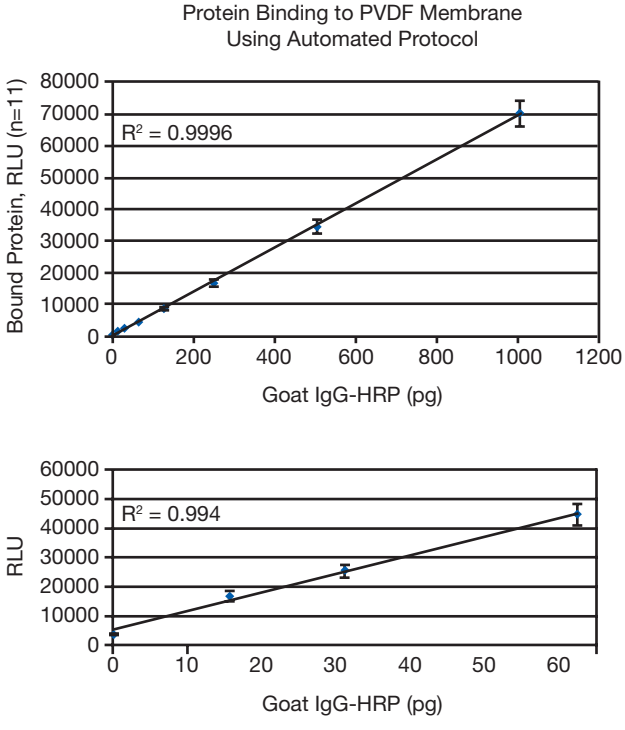
Development of an Automated AcroWell™ Filter Plate Based ELISA Assay

One classic technique for protein analysis is the western immunoblot that, although highly specific, is time-consuming, technically difficult, and not very sensitive. Moreover, the technique is low-throughput and difficult to automate. In response to these various technical difficulties and limitations associated with the western immunoblot, Pall Corporation (East Hills, NY, USA), a leader in membrane filtration and product design, teamed up with PerkinElmer Life Sciences (Boston, MA, USA), an industry leader in the field of automation and equipment, to develop an alternative assay for the detection of protein-protein interactions. The alternative assay that is described in this study uses 96-well membrane-bottom plates to specifically separate and capture proteins of interest that are then detected by a chemiluminescent or fluorescent reader.

Protein Binding Capacity and Detection Sensitivity

The first step in the dot-blotting procedure is the binding of the target protein to the membrane. For our first experiment, varying amounts (2.5-1,000 pg) of HRP-labeled goat IgG were added to pre-wetted, white AcroWell 96 filter plates with BioTrace™ PVDF membrane for 30 minutes and washed. LumiGLO* was added for 5 minutes. The plates were read automatically using the integrated Fusion* reader (luminescence mode) or manually using a PerkinElmer MICROBETA* Counter.[4] The results are summarized in Figure 3.12 and indicate that as little as 2.5 pg of goat IgG can be reliably detected using the AcroWell 96 filter plate as described. Moreover, the linear range of binding and detection is over 3 orders of magnitude.

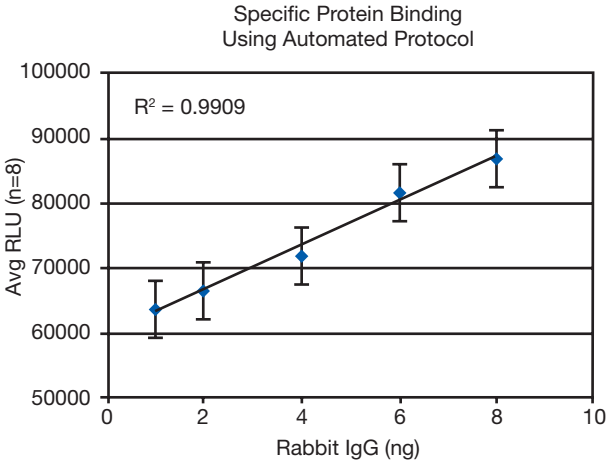
Figure 3.12
Protein Binding to BioTrace™ PVDF Membrane



Specific Protein Binding

The second experiment demonstrates the use of the AcroWell™ 96 filter plate for the immunodetection of an abundant, purified protein. Varying amounts (1-8 ng) of rabbit IgG (Kirkegaard & Perry Laboratories) were added to prewetted, white AcroWell 96 filter plates with BioTrace PVDF membrane for 30 minutes and washed. Following incubation with block (30 minutes) and washing, HRP-labeled goat anti-rabbit IgG was added for 60 minutes and washed. Following LumiGLO* addition for 5 minutes, the plates were read as before. The results are summarized in Figure 3.13.

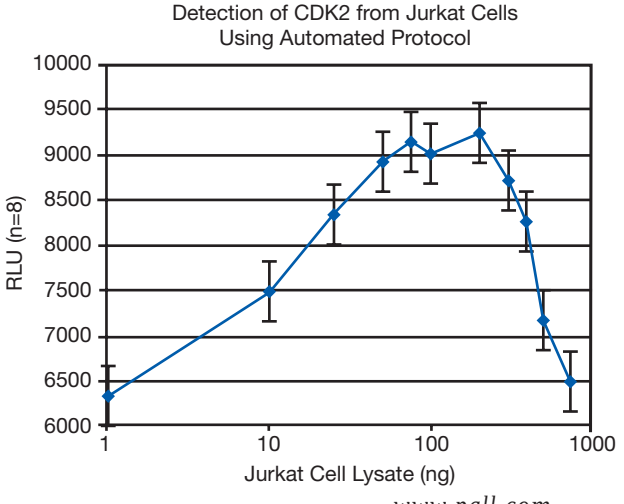
Figure 3.13
Specific Protein Binding to AcroWell™ Filter Plate with BioTrace™ PVDF Membrane



Detection of CDK2 from Jurkat Cells

To more rigorously test the sensitivity and dynamic range of the AcroWell 96 filter plate system, a third experiment was performed to specifically bind and detect cyclin-dependent kinase 2 (CDK2) in Jurkat cells. Varying amounts (1-5,000 ng) of Jurkat cell lysate (BD Biosciences) were added to an AcroWell 96 filter plate with BioTrace PVDF membrane for 30 minutes and washed. Anti-CDK2 mAb (BD Biosciences) was then added for 60 minutes and washed. HRP-labeled goat anti-mouse IgG was added for 60 minutes and washed. LumiGLO* was added and plates were read after 5 minutes as before. The results are summarized in Figure 3.14. The data show that using the AcroWell 96 filter plate system, CDK2 protein kinase from crude cell lysates can be specifically detected linearly over one order of magnitude.

Figure 3.14
Detection of CDK2 from Jurkat Cells Using an AcroWell Filter Plate



In summary, the AcroWell™ filter plates offer the following advantages in automation of a sensitive ELISA assay compared to conventional assay formats such as Western blotting.

- The use of the AcroWell 96 filter plate is an attractive alternative to western immunoblot because of its simplicity, rapidity, automation capability, and increased sensitivity.
- Compared to classical western immunoblot, the AcroWell 96 filter plate technique can be up to 100-fold more sensitive.
- AcroWell 96 filter plate assay takes approximately half the time or less to complete compared to western immunoblot assay.
- AcroWell 96 filter plate polypropylene housing and low hold-up configuration using SBS robotic guidelines result in low, non-specific binding, consistent CV's, greater detection accuracy, and automated processing.
- The variety of plate housings available for AcroWell 96 filter plates allows optimized detection by chemiluminescent, radiometric, and fluorescent techniques.
- The use of high-performance binding membranes, such as BioTrace™ PVDF and NT membrane, permits strong, reproducible binding resulting in improved specific detection of biomolecules.

Ordering Information for ELISA and Immunoassay Using AcroWell™ 96 Multi-Well Filter Plates with BioTrace PVDF and NT Membranes

AcroWell 96 Filter Plates, 350 µL Well

Part Number	Description	Pkg
5020	0.45 µm GHP membrane, natural	10/pkg
5021	0.45 µm GHP membrane, white	10/pkg
5022	0.2 µm BioTrace NT membrane, white	10/pkg
5025	0.2 µm BioTrace NT membrane, black	10/pkg
5023	0.45 µm BioTrace PVDF membrane, natural	10/pkg
5026	0.45 µm BioTrace PVDF membrane, black	10/pkg
5027	0.45 µm BioTrace PVDF membrane, white	10/pkg

References for ELISA and Immunoassay Using AcroWell™ 96 Multi-Well Filter Plates with BioTrace™ PVDF and NT Membranes

1. Valenzano, K.J., Miller, W., Kravitz, J., Samama, P., Fitzpatrick, D., & Seeley, K. (2000). Development of a fluorescent ligand-binding assay using the AcroWell filter plate. *J. Biomolecular Screening*, 5(6), 455–461.
2. Tang, N. (2002).Chemiluminescent detection of protein binding. *Genetic Engineering News*, 22(8), 34.
3. Protocol: Biomolecule binding and blocking procedures for AcroWell filter plates with BioTrace NT and BioTrace PVDF membranes, PN 33189.
4. Technical Report: The AcroWell plate: low fluorescence background using the DELFIA* system, PN 33137.
5. Technical Report: The AcroWell filter plate minimizes crosstalk, PN 33177.
6. Technical Report: Automated plate ELISA and dot-blot assays using AcroWell 96 filter plates and a robotic workstation with integrated plate reader, PN 33293.

3.2.5 ELISA and Immunoassay Using AcroWell™ 96 Multi-Well Filter Plates with Chemiluminescent (DELFIA) Detection

A sensitive and reliable labeling and detection system has been designed and patented by Wallac (PerkinElmer, Boston, MA, USA). It uses lanthanide chelates that give an intense and long-lived fluorescence emission (millisecond range), making it possible to measure fluorescence after a delay time. This eliminates the background counts from short-lived fluorescent emissions from organic fluorophores that accompany the sample because they decay prior to detection. This time-delayed fluorescence, in combination with a large stokes shift (340 to 615 nm), effectively reduces background emissions to a level that allows measurement sensitivity to rival and possibly exceed that achieved using expensive and dangerous radioactive tags.

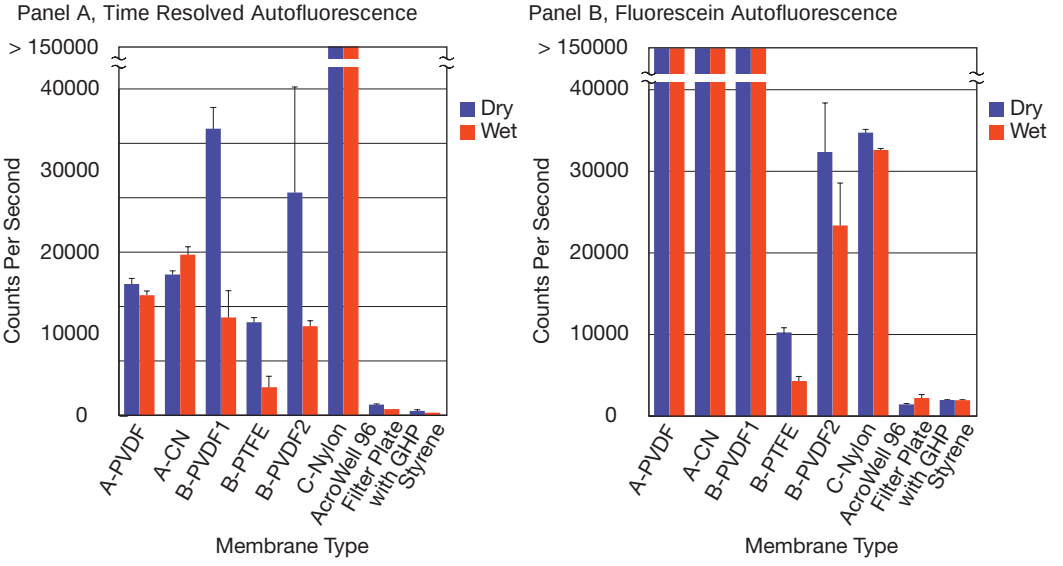
Numerous reports exist discussing the successful use of the lanthanide chelate labeling system to label antibodies for immunoassays as well as ligands for HTS receptor binding assays.[1,2,3,4] The DELFIA system allows researchers to detect binding of a labeled ligand to a receptor after washing to remove unbound-labeled ligand. Cell-based assays are particularly well suited to the DELFIA system because of its broad detection range and high sensitivity (below 1.0 fmol Europium). This degree of sensitivity can only be achieved when wash steps effectively remove unbound ligand and background is low. The most efficient way to remove binding and wash solutions, and minimize target loss, is through the use of a filter plate. Use of filter plates in fluorescent assay systems has, however, been limited because of high background fluorescence from the filter material.

The AcroWell 96 filter plates have been optimized for retentate and hybridization binding applications. The plates consist of a chemically resistant/biologically inert polypropylene filter plate assembly with two membrane layers sealed to the bottom of the plate using a patented sealing process that minimizes crosstalk. The bottom membrane support layer is hydrophobic PTFE that protects the upstream functional membrane. The PTFE acts as a barrier to passive flow, allowing lengthy or high temperature incubations with the wells filled with hybridization and immunodetection solutions. Small holes in the center of each well allow the fluid to pass under applied vacuum or centrifugation. Minimal hold-up allows for greater detection accuracy. A serialized barcode label allows for the use of automated tracking systems and identifies the membrane type. AcroWell 96 filter plates are available in clear, white, and black housing colors. Choose clear plates for time-resolved fluorescence, white plates for chemiluminescence/radioactivity, and black plates for fluorescein detection assays.

Tip: *The AcroWell 96 filter plates do not incorporate filtrate flow directors and should not be used when the filtrate needs to be collected for downstream analysis. For applications that require recovery of the filtrate, see our AcroPrep™ line of filter plates.*

A plate selection guide is shown in Figure 3.9 for the range of membranes available in this format. A summary of properties of the AcroWell™ 96 multi-well devices is presented in Table 3.6.

Figure 3.15
Background Fluorescence of AcroWell Plates



All 96 wells of the AcroWell 96 filter plate and competitor plates (A, B, or C) were read on a VICTOR multilabel counter using wavelength settings optimized for either fluorescein (excitation at 485 nm, emission at 535 nm) or time-resolved fluorescence (excitation at 340 nm, emission at 615 nm). Dry plates were read directly, while wet plates were read after vacuum (10 in. Hg) filtration of 200 µL distilled sterile water. Autofluorescence values for the AcroWell 96 filter plate with GHP membrane were not significantly higher than values for the plain styrene plates (no filtration membrane). A-CN = 0.45 µm Cellulose Nitrate; A-PVDF = 0.45 µm PVDF; B-PVDF1 = 0.22 µm PVDF with an opaque plate; B-PTFE = 5.0 µm hydrophilic PTFE; B-PVDF2 = 0.22 µm PVDF; C-Nylon = 1.2 µm Nylon; AcroWell 96 Filter Plate with GHP membrane = AcroWell 96 filter plate with 0.45 µm GHP membrane; Styrene = standard microtiter plate without a filtration membrane.*

Table 3.6
Properties of the AcroWell™ 96 Multi-Well Filter Plates

Specification	Parameter
Materials of Construction	
Membrane	GHP (hydrophilic polypropylene), BioTrace™ PVDF (hydrophobic), BioTrace Nitrocellulose
Media Support	Emflon membrane (PTFE) backed with non-woven polypropylene
Device	Polypropylene (lid polystyrene)
Effective Membrane Area	0.30 cm^2
Dimensions	
Length	12.78 cm (5.03 in.)
Width	8.51 cm (3.35 in.)
Height (without Lid)	1.44 cm (0.656 in.)
Height (with Lid)	1.70 cm (0.7 in.)
Tip Length	0.53 cm (0.21 in.)
Capacities	
Maximum Well Volume	0.30 mL
Recommended Volume	0.25 mL
Hold-up Volume (Membrane/Support)	< 0.010 mL
Maximum Centrifugal Force	3,000 x g
Centrifuge	Swinging bucket rotors
Operating Vacuum	25.4 cm Hg (10 in. Hg)
Typical Sample Processing Times for 0.25 mL Volume at 25.4 cm Hg (10 in. Hg)	GHP membrane: 40 seconds BioTrace PVDF membrane: 10 seconds BioTrace NT membrane: 50 seconds
Fluorescence Detection (GHP Plates)*	Cy5 fluor < 25,000 (natural) < 40,000 (white) Fluorescein < 2,000 (natural) < 40,000 (white) Time-resolved fluorescence (DELFIA) < 2,000 < 3,000
Light Emitting Detection (GHP Plates)**	Signal to Noise 5:1 (natural) Signal to Noise 50:1 (white)

**Fluorescence was detected using the VICTOR* multilabel counter (PerkinElmer, Wallac) using standard filters and settings for each AcroWell 96 filter plate with GHP membrane.*

***Luminometry, 20 pg of an alkaline phosphatase-labeled antibody was placed in the AcroWell 96 filter plate with GHP membrane and assayed using LumiGLO* reagent. Adjacent wells were counted for light crosstalk and background.*

Protocol for ELISA and Immunoassay Using AcroWell™ 96 Multi-Well Filter Plates with Chemiluminescent (DELFIA) Detection

A. Materials Required

1. AcroWell 96 filter plates with BioTrace™ PVDF membrane (PN 5023, 5026, or 5027) or BioTrace NT membrane (PN 5022 or 5025). For specifications, see Table 3.6.
2. High purity water or buffer, such as phosphate buffered saline (PBS)
3. Source of vacuum [25.4 cm Hg (10 in. Hg), (Pall vacuum manifold, PN 5017)]
4. Liquid handling source, such as the MultiPROBE* II HT EX workstation with Gripper* Integration Platform and Fusion* Universal Analyzer. The vacuum filtration option was also included.

B. Vacuum Manifold Filtration

1. During use, hold the filter plate so the membrane on the bottom is not touched or scratched. (Avoid contact between the membrane and other surfaces during incubation to prevent liquid flow due to contact wicking).
2. The AcroWell 96 filter plate with BioTrace PVDF membrane requires pre-wetting with 100 µL pure methanol, isopropanol, or 70% ethanol.

Tip: *Do not let the membrane dry or pre-wetting will need to be repeated. After wetting, apply vacuum and rinse with 100 µL water or buffer.*

3. Place the filter plate on the vacuum manifold. Rinse with 100 µL water or buffer.
4. Apply vacuum to manifold to initiate liquid flow. Recommended vacuum is 25.4 cm Hg (10 in. Hg).

Tip: *DO NOT exceed 38.1 cm Hg (15 in. Hg); most house vacuum and aspirators do not exceed 38.1 cm Hg (15 in. Hg).*

5. Add the sample to be filtered to the plate. Incubate if required.
6. Apply vacuum.
7. Gently tap the plate to remove any hanging droplets.
8. Release vacuum from the manifold.

Tip: *Do not release the vacuum by pulling the corner of the plate. The manifold gasket will degrade.*

9. If needed, add wash solutions and filter.

C. Centrifugation in a Swinging Bucket Rotor

1. Place the AcroWell 96 filter plate on top of a receiver plate.
2. Insert the plates into a standard multi-well plate swinging bucket rotor assembly.
3. Place a duplicate pair of plates matching the weight of the test plate (add water to the receiver plate and match weight of the test plate).

Tip: *An imbalance can result with a single test plate if no counter balancing plate is used. If different volumes of sample are used in multiple plates, they will need to be balanced in pairs by addition of water to empty wells.*

4. Centrifuge at 500-3,000 x g for 1-2 minutes.

Tip: *The centrifugal force and time parameters can be varied to optimize the filtration rate of fluids in contact in the well of the plate.*

5. If needed, add wash solution(s) and repeat. Ensure that plates are in balance before centrifugation.

D. Immunodetection Protocol

1. Add the sample containing proteins to be bound to the AcroWell™ 96 filter plate with BioTrace™ NT membrane (PN 5022, 5025) or the pre-wetted AcroWell 96 filter plates with BioTrace PVDF membrane (PN 5023, 5026, 5027).
2. Incubate for 5-30 minutes and filter as directed.
3. Effective blocking can be achieved by adding a dilution of a standard protein blot blocking solution. A five- to ten-fold dilution of a standard casein blocking solution has been shown to completely prevent further binding of biomolecules while avoiding filter fouling. Blocking may have to be individually optimized depending on which blocking solution is used.
4. To avoid sample loss or dehydration during high-temperature incubations, it is recommended that the AcroWell 96 filter plate be placed on a receiver plate and sealed into a bag containing a moist paper towel. Long incubations at high temperature may result in a small amount of weeping, usually less than 5% of the volume (20-40 µL).
5. Add wash solutions and filter as needed. Repeat two to four times.
6. Add detection solutions and place the AcroWell 96 filter plate directly into the detector.

Application Data for ELISA and Immunoassay Using AcroWell 96 Multi-Well Filter Plates with Chemiluminescent (DELFIA) Detection

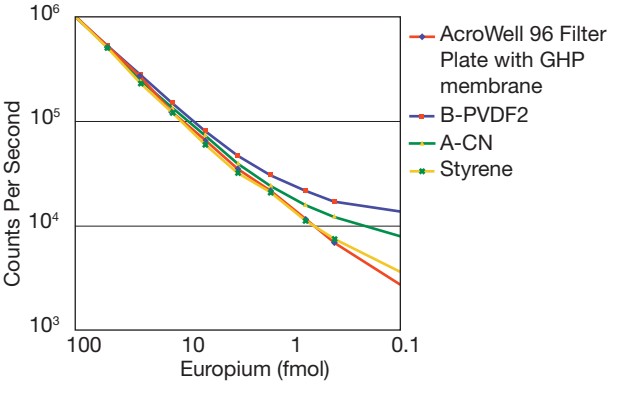
Background Fluorescence in AcroWell Plates

The background fluorescence of the AcroWell 96 and competitor filter plates was determined. Measurements were made before (dry) and after (wet) filtration of water to determine if any extractables were present that either masked or caused autofluorescence. Some of the competitor PVDF plates did have fluorescent extractables; but even after rinsing, the background was still extremely high with some background emissions consistently above 150,000 counts per second. The data summarized in Figure 3.14 shows that the AcroWell 96 filter plate with GHP membrane has a background fluorescence that is many times lower than the nearest competitor. Its fluorescent background emissions are similar to that of a plain styrene 96-well plate without a filter membrane. The extremely low fluorescence background for the AcroWell 96 filter plate was seen for both time-resolved fluorescence (Panel A, excitation at 340 nm, emission at 615 nm) and fluorescein (Panel B, excitation at 485 nm, emission at 535 nm) wavelengths.

Europium Detection

The benefit of extremely low background fluorescence is that significantly lower concentrations of fluorescent label can be detected. This was verified by comparing the ability of the AcroWell 96 filter plate and competitor plates to detect serial dilutions of Europium Standard Solution (PerkinElmer, Boston, MA, USA). The data are summarized in Figure 3.16 and data show that at Europium levels above 100 fmol/well, the competitor and AcroWell 96 filter plates perform equivalently. However, at levels below 100 fmol/well, the linearity of the measurements, and the ability to accurately detect trace Europium is impaired by the high background emissions of competitor plates.

Figure 3.16
AcroWell™ 96 Filter Plate Dilution Series



Serial dilutions of 1.0 nmol/L Europium Standard Solution were read in AcroWell™ 96 and competitor filter plates using a VICTOR multilabel counter. A master mix containing a stepwise dilution series beginning at 200 fmol/well and ending at 0.4 fmol/well was placed in duplicate in rows B and C of each plate. AcroWell 96 filter plate with GHP membrane = filter plate with 0.45 µm GHP membrane; B-PVDF2 = 0.22 µm PVDF; A-CN = 0.45 µm Cellulose Nitrate; Styrene = standard multi-well solid bottom plate without a filtration membrane.*

An equally important factor in low level fluorescence detection is the signal-to-noise ratio. At signal-to-noise ratios below 5:1, it is difficult to be sure that the measurement is a real signal above background. The AcroWell 96 filter plate and styrene plate do not have a signal-to-noise ratio below 5:1 until Europium levels are below 1 fmol/well (see Table 3.7). Competitor filter plate signal-to-noise ratios drop below 5:1 at Europium levels below 13 fmol/well.

Table 3.7
*Signal-to-Noise Ratios of Dilute Europium Samples**

Europium	Plate Type			
(fmol/well)**	AcroWell 96 Plate	B-PVDF	A-CN	Styrene
13	53:1	8:1	6:1	60:1
6	27:1	4:1	3:1	30:1
3	14:1	3:1	2:1	16:1
2	8:1	2:1	1:1	10:1
1	5:1	1:1	1:1	5:1

**Signal-to-noise ratios were calculated from the measurements illustrated in Figure 3.16 by dividing the total activity at each Europium concentration by the fluorescence background activity of the respective plates.*

***Based on the assumption that when using the DELFIA system, each labeled antibody has an average of 10 Europium conjugates bound, detection of 1.0 fmol of Europium should be equivalent to the detection of 0.1 fmol of labeled antibody.*

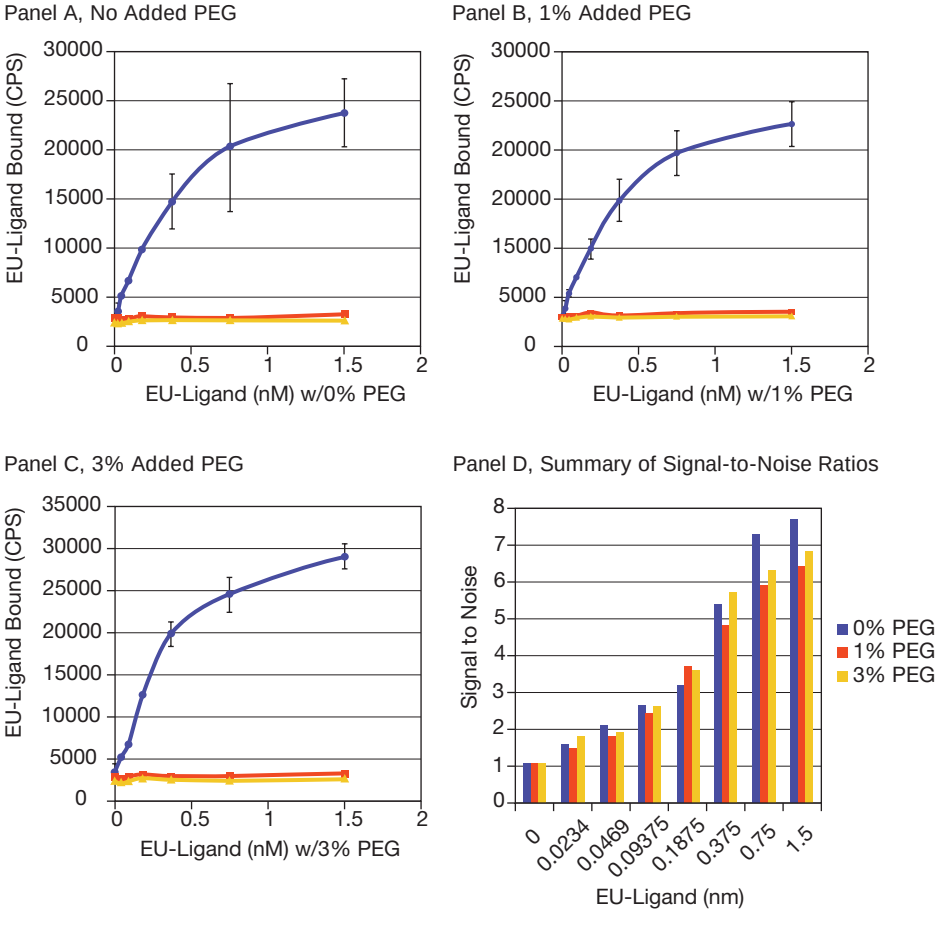
Receptor/Ligand Binding Assays

A binding assay was set up using targets embedded in cell membrane fragments. Three concentrations of polyethylene glycol (PEG) were added to the assay buffer and, following the binding of Europium labeled ligand, the samples were washed thoroughly. Increasing concentrations of labeled ligand were added from 0 to 24 nM. Saturation was achieved at 1.5 nM and the data at higher concentrations are not shown (see Figure 3.17). Filtration

was performed at 1,000 x g using a low-speed centrifuge with a rotor designed for 96-well plates. Subsequent ligand binding assays have been performed using vacuum (10 in. Hg) with similar results (data not shown).

Figure 3.17

DELFIA Assays Using the AcroWell™ 96 Filter Plate



Europium-labeled ligand was bound to a target receptor found in lysed cellular membrane fragments (specific receptor and ligand identities are currently confidential). Three independent readings for each data point were made on AcroWell 96 filter plates. Increasing concentrations of labeled ligand were added to determine signal-to-noise at saturation in three solutions (Panels A-C), each containing the indicated concentration of polyethylene glycol (PEG). The total binding activity (blue) approached saturation at 1.5 nM EU-ligand while the non-specific binding (red) and background plate fluorescence (yellow) remained extremely low. Signal-to-noise ratios were calculated as total binding/non-specific binding and plotted for all three PEG concentrations in Panel D.

Signal uniformity was confirmed by adding a constant quantity of target and ligand across the plate during binding assays. Under these conditions, the AcroWell™ 96 filter plate exhibited a well-to-well variation of less than 10%. Overall, the data demonstrate tight standard errors and saturation binding kinetics curves similar to those seen previously using a plain styrene plate for detection (data not shown).

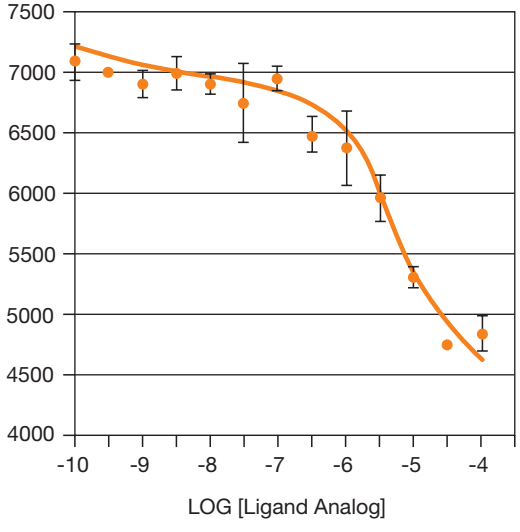
Using AcroWell 96 filter plates, the signal-to-noise ratio was 7:1 at 1.5 nM labeled ligand. This signal-to-noise ratio was achieved at a fluorescent count of around 18,000 CPS using a VICTOR* multilabel counter. It is important to note that following filtration washes and addition of enhancement solution, competitor plates produce background fluorescence counts near 15,000 CPS while the AcroWell 96 filter plate background fluorescence remains below 2,500 CPS. Therefore, under these typical assay conditions, a competitor plate would only give a 1:1 signal-to-noise ratio, making detection of positive hits unlikely. In contrast, the use of the AcroWell 96 filter plate allows researchers to easily detect new drug candidates.

Competitive Inhibition

When a new drug lead is detected, it is always important to verify that the positive hit is actually binding to the target and not to some other receptor or non-specific binding activity. Because the AcroWell 96 filter plate is made of inert polypropylene, it is unlikely that the plate itself will bind labeled ligands. To eliminate the contribution of other non-specific interactions, a second assay must be performed. The most effective way to verify target-binding specificity is to set up an assay series at ligand concentrations well below saturation and add increasing concentrations of a second molecule that is known to compete for binding to the specific target receptor. In order to accurately measure competitor inhibition, it is critical that filter plate background fluorescence does not mask the Europium signal as signal strength declines.

A low affinity binding assay was set up using labeled ligand concentration of 0.2 nM and 3% PEG. Increasing concentrations of inhibitor (ligand analog) were added to the assay mixture and allowed to interact. Following washes and the addition of enhancement solution, the assays were counted directly in the AcroWell 96 filter plate. The resulting data indicate that the analog specifically competes with the labeled ligand binding and the binding constant was calculated at 1.27×10^6 nM inhibitor (see Figure 3.18). Note that the detection of Europium labeled ligand ranged from 7,300 to 5,000 CPS. Competitor plates would not allow detection of binding and inhibition at this level due to their high background fluorescence.

Figure 3.18
Binding Inhibition Using Ligand Binding Agonists



Europium-labeled ligand was bound to the same target receptors at a ligand concentration of 0.2 nM EU-ligand. Increasing concentrations of a ligand analog were added to separate wells and each data point was assayed, as before, in triplicate directly in the AcroWell™ 96 filter plate.

Ordering Information for ELISA and Immunoassay Using AcroWell™ 96 Multi-Well Filter Plates with Chemiluminescent (DELFIA) Detection

AcroWell 96 Filter Plates, 350 µL Well

Part Number	Description	Pkg
5020	0.45 µm GHP membrane, natural	10/pkg
5021	0.45 µm GHP membrane, white	10/pkg
5022	0.2 µm BioTrace™ NT membrane, white	10/pkg
5025	0.2 µm BioTrace NT membrane, black	10/pkg
5023	0.45 µm BioTrace PVDF membrane, natural	10/pkg
5026	0.45 µm BioTrace PVDF membrane, black	10/pkg
5027	0.45 µm BioTrace PVDF membrane, white	10/pkg

References for ELISA and Immunoassay Using AcroWell 96 Multi-Well Filter Plates with Chemiluminescent (DELFIA) Detection

1. Liu, J., Gallagher, M., Horlick, R.A., Robbins, A.K., & Webb, M.L. (1998). A time resolved fluorometric assay for galanin receptors. *J. Biomol. Screening*, (3), 199–205.
2. Appell, K., Chung, T., Solly, K., & Chelsky, D. (1998). Biological characterization of neurokinin antagonists discovered through screening a combinatorial library. *J. Biomol. Screening*, (3), 19–27.
3. Hemmila, I.A., & Webb, S. (1997). Time-resolved fluorometry: An overview of the labels and core technologies for drug screening applications. *Drug Discovery Today*, (2), 373–381.
4. Inglese, J., Samama, P., Patel, S., Burbaum, J., Stroke, I., Appell, K. (1998). Chemokine receptor-ligand interactions measured using time-resolved fluorescence. *Biochemistry*, (37), 2372–2377.
5. Rogers, M.V. (1997). Light on high throughput screening: Fluorometric-based assay technology. *Drug Discovery Today*, (2), 156–160.

3.2.6 Activation Protocol for Biodyne® C Membrane for Subsequent Covalent Linking of Ligands

Certain procedures require that molecules be covalently immobilized on membranes. Typical applications requiring covalently immobilized ligands are immuno-capture assays, ELISAs, immobilization of amino-terminated oligonucleotides, and gene-probe tests. Several patented (U.S. #4,693,985 and 4,886,836) activation protocols for the covalent attachment of ligands to Biodyne C membrane are described. Please note that the procedures that use methylene chloride as a solvent must be performed beneath a fume hood. Also, membranes activated in methylene chloride should be protected against exposure to water or water vapor until use. In addition, glutaraldehyde can be added to the membranes in water or buffer, like phosphate buffered saline (PBS).

Protocol for Activation Protocol for Biodyne C Membrane for Subsequent Covalent Linking of Ligands

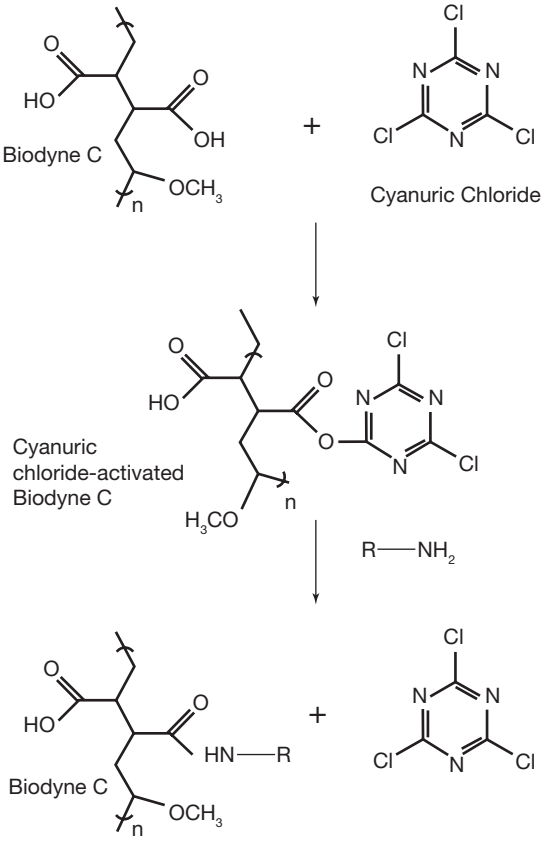
A. Materials Required

1. Biodyne C membrane
2. Cyanuric Chloride (TsT)
3. Methylene Chloride (CH_2Cl_2)
4. Triethylamine (TEA)
5. Dicyclohexylcarbodiimide (DCCD)
6. Phosphate buffered saline
7. Glutaraldehyde
8. Glass containers or trays
9. Ventilated chemical hood

B. Activation of Biodyne C Membrane

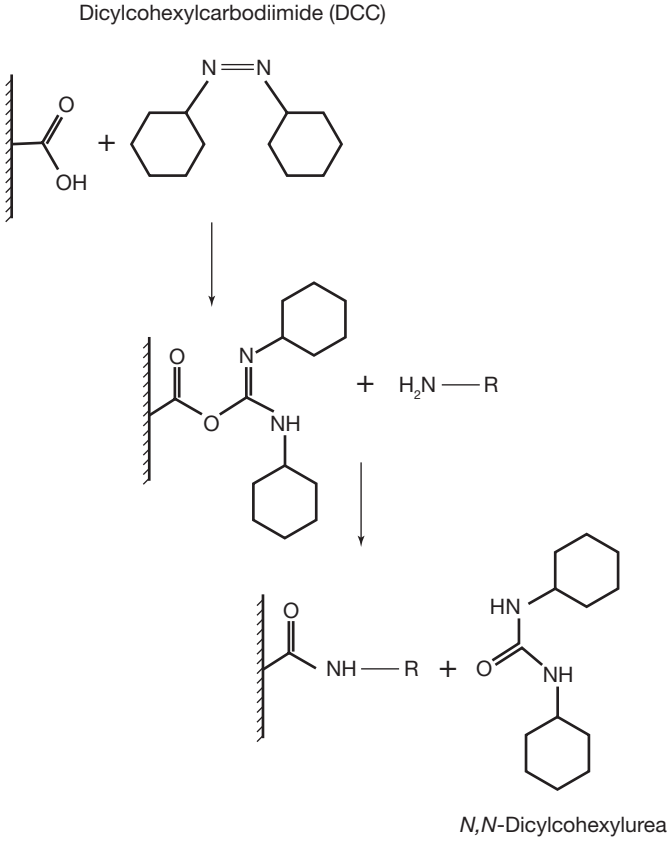
1. TsT (Trichloro-s-Triazine or Cyanuric Chloride). See Figure 3.19.
   - **a.** Cut pieces of Biodyne C membrane
   - **b.** Soak membrane in 4.3 mL TEA and 65 mL of $MeCl_2$ for 2 minutes.
   - **c.** Add 2g TsT, agitate gently, and then incubate for 15 minutes at room temperature.
   - **d.** Wash membrane 4x for 5 minutes per wash with 50 mL $MeCl_2$.
   - **e.** Air dry 2 minutes; place in vacuum desiccator until use.

Figure 3.19
Activation of Biodyne® C Membrane with Cyanuric Chloride



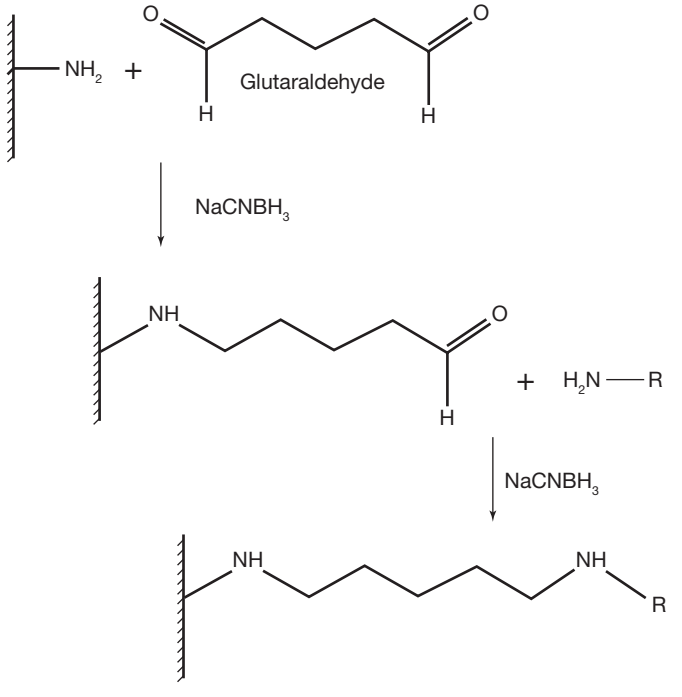
2. Dicyclohexylcarbodiimide (DCC). See Figure 3.20.
- **a.** Soak membrane in 50 mL of $MeCl_2$ containing 5g (10% w/v) DCC for 30 minutes.
- **b.** Wash membrane 4x for 5 minutes per wash with 50 mL $MeCl_2$.
- **c.** Air dry 2 minutes; place in vacuum desiccator until use.

Figure 3.20
Activation of Biodyne® C Membrane with Dicylcohexylcarbodiimide (DCC)



3. Glutaraldehyde. See Figure 3.21.
- **a.** Cut 13 mm discs of sample media.
- **b.** Soak discs in 50 mL of PBS containing 0.5%-5% (2.5 mL) glutaraldehyde for 2 hours.
- **c.** Wash membranes 4x for 5 minutes per wash with 50 mL PBS.
- **d.** Air dry 10 minutes and place in vacuum desiccator or store in PBS until use.

Figure 3.21
Activation of Biodyne® C Membrane with Glutaraldehyde



Ordering Information for Activation Protocol for Biodyne® C Membrane for Subsequent Covalent Linking of Ligands

Biodyne C Membrane, 0.45 µm

Part Number	Description	Pkg
60316	82 mm discs	50/pkg
60317	85 mm discs	50/pkg
60318	132 mm discs	50/pkg
60319	137 mm discs	50/pkg
60315	7 x 8.5 cm sheets	10/pkg
60314	20 x 20 cm sheets	10/pkg
60251	29 cm x 3 m roll	1/pkg

Notes