something about us

Vision

AppliChem was founded with the aim of supplying chemicals for chemical, biological, pharmaceutical and clinical research. It was also intended that AppliChem's products should be available worldwide.

Experience

Our chemists have had many years of in-depth experience and offer a sound partnership in helping to solve your problems in the lab. With you or for you – we want to develop new products. As well as flexibility, we assure you of strict confidentiality in all your projects.

Assortment

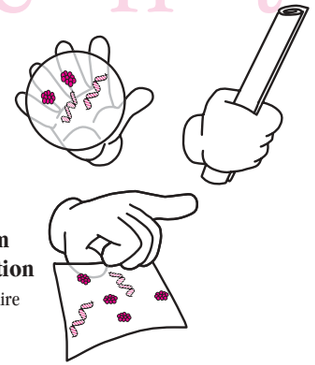
We prepare and provide you with chemicals and reagents including even those not listed in our current catalogs. When talking of “chemicals” in the widest sense of the word, we offer the service ‘all products – one supplier’.

Quality

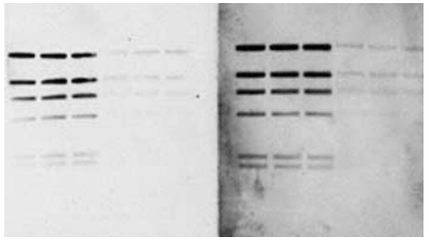
Thanks to our quality management system, with AppliChem as your supplier you gain a decisive advantage over your competitors. Our products will fulfil your expectations and your individual, particular requirements are our business.

AppliChem is continuously gaining new customers, due to the exact and constant quality, as well as to the advantageous prices, of our products and services. AppliChem is a reliable partner. Our quality control department provides detailed documentation on request.

contents



Introduction



AppliChem supplies a range of transfer membranes designed and tested specifically for RNA, DNA and protein analysis. AppliChem also provides our customers with tried and proven protocols developed to obtain consistent reproducible and dependable results when used with AppliChem membranes. AppliChem highly recommends that you follow these protocols for optimal transfers.

Products

Reprobe Nitrocellulose Supported Transfer Membrane
Outperforms unsupported nitrocellulose when extensively reprobing or performing colony and plaque lift. Some of the applications include DNA, RNA, and protein assays.

Pure Nitrocellulose Unsupported Transfer Membrane
Unsupported membrane of choice used for all protein or immunoblotting applications.

PVDF-Star (Polyvinylidene Fluoride) Transfer Membrane
Naturally hydrophobic, unsupported PVDF has the highest binding capacity for western transfers, amino acid analysis and protein sequencing.

Reprobe Nylon Positively Charged Transfer Membrane
Next generation and outperforms previous membranes with high signal strength and low background attributes. Inherently charged nylon membrane. Tested and performs well with radioactive or non-radioactive procedures.

Pure Nylon Neutral Transfer Membrane
Used where a neutral or single probe test is desired.

Reprobe Nitrocellulose Supported Transfer Membrane

- Supported for procedures requiring rigorous handling
- Strong – will not curl, bend or crack after baking
- High sensitivities, low backgrounds
- Multiple reprobings
- Nucleic acid binding is 100 µg/cm²

Applications
- Northerns, Southerns
- Multiple re-hybridizations
- Colony/plaque lifts
- Dot/slot blots
- Radiolabeled detection systems
- Chemiluminescent
- Biotinylated detection systems

Product Description
Reprobe Nitrocellulose Supported is an internally supported nitrocellulose membrane that combines the binding characteristics of nitrocellulose with the strength of nylon. Reprobe Nitrocellulose Supported is ideal for applications where nitrocellulose has been used in the past such as DNA/RNA/Protein transfers. However, it outperforms unsupported nitrocellulose when extensive handling is required such as reprobing or colony and plaque lifts.
The applied, unique process of impregnating pure nitrocellulose polymer onto an inert polyester web produces a very strong, dimensionally stable hybridization membrane. The inert web provides dimensional strength to prevent curling, cracking and tearing even after baking. This ensures the highest binding nitrocellulose membrane, approximately 100 µg/cm². There is nothing to lower the binding capacity such as cellulose acetate or other additives.
This combination is especially important in colony and plaque lifts and multiple reprobings or any other procedure requiring rigorous handling. Membrane breakage is eliminated, reducing the costs due to wasted time, materials, and lost data. Many researchers have reprobed Reprobe Nitrocellulose Supported as many as five times.
In all protocols using nitrocellulose, Reprobe Nitrocellulose Supported may be substituted without making any changes in the protocol while maintaining the same high signal and low backgrounds.
All membranes are Triton free and do not contain any other cytotoxins.

Pure Nitrocellulose Unsupported Transfer Membrane

- For procedures that require optimum resolution
- Membrane of choice for all protein or immunoblotting applications
- Cellulose acetate free – assuring high binding and sensitivity
- Low background
- Easily blocked
- Nucleic acid binding is 100 µg/cm²

Applications
- Westerns
- Protein & immunoblotting
- Northerns
- Southerns
- Dot/slot blots
- Radiolabeled chromogenic and chemiluminescent detection systems

Product Description
Pure Nitrocellulose is a pure unsupported nitrocellulose and is the membrane of choice for all protein or immunoblotting applications. Pure Nitrocellulose unsupported does not contain cellulose acetate, a low binding polymer. This assures the highest binding and sensitivity, 100 µg/cm².
Nitrocellulose exhibits the highest sensitivity with very low backgrounds in all transfers, especially in protein blotting. Unlike PVDF, nitrocellulose wets out naturally, does not require methanol, and will not turn hydrophobic during the transfer process. Nitrocellulose is very easily blocked and does not need the many blocking steps required with PVDF.
Excellent results will be obtained with all detection systems: antibody/antigen, radiolabeled, biotinylated, and chemiluminescent, giving you a great amount of flexibility in designing your procedure.

PVDF-Star (Polyvinylidene Fluoride) Transfer Membrane

- Ideal for use in protein binding applications such as western blots, solid phase assays and immunoblotting procedures
- Naturally hydrophobic unsupported membrane
- Superior strength, can withstand aggressive handling or be used with automated equipment without breaking or tearing
- High range of chemical compatibility, resistant to most commonly used chemicals and chemically aggressive solvents. Chemical compatibility allows the use of all commonly used stains
- Low backgrounds, high sensitivity detects low-level components
- Highest binding capacity eliminating sample "blow through", binds a wide range of fragment sizes
- BSA binding capacity is 125 µg/cm²
- Low extractables, ensures tests will be clean with consistent results
- Hydrophobic, resists water
- Lot-to-lot consistency, quality checks ensure consistent binding for dependable results every time

Applications

- Western transfers
- Protein sequencing
- Peptide mapping
- Amino acid analysis
- Dot/slot blots

Product Description

PVDF-Star (Polyvinylidene Fluoride) membrane is a naturally hydrophobic, unsupported transfer membrane. It has a high binding capacity and low backgrounds and is ideal for use in protein binding applications such as Western blots, solid phase assays, immuno blotting procedures, amino acid analysis and protein sequencing.

Exceptional quality control procedures throughout the manufacturing process eliminates "lot-to-lot" variation and greatly minimizes "bald spot" problems. PVDF assures reproducible results with maximum sensitivity.

Protein can be electroblotted from a variety of gel matrices onto PVDF-Star. Because of its high sensitivity and strong binding affinity, protein will not pass through the membrane. The broad chemical compatibility of PVDF-Star allows the use of all commonly used stains such as: Amido Black, Colloidal Gold, Coomassie Blue, India Ink and Ponceau-S. PVDF-Star will not degrade, distort or shrink when using high concentrations of methanol for destaining. The exceptional tensile strength allows for multiple reprobings and easy removal of target bands without concern for the membrane tearing, fracturing or curling.

PVDF-Star exhibits efficient transfer of proteins in a wide range of molecular weights, from 5k Daltons to 700k Daltons. The binding capacity averages 125 µg/cm² for larger globular proteins such as immunoglobulins and higher binding capacities for small peptides.

Specifications

Thickness	140 – 250 µm
BSA Protein Binding	125 µg/cm² for Immunoglobulins
Pore Size Range	0.2 and 0.45 µm

Reprobe Nylon Positively Charged Transfer Membrane

- Ideal for use in multiple reprobings and nucleic acid transfers under alkaline conditions
- Specifically designed for multiple reprobings: Inherently charged; enhances nucleic acid binding – even under alkaline conditions. Supported, has added strength and durability preventing distortion or contamination in multiple reprobings
- Greater binding capacity with a binding capacity of 450 mg/cm², Reprobe Nylon Positively Charged Transfer Membrane can bind a wide range of fragment sizes and can still be easily blocked for low background
- Excellent signal better retains DNA resulting in a strong signal using smaller quantities of DNA
- Hydrophilic, eliminates the need for wetting agents that can potentially interfere with biological processes
- Lot-to-lot consistency, quality checks ensure lot-to-lot consistency, both down and across the polyester web, for dependable results every time

Applications

- Radiolabeled & non-radiolabeled detection systems
- Southern transfers
- Northern transfers
- Micro arrays
- Macro arrays
- Chemiluminescent systems
- Multiple Reprobings
- Alkaline Blotting
- UV Crosslinking

Product Description

Reprobe Nylon Positively Charged transfer membrane is an inherently charged, naturally hydrophilic nylon membrane, a pure polymer impregnated by an inert polyester web. It is specifically designed to allow for numerous reprobings and use in nucleic acid transfers under alkaline conditions. Reprobe Nylon Positively Charged transfer membrane has been tested to provide consistent results through 12 or more reprobings.

The high binding capacity of 450 mg/cm² makes Reprobe Nylon Positively Charged transfer membrane ideal for all Southern and Northern applications, including alkaline blotting. Reprobe Nylon Positively Charged transfer membrane is ideally suited for all probes both radioactive and non-radioactive, including chemiluminescent and biotinylated detection systems.

Reprobe Nylon Positively Charged offers significantly increased binding, maximum "lot-to-lot" consistency, and excellent signal retention. This higher sensitivity enables researchers to obtain reliable results using smaller quantities of DNA samples and allows them to conserve valuable or expensive samples and save them for future use.

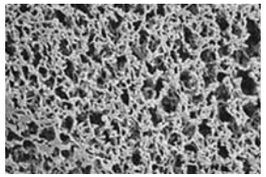
Given that smaller samples and higher density formats or micro arrays are becoming more common, the Reprobe Nylon Positively Charged Transfer Membrane is especially well suited for genomic research. Even with small DNA samples, Reprobe Nylon Positively Charged Transfer Membrane is able to retain a strong signal after multiple reprobings, providing easier automated reading of signals and more discrimination.

The unique qualities of Reprobe Nylon Positively Charged Transfer Membrane provide a lower background and higher signal assuring reliable results the first time a transfer is executed.

Product Characteristics

Sterilization	Gamma irradiation or Ethylene Oxide (EtO)
USP Class VI Testing	Passed
Thickness	65 – 125 µm
Extractables	<0.2 % (<0.0015 mg/cm²)
Nucleic Acid Binding	450 µg/cm²
Maximum Operating Temperature	180°C (356°F)
Pore Size Range	Optimized for DNA Binding
Sealing Compatibility	Ultrasonic, heat, radio frequency and insert molding

Pure Nylon Neutral Transfer Membrane



The purity and consistency of the Pure Nylon Neutral Transfer Membrane, coupled with its added durability and signal retention, make it an ideal membrane for use in medical research, scientific studies or test confirmations where precise biological pattern replications, such as DNA and RNA transfers, are integral to the success of the procedure.

- Untreated nylon performs optimally in standard techniques
- Single probes
- Ideal for use in nucleic acids transfers and protein binding
- Supported Nylon membrane, has added strength and durability preventing distortion or contamination in multiple reprobings
- High binding capacity, with a nucleic acid binding capacity of approx. 350 µg/cm², Pure Nylon Neutral Transfer Membrane can bind a wide range of fragment sizes, increasing the efficiency of transfers
- Hydrophilic, which eliminates the need for wetting agents that can potentially interfere with biological processes
- Lot-to-lot consistency, quality checks ensure lot-to-lot consistency, both down and across the polyester web, for dependable results every time

Applications

● Radiolabeled & non-radiolabeled detection systems ● Southern transfers ● Northern transfers
● UV Crosslinking ● Colony lifts ● Plaque lifts ● Library Screenings ● Single Probe ● Protein binding
● Micro arrays ● Macro arrays ● Dot/Slot blots

Product Description

Pure Nylon Neutral is a pure polymer impregnated by an inert polyester web. It is naturally hydrophilic and optimized for protein binding and for high, reproducible binding of nucleic acids. This supported nylon membrane, is optimal for all traditional DNA and RNA transfers. Pure Nylon Neutral has a higher binding capacity (approx. 350 µg/cm²) than nitrocellulose membrane and binds a greater range of fragment sizes.

Pure Nylon Neutral membrane is naturally hydrophilic, eliminating the need for wetting agents that can potentially interfere with biological processes. Pure Nylon Neutral membrane wets out quickly and evenly and will never turn hydrophobic during hybridization.

UV crosslinking Pure Nylon Neutral is an excellent way to attach nucleic acids. By exposing a nylon membrane to a UV source, a covalent bond forms between the membrane and the DNA or RNA, which maximizes signal retention after reprobing.

By the unique impregnation process, Pure Nylon Neutral has many advantages. Nylon is impregnated onto an integral polyester support web giving the nylon added dimensional strength, which prevents cracking, tearing, curling, and breaking. This is essential to protocols requiring aggressive handling, such as colony and plaque lifts and multiple reprobings. Using Pure Nylon Neutral by avoiding membrane breakage can save a significant amount of time and money.

Product Characteristics

Sterilization	Gamma irradiation or Ethylene Oxide (EtO)
USP Class VI Testing	Passed
Thickness	65 – 125 µm
Extractables	<0.2 % (<0.0015 mg/cm²)
BSA Protein Binding	350 ug/cm²
Maximum Operating Temperature	180°C (356°F)
Pore Size Range	0.1 to 20 µm
Sealing Compatibility	Ultrasonic, heat, radio frequency and insert molding

Performance Characteristics

Pore Size	0.22 µm	0.45 µm
Minimum Bubble Point kg/cm² (psi)	40 (2.80)	35 (2.45)
Typical Flow Rate ml/min/cm²@0.7 kg/cm² (10psi)	9.7	17.0

product information

Transfer Membranes Supplied by AppliChem

Membrane	Features	Benefits	Choose By Detection Systems	Choose By Procedures
Nylon				
Pure Nylon Neutral	Pure Nylon Membrane	Untreated nylon performs optimally in standard techniques like Northerns and Southerns. Binding capacity 350 µg/cm².	Radiolabeled & Non-Radiolabeled Detection Systems	Northerns Southerns UV Crosslinking Colony/Plaque Lifts Library Screenings
Reprobe Nylon Positively Charged	Positively Charged Nylon Membrane	Inherently charged nylon membrane specifically designed for Multiple Reprobings. Binding capacity 450 µg/cm².	Radiolabeled Detection Systems	Northern Southerns Multiple Reprobings Alkaline Blotting
Nitrocellulose				
Pure Nitrocellulose Unsupported	Pure Nitrocellulose	Pure nitrocellulose is the membrane of choice for protein and immunoblotting techniques, as well as any other procedures that require optimum resolution. Binding capacity 100 µg/cm².	Radiolabeled, Chromogenic and Chemiluminescent Detection Systems	Westerns Protein & Immunoblotting Northerns Southerns
Reprobe Nitrocellulose Supported	Supported Pure Nitrocellulose	Supported pure nitrocellulose is used in procedures requiring the highest sensitivities, low backgrounds and rigorous handling. The membrane can be reprobed many times. Binding capacity 100 µg/cm².	Radiolabeled Detection Systems Chemiluminescent and Biotinylated Detection Systems	Northerns Southerns Multiple Rehybridizations Colony and Plaque Lifts
Polyvinylidene Fluoride				
PVDF-Star	Hydrophobic PVDF Membrane	Hydrophobic PVDF membrane is designed for protein sequencing, western transfers and amino acid analysis. Binding capacity 125 µg/cm².	Chemical compatibility allows the use of all commonly used stains	Western Transfers Protein Sequencing Amino Acid Analysis

product info

AppliChem Transfer Membranes by Application

Application	Membrane	Catalog Number
Alkaline Blotting	Reprobe Nylon Positively Charged	A5255
Amino Acid Analysis	Pure Nitrocellulose Unsupported	A5250 or A5239
	PVDF-Star	A5243
Biotinylated Probes	Pure Nylon Neutral	A4399 or A5248
	Reprobe Nitrocellulose Supported	A5237 or A5242
Cell Blotting	Reprobe Nylon Positively Charged	A5255
	Pure Nylon Neutral	A4399 or A5248
Cell Labeling	Pure Nylon Neutral	A4399 or A5248
Chemilumines-cent Probes	Pure Nylon Neutral	A4399 or A5248
	Reprobe Nitrocellulose Supported	A5237 or A5242
Chromogenic	Pure Nitrocellulose Unsupported	A5250 or A5239
	Reprobe Nitrocellulose Supported	A5237 or A5242
Colony Lift	Pure Nylon Neutral	A4399 or A5248
	Reprobe Nitrocellulose Supported	gridded discs *
DNA Hybridization	Reprobe Nylon Positively Charged	A5255
	Pure Nylon Neutral	A4399 or A5248
	Reprobe Nitrocellulose Supported	A5237 or A5242
Dot/Slot Blotting	Reprobe Nylon Positively Charged	A5255
	Reprobe Nitrocellulose Supported	A5237 or A5242
Drug Binding Assay	Pure Nitrocellulose Unsupported	A5250 or A5239
	Reprobe Nitrocellulose Supported	A5237 or A5242
Hormone Binding	Pure Nitrocellulose Unsupported	A5250 or A5239
	Reprobe Nitrocellulose Supported	A5237 or A5242
Immuno-blotting	Pure Nitrocellulose Unsupported	A5250 or A5239
	PVDF-Star	A5243
Library Screening	Pure Nylon Neutral	A4399 or A5248
	Reprobe Nitrocellulose Supported	A5237 or A5242
Multiple Reprobings	Reprobe Nylon Positively Charged	A5255
Northern Transfers	Reprobe Nylon Positively Charged	A5255
	Pure Nylon Neutral	A4399 or A5248
	Reprobe Nitrocellulose Supported	A5237 or A5242
	Pure Nitrocellulose Unsupported	A5250 or A5239
Non-Radio-active Probes	Pure Nylon Neutral	A4399 or A5248
	Reprobe Nitrocellulose Supported	A5237 or A5242
Nucleic Acid Transfer	Reprobe Nylon Positively Charged	A5255
	Pure Nylon Neutral	A4399 or A5248
	Reprobe Nitrocellulose Supported	A5237 or A5242
	Pure Nitrocellulose Unsupported	A5250 or A5239
Plaque Lifts	Reprobe Nitrocellulose Supported	gridded discs *
Protein Blotting	Pure Nitrocellulose Unsupported	A5250 or A5239
	PVDF-Star	A5243
Protein Precipitation	Pure Nitrocellulose Unsupported	A5250 or A5239
	Reprobe Nitrocellulose Supported	A5237 or A5242
Protein Sequencing	PVDF-Star	0.1 µm pore size *
Protein Transfer	Pure Nitrocellulose Unsupported	A5250 or A5239
	PVDF-Star	A5243
Radiolabeled Probe	Reprobe Nylon Positively Charged	A5255
	Reprobe Nitrocellulose Supported	A5237 or A5242
RNA Hybridizations	Reprobe Nylon Positively Charged	A5255
	Reprobe Nitrocellulose Supported	A5237 or A5242
Solid Phase Assay	PVDF-Star	A5243
Southern Transfers	Reprobe Nylon Positively Charged	A5255
	Reprobe Nitrocellulose Supported	A5237 or A5242
	Pure Nylon Neutral	A4399 or A5248
UV Crosslinking	Pure Nylon Neutral	A4399 or A5248
Vacuum Blotting	Reprobe Nylon Positively Charged	A5255
	Reprobe Nitrocellulose Supported	A5237 or A5242
Westerns	Pure Nitrocellulose Unsupported	A5250 or A5239
	PVDF-Star	A5243

* available on request

applications

selection

Selecting a Transfer Membrane

Selecting the appropriate membrane is critical to the success of a nucleic acid or protein transfer procedure. AppliChem supplies several types of membranes for hybridization technology, each exhibiting different performance characteristics which can directly affect the outcome of a specific technique. Below are some of the more frequently performed procedures and features of hybridization membranes.

Rehybridizations AppliChem offers membranes recommended for rehybridization procedures: Pure Nylon Neutral, Reprobe Nylon Positively Charged and Reprobe Nitrocellulose Supported, a supported pure nitrocellulose.
Reprobe Nylon Positively Charged membranes can be most frequently reprobed. On nylon membranes, the number of reprobing steps is a function of the amount of hydrolysis to which the membrane is exposed during the protocol, and the additive effects of hot water, sodium hydroxide and an acidic environment. Sodium hydroxide solutions deteriorate the nylon matrix and are not recommended in procedures where reprobing steps are required.
The polyester support web used in manufacturing Reprobe Nitrocellulose Supported allows the membrane to be reprobed several times. Because the binding capacity of nitrocellulose is less than that of nylon (100 µg/cm² vs. 400 µg/cm²), the potential number of rehybridizations is fewer. See below for more details (Southern (DNA) and Northern (RNA) Hybridizations)

Non-Radioactive Probes Reprobe Nylon Positively Charged may be used particularily where multiple probing is involved.

UV Crosslinking For covalent binding of nucleic acids to a transfer membrane, AppliChem membranes can be UV Crosslinked by following the manufacturer's instructions. It is particularly recommended when working with short fragments, small samples, or low numbers of base pairs, because of the improved resolution this technique offers.

Protein Blotting Pure Nitrocellulose Unsupported and PVDF-Star membranes are recommended for use in protein blotting. Nitrocellulose membranes are able to be more thoroughly blocked, reducing the high background potential associated with protein blotting. PVDF membranes are more resistant to the harsh chemicals used in Edman degradation.

Alkaline Blotting For more rapid transfers, an alkaline blotting procedure can be used with Reprobe Nylon membranes. Alkaline blotting is not recommended when reprobing is required. See Protocol 6 for more details on Alkaline Blotting with Reprobe Nylon Positively Charged Transfer Membrane.

Staining Procedures Reprobe Nitrocellulose Supported, Pure Nitrocellulose Unsupported and PVDF-Star membranes are recommended for procedures that require a staining step with India Ink, Coomassie Blue, Colloidal Gold, or any other commonly used stain. Nylon membranes irreversibly bind many stains.

Reducing Backgrounds There are many sources of background problems, or low signal-to-noise ratios. Some of the most common include: contaminated probes, contaminated hybridization solutions, and incorrectly chosen stringency levels. Nonfat milk should not be used as a blocking agent as it may increase nonspecific binding. Nylon membranes have a much higher binding capacity than nitrocellulose membranes, and a greater potential for backgrounds. AppliChem membranes are all manufactured by strict quality control procedures, ensuring a uniform membrane with consistently low backgrounds.

Troubleshooting Common Blotting Problems Many blotting problems can be eliminated by observing the following recommendations.
Blotchy or incomplete transfers are caused by poor contact between the gel and the membrane. Even after careful smoothing of the membrane to the gel, incomplete degassing of transfer solutions can cause air pockets to form. Evolving gas from Tris or, in the case of protein transfers, methanol, can disrupt the tight contact necessary between the membrane and the gel for successful transfers.
Smeared or skewed bands are often caused by uneven contact between the gel and the membrane, or the membrane and the chromatography paper. To avoid this problem, roll a pipet down the membrane after it has been applied to the gel, and once again over the chromatography paper after it has been applied to the membrane. Do not move the membrane until the transfer is complete, as this will cause smearing.

Troubleshooting Guide and Application Tips

Problems and Solutions

Unsuccesful Rehybridizations

My membrane is deteriorating during the rehybridization procedure?

If so, what type of membrane are you using? AppliChem offers three types of membranes recommended for rehybridization procedures: Pure Nylon Neutral, Reprobe Nylon Positively Charged, and Reprobe Nitrocellulose Supported, a supported pure nitrocellulose.
Pure Nylon Neutral membranes have largely replaced nitrocellulose because they are more resilient during applications requiring multiple reprobes. Reprobe Nitrocellulose Supported (a supported nitrocellulose) was developed for the same reason. The polyester support web used in manufacturing Reprobe Nitrocellulose Supported allows the membrane to be reprobed several times. Reprobe Nylon Positively Charged is similar in resiliency to Pure Nylon Neutral.

My application demands an extensive number of reprobes and I'm losing signal?

If so, what type of membrane are you using? Because the binding capacity of nitrocellulose is less than that of nylon (100 µg/cm² vs. 400 µg/cm²), the potential number of rehybridizations is fewer as compared to nylon membranes. Likewise, the binding capacity of charged nylon (Reprobe Nylon Positively Charged) membranes is generally greater than standard nylon, and subsequently charged nylon may have advantages during multiple reprobes. The number of reprobing steps is a function of the amount of hydrolysis to which the membrane is exposed during the protocol, and the additive effects of hot water, sodium hydroxide and an acidic environment.

My probe is not stripping from the membrane, how should I change my procedure?

Did you let the membrane dry after the initial probe was applied? Drying causes irreversible binding of DNA to microporous membranes. If this has occurred, look through the helpful tips listed below.

My probe won't strip from the membrane, how can I rescue this blot?

Try preparing a new probe and using a different detection protocol. For example, if you prepared a biotinylated probe and detected with a streptavidin conjugate, omit the biotin-streptavidin step during rehybridization by using a directly conjugated probe, such as an alkaline phosphatase conjugated probe. If you used a radioactive probe, use a chemiluminescent system to detect after the next hybridization (or vice versa). If you have enough time and are using radioactive probes (e.g. Protocol 11), simply let your first probe decay before the second round of hybridization.

Signal Problems

The nucleic acid did not transfer completely to the membrane, what should I do?

Blotchy or incomplete transfers are caused by poor contact between the gel and the membrane. Even after careful smoothing of the membrane to the gel, incomplete degassing of transfer solutions can cause air pockets to form. Evolving gas from Tris or, in the case of protein transfers, methanol, can disrupt the tight contact necessary between the membrane and the gel for successful transfers.

My Signal is low, what are the common reasons for this?

When you have low signal, it is best to check your reagents by performing extra controls. The most common reason for poor signal is a bad probe. Prepare a new probe and perform a dot blot comparing the old and new probes. Do you see a difference between the probes? Even nonradioactive probes can deteriorate during storage. Is the signal weak for the new probe as well? Then your detection enzymes may be bad or the reagents used to prepare the probe are bad. You might also blot a small amount of unlabeled complementary DNA and hybridize to the new probe. Are you seeing signal from the blotted probe but not the hybridized DNA? If so there could be a problem with your hybridization protocol, such as the wash temperature or your buffers. If you're using nonradioactive detection methods, test your enzymes and substrates as well.

Background Problems

I switched to a non-radioactive detection system, and now I have high background, what should I do?

Reprobe Nylon Positively Charged may be used particularily where multiple probing is involved. Additionally, AppliChem's positively charged Reprobe Nylon membrane shows superior results as compared to competitor membranes when non-radioactive detection is used.

Everything was working fine and now suddenly I have high backgrounds, Why?

Did you make up a new probe? If so, was there adequate separation of the unincorporated label from the incorporated? Are you using old solutions? There may be contamination. Usually in these cases it is best to prepare new solutions, new probes and use new reagents. This is often the fastest way to get your system working again.

Miscellaneous

My membrane changed color during my blotting procedure, should I be concerned?

No. Slight color changes in AppliChem's new positively charged membranes are expected and have no effect on results. These color changes will vary according to the blotting procedure used and the pH of solutions. AppliChem uses this color change to ensure quality during the manufacturing procedure.

High Backgrounds

Poor agitation during prehybridization and hybridization steps can lead to insufficient blocking of the entire membrane. Due to the strength of the internal support web, Reprobe Nitrocellulose Supported, Pure Nylon Neutral, and Reprobe Nylon Positively Charged transfer membranes can withstand higher levels of agitation without tearing or ripping. Incorrect probe concentration can occur when using dextran sulfate in hybridization or prehybridization solutions. Dextran sulfate causes the effective concentration of the probe to increase because it excludes the probe from the volume of solution the dextran sulfate polymer occupies. When using dextran sulfate, lower the probe solutions to less than 10 ng/ml of the solution. When not using dextran sulfate, maintain the optimum probe concentration at 25 – 40 ng/ml of solution.
Residual agarose on membranes can cause a fuzzy background to appear on blots. Be sure to wash nylon membranes with 5X SSPE at 60°C, after the immobilization step. Due to the strength of the membrane, supported membranes (Reprobe Nitrocellulose Supported, Pure Nylon Neutral and Reprobe Nylon Positively Charged Transfer Membranes) can be more easily washed without tearing or ripping.

Troubleshooting Gel Casting Procedures

Troubleshooting blotting problems begins with the correct gel casting procedures. Skewed, streaked, incomplete, or non-uniform transfers can be the results of poorly cast gels. The following recommendations are made for setting up the gel. Gels greater than 4mm thick can interfere with the free transfer of nucleic acids.
Be sure that the gel tray is level before casting the gel. If the surface is not level, non-uniform transfers may result. Maintain a gel casting temperature of 55°C – 70°C degrees, and be sure that the gel particles are completely dissolved. Undissolved agarose particles can result in streaked or skewed bands.
Immediately after gel casting and solidification, submerge the gel slab in electrophoresis buffer. This will prevent the formation of an impermeable "skin" over the surface of the gel which can inhibit transfer of nucleic acids from the gel.
After setting up the blotting assembly, be sure to:

- Invert the gel so that the underside of the gel is the side in contact with the membrane.
- Allow the transfer solutions enough time to "breathe", so that they may degas completely. Incompletely degassed transfer solutions evolve gas after the blotting assembly is set up, and can cause air bubbles between the membrane and gel that can impede the transfer of nucleic acids.

t r o u b l e s h o o t i n g & t i p s

Probe Related Background Problems

While there are several ways to decontaminate probe solutions, the following methods are two of the most efficient. The second method can be rapidly performed with minimum effort.

Method 1:
Phenol/Chloroform extract the probe to remove unincorporated nucleotides, proteins, and other contaminants.
Method 2:
Clean the probe by adding a small volume of the hybridization buffer to the probe and filtering it through a Cameo 25AS low protein binding cellulose acetate syringe filter (GE Osmonics Catalog #DDA02025S0; available at AppliChem). Contaminants in the probe solution will be held back by the 0.2 µm filter with no probe loss caused by nonspecific binding to the filtration membrane.
Probe length is also a factor contributing to background levels seen on transfer membranes. Between 250 – 800 base pairs is the recommended optimum length of a probe; probe lengths smaller or larger than this can lead to a low signal-to-noise ratio. Probes smaller than 250 base pairs often bind poorly and may require less stringent hybridization and wash procedures. Probes larger than 800 base pairs may contain a wider variety of size classes, which can lead to extraneous binding to the transfer membrane.

Hybridization Solution Related Background Problems

Contaminated hybridization solutions are another common source of background problems. Hybridization solutions should be filtered with a pure cellulose acetate Cameo 25AS syringe filter (GE Osmonics Catalog #DDA02025S0; available at AppliChem), to remove contaminants.
Additionally, all solutions and buffers should be made fresh before each transfer with sterile, double-distilled, deionized water, and very high grade reagents. After fresh buffers are made, they should be filtered with a Cameo 25AS syringe filter (GE Osmonics Catalog #DDA02025S0; available at AppliChem) to ensure that no contaminants remain in the solution. Formamide-based hybridization solutions are a frequent source of background noise, and the formamide must be freshly made and deionized.

DNA Dependent Background

This type of background is caused by nonspecific annealing of the probe to the bound DNA or RNA, and can be eliminated by adjusting the stringency of both the hybridization and wash steps, and by the addition of heterologous DNA or RNA to the hybridization solutions. During the hybridization and prehybridization steps, a concentration of 0.1 – 1 % SDS is recommended for nylon membranes. Because the final wash step after the hybridization is the most important, AppliChem recommends:
Northerns: 0.1X SSPE, 0.1 – 0.5 % SDS, for 30 minutes at 65°C.
Southerns: 0.1X SSPE, 0.5 – 1 % SDS, for 30 minutes at 65°C.

Optimized Blocking Solutions

A concentration of 5 – 7X Denhardt's solution is recommended for use with nylon membranes. Exceeding this level can lead to quenching of the signal.

Backgrounds Associated with Reprobing

A follow-up autoradiograph after probe removal is strongly recommended to determine if the probe has been fully stripped. Otherwise, backgrounds can appear in blots that have not been fully erased.

Protocols and Technical Papers – Survey

Selecting the appropriate membrane is critical to the success of protein transfer and nucleic acid procedures. Using the correct protocol with AppliChem membranes insures you that your procedure will perform with optimal results.
AppliChem provides the following tried and proven protocols developed by MSI to obtain consistent reproducible and dependable results when used with AppliChem' membranes.

1 Southern (DNA) and Northern (RNA) Hybridizations with Reprobe Nylon Positively Charged Membranes
Southern (DNA) and northern (RNA) protocol for our charged nylon – Reprobe Nylon Positively Charged Membrane – a membrane manufactured for your multi-probe needs.

2 Southern (DNA) and Northern (RNA) Hybridizations
Run Southern DNA and Northern (RNA) Hybridizations with Pure Nylon Neutral membranes

3 Southern (DNA) and Northern (RNA) Hybridizations
Protocol for use with Reprobe Nitrocellulose Supported and Pure Nitrocellulose Unsupported membranes.

4 Non-Radioactive Detection Systems with Pure Nylon Neutral
Procedure for non-radioactive systems – Reprobe Nylon Positively Charged may be used particulary where multiple probing is involved.

5 Non-Radioactive Detection Systems with Nitrocellulose Membranes
with Reprobe Nitrocellulose Supported Membranes and Pure Nitrocellulose Unsupported

6 Alkaline Blotting with Reprobe Nylon Positively Charged Transfer Membrane
Membranes with very high binding capacities are necessary for the success alkaline blotting

7 Colony Hybridizations and Plaque Lifts with Nylon Membranes
Perform colony hybridizations and plaque lifts with Pure Nylon and Reprobe Nylon Membranes

8 Colony Hybridizations and Plaque Lifts with Nitrocellulose Transfer Membranes
Perform colony hybridizations and plaque lifts with Reprobe Nitrocellulose Supported and Pure Nitrocellulose Unsupported Transfer Membranes.

9 Colony Hybridizations and Plaque Lifts with Pure Nylon Neutral Membranes
Perform colony hybridizations and plaque lifts with Pure Nylon Neutral Membranes

10 RNA Probes to be Used in Northern and Southern Blotting
Use Pure Nylon Neutral in northern and southern blotting

11 Oligonucleotide Probes to be Used in Northern and Southern Blotting
Use Pure Nylon Neutral membranes in Northern and Southern Blotting

12 Nucleic Acid Dot/Slot Blotting with Nylon Membranes
Purify DNA or RNA with Pure Nylon Neutral membranes

13 Nucleic Acid Dot/Slot Blotting with Nitrocellulose Membranes
Purify DNA or RNA with Reprobe Nitrocellulose Supported and Pure Nitrocellulose Unsupported Transfer Membranes.

14 Western (Protein) Blotting with Nitrocellulose Membranes
For use with Reprobe Nitrocellulose Supported and Pure Nitrocellulose Unsupported Transfer Membranes.

Protocols for DNA and RNA Applications

Protocol 1: Southern (DNA) and Northern (RNA) Hybridizations
with Reprobe Nylon Positively Charged Membranes

01 protocol

Gel Preparation

Southerns

Run DNA on an agarose gel with a running buffer of TAE or TBE. Rinse gel with dH_2O. Fragment DNA by immersing the gel in 0.2 N HCl for 10 minutes. Denature DNA by soaking gel in 1.0 M NaCl/0.5 M NaOH for 30 minutes. Neutralize the gel by soaking in 0.5 M Tris · HCl (pH 8.0), 0.5 M NaCl for 30 minutes.

Northerns

Run RNA under denaturing conditions in a Glyoxal, Formaldehyde, or Methyl Mercuric Hydroxide gel. Gel should be 0.8 – 1.5 % agarose, 2.5 – 5.0 mm thick. Stain with 33 µg/ml acridine orange in 10 mM $NaPO_4$ (pH 6.5), then destain 3 x 15 minutes in buffer. Or, stain with ethidium bromide, 1 µg/ml in 50 mM NaOH for 25 minutes, then destain in 200 mM NaOAc (pH 4.0), 2 x 20 minutes.

Membrane Preparation

Float membrane on distilled water, then immerse until thoroughly wet. Transfer the membranes to 20X SSC prior to blotting.

Capillary Transfer

When starting new experiments, it is highly recommended that controls be performed to ensure complete transfer. Stain the gel with ethidium bromide before and after transfer. Add 10 ml of a 10 mg/ml Ethidium Bromide stock to about 500 ml of Running Buffer. Soak the gel with gentle agitation for 20 minutes. If low levels of DNA need be visualized, destain the gel for 20 to 40 minutes.

Use a Transfer Buffer of 20X SSC. Cut three pieces of filter paper (chromatography grade) 7.6 cm (three inches) longer than the glass plate to be used for the capillary transfer. Place on top of a glass plate and then saturate it with Transfer Buffer. Place the gel on top of the filter paper. The bottom surface of the gel should face up. Remove all air bubbles between the gel and blotting surface with a gloved hand or by rolling a pipette across the surface. Place the membrane over the gel, again removing all air bubbles. Place 2 – 5 pieces of filter paper cut to the size of the gel over the assembly. Place the glass plate and the gel assembly on top of a glass baking tray filled with transfer buffer. Allow the bottom layer of filter paper to overhang into the transfer solution in the glass baking tray.

Place a 5 cm (2 inch) stack of paper towels on top of the gel assembly and secure it with a light weight. Make sure that the filter paper under the gel is completely saturated. The wicking action of the solution through the gel and up the paper towels allows the solution to transfer the DNA or RNA molecules to the membrane. Throughout the transfer, do not allow the paper on top of the gel to contact the paper below the gel. If the paper towels become saturated with transfer buffer, replace them with dry ones. After transfer, stain the gel with 0.5 µg/ml ethidium bromide to check transfer efficiency.

Alternative Transfer Systems

Vacuum blotting, semi-dry electroblotting, bi-directional transfers, and positive pressure blotting systems can all be used with AppliChem nylon membranes. Follow manufacturers instructions.

Immobilization

After blotting, wash the membranes in 5X SSPE at 60°C for 5 minutes. This is an optional step to remove residual agarose. The nucleic acid on the membrane is immobilized by drying thoroughly. This is accomplished by baking the membrane for 15 minutes at 80°C. It is not necessary to use a vacuum oven.

As an additional immobilization step, the dry membrane may be exposed to 5 mJ/cm^2 260 nm UV irradiation using a UV crosslinker. A slight improvement in signal may be observed after UV irradiation. If the immobilization procedure is skipped, the signal will be reduced. For applications involving large amounts of applied DNA (e.g., 10 ng of *Hind* III digested Lambda DNA), drying may not be required.

Hybridization Procedure

There are various formats used for hybridization: tubes in a hybridization oven, Tupperware in a shaking waterbath, or sealed bags in an oven. Heat-sealable bags conserve solution and generate less volume of radioactive waste (if radioactive detection procedures are employed.) Tubes within hybridization ovens also have this advantage. Tupperware use within a shaking waterbath uses more hybridization solution, but may help to achieve complete hybridization. The choice depends on available resources within the laboratory and on individual preference.

All hybridization solutions should be filtered before use with a sterile Cameo 25AS, 0.22 µm supported cellulose acetate filter (GE Osmonics catalog #DDA02025S0; available at AppliChem). (NOTE: Use only cellulose acetate filters; other membrane types may not perform comparably). The low binding Cameo 25AS will filter prehybridization and hybridization solutions without nonspecifically binding essential components of these solutions.
Before prehybridization wet the blot in dH_2O and then transfer the blot to whichever salt solution is used for hybridization. (For example, if 6X SSPE is used during hybridization, then wet the blot in dH_2O and transfer to 6X SSPE.

Prehybridization

AppliChem recommends that blots be placed in prehybridization solution for 30 minutes at 65°C. Place blots individually into prehybridization solution if many blots are being processed simultaneously. Use at least 0.25 ml/cm² of the following prehybridization buffers, complementary to the probe. Typically Salmon Sperm DNA is phenol/chloroform extracted, sheared, and heat denatured.

Southern Prehybridization Solution	Northern Prehybridization Solution
Denhardt's solution	5x Denhardt's solution
6X SSC	5x SSPE
1.0 % SDS	0.1 – 0.5 % SDS
100 µg/ml denatured DNA*	100 µg/ml denatured DNA*

* Denatured DNA is typically Salmon Sperm DNA, phenol/chloroform extracted, sheared, and heat denatured.

There are many variations on the hybridization procedure. AppliChem does not recommend inclusion of 10 % dextran sulfate or 10 % PEG unless fast hybridization times (e.g., 4 hours) are required. Also, dextran sulfate should not be used with oligonucleotide probes. Solutions containing 7 % SDS are not recommended. SSPE or SSC may be used, and the concentration may be as low as 2X or as high as 6X. The denatured DNA used depends on the application; it must not be complementary to the probe.

Hybridization

Remove prehybridization solution and replace with hybridization solution.
Hybridization temperature should be determined by the presence of formamide in the hybridization solution.

Hybridization Temperature Chart

Temperature	% Formamide	Hybridization Solution
42°C	50 %	Low Temperature Hybridization Solution
65°C	0 %	High Temperature Hybridization Solution

Low Temperature Hybridization Solution

Southerns	Northerns
50 % formamide	50 % formamide
5X Denhardt's solution	5X Denhardt's solution
6X SSPE	5X SSPE
0.2 % SDS	0.2 % SDS
100 µg/ml denatured DNA*	100 µg/ml denatured DNA*

* Denatured DNA is typically Salmon Sperm DNA, phenol/chloroform extracted, sheared, and heat denatured.

High Temperature Hybridization Solution

Southerns	Northerns
5X Denhardt's solution	5X Denhardt's solution
6X SSC	5X SSPE
1 % SDS	0.5 % SDS
100 µg/ml denatured DNA*	100 µg/ml denatured DNA*

* Denatured DNA is typically Salmon Sperm DNA, phenol/chloroform extracted, sheared, and heat denatured.

Alternative probes (oligonucleotide, RNA, or biotinylated probes) may also be used. See Protocol 11 for "Oligonucleotide Probes to be Used in Northern and Southern Blotting". Clean probe solutions by adding a small amount of hybridization solution to the probe, and filter it through a Cameo 25AS supported cellulose acetate syringe filter (GE Osmonics catalog #DDA02025S0; available at AppliChem) to eliminate any contaminants before they come into contact with the transfer membrane. The probe is typically used in excess during hybridization procedures. Calculate the amount of bound target DNA and be sure the total amount of probe is at least 10 fold greater. Probe concentration should not exceed 20 ng/ml. Increase the volume of the hybridization reaction if necessary so probe is in excess. Single copy genes or low copy message may require $1 - 5 \times 10^6$ cpm/ml. Radioactive probes should be labeled no more than 24 hours before hybridization. Nonradioactive probes may be stored at -20°C for up to 6 months. Denature the probe by placing it into 100 to 500 ml prehybridization solution (or TE buffer) and heating to 95°C for 5 minutes. Place on ice. Add the probe to the prehybridization solution to make hybridization solution. Hybridize at least 12 hours if PEG or dextran sulfate are not used. Usually this step is carried out overnight.

Post Hybridization Washing Steps

Briefly rinse the blots in 2X SSC. Then, wash twice 15 minutes with 2X SSC, 1 % SDS at 65°C and twice 15 minutes in 0.1X SSC, 1 % SDS at 65°C. Transfer to 2X SSC after washing. Use at least 1 ml of wash solution per square centimeter for all membranes. There are many variations on washing procedures. More or less stringent conditions may be used depending on the application. The washing procedure works for most applications.
After washing, remove excess moisture with paper towels. Washes for northern blots sometimes substitute SSPE for SSC.

Autoradiography and Detection

Important: If the membrane is to be rehybridized, do not allow it to dry past this point. This will cause irreversible binding of probe to the membrane. For radioactive probes, wrap the membrane in plastic wrap and expose film at -70°C in a cassette with an intensifying screen for 25 – 60 hours. For nonradioactive probes, follow the manufacturer's recommended procedure. Alkaline phosphatase conjugated streptavidin enzymes with biotin-labeled probes work well with AppliChem's Reprobe Nylon membranes.

Probe Removal (Stripping)

Important: Do not allow the membrane to dry. Wash in 0.4 M NaOH for 30 min at 45°C with moderate agitation. Wash in Membrane Neutralization Solution (1.5 M NaCl; 1 M Tris · HCl, pH 7.4) for 15 minutes at room temperature.
When using this procedure for the first time, confirm probe removal by re-exposing film. If non-radioactive methods are used to confirm probe removal, the membrane should be blocked with prehybridization solution for 30 minutes before detection, otherwise high background levels will be encountered.
The success of probe removal depends on the amount of applied DNA. This procedure has been tested for 12 to 15 rounds of stripping and reprobing with up to 1 ng of applied DNA.

Protocol 2: Southern (DNA) and Northern (RNA) Hybridizations

with Pure Nylon Neutral Membranes

Gel Preparation

Southerns

Run DNA on an agarose gel with a running buffer of TAE or TBE. Rinse gel with dH_2O. If necessary, fragment DNA by immersing the gel in 0.25 N HCl for 8 – 10 minutes. Denature DNA by soaking gel in 1.0 M NaCl/0.5 M NaOH two times for 20 minutes each. Neutralize the gel by soaking in 0.5 M Tris (pH 7.5), 1.5 M NaCl two time for 20 minutes each.

Northern

Run RNA under denaturing conditions in a Glyoxal, Formaldehyde, or Methyl Mercuric Hydroxide gel. Gel should be 0.8 – 1.5 % agarose, 2.5 – 5.0 mm thick. Stain with 33 µg/ml acridine orange in 10 mM $NaPO_4$ (pH 6.5), then destain 3 x 15 minutes in buffer. Or, stain with ethidium bromide, 1 µg/ml in 50 mM NaOH for 25 minutes, then destain in 200 mM NaOAc (pH 4.0), 2 x 20 minutes.

Membrane Preparation

Float membrane on distilled water, then immerse until thoroughly wet. Soak the membrane in the transfer buffer until use.

Capillary Transfer

Use a transfer buffer of 10X SSPE or SSC.

Cut three pieces of filter paper (chromatography grade) 7.5 cm (3 inches) longer than the glass plate to be used for the capillary transfer. Saturate the filter paper with transfer buffer and place on top of a glass plate.

Place the gel on top of the filter paper, and the membrane over the gel. Place 5 pieces of filter paper cut to the size of the gel over the assembly. Throughout the transfer, do not allow the paper on top of the gel to contact the paper below the gel. This is done by placing strips of Parafilm around the sides of the gel.

Place the glass plate and the gel assembly on top of a glass baking tray filled with transfer buffer. Allow the bottom layer of filter paper to overhang into the transfer solution in the glass baking tray. Place a 5 cm (2 inch) stack of paper towels on top of the gel assembly and secure it with a light weight. Make sure that the filter paper under the gel is completely saturated. The wicking action of the solution through the gel and up the paper towels allows the solution to transfer the DNA or RNA molecules to the membrane.

Secure plastic wrap over the entire assembly and place in a cold room for 3 hours to overnight. If the paper towels become saturated with transfer buffer, replace them with dry ones. After transfer, stain the gel with 0.5 µg/ml ethidium bromide to check transfer efficiency.

Alternative Transfer Systems

Vacuum blotting, semi-dry electroblotting, bi-directional transfers, and positive pressure blotting systems can all be used with AppliChem nylon membranes. Follow manufacturers instructions.

Immobilization

After blotting, wash the membranes in 5X SSPE at 60°C for 5 minutes. This is an optional step to remove residual agarose. The membrane can be immobilized by baking or UV crosslinking.

To bake the membrane a vacuum oven or convection oven may be used, place the membrane in a 65° – 80°C oven for 1 hour or until the membrane is completely dry.

The membrane may be UV crosslinked by exposing the membrane to a controlled UV source. Follow the instructions of the manufacturer of the crosslinker or expose a damp membrane to a transilluminator. The total exposure should be 120 mJ/cm. Increased exposure will cause a decrease in signal intensity after reprobing, proportional to the amount of over-exposure.

Hybridization Procedure

Hybridization is most commonly done in heat-sealable bags in order to conserve solution and protect researchers from exposure to radioactivity. All hybridization solutions should be filtered before use with a Cameo 25AS, 0.22 µm supported cellulose acetate filter (GE Osmonics catalog #DDA02025S0; available at AppliChem). (NOTE: Use only cellulose acetate filters; other membrane types may not perform comparably). The low binding Cameo 25AS will filter prehybridization and hybridization solutions without nonspecifically binding essential components of these solutions.

Prehybridization

This step should always be carried out at the temperature of the hybridization. Place the membrane in a heat-sealable bag without the probe in 0.25 ml/cm² of the following prehybridization buffer:

Southern Prehybridization Solution	Northern Prehybridization Solution
6X SSPE	5X SSPE
5X Denhardt's solution	50 % Formamide
0.5 – 1.0 % SDS	0.1 – 0.5 % SDS
50 µg/ml denatured DNA	100 µg/ml denatured DNA
10 % Dextran Sulfate	5X Denhardt's solution
	Shake one to two hours, at 42°C.

Hybridization

Remove prehybridization solution completely from bag, and add the hybridization solution.

Hybridization temperature should be determined by the presence of formamide in the hybridization solution.

Hybridization Temperature Chart

Temperature	% Formamide	Hybridization Solution
42°C	50 %	Low Temperature Hybridization Solution
65°C	0 %	High Temperature Hybridization Solution

Low Temperature Hybridization Solution

Southern	Northern
50 % formamide	50 % formamide
5X Denhardt's solution	5X Denhardt's solution
6X SSPE	5X SSPE
0.2 % SDS	0.2 % SDS
100 µg/ml denatured DNA	100 µg/ml denatured DNA
10 % dextran sulfate	10 % dextran sulfate

High Temperature Hybridization Solution

Southern	Northern
5X Denhardt's solution	5X Denhardt's solution
6X SSPE	5X SSPE
0.5 % SDS	0.5 % SDS
50 µg/ml denatured DNA	100 – 200 µg/ml denatured DNA
10 % dextran sulfate	10 % dextran sulfate

NOTE: When using nylon membranes, the potential for backgrounds is greater. Increased volumes of Denhardt's solution and SDS can help further block the membrane.

Dextran sulfate is a rate enhancer for probes larger than 200 base pairs and should not be used with oligonucleotide probes.

Alternative probes (oligonucleotide, RNA, or biotinylated probes) may also be used.

Clean probe solutions by adding a small amount of hybridization solution to the probe, and filter it through a Cameo 25AS supported cellulose acetate syringe filter (GE Osmonics catalog #DDA02025S0; available at AppliChem), to eliminate any contaminants before they come into contact with the transfer membrane.

Denature the probe by boiling in TE buffer for 5 minutes, or incubate with 0.1 volume of 1 N NaOH at 37°C for 5 minutes. Place on ice.

protocol 02

Add the decontaminated probe to the hybridization solution in the heat-sealable bag and reseal. Probe concentration should not exceed 20 ng/ml. Single copy genes or low copy message may require 1 – 5 x 10^6 cpm/ml. Probes should be labeled no more than 24 hours before hybridization.
Hybridize 12 hours to overnight. IMPORTANT: If the membrane is to be rehybridized, do not allow it to dry past this point. This will cause irreversible binding to the membrane.

Post Hybridization

Wash temperature should be 25°C below the Tm (melting temperature of the hybrid). If the homology between the probe and membrane-bound DNA is inexact, the wash temperature should be lower.

Stringency Washing Procedure

Low Stringency (for inexact matching)
2 x 15 minutes with 1X SSC, 0.1 % SDS at room temperature
2 x 15 minutes with 1X SSC, 0.1 % SDS at 37°C

Medium Stringency
2 x 15 minutes with 5X SSC, 0.5 % SDS at room temperature
2 x 15 minutes with 1X SSC, 0.5 – 1.0 % SDS at 37°C
1 x 15 minutes with 0.1X SSC, 1.0 % SDS at 37°C

High Stringency (for perfect hybrids)
2 x 15 minutes with 5X SSC, 0.5 % SDS at room temperature
2 x 15 minutes with 1X SSC, 0.5 – 1.0 % SDS at 37°C
3 x 15 minutes with 0.1X SSC, 1.0 % SDS at 65°C

NOTE: With nylon membranes, a medium or high stringency washing procedure is recommended to control potential background problems.
Use 0.5 ml of wash solution per square centimeter for all membranes. After washing, remove excess moisture with paper towels. Washes for Northern blots should substitute SSPE for SSC in all wash steps.

Autoradiography

Wrap the membrane in plastic wrap and autoradiograph at -70°C in a cassette with an intensifying screen while slightly damp. Expose the membrane for 25 – 60 hours.

Probe Removal

Do not allow the membrane to dry if a rehybridization step is intended. Wash in 5 mM Tris · HCl (pH 8.0), 0.2 mM EDTA, 0.05 % pyrophosphate, 0.1 Denhardt's for 1 – 2 hours at 65°C. Rinse in 1X SSPE. Or, wash in 50 % formamide, 6X SSPE at 65°C for 30 minutes. Rinse in 2X SSPE.

protocols

Protocol 3: Southern (DNA) and Northern (RNA) Hybridizations Nitrocellulose Membranes with Reprobe Nitrocellulose Supported, Pure Nitrocellulose Unsupported Transfer Membranes

protocol 03

protocols

Gel Preparation

Southerns
Run DNA on an agarose gel with a running buffer of TAE or TBE. If necessary, fragment gel in 0.25 N HCl. Rinse gel with dH_2O and denature DNA by soaking gel in 1.0 M NaCl/0.5 M NaOH 2 times for 20 minutes each. Neutralize the gel by soaking in 0.5 M Tris (pH 7.5), 1.5 M NaCl two times for 20 minutes each.

Northerns
Run RNA under denaturing conditions in a Glyoxal, Formaldehyde, or Methyl Mercuric Hydroxide gel. Gel should be 0.8 – 1.5 % agarose, 2.5 – 5.0 mm thick. Stain with 33 µg/ml acridine orange in 10 mM $NaPO_4$ (pH 6.5), then destain 3 x 15 minutes in buffer, or stain with ethidium bromide, 1 µg/ml in 50 mM NaOH for 25 minutes, then destain in 200 mM NaOAc (pH 4.0), 2 x 20 minutes.

Membrane Preparation

Float membrane on distilled water, then immerse until wet thoroughly. If wetting is not immediate, heat water until just under boiling temperature. Soak in the transfer buffer until use.

Gel Pretreatment

Southerns: Not necessary.
Northerns: Pretreatment, such as fragmentation of RNA, is unnecessary.

Capillary Transfer

Use a transfer buffer of 20X SSPE or SSC.
Cut 3 pieces of filter paper (chromatography grade) 7.5 cm (3 inches) longer than the glass plate to be used for the capillary transfer. Saturate the filter paper with transfer buffer and place on top of a glass plate.
Place gel on top of the filter paper, and the membrane over the gel. Place 5 pieces of filter paper cut to the size of the gel over the assembly. Throughout the transfer, do not allow the paper on top of the gel to contact the paper below the gel. This is done by placing strips of Parafilm around the sides of the gel.
Place the glass plate and the gel assembly on top of a glass baking tray filled with transfer buffer. Allow the bottom layer of filter paper to overhang into the transfer solution in the glass baking tray. Place a 5 cm (2 inch) stack of paper towels on top of the gel assembly and secure with a light weight. Make sure that the filter paper under the gel is completely saturated. The wicking action of the solution through the gel and up the paper towels allows the solution to transfer the DNA or RNA molecules to the membrane.
Secure plastic wrap over the entire assembly and place in a cold room for 3 hours to overnight. If the paper towels become saturated with transfer buffer, replace them with dry ones. After transfer, stain the gel with 0.5 µg/ml ethidium bromide to check transfer efficiency.

Alternative Transfer Systems

Vacuum blotting, semi-dry electroblotting, bidirectional transfers, and positive pressure blotting systems can all be used with AppliChem nitrocellulose membranes. Follow manufacturers instructions.

Immobilization

After blotting, wash the membranes in 5X SSPE at 60°C for 5 minutes. This is an optional step to remove residual agarose. The membrane can be immobilized by backing or UV crosslinking.
To bake the membrane a vacuum oven or convection oven may be used, place the membrane in a 65° – 80°C oven for 1 hour or until the membrane is completely dry.
The membrane may be UV crosslinked by exposing the membrane to a controlled UV source. Follow the instructions of the manufacturer of the crosslinker or expose a damp membrane to a controlled UV source, total exposure should be 120 mJ/cm. Increased exposure will cause a decrease in signal intensity after reprobing, proportional to the amount of over-exposure.

Hybridization Procedure

Hybridization is most commonly done in heat-sealable bags in order to conserve solution and protect researchers from exposure to radioactivity. All hybridization solutions should be filtered before use with Cameo 25AS, 0.22 µm supported cellulose acetate filter (GE Osmonics catalog #DDA02025S0; available at AppliChem) (NOTE: Use only cellulose acetate filters; other membrane types may not perform comparably). The low binding Cameo 25AS will filter prehybridization and hybridizaton solutions without nonspecifically binding essential components of these solutions.

Prehybridization

This step should always be carried out at the temperature of the hybridization. Place the membrane in a heat-sealable bag without the probe in 0.1 ml/cm² of the following prehybridization buffers.

Southern Prehybridization Solution	Northern Prehybridization Solution
5X SSPE	5X SSPE
50 % Formamide	50 % Formamide
5X Denhardt's solution	5X Denhardt's solution
0.5 % SDS	0.1 – 0.5 % /SDS
100 µg/ml denatured DNA	100 µg/ml denatured DNA
10 % dextran sulfate	10 % dextran sulfate

Nitrocellulose membranes should be wet with 1X SSPE, 0.1 % SDS before prehybridization step.
Shake 1 to 2 hours.

Hybridization

Remove prehybridization solution completely from bag, and add the hybridization solution.
Hybridization temperature should be determined by the presence of formamide in the hybridization solution.

Hybridization Temperature Chart

Temperature	% Formamide	Hybridization Solution
42°C	50 %	Low-Temperature Hybridization Solution
65°C	0 %	High-Temperature Hybridization Solution

Low-Temperature Hybridization Solution

Southerns	Northerns
50 % Formamide	50 % Formamide
5X Denhardt's solution	5X Denhardt's solution
6X SSPE	5X SSPE
0.2 % SDS	0.2 % SDS
100 – 200 µg/ml denatured DNA	100 – 200 µg/ml denatured DNA
10 % dextran sulfate	10 % dextran sulfate

High-Temperature Hybridization Solution

Southerns	Northerns
5X Denhardt's solution	5X Denhardt's solution
6X SSPE	5X SSPE
0.5 % SDS	0.5 % SDS
50 µg/ml denatured DNA	100 µg/ml denatured DNA
10 % dextran sulfate	10 % dextran sulfate

Dextran sulfate is a rate enhancer for probes larger than 200 base pairs and should not be used with oligonucleotide probes. Alternative probes (oligonucleotide, RNA, or biotinylated probes) may also be used.

Clean probe solutions by adding a small amount of hybridization solution to the probe, and filter it through a Cameo 25AS supported cellulose acetate syringe filter (GE Osmonics catalog #DDA02025S0; available at AppliChem), to eliminate any contaminants before they come into contact with the transfer membrane.
Denature the probe by boiling in TE buffer for 5 minutes, or incubate with 0.1 volume of 1 N NaOH at 37°C for 5 minutes. Place on ice.
Add the decontaminated probe to the hybridization solution in the heat-sealable bag and reseal. Probe concentration should not exceed 20 ng/ml. Single copy genes or low copy message may require 1 – 5 x 10^6 cpm/ml. Probes should be labeled no more than 24 hours before hybridization.
Hybridize 12 hours to overnight. Important: If the membrane is to be rehybridized, do not allow it to dry past this point. This will cause irreversible binding to the membrane.

Post Hybridization

Wash temperature should be 25°C below the Tm (melting temperature of the hybrid). If the homology between the probe and membrane-bound DNA is inexact, the wash temperature should be lower.

Stringency Washing Procedure

Low Stringency (for inexact matching)
2 x 15 minutes with 1X SSC, 0.1 % SDS at room temperature
2 x 15 minutes with 1X SSC, 0.1 % SDS at 37°C

Medium Stringency
2 x 15 minutes with 5X SSC, 0.5 % SDS at room temperature
2 x 15 minutes with 1X SSC, 0.5 – 1.0 % SDS at 37°C
1 x 15 minutes with 0.1X SSC, 1.0 % SDS at 37°C

High Stringency (for perfect hybrids)
2 x 15 minutes with 5X SSC, 0.5 % SDS at room temperature
2 x 15 minutes with 1X SSC, 0.5 – 1.0 % SDS at 37°C
3 x 15 minutes with 0.1X SSC, 1.0 % SDS at 65°C

NOTE: With nitrocellulose membranes, an initial low stringency washing procedure is recommended.
Use 0.5 ml of wash solution per square centimeter for all membranes. After washing, remove excess moisture with paper towels.

Autoradiography

Wrap the membrane in plastic wrap and autoradiograph at -70°C in a cassette with an intensifying screen while slightly damp. Expose the membrane for 25 – 60 hours.

Probe Removal

Do not allow the membrane to dry if a rehybridization step is intended.
For Pure Nitrocellulose Unsupported: Boil 0.1X SSPE or SCC, 0.1 % SDS and add blot after removing from heat. Check for residual probe activity.
For Reprobe Nitrocellulose Supported: Boil in distilled water for 5 minutes. Check for residual probe activity. If necessary, boil for additional 5 minutes.

Protocol 4: Non-Radioactive Detection Systems with Pure Nylon Neutral

There are many advantages when hybridizing with non-radioactive probes. The disposal problems associated with radioactive materials are eliminated with the use of nonisotopic probes. Biotinylated probes are more stable than isotopic probes, and have better signal-to-noise ratios. Increased amounts of probe can be used, cutting hybridization times by several hours. Pure Nylon Neutral is a chemically optimized hybridization membrane that demonstrates superior binding and reduced backgrounds when using biotinylated probe systems. Pure Nylon Neutral is the recommended membrane when working with chemiluminescent detection systems. Charge-modified nylon membranes are difficult to use with non-radioactive detection systems because background noise can obscure the bands. Reprobe Nylon Positively Charged may be used particularly where multiple probing is involved.

Gel

Run an agarose gel in TBE buffer, and stain with ethidium bromide (1.0 µg/ml) for 15 minutes.

Membrane Preparation

Submerge the membrane in deionized water to moisten, and soak in 10X SSC for 30 minutes prior to transfer.
For electrophoretic transfers, soak in 1X TAE.

Gel Preparation

Depurinate the gel in 0.25 M HCl for 30 minutes while gently agitating, with one exchange at 15 minutes. For preparations involving whole plasmids, extend the depurination time to 40 minutes, with exchanges at 15 and 30 minutes. Denature the gel in 1.5 M NaCl, 0.5 M NaOH for 1 hour with one exchange at 30 minutes. Place the gel in 1.5 M NaCl, 1 M Tris · HCl (pH 8.0) for 1 hour with one exchange at 30 minutes in order to neutralize the gel.

Transfer

Transfer the DNA to the membrane by Southern blotting (either vacuum or capillary) and restain the gel with ethidium bromide to determine transfer efficiency. See Protocol 1 for Southern (DNA) and Northern (RNA) Hybridizations with Reprobe Nylon Positively Charged Membranes.

Immobilization

After blotting, wash the membrane in 5X SSPE at 60°C for 5 minutes. This is an optional step to remove residual agarose. Bake membrane at 80°C until dry or UV crosslink per manufacturer's instructions. Do not exceed 120 mJ/cm ^{2} . Increased exposure will cause a decrease in signal intensity after reprobing, proportional to the amount of overexposure.

Probe Preparation

Ideally, probes should be between 400 – 600 base pairs. Probe sizing gels, using the appropriate size standards, should be run with the probes after labelling.

Prehybridization

Prewash the membrane in 2X SSC, 1 % SDS at 42°C for 1 hour.
Block membranes in the prehybridization solution for 30 – 40 minutes at room temperature.

Prehybridization Buffer

Tris-buffered saline/pH 7.5 (0.1 M Tris · HCl, 0.5 M NaCl)
1.0 % Casein
3.0 % Liquid Hipure Gelatin
0.05 % Tween-20

Hybridization

Convert the probe to its single stranded form by adding an equal volume of 0.2 N NaOH and incubate at 37°C 1 – 2 hours. Remove the prehybridization buffer, add the hybridization buffer with the probe, and incubate at 42°C 12 hours or overnight with gentle agitation.

Hybridization Buffer

45 % deionized formamide
1X Denhardt's solution
5X SSC
0.1 % SDS
10 % Dextran Sulfate
20 mM Phosphate buffer/pH 7.0

Washes

When the hybridization is complete, wash the membrane in small trays in the following wash solutions:
0.16X SSC, 0.1 % SDS, 2 times for 3 minutes each at room temperature
0.16X SSC, 0.1 % SDS, 2 times for 3 minutes each at 64°C;
Rinse in 2X SSC, 2 times for 3 minutes each at room temperature.

Post Hybridization

Block the filter by rinsing for one minute in Buffer 1, followed by an incubation step in 3 % Fraction V BSA in freshly made Buffer 1 for one hour at 65°C. Repeat incubation step with fresh buffer for an additional 30 minutes.

Buffer 1

0.1 M Tris · HCl (pH 7.5)
0.1 M NaCl
2 mM Magnesium Chloride
0.05 % Triton-X 100

Detection

Remove the membrane from the hybridization solution, and wash in TBS buffer with 5 % Tween-20 for 5 minutes. Additional washes in TBS buffer may be required.
Add the streptavidin-enzyme complex according to manufacturer's instructions.

Recommended Buffer Solutions

Alkaline Phosphatase Buffer Solution	Horseradish Peroxidase Substrate Buffer Solution
100 mM Tris · HCl (pH 9.1)	2 mg/ml Tetramethyl Benzidene in
100 mM NaCl	100 % Ethanol and H_2O_2 at 0.0014 %
5 mM $MgCl_2$	0.01 M Sodium Citrate
5 mM Nitro Blue Tetrazolium	0.01 M EDTA (pH 5.0)
3 mM 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)	

Post Detection

After the membranes are adequately stained, rinse briefly in deionized water. Dry the membranes between filter paper, or bake in a vacuum oven.

protocol 05

Protocol 5: Non-Radioactive Detection Systems with Nitrocellulose Membranes

with Reprobe Nitrocellulose Supported Membranes and Pure Nitrocellulose Unsupported

There are many advantages when hybridizing with non-radioactive probes. The disposal problems associated with radioactive probes are eliminated with the use of non-isotopic probes. Biotinylated probes are more stable than isotopic probes, and have better signal-to-noise ratios. Increased amounts of probe can be used, cutting hybridization times by several hours. Reprobe Nitrocellulose Supported membranes are strong and easy to use with biotinylated detection systems. Traditional nitrocellulose membranes can sometimes be difficult to use because the fragility of the membrane can cause blue lines and spots when using biotinylated probes. However, they require no excessive blocking steps.

Gel

Run an agarose gel in TBE buffer, and stain with ethidium bromide (1.0 µg/ml) for 15 minutes.

Membrane Preparation

Submerge the membrane in deionized water to moisten, and then soak in 20X SSC, for 30 minutes before transfer.

Gel Preparation

Depurinate the gel in 0.25 M HCl for 30 minutes while gently agitating, with one exchange at 15 minutes. For preparations involving whole plasmids, extend the depurination time to 40 minutes, with exchanges at 15 and 30 minutes. Denature the gel in 1.5 M NaCl, 0.5 M NaOH for 1 hour with one exchange at 30 minutes. Place the gel in 1.5 M NaCl, 1 M Tris · HCl (pH 8.0) for 1 hour with one exchange at 30 minutes in order to neutralize the gel.

Transfer

Transfer the DNA to the membrane by Southern blotting, (either vacuum or capillary) and restain the gel with ethidium bromide to determine transfer efficiency.

Immobilization

Bake the membrane in a vacuum oven at 80°C for two hours.

Probe Preparation

Ideally, probes should be between 400 – 600 base pairs. Probe sizing gels, using the appropriate size standards, should be run with the probes after labelling.

Prehybridization

Incubate the blot in a heat-sealable bag with 0.1 ml/cm² prehybridization buffer (without the probe) for four hours at 42°C.

Prehybridization Solution

50 % Formamide
5X SSC
5X Denhardt's solution
25 mM sodium phosphate (pH 6.5)
Denatured salmon sperm DNA (0.5 mg/ml)

Hybridization

Convert the probe to its single stranded form by adding an equal volume of 0.2 N NaOH and incubate at 37°C. Remove the prehybridization buffer, add the hybridization buffer with the probe, and incubate at 42°C overnight with gentle agitation.

Hybridization Buffer

45 % Formamide
5X SSC
5X Denhardt's solution
20 mM sodium phosphate (pH 6.5)
Denatured salmon sperm DNA (0.2 mg/ml)
Biotinylated probe (heat denatured)

Washes

When the hybridization is complete, wash the membrane in small trays in the following wash solutions:

2X SSC, 0.1 % SDS	6 minutes with one exchange at 3 minutes at room temperature
0.2X SSC, 0.1 % SDS	6 minutes with one exchange at 3 minutes at room temperature
0.16X SSC, 0.1 % SDS	30 minutes with one exchange at 15 minutes at room temperature

Post Hybridization

Block the filter by rinsing for one minute in Buffer 1, followed by an incubation step in 3 % Fraction V BSA in freshly made Buffer 1 for one hour at 65°C.

Buffer 1	Buffer 2
2 mM Magnesium Chloride	0.1 M Tris · HCl (pH 9.5)
0.1 M Tris · HCl (pH 7.5)	0.1 M NaCl
0.1 M NaCl	50 mM Magnesium Chloride
0.05 % Triton-X 100	

Chromogenic Detection

Expose the blot to 7.0 ml of streptavidin detection solution (prepare using 1.0 µl streptavidin-alkaline phosphatase stock solution per 1.0 ml of Buffer 1) per 100 cm²of filter area for 5 minutes/side with gentle mixing. Wash the membrane in Buffer 1 for 30 minutes (with one exchange at 15 minutes) and Buffer 2 for 10 minutes.

Visualization

For each 100 cm² of membrane, prepare a solution containing 33 µl of Nitro Blue Tetrazolium (75 mg/ml in 70 % dimethylformamide) and 25 µl of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; 50 mg/ml in dimethylformamide) in 7.5 ml of the incubation mixture referenced above (Buffer 1 with 3 % Fraction V BSA). Place the membrane and solution in a heat-sealable bag in the dark. Color development should occur within 30 minutes.

Post Detection

Stop the reaction by allowing the membrane to soak for 1 – 2 minutes in a solution of 20 mM Tris (pH 7.5), 0.5 mM disodium EDTA, followed by baking for 1 – 2 minutes at 80°C.

Alkaline Phosphatase Buffer Solution	Horseradish Peroxidase Substrate Buffer Solution
100 mM Tris · HCl (pH 9.1)	2 mg/ml Tetramethyl Benzidene in
100 mM NaCl	100 % Ethanol and H_2O_2 at 0.0014 %
5 mM $MgCl_2$	0.01 M Sodium Citrate
5 mM Nitro Blue Tetrazolium	0.01 M EDTA (pH 5.0)
3 mM 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)	

After membranes are adequately stained, rinse briefly in deionized water. Dry the membranes between filter paper, or bake in a vacuum oven.

protocol 06

Protocol 6: Alkaline Blotting with Reprobe Nylon Positively Charged Transfer Membrane

Membranes with very high binding capacities are necessary for the success of this technique. For this reason, Reprobe Nylon membranes are recommended for use in alkaline blotting.

Membrane Preparation

Wet Reprobe Nylon in dH_2O, then soak in freshly prepared transfer buffer (0.4 M NaOH/0.6 M NaCl).

Gel Preparation

Soak the agarose gel in transfer buffer for 15 minutes with gentle agitation. Use a 4:1 ratio of buffer to gel. Fragment the DNA by incubation in 0.25 N HCl for 15 minutes (use a 2:1 acid-to-gel ratio) and then soak in transfer buffer for 30 minutes.

Transfer

Capillary blot DNA to Reprobe Nylon membrane (see Protocol 1 for the Southern blotting procedure).

Neutralization

Neutralize membranes in 0.5 M Tris · HCl (pH 7.0), 1 M NaCl for 15 minutes at room temperature.

Immobilization

Bake membrane at 80°C until dry or UV crosslink per manufacturer's instructions. Do not exceed 120 mJ/cm². Increased exposure will cause a decrease in signal intensity after reprobing, proportional to the amount of overexposure.

Hybridization

Hybridize by standard Southern procedures for nylon membranes (see Protocol 1 for additional information).

protocols

Protocol 7: Colony Hybridizations and Plaque Lifts with Nylon Membranes

with Pure Nylon Neutral and Reprobe Nylon Positively Charged Transfer Membranes.

protocol 07

Membrane Preparation

If necessary, sterilize the membrane between two pieces of filter paper in the autoclave for 15 minutes. Allow the membrane to wet on the agar surface (or appropriate medium) on the petri dish. Mark the membrane so that future orientation is assured.

Colony Transfer

Step 1:

Incubate cell colonies and plaques at 37°C until they are 0.5 – 1.0 mm in diameter. Alternatively, the cell colonies may be grown after the membrane and appropriate cell media have been added to the plate.

Step 2:

Replication. With forceps, lift the membrane from the surface of the plate and place it colony/plaque side up on two sheets of dry filter paper. Carefully place a fresh membrane over the one with the colonies, and apply gentle pressure with a replication tool or glass plate. Avoid smearing the colonies when separating the membranes. To amplify plasmids, transfer the membrane to an agar plate with 200 – 250 µg/ml of chloramphenicol at 37°C for 10 hours.

Plaque Transfer

Step 1:

Cells should be plated with phage in soft agarose and incubated at 37°C until plaques are 0.2 mm. After incubation, chill plate at 4°C for 15 minutes to set the agarose.

Step 2:

Place membrane on the plate in complete contact with the agarose and assure orientation by marking. Allow phage to transfer for 2 minutes. Increase the transfer time if many transfers are to be performed.

Isolation

Lay membrane on filter paper prewet with 0.5 N NaOH (add 1.5 M NaCl for plaques) for 5 – 7 minutes to denature the nucleic acid.

Immobilization Colonies

Bake at 80°C until dry or UV crosslink per manufacturer's instructions. Neutralize the membrane on filter paper saturated with 1 M Tris · HCl (pH 8.0) for 5 minutes. Incubate on filter paper saturated with 1 M Tris · HCl (pH 8.0), 1.5 M NaCl for 5 minutes. Wash in 2X SSC. Bake membranes for 15 – 30 minutes until dry at 80°C.

Plaques

Neutralize and incubate the membrane on filter paper saturated with 0.5 M Tris · HCl (pH 8.0), 1.5 M NaCl. Place the membrane on sheets wet with 2X SSC and blot dry. Bake membranes until dry for 15 – 30 minutes at 80°C.

Hybridization

Use 5 – 10 ml of prehybridization buffer solution per membrane, with gentle agitation. Prehybridize for 1 hour in a heat-sealable bag.

Prehybridization Buffer

50 % Formamide

1 – 3X Denhardt's solution

5X SSPE

0.1 – 0.5 % SDS

100 – 200 µg/ml denatured DNA*

Hybridize in freshly made prehybridization buffer with 1 – 5 x 10^6 cpm/ml probe, or approximately 5 – 20 ng/ml. Incubate for 12 – 20 hours at 45°C.

* Denatured DNA is typically Salmon Sperm DNA, phenol/chloroform extracted, sheared, and heat denatured.

Detection

Autoradiograph on X-ray film at -70°C for 48 hours or more.

Probe Removal (Stripping)

See probe removal details in the appropriate sections of this protocol please see Protocol 1.

protocols

protocol 08

Protocol 8: Colony Hybridizations and Plaque Lifts with Nitrocellulose Transfer Membranes with Reprobe Nitrocellulose Supported, Pure Nitrocellulose Unsupported Membranes

Membrane Preparation
If necessary, sterilize the membrane between 2 pieces of filter paper in the autoclave for 15 minutes. Allow the membrane to wet on the agar surface. Mark the membrane so that future orientation is assured.
NOTE: Do not autoclave the membrane with the separator paper.

Colony Transfer
Step 1:
Incubate cell colonies and plaques at 37°C until they are 0.5 – 1.0 mm in diameter. Alternatively, the cell colonies may be grown after the membrane and appropriate cell media have been added to the plate.
Step 2:
Replication. With forceps, lift the membrane from the surface of the plate and place it colony/plaque side up on two sheets of dry filter paper. Carefully place a fresh membrane over the one with the colonies, and apply gentle pressure with a replication tool or glass plate. Avoid smearing the colonies when separating the membranes. To amplify plasmids, transfer the membrane to an agar plate with 200 – 250 µg/ml of chloramphenicol at 37°C for 10 hours.

Plaque Transfer
Step 1:
Cells should be plated with phage in soft agarose and incubated at 37°C until plaques are 0.2 mm. After incubation, chill plate at 4°C for 15 minutes to set the agarose.
Step 2:
Place membrane on the plate in complete contact with the agarose and assure orientation by marking. Allow phage to transfer for 5 minutes. Increase the transfer time if many transfers are to be performed.

Isolation and Immobilization
Lay membrane on filter paper prewet with 0.5 N NaOH (add 1.5 M NaCl for plaques) for 5 – 7 minutes to denature the nucleic acid.

Colony Hybridization
Neutralize the membrane on filter paper saturated with 1 M Tris · HCl (pH 8.0) for 5 minutes. Incubate on filter paper saturated with 1 M Tris · HCl (pH 8.0), 1.5 M NaCl for 5 minutes. Wash in 2X SSC.

Plaques
Neutralize and incubate the membrane on filter paper saturated with 0.5 M Tris · HCl (pH 8.0), 1.5 M NaCl. Place the membrane on sheets wet with 2X SSC and blot dry.

Immobilization
Bake membranes until dry for 15 – 30 minutes at 80°C. Nitrocellulose membranes must always be baked in a vacuum oven.

Hybridization
Use 5 – 10 ml of prehybridization buffer solution per membrane with gentle agitation. Prehybridize for 1 hour in a heat-sealable bag.

Prehybridization Buffer
50 % Formamide
5X SSPE
1 – 3X Denhardt's solution
0.1 – 0.5 % SDS
100 – 200 µg/ml denatured DNA
Hybridize in freshly made prehybridization buffer with 1 – 5 x 10^6 cpm/ml probe, or approximately 5 – 20 ng/ml. Incubate for 12 – 20 hours at 45°C.

Detection
Autoradiograph on X-ray film at -70°C for 48 hours or more.

Probe Removal
See Southern and Northern Hybridizations for reprobing procedures (see Protocol 3).

Protocol 9: Colony Hybridization and Plaque Lifts with Pure Nylon Neutral Membranes

protocol 09

Membrane Preparation
If necessary, sterilize the membrane between two pieces of filter paper in the autoclave for 15 minutes.

Colony Transfer
Step 1:
Incubate cell colonies and plaques at 37°C until they are 0.5 – 1.0 mm in diameter. Alternatively, the cell colonies may be grown after the membrane and appropriate cell media have been added to the plate. Place membranes on the plate with the center touching first so that the membranes wet from the center out to the edge. Assure orientation by marking.
Step 2:
Replication. With forceps, lift the membrane from the surface of the plate and place it colony/plaque side up on two sheets of dry filter paper. Carefully place a fresh membrane over the one with the colonies, and apply gentle pressure with a replication tool or glass plate. Avoid smearing the colonies when separating the membranes. To amplify plasmids, transfer the membrane to an agar plate with 200 – 250 µg/ml of chloramphenicol at 37°C for 10 hours.

Plaque Transfer
Step 1:
Cells should be plated with phage in soft agarose and incubated at 37°C until plaques are 0.2 mm. After incubation, chill plate at 4°C for 15 minutes to set the agarose.
Step 2:
Place membrane on the plate in complete contact with the agarose and assure orientation by marking. Allow phage to transfer for 5 minutes. Increase the transfer time if many transfers are to be performed.

Isolation and Immobilization
Lay membrane on plastic wrap with a pool of 0.5 N NaOH (add 1.0 M NaCl for plaques) for 10 minutes to denature the nucleic acid. Remove circles from plastic wrap and transfer them to filter paper to absorb excess moisture for 2 minutes. If filter paper absorbtion is incomplete, transfer the circles to fresh filter paper.

Colonies
Neutralize the membrane on plastic wrap with pools of 0.5 M Tris · HCl (pH 8.0), 0.5 M NaCl for 2 minutes and transfer them to the filter paper to absorb excess moisture. If filter paper absorbtion is incomplete, transfer the circles to fresh filter paper. Rinse in 2X SSC.

Plaques
Neutralize and incubate the membrane on filter paper saturated with 0.5 M Tris · HCl (pH 8.0), 1.5 M NaCl. Place the membrane on sheets wet with 2X SSC and blot dry. Bake membranes until dry for 15 – 30 minutes at 80°C.

Hybridization
See prehybridization, hybridization, and post-hybridization procedures within the Southern and Northern Hybridization section.

Detection
Autoradiograph on X-ray film at -70°C for 48 hours or more.

Probe Removal
See Southern and Northern Hybridizations for probe removal details.

protocol 10

Protocol 10: RNA Probes to be Used in Northern and Southern Blotting

RNA Probes with Pure Nylon Neutral Membranes

1. **Pre-Hybridization Conditions:**

   5X SSPE

   1.0 % SDS

   50 % Formamide

   10X Denhardt's Reagent

   Pre-Hybridization be performed for 1 – 2 hours between 50 – 60°C.

   NOTE: For probe denaturation, incubate the probe at 100°C for 5 minutes, then immediately chill on ice.
2. **Remove the Pre-Hybridization Buffer**
3. **Hybridization Conditions:**

   5x SSPE

   1.0 % SDS

   50 % Formamide

   20 ng/ml RNA Probe

   Hybridization should be performed in a shaker bath for 1 hour at TH.
4. **Washing Conditions: Wash the blot three times in 1X SSPE and 0.1% SDS for 20 minutes each at 65° C.**
5. **Final Wash Conditions: Wash the blot in 0.1X SSPE and 0.1% SDS for 20 minutes at 65° C.**

RNA Probes with Pure Nitrocellulose Unsupported and Reprobe Nitrocellulose Supported Membranes

1. **Pre-Hybridization Conditions:**

   6X SSPE

   0.5 % SDS

   5X Denhardt's Reagent

   Pre-Hybridization should be performed at 60°C for 30 minutes to 1 hour.
2. **Remove the Hybridization Buffer**
3. **Hybridization Conditions:**

   6X SSPE

   0.5 % SDS

   5X Denhardt's Reagent

   100 µg/ml RNA

   Hybridization should be carried out with 10^6 – 10^7 cpm/ml end labeled probe at TH for up to 20 hours.
4. **Washing Conditions: Wash the blot three times in 1X SSPE and 0.1 % SDS for 20 minutes at 65°C.**
5. **Final Wash Conditions: Wash the blot in 0.1X SSPE and 0.1 % SDS for 20 minutes at 65°C.**

NOTE: Background removal is accomplished by incubating the blot in 1.0 µg/ml RNase A in 2X SSC for 20 minutes at room temperature. Next rinse the blot in 1X SSPE and 0.1 % SDS.

Protocol 11: Oligonucleotide Probes to be Used in Northern and Southern Blotting

For Pure Nylon Neutral Membranes

protocol 11

Non Isotopic Detection Systems

1. **Pre-Hybridization Conditions:**

   5X SSPE

   1.0 % SDS

   50 % Formamide

   5X Denhardt's Reagent

   Pre-Hybridization be performed at TH (Hybridization Temperature), for 1 – 2 hours.

   NOTE: High concentrations of blocking reagent such as Denhardt's will quench signal. Concentrations of up to 10X can be used. Some common substitutions for Denhardt's Reagent are Heparin, Gelatin, and commercially produced blocking powders. Non-fat dry milk is not recommended for use due to it's potential for quenching signal.
2. **Remove the Pre-Hybridization Buffer**
3. **Hybridization Conditions:**

   5X SSPE

   1.0 % SDS

   50 % Formamide

   20 µg/ml oligonucleotide

   Hybridization should be performed in a shaker bath for 1 hour at TH.
4. **Washing Conditions: Wash the blot twice for 5 minutes each in 500 ml 2X SSPE and 0.1% SDS at room temperature.**
5. **Final Wash Conditions: Wash the blot twice for five minutes each in 500 ml of 0.1X SSPE at TH.**

Isotopic Detection Systems

1. **Pre-Hybridization Conditions:**

   6X SSPE

   0.5 % SDS

   5X Denhardt's Reagent

   Pre-Hybridization should be carried out at TH for 30 minutes to 1 hour
2. **Remove the Pre-hybridization Buffer**
3. **Hybridization Conditions:**

   6X SSPE

   0.5 % SDS

   5X Denhardt's Reagent

   100 – 200 µg/ml tRNA

   Hybridization should be carried out with 10^6 – 10^7 cpm/ml end labeled probe at TH.
4. **Washing Conditions: Wash the blot three times in 1X SSPE and 0.1 % SDS for 20 minutes at 65°C.**
5. **Final Wash Conditions: Wash the blot in 0.1X SSPE and 0.1 % SDS for 20 minutes at TH.**

p r o t o c o l s

protocol 12

Protocol 12: Nucleic Acid Dot/Slot Blotting with Nylon Membranes
with Pure Nylon Neutral Membranes

Sample Preparation
Purify DNA or RNA by cell lysis or by standard methods, and suspend in 50 µl of TE buffer (pH 8.0). For RNA, adjust the pH to 7.5.
DNA: Denature DNA with the addition of 0.1 volume of 3 M NaOH. Incubate for 1 hour at 60°C. Cool and add 1 volume 2 M NH_4OAc (pH 7.0), or 6X SSC.
RNA: Denature RNA by adding 35 µl of 20X SSC, and 20 µl of 37 % formaldehyde. Incubate for 15 minutes at 60°C.

Membrane Preparation
Wet membranes in dH_2O, then soak in the appropriate buffer.
DNA - Soak in 1 M NH_4OAc (pH 7.0), or 6X SSC.
RNA - Soak in 20X SSC.

Sample Application
If using a filtration manifold, cut 2 pieces of filter paper to the size of the manifold plate and soak them in:
DNA - 6X SSC
RNA - 20X SSC
Place filter paper, then membrane onto the sample plate and secure the dot blotting or slot blotting unit. Apply low vacuum, and wash the wells with either 500 µl of 1 M NH_4OAc (pH 7.0), or 6X SSC for DNA, 500 µl of 20X SSC for RNA. Apply sample (not to exceed 10 µg DNA or RNA/well), under low vacuum. Remove membrane. If working with RNA, rinse each well again with 500 µl of 20X SSC.
If dotting directly onto the membrane, apply 2 – 5 µl samples to membrane placed on 2 sheets of dry filter paper. Allow to dry.

Immobilization
After blotting wash the membrane in 5X SSPE at 60°C for 5 minutes. This is an optional step to remove residual agarose. The membrane can be immobilized by baking or UV crosslinking.
To bake the membrane a vacuum or convection oven may be used, place the membrane in a 65°– 80°C oven for 1 hour or until membrane is completely dry.
The membrane may be UV crosslinked by exposing the membrane to a controlled UV source such as a transilluminator. Follow the instructions of the manufacturer of the transilluminator or expose a damp membrane to 120 mJ/cm. Increased exposure will cause a decrease in signal intensity after reprobing, proportional to the amount of the overexposure.

Hybridization
Hybridize by standard methods. See 9.) Southern and Northern procedures for more information.

Detection
Detect with isotopic or colorimetric probes. Nylon membranes can be scanned by laser densitometry.

Protocol 13: Nucleic Acid Dot/Slot Blotting with Nitrocellulose Membranes
with Reprobe Nitrocellulose Supported, Pure Nitrocellulose Unsupported Membranes

protocol 13

Sample Preparation
Purify DNA or RNA by cell lysis or by standard methods, and suspend in 50 µl of TE buffer (pH 8.0). For RNA adjust the pH to 7.5.
DNA: Denature DNA with the addition of 0.1 volume of 3 M NaOH. Incubate for 1 hour at 60°C. Cool and add 1 volume 2 M NH_4OAc (pH 7.0), or 6X SSC.
RNA: Denature RNA by adding 35 µl of 20X SSC, and 20 µl of 37 % formaldehyde. Incubate for 15 minutes at 60°C.
Wet membrane in dH_2O, then soak in the appropriate buffer.
DNA - Soak in 1 M NH_4OAc (pH 7.0), or 6X SSC.
RNA - Soak in 20X SSC.

Sample Application
If using a filtration manifold, cut 2 pieces of filter paper to the size of the manifold plate and soak them in:
DNA - 6X SSC
RNA - 20X SSC
Place filter paper, then membrane onto the sample plate and secure the unit. Apply low vacuum, and wash wells with 500 µl of 1 M NH_4OAc (pH 7.0), or 6X SSC for DNA, 500 µl of 20X SSC for RNA. Apply sample (not to exceed 10 µg DNA or RNA/well), under low vacuum. Remove membrane. If working with RNA, rinse each well again with 500 µl of 20X SSC.
If dotting directly onto the membrane, apply 2 – 5 µl samples to membrane placed on 2 sheets of dry filter paper. Allow to dry.

Immobilization
Immobilize DNA by baking in a vacuum oven at 80°C for 15 minutes – 1 hour. Remove when dry.

Hybridization
Hybridize by standard methods. See Protocol 3 Southern and Northern procedures for more information.

Detection
Detect with isotopic or colorimetric probes. Densitometric analysis can be carried out on nitrocellulose membranes after clearing with paraffin oil.

Protocols for Protein Applications

Protocol 14: Western (Protein) Blotting with Nitrocellulose Membranes

with Reprobe Nitrocellulose Supported, Pure Nitrocellulose Unsupported Membranes

Protocols are often provided for Western blotting onto nitrocellulose and nylon membranes, but AppliChem recommends that most Western blot procedures be performed on AppliChem Pure Nitrocellulose Unsupported or Reprobe Nitrocellulose Supported membranes to reduce the potential for high backgrounds.

Gel Preparation

Gels may be stained before transfer with Coomassie Blue, or after transfer with Fast Green, Amido Black, or any other appropriate stain. Soak the gel for 1 hour in a transfer buffer made of: 25 mM Tris · HCl (pH 8.0), 0.15 M glycine, 20 % methanol.

Membrane Preparation Transfer

Completely soak the membrane in deionized water, and then in transfer buffer.

Electroblotting

Assemble the membrane and gel in the electroblotting unit.

Place the membrane on the anode (positive) side of the gel. Transfer according to manufacturer's instructions. Remove and wash thoroughly with transfer buffer.

Capillary Blotting

Prepare gel assembly by the method of Southern. Transfer for 2 hours to overnight. Use transfer buffer of 10 mM Tris · HCl (pH 7.5).

After the transfer step, determine transfer efficiency by staining the blot or gel by standard methods.

Blocking Procedures

Step 1: First Wash

Block the blot in PBS buffer (0.9 % NaCl, 10 mM sodium phosphate, pH 7.2) containing 5 % nonfat dry milk for 1 hour, with gentle agitation. Tween-20 may also be added to enhance blocking.

Step 2: Primary Antibody Binding

Remove the PBS buffer solution from blot completely. Dilute the first antibody in 50 ml of fresh PBS buffer solution. Incubate the blot in the PBS blocking buffer/antibody solution for 1 hour at 37°C with gentle agitation. Use a ratio of 5 – 10 ml of solution to 100 cm^2 of membrane.

Step 3: Second Wash

Wash the membrane in 100 ml of fresh PBS buffer solution (without antibody) with 0.1 – 0.3 % Tween-20. Agitate in a shaker for 5 minutes. Repeat the wash step 2 times. (Note: Increasing the number of short washes reduces the potential for high backgrounds).

Detection

Thoroughly remove the PBS buffer solution and overlay the blot with an antispecies (second) antibody, or with protein A (radiolabeled or enzyme linked) for 1 – 2 hours at room temperature with gentle agitation.

The final concentration of radiolabeled second antibody solution should be 1 – 2 x 10^5 dpm/ml of PBS buffer solution. Enzyme-linked second antibody solutions should be made at a 1:1000 titer in PBS buffer solution.

Repeat the wash step described in the procedure above.

Signal Development

The choice of signal development method is dependent on the type of probe used. Radiolabeled probes are developed and quantitated by autoradiography. Enzyme-conjugated labels (horseradish peroxidase or alkaline phosphatase) are developed and quantitated with the appropriate substrate solution.

Probe Removal

Do not allow the filter to become dry, or irreversible binding of the probe will result.

Wash the membrane at 60°C for 30 minutes in 0.05 M sodium phosphate (pH 6.5), 10.0 M urea, 0.1 M 2-mercaptoethanol, or wash the membrane in 0.2 M glycine · HCl, 0.5 M NaCl for 5 minutes. Rinse in 0.1 M NaOH or 0.5 M Tris for 10 minutes.

Protocol 15: Western Blotting from a SDS-PAGE System

Tank Electroblotting

After electrophoresis, place the SDS-PAGel in a transfer buffer of 25 mM Tris/192 mM Glycine in 15 % methanol, pH 8.2, to equilibrate for 15 – 20 minutes before blotting. Size and cut the PVDF-Star membrane to fit the SDS-PAGel. Pre-wet the membrane in 5 – 10 ml of 100 % methanol for 5 seconds. Place the membrane in 500 ml of water for 5 minutes to remove the methanol. Equilibrate with transfer buffer (500 ml, as described above) for 10 – 15 minutes prior to use in blotting.

The PVDF-Star membrane must be kept wet at all times. If the membrane dries out, re-wet in methanol and water as described above, and proceed with the transfer. Assemble the blotting cassette as follows:

Insert one half of the plastic cassette in a large dish containing transfer buffer. Place a piece of moistened filter paper on the cassette. Carefully put the gel onto the wet filter paper, and place the wet PVDF-Star membrane onto the gel, removing any trapped air bubbles. Overlay a second sheet of moistened filter paper on top of the PVDF-Star membrane, again removing any trapped air bubbles. Place a porous foam sheet on top to complete the sandwich and ensure uniform membrane contact with the gel, and insert the assembled cassette into the blotting apparatus. Be sure that the membrane side of the assembly faces the anode electrode in order that transfer from the gel to the membrane will take place.

Fill the electroblotting apparatus with 4 – 5 liters of transfer buffer and connect the cooling coil to a suitable circulation cooling water bath. Transfer proteins from the SDS-PAGel to the PVDF-Star membrane at 70 V for 1 – 2 hours. After transfer, stain the membrane with Amido Black or Coomassie Blue for 10 minutes followed by rapid destaining in 50 % methanol, 10 % acetic acid for 10 minutes, followed by a wash in distilled water.

Semi-Dry Electroblotting

After electrophoresis, place the SDS-PAGel in transfer buffer for 15 – 20 minutes to equilibrate before blotting. See the semi-dry electroblotter manufacturer's instructions for details on assembly and recommended buffers.

Transfer times are dependent on the size of the proteins and percentage of gel used. In most cases, complete transfer is achieved in 30 minutes. Transfer times should never exceed one hour.

Protein MW Range (Daltons)	Estimated Transfer Time
50,000	15 minutes
50,000 – 200,000	30 minutes
Up to 250,000	40 minutes

NOTE: Be sure to remove all trapped air bubbles to avoid bald spots in the blotted membrane.

Also, when transferring from thin polyacrylamide gels, place the wetted transfer membrane onto the gel while still on the electrophoresis plate. Invert the plate and carefully place the membrane/gel sandwich onto wetted filter paper, then remove the glass plate and continue.

Storage

Membrane blots can be dried and stored at 4°C for use at a later date. Rewet the membrane by prewetting it in a small volume of 100 % methanol for 2 – 4 seconds then placing it in a large volume of deionized water to remove the methanol or, if exposure to methanol is to be avoided, the membrane blot can be taken directly to protein background "blocking" and then air dried. This coats the membrane with hydrophilic protein and allows easy rewetting in antibody incubation solution. Using this method requires that the membrane strips be stored at 4°C. The blots are stable for up to 1 year when stored dry.

Immunostaining

Begin by incubating blots in a protein blocking solution of 5 % BSA in TBS for 1 hour at 37°C or, by incubating blots in 0.5 % non-fat milk in PBS at 37°C for 1 hour. After incubation with the protein blocking solution, the blots should be washed for 5 minutes in a wash solution of 0.1 % (w/v) BSA in TBS. Repeat the wash cycle 3 times. Incubate with the primary monoclonal antibody, which has been diluted 1:1000 with an antibody incubation solution of 1 % BSA, 0.05 % Tween-20 in TBS, for 2 hours at room temperature with gentle agitation. Repeat the wash step and incubate with one of the following enzyme-conjugated second antibody working solutions for 2 hours at room temperature.

Horseradish Peroxidase (HRP) conjugated rabbit anti-mouse IgG (1:500 dilution in 1 % BSA, 0.05 % Tween-20 in TBS).

Alkaline Phosphatase conjugated goat anti-mouse IgG (1:500 dilution in 1 % BSA, 0.05 % Tween-20 in TBS).

Colloidal Gold labeled goat anti-mouse IgG (1:100 dilution in 1 % BSA, 0.05 % Tween-20 in TBS overnight at room temperature).

protocol 15

Repeat wash step and then incubate with the appropriate substrate system to develop the colored reaction product. The development of the color can take 10 – 20 minutes at room temperature. Horseradish Peroxidase Substrate development is stopped by rinsing in water followed by drying the membrane. The developed blots should be stored dry, and protected from light.

Enzyme Conjugate Substrates

Horseradish Peroxidase
0.8 mM AEC (3-amino-9-ethylcarbazole)
29 mM sodium acetate
0.2 mM thimerosal
0.02 % hydrogen peroxide
4CN (4-chloro-1-napthol)
DAB (3, 3'-diaminobenzidine)

Alkaline Phosphatase

0.56 mM 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)
0.45 mM NitroBlue tetrazolium (NBT) in 10 mM Tris · HCl, pH 9.5

Colloidal (silver enhancement)

77 mM hydroquinone
5.5 mM silver lactate in 100 mM citrate buffer pH 3.85 (make reagent in a darkened container).
After silver enhancement is complete, soak the blot in fixing solution, for 5 minutes followed by a rinse in water.

Storage

Store blots dry. Avoid fading by protecting the blot from light.

Protein Staining

Low Sensitivity Detection (1 – 5 ng/band)

Ponceau-S Dye

After blotting, rinse PVDF-Star with dH_2O. Stain with 0.2 % of Ponceau-S for 1 minute. Rinse briefly with dH_2O to remove excess stain.
To remove stain from protein bands, rinse briefly (5 minutes) in 0.1 N NaOH. This may be less successful with basic proteins and could lead to protein loss from the membrane.

Amido Black or Coomassie Blue

Membrane strips should be stained in Amido Black or Coomassie Blue for 10 to 15 minutes, followed by destaining for 10 to 15 minutes in 45 % methanol/7 % acetic acid for Amido Black and 50 % methanol/10 % acetic acid for Coomassie Blue. Place the blots in 90 % methanol for 1 to 2 minutes to remove any residual stain.

High Sensitivity Detection

0.005 – 0.1 µg/band

India Ink

Place the blots in 0.1 M sodium phosphate buffered saline containing 0.3 % Tween-20 at 37°C for 1 hour. Wash in this buffer for 1/2 hour at room temperature, repeating the process 3 times. Stain overnight in 0.1 % India ink in the buffer solution and air dry for storage.

Colloidal Gold

Follow the above procedure using Colloidal Gold undiluted according to the manufacturer's instructions.

Protocol 16: Protein Sequencing From A Western Blot

protocol 16

AppliChem PVDF-Star membrane is preferentially chosen for applications requiring direct protein sequencing from a Western Blot because of the membrane's chemical compatibility with the acidic environment necessary for use with protein sequencing equipment.
For optimal results, prepare the samples and run protein samples on a polyacrylamide gel as indicated in Western Blotting PVDF-Star Transfer Membrane protocol.
After electrophoresis is complete, prepare the membrane according to protocol and electroblot the proteins to the PVDF-Star membrane. Remove the membrane from the gel, rinse with dH_2O and stain with Coomassie Blue, Amido Black, or Ponceau-S to locate the bands of interest.
Destain the membrane and carefully excise the band from the PVDF-Star membrane. Load the band into the sequencing cup of the sequencer apparatus according to manufacturer's instructions. The use of polybrene is not necessary. This eliminates precycling and shortens run times while reducing sequencer background. Operate the sequencer according to manufacturer's instructions. Yields should be in excess of 75 % of estimated blotted protein.

Stain	Staining Solution	Destain
Coomassie Blue	0.1 % Coomassie Blue R-250 in 1 % acetic acid/40 % MeOH	50 % MeOH in dH_2O
Amido Black	0.1 % Amido Black in 1 % acetic acid/40 % MeOH	dH_2O
Ponceau-S	0.2 % Ponceau-S in 1 % acetic acid	dH_2O

Protocol 17: Stripping PVDF-Star (Polyvinylidene Fluoride) Transfer Membrane

protocol 17

This stripping procedure will not adversely alter the membrane bound protein and is especially recommended when studying reduced or denatured proteins.

1. Prewet the membrane with 100 % methanol, then incubate in 0.2 M glycine · HCl, pH 2.5 with 0.05 % Tween 20.
2. Place the membrane in a sealed plastic container, and place in an agitating water bath at 80°C for one hour. Decant the old solution and replenish the bag with the same volume of fresh solution. Repeat the agitation for one hour.
3. Wash the membrane in BBS-Tween 20, then re-block in 5 % BSA with BBS.

protocol 18

Protocol 18: Transfer of High Molecular Weight Proteins from a Non-Denaturing Gel System to the PVDF-Star (Polyvinylidene Fluoride) Membrane

PVDF-Star transfer membrane is an intelligent alternative to nitrocellulose in Western blotting applications. It offers greater mechanical strength, high protein binding capacity, and compatibility with moist protein staining protocols. Its greater chemical compatibility allows for use of a wide range of solvents for rapid destaining. A non-denaturing gel system in conduction with PVDF-Star makes it possible to quantitatively transfer high molecular weight proteins in the size range of 67,000 to 669,000 daltons.

protocols

Electrophoresis Under non-Denaturing Conditions

Dissolve samples in the electrophoresis buffer consisting of 90 mM EDTA, 0.005 % sodium azide, 10 % glycerol, and 0.01 % bromophenol blue as tracking dye. Electrophoresis is carried out using 3 – 27 % T linear gradient gels. These gels are initially run at a constant voltage of 150 V for 1 hour, then increased to 200 V until the dye front reaches the end of the gel slab.

Electroblotting

After PAGE, electrotransfer is carried out in a Tris/glycine buffer system in the presence of methanol. The PVDF-Star membrane must be wet in methanol prior to transfer for 1 to 2 seconds to allow the hydrophobic surfaces to become fully wet in aqueous solutions. This is followed by a rinse in water and finally in transfer buffer. The membrane must remain wet at all times. If the membrane dries out, re-wet in methanol and water as described above and proceed with the transfer. Next, assemble the blotting cassette following the manufacturer's instructions. Transfer proteins from the gel to the membrane at 200 V for 18 hours at 18°C. After electrotransfer the blot is washed four times, for 30 minutes in phosphate buffered saline solution containing Twen-20. The first wash should be done at 37°C, while the remaining three are at room temperature. Stain the blots according to the manufacturer's instructions. For general guidelines on Western blotting with AppliChem PVDF-Star membrane see Protocol 15.

Storage

Membrane blots can be dried and stored at +4°C for use at a later date. Rewet the membrane by placing the membrane in a small volume of 100 % methanol for 1 – 2 seconds, then placing it in a large volume of deionized water to remove the methanol. If exposure to methanol is to be avoided, the blot can be taken directly to protein "blocking" and then air dried. This will coat the membrane with hydrophilic protein and allows for rewetting in antibody incubation solution. When stored dry, the blots will remain stable for up to one year.

protocol 19

Protocol 19: Transfer of Basic Proteins from an Acid/Urea Gel System at Acidic pH to the PVDF-Star (Polyvinylidene Fluoride) Transfer Membrane

PVDF-Star transfer membrane is an intelligent alternative to nitrocellulose in Western blotting applications. It offers greater mechanical strength, while combining high protein binding capacity with low backgrounds. Due to the chemical compatibility of the PVDF-Star membrane, a wide range of solvents can be used for destaining.

Protocol

Begin by dissolving the protein in a solution of 0.9 M acetic acid, and 6.25 M urea. Volumes will depend on the total volume of the samples being used. It may also be beneficial to add 0.1 % of tracking dye, for example Pyronin-Y. This will make it easier to trace the proteins as they run down the gel.

Load the solution onto a PAGel, and run the electrophoresis in 0.9 M acetic acid at 35 mA for 4 hours or until the dye reaches the bottom of the gel. Prior to transfer it is imperative to wet the PVDF-Star membrane for 1 – 2 seconds in 100 % methanol, followed by a rinse in water, and finally with transfer buffer. The membrane must remain wet at all times. If it dries out, the transfer can be adversely affected. The wetting steps can be repeated as necessary to prevent this from happening. Electroblotting can then be carried out at 150 V for 2 hours. For best results follow the manufacturing instructions accompanying your equipment. Membrane blots should then be washed in phosphate buffered saline containing 0.3 % Tween-20 at 37°C. Two additional washes should follow both at room temperature for 30 minutes. Staining with any of the commonly used dyes can then be accomplished easily with PVDF-Star.

protocol 19

Colloidal Silver Enhancement

Use 77 mM hydroquinone, 5.5 mM silver lactate in 100 mM citrate buffer at pH 3.85. Make reagent in a darkened container as it is photosensitive. After the enhancement is complete, soak the blot in fixing solution for 5 minutes followed by a rinse in water.

Coomassie Blue or Amido Black

Soak the membrane in either dye for 10 to 15 minutes. Destaining should immediately follow with 45 % methanol and 7 % acetic acid for Amido Black, or 50 % methanol and 10 % acetic acid for Coomassie blue. Destaining should continue for 10 to 15 minutes. Placing the blots into 90 % methanol for 1 to 2 minutes after destaining will remove any residual stain.

Ponceau S

After blotting, rinse the PVDF-Star with deionized water. Apply 0.2 % Ponceau-S for 1 minute then rinse with deionized water to remove excess stain.

Storage

Membrane blots can be dried and stored at 4°C for use at a later date. Rewet the membrane by placing the membrane in a small volume of 100 % methanol for 1 – 2 seconds, then placing it in a large volume of deionized water to remove the methanol. If exposure to methanol is to be avoided, the blot can be taken directly to protein "blocking" and then air-dried. This will coat the membrane with hydrophilic protein and allows for rewetting in antibody incubation solution. When stored dry, the blots will remain stable for up to one year

protocol 20

Protocol 20: Protein Dot Slot Blotting with PVDF (Polyvinylidene Fluoride) Transfer Membranes

Sample Preparation

Suspend purified samples in a buffer solution such as PBS or TBS. If a filtration manifold is being used, the samples should be diluted to between 300 – 500 µl for a concentration of between 1 – 10 µg/spot. Serial dilutions of the sample can be performed to determine the optimal concentrations for subsequent binding assays. If a filtration manifold is not being used, volumes should be between 2 – 5 µl. Increase the sample concentration and reapply until the appropriate amount of sample is immobilized.

Membrane Preparation

Soak the membrane in 100 % methanol until complete saturation is reached, then soak the membrane in PBS or TBS until it is used.

Sample Application

Place the membrane as flat as possible on dry sheets of absorbent blotting paper. Apply 2 – 5 ml of sample to the membrane. If larger volumes are used, the samples should be allowed to spot dry prior to subsequent sample application. If a filtration manifold is being used, place two sheets of absorbent blotting paper in the filter support plate. The membrane should be placed on top of the absorbent paper and clamp the sample well plate into place. Apply low vacuum and wash the individual wells with 500 µl of sample buffer. Add sample to the well and filter through. Follow sample filtration with a wash of 500 µl of sample buffer.

protocols

protocol 21

Protocol 21: Blocking with Blocking Reagent CA (A3409,0010)

Blocking Reagent for Hydridization Assays and Western Blots

Storage

Room Temperature

Introduction

The Blocking Reagent CA is used in hybridization and detection procedures using non-radioactive nucleic acid probes, and for Western blots.

When immunoassays and hybridization assays, such as dot blots, Western blots, Southern blots, or Northern blots are performed, there is nonspecific binding resulting in high background. In order to reduce the nonspecific binding, Blocking Reagent CA is used to "block" unbound sites left after immobilization of the specific protein or after the hybridization with non-radioactive probe. The Blocking Reagent CA improves sensitivity and reduces background.

NOTE: Nonfat dry milk inhibits the streptavidin-biotin interaction due to its content of biotin

1. Procedure

Proteins

For blotting applications such as Western blots and dot blots, add 0.2 % (w/v) Blocking Reagent CA into TBST or PBST, heat to 75 – 80°C in a water bath or microwave oven, and stir well until dissolved. The Blocking Reagent CA dissolves to give a milky solution. Use for blocking and for dilutions of antibodies.

NOTE: Do not use the Blocking Reagent CA in PBST for alkaline phosphatase conjugate dilutions

Nucleic Acids

For hybridization applications add 0.2 % (w/v) Blocking Reagent CA to Tris-Saline buffer (100 mM Tris-Cl pH 7.5, 600 mM NaCl), heat to 60 – 65°C in a water bath or microwave oven, and stir well until dissolved. The Blocking Reagent CA dissolves to give a clear solution. Use for blocking after the wash steps, and before incubation in any enzyme-conjugate solution (e.g. Streptavidin-HRP, Streptavidin-AP).

2. Optimization of Time Required for Blocking with the Blocking Reagent CA

2.1. Cut 7 small squares of nitrocellulose or other suitable membrane.
2.2. Label each square with a ball point pen in 10 minute increments (60, 50, 40, 30, 20, 10), and one without blocking.
2.3. Place the first square (60) in a few ml of Blocking Reagent CA solution, and add successive squares at 10-minute intervals.
2.4. Wash all squares in TBST, PBST or Tris-Saline buffer (100 mM Tris-Cl pH 7.5, 600 mM NaCl).
2.5. Dilute the secondary antibody or streptavidin (HRP-conjugated) in Blocking Reagent CA solution.
2.6. Incubate on shaker for 1 hour.
2.7. Rinse in TBST, PBST or Tris-Saline buffer three times, 10 minutes each time.
2.8. Detect with the Chemiluminescent Detection Kit for horseradish peroxidase (Order-No. A3417,1200).
2.9 Evaluate background intensity in each square. Select the incubation time that gives the lowest background.

Protocol 22: Blocking with Blocking Reagent GE (A4879)

Blocking Reagent for Western Blots, ELISAs, surface coating & Gene-chip Technology (replaces acetylated BSA)

protocol 22

CAS-No.:	68410-45-7
pH-Value	5.0 – 7.0 (20 %, RT)
Source	bovine
Description	highly purified gelatin proteins in pharmaceutical quality in accordance with DAC 1986, 1st. supplement 1989
Storage	room temperature, dry place
Stability	minimum 2 years at room temperature

Introduction

The Blocking Reagent GE is a fat-free and carbohydrate-free gelatin derivative. It has a better blocking capacity than bovine serum albumin (BSA), there is virtually no background in western blotting, especially in combination with enhanced chemiluminescence detection, and it increases the signal-to-noise ratio in ELISAs. The Blocking Reagent GE has an average molecular weight of 3 kD. It his highly soluble in aqueous solutions (up to 50 %) and it doesn't precipitate at +4°C in aqueous solutions.

Standard Western-Blot Protocol

- Perform SDS-polyacrylamide gel elctrophoresis (SDS-PAGE) on your sample of choice and transfer the proteins to either nitrocellulose or PVDF membranes.
- Block residual binding sites by incubating the membrane in blocking buffer with the appropriate detergent and 1 – 3 % of Blocking Reagent GE in either PBS (phosphate buffered saline) or TBS (Tris buffered saline) for 25 – 60 minutes at room temperature and constant agitation.
- Incubate the membrane with the appropriate first antibody in freshly prepared blocking solution either at room temperature for several hours or over night at +4°C with constant agitation.
- After first antibody incubation wash the membrane several times with blocking buffer.
- Incubate the membrane with secondary antibody of choice in blocking buffer for 1 – 2 hours at room temperature and consistent agitation.
- Wash membrane several times in blocking buffer.
- Wash membrane twice in PBS or TBS.
- Use detection method of choice.

Recipes for Buffers used in Nucleic Acid Transfers *

Electrophoresis Buffers

1. TAE (1X)	40 mM Tris-acetate, 1 mM EDTA, pH 8.0 pH adjustments can be made with glacial acetic acid
2. TBE (1X)	45 mM Tris-base, 45 mM boric acid, 2 mM EDTA, pH 8.0
3. MOPS Buffer (10X)	50 mM sodium acetate, 10 mM EDTA, 0.2 M 3-morpholinopropanesulfonic acid, pH 7.0
4. E Buffer	5 mM sodium borate, 10 mM sodium sulfate, 1 mM EDTA, 50 mM boric acid, pH 8.2

Transfer and Washing Buffers

1. SSPE (1x)	0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM EDTA. To bring pH to 7.7, Na_2HPO_4 is added to NaH_2PO_4
2. SSC (1x)	0.15 M NaCl, 15 mM sodium citrate, pH 7.0
3. TBS (1x)	50 mM Tris, 150 mM NaCl

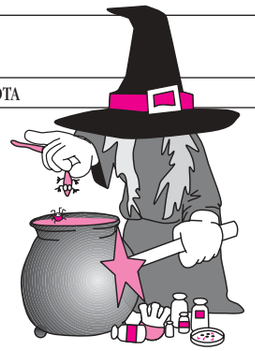
Blocking Agents

1. Denhardt's Reagent	0.02 % Ficoll (mw = 400,000), 0.02 % polyvinylpyrolidone (mw = 40,000), 0.02 % BSA(Bovine Serum Albumin)

Sample Buffers

1. TE Buffer (1x)	10 mM Tris · HCl, pH 8.0, 1 mM EDTA
2. SET Buffer (1x)	0.1 M NaCl, 10 mM Tris · HCl, pH 8.0, 1 mM EDTA

pH adjustment to EDTA are made with NaOH
pH adjustments to Tris solution is made with HCl
* formulations recommended by AppliChem



Related Products: Chemicals Used For Blotting

AppliChem's General Catalog lists more than 2,700 biochemicals. Ask your local distributor for a copy of our catalog or order it at www.applichem.de.

Acids and bases
Albumins
Amino acids
Antibiotics/Antimycotics
Biological Buffers
Cell culture powder media
Detergents
DNA and RNA isolation kits
Electrophoresis Chemicals
Enzyme inhibitors
Enzyme substrates
Enzymes
Indicators
Molecular biology grade reagents
Natural substance standards
PCR grade reagents
ready-to-use solutions and reagents
Salts
Solvents
Stains
Standard volumetric solutions
Vitamins
and many more…

Prod. No.	Description	Comments
A1398	Acridine orange (C.I. 46005)	
	Acrylamide, Bisacrylamide	Probably the widest range of acrylamide – bisacrylamide mixtures available in the market.
	Agarose	AppliChem offers 11 different types of agaroses
A1399	Amido black 10 B (C.I. 20470)	
A2936	Ammonium acetate Molecular biology grade	
A4716	Ammonium acetate – Solution (5 M) Molecular biology grade	
A1117	BCIP	5-Bromo-4-chloro-3-indolyl phosphate p-toluidine salt
	Blocking reagents	see next pages
A3786	4-Chloro-1-naphthol	
	Coomassie, Brilliant blue	G-250 or R-250
A0596	3,3'-Diaminobenzidine tetrahydrochloride	DAB
A4892	EDTA – Solution pH 8.0 (0.5 M) Molecular biology grade	
A1151	Ethidium bromide	
A2273	Ethidium bromide – Solution 0.07 %	*ready-to-use* solution in dropper bottles (concentration 0.7 mg/ml)
A1152	Ethidium bromide – Solution 1 %	*ready-to-use* solution (concentration 1 mg/ml)
A1401	Fast Green FCF (C.I. 42053)	
A2156	Formamide Molecular biology grade, deionized	
A1067	Glycine Molecular biology grade	
A2169	Glyoxal – Solution 40 % Molecular biology grade	*ready-to-use* solution
A3728	Hybridization Solution	for Southern and Northern blots
	Loading buffers	10 different loading buffers
A4998	Magnesium chloride solution (1 M) Molecular biology grade	
A1108	β-Mercaptoethanol Molecular biology grade	
A1243	NBT	Nitro blue tetrazolium chloride
	PBS buffer (Dulbecco's) – Powder	powder mixtures (10X and 1X)
	Phosphate buffer	see the different Sodium phosphates in the catalog
A3099	Polyethylene glycol 6000 Molecular biology grade	other PEG's available: PEG 200 – PEG 8000
A1405	Ponceau S (C.I. 27195)	
A1406	Pyronin Y (C.I. 45005)	
	SDS – Solutions Molecular biology grade	*ready-to-use* solutions (10 % or 20 %)
A2942	Sodium chloride Molecular biology grade	
A4522	*tri*-Sodium citrate dihydrate Molecular biology grade	
A3910	Sodium hydroxide pellets *BioChemica*	
A2197	Sodium pyrophosphate decahydrate *BioChemica*	
A4506	SSC buffer (20X) – Powder Molecular biology grade	
A1396	SSC buffer (20X) Molecular biology grade	*ready-to-use* solution
A1397	SSPE buffer (20X) Molecular biology grade	*ready-to-use* solution
	TAE buffer	*ready-to-use* solutions (50X or 10X)
	TBE buffer	*ready-to-use* solutions (10X or 5X) or as powder
A3840	3,3',5,5'-Tetramethyl benzidine	TMB
A1278	Thimerosal	
	Tris buffer (1 M)	*ready-to-use* solutions (pH 6.8 – pH 8.8)
A1287	Triton, X-100 – Solution 10 % peroxide-free	
A4975	Triton, X-100 Molecular biology grade	
A1284	Tween, 20 – Solution 10 % peroxide-free	
A4974	Tween, 20 Molecular biology grade	
A1049	Urea Molecular biology grade	

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Triton® X-100 is a registered trademark of Union Carbide Company

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