Amersham™ Hybond™ and Amersham Protran™ Membranes

Optimized for protein transfer

Product Booklet

Page Finder

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2. Handling

2.1. Safety warnings and precautions

Caution: *For research use only*. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

GE recommend that this product and components are handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. As all chemicals should be regarded as potentially hazardous, it is advisable when handling chemical reagents to wear suitable protective clothing and work a powder-free laboratory environment.

Care should be taken to avoid contact with skin or eyes. In case of contact with skin or eyes wash immediately with water. Note that the procedures require the use of: harmful, toxic, irritant and flammable substances.

2.2. Storage

Membranes should be stored in a clean, dry atmosphere away from noxious fumes. In order to preserve optimum performance, avoid conditions of extreme humidity. Membranes are affected by the environment and so should be kept in the bags and boxes in which they are received, protected from exposure to sunlight at 18°C to 25°C, and at 40% to 60% relative humidity.

2.3. Stability

Before opening, membranes are stable for two years. Once open, keep in the bags in which they were received. Performance is consistent for up to twelve months when stored under the recommended conditions.

The membrane's shelf life is indicated on the individual packaging.

2.4. Handling the product

Membranes should be handled wearing powder-free gloves or using blunt-ended forceps. All membranes should be cut using clean, sharp scissors to avoid damage to the membrane edges.

If a transfer membrane dries out at any time the wetting procedure must be repeated.

3. Components

3.1. Amersham Protran Nitrocellulose Membranes

Nitrocellulose Membranes	Quantity	0.1 µm	0.2 µm	0.45 µm
30 cm × 4 m	1 roll	10600000	10600001	10600002
20 cm × 4 m	1 roll	10600005	10600006	10600007
15 cm × 4 m	1 roll	10600010	10600011	10600012
300 × 600 mm	5 sheets	10600031	10600032	10600033
200 × 200 mm	10 sheets	10600046	10600044	10600042
200 × 200 mm	25 sheets	10600045	10600043	10600041
140 × 160 mm	25 sheets	10600064	10600063	10600062
102 × 133 mm	10 sheets	10600126	10600125	10600124
100 × 100 mm	10 sheets	10600077	10600075	10600073
100 × 100 mm	25 sheets	10600076	10600074	10600072
80 × 90 mm	25 sheets	10600095	10600094	10600093

3.2. Amersham Protran Premium Nitrocellulose Membranes

Product	Quantity	0.2 µm	0.45 µm
30 cm × 4 m	1 roll	10600004	10600003
20 cm × 4 m	1 roll	10600009	10600008
15 cm × 4 m	1 roll	10600014	10600013
300 × 600 mm	5 sheets	10600035	10600034
200 × 200 mm	10 sheets	10600050	10600048
200 × 200 mm	25 sheets	10600049	10600047
140 × 160 mm	25 sheets	10600066	10600065
100 × 100 mm	10 sheets	10600081	10600079
100 × 100 mm	25 sheets	10600080	10600078
80 × 90 mm	25 sheets	10600097	10600096

3.3. Amersham Protran Supported Nitrocellulose Membranes

Product	Quantity	0.2 µm	0.45 µm
30 cm × 4 m	1 roll	10600015	10600016
20 cm × 4 m	1 roll	10600017	10600018
15 cm × 4 m	1 roll	10600019	10600020
300 × 600 mm	5 sheets	10600037	10600036
200 × 200 mm	10 sheets	10600054	10600052
200 × 200 mm	25 sheets	10600053	10600051
140 × 160 mm	25 sheets	10600068	10600067
102 × 133 mm	10 sheets	10600128	10600127
100 × 100 mm	10 sheets	10600083	10600085
100 × 100 mm	25 sheets	10600082	10600084
80 × 90 mm	25 sheets	10600099	10600098

3.4. Amersham Hybond PVDF Membranes

Product	Quantity	P 0.45 µm	P 0.2 µm	LFP 0.2 µm	SEQ 0.2 µm
26 cm × 4 m	1 roll		10600021		10600030
25.4 cm × 4 m	1 roll			10600022	
30 cm × 4 m		10600023			
15 cm × 4 m		10600029			
300 × 600 mm		10600038			
260 × 600 mm	5 sheets	10600029	10600039		
265 × 600 mm				10600040	
200 × 200 mm	10 sheets		10600058	10600060	10600061
200 × 200 mm	25 sheets		10600057		
140 × 160 mm	25 sheets	10600069		10600090	
100 × 100 mm	10 sheets	10600086	10600089	10600091	10600092
100 × 100 mm	25 sheets	10600087	10600088		
80 × 90 mm	25 sheets	10600100	10600101	10600102	

3.5. Amersham Protran Western Blotting Sandwiches

Product	Quantity	0.1 µm	0.2 µm	0.45 µm
Amersham Protran Sandwiches				
140 × 160 mm	10 sandwiches	10600105	10600104	10600103
80 × 90 mm	10 sandwiches	10600116	10600115	10600114
Amersham Protran Premium Sandwiches				
140 × 160 mm	10 sandwiches	-	10600107	10600106
80 × 90 mm	10 sandwiches	-	10600118	10600117
Amersham Protran Supported Sandwiches				
140 × 160 mm	10 sandwiches	-	10600109	10600108
80 × 90 mm	10 sandwiches	-	10600120	10600119

3.6. Amersham Hybond PVDF Western Blotting Sandwiches

Product	Quantity	P 0.45 µm	P 0.2 µm	LFP 0.2 µm	SEQ 0.2 µm
Amersham Hybond Sandwiches					
140 × 160 mm	10 sandwiches	10600110	10600111	-	-
80 × 90 mm	10 sandwiches	10600121	10600122	10600123	-

4. Descriptions

In addition to buffer characteristics such as pH, salt type, salt concentration, and the presence of detergents such as SDS, the degree to which molecules bind to a membrane is influenced by the physical and chemical characteristics of the membrane itself. A protein's properties affect its ability to bind to membrane surfaces. Finding the right membrane may require experimenting with your specific protein on different membranes.

In general, membranes are porous materials with pore sizes from 0.05 to 10 µm in diameter. The binding capacity of a membrane depends primarily on the pore size. A membrane with many small pores has a larger binding surface than one with larger pores, and thus generally has a higher binding capacity. The exact mechanism by which biomolecules interact with the membrane is not known, but it is assumed to be a combination of non-covalent and hydrophobic forces. It should be noted that although protein conformation and buffer composition also affect binding capacity, the overall sensitivity of a Western blot depends on the amount of protein immobilized on the membrane and presented to the primary antibody. Note, however, that excessive protein binding does not necessarily lead to improved signals in immunoblotting and can even have the opposite effect. This is because proteins concentrated at high density tend to self-associate through weak interactions rather than interacting with the membrane surface. This means that protein-antibody complexes, the formation of which lies at the heart of the detection principle in Western blotting, are easily lost from the membrane during washing steps.

4.1. Nitrocellulose membranes

Nitrocellulose membranes are the most frequently used membranes for Western blotting with their main advantage being a low background, no matter the detection method applied. The Amersham Protran transfer membranes consist of 100% pure nitrocellulose with pore sizes ranging from 0.1 to 0.45 µm. Inclusion of methanol in the transfer buffer improves protein binding to nitrocellulose membranes. Nitrocellulose membranes are not recommended for stripping and reprobing, as they become brittle and difficult to handle when dry. However, Amersham Protran Supported membranes have improved mechanical strength through the incorporation of a polyester support web.

The Amersham Protran Western blotting transfer membrane family is composed of the high-quality 100% pure nitrocellulose membrane legacy of Schleicher & Schuell™. The sheets and rolls are available in three pore sizes with high surface area and excellent uniformity. The Amersham Protran Western blotting membranes have good binding properties for Western blot, dot-blot assays and other protein or nucleic acid methods.

Amersham Protran nitrocellulose membranes are available in 0.1, 0.2 and 0.45 µm pore sizes in a variety of package sizes containing rolls, pre-cut sheets and sandwiches for nearly any application. The Amersham Protran nitrocellulose membrane is a binding matrix for Western blotting with high affinity for proteins, blocking ability, and compatibility with a range of detection methods (e.g. chemiluminescence, chromogenic, fluorescence).

In general, one can say that the binding affinity of small peptides increases with decreasing pore size. Consequently, both Amersham Protran 0.2 and Amersham Protran 0.1 are good choices for working with small proteins and peptides. The Amersham Protran 0.1 membrane has a minimal pore size of 0.1 µm, exhibits excellent binding affinity for small peptide (M_r < 10 000) and nucleic acid (< 300 bp) blotting purposes and produces very low background in chemiluminescent Western blotting.

Amersham Protran Premium (0.45 and 0.2 µm pore size) membranes give high sensitivity, resolution, and low background for all labeling and detection systems (chemiluminescence, fluorescence, colorimetric and radioactive). The low background enables a dynamic range for complicated samples.

4.2 Supported nitrocellulose membranes

Amersham Protran Supported (0.45 and 0.2 µm pore size) membranes consist of nitrocellulose supported by an inert polyester non-woven material within the membrane. The polyester support does not affect transfer conditions or results and gives the membrane exceptional handling characteristics, allowing reprobing.

Amersham Protran membranes are compatible with several different staining methods such as Amido black, Aniline blue black, Ponceau S, Colloidal gold, Fast Green and Toluidine blue.

4.3. PVDF membranes

PVDF (polyvinylidene fluoride) membranes have, in general, a high protein binding capacity, high mechanical strength, and are well suited for

Western blotting applications where stripping and reprobing are needed. As PVDF membranes are highly hydrophobic, they need to be prewetted in either methanol or ethanol before use to be compatible with aqueous solutions. Proteins bind to PVDF membranes via a combination of dipole and hydrophobic interactions. PVDF membranes tend to have a higher background due to their higher protein binding capacity.

Amersham Hybond P 0.2 µm and Amersham Hybond P 0.45 µm are robust and chemically stable membranes, well suited for stripping and reprobing. Amersham Hybond P 0.45 is well suited for screening applications of proteins Mr>20000. The smaller pore size of the 0.2 µm membrane eliminates blow-through and increases protein binding over a wide range of molecular weights. These membranes are compatible with a range of solvents used for rapid destaining. Amersham Hybond should be immersed in 100% methanol, and then soaked in ultrapure water before use.

Amersham Hybond LFP 0.2 µm is a hydrophobic PVDF membrane specifically optimized for fluorescence detection in Western blotting applications. It has low background fluorescence, resulting in high sensitivity. This low fluorescence PVDF membrane offers the same benefits as standard PVDF membranes in addition to minimal autofluorescence and high sensitivity. This membrane must be activated with methanol and is particularly recommended in combination with detection using Amersham ECL Plex™.

Amersham Hybond SEQ 0.2 µm has a minimal pore size of 0.2 µm and is specifically designed for protein sequencing applications and solid phase amino acid analysis. The small pore size of this membrane eliminates blow-through and increases protein binding over a wide range of molecular weights. Hybond SEQ 0.2 offers excellent blotting for proteins M_r < 20 000.

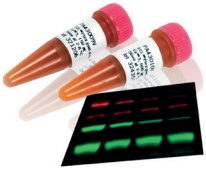
4.4. Membrane sandwiches

Amersham Protran sandwiches and Amersham Hybond sandwiches are precut, preassembled high-quality nitrocellulose/PVDF membrane/filter paper sandwiches designed to make Western blotting easier and to improve results. Membranes are precut to either 8 cm × 9 cm or to 14 cm × 16 cm to fit most precast gel formats and are preassembled with two Whatman™ medium thickness 3 MM Chr (0.34 mm) filter papers to simplify blotting set up.

Sample preparation

Gel electrophoresis

Transfer



Antibody probing

Detection

Imaging

Analysis

GE offers a complete Western blotting solution

5. Western blotting

5.1. Basics

Western blotting, also known as immunoblotting, is a well-established and widely used technique for the detection and analysis of proteins. The method is based on building an antibody-protein complex via specific binding of antibodies to proteins immobilized on a membrane and detecting the bound antibodies with one of several detection methods. The Western blotting method was first described in 1979[1] and has since become one of the most commonly used methods in life science research.

The samples used for electrophoresis normally consist of a complex protein mixture such as a cell or tissue extract, but it can also be a sample of purified proteins, such as a fraction from a purification procedure. The sample undergoes gel electrophoresis for protein separation and the proteins are then immobilized onto a membrane following electrophoretic transfer from the gel. The membrane is blocked to prevent non-specific binding of antibodies, followed by incubation with a primary antibody that specifically binds to the protein of interest. Unbound antibodies are removed by washing and a secondary antibody, conjugated to an enzyme, a fluorophore, or an isotope, is used for detection. The detected signal from the protein-antibody-antibody complex is proportional to the amount of protein on the membrane.

The most commonly used method for detection is chemiluminescence, based on secondary antibodies conjugated with horseradish peroxidase enzyme. On the addition of a peroxide-based reagent, the enzyme catalyzes the oxidation of luminol, resulting in the emission of light. The light signal can be captured either using a charge-coupled device (CCD) camera-based imager or by exposure to X-ray film.

A more recent detection method is fluorescence. The secondary antibodies are labeled with a fluorophore such as a CyDye™ fluorescent dye. A fluorescent light signal can be detected directly using a laser scanner or a CCD camera-based imager equipped with appropriate light sources and filters. Regardless of the detection method, the signal intensity correlates with the amount of protein and can be visually estimated as well as quantitated using analysis software.

[1]Towbin H, et al. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. Proc Natl Acad Sci USA; **76**: 4350-4. 1979

5.2. Electrotransfer

Electrotransfer is almost exclusively the contemporary transfer method of choice due to its speed, uniformity of transfer, and transfer efficiency. Electrotransfer relies on the same electromobility principles that drive the migration of proteins during separation in PAGE. The gel, membrane, and electrodes are assembled in a sandwich so that proteins move from gel to membrane, where they are captured in a pattern that mirrors their migration positions in the gel. Wet and semidry electrotransfer are used frequently.

In the choice of wet transfer, the gel and membrane are both fully immersed in transfer buffer and a current is applied in the direction of the gel to the membrane. Generally, wet transfer requires cooling of the unit and internal circulation of the transfer buffer by the presence of a stirring magnet. Wet transfer is recommended for large proteins, but it is a relatively slow technique, requiring large volumes of buffer. Wet transfer should be applied in preference to semidry transfer when it is important to obtain blots of the highest quality in terms of distinct, sharp bands and efficient transfer.

Semidry transfer is faster than wet transfer and, in addition, consumes less buffer. The membrane is placed in direct contact with the gel and several layers of filter paper soaked in transfer buffer are placed above and below the gel and membrane. Semidry transfer is usually less efficient than wet transfer, especially for large proteins. Heating is less of a problem with semidry transfer for normal transfer times, as the electrode plates absorb heat, but semidry systems should be avoided for extended transfer times as this may lead to overheating and gel drying due to buffer depletion.

For more information please see the Western blotting handbook (code number 28-9998-97), which is available at www.gelifesciences.com/amershamwb

6. Additional materials needed to perform Western blotting

- PAGE gels
- Electrophoresis system
- Protein transfer unit
- Blotting Paper
- Trays/dishes
- Assorted laboratory glassware
- Orbital shaker/rocking platform or equivalent
- Plastic wrap or equivalent
- Timer
- Detection facilities depending on the choice of the used detection instrumentation:
  - Chemiluminescence: autoradiography/X-ray film equipment or imaging system
  - Fluorescence: imaging system
  - Colorimetric: imaging system/scanner
- Image analysis software (e.g. ImageQuant™ TL software) for quantitative Western blotting experiments
- Reagents required for electrophoresis, blotting and immunodetection of proteins should be analysis grade where possible:
  - Methanol
  - Tris
  - Glycine
  - Tween™ 20
  - Sodium dodecyl sulfate (SDS)
  - Dithiothreitol (DTT)
  - Glycerol
  - Bromophenol blue
  - Phosphate buffered saline (PBS) or Tris-buffered Saline (TBS)

7. Recommended Western blotting protocol

Step	Action
1.	Separate the protein samples and molecular weight markers using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). • Run an SDS-PAGE gel loaded with your samples diluted in sample loading buffer and heated to 96°C for 5 min. • Apply 1.5–3 µl Amersham ECL Plex Rainbow™ or 5 µl Amersham Rainbow markers. The markers can be applied without preheating.
Note	*Amersham Protran and Amersham Hybond membranes are compatible with Amersham ECL™ gel box system, Amersham ECL Rainbow and Amersham ECL DualVue™ molecular weight markers.*
2.	Carefully remove bromophenol blue front and the stacking gel (if applicable) and equilibrate the gel in cold transfer buffer for 20 min.
3.	Cut Amersham membrane and blotting paper into suitable size for blotting (e.g. 8 × 8 cm) or used any of pre-cut membranes or sandwiches. If applicable mark membranes by cutting one or more corners of the membrane. If using a PVDF membrane, prewet the membrane in methanol for 20 s. Next, wet the membrane in ultrapure water for 10 s and soak the membrane in transfer buffer for 5 min.

Amersham Hybond PVDF membranes	Amersham Protran Nitrocellulose membranes
• prewet in methanol for 20 s	• prewet in ultrapure water for 20 s
• wash in ultrapure water for 20 s	• equilibrate in cold transfer buffer for 5 min
• equilibrate in cold transfer buffer for 5 min	

4.	Assemble the blotting cassette for transfer.
Note	*Both wet and semidry transfer methods are compatible with Amersham Protran and Amersham Hybond membranes. However, we recommend the wet transfer method.*

Wet/tank transfer

- Place the electrocassette anodic side facing down in a tray filled 3 cm deep with prechilled transfer buffer.
- Load the cassette starting at the anodic side with a foam sponge, followed by two prewetted Amersham Hybond blotting papers, the prepared membrane, the gel, and again two wet Amersham Hybond blotting papers and a second foam sponge.
- Make sure to remove any trapped air between filter papers, membrane and gel.
- Close the cassette and place the anodic side (+) in the same orientation as the following cassettes in the electroblotting tank filled with cold transfer buffer.
- Connect color-coded leads to the power supply.
- Choose transfer conditions based on the instrument manufacturer's instructions (typically 25 V at 4°C with stirring fly for 2.5 h).

Semidry transfer

- The sandwich is built on the lower electrode (anode [+]).
- Place the prewetted membrane onto the stack of three wetted Amersham Hybond blotting papers.
- The blotting paper and membrane must be the same size as the gel or 1 to 2 mm smaller in length and breadth.
- As each layer is added, take care to avoid wrinkles, folds or trapped air bubbles.
- Place the gel on the membrane.
- Cover the gel with three layers of saturated blotting paper.
- This sandwich is placed and compressed between two flat conduction plates that also serve as cathode (-) and anode (+).
- The orientation of the sandwich must be so that the membrane is on the anode (+) side of the gel.
- Connect color-coded leads to the power supply.
- Choose transfer conditions based on the instrument manufacturer's instructions (typically 1 h).

7.1. Recommended chemiluminescence detection protocol

Step	Action
1.	Incubate the membrane in a suitable blocking solution on an orbital shaker for 1 h at room temperature or overnight at 2°C to 8°C.
2.	Briefly rinse the membrane with three changes of wash buffer.
3.	Incubate the membrane in the primary antibody solution on an orbital shaker for 1 h at room temperature or overnight at 2°C to 8°C.
4.	Briefly rinse the membrane with two changes of wash buffer.
5.	Wash the membrane 4 to 6 times in wash buffer for 5 min each at room temperature on an orbital shaker.
Note	*Exposure to X-ray film requires up to 6 wash steps, to avoid background.*
6.	Dilute the secondary antibody (e.g. HRP-conjugated) in wash buffer.
7.	Incubate the membrane in the secondary antibody solution for 1 h at room temperature on an orbital shaker.
8.	Briefly rinse the membrane with two changes of wash buffer.
9.	Wash the membrane 4 to 6 times in wash buffer for 5 min each at room temperature on an orbital shaker.
10.	Detection using HRP and Amersham ECL Select Western Blotting Detection Reagent: Allow the detection solutions to equilibrate to room temperature for 20 min.
11.	Mix detection solutions A (luminol) and B (peroxide) in a ratio of 1:1 to a working solution. The final volume of detection reagent required is 0.1 ml/cm^2 membrane.
Note	*If the mixed reagent is not to be used immediately, protect it from exposure to the light either by wrapping in foil or storing in a dark place.*
12.	Drain the excess wash buffer from the washed membrane and place it protein side up in a suitable box or on a sheet of plastic wrap or other suitable clean surface. Add detection reagent onto the membrane and make sure it completely covers the membrane.
13.	Incubate for 5 min at room temperature.
14.	Drain off excess detection reagent by holding the membrane edge gently against a tissue paper.

15.

Detection using a CCD camera based system (e.g. ImageQuant LAS product family):

- Place the blot protein side up on a sample tray.
- The blot can be placed on a piece of plastic wrap, protein side up, to facilitate easy movement of the film on the sample tray.
- Place the sample tray in the CCD camera compartment and select suitable exposure time and/or function
- Use the automatic exposure function or select exposure time manually. Recommended starting exposure time is 60 s. Increase or decrease exposure time depending on the obtained signal intensity.

Detection using X-ray film (e.g. Amersham Hyperfilm™):

- Place the blot protein side down on to a fresh piece of plastic wrap, wrap the blots and gently smooth out any air bubbles.
- Place the wrapped blot with protein side up in an X-ray film cassette.
- Make sure there is no free liquid in the cassette; the film must not get wet.
- Place a sheet of X-ray film on top of the membrane. Close the cassette and allow exposure. Suitable exposure start time is 1 min.
- This stage should be carried out in a dark room using red safe lights. Do not move the film while it is being exposed.
- Develop the film immediately and, on the basis of the signal intensity obtained, estimate exposure time for a second sheet of film.

7.2. Recommended fluorescence detection protocol

Step	Action
1.	Block the membrane after transfer in 2% Amersham ECL Prime blocking agent in wash buffer, shaking for 1 h at room temperature or overnight at 2°C to 8°C.
2.	After blocking, rinse the membrane twice in PBS-T and then wash for 2 × 5 min. Keep the membrane in wash buffer or wash buffer without Tween 20 (for longer times than 3 days) until probed with primary and secondary antibodies.
3.	For single protein detection dilute the primary antibody to optimal concentration in wash buffer or blocking solution.
4.	Incubate a blocked membrane (protein side up) with the diluted primary antibody for 1.5 h at room temperature, shaking on a shaking platform.
5.	Rinse the membrane twice in wash buffer, then wash the membrane for 2 × 5 min in wash buffer shaking at room temperature.
6.	Dilute the ECL Plex CyDye conjugated secondary antibodies (1 µg/µl) to optimal concentration (between 1:1250 and 1:4000, we recommend 1:2500) in wash buffer.
7.	Incubate the washed membrane protected from light for 1 h at room temperature, shaking.
8.	Rinse the membrane three times in wash buffer, followed by 4 × 5 min in wash buffer, shaking at room temperature and protected from light.
9.	Rinse the membrane three times in wash buffer without Tween 20.
10.	Detect the secondary antibody signal by scanning the membrane using a fluorescent laser scanner.
Note	*If stripping is planned, do NOT allow the membrane to dry out*

For more information on fluorescence detection, see the Amersham ECL Plex Western Blotting System product booklet (search for code number 28994582 at www.gelifesciences.com) or download the Western Blotting handbook at www.gelifesciences.com/amershamwb

8. Additional information

8.1. Handling of membranes

1.	Always use powder-free gloves. Powder from gloves can give rise to contamination on fluorescent images of membranes
2.	Do not use a ballpoint pen on the membrane, since it can contaminate other membranes in the same tray and the ink is visible upon fluorescent scanning. If you need to write something on the membrane use a pencil.
3.	Do not touch membranes probed with the secondary antibody with gloved hands. This could leave marks on the fluorescent image.
4.	Always use forceps (slide forceps are recommended) when handling the membranes.
5.	Make sure to always keep the protein side of the membranes up throughout the procedure. Remember to scan the membranes with protein side facing down on the scanner bed if imaging system is used.
6.	To avoid mixing up your membranes, identify them by cutting, for example, one or more corners on each membrane. This is also very useful in a multi-membrane scan to be able to discriminate them from each other during image analysis.

8.2. Recommended Buffers and Solutions

Sample loading buffer for protein samples in SDS PAGE:
120 mM Tris pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 200 mM DTT, trace amount of bromophenol blue

SDS PAGE Running buffer:
25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS

Transfer buffer (wet transfer):
25 mM Tris, 192 mM glycine, 20% (v/v) methanol

Phosphate buffered saline (PBS), pH 7.4:
137 mM NaCl, 10 mM Na_2HPO_4 KH_2PO_4 H_2O

Tris buffered saline (TBS), pH 7.6:
50 mM Tris, 150 mM NaCl

Wash buffer: PBS-T and TBS-T:
- PBS or TBS with 0.05–0.1% (w/v) Tween 20
- Suitable for antibody dilutions
- Usage of TBS is recommended for detection of phosphorylated proteins

Blocking solution:
- % (w/v) Amersham ECL Prime or Amersham ECL Select Blocking Agent in wash buffer

Membrane stripping buffer (for PVDF membranes):
100 mM β-mercaptoethanol
2% (w/v) SDS
62.5 mM Tris-HCl pH 6.8

9. Related Western Blotting products

GE offers a complete range of products to facilitate Western blotting, including:

9.1. Molecular weight markers

Molecular weight markers are used to determine protein size. In addition, prestained markers allow confirmation of protein transfer and orientation (as the colored bands transfer to the membrane).

- Amersham ECL Rainbow Markers are prestained multicolored markers for monitoring progress of protein electrophoresis, confirming transfer efficiency and estimation of molecular weight of blotted proteins.
- Amersham ECL Plex Rainbow Markers are prestained multicolored markers, proteins are directly visible on gel and blot. Fluorescent marker bands are visible in both the Cy™3 and the Cy5 channel.
- Amersham ECL DualVue Markers are prestained two-colored markers, proteins directly visible on gel and blot. Tagged recombinant proteins generate chemiluminescent bands visible on film or with a CCD imager. DualVue markers are optimized for use with Amersham ECL, Amersham ECL Prime, and Amersham ECL Select.

9.2. Protein electrophoresis system

Amersham ECL Gel and Amersham ECL Gel Box constitute a horizontal mini-gel system for high-quality protein electrophoresis. The gel is compatible with standard Tris-Glycine buffers, and has a shelf life of up to 12 months. The gel contains no sodium dodecyl sulfate (SDS), making it an excellent choice for protein analysis under both native and denaturing conditions, depending on the choice of running buffer. Amersham ECL Gel reproducibly resolves complex samples, and works well with standard protein detection protocols. The gel enables high electrotransfer efficiency of proteins, and convenient integration into the Amersham ECL Western blotting workflow.

9.3. Blocking agents

After protein transfer the membrane need to be incubated in a blocking solution to prevent non-specific binding of antibodies, which can cause background and non-specific protein bands on the blot. The blocking agent should be optimized for best results. No single blocking agent is optimal for all proteins and antibodies. Blocking reagents are typically diluted to 2% to 5% (w/v) in PBS-T or TBS-T buffer. We recommend the following blocking agents compatible with Amersham ECL, ECL Prime and ECL Select: Amersham ECL Prime Blocking Agent, Amersham ECL Blocking Agent, BSA Blocking Agent, Non-fat dry milk.

9.4. Immunodetection reagents

- Primary antibody specific to the target protein(s)
- Reporter protein conjugated secondary antibody specific to the primary antibody. Dependent on the detection mechanisms, a secondary antibody conjugated to horseradish peroxidase, alkaline phosphatase, or a fluorescent molecule is possible.

9.5. Stripping and reprobing membranes

In some applications, it is necessary to detect more than one protein on the same membrane, particularly if you are performing a quantitative analysis. This can be achieved by stripping and reprobing your membrane, but this carries the risk of loss of protein from the membrane. Stripping and reprobing blots saves time and samples by enabling you to reprobe a single blot with different primary antibodies. It is therefore critical to use conditions that release antibodies from antigen while minimizing the elution of protein sample bound to the membrane.

By using a combination of detergents, reducing agents, heat and/or low pH, it is usually possible to find conditions that lead to acceptable results. Note that reblocking may be required prior to antibody incubation.

An alternative to stripping and reprobing is to use secondary antibodies labeled with fluorophores for simultaneous detection of more than one protein. With Amersham ECL Plex, two proteins can be simultaneously detected on a single blot with minimal cross-reactivity between antibodies or dyes. Even three target proteins can be detected simultaneously if a third species of primary antibody is used.

10. Visualization of Western blotting results

GE offers a broad portfolio around imaging of Western blotting results.

10.1. Chemiluminescence detection

Chemiluminescence is defined as light emission produced in a multistep reaction whereby peroxidase catalyzes the oxidation of luminol. In the presence of chemical enhancers and catalysts, the light intensity and the duration of light emission is greatly increased in a process known as enhanced chemiluminescence (ECL). ECL based on horseradish peroxidase (HRP)-conjugated secondary antibodies is a sensitive detection method where the light emission is proportional to protein quantity.

Amersham Hyperfilm ECL offers excellent sensitivity for low concentration protein detection, excellent band resolution, and the detection of low intensity bands. The film is also artefact-free due to an anti-static layer and produces publication-quality images due to a clear background. Amersham Hyperfilm ECL is suitable for use with Amersham ECL and Amersham ECL Prime and it can be processed in automatic processors or manually using most common X-ray film developers and fixers.

10.2. Fluorescence detection

The fluorescently-labeled secondary antibody is excited by light and the emission of the excitation is then detected by a photo sensor. Fluorescence is considered to be an excellent method for quantitation, but is less sensitive than chemiluminescence. One advantage of fluorescence detection is that two proteins can be detected at the same time using antibodies with different fluorophores.

10.3. Colorimetric detection

Colorimetric detection is a very simple and cost-effective method for visualizing protein signals. Enzymes like alkaline phosphatase (AP) and HRP convert substrates to a colored precipitate. Since the accumulated precipitate is visible to the human eye no further work in the dark room with costly materials is needed. On the other hand colorimetric detection is considered less sensitive than chemiluminescence detection.

10.4. Radioactive detection

Chemiluminescent and fluorescent methods for the detection of enzyme-labeled antibodies have largely replaced radioactivity in the immunodetection of proteins. This is because non-radioactive systems are highly sensitive, yield hard-copy results for subsequent analysis after as little as 1 min exposure to autoradiography film, and are safer to handle than radioisotopes.

11. Related applications

Eastern Blotting and Far Western Blotting applications are related to Western blotting. Special protocols are needed to be followed for these applications.

Dot blot is an application that can be used to optimize the ideal antibody concentrations for detection results of highest quality. This method can also be used for optimizing additional parameters, such as blocking agents, species of primary antibody, and quality of primary antibody. For more information please see our GE Western Blotting handbook, available from www.gelifesciences.com/westernblotting

Southern and Northern Blots were developed for analysis of nucleic acids. After electrophoresis the nucleic acid sample is usually transferred to nylon membranes. For more information please see the GE Handbook Nucleic Acid Sample Preparation for Downstream Analyses available from www.gelifesciences.com/sampleprep

12. Trouble shooting guide

Problem	Possible causes	Action to take
No signal	• Nondetectable amounts of target protein	• Use appropriate amounts of protein
	• Primary antibody is not binding to target protein, which may be due to bad quality and/or nonspecific primary antibody.	• Use appropriate and high quality primary antibody with a good binding capacity.
	• Incorrect species of secondary antibody has been used.	• Check that secondary antibody is from appropriate species
	• Transfer did not work efficiently	• Check that transfer equipment is working properly and that the correct procedure has been followed. • Check protein transfer by staining the membrane and/or gel. • Confirm transfer efficiency using prestained Rainbow marker.
	• Some proteins may be affected by the treatments required for electrophoresis.	• For some proteins special Western blotting procedures are needed.
	• Detection reagents do not function properly • Incorrect storage of detection reagent may cause a loss of signal. Bacterial growth may inhibit the reagent.	• To test the detection reagent activity, in a darkroom prepare 1 to 2 ml of detection reagent working solution in a clear test tube. Add 1 µl of undiluted HRP-conjugated antibody solution. • The solution should immediately emit a visible blue light that fades during the next several minutes.
Weak signal	• Transfer efficiency may have been poor.	• Optimize transfer conditions
	• Insufficient protein was loaded on to the gel.	• Check protein concentration
	• The antibody concentration or quality could be too low.	• Optimize primary and secondary antibody dilutions. • Always use high quality antibodies

Problem	Possible causes	Action to take
Weak signal, continued	• Exposure time may have been too short.	• Try different exposure times to acquire optimal result
High background	• Too high concentrations of primary and secondary antibodies.	• Optimization is required.
	• Transfer and incubation buffers may have become contaminated and require replacing.	• Always use fresh solutions • Insufficient blocking. Optimize blocking protocol.
	• Insufficient washing.	• Add washing steps and use sufficient amount of wash buffer and add additional washing steps.
	• The blocking agent used was not freshly prepared or was too dilute or was incompatible with the application.	• Always use freshly prepared and completely resolved blocking agent. • If phosphorylated proteins are to be stained use TBS-T instead of PBS-T to prepare antibody dilutions. • The level of Tween used in the blocking agent was not sufficient for the application performed.
	• The membrane was allowed to dry (or dry partially) during some of the incubations.	• Poor gel quality. • Nonspecific and bad quality of antibodies. • The film detection of the signal was allowed to over expose.
	• Non compatible products	• Always check product compatibilities
	• The level of signal is so high that the film has become completely overexposed.	• Optimize the signal intensity: use appropriate protein amounts, optimize antibody dilutions.
Negative white bands on the film	• Negative bands generally occur when protein target is in excess and antibody concentrations are too high. The effect is caused by substrate depletion.	• Load less amount of protein. • Dilute both primary and secondary antibody further

Problem	Possible causes	Action to take
Excessive, diffuse signal	• Insufficient protein was loaded on to the gel.	• Load appropriate amounts of protein sample.
	• Bad quality and/or unspecific primary antibody.	Always used high quality antibodies and optimized concentration
	• Transfer efficiency may have been poor.	• Optimize transfer conditions
	• Exposure time may have been too short	• Try different exposure times to acquire optimal result
Uneven, spotted background	• Insufficient washing.	• Add additional washing steps
	• The blocking agent is not completely dissolved in the buffer.	• Make sure that the blocking agent is completely dissolved, prepare blocking agent 30 min before usage
	• Incorrect handling can lead to contamination on the blots and/ or membrane damage which may cause non-specific signal.	• Avoid contaminations with e.g. dyes, latex gloves powder.
	• Areas of the blot may have dried during some of the incubations.	• Avoid membrane and partial membrane drying
	• Antibody aggregates	• Always use high quality antibodies

13. Related products

This chapter presents a subset of related products. For more information, refer to www.gelifesciences.com/westernblotting

13.1. Sample preparation

Product	Quantity	Code no.
SDS-PAGE Clean-up kit	50 samples	80-6484-70
Mammalian Protein Extraction Buffer	1 × 500 ml	28-9412-79
2-D Quant Kit	500 assays	80-6483-56

13.2. Molecular weight markers

Product	Quantity	Code no.
Amersham ECL Rainbow Marker – Low range	250 µl	RPN755E
Amersham ECL Rainbow Marker – High range	250 µl	RPN756E
Amersham ECL Rainbow Marker – Full range	250 µl	RPN800E
ECL DualVue Western Blotting Markers (25 loadings / pack)	1 pack	RPN810
ECL Plex Fluorescent Rainbow Markers	120 µl	RPN850E
ECL Plex Fluorescent Rainbow Markers	500 µl	RPN851E

13.3. Amersham ECL protein electrophoresis gels

Product	Wells	Quantity	Code no.
Amersham ECL Gel Box	-	1	28-9906-08
Amersham ECL Gel 10%	10	10	28-9898-04
Amersham ECL Gel 12%	10	10	28-9898-05
Amersham ECL Gel 4-12%	10	10	28-9898-06
Amersham ECL Gel 8-16%	10	10	28-9898-07
Amersham ECL Gel 4-20%	10	10	28-9901-54
Amersham ECL Gel 10%	15	10	28-9901-55
Amersham ECL Gel 12%	15	10	28-9901-56
Amersham ECL Gel 4-12%	15	10	28-9901-57
Amersham ECL Gel 8-16%	15	10	28-9901-58
Amersham ECL Gel 4-20%	15	10	28-9901-59

13.4. Electrophoresis and Western blotting instruments

Product	Quantity	Code no.
Tank transfer units		
TE 22 Mini Tank Transfer Unit	1	80-6204-26
TE 62 Transfer Cooled Unit	1	80-6209-58
ECL Semidry Blotters		
TE 70 Semidry Transfer Unit, 14 × 16 cm	1	80-6210-34
TE 70 PWR Semidry Transfer Unit, 14 × 16 cm (Including Power Supply)	1	11-0013-41
TE 77 Semidry Transfer Unit, 21 × 26 cm	1	80-6211-86
TE 77 PWR Semidry Transfer Unit, 21 × 26 cm	1	11-0013-42

13.5. Blotting paper

Product	Quantity	Code no.
Amersham Hybond Blotting Paper, 20 × 20 cm	100 sheets	RPN6101M
Whatman 3MM Chr Blotting Papers, 20 × 20 cm	100 sheets	3030-861

13.6. Blocking agents

Product	Quantity	Code no.
Amersham ECL Blocking Agent	40 g	RPN2125
Amersham ECL Prime Blocking Agent	40 g	RPN418

13.7. HRP-linked secondary antibodies

Product	Host	Quantity	Code no.
Amersham ECL sheep-α-mouse IgG-HRP, whole Ab	sheep	1 ml	NA931-1ML
Amersham ECL sheep-α-mouse IgG-HRP, $F(ab)_2$ fragment	sheep	1 ml	NA9310-1ML
Amersham ECL donkey-α-rabbit IgG-HRP, $F(ab)_2$ fragment	donkey	1 ml	NA9340-1ML
Amersham ECL donkey-α-rabbit IgG-HRP, whole Ab	donkey	1 ml	NA934-1ML
Amersham ECL sheep-α-human IgG-HRP, whole Ab	sheep	1 ml	NA933-1ML

13.8. Amersham ECL Plex CyDye conjugated antibodies

Product	Host	Quantity	Code no.
Amersham ECL Plex goat-α-mouse IgG-Cy2	goat	150 µg	28-9011-08
Amersham ECL Plex goat-α-rabbit IgG-Cy2	goat	150 µg	28-9011-10
Amersham ECL Plex goat-α-rabbit IgG-Cy3	goat	150 µg	28-9011-06
Amersham ECL Plex goat-α-mouse IgG-Cy3	goat	150 µg	PA43009
Amersham ECL Plex goat-α-rabbit IgG-Cy5	goat	150 µg	PA45011
Amersham ECL Plex goat-α-mouse IgG-Cy5	goat	150 µg	PA45010

13.9. Western Blotting Combination Packs

Product	Quantity	Code no.
Amersham ECL Plex Western Blotting Combination Pack Cy3, Cy5, Hybond ECL	1	RPN998
Amersham ECL Plex Western Blotting Combination Pack Cy3, Cy5, Hybond LFP for two slab gels	1	RPN999

13.10. Detection reagents

Product	Quantity	Code no.
Amersham ECL Select Western Blotting Detection Reagent	for 1000 cm^2	RPN2235
Amersham ECL Prime Western Blotting Detection Reagent	for 1000 cm^2	RPN2232
Amersham ECL Western Blotting Detection Reagents	for 1000 cm^2	RPN2109
Amersham ECL Western Blotting Detection Reagents	for 4000 cm^2	RPN2106
Amersham ECL Western Blotting Detection Reagents	for 6000 cm^2	RPN2134

13.11. Amersham Hyperfilm X-ray films

Product	Quantity	Code no.
Hyperfilm ECL (5 × 7 inches)	50 sheets	28-9068-35
Hyperfilm ECL (18 × 24 cm)	50 sheets	28-9068-36
Hyperfilm ECL (8 × 10 inches)	50 sheets	28-9068-38
Hyperfilm ECL (24 × 30 cm)	50 sheets	28-9068-40

13.12. Imaging systems

Product	Quantity	Code no.
ImageQuant LAS 4000	1	28-9558-10
ImageQuant LAS 4010	1	28-9558-11
ImageQuant LAS 4000 mini	1	28-9558-13
ImageQuant LAS 500	1	29-0050-63

13.13. Software and accessories

Product	Quantity	Code no.
CD and getting started: ImageQuant TL and IQTL SecurITy Software package (with Getting Started Guide)		28-9380-94
Licenses for ImageQuant TL only	1 user	28-9236-62
ImageQuant TL and ImageQuant TL SecurITy	1 user	28-9332-73

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