GE Healthcare

Amersham Hybond ECL Nitrocellulose Membrane

Product Booklet

Codes:		
	RPN68D	6 × 8 cm, 50 sheets
	RPN2020D	20 × 20 cm, 10 sheets
	RPN78D	7 × 8 cm, 50 sheets
	RPN910D	9 × 10.5 cm, 10 sheets
	RPN82D	82 mm, 50 discs
	RPN132D	132 mm, 50 discs
	RPN203D	20 cm × 3 m, 1 roll
	RPN1010D	10 × 10 cm, 10 sheets
	RPN1520D	15 × 20 cm, 10 sheets
	RPN3032D*	30 cm × 3 m, 1 roll
	RPN303D	30 cm × 3 m, 1 roll

* 0.20 µm pore size

Page finder

1. Legal

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ECL Direct (RPN3000,3001,3005) is covered by US patent numbers 373017 and 513040 and foreign equivalents and is sold under license from Institut Pasteur.

ECL Plus contains Lumigen PS3 substrate and is sold under exclusive license from Lumigen Inc.

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2. Handling

2.1. Safety warnings and precautions

Warning: ***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.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is 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. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water (see safety data sheet for specific advice).

Note that the procedures require the use of:
Acrylamide/NN,-methylene-bis-acrylamide: toxic substance
Ammonium Persulphate: harmful TEMED: highly flammable, irritant
β-Mercaptoethanol: poisonous substance
Methanol: toxic substance
Sodium Dodecyl Sulphate: irritant
Please follow the manufacturers' safety data sheet relating to the safe handling and use of these materials.

2.2. Storage

Membranes are provided in airtight resealable aluminium bags to prevent absorption of moisture and other airborne contaminants. Membrane should be stored in a clean, dry atmosphere away from noxious fumes. Avoid conditions of extreme humidity. Due to their

flammable nature nitrocellulose membranes should be stored in a dedicated flame-proof cabinet.

2.3. Stability

Before opening, membranes are stable for one year. Once open keep in the resealed bag at ambient. Performance is consistent for up to three months when stored under the recommended conditions.

3. Components

RPN68D: 6 × 8 cm, 50 sheets

RPN2020D: 20 × 20 cm, 10 sheets

RPN78D: 7 × 8 cm, 50 sheets

RPN910D: 9 × 10.5 cm, 10 sheets

RPN82D: 82 mm, 50 discs

RPN132D: 132 mm, 50 discs

RPN203D: 20 cm × 3 m, 1 roll

RPN1010D: 10 × 10 cm, 10 sheets

RPN1520D: 15 × 20 cm, 10 sheets

RPN3032D*: 30 cm × 3 m, 1 roll

RPN303D: 30 cm × 3 m, 1 roll

* 0.20 µm pore size

4. Other materials required

Equipment

The following materials and equipment are required but not supplied:

- Vertical gel electrophoresis apparatus, for example Hoefer™ mini VE and SE200/600 electrophoresis systems
- Hybond™ Blotting Paper from GE Healthcare
- Trays/dishes
- Pipettes, for example, Gilson™ Pipetman™ P20, P200, P1000 and P5000.
- Assorted laboratory glassware
- Orbital shaker
- SaranWrap™ or similar cling film
- X-ray film cassette, for example Hypercassette™ from GE Healthcare
- Timer
- Autoradiography film, for example Hyperfilm™ ECL™
- X-ray film developing facilities.

Solutions

- **Reagents required for electrophoresis, blotting and immunodetection of proteins**

All reagents should be AnalR™ grade where possible.

Protein sample loading buffer

4.0 ml Distilled water
1.0 ml 0.5M Tris-HCl, pH 6.8
0.8 ml Glycerol
1.6 ml 10%(w/v) Sodium Dodecyl Sulphate (SDS)

0.4 ml β-Mercaptoethanol
0.5 ml 0.05% (w/v) Pyronin Y (e.g. Sigma™ code P-7017)
Store in dark at room temperature for a maximum of 2 weeks.

0.5 M Tris-HCl, pH 6.8
6.0 g Trizma™ base
Add approximately 90 ml distilled water. Mix to dissolve. Adjust pH to 6.8 with Hydrochloric acid. Make up to a final volume of 100 ml. Store at 2–8°C.

Resolving gel buffer
90.8 g Trizma base
2.0 g Sodium Dodecyl Sulphate (SDS)
Add approximately 900 ml distilled water. Mix to dissolve. Adjust pH to 8.8 with Hydrochloric acid. Make up to a final volume of 1000 ml. Store at 2–8°C.

Acrylamide stock solution (30% (w/v))
29.2 g Acrylamide
0.8 g N, N-methylene-bis-acrylamide
Add to approximately 50 ml of distilled water. Mix to dissolve. Make up to a final volume of 100 ml. Store at 2–8°C for a maximum of 2 weeks.

Warning: Acrylamide and N,N-methylene-bis-acrylamide (monomers) are toxic. Please follow the manufacturer's instructions relating to the safe handling and use of these materials.

Ammonium persulphate stock solution (10% (w/v))
0.1 g Ammonium Persulphate
1.0 ml Distilled water
Mix well. Prepare fresh as required.

Resolving gel (12%)
7.5 ml Acrylamide stock solution (30%(w/v))
9.0 ml Resolving gel buffer

2.0 ml Distilled water
180 µl Ammonium Persulphate stock solution (10% (w/v))
18 µl N,N,N,N-Tetramethylethylenediamine (TEMED)

Stacking gel (4%)
1.8 ml Acrylamide stock solution (30% (w/v))
5.0 ml Stacking gel buffer
4.0 ml Distilled water
50 µl Ammonium Persulphate stock solution (10% (w/v))
10 µl N,N,N,N-Tetramethylethylenediamine (TEMED)

Stacking gel buffer
30.3 g Trizma base
2.0 g Sodium Dodecyl Sulphate (SDS)
Add approximately 900 ml of distilled water. Mix to dissolve. Adjust pH to 6.8 with Hydrochloric acid. Make up to a final volume to 1000 ml. Store at 2–8°C.

Protein transfer buffer
3.03 g Trizma base
14.4 g Glycine
200 ml Methanol (optional)
Add approximately 650 ml of distilled water. Mix to dissolve. Make up to a final volume of 1000 ml. Store at 2–8°C. Inclusion of methanol in the buffer minimizes swelling of the gel during blotting and increases the binding capacity of nitrocellulose membrane.

Tris buffered saline (TBS), pH7.6
12.1 g Trizma base
40.0 g Sodium Chloride
Dilute to 5000 ml with distilled water. Adjust pH to 7.6 with Hydrochloric acid. Store at 2–8°C.

TBS-Tween (TBS-T)
Dilute required volume of Tween™-20 in TBS to give a 0.1%(v/v) solution. Store at 2–8°C.

Phosphate Buffered Saline (PBS), pH7.5
11.5 g Di-Sodium Hydrogen Orthophosphate, Anhydrous
2.96 g Sodium di-Hydrogen Orthophosphate
5.84 g Sodium Chloride Dilute to 1000 ml with distilled water. Adjust pH to 7.5. Store at 2–8°C

PBS-Tween (PBS-T)
Dilute required volume of Tween-20 in PBS to give a 0.1%(v/v) solution. Store at 2–8°C.

5. Description

All Hybond membranes from GE Healthcare are manufactured to strict specifications based on our extensive experience in Life Science. Production runs are carefully controlled and the product exhaustively screened to ensure that only the most consistent product reaches the user. Hybond ECL has been extensively tested in combination with the GE Healthcare ECL labelling and detection systems for both protein and nucleic acid applications. Hybond ECL is recommended for use in protein studies. Significant advances in membrane technology over recent years means that more suitable membranes are available for nucleic acid work.

GE Healthcare offers scientists the choice of three chemiluminescent substrates each with distinct advantages. Launched over 10 years ago, ECL, the original chemiluminescent reagent in which the oxidation of luminol is catalyzed by Peroxidase/Hydrogen (1,2), revolutionized Western blotting. ECL Plus™ addressed the need for increased sensitivity whilst also offering both chemiluminescent and chemifluorescent detection options. Continually developing innovative products GE Healthcare then introduced ECL Advance™, offering the ultimate in sensitivity and increased light output thereby minimizing the use of valuable antibodies. Emitted light allows for the rapid capture of results on X-ray film, for example Hyperfilm ECL. Multiple exposures are possible and a hard copy result ensures no fading of results, a problem with some colorimetric systems. ECL Plus offers the added advantage of chemifluorscent detection on an appropriate fluorescent scanner. Hybond ECL is the ideal membrane for use with the ECL Western blotting system.

In early nucleic acid studies, described by Edwin Southern (3), nitrocellulose membranes were used for DNA immobilization. Today more robust nylon membranes such as Hybond-NX, Hybond-N+ and Hybond-XL are available for this type of work. However, many researchers continue to use nitrocellulose in their own optimized procedures. This booklet restricts itself to information on the use of Hybond ECL for Western blotting applications.

6. Critical parameters

Storage

GE Healthcare has addressed the special storage requirements of nitrocellulose membranes by providing the membrane in an airtight resealable aluminium bag. This prevents absorption of moisture and other airborne contaminants which would otherwise have an adverse effect on the performance of the membrane. Space is provided on the bag label for the user to insert an '*opened on*' date.

Membranes should be stored under the conditions stated on the front of this booklet. Nitrocellulose membranes will set up an equilibrium with the environment. A key factor in storage is humidity. In conditions of high humidity the membrane will absorb moisture and contaminants, causing folding and curling as the membrane expands. This will affect performance reducing sensitivity and wettability. In conditions of low humidity the membrane will lose moisture, this causes the membrane to become brittle and increasingly hydrophobic making wetting difficult.

Handling

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

Wettability

The wettability of the membrane is important in achieving a consistent performance. Nitrocellulose membrane should be pre-wet before use and wetting should be even and rapid.
Pre-wet first in distilled water, then transfer the membrane to the appropriate transfer buffer and equilibrate for at least 10 minutes before use.

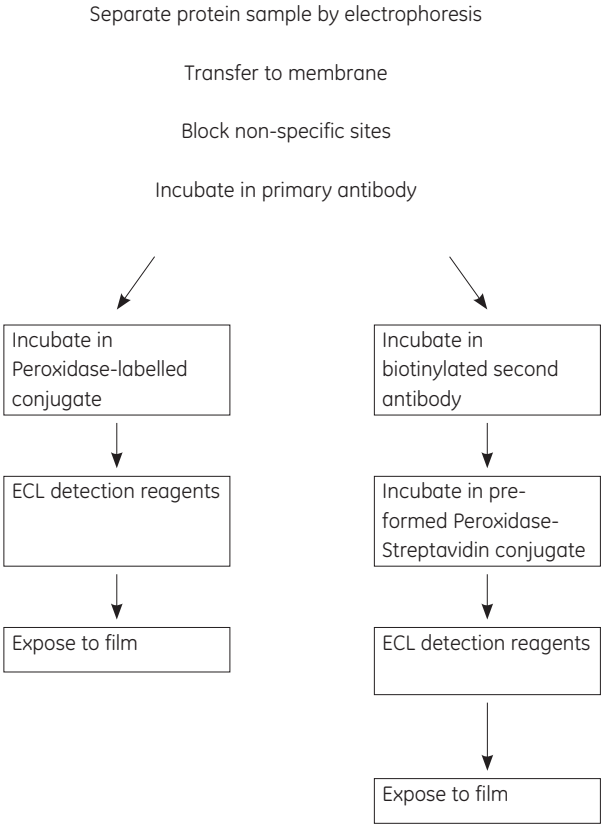

Figure 1. Schematic representation of possible detection strategies

7. Protocols

7.1. Protocol for Western blotting

There are three basic methods for transferring proteins to membranes, capillary blotting, diffusion blotting, and electroblotting. Capillary and diffusion blotting are relatively slow procedures. The latter is by far the most widely used (4).

Protocol	Notes
1. Separate the protein samples using gel electrophoresis or isoelectric focusing.	
2. Remove the stacking gel and remove one corner to orientate the resolving gel.	**2.** A clean sharp razor blade or scalpel should be used.
3. Soak the gel in the protein transfer buffer for at least 10–20 minutes.	**3.** For transfer buffers without methanol it is essential that complete equilibration of the resolving gel is achieved to prevent distortions within the gel which would cause band smearing. Only a brief rinse is required to achieve equilibration if the transfer buffer contains methanol.
4. Prepare a sheet of Hybond ECL. Cut the membrane to size. Pre-wet the membrane in distilled water and then equilibrate in the protein transfer buffer for at least 10 minutes.	**4.** Fresh membrane should be used if rehydration is not even and/or rapid. Hydration must be complete before the membrane is used.

Protocol	Notes
5. Assemble the electroblotting cassette and place between the electrodes in the blotting unit, according to the manufacturer's instructions.	**5.** The Hybond ECL must be on the anode side of the gel.
6. Transfer for 1–4 hours at 8–10 v/cm.	**6.** Extended transfer times, overnight for example, may require cooling.
7. Following transfer remove the membrane from the blotting cassette, mark the orientation of the gel on the membrane and rinse briefly.	**7.** Membranes should be air dried before storage. Blots may be stored between sheets of Whatman™ 3 MM paper wrapped in SaranWrap at 2–8°C for up to 3 months.
8. Blots may be used immediately or allowed to air dry and stored.	

7.2. Protocol for immunodetection

Please note: Protein detection using ECL reagents is extremely sensitive. For results with high signal and low background it is essential to optimize the concentrations of both primary and secondary antibodies. The high sensitivity means that much higher dilutions of antibodies than used with conventional systems are required. See the Additional Information section, page 27 for details of optimization experiments that can be performed to determine the best concentrations of primary and secondary antibodies.

During immunodetection, sufficient solution should be used to adequately cover the membrane and the containers should be agitated gently. Details of a recommended rolling cylinder system can be found on page 30.

Protocol	Notes
1. Blocking the membrane Non-specific binding sites are blocked by immersing the membrane in 5% (w/v) blocking reagent in TBS-T or PBS-T for one hour at room temperature on an orbital shaker.	**1.1.** The combination of Tween and a blocking reagent should be suitable for most protein blotting work. Optimum Tween concentrations will vary to suit specific experiments, but a 0.1% (v/v) Tween-20 concentration in PBS or TBS is suitable for most ECL Western blotting work on nitrocellulose membranes. Certain experimental situations may require alteration of the duration and temperature of the blocking incubation. **1.2.** Alternative blocking buffers are given on pages 28–29. **1.3.** Membranes may be left in the blocking solution overnight at 2 to 8°C if more convenient.
2. Washing PBS-T or TBS-T is recommended for washing. Briefly rinse the membrane using two changes of washing buffer then wash once for 15 minutes and	**2.** As a general rule, as large a volume as possible of washing buffer should be used each time, 4 ml of buffer per cm^2 is suggested. Brief rinses of the membrane before incubating in wash

Protocol	Protocol
2. Washing *continued.* twice for 5 minutes, with fresh changes of the washing buffer at room temperature.	**2.** *continued.* buffer will improve washing efficiency.
3. Dilution of primary antibody During the washing step dilute the primary antibody to an appropriate dilution.	**3.** Dilution of the primary antibody required to give optimum results will vary and should be determined for each antibody used. These optimization experiments may be performed by dot blot analysis. (See Additional Information page 27).
4. Incubation Incubate the membrane in diluted primary antibody for 1 hour at room temperature.	**4.** Incubation times and temperatures will vary and should be optimized for each antibody. The conditions indicated are recommended starting points.
5. Washing Wash the membrane as detailed in step 2.	
6. Dilution During the washing step dilute the biotinylated antibody or the peroxidase labelled secondary antibody to an appropriate dilution (see Additional Information page 27).	**6.** The ECL Western blotting detection reagent can be used with any Peroxidase labelled second antibody. Due to the sensitivity of the ECL Western blotting detection reagents the dilution of the second antibody should be optimized to give the

Protocol	Notes
	6. *continued* highest signal with minimum background (see page 27).
7. Incubation Incubate the membrane in the diluted second antibody for 1 hour at room temperature.	**7.** Incubation times and temperatures may vary for individual antibodies.
8. Washing Wash the membrane as detailed in step 2.	**8.** If a Peroxidase labelled second antibody is used, proceed to step 10 for the washing step.
9. Incubation If a biotinylated second antibody was used then dilute the Peroxidase-Streptavidin conjugate to an appropriate dilution, and incubate for 1 hour at room temperature.	**9.** Incubation times may vary.
10. Washing Wash the membrane as detailed in step 2.	**10.** Thorough washing of the membrane will minimize background.
11. Detection Read through this whole section before proceeding. It is necessary to work quickly once the blots have been exposed to the detection solution. All steps can be	**11.** Information provided here refers to the use of ECL detection reagents. Other detection reagents in the series, ECL Plus and ECL Advance for example have variations to this protocol.

Protocol	Notes
11. Detection *continued* carried out in a dark room; it is only necessary to switch off the light after section 11.5.	**11.** *continued* Details are provided in the pack literature provided with these products.
11.1. Take the ECL detection reagents supplied and mix an equal volume of detection solution 1 with detection solution 2 to give sufficient liquid to cover the membranes.	**11.1.** The final volume required is 0.125 ml/cm^2 membrane.
11.2. Drain the excess wash buffer from the washed blots and place **protein side uppermost** on a sheet of SaranWrap spread over the bench. Add the prepared ECL detection reagent directly to the side carrying the protein; do not leave the blots to dry out.	**11.2**. An alternative method is to equilibrate the drained blots in a fresh container in the required quantity of solution 2 (0.0625 ml/cm^2), and then add to this a similar quantity of solution 1 and mix by gentle shaking.
11.3. Incubate for 1 minute at room temperature.	
11.4. Drain off excess detection reagent. Place the blot, **protein side down**, onto a fresh piece of SaranWrap. Fold the SaranWrap over	**11.4.** Drain off excess detection reagent by holding the blot vertically and touching the edge of the blot against tissue

Protocol	Notes
11.4. Continued. the back of the blots to form an envelope.	**11.4.** Continued. paper. Smooth out any air bubbles that may form.
11.5. Place the blots, **protein side up**, in the film cassette. Work as quickly as possible; minimize the delay between incubating the blots in the detection reagent and exposing them to the film (next step).	**11.5.** Ensure that there is no free detection reagent in the film cassette; the film must not get wet.
11.6. Switch off the lights and carefully place a sheet of autoradiography film, for example Hyperfilm ECL on top of the blots, close the cassette and expose for 15 seconds.	**11.6.** Do this in a dark room, using an appropriate red safelight. Do not move the film while it is being exposed.
11.7. Remove and develop the film. If required expose a second piece of film for an appropriate length of time.	**11.7.** On the basis of the first exposure's appearance estimate how long to continue the exposure of the second piece of film. Second exposures can vary from 1 minute to 1 hour. This will depend on the amount of target protein on the blot. If background is high the

Protocol	Notes
	11.7. *Continued.* blot may be rewashed twice for 10 minutes with wash buffer and re-detected following steps 11.1–11.7, some loss of sensitivity may result. If over-exposure occurs because of high target antigen concentration, leave blots for 5–10 minutes before re-exposing to film. If exposure times of less than 5 seconds are routinely required it is recommended that the antibodies used are further diluted as it is difficult to perform such exposures.

7.3. Protocol for dot blotting (manual)

The following is a general protocol for dot blotting proteins. A number of commercially available devices are also available. These provide for a more consistent and even application of the sample than the manual procedure described below. This parameter is particularly important in those experiments requiring quantification.

Protocol	Notes
1. Cut the Hybond ECL membrane to size using a clean scalpel.	
2. Using a pencil, mark the membrane lightly with a grid or dots to guide subsequent sample application. There should be a minimum distance of 1 cm between samples applied in a volume of 5 µl or less.	
3. Pre-wet the membrane in distilled water followed by TBS or PBS for protein samples.	**3.** Samples can either be applied directly to the wet membrane or the membrane can be allowed to air dry before sample application.
4. Dilute the samples in an appropriate buffer, for example TBS or PBS to the required concentration. A sample size of 1–2 µl is ideal for manual dot blotting.	**4.** Carrier substances, for example bovine serum albumin, may be included in the diluent buffer to improve retention of very small amounts of target on the membrane.

Protocol	Notes
	4. *Continued.* Larger sample volumes of 50–200 µl are common for commercial apparatus. This ensures an even application of the sample over the whole dot or slot.
5. Carefully apply the 2 µl sample to the membrane, avoiding touching the membrane with the pipette tip. Leave the membrane to air dry.	**5.** If the sample volume is greater than 2 µl, then apply in successive 2 µl aliquots to the same position on the membrane, allow the aliquot to dry between each application. This will prevent the sample spreading.
6. No further treatment is required for protein samples.	
7. Blots may be used immediately or stored at 2–8°C wrapped in SaranWrap for up to 3 months.	

7.4. Protocol for reprobing membranes

Following ECL detection it is possible to reprobe the membrane several times to either clarify or confirm results or when small or valuable samples are being analyzed (5). Sequential reprobing of membranes with a variety of antibodies is possible. The blots must be stored wet wrapped in SaranWrap at 2–8°C after each immunodetection.

Protocol one

This procedure is suitable for sequential reprobings using primary and secondary antibodies raised in different species or where different immunodetection systems have been used.

Protocol	Notes
1. Reapply prepared ECL detection reagents to the blots and re-expose to a sheet of autoradiography film.	**1.** Refer to step 11 on pages 20–23.
2. If a signal is detected incubate the blot in prepared ECL detection reagents for 30 minutes. Repeat step 1. If no signal is detected proceed to step 3.	**2.** Do not allow the blots to dry out. Excessive incubation in ECL reagents will inhibit peroxidase activity.
3. Perform immunodetection using a primary antibody raised in a different species to the first. Secondary antibodies must demonstrate no cross reactivity.	

Protocol two

The complete removal of primary and secondary antibodies from membranes is possible following the method outlined below. The membranes may be stripped of bound antibodies and reprobed several times. This procedure is not suitable for enyzme substrates where insoluble reaction products are deposited on the membrane. Membranes should be stored wet wrapped in SaranWrap in a refrigerator (2–8°C) after each immunodetection.

In excess of 50% of some target proteins can be lost when performing experiments where blots are stripped and reprobed. It is therefore important to consider which antigen is present in least abundance and probe for this first.

Protocol	Notes
1. Submerge the membrane in stripping buffer (100 mM b-mercaptoethanol, 2% (w/v) Sodium Dodecyl Sulphate, 62.5 mM Tris-HCl pH 6.7) and incubate at 50°C for 30 minutes with occasional agitation.	**1.** Different antibodies may require different stripping temperatures and/or incubation periods. Two Tech Tips are available that detail strategies for stripping Western blots and reprobing.
2. Wash the membrane for 2 x 10 minutes in TBS-T or PBS-T at room temperature using large volumes of wash buffer.	**2.** Membranes may be incubated with the ECL detection reagents and exposed to film to ensure removal of antibodies.
3. Block the membrane by immersing in 5% (w/v) blocking reagent in TBS-T or PBS-T for 1 hour at room temperature.	**3.** Refer to note 1.1. on page 17.
4. Perform immunodetection as described in page 17.	

8. Additional information

8.1. Determination of optimum antibody concentration

Due to the sensitivity of ECL Western blotting, optimization of antibody concentrations is necessary to ensure the best results. Generally, lower concentrations of both primary and secondary antibodies are required with ECL compared to colorimetric detection. Outlined below are protocols for determining optimal antibody concentrations.

Dot blots are a quick and effective method for determining the optimum dilution of a primary antibody of unknown concentration. Alternatively, a Western blot can be prepared and then cut into several strips. It should be noted that some antibodies may require alternative blocking and washing steps to the ones suggested below.

1. Primary antibodies

1.1. Spot a serial dilution of the antigen on to prepared Hybond ECL, and allow to dry. The dilution range should be representative of the likely quantity of antigen requiring detection. Prepare one blot for each primary antibody dilution to be tested.

1.2. Incubate in block solution for 1 hour at room temperature with agitation.

1.3. Wash the membranes briefly in two changes of washing buffer then wash once for 15 minutes and twice for 5 minutes, with fresh changes of the washing buffer at room temperature.

1.4. Prepare several dilutions of primary antibody. Incubate one blot in each antibody dilution for 1 hour at room temperature.

1.5. Wash as detailed in step 1.3.

1.6. Dilute the secondary antibody and incubate the membranes for 1 hour at room temperature.

1.7. Wash as detailed in step 1.3.

1.8. Detect using ECL detection reagents as detailed on pages 17–22. The antibody dilution that gives the maximum signal with minimum background should be selected.

2. Secondary antibodies

2.1. Prepare dot blots and block the membranes as described in 1.1 and 1.2.

2.2. Incubate in an optimized dilution of primary antibody for 1 hour at room temperature.

2.3. Wash as detailed in step 1.3.

2.4. Prepare several dilutions of secondary antibody. Incubate one blot in each antibody dilution for 1 hour at room temperature.

2.5. Wash as detailed in step 1.3.

2.6. Detect using ECL detection as detailed on pages 17–22. The antibody dilution that gives maximum signal with minimum background should be selected.

8.2. Blocking buffers used in Western blotting

Blocking agent	Characteristics
ECL Blocking Agent	Optimized for use with ECL immunodetection Gives maximum signal:noise ratio Recommended for most applications
ECL Advance blocking agent	Optimized for use with ECL Advance only
Dried milk For example, 5%(w/v) non-fat dried milk in PBS or TBS	Clean background Deteriorates rapidly Disguises some antigens

Blocking agent	Characteristics
	Variable depending upon brand
Milk/Tween-20 For example, 5%(w/v) non-fat dried milk, 0.1%(v/v) Tween-20 in PBS or TBS	Clean background Deteriorates rapidly Disguises some antigens
Tween-20 For example, 0.1%(v/v) Tween-20, 0.02%(w/v) Sodium Azide in PBS or TBS	Allows staining after antigen detection May give rise to residual background Azide is an inhibitor of Peroxidase. Use of formulation containing azide as a diluent for Peroxidase labelled antibodies or streptavidin is not advised.
BSA For example, 3%(w/v) BSA, 0.02%(w/v) Sodium Azide in PBS or TBS	Good signal strength Azide is an inhibitor of Peroxidase. Use of formulation containing azide as a diluent for Peroxidase labelled antibodies or streptavidin is not advised.
Horse serum For example, 10%(v/v) horse serum, 0.02%(w/v) Sodium Azide in PBS or TBS	Clean background Incompatible with some anti-immunoglubin antibodies Azide is an inhibitor of Peroxidase. Use of formulations containing azide as a diluent for Peroxidase labelled antibodies or streptavidin is not advised.

8.3. Details of the container recommended for immunodetection

For optimal processing of blots we recommend the use of a rolling cylinder. This technique can be used with any suitable container of sufficient length and circumference to accommodate the blot. The lid should seal well and fit such that while being rolled on its side on a roller incubator, the inner walls of the container are uniformly covered with solution. Blots are placed against the inner wall of the container with the side of the membrane carrying the transferred proteins facing into the centre. The natural spring of the membrane and, once wet, surface tension is sufficient to adhere the blot to the wall of the container. Avoid overlapping of the blot.

The rolling cylinder procedure allows the use of the small volumes of reagents recommended in the protocol. Since processing is dynamic, i.e. the membrane is constantly moved through the reagent solution, no localized depletion of reagent solution occurs and the washing steps are very thorough. This directly results in increased sensitivity of detection and strength of signal, and reduced backgrounds. As the blot is effectively immobilized on the inner surface of the cylinder there is no risk of uneven detection, or scratching or tearing of the membrane during immunodetection.

In our laboratories, we routinely use a sealable screw-cap cylinder of 2.8 cm diameter and 11.2 cm height, to process 8 x 6 cm blots.

A schematic representation of the processing technique is shown in Figure 2.

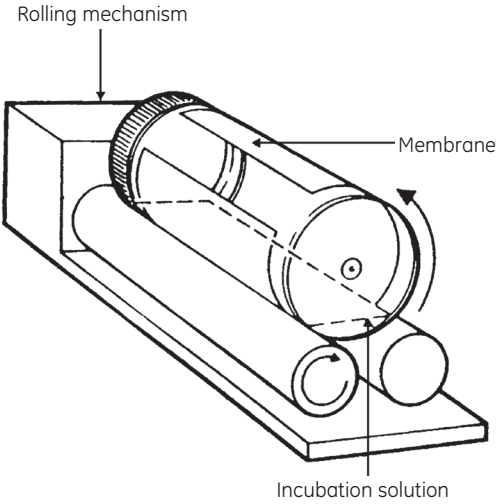

Figure 2. Immunodetection container

9. Related products

Hybond membranes

Size	Pack size	Code		
		Hybond-NX (nylon)	Hybond-N+ (positively charged nylon)	Hybond-XL (positively charged nylon)
82 mm diam	50 discs		RPN82B	RPN82S
87 mm diam	50 discs		RPN87B	RPN87S
132 mm diam	50 discs		RPN132B	RPN132S
137 mm diam	50 discs		RPN137B	RPN137S
11.9 x 7.8 cm	50 sheets		RPN119B	RPN119S
22.2 x 22.2 cm	50 sheets		RPN2250B	
22.5 x 22.5 cm	50 sheets		RPN225B	
15 x 73 mm	50 sheets		RPN1576B	
12 x 10 cm	20 sheets		RPN1210B	RPN1210S
15 x 10 cm	20 sheets		RPN1510B	RPN1510S
15 x 20 cm	10 sheets		RPN1520B	RPN1520S
20 x 20 cm	10 sheets		RPN2020B	RPN2020S
22 x 22 cm	10 sheets		RPN2222B	RPN2222S
30 x 50 cm	5 sheets		RPN3050B	RPN3050S
20 cm x 3 m	1 roll		RPN203B	RPN203S
30 cm x 3 m	1 roll	RPN303T	RPN303B	RPN303S
82 mm	50 gridded discs		RPN1782B	
87 mm	50 gridded discs		RPN1787B	
132 mm	50 gridded discs		RPN1732B	
137 mm	50 gridded discs		RPN1737B	

Size	Pack size	Code		
		Hybond-C Extra	Hybond-N (neutral nylon)	Hybond-P (hydro-phobic PVDF)
82 mm diam	50 discs	RPN82E	RPN82N	
87 mm diam	50 discs		RPN87N	
132 mm diam	50 discs		RPN132N	
137 mm diam	50 discs	RPN137E	RPN137N	
11.9 x 7.8 cm	50 sheets		RPN119N	
22.2 x 22.2 cm	50 sheets			
22.5 x 22.5 cm	50 sheets			
15 x 73 mm	50 sheets			
12 x 10 cm	20 sheets		RPN1210N	
15 x 10 cm	20 sheets		RPN1510N	
14 x 16 cm	15 sheets			RPN1416F
15 x 20 cm	10 sheets		RPN1520N	
20 x 20 cm	10 sheets	RPN2020E	RPN2020N	RPN2020F
22 x 22 cm	10 sheets		RPN2222N	
30 x 50 cm	5 sheets		RPN3050N	
20 cm x 3 m	1 roll	RPN203E	RPN203N	
30 cm x 3 m	1 roll	RPN303E	RPN303N	RPN303F

Hybond blotting paper

Size	Pack size	Code
20 x 20 cm	100 sheets	RPN6101M

10. Protein studies

ECL Western blotting detection reagents

– for 4000 cm^2 membrane	RPN2106
– for 2000 cm^2 membrane	RPN2209
– for 1000 cm^2 membrane	RPN2109
ECL Western blotting analysis system	RPN2108

For the detection of either mouse or rabbit membrane bound primary antibodies.

Sufficient for 1000 cm^2 membrane.

ECL Advance Western blotting detection reagents	
– for 1000 cm^2 membrane	RPN2135
ECL Plus Western blotting detection reagents	
– for 1000 cm^2 membrane	RPN2132
– for 3000 cm^2 membrane	RPN2133
Streptavidin-horseradish peroxidase conjugate	RPN1231-2 ml

Horseradish peroxidase-labelled second antibody conjugates

Mouse Ig. HRP-linked whole antibody (from sheep)	NA931-1 ml
Rabbit Ig. HRP-linked whole antibody (from donkey)	NA934-1 ml
Human Ig. HRP-linked whole antibody (from sheep)	NA933-1 ml

Molecular weight markers

ECL DualVue™ Western blotting markers (molecular weight range 15 000–150 000 Da)	RPN810
ECL protein molecular weight markers (molecular weight range 14 400–97 400 Da)	RPN2107

Low-Range Rainbow™ markers (M_r 3 500–38 000) use seven seperate protiens with five different colors		RPN755E
High-Range Rainbow markers (M_r 12 000–225 000) use eight seperate protiens with six different colors		RPN756E
Full-Range Rainbow markers (M_r 12 000–225 000) use ten seperate protiens with six different colors		RPN800E
For use on SDS-PAGE		
ECL Blocking Agent (40 g membrane blocking agent, sufficient for at least 20 miniblots)		RPN2125
Hyperfilm ECL		
25 sheets	18 x 24 cm	RPN2103K
75 sheets	18 x 24 cm	RPN3103K
25 sheets	30 x 40 cm	RPN2104K
25 sheets	5 x 7 inches	RPN1674K
25 sheets	8 x 10 inches	RPN2114K
75 sheets	8 x 10 inches	RPN3114K
25 sheets	10 x 12 inches	RPN1681K
Hypercassette, neutral (standard)		
1 cassette	18 x 24 cm	RPN11642
1 cassette	24 x 30 cm	RPN11643
1 cassette	30 x 40 cm	RPN11644
1 cassette	35 x 43 cm	RPN11645
1 cassette	18 x 43 cm	RPN11646
1 cassette	20 x 40 cm	RPN11647
1 cassette	5 x 7 inches	RPN11648
1 cassette	8 x 10 inches	RPN11649
1 cassette	10 x 12 inches	RPN11650
Hypercassette, blue (standard)		
1 cassette	18 x 24 cm	RPN13642

1 cassette	24 x 30 cm	RPN13643
1 cassette	35 x 43 cm	RPN13645
1 cassette	8 x 10 inches	RPN13649

Hypercassette, red (standard)

1 cassette	18 x 24 cm	RPN12642
1 cassette	24 x 30 cm	RPN12643
1 cassette	35 x 43 cm	RPN12645
1 cassette	8 x 10 inches	RPN12649

Hypercassette, neutral (deep)

1 cassette	18 x 24 cm	RPN11628
1 cassette	30 x 40 cm	RPN11627
1 cassette	8 x 10 inches	RPN11629

11. References

1. Isacsson, U. and Watermark, G., *Anal. Chim. Acta*. **68**, 339–362 (1974).

2. Whitehead, T.P. *et al., Clin. Chem*. **25**, 1531–1546 (1979).

3. Southern, E.M., *J. Mol. Biol*. **98**, 503–517 (1975).

4. Thomas, N. *et al., Anal. Biochem*. **170**, 393–396 (1988).

5. Kaufmann, S.H. et al., *Anal. Biochem*. **161**, 89–95 (1987).

12. Recommended applications for blotting membranes

Hybond membranes for binding nucleic acid

Applications	Hybond-NX (nylon)	Hybond-XL (positively charged nylon)	Hybond-N+ (positively charged nylon)	Hybond-N (neutral nylon)
Southern blotting				
DNA fingerprinting	+	+	+	+
Radioactive detection	++	+++	++	++
ECL detection	-	-	+++	-
AlkPhos Direct™ detection	-	-	+++	-
Gene Images™ detection	-	-	+++	-
Alkali blotting/fixation	--	+++	++	--
Low volume hybridizations	+++	+++	+	--
Rapid-hyb™ buffer	+	+++	++	+
Northern blotting				
Radioactive detection	++	+++	+	++
Non-radioactive detection	-	-	++	-
Dot/slot blots				
Radioactive detection	++	+++	++	++
Non-radioactive detection	-	-	++	-
Colony/plaque lifts				
Radioactive detection	+++	++	+	++
Non-radioactive detection	+	-	++	+

Hybond membranes for binding protein

Applications	Hybond-P	Hybond-ECL	Hybond-C Extra
Western blotting			
ECL detection	+++	+++	+
ECL Advance detection	+++	+++	+
ECL Plus detection	+++	++	+
Chromogenic detection	++	++	+
Colloidal gold detection	++	++	-
ECF detection	+++	+	-
Radioactive detection	+	+	++
Glycoprotein detection	+++	+	+
Reprobing Westerns	+++	-	+
Expression screening	+	-	+++

Key: Suitable = +, Recommended = ++, Highly recommended = +++
Not recommended = -, Unsuitable = --

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