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.

This product contains no toxic or corrosive substances. However, we 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.

This product is intended only for use in blotting and hybridization applications as outlined in this booklet.

Certain reagents indicated for use with this product are hazardous. Users are cautioned to follow the manufacturer’s safety recommendations.

Warning: Hybond-C extra is a nitrocellulose membrane.
Flammable: Do not heat above 50°C, unless fixing in a vacuum oven.
Do not expose to UV transilluminators. Do not bring into contact with naked flames.

It is recommended that you store this product in a dedicated steel flameproof cabinet in your laboratory.

Handling instructions

Hybond-C extra should be stored in a dry and clean environment.

The membrane should be handled using gloves or forceps to prevent membrane contamination.

Hybond-C extra should be rinsed well in distilled water before use. Sharp scissors or a scalpel must be used to cut the membrane.
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The Hybond range

All Amersham International membranes are manufactured specifically for life science applications, and are not standard filtration products. There are two distinct types of membrane:

a) **Unsupported**, where the active substrate is present as a pure, cast sheet. Examples are Hybond-ECL and Hybond-C.

b) **Supported**, where the active substrate is cast onto an inert 'web' or support. Hybond-N, -N+, -C super and -C extra all fall into this class.

All Amersham membranes are identical on both sides so that it does not matter which side you use.

Production runs are carefully controlled and the end product exhaustively screened to make sure that only the most consistent product reaches you.

Membranes, particularly those made from nitrocellulose, are affected by the environment and so should be kept in the bags in which they are received. They should also be kept away from excessive heat, light, moisture and noxious fumes in order to preserve optimum performance.

Introduction to Hybond-C extra

Hybond-C extra is a supported membrane made from mixed ester nitrocellulose. It has the same physical strength as nylon membranes, and can be used in all DNA, RNA and protein transfer and detection procedures. It offers excellent band resolution and sensitivity.

Hybond-C extra is particularly recommended for use in Northern and Western blotting. Its high physical strength facilitates reprobing.

Blotting protocols: Western Blotting

Protocol for electroblotting proteins

The following protocol is for the transfer to Hybond-C extra membranes of proteins separated on standard SDS-containing polyacrylamide gels. For transfers involving urea gels see Towbin, Staehelin and Gordon[1]. For non-denaturing gels see Renart, Reiser and Stark[2].

1) Gels may be stained, prior to use, with Coomassie brilliant blue R. However, in some cases fixing of the gels may affect the antigenicity of a protein. Alternatively, filters may be stained after transfer using fast green, amido black or a variety of other stains.

2) Soak gel in transfer buffer for at least 10-20 minutes.

3) Equilibrate membrane by briefly soaking membrane completely in deionized water and then in transfer buffer for at least 10 minutes.
4) Using a commercial electroblotting unit make up a cassette as described in the manufacturer's literature.

5) Transfer the complete assembly to the electroblotting unit. Ensure that the Hybond membrane is on the anode side of the gel.

6) Transfer for 1-2 hours in protein transfer buffer at up to 400 mA, or overnight with cooling at 20 mA. Follow manufacturer's instructions.

7) Remove membrane and wash with PBS to remove residual acrylamide. Allow to air dry. Blots may be used immediately or stored in a dessicator between sheets of filter paper at 2-8°C for up to 3 months.

**Blocking protein blots**

1) After transfer, place the membrane in a suitable container, such as a roller drum or sandwich box, and block for 1 hour at room temperature in PBS or TBS containing 0.1% Tween + 5% dried skimmed milk (or 0.5% gelatin or 0.25%-3% BSA). Incubate with gentle shaking or agitation.

2) At the end of the blocking incubation, wash the membrane twice for 5 minutes with fresh changes of washing buffer (0.1% Tween in PBS or TBS) at room temperature.

**Probing protein blots**

1) Dilute primary antibody in PBS or TBS and incubate the blot in this solution for 1 hour at room temperature*.

2) Wash the membrane as described in step 2 of 'blocking'.

3) Dilute secondary antibody in PBS or TBS and add to the blot. Incubate for 1 hour at room temperature*.

4) Wash the membrane as in step 2.

5) Detect the signal using the detection system of choice.

*(0.1% Tween or 0.1% dried skimmed milk or 0.25% gelatin or 1% BSA may be added to the diluent buffer if high background problems persist).

**Immunodetection of protein blots**

Amersham's extensive range of immunodetection products include the immunogold system which can be readily intensified using the matched silver enhancement reagents. Our other products make use of colorimetric detection and are based on either directly labelled second antibodies or the streptavidin/biotin system.

The choice of PBS or TBS buffer system will depend on the detection system of choice, users should refer to the relevant protocol booklet. Also, the concentration of Tween-20™ in the washes and in the diluent should be
optimized, as this affects the binding of the antibodies, especially those of low affinity. For most protein blotting work, 0.1% Tween is adequate.

Tween-20 is a registered trademark of Atlas Chemical Co.

Membrane stripping protocol

Incubate the membrane at 60°C for 30 minutes in the following solution:
- 0.05 M sodium phosphate, pH 6.5
- 10 M urea
- 0.1 M 2-mercaptoethanol

The blot may then be reprobed. Reprobing is not recommended when enzyme-linked antibodies have been used, since substrate deposits are difficult to remove.

Blotting Protocols: Nucleic Acid blotting

Northern blotting

1) Electrophorese RNA in a denaturing system. Formaldehyde/agarose is recommended for use with Hybond-N as follows:

   a. Incubate RNA at 65°C for 5 minutes in the following solution:
      RNA (final volume) 6 l
      Formamide (deionized) 12.5 l
      10 x MOPS 2.5 l
      Formaldehyde (37%) 4 l

   b. Chill on ice and add 2.5 l 50% (v/v) glycerol, containing 0.1 mg/ml bromophenol blue.

   c. Run on a 1-1.5% agarose gel prepared as follows:
      Agarose 1-1.5 g
      10 x MOPS 10 ml
      Water 73 ml

      Dissolve agarose and cool to 50°C. Check volume and if necessary make up to 83 ml. Add 17 ml formaldehyde (37% v/v solution), mix and pour immediately. Running buffer is 1 x MOPS buffer.

2) Set up capillary blot as described below. Do not wash filter.

Setting up a capillary blot

1) Fill tray or glass dish with blotting buffer (20x SSC or 20x SSPE). Make a platform and cover it with a wick made from three sheets of Whatman 3MM filter paper, saturated with blotting buffer.
2) Place the gel on the wick and avoid trapping air bubbles beneath it. Surround it with cling film to prevent the blotting buffer being absorbed directly into the paper towels above.

3) Cut a sheet of Hybond-C extra membrane to the exact size of the gel (prewet by rinsing in distilled water followed by soaking in 20x SSC or 20x SSPE for at least 10 minutes) and place on top of the gel. Avoid trapping bubbles beneath the membrane. If bubbles appear, they should be squeezed out using a glass rod or pipette.

4) Place three sheets of 3MM paper cut to size and wetted with blotting buffer, on top of the Hybond-C extra membrane.

5) Place a stack of absorbent paper towels on top of the 3MM paper (approximately 5 cm high).

6) Place a glass plate on top of the paper towels and put a 0.75-1 kg weight on top. Allow transfer to proceed for 2-3 hours or overnight.

7) After blotting carefully dismantle the apparatus. Before removing from the gel, mark the membrane with pencil to allow later identification of tracks. For DNA blots only, wash the membrane briefly and carefully in 2x SSC or 2x SSPE to remove any adhering agarose.

8) Fix the sample to the membrane as described on page 7.

Dot blotting of RNA

1) Incubate RNA at 65°C for 5 minutes in three volumes of the following solution:
   Formamide 500 l
   Formaldehyde (37% solution) 162 l
   10 x MOPS buffer 100 l

2) Chill on ice. Add 1 volume of cold 20x SSC.

3) Spot samples on to Hybond-C extra membrane, pre-wetted in 10x SSC, in 2 l aliquots. Allow to dry between each application. Alternatively, a commercial dot blotting apparatus may be used.

4) Dry and fix the RNA to the membrane as described on page 7.

Colony/plaque blotting

1) Select correct membrane size and pre-wet on an unused agar plate.

2) Carefully place membrane on to the agar surface. Mark the membrane and agar using a sterile needle or other means to ensure correct orientation of colonies/plaques.*

3) Remove membrane after one minute and place, colony side up, on sterile filter paper. The membrane may then be treated as in steps 7-9 below. If replicas are required proceed as follows:

4) Prepare a second filter as described in step 1. Place it on the template filter. Press firmly together
using a replica plating tool, take care not to allow lateral movement. Mark the replica filter through the holes used to align the template.

5) Incubate all replica filters on fresh agar at 37°C until colonies of 0.5-1 mm diameter are obtained. Gridded, circular membranes are available from Amersham that provide an easy and simple means of realigning positives.

6) If the sequence to be screened is contained in an amplified plasmid such as pBR322, transfer the membrane to agar plates containing 500 μg/ml chloramphenicol. Incubate for a further 16-24 hours.

7) Remove the membrane and place, colony side up, on a pad of absorbent filter paper soaked in denaturing solution. Leave for 7 minutes.

8) Place membrane, colony side up, on a pad of absorbent filter paper soaked in neutralizing solution. Leave for 3 minutes then repeat with a fresh pad soaked in the same solution.

9) Wash filters in 2x SSC. Transfer to dry filter paper and air dry, colony side up. Fix sample to the membrane as described on below.

Fixation protocols for DNA and RNA blots

Warning: Do not expose nitrocellulose to UV light.

Oven baking: Bake in a vacuum oven at 80°C for 2 hours.

Hybridization protocols

The following procedure is suitable for DNA and RNA blots, abut see note on RNA blots below:

1) Make up a prehybridization solution as follows (for 25 ml)

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume</th>
<th>Final Concentration</th>
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<tr>
<td>20x SSPE</td>
<td>6.25 ml</td>
<td>5x SSPE</td>
</tr>
<tr>
<td>100x Denhardt’s solution</td>
<td>1.25 ml</td>
<td>5x Denhardt’s solution</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>1.25 ml</td>
<td>0.5% (w/v) SDS</td>
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Make up to 25 ml with sterile water. Add to the membrane in a hybridization box or bag.

2) Denature 0.5 ml of the 1 mg/ml solution of sonicated non-homologous DNA by heating in 100°C for 5 minutes. Chill on ice and add to prehybridization solution.

3) Prehybridize in a shaking water bath at 65°C for 1 hour.

4) Denature labelled probe (unless using an RNA or ssDNA probe) by heating to 100°C for 5 minutes. Add the probe to the prehybridization solution.
5) Incubate for at least 12 hours at 65°C.

6) Following hybridization, wash the filters by incubating them in 2x SSPE, 0.1% (w/v) SDS at room temperature for 10 minutes. Repeat.

7) Replace the solution with 1x SSPE, 0.1% (w/v) SDS. Incubate at 65°C for 15 minutes.
8) Replace the solution with 0.1x SSPE, 0.1% (w/v) SDS. Incubate at 65°C for 10 minutes. Repeat if necessary. **Note:** This is a high stringency wash and should be used omitted if partially related sequences are to be probed.

9) Remove filter, wrap in SaranWrap™ and carry out autoradiography. SaranWrap should not be used if the probe is labelled with sulphur-35.

*Amersham supplies a rapid hybridization system that allows hybridization to take place in 1-2 hours with no loss in sensitivity. *™SaranWrap is a registered trademark of Dow Chemical Company.

**RNA blot hybridization**

The hybridization protocol given is suitable for Northern blots or RNA probes. In some instances, it may be beneficial to include formamide at the final concentration of 50% in the hybridization step and an incubation temperature of 42°C. However, the temperature may need to be raised for some sequences to avoid non-specific hybridization.

Other published protocols may also be successfully used with Hybond-C extra.

**Membrane stripping protocols**

For successful removal of probes, membranes must never be allowed to dry during or after hybridization and washing.

**Method 1:**

Wash blot for 1-2 hours at 65°C in:
- 0.005 M Tris-HCl, pH 8.0
- 0.002 M Na₂EDTA
- 0.1 x Denhardt's solution

**Method 2:**

Boil a solution of 0.1% SDS. Pour on the membrane and allow to cool to room temperature.

Autoradiograph for your normal exposure time to check that the probe has been removed. The blot may then be pre-hybridized and hybridized using a new probe.

**Solutions required**

- 20x SSPE
- 3.6 M NaCl
0.2 M sodium phosphate
0.02 M EDTA pH 7.7

**Denaturing solution:**
1.5 M NaCl
0.5 M NaOH

**10x MOPS buffer:**
0.2 M 3-[N-Morpholino] propane-sulphonic acid
0.05 M Na acetate pH 7.0
0.1 M Na$_2$EDTA

**PBS pH 7.5**
80 mM Na$_2$HPO$_4$
20 mM NaH$_2$PO$_4$.2H$_2$O
100 mM NaCl
in 1 liter distilled water

**TBS pH 7.6**
200 mM Tris
137 mM NaCl
Adjust to pH 7.6 with 1 M HCl

**20x SSC:**
3 M NaCl
0.3 M Na$_3$citrate

**Neutralizing solution:**
1.5 M NaCl
0.5 M Tris-HCl, pH 7.2
0.001 M EDTA

**100x Denhardt’s solution**
2% (w/v) BSA (bovine serum albumin)
2% (w/v) Ficoll™
2% (w/v) PVP (polyvinylpyrrolidone)

**Protein transfer buffer:**
0.025 M Tris-HCl, pH 8.3
0.15 M Glycine
20% (v/v) Methanol

™Ficoll is a trademark of Pharmacia

**References**


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