

## Choice of membrane

Choose the material for the blot membrane carefully.

Different membranes (PVDF, nylon, nitrocellulose, etc.) may not perform equally in a Western blot. They give varying amounts of specific antibody-antigen signal and varying amounts of nonspecific background.

**Note:** For Westerns using most tag-specific antibodies, we generally recommend **PVDF membranes** for optimum signal and minimum background.

## Optimal dilution of antibody reagents

Detection of different tagged proteins may require different concentrations of tag-specific antibody reagents. To be sure the procedure works optimally for a given tagged protein, the optimal concentration of antibody must always be determined experimentally. If you are working with c-myc-, HA-, or VSV-G tagged proteins and BM tag-specific antibody reagents, the antibody concentrations and dilutions listed in Table 1C.2 (on page 1.9 of this manual) may be used as starting points for optimization experiments.

**Note:** The table in Procedure III (later in this section) also gives concentration ranges for tag-specific antibody reagents that may be used in initial experiments.

The concentration of tag-specific antibody required to detect an epitope-tagged protein depends on many factors, including:

- ▶ The affinity of the tag-specific antibody
- ▶ How many copies of the tag sequence occur in the target protein
- ▶ The abundance (expression level) of the tagged protein in the sample
- ▶ The specificity of antibody-antigen reaction
- ▶ The experimental conditions under which the antibody-antigen binding occurs.

## Choice of blocking reagent

In the Western blot procedure, the blot is incubated with blocking reagent before it is incubated with the detecting antibodies. In addition, the detecting antibody is usually mixed with a more dilute solution of blocking reagent. These steps are necessary to minimize non-specific binding of the detecting antibody to sticky proteins present in the cell extracts.

A variety of blocking reagents are available for Western blotting. We recommend using **Boehringer Mannheim Blocking Reagent**, the **BM Chemiluminescence Western Blotting Substrate (POD) reagent set**, and several **BM Western Blotting Kits**. If performing chemiluminescent detection with **CDP-Star™** and AP-conjugated antibodies, Tropix's **I-Block™ Blocking Reagent** (Cat. No. A1300) must be used.

**Note:** In certain cases, solutions of nonfat dry milk may be substituted for **Boehringer Mannheim Blocking Reagent**. See the topic, "Troubleshooting the Western blot" later in this section for details.

## Proper washing between antibody incubations

The washes after each incubation with antibody are important for removing unbound antibody from the blot and for reducing background due to nonspecific antibody-protein binding. Always follow the instructions for the wash steps in the procedures below to get optimal results. Only if the procedure results in a low signal or a high background should you alter these wash steps.

## Getting Started: Procedures for Western blotting

This section describes both indirect and direct procedures for analyzing tagged proteins on a Western blot. These procedures were developed for detection of proteins tagged with c-myc, HA, or VSV-G epitopes.

**Caution:** These procedures must be optimized empirically for each experimental system (tagged protein, cell in which protein is expressed, tag-specific antibody, secondary antibody). Use the parameters listed in these procedures (antibody concentrations, incubation times, wash conditions, and so forth) only for initial experiments or for determining optimal ranges for each parameter. Pay particular attention to those procedural steps marked with a **Note** or **Caution** statement, since these are critical steps that must be optimized if the procedure is to produce meaningful results.

**Note:** The products printed in colored type are available from **Boehringer Mannheim**. For detailed ordering information on these and related products, see the **Boehringer Mannheim Product Ordering Guide**, Section 5B of this manual.

## I. Preparing the sample

1 To prepare samples for Western blotting, do the following:

▶ Mix the protein sample with concentrated electrophoresis sample buffer to produce a protein sample containing 2% SDS, 100 mM DTT, 60 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.01% bromphenol blue.

▶ Heat the protein samples to 100°C for 5 min in a boiling water bath or other heating device.

**Note:** Some tagged proteins precipitate when boiled in electrophoresis sample buffer. If initial experiments produce no signal, try denaturing the samples at a lower temperature. For details, see Sambrook, Fritsch, and Maniatis, p. 18.45–18.54.

▶ If the protein samples contain insoluble material, clarify them by centrifugation.

**Caution:** If not removed, insoluble material will cause poor resolution or streaking of the proteins on the gel.

**Note:** In many cases, samples for electrophoresis may be stored frozen at –20°C or –70°C and thawed just before electrophoresis. Thawed samples should be centrifuged briefly to remove any insoluble materials.

Before centrifugation, the thawed sample may also need to be sonicated briefly to disrupt aggregates that formed during storage.

2 Run the denatured samples on an acrylamide gel with a Tris-glycine-SDS running buffer according to standard electrophoresis protocols (Sambrook, Fritsch and Maniatis, 1989, p. 18.47–18.54).

**Note:** Prestained molecular weight markers can be electrophoresed on the same gel with the experimental samples and transferred to the blot to help determine the molecular weight of the experimental bands on your blot.

Alternatively, run samples on duplicate gels, then detect tagged proteins on one blot with antibody while staining the second gel and/or blot for protein (for example, with Coomassie Blue, Ponceau S, or silver stain (Sambrook, Fritsch and Maniatis, 1989, p. 18.55–18.57).

## II. Western transfer

**Caution:** Always wear gloves when handling the gel or membrane. Maneuver the gel with forceps or gloved hands to minimize background.

1 Prepare Western Transfer Buffer (10% methanol, 24 mM Tris, and 194 mM glycine) (Towbin and Gordon, 1984) in advance and refrigerate.

**Caution:** The buffer must be at 4°C for electrophoretic transfer.

2 Prepare a polyvinyl-difluoride (PVDF) membrane as follows:

▶ Pre-wet the membrane with 100% methanol.

▶ Equilibrate the membrane for at least 5 minutes in Western Transfer Buffer.

**Note:** Different membranes may not perform equally. For westerns using most tag-specific antibodies, we recommend PVDF membranes.

3 Remove the gel from the electrophoresis apparatus and incubate it in Western Transfer Buffer for approximately 10 min to remove detergent.

4 As instructed by the manufacturer of your transfer apparatus, do the following:

▶ Place the gel in contact with the PVDF membrane.

**Caution:** Remove any air bubbles that form between the membrane and the gel by rolling a pipet over the membrane-gel sandwich.

▶ Inset the gel-membrane sandwich into the transfer apparatus.

▶ Assemble the remaining components of the transfer apparatus.

5 Add prechilled Western Transfer Buffer to the transfer apparatus, then transfer the protein from the gel to the membrane electrophoretically.

**Note:** Follow previously established protocols or the manufacturer's recommendations for the blot transfer. For transfer in the Mini Trans-Blot® apparatus (Bio-Rad) or an equivalent apparatus, we recommend a 1 h transfer at 40 V.

6 During the blotting procedure, prepare the detection reagents for the next procedure (Procedure III, Steps 1 and 2).

- 7 After the transfer, do the following:
- ▶ Unclamp the blot sandwich and remove the sheets of blotting paper, exposing the blot membrane.
  - ▶ Mark the side of the membrane that was facing the gel.
  - ▶ Mark the position of the prestained markers, since they may fade away during detection.
- 8 The blot membrane is now ready for the detection procedure (Procedure III below). Do either of the following:

If you want to	Then
Perform the detection procedure immediately	Keep the blot membrane moist until you incubate it with Blocking Reagent (Step 4 of Procedure III)
Store the PVDF* membrane for a time before performing the detection procedure	a) Air dry the membrane. b) Protect the dried membrane from moisture and store it in the refrigerator. c) Just before incubating the blot with Blocking Reagent (Step 4 of Procedure III), rewet the membrane with 100% methanol.

\* Do not store other types of membrane prior to Procedure III.

### III. Detecting the epitope-tagged protein with tag-specific antibodies

- 1 Prepare about 1 liter of Reagent Diluent. Reagent Diluent may be either phosphate buffered saline (PBS) or Tris buffered saline (TBS):

▶ **PBS:** 1 mM  $\text{KH}_2\text{PO}_4$ , 10 mM  $\text{Na}_2\text{HPO}_4$ , 137 mM NaCl, 2.7 mM KCl; pH 7.4

**Caution:** If you are using antibodies conjugated with alkaline phosphatase (AP) in the detection procedure, do not use PBS or any other phosphate-containing buffer as Reagent Diluent; use TBS instead. Phosphate inhibits the enzymatic activity of AP.

▶ **TBS:** 50 mM Tris-HCl, 150 mM NaCl; pH 7.5

**Caution:** If you are using antibodies conjugated with peroxidase (POD) in the detection procedure, do not add sodium azide to Reagent Diluent or any reagent used in the procedure. Azide inhibits the enzymatic activity of POD.

- 2 For detection with Anti-TAG antibody or Anti-TAG-POD (where TAG = c-myc, HA or VSV-G), prepare the following reagents:

Step	Reagent required	Reagent composition	Volume needed* (direct–indirect)
Blocking	Blocking Solution	1% Blocking Reagent [a 1:10 dilution of Stock 10% Blocking Reagent <sup>††</sup> in Reagent Diluent]	10 ml
Dilution	Antibody Diluent	0.5% Blocking Reagent (a 1:20 dilution of Stock 10% Blocking Reagent <sup>††</sup> in Reagent Diluent)	10–20 ml
Indirect detection	Working Concentration Tag-specific Antibody	Anti-TAG 1–5 $\mu\text{g}/\text{ml}$ <sup>§</sup> , prepared in Antibody Diluent	10 ml
	Working Concentration Secondary Antibody	Anti-Mouse IgG (H&L)-POD (Cat. No. 605 250) OR Anti-Mouse IgG (H&L)-AP (Cat. No. 605 260), diluted <sup>†</sup> in Antibody Diluent	10 ml
Direct detection	Working Concentration Tag-specific Antibody-POD	Anti-TAG-POD, diluted <sup>§</sup> to 0.1–1.0 $\mu\text{g}/\text{ml}$ in Antibody Diluent	10 ml
Wash	Wash Buffer	Reagent Diluent with 0.1% Tween <sup>®</sup> 20	100–200 ml

\* All reagent volumes are the amounts needed to process a 100 cm<sup>2</sup> (10 cm x 10 cm) blot membrane. Adjust these volumes to accommodate smaller or larger membranes.

<sup>§</sup> The optimal concentration of Anti-TAG or Anti-TAG-POD will vary with the identity of the tagged protein and must be determined experimentally. For initial experiments and for TAG = c-myc, HA, or VSV-G, use the concentrations recommended in this table.

<sup>†</sup> The optimal concentration will vary from lot to lot of secondary antibody. For initial experiments, use the dilution recommended by the antibody manufacturer (see Table 1C.2 on page 1.9).

<sup>††</sup> Stock 10% Blocking Reagent is available in the [BM Chemiluminescence Western Blotting Substrate \(POD\)](#).

3 Transfer the membrane (protein side up) to a container large enough to hold it.

**Caution:** Be sure the side of the membrane that contains protein faces up so the detection reagents will have maximum access to the antigens.

**Note:** A disposable, square petri dish (100 x 15 mm) makes a convenient container for a 10 x 10 cm blot.

4 Cover the membrane with 10 ml of Blocking Solution.

5 Place container on a rotating platform and rotate gently for 1 h at room temperature (RT).

**Caution:** Make sure the reagent completely and constantly covers the membrane during this incubation and all incubation steps below.

**Note:** This incubation step may also be performed at 4°C overnight, if convenient.

6 Drain the Blocking Solution from the container

7 Cover the blocked membrane with 10 ml of Working Concentration Tag-specific Antibody reagent (prepared in Step 2).

8 Place the container on a rotating platform and incubate for 1 h at RT.

9 Drain the antibody solution from the container and rinse the membrane with approximately 20 ml of Wash Buffer.

10 While rotating the container gently, wash the membrane 4 times at RT with Wash Buffer (20 ml per wash, 10 min incubation per wash).

11 Depending on the antibody reagent you used in Step 7 above, do one of the following:

In procedure III, if you used	Then go to Procedure
Indirect detection with Anti-c-myc, Anti-HA, or Anti-VSV-G	IV
Direct detection* with Anti-c-myc-POD, Anti-HA-POD, or Anti-VSV-G-POD	V

\* If you are using a direct detection procedure, the antibody-antigen complexes are now ready to be visualized.

## IV. Indirect detection of antibody-antigen complexes with secondary antibody

1 Discard the last wash and cover the membrane with 10 ml of Working Concentration Secondary Antibody.

2 Place the container on a rotating platform and incubate, while rotating, for 1 h at RT.

3 Drain the antibody solution from the container and rinse the membrane with Wash Buffer.

4 While gently rotating the container, wash the membrane 4 times with Wash Buffer (as in Step 10 of Procedure III).

5 Go to Procedure V below.

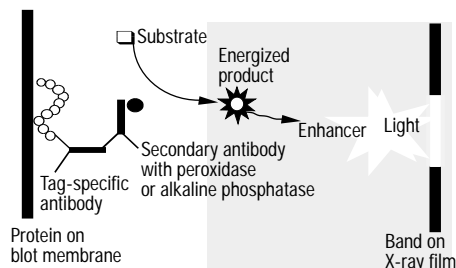
## V. Visualizing antibody-antigen complexes

**Caution:** All reagent volumes in these procedures are the amounts needed for a 10 x 10 cm blot. Adjust these volumes to accommodate smaller or larger membranes.

**Note:** The procedures describe chemiluminescent and chromogenic procedures for visualization of peroxidase- and alkaline phosphatase-conjugated antibodies. Depending on the antibody conjugate you are using and the type of detection procedure you wish to use, follow one of the four procedures:

If your antibody is conjugated with	AND you want to visualize the complexes	Then follow Procedure
POD	Chemiluminescently	VA
POD	Chromogenically	VB
AP	Chemiluminescently	VC
AP	Chromogenically	VD

**Note:** Visualization is different for chemiluminescent and chromogenic procedures. Whereas the chromogenic signal (a colored band) appears directly on the membrane, the chemiluminescent signal (Figure 3A.4) is emitted light and must be recorded on a sheet of film placed in contact with the membrane.



**Figure 3A.4: Chemiluminescent visualization for Western blots.** Chemiluminescent visualization requires an enzyme substrate that can be converted to an energized product. The energized product then decays, emitting light that, after amplification by a chemical enhancer, makes a visible band on a sheet of film.

#### VA. Chemiluminescent visualization for peroxidase-conjugated antibodies

**Caution:** Do not let the membrane go dry during any step in Procedure VA.

- 1 Prepare 10 ml Detection Solution according to the package insert provided with the **Chemiluminescence Blotting Substrate (POD) reagent set**.
- 2 Discard the last wash (from Procedure III or Procedure IV).
- 3 Cover the blot membrane with Detection Solution and incubate the membrane for 1 minute.
- 4 Drain excess Detection Solution from the membrane, and wrap the blot in plastic wrap.
- 5 In a dark room, place a sheet of X-ray film (for example, **Lumi-Film Chemiluminescent Detection Film**) over the plastic-wrapped membrane in a film cassette.

**Caution:** Make sure the protein side of the membrane faces the X-ray film. This ensures maximum sensitivity.

- 6 Close the cassette and expose the membrane to the X-ray film for 60 s.  
**Note:** Incubation time will vary with the intensity of the protein bands and the type of film used. Use 60 seconds as a starting point for a typical experiment.

- 7 Remove the X-ray film from the cassette and develop the film according to the manufacturer's instructions.
- 8 Insert a new sheet of X-ray film over the plastic-wrapped membrane in the film cassette, then on the basis of the band intensity on the first film, adjust the exposure time of the second film to improve the clarity and intensity of the bands, as follows:

- ▶ If the first film is too dark (overexposed), decrease the exposure time to 10–50 s.

- ▶ If the first film is too light (underexposed), increase the exposure time to 5–60 min.

**Note:** The luminescence reaction reaches its maximum after 1–2 min substrate development and is relatively constant for 20–30 min. After 1 h, the signal intensity decreases to about 60–70% of maximum.

#### VB. Chromogenic visualization for peroxidase-conjugated antibodies

- 1 Prepare POD Staining Solution by adding 50  $\mu$ l of stock **BM Teton** substrate (50 mg/ml) and 6  $\mu$ l  $H_2O_2$  to 10 ml of substrate buffer (100 mM **Tris**, 50 mM  $MgCl_2$ ; 100 mM NaCl; pH 8.0).
- 2 Discard the last wash (from Procedure III or Procedure IV) and rinse blot membrane once (for 5 min) in TBS (50 mM **Tris**, 150 mM NaCl; pH 7.5).
- 3 Cover blot membrane with 10 ml POD Staining Solution.
- 4 Incubate membrane at RT without agitation, until the antigen bands are stained to the desired intensity (usually 10–60 min).
- 5 Stop the color reaction by rinsing the membrane several times with distilled  $H_2O$ .
- 6 Photograph the blot membrane to record the results.
- 7 Dry the membrane on paper towels and store away from bright light.

**Note:** The colored precipitate is stable if stored away from light.

#### VC. Chemiluminescent visualization for alkaline phosphatase-conjugated antibodies

**Note:** If performing chemiluminescent detection with **CDP-Star™** and AP-conjugated antibodies, Tropix's I-Block™ Blocking Reagent (Cat. No. A1300) must have been used.

**Caution:** Do not let the membrane go dry during any step in Procedure VC.

- 1 Discard the last wash (from Procedure III or Procedure IV) and rinse the membrane once with TNM (100 mM **Tris-HCl**, 100 mM NaCl, 50 mM  $MgCl_2$ ; pH 9.5)
- 2 Place the rinsed blot membrane in a sealable plastic bag.
- 3 Add 2–3 ml of 0.25 mM **CDP-Star™** (prepared with TNM) to the bag containing the membrane and seal the bag.
- 4 Spread the CDP-Star gently over the membrane in the sealed bag for 3–5 min.

- 5 Open the bag and pour the CDP-*Star* off the membrane. Let all the excess liquid drip out of the bag.
- 6 Using blunt-ended forceps, transfer the membrane to a plastic coated piece of cardboard. Make sure the protein side of the membrane faces up.
- 7 Cover the membrane with clear plastic. Be careful to remove any air bubbles trapped between the plastic and the membrane.
- 8 In the dark room, place a sheet of X-ray film over the membrane. Make sure the film faces the protein side of the membrane.
- 9 Incubate the film-membrane sandwich for 60 s.  
*Note: Exposure time will vary with the intensity of the bands and the type of film used. Use 60 s as a starting point for a typical experiment).*
- 10 Remove the X-ray film from the cassette and develop the film according to the manufacturer's instructions.
- 11 Insert a new sheet of X-ray film over the blot membrane in the film cassette, then on the basis of the band intensity on the first film, adjust the exposure time of the second film to improve the clarity and intensity of the bands, as follows:
  - ▶ If the first film is too dark (over-exposed), decrease the exposure time to 10–50 s.
  - ▶ If the first film is too light (under-exposed), increase the exposure time to 2–5 min.

#### VD. Chromogenic visualization for alkaline phosphatase-conjugated antibodies

- 1 Warm **BM Purple AP Substrate** to RT and invert once to mix.
- 2 Rinse blot membrane once with distilled H<sub>2</sub>O.
- 3 Cover blot membrane with 10 ml **BM Purple AP Substrate**.
- 4 Place container on a rotating platform and rotate gently at RT, until the antigen bands are stained to the desired intensity (usually 10–30 min).
- 5 Stop the color reaction by rinsing the membrane several times with distilled H<sub>2</sub>O.
- 6 Photograph the blot membrane to record the results.
- 7 Dry the membrane on paper towels and store away from bright light.  
*Note: The colored precipitate is stable if stored dry and away from light.*