

Section 3A

Analysis on a Western Blot

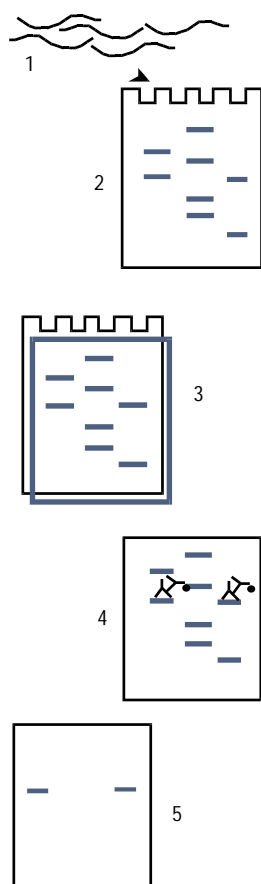


Figure 3A.1: Steps in a Western blot.
The numbered steps are:

1. apply protein samples to gel;
2. separate the proteins electrophoretically;
3. blot the proteins from a gel onto a flexible membrane;
4. probe the membrane with a primary antibody that recognizes a specific (target) protein (followed in some cases by a secondary antibody that recognizes the primary antibody);
5. visualize the antibody-antigen complexes with an indicator molecule that produces a visible signal.

Overview of technique

Western blot analysis is the most commonly used immunochemical technique for detection of epitope-tagged proteins. It allows the detection of epitope-tagged proteins in complex mixtures such as cell or membrane extracts (for instance, Canfield and Levenson, 1993; Duden *et al.*, 1991). When combined with immunoprecipitation (as described in Section 4.D of this manual), it can reveal information about the interaction of the tagged protein with other cell components (for instance, as in Dietzen, Hastings and Lublin, 1995).

In a Western blot, proteins are electrophoretically separated on an acrylamide gel, then transferred to a membrane detected with one or more antibodies (Figure 3A.1). The antibody detection technique may be:

- ▶ **Direct:** The membrane is incubated with an enzyme-conjugated tag-specific antibody. [Antibodies for direct detection are often conjugated with horseradish peroxidase (POD) or alkaline phosphatase (AP).]
- ▶ **Indirect:** The membrane is incubated first with an unconjugated tag-specific antibody (primary antibody), then with an enzyme-conjugated antibody (secondary antibody) that recognizes the tag-specific antibody. (Secondary antibodies for indirect detection of primary antibodies are usually conjugated with POD or AP.)

Suitable enzyme substrates for Western blotting must produce a signal on the membrane at the site of the enzyme-conjugated antibody (and thus, the tagged protein). Examples of Western blotting substrates include:

- ▶ **Chemiluminescent Western Blotting Substrate (POD)** for chemiluminescent visualization of peroxidase-conjugated antibodies (Signal recorded on X-ray film)
- ▶ **BM Teton** for chromogenic visualization of peroxidase-conjugated antibodies (Signal recorded on the membrane)

- ▶ **CDP-Star** for chemiluminescent visualization of alkaline phosphatase-conjugated antibodies (Signal recorded on X-ray film)
- ▶ **BM Purple** for chromogenic visualization of alkaline phosphatase-conjugated antibodies (Signal recorded on the membrane)

Critical factors for successful Western blots

In a Western blotting procedure, many variables affect the outcome. We have listed below a few controllable factors that, in our experience, most affect the success of the Western blot procedure. Use these as guidelines for designing your own Western.

Note: For general information on the Western blotting procedure, see Towbin and Gordon (1984).

Preparation of a sample containing a tagged protein

The most common, convenient way to obtain a protein sample for a Western blot is to resuspend and lyse cells directly in an electrophoresis sample buffer (as in Procedure I later in this section), then remove insoluble cell debris by centrifugation. The clarified sample can be loaded directly on an acrylamide gel.

Alternative sources of Western blot samples (which may be mixed with electrophoresis sample buffer) include:

- ▶ Cell extracts
- ▶ Fractions from chromatography column eluates
- ▶ Immunoprecipitates

Note: Buffers for these samples must be compatible with the electrophoresis sample buffer. For instance, in the samples, avoid using buffers containing potassium salts since potassium will form a precipitate with the SDS in the electrophoresis sample buffer.

In most cases, protein samples may be run on an acrylamide gel without further preparation. However, if the epitope-tagged protein is expressed only at low levels in the cell, there may not be enough tagged protein in the sample to be detected. In this case, you should concentrate the tagged proteins in the sample by immunoprecipitating them before applying them to the electrophoretic gel. See Section 4D of this manual for a description of the immunoprecipitation procedure.

Control samples

In some Western blots, especially under non-optimal conditions, the detecting antibody may bind non-specifically to non-tagged proteins in the sample. To avoid confusion in interpreting the band pattern, always include proper controls on the blot. These controls should *not* contain the tagged protein, but should be prepared in the same way as samples that do contain the tagged protein. Any bands on the Western blot that appear in both the control and experimental sample are proteins that the antibody binds non-specifically.

For example, suppose you want to confirm the expression of an epitope-tagged protein from a plasmid transformed or transfected into a particular organism. The proper control would be an extract from cells of that organism that did not contain the plasmid. An even better control would be an extract from cells that contained a version of the plasmid without the gene for the tagged protein.

Direct vs. indirect detection

In Western blotting, antibodies detect a specific tagged protein from a complex mixture of proteins (cell extract). As noted above, the detection may involve one antibody (direct detection) or two antibodies (indirect detection).

The principal advantages of the direct method are:

- ▶ **Time savings** (up to 2 hours), since the direct procedure takes fewer steps than the indirect procedure (See the “Procedures for Western blotting” topic below.)

- ▶ **Increased specificity** in analysis of immunoprecipitated samples, since the direct method visualizes only the target protein (Figure 3A.2), eliminating bands that can potentially interfere with the interpretation of results
- ▶ **Lower cost**, since direct procedures
 - 1) require much lower levels of tag-specific antibody than indirect procedures, and
 - 2) do not require a secondary antibody

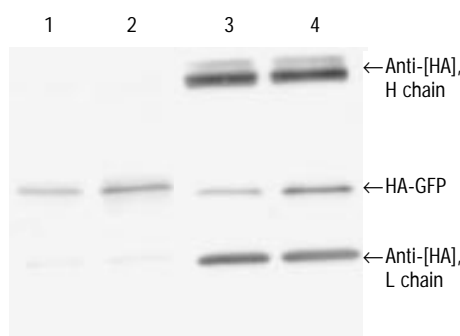


Figure 3A.2: Western analysis of immunoprecipitated HA-tagged Green Fluorescent Protein (HA-GFP). HA-GFP was immunoprecipitated from an *E. coli* crude extract with unconjugated Mouse monoclonal Anti-HA and Protein A-agarose as described in Section 4°C of this manual. The immunoprecipitate was analyzed on a Western blot with both direct (Lanes 1 and 2) and indirect (Lanes 3 and 4) detection of HA-GFP. Lanes 1 and 2: 5 μ l and 15 μ l of *E. coli* extract (3.3 mg/ml total protein) immunoprecipitated and analyzed with Mouse monoclonal Anti-HA-peroxidase. Lanes 3 and 4: The same immunoprecipitated samples were analyzed with unconjugated Mouse monoclonal Anti-HA (primary antibody) and Anti-Mouse IgG (H&L)- peroxidase (secondary antibody). Note heavy and light chains of the mouse monoclonal primary antibody that were detected with the Anti-Mouse IgG secondary antibody conjugate.

The principal advantages of the indirect method are:

- ▶ **Greater flexibility**, since the indirect method does not require a conjugated primary antibody (which may not be readily available)
- ▶ **Sensitivity**, since the indirect method can be more sensitive than a direct method

Note: Some direct detection systems, however, are equal in sensitivity to indirect detection systems (Figure 3A.3).

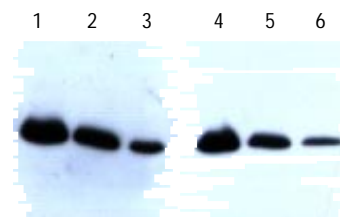


Figure 3A.3: Sensitivity of direct and indirect methods for detection of an HA-tagged protein. HA-GFP was expressed in *E. coli* and purified from cell extracts as previously described (Gill *et al.*, 1996). Three amounts of the purified protein were analyzed on a Western blot using direct (Lanes 1–3) or indirect (Lanes 4–6) detection procedures. The six samples contained HA-GFP protein at 6 ng (Lanes 1 and 4), 3 ng (Lanes 2 and 5), or 1 ng (Lanes 3 and 6). The overall sensitivity (signal to noise) of the two detection methods is equivalent.

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.