BioFeedback

Rapid High Resolution Western Blotting: From Gel to Image in a Single Day

ABSTRACT

A streamlined protocol is described that allows high sensitivity antigen detection by Western blotting in a single day. The choice of membrane blotting matrix, as well as blocking reagents, has been optimized in order to allow rapid development of the blot with chemiluminescent reagents. The entire process, from gel to blot to a permanent, hard copy image on x-ray film, can be accomplished within six hours.

INTRODUCTION

Antigen detection by Western blotting has become an increasingly popular method for identifying and characterizing nanogram amounts of specific proteins (7). A variety of detection methods are available, including colorimetric substrates and radiolabeled antibodies/protein A. While colorimetric methods are often limited in their sensitivity, the use of [^{125}I]labeled reagents is associated with higher background levels and problems inherent in radioisotope shielding and disposal. Recently, nonradioactive chemiluminescent reagents have become commercially available that can be used with high sensitivity for Western blotting. These reagents provide relatively rapid signal generation that can be recorded as a hard copy on x-ray film.

We have adopted the chemiluminescent technology as a means for studying the oncogene product of Rous sarcoma virus (RSV), pp60$^{v\text{-}src}$, as well as the substrates for its tyrosine protein kinase activity. Initial results obtained from Western blots using anti-src (3) or anti-phosphotyrosine (anti-ptyr) (1) antibodies yielded blots with extremely high background levels. We, therefore, modified the blotting protocol and the detection reagents to allow for reproducible, high-resolution blots. In the process, we were able to streamline the protocol into a one-day procedure.

MATERIALS AND METHODS

To probe for the presence of pp60$^{v\text{-}src}$, plasma membranes prepared from RSV-transformed vole cells (clone 1T) as described (4) were solubilized by boiling in sodium dodecyl sulfate (SDS)/sample buffer and were subjected to SDS polyacrylamide gel electrophoresis according to Laemmli (2). Samples for analysis of total cellular tyrosine phosphoproteins were prepared by lysing RSV-transformed vole cells directly in SDS/sample buffer containing 1 mM Na_3VO_4 (5). Gel electrophoresis was conducted at 25-30 mA for approximately 1.5 h. The wet gel was transferred onto Whatman 3MM paper. A piece of Immobilon™-P membrane (Millipore, Bedford, MA) was cut to the same size as the gel, wetted with methanol, soaked in H_2O for 5 min and then overlaid onto the wet gel. A "sandwich" was made as described by Towbin (7) and placed into a Mini-Genie Electroblotter (Idea Scientific, Corvallis, OR); blotting was carried out at 480 mA for 30 min at room temperature.

All subsequent steps were performed with Tris-buffered saline (0.9% NaCl, 10 mM Tris-HCl, pH 7.4) that contained 0.5 g/l $MgCl_2$. Nonspecific binding sites were blocked by incubating the blot either overnight at 4°C (2-day protocol) or for 1 h at 37°C as described in Table 1 (1 -day protocol). Incubation with the primary antibody was performed by sealing the blot in a "Seal-A-Meal™" (Dazey, Industrial Airport, KS) bag containing 10 ml saline + bovine serum albumin (BSA)

Table 1. One-Day Chemiluminescent Western Blot Protocol

Step	Time and Temperature	Conditions
1. Gel Electrophoresis	l-2 h; R.T.	Standard Laemmli conditions (2)
2. Electrobiotting	30 min; R.T.	25 mM Tris-HCI/192 mM glycine/ 20% MeOH 480 mA; Immobilon-P PVDF
3. Blocking	1 h; 37°C	anti-src: 3% BSA in saline anti-ptyr: 1% BSA in saline
4. 1° Antibody	1 h; 4°C	anti-src: 1 :1 000 dilution into saline + 3% BSA + 10% CS anti-ptyr: 1:2000 dilution into saline + 1% BSA
5. Wash	30 min; R.T.	5 min each: twice with saline; twice with saline + 3% Tween 20; twice with saline
6. 2° Antibody (HRP conjugated)	30 min; 4°C	anti-src blots: 1:2000 dilution into saline + 3% BSA + 10% CS anti-ptyr blots: 1:2000 dilution into saline + 1% BSA
7. Wash	30 min; R.T.	as in step #5 above
8. Detection	1 min; R.T.	Mix Amersham ECL reagents 1 :1 per manufacturer's specifications; add BSA to 3% (anti-src) or 1% (anti-ptyr) (wt/vol)
9. Exposure	1 O-60 s; R.T.	Dark room, red safe light; Kodak XAR x-ray film

Abbreviations: BSA = bovine serum albumin, Fraction V; CS = calf serum; HRP = horse radish peroxidase; R.T. = room temperature; saline = 0.9% NaCl, 10 mM Tris-HCI, pH *7.4, 0.5* g/l $MgCl_2$.

± calf serum + antibody (see Table 1). The source of the antisera was either homemade: rabbit anti-src polyclonal (3) and rabbit anti-ptyr, prepared as in (l), or commercial: anti-src monoclonal and anti-ptyr monoclonal (Oncogene Science, Manhasset, NY).

The blots were washed, incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (HRP) (anti-rabbit Ig, HRP, from donkey; or anti-mouse Ig, HRP, from sheep; Amersham, Arlington Heights, IL) and washed again as indicated in Table 1. Equal volumes of detection reagents 1 and 2, provided for enhanced chemiluminescence (ECL) Western blotting (Amersham), were mixed and BSA was added to a final concentration of 1% or 3% (wt/vol). Detection was accomplished by incubating the blot with this mixture for 1 min. After the excess fluid was drained, the blot was covered with Saran Wrap™ and exposed to x-ray film.

RESULTS AND DISCUSSION

Our initial studies were carried out using plasma membrane fractions from RSV-transformed cells and antiserum directed against $pp60^{v\text{-}src}$ (3). We first optimized the source of membrane blotting matrix for use with chemiluminescent substrates. Polyvinylidene fluoride (PVDF) membranes were found to give higher signals and lower background when compared with nitrocellulose membranes from a variety of manufacturers (Bio-Rad, Richmond, CA, and Schleicher & Schuell, Keene, NH). Nylon membranes yielded unacceptable results (weak signal, high background). For all subsequent experiments, Immobilon-P PVDF membranes (Millipore) were used. Due to the hydrophobic nature of the PVDF matrix, complete transfer of nearly all proteins could be achieved in only 30 min.

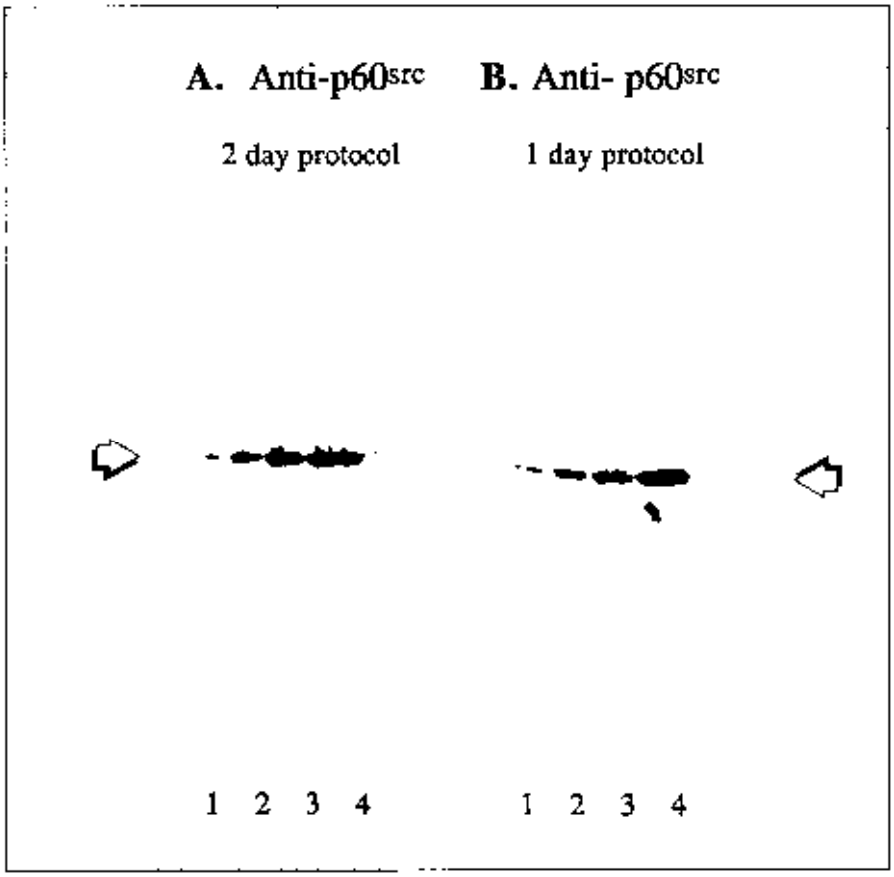

Figure 1. Comparative Western blot analysis of $pp60^{v\text{-}src}$ using 2-day and l-day protocols. Plasma membrane fractions from RSV-transformed vole cells were analyzed by gel electrophoresis and Western blotting as described in Materials and Methods. The primary antibody was rabbit anti $p60^{src}$ (3), and the secondary antibody was donkey anti-rabbit Ig, HRP-conjugated. A: The blocking step was carried out at 4°C overnight. B: Blocking was carried out for 1 h at 37°C. as in Table 1. Both blots were exposed to x-ray film for 10 s. Lane 1 (2.5 µg), lane 2 (12.5 µg), lane 3 (25 µg) and lane 4 (50 µg) of membrane protein. The bands representing $pp60^{v\text{-}src}$ (approximately 0.1% of the total membrane protein) are indicated by the arrows. The signal obtained in lane 1, therefore, represents about 2.5 ng of antigen.

Several other factors were important for reducing background on the blots. We found that inclusion of 0.5 g/l $MgCl_2$ in the saline solutions and 3% Tween 20 in two of the saline washes (Table 1) helped to eliminate the "blotchy" background problems. Moreover, the presence of BSA in the detection reagent reaction also considerably improved the appearance of the blots. A typical Western blot using anti-src antiserum is shown in Figure 1A. It should be noted that the signal was obtained by exposing the film for only 10 s. Exposure for up to 35 s was possible without a significant increase in background.

Based on the rapidity with which a signal was obtained, we reasoned that it should be possible to condense the entire Western blotting procedure into a single day. Typically, the blocking step had been performed overnight at 4°C (2-day protocol) (Figure 1A). We, therefore, modified the blocking step to a l-h incubation at 37°C. When the l-day protocol outlined in Table 1 was followed, the results of the l-day Western blotting were nearly indistinguishable from those obtained in two days (compare Figure 1B with Figure IA). The total amount of time required was approximately 6 h. This could be shortened by the use of mini-gels for electrophoresis. In addition, the sensitivity of the assay allowed detection of antigen in the nanogram range, similar to that recently reported by Sandhu et al. (6) using a lengthier chemiluminescent protocol.

The l-day protocol was also adaptable for use with other antigens and antibodies. Phosphotyrosine-containing proteins from RSV-transformed cells were readily detectable by anti-phosphotyrosine antibodies, as illustrated in Figure 2. Slight modifications,

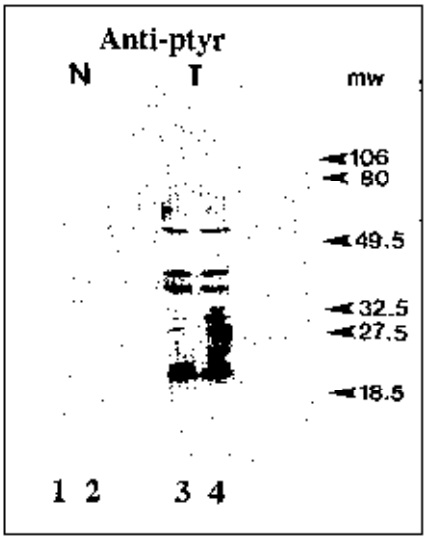

Figure 2. Detection of phosphotyrosine-containing proteins using the l-day Western blot protocol. Whole cell lysates from normal cells (lanes 1 and 2) and RSV-transformed vole cells (lanes 3 and 4) were analyzed by Western blotting using a monoclonal anti-phosphotyrosine antibody and the l-day protocol outlined in Table 1. Lanes 1 and 3 contained approximately 20 μg of total cell protein; lanes 2 and 4, approximately 40 μg protein. Exposure time was 20 s. The positions of the molecular weight markers in kDa are indicated by arrowheads in the right margin. Identical results were obtained with rabbit polyclonal anti-ptyr antibody.

including reduction in the amount of BSA to 1% and removal of the calf serum during antibody incubations (Table l), were made to enhance the signal-to-noise ratio.

In summary, using appropriate modifications, Western blotting can be achieved in a single day. We have successfully employed both monoclonal and polyclonal antibodies in this manner. The speed and sensitivity of this method make it ideal for applications requiring rapid detection of antigenic proteins.

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Andrew Klapper, Barbara and Marilyn D. Resh
Department of Cell Biology and Genetics
Sloan-Kettering Institute
1275 York Avenue
New York, NY 10021