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Blue Dry Western: Simple, economic, informative, and fast way of immunodetection

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ABSTRACT

The analysis by electrophoresis followed by transfer to membranes and immunodetection (Western blot) is probably the most popular technique in protein study. Accordingly, it is a time- and money-consuming procedure. Here a protocol is described where immunodetection can be accomplished in 30 min. This approach also allows permanent staining of proteins by Coomassie Blue R on the membrane before immune staining with clear background and high sensitivity.

Western blot analysis is a widely used procedure and a key workflow in many laboratories. Proteins from different biological samples are usually analyzed following separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE),[1] according to their mass, or by two-dimensional gel electrophoresis (2-DE), according to their p*I* and mass. The separated proteins are then transferred to membranes, where specific proteins of interest can be detected using antibodies that recognize the target. To minimize the amount of background noise and to increase the sensitivity for detecting the protein band or spot on the membrane, different blocking reagents (e.g., bovine serum albumin [BSA], skimmed milk) are used to prevent nonspecific antibody binding to the membranes. Specific primary antibodies are incubated with the membrane, and then the bound antibody is detected by incubation with a secondary antibody usually conjugated with horseradish peroxidase (HRP). To increase the signal/background ratio, antibodies are diluted in phosphate-buffered saline (PBS) or Tris-buffered saline (TBS) containing blocking agents (milk or BSA and 0.1% [v/v] Tween 20). This process can be time-consuming because most protocols recommend that the antibody solutions be incubated with the membrane for relatively long time periods. Usually, each blocking or antibody solution is incubated with the membrane for at least 1 h, although some protocols recommend overnight treatment. Finally, the position of the secondary antibody is detected by incubation with a substrate that generates a luminescent product following the activity of HRP. This signal can then be visualized by exposition to X-ray film or by collection of the light using a charge-coupled device (CCD) camera in a gel documentation system. In many cases, the relative expression of the protein is determined by comparison with a control protein (actin, tubulin, or glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) by "reprobing" the blot with the relevant control antibody. What is interesting is that usually the same protocol is applied for both nitrocellulose and polyvinylidene difluoride (PVDF) membranes, although the chemistry of the PVDF membrane is quite different. The advantage of using PVDF membranes is not only their durability but also their high hydrophobicity, which can allow immunodetection without blocking [1]. So far, this approach has not been very popular, possibly because of a chance of high background. Here a strategy that exploits the hydrophobic qualities of PVDF membranes to allow very fast and simple immunodetection with clear background is presented. Furthermore, this approach allows the proteins on the membrane to be permanently stained by Coomassie R prior to immunodetection. This makes the procedure very informative, allowing reliable normalization of analyzed samples and identification of specific proteins on the membrane.

Materials and methods

Reagents

All of the reagents used were from Sigma (St. Louis, MO, USA) unless stated otherwise. The suppliers of other reagents were as follows. Dithiothreitol (DTT) and protease and phosphatase

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[1] *Abbreviations used:* SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; 2-DE, two-dimensional gel electrophoresis; BSA, bovine serum albumin; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; CCD, charge-coupled device; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PVDF, polyvinylidene difluoride; DTT, dithiothreitol; ECL, enhanced chemiluminescence; PCNA, proliferating cell nuclear antigen; LDH, lactate dehydrogenase; EF–Tu, elongation factor Tu; PDI, protein disulfide isomerase; IPG, immobilized pH gradient; IEF, isoelectrofocusing; CHAPS, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate.

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inhibitor cocktails were obtained from Roche Diagnostics (Mannheim, Germany). PVDF membrane, solution for the enhanced chemiluminescence (ECL) reaction, and rainbow protein markers were obtained from Amersham Biosciences (Montreal, Quebec, Canada). Methanol and acetic acid were obtained from Fisher Scientific (Nepean, Ontario, Canada). Tris base, 10× TGS buffer (Tris, glycine, and SDS), and molecular mass marker (Precision Plus Protein Standards, dual color) were obtained from Bio-Rad (Hercules, CA, USA). RPMI-1640 medium was obtained from HyClone (Logan, UT, USA). Anti-PCNA (proliferating cell nuclear antigen) monoclonal antibody PC10 (sc-56), anti-PCNA goat polyclonal antibody C-20 (sc-9857), anti-aldolase A goat polyclonal antibody (sc-12059), anti-LDH (lactate dehydrogenase) goat polyclonal antibody (sc-27232), monoclonal antibody against elongation factor Tu (EF–Tu, sc-21758), rabbit polyclonal anti-enolase antibody (sc-15343), rabbit polyclonal anti-PDI (protein disulfide isomerase) antibody (sc20132), anti-GAPDH mouse monoclonal antibody (sc-47724), mouse monoclonal anti-Rb (sc-102), and mouse monoclonal anti-annexin II (sc-28385) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti-γ-tubulin (T6557) and goat polyclonal anti-vimentin (V 4630) antibodies were obtained from Sigma. Mouse monoclonal antiphosphotyrosine antibody 4G10 (05-321X) was obtained from Upstate Biotechnology (Lake Placid, NY, USA).

Cells and cell extraction

MCF7 or HEK 293 cells were maintained in RPMI-1640 medium supplemented with 10% (w/v) fetal calf serum. Cell cultivation and protein extraction were as described previously [2–6]. Briefly, cells in monolayer were washed once with PBS. The cells were then scraped into PBS using a plastic cell lifter and collected by centrifugation at 900*g* for 5 min. The cell pellet was treated with hypotonic buffer (10 mM Tris [pH 7.5], 25% [v/v] glycerol, 1 mM DTT, and protease and phosphatase inhibitor cocktails). The final concentration of the cell extracts was usually approximately 2 mg/ml (equivalent to 10^7 cells/0.5 ml buffer). For 2-DE, cells were solubilized in a lysis buffer (7 M urea, 2 M thiourea, 4% [w/v] CHAPS, 1% [w/v] DTT, 2% [v/v] immobilized pH gradient [IPG] buffer [pH 3–10], protease inhibitor cocktail, and 0.001% [w/v] bromophenol blue). Protein concentration was determined using the Coomassie Protein Assay Reagent Kit as suggested by the supplier (Pierce, Rockford, IL, USA).

One-dimensional electrophoresis

Samples were analyzed on 10% (w/v) SDS-denaturing gels according to Laemmli with some modifications using a Hoefer miniVE electrophoresis apparatus [2,5,6].

Two-dimensional protein gel electrophoresis

The first dimension, isoelectrofocusing (IEF), was done using a DryStrip Kit as suggested by the manufacturer (Amersham Biosciences) with some modifications. Briefly, 50 to 100 μg of protein extract was mixed with rehydration solution (7 M urea, 2 M thiourea, 2% [w/v] CHAPS, 0.3% [w/v] DTT, 0.5% [v/v] IPG buffer [pH 3–10], and 0.001% [w/v] bromophenol blue) in a total volume of 150 μl. To prepare the first-dimensional gel, 7-cm IPG gel strips (pH 3–10) were rehydrated overnight at 4 °C by placing them gel side down in rehydration solution in the Immobiline DryStrip Reswelling Tray (Amersham Biosciences). IEF was conducted at 20 °C using a Multiphor II unit (Amersham Biosciences). The EPS 3500 XL power supply was programmed in gradient mode with voltages from 300 V (0.01 h) to 3500 V (1.5 h), which was then changed to a single mode at 3500 V for 2.5 h. The total voltage hours was 14,000. Prior to the second dimension, the IPG gel strips were soaked for 10 min in equilibration solution (50 mM Tris–HCl [pH 6.8], 6 M urea, 2% [w/v] SDS, and 30% [v/v] glycerol) containing

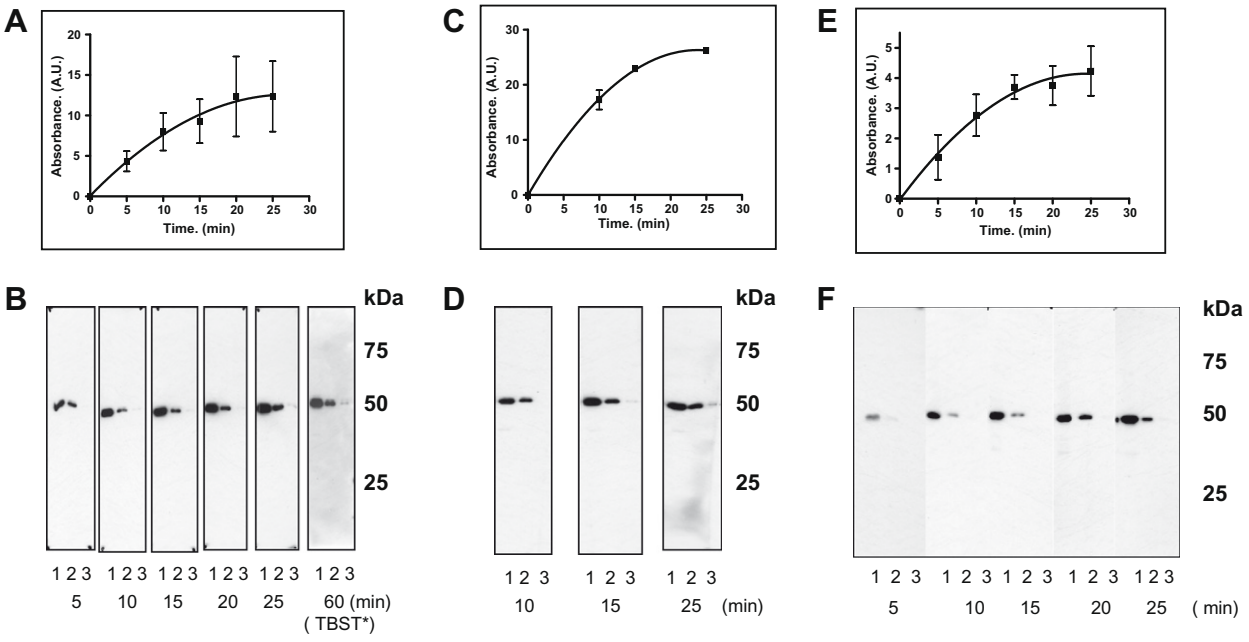

Fig. 1. Effect of incubation time with antibodies on the signal production. Proteins of HEK 293 extract (1–10, 1–2, and 0.1–3 μg) were separated by SDS–PAGE and transferred to Immobilon-P. The membranes were dried and treated by antibodies. (A and B) Incubation time with the primary antibody was 5, 10, 15, 20, or 25 min. Incubation time with the secondary antibody was 30 min in all cases (effect of incubation time with primary antibody). *This membrane was not dried after transfer but rather was treated by the classical method (see Materials and methods). (C and D) The membranes were incubated with the primary antibody for 25 min. The following incubation time with the secondary antibody was 10, 15, or 25 min (effect of incubation time with secondary antibody). (E and F) Effect of incubation time on signal production when the primary and secondary antibodies were combined in the same solution. The membranes were incubated in solution containing the primary and secondary antibodies for 5, 10, 15, 20, or 25 min. In all cases, EF–Tu mouse monoclonal antibody (dilution 1:500 in TBS with 3% [w/v] BSA) and HRP rabbit anti-mouse (dilution 1:10,000 in TBS with 3% [w/v] milk) were used. (A, C, and E) Graphical representation of the signal intensity. (B, D, and F) Actual films.