Video Article

Western Blotting Protocol - ADVERTISEMENT

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Abstract

The western blotting technique measures protein expression in a cell or tissue extract using antibody-based detection of a target protein. The antibodies used in a western blot can be specific to a total protein or to unique post-translational modifications of a protein, such as sites of phosphorylation, acetylation, methylation, or ubiquitination. The broad range of available total protein and modification-specific antibodies allows researchers to study cellular signaling events involved in many biological contexts and processes. Here we provide a comprehensive western blot protocol developed and used at Cell Signaling Technology that includes optimal reagents and support information to ensure optimal results.

Video Link

The video component of this article can be found at http://www.jove.com/video/5072/

Introduction

Western blotting, also called immunoblotting, is a widely used technique to monitor protein expression in a cell or tissue extract based upon antibody binding to a specific protein of interest. Antibodies used in western blotting can be specific to total protein or to a post-translational modification of a protein, such as sites phosphorylation, acetylation, methylation, or ubiquitination.

At Cell Signaling Technology, we perform western blots daily to validate our existing and new antibodies. Here, we provide a complete protocol for the technique based upon a decade of our experience. We also provide a list of commonly used reagents that are used by CST scientists and work optimally with CST antibodies. Therefore, using additional directions found on your antibody product datasheet, you can simply replicate what we are doing here and get the expected results in the shortest amount of time. We will also talk about the critical steps in western blotting and how protocol changes can affect the final outcome of your blot.

Protocol

A. Solutions and Reagents

Before you begin, you should have a number of solutions on hand. Directions to prepare these solutions can be found on the protocol page of our website.

[Editor note: we would like to a have a video shot of our companion reagents and their packaging.]

- 1X PBS, prepared using 20X PBS stock from CST #9808
- 1X SDS Sample Buffer, using either Blue Loading Buffer Pack #7722 or Red Loading Buffer Pack #7723
- Transfer Buffer
- 1X TBST, prepared from 10X TBST stock from CST #9997
- Nonfat Dry Milk from CST #9999
- Blocking Buffer
- BSA from CST #9998
- Primary Antibody Dilution Buffer containing either 5% BSA or 5% nonfat dry milk as indicated on product datasheet
- SignalFire ECL Reagent from CST #6883
- Prestained Protein Marker from CST #7720
- Blotting membrane: This protocol has been optimized for nitrocellulose membranes. PVDF membranes may also be used, but we find they generally result in a higher background.

B. Sample Preparation and Protein Blotting

1. Treat cells by adding fresh media containing regulator for desired time.

2. Aspirate media from cultures; wash cells with 1X PBS; aspirate.
3. Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl per plate of 10 cm diameter plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice. Alternatively, samples can be lysed using 1X Cell Lysis Buffer (#9803) or 1X RIPA Buffer (#9806), which allows protein quantitation using Bradford assay or other methods.
4. Sonicate for 10-15 sec to complete cell lysis and shear DNA (to reduce sample viscosity). This is especially important for membrane-bound and nuclear proteins.
5. Heat a 20 µl sample to 95-100 °C for 5 min; cool on ice.
6. Microcentrifuge for 5 min.
7. Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm). **NOTE:** Loading of prestained molecular weight markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 10 µl/lane) to determine molecular weights are recommended.
8. Electrotransfer to nitrocellulose or PVDF membrane.

C. Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm^2) of membrane; for different sized membranes, adjust volumes accordingly.

I. Membrane Blocking

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 min at room temperature.
2. Incubate membrane in 25 ml of blocking buffer for 1 hr at room temperature.
3. Wash once for 5 min with 15 ml of TBST.

II. Primary Antibody Incubation

For Unconjugated Primary Antibodies

1. Incubate membrane and primary antibody (at the appropriate dilution as recommended in the product datasheet) in 10 ml primary antibody dilution buffer (as indicated on the product datasheet) with gentle agitation **overnight** at 4 °C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate membrane with the species appropriate HRP-conjugated secondary antibody (#7074 or #7076) (1:2000) and Anti-biotin, HRP-linked Antibody (#7075) (1:1000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
4. Wash three times for 5 min each with 15 ml of TBST.
5. Proceed with detection (Section D).

D. Detection of Proteins

1. Incubate membrane with 10 ml SignalFire ECL Reagent #6883 (mix 1 part 2X Reagent A and 1 part 2X Reagent B) with gentle agitation for 1 min at room temperature.
2. Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap, and expose to x-ray film. An initial 10 sec exposure should indicate the proper exposure time.

Discussion

Importance of following CST recommended protocol

Cell Signaling Technology (CST) antibodies are validated using our recommended western blotting protocol, which has been developed and optimized by CST scientists. All data shown on product webpages and datasheets was generated using these protocols. We strongly recommend following our protocols to achieve optimal results with all of our antibodies. Here are a few examples of the effects minor changes to the standard protocol may have on your results.

[Images from http://www.cellsignal.com/support/protocols/western-variation.html will be shown here.]

Lysate Preparation

Shown here are the effects of sonication in conjunction with sorbitol lysis of CKR/PAEC cells using Phospho-Histone H3 (Ser10) Antibody #9701. CST-recommended sonicated extracts display a greatly enhanced signal as compared to nonsonicated extracts using this antibody.

Primary Antibody Dilution Buffer

Primary antibodies can be diluted in a TBST buffer containing either 5% BSA or 5% milk. The optimal dilution buffer has been predetermined for each antibody and included on the product datasheet. Failure to follow the recommended dilution buffer can result in less-than-optimal signal. For example, dilution of the Phospho-Akt (Ser473) Antibody #9271 with a BSA-based buffer provides a stronger signal as compared to a milk-based buffer in this western blot analysis of extracts from C2C12 cells treated with insulin.

Primary Antibody Incubation

The primary antibody incubation period can vary greatly depending upon which protocol a researcher is using. CST recommends incubating the primary antibody overnight at 4 °C for all CST antibodies. Shown here are several examples of antibody performance using overnight incubations

at 4 °C as compared to 2-hour incubation at room temperature. Phospho-Akt (Ser473) Antibody #9271, Phospho-GSK-3β (Ser9) Antibody #9336, and PKCδ Antibody #2058 all perform better with an overnight incubation at 4 °C.

Wash and Dilution Buffer

Another area where western blot protocols can vary is with the wash and dilution buffers. CST recommends using Tris buffered saline with Tween-20 (TBST; see above for concentration) for antibody dilution buffers and wash steps. Shown here is a comparison of antibody performance using TBST-based dilution and wash buffers versus those made with phosphate buffered saline with Tween (PBST). For all antibodies shown, TBST provided a stronger signal as compared to PBST.

Transfer and Antibody Incubations

CST recommends wet transfer followed by 1-hour blocking and overnight primary antibody incubation at 4 °C. iBlot is a dry blotting system that completes transfer in 7 min. SNAPi.d. is a vacuum operated incubation system, which reduces antibody incubation times to less than 30 min. The western blot shown here depicts the benefits of using the CST-recommended transfer and incubation conditions for Phospho-p38 MAPK (Thr180/Tyr182) (3D7) Rabbit mAb #9215.

In closing, we hope this video is a helpful resource for performing western blots using CST antibodies in your own lab. Please view our second video, Western Blotting Troubleshooting Guide, where we discuss common issues with western blotting and how to troubleshoot. Cell Signaling Technology prides itself in providing you with exceptional customer service and support. Since all of our antibodies are produced in house, the same scientists who develop and assay these reagents are available as technical resources for our customers. These scientists can be contacted directly and will personally provide technical assistance to you, our customer.

[show info below with voiceover]

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