Video Article

Western Blotting Troubleshooting Guide by Cell Signaling Technology - ADVERTISEMENT

Minh-Ha Lieu[1], Troy Humphreys[1], Elisabeth Antanavich[1], Susan Chow[1]

[1]Cell Signaling Technology, Inc.

URL: http://www.jove.com/video/5071
DOI: doi:10.3791/5071

Keywords: Advertisement, Basic Protocols, Molecular Biology, Cellular Biology, Biochemistry, Genetics, Proteins, Antibodies, Western Blotting, Western Blot, DNA

Date Published: 6/12/2013

Citation: Lieu, M.H., Humphreys, T., Antanavich, E., Chow, S. Western Blotting Troubleshooting Guide by Cell Signaling Technology - ADVERTISEMENT. *J. Vis. Exp.* (), e5071, doi:10.3791/5071 (2013).

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 an extensive western blot troubleshooting guide based on our many years of experience, offering solutions to save you valuable time and reagents.

Video Link

The video component of this article can be found at http://www.jove.com/video/5071/

Introduction

Western blotting, also called immunoblotting, allows researchers to determine levels of protein expression in a cell or tissue extract through antibody binding to a specific protein of interest.

Although this technique is widely used and accepted, problems can occur that lead to suboptimal results, which can be time consuming and frustrating. Common issues include low signal and/or high background, both resulting in a blot that is difficult to interpret or quantitate.

This is the second video in our 2-part series on western blotting. In our first video, Western Blotting Protocol, we provide a comprehensive western blot procedure used in-house by CST scientists to validate our CST antibodies. In this second video, we provide a guide to help you troubleshoot, suggesting tips to help you diagnose the problem and providing solutions to ensure you get the expected results in the shortest amount of time.

Discussion

Before we begin, it is important to note that the single biggest contributing factor to western blot success is the quality of the primary antibody. Antibodies that are not properly validated and lack specificity will generate a poor signal or result in multiple bands on a blot, some of which are nonspecific. This can be the cause of misleading results and uncertainty as to whether you are detecting the correct protein or not.

This is why we take antibody validation seriously. Antibodies from Cell Signaling Technology are produced and thoroughly validated in-house. The western blots we show on every product web page are performed in a number of cell lines and tissue samples with varying expression levels known from the literature. First we ensure that the banding pattern corresponds to the molecular weight(s) for the protein described in the literature. We also treat cells with growth factors, chemical activators or inhibitors, or siRNA to induce or inhibit expression to verify whether bands can be induced or knocked down appropriately. We also confirm phospho-specificity by using phosphatase treatments that should eliminate all signal. Using all of these controls, we at CST convince ourselves first that we are providing you with the most specific and sensitive antibody possible for western blotting before we release it. We hope this careful validation will allow you in turn to be confident in your results and to quickly get to a publication quality western blot.

CST recommends always using a positive and negative control with each experiment. CST scientists have compiled a useful Controls Table that can be used as a starting point for many of our phospho-antibodies. Control cell extracts are also available. These will help identify any potential problem with the technique and serve as a valuable tool for potential troubleshooting.

[Display controls table web page]

Common Problems

Poor western blot results can be generally classified as either low signal or high background. The signal is considered low for most targets if the protein of interest cannot be detected after a 1 - 30 sec exposure of the blot to film. High background is defined as having a generally dark background or nonspecific bands appear after a 1 - 30 sec exposure of the blot to film.

Low Signal

There are a number of causes for low signal. The first cause of low signal can be the protein of interest is below detectable levels. If this is due to minimal expression in the starting cell line or tissue sample, an alternate cell line may be necessary or a chemical stimulation to induce expression may be needed.

Secondly, undetectable protein can be caused by suboptimal sample preparation due to lack of recommended lysis buffer (with required phosphatase inhibitors) or sonication conditions that result in poor extraction of target proteins or sample degradation. CST offers an optimized Cell Lysis Buffer that already contains all phosphatase inhibitors. We always recommend sonication to ensure total cell lysis and to shear the chromosomal DNA. We recommend 3 pulses for 10 sec at 35% - 40% power. Allow 10 sec between pulses and keep samples on ice while sonicating. If you do not have access to a probe sonicator, you may pass your samples through a fine gauge needle. CST also offers positive control cell lysates for many of our antibodies to determine if low signal is due to sample preparation or subsequent blotting steps.

Thirdly, undetectable protein can also result from inadequate loading of lysate onto the gel. CST recommends loading 20 µg of protein lysate per lane; however, if protein levels are below detection, more protein (up to 40 µg) can be loaded or immunoprecipitation prior to SDS-PAGE may be necessary. When working with tissue, we generally recommend loading 80-100 µg of protein.

Low signal can stem from issues with the transfer step. CST recommends always performing a wet transfer over iBlot or other transfer systems. Please view our Western Blotting Protocol movie for a comparison of antibody performance with wet transfer versus iBlot. When troubleshooting a wet transfer, the problem can be either incomplete transfer or overtransfer. Incomplete transfer can be corrected by increasing the time and/or voltage. Prestained molecular weight marker can be a useful tool to visually check if complete transfer has occurred. Overtransfer is common with very low molecular proteins, particularly if a 0.45 µm membrane is used. CST recommends a 2-hour transfer with 0.22 µm membrane.

Excessive blocking or washing can also cause low signal. Blocking the membrane for too long can obscure antigenic epitopes and prevent the antibody from binding. Block for 1 hr at room temperature, never overnight. Washing for longer than the recommended 3 x 5 min is common and can result in reduced signal. This applies to washing steps after both primary and secondary antibody incubations.

When using a phospho-specific antibody, insufficient incubation with primary antibody can also result in low signal. Phospho-antibodies generated against a single or dual phosphorylation site are highly specific, but generally result in lower signal than total protein antibodies. It is critical that these antibodies be incubated with blots overnight at 4 °C in the buffer recommended on the datasheet. It is also important to add phosphatase inhibitors to the cell lysis buffer when using a phospho-antibody.

Lastly, low signal can be the result of improperly prepared SignalFire chemiluminescent detection reagent. Remake the solution if necessary, and use biotinylated protein markers detected with a streptavidin-HRP secondary antibody as a positive control.

High Background

The starting material can be one of several causes of high background. In general, primary cell and tissue extracts tend to contain more background bands and degradation products than cell line extracts. Using fresh, sonicated, and clarified tissue extracts may lessen background. Lysing in RIPA Buffer #9806 may provide a more thorough and consistent lysis of tissue.

Choice of membrane can also greatly impact the background levels. Use only high quality nitrocellulose or PVDF membranes. CST recommends using nitrocellulose membranes, as PVDF tends to result in higher background. Pore size of 0.2 µm is generally recommended; membranes with a pore size of 0.45 µm are not recommended for proteins smaller than 30 kDa.

Insufficient membrane blocking and inadequate washing can result in high background. CST recommends blocking for 1 hr at room temperature in 5% milk in TBST. Blocking for less than 1 hr or using another blocking buffer can result in nonspecific binding of primary and secondary antibody. CST recommends washing for 3 x 5 min with TBST. Use of the mild detergent Tween-20 helps remove nonspecifically bound proteins.

Proper dilution and incubation of primary and secondary antibodies are important to minimize high background. Incubate primary antibody overnight at 4 °C in TBST at the recommended dilution with the recommended dilution buffer. Please consult the product datasheet for recommended dilution and dilution buffer specific to each primary antibody. Issues can also occur from the secondary antibody. Some secondary antibodies bind nonspecifically to proteins in cell extracts. To assess the quality of a secondary antibody, perform a blot (through to film exposure) without primary antibody. Serial dilutions of the secondary antibody can be performed on blots with the same cell extracts and primary antibody to optimize secondary antibody concentration. Always incubate secondary antibody in 5% milk in TBST for 1 hr at room temperature. CST offers secondary antibodies with optimal dilutions predetermined, saving you this troubleshooting step. These secondary antibodies are used in-house for antibody validation and are optimized to work with CST primary antibodies.

[show secondary antibody listing web page]

Finally, high background can occur at the detection step. SignalFire ECL Reagent should be prepared immediately before use. Also, long film exposure time due to low protein expression can also result in high background noise. It is never necessary to expose the film for more than 30 sec. Using a higher expressing cell line or inducing expression with chemical treatment can correct this problem.

In closing, we hope this video is a helpful resource for performing western blots using CST antibodies in your own lab. Please view our first video, Western Blotting Protocol, for a comprehensive western blot procedure. 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]

Technical Support

Hours: 9:00 AM - 6:00 PM (EST)

Toll free phone: 877-678-8324 or 877-678-TECH

E-mail: support@cellsignal.com

Suggestions or comments e-mail: tech@cellsignal.com