



A GUIDE TO SUCCESSFUL WESTERN BLOTTING

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WB



The western blot technique is a powerful tool to elucidate the complex signaling events that underlie biological processes and disease. This paper highlights critical steps in the western blot protocol and demonstrates how protocol changes can affect the final outcome of your blot.



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Introduction

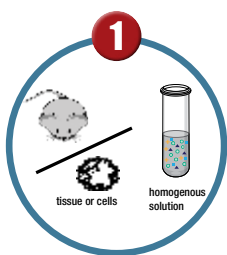
Western blotting, also called immunoblotting, is a widely used and accepted technique to detect levels of protein expression in a cell or tissue extract. This technique measures protein levels in a biological sample through antibody binding to a specific protein of interest. The precise binding that occurs between an antibody and its target protein epitope allows detection of highly specific amino acid sequences within a protein. Antibodies can also detect specific post-translational modifications (PTM) of a protein. Phospho-specific antibodies have been used to identify components of specific signaling pathways and to study changes in phosphorylation events in various biological contexts. Antibodies specific to other PTMs have been developed recently, allowing researchers to monitor changes in acetylation, methylation, and ubiquitination status of a protein.

At Cell Signaling Technology (CST), scientists perform more than one thousand western blots daily to validate our existing and new antibodies. The western blot protocol we have been optimizing for over a decade can be seen on page 13 and is also available online (see link below) so you can replicate the procedure and get reproducible and reliable results.

Here, we will highlight the critical steps in the western blot protocol and demonstrate how protocol changes can affect the final outcome of your blot. We will also discuss the importance of using a well-validated antibody in your western blot experiments. Finally, we will provide a list of commonly used reagents that are utilized by CST scientists and work optimally with our antibodies.

The western blot technique is a powerful tool to help us understand the complex signaling events that underlie biological processes and disease.

www.cellsignal.com/WBProtocol



Sample Preparation

Lysis buffer & sonication =
better release of proteins

10 Step Protocol to a better western blot

Here we give an overview of the protocol we recommend and discuss which steps are key to a successful experiment. Within, we provide recommendations on reagents and procedures based on our extensive experience with western blotting as part of our validation, lot testing, and technical support process. Although the western blotting technique is widely used and accepted, problems can occur that lead to suboptimal results, which can be time consuming and frustrating. This paper aims to help you improve your western blotting by providing suggestions to allow you to get the expected results in the shortest amount of time with minimal end-user optimization.



SDS-PAGE

Run with appropriate controls



Wet Transfer

To nitrocellulose membranes



Blocking

5% milk in TBST



Primary Antibody Incubation

At 4°C overnight in 5%
BSA or nonfat milk* in TBST

*as recommended on
product datasheet



Detection

Mix reagents immediately before
use for chemiluminescent detection



Washing

3 x 5 min in TBST



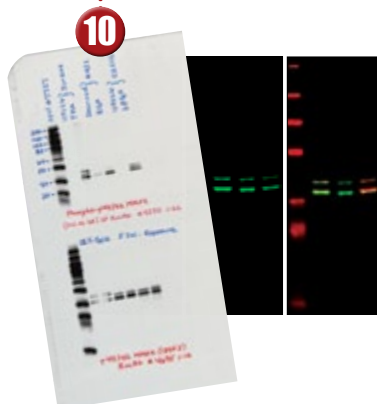
Secondary Antibody Incubation

For 1 hr at room temperature
in 5% nonfat milk in TBST



Washing

3 x 5 min in TBST



Imaging

Chemiluminescent imaging
offers greater flexibility and
ease of use

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The importance of a well-validated antibody.

The primary antibody is the key reagent which impacts data quality. A poor primary antibody can result in dirty, uninterpretable or misleading results. To be confident that an antibody is specific to the target of interest, the antibody specificity should be validated. It takes more than just seeing a band at the expected molecular weight to validate antibody specificity. Antibodies should undergo a stringent validation procedure using a number of different approaches to ensure that the antibody detects the target accurately.

Antibody validation includes:

- Specificity testing on cell and tissue extracts with documented protein expression levels—not just on recombinant protein.
- Specificity confirmation through the use of siRNA transfection or knockout cell lines.
- Specificity testing of antibodies directed against a post-translational modification by treatment of cell lines with growth factors, chemical activators, or inhibitors, which induce or inhibit target expression.
- Phospho-specificity testing of phospho-antibodies by phosphatase treatment.

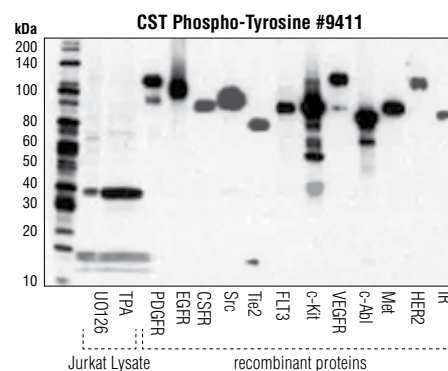
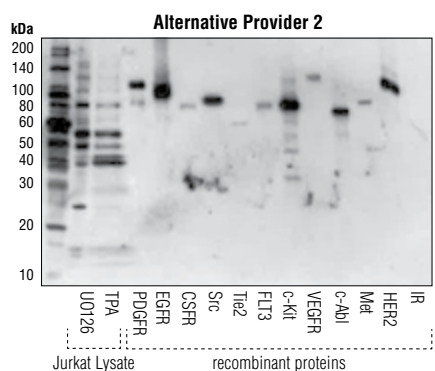
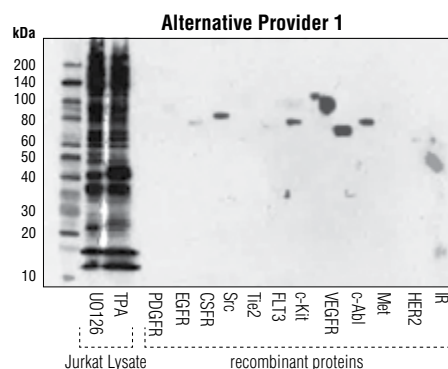
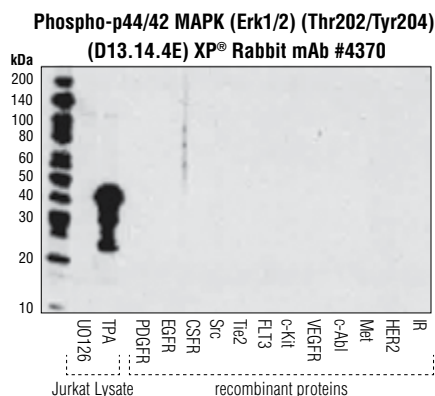
Be in Control of Your Western Blot

An important consideration in any experiment is the inclusion of appropriate controls. Positive and negative controls ensure confidence that your antibody is detecting a specific signal. In the figure below, Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibodies are evaluated using negative (U0126-treated) and positive (TPA-treated) Jurkat lysate, as well as purified recombinant tyrosine-phosphorylated proteins. The importance of using a well-validated antibody and the inclusion of proper experimental controls is demonstrated by the cross-reactivity of the alternate providers' antibodies in both negative control lysate and with non-target phospho-tyrosine proteins.

Loading controls are used to ensure equal loading of a gel as well as the integrity of the sample and are important when western blot results are being compared quantitatively. When a modification state specific antibody is used to assess changes in activation state of a protein, the corresponding total protein antibody should be used to ensure equal loading and sample integrity. Proteins that express well across many cell lines and tissues, such as β -actin, α -tubulin, and GAPDH are often used as loading controls in experiments designed to compare total protein levels across multiple samples.

Cellular fractionation markers should be used when you are preparing nuclear and cytoplasmic extracts to confirm that the lysate was prepared appropriately. Histone H3 is a nuclear protein and Lamin A is a nuclear membrane protein; both of these offer strong nuclear fraction markers. Cytoplasmic proteins like MEK1/2 and cytoskeletal proteins like β -actin and α / β -tubulin are common cytoplasm markers.

These results demonstrate that #4370 displays exceptional specificity. Western blot analysis using a panel of recombinant tyrosine-phosphorylated proteins shows no detectable cross-reactivity using Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370 and significant cross-reactivity with other tyrosine phosphorylated proteins using alternative provider antibodies. Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 was used to demonstrate protein loading and verify molecular weight of the tagged recombinant proteins.



See our Controls Table to learn more about specific cell line and treatment information so you can make effective control lysates.

www.cellsignal.com/controls

Critical steps in the recommended protocol—how critical are they?

Protocol changes can have a significant effect on western results. We have tested common variations of lysate preparation, gels, transfer, membrane, buffers, blocking, and antibody dilution and incubation protocol steps and identified those which yield optimal results. In the pages that follow, you will find the data that was used to determine procedure for critical steps in our recommended western blotting protocol.

Lysate Preparation

Ensuring Complete Lysis and Using Fresh Samples

To prepare samples for analysis, it is recommended that chemical and physical methods are used to disrupt membranes and ensure cell lysis and the release of proteins of interest. For sample lysates from tissues, a homogenization step is recommended in most cases, and a protease inhibitor cocktail is usually added to the lysis buffer prior to homogenization. Lysis buffers contain varying degrees of detergents, salts, and enzymatic inhibitors (such as phosphatase or protease inhibitors **(A)**) to break apart cells, solubilize proteins, and prevent degradation. Shown here are the effects of sonication in conjunction with chemical cell lysis. We recommend sonication of cell and tissue extracts in general, but it is crucial for the study of nuclear and chromatin associated proteins such as Histone H3. Here **(B)** we observe a greatly enhanced signal in sonicated compared to nonsonicated extracts. This is because sonication disrupts membranes and chromatin to a greater extent than lysis buffer alone. We always recommend sonication to ensure total cell lysis and to shear the chromosomal DNA. We recommend 3 pulses for 10 seconds at 35%–40% power, though it is important to remember that different sonicators perform differently and should be individually optimized per manufacturer's recommendations. Allow 10 seconds between pulses and keep samples on ice while sonicating. If you do not have access to a probe sonicator, passing your samples through a fine gauge needle will also serve to break membranes and shear DNA.

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. Additionally, lysing in high-detergent RIPA buffer may provide a more thorough and consistent lysis of tissue compared to standard lysis buffers. Here **(C)** we compare old and new mouse brain lysate. The older lysate yields a weaker specific signal and higher levels of background.

Gel Electrophoresis

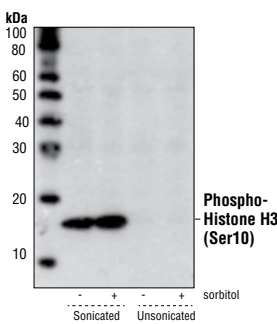
Including Adequate Sample and Molecular Weight Markers

SDS-PAGE is used to separate proteins according to their electrophoretic mobility, which is a function of protein size and charge. We recommend loading 20–30 µg of total protein on precast Tris-Glycine mini-gels. For high molecular weight targets, we recommend Tris-Acetate gels and associated buffers. Molecular weight markers should always be included in a lane near the samples of interest as a point of reference. Molecular weight markers (or ladders) are made from a mixture of purified proteins of known molecular weight. Using a prestained marker allows visualization of how far proteins have migrated during electrophoresis and later confirms electrotransfer to the membrane. Using a biotinylated marker offers visualization of the ladder along with antibody targets on film. It is useful to include both types of markers for both monitoring experiment progression and ease of interpreting results.

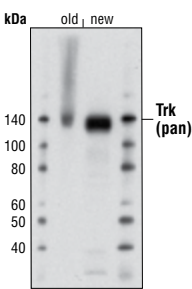
(A) Common Phosphatase and Protease Inhibitors

Inhibitor	Target	Final Concentration
Aprotinin	Trypsin, Chymotrypsin, Plasmin	2 µg/mL
Leupeptin	Lysosomal	1–10 µg/mL
Pepstatin A	Asp proteases	1 µg/mL
PMSF	Asp proteases	1 mM
EDTA	Mg & Mn metalloproteases	1–5 mM
EGTA	Ca metalloproteases	1 mM
Na Fluoride	Ser & Thr phosphatases	5–10 mM
Orthovanadate	Tyr phosphatases	1 mM
Pyrophosphate	Ser & Thr phosphatases	1–2 mM
β-glycerophosphate	Ser & Thr phosphatases	1–2 mM

(B) Lysate sonication is critical to the observation of nuclear and chromatin-bound proteins. Western blot analysis of extracts from CKR/PAEC cells, untreated or treated with sorbitol and either sonicated or without sonication, using Phospho-Histone H3 (Ser10) Antibody #9701.



(C) Fresh lysate yields a significantly stronger specific signal and lower background due to lower degradation levels. Western blot analysis of extracts from mouse brain, either prepared three months prior to use (new) or one year prior to use (old), using Trk (C17F1) Rabbit mAb #4609.



TROUBLESHOOTING TIP

Lack of signal because the concentration of the protein of interest is below detectable levels. If this is due to minimal expression in the starting cell line or tissue sample, chemical stimulation to induce expression may be needed or an alternate cell line, expressing higher levels, may be considered if possible. A gene expression database should be consulted to estimate protein expression levels in specific cell lines and tissues.

Transfer...

How Much Time Are You Really Saving?

Wet Transfer

Optimal Transfer Method

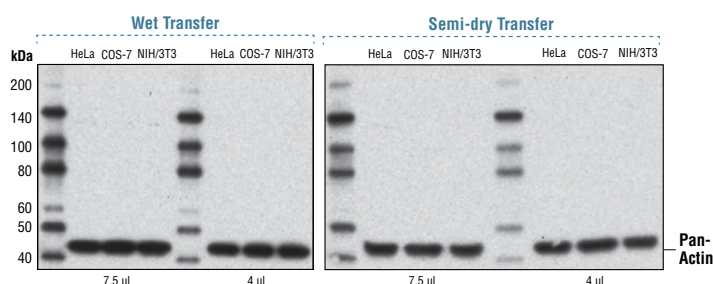
Wet transfer of proteins from gel to membrane refers to the full immersion of the filter paper-gel-membrane-filter paper “sandwich” in buffer. In this method, electrophoretic transfer to 0.2 µm pore-size nitrocellulose membrane is performed submerged and under cooling conditions for 1.5 hours at 70 V in transfer buffer containing 20% methanol.

Semi-Dry Transfer

Alternative Transfer Method

Semi-dry transfer of proteins from gel to membrane refers to a system in which the filter paper-gel-membrane-filter paper “sandwich” is saturated with buffer, and is placed between otherwise dry anode and cathode plates. In this system, transfer is performed on the benchtop at room temperature for 15–60 minutes. CST has also tested the Trans-blot® Turbo™ transfer system from Bio-Rad. This system works well for the transfer of both low and high molecular weight proteins and requires less buffer than wet transfer, however in some instances it can yield higher background staining. Shown here **(A)** is a side-by-side comparison of semi-dry transfer and wet transfer, which offer similar transfer of low to mid-weight protein.

(A) For low molecular weight targets, results obtained with wet and semi-dry transfer methods are comparable. Western blot analysis of titrations of extracts from HeLa, COS-7 and NIH/3T3 cells using Pan-Actin (D18C11) Rabbit mAb #8456. Transfer was performed using either wet transfer (left) or Trans-Blot® Turbo™ semi-dry transfer (right).

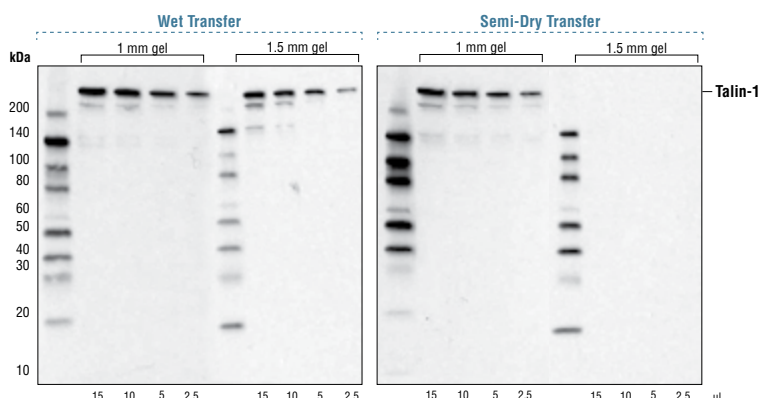


Gel Thickness

Hidden variable in electrophoretic transfer

The thickness of the gel used for electrophoresis introduces yet another transfer efficiency variable. CST scientists find that while proteins transfer well from all gels tested using the wet transfer method, the degree of gel thickness can affect transfer efficiency using semi-dry transfer systems. Shown here **(B)** are various combinations of 1 mm and 1.5 mm gels, and wet and semi-dry transfer methods. While gel selection has little effect on the semi-dry transfer of low molecular weight proteins, larger molecular weight proteins transfer more effectively from thinner gels than from thicker varieties. There is less distance for proteins to migrate during electrophoretic transfer in a 1 mm gel; transfer from a 1.5 mm gel may require longer transfer and high molecular weight proteins may transfer less efficiently.

(B) For high molecular weight targets, gel thickness is an important consideration in choosing a transfer method. Overall, wet transfer yields stronger signal regardless of gel thickness. Western blot analysis of titrations of extracts from C6 cells using Talin-1 (C45F1) Rabbit mAb #4021. The gels used for SDS-PAGE were either Mini-PROTEAN® gels (1 mm) from Bio-Rad or Novex® gels (1.5 mm) from Life Technologies, and the transfer method employed was either wet transfer (left) or Trans-Blot® Turbo™ semi-dry transfer (right).



TROUBLESHOOTING TIP

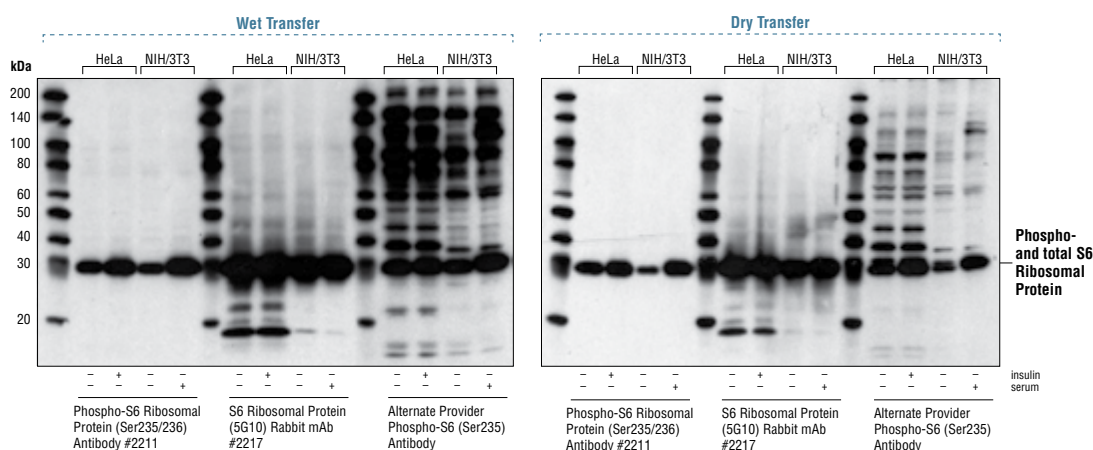
Low signal from inadequate loading of lysate onto the gel. We recommend loading 20–30 µg of total protein lysate per lane; however, if protein levels are below detection, immunoprecipitation prior to SDS-PAGE may be necessary to enrich the protein of interest. When working with tissue, we generally recommend loading up to 100 µg of total protein depending on target expression level.

Dry Transfer

Limited Sensitivity Transfer Method

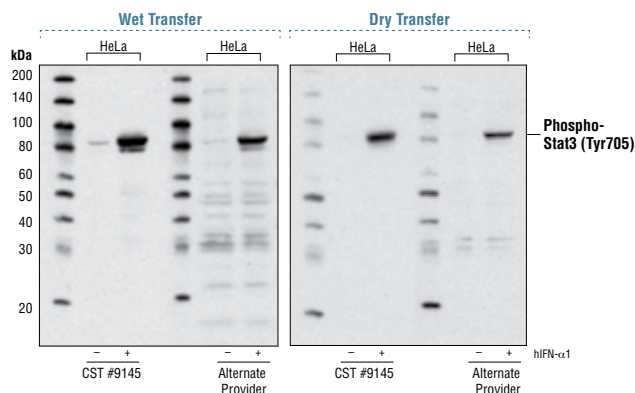
Dry transfer of proteins from gel to membrane refers to a system in which no additional buffer is added because the anode and cathode plate system contains a buffer matrix. iBlot® blotting system from Life Technologies is one example of a dry blotting system and completes a dry transfer in 7 minutes. In our hands, this system has yielded lower signal levels ranging from slightly to dramatically lower signal depending on the individual primary antibody. The largest differences in signal are observed for larger molecular weight proteins, suggesting inefficient transfer. Shown here **(A,B)** are comparisons of wet transfer and iBlot® dry transfer system using both low **(A)** and mid-weight **(B)** targets as well as CST antibodies and antibodies from alternative providers.

PLEASE NOTE: In a situation where sample amount is limiting or low endogenous levels of a protein need to be detected, the dry transfer method may limit the sensitivity of your assay.



(A) For low molecular weight targets in highly expressing cell lines, dry transfer results in reduced specific signal. Western blot analysis of extracts from HeLa and NIH/3T3 cells, untreated or treated with insulin or serum as indicated, using Phospho-S6 Ribosomal Protein (Ser235/236) Antibody #2211, S6 Ribosomal Protein (5G10) Rabbit mAb #2217, or an alternate provider's phospho-S6 (Ser235) antibody. Blots were transferred using either traditional wet transfer methods (left) or iBlot® dry transfer system (right) and exposed to film for the same amount of time.

(B) For mid- to high molecular weight targets, dry transfer yields significantly lower specific signal, likely due to inefficient transfer. Western blot analysis of extracts from HeLa cells, untreated or treated with Human Interferon- α 1 (hIFN- α 1) #8927, using Phospho-Stat3 (Tyr705) (D3A7) XP® Rabbit mAb #9145 or a phospho-Stat3 antibody from an alternate provider. Blots were transferred using either traditional wet transfer methods (left) or iBlot® dry transfer system (right).



TROUBLESHOOTING TIP

Incomplete transfer or over transfer. Incomplete transfer can be visually confirmed using a pre-stained molecular weight marker and can be corrected by increasing the time and/or voltage of electrophoretic transfer. Over transfer is common with very low molecular proteins, particularly if a 0.45 μ m pore-size membrane is used. We recommend a 1.5 hour transfer with 0.2 μ m pore-size membrane. Incomplete transfer of high molecular weight proteins can be corrected by adjusting the methanol concentration in the transfer buffer, as methanol has a fixative effect. We find decreasing the methanol concentration in the transfer buffer from 20% to 5% and increasing transfer time to 3 hours are helpful for the transfer of proteins over 200 kDa.

Blocking

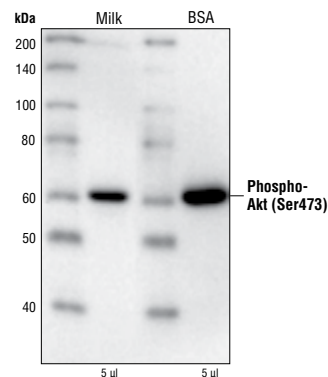
We recommend a quick wash of the membrane in Tris-Buffered Saline (TBS) following transfer to remove residual transfer buffer, followed by blocking in 5% nonfat milk in Tris Buffered Saline with Tween® 20 detergent (TBST) for one hour at room temperature. This blocking step helps reduce non-specific primary antibody binding and reduces background. Blocking the membrane for too long should be avoided as it can obscure antigenic epitopes and prevent the antibody from binding. Blocking is followed by a 5 minute wash in TBST.

Primary Antibody

Dilution Buffer

Dependent upon Antibody Specificity

Primary antibodies should be diluted in TBST buffer containing either 5% BSA or 5% nonfat milk. The optimal dilution buffer has been predetermined for each CST™ antibody and is included on the individual product datasheet. Alternative dilution buffers can result in less-than-optimal signal. For example **(A)**, dilution of the Phospho-Akt (Ser473) Antibody with a BSA-based buffer provides a stronger signal than a milk-based buffer in this western blot analysis of extracts from C2C12 cells treated with insulin.



(A) Diluting antibodies in milk generally yields a lower specific signal than BSA because milk is a stronger blocking agent. Western blot analysis of extracts from insulin-treated C2C12 cells using Phospho-Akt (Ser473) Antibody #9271. Primary antibody dilution buffer was composed of either 5% nonfat dry milk in TBST or 5% BSA in TBST, as indicated.

Incubation

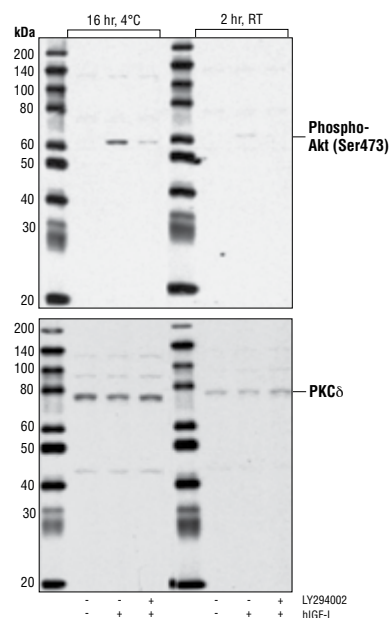
Overnight Incubation yields Improved Antibody Binding

The primary antibody incubation period can vary greatly depending upon the protocol a researcher is using. We recommend incubating the primary antibodies overnight at 4°C. Shown here **(B,C)** are two examples of antibody performance using overnight incubations at 4°C compared to 2 hour incubation at room temperature. Phospho-Akt (Ser473) Antibody and PKCδ Antibody both perform better with an overnight incubation at 4°C.

Abbreviated Incubation Systems

Less Incubation Time yields Less Signal

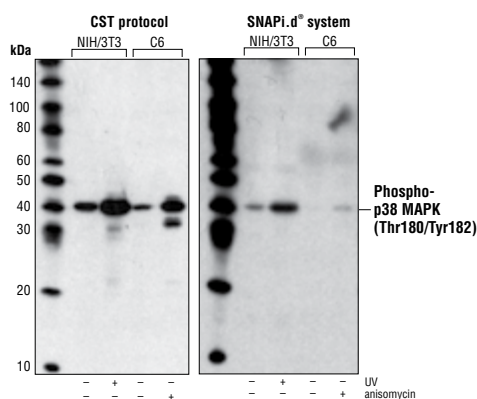
We recommend one hour blocking followed by overnight primary antibody incubation at 4°C after transfer. Systems advertising abbreviated antibody incubation periods are becoming increasingly popular as they can speed western blotting results. When using these systems, the manufacturer's recommended protocols should be followed and optimized. These protocols often require weaker blocking buffers and higher concentrations of primary and secondary antibodies to achieve necessary antibody binding levels in abbreviated time periods. CST scientists compared SNAPi.d.® from Millipore, a vacuum operated incubation system that reduces antibody incubation times to less than 30 minutes, to the CST-recommended antibody incubation protocol. In general, the expedited antibody incubation system resulted in decreased protein target signal **(C)**.



(B) Primary antibody incubation overnight at 4°C yields significantly increased antibody binding compared to a 2 hr incubation. Western blot analysis of extracts from HeLa cells, untreated or treated with LY294002 #9901 or Human Insulin-like Growth Factor I (hIGF-I) #8917, using Phospho-Akt (Ser473) Antibody #9271 (top) or PKCδ Antibody #2058 (bottom). Primary antibody incubation was performed overnight at 4°C or for 2 hr at room temperature, as indicated.

(C) Abbreviated incubation systems allow less time for antibody binding, and therefore yield a weaker specific signal than the recommended incubation.

Western blot analysis of extracts from NIH/3T3 and C6 cells, untreated, UV irradiated or treated with anisomycin as indicated, using Phospho-p38 MAPK (Thr180/Tyr182) (3D7) Rabbit mAb #9215. Blots were incubated using either the CST-recommended protocol (left) or SNAPi.d.® incubation system (right).



Wash and Dilution Buffer

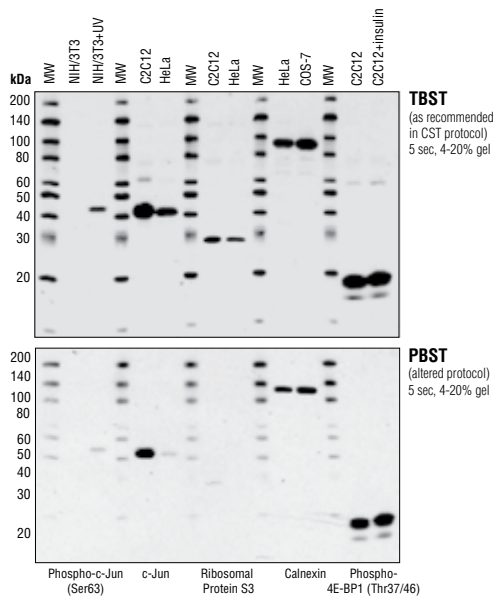
TBST Buffers yield Stronger Signal

Another area where western blot protocols can vary is with the wash and dilution buffers. We recommend using TBST for antibody dilution buffers and wash steps. Shown here **(A)** is a comparison of antibody performance using TBST-based dilution and wash buffers versus those made with Phosphate Buffered Saline with Tween® 20 detergent (PBST). For all antibodies shown, TBST-based buffers provided a stronger signal than PBST-based buffers. Washing for longer than the recommended 3 x 5 minutes is common and can result in reduced signal, because the antibody may be dislodged from the bound epitope. This applies to washing steps after both primary and secondary antibody incubations.

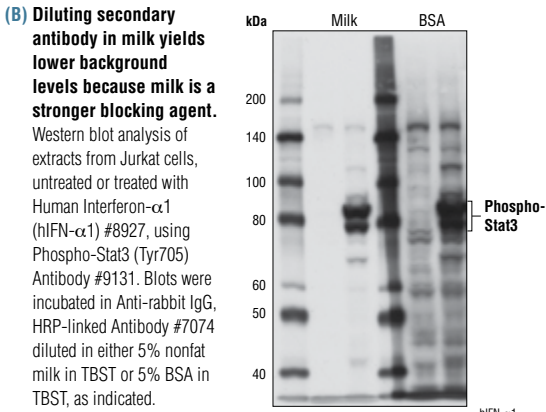
Secondary Antibody

Dilution in Stronger Blocking Agents yields Less Background

Secondary antibody should be used according to the manufacturer's recommendations. Frequently, these recommendations are provided as a wide dilution range. To obtain the optimal result with the secondary antibody, you should perform a titration experiment with the antibody to determine maximum signal and minimal background. We suggest diluting the secondary antibody in 5% nonfat milk in TBST. Here **(B)** we compare secondary antibody dilution in 5% nonfat milk with dilution in 5% BSA. Because the nonfat milk offers stronger blocking, the BSA-based dilution yields significantly higher background than the milk-based dilution. If performing a western blot of immunoprecipitated lysates, you may want to consider using either conformation or chain-specific secondary antibody to avoid signal from IgG heavy or light chains. This is especially important if your target of interest has a molecular weight near 50 or 25 kDa, as these are the weights of the immunoglobulin heavy and light chain, respectively.



(A) TBST buffer yields overall stronger signal than PBST buffer. Western blot analysis of extracts from various cell lines using Phospho-c-Jun (Ser63) II Antibody #9261, c-Jun (60A8) Rabbit mAb #9165, Ribosomal Protein S3 Antibody #2579, Calnexin (C5C9) Rabbit mAb #2679, and Phospho-4E-BP1 (Thr37/46) (236B4) Rabbit mAb #2855. Antibody dilution and wash steps were performed using either TBST (top) or PBST (bottom), as indicated.



(B) Diluting secondary antibody in milk yields lower background levels because milk is a stronger blocking agent. Western blot analysis of extracts from Jurkat cells, untreated or treated with Human Interferon-α1 (hIFN-α1) #8927, using Phospho-Stat3 (Tyr705) Antibody #9131. Blots were incubated in Anti-rabbit IgG, HRP-linked Antibody #7074 diluted in either 5% nonfat milk in TBST or 5% BSA in TBST, as indicated.

TROUBLESHOOTING TIP

Some secondary antibodies bind nonspecifically to various proteins in cell extracts, leading to high background. To assess the quality of a secondary antibody, perform a blot (through to film exposure) without primary antibody.

Detection

Chemiluminescent

Offers Ease of Use

To observe maximum signal, these reagents should be mixed directly prior to use to minimize signal reduction due to light exposure and degradation over time. The quality and strength of the detection reagent can enhance a weak signal and reduce background staining. Shown here **(A)** are two chemiluminescent detection reagents that provide robust and specific signals.

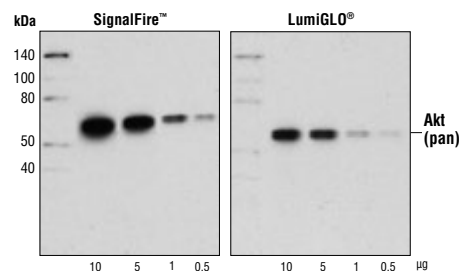
Fluorescent

Offers Digital Data Storage and Ease of Quantification

Western detection using fluorescent labeled secondary antibodies requires specialized instrumentation, such as a fluorescent imager. Fluorescent detection is becoming increasingly popular as it provides faster visualization and quantification of results, as well as digital data storage. Fluorescent detection allows phospho- and total protein levels to be determined on the same membrane, and regulation of different targets at different molecular weights can be examined simultaneously. It is important to remember, however, that primary antibody affinities vary, and no two individual antibodies can be directly compared to one another.

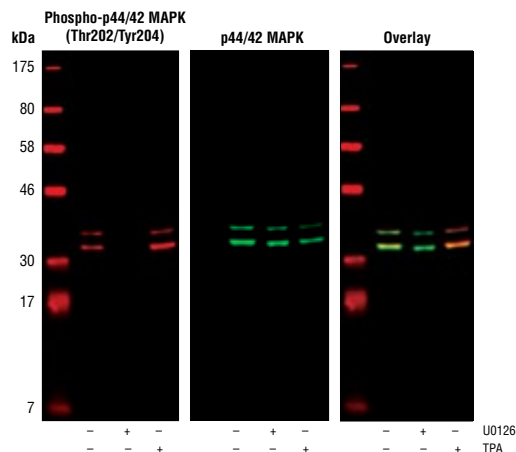
A modified immunoblotting protocol should be used for fluorescent western blotting. We find that omitting Tween® 20 detergent during the blocking step (using only 5% nonfat dry milk in TBS) and drying the membrane prior to imaging are the only necessary changes from our standard protocol. Full details can be found in our Fluorescent Western Immunoblotting Protocol (Primary Antibody Incubation In BSA) and our Fluorescent Western Immunoblotting Protocol (Primary Antibody Incubation In Milk) available online. Here we show a fluorescent western performed for the simultaneous detection of phospho- and total p44/42 MAPK **(B)**.

Two-color western blots require primary antibodies from different species and appropriate secondary antibodies labeled with different dyes. If the primary antibodies require different primary antibody incubation buffers, each primary antibody should be tested individually in both buffers to determine the optimal buffer for the dual-labeling experiment. Some antibody pairs may not work together due to overlapping epitopes, interference caused by primary and/or secondary antibodies, or incompatibilities in primary antibody incubation buffers.



(A) Detection Reagents

Western blot analysis of titrations of extracts from Jurkat cells using Akt (pan) (C67E7) Rabbit mAb #4691. Blots were detected using SignalFire™ ECL Reagent #6883 (left) or LumiGLO® Reagent #7003 (right).



(B) Fluorescent Western Blotting

Western blot analysis of extracts from COS-7 cells, untreated or treated with either U0126 #9903 (10 µM for 1 hr) or TPA (12-O-Tetradecanoylphorbol-13-Acetate) #4174 (200 nM for 10 min), using Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370 and p44/42 MAPK (Erk1/2) (3A7) Mouse mAb #9107.

Western Immunoblotting Protocol

For western blots, incubate membrane with diluted primary antibody in either 5% w/v BSA or nonfat dry milk, 1X TBS, 0.1% Tween® 20 detergent at 4°C with gentle shaking, overnight. **NOTE:** Please refer to each primary antibody datasheet for recommended primary antibody dilution buffer and recommended antibody dilution.

A. Solutions and Reagents

NOTE: Prepare solutions with RODI or equivalent grade water.

- 20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH₂O, mix.
- 10X Tris Buffered Saline (TBS):** To prepare 1 L 10X TBS: 24.2 g Tris base, 80 g NaCl; adjust to pH 7.6 with HCl.
- 1X SDS Sample Buffer:** (#7722, #7723) 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red.
- Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5).
- 10X Tris Buffered Saline with Tween® 20 detergent (TBST-10X):** (#9997) To prepare 1 L 1X TBST: add 100 ml 10X TBST to 900 ml dH₂O, mix.
- Nonfat Dry Milk:** (#9999)
- Blocking Buffer:** 1X TBST with 5% w/v nonfat dry milk; for 150 ml, add 7.5 g nonfat dry milk to 150 ml 1X TBST and mix well.
- Wash Buffer:** 1X TBST
- Bovine Serum Albumin (BSA):** (#9998)
- Primary Antibody Dilution Buffer:** 1X TBST with 5% BSA or 5% nonfat dry milk as indicated on primary antibody datasheet; for 20 ml, add 1.0 g BSA or nonfat dry milk to 20 ml 1X TBST and mix well.
- Phototope®-HRP Western Blot Detection System:** (anti-rabbit #7071) (anti-mouse #7072) Includes biotinylated protein ladder (#7727), secondary antibody conjugated to horseradish peroxidase (HRP) (anti-rabbit #7074) (anti-mouse #7076), anti-biotin HRP-linked Antibody (#7075), LumiGLO® chemiluminescent reagent and peroxide (#7003).
- Prestained Protein Marker, Broad Range (Premixed Format):** (#7720)
- Blotting Membrane:** This protocol has been optimized for Nitrocellulose Sandwiches (#12369). PVDF membranes may also be used. Pore size 0.2 µm is generally recommended.

B. Protein Blotting

A general protocol for sample preparation is described below.

- Treat cells by adding fresh media containing regulator for desired time.
- Aspirate media from cultures; wash cells with 1X PBS; aspirate.
- 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.
- Sonicate for 10–15 sec to complete cell lysis and shear DNA (to reduce sample viscosity).
- Heat a 20 µl sample to 95–100°C for 5 min; cool on ice.
- Microcentrifuge for 5 min.
- 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.
- Electrotransfer to nitrocellulose (#12369) membrane.

C. Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm²) of membrane; for different sized membranes, adjust volumes accordingly.

I. Membrane Blocking

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

II. Primary Antibody Incubation

Proceed to one of the following specific set of steps depending on the primary antibody used.

For Unconjugated Primary Antibodies

- Incubate membrane and primary antibody (at the appropriate dilution and buffer as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
- Wash three times for 5 min each with 15 ml of TBST.
- 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.
- Wash three times for 5 min each with 15 ml of TBST.
- Proceed with detection (Section D).

For HRP Conjugated Primary Antibodies

- Incubate membrane and primary antibody (at the appropriate dilution as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
- Wash three times for 5 min each with 15 ml of TBST.
- Incubate with 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.
- Wash three times for 5 min each with 15 ml of TBST.
- Proceed with detection (Section D).

For Biotinylated Primary Antibodies

- Incubate membrane and primary antibody (at the appropriate dilution as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
- Wash three times for 5 min each with 15 ml of TBST.
- Incubate membrane with Streptavidin-HRP (#3999) in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
- Wash three times for 5 min each with 15 ml of TBST.
- Proceed with detection (Section D).

Do not add Anti-biotin, HRP-linked Antibody for detection of biotinylated protein markers. There is no need. The Streptavidin-HRP secondary antibody will also visualize the biotinylated markers.

D. Detection of Proteins

- Incubate membrane with 10 ml LumiGLO® (0.5 ml 20X LumiGLO® #7003, 0.5 ml 20X Peroxide, and 9.0 ml purified water) or 10 ml SignalFire™ #6883 (5 ml Reagent A, 5 ml Reagent B) with gentle agitation for 1 min at room temperature. **NOTE:** LumiGLO® substrate can be further diluted if signal response is too fast.
- 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. **NOTE:** Due to the kinetics of the detection reaction, signal is most intense immediately following incubation and declines over the following 2 hr.

*Membranes can be stripped of antibodies and reused for the detection of a second protein. CST's reprobing protocol is available online. It is not recommended to strip and re-probe a blot multiple times. **NOTE:** The best possible results are always obtained using fresh western blots.*

www.cellsignal.com/WBReprobing



Recommended CST Reagents

Cell Signaling Technology offers a wide selection of standard buffers, molecular weight markers, protease and phosphatase inhibitors, secondary antibodies, detection reagents, and experimental controls to support your western blotting experiments. These reagents are also used in-house for antibody validation in western blotting therefore work optimally with our primary antibodies.

Reagents available from CST

Buffer Reagents

1. Tris-Glycine SDS Running Buffer (10X) #4050
2. Tris Buffered Saline with Tween® 20 detergent (TBST-10X) #9997
3. Nonfat Dry Milk #9999
4. BSA #9998

Sample Preparation Buffers

1. Blue Loading Buffer Pack #7722
2. Red Loading Buffer Pack #7723
3. RIPA Buffer (10X) #9806*
4. Chaps Cell Extract Buffer (10X) #9852*
5. Cell Lysis Buffer (10X) #9803*

Inhibitors For Sample Preparation

1. Protease/Phosphatase Inhibitor Cocktail (100X) #5872
2. PMSF #8553
3. Protease Inhibitor Cocktail (100X) #5871
4. Phosphatase Inhibitor Cocktail (100X) #5870

Protein Markers/Standards

1. Biotinylated Protein Ladder Detection Pack #7727
2. Prestained Protein Marker, Broad Range (Premixed Format) #7720

Filter Paper and Membrane

1. Nitrocellulose Sandwiches #12369

Secondary Antibodies

1. Anti-mouse IgG, HRP-linked Antibody #7076
2. Anti-rabbit IgG, HRP-linked Antibody #7074
3. Anti-rat IgG, HRP-linked Antibody #7077
4. Anti-biotin, HRP-linked Antibody #7075
5. Anti-mouse IgG, AP-linked Antibody #7056
6. Anti-rabbit IgG, AP-linked Antibody #7054
7. Anti-biotin, AP-linked Antibody #7055
8. Mouse Anti-rabbit IgG (Conformation Specific) (L27A9) mAb #3678
9. Mouse Anti-rabbit IgG (Conformation Specific) (L27A9) mAb (HRP Conjugate) #5127
10. Mouse Anti-rabbit IgG (Light-Chain Specific) (L57A3) mAb #3677
11. Anti-mouse IgG (H+L) (DyLight® 680 Conjugate) #5470
12. Anti-mouse IgG (H+L) (DyLight® 800 Conjugate) #5257
13. Anti-rabbit IgG (H+L) (DyLight® 680 Conjugate) #5366
14. Anti-rabbit IgG (H+L) (DyLight® 800 Conjugate) #5151
15. Streptavidin-HRP #3999

Detection Reagents

1. SignalFire™ ECL Reagent #6883
2. 20X LumiGLO® Reagent and 20X Peroxide #7003

*All CST™ lysis buffers contain protease and phosphatase inhibitors. We recommend adding 1 mM PMSF immediately before use.

Other Suppliers

Precast Gels

We currently use Novex® (from Life Technologies) Tris-Glycine (1.5 mm x 15 well sized) precast gels. For select large molecular weight proteins we use the Tris-Acetate precast gels and associated buffers, also Novex® from Life Technologies.

Apparatus

1. Novex® by XCell SureLock® (gel box) from Life Technologies
2. Mighty Small Transphor (transfer box - Cat# 20152156) from GE Healthcare

Foam Sponges

1. Foam Sponge (Cat# TE25F-1/8) from GE Healthcare

Film

1. Amersham™ Hyperfilm™ ECL (Cat# 28906838) from GE Healthcare
2. BioMax® Light Film (Cat# 178-8207) from Eastman Kodak Company

CST offers companion products to aid in your sample preparation and western blotting.

www.cellsignal.com/WBCompanion

Technical Support

We hope this paper is a helpful resource for performing western blots in your own lab. Cell Signaling Technology prides itself in providing you with exceptional customer service and support, and we are happy to share our experience with you. 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.



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