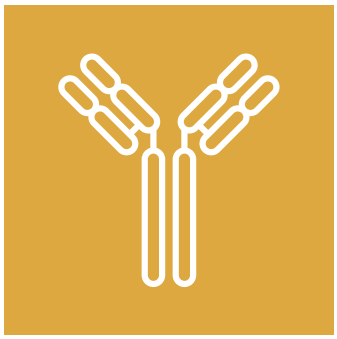
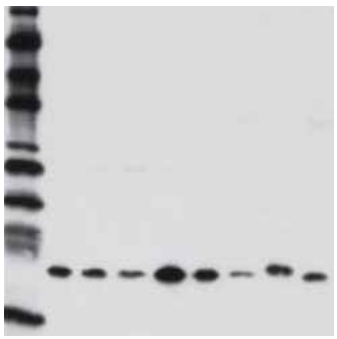
Cell Signaling TECHNOLOGY®

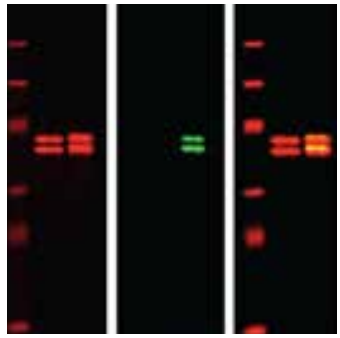
APPLICATION GUIDE

A Guide to Successful

Western Blotting







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 (PTMs) on 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, allowing researchers to monitor changes in the acetylation, methylation, and ubiquitination status of a protein.

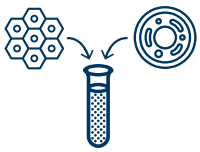
At Cell Signaling Technology (CST), scientists perform more than one thousand western blots daily to validate our existing and new antibodies. The western blot protocol we have been optimizing for over two decades can be found towards the end of this guide and is also available online so you can replicate 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.

10 steps 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. 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. The information and suggestions contained within this guide empower you to achieve the expected results with minimal optimization, saving you time, resources, and frustration.

1



Sample Preparation
Lysis buffer + sonication = better release of proteins

2

SDS-PAGE
Run with appropriate controls

3

Transfer
To nitrocellulose membranes

4

Blocking
5% milk in TBST

5

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

** as recommended on product datasheet*

6

Washing
3 x 5 min in TBST

7

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

8

Washing
3 x 5 min in TBST

9

Detection
Mix reagents immediately before use for chemiluminescent detection.

10

Imaging
For best results, we recommend chemiluminescent imaging.

The importance of a well-validated antibody

The primary antibody is the key reagent that 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. CST® antibodies undergo a stringent validation procedure using a number of different approaches to ensure that the antibody detects the target accurately.

Antibody validation at CST 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, that induce or inhibit target modification.
- Phospho-specificity testing of phospho-antibodies using a phosphatase treatment.

cst-science.com/WBvalidation

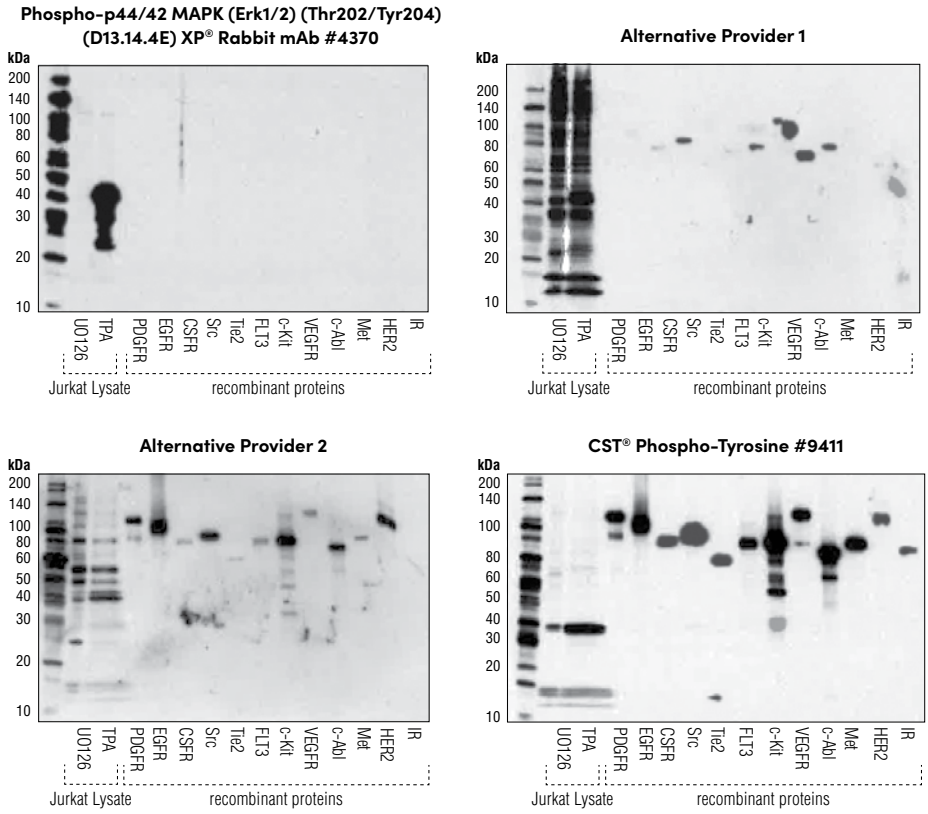
Be in control of your western blot

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 highlighted when observing the figure below, in which alternate providers' antibodies display cross-reactivity with both negative control lysates and 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 an antibody specific to a post-translational modification is used to assess changes in the activation state of a protein, the corresponding total protein antibody should be used to ensure equal loading and sample integrity. Additionally, 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 act as strong nuclear fraction markers. Cytoplasmic proteins like MEK1/2 and cytoskeletal proteins like β-actin and α/β-tubulin are common cytoplasmic markers.

These results demonstrate that #4370 displays exceptional specificity. WB 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 select effective control lysates at **cst-science.com/WBcontrols**

Critical steps in the recommended protocol... how crucial 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 incubation protocol steps. We have identified the parameters that yield optimal results. In the pages that follow, you will find the data that was used to determine the procedure for critical steps in our recommended western blotting protocol.

cst-science.com/westernprotocol

Lysate Preparation

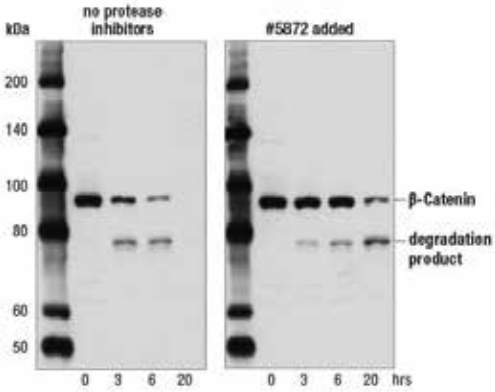
Ensuring Complete Lysis and Using Fresh Samples

To prepare samples for analysis, it is recommended that chemical and physical methods are used to disrupt membranes and ensure cell lysis, allowing for 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 **(1A)**) to break apart cells, solubilize proteins, and prevent degradation **(1B)**. 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 **(1C)** 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. Sonicators perform differently and sonication parameters should be individually optimized per the 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.

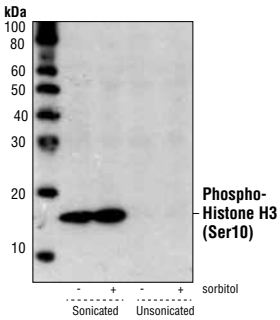
In general, primary cell and tissue extracts tend to produce more background bands and degradation products than cell line extracts. Using fresh, sonicated, and clarified tissue extracts may lessen background. Additionally, lysing in high-detergent RIPA buffer may provide a more thorough and consistent lysis of tissue compared to standard lysis buffers.

(1A) 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^{2+} & Mn metalloproteases	1–5 mM
EGTA	Ca^{2+} 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



(1B) Protein Degradation: Western blot analysis of extracts from NIH/3T3 cells, prepared in lysis buffer in the absence of protease inhibitors (left) or with Protease/Phosphatase Inhibitor Cocktail (100X) #5872 added (right), and incubated at 37°C for the indicated time points, using β-Catenin (D10A8) XP® Rabbit mAb #8480. In the absence of protease inhibitors, β-Catenin signal fades within 3 hr after harvest, indicating protein degradation. In the presence of the protease inhibitor cocktail, the β-Catenin degradation is slowed significantly and signal is still present at 20 hr following harvest.



(1C) Lysate sonication is critical for detecting 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.

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. Alternatively, another cell line or tissue type that has higher levels of the target may be considered. A gene expression database should be consulted to estimate protein expression levels in specific cell lines and tissues.

Gel Electrophoresis

Including Adequate Sample and Molecular Weight Markers

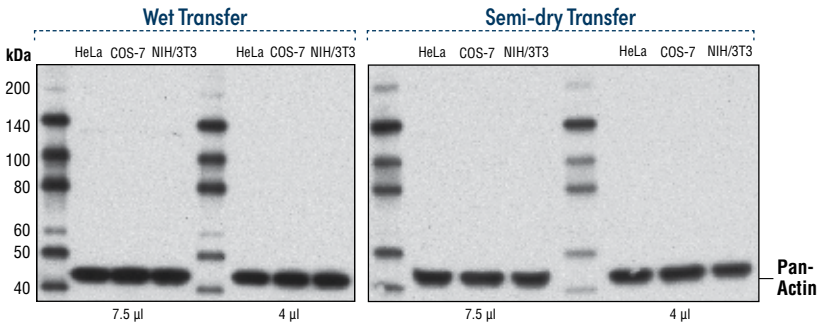
SDS-PAGE is used to separate proteins according to their electrophoretic mobility, which is a function of protein size and charge. We recommend loading 20–30 µg of total protein on precast tris-glycine mini-gels. For high molecular weight targets, we recommend tris-acetate gels and associated buffers. 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 the antibody targets. Using both types of markers allows you to monitor gel progression, determine transfer success, and aides in determining band sizes.

Transfer

Semi-Dry Transfer

Optimal 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, a transfer is performed on the benchtop at room temperature. CST routinely uses the Trans-blot Turbo transfer system from Bio-Rad at 25V for 7-10 minutes. Please refer to the system manufacturer's recommendations for the best settings to use with your equipment. This system works well for the transfer of a wide molecular weight range of proteins and requires less buffer than a wet transfer. Shown here **(2A)** is a side-by-side comparison of semi-dry transfer and wet transfer, which offer similar transfer of low to mid-weight protein.



(2A) 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).

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 for the protein of interest. When working with tissue, we generally recommend loading up to 100 µg of total protein depending on target expression level.

Wet Transfer

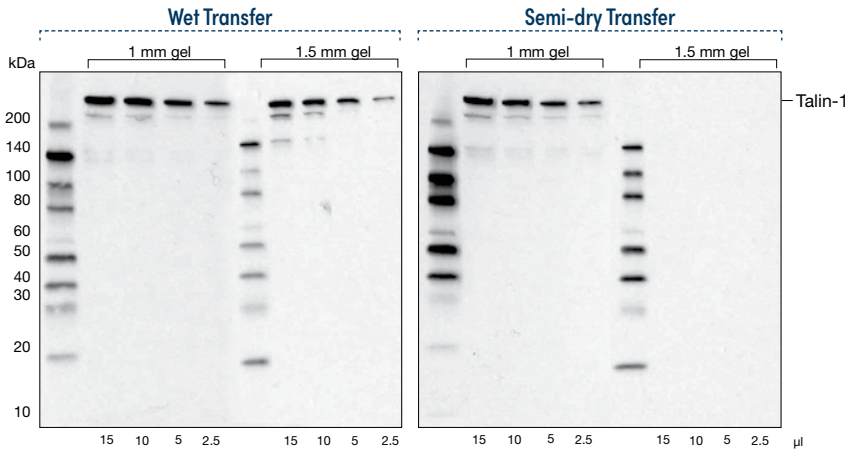
Alternative Transfer Method

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 2 hours at 70 V in transfer buffer containing 20% methanol. For some larger proteins (> 200 kDa), reducing the amount of methanol to 5% and running the transfer for 3 hours may improve transfer and detection.

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 **(2B)** 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.



(2B) 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).

Dry Transfer

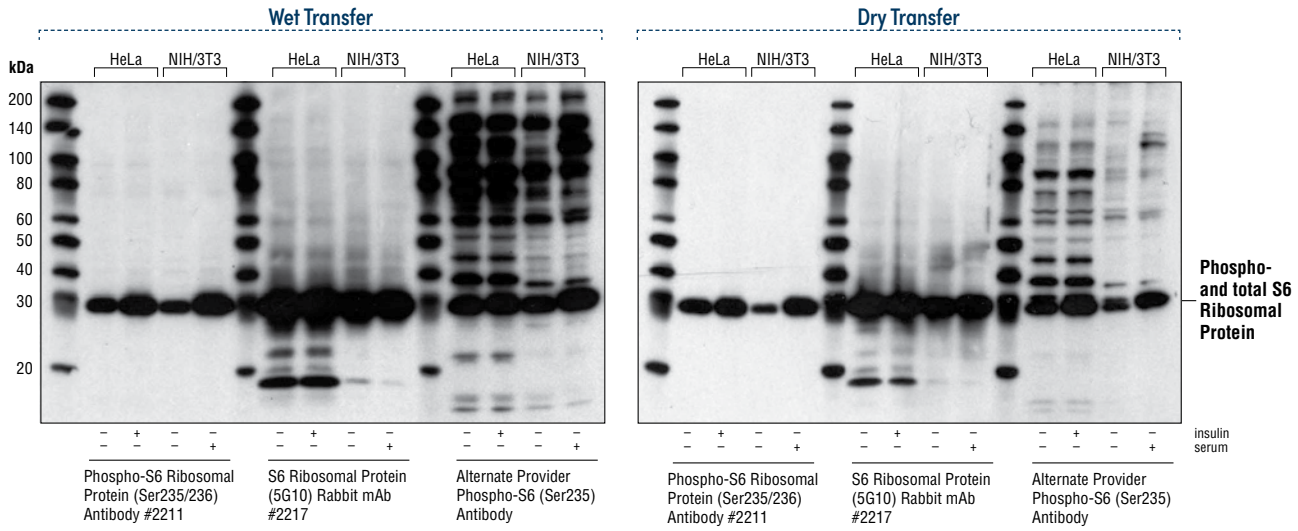
Limited Sensitivity Transfer Method

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 **(3A, 3B)** are comparisons of wet transfer and iBlot dry transfer system using both low **(3A)** and mid-weight **(3B)** targets as well as CST® antibodies and antibodies from alternative providers.

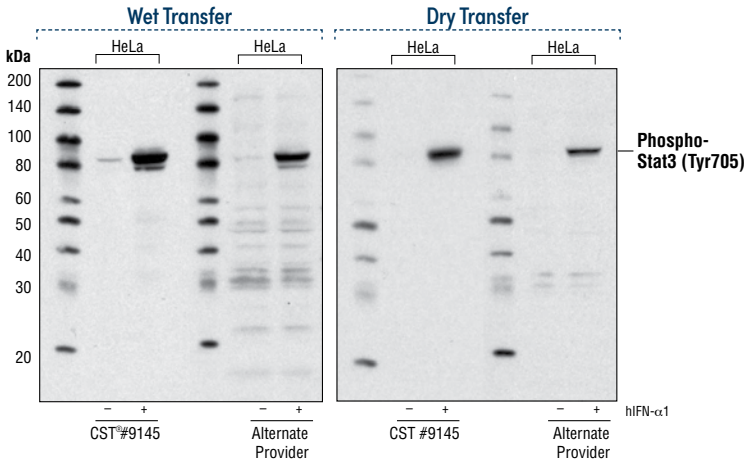
PLEASE NOTE: In a situation where sample amount is limiting or low endogenous levels of a protein need to be detected, the dry transfer method may limit the sensitivity of your assay.

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 weight proteins, particularly if a 0.45 µm pore-size membrane is used. We recommend using a shorter transfer with 0.2 µm pore-size membrane. Incomplete transfer of high molecular weight proteins may be improved by increasing transfer time. Some large molecular weight proteins (>200 kDa) transfer more efficiently under certain wet transfer conditions, and may benefit from decreasing the methanol concentration in the transfer buffer from 20% to 5%. We have also observed that some large proteins may better resolve and transfer when samples are run on 3-8% tris-acetate gels.



(3A) For low molecular weight targets in high-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.



(3B) 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-α1 (hIFN-α1) #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).

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 1 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 **(4A)**, 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. Milk is a stronger blocking agent than BSA and is recommended to reduce background signal when a strong specific signal can be maintained.

Incubation

Overnight Incubation Yields Improved Antibody Binding

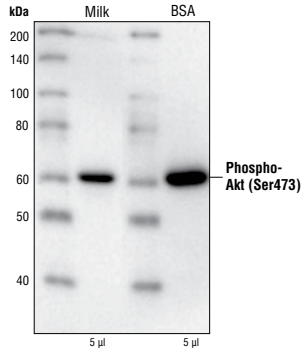
The primary antibody incubation period can vary greatly depending upon the protocol a researcher is using. We recommend incubating the primary antibodies overnight at 4°C. Shown here **(4B, 4C)** are two examples of antibody performance using overnight incubations at 4°C compared to 2 hour incubation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

Abbreviated Incubation Systems

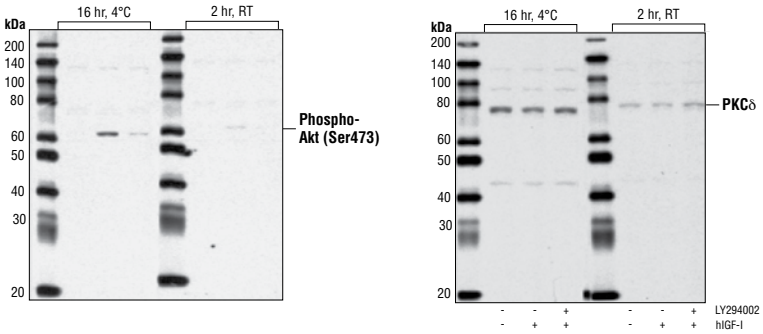
Less Incubation Time Yields Less Signal

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 **(4C)**.

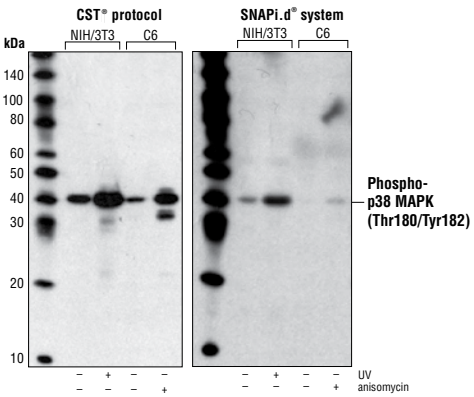
PLEASE NOTE: CST validates antibodies for Simple Western™ by ProteinSimple™. Simply dilute the antibody to the recommended dilution found on the data sheet and product page, then run the standard Simple Western protocol. View the complete list of Simple Western approved primary antibodies here: cst-science.com/simplewestern



(4A) 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



(4B) 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 (left) or PKCδ Antibody #2058 (right). Primary antibody incubation was performed overnight at 4°C or for 2 hr at room temperature, as indicated.

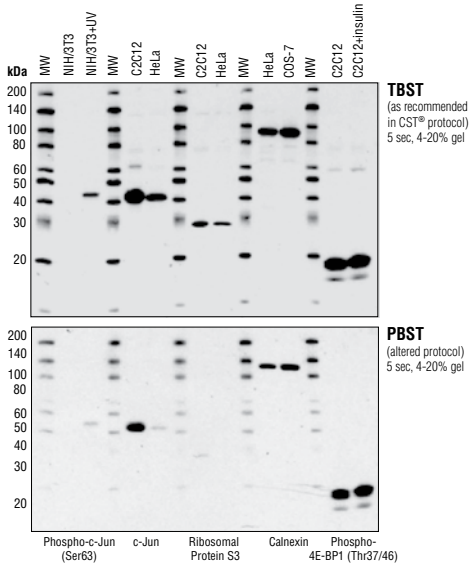


(4C) Abbreviated incubation systems allow less time for antibody binding, and therefore yield a weaker specific signal than the recommended incubation. Western blot analysis of extracts from NIH/3T3 and C6 cells, untreated, UV irradiated or treated with anisomycin as indicated, using Phospho-p38 MAPK (Thr180/Tyr182) (3D7) Rabbit mAb #9215. Blots were incubated using either the CST-recommended protocol (left) or SNAPi.d incubation system (right).

Washing and Dilution Buffer

TBST Buffers Yield Stronger Signal

Another area where western blot protocols can vary is with the washing and dilution buffers. We recommend using TBST for antibody dilution buffers and washing steps. Shown here **(5A)** is a comparison of antibody performance using TBST-based dilution and washing 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.

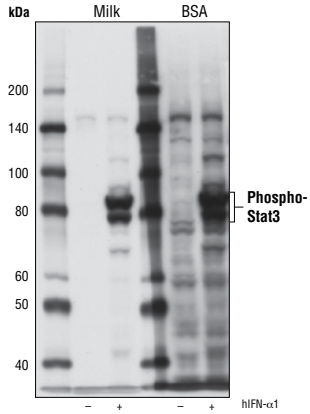


(5A) TBST buffer yields overall stronger signal than PBST buffer. Western blot analysis of extracts from various cell lines using Phospho-c-Jun (Ser63) II Antibody #9261, c-Jun (60A8) Rabbit mAb #9165, Ribosomal Protein S3 Antibody #2579, Calnexin (C5C9) Rabbit mAb #2679, and Phospho-4E-BP1 (Thr37/46) (236B4) Rabbit mAb #2855. Antibody dilution and wash steps were performed using either TBST (top) or PBST (bottom), as indicated.

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 **(5B)** we compare secondary antibody dilution in 5% nonfat milk with dilution in 5% BSA. Because the nonfat milk offers stronger blocking, the dilution in BSA yields significantly higher background than the dilution in milk. 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.



(5B) 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

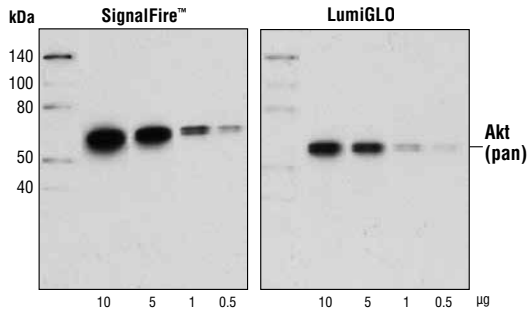
Some secondary antibodies bind nonspecifically to various proteins in cell extracts, leading to high background. To assess the quality of a secondary antibody, perform a blot (through to detection) without primary antibody.

Detection

Chemiluminescent

Offers Cost-Effective Visualization

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 **(6A)** are two chemiluminescent detection reagents that provide robust and specific signal.



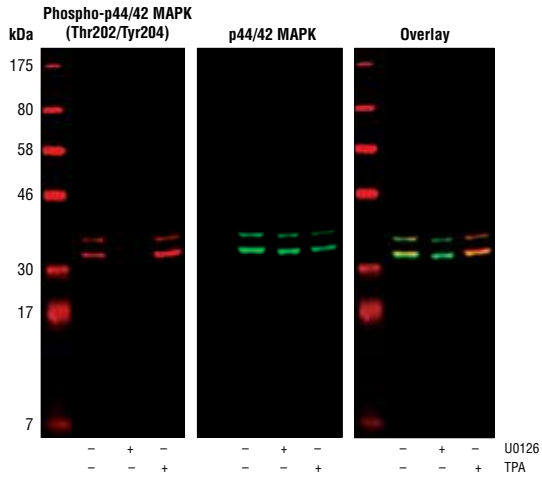
(6A) Detection Reagents
Western blot analysis of titrations of extracts from Jurkat cells using Akt (pan) (C67E7) Rabbit mAb #4691. Blots were detected using SignalFire™ ECL Reagent #6883 (left) or LumiGLO Reagent #7003 (right).

Fluorescent

Offers Instant Plexing

Western detection using fluorescent labeled secondary antibodies requires specialized instrumentation, such as a fluorescent imager. It also is popular as it provides faster visualization and quantification of results. Fluorescent detection allows phospho- and total protein levels to be determined on the same sample lanes, 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, even when they recognize the same target protein.

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 **(6B)**.



(6B) 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.

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.

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 at 4°C with gentle shaking, overnight.

NOTE: Please refer to primary antibody datasheet or product webpage for recommended primary antibody dilution buffer and recommended antibody dilution.

A. Solutions and Reagents

NOTE: Prepare solutions with RODI or equivalent grade water.

1. **20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH_2O, mix.
2. **10X Tris Buffered Saline (TBS):** (#12498) To prepare 1 L 1X TBS: add 100 ml 10X to 900 ml dH_2O, mix.
3. **1X SDS Sample Buffer:** Blue Loading Pack (#7722) or Red Loading Pack (#7723) Prepare fresh 3X reducing loading buffer by adding 1/10 volume 30X DTT to 1 volume of 3X SDS loading buffer. Dilute to 1X with dH_2O.
4. **10X Tris-Glycine SDS Running Buffer:** (#4050) To prepare 1 L 1X running buffer: add 100 ml 10X running buffer to 900 ml dH_2O, mix.
5. **10X Tris-Glycine Transfer Buffer:** (#12539) To prepare 1 L 1X Transfer Buffer: add 100 ml 10X Transfer Buffer to 200 ml methanol + 700 ml dH_2O, mix.
6. **10X Tris Buffered Saline with Tween® 20 detergent (TBST):** (#9997) To prepare 1 L 1X TBST: add 100 ml 10X TBST to 900 ml dH_2O, mix.
7. **Nonfat Dry Milk:** (#9999)
8. **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.
9. **Wash Buffer:** (#9997) 1X TBST
10. **Bovine Serum Albumin (BSA):** (#9998)
11. **Primary Antibody Dilution Buffer:** 1X TBST with 5% BSA or 5% nonfat dry milk as indicated on primary antibody product webpage; for 20 ml, add 1.0 g BSA or nonfat dry milk to 20 ml 1X TBST and mix well.
12. **Biotinylated Protein Ladder Detection Pack:** (#7727)
13. **Blue Prestained Protein Marker, Broad Range (11-250 kDa):** (#59329)
14. **Blotting Membrane and Paper:** This protocol has been optimized for nitrocellulose membranes. Pore size 0.2 µm is generally recommended.
15. **Secondary Antibody Conjugated to HRP:** anti-rabbit (#7074); anti-mouse (#7076)
16. **Detection Reagent:** LumiGLO chemiluminescent reagent and peroxide (#7003) or SignalFire™ ECL Reagent (#6883)

B. Protein Blotting

A general protocol for sample preparation is described below.

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.
4. Sonicate for 10–15 sec to complete cell lysis and shear DNA (to reduce sample viscosity).
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 (#59329, 5 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 10 µl/lane) to determine molecular weights are recommended.
8. Electrotransfer to nitrocellulose membrane (#12369).

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 three times for 5 min each 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

1. 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.
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 at 1:2000) and anti-biotin, HRP-linked Antibody (#7075 at 1:1000–1:3000) 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).

For HRP Conjugated 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 with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate with Anti-biotin, HRP-linked Antibody (#7075 at 1:1000–1:3000), 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).

For Biotinylated 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 with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate membrane with Streptavidin-HRP (#3999 at the appropriate dilution) 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).

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

1. 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.
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.

NOTE: Due to the kinetics of the detection reaction, signal is most intense immediately following incubation and declines over the following 2 hr.

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

Tris-Glycine SDS Running Buffer (10X) #4050

Tris-Glycine Transfer Buffer (10X) #12539

Tris Buffered Saline (10X) #12498

Tris Buffered Saline with Tween® 20 detergent (TBST-10X) #9997

Nonfat Dry Milk #9999

BSA #9998

Sample Preparation Buffers

Blue Loading Buffer Pack #7722

Red Loading Buffer Pack #7723

RIPA Buffer (10X) #9806*

Chaps Cell Extract Buffer (10X) #9852*

Cell Lysis Buffer (10X) #9803*

Inhibitors For Sample Preparation

PMSF #8553

Protease Inhibitor Cocktail (100X) #5871

Phosphatase Inhibitor Cocktail (100X) #5870

Protease/Phosphatase Inhibitor Cocktail (100X) #5872

Protein Markers/Standards

Biotinylated Protein Ladder Detection Pack #7727

Color-coded Prestained Protein Marker, Broad Range (10-250 kDa) #74124

Blue Prestained Protein Marker Detection Pack #86810

Filter Paper and Membrane

Nitrocellulose Sandwiches #12369

Secondary Antibodies

Anti-mouse IgG, HRP-linked Antibody #7076

Anti-rabbit IgG, HRP-linked Antibody #7074

Anti-rat IgG, HRP-linked Antibody #7077

Anti-biotin, HRP-linked Antibody #7075

Anti-mouse IgG, AP-linked Antibody #7056

Anti-rabbit IgG, AP-linked Antibody #7054

Anti-biotin, AP-linked Antibody #7055

Mouse Anti-rabbit IgG (Conformation Specific) (L27A9) mAb #3678

Mouse Anti-rabbit IgG (Conformation Specific) (L27A9) mAb (HRP Conjugate) #5127

Mouse Anti-rabbit IgG (Light-Chain Specific) (L57A3) mAb #3677

Anti-mouse IgG (H+L) (DyLight 680 Conjugate) #5470

Anti-mouse IgG (H+L) (DyLight 800 Conjugate) #5257

Anti-rabbit IgG (H+L) (DyLight 680 Conjugate) #5366

Anti-rabbit IgG (H+L) (DyLight 800 Conjugate) #5151

Streptavidin-HRP #3999

Detection Reagents

20X LumiGLO Reagent and 20X Peroxide #7003

SignalFire™ ECL Reagent #6883

SignalFire Plus ECL Reagent #12630

SignalFire Elite ECL Reagent #12757

*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 Mini-PROTEAN TGX Precast Protein Gel from BioRad. For select large molecular weight proteins we use the NuPAGE 3 to 8%, Tris-Acetate gels and associated buffer from ThermoFisher Scientific.

Apparatus

Mini-PROTEAN Tetra Cell (gel box - Cat #1658004EDU) from BioRad

Trans-Blot Turbo Transfer System (Cat #1704150) from BioRad

CST offers companion products to aid in your sample preparation and western blotting. **cst-science.com/WBcompanion**

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Doing good science is important. So is just doing good.

Cell Signaling Technology (CST) is a *different* kind of Life Sciences company—one founded, owned, and led by active research scientists, with the highest standards of product and service quality, technological innovation, and scientific rigor for over 20 years. We consistently provide fellow scientists around the globe with best-in-class products and services to fuel their quests for discovery.

Helping researchers find new solutions is our main mission every day—but it's not our only mission. We're also dedicated to helping identify solutions to other problems facing our world. We believe that all businesses must be responsible and work in partnership with local communities, while seeking to minimize their environmental impact. That's why we joined 1% for the Planet as its first Life Sciences member, and have committed to achieving net-zero emissions by 2029.

At CST, we believe in the power and promise of science to solve the challenges we face as a global community. We're a company of caring people driven by a devotion to facilitating good science—a company committed to doing the right thing for our Customers, our communities, and our planet.

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