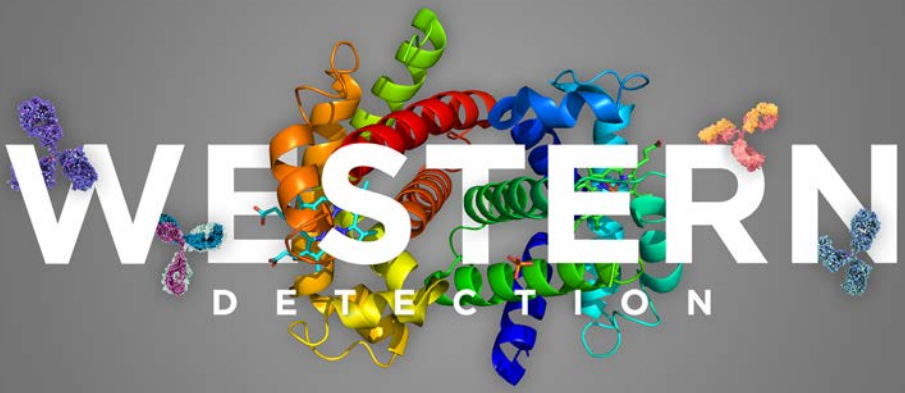
western blotting

thermo scientific invitrogen



A guide to better blots

Tips | Tricks | Troubleshooting

Find it at **fishersci.com**

fisher scientific
part of Thermo Fisher Scientific

Achieve successful blots

Western blotting is an extremely useful tool for protein biology. We offer a comprehensive suite of solutions for every step of the western blotting workflow to help you obtain high-quality, publishable results with minimal time and effort.

This handy guide will give you tips and tricks for making better blots. The reagents you use and the steps you take are important factors in creating high-quality data. Keep this guide at your lab bench to give you tips as you perform the steps of your western blots.

Consult the troubleshooting section that will help you visually identify specific problems on your blots, and suggest what may be causing them and some solutions to remedy them.

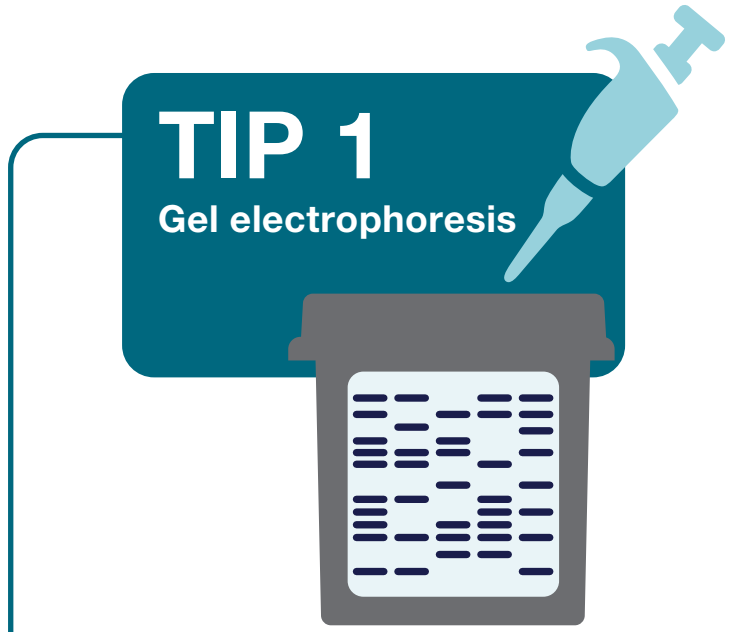
We understand the importance of great data and want you to be successful and proud of what you discover from your proteins, advancing science for us all. Count on Thermo Fisher Scientific to help you achieve the results you want and need.

Tips, tricks, and troubleshooting

Contents

Tips and tricks
for making better blots

General western blotting

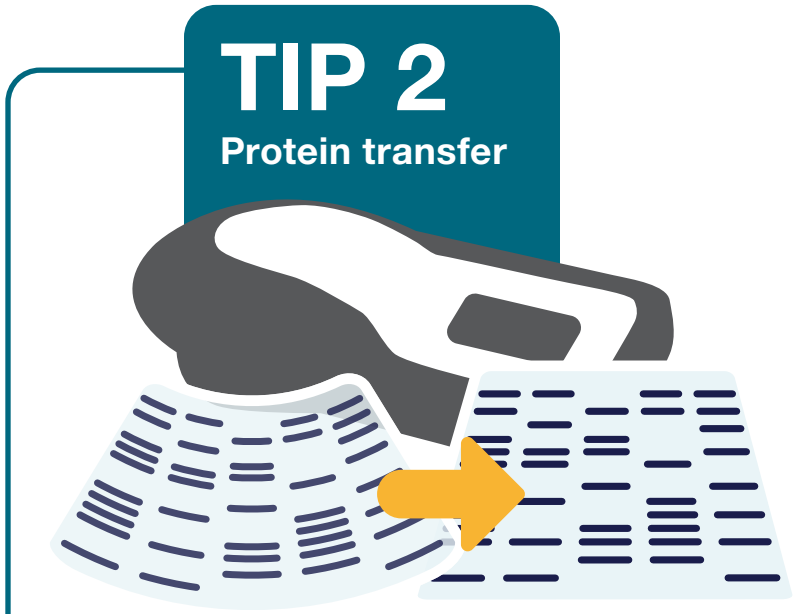


Loading sample in your gel an issue?

Use gel loading tips for a better experience. These tips are thinner, allowing you to insert the tip deeper into the well and not cause overflow.

Want an even better experience? Use Invitrogen™ precast gels with WedgeWell™ format. These gels allow you to load up to 60 µL of sample with a typical pipette tip—no need for special tips.

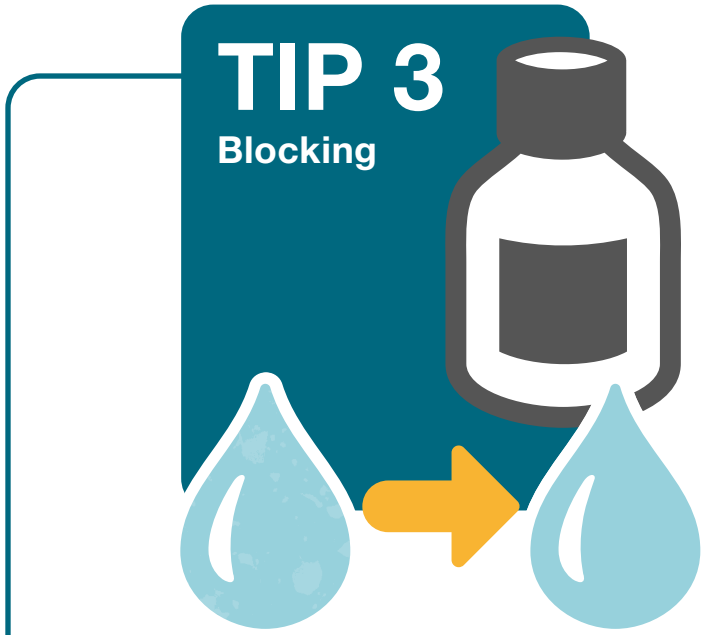
Learn more at **thermofisher.com/bolt**



High molecular weight protein transfers an issue?

Add 0.01–0.05% SDS to the transfer buffer to help pull proteins from the gel onto the membrane.

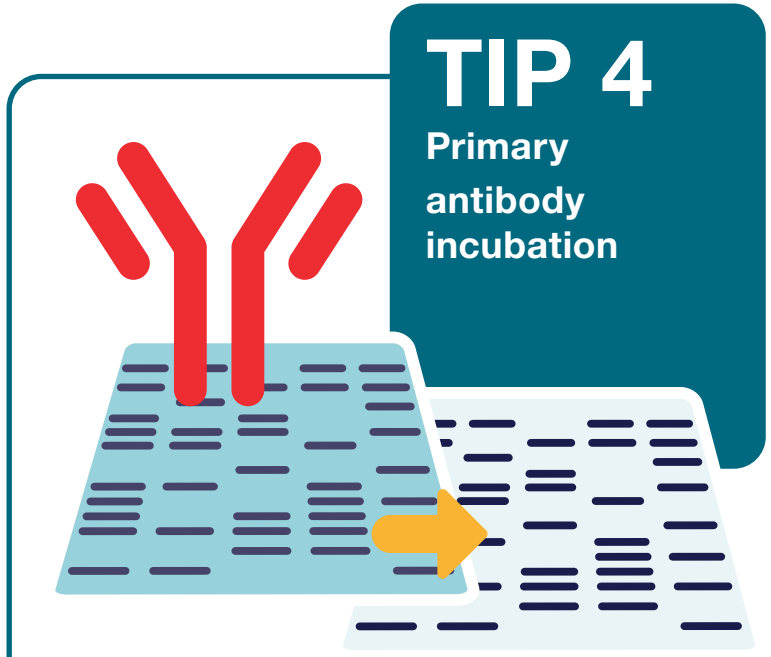
Want a better way? Use our Invitrogen™ NuPAGE™ Tris-Acetate Protein Gels (Cat. No. EA0375BOX), recommended for better resolution of proteins 150 kDa and above, and our Invitrogen™ iBlot™ 2 Gel Transfer Device (Cat. No. IB21001) for efficient 7-minute transfer of high molecular weight proteins.



Blotchy blot?

Try filtering your milk blocking buffer for better results. Dry milk powder often does not dissolve completely, and milk solids can interfere with your western detection.

Want to try something easier? Use our Thermo Scientific™ Pierce™ Clear Milk Blocking Buffer (Cat. No. PI37587), which does not contain milk solids that can cause interference.



Not getting a clean western signal?

If you are using an antiserum instead of a purified primary antibody, this might cause high background. Clean it up using a lysate that does not contain your target protein. Simply add 100 µL of target-free lysate to your antiserum, shake, and incubate overnight at 4°C.

An alternative approach—Use Thermo Scientific™ SuperSignal™ Western Blot Enhancer (Cat. No. PI46640). This not only improves your signal but also decreases nonspecific binding, yielding much better results.

Want to detect multiple targets by chemiluminescence?

Detect 2 or more targets on the same blot by incubating the blot with 2 or more primary antibodies. This will work provided the molecular weights of the targets are different and the gel conditions are optimized to separate the targets from each other.

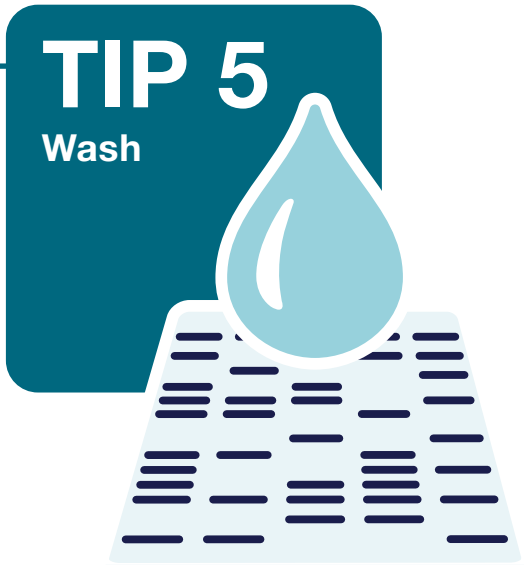
Want a better experience? Switch to fluorescence mode and use our Invitrogen™ Alexa Fluor™ Plus Secondary Antibodies for true multiplex detection.

Learn more at **thermofisher.com/alexafluorplus**

Reusing your primary antibodies over and over again?

Make sure you add an antimicrobial agent to prevent microbial growth. Sodium azide is not recommended for HRP detection. Use alternative antimicrobial agents such as KATHON™ or ProClin™ preservative at concentrations up to 0.1%. Use your primary antibodies no more than 3 times.

Want a better way? Use the Invitrogen™ iBind™ Western System (Cat. No. SLF1000), our automated western blot processing device, for primary antibody savings. For best results, use fresh primary antibody every time. You end up using the same amount of primary antibody.

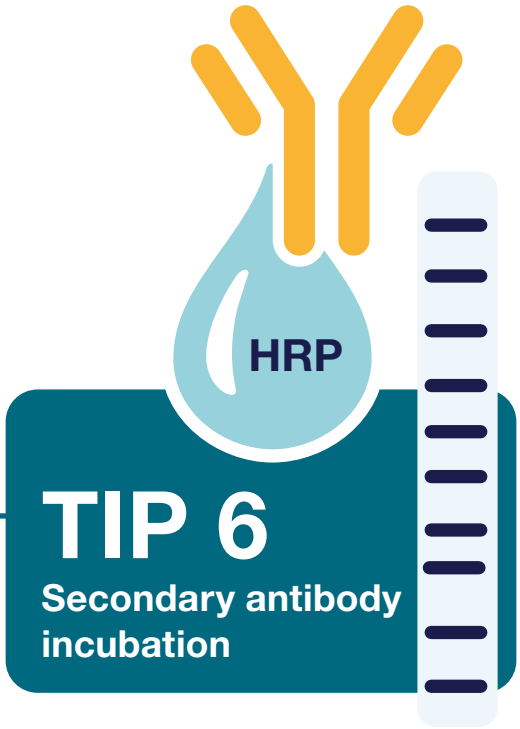


Nonspecific bands plaguing your western?

Try using up to 0.05% Tween™ 20, Triton™ X-100, or NP-40 detergent in your wash and blocking buffers.

Try an alternative—Use our Thermo Scientific™ blocking buffers that already have detergent in them.

Learn more at **thermofisher.com/blockingbuffers**



Want to detect PageRuler unstained protein ladders in your chemiluminescence blot?

Just add 1 µL of Strep-Tactin™ conjugate per 10 mL of your secondary antibody incubation solution. Your Thermo Scientific™ PageRuler™ unstained protein ladders (Cat. No. PI26614, PI26630, PI26632 and PI26637) can be detected on X-ray film or using imaging equipment.

Want a better way? Use our Invitrogen™ iBright™ Prestained Protein Ladder (Cat. No. LC5615) for both chemiluminescent and fluorescent detection.

TIP 7
Target detection

Detecting peptides on westerns?

Add up to 20% methanol to transfer buffer to boost peptide binding to the membrane. Use a 0.2 µm PVDF membrane to prevent blow through of the peptides.

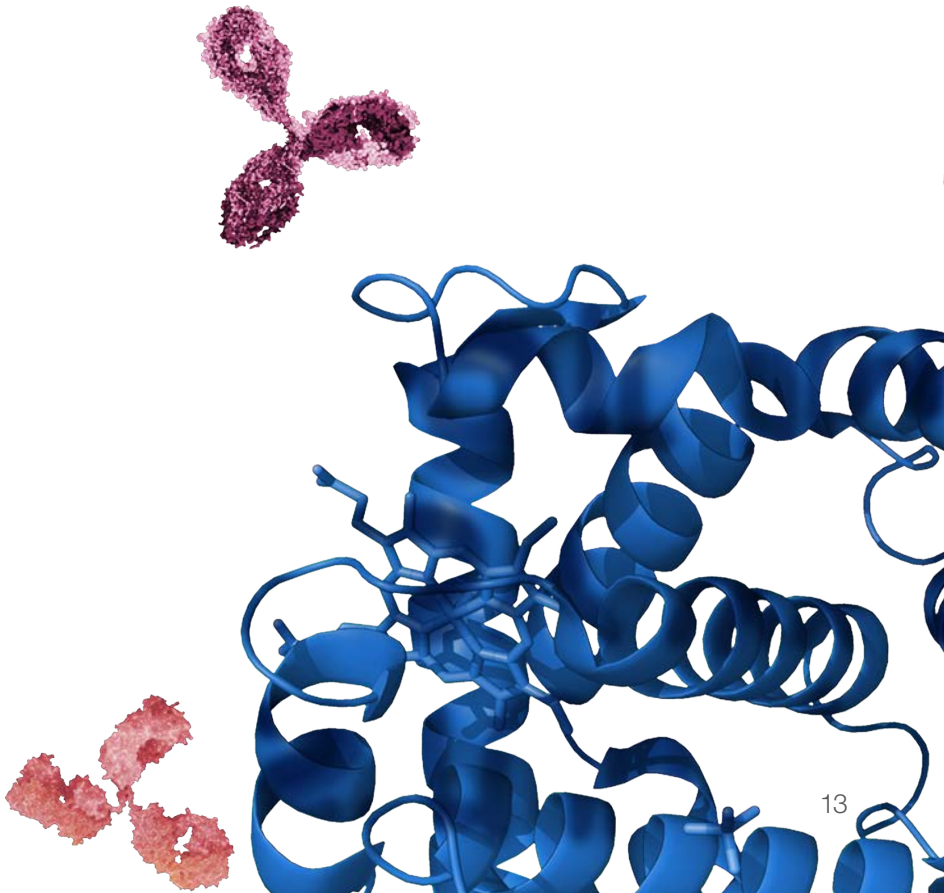
Want a better way? Use our Invitrogen™ Novex™ Tricine Gels for optimum resolution of peptides by gel electrophoresis and our iBlot 2 Gel Transfer Device (Cat. No. IB21001) for efficient transfer of peptides in just 7 minutes.

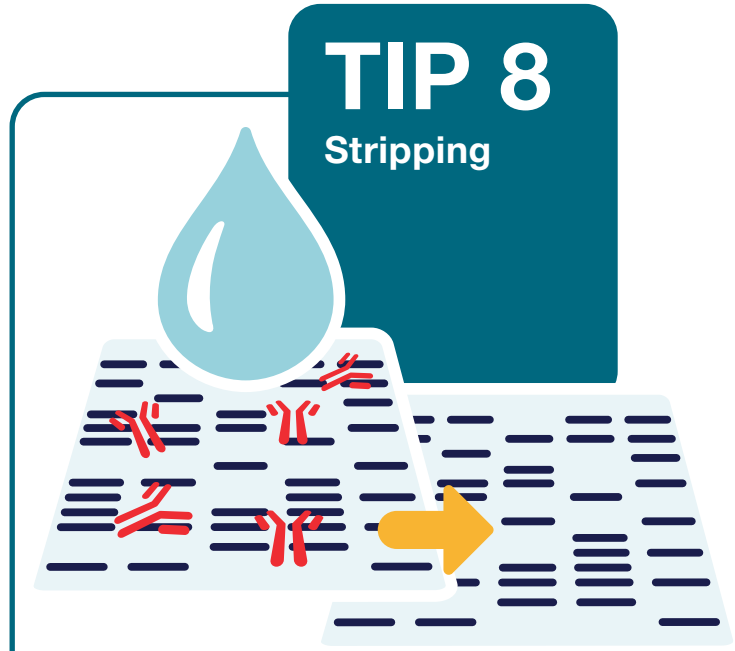
Not sure if your chemiluminescence substrate is working?

Do a quick darkroom test. Add 1 µL of HRP-conjugated secondary antibody to a working solution of your substrate in the dark. If it glows, your substrate is functional. If not, you need new substrate.

Want a better way? Use our Thermo Scientific™ SuperSignal™ West Substrates to obtain fast, reproducible results you can count on.

Learn more at **thermofisher.com/chemisubstrates**



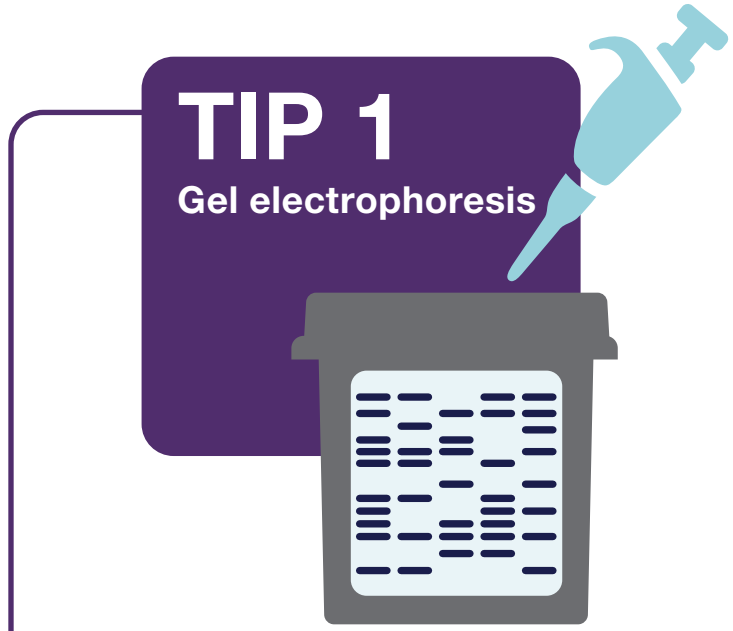


Stripping blots without getting desired results?

Always wet the blot with wash buffer for 5–10 minutes before adding stripping buffer. Use homemade stripping buffer and heat the blot at 37°C for up to 30 minutes.

Want a better way? Try our Thermo Scientific™ Restore™ PLUS Western Blot Stripping Buffer (Cat. No. PI46430) for efficient stripping in just 10 minutes at room temperature.

Fluorescent western blotting



Want to avoid high background?

Sample buffers containing bromophenol blue will fluoresce and can contribute to increased background. If using sample buffers with bromophenol blue, the dye front may be run off the gel prior to transfer or cut from the membrane after transfer to avoid background fluorescence signal.

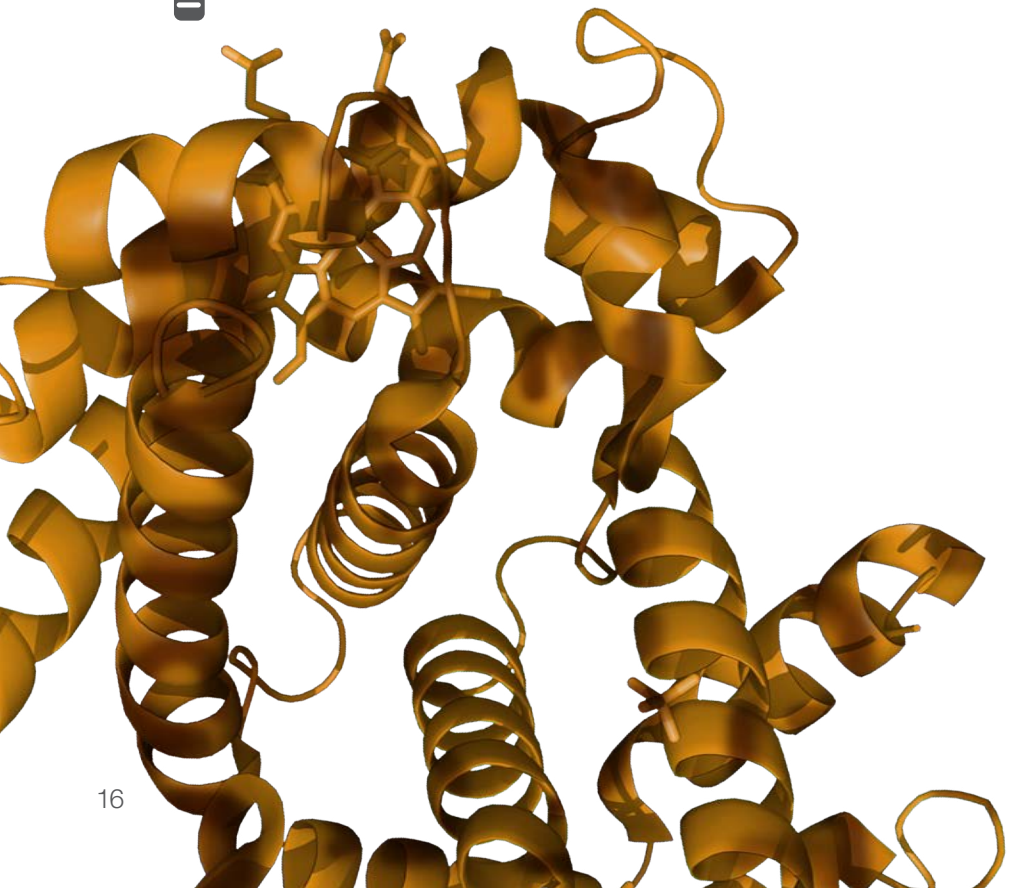
Want a better way? Consider using fluorescence-compatible sample buffers without bromophenol blue, such as Invitrogen™ Fluorescent Compatible Sample Buffer (Cat. No. LC2570).

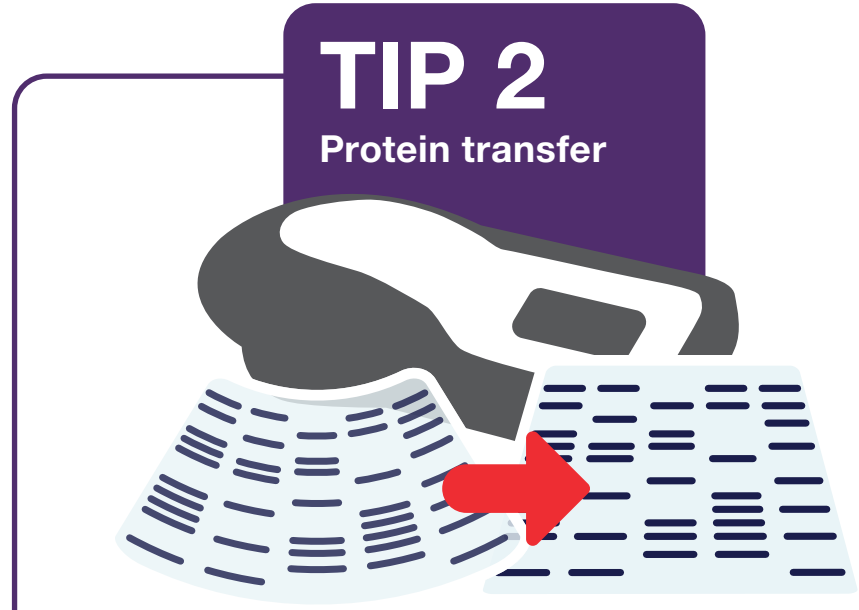
TIP 1 continued

Want to decrease background fluorescence and signal bleed-through to adjacent lanes?

Decrease the amount of molecular weight markers loaded onto the gel. Use single-color, prestained molecular weight markers, as multicolor markers can appear in distinct channels.

Want a better way? Use protein ladders specifically designed for western blotting, such as Invitrogen™ iBright™ Prestained Protein Ladder (Cat. No. LC5615), which provide prestained proteins as well as fluorescent bands for detection. Typically, 2–4 µL is sufficient.

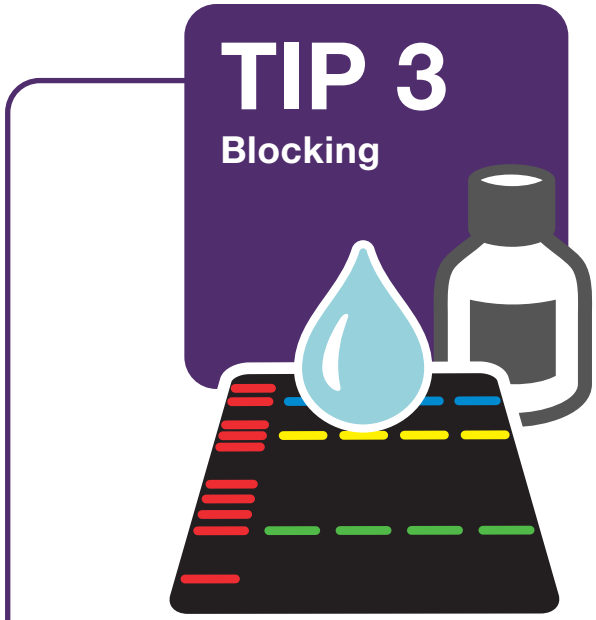




High background due to incorrect choice of membrane?

The nature of the membrane can affect the background. Choose membranes with low autofluorescence, including nitrocellulose and specialty low-fluorescence PVDF membranes. Also, avoid using pens on membranes, as many inks fluoresce; use a pencil instead.

Want a better way? Use Invitrogen™ Nitrocellulose/Filter Paper Sandwich (Cat. No. LC2009) and Thermo Scientific™ Low-Fluorescence PVDF Transfer Membrane (Cat. No. PI22860) to overcome this issue.



Is membrane contamination (speckles and fingerprints on the membrane) an issue?

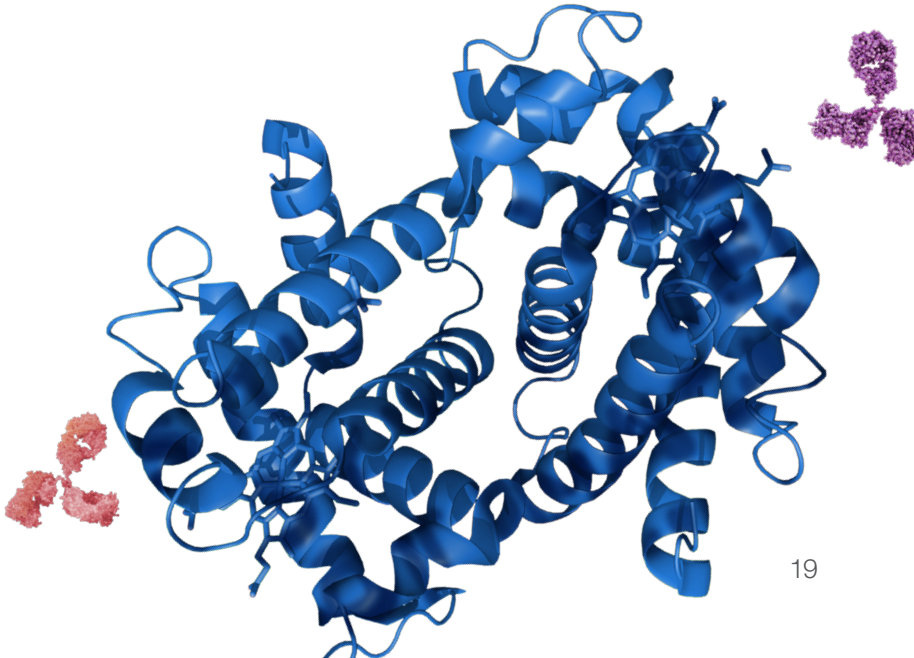
Handle membranes with gloved hands and clean blunt forceps. Also, use only high-quality filtered blocking buffer to help prevent particles and contaminants from settling on membranes.

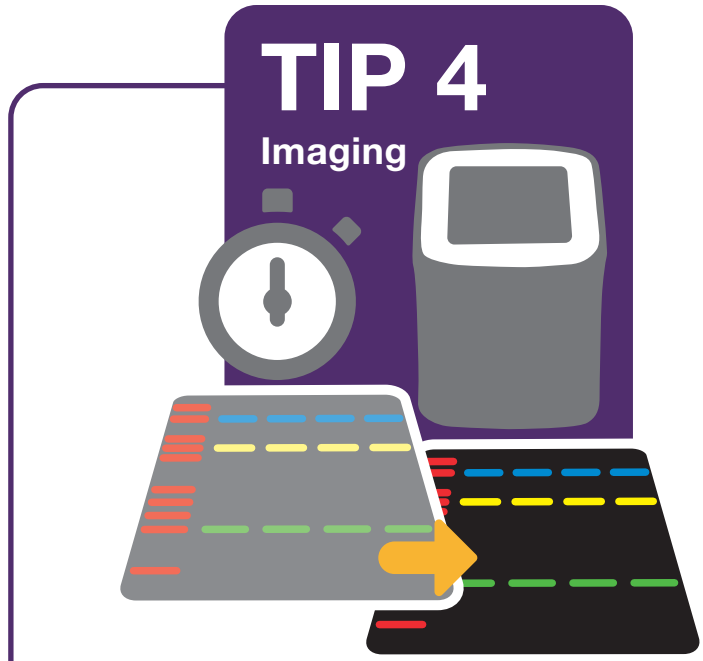
Want a better way? Consider using Thermo Scientific™ Blocker™ FL Fluorescent Blocking Buffer (Cat. No. PI37565), which is designed to reduce cross-reactivity and produce high signal-to-noise ratios. Do not add detergent to blocking buffer, as this may increase background fluorescence.

Fluorescence detection a pain with mammalian samples?

Use a nonmammalian blocker like salmon serum to maintain low backgrounds and high signal-to-noise ratios.

Want a better experience? Try our Thermo Scientific™ SEA BLOCK Blocking Buffer (Cat. No. PI37527) for fluorescence detection. Made from steelhead salmon serum, SEA BLOCK Blocking Buffer is especially useful as a blocking agent in fluorescence detection methods involving mammalian samples.

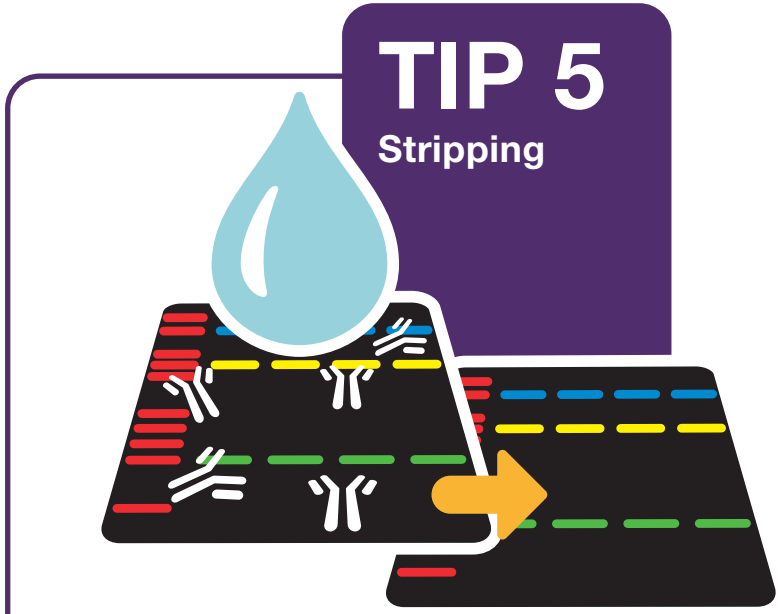




Don’t see signal or have weak signal in blot?

Increase exposure time to obtain a better signal.

Want a better way? Utilize the Smart Exposure feature to obtain an optimal image on the Invitrogen™ iBright™ FL Imaging System.



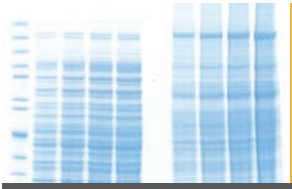
Stripping blots without getting desired results?

Traditional stripping buffers may only be effective for removing low-affinity antibodies, leaving behind dye-labeled secondary antibodies that can overwhelm the target signal when reprobing. Choose a stripping buffer that is specific for use in fluorescent western blotting.

Want a better way? Try our Thermo Scientific™ Restore™ Fluorescent Western Blot Stripping Buffer (Cat. No. PI62299), which is gentle and designed for quickly removing primary and near-infrared (IR) dye–labeled secondary antibodies from western blots. This stripping buffer is for use with low-fluorescence PVDF membranes only.

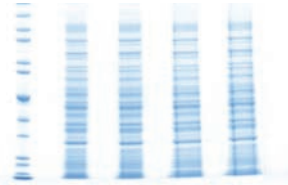
Troubleshooting

Problems, causes, and solutions



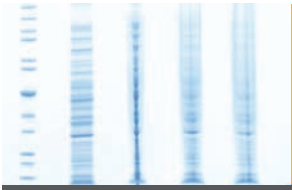
Protein bands lose resolution, lanes have streaks and are not straight

Possible cause	Solutions
Too much protein loaded per lane	Reduce the sample loads. The maximum recommended sample load for optimal resolution in mini gels with 10, 12, 15, or 17 wells is 0.5 µg per band or about 10–15 µg of cell lysate per lane.



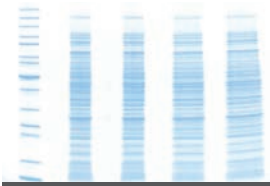
Viscous samples, streaks at sample lane edges, dumbbell-shaped bands, lane widening

Possible cause	Solutions
Excess salt (ammonium sulfate) in sample during gel electrophoresis	Perform dialysis to decrease salt concentration. Use a small dialysis device such as the Thermo Scientific™ Slide-A-Lyzer™ MINI Dialysis Device, 0.5 mL (Cat. No. PI88401).
	Concentrate and resuspend samples in lower-salt buffer prior to electrophoresis. Use small-volume concentrators such as Thermo Scientific™ Pierce™ Protein Concentrators PES, 0.5 mL (Cat. No. PI88513).
	Make sure that the salt concentration does not exceed 100 mM.



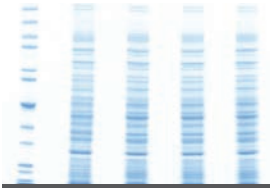
Protein aggregation resulting in narrow lanes that cannot be interpreted

Possible cause	Solutions
DNA contamination—genomic DNA in the cell lysate may cause the sample to become viscous, resulting in protein aggregation, which can affect protein migration patterns and resolution	Shear genomic DNA to reduce viscosity before loading the sample.



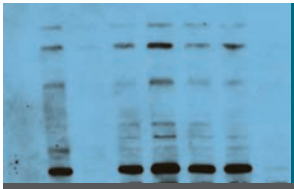
Uneven sample lanes, lane widening

Possible cause	Solutions
Excess salt (sodium chloride) in sample during gel electrophoresis. High salt concentrations result in increased conductivity, which affects protein migration and can result in protein bands spreading into adjacent lanes containing samples with normal salt concentrations.	Perform dialysis to decrease salt concentration. Use a small dialysis device such as the Slide-A-Lyzer MINI Dialysis Device, 0.5 mL (Cat. No. PI88401).
	Concentrate and resuspend samples in lower-salt buffer prior to electrophoresis. Use small-volume concentrators such as Pierce Protein Concentrators PES, 0.5 mL (Cat. No. PI88513).
	Make sure that the salt concentration does not exceed 100 mM.
High detergent concentration (e.g., SDS or Triton X-100 detergent) in gel electrophoresis. Detergents form mixed micelles with the anionic detergent SDS in the gel and migrate down into the gel; they interfere with the SDS–protein binding equilibrium.	Most of the nonionic detergents (e.g., Triton X-100, NP-40, and Tween 20 detergents) interfere with SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Keep the ratio of SDS to nonionic detergent at 10:1 or greater to minimize these effects.
	Use detergent removal columns or the Thermo Scientific™ Pierce™ SDS-PAGE Sample Prep Kit (Cat. No. PI89888) to remove excess detergent.
High concentration of RIPA (radioimmunoprecipitation assay) buffer results in widening of lanes and significant streaking during electrophoresis.	Dilute samples before electrophoresis to lower the final concentration of lysis buffer to prevent buffer-related defects.



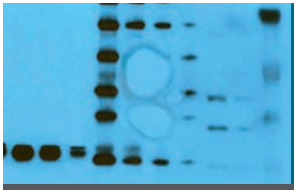
Shadow at lane edges

Possible cause	Solutions
Excess reducing agent in the lysis or sample buffer.	The final concentration of reducing agents for SDS-PAGE should be less than 50 mM for DTT (dithiothreitol) and TCEP (tris(2-carboxyethyl)phosphine), and less than 2.5% for β-ME (β-mercaptoethanol).



Nonspecific or diffuse bands

Possible cause	Solutions
Antibody concentration too high	Reduce concentrations of antibodies, particularly of primary antibody.
Too much protein loaded on gel	Reduce the amount of sample loaded on gel.
Signal from chemiluminescent substrate too strong	Reduce the length of time the blot is exposed to film.
	Reduce the concentration of the substrate.
	Shorten incubation time of membrane with substrate.
	Completely remove substrate after incubation period.
	Decrease the concentration of antibodies, particularly HRP- and AP-conjugated antibodies.



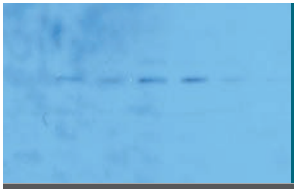
Partially developed areas or blank areas

Possible cause	Solutions
Incomplete transfer	Ensure sufficient contact between the gel and membrane during transfer by using a gel roller across the transfer stack.
	Wet and activate membrane according to manufacturer's instructions.
	Always wear clean gloves or use forceps when handling membrane.

High background

Possible cause	Solutions
Antibody concentration too high	Decrease concentration of primary and/or secondary antibody.
Incompatible blocking buffer	Do not use milk with avidin–biotin system. Milk contains biotin, which will result in high background.
	When probing for phosphoproteins, avoid phosphate-based buffers like PBS and phosphoprotein-containing blockers like milk or casein. Instead, block with BSA in Tris-buffered saline.
	Test for cross-reactivity in blocking buffer by blocking a clean piece of membrane, incubating with antibodies, and then detecting with the substrate of choice.
	When using an alkaline phosphatase (AP) conjugate, a blocking buffer in Tris-buffered saline (TBS) should be selected because phosphate-buffered saline (PBS) interferes with AP activity.
	Try a different blocking buffer. Use our blocking buffer selection guide at **thermofisher.com/blockingbuffers** to find the most compatible blocking buffer for your experiment.
Insufficient blocking of nonspecific sites	Increase the concentration of protein in the blocking buffer.
	Optimize blocking time and/or temperature. Block for at least 1 hour at room temperature (RT) or overnight at 4°C.
	Adding Tween 20 detergent to the blocking buffer can help minimize background. However, too much detergent can interfere with antibody binding. A final concentration of 0.05% often works well. For ease of use, choose a blocking buffer that already contains 0.05% Tween 20 detergent, such as Thermo Scientific™ StartingBlock™ T20 Blocking Buffer (Cat. No. PI37543 or PI37539) or SuperBlock™ T20 Blocking Buffer (Cat. No. PI37536 or PI37516).
	Prepare antibody dilutions in a blocking buffer that contains 0.05% Tween 20 detergent.
	Use Thermo Scientific™ SuperSignal™ Western Blot Enhancer (Cat. No. 46640) to reduce background and enhance detection of low-abundance and weakly immunoreactive antigens.

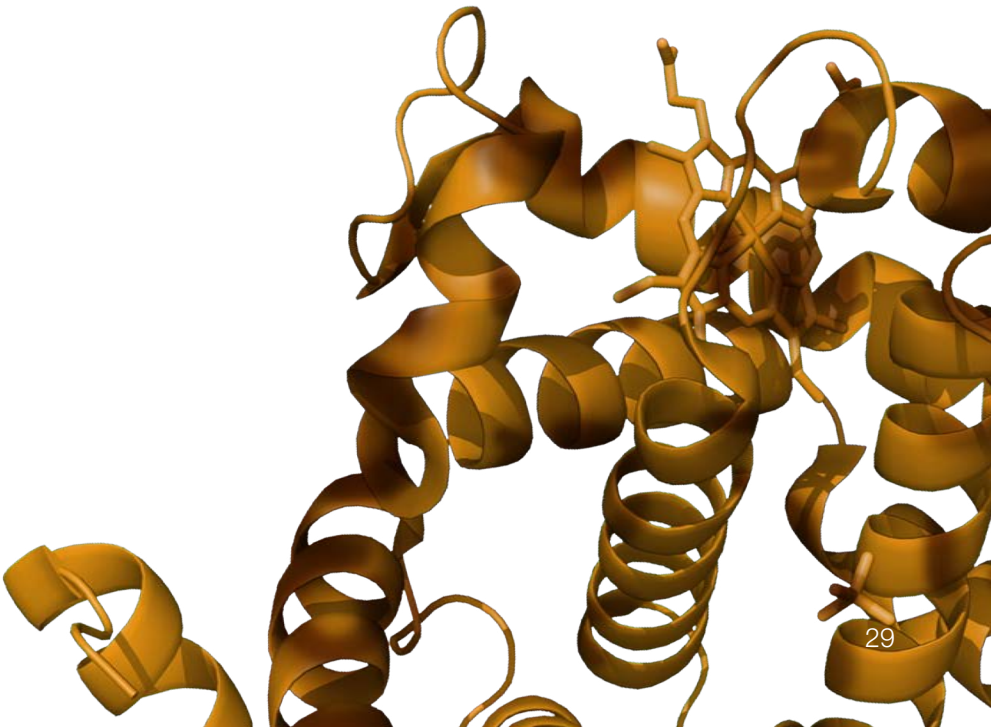
Possible cause	Solutions
Insufficient washing	Increase the number of washes and/or the volume of buffer used.
	Add Tween 20 detergent to the wash buffer to a final concentration of 0.05%. If the concentration of Tween 20 detergent is too high, it can strip proteins off the membrane.
Membrane handled improperly	Wet and activate membrane according to manufacturer's instructions.
	Always wear clean gloves or use forceps when handling membrane.
	Cover the membrane with liquid at all times to prevent drying.
	Use agitation during all incubations.
	Handle membrane carefully—damage to the membrane can cause nonspecific binding.
Contamination of equipment or materials	Prepare fresh buffers and filter them before use.
	Use only clean and contaminant-free electrophoresis equipment, blotting equipment, and incubation trays.
Signal from chemiluminescent substrate too strong	Reduce the length of time the blot is exposed to film.
	Reduce the concentration of the substrate.
	Shorten incubation time of membrane with substrate.
	Completely remove substrate after incubation period.
	Decrease the concentration of antibodies, particularly HRP- and AP-conjugated antibodies.

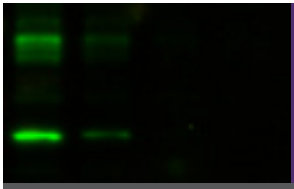


Weak signal or no signal

Possible cause	Solutions
Incomplete or inefficient transfer	After transfer, stain the gel with a total protein stain to determine transfer efficiency.
	After transfer, assess transfer efficiency by staining the membrane with the Thermo Scientific™ Pierce™ Reversible Protein Stain Kit (Cat. No. PI24585 or PI24580).
	Ensure sufficient contact between the gel and membrane during transfer by using a gel roller across the transfer stack.
	Ensure that the stack is placed in the transfer apparatus in the proper orientation such that proteins will migrate onto the membrane.
	Wet and activate the membrane according to the manufacturer's instructions.
	Use a positive control, such as prestained molecular weight markers, to assess transfer efficiency.
	Use molecular weight markers compatible with a western-imaging substrate, such as the Invitrogen™ iBright™ Prestained Protein Ladder (Cat. No. LC5615) or Invitrogen™ MagicMark™ XP Western Protein Standard (Cat. No. LC5602), as a positive control.
	Increase transfer time and/or voltage.
	Make sure sample preparation conditions have not destroyed the antigenicity of the sample. (Some proteins cannot be run under reducing conditions.)
Insufficient binding to membrane	For low molecular weight (MW) antigens, add 20% methanol to the transfer buffer to help binding and prevent proteins from passing through membrane.
	Reduce transfer time. Low MW antigens may pass through membrane.
	For high MW antigens, add 0.01–0.05% SDS to transfer buffer to pull proteins from the gel onto membrane.
	Change membrane type (NC vs. PVDF).
	Change to membrane with smaller pore size.
Antibody concentration too low	Increase antibody concentrations. Antibody may have poor affinity for the target protein.
	Antibody may have lost activity. Perform a dot blot to determine activity.
Insufficient antigen present	Load more protein onto the gel.

Possible cause	Solutions
Antigen masked by blocking buffer	Decrease concentration of protein in blocking buffer.
	Try a different blocking buffer. Use our blocking buffer selection guide at **thermofisher.com/blockingbuffers** to find the most compatible blocking buffer for your experiment.
Buffer contains sodium azide	Sodium azide inhibits HRP. Do not use it with HRP-conjugated antibodies.
Signal from chemiluminescent substrate too weak	Increase incubation time of membrane with substrate.
	Increase film exposure time.
	Ensure that the substrate is not expired.
	When you have minimal protein, use Thermo Scientific™ SuperSignal™ West Femto Maximum Sensitivity Substrate (Cat. No. PI34096) to maximize your western blot signals.
Membrane has been stripped and reprobed	Avoid repeated stripping of the same membrane.
	Shorten incubation time in stripping buffer to prevent loss of antigen.
Digestion of antigen on membrane	Blocking substance may have proteolytic activity (e.g., gelatin).
Protein degradation from prolonged blot storage	Prepare new blot.

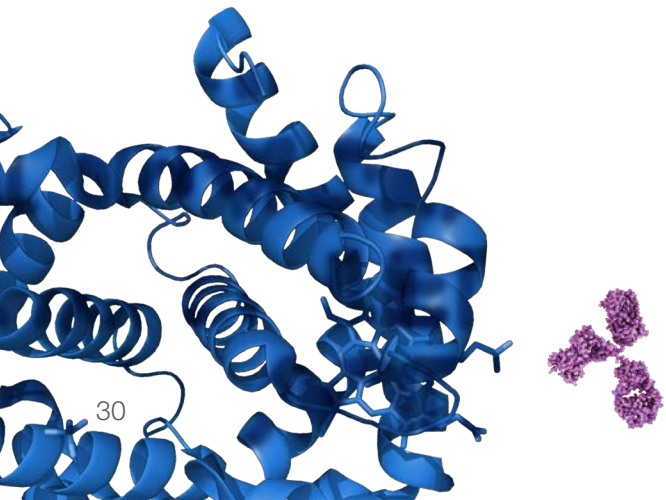


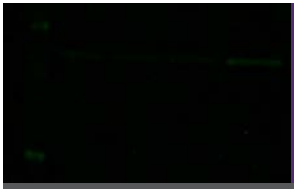


Nonspecific or diffuse bands

Possible cause	Solutions
Poor antibody specificity for the target of interest	Evaluate additional primary antibodies.
	Use only primary antibodies validated* for western blots.
Poor sample integrity	Sample degradation due to overheating or protease activity results in target breakdown and low target recognition by the antibody. For example, do not boil SDS-PAGE samples in SDS sample buffer, but rather heat them at 70°C for 10 minutes to avoid proteolysis.
Antibody cross-reactivity in multiplex detection	Choose primary antibodies raised in distantly related host species.
	Use highly cross-adsorbed secondary antibodies.
	Reduce the amount of the secondary antibody used, to remain within the optimal performance range.
Fluorescent bleed-through from another channel when multiplexing (appearance of an unexpected band)	Avoid spectrally close conjugates, especially when the signal is very strong.
	Ensure that your fluorescent dyes can be distinctly detected on your imager.
	Use the autoexposure feature on the instrument to determine the optimal exposure time for each channel.

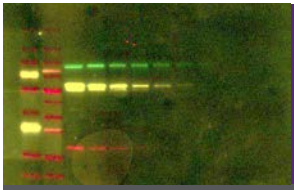
*The use or any variation of the word "validation" refers only to research use antibodies that were subject to functional testing to confirm that the antibody can be used with the research techniques indicated. It does not ensure that the product(s) was validated for clinical or diagnostic uses.





Weak or no signal

Possible cause	Solutions
Insufficient amount of primary antibody	Increase primary antibody concentration.
	Ensure primary antibody has a good titer and is specific for the antigen to be detected.
	For a low-abundance target in a cell or tissue lysate, increase the amount of primary antibody or the amount of sample loaded on the gel.
	Extend the incubation time to overnight at 4°C, or 3–6 hours at room temperature.
	Try using an antibody enhancer.
Lost activity of antibody	Ensure the antibody was stored appropriately.
	Check the expiration date of the antibody.
	Avoid multiple uses of prediluted antibodies.
Imaging exposure time is too short	Increase exposure time.
	Utilize the Smart Exposure feature to obtain an optimal image on the iBright FL1000 sytem.
Incorrect instrument settings	Ensure the correct excitation and emission ranges are selected for the intended fluorophore.
Use of detergent	Too much detergent or the nature of the detergent can result in washing away the signal—decrease or eliminate detergent.
Blocking buffer blocks antigen	Some blocking soutions can mask the blot and reduce the availability of the antigen to the antibody, especially if the blocking step is >1 hour.
	Dilute the primary antibody in wash buffer.
	Evaluate another blocking buffer.
Quantity of sample loaded on the gel	Too much lysate can overcrowd your specific target and reduce the antibody sensitivity.
	Too little lysate leads to insufficient availability of the target of interest.
	Perform serial dilutions of the lysate or sample to determine the optimal amount of protein to load.
Poor transfer of protein, or loss of the protein after transfer	Check transfer conditions to confirm protein transfer.
	Reoptimization may be required when probing for a new protein.



Background issues (high, uneven, or speckled)

Possible cause	Solutions
High background due to membrane contamination	Handle the membrane using clean forceps and clean incubation trays or dishes.
	Determine the best blocking buffer for your application—primary antibodies will react differently in different blocking buffers. Blocking buffers like normal animal sera or milk may result in cross-reactivity.
Artifacts from overloading the protein marker or ladder	Load less of the molecular weight marker onto the gel.
Nonoptimal wash or diluent solutions	Use a wash buffer with 0.1–0.2% Tween 20 detergent.
	Prepare the secondary antibody dilution with 0.05% Tween 20 detergent.
	Increase the number or duration of wash steps.
High background from an excess of secondary antibody	Optimize the secondary antibody dilution depending on the dye being used, following the vendor-recommended dilution and adapting accordingly.
Blotchy or uneven background due to the membrane drying out	Ensure good coverage of the whole blot during all incubation steps.
	Ensure consistent agitation during every incubation step.
Incorrect choice of membrane	The nature of the membrane can affect the background; for example, PVDF membranes can autofluoresce and cause high background, so use low-fluorescence PVDF membranes.
Speckles and fingerprints on the membrane	Use clean forceps to handle the membrane, and avoid directly touching the membrane; particulates and contaminants from unclean tools may fluoresce.
	Use clean incubation trays or dishes—rinsing with methanol followed by water will help dissolve residual dried dyes from previous uses.
	Clean transfer devices and dusty consumables if using a wet transfer method, as they can introduce speckles.
	Clean the imager surface with ethanol to remove dust, lint, and residue before capturing the image.

Notes

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