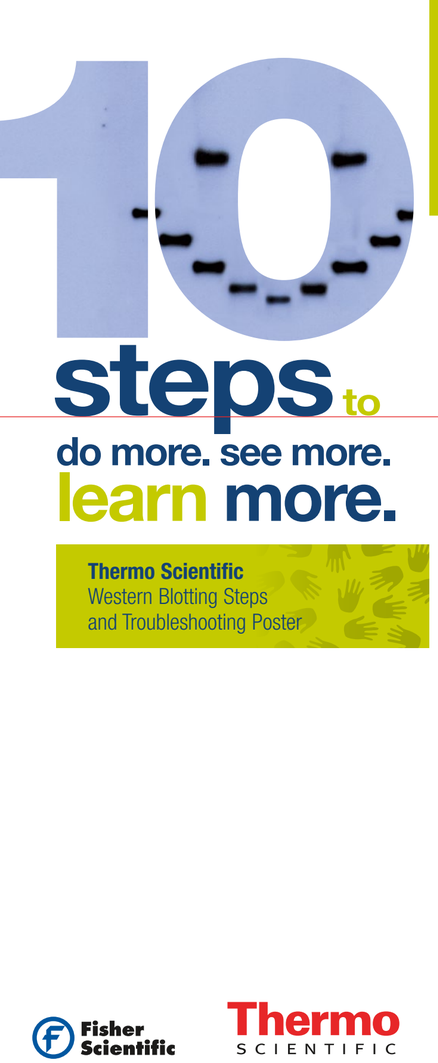
10 steps to do more. see more. learn more.

Thermo Scientific
Western Blotting Steps
and Troubleshooting Poster



step 1

Gel Electrophoresis

The first step to a successful Western blot is to separate the proteins in your sample using gel electrophoresis.

We carry a full line of pre-cast gels that make sample loading easier, run more quickly and give you excellent resolution of your proteins. Their one-year shelf life and compatibility with a range of gel tanks make them the clear choice for your lab.

- **Gels** – Thermo Scientific™ Precise™ Protein Gels (# PI25200 and others) are cast in a durable plastic cassette with a neutral-pH buffer that prevents polyacrylamide breakdown and results in a long shelf life.
- **Gel Electrophoresis Buffers** – we offer a variety of ready-to-use, pre-formulated buffers for protein methods, such as Thermo Scientific™ BupH™ Tris-Hepes-SDS Running Buffer (# PI28398), Lane Marker Reducing Sample Buffer (5X) (# PI39000), Lane Marker Non-Reducing Sample Buffer (5X) (# PI39001) and LDS Sample Buffer (# PI84788).
- **Molecular Weight Protein Ladders** – Thermo Scientific™ SuperSignal™ Molecular Weight Ladders (#s PI84785, PI84786) provide reliable and proportional band intensities in stained gels and immunoblots developed with chemiluminescent, fluorescent, chromogenic or other detection systems. See our full line of prestained and unstained protein ladders at thermoscientific.com/protein-ladder.

step 2

Protein Transfer

After electrophoresis, transfer protein from the gel to a membrane using electrophoretic transfer.

This step is critical to ensure that the protein adheres to the membrane. The unique Thermo Scientific™ Pierce™ G2 Fast Blotter allows for the fast and efficient transfer of proteins ranging from 10-300kDa in as few as 10 minutes. Consider using our signal enhancers to increase the sensitivity of your Western blot after this step.

- **Blotter** – the Thermo Scientific™ Pierce™ G2 Fast Blotter (# PI62288) can transfer proteins in as few as 5 to 10 minutes when used with our Thermo Scientific™ Pierce™ 1-Step Transfer Buffer.
- **Transfer Buffers and Accessories** – we offer a variety of transfer buffers and accessories to help your blot look its best. Our transfer buffers include Methanol-free Transfer Buffer (# PI35040), Tris-Glycine Transfer Buffer (# PI28380) and our 1-Step Transfer Buffer (# PI84731). The Thermo Scientific™ Pierce™ Reversible Protein Stain Kit (#s PI24585, PI25480) offers a non-destructive, reversible, reliable and sensitive method to stain and detect proteins on nitrocellulose and PVDF membranes. The Thermo Scientific™ SuperSignal™ Western Blot Enhancer (# PI46640) helps increase signal-to-noise ratio by reducing any background noise caused by 'dirty' primary antibodies for better sensitivity.
- **Transfer Membranes and Filter Paper** – our transfer membranes are available in a variety of types, including nitrocellulose (# PI88018), PVDF (# PI88518) and low-fluorescence PVDF (# PI22860). We also offer Western blotting filter paper in regular (#s PI88600, PI84783 and PI84784) and extra thick (# PI88605, PI88610, PI88615 and PI88620) sheets.

step 3

Blocking

Next, block the unreacted sites on the membrane to reduce the amount of nonspecific binding.

We have a complete selection of blocking buffers to improve the sensitivity of your Western blot. The proper choice of buffer depends on the antigen and type of enzyme conjugate to be used. With the wide range we offer, choose the one that delivers the highest signal-to-noise ratio possible for your blots.

We have a wide selection of Thermo Scientific blocking buffers available to meet the need of just about any Western blot protocol. Visit thermoscientific.com/blockingbuffers for our blocking buffer selection guide.

- Blocker BLOTTO Blocking Buffer
- Pierce Clear Milk Blocking Buffer
- Pierce Fast Blocking Buffer
- SEA BLOCK Blocking Buffer
- StartingBlock Blocking Buffers
- SuperBlock Blocking Buffers
- Blocker BSA Blocking Buffers
- Blocker Casein Blocking Buffers
- Protein-free Blocking Buffer
- Normal Serum

step 4

Primary Incubation

Incubate the membrane with primary antibody.

Our antibodies are fully validated, eliminating the need to screen numerous antibodies to find the correct one.

Visit thermoscientific.com/pierce-abs to find your antibody.

Thermo Scientific™ Pierce™ Antibodies are developed for a wide variety of application needs. We offer over 40,000 antibodies for over 50 research areas and all of our antibodies are validated and guaranteed to perform in the stated application and species. We also offer antibody conjugates available with Thermo Scientific™ DyLight™ Dyes, biotin, horseradish peroxidase (HRP), alkaline phosphatase (AP) and more. Our website enables you to easily search by protein target and then filter by the specific assays that interest you.

Our custom antibody service leverages our experience and proprietary antigen design tools to produce more robust antibodies.

thermoscientific.com/pierce-abs

step 5

Wash

Remove unbound primary reagents and reduce background.

Our dry blend buffers and high-purity detergents all serve to enhance your signal-to-noise ratio.

Powerful, easy-to-use Thermo Scientific™ solutions help achieve the cleanest results possible.

Buffered Saline Solutions	Thermo Scientific™ Surfact-Amps™ Detergents
• BupH Phosphate Buffered Saline Packs (# PI28372)	• Tween™-20 Detergent (# PI28320)
• Pierce 20X Phosphate Buffered Saline (# PI28348)	• Tween™-80 Detergent (# PI28328)
• BupH Tris Buffered Saline (#s PI28376, PI28358)	• Triton™ X-100 Detergent (# PI28314) NP-40 (# PI28324)
• Modified Dubecco's PBS Buffer (#s PI28344, PI28374)	

Skip this step if you use Thermo Scientific™ StartingBlock™ T20 Blocking Buffer in PBS or TBS or Thermo Scientific™ SuperBlock™ T20 Blocking Buffer in PBS or TBS. These buffers already contain Tween-20 Detergent at optimized concentrations.

Fisher Scientific

Thermo SCIENTIFIC

For technical information, video tutorials and selection guides visit **thermoscientific.com/Western**

step 6

Secondary Incubation

Incubate the membrane with secondary antibody.

Choose an appropriate secondary detection probe for your Western blot. Our secondary antibodies and detection reagents are available in a variety of formats and conjugated types including HRP, AP, DyLight Dyes and others. Check out our secondary antibody selection guide to find the secondary antibody or detection reagent that is right for you.

We also offer a complete line of biotin-binding proteins and conjugates (Avidin, Streptavidin, etc.), antibody-binding proteins (Protein A, Protein G, etc.) and specialized detection probes and kits. Visit thermoscientific.com/Western for a complete list.

thermoscientific.com/pierce-abs

step 7

Wash

Remove unbound secondary reagents and reduce background.

Our dry buffers and high-purity detergents all serve to enhance your signal-to-noise ratio.

Powerful, easy-to-use solutions help achieve the cleanest results possible.

Buffered Saline Solutions	Surfact-Amps Detergents
• BupH Phosphate Buffered Saline Packs (# PI28372)	• Tween-20 Detergent (# PI28320)
• 20X Phosphate Buffered Saline (# PI28348)	• Tween-80 Detergent (# PI28328)
• Tris Buffered Saline (#s PI28376, PI28358)	• Triton X-100 Detergent (# PI28314) NP-40 (# PI28324)

step 8

Incubation with Substrate

Add the detection reagent to your secondary HRP or AP conjugate.

Choose the appropriate substrate for your needs from the Thermo Scientific™ Pierce™ ECL and Thermo Scientific™ SuperSignal™ families of chemiluminescent HRP substrates. All of our substrates offer excellent performance in Western blotting with long light emission and strong signal intensity.

We offer five types of chemiluminescent substrates for Western blot detection with HRP:

- **Pierce ECL Substrate (# PI32106)** An entry-level substrate with low-picogram level sensitivity. Select when the sample target is abundant.
- **Pierce ECL Plus Substrate (# PI32132)** Detect down to 0.5pg of your target. Select when target is less abundant and sample is limited.
- **SuperSignal West Pico Chemiluminescent Substrate (# PI34078)** Our most popular substrate can be easily optimized to detect targets with greater sensitivity than ECL substrates.
- **SuperSignal West Dura Extended Duration Substrate (# PI34076)** Offers high sensitivity and 24-hour signal output that is ideal for CCD-camera and other digital imaging systems.
- **SuperSignal West Femto Maximum Sensitivity Substrate (# PI34096)** Our most sensitive substrate! Select when sample is limited and/or target is less abundant.

Visit www.thermoscientific.com/Western to see our complete line of chemiluminescent and colorimetric substrates.

step 9

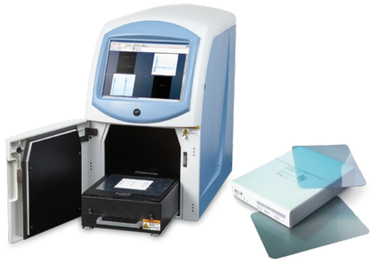
Target Detection

Capture and analyze your image.

Select your mode of target detection. Choose between traditional detection using X-ray film or the more quantitative cooled-CCD camera imaging technology. We offer both!

Thermo Scientific™ CL-XPosure™ Film (#s PI34089, PI34090, PI34091) is economically priced, clear-blue film for detection of chemiluminescent Western blots. Or, take your imaging to the next level with the **Thermo Scientific™ myECL™ Imager** for one-touch image capture of Western blots. With the **Thermo Scientific™ myImageAnalysis™ Software (# PI62237)**, the myECL Imager is a powerful tool to analyze and quantify your target bands. The **myECL Imager (# PI62236)** is a powerful and easy-to-use blot and gel documentation instrument for sensitive, multimode image capture and analysis via an intuitive touchscreen interface and advanced integrated software. The myECL Imager works with chemiluminescent, colorimetric or UV light-activated fluorescent substrates or stains.

If you are detecting with X-ray film, use **Thermo Scientific™ Pierce™ Background Eliminator Kit (# PI32065)** for fast, easy removal of artifacts to correct for overexposure of blots.



step 10

Stripping (if necessary)

Reprobe the blot if needed.

Using our Thermo Scientific™ Restore™ Products you can quickly strip and reprobe, as well as reuse the blot again and again. Strip time off your research processes!

Reprobing a Western blot saves time and conserves sample while allowing optimization to be performed as needed. Reprobing also allows the same blot to be probed for different target proteins. We offer specially formulated buffers that are developed to efficiently strip primary and secondary antibodies from Western blots so that membranes can be reprobed under alternate conditions or with another antibody to detect a different protein target.

Thermo Scientific Stripping Buffers

- Restore Western Blot Stripping Buffer (# PI21059)
- Restore PLUS Western Blot Stripping Buffer (# PI46430)
- Restore Fluorescent Western Blot Stripping Buffer (# PI62300)

Troubleshooting

Tips to better **Western blots** and better data thermoscientific.com/Western

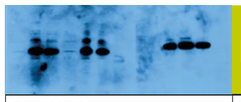
do more
see more
learn more

Thermo Scientific
Tips for trouble-free
Western blots

Hang this poster in your lab to help you avoid or overcome problems in your Western blotting application.

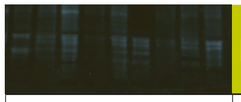
High Background that is Uniformly Distributed

Possible Causes	Precautions/Solutions
Antibody concentrations are too high	• High concentrations of primary and/or secondary antibody can cause high background. • Decrease antibody concentrations.
Incompatible blocking buffer was used	• Optimize blocking buffer. The best blocking buffer is system-dependent. • Increase the concentration of protein in the blocking buffer. • Optimize blocking time and/or temperature. Block for at least 1 hour at RT or overnight at 4°C. • Add 0.05% Tween-20 Detergent to the blocking buffer at a final concentration of 0.05%. This is not applicable to Thermo Scientific™ StartingBlock™ T20 Blocking Buffer in PBS or TBS or Thermo Scientific™ SuperBlock™ T20 Blocking Buffer in PBS or TBS. • Prepare antibody dilutions in blocking buffer that contains 0.05% Tween-20 Detergent.
Cross-reactivity of antibody with other proteins in blocking buffer	• Use a different blocking buffer. Thermo Scientific Pierce Protein-free Blocking Buffers are PBS and TBS formulations of a non-protein compound for effective membrane and plate blocking with extremely low background. • Do not use milk with avidin-biotin systems. Milk contains biotin. • Test for cross-reactivity. Block a clean piece of membrane, incubate with antibodies and then detect with Thermo Scientific™ SuperSignal™ Chemiluminescent Substrate. • Reduce the concentration of the HRP conjugate.
Insufficient washing	• Increase number of washes and the volume of buffer used. • Add Tween-20 Detergent to wash buffer at a final concentration of 0.05%. (Note: If the concentration of Tween-20 is too high, it can strip proteins off the membrane.) Skip this step if you use StartingBlock T20 Blocking Buffer in PBS or TBS or SuperBlock T20 Blocking Buffer in PBS or TBS.
Exposure time is too long	• Reduce the time the blot is exposed to film.
Membrane problems	• Wet membranes thoroughly according to the manufacturer's instructions. • Use new membranes. • Cover the membrane with liquid at all times to prevent it from drying. • Use agitation during all incubations. • Handle membranes carefully – damage to the membrane can cause nonspecific binding. • Do not handle membrane with bare hands. Always wear clean gloves or use forceps.
Contamination or growth in buffers	• Prepare new buffers.



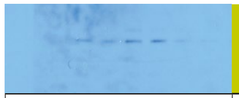
High Background that is Blotchy or Speckled

Possible Causes	Precautions/Solutions
Antibody concentrations are too high	• High concentrations of primary and/or secondary antibody can cause high background. • Decrease antibody concentrations.
Aggregate formation in the HRP conjugate can cause speckling	• Filter the conjugate through a 0.2µm filter. • Use a new, high-quality conjugate.
Incompatible blocking buffer was used	• Compare different blocking buffers.
Insufficient blocking of nonspecific sites	• Optimize blocking buffer. The best blocking buffer is system-dependent. • Increase concentration of protein in the blocking buffer. • Optimize blocking time and/or temperature. Block for at least 1 hour at RT or overnight at 4°C. • Add Tween-20 Detergent to the blocking buffer to a final concentration of 0.05%. Skip this step if you use StartingBlock T20 Blocking Buffer in PBS or TBS or SuperBlock T20 Blocking Buffer in PBS or TBS. • Make up antibody dilutions in blocking buffer with 0.05% Tween-20 Detergent.
Cross-reactivity of antibody with other proteins in blocking buffer	• Use a different blocking buffer. • Do not use milk with avidin-biotin systems. Milk contains biotin. • Test for cross-reactivity. Block a clean piece of membrane, incubate with antibodies and then detect with SuperSignal Chemiluminescent Substrate. • Reduce the concentration of the HRP conjugate.
Membrane was not wetted properly	• Wet membrane according to the manufacturer's instructions. • Do not handle membrane with bare hands. Always wear clean gloves or use forceps. • Use a new membrane. • Cover the membrane with liquid at all times to prevent it from drying. • Use agitation during all incubations. • Incubate membranes separately to ensure that membrane strips are not covering one another during incubations. • Handle membranes carefully – damage to the membrane can cause nonspecific binding.
Contamination in buffers	• Use new buffers. • Filter buffers before use.
Contaminated equipment	• Use only clean and contaminant-free electrophoresis equipment, blotting equipment and incubation trays. • No pieces of gel should be left on the membrane after transfer because proteins can stick to them, causing background.



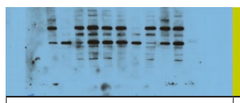
Black Blots with White Bands or Signal That Decreases Quickly

Possible Causes	Precautions/Solutions
Antibody concentrations are too high	• Reduce antibody concentrations, especially the HRP conjugate. Signal that decreases quickly and the appearance of white bands are indications that there is too much HRP in the system.



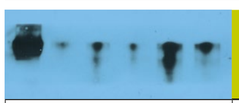
Weak Signal or No Signal

Possible Causes	Precautions/Solutions
Proteins did not transfer properly to the membrane	• After transfer, stain the gel with a total protein stain to determine transfer efficiency. *(Note: Total protein stains may not be able to detect low quantities of antigen.)* • Use Pierce Reversible Membrane Stain to check membrane for transfer efficiency. • Ensure sufficient contact between the gel and membrane during transfer. • Make sure the transfer sandwich is assembled correctly. • Wet membrane according to manufacturer's instructions. • Make sure transfer unit does not overheat during electroblotting procedure. • Use positive control and/or molecular weight markers. • Optimize transfer time and current. • Use Thermo Scientific™ Pierce™ Lane Marker Sample Buffer. The tracking dye transfers to the membrane. • Make sure sample preparation conditions have not destroyed antigenicity of the sample. *(Note: Some proteins cannot be run under reducing conditions.)*
Insufficient binding to membrane	• Add 20% methanol to the transfer buffer to help binding. Low MW antigen may pass through the membrane. Use a membrane with a smaller pore size.
Insufficient amount of antibodies	• Increase antibody concentrations. Antibody may have poor affinity for the target protein. • Antibody may have lost activity. Perform a dot blot to determine activity.
Antibody concentrations are too high	• Using too much primary or secondary antibodies can cause the signal to fade quickly.
Insufficient amount of antigen present	• Load more protein onto the gel.
The antigen is masked by the blocking buffer	• Try different blocking buffers. • Optimize blocking buffer protein concentration.
Buffers contain sodium azide	• Do not use sodium azide, an inhibitor of HRP, as a preservative in buffers.
Exposure time is too short	• Lengthen the film exposure time.
Substrate incubation is too short	• SuperSignal Substrates require a 5-minute substrate incubation.
Inactive substrate	• Thermo Scientific™ SuperSignal™ West Pico Chemiluminescent Substrate and Thermo Scientific™ SuperSignal™ West Dura Chemiluminescent Substrate are stable for up to 12 months at RT. Thermo Scientific™ SuperSignal™ West Femto Chemiluminescent Substrate is stable for at least six months at RT. • To evaluate the substrate activity, prepare a small amount of working solution. In a darkroom, add a small amount of HRP conjugate. A blue light should be observed. If no glow is observed, either the substrate or the HRP conjugate is inactive. • Ensure there is no cross-contamination between the two bottles of substrate, which can cause a decline in activity.
Membrane has been stripped and reprobed	• Optimize stripping procedure to prevent any loss of antigen or denaturation. • Reprobe only when necessary. • Avoid repeated reprobing of the same membrane.
Digestion of antigen on the membrane	• Blocking substance may have proteolytic activity (e.g., gelatin).
Protein degradation from blot storage	• Prepare a new blot.



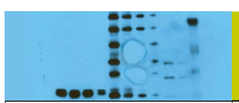
Nonspecific Bands

Possible Causes	Precautions/Solutions
Antibody concentrations are too high	• Reduce antibody concentrations.
SDS caused nonspecific binding to immobilized protein bands	• Wash blots after transfer. • Do not use SDS during immunoassay procedure.



Diffuse Bands

Possible Causes	Precautions/Solutions
Antibody concentrations are too high	• Reduce antibody concentrations.
Too much protein is loaded onto the gel	• Reduce the amount of protein loaded onto the gel.



Partly Developed Area or Blank Areas

Possible Causes	Precautions/Solutions
Incomplete transfer of proteins from the gel	• Make sure there are no air bubbles between the gel and membrane during transfer. • Wet membrane according to the manufacturer's instructions. • Do not handle the membrane with bare hands. Always wear clean gloves or use forceps. • Use a new membrane. • Incubate membranes separately to ensure that membrane strips are not covering one another during incubations.

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