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[Home](#) > [Support](#) > Western Blot Troubleshooting

Western Blot FAQ

Troubleshooting Guide

Difficulties with Western blot assays can generally be broken down into three categories:

- [No Signal or Weak Signal](#)
- [Nonspecific Bands](#)
- [High Background](#)

Can't find your answer? [Contact Us](#) ^[1]

1. No Signal or Weak Signal

Possible Cause	Solution
<i>Cell or tissue type does not express the protein of interest</i>	<ul style="list-style-type: none">• Perform a positive control• Preferably from cell or tissue lysate already verified to express target protein
<i>Improper cell treatment</i>	<ul style="list-style-type: none">• Stimulate cells with the appropriate chemical, protein, or growth factor• Verify that the stimulation works
<i>Improper sample preparation for gel loading</i>	<ul style="list-style-type: none">• Ensure that the protein in the lysate is stable.• Use appropriate protease and phosphatase inhibitors• Ensure protein samples contain SDS, and have been added to gel loading• Include a reducing agent such as dithiothreitol (DTT) or mercaptoethanol

Specific antigen concentration is too low

- Load more protein on gel
- Enrich the antigen by fractionation or by immunoprecipitation

Proteins did not transfer properly to the membrane

- Wet PVDF membrane in methanol or nitrocellulose membrane transfer buffer before use
- Ensure there is good contact between the membrane and the gel
- Optimize the transfer time
- After transfer, ensure molecular weight markers were transferred
- Stain the membrane with Ponceau red, and the gel with Coomassie blue

Insufficient antigen binding to membrane

- If the antigen has low molecular mass, it may pass through the membrane
- Switch to a membrane with a smaller pore size
- Switch to a different type of membrane

The antigen is masked by the blocking buffer

- Test different blocking buffers
- Try milk, serum, BSA in Tris-buffered saline & PBS
- Test different concentrations of each

Insufficient amount of antibodies present

- Increase concentration of primary and/or secondary antibodies

Antibody exposure time is too short

- Increase the exposure time

Antibodies may have lost activity

- Test antibodies by performing a Dot Blot

Excessive washing of the membrane

- Reduce the number of washes

Substrate incubation is too short

- Increase substrate incubation time

Substrate has lost activity

- Test substrate using a positive control

Na azide inhibits enzyme reaction of HRP-conjugated Ab

- Do not use sodium azide together with HRP-conjugated antibodies

TOP

2. Nonspecific Bands

Possible Cause	Solution
<i>Non-specific antibody binding</i>	<ul style="list-style-type: none">• Reduce primary antibody concentration• Decrease the amount of total protein loaded on gel• Adjust membrane blocking conditions• Increase number of washes• Verify the specificity of the antibody• Blot with the secondary antibody alone.• If bands develop, choose an alternate secondary antibody
<i>Degradation of protein</i>	<ul style="list-style-type: none">• Prepare fresh samples• Use protease inhibitors during sample preparation• Minimize freeze/thaw cycles of sample
<i>Aggregation of analyte</i>	<ul style="list-style-type: none">• Increase the amount of DTT (20 -100mM) to ensure complete reduction of disulfide bonds• Heat in boiling water bath for 5-10 minutes before loading onto gel
<i>Cell lines have been passaged extensively Differences in protein expression profiles result</i>	<ul style="list-style-type: none">• Go back to the original non-passaged cell line• Run the current and original cell line samples side-by-side
<i>Protein has multiple modifications in vivo Acetylation, methylation, glycosylation, phosphorylation, etc</i>	<ul style="list-style-type: none">• Review the literature for modified protein variants• Adjust sample preparation accordingly
<i>Target protein has multiple isoforms Other proteins share similar epitopes</i>	<ul style="list-style-type: none">• Check the literature for target protein isoforms• Perform a BLAST search to check for possible cross-reactions• Include other cell or tissue types

TOP

3. High Background

Possible Cause

Solution

Too much protein per lane

- Titrate down the amount of protein loaded per lane

Insufficient blocking of non-specific binding

- Adjust blocking conditions
- Include blocking agent in the antibody buffers as well

The primary antibody concentration may be too high

- Titrate the antibody to find the optimal concentration

The secondary antibody may be binding non-specifically

- Blot with the secondary antibody alone
- If bands develop, choose an alternate secondary antibody

Incubation temperature may be too high

- Incubate blot at 4°C

Cross-Rxn between blocking agent & primary or secondary Ab
Antibody cross-reacts with casein, a milk phosphoprotein

- Add a mild detergent, e.g. Tween® 20, to incubation & washing buffers
- Recommended for phosphoprotein specific antibodies
- Use BSA as a blocking reagent instead of milk

Washing of unbound antibodies may be insufficient

- Increase the number of washes

The membrane may give high background

- Nitrocellulose membrane may give less background than PVDF

The membrane has dried out

- Avoid drying out the membrane during processing and incubation

TOP

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Cell Applications, Inc
6455 Weathers Place
San Diego, CA 92121
Open M-F, 8am-5pm PST

858-453-0848
info@cellapplications.com

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