



# APPLICATIONS, INC.

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## 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](#) <sup>[1]</sup>

### 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"><li>• Perform a positive control</li><li>• Preferably from cell or tissue lysate already verified to express target protein</li></ul>
<i>Improper cell treatment</i>	<ul style="list-style-type: none"><li>• Stimulate cells with the appropriate chemical, protein, or growth factor</li><li>• Verify that the stimulation works</li></ul>
<i>Improper sample preparation for gel loading</i>	<ul style="list-style-type: none"><li>• Ensure that the protein in the lysate is stable.</li><li>• Use appropriate protease and phosphatase inhibitors</li><li>• Ensure protein samples contain SDS, and have been denatured prior to gel loading</li><li>• Include a reducing agent such as dithiothreitol (DTT) or mercaptoethanol</li></ul>

*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

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## 2. Nonspecific Bands

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

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## 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

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

**800-645-0848**  
**[info@cellapplications.com](mailto:info@cellapplications.com)**

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