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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** [1]

1. No Signal or Weak Signal

Possible Cause	Solution
Cell or tissue type does not express the protein of interest	• Perform a positive control • Preferably from cell or tissue lysate already verified to target protein
Improper cell treatment	• Stimulate cells with the appropriate chemical, protein • Verify that the stimulation works
Improper sample preparation for gel loading	• Ensure that the protein in the lysate is stable. • Use appropriate protease and phosphatase inhibitors • Ensure protein samples contain SDS, and have been to gel loading • Include a reducing agent such as dithiothreitol (DTT) mercaptoethanol

Specific antigen concentration is too low	• Load more protein on gel • Enrich the antigen by fractionation or by immunoprec
Proteins did not transfer properly to the membrane	• Wet PVDF membrane in methanol or nitrocellulose m transfer buffer before use • Ensure there is good contact between the membrane • Optimize the transfer time • After transfer, ensure molecular weight markers were • Stain the membrane with Ponceau red, and the gel w Coomassie blue
Insufficient antigen binding to membrane	• If the antigen has low molecular mass, it may pass th 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 a
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-conjugat

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2. Nonspecific Bands

Possible Cause	Solution
Non-specific antibody binding	• 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
Degradation of protein	• Prepare fresh samples • Use protease inhibitors during sample preparation • Minimize freeze/thaw cycles of sample
Aggregation of analyte	• 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
Cell lines have been passaged extensively *Differences in protein expression profiles result*	• Go back to the original non-passaged cell line • Run the current and original cell line samples side-by-side
Protein has multiple modifications in vivo *Acetylation, methylation, glycosylation, phosphorylation, etc*	• Review the literature for modified protein variants • Adjust sample preparation accordingly
Target protein has multiple isoforms *Other proteins share similar epitopes*	• Check the literature for target protein isoforms • Perform a BLAST search to check for possible cross-reactions • Include other cell or tissue types

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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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