

Immunoblotting Troubleshooting Guide

| Problem | Possible Cause | Solution |
|--|---|---|
| No signal or weak signal or non-specific bands | Substrate or conjugate weak or no longer active due to age or improper storage | Test conjugate and substrate for activity. For example, add enzyme conjugate to substrate solution. It should change color. |
| | If chemiluminescent detection is being used, the film development solution may have expired | Use fresh film development solutions. |
| | Incorrect substrate for application | Make sure that the substrate selected is appropriate for the enzyme conjugate. |
| | Substrate prepared incorrectly | Follow instructions that accompany the substrate. Make sure that fresh H ₂ O ₂ is added if necessary. |
| | Incorrect dilution of primary or secondary antibody | Check the literature or data sheet for recommended dilutions for the antibodies being used. Try a range of dilutions. More is not always better, especially with sensitive detection systems such as chemiluminescence. Most of Sigma's antibodies are tested with colorimetric substrates that are not as sensitive. For this reason, the antibody may need to be diluted 5-10 times more if chemiluminescent detection is being used. |
| | Incorrect primary antibody for the application | Make sure the antibody has been shown to work in immunoblotting. Not all antibodies work in all applications. Primary antibody may not be capable of reacting with the protein of interest from the species being studied. Check the literature/data sheet and protein sequence information. |
| | Protein of interest is not present or is present in low amounts | If the positive control worked, check the amount of protein loaded. If necessary, try enriching the amount of protein of interest in the sample loaded by immunoprecipitation or by purification. Consult the literature for the best source for the protein of interest. See "Poor protein transfer" below |
| | Inappropriate secondary antibody for the application | The secondary antibody may not be capable of binding to the primary antibody. Test this by spotting primary antibody on a small piece of membrane. When the spot dries, block, then probe with diluted secondary, wash and develop with substrate. A spot should appear if the secondary bound to the primary. |
| | Incubation times inadequate | Incubate at least one hour with primary antibody. |
| | Enzyme inhibitor present | Sodium azide will inhibit peroxidase reactions. |
| | Over washing | Shorten wash times, omit detergents from washing buffers |
| | Poor protein transfer | Check transfer of the proteins to the membrane by staining the membrane with Ponceau S (Product No. P7170) prior to blocking. Make sure membrane wets uniformly before transfer. Test transfer times. Small proteins (< 10,000) may have transferred through the membrane (try including a second membrane behind the first). Larger proteins may require longer transfer times. Check transfer buffer - high methanol concentrations may prevent transfer of the protein from the gel. 0.005-0.01% SDS in the transfer buffer may increase the transfer of protein from the gel, but it can also interfere with protein binding to the membrane. If the pI of the protein is >9.0 try using CAPS, pH 9 as the transfer buffer. |
| High background or Non-specific bands | Insufficient blocking | Try different blocking strategies: Longer blocking times, higher % of blocker, a different blocker, inclusion of blocker protein in antibody dilutions or try a fresh batch of the same blocker. |
| | Primary antibody may not be specific enough | Try a monoclonal or an affinity purified polyclonal antibody. |
| | High concentration of primary antibody | Check literature or data sheet for recommended dilution of primary. Try a range of dilutions in order to optimize your system. |
| | High concentration of secondary antibody | Test this by running an extra sample lane and omitting the primary antibody incubation from the procedure. If this "secondary only" control results in non-specific staining, try further dilutions of the secondary antibody or try another secondary. |
| | Secondary antibody is cross-reacting with other proteins in the sample | Dilute the secondary antibody in buffer containing 1-5% normal serum from the same species as the sample |
| | Multiple bands may be proteolytic fragments of the protein of interest | Make sure protease inhibitors are present from the first step of sample preparation. Store and handle sample preparations to reduce the chance of proteolysis. Try fresh samples when possible. |
| | Antibody incubation times longer than necessary | Shorten the incubation times. |
| | Overincubation with colorimetric substrate solution | Decrease the staining time. Expose the membrane to substrate until a positive signal is seen. Stop the reaction by washing the membrane before the background develops. |
| | Inadequate washing | Increase the number or stringency of the washes |
| | Contaminating enzymes present in sample | Test sample with substrate alone to check for contaminating enzyme activity in protein sample. |