



## TROUBLESHOOTING GUIDE – WESTERN BLOT

<b>Problem</b>	<b>Possible Cause</b>	<b>Possible Solutions</b>
Weak or No Signal	Antibody concentrations too low	<ul style="list-style-type: none"> <li>• Increase amount of antibody</li> </ul>
	Primary antibody does not recognize antigen	<ul style="list-style-type: none"> <li>• Check data sheets/references to verify that antibody does detect the antigen in the particular species</li> </ul>
	Antibody may not recognize a denatured form of the antigen	<ul style="list-style-type: none"> <li>• Dot blot native and denatured forms, then prove with antibody</li> </ul>
	Secondary antibody may be inhibited	<ul style="list-style-type: none"> <li>• HRP should not be used in conjunction with sodium azide or hemoglobin. Thimerosal can be substituted.</li> <li>• Biotinylated antibodies should not be used with milk or casein</li> </ul>
	Inappropriate secondary antibody	<ul style="list-style-type: none"> <li>• Check research/data sheets to make sure the secondary is directed against the host species and isotype of the primary antibody</li> </ul>
	Constant freezing/thawing can affect antibody	<ul style="list-style-type: none"> <li>• The antibody has most likely degraded. In the future, it is recommended that aliquots of smaller amounts be made.</li> </ul>
	Antibody not stored as recommended	<ul style="list-style-type: none"> <li>• The antibody has most likely degraded. Unfortunately a new vial must be used.</li> </ul>
	Protein is undetectable	<ul style="list-style-type: none"> <li>• Load more protein onto the gel</li> <li>• May be necessary to induce cells before harvest</li> </ul>
	Detergents can disrupt protein binding	<ul style="list-style-type: none"> <li>• Use a milder detergent such as Tween-20</li> </ul>
	Too many proteins in sample cause competition	<ul style="list-style-type: none"> <li>• Centrifuge lysate to enrich the sample with the protein of interest</li> </ul>
	Insufficient Development Time	<ul style="list-style-type: none"> <li>• Increase development time</li> </ul>
	Sensitivity issues of detection system	<ul style="list-style-type: none"> <li>• Use a more sensitive system- try ECL rather than colorimetric</li> </ul>
	Wrong type of membrane	<ul style="list-style-type: none"> <li>• Use PVDF membranes for a maximum signal. Remember to use more stringent blockers.</li> </ul>

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Diffuse Background Staining	Insufficient Blocking	<ul style="list-style-type: none"> <li>• Block overnight</li> <li>• Increase amount of blocker</li> <li>• Try a different blocker</li> <li>• Repeat with a freshly made blocker</li> <li>• Are the antibodies in the buffer too?</li> </ul>
	Inadequate Washing	<ul style="list-style-type: none"> <li>• Increase stringency and number of washes</li> </ul>
	Contaminated solutions, membrane mishandling, and/or dirty gel plates can lead to spots and streaks	<ul style="list-style-type: none"> <li>• Be aware of potential contaminants- use clean equipment, fresh solutions, wear gloves</li> </ul>
Non-Specific Bands	Incomplete protein reduction causing non-specific bands with a high molecular weight	<ul style="list-style-type: none"> <li>• Use a stronger and more stable reducing agent such as DTT over <math>\beta</math>-mercaptoethanol</li> <li>• Purified protein is more prone to aggregation. A high molecular weight laddering pattern may be showing</li> </ul>
	Sample degradation causing non-specific bands with low molecular weights	<ul style="list-style-type: none"> <li>• Add a sample buffer with protease inhibitor immediately after harvest</li> <li>• Always use fresh samples</li> <li>• Store samples at <math>-70^{\circ}\text{C}</math> or below</li> </ul>
	Protein overloading	<ul style="list-style-type: none"> <li>• Use less protein</li> </ul>
	Loading whole cell homogenate	<ul style="list-style-type: none"> <li>• Use cell lysate supernatant for non-membrane bound proteins</li> </ul>
	Antibody concentration too high	<ul style="list-style-type: none"> <li>• Decrease concentration</li> </ul>
	Over-incubation with secondary antibody enzyme substrate	<ul style="list-style-type: none"> <li>• Decrease staining time</li> </ul>
	Antibody-antigen cross reactions	<ul style="list-style-type: none"> <li>• Determine which bands are being caused by the secondary antibody by probing the membrane</li> </ul>
	Additional proteins are being recognized by polyclonal antiserum and causing bands	<ul style="list-style-type: none"> <li>• Use a monoclonal or affinity purified antibody</li> </ul>
	Bands generated by recognition of homologous amino acid sequences	<ul style="list-style-type: none"> <li>• Use a monoclonal antibody specific for a different epitope</li> </ul>
	Extended incubation periods	<ul style="list-style-type: none"> <li>• One hour should be sufficient for most proteins</li> <li>• Try raising the temperature, rather than increasing the time</li> </ul>

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Poor Protein Transfer	Protein won't transfer	<ul style="list-style-type: none"> <li>• Use a nylon membrane</li> <li>• Increase field strength</li> <li>• Increase transfer time</li> </ul>
	Protein transfers through membrane	<ul style="list-style-type: none"> <li>• Shorten transfer time</li> <li>• Use a double layer of membrane</li> <li>• Use a PVDF membrane</li> </ul>
	Protein lost from membrane after transfer	<ul style="list-style-type: none"> <li>• Treat with isopropanol for one minute</li> <li>• Allow membrane to completely dry before blocking</li> </ul>
	Protein binding is blocked	<ul style="list-style-type: none"> <li>• Use brand new nitrocellulose sheets</li> <li>• Always wear gloves when handling membranes</li> </ul>