GENERAL WESTERN BLOT TROUBLESHOOTING TIPS

SYMPTOM	ISSUE	RECOMMENDATIONS
 Wrong Band Size or Multiple Bands	Protein Variants	Many factors can alter the predicted molecular weight of a protein and include post-translational modifications (such as glycosylation), protein processing (cleavage from a pro-form to a mature form), isoforms from alternative splicing, multimer formation, and amino acid charge. To confirm specificity, use a control such as recombinant protein or overexpression lysate, a downregulated knockdown/knockout lysate, a peptide competition of the primary antibody, or a treatment known to effect the expression of the protein.
No Bands/Signal	Antibody Concentration	Increase the concentration of the primary or secondary antibody.
	Protein Concentration	Titer total protein loaded until signal appears.
		Use a positive control lysate known to express the target protein, an overexpression lysate, or a recombinant protein.
		Use a fraction specific prep to increase the concentration of the protein such as a nuclear prep vs. a whole cell prep.
		Confirm the lysis buffer used was strong enough to disrupt the cell's membrane, nucleus , etc., where the target is localized. Use appropriate protease inhibitors to prevent degradation.
		Confirm that the separated proteins successfully transferred to the membrane by Ponceau staining the membrane, and that they were completely transferred to the membrane by Coomassie staining of the gel.
	Antibody Compatibility	Ensure that the primary will react with the species of your lysate and is suitable for western blot.
		Confirm the secondary is compatible with the primary.
	Blocking	Do not block the membrane for more than 1 hour. Lower the concentration of your blocking solution or change blocking solutions.
	ECL Reaction	Make a fresh preparation of the two reagents. Test by dot blotting secondary onto membrane and incubating with ECL.
		Use more sensitive ECL reagents designed for working with proteins of low abundance.
	Image Exposure	Increase the exposure time of the film.
	Native Proteins	Some antibodies are not designed for use with reduced and denatured proteins. Run blot with proteins in native conformation.
	Small Proteins	Use 0.2 µm membranes instead of 0.45 µm to prevent transfer through the membrane.
		Remove SDS from transfer buffer, increase methanol, and reduce transfer time.
		Use a high percentage SDS-PAGE gel.
	Large Proteins	Use a low percentage SDS-PAGE gel and use a lower voltage to transfer in the cold overnight.
		Add SDS to the transfer buffer and lower the concentration of methanol.
	Sodium Azide	Remove from all buffers as it inhibits the ECL reaction.
 High Uniform Background	Insufficient Blocking	Block for one full hour at room temperature. Use 3% BSA instead of milk.
	Insufficient Washing	Wash membranes on an orbital shaker at high speed with a large volume of washing buffer. Wash for a full 5 minutes for 5 times
	Non-Specific Binding	Lower the concentration of your primary or secondary antibody. Dilute antibodies in blocking solution.
		Always incubate your primary antibody at 4°C overnight and not at room temperature.
		Confirm the secondary is specific by omitting the primary and running a secondary only blot.
	Incompatible Block	Do not use milk if you're using a phospho-specific antibody or an avidin/streptavidin secondary as milk contains casein and biotin.
		Do not use milk or BSA if your secondary is anti-bovine, anti-goat, or anti-sheep as these will recognize the bovine IgG in the block. Instead, use 5% serum from the host species of the secondary antibody.
	Dry Membrane	Take care to prevent drying of the membrane after applying the antibodies.
	Film Exposure	Lower the exposure time of the film or wait 15 minutes before exposing.
	ECL Incubation	Lower the incubation time to 1-3 minutes.
 Speckled or Swirly Background	Mishandling of Membrane	Minimize any contact with the membrane or gel. Use only clean tools to manipulate them. Never directly touch with your hands.
	Buffer Contamination	Use fresh buffers.
	Uneven Plastic Wrap	Plastic food wraps are difficult to uniformly flatten and cause swirling patterns from excess ECL. Use sheet protectors which are made of thicker plastic.
	Air Bubbles	Bubbles between the membrane and gel during transfer can cause spots. Roll out any bubbles before closing sandwich cassette.
	HRP Aggregation	Filter the secondary with a 0.2 µm filter to remove any aggregates.
	Insufficient Washing	Wash membranes on an orbital shaker at high speed with a large volume of washing buffer. Wash for a full 5 minutes for 5 times.
 White Bands	Too Much Antibody/Protein	White bands surrounded by black (ghost bands) are caused by an intense localized signal that completely exhausts the ECL reaction with a quick burst of light. Therefore there is no light produced during development and a white band occurs. Use less primary, secondary, or protein.
 Smeared Bands/Lanes	Protein Load	Use less total protein loaded into each lane. You may perform an immunoprecipitation first to enrich your target in the lysate.
	Antibody Concentration	Lower the concentration of your primary antibody

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