Western Blot Troubleshooting Guide

Problem	Possible Cause	Solution
Diffuse bands	1. Antibody concentration too high	1. Decrease the antibody concentration
	2. Excess protein on gel	2. Reduce the amount of total protein loaded on the gel
	3. Protein transfer was too fast and/or the gel was over-heated during electrophoresis	3. Increase transfer time *and* Apply cooling system for electrophoresis
Nonspecific bands	1. Non-specific binding to immobilized protein bands caused by SDS	1. Wash blots after transfer and don't use SDS
	2. Gradually accumulative differences of protein expression profiles due to frequent passage of cell lines	2. Go back to original non-passaged cell line and then run current and original cell line samples in parallel
	3. Degraded protein sample	3. Use fresh sample and add protease inhibitors
	4. Presence of new proteins or different splice variants that share similar epitopes	4. Check literature for other reports Perform a BLAST search Use cell line or tissue reported on the datasheet
	5. Presence of impurities in the antibody preparation	5. Use monoclonal antibodies Purify the antibody by affinity method
	6. Formation of multimers on protein target	6. Before running SDS PAGE gel, boil protein for 10 minutes to disrupt multimers
	7. Formation of different protein subtypes which have different molecular weights	7. Examine literature and use bioinformatics analysis to estimate correct size of protein
	8. Presence of multi-modifier locus in protein	8. Examine literature and use bioinformatics analysis to confirm presence of modifier locus and its molecular weight
	9. Too high primary antibody concentration	9. Decrease primary antibody concentration
	10. Non-specific bands caused by secondary antibody	10. Use fluorescence labels on primary antibody
	11. Excess protein on gel	11. Reduce amount of total protein loaded on gel
	12. Insufficient washing	12. Increase number of washes
	13. Blocking problem	13. Increase blocking time *and* Optimize choice of blocking agent

Problem	Possible Cause	Solution
Weak or no signal from the blot	1. Detection step missed or detection reagents not working	1. After the blot processing is complete, perform the detection step using your standard detection reagents and protocol manually. Make sure the detection reagents are functional
	2. Insufficient incubation with detection reagent	2. Remove blot from detection reagent when signal-to-noise ratio is acceptable
	3. Poor or incomplete transfer	3. Make sure transfer apparatus and membrane sandwiches are assembled correctly Use appropriate transfer times After blotting, stain membrane to measure transfer efficiency
	4. Protein of interest ran off the gel	4. Use positive control and/or molecular weight marker to match gel separation range to size of protein being blotted. After blotting, stain membrane to measure transfer efficiency.
	5. Incorrect reagents added or incorrect containers are filled	5. Make sure that a. primary and secondary antibodies are added to correct containers b. the numbers on the antibody container in the tank and tray match each other c. primary and secondary antibodies, substrates, enzyme system, and sample are compatible
	6. Sample is too dilute	6. Load a larger amount of protein onto the gel or increase the concentration of proteins
	7. Poor retention of proteins or the protein is weakly bound to membrane	7. Use membranes with appropriate binding capacity Dry the PVDF membrane after protein transfer to ensure strong binding of the proteins
	8. Inactive or overly dilute primary or secondary antibody	8. Determine antibody activity by performing a serial dilution using six trays or by using dot blot Increase antibody concentration if necessary
High background on the blot	1. Film overexposed or became wet during exposure	1. Decrease exposure time or allow signal to further decay Prevent leakage of solutions by encasing membrane in transparency film and blotting excess substrate from edge before the exposure
	2. Suboptimal blocking time or washing intensity	2. Increase the blocking time and the number of washes
	3. High concentration of primary and/or secondary antibody	3. Determine optimal antibody concentration by performing dilution series using all six trays of the BlotCycler™. Decrease antibody concentration as necessary
	4. Protein is overloaded	4. Reduce the amount of protein loaded on the gel or dilute the sample
	5. Membrane, solutions, trays, or antibody containers are contaminated.	5. Use clean glassware and purified water to prepare solutions Wear clean gloves at all times Use the forceps when handling membranes Run the cleaning protocol using the recommended cleaning buffer while increasing the concentration twofold
Non-specific binding too high	1. Insufficient removal of SDS or weakly bound proteins on the membrane after blotting	1. Follow proper protocol for membrane preparation before the immunodetection step
	2. The blocking time is too short	2. Increase the blocking time
	3. Affinity of the primary antibody for the protein standards	3. Check with protein standard manufacturer for homologies with primary antibody
	4. Protein is overloaded	4. Reduce the amount of protein loaded on the gel or dilute the sample