Western Transfer Troubleshooting Guide

Problem	Possible Source	Suggestion
Weak/No Signal	Insufficient amount of protein present	Increase amount of protein in sample. Confirm protein is present through an alternate method.
	Gel ran too long	Decrease running time and monitor the gel by watching dye front and ensuring the current is turned off when dye front is near bottom of gel.
	Old or degraded protein samples	Test a fresh sample of target protein.
	Protein over-transferred/did not transfer correctly	Use a membrane with a smaller pore size. Decrease voltage for lower MW proteins (less than 10kDa). Make sure membrane is pre-soaked according to manufacturer's instructions. Check and optimize transfer time, as appropriate transfer time will vary with MW of the target protein. Make sure transfer "sandwich" is set up correctly and there is sufficient contact between gel and membrane. (Please see our Western Transfer protocol for correct transfer "sandwich" set up.) Ensure the correct transfer buffer is being used. The presence of SDS during transfer can reduce the binding of the protein when using a nitrocellulose membrane. Add 20% methanol to transfer buffer to increase binding. Check protein transfer by staining the membrane with a reversible stain, such as Ponceau S, to visualize major bands.
	Protein has a pI greater than 9	Substitute buffer with a higher pH buffer (i.e. CAPS buffer, pH 10.5).
	Protein masked by blocking buffer	Test using a different blocking buffer. Optimize concentration of protein in blocking buffer. We recommend 10% Seablock in diH_2O.
	Enzyme inhibitor present (i.e. azide)	Make sure buffers do not contain sodium azide, as this will interfere with the HRP signal.
	Old or degraded primary antibody	Check manufacturer's recommendations for storage and/or expiration date. If expired or past storage time, purchase new antibody. If antibody was subjected to repeated freeze/thaw cycles purchase new antibody, as this can affect the structure and protein:antibody binding. Perform a Dot Blot to ensure antibody is still active.
	Insufficient amount of primary and/or secondary antibody	Check manufacturer's recommendation for antibody concentration. Test a range of concentrations to find the optimal condition for individual assay.

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Problem	Possible Source	Suggestion
Weak/No Signal (continued)	Incorrect primary antibody	Ensure primary antibody reacts with the target protein from the species being studied (i.e. Rabbit anti-human VEGF ➜ Human VEGF).
	Incorrect secondary antibody	Ensure the secondary antibody being used is against the species of the primary antibody (i.e. Donkey anti-rabbit IgG ➜ Rabbit anti-human VEGF).
	Insufficient incubation of primary antibody	Increase incubation period of primary antibody. Antibody can be incubated for at least one hour at room temperature or overnight at 4°C.
	Insufficient incubation of secondary antibody	Increase incubation period of secondary antibody (please see manufacturer's recommendations for incubation time and temperature).
	Membrane over-washed	Shorten wash times and/or reduce number of washes. Use a washing buffer that does not contain a detergent, or that has a lower concentration of detergent present.
	Incompatible developing reagent	Make sure the developing reagent being used is compatible with the enzyme conjugate. Ensure substrate is made correctly by following the manufacturer's instructions.
	Excess salt present	Decrease amount of salt present in wash buffer. Decrease amount of salt present in antibody dilution buffer.
	Degraded/Incorrect developing reagent	Ensure the developing reagent was prepared correctly. Check that the reagent is still active. If not, purchase new reagent.
	For ECL method, film is bad/expired	Ensure film is not expired or exposed. If so, purchase new film.
	For ECL method, ECL developing solutions are old/expired	Use fresh ECL developing solutions.
	For ECL method, film exposure time is too short	Increase exposure time of the film.
	For ECL method, cling film interfering with reaction	Use different brand of cling film.
High Background	Antibody concentration too high	Check manufacturer's recommendations for primary and/or secondary antibody concentrations. Test a range of concentrations to find optimal condition for individual assay.
	Over-incubation of antibodies	Check manufacturer's protocol to ensure the correct incubation time and temperature is used. If this information is not supplied by the manufacturer, optimize incubation time through testing.

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Problem	Possible Source	Suggestion
High Background (continued)	Non-specific binding of antibodies	Ensure the primary antibody is specific only for the protein that is being targeted. Run a control, omitting the primary antibody incubation. If bands develop, choose a different secondary antibody. Choose a different host species for secondary antibody.
	Cross reaction of antibodies with proteins in blocking buffer and/or sample	Use a different blocking buffer formulation. Goat antibodies will react with BSA due to a slight recognition of the bovine protein by the goat antibody. This will lead to a purple sheen on your membrane. If this occurs, use a different species antibody or a different blocking buffer. If secondary antibody is suspected, dilute antibody in buffer containing 1-5% normal serum from the same species as target protein.
	Ineffective blocking buffer	Use a different blocking buffer formulation. Add detergent to blocking buffer, such as TWEEN-20.
	Insufficient blocking	Use a different blocking buffer formulation. Batch of blocking buffer may be inactive. Use fresh blocking buffer. If blocking overnight at 4°C, this may decrease blocking efficiency since lower temperatures may lessen the effectiveness. Try incubation for one hour at room temperature. Optimize incubation time and/or temperature of blocking step.
	Insufficient washing	After each step, wash membrane 3 times for 10 minutes each. Increase the duration of the washing step if this is not sufficient. Try a different detergent in washing buffer or add TWEEN-20 if not already present. Increase volume of washing buffer used.
	Over-incubation in color development substrate	Shorten incubation time in color development solution. Monitor development until bands are present and stop reaction by washing membrane in diH_2O.
	Excess enzyme present	Reduce concentration of selected enzyme conjugate.
	Interference by endogenous enzyme	Non Fat Dry Milk contains endogenous biotin and may interfere with avidin/biotin detection system. Use different blocking buffer, such as 3% BSA.
	Contaminated reagents	Filter buffers before use to remove contaminant. Make fresh buffers and re-run Western Transfer.

Western Transfer Troubleshooting Guide continued...

Problem	Possible Source	Suggestion
High Background (continued)	Membrane problems	Test new membranes. During the entire procedure the membrane can only be dried between transfer and immunostaining. Once immunostaining has begun, the membrane cannot be dried until completion of immunostaining procedure. Re-run Western Transfer if membrane was dried at any other point during immunostaining procedure.
	For ECL method, ECL film overexposed	Shorten film exposure time. If signal from target protein is too strong, wait 5-10 minutes and re-expose to film.
Splotchy Background	Uneven agitation during incubations	Ensure the membrane is agitated evenly by placing on a rocker or shaker during incubation.
	Insufficient amount of incubation buffer	Ensure enough solution is present during each incubation period to fully submerge the membrane and allow it to float freely in the solution.
	Air bubbles present during transfer	Gently remove all air bubbles from transfer "sandwich" before transfer is started. This can be done by using a roller or clean glass rod/Pasteur pipette.
	Membrane problems	Ensure membrane is wetted thoroughly according to the manufacturer's protocol. Handle membranes with extreme care. Mishandled membranes can cause non-specific binding. Handling membranes with bare hands can lead to contamination of membrane. Use forceps or wear gloves when handling membrane.
	Aggregation of HRP conjugate	If using HRP, filter conjugate to remove HRP aggregates. Use a fresh sample of HRP conjugate.
	Contamination of reagents	Filter buffers before use to remove contaminant. Make fresh buffers and re-run Western Transfer.
Non-specific bands	Antibody concentration too high	Check manufacturer's recommendations for primary and/or secondary antibody concentrations. Test a range of concentrations to find optimal condition for individual assay.
	Old or degraded antibody	Check manufacturer's recommendations for storage and/or expiration date. If expired or past storage time, purchase new antibody. If antibody was subjected to repeated freeze/thaw cycles purchase new antibody, as this can affect the structure and protein:antibody binding.

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Problem	Possible Source	Suggestion
Non-specific bands (continued)	Non-specific binding of primary antibody	Decrease concentration of primary antibody. Check concentration of protein being added to gel. Use a more specific antibody, such as a monoclonal antibody or Antigen Affinity Purified polyclonal antibody. Add a small amount of detergent, such as TWEEN-20 (0.1-0.5%) to the primary antibody solution and/or blotting buffer. Increase number/duration of washes.
	Non-specific binding of secondary antibody	Decrease concentration of secondary antibody. Run a control, omitting the primary antibody incubation. If bands develop, choose a different secondary antibody. Add a small amount of detergent, such as TWEEN-20 (0.1-0.5%) to the secondary antibody solution. Increase number/duration of washes.
	Aggregation of target protein	Add or increase a reducing agent, such as DTT or BME, to ensure all disulfide bonds are reduced. Heat protein sample in a hot water bath for 5-10 minutes before loading onto gel. Check concentration of protein being added to gel. A concentration that is too high can cause protein aggregation. Re-run Western Transfer with a lower concentration of protein.
	Degradation of protein sample	Make fresh samples and minimize freeze/thaw cycles of samples. Add protease inhibitor to samples prior to storage. Keep samples stored between -20°C and -80°C.
	Insufficient blocking	Test a different blocking buffer formulation. Batch of blocking buffer may be inactive. Use a fresh blocking buffer. If blocking overnight at 4°C, this may decrease blocking efficiency since lower temperatures may lessen the effectiveness. Try incubation for one hour at room temperature. Add TWEEN-20 to blocking buffer. Optimize incubation time and/or temperature of blocking step.
	SDS facilitates binding to immobilized protein bands	Ensure membrane is sufficiently washed after transfer of proteins. Do not use SDS during immunostaining when using a nitrocellulose membrane.
	Excess enzyme present	Reduce concentration of the selected enzyme conjugate.
	Contamination of reagents	Filter buffers before use to remove contaminant. Make fresh buffers and re-run Western Transfer.

Western Transfer Troubleshooting Guide continued...

Problem	Possible Source	Suggestion
Diffused/Fuzzy bands	Antibody concentration too high	Check manufacturer's recommendations for primary and/or secondary antibody concentrations. Test a range of concentrations to find optimal condition for individual assay.
	Excess protein on gel	Further dilute protein samples before loading onto gel.
	Gel over-heated/high voltage	Heat causes the gel to lose its structure and resolving power. Be sure the transfer reservoir is run in an ice bath. Reduce voltage or current used during transfer
	Incorrect preparation of membrane	Pre-soak membrane in correct transfer solution (see membrane manufacturer's protocol). Ensure it is soaked for the correct amount of time.
White Bands (ECL visualization method)	Antibody or protein concentration too high/Excessive signal generated	White bands are the result of rapid consumption of the substrate caused by the presence of too much conjugate. This is the result of high concentrations of the antibodies and/or proteins. Check manufacturer's recommendations for antibody and/or protein concentrations.