TROUBLESHOOTING WESTERN BLOTTING

Troubleshooting *This troubleshooting document gives the problem, possible cause and suggested solution for problems during the Western blotting application:*

Problem: No signal/No bands observed

The primary antibody and the secondary antibody are not compatible	An incorrect secondary antibody is used, it might be raised against the species in which the primary was raised (e.g. primary is raised in rabbit, use secondary antibody raised in anti-rabbit).
Insufficient primary or secondary antibody is bound to the protein of interest	The antibody has low affinity with protein of interest. Use a more concentrated antibody lysate. The antibody may have lost its activity, perform a Dot Blot. If incubation time is insufficient, increase incubate time (e.g. overnight at 4^{O}C).
There is insufficient protein/antigen	Increase the amount of protein that is loaded on the gel. Confirm the presence of protein by another method (e.g. Dot Blot). Use protease inhibitors. Run the recommended positive control.
Incorrect storage	Ensure that all antibodies have been stored correctly according to the manufacturer's instructions.
The antibodies are too old	Make sure that the antibodies have not exceeded their date of expiration.
A cross-reaction between the blocking agent and primary or secondary antibody has taken place	Use a mild detergent such as Tween-20 or switch the blocking reagent.
The primary antibody does not recognize the protein in the species being tested	Check the manufacturers datasheet to make sure that the antibody should react with the target protein. Run the recommended positive control.
The transfer of the protein to the membrane is poor	Check the transfer to ensure it is complete with Ponceau S, Amido Black or India Ink. Check whether the transfer was not performed the wrong way. If using PVDF membrane make sure you pre-soak the membrane in methanol then in transfer buffer. Soak a nitrocellulose membrane in transfer buffer. Optimize the transfer time, high weight protein may require more time for transfer.
Excessive washing of the membrane	Do not over wash the membrane.
The washing is incorrect	Make sure that correct washing steps are included.
The detection kit is old and the substrate is inactive	Purchase fresh antibody when the antibody is expired or past the manufacturing date.
Incorrect storage of antibody	Follow the manufacturer's storage recommendations and avoid freeze/thaw cycles.
Because of too much blocking the protein of interest cannot be visualized	Instead of using 5% milk in the antibody buffers try removing the milk or using 0.5%. Switch blocking reagents or block for less time.
Over-use of the primary antibody	Use fresh antibody as the effective concentration is lowered upon each re-use.
The secondary antibody is inhibited by sodium azide	Make sure that the buffers do not contain sodium azide when working with HRP-conjugated antibodies.

Problem: Faint Bands

The protein-antibody binding is low	The number of washes should be reduced to a minimum. Reduce the NaCl concentration in the blotting buffer of antibody solution.
Insufficient primary or secondary antibody is bound to the protein of interest	The antibody has low affinity with protein of interest. Use a more concentrated antibody lysate. The antibody may have lost its activity, perform a Dot Blot. If incubation time is insufficient, increase incubate time (e.g. overnight at 4℃).
There is insufficient protein/antigen	Increase the amount of protein that is loaded on the gel. Use protease inhibitors.
The conjugate is inactive	Mix the enzyme and its substrate in a tube. If no color develops or if it is weak make fresh reagents.
Non-fat dry milk may mask some antigen	Decrease the milk percentage in blocking buffer and in the antibody solutions. Instead of using 5% milk in the antibody buffers try removing the milk or using 0.5%, or substitute with 3% BSA.

Problem: High background

The blocking of non-specific binding might be absent or insufficient	Increase the blocking incubation period and consider changing the blocking reagent. Adjust the concentration of the blocking reagent up or down as needed.
Incubation temperature too high	Incubate the blot at 4℃.
The primary antibody may be binding non-specifically or the concentration of the primary antibody may be too high	Use mono-specific or antigen affinity-purified antibodies. Titrate the antibody to the optimal concentration, incubate for longer time but in more diluted antibody solution (a slow but targeted binding is best).
The secondary antibody may be binding non-specifically or reacting with the blocking reagent	Run a secondary control without the primary antibody, choose an alternative secondary antibody if bands develop.
A cross-reaction between the blocking agent and primary or secondary antibody has taken place	Add a mild detergent such as Tween-20 to the incubation and the washing buffer.
Non-fat dry milk may contain the target antigen (phosho-specific protein)	Substitute the milk with 3% BSA as blocking reagent.
The washing of unbound antibodies may be insufficient	Increase the number of washes. Increase the Tween-20 concentration in the wash-buffer (0.1%-0.5%).
The choice of membrane may give a high background	PVDF is considered to give higher background than nitrocellulose membrane.
The membrane has dried out	Make sure that the membrane does not dry out during incubation.
The film is overexposed	Reduce the exposure time. If the target is too strong, wait for 5-10 minutes and re-expose to film.

Problem: Multiple/Extra bands

Cell lines that have been frequently passaged, gradually accumulate differences in their protein expression profiles	Go back to the original non-passaged cell line and run the current and original cell line samples in parallel.
The protein sample has multiple modified forms in vivo such as acetylation, myristylation, phosphorylation, glycosylation etc	Check the literature and use an agent to dephosphorylate, de-glycosylate, etc. the protein of interest and bring it to the correct size.
The target protein has been digested (more likely if the bands are of lower molecular weight)	Ensure that there are incorporate sufficient protease inhibitors in the sample buffer.
Detection of unreported novel proteins or different splice variants, which share similar epitopes and could possibly be from the same protein family	Check the literature for other reports and also perform a BLAST search. Use the cell line or tissue reported on the datasheet.
The primary antibody concentration is too high (at high concentration multiple bands are often seen)	Dilute the antibody solution. Decrease the incubation period. Reduce the amount of protein loaded on the gel.
The secondary antibody concentration is too high (at high concentration secondary antibodies will bind non-specifically)	Dilute the antibody solution. Run a control with secondary antibody alone. If bands develop choose an alternative secondary antibody.
The antibody has not been purified	Use affinity purified antibody, this will often remove non-specific bands.

Reagents are contaminated	Check the buffers for particulate or bacterial contamination. Make fresh reagents.
There is a non-specific binding for primary or secondary antibody	Use blocking peptides (Tween-20) to primary or secondary antibody solution to differentiate specific and non-specific bands. Only specific bands should be blocked (and thus disappear). Increase number of washes. Increase concentration Tween-20 in the buffer used for washing.
The protein target may form multimers	Try boiling in SDS-Page for 10 minutes instead of 5 minutes to disrupt multimers.

Problem: Spots on the blot.

White spots: Air bubbles that are trapped against the membrane	Make sure you remove bubbles when preparing the gel for transfer.
There is an uneven agitation during incubation	Incubate antibodies under agitation by placing on a rocker/shaker.
Black dots: The antibodies are binding to the blocking agent	Filter the blocking agent.
reagents/equipment are contaminated	Check buffer/electrophoresis unit for bacterial contamination. Make fresh reagents, wash membrane thoroughly.

Problem: White bands

Excessive signal generated	Dilute the antibodies or protein more. Excessive antibody or protein can cause extremely high levels of localized signal.

Problem: Moleculare weight marker lane is black

The antibody is reacting with the molecular weight marker	Add a blank lane between the molecular weight marker and the first sample lane.

Problem: The band of interest is very low/high on the blot

Separation is not efficient	Change the gel percentage: a higher percentage for small proteins, a lower percentage for large proteins.

Problem: The bands have a smile effect

The migration was too fast	Slow down the migration.
The migration was too hot	Run the gel in the cold room or on ice.

Problem: There is an uneven band size in the lanes probed for the same protein

The gel has set too quickly while casting	The recipe of the gel should be reviewed and the addition of TEMED to the gels especially, add a little 0.1% SDS in water to the top of the migrating gel while it sets to stop it from drying.
The acrylamide percentage is not even along the lanes	

Helpful links / references

W. Neal Burnette (April 1981). "'Western blotting': electrophoretic transfer of proteins from sodium dodecyl sulfate — polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A". *Analytical Biochemistry* **112** (2): 195-203. United States: Academic Press.

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