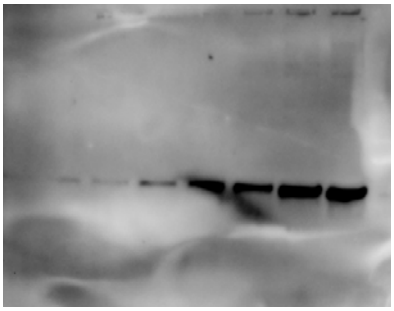
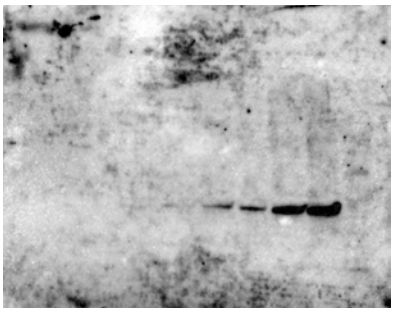
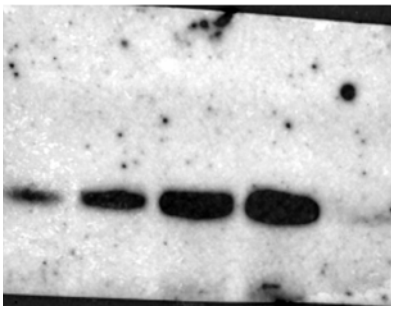
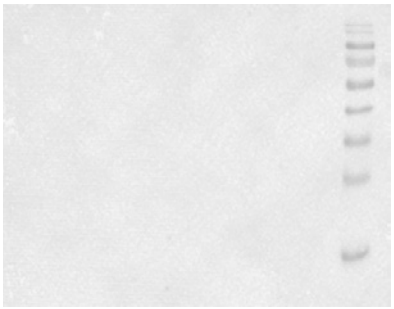
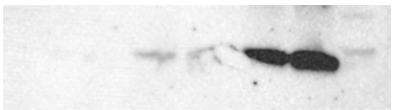
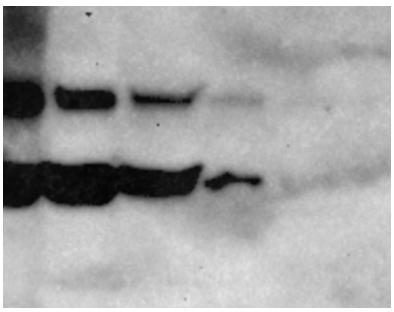
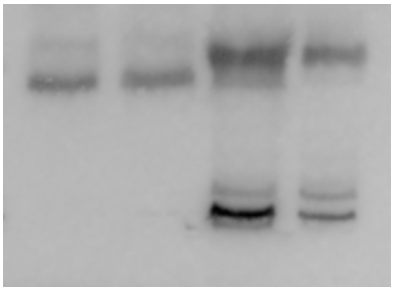
Troubleshooting

Problem	Cause	Solution
High background 	Insufficient bloking	- Block the membrane for at least one hour with skim milk or BSA depending on the type of protein.
	Blocking is not compatible	- For phosphorylated protein detection, milk should not be used (milk and casein are phospho-protein rich).
	High concentration of primary antibody	- Reduce and/or optimize the concentration of the primary antibody.
	High concentration of secondary antibody	- Reduce and/or optimize the concentration of the secondary antibody.
	Long exposure time with chemiluminescent substrate	- Decrease the membrane incubation time in the substrate. - Decrease the blot detection time, e.g. 120 or 60 seconds instead of 180 seconds.
	Too sensitive Detection reagents	- In the case of using a strong chemiluminescent substrate for the detection step, in order to reduce the background while having the lanes, dilute the substrate with PBS (or other buffers you are using for washing without detergent) with a 2: 1 proportion. - Decrease the quantity of the substrate (put less volume of each solution but still a 1:1 proportion). Follow the substrate supplier's instructions.
	The membrane has dried out	- Never let the membrane dry out during the Western Blotting workflow in the case of hydrophobic membranes.
	Membrane with an excess of chemiluminescence substrates	- Before imaging, carefully drip out the excess substrate from the membrane.
	The membrane was in contact with the container in the equilibrating or washing step	- Keep the protein side of the blot upward and in contact with the solution.
	Insufficient washing	- Wash the membrane at least three times, each for a minimum of 5 minutes. - Insufficient volume of washing buffer. - Insufficient concentration of the detergent in the buffer. Use PBS containing >0.05% Tween 20.

Problem	Cause	Solution
Patchy or Uneven black Spots on the Blot  	The membrane got dried during the detection stage	- Incubate the membrane in the detection substrate according to the producer's recommendation. - To avoid drying during the detection step, place the blot in between the development folder and gently smooth out the air bubbles. Be careful not to dry the membrane.
	Contaminated membrane	- Use clean buffers in each step. - Be careful about the residues of the gel on the membrane.
	The secondary antibody is aggregated	- Filter the secondary antibody using a 0.22 µm low protein binding filter (e.g. SEPARA 0.2 µm RC, GVS).
	Antibodies are binding to the blocking buffer	- Filter the blocking solution antibody using a 0.22 µm filter to remove undissolved blocking agent or any contamination. - Use an alternative blocking agent.
	Insufficient blocking	- Block the membrane for at least one hour with skim milk or BSA depending on the type of protein.
	Contaminated equipment	- Do not use metallic forceps, which can cause speckling due to rust contamination. - Use clean plastic containers to avoid any type of cross-reaction.
	Contaminated reagents	Make sure that all solutions, buffers, and chemiluminescence substrates are clean - Avoid repetitive freezing/thawing of the proteins.
No band (including protein marker) or fade bands 	No more activity of reagents	- Check the datasheet and expiration date of reagents.
	Too strong washing	- Try to reduce repetition and/or time of washing. - Reduce the concentration of detergent (e.g. Tween 20) in the washing buffer.
	No transfer	- Check the transfer efficacy using Ponceau S as a reversible stain on the membrane. - Check the transfer efficacy using coomassie Blue on the gel. - Check if the "sandwich" (sponge, filter paper, gel, and membrane) is assembled and placed correctly.
No band (but protein marker present) 	Wrong antibodies	- Check the compatibility of the primary antibody with your target protein. - Check the compatibility of the primary antibody with the secondary antibody.
	The activity of antibodies is reduced	- Try a fresh antibody. - Check the storage condition. - Avoid repetitive freezing/thawing of the antibodies.
Uneven white spots 	Presence of air bubbles during the transfer	- Air bubbles between gel and membrane can cause white spots. Use a proper roller to smooth out all the bubbles before starting the transfer.

Problem	Cause	Solution
Smear bands 	Too much sample is loaded	- Dilute more the loading sample.
	Poor quality of the sample	- Contaminated, degraded, or denatured sample.
	Poor gel preparation (uneven gel)	- Fill the cassette to the volume before the gel starts polymerization.
White/hollow bands 	High concentration of reagents	- Decrease the concentration of primary/secondary antibodies or use less protein.
Bands smile within the gel lanes 	The running condition was too fast	- Reduce voltage during electrophoresis.
	Migration was too hot	- Run the gel in the cold room.
Broad or misshapen bands 	Gel electrophoresis problems	- Poor gel polymerization. - Inappropriate running condition.

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