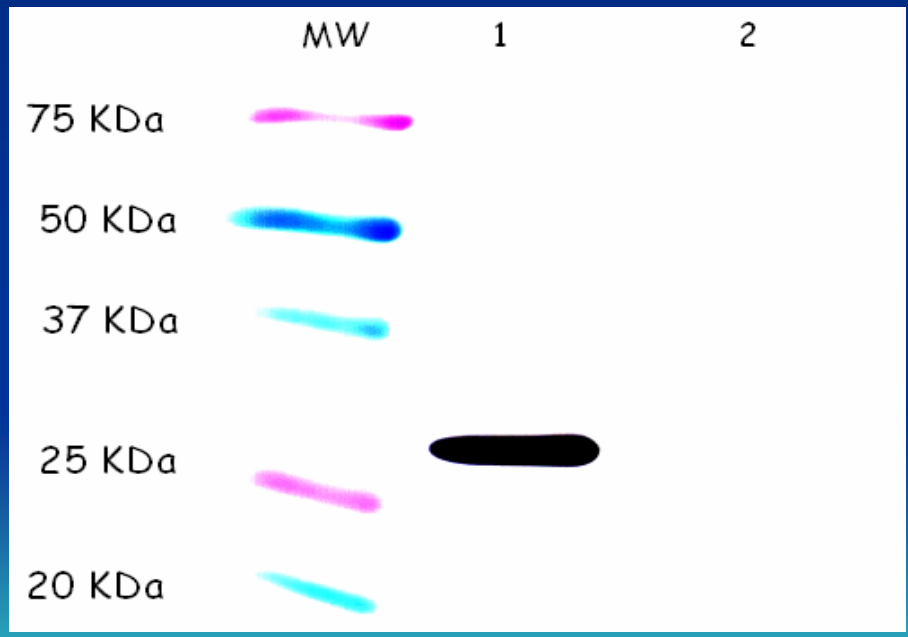
Protocol for Western blot and troubleshooting



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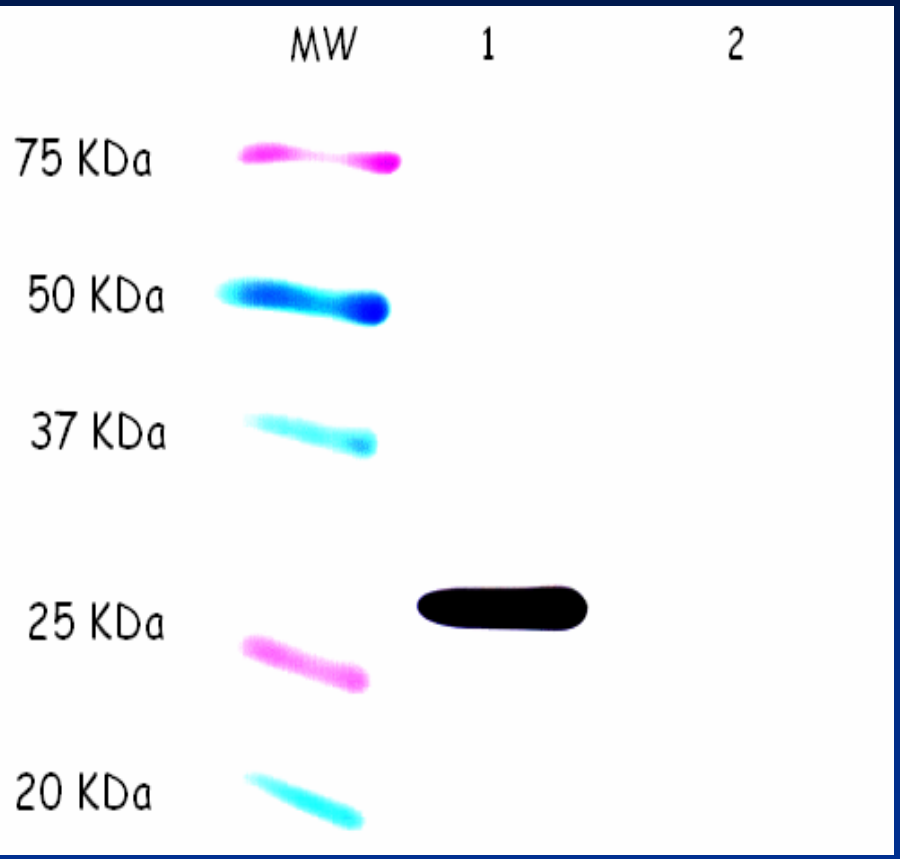
1. After blocking, wash the membrane in washing buffer for one min
2. Incubate the membrane with diluted antibody for at least 1 hr at RT or overnight on a rocker or shaker at 4 C (Use 3-5 ml)(the primary should be diluted to their optimal concentration in 1X TBS+ 0.1 % tween-20+ 5% Bovine Serum Albumin)
3. Remove the primary antibody and rock the membrane in DW the three times in washing buffer (TBST) for 5 min

4. Wash 3x ~15 min in TBST + 0.5% milk.
5. Incubate with secondary antibody at appropriate dilution in TBST + 0.5% milk at least 1 hr RT
6. Remove the secondary antibody
7. Wash 2x 15 min ea in TBST + 0.5% milk
8. Wash 3x 15 min ea in TBST
9. Wash the membrane in DW for 5 min

visualization by color reaction or by chemiluminiscence

10. Detect the band using regents such as Chloranaphthol, Aminoethyl carbazole or Diaminobenzidin
11. Prepare the suitable Luminal Reagent.
12. Incubate the membrane in the Luminal Reagent for one min ar RT

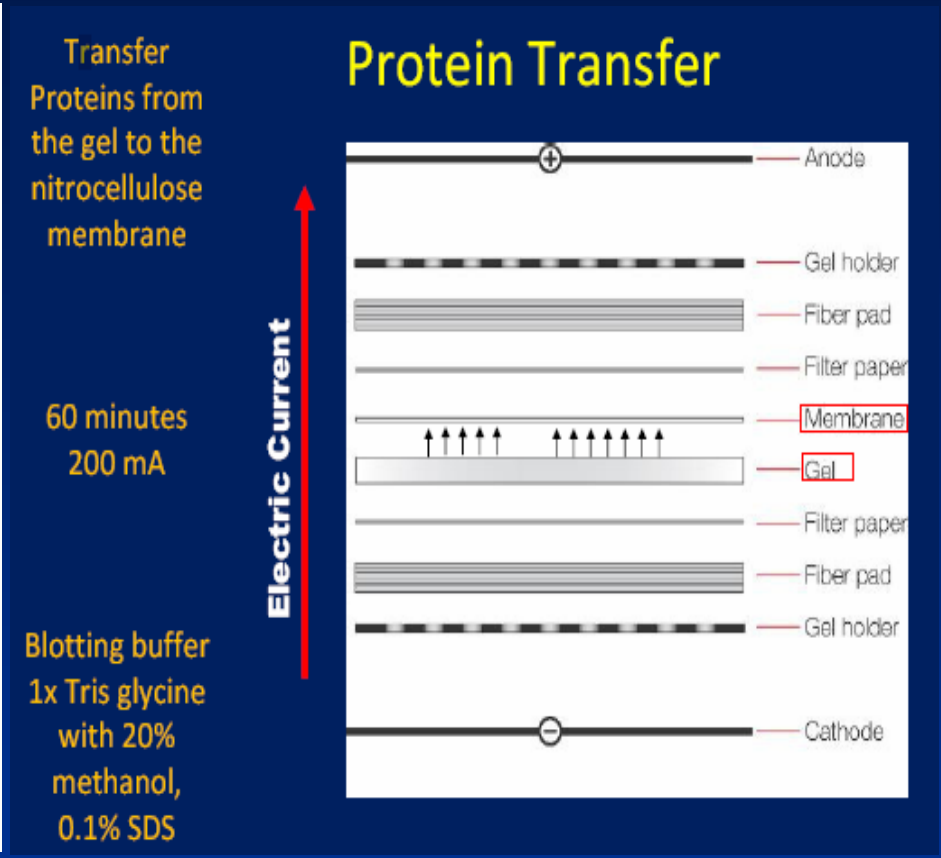
13. Place the membrane into the cassette and cover it by a plastic sheet.
14. In a dark room, qulickly, place an ECL hyper film over the memnrane in the cassette, then close the cassette and wait for 1 min
15. Develop the film by rinsing it for 30 seconds in developer solution, then for 30 sec in fixative solution
16. Wash the film in tap water for 1 min and then dry it.



Transfer Proteins from the gel to the nitrocellulose membrane

60 minutes
200 mA

Blotting buffer
1x Tris glycine with 20% methanol, 0.1% SDS



Troubleshooting of Western blot

Problem: No Bands Observed

Possible Source	Suggestion
Insufficient antibody	Antibody may have low affinity to protein of interest. Increase antibody concentration (2-4 fold higher than recommended starting concentration).
	Antibody may have lost activity. Perform a Dot Blot.
Insufficient protein	Increase the amount of total protein loaded on gel.
	Confirm the presence of protein by another method.
	Use a positive control (recombinant protein, cell line or treat cells to express analyte of interest).
	Perform a Dot Blot.
Poor transfer	Wet PVDF/Immobilon-P membrane in methanol or nitrocellulose membrane in transfer buffer.
	Ensure that there is good contact between PVDF membrane and gel.
Incomplete transfer	Optimize transfer time. High MW protein may require more time for transfer.

Problem: Faint Bands (Weak Signal)

Possible Source	Suggestion
Low protein-antibody binding	Reduce the number of washes to minimum.
	Reduce NaCl concentration in Blotting Buffer used for wash steps (recommended range 0.15M - 0.5M).
	Reduce NaCl concentration in Antibody Solution (recommended range 0.15M - 0.5M).
Insufficient antibody	Antibody may have low affinity to protein of interest. Increase antibody concentration (2-4 fold higher than recommended starting concentration).
Insufficient protein	Increase the amount of total protein loaded on gel.
Inactive conjugate	Mix enzyme and substrate in a tube. If color does not develop or, it is weak. Make fresh or purchase new reagents. Switch to ECL.
Weak/Old ECL	Purchase new ECL reagents.
Non-fat dry milk may mask some antigen	Decrease milk percentage in Block and Antibody Solutions or substitute with 3% BSA.

Problem: Extra Bands

Possible Source	Suggestion
Non-specific binding of primary antibody	Reduce primary antibody concentration. (See Figure 1)
	Reduce the amount of total protein loaded on gel.
	Use monospecific or antigen affinity purified antibodies (such as R&D Systems "MAB" or "AF" designated antibodies).
Non-specific binding of secondary antibody	Run a control with the secondary antibody alone (omit primary antibody). If bands develop choose an alternative Secondary Antibody.
	Use monospecific or antigen affinity purified antibodies (such as R&D Systems "BAF" or "HAF" designated secondary antibodies).
Non-specific binding of primary or secondary antibodies	Add 0.1 - 0.5% Tween® 20 to primary or secondary Antibody Solution.
	Use 2% non-fat dry milk in Blotting Buffer as a starting point to dilute primary and secondary antibodies. Adjust antibody concentration up or down as needed. (See Figure 2a & 2b)
	Increase number of washes.
	Increase NaCl concentration in Blotting Buffer used for antibody dilution and wash steps (recommended range 0.15M - 0.5M). (See Figure 2c)
	Increase Tween® 20 concentration in Blotting Buffer used for wash steps (0.1%-0.5%).
Aggregation of analyte	Increase amount of DTT to ensure complete reducing of disulfide bonds (20 - 100mM DTT). Heat in boiling water bath 5-10 minutes before loading onto gel.
	Perform a brief centrifugation.

Problem: High Background

Possible Source	Suggestion
Non-specific binding of primary antibody	Use monospecific or antigen affinity-purified antibodies (such as R&D Systems "MAB" or "AF" designated antibodies).
	Block in 5% milk. Adjust the milk (2-5%) or NaCl (0.15-0.5M) concentrations of primary Antibody Solution. (See Figure 3).
	Decrease antibody concentration.
Non-specific binding of secondary antibody	Run a control with the secondary antibody alone (omit primary antibody). If bands develop choose an alternative Secondary Antibody.
Insufficient blocking	Start with 5% dry milk with 0.1%- 0.5% Tween 20, 0.15 -0.5M NaCl in 25mM Tris (pH 7.4). Incubation time may be extended. Adjust milk concentration up or down as needed.
	Overnight blocking at 4°C may decrease blocking efficiency since detergents might not be effective at lower temperatures.
Non-fat dry milk may contain target antigen	Substitute with 3% BSA.

Problem: Diffuse Bands

Possible Source	Suggestion
Excessive protein on gel	Reduce amount of protein loaded

Problem: White Bands (ECL method)

Possible Source	Suggestion
Excessive signal generated	Reduce antibody or protein concentration. Excessive antibody or protein can cause extremely high levels of localized signal (usually at a single band). This results in rapid, complete consumption of substrate at this point. Since there is no light production after the completion of this reaction, white bands are the result when exposed to film.

Problem: Patchy uneven spots all over the blot

Possible Source	Suggestion
Contamination of reagents	Check buffers for particulate or bacterial contaminate. Make fresh reagents.
Not enough solution during incubation or washing	Make sure membrane is fully immersed during washes and antibody incubations.
Air bubble trapped in membrane	Gently remove any air bubbles. Especially during transfer.
Uneven agitation during incubations	Ensure uniform agitation by placing on a rocker/shaker.
Contaminated equipment	Make sure that the electrophoresis unit is properly washed. Protein or pieces of gel remaining on the unit may stick to the membrane. Wash membrane thoroughly.
HRP aggregation	Filter conjugate to remove HRP aggregates.
Long exposure	Reduce exposure time.


Questions
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