Western blot trouble shooting

A. Can't visualize protein of interest

- Sample preparation (see 1)
- Inadequate transfer (see 2)
- Inefficient antibody binding (see 3, 4)
- Problems with reagents (see 5, 6)

B. Band incorrect size

- Protein is smaller than expected (see 7)
- Protein is larger than expected (see 8, 9)
- Multiple bands (see 10)
- Bands are very high or low on gel (see 11)

C. Band artifacts

- Incomplete bands (see 12)
- Diffuse bands (see 13)
- Streaking (see 14)
- Lateral spreading (see 15)
- Band distortion (see 16)

D. Electrophoresis troubles

- Gel polymerizes too quickly or slowly (see 17, 18, 19)
- Gel runs too quickly or slowly (see 20, 21)
- Smiling dye front (see 22)
- Slanted dye front (see 23)

E. High background

- Insufficient blocking (see 24)
- Inappropriate washing (see 25)
- Reagent contamination (see 26)
- Membrane choice (see 27)
- Non-specific antibody binding (see 28)
- Film developing (see 29)

A. Problem	Cause(s)	Solution
1. Sample preparation	Inefficient extraction	Try alternate methods; include positive control on gel
	Protein expressed at low levels in tissue or cells	Load more total protein on gel; concentrate using Afyon, or pool multiple samples
	Protein was degraded during extraction	Use protease inhibitors in lysis buffer
2. Inadequate transfer	Transfer buffer incorrectly prepared/ too much methanol in buffer	Check protocol, decrease methanol
	Larger proteins may require more time/current	Repeat with longer time/higher voltage
	Insufficient contact between gel and membrane	Check fiber pad thickness; replace if too thin
3. Inefficient binding of primary antibody	Low affinity of antibody for protein or antibody is old/weak	Increase concentration of primary antibody; purchase new antibody and maintain proper storage
	Antibody has weak cross-reactivity with species of interest	Try alternate primary antibody source
	Antibody removed with washing	Use minimum number of washes; decrease salt concentration in wash
	Antigen masked by blocking agent (ex., milk)	Try alternative blocker (ex., BSA)
4. Inefficient binding of secondary antibody	Incorrect species chosen	Use antibody directed against primary antibody species
	Insufficient antibody concentration or antibody is old	Increase dilution or obtain new antibody
5. Conjugate/ substrate inactive	Reagents old or unstable	Mix conjugate + substrate in a tube; or luminescence in dark for ECL – obtain new reagent if no signal
	HRP inactivated by sodium azide	Avoid using solutions containing this preservative
6. Detection reagent (ECL)	Solution is old or stored improperly	Purchase new reagent

B. Problem	Cause(s)	Solution
7. Protein smaller than expected	Proteolysis; sample freeze/thaw	Use protease inhibitors/fresh samples
	Splice variant	Consult literature/use appropriate controls
8. Protein larger than expected and/or multiple bands	Naturally occurring protein modifications (glycosylation, phosphorylation, acetylation, etc.)	Consult literature to find additives to remove chemical groups
	Protein expression changing in overpassaged cell line	Use earlier passages; include positive control
9. Protein larger than expected	Protein aggregates – disulfide bonds intact	Use DTT in sample buffer; briefly spin samples prior to loading
10. Extra bands	Non-specific binding of primary or secondary antibody	Decrease concentrations; try blocking peptide experiment (will remove protein of interest)
11. Band appears very high or low on the blot	Gel percentage is not optimum	Increase gel percentage for smaller protein; decrease for larger protein

C. Problem	Cause(s)	Solution
12. Incomplete bands	Bubbles between gel and membrane	Using a pipette, roll over the gel/ membrane sandwich to force air bubbles out
13. Diffuse bands	Slow migration	Increase voltage; ensure proper buffer preparation
	Sample not heated correctly	Make sure sample is heated to 90°C for 2 min. prior to loading
	SDS in sample buffer is too old	Prepare new SDS for sample buffer
14. Streaking in lanes	High salt concentration in sample	Decrease salt concentration in sample buffer
	Sample too concentrated or insufficient SDS	Increase dilution/use more SDS
15. Lateral spreading of bands	Sample diffusion from wells during loading	Minimize loading time

16. Band distortion	Gel failed to polymerize completely around sample wells	Increase TEMED/AP
	Too much pressure applied to gel when pouring	Screws on the gel assembly apparatus should not be more than "thumb tight"
	Particulate matter in gel	Filter and mix gel reagents prior to preparing gel
	Excessive/uneven heating of gel	Decrease running voltage/provide cooling
	Bubbles in gel due to dirty plates	Wear gloves when handling plates
		Clean plates with ethanol and deionized H_20
	Bubbles in gel from air introduced from pouring device (syringe or pipette)	Do not expel entire volume of gel mix
	Bubbles under comb from trapped air	Insert comb at an angle and reposition before gel solidifies

D. Problem	Cause(s)	Solution
17. Gel fails to polymerize	Failure to add TEMED and AP	Repeat with TEMED and AP
	AP solution is stable only a few days at 4°C	Prepare fresh AP
	Oxygen inhibits polymerization	Layer gel with isopropanol before pouring stacker
18. Gel polymerizes too slowly	Insufficient TEMED/AP	Increase amount of TEMED/AP
	AP solution losing activity	Prepare fresh AP
19. Gel polymerizes too quickly	Excessive amount of TEMED/AP	Reduce amount of TEMED and AP, keeping the ratio the same
20. Run time unusually long	Running buffer too concentrated	Check protocol; dilute buffer if necessary
	Insufficient current	Increase voltage
21. Run time unusually short	Buffer too dilute	Check protocol; replace buffer if necessary
22. Dye front "smiling"	Migration too fast	Decrease voltage
	Heat generated	Decrease voltage; provide cooling
23. Dye front slanted	Bubble trapped between glass plates at the bottom of gel	Hold gel at an angle; place corner into lower buffer chamber; slowly move to horizontal position

E. Problem	Cause(s)	Solution
24. Insufficient blocking	Biotin in milk incompatible with streptavidin system, or milk contains antigen of interest	Use BSA
	If using AdvanBlock-PF with WesternBright MCF, some primary antibodies may require a protein blocker	Include BSA or milk in AdvanBlock-PF solution used to dilute the primary antibody
	Milk solution diluted too much	Increase to 5% milk solution
	Blocking time too short	Increase incubation time
	Some detergents not as effective in cold temperatures	Use 1 hr RT incubation instead of overnight at 4°C
25. Inappropriate wash conditions	Insufficient number of washes	Increase number of washes or duration of each wash step
	Insufficient detergent concentration	Increase detergent concentration or use stronger detergents (SDS, NP-40)
26. Reagent contamination	Bacterial or fungal growth in buffers	Check all buffers for turbidity; prepare new
27. Membrane choice	PVDF membranes may have higher background than nitrocellulose	Try nitrocellulose membranes
	Some membranes have high autofluorescence	Use only low-autofluorescence PVDF membranes with fluorescent Western blots
	Membrane dried out	Ensure membrane is hydrated during all steps
28. Non-specific binding of primary or secondary antibody	Concentration of antibody too high or antibody not affinity purified	Decrease antibody concentration; try monoclonal antibody or affinity purified
	Too much protein on gel	Decrease amount of protein loaded
29. Image overexposed	Time of exposure to film or CCD camera is too long	Reduce exposure time; if not possible, increase antibody dilutions or load less sample

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