101 western blotting troubleshooting tips

OVER 16,000 ELISA KITS

ASPIRE
TO CURE

NO BANDS OBSERVED

Problems	Explanation
1. Incorrect primary antibody	Antibody has low to no affinity.
2. Inactive antibody	Perform a dot blot.
3. Insufficient protein concentration	Increase the amount of protein and use a positive control.
4. Poor transfer	Make sure the membrane is activated. Transfer buffer must contain methanol when using nitrocellulose membranes. PVDF membranes must be pre-soaked with methanol.
5. Incomplete Transfer.	To ensure the transfer is complete, stain the membrane with India Ink, Amido Black or Ponceau S.
6. Over Transfer.	Reduce time of transfer or voltage.
7. Incorrect secondary antibody	Confirm host species and IgG type of primary.
8. Antibodies expired	Check that all antibodies are in date.
9. Incorrect storage of antibodies	Ensure all antibodies are stored as per manufacturer's instructions.
10. Suboptimal primary antibody incubation time	Increase incubation time with the primary antibody.
11. Incompatible primary and secondary antibody	Maintain a consistent species in both antibodies.
12. Insufficient secondary antibody concentration	Increase the concentration of primary/secondary antibody.
13. Excessive washing	Reduce the number and duration of washes.
14. Incorrect orientation	Mark your membrane to ensure correct orientation.
15. Contaminated wash or incubation buffer.	Use fresh, sterile buffer.
16. Insufficient exposure time when imaging.	Re-image the blot with a longer exposure time.

NO BANDS OBSERVED

Problems	Explanation
17. Incorrect filter settings.	Ensure the detection instrument is set to read the correct wavelengths.
18. Reduced efficacy of antibodies due to overuse.	Use fresh primary and secondary antibodies for each experiment.
19. Absence of protein of interest.	Run a positive control.
20. Sodium Azide contamination.	Sodium Azide contamination will quench HRP signal.
21. Isoelectric point >9.	Use a different buffer system with higher pH, e.g. CAPS (pH 10.5).

FAINT BANDS (WEAK SIGNAL)

Problems	Explanation
22. Insufficient antibody concentration.	Increase the antibody concentration 2-4 fold higher than starting concentration.
23. Insufficient protein concentration.	Increase total protein loaded on the gel.
24. Suboptimal antibody binding.	Reduce number of washes. Reduce NaCl concentration in the blotting buffer and in the antibody solution (recommended range = 0.15M - 0.5M).
25. Inactive conjugate.	Purchase new reagents or switch to ECL.
26. Old or weak ECL.	Use new ECL reagents.

TOO MANY BANDS

Problems	Explanation
27. Non-specific antibody.	Ensure the antibody used is specific for the protein of interest.
28. Proteolytic breakdown	Use protease inhibitors to prevent the proteolytic breakdown of the antigen.
29. Gel overloading.	Overloading the gel with too much protein can cause the development of "Ghost bands." Optimise protein concentration.
30. Insufficient blocking.	Extend the blocking time.
31. Low antigen concentration.	Consider immunoprecipitating target protein.
32. Non-specific secondary antibody binding.	Use secondary antibody only control. If bands develop use a different secondary antibody.
33. Analyte aggregation.	Increase DTT concentration.
34. Analyte degradation.	Make fresh samples. Reduce the number of freeze/thaw cycles of the sample. Add protease inhibitors to a sample before it is stored.
35. Protein degradation.	Target protein of interest degraded.
36. Splice variants.	Could lead to the visualisation of multiple bands.
37. High primary antibody concentration.	Use a lower concentration of primary antibody.
38. Protein may form multimers.	If samples are insufficiently reduced, proteins will form dimers, trimers or multimers due to disulfide bond formation. During sample preparation, boil the sample for longer in Laemmli buffer to prevent this.
39. Cells passaged too many times.	Use the original non-passaged cell line.

TOO MANY BANDS

Problems	Explanation
40. Antibodies are not purified.	Use affinity purified antibodies.
41. The bands are non-specific.	Use blocking peptides to differentiate between specific and non-specific bands.
42. Post translational modification.	Protein sample has multiple modified forms e.g. acetylation, methylation and phosphorylation. Check the literature to see if multiple bands have been reported.
43. Ionic interactions.	Increase salt concentration of incubation buffers. Include stronger detergent in washing step.

WRONG BAND SIZES

Bands have lower MW than expected

Problems	Explanation
44. Samples have been digested/degraded.	Use fresh sample. Use lysis buffer with proteinase inhibitors.
45. Primary antibody detecting splice variants.	Identify splice variants for your protein. Try a different primary antibody.
46. Primary antibody binding to a similar epitope on a different protein.	Run negative control to detect proteins that react with your antibody.

Bands have higher MW than expected

Problems	Explanation
47. Protein aggregation.	Decrease protein concentration. Prepare new sample with fresh loading buffer.
48. Incomplete denaturation.	Denature protein with urea.
49. Proteins are glycosylated.	Use enzymatic treatment to strip post-translational modifications.

POOR QUALITY TRANSFER

Problems	Explanation
50. Membrane choice.	Choose either PVDF/nitrocellulose (NC) membranes according to the target protein molecular weight.
51. Dry membrane.	It is important not to let the membrane or filter paper dry out.
52. Incomplete protein resolution.	Ensure optimal gel concentration is used for the protein of interest.
53. Incorrect sample preparation.	The sample must contain DTT or B-Mercaptoethanol and be heated prior to loading.
54. Incorrect assembly of membranes.	PVDF and NC membranes should be oriented on the anode (+) side of the gel.
55. Incorrect transfer time.	Increasing transfer time can improve protein molecule transfer.
56. Insufficient power supply.	Membranes can be placed on either side of the gel in the case that the power supply is incorrectly connected.

HIGH BACKGROUND

Problems	Explanation
57. Non-specific antibody binding.	Ensure the correct and most specific primary antibody is used.
58. Insufficient blocking.	Optimise blocking time duration.
59. Incomplete blocking.	Optimise choice of blocking buffer. Increase protein concentration in blocking agent.
60. Incompatible blocking agent.	Compare different blocking buffers.
61. Incorrect membrane choice.	Nitrocellulose membranes generally have less background compared to PVDF.

HIGH BACKGROUND

Problems	Explanation
62. Film overexposed.	Ensure the correct and most specific primary antibody is used.
63. Secondary antibody binding to the blocking reagent.	Optimise blocking time duration.
64. Incubation temperature is too high.	Optimise choice of blocking buffer. Increase protein concentration in blocking agent.
65. Excessive incubation.	Compare different blocking buffers.
66. Too much substrate (when using enzyme-conjugated antibody).	Dilute substrate. Reduce substrate incubation time.
67. Protein is overloaded.	Dilute sample concentration or reduce load.
68. Contamination of membranes, solutions, antibody containers or trays.	Wear clean gloves. Use forceps to handle membranes. Use clean glassware and distilled water to prepare solutions. Run cleaning protocol with cleaning buffer.
69. Non-fat dry milk may contain the target antigen.	Substitute with 3% BSA.
70. Suboptimal antibody concentration.	Optimise antibody concentration.
71. Insufficient washing.	Increase the number of washes performed. Increase the concentration of Tween 20 used in wash buffer.
72. Blot has dried out.	Cover the membrane in buffer during incubation.
73. Cross-reactivity of antibody with other proteins.	Reduce secondary antibody concentration. Use different blocking agent.

SPOTS OR SMUDGES ON THE GEL

Black dots or speckled background

Problems	Explanation
74. Blocking reagent has clumped together. The antibodies are binding to it.	Binding will appear as dots. Filter the blocking agent.
75. Contamination of gel or reagents.	Use fresh, sterile buffer.
76. Exposure time is too long.	Reduce the exposure time.
77. Insufficient amount of solution during washing or incubation.	Fully immerse the membrane during antibody incubations and washes.
78. Contamination of equipment.	Ensure the electrophoresis equipment is properly washed. Wash the membrane thoroughly.
79. Uneven agitation.	Place on a rocker or shaker to ensure uniform agitations during incubations.
80. Secondary antibody aggregation.	Increase secondary antibody dilution. Spin down antibody aggregates.
81. Membrane dried unevenly.	Thoroughly wet the membrane before starting the protocol. Ensure the membrane does not dry out.

White spots or smudges

Problems	Explanation
82. Air bubbles trapped against the membrane.	Remove any air bubbles trapped between the membrane and the gel during transfer.

BANDS APPEAR VERY LOW

Problems	Explanation
83. The gel has been running for too long.	Try running the gel for a shorter period of time.
84. Insufficient amount of acrylamide in the gel.	Run lower molecular weight proteins in gels with a higher percentage of acrylamide.

BANDS APPEAR VERY HIGH

Problems	Explanation
85. The gel has not been running for long enough.	Try running the gel for a longer period of time.
86. Too much acrylamide in the gel.	Run higher molecular weight proteins in gels with a lower percentage of acrylamide.

DISTORTED BANDS

Smile/Curve effect on the gel

Problems	Explanation
87. Voltage was too high during migration.	Check the protocol for the suggested voltage.
88. Gel was too hot during migration.	Run the gel at 4° C, on ice or in a cold room.

Uneven bands

Problems	Explanation
89. Gel has polymerized unevenly.	Check the gel recipe. Make sure the correct amount of TEMED has been added. Ensure the gel is covered entirely in buffer when setting.
90. Salt concentration varies between wells.	Ensure that the salt concentration is similar across different samples.

DISTORTED BANDS

Diffused or Streaked bands

Problems	Explanation
91. Excessive amount of protein on the gel.	Reduce the amount of protein loaded on the gel.
92. Membrane slipped during transfer.	Avoid moving the gel or membrane during transfer.
93. Contact between the membrane and the gel is incomplete during transfer.	Use thicker filter paper. Squeeze to remove excess buffer and air bubbles from between the gel and membrane.

Blurry bands

Problems	Explanation
94. Voltage for electrophoresis is too high.	Run gel for longer at a lower voltage.
95. Air bubbles trapped between membrane and gel.	Remove air bubbles by squeezing with a sterile glass rod.
96. Incorrect loading buffer composition.	Mix a new loading buffer.

WHITE BANDS (IF USING ECL METHOD)

Problems	Explanation
97. Antibody concentration is too high.	Dilute the antibody to the optimal concentration.
98. Generation of excessive signal.	Reduce the concentration of the protein or antibody.

WHITE BANDS (IF USING ECL METHOD)

Problems	Explanation
99. Overexposure during visualization.	Decrease exposure time.
100. The blot was moved during transfer.	Avoid moving the membrane or gel during transfer.
101. Loading sample was too concentrated.	Reduce the amount of sample loaded.

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Dublin, Ireland | London, UK | +353 1 887 9802 (IRE) | T: +44 20 8123 7624 (UK)