

# WESTERN BLOTTING TIPS AND TROUBLESHOOTING GUIDE

## TIPS FOR SUCCESSFUL WESTERN BLOTS

### TROUBLESHOOTING GUIDE

1. **Suboptimal protein transfer.** This is the most common complaint with western blotting and could be due to many different problems listed below.

- a) Low quality membrane used for transfer. High quality PVDF membrane is the blotting membrane of choice as low molecular weight proteins are sometimes transferred through nitrocellulose membranes and therefore may be not visible on the blot.
- b) Improper Western transfer procedure.
- c) For semi-dry Western transfers make sure the filter paper is wet.
- d) SDS-PAGE gel incorrectly made and/or run.
- e) Use unstained gels for transfer, as stained proteins are transferred with lower efficiency.
- f) Improper sample preparation for gel loading. Proper migration depends on protein samples containing SDS, dithiothreitol (DTT) or 2-mercaptoethanol and must be heated prior to loading. Use prestained protein to monitor of the transfer.

2. **Current is much higher than the expected start current.**

- a) Transfer buffer too concentrated. Prepare fresh buffer or dilute concentrated solution.

**Important- Make sure Tris base was used and not Tris-HCl when transfer solution was made.**

3. **Power supply current reading on power supply is zero when switched on.**

- a) Connection to power supply is not complete. Check connections between lid of apparatus and the electrode assembly jacks. Check connections between lid of apparatus and power supply.
- b) Insufficient buffer level. Make sure the upper buffer chamber doesn't leak. Verify that the assembly was properly done.
- c) Ionic strength of the buffer is too high.

- d) Power supply is operating at a current close to its limit. Use a power supply with higher limits.
- e) Broken electrode. Check wire electrodes to make sure they are not broken.
- f) Blown fuse on power supply. Check fuse and replace, if necessary.

### **3. Too many bands on a Western blot.**

- a) Antibody is not specific for the protein of interest. Reduce primary antibody concentration. Reduce the amount of total protein loaded on gel. Use monospecific or antigen affinity purified antibodies.
- b) Proteolytic breakdown of the antigen. This is not uncommon, particularly if samples are stored for prolonged time or if proteins or membranes are fractionated after homogenization of the starting tissue. All additional bands are of lower apparent molecular mass than the full-length protein. Addition of protease inhibitors could help prevent this.
- c) Too much protein per lane or detection system too sensitive. Overloading of the gel is one of the most common reasons for "ghost bands". Immobilized proteins may provide a concentrated adsorptive surface to which certain IgG may bind nonspecifically. Similarly, such nonspecific binding may be uncovered when highly sensitive detection systems such as enhanced chemoluminescence are employed. A dilution series of the starting material usually clarifies which of the signals are artefactual.
- d) Inefficient blocking. A variety of different blocking agents are described in the literature including nonionic detergents and/or proteins. Change of the blocking conditions may remedy the problem.
- f) Concentration of antigen too low. The resolution of SDS-PAGE is limited. If the relative concentration of the antigen of interest is too low (less than 0.2% of total protein), it may be difficult to detect. Signal enhancement may then lead to the appearance of artificial bands. Enrichment of the antigen by fractionation or by immunoprecipitation should be considered.
- g) 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. Add 0.1 - 0.5% Tween® 20 to 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. Increase number of washes. Increase NaCl concentration in Blotting Buffer used for antibody dilution and wash steps (recommended range 0.15M - 0.5M).
- h) 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.

- i) Degradation of Analyte. Minimize freeze/thaw cycles of sample. Add protease inhibitors to sample before storage. Make fresh samples.
- j) Contamination of reagents. Check buffers for particulate or bacterial contamination. Make fresh reagents.

#### **4. No Bands Observed**

- a) Insufficient antibody. Some antibodies have low affinity to the protein of interest. Increase antibody concentration (2-4 fold higher than recommended starting concentration).
- b) Antibody may have lost activity. Perform a Dot Blot.
- c) Insufficient protein. Increase the amount of total protein loaded on gel. Use a positive control (recombinant protein, cell line or treat cells to express analyte of interest).
- d) Poor transfer. Wet PVDF/Immobilon-P membrane in methanol or nitrocellulose membrane in transfer buffer before use. Ensure that there is good contact between PVDF or nitrocellulose membrane and gel.
- e) Incomplete transfer. Optimize transfer time. High MW protein may require more time for transfer. To ensure transfer is complete, stain the membrane with Ponceau S, Amido Black or India Ink. Use prestained MW marker to help optimize transfer.
- d) Over transfer. Reduce voltage or time of transfer for low molecular weight proteins (< 10 kDa).
- e) Isoelectric point of protein of interest is >9. Use alternative buffer system with higher pH such as CAPS (pH 10.5).
- f) Incorrect secondary antibody used. Confirm host species and Ig type of primary antibody.
- g) Old antibody. If antibody is expired or past manufacturer warranty, purchase fresh antibody.
- h) Incorrect storage of antibodies. Follow manufacturer's recommended storage and avoid freeze/thaw cycles.
- i) Sodium Azide contamination. Make sure buffers do not contain Sodium Azide as this will quench HRP signal.
- j) Insufficient incubation time with primary antibody. Extend incubation time to overnight at 4°C.

#### **5. Faint Bands (Weak Signal)**

- a) 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).
- b) Insufficient antibody. Antibody may have low affinity to protein of interest. Increase antibody concentration (2-4 fold higher than recommended starting concentration).
- c) Insufficient protein Increase the amount of total protein loaded on gel
- d) Weak/Old ECL. Use new ECL reagents.
- f) Non-fat dry milk may mask some antigens. Decrease milk percentage in Block and Antibody Solutions or substitute with 3% BSA.

## 6. High Background

- a) Non-specific binding of primary antibody. Use monospecific or antigen affinity-purified antibodies. Block in 5% milk. Adjust the milk (2-5%) or NaCl (0.15-0.5M) concentrations of primary Antibody Solution.
- b) 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.
- c) 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. Non-fat dry milk contains endogenous biotin and is incompatible with avidin/streptavidin. Substitute with 3% BSA. Some IgY antibodies may recognize milk protein Substitute with 3% BSA.
- d) Insufficient wash. Increase number of washes. Increase Tween 20 concentration in Wash Buffer (0.1%-0.5%).
- e) Non-Specific Binding of Primary Antibody. Increase NaCl concentration in primary Antibody Solution and Blotting Buffer used for dilution of primary antibody and wash steps (recommended range 0.15M - 0.5M).
- f) Film overexposed. Reduce exposure time. If target signal is too strong wait 5-10 minutes and re-expose to film.

## 7. Diffuse Bands

a) Excessive protein on gel. Reduce amount of protein loaded

### **8. White Bands (ECL method)**

a) 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.

### **9. Patchy uneven spots all over the blot**

a) Contamination of reagents. Check buffers for particulate or bacterial contaminate. Make fresh reagents.

b) Not enough solution during incubation or washing. Make sure membrane is fully immersed during washes and antibody incubations.

c) Air bubble trapped in membrane. Gently remove any air bubbles. Especially during transfer.

d) Uneven agitation during incubations. Ensure uniform agitation by placing on a rocker/shaker.

e) 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.

f) HRP aggregation. Filter conjugate to remove HRP aggregates.

g) Long exposure. Reduce exposure time.