

Western Blot Troubleshooting Guide

Problem: Spots on protein bands or uneven spots formed on the membrane

Causes:

- Trapped air bubbles against the membrane and the gel during transfer
- Air bubbles between X-ray film and membrane during exposure
- Membrane not hydrated evenly

Solutions:

- Use a roller to smooth out any trapped air bubbles when preparing for transfer
- Remove air bubbles before exposing blot to film
- Keep membrane hydrated

Problem: Excess signal (e.g: blot glows in the dark) or high background

Causes:

- Too much HRP
- Inadequate blocking or washing of membrane
- Overexposed film
- Too much antigen or primary antibody being used

Solutions:

- Further dilute or decrease the amount of HRP-conjugate concentration
- Increase the duration for blocking and increase the number and volume of washes
- Change or adjust primary antibody concentration (for example: use 0.5-4µg/ml of primary antibody)

Problem: Non-specific binding or multiple bands observed

Causes:

- Too much HRP
- Primary antibody concentration is too high
- SDS from gel may cause nonspecific binding
- The target in protein sample has been digested
- Protein samples has multiple modified forms due to acetylation, methylation, myristylation, phosphorylation and glycosylation

Solutions:

- Further dilute or decrease the amount of HRP-conjugate concentration
- Change or adjust primary antibody concentration (e.g.: use 0.5-4µg/ml of primary antibody)
- Allow gel to immerse in transfer buffer for a few minutes (3-5minutes) before transferring
- Use fresh antibody and keep antibody at 4°C or include adequate protease inhibitor in sample buffer

- Use an agent to dephosphorylate and de-glycosylate, etc. the protein to obtain the right size

Problem: **Weak or no signal**

Causes:

- Insufficient antigen
- Not enough primary antibodies
- Inefficient protein transfer
- Low HRP or substrate activity
- Excessive washing of the membrane
- Protein of interest is found abundantly in the tissue
- Secondary antibody inhibited by Sodium Azide

Solutions:

- Load at least 20-50µg protein per lane
- Adjust primary antibody concentration (e.g., use 4 µg/ml of primary antibody) or incubate longer with primary antibody (e.g. overnight at 4°C)
- Optimize transfer procedure, use Ponceau S. (Cat.# 6411-200) to check whether protein is transferred to the membrane
- Make sure HRP-conjugate is diluted properly
- Do not over wash the membrane
- Prepare nuclear lysate for protein found in the nuclear
- Sodium Azide inhibits peoxidase activity

Problem: **Speckled background**

Causes:

- Over-heating during electrophoresis or transfer
- Aggregate formation in the HRP-conjugate

Solutions:

- Control temperature during electrophoresis or transfer
- Filter HRP-conjugate before using

Problem: **Molecular marker lane is black**

Cause:

- Antibody reacting with molecular marker

Solution:

- Add a blank lane between marker and the first sample

Problem: **White bands with black background, yellow or black bands form on the membrane**

Cause:

- Too much HRP

Solution:

- Further dilute or decrease the amount of HRP-conjugate concentration