CD Creative Diagnostics®

WB TROUBLESHOOTING TIPS

Creative Diagnostics

Abstract

Western blot is a widely used analytical technique used to detect specific proteins in a sample of tissue homogenate or extract.

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Western blot troubleshooting tips

Solve your western blot problems with these troubleshooting tips, covering common causes of unusual or unexpected bands, no bands, faint bands or weak signal, high background on the blot and more.

Problems Index

Western blot is a technique that is very useful for protein detection as it allows the user to quantify the protein expression as well.

- **Unusual or unexpected bands**

  Possible causes and corresponding solutions

  1. Protease degradation produces bands at unexpected positions

     Use a fresh sample which had been kept on ice or alter the antibody.
  2. Protein quaternary structure not broken

     If the protein seems to be in too high of a position, then reheating the sample can help to break the quaternary protein structure.
  3. Non-specific protein

     Many factors can alter the predicted molecular weight of a protein, including post-translational modifications (such as glycosylation), protein processing (cleavage from a pro-form to a mature form), isoforms from alternative splicing, multimer formation, and amino acid charge.

     To confirm specificity, use a control such as recombinant protein or overexpression lysate, a downregulated knockdown/knockout lysate, a peptide competition of the primary antibody, or a treatment known to effect the expression of the protein.

4. High voltage or air bubbles present during transfer causing blurry bands

   Ensure that the gel is run at a lower voltage, and that the transfer sandwich is prepared properly. In addition, changing the running buffer can also help the problem.

5. Too fast of a travel through the gel results in nonflat bands

   To fix this the gel should be optimized to fit the sample.

6. White (negative) bands on the film

   Often due to too much protein or antibody.

➢ **No bands, faint bands or weak signal**

- No or faint bands can cause by many reasons related to antibody, antigen, or buffer used.

1. Improper antibody or antibody concentration

   If an improper antibody is used, either primary or secondary, the band will not show. In addition, the concentration of the antibody should be appropriate as well; if the concentration is too low, the signal may not be visible. It is important to remember that some antibodies are not to be used for western blot.

2. Very low concentration or absence of the antigen causing no visible bands

   Double check that the protein you are studying really is the species you think it is. Antibody epitopes are species specific. Antigen from another source can be used to confirm whether the problem lies with the sample or with other elements, such as the antibody. Checking this may require some notebook and paper digging, sequencing of vectors or other types of controls.

   If weak signals caused by low concentration of antibody or antigen, increasing exposure time can also help to make the band clearer.

3. Prolonged washing decreased the signal

   Control washing time.

4. Methanol concentration may be too high.

   Too high concentration of methanol may result in the separation of protein and SDS and thus cause protein precipitation in the gel. At the same time, it may cause shrinking or hardening of the gel to inhibit transferring of high molecular weight proteins. As a result, choose suitable methanol concentration according to different molecular weight.

5. Problematic Buffers

   Buffers can also contribute to the problem. Make sure that buffers like the transfer buffer, TBST, running buffer and ECL are all new and non-contaminated. If the buffers are contaminated with sodium azide, it can inactivate HRP.

6. Nonfat dry milk masking the antigen

   Use BSA or decrease the amount of milk used.

- A bad gel

  Even if you do everything right - you followed the recipes carefully, minded you pH, used fresh reagents, meticulously cleaned your plates - things can still go wrong with your gel.

1. Messed up wells

Properly made wells should be uniform and rinsed.

2. Bubbles or blotting paper in the gel

   These blemish will impair protein migration.

3. Gel dried out

   If your gel can be seen shrinking from the edge, it is drying out. Such a gel will not run properly.

4. Gel is runny

   Before you use your gel, gently tilt it to ensure that it is completely set up.

- Transfer problems

✓ Some stupid mistakes in transfer, for example:

1. Wrong choice of membrane

   Choose suitable pore size membrane. Use 0.45um size membrane for proteins larger than 22KD. Use 0.2 μm size membrane for proteins smaller than 22 KD.

2. Handle the membrane improperly

   Handle membrane with gloves, and make sure that there are no pressed marks if using laser capture.

3. Bubbles exist in the sandwich

   Make sure there are no bubbles, including on the hydrophobic membrane, and everything in the sandwich well hydrated.

4. Gel not put in the correct place

   Double (or triple) check that the gel is in the correct place relative to the membrane, and that you know the orientation of your lanes.

5. Watch the voltage and amps

   Do not just hit “transfer” and walk away. Stay a few minutes to make sure that your voltage and amps are okay.

✓ Disappearing Transfer

6. Transferring the gel too quickly and hot

   To avoid this problem, simply run your transfer at a lower voltage for a longer time.

7. Poorly fitting sandwich

   A common source of transfer problems is the tightness of the sandwich. A good sandwich is tight. Make sure yours is too, by using extra sponges if needed; using appropriately thick filter paper (too thick can cause issues); and as always insure that all of your sandwich components are clean, hydrated and free from bubbles.

8. Small proteins ran away

   Small proteins move faster than large proteins during transfer and can actually transfer through the membrane. If you are working with a small protein, test different transfer times and use two membranes during the transfer.

9. Large proteins never moved

   Large proteins have the opposite problem. Some large proteins may not transfer at all. Consider adding a small amount of SDS to the transfer buffer and increasing the transfer time if this is your problem.

- Poor Development

10. Developing reagent gone bad

The most common problem when using HRP conjugated secondary antibodies is that the developing reagent, which contains hydrogen peroxide, has gone bad due to age or contamination. Make sure the developing reagent isn't having problems.

➢ **Smeared bands/lanes**

Possible causes and corresponding solutions

1. Protein load

Use less total protein loaded into each lane. You may perform an immunoprecipitation first to enrich your target in the lysate.

2. Antibody concentration

Lower the concentration of your primary antibody.

➢ **High background on the blot**

Possible causes and corresponding solutions

1. Too much antibody

High background is often caused by too high concentration of the antibody, which can bind to PVDF membranes.

2. Buffers are too old

Replace new buffers.

3. Insufficient blocking

Block for one full hour at room temperature. Use 3% BSA instead of milk.

4. Incompatible blocking buffer

(a) Do not use milk if you're using a phospho-specific antibody or an avidin/streptavidin secondary as milk contains casein and biotin.

(b) Do not use milk or BSA if your secondary is anti-bovine, anti-goat, or anti-sheep as these will recognize the bovine IgG in the block. Instead, use 5% serum from the host species of the secondary antibody.

5. Not enough washing time

Increasing the washing time help to decrease the background.

6. Dry Membrane

Take care to prevent drying of the membrane after applying the antibodies.

7. Too high of an exposure can lead to this problem

It is advisable to check different exposure times to achieve an optimum time.

➢ **Speckled or swirly background**

Possible causes and corresponding solutions

1. Membrane polluted during handling

Minimize any contact with the membrane or gel. Use only clean tools to manipulate them. Never directly touch with your hands.

2. Buffer contamination

Use fresh buffers.

3. Air Bubbles

Bubbles between the membrane and gel during transfer can cause spots. Roll out any bubbles before closing sandwich cassette.

4. HRP Aggregation
   Filter the secondary with a 0.2 μm filter to remove any aggregates.
5. Insufficient Washing
   Wash membranes on an orbital shaker at high speed with a large volume of washing buffer. Wash for a full 5 minutes for 5 times.

- **Invisible dots on the bands**

Air bubbles were trapped in the gap of gel and membrane during transferring

Remove bubbles in the gap of gel and membrane when preparing for transferring.

- **Incomplete bands**

Substrate is not well-distributed during incubation

Even the substrate during incubation.

- **Smile effect of the bands**

1. Migration was too fast during electrophoresis
   Reduce the voltage to slow down the migration.
2. Migration was too hot
   Run the gel in the cold room or in ice.

- **Patchy or uneven spots on the blot**

Possible causes and corresponding solutions

1. Improper transfer
   If there are air bubbles trapped between the gel and the membrane, it will appear darker on the film.
2. Uneven agitation during the incubation
   It is important to use a shaker for all incubation.
3. Insufficient washing
   Washing is of utmost importance as well to wash the background.
4. Antibodies binding to the blocking agents
   In this case another blocking agent should be tried. Filtering the blocking agent can also help to remove some contaminants.
5. Aggregation of the secondary antibody
   Centrifuged and filtered the secondary antibody to remove the aggregated.

- **Black dots on the blot**

Possible causes and corresponding solutions

1. Air bubbles were trapped against the membrane during transferring or the antibody is not well distributed during incubation
   Try to remove bubbles. Keep shaking when incubating the antibody.
2. The blocking agent was not well dissolved
   Filter the blocking agent.

3. The antibody reacts with the blocking solution

   Choose suitable blocking solution.

4. There are aggregates in the HRP conjugated secondary antibody

   Filter the secondary antibody agent and remove the aggregates.

- **Marker lane is black**

  The antibody reacts with the MW marker

  Add a blank lane between the marker and the adjacent sample lane.