

abcam

# Western blot troubleshooting tips

Solve common western blot problems

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## No signal

### The primary antibody and the secondary antibody are not compatible

Use a secondary antibody that was raised against the species in which the primary was raised (eg if the primary is raised in rabbit, use an anti-rabbit secondary).

### Not enough primary or secondary antibody is bound to the protein of interest

Use a higher concentration of antibody or incubate longer (eg overnight) at 4°C.

### There is cross-reactivity between the blocking agent and the primary or secondary antibody

Use a mild detergent such as Tween 20 or switch blocking reagent (ie commonly used blocking reagents are milk, BSA, serum or gelatin).

### The primary antibody does not recognize the protein in the species being tested

Check the datasheet or perform a BLASTp alignment to see whether your antibody should react with the target protein. Run the recommended positive control.

### There is insufficient antigen

Load at least 20–30 µg protein per lane, use protease inhibitors and run the recommended positive control.

### The protein of interest is not abundantly present in the tissue

Use an enrichment step to maximize the signal (eg prepare nuclear lysates for a nuclear protein).

### There is a poor transfer of protein to membrane

Check the transfer with a reversible stain such as Ponceau S. If proteins have not transferred effectively, check the transfer was not performed in the wrong direction. If using PVDF membrane, make sure that you pre-soak the membrane in methanol and then in transfer buffer.

### Excessive washing of the membrane

Reduce the number or duration of washing steps.

### Overuse of the primary antibody

Use fresh antibody as the effective concentration is lowered upon each use.

### The secondary antibody is inhibited by sodium azide

Do not use sodium azide together with HRP-conjugated antibodies.

### The detection kit is old and the substrate is inactive

Use fresh substrate.

## High background

### Blocking of non-specific binding might be absent or insufficient

Increase the blocking incubation period and consider changing the blocking agent, we recommend 3–5% non-fat dry milk, BSA, or normal serum for 1 h at room temperature. These can be included in the antibody buffers as well.

### The primary antibody concentration may be too high

Titrate the antibody to the optimal concentration. Incubate for longer but in more dilute antibody (a slow but targeted binding is best).

### The incubation temperature may be too high

Incubate membrane at 4°C.

### The secondary antibody may be binding non-specifically or reacting with the blocking reagent

Run a secondary control without the primary antibody.

### Cross-reactivity between the blocking agent and primary or secondary antibody

Add a mild detergent such as Tween 20 to the incubation and washing buffer.

For phospho-specific antibodies: milk contains casein which is a phospho-protein; the phospho-specific antibody will detect casein present in the milk causing high background. Use BSA as a blocking reagent instead of milk.

### The washing of unbound antibodies may be insufficient

Increase the number and time of washes.

### Your choice of membrane may give high background

Nitrocellulose membranes are considered to give less background than PVDF.

### The membrane has dried out.

Care should be taken to prevent the membrane from drying out during incubation.

## Multiple bands

### Cell lines that have been frequently passaged gradually accumulate differences in their protein expression profiles

Go back to the original non-passaged cell line and run these samples in parallel.

### The protein sample has multiple modified forms in vivo such as acetylation, methylation, myristylation, phosphorylation, glycosylation etc

Examine the literature and use an agent to remove modifications where possible so that the protein runs at the expected size.

### The target in your protein sample has been digested (more likely if the bands are of lower molecular weight)

Make sure that you incorporate sufficient protease inhibitors in your sample buffer.

### Unreported novel proteins or different splice variants that share similar epitopes and could possibly be from the same protein family are being detected

Check the literature for other reports and also perform a BLAST search; use the cell line or tissue reported on the datasheet.

### Primary antibody concentration is too high

Try decreasing the concentration of the primary antibody. Run a secondary antibody control (without the primary).

### The antibody has not been purified

Try to use affinity purified antibody. This will often remove non-specific bands.

### The bands may be non-specific

Where possible use blocking peptides to differentiate between specific and non-specific bands. Only specific bands should be blocked (and thus disappear).

### The protein target may form multimers

Try boiling in Laemmli buffer for 10 min rather than 5 min to disrupt multimers.

## Uneven white spots in the blot

Air bubbles were trapped against the membrane during transfer or the antibody is not evenly spread on the membrane

Make sure you remove bubbles when preparing the gel for transfer. Incubate the antibodies while agitating.

## Black dots on the blot

The antibodies are binding to the blocking agent

Filter the blocking agent

## White bands on a black blot (negative of expected blot)

Too much primary and/or too much secondary antibody

Dilute the antibodies more.

## Molecular weight marker lane is black

The antibody is reacting with the molecular weight marker

Add a blank lane between the molecular weight marker and the first sample lane.

## Band of interest is very low/high on the blot

Separation is not efficient

Change the gel percentage: use a higher percentage for small proteins and a lower percentage for large proteins.

## Smile effect on the bands

Migration was too fast

Decrease the voltage while running the gel.

Migration was too hot (changing the pH and altering the migration)  
Run the gel in the cold room or on ice.

## Uneven band size in lanes probed for the same protein

Gel has set too quickly while casting and the acrylamide percentage is not even throughout the gel

Review the recipe of the gel and the addition of TEMED to the gels, add some 0.1% SDS in water to the top of the migrating gel while it set to stop it from drying.

## Uneven staining of the gel

Contamination from bacteria

Keep antibodies at 4°C and use fresh buffers when covering the gel.

Not enough antibody volume

Make sure the membrane is covered with the antibody and incubate while agitating