Western Blot FAQs

Troubleshooting guide to western blot.

1. 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 (e.g. 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 more concentrated antibody. Incubate longer (e.g. overnight) at 4°C.

The cross-reaction between the blocking agent and the primary or secondary antibody.

Use a mild detergent such as Tween20 or switch blocking reagent (i.e. 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 Clustal Omega/ClustalW alignment to ensure 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; run the recommended positive control.

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

Use an enrichment step to maximize the signal (e.g. prepare nuclear lysates for a nuclear protein, etc.).

There is a poor transfer of protein to membrane.

Check the transfer with a reversible stain such as Ponceau S; check that the transfer was not performed the wrong way; if using PVDF membrane make sure you pre-soak the membrane in MeOH then in transfer buffer.

Excessive washing of the membrane.

Do not over-wash the membrane.

Too much blocking does not allow you to visualize your protein of interest.

Instead of using 5% milk in the antibody buffers try removing the milk or using 0.5%. Switch blocking reagents or block for less time.

Over-use of the primary antibody.

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

The secondary antibody is inhibited by sodium azide.

Do not sodium azide together with HRP-conjugated antibodies.

Detection kit is old and substrate is inactive.

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Use fresh substrate.

2. High background

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

Increase the blocking incubation period and consider changing the blocking agent, Abcam recommends 5% non-fat dry milk, 3% BSA, or normal serum for 30 min. 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 blot 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-reaction between blocking agent and primary or secondary antibody.

Add a mild detergent such as Tween20 to the incubation and washing buffer (phospho-specific protein). Milk contains casein which is a phospho-protein; this is why it causes high background because the phospho-specific antibody detects the casein present in the milk. Use BSA as a blocking reagent instead of milk.

The washing of unbound antibodies may be insufficient.

Increase the number of washes.

Your choice of membrane may give high background.

Nitrocellulose membrane is 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.

Your sample contains high levels of endogenous immunoglobulins.

Use our VeriBlot secondary antibodies which enable the trouble-free detection of immunoblotted target protein bands, without interference from denatured IgG.

3. 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 the original cell line samples in parallel.

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

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Examine the literature and use an agent to dephosphorylatem, etc. the protein to bring it to the correct 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 - at high concentration multiple bands are often seen.

Try decreasing the concentration. 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 SDS-PAGE for 10 minutes rather than 5 minutes to disrupt multimers.

4. Uneven white "spots" on 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 under agitation.

5. Black dots on the blot

The antibodies are binding to the blocking agent.

Filter the blocking agent.

6. White bands on a black blot (negative of expected blot)

Too much primary and/or too much secondary antibody.

Dilute the antibodies more.

7. MW marker lane is black

The antibody is reacting with the MW marker.

Add a blank lane between the MW marker and the first sample lane.

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8. The band of interest is very low/high on the blot

Separation is not efficient.

Change the gel percentage: a higher percentage for small protein, a lower percentage for lower proteins.

9. Smile effect on the bands

Migration was too fast.

Migration was too hot (changing the pH and altering the migration).

Slow down the migration or run the gel in the cold room or on ice.

10. Uneven band size in lanes probed for the same protein

Gel has set too quickly while casting and the acrylamide percentage is not even along the lanes.

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

11. Uneven staining of the gel

Contamination from bacteria

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

Not enough antibody

Make sure the membrane is covered with the antibody and incubate under agitation.

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