The western blot guide

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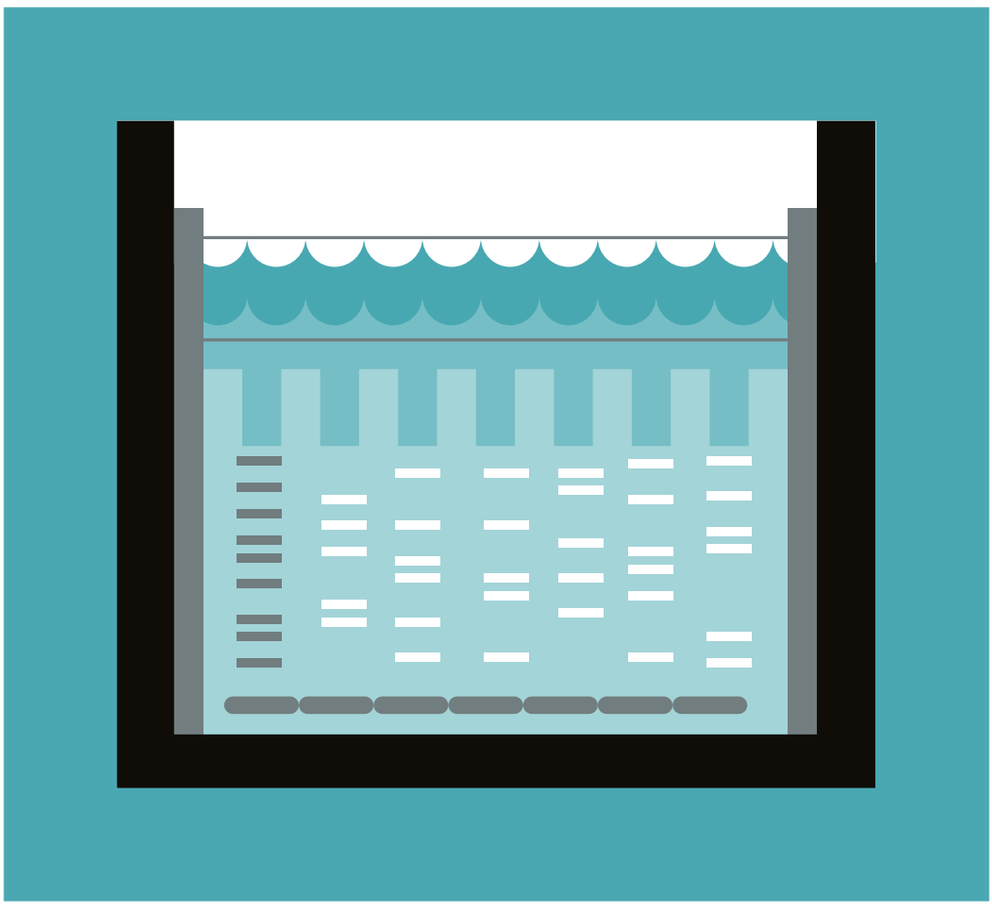

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Contents

An introduction to western blot

Here we introduce you to western blot basics, including choosing antibodies, the difference between indirect and direct labeling, and various methods of immunoblot detection.

What is a western blot?

Western blot, or western blotting, is a technique widely used in research to separate and identify specific proteins within a complex mixture. Western blot allows us to determine the relative protein levels between samples and establish the molecular weight of the target, which can provide insight into its post-translational processing. To achieve this, western blot implements three steps: (1) separation by size, (2) transfer to a solid support, and (3) visualizing target protein using primary and secondary antibodies.

In the first step, the proteins are separated based on size by gel electrophoresis (Figure 1). The gel is then placed in contact with the membrane, and the use of an electrical current induces the proteins to migrate from the gel to the membrane. Protein transfer to the membrane is essential because gels used for electrophoresis provide a poor surface for immunostaining, ie, antibodies don't stick to the proteins in the gel. The membrane can be further processed with antibodies specific to the target of interest and visualized using enzyme-linked or fluorophore-conjugated secondary antibodies and detection reagents.

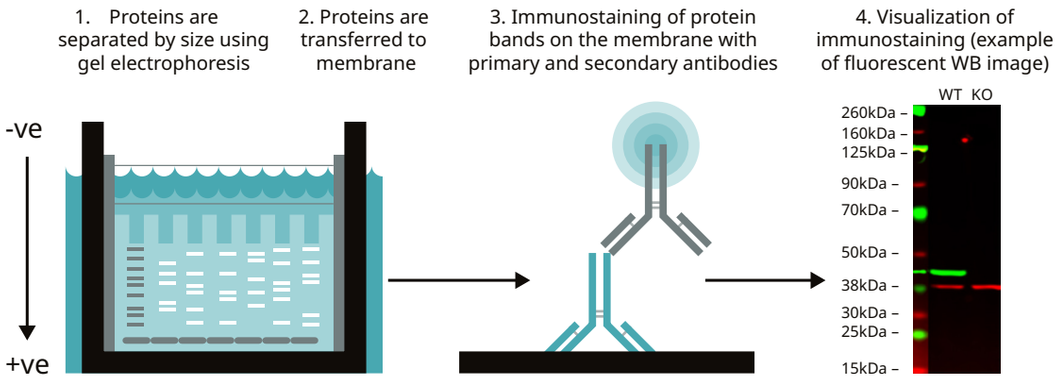

Figure 1. A simplified diagram of western blotting.

Choosing antibodies for western blot

Your western blot's sensitivity and specificity depend on the antibodies' quality and the experimental conditions they are used in. When working with tissue lysates or tissue culture supernatants containing serum and, therefore, endogenous immunoglobulins, you should select a primary antibody raised in species different from that of your sample. For example, if you are studying a mouse protein, choose a primary antibody raised in a species other than mouse (eg, primary antibody raised in rabbit). This is to avoid cross-reactivity of the secondary anti-immunoglobulin antibody with endogenous immunoglobulins in the sample. The choice of host species of the primary antibody is less critical when using samples that don't contain endogenous immunoglobulin.

Whenever possible, choose a primary antibody that has been knock-out (KO) validated to ensure it binds specifically to the intended target.

To visualize your protein, select a secondary antibody (against the host species of the primary antibody) that will bind to the primary antibody. Using an enzyme-linked secondary antibody, such as horseradish peroxidase (HRP)- or alkaline phosphatase (AP)-conjugate antibody, or a western blot-optimized fluorescence conjugate, offers a high level of sensitivity. Remember to check that the light emission wavelength of a conjugate is compatible with your reading device/scanner.

Compared to the primary antibody only, the increased sensitivity of conjugated secondary antibodies results from these antibodies binding to the primary antibody at multiple locations, which amplifies the signal. Therefore, using secondary antibodies is ideal for western blot since their signal amplification allows for easier detection of the protein of interest in a complex protein mixture.

Using directly conjugated primary antibodies (eg HRP-conjugates) in a western blot allows you to speed up and simplify the protocol, omitting the need for the secondary antibody step. When choosing primary antibody conjugates, pay attention to antibody specificity. Ideally, go for recombinant monoclonal antibodies, which provide high specificity and batch-to-batch consistency.

Compared to secondary antibodies, primary conjugates don't provide signal amplification, so your protein of interest should be abundant in the sample. Abcam offers a wide range of primary recombinant antibodies directly conjugated to HRP suitable for western blot. If your antibody of choice is not available in a suitable conjugated format, you can use abcam's antibody conjugation kits.

Western blot indirect and direct labeling

Before running a western blot, it is extremely important to research the target protein thoroughly. To learn more about the procedure, refer to our western blot protocol.

1. In a traditional western blot (indirect labeling), protein samples are first resolved by SDS-PAGE and then electrophoretically transferred to the membrane.
2. After a blocking step, the membrane is probed with a primary antibody that was raised against the antigen in question.
3. Following a washing step, the membrane is typically incubated with an enzyme-conjugated secondary antibody directed against the primary antibody. In case of fluorescent detection, a fluorophore-conjugated secondary antibody is used instead.
    - The fluorescence of the dye or activity of the enzyme, such as alkaline phosphatase (AP), glucose oxidase (GO) or horseradish peroxidase (HRP), is necessary for signal generation.
4. Finally, the membrane is washed again and incubated with an appropriate enzyme substrate (if necessary), producing a reportable signal.
    - Direct labeling analysis uses conjugated primary antibodies (eg, HRP-conjugated), which eliminates the need for the secondary antibody step, thereby simplifying the procedure, shortening the protocol, and expediting the time to results.

You can watch our on-demand western blot webinar for more information on the western blot procedure.

Immunoblot detection

There are several different choices of readout when western blotting. Each has advantages and disadvantages, which depend on your needs and the equipment available in your lab.

Colorimetric western blotting

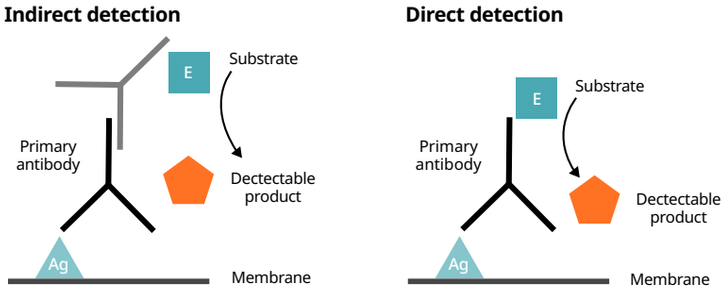

Figure 2. Schematic representation of colorimetric western blot detection. The left panel demonstrates indirect detection, while the right panel shows direct detection.

Colorimetric detection relies on generating a colored product that becomes deposited on the western blot, which is formed following the conversion of a chromogenic blotting substrate by an appropriate enzyme. The limited sensitivity of chromogenic substrates can make it difficult to optimize them for detecting proteins of low abundance. However, the chromogenic reaction can be allowed to develop for several hours (or even overnight) to allow the background signal to develop simultaneously.

In contrast, colorimetric substrates are perfect for detecting abundant proteins since the reaction can be monitored visually and allowed to progress until there is adequate color development before being stopped. No specialized equipment is required to visualize the colored precipitate, and the produced signal is highly stable.

Fluorometric western blotting

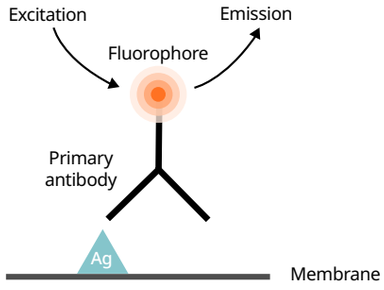

Figure 3. Schematic representation of fluorescent western blot detection.

Fluorometric detection requires the use of an antibody, which has been labeled with a fluorophore. A light source is used to excite the fluorophore, producing a transient light emission as it returns to its ground state. The light is emitted at a higher wavelength than that used for excitation and is detected with a specialized reader, such as a fluorimeter scan.

Chemiluminescent western blotting

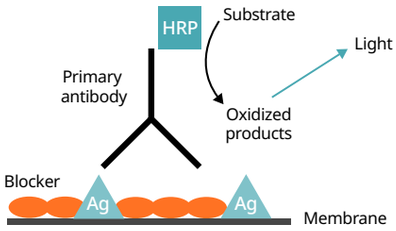

Figure 4. Schematic representation of fluorescent western blot detection.

Chemiluminescence occurs when a substrate is catalyzed by an enzyme and produces light as a by-product of the reaction. The limiting reagent in the reaction is the substrate – as this is exhausted, the light production decreases and eventually stops. However, a well-optimized procedure should produce a stable light output for several hours, allowing consistent and sensitive protein detection.

To visualize the light signal, you can use a developer machine if working with X-ray films or a western blot camera device, which requires no X-ray films.

Buffers and stock solutions for western blot

Your western blot experiment will require several buffers and stock solutions. Getting these right and prepared in advance, where possible, will save you time further along in the process.

Lysis buffers

Lysis buffers differ in their ability to solubilize proteins. The buffers containing sodium dodecyl sulfate (SDS) and other ionic detergents are the most efficient in extracting membrane proteins from lipid bilayers at a high yield.

When choosing a lysis buffer, the main consideration is whether the selected antibody will recognize denatured samples. When this is not the case, it will be noted on the antibody datasheet, and you should use buffers without detergent or with relatively mild non-ionic detergents (NP-40, Triton X-100).

Table 1. Protein location and lysis buffer choice.

Protein location	Buffer recommended
Whole cell	NP-40
Cytoplasmic (soluble)	Tris-HCl
Cytoplasmic (cytoskeletal bound)	Tris-Triton
Membrane bound	NP-40 or RIPA
Nuclear	RIPA or use nuclear fraction protocol*
Mitochondria	RIPA or use mitochondrial fraction protocol*

*Proteins found exclusively or predominantly in a subcellular location will be more enriched in a lysate of the subcellular fraction compared with whole cell or tissue lysates. This can be useful when trying to obtain a signal for a weakly-expressed protein. Please consult our separate protocols for subcellular fractionation.

All four of these buffers below can be kept at 4°C for several weeks or for up to a year if divided into aliquots and stored at -20°C.

NP-40 buffer

NP-40 is a popular buffer for studying cytoplasmic and membrane-bound proteins and for whole-cell extracts. Suppose you are concerned that the protein of interest is not being completely extracted from insoluble material or aggregates. In that case, RIPA buffer may be more suitable as it contains ionic detergents that will solubilize proteins more efficiently.

- 150 mM NaCl
- 1.0% NP-40 (possible to substitute with 0.1% Triton X-100)
- 50 mM Tris-HCl, pH 8.0
- Protease inhibitors

RIPA buffer (radioimmunoprecipitation assay buffer)

RIPA buffer contains the ionic detergent sodium deoxycholate as an active constituent and is useful for the lysis of whole-cell extracts and membrane-bound proteins. Also, RIPA buffer may be preferable to NP-40 or Triton X-100-only buffers for extracting nuclear proteins.

A RIPA buffer will disrupt protein-protein interactions and may, therefore, be problematic for immunoprecipitation (IP) and pull-down assays prior to western blot. When it's crucial to preserve protein-protein interactions or to minimize denaturation, you should use a buffer without ionic detergents (eg, SDS) and ideally without non-ionic detergents (eg, Triton X-100).

Cell lysis with detergent-free buffer is achieved by mechanical shearing, often with a Dounce homogenizer or by passing cells through a syringe needle. In these cases, a simple Tris buffer will suffice, but as noted above, buffers with detergents are required to release membrane- or cytoskeleton-bound proteins.

- 50 mM Tris-HCl, pH 8.0
- 150 mM NaCl
- 1% NP-40
- 0.5% sodium deoxycholate - can be prepared as a 10% sodium deoxycholate stock solution (5 g into 50 mL), which must be protected from light.
- 0.1% SDS (sodium dodecyl sulfate)
- Protease inhibitors
- Phosphatase inhibitors – if the samples will be used for IP before proceeding with western blot. Note that using phosphatase inhibitors will interfere with phosphatase treatment if this is required before WB.
- 1mM EDTA – optional*

*Some RIPA buffer recipes include 1mM EDTA – a chelator of divalent cations (an important cofactor of many enzymes, such as DNases and proteases) – to inhibit proteases, help dissociate ribosomal subunits and protein-RNA complexes, etc.

Tris-HCl

- 20mM Tris-HCl, pH 7.5
- Protease inhibitors

Tris-Triton buffer (for cytoskeletal-bound proteins extraction)

- 10 mM Tris, pH 7.4
- 100 mM NaCl
- 1 mM EDTA
- 1 mM EGTA
- 1 mM NaF

— 20 mM $Na_4P_2O_7$

— 2 mM Na_3VO_4

— 1% Triton X-100

— 10% glycerol

— 0.1% SDS

— 0.5% deoxycholate

Protease and phosphatase inhibitors

As soon as lysis occurs, proteolysis, dephosphorylation, and denaturation begin. These events can be slowed down significantly if samples are kept on ice or at 4°C at all times and appropriate inhibitors are added fresh to the lysis buffer.

Ready-to-use cocktails of inhibitors are available from various suppliers, but you can prepare your own inhibitor cocktail (Table 2).

Table 2. Preparation of your own protease/phosphatase inhibitor cocktails.

Inhibitor	Protease/ phosphatase inhibited	Final concentration in lysis buffer	Stock (store at -20°C)
Aprotinin	Trypsin, chymotrypsin, plasmin	2 µg/mL	Dilute in water, 10 mg/mL. Do not re-use thawed aliquots.
Leupeptin	Lysosomal	5–10 µg/mL	Dilute in water. Do not re-use thawed aliquots.
Pepstatin A	Aspartic proteases	1 µg/mL	Dilute in methanol, 1 mM.
PMSF	Serine, cysteine proteases	1 mM	Dilute in ethanol. You can re-use the same aliquot.
EDTA	Metalloproteases that require Mg^{2+} and Mn^{2+}	5 mM	Dilute in dH_20, 0.5M. Adjust pH to 8.0.
EGTA	Metalloproteases that require Ca^{2+}	1 mM	Dilute in dH_20, 0.5M. Adjust pH to 8.0.
Sodium fluoride	Serine/threonine phosphatases	5–10 mM	Dilute in water. Do not re-use once defrosted.
Sodium orthovanadate	Tyrosine phosphatases	1 mM	Dilute in water. Do not re-use once defrosted.
Sodium pyrophosphate	Serine/threonine phosphatase inhibitor	20 mM	Dilute in water.

Sodium orthovanadate preparation

Perform all the steps under the fume hood.

1. Prepare a 100 mM sodium orthovanadate solution with double distilled water.
2. Set pH to 9.0 with HCl.
3. Boil until colorless*.
4. Cool to room temperature. Minimize volume change due to evaporation by covering loosely.
5. Set pH to 9.0 again.
6. Boil again until colorless*.
7. Repeat this cycle until the solution remains at pH 9.0 after boiling and cooling*.
8. Bring up to the initial volume with water.
9. Store in aliquots at -20°C.
10. Discard if the samples turn yellow.

*Avoid large changes in volume during boiling; put a loose lid on the container to protect it from evaporation.

Soluble protein buffer

- 20 mM Tris-HCl, pH 7.5
- 1 mM EGTA (Ca2+ chelator)

Loading, running, transfer, and blocking buffers

Loading buffer/Laemmli 2X buffer

- 4% SDS
- 10% 2-mercaptoethanol
- 20% glycerol
- 0.004% bromophenol blue
- 0.125 M Tris-HCl
- Check the pH and adjust it to 6.8

Running buffer (Tris-Glycine/SDS)

- 25 mM Tris base
- 190 mM glycine
- 0.1% SDS
- Check the pH and adjust to 8.3

Transfer buffer (wet)

- 25 mM Tris base
- 190 mM glycine
- 20% methanol
- Check the pH and adjust to 8.3
- For proteins >80 kDa, we recommend including SDS at a final concentration of 0.1%

Transfer buffer (semi-dry)

- 48 mM Tris
- 39 mM glycine
- 20% methanol
- 0.04% SDS

Blocking buffer

- 3–5% milk or BSA (bovine serum albumin)
- Add BSA to Tris-buffered saline (TBS) or TBST (TBS containing 0.1% Tween 20)
- Mix well and filter. Failure to filter can lead to spotting, with tiny dark dots contaminating the blot during signal development and interfering with protein band visualization.

Tris-buffered saline (TBS) recipes

10X TBS (10X stock solution Tris-buffered saline)

This 10X TBS stock solution contains 200 mM Tris and 1500 mM NaCl.

For 1 L:

- 24 g Tris base (formula weight, FW: 121.1 g)
- 88 g NaCl (FW: 58.4 g)
- Dissolve in 900 mL of distilled water
- pH to 7.6 with 12 N HCl
- Add distilled water to a final volume of 1 L

For a 1X solution, mix 1 part of the 10X solution with 9 parts distilled water and adjust pH to 7.6 again. The final molar concentrations of the 1X solution are 20 mM Tris and 150 mM NaCl.

An alternative recipe for Tris buffer combines Tris base and Tris-HCl. This avoids the large volume of potentially hazardous hydrochloric acid that is needed to neutralize a solution of Tris base alone.

10x TBS alternative recipe

For 1 L:

— 24 g Tris-HCl (FW: 157.6 g)

— 5.6 g Tris base (FW: 121.1 g)

— 88 g NaCl (FW: 58.4 g)

— Dissolve in 900 mL of distilled water

1. The pH of the solution should be about 7.6 at room temperature. If too basic, adjust to pH 7.6 with concentrated HCl, and if too acidic, adjust with concentrated NaOH.
2. Add distilled water to a final volume of 1 L.
3. For a 1X solution, mix 1 part of 10X solution with 9 parts distilled water and pH to 7.6 again.
4. The final molar concentrations of the 1X solution are 20 mM Tris and 150 mM NaCl.

TBST (Tris-buffered saline, 0.1% Tween 20)

For 1 L:

— 100 mL of TBS 10x

— 900 mL distilled water

— 1 mL Tween 20

TBS 0.025% Triton X-100

For 1 L:

— 250 µL Triton X-100

— 1 L TBS pH 7.6–7.8

Stripping buffers

Medium stripping buffer

— 15 g glycine
— 1 g SDS
— 10 mL Tween 20

1. Adjust the volume to 800 mL with distilled water.
2. Adjust pH to 2.2.
3. Bring volume up to 1 L with distilled water.

Harsh stripping buffer

Prepare buffer and strip membranes under a fume hood.

For 100 mL:

— 20 mL SDS 10%
— 12.5 mL Tris HCl, pH 6.8, 0.5 M
— 67.5 mL distilled water
— Add 0.8 mL β-mercaptoethanol under the fume hood

Nuclear fractionation buffers

The following nuclear fractionation buffers are used for extracting and fractionating the nuclear fraction of cells. The main difference between the buffer recipes below is that buffer A contains a detergent, NP-40.

Nuclear fractionation protocol reagents buffer A

— 10 mM HEPES
— 1.5 mM $MgCl_2$
— 10 mM KCl
— 0.5 DTT
— 0.05% NP-40 (or 0.05% Igepal or Tergitol) pH 7.9

To prepare 250 mL stock of buffer A:

— HEPES: 1 M = 238.3 g/L, therefore 10 mM = 0.59 g/250 mL
— $MgCl_2$: 1 M = 203.3 g/L, therefore 1.5 mM = 0.076 g/250 mL
— KCl: 1 M = 74.5 g/L, therefore 10 mM = 0.187 g/250 mL
— DTT: 1 M = 154.2 g/L, therefore 0.5 mM = 0.019 g/250 mL
— NP-40: 0.05%

Nuclear fractionation protocol reagents buffer B

- 5 mM HEPES
- 1.5 mM $MgCl_2$
- 0.2 mM EDTA
- 0.5 mM DTT
- 26% glycerol (v/v) pH 7.9

To prepare 250 mL stock of buffer B:

- HEPES: 1 M = 238.3 g/L, therefore 5 mM = 0.295 g/250 mL
- $MgCl_2$: 1 M = 203.3 g/L, therefore 1.5 mM = 0.076 g/250 mL
- EDTA: 1 M = 372.2 g/L, therefore 0.2 mM = 0.0186 g/250 mL
- DTT: 1 M = 154.2 g/L, therefore 0.5 mM = 0.019 g/250 mL
- 26% glycerol (v/v) = 65 mL

Primary antibody solution in 1% BSA/TBS

Example of 10 mL primary antibody solution containing any primary antibody at 1:1000 dilution:

- 10 µL primary antibody
- 0.1 g BSA
- 9.90 mL 1X TBS, pH 7.6–7.8

ABC (avidin-biotin complex) in TBS

An example of ABC solution, with each part used at a dilution of 1:100.

For 1 mL:

- 10 µL Streptavidin
- 10 µL HRP (or AP)-biotin
- 980 µL 1X TBS pH 7.6–7.8

Bicarbonate/carbonate coating buffer (100 mM)

- 3.03 g Na_2CO_3
- 6.0 g $NaHCO_3$ (1 L distilled water) pH 9.6

Recommended controls for western blot

Including controls is essential for every experiment, and western blot is no exception. Carefully chosen controls help you recognize unexpected sources of error, both random and systemic, and fix these before they compromise your results.

Positive control lysate

A positive control lysate is a lysate from a cell line or tissue sample known to express the protein you are detecting. A positive result from the positive control, even if the test samples are negative, will indicate that the procedure and reagents are optimized and working. It will, therefore, verify the validity of any negative results. On the contrary, a negative result in the positive control lane suggests that at least one of the steps in your protocol didn't work correctly, or there's a problem with your antibody.

We strongly recommend using a positive control lysate when setting up a new experiment; this will give you immediate confidence in the protocol.

Tip: *If the expression of your target protein isn't well characterized, use a lysate from cell lines or tissues overexpressing your protein of interest as a positive control. We prefer using cell lysate to tissue lysate as a positive control because protein expression levels in tissue may be easily affected by individual differences or heterogeneity and easily cause lysate batch differences.*

For modified targets, like phosphorylation-modified proteins, we suggest using a total antibody as a control. For proteins regulated by drug treatment, we suggest using a control antibody from the same signaling pathway to validate that your signaling pathway has been activated successfully.

We recommend checking the antibody datasheet, which will often provide a suggested positive control. Always ensure the tissue or cell line you use is from a tested species. Not all the datasheets will have a suggested suitable control, and we recommend the following in these circumstances:

— Check if there are any citations and product reviews for the antibody. Any tissues, cells, or lysates that have been used successfully by these customers can be considered a suitable positive control.

— Check the Swiss-Prot or Omnigene database links on the datasheet. These databases will often list tissues expressing your protein of interest. These can also be considered suitable positive controls.

— Check the GeneCards or Human Protein Atlas entry for the protein. This will usually provide you with relative protein expression levels in various tissues and/or cell lines.

— If you still have difficulty finding a suitable control, we recommend doing a quick literature search on PubMed to see which tissues and cells express the protein of interest.

If you are using online databases, such as Swiss-Prot, Omnigene, or Human Protein Atlas, consider where the data has come from. Thus, the data based on mRNA expression may not correlate well with detectable protein levels.

Negative control lysate

Negative control lysate is a lysate from a cell line or tissue sample known not to express the protein you are detecting. A negative control will allow you to check for non-specific binding of your antibody and false-positive results.

Tip: *Use a lysate from a validated knock-out cell line or tissue sample as a negative control.*

Loading controls

Loading controls are proteins that exhibit high-level, constitutive expression in the cell type or sample that you are studying. Housekeeping genes, such as actin, GAPDH, tubulin, or mitochondrial proteins, are usually good choices.

Loading controls confirm that protein loading is the same across the gel and, therefore, help to normalize the protein levels you detect. Loading controls also ensure the reliability of your data between samples since the expression levels of the loading control should remain consistent between the different sample types.

Below is an example of negative and loading controls in western blotting (Figure 5).

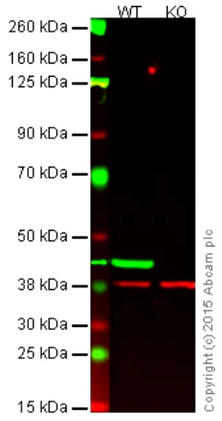

Figure 5. Western blot with anti-beta actin antibody [AC-15] (ab6276).

Lane 1: Wild-type HAP1 cell lysate (20 µg).

Lane 2: Beta-actin knock-out HAP1 cell lysate (20 µg), used as a negative control.

Lanes 1 and 2: Merged signal (red and green). Green - beta-actin (ab6276) observed at 42 kDa. Red - loading control (ab181602) observed at 37 kDa. Ab6276 was shown to specifically react with beta-actin in wild-type HAP1 cells (lane 1).

No band was observed in the negative control (beta-actin knock-out cell lysate in lane 2).

Here are the main reasons you need to be using loading controls:

— **Quantification:** when lanes have not been loaded evenly, loading controls can be used to quantify the protein amounts in each lane by using the density of the loading control band to correct for the differences in loading.

— **Equal transfer:** loading controls have a second role as a control in western blots. They can be used to check that there has been even transfer from the gel to the membrane across the whole gel. This is imperative when comparing the protein expression levels between samples.

— **Edge effect:** this is an issue that is particularly important in signaling assays or experiments where a large number of lanes are being run at once. Proteins in the outer lanes of the gel are transferred to the membranes in a position close to the frame. This may result in more variation in binding compared to other areas of the gel. Loading controls can show if this effect has occurred and allows us to correct for the variation in binding.

— **Requested by referees:** using loading controls is essential for publication-quality work. As an example, to be published in many Nature journals, a paper must meet four general criteria, the first of which is that it must "provide strong evidence for its conclusions." This directly correlates to the necessity of controls to prove that the results obtained are valid.

Use the table below to select the right loading control for your sample type. Note it's essential to choose a loading control with a different molecular weight than the protein of interest. This ensures that you will be able to distinguish between the bands.

Table 3. Suitable loading controls for various sample types.

kDa	Whole cell	Mitochondrial	Nuclear	Membrane	Cytoskeleton	Serum
125	Vinculin					
110				NaK ATPase		
75						Transferrin
66			Lamin B1*			
60		HSP60				
55	Alpha tubulin**		HDAC1		Alpha tubulin**	
50	Beta tubulin**		YY1		Beta tubulin**	
42	Actin				Actin	
40	Beta-actin***				Beta-actin***	
35	GAPDH†		TBP‡			
30		VDAC1/Porin	PCNA			
24	Cyclophilin B					
20	Cofilin	COX IV§			Cofilin	
15			Histone H3			

*Not suitable for samples where the nuclear envelope is removed.

**Tubulin expression may vary according to resistance to antimicrobial and antimiotic drugs (Sangrajang S *et al.*, 1998; Prasad V *et al.*, 2000).

***Not suitable for skeletal muscle samples. Changes in cell-growth conditions and interactions with extracellular matrix components may after actin protein synthesis (Farmer *et al.*, 1983).

†Some physiological factors, such as hypoxia and diabetes, increase GAPDH expression in certain cell types.

‡Not suitable for samples where DNA is removed.

§Many proteins run at the same 16 kDa size as COX IV.

Endogenous control lysate

We recommend including an endogenous control if you test a recombinant protein sample. Folding of the recombinant protein may differ from the endogenous native form and may prevent antibody access to the epitope.

Always ensure the recombinant protein includes the immunogen sequence for the antibody you are using. An endogenous positive control is important to validate the results and indicate how well the reagents (eg, antibodies) and protocol are working.

Recombinant protein and overexpression lysate antibody control

We recommend using an anti-Tag antibody to validate recombinant or overexpressed proteins. Additionally, assessing mRNA levels is also encouraged to validate protein overexpression. Be careful when the immunogen is located at the same end as the tag of the protein because the tag may potentially block the epitope recognized by the antibody.

Always ensure the recombinant or overexpressed protein includes the immunogen sequence for the antibody you are using.

No primary antibody control

This is when you add only a secondary antibody. This control can indicate the occurrence of non-specific binding of the secondary antibody. This is a critical step when optimizing a multiplexed western blot, as it confirms secondary antibodies don’t interfere with each other and cause off-target binding.

Antibody dilution buffer containing no antibody is used instead of the primary antibody solution at this point in the procedure. The secondary antibody is incubated on the sample in the same way as usual.

Western blot sample preparation

Here we cover all essential details of sample preparation for western blot, including obtaining lysate from cell culture and tissues, measuring protein concentration, and reducing and denaturing your sample.

Understanding your protein of interest's characteristics

Before setting up a western blot experiment, inform yourself about the characteristics of the protein of interest. Those include:

— Size

— Cellular localization

— Solubility (membrane integrated vs soluble)

— Modifications (protease processing, post-translational modifications like glycosylation)

— Known isoforms

— Protein abundance

Depending on those specific characteristics, you might need to set up a suitable strategy to be able to detect the target protein using western blotting.

Sample preparation overview

The preparation of your sample for western blot involves four main steps:

1. Sample isolation – Fractions, organelles, cells, or tissue must be individually isolated from a certain source of interest. It's essential to consider additional requirements based on the source type. For adherent cells, a simple trypsin treatment may suffice, while uneven tissues may require the removal of unwanted components, such as fat or collagen. Removing these unwanted components may be necessary to enhance the quality of the experiment.
2. Lysis – The organelles, cells or tissue samples are treated with buffers and mechanically agitated to release proteins. Selecting a suitable lysis strategy is critical for obtaining good-quality results. In some cases, samples like cell culture supernatant may not require lysis, but protein abundance might be low, making sample loading challenging. In such scenarios, protein precipitation techniques may be necessary, like TCA precipitation or protein concentration, using specific spin columns.
3. Measuring protein concentration – This step is crucial to ensure the loading of sufficient protein and equal amounts of the sample. Keep in mind that some protein concentration measurement techniques may not be compatible with detergent in the lysis buffer or buffer constituents like EGTA. Checking compatibility beforehand helps in selecting the appropriate method for protein determination.
4. Reducing and denaturing – Samples are also usually treated with agents to disrupt higher-level protein structures (secondary and tertiary structures) and

linearize the proteins, ensuring protein separation exclusively by size. The sample buffer often contains DTT or beta-mercaptoethanol to reduce disulfide bonds and SDS to form denatured protein micelles coated with SDS molecules. Additionally, the sample is boiled, or at least heated, for a short time.

Selecting a suitable lysis buffer and including protease inhibitors

Selecting a specific lysis buffer is vital for targeting a protein located in a particular compartment. The components of the suitable lysis buffer should ensure effective solubilization and detection of the target protein in the sample. For more detailed information and recipes for lysis buffers, refer to Buffers and stock solutions for western blot.

Enriching a specific organellar fraction, such as mitochondria, may be necessary if working with low-abundance proteins or difficult target proteins with high background. This approach, called cellular fractionation, improves the signal-to-noise ratio by excluding other proteins and reducing sample diversity. Cellular fractionation, typically achieved through a differential centrifugation process, may involve using sugar-based materials for density gradient centrifugation, such as a sucrose gradient or a sucrose cushion.

Adding protease inhibitors is essential to monitor protein levels and prevent protein degradation by proteases. Many protease inhibitors represent a mix of several compounds targeting various cellular proteases. However, for some specific targets, it may be necessary to include additional specific protease inhibitors. Note that some proteins only interact with individual proteases upon lysis of membranes.

Preparation of lysate from cell culture

1. Place the cell culture dish on ice and wash the cells with ice-cold PBS.
2. Aspirate the PBS, then add ice-cold lysis buffer (1 mL per 10^7 cells/100 mm dish/150 cm^2 flask; 0.5 mL per $5x10^6$ cells/60 mm dish/75 cm^2 flask).
3. Scrape adherent cells off the dish using a cold plastic cell scraper, then gently transfer the cell suspension into a pre-cooled microcentrifuge tube. Alternatively, cells can be trypsinized and washed with PBS before resuspension in lysis buffer in a microcentrifuge tube.
4. Maintain constant agitation for 30 min at 4°C.
5. Centrifuge in a microcentrifuge at 4°C. You may have to adjust the centrifugation force and time depending on the cell type. A general guideline is 20 min at 12,000–16,000 x g, but this must be determined for your experiment (eg, leukocytes need very light centrifugation).
6. Gently remove the tubes from the centrifuge and place them on ice. Aspirate the supernatant and transfer it to a fresh tube kept on ice while discarding the pellet.

Preparation of lysate from tissues

1. Dissect the tissue of interest with clean tools, preferably on ice, and as quickly as possible to prevent protease degradation.
2. Place the tissue in round-bottom microcentrifuge tubes or Eppendorf tubes and immerse it in liquid nitrogen to snap freeze. Store samples at -80°C for later use or keep them on ice for immediate homogenization.
3. For a ~5 mg piece of tissue, add ~300 µL of ice-cold lysis buffer rapidly to the tube, homogenize with an electric homogenizer, rinse the blade twice with another 2 x 300 µL of lysis buffer, then maintain constant agitation for 2 hours at 4°C (eg, place your sample on an orbital shaker in the fridge). The volumes of lysis buffer must be determined depending on the amount of tissue present. Protein extract should not be too diluted to avoid protein loss and large volumes of samples to be loaded onto gels. The minimum recommended concentration is 0.1 mg/mL, while the optimal concentration is 1–5 mg/mL).
4. Centrifuge the tubes for 20 min at 12,000–16,000 x g at 4°C in a microcentrifuge. Gently remove the tubes from the centrifuge and place them on ice; then aspirate the supernatant and transfer it to a fresh tube kept on ice. Discard the pellet.

Determination of protein concentration

Once the samples are fully lysed, measure protein concentration.

1. Perform a Bradford assay, a Lowry assay, or a bicinchoninic acid (BCA) assay. Bovine serum albumin (BSA) is a frequently used protein standard.
2. Once you have determined the concentration of each sample, you can freeze them at -20°C or -80°C for later use or prepare them for immunoprecipitation or loading onto a gel.

Preparation of samples for loading into gels

Denatured, reduced samples

To ensure proper detection of the protein of interest, it is necessary to denature it, as antibodies typically recognize a specific region called an epitope, which may be located within the protein's 3D conformation. Denaturation involves unfolding the protein to expose the epitope.

To denature the protein, we use a loading buffer with the anionic detergent sodium dodecyl sulfate (SDS). The sample is then heated by boiling the mixture at 95–100°C for 5 minutes. Alternatively, heating at 70°C for 5–10 minutes is acceptable, especially when studying multi-pass membrane proteins that tend to aggregate when boiled, hindering efficient gel entry.

The standard loading buffer is called 2X Laemmli buffer[1]. It can also be prepared at 4X and 6X strength to minimize the dilution of the samples. The 2X Laemmli buffer is to be mixed with the sample in 1:1 ratio. For the 2X Laemmli buffer recipe, refer to Buffers and stock solutions for western blot.

When SDS is used with proteins, it imparts a negative charge to all proteins by binding to them. SDS attaches to proteins in a mass ratio of 1.4:1, resulting in negatively charged denatured polypeptides that migrate based on their molecular weight rather than their intrinsic charge density.

Using high-quality SDS grade is crucial for obtaining clear protein separation.

A protein-stained background, along with indistinct or slightly distinct protein bands, indicates old or poor-quality SDS. Including 2-mercaptoethanol or dithiothreitol in the buffer reduces disulfide bridges, which is necessary for separation by size.

Glycerol is added to the loading buffer to increase the sample's density, keeping it at the bottom of the well and preventing overflow and uneven gel loading.

To visualize the protein migration, it is common to include a small anionic dye molecule in the loading buffer (eg, bromophenol blue). The dye, being anionic and small, migrates the fastest among the components in the mixture, serving as a migration front to monitor the separation progress.

During the protein sample treatment, the sample should be mixed by vortexing before and after the heating step for optimal resolution. After vortexing, we recommend a short centrifugation step to remove residual liquid from the cap or tube walls. This step is especially critical when loading the entire volume. To ensure equal loading and avoid loading bubbles, we suggest preparing a higher sample volume than necessary and not loading the whole volume.

Native and non-reduced samples

In some cases, antibodies may recognize epitopes composed of non-contiguous amino acids. Although these amino acids are not sequential in the protein's primary sequence, they are in close proximity within the folded three-dimensional structure. Antibodies can only recognize such epitopes as they appear on the surface of the folded structure.

In these circumstances, we must run a western blot under non-denaturing conditions, as indicated in the datasheet's applications section. In non-denaturing conditions, the sample and migration buffers do not contain sodium dodecyl sulfate (SDS), and the samples are not heated.

Furthermore, certain antibodies only recognize proteins in their non-reduced form, particularly on cysteine residues. When using such antibodies, omit the reducing agents, such as β-mercaptoethanol and dithiothreitol (DTT), from the loading and migration buffers.

Table 4. Protein state and corresponding gel and buffer conditions.

Protein state	Gel condition	Loading buffer	Migration buffer
Reduced, denatured	Reducing and denaturing	With 2-mercaptoethanol or DTT and SDS	With SDS
Reduced, native	Reducing and native	With 2-mercaptoethanol or DTT and SDS	No SDS
Oxidized, denatured	Non-reducing and denaturing	No 2-mercaptoethanol or DTT, with SDS	With SDS
Oxidized, native	Non-reducing and native	No 2-mercaptoethanol or DTT, with SDS	No SDS

Rule of thumb: reduce and denature unless the datasheet specifies otherwise.

Electrophoresis for western blot

Electrophoresis is a method used in western blot to separate and analyze proteins based on their size and charge. Electrophoresis can be one-dimensional (ie, one plane of separation) or two-dimensional. For most routine protein separations, one-dimensional electrophoresis suffices, while two-dimensional separation is used for more advanced proteomic studies in cells and involves isoelectric focusing in the first dimension.

Here we will focus on one-dimensional electrophoresis techniques. For those seeking a basic understanding of electrophoresis protocols for proteins, we recommend referring to the book "Gel Electrophoresis of Proteins: A Practical Approach"[2].

Introduction to SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is a widely used electrophoretic technique that separates proteins based on their molecular weight. By using sodium dodecyl sulfate (SDS) and polyacrylamide gel, this method eliminates the influence of protein structure and charge, enabling a focus solely on molecular weight differences.

SDS acts as a detergent, breaking down the tertiary structure of proteins and converting them into linear molecules for easier separation. Polyacrylamide, a chemically inert substance, is chosen for its versatility in creating gels with varying concentrations, resulting in different pore sizes that cater to specific separation needs. This flexibility allows researchers to tailor the conditions of SDS-PAGE according to their experimental requirements.

Preparation of polyacrylamide (PAGE) gels

Polyacrylamide gels are essential components formed by the polymerization of acrylamide and N, N'-methylene bisacrylamide (or bis for short). The cross-linking agent for the gels is bis. Polymerization is initiated by adding ammonium persulfate (APS) along with either DMAP or TEMED. The gels are neutral and hydrophilic three-dimensional networks of long hydrocarbons cross-linked by methylene groups.

The separation of molecules within a gel is determined by the relative size of the pores formed within it. The pore size depends on two factors: the total amount of acrylamide present (%T) and the amount of cross-linker (%C). As the total amount of acrylamide increases, the pore size decreases. The smallest pore size is achieved with 5%C, while any increase or decrease in %C increases the pore size.

Gels can be purchased pre-made or produced in the laboratory using specific recipes found in laboratory handbooks.

It's crucial to carefully choose the percentage of acrylamide in your gel, as it determines the rate of migration and the degree of separation between proteins. For smaller proteins, you'll need a higher percentage of acrylamide and vice versa. Refer to Table 5 for guidance on selecting the appropriate gel percentage based on protein size.

Table 5. Protein sizes resolved by different gel percentages.

Protein size, kDa	Gel acrylamide, %
4–40	20
12–45	15
10–70	12.5
15–100	10
25–200	8

⚠ Note that acrylamide is a potent cumulative neurotoxin: always wear gloves when handling it.

For enhanced protein separation and resolution, consider using gradient gels. Unlike fixed-concentration PAGE gels, gradient gels have a continuous range of polyacrylamide concentrations, allowing for the resolution of a broader range of protein sizes on a single gel. Moreover, gradient gels yield sharper protein bands, facilitating better separation of similar-sized proteins and producing easily discernible data.

Once you prepared the suitable PAGE gel for your protein size, place the gel in the electrophoresis tank as instructed by the manufacturer and bathe in the migration (or running) buffer.

Molecular weight markers for electrophoresis

Molecular weight markers are essential tools that allow us to extrapolate the protein size of the sample (Figure 6) and monitor the progress of an electrophoretic run. There are various commercially available molecular weight markers, including prestained and unstained markers suitable for western blot. Prestained markers have the advantage of being transferred to the western blot membrane, making them more convenient to handle. In contrast, unstained markers require manual labeling as they are not visible without further staining.

Abcam provides several prestained protein ladders for different molecular weight ranges: mid-range of 10–180 kDa (ab116027 or ab234617), broad range of 10–245 kDa (ab116028), and extra broad of 5–245 kDa (ab116029) or 6.5–270 kDa (ab234592).

Keep in mind that the apparent molecular weight of markers can change depending on the running buffer chosen and the consequent pH of the system. The variance in pH between SDS-PAGE running buffers can affect the charge of the labeled protein standard and its binding capacity for SDS, causing a shift in mobility and an apparent change in molecular weight (as shown in Figure 6).

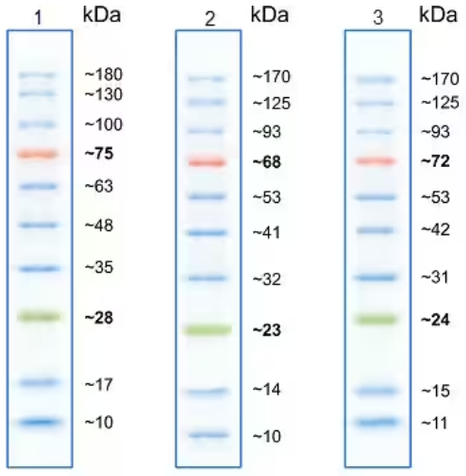

Figure 6. SDS-PAGE with prestained protein ladder – mid-range molecular weight (10–180 kDa) (ab116027) run with different SDS-PAGE buffer chemistries. Gel 1: Tris-Glycine (~4-20%), Gel 2: Bis-Tris (12%) MOPS buffer, Gel 3: Bis-Tris (10%) MES buffer.

Tips for loading samples and running the gel

To ensure accurate results, follow these tips when loading samples and running the gel:

— Include appropriate positive and loading controls in your experimental setup. See Recommended controls for western blot for more information.

— Use special gel loading tips or a micro-syringe to load the complete sample into wells.

— Avoid introducing bubbles during sample loading to prevent unequal loading. Load around 80% or less of the sample volume to avoid bubbles.

— Take care not to touch the bottom of the wells with the tip to avoid creating distorted bands.

— Avoid overfilling wells to prevent spillage into adjacent wells, which could lead to poor data and poorly resolved bands. When working with unique samples (eg, wild type vs knock-out), avoid loading samples in adjacent pockets to prevent spillover.

— Load 15–40 µg total protein per mini-gel well. If loading purified proteins, you might reduce the loaded amount.

— Submerge the gels in the running buffer (see a buffer recipe here), normally containing SDS, except in native gel electrophoresis.

— Run the gel for the recommended time as instructed by the manufacturer, as this can vary from machine to machine (eg, 30 minutes to overnight, depending on the voltage).

— When the dye (the migration front) reaches the bottom of the gel, turn the power off. Proteins will slowly elute from the gel at this point, so do not store the gel but proceed immediately to transfer.

Protein transfer and visualization

Here we provide essential tips for the successful transfer and staining of proteins in western blot, including visualization of proteins and development methods for visualization.

Visualization of proteins in western blot

After separating proteins through gel electrophoresis, the next step is to verify if the proteins have migrated uniformly and transfer them onto a specialized membrane. The protein visualization stage is crucial for accessing the quality of protein migration, ensuring uniform and even distribution. Two commonly used methods for protein visualization are copper stain and Coomassie stain.

If we intend to transfer the separated proteins to a membrane later on, copper stain is recommended. On the other hand, the Coomassie stain is irreversible and fixes the proteins in the gel, making it less suitable for subsequent protein transfer. However, if our main objective is to observe the results of the SDS-PAGE separation without protein transfer, we can employ the Coomassie stain. Alternatively, we can apply the Coomassie stain to gels post-transfer to check for transfer efficiency or use the PVDF membranes for Coomassie staining as they can be destained.

Using Coomassie stain on a PVDF membrane offers several benefits. Firstly, it provides a comprehensive overview of the protein loading on the gel, serving as a potential loading control in addition to any housekeeping proteins that need to be detected. Secondly, after scanning, the PVDF membrane can be effortlessly destained using methanol and automatically activated for further staining.

Using the appropriate staining method and membrane, protein visualization in western blot can effectively evaluate protein migration and loading, ensuring reliable results in downstream analysis.

Coomassie stain

As soon as the power is turned off, the separated protein bands will naturally begin to diffuse since they are freely soluble in an aqueous solution. The crucial step is to prevent protein diffusion by treating the gel with 40% distilled water, 10% acetic acid, and 50% methanol solution. This treatment causes almost all proteins to precipitate, ie, become insoluble.

To visualize the fixed proteins, place the gel in the same solution of water/acetic acid/methanol, but this time add 0.25% Coomassie Brilliant Blue R-250 by weight. Incubate the gel for 4 hours to overnight at room temperature on a shaker. Afterward, transfer the gel (remember to save the dye mixture as it can be reused many times) to a mix of 67.5% distilled water, 7.5% acetic acid, and 25% methanol. Place the gel on a shaker and replace the rinse mixture with a fresh solution until the excess dye has been removed.

The Coomassie stain will not bind to the acrylamide in the gel and will wash out, leaving a clear gel. However, the stain strongly binds to the gel's proteins, resulting in a deep blue color.

Copper stain

Briefly rinse freshly-electrophoresed gels in distilled water for a maximum of 30 seconds and then transfer them to a solution of 0.3 M $CuCl_2$ for 5–15 min. After that, wash the gels briefly in de-ionized water and view them against a dark-field background.

Proteins will appear as clear zones against a translucent blue background. To completely destain the gels, perform repeated washes in a solution of 0.1–0.25 M Tris/0.25 M EDTA pH 8.0. Then, move the gel to a dish of transfer buffer before proceeding with transfer according to the transfer apparatus manufacturer's instructions.

Protein transfer

Protein transfer is the process of transferring proteins from a gel onto a membrane. Manufacturers of transfer apparatus typically provide detailed instructions for the transfer process on their websites. These details vary depending on the system, but the principle remains the same in each case.

Protein transfer is achieved by applying an electrical field, similar to gel electrophoresis, which induces proteins with an electrical charge to migrate from the gel onto a sturdy support, such as a membrane (Figure 7). This process, known as blotting, has evolved from early methods relying on protein diffusion to the now standard practice of blotting in an electrical field, which yields more reliable results.

Figure 7. A schematic representation of protein transfer from the gel to a membrane. Negatively charged proteins move up towards the positive cathode and onto the membrane.

Transfer can be done using a wet or semi-dry system. The wet transfer is less prone to failure due to membrane drying and is especially recommended for transferring large proteins. For both kinds of transfer, the membrane is placed next to the gel. The two are sandwiched between absorbent materials, and the sandwich is clamped between solid supports to maintain tight contact between the gel and membrane.

Wet protein transfer

During wet protein transfer, the gel and membrane are sandwiched between sponge and paper, arranged in the following order: sponge > paper > gel > membrane > paper > sponge. This sandwich is tightly clamped to ensure no air bubbles are trapped or formed between the gel and membrane. The sandwich is then submerged in the transfer buffer, to which an electrical field is applied. The negatively-charged proteins travel toward the positively-charged electrode but are bound by the membrane, preventing them from further migration.

A standard buffer for wet transfer is the same as the 1x Tris-glycine buffer used as the gel running buffer, but without SDS and with the addition of methanol to a final concentration of 20%. However, for proteins larger than 100 kDa, including SDS at a final concentration of 0.1% is recommended.

Semi-dry protein transfer

In a semi-dry transfer, a sandwich consisting of paper > gel > membrane > paper wetted in transfer buffer is placed directly between positive and negative electrodes (cathode and anode, respectively). Similar to the wet transfer, it is crucial that the membrane is closest to the positive electrode and the gel closest to the negative electrode.

The composition of the transfer buffer for semi-dry transfer may differ from that of wet transfer, so we advise you to consult the apparatus manufacturer's protocol. A standard recipe for semi-dry transfer buffer includes 48 mM Tris, 39 mM glycine, 0.04% SDS, and 20% methanol.

There are two commonly used membrane types: nitrocellulose (eg, 0.45 µm or 0.22 µm membranes) and PVDF (positively charged nylon, eg, low fluorescent western membrane). Both types work effectively, and the choice depends on personal preference. An advantage of the PVDF membrane is that it allows for easy staining and destaining with Coomassie.

Note that PVDF membranes require careful pre-treatment: cut the membrane to the appropriate size and soak it in methanol for 1–2 min. Then, incubate the membrane in an ice-cold transfer buffer for 5 min. Failure to equilibrate the membrane in an ice-cold transfer buffer can lead to shrinking during transfer and a distorted transfer pattern.

Transfer of large and small proteins

Several factors can affect transfer efficiency, like the balance of SDS and methanol in the transfer buffer, protein size, and gel percentage. The following modifications will encourage efficient transfer for different protein types.

Transfer tips for large proteins (>100 kD)

— For large proteins, transfer out of the gel may be very slow, as they run slowly within the gel during separation. If blotting a large protein, run your samples in a low-concentration gel, 8% or less. These will be very fragile, so handle them carefully.

— Large proteins will tend to precipitate in the gel, hindering transfer. Adding SDS to a final concentration of 0.1% in the transfer buffer will discourage this. Methanol tends to remove SDS from proteins, so reducing the methanol percentage to 10% or less will also guard against precipitation.

— Lowering the methanol percentage in the transfer buffer also promotes gel swelling, allowing large proteins to transfer more easily.

— Methanol is only necessary if using nitrocellulose. If using PVDF, methanol can be removed from the transfer buffer altogether and is only needed to activate the PVDF before assembling the gel/membrane sandwich.

— Choose wet transfer overnight at 4°C instead of semi-dry transfer.

Transfer tips for small proteins (<100 kD)

— SDS hinders all proteins from binding to membranes, but small proteins are more affected by this than large ones. If your protein of interest is small, omit SDS from the transfer buffer.

— Keep the methanol concentration at 20%.

General transfer tips:

— Avoid touching the membrane with your fingers; use tweezers instead and only touch the edge of the membrane. Oils and proteins on fingers will block efficient transfer and create dirty blots.

— After sandwiching the gel and membrane between paper, we can eliminate air bubbles between the gel and membrane by rolling them out with a roller, pipette, or 15 mL tube. Also, we could assemble the sandwich in a dish of transfer buffer to prevent the formation of bubbles in the first place.

— Make sure the paper and membrane are cut to the same size as the gel. Large overhangs may prevent a current from passing through the membrane in semi-dry transfers.

— Chicken antibodies tend to bind PVDF and other nylon-based membranes, leading to high background. In this case, switching to a nitrocellulose membrane should help reduce background staining.

The following reference discusses a gel and buffer system that allows the transfer of proteins as large as 500 kD: Bolt MW and Mahoney PA (1997)[3].

Visualization of proteins in membrane with Ponceau Red

Visualizing the proteins using Ponceau Red allows us to check for transfer success. We can implement the following protocol:

1. Wash the membrane in TBST.
2. Dilute the stock of Ponceau Red (example: ab270042 or ab146313) 1:100. The stock is made of 2% Ponceau S in 30% trichloroacetic acid and 30% sulfosalicylic acid.
3. Incubate on an agitator for 5 min, then wash extensively in water until the water is clear and the protein bands are well-defined.
4. The membrane may be destained completely by repeated washing in TBST or water. When using a PVDF membrane, re-activate it with methanol, then wash it in TBST.

Check out the details recipes for TBS 10x and TBST in Buffers and stock solutions for western blot.

Blocking the membrane

Blocking the membrane prevents non-specific background binding of the primary and/or secondary antibodies to the membrane, which has a high capacity for binding proteins and, therefore, antibodies.

Two blocking solutions are traditionally used: non-fat milk or BSA (Cohn fraction V). Milk is cheaper but not recommended for studies of phospho-proteins because it contains a phospho-protein casein. Casein will cause high background as the phospho-specific antibodies will detect it.

Some antibodies give a stronger signal on membranes blocked with BSA versus milk for unknown reasons. Check the application notes on the datasheet for specific instructions on how to block the membrane.

To prepare a 5% milk or BSA solution, weigh 5 g per 100 mL TBS with Tween 20 (TBST) buffer. Mix well and filter. Failure to filter can lead to spotting where tiny dark grains will contaminate the blot during development.

Incubate for 1 hr at 4°C under agitation. Rinse for 5 s in TBST after the incubation.

Below are some of abcam's recommended blocking solutions:

— Protein Block (ab64226)

— 10X Blocking Buffer (ab126587)

— Protein Block (ab156024)

Incubation with primary antibody

Incubation buffer

Dilute the antibody in TBST at the dilution suggested in the antibody datasheet. If the datasheet does not specify a dilution, try a range of dilutions (1:100–1:3000) and optimize the dilution according to the obtained results. Keep in mind that too much antibody will result in non-specific bands. Certain antibodies may require a different buffer, such as PBS. Regardless of the buffer type selected for the primary antibody, we recommend using the same buffer for the secondary antibody solution.

Some laboratories prefer incubating primary antibodies in a blocking buffer, while others use TBST without a blocking agent. The results vary from antibody to antibody, and you may find it makes a difference to use a non-blocking agent in the antibody buffer or the same agent as the blocking buffer.

If the high background is not an issue, some antibodies produce a much stronger signal if diluted in a buffer with low concentrations (0.5–0.25%) of milk or BSA or even without these additives.

Incubation time

The incubation time can vary between a few hours to overnight (rarely more than 18 h) and depends on the antibody's binding affinity for the target protein and the protein's abundance. We recommend trying a more diluted antibody and a prolonged incubation time to ensure specific binding.

Incubation temperature

The incubation should be carried out at a cold temperature. If incubating in a blocking buffer overnight, do it at 4°C to avoid contamination and the following protein destruction (especially for phospho groups).

Agitating the antibody solution is recommended to enable adequate homogenous membrane covering and prevent uneven binding. During the incubation, ensure you add a sufficient solution volume to accommodate your container size. The container itself should be suitable for the size of your membrane, allowing some space. Additionally, covering the container with a lid can help prevent evaporation.

Incubation with secondary antibody

Wash the membrane several times in TBST while agitating, 5 min or more per wash, to remove residual primary antibody. You might also use a pipette to remove residual liquid from the corner of your container.

Incubation buffer and dilution

Dilute the antibody in TBST at the dilution suggested in the antibody datasheet. If the datasheet does not have a recommended dilution, try a range of dilutions (1:1,000–1:2,0000) and optimize the dilution according to the results. Remember that excess of antibody will result in non-specific bands.

To reduce the background, you may incubate the secondary antibody in the blocking buffer. But a background reduction may come at the cost of a weaker specific signal, presumably because the blocking protein hinders the binding of the antibody to the target protein.

Incubation time and temperature

1–2 h at room temperature with agitation.

Which conjugate and target?

We recommend using horseradish peroxidase (HRP)-conjugated secondary antibodies due to their high sensitivity. In contrast, alkaline phosphatase (AP)-conjugated secondary antibodies are less sensitive and not recommended.

It is crucial to select the appropriate type of secondary antibody that matches the type of primary antibody. Various secondary antibody types are available – H&L chain, Fc, heavy chain, mu chain, F(ab')2, alpha chain, and light chain – which may exhibit different behaviors.

Development methods

Development methods in western blot include using detection kits, X-ray films, and digital images.

Detection kits

For HRP-conjugated antibodies, enhanced chemiluminescence (ECL) kits are traditionally used as substrates. We offer ECL substrate kits with varying detection limits, such as high-sensitivity kits to detect 23 pg–187 ng of protein per band (eg, ab133406) or very high-sensitivity kits to detect 4.6 pg–4.7 ng of protein per band (eg, ab133408).

X-ray films

Many labs work with easy-to-use automated X-ray film developers. Remember that an over-exposed film is unsuitable for analysis as it makes it impossible to determine the relative amount of protein. Over-exposed films show completely black bands with no contrast and/or numerous non-specific bands.

In some cases, multiple exposures might be necessary to identify the optimal exposure time. Weak signals may require longer exposure to the film, while strong

signals develop quickly. If signals vary significantly in intensity, exposing them to separate films may be necessary to ensure optimal visualization.

Transferring the marker band carefully and accurately is crucial to ensure reliable data.

Digital images

The new generation of film developers are units with a camera inside an enclosure, removing the need for a darkroom. The camera detects the chemiluminescence emanating from the membrane, transforming the signal into a digital image for rapid analysis with software provided by the detection machine.

Compared to traditional X-ray films, digital imaging offer a significant advantage – the ability to perform preliminary exposure testing without wasting films. Before capturing the image, we can assess signal intensities, adjust the exposure time and select a suitable camera sensitivity. Moreover, we can monitor the signal capture process by using a shorter exposure time and stopping when the desired signal level is reached. Also, the software can sum up the signals from the previous images, aiding in the analysis.

During detection, it is crucial to stop when the signal intensity has reached its maximum. If the entire ECL solution has been used up, the signal might appear inverse, resulting in a white dot in the middle of the band. Many detection systems will automatically stop detection when a signal has reached its maximum on the blot. However, we advise caution as such a strong signal might lead to the underexposure of other bands on the same blot, which would have required further exposure.

Various machines are now commercially available, including systems that do not use HRP-conjugated antibodies (ie, chemiluminescence). For example, STORM Analyzers detect fluorescence from fluorochrome-conjugated secondary antibodies, while the Odyssey Infrared Imaging System detects infrared fluorescence.

Fluorescent western blotting

Here are a few useful tips on how to get the best possible results out of your fluorescent western blot experiment.

Choosing antibodies for fluorescent western blot

— To obtain clear and bright bands, use antibodies that have been properly validated in western blot.

— When multiplexing, ensure that each primary antibody used is from a different species or isotype to prevent cross-reactivity.

— Select appropriate internal reference antibodies based on the type of lysate. Keep in mind that certain lysate types, such as plasma, are not compatible with using GAPDH as the loading control.

— Use secondary antibodies that are highly cross-adsorbed to minimize cross-species reactivity.

— Alternatively, primary antibodies conjugated to the correct fluorescent dye can also be used. However, as with ECL western blot, expect a weaker signal than when using a secondary antibody for detection.

— When selecting a loading control, avoid choosing antibodies with molecular weights that overlap with those of your primary antibodies.

Optimizing your antibodies for fluorescent western blot

Optimize the primary and secondary antibody concentrations to get the best signal-to-noise ratio:

— Find the optimal antibody concentration by individually titrating your primary and secondary antibodies. Test several dilutions and select the one that yields the highest signal-to-background ratio.

— Before attempting multi-color analysis, optimize conditions for individual antibody pairs (primary and conjugated secondary) separately.

Steps and reagents to pay attention to in your fluorescent western blot protocol

— Membranes can autofluoresce, which may result in high background. Nitrocellulose membranes are considered to be the best option for low background compared to traditional PVDF membranes. If PVDF membranes are required, there are low-fluorescence PVDF membranes available from some vendors.

— Like membranes, blocking buffers can autofluoresce, thereby giving a higher background. Many vendors supply fluorescence-optimized western blot blocking buffer in both PBS- and TBS-based formats.

— Be wary of anything that can settle on the membrane and create fluorescent artifacts:
  — Pen marks can have some autofluorescence, so use a pencil.
  — Handle the membrane with care; use blunt forceps and avoid scratching/creasing to prevent fluorescent artifacts.
  — Undissolved particles within buffers, such as milk powder in a blocking buffer, can potentially settle on the membrane and create fluorescent artifacts. Therefore, we suggest using high-quality reagents, allowing suitable time for all components to fully dissolve, and filter sterilize all buffers.
— Avoid membrane stripping if the experiment's goal is accurate quantification or multiplexing.
— To avoid any chance of potential photobleaching, protect the membrane from the light during incubation and wash steps with aluminum foil.
— If using bromophenol blue in your loading dye, you should ensure your dye front is cut from the gel before the transfer, as this dye will transfer to the membrane and autofluoresce. If blotting a low molecular weight protein, we recommend using fluorescent western blot loading dyes that lack bromophenol blue and don't autofluoresce.

Figure 8 shows an example of the fluorescent western blot performed with IRDye® secondary antibodies.

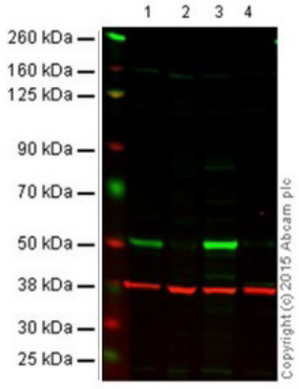

Figure 8. Western blot with goat anti-mouse IgG H&L (IRDye® 800CW) preadsorbed (ab216772).
All lanes: Anti-p53 antibody [DO-1] - ChIP Grade (ab1101) at 1/1000 dilution (Anti-p53 antibody [DO-1]).
Lane 1: Wild-type HAP1 cell lysate (20 µg)
Lane 2: p53 knock-out HAP1 cell lysate (20 µg)
Lane 3: A431 cell lysate (20 µg)
Lane 4: Saos-2 cell lysate (20 µg)
Secondary in all lanes: Goat anti-Mouse IgG H&L (IRDye® 800CW) preadsorbed (ab216772) at 1/10000 dilution.

This blot was produced using a 4–12% Bis-tris gel under the MES buffer system. The gel was run at 200V for 50 minutes before being transferred onto a nitrocellulose membrane at 30V for 70 minutes. The membrane was then blocked for an hour before being incubated with ab1101 overnight at 4°C. Antibody binding was detected using the Goat anti-Mouse IgG H&L (IRDye® 800CW) preabsorbed ab216772 at a 1/10,000 dilution for 1hr at room temperature and then imaged using the Licor Odyssey CLx.

The image shows a merged signal (red and green). Green – p53 (ab1101) observed at 53 kDa. Red - GAPDH loading control (ab181602), observed at 37 kDa using as secondary Goat Anti-Rabbit IgG H&L (IRDye® 680RD)- ab216777.

Multiplexing in fluorescent western blotting

Here we discuss the advantages of detecting multiple targets on the same blot at the same time with fluorescent western blotting.

Multiplexing in fluorescent western blotting allows you to:

1. Quantify relative protein abundance.

Compare the abundance of one protein over another. For instance, you can compare the abundance of a phosphorylated form of your protein of interest to the total amount of protein.

2. Normalize in the same gel.

Do normalization of band intensity with an internal control in the same blot without the inconveniences of stripping and reprobing again.

Why is quantification and multiplexing possible with fluorescent WB?

— Fluorescent applications are more quantitative than enzyme-based approaches (eg HRP), making normalization against an internal control (eg housekeeping gene) easier and more accurate.

— Fluorescent western blotting provides the widest dynamic range , so both low- and high-abundant proteins can be detected in the same experiment.

Western blot membrane stripping for restaining

Essential guidance for removing antibodies from western blot membranes.

What is membrane stripping?

Membrane stripping is the process of removing primary and secondary antibodies from a western blot membrane. Its purpose is to allow the investigation of multiple proteins on the same blot without cutting the membrane. Membrane stripping is particularly useful when examining a protein of interest alongside a suitable loading control or when studying two proteins of similar molecular weight.

There are alternative scenarios where membrane stripping proves beneficial. For instance, it's helpful when an incorrect antibody has been used or when membrane blocking must be repeated due to high background signals. By stripping and reprobing a single membrane, you can save samples and time, avoiding the need to run and blot multiple gels.

Avoid making quantitative comparisons of targets probed before and after stripping since the procedure removes some sample protein from the membrane. For the same reason, a stripped membrane should not be probed to demonstrate the absence of a protein, like for knock-out testing or an RNAi experiment.
We recommend using a PVDF membrane to minimize the loss of sample protein, as nitrocellulose membranes tend to have a more significant protein loss.

Membrane stripping protocols

In the table below, we outline two stripping protocols that differ in their treatment's harshness. As a general rule, try the mild stripping first and then proceed to the harsh one if the signal from the targeted antibody persists.

These steps can be repeated for probing with several antibodies. However, remember that after each stripping round, the potential signal may become weaker, and the background could increase.

Table 6. Buffer recipes and step-by-step protocols for mild and harsh membrane stripping.

	Mild stripping	**Harsh stripping**
Buffer	Buffer, 1L:	Prepare buffer and strip membranes under a fume hood.
	15 g glycine	
	1 g SDS	Buffer, 0.1 L:
	10 mL Tween 20	20 mL SDS 10%
	Dissolve in 800 mL distilled water.	12.5 mL Tris HCl, pH 6.8, 0.5 M
	Adjust pH to 2.2.	67.5 mL distilled water
	Bring volume up to 1 L with distilled water.	Add 0.8 mL ß-mercaptoethanol under the fume hood.

	Mild stripping	Harsh stripping
Procedure	1. Use buffer volume covering the membrane and incubate at room temperature for 5–10 min.	1. Warm the buffer to 50°C.
	2. Discard the buffer.	2. Add the buffer to a small plastic box with a tight lid; use a buffer volume that will cover the membrane.
	3. Repeat incubation for 5–10 min with fresh stripping buffer.	3. Add the membrane. Incubate at 50°C for up to 45 min with some agitation.
	4. Discard the buffer.	4. Dispose of the solution as required for ß-mercaptoethanol based buffers.
	5. Wash for 10 min in PBS x 2 times.	5. Rinse the membrane under a running water tap for 1–2 min.
	6. Wash for 5 min in TBST x 2 times.	6. Traces of ß-mercaptoethanol will damage the antibodies. Wash extensively for 5 min in TBST.
	7. Ready for blocking	7. Ready for blocking.

Determining the membrane stripping efficiency

To determine the membrane stripping efficiency, you can incubate the membrane with a chemiluminescent detection reagent after the stripping procedure. Avoid using colorimetric/chromogenic detection reagents, as they leave a permanent visible stain on the membrane, which can interfere with the subsequent detection of targets with similar molecular weights. Chemiluminescent reagents like ECL are preferable since they are more sensitive than colorimetric reagents and won't leave any stain.

The most reliable way to test the success of stripping is by re-blocking the membrane and then using a secondary antibody. No signal bands should be detected if the primary antibody was successfully removed. If the membrane is clean, you can wash it to remove any residual secondary antibodies before starting the next primary antibody incubation. If signals still appear, you may need to escalate from mild to harsh stripping or repeat the entire procedure.

Lastly, it is crucial to rinse the membrane thoroughly with buffer after achieving satisfactory stripping efficiency. The residual stripping buffer should be completely removed, as any leftover may interfere with the follow-up immunodetection process.

Western blot troubleshooting and optimization

Here we share troubleshooting tips for the most common western blot issues and guidance on tackling different western blot types. You'll have a much better understanding of the following:

— What the most common problems in western blot are, and how to troubleshoot them

— How to keep the proteins in their phosphorylated state

— How to work with histones

Western blot troubleshooting tips

All experiments and model systems are different, so it may not be enough to follow a standard protocol to get the results you are expecting. If you are having difficulties getting your western blots exactly how you want them, take a look over our troubleshooting tips. Here we cover all common causes of no signal, high background, and multiple bands.

Detection problems

No signal or faint bands

— All bands, including the ladder, are faint or have no signal.

If all the bands on your blot, including the molecular weight ladder, are difficult to see, it could indicate a problem with your technique rather than the protein you're trying to detect. Familiarize yourself with the western blot protocol and check the common pitfalls below.

Possible causes	Solution
Problems with transferring proteins to the membrane.	Check the transfer was successful using a reversible stain, such as Ponceau S, before immunostaining. If the proteins have not transferred effectively, check the transfer was performed in the right direction (see diagram). If using a PVDF membrane, make sure you pre-soak the membrane in methanol and then in transfer buffer.
Too much washing between steps.	Washing with a buffer between steps is necessary, but washing too aggressively can sometimes remove detection reagents. Reduce the duration or number of washing steps.
The wash or incubation buffer is contaminated with bacteria.	Use fresh, sterile buffer (eg, our sterile PBS).
Reagents may have lost activity due to improper storage and handling.	Check the storage instructions for your products on the datasheet. Avoid excessive freezing/thawing. If using fluorescent detection, the fluorophore may have been damaged by too much light exposure. Store and handle fluorophores and fluorophore-conjugated antibodies in the dark and minimize light exposure by wrapping the vial in foil.
You may have used the wrong filter settings for detection.	Ensure you set the instrument to read the correct wavelengths.
There may not be enough exposure time when imaging the blot.	Try imaging the blot again with a longer exposure time. This may require some optimization to get right.

— Bands in the sample lanes are faint or have no signal

If only the sample lanes are difficult to see and the molecular weight ladder is unaffected, this suggests issues with detecting the protein of interest.

Possible causes	Solution
The primary antibody and the secondary antibody are not compatible.	Make sure you use a secondary antibody raised against the primary antibody species. Make sure that the isotypes of the primary and secondary are compatible.
Not enough antibody is bound to the protein.	Add a higher concentration of primary antibody. Incubate the sample for longer with the antibody (eg, overnight) at 4°C.
The protein of interest isn't present.	Run a positive control. Check the scientific literature to see if the protein is expected in your cell line.
Not enough protein is present.	Make sure you load at least 20–30µg protein per lane, use protease inhibitors, and run the recommended positive control. Use an enrichment step to maximize the signal (eg prepare nuclear lysates for a nuclear protein, use lower antibody dilution, higher sensitivity ECL substrate).
Overuse of antibodies has reduced their effectiveness.	Make sure you use fresh primary and secondary antibodies for each experiment; the effective antibody concentration is lowered after each use.
Buffers may be incompatible with the detection method.	Some buffers contain reagents that may interfere with detection. For example, sodium azide is an inhibitor of HRP, so it is unsuitable for use with HRP-conjugated antibodies. Check your buffers don't contain any incompatible reagents, and change the buffer if needed.

Membrane protein.	Use un-boiled lysate. As shown in Figure 9 below, you can receive different western blot results when using boiled vs unboiled samples.

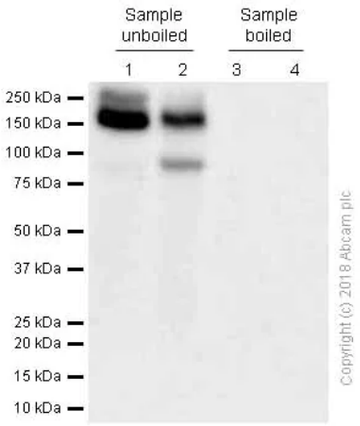

Figure 9. Western blot with Anti-P Glycoprotein antibody [EPR10364-57] (ab170904). All lanes: Anti-P Glycoprotein antibody [EPR10364-57] (ab170904) at 0.1 µg/mL (purified). Lanes 1 & 3 : C6 (Rat glial tumor glial cell) whole cell lysates prepared using RIPA lysis method. Lanes 2 & 4 : C6 (Rat glial tumor glial cell) whole cell lysates prepared using 1%SDS Hot lysis method.

— No bands when testing recombinant protein or over-expression lysate.

Possible causes	Solution
Unqualified recombinant protein or over-expression vector.	Use Coomassie blue staining or silver staining to validate or test with a tag antibody.
It's not a full-length protein, and the immunogen sequence of this antibody is not included in this partial-length recombinant protein or over-expressed protein.	Use a new antibody with the immunogen located in this protein.
Immunogen is located at the same end as the tag, so the tag may block the recognition of the antibody.	Remake a recombinant protein or over-expression lysate to avoid immunogen and tag being positioned at the same end. Alternatively, use a new antibody with an immunogen not located close to the tag.

High background

Possible causes	Solution
The secondary antibody may be binding non-specifically.	Run a control without any primary antibody. Make sure you use a secondary antibody raised in a different species to your sample. Try a secondary antibody that has been pre-adsorbed against the lg of the species of your samples.
Primary antibody concentration may be too high.	Dilute the antibody further to its optimal concentration.
The secondary antibody may be binding to the blocking reagent.	Add a mild detergent such as Tween 20 to the incubation and washing buffer. Note that phospho-specific antibodies may react with a milk-blocking agent due to the presence of the phosphoprotein casein. If using phospho-specific antibodies, block with BSA instead of milk.
Blocking of non-specific binding may be insufficient.	Increase the blocking incubation period and consider changing the blocking agent. We recommend blocking 3–5% non-fat dry milk, BSA, or normal serum for 1 hr at room temperature.
The incubation temperature may be too high.	Make sure you incubate samples at 4°C. Keep on ice throughout the western blot process.
Not enough washing between steps.	Residual unbound antibodies or other reagents remaining between steps can produce a high background. Wash extensively in the buffer between all steps. If using fluorescent detection, be sure to remove Ponceau S before immunostaining as this can autofluoresce.
Too much substrate (if using enzyme-conjugated antibody).	Dilute the substrate and reduce substrate incubation time.

Your substrate has too high sensitivity.	Replace it with a milder substrate.
Signal amplification may be too high (if using a signal amplification technique).	Reduce the amount of signal amplification (eg, conjugate less biotin to secondary antibody if using biotinylation).
The blot has dried out.	Prevent the membrane from drying out during incubation by keeping it covered in the buffer.
The membrane was contaminated during the experiment process.	Always keep the membrane clean.
Incubating two or more membranes in one container.	Make sure to place them back to back.
Exposure time may be too high when imaging the blot.	Try imaging the blot again with a longer exposure time. This may require some optimization to get right.
Your choice of the membrane may give a high background.	Nitrocellulose membranes generally give less background than PVDF; consider using a nitrocellulose membrane instead if high background persists.
Your lysate method is not strong enough.	Use a stronger lysate method (for example, 1%SDS hot method) to get a stronger signal and reduce non-specific bands.

Unexpected or multiple bands

— Multiple bands at various molecular weights

Possible causes	**Solution**
Proteases may have digested the protein.	This is likely if you see multiple bands at low molecular weight. Add protease inhibitors to prevent protein degradation. Use fresh lysate.

The protein may form multimers.	This is likely if you see extra bands at high molecular weights that are 2x or 3x the weight of the expected bands. Some proteins will form dimers, trimers, or larger multimers due to disulfide bond formation if the samples are insufficiently reduced. To prevent this, try boiling the sample for longer in Laemmli buffer during sample preparation.
Modifications like heavy glycosylation.	This is likely if you see multiple bands at high molecular weight. Use un-boiled lysate.
The protein may be cleaved, activated form.	Check the literature to see where is the cleavage site and which fragment includes the immunogen sequence of your antibody.
The cell line may have been passaged too many times.	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 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). Use knock-out lysates or cells that do not express endogenous target protein.
The antibodies are not purified.	Some antibody formats are relatively impure and may contain additional proteins. If possible, use antibodies that have been affinity purified.

<u>Unusual gel or band appearance</u>

Spots or smudges

— Black dots or speckled background

Possible causes	Solution
The blocking reagent has clumped together, and antibodies are binding to it.	This binding will appear as dots of positive signal. Filter the blocking agent.
The gel or reagents are contaminated with bacteria.	Make sure you use fresh, sterile buffer (eg our sterile PBS).

— White spots or smudges

Possible causes	Solution
Air bubbles were trapped against the membrane during transfer.	Bubbles will appear as uneven white spots. Make sure you remove any air bubbles caught between the gel and the membrane during transfer. You can do this by lightly pressing down on the stack with a small roller. You should be able to see any bubbles after checking the success of the transfer with Ponceau S.

Bands appear very low or very high

— Bands very low

Possible causes	Solution
The gel has been run for too long.	Before proceeding with blocking and immunostaining, check the transfer of proteins to the membrane with Ponceau S. If all bands appear very low, you may have left the proteins too long to migrate through the gel. Try running a gel again for a shorter time before proceeding.

Not enough acrylamide in the gel.	The bands may be very low on the blot if there's not enough acrylamide in the buffer. This is because the proteins do not experience enough resistance, so migrate too quickly across the gel. You should generally run lower molecular weight proteins in gels with a higher percentage of acrylamide. Check this table for suggested gel recipes, and increase the amount of acrylamide if necessary.

— Bands very high

Possible causes	**Solution**
The gel has not been run for long enough.	Before proceeding with blocking and immunostaining, check the transfer of proteins to the membrane with Ponceau S. If all bands appear very high, the proteins may not have had enough time to migrate across the gel. Try running the gel for longer before proceeding.
Too much acrylamide in the gel.	The bands may be very high on the blot if there's too much acrylamide in the buffer. This is because a high acrylamide density can block effective migration of proteins through the gel. You should generally run higher molecular weight proteins with a lower percentage of acrylamide. Check this table for suggested gel recipes, and reduce the amount of acrylamide if necessary.

Bands are misshapen or uneven

— Smile effect on bands

Possible causes	Solution
Voltage may have been too high during migration.	If the voltage is too high, migration will occur too quickly. Check the protocol for the suggested voltage and decrease if necessary.
Gel may have been too hot during migration.	If the temperature is too high, the pH of the buffer may be slightly altered, which could affect migration. Run the gel at 4°C: on ice or in a cold room.

— Bands are uneven

Possible causes	Solution
The gel has polymerized unevenly.	When the gel has not polymerized properly, bands can appear wonky or uneven. In extreme cases, lanes probed for the same protein can appear at different molecular weights (see image above). Check your gel recipe to see if you've added the right amount of TEMED. Ensure the gel is covered entirely in buffer while it is setting.

Bands appear white (if using ECL detection)

Possible causes	Solution
Primary and secondary antibody concentration may be too high.	If the antibody concentration is very high, then the substrate is consumed very quickly. This means very little light is absorbed at this point, leading to a white band when you image the blot. Dilute the antibody to its optimal concentration.

Western blot optimization

Working with specific proteins, such as phosphorylated proteins or histones, may require some protocol optimization. Below we highlight the most important tips for performing western blot with phosphorylated proteins and histones.

Western blot protocol for phosphorylated proteins

If you're working with post-translational modifications of proteins, such as phosphorylation, it's essential to handle your samples with care and use the right reagents to maintain the structural integrity of your proteins. To keep the proteins in their phosphorylated state, add phosphatase inhibitors to the working stocks of your buffers and always keep samples on ice.

It is also important to block the membrane in BSA and avoid using casein or milk-blocking buffers. Casein is a phosphoprotein present in milk, which causes high background signals. A starting BSA concentration of 5% w/v in TBS-T is suggested; however, the optimal concentration is antibody-dependent and should be empirically determined. When using abcam recombinant antibodies, 5% non-fat dry milk can be a good alternative blocking buffer.

For step-by-step guidance, check our western blot protocol for phosphorylated proteins.

Histone western blot protocol

Histones have a very low molecular weight, usually around 10–20 kDa, and due to their small size, you need to be careful when carrying out your western blot.

Tips for successful western blotting with histones:

— Use a high-percentage gel for a clear resolution of histone proteins.

— Use a nitrocellulose membrane with a pore size of 0.2 µm to ensure the optimal capture of histone proteins.

— We recommend using high-quality BSA in your blocking solutions over dried milk powders. However, the optimal blocking is antibody-dependent and should be empirically determined.

— Always use loading control antibodies to standardize your experiments.

For more details, refer to our step-by-step histone western blot protocol.

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