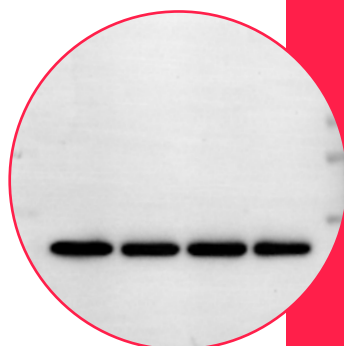
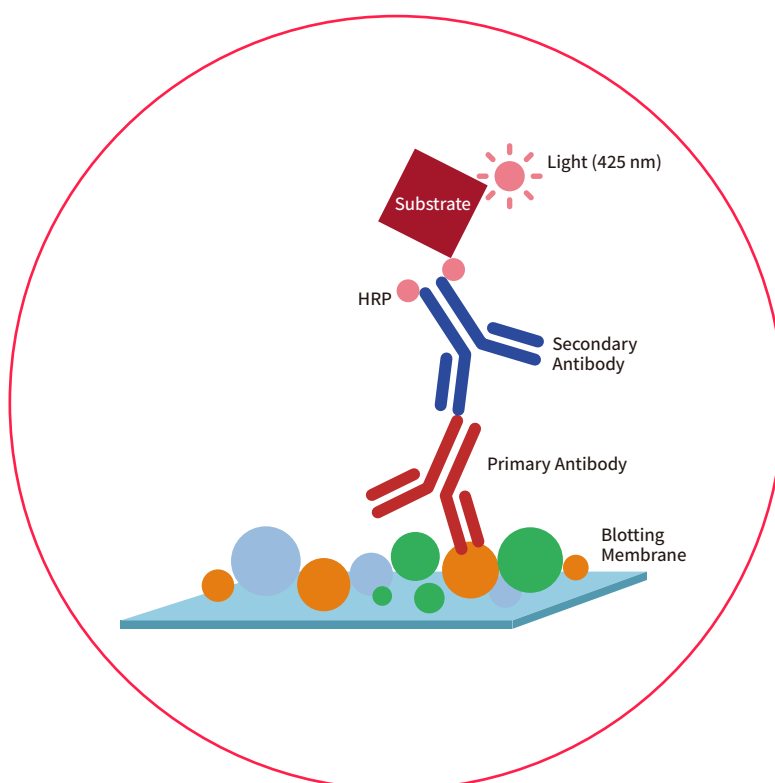


Western Blot (WB) Technical Manual

Western Blot Workflow
From Sample Preparation to Result Analysis



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I. Technical Background

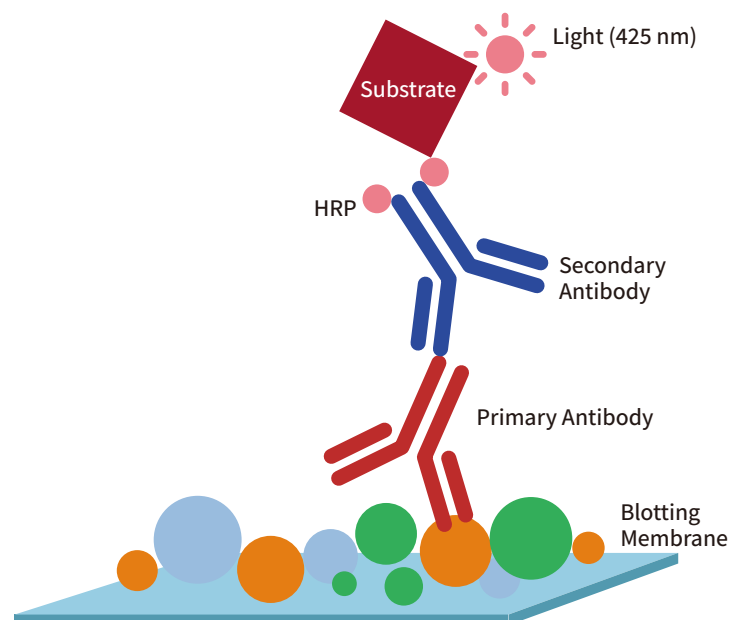
Western Blot (WB), also known as protein immunoblotting, is used to separate and identify specific proteins from complex mixtures and is a widely used basic protein analysis method. This technique was established by Towbin et al. in 1979. The earliest established core process of electrophoresis transfer involved separating proteins using polyacrylamide gel electrophoresis (SDS-PAGE), then transferring the proteins from the gel to a nitrocellulose membrane, and finally detecting the target protein with the help of antibodies. W. Neal Burnette of the Tobert Nowinski Laboratory in Seattle was the first to coin the term "western blotting." The term is named in honor of Edwin Southern, who named DNA blotting, and also refers to the location of Nowinski's laboratory on the West Coast.

Technical Applications

- Qualitative protein analysis
- Semi-quantitative protein analysis
- Protein expression quantification
- Validation of post-translational modifications
- Fundamental technique in life sciences research

II. Technical Principle

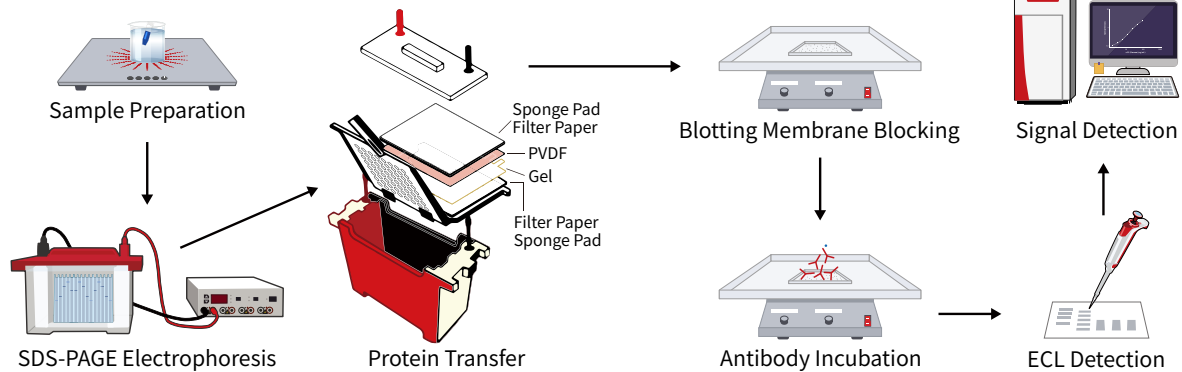
Similar to Southern or Northern hybridization methods, but Western blot uses SDS-PAGE, the analyte is protein, the "probe" is an antibody, and the "color development" is achieved using a labeled secondary antibody. Protein samples are first separated using SDS-PAGE. Since gels are not conducive to antibody binding to proteins, the proteins need to be transferred to a solid support (e.g., a PVDF membrane). The solid support adsorbs proteins non-covalently, maintaining the type and biological activity of the peptides separated by electrophoresis. The proteins or peptides on the solid support serve as antigens, reacting with the corresponding primary antibody in an immunoreaction, followed by a reaction with an enzyme-or isotope-labeled secondary antibody. The separated proteins are then detected using methods such as substrate color development or fluorescence imaging, enabling qualitative and semi-quantitative analysis of the target protein.



Schematic diagram of the enzyme-substrate reaction during chemiluminescent western blot detection.

III. Operating Steps

Western Blot (WB) Workflow



Stage 1 - Sample preparation

Key reagents

- Lysis buffer (e.g., RIPA) • Protease inhibitor cocktail • BCA or Bradford protein assay kit
- Phosphatase inhibitor cocktail (optional, for phosphorylated proteins) • 5X loading buffer

1. Extract total protein

(1) For tissue samples:

- Wash the tissue block with pre-cooled PBS to remove blood stains, cut it into small pieces and place it in a homogenizer.
- The amount of RIPA lysis buffer used can be added at a ratio of approximately 30 mg tissue to 300 μ L of lysis buffer, followed by low-temperature homogenization. If the lysis buffer does not contain protease inhibitor cocktail, which should be added before use. For phosphorylated proteins, phosphatase inhibitor cocktail should be added before use.

Tip1: If lysis is insufficient, you can add lysis buffer as needed; if the protein content of the tissue is low, you can reduce the amount of lysis buffer to increase the protein concentration in the crude extract solution.

- Transfer the homogenate to a 1.5 mL centrifuge tube and vortex. Incubate on ice for 30 min, repeatedly pipetting every 10 min to ensure complete lysis of the tissue cells.

- Centrifuge at 12000 g for 5 min, collect the supernatant, which is the total protein solution, and store at -80 $^{\circ}$ C for later use.

(2) For adherent cell samples:

- Wash the cells 2-3 times with pre-cooled PBS, and thoroughly aspirate any remaining solution on the last wash.
- Add RIPA lysis buffer into the cell culture plate / flask at a ratio of 250 μ L of lysis buffer per well in a 6-well plate. Shake the culture plate / flask repeatedly to ensure that the lysis buffer is in full contact with the cells for 3-5 min.
- Use a cell scraper to scrape off the cells and collect them into a centrifuge tube.
- On ice, lyse for 30 min.
- Centrifuge at 12000 g for 5 min, collect the supernatant, which is the total protein solution, and store at -80 $^{\circ}$ C for later use.

(3) For suspended cell samples:

- Transfer the suspended cell culture medium to a centrifuge tube, centrifuge at 4 $^{\circ}$ C, 1000-3000 rpm for 5 min, and discard the supernatant.

- b. Add pre-cooled PBS (approximately 5-10 times the volume of the cell pellet), gently pipette to mix, centrifuge at 4 °C, 1000-3000 rpm for 5 min, and discard the supernatant. Repeat the washing process 2-3 times to thoroughly remove serum proteins from the culture medium.
- c. Mix the cells with RIPA lysis buffer at a ratio of 250 µL of lysis buffer per well in a 6-well plate and shake.
- d. Incubate on ice for 30 minutes, and repeatedly pipette several times every 10 minutes to ensure complete cell lysis.
- e. Centrifuge at 12000 g for 5 min, collect the supernatant, which is the total protein solution, and store at -80 °C for later use.

2. Measure protein concentration

The protein concentration in the lysate was determined using the Bradford assay or BCA assay. (If the protein concentration is low and the protein resides in the nucleus or mitochondria, separation of cellular components to enrich the target protein may be considered.)

3. Add loading buffer for denaturation

Add the sample and 5X loading buffer at a ratio of 4 : 1, mix well, and heat in a 95 °C water bath or metal bath for 10 minutes to fully denature the protein, then store at -20 °C for later use.

(For details on lysing different sample types, please refer to the EnkiLife Comprehensive Guide to Lysis Protocols for Different Sample Types.)

Stage 2 - Sample loading and gel running

Key reagents

- Prestained protein marker
- SDS-PAGE gels (such as Tris-Glycine gel, Bis-Tris gel, Tris-Tricine gel.)
- Electrophoresis buffer (such as Tris-Glycine buffer, MOPS / MES buffer, Tris-Tricine buffer.)

1. Gel preparation

Take Tris-Glycine gel as an example. The formula is as follows:

Separating gel (taking 1.0 mm, 2-plate gel casting as an example)

Applicable molecular weight range reference	>100 kDa	50–100 kDa	20–80 kDa	<40 kDa
Separating gel concentration	8%	10%	12%	15%
30% Acr-Bis (29:1)	2.7 mL	3.3 mL	4.0 mL	5.0 mL
1.5M Tris-HCl pH8.8	2.5 mL	2.5 mL	2.5 mL	2.5 mL
10% SDS	0.1 mL	0.1 mL	0.1 mL	0.1 mL
10% Ammonium Persulfate (APS)	0.1 mL	0.1 mL	0.1 mL	0.1 mL
TEMED	4 µL	4 µL	4 µL	4 µL
ddH ₂ O	4.6 mL	4.0 mL	3.3 mL	2.3 mL
Total volume	10 mL	10 mL	10 mL	10 mL

Stacking gel (taking 1.0 mm, 2-plate gel casting as an example)

Stacking gel concentration	4%
30% Acr-Bis (29:1)	0.83 mL
1.5M Tris-HCl pH6.8	1.25 mL
10% SDS	0.05 mL
10% Ammonium Persulfate (APS)	0.05 mL
TEMED	5 μ L
ddH ₂ O	2.82 mL
Total volume	5 mL

Tip1: The glass plate used for making SDS-PAGE gels should be cleaned and dried before use.

Tip2: Add TEMED and APS last, and mix thoroughly before pouring the SDS-PAGE gels.

Tip3: The larger the protein, the lower the percentage of acrylamide in the gel. This creates a lower density polymer, making it easier for the protein to pass through.

Prepare a separating gel of appropriate concentration and pour it into the gel casting plate to about 1.5 cm from the top edge of the short gel plate (do not pour in the full amount if the prepared solution is excessive). Gently pour ddH₂O or isopropanol into the gel casting plate to act as a liquid seal and promote the solidification of the separating gel. The separating gel will solidify in about 20 min. Discard the ddH₂O or isopropanol used for liquid sealing, and tilt the gel casting plate for a while to allow the remaining ddH₂O to gather together for easy absorption with filter paper. Lay the gel casting plate flat and continue to pour the prepared stacking gel into the gel casting plate until it reaches the top edge of the thin glass plate, trying to avoid generating air bubbles. Then gently insert a comb. The stacking gel will solidify and be ready for use in about 20 min.

Tip1: Tris- Tricine gels are better at separating low molecular weight proteins (<10 kDa) than Tris - Glycine gels.

Tip2: Gradient gels can provide clearer bands and offer a wider protein detection range on a single gel, such as 10-250 kDa.

Tip3: Prepare the SDS-PAGE gel according to the molecular weight of the target protein. Alternatively, you can choose a pre-cast gel or a rapidset broad-range gradient gel kit to reduce gel preparation time and provide a wider protein separation range.

2. Sample loading

Add an equal volume of the denatured protein sample to the gel. The typical protein loading volume is 10–20 μ g.

Tip1: Prevent overload; otherwise, the sample may overflow into adjacent wells.

Tip2: Be careful not to let the pipette tip touch the bottom of the sample well to prevent strip deformation.

3. Electrophoresis

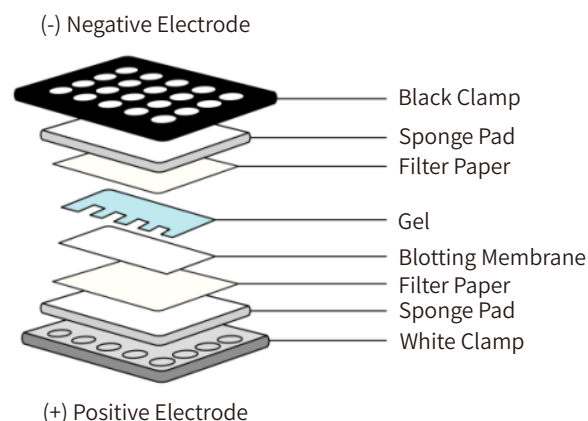
Match the appropriate electrophoresis buffer to the SDS-PAGE gels, or choose a universal rapid electrophoresis buffer. There is no need to change the electrophoresis buffer due to changes in gel type, and electrophoresis can be completed in 30 minutes, which greatly shortens the electrophoresis time.

Tip1: The gel running time will vary depending on the instrument, gel composition, buffer system, and target protein used. Larger protein molecules require longer running times. The actual electrophoresis time should be determined when bromophenol blue reaches the bottom of the gel or when the prestained marker bands are fully separated.

Stage 3 - Protein transfer (taking wet transfer as an example)

Key reagents

- SDS-PAGE gel
- Transfer buffer
- Washing buffer (e.g., TBST)
- Blotting membrane (NC membrane or PVDF membrane)
- Methanol /ethanol
(required for activating the PVDF membrane)
- Sponge pad
- Filter paper (optional)
- Ponceau S staining solution (optional)



Schematic diagram of gel-blotting membrane sandwich assembly for wet transfer.

1. Soaking blotting membrane and sponge pad

Because PVDF membranes are naturally hydrophobic, they must be soaked in methanol/ethanol for 30 s before use. (This step is necessary for PVDF membranes, but not for NC membranes.) After activation, the PVDF membrane, along with the sponge pad, should be soaked in transfer buffer at room temperature for 3-5 min to equilibrate.

Tip1: The pore sizes of the blotting membrane required for western blotting experiments are mostly 0.45 μm and 0.22 μm . 0.45 μm is suitable for proteins with a molecular weight greater than 20 kDa, while 0.22 μm is suitable for low molecular weight proteins with a molecular weight less than 20 kDa.

2. Transfer membrane

(1) Remove the gel from the electrophoresis tank and immerse it in transfer buffer at room temperature for 3-5 min to equilibrate.

(2) Arrange them tightly in the manner of sponge pad-filter paper (optional)-gel-blotting membrane-filter paper (optional)-sponge pad to ensure that there are no air bubbles between each layer.

Tip1: Note that the gel should be close to the negative electrode and the blotting membrane should be close to the positive electrode.

Tip2: Transfer time may vary depending on the instrument, transfer buffer, and the molecular weight of the target protein. Traditional transfer buffers require a longer transfer time and an ice bath. Alternatively, an ice-free rapid transfer buffer can be selected, which allows transfer to be completed at room temperature for 25 min at 400 mA (for large molecular weight proteins, the transfer time should be appropriately extended).

(3) After the transfer is complete, remove the blotting membrane and rinse it once in TBST.

Tip1: If you need to observe protein transfer, you can use Ponceau S to stain the blotting membrane, and then clean it with TBST after observation.

Stage 4 - Blotting membrane blocking and antibody incubation

Key reagents

- Blocking buffer (such as TBST containing 3–5% milk or BSA)
- Washing buffer (e.g., TBST)
- Antibody diluent (such as TBST containing 3–5% milk or BSA)
- Primary antibody
- Secondary antibody

1. Blotting membrane blocking

Place the blotting membrane in an incubation box and cover it with blocking buffer. Incubate with shaking at room temperature for 1–2 h.

Tip1: To shorten the blocking time, consider a 5 min protein-free rapid blocking buffer that is also highly compatible with modified antibodies for western blot detection.

2. Antibody incubation

- (1) Dilute the primary and secondary antibodies according to the dilution recommended in the antibody instructions.
- (2) Primary antibody incubation: Incubate with shaking overnight at 4 °C, or for 2 h at room temperature. The incubation time needs to be optimized and adjusted based on the expression of the target in the samples used.
- (3) Wash the blotting membrane three times with washing buffer, each time for 5 min.
- (4) Secondary antibody incubation: Incubate with shaking at room temperature for 1–2 h.
- (5) Wash the blotting membrane three times with washing buffer, each time for 5 min.

Tip1: To avoid overnight incubation and cross-day experiments, you can consider using a rapid antibody dilution buffer. For the first time, blocking steps can be skipped, and primary and secondary antibody incubations can be completed within 40 min at room temperature.

Stage 5 - Imaging

Key reagents

- Chemiluminescent substrates (e.g., ECL substrate)

After incubation, the blotting membrane can be imaged. Chemiluminescence detection involves incubating the blotting membrane in a chemiluminescent substrate, causing luminescence at the sites where the HRP-conjugated antibody is present.

Stage 6 - Antibody stripping (optional)

Key reagents

- Antibody stripping buffer
- Washing buffer (e.g., TBST)
- Chemiluminescent substrates (e.g., ECL substrate)
- Blocking buffer

After imaging, antibody stripping can be performed when other antibody tests are required on the blotting membrane.

1. Antibody stripping

- (1) In a suitable container, immerse the exposed blotting membrane in 10-15 mL of antibody stripping buffer (enough to completely submerge the blotting membrane), and incubate with shaking at room temperature for 10 min. (The stripping time is subject to the product manual).
- (2) Wash the blotting membrane three times with washing buffer, each time for 5 min.
- (3) Imaging: If the signal is still present, repeat steps 1 and 2 until there is no signal on the blotting membrane.

2. Begin a new round of western blotting experiments.

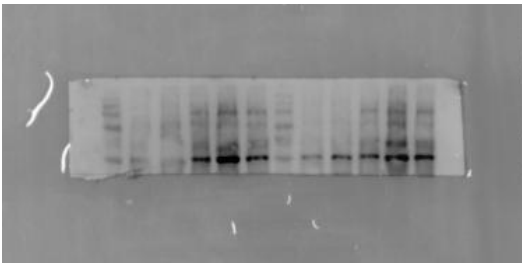
Reseal the cleaned blotting membrane and incubate it with a new primary antibody.

IV. Analysis of Abnormal Results

This paper analyzes common WB problems such as high background, black spots in the background, no signal or weak bands, multiple bands, and abnormal band shapes.

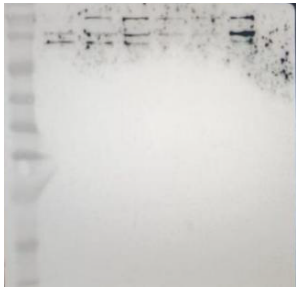
1. High background

Possible reasons	Solutions
Inadequate washing	Unbound antibodies or other reagents remaining between steps can produce high background. Increase the number of washes and/or the amount of wash buffer. Ensure thorough washing with wash buffer between all steps .
Insufficient blocking of nonspecific sites	(1)Increase the protein concentration in the blocking buffer. (2)Optimize the blocking time and/ or temperature. Incubate at room temperature for at least 1 h or perform overnight blocking at 4 °C. (3)Antibody dilutions were prepared in blocking buffer containing 0.05% Tween 20 detergent. Adding Tween 20 to the blocking buffer helps reduce background; however, excessive detergent can interfere with antibody binding. A final concentration of 0.05% Tween 20 typically provides optimal results.
Primary antibodies may bind to blocking buffer	When detecting phosphorylated proteins, phospho-specific antibodies may cross-react with milk-based blocking buffer. Avoid using phosphate-containing buffers (e.g., PBS) and blocking buffers containing phosphorylated proteins (e.g., milk or casein). Use BSA or protein-free blocking buffer for blocking.
Antibody concentration too high	Reduce the concentration of primary and/or secondary antibodies appropriately.



2. Black dots in the background

Possible reasons	Solutions
The blocking agent was not completely dissolved	Before blocking, ensure the blocking agent is completely dissolved. It can be prepared in advance and placed on a shaker to accelerate dissolution.



3. No or weak signal

3.1 Both the prestained marker and the target protein bands showed no or weak signal

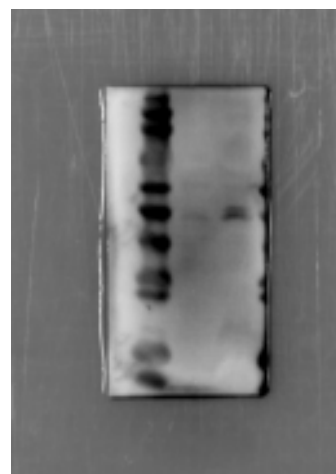
If all bands on the blotting membrane, including the prestained marker, are difficult to see, it is likely a problem with the experimental procedure rather than a problem with protein detection.

Possible reasons	Solutions
The signal from the chemiluminescent substrate is too weak	(1)Increase the incubation time between the blotting membrane and the substrate. (2)Try extending the exposure time and then taking another image. (3)Replace with a high-sensitivity ECL substrate.
Improper storage and handling may cause reagents to lose their activity	Refer to the product storage instructions in the instruction manual. Avoid repeated freeze-thaw cycles.
Low molecular weight proteins may be over-transferred	(1)Low molecular weight proteins may have crossed the blotting membrane, shortening the transfer time. (2)Replace with a smaller pore size blotting membrane (e.g., from 0.45 μm to 0.22 μm).
Large molecular weight proteins may be incompletely transferred to the blotting Membrane	(1)After transfer , use a reversible dye, such as Ponceau S staining buffer, to check if the transfer was successful. If the protein was not transferred effectively, check if the transfer direction was correct. (2)If the transfer direction is correct, increase the transfer time appropriately. (3)If a PVDF membrane is selected, it needs to be activated by immersing it in methanol/ethanol first, and then immersed in the transfer buffer.



3.2 The target protein bands showed no or weak signal

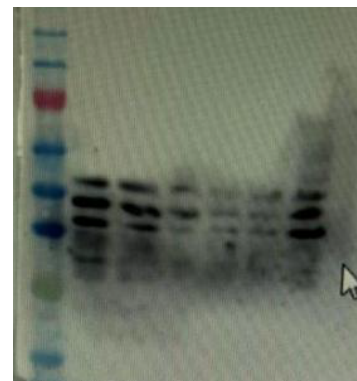
If only the target protein band is weak, while the marker band is unaffected, there may be a problem with the detection of the target protein.



Possible reasons	Solutions
Primary antibody concentration too low	(1) Increase the concentration of the primary antibody. (2) Extend the incubation time (e.g., incubate at 4 °C overnight).
The target protein is not present	Consult scientific literature to see if the target protein is present in the sample used.
Insufficient sample loading of target protein	(1) 10-20 µg of protein is loaded into each lane. If the target protein band is weak, the loading amount can be increased appropriately. (2) Adding enrichment steps maximizes the signal (e.g., for nuclear proteins, using nuclear lysate and increasing antibody concentration, using highly sensitive ECL substrates).
Primary and secondary antibodies mismatch	(1) Ensure that the species of the secondary antibody matches the host of the primary antibody. (2) Ensure that the primary and secondary antibodies are of the same type.
Repeated use of antibodies reduces their effectiveness	Replace with freshly prepared primary and secondary antibodies, as the concentration of effective antibodies decreases with each use.

4. Multiple bands

Possible reasons	Solutions
Protein degradation	If you see multiple bands at low molecular weight regions, this is very likely the cause. Add fresh protease inhibitors to prevent protein degradation.
Protein forms polymers	If you see extra bands at high molecular weights (2 or 3 times the expected band molecular weight) (some protein forms dimers, trimers, or larger polymers due to disulfide bond formation), this may be the cause.
Modifications, such as heavy glycosylation	If you see multiple bands at high molecular weights, this may be the cause.
Proteins may be cut	Review the literature to understand the location of the cleavage site and the fragment containing the antibody immunogen sequence.
The bands are nonspecific bands	Replace with a highly specific antibody.



5. Abnormal band shapes

5.1 Band tailing

Possible reasons	Solutions
Excessive sample loading	Reduce the protein loading amount (e.g., from 40 μg to 20 μg), or perform a loading gradient test.
Proteins were not adequately denatured/reduced during sample preparation	(1) Boil samples at 95–100 °C for 5–10 min to ensure complete denaturation. (2) Add sufficient reducing agent (β -mercaptoethanol or DTT).
High sample viscosity	Ultrasonic disruption or increasing the number of pipetting can reduce sample viscosity.
Improper electrophoresis conditions (excessive voltage/heat generation)	Reduce the voltage, use a pre-cooled buffer, or perform electrophoresis at a low temperature.
Improper gel concentration	Choose the proper concentration based on the molecular weight of the target protein: • Small molecular weight protein (<30 kDa): 12–15% gel; • Medium molecular weight protein (30–100 kDa): 10% gel; • Large molecular weight protein (>100 kDa): 6%–8% gel.
Sample salt concentration too high	If the lysis buffer has a high salt concentration, dilute the sample with PBS or replace it with a low-salt lysis buffer.



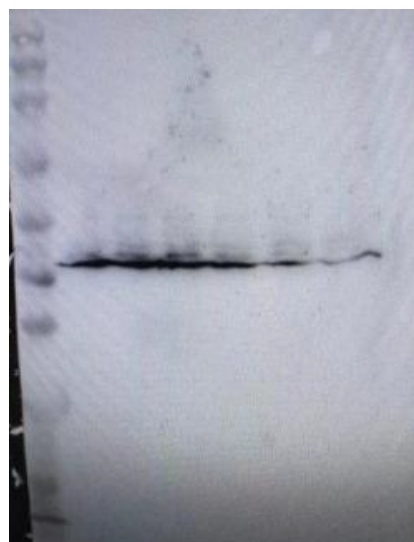
5.2 Band overload

Possible reasons	Solutions
Excessive sample loading	Reducing the sample loading amount allows for preliminary experiments using sample loading gradients (e.g., 5, 10, 20 μg for comparison).
Primary antibody concentration too high	Reduce the concentration of the primary antibody.



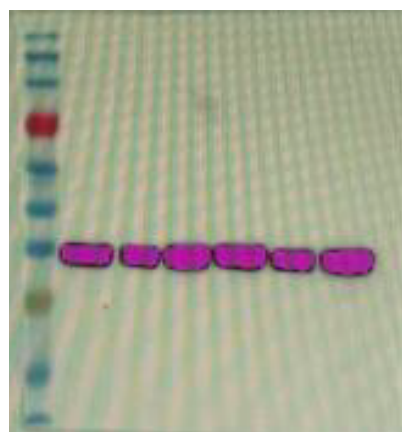
5.3 Horizontal band spreading

Possible reasons	Solutions
Gel preparation/ coagulation issues	(1)Ensure the separating gel has fully solidified (it is recommended to allow it to solidify for at least 20 min). (2)Use appropriate amounts of APS and TEMED to ensure uniform polymerization.
Stale electrophoresis buffer / a low buffer level	(1)Replace with fresh electrophoresis buffer to avoid repeated use. (2)Ensure the buffer level is high, the inner tank is filled with fresh electrophoresis buffer, and the outer tank level also reaches the mark.
Excessive sample loading	Reduce the sample loading amount to avoid excessively wide stripes that exacerbate lateral tailing.
Overheating at the center of the gel	(1)Reduce the running voltage to 80–100 V constant throughout, or run at 80 V initially and increase to 120 V once the samples have entered the separating gel. (2)Use pre-cooled electrophoresis buffer or perform electrophoresis at a low temperature.



5.4 Band whitening

Possible reasons	Solutions
Primary and secondary antibodies concentration too high	If the antibody concentration is too high, the substrate will be consumed quickly. This means that the substrate is depleted rapidly and the signal becomes oversaturated, resulting in white bands when imaging the blotting membrane. In this case, it is necessary to consider reducing the antibody concentration.
Excessive sample loading	The target protein is in excess, and the substrate is depleted instantly. Reduce the protein loading amount.
Excessive exposure time	If the signal is oversaturated during imaging, significantly shorten the exposure time or perform gradient exposure at multiple time points.
Substrate sensitivity too high	The chemiluminescent substrate is too sensitive, and the signal exceeds the detection linear range. It can be replaced with a mild, low-sensitivity ECL substrate.



5.5 Bands with multiple defects

Band issues	Possible reasons	Solutions
High background	(1) Insufficient blocking (2) Primary/secondary antibody concentration too high (3) Inadequate washing	(1) Extend the blocking time. (2) Reduce the concentration of primary and/or secondary antibodies appropriately. (3) Increase the number of washes and/or the amount of wash buffer.
Nonspecific bands	Primary antibody lack of specificity	Change the antibody or try reducing the primary antibody concentration.
Band diffusion/trailing	(1) Gel preparation/coagulation issues (2) Excessive sample loading (3) Sample salt concentration too high (4) High sample viscosity (5) Excessive transfer time or excessive current (especially during wet transfer, small protein molecules are prone to diffusion)	(1) Ensure the separating gel has fully solidified (it is recommended to allow it to solidify for at least 20 min). Use appropriate amounts of APS and TEMED to ensure uniform polymerization. (2) Reduce the sample loading amount. (3) If the lysis buffer has a high salt concentration, dilute the sample with PBS or replace it with a low-salt lysis buffer. (4) Ultrasonic crushing reduces viscosity. (5) Adjust transfer conditions according to protein size to avoid using the same fixed conditions for all proteins.



WB Technical Q&A

1. Common sample issues

Q1: How are nucleoproteins and membrane proteins extracted?

A1: Nuclear proteins and membrane proteins, most of which have low endogenous expression levels, need to be enriched using specialized extraction methods or extraction kits, and then lysed to obtain the sample to be tested.

Q2: For western blotting at the cellular level, how many cells are needed to extract enough protein for a western blotting sample?

A2: Generally, for a single well of a 6-well plate, 5×10^5 - or 1×10^6 cells are sufficient.

Q3: If it is necessary to increase the sample loading amount to make the originally weak bands clearer, but at the same time avoid overloading the sample loading amount, how should it be handled?

A3: Concentrated sample; use 1.5 mm thick gel and a large-pore comb; overload within 30% has minimal impact.

Q4: How long can denatured proteins be stored?

A4: Can be stored for one to two years at -80°C ; two key points: avoid protease degradation and avoid microbial contamination.

Q5: What is the total amount of protein loaded in a single loading, and is it related to the expression level of the target protein?

A5: The standard loading amount is 10-20 μg ; the loading amount, protein abundance, antibody dilution, and exposure time all affect the final band intensity. When exploring conditions, the loading amount can be increased appropriately, but the background may increase simultaneously.

2. Common gel problems

Q1: I want to separate a protein with a molecular weight of 260 kDa. What is the appropriate separating gel concentration for SDS-PAGE electrophoresis? What should the concentration of the stacking gel be? Is it easy to perform western blot on a protein with such a large molecular weight?

A1: For a 260 kDa ultra-large protein, use a 6% separating gel and a 3.5% stacking gel. Be gentle when peeling the gel to avoid breakage. Transferring large protein molecules is more difficult and requires a longer transfer time.

Q2: The molecular weight of the protein I measured is 105 kDa. Theoretically, the separating gel should be 7.5%, but the information I found requires a 10% for the separating gel. I don't know why.

A2: Both gel systems can be used. The 105 kDa protein is separated within the linear separation range of both gels, with the only difference being the position of the band migration.

Q3: Why is the separation effect of small molecule weight proteins poor?

A3: A possible reason is that the standard Tris - Glycine gel has limited resolution for small protein molecules. Try using a Tris- Tricine gel, which is particularly suitable for proteins below approximately 10 kDa.

Q4: Why are the bands in electrophoresis so thick?

A4: Thick bands are common in electrophoresis. This is mostly caused by inefficient stacking in the stacking gel. Consider increasing the length of the stacking gel; ensuring the pH of the stacking gel buffer is correct; and appropriately reducing the voltage during electrophoresis.

3. Common antibody problems

Q1: The marker tested positive?

A1: Because polyclonal antibodies recognize a variety of antigenic epitopes, and pre-stained markers are essentially fusion proteins of different molecular weights, polyclonal antibodies may recognize these proteins, thus producing signals during imaging.

Q2: With so many internal control antibodies, is it sufficient to choose the commonly used GAPDH/ β -actin?

A2: When selecting an internal control antibody, the following core principles should be followed:

(1) Stable expression: The internal reference protein should be relatively constant in different samples and its expression should not fluctuate due to experimental treatment (such as hypoxia or drug stimulation).

(2) Molecular weight difference: The molecular weight of the internal reference protein should be significantly different from that of the target protein (it is recommended to be at least 5 kDa different) to avoid band overlap.

(3) Cellular localization: It should be consistent with the subcellular localization of the target protein. For example, when detecting nuclear proteins, it is not recommended to use cytoplasmic proteins as loading control. (For more details, please refer to the EnkiLife Western Blot (WB) Internal Control Selection Guide)

Commonly used internal control antibodies quick check list:

Internal control protein	Molecular weight	Localization	Suitable scenarios	Precautions
β -Actin	~42 kDa	Cytoplasm /Cytoskeleton	Most universal whole-cell loading control; first choice for non-muscle tissues	Avoid in adipose tissue, muscle tissue, and cytoskeleton-related experiments
GAPDH	~36 kDa	Cytoplasm (glycolytic enzyme)	Routine cell/tissue samples	Avoid in hypoxia, diabetes, and metabolic stress experiments
β -Tubulin	~55 kDa	Cytoplasm (microtubules)	Cytoskeleton research; stable under stress conditions	Avoid in anti-mitotic drug treatment and cell cycle studies
α -Tubulin	~55 kDa	Cytoplasm	Similar to β -Tubulin; can be used interchangeably	Avoid in anti-mitotic drug treatment and cell cycle studies
Vinculin	~117 kDa	Focal adhesion /Cytoplasm	High-molecular-weight target proteins; cell adhesion/migration studies	/

Internal control protein	Molecular weight	Localization	Suitable scenarios	Precautions
Histone H3	~17 kDa	Nucleus /Chromatin	Nuclear protein and chromatin-associated protein detection	/
Lamin B1	~66-70 kDa	Nuclear membrane	Nuclear membrane protein and nucleocytoplasmic fraction samples	Not applicable to nuclear membrane-removed samples
VDAC1	~30-35 kDa	Mitochondria	Mitochondrial outer membrane protein detection	Mainly for mitochondrial fractions
COX IV	~17 kDa	Mitochondria	Mitochondrial inner membrane protein detection	Mainly for mitochondrial fractions
Na ⁺ /K ⁻ ATPase	~110 kDa	Cell membrane	Membrane protein detection	Mostly for membrane fractions
Hsp70/90	~70/90 kDa	Cytoplasm	Relatively stable under non-stress conditions	Expression may be upregulated in heat shock/stress experiments

Q3: How to choose a suitable secondary antibody ?

A3: 1. Antibody considerations:

(1) Determine the host species of the primary antibody (e.g., mouse anti-tubulin, rabbit anti-CD4); most primary antibodies are prepared in mouse or rabbits.

(2) Select appropriate host species and responding species for the secondary antibody [e.g., goat anti-mouse IgG (H+L), donkey anti-rabbit IgG (H+L)].

The species source (host) refers to the species from which this secondary antibody is prepared, while the reaction species refers to the species targeted by the primary antibody.

Tip1: The species of the secondary antibody must differ both from the host species of the primary antibody and from the species of the test sample.

Tip2: When describing anti-mouse antibodies, use specific species whenever possible, such as anti-rat secondary antibody, anti-mouse secondary antibody, anti-hamster secondary antibody, etc.

Tip3: In addition to paying attention to the source of the primary antibody, it is sometimes also necessary to pay attention to the source of the sample used in the reaction.

(3) Consider the specific response of the secondary antibody, i.e. the type and subtype of the target immunoglobulin (Ig).

For example, anti-mouse IgG (H+L) means that the secondary antibody can bind to all mouse IgG (because the homology of IgG from certain species is close, it usually also has a certain degree of cross-reactivity with IgG from other species).

Anti-mouse IgG1, this secondary antibody has relatively higher specificity (it only binds to the mouse IgG subtype IgG1).

Anti-mouse IgG F(ab')₂, this secondary antibody binds only to the F(ab')₂ fragment of mouse IgG antibody.

(4) Considering cross-adsorption, for example, some goat anti-mouse IgG secondary antibodies may bind to rabbit primary antibodies to a certain extent. Cross-adsorption purification of secondary antibodies can effectively remove cross-reactivity from non-target antibodies and proteins, thereby improving antibody specificity.

2. Marker considerations:

The choice of marker depends on the intended experimental application and the method of secondary antibody detection (related to the detection instruments, equipment, and detection system) . (For more details, please refer to the EnkiLife Introduction to Antibody Labeling.)

Q4: The issue of antibody reuse in western blotting.

A4: Diluted antibody working solution is not recommended for long-term repeated use; precious antibodies can be recycled and reused 2-3 times. Store at 4 °C away from light and use within one week if possible. Do not repeatedly freeze and thaw.

4. Abnormal band issues

Q1: Why does the observed band size sometimes deviate from its predicted molecular weight?

A1: When the observed band size deviates from the predicted molecular weight, several factors may account for this discrepancy:

- (1) Post-translational modifications such as phosphorylation or glycosylation can cause the band to migrate slower (apparent higher molecular weight, upward shift).
- (2) Proteolytic cleavage (e.g., precursor processing) can cause the band to migrate faster (apparent lower molecular weight, downward shift).
- (3) Alternative splicing or isoforms of the target protein may produce bands at unexpected positions.
- (4) Incomplete denaturation due to strong intermolecular interactions (e.g., protein oligomers or complexes) can result in aberrant migration.
- (5) Calibration deviation of pre-stained protein markers from different manufacturers may also contribute to apparent discrepancies between observed and expected molecular weights.

Q2: Why do extra bands sometimes appear besides the main band?

A2: Possible causes of extra bands include: insufficient antibody specificity, different modified states or cleavage degradation of the target protein, excessively high concentration of primary or secondary antibody, excessive protein loading, excessive exposure time, and insufficient membrane rinsing.

Q3: Why do bands sometimes appear tailed or curved?

A3: When using specific lysis buffers, or when working with samples of complex composition such as plant or high-fat tissues, interference with bands. Incomplete sample denaturation, degradation, post-translational modifications, and excessive loading can all lead to band smearing or tailing. Non-uniform gel polymerization, leakage in the electrophoresis apparatus, excessive voltage or current, and unevenly formed wells can cause bands to curve or distort. Additionally, band warping may occur when very low molecular weight proteins migrate near the bottom edge of the gel. (For common abnormal band analysis, please refer to Part IV of this article.)

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EnkiLife Western Blot Product Recommendation

EnkiLife focuses on the research and development of life science reagents, providing efficient overall western blot solutions. From sample preparation to imaging, the entire product line has undergone comprehensive and in-depth optimization in terms of both safety and efficiency. The core product, the 2-Hour Rapid Western Blot Ready-to-Use Complete Workflow Kit, compresses the traditional 1-2 day experiment into 2 h. Its out-of-the-box design brings great convenience to researchers and accelerates research output.

Catalog No.	Product name
RA10020	2-Hour Rapid Western Blot Ready-to-Use Complete Workflow Kit
RA10021	4-Hour Fast Western Blot Ready-to-Use Complete Workflow Kit
RA10042	Streamlined Western Blot Complete Kit
RC0160	RIPA Lysis Buffer (strong)
RC0161	RIPA Lysis Buffer (middle)
RC0162	RIPA Lysis Buffer (weak)
RC0005	Cell/Tissue Lysis Buffer
BC00006	Protein Assay Kit (BCA Method)
RA10055	5×Loading Buffer
RA10066	Protease Inhibitor Cocktail (100×, EDTA-free)

Catalog No.	Product name
RA10067	Phosphatase Inhibitor Cocktail II (50x)
RA10063	Protease & Phosphatase Inhibitor Cocktail (100x, EDTA-free)
G0142012	Bis-Tris, 4-20%, 12 wells
G0142015	Bis-Tris, 4-20%, 15 wells
RA10043	RapidSet Broad-Range Gradient Gel Kit
RA10037	Calibrated Color Prestained Protein Marker (8-180 kDa)
RA10038	Calibrated Color Prestained Protein Marker (10-250 kDa)
RA10039	Calibrated High Molecular Weight Color Prestained Protein Marker (25-400 kDa)
RA10070	Pre-stained Protein Ladder (10-180 kDa)
RA10071	Pre-stained Protein Ladder (10-250 kDa)
RA10025	Tris-MOPS-SDS
RA10044	20x Universal Rapid Electrophoresis Buffer
RA10051	20x Ice-Free Rapid Transfer Buffer
RA02202R	PVDF (0.22 μ m, 28x400 cm)
RA02202P	PVDF(0.22 μ m, 6x9 cm, 20 pcs)
RA022	PVDF Membrane (0.22 μ m)
RA04502R	PVDF (0.45 μ m, 28x400 cm)
RA04502P	PVDF(0.45 μ m, 6x9 cm, 20pcs)
RA045	PVDF Membrane (0.45 μ m)
RA10052	5x Protein-Free Rapid Blocking Diluent
RA10058	5min Protein-Free Rapid Blocking Solution
RA10059	1min Protein-Free Rapid Blocking Solution
RA10056	No-Blocking Rapid Antibody Diluent
RA10001	Super-sensitive ECL chemiluminescent reagent
RA10041	Antibody Stripping Buffer
RA10061	10xTBST
RA10057	Coomassie Brilliant Blue Fast Staining Solution (Non-Dehydration)
RA20108	Ponceau S Staining Kit,1x

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for choosing
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