CUSABIO®

Laboratory Technical Manual

Western Blotting

Contents

Western Blotting PROTOCOL WB

Western Blotting (WB) Protocol

Western blotting uses antibodies to identify individual proteins from complex samples and to perform a semi-quantitative analysis. First, proteins are separated from each other based on their size by SDS-PAGE. Next, the proteins are transferred from the gel to membrane by application of an electrical current. Finally, the antigen-loaded blotting membrane could be detected and analyzed according antigen-antibody specific binding by a specific primary antibody.

Western blotting is mainly used for qualitative or semi-quantitative analysis of target protein-specific expression, subsequent analysis of protein-protein or protein-DNA interaction, and identification analysis of protein modification.

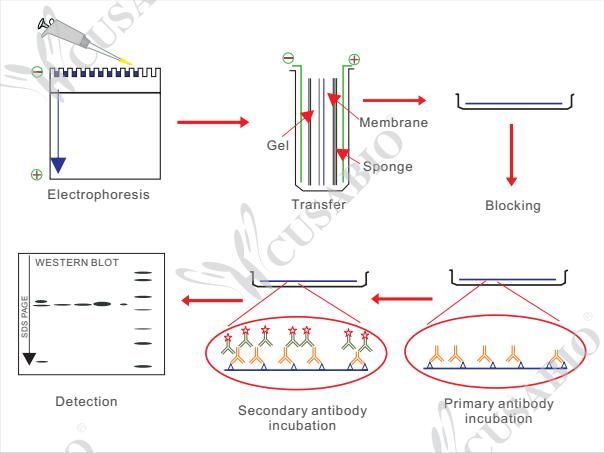


Fig. 1. The brief processes of WB

1 Protein Sample Preparation

1.1 Source of Protein Sample

Protein samples for western blotting can be soluble protein fluids, cell/tissue lysates or immunoprecipitated proteins. The protein loading differs from different samples. Basically, the recommended protein loading of purified protein is no more than 100 ng, and the loading of cell/tissue lysate could be 10-40 μg.

1.2 Protein Sample Preparation

Generally, complex protein components are extracted from animal or plant tissues or cells, and the following principles should be observed during the extraction process:

a. Decide the appropriate extraction method based on the characters of individual protein.

b. Use the appropriate method to maximize the extraction of target protein.

c. Perform under low temperature and add protease inhibitors to prevent protein degradation.

d. Choose the appropriate protein lysate to maintain protein solubility.

Store Protein samples at -80°C, avoid repeated freezing and thawing, detect as soon as possible.

• Preparation of lysate from cell culture

a. After the cell confluence reaches 80%, place the cell culture dish on ice and wash the cells with ice-cold PBS for 3 times.

b. Prepare lysates which containing protease inhibitors. The commonly used protease inhibitors are shown in the table below (Table 2). Appropriate protease inhibitors should be selected according to the experimental requirements. The most commonly used protease inhibitor is PMSF (working concentration is 1 mM), which is highly toxic, so it should be self-protected when used. Its half-life in water is extremely short, so it should be added before use.

c. Add 1 mL of protein lysate containing protease inhibitor to a 10 cm culture dish, shake gently, and lyse on ice for 15-30 min.

d. Scrape adherent cells off the dish using a cold plastic cell scraper, then gently transfer the cell suspension into a 1.5 mL EP tube, then place the tube on ice. Bubbles should be avoided at this time.

Notes

The collected cells can also be fully lysed by sonication. Place the ultrasound probe in the middle of the sample lysate, but do not touch the tube wall or tube bottom for ultrasound. The sonicator we used is Scientz JY92-IIN, with 10% power (650 W), over 2 sec, stop for 3 sec. Basically, the intracellular suspension of 1ml should be sonicated for 10-25 cycles.

e. Centrifuge at 12000 rpm for 10-15 min at 4°C.

f. Gently aspirate the supernatant to another fresh tube and place on ice for later use. Be careful not to absorb impurities such as lipids floating in the upper layer.

g. After protein quantification, add appropriate amount of 6 × sample loading buffer, and boil at 95°C for 5 min, then centrifuge at 12000 rpm for 30 sec, lastly, store at -20°C.

• Sample preparation notes:

All steps must be operated at low temperature! Low temperature! Low temperature!

a. For the cells grown in suspension, collect by centrifugation at 2500 rpm for 3 min, followed by cell washing and lysis procedures.

b. For drug-treated cells, especially samples from apoptosis related studies, media supernatants should also be collected.

c. It is not recommended to use protease to digest and collect cells. Because it may introduce protein impurities or cause damage to some certain proteins, especially the membrane surface proteins, to interfere the experimental results.

d. A viscous transparent gel may appear in the lysate. The transparent gel is a genomic DNA component. Take the supernatant for experiments. However, when the target protein is tightly bound to the genome, the gel needs to be ultrasonically disrupted or syringe-sucked, then take supernatant for subsequent experiments to avoid protein loss.

e. PMSF is unstable in aqueous solution, usually it degrades by half in 30 min. The rate of loss of activity increases with the increase of pH value, and the deactivation rate at 25°C is higher than 4°C. When the sample is processed for more than 1 h, it needs to be added once more.

f. Pay attention to the influence of cell state and the number of cell passages. Heterogeneity exists in cancer cells of different algebras, so the cell morphology, migration and invasion ability may change, thereby make some gene expression change as well.

On one hand, due to the certain heterogeneity of the cells themselves, after a period of cultivation, the overall characteristics of the cells are gradually changed in a way of survival of the fittest.

On the other hand, in the process of cell culture, due to changes in culture conditions or the presence of external stimuli, such as replacement of culture reagents, digestion and passage, cell contamination, and some chemical and physical stimuli, the expression of related genes in cells may be affected. Ultimately affect the experimental results.

When using tumor cells for experiments, it should be preserved first, and try to use relevant cells in the same algebra to carry out relevant experimental research to avoid the occurrence of cell heterogeneity due to excessive passage times, and ultimately lead to inconsistencies in experimental results.

• Preparation of lysate from tissues

a. Collect fresh samples and wash with saline or PBS, then cut into appropriate sizes. You can use a 1-2 mL homogenizer for tissue homogenate on ice, or add liquid nitrogen for grinding. It is recommended to use liquid nitrogen grinding, for the tissue block is not easily damaged and there is frictional heat generation during the homogenization process.

b. Prepare the lysate containing protease inhibitor.

c. Add appropriate amount of lysate containing protease inhibitor (50 mg/500 μL) into the grinded tissue sample, and place the tube on ice for 15-30 min for lyse, meanwhile, intermittently mix to fully lyse.

Notes

To ensure adequate lysis of tissue cells, sonication is recommended. Adjust the ultrasound system to the appropriate frequency and power (the ultrasonic power should not be too large, and set the ultrasonic intermittent to prevent the ultrasonic probe from overheating). Place the ultrasonic probe in the middle of the sample lysate, but do not touch the tube wall or the bottom of the tube, for ice bath ultrasound.

d. Centrifuge at 12000 rpm for 10-15 min at 4°C.

e. Remove the EP tube gently, and absorb the supernatant into a fresh tube. Be careful not to absorb impurities such as lipids floating in the upper layer, then place on ice for later use.

f. After protein quantification, add appropriate amount of 6 × sample loading buffer, and boil at 95°C for 5 min, then centrifuge at 12000 rpm for 30 sec, lastly, store at -20°C.

Notes The tissue sample must be cleaned, so remove the blood vessels, and wash the blood away to avoid interference of IgG in the sample.

Fig. 2. The processes of lysate preparation

1.3 Selection of Protein Lysate

For most samples, RIPA lysis buffer can be used for rapid cell lysis.

Table 1. The components of RIPA lysis buffer

RIPA Lysis Buffer	
Tris-HCl	50 mM
NaCl	150 mM
EDTA	1 mM
SDS (W/V)	0.1% (W/V)
Sodium deoxycholate	1% (W/V)
Triton X-100	1% (V/V)
Appropriate protease inhibitors can be added to the RIPA lysis buffer according to the purpose of experiment.	

The main components of the protein lysate and their effects are as follows:

• Buffer

A buffer with a certain pH range could provide a stable environment for proteins and increase protein solubility as well. The buffers of Tris-HCl or HEPES, pH 7.4 with similar physiological pH are commonly used. The buffer of Tris-HCl (pKa = 8.1) has a pH range of 7.0-9.2, which is sensitive to temperature. The pH value range of HEPES (pKa = 7.55) is 6.5-8.5.

• Salt ion

The appropriate salt ion concentration could maintain protein solubilization. The selection of 150 mM NaCl in an approximate physiological state will not affect the disruption of proteins and protein interactions.

• Chelating agent

Chelate metal ions are used to prevent protein extracts from becoming too viscous, resulting in reduced solubility. In addition, chelating agents can also interact with certain enzymes to inhibit enzyme activity.

• Reducing agent

The addition of a certain amount of reducing agent could protect the free sulfhydryl groups on the protein from oxidation, thereby avoiding protein aggregation or denaturation. The β-mercaptoethanol and dithiothreitol (DTT) are commonly used as reducing agents. The latter one is more powerful than the former one. Usually, the β-mercaptoethanol is volatile and will be oxidized in a short time after being added to the buffer, which could affect the activity of the protein, and its working concentration is 5-20 mM. The DTT has a stronger reducing ability, which can form a stable intramolecular disulfide bond after oxidation without any affect to the protein sulfhydryl group. Its working concentration is 0.5-1 mM. Basically, DTT is recommended for long-term storage, but the DTT solution is not stable and needs to be used right after it was ready.

• Detergent

The detergent is a kind of surfactant, and the hydrophobic segment of the surfactant molecule is inserted into the phospholipid bilayer of the membrane, thereby changing its permeability and ultimately destroying the membrane structure. Therefore, the strength of the surfactant directly determines the ability of lysating cells. The surfactants used in the lysate can be mainly divided into two major kinds: anionic surfactants and nonionic surfactants. Commonly used surfactants are as follows:

SDS: The anionic surfactant, has a strong destructive power, which can basically dissolve all proteins and destroy their natural conformational structure. SDS binds with protein in a ratio of 1.4:1, which can effectively cover the charge of the protein itself. The critical micelle temperature of SDS is a little high, so precipitation could occur at low temperatures, and the precipitation will be more obvious in the presence of potassium salts. In addition, the stronger the ionic strength of the solution, the lower the critical micelle concentration of the ionic detergent, making the protein more soluble.

NaDOC: A kind of ionic surfactant, which is weaker than SDS.

Triton X-100: A kind of non-ionic surfactant. It can destroy the interaction between protein and lipid, but it does not denature the protein, or break the connection between protein and protein neither. It can preserve the natural conformation of the protein. It has a lower critical micelle concentration and two-phase separation can be observed at 64°C.

NP-40: A kind of non-ionic surfactant, it has weak damage to the nuclear membrane, however, it has strong binding ability to proteins, and could ensure sufficient solubility and structural stability of the protein, so it is particularly suitable for dissolution of membrane proteins under non-deformation conditions.

Tween 20: A kind of mild non-ionic surfactant with poor ability for protein solubilization, which does not destroy protein structure and is not a common component of protein lysates.

The selection of detergent depends on the nature of the protein to be extracted and the purpose of the experiment. There are many factors should be considered when choosing the detergent, including fully lysating the cells and dissolving the protein, for the state of the extracted protein (denatured or retained in a natural state).

• Protease inhibitors

A large amount of protease is released when cells and tissues are destroyed during protein extraction process. In order to inhibit protease activity, the samples must be kept at low temperature and an appropriate amount of protease inhibitor should be added to prevent degradation of the protein of interest.

Table 2. Commonly used protease inhibitors

Protease inhibitor	Function	Working concentration	Characters
PMSF	Serine proteases inhibitor Cysteine proteases inhibitor	0.5-1 mM	PMSF has a short half-life in water and needs to be added shortly before use. Very toxic, should pay attention to self-protection during experimental operation.
APMSF	Serine proteases inhibitor	0.4-4 mM	-
Pepstatin	Aspartyl proteases inhibitor	1 μM	Store at -4°C for 1 week, -20°C for up to 1 month; avoid repeated freeze/thaw cycle.
Leupeptin	Serine proteases inhibitor and Cysteine proteases inhibitor	10-100 μM	Store at -4°C for 1 week, -20°C for up to 1 month; avoid repeated freeze/thaw cycle.
Aprotinin	Serine proteases inhibitor	0.01-0.03 μM	Store at -4°C for 1 week, -20°C for up to 1 month; avoid repeated freeze/thaw cycle.
Na_3VO_4	Phosphatases inhibitor	1 mM	Need to be activated. Add acid to adjust the pH to 10 after dissolving, and heat to boil to colorless, cool at room temperature, then adjust the pH to 10 again. Repeat the steps until the solution remains colorless and the pH is stable at 10, aliquot and store at -20°C.
NaF	Phosphatases inhibitor	10-20 mM	-

2 Protein Quantification

In order to quantify the protein of interest in samples, it is necessary to determine the amount of total protein in samples. The difference in the expression level of target protein is reflected when the content of total protein remains constant.

Table 3. Common chemical quantification method

Method	Principle	Interference factors	Characters
Bradford Assay	Under acidic conditions, the binding of the protein to G-250 causes a shift of maximum absorption wavelength of the dye. Within a certain range, the protein content is linear with the 595 nm absorption peak.	The assay is interfered with strong alkaline buffers and high concentration detergents.	Rapid and highly sensitive, minimum detection limit is 1 μg. The protein-dye complex has a high extinction coefficient and the color is stable. The assay is mainly used for the detection of basic or aromatic amino acids because of its high selectivity for proteins.
BCA assay	Cu^{2+} is reduced to Cu^{+} by protein under alkaline conditions, the Cu^{+} then reacts with BCA to form a purple colored complex. The complex has an absorbance at 562 nm that is linear with protein concentration.	The assay is compatible with high concentration detergents, and can tolerate chelating and reducing agents of certain concentrations.	Rapid and highly sensitive with great anti-interference capacity, limit of detection reaches 0.5 μg. There is less protein to protein variation compared with the Bradford assay
Lowry Assay	Under alkaline conditions, Cu^{2+} reacts with the peptide bonds in proteins to form a complex that reduces Folin–Ciocalteu reagent, which results in a blue color complex. There's a linear relationship between the shade of the color and protein concentration.	The assay is suitable for the detection of samples with high lipid content and can tolerate detergent of certain concentrations.	Standard curve is not a straight line, the shade of color varies from protein to protein, and the detection takes longer time.
Ultraviolet-visible spectrophotometry(UV–Vis or UV/Vis)	Based on the physical properties of the protein and Lambert Beer's law, the absorbance at a given wavelength is linear with the protein concentration.	-	The assay is interfered with tryptophan and tyrosine levels in different proteins

BCA and Bradford assay are the most commonly used methods for protein quantification. However, BCA assay is recommended in the presence of high concentration detergent.

2.1 Standard Curve Drawing with Bradford Method

a. Prepare bovine serum albumin (BSA) standard:

Dissolve 0.05 g BSA in 5 mL PBS to obtain 10 mg/mL BSA standard.

b. Prepare Coomassie Brilliant Blue G-250 staining solution:

Dissolve 50 mg Coomassie Brilliant Blue G-250 in 25 mL 90% ethanol, add 50 mL phosphoric acid (85%), and dilute with pure water to 500 mL. Keep away from light.

c. Dilute protein samples:

Perform 1:10, 1:20, and 1:40 dilutions for protein samples.

d. Dilute BSA standard to the following concentrations.

	1	2	3	4	5	6	7	8
Concentration(mg/mL)	0	0.05	0.075	0.1	0.15	0.2	0.3	0.4

e. Add 20 μL each of diluted BSA standard solution and protein sample to the wells of microplate strip. Then add 180 μL of G250 staining solution to each well and mix thoroughly.

f. Measure absorbance with spectrophotometer at a wavelength of 595 nm and make a standard curve to calculate the protein concentration.

2.2 BCA Method (Please Refer to the Corresponding Kit for Instructions)

a. Prepare the BCA working reagent by mixing BCA reagent A with reagent B in a ratio of 50:1 (V/V), and incubate for 24 h at room temperature.

b. Dissolve the standard to a final concentration of 0.5 mg/L with the same solvent used for samples.

c. Pipette gradient volume of standard solution (0 μL, 1 μL, 2 μL, 4 μL, 8 μL, 12 μL, 16 μL, 20 μL) to microplate wells, and add standard diluent to each well to final volume 20 μL.

d. Pipette 200 μL BCA working reagent to each well and incubate at 37°C for 30 min.

e. Measure absorbance at 562 nm using spectrophotometer.

f. Calculate protein concentration based on the standard curve.

3 Protein Sample Preparation

Denaturation of protein, which involves destructing protein tertiary structure and exposing antigenic epitopes, facilitates binding of antibody to the target protein and subsequent detection. To denature protein, mix the protein sample with 2 x sample loading buffer at volume ratio of 1:1 or 6 x sample loading buffer at volume ratio of 5:1, boil the mixed solution at 95°C for 5 min.

Membrane proteins are often aggregated and precipitated under high temperature, they should be treated at 37°C for 30 min.

Table 4. The component of loading buffer

The 6×Sample loading buffer	
Tris-HCl (pH 6.8)	6% (V/V)
SDS	4% (W/V)
Bromophenol blue	0.2% (W/V)
Glycerol	20% (V/V)
DTT	9% (V/V)
Note: loading amount of protein sample should be 10-40 μg per well. Overloading protein can cause smearing.	

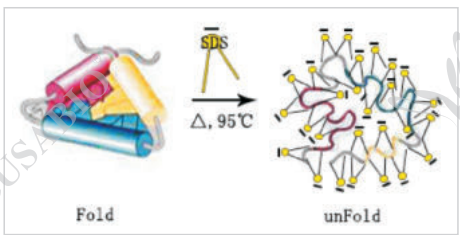
4 SDS-PAGE

4.1 SDS-PAGE Gel

The polyacrylamide gel is formed by the polymerization of acrylamide and methylene bisacrylamide, which results in a network of gel structures with molecular sieving property. Proteins are encapsulated by a large number of SDS micelles with negative charge, covering the proteins' intrinsic charge and providing the proteins with uniform charge-to-mass ratio. The resolution of SDS-PAGE correlates with the concentration of the cross-linker acrylamide and methylidene bisacrylamide used. The molecular sieves formed by different concentrations of cross-linking agents have different pore sizes, giving a variety of separating conditions based on protein molecular weight (details shown below)

Table 5. Gel percentage and corresponding protein size

Gel percentage	Protein size(kDa)
8%	70-200
10%	25-70
12%	20-55
15%	15-45



• SDS charge effect

Table 6. Composition and function of acrylamide gel

Composition	**Function**
Acrylamide	Acrylamide monomers can polymerize to form polyacrylamide gel.
N,N-Methylenebisacrylamide	Induce cross-linking between long polymer chains to form a three-dimensional network.
Tris-HCl Buffer	Maintain a stable pH.
APS	Facilitate cross-linking and provide free radicals that promote the polymerization of acrylamide and N,N-Methylenebisacrylamide.
TEMED	Catalyze the formation of free radicals and accelerate polymerization.

4.2 Electrophoresis Buffer Preparation

Conventional SDS-PAGE is a robust tool for separating proteins with molecular weights (MWs) ranging from 30 kDa to 250 kDa. Choose appropriate separation gel concentration according to the molecular weights of the proteins (Table 5).

Table 7. The components of electrophoresis buffer (1L)

Reagents	Mass(g)
Glycine	14.4
Tris	3
SDS	1
Add ddH_2O and mix thoroughly, pH 8.3	

Use constant power in the Glycine-Tris-gel electrophoresis system, run 60-80 V for the concentrated gel, and 100-120 V for the separation gel. The lower the voltage, the slower it runs, and a better separation can be obtained.

However, the conventional Glycine-Tris-gel system does not result in a good resolution for the separation of small molecular proteins with low molecular weights (<30 kDa). Tricine has better electron mobility and dissociation constant than Glycine, which offers small molecule proteins better concentration effect in the concentrated gel and higher resolution in the separation gel.

Table 8. Recommended electrophoresis buffers for small molecule proteins

	Reagent	Mass(g)	Comments
Anode buffer(1L buffer system)	Tris	24.228	Dissolve Tris in ddH_2O, mix thoroughly and adjust pH to 8.9.
Cathode buffer(1L buffer system)	Tricine	17.92	Dissolve reagent in ddH_2O, mix thoroughly.
	Tris	12.114	
	SDS	1	

We recommend running Tricine-Tris-gel at constant voltage (60-100 V).

4.3 Protein Marker and Loading Control

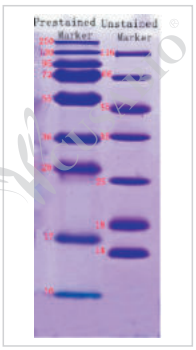
It's important to use appropriate controls based on experimental requirement.

Indicator of molecular weight: Protein marker that covers an appropriate range of molecular weights can indicate the molecular weight of the protein and to some extent reflect the electrophoresis effect and the membrane transfer efficiency. Based on different features, the commonly used markers can be roughly divided into three types: unstained marker, pre-stained marker, and exposure marker.

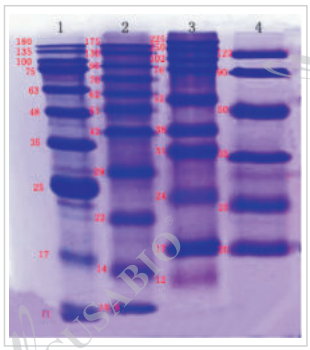
Table 9. Comparison of different protein markers

Protein Marker	Advantage	Disadvantage
Unstained marker	Unstained marker is the most accurate protein marker, it does not carry dye molecules or labeling molecules and can accurately determine protein size.	Unstained markers are not visible during electrophoresis, electrophoresis and transfer process cannot be monitored in real time. It must be stained to be seen.
Prestained marker	Pre-stained marker is a mixture of proteins covalently coupled to dye, it can be directly observed by the naked eye during the experiment, and serves as a reference in electrophoresis and transfer process.	Due to the dye coupling, migration efficiency of the protein molecules after dyeing in electrophoresis is changed, resulting in a shift of the indicated molecular weight, which can cause inaccurate protein sizing. The final result requires a comparison between the Marker and target bands, during which human errors may occur.
Exposure marker	The target bands and the marker bands can be simultaneously exposed to reduce errors and the mobility of proteins.	High cost.

This explains why the observed band size is different from the predicted size in western blot. To minimize the impact of molecular weight shift caused by prestained marker, compare it with unstained marker to identify the difference in molecular weight for accurate molecular weight sizing of the target band (The left figure below indicates the difference between prestained marker and unstained marker). In addition, the protein marker varies widely from manufacturer to manufacturer, and customers should be cautious when choosing protein marker (The right figure below indicates the difference between prestained markers from different manufacturers). When identifying proteins in positive western blot result, the molecular weight shift caused by prestained marker should be taken into consideration.



Prestained maker and unstained marker



Prestained markers from different manufacturers

Positive Control: tissues or lysate from cell lines that express the protein of interest.

Loading Control: level of the proteins encoded by the housekeeping genes are relatively constant in various tissues and cells, such protein can serve as loading control during the quantification of the protein of interest to ensure the same amount of protein is loaded into each lane. In addition, the loading control protein can be used to assess whether the experiment runs successfully.

Common internal reference gene (Housekeeping gene): It is crucial to choose the right loading control protein according to the purpose of the experiment when different proteins are studied.


Fig. 3. The simply structure of cell

The following factors should be considered when choosing loading control protein:

Table 10. The genes in different location of cell

locality	Nuclear	Nuclear membrane	Cytoplasm	Cellular membrane	Mitochondrion	Membrane
Animal Tissue/ Cell	Histone H3 (17 kDa)、PCNA (29 kDa)	Lamin B (66 kDa)	β -actin (43 kDa)、GAPDH (36 kDa)、Tubulin (5 kDa)	Na^+/K^+-ATPase (120 kDa)	CoX IV (17 kDa)、VDAC1 (30 kDa)	ATP1A1 (113 kDa)

a. The expression level of some housekeeping genes may vary in response to certain experimental conditions, such as external stimulation or drug treatment. When choosing a loading control protein, it's important to consult relevant literature and validate that its expression is constant across samples and not affected by certain experimental conditions.

b. The molecular weight of the protein of interest should be different forms that of the loading control protein to enable distinct detection and differentiation. If the loading control protein has a similar molecular weight to the protein of interest, visualize the band of protein of interest first, then wash the antibodies away with a primary/secondary antibody removal solution, followed by the incubation with loading control antibody and the visualization of loading control protein.

4.4 Running the Gel

a. After flash spinning the samples to remove impurities, 10-40 μg of total protein is recommended to load into the wells. The recommended quantity of purified protein is 10-100 ng. Loading quantities should be adjusted according to the result.

b. Molecular weight markers should be included in a lane to indicate the protein of interest.

c. Run with constant voltage (voltage set at 120 V or lower). To obtain a better experimental result, 80 V is recommended when the whole protein migrates in concentration gel. While the proteins migrate into separation gel, voltage should be boosted up.

d. Usual running time is about 1.2 h. However, the protein which weighs lower than 20 kDa should shorten the running time according to the interesting protein. While the protein is bigger than 100 kDa, running time should be extended to get a better separation of protein.

5 Transfer

5.1 Transfer Methods

To transfer the separated proteins from the gel to the solid phase medium, wet transfer and semi-dry transfer methods are commonly used. The two transfer methods are identical in principle, except that the mechanical devices for applying the electric field and the approaches to immobilize gels and membranes are different, semi-dry transfer employs multi-layer filter paper infiltrated with buffer solution.

Table 11. Comparison between types of transferring methods

Methods	Advantages	Disadvantages
Tank transfer	Membrane transfer result is good, the temperature is controllable while transferring.	It takes longer time, and need a large quantity of transfer buffer.
Semi-dry transfer	Membrane transfer effectiveness is high within a short time, and it costs less transfer buffer.	The temperature can't be controlled while membrane transferring, thus the high temperature causes high background eventually.

While membrane transferring, the heat generates rapidly in the assembly within a short time under high current. So it's necessary to take measures while membrane transferring to keep a low temperature condition. While tank transferring, the assembly can be ice bathed for heat dissipation, while semi-dry transferring, it's unsuitable to have a long time electric turn, so that we recommend tank transfer for high molecular weight protein (Above 100 kDa).

As for low molecular weight and middle molecular weight protein, the effectiveness of semi-dry transfer and tank transfer nearly same.

Note

We recommend semi-dry transfer for small piece of gel with high abundance.
We recommend tank transfer for big piece of gel with low abundance.

5.2 Selection for Membranes

The nitrocellulose (NC) and polyvinylidene difluoride (PVDF) are the most commonly used membranes.

Table 12. Comparison between NC and PVDF membrane

	NC membrane	PVDF membrane
Protein binding ability	80-100 μg/cm^2	100-300 μg/cm^2
Binding Strength	Low	High
Physical characteristics	Fragile	Durable and resilient
Whether activation is needed	No need activation	Alcohol activation

The binding ability of NC membrane is mainly related to its purity, the higher purity is, the stronger protein binding capacity will be. However, the high purity NC membrane is fragile and easy to break. Compared with NC membrane, PVDF membrane not only has stronger protein binding ability, but also owns better chemical resistance.

Please note that before using PVDF membrane, it should be soaked in methanol (>15 sec) to activate the positively charged groups on the membrane and equilibrated in the membrane buffer for a period of time. Besides, the PVDF membrane and NC membrane have different pore sizes.

For small molecular protein (<20 kDa), we advise to adopt 0.22 μm of membrane pore size to avoid exceed turn around.

For most of situation, 0.45 μm of membrane pore size is suggested.

5.3 Protein Separation and Membrane Transfer Condition Optimization

Regarding to membrane transfer for small molecular proteins, we are capable to optimize based on the below aspects:

a. Increase the concentration of cross-linking agent, and adopt 15% acrylamide gel to have electrophoresis. However, aimed at the proteins that below 15 kDa, the resolution of 15% acrylamide gel is low, so we advise to add 10% of interlayer gel between the stacking gel and separating gel to increase the resolution of small molecular proteins.

b. Replace Tris-Glycine buffer system with Tris-Tricine electrophoresis system to achieve better concentration and separation effects. Please note that the voltage should be decent while using Tris-Tricine, 60 V-80 V will be good.

c. Choose 0.22 μm of pore size membrane.

d. Shorten the membrane transfer time.

Regarding to membrane transfer for large molecular proteins, we are capable to optimize based on the below aspects:

a. Decline the concentration of cross-linking agent, process the electrophoresis with 8%-5% acrylamide gel. Please note that the lower gel concentration is, the more fragile the membrane will be, please take careful while operation.

b. While membrane transferring, please decently turn up the current, delay the membrane transfer time and avoid heat generated. Tank transfer at 4°C temperature for overnight is recommended.

c. Declining the methanol in the transfer buffer facilitates to the separation of SDS molecule from the protein. Methanol with high concentration fixates the protein and it's not good for large molecular protein, it's advised to decline methanol concentration to 10%.

5.4 Transfer Efficiency Monitoring

a. The transfer result of prestained marker reflects protein transfer efficiency.

b. Stain the gel with Ponceau, to judge whether the transfer is successful from stained bands. The process is reversible, but it's not suitable for Nylon.

Fermentation of ponceau staining solution: mix the 5% (V/V) acetic acid, 0.1% (W/V) Ponceau, ddH_2O well, and stored at 4°C

Ponceau staining process:

• Soak the transferred PVDF or NC membrane to the Ponceau staining solution and oscillate for 5-10 min.

• Take out the imprinted membrane, wash 3 times × 5min with PBS.

• Observe the stained red bands and make record to the transfer results.

• Wash 3 times × 5min with PBS again to remove the combined Ponceau in order to have a further WB analysis.

c.Stain the gel with Coomassie Blue Staining Solution, the process is irreversible, but the sensitivity of Coomassie Blue Staining Solution is higher than the sensitivity of Ponceau.

Fermentation for coomassie blue staining solution: Add 10% (V/V) glacial acetic acid, 45% (V/V) methanol, 0.25% (W/V), ddH_2O and mix well.

Fermentation for coomassie blue staining destaining solution: Add 25% (V/V) methanol, 8% (V/V) glacial acetic acid, ddH_2O and mix well.

Coomassie staining process:

• Soak the transferred PVDF and NC membrane to the coomassie blue staining solution, use a shaker to shake slowly at room temperature for 1 h (Advice to make some adjustments according to the gel dimension, thickness, and temperature) until the gel turns blue.

• Pour out the staining solution and soak the gel to the destaining solution, shake slowly with a shaker at room temperature for 4 h until the blue background is destained and the protein bands are visible.

5.5 Preparation of Transfer Buffer

The fermentation of 1L transfer buffer as below.

Table 13. The components of 1L transfer buffer

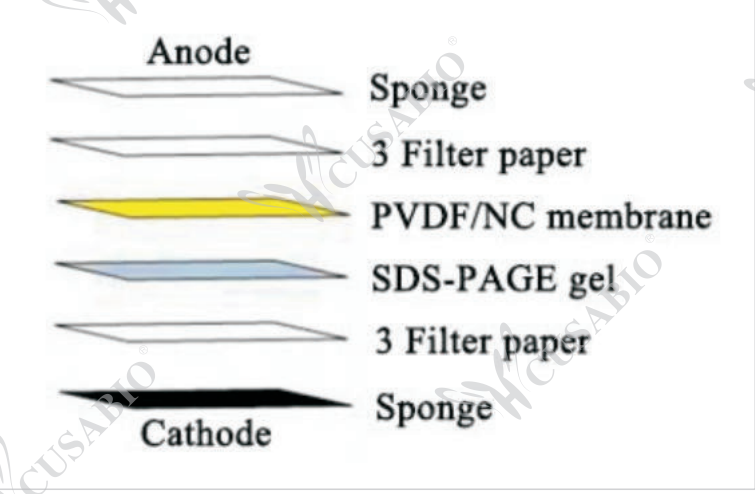
Reagent	Mass (g)
Glycine	11.26
Tris	2.43
Methanol	200 mL
*Add ddH_2O and mix well to dissolve sufficiently	

The transfer buffer needs to be stored away from light, it can be repeated using.
However, due to the methanol is volatile, it's necessary to change to the fresh transfer buffer in time.

5.6 Transfer

a. Be careful to separate the separation gel.

b. Make the "sandwich" by filter paper-gel- filter paper, keep the "sandwich" hydrated and avoid bubble between it. Prepare the "sandwich" as follows:



c. Assemble the transfer device according to manufacturer's manual. Transfer proteins to nitrocellulose or PVDF membrane. 0.22 μm membrane is recommended for the protein which molecular weight lower than 20 kDa.

d. There are usually two devices that you can choose in the light of actual conditions, wet transfer or semi-dry-transfer. Both wet transfer and semi-dry-transfer can work well for conventional size protein (20-100 kDa), while wet transfer is more suitable for high molecular weight proteins for the reason that semi-dry-transfer can yield higher background staining.

e. Rinse the blot in PBS for approximately 5 min.

6 Blocking

Block the membrane using blocking buffer for 1h at room temperature.

• Blocking buffer selection

The binding surface is uneven, there are lots of small holes. While the protein is being transferred to binding surface, it absorbs to the binding surface through mutual effect. However, not all of the sites are being absorbed to proteins, so that the blocking buffer is needed to absorb to the rest of binding sites, in order to prevent the antibody molecular from absorbing to the membrane directly and cause the fake positive or high background result.

The principle for selecting a blocking buffer should be:

a. The blocking buffer is capable to block all of the uncombined sites on the blocking membrane.

b. The blocking buffer doesn't interfere the combination of target proteins. It doesn't combine with the epitopes of target proteins, or cross-react with other reagents.

Here are specific details of blocking buffers which are commonly used:

Table 14. Comparison of various blocking buffer

Blocking buffer	Advantages	Disadvantages
5% non-fat dry milk	The component is complex, it contains many proteins with different molecular weight, which can sufficiently blocking.	It doesn't suitable for biotin-avidin and alkaline phosphatase detection systems (due to small amounts of biotin and alkaline phosphatase residues existed in non-fat dry milk).
1% Casein	It has negative charge in Neutral and alkaline condition and has interaction with positively charged membranes.	The solubility is relatively bad, and it's not good for blocking.
5% BSA	The component is simple, it's compatible for most of situations.	When the immunogen is coupled with BSA, it may cross-react with the residual BSA in the antibody due to its certain immunogenicity, resulting in a certain background.
Serum	Not only blocks non-specific binding, but also the antibodies in serum block FC receptors that possibly exist in the sample to avoid the primary and secondary antibody react against the FC receptor.	The cost is relatively higher.
Non-protein compound	Gelatin, Tween-20, etc. can reduce the hydrophobic interaction between proteins, elute non-specific adsorption, and improve the specific recognition ability of antibodies.	-

A comparative experiment for different blocking buffers is performed as below:

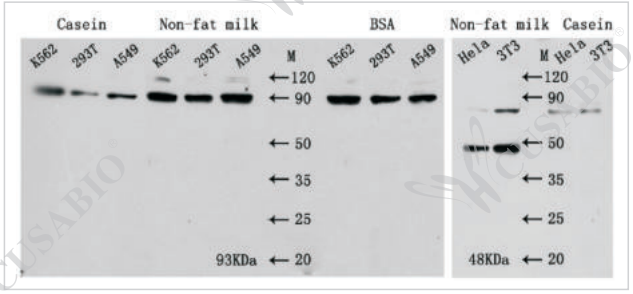


Fig. 4. Comparison of different blocking buffer

Note

It is important to note that for phosphorylated proteins, skim milk powder and casein are not recommended as blocking buffer, and we don't suggest to replace PBST with TBST either. The selection for blocking buffers should have some adjustments according to the different results. For most of antibodies, adopt skim milk powder as blocking buffer reaches good blocking effect, however, adopt BSA as blocking buffer for some of the antibodies will be good for reducing the background. Furthermore, usually the blocking condition is under the room temperature for 1 h.

7 Primary Antibody Incubation

Obey the product protocol and dilute the primary antibody, usually the components of antibody dilution buffer and blocking buffer are same. Besides, we advise to choose validated primary antibodies, and incubation overnight at 4°C is recommended, so that the antigen and antibody could combine sufficiently.

We advise to have a gradient preliminary experiment to determine the best dilution ratio of primary antibodies, like Dot blot method.

a. Upload the samples with different loading amount sequentially on the NC membrane and air dried naturally.

b. Block the membrane after absorbing the sample completely.

c. Cut the membrane according to the loading gradient.

d. Incubate the primary and secondary antibodies with different concentration gradient respectively.

e. At last, adopt ECL luminescent substrate incubation and development to preliminary determine the dilution ratio range based on the development result.

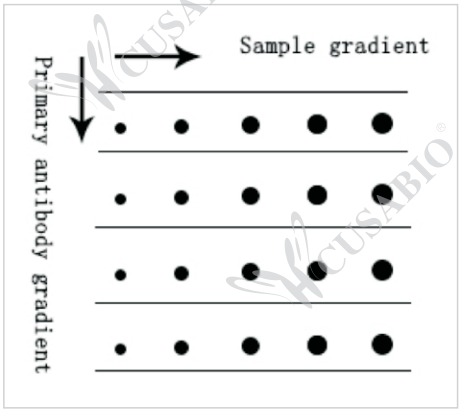


Fig.5. Dot Blot

8 Secondary Antibody Incubation

8.1 Experimental Operation

a. Before the secondary antibody incubation, wash the membrane 3 times for 10 min with PBST/TBST to eliminate the uncombined primary antibodies.

b. Properly dilute the secondary antibody, incubate at room temperature for 1 h.

c. After the secondary antibody incubation finished, wash the membrane 3 times for 10 min with PBST/TBST to eliminate the uncombined secondary antibodies.

8.2 Selection for Secondary Antibody

• Species resource

We do not advice to select secondary antibodies with rabbit, rat or mouse species, because their homology is much similar with human species and easy to have cross-reactivity, then caused high background eventually. Secondary antibodies in goat or donkey species are commonly used. Please note that species resource of the selected secondary antibodies must be different with the selected primary antibody, the secondary antibody species resource selection depends on the species of primary antibody.

Besides, it's also necessary to pay attention on the subtype if you were using a monoclonal primary antibody, and the selected secondary antibody which against the subtype of primary antibody.

• Purification method

The mainly purification methods are Protein G/A purification and Antigen affinity purification. Protein G/A purification method combines all of the antibody IgG molecular in serum, there is no distinguish with antigen specificity.

Affinity purification is a method of eluting by binding to a ligand or receptor specifically recognized by an antibody. And a specific antibody component in serum can be purified via the method. So secondary antibodies with antigen affinity method will decline the unspecific binding and improve the specificity of detected proteins.

• Suitable conjugations.

In WB validation, the most commonly used conjugation for secondary antibody is enzyme, such as Horseradish Peroxidase (HRP) and Alkaline Phosphatase (AP).

Commonly used as substrate, HRP has the characteristic of high specificity, stable, rapid and economical. Although AP is more sensitive, the background is usually high, and the endogenous phosphatase that may exist in the experimental sample and interfere result.

Furthermore, while using AP conjugation for secondary antibody, please be carefully to choose the blocking buffer in order to avoid phosphatase interference.

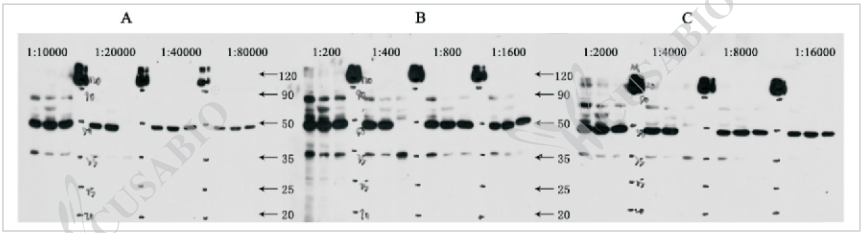


Fig. 6. The dilution ranges are according to instructions of different manufactures

9 Image Development

• Chemiluminescence development

Luminol, which is one of the most classical HRP chemiluminescent substrate, can generate enzyme catalysis reaction with horseradish peroxidase in the presence of H_2O_2. It has high sensitivity and good imaging characteristic, which can develop on the film.

• Substrate development

There are many kinds of HRP chromophoric substrates, the most commonly used is DAB. It develops by reacting with HRP to form an insoluble brown precipitate, and owns high sensitivity as well.

However, it needs to be used on spot and it is carcinogenic, so please watch out while operating.

• Fluorescent development

By using a suitable fluorescent secondary antibody, fluorescence secondary antibody development can be achieved, which makes up the quantitative defects of chemiluminescence and substrate development.

10 Frequently Asked Questions

10.1 High Background

Possible Cause	Solution
Insufficient blocking or inappropriate blocking buffer.	Optimize the blocking effect and select a correct blocking buffer.
The concentration of incubated antibody is too high, incubation time is too long or the temperature is too high.	Adjust the antibody incubation concentration and incubation time.
Inadequate washing.	Increase the washing times, and extend the washing time.
Secondary antibody non-specifically binding.	Set a control for the secondary antibody and select an appropriate secondary antibody.
Membrane is dry.	Keep the membrane wet while operating.
Membrane is contaminated.	Keep the membrane clean while operating, do not press by hand.
Excess chemiluminescent substrate residue.	Drain the excess chemiluminescent liquid then develop.
Film exposure time is too long.	Check several times to confirm optimal exposure time.

10.2 No Target Bands

Possible Cause	Solution
The target protein in sample expresses with low abundance, or it has no expression at all.	Double confirm the feasibility of detected sample, enrich the abundance of the target protein before detecting.
Protein degradation during extraction.	During protein extraction, keep protein at low temperature and add protease inhibitor.
The incorrect storage condition for protein samples and cause the protein degradation.	The protein samples are suggested to store after thermal denaturation with SDS. Valuable samples are suggested to store at -80°C.
Protein transfer efficiency is low.	Adopt Ponceau to confirm if the transfer system is normal.
Antibody incubation concentration is too low, or incubation time is too short.	Optimize the amount of antibody incubation, primary antibody is recommended to incubate overnight at 4°C.
Primary and secondary antibody are not compatible.	Select correct primary antibody and secondary antibody.
The antibody is inactivated.	Store the antibodies properly.
The film development solution may have expired.	Use fresh film development solutions, and prevent from light.

10.3 The Observed Band Size Don't Match with the Predicted Band Size

Possible Cause	Solution
Marker's indicated size has deviation.	Choose a suitable prestained marker and observe the difference with the unprestained marker.
The influence of electrophoresis system.	To avoid the instability factors of the edge wells, load the sample to the middle wells, and add the same amount of sample loading buffer into the edge wells to balance system electrophoresis.
Post-translational modifications.	Look through the literature to double confirm if the protein is phosphorylated or glycosylated and so on.
Post-translational shear and isomers.	Look through the literature to see if the protein has multiple splicing active forms.
Formation of protein polymer.	Keep protein monomer status by using fresh DTT or β-mercaptoethanol during sample preparation.
Relatively change of protein charges.	The amino acid composition of the protein is different, and the charge of some proteins is not completely being covered by negatively charged SDS, resulting in the protein migration rate is not proportional to the protein size.

10.4 Other Issues

Possible Cause	Solution
Reflection or yellow bands on membrane is being found.	The concentration of primary or secondary antibody is too high, or the sample loading quantity is too much, caused the enzymes is being consumed instantaneously.
White blank point.	There are bubbles remains in the transmembrane for sandwich, or the antibody is not evenly incubated, so it should be oscillated incubate.
Black dots on the background.	The granule of blocking buffer residues, please stir and dissolve the granule sufficiently before using.
Smiling bands.	The migration was too fast, decrease the voltage while running the gel. The gel solidifies unevenly, it should be prepared correctly.
Frowning bands.	When electrophoresis, the substrate may aggregate lots of bubbles, it's easy to lead voltage imbalance and cause frown bands eventually.
Smeared bands.	The sample contains insolubles, we advise to centrifuge or optimize the protein extraction. Sample overload, please load less protein into each lane. The concentration of the gel is not suitable, the protein resolution is low. We advise to adjust the ratio of cross-linking agent. The electrophoresis buffer may be repeat using, please change to fresh electrophoresis buffer.
Distorted bands.	The surface of gel is uneven and or the gel is prepared unevenly. The concentration of salt ion in sample is too high, it interferes the electrophoresis. The voltage is too high, so that it leads to fast migration.

CUSABIO TECHNOLOGY LLC

Postal Address: 7707 Fannin St., Ste 200-V126, Houston, TX 77054, USA

Tel : 301-363-4651（Available 9 a.m. to 5 p.m. CST from Monday to Friday）

Email: support@cusabio.com

Web: www.cusabio.com