2. Transfer

2.1 Carefully remove the gel, rinse with pure water, gently scrape off the stacking gel, and equilibrate the gel in transfer buffer for 5 minutes.

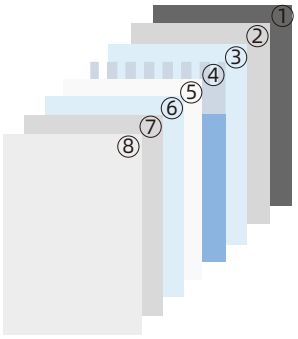
2.2 Soak the membrane in absolute methanol or absolute ethanol for 1 minute until the entire membrane changes from white opaque to semi-transparent. Then equilibrate the membrane in transfer buffer for at least 1 minute before assembly.

2.3 Cut 6 pieces of filter paper to a size 1mm narrower than the gel, and soak them in transfer buffer for later use.

2.4 Place the transfer cassette, two sponge pads, a glass rod, the pre-wetted filter papers, and the activated membrane in a suitable tray filled with transfer buffer.

2.5 Open the cassette and place it flat with the black side facing down. Place one sponge pad on it, and roll over it several times with the glass rod to remove any trapped air bubbles. Place three layers of filter paper on the sponge pad. Secure the filter paper with one hand and use the glass rod with the other to roll out air bubbles. Place the gel on the filter paper, align it carefully, and gently roll a rod over the surface to remove trapped air bubbles. Place the membrane on top of the gel, ensuring it completely covers the gel surface (do not reposition the membrane once placed) and remove any trapped air bubbles. Place another three layers of filter paper on the membrane and remove bubbles. Finally, place the second sponge pad on top, roll a few times, and close the cassette.
Note: Perform the entire operation in transfer buffer. Avoid introducing air bubbles, as they will affect transfer efficiency.

2.6 Place the cassette into the transfer tank, ensuring the black side of the cassette faces the black side of the tank. Add 1 L of transfer buffer. Place the transfer tank in an ice-water bath to prevent excessive heat during transfer. Run at 300 mA constant current for 30–90 minutes.
Note: It is recommended to use a prestained protein marker to evaluate transfer efficiency.



① Cathode (-)
② Sponge Pad
③ Filter Paper
④ Gel
⑤ PVDF
⑥ Membrane
⑦ Sponge Pad
⑧ Anode (+)

Note: The tightness of the transfer sandwich directly affects band quality. Ensure the transfer sandwich is assembled tightly and evenly.

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3. Immunodetection

The following is a general immunodetection protocol. For optimal results, please refer to the protocol provided by the immunodetection reagent manufacturer.

3.1 Rinse the membrane with TBST to remove any residual gel. Transfer the membrane, protein-side up, to a container containing blocking buffer. Block for at least 1 hour (2 hours optimal) at room temperature on a shaking platform.

3.2 Discard or aspirate the blocking buffer. Add the primary antibody diluted to the appropriate concentration in TBST. Incubate at room temperature for 1~2 hours (or incubate at 4 °C overnight). Discard the primary antibody solution, or collect it for reuse. Wash the membrane three times with TBST for 10 minutes each at room temperature on a shaking platform.

3.3 Add the secondary antibody diluted to the appropriate concentration in TBST. Incubate at room temperature for 1~2 hours. Discard or recover the secondary antibody solution. Wash the membrane three times with TBST for 10 minutes each at room temperature on a shaking platform.

3.4 Chemiluminescence / Chromogenic Detection
Proceed according to the instructions of the detection reagent used. Capture images of the membrane using an appropriate imaging system.
Note: This product is intended for research use by qualified professionals only. Its use for clinical diagnosis, therapeutics, or in food/drug applications has not been validated. Please use with caution. For your safety and health, please wear a lab coat and disposable gloves during operation.

Product Specifications

	Model	Pore Size	Length(mm)	Width(mm)
Roll	**3550YH**		3750	265
Sheet	**3550YH**	0.2μm	297	210
			210	148
			200	200
			150	150
			84	70
Roll	**2770H**		3750	265
Sheet	**2770H**	0.45μm	297	210
			210	148
			200	200
			150	150
			84	70

* Sheet membrane dimensions can be customized.

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UM5658E Rev.01-b

Introduction

The immunoblotting assay, also known as Western Blot, is a analytical technique that combines high-resolution gel electrophoresis with immunochemical analysis. The key to the immunoblotting assay is immobilizing the target proteins on a solid support (membrane) for subsequent detection.

Polyvinylidene fluoride (PVDF) membrane is a typical immunoblotting membrane suitable for binding various proteins. PVDF membranes are inherently hydrophobic, with uniformly distributed pore structure and strong protein-binding capacity. Cobetter transfer membranes are available in two pore sizes: 0.45 μm and 0.2 μm, both are hydrophobic membranes. The model 2770H has a pore size of 0.45 μm, suitable for proteins of most molecular weights; while the model 3550YH has a pore size of 0.2 μm, more suitable for proteins smaller than 20 kDa.

Cobetter transfer membranes possess high mechanical strength, are less prone to irregular breakage during cutting, and are compatible with both chemiluminescent and immunofluorescence detection. The membrane has no designated front or back side; either surface may face the gel during transfer.

This manual provides a recommended wet transfer protocol. To achieve optimal transfer results for different proteins, experimental conditions may require appropriate adjustment.

Recommended Materials for Western Blotting

PVDF membrane, filter paper, sponge pads

Pure water, 100% methanol

30% (w/v) Acrylamide monomer solution
-Acrylamide 29 g, N,N'-Methylenebisacrylamide 1 g
-Add deionized water, bring volume to 100mL, adjust solvent pH to no more than 7.0, store in a brown bottle at 4 ℃

10% Ammonium persulfate (store at -20 ℃)
-Ammonium persulfate 1 g
-Bring volume to 10 mL with deionized water, aliquot into tubes (1mL per tube, 10 tubes total)

1.5M Tris-HCl (pH 8.8)
-Tris 18.2g
-Adjust pH to 8.8 with HCl,, bring volume to 100 mL with deionized water, readjust pH to 8.8

1M Tris-HCl (pH 6.8)
-Tris 12.12g
-Adjust pH to 6.8 with HCl, bring volume to 100 mL with deionized water, readjust pH to 6.8

10x Protein electrophoresis buffer
-Tris 30g
-Glycine 144g pH8.3
-SDS 10g
-Bring to volume with to 1000 mL deionized water

6×SDS loading buffer

-0.5M tris, pH6.8	0.35M	7.0mL
-SDS	0.35M	1.0g
-Glycerol 30%V/V		3.0mL
-DTT	0.6M	0.93g
-Bromophenol Blue	0.175mM	1.2mg

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Transfer buffer
-25 mM Tris, 192 mM glycine, 20% (v/v) methanol
-Dissolve 3.03 g Tris and 14.4 g glycine in 500 mL deionized water
-Add 200 mL methanol, then bring to final volume of 1 L with deionized water

TBS solution
-20 mM Tris-HCl, 500 mM NaCl
-Dissolve 4.84 g Tris and 58.48 g NaCl in 1.5 L water, adjust pH to 7.5 with HCl
-Bring to final volume of 2 L with deionized water

TBST washing buffer
-20 mM Tris-HCl, 500 mM NaCl, 0.1% Tween-20
-Add 1 mL Tween-20 to 1 L TBS solution

Blocking buffer
-5% Skim milk powder - TBS, weigh 5.0 g skim milk powder into 100ml TBS, stir until completely dissolved

Antibody dilution buffer
-5% Skim milk powder - TBST, weigh 5.0 g skim milk powder into 100 mL TBST, stir until completly dissolved

Primary antibody (specific to the target protein)
-Use according to the supplier's instructions, typically diluted in antibody dilution buffer

Secondary antibody (specific to the primary antibody), HRP-conjugated
-Use according to the supplier's instructions, typically diluted in antibody dilution buffer

Usage Notes

1. Cobetter transfer membranes are hydrophobic; therefore, they need to be activated with absolute methanol/absolute ethanol before use. The activation procedure is as follows: gently grasp the membrane cut to the required size with tweezers and place it into a container containing absolute methanol/absolute ethanol, ensuring the membrane is completely submerged. This step should be performed for at least 1 minute to ensure complete activation. A successful activation is indicated by the membrane changing from white opaque to a semi-transparent state. The membrane can then be placed into the transfer buffer for further equilibration.
2. During the equilibration process, the membrane may float on the surface of the liquid due to changes in its surface hydrophobicity. At this point, gently press the membrane into the transfer buffer using forceps to ensure complete replacement of the absolute methanol/absolute ethanol with transfer buffer. This process should ideally last for more than 1 minute.
3. Once the membrane is activated, it is essential to keep it moist throughout the subsequent transfer and antibody incubation processes. If the membrane dries out, it will directly affect the final band appearance, potentially leading to missing bands, high background, or other artifacts.

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Protein Transfer Operation

1. SDS-PAGE Electrophoresis

1.1 After aligning the glass plates, vertically clamp them onto the casting stand in preparation for pouring the gel.
Note: During operation, ensure the two glass plates are properly aligned to prevent gel leakage.

1.2 Prepare the separating gel of a specific concentration according to the specified ratios. Add TEMED and mix immediately, then pour the gel. When pouring, use a pipette to draw up the gel solution and dispense it along the glass plate. Stop when the gel surface is approximately 2 cm from the top of the glass plate. Then, carefully overlay the gel with a thin layer of water to ensure even polymerization.
Note: When pouring the gel, ensure the solution flows down along the glass plate to avoid generating bubbles in the gel. When overlaying with water, proceed slowly to avoid distorting the gel.

1.3 When a clear refractive interface is observed between the water and the gel, it indicates the gel has polymerized. Wait an additional 5 minutes for the gel to fully solidify, then pour off the overlay water and blot away residual moisture using filter paper.

1.4 Prepare a 5% stacking gel according to the specified ratios. Add TEMED and mix immediately, then pour the gel. Fill the remaining space with the stacking gel and insert the comb into the stacking gel. Keep the comb level when inserting. After the stacking gel has fully solidified, gently grasp the comb on both sides and pull it straight up vertically to remove it. The gel is now ready for electrophoresis.

Preparation Ratios for Separating Gels of Different Concentrations					
Reagents	6%	8%	10%	12%	15%
H_2O(ml)	3.18	2.78	2.38	1.98	1.38
30% Acrylamide(29:1)/mL	1.2	1.6	2.0	2.4	3.0
1.5M Tris-Hcl(pH8.8)/mL	1.5	1.5	1.5	1.5	1.5
10%SDS /uL	60	60	60	60	60
10%AP(Ammonium Persulfate)/uL	60	60	60	60	60
TEMED /uL	3	3	3	3	3
Total Volume /mL	6.0	6.0	6.0	6.0	6.0

Reagents	5% Stacking Gel Preparation Ratio
H_2O(ml)	2
30%Acrylamide(29:1)/mL	0.5
1.5M Tris-Hcl(pH8.8)/mL	0.5
10%SDS /uL	40
10%AP(Ammonium Persulfate)/uL	30
TEMED /uL	4
Total Volume /mL	3

1.5 Measure the protein concentration in the samples. Calculate the volume of sample solution required to load the desired amount of protein (e.g., 1 μg, 0.5 μg, etc.), which is the loading volume. Transfer the calculated sample volume to a 0.5 mL centrifuge tube and add 6x SDS loading buffer.

1.6 Prior to loading, denature the protein by boiling the samples in a water bath for 5 minutes.

1.7 Fill the electrophoresis tank with running buffer until the inner short glass plate is fully submerged, then prepare to load the samples. Use a pipette to aspirate the sample along the tube wall, carefully avoiding air bubbles. Insert the pipette tip into the loading well and dispense the sample slowly.

1.8 Select an appropriate voltage for electrophoresis. Typically, constant voltage electrophoresis is used (e.g., 90 V for stacking gel, 150 V for separating gel). Electrophorese until the bromophenol blue dye front reaches approximately 1 cm from the bottom of the gel. Then, stop the electrophoresis and proceed to transfer.