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Protocol

Western Blotting

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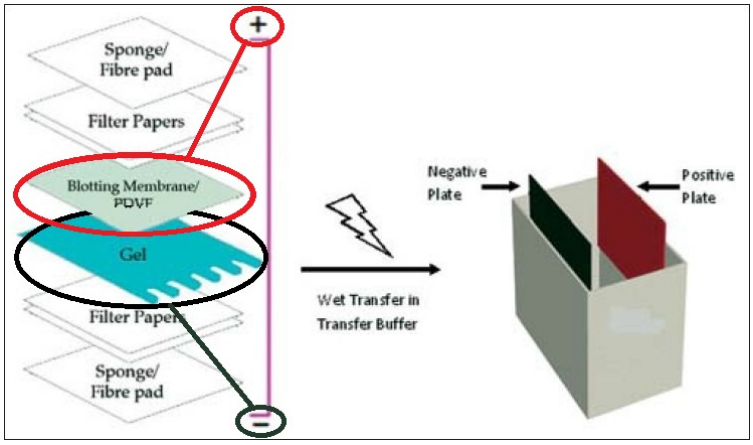
OVERVIEW

Western blotting is an important technique used in cell and molecular biology. By using a western blot, researchers are able to identify specific proteins from a complex mixture of proteins extracted from cells. The technique uses three elements to accomplish this task: (1) separation by size, (2) transfer to a solid support, and (3) marking target protein using a proper primary and secondary antibody to visualize.

RECIPE

1. Dissolve the following in 800 ml of distilled H_2O
    - 8.8 g of NaCl
    - 0.2g of KCl 3g
    - of Tris base
2. Add 500ul of Tween-20
3. Adjust the pH to 7.4
4. Add distilled H_2O to 1L
5. Sterilize by filtration or autoclaving



The most important troubleshooting image for Western Blots

Western Blotting

TECHNIQUE

Cell lysis to extract protein

Protein can be extracted from different kind of samples, such as tissue or cells. Below is the protocol to extract proteins from adherent cells.

Adherent cells:

1. Wash cells in the tissue culture flask or dish by adding cold phosphate buffered saline (PBS) and rocking gently. Discard PBS. (Tip: Keep tissue culture dish on ice throughout).
2. Add PBS and use a cell scraper to dislodge the cells. Pipette the mixture into microcentrifuge tubes.
3. Centrifuge at 1500 RPM for 5 minutes and discard the supernatant.
4. Add 180 µL of ice cold cell lysis buffer with 20 µL fresh protease inhibitor cocktail. (Tip: If protein concentration is not high enough at the end, it is advised to repeat the procedure with a higher proportion of protease inhibitor cocktail).
5. Incubate for 30 minutes on ice, and then clarify the lysate by spinning for 10 minutes at 12,000 RPM, at 4°C.
6. Transfer supernatant (or protein mix) to a fresh tube and store on ice or frozen at -20°C or -80°C.
7. Measure the concentration of protein using a spectrophotometer.

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Sample preparation

1. $$\text{Using, concentration} = \frac{\text{mass}}{\text{volume}}$$

   determine the volume of protein extract to ensure 50 µg in each well.

2. Add 5 µL sample buffer to the sample, and make the volume in each lane equalized using double distilled H_2O (dd H_2O). Mix well. (Tip: Total volume of 15 µL per lane is suggested).
3. Heat the samples with dry plate for 5 minutes at 100°C.

Gel preparation

1. After preparing the 10% stacking gel solution, assemble the rack for gel solidification [Figure 1]. (Tip: 10% AP and TEMED solidify the solution; therefore, both gels can be prepared at the same time, if the abovementioned reagents are not added until the end).

10% Stacking gel	dd H_2O	3 mL
	1 M Tris-HCl	2.1 mL (pH 8.9)
	30% Acr Bis	2.8 mL
	10% SDS	80 µL
	10% APS*	56 µL
	TEMED*	6 µL
6% Separating gel	dd H_2O	2 mL
	1M Tris-HCl	400 µL (pH 6.7)
	30% Acr Bis	600 µL
	10% SDS*	36 µL
	10% APS*	24 µL
	TEMED	4 µL

*, APS: Ammonium Persulfate ; TEMED: Tetramethylethylenediamine; SDS: Sodium dodecyl sulfate

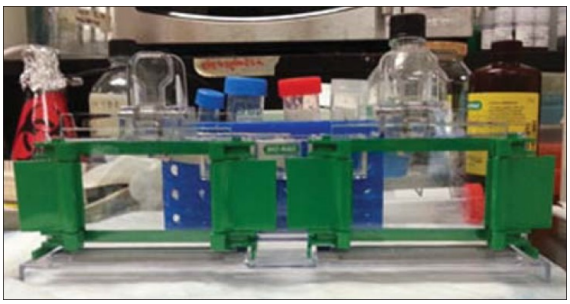

Fig 1. Assembled rack for gel solidification

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2. Add stacking gel solution carefully until the level is equal to the green bar holding the glass plates [Figure 2]. Add H_2O to the top. Wait for 15–30 minutes until the gel turning solidified. (Tip: Using a suction pipette can make the process of adding the gel to the glass plate easier).
3. Overlay the stacking gel with the separating gel, after removing the water. (Tip: It is better to tilt the apparatus and use a paper towel to remove the water).
4. Insert the comb, ensuring that there are no air bubbles.
5. Wait until the gel is solidified. (Tip: Solidification can be easily checked by leaving some gel solution in a tube).

Fig. 2 Add gel solution using a transfer pipette

Electrophoresis

1. Pour the running buffer into the electrophorator [Figure 3].
2. Place gel inside the electrophorator and connect to a power supply. (Tip: When connecting to the power source always connect red to red, and black to black).
3. Make sure buffer covers the gel completely, and remove the comb carefully.
4. Load marker (6 µL) followed by samples (15 µL) in to each well [Figure 4].

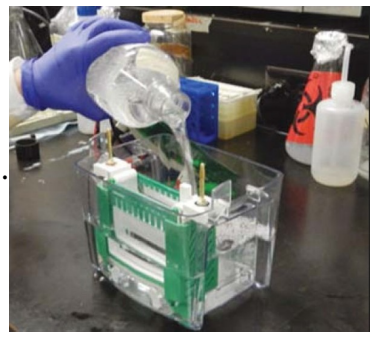

Fig. 3 Add running buffer to the electrophorator

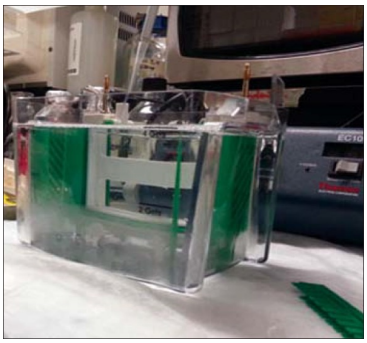

Fig. 4 Add samples and molecular marker to the gel, after removing the combs

5. Run the gel with low voltage (60 V) for separating gel; use higher voltage (140 V) for stacking gel [Figure 5a and b].
6. Run the gel for approximately an hour, or until the dye front runs off the bottom of the gel [Figure 6].

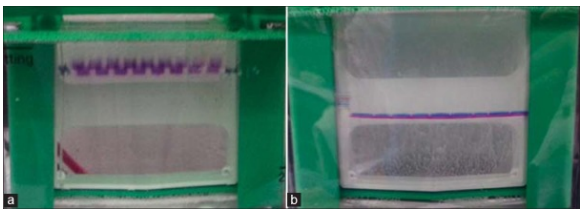

Fig. 5 (a) Samples running through the stacking gel (lower voltage). (b): Samples running through the separating gel (higher voltage)

Electrotransfer

1. Cut 6 filter sheets to fit the measurement of the gel, and one polyvinylidene fluoride (PDVF) membrane with the same dimensions.
2. Wet the sponge and filter paper in transfer buffer, and wet the PDVF membrane in methanol.
3. Separate glass plates and retrieve the gel.
4. Create a transfer sandwich as follows:
   Sponge
   3 Filter Papers
   Gel PVDF
   3 Filter Papers
   (Tip: Ensure there are no air bubbles between the gel and PVDF membrane, and squeeze out extra liquid).
5. Relocate the sandwich to the transfer apparatus, which should be placed on ice to maintain 4°C. Add transfer buffer to the apparatus, and ensure that the sandwich is covered with the buffer. Place electrodes on top of the sandwich, ensuring that the PVDF membrane is between the gel and a positive electrode [Figure 7].

Fig. 6 Run the gel to the bottom of the electrophorator

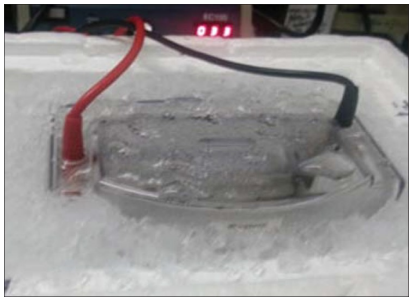

Fig 7. Transfer should be done on ice

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6. Transfer for 90 minutes [Figure 8]. (Tip: The running time should be proportional to the thickness of the gel, so this may be reduced to 45 minutes for 0.75 mm gels).

Fig 8. Membrane after transfer

Blocking and antibody incubation

1. Block the membrane with 5% skim milk in TBST* for 1 hour.
2. Add primary antibody in 5% bovine serum albumin (BSA) and incubate overnight in 4°C on a shaker [Figure 9].
3. Wash the membrane with TBST for 5 minutes. Do this 3 times. (Tip: All washing and antibody incubation steps should be done on a shaker at room temperature to ensure even agitation).
4. Add secondary antibody in 5% skim milk in TBST, and incubate for 1 hour.
5. Wash the membrane with TBST for 5 minutes. Do this 3 times
6. Prepare ECL mix (following the proportion of solution A and B provided by the manufacturer). Incubate the membrane for 1–2 minutes [Figure 10]. (Tip: Use a 1000 µL pipette to ensure that ECL covers the top and bottom of the membrane).
7. Visualize the result in the dark room [Figure 11]. (Tip: If the background is too strong, reduce exposure time).

Fig 9. Use a shaker to incubate the membrane with antibody

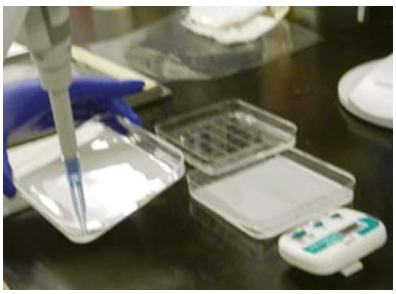

Fig. 10 Incubate the membrane with ECL mix using a 1000 µL pipette to help the process

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Fig. 11 Use the cassette to expose the membrane in the dark room

Blotting

After separating the protein mixture, it is transferred to a membrane. The transfer is done using an electric field oriented perpendicular to the surface of the gel, causing proteins to move out of the gel and onto the membrane. The membrane is placed between the gel surface and the positive electrode in a sandwich. The sandwich includes a fiber pad (sponge) at each end, and filter papers to protect the gel and blotting membrane [Figure 12]. Here two things are very important: (1) the close contact of gel and membrane to ensure a clear image and (2) the placement of the membrane between the gel and the positive electrode. The membrane must be placed as such, so that the negatively charged proteins can migrate from the gel to the membrane. This type of transfer is called electrophoretic transfer, and can be done in semi-dry or wet conditions. Wet conditions are usually more reliable as it is less likely to dry out the gel, and is preferred for larger proteins.

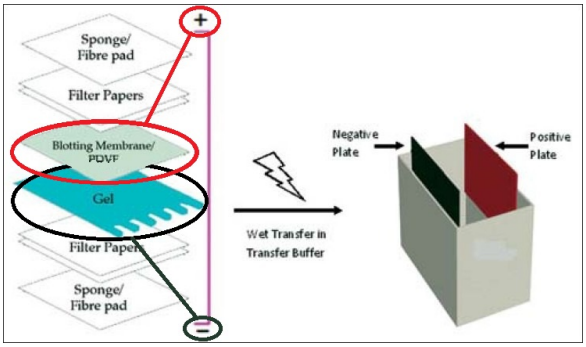

Fig. 12 Assembly of a sandwich in western Blot