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Western Blotting Protocol

Introduction

Western blotting, also called protein blotting or immunoblotting, is a method of detecting certain proteins in complex samples based on the specific combination of antigens and antibodies.Owing to the high resolution of SDS-PAGE and the high specificity and sensitivity of solid phase immunoassay, western blotting has become a routine technique for protein analysis. Western blotting is frequently employed for protein identification and enables qualitative and semi-quantitative analysis of the protein. Combined with chemiluminescence detection, the difference of the same protein expression in multiple samples can be compared at the same time.

General rule: Blocking 1h in 5% nonfat milk/PBST, primary antibody overnight in primary antibody dilution buffer, wash in PBST and secondary antibody in 5% nonfat milk/PBST for 1h RT.

Sample preparation.

1. Prepare the IntactProtein™ lysis buffer by adding 2 μL of Reagent A into 1 mL of Reagent B immediately before use. Mix thoroughly by vortexing and place on ice.Tips: Calculate the volume of the lysis buffer you need as per Step 3; discard the unused buffer after use.
2. Discard the cell culture medium and wash the cells twice with ice-cold PBS.
3. Place the culture dish/plate on ice or ice water and add 1 mL of the premixed lysis buffer per $5x10^6$ cells (e.g. add 300 μL of lysis buffer to a 35 mm dish containing $1x10^6$ cells). Keep the dish/plate on ice for an additional 5 min and swirl occasionally to allow the lysis buffer to completely cover the cells.
4. After 5 min of lysis, scrape the cells off the dish/plate using a clean plastic scraper and collect the lysate into a centrifuge tube.
5. Vortex the lysates thoroughly (3 x 10 sec) and place the lysates on ice or ice water for another 10 min to complete the lysis.
6. Heat the lysates on a 95°C heat block for 5 min.
7. Cool the lysates on ice or ice water for 3 min.
8. Centrifuge the lysates at 13, 000g for 5 min at 4°C.
9. Measure the protein concentration using a NanoDrop spectrophotometer or SDS compatible protein assay.
10. Store the lysates at -20°C for future use or use immediately for further analysis.

Membrane Blocking

1. Incubate membrane in 25 ml of blocking buffer for 1 hr at room temperature.
2. Wash four times for 10 min each with 25 ml of PBST.

Antibody Incubation

1. Incubate membrane and primary antibody (at the appropriate dilution) in 5 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
2. Wash four times for 10 min each with 25 ml of PBST.
3. Incubate membrane and secondary antibody (such as HRP-conjugated goat anti-rabbit secondary antibody,Cat#201) in 5 ml of blocking buffer with gentle agitation for 1 hr at room temperature.

Detection of Proteins

1. Wash the membrane bound with antibodies four times for 10 min each with 25 ml of PBST.
2. Prepare ECL Reagent (such as FeQ™ ECL Substrate Kit,Cat#226) . Add equal volumes of Reagent A and Reagent B in a small tray. Mix well.
3. Immerse the membrane in the small tray containing the ECL Reagent, ensuring it is fully exposed to the reagent, and then expose to X-ray film or BioRad imaging system.