ClearBand ECL Substrate for Western Blotting

Abstract

Western blotting is a powerful and commonly used tool to identify and quantify a specific protein in a complex mixture. The technique enables indirect detection of protein samples immobilized on a nitrocellulose or polyvinylidene fluoride (PVDF) membrane.

To detect the target protein, a primary antibody against the target antigen is applied to the membrane as a probe. The membrane is washed and incubated with a horseradish peroxidase (HRP) conjugated secondary antibody that is reactive toward the primary antibody. The membrane is washed again and incubated with an appropriate luminol-based chemiluminescent substrate. In the presence of HRP and a peroxide buffer, luminol oxidizes and produces a weak flash of light at 425 nm. The signal is evaluated with X-ray film or imaging instrumentation.

Chemiluminescent detection

1. Allow the detection solutions (solution A and B) to equilibrate to room temperature for 15 min.
2. Prepare the working solution by mixing detection solutions A and B in a 1:1 ratio. The final volume of detection reagent is 0.1 ml/cm^2 membrane.
3. Drain excess wash buffer from the washed membrane and place it protein side up. Do not let the membrane dry out.
4. Add detection solution onto the membrane and make sure it completely covers the membrane.
5. Incubate for 2 minutes at room temperature.
6. Drain off excess detection reagent.
7. Acquire the signal with x-ray film or imaging devices.

Troubleshooting

- High membrane background
  1. High concentration of Antibody: Optimize antibody concentration, further dilute primary and secondary antibody.
  2. Inefficient blocking:
     a. Increase non-fat dried milk up to 5% if possible.
     b. Increase Tween 20 concentration (Tween 20 may reduce the binding of antibodies, especially of low affinity primary antibodies).
     c. Use InstantBlock blocking buffer.
  3. Insufficient washing:
     a. Increase both the volume, length and number of wash steps.
  4. Incompatible blocking agent: Non-fat dry milk contains endogenous biotin and is incompatible with avidin/streptavidin systems. Substitute with InstantBlock blocking buffer or 5% BSA.
  5. Non-specific binding of secondary antibody: Confirm the secondary is specific by omitting the primary and running a secondary only blot. If bands develop choose an alternative secondary antibody.
  6. Contaminated buffer solutions: Check buffers for particulate or bacterial contaminate. Replace old buffers.

- No bands or weak bands
    1. Excessive signal generated. The enzyme in the system depleted the substrate and caused the signal to fade quickly. Further dilute secondary Antibody.
    2. Antibodies may have lost activity. Perform a Dot Blot. Follow manufacturer's recommended storage and avoid freeze/thaw cycles.
    3. Incorrect secondary antibody used. Confirm host species/Ig type of primary Antibody.
    4. Non-fat dry milk may mask some antigens: Decrease milk percentage in Blocking Buffer or substitute with InstantBlock blocking buffer or 5% BSA Blocking Buffer.
    5. Sodium azide contamination: Make sure buffers do not contain sodium azide as this will quench HRP signal.
    6. Contaminated stock solutions: Do not contaminate the chemiluminescent substrate stock solutions using the same pipette tip.
- White bands or “ghost bands”
    1. Excessive signal generated: Excessive antibodies or loaded protein can cause high levels of localized signal. This results in rapid consumption of substrate at this point. Since there is no light production after the completion of this reaction, white bands are the result. Try first to further dilute secondary antibody.