Chemiluminescent Detection System for Nucleic Acid Blotting

Code K0626    20 x 150 cm² Blots

Intended Use
FOR LABORATORY USE.

Summary and explanation
The Chemiluminescent Detection System for Nucleic Acid Blotting is a complete kit for the detection of Southern and Northern blot hybridizations using fluorescein-labelled probes. Hybridized probes are detected by binding of an alkaline phosphatase-conjugated anti-fluorescein antibody fragment.

The immobilized alkaline phosphatase activity is used to hydrolyze a chemiluminescent dioxetane-phosphate substrate, resulting in sustained light emission at the site of hybridization on the blot. The light signal emitted from the blot is captured by overlaying the blot with photographic film, or by imaging using a sensitive CCD camera system.

Reagents provided

Quantity Description
1x10 mL 10X Blocking Solution: Proteinaceous concentrate containing 15 mM sodium azide as preservative. Dilute in water at 1:10.
1x0.4 mL Anti-FITC AP Conjugate: Antibody to fluorescein, conjugated to alkaline phosphatase. Contains 15 mM sodium azide. Dilute in 1X blocking solution at 1:3200.
10 packets TBST Wash Buffer: Each packet dissolved in 2 L of water makes a solution of 50 mM Tris-HCl, 0.3 M NaCl, 0.1% Tween®20, pH 7.7.
1x160 mL Chemiluminescent Substrate: 0.25 mM CDP-Star® dioxetane alkaline phosphatase substrate in buffer. * Ready to use.

1 Instructions
1 Laminated Protocol Card

*CDP-Star® is a registered trademark of Tropix, Inc. Protected under one or more of U.S. Patents 5,582,980; 5,851,771; 5,538,882; 5,145,772; 4,978,614; and 4,931,569.

Materials Required but Not Supplied
Stringent wash solution (code S1802 for long probes or S1803 for oligonucleotide probes)
TE Buffer pH 8.0 (10 mM Tris-HCl, 1 mM EDTA)
Forceps
Water bath, 55–65°C
Platform shaker
Incubation trays
Heat-sealable bags or plastic folders
Heat sealer
X-ray film and film exposure holders
Film processing equipment

Precautions
1. FOR LABORATORY USE.
2. Do not let blots dry out at any time during the detection procedure, as this will cause non-specific binding of the detection components resulting in very high background.
3. Improper handling of the blots may cause background spots and smudges. Handle the blot by the edges only using clean forceps. Be careful not to scratch the membrane during handling.
4. The chemiluminescent substrate is very sensitive to alkaline phosphatase activity from microbial contamination. All reagents and materials coming into contact with the blot, including the glassware used for reagent preparation, incubation trays, and forceps must be very clean or sterile. Highly purified water from Nanopure® of Milli-Q® equipment is normally sufficient for reagent preparation, but if contamination is suspected, water should be sterilized by filtration using 0.45 µm or smaller pore diameter filters.
5. The 10X Blocking Buffer contains 15 mM sodium azide, which is highly toxic in pure form. Though not classified as hazardous at product concentrations, build-ups of sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent azide build-up in plumbing.
6. Wear appropriate personal protective equipment to avoid contact with eyes and skin.

Reagent preparation and storage
All kit components should be stored at 2–8 °C.

Stringent Wash:
Calculate the volume of stringent wash (code S1802 for long probes or S1803 for oligonucleotide probes) required for the blots being processed (a total of 3 mL per cm² of membrane). Warm the required volume of solution to the stringent wash temperature appropriate for the specific probe used.
Blocking Solution:
This reagent should be prepared just prior to use by adding one volume of 10x Blocking Solution to nine volumes of water in a clean incubation tray that closely matches the size of the blot. Invert the bottle several times to mix the contents prior to dilution. The tray size must be such that the blot can be fully immersed in blinking buffer using 0.2–0.3 mL of diluted blocking solution per cm² of membrane. Calculate the volume of blocking solution required for the blots being processed. For example, a 150 cm² membrane requires 0.3 mL x 150 = 45 mL blocking solution, which is prepared by diluting 4.5 mL of 10X Blocking Solution in 40.5 mL of water.

Anti-fluorescein alkaline phosphatase antibody conjugate:
This reagent is prepared by dilution directly into the blocking solution after the blocking stop is completed. The conjugate is used at a dilution of 1:3200. For example, if 45 mL of blocking buffer was used to block the membrane, the volume of conjugate to add would be: 45 mL ÷ 3200 ≈ 0.014 mL, or 14 µL. Discard the diluted solution after use.

TBST Wash Buffer:
Dissolve the entire contents of one packet in 2 L of water while stirring. Unused solution may be stored at 2–8 °C for up to two weeks.

Detection procedure
DO NOT ALLOW BLOTS TO DRY OUT DURING THE DETECTION PROCEDURE.

STEP 1 STRINGENT WASHES
Remove blots from hybridization solution and immerse in stringent wash solution at the appropriate temperature for the probe used.
Wash the blots 3 x 10 minutes while maintaining the stringent temperature, using 1 mL per cm² of membrane for each wash.
Gently shake or rock the blot during the wash.
Blots can be washed in roller tubes or sealed bags incubated in a hybridization oven or water bath to maintain temperature.

STEP 2 BLOCKING
During the stringent wash step, prepare the blocking butter in an incubation tray.
Remove blots from the stringent wash and immerse in the diluted blocking solution at room temperature.
Incubate the blots for 30 minutes while gently shaking or rocking the tray.

STEP 3 ANTI-FITC AP CONJUGATE INCUBATION
Leaving the blots in the tray, decant the blocking solution into a clean incubation tray.
Add anti-FITC AP conjugate to the blocking solution at a final dilution of 1:3200.
Mix the solution in the tray, then transfer the wet, blocked blots to the tray containing the conjugate solution.
Work quickly to avoid drying of the blots.
Incubate the blots in the conjugate solution for 30 minutes at room temperature while gently shaking or rocking the tray.

STEP 4 TBST WASH
Decant the conjugate solution from the tray and rinse the blots twice using 25 mL of TBST solution per blot for each rinse.
Transfer the blots to a larger tray and wash an additional 3 x 10 minutes at room temperature, using 2 mL of fresh TBST per cm² of membrane for each of the three washes.
Gently shake or rock the tray during the washes.

STEP 5 CHEMILUMINESCENT SUBSTRATE INCUBATION
Transfer the washed blots to a clean hybridization tube, bag, or tray containing 0.05 mL of chemiluminescent substrate per cm² of membrane, and incubate for five minutes at room temperature with gentle shaking or rocking.

STEP 6 SIGNAL DEVELOPMENT
Drain the majority of the substrate from the blots by briefly holding each blot up by a corner. Do not let blots dry out.
Place the drained blots in individual heat-seal bags or plastic folders.
Gently squeeze excess substrate and bubbles from the bags and seal the edges. The blots should still appear damp.
Let the sealed blots incubate for one hour at room temperature.

STEP 7 FILM EXPOSURE
Place the sealed blots in a film exposure holder or cassette.
Tape may be used to secure the corners of the bag within the holder to prevent shifting of the blot.
Working in the dark, overlay the blot with X-ray film and close the holder.
Do not move the film after placing it over the blot. If necessary, place a flat weight (such as a book) on the film holder to press the film to the blot. This will ensure a sharp image.
Typical exposure times are 5–45 minutes, which are sufficient for detecting single-copy genes in Southern blots. Weak signals may be improved by lengthening exposure times. Light emission peaks 1–2 hours after substrate incubation and will decrease slowly over the next 24–48 hours. Process the film according to the manufacturer's instructions.
## Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Probable Cause</th>
<th>Suggested Action</th>
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</thead>
<tbody>
<tr>
<td>1. No signal or weak signal.</td>
<td>1a. Probe not denatured (Southern)</td>
<td>1a. Boil (long probes) or heat at 60°C (oligo probes) prior to hybridization.</td>
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<tr>
<td></td>
<td>b. Inefficient transfer to membrane.</td>
<td>b. Check that transfer solution and times are adequate for transfer system and membrane.</td>
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<td></td>
<td>c. Inadequate immobilization of DNA or RNA.</td>
<td>c. Consult membrane manufacturer’s recommendations for UV cross-linking or baking; periodically calibrate light source used for UV cross-linking.</td>
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<td></td>
<td>d. Poor membrane quality.</td>
<td>d. Avoid use of membranes that are discolored or do not wet evenly.</td>
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<td></td>
<td>e. Membrane is incompatible with chemiluminescent detection</td>
<td>e. Use Schleicher &amp; Schuell Nytran, Amersham Pharmacia Hybond-N+ or Pall Biodyne membranes. Nylon membranes from other manufacturers may also be suitable; consult membrane manufacturer for compatibility with chemiluminescent detection.</td>
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<tr>
<td></td>
<td>f. Washes too stringent.</td>
<td>f. Decrease stringent wash temperature.</td>
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<tr>
<td>2. Overall background.</td>
<td>2a. Blot was allowed to dry during detection.</td>
<td>2a. Keep blot wet at all times.</td>
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<td></td>
<td>b. Dirty incubation trays.</td>
<td>b. Clean trays with detergent.</td>
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<tr>
<td></td>
<td>c. Insufficient prehybridization.</td>
<td>c. Increase prehybridization time to at least 30 minutes; increase prehybridization volume to at least 0.1 mL per cm² of membrane; use hybridization buffer containing adequate blocking agents.</td>
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<tr>
<td></td>
<td>d. Membranes adhered to each other during hybridization or washes.</td>
<td>d. Decrease the number of membranes processed at one time.</td>
</tr>
<tr>
<td></td>
<td>e. Membrane is incompatible with chemiluminescent detection.</td>
<td>e. Use Schleicher &amp; Schuell Nytran, Amersham Pharmacia Hybond-N+ or Pall Biodyne membranes. Nylon membranes from other manufacturers may also be suitable; consult membrane manufacturer for compatibility with chemiluminescent detection.</td>
</tr>
<tr>
<td>3. Spotty background.</td>
<td>3a. Solutions are contaminated with bacteria or particles.</td>
<td>3a. Filter solutions at 0.45 microns.</td>
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<td></td>
<td>b. Anti-FITC AP conjugate has precipitated.</td>
<td>b. Centrifuge tube of conjugate for 5 minutes in a microcentrifuge at maximum speed to pellet precipitates. Use clarified supernatant for dilution.</td>
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