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Southern-Light™ & Southern-Star™ Systems

Chemiluminescent Detection System for Fluorescein-Labeled DNA with CSPD® or CDP-*Star*® Substrate

Cat. Nos. ST100CF, SL100CF, SL100MCF
ST100SF, SL100SF, SL100MSF

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Version 9012-B.1

I. INTRODUCTION

The Tropix® Southern-Light™ and Southern-*Star*™ chemiluminescent detection systems are designed for rapid, sensitive detection of DNA or RNA immobilized on membranes with biotin-, fluorescein-, or alkaline phosphatase (AP) - labeled probes, using Tropix CSPD® or CDP-*Star*® chemiluminescent substrates for alkaline phosphatase (1-3).

Detection and quantitation of a specific DNA sequence can be achieved with the technique originally described by Southern (4). The degree of hybridization of a complementary probe to the DNA target is a measure of the amount of the specific target sequence. Detection of DNA is most often performed with radioisotopes such as ^{32}P and ^{35}S. Nonisotopic DNA detection techniques have principally incorporated alkaline phosphatase as the preferred label due to its thermal stability and high turnover rate. In these systems, the nucleic acid probe is labeled with biotin-, fluorescein-, or digoxigenin-labeled nucleotides, and detected indirectly with a streptavidin-, avidin- or other secondary antibody-alkaline phosphatase conjugate. Oligonucleotide probes may also be directly labeled with alkaline phosphatase.

Nonisotopic detection procedures based on alkaline phosphatase are limited by the ability to measure a low concentration of enzyme. CSPD® and CDP-*Star*® substrates are novel, direct chemiluminescent substrates for alkaline phosphatase. Upon enzymatic dephosphorylation, the substrates decompose, resulting in a prolonged, constant emission of light with a maximum of 461 nm on nylon membrane. Detection with the Southern-Light™ and Southern-*Star*™ systems requires no specialized equipment and results can be conveniently imaged on X-ray film with exposure times of 5 min to 2 hr. The Southern-Light™ and Southern-*Star*™ systems may also be used to detect DNA in colony and plaque hybridizations. Northern blotting can also be successfully performed using these reagents (5-7).

II. SYSTEM COMPONENTS

Small size kits (ST series) contain materials for 10 blots (10 x 10 cm). Standard size kits (SL series) contain materials for 30 blots. Shelf-life for all components is 1 year when stored as indicated.

	ST100CF	SL100CF	SL100MCF	ST100SF	SL100SF	SL100MSF
Tropilon-Plus™ Membrane	-	-	1 roll	-	-	1 roll
I-Block™ Reagent	7.5 g	30 g	30 g	7.5 g	30 g	30 g
Fluor*x*-AP™ Conjugate	20 µL	60 µL	60 µL	20 µL	60 µL	60 µL
10X Assay Buffer	50 mL	150 mL	150 mL	50 mL	150 mL	150 mL
CSPD® Substrate	30 mL	100 mL	100 mL	-	-	-
CDP-*Star*® Substrate	-	-	-	30 mL	100 mL	100 mL
Development Folders	10 ea	30 ea	30 ea	10 ea	30 ea	30 ea

1. Tropilon-Plus™ Membrane: 0.45 µm, positive nylon membrane, 30 cm x 2 m.

2. I-Block™ Reagent: Purified casein. Store at room temperature.

3. Fluor*x*-AP™ Conjugate: Anti-Fluorescein-AP conjugate. Store at 4°C or -20°C.

4. 10X Assay Buffer: 200 mM Tris (pH 9.8), 10 mM $MgCl_2$. Store at 4°C.

5. Chemiluminescent Substrate: CSPD® or CDP-*Star*® solution, 0.25 mM. Store at 4°C.

6. Development Folders: 14 cm x 19 cm clear polypropylene folders.

III. MEMBRANE SELECTION & PREPARATION

Neutral or positively charged nylon membranes may be used with the Southern-Light™ or Southern-*Star*™ system (8). Tropix Tropilon-Plus™ positively charged nylon membrane provides optimal results and is recommended. The use of CSPD® or CDP-*Star*® substrate on nitrocellulose membranes requires the use of Tropix Nitro-Block-II™ luminescence enhancer (Cat. No. LNX200). Nitro-Block-II™ enhancer is a polymeric material designed to increase the chemiluminescent signal on nitrocellulose membranes. Nitro-Block™ enhancer (Cat. No. LN200) may also be used with CSPD® substrate.

When using nylon membranes, such as Tropilon-Plus™ membrane, best results are obtained when DNA is fixed to the membrane by UV cross-linking. UV crosslinking is performed on a dry membrane with a shortwave UV (254 nm) light source. A total UV exposure of 120 millijoules is recommended (e.g. 1.2 milliwatts/cm^2 x 100 sec = 120 millijoules/cm^2).

Note: Prior to UV cross-linking, a short wash (5 to 10 min) in 5X SSC may reduce background. UV cross-linking is best performed on a dry membrane.

IV. HYBRIDIZATION ON NYLON MEMBRANE

The following hybridization and stringency wash procedures have been developed and are suggested for use on nylon membranes with either short (oligonucleotide) or long (random-primed or nick-translated) probes. Other solutions and procedures may be substituted. Consult membrane manufacturer for buffers suitable for nitrocellulose membranes.

A. Hybridization of Oligonucleotide Probes

Oligonucleotide probes can be labeled with fluorescein using several methods. Oligonucleotides can be synthesized with an amine group attached to the 5' end and subsequently reacted with fluorescein isothiocyanate. Direct labeling with fluorescein during synthesis can be done using fluorescein-labeled phosphoramidites (9). Alternatively, terminal deoxynucleotidyl transferase can be used to attach fluorescein-labeled nucleotides to the 3' end of an existing oligonucleotide. Oligonucleotide probes may also be directly labeled with alkaline phosphatase (10). The recipes for buffers for this procedure are described in Appendix A.

1. Wet blot in 0.25 M sodium phosphate, pH 7.2.

2. Prehybridize in Hybridization Buffer for 1 hr at 55°C or an appropriate hybridization temperature. Drain buffer.

3. Dilute biotinylated probe (0.1-5 pmol/mL) in fresh Hybridization Buffer (10-100 µL/cm^2) and add to blot. Incubate 2 hr at the appropriate temperature.

4. Wash 2 x 5 min at room temperature in 2X SSC/1% SDS (1 mL/cm^2).

5. Wash 2 x 15 min at the hybridization temperature in 1X SSC/1% SDS.

6. Wash 2 x 5 min at room temperature in 1X SSC. Proceed to Detection (Section V).

B. Hybridization of Random-Primed or Nick-Translated Probes

The recipes for buffers for this procedure are described in Appendix A.

1. Wet membrane in 0.25 M sodium phosphate, pH 7.2.

2. Prehybridize with Hybridization Buffer for 1 hr at 65°C. Drain buffer.

3. Dilute heat-denatured probe in fresh Hybridization Buffer (10-100 ng/mL) and add to blot (10-100 µL/cm^2). Incubate overnight at 65°C.

4. Wash 2 x 5 min at room temperature with 2X SSC/1% SDS (1 mL/cm^2).

5. Wash 2 x 15 min at 65°C with 0.1X SSC/1% SDS.

6. Wash 2 x 5 min at room temperature in 1X SSC. Proceed to detection (Section V).

V. CHEMILUMINESCENT DETECTION OF PROBE DNA

This procedure has been optimized for detection of fluorescein-labeled DNA. All steps should be performed at room temperature unless stated otherwise. Buffer recipes are described in Appendix Section B. Never touch membranes with ungloved hands.

A. Fluorescein-Labeled Probes on Nylon Membranes

1. Wash blot for 2 x 5 min in Blocking Buffer (0.5 mL/cm^2), then incubate blot for 25 min in Blocking Buffer (1 mL/cm^2).

2. Dilute Fluor*x*-AP™ conjugate 1:5,000 in Blocking Buffer (0.1 mL/cm^2).

3. Incubate blot in conjugate solution for 25 min with constant agitation.

4. Wash for 5 min in Blocking Buffer (0.5 mL/cm^2), then 3 x 5 min in Wash Buffer (1 mL/cm^2).

5. Wash 2 x 2 min in 1X Assay Buffer (0.25 mL/cm^2; dilute 10X Assay Buffer 1:10 in H_2O).

6. Drain blots by touching a corner on a paper towel and place on plastic wrap on a flat surface (without letting blots dry).

7. Pipette a thin layer of CSPD® or CDP-*Star*® Ready-To-Use substrate solution onto the blot (3 mL/100 cm^2) and incubate for 5 min.

8. Drain excess CSPD® or CDP-*Star*® solution, and place blot in a Development Folder, after removing anti-static sheet. Alternatively, blot can be placed in a hybridization bag. Smooth out bubbles or wrinkles.

9. Membranes may be imaged by placing them in contact with X-ray film. Initial exposures of 1-10 min for CDP-*Star*® substrate or 5-30 min for CSPD® substrate are recommended.

B. Fluorescein-Labeled Probes on Nitrocellulose Membranes

The following change is necessary when using nitrocellulose membrane:

Step 7: Incubate blot for 5 min in CSPD® or CDP-*Star*® Ready-To-Use substrate solution containing 1:20 Nitro-Block-II™ enhancer (150 µL enhancer + 3 mL CSPD® or CDP-*Star*® substrate).

NOTE: The Cat. No. for Nitro-Block-II™ luminescence enhancer is LNX200. Nitro-Block™ enhancer (Cat. No. LN200) may also be used with CSPD® Ready-To-Use solution.

C. Biotinylated Probes

Detection of biotinylated probes is performed by substituting a streptavidin-AP conjugate (Avid*x*-AP™ conjugate, Cat. No. APA10) for the Fluor*x*-AP™ conjugate. For detection of other haptens such as digoxigenin (11), the corresponding antibody-alkaline phosphatase conjugate should be used. The following buffer changes must be made for the Avid*x*-AP™ conjugate:

Steps 1-4: Substitute 0.5% SDS for Tween®-20 detergent in the Blocking / Wash Buffers.

Step 2: Dilute Avid*x*-AP™ conjugate 1:5,000 in Blocking Buffer and incubate with blot for 20 min.

Proceed with washing and detection (Steps 4-9).

D. Alkaline Phosphatase-Labeled Probes

The Southern-Light™ and Southern-*Star*™ detection systems can also be used to detect alkaline phosphatase-labeled oligonucleotide probes (10). Solution recipes are in Appendix Section C.

Hybridization Protocol

1. Prehybridize for 30 min in Hybridization Buffer at a temperature optimum for the probe.

2. Hybridize for 30 min with 0.25-1 nM probe in fresh Hybridization Buffer.

3. Wash 4 x 5 min with 5X SSC/1% SDS at 45°C.

4. Wash 2 x 15 min with 1X SSC/1% SDS at the hybridization temperature.

5. Rinse membrane in 1% Triton X-100, 125 mM NaCl, 50 mM Tris (pH 8.0). Wash for 20 min with the same buffer at room temperature.

6. Wash 2 x 1 min at room temperature in 1X SSC.

7. Proceed with detection (Section VA), omitting Steps 2 and 3.

E. Reprobing Procedures

Blots which have remained wet may be stripped and reprobed (12) as follows:

1. Wash 2 x 20 min in 0.1X SSC/1% SDS at 95°C.

2. Wash 2 x 5 min in 1X SSC at room temperature and air dry. Alternatively, store wet, sealed in a hybridization bag or plastic wrap at 4°C.

NOTE: Successful stripping may be confirmed by repeating Steps 5-9 of Section VA. If signal is observed, the probe was not completely stripped and more rigorous treatment is necessary.

VI. TROUBLESHOOTING

Tropix has optimized the above protocols using the Tropilon-Plus™ membrane and the reagents supplied. With other materials, results may vary. Since CSPD® and CDP-*Star*® substrates provide extremely sensitive detection of alkaline phosphatase activity, it is important that only ultrapure water and other reagents free of alkaline phosphatase contamination be used.

If the expected sensitivity is not attained:

1. For best results, prepare all buffers fresh daily.

2. Increase film exposure as much as possible.

3. Increase hybridization time to overnight and/or conjugate incubation to 60 min.

4. Increase the concentration of labeled DNA and/or alkaline phosphatase conjugate. However, this may cause increased nonspecific background.

5. Check that the probe is effectively labeled and denatured prior to use. Spot serial dilutions on a membrane and detect.

If nonspecific background is too high:

1. Decrease the exposure time until appropriate resolution is achieved.

2. Splotchy images may result from bacterial contamination. Make sure that all buffers are free of contamination and that the blot and anything that contacts the blot are clean.

3. If background appears evenly across the blot but obscures the specific signal, perform blocking overnight at 4°C, or increase the number of washes after conjugate incubation.

4. To reduce nonspecific binding of the conjugate, increase the dilution to 1:10,000 or 1:15,000 and spin down any particulate material prior to use.

5. To reduce nonspecific binding of DNA probe, reduce the probe concentration or increase the duration of the final two stringency washes.

6. If the background signal is spotty, precipitate the probe with ethanol.

APPENDICES

A. Solutions for Hybridization on Nylon Membranes

20% SDS 20 g/100 mL

0.2 M EDTA (pH 8.0) * 7.4 g/100 mL
(dihydrate)

0.5 M Sodium Phosphate, pH 7.2

0.5 M Na_2HPO_4-$7H_2O$	13.4 g/100 mL
85% H_3PO_4	0.4 mL/100 mL

25X SSC (pH 7.0) *

3.75 M NaCl	21.9 g/100 mL	* Adjust pH with HCl or NaOH,
0.375 M Sodium Citrate dihydrate	11 g/100 mL	then sterile filter (0.45 µm).

Hybridization Buffer

1 mM EDTA	0.25 mL, 0.2 M
7% SDS	17.5 mL, 20%
0.25 M Sodium Phosphate	25 mL, 0.5 M

Add deionized H_20 to 50 mL. If the SDS precipitates, place the buffer in a 50°C water bath to redissolve. Dextran sulfate (5%) may be added for long DNA probes.

Stringency Wash Buffers

2X SSC/1% SDS	16 mL, 25X 10 mL, 20%
1X SSC/1% SDS (for oligonucleotides)	8 mL, 25X 10 mL, 20%
0.1X SSC/1% SDS (for long DNA probes)	0.8 mL, 25X 10 mL, 20%
1X SSC	8 mL, 25X

Adjust the volumes to 200 mL with deionized H_2O.

NOTE: Long DNA probes are prepared by nick translation or random primer labeling.

B. Solutions for Chemiluminescent Detection of Fluorescein-Labeled Probes

10X PBS

0.58 M Na_2HPO_4	82.3 g
0.17 M NaH_2PO_4-H_2O	23.5 g
0.68 M NaCl	40.0 g

Add deionized H_2O to 1000 mL.

Wash Buffer

1X PBS	50 mL, 10X
0.1% Tween®-20 detergent	0.5 mL

Add deionized H_2O to 500 mL

Blocking Buffer

1X PBS	30 mL, 10X
0.2% I-Block™ Reagent	0.6 g
0.1% Tween®-20 detergent	0.3 mL

Add 30 mL of 10X PBS to 270 mL of deionized H_2O. Microwave for 80 sec. Add I-Block™ reagent slowly while stirring. Do not boil. Add Tween®-20 detergent. Cool to room temperature before use.

C. Solutions for Use with Alkaline Phosphatase Labeled Probes

Hybridization Buffer

7% SDS	17.5 mL, 20%
0.25 M Sodium Phosphate	25 mL, 0.5 M
1% I-Block™ Reagent	0.5 g

Add deionized H_2O to 50 mL. Heat to dissolve I-Block™ reagent. If SDS precipitates, place at 50°C to redissolve.

Stringency Wash Buffers

5X SSC/1% SDS	40 mL, 25X 10 mL, 20%
1X SSC/1% SDS	8 mL, 25X 10 mL, 20%
1% Triton X-100	2 mL
125 mM NaCl	5 mL, 5 M
50 mM Tris (pH 8)	10 mL, 1 M Tris (pH 8)
1X SSC	8 mL, 25X

Adjust volumes to 200 mL with deionized H_2O.

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