Catalog Number: 821559, 821560

Aurora Southern Blot Kit

Southern Blot Chemiluminescent Detection System For Biotin Labeled Probes

Protocol for Detecting Membrane-Bound DNA with Starlight Chemiluminescent Substrate

Catalog # 821559 10-20 blots
Catalog # 821560 50-100 blots

1. INTRODUCTION

Detection and quantitation of a specific DNA sequence in a sample can be achieved by using the technique originally described by Southern[1]. The degree of hybidization of a complementary probe to the DNA target is a measure of the amount of the specific target sequence in the sample. 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, digoxigenin or fluorescein labeled nucleotides and detected via a streptavidin or secondary antibody alkaline phosphatase conjugate. Oligonucleotide probes may be labeled directly with alkaline phosphatase.

Non-isotopic detection procedures based on alkaline phosphatase are limited by the ability to measure a low concentration of enzyme. StarLight™ substrate is a novel, direct chemiluminescent substrate for alkaline phosphatase. Upon enzymatic dephosphorylation, StarLight™ decomposes, resulting in a prolonged, constant emission of light with a maximum at 477 nm. The Aurora™ Southern Blot system is the first test kit to incorporate StarLight™ substrate in an ultrasensitive, rapid detection kit for membrane-bound DNA. It requires no specialized equipment and results can be conveniently imaged on instant photographic film or x-ray film with exposure times of 5-120 minutes. Aurora™ test kits may also be used to detect DNA in colony hybridizations and plaque lifts.

2. KIT COMPONENTS

Aurora™ Southern Blotting kits are available in 2 sizes and sufficient for either 10 or 30 blots (10 cm x 10 cm). The reagents included in each kit are described below.

> **NOTE:** In order to assure low nonspecific background signal, all reagents must be kept free from alkaline phosphatase and bacterial contamination.

1. **Chemiluminescent Substrate:** Ultra Pure StarLight™ chemiluminescent substrate is supplied as a 0.25 mM ready-to-use solution. Store at 2-8°C in the dark.

2. **Aurora™ Blocking Reagent:** This protein-based membrane blocking reagent is supplied as a dry powder. Aurora™ Blocking Reagent is a highly purified casein which has been screened for low alkaline phosphatase contamination and has been shown to be an efficient blocking reagent. Store dry at room temperature.

3. **10X Assay Buffer:** 200 mM Tris-HCl, pH 9.8, 10 mM $MgCl_2$. Dilute 1:10 with deionized water. Store at 4°C.

4. **ActiBind-AP™:** Alkaline phosphatase conjugated streptavidin that has been selected and assayed for high specific activity and low nonspecific binding. Supplied in buffered glycerol solution, it should be diluted 1:5000 prior to use. Store at 4°C (or –20°C for long term storage).

5. **Development Folders:** 14 cm x 19 cm clear polypropylene sheets for membrane blot handling during imaging.

3. SELECTION AND PREPARATION OF MEMBRANES

Neutral or positively charged nylon membranes may be used with the Aurora™ Southern Blot chemiluminescent detection system. Biotrans(+)™ positively charged nylon membrane (available from MP) provides optimal results and is highly recommended. The use of StarLight™ chemiluminescent substrate on nitrocellulose membranes requires the use of Opti-Membrane™ Reagent (Cat. No. 821549). Opti-Membrane™ Reagent is a polymeric material designed

to increase the chemiluminescent signal on nitrocellulose membranes.

When using nylon membranes, such as Biotrans(+)™, best results are obtained when DNA is fixed to the membrane by UV cross-linking. UV cross-linking 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 seconds = 120 millijoules/cm^2).

4. HYBRIDIZATION OF HAPTEN LABELED PROBES OF NYLON MEMBRANE

The following hybridization and stringency wash procedures are suggested for use on nylon membranes. Other hybridization solutions and wash procedures may be substituted. Consult membrane manufacturer for hybridization buffers that are suitable for nitrocellulose membranes.

HYBRIDIZATION OF OLIGONUCLEOTIDE PROBES

Oligonucleotide probes can be biotinylated using several methods. Oligonucleotides can be synthesized with an amine group attached to the 5' end and subsequently reacted with NHS-biotin. Direct labeling with biotin during synthesis can be accomplished using biotin labeled phosphoramidites. Alternatively, terminal deoxynucleotidyl transferase can be used to attach one or more biotinylated nucleotides to the 3' end of an existing oligonucleotide. Recipes for the recommended hybridization and wash buffers for oligonucleotide probes are described in Section 12.A.

1. Wet membrane in **0.25 M Disodium Phosphate**, pH 7.2.

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

3. Dilute biotinylated probe (0.1 - 5.0 pmol/ml) in fresh **Hybridization Buffer** and add to membrane. Incubate 2 hours at the appropriate temperature.

4. Wash 2X 5 minutes at room temperature in 2X SSC,
1% SDS (1 ml/cm^2).
Wash 2X 15 minutes at the hybridization temperature in 1X SSC, 1% SDS.
Wash 2X 5 minutes at room temperature in 1X SSC.

5. Proceed to chemiluminescent detection of biotinylated DNA. **(See Section 5)**

HYBRIDIZATION OF HAPTEN LABELED DNA PROBES PREPARED BY RANDOM PRIMING OR NICK TRANSLATION

The following protocol has been developed for hybidization of DNA probes. The recipes for the recommended hybridization and wash buffers for long DNA probes are described in Section 12.A.

1. Wet membrane in **0.25 M Disodium Phosphate**, pH 7.2.
2. Prehybridize with **Hybridization Buffer** for 60 minutes at 65°C. Drain buffer.
3. Dilute heat denatured, biotin-labeled probe in fresh **Hybridization Buffer** (10 - 100 ng/ml) and add to blot (10 - 100 µl per cm^2). Incubate overnight at 65°C.
4. Wash 2X 5 minutes at room temperature in 2X SSC,
1% SDS (1 ml/cm^2).
Wash 2X 15 minutes at 65°C with 0.1X SSC, 1% SDS.
Wash 2X 5 minutes at room temperature in 1X SSC.
5. Proceed to chemiluminescent detection of biotinylated DNA. **(See Section 5)**

5. CHEMILUMINESCENT DETECTION OF BIOTINYLATED DNA ON NYLON MEMBRANE

The following protocol has been optimized for the detection of immobilized biotinylated or hybridized biotinylated DNA on neutral and positively charged nylon membranes. All procedures should be performed at room temperature unless stated otherwise.

Recipes for solutions required for chemiluminescent detection are found in Section 12.B. Refer to **Section 6** for chemi-luminescent detection on nitrocellulose membrane.

MEMBRANE BLOCKING AND CHEMILUMINESCENT DETECTION

1. Wash blot 2X 5 minutes in **Blocking Buffer** (0.5 ml/cm^2).
2. Incubate for 10 minutes in **Blocking Buffer** (1 ml/cm^2).
3. Dilute **ActiBind-AP™** conjugate 1:5000 in **Blocking Buffer**. Use 2.0 µl of **ActiBind-AP™** conjugate in 10 ml **Blocking Buffer** per 100 cm^2 (0.1 ml/cm^2).
4. Incubate blot for 20 minutes at room temperature with constant agitation in conjugate solution.
5. Wash 1X 5 minutes in **Blocking Buffer** (0.5 ml/cm^2).
6. Wash 3X 5 minutes in **Wash Buffer** (1 ml/cm^2).
7. Wash 2X 2 minutes in **Assay Buffer** (0.5 ml/cm^2).
8. Add **Chemiluminescent Substrate Solution** (StarLight™) to blot (3 ml/100 cm^2).
9. Slowly agitate for 5 minutes.
10. Drain excess substrate solution, but do not allow blot to dry.
11. Place blot in **Development Folder** (after removing the anti-static sheet). Alternatively, the blot can be wrapped in Saran wrap or a hybridization bag. Proceed to Section 10 Film Exposure and Development. ***Never touch blot with ungloved hands. Mishandled blots exhibit high nonspecific background signals.***

6. CHEMILUMINESCENT DETECTION OF NITROCELLULOSE MEMBRANE

The following buffer changes are necessary when using nitrocellulose membrane: **Tween-20 should be substituted for SDS in the Blocking Buffer** (0.1% Tween-20) and **Wash Buffer** (0.1% Tween-20). Dilute the ActiBind-AP™ conjugate 1:5000 in Blocking Buffer (0.2% Aurora™ Blocking Reagent/0.1% Tween-20 in 1X PBS) and incubate with the blot for 20 minutes. Then proceed with washing and chemiluminescent detection (Section 5, Steps 5-11) with the following addition: Incubate blot for 5 minutes in Chemiluminescent Substrate Solution containing 1:20 dilution of Opti-Membrane™ Reagent (Cat. No. 821549) without agitation (20 ml/cm^2). Proceed with Step 10.

> **NOTE**: A separate container should be reserved for incubation with Opti-Membrane™ Reagent as this material is difficult to remove and may cause problems if allowed to contaminate the membrane during other steps. Alternatively, a sealed plastic bag may be used.

7. CHEMILUMINESCENT DETECTION OF FLUORESCEIN AND OTHER HAPTEN LABELED DNA PROBES.

Detection of fluorescein labeled probes is performed by substituting an anti-fluorescein-alkaline phosphatase conjugate for the ActiBind-AP™ conjugate. For detection of other haptens such as digoxigenin or 2,4-dinitrophenyl (DNP), the corresponding antibody-alkaline phosphatase conjugate should be used in place of the ActiBind-AP™. The following buffer changes must be made when using antibody conjugates: **Tween-20 should be substituted for SDS in the Blocking Buffer** (0.1% Tween-20) and **Wash Buffer** (0.1% Tween-20). Dilute the antibody-alkaline phosphatase conjugate 1:5000 (consult manufacturer's recommendations) in Blocking Buffer (0.2% Aurora™ Blocking Reagent/0.1% Tween-20 in PBS) and incubate with blot for 30 minutes. Then proceed with washing and chemiluminescent detection (See Section 5, Steps 5-11).

8. HYBRIDIZATION OF ALKALINE PHOSPHATASE LABELED PROBES

The Aurora™ Southern Blot kit can also be used to detect hybridization of alkaline phosphatase labeled oligonucleotide probes to target DNA. Recipes for recommended solutions for hybridization and stringency washes of alkaline phosphatase labeled probes are found in Section 12.C.

HYBRIDIZATION PROTOCOL

1. Prehybridize for 30 minutes in **Hybridization Buffer**. Use temperatures that are optimum for your probe. Hybridize for 30 minutes with 0.25 - 1.0 mM probe in fresh hybridization buffer.
2. Wash 4X 5 minutes with 5X SSC, 1% SDS at 45°C.
3. Wash 2X 15 minutes with 1X SSC,
1% SDS at the hybridization temperature.
4. Rinse membrane in 1% Triton X-100, 125 mM NaCl, 50 mM Tris-HCl, pH 8.0. Wash for 20 minutes with the same buffer at room temperature.
5. Wash 2X 1 minute with 1X SSC at room temperature.
6. Proceed with chemiluminescent detection (see Section 5, Steps 7-11).

9. REPROBING PROCEDURES

Blots which have remained wet following hybridization may be stripped of hybridized DNA and used for subsequent reprobing with the following procedure:

1. Wash 2X 20 minutes in 0.1X SSC, 1% SDS at 95°C.

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

Successful removal of biotinylated probes and ActiBind-AP™ conjugate may be confirmed by following the chemiluminescent detection steps described in Section 5 (Steps 7-11). If a detectable signal is observed, the biotin-labeled probe was not fully stripped and more rigorous treatment is necessary.

10. FILM EXPOSURE AND DEVELOPMENT

Membranes may be imaged by placing them in direct contact with standard Kodak XAR x-ray film or on Polaroid instant Photographic Black and White film. Initial exposures of 5 to 30 minutes are recommended to assess the optimal exposure time for your application. Imaging of small blots (up to 7.3 cm x 9.5 cm) may be performed with an instant film format luminometer using Polaroid Type 612 Instant B/W film ASA 20,000 or Polaroid Type 667 film, ASA 3000. The recommended processing time for instant film in this application is 45 seconds.

11. TROUBLESHOOTING GUIDE

Since StarLight™ substrate provides extremely sensitive detection of alkaline phosphatase activity, it is important that only ultrapure water and other reagents which are free of alkaline phosphatase contamination be used.

For the detection of DNA with biotinylated probes, MP has optimized the above protocols using Biotrans(+)™ membrane, Aurora™ Blocking Reagent, ActiBind-AP™ conjugate and StarLight™ substrate. With other membranes or enzyme conjugates, results may vary. Please call MP Technical Service for further information.

If the expected sensitivity is not attained, you should try the following:

1. For best results, all buffers should be prepared daily.

2. To detect lower levels of DNA, lengthen the film exposure time until nonspecific background signal obscures the image.

3. Increase the incubation time during the hybridization step to overnight and/or conjugate incubation to 60 minutes.

4. Increase the concentration of labeled DNA and/or alkaline phosphatase conjugate. This may, however contribute to increased nonspecific binding.

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

If the nonspecific background signal is too high (e.g. the film appears overexposed or the image is uneven or spotty) you should try the following:

1. Splotchy images may result from bacterial contamination of the membrane. Make sure that all buffers are free of contamination prior to use and that the blot and anything that contacts the blot, such as blotting paper and hybridization bags are clean and fingerprint free.

2. Decrease the film exposure time until appropriate resolution is achieved.

3. If the background signal appears evenly across the membrane but obscures the specific signal, incubate the blot in the Blocking Buffer overnight at 4°C or increase the number of wash steps after conjugate incubation.

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

5. In order to reduce nonspecific binding of DNA probe to the blot, reduce the biotin-labeled probe concentration in the hybridization buffer, or increase the duration of the final two stringency washes.

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

12. SOLUTIONS

12.A SOLUTIONS FOR HYBRIDIZATION AND STRINGENCY WASHES OF HAPTEN LABELED PROBES
FORMULA TO MAKE
COMPOSITION: WEIGHT 100 ml USE:
20% Sodium Dodecyl Sulfate 228.38 20.0 g
0.5 M Disodium Phosphate, pH 7.2
0.5 M Na_2HPO_4•$7H_2O$ 268.07 13.4 g 85% H_3PO_4 98.00 0.4 ml
25X SSC, *pH 7.0
3.75 M Sodium Chloride 58.44 21.9 g
0.375 M Sodium Citrate
dihydrate 294.10 11.0 g
0.2 M Ethylenediaminetetraacetic acid
(EDTA) disodium dihydrate, *pH 8.0 372.20 7.4 g

*Adjust pH with HCl or NaOH and sterile filter (0.45 µ) prior to use.

Hybridization Buffer
COMPOSITION: TO MAKE 50 ml, USE:
1 mM EDTA 0.25 ml of 0.2 M EDTA
7% SDS 17.5 ml of 20% SDS
0.25 M Disodium Phosphate, 25 ml of 0.5 M Disodium
pH 7.2 Phosphate, pH 7.2

Add deionized H_2O 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

COMPOSITION: TO MAKE 200 ml, USE:
2X SSC, 1% SDS 16 ml of 25X SSC,
10 ml of 20% SDS
1X SSC, 1% SDS 8 ml of 25X SSC,
(Oligonucleotides) 10 ml of 20% SDS
0.1X SSC, 1% SDS 0.8 ml of 25X SSC,
(Long DNA probes) 10 ml of 20% SDS
1X SSC 8 ml of 25X SSC

Adjust the volume of each buffer to 200 ml with deionized H_2O.

> **NOTE:** Long DNA probes include probes that have been prepared by nick translation and random primer labeling.

12.B SOLUTIONS FOR CHEMILUMINESCENT DETECTION

> **NOTE:** Prepare all solutions with filter sterilized, deionized H_2O. Unless stated otherwise, the volume of each solution is sufficient to process one blot (100 cm^2). See Sections 6 and 7 for modifications required in the detection reagents when using nitrocellulose membranes or anti-digoxigenin conjugates.

10X Phosphate Buffered Saline (PBS)
COMPOSITION: TO MAKE 1000 ml, USE:
0.58 M Na_2HPO_4 82.3 g of Na_2HPO_4

0.17 M NaH_2PO_4•H_2O 23.5 g of NaH_2PO_4•H_2O

0.68 M NaCl 40.0 g of NaCl

Add deionized H_2O to 1000 ml. **Note:** A 1X PBS solution should have a pH of 7.3 to 7.4. This amount of stock solution is sufficient to process approximately 20 blots.

Blocking Buffer
COMPOSITION: TO MAKE 300 ml, USE:
0.2% Aurora™ Blocking 0.6 g of Aurora™
Reagent Blocking Reagent

1X PBS 30 ml of 10X PBS

0.5% SDS 7.5 ml of 20% SDS

Add 30 ml of 10X PBS to 200 ml of deionized water. Add Aurora™ Blocking Reagent and microwave for approximately 80 seconds (35 seconds/100 ml). Alternatively, heat to 70°C with stirring for 5 minutes. Do not boil. Add SDS and deionized H_2O to 300 ml. The solution may remain slightly opaque. Cool to room temperature before use.

NOTE: Pretreatment of Aurora™ Blocking Reagent with avidin-agarose for 16 hours at 4°C (10 ml per liter) may help to reduce background for ultrasensitive detection[9].

Wash Buffer
COMPOSITION: TO MAKE 500 ml, USE:
1X PBS 50 ml of 10X PBS
0.5% SDS 12.5 ml of 20% SDS
Add deionized H_2O to 500 ml.

Assay Buffer
The kit is supplied with 10X Assay Buffer:
200 mM Tris-HCl, pH 9.8, 10 mM $MgCl_2$.
Dilute 1:10 with deionized water. Store at 4°C.

Chemiluminescent Substrate Solution
The chemiluminescent substrate, StarLight™, is supplied as a ready-to-use solution.

12.C SOLUTIONS FOR HYBRIDIZATION OF ALKALINE PHOSPHATASE LABELED PROBES

Hybridization Buffer
COMPOSITION: TO MAKE 50 ml, USE:
7% SDS 17.5 ml of 20% SDS
0.25 M Disodium 25 ml of 0.5 M Disodium
Phosphate, pH 7.2 Phosphate, pH 7.2
1% Aurora™ Blocking 0.50 g of Aurora™
Reagent Blocking Reagent
Add deionize H_2O to 50 ml. If SDS precipitates, place at 50°C to redissolve.
Stringency Wash Buffers
COMPOSITION: TO MAKE 200 ml, USE:
5% SSC, 1% SDS 40 ml of 25X SSC,
10 ml of 20% SDS
1X SSC, 1% SDS 8 ml of 25X SSC,
10 ml of 20% SDS
1% Triton X-100, 125 mM 2 ml of Triton X-100,
125 mM NaCl, 5 ml of 5M NaCl,
50 mM Tris, pH 8.0 10 ml of 1M Tris, pH 8.0
1X SSC 8 ml of 25X SCC
Adjust the volume of each buffer to 200 ml with deionized H_2O.

Assay Buffer and Substrate Solution
Prepare as described in Section 12.B.

13. REFERENCES

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14. WARRANTY

MP warrants its products to the original purchaser against defects in materials and workmanship under normal use and application. MP's sole obligation under this warranty shall be to replace defective products.

All products are supplied **For Research Use Only** and are **Not For Resale.** Commercialization of products using these components requires an express license under applicable patents and intellectual property from MP.

Our preparations are intended exclusively for *in vitro* use only. They are not for diagnostic or therapeutic use in humans or animals. Those preparations with known toxicity are sent with an information sheet which describes, to our knowledge, the potential dangers in handling. The absence of a toxicity warning with one of our products does not, however, preclude a possible health hazard. With all of our products, due care should be exercised to prevent human contact and ingestion. All preparations should be handled by trained personnel only.

This warranty is in lieu of all other warranties, express or implied, including the warranties of merchantability and fitness for a particular purpose. In no case shall MP be liable to incidental or consequential damages.