BrightStar® BioDetect™ Kit

(Part Number AM1930)

Protocol

P/N 1930M Revision B **Revision Date: October 1, 2008**

For research use only. Not for use in diagnostic procedures.

Literature Citation: When describing a procedure for publication using this product, please refer to it as the BrightStar® BioDetect™ Kit.

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I. Introduction

A. Overview

The BrightStar® BioDetect™ Kit for nonisotopic detection of biotinylated RNA and DNA probes is a result of research efforts at Ambion to develop a nonisotopic chemiluminescent detection system that does not suffer from the high background and low sensitivity typical of these systems. The result is a kit that yields extremely high signal to noise ratios with a limit of detection of about 100 fg.

The kit is designed to detect biotinylated RNA or DNA probes on positively charged nylon membrane. Ambion® BrightStar-Plus positively charged nylon membrane will give optimal performance with this chemiluminescent detection system.

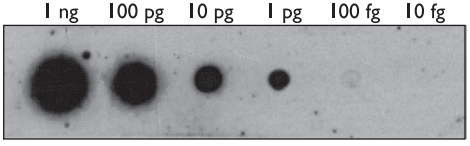

Figure 1. Detection of Biotinylated DNA with BioDetect

A dilution series of a 1 kb biotinylated DNA probe, as indicated in the figure, was spotted on BrightStar®-Plus nylon membrane and then detected using the BrightStar® BioDetect™ procedure. A 45 min exposure to film revealed a limit of sensitivity of about 100 fg.

B. Reagents Provided with the Kit and Storage

Amount	Component	Storage
1 L	5X Wash Buffer	RT
3 L	1X Blocking Buffer	RT
125 mL	10X Assay Buffer	4°C
20 µL	Streptavidin-Alkaline Phosphatase*	4°C
50 mL	CDP-*Star*®	4°C
20 µL	Labeled Control DNA (1 ng/µL)*	–20°C

* These components are shipped in the slit of the grey foam inside the styrofoam cooler.

IMPORTANT

Do not freeze the Strepavidin-Alkaline Phosphatase. Repeated freezing and thawing can compromise its performance.

C. Materials Not Provided with the Kit

- Positively charged nylon membrane
  We recommend BrightStar®-Plus Membranes (P/N AM10100, AM10102, AM10104)
- Electrotransfer device
  Although virtually any electroblotter should function well, we recommend the Panther™ Semi-Dry Electroblotter (Model #HEP-1) from OWL Scientific as an affordable device that has been tested at Ambion for this application.
- Autoradiographic film

D. Related Products Available from Applied Biosystems

BrightStar®-Plus Membranes P/N AM10100–AM10104	Positively-charged nylon membranes recommended for use with Ambion BrightStar® nonisotopic labeling and detection products. These membranes are an excellent choice for Northerns and other blot hybridizations.
BrightStar® Psoralen-Biotin P/N AM1480	Nonisotopic labeling kit for fast efficient biotin labeling of RNA, dsDNA, and oligonucleotides longer than 47mers

II. BioDetect Procedure

A. When to Use BioDetect

Biotinylated nucleic acids must be bound to positively-charged nylon membranes before beginning the detection procedure. This is typically achieved by hybridization of the probe with a target that is cross-linked to the blot.

Blot hybridizations (e.g. Northerns, Southerns, Dot Blots)

Northern or Southern blots (preferably using BrightStar-Plus membrane from Ambion) on positively charged nylon membrane can be directly processed as described in section *II.C. Detection Procedure* on page 4. Be sure to complete the post-hybridization washes before starting the BioDetect procedure. To control background, it is important to keep the membrane damp after the post-hybridization wash steps. Background may be higher using membranes that have been allowed to dry.

Gel separated probe (e.g. RPAs, S1 Nuclease Assays)

The transfer of polyacrylamide gel-resolved probes to the BrightStar-Plus membrane, as in a ribonuclease protection assay, is the first step that differs from a typical ^{32}P-based assay. Electroblotting (instead of passive transfer) from the polyacrylamide gel onto the membrane is the best approach. After the transfer, the biotinylated nucleic acids are crosslinked to the membrane. Methods for both the membrane transfer and crosslinking are described in sections IV.A on page 10, and IV.B on page 11.

B. Buffer and Equipment Preparation

- The BioDetect procedure has been optimized for a 100 cm^2 membrane. Smaller or larger membranes can be used with appropriate (proportional) adjustments to solution volumes.
- The containers should be clean and RNase-free. We recommend treating the containers with Ambion RNase*Zap*® Solution (P/N AM9780), a strong RNase denaturing solution, followed by rinsing with high quality, RNase-free water.
- It is important that the Wash and Blocking Buffers are in solution for use. If any precipitate is visible, resolubilize it by warming to 37–65°C. Do not, however, heat the Streptavidin-Alkaline Phosphatase or the CDP-*Star*.
- Use good sterile technique when handling the Wash and Blocking Buffers. Bacterial contamination will cause high background.

- Dilute the needed amount of 5X Wash Buffer and 10X Assay Buffer to 1X with ddH_2O before use.
  Prepare 5 mL of 1X Wash Buffer per cm^2 of membrane, or 500 mL of Wash Buffer for 100 cm^2 membrane.
  Prepare 2 mL of 1X Assay Buffer per cm^2 of membrane, or 200 mL of Assay Buffer for 100 cm^2 membrane.
- Discard solutions after each incubation. The solutions are not reusable.

C. Detection Procedure

IMPORTANT

All incubations described below should be done at room temperature with constant, gentle agitation in containers that allow free movement of the membrane (e.g. in flat trays).
Also, do not allow the membrane to dry! Once begun, the membrane must remain wet throughout the procedure.

1. Wash membrane 2 x 5 min in 1X Wash Buffer

Do two, 5 min washes in approximately 1 mL Wash Buffer/cm^2 membrane/wash.

The Wash Buffer is provided at 5X, so it must be diluted prior to use. Generally, dilution in ddH_2O is sufficient (DEPC treatment and/or autoclaving of the water is not necessary).

The end of this washing period is good stopping point if there is not enough time to complete the detection procedure. At this point, the membrane can be stored wet, wrapped in plastic, for several days at 4°C.

2. Incubate membrane 2 x 5 min in Blocking Buffer

Do two, 5 min incubations in approximately 0.5 mL Blocking Buffer/cm^2 membrane/incubation.

Do not dilute the Blocking Buffer as it is provided as a ready-to-use solution. As mentioned above, make sure to resolubilize any precipitate prior to use.

3. Incubate membrane 30 min in Blocking Buffer

For this incubation, use approximately 1 mL Blocking Buffer/cm^2 membrane.

4. Incubate membrane 30 min in diluted Strep-AP

Prepare the diluted Strep-Alkaline Phosphatase (Strep-AP) by gently and thoroughly mixing together 10 mL Blocking Buffer and 1 µL Strep-AP per typical size membrane (100 cm^2).

Prepare only the volume needed for the experiment at hand, because Strep-AP is not stable for storage when it is diluted in Blocking Buffer.

It is important to mix the Strep-AP with the Blocking Buffer before adding it to the membrane, because if the Strep-AP is added directly to the container with the membrane and Blocking Buffer, heavy and uneven background may result.


NOTE

Vigorous agitation of enzymes can lead to partial denaturation.

5. Incubate membrane 10 min in Blocking Buffer

Use 0.5 mL Blocking Buffer/cm^2 membrane.

(optional) This incubation can be extended to 15 min to further decrease nonspecific background.

6. Wash membrane 3 x 5 min in 1X Wash Buffer

Do three, 5 min washes in approximately 1 mL Wash Buffer/cm^2 membrane/wash.

(optional) These washes can be extended to 15 min each to further decrease nonspecific background.

7. Incubate membrane 2 x 2 min in 1X Assay Buffer

Do two, 2 min incubations in approximately 0.5 mL 1X Assay Buffer/cm^2 membrane/incubation.

The Assay Buffer is provided at 10X, so it must be diluted in high quality water before use.

8. Incubate membrane 5 min in CDP-*Star*

Use very little CDP-*Star*: only about 5 mL/100 cm^2. The CDP-*Star* is provided as a ready-to-use solution; do not dilute it.

9. Shake off excess CDP-*Star* and expose membrane to film

Quickly blot the membrane on a piece of filter paper to remove excess CDP-*Star*, without letting the membrane dry. If any free-floating CDP-*Star* is left on the membrane, it may generate blotchy background. Wrap in a single layer of plastic, and expose to film at room temperature.

10. About the light emission from CDP-*Star*

CDP-*Star* reaches peak light emission in 2–4 hr, then emission falls to a plateau which persists for several days. Try initial exposures of 1–2 hr followed by shorter exposures (5 min to 2 hr) the following day if necessary to obtain the desired image.

III. Troubleshooting

A. Use of the Labeled Control DNA

The Psoralen-Biotin Labeled Control DNA is a linear 4 kb plasmid (1 ng/µL in 1X TE) containing a 1 kb insert of the mouse Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) gene with biotin molecules covalently bound throughout. It is provided as a positive control for dot detections to ensure that the BioDetect Kit is functional. Typically, 100 fg to 1 pg of Labeled Control DNA can be detected using a dot detection assay.

Dot detection

To ensure the BioDetect Kit is performing well, detect dots of the Labeled Control DNA. To do this, make 10-fold serial dilutions of the Labeled Control DNA in molecular biology-grade water from 1 ng/µL to 100 fg/µL. Make the dots by simply pipetting 1 µL of each dilution onto dry BrightStar-Plus or another positively-charged nylon membrane. Allow the membrane to dry completely at room temperature.

Detect the dots by following the procedure described in section *II.C. Detection Procedure* starting on page 4.

B. High Background

The most likely problems to occur in a nonisotopic detection procedure relate to background. Note that film exposure times using the BrightStar chemiluminescent system are significantly shorter (6 hr or less) than those when radiolabeled probes are used. An overnight exposure will produce high background. However if background comes up to unacceptable levels before the desired signal is detected, or the film is littered with spots and/or blotchiness, there are several possible sources and solutions for this problem that should be considered.

1. Precipitation of Wash or Blocking Buffers

The Wash and Blocking Buffers contain salts and detergents that may precipitate at room temperature. Accumulation of these precipitates on the membrane can result in increased background. Simply warming these solutions to between 37°C and 65°C to redissolve precipitates before use can prevent this problem.

Do ***not*** warm the Strep-AP or CDP-*Star* solutions.

2. Gel pieces and/or polyacrylamide residue on the membrane

Directly after transfer, remove the membrane from the gel while submerged in transfer buffer. Then briefly rinse the membrane in the transfer buffer to wash off any loose gel fragments. Extending the length of washes in the detection procedure to 15 min each (or longer) may also help.

3. Insufficient exposure to buffers and reagents

If the membrane is not freely moving, it will not be fully exposed to the wash solutions, blocking buffers, and reagents. We have found that membranes processed in the conical tubes used in rotatory hybridization cabinets sometimes stick to the sides of these tubes. Although such tubes can be used successfully, we recommend using flat trays (e.g. the lids from pipet tip boxes). Washes following the incubation in diluted Strep-AP (steps 5–6 on page 5) may be extended to 15 min each to further decrease non-specific background when there is no other attributable source.

4. Failure to mix Strep-AP with Blocking Buffer before adding it to the membrane

If the Strep-AP is added directly to the container with the membrane and Blocking Buffer, instead of mixing the solutions before pouring them into the container with the membrane, high background can result. This is probably due to localization of high concentrations of Strep-AP on the membrane surface. This problem can be avoided by thoroughly mixing the Strep-AP and Blocking Buffer before adding the solution to the membrane. We recommend mixing the Strep-AP and Blocking Buffer in a graduated cylinder with repeated, gentle inversion.

5. Excess CDP-*Star* not drained and blotted from membrane

Since the CDP-*Star* reaction products are not bound by the alkaline-phosphatase, they can float freely on the membrane, creating blotchy background. After incubating the membrane with CDP-*Star*, hold the membrane up over the tray to allow any excess CDP-*Star* to drain off. Afterwards, briefly blot the membrane onto a piece of filter paper, making sure not to dry it.

6. Folds or multiple layers of plastic wrap

Folds in the plastic wrap covering the membrane during exposure can trap CDP-*Star* causing "veins" and blotchiness. Also, multiple layers and plastic wrap edges can prevent the light from penetrating fully. Use a single plastic wrap layer and avoid creating wrinkles.

7. Insufficient liquid in filter papers during electroblotting

The filter papers used to form the "sandwich" in the electroblotter must be dripping wet so that equal current can move through all parts of the transfer stack.

8. Bacterial/nuclease contamination

Poor signal detection coupled with an even, high background may indicate contamination of solutions and/or trays with nucleases or alkaline phosphatase. If you suspect contamination, thoroughly clean all vessels and trays with Ambion RNase*Zap* Solution (P/N AM9780) and rinse with RNase-free ddH_2O. Note, that if any of the solutions in the Bio-Detect Kit become contaminated with bacteria, they must be replaced.

C. Low Sensitivity

Low sensitivity usually results from problems preparing the probe or inefficient transfer of nucleic acids to the nylon membrane. The probe and the sensitivity of the detection procedure can be tested by spotting dilutions of the probe on a membrane strip, crosslinking, and following the detection procedure.

1. Low specific activity probe synthesized

At Ambion we get the highest specific activity biotinylated probes by post-synthesis labeling with Psoralen-Biotin. The Ambion BrightStar Psoralen-Biotin Labeling Kit (P/N AM1480) can be used for this purpose.

The most likely factors that compromise specific activity of Psoralen-Biotin labeled probes are whether the correct wavelength was used for labeling and whether the UV source was too far from the sample. The purity of the sample and salt concentration can also be a factor.

Labeling reactions that incorporate biotinylated nucleotides enzymatically should contain a 2:3 ratio of biotin-CTP (or biotin-dCTP) to non-biotinylated CTP or dCTP (a higher ratio will inhibit hybridization). Since biotinylated-NTPs are not incorporated as efficiently as unmodified nucleotides, further dilution will result in much less incorporation and a lower sensitivity probe. See Ambion's Technical Bulletin #173 for sources of modified NTPs and suggestions for setting up the reactions.

2. Inaccurate quantitation of probe

Contaminating salts, protein, and possibly acrylamide, can affect spectrophotometric readings. A_{260}/A_{280} ratios should fall between 1.8 and 2.1. If they do not, clean up biotinylated probes (e.g. by phenol extraction and/or precipitation and an EtOH wash step) to get a more accurate reading. Being aware of the maximum theoretical yield of a labeling reaction can also serve as a warning of inaccurate quantitation.

3. Degradation of probe

Probe degradation can result in lower sensitivity. Probe integrity can be assessed by running “probe alone” on a denaturing gel, transferring to membrane, crosslinking, and following the detection procedure.

4. Inefficient transfer to membrane

Inefficient transfer to membrane vs. “probe problems” can be assessed by staining the gel with EtBr after transfer to visualize any residual nucleic acid. In an RPA, if transfer is incomplete, a smear from the yeast RNA in the control lane should be visible; in a Northern, rRNAs should be visible. Alternatively, several transfers or similar dilutions of two probes transferred on the same membrane can be compared. Be sure to follow the manufacturer's instructions when electroblotting.

5. Inefficient or lack of crosslinking

Biotinylated probes transferred to a membrane from a nuclease protection assay gel must be crosslinked to the membrane before beginning the detection procedure; if left uncrosslinked, probes may wash off,

reducing signal. However, this is not necessary for biotinylated probes used in blot hybridizations (e.g. Northerns, Southerns). These probes are bound by hybridization with the nucleic acid that is crosslinked to the membrane, and will not be removed by the BioDetect wash steps.

6. Exposure to film at reduced temperature

Exposure of the membrane to film below room temperature will inhibit or prevent the enzymatic reaction that produces light emission and will result in signal reduction or loss.

IV. Additional Procedures

A. Electroblotting from Polyacrylamide Gels

Electroblotting from polyacrylamide gels is best accomplished using a commercially available active transfer apparatus (tank electroblotter, semi-dry gel blotter, vacuum blotter, pressure blotter etc.). At Ambion we routinely use the OWL Scientific Panther™ Semi-Dry Electroblotter (Model # HEP-1), a relatively inexpensive and efficient electroblotter. The procedure is as follows:

1. Cut six pieces of filter paper and one BrightStar-Plus or other positively-charged nylon membrane to size of gel.
2. Thoroughly wet two pieces of the filter paper in 0.5X TBE and lay them over the bottom electrode. Separate the glass plates of the gel-plate sandwich and use a third piece of filter paper (wet) to lift the gel off the bottom plate.
3. Lay the wet membrane on the gel and wet the entire "sandwich" in 0.5X TBE, removing any bubbles that might have formed (a long plastic pipet can be rolled over the surface for this purpose).
4. Lay the "sandwich" carefully on the wet pieces of filter paper.
5. Thoroughly wet the remaining three pieces of filter paper and lay them carefully on the membrane surface. Again, it is important to avoid forming air bubbles.
6. Close the apparatus and apply current of 200 milliamperes for a period of 1 hr or 400 mA for 30 min.
7. After transfer is complete, remove the gel and membrane from the stack together and submerge them in transfer buffer. Peel the gel from the membrane and discard it. Rinse the membrane by briefly shaking it in the transfer buffer to dislodge any acrylamide fragments.
8. Immobilize the nucleic acid on the membrane by UV crosslinking (see below) and then proceed to the detection procedure (section *II.C. Detection Procedure* starting on page 4.).

B. Crosslinking

The membrane should be treated to immobilize the nucleic acid immediately after transfer and rinsing. This can be done by either one of the following methods:

UV crosslinking

If using a commercial crosslinker, the manufacturer's recommendations should be followed (i.e. UV exposure of approximately 1.2 millijoules). The membrane should be kept wet during this process. Ultraviolet crosslinking may also be accomplished by using a transilluminator or a handheld UV light source.

Baking

The membrane may be treated by baking at 80°C for 15 min. Since the membrane is not nitrocellulose, it is not necessary to use a vacuum oven. A conventional oven or convection oven is suitable.

V. Appendix

A. Quality Control

The Labeled Control DNA is spotted onto Ambion BrightStar-Plus Membrane, and subjected to the procedure outlined in section *II.C. Detection Procedure* starting on page 4 of this protocol. Immediately after removing the CDP-*Star*, 10 pg of the Labeled Control DNA can be detected in a 30 min exposure to X-ray film. The membrane is exposed to x-ray film for 30 min again at 2 hr after removing the CDP-*Star*, and 1 pg of Labeled Control DNA can be detected.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with super-coiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

Protease testing

Meets or exceeds specification when a sample is incubated with protease substrate and analyzed by fluorescence.

B. Safety Information

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.

- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.