BD SpotLight™ Random Primer Labeling Kit User Manual

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I. Introduction

Northern and Southern blots are useful tools for analyzing gene structure and expression, for characterizing genomes, and for medical diagnostics. Conventional hybridization methods that employ radioactively labeled probes pose drawbacks such as isotope instability (i.e., short half-life), long autoradiograph exposure times, and hazards in material handling and disposal. BD Biosciences Clontech **SpotLight™ Chemiluminescent Labeling and Detection System** provides an alternative method that involves labeling hybridization probes with biotin followed by visualization with a chemiluminescent detection method.

BD Biosciences Clontech **SpotLight™ Random Primer Labeling Kit** is a complete system for the incorporation of biotinylated dCTP into double-stranded DNA (50–300 bp) using the random-prime method of Feinberg & Vogelstein (Feinberg & Vogelstein ,1984). This technique uses the Klenow fragment of DNA polymerase I and a mixture of random primers (6–10 nucleotides long) to prime DNA synthesis *in vitro* along a double-stranded DNA template. Applications using biotinylated probes include Northern blots such as BD Biosciences Clontech MTN® blots Southern blots, and dot blots, including BD Biosciences Clontech MTE™ arrays.

To hybridize your biotinylated probe use our BD Biosciences Clontech SpotLight™ Chemiluminescent Hybridization & Detection Kit (K1032-1). The specially designed BD Biosciences Clontech SpotHyb™ hybridization buffer contained in the kit has been optimized for use with nylon membranes, minimizing nonspecific hybridization and general background problems. The high affinity of biotin for streptavidin (k_d = ~10^{-15}) permits sensitive detection of probe-target hybrids. Nonisotopic detection is accomplished by binding of streptavidin-horse radish peroxidase (HRP) complexes to the labeled probe's biotin groups followed by reaction with an enhanced luminol substrate, which results in light emission localized to the hybridized probe. For users not familiar with performing and troubleshooting Northern and Southern blots, we recommend the following references: *Molecular Cloning: A Laboratory Manual,* 2nd ed.1 and *Current Protocols in Molecular Biology* (chapters 3 and 7) .

II. List of Components

Store all components at –20°C.

The following reagents are suitable for 10 labeling reactions.

- **50 µl** **10X Random Primer Mix** (1 µg/ml)
- **50 µl** **10X Klenow Labeling Mix**
- **50 µl** **10X Klenow Reaction Buffer**
- **30 µl** **EDTA** (500 mM)
- **10 µl** **Klenow Enzyme** (10 units/µl)
- **10 µl** **β-actin Control cDNA** (10 ng/µl)
- **5 µl** **Biotin dT_{100} Control** (10 ng/µl)
- **500 µl** **Nuclease-free Water**
- **1** **NucleoSpin® Extraction Kit**

III. Additional Materials Required

The following materials are required but not supplied:

- **Microcentrifuge**
- **Thermal cycler, heatblock or waterbath**
- **SpotLight™ Chemiluminescent Hybridization & Detection Kit** (#K1032-1)
- **Deionized H_2O**
- **Nylon Membrane** (for teststrip)
- **X-ray film** (Kodak BioMax MS or MR or similar high-sensitivity film)
- **Plastic wrap or acetate page protectors**

IV. Preparation of Biotin-Labeled cDNA Probes

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

The 50-µl reaction described below converts 100 ng of linear DNA into biotin-labeled cDNA. To confirm that the probe preparation protocol works in your hands, we recommend that you perform a parallel labeling with the β-actin Control cDNA.

A. Probe Labeling

1. In PCR tube: combine: 10–100 ng of linear DNA* (template DNA), 5 µl of 10X Random Primer Mix, and bring volume up to 31 µl with nuclease-free water (if necessary).

   *For the control reaction, add 2.5 µl of β-actin Control cDNA (10 ng/µl).

2. Heat mix to 97°C in thermal cycler or boil for 3 min. Remove tubes and chill quickly on ice.
3. Spin briefly at room temperature to collect droplets formed by condensation, then return tube to ice.
4. Add to template/primer mix:

5 µl	10X Klenow Reaction Buffer
5 µl	10X Klenow Labeling Mix
8 µl	ddH_2O
1 µl	Klenow Enzyme
50 µl	Total volume

5. Incubate at 37°C for 30 min in a thermal cycler, water bath, or heat block.
6. Remove tube from the thermal cycler and stop the reaction by adding 2 µl of 0.5 M EDTA (pH 8.0).

B. Purification of Biotinylated Probe

To purify the labeled cDNA from unincorporated biotin-labeled nucleotides and small (<0.1 kb) cDNA fragments, use the NucleoSpin Extraction columns provided and follow the procedure described below. Before use, be sure to add 95% ethanol directly to Buffer NT3 as specified on the bottle.

1. Dilute probe synthesis reactions to 400 µl total volume by adding 350 µl of Buffer NT2; mix well by pipetting.
2. Place a NucleoSpin Extraction Spin Column into a 2-ml Collection Tube, and pipet the sample into the column. Centrifuge at 14,000 rpm for 1 min. Discard Collection Tube and flowthrough.
3. Insert the NuceloSpin column into a fresh 2-ml Collection Tube. Add 400 µl Buffer NT3 to the column. Centrifuge at 14,000 rpm for 1 min. Discard Collection Tube and flowthrough.
4. Repeat Step 3 two more times.

IV. Preparation of Biotin-Labeled cDNA Probes *continued*

5. After the last wash, discard flowthrough, place tube back into an empty collection tube and spin again for 1 min at 14,000 rpm to remove traces of buffer.
6. Transfer the NucleoSpin column to a clean 1.5-ml microcentrifuge tube. Pipette 50 µl Buffer NE directly onto the filter, being careful not to touch the surface of the filter with the pipette tip. Allow filter to soak for 2 min.
7. Centrifuge at 14,000 rpm for 1 min to elute purified probe.
8. Repeat Steps 6 and 7.
9. Continue with Section C to determine concentration of the newly synthesized biotinylated probe by UV spectroscopy. If necessary, you can store your probe on ice or at 4°C for a few hours. Probes may also be stored at –20°C for up to six months.

C. Quantification of Synthesized Probe

For chemiluminescent detection of nucleic acids, it is important to accurately quantitate the amount of probe that is synthesized. Because this is an enzymatic reaction, and probe is synthesized in excess of template, we highly recommend that the purified probe be quantitated spectrophotometrically. Typical yield using the control DNA is 5–15 ng/µl, which is a total amount of 500 ng to 1.5 µg of biotinylated probe per reaction.

1. Blank spectrophotometer at 260 nm with a quartz cuvette filled with 500 µl of nuclease-free water.
2. In a sterile microcentrifuge tube, add 2.5 µl of biotinylated DNA probe to 497.5 µl of nuclease-free water. Vortex and transfer to empty quartz cuvette.
3. Read sample.
4. To determine concentration:
   a. Total OD_{260} = (OD_{260}) x (200)
   b. Concentration (µg/ml) = (total OD_{260}) x (50)
   c. Concentration (µg/µl) = (concentration [µg/ml]) ÷ 1000

IV. Preparation of Biotin-Labeled cDNA Probes *continued*

D. Determining Sensitivity of Biotinylated Probes

The sensitivity of biotin-labeled nucleic acid probes may be assessed via chemiluminescent detection using BD Biosciences Clontech SpotLight™ Chemiluminescent Hybridization & Detection Kit (#K1032-1).

Note: The volume of solution given for each step is approximate and may need to be adjusted depending on the size of container used for the incubations. In general, use enough of each solution to keep the membrane fully submerged during each incubation step. If the sides of the membrane stick out from the liquid, high background will appear at the edges of the membrane

1. Prepare dilutions of the Biotin dT_{100} Control with deionized H_2O. From the 10 ng/µl Biotin dT_{100} stock, make an initial dilution to 2 ng/µl (dilution A), then make the following 5-fold serial dilutions: 400 pg/µl, 80 pg/µl, and 16 pg/µl (see Table I).

TABLE I. DILUTION OF BIOTINYLATED CONTROL

Biotin dT_{100} Control Starting concentration	Dilution (µl stock + µl H_2O)	Final Concentration (dilution name)	Total Dilution
10 ng/µl	1 µl + 4 µl	2 ng/µl (A)	1:5
2 ng/µl (dilution A)	1 µl + 4 µl	400 pg/µl (B)	1:25
400 pg/µl (dilution B)	1 µl + 4 µl	80 pg/µl (C)	1:125
80 pg/µl (dilution C)	1 µl + 4 µl	16 pg/µl (D)	1:625

2. Prepare dilutions of the labeled DNA from both the β-actin Control cDNA and your experimental probe with deionized H_2O. Make three dilutions at 1:5, 1:25, and 1:125 (Figure 1).
3. Spot 1 µl of each of your probe dilutions, plus 1 µl of Biotin dT_{100} dilutions A–D, sequentially onto a 3 x 7 cm strip of pre-marked nylon membrane (Figure 2). Let this “teststrip” air dry for 30 min before continuing.
4. Incubate the membrane in 5 ml Blocking Buffer with gentle agitation for 15 min. Ensure that the teststrip is entirely covered by the Blocking Buffer.
5. Add 8 µl of Stabilized Streptavidin-Horseradish Peroxidase Conjugate to the Blocking Buffer (a 1:300 final dilution) being careful to avoid pipetting directly onto the membrane. Incubate for 5 min with gentle agitation.
6. Meanwhile, dilute an aliquot of the Wash Buffer (4X) included in the SpotLight Hybridization & Detection Kit to 1X with sterile H_2O. Prepare approximately 30 ml of 1X Wash Buffer.

IV. Preparation of Biotin-Labeled cDNA Probes *continued*

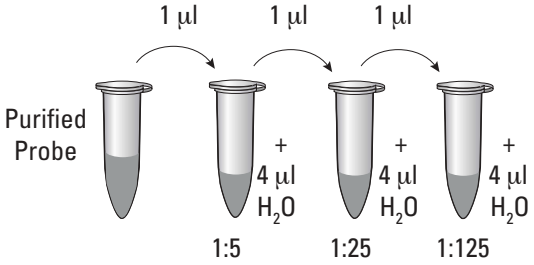

Figure 1. Dilutions of probes to quantitate activity. Dilute your purified probe as indicated with sterile, distilled H_2O.

7. Pour off the Blocking Buffer and wash the membrane in 10 ml of 1X Wash Buffer for 2 min with constant, gentle agitation. Perform wash a total of three times.
8. Pour off Wash Buffer and transfer the teststrip to a clean container. Equilibrate the membrane in 5 ml Substrate Equilibration Buffer for 1 min with gentle agitation.
9. Remove the teststrip from the Substrate Equilibration Buffer and transfer to a clean container. Pipette 1 ml of Working Solution (consisting of equal amounts of Luminol/Enhancer Solution and Stable Peroxide Solution) onto the membrane. Let signal develop for 5 min.
10. Drain off the Working Solution from membrane surface and blot the membrane briefly on filter paper. Do not allow membrane to dry.

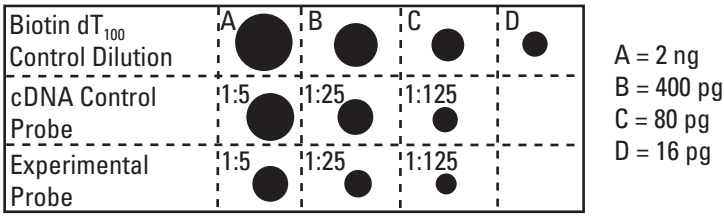

Figure 2. Diagram of probe activity teststrip. Mark a 3 x 7 cm strip of nylon membrane as shown with pencil. Spot 1 µl of each probe dilution plus 1 µl of Biotin dT_{100} dilutions A-D. After performing chemiluminescent detection, the 1:5 Control cDNA probe dilution typically results in a signal similar to Biotin dT_{100} dilution B.

IV. Preparation of Biotin-Labeled cDNA Probes *continued*

11. Wrap the moist membrane in clear plastic wrap or put into an acetate page protector.
12. Place the membrane into a film cassette, without an intensifying screen, and expose it to regular x-ray film for 1 min. Shorter or longer exposure may be used for appropriate signal intensity.
13. Note orientation of film. Remove the film from the cassette and develop the film according to the manufacturer's instructions.
14. Compare the spot intensities of your labeled probes to the dilutions of the Biotin dT_{100} control. The 1:5 dilution of you experimental probe must be **at least** strong as Biotin dT_{100} dilution **D** to yield satisfactory hybridization results. If the probe signal is weaker than this, it will be difficult or impossible to detect signals from low abundance species.
15. The 1:5 dilution of the probe synthesized from the β-actin Control cDNA should yield a signal at least as strong as Biotin dT_{100} dilution **C**. If the signal is significantly weaker than this, then the probe labeling reaction has failed (see Troubleshooting).

V. Troubleshooting Guide

A. Little or No Probe Synthesized

If little or no probe is synthesized, test the probe synthesis reagents by performing a control reaction.

- The Control β-actin cDNA is a good positive control for preparing your hybridization probe. Compare the incorporation of biotin for probe made from the Control cDNA and your experimental template. Control cDNA probes should produce a 1:5 dilution signal that corresponds to the 400 pg Biotin dT_{100} dilution B (Figure 2). If you obtain this level of incorporation with the Control cDNA, but cannot obtain a signal from your experimental template equivalent to the 80 pg Biotin dT_{100} dilution C, your DNA may contain inhibitory contaminants (see below) or the concentrations of your template is significantly lower than you expected. On the other hand, if the Control cDNA fails to yield good incorporation, you should troubleshoot the labeling procedure or check the quality of the reagents.
- Be sure that you are using the optimum amount of DNA to prepare your hybridization probe. We typically use 100 ng of DNA for each labeling reaction.
- Impurities in template DNA samples, such as protein, can inhibit the labeling reaction. In this case, you may need to perform additional steps to purify your total cDNA starting material. Try treating your DNA twice with phenol:chloroform and once with chloroform, followed by precipitation with 1/10 volume of 2 M NaOAc (pH 4.5) and 2.5 volumes of ethanol. This will help ensure the removal of any protein and other impurities that may not have been removed effectively during initial DNA purification.

B. Low Probe Activity

Your Klenow may be degraded

- This can happen if Klenow is not kept on ice at all times, or it is not returned to the freezer promptly after use.

Your probe was lost during spin column chromatography

- This can happen if an inappropriate amount of ethanol is added to Buffer NT3 or if too much or too little Buffer NT2 is mixed with the probe (Section IV.B)

VI. References

Ausubel, F. M., Brent, R., Kingdom, R. E., Moore, D. M., Seidman, J. G., Smith, J. A. & Struhl, K., Eds. (1995) Current Protocols in Molecular Biology. (John Wiley & Sons, NY).

Feinberg, A. P. & Vogelstein, B. (1984) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **137**:266–267.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual, Second Edition* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

VII. Related Products

For a complete listing of all BD Biosciences Clontech products, please visit **www.bdbiosciences.com/clontech**

	Cat. No.	New Cat. No.
• BD Spotlight™ Chemilumniscent Hybridization and Detection Kit	K1032-1	634802
• BD Atlas™ SpotLight™ Labeling Kit	K1033-1	634803
• BD MTN™ Multiple Tissue Northern Blots	many	many
• Control cDNA	many	many
• Poly A^+ and Total RNAs	many	many