INSTRUCTIONS

Biotin 3´ End DNA Labeling Kit

89818

1290.8

Number	Description
89818	**Biotin 3´ End DNA Labeling Kit**, sufficient reagents to perform 20 labeling reactions (5pmol each)
	Kit contents:
	5X TdT Reaction Buffer, 1mL, 500mM cacodylic acid, 10mM $CoCl_2$, 1mM DTT, pH 7.2; store at -20°C.
	Terminal Deoxynucleotidyl Transferase (TdT), 50µL; ~15 U/µL in 60mM potassium phosphate, 150mM potassium chloride, 1mM 2-mercaptoethanol, 1% Triton™ X-100, 50% glycerol, pH 7.2; store at -20°C
	Biotin-11-UTP, 100µL; 5µM in 10mM Tris•HCl, 1mM EDTA, pH 7.5; store at ≤ 4°C and minimize freeze-thaw cycles and exposure to light
	Unlabeled Control Oligo, 140µL; 1µM in 10mM Tris•HCl, 1mM EDTA, pH 7.5; store at -20°C
	Biotin-Control Oligo, 40µL; 1µM in 10mM Tris•HCl, 1mM EDTA, pH 7.5; store at -20°C
	Storage: Upon receipt store individual components as indicated. Product is shipped with dry ice.

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Introduction

The Thermo Scientific™ Biotin 3´ End DNA Labeling Kit uses TdT to incorporate 1-3 biotinylated ribonucleotides onto the 3´ end of DNA strands. This labeling strategy has the advantage of localizing the biotin to the 3' end of the probe where it is less likely to interfere with hybridization or sequence-specific binding of proteins. Biotin-labeled DNA probes can be used to facilitate non-isotopic detection in a variety of applications including electrophoretic mobility shift assays (EMSA), Northern or Southern blots, colony hybridizations, or *in situ* hybridizations.

The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes non-template-directed deoxynucleotide incorporation onto the 3´-OH end of DNA.[1-2] TdT exhibits substrate preference for single-stranded DNA, but it will also label duplex DNA with 3´ overhangs and blunt duplexes, albeit with lower efficiency.[3] Under the appropriate conditions (in the presence of Co^{2+}), TdT will incorporate ribonucleotides onto the 3´ end of DNA as well.[2]

For nonisotopic EMSA applications, use the Thermo Scientific™ LightShift™ Chemiluminescent EMSA Kit (Product No. 20148). For nonisotopic Northern or Southern blotting applications, use the Thermo Scientific™ North2South™ Chemiluminescent Nucleic Acid Hybridization and Detection Kit (Product No. 17097). For other types of nucleic acid detection by means of the biotin-labeled DNA probe, use the Chemiluminescent Nucleic Acid Detection Module (Product No. 89880).

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Labeling Procedure

Note: This procedure is optimized for labeling 5pmol of 3´-OH ends in each reaction. The presence of Mg^{2+}, protein and residual agarose may decrease labeling efficiency. Use only purified DNA in the following reaction. For applications requiring double-stranded DNA oligos, end-label both complementary oligonucleotides separately and then anneal them before use (see note after Step B.8).

A. Additional Materials Required

- Ultrapure water
- 1µM of oligonucleotide for labeling
- 37°C heat block or water bath
- 0.2M EDTA, pH 8.0
- Chloroform:isoamyl alcohol (24:1)
- Microcentrifuge tubes and microcentrifuge

B. Labeling Reaction

1. Thaw all kit components except the TdT, and place them on ice. Keep TdT at -20°C until needed.
2. Just before use, dilute a portion of the TdT stock in 1X TdT Reaction Buffer to a working concentration of 1.5 U/µL. For example, prepare enough diluted TdT for each reaction by combining 2µL of 5X TdT Reaction Buffer, 7µL of ultrapure water and 1µL of 15 U/µL stock of TdT.

   Note: Use the diluted TdT stock immediately. Do not store the diluted enzyme.
3. Prepare the labeling reaction for the control system by adding components in the order listed below. Prepare the sample labeling reaction(s) in the same manner, substituting 5pmol 3'-OH ends of the sample DNA in place of the Unlabeled Control Oligo.

   Note: Mix reactions gently. Do not vortex.

Component	Volume (µL)	Final Concentration
Ultrapure water	25	---
5X TdT Reaction Buffer	10	1X
Unlabeled Control Oligo (1µM)	5	100nM
Biotin-11-UTP (5µM)	5	0.5µM
Diluted TdT (1.5U/µL)	5	0.15 U/µL
Total Volume	50	---

4. Incubate reactions at 37°C for 30 minutes.
5. Add 2.5µL 0.2M EDTA to stop each reaction.
6. Add 50µL chloroform:isoamyl alcohol to each reaction to extract the TdT. Vortex the mixture briefly, then centrifuge 1-2 minutes at high speed in a microcentrifuge to separate the phases. Remove and save the top (aqueous) phase.
7. To determine labeling efficiency, see the Procedure for Estimating Labeling Efficiency on the next page. At this point, the DNA is ready to be used in the end application.

Note: For applications such as electrophoretic mobility shift assays (EMSA) that require biotin-labeled double-stranded DNA, complementary oligos should be end-labeled separately and then annealed before use. Anneal oligos by mixing together equal amounts of labeled complementary oligos and incubating the mixture for 1 hour at room temperature. Oligonucleotides with high melting temperatures or secondary structure may require denaturation and slow cooling for optimal annealing (e.g., denature at 90°C for 1minute, then slowly cool and incubate at the melting temperature for 30 minutes). Freeze annealed oligos that will not be used immediately, and thaw on ice for use. Removal of the unincorporated Biotin-11-UTP is not necessary for use in EMSA but may be necessary for other applications.

Procedures for Estimating Labeling Efficiency

Labeling efficiency can be determined by dot blots using either a dot/slot blotting apparatus (Option 1) or by hand spotting (Option 2). The following dot blot protocols were developed for use with the detection reagents in the LightShift Chemiluminescent EMSA Kit (Product No. 20148). Sample results appear in the Appendix of these instructions.

A. Additional Materials Required

- Positively-charged nylon membrane (e.g., Biodyne™ B Membrane, Product No. 77016)
- Dot blotting apparatus
- TE Buffer: 10mM Tris•HCl, 1mM EDTA, pH 8.0
- 96-well microplate (low adsorption) for preparing dilutions
- Multi-channel pipette
- Microcentrifuge tubes and microcentrifuge
- UV lamp or cross-linking device equipped with 254nm bulbs or 312nm transilluminator
- Streptavidin-HRP and chemiluminescent substrate reagents (as in LightShift Chemiluminescent EMSA Kit, Product No. 20148)
- X-ray film or CCD camera

B. Option 1: Dot Blot Using a Dot/Slot Blotting Apparatus

1. Hydrate/equilibrate a positively-charged nylon membrane in TE Buffer for at least 10 minutes.
2. Pipette 125µL TE Buffer into wells B1 through H12 of a 96-well dilution plate (leave wells A1-A12 empty).
3. Dilute a sample of the Biotin Control Oligo and the Unlabeled Control Oligo stocks 200-fold in TE Buffer to make 5 nM oligo working stocks (e.g., 2µL Control Oligo + 398µL TE Buffer).
4. In microcentrifuge tubes, prepare a series of oligo standards according to the following table:

	% Biotin				
Component	**100**	**75**	**50**	**25**	**0**
Biotin Control Oligo (5nM)	15	11.25	7.5	3.75	0
Unlabeled Control Oligo (5nM)	0	3.75	7.5	11.25	15
TE Buffer (pH 8.0)	285	285	285	285	285
Total Volume	300µL	300µL	300µL	300µL	300µL

5. Pipette 250µL of the above oligo standards into wells A1-A5 of the 96-well microplate.
6. In a microcentrifuge tube, prepare a 20-fold dilution of the test TdT labeling reaction in TE Buffer to achieve a final concentration of 5nM (e.g., 2µL TdT reaction + 38µL TE Buffer).
7. In another microcentrifuge tube, combine 15µL of 5nM dilution from Step 6 with 285µL TE Buffer. Pipette 250µL of this sample into an available "A" well in the unused portion (A6-A12) of the 96-well microplate above.
8. Prepare a series of two-fold dilutions of standards and samples by removing 125µL aliquots from all "A" wells and mixing them with the TE Buffer in corresponding "B" wells, continuing down the plate through the "H" wells.
9. Place the hydrated nylon membrane into the dot blot apparatus. Add 100µL TE into wells of dot blotting apparatus.
10. Apply vacuum or attach to a peristaltic pump to establish a flow rate of 100µL/~5 min/well.
11. When the solution has been drawn through the membrane, break the vacuum to stop flow.
12. Apply 100µL per well of samples from the microplate (results in 25fmol 3'-OH ends in spot A to 0.2fmol in spot H).
13. Apply vacuum to draw samples through the membrane.
14. Wash wells three times with 125µL TE Buffer, breaking the vacuum between each application.
15. After the final wash, remove the membrane from the blotting device and place it on a clean paper towel. Allow the residual buffer to absorb into the membrane.
16. Immediately UV crosslink the membrane as described in Section D below.

C. Option 2: Dot Blot by Hand Spotting

Note: The same quantity of DNA per spot will be applied as in Option 1 but in a smaller total volume.

1. Hydrate/equilibrate a positively-charged nylon membrane in TE Buffer for at least 10 minutes.
2. Dilute a sample of the Biotin Control Oligo and the Unlabeled Control Oligo stocks 20-fold in TE Buffer to make 50nM oligo working stocks (e.g., 2µL Control Oligo + 38µL TE, pH 8.0).
3. In microcentrifuge tubes, prepare a series of oligo standards according to the following table:

	% Biotin				
Component	**100**	**75**	**50**	**25**	**0**
Biotin Control Oligo (50nM)	12	9	6	3	0
Unlabeled Control Oligo (50nM)	0	3	6	9	12
TE, pH 8.0	48	48	48	48	48
Total Volume	60µL	60µL	60µL	60µL	60µL

4. Pipette 50µL of the oligo working stocks into wells A1-A5 of a 96-well dilution plate.
5. In a microcentrifuge tube, make a 10-fold dilution of the test TdT labeling reaction in TE Buffer to achieve a final concentration of 10nM (e.g., 6µL TdT reaction + 54µL TE, pH 8.0).
6. Place 50µL of each 10nM test DNA samples into unused "A" wells of the 96-well plate. Prepare a series of two-fold dilutions of standards and samples by removing 25µL aliquots from all "A" wells and mixing them with the 25µL TE Buffer in corresponding "B" wells, continuing down the plate through the "H" wells.
7. Place the equilibrated membrane onto a clean, dry paper towel. Allow excess buffer to absorb into the membrane, but do not allow the membrane dry out.
8. Spot 2µL of samples and standards onto the membrane (resulting in 20fmol 3'-OH ends in "A" spots and 0.16fmol in "H" spots). Allow the samples absorb into the membrane.

D. UV Crosslink the Dot blot

1. Immediately UV crosslink the membrane by one of the following three methods:
   - **Option 1**: Crosslink at 120mJ/cm^2 using a commercial UV-light crosslinking instrument equipped with 254nm bulbs (45-60 second exposure using the auto crosslink function).
   - **Option 2**: Crosslink at a distance of approximately 0.5cm from the membrane for 5-10 minutes with a hand-held UV lamp equipped with 254nm bulbs.
   - **Option 3**: Crosslink for 10-15 minutes with the membrane face down on a transilluminator equipped with 312nm bulbs.
2. Continue with the detection and analysis immediately, or store the membrane dry at room temperature until the detection protocol can be performed.

E. Detection and Analysis

Detect the spotted standards and samples using the procedure and reagents for the LightShift Chemiluminescent EMSA Kit (Product No. 20148) or Chemiluminescent Nucleic Acid Detection Module (Product No. 89880). To determine the labeling efficiency, compare spot intensities of the sample lanes to those of the Biotin Control Oligo standards.

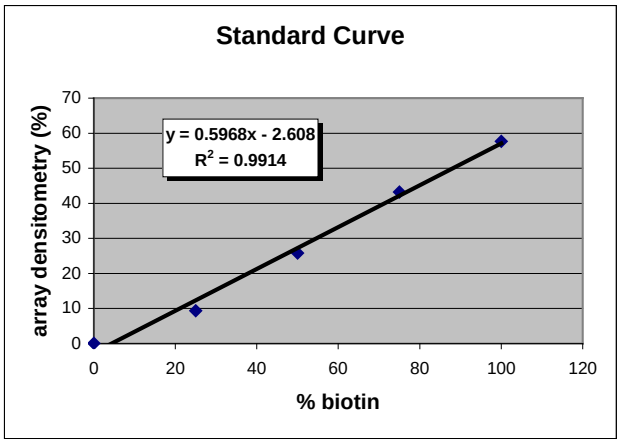
- If using X-ray film to document results, compare the row of sample spots to the row of standard spots to estimate efficiency. Densitometry of the film also can be performed.
- If using a cooled CCD camera to document results, spot densitometry can be performed (choose a dilution that is within the linear exposure range) and a standard curve constructed from which to extrapolate the labeling efficiency of the unknown samples.

The Unlabeled Control Oligo should be labeled with > 70% efficiency as determined by the dot blot analysis described here. The efficiency of TdT labeling is somewhat variable, even among different single-stranded oligonucleotides. This variability does not correlate directly to the identity of the 3' terminal base, but is related to the overall sequence. Typically, oligonucleotides with relatively inefficient labeling (< 50%) still produce good signal in subsequent chemiluminescent EMSA detection procedures.

Appendix

A sample dot blot to estimate labeling efficiency appears below. The blot includes the dilution series for each of the standards made in Option 1 of the Procedures for Estimating Labeling Efficiency as well as three different test oligos. A dot blotting apparatus and vacuum were used to spot the samples. The blot was detected using the reagents supplied in the LightShift Chemiluminescent EMSA Kit (Product No. 20148).

	Standards (% Biotin)					Oligo		
fmol	100	75	50	25	0	A	B	C
25								
12.5								
6.2								
3.1								
1.6								
0.8								
0.4								
0.2								



	Density	% Biotin
Oligo A	37.30	66.9
Oligo B	51.00	89.8
Oligo C	32.98	59.6

Related Thermo Scientific Products

20148	**LightShift Chemiluminescent EMSA Kit**
89880	**Chemiluminescent Nucleic Acid Detection Module**
77016	**Biodyne B Nylon Membranes,** 8 × 12cm, 25 sheets
78833	**NE-PER™ Nuclear and Cytoplasmic Extraction Reagents**
17097	**North2South Chemiluminescent Hybridization and Detection Kit**
17075	**North2South Biotin Random Prime DNA Labeling Kit**

Cited References

1. Roychoudhury, R. and Wu, R. (1980). Terminal transferase-catalyzed addition of nucleotides to the 3´ Termini of DNA. *Methods Enzymol* **65:**43-62.
2. Roychoudhury, R., *et al.* (1976). Terminal labeling and addition of homopolymer tracts to duplex DNA fragments by terminal deoxynucleotidyl transferase. *Nucl Acids Res* **3:**863-77.
3. Michelson, A.M. and Orkin, S.H. (1982). Characterization of the homopolymer tailing reaction catalyzed by terminal deoxynucleotidyl transferase. *J Biol Chem* **257:**14773-82.

Product Reference

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