INSTRUCTIONS

Pierce™ RNA 3' End Biotinylation Kit

20160

2226.5

Number	Description
20160	**Pierce RNA 3' End Biotinylation Kit**, sufficient for 20 biotinylation reactions

Kit Contents:

T4 RNA Ligase Reaction Buffer (10X), 100µL, 0.5M Tris•HCl, 0.1M $MgCl_2$, 0.1M DTT, 10mM ATP; pH 7.8 at 25°C

T4 RNA Ligase, 40µL, 20,000 U/mL

Non-labeled RNA Control, (10µM), 100µL

5'-CCUGGUUUUUAAGGAGUGUCGCCAGAGUGCCGCGAAUGAAAAA-3'

Biotinylated IRE RNA Control (125nM), 35µL

5'-UCCUGCUUCAACAGUGCUUGGACGGAAC-3'-Biotin

RNase Inhibitor (40U/µL), 2 × 10µL

Nuclease-free Water, 1.5mL

DMSO, 200µL

PEG 30%, 300µL

Biotinylated Cytidine (Bis)phosphate, (1mM), 40µL

Glycogen, 20mg/mL, 20µL

Storage: Upon receipt store at -20°C. Product shipped on dry ice.

Introduction

The Thermo Scientific™ Pierce™ RNA 3´ End Biotinylation Kit uses T4 RNA ligase to attach a single biotinylated nucleotide to the 3´ terminus of an RNA strand. The unique feature of this kit is the biotinylated cytidine (bis)phosphate nucleotide that contains a 3´,5´ phosphate on the ribose ring to accommodate the ligation and a biotin linker on the cytidine for detection. The kit contains T4 RNA ligase reaction components, a positive control non-labeled RNA strand and a biotinylated RNA probe to quantitate labeling. To enhance biotinylation efficiency and RNA stability, RNase inhibitor, glycogen and ligation-enhancing reagents are also included. Once biotinylated, the labeled RNA probe is easily prepared for use in downstream applications such as RNA electrophoretic mobility shift assays (RNA EMSA, Product No. 20158), RNA pull-down assays or miRNA profiling.

Regulation of cellular function is dependent on critical RNA interactions with proteins and other RNA, including miRNA. These interactions have been difficult to isolate and are highly dependent on maintaining RNA secondary structure. To enrich for these interactions it is often necessary to label the RNA; however, nonradioactive labeling methods can compromise RNA secondary structure or interfere with protein-RNA interactions. The Pierce RNA 3' End Biotinylation Kit provides a rapid nonradioactive method for RNA labeling that results in minimal interference of RNA secondary structure. The optimized labeling conditions accommodate RNA of different sizes, complexity and sources and produce ligation efficiencies > 75%.

Additional Materials Required

- 1-50pmol of RNA for labeling
- Heated mixer or chiller for incubation at 16°C or 37°C
- Chloroform:isoamyl alcohol (24:1)
- Nuclease-free pipette tips and tubes
- Microcentrifuge
- Heating block
- 5M NaCl
- Ultrapure water
- 100% ethanol, ice-cold
- 70% ethanol, ice-cold

Procedure for RNA Ligation

Note: This kit is optimized for 50pmol of RNA; however, RNA amounts from 1 to 50pmol generally produce similar ligation efficiencies. Purified RNA produces the most robust results. Optimize as needed to achieve optimal efficiency. Maintain a nuclease-free environment during this procedure and when working with the RNA that is intended for labeling.

A. RNA Ligation Reaction

1. Thaw all kit components except the 30% PEG and DMSO on ice. Thaw DMSO at room temperature and warm the 30% PEG at 37°C for 5-10 minutes until volume is fluid.
2. Adjust the heating block to 85°C.
3. Transfer 5µL of the Non-labeled RNA Control to a microcentrifuge tube. Heat the RNA for 3-5 minutes at 85°C. Place RNA immediately on ice.

   Note: The RNA might require heating to relax the secondary structure. Also, heating the RNA in the presence of ~25% DMSO might increase efficiency for RNA with significant secondary structure.
4. Prepare the labeling reaction for the control system or test RNA by adding components in the order listed in Table 1.

   Note: 30% PEG is the last reagent added. Carefully pipette the 30% PEG into the reaction mixture. Use a new pipette tip to mix ligation reaction after the PEG addition.

Table 1. Preparation of the RNA ligation reaction.

Component	Volume (µL)	Final Concentration (Amount)
Nuclease-free Water	3	---
10X RNA Ligase Reaction Buffer	3	1X
RNase Inhibitor	1	1.33U/µL (40U)
Non-labeled RNA Control or Test RNA	5	1.67µM (50pmol)
Biotinylated Cytidine (Bis)phosphate	1	33.3µM (1nmol)
T4 RNA Ligase	2	1.33U/µL (40U)
30% PEG	15	15%
Total	30	---

5. Incubate the reactions at 16°C for 2 hours for the control RNA. Ligation might require overnight incubation to increase efficiency.
6. Add 70µL of nuclease-free water to the ligation reaction.
7. Add 100µL of chloroform:isoamyl alcohol to each reaction to extract the RNA ligase. Vortex the mixture briefly, then centrifuge 2-3 minutes at high speed in a microcentrifuge to separate the phases. Carefully remove the top (aqueous) phase and transfer to a nuclease-free tube.

8. Add 10µL of 5M NaCl, 1µL of glycogen, and 300µL of ice-cold 100% ethanol. Precipitate for ≥ 1 hour at -20°C.
9. Centrifuge for 15 minutes at ≥ 13,000 × *g* at 4°C. Carefully remove the supernatant, taking care not to disturb the pellet.
10. Wash the pellet with 300µL of ice cold 70% ethanol. Carefully remove ethanol, and air-dry pellet (~5 minutes).
11. Resuspend the pellet in 20µL of nuclease-free water or buffer of choice.

Procedure for Determining Labeling Efficiency by Dot Blotting

To detect the biotinylated RNA, the following protocol uses the Thermo Scientific Chemiluminescent Detection Module (Product No. 89880), which is available separately.

1. Hydrate/equilibrate a positively charged nylon membrane in TE Buffer for at least 10 minutes.
2. Dilute the Biotinylated IRE RNA Control 25-fold in ultrapure water to make a 5nM stock solution (e.g., 2µL of RNA + 48µL of water)
3. In microcentrifuge tubes, prepare a series of RNA standards according to the table below.

Table 2. Preparation of the RNA standards.

	Biotin (%)				
	100	75	50	25	0
Component (µL)					
Biotinylated IRE RNA Control	12	9	6	3	0
Water	48	51	54	57	60
Total Volume (µL)	60	60	60	60	60

4. Add 50µL of the Biotinylated IRE RNA Control stock solution into wells A1-A5 of a 96-well plate.
5. In a microcentrifuge tube, make a 2500-fold dilution of the test RNA labeling reaction in water to achieve a final concentration of 1nM (e.g., first add 10µL of test RNA + 240µL of water, then 1µL of the diluted test RNA + 99µL of water).
6. Place 50µL of each 1nM test RNA sample into unused "A" wells of the 96-well plate. Prepare a series of two-fold dilutions by removing 25µL from all "A" wells and mixing them with the 25µL of water in corresponding "B" wells, and continuing down the plate through the "E" wells.
7. Place the equilibrated membrane onto a clean, dry paper towel. Allow excess buffer to absorb into the membrane, but do not allowthe membrane to dry out.
8. Spot 2µL of the Biotinylated IRE RNA Control and test RNA onto a positively charged nylon membrane and allow it to absorb.
9. Immediately UV crosslink the membrane by one of the following three methods:

   Option 1: Use a commercial UV-light crosslinking instrument equipped with 254nm bulbs (45-60 second exposure using the auto crosslink function) and crosslink at 120mJ/cm^2.

   Option 2: Use a hand-held UV lamp with 254nm bulbs positioned ~0.5cm from the membrane and crosslink for 5 minutes.

   Option 3: Place the membrane face down on a transilluminator equipped with 312nm bulbs and crosslink for 10-15 minutes.
10. Continue with the detection analysis immediately or store the membrane dry at room temperature.
11. Detect the spotted RNA controls and test samples using the Chemiluminescent Detection Module. To determine labeling efficiency, compare spot intensities of the test RNA to the Biotinylated IRE RNA Controls.
12. For quantitation, identify the non-saturated spots with the same RNA concentration for the Biotinylated IRE RNA Control and test RNA for spot densitometry.

    Note: Typical labeling efficiencies of the control RNA are ≥ 75%. Labeling efficiency varies depending on the length and complexity of the RNA. Typically, RNA with low labeling efficiency (~20%) will produce adequate signal intensity in chemiluminescent EMSAs. Use RNA probes with ≥ 75% labeling in the low nanomolar range. Probes with lower labeling efficiency will require a higher concentration.

Troubleshooting

Problem	Possible Cause	Solution
Inefficient Ligation	RNA quality was poor	Gel purify RNA
	RNA had complex secondary structure	Heat at 85°C with 25% DMSO for 5 minutes and fast cool; increase incubation time and temperature; and optimize DMSO concentration
	RNA was not within the proper concentration range	Determine RNA concentration by $A_{260/280}$ and convert to pmol as follows: (µg × 10^{-6})(1 × 10^{12} pmol/mol)/ (330g/mol) (# bases) = pmol nucleic acid
	In vitro transcription reaction was inefficient or produced multiple transcripts	Before labeling, evaluate RNA by gel electrophoresis
	Improper ligation ratio was used	Determine RNA concentration by $A_{260/280}$ and increase or decrease amount of biotinylated nucleotide or RNA
	Ligation time was not sufficient	Some RNAs effectively ligate within 30 minutes; highly complex and lengthy RNAs will require longer incubation (e.g., overnight)
	Ligation temperature was not optimal	Increase incubation temperature to 37°C
Degraded RNA	A nuclease-free environment was compromised	Clean work area and make sure all plastics are from unopened packages
	mRNA from *in vitro* transcription reaction was degraded	After transcription, ensure RNA is intact by gel electrophoresis
Non-functional RNA in downstream application	RNA was improperly folded	Re-fold RNA by heating at 85-90°C for 2-5 minutes and then slowly cool to room temperature

Related Thermo Scientific Products

20158 **LightShift Chemiluminescent RNA EMSA Kit**

89880 **Chemiluminescent Detection Module**

89880A **Nucleic Acid Detection Blocking Buffer**

20159 **tRNA**

77016 **Biodyne™ B Nylon Membranes,** 0.45µm, 8cm × 12cm, 25 sheets

General References

England, T.E., *et al.* (1980). Specific labeling of 3' termini of RNA with T4 RNA ligase. *Methods Enzym* **65**:65-74.

Keith, G. (1983). Optimization of conditions for labeling the 3' OH end of tRNA using T4 RNA ligase. *Biochimie* **65**:367-70.

McKinley, B.A. and Sukhodolets, M.V. (2007). *Escherichia coli* RNA polymerase-associated SWI/SNF protein RapA: evidence for RNA-directed binding and remodeling activity. *Nucleic Acids Res* **35**:7044-60.

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