Oligonucleotide 3' End Labeling

1. Introduction

The techniques for end labeling oligonucleotides with radioisotopes have driven nucleic acid probe technology. Oligonucleotide probes can be custom made based on sequence information of the target DNA or RNA in several hours on a DNA synthesizer. Use of a DNA synthesizer eliminates the usual cumbersome and time consuming steps involved in cloning and isolation of restriction fragments to be used as probes. Oligonucleotide probes are highly specific and can be designed to detect single base changes in a gene.

Synthetic oligonucleotides prepared with free 5' and 3' hydroxyl groups will not require pretreatment with bacterial alkaline or calf intestinal phosphatise. End labelling of oligos is also much simpler than double stranded DNA molecules because there is no need to modify reaction conditions depending upon whether the fragment has 5' or 3' overhangs or blunt ends, as with restriction fragments. Oligos can be labeled at either the 3' or the 5' end. Using polynucleotide kinase and ATP (γ-^{32}P), the 5' end is labeled. Using terminal transferase and deoxynucleotide triphosphate the 3' end is labeled. ^{32}P or ^{35}S nucleotides can be used for labeling. Oligos can also be labeled on the 3' end using 5-iododeoxycytidine 5'-triphosphate (^{125}I). Use of ^{35}S and ^{125}I labeled oligos are especially useful when high resolution (as in *in situ* hybridization) or when long probe stability is needed.

2. Principle

Terminal deoxynucleotidyl transferase catalyzes the addition of deoxynucleotide 5' triphosphates to the 3' end of nucleic acids in a template-independent reaction and produces a homopolymeric tail. If nucleotide analogs which lack a 3' hydroxyl are used (NEG026), the result is that a single nucleotide is added to the 3' end of the nucleic acid. Labeling of the 3' end using 2'deoxynucleotides results in a polymeric tail ("tailing" – the addition of multiple labeled nucleotides at the end of the oligo). The extent of the 3' tail elongation can be controlled by keeping a low molar excess of nucleotide over 3' ends.

The labelling procedures described below detail the use of chain terminators (NET026) and 2' deoxynucleotides (NEG012Z, NEG034H, NEX074) for 3' end labelling of oligonucleotides. The choice of labelling technique depends on 1) specific activity of the probe needed, 2) time of autoradiography desired (^{32}P faster than ^{125}I, faster than ^{35}S), and 3) degree of resolution needed in autoradiography (^{35}S and ^{125}I give higher resolution than ^{32}P).

3. Explanation

This protocol is based on the observations by Bollum on the use of terminal deoxynuleotidyl transferase for labelling oligonucleotides. The choice of radioactive nucleotide depends on the type of isotope desired (^{32}P, ^{125}I, or ^{35}S) and whether to singly or multiply label the oligonucleotide (chain terminator or normal 2' deoxynucleotide).

Specific activity of labeled oligonucleotides:

Initial length of oligo	# labeled nucleotides added (tails)	Isotope[1]	DPM/µg labeled oligo[2]
20	1	^{32}P	1.6×10^9
30	1	^{32}P	1.1×10^9
20	1	^{35}S, ^{125}I	3.8×10^8
30	1	^{35}S, ^{125}I	2.6×10^8
20	5	^{32}P	6.7×10^9
20	5	^{35}S, ^{125}I	1.6×10^9
20	10	^{32}P	1.1×10^{10}
20	10	^{35}S, ^{125}I	2.7×10^9
20	100	^{32}P	2.8×10^{10}
20	100	^{35}S, ^{125}I	6.7×10^9
Theoretical maximum[3]		^{32}P	3.4×10^{10}
		^{35}S, ^{125}I	8.1×10^9

[1]Assumes a specific activity of ^{32}P of 5000 Ci/mmol and 1200 Ci/mmol for ^{35}S and ^{125}I.

[2]Formula used to calculate specific activity of labeled oligonucleotide:

$$\text{DPM}/\mu g = \text{specific activity (Ci/mmol)} \times \frac{\text{Tails (average number of labels)}}{\text{Tails + oligo initial length}} \times 6.72 \times 10^6$$

[3]Specific activity of 1 g of labeled nucleotide monophosphate at given specific activity in Ci/mmol

4. Materials needed

- Labeled nucleotide appropriate for assay
  - 3' dATP (α-^{32}P): NEG026
  - dATP(α-^{32}P):NEG012Z/BLU012Z/NEG512Z/BLU512Z
  - dATP (α-^{35}S): NEG034H/NEG734H
  - 5-^{125}I iodo-dCTP: NEX074
- Terminal transferase (18 U/µL)
- Reaction buffer
- Oligonucleotide
- Purified water
- Filters, vials, and scintillation cocktail
- Liquid scintillation counter, such as a Tri-Carb

5. 3' end labeling reaction

1. Preparation of ligo for end labelling: prior to the end labelling, it is recommended that the oligonucleotide by purified to remove any contaminating salts, reagents, organic solvents, or protein which might affect the reaction.
2. Add 23 µL total of oligonucleotide + reaction buffer (10 pmoles of oligo, unless using NEG012Z – 4 pmoles) on ice in a tube. Mix and briefly centrifuge components to bottom of tube.
3. Add 10 µL tracer to the reaction tube. For optimal labelling, the excess of tracer over 3' ends is critical and these conditions describe the desired excess. The reaction conditions are based on using 100 µCi of ^{32}P at 5000 Ci/mmol or 50 µCi of ^{35}S or ^{125}I at 1000 Ci/mmol per reaction. Note that NEX074 is shipped in ethanol – the material must be dried and reconstituted in water prior to use. Mix well.
4. Add 2 µL (36 units) of terminal transferase. Mix the reaction and incubate at recommended temperature for recommended length of time, according to enzyme supplier.
5. Stop reactions by adding 5 µL of 0.1 M EDTA. Keep on ice.

Reaction summary:

Method	Reagent	End labelling with chain terminator	End labelling with 2'deoxynucleotide[2]
Labeling with ^{32}P	Oligonucleotide	10 pmoles in 23 µL	4 pmoles in 23 µL
	Tracer	20 pmoles – 100 µCi (10 µL of NEG026A)	20 pmoles – 100 µCi (10 µL of NEG012Z)
	Terminal transferase	2 µL (36 units)	2 µL (36 units)
Labeling with ^{35}S or ^{125}I	Oligonucleotide	n/a	10 pmoles in 23 µL
	Tracer		20 pmoles – 100 µCi (10 µL of NEG034H or NEX074)
	Terminal transferase		2 µL (36 units)

6. Analysis of probe

1. 1 O.D. = 20 µg/mL; average molecular weight dNMP: 325
2. The extent of oligonucleotide labelling is controlled by the molar ratio of labeled nucleotide over 3'-hydroxyls of the oligo. If more extensive labelling of the oligonucleotide is desired, decrease the molar amount of oligonucleotide used in the reaction, then proceed as directed.
3. The amounts of labeled nucleotide (tracer) are based on the following specific activities: ^{32}P = 5,000 Ci/mmol, ^{35}S and ^{125}I = 1,000 Ci/mmol. If the specific activity of the tracer used differs significantly from these values, adjust the amount of radioactivity used in a reaction based on the molar excess of labeled nucleotide desired.