

End Labeling Procedures

An Overview

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1. Introduction

The purpose of this chapter is to give an overview of the different end labeling procedures. These protocols have been standardized and optimized by several biotechnology companies and are available in kits. Unless your laboratory is involved in producing large quantities of many different probes (e.g., micrograms of each probe), it is impractical, and perhaps more expensive, to set up your own protocol. However, a good understanding of the type of probe, the location of the label (3'-, 5'-end or distributed all along the molecule), and the number of labeled nucleotides incorporated should be considered when planning an experiment.

2. Label Location

2.1. 3'-End Labeling

The efficiency of labeling the 3'-end of a DNA molecule depends on the type of enzyme used, the type of DNA molecule (single- or double-stranded), and the length of the 3'-end (recessed, blunt end, or protruding).

DNA polymerase I Klenow fragment (exo⁻) fills in the 3'-recessed ends of restriction fragments in the presence of radiolabeled nucleotides (*1*). The number of labeled molecules will depend on the type of labeled nucleotide added and the sequence of the complementary strand. Blunt end fragments can also be labeled by replacing the unlabeled 3'-end nucleotide by a labeled molecule.

Klenow fragment does not label 3'-protruding ends efficiently. Labeling with Klenow fragment is an appropriate method for producing DNA size markers.

Bacteriophage T4 DNA polymerase synthesizes DNA on a 5'—3' direction. The enzyme also has exonuclease activity 3'—5' but not on the 5'—3' direction. Two steps are involved in labeling probes with bacteriophage T4 DNA polymerase (2): a replacement reaction using the 3'—5' exonuclease activity of the enzyme in the absence of dNTPs to generate 3'-protruding ends, and then filling in the ends with a mixture of unlabeled and labeled dNTPs. The resulting labeled fragment can be further digested with endonucleases and generate a mixture of probes of different sizes. Alternatively, the 3'-protruding tails of double-stranded DNA, previously digested with endonucleases, are regenerated by the bacteriophage T4 DNA polymerase in the presence of all four dNTPs (including the desired labeled dNTP). In the presence of dNTPs, T4 DNA polymerase 3'—5' exonuclease activity is inhibited and the polymerase activity predominates. However, caution should be taken to avoid long incubations, as the dNTPs could be exhausted, and the 3'—5' exonuclease activity of T4 DNA polymerase will resume and degrade double-stranded DNA as well as single-stranded DNA. Keep in mind that T4 DNA polymerase has a higher rate of 3'—5' exonuclease activity on single-stranded DNA than on double-stranded DNA. The replacement method can be difficult to control; therefore, filling in previously endonuclease digested DNA with 3'- or 5'-protruding ends in the presence of dNTPs is the best alternative when using bacteriophage T4 DNA polymerase for labeling DNA.

Terminal deoxynucleotidyl transferase (TdT) is a template independent DNA polymerase that incorporates dNTPs to the 3'-OH end of single- or double-stranded DNA, and RNA in an irreversible manner (3). This enzyme is used for the production of synthetic homo- or heteropolymers (4), for incorporating a homopolymeric tail to any type of DNA 3'-end (3,5—8) and for incorporating a single nucleotide analog such as [α -³²P]cordycepin-5'- triphosphate (Promega) (6,9) or digoxigenin-11-ddUTP (Roche Molecular Biochemicals) (10). Terminal dideoxynucleotidyl transferase labels 3'-protruding ends more efficiently than blunt ends or 3'-recessed ends. The incorporation of dA or dT residues is more favorable than incorporating dC or dG. The type of method chosen for incorporating labeled nucleotides to the 3'-end of a DNA molecule depends on the required probe sensitivity and specificity. The 3'-end tailing reaction synthesizes highly sensitive probes owing to the addition of several labeled molecules, but the specificity decreases owing to unspecific binding of the added nucleotide tail. This inconvenience can be solved by changing the stringency conditions. If probe specificity is the priority, 3'-end labeling of the DNA molecule should be performed.

2.2. 5'-End Labeling

There are three ways of labeling DNA molecules at the 5'-end: enzymatic, chemical, or combined methods. A brief description of each method is given.

2.2.1. Enzymatic Methods

The bacteriophage T4 polynucleotide kinase catalyzes two reactions: forward and exchange. In the forward reaction, the enzyme transfers the γ phosphate of [γ - ^{32}P]ATP to the 5'-hydroxy group of a DNA molecule (oligonucleotides or nucleoside 3'-monophosphates) or RNA, previously dephosphorylated with alkaline phosphatase. In the exchange reaction, T4 polynucleotide kinase transfers the 5'-terminal phosphate group of the DNA molecule to ADP. Then, the enzyme transfers the γ phosphate of [γ - ^{32}P]ATP to the 5'-hydroxy group of a DNA molecule. The forward and the exchange reactions depend on the amount of ATP available (*13,14*). The wild-type bacteriophage T4 polynucleotide kinase has 3'-phosphatase activity (*11*); however, this unwanted property has been engineered and a mutant T4 polynucleotide kinase 3'-phosphatase minus enzyme is now available (MBI Fermentas, Roche Molecular Biochemicals) (*12*). T4 polynucleotide kinase preferentially labels protruding 5'-ends over blunt ends or recessed 5'-ends, but in the presence of polyethylenglycol 8000 the reaction conditions for labeling blunt ends or recessed 5'-ends can improve (*13*). Precaution should be taken in avoiding ammonium and phosphate ions during any purification procedure, as T4 polynucleotide kinase is strongly inhibited by these ions (*13,14*).

2.2.2. Chemical Methods

This approach is suitable for synthetic oligonucleotides with a modified 5'-end. Terminal amino function is incorporated to the 5'-end after synthesizing the oligonucleotide by adding a phosphoroamidite group. After cleavage from the synthesis support and activation, a digoxigenin molecule is covalently linked (Roche Molecular Biochemicals) (*15*). Large quantities of oligonucleotide can be labeled per reaction. The 3'-end remains undisturbed and available for primer extension.

2.2.3. Combined Method

Two methods have been developed by Promega to incorporate nonradioactive labels to the 5'- or/and 3'-end of unmodified or modified oligonucleotides. The T4 polynucleotide kinase incorporates a thiophosphate from adenosine-5'-*O*-(3-thiotriphosphate) to an unmodified oligonucleotide. Then, the activated thiol group of the oligonucleotide reacts with a maleimide modified hapten (fluorescein or biotin), leaving the 3'-end of the molecule unaltered

(FluoroAmpJ T4 Kinase System, Promega). The alternative method generates alkaline phosphatase conjugates at the 5'- or 3'-end of unmodified or amino modified oligonucleotides (LIGHTSMITH[®] II System, Promega Corp.). If the starting oligonucleotide is not modified, terminal dideoxynucleotidyl transferase incorporates an amino modified ATP to the 3'-end before the activation and conjugation of the enzyme hapten.

3. Purification of Labeled Probes

Radiolabeled probes are usually not purified after synthesis; however, if the incorporation yield is low, removing the unincorporated label might help to avoid a high background noise.

There are four methods to purify labeled probes: spin column chromatography, membrane filtration, adsorption to silica gel membranes, and ethanol precipitation. Gel size exclusion properties in spin column chromatography are not the same as in flow-dependent fractionation. In spin column chromatography, *g* force applied to the column and the centrifugation time are important factors during the exclusion process. Using prepacked and equilibrated columns prevents dilution of the applied sample. Reproducibility during purification is achieved with commercially prepacked columns (e.g., Amersham Pharmacia, Roche Molecular Biochemicals). Specific resins are used for particular purification procedures such as buffer exchange and desalting, and removal of excess primers or free nucleotides.

Membrane filtration is a fast and reliable way of removing excess label, exchanging buffers, and concentrating a sample. Microcentrifuge devices are commercially available with different cut off ranges (e.g., Microcon[®], Millipore). This method is appropriate to remove primers, linkers, labeled nucleotides and desalting samples; however, modifying enzymes are usually retained together with the labeled DNA molecule. Therefore, membrane filtration is recommended mainly for chemical labeling methods.

Adsorption of DNA molecules of certain size ranges to silica-gel membrane occurs at high ionic strength and is eluted at lower ionic strength (Qiagen, Germany). No ethanol precipitation is required. Silica-gel membranes assembled on spin columns overcome the problems associated with silica-gel slurries (low yields, slurry carried over with eluted DNA, etc.). Free labeled nucleotides, modifying enzymes, reaction buffers, and other components of the labeling reaction mixtures are easily removed by this method. Recovery of oligonucleotides (17—40 bases long) and double- or single-stranded DNA fragments up to 10 kb long is feasible. This is the most efficient way to clean up any modification reaction.

Ethanol precipitation with ammonium or sodium acetate can be performed for most labeling procedures (*13,14*); however, for Digoxigenin-labeled probes,

lithium chloride (final concentration 0.4 M) should be used instead of sodium acetate. Oligonucleotides and low concentration of labeled probes are easily precipitated with carrier molecules such as glycogen (final concentration 0.4 mg/ μ L). Alkaline conjugates and fluorescein- or biotin-labeled probes require a combination of spin column chromatography and ethanol precipitation. The storage temperature for most probes is -20°C , or temporarily 4°C . Specific storing buffers are recommended for each method, and special care should be taken regarding pH conditions, light exposure, stabilizers (e.g., glycerol), and half-life of the probe. Storing the synthesized probe in small aliquots prevents degradation by repeated freeze—thaw cycles, and the possibility of accidental cross contamination with other probes.

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<http://www.springer.com/978-0-89603-885-1>

Gene Probes

Principles and Protocols

Aquino do Muro, M.; Rapley, R. (Eds.)

2002, XI, 272 p., Hardcover

ISBN: 978-0-89603-885-1

A product of Humana Press