

## Preparation of Template DNA and Labeling Techniques

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### Summary

A variety of probes can be used for *in situ* hybridization, depending on the application and the labeling strategy. In general, RNA probes (riboprobes) are now more commonly used. However, some laboratories may still prefer to use DNA probes for *in situ* hybridization, and a number of techniques for this type of labeling have been described in this chapter. The preparation of plasmid DNA and the subsequent use of polymerase chain reaction products as labeling templates are discussed in detail. Both radioactive and nonradioactive labeling procedures are described and the latest nonradioactive detection methods are outlined.

**Key Words:** Random primed labeling; PCR template; oligonucleotide probe; DIG labeling; alkaline phosphatase; chemiluminescence.

### 1. Introduction

A variety of probes can be used for *in situ* hybridization, depending on the application and the labeling strategy. In general, RNA probes (riboprobes) are now used more commonly. However, some laboratories may still prefer to use DNA probes for *in situ* hybridization, and a number of techniques for this type of labeling are given in this chapter.

The two main procedures for labeling a double-stranded DNA fragment are random primed synthesis (**1**) and nick translation (**2**). The random primed method is the most commonly used protocol because of its simplicity and highly efficient labeling process. The random primed method is extremely robust and will generate probes with very high specific activity. Nick translation is used less commonly because several critical parameters need to be carefully optimized for successful labeling.

Random primed labeling involves the denaturation of the double-stranded DNA fragment and annealing of short oligonucleotide primers of random

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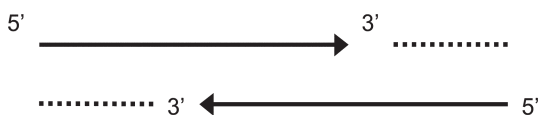


Fig. 1. Overlapping oligonucleotide probe. Two oligonucleotides of 30–35 bases (solid arrows) are designed to the gene of interest with the shown 5' to 3' orientation. The region of overlap is 10–15 bases. The dotted line indicates the fill-in reaction by Klenow fragment in the presence of [ $\alpha$ - $^{32}\text{P}$ ] dCTP, which will generate a probe of 50–55 bases (*see Subheading 3.5.*).

sequence. These random sequence oligonucleotides are usually 8–10 bases long. The oligonucleotides prime the synthesis of a new DNA strand by the Klenow fragment of DNA polymerase I. The inclusion of [ $\alpha$ - $^{32}\text{P}$ ] dCTP will result in uniformly labeled DNA on both strands.

Traditionally, the DNA fragment to be labeled has been a DNA insert from a recombinant plasmid, which is purified using agarose gel electrophoresis after restriction enzyme digestion. This multistep procedure is time-consuming, cumbersome, and routinely results in a DNA preparation containing impurities that reduce the efficiency of DNA labeling. This traditional source of template DNA should be replaced by a specific polymerase chain reaction (PCR) product generated from the recombinant plasmid.

A specific PCR product can be easily generated from a recombinant plasmid using primers to the polylinker region or gene specific primers. The PCR product can then be cleaned up using a commercial mini-column. The resulting template DNA is extremely clean and available in large amounts. This DNA is an excellent template for labeling reactions, which will result in a high specific activity probe (*see Note 1*). This type of DNA template also fully complies with Arthur Kornberg's fifth law of enzymology: "thou shalt not waste clean enzymes on dirty substrates" (3).

Another source of DNA template for labeling is two overlapping oligonucleotides (30–35 nucleotides long). These oligonucleotides can be designed to the gene of interest and they can generate a labeled double-stranded DNA probe of 50–55 bases long (*see Fig. 1*). This type of probe is particularly useful if a cloned DNA fragment is not available (*see Note 2*).

An alternative to radioactive labeling of DNA is nonradioactive labeling using digoxigenin (DIG)-labeled nucleotides. In the past, nonradioactive DNA probes have suffered from a lack of sensitivity compared with  $^{32}\text{P}$ - or  $^{33}\text{P}$ -labeled probes. However, recent advances in chemiluminescent and antibody/colorimetric detection methods have made this approach far more feasible. DIG-labeled DNA probes are safer and relatively stable, which means that a large

batch of probe can be synthesized and stored for at least 1 yr. This has advantages for reproducibility and efficiency of DNA detection. For these safety and practical issues, nonradioactive labeling of DNA should be seriously considered for each experimental application.

## 2. Materials

### 2.1. Preparation of Template DNA

1. Competent *Escherichia coli* cells (for example, DH5 $\alpha$  strain) containing the plasmid with the insert to be labeled (4).
2. TB (terrific broth) (4): 12 g of Bacto tryptone, 24 g of Bacto yeast extract, 4 mL of glycerol, water to 900 mL. Autoclave and make up to 1 L with sterile phosphate buffer (0.17 M KH<sub>2</sub>PO<sub>4</sub> and 0.72 M K<sub>2</sub>HPO<sub>4</sub>).
3. Plasmid purification kit: silica membrane kits are available from Qiagen (QIAfilter plasmid kit; Germantown, MD), Promega (Madison, WI) (PureYield™ Plasmid System), and Marligen Biosciences (Ijamsville, MD; Rapid Plasmid System). Anion-exchange kits include QIAGEN plasmid kit (Qiagen), and High Purity Plasmid System (Marligen Biosciences).
4. Taq PCR Master Mix Kit (Qiagen).
5. PCR clean-up kit; available from Promega (Wizard® PCR Prep DNA Purification System) or Qiagen (QIAquick PCR Purification Kit).

### 2.2. Radioactive Labeling of DNA

1. Random oligonucleotide primer; the original protocol used hexamers (6' mer); however, the efficiency of labeling is dramatically improved if a longer primer is used. Random oligonucleotides should be 10 bases long (10' mer). These can be ordered from any Custom Oligonucleotide manufacturer (see Note 3).
2. DNA polymerase I "Klenow fragment" (approx 5 U/ $\mu$ L), and 10X reaction buffer (provided by supplier).
3. [ $\alpha$ -<sup>32</sup>P] dCTP (3000 Ci/mmol, 10  $\mu$ Ci/ $\mu$ L).
4. dNTP stock solutions; 10 mM stocks of dATP, dGTP, and dTTP.
5. Sephadex G-25 Medium.
6. 1X TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM ethylene diamine tetra-acetic acid (EDTA).
7. Dry block heater.

### 2.3. Nonradioactive Labeling of DNA

1. The materials for nonradioactive labeling are similar to radioactive labeling, except for the substitution of [ $\alpha$ -<sup>32</sup>P] dCTP with DIG-11-dUTP. Components can be bought separately or a DIG labeling kit can be purchased from commercial suppliers (Roche Applied Science, Basel, Switzerland).
2. 10X DIG labeling mix (1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, and 0.35 mM DIG-11-dUTP).

## 2.4. Detection of DIG-Labeled DNA

1. For color detection; NBT/BCIP solution (Roche Applied Sciences).
2. For chemiluminescent detection; CDP-Star solution (available from both Applied Biosystems, Foster City, CA, and Roche Applied Science).

## 3. Methods

### 3.1. Plasmid and Template Preparation

#### 3.1.1. Preparation of Plasmid DNA

1. An *E. coli* colony containing the plasmid DNA of interest should be placed in 2 mL of TB with appropriate antibiotic selection (for example: ampicillin at 100 µg/mL) and grown at 37°C for 16 h with shaking (4).
2. 0.5 mL of the overnight bacterial culture is then placed in 50 mL of TB (with antibiotic) and grown at 37°C with vigorous shaking to achieve high density (approx 4–5 h). The remainder of the overnight culture can be stored frozen as a glycerol stock. It is important that plasmid DNA for a preparative purpose (e.g., riboprobe template) is isolated from cultures that have just reached saturation, not from an overnight incubation.
3. Plasmid DNA should be isolated using a commercially available kit from a molecular biology supplier. There are two types of kit based on the purification method; silica membrane spin column (e.g., Qiagen, Promega, Marligen Biosciences) and anion-exchange chromatography (e.g., Qiagen, Marligen Biosciences; see **Note 4**).

#### 3.1.2. Preparation of Template DNA for Random Primed Labeling

1. A PCR DNA fragment can be generated from plasmid DNA that contains the gene of interest. A 50 or 100-µL PCR should be performed according to the manufacturers protocol (Qiagen) with approx 1.0 pg of plasmid DNA. The PCR primers can be either specific to the gene of interest or designed to the polylinker region (e.g., nucleotide sequencing primers). The optimal size of the PCR fragment should be 200–500 bp.
2. The PCR product should then be purified using a PCR clean-up kit. This procedure will remove excess primers and nucleotides.
3. The PCR product is then analysed by agarose gel electrophoresis (1.5–2.0% agarose) with appropriate DNA MW markers (4). The PCR product should be the expected size, based on primer location, and free of any nonspecific DNA fragments.
4. Quantitation of the PCR fragment should be performed by measuring the absorbance at 260 nm (1 A<sub>260</sub> unit = 50 µg/mL). Alternatively the PCR product can be quantitated by fluorescence using SYBR Green and a DNA standard curve. This fragment can then be used as a template for radioactive (see **Subheading 3.4.**) or DIG labeling (see **Subheading 3.7.**).

### 3.1.3. Preparation of Template DNA for Riboprobe Synthesis

1. Plasmid DNA (5–10  $\mu\text{g}$ ) containing the gene of the interest is digested with an appropriate restriction enzyme, which cuts at the 3' end of the gene (4).
2. Digested DNA should be analyzed on a 0.8% agarose gel with suitable DNA MW markers to confirm complete digestion. This is particularly important when a large amount of DNA is digested.
3. The linearized plasmid DNA should then be cleaned up with a PCR clean-up kit (e.g., QIAquick PCR Purification Kit; Qiagen). This kit will remove enzyme and result with the DNA being in a suitable solution for further reactions (10 mM Tris-HCl, pH 8.0). The use of phenol extractions and ethanol precipitations to clean up the DNA should be avoided at all cost. These methods usually result in poor efficiency and considerable inconsistency in subsequent reactions.
4. The purified linear plasmid DNA can then be used as a template for riboprobe synthesis. A detailed protocol for riboprobe synthesis after plasmid preparation can be found in this book (see Chapter 3).

## 3.2. Radioactive Probe Labeling

### 3.2.1. Random Primed Radioactive Labeling of a DNA Fragment

1. Prepare the DNA/random primer mixture as follows: 2  $\mu\text{L}$  of DNA (e.g., PCR product, 25–50 ng), 1  $\mu\text{L}$  of random primer (50 ng), and 3  $\mu\text{L}$  of sterile water to a final volume of 6  $\mu\text{L}$ .
2. Incubate the DNA mixture at 95°C for 5 min, then chill on ice. A dry block heater is a safe and convenient method of heating the sample compared with a boiling water bath, which can result in contamination of the work area.
3. Add the following reaction mixture to the denatured DNA/primer solution: 1  $\mu\text{L}$  of nucleotide mix, (3.3 mM each of dATP, dGTP, dTTP), 3  $\mu\text{L}$  [ $\alpha$ -<sup>32</sup>P] dCTP (3000 Ci/mmol, 10  $\mu\text{Ci}/\mu\text{L}$ ), 1.5  $\mu\text{L}$  10X Klenow enzyme buffer, 3  $\mu\text{L}$  of sterile water, and 0.5–1  $\mu\text{L}$  of DNA polymerase I Klenow fragment (5 U/ $\mu\text{L}$ ).
4. Incubate the final reaction solution (15  $\mu\text{L}$ ) at 37°C for 15–30 min.
5. The labeled DNA fragment can be separated from unincorporated radioactive nucleotides using Sephadex G-25 (see **Subheading 3.6.**).

### 3.2.2. Radioactive Labeling of Double-Stranded Oligonucleotide Probes

1. Prepare the oligonucleotide mixture as follows: 1  $\mu\text{L}$  of forward 30'mer oligonucleotide (25 ng), 1  $\mu\text{L}$  of reverse 30'mer oligonucleotide (25 ng), and 4  $\mu\text{L}$  of sterile water to a final volume of 6  $\mu\text{L}$ .
2. Incubate the oligonucleotide mixture at 80°C for 5 min, then allow to slowly cool to room temperature.
3. Add the following reaction mixture to the oligonucleotide solution: 1  $\mu\text{L}$  of nucleotide mix, (3.3 mM each of dATP, dGTP, and dTTP), 3  $\mu\text{L}$  [ $\alpha$ -<sup>32</sup>P] dCTP (3000 Ci/mmol, 10  $\mu\text{Ci}/\mu\text{L}$ ), 1.5  $\mu\text{L}$  10X Klenow fragment buffer, 3  $\mu\text{L}$  of sterile water, and 0.5–1  $\mu\text{L}$  of DNA polymerase I Klenow fragment (5 U/ $\mu\text{L}$ ).

4. Incubate the final reaction solution (15  $\mu$ L) at 37°C for 15–30 min.
5. The labeled DNA fragment can be separated from unincorporated radioactive nucleotides using Sephadex G-25 (*see Subheading 3.6.*).

### 3.2.3. Removal of Unincorporated Radioactive Nucleotides

1. Add 35  $\mu$ L of 1X TE buffer to the reaction mixture and load onto a small Sephadex G-25 Medium column (70  $\times$  10 mm) equilibrated in 1X TE buffer.
2. Collect approx 0.3-mL fractions in sterile microcentrifuge tubes.
3. Identify fractions containing the labeled DNA (first peak) using a hand-held radioactive monitor.

## 3.3. Nonradioactive Labeling of DNA

### 3.3.1. Random Primed DIG Labeling of a DNA Fragment

1. Prepare the DNA/random primer mixture as follows: 2  $\mu$ L of DNA (e.g., PCR product, 25–50 ng), 1  $\mu$ L of random primer (50 ng), and 9  $\mu$ L of sterile water to final volume of 12  $\mu$ L.
2. Incubate the DNA mixture at 95°C for 5 min, then chill on ice (*see Note 5*).
3. Add the following reaction mixture to the denatured DNA/primer solution: 2  $\mu$ L 10X DIG labeling mix (*see Subheading 2.3.*), 2  $\mu$ L 10X Klenow enzyme buffer, 3  $\mu$ L of sterile water, and 1  $\mu$ L of DNA polymerase I Klenow fragment (5 U/ $\mu$ L).
4. Incubate the final reaction solution (20  $\mu$ L) at 37°C for 1 h or 20 h. Time course studies by Roche Applied Science indicate that higher yields of DIG-labeled DNA are obtained with longer incubation times (approximately fivefold increase in yield).
5. The labeled DNA fragment can then be used in hybridization techniques. It may be necessary to optimize the concentration of DIG-labeled DNA in the hybridization solution. If the concentration of probe is too high, then background problems may appear, whereas low concentrations may result in weak signals.

### 3.3.2. Detection of DIG-Labeled DNA

DIG-labeled DNA is detected by a DIG antibody that is conjugated to alkaline phosphatase. The enzyme will then catalyze a color or chemiluminescent reaction, depending on the type of substrate added. Chemiluminescent substrates are  $10^4$  to  $10^5$  more sensitive than colorimetric substrates. However, the colorimetric systems are generally more appropriate for *in situ* hybridization methods, whereas the chemiluminescent methods are more appropriate for blots.

Two chemiluminescent substrates are available: CSPD and CDP-Star (both available from Applied Biosystems and Roche Applied Science). CDP-Star produces a more intense and rapid signal compared to CSPD. DIG-labeled DNA also can be detected by DIG antibodies conjugated to different fluoro-

chromes, which can be visualized directly. This method is more suitable for *in situ* hybridization applications, however, sensitivity will be lower compared with chemiluminescence.

#### 4. Notes

1. It is recommended that the PCR fragment for DNA labeling is generated from a well-characterized source to ensure that the valid gene sequence is used as a probe. This is the reason that a characterized recombinant plasmid containing the gene sequence of interest is suggested as the template for generating the PCR product. It is possible to label a PCR fragment generated from cellular mRNA or genomic DNA, however, they should be used with caution until their identity is validated. Uncharacterized PCR products should be subcloned and subjected to partial DNA sequencing. The subcloned PCR fragment can then be used as the source for generating the labeling template.
2. Overlapping oligonucleotide probes are particularly useful if a cloned DNA fragment is not available. A labeled DNA probe of 50–55 bases can easily be produced that can be used with traditional hybridization conditions. These probes are ideal for examining alternatively spliced mRNA transcripts. The fill-in reaction will add approx 20 nucleotides to each strand of the template, thus, ensuring a high level of radionucleotide incorporation. The specific activity of the probe will be far greater compared to 5' end labeling of oligonucleotides with [ $\gamma$ - $^{32}\text{P}$ ] dATP.
3. Commercial random primed labeling kits are available from many suppliers; however, they can be expensive. The technique described here is simple and only a few components are required, therefore, it is quite easy to label DNA using individually purchased enzyme, random primers, and nucleotide stocks.
4. The silica membrane kits are rapid, convenient, and can produce high-quality DNA. This DNA is suitable for all standard protocols, for instance, PCR template, restriction enzyme digestions, DNA sequencing, and template for riboprobe synthesis. The anion-exchange kits produce excellent quality DNA for the more demanding applications, for instance, mammalian cell transfections. These kits should be considered for riboprobe templates because the DNA will be of the highest quality.
5. A dry block heater is a safe and convenient method of heating the sample, compared with a boiling water bath, which can result in contamination of the work area.

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