PCR
PROTOCOLS

A GUIDE TO METHODS AND APPLICATIONS

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that only four different oligonucleotides will be required for the distinction of two allelic sequences. Direct detection of fluorescent groups incorporated into one of the oligonucleotides will offer advantages as a means of detection. Fluorophores are stable and safe moieties that may be detected instantaneously at the conclusion of an assay. Their major drawback is the limited detection sensitivity. We have demonstrated that combined with amplification by PCR, fluorophores afford a readily detectable signal in the analysis of single-copy genes (Landegren *et al.* 1988a). Gene detection techniques will attain their greatest importance if the procedure can be fully automated from the introduction of the DNA or tissue sample to the printout of the interpretation of the outcome. We are presently adapting a robotic workstation to perform the described analysis in large numbers and with a short turn-around time. Such an instrument, along with a large panel of ready-to-use detection reagents, could form the basis for a greatly simplified analysis of large numbers of DNA samples for the presence of gene variants that cause or predispose to disease, or that are associated with particular malignancies. The same technique also lends itself to the analysis of DNA sequences that vary among different individuals and facilitates the analysis of genetic linkage or the identification of individuals.

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NONISOTOPICALLY LABELED PROBES AND PRIMERS

Corey Levenson and Chu-an Chang

The PCR produces sufficient quantities of amplified DNA to enable the use of nonisotopic labels for the detection of target sequences. Labels may be incorporated directly into the PCR product via modified nucleoside triphosphates or primers that are labeled at their 5′ termini. Alternatively, the PCR may be performed by using conventional oligonucleotide primers and triphosphates, and the product sequences can be detected by hybridization to an appropriately labeled nonradioactive probe. For primers, the most commonly employed 5′-terminal labels are biotin and a variety of fluorescent dyes (Chollet and Kawashima 1985; Smith *et al.* 1985; Smith *et al.* 1986; Adarichev *et al.* 1987; Smith *et al.* 1987; Brosalina and Grachev 1986; Ansorge *et al.* 1986; Ansorge *et al.* 1987). The most sensitive nonisotopic probes are conjugates between oligonucleotides and enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (Chu and Orgel 1988; Jablonski *et al.* 1986; Li *et al.* 1987; Urdea *et al.* 1988). The synthesis and purification of biotinylated, fluorescent-, and HRP-labeled oligomers are described below. The incorporation of labels via modified triphosphates will not be discussed in this chapter.

PCR Protocols: A Guide to Methods and Applications

Chemistry

A successful primer- or probe-labeling reaction depends upon the presence of a unique functional group at the terminus of the oligomer to be labeled. This functional group must be inherently more reactive toward labeling reagents than the other reactive groups (e.g., phosphates, exocyclic amino groups) are that are normally present in oligonucleotides. Generally, this functional group is a primary amine or thiol group that is introduced during the synthesis of the oligomer via an appropriately protected phosphoramidite. The incorporation of protected amines (Lin and Prusoff 1978; Smith *et al.* 1985; Coull *et al.* 1986; Wachter *et al.* 1986; Connolly 1987; Bischoff *et al.* 1987) and thiols (Connolly and Rider 1985) into oligonucleotides and their subsequent modification has been described by a number of groups. We have developed reagents that incorporate a tetraethylene glycol spacer between the amine (or thiol) and the 5' hydroxyl of the oligomer; commercially available labeling reagents that incorporate linkers of six to twelve atoms have been found to be adequate. Once a primary amino group has been successfully introduced, reaction with a variety of commercially available acylating reagents will yield the desired 5'-labeled oligomer. In the case of the 5'-thiolated oligomers, conjugates are produced via a reaction with an appropriately modified enzyme (i.e., maleimido labeled).

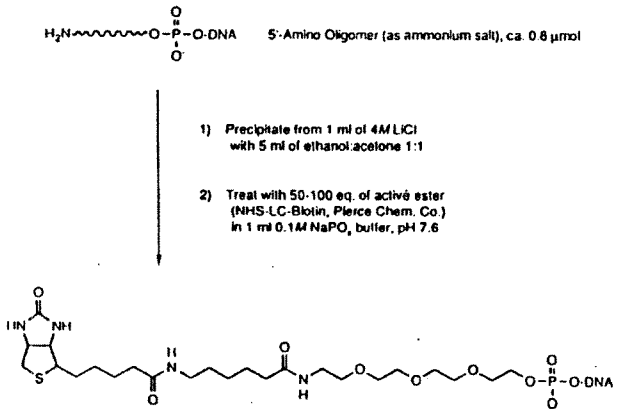

Figure 1 Scheme for the synthesis of 5'-biotinylated PCR primers.

The synthesis of a biotinylated oligomer and the structure of our tetraethylene glycol-based linker are illustrated in Fig. 1. The reagents we use for the introduction of the 5'-amino or -thiol group are not currently commercially available; however, phosphoramidite reagents suitable for the incorporation of these functional groups are available from a variety of vendors (including Clonetech, Applied Biosystems, Glen Research, and ChemGenes). The protocol provided by the manufacturers should be adhered to during the coupling of the reagent to the oligonucleotide being synthesized. Once the addition of the modified amidite is complete, the oligomer should be deprotected (again following the protocol of the reagent supplier) and the crude oligomer should be treated as described in the protocol outlined as follows.

Protocols

Synthesis of Labeled PCR Primers from 5'-Amino Oligonucleotides

Conversion of the Ammonium Salt of the DNA to the Lithium Salt

1. Add 1 ml of 4 *M* lithium chloride to the dried residual deprotected oligomer generated from a 1-µmol-scale synthesis.
2. Sonicate (or vortex) briefly and transfer the suspension to a 1.5-ml Eppendorf tube and spin for about 2 minutes in a microfuge.
3. Filter the supernatant through 0.45-µm syringe filters (small diameter) into clean, labeled 12-ml silanized glass screw-cap tubes.
4. Add 5 ml of cold absolute ethanol : acetone (1 : 1) to each tube, mix, and chill for 1 hour in the freezer.
5. Centrifuge at about 4000 × *g* for 10 to 15 minutes.
6. Decant (discard) the supernatant.

Acylation of the Lithium Salt of the 5'-Amino-Labeled DNA

1. Using water-soluble acylating reagents (such as sulfo-N-hydroxysuccinimide esters), add to the pellet a solution of 50 to 100 μmol of labeling reagent (such as LC-NHS-biotin from Pierce Chemical Co.) dissolved in 1 ml of 0.1 *M* sodium phosphate buffer (pH 7.6), agitate to dissolve the pellet, and let the solution sit overnight.
2. Using water-insoluble acylating reagents (such as isothiocyanates or N-hydroxysuccinimide esters), dissolve the pellet in 0.9 ml of 0.1 *M* sodium phosphate buffer (pH 7.6), add 50 to 100 μmol of labeling reagent dissolved in 0.1 ml of DMSO or DMF, agitate, and let the solution sit overnight.

Removal of Excess Labeling Reagent

1. Pipette the reaction mixture onto the top of a short G-25 column (disposable NAP-10 columns from Pharmacia work well for this step) that has been pre-equilibrated with 20 ml of 0.1 *M* triethylammonium acetate [TEAA (pH about 7.4)]. Allow the mixture to enter the bed of the column.
2. Apply 1.5 ml of 0.1 *M* TEAA to the top of the column and collect the eluate.

Purification of the Labeled Primers

The labeled primers may be purified by HPLC or PAGE. It is impossible to provide a detailed protocol that is suitable for all labeled primers since the nature of the label and its linkage to the oligomer will alter the mobility of the oligomer in both chromatographic and electrophoretic systems.

In general, labeled primers will migrate more slowly on PAGE than unlabeled control oligomers do (unless, of course, the label is strongly anionic). Fluorescent primers can be easily differentiated on gels (unlike biotinylated primers) and will be much more strongly retained on reversed-phase HPLC columns than will either biotinylated or unmodified primers. When the labeling reagent exists as a mixture of isomers (i.e., N-hydroxysuccinimide esters of 5- and 6-carboxy fluorescein), the labeled primers resulting from each label isomer may often be resolved.

The linkers we have employed are based on tetraethylene glycol, and the following HPLC protocol works well with biotinylated primers.

HPLC Purification of Biotinylated Primers

Column:	PRP-1 (7 by 305 mm, from The Hamilton Co.) with guard cartridge (from Alltech Associates, Inc.)
Flow Rate:	2 ml/minute
Solvent A:	0.1 *M* Triethylammonium Acetate [TEAA (pH about 7.4)], containing 5% acetonitrile
Solvent B:	Acetonitrile
Gradient:	30 minutes for equilibration 5 to 10% B over 30 minutes 10 to 50% B over 10 minutes
Detection:	UV at 260 nm

The biotinylated oligomers should elute as large peaks at about 25 minutes (after the unmodified DNA peaks; see Fig. 2). Elution time may vary with the nature of the linker between the DNA and the biotin. The same column and solvent system have been used successfully with primers labeled with fluorescein, rhodamine, and Texas Red, although acetonitrile concentrations as high as 30% may be required to elute the labeled oligomers. Therefore, gradients need to be adjusted according to the label used.

Synthesis of HRP-Labeled Probes from 5'-Mercapto Oligomers

During solid-phase DNA synthesis (Fig. 3), sulfhydryl groups are introduced at the 5' termini of oligonucleotides as trityl mercaptans (Connolly and Rider 1985). Again, we have used phosphoramidite reagents that incorporate a tetraethylene glycol linkage between the tritylthiol and the 5' hydroxyl, although commercially available reagents that incorporate linkers of six to twelve carbon atoms work as well (e.g., ThioModifier from Clonetech, Palo Alto, California). The protocols provided by the manufacturer should be followed during the coupling of the reagent to the oligonucleotide being synthesized. The final detritylation step is omitted after addition of the modified

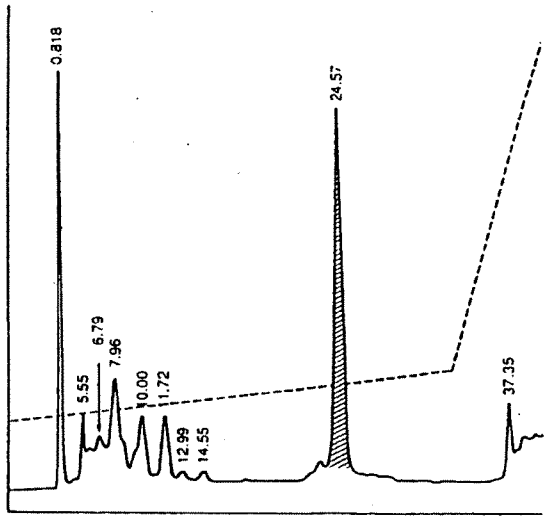

Figure 2 HPLC purification of biotinylated oligonucleotides. Vertical scale is absorbance at 260 nm. Retention times shown are in minutes. The dashed line shows the percentage of buffer B in the elution solvent. Details are given in the text.

amidite. The oligomer is deprotected with concentrated ammonia according to standard procedures and treated as described in the following procedure.

Purification of the 5'-Tritylmercapto Oligonucleotide

1. The ammoniacal solution of the crude oligomer is dried under a stream of air, and 1 ml of glass-distilled water is added to the residual crude oligomer. Vortex or sonicate the tube if necessary to suspend the residue and incubate for 5 minutes.
2. Filter the supernatant through a 0.45-μm syringe filter (Nylon Acrodisc from Gelman Sciences, Inc.). Rinse the filter with 0.8 ml of water and combine the washings with the supernatants.

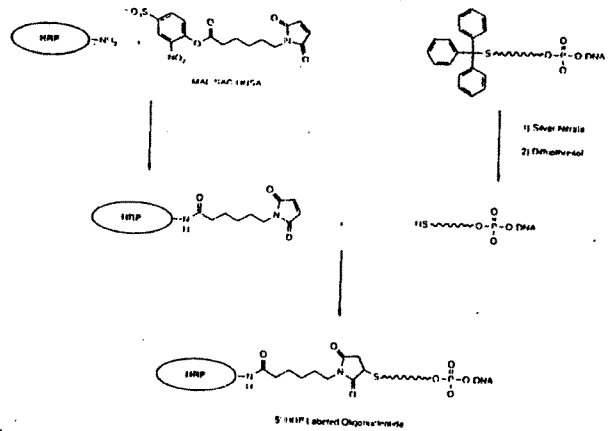

Figure 3 Scheme for the preparation of 5'-horseradish peroxidase (HRP)-labeled probes.

3. The filtrate is fractionated on an HPLC fitted with a 2-ml sample loop. The following HPLC protocol is used:

Column:	PRP-1 (7 by 305 mm, Hamilton) with guard cartridge (4.6 by 10 mm, Alltech)
Flow rate:	2 ml/minute
Solvent A:	0.1 *M* TEAA (pH about 7.4) containing 5% acetonitrile
Solvent B:	Acetonitrile
Gradient:	30 minutes for equilibration 10 to 50% B over 25 minutes 50 to 100% B over 5 minutes 100% B for 5 minutes 100 to 10% B over 5 minutes
Detection:	UV at 260 nm

The trityl-containing fraction should elute last from the column as a major peak at about 18 minutes (see Fig. 4). A good synthesis usually results in about 40 A_{260} units of tritylated oligomer.

The HPLC profiles of the oligomers are almost identical over a wide range of sizes of oligomers (13 to 59 bases). The purified oligomer is

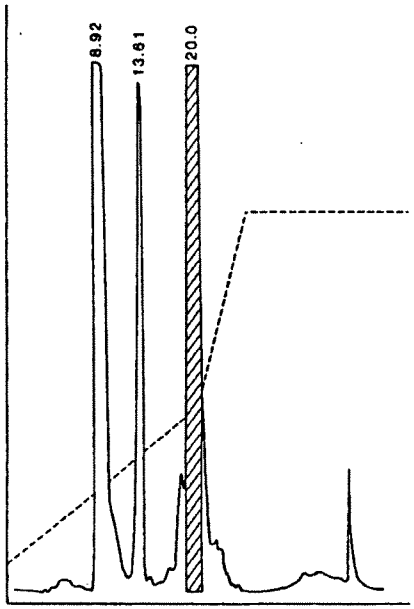

Figure 4 HPLC purification of 5-tritylmercapto oligonucleotides. Vertical scale is absorbance at 260 nm. Retention times shown are in minutes. The dashed line shows the percentage of buffer B in the elution solvent. Details are given in the text.

aliquoted out for conjugation, and the remainder is dried and stored at −20°C. The oligomer is stable for at least a year under these conditions.

Removal of the Trityl Group from the Oligonucleotide

1. Dry an aliquot containing 10 A_{260} units of the HPLC-purified tritylthiol oligomer in a Speed Vac.
2. Dissolve the oligomer in 0.1 ml of 0.1 *M* TEAA buffer (pH 6.0).

3. Add 10 μl of 0.1 *M* silver nitrate stock solution (in water, protected from light) to the oligomer. The solution should turn cloudy with the appearance of a white precipitate, indicating the release of a trityl group. After briefly vortexing the mixture, incubate it at room temperature for 1 hour.
4. Add 10 μl of freshly prepared dithiothreitol (DTT) solution [0.15 *M* in TEAA buffer (pH 6.0)] to the mixture. A thick yellowish precipitate forms immediately. The reaction mixture is incubated for another hour at room temperature with occasional vortexing.
5. Dilute the mixture to 1 ml with TEAA buffer and transfer it to the top of a short, disposable G-25 Sephadex column (NAP-10 from Pharmacia) that has been pre-equilibrated with 30 ml of 0.1 *M* sodium phosphate buffer (pH 6.0). Allow the mixture to enter the bed of the column.
6. Apply 1.5 ml of phosphate buffer to the column and collect the eluate. (The thiolated DNA is contained in this 1.5-ml eluate.)
7. The detritylated 5′-thiolated oligomer is combined immediately with the maleimido-modified HRP prepared as follows.

Preparation of Maleimido-HRP

A heterobifunctional crosslinking reagent, i.e., mal-sac-HNSA (maleimido-6-aminocaproyl ester of 1-hydroxy-2-nitrobenzene-4-sulfonic acid sodium salt), is used to introduce maleimidyl moieties to HRP through amide linkages to its lysine residues. Mal-sac-HNSA can be prepared (Aldwin and Nitecki 1987) or purchased (Bachem Bioscience Inc., Philadelphia, Pennsylvania). HRP (Type VI, Sigma Chemical Co., St. Louis, Missouri) can be used without further purification.

The maleimido-HRP prepared according to the following protocol is sufficient for conjugation to four 5′-thiolated oligomers obtained from the previous procedure. Since better results are obtained when the modified HRP is used fresh, the following protocol should be carried out concurrently with the detritylation of protected thiolated oligomers.

1. HRP (40 mg) is dissolved in 1.5 ml of 0.1 *M* sodium phosphate buffer (pH 7.5) with gentle stirring. Keep the solution in the dark. The dissolution takes about 10 minutes.
2. Mal-sac-HNSA (3 mg) is added to the dark red-brown HRP solution. Stir for 30 minutes at room temperature.
3. Transfer the mixture to the top of a disposable G-25 Sephadex column (NAP-25, Pharmacia) that has been pre-equilibrated with 30 ml of 0.1 *M* sodium phosphate buffer (pH 6.0). Allow the dark solution to enter the bed of the column.
4. Apply about 3.5 ml of the phosphate buffer (pH 6.0) to the top of the column and collect the brown HRP-containing fraction (which elutes out of the column first) in about 2 ml of buffer.
5. Add a 0.5-ml aliquot of this maleimido-HRP solution to the 5'-thiolated oligomer obtained above in 1.5 ml of buffer. Mix this solution briefly and concentrate the volume to about 1 ml in a Speed Vac.
6. The reaction mixture is kept in a refrigerator (4°C) for 24 hours before purification.

Purification of the HRP-Oligomer Conjugate

Weak anion-exchange (DEAE) column chromatography is used to purify the conjugate because of (1) its high capacity: the conjugate prepared as described can be purified in one run; (2) mild running conditions: since a salt (sodium chloride) gradient is used to elute the conjugate from the column, no post-column manipulation is required for the use and storage of the conjugate; and (3) high resolution: the desired conjugate can be easily separated from the excess uncoupled HRP, which elutes at the void volume, or the unreacted oligomer, which elutes after the conjugate peak (see Fig. 5).

HPLC Purification Protocol

Column:	Nucleogen DEAE 500–10 (6 by 125 mm, Macherey Nagel), with guard cartridge (4.6 by 10 mm, Alltech)
Flow rate:	1.5 ml/minute
Buffer A:	20 m*M* sodium phosphate (pH 6.0)
Buffer B:	Buffer A plus 1 *M* sodium chloride

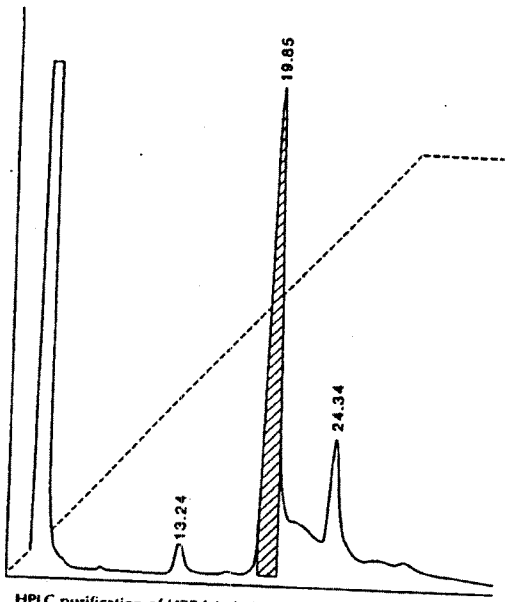

Figure 5 HPLC purification of HRP-labeled DNA probe. Vertical scale is absorbance at 260 nm. Retention times shown are in minutes. The dashed line shows the percentage of buffer B in the elution solvent. Details are given in the text.

Gradient:	15 minutes for equilibration with Buffer A 0 to 100% B over 30 minutes 100% B for 10 minutes 100 to 0% B over 5 minutes
Detection:	UV at 260 nm

Because of the anion-exchange nature of this purification method, the retention time of the conjugate is proportional to its negative charge. The uncoupled HRP is not retained by the column and elutes almost at the void volume of the column (at about 2.2 minutes). The conjugate elutes after the HRP according to the size of the oligomer (for instance, the observed retention times for several 15-mers ranged from 19 to 21 minutes). The unconjugated oligomers

and their dimers (generated through disulfide formation) elute from the column after elution of the desired conjugate. The separation between the conjugate and the uncoupled oligomer on the column is usually 2 to 3 minutes. The dimers are eluted even later (Fig. 5).

The purified conjugates are stored at 4°C in the HPLC elution buffer without further manipulation. Under these conditions, the conjugates are stable for at least 12 months. The biological activity of the HRP is not adversely affected by either the conjugation to the oligomer or storage.

Stoichiometry and Quantitation of the Purified HRP–Oligomer Conjugate

The ratio of HRP to oligonucleotide can be determined from the ratio of UV absorbance at 402 and 260 nm. The major product formed in the conjugation reaction described above is a 1 : 1 adduct of enzyme to nucleic acid.

The concentration of the conjugate in the elution buffer is determined spectrophotometrically using the absorbance of the HRP at 402 nm. A molar absorption coefficient of 100,000 is used for this determination (Shannon *et al.* 1966).

Summary

Synthetic oligonucleotide probes and primers may be prepared to which are attached a variety of nonisotopic "reporter groups." Labeled primers (i.e., where the label is biotin or fluorescein) have been used successfully in otherwise conventional PCRs to yield PCR products that incorporate these labels, eliminating the need for radioactive materials. In the case of primers, the nature of the PCR precludes the attachment of labels to the 3' termini. Although labels attached to the bases would probably be tolerated, provided they do not interfere with hybridization of the primer to its template, we have used 5'-end-labeled probes and primers extensively, and it is their preparation that has been described here.

PCRs conducted with conventional (nonlabeled) primers yield products that can be probed using enzyme-labeled oligonucleotide probes. Using commercially available phosphoramidite reagents, one can produce oligomers containing functional groups (i.e., thiols or primary amines) at either terminus and can label them using the protocols described in this chapter. Once a primary amine (or thiol) has been introduced, one can select from a vast array of commercially available labeling reagents (most of which were originally designed to be used for protein modification). By using such reagents, biotin, fluorescent dyes, photoreactive groups, spin labels and other moieties may be attached to synthetic oligonucleotides. Indeed, primary amines that have been introduced can be converted to thiols by using such reagents (Bischoff *et al.* 1987). Control reactions using unmodified oligomers should always be conducted to ensure that side reactions are not occurring between the particular labeling reagent employed and the exocyclic amines on dA, dC, and dG.

Acknowledgments

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