Synthesis and Characterization of 5′-Fluorescent-dye-labeled Oligonucleotides

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Fluorescent-dye-labeled oligonucleotides are used in many procedures, including DNA sequencing, PCR, restriction mapping, the study of genetic disease, and forensics identification. In this paper, we describe detailed methods for the synthesis, purification, and quantification of 5′-fluorescent-dye-labeled oligonucleotides. The relationship of specific and nonspecific dye attachment to synthetic oligonucleotides is discussed, and the importance of primer design is considered as a way to prevent chemical problems from interfering with biological reactions.

New chemistries and techniques developed over the last decade have greatly simplified the synthesis of oligonucleotides and their conjugates, and the current procedures are more economical, less tedious, and more reliable than the protocols of a few years ago. Techniques for labeling oligonucleotides with such moieties as fluorescent dyes, biotin, and digoxigenin have been developed. This progress in developing easier synthesis and effective labeling methods has also contributed to a much-expanded role for synthetic oligonucleotides in scientific research.

This report outlines faster and more refined methods for synthesis, labeling, purification, and quantitation of 5′-fluorescent dye oligonucleotides using a 6-carboxy 4′,5′-dichloro-2′,7′-dimethoxy fluorescein label (JOE) as an example. We describe the synthesis of the oligonucleotide with conventional cyanoethyl phosphoramidite chemistry at a 1 μmole scale. We also describe reaction conditions for dye attachment to the synthesized oligonucleotide, emphasizing the optimal proportions of required reagents. HPLC isolation methods and conditions for purification of dye-labeled oligonucleotides are also described (Fig. 1). In addition, the methods for quantitation of the fluorescent-dye oligonucleotide are reviewed with emphasis on conversion of OD units to picomoles, and information is provided regarding primer design and oligonucleotide concentration versus dye concentration.

MATERIALS AND METHODS

Synthesis

For demonstration, a 6-carboxy 4′,5′-dichloro-2′,7′-dimethoxy fluorescein-labeled (JOE is one of the fluorescein dye lambda max 526 nm used in four-color sequencing reactions) 18-residue (the universal priming site sequence for −21M13 DNA) deoxyoligonucleotide is synthesized using conventional cyanoethyl phosphoramidite chemistry on a commercial DNA synthesizer at a 1.0 μmole scale with Aminolink 2 (ABI). Aminolink 2 reagent is used to place an aliphatic-linked primary amine at the 5′ terminus of the oligonucleotide during synthesis. Oligonucleotides are automatically cleaved from the solid support with concentrated ammonia and collected in a vial on the synthesizer. The cleaved oligonucleotide in the collection vial is diluted to 3 ml with concentrated ammonia. Deprotection is complete after incubation for 8–16 hr at 55°C.

Dye Attachment

An aliquot (200 μl) of the 5′-aminolinked oligonucleotide in a 1.5-ml microfuge tube is evaporated in vacuo until a dry pellet is visible (~2 hr). The resulting residue is dissolved in 100 μl of 1 M NaCl and then precipitated with 400 μl of ethanol. The mixture is vortexed, cooled to −20°C for 5 min, centrifuged for 2 min, and the supernatant discarded. The precipitate is dissolved in 42 μl of 0.25 M Na_2CO_3/$NaHCO_3$ buffer (pH 9.0) (adjust 0.25 M sodium bicarbonate to pH 9.0 with 0.25 M sodium carbonate). A 4-μl aliquot of one of four commercially available fluorescent-dye *N*-hydroxysuccinimide ester solutions in DMSO (FAM, P/N 400985; JOE, P/N 400986; ROX, P/N 400980; and TAMRA, P/N 400981) is added. The mixture is vortexed and allowed to react, in the absence of light, for 1 hr at 37°C.

Purification

The crude dye reaction mixture contains

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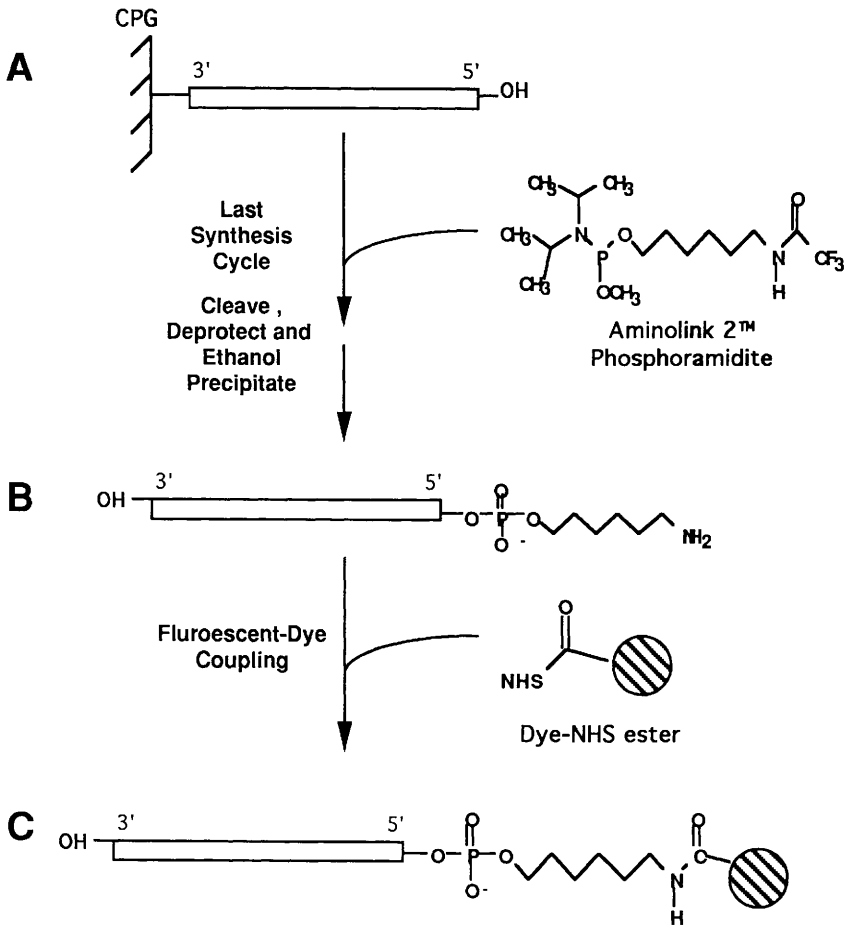

FIGURE 1 Three main procedures are involved in the synthesis and purification of fluorescent-dye-labeled oligonucleotides. An amino-linker residue is appended to the 5′ termini of a synthetic oligonucleotide during the penultimate coupling cycle of automated DNA synthesis (*A*). The oligonucleotide is automatically cleaved from its support with concentrated ammonium hydroxide. Exocyclic amine protecting groups are removed by incubation at 55°C for 16 hr. The deprotected oligonucleotide is ethanol-precipitated to remove any salts and to exchange the counteraction on the phosphate backbone with natural sodium ions. The amine-linked oligonucleotide is reacted at pH 9.0 with an *n*-hydroxysuccinimide ester of one of four fluorescent dyes (*B*). The dye-labeled product is then purified using G-25 Medium Sephadex size-exclusion chromatography resin, followed by a final reverse-phase HPLC purification (*C*).

dye-labeled product, unreacted amino-linked oligonucleotide, excess dye species, and salts (Fig. 2A). The mixture is first purified by size-exclusion chromatography using commercially available PD-10 Columns (Pharmacia, no. 17-0851-01). Columns are equilibrated with 12 ml of 0.1 M triethylammonium acetate (TEAA) (pH 7.0). The dye reaction mixture is loaded and chromatographed to the column's bottom with 2.4 ml of 0.1 M TEAA. A colored product band is eluted and collected with 1 ml of 0.1 M TEAA. [One can also fabricate a size-exclusion chromatography column from Sephadex G-25 Medium (Pharmacia, no. 17-003-02) that is preswollen in deionized water and packed into 10-ml graduated glass pipettes.]

The Sephadex-purified dye-labeled oligonucleotide is further purified by reverse-phase HPLC (Fig. 2B). To prepare the Sephadex-purified material of preparative HPLC, the sample is either evaporated in vacuo or injected without concentration onto a Model 152 HPLC Chromatograph System (ABI). The first set of peaks represents failure sequences and unreacted starting material. The desired product is the major peak eluting later in the chromatography and is collected during the purification procedure. For further characterization, an HPLC analysis is performed of the purified product (Fig. 2C).

Quantification

The purified product is dried in vacuo, resuspended in 300 μl of 1 M NaCl, and ethanol-precipitated as above. The product is dissolved in deionized water and quantitated spectrophotometrically (Fig. 3). Typically the molar concentration is determined according to the following formula: The number of moles of oligonucleotide in one OD unit = 33 μg of DNA/molecular weight of the oligonucleotide. One OD unit = 33 μg of DNA. The molecular weight of the oligonucleotide equals 330 grams × the number of bases in the oligonucleotide + 700 grams for the dye attached. An example for an 18-mer is 1 OD = 33 μg/5940 + 700 = 4970 pmoles.[1] The example of the 18-mer synthesized dye-labeled oligonucleotide has one optical density unit equal to about 5000 pmoles. A more precise molar concentration determination can be obtained with the use of other formulas.[2]

RESULTS

The synthesis of a 5′ amino-linked oligonucleotide is described using standard cyanoethyl phosphoramidite chemistry with a 1 μmole synthesis column. The 1 μmole cycle will typically yield 100 OD units of crude DNA.

A 6 OD aliquot from the 1 μmole synthesis is typically used for dye labeling. A larger amount of oligonucleotide produces proportionally greater quantities of product up to 100 OD units. The dye reaction steps are quantitated to determine overall yield of dye-labeled oligonucleotide from amino-linked oligonucleotide (Table 1). Typically, a 30% overall yield can be expected from a dye-labeled oligonucleotide compared to the starting amino-linked oligonucleotide. The shortened reaction time at the elevated temperature is quite successful in driving the reaction to completion. We find that the greatest loss of product occurs in the HPLC purification, and that the losses are due primarily to the technique for collecting fractions of the eluting peak. The quantitation of the exam-

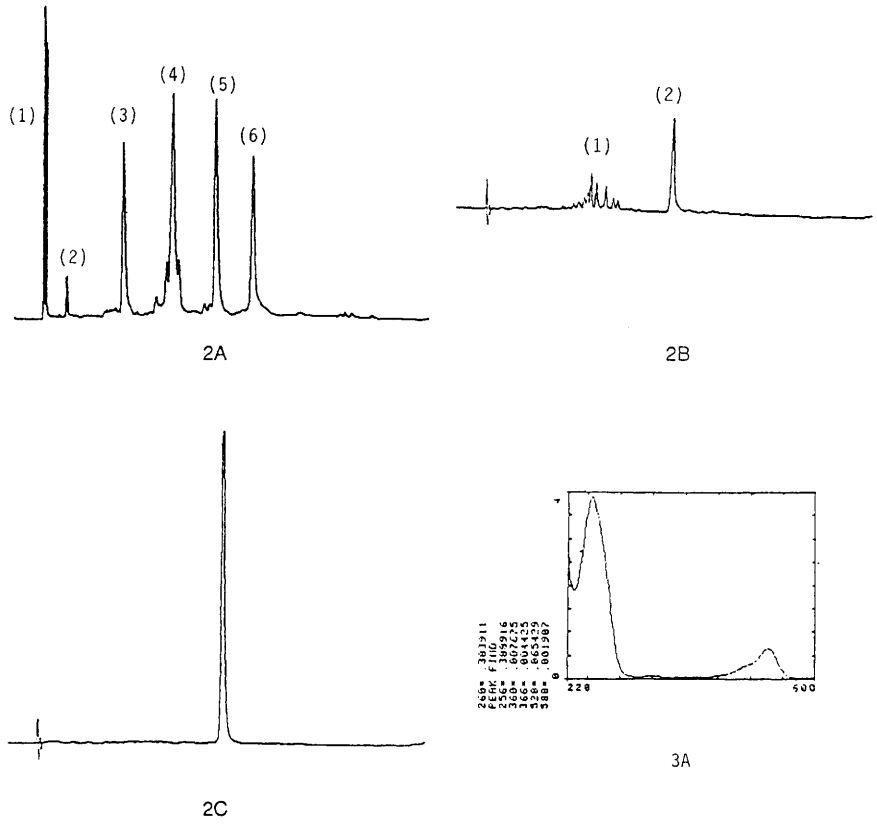

FIGURE 2 HPLC chromatograms (HPLC system and conditions) show crude synthetic oligonucleotide, crude dye-labeled oligonucleotide, Sephadex-purified dye-labeled oligonucleotide, and HPLC-purified final product. (*A*) Chromatograph of the dye–oligonucleotide reaction mix: (peak 5) the dye-labeled product; (peak 4) unreacted amino-linked oligonucleotide; (peaks 3 and 6) spent dye species; (peak 1) *N*-hydroxysuccinimide; and (peak 2) benzamide. (*B*) Chromatograph of the post-Sephadex purification. (Peaks 1) Unreacted amino-linked oligonucleotide and assorted failure sequences; (peak 2) the desired dye-labeled product. HPLC conditions are 1.0 ml/min, Aquapore C8 reverse-phase column; buffer A, 0.1 M triethylammonium acetate; buffer B, 100% acetonitrile. The gradient conditions are 8% B to 20% B in 24 min and then to 40% B in 10 min for a total of 34 min. (*C*) An analytical chromatograph of the purified dye-labeled oligo. The HPLC conditions are the same as those described for the Fig. 2B legend.

ple 18-mer dye-labeled oligonucleotide shows that the overall yield is 30%.

An important concern in calculating absorbance is the use of a proper dilution protocol, and here the use of precise and accurate protocols cannot be overemphasized.

DISCUSSION

It is clear that dye labeling of synthetic oligonucleotides is an important advance in analytical biotechniques. The procedures described here produce fluorescent-dye-labeled synthetic oligonucleotides in a shorter time and in high yield and purity. Coupling efficiencies, however, vary depending upon which fluorescent dye is used. FAM, JOE, TAMRA, and ROX have approximate coupling efficiencies of 80, 60, 70, and 70% respectively.

The contribution to absorbance at 260 nm is due mostly to the oligonucleotide, and the absorbance at 526 nm is due exclusively to dye species. Spectral analysis is a complement to the analytical HPLC. Thus, the HPLC will exhibit the purity of the dye-labeled oligonucleotide (one peak), whereas the spectral analysis will qualitatively exhibit the 260-nm/526-nm absorbance ratio.

The HPLC profile in Figure 2B shows the failure sequences, unreacted amino-linked oligonucleotide, and the dye-labeled oligonucleotide. If excess dye species were also present, other peaks could elute near the desired product peak. If these peaks were collected, the HPLC may still show one peak but the spectral analysis would show very little absorbance at 260 nm and a large absorbance at 528 nm. An ethanol precipitation of the product would probably yield no dye-labeled oligonucleotides. In the other extreme, if there were poor dye coupling and very little product is present, the collected product may have other failure sequences present, especially if there is poor resolution in the HPLC. This could lead to a broad, poorly resolved set of peaks in the chromatograph, measurable absorbance at 260 nm, and no absorbance at 528 nm.

Additionally, the FAM and JOE dye stability is sensitive to low pH. If the samples are at or near pH 4, the dyes do not have an absorbance. It is best to maintain a neutral pH environment during analysis.

These two results (low and high dye absorbance) are extreme and require reexamination of the synthesis protocols to discover the problem. A less obvious problem, which can be serious, is seen with the dye TAMRA and to a lesser extent with ROX. Both of these free dyes tend to complex with the labeled oligonucleotide. These species, with varying amounts of nonspecifically attached dye, will not resolve by HPLC under the stated conditions. The desired product peak will be collected and show one peak on the analytical HPLC but will show a higher amount of absorbance at 588 nm (TAMRA absorbance). This greater absorbance can be a low percent-

TABLE 1 Recovery of Dye-labeled Oligonucleotide Through Multiple Synthesis and Characterization Steps

Purification Step	Starting material (ODs)	Remaining material (ODs)	Step recovery (%)	Overall yield (%)
Automated synthesis	—	6.0	—	—
Dye-coupling	6.0	5.4	90	90
Exclusion chromatography	5.4	3.8	70	63
HPLC	3.8	2.0	52	33

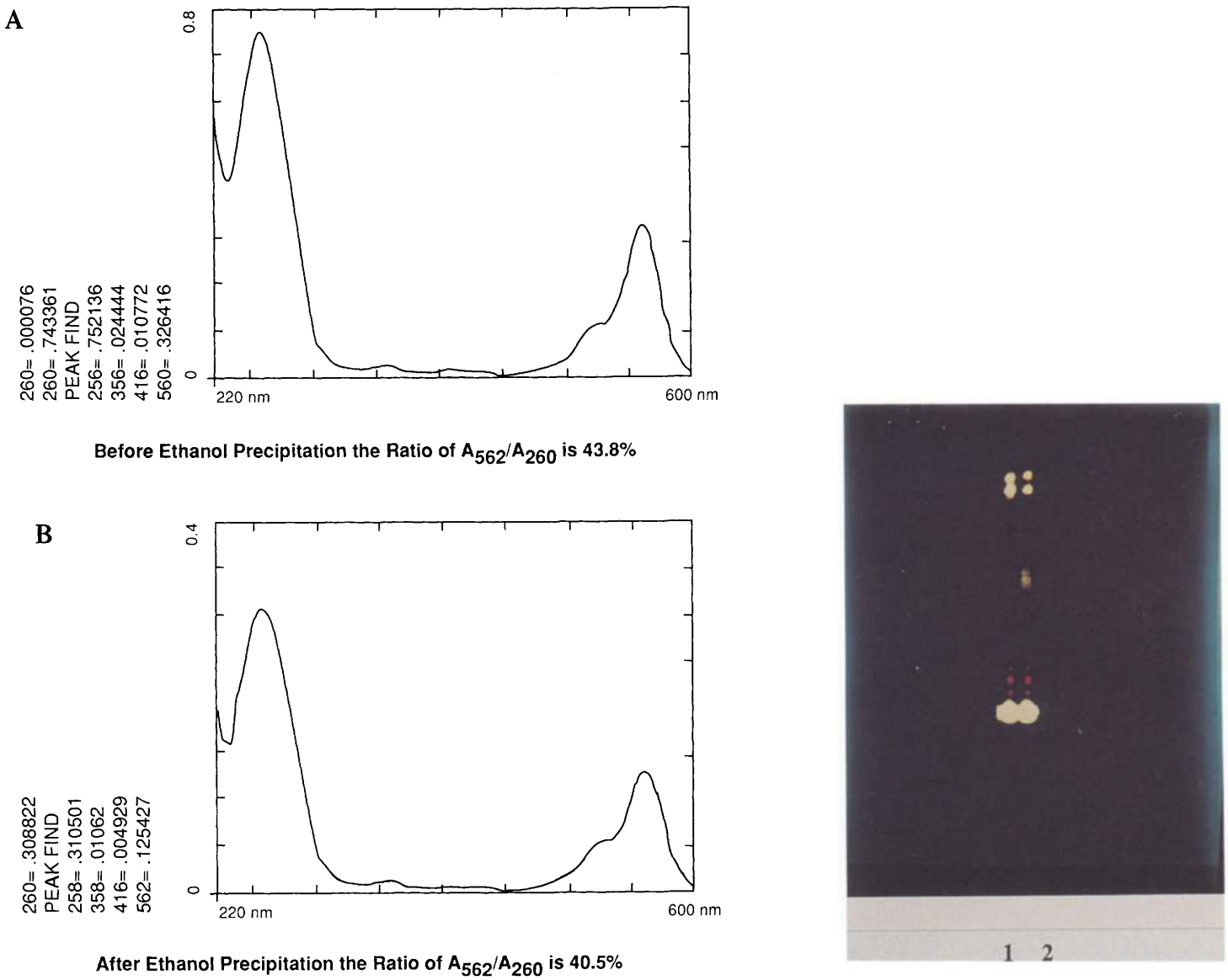

FIGURE 3 (*A*) Purified dye-labeled oligonucleotide is quantitated using UV spectroscopy (HP8451A Photodioarray Spectrophotometer, Hewlett-Packard, Palo Alto, CA) over the range of 220–600 nm. The oligonucleotide moiety contributes to most of the absorbance at 260 nm, whereas the absorbance at 528 nm is specifically due to the dye label. This spectrum is also used to quantitate the recovered dye-labeled product. The optical density unit is determined by the absorbance reading at $A_{260\ nm}$. Here, 1 OD unit equals 5000 picomoles. (*B*) The spectra of the TAMRA-labeled oligonucleotide shows the amount of dye present before and after ethanol precipitation. As described in the text, the ethanol precipitation removes any nonspecific free dye species. Before ethanol precipitation, the ratio of A_{562}/A_{260} is 43.8%. After the ethanol precipitation, the ratio decreases to 40.5%. This 3.3% is the amount of free dye that complexed with the DNA. (*C*) PCR product showing the free dye smear present in lane *2*. (Lane *1*) Removal of the free dye by ethanol precipitation.

age (as low as 3.3%) of the dye-linked oligonucleotide. The presence of the complexed species is seen in a gel where the dye will dissociate from the oligonucleotide due to salts in the loading and running buffers. This causes a large colored smear to migrate randomly in the gel. The ethanol precipitation procedure will remove any complexed free dye species. Figure 3A shows the spectra before and after ethanol precipitation. There is only 3.3% excess dye present, yet this level could cause serious problems with automated gel scanning (Fig. 3B).

The quantification of dye-labeled oligonucleotides involves standard techniques. Consistency of method, rather than strict adherence to a single prescribed set of parameters, is the most important consideration. Optical density (OD_{260} units) measured on a UV spectrophotometer is used to establish concentration. However, the methods of dilution and other parameters are sometimes varied. Individuals should rely on their own techniques for establishing the specific parameters for oligonucleotide quantification. Table 1 offers a quick reference for conversion of OD units to picomole quantities for various length dye-labeled oligonucleotides.

Dye-labeled oligonucleotides have found many uses, including DNA se-

quencing, PCR, restriction/ligation, VNTR, microsatellites, forensics, and diagnostics to name a few. Carrano et al. have demonstrated the use of a restriction/ligation labeling technique for cosmid clone fingerprinting and ordering that uses fluorescent-dye-labeled oligonucleotides.[3] In a screening assay for carriers of muscular dystrophy, deletions with the dystrophin coding region have been detected using multiplexed PCR with fluorescent-tagged oligonucleotide primers.[4] Fluorescent gel scanning has also been demonstrated to be very useful to human identification, e.g., war casualties and maternity/paternity cases. The D1S80 locus (a VNTR) was examined in approximately 100 samples and the results were compared precisely with published results obtained by electrophoresis on polyacrylamide gels and detected by silver staining.[5] The level of expression of the T-cell receptor (TCR) gene has been measured using fluorescent gel scanning detection of PCR-amplified TCR mRNA transcripts.[6] Polymorphic single tandem repeats (STRs) have been studied for the purpose of linkage analysis using fluorescent gel scanning.[7]

For effective use of any oligonucleotide in PCR, including the dye-labeled molecules described here, proper primer design is important. More than three consecutive G residues should be avoided, because these can cause unwanted secondary structures and hinder the expected annealing. In general, any self-complementary sequence should also be avoided. The length of a primer can be of critical importance; with the use of shorter-length primers, the number of statistical binding sites is greater, whereas with the use of longer-length primers, the T_m may be too high for proper annealing. In our experience, primers of 20–30 nucleotides work best in applications such as PCR and sequencing.

There are new chemistries appearing that will further ease the techniques for labeling oligonucleotides. Currently, several biotin and fluorescent dye phosphoramidites are commercially available, and these compounds show promise in the case of labeling synthetic DNA. The procedure of using dye-*N*-hydroxysuccinimide (NHS) ester solutions for dye attachment to synthetic DNA is still the most economical method. Also, there are numerous compounds in NHS ester form that can be used to label synthetic DNA.

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