1026–1031 *Nucleic Acids Research, 1998, Vol. 26, No. 4*

Efficient synthesis of double dye-labeled oligodeoxyribonucleotide probes and their application in a real time PCR assay

Bashar Mullah*, Ken Livak, Alex Andrus and Paul Kenney

PE Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404, USA

Received October 7, 1997; Revised and Accepted December 16, 1997

ABSTRACT

A fast cleaving non-nucleosidic tetramethylrhodamine dye-labeled support has been developed for automated synthesis of double dye-labeled oligodeoxyribonucleotides in high yield. A mixture (1:1:2) of t-butylamine:methanol:water is used for cleavage and deprotection of dye-labeled oligodeoxyribonucleotides without any degradation or modification of dyes and nucleobases. The cleavage rate of oligodeoxyribonucleotides is significantly increased by using a diglycolate ester linkage instead of the commonly used succinate linkage. These double dye-labeled probes are used in PCR for real time detection of a specific PCR product. Using a 5′-exonuclease assay, detected on the ABI PRISM 7700 Sequence Detection System, there was no distinguishable difference in performance of probes synthesized using the dye-labeled support compared with traditional post-synthetic attachment of rhodamine.

INTRODUCTION

Real time detection and quantitation of specific PCR products are accomplished using double dye-labeled fluorogenic oligodeoxyribonucleotide probes (1–3) during PCR. The fluorogenic probe has a fluorescent reporter dye at the 5′-end and a quencher dye at the 3′-end or at an internal site. When the intact probe is excited by irradiation the reporter fluorescence is greatly reduced by quenching through the process of fluorescence resonance energy transfer (4,5). During PCR Taq DNA polymerase cleaves the probe if the probe hybridizes with the target sequence by virtue of its 5′-exonuclease activity. This cleavage of the probe separates the reporter dye from quencher dye, increasing the reporter dye fluorescence signal. Probe design and synthesis has been simplified by the discovery of quenching probes with fluorescein reporters at the 5′-end and a tetramethylrhodamine (TMR) quencher at the 3′-end (2). The 5′-fluorescein dyes are incorporated by coupling respective fluorescein dye phosphoramidites at the 5′-end during probe synthesis. For incorporation of the 3′-terminus dye an isothiocyanate or *N*-hydroxysuccinimide ester derivative of the dye was reacted with deprotected oligodeoxyribonucleotide having a primary amine functionality on a linker arm at pyrimidine C-5. The post-synthesis coupling of dye with oligodeoxyribonucleotide requires a large excess of reactive dye derivatives and is not very efficient. Labor-intensive purification is necessary for separation of double dye-labeled oligodeoxyribonucleotides from single-labeled impurities. We have reported that dye-labeled supports could be used for automated incorporation of dyes at the 3′-end of oligodeoxyribonucleotides (6,7). Here we describe in detail the derivatization of non-nucleosidic TMR-labeled solid supports for automated synthesis of double dye-labeled oligodeoxyribonucleotide probes and application of these probes in a real time PCR detection assay.

MATERIALS AND METHODS

Fluorescein dye phosphoramidites, phosphoramidites, phosphalink, other ancillary reagents, tetramethylrhodamine *N*-hydroxysuccinimide ester and aminopropyl CPG were from PE Applied Biosystems (Foster City, CA). Other chemicals were purchased from Aldrich Chemical Company and used as received. Oligodeoxyribonucleotide syntheses were performed on an ABI 394 DNA/RNA synthesizer according to the operator's manual. HPLC analyses were conducted with a Perkin-Elmer series 200 LC pump equipped with an ABI 783A programmable detector, Perkin-Elmer ISS200 autosampler and PE Nelson 900 series data system. An RP-18 reverse phase column (220 × 4.6 mm) from Perkin-Elmer Corporation and a Nucleopac-100 anion exchange column (250 × 4 mm) from Dionex Corporation were used. The purity of compounds **2b**, **5**, **6**, **7a** and **7b** was checked by reverse phase HPLC with solvent A [3% acetonitrile in 0.1 M triethylammonium acetate (TEAA)] and solvent B (90% acetonitrile in water); gradient, 30–80% B over 25 min, 80% B for 10 min, flow rate 1 ml/min. Real time PCR detection was carried out on the ABI PRISM[a] 7700 Sequence Detection System. Snake venom phosphodiesterase (SVP, *Crotalus adamanteus* venom) and alkaline phosphatase (*Escherichia coli*) were purchased from Pharmacia. SVP was obtained as a powder, which was dissolved in water (1 mg/ml).

2-*N*-(*N*-Fmoc-6-aminohexanoyl)-2-amino-1,3-propanediol (5)

Serinol (773 mg, 8.50 mmol), 1-hydroxybenzotriazol (574 mg, 4.25 mmol), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (1.61 g, 4.25 mmol) and *N*,*N*-

*To whom correspondence should be addressed. Tel: +1 650 638 5745; Fax: +1 650 572 2743; Email: mullahbk@perkin-elmer.com

diisopropylethylamine (1.68 g, 13 mmol) were added to a stirred solution of 6-*N*-Fmoc-ε-aminocaproic acid (1.5 g, 4.25 mmol) in DMF (20 ml). The reaction mixture was stirred at room temperature under an argon atmosphere for 2 h. DMF was removed under reduced pressure. The residue was dissolved in $CHCl_3$ (100 ml) and washed with 5% aqueous HCl (1 × 50 ml), H_2O (1 × 50 ml) and saturated brine (1 × 50 ml). The organic layer was dried ($MgSO_4$) and evaporated to give an oil which was dissolved in EtOH (10 ml) and kept in a refrigerator. Compound **5** crystallized as colorless fine needles (1.2 g, 66%; 98.7% pure by HPLC). ^{1}H NMR ($CDCl_3$) δ: 1.35 (m, 2H), 1.45 (m, 2H), 1.66 (m, 2H), 2.23 (t, *J* = 7.2 Hz, 2H), 3.18 (m, 2H), 3.74 (dd, *J* = 11.1, 4.2 Hz, 2H), 3.82 (dd, *J* = 11.1, 3.9 Hz, 2H), 3.95 (m, 1H), 4.20 (t, *J* = 6.6 Hz, 1H), 4.39 (d, *J* = 6.6 Hz, 2H), 5.00 (bs, 1H), 6.40 (d, *J* = 7.4 Hz, 1H), 7.28–7.43 (m, 4H), 7.58 (d, *J* = 7.2 Hz, 2H), 7.76 (d, *J* = 7.2 Hz, 2H). High resolution mass: $M+Cs^+$, calculated 559.1209, found 559.1193.

1-*O*-DMT-2-*N*-(*N*-Fmoc-6-aminohexanoyl)-2-amino-1,3-propanediol (6)

A solution of dimethoxytrityl chloride (1.16 g, 3.43 mmol) in dry pyridine (20 ml) was dropwise added to a stirred solution of compound **5** (1.33 g, 3.12 mmol) in pyridine (20 ml) at room temperature under an argon atmosphere. The addition was complete in 30 min. The flask was stoppered and stirred at room temperature for 46 h. Pyridine was removed under reduced pressure, the residue dissolved in $CHCl_3$ (100 ml) and washed with H_2O (1 × 100 ml) and saturated brine (1 × 100 ml). The organic layer was dried ($MgSO_4$) and evaporated to give a yellowish oil. The product was isolated by column chromatography on silica gel eluting with 1–5% MeOH in $CHCl_3$. Appropriate fractions were combined and evaporated to a colorless foam (1.31 g, 57%; 97% pure by HPLC). ^{1}H NMR ($CDCl_3$) δ: 1.26 (m, 2H), 1.45 (m, 2H), 1.62 (m, 2H), 2.16 (t, *J* = 7.2 Hz, 2H), 2.85 (bs, 1H), 3.16 (m, 2H), 3.28 (dd, *J* = 9.9, 4.8 Hz, 1H), 3.33 (dd, *J* = 9.9 Hz, 4.5 Hz, 1H), 3.68 (m, 1H), 3.74 (s, 6H), 3.81 (m, 1H), 4.09 (m, 1H), 4.19 (t, *J* = 6.6 Hz, 1H), 4.39 (d, *J* = 6.6 Hz, 2H), 4.91 (bs, 1H), 5.95 (d, *J* = 7.8 Hz, 1H), 6.82 (d, *J* = 8.7 Hz, 4H), 7.20–7.42 (m, 13H), 7.58 (d, *J* = 7.2 Hz, 2H), 7.75 (d, *J* = 7.2 Hz, 2H).

Succinate esters 2a and 7a

Succinic anhydride (0.96 mmol), Et_3N (0.74 mmol) and 4-dimethylaminopyridine (0.37 mmol) were added to a solution of 0.74 mmol precursor (**1** or **6**) in CH_2Cl_2 (20 ml). The reaction mixture was stirred at room temperature for 3 h. The reaction mixture was diluted with CH_2Cl_2 (30 ml) and washed with 5% aqueous citric acid (1 × 50 ml) and saturated brine (2 × 50 ml). The organic layer was dried ($MgSO_4$) and evaporated to give a foam. The product was purified by column chromatograghy on silica gel eluting with a $CHCl_3$/MeOH gradient (0–5% MeOH). Appropriate fractions were combined and evaporated to give the succinates (**2a**, **7a**) as a colorless foam. **2a** (418 mg, 83%) ^{1}H NMR ($CDCl_3$) δ: 1.13–1.45 (m, 6H), 1.95 (m, 1H), 2.58 (s, 4H), 3.10 (m, 4H), 3.80 (s, 6H), 4.15–4.40 (m, 5H), 4.85 (bs, 1H), 6.81 (d, *J* = 8.7 Hz, 4H), 7.25–7.42 (m, 13H), 7.56 (d, *J* = 7.2 Hz, 2H), 7.74 (d, *J* = 7.2 Hz, 2H). **7a** (439 mg, 78%; 98% pure by HPLC) ^{1}H NMR ($CDCl_3$) δ: 1.25 (m, 2H), 1.45(m, 2H), 1.60 (m, 2H), 2.18 (t, *J* = 7.5 Hz, 2H), 2.52 (s, 4H), 3.13 (m, 3H), 3.25 (dd, *J* = 8.7, 4.0 Hz, 1H), 3.78 (s, 6H), 4.22 (m, 2H), 4.41 (m, 4H), 5.00 (unresolved t, 1H), 6.10 (d, *J* = 7.2 Hz, 1H), 6.80 (d, *J* = 8.5 Hz, 4H), 7.19–7.41 (m, 13H), 7.56 (d, *J* = 7.5 Hz, 2H), 7.75 (d, *J* = 7.2 Hz, 2H). High resolution mass: $M+Cs^+$, calculated 961.2676, found 961.2699.

Diglycolate esters 2b and 7b

A solution of diglycolic anhydride (0.94 mmol) in CH_2Cl_2 (5 ml) was dropwise added to a mixture of Et_3N (0.89 mmol), 4-dimethylaminopyridine (0.37 mmol) and compound **1** or **6** (0.74 mmol) in CH_2Cl_2 (15 ml) at 0°C (ice bath) under an argon atmosphere. After addition was complete (10 min) the ice bath was removed and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was diluted with CH_2Cl_2 (30 ml) and washed with 5% aqueous citric acid (1 × 50 ml) and saturated brine (2 × 50 ml). The organic layer was dried ($MgSO_4$) and evaporated to give a foam. The product was purified by column chromatograghy on silica gel eluting with a $CHCl_3$/EtOH gradient (2–10% EtOH). Appropriate fractions were combined and evaporated to give diglycolate esters **2b** and **7b** as a colorless foam. **2b** (354 mg, 60%; 98% pure by HPLC) ^{1}H NMR ($CDCl_3$) δ: 1.00–1.25 (m, 6H), 1.75 (m, 1H), 2.88 (m, 4H), 3.70 (s, 6H), 3.96 (s, 2H), 4.04 (s, 2H), 4.13 (m, 3H), 4.31 (d, *J* = 6.9 Hz), 5.18 (bs, 1H), 6.74 (d, *J* = 8.7 Hz, 4H), 7.18–7.34 (m, 13H), 7.53 (d, *J* = 7.5 Hz, 2H), 7.67 (d, *J* = 7.5 Hz, 2H). High resolution mass: $M+Cs^+$, calculated 920.2411, found 920.2401. **7b** (260 mg, 56%; 98% pure by HPLC) ^{1}H NMR ($CDCl_3$) δ: 1.20 (m, 2H), 1.39 (m, 2H), 1.58 (m, 2H), 2.18 (t, *J* = 7.5 Hz, 2H), 2.90–3.25 (m, 4H), 3.80 (s, 6H), 3.86 (s, 4H), 4.00–4.40 (m, 6H), 4.85 (unresolved t, 1H), 5.92 (d, *J* = 7.2 Hz, 1H), 6.75 (d, *J* = 8.1 Hz, 4H), 7.20–7.40 (m, 13H), 7.52 (d, *J* = 7.2 Hz, 2H), 7.69 (d, *J* = 7.2 Hz, 2H). High resolution mass: $M+Cs^+$, calculated 977.2625, found 977.2658.

General procedure for derivatization of TMR-labeled CPG support (4a, 4b, 9a, 9b)

A mixture of CPG (500 , 40 µmol/g amine loading, 2 g, 80 µmol), succinates or diglycolates (**2a**, **2b**, **7a** or **7b**, 160 µmol), 1-hydroxybenzotriazol (160 µmol), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (160 µmol) and *N*,*N*-diisopropylethylamine (270 µmol) in DMF (15 ml) was shaken on a wrist action shaker for 4 h at room temperature. The support was washed with DMF (3 × 15 ml), CH_3CN (2 × 15 ml) and dried under vacuum overnight. The trityl cation assay gave a loading of 30–35 µmol/g. The support was capped with acetic anhydride/lutidine in THF (10% solution, 5 ml) for 1 h at room temperature and was washed with CH_3CN (3 × 10 ml). The support (**3a**, **3b**, **8a** or **8b**) was treated with 20% piperidine in DMF (3 × 10 ml, 10 min each time) to remove the Fmoc protecting group. Removal of the Fmoc group was monitored by measuring UV absorbance of the solution at 302 nm. The support was washed with DMF (3 × 10 ml) and was then treated with TMR *N*-hydroxysuccinimide ester (192 µmol) and Et_3N (384 µmol) in DMF (10 ml) for 24 h on a shaker. The support was washed with DMF (3 × 10 ml) and CH_3CN (2 × 10 ml). The support was capped with acetic anhydride/lutidine in THF (10% solution, 5 ml) for 1 h and then washed with CH_3CN (3 × 15 ml) and dried under high vacuum for 24 h.

Double dye-labeled oligodeoxyribonucleotide synthesis

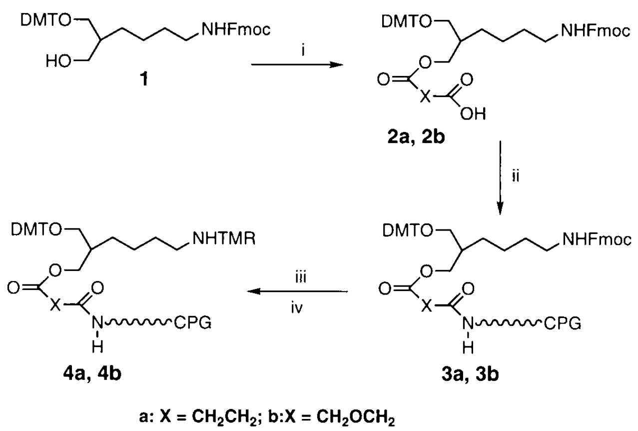
TMR-labeled supports **4b** and **9b**, fluorescein dye amidites (8) and dA^{bz}, dC^{bz}, dG^{dmf} and T phosphoramidites were used in automated synthesis of double dye-labeled oligodeoxyribonucleotide on a ABI 394 DNA/RNA synthesizer at 0.2 and 1.0 µmol scales. The standard synthesis cycle was slightly modified by extending the coupling time of fluorescein amidites by an additional 120 s. Cleavage of double dye-labeled oligodeoxyribonucleotides was accomplished by treating with a mixture (1:1:2) of t-butylamine:methanol:water for 1 h on the synthesizer for support **4b** and for 20 min for support **9b**. Deprotection was performed by heating at 65°C for 3 h or at 85°C for 1 h. For post-synthesis addition of TMR dye the probe was synthesized by coupling FAM amidite (8) at the 5′-end and phosphalink (9) followed by a linker arm nucleotide (LAN) phosphoramidite (10) at the 3′-end. LAN replaced a T nucleotide at the 3′-end of the probe and offered a primary amine group to couple with TMR *N*-hydroxysuccinimide ester after deprotection of the probe. Phosphalink provided the 3′-end blocking phosphate group. Oligodeoxyribonucleotides were analyzed by both reverse phase and anion exchange HPLC. Double dye-labeled probes used in PCR assay were purified by reverse phase HPLC. Reverse phase HPLC: RP-18 column (220 × 4.6 mm), flow rate 1 ml/min, gradient 0–20% B over 24 min followed by 20–40% B over 10 min; solvent A, 0.1 M TEAA; solvent B, acetonitrile. Anion exchange HPLC: Nucleopac PA-100 column (250 × 4 mm), flow rate 1 ml/min, gradient 0–60% B over 25 min; solvent A, 20 mM $LiClO_4$ and 20 mM NaOAc in H_2O:CH_3CN (9:1, pH 6.5); solvent B, 600 mM $LiClO_4$ and 20 mM NaOAc in H_2O:CH_3CN (9:1, pH 6.5).

Enzymatic digestion of double dye-labeled oligodeoxyribonucleotides

A digestion cocktail (55 µl) for each sample was prepared by mixing the following reagents; water (44 µl), 1 M $MgCl_2$ (0.8 µl), 0.5 M Tris buffer, pH 7.5 (3.5 µl), alkaline phosphatase (4.0 µl) and snake venom phosphodiesterase (2.4 µl). Typically 0.4 ODU of oligodeoxyribonucleotide were dissolved in digestion cocktail and heated at 37°C for 8 h. A RP-18 reverse phase column was used for analysis with the detector set at 260 nm. Solvent A was 3% acetonitrile in 0.1 M triethyammonium acetate and solvent B was 90% acetonitrile in water. The gradient was 100% A for 5 min, 100–90% A over 30 min, 90–0% A over 30 min, 100% B for 5 min, 0–100% A over 2 min, flow rate 0.5 ml/min. The order of elution was C, G, T, A, 6-carboxyfluorescein, 3′-nucleotide–TMR linker conjugate. The 3′-nucleotide–TMR linker conjugate was confirmed by enzymatic digestion of 3′-TMR-labeled oligodeoxyribonucleotide 5′-d(ATGCCCTCCCCCATGCCAT-CCTGCGT)-3′-TMR. The 3′-thymidine nucleotide–TMR linker conjugate was isolated by reverse phase HPLC of the digestion mixture. A sample of thymidine nucleotide–TMR linker conjugate was synthesized by coupling thymidine amidite with TMR-labeled support **4b** followed by detritylation and cleavage of the conjugate from the support. These two samples of thymidine nucleotide–TMR conjugate showed the same retention time in reverse phase HPLC analysis. The identity of the thymidine nucleotide–TMR linker conjugate isolated from enzymatic digestion experiments was further confirmed by high resolution mass spectral analysis. Thymidine nucleotide–TMR conjugate ($C_{42}H_{50}N_5O_{13}P$), HRMS: *m*/*z* calculated 864.3221 (MH^+), observed 864.3256 (MH^+).

PCR reaction and assay

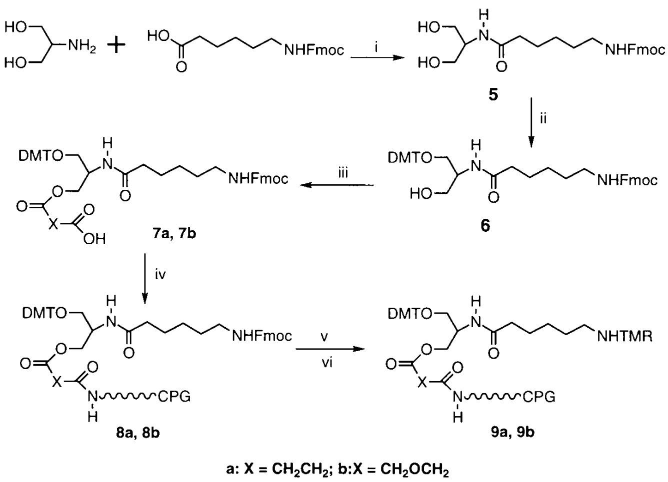
Reactions were assembled using components of the TaqMan® PCR Core Reagent Kit (Perkin Elmer, N808-0228) and the TaqMan β-actin Detection Reagents (Perkin Elmer, P/N 401846). Reactions contained 1× TaqMan buffer A (containing ROX-labeled passive reference dye), 3.5 mM $MgCl_2$, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 400 µM dUTP, 300 nM β-actin forward primer, 300 nM β-actin reverse primer, 200 nM double dye-labeled oligodeoxyribonucleotide 5′-FAM-d(ATGCCCTCCCCCATG-CCATCCTGCGT)-3′-TMR, 0.02 ng/µl human male DNA (Raji cell DNA included in β-actin detection reagent kit), 0.05 U/µl AmpliTaq Gold™ DNA polymerase and 0.01 U/µl uracil-*N*-glycosylase in a total volume of 50 µl. The fluorescence of FAM in this double dye-labeled 26mer probe was one seventh of the fluorescence of an identical 26mer labeled with FAM only. The thermal cycling protocol was 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Thermal cycling, fluorescence detection and data analysis were performed on the ABI PRISM™ 7700 Sequence Detector using the software provided with the instrument.



Scheme 1. Derivatization of TMR labeled solid supports (**4a** and **4b**). Reagents: (i) succinic or diglycolic anhydride, DMAP, TEA; (ii) HOBT, HBTU, DIPEA, aminopropyl CPG; (iii) 20% piperidine in DMF; (iv) TMR NHS ester, TEA.

RESULTS

Derivatization of TMR-labeled supports are shown in Schemes 1 and 2. Partially protected amino diol **1** (11) was reacted with succinic anhydride in the presence of Et_3N and DMAP to give succinate **2a** in 83% yield after silica gel chromatography. Succinate **2a** was loaded on a aminopropyl-CPG solid support in the presence of 1-hydroxybenzotriazol (HOBT), 2-(1*H*-benzotriazol-1-yl)-1, 1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (12) and diisopropylethyl amine (DIPEA). The loading of **2a** onto the CPG support was determined to be 30–35 µmol/g as quantitated by trityl cation assay. The Fmoc group was removed by treating support **3a** with 20% piperidine in DMF and was followed by UV spectroscopy. Support **3a** was reacted with TMR *N*-hydroxysuccinimide ester in DMF for 24 h at room temperature to give TMR-labeled support **4a**. Dye coupling was determined to be 98–99%. Support **4a** was used for synthesis of oligodeoxyribonucleotides. Synthesis of oligodeoxyribonucleotide using support **4a** proceeded in high yield as judged by trityl cation measurement and by HPLC analysis of the oligodeoxyribonucleotide. However, cleavage of dye-labeled oligodeoxyribonucleotide was very slow; only 27% cleaved in 1 h and 50% cleaved in 2 h with a mixture (1:1:2) of t-butylamine: methanol:water (13) at room temperature. In order to increase the cleavage rate linker **5** was synthesized from serinol and *N*-Fmoc-ε-aminocaproic acid in 66% crystallized yield. Protection of one of the hydroxyl groups with dimethoxytrityl gave **6**, which was converted to succinate **7a** in 78% yield after chromatographic purification. Aminopropyl-CPG was derivatized with **7a** to give **8a**, which was finally converted to TMR-labeled support **9a** as described above. The cleavage rate of oligodeoxyribonucleotides synthesized with support **9a** was improved; 60% cleaved in 1 h and 90% cleaved in 2 h at room temperature. To further enhance the cleavage rate diglycolate ester derivatives **2b** and **7b** were synthesized in 60 and 56% yield respectively. Diglycolates **2b** and **7b** were used to derivatize TMR-labeled supports **4b** and **9b**. When **4b** was used for oligodeoxyribonucleotide synthesis >98% oligodeoxyribonucleotide was cleaved in 1 h at room temperature. With support **9b** 98% of oligodeoxyribonucleotide was cleaved in 20 min.



Scheme 2. Derivatization of TMR labeled solid supports (**9a** and **9b**). Reagents: (i) HOBT, HBTU, DIPEA; (ii) dimethoxytrityl chloride, pyridine; (iii) succinic or diglycolic anhydride, DMAP, TEA; (iv) HOBT, HBTU, DIPEA, aminopropyl CPG; (v) 20% piperidine in DMF; (vi) TMR, NHS ester, TEA.

Double dye-labeled oligodeoxyribonucleotides were synthesized at both 0.2 and 1.0 μmol scales using solid supports **4b** and **9b**. Oligodeoxyribonucleotides were cleaved on the instrument using a mixture (1:1:2) of t-butylamine:methanol:water and protecting groups were removed by heating at 65°C for 3 h or at 85°C for 1 h. Oligodeoxyribonucleotides were analyzed by both reverse phase and anion exchange HPLC (Fig. 1). Purification of the oligodeoxyribonucleotides was carried out by reverse phase

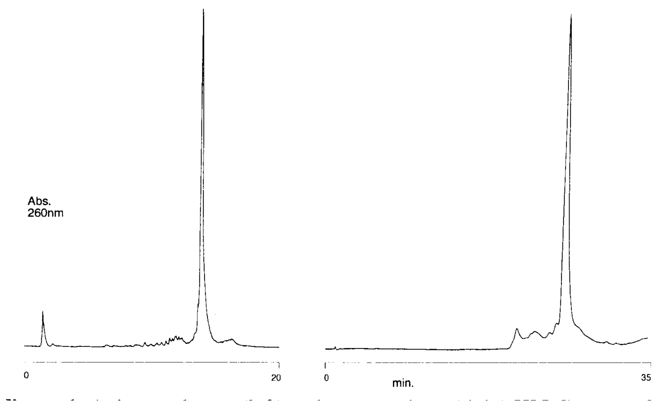


Figure 1. Anion exchange (left) and reverse phase (right) HPLC traces of a crude sample of a double dye-labeled oligodeoxyribonucleotide, 5′-FAM-d(TCACAGTCTGATCTCGAT)-3′-TMR.

HPLC. Double dye-labeled oligodeoxyribonucleotides were enzymatically digested with snake venom phosphodiesterase and alkaline phosphatase. The digestion mixture was analyzed by reverse phase HPLC using a C-18 column and no modifications of nucleobases or dyes were observed. After enzymatic digestion TMR dye remained attached to the 3′-end nucleotide. The 3′-end nucleotide–TMR conjugate was identified by comparison with a known sample of nucleotide–TMR conjugate by reverse phase HPLC analysis. Both conjugates showed the same retention time in two different gradients. The identity of the nucleotide–TMR conjugate was further confirmed by mass spectral analysis.

The performance of the oligodeoxyribonucleotides synthesized with different solid supports was compared by using them as probes in the 5′-nuclease TaqMan assay to detect PCR amplification of DNA. The nucleotide sequence used in synthesis of the oligodeoxyribonucleotides corresponds to a segment of the human β-actin gene. Reactions were run containing each of the double dye-labeled oligodeoxyribonucleotides, forward and reverse primers specific for the human β-actin gene and all the other components required for PCR amplification. The reactions were run in an ABI PRISM 7700 Sequence Detector, which monitors fluorescence from each reaction during the thermal cycling of PCR. Figure 2 shows the structures of different linkers used to attach TMR dye to the probes. Figure 3 shows the fluorescence profiles for representative reactions. During each cycle hybridization of the double dye-labeled probe to the β-actin DNA segment being amplified results in cleavage of the probe by the 5′-exonuclease activity of Taq DNA polymerase. Cleavage of the probe frees the fluorescein from the quenching effects of the TMR also attached to the oligodeoxyribonucleotide. Thus accumulation of β-actin-specific amplified product is detected by an increase in fluorescein emission. As shown in Figure 3, regardless of the linker attaching TMR, the double dye-labeled oligodeoxyribonucleotides behave nearly identically in detection of PCR product accumulation. Two parameters are used to characterize the results obtained using fluorogenic probes and the 5′-nuclease assay: ΔR_n, the change in normalized reporter fluorescence, and C_T, the threshold cycle. For each fluorescence measurement R_n is determined by calculating the contribution of the reporter fluorescein and dividing by the

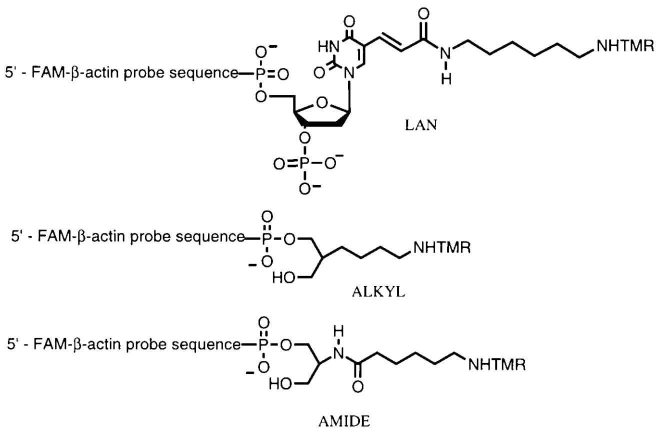


Figure 2. Structures of linkers used to couple the TMR dye to β-actin probes.

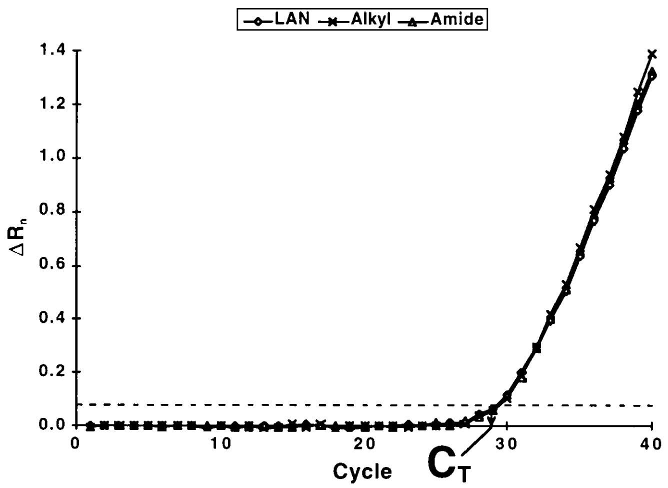


Figure 3. Amplification plots showing accumulation of an amplified human β-actin DNA segment detected using the 5′-nuclease assay and fluorogenic probes. As indicated in the legend, this figure compares double dye-labeled oligonucleotides with TMR attached via a LAN (linker arm nucleotide), alkyl linker or amide linker. For each fluorescence measurement made by the ABI PRISM 7700 Sequence Detector the instrument software calculates R_n by determining the contribution of the reporter fluorescein and dividing by the contribution of an internal reference dye ROX, included in the reaction. ΔR_n is the value of R_n in any cycle minus the R_n prior to PCR amplification. C_T is the fractional cycle number at which ΔR_n crosses some fixed threshold above baseline. The threshold is indicated by the dotted line. In this case the threshold was determined by calculating the average standard deviation of ΔR_n between cycles 3 and 24 for all the plots and multiplying this standard deviation by a factor of 20. Average standard deviation, 0.0039; threshold, 0.078.

contribution of an internal reference dye ROX included in the reaction. ΔR_n is the value of R_n at any cycle minus R_n prior to PCR amplification. In Figure 3 ΔR_n is plotted on the *y*-axis. C_T is the fractional cycle number at which ΔR_n crosses some fixed threshold above baseline, as depicted in Figure 3. The C_T value is predictive of the input amount of target and thus is useful in using the 5′-nuclease assay for quantitation of DNA and RNA (14). For each of the double dye-labeled oligodeoxyribonucleotides eight replicate reactions were run. Table 1 shows that there are no significant differences in the average ΔR_n and C_T values observed in these replicates.

DISCUSSION

We have developed TMR dye-labeled non-nucleosidic supports for automated synthesis of double dye-labeled oligodeoxyribonucleotides in high yield. TMR dye is stable in all currently used ancillary DNA synthesis reagents, except concentrated ammonium hydroxide, which is widely used for cleavage and deprotection of oligodeoxyribonucleotides. The cleavage and deprotection of TMR-labeled oligodeoxyribonucleotides was carried out with a mixture (1:1:2) of t-butylamine:methanol:water without any detectable degradation or modification of dyes and nucleobases. The cleavage of dye-labeled oligodeoxyribonucleotides from the support was significantly improved by anchoring non-nucleosidic linkers to the solid support through a diglycolate moiety instead of succinate and also by introducing an amide functionality in one of the linker arms. The diglycolate ester linkage was found to be more labile than the succinate ester linkage. However, premature cleavage of the linkers during oligodeoxyribonucleotide synthesis was not observed. The exocyclic amine protecting groups were completely removed by treating dye-labeled oligodeoxyribonucleotides with a mixture (1:1:2) of t-butylamine:methanol:water without any adverse effect on the quality of the oligodeoxyribonucleotides. Use of a TMR-labeled support simplifies probe synthesis and purification protocols and significantly improves yield and quality of double dye-labeled probes. The results in Figure 3 and Table 1 show that variation of the linker arm had little, if any, effect on the performance of these oligodeoxyribonucleotides in the 5′-nuclease assay. Thus the linker with the diglycolate ester and the amide functionality is preferred because it improves yield and produces a more rapid and complete cleavage.

Table 1. Comparison of average ΔR_n and C_T values for double dye-labeled oligonucleotides synthesized with TMR attached via a LAN, alkyl linker or amide linker

	ΔR_n	C_T
LAN	1.33 ± 0.05	29.22 ± 0.07
Alkyl	1.34 ± 0.09	29.34 ± 0.18
Amide	1.24 ± 0.06	29.42 ± 0.15

Eight replicates were run to determine the average and standard deviation for each value. ΔR_n and C_T are defined in the legend to Figure 3. The ΔR_n values reported here are after 40 cycles of PCR.

Using the 5′-exonuclease assay for quantitation depends on the ability to determine accurate and precise C_T values. Low initial background fluorescence enhances the ability to determine C_T because it enables detection of a significant increase in fluorescence earlier in the cycling process. Conversely, the presence of oligodeoxyribonucleotides labeled only with the reporter fluorescein impairs C_T determination because of the increased initial fluorescence (background). The performance of probes labeled by post-synthetic reaction with TMR NHS ester depends critically on purification of double dye-labeled oligodeoxyribonucleotides from oligodeoxyribonucleotides labeled only with the reporter dye. By incorporating the TMR using a labeled support, generation of oligodeoxyribonucleotides labeled only with fluorescein at the 5′-end is greatly reduced. This simplifies purification and enhances the ability to synthesize probes with lower initial fluorescence. Thus this new method for synthesis of double dye-labeled oligodeoxyribonucleotides enhances the use of the 5′-nuclease assay for sensitive detection and accurate quantitation of specific DNA and RNA sequences.

ACKNOWLEDGEMENTS

We thank Dr Susan MacWhorter, Dr Paul Wyatt and Ms Shannan Downey for their assistance.

REFERENCES

1 Lee,L.G., Connell,C.R. and Bloch,W. (1993) *Nucleic Acids Res*., **21**, 3761–3766.
2 Livak,K.J., Flood,S.J.A., Marmaro,J., Giusti,W. and Deetz,K. (1995) *PCR Methods Applicat*., **4**, 357–362.
3 Mullah,B., Wyatt,P., Stevens,J., Andrus,A. and Livak,K.J. (1996) *Collect. Czech. Chem. Commun*., **61**, 287–289.
4 Forster,V.T. (1948) *Annln Phys.* (*Leipzig)*, **2**, 55–75.
5 Stryer,L. (1978) *Annu. Rev. Biochem*., **47**, 819–846.
6 Mullah,B. and Andrus,A. (1997) *Tetrahedron Lett*., **38**, 5751–5754. (US patent application no. 08593031)
7 Ota,N., Hirano,K., Warashina,M., Andrus,A., Mullah,B., Hatanaka,K. and Taira,K. (1998) *Nucleic Acids Res*., in press.
8 Applied Biosystems (1994) *User Bulletin No. 78*. Applied Biosystems, Foster City, CA. (Taqman probes are available from PE Applied Biosystems, http://oligos.abd.perkin-elmer.com/oligo/worldwide.html)
9 Horn,T. and Urdea,M.S. (1986) *Tetrahedron Lett*., **27**, 4705–4708.
10 Ruth,J.L., Morgan,C. and Pasko,A. (1985) *DNA*, **4**, 93.
11 Nelson,P.S., Kent,M. and Muthini,S. (1992) *Nucleic Acids Res*., **20**, 6253–6259.
12 Knorr,R.A., Trzeciak,A., Bannwarth,W. and Gillessen,D. (1989) *Tetrahedron Lett*., **30**, 1927–1930.
13 Woo,S.L., Menchen,S.M. and Fung,S. (1993) US patents 5,231,191 and 4,965,349.
14 Heid,C.A., Stevens,J., Livak,K.J. and Williams,P.M. (1996) *Genome Res*., **6**, 986–994.