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

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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 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 5′-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, phosphodiesterase (SVP, Crotalus adamanteus venom) and alkaline phosphatase (Escherichia coli) 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 PRISMª 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-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (1.61 g, 4.25 mmol) and N,N-

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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 CHCl₃ (100 ml) and washed with 5% aqueous HCl (1 x 50 ml), H₂O (1 x 50 ml) and saturated brine (1 x 50 ml). The organic layer was dried (MgSO₄) 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). ¹H NMR (CDCl₃): δ: 1.35 (m, 2H), 1.45 (m, 2H), 1.66 (m, 2H), 2.25 (t, J = 7.2 Hz, 2H), 3.18 (m, 2H), 3.74 (s, 6H), 3.81 (m, 1H), 4.00–4.40 (m, 6H), 4.85 (s, 2H), 4.04 (s, 2H), 4.13 (m, 3H), 4.31 (d, J = 6.9 Hz, 1H), 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 599.129, found 599.1193.

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 (1.35 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 CHCl₃ (100 ml) and washed with H₂O (1 x 100 ml) and saturated brine (1 x 100 ml). The organic layer was dried (MgSO₄) and evaporated to give a yellowish oil. The product was isolated by column chromatography on silica gel eluting with 1–5% MeOH in CHCl₃. Appropriate fractions were combined and evaporated to give succinate esters 2a and 7a as a colorless foam.

Succinate esters 2a and 7a

Succinic anhydride (0.96 mmol), Et₃N (0.74 mmol) and 4-dimethylaminopyridine (0.37 mmol) and compound 1 or 6 (0.74 mmol) in CH₂Cl₂ (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 CH₂Cl₂ (30 ml) and washed with 5% aqueous citric acid (1 x 50 ml) and saturated brine (2 x 50 ml). The organic layer was dried (MgSO₄) and evaporated to give a foam. The product was purified by column chromatography on silica gel eluting with a CHCl₃/EtOH gradient (2–10% EtOH). Appropriate fractions were combined and evaporated to give diglycolate esters 2b and 7b as a colorless foam.

Diglycolate esters 2b and 7b

A solution of diglycolic anhydride (0.94 mmol) in CH₂Cl₂ (5 ml) was dropwise added to a mixture of Et₃N (0.89 mmol), 4-dimethylaminopyridine (0.37 mmol) and compound 1 or 6 (0.74 mmol) in CH₂Cl₂ (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 CH₂Cl₂ (30 ml) and washed with 5% aqueous citric acid (1 x 50 ml) and saturated brine (2 x 50 ml). The organic layer was dried (MgSO₄) and evaporated to give a foam. The product was purified by column chromatography on silica gel eluting with a CHCl₃/EtOH gradient (2–10% EtOH). Appropriate fractions were combined and evaporated to give diglycolate esters 2b and 7b as a colorless foam.

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-((H-benzotriazol-1-yl)-1,1,3,3-tetramethyloxiran hexafluorophosphate (160 µmol) and N,N-diisopropylpropylenylide (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 x 15 ml), CH₂CN (2 x 15 ml) and dried under vacuum overnight. The trityl cation assay gave a loading of 40–35 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 CH₂CN (3 x 10 ml). The support (3a, 3b, 8a or 8b) was treated with 20% piperidine in DMF (3 x 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 x 10 ml) and was then treated with TMR N-hydroxysuccinimide ester (192 µmol) and Et₃N (384 µmol) in DMF (10 ml) for 24 h on a shaker. The support was washed with DMF (3 x 10 ml) and CH₂CN (2 x 10 ml). The support was capped with acetic anhydride/lutidine in THF (10% solution, 5 ml) for 1 h and then washed with CH₂CN (3 x 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 dAβz, dCβz, dGβz 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 provided 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 LiClO4 and 20 mM NaOAc in H2O:CH3CN (9:1, pH 6.5); solvent B, 600 mM LiClO4 and 20 mM NaOAc in H2O:CH3CN (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 MgCl2 (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. Sample 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(ATGCCCTCCCCCTATGCCCAT- CCTGCCCT))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 (C24H30N6O13P), 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 MgCl2, 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-CATCTGCGT)-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.

RESULTS

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.
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 oligodeoxyribonucleotides 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 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.

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 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: ΔR0, the change in normalized reporter fluorescence, and C0, the threshold cycle. For each fluorescence measurement R0 is determined by calculating the contribution of the reporter fluorescein and dividing by the
contribution of an internal reference dye ROX included in the reaction. \( \Delta R_0 \) is \( R_0 \) at any cycle minus \( R_0 \) prior to PCR amplification. In Figure 3 \( \Delta R_0 \) is plotted on the y-axis. \( C_T \) is the fractional cycle number at which \( \Delta R_0 \) 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 \( \Delta R_0 \) 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 nucleotides in high yield. TMR dye is stable in all currently used for automated synthesis of double dye-labeled oligodeoxyribonucleotides. We have developed TMR dye-labeled non-nucleosidic supports

Amide functionality is preferred because it improves yield and produces a more rapid and complete cleavage.

**Table 1.** Comparison of average \( \Delta R_0 \) and \( C_T \) values for double dye-labeled oligonucleotides synthesized with TMR attached via a LAN, alkyl linker or amide linker

<table>
<thead>
<tr>
<th>Linker</th>
<th>( \Delta R_0 ) (±SD)</th>
<th>( C_T ) (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAN</td>
<td>1.33 ± 0.05</td>
<td>29.22 ± 0.07</td>
</tr>
<tr>
<td>Alkyl</td>
<td>1.34 ± 0.09</td>
<td>29.34 ± 0.18</td>
</tr>
<tr>
<td>Amide</td>
<td>1.24 ± 0.06</td>
<td>29.42 ± 0.15</td>
</tr>
</tbody>
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Eight replicates were run to determine the average and standard deviation for each value. \( \Delta R_0 \) and \( C_T \) are defined in the legend to Figure 3. The \( \Delta R_0 \) 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.

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REFERENCES