Chem. Eur. J. **2003**

Supporting Information

for

Rigid, Conjugated, Fluoresceinated Thymidine Triphosphates: Syntheses and Polymerase Mediated Incorporation Into DNA

Lars H. Thoresen, Guan-Sheng Jiao, Wade C. Haaland,
Michael L. Metzker, and Kevin Burgess*

- procedures for the organic syntheses
- spectroscopic data for compounds **12 - 14**
- overlay for donor and acceptor components of cassette **12**
- protocols for the enzyme incorporation experiments, and gel showing incorporation of compounds **3a** by TaqFS DNA polymerase

General Synthetic Procedures. Melting points are uncorrected. High field NMR spectra were recorded on Varian Unity Plus (^{1}H at 300 MHz, ^{13}C at 75 MHz, ^{31}P at 121 MHz) or Inova (^{1}H at 500 MHz, ^{13}C at 125 MHz) NMR spectrometers. Chemical shifts are reported in units of ppm relative to the solvent peak ($CDCl_3$: 7.27 ppm for ^{1}H and 77.0 ppm for ^{13}C; D_2O: 4.63 ppm for ^{1}H; Acetone-d_6: 2.04 ppm for ^{1}H and 29.9 ppm for ^{13}C; DMSO-d_6: 2.50 ppm for ^{1}H and 39.5 ppm for ^{13}C; CD_3OD: 3.30 ppm for ^{1}H and 49.0 ppm for ^{13}C; CD_3CN: 1.93 ppm for ^{1}H and 1.3 ppm for ^{13}C). FT-IR spectra were obtained as neat films using NaCl plates and a 4021 GALAXY series instrument. Mass spectra were obtained from the Mass Spectrometry Applications Laboratory at Texas A&M University. Thin layer chromatography was performed using silica gel 60 F254 plates. Flash chromatography was performed using silica gel (230-600 mesh). CH_2Cl_2, THF, DMF, triethylamine, and toluene were distilled from appropriate drying agents. Other chemicals were purchased from commercial suppliers and used as received. UV spectra were recorded in 1 cm path length quartz cuvettes on a Milton Roy Spectronic 3000 diode array UV spectrometer at concentrations between of 10 µM. Fluorescence emission spectra were recorded in 1 cm path length quartz cells on a SLM Aminco Fluorimeter. All fluorescence spectra were recorded at a concentration of 1.0 µM. Analytical HPLC were run with two SSI 222C pumps controlled by a SSI 232C gradient controller using a Supelcosil LC-18-T (4.6 x 150 mm) C-18 column. Gradient elution was used (A = 0.1 M TEAA pH 7, B = 0.1 M TEAA in 70% $MeCN_{(aq)}$) with a constant flow rate of 1.0 mL/min. Preparative HPLC were run on a Beckman System Gold 125P Solvent Module and 166P UV-vis detector using a Vydac semi-preparative C-18 column (cat # 218TP101522, 22 x 250 mm). The solvent system described above was used at a flow rate of 10 mL/min. ^{31}P NMR spectra were externally referenced to H_3PO_4 (0 ppm) and spectra recorded at 121 MHz with broadband proton decoupling. In some cases where exchange of the counterions caused peak-broadening, ^{31}P NMR spectra were recorded in 0.1 M EDTA/D_2O in order to obtain sharp, resolved peaks.

2’-Deoxy-5-iodouridine Triphosphate. 2'-Deoxy-5-iodouridine -3'-acetate (prepared as described in *J. Med. Chem.* 1967, p 1163. 117 mg, 0.30 mmol) was dissolved in 5 mL dry prydine then concentrated *in vacuo* three times then dissolved in 5 mL 1:4 pridine/DMF. 2-Chloro-4H-1,3,2-benzodioxaphosphorin-4-one (72 mg, 0.35 mmol) was added and the reaction stirred under nitrogen for 30 min at 25 ºC. Tributylammonium pyrophosphate (216 mg, 0.47 mmol, solution in 1 mL DMF) and tributylamine (230 mg, 1.24 mg) were added simultaneously. The reaction was stirred another 30 min at 25 ºC. Iodine (120 mg, 0.47 mmol) was added as a solution in 5 mL 95:5 pyridine/water. Stirring was continued another 20 min. Sodium thiosulfate was added dropwise as a saturated aqueous solution (*ca*. 30 drops) until the iodine color disappeared. The reaction mixture was concentrated *in vacuo* then partitioned between dichloromethane and water. The layers were separated and the organic extracts discarded. The aqueous portion was concentrated *in vacuo* then stirred in 20% ammonium hydroxide(aq.) at 25 ºC for 1 h then concentrated *in vacuo*. The crude reaction mixture was dissolved in 5 mL water then applied to a DEAE Sephadex column (1x25 cm) and eluted with 0 to 1 M TEAB buffer (pH 7.3). Absorbance of the eulate was monitored at 310 nm. The desired product eluted between 0.4 and 0.55 M TEAB. Product containing fractions were concentrated *in vacuo* to yield 2’-deoxy-5-iodouridine triphosphate as a clear film (tetra(triethylammonium) salt, 83 mg, 28%): ^{1}H-NMR (D_2O, 300 MHz): δ (ppm) 1.23 (t, *J* = 7.2 Hz, 36H), 2.33 (t, *J* = 5.7 Hz, 2H), 3.15 (q, *J* = 7.2 Hz, 24H), 4.15 (m, 3H), 4.57 (m, 1H), 6.22 (t, *J* = 6.9 Hz, 1H), 8.21 (s, 1H); ^{31}P-NMR (D_2O, 121 MHz): δ (ppm) -20.3 (t, *J* = 18.3 Hz, 1P), -10.4 (d, *J* = 15.9 Hz, 1P), -6.0 (d, *J* = 16.5 Hz, 1P); MS (ESI): m/z 593 (M-H).

2’,3’-Dideoxy-5-iodouridine Triphosphate. 2',3’-Dideoxy-5-iodouridine (62 mg, 183 μmol) was dissolved in 5 mL dry pyridine then concentrated *in vacuo* three times then dissolved in 1 mL 1:4 pyridine/DMF. 2-Chloro-4H-1,3,2-benzodioxaphosphorin-4-one (41 mg, 202 μmol) was added as a solution in 300 μL DMF and the reaction stirred under nitrogen for 10 min at 25 ºC.

Tributylammonium pyrophosphate (125 mg, 275 μmol) and tributylamine (143 mg, 770 μmol) were added together as a solution in 400 μL DMF. The reaction was stirred another 10 min at 25 ºC. Iodine (73 mg, 288 μmol) was added as a solution in 3 mL 98:2 pyridine/water. Stirring was continued another 15 min. Sodium thiosulfate was added dropwise as a saturated aqueous solution (*ca.* 10 drops) until the iodine color disappeared. The reaction mixture was concentrated *in vacuo* then partitioned between dichloromethane and water. The layers were separated and the organic extracts discarded. The crude reaction mixture was dissolved in 5 mL water then applied to a DEAE Sephadex column (1x30 cm) and eluted with 0 to 1 M lithium chloride. The absorbance of the eluate was monitored at 310 nm. The desired product eluted between 0.4 and 0.5 M LiCl. Product containing fractions were concentrated *in vacuo* and the product precipitated from acetone to yield 2',3'-dideoxy-5-iodouridine triphosphate as a white solid (tetralithium salt, 60 mg, 54%): ^{1}H-NMR (D_2O, 300 MHz): δ (ppm) 1.98 (m, 1H), 2.14 (m, 2H), 2.45 (m, 1H), 4.13 (m, 1H), 4.27 (m, 1H), 4.38 (m, 1H), 6.07 (dd, *J* = 6.6 Hz, 3.6 Hz, 1H), 8.22 (s, 1H); ^{31}P-NMR (D_2O, 121 MHz): δ (ppm) -20.4 (t, *J* = 19.1 Hz, 1P), -10.2 (d, *J* = 17.8 Hz, 1P), -5.8 (d, *J* = 19.1 Hz, 1P); MS (ESI): m/z 577 (M-H).

Preparation of Water-soluble Palladium Catalysts. *Method A* (adapted from Ghadiri *et al.*)[1]: Palladium (II) acetate (4.4 mg, 19.6 μmol) and 3,3'3"-phosphinidyne-tris(benzenesulfonic acid) trisodium salt (56.8 mg, 99.9 μmol) were combined in 1.0 mL 32 mM phosphate buffer (pH 7.2). The mixture was sonicated for 10 min then nitrogen was bubbled through the yellow solution for 45 min. The solution was "aged" under nitrogen at 4 ºC for 24 h before use.

Method B (adapted from Casalnuovo *et al.*)[2]: Na_2PdCl_4 (250 mg, 0.85 mmol) and 3,3',3"-phosphinidyne-tris(benzenesulfonic acid) trisodium salt (2.17 g, 3.82 mmol) were combined in 5 mL degassed water. To this orange solution was added $NaBH_4$ (71 mg, 1.87 mmol); this resulted in vigorous gas evolution. The bright yellow solution was stirred under nitrogen at 25 ºC for 5 min then concentrated in vacuo. The yellow solid was triturated with tetrahydrofuran, dried in vacuo. This solid was routinely stored at –20 °C.

Triphosphate 1a. 5-Ethynylfluorescein **7** (7.0 mg, 20 μmol), 5-iodo-2'3'-dideoxyuridine triphosphate tetralithium salt (6.0 mg, 10 μmol), copper iodide (0.1 mg, 0.5 μmol), triethylamine (36 mg, 359 μmol), and 20 mM palladium catalyst solution (Method A, 100 μL, 2 μmol) were combined in 400 μL degassed 30 mM phosphate buffer (pH 7.2). The yellow solution was stirred 24 h at 25 ºC under nitrogen at which time analytical HPLC showed disappearance of the starting triphosphate. The product was purified by preparative HPLC then lyophilized to give the title compound as a fluffy orange solid (tetra(triethylammonium) salt, 2.8 mg, 17%): ^{1}H NMR (D_2O, 300 MHz): δ (ppm) 1.12 (t, *J* = 7.0 Hz, 36H), 1.75 (m, 1H), 2.01 (m, 2H), 2.34 (m, 1H), 3.04 (q, *J* = 7.0 Hz, 24H), 4.00 (m, 1H), 4.12 (m, 1H), 4.25 (m, 1H), 6.11 (dd, *J* = 6.9 Hz, 3.9 Hz, 1H), 6.75 – 6.85 (m, 4H), 7.13 (d, *J* = 8.4 Hz, 2H), 7.28 (d, *J* = 6.3 Hz, 1H), 7.85 (d, *J* = 7.5 Hz, 1H), 8.11 (s , 1H), 8.15 (s, 1H); ^{31}P-NMR (0.1 M EDTA/D_2O, 121 MHz): δ (ppm) -22.2 (t, *J* = 20.7 Hz, 1P), -10.3 (d, *J* = 19.4 Hz, 1P), -10.0 (d, *J* = 19.5 Hz, 1P); MS (ESI): m/z 805 $(M-H)^-$.

Triphosphate 1b. 5-Ethynylfluorescein **7** (7.0 mg, 20 μmol), 5-iodo-2'-deoxyuridine triphosphate tetralithium salt (6.2 mg, 10 μmol), copper iodide (0.1 mg, 0.5 μmol), triethylamine (36 mg, 359 μmol), and 20 mM palladium catalyst solution (Method A, 100 μL, 2 μmol) were combined in 400 μL degassed 30 mM phosphate buffer (pH 7.2). The yellow solution was stirred 17 h at 25 ºC under nitrogen at which time analytical HPLC showed disappearance of the starting triphosphate. The product was purified by preparative HPLC then lyophilized to give the title compound as a fluffy orange solid (tetra(triethylammonium) salt, 5 mg, 41%): ^{1}H-NMR (D_2O, 500 MHz): δ (ppm) 1.25 (t, *J* = 7 Hz, 36H), 2.45 (t, *J* = 5.5 Hz, 2H), 3.17 (q, *J* = 7 Hz, 24H), 4.22 (m, 3H), 4.66 (m, 1H), 6.29 (t, *J* = 6.5 Hz, 1H), 6.77 – 6.80 (m, 4H), 7.32 (dd, *J* = 9.0 Hz, 5.5 Hz, 2H), 7.39 (d, *J* = 7.5 Hz, 1H), 7.86 (d, *J* = 8.0 Hz, 1H), 8.04 (d, *J* = 0.5 Hz, 1H), 8.24 (s, 1H); ^{31}P-NMR (D_2O, 121 MHz): δ (ppm) -22.5 (t, *J* =

18.8 Hz, 1P), -10.7 (d, *J* = 19.5 Hz, 1P), -9.6 (d, *J* = 19.0 Hz, 1P); MS (ESI): m/z 821 $(M-H)^-$; HRMS (ESI): m/z 821.0140 $(M-H)^-$,calc for $C_{31}H_{24}N_2O_{19}P_3^-$: 821.0186.

Triphosphate 2a. 5-(4-Ethynylphenyl)fluorescein **9** (5.8 mg, 13.5 μmol), 5-iodo-2'-3'-dideoxyuridine triphosphate tetralithium salt (5.4 mg, 9.0 μmol), copper iodide (0.2 mg, 0.9 μmol), triethylamine (45 mg, 45 μmol), and palladium complex **B** (2 mg, 0.9 μmol) were combined in 400 μL degassed 30 mM phosphate buffer (pH 7.2). The solution was stirred at 25 ºC under nitrogen for 3 d. Purification by preparative HPLC yielded the title compound as a fluffy orange solid (tetra(triethylammonium) salt, 2.0 mg, 17%): ^{1}H-NMR (D_2O, 500 MHz): δ (ppm) 1.07 (t, *J* = 7.3 Hz, 36H), 2.02 (m, 2H), 2.34 (m, 1H), 3.00 (q, *J* = 7.3 Hz, 24H), 3.98 (m, 1H), 4.11 (m, 1H), 4.24 (m, 1H), 5.97 (dd, *J* = 7.1 Hz, 3.4 Hz, 1H), 6.65 – 6.50 (m, 4H), 7.13 (d, *J* = 10.0 Hz, 2H), 7.27 (d, *J* = 8.0 Hz, 1H), 7.60 (d, *J* = 8.5 Hz, 2H), 7.70 (d, *J* = 8.5 Hz, 2H), 7.77 (dd, *J* = 8.0 Hz, 1.5Hz, 1H), 8.00 (d, *J* = 1.5 Hz, 1H), 8.01 (s, 1H); ^{31}P-NMR (0.1 M EDTA/D_2O, 121 MHz): δ (ppm) -21.6 (t, *J* = 20.0 Hz, 1P), -10.3 (d, *J* = 19.2 Hz, 1P), -9.7 (d, *J* = 18.8 Hz, 1P); MS (ESI): m/z 293 $(M-3H)^{-3}$, 440 $(M-2H)^{-2}$, 881 $(M-H)^-$.

Triphosphate 2b. Following the procedure described for **2a**, 5-(4-ethynylphenyl)fluorescein **9** (5.8 mg, 13.5 μmol), 5-iodo-2'-deoxyuridine triphosphate tetralithium salt (5.5 mg, 9.0 μmol), copper iodide (0.2 mg, 0.9 μmol), triethylamine (118 mg, 1.17 mmol), and palladium complex **B** (2 mg, 0.9 μmol) were reacted for 12 h to yield the title compound as a fluffy orange solid (bis(triethylammonium) salt, 4.1 mg, 42%): ^{1}H-NMR (D_2O, 500 MHz): δ (ppm) 1.09 (t, *J* = 7.5 Hz, 18H), 2.24 (m, 2H), 3.01 (q, *J* = 7.5 Hz, 12H), 3.93 – 4.03 (m, 3H), 4.48 (m, 1H), 6.05 (t, *J* = 6.5 Hz, 1H), 6.63 – 6.71 (m, 4H), 7.18 – 7.26 (m, 3H), 7.52 (d, *J* = 8.5 Hz, 2H), 7.61 (d, *J* = 8.5 Hz, 2H), 7.67 (d, *J* = 7.0 Hz, 1H), 7.93 (s , 1H), 8.01 (s, 1H); ^{31}P-NMR (0.1 M EDTA/D_2O, 121 MHz): δ (ppm) -21.8 (t, *J* = 20.2 Hz, 1P), -10.4 (d, *J* = 19.7 Hz, 1P), -9.7 (d, *J* = 18.9 Hz, 1P); MS (ESI): m/z 298 $(M-3H)^{-3}$, 448 $(M-2H)^{-2}$, 897 $(M-H)^-$.

Triphosphate 3a. Following the procedure described for **2a**, 5-(2-(4-ethynylphenyl)-ethynyl-fluorescein **11** (7.5 mg, 16.4 μmol), 5-iodo-2'-3'-dideoxyuridine triphosphate tetralithium salt (5.4 mg, 9.0 μmol), copper iodide (0.2 mg, 0.9 μmol), triethylamine (118 mg, 1.17 mmol), and palladium complex **B** (2 mg, 0.9 μmol) were reacted for 3 d to yield the title compound as a fluffy orange solid (tetra(triethylammonium) salt, 5.0 mg, 42%): ^{1}H-NMR (D_2O, 500 MHz): δ (ppm) 1.07 (t, *J* = 7.0 Hz, 36H), 1.73 – 1.80 (m, 1H) 1.92 – 2.02 (m, 2H), 2.27 – 2.34 (m, 1H), 2.98 (q, *J* = 7.0 Hz, 24H), 3.95 - 4.00 (m, 1H), 4.09 – 4.13 (m, 1H), 4.20 – 4.24 (m, 1H), 5.96 (dd, *J* = 7.0 Hz, 4.0 Hz, 1H), 6.45 – 6.47 (m, 4H), 7.07 (d, *J* = 9.5 Hz, 2H), 7.19 (d, *J* = 8.0 Hz, 1H), 7.48 (m, 4H), 7.62 (dd, *J* = 8.0 Hz, 1.5 Hz, 1H), 7.83 (dd, *J* = 1.5Hz, 0.5 Hz 1H), 7.95 (s, 1H); ^{31}P-NMR (D_2O, 121 MHz): δ (ppm) -21.1 (t, *J* = 20.1 Hz, 1P), -10.2 (d, *J* = 20.1 Hz, 1P), -5.5 (d, *J* = 18.9 Hz, 1P); MS (ESI): m/z 301 $(M-3H)^{-3}$, 452 $(M-2H)^{-2}$, 905 $(M-H)^{-}$.

Triphosphate 3b. Following the procedure described for **2a**, 5-(2-(4-ethynylphenyl)-ethynyl-fluorescein **11** (6.1 mg, 13.5 μmol), 5-iodo-2'-deoxyuridine triphosphate tetralithium salt (5.5 mg, 9.0 μmol), copper iodide (0.2 mg, 0.9 μmol), triethylamine (45 mg, 45 μmol), and palladium complex **B** (2 mg, 0.9 μmol) were reacted for 12 h to yield the title compound as a fluffy orange solid (tetra(triethylammonium) salt, 3.0 mg, 26%): ^{1}H-NMR (D_2O, 500 MHz): δ (ppm) 1.10 (t, *J* = 7.0 Hz, 36H), 2.26 (t, *J* = 6.5 Hz, 2H), 3.01 (q, *J* = 7.0 Hz, 24H), 4.06 (m, 3H), 4.51 (m, 1H), 6.14 (t, *J* = 7.0 Hz, 1H), 6.48 – 6.50 (m, 4H), 7.10 (d, *J* = 10.0 Hz, 2H), 7.22 (d, *J* = 7.5 Hz, 1H), 7.51 (m, 4H), 7.65 (dd, *J* = 8.0 Hz, 1.5 Hz, 1H), 7.85 (d, *J* = 1.5Hz, 1H), 8.05 (s, 1H); ^{31}P-NMR (D_2O, 121 MHz): δ (ppm) -21.6 (t, *J* = 19.7 Hz, 1P), -10.4 (d, *J* = 18.6 Hz, 1P), -5.8 (d, *J* = 19.0 Hz, 1P); MS (ESI): m/z 306 $(M-3H)^{-3}$, 460 $(M-2H)^{-2}$, 921 $(M-H)^{-}$.

5-Iodofluorescein Diacetate (5). 5-Iodofluorescein (410 mg, 0.89 mmol) was dissolved in 5 mL dry pyridine. DMAP (1 mg, 0.009 mmol) was added followed by acetic anhydride (206 mg, 2.01 mmol). The solution was stirred 4 h at 25 °C under N_2 then concentrated *in vacuo*. The crude product was purified by flash chromatography eluting with 30% EtOAc/hexanes yielding 337 mg (69%) of 5-iodofluorescein diacetate **4** which crystallized from the product-containing fractions as a white solid: mp 223–224 °C (dec); R*f* 0.39 (35 % EtOAc/hexanes); ^{1}H-NMR ($CDCl_3$, 500 MHz): δ (ppm) 2.33 (s, 6 H), 6.84 (m, 4 H), 6.96 (dd, *J* = 8 Hz, 0.8 Hz, 1 H), 7.10 (m, 2H), 8.00 (dd, *J* = 8 Hz, 1.3 Hz, 1H), 8.38 (m, 1 H); ^{13}C-NMR ($CDCl_3$, 125 MHZ): δ (ppm) 21.1, 81.9, 95.3, 110.5, 115.7, 117.9, 125.8, 128.2, 128.9, 134.2, 144.0, 151.5, 152.1, 152.2, 167.3, 168.8; IR(film): ν (cm^{-1}) 1427, 1616, 1766, 2936, 3067; MS (ESI): m/z 543 $(M+H)^+$.

5-(2-Trimethylsilylethynyl)fluorescein Diacetate (6). 5-Iodofluorescein diacetate **5** (0.87 g, 1.60 mmol), trimethylsilylacetylene (0.79 g, 8.02 mmol), tetrakis(triphenylphosphine)palladium (93 mg, 0.08 mmol), copper(I) iodide (15 mg, 0.08 mmol), and triethylamine (0.81 g, 8.02 mmol) were dissolved in 5 mL freshly distilled THF in a Schlenk tube. The solution was freeze-pump-thawed three times, then stirred 1 h at 55 °C under N_2. The solution was concentrated *in vacuo* then purified by flash chromatography eluting with 30% EtOAc/hexanes to yield 5-(2-trimethylsilyl-alkynyl)fluorescein diacetate **6** as a pale yellow film (0.78 g, 95% yield). R*f* 0.55 (35 % EtOAc/hexanes); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ (ppm) 0.29 (s , 9H), 2.33 (s, 6H), 6.82 (m, 4H), 7.10 (m, 2H), 7.13 (d, *J* = 8.1 Hz, 1H), 7.75 (dd, *J* = 8.1 Hz, 1.2 Hz, 1H), 8.11 (s, 1H); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ (ppm) -0.3, 21.1, 81.8, 102.6, 110.5, 116.0, 117.8, 124.1, 125.6, 126.5, 128.5, 128.9, 138.5, 151.5, 152.1, 186.8; IR(film): ν (cm^{-1}) 1417, 1611, 1770, 2960; MS (ESI): m/z 513 $(M+H)^+$.

5-Ethynylfluorescein (7). 5-(2-Trimethylsilylethynyl)fluorescein diacetate **6** (318 mg, 0.62 mmol) and potassium carbonate (428 mg, 3.10 mmol) were combined in 6 mL 1:1 MeOH/THF. The bright orange mixture was stirred for 20 h at 25 °C. The reaction mixture was poured into 40 mL water then acidified to pH 2 with concentrated HCl. The resulting precipitate was collected by filtration, rinsed with water, then dried *in vacuo* to give 5-ethynylfluorescein **7** (200 mg, 90%) as an orange solid: mp >400 °C (dec); R*f* 0.30 (50% EtOAc/hexanes); ^{1}H-NMR (acetone-*d*$_6$, 500 MHz): δ (ppm) 3.91 (s, 1H), 6.62 (dd, *J* = 8.5 Hz, 2.5 Hz, 2H), 6.70 (d, *J* = 8.5 Hz, 2H), 6.75 (d, *J* = 2.5 Hz, 2H), 7.31 (dd, *J* = 8 Hz, 1 Hz, 1H), 7.87 (dd, *J* = 8 Hz, 1.5 Hz, 1H), 8.01 (dd, *J* = 1.5 Hz, 0.5 Hz, 1H), 9.16 (bs, 2H); ^{13}C-NMR (acetone-d_6, 125 MHz): δ (ppm) 68.0, 81.3, 82.5, 103.3, 110.8, 113.4, 124.9, 125.4, 128.4, 128.5, 130.0, 139.2, 153.3, 153.8, 160.6, 168.6; IR(film): ν (cm^{-1}) 1258, 1606, 1693, 1756, 3071, 3279; MS (ESI): m/z 357 $(M+H)^+$; HRMS (ESI): m/z 357.0751 $(M+H)^+$, calc for $C_{22}H_{13}O_5^+$: 357.0463.

4-(Trimethylsilylethynyl)phenylboronic Acid (8). Following the procedure described in *J. Org. Chem.* **2000**, 2837, 1-bromo-4-(trimethylsilyl)benzene (4.5 g, 17.8 mmol) was dissolved in 40 mL distilled ether then cooled in a dry ice/acetone bath. 2.2 M *n*-BuLi (10.5 mL, 23.1 mmol) was added dropwise. The reaction was stirred 30 min at –78 ºC. This mixture was then added dropwise to an ether (30 mL) solution of trimethylborate (9.23 g, 103.9 mmol) at –78 ºC. The resulting mixture was stirred at –78 ºC for 30 min then at 25 ºC for 14 h. To this solution was added 100 mL 2 M HCl. After stirring vigorously at 25 ºC for 1 h, the layers were separated and the organic layer washed once with brine. The combined aqueous layers were extracted once with ether, the combined organic layers were dried ($MgSO_4$) then concentrated *in vacuo*. Purification by flash chromatography (1:1 ether/hexanes) yielded the title compound as a white solid (2.82 g, 73%): ^{1}H-NMR (acetone-d_6, 500 MHz): δ (ppm) 0.22 (s, 9H), 7.34 (m, 2H),7.42 (d, *J* = 8.0 Hz, 2H), 7.84 (d, *J* = 8.5 Hz, 2H); ^{13}C-NMR (acetone-d_6, 125 MHz): δ (ppm) –0.1, 95.2, 106.1, 125.5, 131.5, 134.9; MS (ESI): m/z 217 $(M-H)^-$;

5-(4-Ethynylphenyl)fluorescein (9). 5-Iodofluorescein diacetate **6** (100 mg, 0.18 mmol), 4-(2-trimethylsilylalkynyl)- phenylboronic acid (48 mg, 0.22 mmol), $Pd_2(dba)_3$ (5 mg, 0.03 mmol) and cesium carbonate (144 mg, 2.4 mmol) were combined in 4 mL 3:1 acetone/water. The reaction mixture was freeze-pump-thawed twice then stirred 19 h under nitrogen at 65 °C. The reaction mixture was acidified with acetic acid (*ca* 0.5 mL) then concentrated *in vacuo*. The crude yellow solid was purified by flash chromatography eluting with 5 to 10% MeOH/CH_2Cl_2 to give 65 mg (81%) of the title compound as an orange solid: mp >400 °C (dec); R*f* 0.43 (10% MeOH/$CHCl_3$); ^{1}H-NMR (acetone-

d_6, 500 MHz): δ (ppm) 3.78 (s, 1H), 6.63 (dd, *J* = 8.5 Hz, 2.5 Hz, 2H), 6.73 (d, *J* = 8.5 Hz, 2H), 6.75 (d, *J* = 2.5 Hz, 2H), 7.37 (d, *J* = 8 Hz, 1H), 7.66 (d, *J* = 8 Hz, 2H), 7.87 (d, *J* = 8.5 Hz, 2H), 8.10 (dd, *J* = 8 Hz, 1.5 Hz, 1H), 8.22 (d, J = 1.5 Hz, 1H); ^{13}C-NMR (acetone-d_6, 125 MHz): δ (ppm) 80.4, 83.8, 103.3, 111.5, 113.4, 123.1, 123.3, 125.5, 128.2, 128.9, 130.1, 133.5, 134.7, 140.4, 142.7, 153.0, 153.4, 160.4, 169.3; IR(film): ν (cm^{-1}) 1461, 1606, 1737, 3284; MS (ESI): m/z 433 $(M+H)^+$; HRMS (ESI): m/z 433.1014 $(M+H)^+$, calc for $C_{28}H_{17}O_5^+$: 433.1076.

5-Ethynylfluorescein Diacetate (10). 5-(2-Trimethylsilylethynyl)fluorescein diacetate **6** (350 mg, 0.68 mmol) was dissolved in 10 mL freshly distilled THF. TBAF (0.68 mL, 1.0 M in THF) was added then the solution immediately concentrated *in vacuo*. The crude product was purified by flash chromatography eluting with 30% EtOAc/hexanes to give 5-ethynylfluorescein diacetate **10** (142 mg 47%) as an off-white solid: mp 193-194 °C (dec), R*f* 0.36 (35 % EtOAc/hexanes); ^{1}H-NMR ($CDCl_3$, 500 MHz): δ (ppm) 2.32 (s, 6H), 3.25 (s, 1H), 6.83 (m, 4H), 7.11 (m, 2H), 7.16 (dd, *J* = 8 Hz, 1 Hz, 1H), 7.78 (dd, *J* = 8 Hz, 1.5 Hz, 1H), 8.13 (dd, *J* = 1 Hz, 0.5 Hz); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ (ppm) 21.1, 79.8, 81.5, 81.8, 110.5, 115.8, 117.8, 124.2, 124.6, 126.5, 128.7, 128.9, 138.7, 151.5, 152.1, 152.5, 168.1, 168.8; IR(film): ν (cm^{-1}) 1161, 1209, 1766, 2360, 3284; MS (ESI): m/z 441 $(M+H)^+$; HRMS (ESI): m/z 441.0976 $(M+H)^+$, *c*alc for $C_{26}H_{17}O_7^+$: 441.0974

5-(2-(4-(2-Trimethylsilyl)-ethynylphenyl)-ethynyl)-fluorescein Diacetate. 5-Ethynylfluorescein diacetate **7** (40 mg, 0.09 mmol), 1-trimethylsilyl-2-(4-bromophenyl)-ethyne (26 mg, 0.10 mmol), tetrakis(triphenylphosphine)palladium (9 mg, 0.008 mmol), copper(I) iodide (3 mg, 0.016 mmol), and triethylamine (80 mg, 0.80 mmol) were dissolved in 3 mL freshly distilled THF in a Schlenk tube. The solution was freeze-pump-thawed three times, then stirred 3 h at 55 °C under N_2. The solution was concentrated *in vacuo* then purified by flash chromatography eluting with 30% EtOAc/hexanes to yield the title compound (36 mg, 65%) as a pale yellow film: R*f* 0.50 (35% EtOAc/hexanes); ^{1}H-NMR (acetone-d_6, 500 MHz): δ (ppm) 0.27 (s, 9H), 2.33 (S, 6H), 6.85 (m, 4H), 7.11 (d, *J* = 2 Hz , 2H), 7.18

(dd, J = 8.0 Hz, 0.5 Hz, 1H), 7.48 (d, J = 9.0 Hz, 2H), 7.51 (d, J = 9.0 Hz, 2H), 7.81 (dd, J = 8.0, 1.5 Hz, 1H), 8.16 (d, J = 0.5 Hz, 1H); ^{13}C-NMR (acetone-d_6, 125 MHz): δ (ppm) –0.1, 21.1, 81.7, 89.0, 91.4, 96.8, 104.3, 110.5, 115.9, 117.8, 122.2, 123.7, 124.2, 125.5, 126.6, 128.0, 128.9, 131.5, 132.0, 138.2, 151.5, 152.08, 152.11, 168.2, 168.8; IR(film): ν (cm^{-1}) 1768, 2956; MS (ESI): m/z 616 $(M+H)^+$; HRMS (ESI): m/z 613.1671 $(M+H)^+$, calc for $C_{37}H_{29}O_7Si^+$: 613.1682.

5-(2-(4-Ethynylphenyl)-ethynyl)-fluorescein (11). 5-(2-(4-(2-Trimethylsilyl)-ethynylphenyl)-ethynyl)-fluorescein diacetate as prepared above (35 mg, 0.06 mmol) and potassium carbonate (39 mg, 0.29 mmol) were combined in 2 mL 1:1 methanol/THF. The bright orange reaction mixture was stirred 7 h at 25 °C then poured into 35 mL water. The aqueous solution was acidified to pH 2 with concentrated HCl then extracted three times with 25% iPrOH/$CHCl_3$. The combined organic extracts were dried ($MgSO_4$) then concentrated *in vacuo*. The crude product was purified by flash chromatography, eluting with 10% MeOH/$CHCl_3$. to give the title compound (24 mg, 92%) as an orange solid: mp >400 °C (dec); R*f* 0.17 (35% EtOAc/hexanes); ^{1}H-NMR (acetone-d_6, 500 MHz): δ (ppm) 3.85 (s, 1H), 6.63 (dd, J = 9.0, 2.5 Hz, 2H), 6.73 (d, J = 8.5 Hz, 2H), 6.75 (d, J = 2.0 Hz, 2H), 7.34 (d, J = 8.0, 1.0 Hz, 1H), 7.56 (d, J = 8.5 Hz, 2H), 7.64, (d, J = 8.5 Hz, 2H), 7.93 (dd, J = 8.0, 1.5 Hz, 1H), 8.10 (dd, J = 1.5, 1.0 Hz); ^{13}C-NMR (acetone-d_6, 125 MHz): δ (ppm) 78.8, 81.4, 83.6, 90.4, 91.2, 103.4, 104.4, 111.2, 113.6, 123.78, 123.84, 125.51, 125.55, 128.3, 128.8, 130.2, 132.7, 133.0, 138.7, 153.5, 160.7, 168.6; IR(film): ν (cm^{-1}) 1598, 1731, 3283; MS (ESI): m/z 457 $(M+H)^+$; HRMS (ESI): m/z 457.1072 $(M+H)^+$, calc for $C_{30}H_{17}O_5^+$: 457.1076.

Nucleoside 12. 5-Ethynylfluorescein **7** (50 mg, 0.14 mmol), 5-iodo-2’-deoxyuridine (50 mg, 0.14 mmol), tetrakis(triphenylphosphine)palladium (8 mg, 0.007 mmol), copper(I) iodide (3 mg, 0.01 mmol) and triethylamine (71 mg, 0.70 mmol) were dissolved in 2 mL DMF. The solution was freeze-pump-thawed three times then stirred under nitrogen for 3 h at 25 °C. The reaction mixture was concentrated *in vacuo* then purified by flash chromatography eluting with 10 to 20% MeOH/CH_2Cl_2 to give the desired product as an orange solid (20 mg, 24%): mp > 400 °C (dec); R*f* 0.20 (5 % MeOH/CH_2Cl_2); ^{1}H-NMR (CD_3OD), 500 MHz): δ (ppm) 2.25 – 2.38 (m, 2H), 3.79 (dd, *J* = 12.0 Hz, 3.5 Hz, 1H), 3.86 (dd, *J* = 12.0 Hz, 3.0 Hz, 1H), 3.96 (q, *J* = 3.0 Hz, 1H), 4.43 (dt, *J* = 6.0 Hz, 2.5 Hz, 1H), 6.26 (t, *J* = 6.0 Hz, 1H), 6.58 (dd, *J* = 9.0 Hz, 2.0 Hz, 2H), 6.66 (d, *J* = 9.0 Hz, 2H), 6.71 (d, *J* = 2.0 Hz, 2H), 7.20 (d, *J* = 8.0, 1H), 7.86 (dd, *J* = 8.5 Hz, 1.0 Hz, 1H), 8.12 (s, 1H), 8.54 (s, 1H); ^{13}C-NMR (CD_3OD, 125 MHz): δ (ppm) 30.7, 41.9, 47.9, 62.5, 71.8, 84.3, 87.2, 89.2, 92.1, 99.7, 103.6, 111.3, 114.0, 125.8, 126.5, 128.7, 128.9, 130.3, 139.0, 146.0, 151.1, 154.3, 161.9, 164.2, 170.4; IR(film): ν (cm^{-1}) 1606, 1708, 2941, 3400; MS (ESI): m/z 581 (M-H)$^-$.

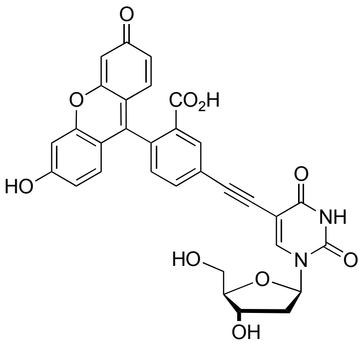
Nucleoside 13. 5-(4-Ethynylphenyl)-fluorescein **9** (71 mg, 0.16 mmol), 5-iodo-2'-deoxyuridine (58 mg, 0.16 mmol), tetrakis(triphenylphosphine)palladium (19 mg, 0.02 mmol), copper(I) iodide (6 mg, 0.03 mmol) and triethylamine (83 mg, 0.82 mmol) were dissolved in 5 mL DMF. The solution was freeze-pump-thawed three times then stirred under nitrogen for 2.5 h at 25 °C. The reaction mixture was concentrated *in vacuo* then purified by flash chromatography eluting with 15% MeOH/CH_2Cl_2 to give the desired product as an orange solid (57 mg, 53%): mp > 400 °C (dec); R*f* 0.31 (10 % MeOH/EtOAc); ^{1}H-NMR (CD_3OD, 500 MHz): δ (ppm) 2.25 – 2.38 (m, 2H), 3.76 (dd, *J* = 12.0 Hz, 3.5 Hz, 1H), 3.76 (dd, *J* = 12.0 Hz, 3.0 Hz, 1H), 3.86 (q, *J* = 3.0 Hz, 1H), 4.33 (dt, *J* = 6.0 Hz, 4.0 Hz, 1H), 6.18 (t, *J* = 6.5 Hz, 1H), 6.45 (dd, *J* = 9.0 Hz, 2.0 Hz, 2H), 6.58 (d, *J* = 2.0 Hz, 2H), 6.61 (d, *J* = 9.0 Hz, 2H), 7.19 (d, *J* = 8.0, 1H), 7.56 (d, *J* = 8.5 Hz, 2H), 7.67 (d, *J* = 8.5 Hz, 2H), 7.93 (dd, *J* = 8.0 Hz, 2.0 Hz, 1H), 8.14 (d, *J* = 1.5 Hz, 1H), 8.38 (s, 1H); ^{13}C-NMR (acetone-d_6, 125 MHz): δ (ppm) 29.7, 41.4, 62.0, 71.4, 83.9, 86.1, 88.5, 92.0, 99.3, 103.0, 111.1, 113.0, 122.9, 123.7, 125.2, 127.9, 128.5, 129.9, 132.5, 134.4, 139.5, 142.4, 144.5, 150.0, 152.7, 153.0, 160.1, 161.5, 169.0; IR(film): ν (cm^{-1}) 1606, 1698, 3062, 3405; MS (ESI): m/z 659 (M+H)$^+$.

Nucleoside 14. 5-(2-(4-Ethynylphenyl)-ethynyl-fluorescein **11** (47 mg, 0.10 mmol), 5-iodo-2'-deoxyuridine (36 mg, 0.10 mmol), tetrakis(triphenylphosphine)palladium (6 mg, 0.005 mmol), copper(I) iodide (2 mg, 0.01 mmol) and triethylamine (52 mg, 0.51 mmol) were dissolved in 2 mL DMF. The solution was freeze-pump-thawed three times then stirred under nitrogen for 2 h at 25 °C. The reaction mixture was concentrated *in vacuo* then purified by flash chromatography eluting with 15% MeOH/CH_2Cl_2 to give the desired product as an orange solid (22 mg, 31%): mp > 400 °C (dec); R*f* 0.30 (10 % MeOH/EtOAc); ^{1}H-NMR (acetone-d_6, 500 MHz): δ (ppm) 2.33 – 2.35 (m, 2H), 3.82 (dd, *J* = 12.0 Hz, 3.0 Hz, 1H), 3.87 (dd, *J* = 12.0 Hz, 3.0 Hz, 1H), 3.99 (q, *J* = 3.0 Hz, 1H), 4.54 (q, *J* = 4.5 Hz, 1H), 6.30 (t, *J* = 6.5 Hz, 1H), 6.64 (dd, *J* = 8.5 Hz, 2.5 Hz, 2H), 6.73 (d, *J* = 8.5 Hz, 2H), 6.75 (d, *J* = 2.5 Hz, 2H), 7.34 (dd, *J* = 8.0 Hz, 0.5 Hz, 1H), 7.53 (d, *J* = 9.0 Hz, 2H), 7.64 (d, *J* = 9.0 Hz, 2H), 7.94 (dd, *J* = 8.0 Hz, 1.5 Hz, 1H), 8.10 (dd, *J* = 1.5 Hz, 0.5 Hz, 1H), 8.53 (s, 1H), 10.35 (s, 1H); ^{13}C-NMR (acetone-d_6, 125 MHz): δ (ppm) 29.9, 41.8, 62.3, 71.7, 84.0, 85.4, 86.4, 88.9, 90.2, 91.4, 92.2, 99.4, 103.3, 111.1, 113.3, 123.1, 124.7, 125.4, 125.6, 125.7, 128.1, 128.5, 130.2, 132.3, 132.7, 138.8, 145.0, 153.3, 153.7, 160.4, 168.6; IR(film): ν (cm^{-1}) 1606, 1693, 2926, 3362; MS (ESI): m/z 683 $(M+H)^+$.

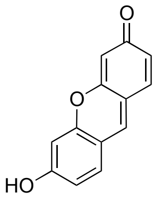
Table S1. Spectroscopic Data for Compounds **12 - 14**

Compound	λ_{max}	λ_{em}	ε	Φ_{fl}
12	320	520	23,900	0.53
	492		56,990	
13	322	516	39,200	ND
	492		63,300	
14	330	520	55,000	ND
	492		63,700	

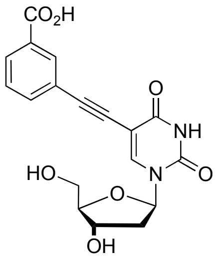
Figure S1. Overlay for Donor and Acceptor Components of Cassette **12**



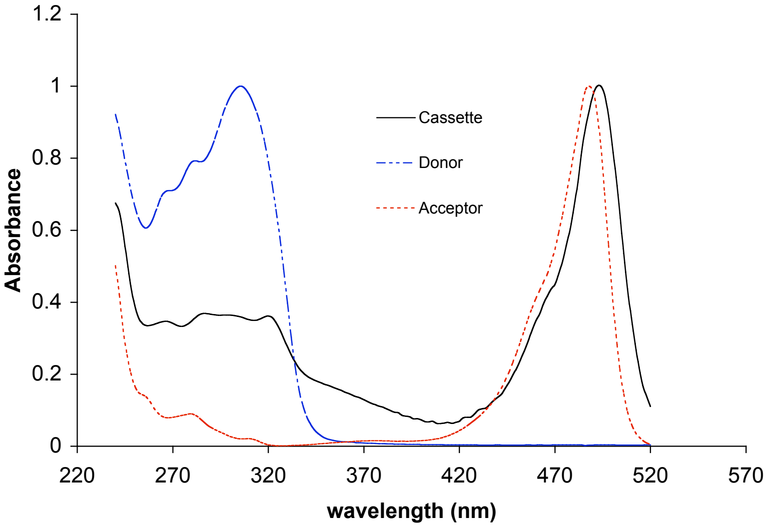
cassette **12**



acceptor part



donor part



General Procedure for Gel Assays. Unlabeled 2'-deoxyribonucleoside triphosphates (dNTPs) and 2'3'-dideoxyribonucleoside triphosphates (ddNTPs) were purchased from Amersham Bioscience Corp. (Piscataway, NJ). Ampli*Taq*FS DNA polymerase (*Taq*FS) and 6-carboxytetramethylrhodamine (6-TAMRA)-ddTTP were purchased from Applied Biosystems, Inc. (ABI, Foster City, CA). *Taq*FS is the full-length form of *Thermophilus aquaticus* DNA polymerase I and has been modified by the substitutions G46D to abrogate the 5'→ 3' exonuclease activity and F667Y to increase ddNTP incorporation [3,4]. DNA synthesis reagents were purchased from Glen Research (Sterling, VA). The Oligo-template (5'-TACGGAGGTGGACTGGCCGTCGTTTTACA) and the R931 universal sequencing primer (5'-NH_2-C_6-TTGTAAAACGACGGCCAGT) were synthesized trityl-on using an ABI model 394 DNA synthesizer and purified by reverse-phase high performance liquid chromatography (RP-HPLC) as previously described [5]. BODIPY-R6G succinimidyl ester (SE) dye was purchased from Molecular Probes (Eugene, OR), coupled to the R931 primer and purified by RP-HPLC as previously described [6].

Incorporation assays. The Oligo-template was standardized for *Taq*FS prior to testing compounds **1**-**3** and modified for direct detection of fluorescent nucleotides using an ABI model 377 DNA sequencer. For comparison of the novel blue fluorescein thymidine nucleotides and yellow 6-TAMRA-ddTTP, the green BODIPY-R6G primer was used to differentiate incorporation events based on gel mobility and color. Briefly, BODIPY-R6G-labeled R931 primer was annealed to the Oligo-template (0.05 pmol to 0.2 pmol, respectively per 5 μL) in 50mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$ by heating to 80^oC for 5 minutes and then slowly cooled to 25^oC. For each reaction, 5 μL aliquots of the primer-template samples were added to enzymatic reactions mixtures containing 6.4 units *Taq*FS and final concentrations of 50 μM ddCTP, dATP, and ddGTP each in 50mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$. Thymidine analogs were added at various concentrations in a final volume of 10 μL at 70^oC for 10 minutes. The samples were stopped by the addition of 10 μL of deionized formamide, 10 mM EDTA, 10 mg/mL blue dextran. The reactions were heated to 65°C for 3 minutes, chilled on ice, and 1 μL was resolved on 10% LongRanger polyacrylamide (BioWhittaker Molecular Applications, Rockland, ME), 6M urea gels and analyzed using ABI Sequencing Analysis version 3.4 software. The ABI run module was modified to collect blue, green, and yellow fluorescent signals in "CCD bins" corresponding to wavelength ranges between approximately 516-527 nm, 540-552 nm, and 570-582 nm, respectively. Fluorescent signals for the desired termination bands were obtained directly from the raw unprocessed data by measuring the peak height and background intensities from the green CCD bin only.

The assignment of the corresponding fluorescent nucleotide termination bands was based on mobility pattern relative to unlabeled dTTP and ddTTP controls and the color of the incorporated product. Fluorescent signals, which contained two different fluorophores (i.e., the green 5'-BODIPY-R6G primer incorporated with the blue compound **3a**) were artificially high primarily because of "cross-talk" of spectrally overlapping neighboring dyes. Signal enhancements due to energy-transfer (ET) was considered negligible because we previously reported no observable enhancement of 5-FAM/BODIPY-R6G when separated 0-to-3 bases apart using the R931 primer [6] and the unusually large distance (25 bases apart) compare to commercially available ET dye-primers (10 bases apart) [7]. We empirically determined the cross-talk correction factor for 5-FAM /BODIPY-R6G and BODIPY-R6G/6-TAMRA dye-labeled termination products to be 1.86 and 1.26, respectively (data not shown).

IC$_{50}$ values and Incorporation ratios. The percentage incorporated ($I_{\%}$) was calculated for each nucleotide tested as follows:

$$I_{\%} = \frac{T_{\mathrm{CT}}}{T_{\mathrm{CT}} + U_{\mathrm{P}}}$$

where T_{CT} is the cross-talk corrected peak height and U_{P} is the unincorporated 5'-BODIPY-R6G extension product. $I_{\%}$ values were plotted against their respective final concentrations to derived an IC$_{50}$ value (effective concentration at 50% incorporation). To express the relative performance of the nucleotides tested, which compete of the same active site of DNA polymerases, an incorporation ratio (I_R) based on IC_{50} values was calculated as follows:

$$I_R = \frac{(IC_{50})_A}{(IC_{50})_B}$$

where $(IC_{50})_A$ and $(IC_{50})_B$ are values for any nucleotide comparison. I_R values greater than one indicate that compound B is incorporated more efficiently than compound A. For example, *Taq*FS that contains the F667Y substitution prefers ddTTP over dTTP approximately 7.6-fold, which is consistent with previous data. [3]

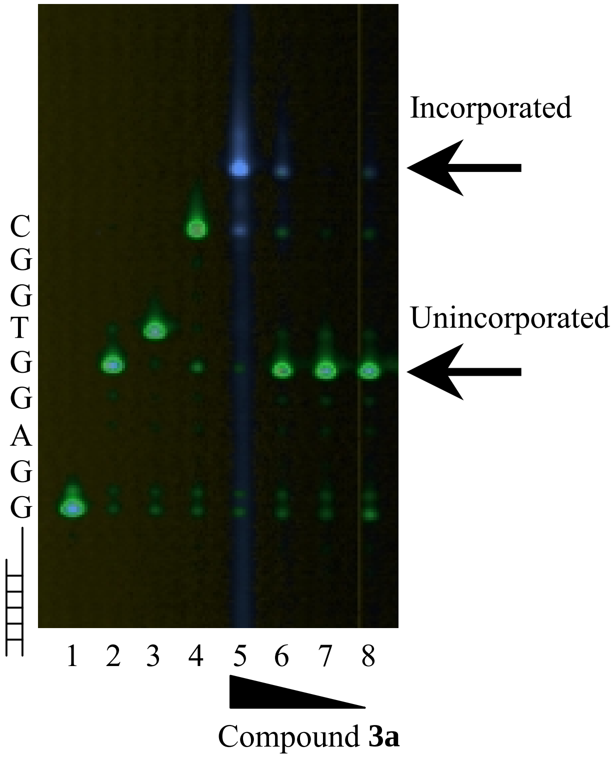


Figure S2. Incorporation of compounds **3a** by *Taq*FS DNA polymerase. All reactions contained *Taq*FS, BODIPY-R6G labeled R931 primer, oligo-template and reaction buffer. Lane 1 contained only the primer; lanes 2-8 contained 50 nM each of dCTP, dATP, and ddGTP. Additionally, lanes 3 and 4 contained 10 nM ddTTP or 10 nM dTTP, respectively and lanes 5-8 contained **3a** at 250 nM, 100 nM, 50 nM, or 25 nM, respectively.

(1) Bong, D. T.; Ghadiri, M. R. *Org. Lett.* **2001**, *3*, 2509-2511.
(2) Casalnuovo, A.; Calabrese, J. C. *J. Am. Chem Soc.* **1990**, *112*, 4324-4330.
(3) Tabor, S.; Richardson, C. C. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 6339-6343.
(4) Brandis, J. W. *Nucleic Acids Res.* **1999**, *27*, 1912-1918.
(5) Metzker, M. L.; Raghavachari, R.; Richards, S.; Jacutin, S. E.; Civitello, A.; Burgess, K.; Gibbs, R. A. *Nucl. Acids Res.* **1994**, *22*, 4259-4267.
(6) Metzker, M. L.; Lu, J.; Gibbs, R. A. *Science* **1996**, *271*, 1420-1422.
(7) Ju, J.; Kheterpal, I.; Scherer, J. R.; Ruan, C.; Fuller, C. W.; Glazer, A. N.; Mathies, R. A. *Anal. Biochem.* **1995**, *231*, 131-140.