3'-O-Modified Nucleotides as Reversible Terminators for Pyrosequencing

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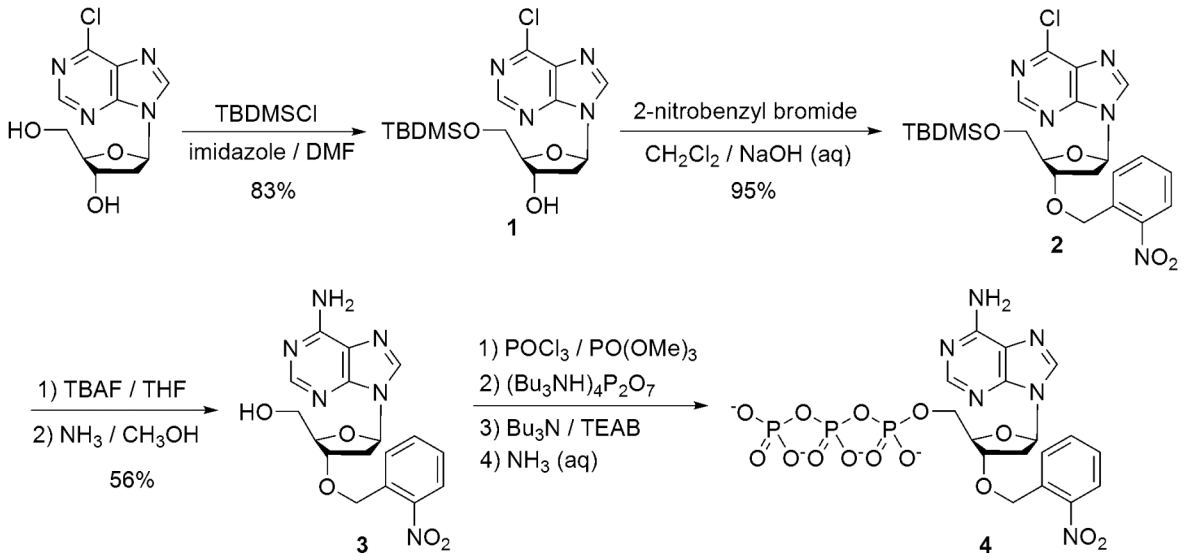
SI Appendix

All chemicals were purchased from Sigma-Aldrich unless otherwise indicated. Oligonucleotides used as primers or templates were synthesized on an Expedite nucleic acid synthesizer (Applied BioSystems) or purchased from Midland. ^{1}H NMR spectra were recorded on a Bruker DPX-300 (300 MHz), DPX-400 (400 MHz) or DPX-500 (500 MHz) spectrometer, and reported in ppm from a $CDCl_3$, CD_3OD or DMSO-d_6 internal standard (7.26, 3.31 or 2.50 ppm respectively). Data were reported as follows: (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, ddd = doublet of doublets of doublets, coupling constant (*J*) in Hz, integration, assignment). Proton decoupled ^{13}C NMR spectra were recorded on a Bruker DPX-300 (75 MHz) or Bruker DPX-400 (100 MHz) spectrometer and reported in ppm from a $CDCl_3$, CD_3OD or DMSO-d_6 internal standard (77.0, 49.0 or 39.5 ppm respectively). Proton decoupled ^{31}P NMR spectra were recorded on a Bruker DPX-300 (121.4 MHz) spectrometer and reported in ppm from an 85% H_3PO_4 external standard. High-resolution Mass Spectra (HRMS) were obtained on a JEOL JMS HX 110A mass spectrometer. Mass measurement of DNA was performed on a Voyager DE MALDI-TOF mass spectrometer (Applied Biosystems). Photolysis was performed by using a Spectra Physics GCR-150-30 Nd-yttrium/aluminum garnet laser that generates light pulses at 355 nm at a frequency of 30 Hz with a light intensity at 1.5 W/cm^2. 9°N polymerase (exo-) A485L/Y409V and Klenow (exo-) fragment DNA polymerases were obtained from New England Biolabs. Starting materials **5**, **12** and **20** were purchased from Berry & Associates (Ann Arbor, Michigan). Phosphoramidites and columns for nucleic acid synthesis were purchased from Glen Research (Sterling, VA). The 3'-O-modified nucleotides [3'-*O*-(2-nitrobenzyl)-dNTPs] were purified with reverse-phase HPLC on a 150 × 4.6-mm C18 column (Supleco Columns), mobile phase: A, 8.6 mM triethylamine/100 mM hexafluoroisopropyl alcohol in water (pH 8.1); B, methanol. Elution was performed with 100% A isocratic over 10 min, followed by a linear gradient of 0-50% B for 20 min and then 50% B isocratic over another 30 min.

I. Synthesis of 3'-*O*-(2-nitrobenzyl)-dATP



9-[β-D-5'-*O*-(tert-butyldimethylsilyl)-2'-deoxyribofuranosyl]-6-chloropurine (Compound 1). To a stirred solution of 9-[*β*-D-5'-hydroxy-2'-deoxyribofuranosyl]-6-chloropurine (Berry & Associates) (1.00 g; 3.69 mmol) and imidazole (554 mg; 8.12 mmol) in anhydrous DMF (18 ml), *tert*-butyldimethylsilyl chloride (TBDMSCl) (611 mg; 3.93 mmol) was added. The reaction mixture was stirred at room temperature for 20 h. After concentration, the residue was purified with flash column chromatography (ethyl acetate/hexane, 2:1) to afford **1** as a colorless oil (1.18 g; 83% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 8.73 (s, 2H, 2-H and 8-H), 6.54 (t, *J* = 6.3 Hz, 1H, 1'-H), 4.61 (dt, *J* = 4.1, 5.9 Hz, 1H, 3'-H), 4.06 (m, 1H, 4'-H), 3.95 (dd, *J* = 3.6, 11.4 Hz, 1H, one of 5'-H), 3.85 (dd, *J* = 3.6, 11.4 Hz, 1H, one of 5'-H), 2.80-2.87 (m, 1H, one of 2'-H), 2.53-2.61 (ddd, *J* = 4.5, 6.5, 13.6 Hz, 1H, one of 2'-H), 0.86 (s, 9H, $C(CH_3)_3$), 0.06 (s, 3H, one of $SiCH_3$), 0.05 (s, 3H, one of $SiCH_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 152.8, 152.3, 151.2, 146.2, 132.7, 89.2, 86.6, 71.9, 64.1, 41.5, 26.4, 19.2, -5.3; HRMS (FAB+) calcd for $C_{16}H_{26}O_3N_4SiCl$ [$(M+H)^+$]: 385.1463, found: 385.1467.

9-[β-D-5'-O-(tert-butyldimethylsilyl)-3'-O-(2-nitrobenzyl)-2'-deoxyribofuranosyl]-6-chloropurine (Compound 2). To a stirred solution of **1** (1.18 g; 3.07 mmol) in CH_2Cl_2 (94 ml), tetrabutylammonium bromide (TBAB) (509 mg; 1.55 mmol), 2-nitrobenzyl bromide (2.03 g; 9.21 mmol) and 40% aqueous NaOH solution (47 ml) were added. The reaction mixture was stirred at room temperature for 1 h. Ethyl acetate (300 ml) was added and the organic layer was separated. The aqueous layer was extracted with ethyl acetate (2×100 ml). The combined organic layer was washed consequently with saturated aqueous $NaHCO_3$ and brine solution, and dried over anhydrous Na_2SO_4. After evaporation, the residue was purified with flash column chromatography (ethyl acetate/hexane, 1:2) to afford **2** as a yellow oil (1.52 g; 95% yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.74 (s, 1H, 2-H), 8.50 (s, 1H, 8-H), 8.10 (dd, *J* = 2.0, 8.2 Hz, 1H, one of C_6H_4), 7.80 (d, *J* = 7.7 Hz, 1H, one of C_6H_4), 7.66-7.71 (t, *J* = 7.6 Hz, 1H, one of C_6H_4), 7.47-7.53 (m, 1H, one of C_6H_4), 6.56 (dd, *J* = 6.1, 7.5 Hz, 1H, 1'-H), 4.92-5.03 (m, 2H, $OCH_2C_6H_4$),

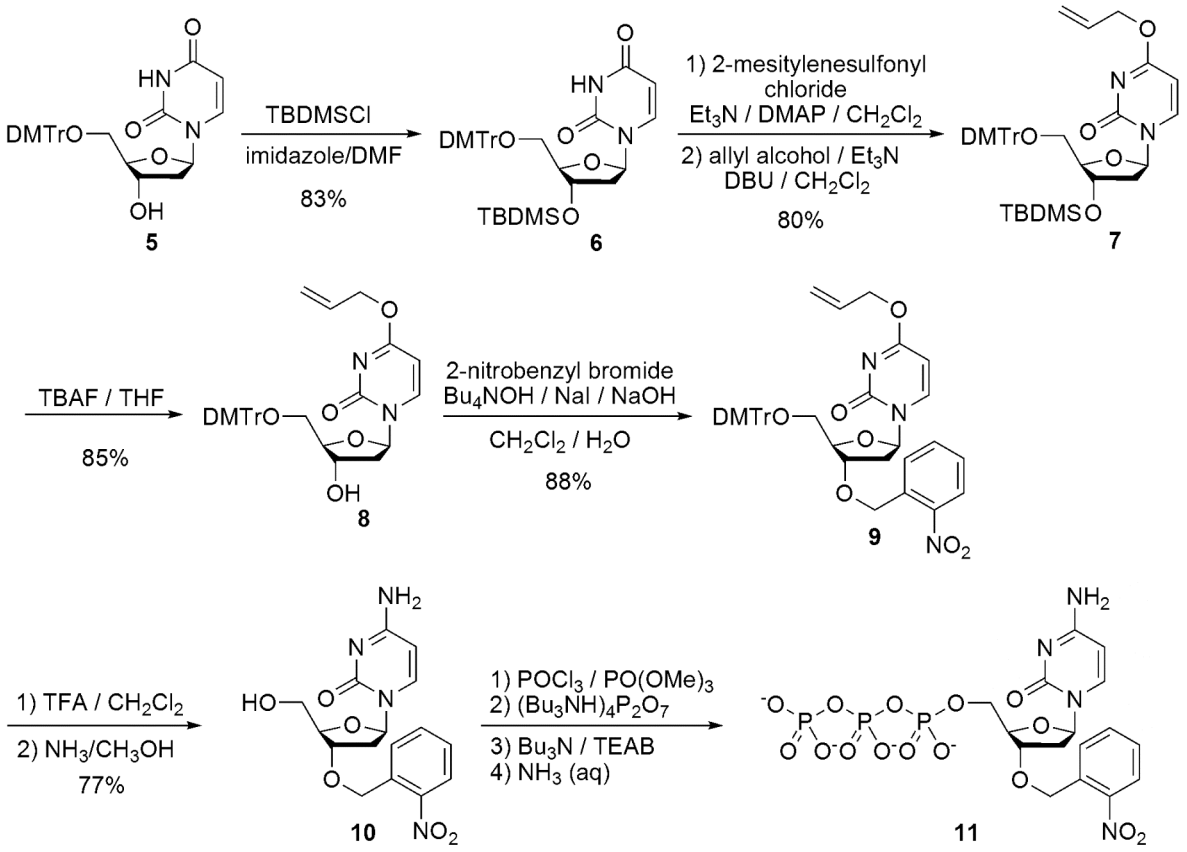
4.45 (m, 1H, 3'-H), 4.34 (m, 1H, 4'-H), 3.94 (dd, *J* = 4.0, 11.2 Hz, 1H, one of 5'-H), 3.86 (dd, *J* = 3.0, 11.2 Hz, 1H, one of 5'-H), 2.67-2.81 (m, 2H, 2'-H), 0.91 (s, 9H, C(CH_3)$_3$), 0.11 (s, 3H, one of SiCH_3), 0.10 (s, 3H, one of SiCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.7, 151.0, 150.8, 147.2, 143.5, 133.8, 133.6, 132.0, 128.7, 128.3, 124.6, 85.5, 85.1, 80.2, 68.0, 63.4, 38.4, 25.8, 18.2, -5.5, -5.6; HRMS (FAB+) calcd for $C_{23}H_{31}O_5N_5SiCl$ [$(M+H)^+$]: 520.1783, found: 520.1804.

3'-O-(2-nitrobenzyl)-2'-deoxyadenosine (Compound 3). To a stirred solution of **2** (1.52 g; 2.92 mmol) in anhydrous THF (70 ml), tetrabutylammonium fluoride (TBAF) in THF solution (1.0 M; 3.2 ml; 3.20 mmol) was added. The reaction mixture was stirred at room temperature for 1 h. After concentration, the residue was dissolved in a mixture of dioxane (25 ml) and 7 M methanolic ammonia (50 ml). The solution was stirred in an autoclave at 85-90°C for another 12 h. After evaporation, the residue was purified with flash column chromatography (CH_3OH/CH_2Cl_2, 1:15) to afford **3** as a white solid (632 mg; 56% yield): ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.34 (s, 1H, 2-H), 8.13 (s, 1H, 8-H), 8.07 (d, *J* = 8.1 Hz, 1H, one of C_6H_4), 7.76-7.84 (m, 2H, two of C_6H_4), 7.57-7.63 (m, 1H, one of C_6H_4), 7.31 (*br* s, 2H, 6-NH_2), 6.34 (dd, *J* = 6.2, 8.0 Hz, 1H, 1'-H), 5.35 (*br* s, 1H, 5'-O*H*), 4.88-4.96 (m, 2H, O$CH_2C_6H_4$), 4.37 (m, 1H, 3'-H), 4.13 (m, 1H, 4'-H), 3.53-3.67 (m, 2H, 5'-H), 2.81-2.90 (ddd, *J* = 5.9, 8.2, 13.6 Hz, 1H, one of 2'-H), 2.49-2.57 (m, 1H, 2'-H, partly superimposed by solvent signal); ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.2, 152.5, 149.0, 147.7, 139.7, 133.8, 133.6, 129.3, 128.8, 124.5, 119.3, 85.3, 84.2, 80.4, 67.1, 62.0, 36.3; HRMS (FAB+) calcd for $C_{17}H_{19}O_5N_6$ [$(M+H)^+$]: 387.1417, found: 387.1432.

3'-O-(2-nitrobenzyl)-2'-deoxyadenosine-5'-triphosphate (Compound 4). Compound **3** (114 mg; 0.29 mmol) and proton sponge (75.8 mg; 0.35 mmol) were dried in a vacuum desiccator over P_2O_5 overnight before dissolving in trimethyl phosphate (0.60 ml). Freshly distilled $POCl_3$ (33 μl, 0.35 mmol) was added dropwise at 0°C and the mixture was stirred for 2 h. Subsequently, a well-vortexed mixture of tributylammonium pyrophosphate (552 mg) and tributylamine (0.55 ml; 2.31 mmol) in anhydrous DMF (2.33 ml) was added in one potion at room temperature and stirred for 30 min. Triethyl ammonium bicarbonate solution (TEAB) (pH 8.0; 15 ml; 0.1 M) was then added and the mixture was stirred for 1 h at room temperature. The resulting mixture was concentrated and the residue was diluted with 5 ml of water. The crude mixture was then purified with anion exchange chromatography on DEAE-Sephadex A-25 at 4°C using a gradient of TEAB (pH 8; 0.1-1.0 M). Further purification by RP HPLC to give compound **4** as colorless syrup: ^{1}H NMR (300 MHz, D_2O) δ 8.07 (s, 1H, 2-H), 8.03 (s, 1H, 8-H), 7.91 (m, 1H, one of C_6H_4), 7.71-7.76 (m, 2H, two of C_6H_4), 7.52-7.59 (m, 1H, one of C_6H_4), 6.56 (dd, *J* = 6.4, 7.7 Hz, 1H, 1'-H), 4.97 (m, 2H, O$CH_2C_6H_4$), 4.58 (m, 1H, 3'-H), 4.40 (m, 1H, 4'-H), 4.05-4.20 (m, 2H, 5'-H), 2.55-2.63 (m, 2H, 2'-H); ^{31}P NMR (121.4 MHz, D_2O) δ -8.0 (d, *J* = 19.8 Hz, 1P, γ-P), -11.2 (d, *J* = 19.1Hz, 1P, α-P), -22.5 (t, *J* = 19.4 Hz, 1P, β-P).

3'-*O*-(2-nitrobenzyl)-dATP was further characterized by single base extension reaction to generate DNA extension product and characterized by MALDI-TOF MS as shown in Fig 3*E* of the main text.

II. Synthesis of 3'-*O*-(2-nitrobenzyl)-dCTP



3'*-O-*(*tert-*butyldimethylsilyl)-5'-O-*(4,4'-dimethoxytrityl)-2'-deoxyuridine (Compound 6). To a stirred solution mixture of **5** (4.00 g; 7.50 mmol) in DMF (15 ml), imidazole (1.90 g; 15.0 mmol) and TBDMSCl (1.80 g; 12.0 mmol) were added. The reaction mixture was stirred for 4 h at room temperature. After concentration, the residue was purified with flash column chromatography (hexane/ethyl acetate, 3:1~1:1) to afford **6** as white foam (4.01 g; 83% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 7.94 (d, *J* = 8.0 Hz, 1H, 6-H), 7.43 (d, *J* = 8.4 Hz, 2H, two aromatic protons of DMTr), 7.33-7.30 (m, 7H, seven aromatic protons of DMTr), 6.88 (d, *J* = 8.4 Hz, 4H, four ortho protons to CH_3O of DMTr), 6.23 (dd, *J* = 4.0, 6.4 Hz, 1H, 1'-H), 5.39 (d, *J* = 8.0 Hz, 1H, 5-H), 4.55 (m, 1H, 3'-H), 3.95 (m, 1H, 4'-H), 3.80 (s, 6H, two CH_3O), 3.51 (m, 1H, one of 5'-H), 3.37 (m, 1H, one of 5'-H), 2.31 (m, 2H, 2'-H), 0.86 (s, 9H, $C(CH_3)_3$), 0.07 (s, 3H, one of $SiCH_3$), 0.01 (s, 3H, one of $SiCH_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 165.1, 159.3, 151.0, 144.9, 141.3, 135.72, 135.67, 130.4, 128.4, 127.9, 127.1, 113.2, 101.6, 87.2, 86.8, 85.6, 71.7, 62.6, 54.8, 41.1, 25.2, 17.8, -5.5, -5.7; HRMS (FAB+) calcd for $C_{36}H_{44}N_2O_7Si$ (M^+): 644.2918, found: 644.2927.

4*-O-*allyl-3'*-O-*(*tert-*butyldimethylsilyl)-5'-O-*(4,4'-dimethoxytrityl)-2'-deoxyuridine (Compound 7). To a solution of **6** (3.90 g; 6.05 mmol), triethylamine (1.28 g; 12.7 mmol) and DMAP (4-dimethylaminopyridine) (80.0 mg; 0.64 mmol) in CH_2Cl_2 (60 ml), 2-mesitylenesulfonyl chloride (2.92 g; 12.7 mmol) were added. The reaction mixture was stirred for 3 h at room temperature and then diluted with CH_2Cl_2 (50 ml) and washed with water (30 ml) and saturated aqueous $NaHCO_3$ solution (30

ml). The organic layer was separated and dried over anhydrous Na_2SO_4. After evaporation, the residue was dissolved in CH_2Cl_2 (30 ml) to which allyl alcohol (6.00 ml; 88.3 mmol) and triethylamine (8.00 ml; 56.7 mmol) were added, and the resulting mixture was stirred at 0°C for 15 min. DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) (1.00 ml; 6.50 mmol) was then added and the reaction temperature was allowed to warm to room temperature and stirred for another 10 h. The reaction mixture was then diluted with CH_2Cl_2 (50 ml) and washed with brine. The organic layer was dried over anhydrous $MgSO_4$. After evaporation, the crude product was further purified with flash column chromatography (hexane/ethyl acetate, 4:1) to afford **7** as white foam (3.28 g; 80% yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.27 (d, *J* = 7.2 Hz, 1H, 6-H), 7.42 (d, *J* = 8.4 Hz, 2H, two aromatic protons of DMTr), 7.34-7.26 (m, 7H, seven aromatic protons of DMTr), 6.85 (d, *J* = 8.4 Hz, 4H, four ortho protons to CH_3O of DMTr), 6.29 (dd, *J* = 4.0, 6.4 Hz, 1H, 1'-H), 6.05 (m, 1H, CH_2=*CH*CH_2), 5.62, (d, *J* = 7.6 Hz, 1H, 5-H), 5.39 (dd, *J* = 1.6, 17.0 Hz, 1H, one of *CH_2*=$CHCH_2$), 5.29 (dd, *J* = 0.8, 10.4 Hz, 1H, one of *CH_2*=$CHCH_2$) 4.90 (d, *J* = 5.5 Hz, 2H CH_2=CH*CH_2*), 4.51 (m, 1H, 3'-H), 3.99 (m, 1H, 4'-H), 3.81 (s, 6H, two CH_3O), 3.51 (m, 1H, one of 5'-H), 3.37 (m, 1H, one of 5'-H), 2.54 (m, 1H, one of 2'-H), 2.06 (m, 1H, one of 2'-H), 0.86 (s, 9H, C(C*H_3*)$_3$), 0.03 (s, 3H, one of $SiCH_3$), -0.03 (s, 3H, one of $SiCH_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4, 159.1, 156.1, 144.7, 143.4, 135.79, 135.69, 132.5, 130.6, 128.6, 128.3, 127.5, 119.0, 113.6, 95.7, 87.2, 86.9, 86.7, 70.7, 68.0, 62.2, 55.7, 42.6, 26.1, 18.3, -4.2, -4.6; HRMS (FAB+) calcd for $C_{39}H_{50}N_2O_7Si$ [$(M+H)^+$]: 685.3309, found: 685.3299.

4*-O-*allyl*-5'-O-*(4,4'-dimethoxytrityl)*-2'-deoxyuridine (Compound 8). To a solution of **7** (1.40 g; 2.04 mmol) in THF (30 ml), TBAF in THF solution (1.0 M; 2.50 ml; 2.50 mmol) was added slowly. The reaction mixture was stirred for 1 h at room temperature. After concentration, the residue was dissolved in ethyl acetate (70 ml) and then washed with brine. The organic layer was dried over anhydrous Na_2SO_4. After evaporation, the crude product was further purified with flash column chromatography (hexane/ethyl acetate, 1:1~1:4) to afford **8** as white foam (986 mg; 85% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 8.22 (d, *J* = 7.6 Hz, 1H, 6-H), 7.44-7.41 (m, 2H, two aromatic protons of DMTr), 7.34-7.26 (m, 7H, seven aromatic protons of DMTr), 6.88 (d, *J* = 8.4 Hz, 4H, four ortho protons to CH_3O of DMTr), 6.20 (dd, *J* = 4.0, 6.4 Hz, 1H, 1'-H), 6.05 (m, 1H, CH_2=*CH*CH_2), 5.72, (d, *J* = 7.2 Hz, 1H, 5-H), 5.40 (dd, *J* = 1.6, 17.6 Hz, 1H, one of *CH_2*=$CHCH_2$), 5.29 (dd, *J* = 1.6, 10.4 Hz, 1H, one of *CH_2*=$CHCH_2$), 4.90 (m, 2H, CH_2=CH*CH_2*), 4.55 (m, 1H, 3'-H), 4.08 (m, 1H, 4'-H), 3.80 (s, 6H, two CH_3O), 3.45 (d, *J* = 3.2 Hz, 2H, 5'-H), 2.54 (m, 1H, one of 2'-H), 2.06 (m, 1H, one of 2'-H); ^{13}C NMR (100 MHz, CD_3OD) δ 171.8, 159.3, 157.0, 145.1, 144.0, 135.9, 135.7, 132.4, 130.4, 128.4, 127.9, 127.0, 118.0, 113.2, 95.8, 87.4, 87.1, 86.9, 70.5, 67.7, 63.0, 54.8, 41.5; HRMS (FAB+) calcd for $C_{33}H_{35}N_2O_7$ [$(M+H)^+$]: 571.2444, found: 571.2457.

4*-O-*allyl*-5'-O-*(4,4'-dimethoxytrityl)*-3'-O-*(2-nitrobenzyl)*-2'-deoxyuridine (Compound 9). To a vigorously stirred mixture of **8** (750 mg; 1.32 mmol), Bu_4NOH (tetrabutylammonium hydroxide) (0.60 ml; 55-60% in water) and NaI (20.0 mg; 0.13 mmol) in CH_2Cl_2/H_2O (4.0 ml/4.0 ml), aqueous NaOH solution (1.0 M; 4.0 ml; 4.0 mmol) was added. The mixture was stirred for 10 min at room temperature, 2-nitrobenzyl bromide (570 mg; 2.64 mmol) in 3.0 ml of CH_2Cl_2 was added and the resulting reaction mixture was stirred for another 3 h at room temperature. The reaction mixture was diluted with CH_2Cl_2

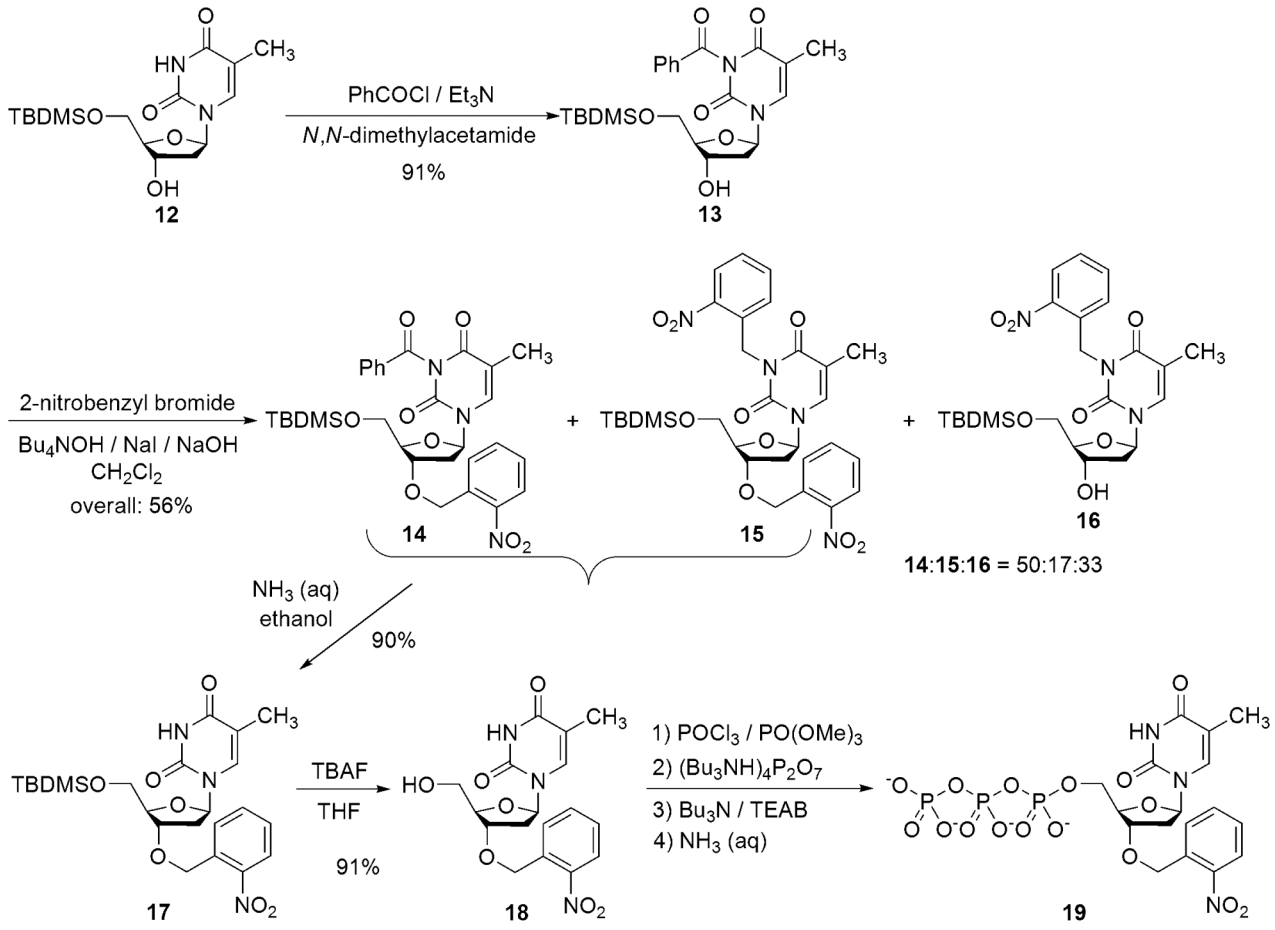
(70 ml) and then washed with brine. The organic layer was dried over anhydrous Na_2SO_4. After evaporation, the crude product was then purified with flash column chromatography (hexane/ethyl acetate, 3:1~1:1) to afford **9** as white solid (819 mg; 88% yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (m, 2H, 6-H and one of C_6H_4), 7.76 (d, J = 8.0 Hz, 1H, one of C_6H_4), 7.64 (m, 1H, one of C_6H_4), 7.46-7.41 (m, 3H, two aromatic protons of DMTr and one of C_6H_4), 7.34-7.26 (m, 7H, seven aromatic protons of DMTr), 6.85-6.83 (m, 4H, four ortho protons to CH_3O of DMTr), 6.35 (dd, J = 4.0, 6.4 Hz, 1H, 1'-H), 6.05 (m, 1H, CH_2=*CH*CH_2), 5.71 (d, J = 7.6 Hz, 1H, 5-H), 5.40 (dd, J = 1.6, 17.6 Hz, 1H, one of *CH_2*=$CHCH_2$), 5.29 (dd, J = 1.6, 10.4 Hz, 1H, one of *CH_2*=$CHCH_2$), 4.90 (m, 4H, CH_2=CH*CH_2* and O*CH_2*C_6H_4), 4.29 (m, 2H, 3'-H and 4'-H), 3.80 (s, 6H, two CH_3O), 3.50 (m, 2H, 5'-H), 2.80 (m, 1H, one of 2'-H), 2.26 (m, 1H, one of 2'-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4, 159.1, 156.1, 147.4, 144.8, 143.1, 135.75, 135.71, 134.7, 134.2, 132.5, 130.4, 129.0, 128.6, 128.5, 128.4, 127.5, 125.1, 119.1, 113.7, 95.9, 87.4, 87.2, 84.7, 79.8, 68.7, 68.1, 63.4, 55.6, 39.4; HRMS (FAB+) calcd for $C_{40}H_{40}N_3O_9$ [$(M+H)^+$]: 706.2765, found: 706.2781.

3'-O-(2-nitrobenzyl)-2'-deoxycytidine (Compound 10). To a solution of **9** (706 mg; 1.0 mmol) in CH_2Cl_2 (30 ml), trifluoroacetic acid (TFA) (0.90 ml) was added slowly at room temperature. The resulting red solution was stirred for 10 min at room temperature and then quenched with saturated aqueous $NaHCO_3$ solution (30 ml). The mixture was diluted with CH_2Cl_2 (30 ml). The organic layer was separated, washed with saturated $NaHCO_3$ solution (2 × 15 ml) and dried over anhydrous Na_2SO_4, and then concentrated. The residue was dissolved in ammonia solution in methanol (7 M; 25 ml) and stirred in a sealed tube for 20 h at 55^{o}C. After evaporation, the crude product was then purified with flash column chromatography (CH_3OH/CH_2Cl_2, 1:10) to afford **10** as white solid (280 mg; 77% yield): ^{1}H NMR (300 MHz, CD_3OD) δ 7.98 (m, 2H, 6-H and one of C_6H_4), 7.77 (d, J = 7.6 Hz, 1 H, one of C_6H_4), 7.70 (t, J = 7.5 Hz, 1H, one of C_6H_4), 7.52 (t, J = 7.5, 1H, one of C_6H_4), 6.27 (dd, J = 6.0, 8.1 Hz, 1H, 1'-H), 5.93 (d, J = 7.6 Hz, 1H, 5-H), 4.91 (s, 2H, O*CH_2*C_6H_4), 4.30 (m, 1H, 3'-H), 4.17 (m, 1H, 4'-H), 3.79 (m, 2H, 5'-H), 2.56 (m, 1H, one of 2'-H), 2.18 (m, 1H, one of 2'-H); ^{13}C NMR (75 MHz, CD_3OD) δ 166.6, 157.2, 148.2, 141.6, 134.2, 133.7, 129.4, 128.7, 124.6, 95.3, 86.8, 85.7, 80.5, 68.1, 62.3, 38.0; HRMS (FAB+) calcd for $C_{16}H_{19}N_4O_6$ [$(M+H)^+$]: 363.1305, found: 363.1317.

3'-O-(2-nitrobenzyl)-2'-deoxycytidine-5'-triphosphate (Compound 11). The preparation procedure was similar to the synthesis of **4**: ^{1}H NMR (300 MHz, D_2O) δ 8.36 (s, 1H, 6-H), 7.96 (d, J = 7.2 Hz, 1 H, one of C_6H_4), 7.62 (m, 2H, two of C_6H_4), 7.47 (m, 1H, one of C_6H_4), 6.28 (t, J = 6.0 Hz, 1H, 1'-H), 5.92 (d, J = 6.0 Hz, 1H, 5-H), 4.88 (s, 2H, O*CH_2*C_6H_4), 4.50 (m, 1H, 3'-H), 4.40 (m, 1H, 4'-H), 4.18 (dd, J = 3.4, 12.0 Hz, 1H, one of 5'-H), 4.10 (dd, J = 3.4, 12.0 Hz, 1H, one of 5'-H), 2.52 (m, 1H, one of 2'-H), 2.17 (m, 1H, one of 2'-H); ^{31}P NMR (121.5 MHz, D_2O) δ -7.9 (d, J = 20.8 Hz, 1P, γ-P), -11.4 (d, J = 19.4 Hz, 1P, α-P), -22.3 (t, J = 20.1 Hz, 1P, β-P).

3'-*O*-(2-nitrobenzyl)-dCTP was further characterized by single base extension polymerase reaction to generate DNA extension product and characterized by MALDI-TOF MS as shown in Fig 3*F* of the main text.

III. Synthesis of 3'-*O*-(2-Nitrobenzyl)-dTTP



3*-N-*benzoyl-5'*-O-*(*tert-*butyldimethylsilyl)thymidine (Compound 13). 5'-*O*-(*tert*-butyldimethyl)-thymidine **12** (1.0 g; 2.81 mmol) was dissolved in *N*,*N*-dimethylacetamide (5.0 ml), and then triethylamine (0.47 ml; 3.37 mmol) was added. Cooled to 0°C, benzoyl chloride (0.36 ml; 3.09 mmol) was added and the reaction mixture was then stirred overnight. After filtration and concentration, the residue was diluted with water and extracted with ethyl acetate (50 ml × 3). The organic layer was combined and dried over anhydrous Na_2SO_4. After concentration, the residue was recrystallized from ethanol to afford the desired compound **13** as colorless solid (1.18 g; 91% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 7.96 (d, J = 7.1 Hz, 1H, one of C_6H_5), 7.95 (d, J = 7.0 Hz, 1H, one of C_6H_5), 7.78 (s, 1H, 6-H), 7.70-7.71 (m, 1H, one of C_6H_5), 7.52-7.56 (m, 2H, two of C_6H_5), 6.25 (dd, J = 6.3, 8.0 Hz, 1H, 1'-H), 4.38-4.39 (m, 1H, 3'-H), 4.00 (m, 1H, 4'-H), 3.92-3.93 (m, 1H, one of 5'-H), 3.87-3.88 (m, 1H, one of 5'-H), 2.31-2.37 (m, 1H, one of 2'-H), 2.21-2.28 (m, 1H, one of 2'-H), 1.94 (s, 3H, 5-CH_3), 0.96 (s, 9H, C(CH_3)$_3$), 0.16 (s, 3H, one of SiCH_3), 0.15 (s, 3H, one of SiCH_3); ^{13}C NMR (75 MHz, CD_3OD) δ 170.3, 164.2, 150.8, 137.8, 136.3, 133.0, 131.5, 130.4, 111.3, 89.2, 87.0, 72.6, 64.6, 41.6, 26.5, 19.3, 12.7, -5.2; HRMS (FAB+) calcd for $C_{23}H_{32}N_2O_6Si$ [$(M+H)^+$]: 461.2108, found: 461.2116.

3*-N-*benzoyl-5'*-O-*(*tert-*butyldimethylsilyl)-3'*-O-*(2-nitrobenzyl)thymidine (Compound 14) & *5'*-O-*(*tert-*butyldimethylsilyl)-3*-N-*(2-nitrobenzyl)-3'*-O-*(2-nitrobenzyl)thymidine (Compound 15). To a solution of 2-nitrobenzylbromide (718 mg; 3.32 mmol) in CH_2Cl_2 (10 ml), a mixture of **13** (1.18 g; 2.56 mmol) in aqueous Bu_4NOH (60%; 10 ml), NaI (76.7 mg; 0.51 mmol), CH_2Cl_2 (10 ml), H_2O (10 ml) and aqueous NaOH solution (1.0 M; 10 ml) was added dropwise and then stirred at room temperature for 6 h with exclusion of light. The reaction mixture was diluted with water and then extracted with CH_2Cl_2 (50 ml × 3). The organic layer was washed with brine and dried over anhydrous Na_2SO_4. After filtration and concentration, the resulting crude product was further purified with flash column chromatography (20-40% ethyl acetate in hexane) to afford a brown solid mixture of **14** and **15** (588 mg; based on ^1H NMR calculation, the ratio of **14** and **15** is about 3:1) and **16** as a pure compound (232 mg; 18%). Without further purification, the mixture of **14** and **15** was directly used as starting material in the next step. Pure compound **15** was recovered in the next step described as follows. HRMS (FAB+) of **14**: calcd for $C_{30}H_{37}N_3O_8Si$ [$(M+H)^+$] 596.2428, found: 596.2426.

5'*-O-*(*tert-*butyldimethylsilyl)-3*-N-*(2-nitrobenzyl)thymidine (Compound 16). ^1H NMR (400 MHz, CD_3OD): δ 8.00 (d, *J* = 8.0 Hz, 1H, one of C_6H_4), 7.59 (s, 1H, 6-H), 7.50 (dd, *J* = 7.2, 7.6 Hz, 1H, one of C_6H_4), 7.37 (dd, *J* = 7.2, 8.0 Hz, 1H, one of C_6H_4), 7.18 (d, *J* = 8.0 Hz, 1H, one of C_6H_4), 6.33 (m, 1H, 1'-H), 5.49 (m, 2H, $NCH_2C_6H_4$), 4.42-4.43 (m, 1H, 3'-H), 3.98-3.99 (m, 1H, 4'-H), 3.86-3.90 (m, 1H, one of 5'-H), 3.80-3.83 (m, 1H, one of 5'-H), 2.29-2.31 (m, 1H, one of 2'-H), 2.02-2.11 (m, 1H, one of 2'-H), 1.94 (s, 3H, 5-CH_3), 0.91 (s, 9H, $C(CH_3)_3$), 0.11 (s, 3H, one of $SiCH_3$), 0.10 (s, 3H, one of $SiCH_3$); ^{13}C NMR (75 MHz, CD_3OD) δ 165.1, 152.2, 150.0, 136.5, 134.7, 133.7, 129.3, 128.7, 125.9, 111.1, 89.1, 87.6, 72.6, 64.6, 42.6, 41.7, 26.5, 19.4, 13.4, -5.3; HRMS (FAB+) calcd for $C_{23}H_{33}N_3O_7Si$ [$(M+H)^+$]: 492.2166, found: 492.2166.

5'*-O-*(*tert-*butyldimethylsilyl)-3'*-O-*(2-nitrobenzyl)thymidine (Compound 17). 30% Ammonium hydroxide solution (1.44 ml; 12.32 mmol) was added to the mixture of **14** and **15** (1.24 g) in ethanol (15 ml). The reaction mixture was stirred for 1 h at room temperature with exclusion of light, and then subjected to evaporation. The residue was extracted with CH_2Cl_2 (50 ml × 3). The organic layer was combined and washed with brine, dried over anhydrous Na_2SO_4. After concentration, the residue was further purified with flash column chromatography (40% to 60% ethyl acetate in hexane) to afford **17** as yellow solid (681 mg; 90% yield) and **15** (311 mg) was recovered as brown solid. For compound **17**: ^1H NMR (300 MHz, $CDCl_3$) δ 9.24 (s broad, 1H, N*H*), 8.06 (d, *J* = 8.1 Hz, 1H, one of C_6H_4), 7.78 (d, *J* = 7.5 Hz, 1H, one of C_6H_4), 7.66 (t, *J* = 7.5 Hz, 1H, one of C_6H_4), 7.49 (s, 1H, 6-H), 7.45 (t, *J* = 7.5 Hz, 1H, one of C_6H_4), 6.35 (m, 1H, 1'-H), 4.90 (m, 2H, $OCH_2C_6H_4$), 4.23-4.25 (m, 1H, 3'-H), 4.19-4.20 (m, 1H, 4'-H), 3.93-3.94 (m, 1H, one of 5'-H), 3.79-3.84 (m, 1H, one of 5'-H), 2.49-2.55 (m, 1H, one of 2'-H), 1.98-2.07 (m, 1H, one of 2'-H), 1.92 (s, 3H, 5-CH_3), 0.91 (s, 9H, $C(CH_3)_3$), 0.11 (s, 3H, one of $SiCH_3$), 0.10 (s, 3H, one of $SiCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.0, 150.6, 147.1, 135.3, 134.3, 133.9, 128.9, 128.4, 124.8, 111.1, 85.1, 80.7, 68.1, 63.9, 38.0, 26.0, 18.4, 12.6, -5.3 and -5.4; HRMS (FAB+) calcd for $C_{23}H_{33}N_3O_7Si$ [$(M+H)^+$]: 492.2166, found: 492.2152. For compound **15**: ^1H NMR (300 MHz, $CDCl_3$) δ 7.95-8.02 (m, 2H, two of Ar*H*), 7.72 (d, *J* = 7.8 Hz, 1H, one of Ar*H*), 7.62 (d, *J* = 7.5 Hz, 1H, one of Ar*H*), 7.58 (s, 1H, 6-H), 7.32-7.50 (m, 3H, three of Ar*H*), 7.17 (d, *J* = 7.8 Hz, 1H, one of Ar*H*), 6.35 (dd,

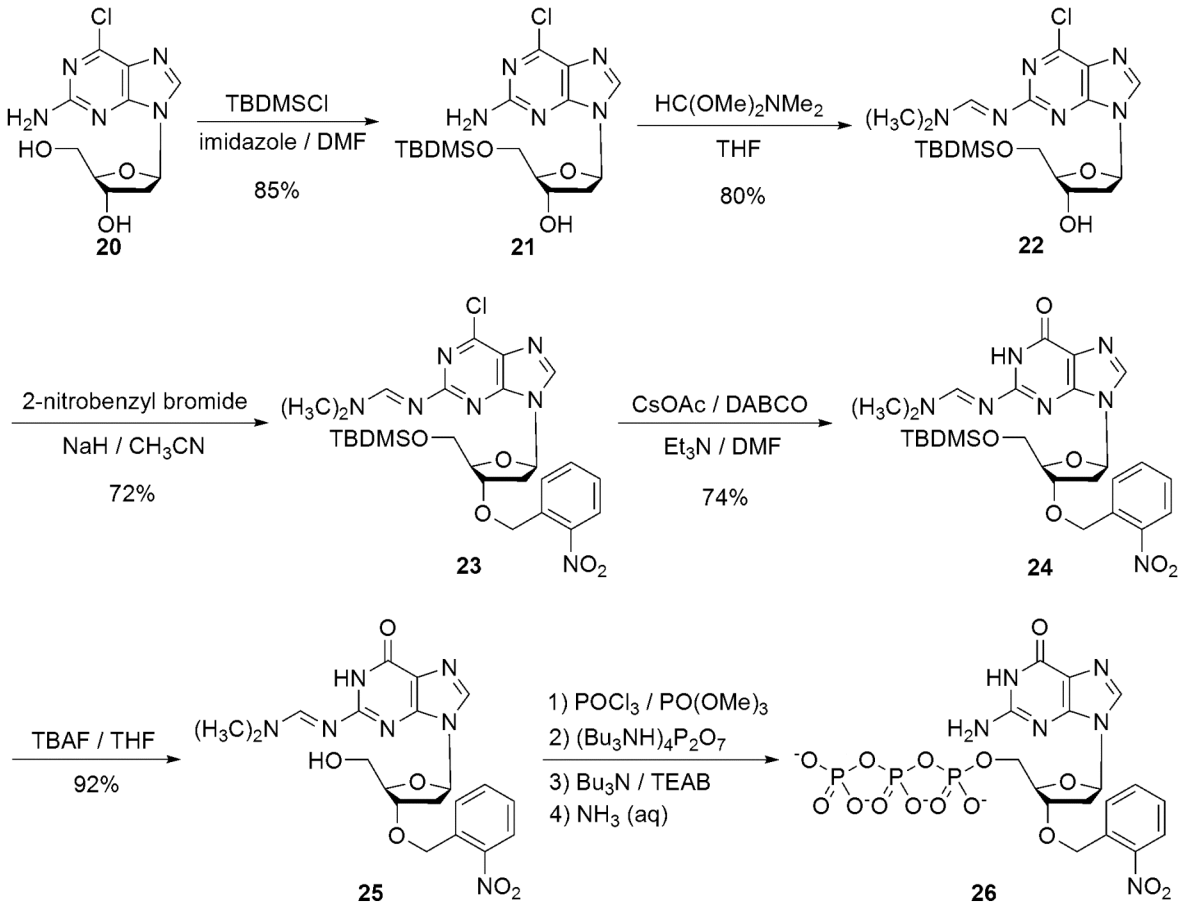
J = 6.0, 7.5 Hz, 1H, 1'-H), 5.47 (s, 2H, NC$H_2C_6H_4$), 4.89 (m, 2H, OC$H_2C_6H_4$), 4.24-4.25 (m, 1H, 3'-H), 4.19 (m, 1H, 4'-H), 3.90-3.94 (m, 1H, one of 5'-H), 3.80-3.84 (m, 1H, one of 5'-H), 2.49-2.56 (m, 1H, one of 2'-H), 2.00-2.09 (m, 1H, one of 2'-H), 1.94 (s, 3H, 5-CH_3), 0.91 (s, 9H, C(CH_3)$_3$), 0.11 (s, 3H, one of SiCH_3), 0.10 (s, 3H, one of SiCH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.2, 150.7, 148.8, 147.1, 134.0, 133.7, 133.5, 132.3, 128.8, 128.3, 127.9, 127.9, 124.8, 124.6, 110.1, 85.8, 85.1, 80.5, 67.9, 63.8, 41.5, 37.9, 25.9, 18.3, 13.2, -5.4, -5.5; HRMS (FAB+) calcd for $C_{30}H_{38}N_4O_9Si$ [$(M+H)^+$]: 627.2486, found: 627.2494.

3'-O-(2-nitrobenzyl)thymidine (Compound 18). To a solution of **17** (681 mg; 1.39 mmol) in 12 ml of anhydrous THF at 0^{o}C, TBAF in THF solution (1.0 M; 2.78 ml; 2.78 mmol) was added. The solution was allowed to warm to room temperature and continue stirring for 2 h with exclusion of air and light. The mixture was poured into cold water (50 ml), and the resulting mixture was extracted with ethyl acetate (50 ml × 3). The organic layer was combined, washed with brine and dried over anhydrous Na_2SO_4. After concentration, the residue was purified with flash column chromatography (1-7% CH_3OH in CH_2Cl_2) to afford **18** (477 mg; 91% yield) as yellow solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.78 (s, 1H, N*H*), 8.00 (d, *J* = 8.1 Hz, 1H, one of C_6H_4), 7.72 (d, *J* = 7.8 Hz, 1H, one of C_6H_4), 7.62 (t, *J* = 7.5 Hz, 1H, one of C_6H_4), 7.49 (s, 1H, 6-H), 7.42 (dd, *J* = 7.5, 7.8 Hz, 1H, one of C_6H_4), 6.35 (t, *J* = 6.9 Hz, 1H, 1'-H), 4.90 (m, 2H, OC$H_2C_6H_4$), 4.34-4.36 (m, 1H, 3'-H), 4.15-4.16 (m, 1H, 4'-H), 3.90-3.94 (m, 1H, one of 5'-H), 3.78-3.83 (m, 1H, one of 5'-H), 3.43 (s, 1H, O*H*), 2.41-2.47 (m, 1H, one of 2'-H), 2.29-2.36 (m, 1H, one of 2'-H), 1.85 (s, 3H, 5-CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.4, 150.7, 147.3, 134.1, 133.8, 129.0, 128.4, 124.8, 124.7, 111.1, 86.6, 85.1, 80.0, 68.2, 62.7, 37.2, 12.5; HRMS (FAB+) calcd for $C_{17}H_{19}N_3O_7$ [$(M+H)^+$]: 378.1301, found: 378.1313.

3'-O-(2-nitrobenzyl)thymidine-5'-triphosphate (Compound 19). The synthetic procedure was similar to the synthesis of **4**. For compound **19**: ^{1}H NMR (300 MHz, D_2O) δ 8.14 (d, *J* = 8.3 Hz, 1H, one of C_6H_4), 7.68-7.74 (m, 3H, 6-H and two of C_6H_4), 7.52-7.58 (m, 1H, one of C_6H_4), 6.22 (dd, *J* = 6.3, 8.0 Hz, 1'-H), 4.89 (m, 2H, OC$H_2C_6H_4$), 4.52 (m, 1H, 3'-H), 4.46 (m, 1H, 4'-H), 4.12-4.19 (m, 2H, 5'-H), 2.42-2.49 (m, 1H, one of 2'-H), 2.22-2.34 (m, 1H, one of 2'-H), 1.89 (s, 3H, 5-CH_3); ^{31}P NMR (121.4 MHz, D_2O) δ -6.2 (d, *J* = 20.6 Hz, 1P, γ-P), -11.2 (d, *J* = 19.4 Hz, 1P, α-P), -22.5 (t, *J* = 20.2 Hz, 1P, β-P).

3'-*O*-(2-nitrobenzyl)-dTTP was further characterized by single base extension reaction to generate DNA extension product and characterized by MALDI-TOF MS as shown in Fig 3*H* of the main text.

IV. Synthesis of 3'-*O*-(2-nitrobenzyl)-dGTP



2-Amino-6-chloro-9-[β-D-5'-O-(*tert*-butyldimethylsilyl)-2'-deoxyribofuranosyl]purine (Compound 21). To a stirred solution of **20** (1.00 g; 3.50 mmol) and imidazole (715 mg; 10.50 mmol) in 10.0 ml anhydrous DMF, TBDMSCl (686 mg; 4.6 mmol) was added. The reaction mixture was stirred at room temperature for 12 h. After evaporation, the residue was purified with flash column chromatography (CH_3OH/CH_2Cl_2, 1:9) to afford **21** as a colorless crystal (1.19 g; 85%): ^{1}H NMR (400 MHz, CD_3OD) δ 8.24 (s, 1H, 8-H), 6.33 (dd, *J* = 6.5, 7.0 Hz, 1H, 1'-H), 4.53-4.54 (m, 1H, 3'-H), 4.00-4.01 (m, 1H, 4'-H), 3.83-3.88 (m, 2H, 5'-H), 2.69-2.73 (m, 1H, one of 2'-H), 2.45-2.48 (m, 1H, one of 2'-H), 0.86 (s, 9H, $C(CH_3)_3$), 0.05 (s, 3H, one of $SiCH_3$), 0.04 (s, 3H, one of $SiCH_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 161.2, 154.5, 151.1, 142.3, 125.2, 89.0, 85.6, 72.1, 64.4, 41.4, 26.4, 19.2, -4.51, -4.50; HRMS (FAB+): calcd for $C_{16}H_{26}ClN_5O_3Si$ [$(M+H)^+$] 400.1572, found: 400.1568.

6-Chloro-2-[(dimethylaminomethylene)amino]-9-[β-D-5'-O-(*tert*-butyldimethylsilyl)-2'-deoxyribofuranosyl]purine (Compound 22). To a solution of **21** (1.19 g; 3.00 mmol) in anhydrous THF (8.0 ml), *N*,*N*-dimethylformamide dimethylacetal (3.10 ml; 18.0 mmol) was added at room temperature and the reaction mixture was then stirred at 40°C under argon atmosphere for 3 h. After evaporation, the resulting residue was purified with flash column chromatography (CH_3OH/CH_2Cl_2, 1:9) to afford **22** as a colorless solid (1.09 g; 80%): ^{1}H NMR (500 MHz, CD_3OD) δ 8.70 (s, 1H, $CHN(CH_3)_2$), 8.45 (s, 1H, 8-H), 6.47 (dd, *J* = 6.5, 7.0 Hz, 1H, 1'-H), 4.56-4.57 (m, 1H, 3'-H), 4.00-4.01 (m, 1H, 4'-H), 3.91-3.94

(m, 1H, one of 5'-H), 3.83-3.86 (m, 1H, one of 5'-H), 3.27 (s, 3H, one of $N(CH_3)_2$), 3.18 (s, 3H, one of $N(CH_3)_2$), 2.72-2.77 (m, 1H, one of 2'-H), 2.53-2.55 (m, 1H, one of 2'-H), 0.95 (s, 9H, $C(CH_3)_3$), 0.08 (s, 3H, one of $SiCH_3$), 0.07 (s, 3H, one of $SiCH_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 163.2, 160.6, 154.1, 151.0, 143.6, 127.9, 88.9, 85.7, 71.9, 64.2, 41.7, 35.6, 26.4, 19.2, -5.5; HRMS (FAB+): calcd for $C_{19}H_{33}ClN_6O_3Si$ [$(M+H)^+$]: 455.1994, found: 455.1999.

6-Chloro-2-[(dimethylaminomethylene)amino]-9-[β-D-5'-O-(*tert*-butyldimethylsilyl)-3'-O-(2-nitrobenzyl)-2'-deoxyribofuranosyl]purine (Compound 23). To a solution of **22** (1.09 g; 2.40 mmol) in anhydrous acetonitrile (3.5 ml), 95% NaH powder (122 mg; 4.80 mmol) was added at 0 °C. After stirring for 50 min at room temperature, 2-nitrobenzyl bromide (1.04 g; 4.80 mmol) in anhydrous acetonitrile (1.5 ml) was added and the reaction mixture was stirred for another 2 h at room temperature with exclusion of air and light. After filtration and concentration, the resulting residue was dissolved in ethyl acetate (100 ml), washed with saturated aqueous $NaHCO_3$ and brine respectively and dried over anhydrous Na_2SO_4. After concentration, the crude product was further purified with flash column chromatography (10-60% ethyl acetate in hexane) to afford **23** (1.02 g; 72%) as yellow oil: ^1H NMR (400 MHz, CD_3OD) δ 8.76 (s, 1H, $CHN(CH_3)_2$), 8.41 (s, 1H, 8-H), 8.02 (d, J = 8.0 Hz, 1H, one of C_6H_4), 7.78 (d, J = 7.6 Hz, 1H, one of C_6H_4), 7.70-7.72 (m, 1H, one of C_6H_4), 7.53-7.54 (m, 1H, one of C_6H_4), 6.44 (dd, J = 6.8, 7.0 Hz, 1H, 1'-H), 4.96 (m, 2H, $OCH_2C_6H_4$), 4.44-4.45 (m, 1H, 3'-H), 4.22 (m, 1H, 4'-H), 3.84-3.87 (m, 2H, 5'-H), 3.21 (s, 3H, one of $N(CH_3)_2$), 3.13 (s, 3H, one of $N(CH_3)_2$), 2.72-2.77 (m, 1H, one of 2'-H), 2.03-2.55 (m, 1H, one of 2'-H), 0.89 (s, 9H, $C(CH_3)_3$), 0.06 (s, 3H, one of $SiCH_3$), 0.05 (s, 3H, one of $SiCH_3$); ^{13}C NMR (75 MHz, CD_3OD) δ 163.2, 160.7, 154.1, 151.0, 149.5, 144.1, 134.9, 134.6, 130.6, 129.8, 125.6, 120.1, 86.8, 86.1, 81.4, 69.8, 64.6, 41.7, 38.6, 35.6, 19.3, -5.9; HRMS (FAB+): calcd for $C_{26}H_{36}ClN_7O_5Si$ [$(M+H)^+$]: 590.2314, found: 590.2291.

5'-O-(t-butyldimethylsilyl)-N^2-[(dimethylamino)methylene]-3'-O-(2-nitrobenzyl)-2'-deoxyguanosine (Compound 24). To a solution of **23** (1.02 g; 1.73 mmol) in anhydrous DMF (15.0 ml), cesium acetate (996 mg; 5.19 mmol), 1,4-diazabicyclo[2.2.2]octane (DABCO) (194 mg; 1.73 mmol) and triethylamine (0.72 ml; 5.19 mmol) were added under argon and stirred overnight at room temperature with exclusion of air and light. Ac_2O (5.0 ml) was added to the above reaction mixture and stirred for another 30 min. The reaction mixture was then quenched with H_2O and extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4. After evaporation, the crude product was further purified with flash column chromatography (1-5% CH_3OH in CH_2Cl_2) to afford **24** (732 mg; 74%) as yellow solid: ^1H NMR (500 MHz, CD_3OD) δ 8.70 (s, 1H, $CHN(CH_3)_2$), 8.03 (d, J = 8.5 Hz, 1H, one of C_6H_4), 8.01 (s, 1H, 8-H), 7.78 (d, J = 7.5 Hz, 1H, one of C_6H_4), 7.72 (dd, J =7.5, 8.0 Hz 1H, one of C_6H_4), 7.55 (dd, J = 7.5, 8.0 Hz, 1H, one of C_6H_4), 6.36 (dd, J = 6.5, 7.0 Hz, 1H, 1'-H), 4.96 (m, 2H, $OCH_2C_6H_4$), 4.42-4.43 (m, 1H, 3'-H), 4.18-4.19 (m, 1H, 4'-H), 3.83-3.84 (m, 2H, 5'-H), 3.20 (s, 3H, one of $N(CH_3)_2$), 3.12 (s, 3H, one of $N(CH_3)_2$), 2.64-2.72 (m, 2H, 2'-H), 0.89 (s, 9H, $C(CH_3)_3$), 0.08 (s, 3H, one of $SiCH_3$), 0.07 (s, 3H, one of $SiCH_3$); ^{13}C NMR (75 MHz, CD_3OD) δ 160.1, 159.9, 159.2, 152.0, 149.6, 138.2, 134.8, 134.6, 130.8, 129.9, 125.7, 120.1, 86.6, 85.3, 81.7, 69.3, 64.8, 41.5, 38.7, 35.3, 26.4, 19.3, -5.3; HRMS (FAB+): calcd for $C_{26}H_{37}N_7O_6Si$ [$(M+H)^+$]: 572.2653, found: 572.2655.

N^2-*[(dimethylamino)methylene]-3'*-O-*(2-nitrobenzyl)-2'-deoxyguanosine (Compound 25).* To a solution of **24** (732 mg; 1.28 mmol) in anhydrous THF (8.0 ml), TBAF in THF solution (1.0 M; 2.56 ml; 2.56 mmol) was added at 0°C. The solution was allowed to warm to room temperature and stirred for 2 h with exclusion of air and light. The reaction mixture was poured into cold water (50 ml) and the resulting mixture was extracted with ethyl acetate (50 ml × 3). The combined organic layer was washed with brine and dried over anhydrous Na_2SO_4. After concentration, the residue was further purified with flash column chromatography (1-7% CH_3OH in CH_2Cl_2) to afford **25** (538 mg; 92%) as yellow solid: ^{1}H NMR (500 MHz, CD_3OD) δ 8.65 (s, 1H, C*H*N$(CH_3)_2$), 8.05 (s, 1H, 8-H), 8.02 (d, *J* = 8.5 Hz, 1H, one of C_6H_4), 7.78 (d, *J* = 7.0 Hz, 1H, one of C_6H_4), 7.72 (dd, J =7.5, 8.0 Hz, 1 H, one of C_6H_4), 7.55 (dd, *J* = 7.5, 8.0 Hz, 1H, one of C_6H_4), 6.34 (m, 1H, 1'-H), 4.96 (m, 2H, O$CH_2C_6H_4$), 4.43-4.44 (m, 1H, 3'-H), 4.20-4.21 (m, 1H, 4'-H), 3.78 (m, 2H, 5'-H), 3.20 (s, 3H, one of N$(CH_3)_2$), 3.11 (s, 3H, one of N$(CH_3)_2$), 2.77 (m, 1H, one of 2'-H), 2.64 (m, 1H, one of 2'-H); ^{13}C NMR (75 MHz, CD_3OD) δ 160.0, 159.9, 159.0, 152.0, 149.6, 138.0, 134.7, 134.5, 130.8, 129.9, 125.6, 120.1, 87.0, 86.0, 82.0, 69.2, 63.6, 41.4, 38.4, 35.3; HRMS (FAB+): calcd for $C_{20}H_{23}N_7O_6$ [$(M+H)^+$]: 458.1788, found: 458.1808.

3'*-O-*(2-nitrobenzyl)-2'-deoxyguanosine-5'-triphosphate (Compound 26). The synthetic procedure was similar to the synthesis of **4**. For compound **26**: ^{1}H NMR (300 MHz, D_2O) δ 8.03 (s, 1H, 8-H), 7.92-7.95 (m, 1H, one of C_6H_4), 7.64-7.65 (m, 2H, two of C_6H_4), 7.46-7.49 (m, 1H, one of C_6H_4), 6.15 (m, 1H, 1'-H), 4.92 (m, 2H, O$CH_2C_6H_4$), 4.54 (m, 1H), 4.35 (m, 1H), 4.07-4.11 (m, 2H, 5'-H), 2.69-2.72 (m, 1H, one of 2'-H), 2.49-2.58 (m, 1H, one of 2'-H); ^{31}P NMR (121.4 MHz, D_2O) δ -7.0 (d, *J* = 19.3 Hz, 1P, γ-P), -11.3 (d, *J* = 19.9 Hz, 1P, α-P), -22.7 (t, *J* = 20.0 Hz, 1P, β-P).

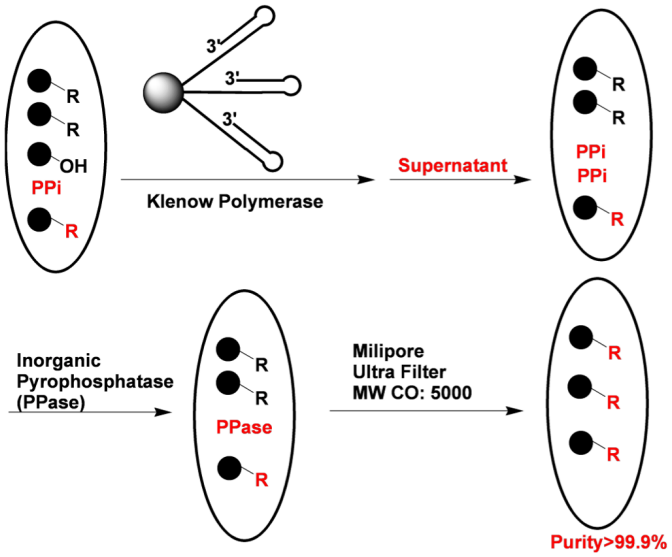
3'-*O*-(2-nitrobenzyl)-dGTP was further characterized by single base extension reaction to generate DNA extension product and characterized by MALDI-TOF MS as shown in Fig 3*G* of the main text.

V. Continuous DNA Polymerase Reaction Using 3'-*O*-(2-nitrobenzyl)-dNTPs as Reversible Terminators in Solution.

We characterized 3'-*O*-(2-nitrobenzyl)-dNTPs, by performing continuous DNA-extension reactions. As an example, for 3'-*O*-(2-nitrobenzyl)-dGTP, we carried out the following continuous DNA extension reactions using a primer (5'-GTTGATGTACACATTGTCAA-3') and a synthetic DNA template (SI Table 1) corresponding to a portion of exon 7 of the human *p53* gene. The two nucleotides in the template immediately adjacent to the annealing site of the primer were 5'-CC-3'. First, an extension reaction using 3'-*O*-(2-nitrobenzyl)-dGTP along with the primer and the template was performed to produce a single base extension product. The reaction mixture (and all the subsequent extension reaction mixture) consisted of 100 pmol of template, 100 pmol of primer, 200 pmol of 3'-*O*-(2-nitrobenzyl)-dGTP, 2 ml of 10X Thermopol II reaction buffer, 2 ml of 20 mM $MnCl_2$ and 2 μl (4 units) 9°N polymerase (exo-) A485L/Y409V in a total reaction volume of 20 μl. After an initial incubation at 95°C for 5 minutes and 4°C for 5 minutes, the reaction was performed at 95°C for 15 seconds, 55°C for 15 seconds, 65°C for 1 minute for 20 cycles. A portion of the resulting DNA products were then precipitated and purified for MALDI-TOF MS analysis using the procedure previously reported (1). For photocleavage, the DNA extension product was purified by HPLC according to

literature (2), and resuspended in 200 ml of deionized water. The mixture was irradiated for 30 seconds using a laser at 355 nm (3 mW/cm^2) and then analyzed by MALDI-TOF MS. After photocleavage, the DNA fragment with the 3'-*O*-(2-nitrobenzyl) group removed was used as a primer for a subsequent extension reaction with 3'-*O*-(2-nitrobenzyl)-dGTP. The second DNA extension product was then purified by ethanol precipitation as described previously and photolyzed. Continuous DNA extension reactions using 3'-*O*-(2-nitrobenzyl)-dATP, 3'-*O*-(2-nitrobenzyl)-dCTP and 3'-*O*-(2-nitrobenzyl)-dTTP were carried out in a same manner thereby confirming these 3'-*O*-(2-nitrobenzyl)-dNTPs can be used as reversible terminators in DNA polymerase reaction.

VI. Enzymatic Purification of the 3'-*O*-Modified Nucleotides

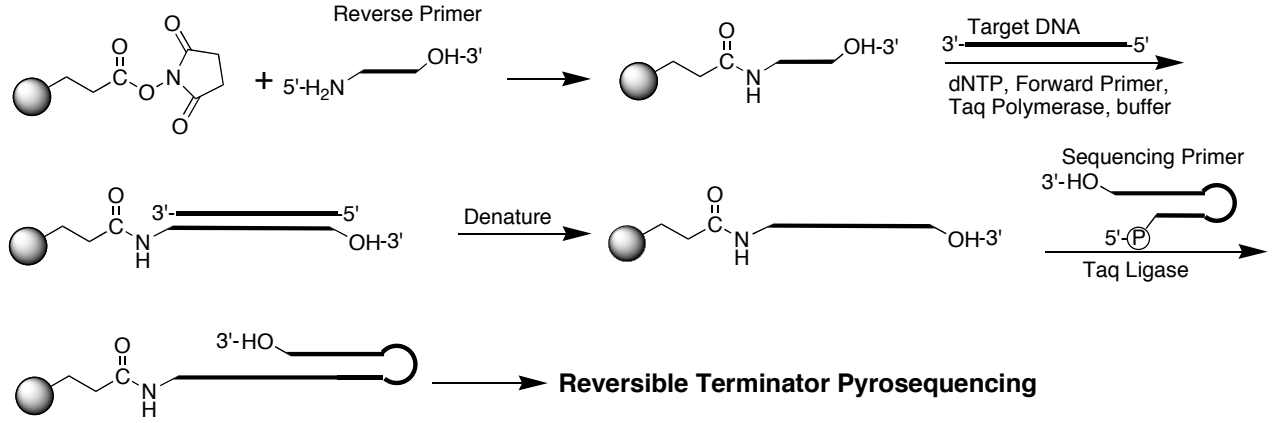


As shown in the scheme above, 5'-amino modified self-priming DNA templates containing long homopolymeric regions were immobilized on sepharose beads using the method described in the manuscript. For purification of 3'-*O*-modified dNTPs (A, C, G, and T), four self-priming templates were used respectively. Sequences are shown as follows: 5'-TTTTTTTTTTTTTTTTTTTTTTTTTT-TTGGAAACAGATGCGCATCTGTTTCC-3', 5'-GGGGGGGGGGGGGGGGGGGGGGGGGG-GGGAAACAGATGCGCATCTGTTTCC-3', 5'-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCGG-AAACAGATGCGCATCTGTTTCC-3' and 5'-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAG-GAAACAGATGCGCATCTGTTTCC-3'. 1 μl of 100 μM 3'-O-modified nucleotide in annealing buffer (20 mM Tris-acetate, 5 mM magnesium acetate, pH 7.6) was added to a reaction mixture containing template immobilized beads, 1 μl of Klenow (exo-) enzyme (2 units) (New England Biolab), and 0.5 unit of inorganic pyrophosphatase (Sigma) and DI H_2O for a total reaction volume of 20 μl. The mixture was shaken at room temperature for 1 hour, and the supernatant was then passed through an ultrafilter (MWCO=5000) (Milipore) by centrifugation to remove the Klenow (exo-) polymerase and inorganic pyrophosphatase to yield pure 3'-*O*-modified nucleotides.

VII. General Procedure of DNA Immobilization on Sepharose Beads.

NHS-ester sepharose beads were washed briefly in a cartridge three times with 1 ml of 1 mM HCl, and 3 times with 1 ml of coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). Then 200 μl of 100 μM amino-modified DNA templates (amino-group on either the 5'-end or on the loop of the DNA hairpin structure) in coupling buffer were added. After the suspension was shaken overnight at room temperature, the beads were washed with 800 μl of washing buffer (20 mM tris-acetate, 5 mM magnesium acetate, 0.1% Tween-20, pH 7.6) for 30 minutes three times, with 800 μl of annealing buffer (20 mM Tris-acetate, 5 mM magnesium acetate, pH 7.6) for 30 minutes three times, and resuspended in storage buffer (50 mM Tris-acetate, 0.02% Tween-20, 0.02% sodium azide, pH 8) at 4°C for future use.

VIII. Construction of PCR Template on the Sepharose Beads



The overall scheme for constructing PCR-amplified DNA template immobilized on sepharose beads is shown above. After immobilizing an M13R primer (5'-NH_2-CAGGAAACAGCTATGAC-3') to sepharose beads, PCR was performed on these beads by adding 3 μl (10 μM) M13F primer with a 5' 11-base overhang (5'-*CACTCGCATGG*GTAAAACGACGGCCAG-3'), 1 μl (2-5 ng) of purified pBluescrip+SK plasmid DNA, 1 μl (10 mM) of dNTPs, 5 units of DNA polymerase (AccuTaq, SIGMA) together with 5 μl of 10×PCR reaction buffer. Eight strip PCR tubes were used for PCR and the total reaction volume in each tube was 50 μl. PCR was started with denature at 95°C for 2 min and then continued for 80 cycles (95°C, 20 seconds, 50°C, 45 seconds, 68°C, 60 seconds) in a PTC-200 Peltier thermal cycler. To avoid pelleting of sepharose beads, the eight strip tubes were taken out and vortexed for 5 seconds when the temperature reached 50°C in each cycle. After PCR, all the sepharose beads were collected, washed with 100 μl of DI H_2O three times, then suspended in five volumes of 0.1 M NaOH for another 10 min at room temperature to denature the double stranded DNA. The beads were pelleted and the supernatant containing forward single-strand DNA was removed. This procedure was repeated one more time to ensure all forward single-strand DNA was removed. The beads with reverse single strand DNA attached were suspended in DI H_2O and washed with 100 μl of DI H_2O three times. The beads with single strand PCR product were ligated to 5'-phosphorylated 40 mer hairpin DNA (5'-GCTGAATTCCGCGTTCGCGGAATTCAGCCACTCGCATGGG-3') with *Taq* ligase (New

England Biolabs) according to the vendor protocol. After ligation the beads were washed three times with 100 μl of DI H_2O and ready for pyrosequencing.

1. Ju J, Kim DH, Bi L, Meng Q, Bai X, Li Z, Li X, Marma MS, Shi S, Wu J, Edwards JR, Romu A, Turro NJ (2006) *Proc Natl Acad Sci USA* 103:19635-19640.

2. Seo TS, Bai X, Kim DH, Meng Q, Shi S, Ruparel H, Li Z, Turro NJ, Ju J (2005) *Proc. Natl. Acad. Sci. USA* 102: 5926-5931.