



Title	Highly efficient enzymatic synthesis of 3'-deoxyapionucleic acid (apioNA) having the four natural nucleobases
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Supplementary Information

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General. ^1H , ^{13}C and ^{31}P NMR spectra were obtained on a JEOL ECX-400P, JEOL ECA-500 or JEOL AL-400 and were reported in parts per million (δ) relative to residual solvent signal for ^1H NMR spectra, 1,4-dioxane signal (67.2 ppm) as internal standard for ^{13}C NMR spectra, and 85% phosphoric acid (0.0 ppm) as external standard for ^{31}P NMR spectra. Coupling constants (J) were reported in Hertz (Hz). Abbreviations of multiplicity were as follow: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad. Data were presented as follows: chemical shift (multiplicity, integration, coupling constant). LR- and HR-MS spectra were obtained on a JEOL JMS-HX110, JEOL JMS-700TZ or JEOL JMS-T100LP. UV spectra were measured with Shimadzu UV Visible Spectrophotometer UV-2450. pH was measured with Beckman Coulter F360 pH meter. DNA oligomers were prepared on an Applied Biosystems 3400 DNA Synthesizer. HPLC was performed with Shimadzu LC-10AD-VP or LC-20AB (pump), Shimadzu SPD-M10A-VP or SPD-M20A (UV-visible detector), Shimadzu CTO-10AS-VP or CTO-20A (column oven), CLASS-VP system, LabSolutions (system controller). Sep-pak Plus C18 Cartridge was purchased from Waters. YMC J'sphere ODS-M80 (150 \times 4.6 mm) was used as reversed-phase C18 HPLC columns. DNA polymerases were purchased from Takara Shuzo (Klenow (exo $^-$), exTaq), Promega (Tfl, Pfu), New England Biolabs (Vent (exo $^-$), Deep Vent (exo $^-$), Therminator, Therminator II) and Toyobo (KOD Dash). PAGE analysis and purification were performed with 220 \times 220 \times 0.5 mm and 220 \times 220 \times 1.5 mm sized gel, respectively. MALDI-TOF mass spectrum was measured with Bruker Daltonics Ultraflex TOF/TOF. Incubation of enzymatic reactions was performed with EYELA MG-1200.

Optimized methods of the primer extension experiments (20mer primer and 27mer

template). The following mixture was used: 0.8 μM duplex consisting of a 5'-FAM labeled primer and a DNA template, Therminator DNA polymerase (0.2 U/ μL , 1.7 μM), 200 μM apioNTPs in the ThermoPol buffer [20 mM Tris-HCl (pH 8.8) containing 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$ and 2 mM MgSO_4 and 0.1% Triton X-100] containing 1.25 mM MnCl_2 in a final volume of 10 μL . The reaction was performed at 44 $^\circ\text{C}$ for 1 h and quenched with the addition of 10 μL loading buffer [1 \times TBE buffer (89 mM Tris/89 mM boric acid/2 mM EDTA), 7 M urea, 0.05% xylene cyanol and bromophenol blue]. The reaction mixture (8 μL) was resolved by electrophoresis at 800 V for 5 h using a 20% (19:1) denaturing polyacrylamide gel containing 7 M urea, and the gels were quantified with FLA-2000 (FUJIFILM).

MALDI-TOF mass spectrum of the elongated product (20mer primer and 27mer template). The following mixture was used: 0.8 μM duplex consisting of a primer and a DNA template, Therminator DNA polymerase (0.2 U/ μL , 1.7 μM), 200 μM apioNTPs in the ThermoPol buffer containing 1.25 mM MnCl_2 in a final volume of 40 μL . The reaction was performed at 44 $^\circ\text{C}$ for 1 h and quenched with the addition of 40 μL of 10 M urea. The mixture was desalted, and counter cations of phosphate groups were exchanged to ammonium salts on ODS column (YMC disposable SPE). The oligonucleotide was then analyzed by MALDI-TOF mass using 3-hydroxypicolinic acid and bis-ammonium citrate as matrix.

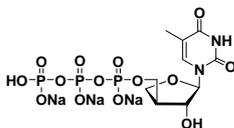
Single nucleotide insertion reactions using the steady-state method (20mer primer and 27mer template). Insertion reactions were initiated by adding 1 μL of dNTP or apioNTP solution (0.001-1 μM) to a reaction mixture containing a mixture of the 0.8

μM duplex consisting of a 5'-FAM labeled primer and a DNA template, and Terminator (0.005 U/ μL , 42.5 nM) in the ThermoPol buffer [and 1.25 mM MnCl_2 for apioNTP (Mn (+))] in a final volume of 10 μL . The amount of dNTP or apioNTP was adjusted to 25% maximum insertion reaction. The reaction was performed at 74 °C for 3 min and quenched by adding 10 μL of the stop buffer. The reaction mixture (8 μL) was resolved by electrophoresis at 800 V for 5 h using a 20% (19:1) denaturing polyacrylamide gel containing 7 M urea, and the gels were quantified with FLA-2000 (FUJIFILM). Reaction velocities were calculated as the yield of reaction divided by reaction time. Kinetic parameters (K_m and V_{max}) were determined by linear regression analysis of a Hanes-Woolf plot^[1] with an average of three independent experiments.

Synthesis and purification of the oligonucleotides. Oligonucleotides were synthesized with a DNA Synthesizer (Applied Biosystem Model 3400) by using 3'-deoxyapionucleoside phosphoramidites or commercially available 2'-deoxyribonucleoside phosphoramidite units at 1 μmol scale following the standard procedure described. Each of 3'-deoxyapionucleoside phosphoramidites was used at concentration of 0.1 M in dry MeCN, and the coupling time was extended to 15 min. After completion of the synthesis, the CPG support was treated with concentrated NH_4OH (55 °C, 12 h) and filtered off, and the filtrate was concentrated. The residue was dissolved in 500 μL of 90% formamide and purified by electrophoresis at 400 V for 8 h using a 20% (29 : 1) denaturing polyacrylamide gel (220 \times 220 \times 1.5 mm). The desired band was cut and extracted in TE buffer at room temperature for overnight. The extract was desalted on Sep-pak C18 column and eluted with aqueous 50% CH_3CN to obtain the desired oligonucleotide.

Single nucleotide insertion reactions using the steady-state method (24mer primer and 27mer template). Insertion reactions were initiated by adding 1 μL of apioATP solution (0.001-1 μM) to a reaction mixture containing a mixture of the 0.8 μM duplex consisting of a 5'-FAM labeled primer and a DNA template, and Terminator (0.2 U/ μL , 1.7 μM) in the ThermoPol buffer [and 1.25 mM MnCl_2 for apioATP (Mn (+))] in a final volume of 10 μL . The amount of apioATP was adjusted to 25% maximum insertion reaction. The reaction was performed at 74 $^\circ\text{C}$ for 3 min and quenched by adding 10 μL of the stop buffer. The reaction mixture (8 μL) was resolved by electrophoresis at 800 V for 4 h using a 20% (29:1) denaturing polyacrylamide gel containing 7 M urea, and the gels were quantified with FLA-2000 (FUJIFILM). Reaction velocities were calculated as the yield of reaction divided by reaction time. Kinetic parameters (K_m and V_{max}) were determined by linear regression analysis of a Hanes-Woolf plot^[1] with an average of three independent experiments.

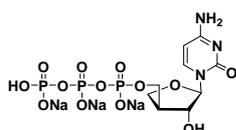
The synthesis of 3'-deoxyapionucleoside 3'-triphosphates (apioNTPs)



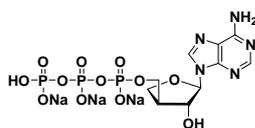
1-((2R,3R,4R)-3-Hydroxy-4-((triphosphoryl)methyl)tetrahydrofuran-2-yl)thymine

sodium salt (**apioTTP**): A solution of 2'-O-acetyl-3'-deoxy-D-apiothymidine^[2] (69 mg, 0.24 mmol) in pyridine/1,4-dioxane (1/3, 960 μ L) was treated with 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (73 mg, 0.36 mmol) in 1,4-dioxane (480 μ L) at room temperature for 10 min. The reaction mixture was treated with 0.5 M solution of bis(tri-*n*-butylammonium)pyrophosphate in dry DMF (960 μ L, 0.48 mmol) and tri-*n*-butylamine (320 μ L, 1.3 mmol) at room temperature for 10 min. The reaction mixture was treated with 1% iodine in pyridine/water (98/2) (ca. 3 mL) for 5 min, and which was treated with 5% aqueous solution of NaHSO₃ (ca. 2 mL) for additional 30 min. The reaction mixture was concentrated *in vacuo*, and the residue was treated with saturated aqueous ammonia solution (20 mL) at room temperature overnight. The reaction mixture was concentrated *in vacuo*, and the residue was dissolved in H₂O (300 mL), and the solution applied to a DEAE Sephadex column, which was eluted with a linear gradient of 750 mL each of H₂O and 1.0 M TEAB (pH 8.0). Fractions containing desired product were concentrated *in vacuo* and coevaporated with H₂O/EtOH (1/1). The residue was dissolved in H₂O (10 mL), and the solution was applied to a column of DIAION PK 212 (H⁺ form), which was eluted with H₂O. The eluate was applied to a DIAION WK 40 (Na⁺ form), which was eluted with H₂O. Fractions containing apioTTP were concentrated *in vacuo* to give **apioTTP** (51%) as a white solid. ¹H NMR (D₂O, 500 MHz) δ 7.55 (1H, s, H-6), 5.84 (1H, d, H-1', $J_{1',2'} = 6.4$ Hz), 4.45 (1H, dd, H-2' $J_{2',1'} = 6.4$, $J_{2',3'} = 6.8$ Hz), 4.30 (1H, dd, H-4'a $J_{gem} = 8.6$, $J_{4'a,3'} = 8.6$ Hz), 4.15 (1H, dd,

H-4'b $J_{\text{gem}} = 8.6$, $J_{4'b,3'} = 9.7$ Hz), 4.13 (2H, dd, H-3", $J_{\text{gem}} = 9.7$, $J_{3'',3'} = 7.4$ Hz), 2.74 (1H, m, H-3'), 1.89 (3H, s, Me). ^{31}P NMR (D_2O , 202 MHz) δ -10.45, -10.67, -22.79. ESIMS-LR m/z 481 [M-H]; ESIMS-HR calcd for $\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_{14}\text{P}_3$ 480.9814, found 480.9802.

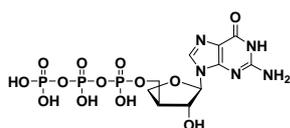


1- $\{(2R,3R,4R)\}$ -3-Hydroxy-4-[(triphosphoryl)methyl]tetrahydrofuran-2-yl} cytosine sodium salt (**apioCTP**, 54%, as a white solid) was obtained from 2'-*O*-acetyl-*N*⁴-benzoyl-3'-deoxy-D-apiocytidine^[2] (129 mg, 0.34 mmol) as described for the synthesis of **apioTTP**. ^1H NMR (D_2O , 500 MHz) δ 7.73 (1H, d, H-6, $J_{6,5} = 7.4$ Hz), 6.05 (1H, d, H-5, $J_{5,6} = 7.4$ Hz), 5.85 (1H, d, H-1', $J_{1',2'} = 5.7$ Hz), 4.41 (1H, dd, H-2', $J_{2',1'} = 5.7$, $J_{2',3'} = 6.3$ Hz), 4.29 (1H, dd, H-4'a $J_{\text{gem}} = 8.6$, $J_{4'a,3'} = 8.0$ Hz), 4.12 (1H, dd, H-4'b $J_{\text{gem}} = 8.6$, $J_{4'b,3'} = 8.0$ Hz), 4.06 (2H, dd, H-3", $J_{\text{gem}} = 5.7$, $J_{3'',3'} = 5.2$ Hz), 2.70 (1H, m, H-3'). ^{31}P NMR (D_2O , 202 MHz) δ -6.33, -10.03, -21.91. ESIMS-LR m/z 466 [M-H]; ESIMS-HR calcd for $\text{C}_9\text{H}_{15}\text{N}_3\text{O}_{13}\text{P}_3$ 465.9818, found 465.9817.



9- $\{(2R,3R,4R)\}$ -3-Hydroxy-4-[(triphosphoryl)methyl]tetrahydrofuran-2-yl} adenine sodium salt (**apioATP**, 55%, as a white solid) was obtained from 2'-*O*-acetyl-*N*⁶-benzoyl-3'-deoxy-D-apioadenosine^[2] (78 mg, 0.20 mmol) as described for the synthesis of **apioTTP**. ^1H NMR (D_2O , 500 MHz) δ 8.35 (1H, s, H-2), 8.11 (1H, s, H-8), 5.94 (1H, d, H-1', $J_{1',2'} = 5.4$ Hz), 4.84 (1H, d, H-2', $J_{2',1'} = 5.4$, $J_{2',3'} = 6.9$

Hz), 4.36 (1H, dd, H-4'a $J_{\text{gem}} = 9.2$, $J_{4'a,3'} = 8.7$ Hz), 4.19 (1H, dd, H-4'b $J_{\text{gem}} = 9.2$, $J_{4'b,3'} = 8.7$ Hz), 4.18 (2H, dd, H-3'', $J_{\text{gem}} = 8.6$, $J_{3'',3'} = 7.4$ Hz), 2.86 (1H, m, H-3'). ^{31}P NMR (D_2O , 202 MHz) δ -8.43, -10.17, -21.68. ESIMS-LR m/z 490 [M-H]; ESIMS-HR calcd for $\text{C}_{10}\text{H}_{15}\text{N}_5\text{O}_{12}\text{P}_3$ 489.9930, found 489.9913.



9- $\{(2R,3R,4R)\}$ -3-Hydroxy-4-[(triphosphoryl)methyl]tetrahydrofuran-2-yl} guanine sodium salt (**apioGTP**, 44%, as a white solid) was obtained from 2'-*O*-acetyl-*N*²-benzoyl-3'-deoxy-D-apioguanosine^[2] (37 mg, 0.09 mmol) as described for the synthesis of **apioTTP**. ^1H NMR (D_2O , 500 MHz) δ 8.00 (1H, s, H-8), 5.78 (1H, d, H-1', $J_{1',2'} = 5.7$ Hz), 4.91 (1H, dd, H-2', $J_{2',1'} = 5.7$, $J_{2',3'} = 7.4$ Hz), 4.31 (1H, dd, H-4'a $J_{\text{gem}} = 8.6$, $J_{4'a,3'} = 8.6$ Hz), 4.21 (2H, dd, H-3'', $J_{\text{gem}} = 8.6$, $J_{3'',3'} = 8.6$ Hz), 4.19 (1H, dd, H-4'b $J_{\text{gem}} = 8.6$, $J_{4'b,3'} = 8.6$ Hz), 2.86 (1H, m, H-3'). ^{31}P NMR (D_2O , 202 MHz) δ -5.28, -10.00, -20.97. ESIMS-LR m/z 506 [M-H]; ESIMS-HR calcd for $\text{C}_{10}\text{H}_{15}\text{N}_5\text{O}_{13}\text{P}_3$ 505.9879, found 505.9874.

A PAGE experiment for the elongation reaction using all four apioNTPs under various concentrations of Therminator DNA polymerase (20mer primer and 27mer template).

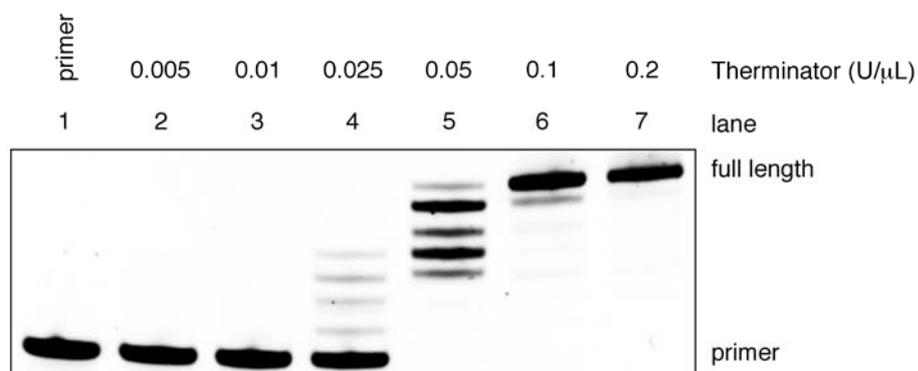


Figure S1. A PAGE experiment for the elongation reaction in the presence of 1.25 mM MnCl_2 using all four apioNTPs at 44 °C for 1 h under various concentrations of Therminator DNA polymerase. Lane 1; primer, lanes 2-7; the elongation product by using apioNTPs under various concentrations of Therminator DNA polymerase. Sequence of the primer-template complex and apioNA elongation product were shown in Figure 2A.

MALDI-TOF mass spectrum of elongated product (20mer primer and 27mer template).

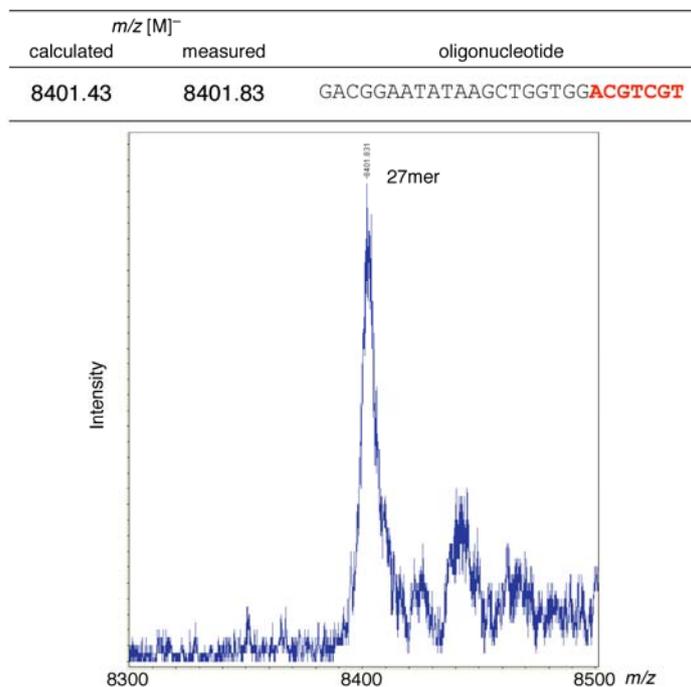


Figure S2. MALDI-TOF mass spectrum of the elongation product by using apioNTPs. The Spectrum was obtained from elongation reaction followed by ODS column purification.

A PAGE experiment for the longer elongation reaction using all four apioNTPs (21mer primer and 43mer template).

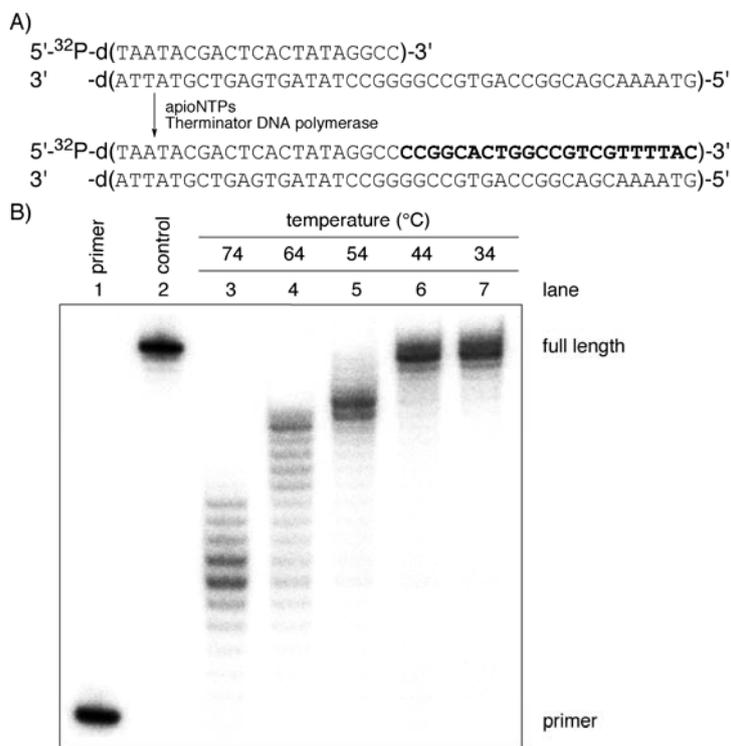
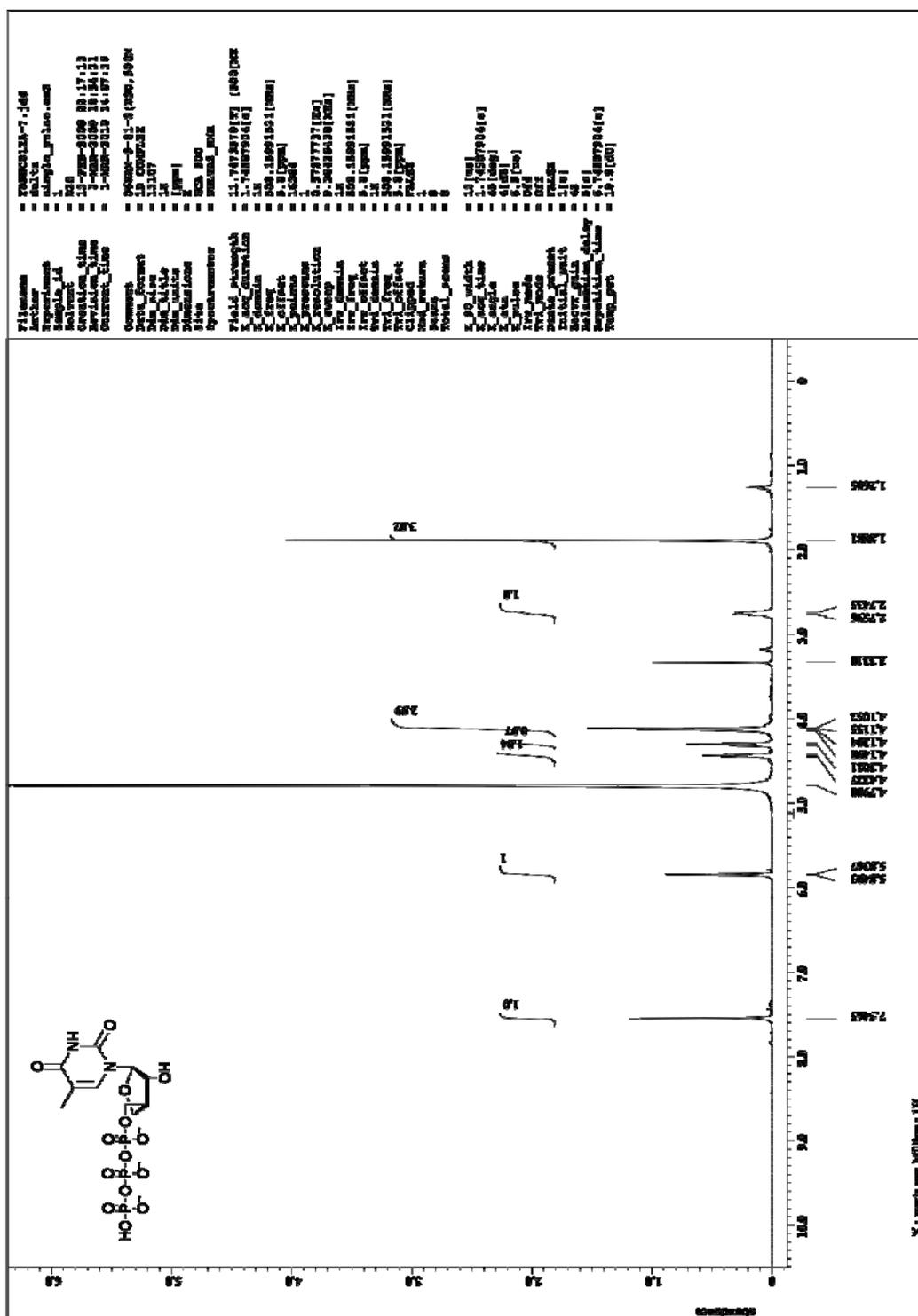
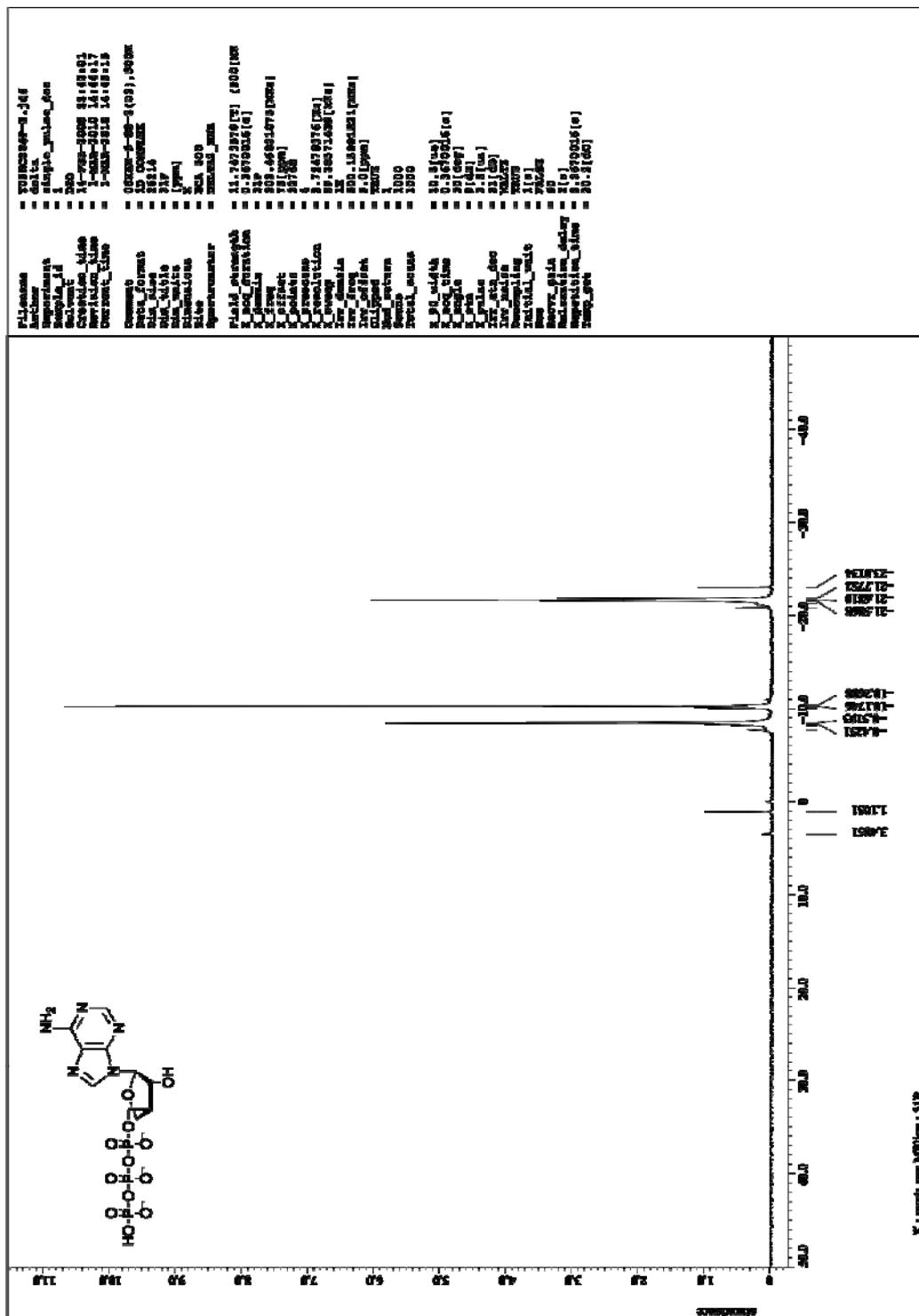
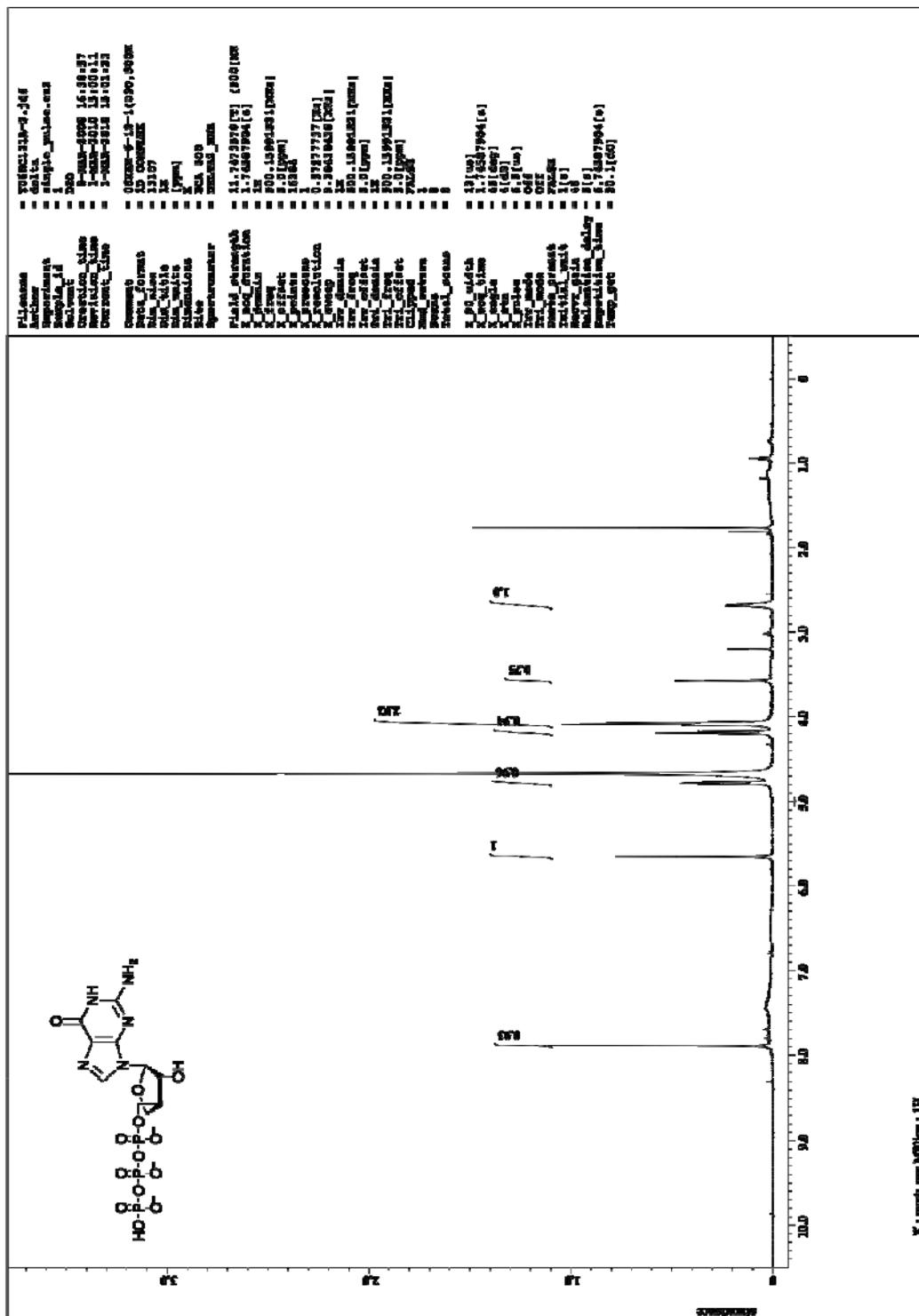


Figure S3. A) Sequence of the primer (21mer)-template (43mer) complex and the apioNA elongation product. Elongated apioNA is shown in bold letters. B) Primer extension experiments: The reactions were performed with same reaction conditions in general method (see supporting information) except for reaction temperature; 74, 64, 54, 44, and 34 °C (lanes 3-7), respectively. Lane 1; primer, lane 2; control.

¹H and ³¹P NMR spectra of apioNTPs.







X : gamma per Million : 101

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