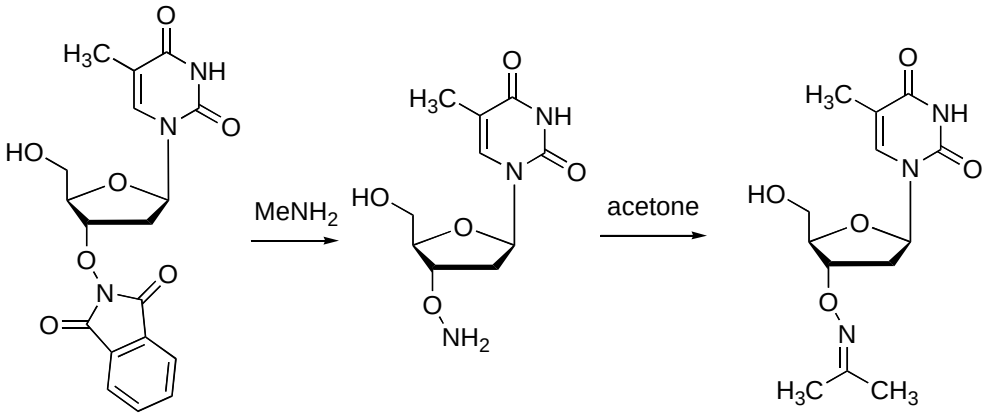
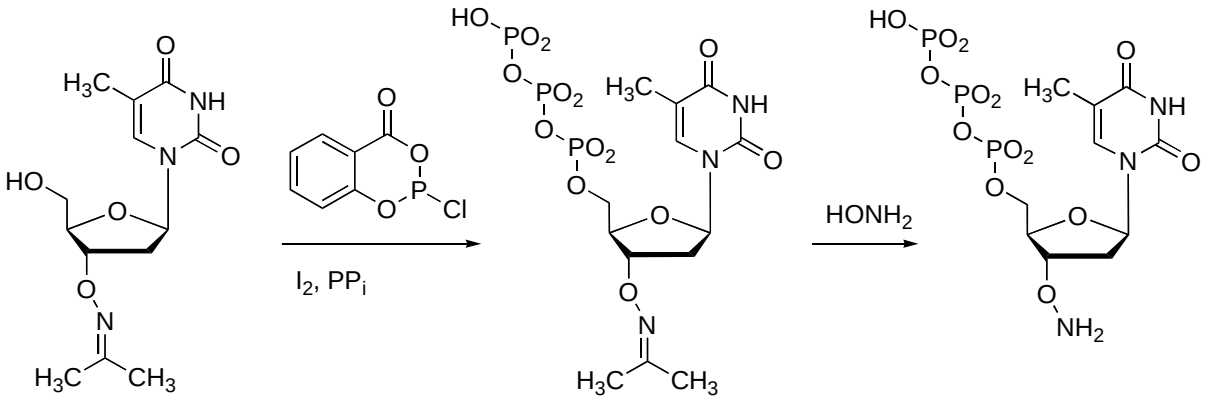
SI Appendix

Synthesis of TTP-ONH$_2$



3'-O-(N-acetone-oxime)-thymidine. 3'-O-phthalimido-thymidine (1.15 g, 3.0 mmol) was dissolved in aqueous methylamine solution (4%, 22 mL, ca. 24 mmol). After 20 min, most of the methylamine was removed *in vacuo* and the remaining solution was treated with acetone (3 mL). After 3 h at room temperature, the solvent was removed *in vacuo*. The residue was redissolved in a mixture of water (25 mL) and acetonitrile (7 mL). Solids were removed from the mixture by filteration (0.2 µm) prior to purification by reverse phase HPLC (Waters Prep Nova-Pak HR C_{18} column, 60 Å, 19 x 300 mm, eluent A = 25 mM TEAA pH 7, eluent B = CH_3CN, gradient from 25 to 50% B in 7 min, then to 80% B in 8 min, flow rate = 5 mL/min, R_t = 14 min) gave 3'-O-(N-acetone-oxime)-thymidine (640 mg; 72%) as a colorless foam after lyophilization (1-4).

^{1}H-NMR (d_6-DMSO, 300 MHz): ∂ (ppm) = 1.79 (d, J = 0.9 Hz, 3H); 1.83 (s, 3H); 1.84 (s, 3H); 2.15-2.35 (m, 2H); 3.55-3.70 (m, 2H); 3.98-4.05 (m, 1H); 4.68-4.72 (m, 1H); 5.15 (br. s, 1H); 6.17 (dd, J = 5.7, 8.7 Hz, 1H); 7.76 (d, J = 0.9 Hz, 1H); 11.3 (br. s, 1H). ^{13}C-NMR (d_6-DMSO, 75 MHz): ∂ (ppm) = 12.3; 15.5; 21.5; 36.4; 61.8; 82.1; 83.9; 84.1; 109.6; 136.0; 150.5; 155.8; 163.7.



3'-O-(N-acetone-oxime)-thymidine-5'-triphosphate. To a solution of 3'-O-(N-acetone-oxime)-thymidine (300 mg, 1.0 mmol) in pyridine (4 mL) and dioxane (3.4 mL) was added a solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (260 mg, 1.4 mmol) in dioxane (2.6 mL) at room temperature. After 10 min a mixture of tributylammonium pyrophosphate in DMF (0.2 M, 10 mL, 2 mmol) and tributylamine (1.2 mL, 4.8 mmol) was added. After 10 min a solution of iodine (360 mg, 1.4 mmol) and water (0.56 mL) in pyridine (28 mL) was added. After 20 min the reaction was quenched by the addition of aqueous Na_2SO_3 (5%, 0.5 mL) and acetone (0.5 mL). The solvents were removed *in vacuo*. Water (50 mL) was added, and the mixture was filtered (0.2 µm). Purification by ion-exchange HPLC (Dionex BioLC DNAPac PA-100, 22 x 250 mm, eluent A = water, eluent B = 1 M aq. NH_4HCO_3, gradient from 0 to 25% B in 16 min, flow rate = 10 mL/min, R_t = 13 min), followed by reverse phase HPLC (Waters Prep Nova-Pak HR C_{18} column, 60 Å, 19 x 300 mm, eluent A = 25 mM TEAA pH 7, eluent B = 50% CH_3CN in A, gradient from 0 to 70% B in 20 min, flow rate = 5 mL/min, R_t = 19 min) gave 3'-O-(N-acetone-oxime)-thymidine-5'-triphosphate as a colorless foam after lyophilization. The yield was determined by UV (260 nm, ext. coeff. = 8800 $Lmol^{-1}cm^{-1}$) to be 450 µmol (45%).

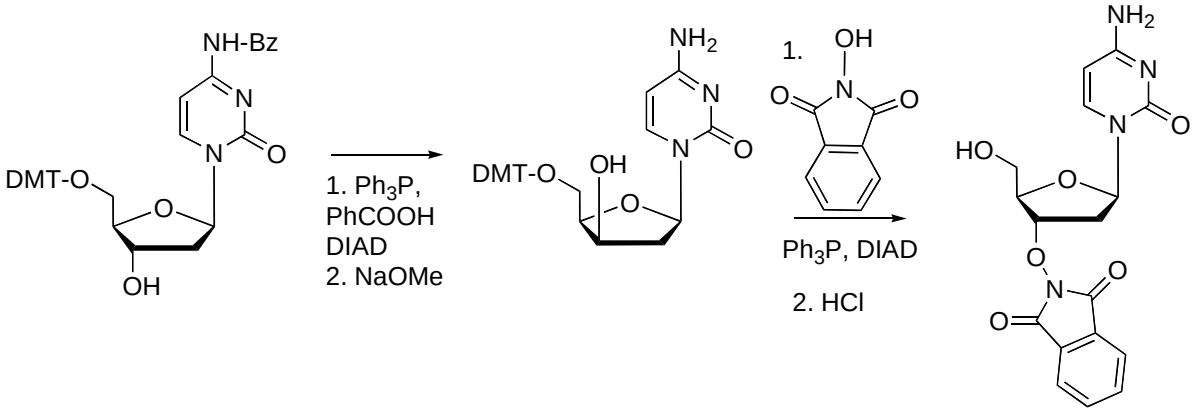
^{1}H-NMR (D_2O, 300 MHz): ∂ (ppm, rel to HDO = 4.65) = 1.75-1.79 (m, 9H); 2.18-2.40 (m, 2H); 4.00-4.15 (m, 2H); 4.22-4.27 (m, 1H); 4.46 (s, 2H); 4.78-4.85 (m, 1H); 6.21 (dd, *J* = 5.7, 9.1 Hz, 1H); 7.67 (s, 1H). ^{31}P-NMR (D_2O, 120 MHz): ∂ (ppm, rel to external H_3PO_4 = 0) = -10.5 (d, *J* = 20.0 Hz, 1P); -11.7 (d, *J* = 20.0 Hz, 1P); -23.3 (t, *J* = 20.0 Hz, 1P).

3'-O-Amino-thymidine-5'-triphosphate. To a solution of 3'-O-(N-acetone-oxime)-thymidine-5'-triphosphate (100 µmol) in water (10 mL) was added aqueous sodium acetate buffer (1M, pH 4.0, 2 mL, 2 mmol) and aqueous hydroxylamine solution (50 wt-%, 100 µL, ca. 1.6 mmol). After 2 h at room temperature, the reaction was diluted with water (20 mL) and filtered (0.2 µm). Purification by ion-

exchange HPLC (Dionex BioLC DNAPac PA-100, 22 x 250 mm, eluent A = water, eluent B = 1 M aq. NH_4HCO_3, gradient from 0 to 30% B in 20 min, flow rate = 10 mL/min, R_t = 15 min) gave 3'-O-amino-thymidine-5'-triphosphate as a colorless foam after lyophilization. The yield was determined by UV (260 nm, ext. coeff. = 8800 $Lmol^{-1}cm^{-1}$) to be 82 µmol (82%).

^{1}H-NMR (D_2O, 300 MHz): ∂ (ppm, rel to HDO = 4.65) = 1.78 (d, *J* = 0.9 Hz, 3H); 2.18-2.29 (m, 1H); 2.37-2.46 (m, 1H); 4.01-4.16 (m, 2H); 4.25-4.29 (m, 1H); 4.61-4.63 (m, 1H); 6.17 (dd, *J* = 5.8, 9.0 Hz, 1H); 7.62 (d, *J* = 1.2 Hz, 1H). ^{31}P-NMR (D_2O, 120 MHz): ∂ (ppm, rel to external H_3PO_4 = 0) = -10.8 (d, *J* = 20 Hz, 1P); -11.7 (d, *J* = 20 Hz, 1P); -23.1 (t, *J* = 20 Hz, 1P).

Synthesis of dCTP-ONH$_2$



5'-O-Dimethoxytrityl-xylo-2'-deoxycytidine. To a solution of N^4-benzoyl-5'-O-dimethoxytrityl-2'-deoxycytidine (8.9 g, 14 mmol), benzoic acid (2.5 g, 20 mmol) and triphenylphosphine (5.2 g, 20 mmol) in THF (150 mL) was added DIAD (3.7 mL, 20 mmol) at 0°C. The reaction was let to warm to room temperature overnight and then was quenched by the addition of water (0.5 mL). The solvents were removed *in vacuo*. Purification by FLC (silica, gradient 50 to 100% EtOAc in hexanes) gave N^4-benzoyl-3'-O-benzoyl-5'-O-dimethoxytrityl-xylo-2'-deoxycytidine (13.7 g) as a colorless foam which, according to NMR, contained significant amounts of triphenylphosphine oxide, as well as some elimination product (2',3'-olefin).

This intermediate was redissolved in methanol (450 mL) and treated with a solution of sodium methoxide in methanol (5.3 M, 4 mL, 21 mmol). After 2 h at room temperature, the reaction was quenched by the addition of AcOH (glacial, 1.25 mL). The solvents were removed *in vacuo* and the residue was

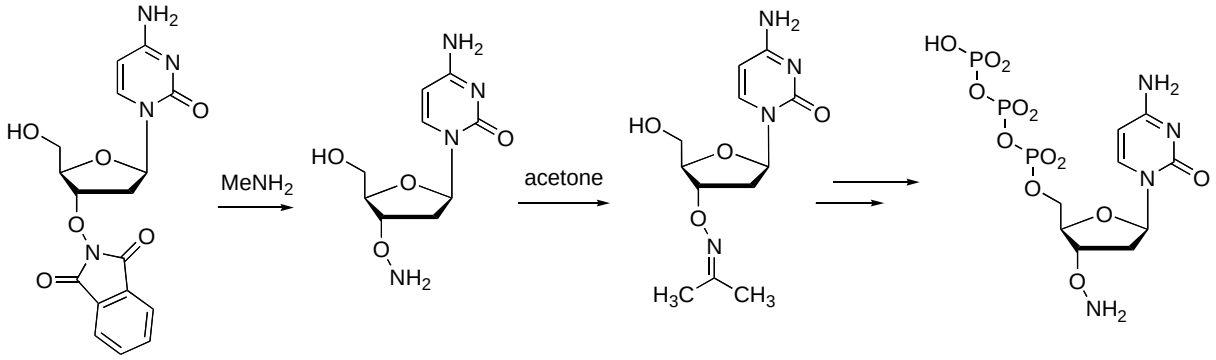
partitioned between DCM (300 mL) and aqueous NaCl (50% sat., 150 mL). The organic phase was separated and the solvent removed *in vacuo*. Purification by FLC (silica, gradient 5 to 10% MeOH in DCM) gave 5'-O-dimethoxytrityl-xylo-2'-deoxycytidine (4.6 g; 62% overall) as a colorless foam.

^{1}H-NMR (d_6-DMSO, 300 MHz): ∂ (ppm) = 1.78-1.87 (m, 1H); 2.46-2.55 (m, 1H); 3.19-3.24 (m, 1H); 3.37-3.43 (m, 1H); 3.76 (s, 6H); 4.07-4.12 (m, 1H); 4.16-4.19 (m, 1H); 5.10-5.20 (m, 1H); 5.66 (d, *J* = 7.4 Hz, 1H); 6.07 (dd, *J* = 1.7, 7.9 Hz, 1H); 6.86-6.92 (m, 4H); 7.16 (br s, 2H); 7.18-7.48 (m, 9H); 7.68 (d, *J* = 7.4 Hz, 1H). ^{13}C-NMR (d_6-DMSO, 75 MHz): ∂ (ppm) = 41.4; 55.0; 62.8; 69.2; 83.4; 85.4; 85.5; 93.0; 113.1; 126.6; 127.8; 129.8; 135.6; 135.7; 141.6; 145.0; 155.2; 158.0; 165.6.

3'-O-Phthalimido-2'-deoxycytidine. To a solution of 5'-O-dimethoxytrityl-xylo-2'-deoxycytidine (3.4 g, 6.4 mmol), N-hydroxy-phthalimide (1.6 g, 10 mmol) and triphenylphosphine (2.6 g, 10 mmol) in THF (180 mL) was added DIAD (1.9 mL, 10 mmol) at 0°C. The reaction was let to warm to room temperature overnight and then was quenched by the addition of water (0.5 mL). The solvents were removed *in vacuo*. Purification by FLC (silica, gradient 3 to 10% MeOH in DCM) gave 5'-O-dimethoxytrityl-3'-O-phthalimido-2'-deoxycytidine (3.7 g) as a colorless foam which, according to NMR, contained significant amounts of triphenylphosphine oxide, as well as some elimination product (2',3'-olefin).

This intermediate was redissolved in methanol (150 mL) and treated with aqueous HCl (conc, 7.5 mL) at room temperature. Within minutes, the product started to precipitate. After 10 minutes, the solids were filtered off and dried at high vacuum to give 3'-O-phthalimido-2'-deoxycytidine (1.5 g, 63% overall) as an off-white powder.

^{1}H-NMR (d_6-DMSO, 300 MHz): ∂ (ppm) = 2.28-2.38 (m, 1H); 2.65-2.74 (m, 1H); 3.62-3.68 (m, 2H); 4.35-4.40 (m, 1H); 4.95-5.00 (m, 1H); 6.20 (d, *J* = 7.9 Hz, 1H); 6.25 (dd, *J* = 6.9, 7.0 Hz, 1H); 7.89 (s, 4H); 8.22 (d, *J* = 7.9 Hz, 1H); 8.71 (s, 1H); 9.83 (s, 1H). ^{13}C-NMR (d_6-DMSO, 75 MHz): ∂ (ppm) = 36.6; 61.0; 84.1; 85.8; 87.7; 94.0; 123.3; 128.6; 134.8; 144.2; 146.9; 159.5; 163.6.



3'-O-(N-acetone-oxime)-2'-deoxycytidine. 3'-O-phthalimido-2'-deoxycytidine (375 mg, 1.0 mmol) was dissolved in aqueous methylamine solution (4%, 11 mL, ca. 12 mmol). After 10 min, most of the methylamine was removed *in vacuo*, and the remaining solution was treated with acetone (2 mL). After 3 h at room temperature, the solvent was removed *in vacuo*. The residue was redissolved in water (30 mL) and the mixture was filtered (0.2 µm). Purification by reverse phase HPLC (Waters Prep Nova-Pak HR C_{18} column, 60 Å, 19 x 300 mm, eluent A = 25 mM TEAA pH 7, eluent B = CH_3CN, gradient from 0 to 50% B in 10 min, then to 85% B in 8 min, flow rate = 5 mL/min, R_t = 17 min) gave 3'-O-(N-acetone-oxime)-2'-deoxycytidine (200 mg; 71%) as a colorless foam after lyophilization.

^{1}H-NMR (d_6-DMSO, 300 MHz): ∂ (ppm) = 1.83 (s, 3H); 1.84 (s, 3H); 1.99-2.09 (m, 1H); 2.30-2.39 (m, 1H); 3.55-3.66 (m, 2H); 4.02-4.06 (m, 1H); 4.65-4.70 (m, 1H); 5.30 (br. s, 1H); 5.77 (d, J = 7.4 Hz, 1H); 6.17 (dd, J = 5.6, 8.7 Hz, 1H); 7.23 (br. s, 2H); 7.84 (d, J = 7.4 Hz, 1H). ^{13}C-NMR (d_6-DMSO, 75 MHz): ∂ (ppm) = 15.5; 21.5; 37.3; 61.9; 82.4; 84.2; 85.2; 94.3; 141.0; 155.1; 155.7; 165.6.

3'-O-(N-acetone-oxime)-2'-deoxycytidine-5'-triphosphate. To a solution of 3'-O-(N-acetone-oxime)-2'-deoxycytidine (170 mg, 0.6 mmol) in pyridine (2 mL) and dioxane (1.5 mL) was added a solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (150 mg, 0.8 mmol) in dioxane (1.5 mL) at room temperature. After 15 min a mixture of tributylammonium pyrophosphate in DMF (0.2 M, 6 mL, 1.2 mmol) and tributylamine (0.7 mL, 2.8 mmol) was added. After 20 min a solution of iodine (210 mg, 0.8 mmol) and water (0.32 mL) in pyridine (16 mL) was added. After 20 min the reaction was quenched by the addition of aqueous Na_2SO_3 (5%, 0.5 mL) and acetone (0.5 mL). The solvents were removed *in vacuo*. Water (30 mL) was added, and the mixture was filtered (0.2 µm). Purification by ion-exchange HPLC (Dionex BioLC DNAPac PA-100, 22 x 250 mm, eluent A = water, eluent B = 1 M aq. NH_4HCO_3, gradient from 0 to 25% B in 16 min, flow rate = 10 mL/min, R_t = 14 min), followed by reverse phase

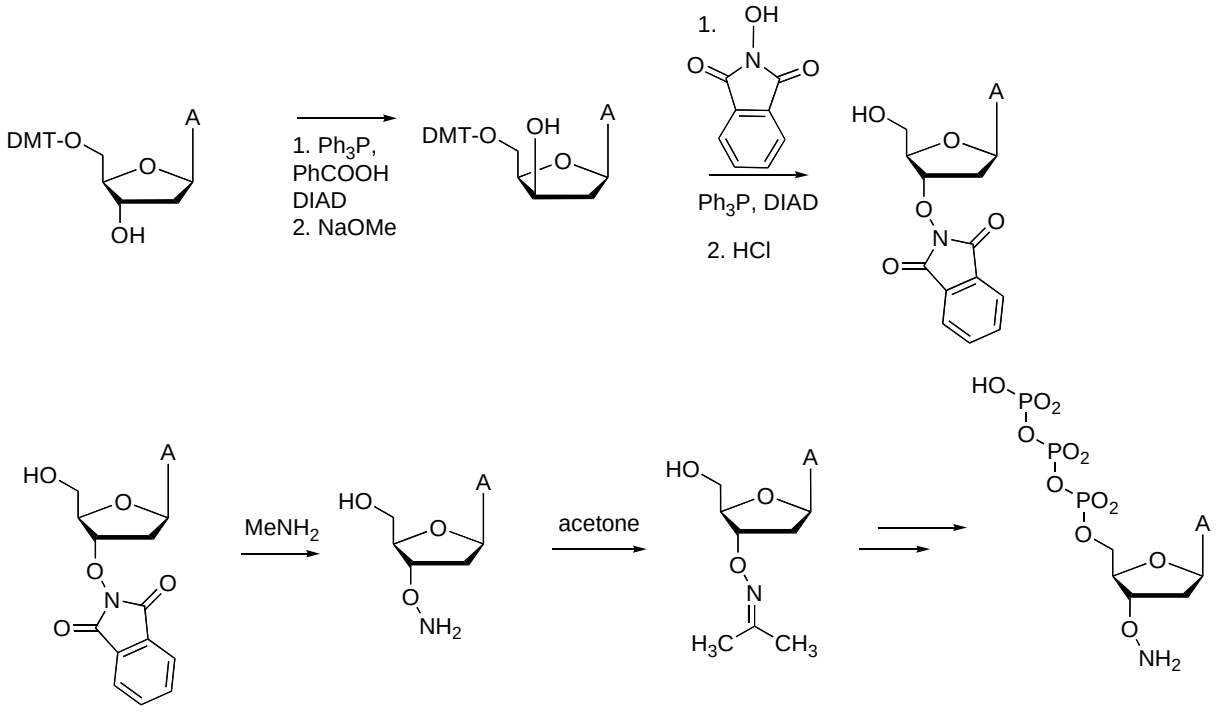
HPLC (Waters Prep Nova-Pak HR C_{18} column, 60 Å, 19 x 300 mm, eluent A = 25 mM TEAA pH 7, eluent B = 50% CH_3CN in A, gradient from 0 to 70% B in 20 min, flow rate = 5 mL/min, R_t = 18 min) gave 3'-O-(N-acetone-oxime)-2'-deoxycytidine-5'-triphosphate as a colorless foam after lyophilization. The yield was determined by UV (260 nm, ext. coeff. = 7300 $Lmol^{-1}cm^{-1}$) to be 225 µmol (38%).

^{1}H-NMR (D_2O, 300 MHz): ∂ (ppm, rel to HDO = 4.65) = 1.77 (s, 3H); 1.79 (s, 3H); 2.12-2.22 (m, 1H); 2.40-2.50 (m, 1H); 4.00-4.16 (m, 2H); 4.28-4.33 (m, 1H); 4.76-4.80 (m, 1H); 6.09 (d, *J* = 7.7 Hz, 1H); 6.18 (dd, *J* = 5.7, 8.4 Hz, 1H); 7.23 (br. s, 2H); 7.96 (d, *J* = 7.7 Hz, 1H). ^{31}P-NMR (D_2O, 120 MHz): ∂ (ppm, rel to external H_3PO_4 = 0) = -10.9 (d, *J* = 19.5 Hz, 1P); -11.4 (d, *J* = 19.5 Hz, 1P); -23.3 (t, *J* = 19.5 Hz, 1P).

3'-O-Amino-2'-deoxycytidine-5'-triphosphate. To a solution of 3'-O-(N-acetone-oxime)-2'-deoxycytidine-5'-triphosphate (100 µmol) in water (10 mL) was added aqueous sodium acetate buffer (1M, pH 4.0, 2 mL, 2 mmol) and aqueous hydroxylamine solution (50 wt-%, 100 µL, ca. 1.6 mmol). After 2 h at room temperature, the reaction was diluted with water (20 mL) and filtered (0.2 µm). Purification by ion-exchange HPLC (Dionex BioLC DNAPac PA-100, 22 x 250 mm, eluent A = water, eluent B = 1 M aq. NH_4HCO_3, gradient from 0 to 30% B in 20 min, flow rate = 10 mL/min, R_t = 16 min) gave 3'-O-amino-2'-deoxycytidine-5'-triphosphate as a colorless foam after lyophilization. The yield was determined by UV (260 nm, ext. coeff. = 7300 $Lmol^{-1}cm^{-1}$) to be 74 µmol (74%).

^{1}H-NMR (D_2O, 300 MHz): ∂ (ppm, rel to HDO = 4.65) = 2.09-2.16 (m, 1H); 2.40-2.50 (m, 1H); 4.00-4.10 (m, 2H); 4.25-4.30 (m, 1H); 4.40-4.45 (m, 1H); 6.02 (d, *J* = 6.5 Hz, 1H); 6.14 (dd, *J* = 6.0, 7.9 Hz, 1H); 7.85 (d, *J* = 6.5 Hz, 1H). ^{31}P-NMR (D_2O, 120 MHz): ∂ (ppm, rel to external H_3PO_4 = 0) = -10.2 (br, 1P); -11.3 (br, 1P); -22.9 (br, 1P).

Synthesis of dATP-ONH_2



5'-O-Dimethoxytrityl-xylo-2'-deoxyadenosine. To a solution of 5'-O-dimethoxytrityl-2'-deoxyadenosine (8.3 g, 15 mmol), benzoic acid (3.0 g, 24 mmol) and triphenylphosphine (6.5 g, 24 mmol) in THF (250 mL) was added DIAD (4.5 mL, 24 mmol) at room temperature. After 1 h the reaction was quenched by the addition of methanol (5 mL). The solvents were removed *in vacuo*. Purification by FLC (silica, gradient 3 to 5% MeOH in DCM) gave 3'-O-benzoyl-5'-O-dimethoxytrityl-xylo-2'-deoxyadenosine (12 g) as a colorless foam which, according to NMR, contained some triphenylphosphine oxide as well as some elimination product (2',3'-olefin).

This intermediate was redissolved in methanol (300 mL) and treated with a solution of sodium methoxide in methanol (5.3 M, 4 mL, 21 mmol). After 16 h at room temperature, the reaction was quenched by the addition of AcOH (glacial, 1.5 mL). The solvents were removed *in vacuo*. Purification by FLC (silica, gradient 3 to 10% MeOH in DCM) gave 5'-O-dimethoxytrityl-xylo-2'-deoxyadenosine (3.7 g; 45% overall) as a colorless foam.

^{1}H-NMR (d_6-DMSO, 300 MHz): ∂ (ppm) = 2.26-2.34 (m, 1H); 2.74-2.84 (m, 1H); 3.18-3.25 (m, 1H); 3.34-3.42 (m, 1H); 3.70-3.74 (2s, 6H); 4.17-4.22 (m, 1H); 4.31-4.36 (m, 1H); 5.95 (d, J = 5.7 Hz, 1H); 6.35 (dd, J = 1.0, 7.8 Hz, 1H); 6.77-6.86 (m, 4H); 7.16-7.44 (m, 11H); 8.16 (s, 1H); 8.27 (s, 1H). ^{13}C-NMR (d_6-DMSO, 75 MHz): ∂ (ppm) = 40.6; 55.0; 55.0; 63.1; 69.6; 82.9; 83.6; 85.5; 113.1; 119.0; 126.6; 127.7; 127.7; 129.7; 135.6; 135.8; 139.8; 145.0; 148.6; 152.3; 156.1; 158.0; 158.0.

3'-O-phthalimido-2'-deoxyadenosine. To a solution of 5'-O-dimethoxytrityl-xylo-2'-deoxyadenosine (3.4 g, 6 mmol), N-hydroxy-phthalimide (1.6 g, 10 mmol) and triphenylphosphine (2.6 g, 10 mmol) in THF (120 mL) was added DIAD (1.9 mL, 10 mmol) at room temperature. After 1 h the reaction was quenched by the addition of methanol (3 mL). The solvents were removed *in vacuo*. Purification by FLC (silica, gradient 3 to 5% MeOH in DCM) gave 5'-O-dimethoxytrityl-3'-O-phthalimido-2'-deoxyadenosine (6.2 g) as a colorless foam which, according to NMR, contained significant amounts of triphenylphosphine oxide, as well as some elimination product (2',3'-olefin).

This intermediate was redissolved in methanol (30 mL) and treated with methanolic HCl (1.25 M, 55 mL, ca. 70 mmol) at room temperature. Within minutes, the product started to precipitate. After 10 minutes, the solids were filtered off and dried at high vacuum to give 3'-O-phthalimido-2'-deoxyadenosine (1.5 g, 63% overall) as an off-white powder.

^{1}H-NMR (d_6-DMSO, 300 MHz): ∂ (ppm) = 2.62-3.02 (m, 2H); 3.60-3.66 (m, 2H); 4.37-4.41 (m, 1H); 5.13-5.18 (m, 1H); 6.59 (dd, J = 6.2, 7.2 Hz, 1H); 7.91 (s, 4H); 8.58 (s, 1H); 8.78 (s, 1H); 8.97 (br s, 1H); 9.60 (br s, 1H). ^{13}C-NMR (d_6-DMSO, 75 MHz): ∂ (ppm) = 36.1; 61.2; 83.8; 84.1; 88.0; 118.5; 123.4; 128.7; 134.9; 142.0; 145.2; 148.1; 150.4; 163.8.

3'-O-(N-acetone-oxime)-2'-deoxyadenosine. 3'-O-phthalimido-2'-deoxyadenosine (790 mg, 2.0 mmol) was dissolved in aqueous methylamine solution (4%, 22 mL, ca. 24 mmol). After 20 min, most of the methylamine was removed *in vacuo*, and the remaining solution was treated with acetone (3 mL). After 3 h at room temperature, the solvent was removed *in vacuo*. The residue was redissolved in water (35 mL) and CH_3CN (15 mL), and the mixture was filtered (0.2 µm). Purification by reverse phase HPLC (Waters Prep Nova-Pak HR C_{18} column, 60 Å, 19 x 300 mm, eluent A = 25 mM TEAA pH 7, eluent B = CH_3CN, gradient from 25 to 50% B in 7 min, then to 80% B in 8 min, flow rate = 5 mL/min, R_t = 14 min) gave 3'-O-(N-acetone-oxime)-2'-deoxyadenosine (465 mg; 76%) as a colorless foam after lyophilization.

^{1}H-NMR (d_6-DMSO, 300 MHz): ∂ (ppm) = 1.86 (s, 3H); 1.87 (s, 3H); 2.47-2.55 (m, 1H); 2.82-2.93 (m, 1H); 3.54-3.72 (m, 2H); 4.11-4.16 (m, 1H); 4.81-4.85 (m, 1H); 5.43 (dd, J = 4.7, 7.0 Hz, 1H); 6.33 (dd, J = 5.9, 8.9 Hz, 1H); 7.34 (br. s, 2H); 8.13 (s, 1H); 8.35 (s, 1H). ^{13}C-NMR (d_6-DMSO, 75 MHz): ∂ (ppm) = 15.5; 21.5; 36.4; 62.2; 82.5; 84.4; 84.9; 119.3; 139.6; 148.8; 152.3; 155.9; 156.2.

3'-O-(N-acetone-oxime)-2'-deoxyadenosine-5'-triphosphate. To a suspension of 3'-O-(N-acetone-oxime)-2'-deoxyadenosine (180 mg, 0.6 mmol) in pyridine (2 mL), dioxane (1.5 mL) and DMF (1 mL) was added a solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (150 mg, 0.8 mmol) in dioxane (1.5 mL) at room temperature, leading to a clear solution. After 15 min a mixture of tributylammonium pyrophosphate in DMF (0.2 M, 6 mL, 1.2 mmol) and tributylamine (0.7 mL, 2.8 mmol) was added. After 20 min a solution of iodine (210 mg, 0.8 mmol) and water (0.32 mL) in pyridine (16 mL) was added. After 20 min the reaction was quenched by the addition of aqueous Na_2SO_3 (5%, 0.5 mL) and acetone (0.5 mL). The solvents were removed *in vacuo*. Water (40 mL) was added, and the mixture was filtered (0.2 µm). Purification by ion-exchange HPLC (Dionex BioLC DNAPac PA-100, 22 x 250 mm, eluent A = water, eluent B = 1 M aq. NH_4HCO_3, gradient from 0 to 25% B in 16 min, flow rate = 10 mL/min, R_t = 13 min), followed by reverse phase HPLC (Waters Prep Nova-Pak HR C_{18} column, 60 Å, 19 x 300 mm, eluent A = 25 mM TEAA pH 7, eluent B = 50% CH_3CN in A, gradient from 0 to 100% B in 20 min, flow rate = 5 mL/min, R_t = 18 min) gave 3'-O-(N-acetone-oxime)-2'-deoxyadenosine-5'-triphosphate as a colorless foam after lyophilization. The yield was determined by UV (260 nm, ext. coeff. = 15400 $Lmol^{-1}cm^{-1}$) to be 240 µmol (40%).

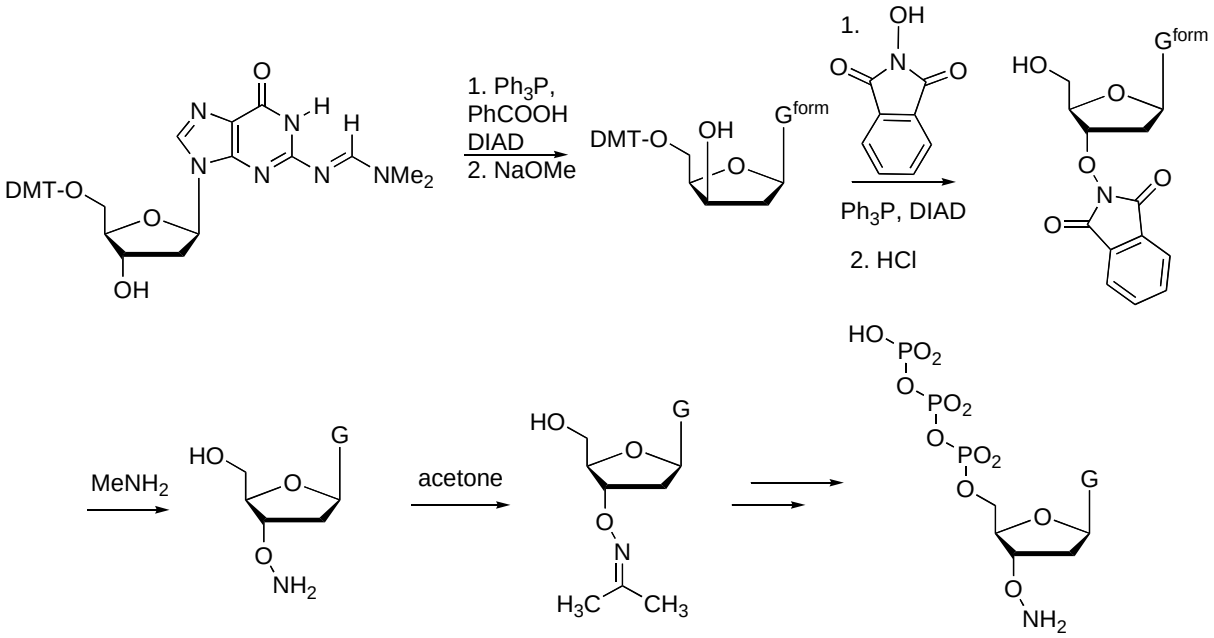
^{1}H-NMR (D_2O, 300 MHz): ∂ (ppm, rel to HDO = 4.65) = 1.78 (s, 3H); 1.83 (s, 3H); 2.55-2.78 (m, 2H); 3.97-4.13 (m, 2H); 4.32-4.37 (m, 1H); 4.90-4.95 (m, 1H); 6.33 (dd, *J* = 5.8, 9.0 Hz, 1H); 8.03 (s, 1H); 8.37 (s, 1H). ^{31}P-NMR (D_2O, 120 MHz): ∂ (ppm, rel to external H_3PO_4 = 0) = -10.4 (d, *J* = 19.5 Hz, 1P); -11.4 (d, *J* = 19.5 Hz, 1P); -23.2 (t, *J* = 19.5 Hz, 1P).

3'-O-Amino-2'-deoxyadenosine-5'-triphosphate. To a solution of 3'-O-(N-acetone-oxime)-2'-deoxyadenosine-5'-triphosphate (100 µmol) in water (10 mL) was added aqueous sodium acetate buffer (1M, pH 4.0, 2 mL, 2 mmol) and aqueous hydroxylamine solution (50 wt-%, 100 µL, ca. 1.6 mmol). After 2 h at room temperature, the reaction was diluted with water (20 mL) and filtered (0.2 µm). Purification by ion-exchange HPLC (Dionex BioLC DNAPac PA-100, 22 x 250 mm, eluent A = water, eluent B = 1 M aq. NH_4HCO_3, gradient from 0 to 30% B in 20 min, flow rate = 10 mL/min, R_t = 15 min) gave 3'-O-amino-2'-deoxyadenosine-5'-triphosphate as a colorless foam after lyophilization. The yield was determined by UV (260 nm, ext. coeff. = 15400 $Lmol^{-1}cm^{-1}$) to be 65 µmol (65%).

^{1}H-NMR (D_2O, 300 MHz): ∂ (ppm, rel to HDO = 4.65) = 2.36-2.43 (m, 1H); 2.57-2.63 (m, 1H); 3.93-4.10 (m, 2H); 4.29-4.34 (m, 1H); 4.50-4.54 (m, 1H); 6.28 (dd, *J* = 7.0, 8.0 Hz, 1H); 8.04 (s, 1H); 8.33 (s,

1H). ^{31}P-NMR (D_2O, 120 MHz): ∂ (ppm, rel to external H_3PO_4 = 0) = -8.8 (d, *J* = 19.5 Hz, 1P); -11.2 (d, *J* = 19.5 Hz, 1P); -22.6 (t, *J* = 19.5 Hz, 1P).

Synthesis of dGTP-ONH$_2$



5'-O-Dimethoxytrityl-N^2-dimethylaminomethylidene-xylo-2'-deoxyguanosine. To a solution of 5'-O-dimethoxytrityl-N^2-dimethylaminomethylidene-2'-deoxyguanosine (9.4 g, 15 mmol), benzoic acid (3.0 g, 24 mmol) and triphenylphosphine (6.5 g, 24 mmol) in THF (250 mL) was added DIAD (4.5 mL, 24 mmol) at room temperature. After 30 min the reaction was quenched by the addition of methanol (2 mL). The solvents were removed *in vacuo*.

This intermediate was redissolved in methanol (600 mL) and treated with a solution of sodium methoxide in methanol (5.3 M, 7.6 mL, 40 mmol). After 16 h at room temperature, the reaction was quenched by the addition of AcOH (glacial, 2.3 mL, 40 mmol). The solvents were removed *in vacuo*. Purification by FLC (silica, gradient 0 to 10% MeOH in DCM) gave 5'-O-dimethoxytrityl-N^2-dimethylaminomethylidene-xylo-2'-deoxyguanosine (5.6 g; 50% overall) as a colorless foam.

^{1}H-NMR (d_6-DMSO, 300 MHz): ∂ (ppm) = 2.18-2.26 (m, 1H); 2.69-2.80 (m, 1H); 3.03 (s, 3H); 3.11 (s, 3H); 3.19-3.25 (m, 1H); 3.34-3.40 (m, 1H); 3.70-3.74 (2s, 6H); 4.16-4.20 (m, 1H); 4.32-4.37 (m, 1H); 5.57-5.61 (m, 1H); 6.29 (dd, *J* = 1.5, 8.4 Hz, 1H); 6.80-6.86 (m, 4H); 7.16-7.44 (m, 9H); 8.00 (s, 1H);

8.54 (s, 1H); 11.38 (s, 1H). ^{13}C-NMR (d_6-DMSO, 75 MHz): ∂ (ppm) = 34.6; 40.6; 40.9; 55.0; 55.0; 63.2; 69.4; 82.0; 83.5; 85.5; 113.1; 119.4; 126.6; 127.7; 129.7; 129.8; 135.6; 135.7; 137.3; 145.0; 149.4; 157.3; 157.7; 157.9; 158.0; 158.0.

5'-O-Dimethoxytrityl-N^2-dimethylaminomethylidene-3'-O-phthalimido-2'-deoxyguanosine. To a solution of 5'-O-dimethoxytrityl-N^2-dimethylaminomethylidene-xylo-2'-deoxyguanosine (4.7 g, 7.5 mmol), N-hydroxy-phthalimide (2.1 g, 13 mmol) and triphenylphosphine (3.4 g, 13 mmol) in THF (150 mL) was added DIAD (2.5 mL, 13 mmol) at room temperature. After 1 h the reaction was quenched by the addition of methanol (2 mL). The solvents were removed *in vacuo*. Purification by FLC (silica, gradient 3 to 10% MeOH in DCM) gave 5'-O-dimethoxytrityl-N^2-dimethylaminomethylidene-3'-O-phthalimido-2'-deoxyguanosine (5.3 g) as a colorless foam which, according to NMR, contained ca 0.25 equivalents of elimination product (2',3'-olefin). An analytical sample was repurified by reverse phase HPLC (Waters Prep Nova-Pak HR C18 column, 60 Å, 19 x 300 mm, eluent A = 25 mM TEAA pH 7, eluent B = CH_3CN, gradient from 50% to 90% B in 18 min, then constant 90% B for 6 min, flow rate = 5 mL/min, Rt = 22 min) to give a colorless foam after lyophilization.

^{1}H-NMR ($CDCl_3$, 300 MHz): ∂ (ppm) = 2.64-2.74 (m, 1H); 2.84-2.94 (m, 1H); 3.09 (s, 3H); 3.16 (s, 3H); 3.31-3.45 (m, 2H); 3.75 (s, 6H); 4.56-4.61 (m, 1H); 5.12-5.16 (m, 1H); 6.53 (dd, J = 5.5, 8.6 Hz, 1H); 6.72-6.78 (m, 4H); 7.12-7.36 (m, 10H); 7.72-7.85 (m, 5H); 8.67 (s, 1H); 10.11 (s, 1H). ^{13}C-NMR ($CDCl_3$, 75 MHz): ∂ (ppm) = 35.3; 41.5; 55.3; 63.6; 82.6; 83.6; 86.7; 88.7; 113.3; 120.4; 123.9; 127.0; 128.0; 128.1; 128.7; 130.0; 130.1; 135.0; 135.5; 135.9; 144.4; 150.4; 157.1; 158.5; 158.6; 158.6; 164.0.

3'-O-(N-acetone-oxime)-2'-deoxyguanosine. To a solution of 5'-O-dimethoxytrityl-N^2-dimethylaminomethylidene-3'-O-phthalimido-2'-deoxyguanosine (900 mg, ca. 1 mmol phthalimido-compound, contains ca. 0.25 eq. 2',3'-olefin) in methanol (7 mL) was added aqueous HCl (conc, 0.4 mL, ca 5 mmol) and TFA (0.1 mL, ca 1.5 mmol). The mixture was shaken for 5 min at room temperature, leading to a clear solution. Ammonium hydroxide (30%, 5 mL, ca 80 mmol) was added, and the resulting suspension was stirred for 1 h. Aqueous methylamine solution (10%, 13 mL, ca. 36 mmol) was added. After 20 min, the supernatant was filtered off and most of the methylamine and ammonia was removed *in vacuo*. The remaining solution was neutralized with dilute aqueous HCl and treated with acetone (3 mL) and CH_3CN (5 mL). After 3 h at room temperature, the mixture was diluted with water (20 mL) and

CH_3CN (20 mL) and filtered (0.2 µm). Purification by reverse phase HPLC (Waters Prep Nova-Pak HR C_{18} column, 60 Å, 19 x 300 mm, eluent A = 25 mM TEAA pH 7, eluent B = CH_3CN, gradient from 25 to 50% B in 5 min, then to 80% B in 12 min, flow rate = 5 mL/min, R_t = 13 min) gave 3'-O-(N-acetone-oxime)-2'-deoxyguanosine (120 mg; 37%) as a colorless foam after lyophilization.

^{1}H-NMR (d_6-DMSO, 300 MHz): ∂ (ppm) = 1.84 (s, 3H); 1.85 (s, 3H); 2.41-2.50 (m, 1H); 2.62-2.72 (m, 1H); 3.52-3.64 (m, 2H); 4.02-4.08 (m, 1H); 4.74-4.78 (m, 1H); 5.05-5.12 (m, 1H); 6.09 (dd, *J* = 5.7, 8.9 Hz, 1H); 6.51 (br. s, 2H); 7.95 (s, 1H); 10.60 (br. s, 1H). ^{13}C-NMR (d_6-DMSO, 75 MHz): ∂ (ppm) =

3'-O-(N-acetone-oxime)-2'-deoxyguanosine-5'-triphosphate. To a suspension of 3'-O-(N-acetone-oxime)-2'-deoxyguanosine (100 mg, 0.3 mmol) in pyridine (1 mL), dioxane (0.8 mL) and DMF (1 mL) was added a solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (75 mg, 0.4 mmol) in dioxane (0.75 mL) at room temperature, leading to a clear solution. After 15 min a mixture of tributylammonium pyrophosphate in DMF (0.2 M, 3 mL, 0.6 mmol) and tributylamine (0.35 mL, 1.4 mmol) was added. After 20 min a solution of iodine (100 mg, 0.4 mmol) and water (0.16 mL) in pyridine (8 mL) was added. After 20 min the reaction was quenched by the addition of aqueous Na_2SO_3 (5%, 0.5 mL) and acetone (0.5 mL). The solvents were removed *in vacuo*. Water (30 mL) was added, and the mixture was filtered (0.2 µm). Purification by ion-exchange HPLC (Dionex BioLC DNAPac PA-100, 22 x 250 mm, eluent A = water, eluent B = 1 M aq. NH_4HCO_3, gradient from 0 to 25% B in 16 min, flow rate = 10 mL/min, R_t = 16 min), followed by reverse phase HPLC (Waters Prep Nova-Pak HR C_{18} column, 60 Å, 19 x 300 mm, eluent A = 25 mM TEAA pH 7, eluent B = 50% CH_3CN in A, gradient from 0 to 100% B in 20 min, flow rate = 5 mL/min, R_t = 18 min) gave 3'-O-(N-acetone-oxime)-2'-deoxyguanosine-5'-triphosphate as a colorless foam after lyophilization. The yield was determined by UV (260 nm, ext. coeff. = 11700 $Lmol^{-1}cm^{-1}$) to be 135 µmol (45%).

^{1}H-NMR (D_2O, 300 MHz): ∂ (ppm, rel to HDO = 4.65) = 1.78 (s, 3H); 1.81 (s, 3H); 2.45-2.55 (m, 1H); 2.65-2.80 (m, 1H); 4.00-4.13 (m, 2H); 4.27-4.32 (m, 1H); 4.87-4.92 (m, 1H); 6.14 (dd, *J* = 5.8, 9.0 Hz, 1H); 7.98 (s, 1H). ^{31}P-NMR (D_2O, 120 MHz): ∂ (ppm, rel to external H_3PO_4 = 0) = -9.7 (d, *J* = 19.5 Hz, 1P); -11.4 (d, *J* = 19.5 Hz, 1P); -23.1 (t, *J* = 19.5 Hz, 1P).

3'-O-Amino-2'-deoxyguanosine-5'-triphosphate. To a solution of 3'-O-(N-acetone-oxime)-2'-deoxyguanosine-5'-triphosphate (50 µmol) in water (5 mL) was added aqueous sodium acetate buffer (1M,

pH 4.0, 1 mL, 1 mmol) and aqueous hydroxylamine solution (50 wt-%, 50 µL, ca. 0.8 mmol). After 2 h at room temperature, the reaction was diluted with water (10 mL) and filtered (0.2 µm). Purification by ion-exchange HPLC (Dionex BioLC DNAPac PA-100, 22 x 250 mm, eluent A = water, eluent B = 1 M aq. NH_4HCO_3, gradient from 0 to 30% B in 20 min, flow rate = 10 mL/min, R_t = 18 min) gave 3'-O-amino-2'-deoxyguanosine-5'-triphosphate as a colorless foam after lyophilization. The yield was determined by UV (260 nm, ext. coeff. = 11700 $Lmol^{-1}cm^{-1}$) to be 36 µmol (72%).

^{1}H-NMR (D_2O, 300 MHz): ∂ (ppm, rel to HDO = 4.65) = 2.50-2.75 (m, 2H); 3.97-4.13 (m, 2H); 4.29-4.34 (m, 1H); 4.55-4.60 (m, 1H); 6.08-6.16 (m, 1H); 8.00 (s, 1H). ^{31}P-NMR (D_2O, 120 MHz): ∂ (ppm, rel to external H_3PO_4 = 0) = -10.6 (br, 1P); -11.2 (br, 1P); -23.0 (br, 1P).

Cleavage chemistry

(a) Cleavage of *O*-(4-nitrobenzyl)hydroxylamine with aqueous HONO at varying dielectric.

To an aqueous solution of *O*-(4-nitrobenzyl)hydroxylamine (1 mM, 300 µL) were added "co-solvent" (brine or water or ethanol or isopropanol or acetonitrile or 1,4-dioxane) (500 µL), aqueous sodium acetate buffer (1 M, 100 µL, pH 3.5 to 6.0), and aqueous sodium nitrite solution (100 mM, 100 µL). The resulting pH was measured with a microelectrode (accuracy ca. ± 0.02). After 1 h at room temperature, and aliquot (100 µL) was removed, neutralized by the addition of K-phosphate buffer (170 mM, 600 µL, pH 7), and analyzed by analytical reverse-phase HPLC (Waters NovaPak C-18 4µm, 3.9x150 mm, with guard column Waters NovaPak C-18 4µm, 3.9x15mm, eluent A = 3% acetonitrile in 25 mM TEAA pH 7, eluent B = acetonitrile, gradient from 20% B to 50% B in 30 min, flow rate = 0.5 mL/min, R_t = product: 8.5 min; starting material: 9.5 min.).

(b1) Cleavage of 3'-O-aminothymidine with aqueous HONO and dioxane as cosolvent.

To an aqueous solution of 3'-O-aminothymidine (**1b**, 20 mM, 50 µL) were added dioxane (300 µL) and aqueous nitrous acid (1 M, 700 µL, pH 5.0 to 6.0, prepared from sodium nitrite and 1 M NaOAc buffer). The resulting pH was measured with a microelectrode (accuracy ca. ± 0.02). After 5 min at room temperature, and aliquot (100 µL) was removed, neutralized by the addition of K-phosphate buffer (1 M, 600 µL, pH 7), and analyzed by analytical reverse-phase HPLC (Waters NovaPak C-18 4µm, 3.9x150 mm, with guard column Waters NovaPak C-18 4µm, 3.9x15mm, eluent A = 25 mM TEAA pH 7, eluent B = acetonitrile, gradient from 3% B to 13% B in 20 min, flow rate = 0.5 mL/min, R_t = product: 8 min; starting material: 11 min.). The amount of cleavage was determined by integrating (267 nm) the peaks of

the remaining starting material (3'-O-aminothymidine) and the product (thymidine). The rates are as follows:

actual pH (± 0.02)	product after 5 min
6.54	17%
6.21	48%
6.00	85%
5.72	>99%

(b) Cleavage of 3'-O-aminothymidine with aqueous HONO and no cosolvent.

To an aqueous solution of 3'-O-aminothymidine (**1b**, 20 mM, 2 µL) was added aqueous nitrous acid (350-700 mM $NaNO_2$/1 M NaOAc, 50 µL, pH 5.5-5.75). The resulting pH was measured with a microelectrode (accuracy ca. ± 0.02). After 1 or 2 min at room temperature, the reaction was quenched by the addition of K-phosphate buffer (1 M, 200 µL, pH 7), and analyzed by analytical reverse-phase HPLC (Waters NovaPak C-18 4µm, 3.9x150 mm, with guard column Waters NovaPak C-18 4µm, 3.9x15mm, eluent A = 25 mM TEAA pH 7, eluent B = acetonitrile, gradient from 3% B to 13% B in 20 min, flow rate = 0.5 mL/min, R_t = product: 8 min; starting material: 11 min.). The amount of cleavage was determined by integrating (267 nm) the peaks of the remaining starting material (3'-O-aminothymidine) and the product (thymidine). The rates are as follows:

conc of $NaNO_2$	actual pH (± 0.02)	product after 1 min	product after 2 min
350	5.50	n/a	90%
700	5.50	98%	>99%
700	5.65	n/a	96%

As control, the natural nucleosides were treated as follows:

An aqueous solution of 2'-deoxyguanosine or 2'-deoxyadenosine or 2'-deoxycytidine (20 mM, 30 µL) was treated with aqueous nitrous acid (700 mM $NaNO_2$, 1 M NaOAc, pH 5.5, 500 µL) at room temperature for 72 h (i.e. 4320 min). An aliquot (50 µL) was removed, neutralized by the addition of K-phosphate buffer (1 M, 200 µL, pH 7), and analyzed by analytical reverse-phase HPLC (Waters NovaPak C-18 4µm, 3.9x150 mm, with guard column Waters NovaPak C-18 4µm, 3.9x15mm. eluent A = 25 mM TEAA pH 7, eluent B = acetonitrile, gradient from 0% B to 3% B in 10 min, then to 30% B in 20 min, flow rate = 0.5 mL/min, R_t = dG: 14 min, dA: 18 min, dC: 8 min). The amount of decomposition was determined by integrating (260 nm) the peaks of the remaining starting material (nucleoside) and the product(s). The results are as follows:

nucleoside	byproducts after 72 h @ 260 nm
dG	20%
dA	13%
dC	15%

References

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4. Burgess, K., Gibbs, R. A., Metzker, M. L., Raghavachari, R. (1994) Synthesis of an oxyamide linked nucleotide dimer and incorporation into antisense oligonucleotide sequences. *J. Chem. Soc. Chem. Commun.* **8**, 915-916.

Methods:

Reconstructing evolutionary adaptive paths (REAP) library creation

Genes encoding polymerases containing amino acid replacements at sites identified using the REAP approach were prepared by DNA2.0 (Menlo Park, California) in a synthetic codon-optimized *Taq* DNA polymerase gene (co-*Taq*). The mutations were distributed throughout 93 sequences (SI Appendix Table 1) to give variant polymerases that each had three or four amino acid replacements. These were cloned into the pASK-IBA43plus vector (IBA GmbH).

Preparation of *Taq* cell-free extract

Bacteria carrying the *Taq* library were grown overnight (37 °C, 250 rpm) in LB medium supplemented with ampicillin. Overnight cultures (40 µL) were used to inoculate LB-Amp cultures (5 mL). These were grown (37 °C, 250 rpm for 3.25 h) to an approximate OD_{550nm} of 1.8. Expression of the vector-borne polymerase variant was then induced with anhydrotetracycline (0.2 ng/ µL final concentration). The cells were allowed to grow for 4 hrs longer to OD_{550nm} ~ 3.5. An aliquot (200 µL) of the induced cell culture was harvested by centrifugation (4500 rpm, 15 min, 4 °C) and stored at -80 °C.

The activity of the endogenously expressed polymerase was tested without purification. Cells were re-suspended in ThermoPol Buffer (50 µL, New England BioLabs) and incubated at 65 °C for 30 min. The suspension was centrifuged and the supernatant, the cell-free extract containing *Taq* variant DNA polymerase, was directly used in primer extension assays.

Standing start primer-extension assays

Gamma-^{32}P-labeled primer (2.5 pmol), cold primer (22.5 pmol) and template (30 pmol) were annealed by incubation at 95 °C for 5 min in NEB ThermoPol Reaction Buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% TritonX-100) and slowly cooled to room temperature, followed by the addition of *Taq* cell-free extract (4 µL, the sole source of polymerase). The mixture (final volume 10 µL) was pre-heated to 72°C (30 sec); extension was initiated by adding either reversibly terminating or irreversibly terminating nucleoside triphosphates (final concentration of 100 µM) or both. After 2 min, the reaction was quenched by 10 mM EDTA in formamide loading buffer (20 µL); products were resolved by 14% PAGE.

Addition of the 3'-ONH_2 reversible terminator followed by cleavage and continued extension

A biotinylated template (30 pmol) was annealed to the complementary 5'-^{32}P-labeled primer (2.5 pmol) and cold primer (22.5 pmol) (Integrated DNA Technologies). The duplex was immobilized onto the magnetic beads loaded with streptavidin (Dynabeads M-270 Streptavidin, Dynal Biotech). All subsequent reactions were done on the magnetic beads.

In the first cycle, beads were incubated (72 °C, 2 min) with REAP-58 in the presence of T-ONH_2 triphosphate (100 μM). Then, the products were treated with buffered sodium nitrite/HONO (700 mM, pH 5.5) to cleave the –ONH_2 group, generating a free 3'-OH group. The cleavage buffer was removed and the beads were washed twice (500 μL of 0.5% $HONH_2$).

A second round of primer extension was then achieved by adding more REAP-58 polymerase and dTTP-ONH_2 into the reaction. The mixtures were incubated at 72 °C for 2 min. Cleavage and washing followed the same steps as described for the first cycle.

Following removal of the reversibly terminating 3'-ONH_2 group, full length product was then obtained by adding a third batch of REAP-58 polymerase and all dNTPs followed by a third incubation at 72 °C for 2 min. The products were then released from the bead (2 min, 90 °C, 10 mM EDTA, 95% formamide) and resolved by 20% denaturing PAGE.

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5'-32P-GCGTAATACGACTCACTATGGACG
    3′-CGCATTATGCTGAGTGATACCTGCAATGTGCTTCTG-Biotin-5′
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SNP detection test using capture of an aldehyde on the 3'-reversibly terminated oligonucleotide

To assess the ability of a combination of reversibly and irreversibly terminating nucleoside triphosphates to perform together in an architecture designed to detect SNPs, 5'-^{32}P-labeled primer (2.5 pmol), cold primer (20 pmol) and different ratios of templates (total 30 pmol) (shown on the Figure 5) were annealed and incubated in 1X NEB ThermoPol Reaction Buffer at 72 °C for 10 min with ddATP (the irreversible terminator), dTTP-ONH_2, dCTP-ONH_2 and dGTP-ONH_2 (the reversible terminators) (1 nmol each, 10 μL reaction volume). Primer extension was initiated by adding REAP-58 (1 μL of a 0.25 μg/μL solution). Reactions were quenched with 10 mM EDTA (5 μL).

The samples were then treated with 300 mole equivalents of PEG aldehyde (HO-CH_2-[CH_2-O-CH_2]$_2$-CH_2-CHO) and incubated at 65 °C for 10 hrs. Samples were resolved on a 20% denaturing PAGE and analyzed with a Molecular Imager FX system.

```
Primer (24 nt):           5′- GCGTAATACGACTCACTATGGACG            -3′
Standard Temp T (36 nt):  3′- CGCATTATGCTGAGTGATACCTGCTATGTGCTTCTG-5′
SNP Temp C (36 nt):       3′- CGCATTATGCTGAGTGATACCTGCCATGTGCTTCTG-5′
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SI Appendix figure legends:

SI Appendix figure 1: A phylogenetic tree of the Family A polymerases. This tree was obtained from the Pfam database, and analyzed for sites that underwent Type II functional divergence.

SI Appendix figure 2: Locations of the 35 sites in the crystal structure of *Taq* polymerase identified by a combination of structural and REAP analyses. These sites hold a total of 57 amino acid replacements, distributed throughout 93 mutant sequences of REAP library. Each mutant had three or four amino acid replacements.

SI Appendix figure 3a and 3b: Incorporation by *Taq* polymerase variants within the REAP library of 3'-modified 2'-deoxynucleoside thymidine triphosphate terminators. (a) Polyacrylamide gel showing extension of a primer using the reversibly blocked 3'-ONH_2 thymidine-5'-triphosphate (dTTP-O-NH_2). (b) Polyacrylamide gel showing extension of a primer using irreversibly blocked 2',3'-dideoxythymidine-5'-triphosphate. Unextended primer is at position N; N+1 is the extended product with reversibly or irreversibly blocked thymidine triphosphate. Co-Taq carries the poly-His tag (ASRGSHHHHHHGAGDRGM), which is presumably absent from NEB Taq; this may account for their different behaviors. The primer and template are shown as below:

Primer (24nt): 5'-^{32}P-GCGTAATACGACTCACTATGGACG
Template (36nt): 3'-CGCATTATGCTGAGTGATACCTGCAATGTGCTTCTG-5'

SI Appendix figure 3c and 3d: Correlation between the ability of various *Taq* variants within the REAP library to accept reversibly and irreversibly blocked thymidine triphosphates. X-axis: Ability of a polymerase to accept dTTP-ONH_2 thymidine triphosphate; Y-axis: Ability of a polymerase to accept ddTTP. (c) Plot that includes variants having a replacement at site 667. (d) Plot that excludes variants having a replacement at site 667.

SI Appendix figure 4a: Incorporation of reversibly blocked 3'-ONH_2 cytosine triphosphates by REAP variants. In a 10 μL reaction volume, γ^{32}P-labeled primer (2.5 pmol), cold primer (22.5 pmol) and the template (30 pmol) (see below) were annealed by incubation at 95 °C for 5 min and slow cooled to room temperature. 4 μL of Taq cell-free extracts were then added to reactions and incubated at 72 °C for 2 min. Reactions were initiated by the addition of dCTP-ONH_2 (Final concentration of 100 μM). The resulting reaction mixtures were separated on 14% PAGE and visualized by autoradiography. Unextended primer is at position N; N+1 is the extended product with dCTP-ONH_2.

Primer (24 nt): 5′- GCGTAATACGACTCACTATGGACG -3′
Template(36 nt): 3′- CGCATTATGCTGAGTGATACCTGCGGTGTGCTTCTG-5′

SI Appendix figure 4b: Correlation between the ability of various Taq polymerase variants of REAP library to accept dTTP-ONH_2 and dCTP-ONH_2. X-axis: the ability of a polymerase to accept a 2'-deoxy-3'-ONH_2 thymidine triphosphate; Y-axis: the ability of a polymerase to accept a 2',3'-dideoxynucleoside cytosine triphosphate.

SI Appendix figure 5: Incorporation of standard deoxythymidine triphosphate by REAP variants. Gamma-^{32}P-labeled primer (2.5 pmol), cold primer (22.5 pmol) and template (30 pmol) were annealed by incubation at 95 °C for 5 min in NEB ThermoPol Reaction Buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% TritonX-100) and slowly cooled to room temperature, followed by the addition of *Taq* cell-free extract (4 μL, the sole source of polymerase). The mixture (final volume 10 μL) was pre-heated to 72°C (30 sec); extension was initiated by adding dTTP (final concentration of 100 μM). After 2 min, the reaction was quenched by 10 mM EDTA in formamide loading buffer (20 μL); products were resolved by 14% PAGE. Unextended primer is at position N; N+2 is the full length extended product with dTTP.

```
Primer (24 nt):       5′- GCGTAATACGACTCACTATGGACG            -3′
Template(36 nt):      3′- CGCATTATGCTGAGTGATACCTGCAATGTGCTTCTG-5′
```

SI Appendix figure 6: Test the fidelity for the L616A and F667Y single replacement variants to incorporate the reversible and irreversible terminators. In a 10 μL reaction volume, γ^{32}P-labeled primer (2.5 pmol), cold primer (22.5 pmol) and four kinds of 36nt long template (30 pmol) (see below) were annealed respectively by incubation at 95 °C for 5 min and slow cooled to room temperature. 1 μL (0.25 μg/μL) of the L616A and F667Y single replacement variants (polymerases were purified by Qiagen Ni-NTA Spin Kit) were then added to reactions and incubated at 72 °C for 2 min. Reactions were initiated by the addition of dNTP-ONH_2 or ddNTP (Final concentration of 100 μM) (refer to gel for specifies). The resulting reaction mixtures were separated on 14% PAGE and visualized by autoradiography. The primer and templates are shown as below:

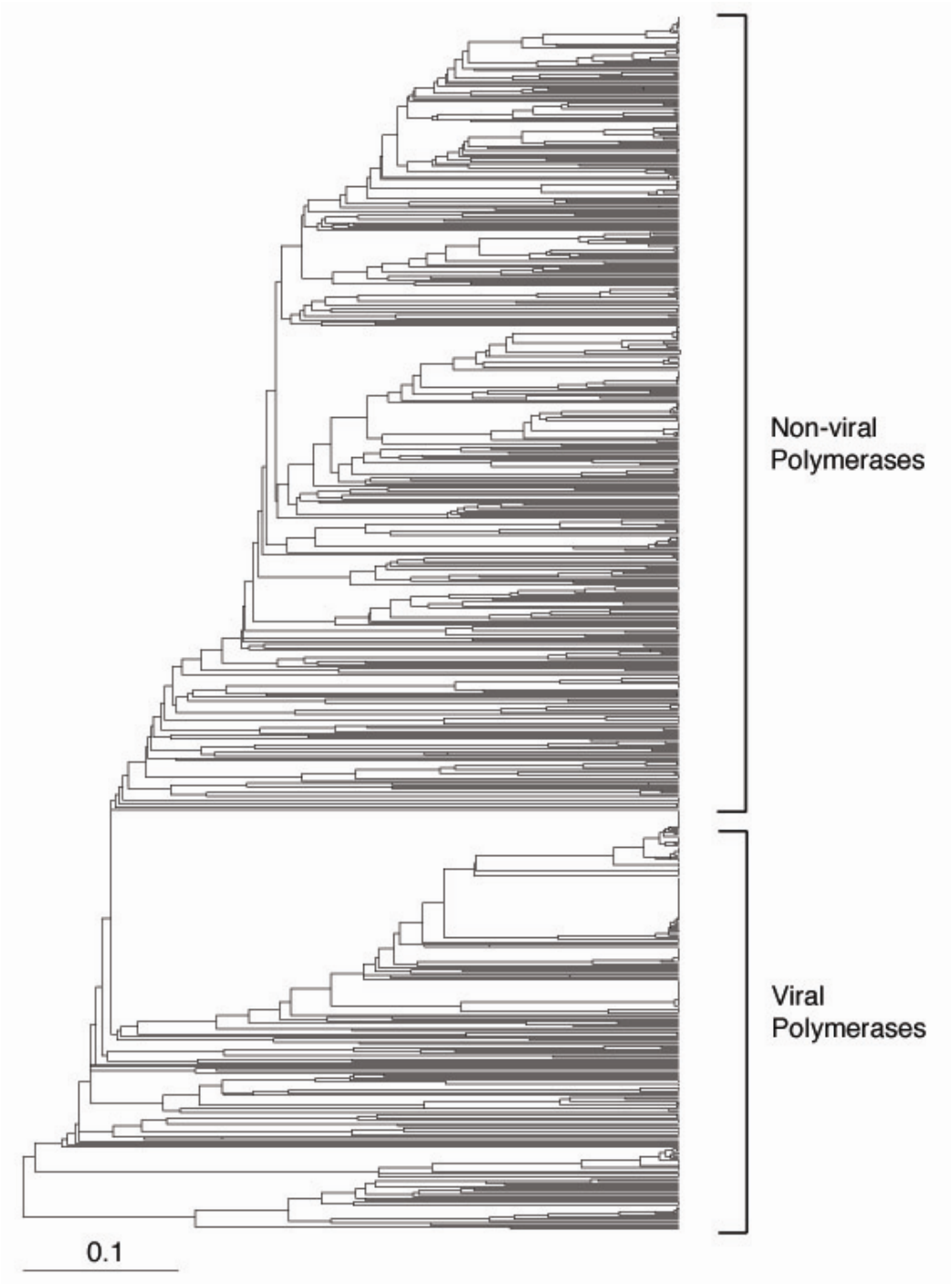
```
Primer (24 nt):       5′- GCGTAATACGACTCACTATGGACG            -3′
Temp A (36 nt):       3′- CGCATTATGCTGAGTGATACCTGCAATGTGCTTCTG-5′
Temp G (36 nt):       3′- CGCATTATGCTGAGTGATACCTGCGGTGTGCTTCTG-5′
Temp T (36 nt):       3′- CGCATTATGCTGAGTGATACCTGCTTTGTGCTTCTG-5′
Temp C (36 nt):       3′- CGCATTATGCTGAGTGATACCTGCCCTGTGCTTCTG-5′
```

SI Appendix figure 7: Gel shift showing the PEG-aldehyde capture 3'-ONH_2 reversible terminator extended on SNP templates. To assess the ability of a combination of reversibly and irreversibly terminating nucleoside triphosphates to perform together in an architecture designed to detect single-nucleotide polymorphisms, 5'-^{32}P-labeled primer (2.5 pmol), cold primer (20 pmol) and template (30 pmol) (sequences shown below) were annealed and incubated in 1X NEB ThermoPol Reaction Buffer at 72 °C for 5 min with ddATP (the irreversible terminator), dTTP-ONH_2, dCTP-ONH_2 and dGTP-ONH_2 (the reversible terminators) (1 nmol each, 10 µL reaction volume). Primer extension was initiated by adding REAP-58 (1 µL of a 0.25 µg/µL solution). Reactions were quenched with 10 mM EDTA (5 µL).

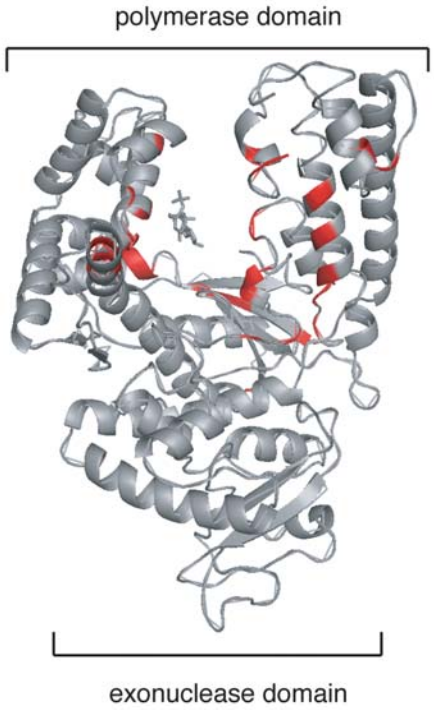
The samples were then treated with 300 mole equivalents of PEG aldehyde (HO-CH_2-[CH_2-O-CH_2]$_2$-CH_2-CHO) and incubated at 65 °C for 10 hrs. Samples were resolved on a 14% denaturing PAGE and analyzed with a Molecular Imager FX system.

```
Primer (24 nt):          5′- GCGTAATACGACTCACTATGGACG  -3′
SNP Temp A (36 nt):      3′- CGCATTATGCTGAGTGATACCTGCAATGTGCTTCTG-5′
SNP Temp G (36 nt):      3′- CGCATTATGCTGAGTGATACCTGCGATGTGCTTCTG-5′
Standard Temp T (36 nt): 3′- CGCATTATGCTGAGTGATACCTGCTATGTGCTTCTG-5′
SNP Temp C (36 nt):      3′- CGCATTATGCTGAGTGATACCTGCCATGTGCTTCTG-5′
```

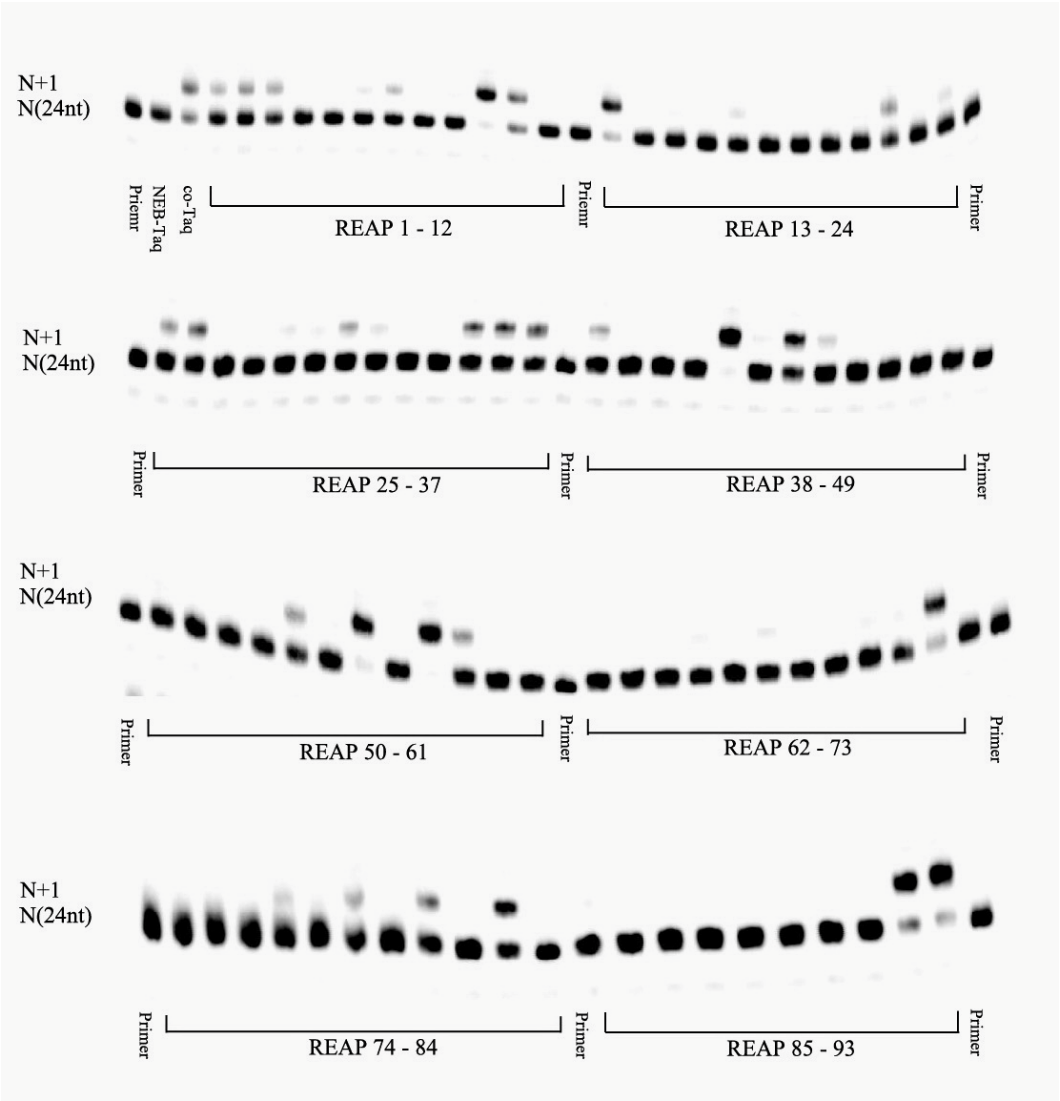
SI Appendix Figure 1:



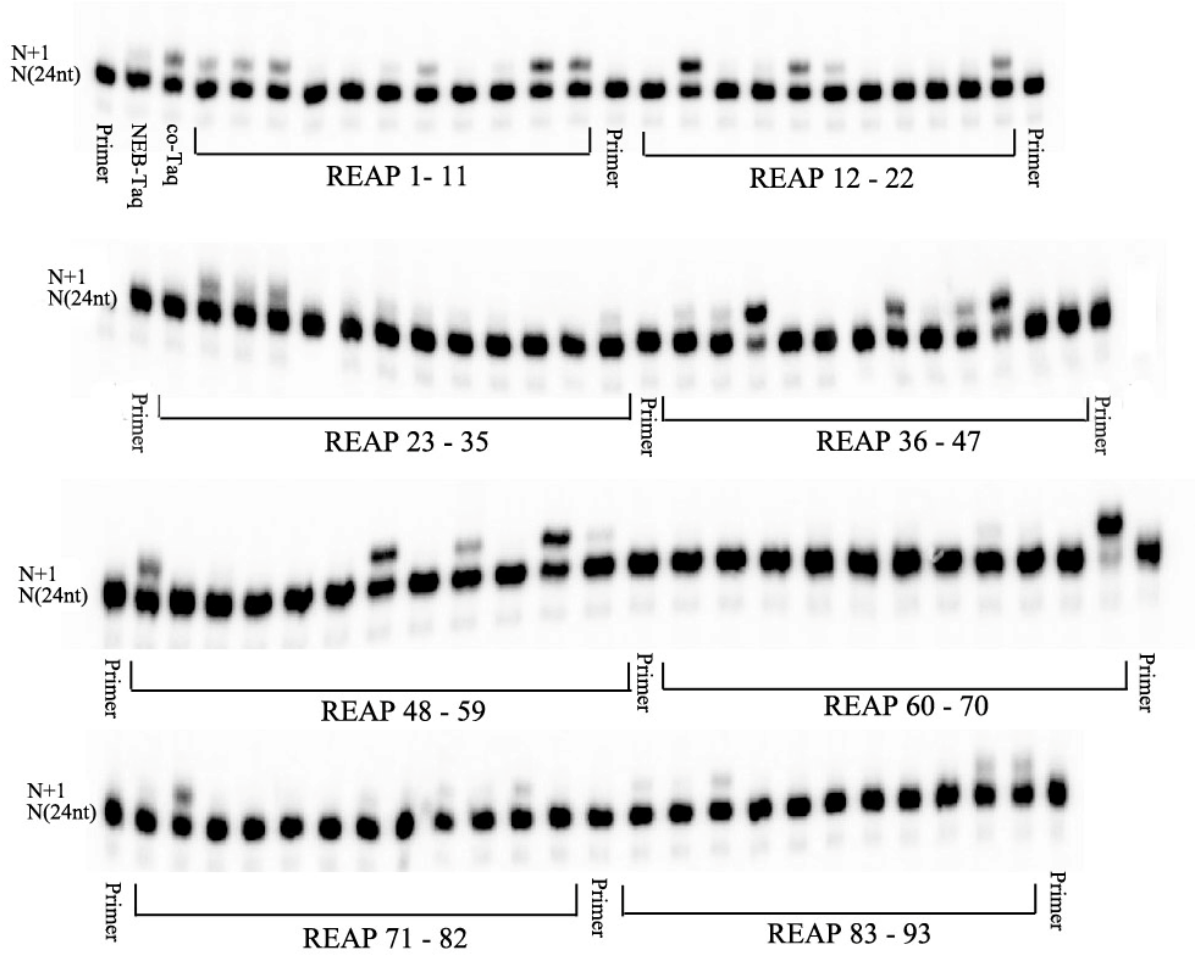
SI Appendix Figure 2:



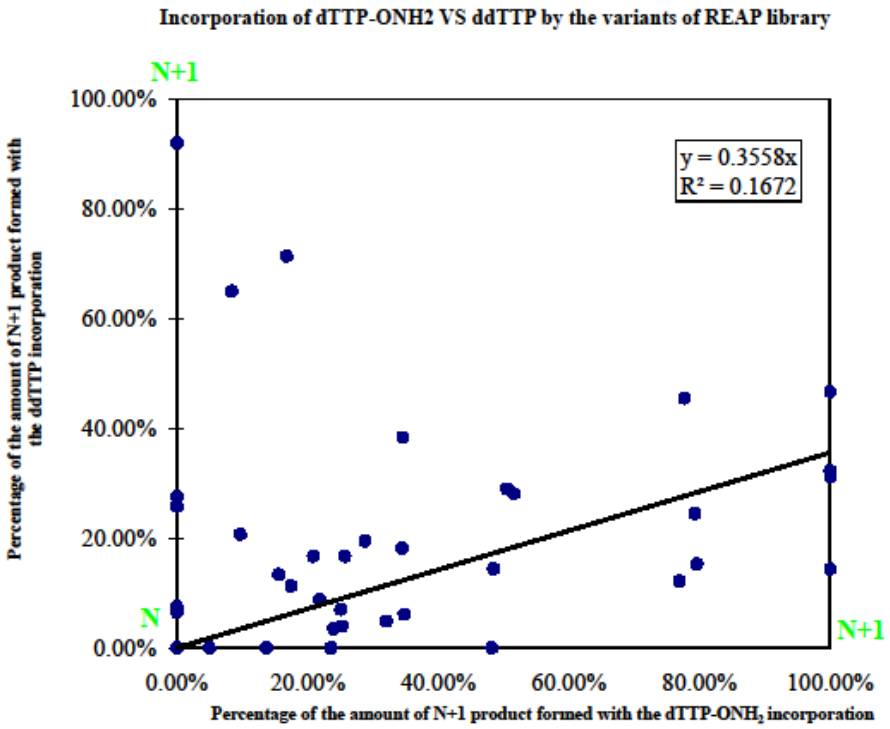
SI Appendix Figure 3a:

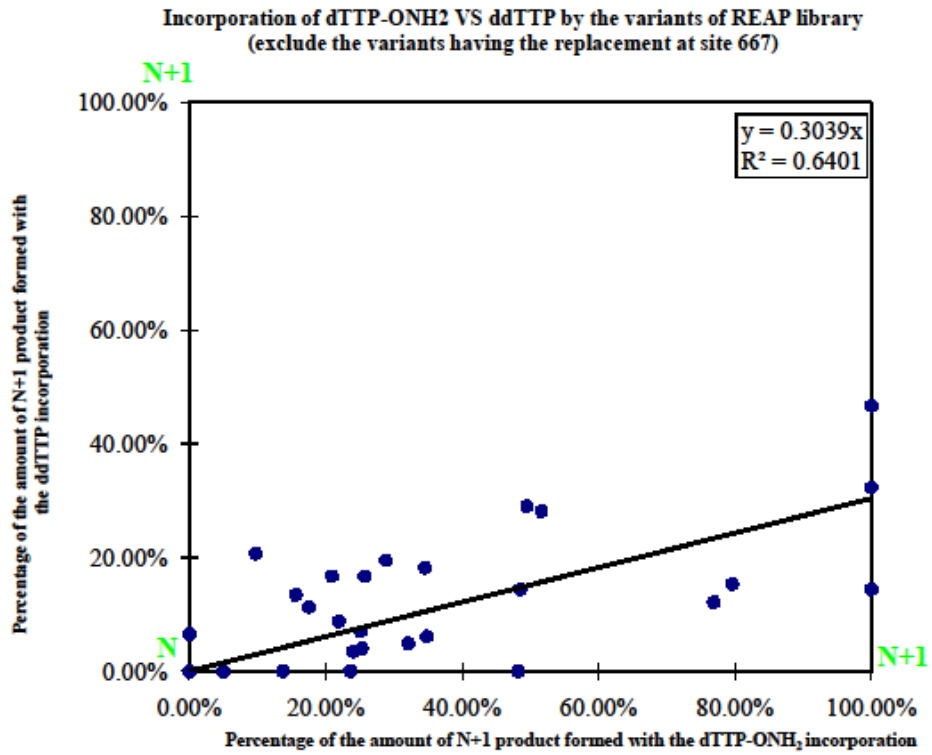


SI Appendix Figure 3b:

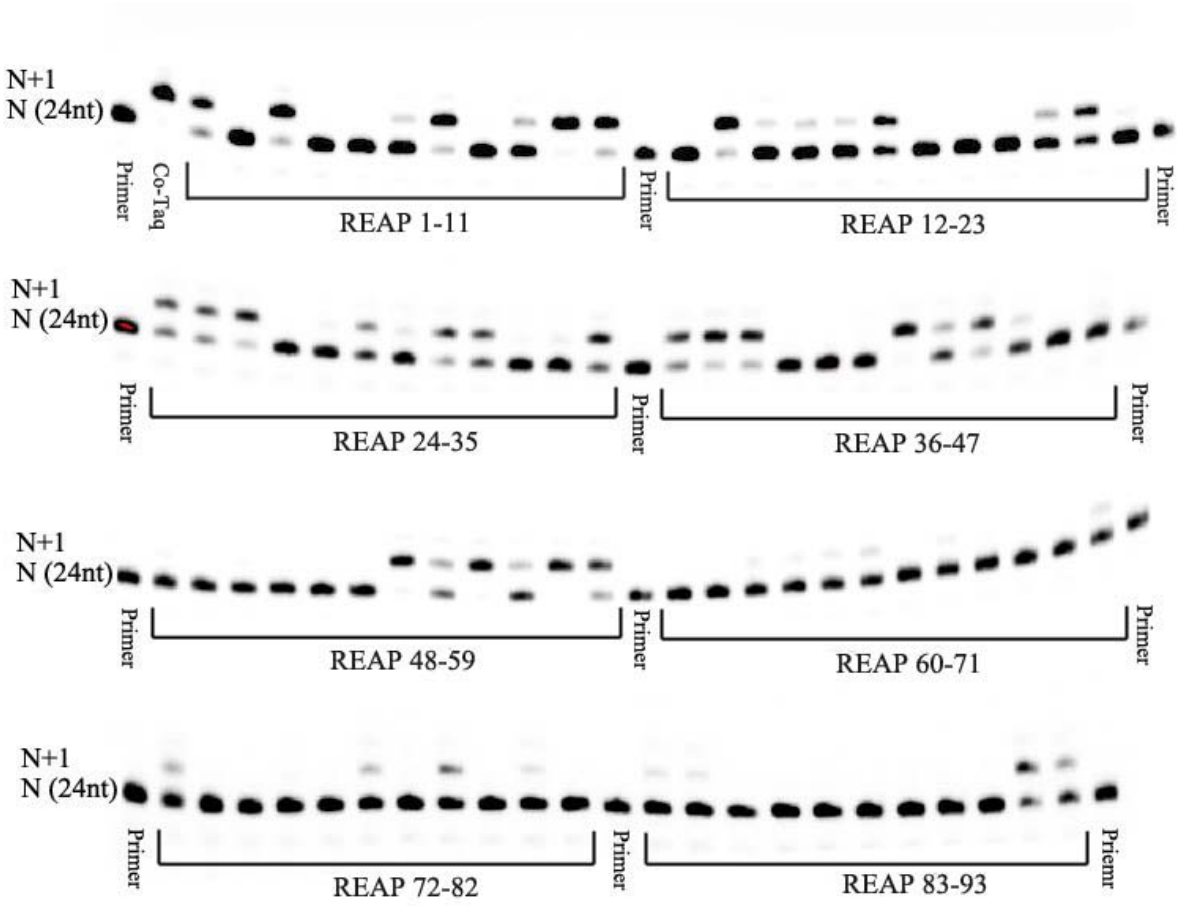


SI Appendix Figure 3c and 3d:





SI Appendix Figure 4a:



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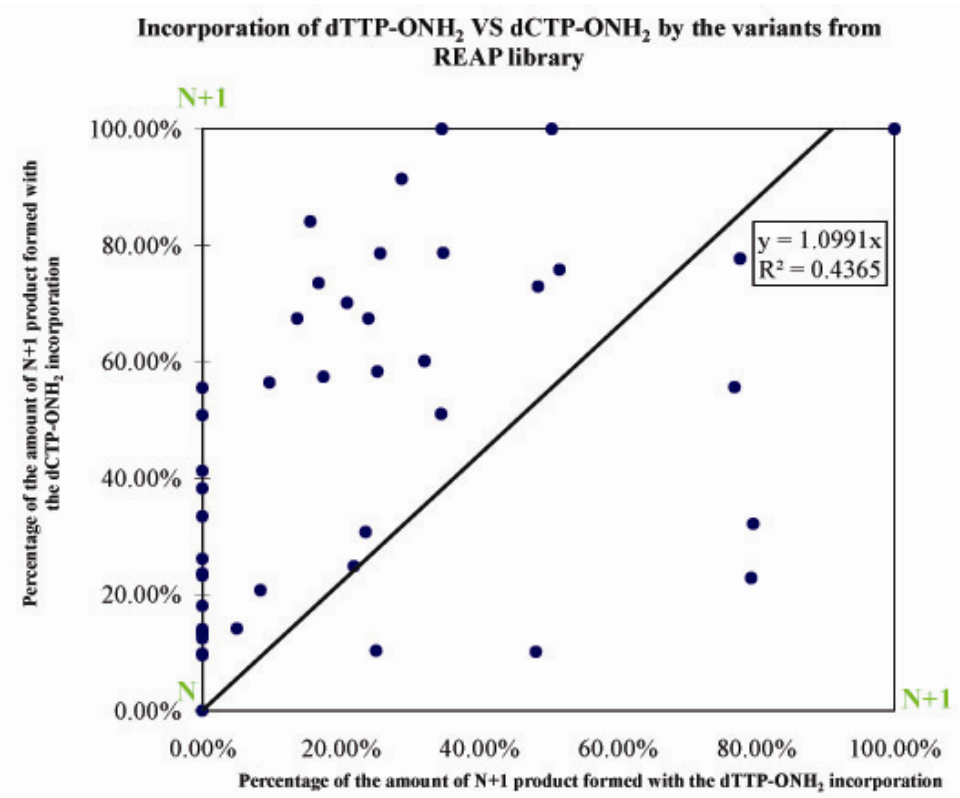
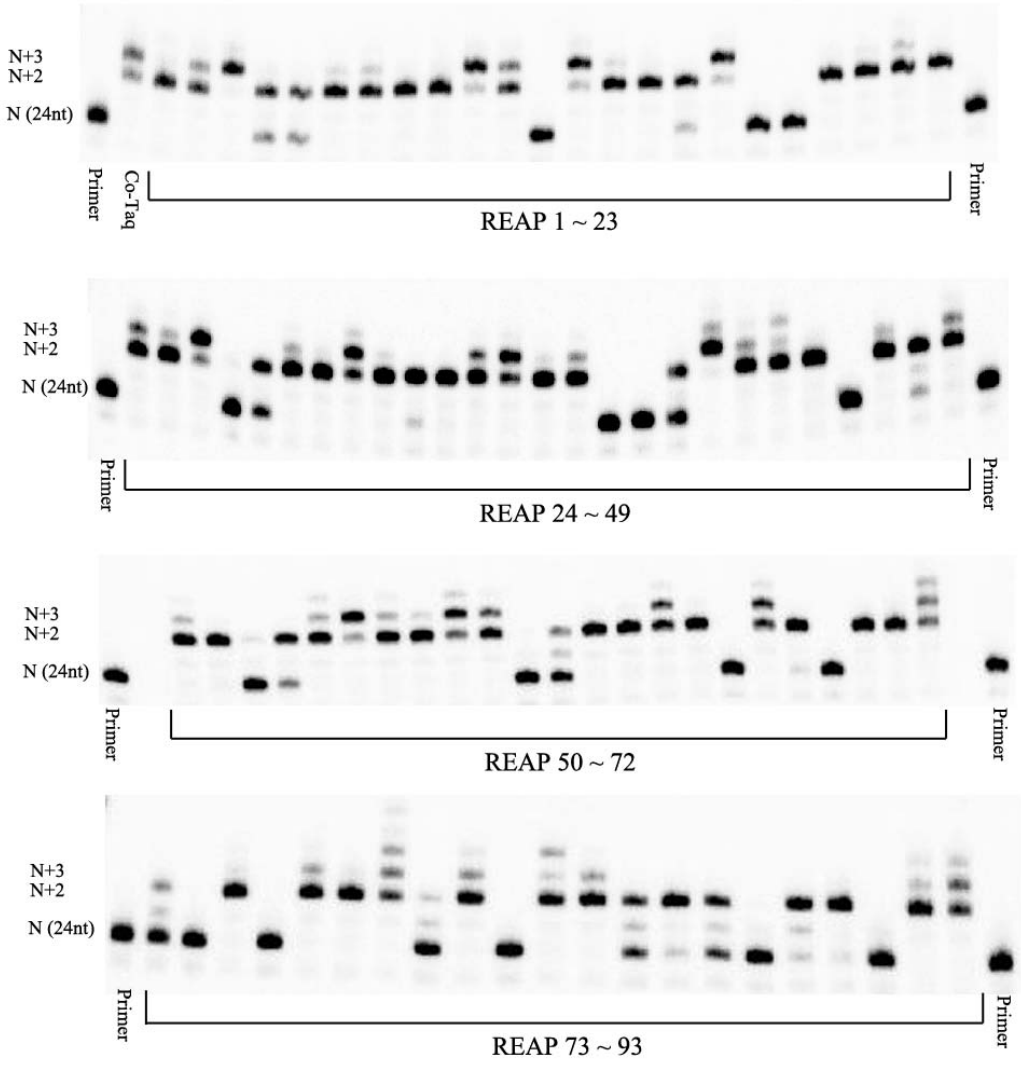
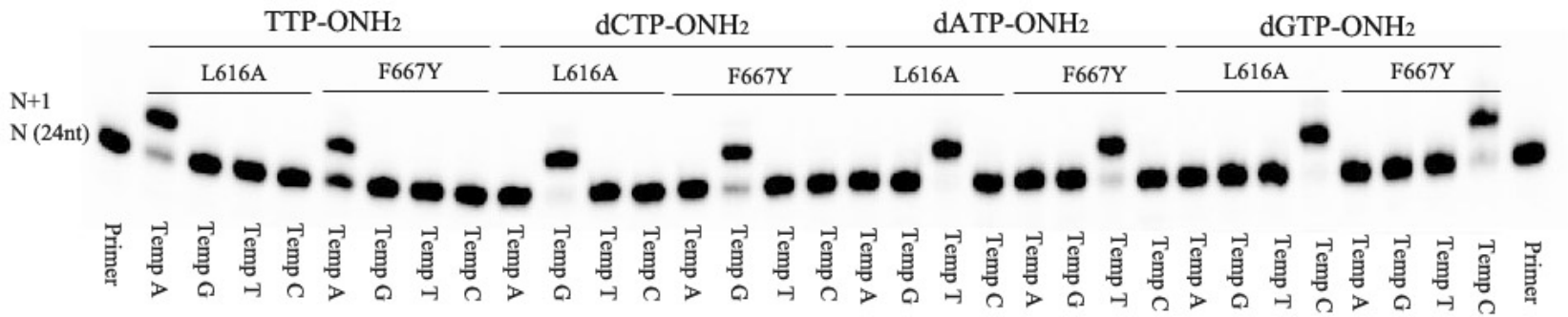


SI Appendix Figure 5:

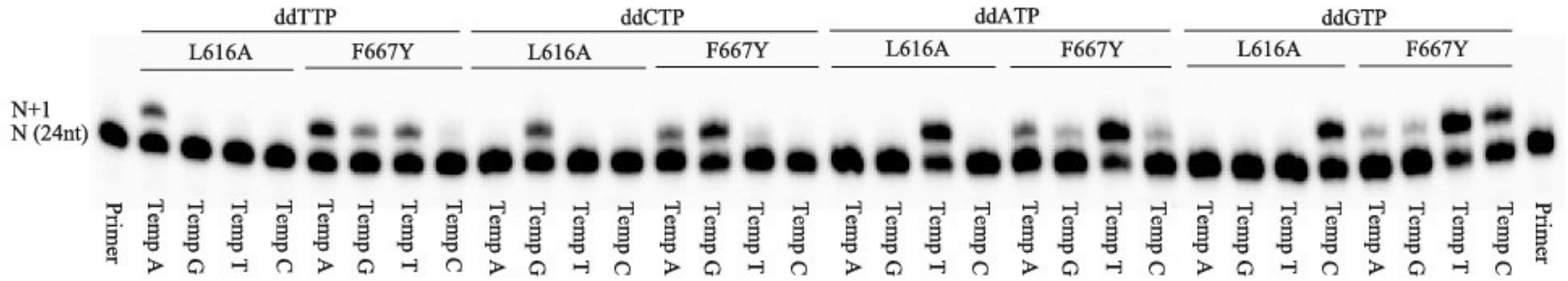


SI Appendix Figure 6:

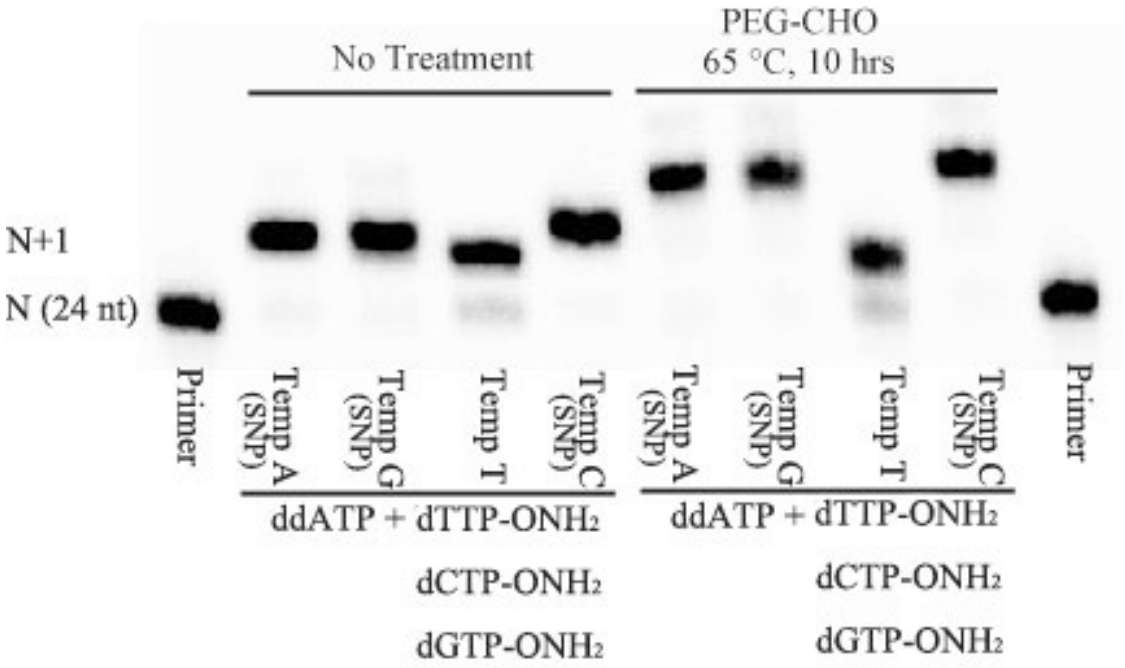
(a)



(b)



SI Appendix Figure 7:



SI Appendix Table 1: Reconstructing Evolutionary Adaptive Paths (REAP) Mutant library:

Culture Number	Mutations Present in REAP Library	Culture Number	Mutations Present in REAP Library
Co-wt-Taq	NONE - *wt* codon-optimized *taq* gene	REAP-47	S513I,V586K,R587V,L609P
REAP-1	Q489H,K540I,M673G	REAP-48	F598W,F667H,Y671F,E742P
REAP-2	D578F,L609C,A743S	REAP-49	L609P,I614E,E742R,R746A
REAP-3	T514V,R588V,I614E	REAP-50	T544A,I614Q,L616I,D625L
REAP-4	E520I,V586K,A600S	REAP-51	K540I,S576H,N583S,D625S
REAP-5	D578F,V586K,M673A	REAP-52	N483R,S576E,D610W,A743R
REAP-6	F598W,L609P,D625S	REAP-53	D578T,L616D,E742R,A777H
REAP-7	S513I,A608K,L609S	REAP-54	A597C,I614E,F667L,A743S
REAP-8	S576E,D625L,E745H	REAP-55	N583S,L616A,A743S,R746A
REAP-9	S576E,Y671F,A743S	REAP-56	S513I,T514V,L616I,E742R
REAP-10	A608G,L616A,E742P	REAP-57	S576E,R587V,A597C,D625S
REAP-11	N483R,F598V,E745H	REAP-58	E520G,K540I,L616A
REAP-12	E520I,D610W,D625S	REAP-59	Q489H,E520G,A608K
REAP-13	A597C,F667Y,A777H	REAP-60	S576H,F667Y,R746A
REAP-14	S576E,D578F,F598V	REAP-61	T544A,F667L,R746A
REAP-15	N483R,T514V,Y545E	REAP-62	Y545E,F598W,L609C
REAP-16	A597C,F667H,M673G	REAP-63	T544A,L609P,L616D
REAP-17	Q489H,D578T,N583S	REAP-64	E520I,F598W,A608E,I614E
REAP-18	S513I,A608E,E615I	REAP-65	N483R,R536I,A600S,M673G
REAP-19	A597C,E615I,M673A	REAP-66	E615I,D625L,F667L,E742P
REAP-20	S513I,Q582A,I614Q	REAP-67	I614Q,M673G,E742P,E745H
REAP-21	A597T,L609C,R660D	REAP-68	A600S,A608G,D625A,F667L
REAP-22	T544A,A608G,L609S	REAP-69	D610W,I614Q,R660D,E745V
REAP-23	K540I,Q582A,E745V	REAP-70	Q582A,R660D,F667Y,A743R
REAP-24	A600S,A743R,E745V	REAP-71	R536I,K540I,A608K,L616I
REAP-25	N583Q,A608E,L616I	REAP-72	T514V,E520G,L609C,F667Y
REAP-26	N583S,F598V,A608G	REAP-73	D578T,F667H,E745V,R746A
REAP-27	N583S,D625L,A777H	REAP-74	V586K,E615I,L616D,Y671F
REAP-28	R536I,R587V,F667L	REAP-75	R536I,I614Q,L616D
REAP-29	D578T,N583Q,R587V	REAP-76	W604G,A608K,D610W
REAP-30	T514V,R536I,D625A	REAP-77	A597T,F598W,W604G
REAP-31	A600S,I614E,Y671F	REAP-78	E520I,Y545E,N583Q,A777H
REAP-32	L609S,R660D,E742R	REAP-79	Q582A,I614G,D625A
REAP-33	S576H,D578T,L616I	REAP-80	S576H,F667H,E742R
REAP-34	Y545E,V586K,A608E	REAP-81	E520G,W604G,E742P
REAP-35	T544A,D578F,L616A,D625A	REAP-82	N583Q,L616D,D625L,M673G
REAP-36	T514V,A597C,L609S,A743R	REAP-83	D578F,L609S,L616I,Y671F
REAP-37	Q489H,R536I,L609C,L616A	REAP-84	A597T,F598V,L609P,M673A
REAP-38	Q489H,F598V,D625A,F667Y	REAP-85	E520G,Q582A,A608G,F667H
REAP-39	E520I,S576H,A608G,E615I	REAP-86	I614G,R660D,M673A,A777H
REAP-40	Y545E,R587V,A608K,E615I	REAP-87	Q582A,W604G,D625L,E742R
REAP-41	D578T,A608E,L609C,D625A	REAP-88	S576E,L616D,M673A,E745V
REAP-42	A597T,L616A,F667Y,E745H	REAP-89	Y545E,D625S,Y671F,M673A
REAP-43	D578F,N583Q,W604G,D625S	REAP-90	S576H,V586K,F598V,I614Q
REAP-44	K540I,L609P,A743S,E745H	REAP-91	A608E,R660D,F667L,M673G
REAP-45	A600S,W604G,L609S,F667H	REAP-92	T544A,F598W,A608K,I614G
REAP-46	S513I,E520G,D610W,I614E	REAP-93	N483R,Q489H,I614G,E742P

*All are derivatives of the co-taq gene and inserted into the pASK-IBA43plus vector. Mutations were designed by using REAP approach and synthesized by DNA 2.0, Inc.