SUPPORTING INFORMATION

Fluorescent xDNA Nucleotides as Efficient Substrates for a Template-independent Polymerase

Sarah K. Jarchow-Choy, Andrew T. Krueger, Haibo Liu, Jianmin Gao, Eric T. Kool
Department of Chemistry, Stanford University, Stanford, CA 94305
*to whom correspondence should be addressed: kool@stanford.edu

CONTENTS:

Synthesis of xDNA nucleoside triphosphates .............................................. p.S2
TdT incorporation of natural and modified dNTPs........................................... p.S6
Table S1. Steady-state kinetic parameters....................................................... p.S8
Fluorescence studies of dxATP and dxCTP incorporation reactions.............. p.S8
Synthesis of DMT-decanoyl-LCAA-CPG solid support............................... p.S9
Fluorescence of TdT reactions on CPG solid support................................. p.S10
31P NMR spectra of xDNA nucleoside triphosphates................................. p.S11
Scheme 1: Synthesis of xDNA triphosphates

((2R,3R,5R)-5-(8-amino-3H-imidazo[4,5-g]quinazolin-3-yl)-3-hydroxytetrahydrofuran-2-yl)methyl triphosphate, Compound 2

30 mg (0.10 mmol) of 1 and 54 mg of proton sponge were dried overnight under vacuum and backfilled with argon. 1.0 mL of anhydrous trimethylphosphate was added and the heterogeneous solution was sonicated and heated until soluble. The solution was cooled to 0°C and 28 µL of a 10% solution of 99.999% POCl₃ (3 eq.) in anhydrous trimethylphosphate was added. The mixture was allowed to stir at 0°C for 4 hours.

After 4 hours, a solution containing 1.5 mL of anhydrous DMF solution containing 0.475 g of tributylammonium pyrophosphate and 0.3 mL tributyl amine was added. After stirring 1 minute at 0°C, 20 mL of 1 M triethylammonium bicarbonate (TEAB) buffer (pH 7.5) was added.
and the reaction was allowed to warm to room temperature over 30 minutes, and then dried on a speed-vac.

The residue was taken up in 0.1 M TEAB buffer and first purified on FPLC via anion exchange on a DEAE-cellulose column with an initial 1 hour at 0.1 M TEAB followed by a 0.1 M to 1 M gradient of TEAB over 3 hours at a flow rate of 1.3 mL/minute. The appropriate fractions (as determined by \(^{31}\)P NMR) were collected, injected into an HPLC equipped with a C18 reverse phase column and purified with a gradient of 10-25% acetonitrile in 0.1 M TEAB over 15 minutes. The appropriate fractions were collected, dried on a speed-vac, and the precipitate was concentrated and converted to the sodium salt (precipitate dissolved in 100 uL methanol followed by adding 400 µL of 0.75 M NaClO\(_4\) in acetone solution, centrifuged for 15 min at 2500 rpm, supernatant decanted, rinsed once more with 500 µL acetone, decanted, then speed vac’d for 1 h).

The yield of the triphosphate product was determined by measuring the absorbance of the solution at 260 nm using the extinction coefficient (for dX \(\varepsilon_{260} = 19,800 \text{ M}^{-1} \text{ cm}^{-1}\)), giving 6.4 mg (12%).

Phosphorus NMR was taken in 15 % D\(_2\)O with 50 mM Tris (pH 7.5) and 2 mM EDTA. \(^{31}\)P-NMR (D\(_2\)O, ppm): -5.3 (1H, d), -10.0 (1H, d), -21.0 (1H, t). ESI-MS cald. for C\(_{14}\)H\(_{14}\)N\(_5\)O\(_{12}\)P\(_3\)\(^4+\) [M\(^+\)H\(^+\)] 536.99; found 536.79.

\(((2R,3R,5R)-5-(4-amino-6-methyl-2-oxo-1,2-dihydroquinazolin-8-yl)-3-hydroxytetrahydrofuran-2-yl)methyl triphosphate, Compound 4\)

52 mg (0.17 mmol) of 3 and 94 mg of proton sponge were dried overnight under vacuum and backfilled with argon. 2.4 mL of anhydrous trimethylphosphate was added and the heterogeneous solution was sonicated and heated until soluble. The solution was cooled to 0 °C, and 0.23 mL of a 20% solution of 99.999% POCl\(_3\) (0.9 eq.) in anhydrous trimethylphosphate was added. The reaction mixture was stirred at 0 °C, and after 60 minutes, an additional 0.2 mL of 20% solution of POCl\(_3\) (1.3 eq.) in anhydrous trimethylphosphate was added. The mixture was allowed to stir at 0 °C for 5 hours.

After 5 hours, 3.5 mL of a DMF solution containing 0.196 g of tributylammonium pyrophosphate and 0.42 mL of tributylamine was added. After stirring for 5 minutes at 0 °C, 3 mL of 1 M triethylammonium bicarbonate (TEAB) buffer (pH 7.5) was added and the reaction was allowed to warm to room temperature over 30 minutes, and then dried on a speed-vac.
The residue was taken up in 0.1 M TEAB and first purified on FPLC via anion exchange on a DEAE cellulose column with an initial 1 hour at 0.01 M TEAB followed by a 0.01 M to 1 M gradient of TEAB over 1 hour, at a flow rate of 1.5 mL/minute. The appropriate fractions were collected, injected into an HPLC equipped with a C18 reverse phase column and purified with a gradient of 10-25% acetonitrile in 0.1 M TEAB over 15 minutes. The appropriate fractions were collected, dried on a speed-vac, and the precipitate was concentrated and converted to the sodium salt.

The yield of the triphosphate product was determined by measuring the absorbance of the solution at 260 nm using the extinction coefficient (for dxC \( \varepsilon_{260} = 5,800 \, \text{M}^{-1}\text{cm}^{-1} \)), giving ~1.7 mg (19%).

Phosphorus NMR was taken in 15% D$_2$O with 50 mM Tris (pH 7.5) and 2 mM EDTA.

$^{31}$P-NMR (D$_2$O, ppm): -5.8 (1H, d), -10.0 (1H, d), -21.4 (1H, t). ESI-MS calcd. for C$_{14}$H$_{16}$N$_3$O$_{13}$P$_3$$^{4-}$ [M$^+$H$^+$K$^+$] 565.95; found 566.04.

((2R,3R,5R)-5-(6-amino-8-oxo-7,8-dihydro-3H-imidazo[4,5-g]quinazolin-3-yl)-3-hydroxytetrahydrofuran-2-yl)methyl triphosphate, Compound 6

8.0 mg (0.025 mmol) of 5 and 13.1 mg of proton sponge were dried overnight under vacuum and backfilled with argon. 376 µL of anhydrous trimethylphosphate was added and the heterogeneous solution was sonicated and heated until soluble. The solution was cooled to 0 °C, and 35 µL of a 10% solution of 99.999% POCl$_3$ (1.5 eq.) in anhydrous trimethylphosphate was added. The reaction mixture was stirred at 0 °C, and after 30 minutes, an additional 30 µL of 10% solution of POCl$_3$ (1.3 eq.) in anhydrous trimethylphosphate was added. The mixture was allowed to stir at 0 °C for 4 hours, after which it became a homogeneous yellow.

After 4 hours, 540 µL of a DMF solution containing 27 mg of tributylammonium pyrophosphate and 60 µL of tributylamine was added. After stirring for 5-7 minutes at 0 °C, 2 mL of 1 M triethylammonium bicarbonate (TEAB) buffer (pH 7.5) was added and the reaction was allowed to warm to room temperature over 30 minutes, and then dried on a speed-vac.

The residue was taken up in water and first purified on FPLC via anion exchange on a DEAE cellulose column with an initial 1 h at 0.01 M TEAB followed by a 0.01 M to 1 M gradient of TEAB over 3 hours, at a flow rate of 1.5 mL/min. The appropriate fractions were injected into HPLC and purified on a C18 reverse phase column with a gradient of 10-25% acetonitrile in 0.1 M TEAB over 15 minutes. The appropriate fractions were collected, injected into an HPLC
equipped with a C18 reverse phase column and purified with a gradient of 10-25% acetonitrile in 0.1 M TEAB over 15 minutes. The appropriate fractions were collected, dried on a speed-vac, and the precipitate was concentrated and converted to the sodium salt.

The yield of the triphosphate product was determined by measuring the absorbance of the solution at 260 nm using the extinction coefficient (for dxT ε_{260} = 5,100 M^{-1}cm^{-1}), giving ~1.0 mg (8%).

Phosphorus NMR was taken in 15% D₂O with 50 mM Tris (pH 7.5) and 2 mM EDTA. \(^{31}\)P-NMR (D₂O, ppm): -6.2 (1H, d), -10.0 (1H, d), -22.0 (1H, t). ESI-MS cald. for C₁₄H₁₄N₅O₁₃P₃⁴⁻·[M⁺H⁺⁺K⁺] 593.08; found 593.31.

\(\text{(2R,3R,5R)-3-hydroxy-5-(6-methyl-2,4-dioxo-1,2,3,4-tetrahydroquinazolin-8-yl)tetrahydrofuran-2-yl)methyl triphosphate, Compound 8}\)

22 mg (0.07 mmol) of 7 and 43 mg of proton sponge were dried overnight under vacuum and backfilled with argon. 1 mL of anhydrous trimethylphosphate was added and the heterogeneous solution was sonicated and heated until soluble. The solution was cooled to 0 °C, and 32 µL of a 20% solution of 99.999% POCl₃ (0.9 eq.) in anhydrous trimethylphosphate was added. The reaction mixture was stirred at 0 °C, and after 60 minutes, an additional 32 µL of 20% solution of POCl₃ (1.3 eq.) in anhydrous trimethylphosphate was added. The mixture was allowed to stir at 0 °C for 5 hours, after which it became a homogeneous yellow.

After 5 hours, 1.5 mL of a DMF solution containing 83 mg of tributylammonium pyrophosphate and 180 µL of tributylamine was added. After stirring for 5 minutes at 0 °C, 2 mL of 1 M triethylammonium bicarbonate (TEAB) buffer (pH 7.5) was added and the reaction was allowed to warm to room temperature over 30 min, and then dried on a speed-vac.

The residue was taken up in water and first purified on FPLC via anion exchange on a DEAE cellulose column with an initial 1 hour at 0.01 M TEAB followed by a 0.01 M to 1 M gradient of TEAB over 1 hour, at a flow rate of 1.5 mL/minute. The appropriate fractions were collected, injected into an HPLC equipped with a C18 reverse phase column and purified with a gradient of 10-25% acetonitrile in 0.1 M TEAB over 15 minutes. The appropriate fractions were collected, dried on a speed-vac, and the precipitate was concentrated and converted to the sodium salt.

The yield of the triphosphate product was determined by measuring the absorbance at 260 nm of a solution (for dxT ε_{260} = 1,200 M^{-1}cm^{-1}), giving ~2.1 mg (6%).

Phosphorus NMR was taken in 15% D₂O with 50 mM Tris (pH 7.5) and 2 mM EDTA. \(^{31}\)P-
NMR (D$_2$O, ppm): -5.8 (1H, d), -10.2 (1H, d), -21.4 (1H, t). ESI-MS calcd. for C$_{14}$H$_{15}$N$_2$O$_4$P$_3$ - [M$^+$H$^+$Na$^+$] 550.97; found 550.41.

**TdT incorporation of dNTPs**

Primers (sequence 5' to 3' ATA CCA AAG T) were 5'-end-labeled with [$\gamma$-32P] ATP (Perkin Elmer) using T4 polynucleotide kinase (New England Biolabs). The radiolabeled primers were purified and desalted using a Microspin™ G-25 microspin column (GE Healthcare).

For TdT reactions with multiple incorporations, a primer and enzyme solution were made. The primer solution consisted of 20 $\mu$M unlabeled primer and ~50 nM labeled primer in a buffer of potassium acetate (200 mM), Tris-acetate (800 mM, pH 7.9), magnesium acetate (40 mM), CoCl$_2$ (1 mM), and dithiothreitol (4 mM). The enzyme solution consisted of TdT (4 U/$\mu$L, cloned from calf thymus; New England Biolabs) in a buffer of potassium acetate (200 mM), Tris-acetate (800 mM, pH 7.9), magnesium acetate (40 mM), CoCl$_2$ (1 mM).

The reactions were initiated by mixing 2.5 $\mu$L of primer solution with 2.5 $\mu$L of enzyme solution. The mixtures were incubated for 3 minutes at 37 °C, then 5 $\mu$L of a 20 $\mu$M dNTP solution was added. Reaction volumes (10 $\mu$L) were incubated at 37 °C for 1 hour and quenched by adding 10 $\mu$L of loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol and bromophenol blue) to the mixture and heating to 95°C for 5 minutes to denature the enzyme. Final unlabeled primer concentrations were 5 $\mu$M. The final concentration of TdT was 1 U/$\mu$L.

It should be noted that for reactions with multiple incorporations, a separate experiment using a different enzyme solution consisting of TdT enzyme (5 U/$\mu$L, recombinant; Affymetrix, Inc.) in a buffer of sodium cacodylate (25 mM, pH 7.2), cobalt chloride (1 mM) and dithiothreitol (0.1 mM) showed more extensive incorporation (Figure 1). The primer solution consisted of 20 $\mu$M unlabeled primer and ~50 nM labeled primer of which 10 $\mu$L was added to 10 $\mu$L of the enzyme solution. The definition for one unit is the same as for the NEB TdT enzyme.

For steady-state experiments, the same primer and enzyme solution were made as above, although the amount of material was increased. The reactions were initiated by mixing 7.5 $\mu$L of primer solution with 7.5 $\mu$L of enzyme solution. The mixtures were incubated for 3 minutes at 37 °C, then 15 $\mu$L of a dNTP solution was added. The dNTP solutions varied in concentration.

Reaction volumes (30 $\mu$L) were incubated at 37 °C for 1 min and quenched by adding aliquots of the mixture to 10 $\mu$L of loading buffer and heating to 95°C for 5 minutes. Final concentrations
of dNTPs ranged from 0.1 - 30 µM. Final unlabeled primer concentrations were 5 µM. The final concentration of TdT was 1 U/µL. These concentrations of reagents and reaction times were adjusted to give less than 20% nucleotide incorporation for analysis by the initial rates method.

Quenched reaction samples (5 µL) were loaded onto a 20% denaturing polyacrylamide gel. Gel electrophoresis was run for approximately 3 hours at 40 watts. The gel was exposed to phosphor screen (10-12 h) at -80 °C, and radioactive DNA was quantified by phosphorimager analysis (Molecular Dynamics Storm 860) using the Image Quant software program.

*Figure 1:* Autoradiogram of a polyacrylamide gel showing TdT insertion of xDNA and DNA triphosphates at the end of a 10mer DNA primer after 1 hour. Lane 1: Unreacted primer strand (sequence 5’-ATACCAAAAGT-3’); M, DNA size marker; Lane 2: Incorporation of dxATP, 10 µM; Lane 3: dxCTP, 10 µM; Lane 4: dxGTP, 10 µM; Lane 5: dxTTP, 10 µM; Lane 6: dATP, 10 µM; Lane 7: dCTP, 10 µM; Lane 8: dGTP, 10 µM; Lane 9: dTTP, 10 µM. (Lengths at left denote approximate numbers of nucleotides.
added to primer; this is an average because different nucleotide homopolymers yield slightly different gel mobilities.)

**Table S1**: Kinetic parameters for single nucleotide insertion of a dNTP or dxNTP on a DNA primer strand by terminal deoxynucleotidyl transferase.

|   | $K_m$ (M)       | $V_{max}$ (% min$^{-1}$) | Frequency $(V_{max}/K_m)$ |
|---|-----------------|---------------------------|--------------------------|
| dATP | 4.8(2.0) x 10$^{-7}$ | 0.06(0.01) | 1.4(0.6) x 10$^5$ |
| dCTP | 2.0(1.5) x 10$^{-4}$ | 0.10(0.04) | 3.1(1.4) x 10$^5$ |
| dGTP | 1.6(0.7) x 10$^{-6}$ | 0.17(0.05) | 1.1(0.6) x 10$^5$ |
| dTTP | 2.6(0.9) x 10$^{-6}$ | 0.19(0.01) | 8.3(0.3) x 10$^4$ |
| dxATP | 7.4(3.1) x 10$^{-8}$ | 0.03(0.01) | 5.1 (2.9) x 10$^5$ |
| dxCTP | 6.3(6.0) x 10$^{-6}$ | 0.08(0.04) | 4.4(1.6) x 10$^5$ |
| dxGTP | 6.1(0.7) x 10$^{-7}$ | 0.14(0.02) | 2.3(0.4) x 10$^5$ |
| dxTTP | 4.1(1.5) x 10$^{-6}$ | 0.05(0.01) | 1.3(0.5) x 10$^4$ |

Conditions: Reactions performed with primer strand (10mer) at 5 µM concentration each, varying dNTP concentrations, potassium acetate (200 mM), Tris-acetate (800 mM, pH 7.9), magnesium acetate (40 mM), CoCl$_2$ (1 mM), and dithiothreitol (4 mM). Enzyme concentration (1 U/µL) and reaction time (1 min) were adjusted to give < 20% incorporation. All values are averaged and values in parentheses represent standard deviations over three data sets.

**Fluorescence studies of dxATP and dxCTP incorporation reactions**

TdT (final concentration 1 U/µL) was added to a 10 µL solution containing 40 µM primer and a buffer of potassium acetate (50 mM), Tris-acetate (200 mM, pH 7.9), magnesium acetate (10 mM), CoCl$_2$ (0.25 mM), and dithiothreitol (1 mM). The mixture was incubated for 3 min at 37 °C. Subsequently, 10 µL of a 20 µM solution of dxATP was added (final [dxATP] was 10 µM) and the mixture was allowed to incubate for 1 h. The reaction was quenched with 10 mM EDTA and heated at 95°C for 5 min to denature the enzyme. The same conditions were used for the dxC fluorescence studies with the exception that a larger amount of dxCTP was added to the enzyme/primer solution (final [dxCTP] was 13 µM).

Purification of products containing either multiple dxC molecules or multiple dxA molecules was done using Sephadex G-10 (NAP$^\text{TM}$-10, GE Healthcare) following kit instructions.

For time course fluorescence studies, TdT reactions were done on a larger scale (100 µL total...
volume) for both dxATP and dxCTP reactions. TdT (200 Units, final concentration 1 U/µL) was added to a reaction containing primer (50 µL total volume) and a buffer of potassium acetate (50 mM), Tris-acetate (200 mM, pH 7.9), magnesium acetate (10 mM), CoCl₂ (0.25 mM), and dithiothreitol (1 mM). The final primer concentration was 0.8 µM for dxATP reactions and 5.8 µM for dxCTP reactions. The mixture was incubated for 3 min at 37 °C. Subsequently, 50 µL of a 5.2 µM dxATP solution and 50 µL 14.8 µM of a dxCTP solution were added (into separate reactions). The final dxATP concentration was 2.6 µM and the final dxCTP concentration was 7.4 µM. Fluorescence spectra were recorded every 1 min for each reaction up to 7 min, then at 15 min, 30 min, and 60 min.

Fluorescence spectra were recorded on a Spex-Fluorolog-3 series fluorimeter. The fluorescence measurements were taken in the right-angle mode on 10 µL solutions in 490 µL PIPES buffer (100 mM NaCl, 10 mM MgCl₂, and 10 mM Na•PIPES, pH 7.0). The excitation and emissions slits were set to 5 nm.

**Synthesis of DMT-decanoyl-LCAA-CPG beads**

To a flask containing methyl-10-hydroxydecanoate (9, 3.0 mL, 14.32 mmol), N-diisopropylethylamine (DIPEA, 4.45 mL, 28.53 mmol), 4-dimethylaminopyridine (DMAP, 0.18 g, 1.43 mmol) and 110 mL of anhydrous dichloromethane was added 7.40 g (21.40 mmol) of dimethoxytrityl chloride. The solution was allowed to stir for ~2 h at room temperature, then washed with NaHCO₃, brine and water. The yellowish product was purified from the starting material using silica column chromatography (3:1, dichloromethane: hexanes) in a 92% yield.

The resulting DMT-protected methyl-10-decanoate (10) was then reduced to methyl-10-decanol. The purified product was dissolved in 73 mL of a 3:1:1 THF/MeOH/H₂O solvent mixture. LiOH•H₂O (4.82 g, 114.75 mmol) was slowly added and the solution was allowed to stir overnight. The orange-colored product was then concentrated and 130 mL of a 0.88 M solution of citric acid was added for neutralization. Product 11 was purified from the starting material using silica column chromatography (19:1, dichloromethane: methanol), giving a 59% yield after removal of solvent under reduced pressure.

To 0.12 g (0.25 mmol) of 11 in 3.50 mL of dimethylformamide was added 0.24 mL (1.40 mmol) of diisopropylethylamine (DIPEA) and 0.26 g (0.70 mmol) of HATU (peptide coupling reagent). The solution was allowed to stir 30 min at room temperature. Subsequently, 1.17 g (0.17 mmol) of long-chain aminoacyl linker controlled pore glass beads (LCAA-500-CPG, 3-
Prime) were added and the reaction was stirred slowly for an additional 4 h. The beads were then filtered, washed with dichloromethane, ethyl acetate, and water, and dried under vacuum overnight in a 75% yield. The loading concentration of DMT (67.6 µmol/g) was determined by measuring absorbance at 498 nm in solution following detritylation of a small weighed sample with dilute trifluoroacetic acid.

**Fluorescence of TdT reactions on CPG solid support**

The TdT enzyme (20 Units, final concentration 1 U/µL) was added to a 10 µL solution containing ~10 µM of CPG-LCAA(dT)$_{25}$ beads (concentration of beads was determined by UV-absorbance of detritylated bead sample) in a buffer of potassium acetate (50 mM), Tris-acetate (200 mM, pH 7.9), magnesium acetate (10 mM), CoCl$_2$ (0.25 mM), and dithiothreitol (1 mM). The solution was allowed to incubate 3 min at 37 °C. Subsequently, 10 µL of dxCTP or dxATP was added and the solutions were allowed to incubate for 1 h with frequent vortexing. The final concentration of dxCTP in solution was 140 µM and the final concentration of dxATP was 68 µM.

The beads were filtered on a CPG column (from Glen Research) and washed three times with deionized water, then dried under vacuum and imaged under an epifluorescence microscope (Nikon Eclipse E800 equipped with 4x and 20x objective). Fluorescence images were taken with a Spot RT digital camera and Spot Advanced Imaging software.
