Supporting Information

Epigenetic Pyrimidine Nucleotides in Competition with Natural dNTPs as Substrates for Diverse DNA Polymerases

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References
1. Synthetical part

1.1. General remarks

Compounds dC\textsuperscript{f}TP\textsuperscript{[1],[2]} and 5-(formyl)-2'-deoxyuridine\textsuperscript{[1],[3]} were prepared according to the literature procedures. 5-(methyl)-2'-deoxycytidine-5'-O-triphosphate (dC\textsuperscript{m}TP) was purchased from New England Biolabs. The NMR spectra were measured on a 400 MHz Bruker AVANCE III spectrometer (\textsuperscript{1}H at 400 MHz, \textsuperscript{13}C at 101 MHz, \textsuperscript{31}P at 162 MHz) equipped with a liquid-nitrogen cryoprobe. Dried solvents were purchased from Acros Organics. Unless stated otherwise, all reactions were performed in a heat gun dried glassware under argon atmosphere, using standard septa techniques. The reactions were monitored by thin-layer chromatography (TLC) using silica gel 60 F254 plates (Merck) and visualized by UV (254 nm). Column chromatography was performed using silica gel (40–63 μm, Fluorochem) by flash liquid chromatography system (FLC) Teledyne ISCO CombiFlash Rf 200. Purification of triphosphates was performed using HPLC (Waters modular HPLC system) on Phenomenex Kinetex column (Kinetex® 5 μm EVO C18 100 Å, AXIA Packed LC Column 250 x 21.2 mm), or Luna column (Luna Omega 5 μm Polar C18 100 Å, AXIA Packed, LC Column 150 x 21.2 mm) or POROS HQ 50 column (lab-packed, 26×120 mm).
1.2. Synthetic scheme of dC\textsuperscript{hm}TP and dU\textsuperscript{hm}TP preparations

**Scheme S1**: Synthesis of dC\textsuperscript{hm}TP (A) and dU\textsuperscript{hm}TP (B). Conditions: i) TBDPSCl, imidazole, DMF, r.t., 18 h; ii) NBS, AIBN, benzene, 80 °C, 3 h, then AcOK, DMF, r.t., 1 h; iii) N-methylpiperidine, Et\textsubscript{3}N, TsCl, MeCN, 0 °C, 4 h, then NH\textsubscript{4}OH, r.t., 30 min; iv) AcOH, TBAF, r.t., 3 h; v) POCl\textsubscript{3}, PO(OMe)\textsubscript{3}, Proton Sponge, then (Bu\textsubscript{3}N)\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7}, Bu\textsubscript{3}N, MeCN, TEAB, 0 °C, 4 h, then K\textsubscript{2}CO\textsubscript{3}, MeOH, H\textsubscript{2}O, r.t., 2 h; vi) (CH\textsubscript{2}O)\textsubscript{n}, Et\textsubscript{3}N, H\textsubscript{2}O, 60 °C, 6 days; vii) AcOH, TFA, 120 °C, 30 min; viii) POCl\textsubscript{3}, PO(OMe)\textsubscript{3}, Proton Sponge, then (Bu\textsubscript{3}N)\textsubscript{2}H\textsubscript{2}P\textsubscript{2}O\textsubscript{7}, Bu\textsubscript{3}N, MeCN, TEAB, 0 °C, 4 h, then NH\textsubscript{4}OH, H\textsubscript{2}O, r.t., 15 min.
1.3. Synthesis of \(3', 5'\)-di(tert-butyldiphenylsilyl)thymidine (1)

![Thymidine structure]

The compound was prepared according to published procedure.[4] A flask was charged with thymidine (5.0 g, 20.64 mmol) together with imidazole (5.62 g, 82.6 mmol). The mixture was dissolved in DMF (40 mL) and TBDPS-Cl (17.0 g, 61.9 mmol) was added slowly with strong stirring and the reaction mixture was stirred at room temperature for 18 hours. Next day, the reaction mixture was diluted with EtOAc (200 mL), washed with saturated solution of \(\text{NH}_4\text{Cl}\) (2 x 80 mL) and brine (2 x 80 mL) and the organic layer was dried over \(\text{Na}_2\text{SO}_4\). Flash column chromatography (20 → 40 % EtOAc in cHex) provided the product 1 (13.36 g, 90%) as a white solid foam. NMR data were in accordance with the literature.[4]

1.4. Synthesis of \(3', 5'\)-di(tert-butyldiphenylsilyl)-5-acetoxymethyl-2'-deoxyuridine (2)

![Deoxyuridine structure]

A solution of 1 (3.0 g, 4.17 mmol) dissolved in benzene (15 mL) was heated to 80 °C, followed by addition of recrystallized NBS (0.75 g, 4.21 mmol) and AIBN (80 mg, 0.49 mmol). After 90 min, another portion of NBS (0.75 g, 4.21 mmol) and AIBN (80 mg, 0.49 mmol) was added followed by another 90 min at 80 °C. The reaction mixture was filtered through fritted glass and the solvent was evaporated. The residue was dissolved in DMF (10 mL), anhydrous potassium acetate (1.0 g, 10.2 mmol) was added and the reaction mixture was stirred for 1 hour at room temperature. The reaction mixture was diluted with EtOAc (100 mL), washed with water (50 mL) and brine (50 mL) and the organic layer was dried over \(\text{Na}_2\text{SO}_4\). Flash
column chromatography (20 → 40 % EtOAc in cHex) provided the product 2 (889 mg, 27 %) as a white solid foam.

**1H NMR** (500.2 MHz, CDCl₃): 0.92, 1.08 (2 × s, 2 × 9H, (CH₃)₂C); 1.90 (s, 3H, CH₃CO); 1.94 (ddd, 1H, J₆₆₃ = 13.1, J₆₂₅ = 8.9, J₂₅₃ = 5.6, H-2'b); 2.40 (ddd, 1H, J₆₆₃ = 13.1, J₆₆₅ = 5.3, J₂₅₃ = 1.4, H-2'a); 3.33, 3.71 (2 × dd, 2 × 1H, J₆₆₅ = 11.6, J₅₄ = 2.8, H-5'); 4.03 (td, 1H, J₄₅ = 2.8, J₄₃ = 1.4, H-4'); 4.37, 4.40 (2 × d, 2 × 1H, J₆₆₃ = 12.5, CH₂O); 4.51 (dt, 1H, J₃₅ = 5.6, 1.4, J₅₃ = 1.4, H-3'); 6.43 (dd, 1H, J₁₂ = 8.9, 5.3, H-1'); 7.27 – 7.34 (m, 6H, 3 × H-m-Ph); 7.36 – 7.42 (m, 5H, 3 × H-p-Ph, H-m-Ph); 7.43 – 7.48 (m, 3H, H-o-Ph, H-p-Ph); 7.48 – 7.52, 7.55 – 7.58, 7.60 – 7.64 (3 × m, 3 × 2H, H-o-Ph); 7.70 (s, 1H, H-6); 8.13 (s, 1H, NH).

**13C NMR** (125.8 MHz, CDCl₃): 18.99, 19.21 ((CH₃)₂C); 20.79 (CH₃CO); 26.86 ((CH₃)₂C); 41.53 (CH₂-2'); 58.88 (CH₂O); 63.93 (CH₂-5'); 73.99 (CH-3'); 85.53 (CH-1'); 88.10 (CH-4'); 109.28 (C-5); 127.90 (CH-m-Ph); 129.99, 130.01, 130.02, 130.06 (CH-p-Ph); 132.17, 132.83, 133.05, 133.12 (C-i-Ph); 135.19, 135.46, 135.65, 135.70 (CH-o-Ph); 140.54 (CH-6); 149.71 (C-2); 161.84 (C-4); 170.40 (CH₃CO).

HR/MS (ESI⁺): m/z: calcd. for C₄₄H₅₂N₂O₇Si₂Na [M-Na]⁺: 799.32053; found: 799.32018 [M-Na]⁺.

1.5. **Synthesis of 3', 5'-di(tert-butyldiphenylsilyl)-5-acetoxyethyl-2'-deoxycytidine (3)**

![Structure of 3']

Compound 2 (388 mg, 0.5 mmol) was dissolved in MeCN (3 mL), cooled down to 0 °C and Et₃N (111 mg, 1.1 mmol) together with N-methylpiperidine (59.5 mg, 0.6 mmol) were added. Next, TsCl (210 mg, 1.1 mmol) dissolved in MeCN (2 mL) was added slowly and the reaction mixture was stirred for 4 hours at 0 °C. NH₄OH (25%, 1.3 mL) was added, the mixture was taken out of the cooling bath and stirred for another 30 min at room temperature. The reaction mixture was diluted with EtOAc (25 mL), washed with brine (25 mL) and dried over Na₂SO₄. Flash column chromatography (2 → 10 % MeOH in DCM) provided the product 3 (272 mg, 70%) as a white solid foam.
**1H NMR** (500.2 MHz, CDCl₃): 0.92, 1.07 (2 × s, 2 × 9H, (CH₃)₂C); 1.90 (ddd, 1H, J_gem = 13.2, J₂b,1' = 8.7, J₂b,3' = 5.7, H-2'b); 1.97 (s, 3H, CH₃CO); 2.59 (ddd, 1H, J_gem = 13.2, J₂'a,1' = 5.4, J₂'a,3' = 1.6, H-2'a); 3.30 (dd, 1H, J_gem = 11.5, J₅'b,₄' = 2.8, H-5'b); 3.73 (dd, 1H, J_gem = 11.5, J₅'a,₄' = 2.4, H-5'a); 4.02 (ddd, 1H, J₄',₅' = 2.8, 2.4, J₄',₃' = 1.6, H-4'); 4.37, 4.40 (2 × d, 2 × 1H, J_gem = 13.2, CH₂O); 4.49 (dt, 1H, J₃',₂' = 5.7, 1.6, J₃',₄' = 1.6, H-3'); 5.68, 6.41 (2 × bs, 2 × 1H, NH₂); 6.46 (dd, 1H, J₁',₂' = 8.7, 5.4, H-1'); 7.25 – 7.34 (m, 6H, 3 × H-m-Ph); 7.34 – 7.48 (m, 8H, 4 × H-p-Ph, H-m-Ph, H-o-Ph); 7.49 – 7.52, 7.55 – 7.58, 7.60 – 7.64 (3 × m, 3 × 2H, H-o-Ph); 7.80 (s, 1H, H-6).

**13C NMR** (125.8 MHz, CDCl₃): 18.95, 19.22 ((CH₃)₂C); 20.77 (CH₃CO); 26.84, 26.85 ((CH₃)₂C); 42.48 (CH₂-2'); 59.83 (CH₂O); 63.92 (CH₂-5'); 74.11 (CH-3'); 86.53 (CH-1'); 88.11 (CH-4'); 101.17 (C-5); 127.79, 127.83, 127.85, 127.87 (CH-m-Ph); 129.90, 129.94 (CH-p-Ph); 132.20, 133.02, 133.04, 133.29 (C-i-Ph); 135.17, 135.41, 135.62, 135.69 (CH-o-Ph); 142.97 (CH-6); 154.76 (C-2); 163.82 (C-4); 171.37 (CH₃CO).

HR/MS (ESI⁺): m/z: calcld. for C₄₄H₅₃N₃O₆Si₂Na [M-Na]⁺: 798.33651; found: 798.33612 [M-Na]⁺.

### 1.6. Synthesis of 5-acetoxy-methyl-2'-deoxycytidine (4)

Compound 3 (651 mg, 0.84 mmol) was dissolved in THF (4.9 mL), the mixture was cooled down to 0 °C and freshly prepared 1 M solution of acetic acid in THF (1.72 mL, 2.05 equiv.) was added, followed by 1 M solution of TBAF in THF (1.72 mL, 2.05 equiv.). The reaction mixture was warmed up to room temperature and left to react for 3 hours, diluted with EtOAc (30 mL) and the product was extracted using H₂O (2 × 20 mL). Combined water layers were washed with EtOAc (10 mL) and evaporated under vacuum. Flash column chromatography (5 → 20% MeOH in DCM) provided the product 4 (228 mg, 91%) as an off-white solid.

**1H NMR** (600.1 MHz, CD₃OD): 2.06 (s, 3H, CH₃CO); 2.14 (dt, 1H, J_gem = 13.6, J₂b,1' = J₂b,3' = 6.5, H-2'b); 2.38 (ddd, 1H, J_gem = 13.6, J₂'a,1' = 6.2, J₂'a,3' = 4.0, H-2'a); 3.74 (dd, 1H, J_gem = 12.1, J₅'b,₄' = 3.8, H-5'b); 3.82 (dd, 1H, J_gem = 12.1, J₅'a,₄' = 3.2, H-5'a); 3.94 (ddd, 1H, J₄',₃' = 4.0, J₄',₅' = 3.8,
3.2, H-4'); 4.37 (dt, 1H, J3',2' = 6.5, 4.0, J3',4' = 4.0, H-3'); 4.86, 4.89 (2 × d, 2 × 1H, Jgem = 12.9, CH2O); 6.22 (dd, 1H, J1',2' = 6.5, 6.2, H-1'); 8.19 (s, 1H, H-6).

13C NMR (150.9 MHz, CD3OD): 20.78 (CH3CO); 42.24 (CH2-2'); 61.55 (CH2O); 62.61 (CH2-5'); 71.77 (CH-3'); 87.65 (CH-1'); 88.97 (CH-4'); 103.11 (C-5); 144.48 (CH-6); 157.88 (C-2); 166.26 (C-4); 172.87 (CH3CO).

HR/MS (ESI+): m/z: calcd. for C12H17N3O6Na [M-Na]+: 322.10096; found: 322.10091 [M-Na]+.

1.7. Synthesis of 5-hydroxymethyl-2'-deoxycytidine-5'-O-triphosphate (dChmTP)

Starting material 4 (40 mg, 0.133 mmol) and Proton Sponge (34 mg, 0.159 mmol) were dried overnight on high vacuum and then dissolved in PO(OMe)3 (1.3 mL). The mixture was cooled down to 0 °C and POCl3 (15 µL, 24 mg, 0.157 mmol) was added dropwise. After 2 hours at 0 °C, a prepared ice-cold mixture of bis(tributylammonium)pyrophosphate (330 mg, 0.6 mmol) and Bu3N (236 µL, 1 mmol) in MeCN (0.9 mL) was added and the resulting mixture was stirred for another 2 hours at 0 °C. A solution of TEAB (2 M in H2O, 2 mL) was added, the reaction was stirred for 15 min at room temperature and then the solvents were evaporated.

The residual liquid was co-distilled with H2O (4 x 10 mL) and the crude product was isolated by HPLC using solvent system A (0.1 M TEAB in H2O) and B (0.1 M TEAB in 50% MeOH) (0 → 20% B in A, Luna column). The crude product was then converted to sodium salt by passing through a column of Dowex 50WX8 and dissolved in MeOH (0.6 mL) and H2O (0.6 mL). Then, K2CO3 (18 mg, 0.130 mmol) was added and the reaction was stirred for 2 hours at room temperature, concentrated under vacuum and purified by HPLC (0 to 30% B in A, Phenomenex Luna Omega Polar). The pure product was converted to sodium salt and lyophilized from water, giving dChmTP (6.8 mg, 10 % over 2 steps) as a white solid. NMR data were in accordance with the literature.[5]
1.8. Synthesis of 5-hydroxymethyl-2′-deoxyuridine (5)

![Chemical Structure](1.8.png)

The compound was synthesized using published procedure.[6] A flask was charged with 2′-deoxyuridine (2.62 g, 11.5 mmol), paraformaldehyde (1.55 g, 51.7 mmol) and a solution of Et₃N in H₂O (0.5 M, 40 mL). The resulting mixture was stirred for 6 days at 60 °C. Every day, additional paraformaldehyde (1.0 g), Et₃N (0.5 mL) and H₂O (5 mL) were added. The solvent was evaporated and the residue was purified by flash column chromatography (15 → 25% MeOH in DCM) followed by crystallization from hot MeOH, giving the product 5 (1.23 g, 41%) as a white solid. NMR data were in accordance with the literature.[6]

1.9. Synthesis of 5-acetoxymethyl-2′-deoxyuridine (6)

![Chemical Structure](1.9.png)

Compound 5 (400 mg, 1.55 mmol) was suspended in glacial acetic acid (20 mL) and TFA (0.04 mL). The resulting mixture was put into an oil bath preheated to 120 °C for 30 minutes before cooling it down to room temperature. Solvents were evaporated and the crude product was purified by flash column chromatography (5 → 15% MeOH in CHCl₃). The reaction gave the product 6 (219 mg, 47%) as a white solid. NMR data were in accordance with the literature.[7]
1.10. Synthesis of 5-hydroxymethyl-2’-deoxyuridine-5’-O-triphosphate (dUhmTP)

![Chemical Structure]

Nucleoside 6 (50 mg, 0.167 mmol) and Proton Sponge (43 mg, 0.200 mmol) were dried overnight on high vacuum and then dissolved in PO(OMe)₃ (0.6 mL). The mixture was cooled down to 0 °C and POCl₃ (27 µL, 44.3 mg, 0.289 mmol) was added dropwise. After 2 hours at 0 °C, a prepared ice-cold mixture of bis(tributylammonium)pyrophosphate (440 mg, 0.800 mmol) and Bu₃N (236 µL, 1 mmol) in MeCN (0.9 mL) was added and the resulting mixture was stirred for another 2 hours at 0 °C. A solution of TEAB (2 M in H₂O, 2 mL) was added, the reaction was stirred for 15 min at room temperature, the solvents were evaporated and the residual liquid co-distilled with H₂O (4 x 10 mL). A solution of NH₃ in H₂O (25%, 5 mL) was added and the mixture was stirred at room temperature for 15 min, followed by evaporation of the solvent. The crude mixture was isolated by HPLC using solvent system A (0.1 M TEAB in H₂O) and B (0.1 M TEAB in 50% MeOH) (0 → 20% B in A, Luna column) and then using POROS 50 HQ column (0 → 100% 400 mM TEAB in H₂O). The purified product was converted to sodium salt by passing through a column of Dowex 50WX8 and lyophilized from water, giving dUhmTP (12.2 mg, 13% over 2 steps) as a white solid. NMR data were in accordance with the literature.[2]
1.11. Synthetic scheme of dUTP preparation

Scheme S2: Synthesis of dUTP. Conditions: i) POCl₃, PO(OMe)₃, Proton Sponge, then (Bu₃N)₂H₂P₂O₇, Bu₃N, MeCN, TEAB, 0 °C, 6 h.

1.12. Synthesis of 5-(formyl)-2'-deoxyuridine-5'-O-triphosphate (dUTP)

5-(formyl)-2'-deoxyuridine (27 mg, 0.11 mmol, 1.0 equiv.) was put into the microwave flask and dried under the vacuum overnight. PO(OMe)₃ (0.7 mL) and POCl₃ (15 µL, 0.17 mmol, 1.55 equiv.) were added by 0 °C. Mixture was stirred for 5 hours at 0 °C. An ice-cold solution of (Bu₃NH)₂ pyrophosphate (1.31 mL, 1.43 mmol, 13.0 equiv., 0.5 M in MeCN) and Bu₃N (0.33 mL, 1.43 mmol, 13.0 equiv.) were added, mixture was stirred for 1 hour at 0 °C, then warmed up to room temperature, stirred extra for 10 minutes and finally treated with TEAB (1 mL, 2 M). The MeCN was removed in vacuo. The concentrated mixture was extracted with water (10 mL) and CHCl₃ (2 x 10 mL). Water phase was evaporated on the rotavapor and crude product was isolated by HPLC using solvent system A (0.1 M TEAB in H₂O) and B (0.1 M TEAB in 50% MeOH) (10 → 100% B in A, Kinetex column). The solvents were evaporated in vacuo and the residue was three times co-evaporated with water. Lyophilization from water gave dUTP (6 mg, 5 %) as a white solid.[2]

¹H NMR (500.0 MHz, D₂O, ref(tBuOH) = 1.24 ppm): 1.27 (t, 36H, Jvic = 7.3, CH₃CH₂N); 2.46 (ddd, 1H, Jgem = 14.1, J₂b,3' = 6.4, J₂b,1' = 6.0, H-2'b); 2.51 (ddd, 1H, Jgem = 14.1, J₂'a,1' = 6.4, J₂'a,3' = 5.1, H-2'a); 3.19 (q, 24H, Jvic = 7.3, CH₃CH₂N); 4.21 – 4.35 (m, 3H, H-4',5'); 4.70 (ddd, 1H,
$J_{3',2} = 6.4, 5.1, J_{3',4'} = 4.0, H-3'$; 6.27 (d, 1H, $J_{1',2'} = 6.4, 6.0, H-1'$); 8.76 (s, 1H, H-6); 9.63 (s, 1H, CHO).

$^{13}C$ NMR (125.7 MHz, D$_2$O, ref(tBuOH) = 30.29 ppm): 8.92 (CH$_3$CH$_2$N); 40.13 (CH$_2$-2'); 47.31 (CH$_3$CH$_2$N); 65.35 (d, $J_{C,P} = 5.5$, CH$_2$-5'); 70.15 (CH-3'); 86.75 (d, $J_{C,P} = 9.1$, CH-4'); 87.33 (CH-1'); 112.58 (C-5); 152.39 (C-2); 154.36 (CH-6); 164.81 (C-4); 190.92 (CHO).

$^{31}$P{$^1$H} NMR (202.4 MHz, D$_2$O): -21.99 (t, $J = 20.5$, P$_\beta$); -10.85 (d, $J = 20.5$, P$_\alpha$); -6.08 (bd, $J = 20.5$, P$_\gamma$).

HR/MS (ESI-): m/z: calcd. for C$_{10}$H$_{11}$N$_2$O$_5$P$_3$$^+\ [M]$: 491.93942; found: 494.96034 [M-3H], 516.94238 [M-2H+Na$^-$].
2. Biochemical part[8–13]

2.1. General remarks

Mass spectra of short DNAs were measured by UltrafleXtreme MALDI-TOF/TOF (Bruker) mass spectrometer with 1 kHz smartbeam II laser technology. The matrix consisted of 3-hydroxypicolinic acid (HPA)/ picolinic acid (PA)/ ammonium tartrate in ratio 9/1/1. Concentrations of DNA were measured on Nanodrop 1000 Spectrophotometer (Thermo Scientific). Synthetic oligonucleotides (templates and 5'-6-FAM-labelled primers) were purchased from Generi Biotech (Table S1). Streptavidin magnetic particles (SMB) were obtained from Roche. DNA polymerases: Vent (exo-), Taq, and Bst Large Fragment were purchased from New England Biolabs, KOD XL DNA polymerase from Novagen, Pwo DNA polymerase from VWR, T4 DNA polymerase, Human DNA polymerase α and Human DNA polymerase β from ChimerX. All restriction endonucleases (REs) and 5-hydroxymethyluridine DNA Kinase (5-HMUDK) were purchased from New England Biolabs. Other chemicals were of analytical grade. All PEX products were purified either by QIAquick Nucleotide Removal KIT (Qiagen) or Monarch PCR & DNA Cleanup Kit (5 μg) (New England BioLabs). Milli-Q water was used for all experiments. Stop solution contained 95% [v/v] formamide, 0.5 mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol FF, 0.025% SDS in Milli-Q water. Aliquots of competitive incorporations samples (10 μL) were analyzed by 12.5 % PAGE (acrylamide/bisacrylamide 19:1, 25% urea) under denaturing conditions (40 mA, 50 °C, 1X TBE buffer). Aliquots of kinetic experiments samples (10 μL) were analyzed by 20 % PAGE (acrylamide/bisacrylamide 19:1, 40% urea) under denaturing conditions (30 mA, 50 °C, 1X TBE buffer). PAGE gels were visualized with Typhoon biological imager FLA 9500 (GE Healthcare Life Sciences).

The PAGE analysis of the products in competitive assays (see below) was followed by densitometric analysis performed by ImageJ software. The percentages of cleaved products (lower bands, natural DNA) and uncleaved products (upper bands, modified DNA) were calculated. The positive control samples (+) and modification control samples (M) gained the values close to 100 % of cleaved products or uncleaved products, respectively. These values represent the efficiency of RE cleavage and efficiency of o-PDA and 5-HMUDK postsynthetic reactions. The 1:1 and 1:10 samples values were double normalized by (+) and (M) control samples. The main value of percentage of uncleaved product (modified DNA) was added by the efficiency of postsynthetic reaction, (M) control sample, and then subtracted by efficiency of RE cleavage, (+) control sample.
Figure S1: dNTPs of interest.

Table S1: The list of used oligonucleotides

| Name                  | Sequence (5’ to 3’)                                | dNTPs used with the sequence |
|-----------------------|----------------------------------------------------|------------------------------|
| Prim248Short-FAM\(^a\) | CATGGGCGGCATGGG                                    | all dNTPs                    |
| PrimScal-T            | CATGGGCGGCATGGGAGTAC                               | dU\(^{hm}\)TP, dU\(^f\)TP    |
| TempScal-T            | TTCGTCTGTCAGT/ACTCCCATGCCGCCCATG                  | dU\(^{hm}\)TP, dU\(^f\)TP    |
| TempSp-T              | TTCGTCTGTCAGT/CCCATGCCGCCCATG                     | dU\(^f\)TP                  |
| TempBglII-C           | AACTACTACA/GATCTCCCATGCCGCCCATG                   | dC\(^{m}\)TP, dC\(^{hm}\)TP, dC\(^f\)TP |
| Prim248Short          | CATGGGCGGCATGGG                                    | dU\(^f\)TP                  |
| Oligo1-T-bio\(^b\)    | CCCACCATTGCCGCCCATG                                | dU\(^f\)TP                  |
| PrimEcoRI-T-FAM\(^a\)| CATGGGCGGCATGGGAAT                                  | dU\(^{hm}\)TP                |
| TempEcoRI-T           | TTCGTCTGTCG/AATTCCCATGCCGCCCATG                   | dU\(^{hm}\)TP                |
| Oligo1-termC          | GCCCATGCCGCCCATG                                   | dC\(^{m}\)TP, dC\(^{hm}\)TP, dC\(^f\)TP |
| Oligo1-termT          | ACCCATGCCGCCCATG                                   | dU\(^{hm}\)TP, dU\(^f\)TP    |

\(^a\) 5’-(6-FAM)-labelled; \(^b\) 5’-biotinylated; target palindromic sequence for restriction endonuclease in bold; /-cleavage site.
2.2. SPO1 polymerase isolation

SPO1 polymerase was cloned and isolated as follows. The gene encoding SPO1 polymerase consists of two exons and one self-splicing intron, belonging to group I (PMID: 1324872). The coding sequence for SPO1 DNA polymerase without the sequence of self-the splicing intron and containing the coding sequence for 6x His-tag at the 3’end was ordered from GeneArt (Invitrogen). This DNA fragment was then inserted into plasmid pBSURNAP (PMID: 34157109) through XbaI and KpnI sites (coding sequences for B. subtilis RNA polymerase subunits were replaced with the SPO1 gene). However, subsequent verification of the gene by sequencing repeatedly showed rearrangements of the coding sequence or T7 promoter sequence in E. coli strains. The SPO1 DNA polymerase seemed to be toxic for E. coli. A different strategy for cloning and overexpression had to be used. As the alternative strategy, a B. subtilis strain containing the SPO1 DNA polymerase gene under the control of the IPTG-inducible Hyperspank promoter integrated at the amyE locus was prepared. The gene for SPO1 DNA polymerase was amplified by PCR (Expand High Fidelity System, Roche) using primers Nos. 3461 (CCGGTCGACGTATTAAAACCTTTAAAGGAGATATATCTC) and 3462 (GCGGCATGCTTAGTGATGGTGATGGTGATGGG) and was inserted into the B. subtilis integrative plasmid pDR110 through XbaI and KpnI sites. The resulting plasmid (LK3255) was then transformed into the B. subtilis BaSysBio strain (LK2504). The resulting strain, LK3256, was used for overexpression of the SPO1 DNA polymerase.

The LK3256 strain was grown in LB medium (1.6 L) containing 0.5 mM IPTG (from start till OD600=2). Purification was performed as follows. Cells were harvested by centrifugation and pellet washed with Lysis buffer P (300 mM NaCl, 50 mM Na2HPO4, 3 mM 2-mercaptoethanol, 5% glycerol). Cells were resuspended in 25 ml Lysis buffer P and lysed by sonication (20x 10” separated by 1’ breaks on ice). The lysate was then cleared by centrifugation and the supernatant was mixed with 1 ml Ni-NTA agarose (equilibrated with Lysis buffer P) and incubated for 2 hours at 4°C with gentle mixing. The mixture was transferred to a Polyprep column (Roche), washed with 30 ml Lysis buffer P and with 30 ml Lysis buffer P with 30 mM imidazole. SPO1 DNA polymerase was eluted with 0.2-ml aliquots of Lysis buffer P with 400 mM imidazole. The fractions from the Ni–NTA containing SPO1 DNA polymerase were dialysed into SPO1 buffer (50mM TrisCl, pH 8, 300 mM NaCl, 3 mM 2-mercaptoethanol and 5% glycerol) and stored at 4°C.
2.3. Incorporation of dUhmTP employing Bst Large Fragment DNA Polymerase in proof-of-principle experiment

Incorporation by PEX: The reaction mixture (60 μL) contained primer PrimEcoRI-T-FAM (0.15 μM), template TempEcoRI-T (0.225 μM), Bst Large Fragment polymerase (0.9 U), natural dNTPs (dGTP, dCTP and dATP, 50 μM), dUhmTP (50 μM) for modification control sample, dTTP (50 μM) for positive control sample or water for negative control sample in reaction buffer provided by supplier. Mixture was incubated at 60 °C for 30 minutes and then divided into 20 μL and 40 μL. The stop solution (20 μL) was added to the first portion (20 μL), mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S2, lanes 2, 3 and 4). The second portion was used in following phosphorylation reaction.

Phosphorylation of dUhm by 5-hydroxymethyluridine DNA Kinase (5-HMUDK): To the mixture after PEX reaction (40 μL) 5-HMUDK (10 U) and T4 DNA Ligase Reaction Buffer (10X) with 10 mM ATP (4 μL) were added. Mixture was incubated at 37 °C for 30 minutes and then divided into 2 × 22 μL. The stop solution (18 μL) was added to the first portion, mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S2, lanes 5 and 6). The second portion was used in following cleavage reaction.

Cleavage by EcoRI: The second portion of PEX product (22 μL) was mixed with CutSmart Buffer (2 μL) and EcoRI-HF (20 U). Mixture was incubated at 37 °C for 30 min and then stopped by addition of stop solution (to reach 40 μL total volume). Products of cleavage were denatured at 95 °C for 5 min and analyzed using 12.5% denaturing PAGE (Figure S2, lanes 7 and 8).
Figure S2: PAGE analysis of PEX experiment with phosphorylation by 5-HMUDK. Lane 1 (P): primer; lane 2 (+): PEX product using natural dNTPs; lane 3 (-): PEX product using only dATP, dCTP and dGTP; lane 4 (H): PEX product using three natural dNTPs and dU^hmTP; lanes 5 (+) and 6 (H): PEX products using natural dNTPs and dU^hmTP, respectively, after phosphorylation; lanes 7 (+) and 8 (H): PEX products using natural dNTPs and dU^hmTP, respectively, after phosphorylation and EcoRI cleavage.

2.4. Incorporation of dU^TP employing Bst Large Fragment DNA Polymerase in proof-of-principle experiment

Incorporation by PEX: The reaction mixture (80 μL) contained primer PrimScal-T-FAM (0.15 μM), template TempScal-T (0.225 μM), Bst Large Fragment polymerase (1.2 U), natural dNTPs (dGTP, dCTP and dATP, 50 μM), dU^TP (50 μM) for modification control sample or dTTP (50 μM) for positive control sample in reaction buffer provided by supplier. Mixture was incubated at 60 °C for 30 minutes and then divided into 2 × 20 μL and 1 × 40 μL. The stop solution (20 μL) was added to the first portion (20 μL), mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S3, lanes 2 and 3). The second portion (20 μL) was used in the cleavage reaction. The third portion (40 μL) was used in the following labelling reaction.
**Labelling of dUf by o-phenylenediamine:** To the mixture after PEX reaction (40 μL) a freshly prepared aqueous solution of o-phenylenediamine (o-PDA) (100 mM, 2 μL, at 37 °C) was added. Mixture was incubated at 37 °C for 5 hours and then divided into 2 × 21 μL. The stop solution (19 μL) was added to the first portion, mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S3, lanes 4 and 5). The second portion was used in the following cleavage reaction.

**Cleavage by Scal:** The second portion of PEX product (20 μL or 21 μL) was mixed with CutSmart Buffer (2 μL) and Scal-HF (20 U). Mixture was incubated at 37 °C for 30 min and then stopped by addition of stop solution (to reach 40 μL total volume). Products of cleavage were denatured at 95 °C for 5 min and analyzed using 12.5% denaturing PAGE (Figure S3, lanes 6, 7, 8 and 9).

**Figure S3:** PAGE analysis of PEX experiment with o-PDA labelling. Lane 1 (P): primer; lane 2 (+): PEX product using natural dNTPs; lane 3 (F): PEX product using three natural dNTPs and dUfTP; lanes 4 (+) and 5 (F): PEX products using natural dNTPs and dUfTP, respectively, after o-PDA labelling; lanes 6 (+) and 7 (F): PEX products using natural dNTPs and dUfTP, respectively, without o-PDA labelling after Scal cleavage; lanes 8 (+) and 9 (F): PEX products using natural dNTPs and dUfTP, respectively, after o-PDA labelling and cleavage by Scal.
2.5. Competitive assay. Incorporation of modified dC<sup>®</sup>TP employing various DNA polymerases

*Competitive incorporation of dCTP vs dC<sup>®</sup>TP (R = f, hm, me)* by PEX: The reaction mixture (40 μL) contained primer **Prim248Short-FAM** (0.15 μM), template **TempBglII-C** (0.225 μM) DNA polymerase (Table S2), natural dNTPs (dGTP, dTTP and dATP, for concentration see Table S2), for ratio 1:1 dCTP (50 μM) and dC<sup>®</sup>TP (50 μM), for ratio 1:10 dCTP (10 μM) and dC<sup>®</sup>TP (100 μM), for positive control sample dCTP (100 μM), for modification control sample dC<sup>®</sup>TP (100 μM), in reaction buffer provided by supplier. Mixture was incubated for 30 min, (for incubation temperatures see Table S2) and then divided into 2 × 20 μL. The stop solution (20 μL) was added to the first portion, mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S4, lanes 1, 2, 3 and 4). The second portion was used in the following cleavage reaction. All experiments were done in triplicate.

*Cleavage by BglII:* The second portion of PEX product (20 μL) was mixed with NEBuffer 3.1 (4 μL) and BglII (20 U). Mixture was incubated at 37 °C for 60 min and then stopped by addition of stop solution (to reach 40 μL total volume). Products of cleavage were denatured at 95 °C for 5 min and analyzed using 12.5% denaturing PAGE (Figure S4, lanes 5, 6, 7 and 8).

**Table S2:** Competitive incorporation conditions of modified dC<sup>®</sup>TP employing various DNA polymerases

| Polymerase       | Units | Incubation temperature | Concentration of dGTP, dTTP and dATP [μM] |
|------------------|-------|------------------------|------------------------------------------|
| Bst Large Fragment | 0.6   | 60 °C                  | 50                                       |
| Taq              | 1     | 60 °C                  | 100                                      |
| KOD XL           | 0.05  | 60 °C                  | 50                                       |
| Pwo              | 0.6   | 60 °C                  | 50                                       |
| Vent(exo-)       | 0.2   | 60 °C                  | 50                                       |
| T4               | 0.6   | 30 °C                  | 50                                       |
### Bst LF

#### A) dC™TP

| Lane | Description |
|------|-------------|
| 1    | PEX         |
| 2    | PEX         |
| 3    | 1. PEX      |
| 4    | 2. BgIII    |

#### B) dC™mTP

| Lane | Description |
|------|-------------|
| 1    | PEX         |
| 2    | PEX         |
| 3    | 1. PEX      |
| 4    | 2. BgIII    |

#### C) dC™TP

| Lane | Description |
|------|-------------|
| 1    | PEX         |
| 2    | PEX         |
| 3    | 1. PEX      |
| 4    | 2. BgIII    |

### Taq

#### A) dC™TP

| Lane | Description |
|------|-------------|
| 1    | PEX         |
| 2    | PEX         |
| 3    | 1. PEX      |
| 4    | 2. BgIII    |

#### B) dC™mTP

| Lane | Description |
|------|-------------|
| 1    | PEX         |
| 2    | PEX         |
| 3    | 1. PEX      |
| 4    | 2. BgIII    |

#### C) dC™TP

| Lane | Description |
|------|-------------|
| 1    | PEX         |
| 2    | PEX         |
| 3    | 1. PEX      |
| 4    | 2. BgIII    |

### KOD XL

#### A) dC™TP

| Lane | Description |
|------|-------------|
| 1    | PEX         |
| 2    | PEX         |
| 3    | 1. PEX      |
| 4    | 2. BgIII    |

#### B) dC™mTP

| Lane | Description |
|------|-------------|
| 1    | PEX         |
| 2    | PEX         |
| 3    | 1. PEX      |
| 4    | 2. BgIII    |

#### C) dC™TP

| Lane | Description |
|------|-------------|
| 1    | PEX         |
| 2    | PEX         |
| 3    | 1. PEX      |
| 4    | 2. BgIII    |
Figure S4: PAGE analyses of PEX experiments with selected DNA polymerases and modified dC^rTPs. Lane 1 (+): PEX product using natural dNTPs; lanes 2 (1:1) and 3 (1:10): PEX products using three natural dNTPs and corresponding ratio of dCTP/dC^rTP; lane 4 (M):
PEX product using dC\textsuperscript{R}TP; lane 5 (+): PEX product using natural dNTPs after cleavage by BglII; lanes 6 (1:1) and 7 (1:10): PEX products using three natural dNTPs and corresponding ratio of dCTP/dC\textsuperscript{R}TP after cleavage by BglII; lane 8 (M): PEX product using dC\textsuperscript{R}TP after cleavage by BglII.

2.6. Competitive assay. Incorporation of modified dC\textsuperscript{R}TP employing Human DNA Polymerase α and β

Primer annealing: The primer Prim248short-FAM was mixed with the template TempBglll-C (1.5-fold excess) in aqueous Tris-HCl buffer (pH 7.5, 50 mM), DTT (5 mM), MgCl\textsubscript{2} (5 mM), to obtain 1.0 μM final concentration of primer. The annealing was performed in a thermal cycler. The sample was incubated at 95 °C for 5 min and then allowed to slowly cool down to 25 °C over 60 min. Prepared primed-TempBglll-C was stored at -20 °C.

Competitive incorporation of dCTP vs dC\textsuperscript{R}TP (R = f, hm, m) by PEX: The reaction mixture (30 μL) contained primed-TempBglll-C (0.1 μM primer, 0.15 μM template), Human polymerase (α or β, 1.5 U), natural dNTPs (dGTP, dTTP and dATP, 100 μM), for ratio 1:1 dCTP (50 μM) and dC\textsuperscript{R}TP (50 μM), for ratio 1:10 dCTP (10 μM) and dC\textsuperscript{R}TP (100 μM), for positive control sample dCTP (100 μM), for modification control sample dC\textsuperscript{R}TP (100 μM), BSA (0.1 mg/mL), glycerol (10 %), DTT (5 mM), MgCl\textsubscript{2} (5 mM) in aqueous Tris-HCl buffer (pH 7.5, 50 mM). Mixture was incubated at 37 °C for either 2 hours (Human polymerase α) or 1 hour (Human polymerase β) and then divided into 2 × 15 μL. The stop solution (15 μL) was added to the first portion, mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S5, lanes 1, 2, 3 and 4). The second portion was used in the following cleavage reaction. All experiments were done in triplicate.

Cleavage by BglII: The second portion of PEX product (15 μL) was mixed with NEBuffer 3.1 (3 μL) and BglII (20 U). Mixture was incubated at 37 °C for 60 min and then the stopped by addition of stop solution (to reach 30 μL total volume). Products of cleavage were denatured at 95 °C for 5 min and analyzed using 12.5% denaturing PAGE (Figure S5, lanes 5, 6, 7 and 8).
**Figure S5**: PAGE analyses of PEX experiments with Human DNA polymerases α or β and modified dC\textsuperscript{R}TPs. Lane 1 (+): PEX product using natural dNTPs; lanes 2 (1:1) and 3 (1:10): PEX products using three natural dNTPs and corresponding ratio of dCTP/dC\textsuperscript{R}TP; lane 4 (M): PEX product using dC\textsuperscript{R}TP; lane 5 (+): PEX product using natural dNTPs after cleavage by BglII; lanes 6 (1:1) and 7 (1:10): PEX products using three natural dNTPs and corresponding ratio of dCTP/dC\textsuperscript{R}TP after cleavage by BglII; lane 8 (M): PEX product using dC\textsuperscript{R}TP after cleavage by BglII.

### 2.7. Competitive assay. Incorporation of modified dU\textsuperscript{R}TP employing various DNA polymerases

*Competitive incorporation of dTTP vs dU\textsuperscript{R}TP (R = f, hm) by PEX*: The reaction mixture (40 μL) contained primer **PrimscaI-T-FAM** (0.15 μM), template **Tempscal-T** (0.225 μM), DNA polymerase (Table S3), natural dNTPs (dGTP, dCTP and dATP, for concentration see Table
S3), for ratio 1:1 dTTP (50 μM) and dURTP (50 μM), for ratio 1:10 dTTP (10 μM) and dURTP (100 μM), for positive control sample dTTP (100 μM), for modification control sample dURTP (100 μM) in reaction buffer provided by supplier. Mixture was incubated for 30 min, (for incubation temperatures see Table S3).

Phosphorylation of dUhm by 5-HMUDK: To the mixture after PEX reaction (40 μL) 5-HMUDK (10 U) and T4 DNA Ligase Reaction Buffer (10x) with 10 mM ATP (4 μL) were added. Mixture was incubated at 37 °C for 30 minutes and then divided into 2 × 22 μL. The stop solution (18 μL) was added to the first portion, mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S6, lanes 1, 2, 3 and 4). The second portion was used in the following cleavage reaction. All experiments were done in triplicate.

Labelling of dUf by o-phenylenediamine: To the mixture after PEX reaction (40 μL) a freshly prepared aqueous solution of o-PDA (100 mM, 2 μL, at 37 °C) was added. Mixture was incubated at 37 °C for 5 hours and then divided into 2 × 21 μL. The stop solution (19 μL) was added to the first portion, mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S6, lanes 1, 2, 3 and 4). The second portion was used in the following cleavage reaction. All experiments were done in triplicate.

Labelling of dUf by o-phenylenediamine employing KOD XL or Pwo polymerase: To the mixture after PEX reaction (40 μL) a freshly prepared aqueous solution of o-PDA (100 mM, 2 μL, at 37 °C) and Thermopol (10X) reaction buffer (4 μL) were added. Mixture was incubated at 37 °C for 5 hours. Mixture was then divided into 2 × 23 μL. The stop solution (17 μL) was added to the first portion, mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S6, lanes 1, 2, 3 and 4). The second portion was used in the following cleavage reaction. All experiments were done in triplicate.

Cleavage by Scal: The second portion of PEX product (21 or 23 μL in case of dUf, 22 μL in case of dUhm) was mixed with CutSmart Buffer (2 μL) and Scal-HF (20 U). Mixture was incubated at 37 °C for 30 min and then stopped by addition of stop solution (to reach 40 μL total volume). Products of cleavage were denatured at 95 °C for 5 min and analyzed using 12.5% denaturing PAGE (Figure S6, lanes 5, 6, 7 and 8).
Table S3: Competitive incorporation conditions of modified dURTP employing various DNA polymerases.

| Polymerase         | Units | Incubation temperature | Concentration of dGTP, dCTP and dATP [μM] |
|--------------------|-------|------------------------|------------------------------------------|
| Bst Large Fragment | 0.6   | 60 °C                  | 50                                       |
| Taq                | 1     | 60 °C                  | 100                                      |
| KOD XL             | 0.05  | 60 °C                  | 50                                       |
| Pwo                | 0.6   | 60 °C                  | 50                                       |
| Vent(exo-)         | 0.2   | 60 °C                  | 50                                       |
| T4                 | 0.6   | 30 °C                  | 50                                       |

2.8. Competitive Assay. Incorporation of dURTP Employing Taq DNA Polymerase

**Competitive incorporation of dTTP vs dURTP by PEX:** The reaction mixture (40 μL) contained primer Prim248Short-FAM (0.15 μM), template TempSp-T (0.225 μM), Taq polymerase (1 U), natural dNTPs (dGTP, dCTP and dATP, 100 μM), for ratio 1:1 dTTP (50 μM) and dURTP (50 μM), for ratio 1:10 dTTP (10 μM) and dURTP (100 μM), for positive control sample dTTP (100 μM), for modification control sample dURTP (100 μM) in reaction buffer provided by supplier. Mixture was incubated at 60 °C for 30 min.

**Labelling of dUR by o-PDA:** To the mixture after PEX reaction (40 μL) a freshly prepared aqueous solution of o-PDA (100 mM, 2 μL, at 37 °C) was added. Mixture was incubated at 37 °C for 5 hours and then divided into 2 × 21 μL. The stop solution (19 μL) was added to the first portion, mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S6, lanes 1, 2, 3 and 4). The second portion was used in the following cleavage reaction. All experiments were done in triplicate.

**Cleavage by SphI:** The second portion of PEX product (21 μL) was mixed with CutSmart Buffer (2 μL) and Sphl-HF (20 U). Mixture was incubated at 37 °C for 30 min and then stopped by addition of stop solution (to reach 40 μL total volume). Products of cleavage were denatured at 95 °C for 5 min and analyzed using 12.5% denaturing PAGE (Figure S6, lanes 5, 6, 7 and 8).
Figure S6: PAGE analyses of PEX experiments with selected DNA polymerases and dURTP.
Lane 1 (+): PEX product using natural dNTPs after o-PDA labelling or phosphorylation; lanes 2 (1:1) and 3 (1:10): PEX products using three natural dNTPs and corresponding ratio of dTTP/dURTP after o-PDA labelling or phosphorylation; lane 4 (M): PEX product using
\( \text{dU}^{\text{RTP}} \) after \( o \)-PDA labelling or phosphorylation; lane 5 (+): PEX product using natural dNTPs after \( o \)-PDA labelling or phosphorylation and cleavage by RE; lanes 6 (1:1) and 7 (1:10): PEX products using three natural dNTPs and corresponding ratio of dTTP/\( \text{dU}^{\text{RTP}} \) after \( o \)-PDA labelling or phosphorylation and cleavage by RE; lane 8 (M): PEX product using \( \text{dU}^{\text{RTP}} \) after \( o \)-PDA labelling or phosphorylation and cleavage by RE.

2.9. Competitive assay. Incorporation of modified \( \text{dU}^{\text{RTP}} \) employing Human DNA Polymerase \( \alpha \) and \( \beta \)

**Primer annealing:** The primer \text{PrimScal-T-FAM} was mixed with the template \text{TempScal-T} (1.5-fold excess) in aqueous Tris-HCl buffer (pH 7.5, 50 mM), DTT (5 mM), MgCl\(_2\) (5 mM), to obtain the 1.0 \( \mu \text{M} \) final concentration of primer. The annealing was performed in a thermal cycler. The sample was first heated at 95 °C for 5 min and then allowed to slowly cool down to 25 °C over 60 min. Prepared \text{primed-TempScal-T} was stored at -20 °C.

**Competitive incorporation of dTTP vs \( \text{dU}^{\text{RTP}} \) (\( R = f, \text{hm} \)) by PEX:** The reaction mixture (30 \( \mu \text{L} \)) contained \text{primed-TempScal-T} (0.1 \( \mu \text{M} \) primer, 0.15 \( \mu \text{M} \) template), Human polymerase (\( \alpha \) or \( \beta \), 1.5 U), natural dNTPs (dGTP, dCTP and dATP, 100 \( \mu \text{M} \)), for ratio 1:1 dCTP (50 \( \mu \text{M} \)) and \( \text{dU}^{\text{RTP}} \) (50 \( \mu \text{M} \)), for ratio 1:10 dCTP (10 \( \mu \text{M} \)) and \( \text{dU}^{\text{RTP}} \) (100 \( \mu \text{M} \)), for positive control sample dTTP (100 \( \mu \text{M} \)), for modification control sample \( \text{dU}^{\text{RTP}} \) (100 \( \mu \text{M} \)), BSA (0.1 mg/mL), glycerol (10 %), DTT (5 mM), MgCl\(_2\) (5 mM) in aqueous Tris-HCl buffer (pH 7.5, 50 mM). Mixture was incubated at 37 °C for either 2 hours (Human polymerase \( \alpha \)) or 1 hour (Human polymerase \( \beta \)).

**Phosphorylation of \( \text{dU}^{\text{hm}} \) by 5-HMUDK:** To the mixture after PEX reaction (30 \( \mu \text{L} \)) 5-HMUDK (10 U) and T4 DNA Ligase Reaction Buffer (10X) with 10 mM ATP (3 \( \mu \text{L} \)) were added. Mixture was incubated at 37 °C for 30 minutes and then divided into 2 \( \times \) 16.5 \( \mu \text{L} \). The stop solution (13.5 \( \mu \text{L} \)) was added to the first portion, mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S7, lanes 1, 2, 3 and 4). The second portion was used in the following cleavage reaction. All experiments were done in triplicate.

**Labelling of \( \text{dU} \) by \( o \)-phenylenediamine:** To the mixture after PEX reaction (40 \( \mu \text{L} \)) a freshly prepared aqueous solution of \( o \)-PDA (100 mM, 2 \( \mu \text{L} \), at 37 °C) and Thermopol (10X) reaction buffer (3 \( \mu \text{L} \)) were added. Mixture was incubated at 37 °C for 5 hours and then divided into 2 \( \times \) 17 \( \mu \text{L} \). The stop solution (13 \( \mu \text{L} \)) was added to the first portion, mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S7, lanes 1, 2, 3 and 4). The second portion was used in the following reaction. All experiments were done in triplicate.
Cleavage by Scal: The second portion of PEX product (16.5 or 17 μL) was mixed with CutSmart Buffer (1.7 μL) and Scal-HF (20 U). Mixture was incubated at 37 °C for 30 min and then stopped by addition of stop solution (to reach 30 μL total volume). Products of cleavage were denatured at 95 °C for 5 min and analyzed using 12.5% denaturing PAGE (Figure S7, lanes 5, 6, 7 and 8).

Figure S7: PAGE analyses of PEX experiments with Human DNA polymerases α or β and dUTP. Lane 1 (+): PEX product using natural dNTPs after o-PDA labelling or phosphorylation; lanes 2 (1:1) and 3 (1:10): PEX products using three natural dNTPs and corresponding ratio of dTTP/dUTP after o-PDA labelling or phosphorylation; lane 4 (M): PEX product using dUTP after o-PDA labelling or phosphorylation; lane 5 (+): PEX product using natural dNTPs after o-PDA labelling or phosphorylation and cleavage by Scal; lanes 6 (1:1) and 7 (1:10): PEX products using three natural dNTPs and corresponding ratio of dTTP/dUTP after o-PDA labelling or phosphorylation and cleavage by Scal; lane 8 (M): PEX product using dUTP after o-PDA labelling or phosphorylation and cleavage by Scal.
2.10. Competitive Assay. Incorporation of modified dC<sup>r</sup>TP employing SPO1 Polymerase

Competitive incorporation of dCTP vs dC<sup>r</sup>TP (R = f, hm, m) by PEX: The reaction mixture (40 μL) contained primer Prim<sub>248Short-FAM</sub> (0.15 μM), template Temp<sub>BglII-C</sub> (0.225 μM), SPO1 polymerase (40 pmol), natural dNTPs (dGTP, dTTP and dATP, 50 μM), for ratio 1:1 dCTP (50 μM) and dC<sup>r</sup>TP (50 μM), for ratio 1:10 dCTP (10 μM) and dC<sup>r</sup>TP (100 μM), for positive control sample dCTP (100 μM), for modification control sample dC<sup>r</sup>TP (100 μM) in 50 mM Tris-HCl buffer, 10 mM MgCl<sub>2</sub> and 1 mM ATP. Mixture was incubated for 1 hour at 37 °C and then purified by Monarch PCR & DNA Cleanup Kit (5 μg) according to standard manufacture’s procedure with the elution volume 40 μL. The mixture was divided into 2 × 20 μL. The stop solution (20 μL) was added to the first portion, mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S8, lanes 1, 2, 3 and 4). The second portion was used in the following cleavage reaction. All experiments were done in triplicate.

Cleavage by BglII: The second portion of PEX product (20 μL) was mixed with NEBuffer 3.1 (4 μL) and BglII (20 U). Mixture was incubated at 37 °C for 60 min and then the stopped by addition of stop solution (to reach 40 μL total volume). Products of cleavage were denatured at 95 °C for 5 min and analyzed using 12.5% denaturing PAGE (Figure S8, lanes 5, 6, 7 and 8).

| SPO1 | A) dC<sup>m</sup>TP | B) dC<sup>hm</sup>TP | C) dC<sup>f</sup>TP |
|------|-------------------|-------------------|-------------------|
| PEX  | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |
| 1  | PEX |  |  |  |  |  |  |  |
| 2  | BglII |  |  |  |  |  |  |  |
| 1  | PEX |  |  |  |  |  |  |  |
| 2  | BglII |  |  |  |  |  |  |  |
| 1  | PEX |  |  |  |  |  |  |  |
| 2  | BglII |  |  |  |  |  |  |  |

Figure S8: PAGE analyses of PEX experiments with SPO1 DNA polymerase and modified dC<sup>r</sup>TPs. Lane 1 (+): PEX product using natural dNTPs; lanes 2 (1:1) and 3 (1:10): PEX products using three natural dNTPs and corresponding ratio of dCTP/dC<sup>r</sup>TP; lane 4 (M): PEX product using dC<sup>r</sup>TP; lane 5 (+): PEX product using natural dNTPs after cleavage by BglII; lanes 6 (1:1) and 7 (1:10): PEX products using three natural dNTPs and corresponding...
ratio of dCTP/dCrTP after cleavage by BglII; lane 8 (M): PEX product using dCrTP after cleavage by BglII.

2.11. Competitive Assay. Incorporation of modified dUrTP employing SPO1 DNA Polymerase

*Competitive incorporation of dTTP vs dUrTP (R = f, hm) by PEX:* The reaction mixture (40 μL) contained primer PrimScal-T-FAM (0.15 μM), template TempScal-T (0.225 μM), SPO1 polymerase (40 pmol), natural dNTPs (dGTP, dCTP and dATP, 150 μM), for ratio 1:1 dTTP (50 μM) and dUrTP (50 μM), for ratio 1:10 dTTP (10 μM) and dUrTP (100 μM), for ratio 1:100 (in the case of dUhmTP) dTTP (1 μM) and dUhmTP (100 μM), for positive control sample dTTP (100 μM), for modification control sample dUrTP (100 μM) in 50 mM Tris-HCl buffer, 10 mM MgCl₂ and 1 mM ATP. Mixture was incubated for 1 hour at 37 °C and then purified by QiAquick Nucleotide Removal KIT according to standard manufacture’s procedure with the elution volume 40 μL.

*Phosphorylation of dUhm by 5-HMUDK:* To the mixture after PEX reaction (40 μL) 5-HMUDK (10 U) and T4 DNA Ligase Reaction Buffer (10x) with 10 mM ATP (4 μL) were added. Mixture was incubated at 37 °C for 30 minutes and then divided into 2 × 22 μL. The stop solution (18 μL) was added to the first portion, mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S9, lanes 1, 2, 3, 4 and 5). The second portion was used in following cleavage reaction. All experiments were done in triplicate.

*Labelling of dUf by o-phenylenediamine:* To the mixture after PEX reaction (40 μL) a freshly prepared aqueous solution of o-PDA (100 mM, 2 μL, at 37 °C) and Thermopol (10X) reaction buffer (4 μL) were added. Mixture was incubated at 37 °C for 5 hours and then divided into 2 × 23 μL. The stop solution (17 μL) was added to the first portion, mixture was denatured at 95 °C for 5 min and further analyzed using 12.5% denaturing PAGE (Figure S10, lanes 1, 2, 3 and 4). The second portion was used in the following cleavage reaction. All experiments were done in triplicate.

*Cleavage by Scal:* The second portion of PEX product (22 or 23 μL) was mixed with CutSmart Buffer (2 μL) and Scal-HF (20 U). Mixture was incubated at 37 °C for 30 min and then stopped by addition of stop solution (to reach 40 μL total volume). Products of cleavage were denatured at 95 °C for 5 min and analyzed using 12.5% denaturing PAGE (Figure S9, lanes 6, 7, 8, 9 and 10; Figure S10, lanes 5, 6, 7 and 8).
**Figure S9**: PAGE analysis of PEX experiments with SPO1 DNA polymerase and \textbf{dU}^{hmTP}. Lane 1 (+): PEX product using natural dNTPs after phosphorylation; lanes 2 (1:1) and 3 (1:10): PEX products using three natural dNTPs and corresponding ratio of dTTP/\textbf{dU}^{hmTP} after phosphorylation; lane 4 (X): PEX product using three natural dTTP and \textbf{dU}^{hmTP} in the ratio 1:100 after phosphorylation; lane 5 (M): PEX product using \textbf{dU}^{hmTP} after phosphorylation; lane 6 (+): PEX product using natural dNTPs after phosphorylation and cleavage by ScaI; lanes 7 (1:1) and 8 (1:10): PEX products using three natural dNTPs and corresponding ratio of dTTP/\textbf{dU}^{hmTP} after phosphorylation and cleavage by ScaI; lane 9 (X): PEX product using three natural dNTPs and dTTP/\textbf{dU}^{hmTP} in the ratio 1:100 after phosphorylation and cleavage by ScaI; lane 10 (M): PEX product using \textbf{dU}^{hmTP} after phosphorylation and cleavage by ScaI.
**Figure S10**: PAGE analysis of PEX experiments with SPO1 DNA polymerase and dU^TP. Lane 1 (+): PEX product using natural dNTPs after o-PDA labelling; lanes 2 (1:1) and 3 (1:10): PEX products using three natural dNTPs and corresponding ratio of dTTP/dU^TP after o-PDA labelling; lane 4 (M): PEX product using dU^TP after o-PDA labelling; lane 5 (+): PEX product using natural dNTPs after o-PDA labelling and cleavage by Scal; lanes 6 (1:1) and 7 (1:10): PEX products using three natural dNTPs and corresponding ratio of dTTP/dU^TP after o-PDA labelling and cleavage by Scal; lane 8 (M): PEX product using dU^TP after o-PDA labelling and cleavage by Scal.

### 2.12. Steady state kinetics assay

Reaction mixtures (10 μL) contained 5'-6-FAM-labelled primer Prim248Short-FAM (1 μM) and template Oligo1-termC (1 μM) for dC^RTP incorporation or Oligo1-termT (1 μM) for dU^RTP incorporation (1 μM) and DNA polymerase (Table S4) in reaction buffer provided by supplier. Reactions were initiated by addition of various concentrations of natural or modified dN^RTPs (N = C or U; R = f, hm, m), mixtures were incubated for 3 min at temperatures corresponding to the DNA polymerase of interest (Table S4). The final dN^RTPs concentrations in the samples were: 0, 0.1, 0.316, 1, 3.16, 10, 31.6 and 100 μM. Reactions were stopped by addition of 10 μL of stop solution. Products were denatured at 95 °C for 5 min and separated using 20% denaturing PAGE (Figures S11 – S17). Kinetic parameters (kcat and Km) were determined by fitting data to the Michaelis-Menten equation using Microsoft Excel and OriginPro 2021 (Figure S18). The ratio of catalytic efficiency of modified dN^RTP in the respect of natural dNTP was calculated as (kcat/Km)_modified/(kcat/Km)_natural. In the cases when double band of product was observed,
the slower band (product N+1 of non-templated addition of another nucleotide) was also included into the calculation. All experiments were performed in triplicate.

**Table S4**: Steady state kinetics assay conditions for selected DNA polymerases.

| Polymerase          | Amount | Incubation temperature |
|---------------------|--------|------------------------|
| Bst Large Fragment  | 0.2 U  | 65 °C                  |
| Taq                 | 0.5 U  | 75 °C                  |
| Vent(exo-)          | 0.1 U  | 75 °C                  |
| Human β             | 1 U    | 37 °C                  |
| SPO1                | 40 pmol| 37 °C                  |
**Figure S11**: PAGE analyses of steady state kinetic experiments with dCTP and selected DNA polymerases. PEX products with the final dCTP concentration of 0 μM (lanes 1), 0.1 μM (lanes 2), 0.316 μM (lanes 3), 1 μM (lanes 4), 3.16 μM (lanes 5), 10 μM (lanes 6), 31.6 μM (lanes 7) and 100 μM (lanes 8).
Figure S12: PAGE analyses of steady state kinetic experiments with dCmTP and selected DNA polymerases. PEX products with the final dCmTP concentration of 0 μM (lanes 1), 0.1 μM (lanes 2), 0.316 μM (lanes 3), 1 μM (lanes 4), 3.16 μM (lanes 5), 10 μM (lanes 6), 31.6 μM (lanes 7) and 100 μM (lanes 8).
Figure S13: PAGE analyses of steady state kinetic experiments with dC<sup>hm</sup>TP and selected DNA polymerases. PEX products with the final dC<sup>hm</sup>TP concentration of 0 μM (lanes 1), 0.1 μM (lanes 2), 0.316 μM (lanes 3), 1 μM (lanes 4), 3.16 μM (lanes 5), 10 μM (lanes 6), 31.6 μM (lanes 7) and 100 μM (lanes 8).
Figure S14: PAGE analyses of steady state kinetic experiments with dC\textsuperscript{TP} and selected DNA polymerases. PEX products with the final dC\textsuperscript{TP} concentration of 0 μM (lanes 1), 0.1 μM (lanes 2), 0.316 μM (lanes 3), 1 μM (lanes 4), 3.16 μM (lanes 5), 10 μM (lanes 6), 31.6 μM (lanes 7) and 100 μM (lanes 8).
Figure S15: PAGE analyses of steady state kinetic experiments with dTTP and selected DNA polymerases. PEX products with the final dTTP concentration of 0 μM (lanes 1), 0.1 μM (lanes 2), 0.316 μM (lanes 3), 1 μM (lanes 4), 3.16 μM (lanes 5), 10 μM (lanes 6), 31.6 μM (lanes 7) and 100 μM (lanes 8).
Figure S16: PAGE analyses of steady state kinetic experiments with dU<sup>hm</sup>TP and selected DNA polymerases. PEX products with the final dU<sup>hm</sup>TP concentration of 0 μM (lanes 1), 0.1 μM (lanes 2), 0.316 μM (lanes 3), 1 μM (lanes 4), 3.16 μM (lanes 5), 10 μM (lanes 6), 31.6 μM (lanes 7) and 100 μM (lanes 8).
Figure S17: PAGE analyses of steady state kinetic experiments with dUTP and selected DNA polymerases. PEX products with the final dUTP concentration of 0 μM (lanes 1), 0.1 μM (lanes 2), 0.316 μM (lanes 3), 1 μM (lanes 4), 3.16 μM (lanes 5), 10 μM (lanes 6), 31.6 μM (lanes 7) and 100 μM (lanes 8).
Figure S18: Example of the curve obtained by fitting the kinetic results for dCmTP and Human β polymerase to the Michaelis-Menten function in the OriginPro 2021.

2.13. Preparation of o-phenylenediamine-labelled oligonucleotide for MALDI-TOF analysis

The reaction mixture (20 μL) contained primer Primer248Short (0.15 μM), 5′-biotinylated template Oligo1T-bio (0.15 μM), Bst Large Fragment DNA polymerase (0.3 U), dATP (50 μM), dGTP (50 μM), dCTP (50 μM) and dUTP (50 μM) in reaction buffer provided by supplier by the manufacturer (2 μL). The mixture was prepared fifteen times in separate Eppendorf tubes. The mixtures were incubated at 60 °C for 40 min. Then 1 μL of o-phenylenediamine (o-PDA) (100 mM) was added to each Eppendorf tube and mixtures were further incubated at 37 °C for 5 hours. Subsequently, all 15 reaction mixtures were combined. Streptavidin magnetic particles (SMB) (280 μL) were prewashed with binding buffer (3 × 500 μL, 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). The PEX reaction mixture (300 μL, prepared as described above) was mixed with prewashed SMB and binding buffer (300 μL) and incubated at 15 °C for 30 min and 1400 rpm. The magnetic beads were collected on a magnet (DynaMag-2, Invitrogen) and washed with wash buffer (3 × 200 μL, 10 mM Tris, 1 mM EDTA, 500 mM NaCl, pH 7.5) and water (5 × 200 μL). Then water (100 μL) was added and the sample was denatured for 2 min at 60 °C and 900 rpm. The beads were collected on a magnet and the solution was transferred into a clean tube. The solution was analyzed by MALDI-TOF mass spectrometry.

MALDI-TOF: calcd. [M]: 6067.96 Da; found: 6070.79 Da [M-3H]⁺, 6094.79 Da [M-2H-Na]⁺, 3035.10 Da [M-2H]²⁺.
3. Supplementary copies of NMR and MS spectra

3.1. $^1$H NMR, $^{13}$C NMR and HR/MS (ESI$^+$) spectra of 3', 5'-di(tert-butyldiphenylsilyl)-5-acetoxyethyl-2'-deoxyuridine (2)
3.2. $^1$H NMR, $^{13}$C NMR and HR/MS (ESI$^+$) spectra of 3', 5'-di(tert-butyldiphenylsilyl)-5-acetoxyethyl-2'-deoxycytidine (3)
3.3. $^1$H NMR, $^{13}$C NMR and HR/MS (ESI$^+$) spectra of 5-acetoxymethyl-2$'$-deoxycytidine (4)
3.4. $^1$H NMR, $^{13}$C NMR, HMBC NMR, $^{31}$P($^1$H) NMR and HR/MS-(ESI-) spectra of 5-(formyl)-2'-deoxyuridine-5'-O-triphosphate (dUTP)
3.5. MALDI-TOF spectrum of o-phenylenediamine-labelled oligonucleotide

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