Nucleotide-Bearing Benzyldene-Tetrahydroxanthylium Near-IR Fluorophore for Sensing DNA Replication, Secondary Structures and Interactions

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1. Additional results, schemes, tables and figures

![Figure S1. Structures of dT^N3 and dT^N3TP](image)

**Figure S1.** Structures of dT^N3 and dT^N3TP

![Normalized absorption and fluorescence spectra](image)

**Figure S2.** A) Normalized absorption (dashed lines) and fluorescence (solid lines) spectra of dT^NNIR in solvents of different polarity. B) Fluorescence spectra of dT^NNIR (λ_ex = 690 nm) in

![Viscosity and fluorescence intensity](image)
glycerol at different temperature (viscosity). C) Emission of $dT^{NNIR}$ ($\lambda_{ex} = 613$ nm) in various dioxane/water ratios.

**Table S1.** Photophysical properties of modified nucleoside, nucleotide and DNA

| Sample            | Solvent    | $\lambda_{abs}^a$ (nm) | $\varepsilon^b$ (M$^{-1}$ cm$^{-1}$) | $\lambda_{em}^c$ (nm) | $\Delta\lambda^d$ (nm) | $\Phi^e$ (%) |
|-------------------|------------|------------------------|--------------------------------------|------------------------|-------------------------|--------------|
| $dT^{NNIR}$       | Water      | 622                    | 20826                                | 749                    | 127                     | 0.33         |
|                   | MeCN       | 664                    | 30703                                | 760                    | 96                      | 0.28         |
|                   | MeOH       | 666                    | 35116                                | 754                    | 88                      | 0.56         |
|                   | 1.4-Dioxane| 694                    | 16160                                | 754                    | 60                      | 4.55         |
| $dT^{NNIR}_{TP}$  | Water      | 623                    | 11746                                | 745                    | 122                     | 0.37         |
| DNA19_1T$^{NNIR}$ | Phosphate  | 690                    | n.d.                                 | 745                    | 55                      | 6.84         |

| Sample            | Phosphate  | 690                    | n.d.                                 | 745                    | 55                      | 6.84         |

$d$ Position of the absorption maximum, $^b$ molar extinction coefficients, $^c$ position of the emission maximum, $^d$ Stokes shift, $^e$ fluorescence quantum yield measured using indocyanine green (ICG) in DMSO ($\Phi = 0.13$ at 25 °C) as reference, n.d. = not determined.

**Table S2.** Fluorescence quantum yields of modified $dT^{NNIR}$ at different viscosity (solution of nucleoside in glycerol was measured at different temperatures)

| Temperature (°C) | Viscosity (cP) | $\Phi^e$ (%) |
|------------------|----------------|--------------|
| 10               | 3900           | 11.0         |
| 20               | 1410           | 9.0          |
| 25               | 945            | 8.2          |
| 30               | 612            | 7.4          |
| 40               | 284            | 6.2          |
| 50               | 142            | 4.6          |
| 70               | 50.6           | 2.5          |
Table S3. Table of oligonucleotide sequences used in enzymatic synthesis

| Oligonucleotide | Length | Sequence (5′→3′) |
|-----------------|--------|-----------------|
| Prim1PEX        | 15-mer | 5′-CATGGGCGGCATGGG-3′ |
| Prim1PEX-FAM (a) | 15-mer | 5′-FAM-CATGGGCGGCATGGG-3′ |
| Temp1PEX        | 16-mer | 5′-ACCCATGCGCCCATG-3′ |
| Temp2PEX        | 19-mer | 5′-CCCACCATGCGCCCATG-3′ |
| Temp2PEX.bio (b) | 19-mer | 5′-bio-CCCACCATGCGCCCATG-3′ |
| Temp2PEX.P (c)  | 19-mer | 5′-P-CCCACCATGCGCCCATG-3′ |
| Temp3PEX        | 31-mer | 5′-CTAGCATGAGCTCGTCCATGCCCATG-3′ |
| Temp3PEX.bio    | 31-mer | 5′-bio-CTAGCATGAGCTCGTCCATGCCCATG-3′ |
| Temp3PEX_TINA (d) | 31-mer | 5′-TINA-CTAGCATGAGCTCGTCCATGCCCATG-3′ |
| Temp4PEX        | 31-mer | 5′-CTAGCATGAGCTCGTCCATGCCCATG-3′ |
| Temp4PEX.bio    | 31-mer | 5′-bio-CTAGCATGAGCTCGTCCATGCCCATG-3′ |
| Prim1PCR        | 20-mer | 5′-GACATCATGAGACATGC-3′ |
| Prim2PCR        | 25-mer | 5′-CGACGACGATACCTGATTC-3′ |
| Prim3PCR        | 22-mer | 5′-GACATCATGAGACATGC-3′ |
| Prim4PCR        | 26-mer | 5′-CARATGTAAASACACTATTAGCATA-3′ |
| Temp1PCR        | 98-mer | 5′-GTGAAATGGTCATTGCTCGTCCATGCCCATG-3′ |
| Temp2PCR        | 100-mer | 5′-GTGAAATGGTCATTGCTCGTCCATGCCCATG-3′ |

Underlined: segments of templates complementary to primers. R is G/A; S is G/C. a 6-carboxyfluorescin (6-FAM) used for oligonucleotide labeling at 5′-end, b template biotinylated at 5′-end, c template phosphorylated at 5′-end, d template labeled by ortho-TINA at 5′-end

Table S4. Table of oligonucleotides prepared in this study

| Oligonucleotide | Sequence (5′→3′), T*: modified nucleotide. | M_calculated (Da) | M_found (Da) |
|-----------------|---------------------------------------------|------------------|--------------|
| ON16_1TNNIR     | 5′-CATGGGCGGACATGGT*3′                      | 5430.6           | 5431.7       |
| ON19_1TNNIR     | 5′-CATGGGCGGACATGGT*GGG-3′                  | 6418.2           | 6419.6       |
| ON19_1TNJ       | 5′-CATGGGCGGACATGGT*GGG-3′                  | 6006.9           | 6007.9       |
| ON31_1TNNIR     | 5′-CATGGGCGGACATGGT*CTGACGTCCATG-3′         | 10060.6          | 10060.6      |
| ON31_4TNJ       | 5′-CATGGGCGGACATGGT*GAGCT*CAGT*GCT*AG-3′    | 9781.4           | 9782.0       |
Figure S3. PAGE analysis of PEX using A) dT<sup>NNIR</sup>TP or B) dT<sup>N3</sup>TP. KOD XL DNA polymerase and Temp<sup>2PEX</sup>, Temp<sup>3PEX</sup> or Temp<sup>3PEX-TINA</sup> templates. Primer (P), positive control (lane 1, 4; PEX with all natural dNTPs), negative control (lane 2, 5; PEX in absence of dTTP). A) PEX with dT<sup>NNIR</sup>TP (lane 3, 6). B) PEX with dT<sup>N3</sup>TP (lane 3, 6).

Scheme S1. Site specific fluorescent labelling of DNA by SNI-PEX
Figure S4. Page analysis of products of polymerase synthesis of oligonucleotides bearing a single modification by SNI-PEX. Primer (P); lane 1: positive control for SNI (only natural dTTP present); lane 2: negative control for SNI (no dNTPs present); lane 3: SNI with modified nucleotide (only dTNNIRTP is present); lane 4: positive control for PEX (all natural dNTPs present); lane 5: negative control for PEX (absence of natural dTTP); lane 6: PEX with primer extended by dTNNIRTP (all natural dNTPs present).

Figure S5. UV melting curves of A) DNA19_1T (Tm: 79.6°C) and B) DNA19_1TNNIR (Tm: 79.5°C).
**Figure S6.** UV-vis absorption spectra (non-normalized) of DNA19_1TNNIR ($\lambda_{abs}$ = 690 nm) compared to DTNNIRTP ($\lambda_{abs}$ = 623 nm).

**Figure S7.** Agarose gel electrophoresis analysis of PCR amplification of 98bp template (Temp1PCR) with KOD XL polymerase and A) dTNNIRTP or B) dTNNIRTP. L: DNA ladder. Positive control (+): all natural dNTPs are present. Negative control (-): absence of natural dTTP. PCR with modified dNTP (*): dTNNIRTP, dATP, dGTP and dCTP is present. PCR with modified dNTP (lane 1-7): dGTP, dCTP, dATP and a mixture of dTTP and dTNNIRTP (the content of modified nucleotide decreases from 100% (lane 1) to 5% (lane 7)) is present.
**Figure S8.** Fluorescence spectra of isolated PCR products and control experiments (PCR mixtures in absence of enzyme). Product of PCR with A) 10% or B) 5% $d^\text{NNIR}TP$ in mixture with natural $dTTP$. Lane 6 – product loaded on gel in Figure S5, B). Lane 7 – product loaded on gel in Figure S5, B).

**Scheme S2.** Scheme of click reaction of azido modified PEX products with alkyne 3.
Figure S9. Page analysis of click reaction performed using ON$_{19\_1T}$ or ON$_{31\_4T}$ with alkyne 3. Lane 1, Nat. ctrl: natural ssDNA (ON$_{19\_1T}$) control before click reaction. Lane 2, Nat.: natural ssDNA (ON$_{19\_1T}$) treated in the conditions of click reaction. Lane 3, T$^{N3}$ ctrl: ON$_{19\_1T}^{N3}$. Lane 4, T$^{N3}$: click reaction with ON$_{19\_1T}^{N3}$. Lane 5, Nat. ctrl: natural ssDNA (ON$_{31\_4T}$) control before click reaction. Lane 6, Nat.: natural ssDNA (ON$_{31\_4T}$) treated in the conditions of click reaction. Lane 7, 4T$^{N3}$ ctrl: ON$_{31\_4T}^{N3}$. Lane 8, 4T$^{N3}$: click reaction with ON$_{31\_4T}^{N3}$.

Figure S10. Fluorescence spectra of isolated products of click reaction using A) ON$_{19\_1T}^{N3}$ or ON$_{31\_4T}^{N3}$ and alkyne 3 (red line, Click reaction). Control - natural oligonucleotide A) ON$_{19\_1T}$ or B) ON$_{31\_4T}$ treated in the conditions of click reaction.
Figure S11. A) Fluorescence microscopy and B) bright field images of U-2 OS cells treated with a mixture of $dT^{NNIRTP}$ (10 µM) and SNTT 1 (10 µM) in tricine buffer (37°C, 5 min) and further incubated in complete medium (37°C, % CO2, 60 min). Shrunk nuclei indicate a significant toxicity of $dT^{NNIRTP}$. Fluorescence of $dT^{NNIRTP}$ is represented with green colour for better visibility.

Figure S12. A) UV-vis absorption spectra ($\lambda_{abs} = 690$ nm) and B) fluorescence spectra ($\lambda_{ex} = 690$ nm, $\lambda_{em} = 745$ nm) of DNA obtained after incubation of PEX reaction mixtures containing $dT^{NNIRTP}$. Red lines represent experiments with and black lines without KOD XL DNA polymerase. The reaction mixtures were incubated at 60°C for 1 hour, then the reactions were stopped by cooling at 4°C. Products were purified using QIAquick Nucleotide Removal Kit (QIAGEN).
**Figure S13.** Fluorescence spectra of dsDNA (DNA19_1\textsuperscript{TNIR}) and nucleotide (dT\textsuperscript{TNIRTP}) at 2 μM concentration. Samples were excited at 720 nm in phosphate buffer (4.5 mM, pH 7.4) at 25°C.

**Scheme S3.** Scheme of digestion by lambda exonuclease
**Figure S14.** A) Fluorescence spectra of reaction mixture (DNA19_1TNNIR-P, buffer for lambda exonuclease and lambda exonuclease) at 0 and after 75 minutes. B) Fluorescence intensity plotted against time. Black line represents digestion reaction mixture with and red line (Control 1) without lambda exonuclease, Control 2 (green line) contains reaction mixture with DNA19_1TNNIR without 5’-P label. Samples were excited at 690 nm at 37°C.

**Figure S15.** Dyes used for determination of mode of interaction between DNA and fluorophore.
Figure S16. Fluorescence spectra of DNA19_1TNNIR with increasing concentration of A) TO, C) DAPI or E) MG and fluorescence intensity plotted against concentration of B) TO, D) DAPI or F) MG. Samples were excited at 690 nm in phosphate buffer (4.5 mM, pH 7.4) at 25°C.
Figure S17. A) Fluorescence spectra of DNA19_1T^{NNIR} after addition of 0-4 equiv. of histone. B) Fluorescence intensity plotted against concentration of histone. Samples were excited at 690 nm in phosphate buffer (4.5 mM, pH 7.4) at 25°C.
**Figure S18.** A) Fluorescence spectra of DNA\(_{19}\)T\(^{NNIR}\) after addition of 0-256 equiv. of spermine. B) Fluorescence intensity plotted against concentration of spermine. Samples were excited at 690 nm in phosphate buffer (4.5 mM, pH 7.4) at 25°C.

**Figure S19.** A) Fluorescence spectra of DNA\(_{19}\)T\(^{NNIR}\) after addition of 0-1.75 equiv. of protamine. B) Fluorescence intensity plotted against concentration of protamine. Samples were excited at 690 nm in phosphate buffer (4.5 mM, pH 7.4) at 25°C.
Figure S20. A) Fluorescence spectra of complex of DNA19_1TNNIR with histone after addition of 0-1.75 equiv. of 98-mer dsDNA. B) Fluorescence intensity plotted against concentration of 98-mer dsDNA. Samples were excited at 690 nm in phosphate buffer (4.5 mM, pH 7.4) at 25°C.

Figure S21. A) Fluorescence spectra of complex of DNA19_1TNNIR with protamine after addition of 0-0.7 equiv. of heparin. B) Fluorescence intensity plotted against concentration of heparin. Samples were excited at 690 nm in phosphate buffer (4.5 mM, pH 7.4) at 25°C.
Figure S22. Monitoring incorporation into DNA in real time using dT<sup>NNIRTP</sup>. A) Fluorescence spectra of SNI (single nucleotide incorporation) reaction at 0 and after 25 minutes, B) fluorescence intensity plotted against time. Red line represents experiment (SNI) with and purple line (Control 1) without KOD XL DNA polymerase, Control 2 (black line) contains reaction mixture without template. Samples were excited at 690 nm at 60°C.

Figure S23. Agarose gel electrophoresis analysis of purified PCR product (Temp2<sup>PCR</sup>) after amplification of RdRP gene region of cDNA. L: DNA ladder. Purified product (+): all natural dNTPs are present.
Figure S24. Agarose gel electrophoresis analysis after rtPCR amplification of Temp2\textsuperscript{PCR} (5.71 x 10\textsuperscript{7} to 5.71 x 10\textsuperscript{10} DNA copies) with KOD XL polymerase, Prim3\textsuperscript{PCR}, Prim4\textsuperscript{PCR}, dGTP, dCTP, dATP and a mixture of dTTP and dT\textsuperscript{NNIR}TP (95:5). L: DNA ladder. Lanes 1-7: Reaction with 5.71 x 10\textsuperscript{7} to 5.71 x 10\textsuperscript{10} DNA copies. NTC = no template control. A) Gel scanned using laser for GelRed, B) gel scanned using 635 nm laser.
**Figure S25.** Results of real-time PCR with dT^{NNIRTP} using 10-fold serial dilutions (5.71 x 10^7 to 5.71 x 10 DNA copies) of Temp2^{PCR} template. A) Standard curve (5.71 x 10^7 to 5.71 x 10^3 DNA copies), B) melting curves, C) melting peaks. NTC = no template control.
Figure S26. Agarose gel electrophoresis analysis after rtPCR amplification of Temp2$^{\text{PCR}}$ (5.71 x 10$^7$ to 5.71 x 10 DNA copies) with KOD XL polymerase, Prim3$^{\text{PCR}}$, Prim4$^{\text{PCR}}$, dGTP, dCTP, dATP, dTTP and SYBR Green. L: DNA ladder. Lanes 1-7: Reaction with 5.71 x 10$^7$ to 5.71 x 10 DNA copies. NTC = no template control. Gel scanned using laser for GelRed.
Figure S27. Results of real-time PCR with SYBR Green using 10-fold serial dilutions (5.71 x 10^7 to 5.71 x 10 DNA copies) of Temp2<sup>PCR</sup> template. A) Amplification curves, B) standard curve (5.71 x 10^7 to 5.71 x 10^3 DNA copies), C) melting curves, D) melting peaks. NTC = no template control.
Table S5. Table of Ct and melting peak values obtained using different concentration of template Temp2\textsubscript{PCR}

| DNA copies   | Ct\textsuperscript{a} \( (dT_{NNIR}^{TP}) \) | Melting peak \( (°C, dT_{NNIR}^{TP}) \) | Ct \( (\text{SYBR Green}) \) | Melting peak \( (°C, \text{SYBR Green}) \) |
|--------------|-----------------------------------------------|----------------------------------------|----------------------------|------------------------------------------|
| \( 5.71 \times 10^7 \) | 19.33                                        | 80.5                                   | 15.01                       | 83.5                                     |
| \( 5.71 \times 10^6 \) | 24.12                                        | 80.5                                   | 18.89                       | 83.5                                     |
| \( 5.71 \times 10^5 \) | 29.33                                        | 80.0                                   | 22.67                       | 83.5                                     |
| \( 5.71 \times 10^4 \) | 34.20                                        | 80.0                                   | 27.15                       | 83.5                                     |
| \( 5.71 \times 10^3 \) | 38.62                                        | 80.0                                   | 31.00                       | 83.5                                     |
| \( 5.71 \times 10^2 \) | below threshold                              | n.d.                                   | 34.18                       | 83.5                                     |
| 5.71 \times 10 \) | below threshold                              | n.d.                                   | 34.74                       | 83.0                                     |

\textsuperscript{a} Threshold cycle, n.d. = not determined (noise)

Figure S28. Agarose gel electrophoresis analysis of products after reverse transcription rtPCR. L: DNA ladder. Reaction was performed in presence (+) and absence (-) of RNA. A) Gel scanned using laser for GelRed, B) gel scanned using 635 nm laser.
Figure S29. Results of one-step RT-rtPCR using dT^{NIR}TP. A) Melting curves, B) melting peaks. Red lines represent experiment with and gray lines without RNA.
2. Experimental part – chemical synthesis

Materials and methods

All materials were purchased from commercial suppliers. The reactions were monitored by thin-layer chromatography (TLC) using Merck silica gel 60 F254 plates and visualized by UV (254 nm). Purification by column chromatography was performed using silica gel (40–63 µm). Separations of nucleotides were performed using HPLC (Waters modular HPLC system) on a column packed with 10 µm C18 reversed phase (Phenomenex, Luna C18). NMR spectra were measured on a Bruker AVANCE III 500 (1H at 500.0 MHz, 13C at 125.7 MHz and 31P at 202.4 MHz) in DMSO-d6 or CD3OD solutions at 25 °C. Chemical shifts (in ppm, δ scale) were referenced to the residual solvent signal in 1H spectra (δ((CH2)SO(CD3)) = 2.5 ppm, δ((CH2)OD) = 3.31 ppm) or to the solvent signal in 13C spectra (δ((CD3)2SO) = 39.7 ppm, δ(CD3OD) = 49.0 ppm). Coupling constants (J) are given in Hz, chemical shifts in ppm (δ scale). Complete assignment of all NMR signals was achieved by using a combination of H,H-COSY, H,C-HSQC and H,C-HMBC experiments. Mass spectra and high resolution mass spectra were measured by ESI ionization technique and spectra were measured on a LTQ Orbitrap XL spectrometer (Thermo Fisher Scientific). Thymidine (dT) was purchased from SANTIAGO. 6-(Diethylamino)-1,2,3,4-tetrahydroxanthylium perchlorate[1] was synthesized as described in literature. 5-(azidomethyl)-2'-deoxyuridine (dTN3)[2], 5-(azidomethyl)-2'-deoxyuridine 5'-O-triphosphate (dTN3TP)[3] and 4-(methyl(prop-2-yn-1-yl)amino)benzaldehyde[4] were prepared following published procedure with minor modifications.

Synthesis and characterization of fluorescent nucleotide and dNTP

6-(Diethylamino)-4-(4-(methyl(prop-2-yn-1-yl)amino)benzylidene)-1,2,3,4-tetrahydroxanthylium (3)

6-(diethylamino)-1,2,3,4-tetrahydroxanthylium perchlorate (1, 887 mg, 3.46 mmol) and benzaldehyde 2 (1349 mg, 7.79 mmol) were dissolved in acetic acid (60 mL). The reaction mixture
was heated to 110°C and further stirred at 110°C for 2h. Then, the mixture was concentrated on rotavap and the crude product was purified by column chromatography using CH₂Cl₂ to CH₂Cl₂/methanol (20:1) as eluent. The alkyne 3 was isolated as a black solid in 50% yield (712 mg) after purification by column chromatography.

¹H NMR (500.0 MHz, DMSO-d₆): 1.24 (t, 6H, Jvic = 7.1, CH₃CH₂N); 1.83 – 1.89 (m, 2H, H-2); 2.84 (t, 2H, J₁,₂ = 5.9, H-1); 2.92 – 2.97 (m, 2H, H-3); 3.05 (s, 3H, CH₃N); 3.23 (m, 1H, J₄ = 2.2, HC≡C); 3.67 (q, 4H, Jvic = 7.1, CH₃CH₂N); 4.30 (d, 2H, J₄ = 2.2, CH₂N); 6.94 – 6.98 (m, 2H, H-m-phenylene); 7.24 (d, 1H, J₅,₇ = 2.5, H-5); 7.37 (dd, 1H, J₇,₈ = 9.4, J₇,₅ = 2.5, H-7); 7.62 – 7.67 (m, 2H, H-0-phenylene); 7.84 (d, 1H, J₈,₇ = 9.4, H-8); 8.07 (s, 1H, CH=C₄); 8.41 (s, 1H, H-9).

¹³C NMR (125.7 MHz, DMSO-d₆): 12.76 (CH₃CH₂N); 21.21 (CH₂-2); 27.12 (CH₂-1); 27.24 (CH₂-3); 38.15 (CH₃N); 41.16 (CH₂N); 45.52 (CH₃CH₂N); 74.97 (HC≡C); 79.81 (HC=C); 95.64 (CH-5); 113.34 (CH-m-phenylene); 117.47 (CH-7); 117.72 (C-8a); 123.18 (C-9a); 123.60 (C-4); 124.37 (C-i-phenylene); 131.76 (CH-8); 133.85 (CH-o-phenylene); 138.64 (CH=C₄); 147.54 (CH-9); 150.54 (C-p-phenylene); 155.28 (C-6); 158.37 (C-4b); 164.03 (C-4a).

HRMS (ESI⁺): calculated for C₂₈H₃₁ON₂: 411.24309; found 411.24333.

6-(Diethylamino)-4-((E)-4-(((1-((1-(2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl)(methyl)amino)benzylidene)-1,2,3,4-tetrahydroxanthylum (dTNNIR)

dTN³ (140 mg, 0.494 mmol), CuSO₄.5H₂O (24.7 mg, 0.099 mmol), sodium ascorbate (39.23 mg, 0.198 mmol) and alkyne 3 (233.8 mg, 0.568 mmol) were placed in a round bottom flask and dissolved in mixture of H₂O/THF (1:3, 8 mL). The reaction mixture was stirred at room temperature until complete consumption of starting material (dTNN³) was observed according to
TLC (CH₂Cl₂/methanol 10:1 as eluent). After evaporation the reaction mixture was purified by column chromatography using CH₂Cl₂ to CH₂Cl₂/methanol (10:1) as eluent. The nucleoside 5'TNIR was isolated in 55% yield (188.7 mg) as a black solid after column chromatography purification.

1H NMR (500.0 MHz, DMSO-d₆): 1.24 (t, 6H, Jvic = 7.1, CH₃CH₂N); 1.82 – 1.89 (m, 2H, H-2''); 2.08 (ddd, 1H, J_gem = 13.3, J₂b,₁' = 7.2, J₂b,₁' = 6.0, H-2'b); H-2''); 2.13 (ddd, 1H, J_gem = 13.3, J₂a,₁' = 6.2, J₂a,₁' = 3.6, H-2'a); 2.83 (t, 2H, J₁₁,₂'' = 6.0, H-1''); 2.90 – 2.95 (m, 2H, H-3''); 3.13 (s, 3H, CH₃N); 3.53 (ddd, 1H, J_gem = 11.9, J₅b,OH = 5.3, J₅b,₄' = 4.3, H-5'b); 3.58 (ddd, 1H, J_gem = 11.9, J₅a,OH = 5.3, J₅a,₄' = 4.0, H-5'a); 3.66 (bq, 4H, Jvic = 7.1, CH₃CH₂N); 3.78 (ddd, 1H, J₄',₅' = 4.3, 4.0, J₄',₃' = 3.7, H-4''); 4.22 (ddd, 1H, J₃',₂'' = 6.0, 3.6, J₃',OH = 4.3, J₃',₂'' = 3.7, H-3''); 4.70 (s, 2H, CH₂NMe); 5.04 (t, 1H, J_OH,₅' = 5.3, OH-5'); 5.16 (s, 2H, CH₂N); 5.28 (d, 1H, J_OH,₃' = 4.3, OH-3'); 6.13 (d, 1H, J₁',₂'' = 7.2, 6.2, H-1'); 6.95 – 7.00 (m, 2H, H-α-phenylene); 7.22 (d, 1H, J₅',₇'' = 2.3, H-5'''); 7.34 (dd, 1H, J₇''',₈'' = 9.4, J₇''',₈'' = 2.3, H-7'''); 7.59 – 7.63 (m, 2H, H-m-phenylene); 7.81 (d, 1H, J₈''',₇'' = 9.4, H-8'''); 8.00 (s, 1H, H-5-triazole); 8.06 (s, 1H, CH=); 8.12 (s, 1H, H-6); 8.36 (s, 1H, H-9'''); 11.58 (s, 1H, NH).

13C NMR (125.7 MHz, DMSO-d₆): 12.77 (CH₃CH₂N); 21.19 (CH₂-2'''); 27.17 (CH₂-1''); 27.31 (CH₂-3'''); 38.46 (CH₃N); 39.94 (CH₂-2''); 45.45 (CH₃CH₂N); 46.36 (CH₂N); 46.74 (CH₂NMe); 61.43 (CH₂-5''); 70.42 (CH-3'''); 84.63 (CH-1''); 87.74 (CH-4''); 95.67 (CH-5''); 107.93 (C-5); 112.64 (CH-α-phenylene); 117.71 (CH-7''); 117.31 (C-8''a); 122.70 (C-4'''); 123.13 (C-9''a); 123.30 (CH-5-triazole); 123.57 (C-α-phenylene); 131.62 (CH-8''); 134.28 (CH-m-phenylene); 139.35 (CH=); 141.20 (CH-6); 143.36 (C-4-triazole); 147.07 (CH-9''); 150.40 (C-2); 150.87 (C-α-phenylene); 155.04 (C-6''); 158.24 (C-4''b); 162.81 (C-4); 164.37 (C-4''a).

HRMS (ESI⁺): calculated for C₃₈H₄₄O₆N₇: 694.3353; found 694.3341.
((2R,3S,5R)-5-((4-(((E)-6-(diethylamino)-2,3-dihydroxanthylum-4(1H)-ylidene)methyl)phenyl)(methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)methyl)-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl triphosphate (dT\textsuperscript{NNIR}TP)

dT\textsuperscript{N3}TP (Et\textsubscript{3}NH\textsuperscript{+} salt; 9.45 mg, 11.4 µmol), CuSO\textsubscript{4}.5H\textsubscript{2}O (1.1 mg, 6.9 µmol), sodium ascorbate (5 mg, 25.2 µmol) and alkyne 3 (13.8 mg, 33.5 µmol) were placed in a round bottom flask and dissolved in mixture of H\textsubscript{2}O/THF (1:3, 1.6 mL). The reaction mixture was stirred at room temperature until complete consumption of starting material (dT\textsuperscript{N3}TP) was observed according to TLC (reverse phase; methanol/water 1:1 as eluent). After evaporation the reaction mixture was purified by HPLC with the use of linear gradient of methanol (5-100%) in water. The nucleotide dT\textsuperscript{NNIR}TP was isolated in 25% yield (3.65 mg) as a dark blue solid after HPLC purification.

Nucleotide was subsequently converted to sodium salt (Dowex 50WX8 in Na\textsuperscript{+} cycle).

\(^{1}\text{H} NMR (500.0 MHz, CD\textsubscript{3}OD): 1.29 (bt, 27H, J\text{vic} = 6.7 \text{CH}_3\text{CH}_2\text{NH}^+); 1.33 (t, 6H, J\text{vic} = 7.1, \text{CH}_3\text{CH}_2\text{N}); 1.90 – 1.97 (bm, 2H, H-2\textsuperscript{″}); 2.26 – 2.35 (bm, 2H, H-2\textsuperscript{′}); 2.80 – 2.86 (bm, 2H, H-1\textsuperscript{″}); 2.92 – 2.97 (m, 2H, H-3\textsuperscript{″}); 3.14 – 3.21 (bm, 21H, CH\textsubscript{3}N, CH\textsubscript{3}CH\textsubscript{2}\text{NH}^+); 3.69 (bic, 4H, J\text{vic} = 7.1, CH\textsubscript{3}CH\textsubscript{2}N); 4.01 (bm, 1H, H-4\textsuperscript{′}); 4.27 – 4.36 (bm, 2H, H-5\textsuperscript{′}); 4.69 (bm, 1H, H-3\textsuperscript{′}); 4.73 (s, 2H, CH\textsubscript{2}NMe); 5.43 (s, 2H, CH\textsubscript{2}N); 6.25 (t, 1H, J\text{H},\text{CH\textsubscript{2}} = 6.2, H-1\textsuperscript{′}); 6.84 – 6.90 (m, 2H, H-o-phenylene); 7.11 (d, 1H, J\text{H} = 2.0, H-5\textsuperscript{″}); 7.22 (bd, 1H, J\text{H} = 9.0, H-7\textsuperscript{″}); 7.51 – 7.58 (m, 2H, H-m-phenylene); 7.60 (bd, 1H, J\text{H} = 9.0, H-8\textsuperscript{″}); 7.99 (s, 2H, H-9\textsuperscript{″}, CH=); 8.27 (H-5-triazole); 8.49 (s, 1H, H-6).

\(^{13}\text{C} NMR (125.7 MHz, CD\textsubscript{3}OD): 9.08 (\text{CH}_3\text{CH}_2\text{NH}^+); 12.89 (\text{CH}_3\text{CH}_2\text{N}); 22.48 (CH-2\textsuperscript{″}); 28.65, 28.73 (CH\textsubscript{2}-1\textsuperscript{″},3\textsuperscript{″}); 39.20 (CH\textsubscript{3}N); 41.20 (CH-2\textsuperscript{′}); 46.83 (CH\textsubscript{3}CH\textsubscript{2}N); 47.32 (CH\textsubscript{3}CH\textsubscript{2}\text{NH}^+); 47.80 (CH\textsubscript{2}N); 48.03 (CH\textsubscript{2}NMe); 66.30 (d, J\text{C-P} = 3.8, CH\textsubscript{2}-5\textsuperscript{′}); 71.53 (CH-3\textsuperscript{′}); 86.66 (CH-1\textsuperscript{′}); 87.59 (d, J\text{C-P} = 8.5, CH-4\textsuperscript{′}); 96.68 (CH-5\textsuperscript{″}); 109.77 (C-5); 113.76 (CH-o-phenylene); 117.65 (CH-7\textsuperscript{″}); 118.53 (C-8\textsuperscript{″}a); 123.56 (C-4\textsuperscript{″}); 124.53 (C-9\textsuperscript{″}a); 124.96 (CH-5-triazole); 125.32 (C-p-phenylene);
132.50 (CH-8”); 135.69 (CH-m-phenylene); 141.72 (CH=); 143.19 (CH-6); 145.10 (C-4-triazole); 147.63 (CH-9”); 152.06 (C-2); 152.50 (C-i-phenylene); 156.56 (C-6”); 159.83 (C-4”b); 164.71 (C-4); 166.53 (C-4”a).

$^{31}$P{¹H} NMR (202.4 MHz, CD$_3$OD): -21.61 (bt, $J$ = 20.9, $P_\beta$); -9.73 (d, $J$ = 20.9, $P_\alpha$); -8.62 (d, $J$ = 20.9, $P_\gamma$).

HRMS (ESI): calculated for (C$_{38}$H$_{44}$O$_{15}$N$_{7}$P$_{3}$/2: 465.60596; found 465.60571.

3. Biochemistry and Cell experiments

Materials and methods
Oligonucleotides used in presented work were purchased from Generi Biotech. Enzyme KOD XL DNA polymerase and corresponding reaction buffer were obtained from Merck Millipore. Natural nucleoside triphosphates (dCTP, dATP, dGTP, dTTP) and histone human recombinant protein (H2A) were purchased from New England Biolabs. Streptavidin coated magnetic beads were obtained from Roche. Isolated RNA and corresponding cDNA were provided by virology department of IOCB Prague. All solutions for biochemistry experiments were prepared in Milli-Q water and in case of RNA experiments RNase free water was used. Stop solution contained 80% (v/v) formamide, 20 mM EDTA, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol in water. Concentration of DNA solutions were calculated using A260 values measured on a Nanodrop 1000 Spectrophotometer (Thermo Scientific) and values obtained with OligoCalc.[5] Mass spectra of oligonucleotides were measured by MALDI-TOF MS, on UltrafleXtreme MALDITOF/TOF mass spectrometer (Bruker Daltonics, Germany), with 1 kHz smartbeam II laser. All gels were analysed by fluorescence imaging using Typhoon FLA 9500 (GE Healthcare) or using transilluminator equipped with GBox iChemi-XRQ Bio imaging system (Syngene, Life Technologies).

Enzymatic synthesis of modified DNA bearing one NNIR modification by primer extension for analysis
The reaction mixture (20 μl) contained FAM labelled primer Prim1$^{PEX}$-FAM (for sequence see Table S3; 3 μM, 1 μL), template Temp2$^{PEX}$ (for sequence see Table S3; 3 μM, 1.5 μL), KOD XL
DNA polymerase (0.25 U/µL, 0.25 µL), natural dGTP (4 mM, 0.6 µL), either natural dTTP (4 mM, 0.3 µL) or dT^{NNIRTP} (4 mM, 0.3 µL) in corresponding reaction buffer (10x, 2 µL) supplied by the manufacturer. The reaction mixture was incubated for 60 min at 60°C in thermal cycler. The reaction was stopped by the addition of PAGE stop solution (20 uL) and the reaction mixture was denatured at 95°C for 5 min and analyzed using 12.5% denaturing PAGE. The gel was visualized by a fluorescent scanner (PAGE gel is shown in Figure S3A).

**Enzymatic synthesis of modified DNA bearing one azido modification by primer extension for analysis**

The reaction mixture (20 µl) contained FAM labelled primer Prim1^{PEX}-FAM (for sequence see Table S3; 3 µM, 1 µL), template Temp2^{PEX} (for sequence see Table S3; 3 µM, 1.5 µL), KOD XL DNA polymerase (0.25 U/µL, 0.125 µL), natural dGTP (4 mM, 0.3 µL), either natural dTTP (4 mM, 0.3 µL) or dT^{NNIRTP} (4 mM, 0.3 µL) in corresponding reaction buffer (10x, 2 µL) supplied by the manufacturer. The reaction mixture was incubated for 30 min at 60°C in a thermal cycler. The reaction was stopped by the addition of PAGE stop solution (20 uL) and the reaction mixture was denatured for 5 min at 95°C and analyzed using 12.5% denaturing PAGE. The gel was visualized by a fluorescent scanner (PAGE gels are shown in Figure S3B).

**Enzymatic synthesis of modified DNA bearing four NNIR modifications by primer extension for analysis**

The reaction mixture (20 µl) contained FAM labelled primer Prim1^{PEX}-FAM (for sequence see Table S3; 3 µM, 1 µL), template Temp3^{PEX} (for sequence see Table S3; 3 µM, 1.5 µL), KOD XL DNA polymerase (0.25 U/µL, 0.3 µL), natural dNTPs (dATP, dGTP, dCTP, 4 mM each, 0.7 µL), either natural dTTP (4 mM, 0.7 µL) or dT^{NNIRTP} (4 mM, 0.7 µL) in corresponding reaction buffer (10x, 2 µL) supplied by the manufacturer. The reaction mixture was incubated for 60 min at 60°C in a thermal cycler. The reaction was stopped by the addition of PAGE stop solution (20 uL) and the reaction mixture was denatured for 5 min at 95°C and analyzed using 12.5% denaturing PAGE. The gel was visualized by a fluorescent scanner (PAGE gel is shown in Figure S3A).

**Enzymatic synthesis of modified DNA bearing four azido modifications by primer extension for analysis**
The reaction mixture (20 μl) contained FAM labelled primer Prim1\textsuperscript{PEX}-FAM (for sequence see Table S3; 3 μM, 1 μL), template Temp3\textsuperscript{PEX} (for sequence see Table S3; 3 μM, 1.5 μL) KOD XL DNA polymerase (0.25 U/μL, 0.3 μL), natural dNTPs (dATP, dGTP, dCTP, 4 mM each, 0.7 μL), either natural dTTP (4 mM, 0.7 μL) or dT\textsuperscript{NNIR}TP (4 mM, 0.7 μL) in corresponding reaction buffer (10x, 2 μL) supplied by the manufacturer. The reaction mixture was incubated for 30 min at 60°C in a thermal cycler. The reaction was stopped by the addition of PAGE stop solution (20 μL) and the reaction mixture was denatured for 5 min at 95°C and analyzed using 12.5% denaturing PAGE. The gel was visualized by a fluorescent scanner (PAGE gel is shown in Figure S3B).

**Single nucleotide incorporation of dT\textsuperscript{NNIR}TP and primer extension (SNI-PEX) for analysis**

The reaction mixture (20 μl) contained FAM labelled primer Prim1\textsuperscript{PEX}-FAM (for sequence see Table S3; 3 μM, 1 μL), template Temp4\textsuperscript{PEX} (for sequence see Table S3; 3 μM, 1.5 μL), KOD XL DNA polymerase (0.25 U/μL, 0.125 μL), either natural dTTP (0.04 mM, 0.5 μL) or dT\textsuperscript{NNIR}TP (0.04 mM, 0.5 μL) in corresponding reaction buffer (10x, 2 μL) supplied by the manufacturer. The reaction mixture was incubated for 10 min at 60°C in a thermal cycler. For the subsequent extension, a mixture of all natural dNTPs (8 mM, 0.5 μL) was added and the reaction mixture was incubated for further 20 min at 60°C. The reaction was stopped by the addition of PAGE stop solution (20 μL) and the reaction mixture was denatured for 5 min at 95°C and analyzed using 12.5% denaturing PAGE. The gel was visualized by a fluorescent scanner (For scheme of reaction see Scheme S1, PAGE gel is shown in Figure S4).

**Preparation of modified oligonucleotide ON19_1T\textsuperscript{NNIR} by primer extension and subsequent magnetic separation**

The reaction mixture (50 μL) contained primer (for sequence see Table S3; Prim1\textsuperscript{PEX}, 100 μM, 1.7 μL), biotinylated template (for sequence see Table S3; Temp2\textsuperscript{PEX}-bio, 100 μM, 1.7 μL), natural dGTP (4 mM, 5 μL), dT\textsuperscript{NNIR}TP (4 mM, 2.7 μL), KOD XL DNA polymerase (2.5 U/μL, 0.9 μL) in corresponding reaction buffer (10x, 5.1 μL) supplied by the manufacturer. The reaction mixture was incubated for 60 min at 60°C in a thermal cycler. The reaction was stopped by cooling at 4°C. Streptavidin magnetic beads (50 μl, Roche) were washed with binding buffer TEN 100 (3 × 300 μl, 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). Then PEX solution (50 μL) and binding buffer (50 μl) were added to the magnetic beads. The mixture was incubated for 30 min at 15°C
and 1400 rpm. Then the magnetic beads were separated and washed with washing buffer TEN 500 (3 × 200 μl, 10 mM Tris, 1 mM EDTA, 1 M NaCl, pH 7.5) and with milli-Q water (4 × 200 μl). Then 50 μl of milli-Q water was added and the sample was denatured for 2 min at 70°C and 900 rpm. The solution containing modified ssDNA was quickly transferred into clean Eppendorf and analysed by MALDI-TOF mass spectrometry (Table S4, for copy of mass spectra see Figure S30).

Preparation of modified oligonucleotide ON19_1T³N³ by primer extension and subsequent magnetic separation

The reaction mixture (200 μL) contained primer (for sequence see Table S3; Prim₁³PEX, 100 μM, 10 μL), template (for sequence see Table S3; Temp₂³PEX-bio, 100 μM, 10 μL), dGTP (4 mM, 5 μL), dT³N³TP (4 mM, 5 μL), KOD XL DNA polymerase (2.5 U/μL, 1.14 μL) in corresponding reaction buffer (10x, 25 μL) supplied by the manufacturer. The reaction mixture was incubated for 30 min at 60°C in a thermal cycler. The reaction was stopped by cooling at 4°C. Streptavidin magnetic beads (100 μl, Roche) were washed with binding buffer TEN 100 (3 × 600 μl, 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). Then PEX solution (200 μL) and binding buffer (200 μl) were added to the magnetic beads. The mixture was incubated for 30 min at 15°C and 1400 rpm. Then the magnetic beads were separated and washed with washing buffer TEN 500 (3 × 400 μl, 10 mM Tris, 1 mM EDTA, 1 M NaCl, pH 7.5) and with milli-Q water (4 × 400 μl). Then 100 μl of milli-Q water was added and the sample was denatured for 2 min at 70°C and 900 rpm. The solution containing modified ssDNA was quickly transferred into clean Eppendorf and analysed by MALDI-TOF mass spectrometry (Table S4, for copy of mass spectra see Figure S31). Product was used for click reactions.

Preparation of modified oligonucleotide ON31_4T³N³ by primer extension and subsequent magnetic separation

The reaction mixture (200 μL) contained primer (for sequence see Table S3; Prim₁³PEX, 100 μM, 10 μL), template (for sequence see Table S3; Temp₃³PEX-bio, 100 μM, 10 μL), dNTPs (dATP, dGTP, dCTP, 4 mM each, 5 μL), dT³N³TP (4 mM, 5 μL), KOD XL DNA polymerase (2.5 U/μL, 1.14 μL) in corresponding reaction buffer (10×, 25 μL) supplied by the manufacturer. The reaction mixture was incubated for 30 min at 60°C in a thermal cycler. The reaction was stopped by cooling at 4°C. Streptavidin magnetic beads (100 μl, Roche) were washed with binding buffer TEN 100
(3 × 600 μl, 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). Then PEX solution (200 μL) and binding buffer (200 μl) were added to the magnetic beads. The mixture was incubated for 30 min at 15°C and 1400 rpm. Then the magnetic beads were separated and washed with washing buffer TEN 500 (3 × 400 μl, 10 mM Tris, 1 mM EDTA, 1 M NaCl, pH 7.5) and with milli-Q water (4 × 400 μl). Then 100 μl of milli-Q water was added and the sample was denatured for 2 min at 70°C and 900 rpm. The solution containing modified ssDNA was quickly transferred into clean Eppendorf and analysed by MALDI-TOF mass spectrometry (Table S4, for copy of mass spectra see Figure S32). Product was used for click reactions.

Preparation of modified oligonucleotides ON16_1T^{NNIR} and ON31_1T^{NNIR} by SNI or SNI-PEX and subsequent magnetic separation

The reaction mixture (100 μl) contained primer Prim1^{PEX} (for sequence see Table S3; 100 μM, 1 μL), template Temp4^{PEX}-bio (for sequence see Table S3; 100 μM, 1.2 μL), KOD XL DNA polymerase (0.25 U/μL, 0.5 μL), dT^{NNIRTP} (0.04 mM, 20 μL) in corresponding reaction buffer (10x, 10 μL) supplied by the manufacturer. The reaction mixture was incubated for 10 min at 60°C in a thermal cycler. For the subsequent extension, a mixture of all natural dNTPs (24 mM, 2 μL) and additional KOD XL DNA polymerase (0.25 U/μL, 0.2 μL) were added and the reaction mixture was incubated for further 60 min at 60°C. Reactions were stopped by cooling at 4°C. Streptavidin magnetic beads (100 μl, Roche) were washed with binding buffer TEN 100 (3 × 450 μl, 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). Then PEX solution and binding buffer (100 μl) were added to the magnetic beads. The mixture was incubated for 30 min at 15°C and 1400 rpm. Then the magnetic beads were separated and washed with washing buffer TEN 500 (3 × 350 μl, 10 mM Tris, 1 mM EDTA, 1 M NaCl, pH 7.5) and with milli-Q water (4 × 350 μl). Then 100 μl of milli-Q water was added and the sample was denatured for 2 min at 70°C and 900 rpm. The solution containing modified ssDNA was quickly transferred into clean Eppendorf and analysed by MALDI-TOF mass spectrometry (Table S4, for copy of mass spectra see Figure S33 and S34).

Preparation of modified dsDNA bearing one NNIR modification by primer extension (DNA19_1T^{NNIR})

The reaction mixture (125 μL) containing primer (for sequence see Table S3; Prim1^{PEX}, 100 μM, 5 μL), template (for sequence see Table S3; Temp2^{PEX}, 100 μM, 5 μL), dGTP (4 mM, 2.5 μL),
**Preparation of modified ssDNA bearing one NNIR modification using lambda exonuclease digestion (ON19_1TNNIR)**

The reaction mixture (100 µL) containing modified dsDNA (prepared using Temp2PEX-P as described above; 0.2 nmol), lambda exonuclease buffer 10x (4 uL) and lambda exonuclease enzyme (10 U/µL, 3 µL) was incubated for 60 min 37°C in a thermal cycler. The reaction was stopped by cooling at 4°C. The modified ssDNA was purified using spin columns (QIAquick® Nucleotide Removal Kit, QIAGEN) and eluted by milli-Q water. Product was analysed by MALDI-TOF mass spectrometry (Table S4, for copy of mass spectra see Figure S38). Prepared ssDNA was used for fluorescence measurements.

**Enzymatic incorporation of dTNNIRTP and dTNNJTP by polymerase chain reaction (PCR)**

The reaction mixture (20 µL) contained primer (for sequences see Table S3; Prim1PCR and Prim2PCR, 10 µM, 4 µL of each), template (Temp1PCR, 10 µM, 0.5 µL), natural dNTPs (dATP, dGTP, dCTP, 0.4 mM each, 1.5 µL) and either dTTP (0.4 mM, 1.5 µL), dTNNIRTP (0.4 mM, 1.5 µL) or mixture of dTNNIRTP with natural dTTP (0-95%), KOD XL DNA polymerase (2.5 U/µL, 1 µL) and corresponding reaction buffer (10×, 2 µL) supplied by the manufacturer. After the initial denaturation for 3 min at 94°C, 30 PCR cycles were run under the following conditions: denaturation for 1 min at 94°C, annealing for 1 min at 51°C, extension for 2 min at 72°C. These PCR process was terminated with a final extension step for 5 min at 72°C. The reaction was stopped by cooling to 4°C. The PCR products were analyzed by agarose gel electrophoresis in 2% agarose gel stained with GelRed™ (Biotium, agarose gels are shown in Figure S7). The PCR products (Figure S7B, lane 6 and 7) were purified using spin columns.
(QIAquick® Nucleotide Removal Kit, QIAGEN) and eluted by milli-Q water. Fluorescence spectra were recorded to prove presence of fluorophore (Figure S8).

**CuAAC reaction with azido modified PEX products**

To ssDNA (2 nmol) in 30 µL H₂O were added 3 µL of DMSO/tBuOH 3:1 mixture, DMSO/tBuOH 3:1 solution of alkyne 3 (50 mM, 2 µL) and a freshly prepared solution of TBTA (50 mM) and CuBr (25 mM) 5:1 (5 µL) in DMSO/tBuOH 3:1. The reaction mixture was incubated at 37°C and 800 rpm overnight. Then the reaction mixtures were freeze-dried, re-suspended in 50 µL water and purified by QIAquick Nucleotide Removal kit (QIAGEN). Fluorescence spectra were recorded in a 100 µL quartz cuvette at 25°C (Figure S10). Control experiments were performed using non-modified ssDNA following the same procedure. Samples of single-stranded DNA for MALDI-TOF MS analysis were prepared following the same procedure (for copy of mass spectra see Figure S35 and S36). To analyze the product of click reaction using PAGE, the reaction was performed as described above using ssDNA (0.5 nmol in 30 µL H₂O) after PEX with FAM labelled primer and DMSO/tBuOH 3:1 solution of alkyne 3 (50 mM, 1 µL). The click reaction products were purified with QIAquick® Nucleotide Removal kit (QIAGEN). The products were analysed using 20% PAGE gel and visualized by a fluorescent scanner (PAGE gel is shown in Figure S9).

**Preparation of template Temp2<sup>PCR</sup> by PCR**

The reaction mixture (20 µL) contained primer (for sequences see Table S3; Prim<sub>3<sup>PCR</sup></sub> and Prim<sub>4<sup>PCR</sup></sub>, 10 µM, 0.8 µL of each), cDNA (1 µL), all natural dNTPs (0.4 mM, 1.5 µL), KOD XL DNA polymerase (2.5 U/µL, 0.5 µL) and corresponding reaction buffer (10x, 2 µL). After the initial denaturation for 3 min at 94°C, 40 PCR cycles were run under the following conditions: denaturation for 20 sec at 94°C, annealing/extension for 10 sec at 64°C. The reaction was stopped by cooling to 4°C. PCR was performed 4 times and products were purified by nucleospin® Gel and PCR Clean-up columns (MACHEREY-NAGEL). Products were subsequently loaded on 2% agarose gel (Figure S23) and then calculated concentration using Nanodrop and OligoCalc.
Real-time PCR using \(dT^{NNIRTP}\)

The reaction mixture (20 µL) contained primer (for sequences see Table S3; Prim\(^3\)PCR and Prim\(^4\)PCR, 10 µM, 0.8 µL of each), template (Temp\(^2\)PCR, 10-fold serial dilutions, 100 pM – 100 aM, 1 µL), natural dNTPs (dATP, dGTP, dCTP, 0.4 mM, 1.5 µL), mixture of dTTP: \(dT^{NNIRTP}\) 95:5 (dTTP, 0.4 mM, 1.43 µL, dT\(^{NNIRTP}\), 0.04 mM, 0.75 µL), KOD XL DNA polymerase (2.5 U/µL, 0.5 µL) and corresponding reaction buffer (10x, 2 µL). After the initial denaturation for 3 min at 94°C, 40 PCR cycles were run under the following conditions: denaturation for 20 sec at 94°C, annealing/extension for 10 sec at 64°C. Samples were excited at 672-684 nm and emission was detected 705-730 nm (as Quasar 705, dye predefined in machine). Then, melting curve analysis was performed under the following conditions: 10 sec at 94°C, 5 sec at 65°C, then followed by a slow increase from 65°C to 95°C with a speed of 0.5°C per second. The reaction was stopped by cooling to 4 °C. Standard curve (established from results obtained using 5.71 x 10\(^7\) to 5.71 x 10\(^3\) DNA copies) was determined to be \(y = -4.8678x + 57.138\) (Figure S25A). The efficiency was calculated to be 60.5% and the correlation coefficient (\(R^2\)) was 0.9993. Melting curve analysis revealed a single peak for product (Figure S25B,C). The PCR products were analyzed by agarose gel electrophoresis in 2% agarose gel stained with GelRed™ (agarose gels are shown in Figure S24). Table S5 shows Ct and melting peak values obtained using different concentration of template Temp\(^2\)PCR.

Real-time PCR using SYBR Green

The reaction mixture (20 µL) contained primer (for sequences see Table S3; Prim\(^3\)PCR and Prim\(^4\)PCR, 10 µM, 0.8 µL of each), template (Temp\(^2\)PCR, 10-fold serial dilutions, 100 pM – 100 aM, 1 µL), natural dNTPs (dATP, dGTP, dCTP, dTTP, 0.4 mM, 1.5 µL), SYBR Green (2 uL of SYBR Green I 10,000x concentrate in DMSO was diluted to 1000 uL water to prepare working solution of the dye, 1 uL of working solution was used for reaction), KOD XL DNA polymerase (2.5 U/µL, 0.5 µL) and corresponding reaction buffer (10x, 2 µL). After the initial denaturation for 3 min at 94°C, 40 PCR cycles were run under the following conditions: denaturation for 20 sec at 94°C, annealing/extension for 10 sec at 64°C. Then, melting curve analysis was performed under the following conditions: 10 sec at 94°C, 5 sec at 65°C, then followed by a slow increase from 65°C to 95°C with a speed of 0.5°C per second. The reaction was stopped by cooling to 4 °C.
Standard curve (established from results obtained using $5.71 \times 10^7$ to $5.71 \times 10^3$ DNA copies) was determined to be $y = -4.021x + 46.082$ (Figure S27B). The efficiency was calculated to be 77% and the correlation coefficient ($R^2$) was 0.9993. Melting curve analysis revealed a single peak for product (Figure S27C,D). The PCR products were analyzed by agarose gel electrophoresis in 2% agarose gel stained with GelRed™ (agarose gels are shown in Figure S26). Table S5 shows Ct and melting peak values obtained using different concentration of template Temp2\textsuperscript{PCR}.

**Reverse transcription real-time PCR using dT\textsuperscript{NNRTP}**

The reaction mixture (20 µL) contained primer (for sequences see Table S3; Prim\textsuperscript{3PCR} and Prim\textsuperscript{4PCR}, 10 µM, 0.8 µL of each), RNA (4 µL), natural dNTPs (dATP, dGTP, dCTP, 0.4 mM, 1.5 µL), mixture of dTTP:dT\textsuperscript{NNRTP} 95:5 (dTTP, 0.4 mM, 1.43 uL, dT\textsuperscript{NNRTP}, 0.04 mM, 0.75 uL), KOD XL DNA polymerase (2.5 U/µL, 0.5 µL) and corresponding reaction buffer (10x, 2 µL), SuperScript III Reverse Transcriptase (200 U/µL, 0.5 µL), DTT (0.1 M, 2 uL), RNase Inhibitor (1 uL). First, reverse transcription was run for 30 min at 50°C followed by denaturation for 3 min at 94°C and then 40 PCR cycles were run under the following conditions: denaturation for 20 sec at 94°C, annealing/extension for 10 sec at 64°C. Samples were excited at 672-684 nm and emission was detected 705-730 nm (as Quasar 705 dye predefined in machine). Then, melting curve analysis was performed under the following conditions: 10 sec at 94°C, 5 sec at 65°C, then followed by a slow increase from 65°C to 95°C with a speed of 0.5°C per second. The reaction was stopped by cooling to 4°C. Melting curve analysis revealed a single peak (at 80.5°C) for product (Figure S29). The PCR products were analyzed by agarose gel electrophoresis in 2% agarose gel stained with GelRed™ (agarose gels are shown in Figure S28).

**Transport of dT\textsuperscript{NNRTP} into U-2 OS cells using SNTT**

U-2 OS cells (ATCC HTB-96) were cultured in a 96-well plate in complete medium [(DMEM high glucose 4.5 g/l (Sigma-Aldrich, D5796) supplemented with 10% FBS (Capricorn, cat.no. FBS-HI-12A) and 1x GlutaMAX-I (Gibco, cat.no. 35050-038)] in an incubator (37°C, 5% CO\textsubscript{2}) to the confluency of about 70%. The medium was aspirated off and the cells were washed with pre-warmed (37°C) tricine buffer. Then the cells were treated with a mixture (40 µL) of dT\textsuperscript{NNRTP} (10 µM) and SNTT 1 (10 µM) in tricine buffer (37°C, 5 min). The mixture was then removed and complete medium (80 µL) was added to the cells. The cell culture was then incubated in complete
medium (37°C, 5% CO₂) for a further 60 min. Prior to microscopy experiments, the medium was exchanged for L-15 medium (w/o serum) and the plate with live cells was mounted onto microscope (Leica SP8). The samples were irradiated with a laser beam at 633 nm, the detector (PMT) was set to 660-700 nm. Negative control experiment was carried out with untreated cells to check for autofluorescence under the same settings.

4. Absorption and Fluorescence measurements

Materials and methods
Chemicals and spectroscopy grade solvents were purchased from Sigma-Aldrich, Alfa Aesar, Acros Organics and used as supplied. UV-visible spectra were measured on a Cary 100 UV-Vis spectrometer (Agilent Technologies). Fluorescence spectra were measured on a Fluoromax 4 spectrofluorimeter (HORIBA Scientific). Melting curves of DNA were measured on a Cary 100 UV-Vis spectrometer in 1 cm path-length quartz cells, by monitoring the absorbance at λ =260 nm while slowly increasing the temperature. All measurements were duplicated or triplicated and values were averaged.

Determination of absorption coefficients
Absorption coefficients were measured using 1 mL quartz cuvettes. The absorption coefficients were calculated using the follow equation \( A = \varepsilon \times c \times l \), where \( A \) is the absorbance of the sample, \( \varepsilon \) is absorption coefficients, \( c \) is the exact concentration of the sample and \( l \) is the length of the path that the light travels through the cuvette.

Determination of fluorescence quantum yields
Relative determination of the fluorescence quantum yields (\( \Phi \)) was performed using indocyanine green (ICQ) in DMSO (\( \Phi = 0.13 \) at 25°C) as reference. The solvents used were either of HPLC or spectroscopy grade. The absorbance of sample solutions at the excitation wavelength were kept below 0.08 to avoid inner filter effects. The quantum yields were calculated using the following equation \( \Phi_f = \Phi_i \times I_f / I_x \times A_x / A_f \times (n_x / n_f)^2 \), where \( \Phi_f \) is the quantum yield, \( I \) is the integrated area under
the fluorescence spectra, \( A \) is the absorbance, \( n \) is the refractive index of the solvent, \( x \) and \( r \) stand for the sample and reference, respectively.

**UV melting curves of DNA19_1T and DNA19_1T^{\text{NNIR}}**

All solutions of DNA (1 nmol) were prepared in 25 mM phosphate buffered saline (1 mL). Melting curve analysis was performed by a slow increase of temperature from 10°C to 90°C with a speed of 1°C per minute. Then samples were cooled to 10°C with a speed of 1°C per minute. The process of denaturation and annealing was repeated three times. Figure S5 shows UV melting curves.

**Kinetics of lambda exonuclease digestion**

Digestion was performed in 100 \( \mu \)L quartz cuvette at 37°C in fluorimeter holder. The initial solution (100 \( \mu \)L) contained DNA19_1T^{\text{NNIR-P}} (0.2 nmol) and buffer (4 \( \mu \)L). The mixture was equilibrated in fluorimeter holder for 3 min at 37°C and then fluorescence spectrum was recorded. Then lambda exonuclease (3 \( \mu \)L) was added and fluorescence spectra were recorded in time intervals (0-75 min). For scheme of digestion see Scheme S3, fluorescence spectra are shown in Figure S14

**Hybridization and digestion studies**

A series of 3 solutions (final volume of each solution was 100 \( \mu \)L) were prepared by mixing ON19_1T^{\text{NNIR}} (104.8 pmol), lambda exonuclease buffer (10x, 1.5 \( \mu \)L) and either just diluting with milli-Q water (solution 1) or adding phosphorylated complementary strand (for sequence see Table S3; Temp2^{\text{PEX-P}}, 100 \( \mu \)M, 1.15 \( \mu \)L, solution 2) or adding phosphorylated complementary strand (Temp2^{\text{PEX-P}}, 100 \( \mu \)M, 1.15 \( \mu \)L) and lambda exonuclease enzyme (10 U/\( \mu \)L, 1.2 \( \mu \)L, solution 3). All solutions were heated to 95°C for 3 min and then left to cool down at room temperature. All solutions were then incubated for 60 min 37°C in a thermal cycler. After cooling down to room temperature each sample was equilibrated for 2 min in fluorimeter holder (100 \( \mu \)L quartz cuvette, 25°C) before recording the fluorescence spectrum (\( \lambda_{ex} = 690 \) nm).

**Interaction of DNA binding dyes with DNA19_1T^{\text{NNIR}}**

Titrations were performed in 100 \( \mu \)L quartz cuvette at 25°C. Solution of modified dsDNA (100 \( \mu \)L, 50 pmol) in phosphate buffer (4.5 mM, pH 7.4) was titrated by either thiazole orange, DAPI
or methyl green. After every addition (0-20 equiv.) the solution was mixed carefully with a pipette and equilibrated for 1-2 min before recording the fluorescence spectrum ($\lambda_{ex} = 690$ nm). For structures of dyes used see Figure S15. Fluorescence spectra are shown in Figure S16.

**Binding of histone to DNA19_1T^NNIR**

Titrations were performed in 100 µL quartz cuvette at 25°C. Solution of dsDNA (100 µL, 2 µM) in phosphate buffer (4.5 mM, pH 7.4) was titrated by H2A histone and by BSA (Bovine serum albumin) as control experiment. After every addition (0-4 equiv.) the solution was mixed carefully with a pipette and equilibrated for 1-2 min before recording the fluorescence spectrum ($\lambda_{ex} = 690$ nm). Fluorescence spectra are shown in Figure S17.

**Binding of spermine to DNA19_1T^NNIR**

Titrations were performed in 100 µL quartz cuvette at 25°C. Solution of modified dsDNA (100 µL, 2 µM) in phosphate buffer (4.5 mM, pH 7.4) was titrated by solution of spermine (0.4 mM, in the same buffer). After every addition (0-256 equiv.) the solution was mixed carefully with a pipette and equilibrated for 1-2 min before recording the fluorescence spectrum ($\lambda_{ex} = 690$ nm). Fluorescence spectra are shown in Figure S18.

**Binding of protamine to DNA19_1T^NNIR**

Titrations were performed in 100 µL quartz cuvette at 25°C. Solution of dsDNA (100 µL, 2 µM) in phosphate buffer (4.5 mM, pH 7.4) was titrated by solution of protamine (protamine sulfate; 0.1 mM, in the same buffer). After every addition (0-1.75 equiv.) the solution was mixed carefully with a pipette and equilibrated for 1-2 min before recording the fluorescence spectrum ($\lambda_{ex} = 690$ nm). Fluorescence spectra are shown in Figure S19.

**Displacement experiment using 98-mer dsDNA**

Titrations were performed in 100 µL quartz cuvette at 25 °C. Solution of dsDNA (DNA19_1T^NNIR) and histone H2A 1:2 was prepared in phosphate buffer (4.5 mM, pH 7.4). Solution of dsDNA-histone complex was titrated by 98-mer dsDNA. After every addition (0-1.75 equiv.) the solution was mixed carefully with a pipette and equilibrated for 1-2 min before recording the fluorescence spectrum ($\lambda_{ex} = 690$ nm). Fluorescence spectra are shown in Figure S20.
Displacement experiment using heparin
Titrations were performed in 100 μL quartz cuvette at 25°C. Solution of dsDNA (DNA19_1T^NNIR) and protamine 1:1.75 was prepared in phosphate buffer (4.5 mM, pH 7.4). Solution of dsDNA-protamine complex was titrated by solution of heparin (40 μM, in the same buffer). After every addition (0-0.7 equiv.) the solution was mixed carefully with a pipette and equilibrated for 1-2 min before recording the fluorescence spectrum (λ<sub>ex</sub> = 690 nm). Fluorescence spectra are shown in Figure S21.

Digestion of histone with Proteinase K
Solution of dsDNA (DNA19_1T^NNIR) and histone H2A 1:2 was prepared in phosphate buffer (4.5 mM, pH 7.4). Equilibrated for 2 min at 25°C and measured fluorescence. Then Proteinase K (40 μM, 0.3 μL) was added and fluorescence spectra (λ<sub>ex</sub> = 690 nm) were recorded in 10 sec intervals reaching peak at 20 min.

Monitoring of single nucleotide incorporation (SNI) in real time
SNI was performed in 100 μL quartz cuvette at 60°C in fluorimeter holder. The initial solution (100 μl) contained primer Prim1^PEX (for sequence see Table S3; 100 μM, 1 μL), template Temp1^PEX (for sequence see Table S3; 100 μM, 1.2 μL), dT^NNIRTP (0.04 mM, 20 μL), corresponding reaction buffer (10x, 10 μL) supplied by the manufacturer and KOD XL polymerase (2.5 U/μL, 0.5 μL). After addition of polymerase the cuvette was placed in fluorimeter holder (preheated at 60°C) and fluorescence spectra were recorded in time intervals (0-25 min). Control experiments contained reaction mixture without enzyme (control 1) or without template (control 2). All samples were excited at 690 nm. Produced dsDNA was purified using spin columns (QIAquick Nucleotidie Removal Kit, QIAGEN) and eluted by milli-Q water. MALDI-TOF mass spectrometry analysis proved formation of desired product (Figure S37). Fluorescence spectra are shown in Figure S22.
5. Copies of NMR spectra

Compound 3

KUBA MKK-231
1H NMR in DMSO-d6
16-07-18 RA

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Compound dT^{NNIR}

KUBA MKK-237
1H NMR in DMSO-d6
11-06-18 RA

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S43
Compound $dT^{NNIRTP}$
6. Copies of MALDI-TOF mass spectra

**Figure S30.** MALDI-TOF MS spectrum of single-modified PEX product (ON19_1T\textsuperscript{NNIR}) obtained using dT\textsuperscript{NNIR}\textsuperscript{TP}. Calculated for [M]: 6418.2 Da; found: 6419.6 Da. The peak at $m/z = 6093.3$ Da represents template Temp\textsuperscript{2PEX-bio}. 
Figure S31. MALDI-TOF MS spectrum of single-modified PEX product (ON19_1T'^N3) obtained using dT'^N3TP. Calculated for [M]: 6006.9 Da; found: 6007.9 Da. The peak at m/z = 6093.1 Da represents template Temp2'^PEX-bio.
Figure S32. MALDI-TOF MS spectrum of the PEX product (ON31_4T<sup>N3</sup>) with 4 modifications obtained using dT<sup>N3</sup>TP. Calculated for [M]: 9781.4 Da; found: 9782.0 Da.
Figure S33. MALDI-TOF MS spectrum of SNI product \((\text{ON16}_1\text{T}^{\text{NNIR}})\) obtained using \(dT^{\text{NNIR}}\text{TP}\). Calculated for \([M]\): 5430.55 Da; found: 5431.7 Da. The peak at \(m/z = 9863.4\) Da represents template Temp\(^4\text{PEX}\)-bio.
Figure S34. MALDI-TOF MS spectrum of SNI-PEX product (ON31_1T<sup>NNIR</sup>) obtained using dT<sup>NNIR</sup>TP. Calculated for [M]: 10060.6 Da; found: 10060.6 Da. The peak at m/z = 9863.7 Da represents template Temp<sup>4PEX-bio</sup>. 
Figure S35. MALDI-TOF MS spectrum of the product (ON19_1T\textsuperscript{NNIR}) of CuAAC between NNIR alkyne 3 and ON19_1T\textsuperscript{N3}. Calculated for [M]: 6418.2 Da; found: 6419.9 Da. The peak at \(m/z = 6094.0\) Da represents template Temp2\textsuperscript{PEX-bio}. No starting material is detected.
Figure S36. MALDI-TOF MS spectrum of the product (ON31_4T^{NNIR}) of CuAAC between NNIR alkyne 3 and ON19_4T^{NN3}. Calculated for [M]: 11426.3 Da; found: 11422.9 Da. No starting material is detected.
Figure S37. MALDI-TOF MS spectrum of SNI product (ON16_1T^{NNIR}) after monitoring single nucleotide incorporation. Calculated for [M]: 5430.6 Da; found: 5431.7 Da. The peak at $m/z = 4788.3$ Da represents template Temp1^{PEX}. 
Figure S38. MALDI-TOF MS spectrum of product (ON19_1TNNIR) after digestion of DNA19_1TNNIR-P. Calculated for [M]: 6418.2 Da; found: 6419.6 Da. No Temp2PEX-P (Calculated for [M]: 5733.74 Da) is detected.
7. References

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