Supporting Information

Influence of major-groove chemical modifications of DNA on transcription by bacterial RNA polymerases

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1. Additional experimental data for biochemistry part

Figure S1. Structures of Modified dNTPs
Table S1. List of oligonucleotides used in the PCR study

| Oligonucleotide | Sequence (5’ → 3’) | Length |
|-----------------|---------------------|--------|
| PrimFOR         | TAGGGGTTCGGGCGACATTTCCCAGG | 25-mer |
| PrimREV         | GGAGAGCCTACACGGAACAAACAG | 26-mer |
| PrimFOR,FAMa    | TAGGGGTTCGGGCGACATTTCCCAGG | 25-mer |
| PrimREV,FAMa    | GGAGAGCCTACACGGAACAAACAG | 26-mer |
| PrimFOR,G5,FAMa | GAGGCCCCCTTCGCTTCAGAAGAA | 20-mer |
| PrimREV,G5,FAMa | GTCAGTGGGAAACCAAGCT | 19-mer |
| PrimFOR,12,FAMa | GACATCATGAGAGACATCGC | 25-mer |
| PrimREV,12,FAMa | GACATCATGAGAGACATCGC | 25-mer |
| PrimFVLAshort   | CAGTGCGATGACTATCG | 16-mer |
| PrimFVLAshort,FAMa | CAGTGCGATGACTATCG | 16-mer |
| TempPveg(b,c)   | TAGGGGTTCGGGCGACATTTCCCAGG | 339-mer |
| TempPveg50/10   | GGAGGCTACACGGAACAAACAG | 87-mer |
| TempFVL-A(b)    | GACATCATGAGAGACATCGC | 98-mer |
| TempL50nt(b)    | CAGTGCGATGACTATCGGACCGTACTGCTAAC | 50-mer |
| TempPb4basll    | CTAAGTGGGTTGGTTTGAAGAT | 31-mer |
| TempPb4basll_Bio(d) | CTAAGTGGGTTGGTTTGAAGAT | 31-mer |

a 6-carboxyfluorescein (6-FAM) used for oligonucleotide labeling at 5’-end
b primer sequences in template are underlined
c promotor sequence is in italics
d 5’-end biotinylated template

1.1. Enzymatic synthesis of template (TempPveg)

The 339-mer template (TempPveg) was prepared by PCR with forward (PrimFOR) and reverse (PrimREV) primers from plasmid containing the Pveg constitutive promoter fragment (cloned in p770 between EcoRI and HindIII). The PCR reaction mixture (200 µL) was prepared by mixing of KOD XL DNA Polymerase (2.5 U/µL, 12 µL), natural dNTPs (4 mM, 7.5 µL), primers (20 µM, 30 µL, PrimFOR and 20 µM, 30 µL, PrimREV) and Pveg-plasmide template (33.81 ng/µL, 11 µL) in KOD XL reaction buffer (20 µL) supplied by the manufacturer. Total volume of reaction mixture was then divided to 4 × 50 µL and inserted into the PCR thermal cycler.

Forty PCR cycles were run in the thermal cycler, preheated to 80 °C, under the following conditions: preheating for 3 min at 94 °C, denaturation for 1 min at 94 °C, annealing for 1 min at 62 °C, extension for 1 min at 72 °C, followed by final extension step of 5 min at 72 °C. PCR products were then purified using QIAquick system according to protocol. In the last step, PCR products were eluted from spin column by 20 µL of MilliQ water and then analyzed on a 1.3 % agarose gel in 0.5 × TBE.
buffer (Figure S2). The final concentrations of prepared templates (Temp<sup>Pveg</sup>) were 270.9, 271.0, 286.7 and 282.6 ng/µL, respectively.

![Agarose gel analysis of PCR template product (Temp<sup>Pveg</sup>), amplified by KOD XL DNA polymerase; Line 1 (L): ladder (mix of dsDNA with specific number of base pairs); Lines 2-5 (A-D): PCR product (339-mer); 1.3 % agarose gel stained with GelRed.](image)

**Figure S2.** Agarose gel analysis of PCR template product (Temp<sup>Pveg</sup>), amplified by KOD XL DNA polymerase; Line 1 (L): ladder (mix of dsDNA with specific number of base pairs); Lines 2-5 (A-D): PCR product (339-mer); 1.3 % agarose gel stained with GelRed.

2. PCR of base-modified dNTPs:

2.1. dU<sup>XT</sup>Ps-general remarks:

For the purposes of transcription experiments, PCR reactions were conducted in a total final volume of 60 µL for U<sup>V</sup>, U<sup>E</sup>, 100 µL for U<sup>Ph</sup>, and 80 µL for U, but before insertion to the thermal cycler each reaction mixture was divided to N × 10 µL (N = 6, 10, 8, respectively) according to the modification. Forty PCR cycles were run in PCR cycler, preheated to 80 °C, under the following conditions: preheating for 3 min at 94 °C, denaturation for 1 min at 94 °C, annealing for 1 min at 70 °C, extension for 2 min at 72 °C, followed by final extension step of 5 min at 72 °C. PCR products were purified using QIAquick system according to protocol. In the last step, PCR products were eluted from spin column by either 20 µL (in the case of U<sup>V</sup>, U<sup>E</sup>, U<sup>Ph</sup>) or 30 µL (in the case of U) of MilliQ water and then analyzed on a 1.3 % agarose gel in 0.5 × TBE buffer (Figure S3). The desired PCR product containing dUTP had to be additionally extracted from agarose gel since secondary shorter (< 100 nt) by-product occurred in the mixture during the amplification and it was not possible to separate it by QIAquick system. The extraction from gel was provided by E.Z.N.A. Gel Extraction Kit according to given protocol. The final concentration of prepared modified DNAs was quantified with NanoDrop instrument and is shown in Table S2.

2.1.1. dU<sup>ETP</sup>, dUTP:

The PCR reaction mixture (10 µL) was prepared by mixing of either KOD XL DNA Polymerase (for U<sup>E</sup>, 2.5 U/µL, 0.4 µL) or DyNazyme II DNA polymerase (for U, 2 U/µL, 0.5 µL) with DMSO (100 %, 0.5 µL), formamide (5 %, 0.5 µL), betaine (0.75 M, 0.5 µL), TMAC (50 mM, 0.5 µL), natural dNTPs (4 mM, 0.5 µL), functionalized dNTPs (4 mM, 1 µL), primers (20 µM, 1 µL, Prim<sup>FOR</sup> and 20 µM, 1 µL, Prim<sup>REV</sup>) and template Temp<sup>Pveg</sup> (20 ng/µL, 1 µL) in appropriate enzyme reaction buffer (1 µL) supplied by the manufacturer.
2.1.2. dU<sup>V</sup>TP, dU<sup>Ph</sup>TP:

The PCR reaction mixture (10 µL) was prepared by mixing of KOD XL DNA Polymerase (2.5 U/µL, 0.4 µL for U<sup>V</sup>, 0.5 µL for U<sup>Ph</sup>), natural dNTPs (4 mM, 0.5 µL), functionalized dNTPs (4 mM, 1 µL), primers (20 µM, 1 µL, Prim<sup>FOR</sup> and 20 µM, 1 µL, Prim<sup>REV</sup>) and template Temp<sup>Pveg</sup> (20 ng/µL, 1 µL) in KOD XL reaction buffer (1 µL) supplied by the manufacturer.

![Figure S3. Agarose gel analysis of PCR products, amplified by: A) and B) KOD XL and C) DyNazymell DNA polymerases; Lines 1 (L): ladder (mix of dsDNA with specific number of base pairs); Lines 2, 5 (T+): natural dNTPs; Lines 3, 6 (T−): dCTP, dGTP, dATP; Lines 4, 7 (U<sup>V</sup>, U<sup>Ph</sup>, U): 1.3 % agarose gel stained with GelRed.](image)

| Entry | Oligonucleotide | Concentration [ng/µL] |
|-------|----------------|-----------------------|
| 1.    | T+             | 274.6                 |
| 2.    | U<sup>V</sup>  | 149.1                 |
| 3.    | T+             | 281.5                 |
| 4.    | U<sup>E</sup>  | 256.6                 |
| 5.    | T+             | 362.0                 |
| 6.    | U<sup>Ph</sup> | 129.4                 |
| 7.    | T+             | 96.6                  |
| 8.    | U              | 54.4                  |

2.2. dA<sup>X</sup>TP—general remarks:

For the purposes of transcription experiments, PCR reactions were conducted in a total final volume of 60 µL for A<sup>Me</sup>, A<sup>E</sup>, A<sup>Ph</sup>, and 100 µL for A<sup>V</sup>, and 40 µL for A<sup>H</sup>, but before insertion to the thermal cycler each reaction mixture was divided to N × 10 µL (N = 6, 10, for A<sup>Me</sup>, A<sup>E</sup>, A<sup>Ph</sup> and A<sup>V</sup>, respectively) and to 2 × 20 µL (for A<sup>H</sup>). Forty PCR cycles were run in PCR cycler, preheated to 80 °C, under the following conditions: preheating for 3 min at 94 °C, denaturation for 1 min at 94 °C, annealing for 1 min at 70 °C, extension either for 2 min at 72 °C (in the case of A<sup>E</sup>, A<sup>Ph</sup>) or 1 min at 72 °C (in the case of A<sup>H</sup>, A<sup>Me</sup>, A<sup>V</sup>), followed by final extension step of 5 min at 72 °C. PCR products were purified using QIAquick system according to protocol. In the last step, PCR products were eluted from spin column by either 20 µL (in the case of A<sup>V</sup>, A<sup>E</sup>) or 30 µL (in the case of A<sup>H</sup>, A<sup>Me</sup>, A<sup>Ph</sup>) of MilliQ water and then analyzed on a 1.3 % agarose gel in 0.5 × TBE buffer (Figure S4). The final concentration of prepared modified DNAs was quantified with NanoDrop instrument and is shown in Table S3.
2.2.1. dA\textsuperscript{M}TP, dA\textsuperscript{E}TP:

The PCR reaction mixture (10 µL) was prepared by mixing of KOD XL DNA Polymerase (2.5 U/µL, 0.4 µL for A\textsuperscript{E} and 0.8 µL for A\textsuperscript{Y}), DMSO (100 %, 0.4 µL), formamide (5 %, 0.5 µL), betaine (0.75 M, 0.5 µL), TMAC (50 mM, 0.5 µL), natural dNTPs (4 mM, 0.5 µL), functionalized dNTPs (4 mM, 1 µL), primers (20 µM, 1 µL, Prim\textsuperscript{FOR} and 20 µM, 1 µL, Prim\textsuperscript{REV}) and template Temp\textsuperscript{Pveg} (20 ng/µL, 1 µL) in KOD XL reaction buffer (1 µL) supplied by the manufacturer.

2.2.2. dA\textsuperscript{H}TP, dA\textsuperscript{Me}TP, dA\textsuperscript{Ph}TP:

The PCR reaction mixture (10 µL) was prepared by mixing of KOD XL DNA Polymerase (2.5 U/µL, 0.4 µL), natural dNTPs (4 mM, 0.5 µL), functionalized dNTPs (4 mM, 1 µL), primers (20 µM, 1 µL, Prim\textsuperscript{FOR} and 20 µM, 1 µL, Prim\textsuperscript{REV}) and template Temp\textsuperscript{Pveg} (20 ng/µL, 1 µL) in KOD XL reaction buffer (1 µL) supplied by the manufacturer.

![Figure S4](image)

Figure S4 Agarose gel analysis of PCR products, amplified by KOD XL DNA polymerase; A) Line 1 (L): ladder (mix of dsDNA with specific number of base pairs); Line 2 (A+) natural dNTPs; Line 3 (A-): dC\textsuperscript{T}, dG\textsuperscript{T}, dT\textsuperscript{T}; Lines 4 (A\textsuperscript{Me}), 5 (A\textsuperscript{H}): dA\textsuperscript{M}TP, dC\textsuperscript{T}, dG\textsuperscript{T}, dT\textsuperscript{T}, X = Me, H; B) Line 1 (L): ladder (mix of dsDNA with specific number of base pairs); Lines 2, 5, 8 (A+): natural dNTPs; Lines 3, 6, 9 (A-): dC\textsuperscript{T}, dG\textsuperscript{T}, dT\textsuperscript{T}; Lines 4 (A\textsuperscript{V}), 7 (A\textsuperscript{E}), 10 (A\textsuperscript{Ph}): dA\textsuperscript{E}TP, dC\textsuperscript{T}, dG\textsuperscript{T}, dT\textsuperscript{T}, X = V, E, Ph; 1.3 % agarose gel stained with GelRed.

Table S3. Concentration of amplified DNAs containing modified dA\textsuperscript{X}TPs

| Entry | Oligonucleotide | Concentration [ng/µL] | Entry | Oligonucleotide | Concentration [ng/µL] |
|-------|-----------------|-----------------------|-------|-----------------|-----------------------|
| 1.    | A+              | 133.9                 | 7.    | A+              | 274.4                 |
| 2.    | A\textsuperscript{H} | 207.2                 | 8.    | A\textsuperscript{E} | 218.5                 |
| 3.    | A+              | 250.0                 | 9.    | A+              | 184.6                 |
| 4.    | A\textsuperscript{Me} | 212.0                 | 10.   | A\textsuperscript{Ph} | 104.4                 |
| 5.    | A+              | 339.3                 |       |                 |                       |
| 6.    | A\textsuperscript{V} | 204.1                 |       |                 |                       |

2.3. dC\textsuperscript{X}TP-general remarks:

For the purposes of transcription experiments, PCR reactions were conducted in a total final volume of 60 µL for C\textsuperscript{E}, C\textsuperscript{Ph}, and 100 µL for C\textsuperscript{V}, and 40 µL for C\textsuperscript{Me}, but before insertion to the thermal cycler each reaction mixture was divided to N × 10 µL (N = 6, 10, 4, respectively) according to the modification. Forty PCR cycles were run in PCR cycler, preheated to 80 °C, under the following
conditions: preheating for 3 min at 94 °C, denaturation for 1 min at 94 °C, annealing for 1 min at 70 °C, extension either for 2 min at 72 °C (in the case of C<sub>Me</sub>, C<sub>E</sub>) or 1 min at 72 °C (in the case of C<sub>V</sub>, C<sub>Ph</sub>), followed by final extension step of 5 min at 72 °C. PCR products were purified using QIAquick system according to protocol. In the last step, PCR products were eluted from spin column by either 20 µL (in the case of C<sub>V</sub>, C<sub>E</sub>) or 25 µL (in the case of C<sub>Ph</sub>) or 30 µL (in the case of C<sub>Me</sub>) of MilliQ water and then analyzed on a 1.3 % agarose gel in 0.5 × TBE buffer (Figure S5). The final concentration of prepared modified DNAs was quantified with NanoDrop instrument and is shown in Table S4.

2.3.1. dC<sub>Me</sub>TP, dC<sub>E</sub>TP:

The PCR reaction mixture (10 µL) was prepared by mixing of Vent(exo-) DNA Polymerase (2 U/µL, 0.8 µL), DMSO (100 %, 0.5 µL), formamide (5 %, 0.5 µL), betaine (0.75 M, 0.5 µL), TMAC (50 mM, 0.5 µL), natural dNTPs (4 mM, 0.5 µL), functionalized dNTPs (4 mM, 1 µL), primers (20 µM, 1 µL, Prim<sup>FOR</sup> and 20 µM, 1 µL, Prim<sup>REV</sup>) and template Temp<sup>Pveg</sup> (20 ng/µL, 1 µL) in Vent(exo-) reaction buffer (1 µL) supplied by the manufacturer.

2.3.2. dC<sub>V</sub>TP, dC<sub>Ph</sub>TP:

The PCR reaction mixture (10 µL) was prepared by mixing of KOD XL DNA Polymerase (2.5 U/µL, 0.4 µL for C<sub>Ph</sub> and 0.8 µL for C<sub>V</sub>), natural dNTPs (4 mM, 0.5 µL), functionalized dNTPs (4 mM, 1 µL), primers (20 µM, 1 µL, Prim<sup>FOR</sup> and 20 µM, 1 µL, Prim<sup>REV</sup>) and template Temp<sup>Pveg</sup> (20 ng/µL, 1 µL) in KOD XL reaction buffer (1 µL) supplied by the manufacturer.

![Figure S5. Agarose gel analysis of PCR products, amplified by A) Vent(exo-) and B) KOD XL DNA polymerases: A) Line 1 (L): ladder (mix of dsDNA with specific number of base pairs); Line 2 (C+): natural dNTPs; Line 3 (C-): dATP, dGTP, dTTP; Lines 4 (C<sub>E</sub>), 5 (C<sub>Me</sub>): dC<sub>E</sub>TP, dATP, dGTP, dTTP, X = E, Me; B) Line 1 (L): ladder (mix of dsDNA with specific number of base pairs); Lines 2, 5 (C+): natural dNTPs; Lines 3, 6 (C-): dATP, dGTP, dTTP; Lines 4 (C<sub>V</sub>), 7 (C<sub>Ph</sub>): dC<sub>V</sub>TP, dATP, dGTP, dTTP, X = V, Ph; 1.3 % agarose gel stained with GelRed.](image-url)
Table S4. Concentration of amplified DNAs containing modified dC<sup>X</sup>TPs

| Entry | Oligonucleotide | Concentration [ng/µL] | Entry | Oligonucleotide | Concentration [ng/µL] |
|-------|----------------|-----------------------|-------|----------------|-----------------------|
| 1.    | C+             | 321.8                 | 5.    | C+             | 308.8                 |
| 2.    | C<sub>Me</sub> | 424.8                 | 6.    | C<sub>E</sub>  | 284.8                 |
| 3.    | C+             | 315.9                 | 7.    | C+             | 356.3                 |
| 4.    | C<sub>V</sub>  | 81.7                  | 8.    | C<sub>Ph</sub>| 282.3                 |

2.4. dG<sup>X</sup>TP-general remarks:

Since 7-deazaguanosin moieties are known to quench fluorescence of intercalators, we also used 5′-6-FAM labeled primers for the visualization of PCR products (Table S1). Anyway, the PCR conditions and preparation of mixtures were the same for both non-labeled and FAM-labeled samples.

For the purposes of transcription experiments, PCR reactions were conducted in a total final volume of 60 µL for G<sup>X</sup> (X = Me, V, E, Ph) and 40 µL for G<sup>H</sup>, but before insertion to the thermal cycler each reaction mixture was divided to 6 × 10 µL (for G<sup>Me</sup>, G<sup>V</sup>, G<sup>E</sup>, G<sup>Ph</sup>) and to 2 × 20 µL (for G<sup>H</sup>). All FAM-labeled samples G<sup>X</sup> (X = Me, V, E, Ph, H) were conducted in total volume of 30 µL and were not divided to smaller reaction volumes. Forty PCR cycles were run in PCR cycler, preheated to 80 °C, under the following conditions: preheating for 3 min at 94 °C, denaturation for 1 min at 94 °C, annealing for 1 min at 70 °C, extension 1 min at 72 °C, followed by final extension step of 5 min at 72 °C. PCR products were purified using QIAquick system according to protocol.

In the last step, PCR products were eluted from spin column by either 25 µL (in the case of G<sup>Me</sup>, G<sup>V</sup>, G<sup>E</sup>, G<sup>Ph</sup>) or 30 µL (in the case of G<sup>H</sup>) of MilliQ water and then analyzed on a 1.3 % agarose gel in 0.5 × TBE buffer (Figure S6-A). Additionally, all the FAM-labeled PCR products were eluted from spin column by 30 µL of MilliQ water and then analyzed on a 1.3 % agarose gel in 0.5 × TBE buffer (Figure S6-B).

The final concentration of all modified DNAs (either non-labeled or labeled) was quantified with NanoDrop instrument and is shown in Table S5-I and Table S5-II, respectively.

The PCR reaction mixture (10 µL) was prepared by mixing of KOD XL DNA Polymerase (2.5 U/µL, 0.4 µL), natural dNTPs (4 mM, 0.5 µL), functionalyzed dNTPs (4 mM, 1 µL), primers (20 µM, 1 µL, Prim<sub>FOR</sub>, or Prim<sub>FOR</sub>-FAM, and 20 µM, 1 µL, Prim<sub>REV</sub>, or Prim<sub>REV</sub>-FAM) and template Temp<sub>Pveg</sub> (20 ng/µL, 1 µL) in KOD XL reaction buffer (1 µL) supplied by the manufacturer.
Figure S6. Agarose gel analysis of PCR products, amplified by KOD XL DNA polymerase: A) 1.3 % agarose gel stained with GelRed; B) 1.3 % plain agarose gel with the use of 5´- 6-FAM labeled primers. A) and B): Lines 1 (L): ladder (mix of dsDNA with specific number of base pairs); Lines 2 (G+): natural dNTPs; Lines 3 (G-): dCTP, dTTP, dATP; Lines 4 (GMe), 5 (G), 6 (G5), 7 (GPh), 8 (G): dGxTP, dCTP, dTTP, dATP, X = Me, V, E, Ph, H.

Table S5-I. Concentration of amplified DNAs containing modified dGxTPs

| Entry | Oligonucleotide | Concentration [ng/µL] |
|-------|-----------------|-----------------------|
| 1. | G+ | 133.9 |
| 2. | GH | 89.8 |
| 3. | G+ | 325.2 |
| 4. | GMe | 277.9 |
| 5. | G+ | 319.4 |
| 6. | GV | 170.0 |
| 7. | G+ | 337.2 |
| 8. | GE | 222.4 |
| 9. | G+ | 302.3 |
| 10. | GPh | 125.4 |

Table S5-II. Concentration of amplified 6-FAM-labeled DNAs containing modified dGxTPs

| Entry | Oligonucleotide – FAM | Concentration [ng/µL] |
|-------|------------------------|-----------------------|
| 1. | G+ – FAM | 290.3 |
| 2. | GH – FAM | 134.0 |
| 3. | GMe – FAM | 132.0 |
| 4. | GV – FAM | 203.0 |
| 5. | GE – FAM | 182.1 |
| 6. | GPh – FAM | 132.2 |

2.5. Sequencing:

The correct sequence of some 339-mer PCR products were confirmed by LIGHTRUN™ Sequencing service (GATC Biotech AG, Germany) using standard Sanger sequencing. Namely PCR products containing dUTP, dUPhTP, dUVTTP, dCMeTP, dC5TP, dAVTP, dATTP, and dAPhTP.
3. Gel Shift assay-promoter fragments:

The list of oligonucleotides used in this PCR study is listed in Table S1. The visualization of PCR products as well as DNA ladder was performed by the use of 5´-6-FAM labeled primers. The FAM-labeled DNA ladder was prepared by mixing of 5 µL of F100nt (38.4 ng/µL), 4 µL of F50nt (35.7 ng/µL), 2 µL of 6X DNA loading dye and 1 µL of H2O. Then, 5 µL of this mixture was loaded to the 2 % agarose gels as well as 5 µL of PCR products (each sample contained 2 µL of purified PCR product, 1 µL of 6X DNA loading dye and 3 µL of H2O).

3.1. Enzymatic synthesis of 100-mer oligonucleotide ladder (F100nt)

The PCR reaction mixture (80 µL) was prepared by mixing of Pwo DNA Polymerase (2 U/µL, 8 µL), DMSO (100 %, 4 µL), formamide (5 %, 4 µL), betaine (0.75 M, 4 µL), TMAC (50 mM, 4 µL), natural dNTPs (4 mM, 2 µL), primers (10 µM, 8 µL, PrimFOR-L20-FAM and 10 µM, 8 µL, PrimREV-L25TH-FAM) and template (1 µM, 2 µL, TempFVL-1) in Pwo reaction buffer (8 µL) supplied by the manufacturer. Total volume of reaction mixture was then divided to 4 × 20 µL and inserted to the PCR thermal cycler. Thirty PCR cycles were run in the thermal cycler, preheated to 80 °C, under the following conditions: preheating for 3 min at 94 °C, denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, extension for 1 min at 72 °C, followed by final extension step of 5 min at 72 °C. PCR products (4 × 20 µL) were then collected together to 2 × 40 µL and purified using QIAquick system according to protocol. In the last step, PCR products were eluted from spin column by 30 µL of MilliQ water. The final concentration of prepared F100nt was 38.4 ng/µL and 39.9 ng/µL, respectively.

3.2. Enzymatic synthesis of 50-mer oligonucleotide ladder (F50nt)

The PCR reaction mixture (80 µL) was prepared by mixing of Pwo DNA Polymerase (2 U/µL, 8 µL), natural dNTPs (4 mM, 4 µL), primers (10 µM, 8 µL, PrimFOR12-FAM and 10 µM, 8 µL, PrimREV12-FAM) and template (1 µM, 4 µL, TempL50nt) in Pwo reaction buffer (8 µL) supplied by the manufacturer. Total volume of reaction mixture was then divided to 8 × 10 µL and inserted to the PCR thermal cycler. Thirty PCR cycles were run in the thermal cycler, preheated to 80 °C, under the following conditions: preheating for 3 min at 94 °C, denaturation for 1 min at 94 °C, annealing for 1 min at 40 °C, extension for 1 min at 72 °C, followed by final extension step of 5 min at 72 °C. PCR products (8 × 10 µL) were then collected together to 2 × 40 µL and purified using QIAquick system according to protocol. In the last step, PCR products were eluted from spin column by 30 µL of MilliQ water. The final concentration of prepared F50nt was 39.9 ng/µL and 35.7 ng/µL, respectively.

3.3. dNTP-general remarks:

PCR reactions were conducted in a total final volume of 60 µL for A'H, A'Me, A'Ph and C'Me, and 80 µL for U, but before insertion to the thermal cycler each reaction mixture was divided to N × 10 µL (N = 6, 8, respectively) according to modification. Thirty PCR cycles were run in PCR cycler, preheated to 80 °C, under the following conditions: preheating for 3 min at 94 °C, denaturation for 1 min at 94 °C, annealing for 1 min at 58 °C, extension for 1 min at 72 °C followed by a final extension step of 5 min at 72 °C. PCR products were purified using QIAquick system according to protocol. In the last step,
PCR products were eluted from spin column by 30 µL of MilliQ water and then analyzed on a 2% agarose gel in 0.5 × TBE buffer (Figures S7, S8, S9). The final concentration of prepared modified DNAs was quantified with NanoDrop instrument and is shown in Tables S6, S7, S8.

The PCR reaction mixture (10 µL) was prepared by mixing of either Vent(exo-) DNA Polymerase (for $A^H$, $A^{Me}$, $A^{Ph}$ and $C^{Me}$, 2 U/µL, 0.5 µL) or DyNazyme II DNA Polymerase (for U, 2 U/µL, 0.5 µL), natural dNTPs (4 mM, 0.5 µL), functionalized dNTPs (4 mM, 1 µL), primers (10 µm, 1 µL, Primer$^{FOR-GS-FAM}$ and 10 µm, 1 µL, Primer$^{REV-GS-FAM}$) and template Temp$^{PEG50/10}$ (1 µm, 0.5 µL) in appropriate enzyme reaction buffer (1 µL) supplied by the manufacturer.

**Figure S7** Agarose gel analysis of PCR products, amplified by Vent(exo-) DNA polymerase; Line 1 (L): ladder (mix of dsDNA with specific number of base pairs); Line 2 (A+): natural dNTPs; Line 3 (A-): dCTP, dGTP, dTTP; Line 4 ($A^H$): $dA^{H}$TP, dCTP, dGTP, dTTP; Line 5 ($A^{Me}$): $dA^{Me}$TP, dCTP, dGTP, dTTP; Line 6 ($A^{Ph}$): $dA^{Ph}$TP, dCTP, dGTP, dTTP; 2% plain agarose gel with the use of 5'-6-FAM labeled primers.

**Table S6. Concentration of amplified DNAs containing modified $d A^X TPs$**

| Entry | Oligonucleotide | Concentration [ng/µL] |
|-------|-----------------|----------------------|
| 1.    | A+              | 141.3                |
| 2.    | $A^H$           | 125.4                |
| 3.    | $A^{Me}$        | 119.1                |
| 4.    | $A^{Ph}$        | 91.2                 |

**Figure S8** Agarose gel analysis of PCR products, amplified by Vent(exo-) DNA polymerase; Line 1 (L): ladder (mix of dsDNA with specific number of base pairs); Line 2 (C+): natural dNTPs; Line 3 (C-): dATP, dGTP, dTTP; Line 4 ($C^{Me}$): $dC^{Me}$TP, dATP, dGTP, dTTP; 2% plain agarose gel with the use of 5'-6-FAM labeled primers.

**Table S7. Concentration of amplified DNAs containing modified $d C^{X}$TP**

| Entry | Oligonucleotide | Concentration [ng/µL] |
|-------|-----------------|----------------------|
| 1.    | C+              | 127.4                |
| 2.    | $C^{Me}$        | 145.3                |
Figure S9 Agarose gel analysis of PCR products, amplified by DyNazyme II DNA polymerase; Line 1 (L): ladder (mix of dsDNA with specific number of base pairs); Line 2 (T+): natural dNTPs; Line 3 (T-): dATP, dGTP, dCTP; Line 4 (U): dUTP, dATP, dGTP, dCTP; 2 % plain agarose gel with the use of 5’-6-FAM labeled primers.

Table S8. Concentration of amplified DNAs containing modified dUTP

| Entry | Oligonucleotide | Concentration [ng/µL] |
|-------|----------------|-----------------------|
| 1.    | U+             | 142.6                 |
| 2.    | U              | 131.4                 |

4. Copies of NMR spectra

MILISAVLJEVIC  NM-dAMeTP_Nasalt_new
1H NMR in D2O
15-02-16 RA
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5.7-methyl-2′-deoxy-7-deazaadenosine 5′-O-triphosphate (dA\textsubscript{Me}TP) – PEX, MALDI-TOF

5.1. Incorporation of 7-Methyl-2′-deoxy-7-deazaadenosine-5′-O-triphosphate into DNA by PEX

The oligonucleotides used in this PEX experiment are listed in Table S1.

The reaction mixture (20 μL) contained 5′-6-FAM labeled primer (3 μM, 1 μL, Prim\textsubscript{248short,FAM}), 31-mer template (3 μM, 1.5 μL, Temp\textsubscript{Prb4basII}), KOD XL DNA polymerase (2.5 U/μL, 1.5 μL), natural dNTPs (dCTP, dGTP, dTTP, 4 mM, 0.5 μL) and dATP and dA\textsubscript{Me}TP (4 mM, 1 μL) in KOD XL reaction buffer (2 μL) supplied by the manufacturer. The reaction mixture was incubated for 30 min at 60 °C in a thermal cycler. Primer extension was stopped by the addition of stop solution (20 μL, 80 % [v/v] formamide, 20 mM EDTA, 0.025 % [w/v] bromophenol blue, 0.025 % [w/v] xylene cyanol) and heated for 5 min at 95 °C. Samples were separated by 12.5 % PAGE (acylamide/bisacrylamide 19:1, 25 % urea) under denaturing conditions (42 mA, 1 h). Visualization was performed by phosphoimagiing (Figure S10).

![Figure S10. PAGE analysis of 31-mer PEX product; 12.5 % denaturating gel; 5′-6-FAM labeled DNAs; (P): primer; (+) positive control: natural dNTPs; (-) negative control: dCTP, dGTP, dTTP; (dA\textsubscript{Me}): dA\textsubscript{Me}TP, dCTP, dGTP, dTTP.]

5.2. Preparation of modified single stranded DNA (ON\textsubscript{Me}) for MALDI TOF analysis

Streptavidin magnetic particles stock solution (Roche, 35 μL) was washed with binding buffer (2 × 100 μL, 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). The PEX solution (prepared as described above using 5′-biotinylated 31-mer template, Temp\textsubscript{Prb4basII-bio}, and non-labeled 15-mer primer, Prim\textsubscript{248short}) and binding buffer (35 μL) were added. Suspension was shaken (1200 rpm) for 30 min at 15 °C. 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 (4 × 200 μL). Then, water (35 μL) was added and the sample was denatured for 2 min at 55 °C and 900 rpm. The beads were collected on a magnet and the solution was transferred into a clean vial. The product was analyzed by MALDI-TOF mass spectrometry (for copy of mass spectrum see Figure S11). The MALDI-TOF spectra were measured on a MALDI-TOF/TOF mass spectrometer.
with 1 kHz smart beam II laser. The measurements were done in reflectron mode by droplet technique, with the mass range up to 30 kDa. The matrix consisted of 3-hydroxypicolinic acid (HPA)/picolinic acid (PA)/ammonium tartrate in ratio 9/1/1. The matrix (1 μL) was applied on the target (ground steel) and dried down at room temperature. The sample (1 μL) and matrix (1 μL) were mixed and added on the top of the dried matrix preparation spot and dried at room temperature.

**Figure S11.** Copy of MALDI-TOF spectrum of ONMe PEX product; M (calc.) = 9669.5 Da, M (found) = 9670.8 Da [M + H]^+. 
Figure S12. Copy of MALDI-TOF spectrum of ONMe PEX product with extension of m/z range 8000 – 12000; M (found) = 9670.8 Da [M + H]⁺; M (found) = 9708.9 Da [M + K]⁺; detail in the black dashed square is depicted in the up right corner.
Figure S13. Copy of MALDI-TOF spectrum of ON-positive control (only natural dNTPs) PEX product; M (calc.) = 9617.3 Da, M (found) = 9618.4 Da [M + H]⁺.
Figure S14. Copy of MALDI-TOF spectrum of ON-positive control (only natural dNTPs) PEX product with extension of m/z range 8000 – 12000; M (found) = 9618.4 Da [M + H]+; M (found) = 9656.4 Da [M + K]+; detail in the black dashed square is depicted in the up right corner.

6. In silico modelling

The crystal structure of the *E. coli* transcription initiation complex with a complete bubble (PDB id 4yln [5]) was used for our models illustrating interactions between RNAP and DNA. Chemical modifications of T7/A11 nucleosides were carried out by the Molefacture plugin from the VMD software package (6). Figures were generated using the ICM Molsoft Browser (7).
7. References

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