Supporting Information I

For

5-(Hydroxymethyl)uracil and –cytosine as epigenetic marks enhancing or inhibiting transcription with bacterial RNA polymerase

Contents

1. Extended results and discussion

Structures of natural pyrimidine dNTPs (dTTP and dCTP) and epigenetic nucleoside triphosphates (dUTP, d5hmUTP, d5mCTP and d5hmCTP) used for the synthesis of fully or partially modified DNAs are shown in *Figure S1.*

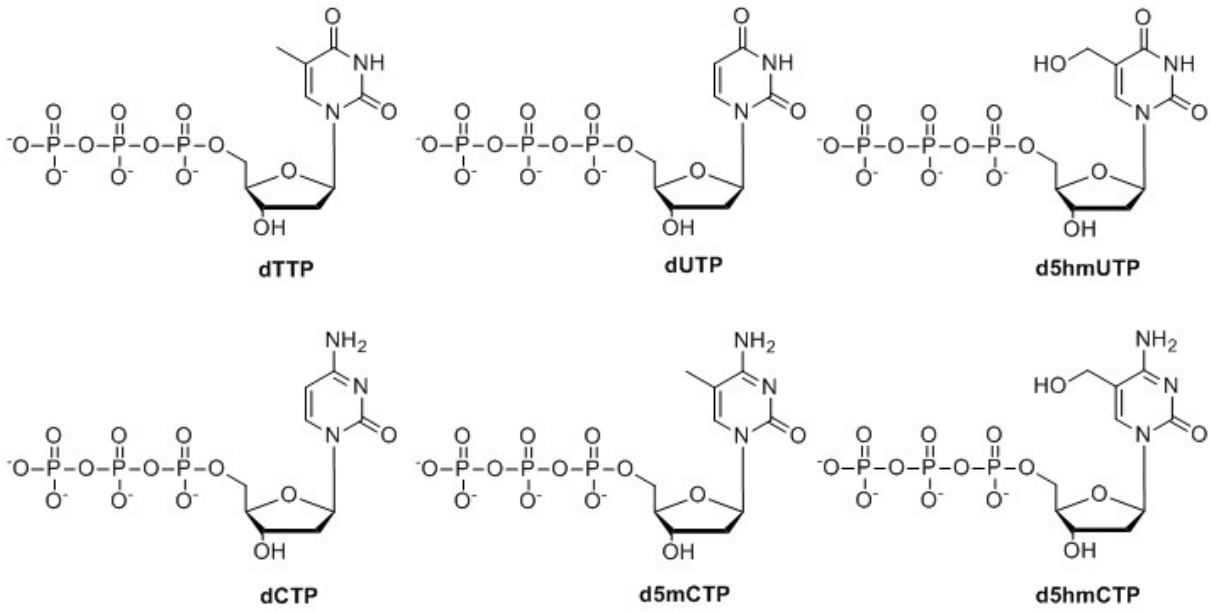


***Figure S1**:* Structures of used triphosphates

Structures of modified nucleotides incorporated into the modified DNA templates are shown in *Figure S2.*

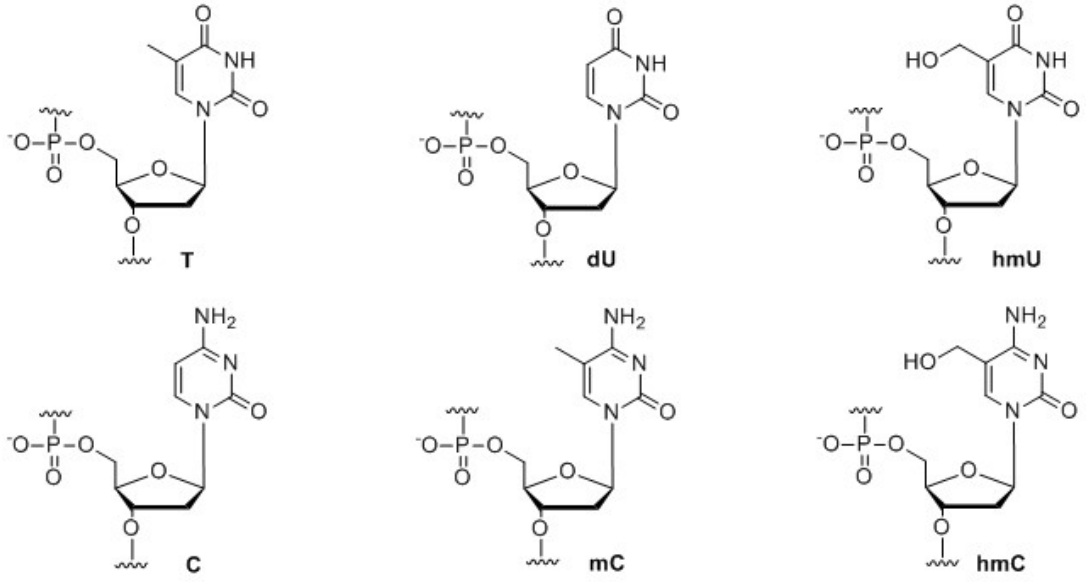


***Figure S2**:* Structures of used canonical and epigenetic nucleotides in DNA (and abbreviations used in gels)

1.1. Synthesis of fully and partially modified DNA templates

1.1.1. Syntheses of fully modified DNA:

A) Synthesis of fully-modified 339-mer templates

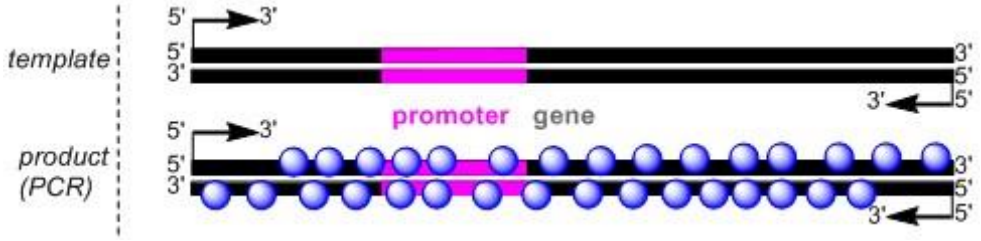


Figure S3. *Synthesis of fully modified 339-mer templates*

Fully modified 339-mer DNA templates with P*veg* or *rrnB P1* promoter sequence were prepared by PCR reactions in the presence of 3 natural dNTPs and either dUTP, d5hmUTP, d5mCTP or d5hmCTP *(Figure S3)*. Templates containing chimeric promoter regions *rrnB P-10Dveg* and *Pveg-10DBP1* were prepared in the same way.

B) Synthesis of modified 235-mer templates

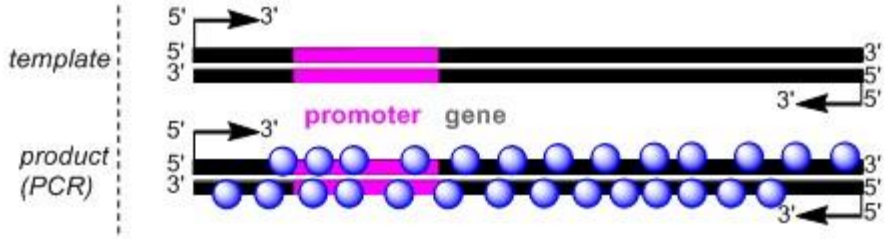


Figure S4. *Synthesis of fully modified 235-mer templates*

To show that the length of the DNA region upstream of the -35 hexamer of the promoter does not significantly affect transcription, a fully modified 235-mer DNA fragment (shortened by 104 bp upstream of the promoter) with the P*veg* promoter region was synthesized in the presence of 3 natural dNTPs and either dUTP, d5hmUTP, d5mCTP or d5hmCTP by PCR *(Figure S4)*. The length of the promoter-upstream region did no have significant effects on transcription (data not shown).

1.1.2. Syntheses of partially modified DNA

235-mers of partially modified DNAs (with P*veg* promoter region) were synthesized to understand which modified part of DNA (promoter, transcribed region, template strand, nontemplate strand) is responsible for the observed effects of transcription.

A) Synthesis of DNA templates modified in the transcribed region

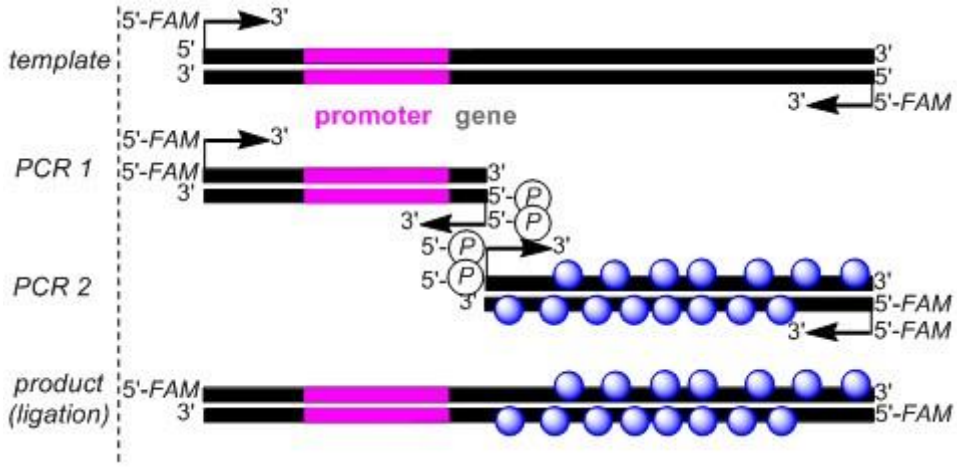


Figure S5: *Synthesis of DNA templates modified in the transcribed region.*

To prepare dsDNA modified in the promoter-downstream part (transcribed region), a natural 69-mer DNA and modified 166-mers DNA were synthesized. The natural 69-mer DNA was synthesized with a 5´-FAM labelled forward primer and a 5´-phosphate modified reverse primer. Modified 166-mers of modified DNAs (representing the promoter-downstream part) were prepared with a 5´-FAM labelled reverse primer and a 5´-phosphate modified forward primer. The 5´-phosphate ends in the primers allowed ligation of only the desired ends of DNA and the 5´-FAM labelled ends prevented undesirable ligations. In the final step, the natural 69bp DNA and modified 166bp DNAs (either dU, hmU, mC or hmC-modified 166-mer) were ligated by T4 DNA Ligase *(Figure S5)*.

B) Synthesis of DNA templates modified in promoter-upstream and promoter regions of dsDNA

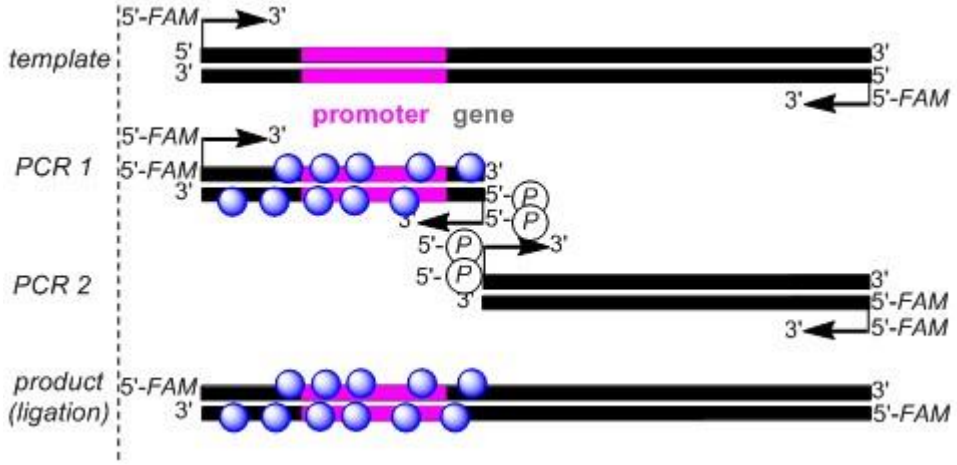


Figure S6. *Synthesis of DNA templates modified in upstream and promoter gene region*

To prepare dsDNA modified in the promoter-upstream and promoter regions, respectively, modified 69-mers of DNA and unmodified 166bp DNA were synthesized by PCR. Modified 69-

bp DNAs (representatives of promoter-upstream and promoter regions) were synthesized with a 5´-FAM labelled forward primer and a 5´-phosphate modified reverse primer. Natural 166-mers of DNA were prepared with a 5´-FAM labelled reverse primer and a 5´-phosphate modified forward primer. 5´-phosphate ends and 5´-FAM labelled ends had the same roles as in (A). In the final step, modified 69bp DNAs of each type (either dU, hmU, mC or hmC-modified 69-mer) and natural 166bp DNA were ligated by T4 DNA Ligase *(Figure S6)*.

C) Synthesis of DNA templates modified in upstream and promoter regions of template strand

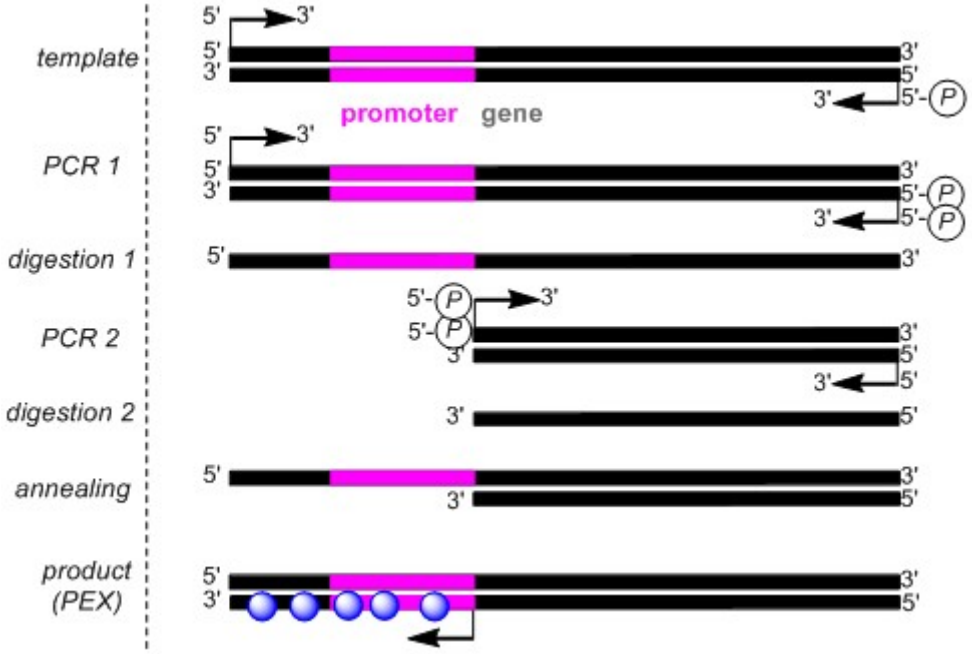


Figure S7. *Synthesis of DNA templates modified in promoter-upstream and promoter regions of the template strand*

To synthetize modified DNAs in the promoter-upstream and promoter regions in the template strand of DNA, natural double stranded 181-, and 235-mers, respectively, were prepared. The 181-mer DNA was synthesized with a 5´-phosphate modified forward primer and an unmodified reverse primer while the 235-mer DNA was prepared with a natural forward primer and a 5´-phosphate modified reverse primer. The DNA synthesis was done by PCR. 5´-A phosphate modification was introduced to facilitate the digestion of the undesired strand by Lambda exonuclease to get the desired single-stranded DNA (ssDNA of 181-mer and ssDNA of 235-mer). Complementary single-stranded DNAs of different length were annealled to obtain dsDNA with a single stranded overhang at the 5´-end of the coding (non-template) strand. The annealed dsDNA was then used for the final PEX reaction in the presence of three natural dNTPs and either of dUTP, d5hmUTP, d5mCTP or d5hmCTP to obtain DNA modified in promoter-upstream and promoter regions in the template strand of DNA *(Figure S7)*.

D) Synthesis of DNA templates modified in promoter-upstream and promoter regions of the coding strand

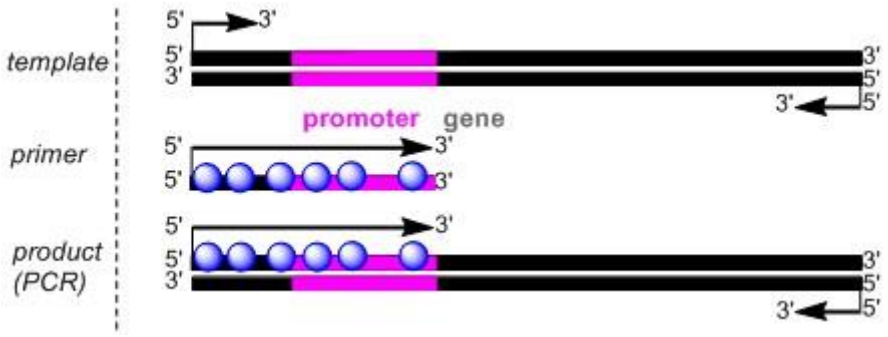


Figure S8. *Synthesis of DNA templates modified in promoter-upstream and promoter regions of the coding strand*

The synthesis of DNA modified in promoter-upstream and promoter regions of the coding strand was performed by PCR reactions using a natural reverse primer and a commercially synthesized dU-, hmU-, mC- or hmC-modified forward primer *(Figure S8)*.

E) Synthesis of DNA templates modified in the promoter region of the coding strand

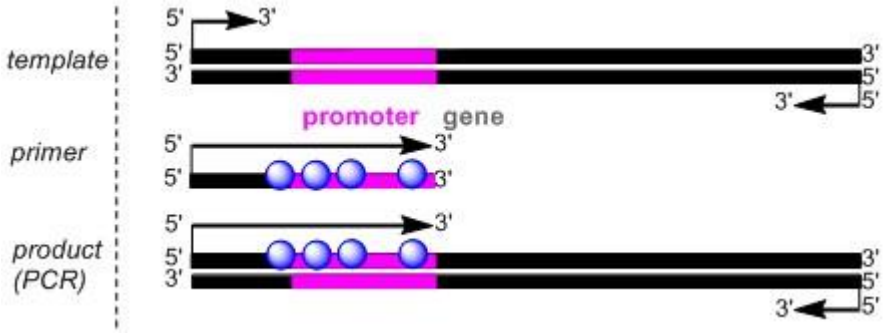


Figure S9. *Synthesis of DNA templates modified in the promoter region of the coding strand*

The synthesis of DNA modified in promoter regions of the coding strand was performed by PCR reaction using natural reverse primer and commercially synthesized dU-, hmU-, mC- or hmC-modified forward primer *(Figure S9)*.

1.2. Determination of (relative) concentrations of modified DNA templates

As the presence of modified nucleobases in the DNA templates presumably could strongly influence both extinction coefficients (for determination of concentration of DNA using NanoDrop) and intercallation and/or possible quenching of fluorescence of the DNA staining fluorescent dyes (i.e. GelRed), we needed to establish an independent and reliable method to determine the concentrations of modified DNA templates prepared by PCR.

To verify the concentrations of modified DNAs for the *in vitro* transcription assay, *^{32}P-labelled* and *NON-labelled* DNA templates were prepared, purified, and diluted to the apparent final concentration of 30 ng/µL according to NanoDrop. Then, 180 ng of DNA was loaded on a 5% PAA gel or 1.3% agarose gel. *^{32}P-labelled* DNA templates loaded on PAA gel were analyzed by gel electrophoresis with detection by radioactivity and *NON-labelled* templates were analysed on agarose gel with detection by GelRed. The relative intensities of the DNA bands on the gels did not correspond to the relative concentrations of the DNA samples as measured by the Nanodrop. Compared to the non-modified DNA, especially the bands of hmU-modified DNA were much weaker showing that its concentration must have been lower. Therefore, the concentration of the modified DNA templates could not be taken from a simple NanoDrop measurement of the UV absorbance.

However, when we compared the intensity of the DNA template spots on the gels using either ^{32}P-radioactivity detection or detection of fluorescence by GelRed staining, the correlation was satisfactory. Therefore, we used either staining by GelRed and fluorescence detection or the radioactive label for determination of relative concentrations of the modified and natural DNA templates in all the transcription studies.

Table S1: Determination of relative concentrations of *NON-labelled* (agarose) and *^{32}P*-radioactive labelled (PAA) DNA templates. T^+, as measured by the NanoDrop was set as 100 %. The percentages in parentheses are the relative amounts of the DNA as detected either by fluorescence or radioactivity compared to T^+. The calculated amounts in [ng] are listed.

Temp^{Pveg}		
	Agarose (*NON-labelled* templates)	**PAA (^{32}P-radioactive labelled templates)**
T^+	180 ng (100 %)	180 ng (100 %)
dU	177.5 ng (98.6 %)	151.7 ng (84.3 %)
hmU	121.6 ng (67.6 %)	96.8 ng (53 %)
C^+	180 ng (100 %)	180 ng (100 %)
mC	144.9 ng (80.5 %)	184 ng (102 %)
hmC	140.9 ng (78.3 %)	160.3 ng (93 %)
Temp^{rrnBP1}		
	Agarose (*NON-labelled* templates)	**PAA (^{32}P-radioactive labelled templates)**
T^+	180 ng (100 %)	180 ng (100 %)
dU	152.6 ng (84.7 %)	120.6 ng (67 %)
hmU	62.2 ng (34.5 %)	90.3 ng (50.2 %)
C^+	180 ng (100 %)	180 ng (100 %)
mC	202.5 ng (112 %)	172 ng (95.7 %)
hmC	195.9 ng (108 %)	217.6 ng (120.9 %)
Temp$^{Pveg\text{-}10DBP1}$		
	Agarose (*NON-labelled* templates)	**PAA (^{32}P-radioactive labelled templates)**
T^+	180 ng (100 %)	180 ng (100 %)
dU	108 ng (60.1%)	139.5 ng (77.5 %)
hmU	67 ng (37 %)	94.8 ng (52.7 %)
C^+	180 ng (100 %)	180 ng (100 %)
mC	146 ng (81 %)	136.9 ng (74.1 %)
hmC	121.6 ng (67.6 %)	121.7 ng (69.6 %)
Temp$^{rrnBP1\text{-}10Dveg}$		
	Agarose (*NON-labelled* templates)	**PAA (*^{32}P-radioactive labelled* templates)**
T^+	180 ng (100 %)	180 ng (100 %)
dU	126.7 ng (70.4 %)	143.4 ng (79.6 %)
hmU	54.8 ng (30.5 %)	77.7 ng (43 %)
C^+	180 ng (100 %)	180 ng (100 %)
mC	111.3 ng (61.8%)	138 ng (76.6 %)
hmC	135 ng (75%)	153 ng (85 %)

2. Experimental part

2.1. General remarks

Synthetic oligonucleotides (*NON-labelled* primers, FAM-labelled primers and *5´-phosphate* modified primers) were purchased from GeneriBiotech. Synthetic modified oligonucleotides (primers ***Prim***$^{FOR\text{-}PgII\text{-}X}$) were purchased from IBA Lifesciences. Natural nucleoside triphosphates (dATP, dCTP, dGTP, dTTP) and d5mCTP were bought from New England Biolabs, dUTP was purchased from Thermo Fischer Scientific, and d5hmCTP was purchased from TriLink Biotechnologies. d5hmUTP was prepared according to published procedure.[1] KOD XL DNA polymerase was purchased from Merck; Dynazyme II from Thermo Fischer Scientific; Vent (exo-) polymerase, Taq DNA polymerase and Taq DNA polymerase for ThermoPol buffer were purchased from New England Biolabs. Enzymes Lambda Exonuclease and T4 DNA Ligase were purchased from New England Biolabs. All PCR products and final DNA templates were purified on columns (QIAquick PCR Purification Kit and QIAquick Nucleotide Removal Kit from QIAGEN; E.Z.N.A. Gel Extraction Kit from Omega Bio-Tek) and/ or on Agencourt AMPure XP magnetic particles (Beckman Coulter Life Science - GE Healthcare).

2.2. Synthesis of fully modified 339-mers of DNA

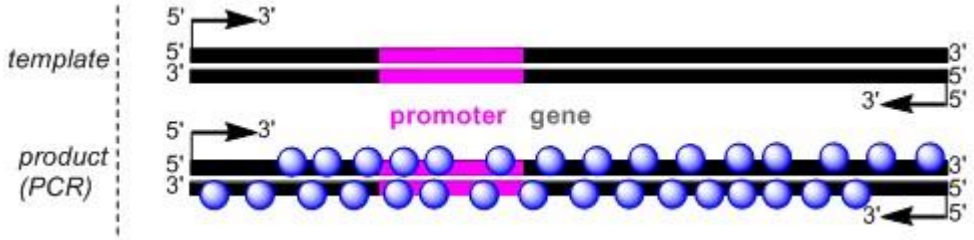


Table S2: *List of oligonucleotides used for synthesis of 339- and 340-mers*

Oligonucleotide-PRIMERs	**Sequence (5´→3´)**	**Length**
Prim^{FOR}	TAGGGGTTCCGCGCACATTTCCCCG	25-mer
Prim^{REV}	GGAGAGCGTTCACCGACAAACAACAG	26-mer
Prim^{FOR} – ^{32}P^{a}	TAGGGGTTCCGCGCACATTTCCCCG	25-mer
Prim^{REV} – ^{32}P^{a}	GGAGAGCGTTCACCGACAAACAACAG	26-mer
Oligonucleotide-TEMPLATEs[b,c]	TAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACC TGACGTCTAAGAAACCATTATTATCATGACATTAACCT ATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCA AGAATTC*NNNNNNNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNN(N)*GTCTAAGCTTGGGTCCCACCT GACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGC GCCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTA GGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCA GTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTG TCGGTGAACGCTCTCC	339-mer or 340-mer
Promoter regions[d]		
Temp^{Pveg}	*TATTTGACAAAAATGGGCTCGTGTTGTACAATAAATGT*	339-mer[e]
Temp$^{rrnB\ P1}$	*CTATTGCAATAAATAAATACAGGTGTTATATTATTAAAC*	340-mer[e]
Temp$^{Pveg\text{-}10DBP1}$	*TATTTGACAAAAATGGGCTCGTGTTGTATATTATTAAAC*	340-mer[e]
Temp$^{rrnB\ P1\text{-}10Dveg}$	*CTATTGCAATAAATAAATACAGGTGTTACAATAAATGT*	339-mer[e]

[a] 5´-end of oligonucleotide is radiolabelled with phosphate from (γ)-^{32}P-dATP

[b] primer sequences in template are underlined

[c] promoter sequence of templates is generally marked by *N*s in italic

[d] all four templates have the same promoter-upstream and -downstream sequences. The specific sequences of promoter region for all four templates (instead of the *N*s in the template sequence) are summarised in the table.

[e] the final length of templates

2.3. Enzymatic synthesis of templates (*Temp^{Pveg}*; *Temp$^{rrnB\ P1}$*; *Temp$^{Pveg\text{-}10DBP1}$*; *Temp$^{rrnB\ P1\text{-}10Dveg}$*)

The 339-mer (in the case of ***Temp^{Pveg}***and ***Temp$^{rrnB\ P1\text{-}10Dveg}$***) and 340-mer (in the case of ***Temp$^{rrnB\ P1}$***and ***Temp$^{Pveg\text{-}10DBP1}$***) templates were prepared by PCR with forward (***Prim^{FOR}***) and reverse (***Prim^{REV}***) *NON-labelled* primers from plasmids containing specific promoter regions cloned in p770 between *Eco*RI and *Hind*III sites.[2] Each PCR reaction mixture (40 µL) contained Taq DNA polymerase for ThermoPol buffer (New England Biolabs; 5000 U/mL; 2.4 µL) with ThermoPol buffer (4 µL), natural dNTPs (4 mM; 1.5 µL), primers (20 µM; 6 µL; ***Prim^{FOR}*** and 20 µM; 6 µL; ***Prim^{REV}***) and appropriate plasmid template (88 ng of ***Pveg***; 86 ng of ***BP1***; 77 ng of ***PgBP1*** and 77 ng of ***BP1Pg***). Total volume of each reaction mixture was divided to 2 Eppendorf tubes and 40 PCR cycles were run in the thermal cycler under the following conditions: preheating for 3 minutes at 94°C, denaturation for 1 minute at 94°C, annealing for

1 minute at 62°C, extension for 1.5 minutes at 75°C, followed by final extension step of 5 minutes at 75°C. PCR products of each type were then mixed together and purified on Agencourt AMPure XP magnetic particles (*see* **2.3.1.**). In the last step, the products were eluted with 40 µL of MilliQ water in final concentrations 99 ng/µL of $\boldsymbol{Temp^{Pveg}}$; 117 ng/ µL of $\boldsymbol{Temp^{BP1}}$; 109 ng/µL of $\boldsymbol{Temp^{PgBP1}}$ and 115 ng/µL of $\boldsymbol{Temp^{BP1Pg}}$. After purification, all templates were run on 1.3% agarose gels and stained with GelRed in 0.5xTBE. 180 ng of DNA was loaded on a gel in all cases *(Figure S10)*.

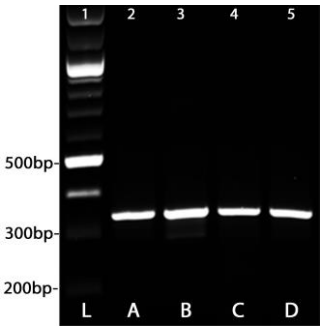


Figure S10: Agarose gel analysis of PCR template products amplified by Taq DNA polymerase. Lane 1 (**L**): ladder (commercial mix of dsDNA with the specific length); Lane 2 (**A**): PCR product of template - $\boldsymbol{Temp^{Pveg}}$); Lane 3 (**B**): PCR product of template - $\boldsymbol{Temp^{rrnB\ P1}}$; Lane 4 (**C**): PCR product of template - $\boldsymbol{Temp^{Pveg\text{-}10DBP1}}$; Lane 5 (**D**): PCR product of template - $\boldsymbol{Temp^{rrnB\ P1\text{-}10Dveg}}$; agarose gel stained with GelRed.

2.3.1. Protocol for purification of DNA on Agencourt AMPure XP magnetic particles

Each DNA sample was transferred after PCR from the PCR tube into a 1.5 mL Eppendorf tube and resuspended Agencourt AMPure XP magnetic particles were added (the volume of Agencourt AMPure XP was 1.8x the volume of the sample). Mixtures of samples and magnetic particles were mixed 10 to 20 times with pipette and incubated at room temperature for 10 minutes. The mixtures were placed on magnet. After separation of magnetic beads from solution (approx. 3 minutes), the solution was discarded and the magnetic beads with bound DNA were washed two times with 200 µL of 80% ethanol. During the washing, the magnetic beads were incubated for 30-60 seconds at room temperature with the ethanol. After the second washing and discarding of ethanol, the DNA product was eluted with 40 µL of MilliQ water from magnetic particles. The mixture of magnetic beads and water was mixed 10 to 20 times with pipette, incubated at room temperature for 3 minutes and placed on a magnet for 1 minute to separate beads from the solution containing DNA. The eluant was transferred into a new Eppendorf tube and its concentration was measured by the NanoDrop spectrophotometer.

2.4. Synthesis of fully modified DNA with different templates ($\boldsymbol{Temp^{Pveg}}$; $\boldsymbol{Temp^{rrnB\ P1}}$; $\boldsymbol{Temp^{Pveg\text{-}10DBP1}}$; $\boldsymbol{Temp^{rrnB\ P1\text{-}10Dveg}}$) – general remarks

To prepare modified templates for transcription, PCR reactions were performed in a total final volume of 20 µL. Natural DNAs (positive controls on gels) were in all cases synthesized

according to a procedure for hmU-modified or xC-modified DNA without any additives and in the presence of all natural dNTPs (4 mM; 0.75 µL) *(Figures S12, S14, S16, S18, S21)*. For xN – modified DNA, where the protocol for synthesis of modified DNA was significantly different, the positive controls were synthesized under the same conditions - in the presence of all natural dNTPs (4 mM; 0.75 µL) *(Figures S11, S13, S15, S17, S20)*.

All samples were prepared in the presence of either *NON-labelled* (***Prim^{FOR}*** and ***Prim^{REV}***) or *^{32}P-labelled* (***Prim^{FOR} – ^{32}P*** and ***Prim^{REV} – ^{32}P***) primers under the same conditions.
The ^{32}P-labelling of primers was achieved by the transfer of the terminal phosphate from (γ)-^{32}P-dATP to the 5´-end of *NON-labelled* primers by T4 polynucleotide kinase.
Forty PCR cycles were run in the thermal cycler under the following conditions: preheating for 3 minutes at 94°C, denaturation for 1 minute at 94°C, annealing for 1 minute at 70°C, extension for 1.5 minutes at 75°C, followed by final extension step of 5 minutes at 75°C.
PCR products of each type were then purified with Agencourt AMPure XP magnetic particles according to the protocol (*see* **2.3.1.**). The concentration of purified samples was measured by the NanoDrop spectrophotometr. All samples were diluted to a final concentration of 20 ng/uL and used for transcription experiments. The exact quantity of DNA for transcription was analysed based on the DNA signals (fluorescence and/or radioactivity) on a gel (*see* **5.2.**).
Control 1.3% agarose gels were stained with GelRed (Biotium) in the case of *NON-labelled* and *^{32}P-labelled* DNA samples *(gels A) andB) in Figures S12, S14, S16, S18, S21)*. Moreover, *^{32}P-labelled* DNA samples were detected by 5% native PAA gel *(gels C) in Figures S12, S14, S16, S18, S21)*. 180ng of each sample was loaded on a 1.3% agarose gel in all cases.

2.4.1. Synthesis of fully modified DNA with the *Temp^{Pveg}* template

2.4.1.1. Synthesis of fully dU-modified DNA with the *Temp^{Pveg}* template

The PCR reaction mixture (20 µL) contained Taq DNA polymerase for ThermoPol Buffer (*New England Biolabs*; 5000 U/mL; 2.4 µL), natural dNTPs (dCTP, dGTP and dATP; 4 mM; 1 µL), dUTP (4 mM; 1 µL), 339-mer template (30 ng/µL; 1.6 µL; ***Temp^{Pveg}***) and primers either *NON-labelled* or *^{32}P-labelled* (20 µM; 3 µL; ***Prim^{FOR}*** and 20 µM; 3 µL; ***Prim^{REV}***) in 10x ThermoPol reaction Buffer (2 µL) supplied by the manufacturer *(Figures S11, S12)*.

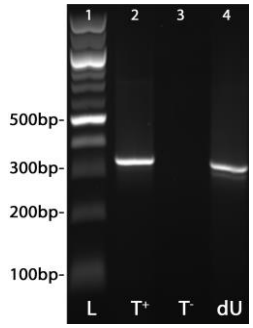


Figure S11: Agarose gel analysis of *NON-labelled* PCR products amplified by Taq DNA polymerase for ThermoPol buffer. The order of samples is as follows: Lane 1 (**L**): ladder (mix of commercial dsDNA with specific length); Lane 2 (**T^+**): PCR was run in the presence of natural dNTPs; Lane 3 (**T^-**): PCR was run in the presence of dCTP, dGTP, dATP; Lane 4, (**dU**): PCR was run in the presence of three natural dNTPs (dCTP, dGTP, dATP) and **dUTP**. 1.3 % agarose gel was stained with GelRed.

2.4.1.2. Synthesis of fully hmU-modified DNA with the *Temp^{Pveg}* template

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 1.2 µL), natural dNTPs (dCTP, dGTP and dATP; 4mM; 0.75 µL), d5hmUTP (4 mM; 1.5 µL), 339-mer template (30 ng/µL; 1.2 µL; ***Temp^{Pveg}***), $MgSO_4$ (100 mM; 2 µL) and primers - either *NON-labelled* or *^{32}P-labelled* (20 µM; 3µL; ***Prim^{FOR}*** and 20 µM; 3 µL; ***Prim^{REV}***) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figure S12)*.

2.4.1.3. Synthesis of fully mC- and hmC-modified DNA with the *Temp^{Pveg}* template

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/ µL; 1.2 µL), natural dNTPs (dTTP, dGTP and dATP; 4 mM; 0.75 µL), d5mCTP (4 mM; 2 µL) or d5hmCTP (4 mM; 1.5 µL), 339-mer template (30 ng/µL; 1.2 µL; ***Temp^{Pveg}***) and primers - either *NON-labelled* or *^{32}P-labelled* (20 µM; 3 µL; ***Prim^{FOR}*** and 20 µM; 3 µL; ***Prim^{REV}***) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figure S12)*.

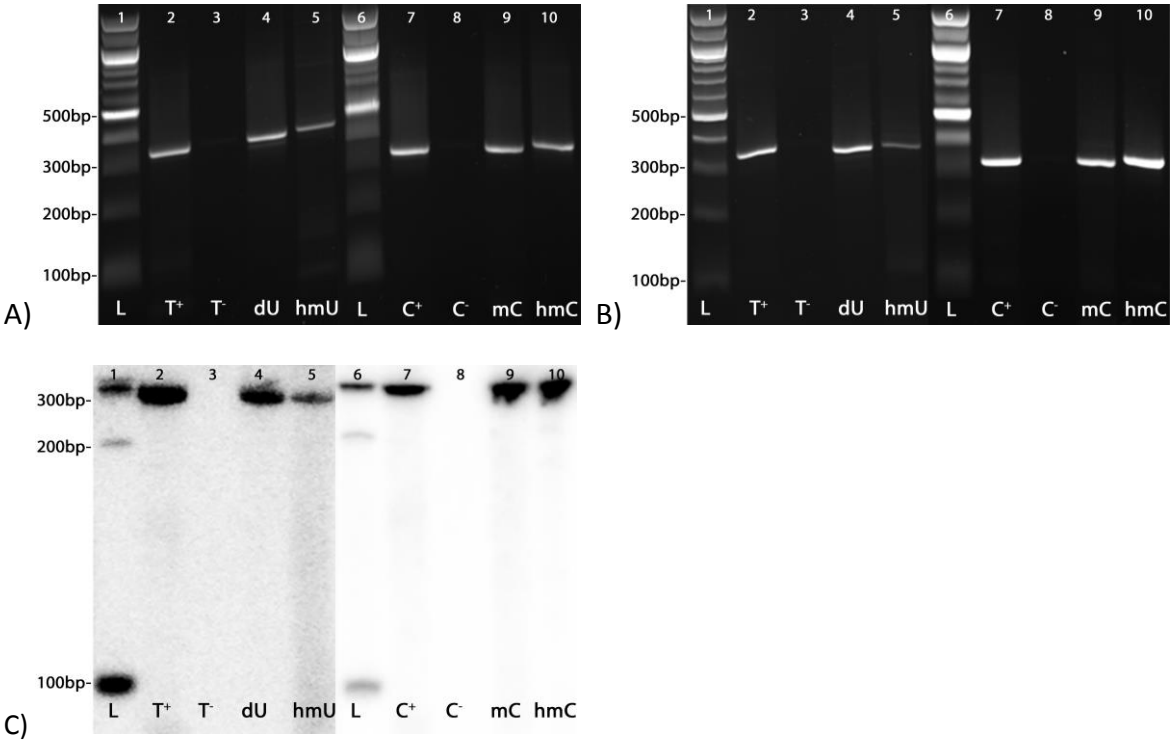


Figure S12: Gel analysis of PCR products that were either *NON-labelled* **A**) or *^{32}P-labelled* **B**) and **C**). In all gels, the order of samples is as follows: Lanes 1,6 (**L**): ladder (mix of commercial dsDNA with specific length); Lanes 2,7 (**T^+** or **C^+**): PCRs were run in the presence of natural dNTPs; Lane 3 (**T^-**): PCR was run in the presence of dCTP, dGTP, dATP; Lane 4, (**dU**): PCR was run in the presence of three natural dNTPs (dCTP, dGTP, dATP) and **dUTP**; Lane 5, (**hmU**): PCR was run in the presence of three natural dNTPs (dCTP, dGTP, dATP) and **d5hmUTP**; Lane 8 (**C^-**): PCR

was run in the presence of dTTP, dGTP, dATP; Lane 9, (**mC**): PCR was run in the presence of three natural dNTPs (dTTP, dGTP, dATP) and **d5mCTP**; Lane 10, (**hmC**): PCR was run in the presence of three natural dNTPs (dTTP, dGTP, dATP) and **d5hmCTP**. The PCR samples were analysed by agarose gel stained with GelRed in the case of **A**) and **B**) and moreover ^{32}*P-labelled* samples were analysed by 5% PAA native gel **C)**.

2.4.2. Synthesis of fully modified DNA with the *Temp$^{rrnB\ P1}$* template

2.4.2.1. Synthesis of fully dU-modified DNA with the *Temp$^{rrnB\ P1}$* template

The PCR reaction mixture (20 µL) contained Taq DNA polymerase for ThermoPol Buffer (*New England Biolabs*; 5000 U/mL; 2.4 µL), natural dNTPs (dCTP, dGTP and dATP; 4 mM; 1 µL), dUTP (4 mM; 1 µL), 340-mer template (24 ng/µL; 1.6 µL; ***Temp$^{rrnB\ P1}$***) and primers - either *NON-labelled* or ^{32}*P-labelled* (20 µM; 3 µL; ***Prim^{FOR}*** and 20 µM; 3 µL; ***Prim^{REV}***) in 10x ThermoPol reaction Buffer (2 µL) supplied by the manufacturer *(Figures S13, S14)*.

2.4.2.2. Synthesis of fully hmU-modified DNA with the *Temp$^{rrnB\ P1}$* template

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 1.2 µL), natural dNTPs (dCTP, dGTP and dATP; 4 mM; 1 µL), d5hmUTP (4 mM; 1 µL), 340-mer template (24 ng/µL; 1.6 µL; ***Temp$^{rrnB\ P1}$***), $MgSO_4$ (100 mM; 0.7 µL) and primers - either *NON-labelled* or ^{32}*P-labelled* (20 µM; 3 µL; ***Prim^{FOR}*** and 20 µM; 3 µL; ***Prim^{REV}***) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figures S13, S14)*.

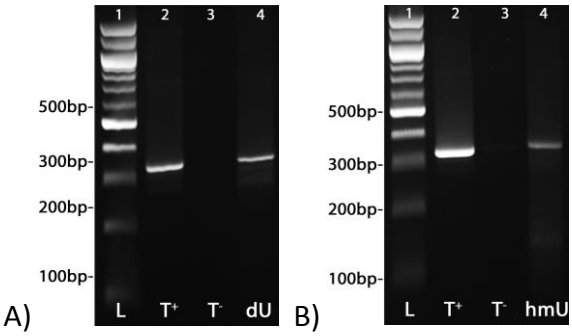


Figure S13: Agarose gel analysis of *NON-labeled* PCR products amplified by Taq DNA polymerase **A**) under the conditions 2.4.2.1 and by KOD XL DNA polymerase **B**) under the conditions 2.4.2.2. The order of samples is as follows: Lanes 1 (**L**): ladder (mix of commercial dsDNA with specific length); Lanes 2 (**T$^+$**): PCR was run in the presence of natural dNTPs; Lanes 3 (**T$^-$**): PCR was run in the presence of dCTP, dGTP, dATP; **A**) Lane 4, (**dU**): PCR was run in the presence of three natural dNTPs (dCTP, dGTP, dATP) and **dUTP**; **B)** Lane 4, (**hmU**): PCR was run in the presence of three natural dNTPs (dCTP, dGTP, dATP) and **d5hmUTP**. 1,3% agarose gel was stained with GelRed.

2.4.2.3. Synthesis of fully mC- and hmC-modified DNA with the *Temp$^{rrnB\ P1}$* template

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µl; 1.2 µL), natural dNTPs (dTTP, dGTP and dATP; 4 mM; 0.75 µL), d5mCTP (4 mM; 2 µL) or d5hmCTP (4 mM; 1.5 µL), 340-mer template (24 ng/µL; 1.6 µL; ***Temp$^{rrnB\ P1}$***) and primers

- either *NON-labelled* or ^{32}*P-labelled* (20 µM; 3 µL; ***Prim^{FOR}*** and 20 µM; 3 µL; ***Prim^{REV}***) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figure S14)*.

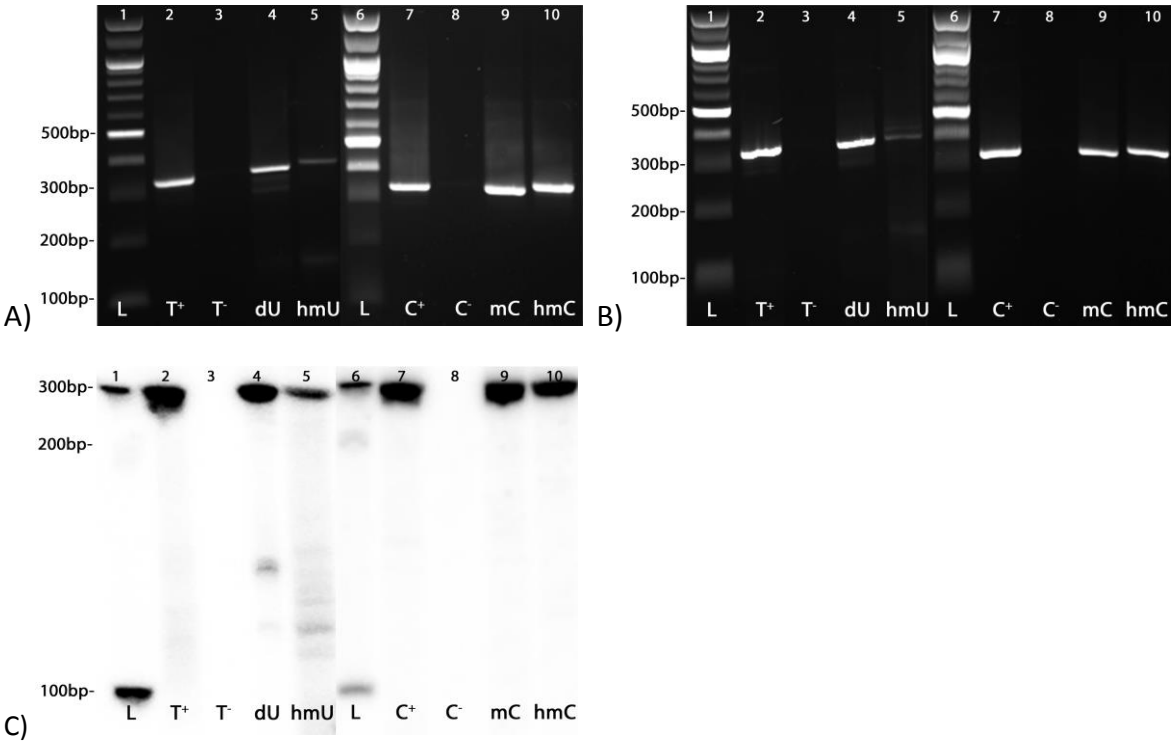


Figure S14: Gel analysis of PCR products that were either *NON-labelled* **A**) or ^{32}*P-labelled* **B**) and **C**). In all gels, the order of samples is as follows: Lanes 1,6 (**L**): ladder (mix of commercial dsDNA with specific length); Lanes 2,7 (**T^{+}** or **C^{+}**): PCRs were run in the presence of natural dNTPs; Lane 3 (**T^{-}**): PCR was run in the presence of dCTP, dGTP, dATP; Lane 4, (**dU**): PCR was run in the presence of three natural dNTPs (dCTP, dGTP, dATP) and **dUTP**; Lane 5, (**hmU**): PCR was run in the presence of three natural dNTPs (dCTP, dGTP, dATP) and **d5hmUTP**; Lane 8 (**C^{-}**): PCR was run in the presence of dTTP, dGTP, dATP; Lane 9, (**mC**): PCR was run in the presence of three natural dNTPs (dTTP, dGTP, dATP) and **d5mCTP**; Lane 10, (**hmC**): PCR was run in the presence of three natural dNTPs (dTTP, dGTP, dATP) and **d5hmCTP**. The PCR samples were analysed by agarose gel stained with GelRed in the case of **A**) and **B**) and moreover ^{32}*P-labelled* samples were analysed by 5% PAA native gel **C)**.

2.4.3. Synthesis of fully modified DNA with the *Temp$^{Pveg-10DBP1}$* template

2.4.3.1. Synthesis of fully dU-modified DNA with the *Temp$^{Pveg-10DBP1}$* template

The PCR reaction mixture (20 µL) contained Taq DNA polymerase for ThermoPol Buffer (*New England Biolabs*; 5000 U/ml; 2.4 µL), natural dNTPs (dCTP, dGTP and dATP; 4 mM; 1 µL), dUTP (4 mM; 1 µL), 340-mer template (24 ng/µL; 1.6 µL; ***Temp$^{Pveg-10DBP1}$***) and primers - either *NON-labelled* or ^{32}*P-labelled* (20 µM; 2 µL; ***Prim^{FOR}*** and 20 µM; 2 µL; ***Prim^{REV}***) in 10x ThermoPol reaction Buffer (2 µL) supplied by the manufacturer *(Figures S15, S16)*.

2.4.3.2. Synthesis of fully hmU-modified DNA with the *Temp$^{Pveg-10DBP1}$* template

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 1.2 µL), natural dNTPs (dCTP, dGTP and dATP; 4 mM; 0.75 µL), d5hmUTP (4 mM; 1.5

µL), 340-mer template (24 ng/µL; 1.6 µL; ***Temp$^{Pveg\text{-}10DBP1}$***), $MgSO_4$ (100 mM; 2 µL) and primers - either *NON-labelled* or *^{32}P-labelled* (20 µM; 2 µL; ***Prim^{FOR}*** and 20 µM; 2 µL; ***Prim^{REV}***) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figure S16)*.

2.4.3.3. Synthesis of fully hmC-modified DNA with the *Temp$^{Pveg\text{-}10DBP1}$* template

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µl; 1.2 µL), natural dNTPs (dTTP, dGTP and dATP; 4 mM; 1 µL), d5hmCTP (4 mM; 2 µL), 340-mer template (24 ng/µL; 1.6 µL; ***Temp$^{Pveg\text{-}10DBP1}$***), $MgSO_4$ (100 mM; 2 µL) and primers - either *NON-labelled* or *^{32}P-labelled* (20 µM; 3 µL; ***Prim^{FOR}*** and 20 µM; 3 µL; ***Prim^{REV}***) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figures S15, S16)*.

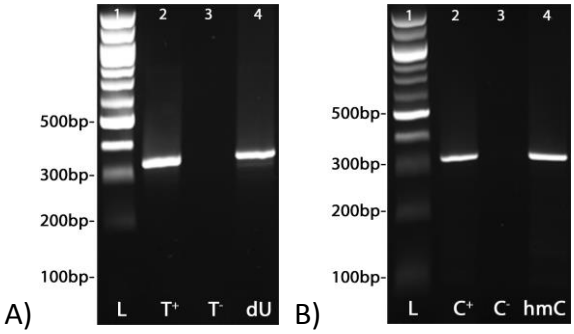
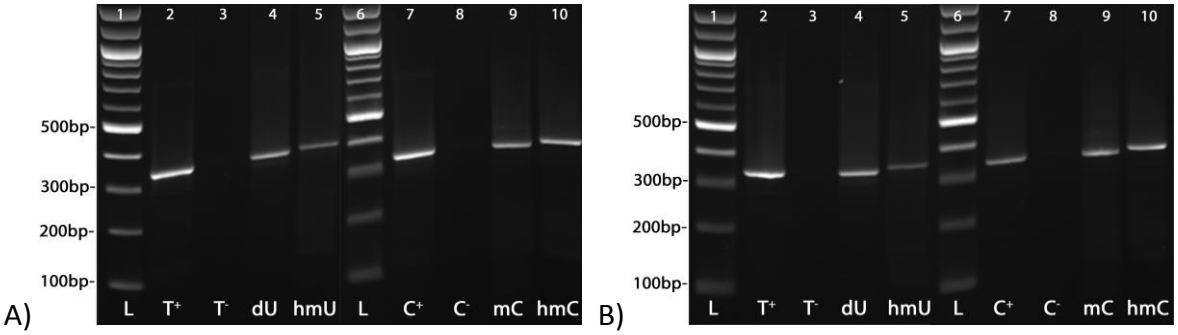
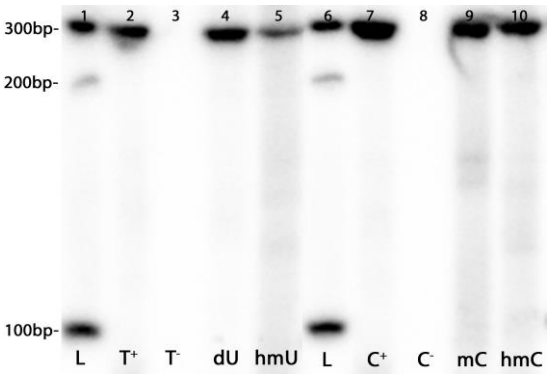


Figure S15: Agarose gel analysis of *NON-labelled* PCR products amplified by Taq DNA polymerase **A**) under the condition 2.4.3.1 and by KOD XL DNA polymerase **B**) under the conditions 2.4.3.3. The order of samples is as follows: Lanes 1 (**L**): ladder (mix of commercial dsDNA with specific length); Lane 2 (**T$^+$ or C$^+$**): PCR was run in the presence of natural dNTPs; **A)** Lane 3 (**T$^-$**): PCR was run in the presence of dCTP, dGTP, dATP; **A)** Lane 4, (**dU**): PCR was run in the presence of three natural dNTPs (dCTP, dGTP, dATP) and **dUTP**; **B)** Lane 3 (**C$^-$**): PCR was run in the presence of dTTP, dGTP, dATP; **B)** Lane 4, (**hmC**): PCR was run in the presence of three natural dNTPs (dTTP, dGTP, dATP) and **d5hmCTP**. 1.3 % agarose gel was stained with GelRed.

2.4.3.4. Synthesis of fully mC-modified DNA with the *Temp$^{Pveg\text{-}10DBP1}$* template

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µl; 1.2 µL), natural dNTPs (dTTP, dGTP and dATP; 4 mM; 0.75 µL), d5mCTP (4 mM; 2 µL), 340-mer template (24 ng/µL; 1.6 µL; ***Temp$^{Pveg\text{-}10DBP1}$***), $MgSO_4$ (100 mM; 1 µL) and primers - either *NON-labelled* or *^{32}P-labelled* (20 µM; 2 µL; ***Prim^{FOR}*** and 20 µM; 2 µL; ***Prim^{REV}***) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figure S16)*.





C)

Figure S16: Gel analysis of PCR products that were either *NON-labelled* **A**) or *^{32}P-labelled* **B**) and **C**). In all gels, the order of samples is as follows: Lanes 1,6 (**L**): ladder (mix of commercial dsDNA with specific length); Lanes 2,7 (**T^{+}** or **C^{+}**): PCRs were run in the presence of natural dNTPs; Lane 3 (**T^{-}**): PCR was run in the presence of dCTP, dGTP, dATP; Lane 4, (**dU**): PCR was run in the presence of three natural dNTPs (dCTP, dGTP, dATP) and **dUTP**; Lane 5, (**hmU**): PCR was run in the presence of three natural dNTPs (dCTP, dGTP, dATP) and **d5hmUTP**; Lane 8 (**C^{-}**): PCR was run in the presence of dTTP, dGTP, dATP; Lane 9, (**mC**): PCR was run in the presence of three natural dNTPs (dTTP, dGTP, dATP) and **d5mCTP**; Lane 10, (**hmC**): PCR was run in the presence of three natural dNTPs (dTTP, dGTP, dATP) and **d5hmCTP**. The PCR samples were analysed by agarose gel stained with GelRed in the case of **A**) and **B**) and moreover *^{32}P-labelled* samples were analysed by 5% PAA native gel **C)**.

2.4.4. Synthesis of fully modified DNA with *the Temp$^{rrnB\ P1\text{-}10Dveg}$* template

2.4.4.1. Synthesis of fully dU-modified DNA with *the Temp$^{rrnB\ P1\text{-}10Dveg}$* template

The PCR reaction mixture (20 µL) contained Taq DNA polymerase for ThermoPol Buffer (*New England Biolabs*; 5000 U/mL; 2.4 µL), natural dNTPs (dCTP, dGTP and dATP; 4 mM; 1 µL), dUTP (4 mM; 1 µL), 339-mer template (24 ng/µL; 1.6 µL; ***Temp$^{rrnB\ P1\text{-}10Dveg}$***) and primers - either *NON-labelled* or *^{32}P-labelled* (20 µM; 2 µL; ***Prim^{FOR}*** and 20 µM; 2 µL; ***Prim^{REV}***) in 10x ThermoPol reaction Buffer (2 µL) supplied by the manufacturer *(Figures S17, S18)*.

2.4.4.2. Synthesis of fully hmU-modified DNA with *the Temp$^{rrnB\ P1\text{-}10Dveg}$* template

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µl; 1.2 µL), natural dNTPs (dCTP, dGTP and dATP; 4 mM; 0.75 µL), d5hmUTP (4 mM; 1.5 µL), 339-mer template (24 ng/µL; 1.6 µL; ***Temp$^{rrnB\ P1\text{-}10Dveg}$***), $MgSO_4$ (100 mM; 2µL) and primers - either *NON-labelled* or *^{32}P-labelled* (20 µM; 2 µL; ***Prim^{FOR}*** and 20 µM; 2 µL; ***Prim^{REV}***) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figure S18)*.

2.4.4.3. Synthesis of fully hmC-modified DNA with *the Temp$^{rrnB\ P1\text{-}10Dveg}$* template

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µl; 1.2 µL), natural dNTPs (dTTP, dGTP and dATP; 4 mM; 1 µL), d5hmCTP (4 mM; 1.5 µL), 339-mer template (24 ng/µL; 1.6 µL; ***Temp$^{rrnB\ P1\text{-}10Dveg}$***), $MgSO_4$ (100 mM; 1 µL) and primers - either *NON-labelled* or *^{32}P-labelled* (20 µM; 2 µL; ***Prim^{FOR}*** and 20 µM; 2 µL; ***Prim^{REV}***) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figures S17, S18)*.

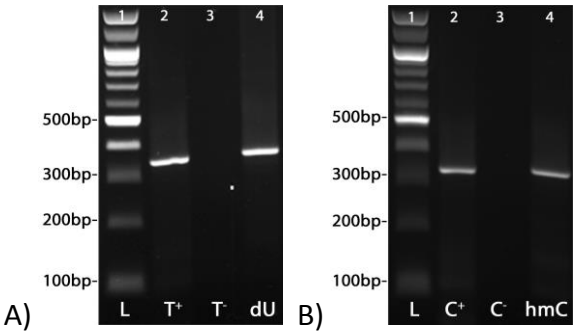


Figure S17: Agarose gel analysis of *NON-labelled* PCR products amplified by Taq DNA polymerase **A**) under the condition 2.4.4.1 and by KOD XL DNA polymerase **B**) under the conditions 2.4.4.3. The order of samples is as follows: Lanes 1 (**L**): ladder (mix of commercial dsDNA with specific length); Lane 2 (**T^+ or C^+**): PCR was run in the presence of natural dNTPs; **A)** Lane 3 (**T^-**): PCR was run in the presence of dCTP, dGTP, dATP; **A)** Lane 4, (**dU**): PCR was run in the presence of three natural dNTPs (dCTP, dGTP, dATP) and **dUTP**; **B)** Lane 3 (**C^-**): PCR was run in the presence of dTTP, dGTP, dATP; **B)** Lane 4, (**hmC**): PCR was run in the presence of three natural dNTPs (dTTP, dGTP, dATP) and **d5hmCTP**. 1,3 % agarose gel was stained with GelRed.

2.4.4.4. Synthesis of fully mC-modified DNA with *the Temp$^{rrnB\ P1\text{-}10Dveg}$* template

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µl; 1.2 µL), natural dNTPs (dTTP, dGTP and dATP; 4 mM; 0.75 µL), d5mCTP (4 mM; 2 µL), 339-mer template (24 ng/µL; 1.6 µL; ***Temp$^{rrnB\ P1\text{-}10Dveg}$***), $MgSO_4$ (100 mM; 1 µL) and primers - either *NON-labelled* or *^{32}P-labelled* (20 µM; 2 µL; ***Prim^{FOR}*** and 20 µM; 2 µL; ***Prim^{REV}***) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figure S18)*.

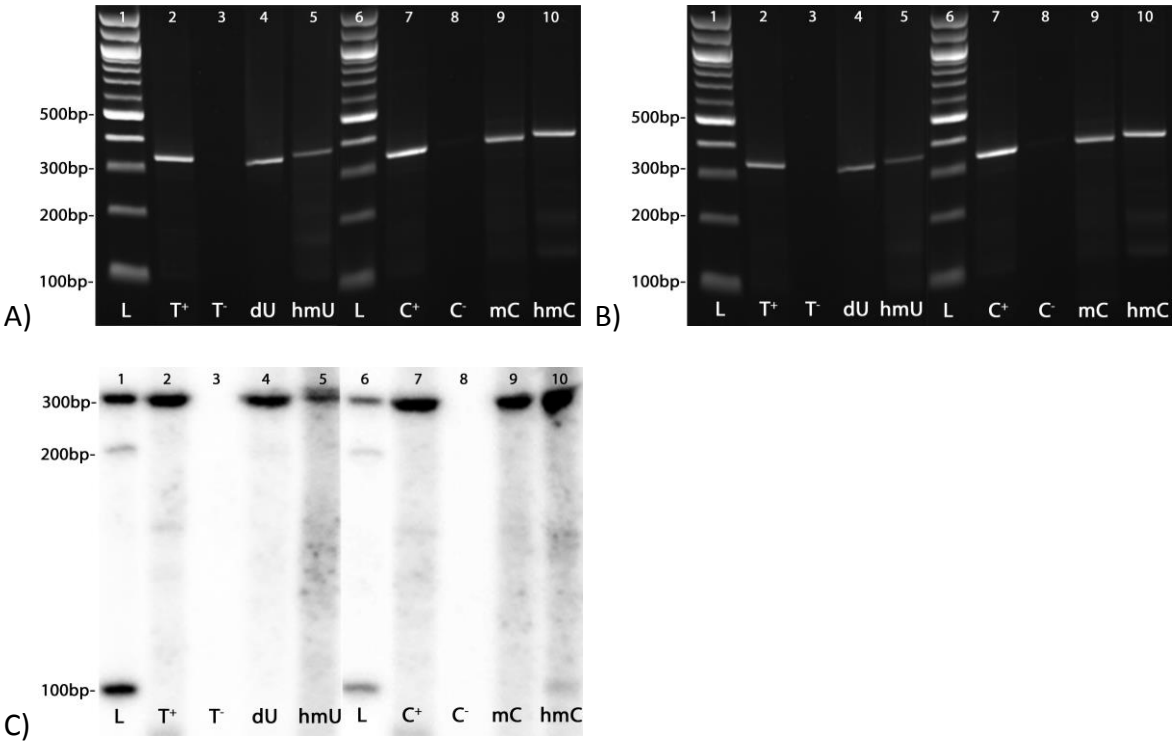


Figure S18: Gel analysis of PCR products that were either *NON-labelled* **A**) or *^{32}P-labelled* **B**) and **C**). In all gels, the order of samples is as follows: Lanes 1,6 (**L**): ladder (mix of commercial dsDNA with specific length); Lanes 2,7 (**T^+** or **C^+**): PCRs were run in the presence of natural dNTPs; Lane 3 (**T^-**): PCR was run in the presence of dCTP, dGTP,

dATP; Lane 4, (**dU**): PCR was run in the presence of three natural dNTPs (dCTP, dGTP, dATP) and **dUTP**; Lane 5, (**hmU**): PCR was run in the presence of three natural dNTPs (dCTP, dGTP, dATP) and **d5hmUTP**; Lane 8 (**C**$^-$): PCR was run in the presence of dTTP, dGTP, dATP; Lane 9, (**mC**): PCR was run in the presence of three natural dNTPs (dTTP, dGTP, dATP) and **d5mCTP**; Lane 10, (**hmC**): PCR was run in the presence of three natural dNTPs (dTTP, dGTP, dATP) and **d5hmCTP**. The PCR samples were analysed by agarose gel stained with GelRed in the case of **A**) and **B**) and moreover ^{32}*P-labelled* samples were analysed by 5% PAGE native gel **C)**.

3. Synthesis of fully modified 235-mers of DNA

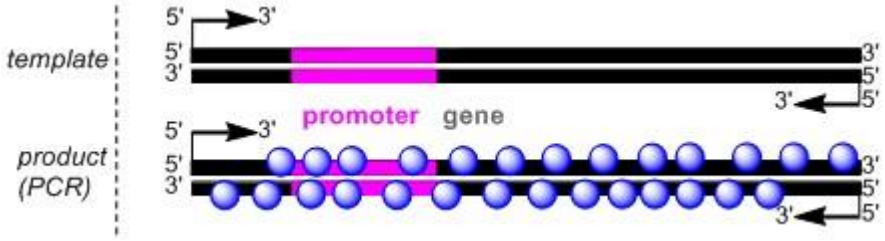


Table S3: *List of oligonucleotides used for synthesis of 235-mers (version Ib)*

Oligonucleotide-PRIMERs	**Sequence (5´→3´)**	**Length**
***Prim*$^{FOR\text{-}PgII}$**	CGT CTT CAA GAA TTC TAT	18-mer
***Prim*$^{REV\text{-}PgII\text{-}R1/R3}$**	GGA GAG CGT TCA CCG ACA	18-mer
Oligonucleotide– TEMPLATE^{a,b}		
***Temp*$^{Pveg\_sh235}$**	CGTCTTCAAGAATTCTAT*TTGACAAAAATGGGCTCGT* *GTTGTACAATAAATGT*GTCTAAGCTTGGGTCCCACCT GACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGC GCCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTA GGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCA GTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTG TCGGTGAACGCTCTCC	235-mer

[a] primer sequences in template are underlined

[b] promoter sequence of template is in italic

3.1. Enzymatic synthesis of template (*Temp*$^{Pveg\_sh235}$)

The 235-mer template (***Temp*$^{Pveg\_sh235}$**) was prepared by PCR with forward (***Prim*$^{FOR\text{-}PgII}$**) and reverse (***Prim*$^{REV\text{-}PgII\text{-}R1/R3}$**) *NON-labelled* primers from the longer ***Temp*^{Pveg}** (339-mer) template containing the P*veg* promoter region. PCR reaction mixture (40 µL) contained Taq DNA polymerase for ThermoPol Buffer (*New England Biolabs*; 5000 U/mL; 2.4 µL) with ThermoPol buffer (4 µL), natural dNTPs (4 mM; 1.5 µL), primers (20 µM, 6 µL, ***Prim*$^{FOR\text{-}PgII}$** and 20 µM, 6 µL, ***Prim*$^{REV\text{-}PgII\text{-}R1/R3}$**) and P*veg* template - ***Temp*^{Pveg}** (72 ng). The reaction mixture was divided into 2 Eppendorf tubes and 40 PCR cycles were run in the thermal cycler as follows: preheating for 3minutes at 94°C, denaturation for 1 minute at 94°C, annealing for 1 minute at 52°C, extension for 1.5 minutes at 75°C, followed by a final extension step of 5 minutes at 75°C. The PCR products were then combined together and purified by Agencourt AMPure XP magnetic

beads according to the protocol (*see* **2.3.1**.). In the last step, the product was eluted with 40 µL of MilliQ water at a final concentration 113 ng/µL. After purification the template was analysed by 1.3% agarose gel stained with GelRed in 0.5xTBE *(Figure S19)*.

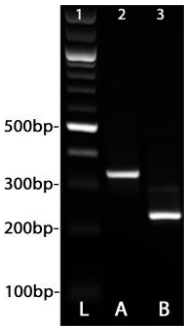


Figure S19: Agarose gel analysis of PCR template product amplified by Taq DNA polymerase. The order of samples is as follow: Lane 1 (**L**): ladder (commercial mix of dsDNA with the specific length); Lane 2 (**A**): 339-mer Pveg template- $\boldsymbol{Temp^{Pveg}}$ used as template for the PCR reaction; Lane 3 (**B**): PCR product of template - 235-mer - $\boldsymbol{Temp^{Pveg\_sh235}}$. 1,3% agarose gel was stained with GelRed

3.2. Synthesis of fully modified DNA with 235-mer template ($\boldsymbol{Temp^{Pveg\_sh235}}$)-general remarks

To prepare modified templates for transcription, PCR reactions were performed in a total final volume of 20 µL. All samples were prepared in the presence of *NON-labelled* commercially available primers ($\boldsymbol{Prim^{FOR\text{-}PgII}}$ and $\boldsymbol{Prim^{REV\text{-}PgII\text{-}R1/R3}}$).
Forty PCR cycles were run in the thermal cycler under the following conditions: preheating for 3 minutes at 94°C, denaturation for 1 minute at 94°C, annealing for 1 minute at 52°C, extension for 1.5 minutes at 75°C, followed by final extension step of 5 minutes at 75°C. PCR products were purified on Agencourt AMPure XP magnetic beads according to the protocol (*see* **2.3.1**.). In the last step, the product was eluted with 40 uL of MilliQ water. Control 1.3% agarose gels were stained by GelRed.

3.2.1. Synthesis of fully dU-modified DNA

The PCR reaction mixture (20 µL) contained Taq DNA polymerase for ThermoPol Buffer (*New England Biolabs*; 5000 U/ml; 2.4 µL), natural dNTPs (dCTP, dGTP and dATP; 4 mM; 1 µL), dUTP (4 mM; 1 µL), 235-mer template (41 ng/µL; 0.88 µL; $\boldsymbol{Temp^{Pveg\_sh235}}$) and primers - either *NON-labelled* (20 µM; 2 µL; $\boldsymbol{Prim^{FOR\text{-}PgII}}$ and 20 µM; 2 µL $\boldsymbol{Prim^{REV\text{-}PgII\text{-}R1/R3}}$) in 10x ThermoPol reaction Buffer (2 µL) supplied by the manufacturer *(Figure S20, S21.)*

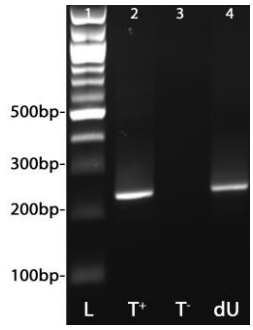


Figure S20: Agarose gel analysis of PCR products amplified by Taq DNA polymerase. The order of samples is as follow: Lane 1 (**L**): ladder (mix of commercial dsDNA with specific length); Lane 2 (**T**$^{+}$): PCR was run in the presence of natural dNTPs; Lanes 3 (**T**$^{-}$): PCR was run in the presence of dCTP, dGTP, dATP; Lane 4, (**dU**): PCR was run in the presence of three natural dNTPs (dCTP, dGTP, dATP) and **dUTP**; agarose gel stained with GelRed.

3.2.2. Synthesis of fully hmU-modified DNA

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 1.2 µL), natural dNTPs (dCTP, dGTP and dATP; 4 mM; 0.75 µL), d5hmUTP (4 mM; 1.5 µL), 235-mer template (41 ng/µL; 0.88 µL; ***Temp***$^{Pveg\ -sh235}$) and *NON-labelled* primers (20 µM; 3µL; ***Prim***$^{FOR\text{-}PgII}$ and 20 µM; 3 µL; ***Prim***$^{REV\text{-}PgII\text{-}R1/R3}$) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figure S21)*.

3.2.3. Synthesis of fully mC- and hmC-modified DNA

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 1.2 µL), natural dNTPs (dTTP, dGTP and dATP; 4mM; 0.75 µL), d5mCTP (4 mM; 2 µL) or d5hmCTP (4 mM; 1.5 µL), 235-mer template (41 ng/µL; 0.88 µL; ***Temp***$^{Pveg\_sh235}$) and *NON-labelled* primers (20 µM; 3 µL; ***Prim***$^{FOR\text{-}PgII}$ and 20 µM; 3 µL ***Prim***$^{REV\text{-}PgII\text{-}R1/R3}$) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figure S21)*.

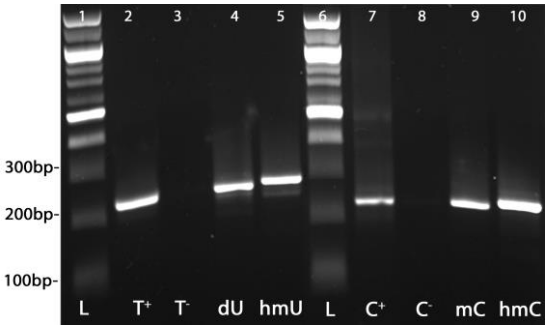


Figure S21: Gel analysis of *NON-labeled* PCR products. The order of samples is as follows: Lanes 1,6 (**L**): ladder (mix of commercial dsDNA with specific length); Lanes 2,7 (**T**$^{+}$ or **C**$^{+}$): PCRs were run in the presence of natural dNTPs; Lane 3 (**T**$^{-}$): PCR was run in the presence of dCTP, dGTP, dATP; Lane 4, (**dU**): PCR was run in the presence of three natural dNTPs (dCTP, dGTP, dATP) and **dUTP**; Lane 5, (**hmU**): PCR was run in the presence of three natural dNTPs (dCTP, dGTP, dATP) and **d5hmUTP**; Lane 8 (**C**$^{-}$): PCR was run in the presence of dTTP, dGTP, dATP; Lane 9, (**mC**): PCR was run in the presence of three natural dNTPs (dTTP, dGTP, dATP) and **d5mCTP**; Line 5, (**hmC**): PCR was run in the presence of three natural dNTPs (dTTP, dGTP, dATP) and **d5hmCTP**. The PCR samples were analysed by agarose gel stained with GelRed.

4. Synthesis of partially modified 235-mers of DNA

Table S4: *List of oligonucleotides used for synthesis of the partially modified 235-mers*

Oligonucleotide-PRIMERs	**Sequence (5´→3´)**	**Length**
***Prim*$^{F2\text{-}PgIII}$**	CGTCTTCAAGAATTCTATTTGACA	24-mer
***Prim*$^{R2\text{-}PgIII}$*_P*^{a}**	GGAGAGCGTTCACCGACA	18-mer
***Prim*$^{F2\text{-}PgIII}$*_F*^{b}**	CGTCTTCAAGAATTCTATTTGACA	24-mer
***Prim*$^{F3\text{-}PgIII}$*_P*^{a}**	TCTAAGCTTGGGTCCCACC	19-mer
***Prim*$^{REV\text{-}PgII/III\text{-}R1/R3}$ *_F*^{b}**	GGAGAGCGTTCACCGACA	18-mer
***Prim*$^{FOR\text{-}PgV}$*_F*^{b}**	CGTCTTCAAGAATTCTAT	18-mer
***Prim*$^{REV\text{-}PgIV\text{-}sh}$*_P*^{a}**	GGACCCAAGCTTAGA	15-mer
***Prim*$^{FOR\text{-}PgIV\text{-}lg}$*_P*^{a}**	CACCTGACCCCATGCCGAAC	20-mer
***Prim*$^{REV\text{-}PgIV\text{-}lg}$*_F*^{b}**	GGAGAGCGTTCACCGACAAACA	22-mer
***Prim*$^{FOR\text{-}PgII\text{-}dU18}$**	CG**dU**C**dU**C**dU**CAAGAA**dU**C**dU**A**dU*dU*C**dU**G*ACAAAAA***dU***GGGC***dU***CG***dU***G***dU*** ***dU***G***dU***ACAA***dU***AAA***dU***G***dU***G*	54-mer
***Prim*$^{FOR\text{-}PgII\text{-}d5hmU18}$**	CG**hmU**C**hmU**C**hmU**CAAGAA**hmU**C**hmU**A**hmU**C**hmU***hmU***GACAAAAA ***hmU***GGGC***hmU***CG***hmU***G***hmU*** ***hmU***ACAA***hmU***AAA***hmU***G***hmU***G*	54-mer
***Prim*$^{FOR\text{-}PgII\text{-}d5mC8}$**	**mC**G**mC**C**mC**AAGAATT**mC**TATTTGA***mC***AAAAATGGG***mC***T ***mC***GTGTTGTA***mC***AATAAATGTG	54-mer
***Prim*$^{FOR\text{-}PgII\text{-}d5hmC8}$**	**hmC**G**hmC**C**hmC**AAGAATT**hmC**TATTTGA***hmC***AAAAATGGG***hmC***T ***hmC***GTGTTGTA***hmC***AATAAATGTG	54-mer
***Prim*$^{REV\text{-}PgII\text{-}R1/R3}$**	GGAGAGCGTTCACCGACA	18-mer
***Prim*$^{FOR\text{-}PgII\text{-}d5hmC8}$**	**hmC**G**hmC**C**hmC**AAGAATT**hmC**TATTTGA***hmC***AAAAATGGG***hmC***T***hmC***G TGTTGTA***hmC***AATAAATGTG	54-mer
***Prim*$^{FOR\text{-}PgII\text{-}d5hmU11}$**	CGTCTTCAAGAATTCTAT***hmU******hmU***GACAAAAA***hmU***GGGC***hmU***CG***hmU***G ***hmU******hmU***G***hmU***ACAA***hmU***AAA***hmU***G***hmU***G	54-mer
***Prim*$^{FOR\text{-}PgII\text{-}d5mC4}$**	CGTCTTCAAGAATTCTATTTGA***mC***AAAAATGGG***mC***T***mC***GTGTTGTA***mC***AA TAAATGTG	54-mer
***Prim*$^{FOR\text{-}PgII\text{-}d5hmC4}$**	CGTCTTCAAGAATTCTATTTGA***hmC***AAAAATGGG***hmC***T***hmC***GTGTTGTA***hmC*** AATAAATGTG	54-mer
Oligonucleotide–TEMPLATE^{c,d,e}		
***Temp*$^{Pveg\ \_sh235}$**	CGTCTTCAAGAATTCTAT*TTGACAAAAATGGGCTCGTGTTGTACAATAAATGTG* TCTAAGCTTGGGTCCCACCTGACCCCATGCCGAACTCAGAAGTGAAACGCCGT AGCGCCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGG CATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGT TGTTTGTCGGTGAACGCTCTCC	235-mer
***Temp*$^{Pveg\ \_sh181}$**	TCTAAGCTTGGGTCCCACCTGACCCCATGCCGAACTCAGAAGTGAAACGCCGT AGCGCCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGG CATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGT TGTTTGTCGGTGAACGCTCTCC	181-mer
***Temp*$^{Us\_69mer}$**	CGTCTTCAAGAATTCTAT*TTGACAAAAATGGGCTCGTGTTGTACAATAAATGTG*T CTAAGCTTGGGTCC	69-mer

[a] 5´-end of oligonucleotide is modified by phosphate

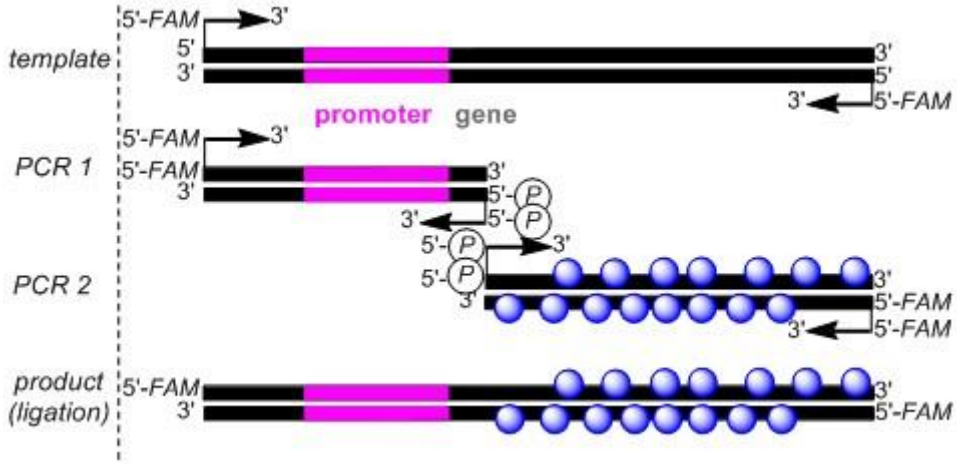
[b] 5´-end of oligonucleotide is labelled by 6-carboxyfluorescein (*6-FAM*)

[c] primer sequences in the template are underlined

[d] promoter sequences in the template are in italics

[e] double underlined primer sequence in the template was *used in section* **4.3.** as ***Prim$^{FOR\text{-}PgV}$_F^{b}***

4.1. Synthesis of dsDNA modified in the promoter-downstream region



4.1.1. Enzymatic syntheses of the natural promoter region and the dU, hmU, mC, hmC modified promoter-downstream region - general remarks

The experiments were designed to obtain blunt-ended DNA with extremities suitable for subsequent ligation. For this purpose, the reactions were performed with a 5'-FAM labelled forward primer and a 5'-phospate reverse primer in the case of the promoter containing portion, and a 5'-phospate forward primer and a 5'-FAM labelled primer for the downstream portion to promote the ligation only between the desired extremities. For the synthesis of the upstream portion, a 69-mer DNA template, purchased from Generi Biotech, was used. The PCR reactions were performed in a final volume of 200 µL.

The obtained products were purified via QIAquick PCR purification kit or QIAquick nucleotide removal kit (*QIAGEN*) according to protocol and eluted in the final step with 30 µL of MilliQ water.

Both downstream and upstream portions were analysed by gel electrophoresis on a 1.3 % agarose gel run in 0.5x TBE buffer and stained with GelRed, *(Figure S22)*.

4.1.1.1. Enzymatic synthesis of the natural promoter region

To obtain the natural upstream portion, the reaction mixture (10 µL) contained *KOD XL DNA polymerase* (*Merck, Novagen;* 2.5 U/µL; 0.25 µL), forward and reverse primers (20 µM; 1 µL; ***Prim$^{FOR\text{-}PgV}$_F*** and 20 µM; 1 µL; ***Prim$^{REV\text{-}PgIV\text{-}sh}$_P***), 69-mer DNA template (1 µM; 0.175 µL; ***Temp^{Us_69mer}***), natural dNTPs (4 mM; 0.075 µL) in the appropriate enzyme reaction buffer (1 µL) supplied by the manufacturer. The reaction was carried out in a PCR thermal cycler as follows: preheating at 95 °C for 1 minutes, denaturation at 95 °C for 0.5 minute, annealing at 46 °C for 0.5 minute, elongation at 72 °C for 0.5 minute and final extension at 72 °C for

1 minute. The amplification was stopped after 25 cycles. The final product was purified by using QIAquick nucleotide removal kit *(QIAGEN*) according to protocol and eluted in the final step with 30 µL of MilliQ water *(Figure S22A)*

4.1.1.2. Enzymatic syntheses of the hmU, mC, hmC modified promoter-downstream regions

The synthesis of the modified downstream portion was performed by using a reaction mixture (10 µL) containing *Vent(exo⁻)* DNA polymerase (*New England Biolabs*; 0.5 µL; 2 U/µL), forward and reverse primers (20 µM; 1 µL ***Prim$^{FOR\text{-}PgIV\text{-}lg}$_P*** and 20 µM; 1 µL; ***Prim$^{REV\text{-}PgIV\text{-}lg}$_F***), either d5hmUTP, d5mCTP or d5hmCTP (4 mM; 0.5 µL), 235-mer template (20 ng/µL; 0.25 µL; ***Temp^{Pveg_sh235}***) and natural dNTPs (dCTP, dGTP and dATP in the case of the d5hmUTP modified portion and dTTP, dGTP and dATP in the case of the d5mCTP or d5hmCTP modified portions; 4 mM; 0.5 µL) in the enzyme reaction buffer (1 µL) supplied by the manufacturer. Forty cycles were run in a PCR thermal cycler using the following protocol: preheating at 94 °C for 3 minutes, denaturation at 94 °C for 1 minute, annealing at 64 °C for 1 minute, elongation at 72 °C for 1.5 minutes and final extension at 72 °C for 5 minutes. The obtained products were purified via QIAquick PCR purification kit (*QIAGEN*) according to protocol and eluted in the final step in 30 µL of MilliQ water *(Figure S22C)*.

4.1.1.3. Enzymatic synthesis of the dU modified promoter-downstream region

The synthesis of the dU modified downstream region was achieved by mixing *Dynazyme II DNA polymerase* (*Thermo Fisher Scientific*; 2 U/µL*;* 0.5 µL), forward and reverse primers (10 µM; 2 µL ***Prim$^{FOR\text{-}PgIV\text{-}lg}$_P*** and 10 µM; 2 µL ***Prim$^{REV\text{-}PgIV\text{-}lg}$_F***), dUTP (4 mM; 1 µL), 235-mer template (20 ng/µL; 0.25 µL; ***Temp^{Pveg_sh235}***), natural dNTPs (dCTP, dGTP and dATP; 4 mM; 0.3 µL) in appropriate enzyme buffer (1 µL) to give a final volume of 10 µL. The reaction was conducted in a PCR cycler under the following conditions: preheating at 94 °C for 3 minutes, denaturation at 95 °C for 1 minute, annealing at 64 °C for 1 minute, elongation at 72 °C for 1.5 minute and final extension at 75 °C for 2 minutes. The amplification was stopped after 30 cycles. The obtained products were purified via QIAquick PCR purification kit (*QIAGEN*) according to protocol and eluted in the final step with 30 µL of MilliQ water *(Figure S22B)*.

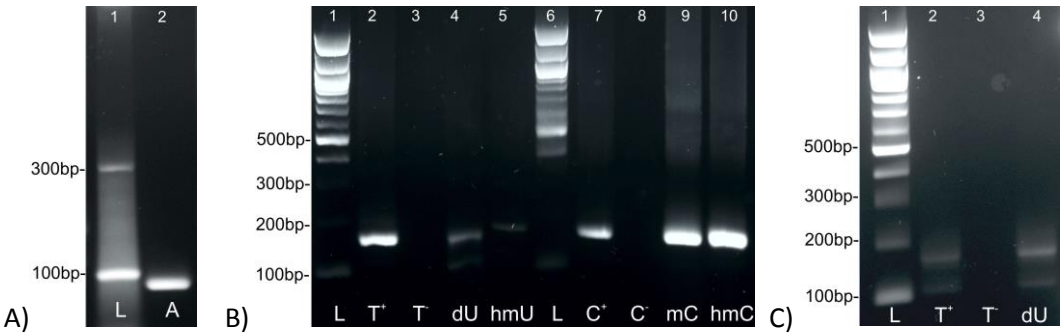


Figure S22: Agarose gel analysis of the downstream and upstream regions obtained by PCR. A) Lane 1 (**L**): ladder (mix of DNA sequences of specific number of base pairs); lane 2 (**A**): upstream unmodified DNA region. B) Lane

1 and 6 (**L**): ladder (mix of DNA sequences of specific number of base pairs); lane 2 and 8: (**T^+, C^+**): positive controls, PCR run in presence of natural dNTPs; lane 3 (**T^-**): negative control, PCR run in the presence of dATP, dGTP, dCTP; lane 4 (**dU**): dU modified PCR product; lane 5 (**hmU**): hmU modified product; lane 8 (**C^-**): negative control, PCR run in presence of dATP, dGTP, dTTP; lane 9 (**mC**): mC modified PCR product; lane 10 (**hmC**): hmC modified product; C) Lane 1 (**L**): ladder (mix of DNA sequences of specific number of base pairs); lane 2 (**T^+**): positive control for the downstream region amplified by Dynazyme DNA polymerase, *i. e.* PCR run in presence of natural dNTPs; lane 3 (**T^-**): negative control, PCR run in the presence of dATP, dGTP, dCTP; lane 4 (**dU**): dU modified PCR product.

4.1.2. DNA Ligation

The ligation between the modified promoter-downstream portion and the natural upstream portion (including the promoter) was performed at a molar ratio of 1:2 (downstream to upstream region), and catalysed by T4 DNA Ligase. The reaction mixture (20 µL) was prepared by mixing T4 DNA Ligase (*New England Biolabs*; 400 U cohesive end units/µL; 2 µL), 0.4 µg of modified downstream portion, 0.33 µg of unmodified upstream portion and dATP (4 mM; 0.5 µL) in the Ligase reaction buffer supplied by the manufacturer (2 µL). The reaction mixture was incubated at 16 °C overnight and then purified through magnetoseparation (*Agencourt AMPure XP magnetic particles, Beckman Coulter*) according the protocol (*see* **2.3.1**.). To allow the isolation of the desired product from the mixture containing downstream-downstream and upstream-upstream portions products, agarose gel purification (E.Z.N.A. Gel Extraction Kit from Omega Bio-Tek) was carried out on 1.3 % agarose gel, stained with GelRed and run in 0.5X TBE buffer, for 90 minutes at 118 V *(Figure S23)*.

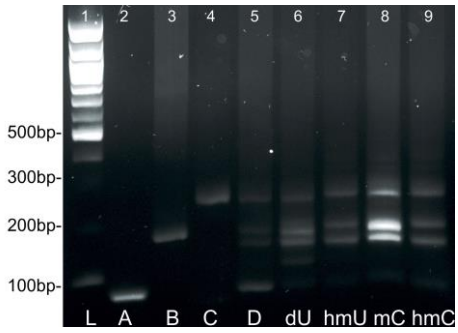


Figure S23: Agarose gel analysis of the ligation between the upstream and downstream DNA region. Lane 1 (**L**): ladder (mix of DNA sequences of specific number of base pairs); lane 2 (**A**): natural DNA upstream region; lane 3 (**B**): natural DNA downstream region; lane 4 (**C**): natural full length DNA template (235-mer); lane 5 (**D**): products after ligation between the unmodified upstream and downstream regions; lane 6 (**dU**): products after ligation between the unmodified upstream region and dU modified downstream region; lane 7 (**hmU**): products after ligation between the unmodified upstream region and hmU modified downstream region; lane 8 (**mC**): products after ligation between the unmodified upstream region and mC modified downstream region; lane 9 (**hmC**): products after ligation between the unmodified upstream region and hmC modified downstream region.

4.1.3. Amplification of the modified templates

Table S5: *List of oligonucleotides used for synthesis of 235-mers amplified from the modified templates extracted from agarose gels*

Oligonucleotide-PRIMERS	Sequence (5´→3´)	Length
$\boldsymbol{Prim^{FOR\text{-}PgIV}}$	CGTCTTCAAGAATTCTATTTGACAAAAATGGGCTCGTG TTGTACAATAAATGTG	54-mer
$\boldsymbol{Prim^{REV\text{-}PgIV}}$	GGAGAGCGTTCACCGACAAACAACAGATAAAACGAAA GGC	40-mer

To verify that we had generated the desired DNA sequence and that the subsequent purificaton from agarose gels did not have any deleteriuous effects, amplifications of the templates extracted from the gels were carried out. The reaction mixture (10 µL) was prepared by mixing *KOD XL DNA polymerase* (*Merck, Novagen*; 2.5 U/µL; 0.5 µL), forward and reverse primers (20 µM; 1 µL $\boldsymbol{Prim^{FOR\_PgVI}}$ and 20 µM; 1 µL $\boldsymbol{Prim^{REV\_PgVI}}$) (*Table S5*), modified template extracted from the gel (20 ng/µL; 1 µL) and natural dNTPs (4 mM; 0.5 µL) in polymerase reaction buffer (1 µL). Forty PCR cycles were run in PCR thermal cycler using the following protocol: preheating at 94 °C for 3 minutes, denaturation at 94 °C for 1 minute, annealing at 68 °C for 1 minute, elongation at 72 °C for 1 minute and final extension at 72 °C for 5 minutes. The PCR products were purified via QIAquick PCR purification kit *(QIAGEN)* and subjected to electrophoresis analysis on 1.3 % agarose gel, run in 0.5x TBE buffer for 90 minutes at 118 V, and stained with GelRed *(Figure S24)*. Subsequently, the PCR products were sequenced and in all cases the correct sequences were detected.

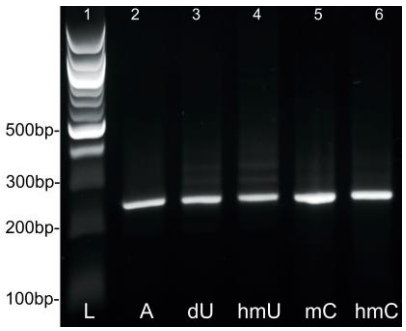
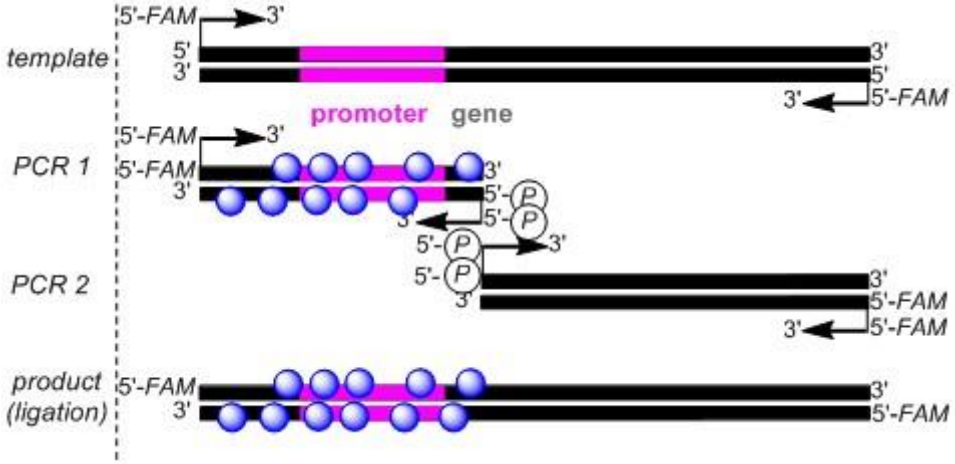


Figure S24: Agarose gel analysis of the products obtained from amplification of the modified templates. Lane 1 (**L**): ladder (mix of DNA sequences of specific number of base pairs); lane 2 (**A**): full length unmodified 235-mer template; lane 3 (**dU**): PCR product obtained from the dU modified template; lane 4 (**hmU**): PCR product obtained from the hmU modified template; lane 5 (**mC**): PCR product obtained from the mC modified template; lane 6 (**hmC**): PCR product obtained from the hmC modified template.

4.2. Synthesis of modified promoter-upstream and promoter regions of dsDNA



4.2.1 Enzymatic syntheses of the dU, hmU, mC, hmC modified promoter region and the natural downstream region – general remarks

The experiments were designed to obtain blunt-ended DNA with extremities suitable for subsequent ligation. For this purpose, the reactions were performed with a 5'-FAM labelled forward primer and a 5'-phospate reverse primer in the case of the upstream promoter containing portion, and a 5'-phospate forward primer and a 5'-FAM labelled primer for the downstream portion to promote the ligation only between the desired extremities. For the synthesis of the upstream portion, a 69-mer DNA template, purchased from Generi Biotech, was used. For technical reasons the template strand contained modifications in both the promoter-upstream and promoter regions whereas the nontemplate strand (coding strand) contained modifications in the promoter region only. The PCR reactions were performed in a final volume of 200 µL.

The obtained products were purified via QIAquick PCR purification kit or QIAquick nucleotide removal kit (*QIAGEN*) according to the protocol and eluted in the final step with 30 µL of MilliQ water.

Both downstream and upstream portions were finally analysed by gel electrophoresis on a 1.3 % agarose gel run in 0.5x TBE buffer and stained with GelRed, *(Figure S25)*.

4.2.1.1. Enzymatic synthesis of the natural downstream region

For the synthesis of the natural portion of the final template, *i.e.* the promoter-downstream region (transcribed region), 10 µL of the reaction mixture were prepared mixing: *Vent(exo$^-$) DNA* polymerase (*New England Biolabs*; 2 U/µL; 0.5 µL), forward and reverse primers (20 µM; 1 µL; ***Prim$^{FOR\text{-}PgIV\text{-}lg}$_P*** and 20 µM; 1 µL; ***Prim$^{REV\text{-}PgIV\text{-}lg}$_F***), 235-mer template (20 ng/µL; 0.75 µL; ***Temp$^{Pveg\ _sh235}$***, natural dNTPs (4 mM; 0.5 µL) in enzyme reaction buffer (1 µL) supplied by the manufacturer. Forty cycles were repeated in a PCR cycler under the following conditions: preheating at 94 °C for 3 minutes, denaturation at 94 °C for 1 minute, annealing at 64 °C for 1 minute, elongation at 72 °C for 1 minute, followed by a final extension step at 72 °C for 5 minutes. The obtained PCR product was purified with QIAquick PCR

purification kit (*QIAGEN*) and eluted in the final step with 30 µL of MilliQ water *(Figure S25A)*.

4.2.1.2. Enzymatic syntheses of the hmU, mC, hmC modified promoter region

The hmU, mC and hmC modified promoter regions were synthesized by using 10 µL of solution containing *KOD XL DNA polymerase* (*Merck, Novagen;* 2.5 U/µL; 0.25 µL), forward and reverse primers (20 µM; 1 µL; ***Prim$^{FOR\text{-}PgV}$_F*** and 20 µM; 1 µL; ***Prim$^{REV\text{-}PgIV\text{-}sh}$_P***), 69-mer DNA template (1 µM; 0.175 µL; ***Temp^{Us_69mer}***), natural dNTPs (dCTP, dGTP and dATP; 4mM; 0.075 µL in the case of the d5hmUTP modified portion and dTTP, dGTP and dATP; 4mM; 0.075 µL in the case of the d5mCTP or d5hmCTP modified portions), functionalized dNTPs, either d5hmUTP, d5mCTP or d5hmCTP (0.5 µL; 4 mM) in appropriate enzyme reaction buffer supplied by the manufacturer (1 µL). The reaction was conducted in a PCR thermal cycler under the following conditions: preheating at 95 °C for 1 minute, denaturation at 95 °C for 0.5 minute, annealing at 46 °C for 0.5 minute, elongation at 72 °C for 0.5 minute and a final extension at 72 °C for 1 minute. The amplification was stopped after 25 cycles. The PCR products were purified with QIAquick nucleotide removal kit (*QIAGENE*) and eluted in the final step with 30 µL of MilliQ water *(Figure S25B)*.

4.2.1.3. Enzymatic syntheses of the dU modified promoter region

For the synthesis of the dU modified promoter region, 0.5 µL of *Dynazyme II DNA polymerase* (*Thermo Fisher Scientific*; 2 U/µL) was mixed with 1 µL of the 10x enzyme reaction buffer, forward and reverse primers (10 µM; 2 µL; ***Prim$^{FOR\text{-}PgV}$_F*** and 10 µM; 2 µL; ***Prim$^{REV\text{-}PgIV\text{-}sh}$_P***), dUTP (4 mM; 1 µL), 69-mer DNA template (1 µM; 0.25 µL; ***Temp^{Us_69mer}***) and natural dNTPs (dCTP, dGTP and dATP; 4 mM; 0.3 µL) in a final reaction volume of 10 µL. The amplification was conducted in a PCR thermal cycler as follows: preheating at 94 °C for 3 minutes, denaturation at 95 °C for 1 minute, annealing at 46 °C for 1 minute, elongation at 72 °C for 1 minute and a final extension step at 72 °C for 5 minutes. The amplification was stopped after 30 cycles. The PCR product was purified with QIAquick nucleotide removal kit (*QIAGENE*) and eluted in the final step with 30 µL of MilliQ water *(Figure S25C)*.

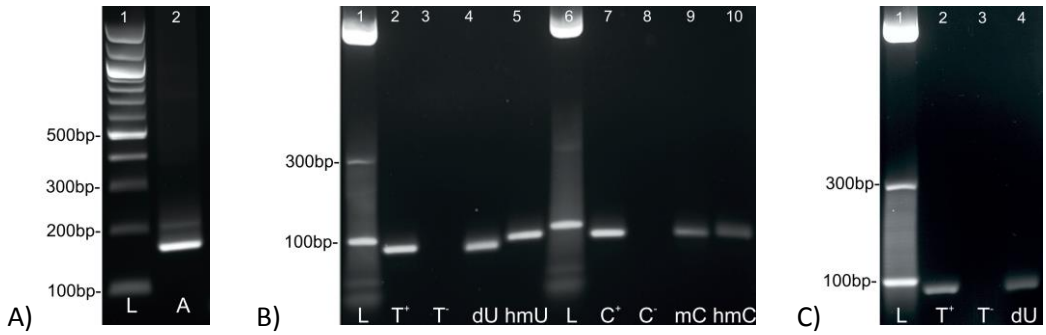


Figure S25: Agarose gel analysis of the enzymatic syntheses of the natural downstream region and modified promoter region. A) Lane 1 (**L**): ladder (mix of DNA sequences of specific number of base pairs); lane 2 (**A**): PCR natural product of the downstream region. B) Lane 1 (**L**): ladder (mix of DNA sequences of specific number of

base pairs); lane 2 (**T**$^{+}$): positive control for the unmodified promoter region, *i.e.* PCR run in the presence of the natural dNTPs, lane 3 (**T**$^{-}$): negative control, PCR run in the presence of dATP, dGTP, dCTP; lane 4 (**dU**): dU modified promoter region; lane 5 (**hmU**): hmU modified promoter region; lane 6 (L): ladder (mix of DNA sequences of specific number of base pairs); lane 7 (**C**$^{+}$): positive control for the unmodified promoter region, *i.e.* PCR run in presence of the natural dNTPs; lane 8 (**C**$^{-}$): negative control, PCR run in presence of dATP, dGTP, dTTP; lane 9 (**mC**): mC modified promoter region; lane 10 (**hmC**): hmC modified upstream region. C) lane 1 (**L**): ladder (mix of DNA sequences of specific number of base pairs); lane 2 (**T**$^{+}$): positive control for the dU modified promoter region amplified by Dynazyme DNA polymerase, i.e. PCR run in presence of the natural dNTPs; lane 3 (**T**$^{-}$): negative control, i.e. PCR run in presence of dATP, dGTP, dCTP; lane 4 (**dU**): dU modified promoter region amplified by Dynazyme DNA polymerase.

4.2.2. DNA Ligation

Ligation between the modified promoter portion and the natural downstream portion was performed with T4 DNA ligase (*New England Biolabs*; 400 U cohesive end units/µL), using a molar ratio of 1:3, downstream to upstream region. To the reaction mixture containing 0.4 µg of downstream portion, 0.5 µg of upstream portion, 0.5 µL of 4 mM ATP and 2 µL of 10x enzyme reaction buffer, 2 µL of T4 DNA Ligase were added to give a final volume of 20 µL. This mixture was incubated at 16 °C overnight. The reaction mixture was then purified through magneto-separation (*Agencourt AMPure XP magnetic particles, Beckman Coulter*) according to the protocol (*see* **2.3.1**.). To allow the isolation of the desired product from the mixture containing upstream-upstream and downstream-downstream portions products, agarose gel purification (E.Z.N.A. Gel Extraction Kit from Omega Bio-Tek) was carried out from a 1.3 % agarose gel run in 0.5x TBE buffer for 90 minutes at 118 V, and stained with GelRed *(Figure S26)*.

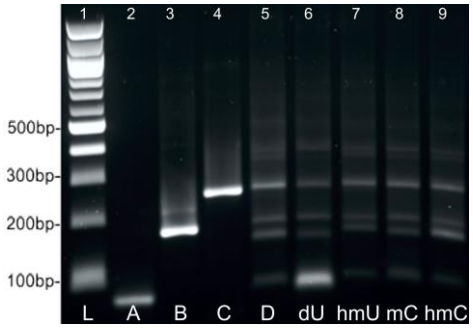


Figure S26: Agarose gel analysis of the ligation between the upstream and downstream DNA portions. Lane 1 (**L**): ladder (mix of DNA sequences of specific number of base pairs); lane 2 (**A**): natural upstream (*i. e.* promoter) region; lane 3 (**B**): natural downstream region; lane 4 (**C**): natural full length DNA template (235-mer); lane 5 (**D**): products after ligation between the unmodified upstream and downstream regions; lane 6 (**dU**): products after ligation between the unmodified downstream region and dU modified upstream region; lane 7 (**hmU**): products after ligation between the unmodified downstream region and hmU modified upstream region; lane 8 (**mC**): products after ligation between the unmodified downstream region and mC modified upstream region; lane 9 (**hmC**): products after ligation between the unmodified downstream region and hmC modified upstream region.

4.2.3 Amplification of the modified templates

As previously described, the amplification of the modified templates extracted from agarose gel was performed to confirm the generation and isolation of the correct sequences. The reaction mixture (10 µL) was prepared by mixing *KOD XL DNA polymerase* (*Merck, Novagen*; 2.5 U/µL; 0.5 µL), forward and reverse primers (20 µM; 1 µL ***Prim^{FOR_PgVI}*** and 20 µM; 1 µL ***Prim^{REV_PgVI}***) *(Table S5)*, modified template extracted from the gel (20 ng/µL; 1 µL) and natural dNTPs (4 mM; 0.5 µL) in polymerase reaction buffer (1 µL). Forty PCR cycles were run in PCR thermal cycler using the following protocol: preheating at 94 °C for 3 minutes, denaturation at 94 °C for 1 minute, annealing at 68 °C for 1 minute, elongation at 72 °C for 1 minute and final extension at 72 °C for 5 minutes. The PCR products were purified via QIAquick PCR purification kit *(QIAGEN)* and subjected to electrophoresis analysis on 1.3 % agarose gel run in 0.5x TBE buffer for 90 minutes at 118 V and stained with GelRed *(Figure S27)*. Subsequently, the PCR products were sequenced and in all cases the correct sequences were detected.

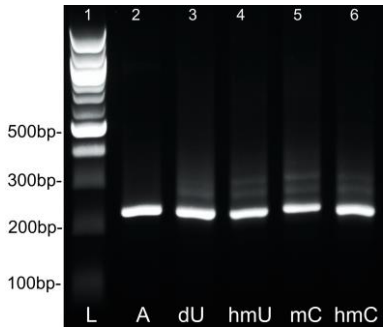
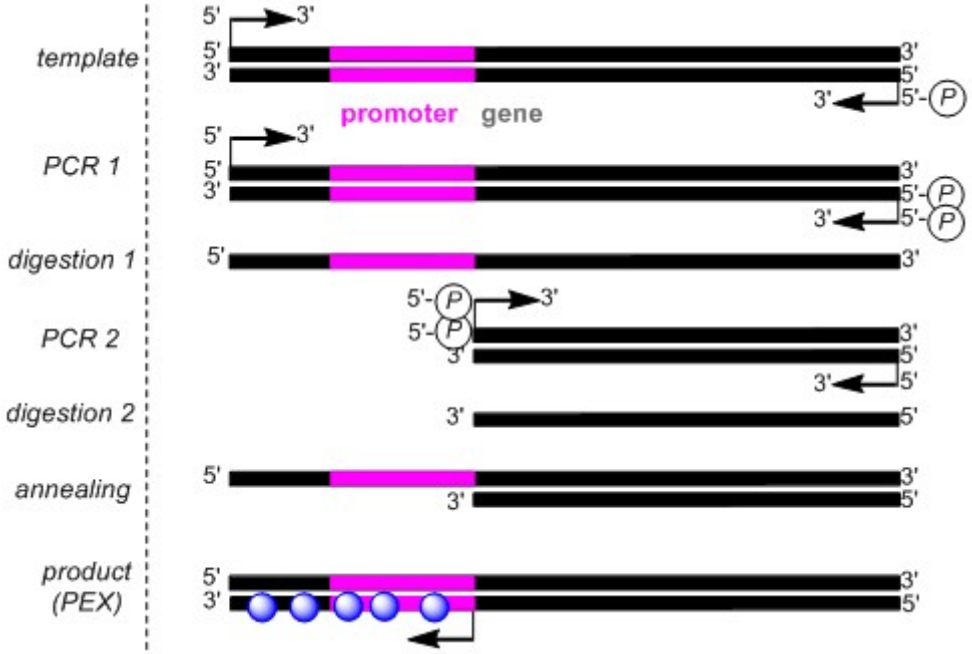


Figure S27: Agarose gel analysis of the products obtained from the amplification of the modified templates extracted from agarose gel. Lane 1 (**L**): ladder (mix of DNA sequences of specific number of base pairs); lane 2 (**A**): full length unmodified 235-mer template; lane 3 (**dU**): PCR product obtained from the dU modified template; lane 4 (**hmU**): PCR product obtained from the hmU modified template; lane 5 (**mC**): PCR product obtained from the mC modified template; lane 6 (**hmC**): PCR product obtained from the hmC modified template.

4.3. Synthesis of modified promoter-upstream and promoter regions in the template strand of DNA



4.3.1. Enzymatic syntheses of *Temp*$^{Pveg\_sh235}$ and *Temp*$^{Pveg\_sh181}$ DNA templates

4.3.1.1. Enzymatic synthesis of the *Temp*$^{Pveg\_sh235}$ DNA template

The non-labelled 235-mer DNA template, ***Temp***$^{Pveg\_sh235}$, was prepared with PCR by mixing *KOD XL DNA polymerase* (*Merck, Novagen*; 2.5 U/ µL; 0.1 µL), forward and reverse *NON- labelled* primers (***Prim***$^{F2\text{-}PgIII}$ and ***Prim***$^{R2\text{-}PgIII}$***_P*** ; 20 µM; 1 µL each), the already described 339-mer template, ***Temp***^{Pveg} (20 ng/µL; 1 µL), natural dNTPs (4 mM; 0.5 µL) and the enzyme reaction buffer supplied by the manufacturer (1 µL) in a final reaction volume of 10 µL. Forty PCR cycles were conducted in a PCR thermal cycler and the reaction was performed as follows: preheating at 94 °C for 3 minutes, denaturation at 94 °C for 1 minute, annealing at 72 °C for 1 minute, elongation at 72 °C for 1 minute, final extension step at 72 °C for 5 minutes. The final product was purified and extracted from 1.3 % agarose gel, stained with GelRed and run in 0.5x TBE buffer for 90 minutes at 108 V, by using the EZNA Gel Extraction kit (*OMEGA Biotek*) according to the protocol and eluted in the final step with 30 µL of MilliQ water. The obtained product was used as a DNA template for the following amplification.

4.3.1.2. Enzymatic synthesis of the *Temp*$^{Pveg\_sh181}$ DNA template

The FAM-labelled 181mer DNA template was prepared with PCR by mixing *Taq DNA Polymerase* (*New England Biolabs*; 5 U/ µL; 1 µL), natural forward primer and 5'-FAM labelled reverse primer (20 µM; 1 µL; ***Prim***$^{F3\text{-}PgIII}$***_P*** and 20 µM; 1 µL ***Prim***$^{REV\text{-}PgII/III\text{-}R1/R3}$ ***_F***), the 339-mer template (20 ng/µL; 1 µL; ***Temp***^{Pveg}**)**, natural dNTPs (4 mM; 0.5 µL) in the enzyme reaction buffer supplied by the manufacturer (1 µL) for a final reaction volume of

10 µL. The amplification was performed in a thermal cycler under the following conditions: preheating at 94 °C for 3 minutes, denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, elongation at 72 °C for 1 minute, followed by final extension step at 72 °C for 5 minutes. The final product was purified and extracted from 1.3 % agarose gel, stained with GelRed and run in 0.5X TBE buffer for 90 minutes at 108 V, by using the EZNA Gel Extraction kit (*OMEGA Biotek*) according to the protocol and eluted in the final step with 30 µL of MilliQ water. The obtained product was used as a DNA template for the following amplification.

4.3.2. Amplification of the natural DNA templates for subsequent Lambda exonuclease digestion – general remarks

For the full length template (the 235-mer), the amplification was performed with a 5'-phosphate-containing reverse primer while for its shorter version (181-mer), the 5'-phosphate-containing forward primer was used to allow, in both cases, the subsequent Lambda exonuclease digestion. The PCR reactions were performed in a final volume of 150 µL. The obtained PCR products were purified with QIAquick PCR purification kit *(QIAGEN)* and eluted in the final step with 30 µL of MilliQ water. The final analysis was performed by gel electrophoresis on a 1.3 % agarose gel stained with GelRed in 0.5x TBE buffer.

4.3.2.1. Amplification of the natural 235-mer DNA template

For the synthesis of the 235-mer template, *Vent(exo$^-$) DNA polymerase* (*New England Biolabs*; 2 U/µL; 1 µL) in appropriate enzyme reaction buffer (1 µL) was added to the mixture (final volume 10 µL) containing 5'-FAM labelled forward primer and 5'-phosphate reverse primer (20 µM; 1 µL; ***Prim$^{F2\text{-}PgIII}$_F*** and 20 µM; 1 µL; ***Prim$^{R2\text{-}PgIII}$_P***), 235-mer DNA template (20 ng/µL; 1 µL; ***Temp^{Pveg_sh235}***), natural dNTPs (4 mM; 0.5 µL), supplied by the manufacturer. Forty PCR cycles were conducted in a PCR thermal cycler and the reaction was performed as follows: preheating at 94 °C for 3 minutes, denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, elongation at 72 °C for 1 minute, followed by final extension step at 72 °C for 5 minutes *(Figure S28)*.

4.3.2.2. Amplification of the natural 181-mer DNA template

The synthesis of the 181-mer template was achieved by preparing a reaction mixture (final volume 10 µL) containing: *Vent(exo$^-$) DNA* polymerase (*New England Biolabs*; 2 U/µL; 1 µL), 10x enzyme reaction buffer (1 µL), forward and reverse primers (20 µM; 1 µL; ***Prim$^{F3\text{-}PgIII}$_P*** and 20 µM; 1 µL; ***Prim$^{REV\text{-}PgII/III\text{-}R1/R3}$_F***), 181-mer DNA template (20 ng/ µL; 1 µL; ***Temp^{Pveg_sh181}***) and standard dNTPs (4 mM; 0.5 µL). The amplification was performed in a PCR cycler under the same conditions as previously described for the 235-mer template *(Figure S28)*.

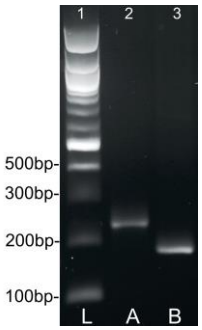


***Figure S28**:* Agarose gel analysis of the natural DNA templates for subsequent Lambda exonuclease digestion. Lane 1 (**L**): ladder (mix of DNA sequences of specific number of base pairs); lane 2 (**A**): 235-mer DNA template; lane 3 (**B**): 181-mer DNA template.

4.3.3. Lambda exonuclease digestion and annealing of the complementary sequences

4.3.3.1. Lambda exonuclease digestion of prepared dsDNA

The generation of the single stranded DNA from the double stranded PCR products was accomplished by using 3.5 U of Lambda exonuclease (*New England Biolabs;* 5 U/µL) per microgram of double stranded DNA. For both templates, the 235-mer and the 181-mer, in a 10 µg of dsDNA were mixed with 7 µL of Lambda exonuclease and 10 µL of enzyme reaction buffer supplied by the manufacturer in a final reaction volume of 100 µL. The mixture was incubated at 37 °C for 1 hour and the DNA then extracted from the mixture by phenol-chloroform. The single stranded DNA was finally recovered through acetone precipitation and dissolved in 30 µL of MilliQ water. The obtained single stranded products were analysed on a 1.3 % agarose gel, stained with GelRed and run in 0.5X TBE buffer for 90 minutes at 118 V *(Figure S29)*.

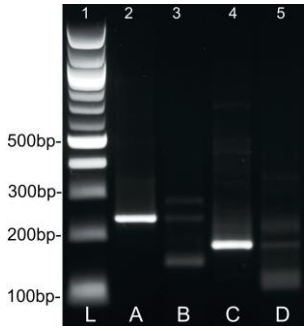


Figure S29: Agarose gel analysis of the natural DNA templates after PCR and after Lambda exonuclease digestion. Lane 1 (**L**): ladder (mix of DNA sequences of specific number of base pairs); lane 2 (**A**): double stranded 235-mer DNA template; lane 3 (**B**): single stranded 235-mer DNA template; lane 4 (**C**): double stranded 181-mer DNA template; lane 5 (**D**): single stranded 181-mer DNA template.

4.3.3.2. Annealing of complementary ssDNA sequences

The subsequent hybridization between the 235-mer ssDNA and the 181-mer ssDNA was performed in Tris-HCl (10 mM Tris, 50 mM NaCl, 1 mM EDTA, pH = 8) containing buffer,

at a molar ratio of 1:1 to yield a final DNA concentration of 0.3 µM, and carried out by heating the mixture at 95 °C and slowly cooling it down to 25 °C for 95 minutes. The resulting duplex was submitted to primer extension experiments *(Figure S30)*.

4.3.4. PEX reactions

The primer extension was performed by mixing *KOD XL DNA polymerase* (*Merck, Novagen*; 2.5 U/ µL; 3 µL), 10X enzyme reaction buffer (6 µL), the complex template and appropriate primer (0.3 µM; 12 µL), the functionalized dNTP (either dUTP, d5hmUTP, d5mCTP or d5hmCTP; 4mM; 3 µL) and suitable natural dNTPs mixture (either dATP, dGTP, dCTP for the dUTP and d5hmUTP modified templates or dATP, dGTP, dTP for the d5mCTP and d5hmCTP modified templates; 4 mM; 3 µL), in a final reaction volume of 60 µL. The mixture was incubated at 65 °C for 30 minutes and purified through magneto separation (*Agencourt AMPure XP magnetic particles, Beckman Coulter*). The samples were finally analysed on a 1.3% agarose gel run in 0.5x TBE buffer at 118 V for 90 minutes and stained with GelRed *(Figure S30)*.

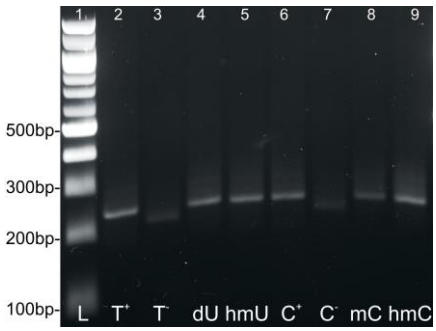
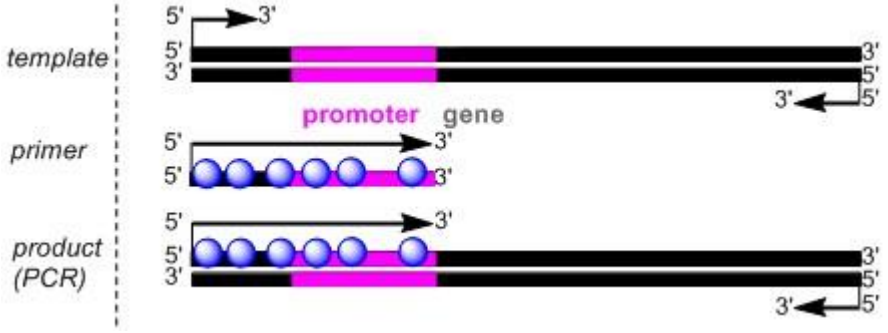


Figure S30: Agarose gel analysis of the DNA templates with modified promoter-upstream and promoter regions in the template strand of DNA. Lane 1 (**L**): ladder (mix of DNA sequences of specific number of base pairs); lane 2 (**T$^+$**): positive control, *i.e.* PEX reaction run with natural dNTP; lane 3 (**T$^-$**): negative control, reaction run with dATP, dGTP, dCTP; lane 4 (**dU**): dU modified template; lane 5 (**hmU**): 5hmU modified template; lane 6 (**C$^+$**): positive control, *i.e.* PEX reaction run with natural dNTP; lane 7 (**C$^-$**): negative control, reaction run with dATP, dGTP, dTTP; lane 8 (**mC**): 5mC modified template; lane 9 (**hmC**): 5hmC modified template.

4.4. Synthesis of modified promoter-upstream and promoter regions in the coding strand of DNA



4.4.1. Synthesis of modified promoter-upstream and promoter regions in the coding strand of DNA-general remarks

To prepare modified templates for transcription, PCR reactions were performed in a total final volume of 60 µL. All samples were prepared in the presence of *NON-labelled* (modified ***Prim***$^{FOR\text{-}PgII}$ and non-modified ***Prim***$^{REV\text{-}PgII\text{-}R1/R3}$) commercially available primers.

Thirty PCR cycles were run in a thermal cycler under the following conditions: preheating for 3minutes at 94°C, denaturation for 1 minute at 94°C, annealing for 1 minute at 68°C, extension for 1 minute at 72°C, followed by final extension step of 5 minutes at 75°C. PCR products were separated on agarose gels and subsequently extracted from the gels (E.Z.N.A. Gel Extraction Kit from Omega Bio-Tek) followed by purification by Agencourt AMPure XP magnetic beads according to the protocol (*see* **2.3.1**.). The product was in the last step eluted by 40 uL of MilliQ water. Control 1.3% agarose gels were stained by GelRed.

4.4.1.1. Synthesis of dU-modified DNA in promoter-upstream and promoter regions of the coding strand

The PCR reaction mixture (20 µL) contained KOD XL (*Merck; Novagen;* 2.5 U/µL; 0.4 µL) or Dynazyme II (*Thermo Fischer Scientific*) DNA polymerase, four natural dNTPs (dCTP, dGTP, dTTP and dATP; 5mM; 0.8 µL), 235-mer template (30 ng/µL; 2 µL; ***Temp***$^{Pveg\_sh235}$) and primers (20 µM; 2 µL; ***Prim***$^{FOR\text{-}PgII\text{-}dU18}$ and 20 µM; 2 µL; ***Prim***$^{REV\text{-}PgII\text{-}R1/R3}$) in 10x reaction Buffer for KOD XL or for Dynazyme (2 µL) supplied by the manufacturer. PCR with KOD XL DNA polymerase was not successful. Therefore, the PCR product for transcription was prepared using Dynazyme II DNA polymerase *(Figure S31 and Figure S32)*.

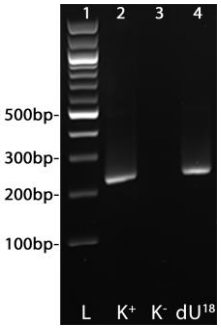


Figure S31: Gel analysis of PCR products. The order of samples is as follows: Lane 1 (**L**): ladder (mix of commercial dsDNA with specific length); Lane 2 (**K+**): PCR was run in the presence of the non-modified primer ***Prim***$^{FOR\text{-}PgII}$; Lane 3 (**K**$^{-}$): PCR was run in the absence of a forward primer; Lane 4, (**dU**^{18}): PCR was run in the presence of ***Prim***$^{FOR\text{-}PgII\text{-}dU18}$.

4.4.1.2. Synthesis of hmU-modified DNA in promoter-upstream and promoter regions of the coding strand

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 0.4 µL), four natural dNTPs (dCTP, dGTP, dTTP and dATP; 5 mM; 0.8 µL), 235-mer template (30 ng/µL; 2 µL; ***Temp***$^{Pveg\_sh235}$) and primers (20 µM; 2 µL; ***Prim***$^{FOR\text{-}PgII\text{-}d5hmU18}$ and

20 µM; 2 µL; ***Prim***$^{REV\text{-}PgII\text{-}R1/R3}$) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figure S32).*

4.4.1.3. Synthesis of mC-modified DNA in promoter-upstream and promoter regions of the coding strand

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase(*Merck; Novagen;* 2.5 U/µL; 0.4 µL), natural dNTPs (dCTP, dGTP, dTTP and dATP; 5 mM; 0.8 µL), 235-mer template (30 ng/µL; 2 µL; ***Temp***$^{Pveg\_sh235}$) and primers (20 µM; 2 µL; ***Prim***$^{FOR\text{-}PgII\text{-}d5mC8}$ and 20 µM; 2 µL; ***Prim***$^{REV\text{-}PgII\text{-}R1/R3}$) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figure S32).*

4.4.1.4. Synthesis of hmC-modified DNA in promoter-upstream and promoter regions of the coding strand

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 0.4 µL), natural dNTPs (dCTP, dGTP, dTTP and dATP; 5 mM; 0.8 µL), 235-mer template (30 ng/µL; 2 µL; ***Temp***$^{Pveg\_sh235}$) and primers (20 µM; 2 µL; ***Prim***$^{FOR\text{-}PgII\text{-}d5hmC8}$ and 20 µM; 2 µL ***Prim***$^{REV\text{-}PgII\text{-}R1/R3}$) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figure S32).*

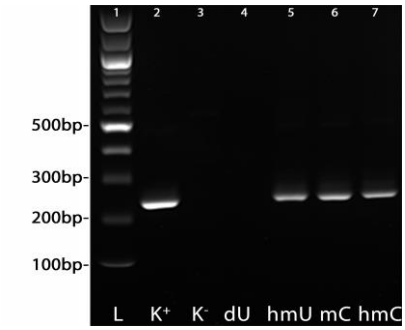
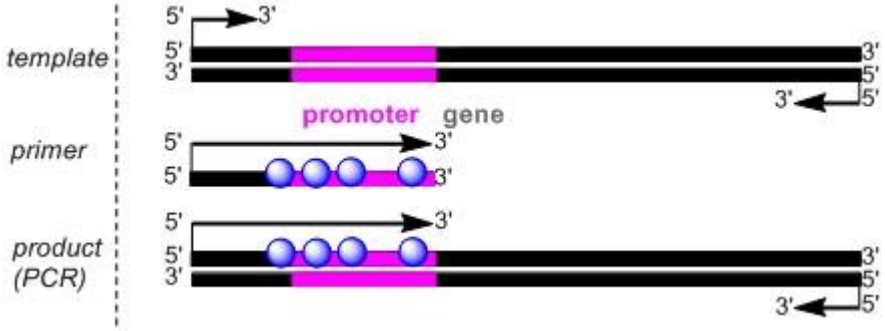


Figure S32: Gel analysis of PCR products modified in promoter-upstream and promoter region of DNA in the coding strand. The order of samples is as follows: Lane 1 (**L**): ladder (mix of commercial dsDNA with specific length); Lane 2 (**K+**): PCR was run in the presence of the non-modified primer ***Prim***$^{FOR\text{-}PgII}$; Lane 3 (**K**$^{-}$): PCR was run in the absence of a forward primer; Lane 4, (**dU**): PCR was run in the presence of ***Prim***$^{FOR\text{-}PgII\text{-}dU18}$; Lane 5, (**hmU**): PCR was run in the presence of ***Prim***$^{FOR\text{-}PgII\text{-}d5hmU18}$; Lane 6 (**mC**): PCR was run in the presence of ***Prim***$^{FOR\text{-}PgII\text{-}d5mC8}$; Lane 7 (**hmC**): PCR was run in the presence of ***Prim***$^{FOR\text{-}PgII\text{-}C8hM}$. PCR samples were analysed by agarose gel stained with GelRed.

4.5. Synthesis of DNA modified in the the coding strand of the promoter region



4.5.1. Synthesis of DNA modified in the the coding strand of the promoter region - general remarks

To prepare modified templates for transcription, PCR reactions were performed in a total final volume of 60 µL. All samples were prepared in the presence of *NON-labelled* (modified ***Prim***$^{FOR\text{-}PgII}$ and non-modified ***Prim***$^{REV\text{-}PgII\text{-}R1/R3}$) commercially available primers.

Thirty PCR cycles were run in a thermal cycler under the following conditions: preheating for 3 minutes at 94°C, denaturation for 1 minute at 94°C, annealing for 1 minute at 68°C, extension for 1 minute at 72°C, followed by final extension step of 5 minutes at 75°C. PCR products were separated on agarose gels, extracted from the gels, and subsequently purified with Agencourt AMPure XP magnetic beads according to the protocol (*see* **2.3.1**.). The product was in the last step eluted with 40 uL of MilliQ water. Control 1.3% agarose gels were stained by GelRed.

4.5.2. Synthesis of dU-modified DNA in the the coding strand of the promoter region

The PCR reaction mixture (20 µL) contained KOD XL (*Merck; Novagen;* 2.5 U/µL; 0.4 µL) or Dynazyme II (*Thermo Fischer Scientific*) DNA polymerase, natural dNTPs (dCTP, dGTP, dTTP and dATP; 5 mM; 0.8 µL), 235-mer template (30 ng/µL; 2 µL; ***Temp***$^{Pveg\_sh235}$) and primers (20 µM; 2 µL; ***Prim***$^{FOR\text{-}PgII\text{-}dU11}$ and 20 µM; 2 µL ***Prim***$^{REV\text{-}PgII\text{-}R1/R3}$) in 10x reaction Buffer for KOD XL or Dynazyme (2 µL) supplied by the manufacturer. PCR with KOD XL DNA polymerase did not proceed. PCR product for transcription was prepared using Dynazyme II DNA polymerase *(Figure S33 and Figure S34)*.

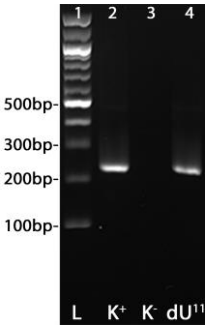


Figure S33: Gel analysis of PCR products. The order of samples is as follows: Lane 1 (**L**): ladder (mix of commercial dsDNA with specific length); Lane 2 (**K+**): PCR was run in the presence of the non-modified primer ***Prim***$^{FOR\text{-}PgII}$;

Lane 3 (**K**$^{-}$): PCR was run in the absence of a forward primer; Lane 4, (**dU**^{11}): PCR was run in the presence of ***Prim***$^{FOR\text{-}PgII\text{-}dU11}$. PCR samples were analysed by 1.3% agarose gel stained with GelRed.

4.5.3. Synthesis of hmU-modified DNA in the the coding strand of the promoter region

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase(*Merck; Novagen;* 2.5 U/µL; 0.4 µL), natural dNTPs (dCTP, dGTP, dTTP and dATP; 5 mM; 0.8 µL), 235-mer template (30 ng/µL; 2 µL; ***Temp***$^{Pveg\_sh235}$) and primers (20 µM; 2 µL; ***Prim***$^{FOR\text{-}PgII\text{-}d5hmU11}$ and 20 µM; 2 µL ***Prim***$^{REV\text{-}PgII\text{-}R1/R3}$) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figure S34).*

4.5.4. Synthesis of mC-modified DNA in the the coding strand of the promoter region

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase(*Merck; Novagen;* 2.5 U/µL; 0.4 µL), natural dNTPs (dCTP, dGTP, dTTP and dATP; 5 mM; 0.8 µL), 235-mer template (30 ng/µL; 2 µL; ***Temp***$^{Pveg\_sh235}$) and primers (20 µM; 2 µL; ***Prim***$^{FOR\text{-}PgII\text{-}d5mC4}$ and 20 µM; 2 µL ***Prim***$^{REV\text{-}PgII\text{-}R1/R3}$) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figure S34).*

4.5.5. Synthesis of hmC-modified DNA in promoter region of coding strand

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase(*Merck; Novagen;* 2.5 U/µL; 0.4 µL), natural dNTPs (dCTP, dGTP, dTTP and dATP; 5 mM; 0.8 µL), 235-mer template (30 ng/µL; 2 µL; ***Temp***$^{Pveg\_sh235}$) and primers (20 µM; 2 µL; ***Prim***$^{FOR\text{-}PgII\text{-}d5hmC4}$ and 20 µM; 2 µL ***Prim***$^{REV\text{-}PgII\text{-}R1/R3}$) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer *(Figure S34).*

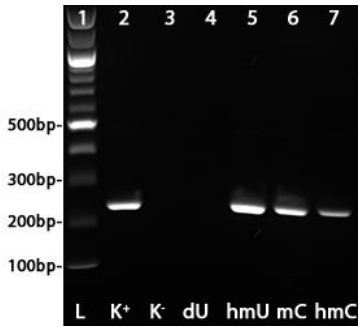


Figure S34: Gel analysis of PCR products modified in promoter region of coding strand. The order of samples is as follow: Lane 1 (**L**): ladder (mix of commercial dsDNA with specific length); Lane 2 (**K+**): PCR was run in the presence of non-modified primer ***Prim***$^{FOR\text{-}PgII}$; Lane 3 (**K**$^{-}$): PCR was run in the absence of forward primer; Lane 4, (**dU**): PCR was run in the presence of ***Prim***$^{FOR\text{-}PgII\text{-}dU11}$; Lane 5, (**hmU**): PCR was run in the presence of ***Prim***$^{FOR\text{-}PgII\text{-}d5hmU11}$; Lane 6 (**mC**): PCR was run in the presence of ***Prim***$^{FOR\text{-}PgII\text{-}d5mC4}$; Lane 7, (**hmC**): PCR was run in the presence of ***Prim***$^{FOR\text{-}PgII\text{-}d5hmC4}$. PCR samples were analysed by agarose gel stained with GelRed.

5. *In vitro* transcription assay

5.1. DNA templates quantification

DNA concentration was measured by the NanoDrop spectrophotometr and subsequently diluted to 5 ng/µL in water. 25 ng of *^{32}P-labelled* DNA were resolved on a 5% polyacrylamide (PAA) gel without urea. 50 ng of *NON-labelled* DNA was analyzed on a 1.5% agarose gel dyed with 10 000x diluted GelRed (Biotium). The PAA gels were dried, exposed to Fuji MS phosphor storage screens and scanned with a Molecular Imager FX (BIORAD) and analyzed with Quantity One program (BIORAD). *NON-labelled* DNA was visualized by UV light and DNA templates were analysed with Quantity One program (BIORAD).

5.2. Multiple round *in vitro* transcription assays

Multiple round transcription assays were performed as described [3], unless stated otherwise. The experiments were carried out in 10 µL with 5 ng of template, 100 mM Tris pH 8, 125 mM $MgCl_2$, 50 mM DTT, 90 mM KCl, 30 nM *Escherichia coli* RNAPσ^{70} holoenzyme (New England Biolabs) and NTPs (200 µM ATP, 1000 µM GTP, 200 µM CTP, 10 µM UTP plus 3.7 kBq [α-^{32}P]UTP). RNAP was used diluted in 50 mM Tris–HCl pH 8.0, 0.1 M NaCl, 50% glycerol. The samples were preheated for 10 min at 37°C and the reactions were initiated with NTPs. The reactions were then allowed to proceed for 10 min at 37°C. Subsequently, the reactions were stopped by the addition of 10 µL of formamide stop solution (95% formamide, 20 mM EDTA, pH 8.0). The samples were loaded onto 7% polyacrylamide gels. The gels were dried and exposed to Fuji MS phosphor storage screens and scanned with a Molecular Imager FX (BIORAD) and analysed with Quantity One program (BIORAD).

Finally, the transcript signals were normalized to signals from the DNA templates and these values were compared to the K+ values (natural DNA template). The natural DNA templates, namely the DNA templates synthesized in the presence of natural dNTPs, reported on the transcription assays gels are unambigously indicated as K^+, differently from the PCR products analisys gels, on which natural DNAs (positive controls for the reactions) were reported like T^+ and C^+.

5.3. *In vitro* transcription assays on U, 5hmU-containing DNA

In vitro transcription asssays with fully modified DNA templates (U, 5hmU) were performed. DNA template quantification and multiple round *in vitro* transcription assays were performed as described in 5. DNA templates were *^{32}P*-labelled. The transcription assays were performed with these samples with concentrations adjusted according to DNA gel electrophoreses and quantitation (*Figure S35*). The two bands in K+ are due to a crack in the gel and not to the presence of two DNA species.

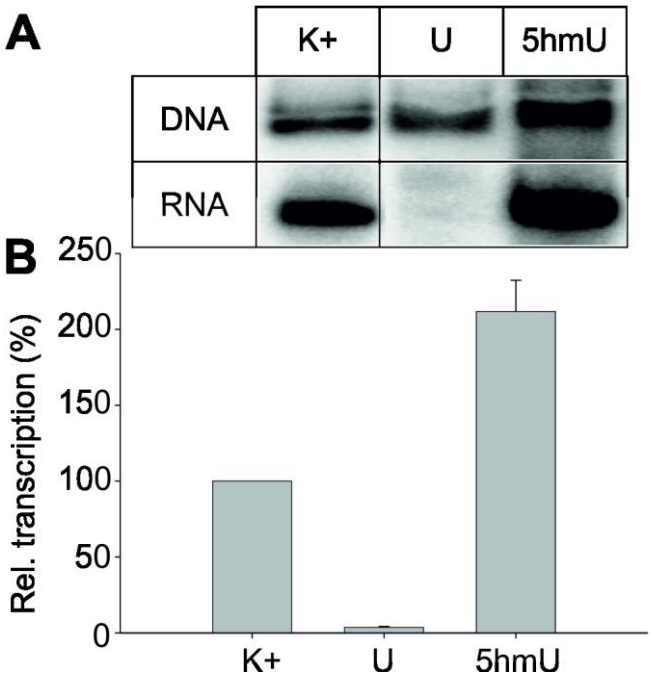


Figure S35: **(A)** Gel electrophoresis of DNA templates (radiolabelled) and RNA transcripts and **(B)** quantitation of transcriptions from full length U or 5hmU modified templates. **K+** is non modified DNA. The bar graph shows the average from two independent experiments. **K+** was set as 100%. The error bars show the range.

6. References

[1] Z. Vaníková, M. Hocek, *Angew.Chem. Int. Ed.* **2014**, *53*, 6734-6737.

[2] L. Sojka, T. Kouba, I. Barvík, H. Šanderová, Z. Maderová, J. Jonák, L. Krásný, *Nucleic Acids Res.* **2011**, *39*, 4598–4611.

[3] J. Wiedermannová, P. Sudzinová, T. Kovaľ, et al. *Nucleic Acids Res.* **2014**; *42(8)*, 5151-5163.

Supporting Information II

For

5-(Hydroxymethyl)uracil and –cytosine as epigenetic marks enhancing or inhibiting transcription with bacterial RNA polymerase

Sequencing of DNA templates:

The correct sequence of all PCR products was confirmed by LIGHTRUN tube Sequencing service (GATC Biotech AG, Germany) using standard Sanger sequencing. The DNA templates with modified dsDNA downstream region and with modified dsDNA upstream region (sections 2.1 and 2.2) were used for their rePCR, according to the protocol reported in the sections 4.1.3. and 4.2.3. of the SI, with natural dNTPs and the resulting products were sent for sequencing.

Red underlined part shows exact match of sequenced DNA with expected sequence in the presence of reverse primer. Blue underlined part shows exact match of sequenced DNA with expected sequence in the presence of forward primer. Double, violet underlined part was correctly sequenced in the presence of either forward or reverse primer.

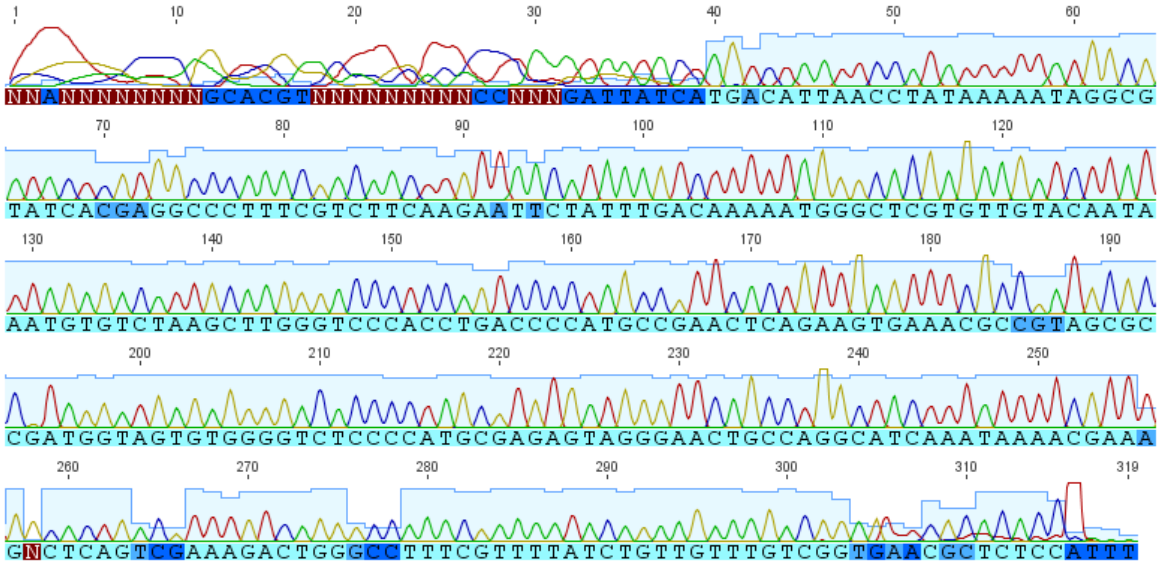
1. Sequencing of fully modified 339-mers, 340-mers and 235-mers of DNA

1.1. Sequencing of fully modified DNA (339-mers) with promotor region of Pveg

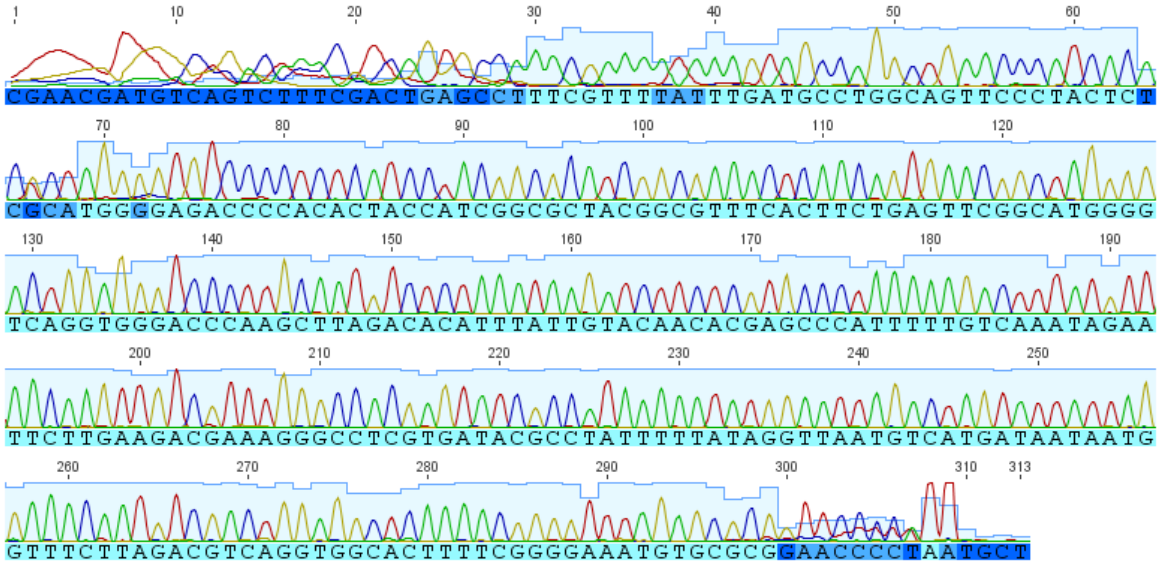
1.1.1. dU-modified DNA

5´-TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TTC AAG AAT TC***T ATT TGA CAA AAA TGG GCT CGT GTT GTA CAA TAA ATG T***GT CTA AGC TTG GGT CCC ACC TGA CCC CAT GCC GAA CTC AGA AGT GAA ACG CCG TAG CGC CGA TGG TAG TGT GGG GTC TCC CCA TGC GAG AGT AGG GAA CTG CCA GGC ATC AAA TAA AAC GAA AGG CTC AGT CGA AAG ACT GGG CCT TTC GTT TTA TCT GTT GTT TGT CGG TGA ACG CTC TCC-3´

Primary strand (exact match for 283 bp):



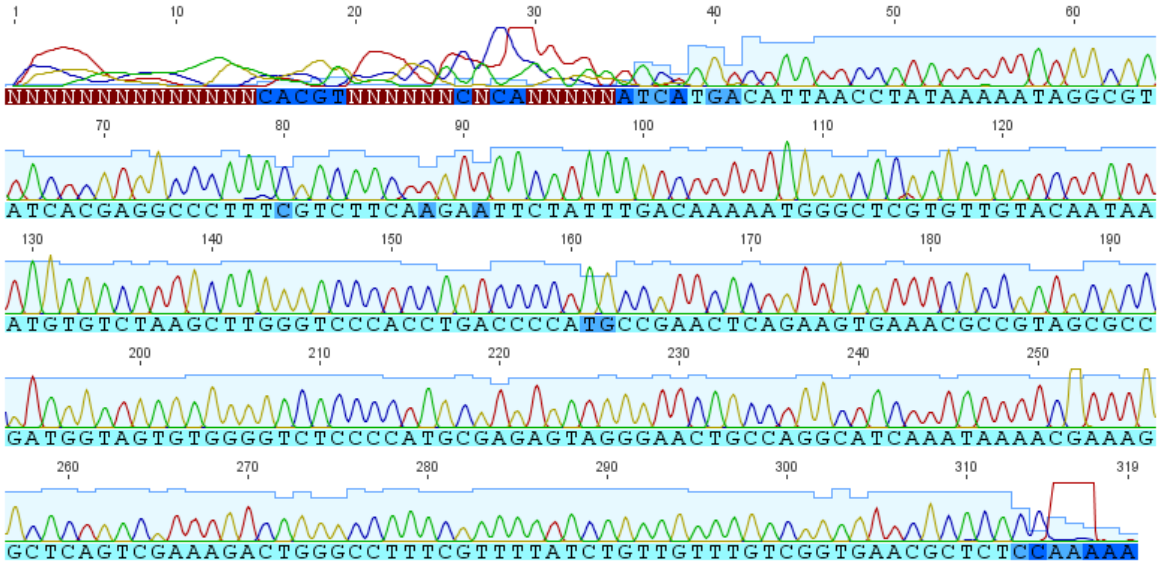
Complementary strand (exact match for 298 bp):



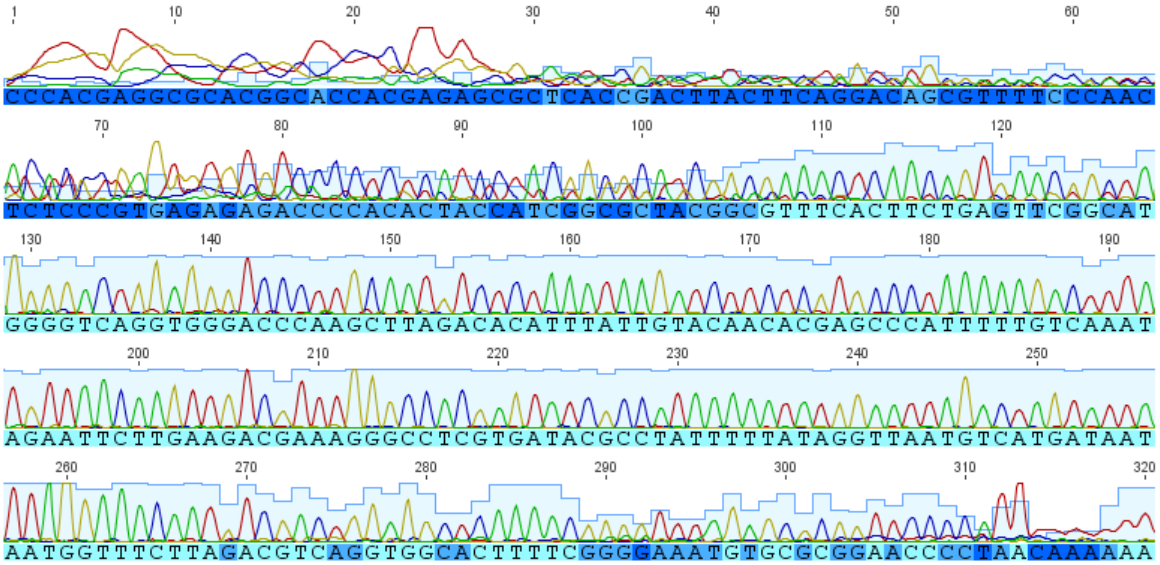
1.1.2. hmU-modified DNA

5´-TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TTC AAG AAT TC***T ATT TGA CAA AAA TGG GCT CGT GTT GTA CAA TAA ATG T***GT CTA AGC TTG GGT CCC ACC TGA CCC CAT GCC GAA CTC AGA AGT GAA ACG CCG TAG CGC CGA TGG TAG TGT GGG GTC TCC CCA TGC GAG AGT AGG GAA CTG CCA GGC ATC AAA TAA AAC GAA AGG CTC AGT CGA AAG ACT GGG CCT TTC GTT TTA TCT GTT GTT TGT CGG TGA ACG CTC TCC-3´

Primary strand (exact match for 280 bp):



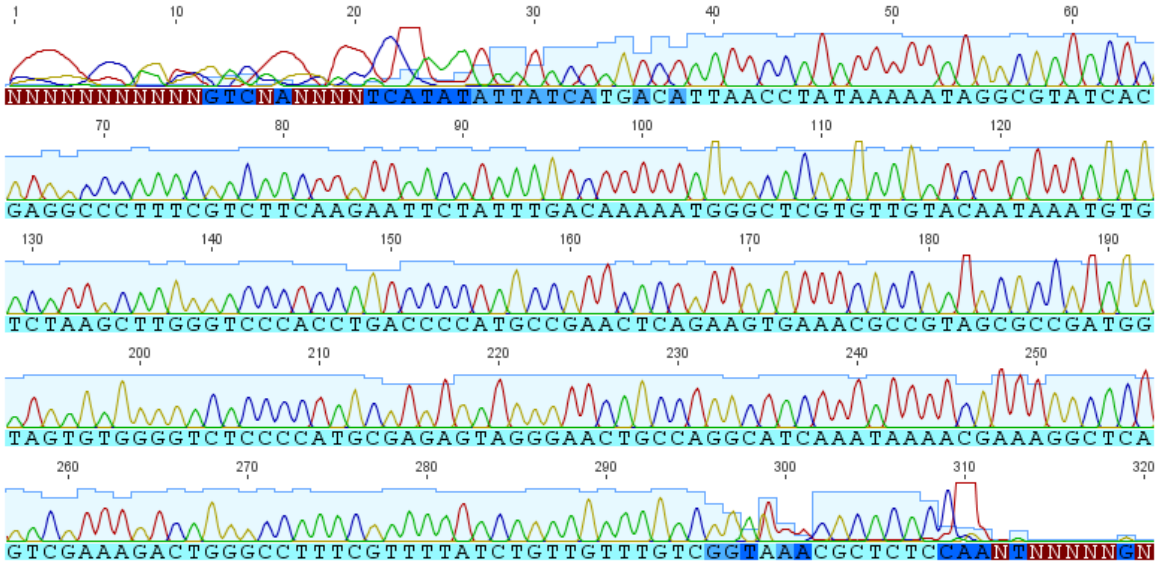
Complementary strand (exact match for 236 bp):



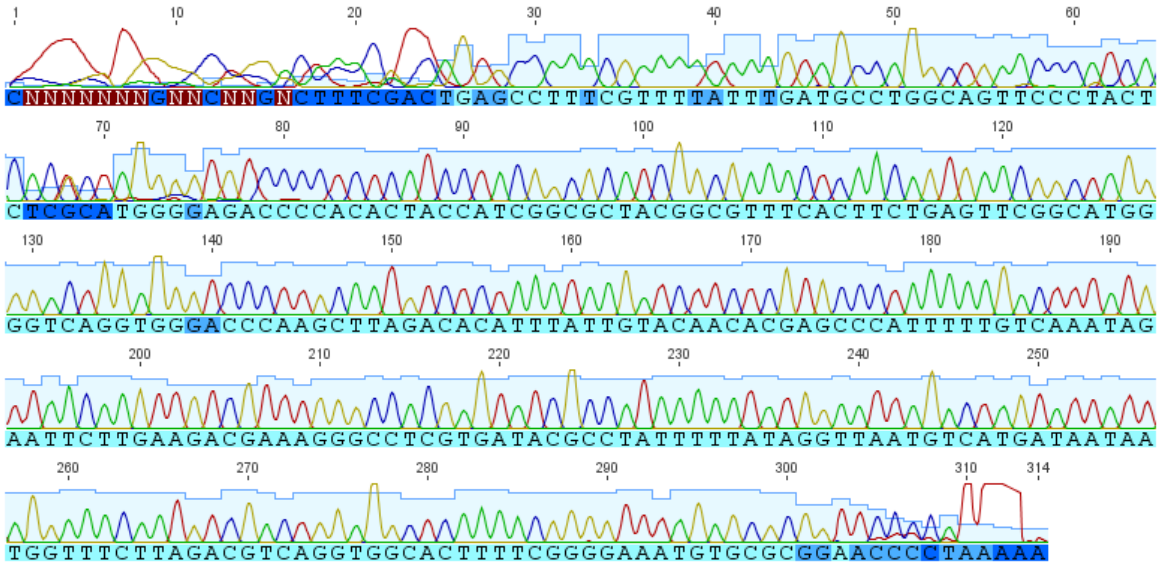
1.1.3. mC-modified DNA

5´-TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TTC AAG AAT TC***T ATT TGA CAA AAA TGG GCT CGT GTT GTA CAA TAA ATG T***GT CTA AGC TTG GGT CCC ACC TGA CCC CAT GCC GAA CTC AGA AGT GAA ACG CCG TAG CGC CGA TGG TAG TGT GGG GTC TCC CCA TGC GAG AGT AGG GAA CTG CCA GGC ATC AAA TAA AAC GAA AGG CTC AGT CGA AAG ACT GGG CCT TTC GTT TTA TCT GTT GTT TGT CGG TGA ACG CTC TCC-3´

Primary strand (exact match for 284 bp):



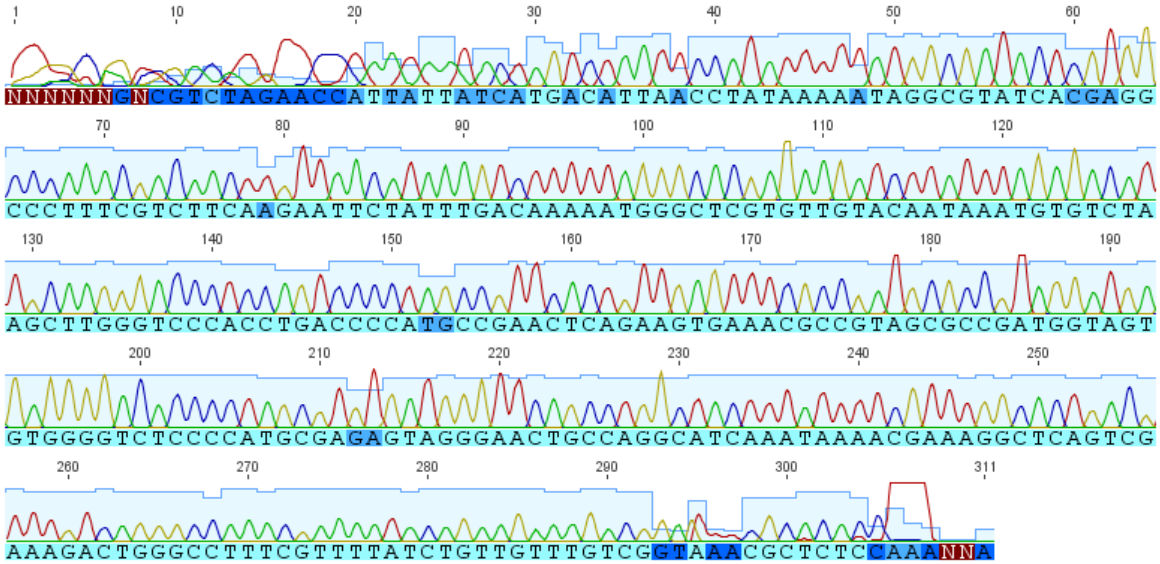
Complementary strand (exact match for 293 bp):



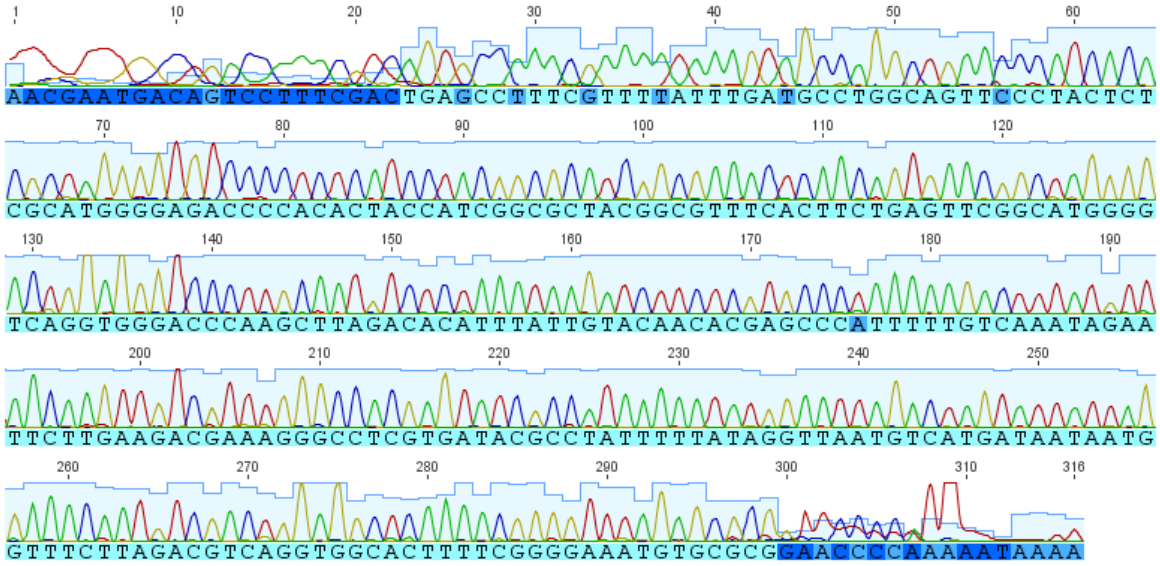
1.1.4. hmC-modified DNA

5´-TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TTC AAG AAT TC***T ATT TGA CAA AAA TGG GCT CGT GTT GTA CAA TAA ATG T***GT CTA AGC TTG GGT CCC ACC TGA CCC CAT GCC GAA CTC AGA AGT GAA ACG CCG TAG CGC CGA TGG TAG TGT GGG GTC TCC CCA TGC GAG AGT AGG GAA CTG CCA GGC ATC AAA TAA AAC GAA AGG CTC AGT CGA AAG ACT GGG CCT TTC GTT TTA TCT GTT GTT TGT CGG TGA ACG CTC TCC-3´

Primary strand (exact match for 290 bp):



Complementary strand (exact match for 294 bp):

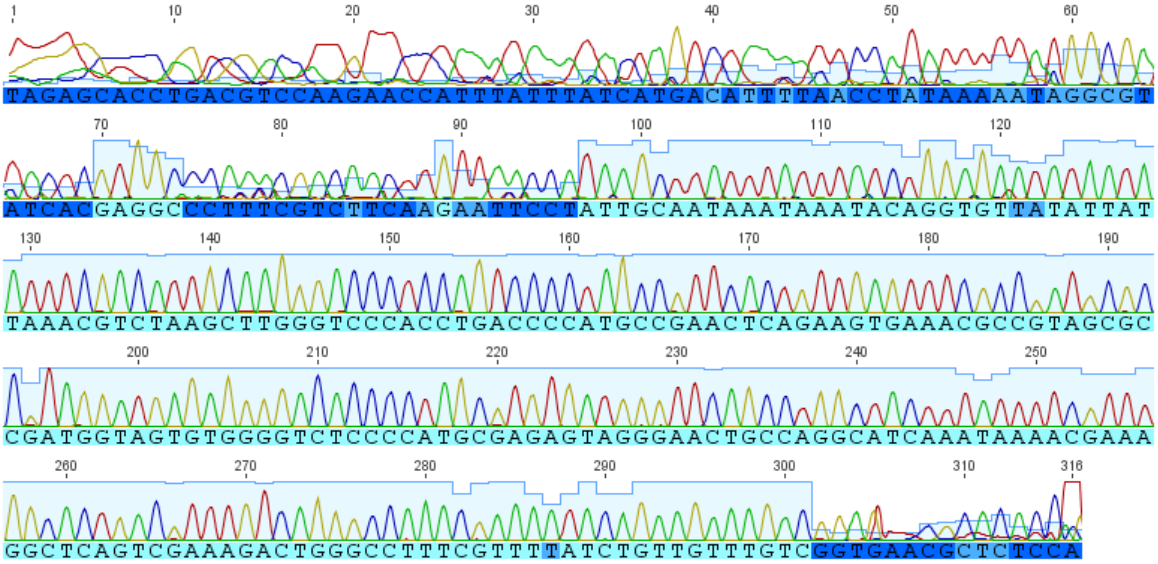


1.2. Sequencing of fully modified DNA (340-mers) with promotor region of BP1

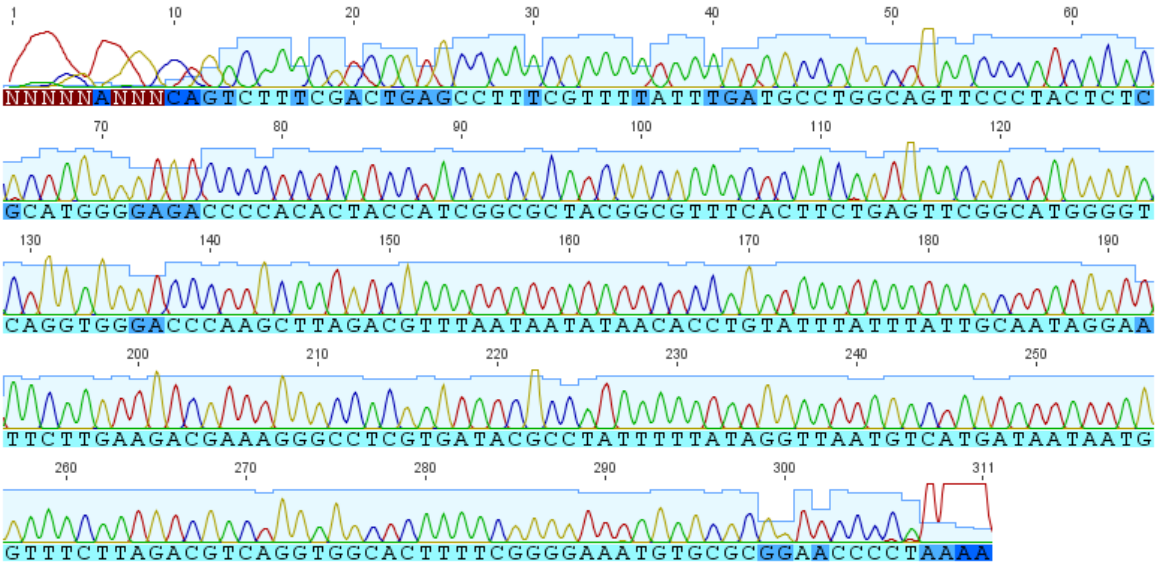
1.2.1. dU-modified DNA

5´-TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TTC AAG AAT TC***C TAT TGC AAT AAA TAA ATA CAG GTG TTA TAT TAT TAA AC***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 272 bp):



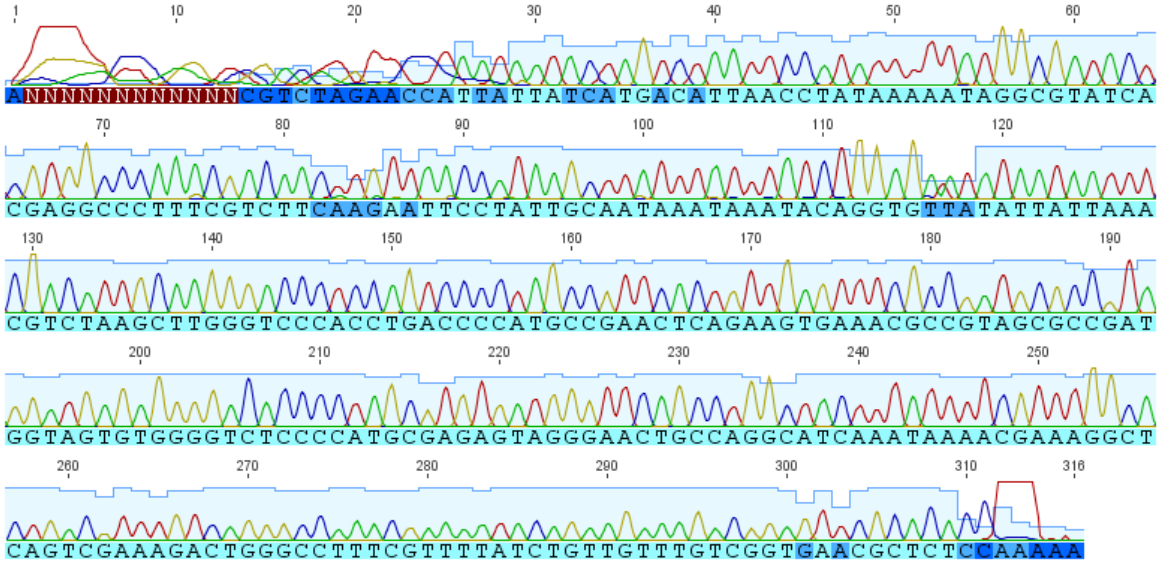
Complementary strand (exact match for 299 bp):



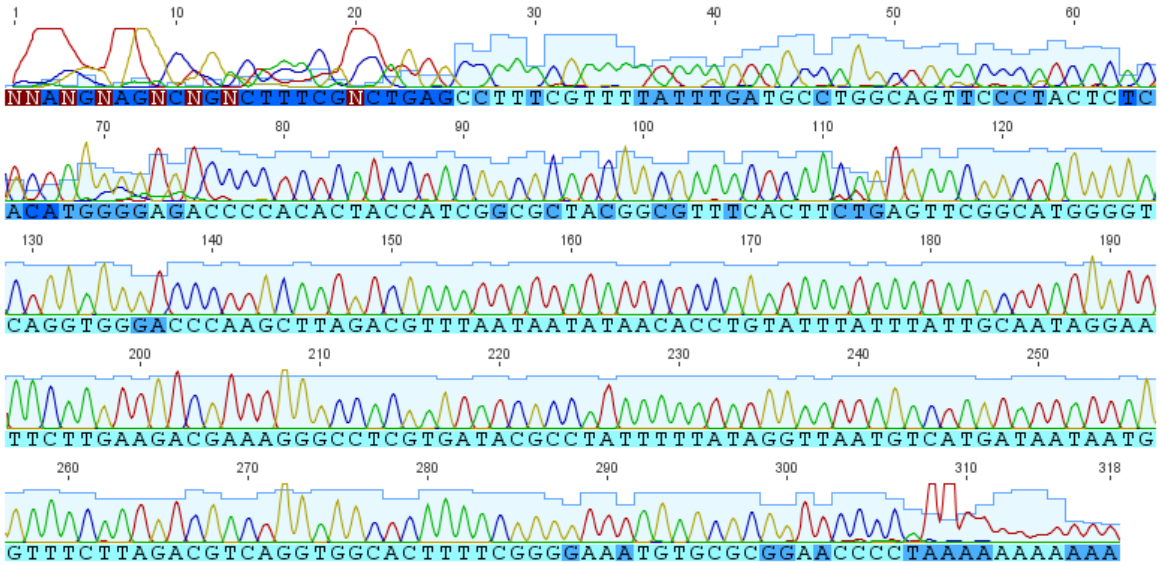
1.2.2. hmU-modified DNA

5´-TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TTC AAG AAT TC***C TAT TGC AAT AAA TAA ATA CAG GTG TTA TAT TAT TAA AC***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 291 bp):



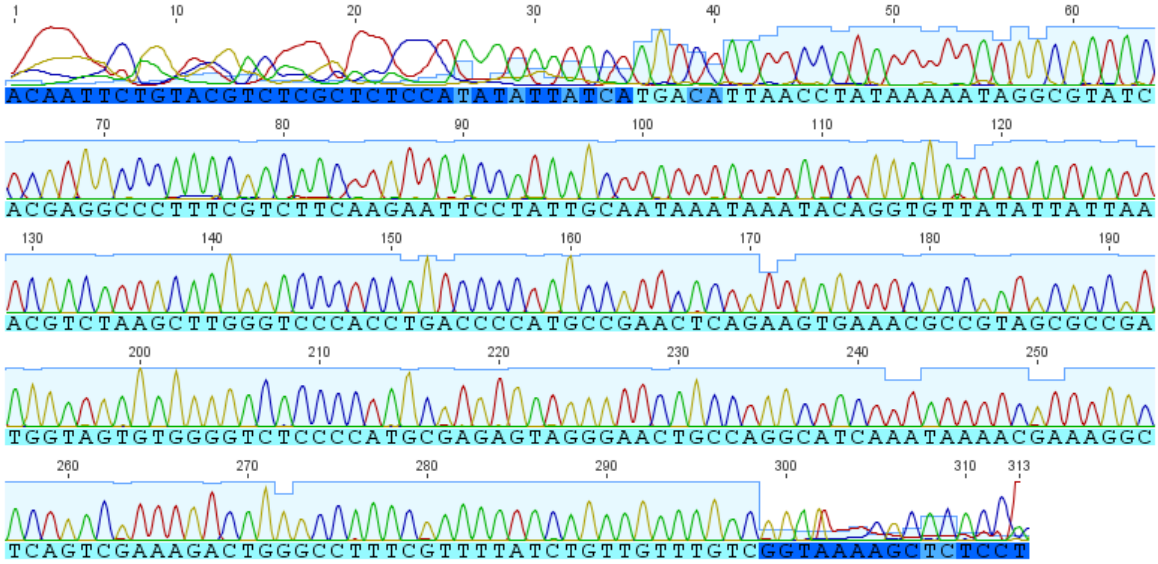
Complementary strand (exact match for 286 bp):



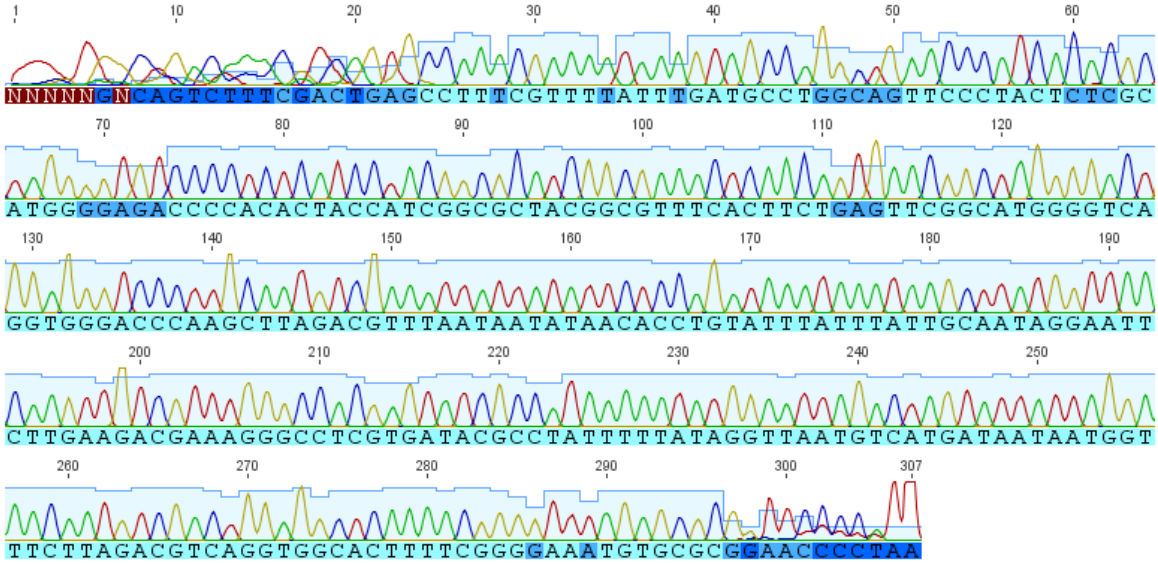
1.2.3. mC-modified DNA

5´-TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TTC AAG AAT TC***C TAT TGC AAT AAA TAA ATA CAG GTG TTA TAT TAT TAA AC***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 285 bp):



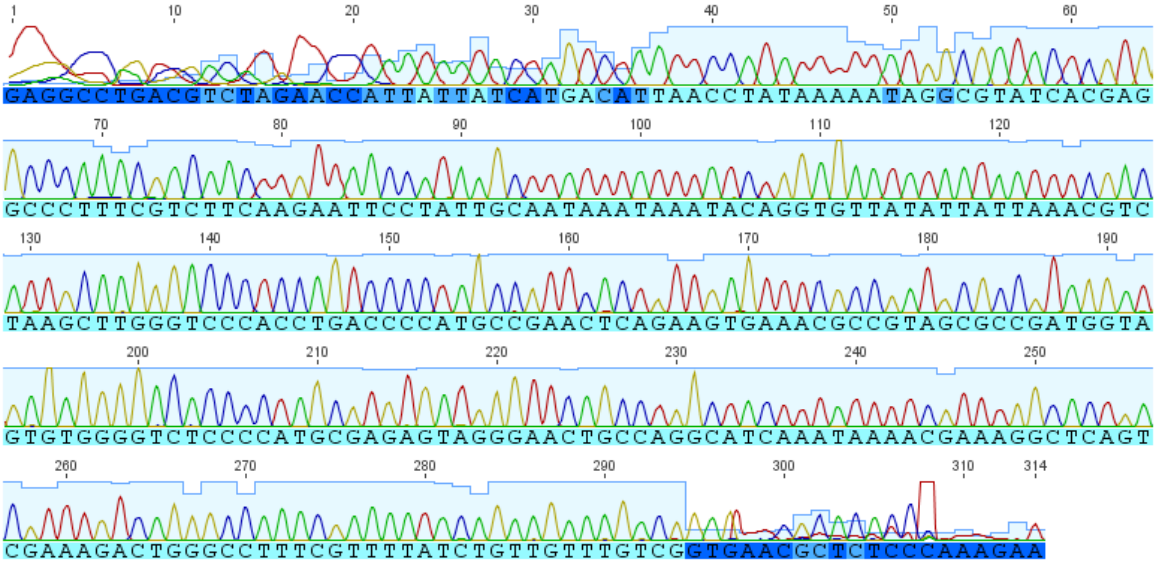
Complementary strand (exact match for 299 bp):



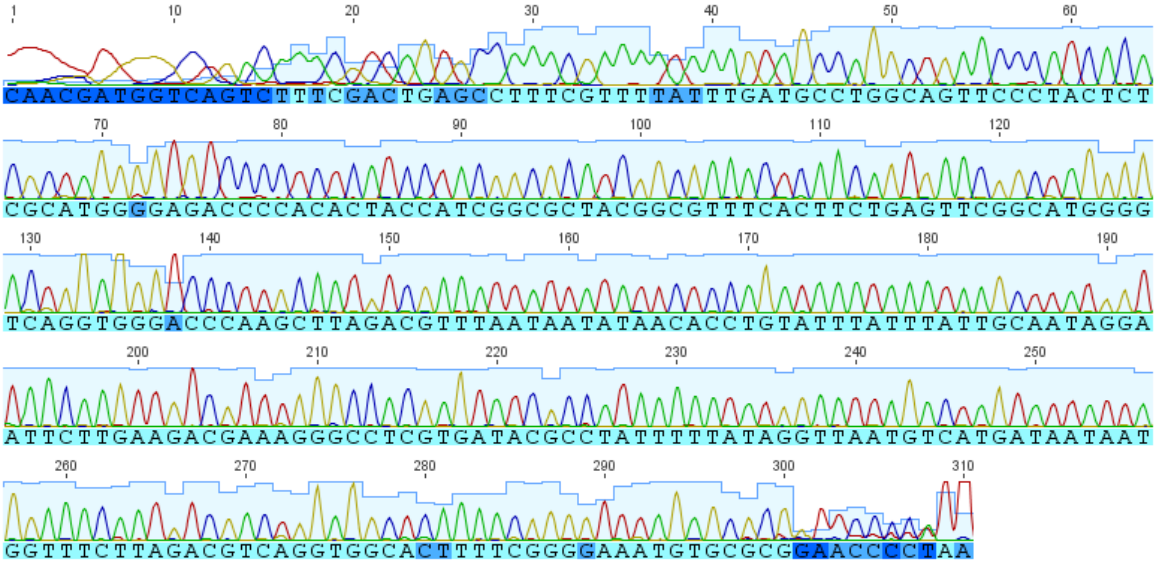
1.2.4. hmC-modified DNA

5´-TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TTC AAG AAT TC***C TAT TGC AAT AAA TAA ATA CAG GTG TTA TAT TAT TAA AC***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 291 bp):



Complementary strand (exact match for 298 bp):

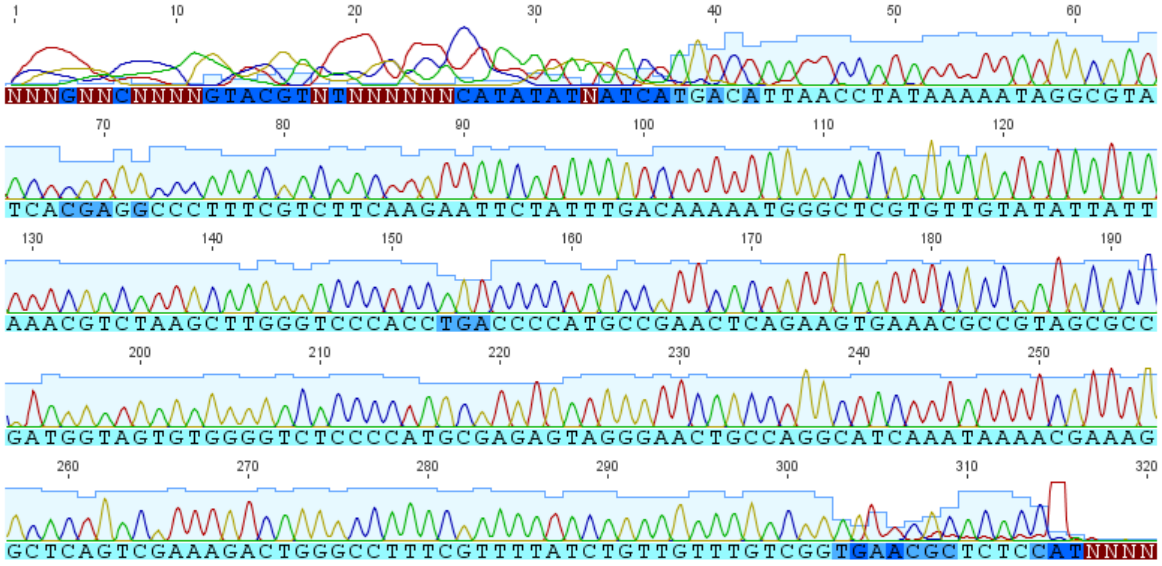


1.3. Sequencing of fully modified DNA (340-mers) with promotor region of PgBP1

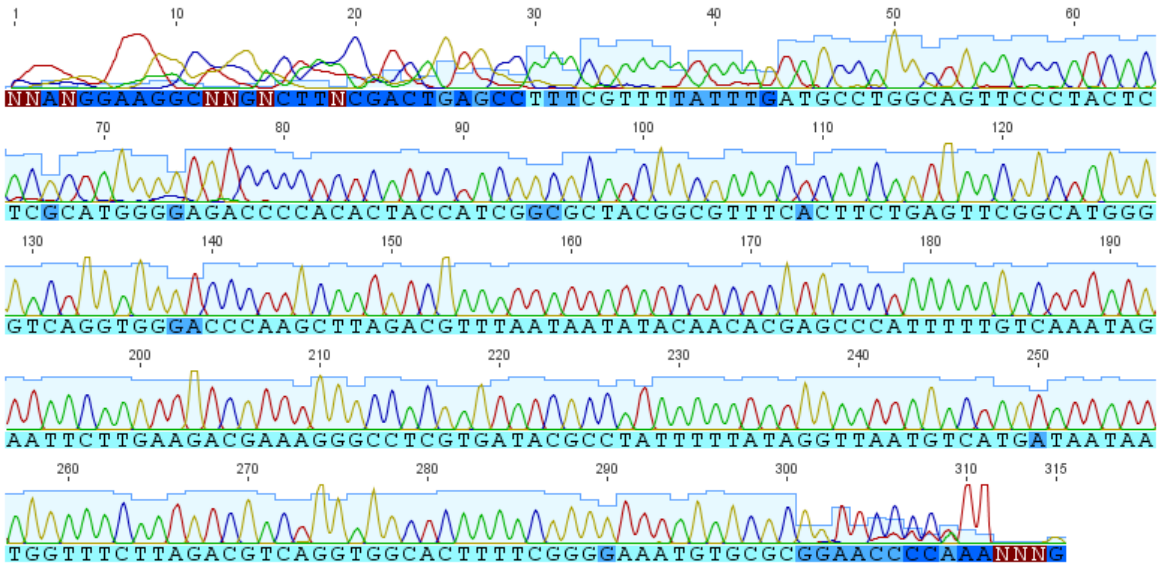
1.3.1. dU-modified DNA

5´-TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TTC AAG AAT TC***T ATT TGA CAA AAA TGG GCT CGT GTT GTA TAT TAT TAA AC***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 281 bp):



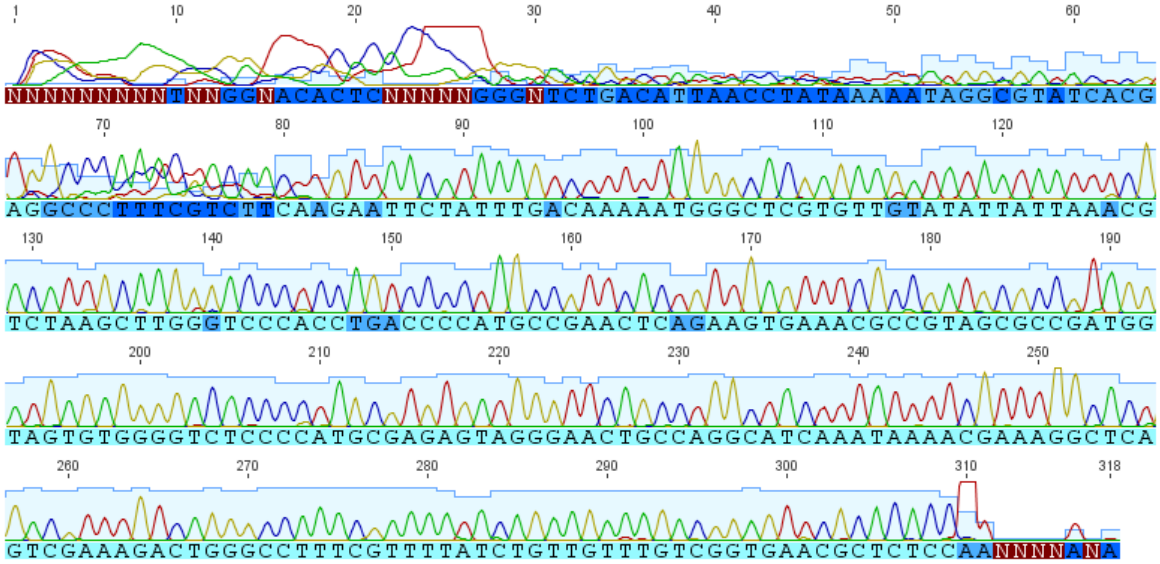
Complementary strand (exact match for 291 bp):



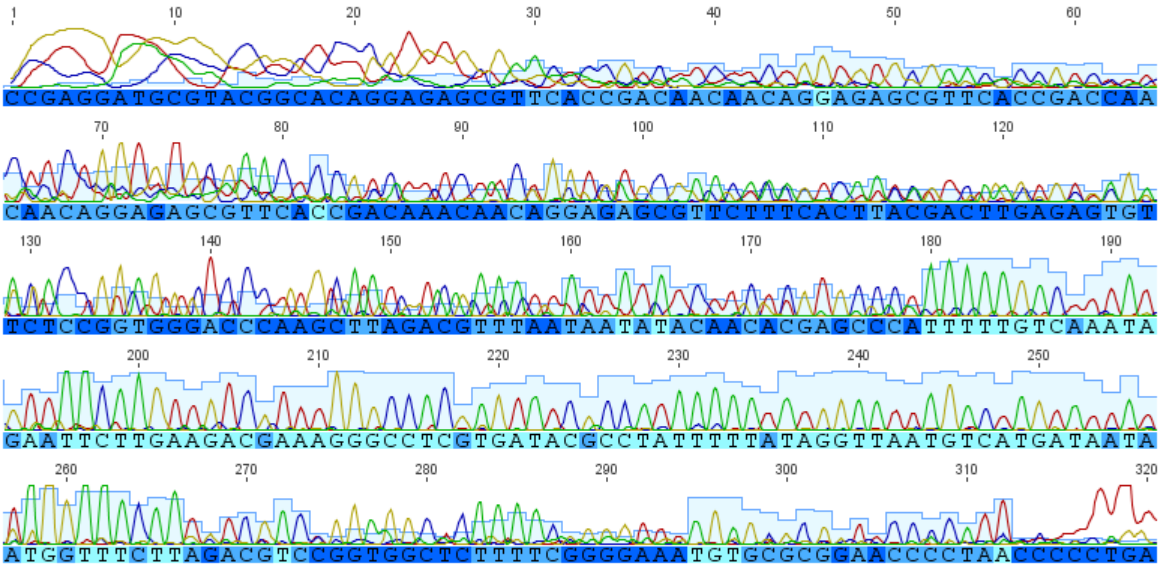
1.3.2. hmU-modified DNA

5´-TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TTC AAG AAT TC***T ATT TGA CAA AAA TGG GCT CGT GTT GTA TAT TAT TAA AC***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 275 bp):



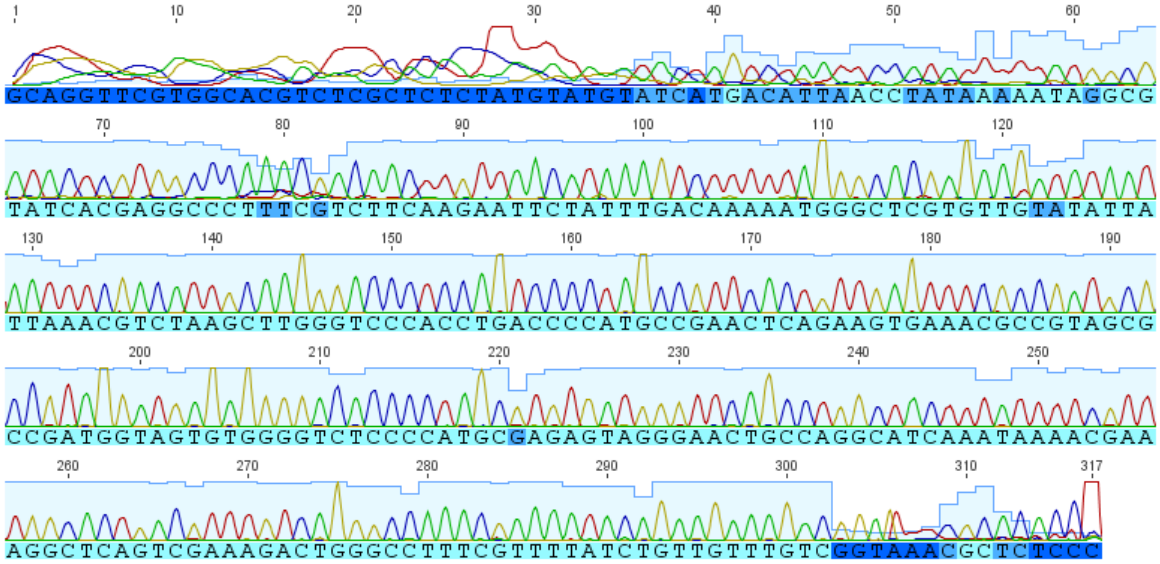
Complementary strand (exact match for 178 bp):



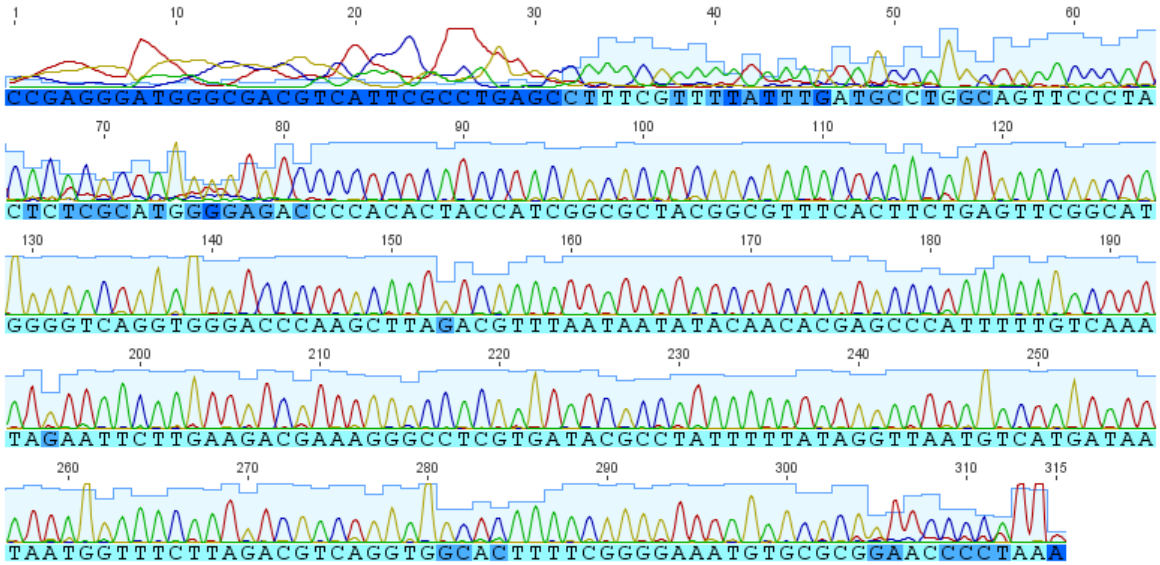
1.3.3. mC-modified DNA

5´-TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TTC AAG AAT TC***T ATT TGA CAA AAA TGG GCT CGT GTT GTA TAT TAT TAA AC***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 281 bp):



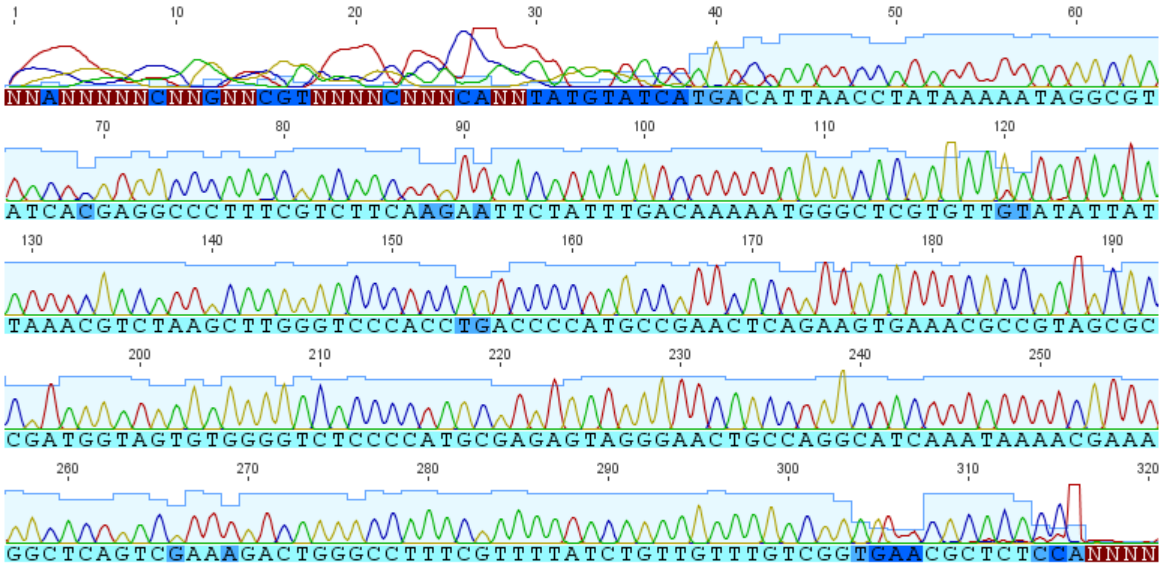
Complementary strand (exact match for 288 bp):



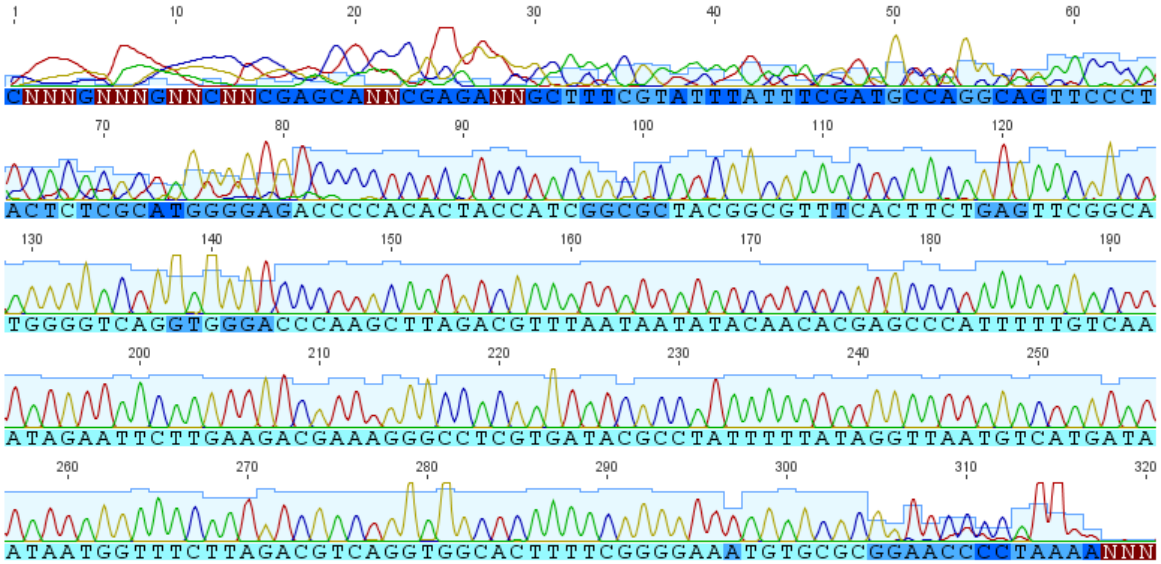
1.3.4. hmC-modified DNA

5´-TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TTC AAG AAT TC***T ATT TGA CAA AAA TGG GCT CGT GTT GTA TAT TAT TAA AC***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 281 bp):



Complementary strand (exact match for 261 bp):

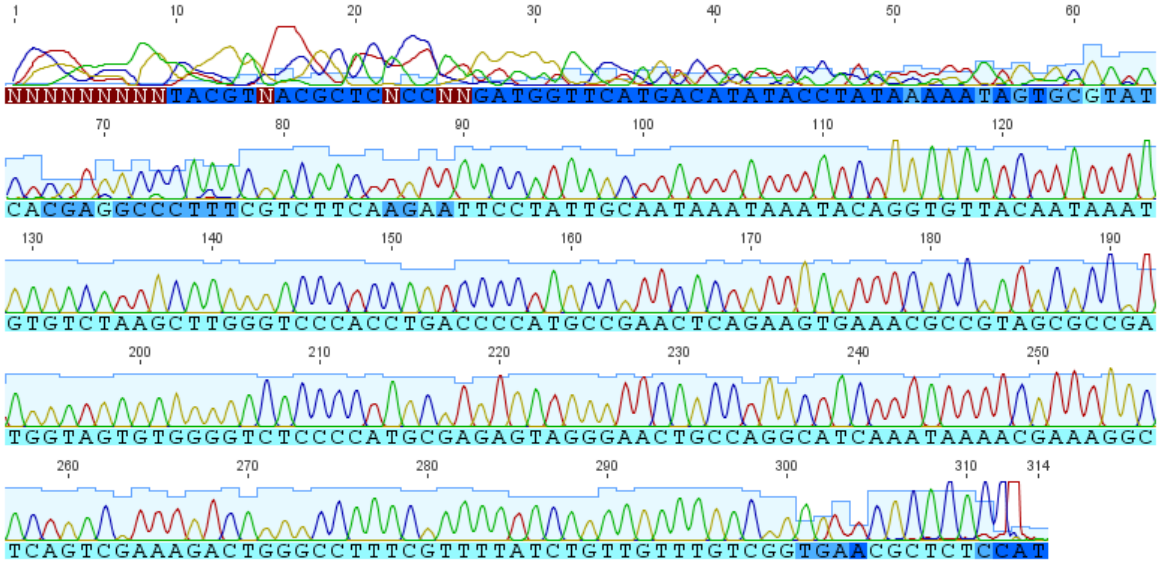


1.4. Sequencing of fully modified DNA (339-mers) with promotor region of BP1Pg

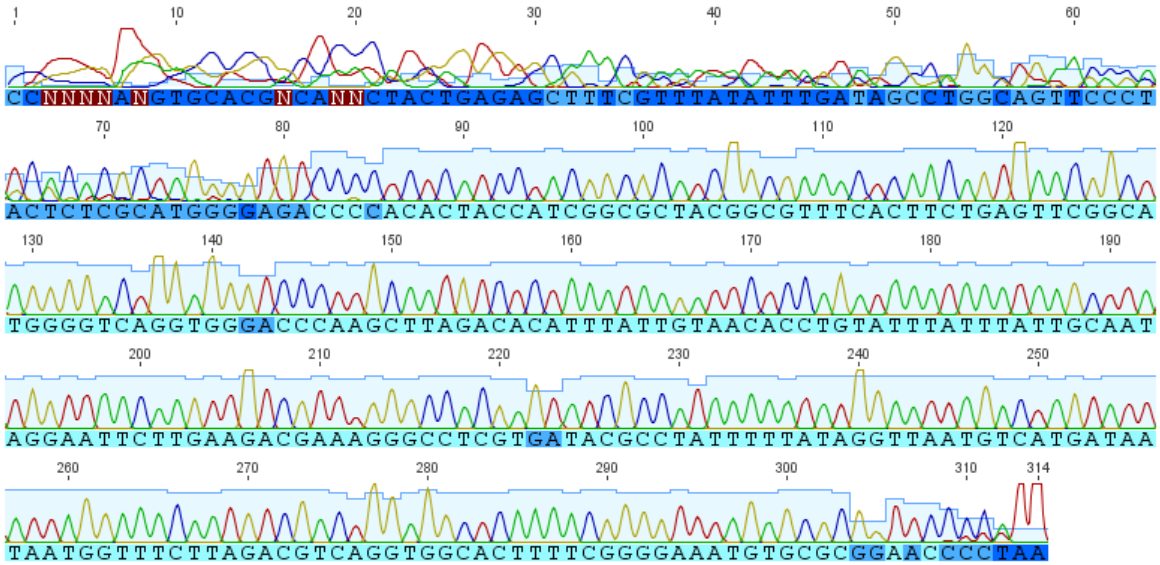
1.4.1. dU-modified DNA

5´-TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TTC AAG AAT TC***C TAT TGC AAT AAA TAA ATA CAG GTG TTA CAA TAA ATG T***GT CTA AGC TTG GGT CCC ACC TGA CCC CAT GCC GAA CTC AGA AGT GAA ACG CCG TAG CGC CGA TGG TAG TGT GGG GTC TCC CCA TGC GAG AGT AGG GAA CTG CCA GGC ATC AAA TAA AAC GAA AGG CTC AGT CGA AAG ACT GGG CCT TTC GTT TTA TCT GTT GTT TGT CGG TGA ACG CTC TCC-3´

Primary strand (exact match for 268 bp):



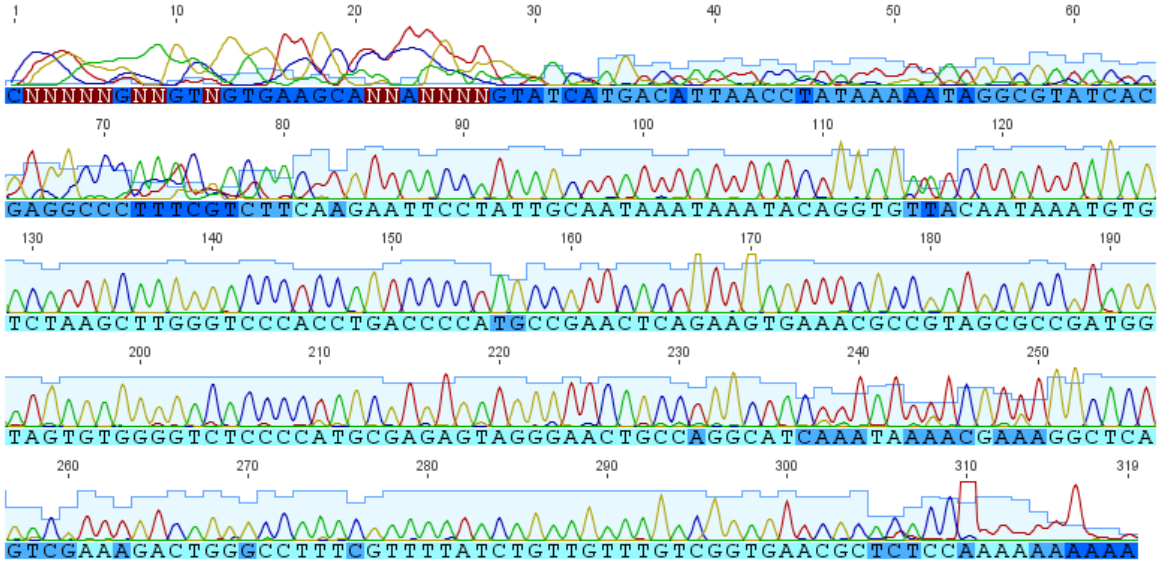
Complementary strand (exact match for 264 bp):



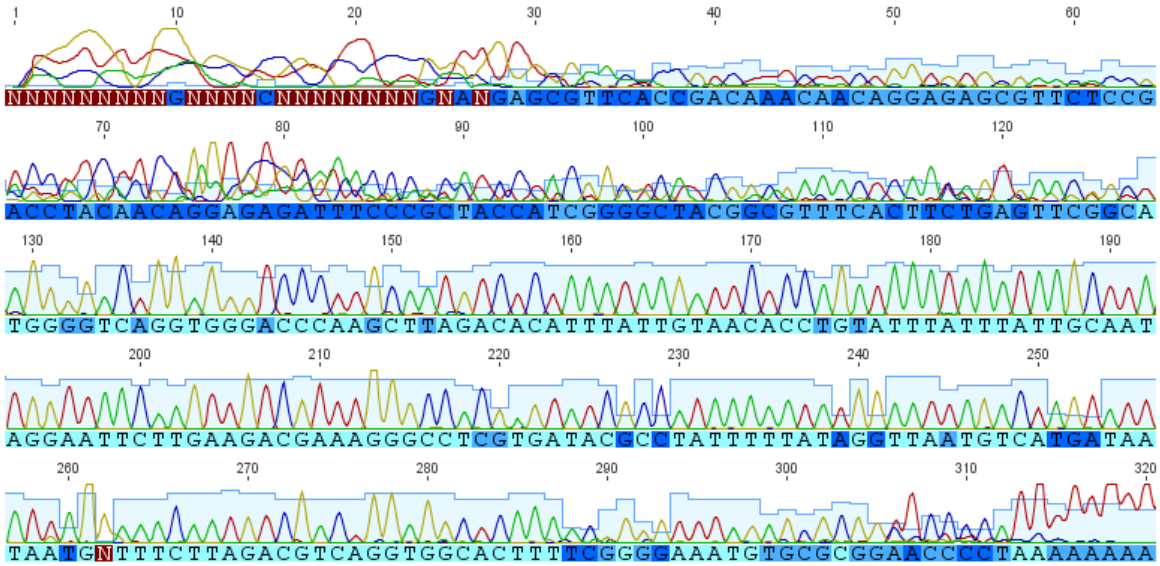
1.4.2. hmU-modified DNA

5´-TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TTC AAG AAT TC***C TAT TGC AAT AAA TAA ATA CAG GTG TTA CAA TAA ATG T***GT CTA AGC TTG GGT CCC ACC TGA CCC CAT GCC GAA CTC AGA AGT GAA ACG CCG TAG CGC CGA TGG TAG TGT GGG GTC TCC CCA TGC GAG AGT AGG GAA CTG CCA GGC ATC AAA TAA AAC GAA AGG CTC AGT CGA AAG ACT GGG CCT TTC GTT TTA TCT GTT GTT TGT CGG TGA ACG CTC TCC-3´

Primary strand (exact match for 281 bp):



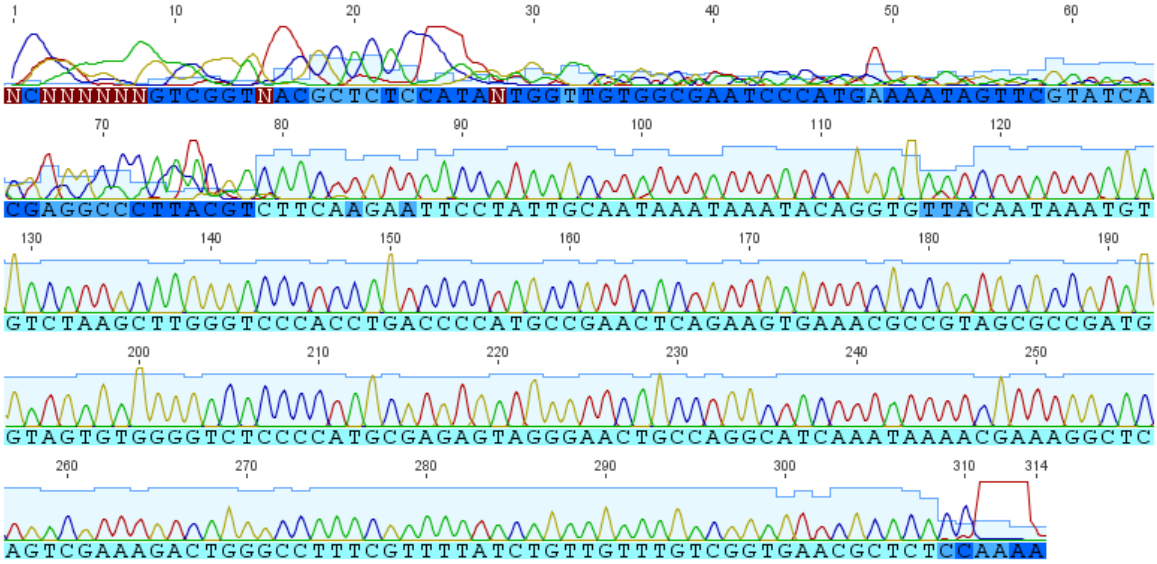
Complementary strand (exact match for 214 bp):



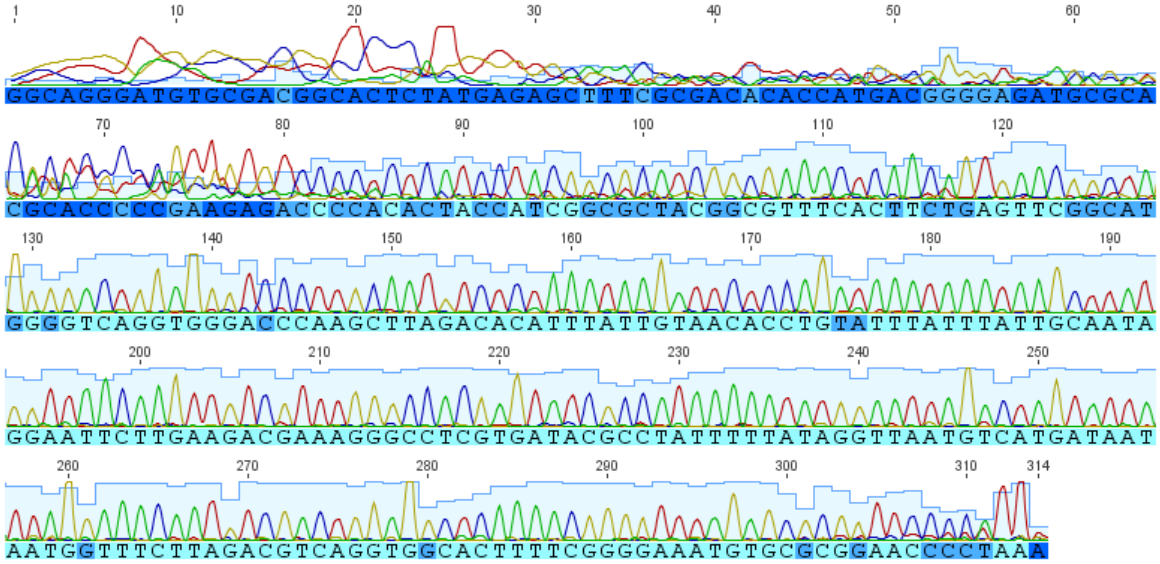
1.4.3. mC-modified DNA

5´-TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TTC AAG AAT TC***C TAT TGC AAT AAA TAA ATA CAG GTG TTA CAA TAA ATG T***GT CTA AGC TTG GGT CCC ACC TGA CCC CAT GCC GAA CTC AGA AGT GAA ACG CCG TAG CGC CGA TGG TAG TGT GGG GTC TCC CCA TGC GAG AGT AGG GAA CTG CCA GGC ATC AAA TAA AAC GAA AGG CTC AGT CGA AAG ACT GGG CCT TTC GTT TTA TCT GTT GTT TGT CGG TGA ACG CTC TCC-3´

Primary strand (exact match for 235 bp):



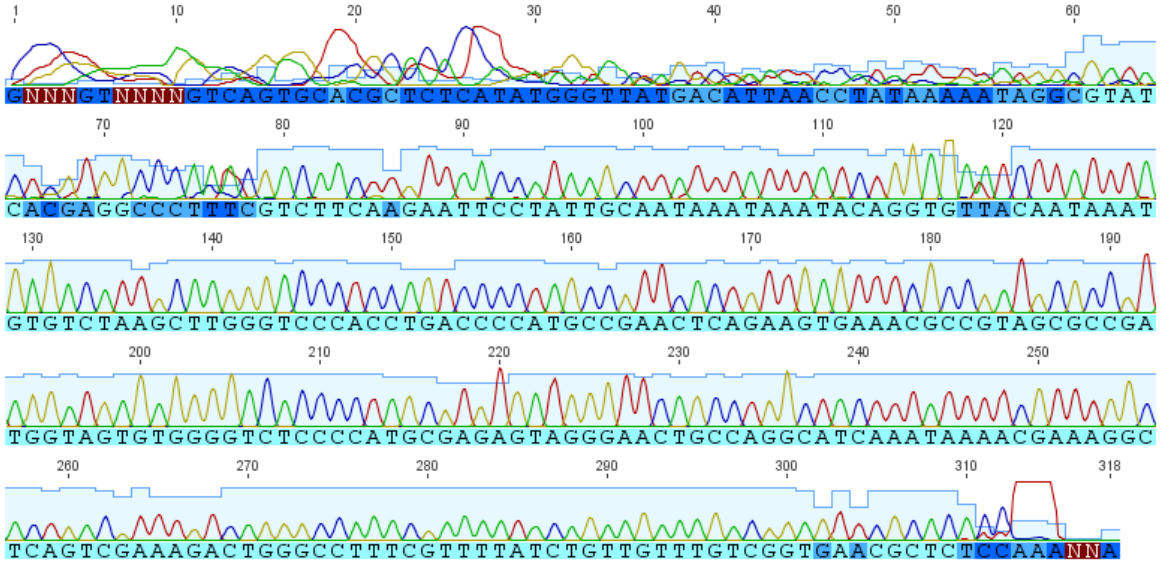
Complementary strand (exact match for 235 bp):



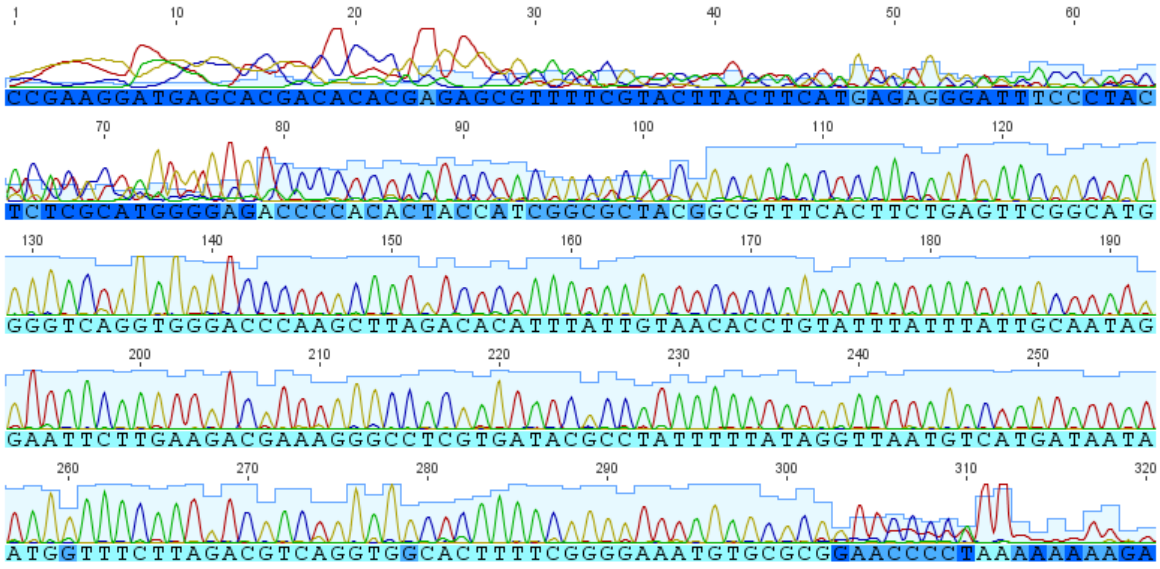
1.4.4. hmC-modified DNA

5´-TAG GGG TTC CGC GCA CAT TTC CCC GAA AAG TGC CAC CTG ACG TCT AAG AAA CCA TTA TTA TCA TGA CAT TAA CCT ATA AAA ATA GGC GTA TCA CGA GGC CCT TTC GTC TTC AAG AAT TC***C TAT TGC AAT AAA TAA ATA CAG GTG TTA CAA TAA ATG T***GT CTA AGC TTG GGT CCC ACC TGA CCC CAT GCC GAA CTC AGA AGT GAA ACG CCG TAG CGC CGA TGG TAG TGT GGG GTC TCC CCA TGC GAG AGT AGG GAA CTG CCA GGC ATC AAA TAA AAC GAA AGG CTC AGT CGA AAG ACT GGG CCT TTC GTT TTA TCT GTT GTT TGT CGG TGA ACG CTC TCC-3´

Primary strand (exact match for 277 bp):



Complementary strand (exact match for 255 bp):

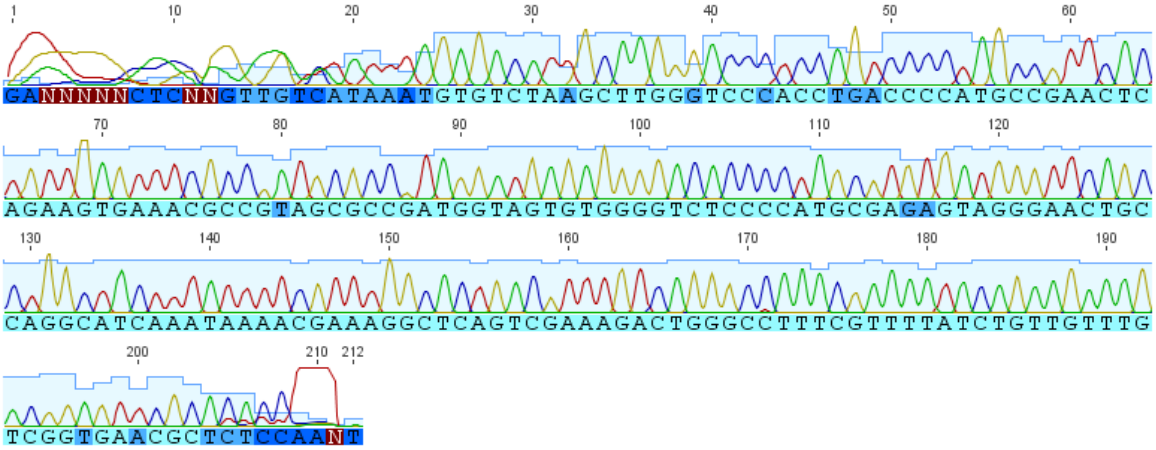


1.5. Sequencing of fully modified DNA (235-mers) with promotor region of Pveg

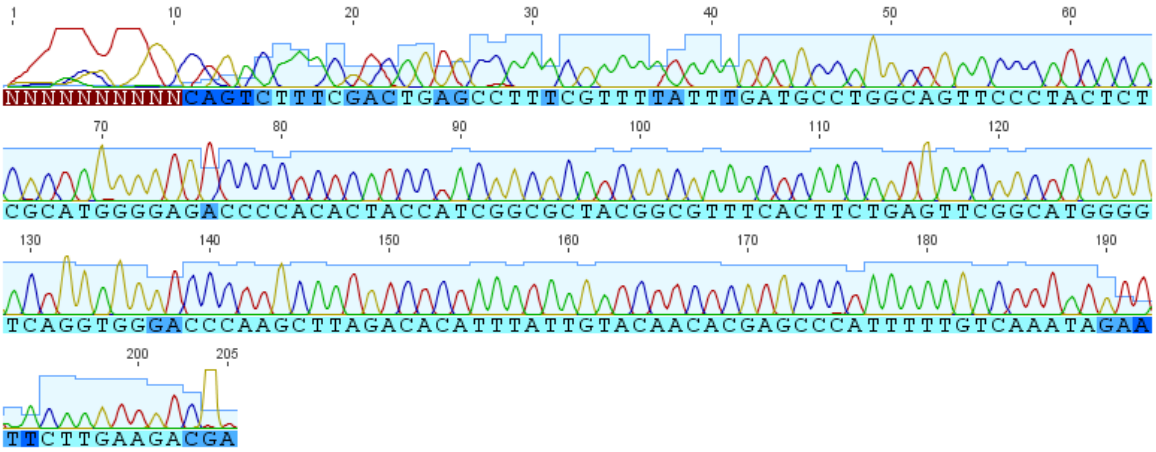
1.5.1. dU-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 190 bp):



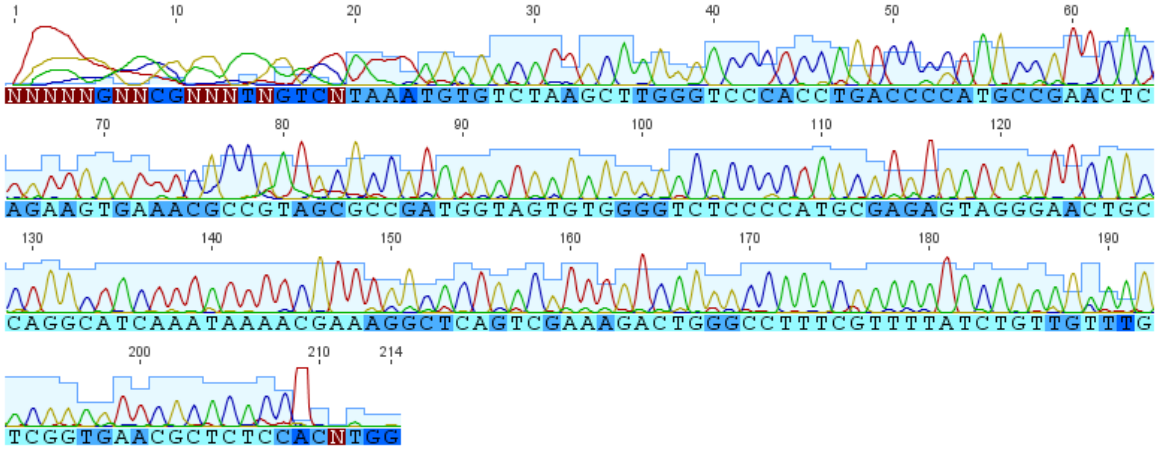
Complementary strand (exact match for 193 bp):



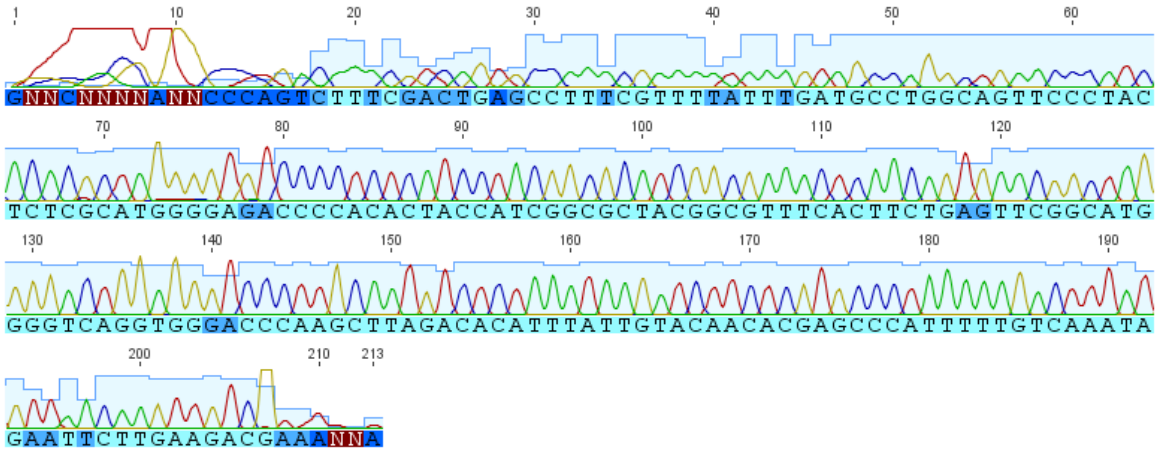
1.5.2. hmU-modified DNA

5′-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3′

Primary strand (exact match for 188 bp):



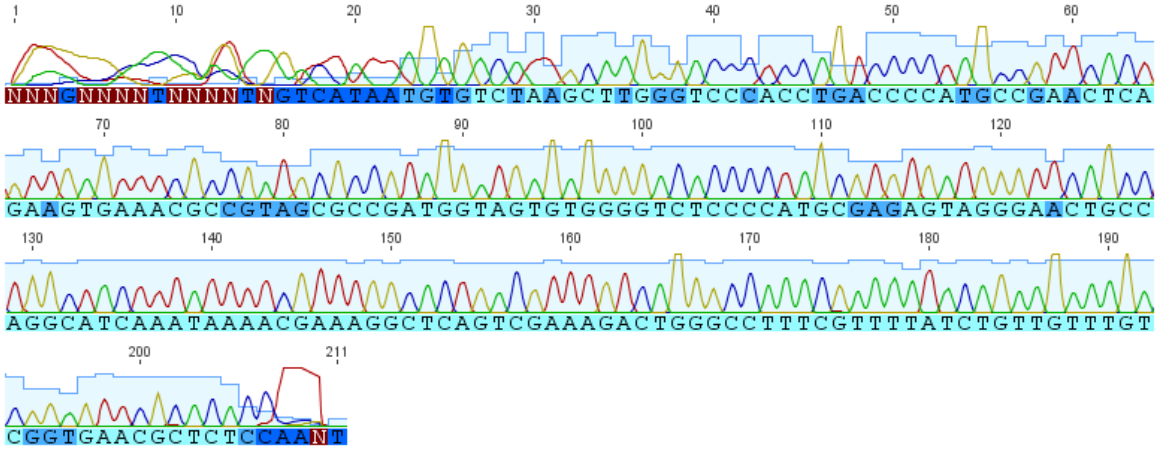
Complementary strand (exact match for 196 bp):



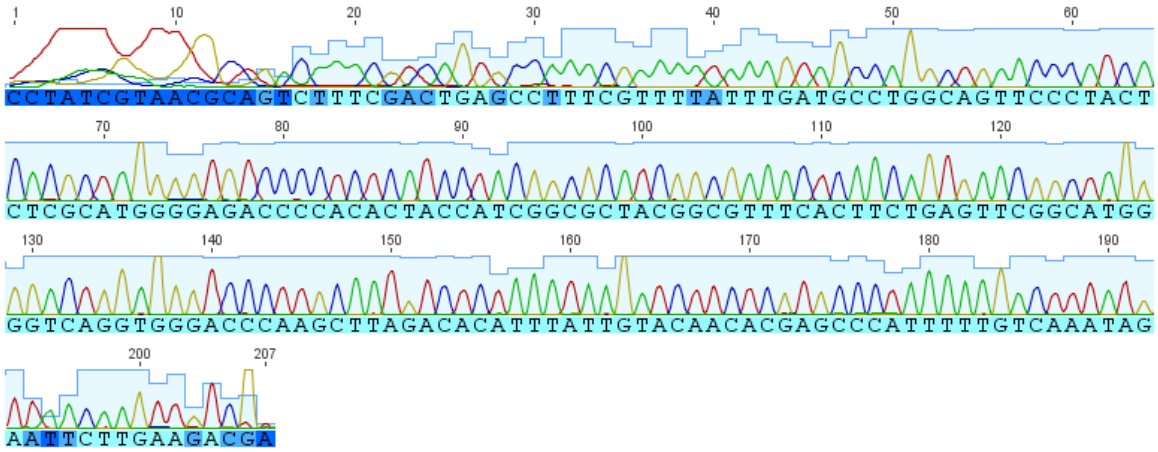
1.5.3. mC-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 187 bp):



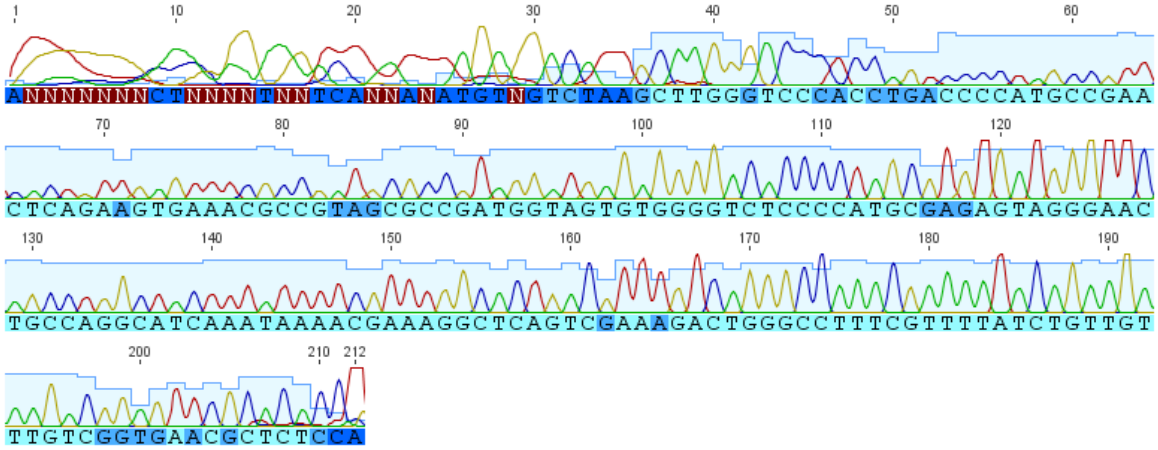
Complementary strand (exact match for 194 bp):



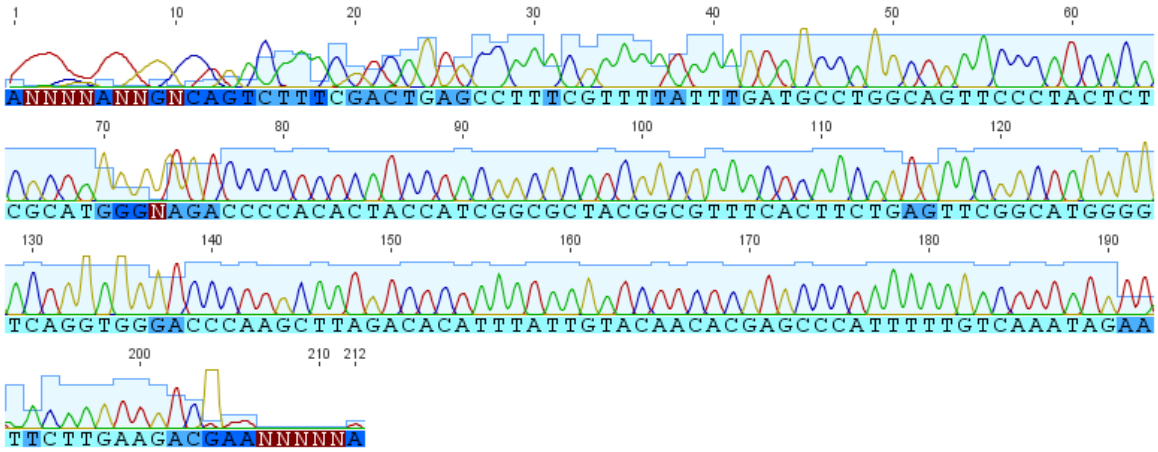
1.5.4. hmC-modified DNA

5′-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3′

Primary strand (exact match for 181 bp):



Complementary strand (exact match for 186 bp):



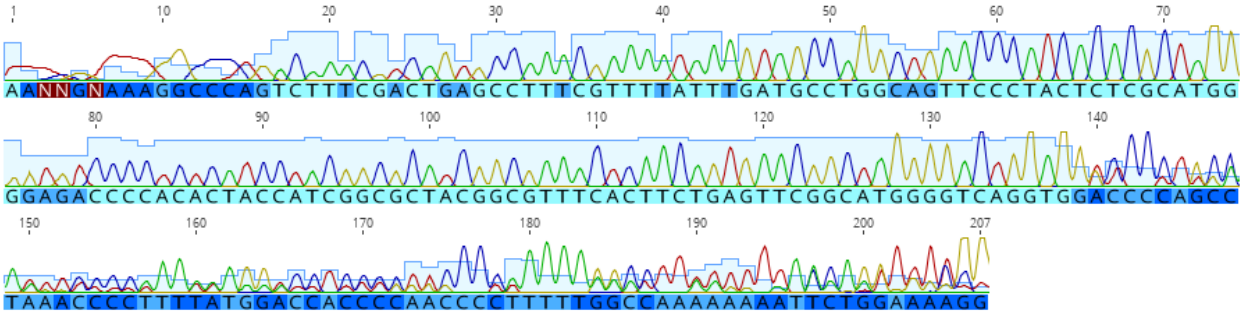
2. Sequencing of partially modified 235-mers of DNA

2.1. Sequencing of modified downstream of dsDNA

2.1.1. dU-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3’

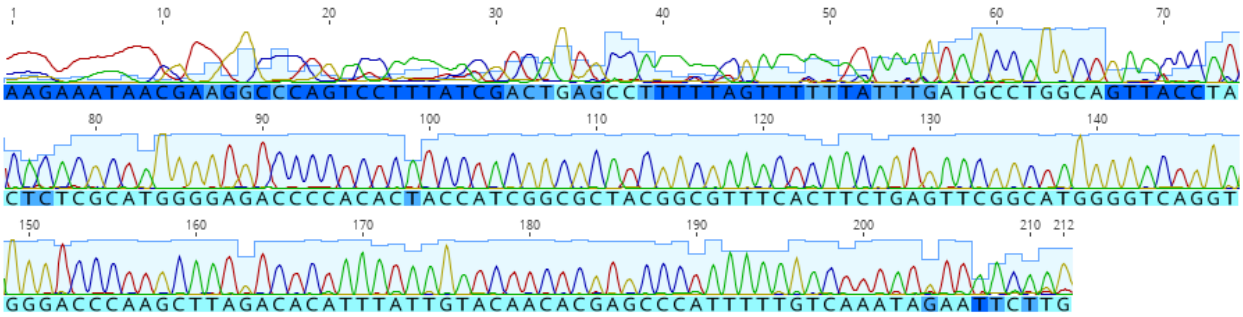
Complementary strand (exact match for 122 bp):



2.1.2. d5hmU-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3’

Complementary strand (exact match for 189 bp):

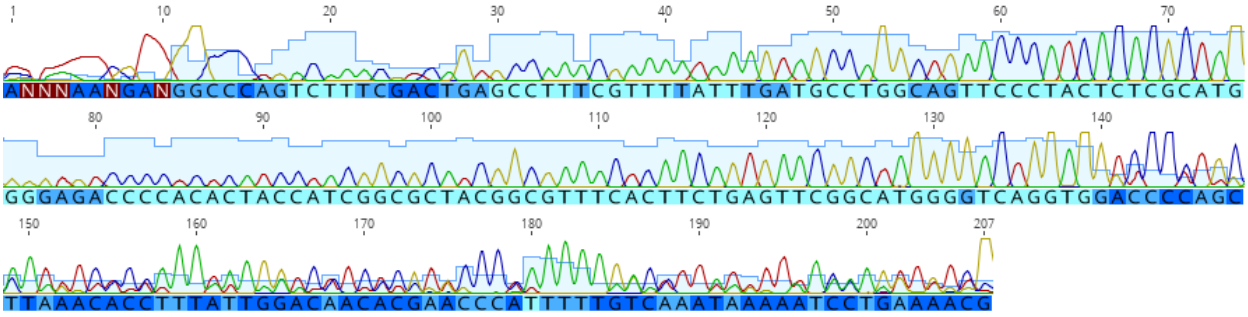


2.1.3. d5mC-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG

GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3'

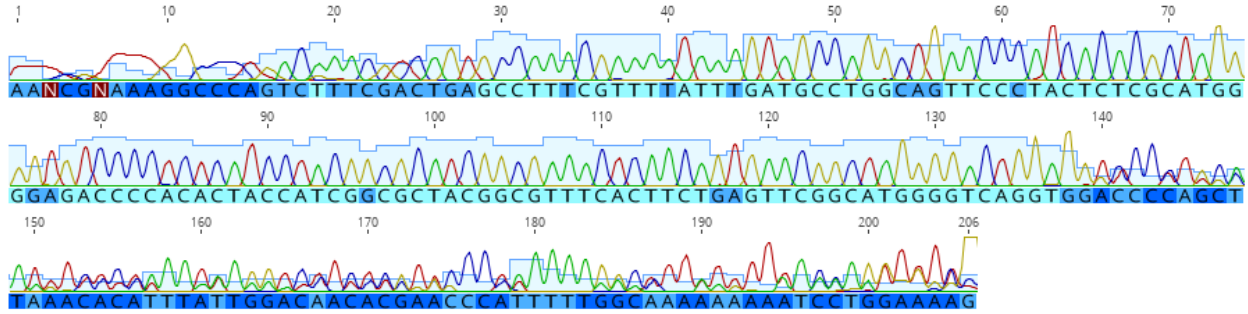
Complementary strand (exact match for 137 bp):



2.1.4. d5hmC-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3'

Complementary strand (exact match for 125 bp):

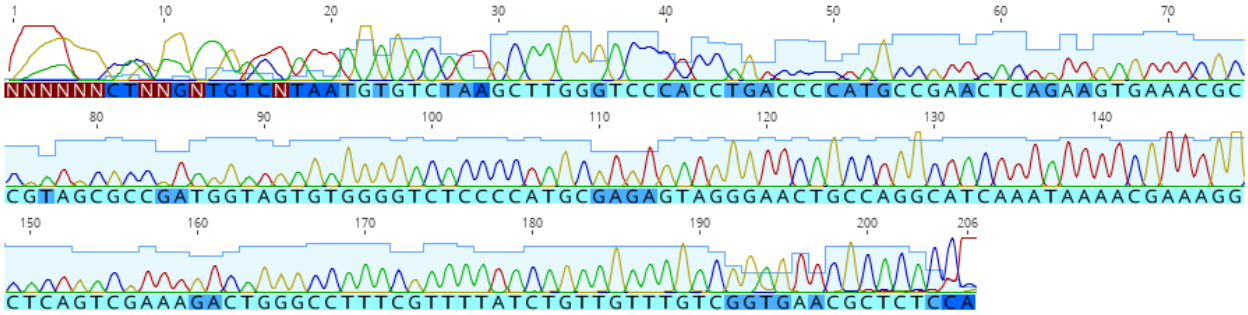


2.2. Sequencing of modified upstream and promotor regions of dsDNA

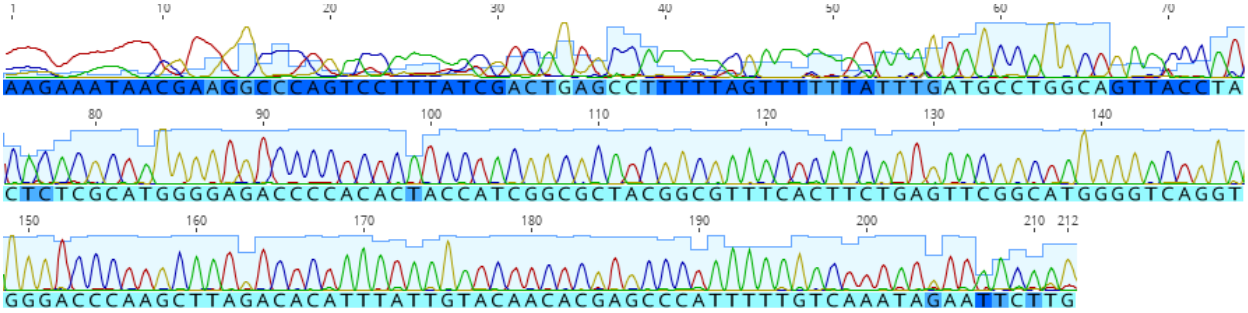
2.2.1. dU-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3'

Primary strand (exact match for 185 bp):



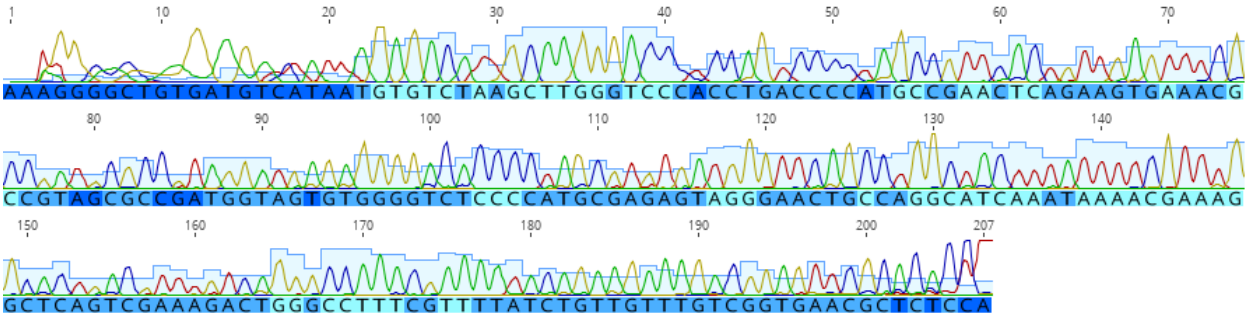
Complementary strand (exact match for 170 bp):



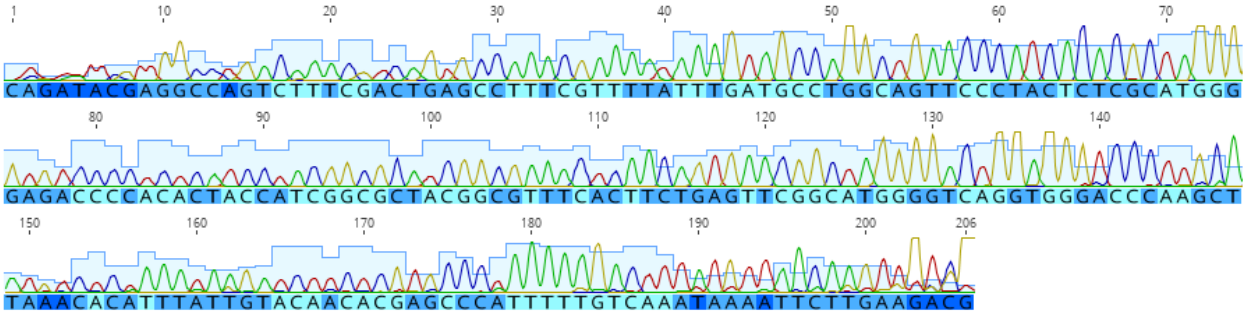
2.2.2. d5hmU-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3’

Primary strand (exact match for 180 bp):



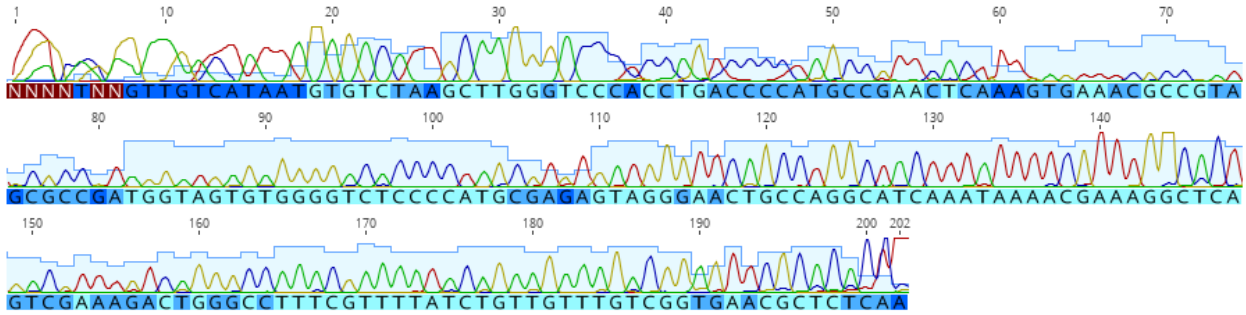
Complementary strand (exact match for 199 bp):



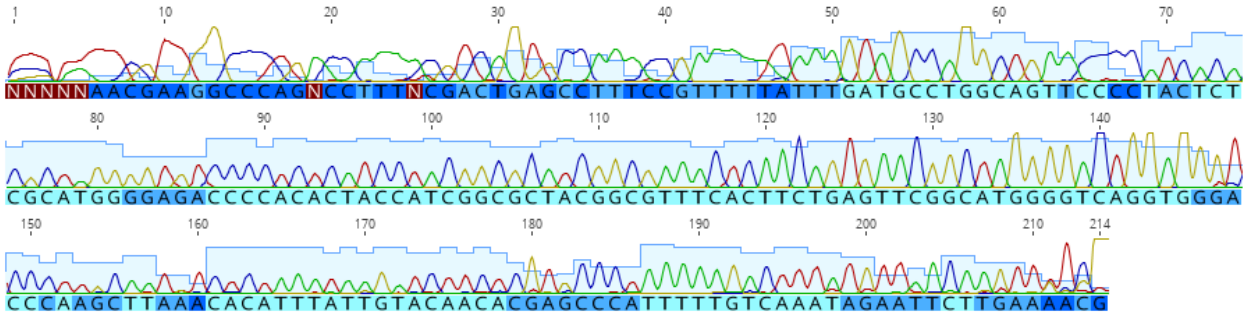
2.2.3. d5mC-modified DNA

5′-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3’

Primary strand (exact match for 184 bp):



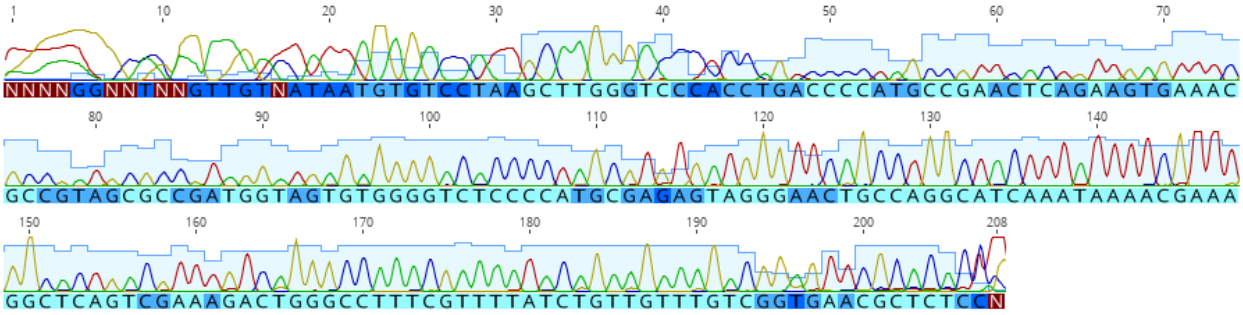
Complementary strand (exact match for 175 bp):



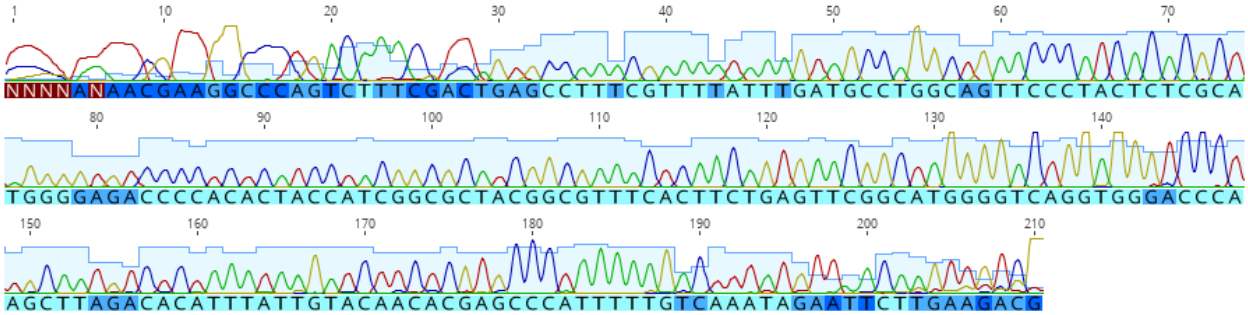
2.2.4. d5hmC-modified DNA

5′-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3’

Primary strand (exact match for 178 bp):



Complementary strand (exact match for 195 bp):

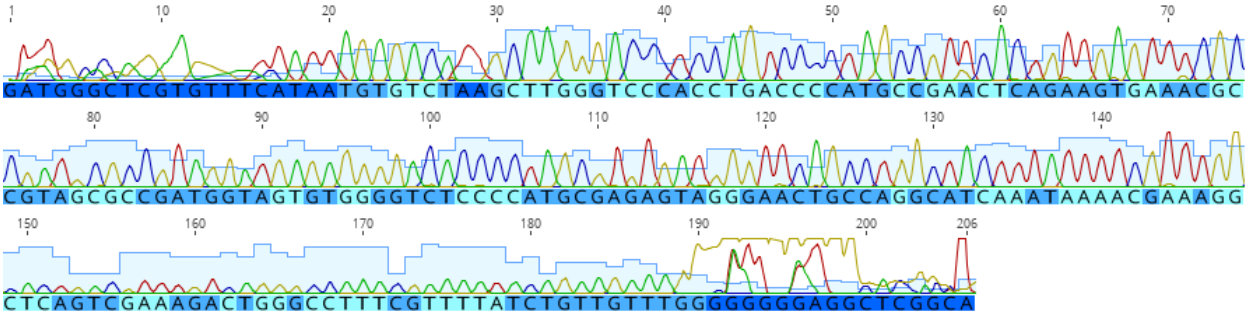


2.3. Sequencing of modified upstream and promotor regions in template strand of DNA

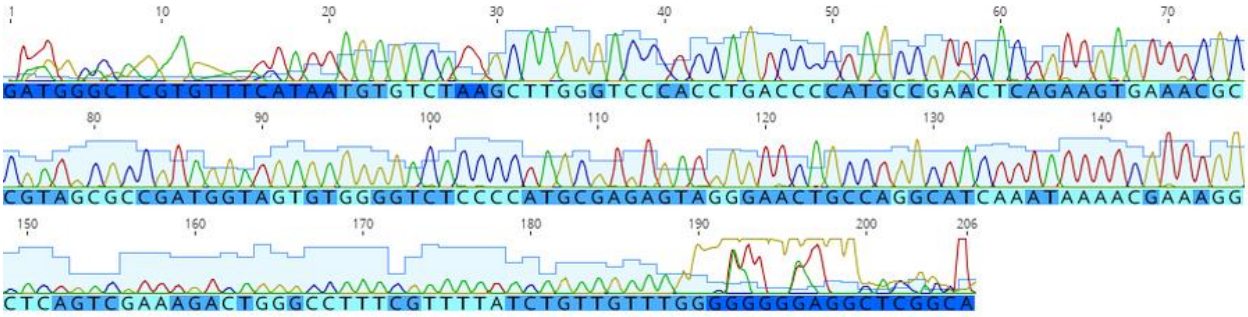
2.3.1. dU-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3’

Primary strand (exact match for 194 bp):



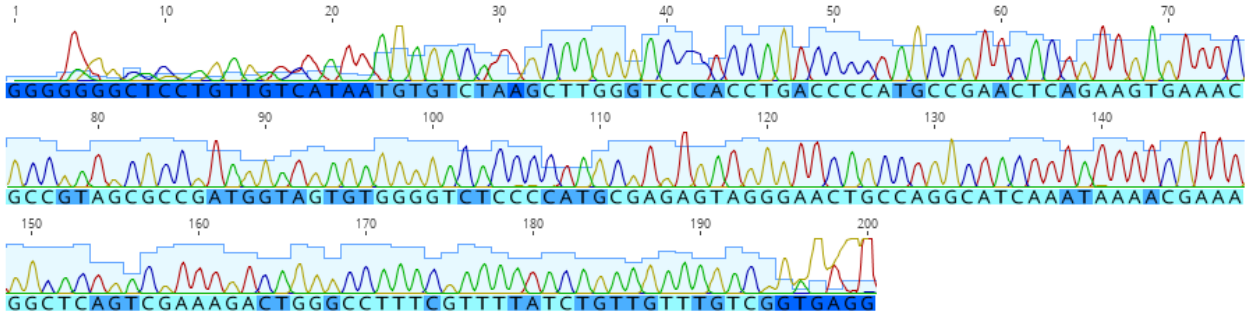
Complementary strand (exact match for 178 bp):



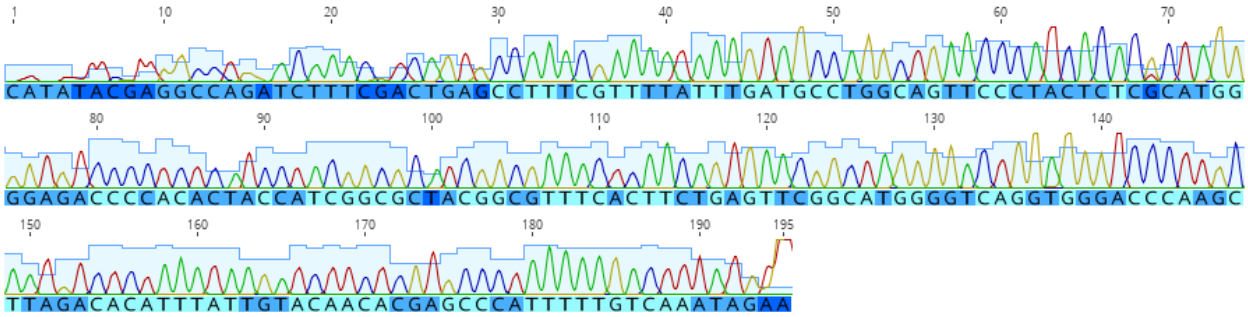
2.3.2. d5hm-U modified DNA

5′-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3′

Primary strand (exact match for 178 bp):



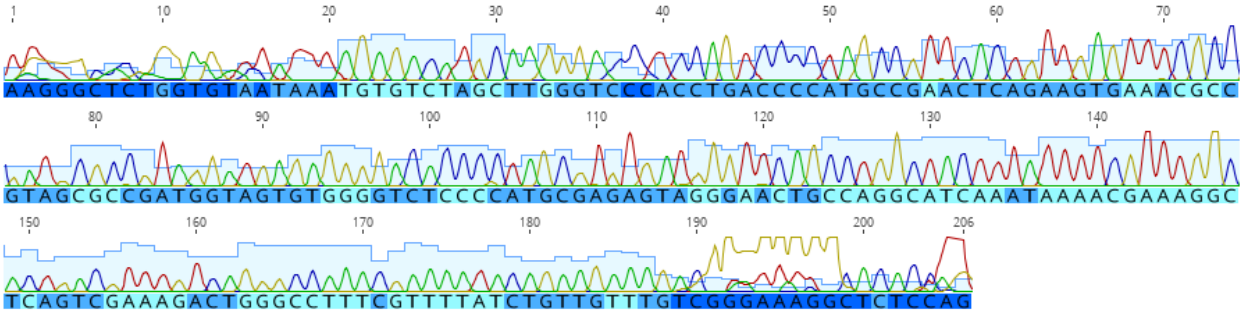
Complementary strand (exact match for 194 bp):



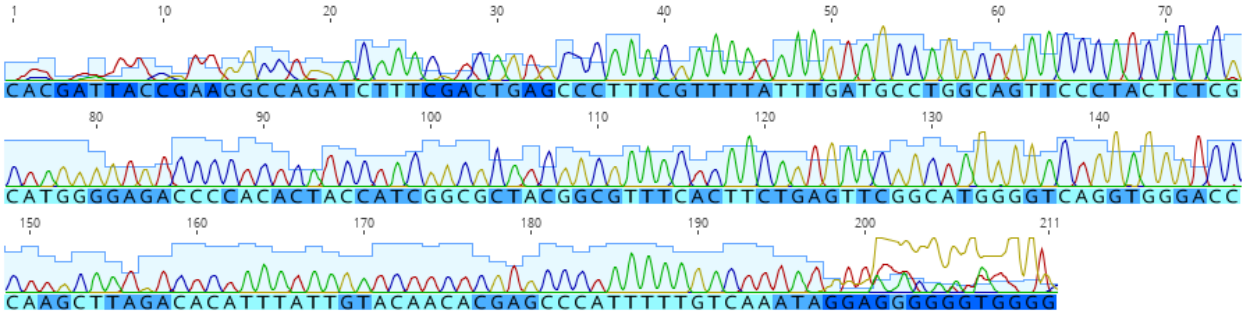
2.3.3. d5mC-modified DNA

5′-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3′

Primary strand (exact match for 200 bp):



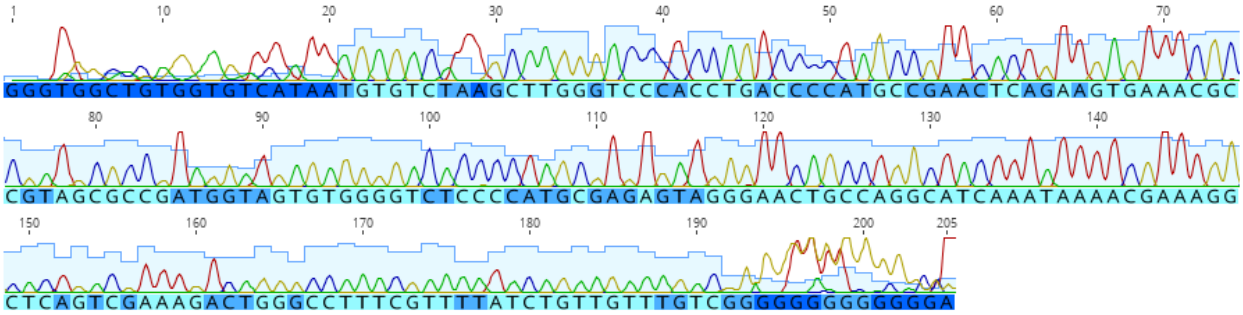
Complementary strand (exact match for 175 bp):



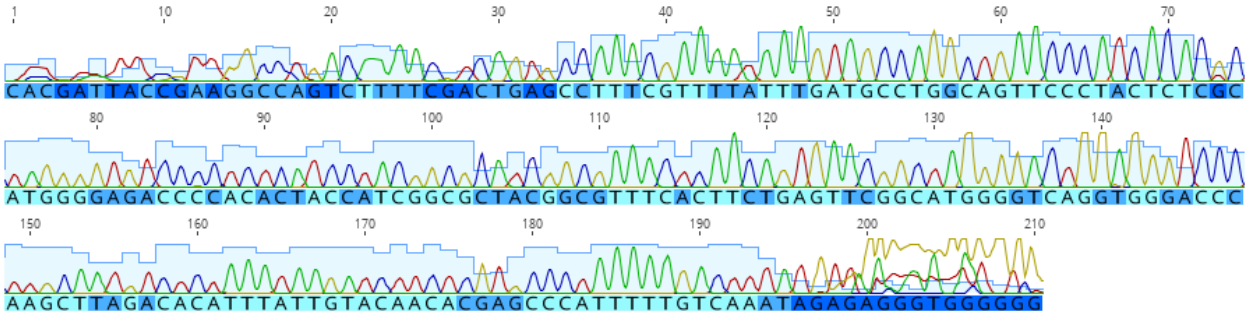
2.3.4. d5hmC-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3’

Primary strand (exact match for 181 bp):



Complementary strand (exact match for 192 bp):

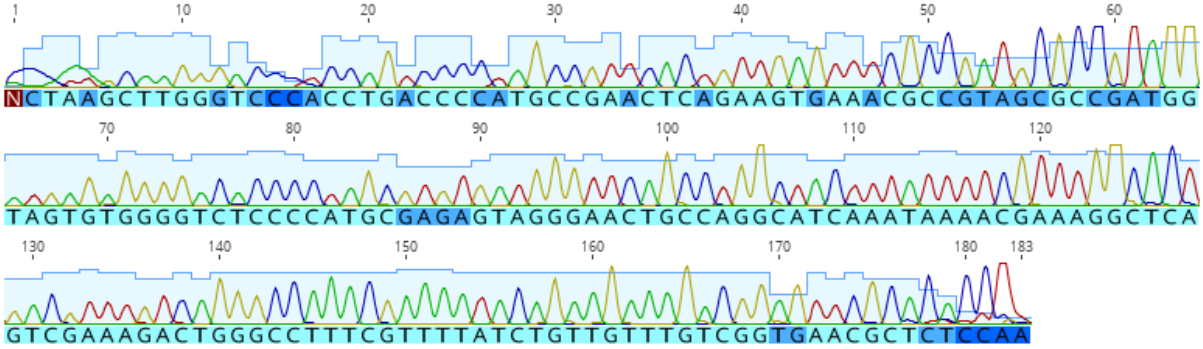


2.4. Sequencing of modified upstream and promotor regions in coding strand of DNA

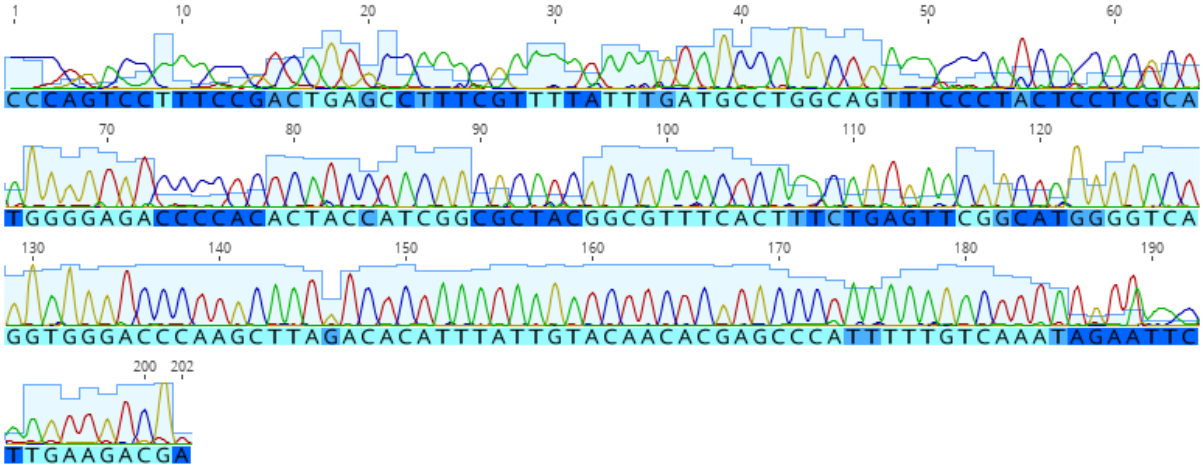
2.4.1. dU-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 181 bp):



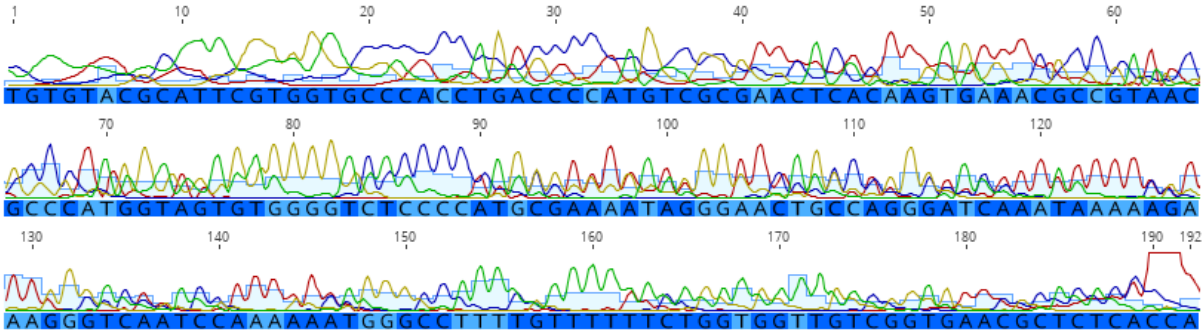
Complementary strand (exact match for 186 bp):



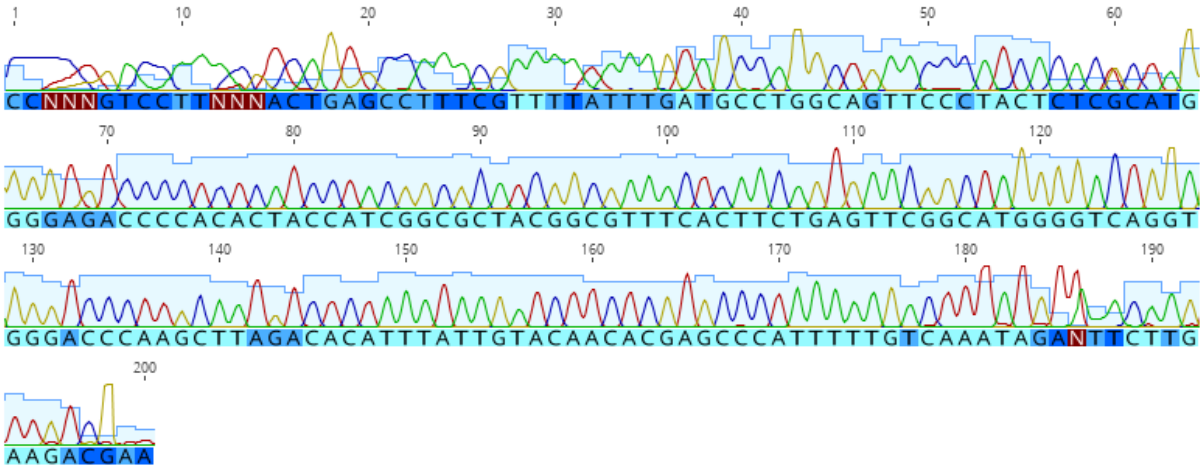
2.4.2. d5hmU-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 151 bp):



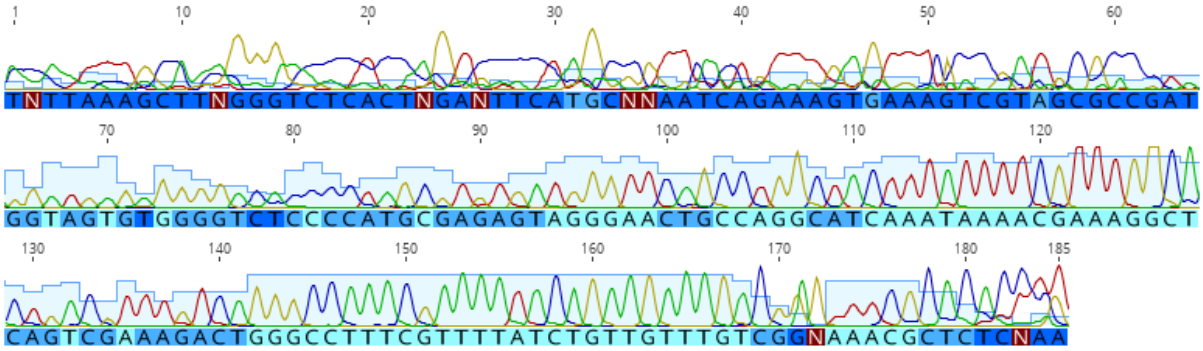
Complementary strand (exact match for 184 bp):



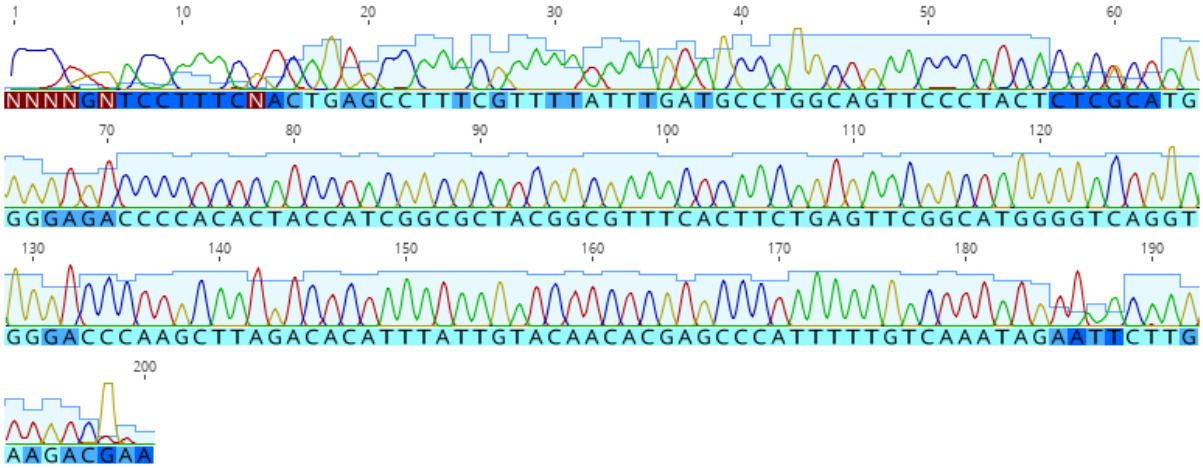
2.4.3. mC-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 148 bp):



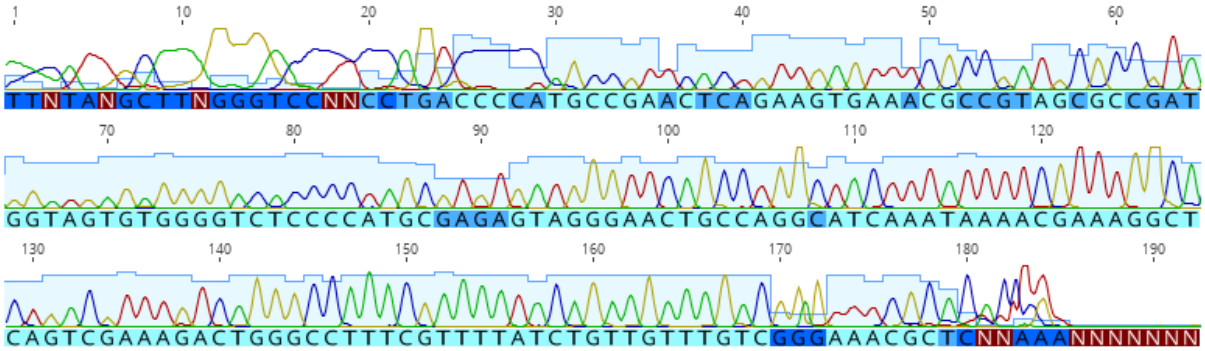
Complementary strand (exact match for 184 bp):



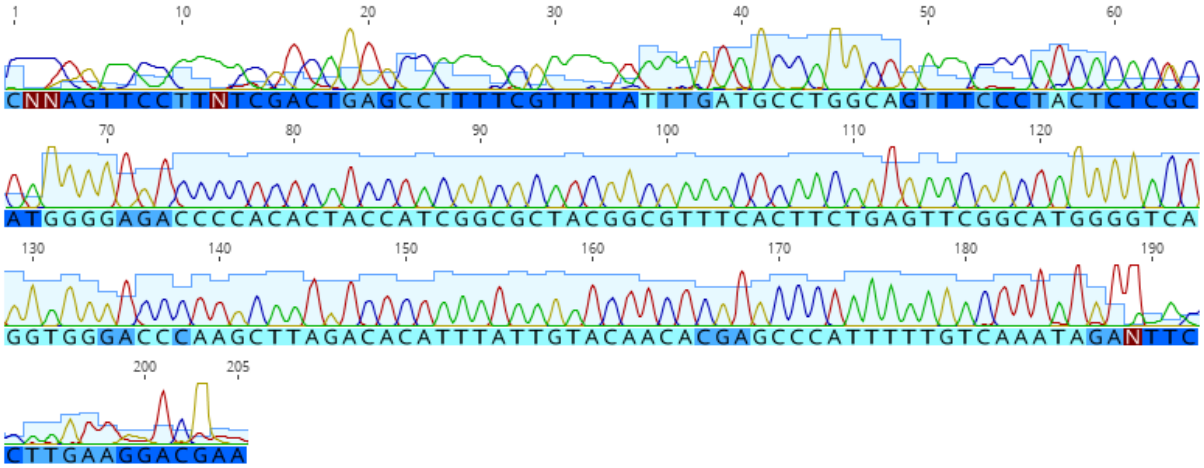
2.4.4. d5hmC-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 154 bp):



Complementary strand (exact match for 182 bp):

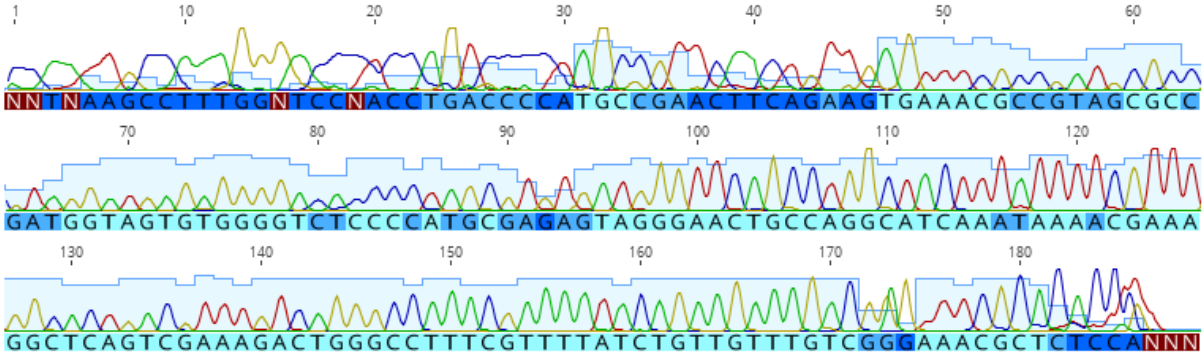


2.5. Sequencing of modified promotor region in coding strand of DNA

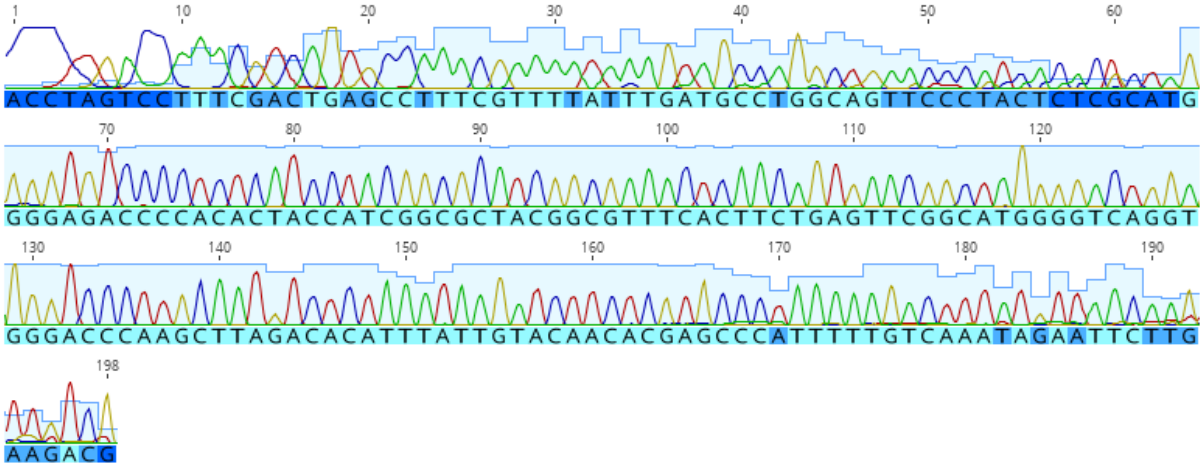
2.5.1. dU-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 166 bp):



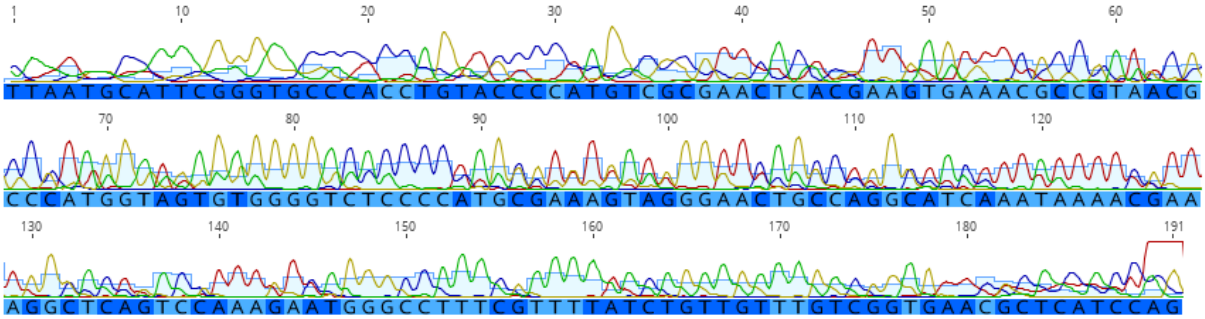
Complementary strand (exact match for 190 bp):



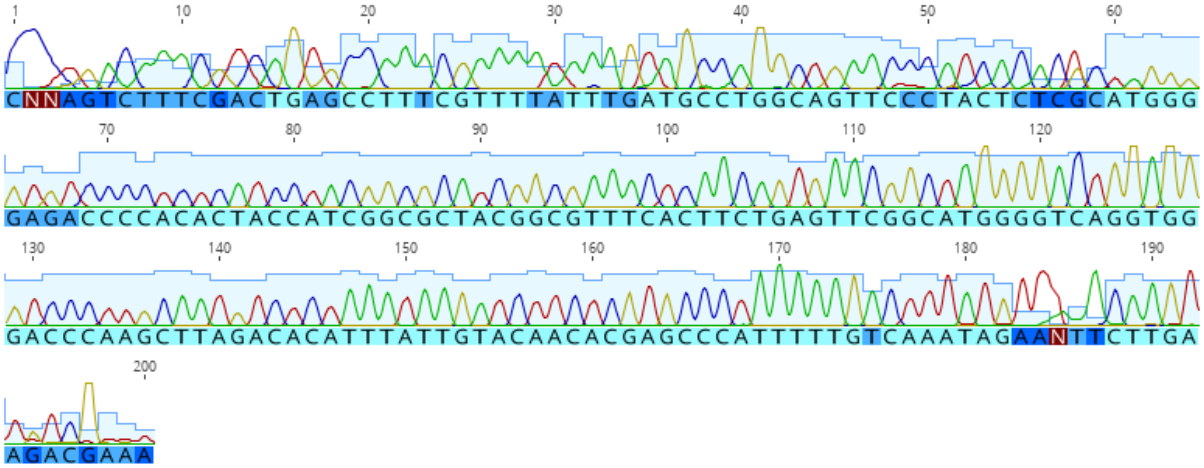
2.5.2. d5hmU-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT*** GTG TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 151 bp):



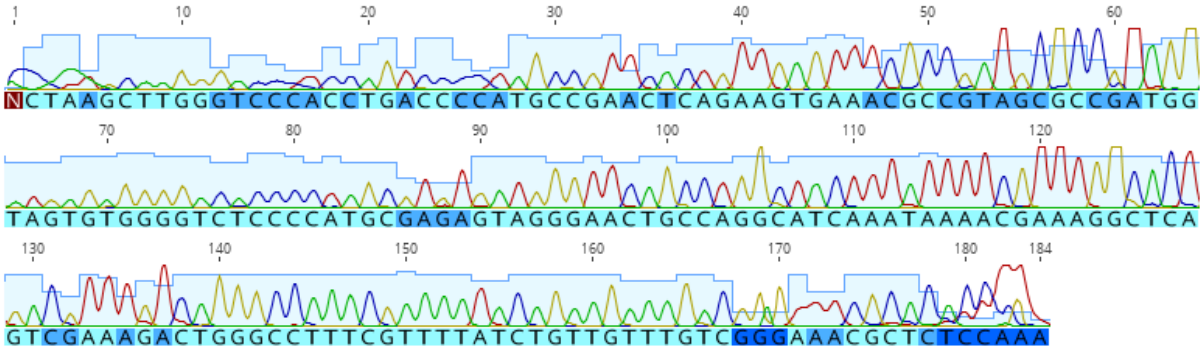
Complementary strand (exact match for 194 bp):



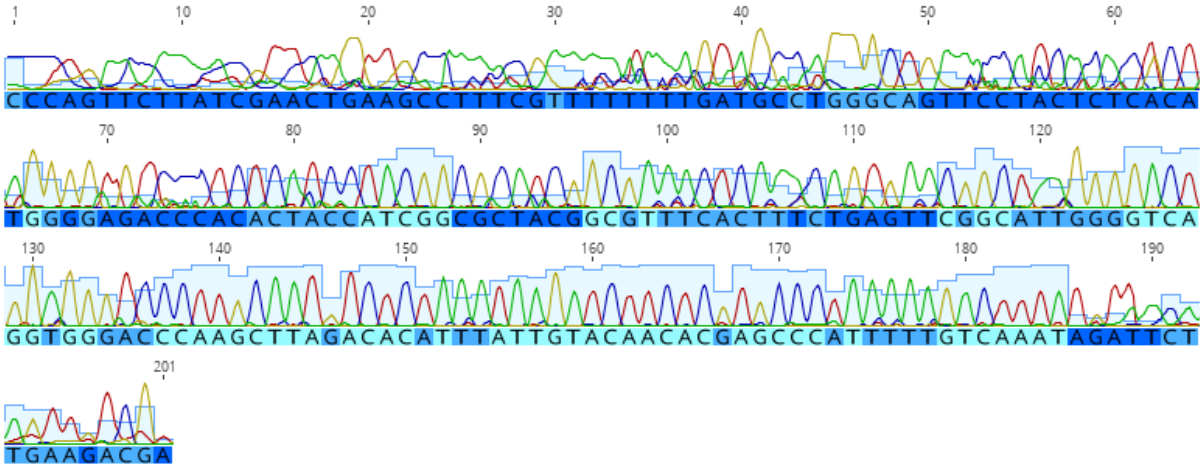
2.5.3. mC-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 180 bp):



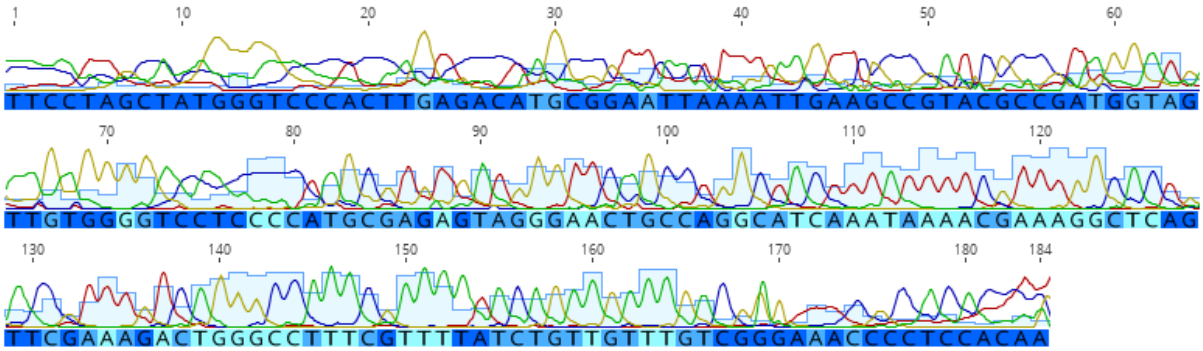
Complementary strand (exact match for 189 bp):



2.5.4. d5hmC-modified DNA

5´-CGT CTT CAA GAA TTC ***TAT TTG ACA AAA ATG GGC TCG TGT TGT ACA ATA AAT GT***G TCT AAG CTT GGG TCC CAC CTG ACC CCA TGC CGA ACT CAG AAG TGA AAC GCC GTA GCG CCG ATG GTA GTG TGG GGT CTC CCC ATG CGA GAG TAG GGA ACT GCC AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT TGT TTG TCG GTG AAC GCT CTC C-3´

Primary strand (exact match for 178 bp):



Complementary strand (exact match for 190 bp):