Univerzita Karlova v Praze

Přírodovědecká fakulta

Studijní program: Organická chemie

Studijní obor: Organická chemie

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Konstrukce modifikovaných DNA s vybranými reaktivními či chránícími skupinami

Construction of modified DNAs with selected reactive or protective groups

Disertační práce

Vedoucí závěrečné práce/ Školitel: prof. Ing. Michal Hocek, CSc., DSc.

Praha, 2020

Disertační práce byla vypracována na Ústavu organické chemie a biochemie, Akademie věd České republiky v Praze v období září 2011 – květen 2019.

Prohlášení:

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V Praze, 28.2.2020

Podpis

Acknowledgement

I would like to thank to my supervisor Prof. Michal Hocek for the opportunity to work on such interesting and challenging research topic, his guidance, useful suggestions and support over the course of my research. My grand gratitude goes to Dr. Libor Krásny for a constructive discussion about gene expression and fruitful collaboration, to Ing. Martina Janoušková for plasmid expression and for her help with DNA transcription experiments. I would like to thank to Dr. Radek Pohl for his help with measuring and interpreting of NMR spectra, to Ing. Kvetoslava Kertisová and Ing. Kateřina Nováková (MALDI-TOF) for measurement of mass spectra, to members of Dr. Mertliková group for helpful biochemical discussions and Prof. P. Klán for help with photocleavage experiments. I am grateful to all people from Development Centre of Institute of Organic Chemistry and Biochemistry AS CR (IOCB AS CR) who created the lighting instruments needed for my research by their original and creative approach to work. I am thankful to all colleagues from our research group for their suggestions, advices and friendship. My great thanks belong to my family and friends for their great support and patience.

My PhD work was a part of a multidisciplinary project performed by Prof. Hocek research group at the IOCB AS CR, partly in collaboration with Dr. Libor Krásny research group from Institute of Microbiology of the CAS. All the synthetic work, most of enzymatic incorporations, part of transcription experiments and all deprotection experiments were performed by me in person. The experiments mentioned in the thesis, which were performed by others are distinctly denoted in the beginning of each chapter and in the particular place of the thesis.

This work was supported by the Academy of Sciences of the Czech Republic (RVO 61388963), by the Czech Science Foundation (14-04289S and 17-03419S) and by the Czech Academy of Sciences (Praemium Academiae award to Michal Hocek). Part of the project was supported by the C4Sys infrastructure.

Abstract

This PhD thesis is focused on the synthesis of DNA modified with photocleavable 2-nitrobenzyl protecting groups in major groove and its applications in the regulation of gene expression in the level of transcription.

In the first part of my thesis, the synthesis of photocaged 2′-deoxyribonucleosides triphosphates and their photolysis to unprotected 5-hydroxymethylated nucleotides is described. All prepared nucleoside triphosphates were good substrates for their enzymatic incorporation into DNA. Synthesized 5-(2-nitrobenzyloxy)methyl-2′-deoxyuridine-5′-monophosphate (**dU^{NB}MP**) and DNA with one 5-(2-nitrobenzyloxy)methyl- modification in the sequence were used for the detailed kinetic studies of photocleavage reactions.

In the second part of the thesis, the series of modified DNAs with specific sequences were prepared by primer extension (PEX) and/or polymerase chain reaction (PCR). A cleavage of prepared modified DNAs was studied by selected restriction endonucleases (REs). In all cases, the nitrobenzylated DNA fully resist the cleavage by REs. The deprotection/ photocleavage conditions for nitrobenzylated DNA were studied in the case of DNAs with positive restriction endonuclease digestion of hydroxymethylated DNA. The resulting photocleaved DNA was fully digested by REs, therefore 2-nitrobenzyl moiety on DNA appears as an applicable, biorthogonal transient protection of DNA against cleavage by REs.

Finally, the synthesized 2′-deoxyribonucleosides triphosphates together with selected epigenetic modifications of pyrimidine triphosphates were used as the building blocks of DNA templates for transcription studies by bacterial RNA polymerase. Systematic transcription study of epigenetic hydroxymethylated and methylated DNA templates showed the strong influence on transcription. Moreover, it was found out that modifications of non-template strand of promoter region in DNA template has crucial effect on the transcription process. Hydroxymethyl moieties in DNA templates display enhancing effect on transcription and as expected nitrobenzylated DNA templates fully inhibited transcription. Transiently protected DNA templates [enzymatically synthesized in the presence of nitrobenzylated uridine and cytidine triphosphates (**dU^{NB}TP** and **dC^{NB}TP**)] with P*veg* promoter region were applied in the regulation studies of transcription process. The inhibition of transcription of nitrobenzylated DNA template (**DNA_N^{NB}**) is fully activated by prior irradiation of **DNA_N^{NB}** with visible light (400 nm, 10 – 30 minutes). Activated transcription of unprotected DNA templates with hydroxymethyl moieties on uridine (**DNA_U^{HM}**) can be blocked (switched off) again by its enzymatic phosphorylation with specific kinase (5-HMUDK).

Abstrakt

Tato dizertační práce je zaměřená na syntézu DNA modifikované pomocí fotolabilních 2-nitrobenzylových chránících skupin a jejich aplikace v procesu regulace genové exprese.

V první části mojí disertační práce je popsaná syntéza 2´-deoxyribonucleozid trifosfátu chráněných fotolabilními chránicími skupinami a jejich fotolýza na odchráněné hydroxymethylové trifosfáty. Všechny připraveny nukleosid trifosfáty byly dobrými substráty pro jejich enzymatickou inkorporaci do DNA. Syntetizovaný 5-(2-nitrobenzyloxy)methyl-2´-deoxyuridin-5´-monofosfát (**dU^{NB}MP**) a DNA s jednou 5-(2-nitrobenzyloxy)methyl-modifikaci v sekvenci byli použity v podrobné studii kinetiky fotolytických reakcí.

V druhé časti disertační práce byla připravena série modifikovaných DNA se specifickou sekvenci pomoci PEX a/nebo PCR. Štěpení modifikované DNA bylo studováno v přítomnosti vybraných restrikčních endonukleáz (RE). Ve všech případech byla DNA modifikovaná nitrobenzylovými skupinami úplně rezistentní vůči štěpení RE. V případě hydroxymethylové DNA s pozitivním štěpením pomoci RE, byly studovaný podmínky ochránění 2-nitrobenzylových zbytku na DNA, tedy 2-nitrobenzylové skupiny se jeví jako využitelné, bioortogonální přechodní chránění DNA proti štěpení restrikčními endonukleázami.

V závěrečné časti, byly syntetizované 2'-deoxyribonukleosidy trifosfáty společně s vybranými epigenetickými modifikacemi pyrimidin trifosfátů použity jako stavební bloky DNA templátů k studiu transkripce pomoci bakteriální RNA polymerázy. Systematická studie transkripce epigenetických hydroxymethylovaných a methylovaných DNA templátů prokázala silný vplyv na proces transkripce. Kromě toho bylo zjištěno, že modifikace ne-templátoveho vlákna promotorové oblasti v DNA templátu silně ovlivňuji proces transkripce. Hydroxymethylové skupiny v DNA templátech transkripci aktivuje a podle očekávání nitrobenzylované DNA templáty transkripci úplně inhibují. V studiu regulace transkripčního procesu bylo využité přechodné chránění DNA templátu s P*veg* promotorovou oblastí [enzymaticky syntetizované v přítomnosti nitrobenzylovaného uridinu a cytidinu trifosfátů (**dU^{NB}TP** a **dC^{NB}TP**)]. Inhibice transkripce nitrobenzylovaného DNA templátů (**DNA_N^{NB}**) je plně aktivována předchozím ozářením **DNA_N^{NB}** templátu pomoci viditelného světla (400 nm, 10-30 minut). Aktivovaná transkripce nechráněných DNA templátů s hydroxymethylovými skupinami na uridinu (**DNA_U^{HM}**) může být opětovně blokována (vypnutá) enzymatickou fosforylací pomoci specifické kinázy (5-HMUDK).

List of abbreviations and symbols

AIBN	Azobisisobutyronitrile
ATP	Adenosine triphosphate
Boc	*tert*-Butyloxycarbonyl
bp	Base pair
BER	Base excision repair
CuAAC	Copper catalysed alkyne-azide cycloaddition
dATP	2'-Deoxyadenosine triphosphate
dCTP	2'-Deoxycytidine triphosphate
dGTP	2'-Deoxyguanosine triphosphate
DIPEA	*N, N*-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	*N, N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
DMT	Dimethoxytrityl
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxyribonucleoside 5'-O-triphosphate
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
dTTP	2'-Deoxythymidine triphosphate
dUTP	2'-Deoxyuridine triphosphate
equiv.	Equivalents
ESI	Electrospray ionization
FAM	Fluorescein
HM	Hydroxymethyl-
5-HMUDK	5- Hydroxymethyluridine DNA kinase
HPLC	High performance liquid chromatography
LED	Light emitting diode
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
NEAR	Nicking enzyme amplification reaction
NB	Nitrobenzyl-
NBS	N-Bromosuccinimide

NMR	Nuclear magnetic resonance
NTP	Ribonucleoside 5'-O-triphosphate
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEX	Primer extension
PPGs	Photoremovable protecting groups
RE	Restriction endonuclease
RNA	Ribonucleic acid
RNAP	RNA polymerase
ssDNA	Single-stranded DNA
TBDMS	*Tert*-butyldimethylsilyl
TDA-1	Tris(3,6-dioxaheptyl)amine
TDG	Thymine DNA glycosylase
TEAB	Tetraethylammonium bicarbonate
TET	Ten-eleven-translocation enzyme
TF	Transcription factor
THF	Tetrahydrofuran
UTP	Uridine triphosphate
UV	Ultraviolet
X	Modification

Contents

1. Introduction

1.1. Deoxyribonucleic acid (DNA) - its discovery and properties

In 1869 a Swiss chemist, Friedrich Miescher discovered a novel molecule, which was isolated from the cells´ nuclei and he named it "nuclein". He showed that "nuclein" was a characteristic component of all nuclei and it was often reported as being closely related to proteins. Today´s utilized term for "nuclein" is deoxyribonucleic acid (DNA).

In the late 1880s, Richard Altmann named DNA separated from proteins as nucleic acid, due to the fact that it behaves like an acid.[1] In 1910, Ludwig Karl Martin Leonhard Albrecht Kossel was awarded the Nobel Prize for his work in identifying the fundamental building blocks of DNA-the purine and pyrimidine bases, one sugar and phosphoric acid.[1, 2] In the first half of the 20th century, it was determined that DNA contains as many purine (adenine-A, guanine-G) as pyrimidine bases (cytosine-C, thymine-T);[1, 3] the molar ratios of A to T and C to G were always very close to one [1, 3] and DNA was confirmed as the genetic material[4]. All acquired facts about DNA and its fundamental studies done by Rosalind Franklin, Raymond Gosling and Maurice Wilkins, were the necessary precursors which led to establishing the structure of DNA by James Watson and Francis Crick in 1953.[5] In 1962, Watson, Crick and Wilkins were jointly awarded the Nobel Prize for their discoveries concerning the molecular structure of nucleic acids and its significance for information transfer in living material.

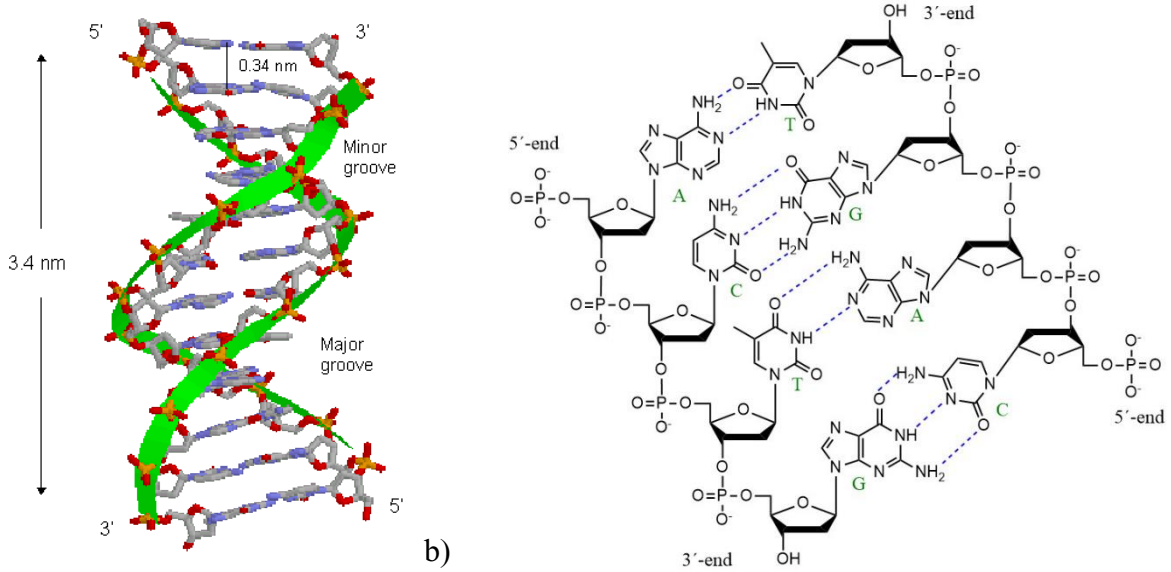
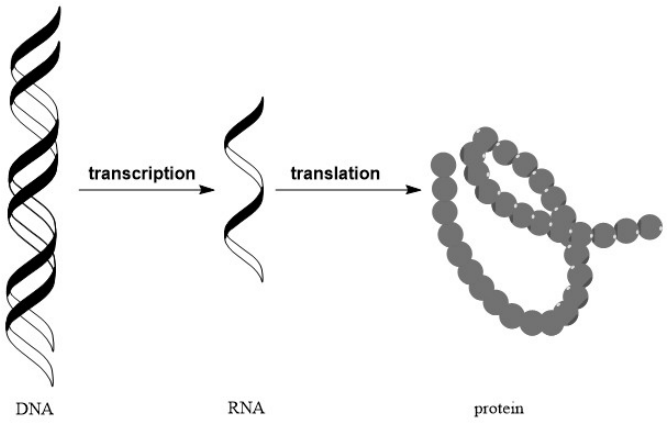


Figure 1: *a) B-DNA double helix (http://www.web-books.com/MoBio/Free/Ch3B3.htm PDB: 1BNA, ref.1b). b) Watson-Crick base pairing of nucleobases in DNA*

From their stories, they conclude that DNA macromolecule exists in the form of a double helix with two antiparallel chains twisted around each other (***Figure 1a***) elucidating, that two

chains of DNA are held together by hydrogen bonds between pairs of complementary nucleobases – adenine (A) with thymine (T), cytosine (C) with guanine (G) and by π-π stacking (***Figure 1b***).[6] Double helical DNA has three major forms: B-DNA (the most common, ***Figure 1a***), A-DNA and Z-DNA. Most DNA double helices are right-handed, only Z-DNA is left-handed.

The sequence of these four nucleobases along the backbone of DNA encodes biological information and can be copied in a process called DNA replication. DNA strands are also used as a template in a process of transcription to create ribonucleic acid (RNA). The RNA strands are translated to specify the sequence of amino acids to make a matching protein in a process called translation. (***Scheme 1***)



Scheme 1*: Simplified diagram of gene expression*

The knowledge of DNA structure, its either physical or chemical properties and its mechanism of function, as well as later discovery of RNA, introduced an opportunity to begin the era of molecular genetics. Discoveries in the field of nucleic acids have affected the development of modern medicine, criminology, molecular biology, chemical biology, biotechnology and nanotechnology.

1.1.1. Epigenetic nucleobases

The genetic information is encoded in DNA with deoxyribonucleosides: deoxyadenosine (dA), deoxycytosine (dC), deoxyguanosine (dG), deoxythymidine (dT) and in RNA with the corresponding ribonucleosides: adenosine (A), cytosine (C), guanosine (G) and uridine (U). (***Figure 2***)

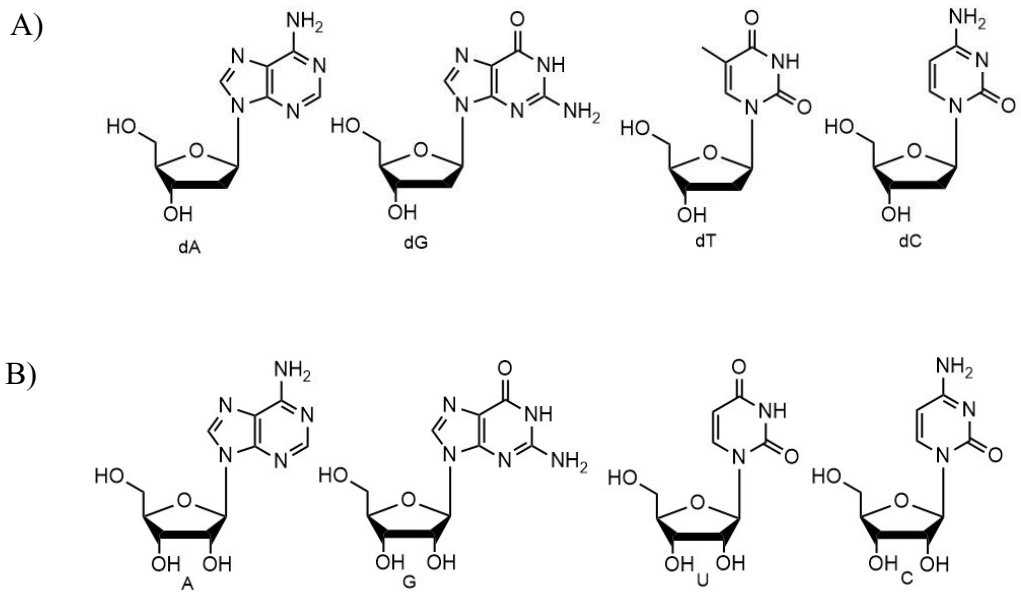


Figure 2: *A) Building blocks of DNA B) Building blocks of RNA*

Other than the four basic canonical nucleobases, DNA and RNA contain a number of modified nucleosides. While DNA stores heritable genetic information inside the cells, the variety of functions were determined for transcribed RNA.[7] Because of the complex function of RNA, RNA is particularly rich in modified – noncanonical nucleosides, which establish the second layer of information coded in RNA. In comparison to RNA, the number of modified nucleobases in DNA is quite small. The most modified DNA bases are formed in response to damaging agents (UV irradiation, O_2) and activated in liver to give intermediates, which react with nucleophilic sites of DNA bases to form DNA lesions, which are mutagenic or cytotoxic.[8, 9] In addition to DNA lesions, DNA modifications enzymatically introduced into the sequence of DNA were discovered. [10]

DNA modifications arise from epigenetic changes and the bases do not change the DNA sequence; instead they affect how cells "read" genes. The DNA modifications occur in both prokaryotic and eukaryotic cells. The most widespread epigenetic change is DNA methylation. (***Figure 3***)

In bacterial genomes, the C5- or N4-methylated deoxycytosine (m^5dC/ 5-mC/ mC or m^4dC) protects the DNA of bacterium from degradation by its own restriction endonucleases, which are produced to degrade foreign DNA and protect bacteria against infection by bacteriophage. N6-methylated deoxyadenosine (m^6dA) is also involved on regulation of virulence, mismatch repair and control of gene expression. [10] [***Figure 3i)-iii)***]

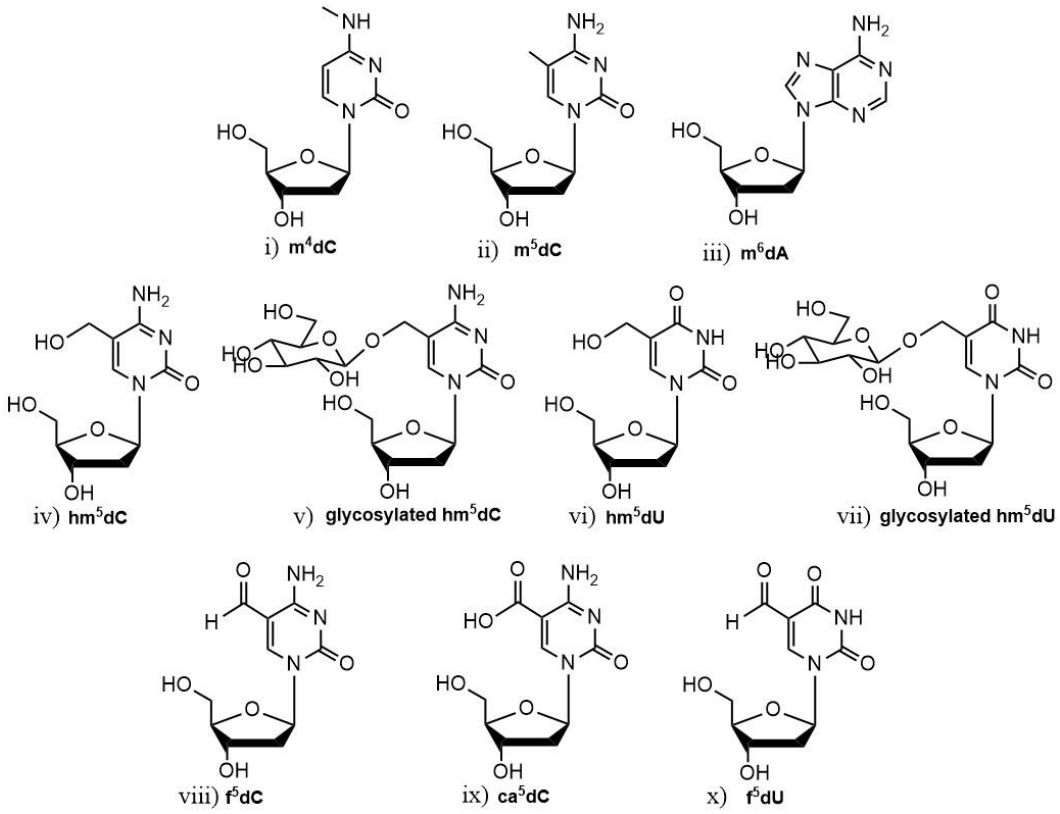


Figure 3: *Noncanonical bases found in DNA*

The best studied epigenetic modification - 5-methyldeoxycytosine (m^5dC/ 5-mC/ mC), is not presented only in bacteria, but it controls gene expression in plants, fungi and animals. The content of mC is approximately constant in all tissues. The mC is involved in many biological processes such as gene expression, genomic imprinting and methylated genes are silenced, thus not transcribed. [11-16][***Figure 3i)***]

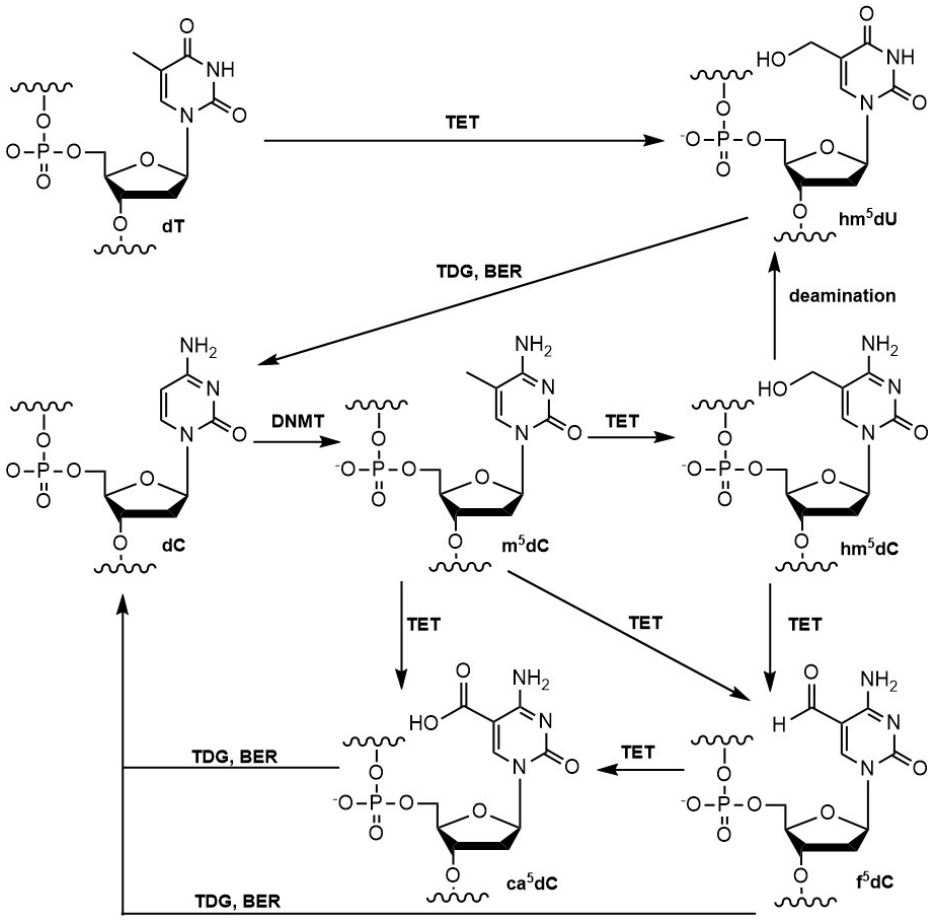
The modified bases 5-hydroxymethyluracil (hm^5dU/ 5-hmU/ hmU) and 5-hydroxymethylcytosine (hm^5dC/ 5-hmC/ hmC) were first determined in T bacteriophages (hmC) and in the species *Trypanosoma brucei* (hmU). In both cases, hydroxymethyl modification is introduced into DNA by incorporation of corresponding hydroxymethyl-modified triphosphates. In the DNA, hydroxymethyl group is glycosylated and glycosylated forms protect the DNA of organism from degradation by restriction enzymes of host cells. [16] [***Figure 3iv)-vii)***]

The hmC was also detected in animal DNA.[17] Later it was found out that hmC is present in mouse stem cell DNA and in neurons. [18, 19] The content of the hmC is variable in all tissues, however it is strongly accumulated in brain tissues and reduced in brain tumor tissues. [20, 21] It was confirmed that hmC is formed post-replicatively. Proteins which are known to bind to mC,

do not bind to hmC therefore the transcriptional activity of hydroxymethylated genes is changed in comparison with transcriptional activity of methylated genes.[22]

The hmC along with 5-formylcytosine (f ^{5}dC/ 5-fC/ fC) and 5-carboxylcytosine (ca^{5}dC/ 5-caC/ caC) are potential intermediates in the process of replacing epigenetic mC with an unmodified dC.[23-25] The hmC, fC and caC are formed by gradual oxidation of mC with ten-eleven-translocation enzyme (TET). The demethylation mechanism of mC is not clear, but it is most likely based on its oxidation[26] and subsequent deamination of hmC to hmU or on decarboxylation reaction of caC.[7] [***Figure 3viii)-ix); Scheme 2***]

The amount of formylated DNA (and RNA) is extremely low and its detection is therefore a challenging task. The level of 5-fdU in DNA is significantly increased in human thyroid carcinoma tissue compared to normal tissues. The results indicate that the DNA and RNA formylation could be additional epigenetic modification and potentially play certain roles on the tumour formation and development. [27] [***Figure 3viii), x)***]



Scheme 2*: DNA demethylation pathway*

It was reported that hmC is present at a level that is approximately 2–3 and 3–4 orders of magnitude greater than 5-fC and 5-caC, respectively, and 35–400 times greater than 5-hmU in the mouse brain and skin, and in human brain.[28]

5-Hydroxymethyluracil was found in genomic DNA of various organisms from bacteriophages to mammals. The post replicative formation of 5hmU occurs via oxidation of thymine by TET in mammalian,[29] or J-binding protein in proteozoan genome.[30] Another way of formation of the 5hmU moiety in DNA is deamination of hmC, situated in base pair with G to hmU:G mismatch. Following mismatch repair (BER) can establish an alternative pathway to demethylation of mC. The hmU:G mismatches together with other oxidized nucleotides 5-fC or 5-caC may be cleaved from DNA by thymine DNA glycosylase (TDG, Smug1) and restored with unmethylated dC.[31] (***Scheme 2***) 5hmU can also be a key intermediate in the site specific mutations since DNA glycosylases make use of 5hmU to create potentially mutagenic non-coding DNA lesions.[32] Moreover the hmU was identified as a base that can influence binding of chromatin, remodelling proteins and transcription factors.[29] (***Scheme 2***)

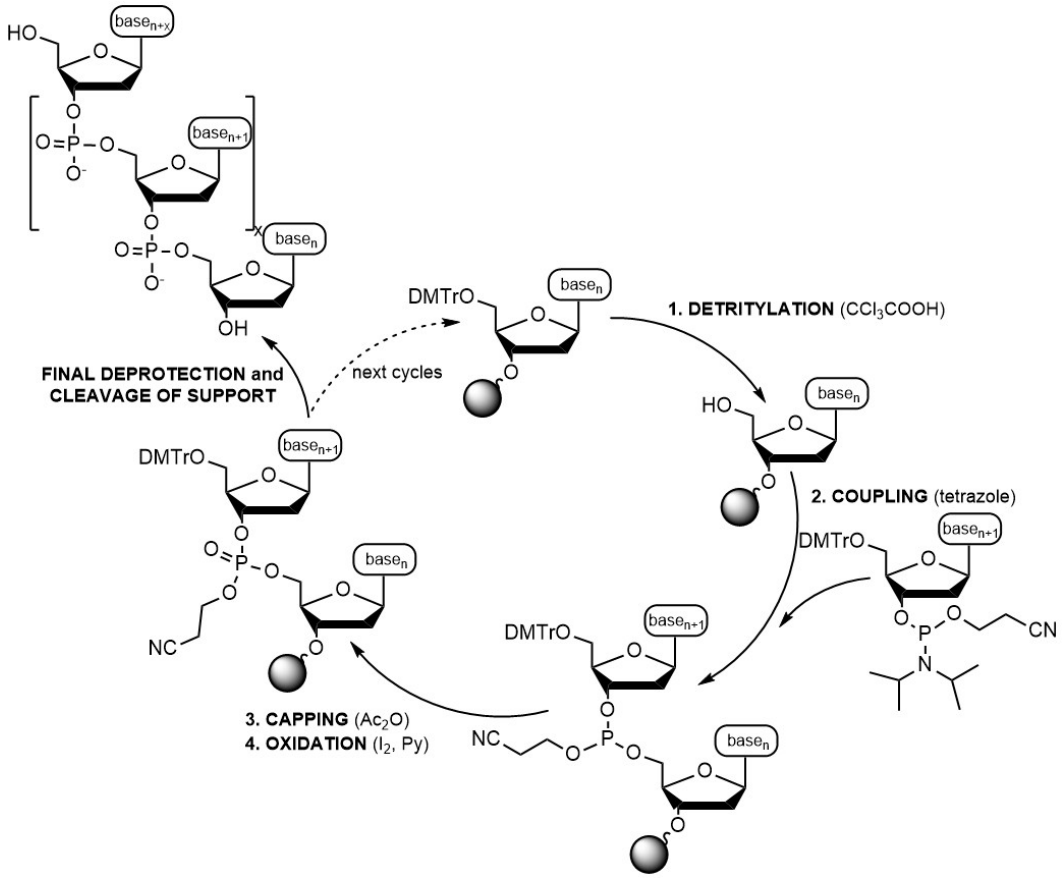
1.2. Synthesis of functionalized or modified DNA

Base-modified oligonucleotides are synthesized either chemically by several methods or by enzymatic incorporation of functionalized 2′-deoxynucleoside 5′-O-triphosphates.

1.2.1. Chemical synthesis of DNA

Short oligonucleotides are usually synthesized chemically on solid support by phosphodiester[33], phosphotriester[34, 35], phosphite triester[36, 37] or the most common phosphor-amidite method[38-40], which uses protected phosphoramidite building blocks (***Scheme 3***).

Phosphoramidite synthesis proceeds in the 3'- to 5'-direction. One nucleotide is added in one four-step synthetic cycle. Generally, the cycle starts with removal of the 5'-dimethoxytrityl (DMT) protecting group from a 2'-deoxynucleoside covalently attached to solid support and it is followed by coupling with protected phosphoramidite in the presence of tetrazole as an activator. In the next step, acetic anhydride in pyridine is used to acetylate any unreacted 2'-deoxynucleoside, followed by oxidation in order to convert the phosphite linkage to phosphate. The cycle is repeated until the oligonucleotide of desired sequence is synthesized. In the final step of synthesis, the 5′-hydroxyl group is detritylated and the oligonucleotide is released from the solid support.



***Scheme 3**: Synthetic cycle for phosphoramidite method*

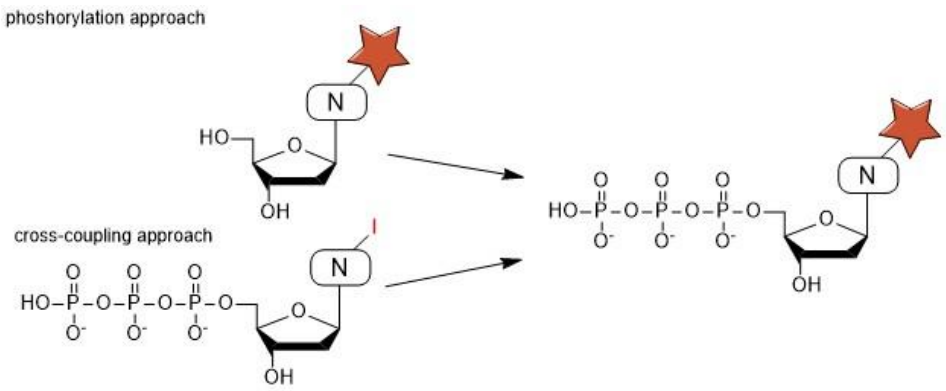
Diverse types of base-modified DNA has been prepared by this method[41-44], however extra functional groups are often limiting factors for the phosphoramidite chemistry.

1.2.2. Enzymatic synthesis of modified DNA

The base-modified DNA can be synthesized either by direct incorporation of functionalized/labelled nucleotide derivatives into DNA by various DNA polymerases, or by postsynthetic modification of halogenated/ alkynylated/ vinylated and otherwise functionalized nucleic acid.

1.2.2.1. Synthesis of modified 2´-deoxyribonucleoside triphosphates (dN^{X}TPs)[45]

Base-modified nucleosides containing functional group or label (typically 5-substituted pyrimidines and 7-substituted 7-deazapurines) established by cross-coupling reactions are the standard substrates for their triphosphorylation and further incorporation to the DNA by polymerase. In addition to nucleobases bearing substituent at position 5 of modified pyrimidines or position 7 of substituted 7-deazapurines, a synthesis of purines, which contain small modification at position 2 was also reported. [45-48]

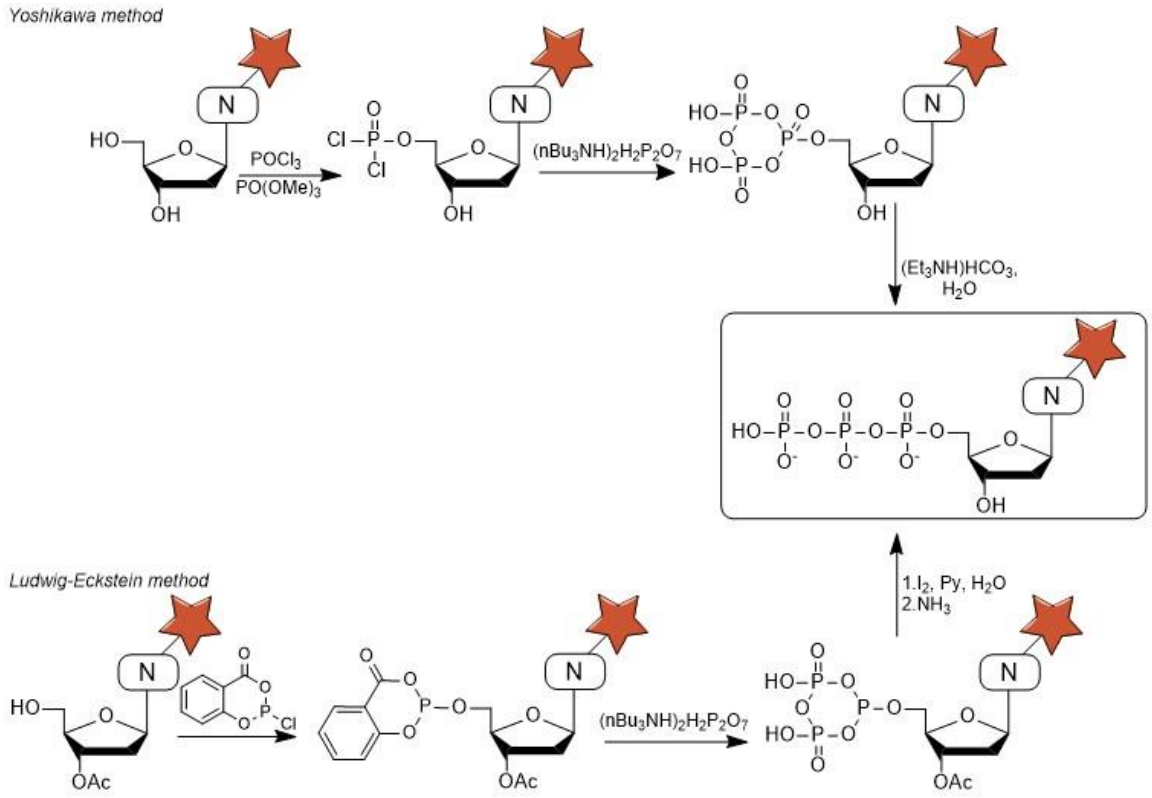


***Scheme 4**: Phosporylation approach and cross-coupling approach for synthesis of dN^XTPs*

Several methods have been developed for the synthesis of modified triphosphates. Phosphorylation approach is suitable for chemically modified nucleosides, usually synthesized by cross-coupling reactions, which are stable under conditions of phosphorylation. Cross-coupling approach for synthesis of modified triphosphates is based on Pd-catalyzed reactions of iodinated (halogenated) nucleotides with chemical label or functionality, which is sensitive to conditions of phosphorylation. Methods for the synthesis of triphosphates are not universal and optimization of conditions is required for each modified nucleoside individually. (***Scheme 4***)

A) *Yoshikawa* method for the synthesis of nucleoside triphosphate involves the simple, selective 5′-monophosphorylation of unprotected nucleoside in the presence of electrophilic phosphorous oxychloride ($POCl_3$). This intermediate reacts *in situ* with pyrophosphate to yield the cyclic triphosphate which is hydrolysed to produce the desired product (***Scheme 5***)[48, 49]

B) The one pot, three-steps method developed by *Ludwig and Eckstein* is one of the most popular procedures, mainly because of its specificity. One disadvantage of protocol is necessity of synthesis of 3′-*O*-acetylated precursor, which reacts with salicyl phosphorochloride to yield activated phosphite intermediate. After two nucleophilic substitution reactions, the cyclic nucleoside triphosphate is formed and subsequently oxidized to form a modified (d)NTP (***Scheme 5***).



Scheme 5*: Yoshikawa method and Ludwig-Eckstein method for the synthesis of dN^{X}TPs*

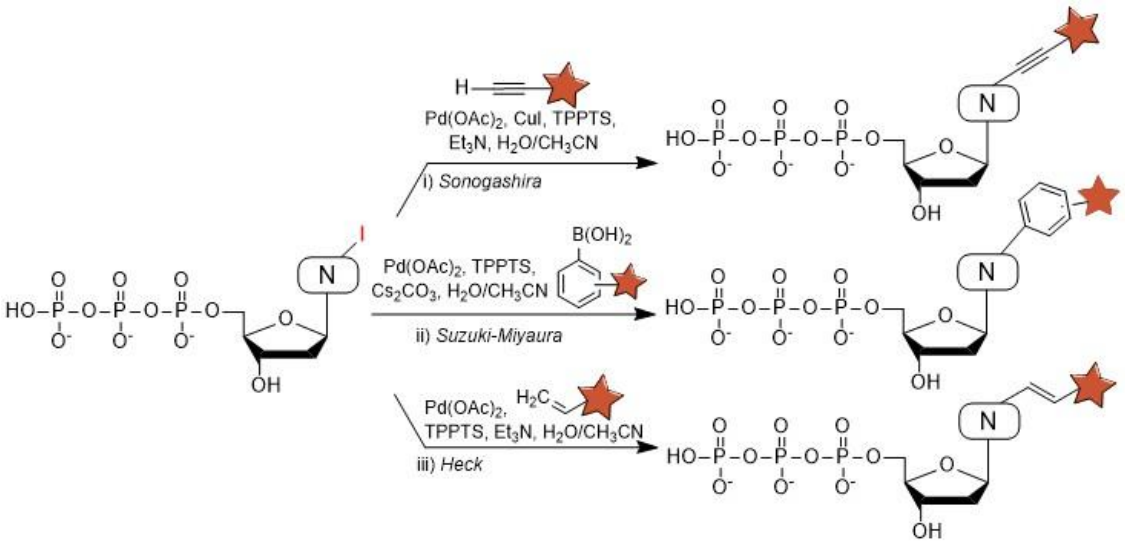
C) - Direct synthesis of modified dN^{X}TPs via aqueous cross-coupling reaction of unprotected iodinated nucleoside triphosphate is used for the synthesis of dNTPs bearing wide spectrum of functional groups (***Scheme 6***). [50-52]The water as co-solvent or solvent is convenient for work with unprotected nucleosides to avoid protection/deprotection steps. Cross-coupling reactions are in general tolerant to most of the reactive functional groups; however, triphosphates easily undergo hydrolysis under increased temperature. The most utilized Pd-catalyzed reactions are Sonogashira reaction, Suzuki-Miyaura coupling and Heck reaction.

- *Sonogashira cross coupling* reaction is used for the synthesis of modified dN^{X}TPs prepared from corresponding dN^{I}TPs in one-step. It has achieved widespread application in nucleoside research and it has been used for the synthesis of fluorescent derivatives[53], redox active antraquinone modified dN^{X}TPs [54] and dN^{X}TPs bearing Michael acceptor moiety.[55] Fluorescently labelled nucleosides and oligonucleotides are widely used for the structural study of DNA, sequencing and other applications in nucleic acid analysis.

- *Suzuki-Miyaura cross-coupling* constitutes the coupling of organoboron compounds with iodinated dN^{I}TPs and is widely used for its arylation. It is one of the most versatile

catalytic processes. The reaction is suitable for obtaining pharmaceutical agents, such as boron derivatives which are stable and nontoxic. *Suzuki-Miyaura cross-coupling* was used for the synthesis of $dN^{X}TPs$ bearing reactive aldehyde moiety[56], fluorescent derivatives[57], redox active benzofurane[58] or even reactive vinyl moiety.[59]

- The *aqueous Heck cross-coupling reaction* is a method of alkenylation to synthesize a wide range of different compounds. Two different approaches have been reported: a) the C-C cross coupling starting from halo-nucleosides and b) the C-C cross coupling starting from vinyl-nucleosides. Most often alkenes have an electron-withdrawing group. Heck alkenylation using palladium complexes of hydrophobic ligands and using ligand free palladium catalysts were developed.[60]



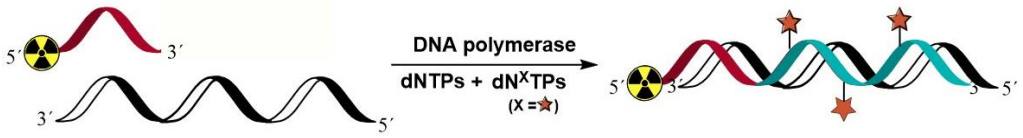
Scheme 6*: Examples for direct synthesis of modified $dN^{X}TPs$ via aqueous cross-coupling reaction*

1.2.2.2. Enzymatic synthesis of functionalized DNA in PEX and PCR reactions

In enzymatic synthesis of base-modified DNA, functionalized/labelled nucleotide derivatives are incorporated into DNA by various DNA polymerases. Further labelling or functionalization of already modified oligonucleotide is possible post-synthetically.

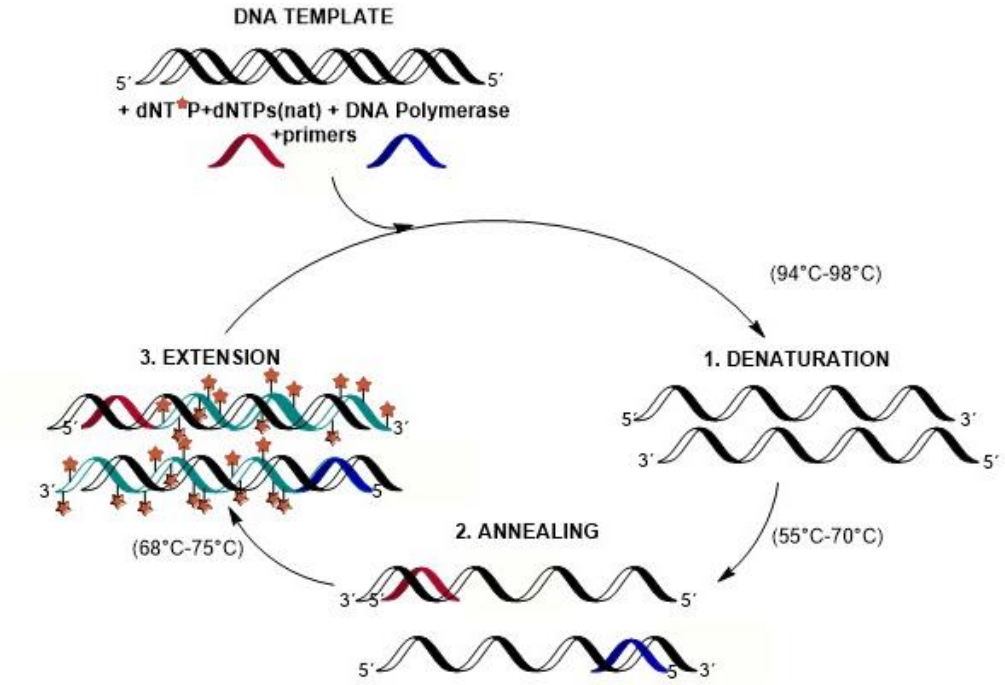
A common approach for the synthesis of oligoribonucleotides is based on an enzymatic incorporation of 2′-deoxynucleoside 5′-O-triphosphates (dNTPs) by DNA polymerase.[61-63] Enzymatic incorporation proceeds in 5′- to 3′- direction. The synthesis of DNA is based on the extension of 3′-end of a primer, which is annealed to a complementary longer template. Two basic methods for the enzymatic synthesis of DNA is primer extension experiment (PEX) and polymerase chain reaction (PCR).[64]

PEX reaction is used for the synthesis of short oligonucleotides (approx. up to 100 bp), which bear modification just in one strand. For visualization of extended product, the primer is usually labelled on the 5′-end with ^{32}P-phosphate or fluorescent probe. The reaction takes place at a temperature suitable for particular DNA polymerase. DNA product is usually determined by polyacrylamide gel electrophoresis. The amount of final product depends on the initial quantity of the template. The PEX reaction is used for the study, when modified nucleotide is tolerated with DNA polymerase and do not restrict the incorporation of further nucleotides (***Scheme 7***).



Scheme 7*: Primer extension experiment*

PCR is the most common method for the synthesis of long DNA (100 bp-1000 bp) containing large amount of modifications in both strands. PCR takes place in the presence of ds DNA template, DNA polymerase, dNTPs and two primers (forward and reverse) in 30-40 cycles. Each cycle consists of three steps: denaturation of double-stranded DNA template at 95°C, annealing of primers (the temperature depends on the melting temperature of primer) and extension reaction (the temperature depends on the type of DNA polymerase). After first cycle, the newly established modified DNA also represents a template for further cycles of the PCR reaction. The PCR reaction is usually terminated by final extension step at 72-75°C (depending on the type of polymerase). (***Scheme 8***) DNA is commonly determined by agarose gel in the presence of fluorescent intercalation reagent (GelRed) and in the case of DNA containing derivatives of 2′-deoxy-7-deazaguanosine the radioactive or fluorescent labelling is necessary.[65, 66] PCR gives ability to produce many copies of target DNA. The limitation of the PCR reaction is mainly the ability of DNA polymerase to incorporate modified nucleotides into the sequence of DNA or to incorporate nucleotides following the modified one, as well as its ability to “read” existing modified DNA strand to create the new one that match the existing ones.



***Scheme 8**: Polymerase chain reaction*

1.2.2.3. Synthesis of modified single-stranded oligonucleotide

Modified single-stranded oligonucleotide can be synthesized by using 5′-end-biotinylated template[67], 5′-end-phosphorylated template[68] or by using nicking enzyme amplification reaction (NEAR).[69]

A) Double stranded DNA (dsDNA) synthesized in the presence of 5′-end-biotinylated template by polymerase reaction is captured to streptavidine magnetic beads. In the next step, all unbound components are washed away except the complex dsDNA-streptavidine, which is attracted to the magnet. The modified single-strand is released by denaturation of the dsDNA when single-stranded template with streptavidine is attracted to magnet and desired modified oligonucleotide is present in solution. (**Scheme 9**)

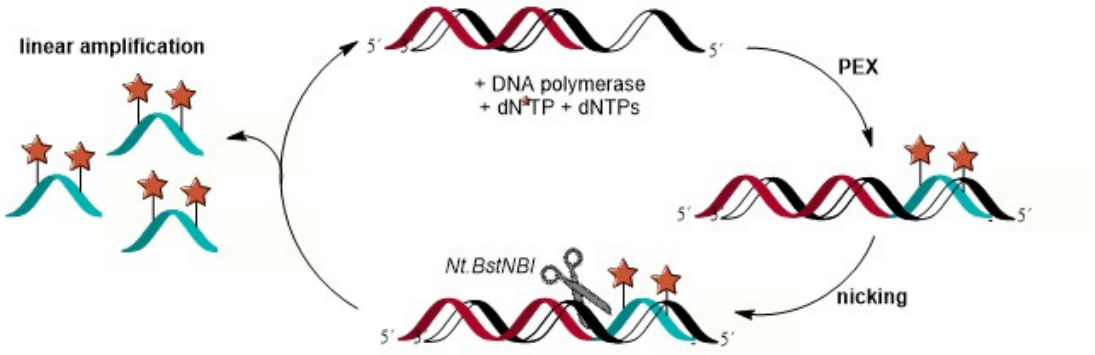
***Scheme 9:** Synthesis of ssDNA by magnetoseparation*

B) Another enzymatic way to obtain modified ssDNA, is to prepare dsDNA in the presence of 5′-phosphorylated oligonucleotide, either primer for PCR or template for PEX

reaction. 5´-phosphorylated dsDNA is a good substrate for Lambda exonuclease, that catalyzes the removal of mononucleotides from dsDNA in 5´-phosphorylated end to 3´direction (***Scheme 10***). [68]

Scheme 10*: Synthesis of ssDNA by Lambda exonuclease digestion*

C) The isothermal method for the synthesis of short modified single-stranded oligonucleotides was recently developed by using a nicking enzyme amplification reaction (NEAR). The reaction proceeds in the presence of DNA polymerase, which elongates the primer annealed with template in the presence of modified dNTPs, and nicking endonuclease (Nt.Bst.NBI), which cleaves the dsDNA in the recognition sequence and the shorter modified oligonucleotide is released to solution (***Scheme 11***). The template sequence and the primer are again available to repeat the reaction. Created shorter modified oligonucleotides can be used as primers for PCR.[70]

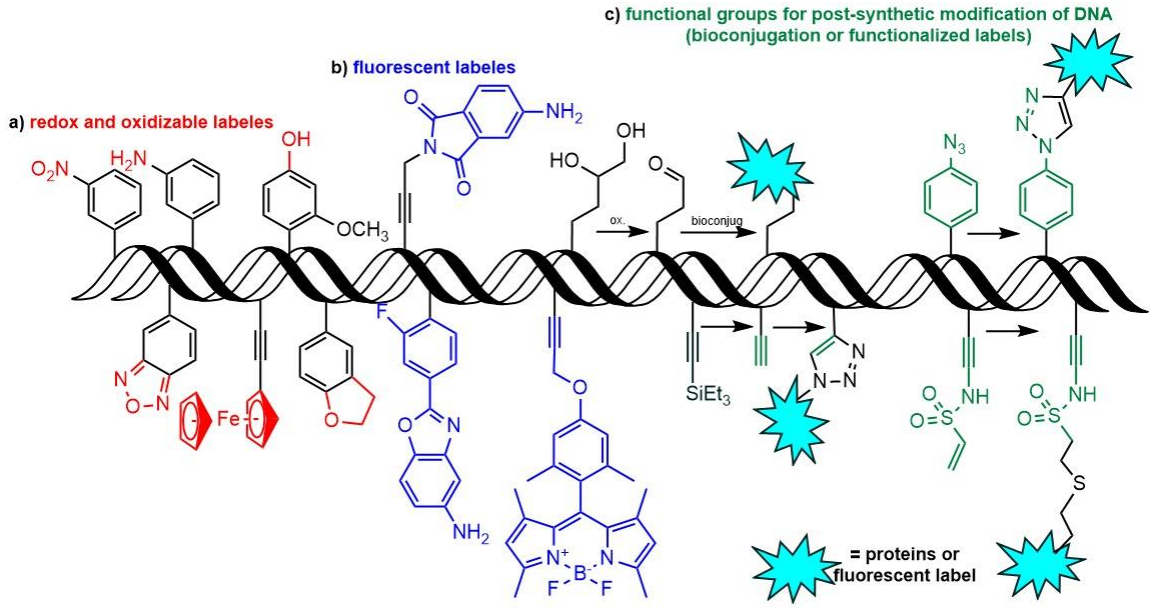


Scheme 11*: Synthesis of ssDNA by NEAR*

Base-functionalized DNA is attractive for a broad range of application. DNAs containing oxidizable or reducing moieties were utilized to study their electrochemical properties.[63, 67, 71, 72] Moreover, in the case of azidophenyl- modified DNA, DNA-protein interaction was determined by electrochemical detection.[73] dNTPs modified with fluorescent labels were incorporated to the DNA to study DNA-protein interaction[74, 75] or to identify the change in secondary structure of the DNA.[76]

Since not all modified building blocks for the synthesis of modified DNA are compatible with the synthetic protocols either for phosphoramidite or enzymatic synthesis, the methodology for the post-synthetical modification of DNA was developed. Other than post-

synthetic coupling reactions of halogenated DNA (on solid support) and Sonogashira coupling of ethynylated DNA, the click reactions were established. Click-reactions are fast, specific with high yields. The most popular click-reaction is azide-alkyne Huisgen cycloaddition with copper (Cu) as the catalyst at room temperature. There are two possible scenarios: DNA contains azido modifications or alkynyl groups in its sequence. The CuAAC click reactions of reactive triple bond modified ONs with different labels are standard in phosphoramidite synthesis. [73, 77-79] The post-synthetical oxidation of dihydroxyalkyl modified DNA to aldehyde linked DNA and its further labelling or bioconjugation through hydrazone formation or reductive aminations was recently published.[80] Post-synthetic deprotection of trimethylsilyl- protected DNA carried out by aqueous ammonia provides ethynyl modified DNA accessible to cleavage by restriction endonucleases.[81] (***Scheme 12***)



Scheme 12: *Representative examples of modifications enzymatically incorporated into DNA and their usage*

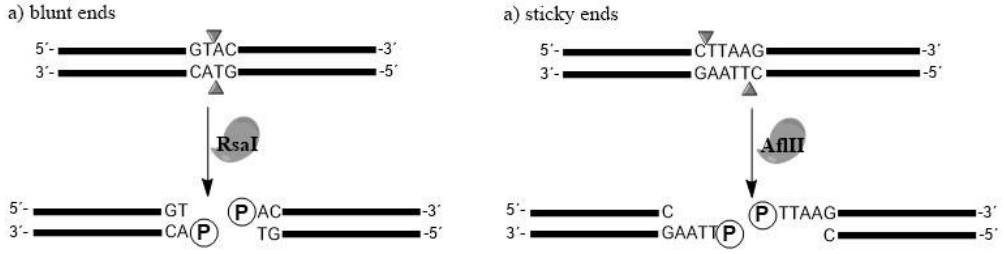
1.3. Restriction endonucleases

1.3.1. Restriction enzymes – general remarks

As it was mentioned in section 1.1.1., restriction endonucleases (REs) have an important function in protection of bacteria from infection by viruses. Except for certain viruses, REs were found only within the prokaryotes. Restriction endonucleases are classified into four main types based on the subunit composition, cleavage position, sequence specificity and cofactor requirements. Type I and III enzymes are combination restriction and modification enzymes.

REs of both types require ATP for restriction. Type I enzymes cut DNA at random far from their recognition sequence. Type II enzymes cut DNA at defined positions close to or inside of their recognition sequences. REs type II are the only class used for DNA gene cloning and analysis.[82] Type III endonucleases cleave DNA outside of their recognition sequences and they rarely yield complete digests. Type IV endonucleases cleave DNA at variable distance from recognition site and preferentially cleave modified, most often methylated DNA. Almost all REs require divalent metal cation (Mg^{2+}) for their activity. REs cut phosphodiester bond in DNA to yield 5´-phosphorylated and 3´-hydroxyl group.

Type II enzymes are the most studied restriction endonucleases and they have mainly homodimeric or homotetrameric structure. They recognize short palindromic sequence, interact symmetrically with a minimum of 10 nucleotide pairs and cut DNA to generate blunt or sticky ends.[6] (***Scheme 13***)



Scheme 13: *Examples of cleavage by restriction endonuclease a) blunt ends -cleavage by RsaI b) sticky ends -AflII*

1.3.2. Digestion of modified DNA by restriction endonucleases

The cleavage of base-modified DNA by restriction endonucleases was studied only slightly. The tolerance of several restriction enzymes to various modifications in major groove was determined.[59, 81, 83, 84] The presence of small substituents (vinyl, ethynyl) attached to A (7-substituted 7-deazaadenines) or U (5-substituted uracils) was tolerated by various REs, however the small modifications on G (7-substituted 7-deazaguanines) or C (5-substituted cytosines) mostly inhibit the cleavage DNA by tested REs. The inhibitive effect on cleavage by REs was observed also in the case of reported 8-modified adenines and guanines[85], 7-deazaadenines[86-91] and 7-deazaguanines[92]. (***Table 1***, ***Figure 4***) The results of cleavage modified DNA by REs are summarized in ***Table 1***. In the case of G-modified triphosphates incorporated into DNA, except the cleavage of DNA with incorporated 7-deaza 2´-deoxyguanosine, only the cleavage of Ac-modified DNA by AfeI was determined. No cleavage by any REs was observed in DNA modified with 5-phenylcytidine.

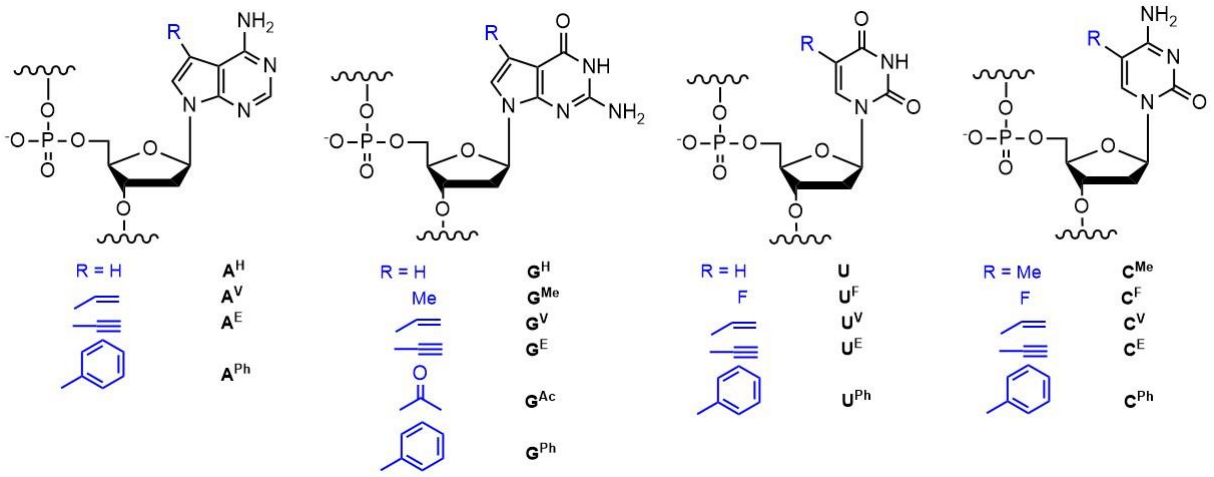


Figure 4: *Structures of dNTPs used for cleavage study*

G* = G^{Ac}, G^{V}, G^{E}, G^{Ph}, G^{Me}

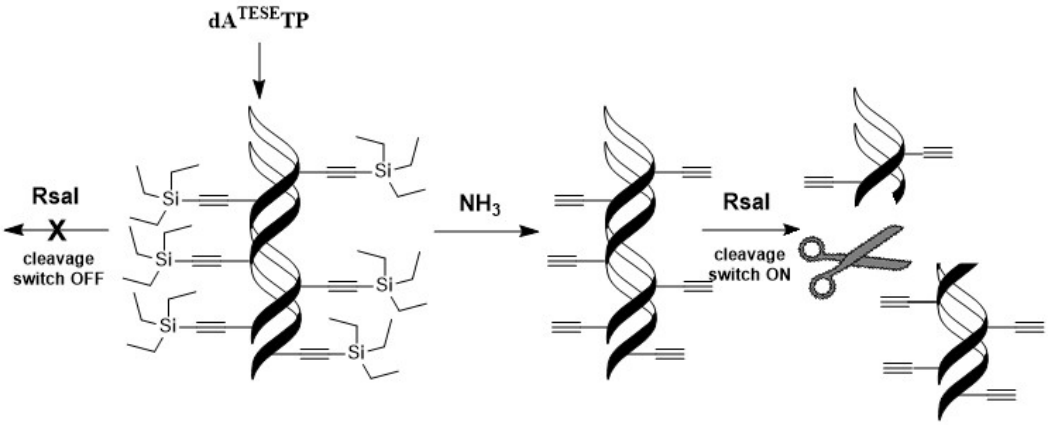
RE	A^H	A^V	A^E	A^{Ph}	G^H	G*	U	U^F	U^V	U^E	U^{Ph}	C^F	C^{Me}	C^V	C^E	C^{Ph}
AfeI	-	-	-	-	-	-/+++^{Ac}	+++	+++	+++	+++	++	+++	-	-	-	-
AflII	x	-	-	-	++	-	+	++	++	++	-	+++	-	-	-	-
ApaLI	x	+	-	-	-	-	+++	+++	++	++	-	+++	-	-	-	-
BamHI	x	-	-	-	-	-	+++	+++	+++	+++	x	+++	-	-	-	-
BglII	x	-	-	-	++	-	-	+++	+++	+++	x	++	-	-	-	-
EcoRI	-	-	-	-	-	-	-	++	-	-	-	+++	++	-	-	-
KpnI	+	++	+++	-	-	-	+++	+++	+++	+	-	+++	+++	+	-	-
NcoI	x	+	+++	x	+	-	+++	+++	+++	+	x	+++	-	-	-	-
PspGI	+	++	+++	+	+	-	+++	+++	++	+	+	+++	-	-	-	-
PstI	+	-	-	-	-	-	-	-	+++	-	-	+++	-	-	-	-
PvuII	+	-	-	-	+++	-	+++	+++	+++	+	-	+++	+++	-	-	-
RsaI	+	+++	+++	-	-	-	+++	+++	+++	++	-	+++	+++	+	-	-
SacI	+	++	+++	-	-	-	+++	+++	+++	+	-	-	-	-	-	-
ScaI	-	-	-	-	-	-	-	+	+++	+++	-	+++	+++	+++	+	-
SphI	+	-	++	+	-	-	+	+++	+++	+	-	+++	-	-	-	-

Approximately yields of cleavage: - = 0-25%; + = 25-50%; ++ = 50-75%; +++ = 75-100%; x = no tested

Table 1: *Overview of cleavage modified DNA by restriction endonucleases*

1.3.3. Transient protection of DNA against cleavage by restriction endonuclease[79, 81]

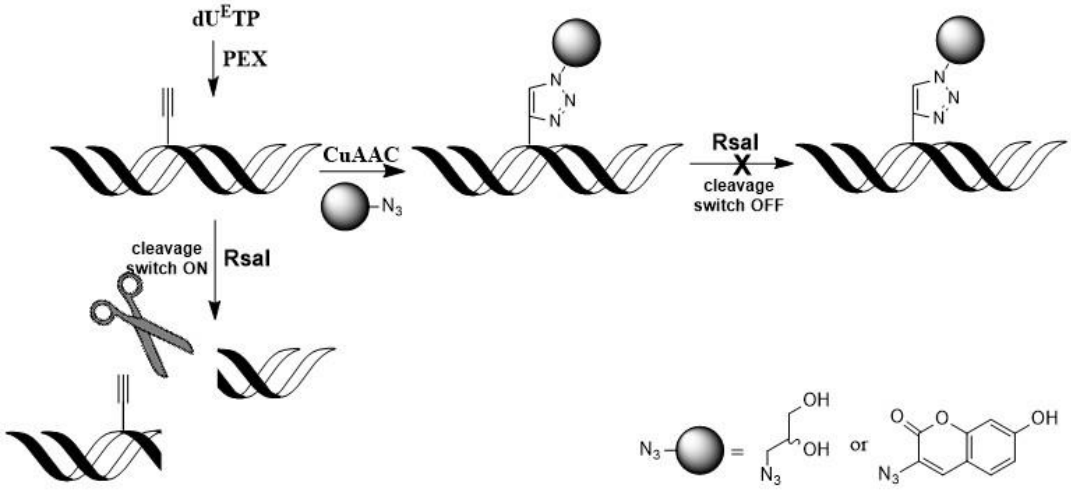
The first case of transient protection of DNA against restriction endonuclease (RE) cleavage was reported for (trialkylsilyl)ethynyl-modified DNA. Three trialkylsilyl groups (trimethylsilyl - TMS; triethylsilyl – TES and triisopropylsilyl - TIPS) were tested as protection groups for 7-acetylene- modified 7-deaza-2´-deoxyadenosine triphosphates incorporated into the DNA by PEX reaction. Full cleavage of natural DNA by KpnI, RsaI and SacI was observed, whereas the TES- and TIPS- protected 7-etynyl- 7-deaza-2´-deoxyadenosines inhibit cleavage by tested REs. In the case of TMS-ethynyl modified DNA, partial cleavage of DNA by RsaI and KpnI was determined. Ethynyl-modified DNA created after deprotection of TES- and TIPS- groups, was fully recognized and cleaved by all three REs. Moreover, **dA^{TESE}TP** was successfully incorporated into 287-mer DNA by PCR reaction. The same trend of cleavage was observed also with TESE- modified 287-mer DNA, when **dA^{TESE}** fully modified DNA was resistant against cleavage by RsaI, however ethynyl- modified DNA, created after deprotection of TESE-modified DNA treated with NH_3, was highly tolerated by the same RE.[81] (***Scheme 14***)



Scheme 14: *Temporary protection of DNA against cleavage by restriction endonucleases-TURN ON*

In 2018, it was shown that ethynyl-modified DNA, synthesized in the presence of 5-ethynyluracil deoxyribonucleoside triphosphate (**dU^{E}TP**), is fully tolerated by restriction endonuclease BamHI. Cleavage of ethynyl-modified DNA was completely inhibited through

the Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC) reaction in the presence of 3-azidopropane-1,2-diol.[93] (***Scheme 15***)



Scheme 15: *Temporary protection of DNA against cleavage by restriction endonucleases-TURN OFF*

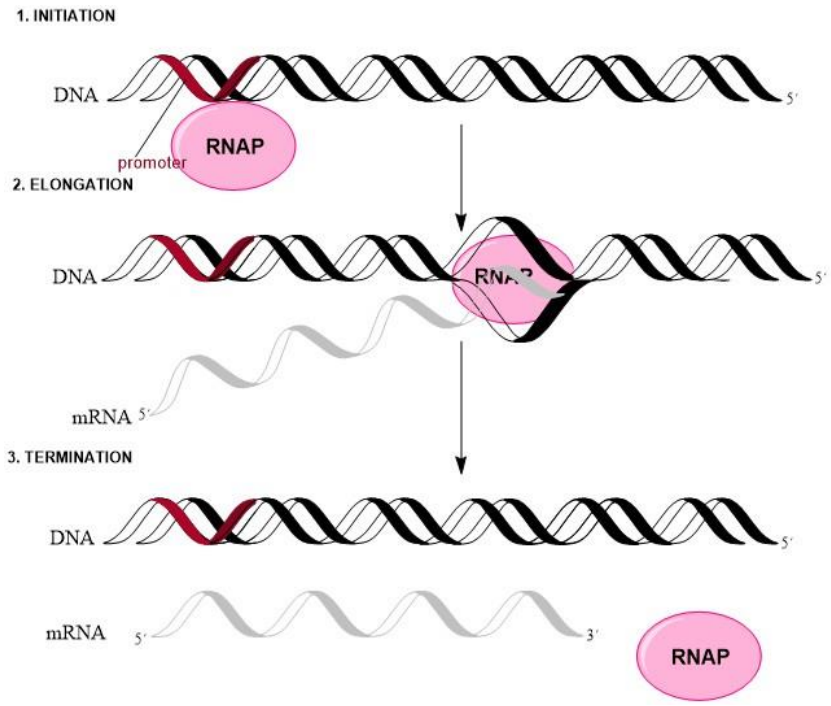
TESE- group can be used for the protection of DNA against RE cleavage; moreover, revealed ethynyl group can be used for further modification of DNA by click reactions and turn off cleavage by RE repetitively.

1.4. Transcription

1.4.1. Transcription-general remarks

Transcription is the first step of gene expression, in which a specific segment of DNA is copied into RNA by enzyme - RNA polymerase (RNAP). The transcription consists of few basic steps: *initiation*, *elongation* and *termination*. In the *initiation* step, the RNAP with transcription factors (TF) bind to the specific DNA sequence called promoter to form a complex (RNA polymerase-promoter open complex), called transcription bubble, in which the DNA is partially unwound and single-stranded. Transcription *initiation* is regulated by additional proteins (activators, repressors), which modulate formation and function of RNAP-DNA complex. In the *elongation* step, RNA synthesis proceeds in the 5′to 3′ direction and incoming nucleotide is added to the 3′-OH group of growing RNA chain. During the *elongation*, the transcription bubble moves along the DNA and RNA strand and is extended. The RNA polymerase selects the nucleotides incorporated into the growing RNA chain through its formation of Watson-Crick base pairing with template DNA strand. A DNA-RNA hybrid helix consist of antiparallel strands and DNA's template strand is read in the 3′to 5′direction.

Transcription *termination* involves cleavage of the newly completed transcript - RNA. The site on the template strand at which RNA polymerase terminates transcription is controlled by base sequence in this region – terminator.[6] (***Scheme 16***)



Scheme 16*: Simple diagram of transcription*

Methylated cytosines of CG sites in the promoter regions of eukaryotic genes give rise to a reduction of gene expression. Inability of transcription factors to bind to methylated target sites, chromatine condensation released by histone deacetylation and binding of 5-methylcytosine proteins on DNA, all the consequences of DNA methylation lead to a strong repression of gene expression.[94] In addition, it was determined that intermediates of active DNA demethylation also influence the process of transcription. The hmC influence binding of transcription factors[95], fC/caC directly influence binding of mammalian RNA polymerase II[96] and incorporated fC modifications can change the shape of DNA[97].

Recently, it was reported that 8-oxo-7, 8-dihydroguanine (8-oxo-G) does not directly block transcription by RNA polymerase II and inhibition of transcription by 8-oxo-G depends on 8-oxoguanine DNA glycosylase.[98]

1.4.2. Study of tolerance modified DNA by RNAP [99]

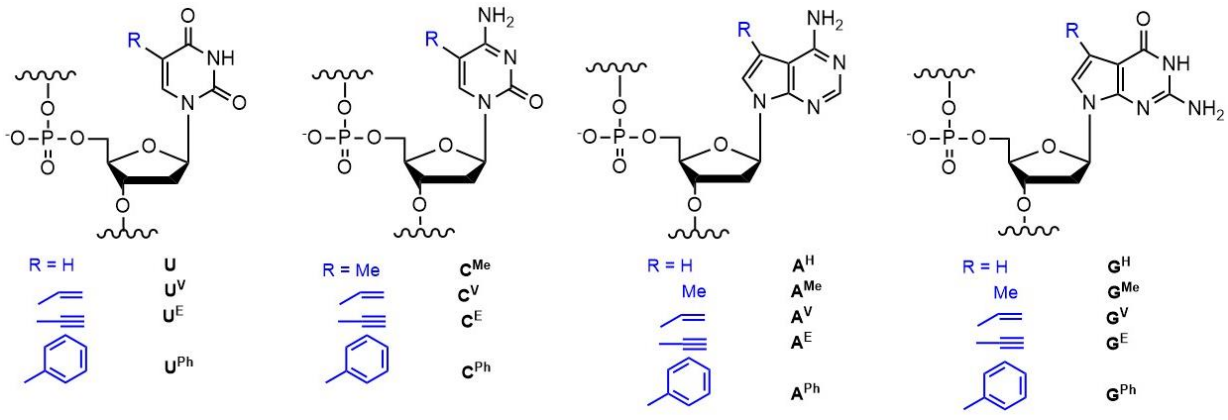
The set of base-modified dNTPs, diverse modifications in the position 5 of pyrimidine bases and in the position 7 of the purine bases, were used for the synthesis of modified DNA with *Pveg* promoter sequence. (***Figure 5A***) Major-groove modified DNA templates were tested in transcription studies by two RNA polymerases (RNAP) - *B.subtilis* RNAP and *E.coli* RNAP. According to quantitation results of transcription by *B.subtilis* RNAP, the transcription was blocked almost in all cases of modified DNA. 7-deazaA ($\mathbf{A^H}$) was fully tolerated and 7-deazaG ($\mathbf{G^H}$) was partially tolerated by *B.subtilis* RNAP.

Both 7-deaza-purine bases ($\mathbf{A^H}$ and $\mathbf{G^H}$) were fully tolerated with *E.coli* RNA polymerase, moreover 7-methyl-7-deazapurines ($\mathbf{A^{Me}}$ and $\mathbf{G^{Me}}$) also gave significant transcription. In the case of modified pyrimidines, reduced amount of transcript was observed for $\mathbf{U^E}$, $\mathbf{C^{Me}}$, $\mathbf{C^V}$ and surprisingly $\mathbf{C^{Ph}}$ modified DNA templates. Inhibitory effect on transcription was determined in all others phenyl-modified DNA templates, $\mathbf{G^V}$, $\mathbf{G^E}$, $\mathbf{U^V}$ and **U** modified DNA templates. The presence of **U** in the DNA template stops the transcription most likely because of inefficient binding RNAP with **U**-modified DNA template during initiation step.

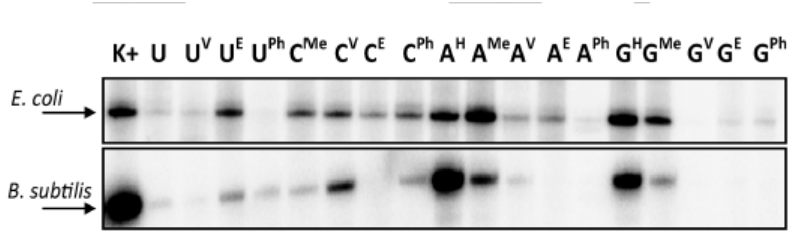
In the case of tested major-groove modifications, tolerated by RNAP, no formation of shorter RNA products was observed, thus the modified dNTPs most probably influence the first step of transcription.

Partial tolerance of smaller major-groove modifications with *E. coli* RNAP gives an assumption to design biorthogonal chemical transformation to convert bulkier substituent to smaller one and therefore to unblock the inhibition of transcription.

A)



B)



C)

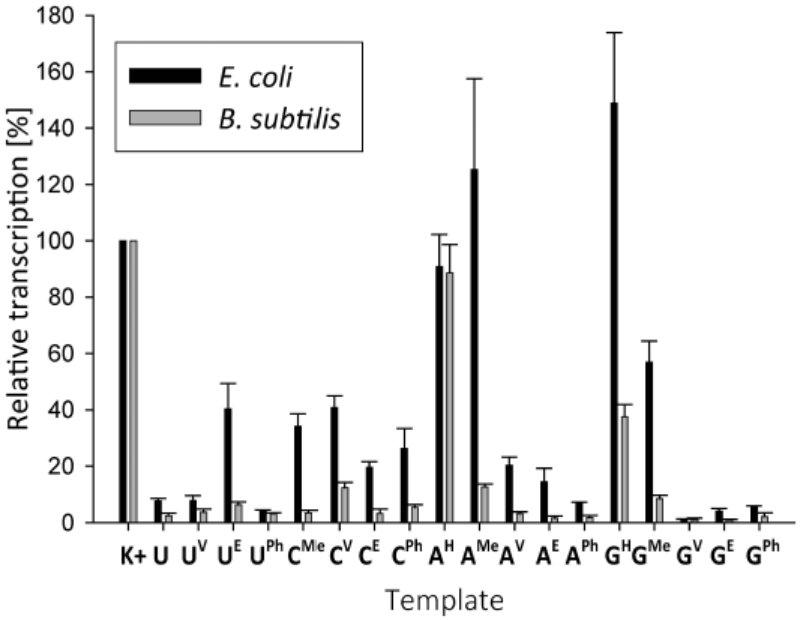


***Figure 5**: A) Structures of modified dNTPs B) Representative results from in vitro multiround transcription* C) Quantitation of in vitro multiround transcription**
(Figures copied from publication)*

In 2018, the first case of bioorthogonal turning off transcription was reported for ethynyl-modified DNA template (**U^{E}**) clicked with water-soluble azides [3-azidopropane-1,2-diol (**APD**) or azidocoumarin (**AC**)].[79] The **U^{E}**-modified DNA template gives approximately 43% transcription in comparison to natural DNA templates, whereas almost the full inhibition reaction of transcriprtion was observed for **U^{E}**-modified DNA clicked with appropriate azides.[100, 101-103]

1.5. Photolabile protecting groups and photocaging of biomolecules

The use of common protecting groups on silent biological reagents or substrates is not fully suitable for rapid biochemical processes. Covalent blocking of the functional groups at the active site of an enzyme can reduce their activity and so to shut down the catalytic cycle. Since the introduction of photoremovable protecting groups (PPGs) in 1970s, PPGs are still in the centre of chemists' interest. PPGs present an ideal alternative to all other methods how to

introduce reagents or substrates into reactions and biological processes, because no reagents other than light are needed, and no further separation of reagent is required. Photolysis reactions enable researchers to control spatial and temporal releasing of desired substrates in synthetic or physiological area.[104] The light releasing of bioactive compounds in living tissues have become an important tool for cell biology[105], biomedicine[106-108], biochemistry[105, 109], neurosiences[105, 109], etc.

The criteria to design a good PPG depends on further application of be released compound. General requirements for a good PPG are: i) the PPG should be soluble in the target media; ii) the released by-product should not react with investigated system and should not absorb the irradiation wavelength of starting compound; iii) PPG should have strong absorption at wavelength above 300 nm and the wavelength must not be absorbed by the media, product, substrate; iv) PPG should be excited by a short light pulse.[104, 105]

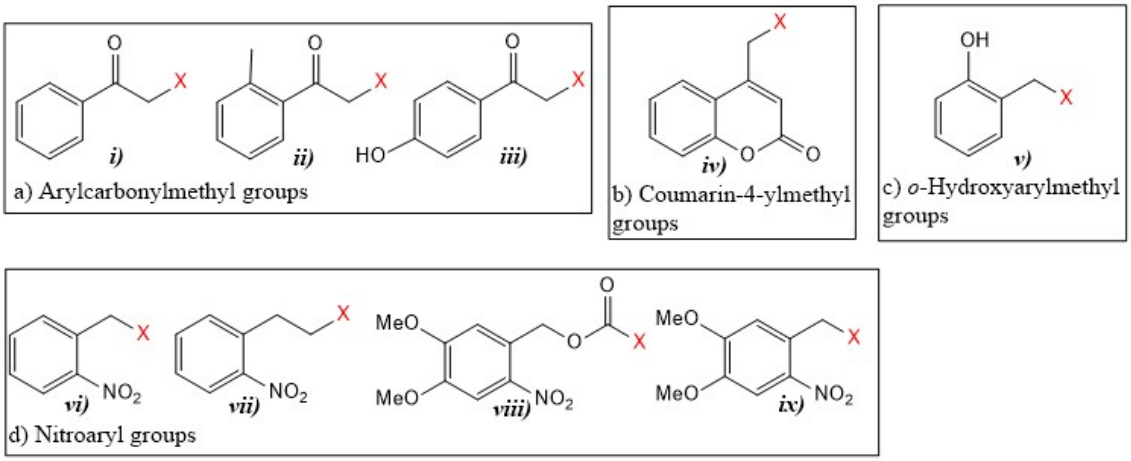


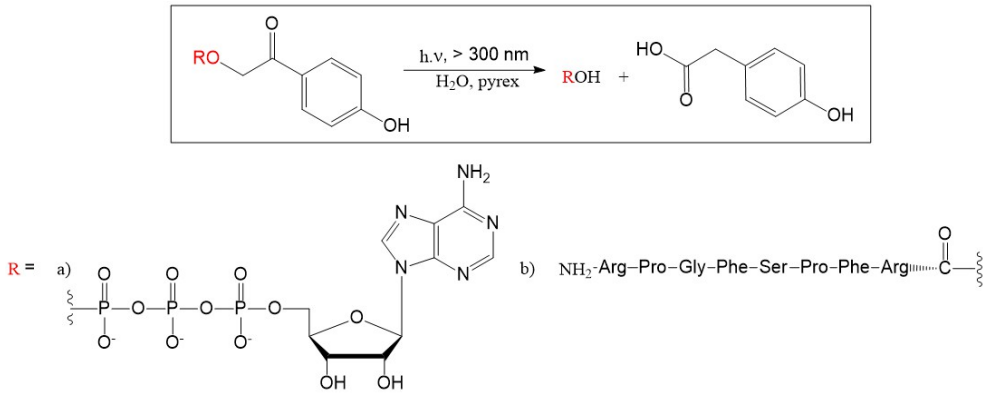
***Figure 7**: Basic structural types of photoremovable protecting groups*

There are several structural types of photoremovable protecting groups and discovery of new photoactive protecting groups is still at the centre of interest. To illustrate the range and variety of its application in chemistry and biology, the most significant groups of PPGs and their aplication are briefly summarized in the following section. (***Figure 7***)

a) Arylcarbonylmethyl groups [*Figure 7a)*]

The photophysical and photochemical properties of thermally stable aromatic ketones are well understood. Its photochemical reactivity proceed usually on carbonyl group of aromatic ketones.[105] *p*-Hydroxyphenacyl protecting group is efficient for protection of carboxylic acids and phosphates.[104] Since in the case of carboxylic acids, no decarboxylation products were observed and side photoproduct in general is biologically

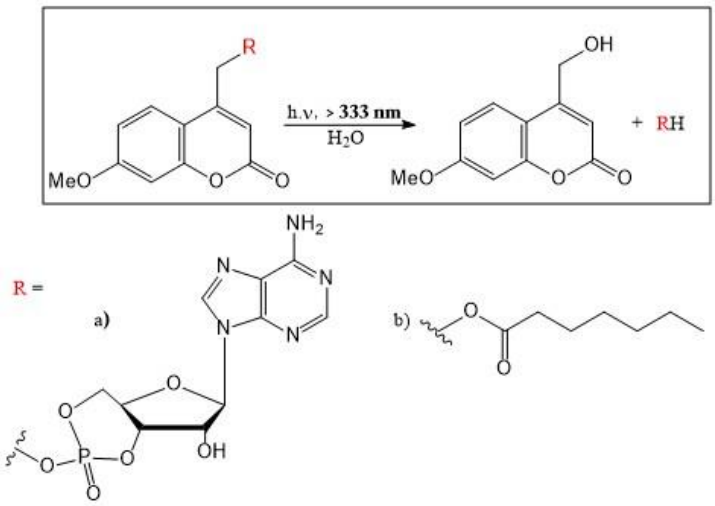
resistant, *p*-hydroxyphenacyl protecting group is an excellent tool to investigate fast biological processes. At the end of the 20th century, *p*-hydroxyphenacyl group impressed as a promising protecting group of phosphates. *p*-Hydroxyphenacyl ester of ATP was formed and its solvolytic stability in various buffers were studied.[110] *p*-Hydroxyphenacyl protecting group was also applied to protect bradykinin [***Scheme 17b)***] from degradation and allow its precise temporal and spatial release at 337 or 300 nm wavelength to activate the bradykinin BK2 receptor.[111] (***Scheme 17***)



Scheme 17*: Examples of p-hydroxyphenacyl protected compounds*

b) Coumarin-4-ylmethyl groups [*Figure 7b)*]

Coumarinyl protecting group is an attractive photoactivable group, because of its stability, large molar absorption coefficient at longer wavelength (320 nm – 490 nm), fast release rate and fluorescent properties. Coumarin-4-ylmethanol is a common precursor of coumarin-caged esters, phosphates, carboxylates, carbonates, carbamates, anhydride derivatives and diols.[105]

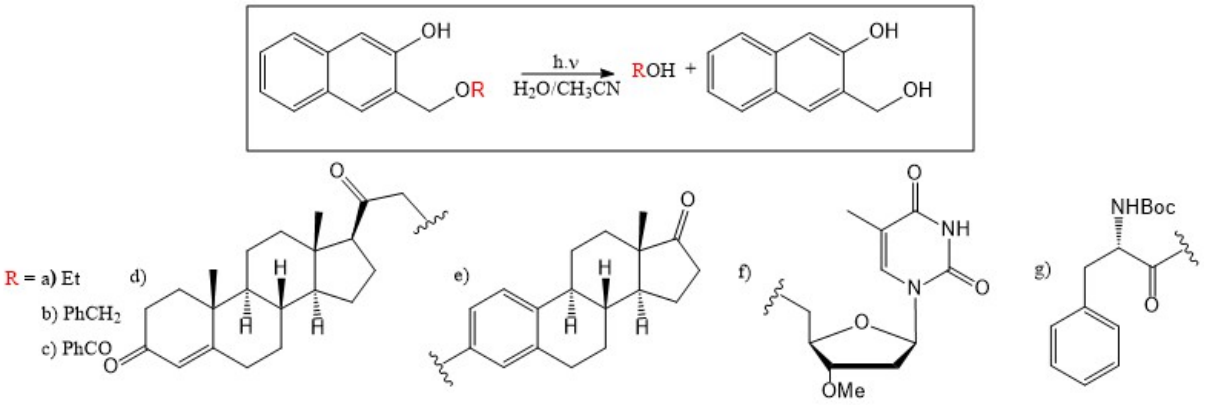


Scheme 18*: Examples of coumarinyl protected compounds*

Uncaging of 4-coumarin-4-yl-1, 3-dioxolanes was successfully obtained under physiological conditions.[112, 113] Coumarin-4-ylmethyl bromide can also efficiently cage carboxylic acids and amines.[114] Methoxy and hydroxyl methylcoumarins were used as a phototrigger for the release of cAMP.(***Scheme 18***)

c) *o*-Hydroxyarylmethyl groups [*Figure 7c)*]

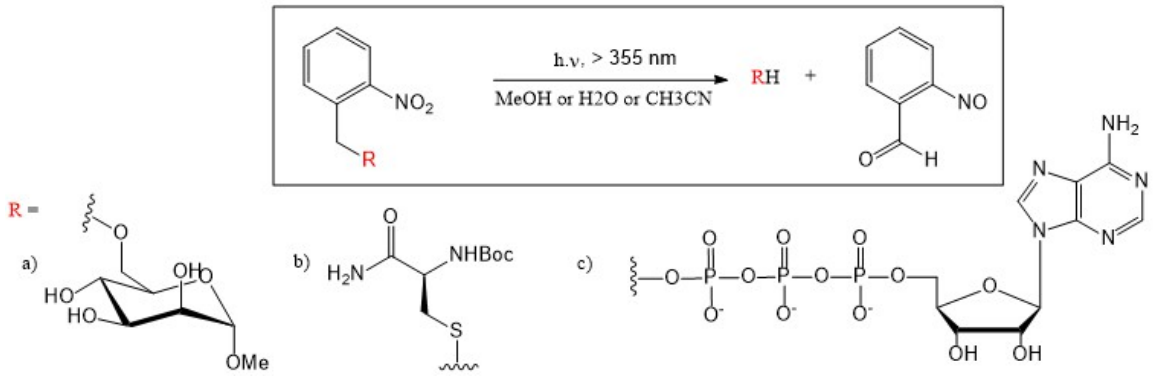
Introduction of *ortho*-hydroxy substituent to the benzylic position change the mechanism of cleavage of C-O bond and enhances the efficiency of deprotection. Irradiation of *ortho*-hydroxybenzyl ethers or their naphthyl analogues in water lead to the release of an alcohol and appropriate parent diol.[115-117] The yields of reaction depend on the nature of appropriate alcohol. In 2008, Popik et al. published the synthesis of alcohols, phenols, and carboxylic acids caged with the (3-hydroxy-2-naphthalenyl)methyl group and the releasing of appropriate alcohols in 91-98% yield upon 300 or 350 nm irradiation.[118] (***Scheme 19***) The disadvantage of such PPGs is that the chromophore of the reaction remains in the photoreaction, behave as an internal filter and thus reduce the efficiency of the photolysis.



Scheme 19*: Examples of (o-hydroxy-2-naphthalenyl)methyl protected compounds*

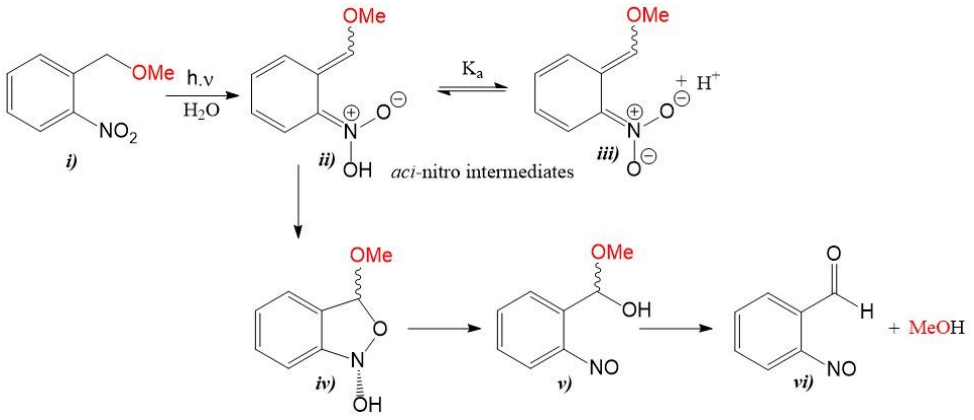
d) Nitroaryl groups [*Figure 7d)*]

The most commonly used PPGs are nitrobenzyl, nitrophenethyl compounds and their dimethoxy derivatives (nitroveratryl).[105] During the photolysis of nitroaryl-protected compounds, a heavily absorbing by-product is formed, which can be toxic for biological systems. Despite the mentioned disadvantages, *o*-nitrobenzylic derivatives are still widely applied PPGs. Nitroaryl PPGs are used to esterify carboxylic groups of natural compounds [104], to cage phosphate group of ATP[119], but mainly to protect the alcohols[120] or thiols [121] and the least to cage amines [122, 123]. (***Scheme 20***)



***Scheme 20**: Examples of: nitroaryl- protected compounds*

Photolysis of *o*-nitrobenzyl- and 1-(2-nitrophenyl)ethyl- derivatives at $\lambda_{max} \approx 400$ nm proceed via *aci*-nitro intermediates.[105] Mechanism of photolysis for *o*-nitrobenzyl protected alcohols was studied for several model compounds.[119, 124] Detailed kinetic and mechanical studies of 1-(methoxymethyl)-2-nitrobenzene were performed by Wirz and co-workers.[124] Deprotection of caged alcohol goes through formation of *aci*-nitro intermediates, irreversible cyclization of *ii)* to *iv)* and hydrolysis of the hemiacetal intermediate *v)* formed by ring opening of *iv)*. The final reaction is releasing of MeOH and nitroso- benzaldehyde from the intermediate *v)*. The ring opening of *iv)* is the rate-determining step. The release of the free final substrate from nitroaryl-protected compound and overall rate of the reaction is slower in comparison to the decay of its primary intermediate. (***Scheme 21***)



***Scheme 21**: Mechanism of photolysis for o-nitrobenzyl protected methanol*

Modifications of the aromatic ring of the *o*-nitrobenzyl chromophore can bring the possibility to modulate its solubility properties or to catch group on a linker, or to tune the absorbance of chromophores. By adding of two methoxy groups to the *o*-nitrobenzyl chromophore the most used 6-nitroveratryl (NV, ***Figure 7d-ix)***) and 6-nitroveratryloxycarbonyl (NVOC, ***Figure 7d-viii)***) PPGs were formed and the change of

the aromatic ring causes an increase of absorbance for new chromophores at longer wavelength λ > 350 nm.[105] Photolysis of NVOC protected alcohol results in the release of a free alcohol substrate, carbon dioxide and photolabile *o*-nitrostyrene through an elimination mechanism. [125, 126]

1.5.1. "Caging" of biologically active substances with PPGs

Caged molecules represent biologically inactive molecules, which can be irreversibly activated by irradiation with light whereas inert photolabile group is removed. In 1978, *o*-nitrobenzyl protected ATP was synthesized as the first "caged molecule" in order to use it for biochemical experiments with spatiotemporal control.[109, 127] Up to now, many different classes of molecules have been protected with PPGs. Caged neurotransmitters[109] and hormones were used for analysis of receptors[109] and their kinetic studies.[128-133] The irreversible photoactivation of proteins has been described for several protein classes. [134]

Caging of nucleic acids is relatively new category of compounds protected by photolabile protecting group. Nucleic acids can be protected with photolabile protecting groups in several ways - *backbone phosphate caging*, *caging of hydroxyl- groups or nucleobases.* (***Figure 8***)

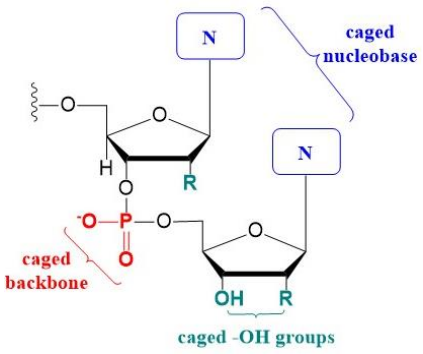


Figure 8*: Caging strategies for nucleic acids (R = H or OH)*

Backbone phosphate caging of DNA [135] or siRNA[136] or mRNA[137] present one form of caged nucleic acids. Coumarinyl- caged mRNA and plasmid DNA were injected to the zebrafish embryos and in both cases, the expression was almost only observed after their activation by light. In the case of siRNA, it was possible to regulate its activity based on the amount of PPG. If siRNA contained 1.4 nitrobenzyl- PPG per duplex, it didn´t cause its complete inactivation however it could be fully activated upon irradiation. Increasing of PPG made the siRNA inactive, but the full activity could not be restored by irradiation. (***Figure 9***)

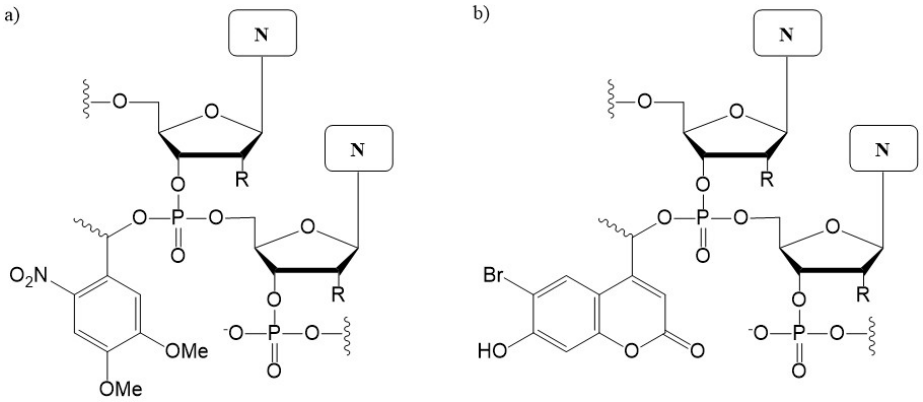
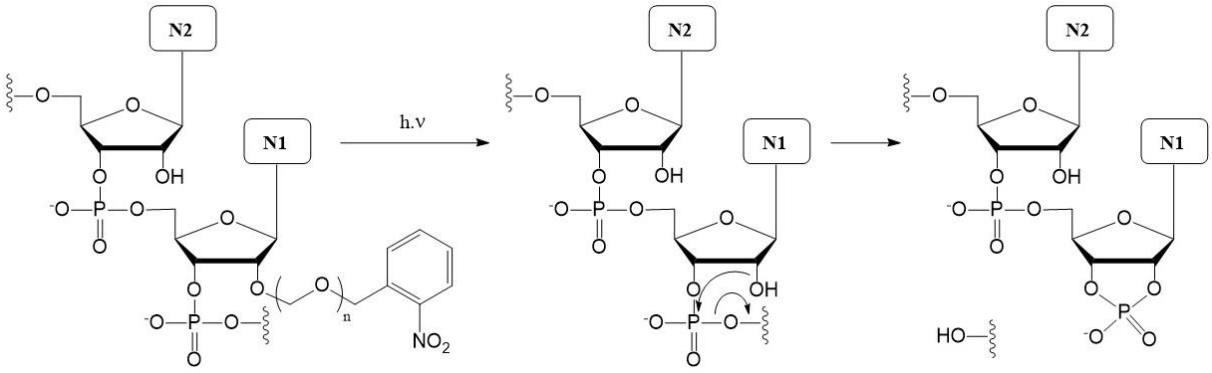


***Figure 9**: Statistical backbone phosphate caging a) of DNA (R=H) or siRNA (R=OH)[136] b) of DNA (R=H) of mRNA (R=OH)[137]*

The plasmid DNA modified with statistical distribution of 1-(4,5-dimethoxy-2-nitrophenyl) diazoethane in DNA backbone was introduced into rat skin cells or HeLa cells and the inhibition of expression in the stage of transcription was determined. Photocleavage of approximately 270 caging groups in plasmid by exposure to light (355 nm), activate production of mRNA, however, the full transcriptional activity was not restored.[138]

The statistical backbone caging is an elegant approach, but the preparation of such caged DNA is not straightforward. Furthermore, mentioned modifying reactions and used caging groups do not lead to clean on/off behaviour of the reaction.

Introduction of ***caging groups on defined position*** of nucleotide is another option to inactivate nucleic acids. ***Caging of 2´-OH group*** of RNA disrupt the Watson-Crick interaction capabilities and so control reaction of a ribozyme or a spliceosome with light.[139] The site-specific incorporation of PPG enables it to obtain a "binary" off/on system before and after irradiation. (***Scheme 22***)



***Scheme 22**: Caging groups on defined position*

The nucleobases play a major role in encoding and storing of genetic information in DNA and RNA. The ***caging of nucleobases*** in the positions essential for the Watson-Crick interaction may be seen as a temporary mismatch and block the interaction with proteins. DNA oligonucleotide with 2-(2-nitrophenyl)propyl modified thymidine at the O^4-position ($\mathbf{T^{NPP}}$) in promoter sequence fully prevent transcription by T7 RNA polymerase.[140] The same caged nucleotide $\mathbf{T^{NPP}}$ was incorporated to DNA aptamer in exact position to avoid interaction with thrombin.[141] DNA modified with few $\mathbf{dG^{NPP}}$ prevent from forming its active conformation and light can initiate the release of an un-modified oligonucleotide and create an active G-quadruplex. [142]

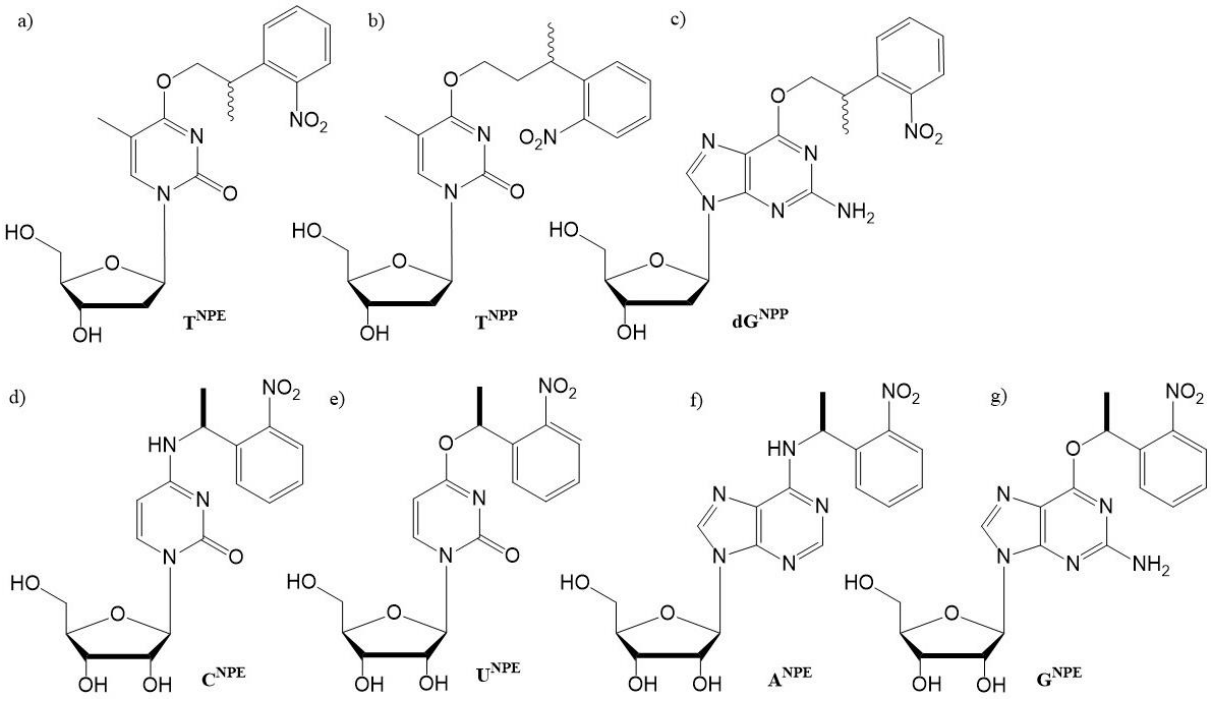


Figure 10*: Examples of residue for making of nucleobase-caged nuclei acids*

To study tertiary folding in RNA, the whole set of nucleobases modified with nitrophenylethyl group ($\mathbf{C^{NPE}}$, $\mathbf{U^{NPE}}$, $\mathbf{A^{NPE}}$, $\mathbf{G^{NPE}}$) in their Watson-Crick interaction surface was examined. Such modified 20-base RNA sequence formed a less stable conformation and light allowed to release and fold an un-modified RNA. [143] (***Figure 10***)

2. Specific Aims of the Thesis

1. Synthesis of photocaged 2'-deoxyribonucleosides and nucleotides derived from 5-hydroxymethylpyrimidines or 7-hydroxymethyl-7-deazapurines and their enzymatic incorporation to DNA.
2. Development of bioorthogonal photochemical deprotection of photocaged DNA.
3. Study of influence of epigenetic nucleobases (5hmU, 5hmC, 5mU a U) and their photocaged derivatives on cleavage of DNA by restriction endonucleases and development of photochemical switch of the DNA cleavage.
4. Synthesis of modified DNA templates containing epigenetic nucleobases (5hmU, 5hmC, 5mU a U) and study of the influence of the modifications on transcription by *E. coli* RNA polymerase.
5. Synthesis of photocaged DNA templates and development of chemical switching of transcription through photochemical deprotection and enzymatic phosphorylation.

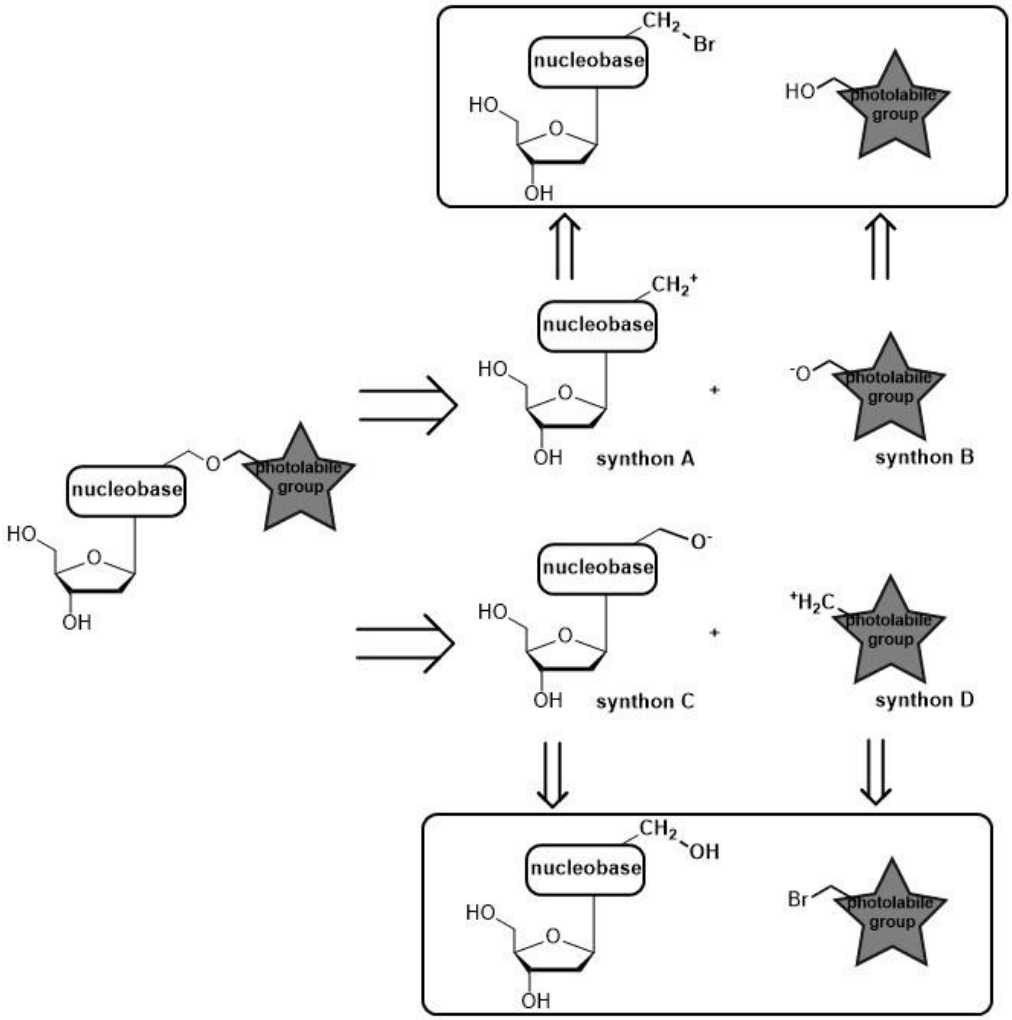
2.1 Rationale of the Specific Aims

Photocaging and photochemical deprotection reactions of nucleobases in DNA were envisaged as suitable bioorthogonal transformations for manipulation of DNA major groove in regulation of protein-DNA binding. Therefore, my first task was to design and synthesize photocaged 5-hydroxymethylpyrimidine and 7-hydroxymethyl-7-deazapurine nucleotides and their enzymatic incorporations to DNA by polymerases. The next logical step was to develop photodeprotection methods under mild conditions and possibly with visible light to avoid damage in live cells. Study of the influence of photocaged or free hydroxymethyl group on cleavage of DNA by restriction endonucleases and switching of the cleavage by deprotection were designed and used as model reactions for modulating of protein-DNA interactions. The next step was the study of the influence of the epigenetic modifications on transcription of DNA templates by bacterial RNA polymerases and it was particularly interesting also from the general point of view of the unknown biological role of some of these natural epigenetic bases. The project should culminate in the development of bioorthogonal set of reactions for switching of bacterial transcription. Based on the previous results, the combination of photocaging of DNA templates and photochemical deprotection with other reactions, e.g. enzymatic phosphorylation of 5hmU in DNA, should have a good potential for the use in artificial chemical epigenetics, i. e. regulation of gene expression through bioorthogonal reactions in the major groove of DNA.

3. Results and Discussion

3.1. Synthesis of photocaged nucleosides and nucleotides

Cross-coupling reactions (aqueous Suzuki, Sonogashira, Heck) are the most efficient and widespread methods for C-C bond formation and synthesis of base-modified pyrimidine and purine nucleo(s)tides. [62, 144, 145]



***Scheme 23**: Retrosynthetic analysis for synthesis of photolabile protected nucleobases*

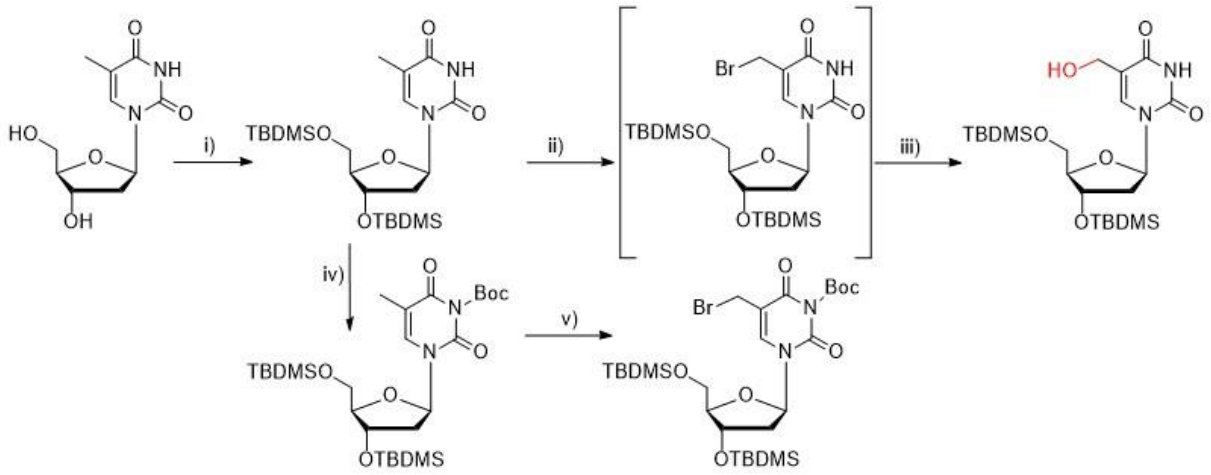
Nucleobases labelled with photolabile protecting groups, through ether functional group, require the basic approach of synthesis including the steps of protection and deprotection of reactive hydroxyl- groups from sugar moiety. According retrosynthetic analysis of desired photolabile protected nucleobases, two possible approaches are considered. In the first case, electrophilic synthon A, with its synthetic equivalent – bromomethyl-modified nucleobase and nucleophilic synthon B with its synthetic equivalent – hydroxymethyl- modified photolabile

moiety were identified. In the second case, hydroxymethyl-modified nucleobase is the synthetic equivalent for synthon C and bromomethyl- modified photolabile moiety is the synthetic equivalent for synthon D. (***Scheme 23***)

The syntheses of all reported intermediates and triphosphates were carried out by me. Parts of the syntheses were performed according to the literature[146-149] but some of the synthetic steps were modified and optimized. The tree step triphosphorylation reactions of caged nucleosides were carried out under the standard Yoshikawa conditions[48, 49] and desired caged triphosphates were obtained in moderate yields. The synthesis of **dU^{X}MP** and **dU^{X}TP** we have reported in my paper *Angew. Chem. Int. Ed*., 2014, **53**, 6734-6737,[150] and some steps of synthesis of **dC^{X}TP** were included in the publication shared with Soňa Boháčová: *Org. Biomol. Chem.*, 2018, **16**, 5427-5432.[151]

3.1.1. Synthesis of hydroxymethyl- or bromomethyl- modified uridine

For a synthesis of pyrimidines nucleosides bearing photolabile protecting group through ether functional group in position 5, hydroxymethyl- or bromomethyl- modified uridines were prepared. The starting hydroxymethyl- or bromomethyl- modified 2'-deoxyuridines were synthesized according to ***Scheme 24***. In the first step, 5'-hydroxyl and 3´-hydroxyl groups of thymidine were protected with *tert*-butyldimethylsilyl (TBDMS) group.[149] Since bromomethyl-derivative of uridine is unstable, hydroxymethyl derivative was synthesized by an one pot, two steps procedure starting from 3', 5'-di-TBDMS-protected thymidine with isolated yield from 14 to 27%. [152] To synthesize the more stable bromomethyl- derivative of uridine as a starting material, the protection of nucleobase's nitrogen in position 3 with tert-butyloxycarbonyl protecting group was required. 5-Bromomethyl 2'-deoxyuridine was prepared by a benzylic bromination of *Boc*-protected 3', 5'-di-TBDMS thymidine in the presence of NBS and catalytic amount of AIBN. [149]

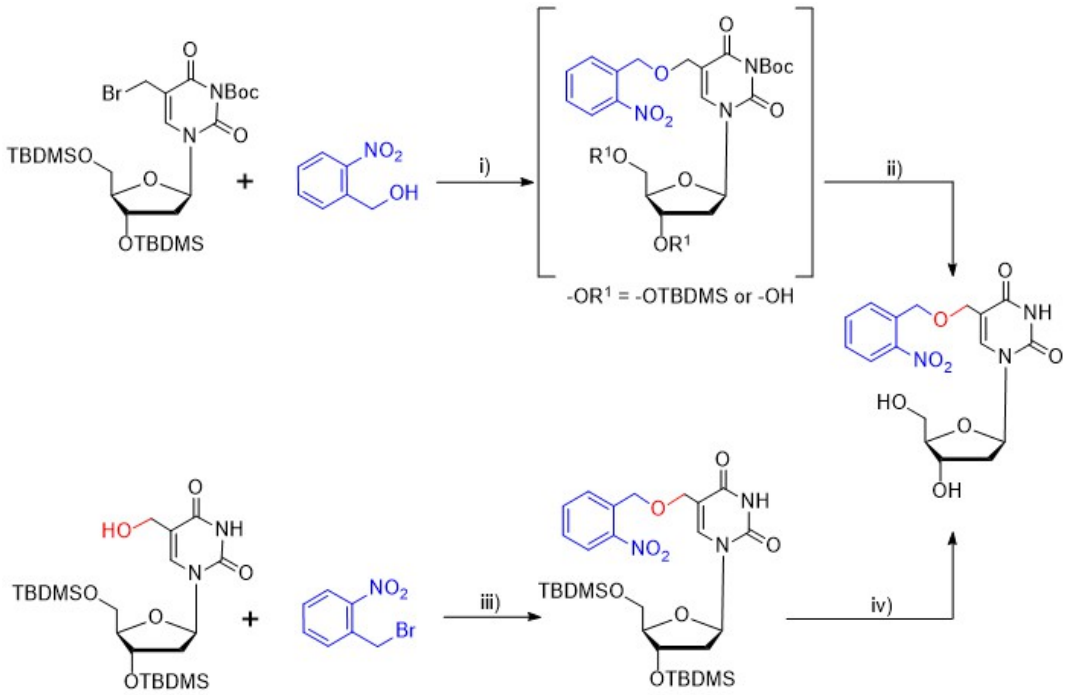


***Scheme 24**: Synthesis of hydroxymethyl- and bromomethyl- modified protected uridine.* Reagents and conditions: i) TBDMSCl, imidazole, DMF, r.t., overnight, 95-99 %; ii) 1. NBS, AIBN, benzene (CCl_4), 85 °C, 30 min; iii) 2. DIPEA, H_2O, DMF, r.t., 5 hours, 14-27 %; iv) Boc_2O, DMAP, dry DMF, r.t., overnight, 66 %; v) NBS, AIBN, benzene, 85 °C, 30 min, 16 %.

3.1.2. Synthesis of photolabile nitrobenzyl- modified and hydroxymethyl-modified nucleos(t)ides

3.1.2.1. Synthesis of NB- modified and HM- modified uridine nucleos(t)ides

Nitrobenzyl- caged uridine nucleobase was prepared by two different approaches either from starting hydroxymethyl- or bromomethyl- modified 2'-deoxyuridines. One pot synthesis represents reaction of freshly prepared 5-bromomethyl 2'-deoxyuridine (prepared by benzylic bromination of *Boc*-protected 3', 5'-di-TBDMS thymidine) with 2-nitrobenzyl alcohol at 110 °C. The cold reaction mixture was dissolved in dry THF incubated with $Et_3N.3HF$ at room temperature. After evaporation of solvent and its double purification by silica gel column chromatography and HPLC purification, 20 % of desired **dU^{NB}** nucleoside was obtained. In the second approach hydroxymethyl- modified 2'-deoxyuridine was incubated with 2-nitrobenzyl bromide, in the presence of hindered base 2,6-di-*tert*-butylpyridine and silver triflate, to yield silyl-protected NB-modified 2´-deoxyuridine (19-35 %). Deprotection step was carried out in THF in the presence of $Et_3N.3HF$ at room temperature to get caged 5-(2-nitrobenzyloxy)methyl-2´-deoxyuridine (**dU^{NB}**) in 49 % yield. (***Scheme 25***) The second approach is more convenient for further usage in synthesis of 5-(2-nitrobenzyloxy)methyl-2´-deoxycytidine - **dC^{NB}** (*section 3.1.2.2*).



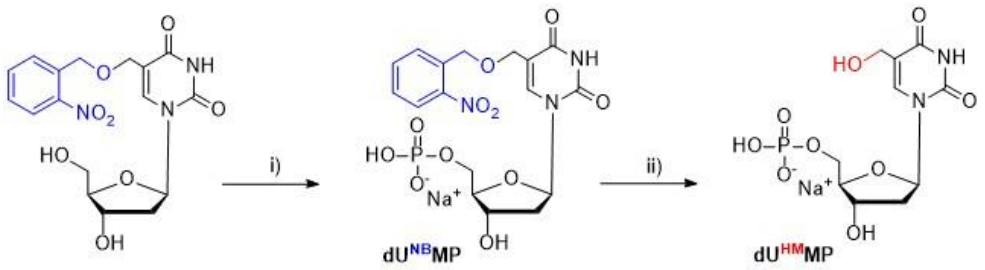
Scheme 25*: Synthesis of NB-modified uridine nucleoside and nucleotide.* Reagents and conditions: i) neat 105 °C, 45 min; ii) $Et_3N.3HF$, dry THF, r.t. 3 hours, 20 %; iii) AgOTf, 2, 6-Di-*tert*-butylpyridine, dry DCM, 3 hours, 19-35 %; iv) $Et_3N.3HF$, dry THF, r.t. 3 hours, 49 %

Unprotected nucleoside **dU^{NB}** was used as a starting material for its monophosphorylation and triphosphorylation. (***Scheme 26***)

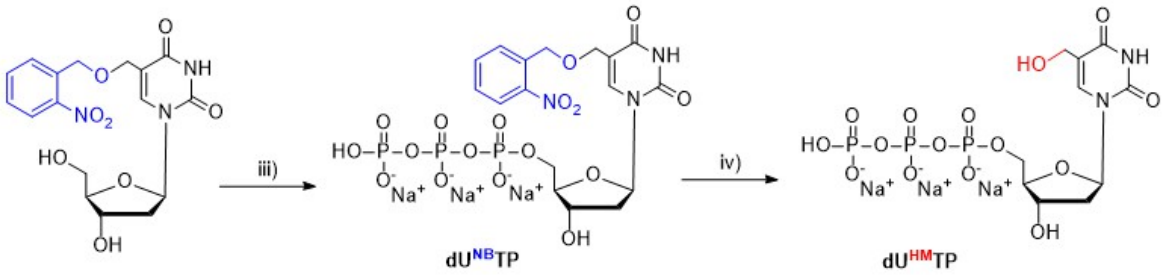
The treatment of **dU^{NB}** with $POCl_3$ in trimethyl phosphate at 0 °C in the presence of 1,8-Bis(dimethylamino)naphthalene followed by quenching the reaction with triethylammonium bicarbonate (TEAB, 2 M) gave expected **dU^{NB}MP** in 62 % yield after HPLC purification. Uncaged **dU^{HM}MP** was prepared by irradiation of caged **dU^{NB}MP** in water with light (366 nm; 0.8 W) for 40 minutes. After reverse-phase HPLC purification of reaction mixture, uncaged monophosphate yielded in 74 %. [***Scheme26a)***] [150]

Triphosphorylation of modified **dU^{NB}** was performed under standard Yoshikawa conditions [48, 49] in the presence of proton sponge. The treatment of **dU^{NB}** with $POCl_3$ in trimethyl phosphate with addition of proton sponge at 0 °C followed by addition of $(NHBu_3)_2H_2P_2O_7$ in dry DMF in the presence of tributylamine. The reaction was quenched with TEAB (2 M) and after purification of reaction mixture by reverse-phase HPLC, appropriate **dU^{NB}TP** was obtained in 35 % yield. In the next step, **dU^{NB}TP** dissolved in water was used for its uncaging by light (366 nm; 0.8W) for 50 minutes. [***Scheme 26b)***] After reverse-phase HPLC purification of reaction mixture, uncaged **dU^{HM}TP** was obtained in 62% yield.[150]

a)



b)



Scheme 26*: Synthesis of NB- and HM- modified uridine nucleotides a) triphosphates; b) monophosphates.* Reagents and conditions: a) i) 1. $POCl_3$, proton sponge, $PO(OMe)_3$, ~ 0°C, 1 hour; 2. 2 M TEAB; 62 %; ii) UV irradiation 366 nm (0.8 W), 40 min, 74 %; iii) 1.$POCl_3$, proton sponge, $PO(OMe)_3$, ~ 0°C, 1 hour; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 °C, 1 hour; 3. 2 M TEAB, 35%; iv) UV irradiation 366 nm (0.8W), 50 min, 62 %

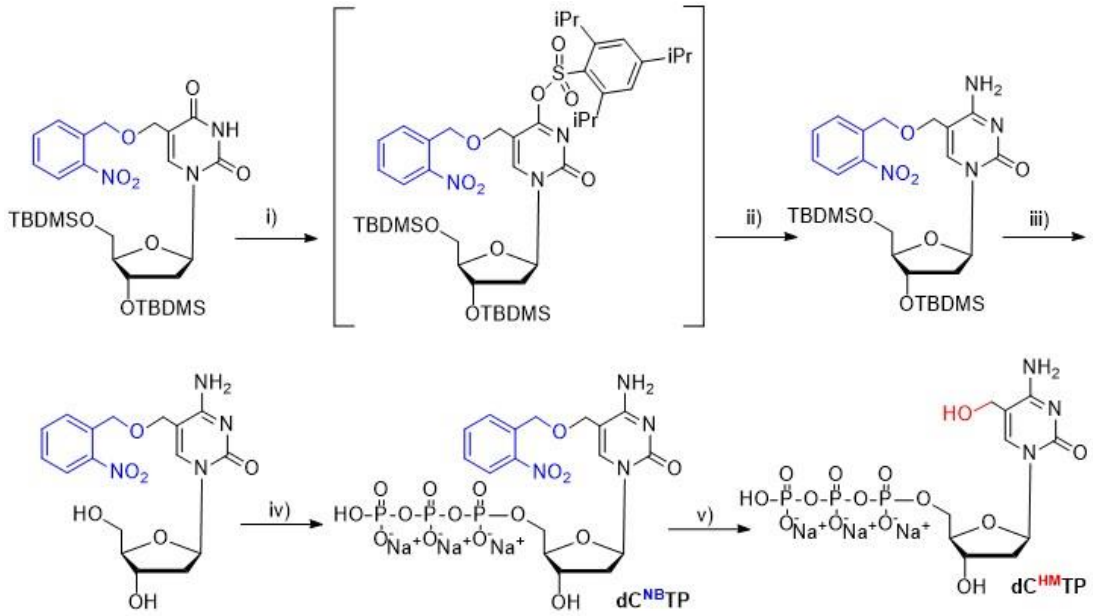
3.1.2.2. Synthesis of NB- modified and HM- modified cytidine nucleos(t)ides [147, 151]

NB-photocaged 2´-deoxycytidine (**dC^{NB}**) was prepared by conversion of silyl-protected NB-modified 2´-deoxyuridine (**dU^{NB}**) in two steps. In the first step, the oxo group in position four of **dU^{NB}** was activated through reaction with 2,4,6-triisopropyl-benzenesulfonyl chloride in the presence of DMAP.[146] By nucleophilic substitution of benzenesulfonyl moiety with gaseous ammonia, silyl-protected NB-photocaged 2´-deoxycytidine was obtained in 80 % yield.[150] The desired nucleoside **dC^{NB}** was prepared by removing of silyl protective groups in the presence of $Et_3N.3HF$ in THF. The yield of deprotection step was moderate (41 %). (***Scheme27***)

Triphosphorylation reaction of **dC^{NB}** was carried out under the same conditions as triphosphorylation of **dU^{NB}**. Unprotected **dC^{NB}** nucleoside was treated with $POCl_3$ in trimethyl phosphate in the presence of proton sponge at 0 °C followed by an addition of pyrophosphate and tributyl amine in DMF. In the final step of triphosphorylation, reaction mixture was treated with triethylammonium bicarbonate (TEAB) to obtain the TEA salt of the desired **dC^{NB}TP**. The target triphosphate - **dC^{NB}TP** in sodium form reached an acceptable 21% yield after isolation by HPLC and ion exchange. (***Scheme27***)

Caged **dC^{NB}TP** was dissolved in water to obtain 12 mM solution, which was irradiated with LED light source (366 nm; 0.8 W) for 40 minutes. Desired deprotected *5-hydroxymethyl-2'-*

deoxycytidine-5'-triphosphate (**dC^{HM}TP**) was isolated by HPLC column chromatography in low 19 % yield. (***Scheme27***) The prepared **dC^{HM}TP** was in the next step used as a substrate for its incorporation to the DNA by PEX or PCR.



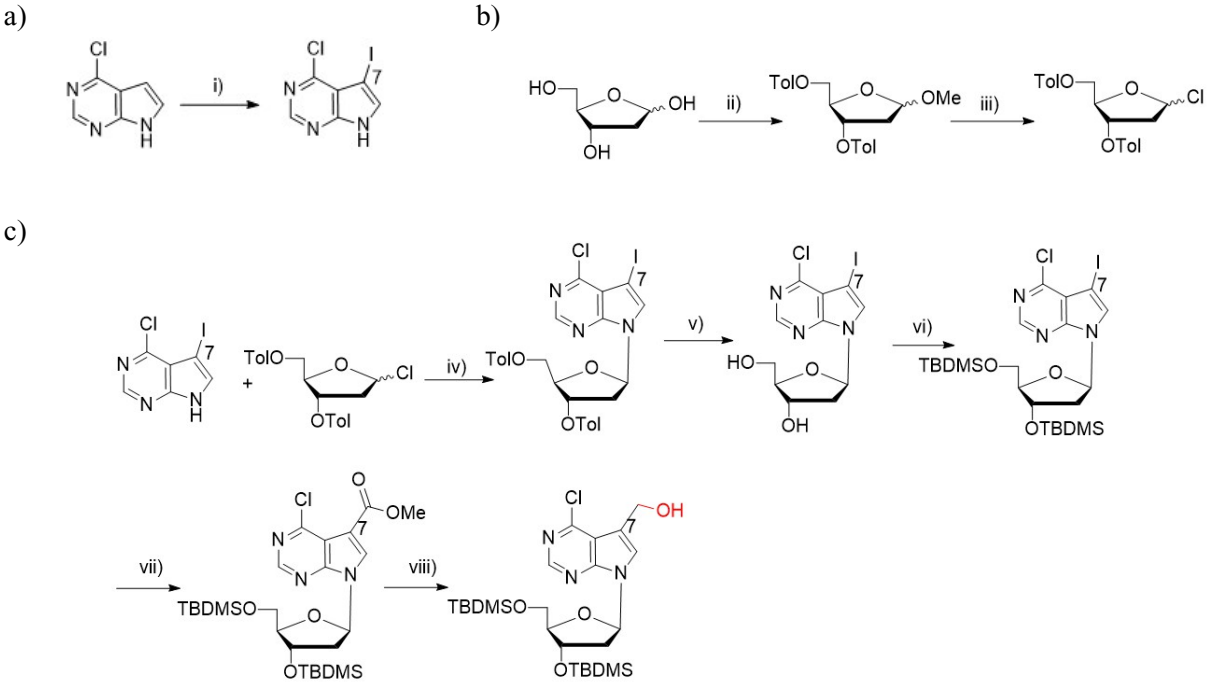
***Scheme 27**: Synthesis of NB-modified cytidine nucleoside and nucleotide.* Reagents and conditions: i) 2,4,6-triisopropyl-benzenesulfonyl chloride, DMAP, Et_3N, dry DCM, r.t., overnight; ii) NH_3 (g), dry dioxane, r.t., 3 hours, 80 %; iii) $Et_3N.3HF$, dry THF, r.t. overnight, 41 %; iv) 1.$POCl_3$, proton sponge, $PO(OMe)_3$, ~ 0 °C, 1 hour; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 °C, 1 hour; 3. 2 M TEAB; 21 % v) UV irradiation 366 nm (0.8 W), 40 min, 19 %

3.1.2.3. Synthesis of NB- modified and HM- modified adenosine nucleos(t)ides [147]

7-deaza-7-iodo-3´, 5´-*di*-*O*-toluoyl-2´-deoxyadenosine was synthesized from a sugar and purine moiety. 6-Chloro-7-iodo-7-deazapurine was prepared by iodination of 6-chloro-7-deazapurine solution in DCM with NIS, in 47-56 % yield after crystallisation. Sugar moiety, 1-chloro-2-deoxy-3, 5-di-*O*-*p*-toluoyl-α-D-erythro-pentofuranose, was synthesized in three step synthesis from 2-deoxy-α-D-erythro-pentofuranose in overall yield 65 % according to the published procedure.[146]

Sugar moiety and purine moiety were incubated in acetonitrile in the presence of base (KOH) and phase-transfer catalyst TDA-1 to yield toluoyl protected nucleoside (49-71 %)[146, 153], which was deprotected by incubation with saturated solution of ammonia at room temperature (69-80 %) [153] and subsequently protected with TBDMSCl in 81 % yield.[147]

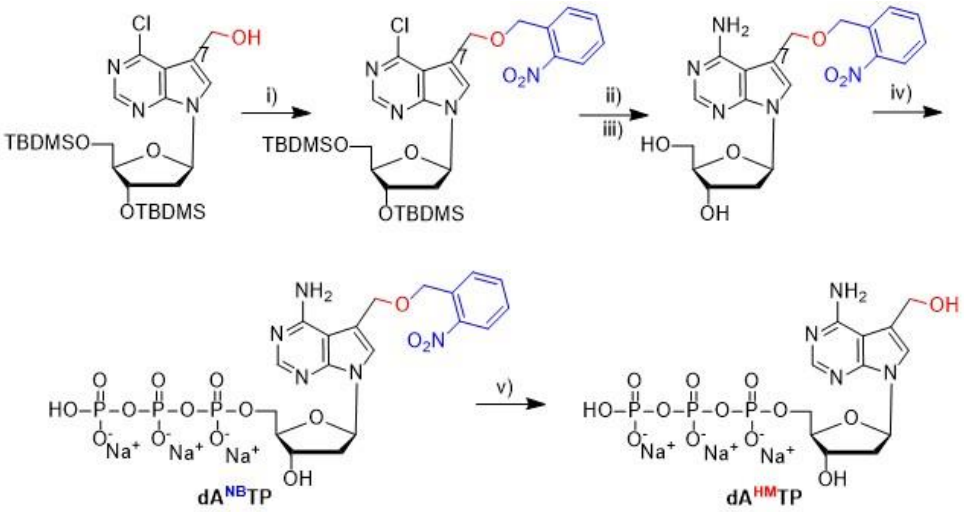
7-Hydroxy-methyl modified protected adenosine was prepared by palladium-catalysed esterification of iodinated nucleoside (75 %) and subsequent reduction of created ester (15-24 %) in the presence of lithium borohydrate ($LiBH_4$). [147](***Scheme 28***)



Scheme 28*: Synthesis of starting nucleoside.* Reagents and conditions: a) purine moiety: NIS, DCM, r.t., 45 min, 47-56 %; b) sugar moiety: ii) 1. 35 % AcCl, dry MeOH, r.t., 1 hour; 2. *p*-TolCl; toluene, r.t., 2 hours, iii) KOH, TDA-1, CH_3CN, r.t., 45 min, 65 % c) nucleoside: iv) KOH, TDA-1, CH_3CN, r.t., 45 min, 49-71 %; v) sat. sol. NH_3/MeOH, r.t., 24 hours, 69-80 %; vi) TBDMSCl, imidazole, dry DMF, r.t., overnight, 81 %; vii) Et_3N, CO (g), $(CH_3CN)_2Cl)Pd(II)$, MeOH/dioxane, r.t. 50 °C, 48 hours, 75 %; viii) $LiBH_4$, dry THF, reflux, 1 hour, 15-24 %.

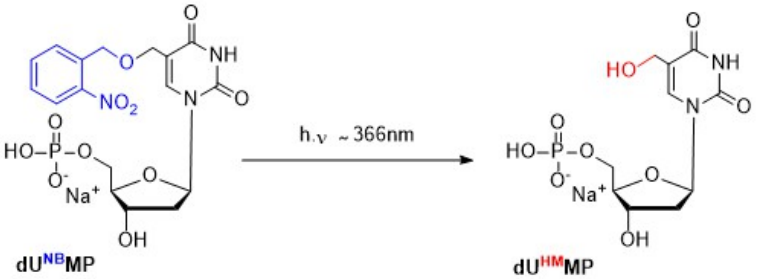
In the next step, silyl protected 6-chloro-7-deaza-7-hydroxy-methyl 2-deoxy-adenosine was incubated with an excess of 2-nitrobenzyl bromide in the presence of base (1 M NaOH) and phase transfer catalyst – Bu_4NBr to yield nitrobenzyl- caged silyl protected 6-chloro-7-deaza-2′-deoxyadenosine (35-48%). To remove silyl groups from prepared nucleobase, protected NB-caged 2′-deoxy-adenosine was incubated in the presence of fluoride anions from Bu_4NF. Subsequent heating up of generated caged 2′-deoxyadenosine in saturated solution of ammonia in MeOH provided desired NB-caged 2′-deoxyadenosine **dA^{NB}** in two steps overall yield 86 %. Such prepared caged nucleoside was triphosphorylated in three steps. In the first step, nucleoside **dA^{NB}** was treated with $POCl_3$ in trimethyl phosphate with addition of proton sponge at 0 °C. After 1 hour 40 minutes of incubation, the reaction mixture was treated by addition of $(NHBu_3)_2H_2P_2O_7$ in dry DMF with tributylamine. Finally, the reaction was quenched with TEAB (2 M) and reaction mixture was purified by reverse-phase HPLC. Desired **dA^{NB}TP** was

obtained in 28 % yield after purification.[147] Uncaged **dA^{HM}TP** was prepared by light irradiation (366 nm, 0.8 W) of NB-caged **dA^{NB}TP** dissolved in water for 1 hour. The yield of deprotection step after reverse-phase HPLC purification of reaction mixture was 39 %. (***Scheme 29***)



Scheme 29: *Synthesis of NB-modified adenosine nucleoside -* ***dA^{NB}*** *and nucleotides -* ***dA^{NB}TP, dA^{HM}TP***. Reagents and conditions: i) 2-nitrobenzyl bromide, n-Bu_4NBr, 1 M NaOH, DCM, r.t., 48 hours; 35-48 %; ii) n-Bu_4NF, THF, 0°C→r.t., 2 hours; iii) sat. solution NH_3/MeOH, 1,4-dioxane, 100 °C, 20 hours, 86 %; iv) 1.$POCl_3$, proton sponge, $PO(OMe)_3$, ~ 0 °C, 1 hour; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 °C, 1 hour; 3. 2 M TEAB; 28 % v) UV irradiation 366 nm (0.8 W), 60 min, 39 %

3.1.3. Kinetic study of deprotection of dU^{NB}MP[150]



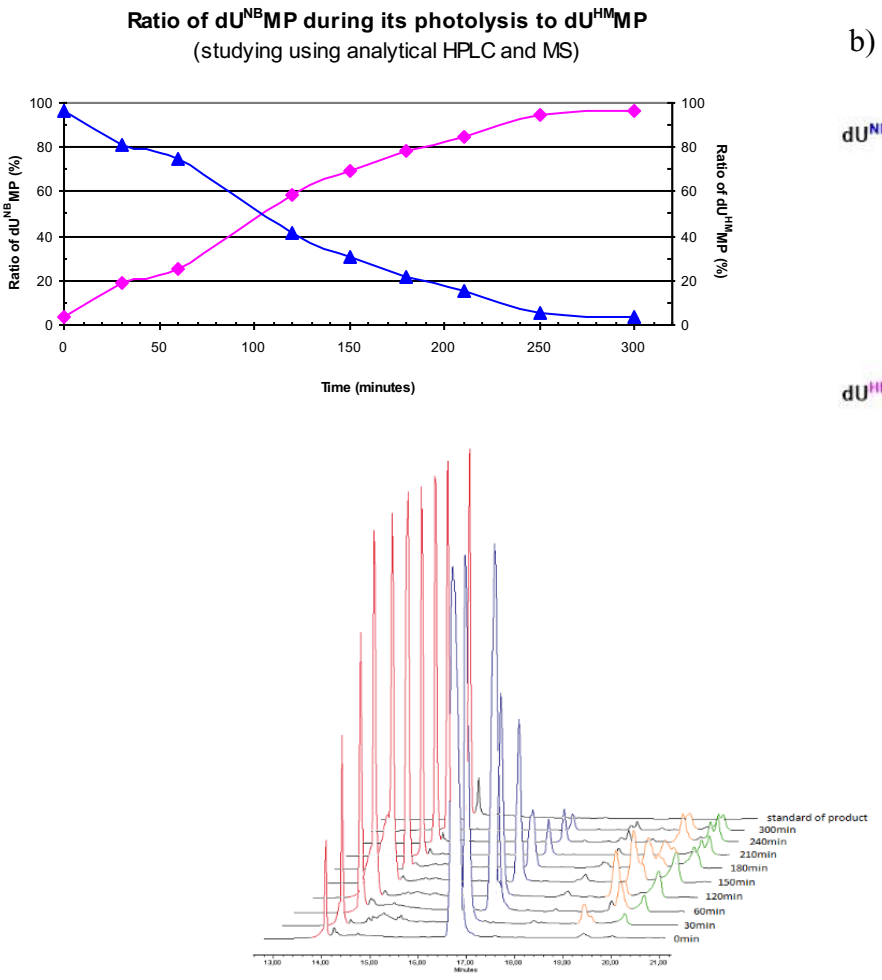
Scheme 30: *Study of* ***dU^{NB}MP*** *photolysis reaction*

Synthesized nitrobenzyl- caged monophosphate **dU^{NB}MP** was used for a kinetic study of deprotection by light. Caged monophosphate was irradiated by UV diode (365 nm, light emitting diode, 1 mW; 1.06 mW/cm^2). The level of deprotection reaction for **dU^{NB}MP** was studied by two different ways. In the first case, photolysis of monophosphate was checked using of analytical HPLC and referent points of starting material-**dU^{NB}MP** and product-**dU^{HM}MP**.

Moreover, the conversion of compound **dU^{NB}MP** to **dU^{HM}MP** after UV exposition was examined using NMR spectroscopy. (***Scheme 30***)

For HPLC analysis of **dU^{NB}MP** photolysis a 3.5 mM solution of **dU^{NB}MP** in water was exposed to light from LED (365 nm, 1 mW; 1.06 mW/cm^2). To study photocleavable behaviour of **dU^{NB}MP**, for each analyse on HPLC, 10 µL of reaction mixture was removed and inject to the analytical HPLC. The analysed solution was removed from stock reaction mixture in different time intervals from 0 to 300 minutes. Retention time of studied compounds in chosen elution system was 17.7 minutes for starting **dU^{NB}MP** and 14.5 minutes for desired **dU^{HM}MP**. Verification of compounds was done by MS. Almost fully (96 %) conversion of starting material **dU^{NB}MP** to desired **dU^{HM}MP** was after the light irradiation for 300 minutes. (***Figure 11***)

a)



b)

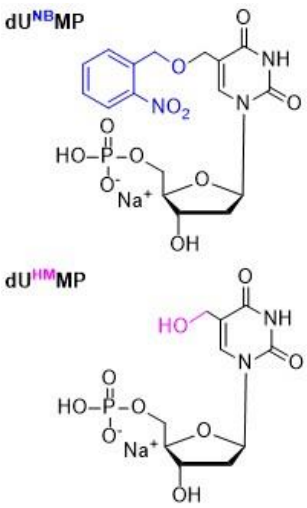


Figure 11**:** *Study of dU^{NB}MP photolysis using analytical HPLC. a) Ratio of disappeared* ***dU^{NB}MP*** *(blue curve) and created product* ***dU^{HM}MP*** *(pink curve). b) Chromatograms of* ***dU^{NB}MP*** *(blue peak) during its photolysis to* ***dU^{HM}MP*** *(red peak) in appropriate time intervals.*

A 16 mM solution of **dU^{NB}MP** in D_2O was prepared for its photolysis kinetic study by NMR. Photocleavable behaviour of **dU^{NB}MP** was detected time intervals of UV irradiation by LED (365 nm, 1 mW; 1.06 mW/cm^2) from 30 minutes to 15 hours. The solution was directly irradiated in Eppendorf vial for definite period and every time of measuring removed to the NMR tube. Percentages of **dU^{NB}MP** disappearance and **dU^{HM}MP** production were obtained after integration of peaks (H-6) from ^{1}H-NMR spectra. The ratio of conversion was reported as the time dependence of the deprotection. Fully conversion of starting **dU^{NB}MP** to **dU^{HM}MP** was observed after 15 hours of irradiation. (***Figure 12***, ***Figure 51***)

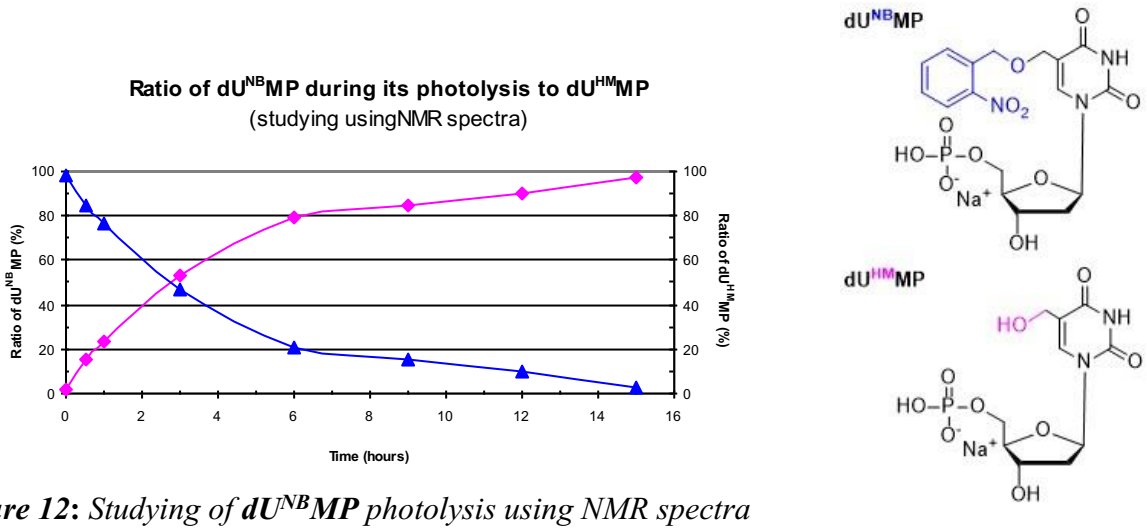
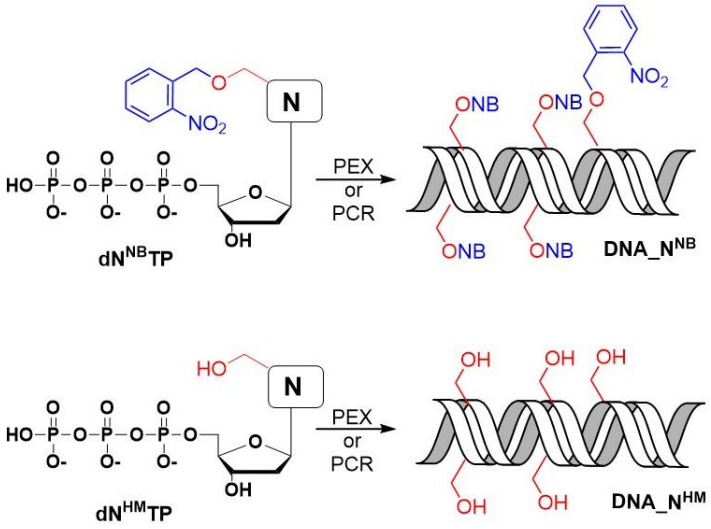


Figure 12*: *Studying of dU^{NB}MP photolysis using NMR spectra

In conclusion, nitrobenzyl- protected nucleosides and nucleotides were prepared largely according to the previously published literature procedures. Some steps of multistep synthesis were modified to reduce the number of steps in the synthesis path, to afford higher yields or more pure products. To have an idea, how the deprotection of NB-caged nucleotide works, study of photocleavage kinetic for **dU^{NB}MP** was used as a model reaction. Photocleavable behaviour of **dU^{NB}MP** was detected either through HPLC analysis or by ^{1}H-NMR analysis. All prepared nucleoside triphosphates were studied as substrates for different DNA polymerases. The caged DNAs were used in photocleavage reactions.

3.2. Enzymatic synthesis of modified DNA

All reported incorporations of hydroxymethylated (**dU^{HM}TP** or **dC^{HM}TP** or **dA^{HM}TP**) and nitrobenzylated (**dU^{NB}TP** or **dC^{NB}TP** or **dA^{NB}TP**) triphosphates by PEX and PCR were performed by me. Some results of this chapter were published in our publications. Incorporation of **dU^{X}TP** into DNA and some of **DNA_U^{X}** screening experiments were included in *Angew. Chem. Int. Ed.*, 2014, **53**, 6734-6737[150]. On the other hand, incorporation of **dC^{X}TP** into DNA and some of restriction endonuclease screening experiments for **DNA_C^{X}** were included in the publication shared with Soňa Boháčová: *Org. Biomol. Chem.*, 2018, **16**, 5427-5432.[151]



Scheme 31: Schematic depiction for enzymatic incorporation of modified nucleotides

3.2.1 Enzymatic incorporation of HM- and NB-modified triphosphates by PEX

The enzymatic synthesis of HM- and NB- modified oligodeoxyribonucleotides (ON^{x}) was studied by primer extension experiment (PEX) using three different DNA polymerases - Vent (exo-), Pwo and KOD XL. Genetically engineered Vent (exo-) and KOD XL (mutant form of KOD polymerase) DNA polymerases are deficient 3′→5′ exonuclease activity. Pwo DNA polymerase was originally isolated from the thermophilic archaebacteria Pyrococcus woesei and possess high level of 3'→5'proofreading activity. The templates and primers for PEX were designed in order to insert one or four modifications to the extended strand of primer (***Table 2***).

oligonucleotide	sequence	Lenght
prim^{Prim248short}	3'-GGGTACGGCGGGTAC-5'	**15-mer**
temp^{Oligo1T}	5'-CCCACCCATGCCGCCCATG-3'	**19-mer**
temp^{Oligo1C}	5'-CCCGCCCATGCCGCCCATG-3'	**19-mer**
temp^{Oligo1A}	5'-CCCTCCCATGCCGCCCATG-3'	**19-mer**
temp^{Prb4basII}	5'-CTAGCATGAGCTCAGTCCCATGCCGCCCATG-3'	**31-mer**

Table 2: *Sequences of oligonucleotides used for the PEX*

In single nucleotide extension experiments, the incorporation of all HM- and NB-modified nucleotides was tested. All hydroxymethyl- modified triphosphates were successfully incorporated into DNA bearing one modification in the presence of all three DNA polymerases. Photolabile nitrobenzyl- modified triphosphates were also found as the good substrates for KOD XL and Vent (exo-) DNA polymerase. In the case of NB- modified oligodeoxyribonucleotides synthesized in the presence of Pwo polymerase and **dC^{NB}TP** or **dA^{NB}TP**, also shorter (n-1) product was observed on PAGE gel. (***Figure 13-15***)

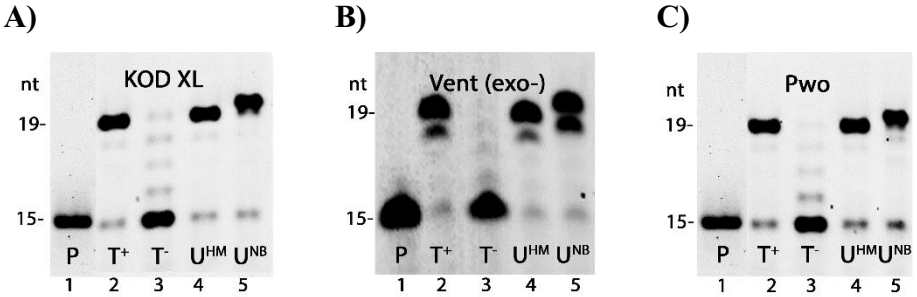


Figure 13**:** *Primer extension experiment for **dU^{HM}TP** and **dU^{NB}TP** with different DNA polymerases: A) KOD XL; B) Vent (exo-); C) Pwo*. Composition of the dNTP mixes and nucleotide labelling are as follows: lanes 1; **P**: primer; lanes 2, **T^{+}**: positive control (product of PEX with natural dTTP and dGTP); lanes 3; **T^{-}**: negative control experiments (absence of natural dTTP); lanes 4; **U^{HM}**: product of PEX with **dU^{HM}TP** + natural dGTP; lanes 5; **U^{NB}**: product of PEX with **dU^{NB}TP** + natural dGTP

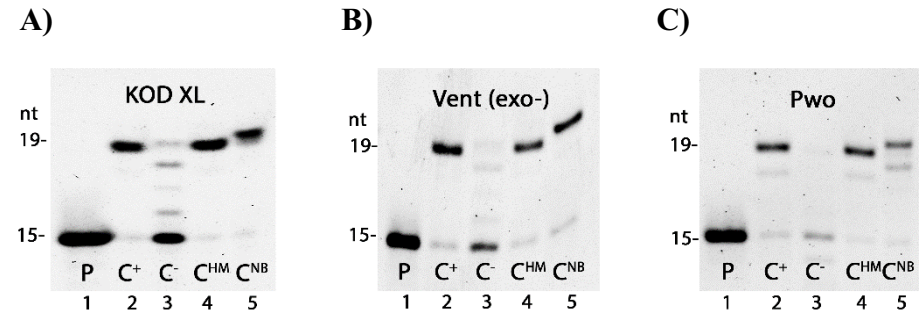


Figure 14**:** *Primer extension experiment for **dC^{HM}TP** and **dC^{NB}TP** with different DNA polymerases: A) KOD XL; B) Vent (exo-); C) Pwo.* Composition of the dNTP mixes and nucleotide labelling are as follows: lanes 1; **P**: primer; lanes 2, **C^{+}**: positive control (product of PEX with natural

dCTP + dGTP); lanes 3; **C**$^{-}$: negative control experiments (absence of natural dCTP); lanes 4; **C**^{HM}: product of PEX with **dC**^{HM}**TP** + natural dGTP; lanes 5; **C**^{NB}: product of PEX with **dC**^{NB}**TP** + natural dGTP

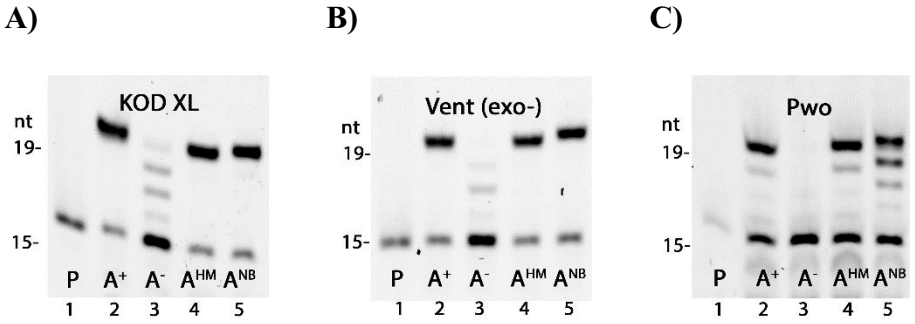


Figure 15**:** *Primer extension experiment for* ***dA^{HM}TP*** *and* ***dA^{NB}TP*** *with different DNA polymerases: A) KOD XL; B) Vent (exo-); C) Pwo.* Composition of the dNTP mixes and nucleotide labelling are as follows: lanes 1; **P**: primer; lanes 2, **A**$^{+}$: positive control (product of PEX with natural dATP + dGTP); lanes 3; **A**$^{-}$: negative control experiments (absence of natural dATP); lanes 4; **A**^{HM}: product of PEX with **dA**^{HM}**TP** + natural dGTP; lanes 5; **A**^{NB}: product of PEX with **dA**^{NB}**TP** + natural dGTP

The hydroxymethyl- and nitrobenzyl- modified triphosphates were also tested for multiple incorporation into DNA, with four modifications. All polymerases gave full-length products. (***Figure 16-18***)

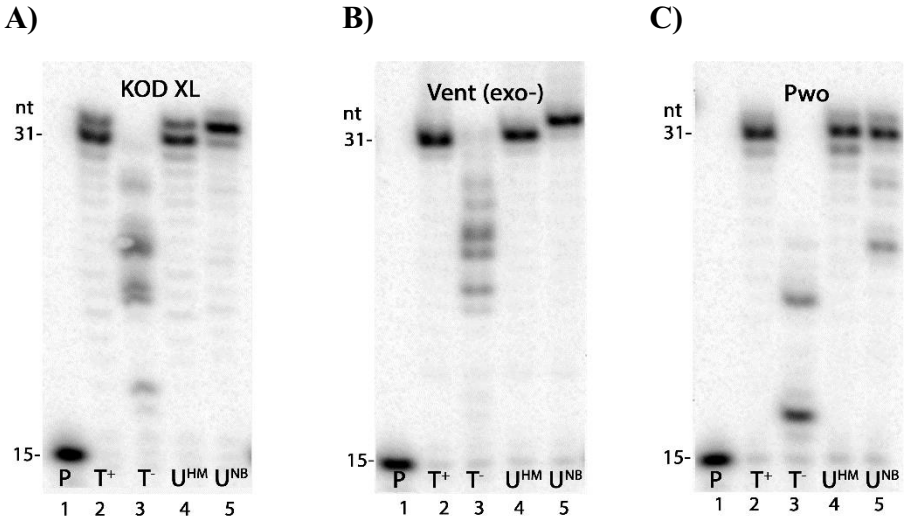


Figure 16**:** *Primer extension experiment for* ***dU^{HM}TP*** *and* ***dU^{NB}TP*** *with different DNA polymerases: A) KOD XL; B) Vent (exo-); C) Pwo.*[150] Composition of the dNTP mixes and nucleotide labelling are as follows: lanes 1; **P**: primer; lanes 2; **T**$^{+}$: positive control (product of PEX with natural dNTPs); lanes 3; **T**$^{-}$: negative control experiments (absence of natural dTTP); lanes 4; **U**^{HM}: product of PEX with **dU**^{HM}**TP** + 3 other natural dNTPs; lanes 5; **U**^{NB}: product of PEX with **dU**^{NB}**TP** + 3 other natural dNTPs

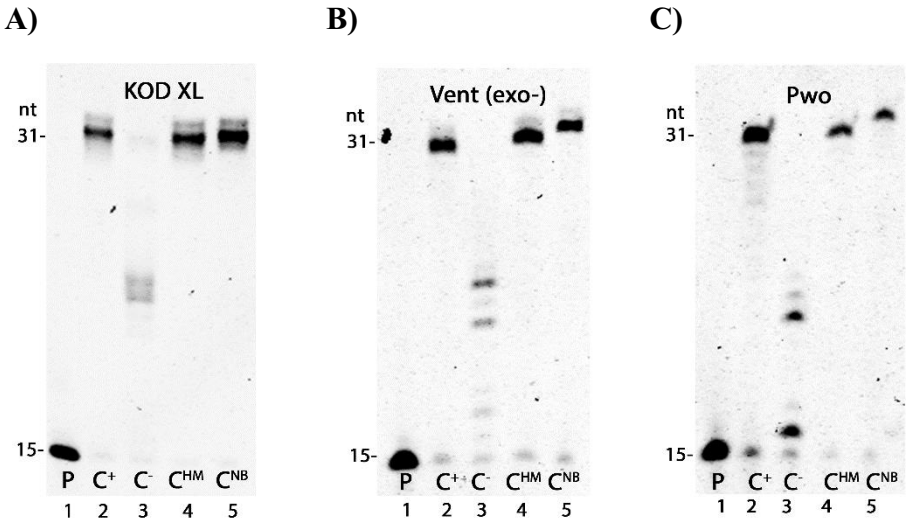


Figure 17*: *Primer extension experiment for dC^{HM}TP and dC^{NB}TP with different DNA polymerases: A) KOD XL; B) Vent (exo-); C) Pwo. Composition of the dNTP mixes and nucleotide labelling are as follows: lanes 1; **P**: primer; lanes 2; **C^{+}**: positive control (product of PEX with natural dNTPs); lanes 3; **C^{-}**: negative control experiments (absence of natural dCTP); lanes 4; **C^{HM}**: product of PEX with **dC^{HM}TP** + 3 other natural dNTPs; lanes 5; **C^{NB}**: product of PEX with **dC^{NB}TP** + 3 other natural dNTPs

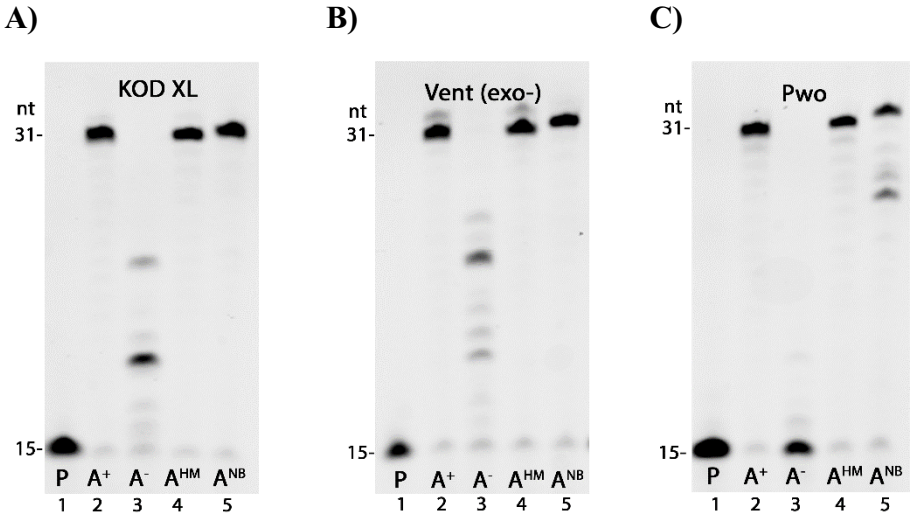


Figure 18*: *Primer extension experiment for dA^{HM}TP and dA^{NB}TP with different DNA polymerases: A) KOD XL; B) Vent (exo-); C) Pwo. Composition of the dNTP mixes and nucleotide labelling are as follows: lanes 1; **P**: primer; lanes 2 **A^{+}**: positive control (product of PEX with natural dNTPs); lanes 3; **A^{-}**: negative control experiments (absence of natural dATP); lanes 4; **A^{HM}**: product of PEX with **dA^{HM}TP** + 3 other natural dNTPs; lanes 5; **A^{NB}**: product of PEX with **dA^{NB}TP** + 3 other natural dNTPs

Nevertheless, in the case of NB- modified oligodeoxyribonucleotides synthesized in the presence of Pwo polymerase and **dN^{NB}TP**, also shorter products were observed on PAGE gels. All tested polymerases were suitable enzymes not only for single incorporation of modified triphosphates but also for their multiple incorporation.

To confirm incorporation of modified triphosphate and exclude a misincorporation of 2´-deoxyribonucleotides, oligonucleotides bearing one modification were prepared by PEX with biotinylated template using KOD XL polymerase. Modified ssDNA was isolated by magnetoseparation and the mass of prepared product was analysed by MALDI-TOF mass-spectrometry. Since NB-protecting group is unstable under the measurement conditions, in all cases the deprotected HM-product was observed as major and NB-modified ssDNA was also detected for NB- oligonucleotides with one modification. Data for single incorporation of modified **dN^{X}TP** are summarized in ***Table 3***.

oligonucleotide	[M] (calc.) (Da)	M (found) (Da)
Oligo1U^{HM}	5981.9	$[M+H]^+$ = 5983.1 Da; Δ = 0.2
Oligo1U^{NB}	6117.0	$[M+H]^+$ = 6118.5 Da; Δ = 0.5; $[M-NB+H]^+$ = 5983.1 Da; Δ = 0.2
Oligo1C^{HM}	5980.9	$[M+H]^+$ = 5981.8 Da; Δ = 0.9
Oligo1C^{NB}	6115.9	$[M+H]^+$ = 6117.8 Da; Δ = 0.9; $[M-NB+H]^+$ = 5982.8 Da; Δ = 0.9
Oligo1A^{HM}	6003.9	$[M+H]^+$ = 6005.7 Da; Δ = 0.8
Oligo1A^{NB}	6138.9	$[M+H]^+$ = 6140.9 Da; Δ = 1; $[M-NB+H]^+$ = 6005.5 Da; Δ = 0.6

Table 3: *MALDI-TOF mass spectrometric data of ONs bearing one photolabile nitrobenzyl-moiety*

3.2.2 Enzymatic incorporation of HM- and NB-modified triphosphates by PCR

In order to clarify, that all HM- and NB- modified triphosphates, are not only good substrates for extension of primer (PEX), but also their incorporation into longer DNA by PCR is possible, the synthesis of modified 98 bp or 287 bp long DNA was performed. The PCR reactions were held in the presence of non-labelled primers, KOD XL DNA polymerase, three natural dNTPs and excess of modified ones. The obtained products were verified by agarose gel analysis.

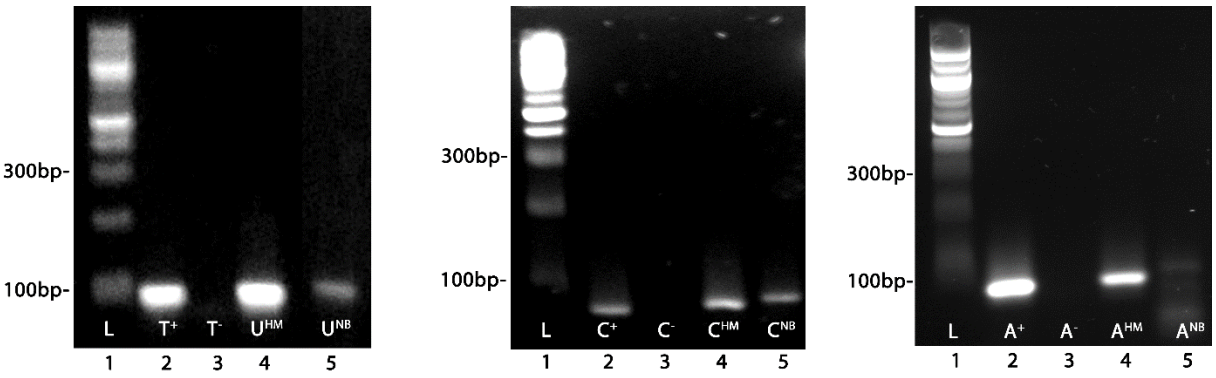
All HM- modified triphosphates were excellent substrates for their incorporation into either 98 bp or 287 bp large DNA. The incorporation of NB- modified nucleoside triphosphates was more problematic. Probably because of difficult ability of reading modified template strand by DNA polymerase, the amount of prepared NB-modified DNA was lower in comparison to natural or HM- modified ones. Moreover, in the case of incorporation **dA^{NB}TP** into 98 bp DNA,

it was not possible to obtain full-length product in the presence of 100% of modified triphosphate. [***Figure 19 a), b)***]

	Sequence	**Lenght**
primers		
LT25TH	3'-TTCCTTATGTCCATAAAACAGGAAC-5'	**25-mer**
L20	3'-CGCTACAGAGAGTACTACAG-5'	**20-mer**
template		
FVL-A	5'-GACATCATGAGAGACATCGCCTCTGGGCTAATAG GACTACTTCTAATCTGTAAGAGCAGATCCCTGGACA GGCAAGGAATACAGGTATTTTGTCCTTG-3'	**98-mer**
primers		
Prim S1-HIV1	5'-GATCACTCTTTGGCAGCGACCCCTCGTCAC-3'	**30-mer**
Prim S2-HIV1	5'-TTAAAGTGCAGCCAATCTGAGTCAACAGAT-3'	**30-mer**
template		
wt-HIV-1PR	5'-CCTCAGATCACTCTTTGGCAGCGACCCCTCGTC ACAATAAAGATAGGGGGGCAATTAAAGGAAGC TCTATTAGATACAGGAGCAGATGATACAGTATT AGAAGAAATGAATTTGCCAGGAAGATGGAAAC CAAAAATGATAGGGGGAATTGGAGGTTTTATCA AAGTAAGACAGTATGATCAGATACTCATAGAAA TCTGCGGACATAAAGCTATAGGTACAGTATTAG TAGGACCTACACCTGTCAACATAATTGGAAGAAA TCTGTTGACTCAGATTGGCTGCACTTTAAATTTT-3'	**287-mer**

Table 4: *Overview of sequences for primers and template used for PCR*

a)



b)

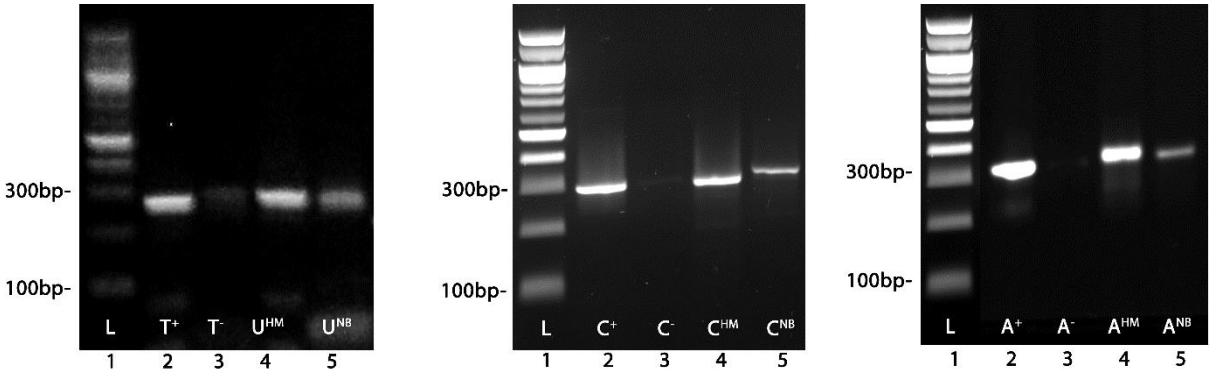


***Figure 19**: Incorporation of HM- and NB- modified triphosphates into a) 98 bp or b) 287 bp long DNA.* Composition of the dNTP mixes and nucleotide labelling are as follows: lanes 1; **P**: primer; lanes 2 $\mathbf{N^{+}}$: positive control (product of PEX with natural dNTPs); lanes 3; $\mathbf{N^{-}}$: negative control experiments (absence of tested natural dNTP); lanes 4; $\mathbf{N^{HM}}$: product of PEX with $\mathbf{dN^{HM}TP}$ + 3 other natural dNTPs; lanes 5; $\mathbf{N^{NB}}$: product of PEX with $\mathbf{dN^{NB}TP}$ + 3 other natural dNTPs.

In order to introduce $\mathbf{dA^{NB}TP}$ into long DNA at least partially, the PCR reaction was run in the presence of different ratios of $\mathbf{dA^{NB}TP}$ to **dATP**. Already in the presence of 90% of $\mathbf{dA^{NB}TP}$, the PCR reaction worked, and the full-length product was determined by agarose gel. (***Figure 20***)

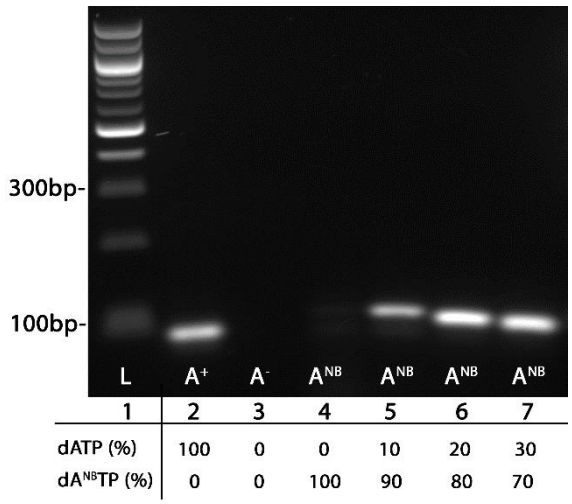


***Figure 20**: Partial incorporation of* $\mathbf{dA^{NB}TP}$ *into 98bp DNA.* Composition of the dNTP mixes and nucleotide labelling are as follows: lane 1; **P**: primer; lane 2; $\mathbf{A^{+}}$: positive control (product of PEX with natural dNTPs); lane 3; $\mathbf{A^{-}}$: negative control experiment (absence of natural dATP); lane 4; $\mathbf{A^{NB}}$: product of PEX with 100% of $\mathbf{dA^{NB}TP}$ + 3 other natural dNTPs; lane 5; $\mathbf{A^{NB}}$: product of PEX with 90% of $\mathbf{dA^{NB}TP}$, 10% of **dATP** + 3 other natural dNTPs; lane 6; $\mathbf{A^{NB}}$: product of PEX with 80% of $\mathbf{dA^{NB}TP}$, 20% of **dATP** + 3 other natural dNTP; lane 7; $\mathbf{A^{NB}}$: product of PEX with 70% of $\mathbf{dA^{NB}TP}$, 30% of **dATP** + 3 other natural dNTPs.

The PCR reaction for $\mathbf{dU^{HM}TP}$ and $\mathbf{dU^{NB}TP}$ was performed also using Pwo and Vent(exo-) polymerase. The incorporation of $\mathbf{dU^{HM}TP}$ to the 287 bp DNA worked reasonably well with Vent(exo-) and weaker with Pwo polymerase. The bulkier $\mathbf{dU^{NB}TP}$ was not tolerated by Pwo

polymerase at all and Vent(exo-) polymerase gave a very little quantity of full-length product. (***Figure 21***)

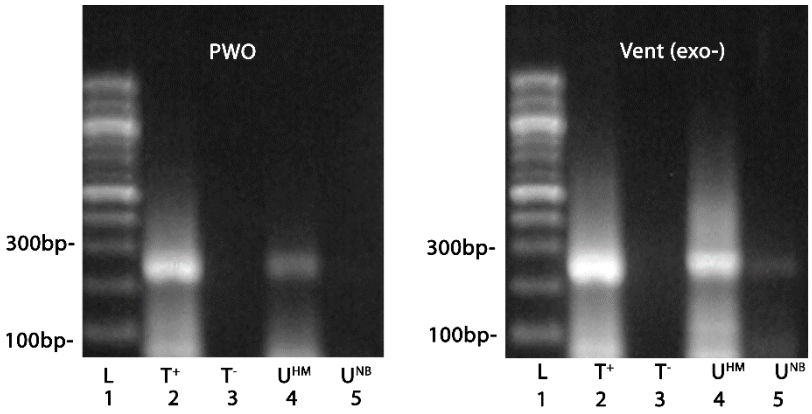
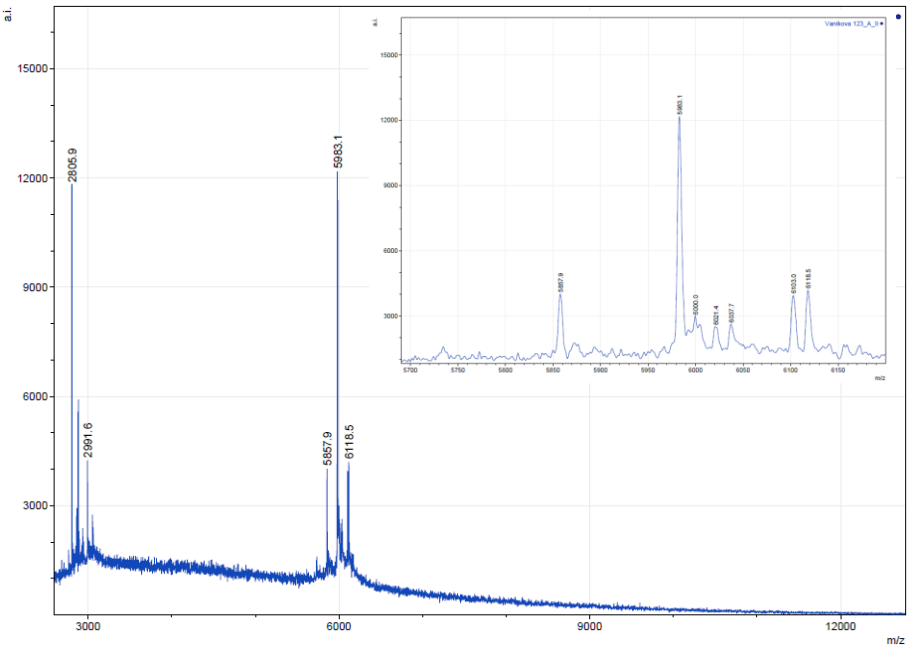


Figure 21*: Incorporation of HM- and NB- modified triphosphates into 287 bp long DNA by a) Pwo or b) Vent(exo-) polymerase.* Composition of the dNTP mixes and nucleotide labelling are as follows: lanes 1; **P**: primer; lanes 2; $\mathbf{T^{+}}$: positive control (product of PEX with natural dNTPs); lanes 3; $\mathbf{T^{-}}$: negative control experiments (absence of natural dTTP); lanes 4; $\mathbf{U^{HM}}$: product of PEX with $\mathbf{dU^{HM}TP}$ + 3 other natural dNTPs; lanes 5; $\mathbf{U^{NB}}$: product of PEX with $\mathbf{dU^{NB}TP}$ + 3 other natural dNTPs

3.2.3 MALDI-TOF analysis of deprotection for NB-modified DNA

A model experiment of deprotection study nitrobenzyl- caged ssDNA by light was performed. Caged ssDNA **Oligo1U^{NB}** with one NB-modification in the sequence was prepared by magnetoseparation in two portions under standard conditions. After magnetoseparation, one portion of caged DNA was directly analysed by MALDI-TOF mass-spectrometry and second portion of ssDNA contained $\mathbf{U^{NB}}$ in the sequence was exposed to UV irradiation at 365 nm (light emitting diode, 1.0 mW; 1.06 mW/cm^2) for 24 hours with occasional stirring. The sample just after UV exposition was also analysed by MALDI-TOF mass spectrometry. In the case of NB- caged ssDNA the mass of NB- modified ssDNA was determined together with deprotected HM- product. After the irradiation of NB- modified sample, the peak of caged product (*m/z* 6118 Da) completely disappeared and only the mass of fully deprotected hydroxymethyl-modified oligo (*m/z* 5983 Da) was observed in the spectra. (***Figure 22***)

a)



b)

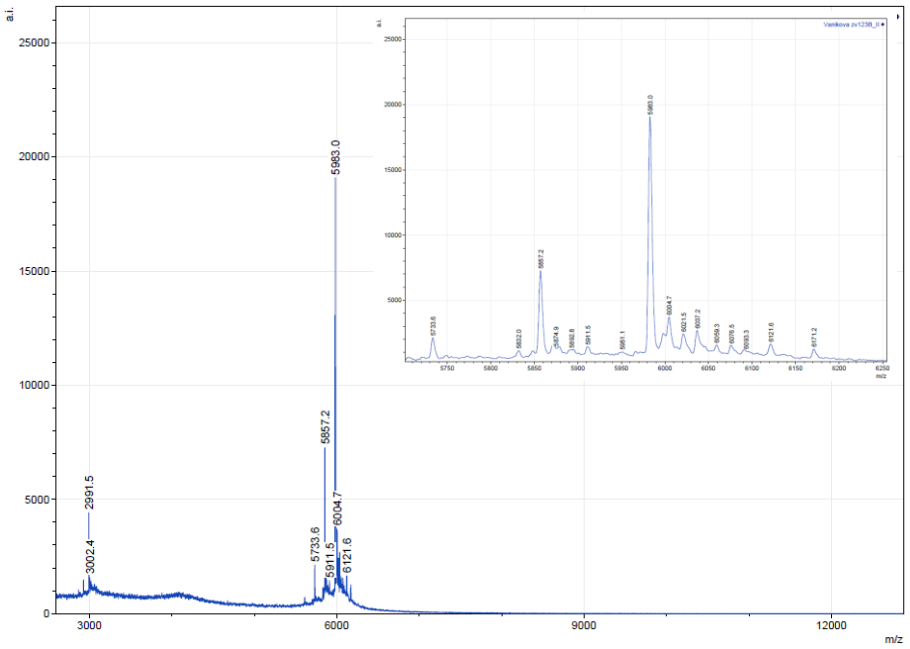
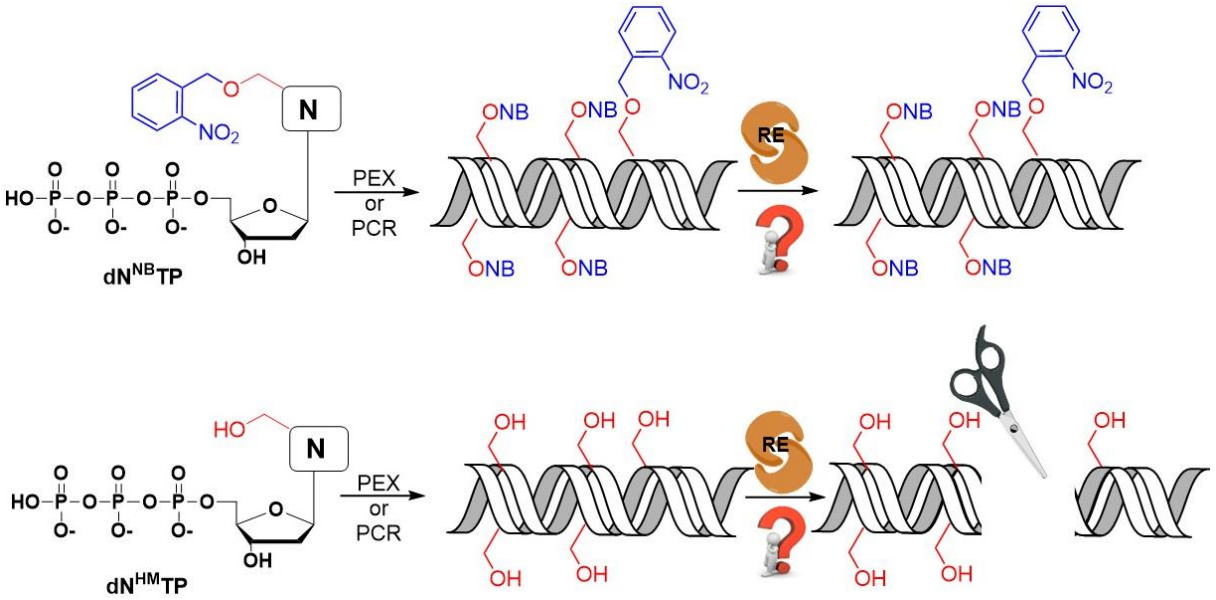


Figure 22: *a) MALDI-TOF spectra of U^{NB}- modified ssDNA*: calculated mass: [M] = 6117.0 Da; found mass: $[M+H]^+$ = 6118.5 Da; Δ = 0.5; $[M-NB+H]^+$ = 5983.1 Da; Δ = 0.2. *b) MALDI-TOF spectra of U^{NB}- modified ssDNA after irradiation by UV light (U^{HM}- modified ssDNA)*: calculated mass: [M] = 5981.9 Da; found mass: $[M+H]^+$ = 5983.0 Da; Δ = 0.1.

3.2.4 Study of cleavage modified DNA with REs

The whole set of HM- and NB- modified triphosphates was used in PEX with Pwo polymerase, which was shown as quite good for efficient incorporation of all modified triphosphates. Based on the previous results of cleavage studies (*section 1.3.2*), we expected, that bulky nitrobenzyl-modified DNA (**DNA_N^{NB}**) would not be cleaved by restriction endonucleases and on the other

hand, small hydroxymethyl modifications would be tolerated by restriction endonucleases and **DNA_N^{HM}** would be successfully cleaved. (***Scheme 32***)



Scheme 32: Schematic representation of expected DNA_N^{X} cleavage by REs

Restriction enzyme	Template	Sequence of template[a, b]
prim^{Prim248short}	-	3'-GGGTACGGCGGGTAC-5'
AflII	**temp$^{AflT\ (c)}$**	5'-TTCGTCGTC**CTTAAG**CCCATGCCGCCCATG-3'
	temp^{AflC}	5'-AACTACTAC**CTTAAG**CCCATGCCGCCCATG-3'
	temp^{AflA}	5'-AACGACGAC**CTTAAG**CCCATGCCGCCCATG-3'
EcoRI	**temp^{EcT}**	5'-TTCGTCGTC**GAATTC**CCCATGCCGCCCATG-3'
	temp^{EcC}	5'-AACTACTAC**GAATTC**CCCATGCCGCCCATG-3'
	temp^{EcA}	5'-AACGACGAC**GAATTC**CCCATGCCGCCCATG-3'
KpnI	**temp$^{KpT\ (c)}$**	5'-TTCGTCGTC**GGTACC**CCCATGCCGCCCATG-3'
	temp^{KpC}	5'-AACTACTAC**GGTACC**CCCATGCCGCCCATG-3'
	temp^{KpA}	5'-AACGACGAC**GGTACC**CCCATGCCGCCCATG-3'
PvuI	**temp$^{PvT\ (c)}$**	5'-TTCGTCGTC**CAGCTG**CCCATGCCGCCCATG-3'
	temp^{PvC}	5'-AACTACTAC**CAGCTG**CCCATGCCGCCCATG-3'
	temp^{PvA}	5'-AACGACGAC**CAGCTG**CCCATGCCGCCCATG-3'
RsaI	**temp$^{RsT\ (c)}$**	5'-TTCGTCGTCG**GTAC**GCCCATGCCGCCCATG-3'
	temp^{RsC}	5'-AACTACTACT**GTAC**ACCCATGCCGCCCATG-3'
	temp^{RsA}	5'-AACGACGACG**GTAC**GCCCATGCCGCCCATG-3'

	temp^{ScT}	5‘-TTCGTCGTC**A**<u>G</u>**T**<u>A</u>**CT**CCCATGCCGCCCATG-3‘
ScaI	**temp^{ScC}**	5‘-AACTACTAC**A**<u>G</u>**TACT**CCCATGCCGCCCATG-3‘
	temp^{ScA}	5‘-AACGACGAC**AG**<u>T</u>**AC**<u>T</u>CCCATGCCGCCCATG-3‘

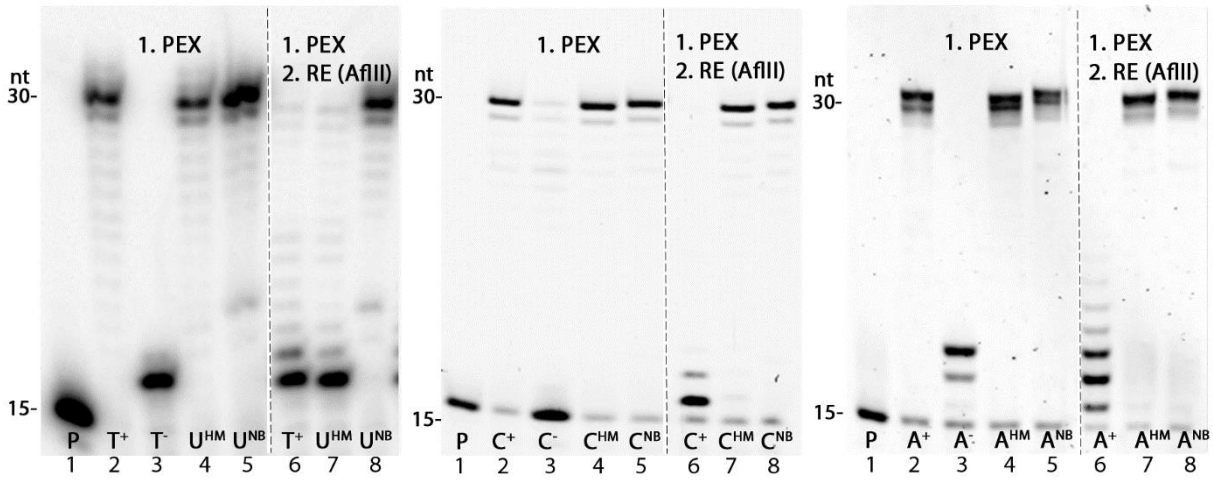
[a] position of modified nucleobases in product strand is underlined
[b] specific sequence for restriction endonuclease is bold
[c] primer is labelled at 5´-end with ^{32}P

Table 5: *Overview of sequences of templates used for cleavage studies*

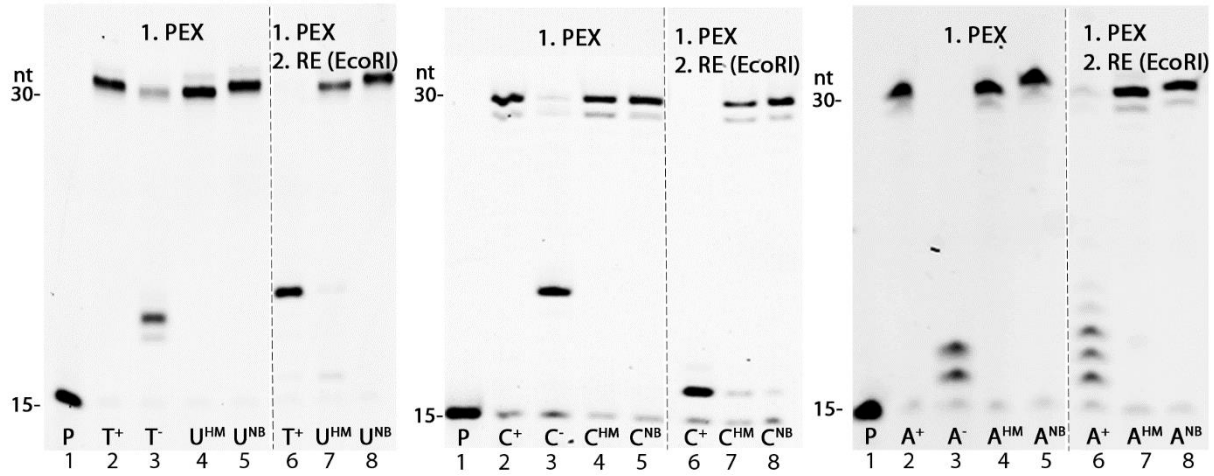
DNA templates were selected according the (mainly) positive results from previous cleavage studies (***Table 1;*** *section 1.3.2*). The sequences of DNA templates for cleavage study were designed in order to contain the modified nucleotide within the recognition sequence for one from six REs (AflII, EcoRI, KpnI, PvuII, RsaI and HF-ScaI). (***Table 5***) In all cases, the PEX proceeded very well giving fully extended products that were analyzed by denaturing PAGE. All products of PEX reaction (either natural DNA or HM-modified DNA or NB-modified DNA) were in the next step exposed to the cleavage reaction with appropriate RE. (***Figure 23***)

It was found out, that any DNA in which palindromic recognition sequence contained nitrobenzyl-group was not recognized by restriction enzyme and NB- modified DNA was not cleaved. Small hydroxymethyl- modification, introduced into DNA either with **dU^{HM}TP** or **dC^{HM}TP** or **dA^{HM}TP** was tolerated and DNA was fully cleaved with KpnI and RsaI. Moreover, fully cleavage of HM-modified DNA, which contained hydroxymetyl moiety on uridine (U^{HM}) in recognition sequence for AflII and PvuI, was also observeed. The partial cleavage (<25%) was noted for HM-modified DNA generated by PEX for **Templ^{ScC}** template. As for the most of modified DNAs with recognition sequence for EcoRI (***Table 1;*** *section 1.3.2*), also HM- or NB-modified DNA were fully inhibited against cleavage by EcoRI.

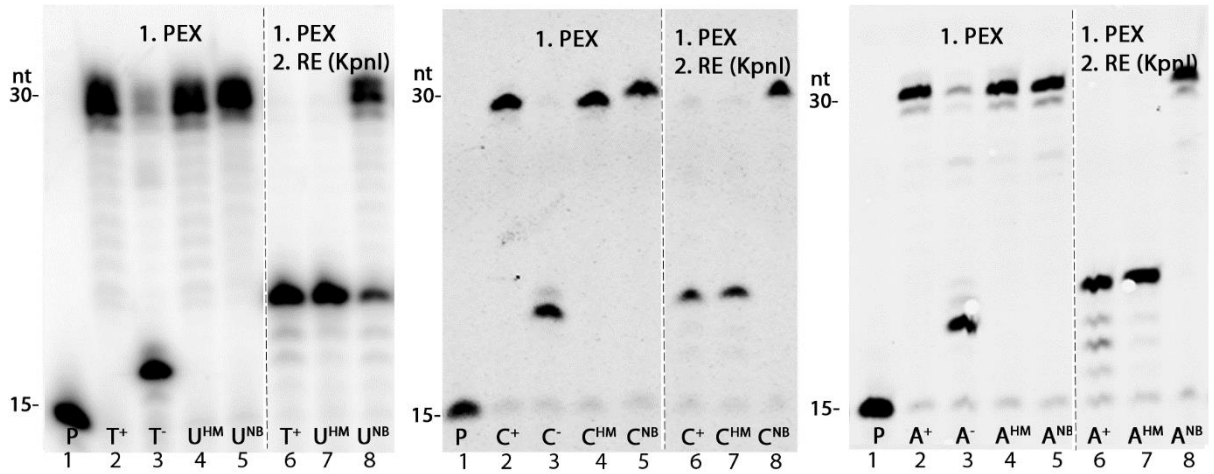
a)



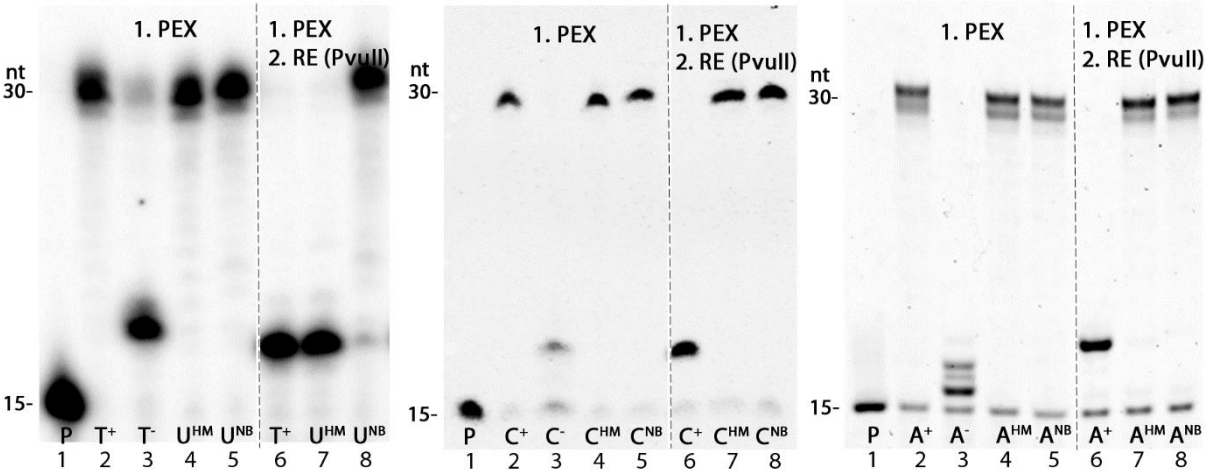
b)


1. PEX
1. PEX
2. RE (EcoRI)
nt
30-
15-
P T+ T- UHM UNB T+ UHM UNB
1 2 3 4 5 6 7 8
1. PEX
1. PEX
2. RE (EcoRI)
nt
30-
15-
P C+ C- CHM CNB C+ CHM CNB
1 2 3 4 5 6 7 8
1. PEX
1. PEX
2. RE (EcoRI)
nt
30-
15-
P A+ A- AHM ANB A+ AHM ANB
1 2 3 4 5 6 7 8

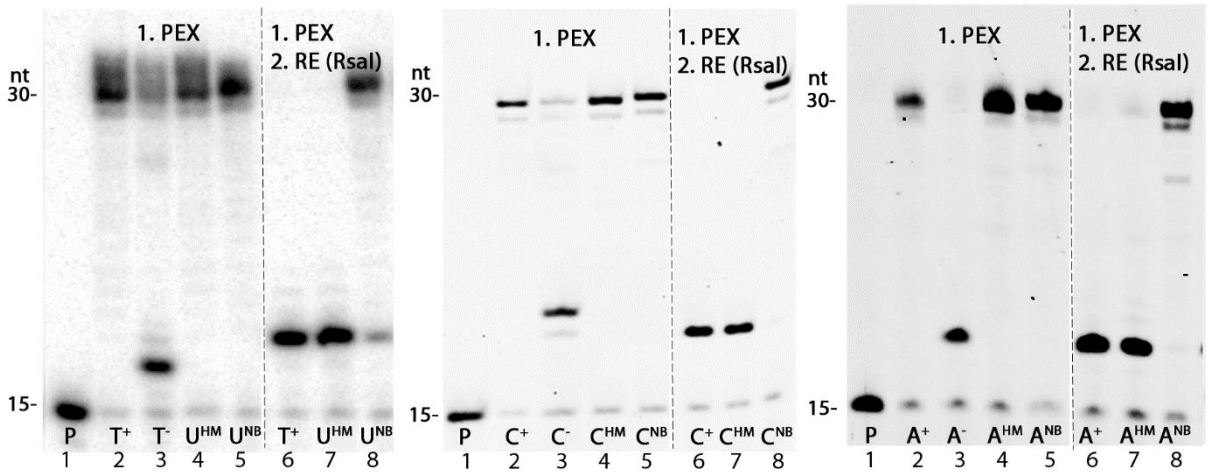
c)


1. PEX
1. PEX
2. RE (KpnI)
nt
30-
15-
P T+ T- UHM UNB T+ UHM UNB
1 2 3 4 5 6 7 8
1. PEX
1. PEX
2. RE (KpnI)
nt
30-
15-
P C+ C- CHM CNB C+ CHM CNB
1 2 3 4 5 6 7 8
1. PEX
1. PEX
2. RE (KpnI)
nt
30-
15-
P A+ A- AHM ANB A+ AHM ANB
1 2 3 4 5 6 7 8

d)


1. PEX
1. PEX
2. RE (PvuII)
nt
30-
15-
P T+ T- UHM UNB T+ UHM UNB
1 2 3 4 5 6 7 8
1. PEX
1. PEX
2. RE (PvuII)
nt
30-
15-
P C+ C- CHM CNB C+ CHM CNB
1 2 3 4 5 6 7 8
1. PEX
1. PEX
2. RE (PvuII)
nt
30-
15-
P A+ A- AHM ANB A+ AHM ANB
1 2 3 4 5 6 7 8

e)



f)

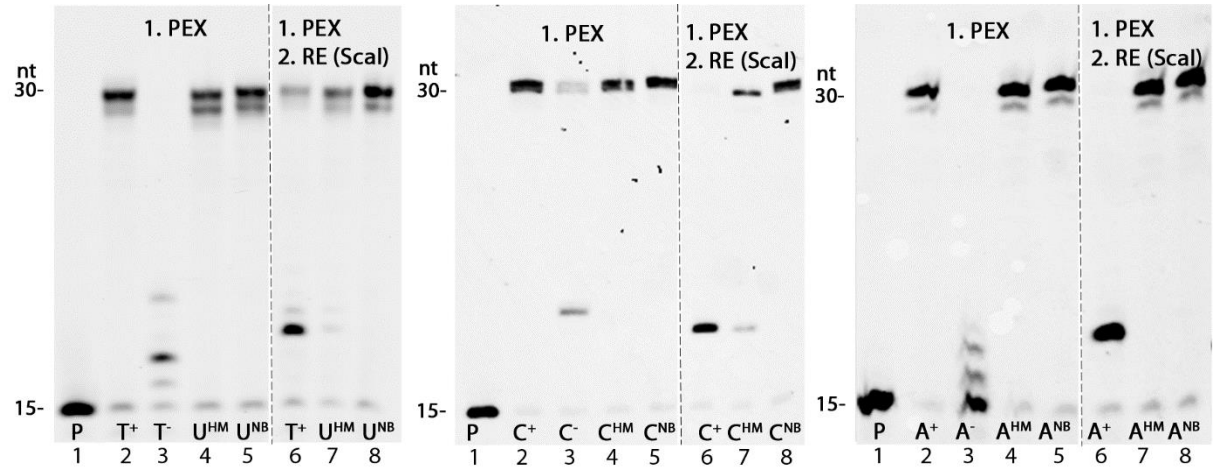
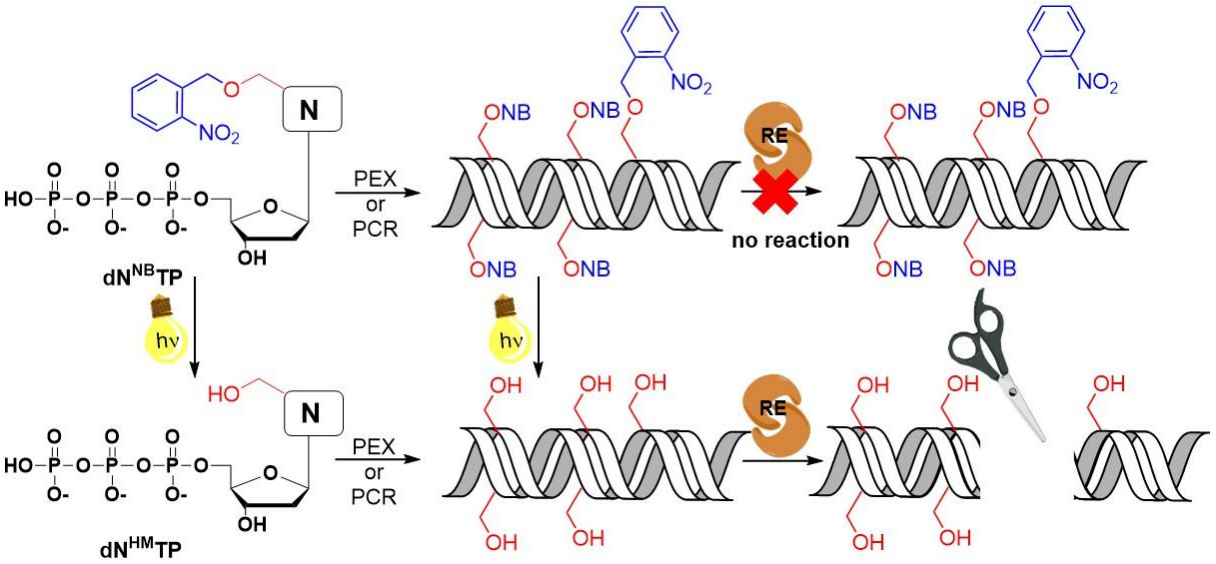


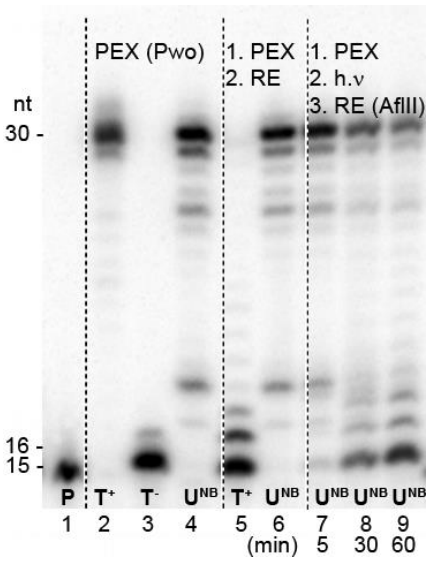
Figure 23*: Study of cleavage HM- and NB-modified DNA with restriction endonucleases: a) AflII or b) EcoRI or c)KpnI or d)PvuII or e)RsaI or f) ScaI.* Composition of the dNTP mixes and nucleotide labelling are as follows: lanes 1, **P**: primer; lanes 2, $\mathbf{N^{+}}$: positive control experiments (product of PEX with natural dNTPs); lanes 3, $\mathbf{N^{-}}$: negative control experiments (absence of examined natural dNTP); lanes 4, $\mathbf{N^{HM}}$: product of PEX with $\mathbf{dN^{HM}TP}$ + 3 other natural dNTPs; lanes 5, $\mathbf{N^{NB}}$: product of PEX with $\mathbf{dN^{NB}TP}$ + 3 other natural dNTPs; lanes 6, $\mathbf{N^{+}}$: natural DNA followed by cleavage with a RE (AflII /EcoRI/ KpnI/ PvuII/ RsaI/ ScaI); lanes 7, $\mathbf{N^{HM}}$: $\mathbf{DNA\_N^{HM}}$ after incubation with appropriate RE; lanes 8, $\mathbf{N^{NB}}$: $\mathbf{DNA\_N^{NB}}$ after incubation with appropriate RE.



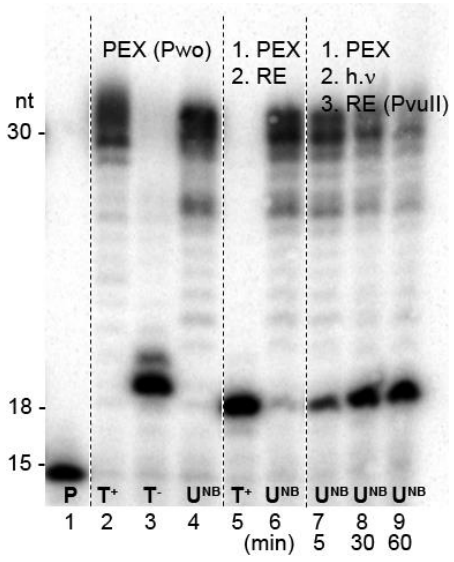
***Scheme 33**: Schematic depiction of photodeprotection and RE cleavage experiments for modified DNA*

In the case of HM- modified DNA (**DNA_N^{HM}**), where the fully cleavage of **DNA_N^{HM}** was observed (AfT/ PvT /KpT/ RsT /KpC /RsC/ KpA/ RsA), the kinetic study of deprotection for modified DNA with bulkier nitrobenzyl- groups (**DNA_N^{NB}**) was performed. Photocleavage of nitrobenzyl- moiety on **DNA_N^{NB}** was proceeded in time intervals 5/ 30/ 60 minutes. In the most cases (except DNA modified for **temp^{KpC}**), the fully cleavage (and therefore fully deprotection) of NB-modified DNA was not observed after its irradiation for 60 minutes. For all **DNA_N^{NB}** prepared by PEX reaction with **temp^{KpN}** (either in the presence of **dU^{NB}TP** or **dC^{NB}TP** or **dA^{NB}TP**) and subsequently irradiated by LED (365 nm, 1 mW) the cleavage of dsDNA with KpnI was close to 100%.

a)



b)



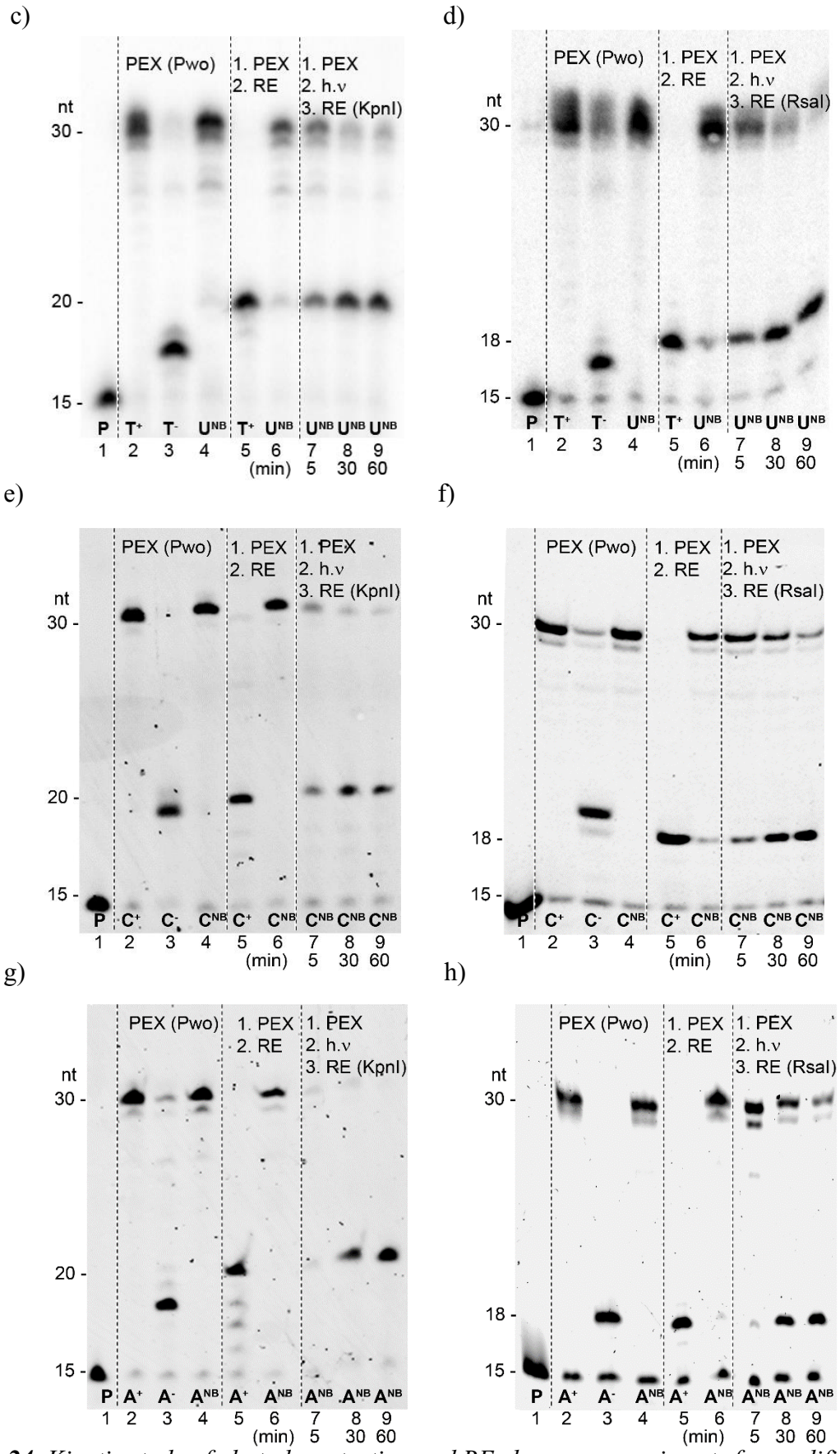
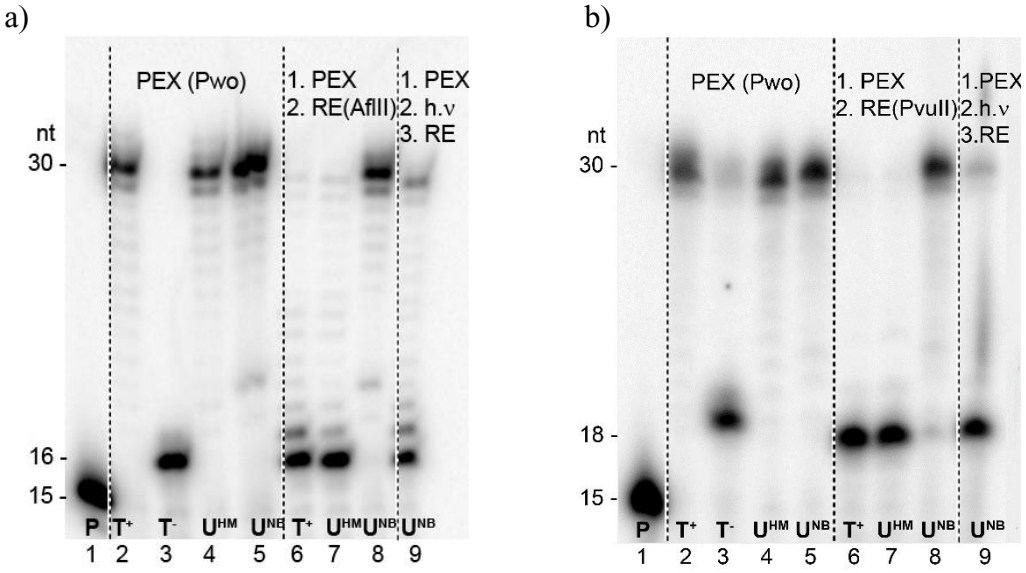


Figure 24: *Kinetic study of photodeprotection and RE cleavage experiments for modified DNA synthesized in the presence of templates: a)* ***temp^{AflT}****, b)* ***temp^{PvT}****, c)* ***temp^{KpT}****, d)* ***temp^{RsT}****,*

e) ***temp^{KpC}****, f)* ***temp^{RsC}****, g)* ***temp^{KpA}****, h)* ***temp^{RsA}***. Composition of the dNTP mixes and nucleotide labelling are as follows: lanes 1, **P**: primer; lanes 2, **T^{+}**: positive control experiments (product of PEX with natural dNTPs); lanes 3, **T^{-}**: negative control experiments (absence of examined natural dNTP); lanes 4, **N^{NB}**: product of PEX with **dN^{NB}TP** + 3 other natural dNTPs; lanes 5, **T^{+}**: natural DNA followed by cleavage with a RE (AflII / PvuII/ KpnI/ RsaI); lanes 6, **N^{NB}**: **DNA_N^{NB}** cleaved with a RE (no cleavage); lanes 7, **N^{NB}**: **DNA_N^{NB}** irradiated for 5 minutes and subsequent cleavaged with appropriate RE; lanes 8, **N^{NB}** : **DNA_N^{NB}** irradiated for 30 minutes and subsequently cleaved with appropriate RE; lanes 9, **N^{NB}** : **DNA_N^{NB}** irradiated fir 60 minutes and subsequently cleaved with appropriate RE.

In order to reach the fully cleavage of NB-modified DNA, the **DNA_N^{NB}** was further exposed irradiation by light (365 nm, 1 mW) for longer time intervals. Almost for all NB-modified DNAs (except **RsA^{NB}**) it was possible to obtain fully deprotection (and therefore fully cleavage by REs) of photocaged DNA after its two hours exposition by light. [***Figure 25 c), d), g)***] Three hours irradiation of **DNA_C^{NB}** was needed to obtain fully cleavage with RsaI. [***Figure 25 f)***] Fully cleavage with PvuII for **DNA_U^{NB}** required four hours irradiation. [***Figure 25 b)***] The **DNA_U^{NB}** with specific sequence for cleavage by AflII, was exposed to UV for six hours. The longer time of deprotection was needed, probably because of two nitrobenzyl- moieties next to cleavage site. [***Figure 25 a)***] Although **DNA_C^{NB}** with specific sequence for cleavage by KpnI contained also two **C^{NB}** nucleobases directly inside the sequence of cleavage, the fully releasing of NB- moiety was achieved after just one-hour irradiation. (***Figure 25 e)***] Although, the both DNA templates (**DNA_C^{NB}, DNA_U^{NB}**) contain two NB- modifications, the orientation of NB-moiety is very likely to be different, and in the case of **DNA_U^{NB}** it seems to be more hidden.



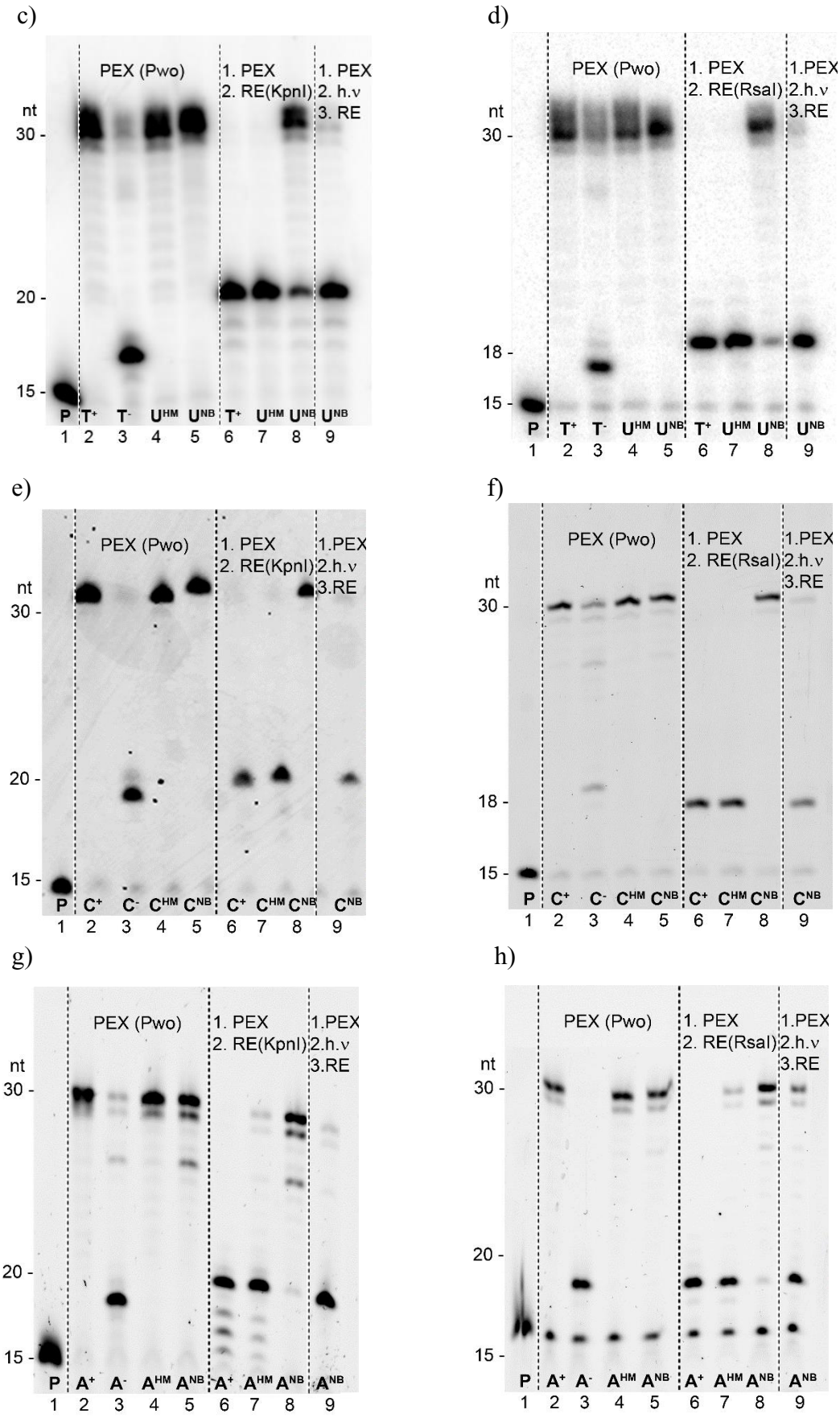


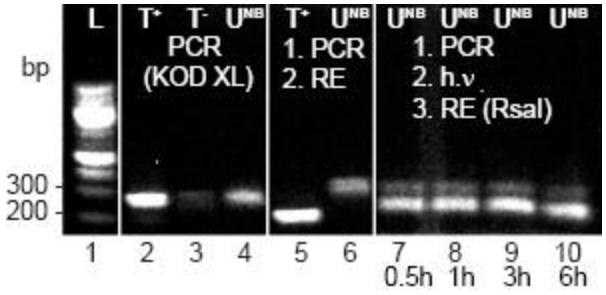
Figure 25: *Overview of PAGE analyses of PEX (Pwo polymerase), deprotection (UV light) and RE cleavage experiments for modified DNA synthesized in the presence of templates:*

a) ***temp^{AflT}***, *b)* ***temp^{PvT}***, *c)* ***temp^{KpT}***, *d)* ***temp^{RsT}***, *e)* ***temp^{KpC}***, *f)* ***temp^{RsC}***, *g)* ***temp^{KpA}***, *h)* ***temp^{RsA}***. Composition of the dNTP mixes and nucleotide labelling are as follows: lanes 1, **P**: primer; lanes 2, **T^{+}**: positive control experiments (product of PEX with natural dNTPs); lanes 3, **T^{-}**: negative control experiments (absence of examined natural dNTP); lanes 4, **N^{HM}**: product of PEX with **dN^{HM}TP** + 3 other natural dNTPs; lanes 5, **N^{NB}**: product of PEX with **dN^{NB}TP** + 3 other natural dNTPs; lanes 6, **T^{+}**: natural DNA followed by cleavage with RE (AflII / PvuII/ KpnI/ RsaI); lanes 7, **N^{HM}** : **DNA_N^{HM}** cleaved by RE (full cleavage); lanes 8, **N^{NB}**: **DNA_N^{NB}** cleaved by RE (no cleavage); lanes 9, **N^{NB}**: photodeprotected **DNA_N^{NB}** cleaved by appropriate RE.

3.2.4.1 Study of cleavage modified PCR products with RsaI

As obvious from the previous study, the bulky NB- modifications in the case of dsDNA modified in one strand (prepared by PEX) with restriction site for RsaI fully blocked the cleavage. The DNAs with small HM- modifications were fully cleaved by RsaI. To verify that the same trend could be observed also in the case of hydroxymethylated or nitrobenzylated DNA modified in both strands, a 287-mer containing recognition sequence for RsaI (5'-GT/AC-3') was synthesized in the presence of **dU^{HM}TP** or **dU^{NB}TP**. The treatment of these DNA constructs with RsaI confirmed the previous observation that bulky **U^{NB}** bases completely inhibit DNA cleavage by RE. (***Figure 26***) Kinetic study of deprotection **U^{NB}** –modified PCR product was further performed in different time intervals (0.5h or 1h or 3h or 6h). At first, the photolysis was checked by treatment with RsaI, where an increasing of cleaved product was determined with time by agarose gel analysis. The clean product of cleavage (214-mer) was determined after previous irradiation of **DNA_N^{NB}** for six hours.

a)



b)

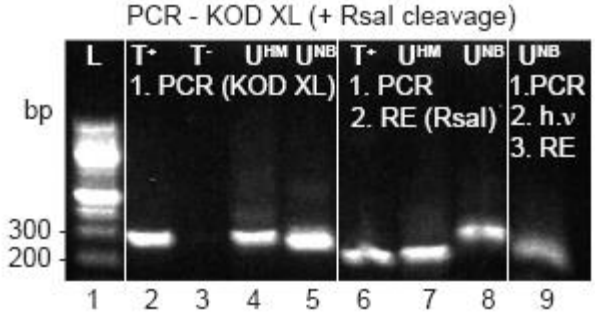


Figure 26: ***a)*** *Agarose gel analysis for kinetic study of photodeprotection of NB-modified DNA:* Composition of the dNTP mixes are as follows: lane 1, **L**: ladder; lane 2, **T^{+}**: natural DNA; lane 3, **T^{-}**: negative control (PCR run in absence of natural dTTP); lane 4, **U^{NB}**: PCR product for **dU^{NB}TP** + 3 other

natural dNTPs; lane 5, **T**$^{+}$: natural DNA cleaved by RsaI; lane 6, **U**^{NB} : NB-modified DNA cleaved by RsaI (no cleavage); lane 7, **U**^{NB}: partially photodeprotected (0.5 hour, 365 nm) NB-modified DNA cleaved by RsaI (partial cleavage); lane 8, **U**^{NB}: partially photodeprotected (1 hour, 365 nm) NB-modified DNA cleaved by RsaI (partial cleavage); lane 9, **U**^{NB}: partially photodeprotected (3 hours, 365 nm) NB-modified DNA cleaved by RsaI (partial cleavage); lane 10, **U**^{NB}: photodeprotected (6 hours, 365 nm) NB-modified DNA cleaved by RsaI (almost full cleavage). ***b)*** *Agarose gel analysis of RE (RsaI) cleavage experiments with 287-mer HM-/ NB- modified DNA:* Lane 1, **L**: DNA ladder; lane 2, **T**$^{+}$: natural DNA; lane 3, **T**$^{-}$: negative control of PCR (product of PCR with dATP, dCTP, dGTP); lane 4, **U**^{HM}: product of PCR with dATP, dCTP, dGTP and **dU**^{HM}**TP**; lane 5, **U**^{NB}: product of PCR synthesized in the presence of dATP, dCTP, dGTP, **dU**^{NB}**TP**; lane 6, **T**$^{+}$: the natural PCR product treated with RsaI; lane 7, **U**^{HM}: the **U**^{HM}-modified PCR product treated with RsaI (full cleavage); lane 8, **U**^{NB}: the **U**^{NB}-modified DNA treated with RsaI (no cleavage); lane 9, **U**^{NB}: photodeprotected (6 hours, 365 nm) **U**^{NB}-modified PCR product treated with RsaI (full cleavage).

Modified PCR products, products of photolysis and products of cleavage with RsaI were used as templates for PCR in the presence of natural dNTPs. The products of rePCR reactions were sequenced and all sequencing results confirmed a fidelity and a specificity of the replication of modified DNA.

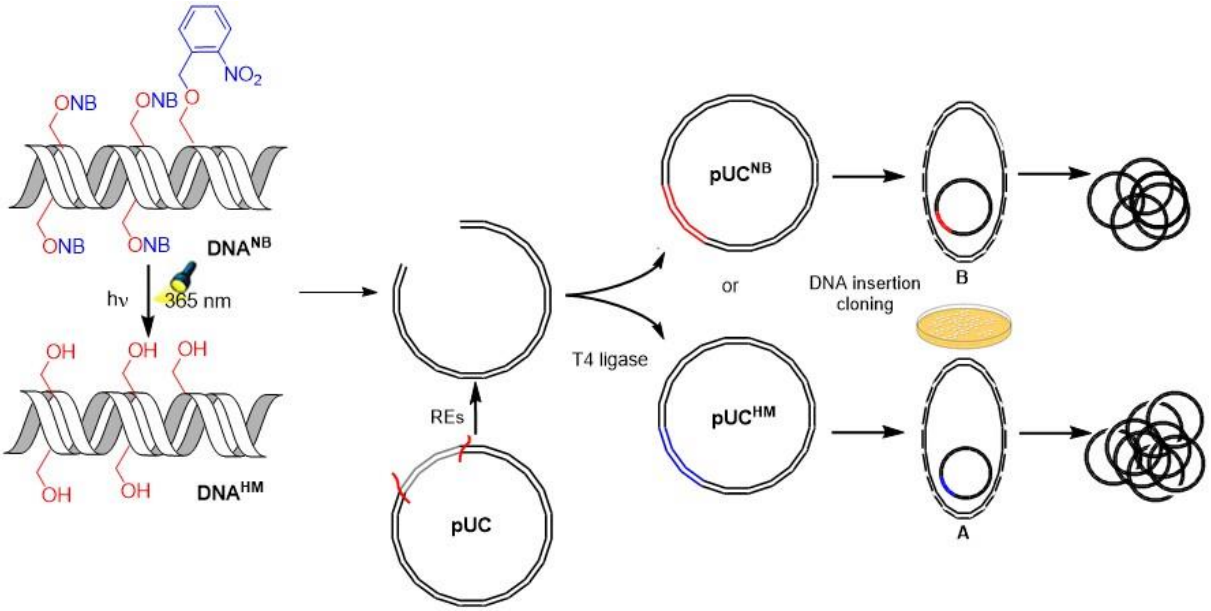
3.2.4.2 Cloning and transfection study for modified pUC plasmids into E. coli

oligonucleotides	**Sequence**	**lenght**
primers		
Prim_L22_XmaI	5‘-GTGTAGGGCC/CAATAGGACTAA-3‘	22-mer
Prim_L19_SfoI	5‘-CTGTAGCCG/CGGTATGTCG-3‘	19-mer
template		
Temp_99_AfeI	5‘-GACATCGGCGCCATACAGCCTCTGGCTAATAGGA CTACTTCTAATCTGTAAGAGCAGATCC/TTAAGCCTGG ACAGGCAATCAGGATAACCCGGGATGTG-3‘	99-mer

Table 6: *Overview of used primers and template sequences*

A 99-bp **U**^{NB}- modified PCR product, with restriction site for AfeI in the middle of the sequence, was prepared by enzymatic reaction. The part of purified NB-modified 99-mer DNA was photodeprotected by UV lamp. The synthesized **U**^{NB} – modified dsDNA, irradiated **U**^{NB} – modified product and unmodified (natural) pUC plasmid were digested by restriction endonucleases XmaI and SfoI. Purified cleaved DNAs, **U**^{NB} – modified dsDNA and dsDNA after photodeprotection were cloned to plasmids (**pUC**^{NB} and **pUC**^{HM}) and plasmids were then transfected to *E. coli*. The replicated plasmids **A** (**pUC**^{HM} was replicated) and **B** (**pUC**^{NB} was replicated) were isolated from transfected colonies. Isolated plasmids with restriction site for AfeI in the middle were cleaved with AfeI [***Figure 27b)***] As it was expected, the isolated

replicated plasmids (**A**, **B**) were successfully cleaved by AfeI restriction endonuclease. Natural plasmid (**N**) isolated from control experiment was not cleaved since it didn't contain the cleavage site for AfeI. In addition, the sequences of both isolated artificial plasmids (**A**, **B**) were checked by Sanger sequencing and experiments confirmed the fidelity of incorporation and absence of DNA damage due to UV irradiation.



Scheme 34: *Simplified schema for transfection of artificial NB- or HM- modified plasmids into E.coli*

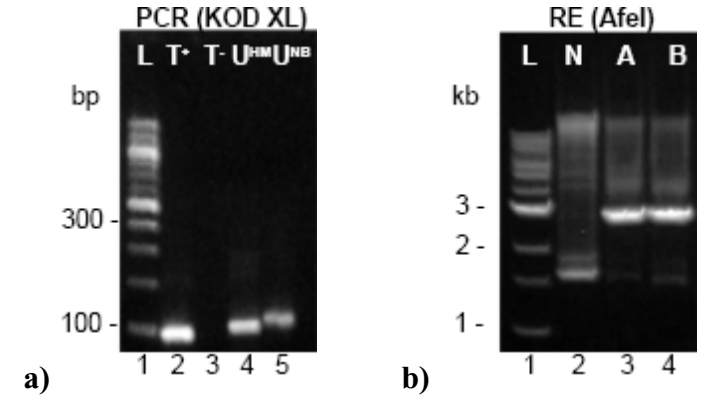


Figure 27**:** *a) Polymerase chain reaction of 99-mer for $dU^{HM}TP$ and $dU^{NB}TP$ with KOD XL DNA polymerase.* Composition of the dNTP mixes and nucleotide labelling are as follows: lane 1, **L**: ladder; lane 2, $\mathbf{T^+}$: positive control (PCR product with natural dNTPs); lane 3, $\mathbf{T^-}$: negative control experiment (absence of natural dTTP); lane 4, $\mathbf{U^{HM}}$: 99-mer product of PCR with $\mathbf{dU^{HM}TP}$ + 3 other natural dNTPs, lane 5, $\mathbf{U^{NB}}$: 99-mer product of PCR with $\mathbf{dU^{NB}TP}$ + 3 other natural dNTPs. *b) Cleavage of natural (2686 bp) and modified (2591 bp) plasmid DNA (pUC) by AfeI to verify the presence of artificial PCR products in the sequence* -. Composition of the dNTP mixes: lane 1, **L**: ladder; lane 2, **N:** negative control (natural plasmid DNA – pUC cleaved by AfeI –no cleavage); lane 3, **A**: amplified plasmid pUC cleaved by AfeI; lane 4, **B**: amplified plasmid pUC cleaved by AfeI (PCR product was before ligation irradiated by UV)

In conclusion, all prepared triphosphates were used as substrates for their single and multi- incorporation into DNA by PEX in the presence of three different DNA polymerases (KOD XL, Vent (exo-), Pwo). Moreover, all modified triphosphates were also successfully incorporated into both strands of dsDNA by PCR and KOD XL polymerase was able to read and amplified modified dsDNA depending on the DNA sequence. The photocleavage studies of NB-modified DNA was monitored by cleavage with restriction endonucleases chosen according the results from the previous cleavage studies (*section 1.3.2*). The systematic study of cleavage by restriction endonucleases elucidate, that **U^{HM}-**modified DNA is the most tolerated by tested REs from all tested hydroxymethyl-modifications (from the point of quantity of REs -AflII, KpnI, PvuII and RsaI). In all cases, The KpnI and RsaI accept HM-modifications in one strand of dsDNA synthesized by PEX. In the cases where fully cleavage of the **DNA_N^{HM}** was observed, the **DNA_N^{NB}** was exposed to irradiation by light (365 nm, 1 mW) for different time intervals. In all cases the fully cleavage of irradiated **DNA_N^{NB}** was reached after its previous irradiation within few hours. The results of our study are summarized in the ***Table 7.***

Enzyme, (3′→5′) sequence	**AflII** G/AATTC	**EcoRI** CTTAA/G′	**KpnI** C/CATGG	**PvuII** GTC/GAC	**RsaI** CA/TG	**ScaI** TCA/TGA
U^{HM}	**+**	**-**	**+**	**+**	**+**	**-**
U^{NB}	**+^{6}**	**-**	**+^{2}**	**+^{2}**	**+^{3}**	**-**
C^{HM}	**-**	**-**	**+**	**-**	**+**	**±**
C^{NB}	**-**	**-**	**+^{1}**	**-**	**+^{3}**	**-**
A^{HM}	**-**	**-**	**+**	**-**	**+**	**-**
A^{NB}	**-**	**-**	**+^{2}**	**-**	**+^{2}**	**-**

+^{X} *– NB-modified DNA cleaved by restriction enzyme after previous irradiation of DNA by UV lamp during X hours*

Table 7: *Outline of results for cleavage with restriction endonucleases*

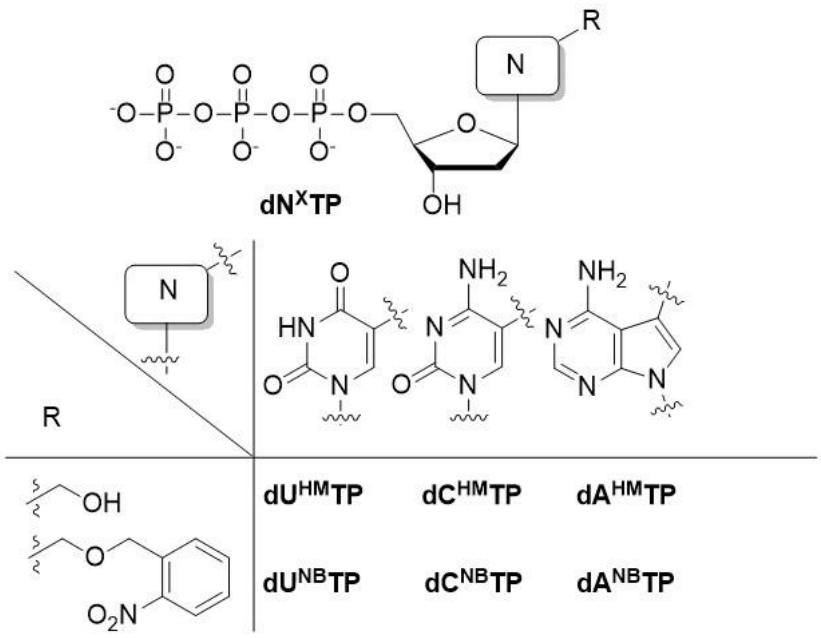
The agarose gel analysis, cleavage studies and sequencing of the prepared DNAs modified in both strands confirmed that fully photocaged **U^{NB}**-modified DNAs are replicable by KOD XL DNA polymerase but are fully protected against cleavage by REs. Simple photolysis of NB-moieties from DNA leads to formation of nucleic acid with small HM-modifications which are tolerated and cleaved by some endonucleases. In addition, the plasmids with **U^{NB}**- and **U^{HM}**-moieties are efficiently replicated in *E. coli*.

3.3. Study of transcription with modified DNA templates

3.3.1 Effect of 5-(hydroxymethyl)-modified and 5-(nitrobenzyloxymethyl)-modified DNA on transcription

The results of our detailed study of HM- and NB- modified DNA cleavage by restriction endonucleases[150, 151] (*section 3.2.3*), the fact that epigenetic nucleobases play critical role in the regulation of gene expression[22, 29, 95] and knowledge that the bulkiness of chemical modifications in major-groove of DNA influence a transcription process[99], all these facts moved our investigation to the more than obvious step of study **DNA_N^{X}**-protein interaction in the level of transcription.

Fully modified DNAs synthesized in the presence of **dU^{NB}TP /dU^{HM}TP** or **dC^{NB}TP /dC^{HM}TP** or **dA^{NB}T /dA^{HM}TP (*Scheme 35*)** were used as templates in *in vitro* transcription assay by RNAP from *E.coli*.



Scheme 35*: *Structures of examined triphosphates

The HM-modified DNA templates displayed a higher level of transcription in comparison to NB-modified DNA templates, which showed strong inhibitory effect on transcription. Moreover, in the case of **U^{HM}**- and **C^{HM}**- modified DNA templates, the significant enhancement of transcription by a factor 3.5 or 2.3 to the natural DNA template was determined.

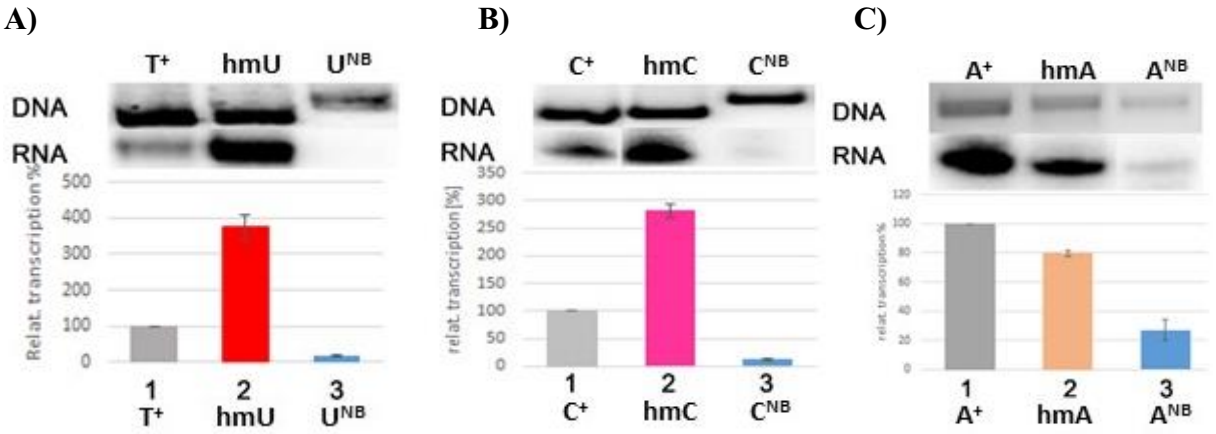


Figure 28: *Relative transcription of modified dsDNA templates* ***A)*** *U^{HM}- and U^{NB}- modified DNA;* ***B)*** *C^{HM}- and C^{NB}- modified DNA;* ***C)*** *A^{HM}- and A^{NB}- modified DNA*

The expected results of the study on one hand and surprisingly high level of transcription for 5-(hydroxymethyl)pyrimidines modified DNA templates on other hand paved the exciting pathway of our further investigation.

3.3.2 Influence of epigenetic hmU, hmC, dU and mC modifications on transcription with bacterial RNA polymerase[154]

The effect of four epigenetic pyrimidine nucleotides on transcription with bacterial RNA polymerase was studied in collaboration with Libor Krásný group from Department of Molecular Genetics of Bacteria, Institute of Microbiology of the Czech Academy of Sciences. Zuzana Vaníková drawn up the strategies for synthesis of partially modified DNA, synthesized $dU^{HM}TP$ and prepared the fully modified DNA templates. Fabrizia Nici synthesized a major part of partially modified DNAs (options A-C) and Soňa Boháčová synthesized partially modified DNAs in promoter region of template strand (option D). Transcription of all modified DNA templates was performed by Martina Janoušková from Libor Krásný group. [154]

Natural DNA templates as a control and fully or partially modified DNA templates containing epigenetic pyrimidine modifications (**dU**, **hmU**, **mC**, **hmC**; ***Figure 29***) were synthesized by the sequence of different enzymatic reactions. In the next step, all synthesized DNAs were used as templates for an *in vitro* transcription studies with bacterial RNA polymerase. [***Figure 29 a), b)***]

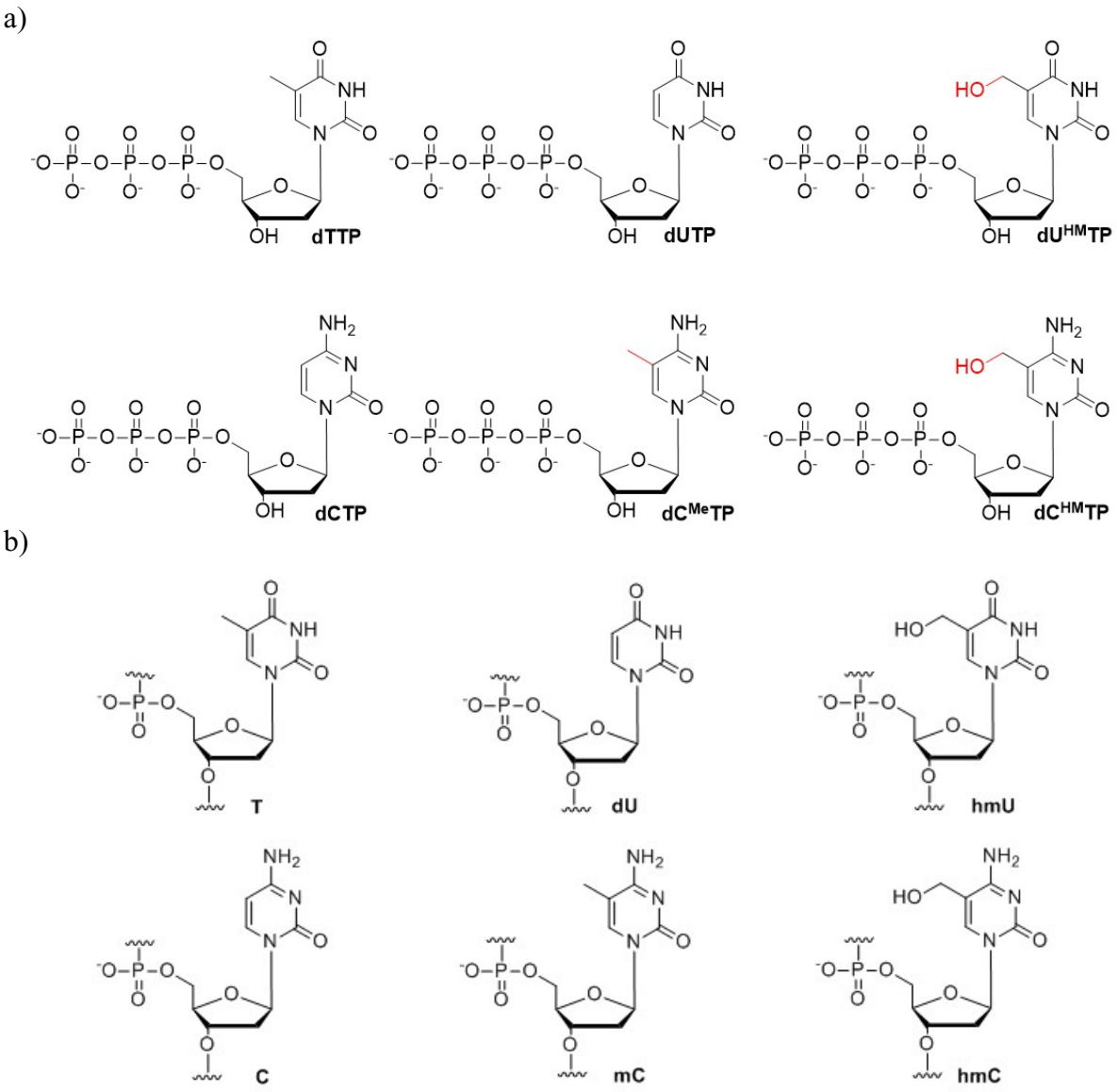


***Figure 29**: a) Structures of examined triphosphates used for preparation of DNA templates and their abbreviations for gels with DNA templates, b) Structures of used nucleotides in DNA and their abbreviations for gels with transcript*

As obvious from the previous results of transcription (*section 3.3.1*), DNA templates with hydroxymethyl moiety on pyrimidine nucleobase with P*veg* promoter increase the amount of transcript in comparison to transcript of natural DNA. To clarify, whether the effect of $\mathbf{U^{HM}}$ and $\mathbf{C^{HM}}$ is general also for other types of promoter we decided to synthesize four kinds of modified DNA templates with different sequence of promoter region and an identical transcribed region. (***Table 8***) All fully modified DNA templates with different promoter region were prepared by PCR. Promoters regions *Pveg* and *rrnB P1* are from Bacillus subtilis and well-recognised by *E. coli* RNAP holoenzyme with primary sigma factor - σ^{70}.[100] The other two promoters are

reciprocal chimeras of *Pveg* and *rrnB P1*, which were designed to identify the modified region of promoter with the most significant influence on transcription.

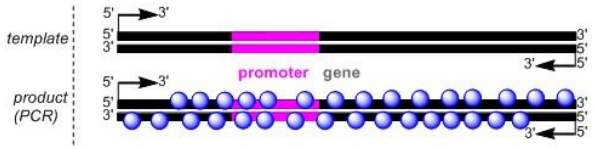
Oligonucleotide-PRIMERs	**Sequence (5′→3′)**	**Length**
***Prim*^{FOR}**	TAGGGGTTCCGCGCACATTTCCCCG	**25-mer**
***Prim*^{REV}**	GGAGAGCGTTCACCGACAAACAACAG	**26-mer**
***Prim*^{FOR} – ^{32}*P*^{a}**	TAGGGGTTCCGCGCACATTTCCCCG	**25-mer**
***Prim*^{REV} – ^{32}*P*^{a}**	GGAGAGCGTTCACCGACAAACAACAG	**26-mer**
Oligonucleotide-TEMPLATEs[b,c]	TAGGGGTTCCGCGCACATTTCCCCGAAAAGTGC CACCTGACGTCTAAGAAACCATTATTATCATGA CATTAACCTATAAAAATAGGCGTATCACGAGGC CCTTTCGTCTTCAAGAATTC*NNNNNNNNNNNNN* *NNNNNNNNNNNNNNNNNNNNN(N)*GTCTAAGC TTGGGTCCCACCTGACCCCATGCCGAACTCAGAA GTGAAACGCCGTAGCGCCGATGGTAGTGTGGGG TCTCCCCATGCGAGAGTAGGGAACTGCCAGGCA TCAAATAAAACGAAAGGCTCAGTCGAAAGACTG GGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAAC GCTCTCC	**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

Table 8: *List of oligonucleotides used for synthesis of 339- and 340-mers*

In fully modified 339-mer or 340-mer DNA templates, natural T or C bases were replaced with non-canonical ones, either **dU**, **hmU** (**U**^{HM}), **mC** (**C**^{Me}) or **hmC** (**C**^{HM}). Since the presence of modifications in DNA templates can influence the extinction coefficients (determination with NanoDrop) and intercalation of fluorescent DNA dyes and reproducibility of results for

quantification of *FAM*-labelled DNA[99] are limited, modified ^{32}P - labelled (using radioactively labelled primers) and *NON*-labelled DNA templates were prepared by PCR to verify the concentration of modified DNAs. Prepared ^{32}P-labelled and *NON*-labelled DNA templates were purified and diluted to the apparent final concentration of 30 ng/μL according to NanoDrop Spectrophotometer. 180 ng of ^{32}P-labelled DNA templates were loaded on a 5 % PAA gel with detection by radioactivity and *NON*-labelled templates were analysed on 1.3 % agarose gel with detection by GelRed. After comparison of the intensity of the DNA template spots on the gels, using either ^{32}P-radioactivity detection or detection by GelRed staining, we found out that concentration of fully modified DNA templates measured by radioactivity reasonably match to relative DNA amounts of *NON*-labelled DNA spots on gels stained by GelRed. For our next investigation, we used either the radioactive labelling or staining by GelRed and fluorescence detection to determine the relative concentrations of the modified and natural DNA templates. (***Table 8***)



Scheme 36: *Synthesis of fully modified templates 339-mers(340-mers)*

At first, fully modified DNA templates were synthesized in the presence of **dUTP** or **dU^{HM}TP** or **dC^{Me}TP** or **dC^{HM}TP** by PCR reaction. KOD XL DNA polymerase was used for the synthesis of **hmC** or **hmU** or **mC** modified DNA templates. **dU** modified DNA templates were synthesized in the presence of Taq DNA polymerase. Purified DNA templates were used in an *in vitro* transcription assay with bacterial RNAP. As expected from previous studies, fully 5-(hydroxymethyl)-modified DNA templates with P*veg* promoter region showed enhancing effect on transcription. The 2.3 times more transcript was determined for DNA template with **hmC** in the sequence and the **hmU**- modified DNA template was transcribed with factor 3.5 in comparison to natural DNA. On the other hand, the presence of **dU** in the sequence of DNA template almost fully inhibited the transcription and the template with **mC** was transcribed with the same efficiency as non-modified DNA, what corresponded to previous studies.[99] The presence of any tested modification (**dU**, **U^{HM}**, **C^{HM}** or **C^{Me}**) in the sequence with *rrnB P1* promoter region inhibits transcription markedly. In order to define which part of promoter region have more impact on transcription process, the DNAs with reciprocal promoter were designed and tested. Modified DNA templates (**hmU**, **mC** or **hmC**) with hybrid sequences of promoter region (P*veg*-10DBP1 and *rrnB* P1-10Dveg) showed the same or slightly lower level

of transcript in comparison to natural DNA. The results of transcription indicate that none "half" of promoter has a major impact in transcription process and both parts of the promoter region are required to acquire observed high level of transcript of hydroxymethylated DNA.

The similar low level of transcript was observed just for **dU**- modified DNA templates with all four types of promoter. (***Figure 30)***

a) b)

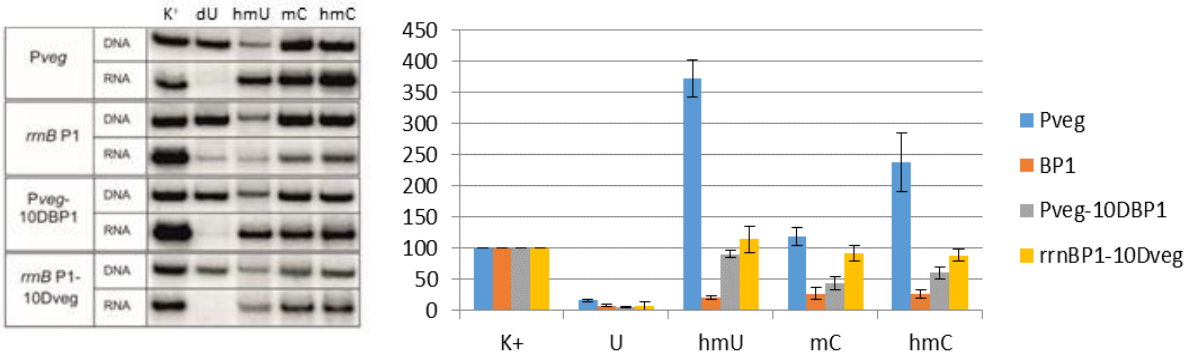


Figure 30: *a) PAA gels of the ^{32}P- labelled DNA templates and RNA transcripts with different promoter region. b) Quantitation of transcriptions from DNA templates:* The labelling is as follows: **K**$^{+}$: transcript of natural DNA templates - set as 100 %.; **dU**: transcript of dU modified DNA templates; **hmU**: transcript of hmU modified DNA templates; **mC**: transcript of mC modified DNA templates; **hmC**: transcript of hmC modified DNA templates. The error bars are ±SD.

To determined which part of modified DNA template with *Pveg* promoter region has major impact on transcription and is responsible for the enhancing effect on transcription of **DNA_C(U)^{HM}** templates, series of partially modified DNA templates were synthesized. Modified 235-mers were systematically prepared in multistep synthesis and tested in transcription assay by *E.coli* RNAP. Four series of partially modified DNA templates were synthesized, in which either transcribed part of DNA template (A) or promoter region in both strands (B) or promoter region of template (C) or non-template strand (D) contained one from four tested epigenetic nucleobases (**dU**, **U^{HM}**, **C^{HM}** or **C^{Me}**). The partially modified DNA templates were prepared enzymatically by a combination of PCR, primer extension, digestion and ligation.

A) ***Synthesis of DNA templates modified in the both strands of transcribed (gene) region***

To prepare ***dsDNA modified in the transcribed region***, a natural 69-mer DNA (promoter region) and modified 166-mer DNAs (representing the promoter-downstream part) were synthesized by PCR. The natural 69-mer DNA was prepared in the presence of a 5´-*FAM*

labelled forward primer, a 5´-*phosphate* modified reverse primer and natural dNTPs. Modified 166-mer DNAs were synthesized in the presence of a 5´-*FAM* labelled reverse primer, a 5´-*phosphate* modified forward primer, 3 natural dNTPs and epigenetic $dN^{X}TP$. The 5´-*phosphate* ends of final DNA fragments allowed ligation of only the desired ends of DNA and vice versa the 5´-*FAM* labelled ends prevented undesirable ligations. To get desired DNA products modified in the promoter-downstream part, the natural 69 bp and modified 166 bp DNAs (modified by either **dU** or **hmU** or **mC** or **hmC**) were ligated by T4 DNA Ligase under the conditions described in supporting information [***Figure 31a)***]**.**

a) ***b)***

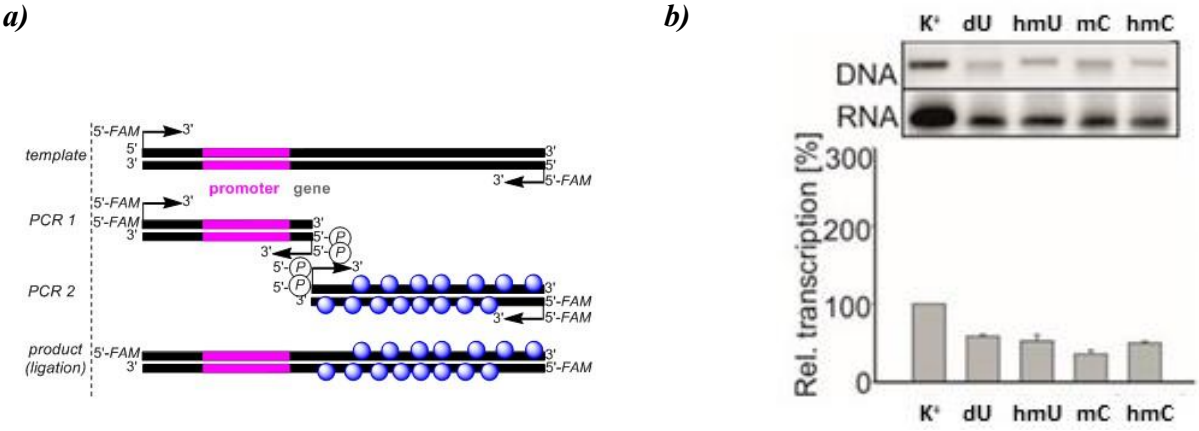


Figure 37: a) *Synthesis of DNA templates modified in the both strands of transcribed region* ***b)*** *Gel electrophoresis of partially modified DNA templates (Gel-Red stained) and RNA transcripts (^{32}P-labelled). Quantitation of relative transcription of dsDNA templates modified in the transcribed (gene) region.*

Such type of partly modified DNA templates was further tested in the transcription assay. The level of transcription for all DNA templates with epigenetic modifications was lower in comparison to natural DNA and nonspecific from the perspective of modifications. [***Figure 31b)***]

B) Synthesis of dsDNA templates modified in the both strands of promoter-upstream and promoter regions

For a synthesis of ***dsDNA templates modified in the promoter-upstream and promoter Pveg regions***, respectively, modified 69-mer of DNAs and natural 166-mer DNA were synthesized by PCR. Modified promoter-upstream and promoter regions (69-mers) were prepared in the presence of 5´-*FAM* labelled forward primer and 5´-*phosphate* modified reverse primer. Natural 166-mers of DNA were synthesized with 5´-*FAM* labelled reverse primer and 5´-*phosphate* modified forward primer. The role of 5´-*phosphate* and 5´-*FAM* labelled ends was the same as

in the previous case. In the final step of preparation partially modified DNA, modified 69 bp DNAs of each type (modified by either **dU** or **hmU** or **mC** or **hmC**) and natural 166 bp DNA were ligated by T4 DNA Ligase under the similar conditions like in previous case. [***Figure 32a)***]

a) ***b)***

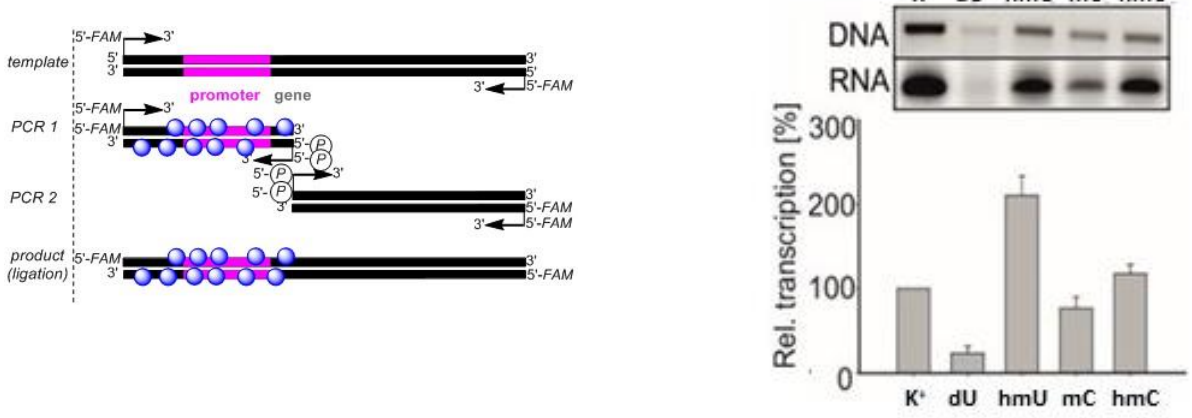


Figure 32: a) *Synthesis of DNA templates modified in the both strands of upstream promoter and promoter region.* ***b)*** *Gel electrophoresis of partially modified DNA templates (Gel-Red stained) and RNA transcripts (^{32}P-labelled). Quantitation of relative transcription of dsDNA templates modified in the promoter-upstream and promoter regions*

Transcription of DNA templates modified in the promoter region were performed under the similar conditions like in the previous cases. Since transcription of the templates displayed the similar trends as transcription of fully modified DNA templates, it confirmed that modification introduced in promoter region of DNA template influence the interaction with RNAP and therefor has the most impact on transcription. DNA templates modified in upstream and promoter regions just in one strand were prepared in the next step to determine whether template or non-template strand of promoter modified DNA control the binding of RNAP. [***Figure 32b)***]

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

Natural double stranded 181-mers and 235-mers, respectively, were prepared by PCR reaction in the presence of all four natural nucleobases and Taq DNA polymerase. The 181 bp DNA was prepared with a 5′-*phosphate* modified forward primer and an unmodified reverse primer while the 235-mer dsDNA was synthesized with a natural forward primer and a 5′-*phosphate* modified reverse primer. 5′-*phosphorylated* primers were applied for PCR, to get the desired natural single-stranded DNAs (ssDNA of 235-mer and ssDNA of 181-mer). Undesired 5′-*phosphorylated* strands of prepared dsDNAs were digested by Lambda exonuclease. Obtained single-stranded DNAs of different length (235 nt and 181 nt), complementary in the sequence,

were annealed to dsDNA with a single stranded overhang at the 5´-end of the non-template strand. To introduce the modifications into the promoter-upstream and promoter regions, the annealed dsDNA was used for the final PEX reaction in the presence of three natural nucleotides and one epigenetic dN^{X}TP (either **dUTP**, **dU^{HM}TP**, **dC^{Me}TP** or **dC^{HM}TP**). [***Figure 33a)***]

a) ***b)***

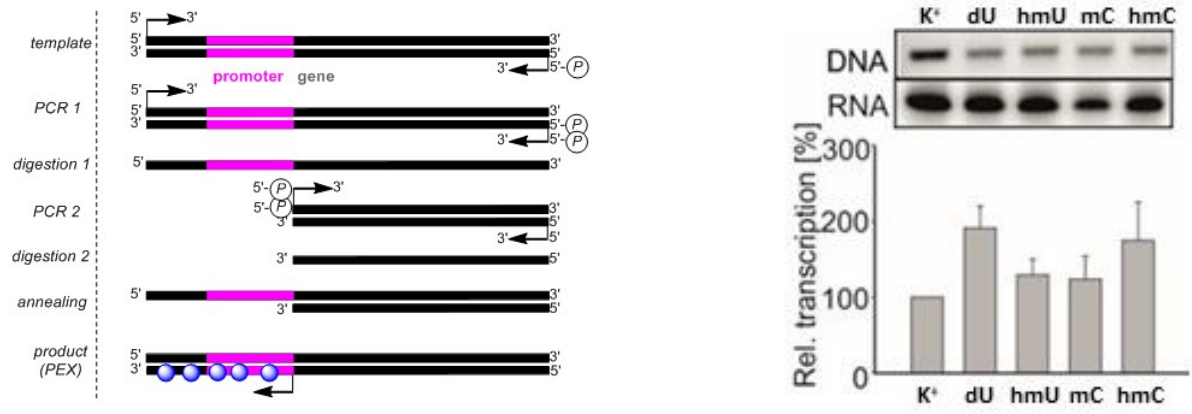


Figure 33: a) *Synthesis of DNA templates modified in the upstream promoter and promoter region of template strand.* ***b)*** *Gel electrophoresis of partially modified DNA templates (Gel-Red stained) and RNA transcripts (^{32}P-labelled). Quantitation of relative transcription of dsDNA templates modified in the promoter-upstream and promoter regions of template strand*

Transcription of the **dU**-modified templates showed surprisingly an increasing effect. The partially **hmU**-modified DNA and partially **hmC**-modified DNA gave enhancing effect on transcription as it was determined in the case of fully modified DNA templates with P*veg* promoter region. However, in the case of HM-modified DNA synthesized in the presence of **dU^{HM}TP**, the enhancement was slighter than for DNA synthesized in the presence of **dC^{HM}TP**. Partially methylated DNA template maintained a similar trend in the amount of transcript as in the case of fully modified DNA. [***Figure 33b)***]

D) Synthesis of DNA templates modified in promoter regions of the non-template strand

The ***DNA modified in promoter region of the non-template (coding) strand*** was prepared by PCR reaction using commercially synthesized **dU**-, **hmU**-, **mC**- or **hmC**-modified forward primers and unmodified (natural) reverse primer. [***Figure 34a)***]

Synthesized DNA templates partially modified in non-template strand showed the similar transcription trends as for fully modified DNA templates and templates modified in both strands of the promoter region. [***Figure 34b)***] The **dU**-modified DNA templates displayed inhibitory effect on transcription as in the case of fully **dU**-modified DNA template. Enhancing effect on

transcription was observed for hydroxymethylated DNAs even though the enhancing effect on transcription for DNA modified in upstream-promoter and promoter region with **U^{HM}-** nucleobase was not so significant. Surprisingly, the level of transcript for **mC**- modified DNA template was a bit higher than for **hmC**- modified DNA template.

a) ***b)***

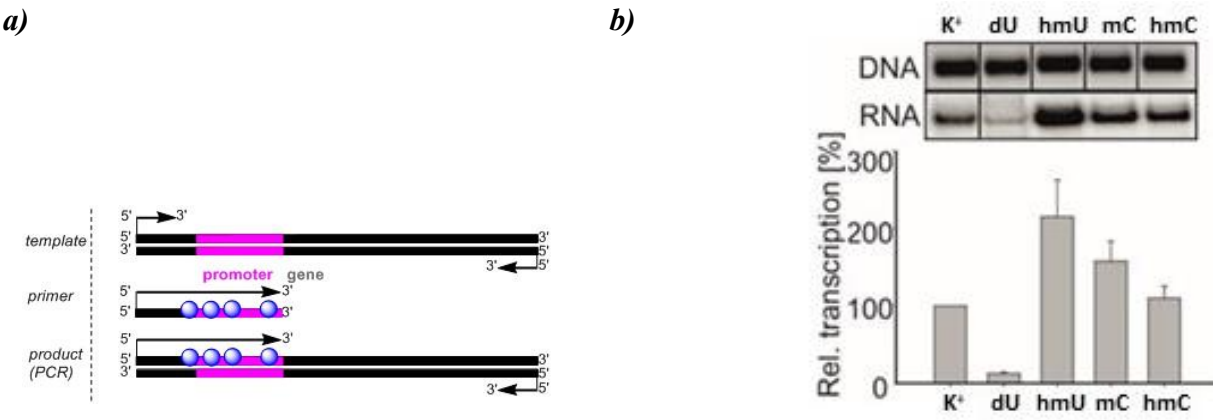


Figure 34: a) *Synthesis of DNA templates modified in the promoter region of non-template strand.* ***b)*** *Gel electrophoresis of partially modified DNA templates (Gel-Red stained) and RNA transcripts (^{32}P-labelled). Relative transcription of dsDNA templates modified in the promoter regions of non-template strand.*

As it was previously published, **hmU** is located in bacteriophages and support the binding of some transcription factors.[10, 20, 22] According the results of the systematic study, **dU**-modified DNA templates have inhibitory effect on transcription in case of P*veg* and *rrnB* promoter region. Depending on the sequence of promoter, **hmU** and **hmC** affect *in vitro* transcription by bacterial *E.coli* RNAP. Strong enhancement of transcription was observed for hydroxymethylated (**hmU** and **hmC**) DNA templates with P*veg* promoter region. The results of transcription assay apparently indicate that non-template strand of the promoter region has crucial role for the process of transcription and the fact can be applied in further study of chemical regulation of transcription.

3.3.3 Switching transcription with bacterial *E.coli* RNA polymerase[155]

The enhanced transcription from hydroxymethylated- (**hmU** and **hmC**) DNA by RNA polymerase from *E.coli* and inhibition of transcription in the case of nitrobenzylated- DNA (***Figure 28***) directed our research to detailed analysis of photocleavage and deprotection of bulky NB-modifications on DNA and consecutive switching ON transcription.

The regulation of transcription by biorthogonal reactions on DNA templates was carried out in cooperation with Libor Krásný group from Department of Molecular Genetics of Bacteria, Institute of Microbiology of the Czech Academy of Sciences. Synthesis of used nucleoside triphosphates (**dU^{HM}TP**, **dU^{NB}TP** and **dC^{NB}TP**), design of 311-mer DNA template containing derived upstream of gene and initial downstream of gene with P*veg* promoter region, an optimization of conditions for synthesis of all DNA templates and their synthesis themselves were done by me as well as part of transcription experiments. Synthesis of desired plasmid with optimized sequence of 311-mer DNA template and most of the transcription experiments of modified DNA templates were performed by Martina Janoušková from Libor Krásný group. [155]

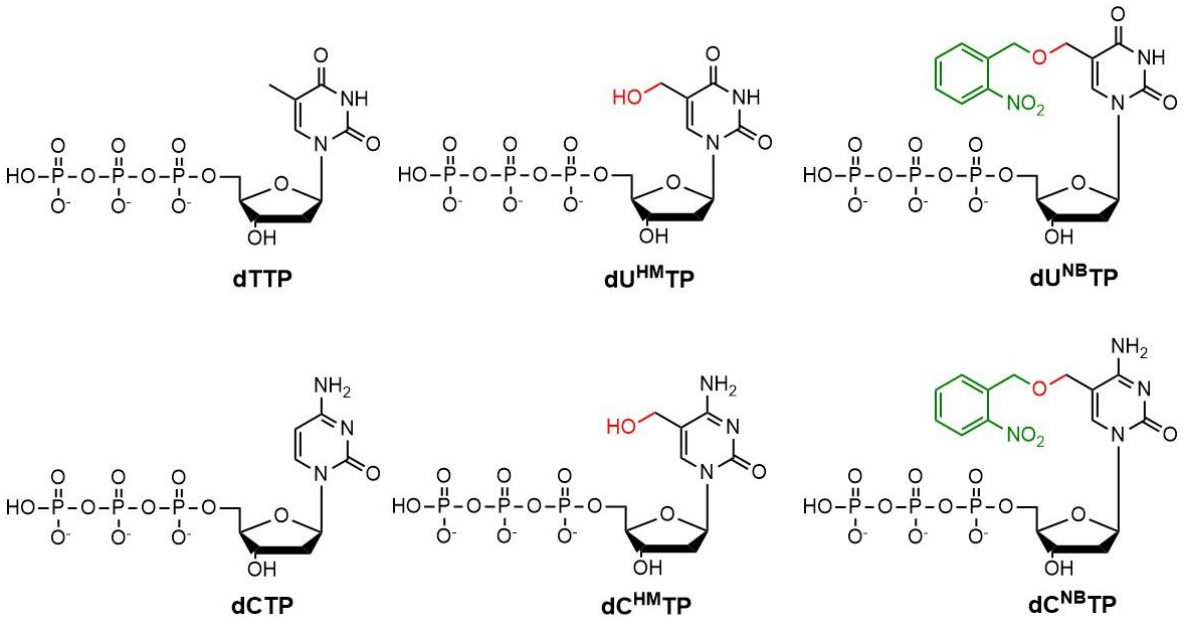


Figure 35*: Structures of triphosphates used for the synthesis of DNA templates*

The structures of used natural triphosphates (**dTTP**, **dCTP**), epigenetic hydroxymethylated triphosphates (**dU^{HM}TP**, **dC^{HM}TP**) and nitrobenzylated triphosphates (**dU^{NB}TP**, **dC^{NB}TP**), which have predisposition for their application in regulation of transcription, are shown in ***Figure 35***.

Oligonucleotides	Sequence (5´→3´)	Length
99-mer ss oligos		
Insert_99_EcoRI[a]	GAC**GAATTC**AGCCATATATCCTCTGGCTAATAG GACTACTTCTAATCTGTACAGCAGATCCATACG CCTGGACAGGCAATCAGGCTAGAG **GAATTC**GTG	**99-mer**
Insert_99_EcoRI_REV[a]	CAC**GAATTC**CTCTAGCCTGATTGCCTGTCCAG GCGTATGGATCTGCTCGTACAGATTAGAAGTA GTCCTATTAGCCAGAGGATATATGGCT**GAAT TC**GTC	**99-mer**
PRIMERs		
***Prim*^{FOR}**	TTCAGCCATATATCCTCTGGCTAATAGG	**28-mer**
***Prim*^{REV}**	GGAGAGCGTTCACCGACAAACAACAG	**26-mer**
TEMPLATE[b,c]		
***Temp*^{Pveg2}**	TTCAGCCATATATCCTCTGGCTAATAGGAC TACTTCTAATCTGTACGAGCAGATCCATAC GCCTGGACAGGCAATCAGGCTAGAGGAAT TC*TA**TTTGACAAAAATGGGCTCGTGTTGTAC AATAAATGT***GTCTAAGCTTGGGTCCCACCT GACCCCATGCCGAACTCAGAAGTGAAAC GCCGTAGCGCCGATGGTAGTGTGGGGTC TCCCCATGCGAGAGTAGGGAACTGCCAGG CATCAAATAAAACGAAAGGCTCAGTCGAA AGACTGGGCCTTTCGTTTTATCTGTTGTTTG TCGGTGAACGCTCTCC	**311-mer**

[a] bold sequence in oligo is site for cleavage with EcoRI
[b] primer sequences in template are underlined
[c] promoter sequence of template is bold in italic

Table 9: *List of used oligonucleotides*

Since NB- modification is bulky and original DNA template (339-mer) contains big number of dA and dT nucleobases in a row in the sequence, the synthesis of fully modified NB- modified DNA with original sequence (used in the *section 3.3.2* for study influence of epigenetic modifications on transcription) in the presence of **dU^{NB}TP** was problematic. Because of facilitate PCR reaction with modified nucleotides, the 311-mer DNA template containing derived upstream of gene and initial downstream of gene with P*veg* promoter region was designed to study the switching transcription. For preparation of 311-mer DNA template, the 1177 plasmid containing the P*veg* promoter region[100] was used to clone an artificial 99-mer

dsDNA sequence upstream of the promoter. Two complementary single strands of commercially available oligos were annealed together, then cleaved with restriction enzyme - EcoRI to create sticky ends. Cleaved dsDNA was ligated into the 1177 plasmid at the upstream EcoRI edge of the promoter. Such changed plasmid was transformed into *E.coli DH5α.* Its colonies with inserts were isolated, verified by sequencing and the final plasmid construct LK2130 was used for its partial replication and production of natural 311-mer dsDNA. The DNA was purified and used as a template for further PCR in the presence of modified dNTPs (**dU^{HM}TP**, **dC^{HM}TP**, **dU^{NB}TP**, **dC^{NB}TP**) to form the modified DNA templates for transcription.

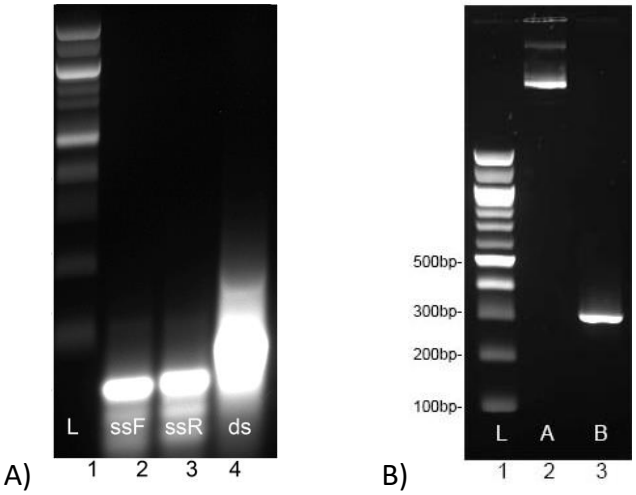


Figure 36: *A) Agarose gel analysis of 99-mer insert:* Lane 1 (**L**): 100bp DNA ladder (commercial mix of dsDNA with size range: 100 bp – 1517 bp); lane 2 (**ssF**): oligo **Insert_99_EcoRI**; lane 3 (**ssR**): oligo **Insert_99_EcoRI_REV**; lane 4 (**ds**): annealed dsDNA product *B) Agarose gel analysis of PCR template.* Lane 1 (**L**): ladder (commercial mix of dsDNA with size range: 100bp - 1517bp); lane 2 (**A**): plasmid construct LK2130; lane 3 (**B**): PCR product of template - ***Temp^{Pveg2}***

3.3.3.1 Synthesis of modified DNA templates

HM- and NB- modified DNA templates for transcription experiments were synthesized by PCR reactions using **dU^{HM}TP**, **dU^{NB}TP**, **dC^{HM}TP** and **dC^{NB}TP** instead of natural **dTTP** or **dCTP**. In all cases, full length, fully modified DNA templates were successfully obtained in good yield. PCR reaction of **U^{NB}** -modified DNA run more slowly than for HM-modified DNA, that's why the elongation time for PCR of NB-modified DNA was prolongated to 2 minutes and final elongation to 7 minutes. Controls for the DNA transcription experiments (positive controls of PCR) were prepared under the condition either for **hmC**- (**C^{HM}-)** or **hmU**- (**U^{HM}-)** modified DNA in the presence of natural dNTPs.

HM- and NB- modified DNA templates prepared under the conditions reported above and in experimental section *6.3.3.1* were used in all following experiments.

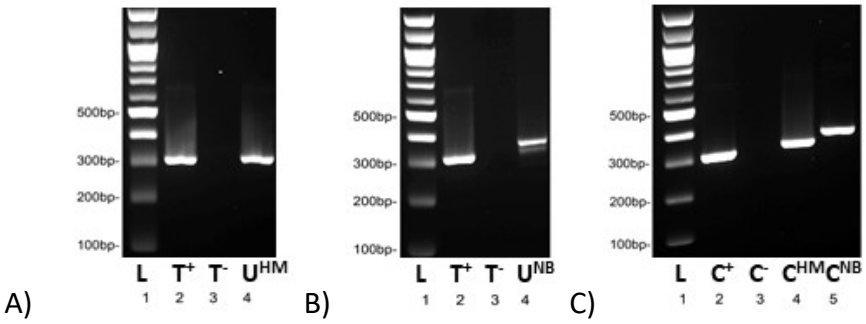
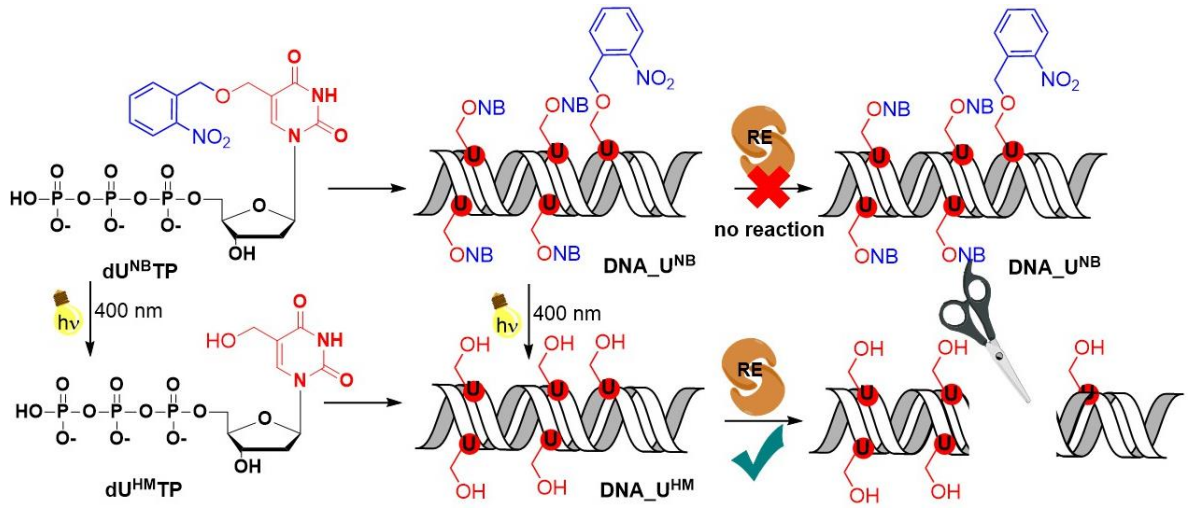


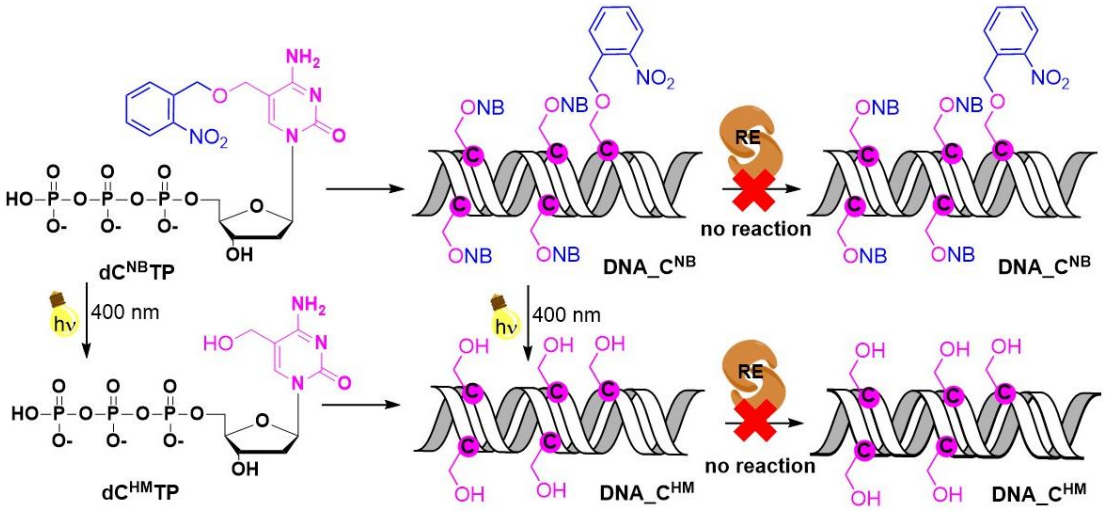
Figure 37: *Agarose gel analysis of modified DNA templates:* The order of samples in gel **A**) is as follows: lane 1, (**L**): ladder (commercial mix of dsDNA); lane 2, ($\mathbf{T^+}$): natural DNA; lane 3, ($\mathbf{T^-}$): PCR was run in absence of dTTP; lane 4, ($\mathbf{U^{HM}}$): $\mathbf{U^{HM}}$ – modified PCR product. The order of samples in gel **B**) is as follows: lane 1, (**L**): ladder (commercial mix of dsDNA); lane 2, ($\mathbf{T^+}$): natural DNA; lane 3, ($\mathbf{T^-}$): PCR was run in absence of dTTP; lane 4, ($\mathbf{U^{NB}}$): $\mathbf{U^{NB}}$ – modified PCR product. The order of samples in gel **C**) is as follows: lane 1, (**L**): ladder (commercial mix of dsDNA); lane 2, ($\mathbf{C^+}$): natural PCR product; lane 3, ($\mathbf{C^-}$): PCR was run in absence of dCTP; lane 4, ($\mathbf{C^{HM}}$): $\mathbf{C^{HM}}$ -modified PCR product; lane 5, ($\mathbf{C^{NB}}$): $\mathbf{C^{NB}}$ -modified PCR product.

3.3.3.2 Study of cleavage of modified dsDNA with AluI and RsaI

A)



B)



Scheme 38: *Cleavage of modified DNA with REs*

First, the cleavage of nucleic acids modified in both strands (hydroxymethylated and nitrobenzylated) was tested with two restriction endonucleases. (***Scheme 38***) Since DNA sequence contain, the specific sites for cleavage with AluI and RsaI, these two endonucleases were chosen for restriction enzyme digestion. Both enzymes contain four nucleobases in their restriction site and generate the blunt ends of dsDNA after cleavage during its incubation at 37 °C. The cleavage of dsDNA was monitored with 1.3% agarose gel stained with Gel Red. As visible by the gels, nitrobenzylated DNA (**DNA_U^{NB}** and **DNA_C^{NB}**) was not tolerated by any RE as expected. HM-modified 311-mer DNA synthesized in the presence of **dU^{HM}TP** was under mentioned conditions fully cleaved by RsaI and AluI. Although complete cleavage of shorter **C^{HM}-** modified DNA with RsaI was nicely observed (*section 6.3.3.2.*), long hydroxymethylated DNA (**DNA_C^{HM}**) modified in both strands was not digested with either AluI or RsaI restriction enzyme. Based on this knowledge, the first kinetic experiments of deprotection of photolabile NB- moieties were focused on uridine modified DNA where the cleavage of NB- group could be monitored by restriction enzyme digestion.

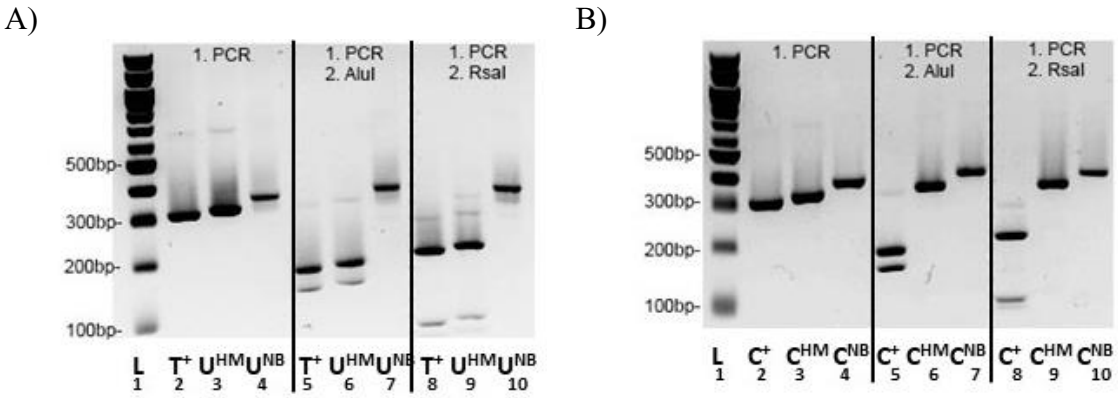


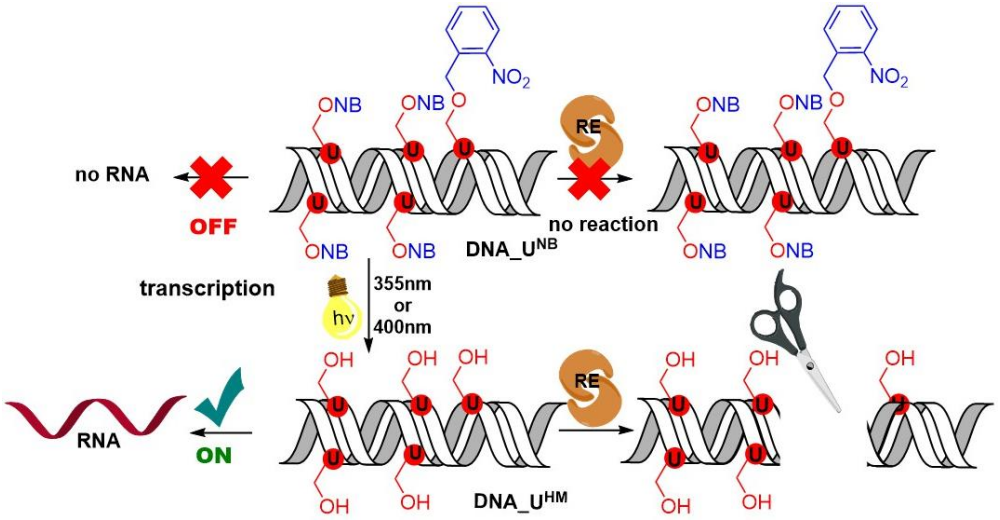
Figure 37: *Study of restriction enzyme digestion (AluI and RsaI):* The order of samples is as follows: Gel **A):** lane 1, (**L**): ladder (commercial mix of dsDNA); lane 2, ($\mathbf{T^{+}}$): natural PCR product; lane 3, ($\mathbf{U^{HM}}$): $\mathbf{U^{HM}}$ – modified PCR product; lane 4, ($\mathbf{U^{NB}}$): $\mathbf{U^{NB}}$ -modified PCR product; lane 5, ($\mathbf{T^{+}}$): natural dsDNA cleaved with AluI; lane 6, ($\mathbf{U^{HM}}$): HM-modified dsDNA cleaved with AluI; lane 7, ($\mathbf{U^{NB}}$): NB-modified dsDNA cleaved with AluI (no cleavage); lane 8, ($\mathbf{T^{+}}$): natural dsDNA cleaved with RsaI; lane 9, ($\mathbf{U^{HM}}$): HM-modified dsDNA cleaved with RsaI; lane 10, ($\mathbf{U^{NB}}$): NB-modified dsDNA cleaved with RsaI (no cleavage). Gel **B)**: lane 1, (**L**): ladder (commercial mix of dsDNA); lane 2, ($\mathbf{C^{+}}$): natural PCR product; lane 3, ($\mathbf{C^{HM}}$): $\mathbf{C^{HM}}$ – modified PCR product; lane 4, ($\mathbf{C^{NB}}$): $\mathbf{C^{NB}}$ -modified PCR product; lane 5, ($\mathbf{C^{+}}$): natural dsDNA cleaved with AluI; lane 6, ($\mathbf{C^{HM}}$): HM-modified DNA incubated with AluI (no cleavage); lane 7, ($\mathbf{C^{NB}}$): NB-modified DNA incubated with AluI (no cleavage); lane 8, ($\mathbf{C^{+}}$): natural DNA cleaved with RsaI; lane 9, ($\mathbf{C^{HM}}$): HM-modified DNA incubated with RsaI (no cleavage); lane 10, ($\mathbf{C^{NB}}$): NB-modified DNA incubated with RsaI (no cleavage).

Since ^{32}P-labelleling of modified DNA is more reliable than its GelRed staining or UV absorption of base-modified nucleic acid, ^{32}P-labelleled DNA templates were prepared by PCR and used to precisely quantify the amount of DNA templates used for their transcription. ^{32}P-labelled primers were used to prepare 311-mer DNA containing modified nucleobases in the sequence. Desired DNA products were prepared by enzymatic reaction in PCR cycler, purified with magnetic beads and diluted to the final concentration approximately 20 ng/µl according to NanoDrop spectrophotometer. Diluted DNAs were used as stock for further reactions and manipulation.

3.3.3.3 Study of transcription for U^{HM}- and U^{NB}- modified DNA

The photocleavage of nitrobenzyl moiety from $\mathbf{U^{NB}}$-modified DNA (**DNA_$\mathbf{U^{NB}}$**) was studied under different conditions. Different kind of lamps, additives and time of irradiation were examined. (***Scheme 39***) In the first trial, the irradiation of nitrobenzylated DNA was performed by LED with maximum emission at 355 nm or 400 nm wavelength and the low power (1 mW for 355 nm; 25 mW for 400 nm). The tested LEDs were used in previous experiments[150, 151] and

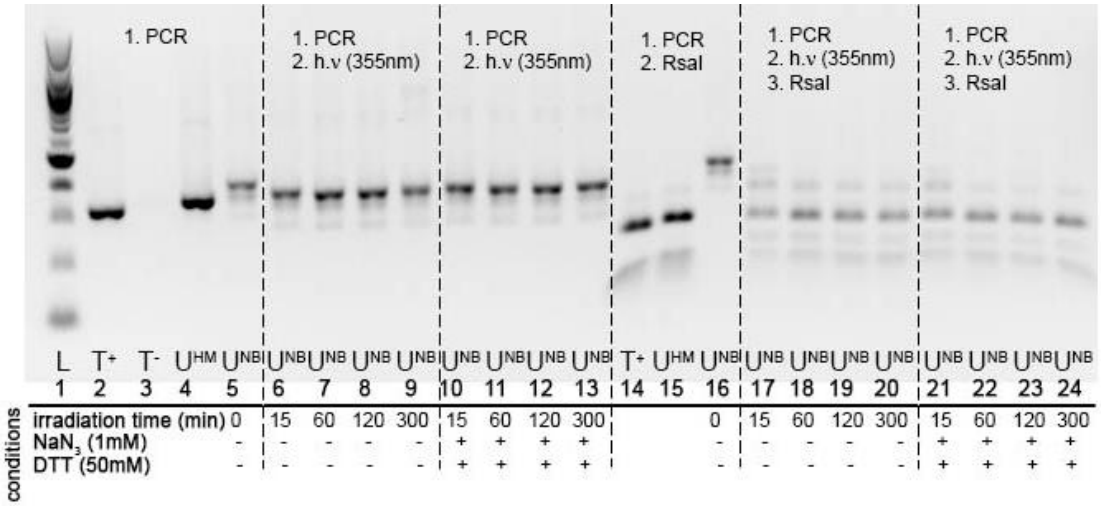
fully cleavage of nitrobenzyl moiety from PEX product was obtained after from 2 to 6 hours of irradiation.



Scheme 39: *Study of transcription for U^{HM} and U^{NB}- modified DNA*

The kinetic study of deprotection was performed, in which approximately 2 x 240 ng of **DNA_U^{NB}** was irradiated in time intervals 15, 60, 120 and 300 minutes. The part of NB-modified DNA was irradiated also in the presence of NaN_3 (1 mM; 1 μL) and DTT (50 mM; 1 μL). To monitor the process of deprotection, the irradiated **DNA_U^{NB}** was digested with RsaI under the same conditions as standards – natural DNA and **DNA_U^{HM}**. In accord with previous results, no cleavage of protected **DNA_U^{NB}** was expected and gradual decreasing with time of full-length irradiated **DNA_U^{NB}** was predicted. As visible from the agarose gel image, the biggest difference of cleavage by RsaI is observed after first 15 minutes of irradiation, however with prolongated irradiation time it is not possible to reach significant difference in digestion by restriction enzyme. Moreover, according to cleavage experiment results, almost fully deprotection of nitrobenzyl moieties would be expected for **DNA_U^{NB}** irradiated for 300 minutes in the presence of additives by lamp with 400 nm wavelength.

a)



b)

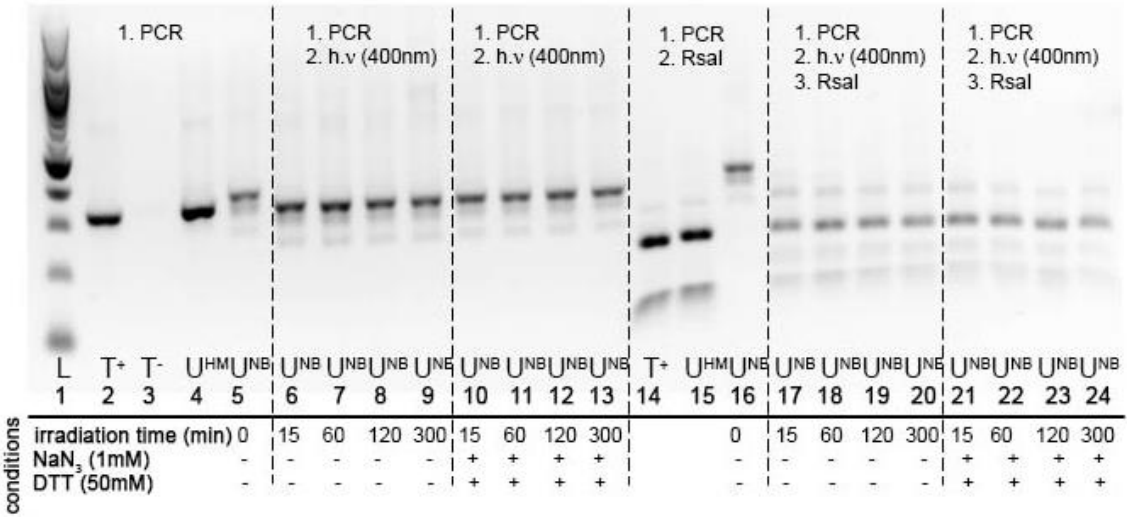


Figure 38**:** *Kinetic study of photocleavage* ***DNA_U^{NB}*** *irradiated by light with wavelength* ***a)*** *355 nm or* ***b)*** *400 nm:* In both gels, the order of samples is: lanes 1, (**L**): ladder (commercial mix of dsDNA); lanes 2, (**T^{+}**): natural PCR product; lanes 3, (**T^{-}**): negative control of PCR in the absence of natural dTTP; lanes 4, (**U^{HM}**): **U^{HM}** – modified PCR product; lanes 5, (**U^{NB}**): **U^{NB}** -modified PCR product; lanes 14, (**T^{+}**): natural PCR product digested with RsaI; lanes 15, (**U^{HM}**): **U^{HM}** -modified DNA digested with RsaI; Lanes 16, (**U^{NB}**): **U^{NB}** -modified DNA digested with RsaI. In gel **A)**: lanes 6-9: **U^{NB}** modified DNA without additives irradiated by LED (355 nm) in the following time intervals: 15/ 60/ 120/ 300 min; lanes 17-20: **U^{NB}-**modified DNA irradiated by LED (355 nm) in the following time intervals: 15/ 60/ 120/ 300 min and digested with RsaI; lanes 10-13: **U^{NB}-**modified DNA irradiated by LED (355 nm) in the presence of additives in the following time intervals: 15/ 60/ 120/ 300 min; lanes 21-24: **U^{NB}-**modified DNA irradiated by LED (355 nm) in the presence of additives in the following time intervals: 15/ 60/ 120/ 300 min and digested with RsaI. In gel **B):** lanes 6-9: **U^{NB} -**modified DNA irradiated by LED (400 nm) in the time intervals: 15/ 60/ 120/ 300 mins; lanes 17-20: **U^{NB}-**modified DNA irradiated by LED (400 nm) in the following time intervals: 15/ 60/ 120/ 300 min and digested with RsaI; lanes 10-13: **U^{NB}-**modified DNA irradiated by LED (400 nm) in the following time intervals: 15/ 60/ 120/ 300 min; lanes 21-24: **U^{NB}-**modified DNA in the presence of additives irradiated by LED (400 nm) in time intervals 15/ 60/ 120/ 300 min and digested with RsaI.

The portions of the same samples were used as templates for transcription studies, which were performed with holoenzyme complexed RNAP with σ^{70} from *Escherichia coli* under the reported conditions (*section 6.3.3.3*). The resulted 145 nucleobase long transcripts (RNA) were checked on 7% polyacrylamide gel. The scanned gels were analysed and quantified with Quantity One program. For a quantification of transcription, the signals of transcript were normalized to the signals of DNA templates. The signals of modified DNA templates were normalized to the signal of natural DNA, which was established as 100%. The results of transcription were set up from three independent experiments (unless otherwise stated). As it is obvious from the gel analysis and evident from graphical figure, in any case of irradiation of **DNA_U^{NB}** (by LED with maximum emission at 400 nm neither 365 nm), the level of transcription of irradiated **DNA_U^{NB}** didn't reach the transcription level of hydroxymethylated DNA. The transcription level was in the range of the transcription for natural DNA, even when digestion by restriction enzyme for 300 minutes indicated more promising results. The transcription results for the **DNA_U^{NB}** irradiated in the presence of additives turned out to double in comparison to the same sample irradiated in absence of scavengers (with water addition).

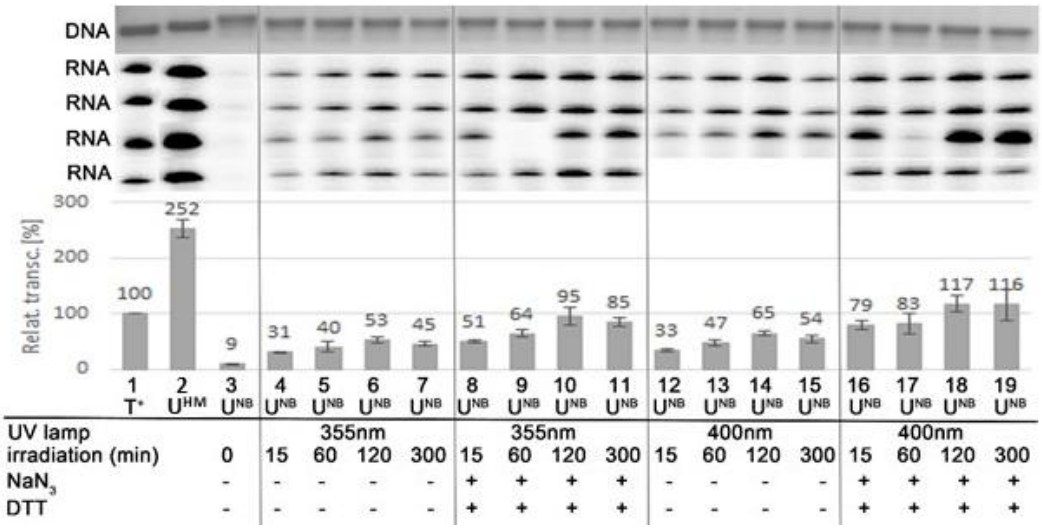


Figure 39: *Transcription of DNA templates reported in Figure 38*

3.3.3.4 The effect of additives on photocleavage of NB- protecting group from DNA

To confirm the observed trend in the presence of scavengers and without additives, 240 ng of stock **DNA_U^{NB}** was irradiated by light with maximum emission at 400 nm in time intervals: 15 minutes or 60 minutes. The irradiation of samples was performed either in the presence of 1 µL of (50 mM) DTT and 1 µL of (1 mM) NaN_3 or in the presence of H_2O (1 µL) to reach the

same dilution of sample as in the case with additives. The results of multi-round *in vitro* transcription assay (***Figure 40***) confirmed the previous observation in which the level of transcript for **DNA_U^{NB}** irradiated in the presence of additives was significantly higher – doubled in comparison to **DNA_U^{NB}** irradiated just in the access of water.

The doubled positive effect on transcription in the presence of additives could be explained by formation of nitrosobenzaldehyde as a by-product during photocleavage, which absorbs the light rather in comparison to the starting nitrobenzyl- modified nucleobase. 1, 4 – dinitrotreitol (DTT) reduce released by-product and in a cooperation with sodium azide (NaN_3) enable faster deprotection of nitrobenzyl moieties from DNA, thus scavengers allow to avoid the DNA damage during its long-term exposure. [156]

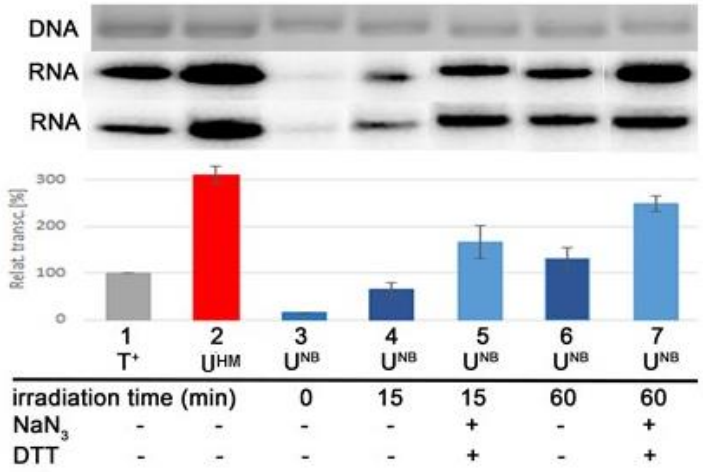


Figure 40: *Comparison of transcription level for **DNA_U^{NB}** irradiated in the presence and in the absence of additives.*

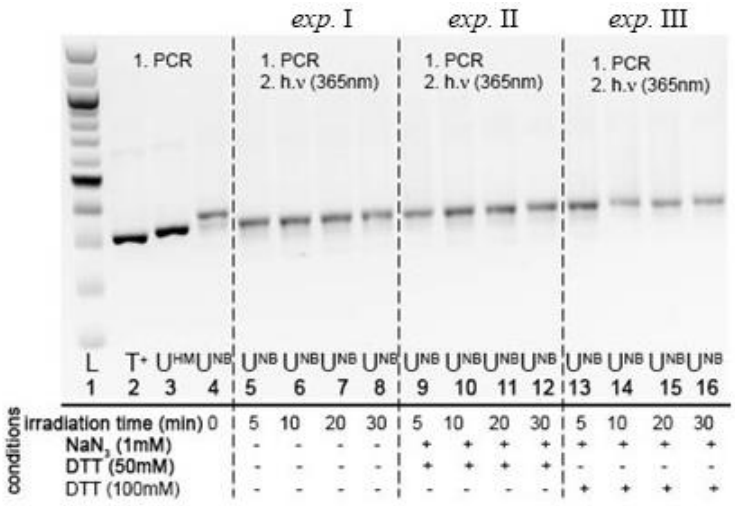
To clarify if it is not possible to achieve higher deprotection of NB-moieties from artificial DNA, the irradiation studies of **DNA_U^{NB}** were performed in the presence of two different concentrations of DTT. Approximately 240 ng of **DNA_U^{NB}** was irradiated in the presence of 1 µL of (1 mM) NaN_3 and 1 µL of DTT (either 50 mM - ***Figure 41;*** lines 9-12 or 100 mM; ***Figure 41*** – lines 13-16). The results of deprotection, therefore level of transcription, were again compared to the results of deprotection without additives and control **DNA_U^{NB}**. (***Figure 41*** – lines 5-8)

The NB-modified DNA was irradiated in different time intervals (5/ 10/ 20/ 30 minutes) by LED with maximum emission at 365 nm (0.8 W). Each irradiation experiment was repeated twice, and both doses where mixed together. The irradiated DNAs from all experiments (I, II and III), were divided into three portions. As previously, the part of the DNA after irradiation (120 ng) was loaded on an agarose gel right after irradiation experiments. Second portion

(120 ng) of the irradiated DNA was firstly incubated with RsaI at 37°C within one hour and then monitored by gel electrophoresis. The third portions of the same irradiated samples (240 ng) were used for transcription studies. The degree of RNA formation was monitored on PAGE. [***Figure 41b)***]

Partial deprotection of nitrobenzylated- DNA was reflected on its migration. As it is obvious from the ***Figure 41 a)*** the irradiated DNA migrates faster in comparison to NB-modified DNA. Moreover, according the image ***B)*** of agarose gels, artificial DNA was partially digested with RsaI however it was not evident difference in DNA cleavage level under the conditions of individual experiments I, II, III.

a)



b)

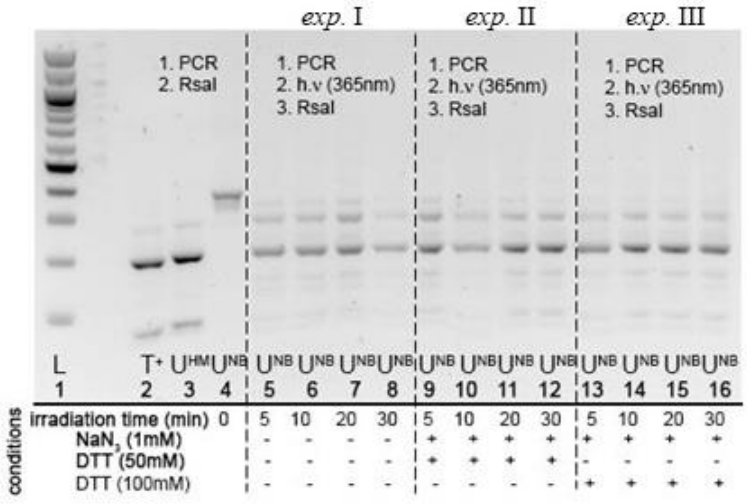


Figure 41**: *a)*** *Agarose gel analysis of NB-modified DNA irradiated under conditions I / II / III by UV lamp 365 nm* ***b)*** *Agarose gel analysis of digested NB-modified DNA irradiated under conditions I / II / III by UV lamp 365 nm.* The order of samples on both gels is as follows: lanes 1, (**L**): ladder (commercial dsDNA with specific length); lanes 2, ($\mathbf{T^{+}}$): natural PCR product; lanes 3, ($\mathbf{U^{HM}}$): $\mathbf{U^{HM}}$ -modified PCR product; lanes 4, ($\mathbf{U^{NB}}$): $\mathbf{U^{NB}}$ -modified PCR product; lanes 5-8 (exp. I): $\mathbf{U^{NB}}$ -modified DNA irradiated by light without additives; lanes 9-12 (exp. II): $\mathbf{U^{NB}}$ - modified DNA

irradiated by light in the presence of additives (50 mM DTT and 1 mM NaN_3); lanes 13-16 (exp. III): **U^{NB}-**modified DNA irradiated by light in the presence of additives (100 mM DTT and 1 mM NaN_3). In all cases (exp. I/ II/ III) the time intervals of irradiations were 5/ 10/ 20/ 30 min.

PAGE analysis of RNA products and its graphical representation again confirmed that the level of transcription for nitrobenzylated DNA irradiated in the presence of additives is significantly higher than in the case of sample irradiated without additives. On the other hand, the results showed no change in the degree of RNA exposed in the presence of different amount of DTT (final concentration 3.57 mM for exp. I or final concentration 7.14 mM for exp. II). Higher concentration of reductant did not hasten the deprotection, thus 1 µL of 50 mM solution of DTT for 240 ng of DNA is suitable and following studies continued under these conditions.

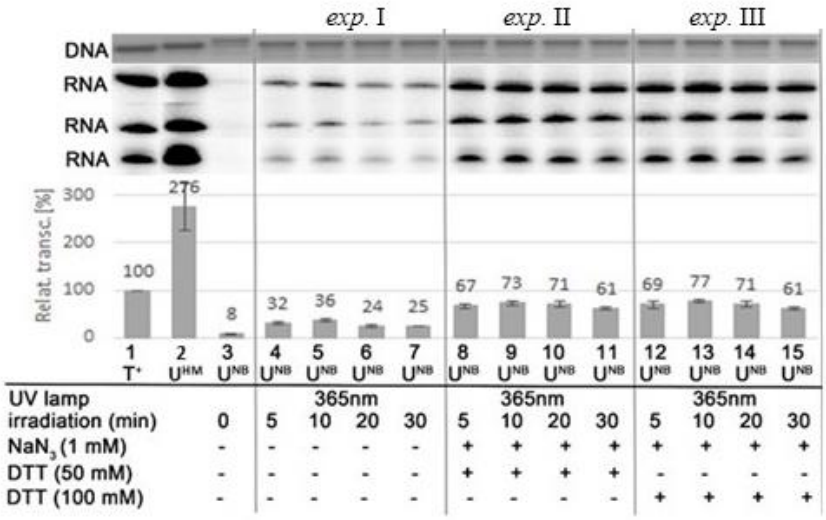


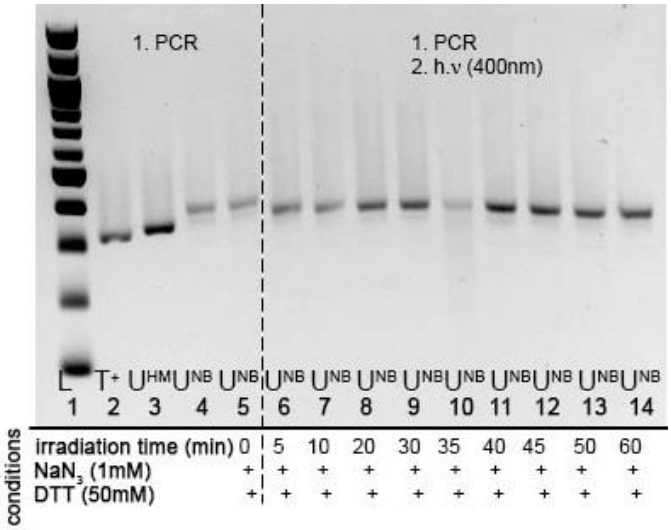
Figure 42**:** *Transcription of deprotected DNA templates prepared in the presence of 50 mM (lines 8-11) or 100 mM (lines 12-15) DTT*

3.3.3.5 The kinetic study of photocleavage under optimized conditions

The next studies were carried out under the conditions optimized in the previous experiments. It means: 240 ng of nitrobenzylated DNA was irradiated in the presence of 1 µL of (1 mM) NaN_3 and 1 µL of (50 mM) DTT. This time a powerful source (3 W) of LED light source with maximum emission at 400 nm was used. The photodeprotection kinetic studies of **DNA_U^{NB}** was performed in time intervals: 5/ 10/ 20/ 30 / 35 /40 /45 /50 /60 minutes. The kinetic studies were performed similarly as in the previous cases. Each irradiation experiment was repeated twice, combined and one quarter of the sample was used for control agarose gels just after irradiation and one quarter of it was loaded on a gel after digestion with RE. The last portion of irradiated **DNA_ U^{NB}** was used as DNA template in the transcription assay.

The image of agarose gel after cleavage with RsaI indicated a progressive decline of full-length starting **DNA_U^{NB}** and gradual gain of digested product with a time. The trend was observed for **DNA_U^{NB}** exposed to irradiation by light for 30-35 minutes (line 10).

A)



B)

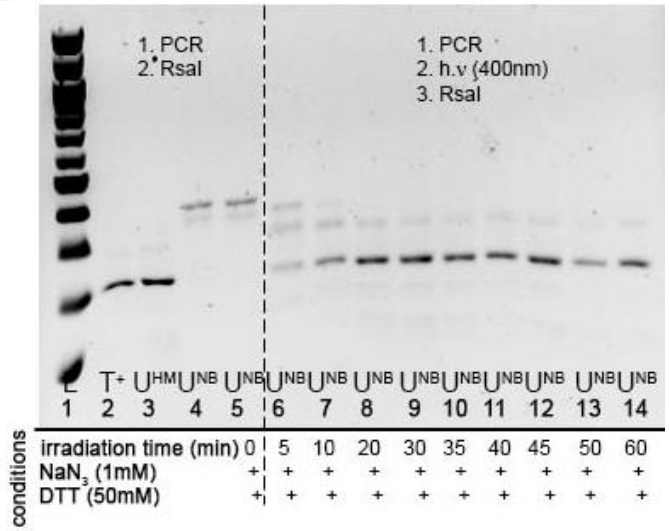


***Figure 43*: *A)** Agarose gel analysis of photodeprotection kinetic study **DNA_U^{NB}** **B)** Agarose gel analysis of digestion **DNA_U^{NB}** with RsaI after its previous irradiation by light.* The order of samples on both gels is as follows: lanes 1, (**L**): ladder (commercial dsDNA with specific length); lanes 2, (**T^{+}**): natural PCR product; lanes 3, (**U^{HM}**): **U^{HM}** -modified PCR product; lanes 4, (**U^{NB}**): **U^{NB}** -modified PCR product; lanes 5: **U^{NB}** **-**modified DNA in the presence of additives (1 µL of 50 mM DTT and 1 µL of 1 mM NaN_3); lanes 6-14: **U^{NB}** **-**modified DNA irradiated by light (400 nm) in the presence of additives (1 µL of 50 mM DTT and 1 µL of 1 mM NaN_3). The time intervals of irradiation were: 5/ 10/ 20/ 30 / 35 /40 /45 /50 /60 minutes.

The results of transcription from kinetic study of photocleavage indicated that irradiation of photocaged **DNA_ U^{NB}** for 20 minutes release the DNA template, transcription of which lead to the approximately the same level of transcript as for the corresponding **DNA_ U^{HM}** template (ca. 370 %).

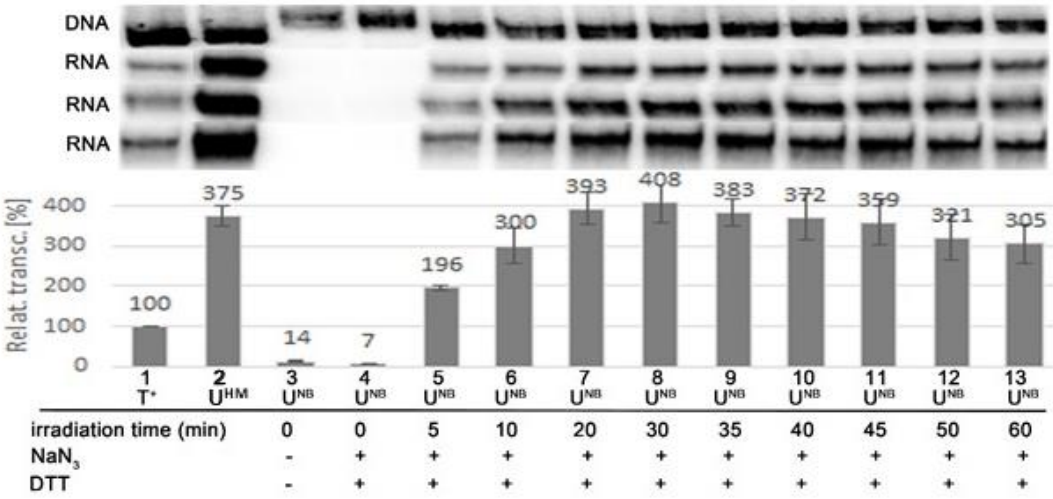


***Figure 44**: Results of in vitro transcription for **DNA_U^{NB}** templates irradiated by light (400 nm) in the presence of additives*

The fully optimized conditions of deprotection were further applied for the kinetic study of photocleavage **DNA_C^{NB}**. The **DNA_C^{NB}** was exposed to the light with maximum emission at 400 nm in time intervals 2/ 5/ 10/ 15/ 17/ 20/ 30 / 40 minutes. Since hydroxymethyl groups on cytidine nucleobase in **DNA_C^{HM}** as well as nitrobenzyl groups in **DNA_C^{NB}**, both are not tolerated with tested restriction endonucleases, the completion of the deprotection was not checked continuously after the sample's irradiation by restriction enzyme digestion.

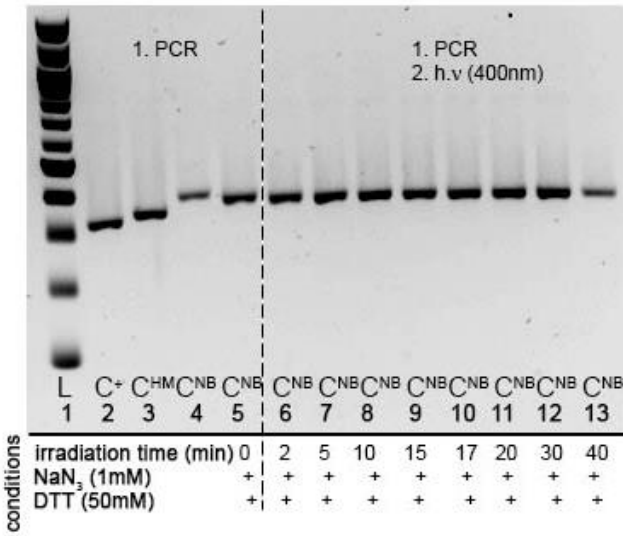


***Figure 45*: *A)** Agarose gel analysis of photodeprotection kinetic study of **DNA_C^{NB}**.* On a gel, the order of samples is: lanes 1, (**L**): ladder (commercial dsDNA with specific length); lanes 2, (**T^{+}**): natural PCR product; lanes 3, (**C^{HM}**): **C^{HM}** -modified PCR product; lanes 4, (**C^{NB}**): **C^{NB}** -modified PCR product; lanes 5: **C^{NB} -**modified DNA in the presence of additives (1 µL of 50 mM DTT and 1 µL of 1 mM NaN_3); lanes 6-14: **C^{NB} -**modified DNA irradiated by light (400 nm) in the presence of additives (1 µL of 50 mM DTT and 1 µL of 1 mM NaN_3). The time intervals of irradiation were: 2 /5/ 10/15 /17 /20 /30 /40 min.

The part of the irradiated **DNA_C^{NB}** samples were loaded on a control 1.3 % agarose gel and the rest was directly used for transcription assay studies by *E.coli* RNAP. The irradiation of **DNA_ C^{NB}** just for 10 minutes released DNA templates, which gave approximately the same level of RNA as corresponding **DNA_ C^{HM}** template (ca. 230 %), what indicated the complete photodeprotection of **DNA_ C^{NB}**.

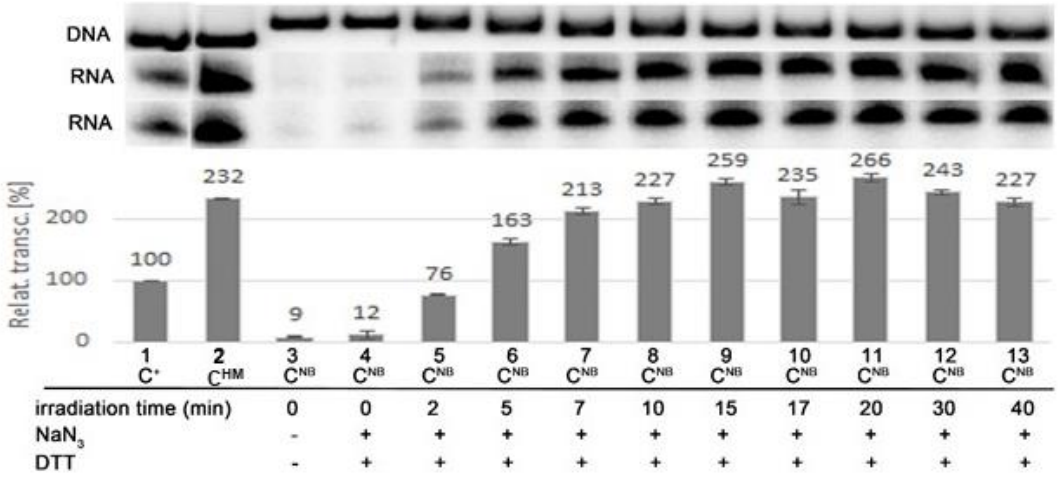
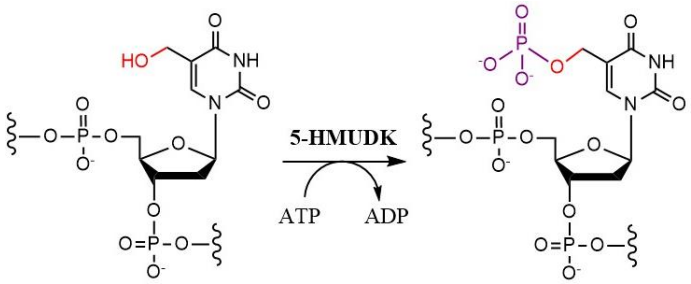


Figure 46: *Results of an in vitro transcription for **DNA_C^{NB}** templates irradiated by light (400 nm) in the presence of additives*

As obvious from the graphical representation of RNA quantity, longer exposition to light of **DNA_N^{NB}** (***Figure 44*** and ***Figure 46***) led to a gradual reduction of transcript concentration. The observed effect was most probably due to DNA damage. The continuous decreasing of RNA concentration was observed after 30 minutes of **DNA_N^{NB}** irradiation. Based on the results above, the irradiation time of **DNA_N^{NB}** for preparative experiments was established as a time when the first maximums of transcripts were observed in the kinetic studies. For the further experiments, the optimized conditions of photocleavage, which present irradiation of **DNA_N^{NB}** in the presence of 1 µL of (1 mM) NaN_3 and 1 µL of (50 mM) DTT by LED light source (400 nm, 3 W) were used. The **DNA_U^{NB}** was exposed to light for 30 minutes and **DNA_C^{NB}** for 10 minutes.

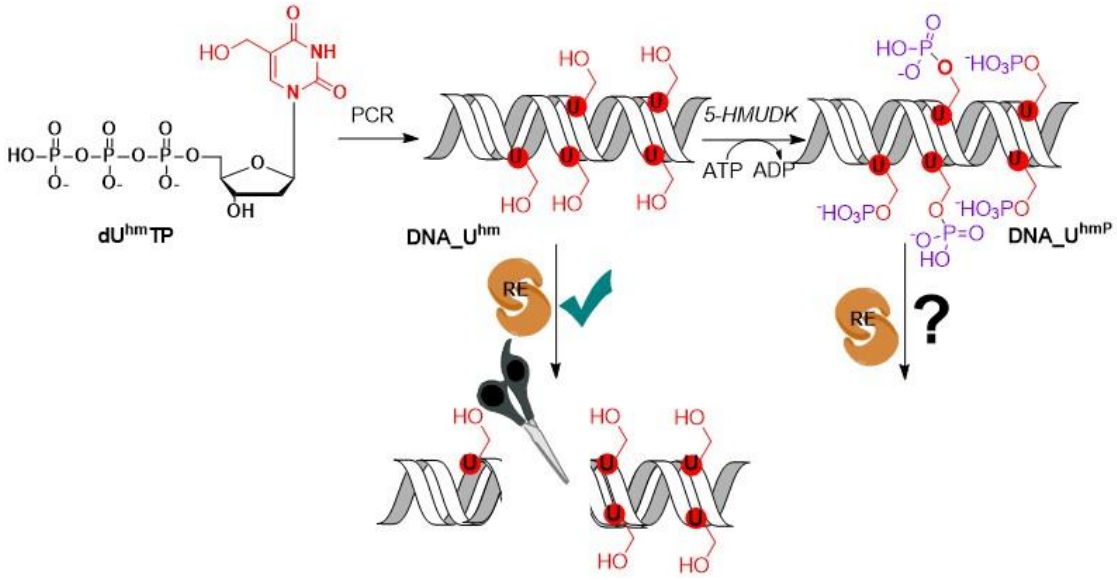
All previous investigations led to the system of photochemical reactions, which allows the light controlled switch ON transcription. Since recent publication with a click reaction of 5-ethynyluracil in major groove DNA represents chemical switch OFF transcription[79], a challenging task of equivalent enzymatically controlled reaction became a centre of our interest. For the purpose an enzyme 5-hydroxymethyluridine DNA Kinase (5-HMUDK) was chosen.

The 5-HMUDK is an enzyme for innovation isolated from a strain of *E. coli* which contains the gene from *Pseudomonas aeruginosa bacteriophage M6*. The DNA kinase transfers the γ-phosphate from ATP to the hydroxymethyl moiety of 5-hydroxymethyluridine nucleobase in DNA.[157-159] (***Scheme 40***)



Scheme 40: *Phoshorylation of U^{HM} moiety on DNA_U^{HM} by 5-HMUDK*

The first investigations for optimization the reaction conditions were taken on enzymatically synthesized **DNA_U^{HM}**.



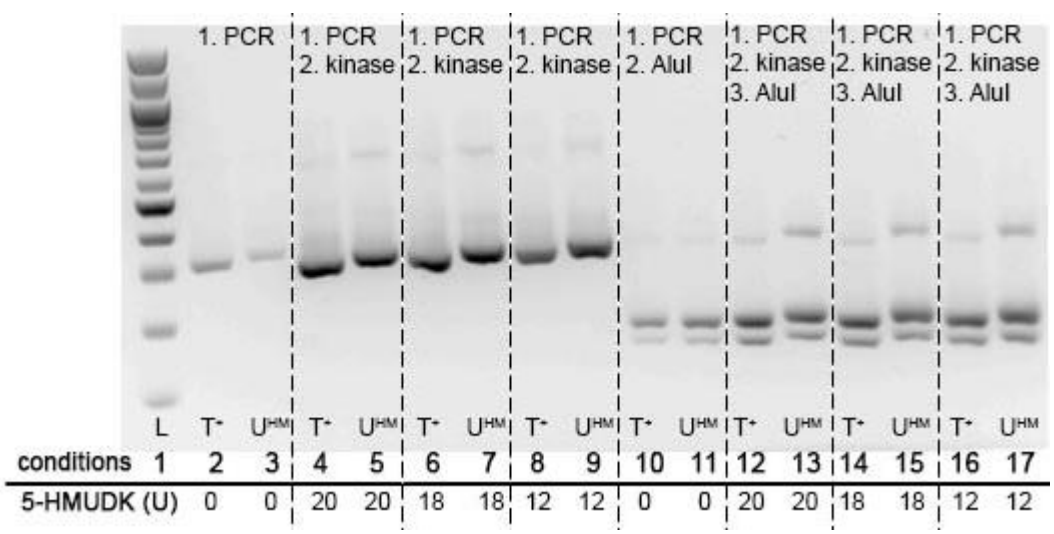
Scheme 41: *Phosphorylation of **DNA_U^{HM}** controlled by cleavage with REs*

The **DNA_U^{HM}** intended for the screening experiments was synthesized by PCR in the presence of KOD XL DNA polymerase under the conditions mentioned in section *6.3.3.1*. 180 ng of **DNA_U^{HM}** was after purification incubated with different amount of 5-HMUDK (20 U for exp. I; 18 U for exp. II and 12 U for exp. III) at 37°C for30 minutes. The DNA from each experiment was divided into few portions. First portions were loaded as a control on agarose gel. The other portions were incubated with AluI restriction enzyme and RsaI restriction enzyme. The rest of DNAs after phosphorylation reaction, from each experiment (exp. I; exp. II; exp. III), were used

as the DNA templates for transcription studies. To show the evidence of selective phosphorylation on **DNA_U^{HM}**, the natural DNA after PCR was examined under the same conditions as **DNA_U^{HM}**.

Since the phosphorylated DNA is resistant against cleavage by NcoI-HF restriction endonuclease, our idea of monitoring the reaction by cleavage with AluI and RsaI was based on it (***Scheme 41***). As readable from the agarose gel analysis (gel A, lines 13, 15, 17 in a gel), the control reaction of cleavage **DNA_U^{HM_P}** with AluI gave almost fully cleaved products same as natural DNA after phosphorylation reaction (gel A, lines 12, 14, 16 in an agarose gel). The results of cleavage reaction did not give the reliable information about the phosphorylation reaction on DNA. On the other hand, the control reaction of cleavage **DNA_U^{HM_P}** with RsaI did not give cleaved 193-mer products in any case. Natural DNA after incubation with 5-HMUDK and subsequent cleavage with RsaI (gel B, lines 12, 14, 16 in an agarose gel) provides cleaved products in all cases. The cleaved products (natural DNAs after incubation with 5-HMUDK) were not phosphorylated and therefore non-cleavage of **DNA_U^{HM_P}** with RsaI indicated successful phosphorylation of 5-hydroxymethylated moieties on **U**s in DNA.

A)



B)

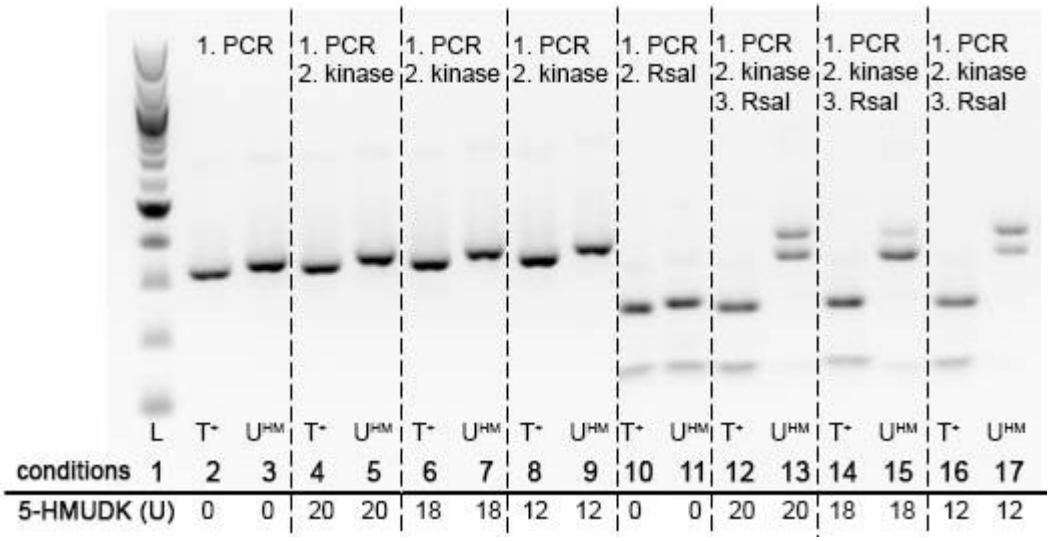
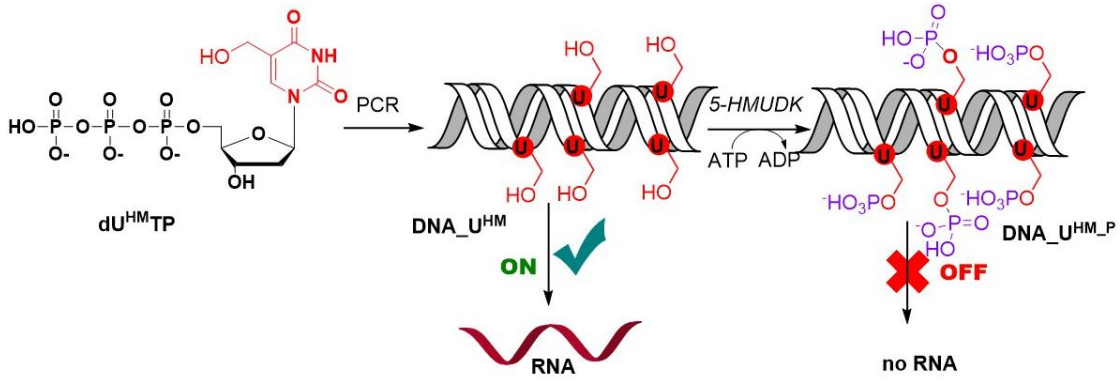


Figure 47: *Cleavage of* ***DNA_U^{HM_P}*** *by restriction enzyme* ***A)*** *AluI or* ***B)*** *RsaI:* On gels, the order of samples is: lanes 1, (**L**): ladder (commercial dsDNA with specific length); lanes 2, (**T^{+}**): natural PCR product; lanes 3, (**U^{HM}**): **U^{HM}** -modified PCR product; lanes 4, 6, 8: natural DNA after incubation with 20U/ 18U/ 12U of 5-HMUDK; lanes 5: HM-modified DNA after incubation with 20U/ 18U/ 12U of 5-HMUDK; lanes 10: natural DNA cleaved by restriction enzyme; lanes 11: HM-modified DNA cleaved by restriction enzyme; lanes 12, 14, 16: natural DNA after incubation with 20U/ 18U/ 12U of 5-HMUDK cleaved by restriction enzyme; lanes 13, 15, 17: **U^{HM}**-modified DNA after incubation with 20U/ 18U/ 12U of 5-HMUDK cleaved by restriction enzyme.

The phosphorylated DNAs from each experiment (exp. I; exp. II; exp. III) were also used as the DNA templates for transcription studies. The transcription experiment of phosphorylated DNAs were repeated three times and results of transcription graphically summarized in ***Scheme 42*** confirmed the observation from RsaI cleavage experiments (***Scheme 41***).



Scheme 42: *Study of transcription for phosphorylated DNA_U^{HM}*

The natural DNA did not undergo the phosphorylation reaction and therefore provided similar amount of transcript as the natural DNA before phosphorylation reaction. Phosphorylation of 5-hydroxymethylated DNA (**DNA_U^{HM_P}**) resulted into complete inhibition of transcription in comparison to the transcription of 5-hydroxymethylated DNA template in all cases

(***Figure 48***). All selected conditions for incubation of **DNA_U^{HM}** with 5-HMUDK were shown as appropriate to inhibit the process of transcription. The incubation of **DNA_U^{HM}** with 18U of 5-HMUDK was chosen for further investigations.

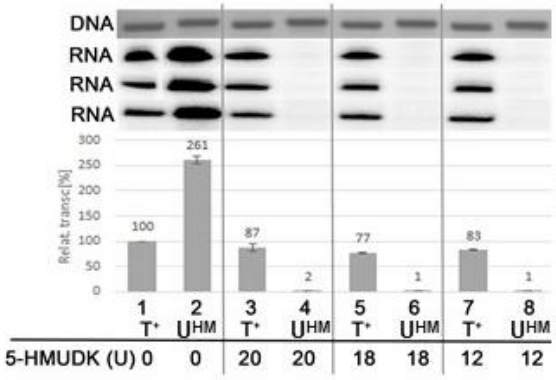
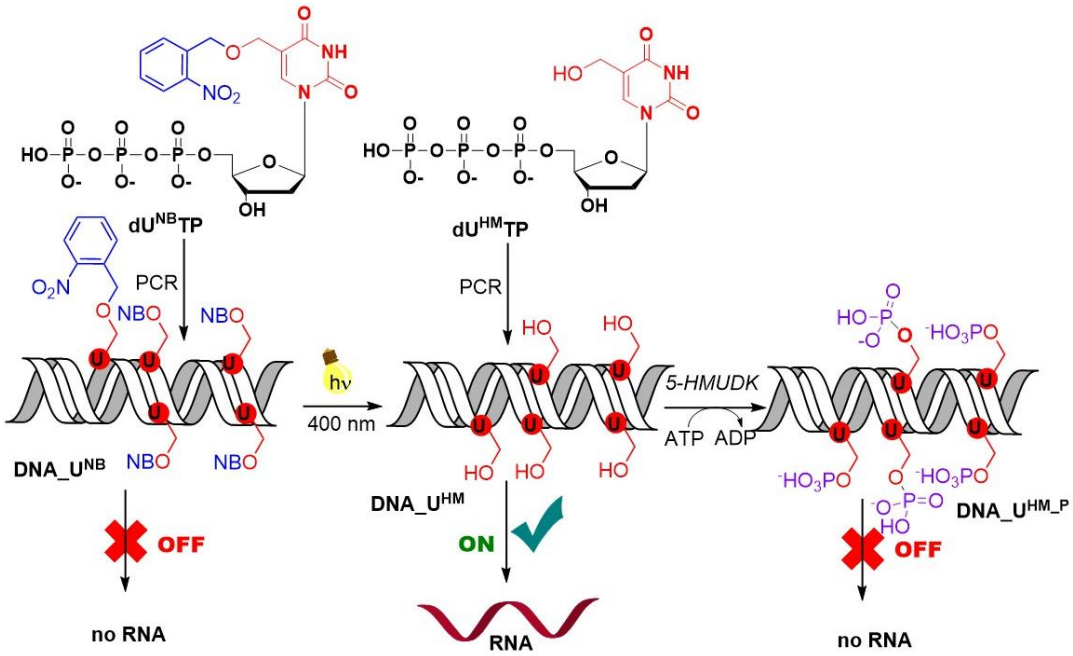


Figure 48: *Agarose gel analysis of phosphorylated DNA templates and results of their transcription study*

3.3.3.6 Controlled switch ON and switch OFF transcription

Based on the results of previous studies we designed the cascade of reactions on nitrobenzylated DNA, which would lead to the controlled regulation of transcription in three-steps.



Scheme 43: *Switching ON and OFF of transcription for U^{X}-modified DNA (X=HM or NB)*

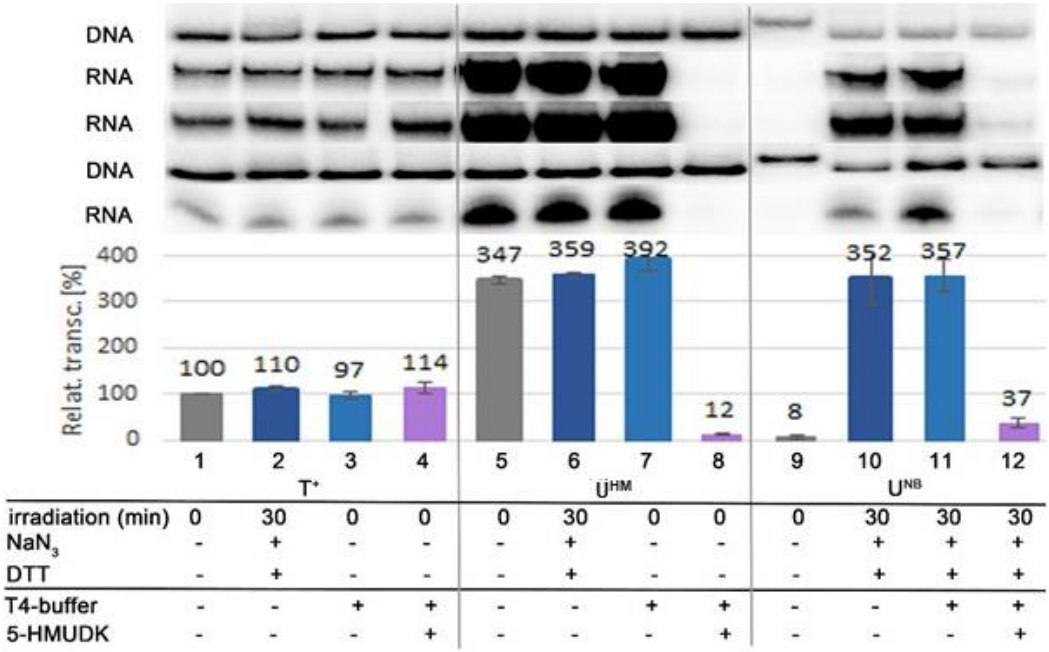
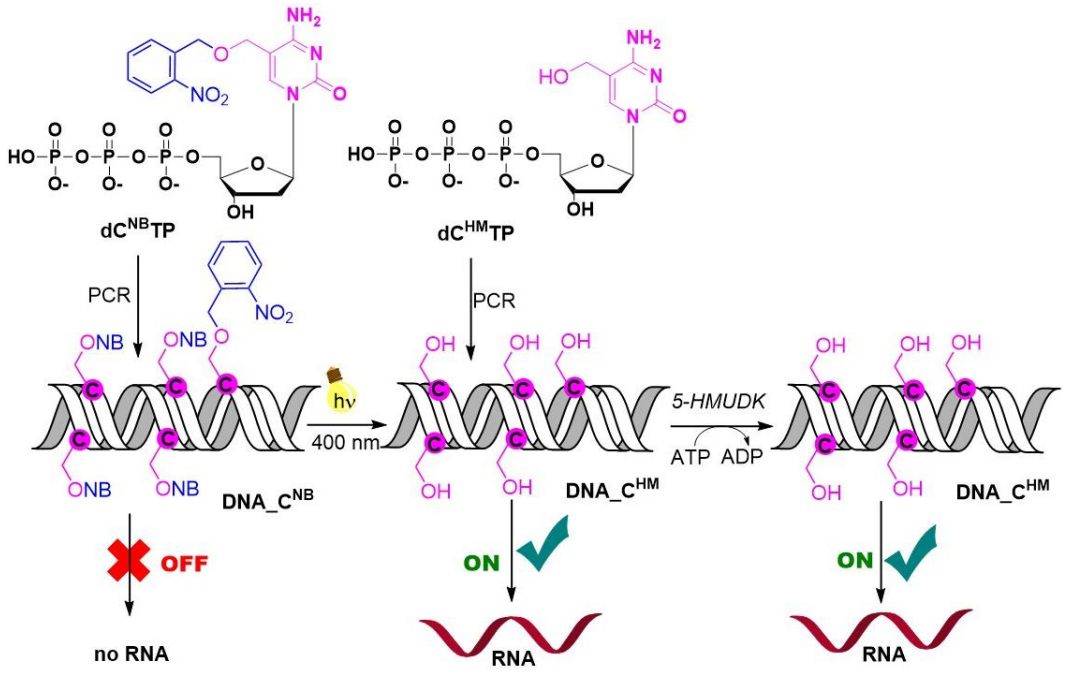


Figure 49: *Results of transcription for DNA templates prepared by light irradiation (400 nm, 3 W) and their incubation with 5-HMUDK (U^X-modified DNA; X=HM or NB)*

The 5-nitrobenzylated DNA - **DNA_U^{NB}** was prepared in preparative scale under optimized conditions by PCR reaction. The **DNA_U^{NB}**, which gives negligible amount of transcript, was irradiated by light with maximum wavelength at 400 nm for 30 minutes. The resulting **DNA_U^{HM}** template increased transcription to the expected approx. 350 % in comparison to the transcription level of natural DNA. The phosphorylation reaction of photodeprotected **DNA_U^{NB}** (**DNA_U^{HM}**) in the presence of 5-HMUDK (18 U) and ATP (**DNA_U^{HM_P}**) repeatedly silenced the transcription process to the 37 % compared to natural DNA. The transcript level of **DNA_U^{HM_P}** decreases to 10 % compared to the transcript level of initially irradiated **DNA_U^{NB}** (**DNA_U^{HM}**). (***Figure 49***)

To confirm, that achieved results related to deprotection and phosphorylation reactions of **DNA_U^{X}** (**DNA_U^{HM}**, **DNA_U^{HM_P}**) were not obtained due to attendance of additional agents, the control experiments were provided for natural and 5-hydroxymethylated (**DNA_U^{HM}**) DNAs. (***Figure 49***) Natural DNA irradiated by light at 400 nm for 30 minutes in the presence of DTT and NaN_3 (lane 2) as well as natural DNA incubated with 5_HMUDK and T4 Buffer with ATP (lanes 3, 4) gave the same level of transcription as natural DNA right after PCR (lane 1). Hydroxymethylated DNA (**DNA_U^{HM}**) irradiated by light at 400 nm for 30 minutes in the presence of DTT and NaN_3 (lane 6) as well as **DNA_U^{HM}** in the presence of T4 Buffer with ATP (lane 7) gave the same (high) level of transcription as **DNA_U^{HM}** right after PCR (lane 5). As obvious from graphical representation the amount of RNA the obtained results of

transcription of $\mathbf{U^X}$-modified DNA are only due to the chemical and enzymatical reactions on $\mathbf{U^X}$-moiety not due to influence of additives.



Scheme 44: *Switching ON of transcription for C^X-modified DNA (X=HM or NB)*

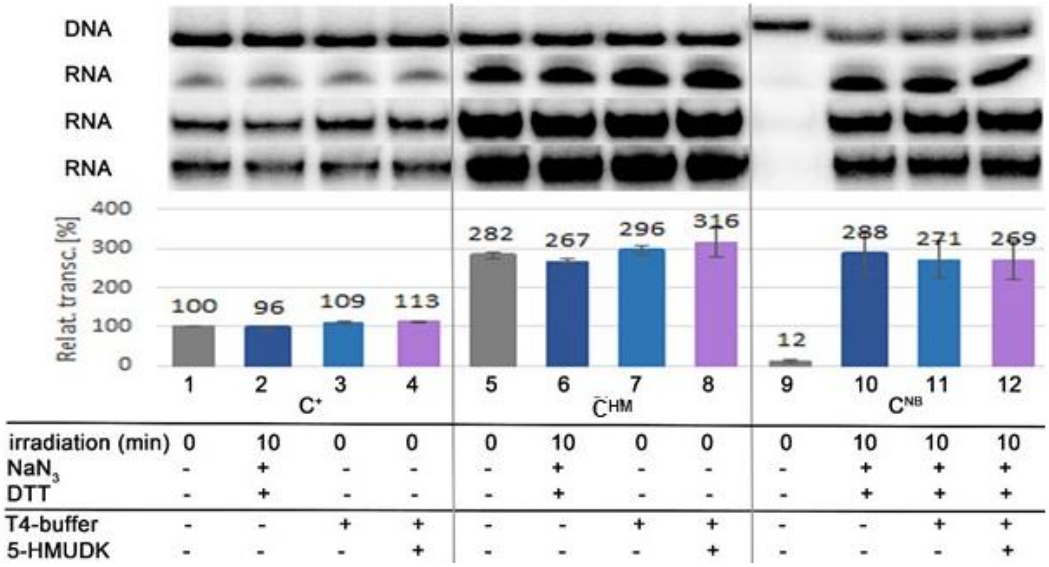


Figure 50: *Results of transcription for DNA templates prepared by light irradiation (400 nm, 3 W) and their incubation with 5-HMUDK (C^X-modified DNA; X=HM or NB)*

Analogously, the $\mathbf{C^X}$-modified DNA was examined under the similar conditions of photocleavage and phosphorylation reactions. The **DNA_C^{NB}** template (which gives negligible amount of RNA) was irradiated by light at 400 nm for 10 minutes. The resulting photodeprotected **DNA_C^{NB}** (**DNA_C^{HM}**) template caused the increasing of transcription level

to ca. 250 % in comparison to the transcription level of natural DNA. Incubation of deprotected **DNA_C^{HM}** in the presence of 5-HMUDK (18 U) and T4 buffer with ATP did not lead to phosphorylation of 5-hydroxymethyl moieties and transcription of resulted **DNA_C^{HM}** template proceeded in the same level as initial **DNA_C^{HM}**. The kinase (5-HMUDK) selectively phosphorylate the 5 - hydroxymethyl moieties just on uridines incorporated to the DNA.

The natural and **DNA_C^{HM}** templates were exposed to the same chemical and enzymatical reactions as **DNA_C^{NB}**. As in the previous case of control reactions with **U^{X}**-modified DNA, no influence of additives on the obtained results of transcription for **C^{X}-**modified DNA was determined.

In conclusion, we optimized the conditions of photocleavage of functionalized nitrobenzylated DNAs (**DNA_N^{NB}**). We found out, that nucleic acids with bulky NB- modifications in the sequence inhibit the process of transcription. Simple photolysis of the nitrobenzyl- moieties in nucleic acids (**DNA_N^{NB}**) lead to the formation of DNAs bearing just small hydroxymethyl-moieties (**DNA_N^{HM}**) which are recognized and tolerated by bacterial RNAP and the process of transcription is activated. Moreover, in the case of **DNA_U^{NB}**, we have developed third step of switching OFF the hmU-containing DNA template through enzymatic phosphorylation reaction - **DNA_U^{HM_P}**. So, in this case we can switch ON the transctiption and then switch it OFF again at will.

4 Conclusions

I have developed the synthesis of 2′-deoxyribonucleoside triphosphates modified with *o*-nitrobenzyl photoremovable protecting group (**dU^{NB}TP**, **dC^{NB}TP**, **dA^{NB}TP**) and demonstrated that they can be efficiently used for polymerase synthesis of major-groove photocaged nucleic acids (DNA) by PEX (for short DNAs containing a few modifications in one strand) and by PCR (for long dsDNAs with many modifications in both strands). Hydroxymethyl-modified 2′-deoxyribonucleoside triphosphates were also prepared by simple photolysis of corresponding nitrobenzyl-caged nucleotides and they were found to be excellent substrates for tested DNA polymerases in PEX and PCR. The nitrobenzyl- modified DNAs (**DNA_N^{NB}**) are fully protected against cleavage by all tested REs. In the case of **DNA_N^{HM}**, where the fully cleavage of **DNA_N^{HM}** was observed (AfT/ PvT /KpT/ RsT /KpC /RsC/ KpA/ RsA), the systematic study of photocleavage of NB- moieties in caged **DNA_N^{NB}** by light (365 nm, 1 mW) was performed. In all cases the fully cleavage of formed **DNA_N^{HM}** by REs was observed after previous several hours irradiation of initial **DNA_N^{NB}**. The same studies of photodeprotection and following cleavage by RsaI were performed also on long **U^{NB}**-modified dsDNA synthesized by PCR. The fully cleavage of long **U^{NB}**-modified DNA was observed after its previous irradiation for 24 hours without any damage in the DNA sequence. Naturally occurring **DNA_N^{HM}** can be synthesized directly by polymerase incorporation of **dN^{HM}TP** or indirectly through biorthogonal photocleavage reaction of **DNA_N^{NB}**. Based on our detailed studies, we found out that photocaged **DNA_U^{NB}** synthesized by PCR is fully resistant against cleavage by REs, it is replicable by KOD XL DNA polymerase in tube end even as a part of plasmid in *E. coli*. It was the first example of photocaging of DNA in the major groove to protect it against RE cleavage.[150]

Using this approach of temporary masking and modulation of DNA templates, the transcription regulation studies has been a major challenge for our further research. In the preliminary study of transcription of **N^{HM}-** and **N^{NB}**- modified DNA templates with P*veg* promoter region (***Figure 28***) we found out, that **DNA_N^{NB}** templates display strong inhibitory effect on transcription in comparison to **DNA_N^{HM}** templates, which are nicely tolerated by RNAP. In addition, **DNA_U^{HM}** and **DNA_C^{HM}** templates unexpectedly showed a significant increase of transcription compared to natural DNA templates. In order to uncover the role of the epigenetic modifications (**hmU**, **hmC**, **mC**, **dU**) in the *in vitro* transcription by bacterial RNAP, we prepared different types of DNA templates with four different promoter regions and the same transcribed region. The results of *in vitro* experiments showed that just

hydroxymethylated **DNA**, either **DNA_U^{HM}** or **DNA_C^{HM},** with P*veg* promoter region have enhancing effect on transcription. The presence of any tested modification in the DNA template with *rrnBP1* promoter region inhibited transcription. Since the amount of transcript for chimeric DNA templates were observed at the lower or at most the same level as for natural DNA template, the results of study suggest that both parts of the promoter region have a decisive influence on transcription. In order to clarify, which part of DNA template have the greatest effect on the transcription process, the partially modified DNA templates with the P*veg* promoter region were synthesized in the presence of four epigenetic modifications by combining several enzymatic reactions: PCR, PEX, ligation, digestion. Based on similar transcription results in the case of fully and partially modified DNA templates, we found out that modifications in the promoter region of DNA templates, especially in its non-template (coding) strand have huge influence on transcription. The results of our systematic study of transcription from fully or partially modified DNA templates containing the epigenetic bases may significantly contribute to the understanding of their biological role.

Finally, we developed an optimized system of photochemical and enzymatic reactions in the major groove of DNA, through which we are able to regulate (switch ON or OFF) an *in vitro* transcription process by bacterial RNAP in three steps. For the purpose, the fully modified DNA templates with P*veg* promoter region were synthesized by PCR in the presence of HM- or NB- modified pyrimidine nucleotides. As obvious, bulky NB- moieties in DNA templates (**DNA_U^{NB}** or **DNA_C^{NB}**) fully inhibits transcription in comparison to natural DNA (switch OFF). Photolysis of either **DNA_U^{NB}** or **DNA_C^{NB}** by light (400 nm, 3 W) lead to deprotection of bulky modifications and consecutive activation of transcription process (switch ON). In addition, enzymatic phosphorylation (by 5-HMUDK) on hydroxymethyl moieties of activated **DNA_U^{NB}** template (**DNA_U^{HM_P}**) switched the transcription off again (switch OFF). The next logical step could be to find the way for enzymatic dephosphorylation of **DNA_U^{HM_P}** (i.e. through phosphatase) and repeated activation of transcription. In the case of activated **DNA_C^{NB}** (**DNA_C^{HM}**), the second switching OFF transcription through phosphorylation step does not work, since 5-HMUDK reacts specifically with **U^{HM}**. Our research demonstrated the proof-of-concept of articifial chemical epigenetics based on biorthogonal chemical and enzymatic reactions in the major groove of DNAs. The results of our systematic investigation open new possibilities for design and synthesis of modified nucleotides, thus further advance research in the field of artificial chemical regulation of transcription and gene expression.

5 List of publications

1. Vaníková, Z.; Hocek, M.: “Polymerase synthesis of photocaged DNA resistant against cleavage by restriction endonucleases.” *Angew. Chem. Int. Ed.* **2014,** *53*, 6734-6737.

2. Janoušková, M.; Vaníková, Z.; Nici, F.; Boháčová, S.; Vítovská, D.; Šanderová, H.; Hocek, M.; Krásný, L.: "5-(Hydroxymethyl)uracil and -cytosine as potential epigenetic marks enhancing or inhibiting transcription with bacterial RNA polymerase" *Chem. Commun.* **2017**, *53*, 13253 - 13255.

3. Boháčová, S.; Ludvíková, L.; Poštová Slavětínská, L.; Vaníková, Z.; Klán, P.; Hocek, M.: "Protected 5-(hydroxymethyl)uracil nucleotides bearing visible-light photocleavable groups as building blocks for polymerase synthesis of photocaged DNA" *Org. Biomol. Chem.* **2018**, *16*, 1527 - 1535.

4. Boháčová, S.; Vaníková, Z.; Poštová Slavětínská, L.; Hocek, M.: "Protected 2'-deoxyribonucleoside triphosphate building blocks for photocaging of epigenetic 5-(hydroxymethyl)cytosine in DNA" *Org. Biomol. Chem.* **2018**, *16*, 5427-5432.

5. Vaníková, Z.; Janoušková, M.; Kambová, M.; Krásný, L.; Hocek, M.: “Switching transcription with bacterial RNA polymerase through photocaging, photorelease and phosphorylation reactions in the major groove of DNA.” *Chem. Sci.* **2019,** *10*, 3937-3942.

6 Experimental section

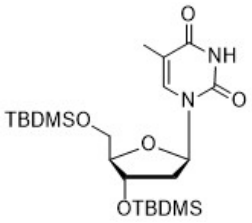
General remarks

Reagents for synthesis all compounds were purchased from commercial suppliers. Compounds protected with photolabile protecting groups were synthesized under argon in glass flasks which walls were covered with aluminium foil. NMR spectra of synthesized compounds were recorded on Bruker Avance 400 MHz spectrometer (400.1 MHz for 1H and 100.6 MHz for 13C) or Bruker Avance 500 MHz spectrometer (499.8 MHz for 1H, 125.7 MHz for 13C, 202.3 MHz for 31P) or 600 MHz (600.1 MHz for ^{1}H, 150.9 MHz for ^{13}C) in DMSO-*d6* or MeOH-*d4* or $CDCl_3$ or in D_2O. Coupling constants (*J*) are given in Hz and chemical shifts in ppm (δ scale). By the combination of measured two-dimensional NMR spectra data (H, H-COSY; H, C-HSQC and H, C-HMBC) achieved the complete assignment of NMR signals and the final structure of synthesized compounds was confirmed. Mass spectra and high-resolution mass spectra of products, monophosphates and triphosphates were measured using electrospray ionization technique (ESI). Mass spectra of modified DNAs were measured by MALDI-TOF, Reflex IV (Bruker) with nitrogen laser. MALDI-TOF mass spectrometry was used also for kinetic photolysis study of U^{NB}-modified DNA. HM-modified triphosphates and monophosphates were prepared by photolysis of corresponding NB-modified nucleotides by light irradiation from Stringer UV LED (LZ4-00U600; 366 nm; 0.8 W; 150.8 mW/cm^2). Nucleosides, nucleoside monophosphates and triphosphates were purified by HPLC on a column packed with 10 μm C18 reversed phase (Phenomenex, Luna C18 (2)). Sample elution was monitored by absorbance at 254 or 264 nm. Primers, templates and biotinylated templates were purchased from commercial suppliers (Generi Biotech, Sigma Aldrich, IBA Lifesciences). Set of natural 2´-deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP; 100 mM) was purchased from New England Biolabs. Polymerases, KOD XL polymerase was purchased from Merck, Vent (exo-) – from New England Biolabs, Pwo from Peqlab. All used restriction endonucleases were bought from New England Biolabs. Streptavidine magnetic particles were purchased from Roche. DNAs after PCR reaction were mostly purified by QIAquick® PCR purification Kit (Qiagen) or by Agencourt AMPure XP magnetic particles (Beckman Coulter Life Science - GE Healthcare).

6.1 Synthesis of photocaged nucleosides and nucleotides

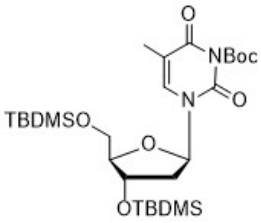
6.1.1 Synthesis of hydroxymethyl- or bromomethyl- modified uridine

3′, 5′-Bis-O-(tert-butyldimethylsilyl)thymidine



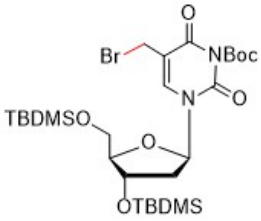
A protection of thymidine was performed under the published conditions and spectral data were in accordance with literature. [149] Yield: 95 %-99 %

3′, 5′-Bis-O-(tert-butyldimethylsilyl)-N^3-(tert-butoxycarbonyl)thymidine



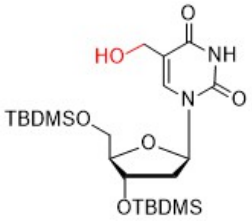
A *Boc*- protection of amino group in TBDMS-protected thymidine was performed under the published conditions and spectral data were in accordance with literature. [149] Yield: 66 %

3′, 5′-Bis-O-(tert-butyldimethylsilyl)- N^3-(tert-butoxycarbonyl)- 5-bromomethyl-2′-deoxyuridine



A bromination of protected thymidine was performed under the published conditions (in benzene or CCl_4 as a solvent). The spectral data were in accordance with literature.[149] Yield: 16 %

3′, 5′-Bis-O-(tert-butyldimethylsilyl)-5-(hydroxymethyl)-2′-deoxyuridine



A hydroxymethyl- modified TBDMS-protected uridine was prepared in two steps through its 5-(bromomethyl) derivative under the published conditions. The spectral data were in accordance with literature.[152]

Yield: 14 %-27 %

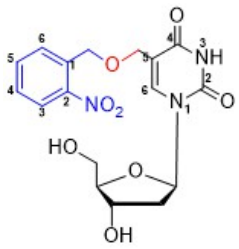
6.1.2 Synthesis of photolabile nitrobenzyl- modified and hydroxymethyl- modified nucleos(t)ides

6.1.2.1 Synthesis of NB- modified and HM- modified uridine nucleos(t)ides

5-(2-Nitrobenzyloxymethyl)-2´-deoxyuridine was prepared by using two approaches. ***Method 1*** describes direct two steps synthesis of fully unprotected **dU^{NB}** from freshly synthesized *3´, 5´-Bis-O-(tert-butyldimethylsilyl)- N^3-(tert-butoxycarbonyl)- 5-bromomethyl-2´-deoxyuridine* without an intermediate isolation. Preparation of *5-(2-nitrobenzyloxymethyl)-2´-deoxyuridine* from *3´, 5´-Bis-O-(tert-butyldimethylsilyl)-5-hydroxymethyl-2´-deoxyuridine* in two steps is described by ***Method 2***. In ***Method 2***, the TBDMS-protected **dU^{NB}** created in the first step was isolated by column chromatography and used as a starting material for its deprotection in the presence of $Et_3N.3HF$. Protected *3´, 5´-Bis-O-(tert-butyldimethylsilyl)-5-(2-nitrobenzyloxymethyl)-2´-deoxyuridine* synthesized by ***Method 2*** was also used as a starting material for the synthesis of *3´, 5´-Bis-O-(tert-butyldimethylsilyl)-5-(2-nitrobenzyloxymethyl)-2´-deoxycytidine*.

Method 1:

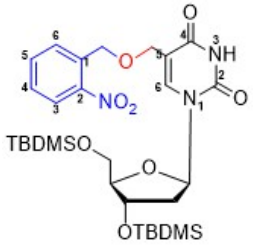
5-(2-Nitrobenzyloxymethyl)-2´-deoxyuridine



3´,5´-Bis-O-(tert-butyldimethylsilyl)-N^3-(tert-butyloxycarbonyl)-5-bromomethyl-2´-deoxyuridine (250 mg; 0.39 mmol) and 2-nitrobenzyl alcohol (447 mg; 2.92 mmol) were heated at 105 °C for 15 min under an argon atmosphere. The cool mixture was dissolved in dry THF (6 mL) followed by addition of $Et_3N.3HF$ (50 μL; 0.307 mmol). Reaction mixture was stirred at room temperature for three hours. After its concentration *in vacuum*, the residue was purified by flash silica gel chromatography (CH_2Cl_2/CH_3OH from 50/1 to 5/1) and next purification by HPLC gradient flash chromatography (dissolved in DMSO/water = 4/1; gradient: water/methanol). Pure product, 5-(2-nitrobenzyloxymethyl)-2´-deoxyuridine, was yielded as a pale yellow solid (30 mg; 20 %). The spectral data were in accordance with literature. [149]

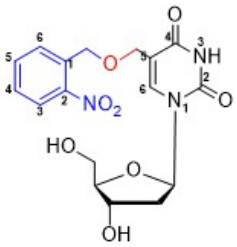
Method 2:

3′, 5′-Bis-O-(tert-butyldimethylsilyl)-5-(2-nitrobenzyloxymethyl)-2′-deoxyuridine



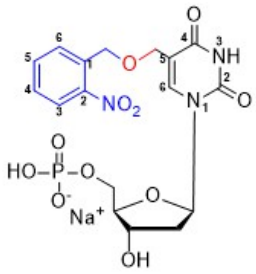
A 2-nitrobenzyl bromide (453 mg; 2.1 mmol) was dissolved in anhydrous DCM (3 ml). The solution of 2-nitrobenzyl bromide was added dropwise at 0 °C into a mixture of *3′, 5′-Bis-O-(tert-butyldimethylsilyl)-5-hydroxymethyl-2′-deoxyuridine* (270 mg; 0.56 mmol) in anhydrous DCM (12 ml) with 2, 6-di-*tert*-butylpyridine (300 µL; 1.34 mmol) and silver triflate (199 mg; 0.78 mmol). The reaction mixture was stirred at room temperature for 3 hours under argon atmosphere and concentrated in vacuum. The residue was purified by gradient column chromatography (gradient from 100 % DCM to 25:1 DCM:MeOH) to yield protected 5-(2-nitrobenzyloxymethyl)-2′-deoxyuridine (130 mg; 19-35 %). The spectral data were in accordance with literature. [147]

5-(2-Nitrobenzyloxymethyl)-2′-deoxyuridine



3′, 5′-Bis-O-(tert-butyldimethylsilyl)-5-(2-nitrobenzyloxymethyl)-2′-deoxyuridine was dissolved in dry THF (4 mL) followed by addition of triethylamine trihydrofluoride ($Et_3N.3HF$, 50 µL; 0.307 mmol). Reaction mixture was stirred in plastic pot at room temperature for three hours. After mixture concentration *in vacuum*, the residue was purified by flash silica gel chromatography (CH_2Cl_2/CH_3OH from 50/1 to 5/1) and afterwards by HPLC gradient flash chromatography (dissolved in DMSO/water; gradient: water/methanol). The *5-(2-nitrobenzyloxymethyl)-2′-deoxyuridine* was yielded as a pale yellow solid (24 mg; 49 %). The spectral data were in accordance with literature. [149]

5-(2-Nitrobenzyloxymethyl)-2′-deoxyuridine-5′-monophosphate ($dU^{NB}MP$)



5-(2-Nitrobenzyloxymethyl)-2′-deoxyuridine (27 mg; 0.069 mmol) was dried at 80 °C for 15 minutes under vacuum. In the next step, the vial with starting material was cooled to room temperature and proton sponge (13 mg; 0.062 mmol) was added to the vial promptly. The content was again evacuated and then phosphorylated with $POCl_3$ (10 µL; 0.103 mmol) in trimethylphosphate (0.3 mL) at 0 °C for 1 hour under an argon atmosphere. After 1 hour of stirring, the reaction was quenched by addition of aqueous TEAB (2 M, 2 mL). The solvents were evaporated in vacuum and the residue was co-distilled several times with water. The product was isolated on HPLC (linear gradient: 0.1 M TEAB 100 % H_2O/ 0.1 M

TEAB 50 % MeOH/ 100 % MeOH), evaporated, co-distilled with water and lyophilized to yield sodium salt of **dU^{NB}MP** as a white powder (21 mg; 62 %).[150]

^{1}H NMR (500.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.34 (ddd, 1H, J_{gem} = 14.0, $J_{2'b,1'}$ = 6.6, $J_{2'b,3'}$ = 4.1, H-2'b); 2.39 (ddd, 1H, J_{gem} = 14.0, $J_{2'a,1'}$ = 7.3, $J_{2'a,3'}$ = 6.0, H-2'b); 3.93 (dd, 2H, $J_{H,P}$ = 5.3, $J_{5',4'}$ = 4.5, H-5'); 4.13 (td, 1H, $J_{4',5'}$ = 4.5, $J_{4',3'}$ = 3.1, H-4'); 4.44 (s, 2H, OCH_2-5); 4.55 (ddd, 1H, $J_{3',2'}$ = 6.0, 4.1, $J_{3',4'}$ = 3.1, H-3'); 4.93, 4.96 (2 × d, 2 × 1H, J_{gem} = 13.4, OCH_2-1); 6.27 (dd, 1H, $J_{1',2'}$ = 7.3, 6.6, H-1'); 7.54 (ddd, 1H, $J_{4,3}$ = 8.2, $J_{4,5}$ = 7.4, $J_{4,6}$ = 1.4, H-4-$C_6H_4NO_2$); 7.66 (dd, 1H, $J_{6,5}$ = 7.7, $J_{6,4}$ = 1.4, H-6-$C_6H_4NO_2$); 7.71 (ddd, 1H, $J_{5,6}$ = 7.7, $J_{5,4}$ = 7.4, $J_{5,3}$ = 1.0, H-5-$C_6H_4NO_2$); 7.97 (s, 1H, H-6); 8.02 (dd, 1H, $J_{3,4}$ = 8.2, $J_{3,5}$ = 1.0, H-3-$C_6H_4NO_2$).

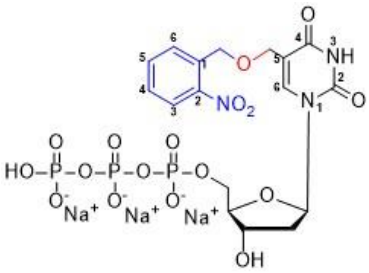
^{13}C NMR (125.7 MHz, D_2O, ref(dioxane) = 69.3 ppm): 41.19 (CH_2-2'); 66.35 (d, $J_{C,P}$ = 4.5, CH_2-5'); 68.37 (OCH_2-5); 71.58 (OCH_2-1); 73.85 (CH-3'); 87.97 (CH-1'); 88.81 (d, $J_{C,P}$ = 8.3, CH-4'); 113.69 (C-5); 127.60 (CH-3-$C_6H_4NO_2$); 131.76 (CH-4-$C_6H_4NO_2$); 133.38 (CH-6-$C_6H_4NO_2$); 135.84 (C-1-$C_6H_4NO_2$); 136.65 (CH-5-$C_6H_4NO_2$); 144.41 (CH-6); 150.60 (C-2-$C_6H_4NO_2$); 155.23 (C-2); 168.97 (C-4).

^{31}P{^{1}H} NMR (202.4 MHz, D_2O): 3.83.

MS (ESI$^-$): *m/z:* 472.3 [M-Na]$^-$

HRMS (ESI$^-$): For the molecular ion $C_{17}H_{19}N_3O_{11}P$ [M-Na]$^-$, the calculated mass was 472.07627, and the observed mass was 472.07637.

5-(2-Nitrobenzyloxymethyl)-2´-deoxyuridine-5´-triphosphate (dU^{NB}TP)



5-(2-Nitrobenzyloxymethyl)-2´-deoxyuridine (23 mg; 0.058 mmol) was dried at 80 °C for 15 minutes under vacuum. Next, the vial with starting material was cooled to the room temperature and proton sponge (11 mg; 0.053 mmol) was added to the vial. The content was again evacuated and suspended in trimethylphosphate (0.17 mL) at 0 °C under argon atmosphere. $POCl_3$ (6 µL; 0.06 mmol) was added to the reaction mixture and the mixture was then stirred at 0 °C for 1 hour. An ice-cooled solution of tri-*n*-butylammonium pyrophosphate (162 mg; 0.29 mmol) in anhydrous DMF (0.5 mL) was added to the reaction mixture. After 1 hour of stirring, the solution was quenched by addition of aqueous TEAB (2 M, 2 mL). The solvents were evaporated in vacuum and the residue was co-distilled several times with water. The product was isolated on HPLC (linear gradient: 0.1 M TEAB 100 % H_2O/ 0.1 M TEAB 50 % MeOH/ 100 % MeOH), evaporated, co-distilled with water and lyophilized to yield sodium salt of **dU^{NB}TP** as a white powder (14 mg; 35 %). [149, 150]

^{1}H NMR (500.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.37 (dd, 2H, $J_{2',1'}$ = 6.6, $J_{2',3'}$ = 5.6, H-2'); 4.19 (m, 1H, H-4'); 4.21 (ddd, 1H, J_{gem} = 11.6, $J_{H,P}$ = 8.1, $J_{5'b,4'}$ = 4.9, H-5'b); 4.27 (ddd, 1H, J_{gem} = 11.6, $J_{H,P}$ = 6.3, $J_{5'a,4'}$ = 3.6, H-5'a); 4.41, 4.47 (2 × d, 2 × 1H, J_{gem} = 12.1, OCH_2-5); 4.67 (td, 1H, $J_{3',2'}$ = 5.6, $J_{3',4'}$ = 3.7, H-3'); 4.91, 4.97 (2 × d, 2 × 1H, J_{gem} = 13.3, OCH_2-1); 6.26 (t, 1H, $J_{1',2'}$ = 6.6, H-1'); 7.54 (ddd, 1H, $J_{4,3}$ = 8.2, $J_{4,5}$ = 7.6, $J_{4,6}$ = 1.3, H-4-$C_6H_4NO_2$); 7.65 (dd, 1H, $J_{6,5}$ = 7.6, $J_{6,4}$ = 1.3, H-6-$C_6H_4NO_2$); 7.71 (td, 1H, $J_{5,4}$ = $J_{5,6}$ = 7.6, $J_{5,3}$ = 1.1, H-5-$C_6H_4NO_2$); 7.95 (s, 1H, H-6); 8.01 (dd, 1H, $J_{3,4}$ = 8.2, $J_{3,5}$ = 1.1, H-3-$C_6H_4NO_2$).

^{13}C NMR (125.7 MHz, D_2O, ref(dioxane) = 69.3 ppm): 41.38 (CH_2-2'); 67.79 (d, $J_{C,P}$ = 5.6, CH_2-5'); 68.39 (OCH_2-5); 71.64 (OCH_2-1); 72.86 (CH-3'); 87.93 (CH-1'); 88.37 (d, $J_{C,P}$ = 8.9, CH-4'); 113.37 (C-5); 127.60 (CH-3-$C_6H_4NO_2$); 131.78 (CH-4-$C_6H_4NO_2$); 133.63 (CH-6-$C_6H_4NO_2$); 135.85 (C-1-$C_6H_4NO_2$); 136.65 (CH-5-$C_6H_4NO_2$); 144.24 (CH-6); 150.66 (C-2-$C_6H_4NO_2$); 154.50 (C-2); 167.96 (C-4).

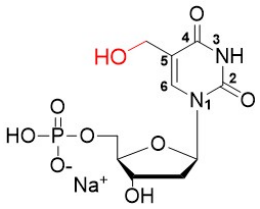
^{31}P{^{1}H} NMR (202.3 MHz, D_2O): -21.41 (bm, Pβ); -10.75 (d, J = 19.3, Pα); -5.44 (bm, Pγ).

MS (ESI$^-$): *m/z:* 472.1 [M-2PO_3Na-Na]$^-$; 552.1 [M- PO_3Na-2Na+H]; 574.1 [M-PO_3Na-Na]$^-$; 676.11[M-Na]$^-$; 698.1 [M-H]$^-$

HRMS (ESI$^-$): For the molecular ion $C_{17}H_{18}O_{17}N_3Na_3P_3$ [M-H]$^-$, the calculated mass was 697.95476, and the observed mass was 697.95460.

For the molecular ion $C_{17}H_{19}O_{17}N_3Na_2P_3$ [M-Na]$^-$, the calculated mass was 675.97282, and the observed mass was 675.97243

5-Hydroxymethyl-2´-deoxyuridine-5´-monophosphate (dU^{HM}MP)



A 16 mM colourless solution of **dU^{NB}MP** (10 mg) in H_2O (1.26 mL) was irradiated by UV light emitting diode (LED; 366 nm; 0.8 W; 150.8 mW/cm^2) in vial from quartz glass with stirring. Kinetic of cleavage was observed by analytical HPLC for 40 minutes. Product was isolated by semipreparative HPLC on C18 column with the use of linear gradient (0.1 M TEAB 100 % H_2O/0.1 M TEAB 50 % MeOH /100 %MeOH). Several co-distillations with water, conversion to sodium salt form (Dowex 50WX8 in Na^+ cycle) followed by freeze drying in water gave the product - **dU^{HM}MP** as a white powder in 74% yield (5.4 mg). [150]

^{1}H NMR (500.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.39 (ddd, 1H, J_{gem} = 14.1, $J_{2'b,1'}$ = 6.5, $J_{2'b,3'}$ = 4.1, H-2'b); 2.43 (ddd, 1H, J_{gem} = 14.1, $J_{2'a,1'}$ = 6.9, $J_{2'a,3'}$ = 5.9, H-2'b); 3.95 (ddd, 1H, J_{gem} = 11.6, $J_{H,P}$ = 4.7, $J_{5'b,4'}$ = 3.1, H-5'b); 4.00 (ddd, 1H, J_{gem} = 11.6, $J_{H,P}$ = 3.9, $J_{5'a,4'}$ = 3.1, H-5'a); 4.18 (qd, 1H, $J_{4',5'}$ = $J_{4',3'}$ = 3.1, $J_{H,P}$ = 1.8, H-4'); 4.39 (s, 2H, CH_2O); 4.61 (m, 1H, H-3'); 6.33 (dd, 1H, $J_{1',2'}$ = 6.9, 5.9, H-1'); 6.19 (s, 1H, H-6).

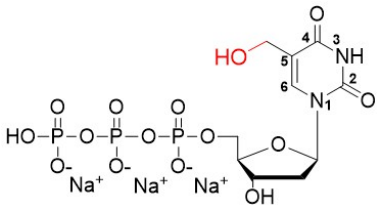
^{13}C NMR (125.7 MHz, D_2O, ref(dioxane) = 69.3 ppm): 41.90 (CH_2-2'); 58.80 (CH_2O); 66.16 (d, $J_{C,P}$ = 4.7, CH_2-5'); 73.77 (CH-3'); 88.27 (CH-1'); 89.27 (d, $J_{C,P}$ = 8.3, CH-4'); 116.74 (C-5); 142.85 (CH-6); 154.41 (C-2); 167.94 (C-4).

^{31}P{^{1}H} NMR (202.4 MHz, D_2O): 3.32.

MS (ESI$^-$): *m/z:* 337.2 [M-Na]$^-$; 359.2 [M-H]$^-$, 360.2 [M];

HRMS (ESI$^-$): For the molecular ion $C_{10}H_{14}N_2O_9Na$ [M-Na]$^-$, the calculated mass was 337.04424, and the observed mass was 337.04439.

5-Hydroxymethyl-2´-deoxyuridine-5´-triphosphate (dU^{HM}TP)



A 16 mM colourless solution of **dU^{NB}TP** (9 mg) in H_2O (0.81 mL) was irradiated by UV (LED; 366 nm; 0.8 W; 150.8 mW/cm^2) under the same conditions as above. Kinetic of cleavage was also observed by analytical HPLC for 50 minutes. Desired **dU^{HM}TP** was isolated by semipreparative HPLC on C18 column with the use of linear gradient (0.1 M TEAB 100 % H_2O/0.1 M TEAB 50 % MeOH /100 % MeOH). After evaporation, several co-distillations with water, conversion to sodium salt form (Dowex 50WX8 in Na^+ cycle) followed by freeze drying in water gave the product - **dU^{HM}TP** as a white powder in 62 % yield (4.5 mg).[150]

^{1}H NMR (500.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.40 (m, 2H, H-2'); 4.18 (m, 1H, H-4'); 4.22 (ddd, 1H, J_{gem} = 11.8, $J_{H,P}$ = 4.2, $J_{5'b,4'}$ = 3.0, H-5'b); 4.27 (ddd, 1H, J_{gem} = 11.8, $J_{H,P}$ = 5.7, $J_{5'a,4'}$ = 3.3, H-5'a); 4.39 (s, 2H, CH_2O); 4.70 (m, 1H, H-3'); 6.33 (t, 1H, $J_{1',2'}$ = 6.5, H-1'); 8.00 (s, 1H, H-6).

^{13}C NMR (125.7 MHz, D_2O, ref(dioxane) = 69.3 ppm): 41.55 (CH_2-2'); 59.22 (CH_2O); 67.69 (d, $J_{C,P}$ = 5.2, CH_2-5'); 72.80 (CH-3'); 87.96 (CH-1'); 88.37 (d, $J_{C,P}$ = 9.1, CH-4'); 116.66 (C-5); 142.54 (CH-6); 154.98 (C-2); 168.67 (C-4).

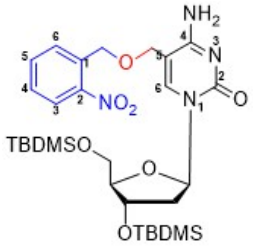
^{31}P{^{1}H} NMR (202.3 MHz, D_2O): -21.36 (dd, J = 20.4, 19.5, P$_\beta$); -10.59 (d, J = 19.5, P$_\alpha$); -5.43 (d, J = 20.4, P$_\gamma$).

MS (ESI$^-$): *m/z:* 337.2 [M-2PO_3Na-Na]$^-$; 417.1 [M+H-PO_3Na-2Na]$^-$; 439.1 [M-PO_3Na-Na]$^-$; 541.1 [M-Na]$^-$; 563.1 [M-H]$^-$; 564.1 [M]

HRMS (ESI$^-$): For the molecular ion $C_{17}H_{19}N_3O_{17}Na_2P_3$ [M-Na]$^-$, the calculated mass was 540.94079, and the observed mass was 540.94107.

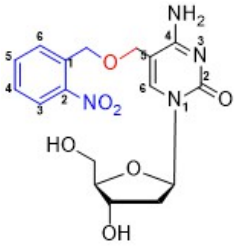
6.1.2.2 Synthesis of NB- modified and HM- modified cytidine nucleos(t)ides

3′, 5′-Bis-O-(tert-butyldimethylsilyl)-5-(2-nitrobenzyloxymethyl)-2′-deoxycytidine



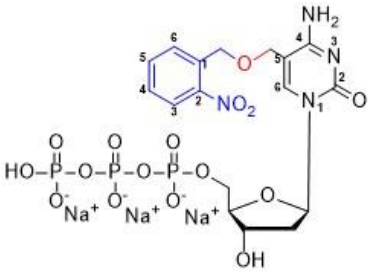
3′, 5′-Bis-O-(tert-butyldimethylsilyl)-5-(2-nitrobenzyloxymethyl)-2′-deoxyuridine (150 mg; 0.24 mmol), DMAP (35 mg; 0.29 mmol) and Et_3N (350 µL; 2.5 mmol) were dissolved in anhydrous DCM (9 ml). The reaction mixture was stirred at room temperature for 10 minutes. 2,4,6-Triisopropyl benzenesulfonyl chloride (350 mg, 1.16 mmol) was added to the reaction mixture, which was subsequently stirred overnight at room temperature under argon atmosphere. The solvent was removed under reduced pressure and residue was dissolved in anhydrous dioxane. [147] The flask with solution was three times evacuated and flushed with gaseous ammonia. The reaction mixture was stirred at room temperature for 3 hours under gaseous ammonia. The residue was concentrated in vacuum and purified by gradient column chromatography (gradient from 100% DCM to 25:1 DCM:MeOH) to yield protected *5-(2-nitrobenzyloxymethyl)-2′-deoxycytidine* (120 mg; 80 % for two steps).[151] The spectral data were in accordance with literature.[147]

5-(2-nitrobenzyloxymethyl)-2′-deoxycytidine



3′, 5′-Bis-O-(tert-butyldimethylsilyl)-5-(2-nitrobenzyloxymethyl)-2′-deoxycytidine (120 mg; 0.19 mmol) was dissolved in dry THF (9 mL). Trimethylamine trihydrofluoride (100 µL; 0.61 mmol) was added to the reaction mixture which was stirred overnight at room temperature. Because of not complete conversion of starting compound, another portion of trimethylamine trihydrofluoride (200 µL; 1.22 mmol) was added, and the stirring was continued overnight. The reaction mixture was concentrated under reduced pressure and residue was purified by gradient column chromatography (gradient from 100 % DCM to 10:1 DCM:MeOH). The desired compound was re-purified by HPLC (gradient H_2O →MeOH) to yield pure 5-(2-nitrobenzyloxymethyl)-2′-deoxycytidine (31 mg; 41 %). The spectral data were in accordance with literature.[147]

5-(2-nitrobenzyloxymethyl)-2´-deoxycytidine triphosphate



5-(2-nitrobenzyloxymethyl)-2´-deoxycytidine (28 mg; 0.071 mmol) was dried at 80 °C for 15 minutes under vacuum. Next, the vial with starting material was cooled to the room temperature and proton sponge (15 mg; 0.071 mmol) was added to the vial. The content was again evacuated and suspended in trimethylphosphate (0.28 mL) at 0 °C under argon atmosphere. $POCl_3$ (8 μL; 0.086 mmol) was added to the reaction mixture and the mixture was then stirred at 0 °C for 1 hour. An ice-cooled solution of tri-n-butylammonium pyrophosphate (198 mg; 0.358 mmol) in anhydrous DMF (0.7 mL) was added to the reaction mixture. After 1 hour of stirring, the solution was quenched by addition of aqueous TEAB (2 M, 2 mL). The solvents were evaporated in vacuum and the residue was co-distilled several times with water. The product was isolated on HPLC (linear gradient: 0.1 M TEAB 100 % H_2O → 0.1 M TEAB 100 % MeOH → 100 % MeOH), evaporated, co-distilled with water; converted into a sodium salt form (Dowex 50 in Na^+ cycle) and lyophilized to yield **dC^{NB}TP** as a white powder (10 mg; 21 %).[147, 151]

^{1}H NMR (500.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.33 (dt, 1H, J_{gem} = 13.8, $J_{2'b,1'} = J_{2'b,3'}$ = 6.5, H-2′); 2.45 (ddd, 1H, J_{gem} = 13.8, $J_{2'a,1'}$ = 6.5, $J_{2'a,3'}$ = 4.6, H-2′); 4.23 (m, 1H, H-4′); 4.27 – 4.35 (m, 2H, H-5′); 4.63, 4.66 (2 × d, 2 × 1H, J_{gem} = 13.1, OCH_2-5); 4.70 (ddd, 1H, $J_{3',2'}$ = 6.5, 4.6, $J_{3',4'}$ = 4.1, H-3′); 4.98, 5.04 (2 × d, 2 × 1H, J_{gem} = 13.0, OCH_2-1); 6.28 (t, 1H, $J_{1',2'}$ = 6.5, H-1′); 7.58 (ddd, 1H, $J_{4,3}$ = 8.2, $J_{4,5}$ = 7.8, $J_{4,6}$ = 1.5, H-4-$C_6H_4NO_2$); 7.65 (dd, 1H, $J_{6,5}$ = 7.8, $J_{6,4}$ = 1.5, H-6-$C_6H_4NO_2$); 7.73 (td, 1H, $J_{5,4}$ = $J_{5,6}$ = 7.8, $J_{5,3}$ = 1.3, H-5-$C_6H_4NO_2$); 7.99 (s, 1H, H-6); 8.06 (dd, 1H, $J_{3,4}$ = 8.2, $J_{3,5}$ = 1.3, H-3-$C_6H_4NO_2$).

^{13}C NMR (125.7 MHz, D_2O, ref(dioxane) = 69.3 ppm): 42.15 (CH_2-2′); 67.6.7 (d, $J_{C,P}$ = 4.2, CH_2-5′); 69.90 (OCH_2-5); 72.11 (OCH_2-1); 72.62 (CH-3′); 88.32 (d, $J_{C,P}$ = 9.3, CH-4′); 88.51 (CH-1′); 107.04 (C-5); 127.81 (CH-3-$C_6H_4NO_2$); 132.04 (CH-4-$C_6H_4NO_2$); 134.32 (CH-6-$C_6H_4NO_2$); 135.54 (C-1-$C_6H_4NO_2$); 136.77 (CH-5-$C_6H_4NO_2$); 144.46 (CH-6); 150.73 (C-2-$C_6H_4NO_2$); 159.88 (C-2); 167.64 (C-4).

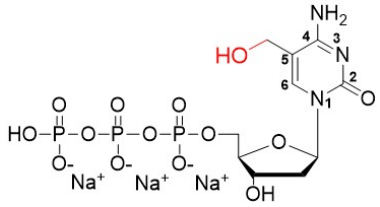
^{31}P{1H} NMR (202.3 MHz, D2O): -22.03 (dd, J = 20.2, 19.8, Pβ); 11.41 (d, J = 19.8, Pα); 6.12 (d, J = 20.2, Pγ).

MS (ESI$^-$): m/z: 471.1 [M-2PO_3Na-Na]$^-$; 551.1 [M-PO_3Na-2Na+H]$^-$; 573.0 [M-PO_3Na-Na]$^-$; 653.0 [M+H-2Na]$^-$; 675.0 [M-Na]$^-$.

HRMS (ESI$^-$): For the molecular ion $C_{17}H_{20}O_{16}N_4Na_2P_3^-$ [M-Na]$^-$, the calculated mass was 674.9888, and the observed mass was 674.9885.

For the molecular ion $C_{17}H_{21}O_{16}N_4NaP_3^-$ [M+H-2Na]$^-$, the calculated mass was 653.00686, and the observed mass was 653.00662.

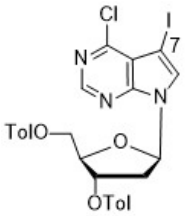
5-Hydroxymethyl-2'-deoxycytidine-5'-triphosphate



A 12 mM colourless solution of **dC^{NB}TP** (10.4 mg) in H_2O (1.2 mL) was irradiated by light (LED; 366 nm; 0.8W; 150.8 mW/cm^2) in glass vial with stirring for 40 minutes. Product was isolated by semipreparative HPLC on C18 column with the use of linear gradient (0.1 M TEAB 100 % H_2O/0.1 M TEAB 50 % MeOH /100 % MeOH). The fractions were evaporated and several times co-distillated with water. The residue was converted to sodium salt form (Dowex 50WX8 in Na^+ cycle) and freeze dried in water. Desired product **dC^{HM}TP** was obtained in low 19 % yield. The spectral data were in accordance with literature. [148]

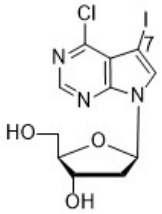
6.1.2.3 Synthesis of NB- modified and HM- modified adenosin nucleos(t)ides

3′, 5′-Bis-O-p-toluoyl-5-chloro-7-iodo-7-deaza-2'-deoxyadenosine



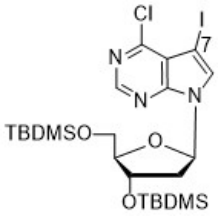
Toluoyl- protected halogenated 7-deaza 2'-deoxyadenosine was prepared by glycosylation reaction of α-L-2-deoxyribofuranosyl chloride with halogenated 7-deaza purine under the published conditions in 49-71 % yield. The spectral data were in accordance with literature. [146, 153]

6-chloro-7-iodo-7-deaza-2'-deoxyadenosine



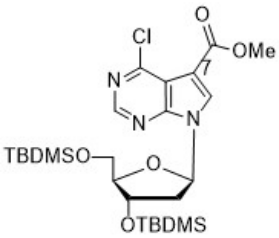
Deprotection of *toluoyl protected 7-deaza purine* was performed in the presence of saturated solution of ammonia in methanol under the published conditions. The spectral data were in accordance with literature. [153] Yield 69-80 %.

3′, 5′-Bis-O-(tert-butyldimethylsilyl)-6-chloro-7-iodo-7-deaza-2'-deoxyadenosine



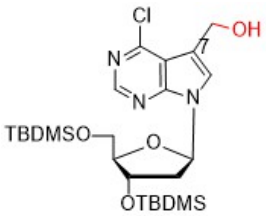
The protection of *5-chloro-7-iodo-7-deaza-2'-deoxyadenosine* was performed in the presence of tert-butyldimethylsilyl chloride according the published procedure. The spectral data were in accordance with literature. [147] Yield 81 %.

3′, 5′-Bis-O-(tert-butyldimethylsilyl)-6-chloro-7-methoxycarbonyl -7-deaza-2'-deoxyadenosine



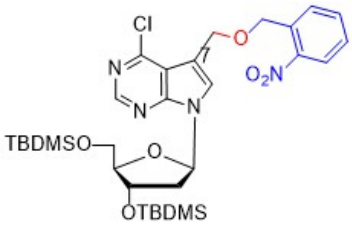
7-methoxycarbonyl-7-deazapurine was synthesized according the published conditions. The reaction was terminated after 27 hours and the product was obtained in 75 % yield. The spectral data were in accordance with literature.[147]

3′, 5′-Bis-O-(tert-butyldimethylsilyl)-6-chloro-7-hydroxymethyl -7-deaza-2'-deoxyadenosine



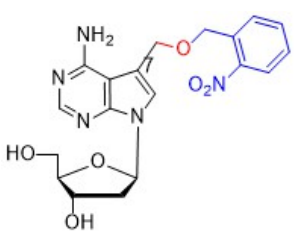
3′, 5′-Bis-O-(tert-butyldimethylsilyl)-6-chloro-7-hydroxymethyl -7-deaza-2'-deoxyadenosine was synthesized under published conditions and the spectral data were in accordance with literature. The product was isolated in 15-24 % yield. [147]

3′, 5′-Bis-O-(tert-butyldimethylsilyl)- 6-chloro-7-(2-nitrobenzyloxymethyl)-7-deaza-2'-deoxyadenosine



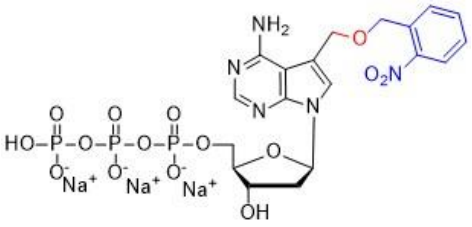
3′, 5′-Bis-O-(tert-butyldimethylsilyl)-6-chloro-7--(2-nitrobenz-yloxymethyl) -7-deaza-2'-deoxyadenosine was synthesized under published conditions and the spectral data were in accordance with literature. [147]Yield 35-48 %.

7-(2-nitrobenzyloxymethyl) -7-deaza-2'-deoxyadenosine



Deprotection and further amination of *3′, 5′-Bis-O-(tert-butyldimethylsilyl)- 6-chloro-7-(2-nitrobenzyloxymethyl)-7-deaza-2'-deoxyadenosine* were held under the published conditions and the spectral data were in accordance with literature.. [147] Yield 86 %.

***7-(2-nitrobenzyloxymethyl)-7-deaza-2'-deoxyadenosine-5'-triphosphate* (dA^{NB}TP)**



7-(2-nitrobenzyloxymethyl)-7-deaza-2'-deoxyadenosine (12 mg; 0.029 mmol) was dried at 80 °C for 15 minutes under vacuum. The vial with starting material was cooled to the room temperature and proton sponge (6.2 mg; 0.029 mmol) was added to the vial. The content was again evacuated and suspended in trimethylphosphate (0.12 mL) at 0 °C under argon atmosphere. $POCl_3$ (3.3 μL; 0.035 mmol) was added to the reaction mixture and the mixture was then stirred at 0 °C for 1 hour 40 minutes. An ice-cooled solution of tri-n-butylammonium pyrophosphate (80 mg; 0.145 mmol) in anhydrous DMF (0.3 mL) was added to the reaction mixture. After 1 hour of stirring, the solution was quenched by addition of aqueous TEAB (2 M, 2 mL). The solvents were evaporated in vacuum and the residue was co-distilled several times with water. The product was isolated by HPLC (linear gradient: 0.1 M TEAB 100 % H_2O → 0.1 M TEAB 100 %/ MeOH → 100 % MeOH), evaporated, co-distilled with water; converted into a sodium salt form (Dowex 50 in Na^+ cycle) and lyophilized to yield **dA^{NB}TP** as a white powder (6.1 mg; 28 %).[147]

^{1}H NMR (500.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.45 (ddd, 1H, J_{gem} = 13.9, $J_{2'b,1'}$ = 6.4, $J_{2'b,3'}$ = 3.2, H-2′b); 2.68 (dt, 1H, J_{gem} = 13.9, $J_{2'a,1'}$ = $J_{2'a,3'}$ = 6.4, H-2′b); 4.12 (m, 1H, H-4′); 4.19 - 4.27 (m, 2H, H-5′); 4.80 (m, 1H, H-3′); 4.86, 4.92 (2 × d, 2 × 1H, J_{gem} = 13.5, CH_2-deazapur); 4.97, 5.04 (2 × d, 2 × 1H, J_{gem} = 13.0, CH_2-$C_6H_4NO_2$); 6.56 (t, 1H, $J_{1',2'}$ = 6.4, H-1′); 7.36 (dd, 1H, $J_{4,3}$ = 8.1, $J_{4,5}$ = 7.8, H-4-$C_6H_4NO_2$); 7.41 (d, 1H, $J_{6,5}$ = 7.8, H-6-$C_6H_4NO_2$); 7.49 (s, 1H, H-6); 7.51 (t, 1H, $J_{5,4}$ = $J_{5,6}$ = 7.8, H-5-$C_6H_4NO_2$); 7.90 (d, 1H, $J_{3,4}$ = 8.1, H-3-$C_6H_4NO_2$); 8.04 (s, 1H, H-2).

^{13}C NMR (125.7 MHz, D_2O, ref(dioxane) = 69.3 ppm): 41.21 (CH_2-2′); 68.13 (d, $J_{C,P}$ = 5.8, CH_2-5′); 68.41 (CH_2-deazapur); 72.11 (CH_2-$C_6H_4NO_2$); 73.79 (CH-3′); 85.32 (CH-1′); 87.95 (d, $J_{C,P}$ = 9.1, CH-4′); 105.16 (C-4a); 115.81 (C-5); 124.49 (CH-6); 127.45 (CH-3-$C_6H_4NO_2$); 131.62 (CH-4-$C_6H_4NO_2$); 134.59 (CH-6-$C_6H_4NO_2$); 135.48 (C-1-$C_6H_4NO_2$); 136.43 (CH-5-$C_6H_4NO_2$); 150.30 (C-2-$C_6H_4NO_2$); 152.92 (C-9a); 154.31 (CH-2); 160.04 (C-4).

^{31}P{^{1}H} NMR (202.3 MHz, D_2O): -22.16 (dd, J =20.4, 19.7, Pβ); -11.17 (d, J = 19.7, Pα); -6.25 (d, J = 20.4, Pγ).

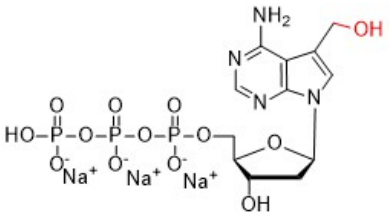
MS (ESI$^-$): m/z: 494.3 [M-2PO_3Na-Na]$^-$; 574.3 [M-PO_3Na-2Na+H]$^-$; 596.3 [M-PO_3Na-Na]$^-$; 698.2 [M-Na]$^-$.

HRMS (ESI$^-$): For the molecular ion $C_{19}H_{21}O_{15}N_5Na_2P_3^-$ [M-Na]$^-$, the calculated mass was 698.00479, and the observed mass was 698.00507.

For the molecular ion $C_{19}H_{22}O_{15}N_5NaP_3^-$ [M-2Na+H]$^-$, the calculated mass was 676.02284, and the observed mass was 676.02307.

For the molecular ion $C_{19}H_{23}O_{15}N_5P_3^-$ [M-3Na+2H]$^-$, the calculated mass was 654.04090, and the observed mass was 654.04099.

7-Hydroxymethyl-7-deaza-2'-deoxyadenosine-5'-triphosphate **(dA^{HM}TP)**



A 11.1 mM solution of *7-(2-nitrobenzyloxy)methyl-7-deaza-2'-deoxyadenosine-5'-triphosphate* **(dA^{NB}TP)** (8 mg) in H_2O (1.0 mL) was irradiated by light (LED; 366 nm; 0.8 W; 150.8 mW/cm^2) in glass vial with stirring during 60 minutes. Product was isolated by semipreparative HPLC on C18 column with the use of linear gradient (0.1 M TEAB 100 % H_2O/0.1 M TEAB 50 % MeOH /100 % MeOH). The fractions were evaporated and several times co-distillated with water. The residue was converted to sodium salt form (Dowex 50WX8 in Na^+ cycle) and freeze dried in water. Desired product **dA^{HM}TP** was obtained in 39 % yield as a white foam.

^{1}H NMR (500.0 MHz, D_2O, ref(dioxane) = 3.75 ppm): 2.44 (ddd, 1H, J_{gem} = 13.9, $J_{2'b,1'}$ = 6.2, $J_{2'b,3'}$ = 3.1, H-2′b); 2.71 (ddd, 1H, J_{gem} = 13.9, $J_{2'a,1'}$ = 7.9, $J_{2'a,3'}$ = 6.3, H-2′b); 4.06 – 4.25 (m, 3H, H-4′,5′); 4.75 – 4.83 (m, 3H, H-3′, CH_2O); 6.65 (dd, 1H, $J_{1',2'}$ = 7.9, 6.2, H-1'); 7.50 (s, 1H, H-6); 8.14 (s, 1H, H-2).

^{13}C NMR (125.7 MHz, D_2O, ref(dioxane) = 69.3 ppm): 41.16 (CH_2-2′); 59.10 (CH_2O); 68.14 (d, $J_{C,P}$ = 5.2, CH_2-5′); 73.93 (CH-3′); 85.47 (CH-1′); 87.90 (d, $J_{C,P}$ = 9.0, CH-4′); 105.34 (C-4a); 118.41 (C-5); 122.81 (CH-6); 153.07 (C-7a); 154.36 (CH-2); 160.31 (C-4).

^{31}P{^{1}H} NMR (202.3 MHz, D_2O): -22.26 (t, J =19.7, Pβ); -11.14 (d, J = 19.7, Pα); -6.88 (bd, J = 19.7, Pγ).

MS (ESI$^-$): m/z: 359.1 [M-2PO_3Na-Na]$^-$; 439.0 [M-PO_3Na-Na+H]$^-$; 461.0 [M-PO_3Na-Na]$^-$; 541.0 [M-2Na+H]$^-$; 563.0 [M-Na]$^-$.

HRMS (ESI$^-$): For the molecular ion $C_{12}H_{17}O_{13}N_4NaP_3^-$ [M-2Na+H]$^-$, the calculated mass was 540.99081, and the observed mass was 540.99067.

For the molecular ion $C_{12}H_{18}O_{13}N_4P_3^-$ [M-3Na+2H]$^-$, the calculated mass was 519.00887, and the observed mass was 519.00874.

6.1.2.4 Kinetic study of deprotection of dU^{NB}MP[150]

a) **Study of deprotection dU^{NB}MP using analytical HPLC**

The 0.7 mg of **dU^{NB}MP** was dissolved in 550 μL of MilliQ water to prepare its 3.5 mM stock solution. The stock solution was directly exposed to UV light (365 nm, light emitting diode, 1 mW; 1.06 mW/cm^2) with stirring at 5-10 °C (cold room). The 10 μL of irradiated solution was removed in time intervals: 0, 30, 60, 120, 150, 180, 210, 240 and 300 minutes and injected to the analytical HPLC. The photocleavable behaviour of **dU^{NB}MP** was studied by gradient reverse phase HPLC analysis done in water/methanol/HCOOH. The retention time of starting **dU^{NB}MP** was 17.7 min and the retention time of desired product **dU^{HM}MP** was 14.5 min. The eluted fractions of product were verified by MS. Percentages of **dU^{HM}MP** production and **dU^{NB}MP** disappearance were obtained by integrating peaks of HPLC traces. (***Figure 11***)

b) **Study of deprotection dU^{NB}MP using NMR spectroscopy**

The 10 mg of starting **dU^{NB}MP** was dissolved in D_2O to prepare a 16 mM solution of **dU^{NB}MP.** The sample was irradiated by LED (365 nm, 1 mW; 1.06 mW/cm^2) and monitored in time intervals: 30 minutes, 1 hour, 3 hours, 6 hours, 9 hours, 12 hours and 15 hours. The prepared solution was stirred, directly irradiated in Eppendorf vial and ^{1}H NMR spectra were measured in particular time intervals. Percentages of **dU^{HM}MP** formation and **dU^{NB}MP** disappearance were obtained by integration of peaks for H-6. (***Figure 12; Figure 51***)

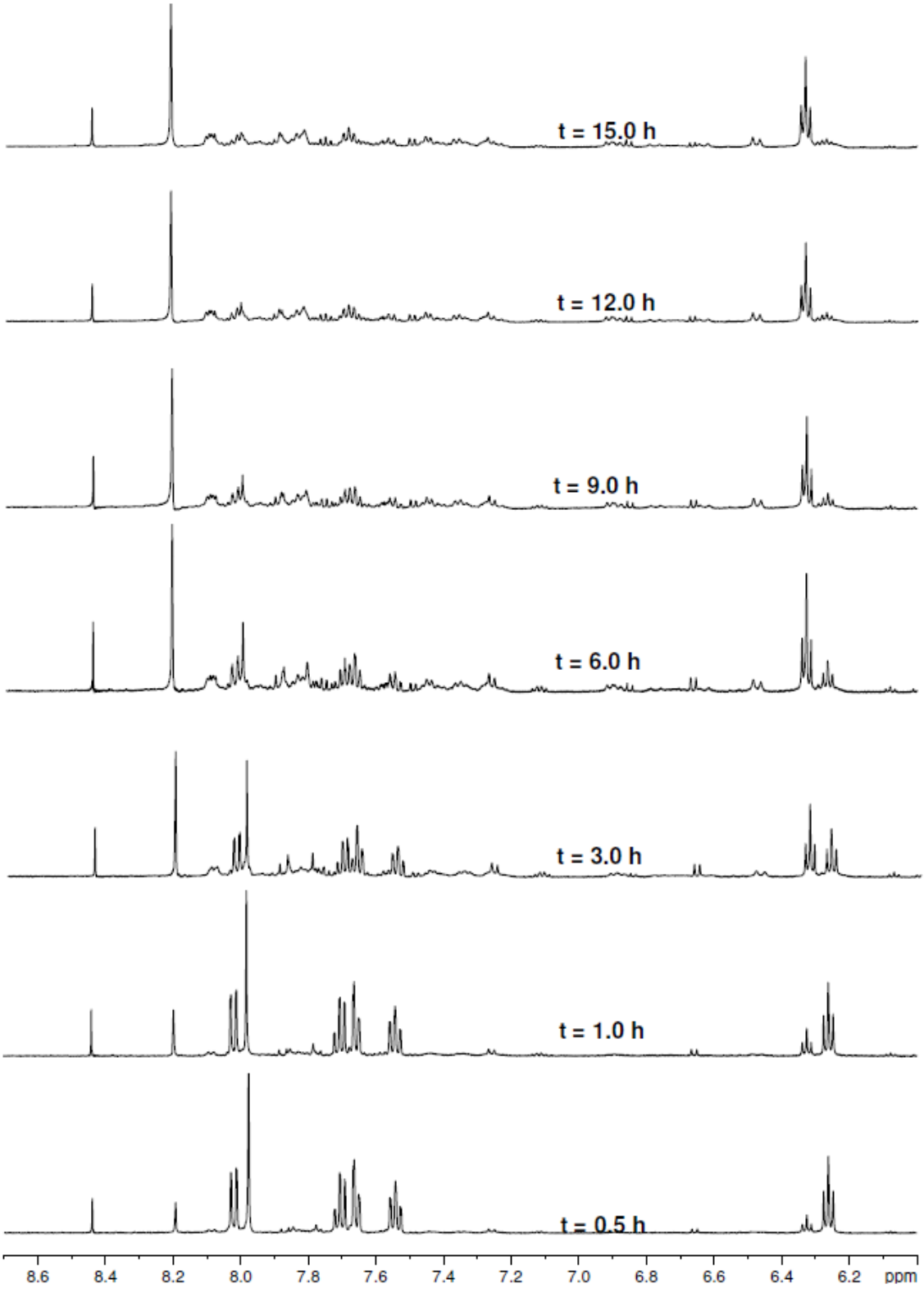


Figure 51: *Study of* ***dU^{NB}MP*** *photolysis using NMR spectra analysis*

6.2 Enzymatic synthesis of modified DNA

General remarks:

NON-labelled and *biotinylated-* oligonucleotides and *FAM*-labelled primers were purchased from GeneriBiotech. Modified nucleoside triphosphates: **dU^{NB}TP**, **dU^{HM}TP**, **dC^{NB}TP**, **dC^{HM}TP**, **dA^{NB}TP** and **dA^{HM}TP** were prepared according to above reported procedures. PEX reaction was stopped by addition of double amount of stop solution (20 mM EDTA, 80 % [v/v] formamide, 0.025 % [w/v] xylene cyanole, 0.025 % [w/v] bromophenol blue) and heated at 95 °C for 5 minutes. Reaction mixtures after PEX (5 µL) were separated by use of a 12 % denaturizing PAGE. Visualization was performed by phosphorimaging or imaging of fluorescence.

6.2.1 Monoincorporation of modified nucleoside triphosphates

Monoincorporation of dU^{HM}TP or dU^{NB}TP by PEX

Reaction mixtures for KOD XL DNA polymerase and Vent (exo-) DNA polymerase contained corresponding DNA polymerase (KOD XL - Merc4biosciences, Novagen, 2.5 U/µL, 0.2 µL or **Vent (exo-) -** New England Biolabs, 2 U/µL, 0.2 µL), natural dGTP (0.4 mM, 0.5 µL), functionalized - **dU^{NB}TP** or **dU^{HM}TP** (4 mM, 1 µL), 19-mer template (3 µM, 1.5 µL, **templ^{Oligo1T}**), *FAM*- labelled primer (3 µM, 1 µL**, prim^{Prim248short}-FAM**:) and 2 µL of KOD XL reaction buffer or ThermoPol reaction buffer for Vent(exo-) supplied by the manufacturer. The total final volume of each reaction was 20 µL.

Reaction mixture for Pwo DNA polymerase consisted of Pwo DNA polymerase (**Pwo** - Peqlab, 1 U/µL, 0.05 µL), natural dGTP (4 mM, 0.7 µL), functionalized - **dU^{NB}TP** or **dU^{HM}TP** (4 mM, 1 µL), 19-mer template (3 µM, 1.5 µL, **templ^{Oligo1T}**), *FAM*- labelled primer (3 µM, 1 µL**, prim^{Prim248short}-FAM**) and 2 µL of Pwo magnesium-containing reaction buffer supplied by the manufacturer. The total final volume of each reaction mixture was 20 µL.

All reaction mixtures were incubated at continuous 60 °C for 30 minutes in a thermocycler. The created DNA was subsequently after PEX denatured by addition of stop solution (40 µL) and heating at 95 °C for 5 minutes. The 5 µL of each reaction mixture was separated by use of a 12 % denaturing PAGE. (***Figure 13***)

Monoincorporation of dC^{HM}TP or dC^{NB}TP by PEX

The reaction mixture either for KOD XL DNA polymerase or Vent (exo-) DNA polymerase or Pwo DNA polymerase (20 µL each) contained particular DNA polymerase (either KOD XL - Merc4biosciences, Novagen, 2.5 U/µL, 0.08 µL or **Vent (exo-) -** New England Biolabs, 2 U/µL, 0.2 µL; or **Pwo** - Peqlab, 1 U/µL, 0.08 µL), *FAM*- labelled primer (3 µM, 1 µL**, prim^{Prim248short}-**

FAM:), 19-mer template (3 µM, 1.5 µL, **templ^{Oligo1C}**), natural dGTP (4 mM, 0.5 µL) and functionalized-**dC^{NB}TP** or **dC^{HM}TP** (4 mM, 1 µL) in 2 µL of appropriate reaction buffer (KOD XL reaction buffer/ ThermoPol reaction buffer for Vent(exo-)/ Pwo magnesium-containing reaction buffer) supplied by the manufacturer. Reaction mixtures were incubated at continuous 60 °C for 30 minutes in thermocycler, and in the next step denatured by addition of stop solution (40 µL) and heated at 95 °C for 5 minutes. The 5 µL of each reaction mixture was separated by use of a 12 % denaturing PAGE. (***Figure 14***)

Monoincorporation of dA^{HM}TP or dA^{NB}TP by PEX

Reaction mixtures for KOD XL DNA polymerase and Vent (exo-) DNA polymerase consisted of 10x diluted appropriate DNA polymerase (either KOD XL - Merc4biosciences, Novagen, 2.5 U/µL, 0.2 µL or **Vent (exo-) -** New England Biolabs, 2 U/µL, 0.2 µL), natural dGTP (4 mM, 0.5 µL), functionalized-**dA^{NB}TP** or **dA^{HM}TP** , 4 mM, 0.5 µL), 19-mer template (3 µM, 1.5 µL, **templ^{Oligo1A}**) and *FAM*- labelled primer (3 µM, 1 µL**, prim^{Prim248short} - FAM**) in 2 µL of appropriate reaction buffer (KOD XL reaction buffer/ ThermoPol reaction buffer for Vent(exo-)) supplied by the manufacturer.

Reaction mixture for Pwo DNA polymerase consisted of Pwo DNA polymerase (**Pwo** - Peqlab, 1 U/µL, 0.5 µL), natural dGTP (4 mM, 0.5 µL), functionalized - **dA^{NB}TP** or **dA^{HM}TP** (4 mM, 0.5 µL), 19-mer template (3 µM, 1.5 µL, **templ^{Oligo1A}**), *FAM*- labelled primer (3 µM, 1 µL**, prim^{Prim248short}-FAM**:) and Pwo magnesium-containing reaction buffer supplied by the manufacturer. The total final volume of each reaction was 20 µL.

Reaction mixtures were incubated at continuous 60 °C for 30 minutes in a thermocycler and in the next step denatured by addition of double amount of stop solution (40 µL) and heating at 95 °C for 5 minutes. Reaction mixtures were separated by use of a 12 % denaturing PAGE. (***Figure 15***)

6.2.2 Multi-incorporation of modified nucleoside triphosphates

Multi-incorporation of dU^{HM}TP or dU^{NB}TP by PEX[150]

The reaction mixture either for KOD XL DNA polymerase or Vent (exo-) DNA polymerase or Pwo DNA polymerase (20 µL each) contained particular DNA polymerase (either KOD XL - Merc4biosciences, Novagen, 2.5 U/µL, 0.05 µL, or **Vent (exo-) -** New England Biolabs, 2 U/µL, 0.05 µL; or **Pwo** - Peqlab, 1 U/µL, 0.08 µL), labelled primer (^{32}P- or *FAM*-labelled, 3 µM, 1 µL**, prim^{Prim248short}**), 31-mer template (3 µM, 1.5 µL, **templ^{Prb4basII}**: 5‘-CTAGCATGAG CTCAGTCCCATGCCGCCCATG-3‘), natural dNTPs (mix of dGTP, dATP, dCTP; 4 mM, 1 µL) and functionalized **dU^{NB}TP** or **dU^{HM}TP** (4 mM, 1 µL) in appropriate

reaction buffer (KOD XL reaction buffer/ ThermoPol reaction buffer for Vent(exo-)/ Pwo magnesium-containing reaction buffer, 2 µL) supplied by the manufacturer. Reaction mixtures were incubated at 60 °C for 32 minutes in a thermomixer and next denatured by addition of stop solution (40 µL) and heated at 95 °C for 5 minutes. Reaction mixtures were separated by use of a 12% denaturing PAGE. (***Figure 16***)

Multi-incorporation of dC^{HM}TP or dC^{NB}TP by PEX

Three reaction mixtures either for KOD XL DNA polymerase, Vent (exo-) DNA polymerase or Pwo DNA polymerase (20 µL each) contained particular DNA polymerase (KOD XL - Merc4biosciences, Novagen, 2.5 U/µL, 0.05 µL; **Vent (exo-) -** New England Biolabs, 2 U/µL, 0.05 µL; or **Pwo** - Peqlab, 1 U/µL, 0.05 µL), *FAM* labelled primer (3 µM, 1 µL**, prim^{Prim248short}-FAM**), and 31-mer template (3 µM, 1.5 µL, **templ^{Prb4basII}**; 5´-CTAGCATGAGCTCAG TCCCATGCCGCCCATG-3´), natural dNTPs (mix of dGTP, dTTP, dATP; 4 mM; 1 µL), functionalized-**dC^{NB}TP** or **dC^{HM}TP** (4 mM, 1 µL), in 2 µL of appropriate reaction buffer (KOD XL reaction buffer/ ThermoPol reaction buffer for Vent(exo-)/ Pwo magnesium-containing reaction buffer) supplied by the manufacturer. Reaction mixtures were incubated at 60 °C for 30 minutes in a thermomixer, subsequently denatured by addition of stop solution (40 µL) and heating at 95 °C for 5 minutes. Reaction mixtures were separated by use of a 12 % denaturing PAGE. (***Figure 17***)

Multi-incorporation of dA^{HM}TP or dA^{NB}TP by PEX

Three reaction mixtures - for KOD XL DNA polymerase, Vent (exo-) DNA polymerase or Pwo DNA polymerase - contained appropriate DNA polymerase (**KOD XL** - Merc4biosciences, Novagen, 2.5 U/µL, 0.05 µL; **Vent (exo-) -** New England Biolabs, 2 U/µL, 0.05 µL; or **Pwo** - Peqlab, 1 U/µL, 0.08 µL), natural dNTPs (mix of dCTP, dGTP and dTTP; 4 mM, 1 µL for KOD XL and Vent (exo-) and 0.8 µL for Pwo), functionalized **dA^{NB}TP** or **dA^{HM}TP** (4 mM, 1 µL), *FAM*- labelled primer (3 µM, 1 µL**, Prim^{248short}-FAM**), and 31-mer template (3 µM, 1.5 µL, **Templ^{Prb4basII}**: 5‘-CTAGCATGAGCTCAGTCCCATGCCGCCCATG-3‘) in 2 µL of appropriate reaction buffer (KOD XL reaction buffer/ ThermoPol reaction buffer for Vent(exo-)/ Pwo magnesium-containing reaction buffer) supplied by the manufacturer. The total final volume of each reaction mixture was 20 µL. Reaction mixtures were incubated at 60 °C for 30 minutes in a thermal cycler, subsequently denatured by addition of stop solution (40 µL) and heating at 95 °C for 5 minutes. Reaction mixtures were separated by use of a 12 % denaturing PAGE. (***Figure 18***)

6.2.3 Magnetoseparation - Isolation of modified oligonucleotides (ssDNA)

For the isolation of ssDNA, the magnetoseparation procedure was used. The magnetic beads with streptavidine moiety were supplied from ROCHE. The composition of binding buffer TEN 100 used in the procedure is as follows: 100 mM NaCl, 10 mM TRIS, 1 mM EDTA, pH 7.5. The buffer used in the washing process, TEN 500 buffer with pH 7.5, contains 500 mM NaCl, 10 mM TRIS, 1 mM EDTA. The magnetic beads were collected on a magnet (DynaMagTM-2, Invitrogen). The mass of ssDNA (MALDI-TOF spectra) was measured on UltrafleXtremeTM MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) with 1 kHz smartbeam II laser. As matrix for measurement a mixture of 3-hydroxypicolinic acid (HPA)/picolinic acid (PA)/ammonium tartrate in ratio 9/1/1 was used. The 2 μL of the matrix and 0.5 μL of the sample were mixed on target by use of anchor-chip desk. The crystallized spots were washed by 0.1 % formic acid and by water. The acceleration tension in reflectron mode was 19.5 kV and range of measurement 3–13 kDa.

Magnetoseparation -General procedure:

The reaction mixture (50 μL) contained KOD XL DNA polymerase (2.5 U/ μL, 0.1 μL), 5'-biotinylated template with one modification (100 μM, 1.6 μL, **Templ^{Oligo1N}**), *NON*-labelled primer (100 μM, 1.6 μL, **Prim^{248short}**), natural dGTP (4 mM, 2.6 μL) and corresponding modified dNTP (4 mM, 2.6 μL) in reaction buffer for KOD XL DNA polymerase (5 μL) supplied by the manufacturer. The reaction mixture was incubated at 60 °C for 40 minutes in a thermomixer. Meanwhile, the 50 μL of properly mixed stock solution of Streptavidine magnetic particles was washed by 200 μL of binding buffer TEN100. The procedure was repeated three times. The PEX reaction was stopped by cooling on ice. The 50 μL of cold reaction mixture was mixed with the prewashed Streptavidine magnetic particles suspended in 50 μL of binding buffer TEN 100. To allow the streptavidine – modified dsDNA to bind to the beads, the suspension (dsDNA + beads) was shaken at 15 ºC and 1200 rpm for 15 minutes. After the binding of dsDNA on the beads, the magnetic beads were collected on a magnet and the solution was discarded. The dsDNA bind on beads was washed with 200 μL of wash buffer TEN 500. The washing of magnetic beads with dsDNA was repeated three times with TEN 500 buffer and then four times with 200 μL of H_2O. After the last washing, 50 μL of water was mixed with dsDNA bind on magnetic beads and the sample was denatured at 75 °C and 900 rpm for 2 minutes. After denaturation reaction, the beads were collected on a magnet and the solution was

immediately transferred into a clean vial. The isolated product ssDNA was analysed by MALDI-TOF mass spectrometry. (***Table 3***)

6.2.4 Enzymatic incorporation of HM- and NB- modified triphosphates by PCR

6.2.4.1 General procedure for synthesis of 98-mer DNA

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase (MErc4biosciences, Novagen, 2.5 U/µL, 1 µL), 0.6 mM natural dNTPs (either 0.06 µL of 10 mM d(C,A,G)TP for U-modified DNA or 0.15 µL of 4 mM d(C,T,G)TP for A- and d(T,A,G)TP for C-modified DNA), functionalized **dN^{HM}TP** (1 µL of 10 mM **dU^{HM}TP** or 1 µL of 4 mM **dA^{HM}TP** or 1 µL of 4 mM **dC^{HM}TP**) or functionalized **dN^{NB}TP** (2 µL of 10 mM **dU^{NB}TP** or 2 µL of 4 mM **dA^{NB}TP** or 2 µL of 4 mM **dC^{NB}TP**), forward and reverse primers (10 µM, 4 µL, **LT25TH** and 10 µM, 4 µL, **L20**) and commercially available single stranded template (1 µM, 0.5 µL, **FVL-A**) in KOD XL reaction buffer (2 µL) supplied by the manufacturer. The PCR reaction was run in 30 PCR cycles in PCR cycler, preheated to 80 °C. The conditions of reaction were as follows: preheating at 94 °C for 3 minutes, denaturation at 95 °C for 1 minute, annealing at 53 °C for 1 minute, extension at 72 °C for 1 minute, followed by the final elongation step at 75 °C for 2 minutes. Impurified PCR products were analysed on a 2 % agarose gel stained with GelRed (Biotium, 10 000X in H_2O) in 0.5x TBE buffer. [***Table 4***; ***Figure 19a)***]

Synthesis of partially A^{NB}-modified 98-mer DNA

The PCR reaction mixture (20 µL) contained KOD XL DNA polymerase (MErc4biosciences, Novagen, 2.5 U/µL, 1 µL), natural dNTPs (0.15 µL of 4 mM d(C,T,G)TPs), forward and reverse primers (10 µM, 4 µL, **LT25TH** and 10 µM, 4 µL, **L20**), commercially available single stranded template (1 µM, 0.5 µL, **FVL-A**) and functionalized **dA^{NB}TP** in different ratios (see table on the bottom) in KOD XL reaction buffer (2 µL) supplied by the manufacturer. The PCR reaction was run in 30 PCR cycles in PCR cycler, preheated to 80 °C. The conditions of reaction were as follow: preheating at 94 °C for 3 minutes, denaturation at 95 °C for 1 minute, annealing at 52 °C for 1 minute, extension at 72 °C for 1.5 minute, followed by the final elongation step at 75 °C for 2 minutes. PCR products were analysed on a 2 % agarose gel stained with GelRed (Biotium, 10 000X in H_2O) in 0.5x TBE buffer. (***Table 4***; ***Figure 20***)

Ratio dA^{NB}TP/ dATP (%)	100/0	90/10	80/20	70/30
Ratio dA^{NB}TP/ dATP (µL)	0.75/0	0,675/0.075	0.6/0.15	0.525/0.225

6.2.4.2 General procedure for synthesis of 287-mer DNA (by KOD XL)

The PCR reaction mixture (20 μL) contained KOD XL DNA polymerase (MErc4biosciences, Novagen, 2.5 U/ μL, 1.5 μL), natural dNTPs (4 mM, 0.5 μL either d(C,A,G)TP for U-modified DNA or d(C,T,G)TP for A-modified DNA or d(T,A,G)TP for C-modified DNA), **dN^{HM}TP** (4 mM, 1 μL for **dC^{HM}TP**, **dU^{HM}TP** and 1.5 μL for **dA^{HM}TP**) or **dN^{NB}TP** (4 mM, 2 μL), forward and reverse primers (20 μM, 2 μL, **Prim S1-HIV1** and 20 μM, 2 μL, **Prim S2-HIV1**) and 287-mer template (94.12 ng/μL, 0.467 μL, **wt-HIV-1PR**) in KOD XL reaction buffer (2 μL) supplied by the manufacturer. The PCR reaction was run in 40 PCR cycles in preheated (80 °C) PCR cycler. The conditions of PCR reaction were as follows: preheating at 94 °C for 3 minutes, denaturation at 94 °C for 1 minute, annealing at 63 °C for 1 minute, extension at 75 °C for 2 minutes, followed by the final elongation step at 75 °C for 2 minutes. PCR products were analysed on 1.3% agarose gel stained with GelRed (Biotium, 10 000X in H_2O) in 0.5 TBE buffer. To verify the sequence fidelity of polymerase incorporation of modified triphosphates, the created modified PCR products (**C^{HM}**-, **C^{NB}**-, **A^{HM}**- and **A^{NB}**- modified 287-mers) or naturally rePCRed **U^{HM}**-, **U^{NB}**- modified 287-mers were purified and used for sequencing both strands. In all cases the expected DNA sequence was confirmed by the analysis. [***Table 4***; ***Figure 19b)***]

Synthesis of U-modified 287-mer DNA by Vent (exo-) and Pwo polymerase[150]

The PCR reaction mixture (20 μL) contained **Vent (exo-)** (New England Biolabs, 2 U/μL, 1.5 μL) or **Pwo** – (Peqlab, 1 U/μL, 1.5 μL) DNA polymerase, natural dNTPs (dCTP, dGTP, dATP 4 mM, 0.5 μL), functionalized **dU^{HM}TP** (4 mM, 1 μL) or **dU^{NB}TP** (4 mM, 2 μL), primers (20 μM, 2 μL, **Prim S1-HIV1** and 20 μM, 2 μL, **Prim S2-HIV1**) and 287-mer template (94.12 ng/μL, 0.467 μL, **wt-HIV-1PR**) in appropriate reaction buffer [10x ThermoPol reaction buffer (2 μL) or 10x Pwo magnesium-containing reaction buffer (2 μL)] supplied by the manufacturer. The PCR reaction was run in 40 PCR cycles in preheated (80 °C) thermal cycler. The conditions of reaction were the same as in the case of reactions with KOD XL DNA polymerase. Reaction mixtures after PCR were analysed on GelRed stained 1.3% agarose gel (Biotium, 10 000X in H_2O) in 0.5 TBE buffer. (***Table 4***; ***Figure 21***)

6.2.5 Study of deprotection by MALDI-TOF analysis[150]

The portion (50 µL) of hydroxymethylated- ssDNA was prepared according to the reported procedure as a standard sample for MALDI-TOF measurement.

The **U^{NB}**-modified DNA with one modification was prepared in two portions. One reaction mixture (50 µL) contained KOD XL DNA polymerase, **Prim^{248short}**, biotinylated **Templ^{Oligo1T},** natural dGTP and functionalized **dU^{NB}TP** (4 mM, 2.6 µL) in KOD XL reaction buffer (5 µL). All reaction mixtures were incubated at 60 °C for 40 minutes in a thermomixer and the separations on magnetic beads were carried out according to standard techniques. The hydroxymethylated- ssDNA and **U^{NB}**-modified ssDNA just after magnetoseparation were analysed by MALDI-TOF mass spectrometry. Another portion of ssDNA after isolation on magnet was exposed to UV irradiation at 365 nm (light emitting diode, 1.0 mW; 1.06 mW/cm^2) for 24 hours with occasional stirring. (***Figure 22***)

6.2.6 Study of cleavage modified DNA with REs (*Scheme 32*)

6.2.6.1 PEX reaction

Method I: The reaction mixtures (20 μL) contained Pwo DNA polymerase (Peqlab, 1 U/μL, 0.2 μL), natural dNTPs (4 mM, 1 μL), functionalized **dN^{X}TP** (4 mM, 1 μL), *^{32}P*- or *FAM*-labelled primer (3 μM, 1 μL**, Prim^{248short}**) and 30-mer template with the specific sequence for the cleavage by restriction endonuclease (3 μM, 1.5 μL; **temp^{KpN}/ temp^{AflN}/ temp^{RsN}/ temp^{PvN}/ temp^{EcN}/ temp^{ScN}**) in Pwo reaction buffer (2 μL) supplied by the manufacturer. Reaction mixtures were incubated at 60 °C for 30 minutes in a thermomixer.

Method II: The reaction mixtures (20 μL) contained Pwo DNA polymerase (Peqlab, 1 U/μL, 0.13 μL), natural dNTPs (4 mM, 0.25 μL), functionalized **dN^{X}TP** (4 mM, 0.5 μL), *^{32}P*- or *FAM*-labelled primer (3 μM, 1 μL**, Prim^{248short}**) and 30-mer template with the specific sequence for the cleavage by restriction endonuclease (3 μM, 1.5 μL; **temp^{KpN}/ temp^{AflN}/ temp^{RsN}/ temp^{PvN}/ temp^{EcN}/ temp^{ScN}**) in Pwo reaction buffer (2 μL) supplied by the manufacturer. Reaction mixtures were incubated at 60 °C for 30 min in a thermomixer. (***Table 10***)

dN^{X}TP	**Method I**	**Method II**
dA^{HM}TP **dA^{NB}TP**	AfA, EcA, KpA, PvA, RsA, ScA	-
dC^{HM}TP **dC^{NB}TP**	KpC, PvC, ScC	AfC, EcC, RsC
dU^{HM}TP **dU^{NB}TP**	AfT, EcT, KpT, PvT, RsT, ScT	

Table 10: *Overview of modified DNA prepared by Method I or Method II*

6.2.6.2 Cleavage by restriction endonucleases-general procedure

The 20 μL portion of modified DNA (either after PEX or after irradiation) was mixed with reaction buffer for corresponding restriction endonuclease (2 μL) supplied by the manufacturer together with one of the restriction endonucleases (AflII/ EcoRI/ KpnI/ RsaI/ ScaI/ PvuII - NewEngland Biolabs). The reaction mixtures were incubated at 37 °C for 60 minutes. The products of cleavage were denatured by addition of stop solution (40 μL, 80 % [v/v] formamide, 20 mM EDTA, 0.025 % [w/v] bromphenole blue, 0.025 % [w/v] xylene cyanol) and heating at 95 °C for 5 minutes. Cold reaction mixtures after cleavage reaction (with other samples of standards) were analysed by 12 % denaturing PAGE. (***Figure 23-25***)

Natural DNA (control samples), HM- modified samples and NB- modified samples were prepared in two portions for each kind of template (2 x 20 µL) according to reported procedure by ***Method I*** or ***Method II***. Both portions were combined and subsequently divided into two portions. The first portion of each sample was denatured by addition of double amount of stop solution (40 µL) and heating at 95 °C for 5 minutes. The second portion was used in the cleavage reaction with corresponding restriction enzyme and denatured with double amount of stop solution at 95 °C for 5 minutes. Reaction mixtures after denaturation reaction were analysed on one PAGE with other samples. (***Table 11***)

ssDNA for template	**Restriction enzyme** (volume)	**Buffer for restriction enzyme**
KpT	**KpnI (**0.5 µL) + BSA (100 mg/mL, 0.2 µL)	NE Buffer-1
RsT, AfT	**RsaI (**0.5 µL)**, AflII (**0.5 µL) + BSA (100 mg/mL, 0.2 µL)	NEBuffer-4
PvT	**PvuII**	NEBuffer-2
KpC, KpA	**KpnI** (0.5 µL)	NEBuffer-1.1
EcC, EcA, EcT	**EcoRI** (0.5 µL)	EcoRIBuffer
AfC, PvC, ScC, ScT, ScA, AfA	**AflII, PvuII, HF-ScaI** (each 0.5 µL)	CutSmart Buffer
RsC, RsA	**RsaI** (0.75 µL)	CutSmart Buffer
PvA	**PvuII** (0.5 µL)	NEBuffer-3.1

Table 11: *Overview of used restriction endonucleases and conditions*

6.2.6.3 Kinetics of photocleavage of nitrobenzyl- modified PEX products

The five portions (5 x 20 µL) of NB- modified PEX products for templates AfT, KpT, PvT, RsT, KpC, RsC, KpA and RsA were prepared by ***Method I*** or ***Method II***. First portion was used like a standard for fully synthesised PEX product. The second one (of each) was cleaved by appropriate restriction endonuclease (as a standard of non-cleaved sample) according to the general procedure immediately after PEX. The remaining portions of the PEX reaction mixtures were exposed to light irradiation for specific time intervals (5 minutes, 30 minutes, 60 minutes) in cold room. The samples after previous irradiation by light were cleaved by RE under the general conditions. (***Figure 24***)

6.2.6.4 Complete deprotection of NB-photocaged PEX products

For complete deprotection of photolabile nitrobenzyl moieties from NB- modified PEX product, the NB-modified DNA was irradiated by light under the general conditions for the specific time intervals from one hour to six hours All portions of samples, including positive and negative controls were separated by use of a 12 % denaturizing PAGE. (***Figure 25***)

6.2.7 Study of cleavage modified PCR products with RsaI [150]

The HM- and NB- modified 287-mers DNA were prepared by PCR according the procedure reported above (*section 6.2.4.2*).

6.2.7.1 General procedure for cleavage of PCR products

The solution of purified **U^{HM}-** or **U^{NB}-** modified 287-mer dsDNA in water (20 μL) was incubated with **RsaI** (2 μL) in the presence of **NEBuffer 4** (2 μL) at 37 °C for 1 hour in thermomixer.

6.2.7.2 Cleavage of PCR products with $dU^{NB}TP$ before and after their deprotection by UV

The PCR reaction mixtures (two portions, each with volume 20μL) were prepared under general conditions. The first portion (directly after PCR) was cleaved by restriction endonuclease Rsa I according to the general procedure for cleavage of PCR products.

The second portion of the PCR reaction mixture was exposed to light irradiation for 6 hours in cold room with occasional stirring. The deprotected dsDNA were cleaved by restriction endonuclease Rsa I according to the general procedure for cleavage of PCR products. (***Figure 26***)

6.2.7.3 Kinetic study of photocleavage for dU^{NB}-modified dsDNA

The PCR reaction mixtures (six portions, every with volume 20 μL) were prepared under the reported conditions. First portion of reaction mixture after PCR was used like a standard for fully synthesised PCR product. The second portion after PCR was incubated with restriction endonuclease RsaI according the general procedure (no cleavage).

The remaining portions of the PCR reaction mixtures were exposed to light irradiation for different time intervals (30 minutes; 1 hour; 3 hours; 6 hours) in cold room. The samples after previous irradiation were cleaved by RsaI under the conditions reported above. (***Figure 26***)

6.2.8 Cloning and transfection study for modified pUC plasmids into *E.coli* [150]

PCR of 99-mer for dU^{HM}- or dU^{NB}- modified DNA

The PCR reaction mixture (20 μL) contained KOD XL DNA polymerase (MErc4biosciences, Novagen, 2.5 U/μL, 1 μL), forward and reverse primers (10 μM, 4 μL, **Prim_L22_XmaI** and 10 μM, 4 μL, **Prim_L19_SfoI**), natural dNTPs (dATP, dCTP, dGTP; 4 mM, 0.15 μL), functionalized **dU^{HM}TP** (4 mM, 2.5 μL) or **dU^{NB}TP** (40 mM, 0.5 μL), 99-mer template with restriction side for AfeI in the middle (1 μM, 1 μL, **Temp_99_AfeI**) in KOD XL reaction buffer (2 μL) supplied by the manufacturer. The PCR reaction was run in 30 PCR cycles in PCR cycler, preheated to 80 °C. The conditions of PCR were as follows: preheating at 94 °C for 3 minutes, denaturation at 95 °C for 1 minute, annealing at 62 °C for 1 minute, extension at 72 °C for 1 minute, followed by the final elongation step at 75 °C for 2 minutes. PCR product was purified on columns (Macherey-Nagel, NucleoSpin Gel and PCR Clean-up) and analysed on a GelRed stained 2% agarose gel (Biotium, 10 000X in H_2O) in 0.5x TBE buffer. [***Figure 27a)***]

Synthesis of 99-mer dU^{X}- modified PCR product ligated to the pUC vector

For ligation, PCR reaction mixture in final volume 80 μL with **dU^{NB}TP** (40 mM, 2 μL) was prepared according the reported procedure. PCR product was purified by Macherey-Nagel spin column. The product eluted in PCR water was concentrated to final volume 40 μL. 20 μL of clean 99-mer **dU^{NB}** – modified product was exposed to light with occasional stirring for 6 hours in a cold room (sample **B**).

Digestion of DNAs by restriction endonucleases: The synthesized PCR product (**dU^{NB}** – modified product), irradiated **dU^{NB}** – modified product and pUC plasmid were digested by restriction endonucleases XmaI and SfoI. The d**U^{NB}** – modified DNA (sample **A**), 99-mer product after 6 hours light exposition (sample **B**) and plasmid DNA pUC (20 μL of each; 25 ng/μL), restriction endonucleases, **XmaI** (1.2 μL) and **SfoI** (1.2 μL), in 10x CutSmart Buffer (4 μL),were incubated at 37 °C for 1 hour in thermomixer. The created linear pUC plasmid was extracted from GelRed stained 1 % agarose gel by spin columns (Macherey-Nagel). Reaction mixtures of sample **A** and **B** were directly purified on spin columns (Macherey-Nagel) and used like inserts for ligation with linear pUC.

Ligation: The insert d**U^{NB}**-modified DNA or insert after irradiation with **U^{HM}** modifications – (sample **A** or **B**) and linear pUC plasmid in molar ratio of 10:1 (insert to vector) were incubated with T4 DNA Ligase (1.2 μL) in 10x T4 DNA Ligase Buffer (2 μL) at room temperature for

15 minutes. The mixtures were chilled on ice and the 7 µL of each **A** or **B** (insert + pUC) was transformed into competent cells (25 µL). The mixtures with cells were incubated on ice for further 30 minutes. Such modified vectors (**A** and **B**) were transformed into the cells by Heat-shock (the cells were incubated at 42 °C for 90 seconds and were instantly cooled on ice for further 90 seconds). The reaction mixtures (**A** and **B**) were incubated with addition of LB medium (300 µL) at 37 °C for 1 hour. The 200 µL from each reaction mixture was spread on a prewarmed selective plate with ampicillin (AMP) and incubated overnight at 37 °C. A few colonies from each plate were transfer into LB medium with AMP (10 mL) and incubated at 37 °C for further 24 hours. The plasmids **A** and **B** with modified sequence were isolated from cells and purified using of Zyppy Plasmid Miniprep Kit. (The plasmid concentration of sample **A** was: 337 ng/ µL in 30 µL of water and 305 ng/ µL for sample **B.**) To verify the presence of plasmids **A** and **B** with artificial sequence, the isolated replicated plasmids **A** and **B** (100 ng/ µL) were exposed to digestion by restriction endonuclease AfeI (0.25 µL) in CutSmart Buffer (1 µL) at 37°C for 1 hour. Both replicated plasmids with artificial fragment sequence (**A** and **B)** were successfully cleaved in comparison to natural plasmid **N**, which was not cleaved. [***Figure 27b)***] Moreover, both plasmids were provided for sequencing, which confirmed the expected sequence of artificial fragment without misincorporations. **(*Scheme 34)***

6.3 Study of transcription with modified DNA templates

6.3.1 Effect of 5-(hydroxymethyl)-modified and 5-(nitrobenzyloxymethyl)-modified DNA on transcription

Synthesis of modified DNA templates

Oligonucleotide-	Sequence (5′→3′)	Length
PRIMERs		
***Prim*^{FOR}**	TTCAGCCATATATCCTCTGGCTAATAGG	**28-mer**
***Prim*^{REV}**	GGAGAGCGTTCACCGACAAACAACAG	**26-mer**
TEMPLATE[a,b]		
***Temp*^{Pveg2}**	TTCAGCCATATATCCTCTGGCTAATAGGAC TACTTCTAATCTGTACGAGCAGATCCATAC GCCTGGACAGGCAATCAGGCTAGAGGAAT TC***TATTTGACAAAAATGGGCTCGTGTTGTAC*** ***AATAAATGT***GTCTAAGCTTGGGTCCCACCT GACCCCATGCCGAACTCAGAAGTGAAAC GCCGTAGCGCCGATGGTAGTGTGGGGTC TCCCCATGCGAGAGTAGGGAACTGCCAGG CATCAAATAAAACGAAAGGCTCAGTCGAA AGACTGGGCCTTTCGTTTTATCTGTTGTTTG TCGGTGAACGCTCTCC	**311-mer**

[a] primer sequences in template are underlined
[b] promoter sequence of template is bold in italic

Table 12: *Overview of used oligonucleotides*

Synthesis of A^{HM}-/ U^{HM}-modified DNA

The reaction mixture (20 µL) for PCR reaction contained *NON*-labelled primers (20 µM; 3 µL; ***Prim*^{FOR}** and 20 µM; 3 µL; ***Prim*^{REV}**), 311-mer template (40 ng/µL; 0.9 µL; ***Temp*^{Pveg2}**), natural dNTPs (for **U^{HM}**- sample: dGTP, dCTP and dATP; for **A^{HM}**- sample: dGTP, dCTP and dTTP; 4 mM; 0.75 µL), modified **dU^{HM}TP** or **dA^{HM}TP** (4 mM; 1.5 µL) and KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 1.2 µL) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer. The PCR reaction was carried out in forty cycles in the thermal cycler. The conditions of PCR reaction were as follows: preheating of reaction mixture at 94 °C for 3 minutes, denaturation at 94 °C for 1 minute, annealing at 68 °C for 1 minute, extension at 75 °C for 1.5 minutes, followed by the final extension step at 75 °C for 5 minutes. ***(Figure 28, Figure 52)***

Synthesis of A^{NB}- / U^{NB}-modified DNA

The reaction mixture (20 µL) for PCR reaction contained *NON*-labelled primers (20 µM; 3 µL; ***Prim*^{FOR}** and 20 µM; 3 µL; ***Prim*^{REV}**), 311-mer template (40 ng/µL; 0.9 µL; ***Temp*^{Pveg2}**), natural

dNTPs (for **U^{NB}**- sample: dGTP, dCTP and dATP; for **A^{NB}**- sample: dGTP, dCTP and dTTP; 4 mM; 0.75 µL), modified **dU^{NB}TP** or **dA^{NB}TP** (4 mM; 2 µL) and KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 1.5 µL) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer. The PCR reaction was carried out in forty cycles in the thermal cycler. The conditions of PCR reaction were as follows: preheating of reaction mixture at 94 °C for 3 minutes, denaturation at 94 °C for 1 minute, annealing at 62 °C for 1 minute, extension at 75 °C for 2 minutes, followed by the final extension step at 75 °C for 7 minutes. ***(Figure 28, Figure 52)***.

Synthesis of C^{HM} and C^{NB}-modified DNA

The reaction mixture (20 µL) for PCR reaction contained either *NON*-labelled or ^{32}P-labelled primers (20 µM; 3 µL; ***Prim^{FOR}*** and 20 µM; 3 µL; ***Prim^{REV}***), 311-mer template (40 ng/µL; 0.9 µL; ***Temp^{Pveg2}***), natural dNTPs (dGTP, dTTP and dATP; 4 mM; 0.75 µL), modified **dC^{HM}TP** (4 mM; 1.5 µL) or **dC^{NB}TP** (4 mM; 2 µL) and KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 1.2 µL) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer. The PCR reaction was carried out in forty cycles in the thermal cycler. The conditions of PCR reaction were as follows: preheating of reaction mixture at 94 °C for 3 minutes, denaturation at 94 °C for 1 minute, annealing at 68 °C for 1 minute, extension at 75 °C for 1.5 minutes, followed by the final extension step at 75 °C for 5 minutes. ***(Figure 28, Figure 52)***

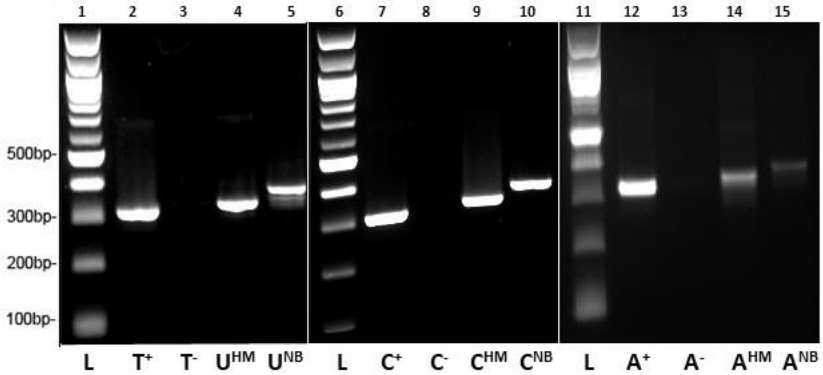


Figure 52: *Agarose gel analysis of modified 311-bp DNA templates.* In the gel, the order of samples is: Lanes 1, 6, 11; (**L**): ladder (commercial dsDNA with specific length); Lanes 2, 7, 12; (**T^{+}** or **C^{+}** or **A^{+}**): natural PCR products; Lane 3; (**T^{-}**): negative control experiment (absence of dTTP); Lane 4; (**U^{HM}**): **U^{HM}** -modified PCR product (PCR was run in the presence of dCTP, dGTP, dATP and **dU^{HM}TP**); Lane 5; (**U^{NB}**): **U^{NB}** -modified PCR product (PCR was run in the presence of dCTP, dGTP, dATP and **dU^{NB}TP)**; Lane 8; (**C^{-}**): negative control experiment (absence of dCTP); Lane 9; (**C^{HM}**): **C^{HM}** -modified PCR product (PCR was run in the presence of dTTP, dGTP, dATP and **dC^{HM}TP**); Lane 10; (**C^{NB}**): **C^{NB}**-modified PCR product (PCR was run in the presence of dTTP, dGTP, dATP and **dC^{NB}TP)**. Lane 13; (**A^{-}**): negative control experiment (absence of dATP); Lane 14; (**A^{HM}**): **A^{HM}**-modified PCR product

(PCR was run in the presence of dTTP, dGTP, dCTP and **dA^{HM}TP**); Lane 15; (**A^{NB}**): **A^{NB}** -modified PCR product (PCR was run in the presence of dTTP, dGTP, dCTP and **dA^{NB}TP)**. 1.3% agarose gels were stained with GelRed.

Multiple round *in vitro* transcriptions: The reaction mixture of each multiple round *in vitro* transcription (10 μL) contained RNA polymerase holoenzyme from *Escherichia coli* (EcoRNAP σ70 ; New England Biolabs; 30 nM), either natural or modified DNA template (5 ng), Tris (pH 8; 100 mM), $MgCl_2$ (125 mM), DTT (50 mM) and KCl (90 mM) was preheated at 37 °C for 10 minutes. The reaction of transcription was started with mixture natural NTPs (ATP - 200 μM, GTP - 1000 μM, CTP - 200 μM, UTP - 10 μM) and [α-32P] UTP (3.7 kBq). The reaction mixture was incubated at 37 °C for 10 minutes and subsequently stopped by the addition of formamide stop solution (10 μL). The reaction of transcription was performed for all DNA templates. The resulted products of transcription (10 μL) were analysed by 7 % native PAA gels. Dried gels were exposed to the Fuji MS phosphor storage screens. The screens were scanned with a Molecular Imager FX (BIORAD) and analysed with Quantity One program (BIORAD). ***(Figure 28)***

Quantification of transcripts: The transcript signals, with subtracted background, were normalized to the signals of DNA templates. In the next step, signals of transcriptions (of non-modified and modified DNA templates) were normalized to the signal of natural DNA template (**T**$^+$ or **A**$^+$ or **C**$^+$) which was set as 100 %. At least three independent transcription experiments were performed. ***(Figure 28)***

6.3.2 Influence of epigenetic hmU, hmC, dU and mC modifications on transcription with bacterial RNA polymerase [154]

General remarks

Synthetic natural oligonucleotides (*FAM*-labelled primers, *NON*-labelled primers and *5´-phosphate* modified primers) were purchased from Generi Biotech. Synthetic modified primers (***Prim$^{FOR\text{-}PgII\text{-}X}$***) were purchased from IBA Lifesciences. Natural deoxynucleotide solution set (dATP, dCTP, dGTP, dTTP) and **dC^{Me}TP** were purchased from New England Biolabs, **dUTP** was purchased from Thermo Fischer Scientific, and **dC^{HM}TP** from TriLink Biotechnologies. **dU^{HM}TP** was prepared according to published [150] and above reported procedure. The Lambda Exonuclease and T4 DNA Ligase were bought from New England Biolabs. All PCR products and final DNA templates were purified using commercially available spin columns (QIAquick

PCR Purification Kit or QIAquick Nucleotide Removal Kit from QIAGEN; E.Z.N.A. Gel Extraction Kit from Omega Bio-Tek) and/ or by Agencourt AMPure XP magnetic particles (Beckman Coulter Life Science - GE Healthcare).

6.3.2.1 Determination of (relative) concentrations of modified DNA templates

Since the presence of modified nucleobases in the DNA templates most likely could influence extinction coefficients (determination of DNA concentration using NanoDrop) and intercalation and/or possible quenching of fluorescence of the DNA staining fluorescent dyes (i.e. GelRed), it was necessary to establish a reliable method for determination of the concentrations of modified DNA templates.

Compared to the non-modified DNA, the bands of $\mathbf{U^{HM}}$-modified DNA gave much weaker signal on agarose gel showing that its concentration must have been lower. Therefore, the concentration of the modified DNA templates could not be simply taken from measurement of the UV absorbance on NanoDrop Spectrophotometer.

The ^{32}P-labelled and *NON*-labelled modified DNA templates were prepared by PCR, purified and diluted to the apparent final concentration of 30 ng/µL according to NanoDrop Spectrophotometer to verify their concentrations. The 180 ng of modified DNAs were loaded on a 5 % PAA gel or 1.3 % agarose gel. The *NON*-labelled DNA templates were analysed on agarose gel with detection by fluorescent dye -GelRed. The ^{32}P-labelled DNA templates were loaded on PAA gel and analysed by detection of radioactivity. The integral values of relative intensities of the DNA bands on the gels did not correspond to the relative concentrations of the modified DNA templates measured by the Nanodrop Spectrophotometer.

By comparing the intensities of the modified DNA template spots on the gels using either fluorescence detection by GelRed staining or ^{32}P-radioactivity detection, the correlation was satisfactory. Based on that, either staining by GelRed and fluorescence detection or ^{32}P -labelling and radioactive detection of DNA signals were used for determination of modified DNA relative concentrations in the transcription studies. (***Table 13)***

$Temp^{Pveg}$		
	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 %)
U^{HM}	121.6 ng (67.6 %)	96.8 ng (53 %)
C^+	180 ng (100 %)	180 ng (100 %)
C^{Me}	144.9 ng (80.5 %)	184 ng (102 %)
C^{HM}	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 %)
U^{HM}	62.2 ng (34.5 %)	90.3 ng (50.2 %)
C^+	180 ng (100 %)	180 ng (100 %)
C^{Me}	202.5 ng (112 %)	172 ng (95.7 %)
C^{HM}	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 %)
U^{HM}	67 ng (37 %)	94.8 ng (52.7 %)
C^+	180 ng (100 %)	180 ng (100 %)
C^{Me}	146 ng (81 %)	136.9 ng (74.1 %)
C^{HM}	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 %)
U^{HM}	54.8 ng (30.5 %)	77.7 ng (43 %)
C^+	180 ng (100 %)	180 ng (100 %)
C^{Me}	111.3 ng (61.8%)	138 ng (76.6 %)
C^{HM}	135 ng (75%)	153 ng (85 %)

Table 13: *Overview of relative concentrations of NON-labelled (agarose) and ^{32}P- labelled (PAA) DNA templates.* Natural DNA template (**T^+**) measured by the NanoDrop Spectrophotometer was set up as 100 %. The percentages listed in parentheses are the relative amounts of the modified DNAs as detected either by fluorescence or radioactivity compared to the signal of natural DNA. The calculated amounts of modified DNAs in [ng] are listed in the table.

6.3.2.2 Synthesis of fully modified 339-mers of DNA

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

The 339-mer (for ***Temp***^{Pveg} and ***Temp***$^{rrnB\ P1\text{-}10Dveg}$) and 340-mer (for ***Temp***$^{rrnB\ P1}$and ***Temp***$^{Pveg\text{-}10DBP1}$) templates were synthesized by PCR in the presence of forward (***Prim***^{FOR}) and reverse (***Prim***^{REV}) *NON*-labelled primers from plasmids, which contained specific promoter regions (either for ***Pveg*** or ***BP1*** or ***PgBP1*** or ***BP1Pg***) cloned in p770 between *Eco*RI and *Hind*III sites.[100] 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), primers (20 µM; 6 µL; ***Prim***^{FOR} and 20 µM; 6 µL; ***Prim***^{REV}), natural dNTPs (4 mM; 1.5 µL) and corresponding plasmid template (88 ng of ***Pveg***; 86 ng of ***BP1***; 77 ng of ***PgBP1*** and 77 ng of ***BP1Pg***). Total volume (40 µL) of each reaction mixture was divided to two Eppendorf PCR tubes and forty PCR cycles were run in the thermal cycler. The reaction mixtures were incubated under the following conditions of PCR reaction: 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 the final extension step of 5 minutes at 75 °C. Created PCR products of each type were mixed together and purified by Agencourt AMPure XP magnetic particles. In the last step of purification, the products were eluted with 40 µL of MilliQ water in final concentrations: 99 ng/µL of ***Temp***^{Pveg}; 117 ng/ µL of ***Temp***^{BP1}; 109 ng/µL of ***Temp***^{PgBP1} and 115 ng/µL of ***Temp***^{BP1Pg}. To check the length and purity of prepared templates, 80 ng of all DNA templates were loaded on 1.3 % agarose gels stained with GelRed. (***Figure 53)***

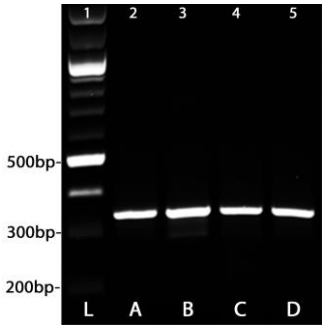


Figure 53: *Agarose gel analysis of PCR template products amplified by Taq DNA polymerase.* Lane 1, (**L**): ladder (mix of natural dsDNA with the specific length); Lane 2, (**A**): PCR product of ***Temp***^{Pveg}; Lane 3, (**B**): PCR product of ***Temp***$^{rrnB\ P1}$; Lane 4, (**C**): PCR product of ***Temp***$^{Pveg\text{-}10DBP1}$; Lane 5, (**D**): PCR product of ***Temp***$^{rrnB\ P1\text{-}10Dveg}$.

Purification of DNA using Agencourt AMPure XP magnetic particles: Synthesized DNA sample after PCR was transferred from the PCR tube (200 μL) into a 1.5 mL Eppendorf tube and resuspended magnetic particles (Agencourt AMPure XP) were added. The volume of added Agencourt AMPure XP magnetic particles was 1.8x the volume of the DNA sample. Mixture of DNA sample and magnetic particles was mixed from 10 to 20 times with pipette and let to stand at room temperature for 10 minutes. The mixture after incubation was placed on magnet. After separation of magnetic beads with bound DNA from solution (approx. 3 minutes), the solution was removed, and the magnetic beads with DNA were washed with 200 μL of 80 % ethanol, two times. During the washing, the magnetic beads with DNA attached to the magnet were incubated at room temperature with the ethanol for 30-60 seconds without breaking of the magnetic beads film attached to the magnet. After the discarding of second portion of ethanol, 40 μL of MilliQ water was added to the magnetic beads with DNA product and mixture was mixed from 10 to 20 times with pipette. After its incubation at room temperature for 3 minutes, mixture was placed on a magnet. After 1 minute the magnetic beads were separated from the solution with DNA. The eluant with DNA was removed into a new Eppendorf tube and used in the next studies.

Synthesis of fully modified DNA with different templates (*Temp^{Pveg}*; *Temp$^{rrnB\ P1}$*; *Temp$^{Pveg\text{-}10DBP1}$*; *Temp$^{rrnB\ P1\text{-}10Dveg}$*)

A total final volume for synthesis of modified DNA templates by PCR was 20 μL. Natural DNAs (**T**$^{+}$ or **C**$^{+}$) were synthesized according to a procedure for **U^{HM}**-modified or **C^{Me}**/**C^{HM}**-modified DNA in the presence of natural dNTPs (4 mM; 0.75 μL) and without any additives. ***(Figures 55, 57, 59, 61, 64)***. When the protocol for synthesis of modified DNA was markedly different, the positive controls were synthesized under the same conditions to show the suitability the conditions also for natural DNA (in the presence of all natural dNTPs - 4 mM; 0.75 μL) ***(Figures 54, 56, 58, 60, 63)***. *

All DNA samples were prepared in the presence of either ^{32}P-labelled (***Prim^{FOR} – ^{32}P*** and ***Prim^{REV} – ^{32}P***) or *NON*-labelled (***Prim^{FOR}*** and ***Prim^{REV}***) primers.

The ^{32}P-labelled primers were freshly prepared by the transfer of the terminal radioactive phosphate from (γ)-^{32}P-dATP to the 5′-end of *NON*-labelled primers by T4 polynucleotide kinase.

The forty cycles of PCR reaction were run in the thermal cycler. The step of preheating was run at 94 °C for 3 minutes, denaturation at 94°C for 1 minute, annealing at 70 °C for 1 minute, extension at 75 °C for 1.5 minutes, followed by final extension step at 75 °C for 5 minutes.
The products of PCR reaction were purified with Agencourt AMPure XP magnetic particles. The concentration of purified DNA samples was determined by measuring of DNA absorbance on NanoDrop Spectrophotometer. All DNA samples were diluted to a final concentration of 20 ng/uL. Such prepared DNA samples were used as templates for transcription studies. The exact quantity of DNA templates was analysed based on the DNA signals (fluorescence and/or radioactivity) on a gel (*section* ***6.3.2.1***).
The 180 ng of *NON*-labelled and ^{32}P-labelled DNA samples *(gels A) and B) in* ***Figures 55, 57, 59, 61, 64)*** were analysed on 1.3 % agarose gels stained with GelRed (Biotium). Moreover, ^{32}P-labelled DNA samples were analysed on 5 % native PAA gel *(gels C) in* ***Figures 55, 57, 59, 61, 64)***. Fidelity of the sequences was confirmed by Sanger sequencing.

**- natural DNA was synthesized under the same conditions as modified DNA reported on a gel, to show, that conditions for the synthesis of modified DNA are suitable also for the synthesis of natural DNA*

I. Synthesis of fully modified DNA with template - $Temp^{Pveg}$

Synthesis of fully dU-modified DNA

The PCR mixture (20 µL) contained P*veg* template (30 ng/µL; 1.6 µL; $\boldsymbol{Temp^{Pveg}}$), either *NON*-labelled or ^{32}P-labelled primers (20 µM; 3 µL; $\boldsymbol{Prim^{REV}}$ and 20 µM; 3 µL; $\boldsymbol{Prim^{FOR}}$), natural dCTP, dGTP and dATP (each 4 mM; 1 µL), **dUTP** (4 mM; 1 µL) and Taq DNA polymerase for ThermoPol Buffer (*New England Biolabs*; 5000 U/mL; 2.4 µL) in ThermoPol Buffer supplied by the manufacturer (2 µL). The products were after PCR reaction purified and analysed by GelRed stained 1.3 % agarose gel ***(Figures 54, 55)***.

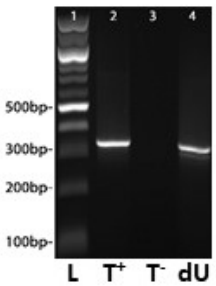


Figure 54: *Control agarose gel analysis of NON-labelled dU-modified DNA*. * Composition of the dNTP mixes and nucleotide labelling are as follows: Lane 1, (**L**): ladder (commercial dsDNA with specific length); Lane 2, ($\mathbf{T^+}$): natural PCR product; Lane 3, ($\mathbf{T^-}$): negative control experiment (absence

of dTTP); Lane 4, (**dU**): **dU**-modified PCR product (PCR reaction was run in the presence of dCTP, dGTP, dATP and **dUTP**).

Synthesis of fully U^{HM}-modified DNA

The PCR mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 1.2 µL), P*veg* template (30 ng/µL; 1.2 µL; ***Temp^{Pveg}***), either ^{32}*P*-labelled *or NON*-labelled primers (20 µM; 3µL; ***Prim^{FOR}*** and 20 µM; 3 µL; ***Prim^{REV}***), natural dCTP, dGTP and dATP (each 4 mM; 0.75 µL), **dU^{HM}TP** (4 mM; 1.5 µL), with addition $MgSO_4$ (100 mM; 2 µL) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer. The products after PCR reaction were purified and analysed by gel analyses ***(Figure 55)***.

Synthesis of fully C^{Me}- and C^{HM}-modified DNA

The PCR mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/ µL; 1.2 µL), P*veg* template (30 ng/µL; 1.2 µL; ***Temp^{Pveg}***), either ^{32}*P*-labelled or *NON*-labelled primers (20 µM; 3 µL; ***Prim^{REV}*** and 20 µM; 3 µL; ***Prim^{FOR}***), natural dTTP, dGTP and dATP (each 4 mM; 0.75 µL), **dC^{Me}TP** (4 mM; 2 µL) or **dC^{HM}TP** (4 mM; 1.5 µL) in 10x reaction Buffer for KOD XL polymerase (2 µL) supplied by the manufacturer ***(Figure 55)***.

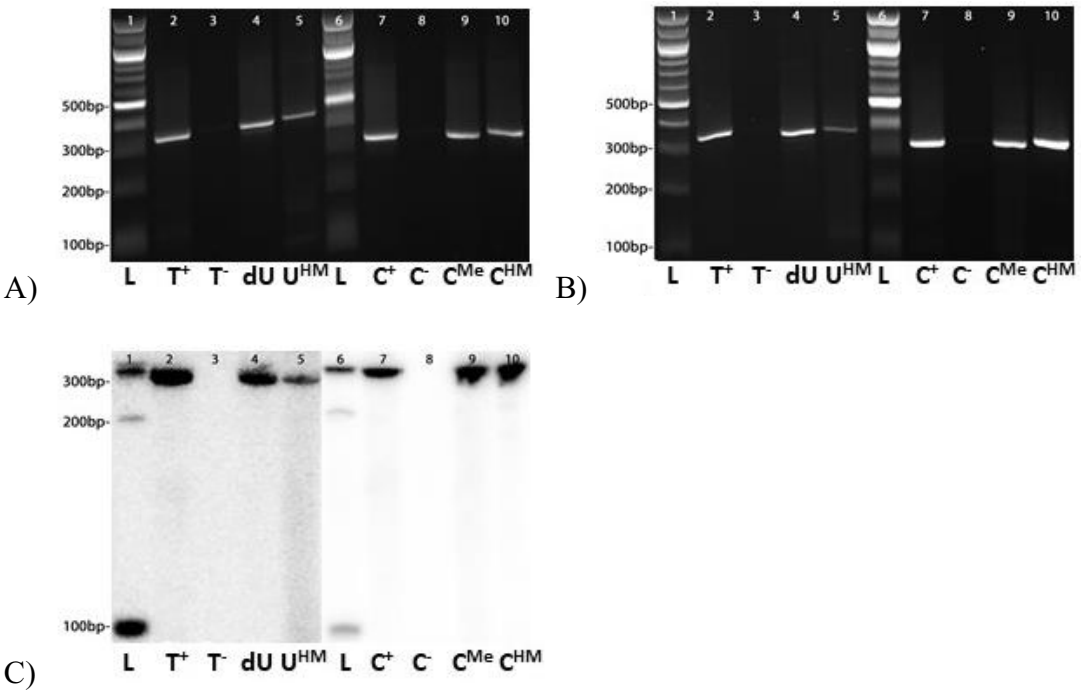


Figure 55: A) *Agarose gel analysis of NON-labelled PCR products.* ***B)*** *Agarose gel analysis of ^{32}P-labelled PCR products.* ***C)*** *Native PAA gel analysis of ^{32}P-labelled PCR products*. In the gels, the order of samples is: Lanes 1,6; (**L**): ladder (commercial dsDNA with specific length); Lanes 2,7; (**T$^+$** or **C$^+$**): natural PCR products; Lane 3, (**T$^-$**): negative control experiment (absence of dTTP);

Lane 4, (**dU**): **dU**-modified PCR product; Lane 5, (**U^{HM}**): **U^{HM}** -modified PCR product (PCR was run in the presence of dCTP, dGTP, dATP and **$dU^{HM}TP$)**; Lane 8, (**C^{-}**): negative control experiment (absence of dCTP); Lane 9, (**C^{Me}**): **C^{Me}** -modifies PCR product (PCR was run in the presence of dTTP, dGTP, dATP and **$dC^{Me}TP$**); Lane 10, (**C^{HM}**): **C^{HM}** -modified PCR product (PCR was run in the presence of dTTP, dGTP, dATP and **$dC^{HM}TP$)**. **A**) and **B**) 1.3% agarose gels were stained with GelRed.

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

Synthesis of fully dU-modified DNA

The PCR mixture (20 µL) contained *rrnB-P1* template (24 ng/µL; 1.6 µL; ***$Temp^{rrnB\ P1}$***), either *^{32}P*-labelled or *NON*-labelled primers (20 µM; 3 µL; ***$Prim^{REV}$*** and 20 µM; 3 µL; ***$Prim^{FOR}$***), natural dCTP, dGTP and dATP (each 4 mM; 1 µL), **dUTP** (4 mM; 1 µL) and Taq DNA polymerase for ThermoPol Buffer (*New England Biolabs*; 5000 U/mL; 2.4 µL) in ThermoPol Buffer supplied by the manufacturer (2 µL). ***(Figures 56, 57)***

Synthesis of fully U^{HM}-modified DNA

The PCR mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 1.2 µL), *rrnB-P1* template (24 ng/µL; 1.6 µL; ***$Temp^{rrnB\ P1}$***), either *^{32}P*-labelled or *NON*-labelled primers (20 µM; 3 µL; ***$Prim^{FOR}$*** and 20 µM; 3 µL; ***$Prim^{REV}$***), natural dCTP, dGTP and dATP (each 4 mM; 1 µL), **$dU^{HM}TP$** (4 mM; 1 µL) and addition $MgSO_4$ (100 mM; 0.7 µL) in 10x KOD XL reaction Buffer (2 µL) supplied by the manufacturer. ***(Figures 56, 57)***

A) B)

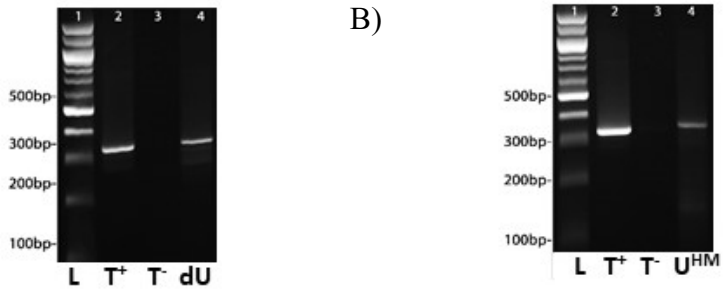


Figure 56: A) *Agarose gel analysis of NON-labelled dU-modified PCR products.* **** B)*** *Agarose gel analysis of NON-labelled U^{HM}-modified PCR products.* ***** In both cases, the order of samples is: Lanes 1, (**L**): ladder (commercial dsDNA with specific length); Lanes 2, (**T^{+}**): natural PCR product; Lanes 3, (**T^{-}**): negative control experiment (PCR was run in absence of dTTP); ***A)*** Lane 4, (**dU**): PCR was run in the presence of dCTP, dGTP, dATP and **dUTP**; ***B)*** Lane 4, (**U^{HM}**): **U^{HM}** -modified PCR product (PCR was run in the presence of dCTP, dGTP, dATP and **$dU^{HM}TP$**. The PCR products were analysed by GelRed stained 1.3% agarose gel.

Synthesis of fully C^{Me}- and C^{HM}-modified DNA

The PCR mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µl; 1.2 µL), *rrnB-P1* template (24 ng/µL; 1.6 µL; ***Temp$^{rrnB\ P1}$***), either ^{32}P-labelled or *NON*-labelled primers (20 µM; 3 µL; ***Prim^{REV}*** and 20 µM; 3 µL; ***Prim^{FOR}***), natural dTTP, dGTP and dATP (each 4 mM; 0.75 µL) and **dC^{Me}TP** (4 mM; 2 µL) or **dC^{HM}TP** (4 mM; 1.5 µL) in 10x KOD XL reaction Buffer (2 µL) supplied by the manufacturer.***(Figures 57)***

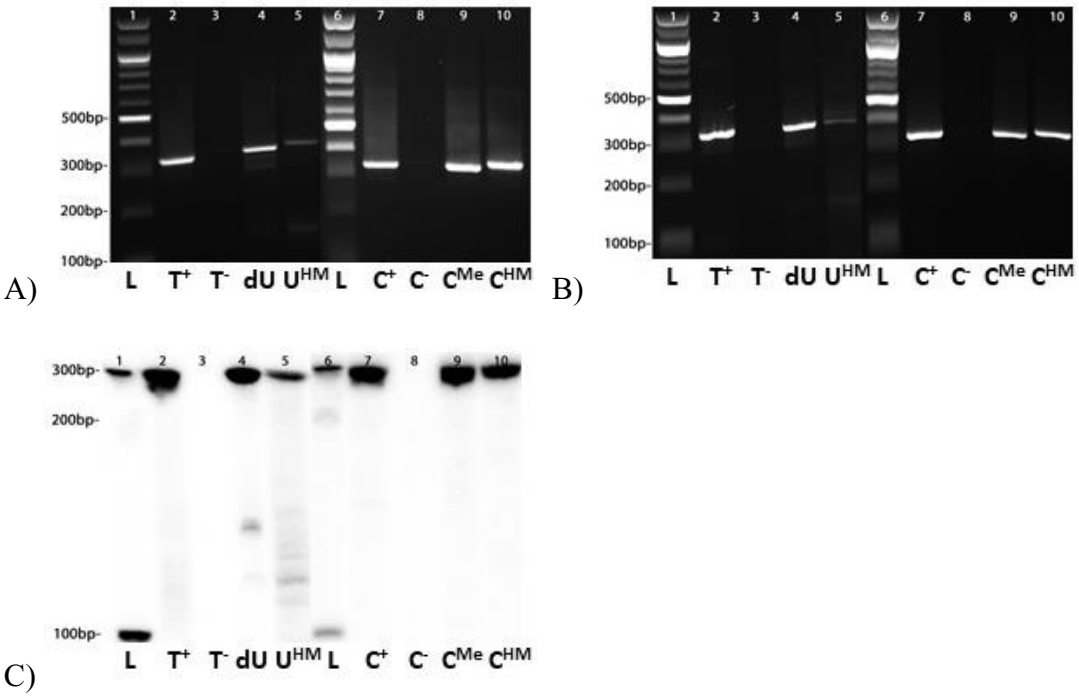


Figure 57: A) *Agarose gel analysis of NON-labelled PCR products.* ***B)*** *Agarose gel analysis of ^{32}P-labelled PCR products.* ***C)*** *Native PAA gel analysis of ^{32}P-labelled PCR products*. In the gels, the order of samples is: Lanes 1,6; (**L**): ladder (commercial dsDNA with specific length); Lanes 2,7; (**T^{+}** or **C^{+}**): natural PCR products; Lane 3, (**T^{-}**): negative control experiment (absence of dTTP); Lane 4, (**dU**): **dU**-modified PCR product; Lane 5, (**U^{HM}**): **U^{HM}** -modified PCR product (PCR was run in the presence of dCTP, dGTP, dATP and **dU^{HM}TP)**; Lane 8 (**C^{-}**): negative control experiment (absence of dCTP); Lane 9, (**C^{Me}**): **C^{Me}** -modified PCR product (PCR was run in the presence of dTTP, dGTP, dATP and **dC^{Me}TP**); Lane 10, (**C^{HM}**): **C^{HM}** -modified PCR product (PCR was run in the presence of dTTP, dGTP, dATP and **dC^{HM}TP)**. **A**) and **B**) 1.3% agarose gels were stained with GelRed.

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

Synthesis of fully dU-modified DNA

The PCR mixture (20 µL) contained *Pveg-10DBP1* template (24 ng/µL; 1.6 µL; ***Temp$^{Pveg\text{-}10DBP1}$***), either ^{32}P-labelled or *NON*-labelled primers (20 µM; 2 µL; ***Prim^{REV}*** and 20 µM; 2 µL;

Prim^{FOR}), natural dCTP, dGTP and dATP (each 4 mM; 1 µL), **dUTP** (4 mM; 1 µL) and Taq DNA polymerase for ThermoPol Buffer (*New England Biolabs*; 5000 U/mL; 2.4 µL) in ThermoPol Buffer supplied by the manufacturer (2 µL). (***Figures 58, 59)***

Synthesis of fully U^{HM}-modified DNA

The PCR mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 1.2 µL), *Pveg-10DBP1* template (24 ng/µL; 1.6 µL; ***Temp$^{Pveg-10DBP1}$***), either ^{32}P-labelled or *NON*-labelled primers (20 µM; 2 µL; ***Prim^{REV}*** and 20 µM; 2 µL; ***Prim^{FOR}***), natural dCTP, dGTP and dATP (each 4 mM; 0.75 µL), **dU^{HM}TP** (4 mM; 1.5 µL) and addition $MgSO_4$ (100 mM; 2 µL) in 10x KOD XL reaction Buffer (2 µL) supplied by the manufacturer. (***Figures 58, 59)***.

Synthesis of fully C^{HM}-modified DNA

The PCR mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µl; 1.2 µL), the *Pveg-10DBP1* template (24 ng/µL; 1.6 µL; ***Temp$^{Pveg-10DBP1}$***), either ^{32}P-labelled or *NON*-labelled primers (20 µM; 3 µL; ***Prim^{REV}*** and 20 µM; 3 µL; ***Prim^{FOR}***), natural dTTP, dGTP and dATP (each 4 mM; 1 µL), **dC^{HM}TP** (4 mM; 2 µL) and addition $MgSO_4$ (100 mM; 2 µL) in 10x KOD XL reaction Buffer (2 µL) supplied by the manufacturer. (***Figures 58, 59)***

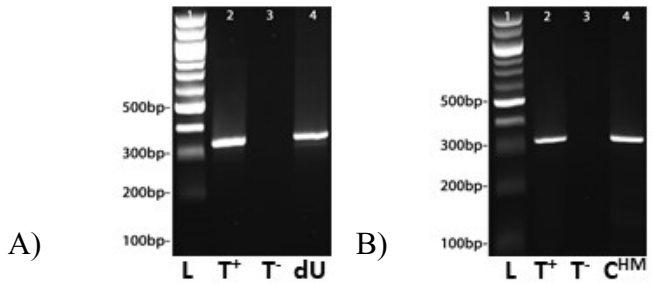


Figure 58: A) *Agarose gel analysis of NON-labelled dU-modified PCR products.** ***B)*** *Agarose gel analysis of NON-labelled C^{HM}-modified PCR products.** In both cases, the order of samples is: Lanes 1, (**L**): ladder (commercial dsDNA with specific length); Lanes 2, (**T$^+$** or **C$^+$**): natural PCR product; Lanes 3, (**T$^-$** or **C$^-$**): negative control experiment (PCR was run in absence of dTTP or dCTP); ***A)*** Lane 4, (**dU**): PCR was run in the presence of dCTP, dGTP, dATP and **dUTP**; ***B)*** Lane 4, (**C^{HM}**): **C^{HM}** -modified PCR product (PCR was run in the presence of dTTP, dGTP, dATP and **dC^{HM}TP**). The PCR products were analysed by GelRed stained 1.3% agarose gel.

Synthesis of fully C^{Me}-modified DNA

The PCR mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µl; 1.2 µL), the *Pveg-10DBP1* template (24 ng/µL; 1.6 µL; ***Temp$^{Pveg-10DBP1}$***), either ^{32}P-labelled or *NON*-labelled primers (20 µM; 2 µL; ***Prim^{REV}*** and 20 µM; 2 µL; ***Prim^{FOR}***), natural

dTTP, dGTP and dATP (each 4 mM; 0.75 µL), **dC^{Me}TP** (4 mM; 2 µL) and addition of $MgSO_4$ (100 mM; 1 µL) in 10x KOD XL reaction Buffer (2 µL) supplied by the manufacturer. (***Figures 59)***

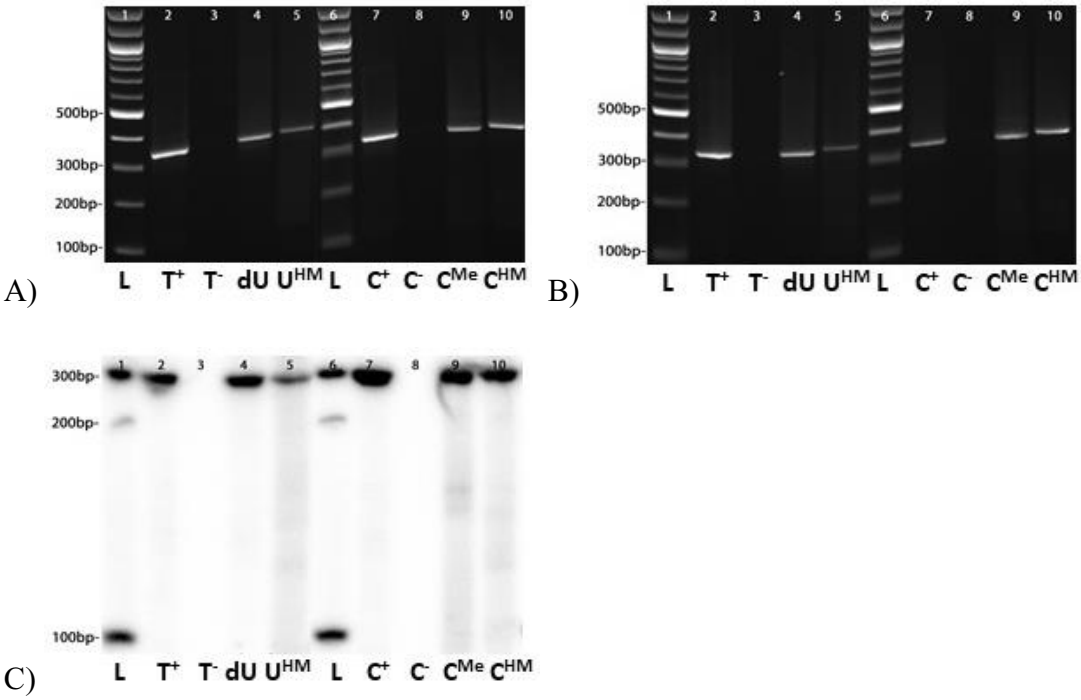


Figure 59: A) *Agarose gel analysis of NON-labelled PCR products.* ***B)*** *Agarose gel analysis of ^{32}P-labelled PCR products.* ***C)*** *Native PAA gel analysis of ^{32}P-labelled PCR products*. In the gels, the order of samples is: Lanes 1,6; (**L**): ladder (commercial dsDNA with specific length); Lanes 2,7; (**T**$^{+}$ or **C**$^{+}$): natural PCR products; Lane 3, (**T**$^{-}$): negative control experiment (absence of dTTP); Lane 4, (**dU**): **dU**-modified PCR product; Lane 5, (**U^{HM}**): **U^{HM}** -modified PCR product (PCR was run in the presence of dCTP, dGTP, dATP and **dU^{HM}TP)**; Lane 8 (**C**$^{-}$): negative control experiment (absence of dCTP); Lane 9, (**C^{Me}**): **C^{Me}** -modifies PCR product (PCR was run in the presence of dTTP, dGTP, dATP and **dC^{Me}TP**); Lane 10, (**C^{HM}**): **C^{HM}** -modified PCR product (PCR was run in the presence of dTTP, dGTP, dATP and **dC^{HM}TP)**. **A**) and **B**) : 1.3 % agarose gels were stained with GelRed.

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

Synthesis of fully dU-modified DNA

The PCR mixture (20 µL) contained *rrnB P1-10Dveg* template (24 ng/µL; 1.6 µL; ***Temp$^{rrnB\ P1\text{-}10Dveg}$***), either *^{32}P*-labelled or *NON*-labelled primers (20 µM; 2 µL; ***Prim^{REV}*** and 20 µM; 2 µL; ***Prim^{FOR}***) natural dCTP, dGTP and dATP (each 4 mM; 1 µL), **dUTP** (4 mM; 1 µL) and Taq DNA polymerase for ThermoPol Buffer (*New England Biolabs*; 5000 U/mL; 2.4 µL) in ThermoPol Buffer supplied by the manufacturer (2 µL). (***Figures 60, 61)***

Synthesis of fully U^{HM}-modified DNA

The PCR mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µl; 1.2 µL), *rrnB P1-10Dveg* template (24 ng/µL; 1.6 µL; ***Temp***$^{rrnB\ P1\text{-}10Dveg}$), either ^{32}P-labelled or *NON*-labelled primers (20 µM; 2 µL; ***Prim***^{REV} and 20 µM; 2 µL; ***Prim***^{FOR}), natural dCTP, dGTP and dATP (each 4 mM; 0.75 µL), **dU^{HM}TP** (4 mM; 1.5 µL) and addition $MgSO_4$ (100 mM; 2 µL) in 10x KOD XL reaction Buffer (2 µL) supplied by the manufacturer. (***Figures 61)***

Synthesis of fully C^{HM}-modified DNA

The PCR mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µl; 1.2 µL), the *rrnB P1-10Dveg* template (24 ng/µL; 1.6 µL; ***Temp***$^{rrnB\ P1\text{-}10Dveg}$), either ^{32}P-labelled or *NON*-labelled primers (20 µM; 2 µL; ***Prim***^{REV} and 20 µM; 2 µL; ***Prim***^{FOR}), natural dTTP, dGTP and dATP (each 4 mM; 1 µL), **dC^{HM}TP** (4 mM; 1.5 µL) and addition $MgSO_4$ (100 mM; 1 µL) in 10x KOD XL reaction Buffer (2 µL) supplied by the manufacturer (***Figures 60, 61)***.

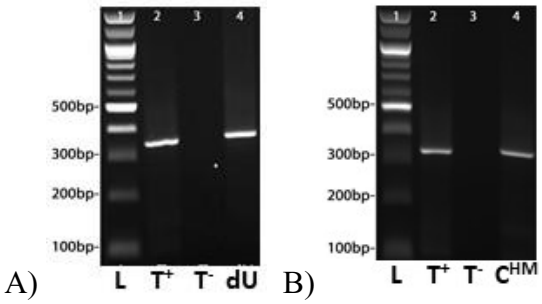


Figure 60: A) *Agarose gel analysis of NON-labelled dU-modified PCR products.** ***B)*** *Agarose gel analysis of NON-labelled C^{HM}-modified PCR products.** In both cases, the order of samples is: Lanes 1, (**L**): ladder (commercial dsDNA with specific length); Lanes 2, (**T**$^+$ or **C**$^+$): natural PCR product; Lanes 3, (**T**$^-$ or **C**$^-$): negative control experiment (PCR was run in absence of dTTP or dCTP); ***A)*** Lane 4, (**dU**): PCR was run in the presence of dCTP, dGTP, dATP and **dUTP**; ***B)*** Lane 4, (**C^{HM}**): **C^{HM}** modified PCR product (PCR was run in the presence of dTTP, dGTP, dATP and **dC^{HM}TP**. The PCR products were analysed by GelRed stained 1.3% agarose gel.

Synthesis of fully C^{Me}-modified DNA

The PCR mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µl; 1.2 µL), *rrnB P1-10Dveg* template (24 ng/µL; 1.6 µL; ***Temp***$^{rrnB\ P1\text{-}10Dveg}$), either ^{32}P-labelled or *NON*-labelled primers (20 µM; 2 µL; ***Prim***^{REV} and 20 µM; 2 µL; ***Prim***^{FOR}), natural dTTP, dGTP and dATP (each 4 mM; 0.75 µL), **dC^{Me}TP** (4 mM; 2 µL) and addition $MgSO_4$ (100 mM; 1 µL) in 10x KOD XL reaction Buffer (2 µL) supplied by the manufacturer. (***Figures 61)***

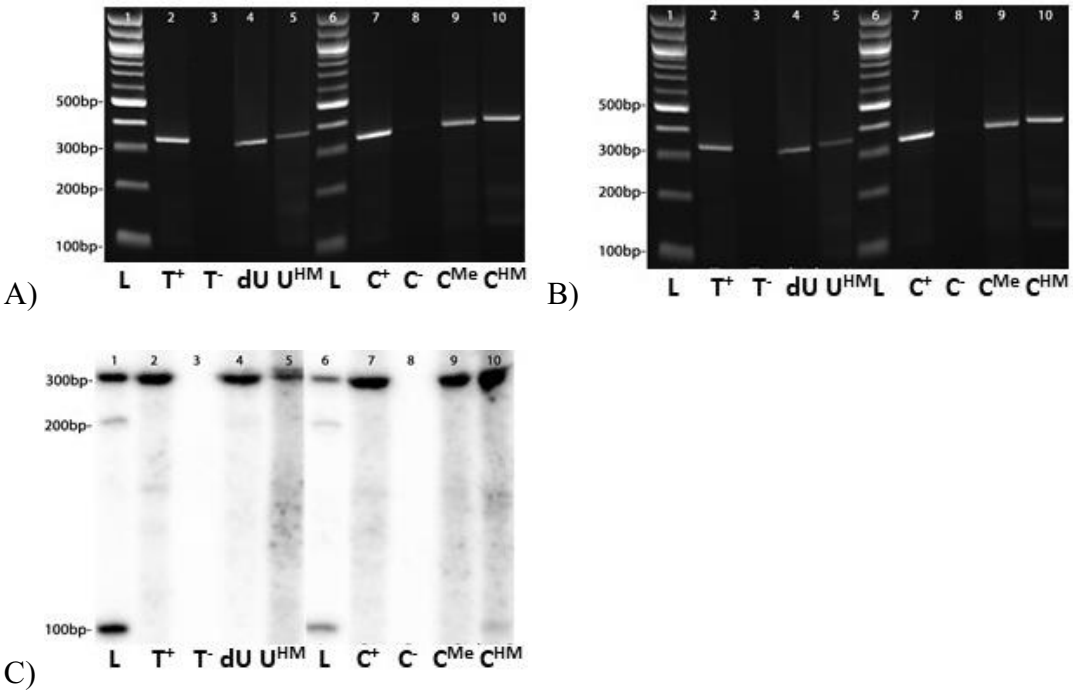


Figure 61: A) *Agarose gel analysis of NON-labelled PCR products.* ***B)*** *Agarose gel analysis of* ^{32}*P-labelled PCR products.* ***C)*** *Native PAA gel analysis of* ^{32}*P-labelled PCR products*. In the gels, the order of samples is: Lanes 1,6; (**L**): ladder (commercial dsDNA with specific length); Lanes 2,7; (**T**$^{+}$ or **C**$^{+}$): natural PCR products; Lane 3, (**T**$^{-}$): negative control experiment (absence of dTTP); Lane 4, (**dU**): **dU**-modified PCR product; Lane 5, (**U**^{HM}): **U**^{HM} -modified PCR product (PCR was run in the presence of dCTP, dGTP, dATP and **dU**^{HM}**TP)**; Lane 8, (**C**$^{-}$): negative control experiment (absence of dCTP); Lane 9, (**C**^{Me}): **C**^{Me} -modified PCR product (PCR was run in the presence of dTTP, dGTP, dATP and **dC**^{Me}**TP**); Lane 10, (**C**^{HM}): **C**^{HM} -modified PCR product (PCR was run in the presence of dTTP, dGTP, dATP and **dC**^{HM}**TP)**. **A**) and **B**) 1.3% agarose gels were stained with GelRed.

6.3.2.3 Synthesis of fully modified 235-mers of DNA with *Pveg* promoter region

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

Table 14: *List of oligonucleotides applied for the synthesis of 235-mers (version Ib)*

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

The PCR reaction mixture (40 µL) contained P*veg* template - ***Temp***^{Pveg} (72 ng), *NON*-labelled primers (20 µM, 6 µL, ***Prim***$^{REV\text{-}PgII\text{-}R1/R3}$ and 20 µM, 6 µL, ***Prim***$^{FOR\text{-}PgII}$), natural dNTPs (4 mM; 1.5 µL) and Taq DNA polymerase for ThermoPol Buffer (*New England Biolabs*; 5000 U/mL; 2.4 µL) with ThermoPol buffer (4 µL) supplied by the manufacturer. The reaction mixture was divided into two PCR tubes and reaction was run in the thermal cycler. The reaction mixture was preheated at 94 °C for 3minutes, denaturation step run at 94 °C for 1 minute, annealing at 52 °C for 1 minute, extension at 75 °C for 1.5 minutes. The reaction run in 40 PCR cycles with a final extension step of 5 minutes at 75 °C. The PCR products were combined and purified by Agencourt AMPure XP magnetic beads. In the last step of purification, the product was eluted with 40 µL of MilliQ water. The final concentration of final product was 113 ng/µL. The purified product was analysed by GelRed stained 1.3 % agarose gel in 0.5xTBE. ***(Figure 62)***

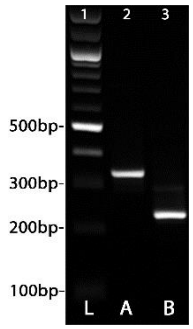


Figure 62: *Agarose gel analysis of 235-mer PCR template product*. The order of samples is: Lane 1, (**L**): ladder (commercial dsDNAs with the specific length); Lane 2, (**A**): 339-mer ***Temp^{Pveg}*** used as template for the PCR reaction; Lane 3, (**B**): 235-mer - ***Temp^{Pveg_sh235}*** (product of described PCR reaction).

Synthesis of fully modified DNA with *Temp^{Pveg_sh235}*-general remarks

The total final volume of all PCR reactions to synthesize modified 235-mer DNAs was 20 µL. The PCR reactions were run in forty PCR cycles in the thermal cycler. The conditions of PCR reactions: preheating at 94 °C for 3 minutes, denaturation at 94 °C for 1 minute, annealing at 52 °C for 1 minute, extension at 75 °C for 1.5 minutes, followed by final extension step of 5 minutes at 75 °C. All PCR products were purified on Agencourt AMPure XP magnetic beads. In the last step of purification, the product was eluted with 40 uL of MilliQ water. The length and purity of final products were checked by GelRed stained 1.3 % agarose gels and fidelity of the sequence was confirmed by Sanger sequencing.

Synthesis of fully dU-modified 235-mer DNA template

The PCR reaction mixture (20 µL) contained 235-mer template (41 ng/µL; 0.88 µL; ***Temp^{Pveg_sh235}***), *NON*-labelled primers (20 µM; 2 µL; ***Prim$^{REV\text{-}PgII\text{-}R1/R3}$*** and 20 µM; 2 µL ***Prim$^{FOR\text{-}PgII}$***), natural dCTP, dGTP and dATP (each 4 mM; 1 µL), **dUTP** (4 mM; 1 µL) and Taq DNA polymerase for ThermoPol Buffer (*New England Biolabs*; 5000 U/ml; 2.4 µL) with ThermoPol Buffer (2 µL) supplied by the manufacturer. ***(Figure 63, 64)***

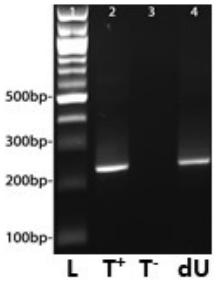


Figure 63: *Agarose gel analysis of dU-modified 235-mer DNA template.* The order of samples is: Lane 1, (**L**): ladder (commercial dsDNAs with specific length); Lane 2, (**T^{+}**): natural PCR product;

Lane 3, (**T**$^{-}$): negative control of PCR (PCR was run in absence of dTTP); Lane 4, (**dU**): dU-modified PCR product.

Synthesis of fully U^{HM}-modified 235-mer DNA template

The PCR mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 1.2 µL), 235-mer template (41 ng/µL; 0.88 µL; ***Temp***$^{Pveg\ \_sh235}$) and *NON*-labelled primers (20 µM; 3µL; ***Prim***$^{REV\text{-}PgII\text{-}R1/R3}$ and 20 µM; 3 µL; ***Prim***$^{FOR\text{-}PgII}$), natural dCTP, dGTP and dATP (each 4 mM; 0.75 µL) and **dU^{HM}TP** (4 mM; 1.5 µL) in 10x KOD XL reaction Buffer (2 µL) supplied by the manufacturer. ***(Figure 64)***

Synthesis of fully C^{Me}- and C^{HM}-modified 235-mer DNA template

The PCR mixture (20 µL) contained KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 1.2 µL), 235-mer template (41 ng/µL; 0.88 µL; ***Temp***$^{Pveg\_sh235}$), *NON*-labelled primers (20 µM; 3 µL; ***Prim***$^{REV\text{-}PgII\text{-}R1/R3}$ and 20 µM; 3 µL ***Prim***$^{FOR\text{-}PgII}$), natural dTTP, dGTP and dATP (each 4 mM; 0.75 µL) and **dC^{Me}TP** (4 mM; 2 µL) or **dC^{HM}TP** (4 mM; 1.5 µL) in 10x KOD XL reaction Buffer (2 µL) supplied by the manufacturer. ***(Figure 64)***

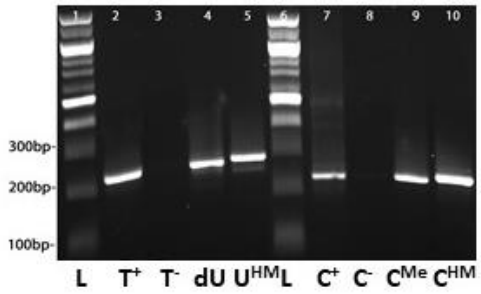


Figure 64: *Agarose gel analysis of NON-labelled modified DNA templates.* The order of samples is: Lanes 1,6; (**L**): ladder (commercial dsDNAs with specific length); Lanes 2,7; (**T**$^{+}$ or **C**$^{+}$): natural PCR products; Lane 3, (**T**$^{-}$): negative control of PCR reaction (PCR was run in absence of dTTP); Lane 4, (**dU**): dU-modified PCR product; Lane 5, (**U^{HM}**): **U^{HM}** -modified PCR product; Lane 8 (**C**$^{-}$): negative control of PCR reaction (PCR was run in absence of dCTP); Lane 9, (**C^{Me}**): **C^{Me}** -modified PCR product; Lane 5, (**C^{HM}**): **C^{HM}** -modified PCR product. The PCR products were analysed by GelRed stained 1.3 % agarose gel.

6.3.2.4 Synthesis of partially modified 235-mers of DNA

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**A**dU***dU*UGACAA*AAA**dU**GGGC**dU**CG**dU**G**dU**C**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**hm***UhmU*GACAAAAA**hmU**GGGC**hmU**CG**hmU**G**hmU**C**hmU**G**hmU**ACAA**hmU**AAA**hmU**G**hmU**G*	**54-mer**
Prim$^{FOR\text{-}PgII\text{-}d5mC8}$	**mC**GT**mC**TT**mC**AAGAATT**mC**TAT*TTGA**mC**AAAAATG G G**mC**T**mC**GTGTTGTA**mC**AATAAATGTG*	**54-mer**
Prim$^{FOR\text{-}PgII\text{-}d5hmC8}$	**hmC**GT**hmC**TT**hmC**AAGAATT**hmC**TAT*TTGA**hmC**AAA AATGGG**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**GT**hmC**TT**hmC**AAGAATT**hmC**TAT*TTGA**hmC**AA AAATGGG**hmC**T**hmC**GTGTTGTA**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*$^{FOR\text{-}PgII\text{-}d5mC4}$**	CGTCTTCAAGAATTCTAT*TTGA**mC**AAAAATGGG**mC* *T**mC**GTGTTGTA**mC**AATAAATGTG*	**54-mer**
***Prim*$^{FOR\text{-}PgII\text{-}d5hmC4}$**	CGTCTTCAAGAATTCTAT*TTGA**hmC**AAAAATGGG* ***hmC**T**hmC**GTGTTGTA**hmC**AATAAATGTG*	**54-mer**
Oligonucleotide–TEMPLATE[c,d,e]		
***Temp*$^{Pveg\_sh235}$**	CGTCTTCAAGAATTCTAT*TTGACAAAAATGGGCTCGT GTTGTACAATAAATGT*GTCTAAGCTTGGGTCCCACCT GACCCCATGCCGAACTCAGAAGTGAAACGCCGTAG CGCCGATGGTAGTGTGGGGTCTCCCCATGCGAGAG TAGGGAACTGCCAGGCATCAAATAAAACGAAAGGC TCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTG T TTGTCGGTGAACGCTCTCC	**235-mer**
***Temp*$^{Pveg\_sh181}$**	TCTAAGCTTGGGTCCCACCTGACCCCATGCCGAACT CAGAAGTGAAACGCCGTAGCGCCGATGGTAGTGTG GGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGC ATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGG GCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTC TCC	**181-mer**
***Temp*$^{Us\_69mer}$**	CGTCTTCAAGAATTCTAT*TTGACAAAAATGGGCTCGT GT TGTACAATAAATGTG*TCTAAGCTTGGGTCC	**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 variant A) and B)* as ***Prim*$^{FOR\text{-}PgV}$*_F*^{b}**

Table 15: *List of oligonucleotides applied for synthesis of the partially modified 235-mer DNA templates*

A) *Synthesis of DNA templates modified in the both strands of transcribed (gene) region*

The dsDNA with natural promoter region and either **dU**, **U^{HM}-**, **C^{Me}-** or **C^{HM}-** modified promoter downstream regions were synthesized in few steps. The PCR reactions to obtain modified 166-mer DNA and natural 69-mer DNA were performed in a total volume of 200 µL. The obtained longer PCR products were purified via QIAquick PCR purification kit and shorter PCR product was purified via QIAquick nucleotide removal kit (*QIAGEN*) according to the protocol. Desired products were eluted in the final step with 30 µL of MilliQ water.

All modified downstream and natural upstream portions were analysed by agarose gel electrophoresis (GelRed stained 1.3 % agarose gel, 0.5x TBE buffer). ***(Figure 65)***

Synthesis of the DNA with natural promoter region

The PCR reaction mixture for synthesis of DNA with natural upstream region (10 µL) contained KOD XL DNA polymerase (*Merck, Novagen;* 2.5 U/µL; 0.25 µL), *NON*-labelled 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 (each 4 mM; 0.075 µL) in the KOD XL reaction buffer (1 µL) supplied by the manufacturer. The amplification reaction was carried out in a PCR thermal cycler. The PCR reaction was run in 25 cycles 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 final extension at 72 °C for 1 minute. The PCR product was purified by using QIAquick nucleotide removal kit *(QIAGEN*). In the final step of purification, the purified DNA was eluted with 30 µL of MilliQ water. [***Figure 65A)***]

Enzymatic syntheses of the U^{HM}-, C^{Me}-, C^{HM}- modified promoter-downstream regions

The PCR reaction mixture for synthesis of the modified downstream portions of DNA (10 µL) contained Vent(exo$^-$) DNA polymerase (*New England Biolabs*; 0.5 µL; 2 U/µL), 235-mer template (20 ng/µL; 0.25 µL; ***$Temp^{Pveg\_sh235}$***), *phosphorylated* forward primer (20 µM; 1 µL ***$Prim^{FOR\text{-}PgIV\text{-}lg}$_P***), *FAM*-labelled reverse primer (and 20 µM; 1 µL; ***$Prim^{REV\text{-}PgIV\text{-}lg}$_F***), either **$dU^{HM}TP$** or **$dC^{Me}TP$** or **$dC^{HM}TP$** (4 mM; 0.5 µL) and natural dCTP, dGTP and dATP in the case of the **$dU^{HM}TP$** modified portion and dTTP, dGTP and dATP in the case of the **$dC^{Me}TP$** or **$dC^{HM}TP$** modified portions (4 mM; 0.5 µL). The reaction was carried out in the ThermoPol reaction buffer (1 µL) supplied by the manufacturer. Forty cycles of PCR were run in a PCR thermal 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 1.5 minutes and final extension at 72 °C for 5 minutes. The obtained products were purified via QIAquick PCR purification kit (*QIAGEN*). In the final step of purification, the purified DNA was eluted in 30 µL of MilliQ water. [***Figure 65C)***]

Enzymatic synthesis of the dU- modified promoter-downstream region

The PCR reaction mixture for synthesis of the **dU**- modified downstream region (10 µL) contained Dynazyme II DNA polymerase (*Thermo Fisher Scientific*; 2 U/µL*;* 0.5 µL), *phosphorylated* forward primer (10 µM; 2 µL ***Prim$^{FOR\text{-}PgIV\text{-}lg}$_P***), *FAM*-labelled reverse primer (10 µM; 2 µL; ***Prim$^{REV\text{-}PgIV\text{-}lg}$_F***), 235-mer template (20 ng/µL; 0.25 µL; ***Temp$^{Pveg\ _sh235}$***), **dUTP** (4 mM; 1 µL), natural dCTP, dGTP and dATP (each 4 mM; 0.3 µL) in appropriate enzyme buffer (1 µL). The reaction was carried out in a PCR cycler under the 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 run in 30 cycles. The obtained PCR product was purified via QIAquick PCR purification kit (*QIAGEN*) according to protocol. In the final step of purification, the modified DNA was eluted with 30 µL of MilliQ water. [***Figure 65B)***]

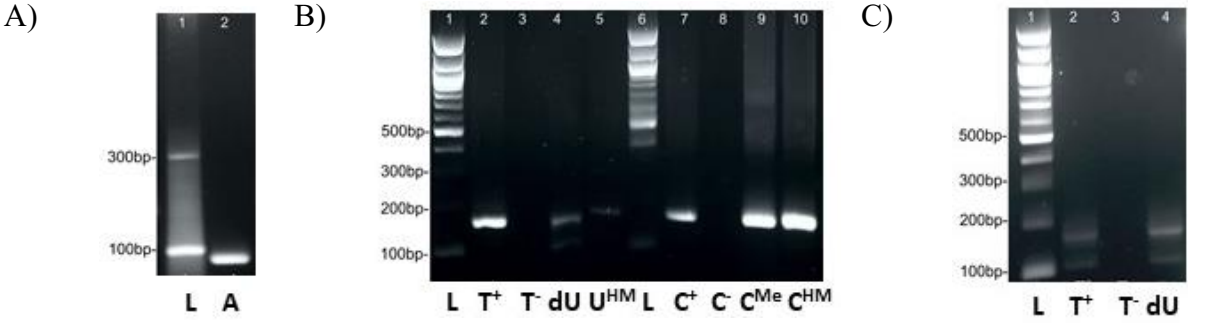


Figure 65: A) *Agarose gel analysis of the natural upstream region*. ***B)*** *Agarose gel analysis of the modified downstream region*. ***C)*** *Agarose gel analysis of the dU-modified downstream region synthesized in the presence of Dynazyme II*. The order of samples is as follows: ***A***) Lane 1, (**L**): ladder (commercial dsDNA with specific length); lane 2, (**A**): natural 69-mer DNA region. ***B***) Lane 1 and 6, (**L**): ladder (commercial dsDNA with specific length); lane 2 and 7, ($\mathbf{T^+}$, $\mathbf{C^+}$): natural PCR product; lane 3 and 8, ($\mathbf{T^-}$ and $\mathbf{C^-}$): negative control of PCR reaction (PCR run in absence dTTP or dCTP); lane 4, (**dU**): **dU** -modified PCR product amplified by Dynazyme II DNA polymerase; lane 5, ($\mathbf{U^{HM}}$): $\mathbf{U^{HM}}$ -modified PCR product; lane 9, ($\mathbf{C^{Me}}$): $\mathbf{C^{Me}}$- modified PCR product; lane 10, ($\mathbf{C^{HM}}$): $\mathbf{C^{HM}}$ -modified PCR product; ***C***) Lane 1, (**L**): ladder (commercial dsDNA with specific length); lane 2, ($\mathbf{T^+}$): natural PCR product amplified by Dynazyme II DNA polymerase; lane 3, ($\mathbf{T^-}$): negative control of PCR reaction (PCR run in absence dTTP); lane 4, (**dU**): **dU** -modified PCR product amplified by Dynazyme II DNA polymerase.

DNA Ligation of natural upstream and modified downstream DNA regions

The ligation of the natural upstream portion with the P*veg* promoter region and the modified promoter-downstream portion was performed at a molar ratio of 2:1 (upstream to downstream region). The ligation reaction was catalysed by T4 DNA Ligase. The reaction mixture (20 µL) for ligation contained T4 DNA Ligase (*New England Biolabs*; 400 U cohesive end units/µL; 2 µL), 0.33 µg of unmodified upstream portion, 0.4 µg of modified downstream 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. The ligated product was purified by *Agencourt AMPure XP magnetic particles (Beckman Coulter*). To isolate the product with desired length and sequence from the mixture containing downstream-downstream and upstream-upstream side-products, agarose gel purification by E.Z.N.A. Gel Extraction Kit from Omega Bio-Tek was carried out. The reaction mixture after ligation and purification through magnetoseparation was loaded on GelRed stained 1.3 % agarose gel and run in 0.5X TBE buffer, for 90 minutes at 118 V ***(Figure 66)***.

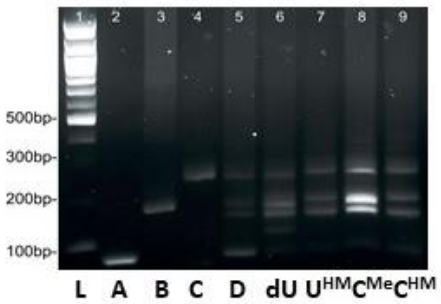


Figure 66: *Agarose gel analysis of the ligated upstream and downstream DNA region*. Lane 1, (**L**): ladder (commercial dsDNAs with specific length); lane 2, (**A**): natural DNA of upstream region with *Pveg* promoter region; lane 3, (**B**): natural DNA downstream region; lane 4, (**C**): natural full length 235-mer DNA template; lane 5, (**D**): products of ligation of the natural upstream and downstream regions; lane 6 (**dU**): products of ligation of the natural upstream region and **dU**-modified downstream region; lane 7 (**U^{HM}**): products of ligation of the natural upstream region and **U^{HM}** -modified downstream region; lane 8 (**C^{Me}**): products of ligation of the natural upstream region and **C^{Me}**-modified downstream region; lane 9 (**C^{HM}**): products of ligation of the natural upstream region and **C^{HM}**- modified downstream region.

Amplification of the modified templates for sequencing

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

Table 16: *List of primers used for amplification of partially modified 235-mers*

To verify the desired DNA sequence of modified 235-mers, amplifications of the modified DNA templates extracted from the gels were carried out in the presence of natural dNTPs. The PCR reaction mixture (10 µL) contained KOD XL DNA polymerase (*Merck, Novagen*; 2.5 U/µL; 0.5 µL), *NON*-labelled forward and reverse primers (20 µM; 1 µL $\boldsymbol{Prim^{FOR\_PgIV}}$ and 20 µM; 1 µL $\boldsymbol{Prim^{REV\_PgIV}}$) (***Table 16***), partially modified DNA template extracted from the gel (20 ng/µL; 1 µL) and set of natural dNTPs (4 mM; 0.5 µL) in KOD XL reaction buffer (1 µL). Forty PCR cycles were run in PCR thermal cycler under the following conditions: 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 natural PCR products were purified via QIAquick PCR purification kit *(QIAGEN)*. The right length of PCR products was determined by GelRed stained 1.3% agarose gel analysis run in 0.5x TBE buffer for 90 minutes at 118 V. ***(Figure 67)*** The obtained PCR products were sequenced, and in all cases, the correct sequences of PCR products were detected.

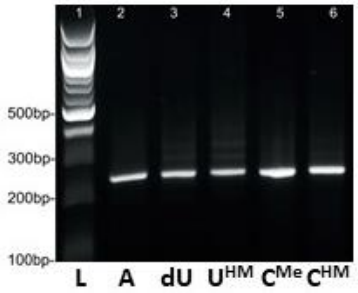


Figure 67: *Agarose gel analysis of the natural PCR products obtained from amplification of the modified templates.* Lane 1, (**L**): ladder (dsDNAs sequences with specific length); lane 2, (**A**): full length unmodified 235-mer template; lane 3, (**dU**): natural PCR product obtained from the **dU**- modified template; lane 4, ($\mathbf{U^{HM}}$): natural PCR product obtained from the $\mathbf{U^{HM}}$- modified template; lane 5, ($\mathbf{C^{Me}}$): natural PCR product obtained from the $\mathbf{C^{Me}}$- modified template; lane 6, ($\mathbf{C^{HM}}$): natural PCR product obtained from the $\mathbf{C^{HM}}$- modified template.

B) *Synthesis of DNA templates modified in the both strands of promoter-upstream and promoter regions*

The dsDNA with natural promoter-downstream region and either **dU**, **U^{HM}-**, **C^{Me}-** or **C^{HM}-** modified promoter-upstream regions were synthesized in few steps. The PCR reactions to obtain modified 69-mer DNA and natural 166-mer DNA were performed in a total volume of 200 µL. The template strand contained modifications in both the promoter and promoter-upstream regions whereas the nontemplate strand of modified DNA contained modifications only in the promoter region. The total volume of PCR reactions was 200 µL.

The obtained PCR products were purified by QIAquick PCR purification kit (*QIAGEN*) or QIAquick nucleotide removal kit (*QIAGEN*) according to the protocol. In the final step of purification, the DNA was eluted with 30 µL of MilliQ water.

Downstream, upstream portions of DNA and final partially modified DNA were analysed by gel electrophoresis on a GelRed stained 1.3 % agarose gel run in 0.5x TBE buffer. ***(Figure 68)***

Enzymatic synthesis of the natural promoter-downstream region (transcribed region)

The 10 µL of the PCR reaction mixture contained *Vent(exo$^-$) DNA* polymerase (*New England Biolabs*; 2 U/µL; 0.5 µL), *phosphorylated* forward primer (20 µM; 1 µL; ***$Prim^{FOR\text{-}PgIV\text{-}lg}$_P***), *FAM*-labelled reverse primer (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 ThermoPol reaction buffer (1 µL) supplied by the manufacturer. The PCR reaction was carried out in a PCR cycler. The 40 cycles of PCR run 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 PCR product was purified with QIAquick PCR purification kit. ***(Figure 68A)***.

Enzymatic syntheses of the U^{HM}-, C^{Me}-, C^{HM}- modified promoter region

The **U^{HM}-**, **C^{Me}-** and **C^{HM}-** modified promoter regions were synthesized by PCR reaction. The PCR reaction mixture (10 µL) contained KOD XL DNA polymerase (*Merck, Novagen;* 2.5 U/µL; 0.25 µL), 69-mer DNA template (1 µM; 0.175 µL; ***$Temp^{Us\_69mer}$***), *FAM*-labelled forward primer (20 µM; 1 µL; ***$Prim^{FOR\text{-}PgV}$_F***), *phosphorylated* reverse primer (20 µM; 1 µL; ***$Prim^{REV\text{-}PgIV\text{-}sh}$_P***), natural dCTP, dATP and dGTP (each 4 mM; 0.075 µL) in the case of the **$dU^{HM}TP$** modified portion or dTTP, dATP and dGTP (each 4 mM; 0.075 µL) in the case of the **$dC^{Me}TP$** or **$dC^{HM}TP$** modified portions and functionalized dNTPs: either **$dU^{HM}TP$** or **$dC^{Me}TP$**

or **dC^{HM}TP** (0.5 µL; 4 mM) in KOD XL reaction buffer supplied by the manufacturer (1 µL). The reaction was run in 25 cycles in a PCR thermal cycler under the 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 PCR products were purified with QIAquick nucleotide removal kit. ***(Figure 68B)***

Enzymatic synthesis of the dU- modified promoter region

The PCR reaction mixture (10 µL) for the synthesis of **dU**-modified promoter region contained 0.5 µL of Dynazyme II DNA polymerase (*Thermo Fisher Scientific*; 2 U/µL), *FAM*-labelled forward primer (10 µM; 2 µL; ***Prim$^{FOR\text{-}PgV}$_F***), *P*-labelled reverse primer (10 µM; 2 µL; ***Prim$^{REV\text{-}PgIV\text{-}sh}$_P***), 69-mer DNA template (1 µM; 0.25 µL; ***Temp^{Us_69mer}***), **dUTP** (4 mM; 1 µL) and natural dCTP, dGTP and dATP (each 4 mM; 0.3 µL) in an appropriate reaction buffer (1 µL). The PCR reaction was carried out in a PCR thermal cycler. The 30 cycles of PCR reaction were run under the conditions: 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 obtained PCR product was purified with QIAquick nucleotide removal kit. ***(Figure 68C)***

Figure 68: A) *Agarose gel analysis of the natural promoter-downstream region*. ***B)*** *Agarose gel analysis of the modified promoter region*. ***C)*** *Agarose gel analysis of the dU-modified upstream-promoter region synthesized in the presence of Dynazyme II*. The order of samples is as follows: ***A***) Lane 1, (**L**): ladder (commercial dsDNA with specific length); lane 2, (**A**): natural promoter-downstream region. ***B***) Lane 1 and 6, (**L**): ladder (commercial dsDNA with specific length); lane 2 and 7, (**T^{+}, C^{+}**): natural PCR product; lane 3 and 8, (**T^{-}** and **C^{-}**): negative control of PCR reaction (PCR run in absence dTTP or dCTP); lane 4, (**dU**): **dU** -modified PCR product amplified by Dynazyme II DNA polymerase; lane 5, (**U^{HM}**): **U^{HM}** -modified PCR product; lane 9, (**C^{Me}**): **C^{Me}**- modified PCR product; lane 10, (**C^{HM}**): **C^{HM}** -modified PCR product; ***C***) Lane 1, (**L**): ladder (commercial dsDNA with specific length); lane 2, (**T^{+}**): natural PCR product amplified by Dynazyme II DNA polymerase; lane 3, (**T^{-}**): negative control of PCR reaction (PCR run in absence dTTP); lane 4, (**dU**): **dU** -modified PCR product amplified by Dynazyme II DNA polymerase.

Ligation of modified promoter region with natural promoter downstream DNA region

Ligation of the modified promoter upstream portion and the natural downstream portion was performed in the presence of T4 DNA ligase (*New England Biolabs*; 400 U cohesive end units/µL). The downstream and upstream regions were ligated in a molar ratio of 1:3. The reaction mixture (20 µL) contained 0.5 µg of upstream portion, 0.4 µg of downstream portion, 0.5 µL of 4 mM ATP, 2 µL of T4 DNA Ligase and 2 µL of 10x reaction buffer for T4 DNA Ligase. The reaction mixture was incubated at 16 °C overnight. The reaction mixture after incubation was purified by *Agencourt AMPure XP magnetic particles (Beckman Coulter*). The purified mixture contained expect product with desired length and sequence also products containing upstream-upstream and downstream-downstream portions products. To obtain the right product, the isolation of desired product through agarose gel purification (E.Z.N.A. Gel Extraction Kit from Omega Bio-Tek) was carried out from a GelRed stained 1.3 % agarose gel run in 0.5x TBE buffer for 90 minutes at 118 V. ***(Figure 69)***

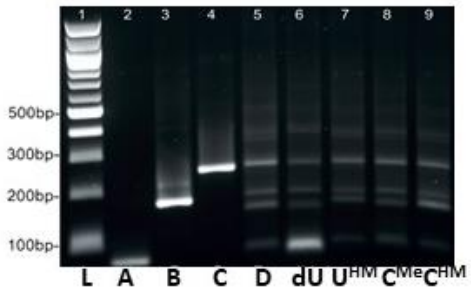


Figure 69: *Agarose gel analysis of the ligated modified upstream and unmodified downstream DNA region*. Lane 1, (**L**): ladder (commercial dsDNAs with specific length); lane 2, (**A**): natural DNA of upstream region with *Pveg* promoter region; lane 3, (**B**): natural DNA downstream region; lane 4, (**C**): natural full length 235-mer DNA template; lane 5, (**D**): products of ligation of the natural upstream and natural downstream regions; lane 6, (**dU**): products of ligation of the **dU**-modified upstream region and non-modified downstream region; lane 7 ($\mathbf{U^{HM}}$): products of ligation of the $\mathbf{U^{HM}}$ -modified upstream region and natural downstream region; lane 8 ($\mathbf{C^{Me}}$): products of ligation of the $\mathbf{C^{Me}}$-modified upstream region and natural downstream region; lane 9 ($\mathbf{C^{HM}}$): products of ligation of the $\mathbf{C^{HM}}$- modified upstream region and natural downstream region.

Amplification of the partially 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 PCR reaction mixture (10 µL) contained KOD XL DNA polymerase (*Merck, Novagen*; 2.5 U/µL; 0.5 µL), *NON*-labelled forward and reverse primers (20 µM; 1 µL $\boldsymbol{Prim^{FOR\_PgVI}}$ and 20 µM; 1 µL $\boldsymbol{Prim^{REV\_PgVI}}$) ***(Table 16)***, partially modified template extracted from the gel (20 ng/µL; 1 µL) and natural dNTPs (4 mM; 0.5 µL) in KOD XL reaction buffer (1 µL). The PCR reaction was carried out in forty PCR cycles in PCR thermal cycler. The conditions of

PCR reaction: 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 natural PCR products were purified via QIAquick PCR purification kit. The PCR products were analysed by GelRed stained 1.3 % agarose gel. ***(Figure 70)*** Moreover, the amplified PCR products were sequenced. In all cases the correct sequences of PCR products were detected.

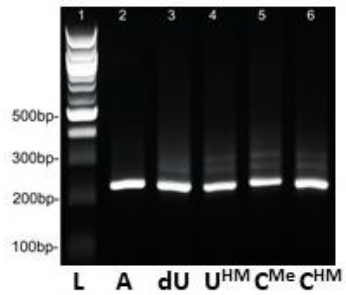


Figure 70: *Agarose gel analysis of the natural PCR products obtained from amplification of the modified templates.* Lane 1, (**L**): ladder (dsDNAs sequences with specific length); lane 2, (**A**): full length unmodified 235-mer template; lane 3, (**dU**): natural PCR product obtained from the **dU**- modified template; lane 4 ($\mathbf{U^{HM}}$): natural PCR product obtained from the $\mathbf{U^{HM}}$- modified template; lane 5 ($\mathbf{C^{Me}}$): natural PCR product obtained from the $\mathbf{C^{Me}}$- modified template; lane 6 ($\mathbf{C^{HM}}$): natural PCR product obtained from the $\mathbf{C^{HM}}$- modified template.

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

PCR syntheses of natural $Temp^{Pveg\_sh235}$ and $Temp^{Pveg\_sh181}$ DNA templates

The natural $Temp^{Pveg\_sh235}$ DNA template was synthesized by PCR reaction in final volume 10 µL. The reaction mixture contained KOD XL DNA polymerase (*Merck, Novagen*; 2.5 U/ µL; 0.1 µL), *NON*-labelled reverse and forward primers ($Prim^{R2\text{-}PgIII}\_P$ and $Prim^{F2\text{-}PgIII}$; 20 µM; 1 µL each), 339-mer template ($Temp^{Pveg}$; 20 ng/µL; 1 µL), mix of natural dNTPs (4 mM; 0.5 µL) and the buffer for KOD XL polymerase supplied by the manufacturer (1 µL). Forty PCR cycles were run in a PCR thermal cycler under the following conditions: 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 and final extension step at 72 °C for 5 minutes. The purified PCR product was moreover extracted from GelRed stained 1.3 % agarose gel (EZNA Gel Extraction kit - *OMEGA Biotek*). In final step of extraction, the *NON*-labelled 235-mer DNA template was eluted with 30 µL of MilliQ water.

The *FAM*-labelled ***Temp^{Pveg_sh181}*** DNA template was prepared by PCR in the presence of Taq DNA Polymerase (*New England Biolabs*; 5 U/ µL; 1 µL). The PCR mixture further contained natural forward primer (20 µM; 1 µL; ***Prim$^{F3\text{-}PgIII}$_P***) and 5'-*FAM* labelled reverse primer (20 µM; 1 µL ***Prim$^{REV\text{-}PgII/III\text{-}R1/R3}$ _F***), the 339-mer template (20 ng/µL; 1 µL; ***Temp^{Pveg}***), mix of natural dNTPs (4 mM; 0.5 µL) and 1 µL of ThermoPol reaction buffer in total reaction volume of 10 µL. The conditions of PCR reaction were 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. The purified 181-mer product was extracted from GelRed stained 1.3 % agarose gel (EZNA Gel Extraction kit - *OMEGA Biotek*). The desired product was in final step eluted with 30 µL of MilliQ water. The obtained product was used as a DNA template for the following amplification.

Synthesis of *NON*-modified ssDNA templates – general remarks

The amplification of both templates, 235-mer and 181-mer, was needed to generate *NON*-modified ssDNA. The amplification of 235-mer dsDNA was performed with a 5'-phosphate-modified reverse primer. The amplification of shorter 181-mer dsDNA was carried out in the presence of the 5'-phosphate-modified forward primer. The phosphate modification on 5´-end of natural dsDNA allowed specific Lambda exonuclease digestion of one DNA strand to generate ssDNA. The final volume of PCR reaction mixtures was 150 µL. The obtained dsDNA products were purified with QIAquick PCR purification kit *(QIAGEN)*. The purified dsDNAs were eluted in the final step of purification with 30 µL of MilliQ water. The length and purity of dsDNA products were checked by agarose gel analysis (1.3 % agarose gel stained with GelRed in 0.5x TBE buffer) and by sequencing.

Amplification of the natural dsDNAs

The 10 µL of reaction mixture for the synthesis of the **235-mer dsDNA** contained Vent(exo$^-$) DNA polymerase (*New England Biolabs*; 2 U/µL; 1 µL), 5'-*FAM* labelled forward primer (20 µM; 1 µL; ***Prim$^{F2\text{-}PgIII}$_F***), 5'-*phosphate* reverse primer (20 µM; 1 µL; ***Prim$^{R2\text{-}PgIII}$_P***), 235-mer DNA template (20 ng/µL; 1 µL; ***Temp^{Pveg_sh235}***), mix of natural dNTPs (4 mM; 0.5 µL) and 1 µL of ThermoPol reaction buffer. The PCR was run in 40 cycles in a PCR thermal cycler under the 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. ***(Figure 71)***

The 10 µL of reaction mixture for the synthesis of the **181-mer dsDNA** contained Vent(exo$^-$) DNA polymerase (*New England Biolabs*; 2 U/µL; 1 µL)*, 5´-phosphate* modified forward primer (20 µM; 1 µL; ***Prim$^{F3\text{-}PgIII}$_P*)**,** *FAM*-labelled reverse primer (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}***), mix of natural dNTPs (4 mM; 0.5 µL) and 10x enzyme reaction buffer (1 µL). The reaction mixture was incubated in PCR cycler under the same conditions as described above for the synthesis of 235-mer dsDNA. ***(Figure 71)***

Digestion of natural dsDNA with Lambda exonuclease

The single stranded DNA was generated from unmodified 235-mer and 181-mer dsDNA. The reaction mixture to create ssDNA included 10 µg of either 235-mer or 181-mer dsDNA, 7 µL of Lambda exonuclease (*New England Biolabs;* 5 U/µL) 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. The single stranded DNAs (235-mer or181-mer ssDNA) were obtained from the reaction mixture by phenol-chloroform extraction and subsequent overnight acetone precipitation. The obtained ssDNA was in the last step of acetone precipitation dissolved in 30 µL of MilliQ water. The obtained ssDNA was analysed on a GelRed stained 1.3 % agarose gel. ***(Figure 71)***

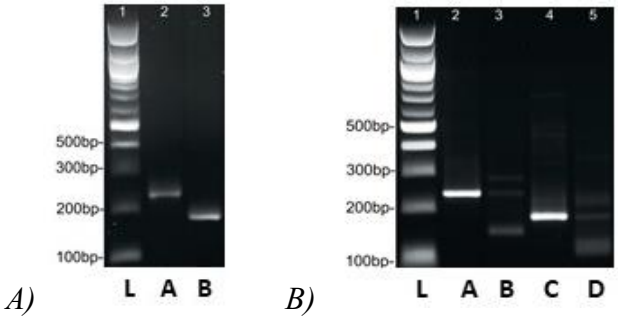


***Figure 71**: **A)** Agarose gel analysis of NON-modified dsDNA templates, **B)** Agarose gel analysis of dsDNA (235-mer and 181-mer) and ssDNA after its Lambda exonuclease digestion. A)* Lane 1, **L**: ladder (commercial mix of dsDNAs with specific length); lane 2, **A**: 235-mer DNA template; lane 3, **B**: 181-mer DNA template. *B)* Lane 1, **L**: ladder (commercial mix of dsDNAs with specific length); lane 2, **A**: 235-mer dsDNA; lane 3, **B**: 235-mer ssDNA; lane 4, **C**: 181-mer dsDNA; lane 5, **D**: 181-mer ssDNA.

Annealing of complementary ssDNA sequences

Obtained the 235-mer ssDNA was hybridized with the 181-mer ssDNA in Tris-HCl containing buffer (10 mM Tris, 50 mM NaCl, 1 mM EDTA, pH = 8) in a molar ratio of 1:1. The reaction

mixture with the final DNA concentration of 0.3 µM was heated at 95 °C for 5 minutes and by its slow cooling to 25 °C for 95 minutes, a complementary duplex of DNA was obtained. The DNA duplex with shorter template strand was submitted for a synthesis of partially modified dsDNA by primer extension experiments. ***(Figure 72)***

Synthesis of DNAs with modified promoter-upstream and promoter regions in the template strand by PEX

The primer extension experiments were performed by mixing KOD XL DNA polymerase (*Merck, Novagen*; 2.5 U/ µL; 3 µL), the DNA duplex with shorter template strand (0.3 µM; 12 µL), the functionalized dNTP (either **dUTP** or **dU^{HM}TP** or **dC^{Me}TP** or **dC^{HM}TP**; 4 mM; 3 µL) and suitable natural dNTPs mixture (either dATP, dGTP, dCTP for the **dUTP** and **dU^{HM}TP** modified templates or dATP, dGTP, dTTP for the **dC^{Me}TP** and **dC^{HM}TP** modified templates; 4 mM; 3 µL) in 10X KOD XL reaction buffer (6 µL). The reaction mixture with total volume of 60 µL was incubated in thermomixer at 65 °C for 30 minutes. The resulting modified DNAs were purified with Agencourt AMPure XP magnetic particles and analysed on a GelRed stained 1.3 % agarose gel. ***(Figure 72)***

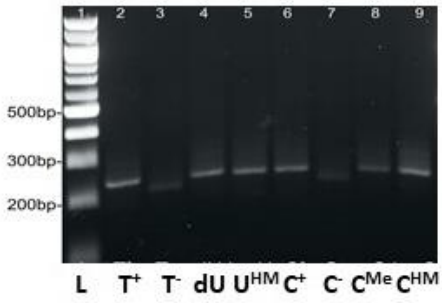


Figure 72: *Agarose gel analysis of partially modified DNA with modified promoter-upstream and promoter regions in the template strand of DNA*. Lane 1, **L**: ladder (commercial mix of dsDNAs with specific length); lane 2, **T^{+}**: unmodified dsDNA synthesized by PEX with natural dNTP; lane 3, **T^{-}** : negative control, PEX run without dTTP; lane 4, **dU**: dU partially modified dsDNA; lane 5, **U^{HM}**: U^{HM} partially modified dsDNA; lane 6, **C^{+}**: unmodified dsDNA, PEX run with natural dNTP; lane 7, **C^{-}**: negative control, PEX run without dCTP; lane 8, (**C^{Me}**): C^{Me} partially modified dsDNA; lane 9, (**C^{HM}**): C^{HM} partially modified dsDNA.

D) *Synthesis of DNA templates modified in promoter region of non-template (coding) strand*

Total final volume of all PCR reactions was 60µL. DNA templates modified in promoter region of the coding strand were synthesized in the presence of commercially available *NON*-labelled

dN-modified forward primer (***Prim***$^{FOR\text{-}PgII}$) and non-modified reverse primer (***Prim***$^{REV\text{-}PgII\text{-}R1/R3}$). The primers were commercially available.

Method A:

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

The partially modified dsDNAs were synthesized in PCR thermal cycler in thirty cycles. The PCR reactions were run under the conditions: preheating at 94°C for 3minutes, denaturation at 94°C for 1 minute, annealing at 68°C for 1 minute, extension at 72°C for 1 minute, followed by final extension step at 75°C for 5 minutes. The PCR products with correct length were separated on agarose gels and extracted from the gels by E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek). The extracted DNA was purified by Agencourt AMPure XP magnetic beads. The product was in the last step of purification eluted with 40 µL of MilliQ water. Obtained partially modified DNAs were analysed on a GelRed stained 1.3 % agarose gels.

dU-partially modified DNA modified in the coding strand of promoter region was synthesized by ***Method A*** described above in the presence of **dU-**modified forward primer (20 µM; 2 µL; *Prim*$^{FOR\text{-}PgII\text{-}dU11}$). PCR reaction with KOD XL DNA polymerase did not provide any product, therefore, the PCR reaction was run in the presence of Dynazyme II DNA polymerase under the same conditions. The PCR reaction with Dynazyme II provided single clean product. ***(Figure 73)***

U^{HM}- partially modified DNA in the coding strand of promoter region was synthesized by ***Method A*** described above in the presence of **U^{HM}**-modified forward primer (20 µM; 2 µL; ***Prim***$^{FOR\text{-}PgII\text{-}d5hmU11}$). The PCR reaction provided single clean product. ***(Figure 73)***

C^{Me}-partially modified DNA in the coding strand of promoter region was synthesized by ***Method A*** described above in the presence of **C^{Me}**-modified forward primer (20 µM; 2 µL; ***Prim***$^{FOR\text{-}PgII\text{-}d5mC4}$). The PCR reaction provided single clean product ***(Figure 73)***

C^{HM}-partially modified DNA in the coding strand of promoter region was synthesized by *Method A* described above in the presence of **C^{HM}**-modified forward primer (20 µM; 2 µL; ***Prim***$^{FOR\text{-}PgII\text{-}d5hmC4}$). The PCR reaction provided single clean product. ***(Figure 73)***

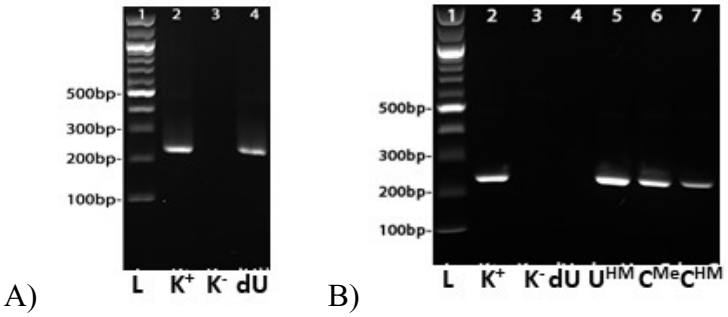


Figure 73: *Agarose gel analysis of partially modified dsDNAs modified in promoter region of the coding strand. A) PCR reaction was run in the presence of dU-modified forward primer with Dynazyme II DNA polymerase*: Lane 1, **L**: ladder (commercial mix of dsDNAs with specific length); Lane 2, $\mathbf{K}^+$: fully natural DNA, PCR was run with natural primer - $\boldsymbol{Prim}^{FOR\text{-}PgII}$; Lane 3, $\mathbf{K}^-$: negative control (PCR was run in the absence of a forward primer); Lane 4, **dU**: **dU**-modified PCR product, PCR was run with of $\boldsymbol{Prim}^{FOR\text{-}PgII\text{-}dU11}$. ***B)*** *PCR reaction was run in the presence of dN-modified forward primer with KOD XL DNA polymerase*: Lane 1, **L**: ladder (commercial mix of dsDNAs with specific length); Lane 2, $\mathbf{K}^+$: fully natural DNA, PCR was run with natural primer - $\boldsymbol{Prim}^{FOR\text{-}PgII}$; Lane 3, $\mathbf{K}^-$: negative control - PCR was run in the absence of a forward primer; Lane 4, **dU**: **dU**-partially modified PCR product, PCR was run with of $\boldsymbol{Prim}^{FOR\text{-}PgII\text{-}dU11}$; Lane 5, $\mathbf{U}^{\mathbf{HM}}$: $\mathbf{U}^{\mathbf{HM}}$-partially modified PCR product, PCR was run with $\boldsymbol{Prim}^{FOR\text{-}PgII\text{-}d5hmU11}$; Lane 6, $\mathbf{C}^{\mathbf{Me}}$: $\mathbf{C}^{\mathbf{Me}}$- partially modified PCR product, PCR was run with $\boldsymbol{Prim}^{FOR\text{-}PgII\text{-}d5mC4}$; Lane 7, $\mathbf{C}^{\mathbf{HM}}$: $\mathbf{C}^{\mathbf{HM}}$-partially modified PCR product, PCR was run with $\boldsymbol{Prim}^{FOR\text{-}PgII\text{-}C4hm}$.

6.3.2.5 *In vitro* transcription assay

Quantification of DNA templates

The concentration of synthesized and purified DNA templates was measured by the NanoDrop spectrophotometer and measured DNA templates were diluted to the concentration 5 ng/µL in water. 25 ng of ^{32}P-labelled DNAs were analysed on a 5% polyacrylamide (PAA) gel without urea. 50 ng of *NON*-labelled DNAs were analysed on a 1.5% agarose gel with GelRed (Biotium). The PAA gels with ^{32}P-labelled DNA samples were dried, exposed to Fuji MS phosphor storage screens overnight and screens were scanned with a Molecular Imager FX (BIORAD). The scans of gel were analysed with Quantity One program (BIORAD). *NON*-labelled DNA samples were visualized by UV light and tiff files of images for DNA templates were analysed with Quantity One program (BIORAD).

***In vitro* transcription assays**

In vitro transcription assays were performed in multiple round as described[160] (unless stated otherwise). The 10 µL of reaction mixture for transcription contained 5 ng of natural/ modified DNA template, 125 mM $MgCl_2$, 100 mM Tris pH 8, 90 mM KCl, 50 mM DTT, 30 nM

Escherichia coli RNAPσ^{70} holoenzyme (New England Biolabs) natural NTPs (200 μM ATP, 1000 μM GTP, 200 μM CTP, 10 μM UTP) and 3.7 kBq [α-^{32}P]UTP. Used RNAP was diluted in 50 mM Tris–HCl pH 8.0, 0.1 M NaCl, 50% glycerol. The samples without NTPs were preheated at 37 °C for 10 minutes. The reactions were initiated by the addition of NTPs and were allowed to proceed at 37°C for 10 minutes. The addition of 10 μL of formamide stop solution (95 % formamide, 20 mM EDTA, pH 8.0) and cooling down of reaction mixture fully stopped the process of reaction. The samples were after reaction loaded onto 7 % polyacrylamide native gels. The PAA gels were dried and exposed to Fuji MS phosphor storage screens overnight and screens were scanned with a Molecular Imager FX (BIORAD). The scans of gel were analysed with Quantity One program (BIORAD).

The transcript signals were normalized to signals from the DNA templates. The acquired values were compared to the values of control sample (K^+ - natural DNA template). The DNA templates synthesized in the presence of natural dNTPs (natural templates) were unambiguously reported as K^+ on the transcription assays gels. Natural DNA templates (positive controls for the PCR reactions) from the PCR products analysis gels were reported as T^+ and C^+.

6.3.3 Switching transcription with bacterial RNA polymerase[155]

General remarks

NON-labelled oligonucleotides (primers) were purchased from GeneriBiotech. Set of natural dNTPs (dATP, dCTP, dGTP, dTTP) was purchased from New England Biolabs. The **dU^{HM}TP**, **dC^{NB}TP** and **dU^{NB}TP** were prepared according to published procedure[150, 151] and **dC^{HM}TP** was purchased from TriLink Biotechnologies. KOD XL DNA polymerase was bought from Merck and Taq DNA polymerase for ThermoPol reaction buffer were purchased from New England Biolabs. Restriction endonucleases AluI and RsaI were bought from New England Biolabs. Agencourt AMPure XP magnetic particles (Beckman Coulter Life Science - GE Healthcare) were used for purification of all PCR products and final DNA templates.

6.3.3.1 Preparation of modified DNA templates

Modified DNA templates for transcription were synthesized by PCR reactions in a total final volume of 20 μL. Natural DNAs, positive controls of PCR reactions and controls for the DNA transcription experiments, were synthesized in the presence of natural dNTPs (4 mM; 0.75 μL) according to a procedure for **U^{HM}**-modified or **C^{HM}**-modified DNA without any additives. (***Figure 37***) Modified dsDNAs were prepared in the presence of either ^{32}P-labelled (***Prim^{FOR} – ^{32}P*** and ***Prim^{REV} – ^{32}P***) or *NON*-labelled (***Prim^{FOR}*** and ***Prim^{REV}***) primers under the reported conditions. The ^{32}P-labelled primers were prepared freshly just before PCR by the transfer of a terminal ^{32}P -labelled phosphate from (γ)-^{32}P-dATP to the 5´-end of *NON*-labelled forward and reverse primers by T4 polynucleotide kinase. Forty PCR cycles were run in the thermal cycler under the conditions reported in each protocol. Obtained PCR products were purified with Agencourt AMPure XP magnetic particles. The concentration of purified DNA templates was measured by the NanoDrop spectrophotometer. All samples of DNA templates for transcription were diluted to a final concentration of 20 ng/L according to NanoDrop spectrophotometer. The exact quantity of DNA templates for transcription was analysed based on the intensity of DNA signals (fluorescence and/or radioactivity) on a gel. *NON*-labelled and ^{32}P-labelled DNA samples were analysed by control 1.3 % agarose gels stained with GelRed (Biotium). (***Figure 37***) Moreover, ^{32}P-labelled DNA templates were determined through 7 % PAGE gels exposed to Fuji MS phosphor storage screens. (***Figure 43***; ***Figure 45***) The sequences of modified *NON*-labelled DNA templates were confirmed by DNA sequencing.

Plasmid with 311-mer region containing P*veg* promoter region

An artificial 99 bp DNA sequence upstream of the promoter was used to clone to a plasmid consists from 1177 nucleotides which carry the P*veg* promoter[99] region. The inserted sequence facilitated subsequent PCR reactions with modified nucleotides.

Two complementary commercially available 99 - mer DNAs [**Insert_99_EcoRI** (100 µL of 0.1 mM) and **Insert_99_EcoRI_REV** (100 µL of 0.1 mM)] were annealed together. First, the DNA was completely denatured at 95 °C for 5 minutes and subsequently annealed at a gradually decreasing of temperature from 95 °C to 25 °C with gradual reduction in the temperature every 2 seconds. The annealed DNA was checked on 2 % GelRed stained agarose gel. [***Figure 36a)***] The prepared 99-bp DNA was digested with EcoRI restriction endonuclease. The dsDNA with created sticky ends was ligated into the EcoRI site of plasmid consists 1177 nucleobases at the upstream edge of the promoter. The adapted plasmid was transformed into *E.coli DH5α*. The replicated colonies with required inserts, yielding the final construct LK2130, were identified and verified by sequencing.

Enzymatic synthesis of natural template (***Temp^{Pveg2}***)

The template ***Temp^{Pveg2}*** containing 311 nucleobases was prepared by PCR in the presence of *NON*-labelled primers (forward - ***Prim^{FOR}*** and reverse - ***Prim^{REV}***) from plasmid (LK2130) containing specific P*veg* promoter region cloned in p770 between *Eco*RI and *Hind*III sites.[79, 99] The PCR reaction was performed in eight portions of 20 µL each. The 20 µL of PCR reaction mixture contained Taq DNA polymerase for ThermoPol buffer (New England Biolabs; 5000 U/mL; 1.2 µL), natural dATP, dTTP, dCTP, dGTP (4 mM; 1.125 µL); forward - ***Prim^{FOR}*** and reverse - ***Prim^{REV}*** primers (20 µM; 3 µL each); the plasmid construct LK2130 (42 ng) in ThermoPol buffer (2 µL). The PCR reaction was run in forty cycles in the thermal cycler under the following conditions: preheating at 94 °C for 3 minutes, denaturation at 94 °C for 1 minute, annealing at 68 °C for 1 minute, extension at 75 °C for 1.5 minutes and final extension step at 75 °C for 5 minutes. All eight portions of PCR reaction mixture (160 µL) were combined into one vial. The 311-mer PCR product was purified using Agencourt AMPure XP magnetic particles according to the published procedure. The final product was eluted with 200 µL of MilliQ water. The final concentration of obtained PCR product - ***Temp^{Pveg2}*** was 132 ng/µL. The identity of obtained dsDNA was confirmed by agarose gel analysis and by DNA sequencing. [***Figure 36B)***]

Synthesis of modified DNA templates

Synthesis of U^{HM}-modified DNA

The reaction mixture (20 µL) for PCR reaction contained either *NON*-labelled or ^{32}P-labelled primers (20 µM; 3 µL; $\boldsymbol{Prim^{FOR}}$ and 20 µM; 3 µL; $\boldsymbol{Prim^{REV}}$), 311-mer template (40 ng/µL; 0.9 µL; $\boldsymbol{Temp^{Pveg2}}$), natural dNTPs (dGTP, dCTP and dATP; 4 mM; 0.75 µL), modified **$dU^{HM}TP$** (4 mM; 1.5 µL) and KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 1.2 µL) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer. The PCR reaction was carried out in forty cycles in the thermal cycler. The conditions of PCR reaction were as follows: preheating of reaction mixture at 94°C for 3 minutes, denaturation step at 94°C for 1 minute, annealing at 68 °C for 1 minute, extension at 75 °C for 1.5 minutes, followed by the final extension step at 75 °C for 5 minutes. [***Figure 37A)***]

Synthesis of U^{NB}-modified DNA

The reaction mixture (20 µL) for PCR reaction contained either *NON*-labelled or ^{32}P-labelled primers (20 µM; 3 µL; $\boldsymbol{Prim^{FOR}}$ and 20 µM; 3 µL; $\boldsymbol{Prim^{REV}}$), 311-mer template (40 ng/µL; 0.9 µL; $\boldsymbol{Temp^{Pveg2}}$), natural dNTPs (dGTP, dCTP and dATP; 4 mM; 0.75 µL), modified **$dU^{NB}TP$** (4 mM; 2 µL) and KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 1.5 µL) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer. The PCR reaction was carried out in forty cycles in a thermal cycler. The conditions of PCR reaction were as follows: preheating of reaction mixture at 94 °C for 3 minutes, denaturation step at 94 °C for 1 minute, annealing at 62 °C for 1 minute, extension at 75 °C for 2 minutes, followed by the final extension step at 75 °C for 7 minutes. [***Figure 37A)***]

Synthesis of C^{HM} and C^{NB}-modified DNA

The reaction mixture (20 µL) for PCR reaction contained either *NON*-labelled or ^{32}P-labelled primers (20 µM; 3 µL; $\boldsymbol{Prim^{FOR}}$ and 20 µM; 3 µL; $\boldsymbol{Prim^{REV}}$), 311-mer template (40 ng/µL; 0.9 µL; $\boldsymbol{Temp^{Pveg2}}$), natural dNTPs (dGTP, dTTP and dATP; 4 mM; 0.75 µL), modified **$dC^{HM}TP$** (4 mM; 1.5 µL) or **$dC^{NB}TP$** (4 mM; 2 µL) and KOD XL DNA polymerase (*Merck; Novagen;* 2.5 U/µL; 1.2 µL) in 10x reaction Buffer for KOD XL (2 µL) supplied by the manufacturer. The PCR reaction was carried out in forty cycles in a thermal cycler. The conditions of PCR reaction were as follows: preheating of reaction mixture at 94 °C for 3 minutes, denaturation at 94 °C for 1 minute, annealing at 68 °C for 1 minute, extension at

75 °C for 1.5 minutes, followed by the final extension step at 75 °C for 5 minutes. [***Figure 37B)***]

6.3.3.2 Study of cleavage of modified dsDNA with AluI and RsaI

For AluI: To a solution of either natural, HM-modified or NB-modified DNA (100 ng), CutSmart Buffer (1 μL) and restriction enzyme AluI (1.8 μL) were added and the mixture was incubated at 37 °C for 1 hour. Then, the resulted reaction mixture and products of cleavage were analysed on GelRed stained 1.3 % agarose gel. (***Scheme 38, Figure 37***)

For RsaI: To a solution of either natural, HM-modified or NB-modified DNA (100 ng), CutSmart Buffer (1 μL) and restriction enzyme RsaI (1.4 μL) were added and the mixture was incubated at 37 °C for 1 hour. In the case of $\mathbf{C^X}$- modified samples, the modified DNA was mixed with 1.8 μL of RsaI. Then, the resulted reaction mixtures and products of cleavage were analysed on GelRed stained 1.3 % agarose gel. (***Scheme 38, Figure 37***)

6.3.3.3 Study of transcription for HM- and NB- modified DNA

Quantification of DNA

DNA templates were quantified according to described procedure[79, 154] (unless stated otherwise). Approximate concentration of purified DNA was measured with a NanoDrop spectrophotometer. The obtained DNAs were diluted in water to the final concentration 5 ng/μL. The *NON*-labelled DNA (50 ng) was analysed on a 1.5 % agarose gel stained with 10,000x diluted GelRed (Biotium) and visualized by UV light. The ^{32}P-labelled DNA (20 – 50 ng) was analysed on a 5 % native PAA gel, dried and exposed to the Fuji MS phosphor storage screens. The screens were scanned with a Molecular Imager FX (BIORAD). The resulted gel documentation was analysed with Quantity One program (BIORAD).

Multiple round *in vitro* transcriptions were performed essentially as described[154] (unless stated otherwise). The reaction mixture of each multiple round *in vitro* transcription (10 μL) contained RNA polymerase holoenzyme from *Escherichia coli* (EcoRNAP σ70 ; New England Biolabs; 30 nM), either natural or modified DNA template (5 ng), Tris (pH 8; 100 mM), $MgCl_2$ (125 mM), DTT (50 mM) and KCl (90 mM) was preheated at 37 °C for 10 minutes. The reaction of transcription was started by the addition of mixture of natural NTPs (ATP - 200 μM, GTP - 1000 μM, CTP - 200 μM, UTP - 10 μM) and [α-32P] UTP (3.7 kBq). The reaction mixture was incubated at 37 °C for 10 minutes and subsequently stopped by the addition of formamide stop

solution (10 μL; 95 % formamide, 20 mM EDTA, pH 8.0). The reaction of transcription was performed for all DNA templates. The resulted products of transcription (10 μL) were analysed by 7 % native PAA gels. Dried gels were exposed to the Fuji MS phosphor storage screens. The screens were scanned with a Molecular Imager FX (BIORAD) and analysed with Quantity One program (BIORAD).

Quantification of product of transcriptions

The transcript signals, with subtracted background, were normalized to the signals average of DNA templates. In the next step, signals of transcriptions (of non-modified and modified DNA templates) were normalized to the signal of natural DNA template ($\mathbf{T}^+$ or $\mathbf{C}^+$) which was set as 100 %. At least two/ three independent transcription experiments were performed.

6.3.3.3.1 Kinetic study of deprotection for NB-modified DNA

Hydroxymethylated and nitrobenzylated DNA samples for the experiments were synthesized according the reported protocols (*chapter 6.3.3.1*). Prepared DNA samples were purified with Agencourt AMPure XP magnetic beads.

The NB-modified DNA samples were irradiated by light with the maximum emission at 355 nm or 400 nm and different source power. The degree of deprotection for **DNA_U^{NB}** was controlled by restriction endonuclease digestion – RsaI (1.4 uL for 100 ng of DNA). The portion of irradiated **DNA_U^{NB}** was used as a template for an *in vitro* transcription assay. The initial experiments were performed with *NON*-labelled DNA samples. The experiments with ^{32}P-labelled **DNA_U^{NB}** samples were performed after optimization of deprotection condition.

Kinetic study of deprotection of DNA_U^{NB} irradiated by light (355 nm; 1 mW or 400 nm; 25 mW)

The **DNA_U^{NB}** prepared and purified according to reported procedure (*section 6.3.3.1*) was diluted to the final concentration of approximately 20 ng/μL (according to NanoDrop). Approximately 240 ng of purified **DNA_U^{NB}** (in 12 μL) was irradiated by light (max. wavelength at 355 nm or 400 nm). Each sample (12 μL) of starting **DNA_U^{NB}** was irradiated at 7-9 °C (cold room) for the following time intervals 15 minutes / 60 minutes / 120 minutes / 300 minutes. Each sample was irradiated either in the presence of additives [1 μL of (1 mM) NaN_3 and 1 μL of (50 mM) DTT] or in the presence of 2 μL H_2O (to obtain the same dilution as in the case of samples irradiated in the presence of additives). The degree of **DNA_U^{NB}**

deprotection was controlled with cleavage by RsaI (***Figure 38***) and used as a template for an *in vitro* transcription assay (***Figure 39***).

6.3.3.4 The effect of additives on photocleavage of NB- protecting group from DNA

The **DNA_U^{NB}** prepared and purified according to reported procedure (*section 6.3.3.1*) was diluted to the final concentration of approximately 20 ng/μL (according to NanoDrop). Approximately 240 ng of purified **DNA_U^{NB}** (in 12 μL) was irradiated by light from LED (400 nm). Each sample (12 μL) of starting **DNA_U^{NB}** was irradiated at room temperature for two-time intervals 15 minutes or 60 minutes. Each sample was irradiated either in the presence of additives (1 μL of (1 mM) NaN_3 and 1 μL of (50 mM) DTT) or in the presence of 2 μL H_2O (to obtain the same dilution as in the case of samples irradiated in the presence of additives). The irradiated **DNA_U^{NB}** was used as a template for an *in vitro* transcription assay in two independent experiments. (***Figure 40***)

6.3.3.4.1 The kinetic study of DNA_U^{NB} deprotection irradiated by light (365 nm; 0.8 W)

The **DNA_U^{NB}** prepared and purified according to reported procedure (*section 6.3.3.1*) was diluted to the final concentration of approximately 20 ng/ μL (according to NanoDrop). Approximately 240 ng of purified **DNA_U^{NB}** (in 12 μL) was irradiated by light from LED light source (365 nm, 0.8 W). Each sample (12 μL) of starting **DNA_U^{NB}** was irradiated at room temperature for the following time intervals: 5 minutes /10 minutes / 20 minutes / 30 minutes. Each sample was irradiated either in the presence of additives [1 μL of (1 mM) NaN_3 and 1 μL of (50 mM) DTT OR 1 μL of (1 mM) NaN_3 and 1 μL of (100 mM) DTT] or in the presence of 2 μL H_2O (to obtain the same dilution as in the case of samples irradiated in the presence of additives). The degree of **DNA_U^{NB}** deprotection was monitored with cleavage by RsaI. (***Figure 41***) The irradiated **DNA_U^{NB}** was used as a template for an *in vitro* transcription assay. (***Figure 42***)

6.3.3.5 The kinetic study of photocleavage under optimized conditions

Deprotection of DNA_U^{NB} by light irradiation (400 nm, 3 W)

The ^{32}P-labelled **DNA_U^{NB}** prepared and purified according to reported procedure (*chapter 6.3.3.1*) was diluted to the final concentration of approximately 20 ng/ μL (according to NanoDrop). Approximately 240 ng of purified **DNA_U^{NB}** (in 12 μL) was irradiated by light

from LED light source (400 nm, 3 W). Each sample (12 µL) of starting **DNA_U^{NB}** was irradiated at room temperature for the following time intervals: 5 minutes /10 minutes / 20 minutes / 30 minutes / 35 minutes / 40 minutes / 45 minutes / 50 minutes / 60 minutes. Each sample was irradiated in the presence of additives [1 µL of (1 mM) NaN_3 and 1 µL of (50 mM) DTT]. The samples were irradiated in 1.5 ml Eppendorf tubes from the top of the opened tube, sides of which were covered with alu-foil. The degree of **DNA_U^{NB}** deprotection was monitored with cleavage by RsaI. (***Figure 43***) The irradiated **DNA_U^{NB}** was used as a template for an *in vitro* transcription assay in at least three independent experiments. (***Figure 44***, ***Scheme 39***)

Deprotection of DNA_C^{NB} by light irradiation (400 nm, 3 W)

The ^{32}P-labelled **DNA_C^{NB}** prepared and purified according to the reported procedure (*chapter 6.3.3.1*) was diluted to the final concentration of approximately 20 ng/ µL (according to NanoDrop). Approximately 240 ng of purified **DNA_C^{NB}** (in 12 µL) was irradiated by light from LED light source (400 nm, 3 W). Each sample (12 µL) of starting **DNA_C^{NB}** was irradiated at room temperature for the following time intervals: 2minutes / 5 minutes / 7 minutes / 10 minutes / 15 minutes / 20 minutes / 30 minutes / 40 minutes. Each sample was irradiated in the presence of additives [1 µL of (1 mM) NaN_3 and 1 µL of (50 mM) DTT]. The samples were irradiated in 1.5 ml Eppendorf tubes from the top of the opened tube, sides of which were covered with alu-foil. The irradiated **DNA_C^{NB}** was loaded on 1.3 % agarose gel or PAA and used as a template for an *in vitro* transcription assay in two independent experiments. (***Figure 45***, ***Figure 46***, ***Scheme 39***)

6.3.3.5.1 Study of enzymatic phosphorylation of U^{HM}- modified DNA

The **DNA_ U^{HM}** synthesized in the presence of **dU^{HM}TP** and after purification was incubated in the presence of 5-hydroxymethyluridine DNA Kinase (5-HMUDK; 20U; 1.2 µL or 18U; 0.9 µL or 12U; 0.6 µL) at 37 °C for 30 minutes. As a control reaction, natural DNA was incubated under the same conditions. The samples after incubation with 5-HMUDK were purified with Agencourt AMPure XP magnetic particles. The conversion of phosphorylation of **DNA_U^{HM}** was monitored by cleavage with restriction endonucleases – AluI or RsaI under the reported conditions (*section 6.3.3.2*). (***Scheme 41, Figure 47***) The phosphorylated DNAs were also used as the DNA templates for transcription studies. (***Scheme 42***, ***Figure 48***)

6.3.3.6 Controlled switch ON and switch OFF transcription

^{32}P-labelled **DNA_U^{NB}** was prepared according to reported procedure (*chapter 6.3.3.1*). Approximately 240 ng of **DNA_U^{NB}** was irradiated by light with maximum absorbance at 400 nm (3 W) in the presence of additives (1 μL of (1 mM) NaN_3 and 1 μL of (50 mM) DTT). The sample was irradiated for 30 minutes at room temperature (***Scheme 43***, ***Figure 49*** - sample no. 10). The modified **DNA_C^{NB}** was irradiated under the same conditions for 10 minutes (***Scheme 44***, ***Figure 50*** - sample no. 10). The irradiation experiments were repeated in six portions for **DNA_U^{NB}** and **DNA_C^{NB}**. After irradiation experiments, the six portions of the same sample (**DNA_U^{NB}** or **DNA_C^{NB}**) were combined. The 400 ng of unpurified, previously irradiated DNA (**DNA_U^{NB}** or **DNA_C^{NB}**) was incubated in the presence of 5-HMUDK (0.3 μL) in 1X T4 DNA Ligase Reaction Buffer at 37 °C for 30 minutes (***Scheme 43, 44; Figure 49, 50 -*** samples no. 12).

Control reaction for phosphorylation: Natural DNA and **DNA_U^{HM}** or **DNA_C^{HM}** were incubated in the presence of 5-HMUDK under the same conditions as irradiated nitrobenzylated **DNA_N^{NB}**. (***Scheme 43, 44; Figure 49, 50 -*** samples no. 4 and 8).

Control reactions for irradiation: Natural DNA and **DNA_U^{HM}** or **DNA_C^{HM}** (approx. 240 ng) were irradiated by light with maximum wavelength at 400 nm (3 W) in the presence of additives [1 μL of (1 mM) NaN_3 and 1 μL of (50 mM) DTT] for appropriate time interval (30 minutes for U-set and 10 minutes for C-set). The samples were irradiated at room temperature as in the case of their nitrobenzylated DNA equivalents (***Scheme 43, 44; Figure 49, 50 -*** samples no.2 and no. 6).

Control reactions for influence of 1X T4 DNA Ligase Reaction Buffer on transcription: The natural DNA, **DNA_N^{HM}** right after PCR (without irradiation prepared according to reported procedure *6.3.3.1*) and irradiated **DNA_N^{NB}** (approx. 240 ng) were incubated in the presence of 1X T4 DNA Ligase Reaction Buffer at 37 °C for 30 minutes (***Scheme 43, 44; Figure 49, 50 -*** samples no. 3, no. 7, no. 11).

All prepared samples and control samples were diluted to the final concentration of 5 ng/ μL and used as DNA templates for transcription experiments under the reported conditions (*section 6.3.3.3*).

7 References

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