

Univerzita Karlova v Praze

Přirodovědecká fakulta / Faculty of Science

Studijní program: Organická chemie / Organic chemistry

Studijní obor: Organická chemie / Organic chemistry



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Enzymatic synthesis of hypermodified DNA bearing anionic or cationic functional groups

Enzymová syntéza hypermodifikovaných DNA nesoucích aniontové nebo kationtové funkční skupiny

DISSERTATION

Disertační práce

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Praha, 2025

This dissertation was worked out at the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, from November 2020 to March 2025.

Declaration

I hereby declare that I have worked on my thesis independently and cited all necessary literature. Neither this work, nor its significant part will be used to obtain another academic degree.

Prague, 2025

M.Sc. Natalia Kuprikova

ACKNOWLEDGEMENT

First of all, I would like to thank my supervisor, Prof. Michal Hocek, for giving me the opportunity to join his group, despite the challenges on the way, both related to the initial lack of knowledge and literal difficulties to arrive to the Czech Republic. Thanks to his guidance, I had a clear direction for my projects while still having enough freedom to become independent.

My sincere thanks to Veronika for her help and patience with me and my manuscripts. Her kindness and involvement supported me throughout my whole PhD journey. I would also like to thank all members of Hocek's group, who are truly great people to be around, for sharing this experience with me. In particular, I must highlight David, Denise, and Filip for their mentorship and support, Arghya for encouraging me to aim high, and Tereza for making the work of our group significantly easier. Among many wonderful people I met during these years, I would like to give special thanks to Tania, Aparna, Marisol, Anna, Norbi, and Luca for sharing both amazing moments and difficult ones with me. I thank Atti and Ivanka for bringing into my life something bigger than I expected to find here.

This work could not be done without all the collaborators. I thank Dr. Lucie Bednářová for conducting CD measurements and their analysis with the help of discussions with Prof. Michaela Vorlíčková, Dr. Marek Ondruš for providing me with hydrophobically modified nucleoside triphosphates, Miguel Riopedre Fernández for performing MD simulations, Dr. Lenka Poštová Slavětínská for measuring and analyzing NMR spectra, Dr. Tomáš Kraus for studying the aggregate formation by confocal microscopy, and Ing. Kateřina Nováková for measuring MALDI-TOF spectra.

Last but definitely not least, I want to thank my family. My dad, who has never doubted me, my mom, who has always supported me, even when it seemed to be 'unsafe', my twin brother for always being my best friend, and my husband Grant for leaving everything behind and stepping into the unknown with me.

This work was supported by the Czech Science Foundation (20-00885X).

SUMMARY

This dissertation describes the synthesis of two sets of base-modified 2'-deoxyribonucleoside 5'-O-triphosphates (dN^RTPs) bearing charged functional groups, their application in enzymatic synthesis to obtain different types of hypermodified DNA, and the subsequent biophysical studies of the resulting DNA species.

Two sets of dN^RTPs, bearing either anionic or cationic modifications, were synthesized by Sonogashira cross-coupling. Anionic (acidic) moieties were represented by carboxylate, sulfonate, phosphonate, and phosphate groups, while cationic (basic) modifications included amino, methylamino, dimethylamino, and trimethylammonium groups. The obtained dN^RTPs were systematically tested as substrates in enzymatic DNA synthesis using two DNA polymerases. All anionically and cationically modified nucleotides were successfully incorporated into DNA by primer extension (PEX), both individually and in different combinations. Moreover, it was possible to replace all four canonical nucleotides in PEX with either anionic or cationic dN^RTPs, leading to the synthesis of hypermodified DNA probes, where each nucleobase displayed a different functional group. The DNA species, bearing all four anionic modifications, was named '*superanionic* DNA' due to its unique nature, as it carries a double negative charge compared to natural DNA. In turn, for hypermodified DNA displaying a whole range of cationic groups, the term '*zwitterionic* DNA' was applied to emphasize the zwitterionic character of the obtained molecule, in which the positive charges of cationic substituents counterbalance the anionic phosphate DNA backbone. To demonstrate the compatibility of different types of modifications in a DNA molecule, PEX was performed with one anionic, one cationic, and two hydrophobic dN^RTPs, resulting in '*mixed*' hypermodified DNA.

Both anionic and cationic series of dN^RTPs were further tested in polymerase chain reaction (PCR). While using modified nucleotides in PCR individually leads to exponential amplification, combinations of nucleotides mostly resulted in low amplification or no PCR product. Nevertheless, hypermodified oligonucleotides (ONs) were successfully obtained by linear amplification (aPCR) and could be further replicated efficiently with natural dNTPs, enabling sequencing and confirming the accuracy of hypermodified DNA synthesis.

Hybridization studies demonstrated that, while the presence of anionic modifications in a DNA duplex decreases its stability, cationic modifications stabilize the duplex. Regarding DNA conformation, all duplexes with one hypermodified strand preserve the typical B-DNA form; however, when both strands are hypermodified, DNA appears to deviate from the B-conformation.

The extreme cases of DNA functionalization, such as superanionic and zwitterionic DNA, expand knowledge about enzymatic DNA synthesis, broadening the boundaries of the field. Combining several types of modifications in a DNA molecule allows for creating a library of peptide-like polymers with expanded chemical diversity, which can be further used in *in vitro* selection of nucleic acids toward various targets. As an example, our group is currently focused on synthesizing a hypermodified aptamer library using modified dN^RTPs bearing carboxylic, dimethylamino, and two hydrophobic groups. Given the interesting properties of hypermodified DNA species, further research in the field of oligonucleotide therapeutics should be pursued.

SOUHRN

Tato disertační práce popisuje syntézu dvou sad na bázi modifikovaných 2'-deoxyribonukleosid-5'-O-trifosfátů (dN^RTP) nesoucích nabitě funkční skupiny, jejich aplikaci v enzymatické syntéze k získání různých typů hypermodifikovaných DNA a následné biofyzikální studie získaných DNA forem.

Dvě sady dN^RTP , obsahující buď aniontové nebo kationtové modifikace, byly syntetizovány pomocí Sonogashirovy reakce. Aniontové (kyselé) skupiny zahrnovaly karboxylát, sulfonát, fosfonát a fosfát, zatímco kationtové (zásadité) modifikace tvořily amino, methylamino, dimethylamino a trimethylamonium skupiny. Získané dN^RTP byly systematicky testovány jako substráty pro enzymatickou syntézu DNA pomocí dvou DNA polymeráz. Všechny aniontové i kationtové modifikované nukleotidy byly úspěšně začleněny do DNA metodou prodloužení primeru (PEX), a to jak jednotlivě, tak v různých kombinacích. Navíc bylo možné nahradit všechny čtyři kanonické nukleotidy v PEXu buď aniontovými, nebo kationtovými dN^RTP , což vedlo k syntéze hypermodifikovaných DNA sond, kde každá nukleobáze nese odlišnou funkční skupinu. DNA nesoucí všechny čtyři aniontové modifikace byla pojmenována 'superaniontová DNA', díky dvojnásobnému zápornému náboji ve srovnání s přirozenou DNA. Naopak pro hypermodifikovanou DNA obsahující celou škálu kationtových skupin byl použit termín 'zwitteriontová DNA', aby byl zdůrazněn zwitteriontový charakter získané molekuly, v níž kladné náboje kationtových substituentů vyvažují aniontovou fosfátovou kostru DNA. Aby byla prokázána kompatibilita různých typů modifikací v molekule DNA, byl PEX proveden s jedním aniontovým, jedním kationtovým a dvěma hydrofobními dN^RTP , což vedlo ke vzniku 'smíšené' hypermodifikované DNA.

Obě série dN^RTP , aniontová i kationtová, byly dále testovány v polymerázové řetězové reakci (PCR). Zatímco použití jednotlivých modifikovaných nukleotidů v PCR umožnilo exponenciální amplifikaci, kombinace nukleotidů většinou vedly k nízké amplifikaci nebo absenci PCR produktu. Přesto byly hypermodifikované oligonukleotidy (ON) úspěšně získány lineární amplifikací (aPCR) a mohly být následně efektivně replikovány pomocí přirozených dNTP, což umožnilo jejich sekvenaci a potvrdilo přesnost syntézy hypermodifikované DNA.

Studie hybridizace ukázaly, že přítomnost aniontových modifikací ve dvoušroubovici DNA snižuje její stabilitu, zatímco kationtové modifikace duplex stabilizují. Co se týče konformace DNA, všechny duplexy s jedním hypermodifikovaným vláknem zachovávají typickou B-DNA formu; avšak v případě, že jsou hypermodifikována obě vlákna, DNA se od B-konformace odchyluje.

Extrémní případy funkcionalizace DNA, jako jsou superaniontová a zwitteriontová DNA, rozšiřují znalosti o enzymatické syntéze DNA a posouvají hranice tohoto oboru. Kombinací několika typů modifikací v molekule DNA lze vytvořit knihovnu polymerů podobných peptidům s rozšířenou chemickou diverzitou, která může být dále využita pro *in vitro* selekci nukleových kyselin vůči různým cílům. Například naše skupina se v současnosti zaměřuje na syntézu hypermodifikované knihovny aptamerů s použitím modifikovaných dN^RTP nesoucích karboxylovou, dimethylaminovou a dvě hydrofobní skupiny. Vzhledem k zajímavým vlastnostem hypermodifikovaných forem DNA by měl být další výzkum v oblasti oligonukleotidových terapeutik nadále rozvíjen.

LIST OF AUTHOR'S PUBLICATIONS

1. N. Kuprikova, M. Ondruš, L. Bednárová, M. Riopedre Fernández, L. Poštová Slavětínská, V. Sýkorová, M. Hocek. Superanionic DNA: enzymatic synthesis of hypermodified DNA bearing four different anionic substituents at all four nucleobases. *Nucleic Acids Res.* **2023**, *51* (21), 11428 – 11438.
2. N. Kuprikova, M. Ondruš, L. Bednárová, T. Kraus, L. Poštová Slavětínská, V. Sýkorová, M. Hocek. Zwitterionic DNA: enzymatic synthesis of hypermodified DNA bearing four different cationic substituents at all four nucleobases. *Nucleic Acids Res.* **2025**, *53* (5), gkaf155.

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1. INTRODUCTION

1.1. DNA – structure and properties

2'-Deoxyribonucleic acid (DNA) plays a vital role in living organisms as it carries genetic information and instructions for all cellular processes. From the chemical point of view, DNA is a polymer composed of monomers – nucleotides. In its turn, a nucleotide is made up of a phosphate group, a sugar moiety (2'-deoxyribose), and one of the four nucleobases – adenine (A), thymine (T), guanine (G), and cytosine (C) (Figure 1). Specific nucleobases form base pairs with each other (A with T, G with C) through hydrogen bonds (Watson-Crick base pairing). In combination with π - π -stacking, such base pairing allows the formation of double-stranded DNA (dsDNA) from two complementary single strands with antiparallel strand orientation.

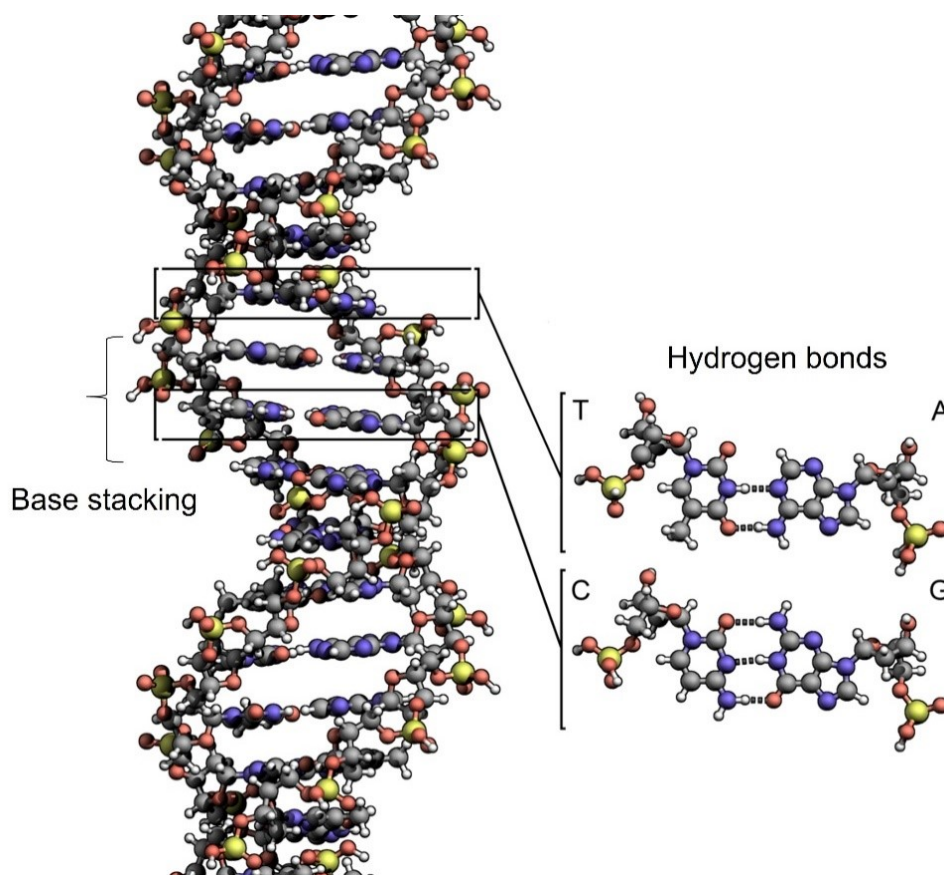


Figure 1. Hydrogen bonds and base-stacking interactions in DNA double helix (taken from <https://en.wikipedia.org/wiki/DNA>).

The stability of dsDNA is primarily determined by hydrogen bonding between base pairs in two complementary strands (Figure 1). Thus, as the length of the duplex increases, the number of hydrogen bonds also increases, leading to greater duplex stability. Another important factor is GC content. Since G-C pairs form three hydrogen bonds while A-T pairs form only two, a duplex with a higher GC content will demonstrate higher stability. Additionally, base-stacking interactions between individual bases are major contributors to duplex stability. Due to relatively rigid structure and mutual orientation of base pairs in dsDNA, base stacking is more prevalent in duplexes than in single strands. High monovalent salt concentration (Na^+ , K^+) masks the destabilizing charge repulsion between two phosphodiester backbones, enhancing base-stacking interactions and further stabilizing the duplex. Interestingly, the effect of divalent ions on DNA structure (Mg^{2+} , Co^{2+} , Zn^{2+} , Cu^{2+}) is highly dependent on their concentration. Since such cations are able to bind to both phosphates and heterocyclic bases, it is possible to regulate the mutual interconversion of double helix, single strand, and random coil structures by selecting an appropriate divalent ion and adjusting its concentration.^{1,2}

To experimentally determine duplex stability, a combination of physical methods including X-ray crystallography, NMR, and UV-vis spectroscopy is employed. The most routinely used method is UV spectroscopy due to its efficiency and affordability. It allows to perform hybridization studies. Heating the DNA duplex disrupts base-stacking interactions and breaks hydrogen bonds between strands, causing the helix to unwind (denature). Once unwound, the bases become unstack and can absorb more light, although the absorption maximum for both double- and single-stranded DNA remains at 260 nm. This effect is called hypochromicity effect.³ As a result, the concerted process of DNA denaturation can be conveniently monitored by an increase in UV absorbance as the double strand unwind into single strands (Figure 2). The mid-point of the transition, when half dsDNA has denatured, is called melting temperature (T_m), which provides valuable information about DNA duplex stability.

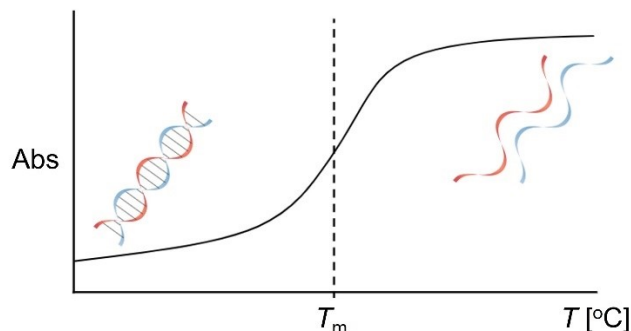


Figure 2. UV melting curve of dsDNA (taken from atdbio.com).

DNA duplexes can adopt various conformations, but three predominant forms are A-, B-, and Z-DNA (Figure 3A). Different DNA conformers are believed to participate in regulation of gene expression by influencing protein binding.⁴ The right-handed B-DNA is considered the most common in living organisms and has two main sites for protein interactions – the major and minor grooves.⁵ The A-form is a right-handed duplex with a more compact helical structure compared to the B-form and typically occurs under dehydrating conditions.⁶ Z-DNA is a left-handed helix with a zigzag pattern, known to be stable in alternating CG sequences at high salt concentrations.⁷

There is a number of methods employed to study DNA conformation, however, the fastest and most inexpensive one is circular dichroism (CD) spectroscopy. This absorption spectroscopy method is commonly used to investigate structural aspects of chiral targets, typically biological molecules. Optically active chiral molecules preferentially absorb circularly polarized light in one direction. The difference in absorption between right- and left-handed polarized light is called CD and can be measured and quantified. For DNA studies, UV light in the 180-350 nm range is used, as DNA bases absorb light in this range. However, a drawback of the method is that it cannot provide structural information at atomic level for large molecules as DNA, unlike NMR spectroscopy or X-ray crystallography. For this reason, CD spectroscopy is primarily used qualitatively in DNA studies. Nevertheless, CD spectroscopy remains an essential tool for the majority of studies concerning DNA secondary structure due to its advantages over other methods. These advantages include extreme sensitivity to low sample concentrations, the ability to study both short and long ONs, the opportunity for *in situ* titration with various agents to induce

conformational DNA isomerization, and, notably, it does not require crystallization or extended experimental times, unlike NMR and X-ray measurements.⁸

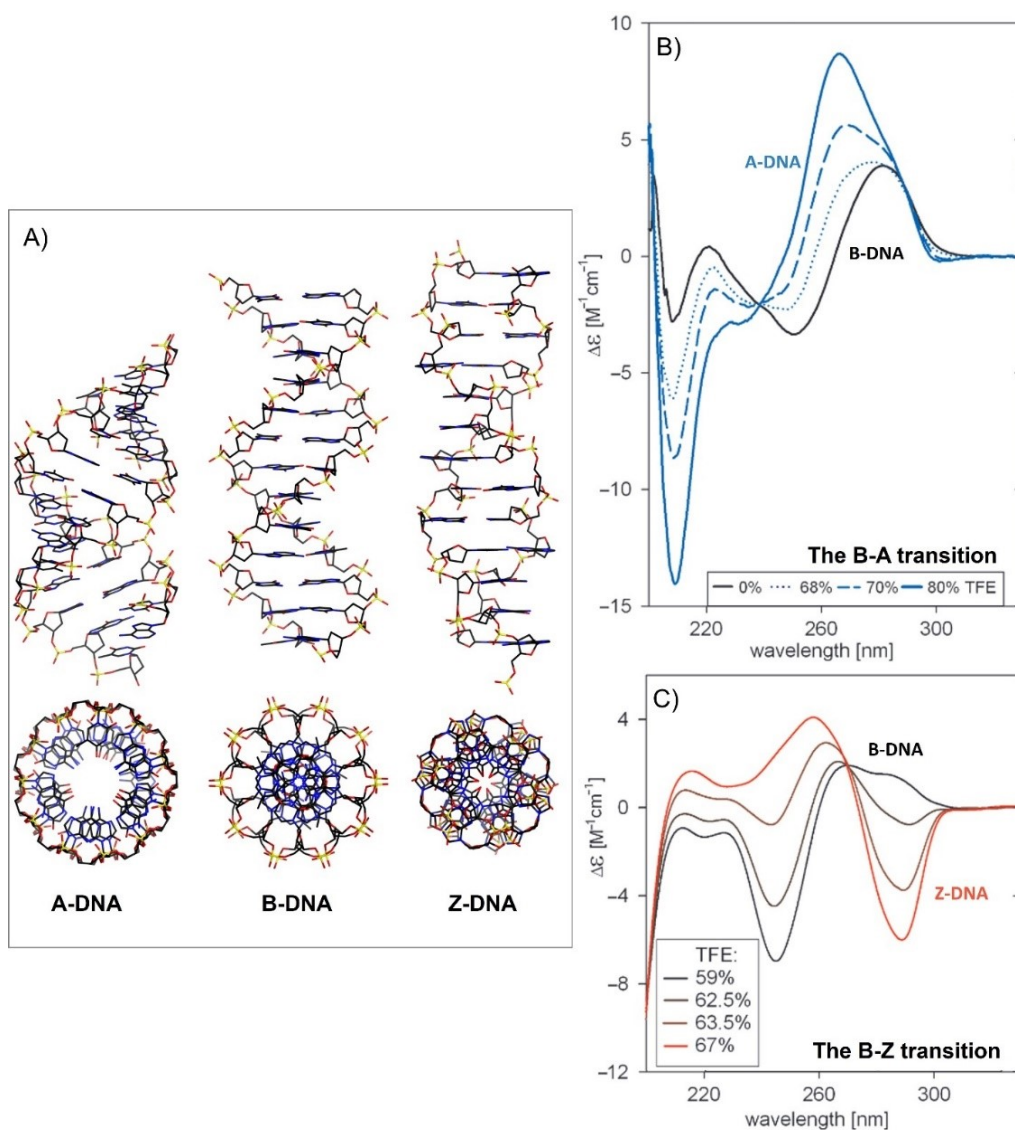


Figure 3. A) A-, B-, and Z-DNA conformers (taken from Wikimedia commons); B) B-A DNA transition of d(GCGGCGACTGGTGAGTACGC) duplex (taken from ref.8); C) B-Z DNA transition of poly[d(GC)] duplex (taken from ref.8).

CD spectrum of the natural right-handed B-DNA is typically characterized by a broad positive band at 260-280 nm and a negative band around 245 nm. Since the base pairs in the B-form are almost perpendicular to the double-helix axis, the chirality of the molecule is weak (Figure 3A). As a result, the intensities of the peaks are relatively small. On the other hand, base pairs in the

right-handed A-DNA form are inclined at an angle of 19° , resulting in higher peak amplitudes. The B-A transition is driven by addition of ethanol,^{9,10} trifluorethanol (TFE),¹¹ or methanol for GC rich DNA¹² (Figure 3B). Regarding the left-handed Z-DNA, its base pairs have an opposite orientation with respect to the backbone than B- and A-forms, so CD spectrum of Z-DNA looks as nearly an inversion of B-DNA spectrum and contains a negative band around 290 nm and a positive band at 260 nm (Figure 3C). Due to the need to flip the base pairs, the B-Z transition is slow compared to the B-A transition. Z-DNA is known to be stable in alternating poly[d(GC)] duplexes at high salt concentrations or TFE.^{13,14}

1.2. Base-modified DNA

As a tool for gene regulation, nature employs naturally occurring modified nucleobases, also known as ‘non-canonical nucleobases’. These DNA modifications comprise an additional layer of information above the basic genetic code. While not interfering with Watson-Crick base pairing, they affect DNA-protein interactions occurring in the major groove of the double helix. As a result, these non-canonical nucleobases, also referred to as epigenetic modifications, alter the phenotype without changing the genotype. Epigenetic modifications play an important role in various biological processes, including development¹⁵ and aging¹⁶. Figure 4 illustrates some examples of non-canonical pyrimidine and purine bases.¹⁷ Given the significance of naturally occurring base-modifications, it should come as no surprise that base functionalization of DNA has become a widely studied research topic.

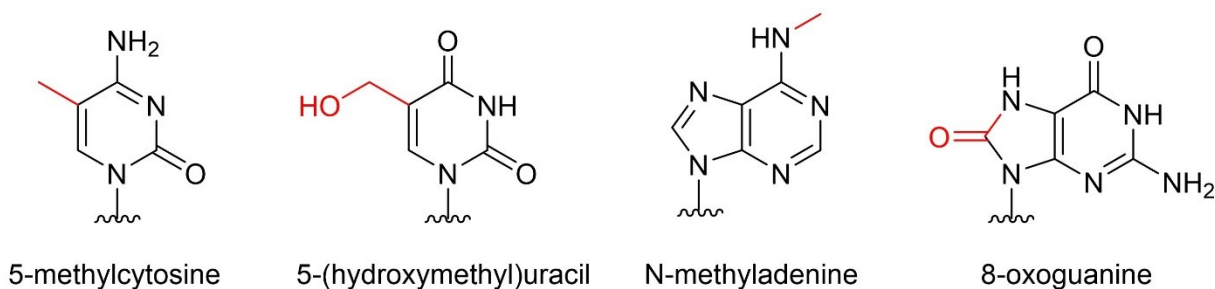


Figure 4. Examples of naturally occurring non-canonical nucleobases.

To modify a nucleobase while preserving DNA’s self-recognition function, it is essential to choose a base site that ensures the introduced modification does not disrupt Watson-Crick base

paring. Following nature's example, the carbon C5 of pyrimidines and C8 of purines could be chosen as optimal positions for derivatization (Figure 5A). Indeed, for pyrimidine derivatives, the C5 position has been proven to be suitable for introducing modifications without disturbing the helical structure of DNA.¹⁸ However, for the purines, modifications at the C8 position have been reported to destabilize dsDNA secondary structure,¹⁹ likely due to nucleoside's transition into an unfavorable *syn*-conformation (Figure 5B). Moreover, several reported C8-modified 2'-deoxyribonucleoside triphosphates (dNTPs) have been found unsuitable as substrates for enzymatic DNA synthesis (see details about enzymatic DNA synthesis in the next chapter).^{20,21} On the other hand, functionalization at the C7-position within the 7-deazapurine structure offers an opportunity to modify purine nucleobases without destabilizing the DNA duplex or disrupting base pairing. Modifications at this position are well accommodated in the DNA major groove²² and 7-modified 7-deazapurines triphosphates serve as efficient substitutes for natural dNTPs in enzymatic DNA synthesis.²³

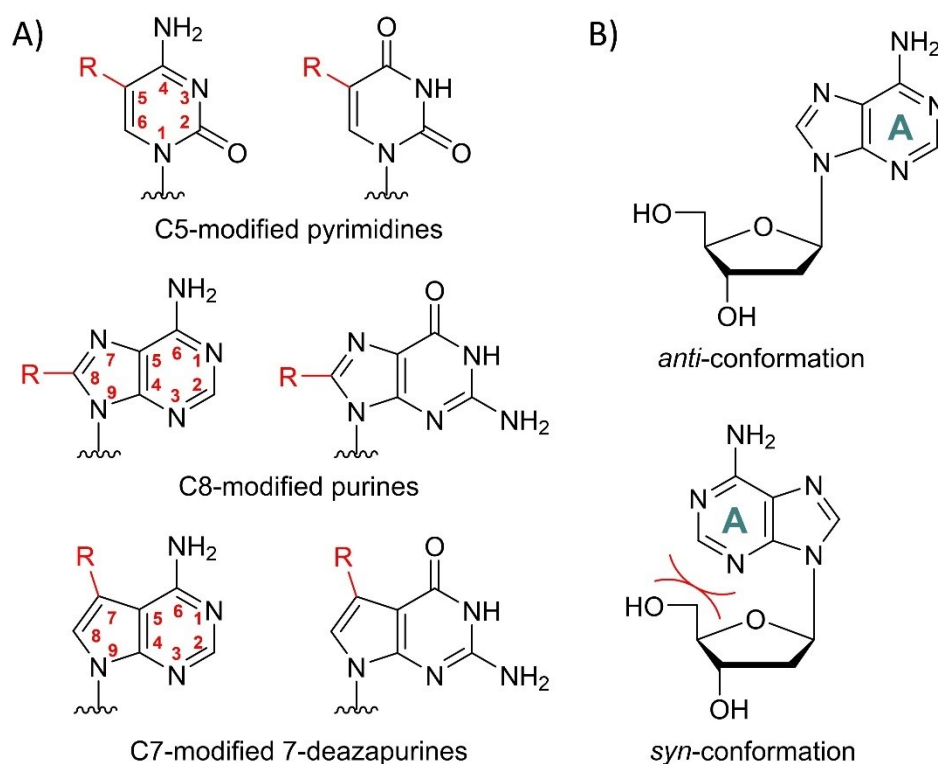


Figure 5. A) Positions of modifications on nucleobases; B) glycosidic bond conformers.

To date, a wide range of functionalities have been introduced into DNA via C5-modified pyrimidines and C7-modified 7-deazapurines including reactive,²⁴ fluorescent,²⁵ epigenetic,²⁶ redox,²⁷ and lipophilic modifications,²⁸ which find potential applications in different fields of biotechnology and chemical biology.

Introducing modifications into DNA duplex can affect both its stability and conformation. It has previously been reported that DNA containing alkynyl-linked modifications^{29,30,37} exhibit a higher stability compared to its natural analog due to the increased polarizability and π - π -stacking effect of the modified nucleobases. Regarding conformational changes upon introduction of functionalities into DNA, it has been shown that incorporating one type of modified base does not alter the overall B-type DNA structure.²¹ Nevertheless, the high-density of major-groove modifications can enforce a transition from the right-handed B-type DNA to a left-handed form similar to Z-DNA.^{31,68}

1.3. Enzymatic DNA synthesis

Non-modified oligonucleotides (ONs) are routinely made by phosphoramidite solid-phase synthesis (SPS), which was developed in the 1980s.³² However, even though it allows large-scale ON synthesis, chemical SPS is highly dependent on a ON length. Even with 99.5% coupling efficiency, the yield of a natural 200-mer ON is about 36%. With dropping the coupling efficiency by 1%, the 200-mer ON yield rapidly decreases to 5%. Thinking about introducing a modification into DNA, one must consider that incorporation of various functionalities can influence coupling efficiency. Moreover, the introduced modifications must tolerate all the SPS steps, including mild acidic, strong basic, and oxidizing conditions. Therefore, chemical ON synthesis remains limited for synthesis of long modified ONs.

Enzymatic synthesis, in turn, offers a feasible approach toward diverse functionalization of DNA. Although the scale of the method is usually limited from pico- to nanomolar quantities, it allows to synthesize long modified DNA threads in the absence of organic solvents. What is needed are primers, templates, DNA polymerase, and a set of four dNTPs, which can be substituted by their modified analogs (dN^RTPs). The primer extension (PEX) reaction is the simplest method for enzymatic DNA synthesis. DNA polymerase elongates a primer along a single-stranded DNA

(ssDNA) template using one or more modified dN^RTPs, resulting in the synthesis of a complementary modified ON (Figure 6). Then, the modified ON can be isolated either as a double strand (dsDNA), together with the template, or as a single strand (ssON) by removing the template strand using separation techniques such as magnetoseparation³³ or λ -exonuclease digestion.³⁴

As one might expect, modified dN^RTPs are generally less efficient substrates for DNA polymerases compared to their natural counterparts, except in rare cases where a modification enhances π - π stacking between adjacent nucleobases.³⁵ When it comes to the choice of DNA polymerases, several studies have shown that B-family DNA polymerases are better suited for incorporating base-modified nucleotides than those from family A.³⁶ Additionally, the lack of 3' to 5' exonuclease activity and thermostability (e. g., in KOD XL and Vent(exo-) DNA polymerases) allows for more relaxed incorporation conditions; however, one should carefully adjust the reaction conditions to avoid misincorporations. The efficiency of DNA functionalization using thermostable DNA polymerases was demonstrated by creating biopolymers containing all four modified nucleotides.^{37,38}

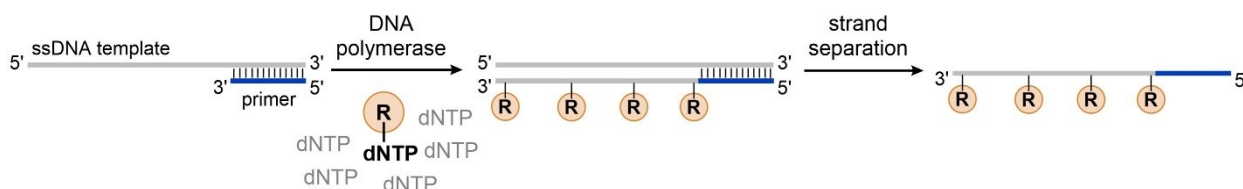


Figure 6. The scheme of PEX reaction followed by ssON generation.

A more challenging task is the application of modified dN^RTPs in polymerase chain reaction (PCR) (Figure 7). For successful exponential amplification, the modified dN^RTPs must not only be good substrates for DNA polymerase but also allow the polymerase to read through the modified template to synthesize another modified strand. This reaction is carried out in cycles and requires an excess of two primers, forward and reverse, unlike PEX, which requires only one. Each cycle consists of strand denaturation, primer annealing, and primer extension, and proceeds within a temperature range from 50 °C to 95 °C. For this reason, the use of thermostable DNA polymerase is advantageous. If the polymerase cannot read through modified template and the forward primer does not extend, the PCR experiment is called asymmetric PCR (aPCR) and results in accumulation of a modified ssON, the product of the reverse primer extension (Figure 7).

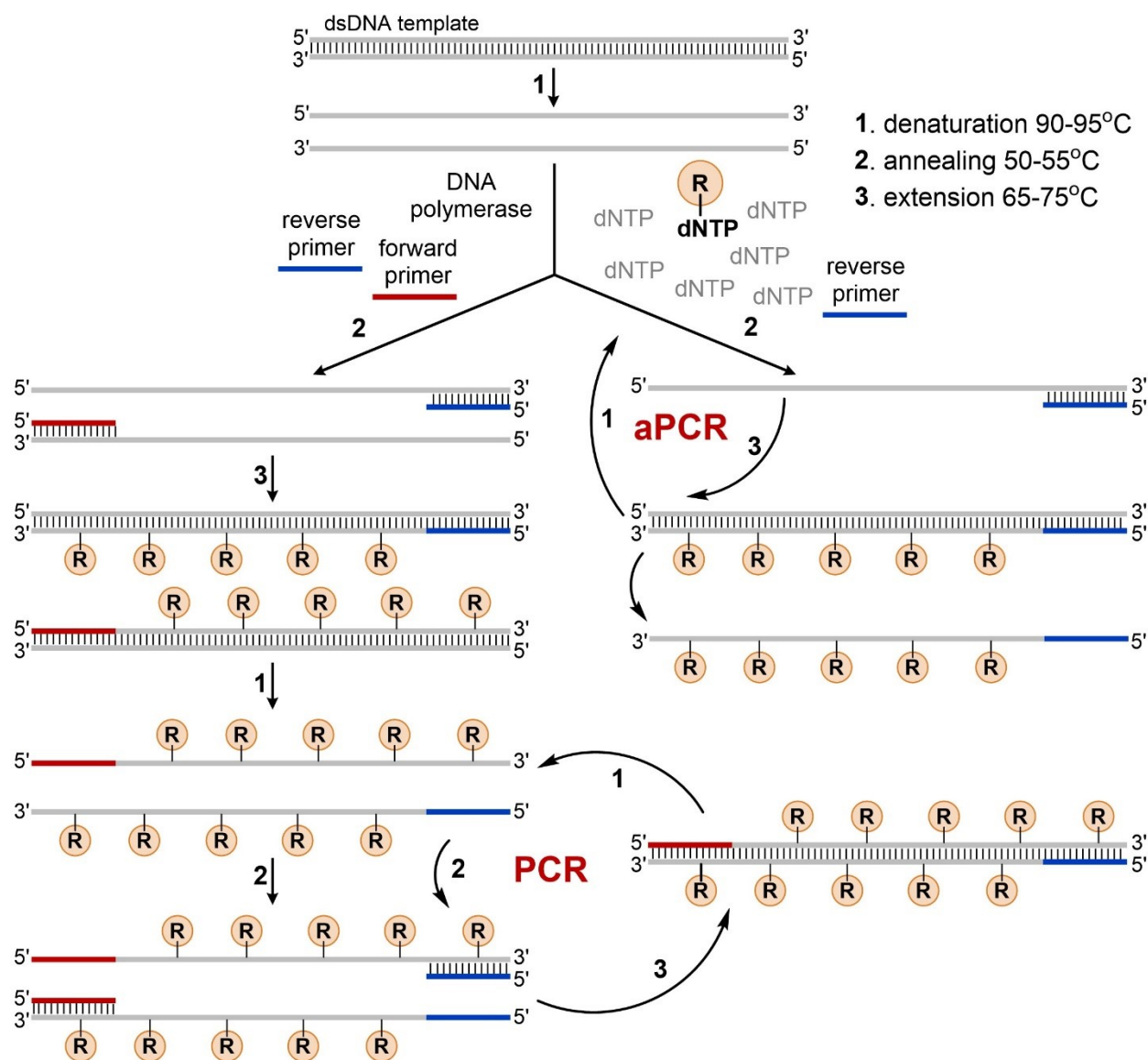


Figure 7. The scheme of polymerase chain reaction (PCR) and asymmetric PCR (aPCR).

The progress of enzymatic reactions is routinely monitored using electrophoretic gel analysis, most commonly with agarose and polyacrylamide (PAGE) gels. The visualization is performed either by initial primer labeling with a fluorophore or a radioactive label, or by subsequent gel post-staining using various DNA intercalators.

1.4. Synthesis of modified nucleoside triphosphates

The general method for obtaining 2'-deoxynucleoside 5'-*O*-triphosphates is the Yoshikawa approach.³⁹ This one-pot reaction includes three steps (Figure 8). First, a 2'-deoxynucleoside (dN) is dissolved in dry trimethylphosphate PO(OMe)₃ under argon atmosphere followed by addition of freshly distilled phosphoryl chloride POCl₃. As a result, a highly reactive 5'-phosphorodichlorate is formed, which further reacts upon addition with tributylammonium pyrophosphate (Bu₃NH)₂H₂P₂O₇, forming a cyclic triphosphate. It is crucial to keep the reaction mixture anhydrous; otherwise, the highly reactive phosphorodichlorate will hydrolyze, forming monophosphate. The obtained cyclic triphosphate is subsequently hydrolyzed by triethylammonium bicarbonate (TEAB), yielding the desired linear dNTP. Another important detail is controlling the duration of the first step of the reaction. Indeed, the primary 5'-OH group is more nucleophilic than the secondary hydroxyl at the C3' position, allowing to avoid extra protection on the 3'-OH group. However, excessive prolongation of the first step in an attempt to fully convert the starting nucleoside into phosphorodichlorate may lead to unwanted phosphorylation of 3'-OH group. To improve the yield and minimize formation of by-products, other methods were developed.⁴⁰ Yet, the Yoshikawa approach remains the most widely used, as it does not require a multistep synthesis of the starting material.

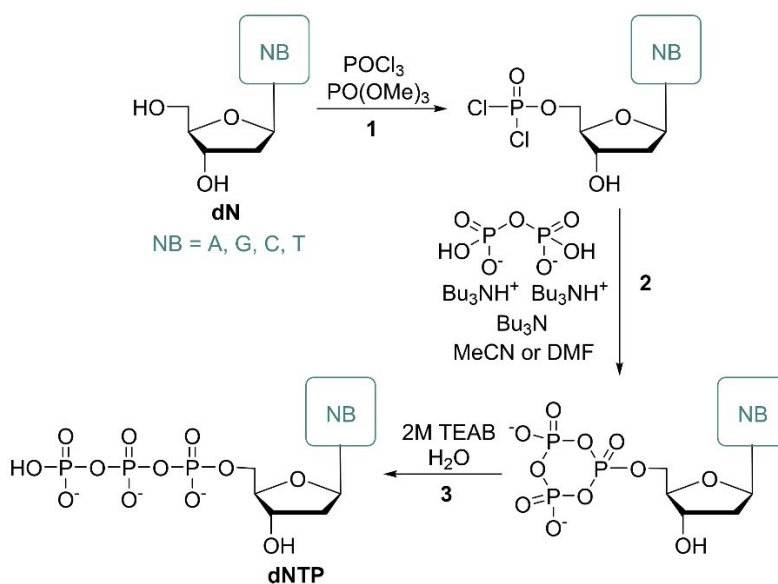


Figure 8. The Yoshikawa triphosphorylation method.

To modify dNTP at a base site, two main synthetic approaches are commonly employed. Either the functional group is first attached to an iodinated nucleoside (dN^{I}) followed by a triphosphorylation reaction, or the modification of interest is directly coupled to an iodinated 2'-deoxyribonucleoside 5'-*O*-triphosphate ($\text{dN}^{\text{I}}\text{TP}$) (Figure 9). The first approach is preferable in cases where harsher coupling conditions are required, as these might lead to the triphosphate group degradation. The second approach is advantageous when the modification of interest can be affected by highly reactive electrophilic POCl_3 during triphosphorylation.

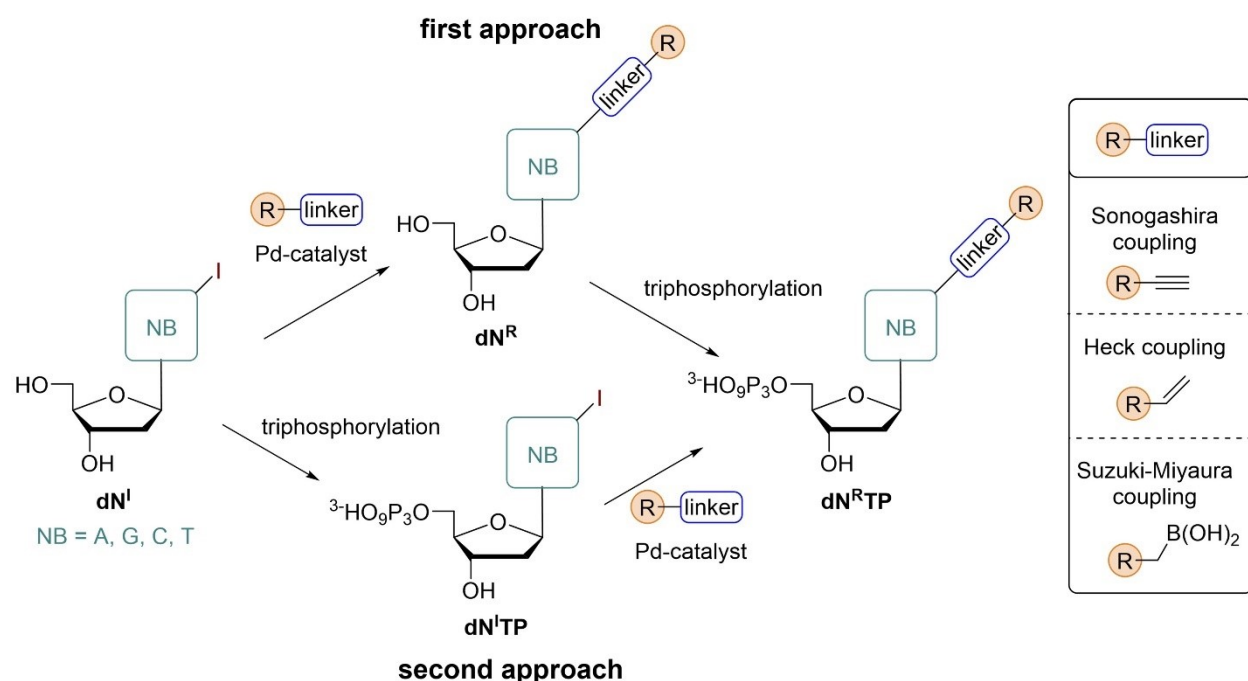


Figure 9. Two main approaches to synthesis of modified $\text{dN}^{\text{R}}\text{TP}$.

As it has been discussed earlier, the most preferable sites for functional group introduction are the C5-position of pyrimidines and the C7-position of 7-deazapurines. The typical way for modifying a nucleobase is through cross-coupling reactions. C5- and C7-iodinated pyrimidines and 7-deazapurines, respectively, are commonly used as starting materials in one-step functionalization via Sonogashira⁴¹ (alkyne-modified moieties), Heck⁴² (alkene-modified moieties) or Suzuki-Miyaura⁴³ (boronic ester-modified moieties) cross-coupling reactions. Given the laborious synthesis of 7-deazapurines,⁴⁴ it is not surprising that the majority of works has been focused on pyrimidines modifications.⁴⁵ Cross-coupling reactions can be directly carried out on

nucleoside triphosphates in DMF or a water/acetonitrile mixture in presence of a palladium catalyst and a water-soluble ligand. The tendency of nucleoside triphosphates to hydrolyze at higher temperatures restricts temperature facilitation of the coupling reaction. And yet, it is possible to achieve the full conversion of the starting material without significant triphosphate degradation by heating the reaction mixture briefly.^{27,28,38}

Purification following Yoshikawa triphosphorylation is generally done by anion-exchange chromatography, which effectively separates residual nucleoside from charged by-products and desired nucleoside triphosphate. Modified dN^RTPs obtained through direct coupling reaction on dN^ITPs are commonly purified by ion-pair reverse-phase high-performance liquid chromatography (IP RP-HPLC) with TEAB, yielding triethylammonium salts. These salts can be further converted into a more stable sodium form using a cation-exchange column.

1.5. Charged modifications on DNA

Since a wide range of modifications can be enzymatically introduced into DNA, one can find among them both hydrophobic and hydrophilic functional groups. Hydrophobic groups, which have been reported previously, include multiple examples, such as carboranes,^{46,38} diamondoids,⁴⁷ grafted polymers,⁴⁸ aromatic hydrocarbons,⁴⁹ and many others. Hydrophilic modifications comprise those that are neutral at physiological pH, such as hydroxyl group,⁵⁰ and those that are charged, either negatively or positively. Since this work focuses on charged modifications, a more detailed discussion on this topic will follow.

Charged functional groups are represented by anionic (acidic) groups (Figure 10) and cationic (basic) ones (Figure 11). The first anionically modified nucleobase to be mentioned is the naturally occurring 5-carboxycytosine, which serves as an intermediate in the active DNA demethylation pathway.⁵¹ Interestingly, among synthesized anionically modified analogs, carboxylic acid is the most studied base modification^{52,53,21} probably due to resembling aspartate or glutamate. Generally, such carboxylate-linked dN^RTPs are efficient substrates in enzymatic DNA synthesis and well accepted by thermostable DNA polymerases. Other reported negatively charged modifications include dicarboxylate-linked dN^RTPs used for constructing polyanionic DNA for potential applications in Si-nanowire biosensors;⁵⁴ phosphate-linked nucleotide, which was

incorporated into DNA to study its influence on DNA's structural properties;⁵⁵ boronic-acid-linked nucleotides, designed for interactions with carbohydrates.^{56,57}

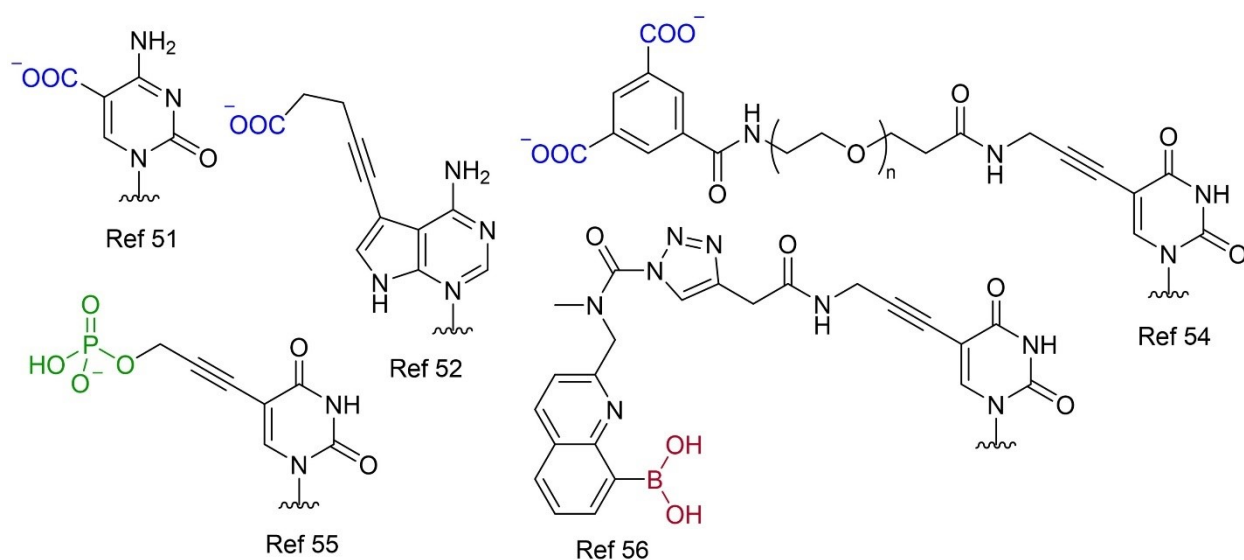


Figure 10. Examples of nucleobases modified with acidic functional groups.

Regarding cationic modifications, there are numerous examples of base-modified dN^RTPs bearing mainly primary amino,⁵⁸ guanidine,^{59,60} and imidazole^{23,61} groups with their application in enzymatic DNA synthesis. These dN^RTPs were reported as rather poor but still useful substrates for DNA polymerases in PEX, though less so in PCR, and were employed in DNazymes selection^{59,60,61} or for further modification of nucleotides or DNA.^{62,63} Interestingly, C7-modified 7-deazapurine triphosphates with an attached 5-aminopentyl moiety demonstrated higher substrate activity than C5-modified pyrimidine analogs.⁶⁴ Among other relevant observations, it was reported^{65,66,67} that a longer linker between the protonated amino group and the nucleobase improves substrate activity. It has also been shown that amino-, guanidine-, and imidazole-linked nucleotides can be simultaneously incorporated into DNA^{59,60} and, moreover, aminopropargyl modification can be introduced into DNA alongside with carboxylate and other hydrophobic amino acid-like functional groups.^{21,52,68}

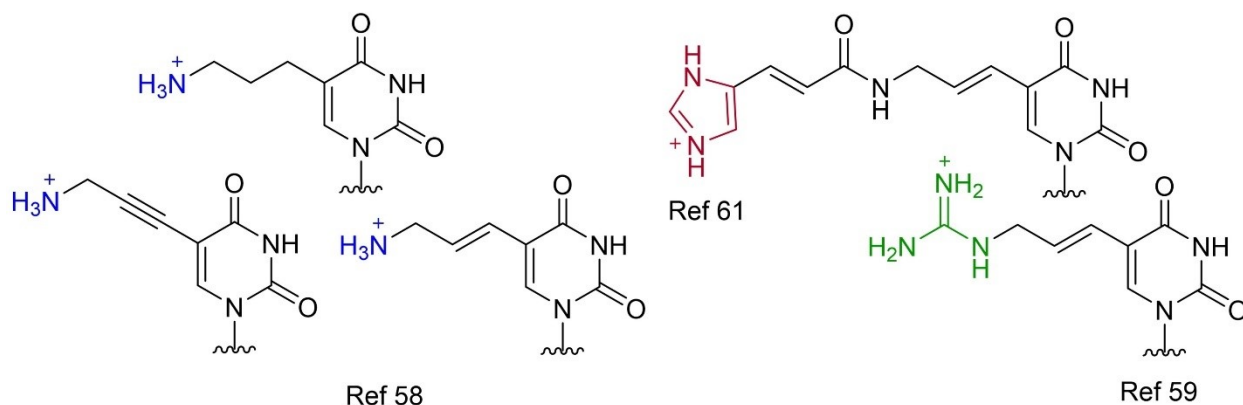


Figure 11. Examples of nucleobases modified with basic functional groups.

One of the promising applications of DNA modified with charged functionalities is *in vitro* selection of aptamers and DNazymes. Aptamers are short single-stranded DNA or RNA molecules that can specifically bind to a cellular target such as protein, peptide, or even cellular surface.⁶⁹ This is possible due to the ability of ssONs to form various 3D structures, allowing them to recognize and bind to their targets with high specificity and affinity. Because of the mechanism of action, aptamers are often called chemical antibodies. DNazymes, or deoxyribozymes, in turn, are ssDNA molecules capable of catalyzing chemical reactions. To date, all DNazymes have been developed synthetically.

Unlike antibodies and protein enzymes, natural DNA does not display a vast variety of functional groups. Introducing suitably modified nucleotides widens chemical diversity of DNA, allowing for other types of interactions. In the case of DNazymes, modifications can improve important catalytic features such as rate, yield, or substrate scope. The first reported modified DNzyme was Zn^{2+} -dependent RNA-cleaving DNzyme with an imidazole moiety attached to the C5 position of 2'-deoxyuridine.⁷⁰ The imidazole moieties on the three modified nucleotides presumably facilitate catalysis by coordinating with the Zn^{2+} cofactor and activating a 2'-OH group located on the RNA substrate (Figure 12). Shortly after, Perrin's group identified M^{2+} -independent RNA-cleaving DNzyme, bearing primary amino and imidazole groups, where cationic moieties supported global folding and general acid-base catalytic mechanism at physiological pH.^{71,72} The advantage of divalent metal ion-independent DNazymes lies in their potential for *in vivo* applications, such as mRNA cleavage.^{73,74} The later M^{2+} -independent generation of DNazymes

incorporated three cationic modifications – imidazole, primary amino, and guanidium groups.^{53,59,60} Introduction of the guanidinium group allowed for the identification of several DNAzymes that catalyze RNA hydrolysis at higher optimal temperatures,⁵⁹ increase first-order rate constant,⁶⁰ accept all-RNA substrates,^{53,75} and enable all-RNA cleavage with multiple turnover kinetics.⁷⁵

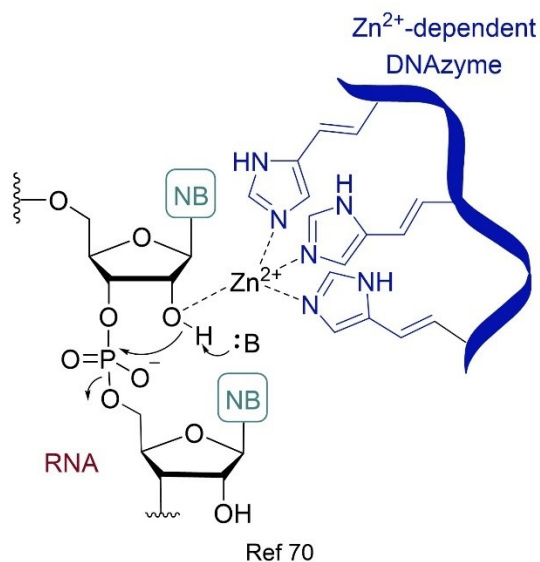


Figure 12. The suggested mechanism of action of Zn^{2+} -dependent RNA-cleaving DNAzyme with imidazole moieties (ref. 70).

So far, almost all reported DNAzymes, obtained by including modified dN^{RTP} s, catalyze RNA hydrolysis. A notable exception is the work of Silverman's laboratory,⁷⁶ where three different modified dU^{RTP} s bearing hydroxyl, carboxyl, or primary amino groups were included in selection experiments to identify amide-hydrolyzing DNAzymes. While unmodified deoxyribozymes for hydrolyzing esters and aromatic amides have been identified,⁷⁷ achieving hydrolysis of aliphatic amides using canonical nucleic acids was yet not impossible. Therefore, functionalizing DNA with protein-like groups provided a feasible solution.

1.6. Hypermodified DNA

As discussed in Section 1.5, nucleotides bearing protein-like functionalities have evoked particular interest due to their possible application in *in vitro* selections. The introduction of

cationic, to a lesser extend anionic, modifications has contributed to the improvement and fine-tuning of DNAzymes properties. Moreover, heavily modified deoxyribozymes, bearing three types of additional functional groups, demonstrated better activity than those with two modifications. In the case of aptamers, it has also been shown that the introduction of amino-acid-like modifications is beneficial for interaction with target proteins.^{78,79} In addition, the use of two modifications was demonstrated to be more effective in selection experiments.⁸⁰

Given that each modification brings an additional function and influences the secondary structure of DNA, the studies of DNA modified at all four nucleobases (further referred to as *hypermodified DNA*) would significantly contribute to the field of DNA functionalization (Figure 13). Previously, there were only a few reports by Famulok group describing hypermodified DNA,^{21,52,68} where various hydrophobic and polar modifications were studied. Our group has explored this direction in greater detail. The studies include enzymatic synthesis of hypermodified DNA with four different hydrophobic arylalkynyl or alkylalkynyl substituents,³⁷ which allowed for expanding chemical diversity of DNA with protein-like groups; monosaccharides⁸¹ for mimicking carbohydrates in lectin-carbohydrate interactions; carboranes³⁸ for redox labeling. Despite the progress in the field, a comprehensive understanding of DNA's functional limits remains incomplete. Studies of hypermodified DNA bearing charged modifications would fill the gap in the scope and limitations of DNA functionalization and open new possibilities for *in vitro* selection experiments.

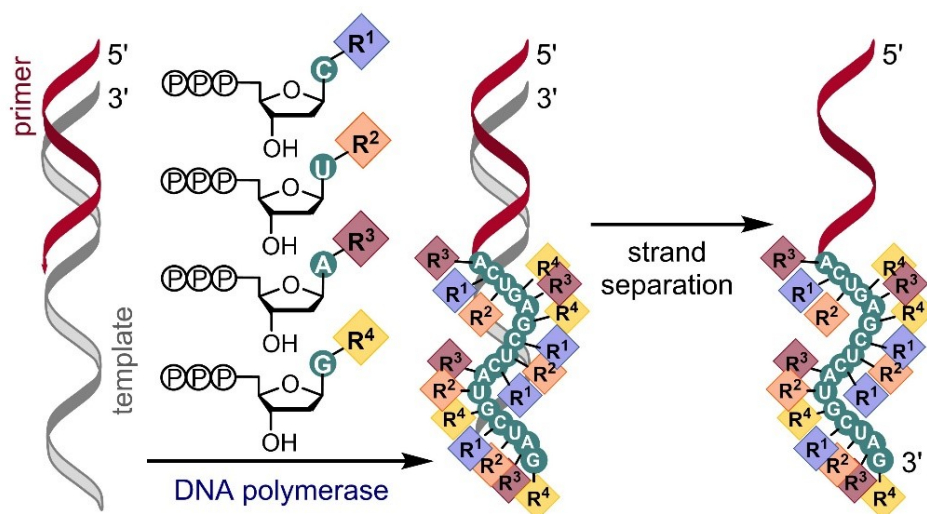


Figure 13. The concept of hypermodified DNA.

2. SPECIFIC AIMS OF THE WORK

1. Design and synthesis of four base-modified dN^RTPs bearing anionic functional groups followed by their use in enzymatic synthesis of hypermodified DNA
2. Design and synthesis of four base-modified dN^RTPs bearing cationic functional groups followed by their use in enzymatic synthesis of hypermodified DNA
3. Enzymatic synthesis of hypermodified DNA displaying both hydrophobic and charged moieties
4. Analysis of biophysical properties of hypermodified DNA duplexes

2.1. Rationale of the specific aims

Unlike proteins, DNA does not exhibit a wide range of functional groups. Introducing modifications into DNA allows for new opportunities for chemical and structural diversity. Aiming to push the limits of DNA functionalization, our group has extensively studied the concept of hypermodified DNA, where each nucleotide carries a different modification. Particular interest was drawn to the work describing hypermodified DNA with hydrophobic protein-like functional groups.³⁷ As a logical follow-up, we decided to study nucleotides with charged modifications as substrates for enzymatic synthesis of modified and hypermodified DNA and their effect on DNA structure.

First, a set of four modified dN^RTPs bearing acidic moieties was designed. Acidic functionalities were represented by carboxylate, sulfonate, phosphonate, and phosphate groups, forming corresponding monoanions under neutral conditions. The functional groups were planned to be introduced by Sonogashira cross-coupling using dN^ITPs and functionalized acetylenes. Once synthesized, the set of dN^RTPs was to be tested as substrates in enzymatic DNA synthesis, replacing one, two, three, or all four canonical nucleotides with their anionically modified analogs. The ultimate goal was to enzymatically synthesize hypermodified DNA bearing all four acidic modifications.

Analogous to anionically modified nucleotides, a set of four dN^RTPs with cationic modifications was planned to be synthesized by Sonogashira cross-coupling. A range of amino derivatives was chosen as modifications, including protonated amino, methylamino, dimethylamino, and trimethylammonium groups. To explore the possible density of cationic modifications on DNA, enzymatic synthesis was to be performed using one, two, three, and, finally, all four cationic dN^RTPs. The main challenge was to create hypermodified DNA displaying a full range of the cationic groups.

For prospective applications in selection of functional nucleic acids (NA), the ability to obtain DNA with various types of modifications is advantageous. For this reason, a useful experiment would be enzymatic synthesis of hypermodified DNA with previously reported³⁷ hydrophobic

nucleotides and charged dN^RTPs of study. Being able to generate such DNA opens up possibilities for fine-tuning functional NA polymers.

Undoubtedly, such unusually highly modified molecules would raise questions about their structure and properties. How does the high density of charged groups affect DNA duplex stability and conformation? This question was addressed using a combination of methods including hybridization studies, confocal microscopy imaging, CD spectroscopy, and molecular dynamics. Additionally, to study the impact of the modifications on DNA resistance to nuclease degradation, digestion experiments were designed with DNase I or human pooled plasma and different types of hypermodified DNA.

The concept of hypermodified DNA broadens our understanding of the world of functional NA. Considering the fact that charged DNA modifications (especially anionic ones) are understudied, the aim of this thesis is to fill the gaps in this area by synthesizing a number of dN^RTPs bearing anionic and cationic groups and thoroughly testing them as substrates in enzymatic DNA synthesis.

3. RESULTS AND DISCUSSION

3.1. Design and synthesis of dN^RTPs bearing anionic functional groups followed by their application in enzymatic DNA synthesis

To increase chemical diversity of modified DNA for further potential application in *in vitro* selection, a set of modified dN^RTPs bearing anionic modifications was designed. The range of functionalities was represented by four acidic groups, namely carboxylate, sulfonate, phosphonate, and phosphate. Then, the anionically modified dN^RTPs were to be studied as substrates in enzymatic DNA synthesis with the final goal of synthesizing hypermodified DNA.

3.1.1. Synthesis of anionically modified dN^RTPs

Four terminal alkynes (**1a-d**) with acidic moieties were designed to be introduced to the C5 position of pyrimidines and the C7 position of 7-deazapurines by their direct Sonogashira cross-coupling on dN^ITPs (Figure 14). Prior to that, dN^ITPs were synthesized from corresponding dN^I by triphosphorylation reaction according to published procedures.^{20,82,83,84}

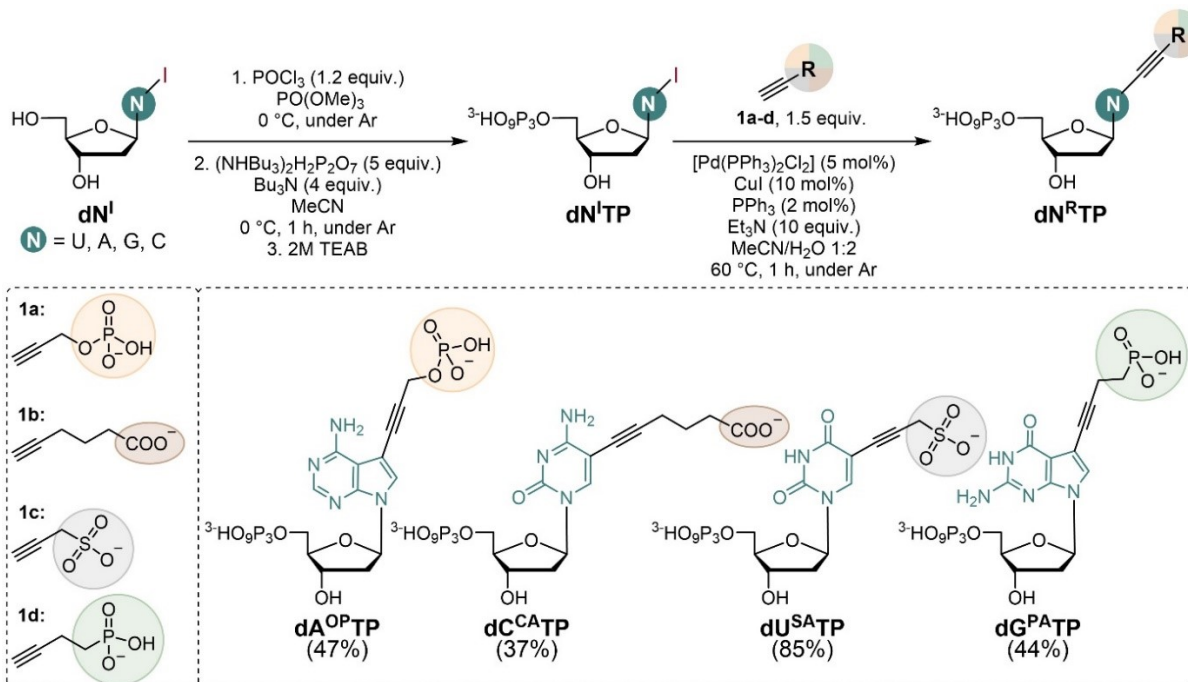


Figure 14. Two-step synthesis of anionically modified dN^RTPs; yields are shown for the cross-coupling step.

The Sonogashira cross-coupling was performed under argon in the presence of [Pd(PPh₃)₂Cl₂] catalyst, CuI, Et₃N, and PPh₃ in acetonitrile/water for 1 h at 60 °C. These conditions were chosen as they had previously been demonstrated to be effective for direct Sonogashira coupling on dN^ITPs.^{27,38} Luckily, the applied conditions proved suitable for obtaining all anionically modified dN^RTPs. The products were purified by HPLC and isolated in moderate to good yields (37–85%). Compared to the previously reported synthesis of modified dN^RTPs bearing carboxylate group,⁵² the direct coupling approach was more straightforward and gave a higher product yield.

3.1.2. Enzymatic incorporation of anionically modified dN^RTPs into DNA

As a first step, all four modified nucleotides dU^{SA}TP, dC^{CA}TP, dA^{OP}TP, and dG^{PA}TP were separately tested as substrates in PEX reactions for two DNA polymerases – Vent(exo–) and KOD XL. The experiments were performed using 15-mer primer Prim^{248short}-FAM, bearing 6-FAM fluorophore at 5'-end, and 19-mer templates Temp^{Oligo1N} (N = T, A, G, C) (Table 1), encoding for one modified nucleotide of choice followed by three natural nucleotides – either dGTP or dTTP, depending on the template (Figure 15). All reactions were carried out at 60 °C for 30 min and terminated by cooling on ice. The product sequences **19DNA_N^R** are specified in Table 2.

Table 1. The primer and templates used in PEX reactions in Sections 3.1.2 and 3.2.2.

ON name	Length (nt)	Sequence (5' → 3')
Prim ^{248short} -FAM ^a	15	CATGGGCGGCATGGG
Temp ^{Oligo1A}	19	CCCT <u>CCC</u> ATGCCG <u>CCC</u> ATG
Temp ^{Oligo1T}	19	CCC <u>ACC</u> ATGCCG <u>CCC</u> ATG
Temp ^{Oligo1C}	19	CCCG <u>CCC</u> ATGCCG <u>CCC</u> ATG
Temp ^{Oligo1G}	19	AAAC <u>CCC</u> ATGCCG <u>CCC</u> ATG
Temp ^{Prb4basII}	31	CTAGCATGAGCTCAGT <u>CCC</u> ATGCCG <u>CCC</u> ATG

Note: 5'-dual-biotinylated versions of the templates were used for magnetoseparation; in the template ONs the segments forming duplex with the primer are underlined; ^a 5'-(6-FAM)-labeled.

Table 2. The list of synthesized dsDNA and ssONs with one anionically modified **dN^RTP**.

Name	Sequence (5'→3')
19DNA_G ^{PA} ^a	<u>CATGGGCGGCATGGG</u> G ^{PA} TTT
19ON_G ^{PA}	<u>CATGGGCGGCATGGG</u> G ^{PA} TTT
19DNA_A ^{OP} ^a	<u>CATGGGCGGCATGGG</u> A ^{OP} GGG
19ON_A ^{OP}	<u>CATGGGCGGCATGGG</u> A ^{OP} GGG
19DNA_U ^{SA} ^a	<u>CATGGGCGGCATGGG</u> U ^{SA} GGG
19ON_U ^{SA}	<u>CATGGGCGGCATGGG</u> U ^{SA} GGG
19DNA_C ^{CA} ^a	<u>CATGGGCGGCATGGG</u> C ^{CA} GGG
19ON_C ^{CA}	<u>CATGGGCGGCATGGG</u> C ^{CA} GGG
31DNA_G ^{PA} ^a	<u>CATGGGCGGCATGGG</u> ACTG ^{PA} AG ^{PA} CTCATG ^{PA} CTAG ^{PA}
31ON_G ^{PA}	<u>CATGGGCGGCATGGG</u> ACTG ^{PA} AG ^{PA} CTCATG ^{PA} CTAG ^{PA}
31DNA_A ^{OP} ^a	<u>CATGGGCGGCATGGG</u> A ^{OP} CTGA ^{OP} GCTCA ^{OP} TGCTA ^{OP} G
31ON_A ^{OP}	<u>CATGGGCGGCATGGG</u> A ^{OP} CTGA ^{OP} GCTCA ^{OP} TGCTA ^{OP} G
31DNA_U ^{SA} ^a	<u>CATGGGCGGCATGGG</u> ACU ^{SA} GAGCU ^{SA} CAU ^{SA} GCU ^{SA} AG
31ON_U ^{SA}	<u>CATGGGCGGCATGGG</u> ACU ^{SA} GAGCU ^{SA} CAU ^{SA} GCU ^{SA} AG
31DNA_C ^{CA} ^a	<u>CATGGGCGGCATGGG</u> AC ^{CA} TGAGC ^{CA} TC ^{CA} ATGC ^{CA} TAG
31ON_C ^{CA}	<u>CATGGGCGGCATGGG</u> AC ^{CA} TGAGC ^{CA} TC ^{CA} ATGC ^{CA} TAG

Note: primer regions are underlined; DNA – a double-stranded modified PEX product (a complementary strand is an unmodified template); ON – a single-stranded modified product; ^a 5'-(6-FAM)-labeled.

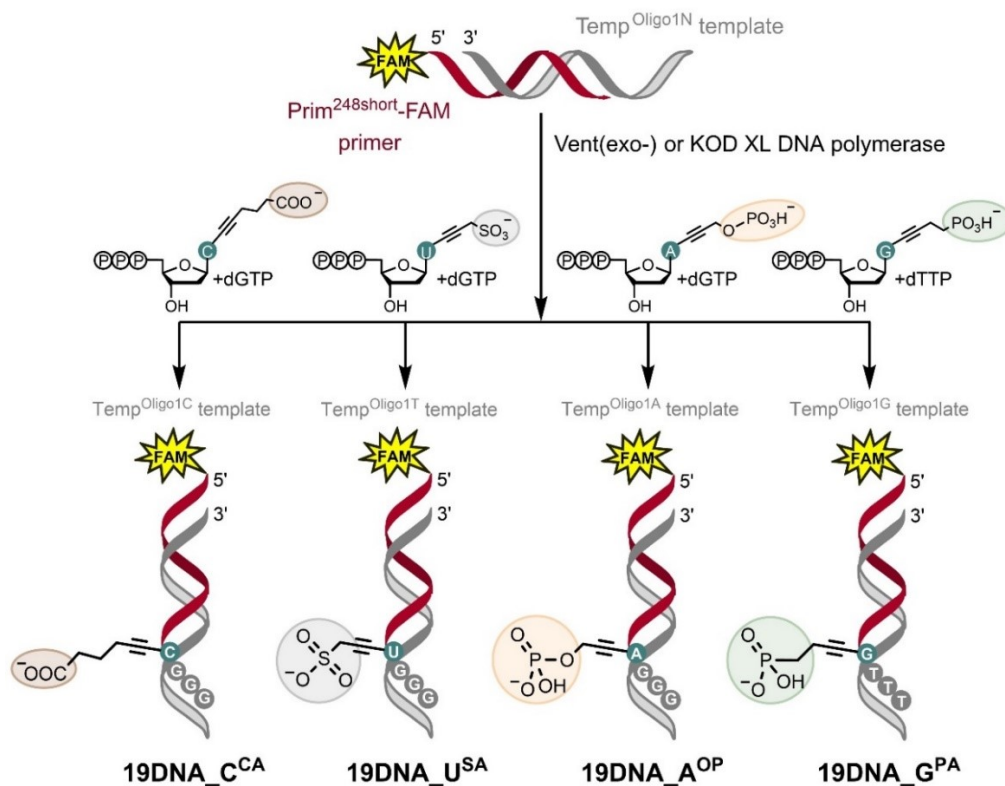


Figure 15. Enzymatic synthesis of 19-mer DNA duplexes (**19DNA_N^R**) containing a single anionic modification.

KOD XL DNA polymerase (a mixture of KOD polymerase from *Thermococcus kodakaraensis* and its exonuclease-deficient mutant)⁸⁵ was chosen as it has previously been reported to efficiently incorporate high density of modifications.^{37,38} Vent(exo–) DNA polymerase was selected as an alternative, more affordable option without exonuclease activity. Although Vent(exo–) DNA polymerase is usually less active than KOD XL, the required amounts of both polymerases was similar in this case (Table 3).

Table 3. Reaction specifications in PEX for single incorporation of anionically modified dN^RTPs.

Template	KOD XL	Vent(exo–)	dN ^R TP
Temp ^{Oligo1T}	0.1 U	0.1 U	dU ^{SA} TP
Temp ^{Oligo1A}		0.1 U	dA ^{OP} TP
Temp ^{Oligo1G}		0.2 U	dG ^{PA} TP
Temp ^{Oligo1C}		0.2 U	dC ^{CA} TP

To qualitatively analyze the results, vertical denaturing polyacrylamide gel electrophoresis (dPAGE) was employed, a technique that separates charged biomolecules based on their size. 5'-(6-FAM)-labeled primer and positive control (using natural dNTPs instead of modified ones) were used as size references, while negative controls (reactions without dN^RTPs) were performed to ensure correct enzymatic behavior. Once the reactions were stopped, formamide-containing PAGE stop solution was added, and the double-stranded PEX products (**19DNA_N^R**) were denatured at 95 °C for 3 min. The samples were further analyzed by dPAGE, which was visualized using fluorescent imaging (Figure 16). The migration character of the modified strands did not significantly differ from positive control, which was expected, as the modified products contained only a single modification without a significant change in mass, thus confirming the proper full-length **19DNA_N^R** products.

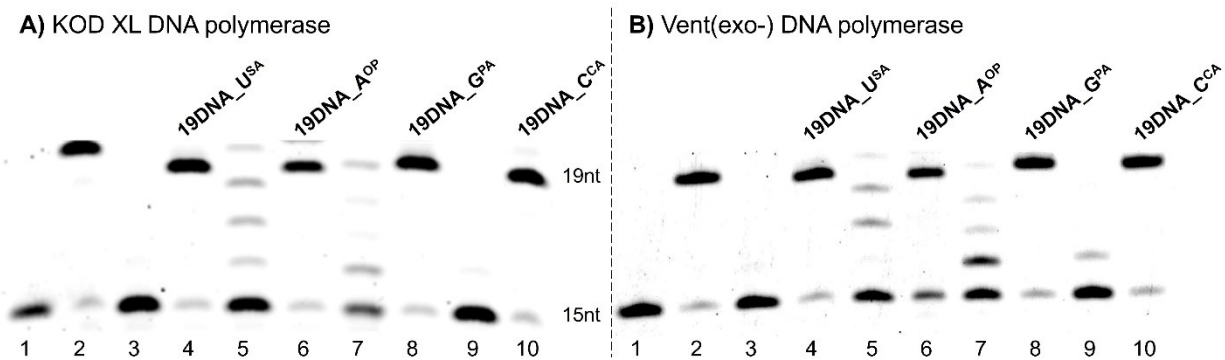


Figure 16. Denaturing PAGE analysis of PEX reactions with one anionically modified **dN^RTP** using templates Temp^{Oligo1T}, Temp^{Oligo1A}, Temp^{Oligo1G}, or Temp^{Oligo1C} and: A) KOD XL DNA polymerase, B) Vent(exo-) DNA polymerase: (lane 1) primer Prim^{248short}-FAM; (lane 2) positive control with all natural dNTPs; PEX reactions with **dU^{SA}TP** (lane 4), **dA^{OP}TP** (lane 6), **dG^{PA}TP** (lane 8), **dC^{CA}TP** (lane 10); (lanes 3, 5, 7, and 9) negative controls missing natural or modified dNTP of interest.

In the next step, each anionically modified **dN^RTP** was tested individually for multiple incorporations into DNA using the same fluorescent primer Prim^{248short}-FAM, 31-mer template Temp^{Prb4basII} (Table 1) encoding for each nucleotide four times, three remaining natural dNTPs, and either Vent(exo-) or KOD XL DNA polymerase (Figure 17). The reactions were carried out at 60 °C for 30 min and terminated by cooling on ice. The product sequences **31DNA_N^R** are specified in Table 2.

Unlike **19DNA_N^R** synthesis, where was no significant difference in DNA polymerase amount (Table 3), the longer modified **31DNA_N^R** required more diverse polymerase conditions (Table 4). While all **dN^RTPs** were well incorporated using the same KOD XL concentration, they demonstrated different substrate activities with Vent(exo-). According to the results, the smallest amount of Vent(exo-) DNA polymerase was required for **dG^{PA}TP**, whereas the largest amount was needed for **dU^{SA}TP**. Overall, Vent(exo-) appeared to be less active than KOD XL, yet still efficient for multiple incorporation of anionically modified **dN^RTPs** of study. The reactions were analyzed by dPAGE and visualized using fluorescence imaging (Figure 18). In this case, the electrophoretic mobility of the modified PEX products differed from both each other and the positive control, which can be explained by the increased number of modifications and the molecular mass of the modified strands.

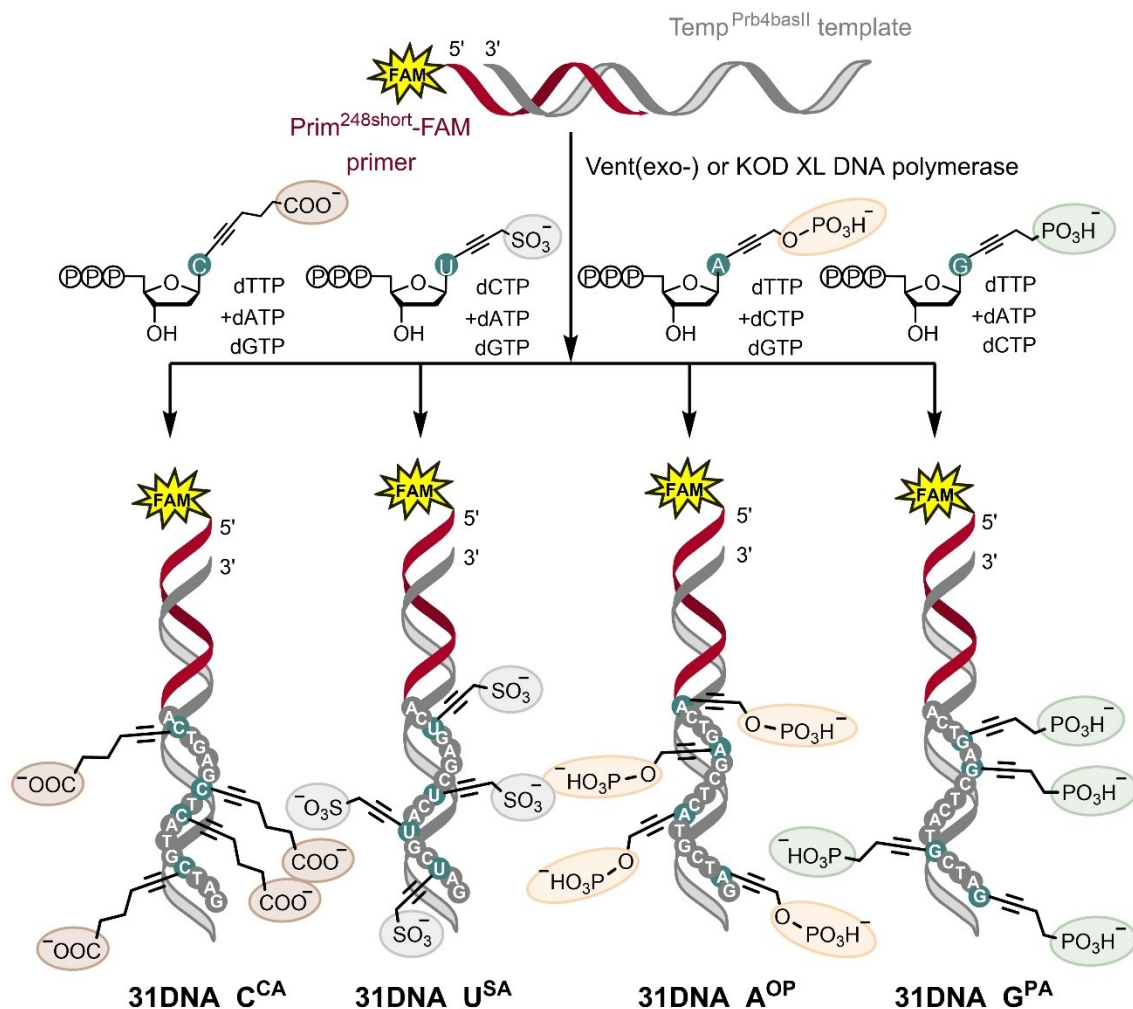


Figure 17. Enzymatic synthesis of 31-mer DNA duplexes (31DNA_N^R) containing four anionic modifications.

Table 4. Reaction specifications in PEX for multiple nucleotide incorporation of anionically modified dN^RTPs.

dN ^R TP	KOD XL	Vent(exo-)
dU ^{SA} TP	0.3 U	1.2 U
dA ^{OP} TP		0.5 U
dG ^{PA} TP		0.4 U
dC ^{CA} TP		1 U

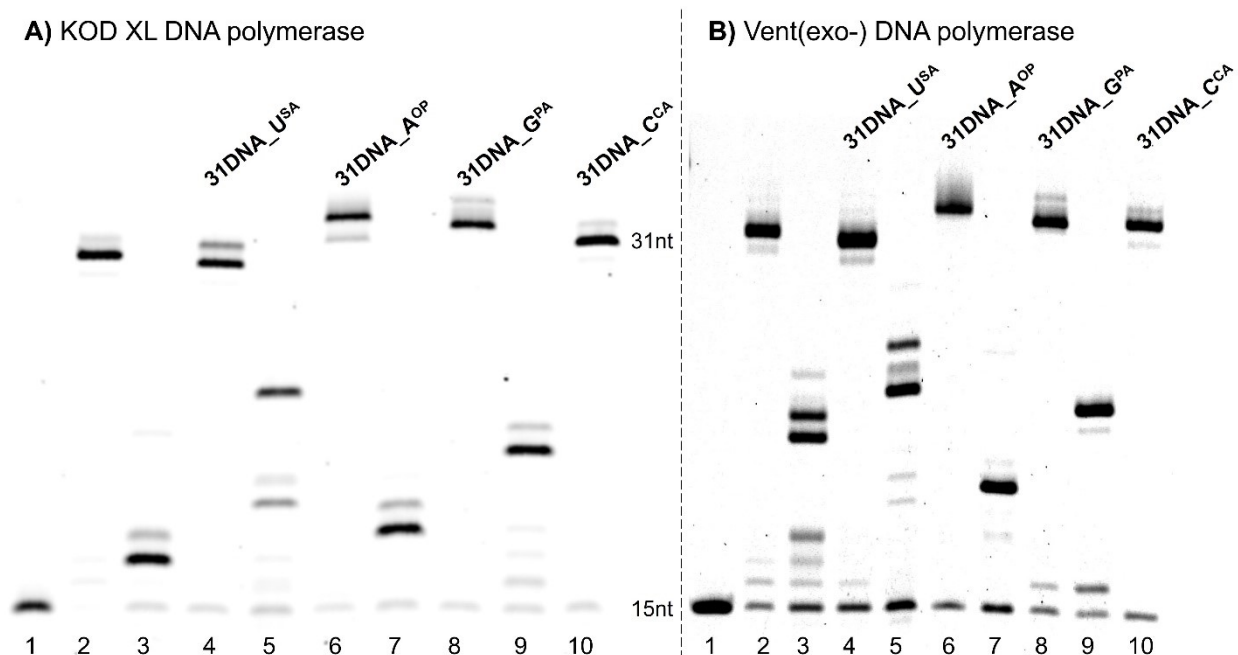


Figure 18. Denaturing PAGE analysis of PEX reactions with one anionically modified **dN^RTP** using template Temp^{Prb4basII} and: A) KOD XL DNA polymerase, B) Vent(exo-) DNA polymerase: (lane 1) primer Prim^{248short}-FAM; (lane 2) positive control with all natural dNTPs; PEX reactions with **dU^{SA}TP** (lane 4), **dA^{OP}TP** (lane 6), **dG^{PA}TP** (lane 8), **dC^{CA}TP** (lane 10); (lanes 3, 5, 7, and 9) negative controls missing natural or modified dNTP of interest.

In order to characterize modified products by mass spectrometry, **19DNA_{N^R}** and **31DNA_{N^R}** were obtained with 5'-biotinylated templates and unlabeled primers. Further, the templates were separated from the modified strands by magnetoseparation (Figure 19). PEX reactions were performed in a larger scale (for the procedure see Section 5.2.7 in ES) and stopped by cooling on ice, followed by mixing with streptavidin-coated magnetic particles and incubating to allow the duplexes to attach to the beads. Then, the double-stranded products were purified from DNA polymerase, non-consumed **dN^RTPs**, dNTPs, and salts. After washing step, modified ssONs were released into water by thermal denaturation, while the beads with attached templates remained on the magnet. Purified **19ON_{N^R}** and **31ON_{N^R}** (Table 2) were subjected to MALDI-TOF analysis, which confirmed successful incorporation of modified **dN^RTPs** and formation of full-length products (Table 32 in ES).

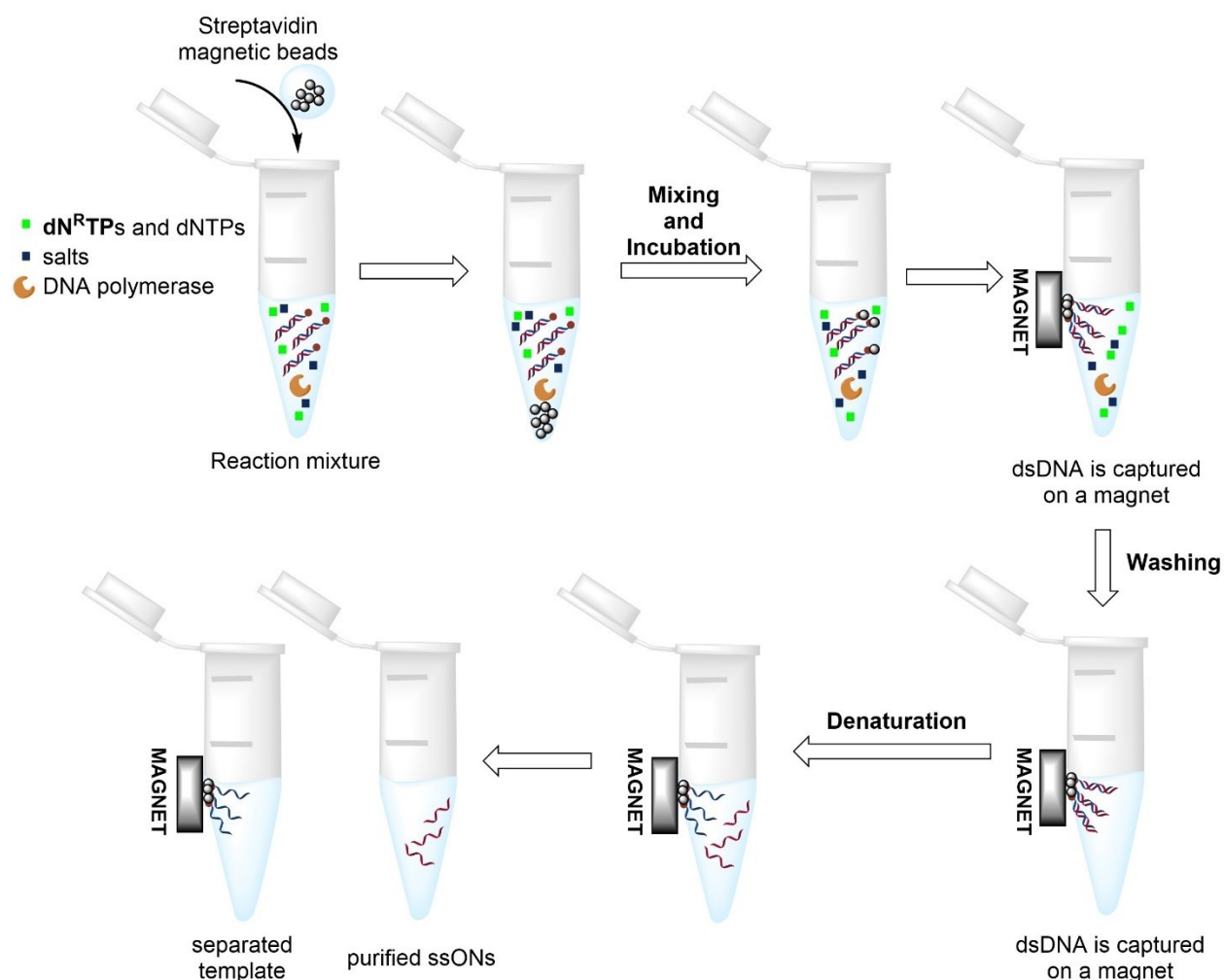


Figure 19. The scheme of magnetoseparation procedure.

3.1.3. Enzymatic synthesis of superanionic DNA by PEX

After demonstrating that anionically modified **dN^RTPs** are good substrates for enzymatic DNA synthesis individually, their various combinations were further tested in PEX. Since both Vent(exo–) and KOD XL DNA polymerases efficiently accept the modified nucleotides, Vent(exo–) was chosen to continue with. Using fluorescent primer Prim^{248short}-FAM and 31-mer template Temp^{Prb4basII} (Table 5), different combinations of two and three **dN^RTPs** (complemented with the remaining natural dNTPs) were applied in PEX (for reaction specifications see Method C1 and Table 28 in Section 5.2.4 in ES). In all cases the formation of full-length products was observed (Figure 20; sequences are specified in Table 6).

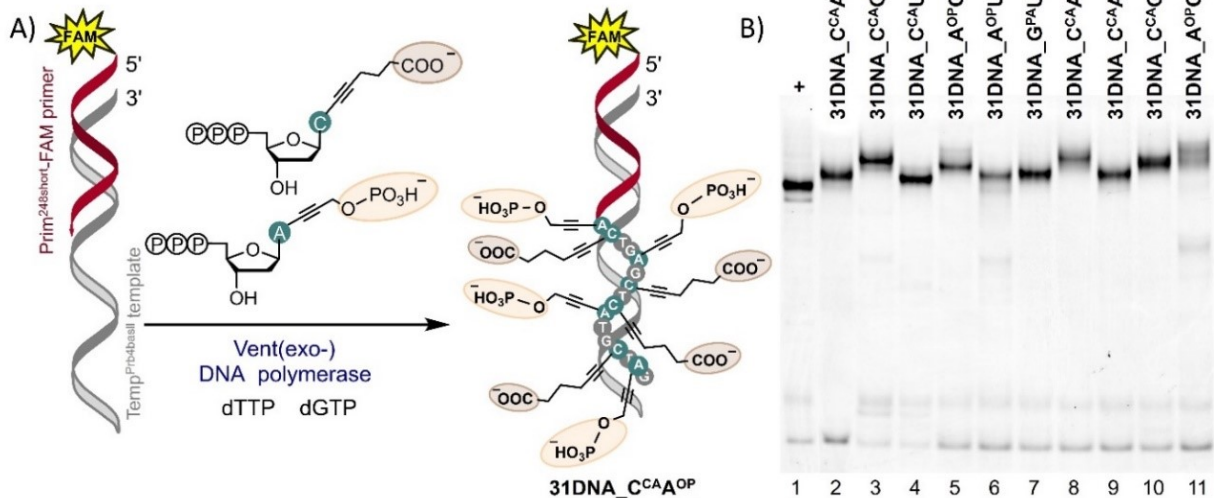


Figure 20. A) An example of PEX reaction with a combination of anionically modified dN^{RTP} s (in this case $\text{dA}^{\text{OP}}\text{TP}$ and $\text{dC}^{\text{CA}}\text{TP}$); B) Denaturing PAGE analysis of PEX reactions with 31-mer template $\text{Temp}^{\text{Prb4basII}}$ and combinations of dN^{RTP} s: (lanes 2-7) two dN^{RTP} s; (lanes 8-11) three dN^{RTP} s. Lane 1 is positive control with all natural dNTPs.

Once the combinations of two and three modified dN^{RTP} s were tested in PEX, all natural nucleotides were replaced with modified analogs, which resulted in hypermodified $31\text{DNA_CCA}^{\text{GPA}}\text{U}^{\text{SA}}\text{A}^{\text{OP}}$ (Table 7), bearing all four anionic modifications (Figure 21A). This DNA was named ‘*superanionic*’ due to its significantly increased number of negative charges compared to natural DNA. For further characterization, superanionic $31\text{DNA_CCA}^{\text{GPA}}\text{U}^{\text{SA}}\text{A}^{\text{OP}}$, obtained with non-labeled primer, was separated from the template according to the procedure described in Section 3.1.2, yielding the single-stranded $31\text{ON_CCA}^{\text{GPA}}\text{U}^{\text{SA}}\text{A}^{\text{OP}}$, which was subsequently analyzed by MALDI-TOF (Table 32 and Figure 54 in ES). Further, a number of templates of different lengths (43, 61, 98, 120, and 150-mers) (Table 5) was designed to determine whether the set of anionically modified dN^{RTP} s is suitable for enzymatic synthesis of longer hypermodified DNA (Figure 21B). The amount of Vent(exo-) DNA polymerase in all cases remained the same as in PEX reactions with a shorter 31-mer $\text{Temp}^{\text{Prb4basII}}$ template, however, the reaction time was prolonged from 30 to 40 min. As shown in Figure 21B, the electrophoretic mobility of superanionic PEX products significantly differed from that of non-modified DNA of the same size, likely due to their increased mass. The smears observed in some of the PEX product bands may result from secondary structures or higher aggregate formation that cannot be resolved under the conditions of standard dPAGE.

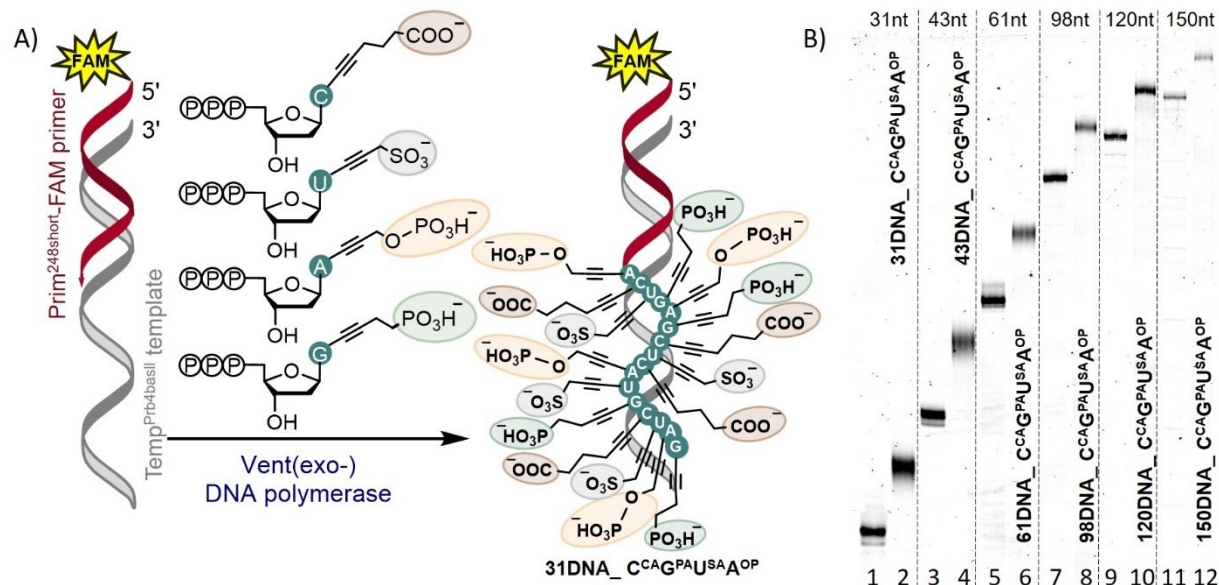


Figure 21. A) PEX reaction using all four anionically modified **dN^RTPs**; B) Denaturing PAGE analysis of PEX with the set of four modified **dN^RTPs** and 31-mer (lane 2), 43-mer (lane 4), 61-mer (lane 6), 98-mer (lane 8), 120-mer (lane 10), and 150-mer (lane 12) templates; lanes 1, 3, 5, 7, 9, and 11: positive controls with all natural dNTPs.

Table 5. The primer and templates used in PEX reactions in Section 3.1.3.

ON name	Length (nt)	Sequence (5' → 3')
Prim ^{248short} -FAM ^a	15	CATGGGCGGCATGGG
Prim ^{LT25TH} -FAM ^a	25	CAAGGACAAAATACCTGTATTCCTT
Temp ^{Prb4basII} ^b	31	CTAGCATGAGCTCAGTCCCATGCCGCCCATG
Temp ^{MO43}	43	CATGAGCTCAGTCTAGCATGAGCTCAGTCCCATGCCGCCCATG
Temp ^{MO61}	61	GACATCATGAGAGACATCGCCTAGCATGAGCTAGCATGAGCTCAGTCCCATGCCGCCCATG
Temp ^{FVL-A}	98	GACATCATGAGAGACATCGCCTCTGGGCTAATAGGACTACTTCTAATCTGTAAGAGCAGATCCCTGGACAGGCAAGGAATACAGGTATTTTGTCTTG
Temp ^{MO120}	120	GACATCATGAGAGACATCGCCTAGCATGAGCTCAGTCTAGCATGAGCTCAGTCTAGCATGAGCTCAGTCTAGCATGAGCTCAGTCTAGCATGAGCTCAGTCTAGCATGAGCTCA
Temp ^{MO150}	150	GTCTAGCATGAAGGAATACAGGTATTTTGTCTTG

Note: in the template ONs the segments forming duplex with the primer are underlined; ^a 5'-(6-FAM)-labeled; ^b 5'-dual-biotinylated version of the template was used for magnetoseparation.

Name	Sequence (5' → 3')
31DNA_C^{CA}A^{OP}	<u>CATGGGCGGCATGGGA</u> ^{OP} C ^{CA} TGA ^{OP} GC ^{CA} TC ^{CA} A ^{OP} TGC ^{CA} TAA ^{OP} G
31DNA_C^{CA}G^{PA}	<u>CATGGGCGGCATGGGAC</u> ^{CA} TG ^{PA} AG ^{PA} C ^{CA} TC ^{CA} ATG ^{PA} C ^{CA} TAG ^{PA}
31DNA_C^{CA}U^{SA}	<u>CATGGGCGGCATGGGAC</u> ^{CA} U ^{SA} GAGC ^{CA} U ^{SA} C ^{CA} AU ^{SA} GC ^{CA} U ^{SA} AG
31DNA_A^{OP}G^{PA}	<u>CATGGGCGGCATGGGA</u> ^{OP} CTG ^{PA} A ^{OP} G ^{PA} CTCA ^{OP} TG ^{PA} CTA ^{OP} G ^{PA}
31DNA_A^{OP}U^{SA}	<u>CATGGGCGGCATGGGA</u> ^{OP} CU ^{SA} GA ^{OP} GCU ^{SA} CA ^{OP} U ^{SA} AGCU ^{SA} A ^{OP} G
31DNA_G^{PA}U^{SA}	<u>CATGGGCGGCATGGGACU</u> ^{SA} G ^{PA} AG ^{PA} CU ^{SA} CAU ^{SA} G ^{PA} CU ^{SA} AG ^{PA}
31DNA_C^{CA}A^{OP}G^{PA}	<u>CATGGGCGGCATGGGA</u> ^{OP} C ^{CA} TG ^{PA} A ^{OP} G ^{PA} C ^{CA} TC ^{CA} A ^{OP} TG ^{PA} C ^{CA} TAA ^{OP} G ^{PA}
31DNA_C^{CA}A^{OP}U^{SA}	<u>CATGGGCGGCATGGGA</u> ^{OP} C ^{CA} U ^{SA} GA ^{OP} GC ^{CA} U ^{SA} C ^{CA} A ^{OP} U ^{SA} GC ^{CA} U ^{SA} A ^{OP} G
31DNA_C^{CA}G^{PA}U^{SA}	<u>CATGGGCGGCATGGGAC</u> ^{CA} U ^{SA} G ^{PA} AG ^{PA} C ^{CA} U ^{SA} C ^{CA} AU ^{SA} G ^{PA} C ^{CA} U ^{SA} AG ^{PA}
31DNA_A^{OP}G^{PA}U^{SA}	<u>CATGGGCGGCATGGGA</u> ^{OP} CU ^{SA} G ^{PA} A ^{OP} G ^{PA} CU ^{SA} CA ^{OP} U ^{SA} G ^{PA} CU ^{SA} A ^{OP} G ^{PA}

Table 7. The list of synthesized superanionic dsDNA and ssONs.

Name	Sequence ($5' \rightarrow 3'$)
31DNA_C^{CA}G^{PA}U^{SA}A^{OP a}	CATGGGCGGCATGGGA ^{OP C^{CA}U^{SA}G^{PA}A^{OP G^{PA}C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}}}}
31ON_C^{CA}G^{PA}U^{SA}A^{OP}	C ^{CA} U ^{SA} A ^{OP G^{PA}}
43DNA_C^{CA}G^{PA}U^{SA}A^{OP a}	CATGGGCGGCATGGGA ^{OP C^{CA}U^{SA}G^{PA}A^{OP G^{PA}C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}}}} C ^{CA} U ^{SA} A ^{OP G^{PA}A^{OP C^{CA}U^{SA}G^{PA}A^{OP G^{PA}C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}}}}}
61DNA_C^{CA}G^{PA}U^{SA}A^{OP a}	CATGGGCGGCATGGGA ^{OP C^{CA}U^{SA}G^{PA}A^{OP G^{PA}C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}}}} C ^{CA} U ^{SA} A ^{OP G^{PA}C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}C^{CA}U^{SA}A^{OP G^{PA}G^{PA}C^{CA}G^{PA}A^{OP U^{SA}}}} G^{PA}U^{SA}C^{CA}U^{SA}C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}A^{OP U^{SA}G^{PA}U^{SA}C^{CA}}}}
98DNA_C^{CA}G^{PA}U^{SA}A^{OP a}	CAAGGACAAAATACCTGTATTTCCTTG ^{PA C^{CA}C^{CA}U^{SA}G^{PA}U^{SA}C^{CA}C^{CA}} A ^{OP G^{PA}G^{PA}G^{PA}A^{OP U^{SA}C^{CA}U^{SA}G^{PA}C^{CA}U^{SA}C^{CA}U^{SA}U^{SA}A^{OP C^{CA}A^{OP G^{PA}A^{OP}}} U^{SA}U^{SA}A^{OP G^{PA}A^{OP A^{OP G^{PA}U^{SA}A^{OP G^{PA}U^{SA}C^{CA}C^{CA}U^{SA}A^{OP U^{SA}U^{SA}A^{OP G^{PA}}}} C^{CA}C^{CA}C^{CA}A^{OP G^{PA}A^{OP G^{PA}G^{PA}C^{CA}G^{PA}A^{OP U^{SA}G^{PA}U^{SA}C^{CA}U^{SA}C^{CA}U^{SA}C^{CA}}} A^{OP U^{SA}G^{PA}A^{OP U^{SA}G^{PA}U^{SA}C^{CA}}}}}}}}}
120DNA_C^{CA}G^{PA}U^{SA}A^{OP a}	CAAGGACAAAATACCTGTATTTCCTTG ^{PA C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}C^{CA}}} U ^{SA} A ^{OP G^{PA}A^{OP C^{CA}U^{SA}G^{PA}A^{OP G^{PA}C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}C^{CA}U^{SA}A^{OP G^{PA}}}} A^{OP C^{CA}U^{SA}G^{PA}A^{OP G^{PA}C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}C^{CA}U^{SA}A^{OP G^{PA}A^{OP C^{CA}U^{SA}}}} G^{PA}A^{OP G^{PA}C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}C^{CA}U^{SA}A^{OP G^{PA}A^{OP C^{CA}U^{SA}G^{PA}A^{OP G^{PA}}}} C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}C^{CA}U^{SA}A^{OP G^{PA}G^{PA}C^{CA}G^{PA}A^{OP U^{SA}G^{PA}U^{SA}C^{CA}U^{SA}}} C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}A^{OP U^{SA}G^{PA}U^{SA}C^{CA}}}}}}}}}}
150DNA_C^{CA}G^{PA}U^{SA}A^{OP a}	CAAGGACAAAATACCTGTATTTCCTTU ^{SA C^{CA}A^{OP U^{SA}G^{PA}C^{CA}U^{SA}A^{OP}}} G ^{PA} A ^{OP C^{CA}U^{SA}G^{PA}A^{OP G^{PA}C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}C^{CA}U^{SA}A^{OP G^{PA}A^{OP C^{CA}}}} U^{SA}G^{PA}A^{OP G^{PA}C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}C^{CA}U^{SA}A^{OP G^{PA}A^{OP C^{CA}U^{SA}G^{PA}A^{OP}}} G^{PA}C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}C^{CA}U^{SA}A^{OP G^{PA}A^{OP C^{CA}U^{SA}G^{PA}A^{OP G^{PA}C^{CA}U^{SA}}}} C^{CA}A^{OP U^{SA}G^{PA}C^{CA}U^{SA}A^{OP G^{PA}A^{OP C^{CA}U^{SA}G^{PA}A^{OP G^{PA}C^{CA}U^{SA}C^{CA}A^{OP U^{SA}}}} G^{PA}C^{CA}U^{SA}A^{OP G^{PA}A^{OP C^{CA}U^{SA}G^{PA}A^{OP G^{PA}C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}C^{CA}U^{SA}}}} A^{OP G^{PA}G^{PA}C^{CA}G^{PA}A^{OP U^{SA}G^{PA}U^{SA}C^{CA}U^{SA}C^{CA}U^{SA}C^{CA}A^{OP U^{SA}G^{PA}A^{OP U^{SA}}}} G^{PA}U^{SA}C^{CA}}}}}}}}}}

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3.1.4. Application of anionic dN^RTPs in PCR

PEX experiments confirmed that the modified anionic dN^RTPs are suitable substrates for DNA polymerases. For potential applications in *in vitro* selection of NA, it was necessary to investigate whether they can be used in PCR. As mentioned in Section 1.3, in PCR, DNA polymerase has to be capable of both incorporating modified nucleotides and reading the resulting modified DNA strand as a template. This is inherently a challenging task, further complicated by the fact that anionic modifications may interact with DNA polymerase. To carefully study the process of amplification on both primers, the reverse primer was labeled with 6-FAM, while the forward primer was labeled with Cy5. Each anionic dN^RTP has been studied in PCR reactions using Vent(exo–) and KOD XL DNA polymerases (Table 8) complemented with a single-stranded 98-mer template Temp^{FVL-A}, 5'-Cy5-labeled forward primer Prim^{L20}-Cy5, 5'-(6-FAM)-labeled reverse primer Prim^{LT25TH}-FAM, and the rest of natural dNTPs (Figure 22A, Table 9). After 30 cycles, PCR products were analyzed by gel electrophoresis using either FAM or Cy5 scan (Table 10, Figure 22B; for uncropped and unmodified gels, see Figure 57 in ES). As a result, all anionic dN^RTPs could be applied in PCR giving full-length amplicon products. Regarding the efficiency of the modified nucleotides as substrates, dC^{CA}TP and dG^{PA}TP have demonstrated high activity with both Vent(exo–) and KOD XL DNA polymerases, although the required amount of Vent(exo–) was twice that of KOD XL (Table 8). In contrast, dU^{SA}TP and dA^{OP}TP appeared to be less effective for Vent(exo–), even when large amounts of the polymerase were used. One of the possible explanations is that these nucleoside triphosphates partially inhibit Vent(exo–) DNA polymerase, since optimal conditions were reached by reducing the triphosphates' final concentration – from 0.4 mM to 0.2 mM of dU^{SA}TP and to 0.05 mM of dA^{OP}TP. Considering this, further PCR experiments involving dU^{SA}TP and dA^{OP}TP in combination with other dN^RTPs were performed using KOD XL DNA polymerase.

Table 8. Reaction condition specifications for PCR (one modified dN^RTP).

Lanes in Figure 22B	dN ^R TP (final concentration)	KOD XL	Vent(exo–)	PCR product
2	dU ^{SA} TP, 0.2 mM	2.5 U	5 U	98PCR_U ^{SA}
3	dA ^{OP} TP, 0.05 mM	1.25 U	5 U	98PCR_A ^{OP}
4	dG ^{PA} TP, 0.4 mM	1 U	2 U	98PCR_G ^{PA}
5	dC ^{CA} TP, 0.4 mM	0.5 U	1 U	98PCR_C ^{CA}

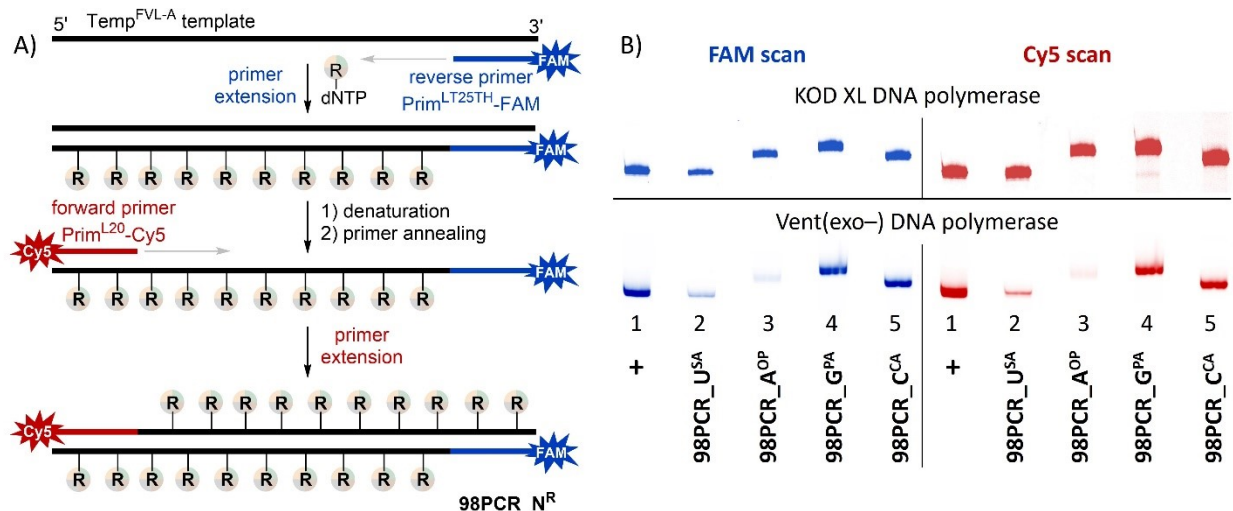


Figure 22. A) The scheme of reverse and forward primers extension in PCR; B) The part of denaturing PAGE displaying full-length PCR products obtained with one anionic **dN^RTP** using 5'-(6-FAM)-labeled reverse primer Prim^{LT25TH}-FAM, 5'-Cy5-labeled forward primer Prim^{L20}-Cy5, 98-mer template Temp^{FVL-A}, and either KOD XL or Vent(exo-) DNA polymerase: (lanes 1) positive control; (lanes 2-5) PCR using **dU^{SA}TP**, **dA^{OP}TP**, **dG^{PA}TP**, or **dC^{CA}TP** in combination with the rest of natural dNTPs.

Based on the results of PCR experiments with each anionically modified **dN^RTP**, a systematic study with different combinations of two, three, and all four **dN^RTPs** in PCR was performed. Only the combination of **dC^{CA}TP** and **dG^{PA}TP** was used with Vent(exo-) DNA polymerase, while KOD XL was applied for all other cases. Figure 23 shows visualization of the PCR products on dPAGE (for uncropped and unmodified gels, see Figure 59 in ES). The 5'-(6-FAM)-labeled products of reverse primer extension were obtained for all combinations, although in several cases the yield was low. Regarding 5'-(Cy5)-labeled forward primer extension, reactions with some **dN^RTP** combinations (Figure 23, lanes 9-12) did not lead to full-length product formation at all. Analyzing the gels, one can conclude that the worst results were observed in most experiments with **dU^{SA}TP**. This can be related to the inhibition of DNA polymerase by the sulfonic group⁸⁶ attached to the modified nucleoside triphosphate.

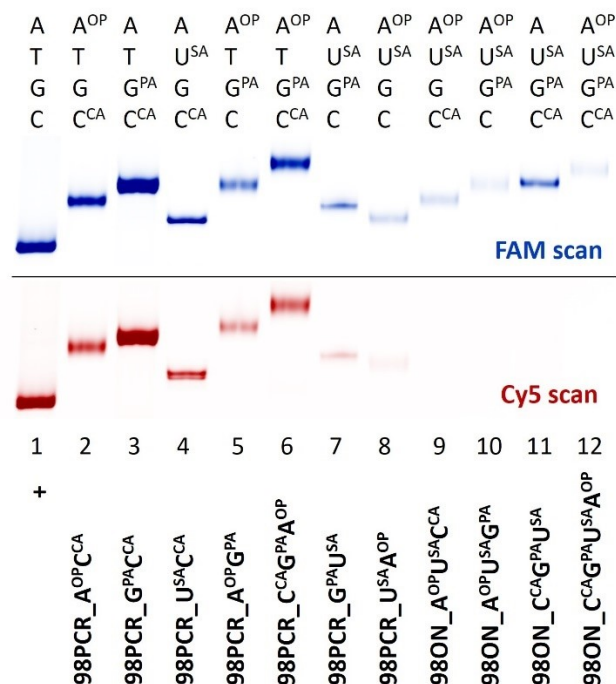


Figure 23. The part of denaturing PAGE displaying full-length PCR products obtained with combinations of anionic **dN^RTPs** (lanes 2-12), used **dN^RTPs** are specified above the gels. Lane 1 is positive control with all natural dNTPs.

Further optimizations of temperature and time of each PCR step, adjusting concentration of enzyme and used **dN^RTPs**, addition of additives (dimethyl sulfoxide (DMSO), formamide, MgSO₄, betaine) did not result in forward primer Prim^{L20}-Cy5 extension. Based on that, it was concluded that PCR with particular **dN^RTP** combinations (Figure 23, lanes 9-12) demonstrate rather asymmetric character giving single-stranded modified products of reverse primer Prim^{LT25TH}-FAM extension (for sequences see Table 10). To improve the yields of aPCR, the amount of template was increased, while forward primer was completely removed (Figure 24; for uncropped and unmodified gels, see Figure 62 in ES).

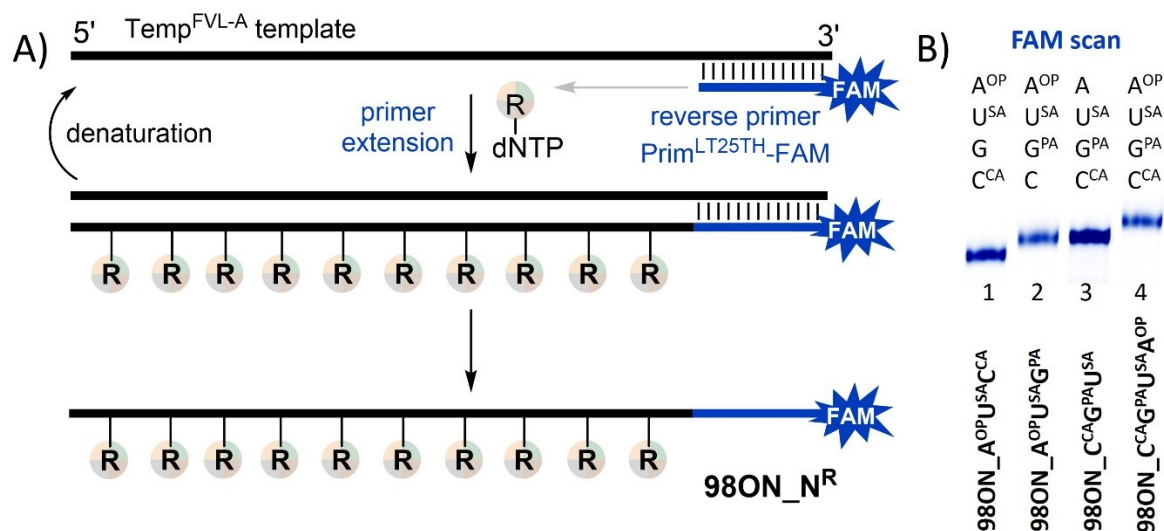


Figure 24. A) The scheme of reverse primer extension in aPCR; B) The part of denaturing PAGE displaying full-length aPCR products obtained with certain combinations of anionic dN^RTPs (lanes 1-4); used dN^RTPs are specified above the gel.

Table 9. Primers and the templates used in PCR experiments in Section 3.1.4

ON name	Length (nt)	Sequence (5'→ 3')
Prim ^{LT25TH} -FAM ^a	25	CAAGGACAAAATACCTGTATTCCTT
Prim ^{L20} -Cy5 ^b	20	GACATCATGAGAGACATCGC
Temp ^{FVL-A}	98	<u>GACATCATGAGAGACATCGCCTCTGGGCTAATAGGACT</u> <u>ACTTCTAATCTGTAAGAGCAGATCCCTGGACAGGCAAG</u> <u>GAATACAGGTATTTTGTCTTG</u>

Note: in the template the segments forming duplex with the primer are underlined;
^a 5'-(6-FAM)-labeled, ^b 5'-Cy5-labeled.

Table 10. The list of obtained modified PCR products.

Name	Sequence (5'→ 3')
98PCR_G ^{PA} ^{a,b}	<u>CAAGGACAAAATACCTGTATTCCTT</u> G ^{PA} CCTG ^{PA} TCCAG ^{PA} G ^{PA} G ^{PA} ATC TG ^{PA} CTCTTACAG ^{PA} ATTAG ^{PA} AAG ^{PA} TAG ^{PA} TCCTATTAG ^{PA} CCCAG ^{PA} A G ^{PA} G ^{PA} CG ^{PA} ATG ^{PA} TCTCTCATG ^{PA} ATG ^{PA} TC
98PCR_A ^{OP} ^{a,b}	<u>CAAGGACAAAATACCTGTATTCCTT</u> GCCTGTCCA ^{OP} GGGA ^{OP} TCTGCT CTTA ^{OP} CA ^{OP} GA ^{OP} TTA ^{OP} GA ^{OP} A ^{OP} GTA ^{OP} GTCCTA ^{OP} TTA ^{OP} GCCCA ^{OP} G A ^{OP} GGCGA ^{OP} TGTCTCTCA ^{OP} TGA ^{OP} TGTC
98PCR_U ^{SA} ^{a,b}	<u>CAAGGACAAAATACCTGTATTCCTT</u> GCCU ^{SA} GU ^{SA} ACCAGGGAU ^{SA} CU ^{SA} GCU ^{SA} CU ^{SA} U ^{SA} ACAGAU ^{SA} U ^{SA} AGAAGU ^{SA} AGU ^{SA} CCU ^{SA} AU ^{SA} U ^{SA} AGCCC AGAGGCGAU ^{SA} GU ^{SA} CU ^{SA} CU ^{SA} CAU ^{SA} GAU ^{SA} GU ^{SA} C
98PCR_C ^{CA} ^{a,b}	<u>CAAGGACAAAATACCTGTATTCCTT</u> GC ^{CA} C ^{CA} TGTC ^{CA} C ^{CA} AGGGAT C ^{CA} TGC ^{CA} TC ^{CA} TTAC ^{CA} AGATTAGAAGTAGTC ^{CA} C ^{CA} TATTAGC ^{CA} C ^{CA} C ^{CA} AGAGGC ^{CA} GATGTC ^{CA} TC ^{CA} TC ^{CA} ATGATGTC ^{CA}

98PCR_A ^{OP} CA ^{a,b}	<u>CAAGGACAAAATACCTGTATTCCTTGC</u> CA ^{CA} TCGTC ^{CA} CA ^{CA} A ^{OP} GGGA ^{OP} TC ^{CA} TGCA ^{TC} CA ^{TTA} OP ^{CA} A ^{OP} GA ^{OP} TTA ^{OP} GA ^{OP} A ^{OP} GTA ^{OP} GTC ^{CA} CCAT A ^{OP} TTA ^{OP} GC ^{CA} CA ^{CA} CA ^{OP} GA ^{OP} GGC ^{CA} GA ^{OP} TGTC ^{CA} TC ^{CA} CA ^{OP} TG A ^{OP} TGTC ^{CA}
98PCR_G ^{PA} CA ^{a,b}	<u>CAAGGACAAAATACCTGTATTCCTT</u> G ^{PA} CA ^{CA} TCG ^{PA} TC ^{CA} CA ^{CA} AG ^{PA} G ^{PA} G ^{PA} ATC ^{CA} TG ^{PA} CA ^{TC} CA ^{TTAC} CA ^{AG} PA ^{ATTAG} PA ^{AAG} PATAG ^{PA} TC ^{CA} C ^{CA} TATTAG ^{PA} CA ^{CA} CA ^{CA} AG ^{PA} AG ^{PA} G ^{PA} CA ^G PA ^{ATG} PA ^{TC} CA ^{TC} CA ^{CA} TG ^{PA} ATG ^{PA} TC ^{CA}
98PCR_USA ^{CA} CA ^{a,b}	<u>CAAGGACAAAATACCTGTATTCCTT</u> GC ^{CA} CA ^{CA} USA ^G USA ^{CA} CA ^{CA} AGGGA USA ^{CA} USA ^G CA ^{USA} CA ^{USA} USA ^{AC} AGA ^{USA} USA ^{AGA} AGU ^{SA} AGU ^{SA} CA ^{CA} C ^{CA} USA ^A USA ^{USA} AGC ^{CA} CA ^{CA} AGAGGC ^{CA} GAU ^{SA} GU ^{SA} CA ^{USA} CA ^{USA} CA ^{USA} CA ^{CA} AU ^{SA} GAU ^{SA} GU ^{SA} CA ^{CA}
98PCR_A ^{OP} G ^{PA} CA ^{a,b}	<u>CAAGGACAAAATACCTGTATTCCTT</u> G ^{PA} CCTG ^{PA} TC ^{CA} OP ^G PA ^G PA ^G PA ^{OP} A ^{OP} TCTG ^{PA} CTCTTA ^{OP} CA ^{OP} G ^{PA} A ^{OP} TTA ^{OP} G ^{PA} A ^{OP} OP ^G PA ^{TA} OP ^G PA ^{TC} CT A ^{OP} TTA ^{OP} G ^{PA} CCCA ^{OP} G ^{PA} A ^{OP} G ^{PA} G ^{PA} CG ^{PA} A ^{OP} TG ^{PA} TCTCTCA ^{OP} TG ^{PA} A ^{OP} TG ^{PA} TC
98PCR_USA ^A OP ^{a,b}	<u>CAAGGACAAAATACCTGTATTCCTT</u> GCCU ^{SA} GU ^{SA} CA ^{CA} OP ^G GGGA ^{OP} USAC USAGCU ^{SA} CU ^{SA} USA ^A OP ^{CA} OP ^{GA} OP ^{USA} USA ^A OP ^{GA} OP ^A OP ^{GU} SA ^A OP ^{GU} SA ^{CC} USAA ^{OP} USA ^{USA} A ^{OP} GCCCA ^{OP} GA ^{OP} GGCGA ^{OP} USA ^{GU} SA ^{CU} SA ^{CU} SA ^{CA} OP ^{USAG} A ^{OP} USAGU ^{SA} CA ^{CA}
98PCR_G ^{PA} USA ^{a,b}	<u>CAAGGACAAAATACCTGTATTCCTT</u> G ^{PA} CCU ^{SA} G ^{PA} USAC ^{CA} AG ^{PA} G ^{PA} G ^{PA} AU ^{SA} CU ^{SA} G ^{PA} CU ^{SA} CU ^{SA} USA ^{AC} AG ^{PA} AU ^{SA} USA ^{AG} PA ^{AAG} PA ^{USA} AG ^{PA} USAC ^{CA} USAAU ^{SA} USA ^{AG} PA ^{CCC} AG ^{PA} AG ^{PA} G ^{PA} CG ^{PA} AU ^{SA} G ^{PA} USA ^{CU} SA ^{CU} SA ^{CA} USA ^{CA} G ^{PA} AU ^{SA} G ^{PA} USAC ^{CA}
98PCR_C ^{CA} G ^{PA} A ^{OP} CA ^{a,b}	<u>CAAGGACAAAATACCTGTATTCCTT</u> G ^{PA} CA ^{CA} CA ^{CA} TCG ^{PA} TC ^{CA} CA ^{CA} A ^{OP} G ^{PA} G ^{PA} G ^{PA} A ^{OP} TC ^{CA} TG ^{PA} CA ^{TC} CA ^{TTA} OP ^{CA} A ^{OP} G ^{PA} A ^{OP} TTA ^{OP} G ^{PA} A ^{OP} A ^{OP} G ^{PA} TA ^{OP} G ^{PA} TC ^{CA} CA ^{CA} TA ^{OP} TTA ^{OP} G ^{PA} CA ^{CA} CA ^{CA} A ^{OP} G ^{PA} A ^{OP} G ^{PA} G ^{PA} CA ^G PA ^{OP} A ^{OP} TG ^{PA} TC ^{CA} TC ^{CA} TC ^{CA} A ^{OP} TG ^{PA} A ^{OP} TG ^{PA} TC ^{CA}
98ON_A ^{OP} USAC ^{CA} a	<u>CAAGGACAAAATACCTGTATTCCTT</u> GC ^{CA} CA ^{CA} USA ^G USA ^{CA} CA ^{CA} A ^{OP} GGG A ^{OP} USAC ^{CA} USA ^G CA ^{USA} CA ^{USA} USA ^A OP ^{CA} A ^{OP} GA ^{OP} USA ^{USA} A ^{OP} GA ^{OP} A ^{OP} G USA ^A OP ^{GU} SA ^{CA} CA ^{USA} A ^{OP} USA ^{USA} A ^{OP} GCA ^{CA} CA ^{CA} A ^{OP} GA ^{OP} GGC ^{CA} GA ^{OP} USAGU ^{SA} CA ^{USA} CA ^{USA} CA ^{CA} A ^{OP} USAGA ^{OP} USAGU ^{SA} CA ^{CA}
98ON_A ^{OP} USAG ^{PA} a	<u>CAAGGACAAAATACCTGTATTCCTT</u> G ^{PA} CCU ^{SA} G ^{PA} USAC ^{CA} OP ^G PA ^G PA ^{OP} G ^{PA} A ^{OP} USACU ^{SA} G ^{PA} CU ^{SA} CU ^{SA} USA ^A OP ^{CA} OP ^{GA} A ^{OP} USA ^{USA} A ^{OP} G ^{PA} A ^{OP} A ^{OP} G ^{PA} USA ^A OP ^G PA ^{USACCU} SA ^A OP ^{USA} USA ^A OP ^G PA ^{CCCA} OP ^G PA ^A OP ^G PA ^G PA ^{CG} PA ^{OP} A ^{OP} USAG ^{PA} USA ^{CU} SA ^{CU} SA ^{CA} OP ^{USAG} PA ^A OP ^{USAG} PA ^{USAC}
98ON_C ^{CA} G ^{PA} USA ^a	<u>CAAGGACAAAATACCTGTATTCCTT</u> G ^{PA} CA ^{CA} CA ^{CA} USA ^G PA ^{USA} CA ^{CA} CA ^{CA} A ^{OP} G ^{PA} G ^{PA} G ^{PA} AU ^{SA} CA ^{USA} G ^{PA} CA ^{USA} CA ^{USA} USA ^{AC} AG ^{PA} AU ^{SA} USA ^{AG} PA ^A AG ^{PA} USA ^{AG} PA ^{USACCA} USA ^{AU} SA ^{USA} AG ^{PA} CA ^{CA} CA ^{CA} AG ^{PA} AG ^{PA} G ^{PA} CA ^{CA} G ^{PA} AU ^{SA} G ^{PA} USAC ^{CA} USA ^{CA} USA ^{CA} AU ^{SA} G ^{PA} AU ^{SA} G ^{PA} USAC ^{CA}
98ON_C ^{CA} G ^{PA} USA ^A OP ^a	<u>CAAGGACAAAATACCTGTATTCCTT</u> G ^{PA} CA ^{CA} CA ^{CA} USA ^G PA ^{USA} CA ^{CA} CA ^{CA} A ^{OP} G ^{PA} G ^{PA} G ^{PA} PA ^{OP} USAC ^{CA} USA ^G PA ^{CA} USA ^{CA} USA ^{USA} A ^{OP} CA ^{OP} G ^{PA} A ^{OP} USAA ^{OP} A ^{OP} G ^{PA} A ^{OP} A ^{OP} G ^{PA} USA ^A OP ^G PA ^{USACCA} CA ^{USA} OP ^{USA} USA ^A OP ^G PA ^{CA} CA ^{CA} CA ^{CA} A ^{OP} G ^{PA} A ^{OP} G ^{PA} G ^{PA} CA ^G PA ^A OP ^{USAG} PA ^{USA} CA ^{USA} CA ^{USA} CA ^{CA} A ^{OP} USAG ^{PA} G ^{PA} A ^{OP} USAG ^{PA} USAC ^{CA}

Note: primer region is underlined; PCR – a double-stranded PCR product with a complementary strand modified in the same manner using modifications under study (primer regions are not modified), ON – a single-stranded product of aPCR; ^a 5'-(6-FAM)-labeled, ^b 5'-Cy5-labeled.

In conclusion, a set of four anionically modified **dN^RTPs** was synthesized by Sonogashira cross-coupling. All modified nucleotides were further applied in enzymatic DNA synthesis and were shown to be good substrates for both tested KOD XL and Vent(exo–) DNA polymerases in PEX, where it was possible to obtain DNA strands containing one, two, three or even all four anionically modified nucleotides with up to 125 anionic modifications in a row. In PCR, the exponential amplification through extension of both forward and reverse primer proceeded mostly with combinations of one or two modified nucleotides. For most combinations of three or four modified **dN^RTPs**, only linear amplification was observed. This suggests that the hypermodified superanionic ON serves as a poor template for enzymatic synthesis of another hypermodified strand, which can be explained by possible interactions between anionic modifications and DNA polymerase.

3.2. Design and synthesis of **dN^RTPs** bearing cationic functional groups followed by their application in enzymatic DNA synthesis

As discussed in Section 1.5, **dN^RTPs** modified with cationic functionalities (imidazole, primary amino, and guanidium groups) have previously been shown to significantly impact the process of RNA-cleaving DNAzymes selection. To further explore the potential application of cationically modified nucleotides in enzymatic DNA synthesis, a set of four **dN^RTPs** displaying primary, secondary and tertiary amino, as well as quaternary ammonium groups was designed. The primary amino group resembled a lysine side-chain, while the other cationic groups were represented by methylamino-, dimethylamino-, or trimethylammonium-propargyl moieties. Once synthesized, the cationically modified **dN^RTPs** were to be tested as substrates in enzymatic synthesis, with the ultimate goal of creating hypermodified DNA.

3.2.1. Synthesis of cationically modified **dN^RTPs**

Similarly to anionic **dN^RTPs**, four cationic groups were designed to be introduced at position 5 of pyrimidines or 7 of 7-deazapurines through an alkyne linker by Sonogashira cross-coupling. In the cases of **dA^{NH2}TP**, **dU^{NMe}TP**, and **dG^{NMe2}TP**, it was possible to perform coupling reactions of functionalized alkynes **2a-c** directly with **dN^ITPs** (Figure 25). The reactions were carried out under argon in presence of Pd(PPh₃)₄ catalyst, CuI, and Et₃N in anhydrous DMF for 5 h at room temperature. These conditions were chosen based on a previously published work,²¹ in which modified propargyl amine moieties were coupled to 5-iododeoxyuridine in the same manner. The modified **dN^RTPs** were purified by HPLC and isolated in moderate yields (27–55%).

However, attempts to obtain **dC^{NMe3}TP** by direct coupling of propargyltrimethylammonium iodide (**2d**) with **dC^ITP** were unsuccessful, even when the reaction time, catalyst amount, or the alkyne **2d** concentration were increased. Presumably, the method failed due to electron-poor nature of the alkyne. To avoid applying harsher conditions that could endanger the triphosphate group, the alkyne **2d** was planned to be attached to 5-iodo-2'-deoxycytidine (**dC^I**) by Sonogashira reaction, followed by triphosphorylation of modified nucleoside **dC^{NMe3}** (Figure 26). Unfortunately, the use of Pd(PPh₃)₄ catalyst was unsuccessful in obtaining **dC^{NMe3}**. After changing

the catalyst to $\text{Pd}(\text{OAc})_2$ and increasing the reaction temperature to $60\text{ }^\circ\text{C}$ for 2.5 h, it was possible to synthesize modified nucleoside dC^{NMe^3} , which was further purified by HPLC and isolated with 40% yield. In the next step, dC^{NMe^3} was converted into modified nucleoside triphosphate $\text{dC}^{\text{NMe}^3}\text{TP}$, which was further purified by HPLC and isolated with 6% yield.

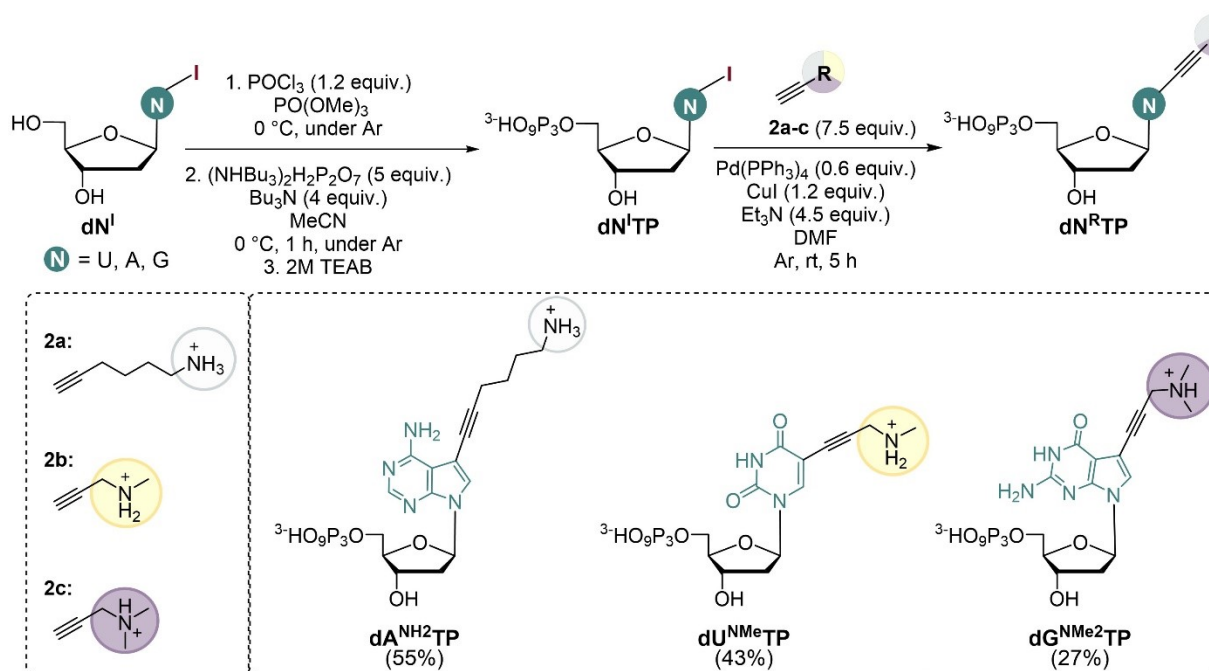


Figure 25. Two-step synthesis of cationically modified $\text{dA}^{\text{NH}_2}\text{TP}$, $\text{dU}^{\text{NMe}}\text{TP}$, and $\text{dG}^{\text{NMe}_2}\text{TP}$; yields are shown for the cross-coupling step.

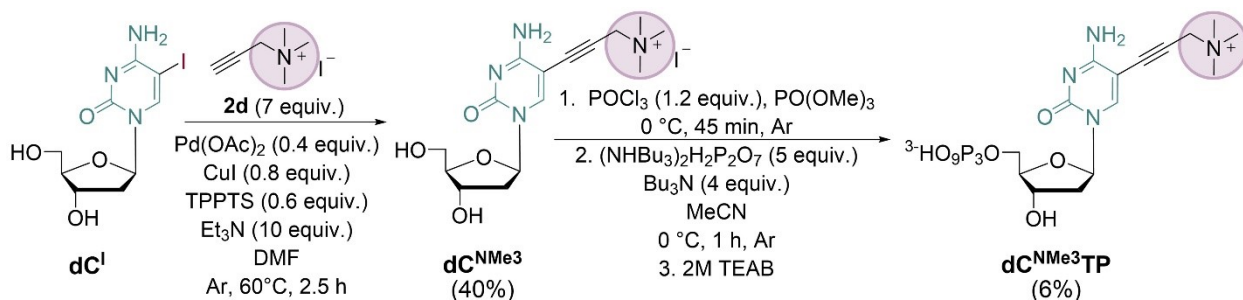


Figure 26. Two-step synthesis of cationically modified $\text{dC}^{\text{NMe}^3}\text{TP}$.

3.2.2. Enzymatic incorporation of cationically modified dN^RTPs into DNA

Once synthesized, the modified nucleoside triphosphates **dA^{NH2}TP**, **dU^{NMe}TP**, **dG^{NMe2}TP**, and **dC^{NMe3}TP** were studied as substrates for two DNA polymerases: Vent(exo–) and KOD XL. The experiments were designed similarly to those with anionic dN^RTPs. First, single incorporation by PEX was performed using 15-mer 5'-(6-FAM)-labeled primer Prim^{248short}-FAM and 19-mer templates Temp^{Oligo1N} (N = T, A, G, C) (Table 1 in Section 3.1.2), complemented with natural nucleotides – either dGTP or dTTP, depending on the template (Figure 27). The reactions were incubated for 30 min at 60 °C and stopped by cooling on ice. The product sequences **19DNA_N^R** are specified in Table 11.

Table 11. The list of 5'-(6-FAM)-labeled synthesized dsDNA and ssONs with one cationically modified dN^RTP.

Name	Sequence (5' → 3')
19DNA_G^{NMe2}	<u>CATGGGCGGCATGGG</u> G ^{NMe2} TTT
19DNA_A^{NH2}	<u>CATGGGCGGCATGGG</u> A ^{NH2} GGG
19DNA_U^{NMe}	<u>CATGGGCGGCATGGG</u> U ^{NMe} GGG
19DNA_C^{NMe3}	<u>CATGGGCGGCATGGG</u> C ^{NMe3} GGG
31DNA_G^{NMe2}	<u>CATGGGCGGCATGGG</u> ACTG ^{NMe2} AG ^{NMe2} CTCATG ^{NMe2} CTAG ^{NMe2}
31ON_G^{NMe2}	<u>CATGGGCGGCATGGG</u> ACTG ^{NMe2} AG ^{NMe2} CTCATG ^{NMe2} CTAG ^{NMe2}
31DNA_A^{NH2}	<u>CATGGGCGGCATGGG</u> A ^{NH2} CTGA ^{NH2} GCTCA ^{NH2} TGCTA ^{NH2} G
31ON_A^{NH2}	<u>CATGGGCGGCATGGG</u> A ^{NH2} CTGA ^{NH2} GCTCA ^{NH2} TGCTA ^{NH2} G
31DNA_U^{NMe}	<u>CATGGGCGGCATGGG</u> ACU ^{NMe} GAGCU ^{NMe} CAU ^{NMe} GCU ^{NMe} AG
31ON_U^{NMe}	<u>CATGGGCGGCATGGG</u> ACU ^{NMe} GAGCU ^{NMe} CAU ^{NMe} GCU ^{NMe} AG
31DNA_C^{NMe3}	<u>CATGGGCGGCATGGG</u> AC ^{NMe3} TGAGC ^{NMe3} TC ^{NMe3} ATGC ^{NMe3} TAG
31ON_C^{NMe3}	<u>CATGGGCGGCATGGG</u> AC ^{NMe3} TGAGC ^{NMe3} TC ^{NMe3} ATGC ^{NMe3} TAG

Note: primer regions are underlined; DNA – a double-stranded modified PEX product (a complementary strand is an unmodified template); ON – a single-stranded modified product.

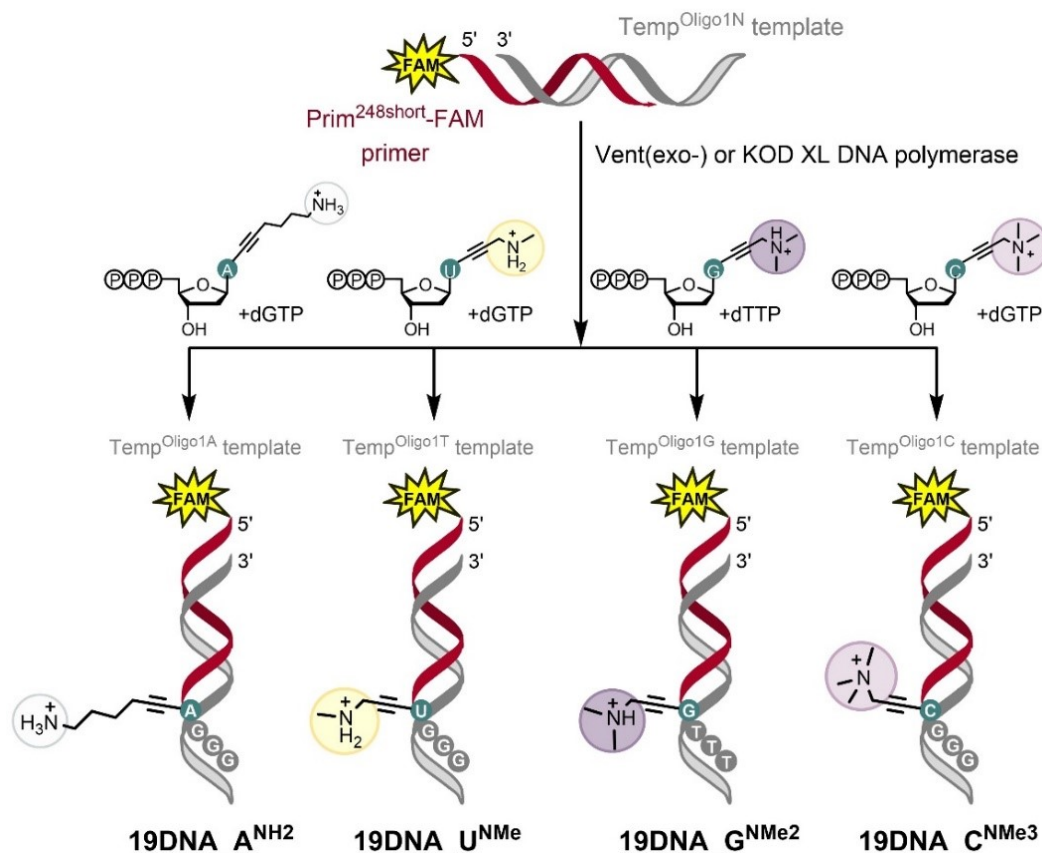


Figure 27. Enzymatic synthesis of 19-mer DNA duplexes (19DNA_N^R) containing a single cationic modification.

Unlike anionic **dN^RTPs**, for which the required amounts of the DNA polymerases for single nucleotide incorporation were approximately the same (Table 3 in Section 3.1.2), some cationically modified nucleoside triphosphates demonstrated higher substrate activity than others. **dA^{NH2}TP** and **dU^{NMe}TP** appeared to be better substrates for both KOD XL and Vent(exo-) DNA polymerases, requiring significantly lower amounts of polymerase than **dG^{NMe2}TP** and **dC^{NMe3}TP** (Table 12). This difference in activity can be attributed to the sterical features of the modifications – primary and secondary amino groups are less branched than tertiary amine and quaternary ammonium groups and might be more easily accepted by DNA polymerase. Although **dG^{NMe2}TP** and **dC^{NMe3}TP** were less active than **dA^{NH2}TP** and **dU^{NMe}TP**, their activity was still comparable to anionic **dN^RTPs**.

Table 12. Reaction specifications in PEX for single incorporation of cationically modified **dN^RTPs**.

Template	KOD XL	Vent(exo-)	dN ^R TP
Temp ^{Oligo1A}	0.01 U	0.05 U	dA^{NH2}TP
Temp ^{Oligo1T}	0.01 U	0.05 U	dU^{NMe}TP
Temp ^{Oligo1G}	0.1 U	0.2 U	dG^{NMe2}TP
Temp ^{Oligo1C}	0.1 U	0.2 U	dC^{NMe3}TP

PEX reactions were stopped by addition of formamide-containing PAGE stop solution, and denatured for 3 min at 95 °C before loading on dPAGE. The products were analyzed along with the positive control, displaying only natural nucleotides, and negative controls, in which the modified or natural nucleotides of study were missing (Figure 28). The migration behavior of the modified strands was similar to that of the positive control, except for **19DNA_A^{NH2}**, which migrated slightly slower, possibly because the less compact attached modification influenced its speed.

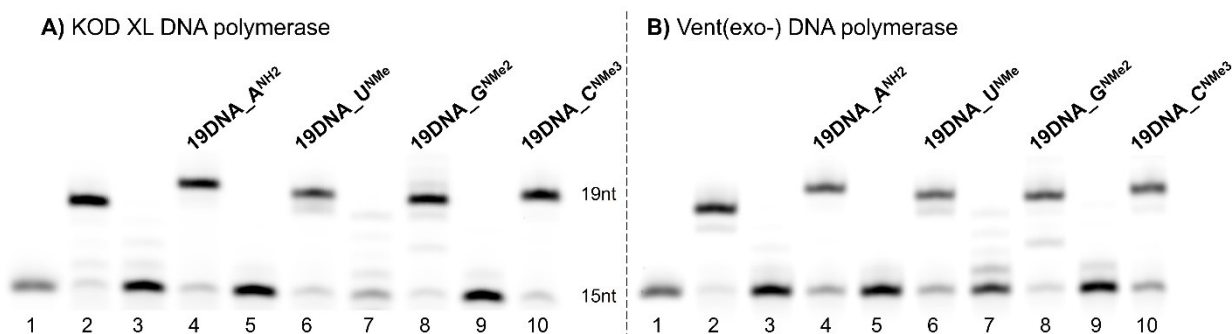


Figure 28. Denaturing PAGE analysis of PEX reactions with one cationically modified **dN^RTP** using templates Temp^{Oligo1A}, Temp^{Oligo1T}, Temp^{Oligo1G}, or Temp^{Oligo1C} and: A) KOD XL DNA polymerase, B) Vent(exo-) DNA polymerase: (lane 1) primer Prim^{248short}-FAM; (lane 2) positive control with all natural dNTPs; PEX reactions with **dA^{NH2}TP** (lane 4), **dU^{NMe}TP** (lane 6), **dG^{NMe2}TP** (lane 8), **dC^{NMe3}TP** (lane 10); (lanes 3, 5, 7, and 9) negative controls missing natural or modified dNTP of interest.

Further, multiple incorporation of each cationically modified nucleotide using a 31-mer template Temp^{Prb4basII} (Table 1 in Section 3.1.2) and either KOD XL or Vent(exo-) DNA polymerase was performed (Figure 29). The reactions were carried out at 60 °C for 30 min and terminated by cooling on ice. The product sequences **31DNA_N^R** are specified in Table 11.

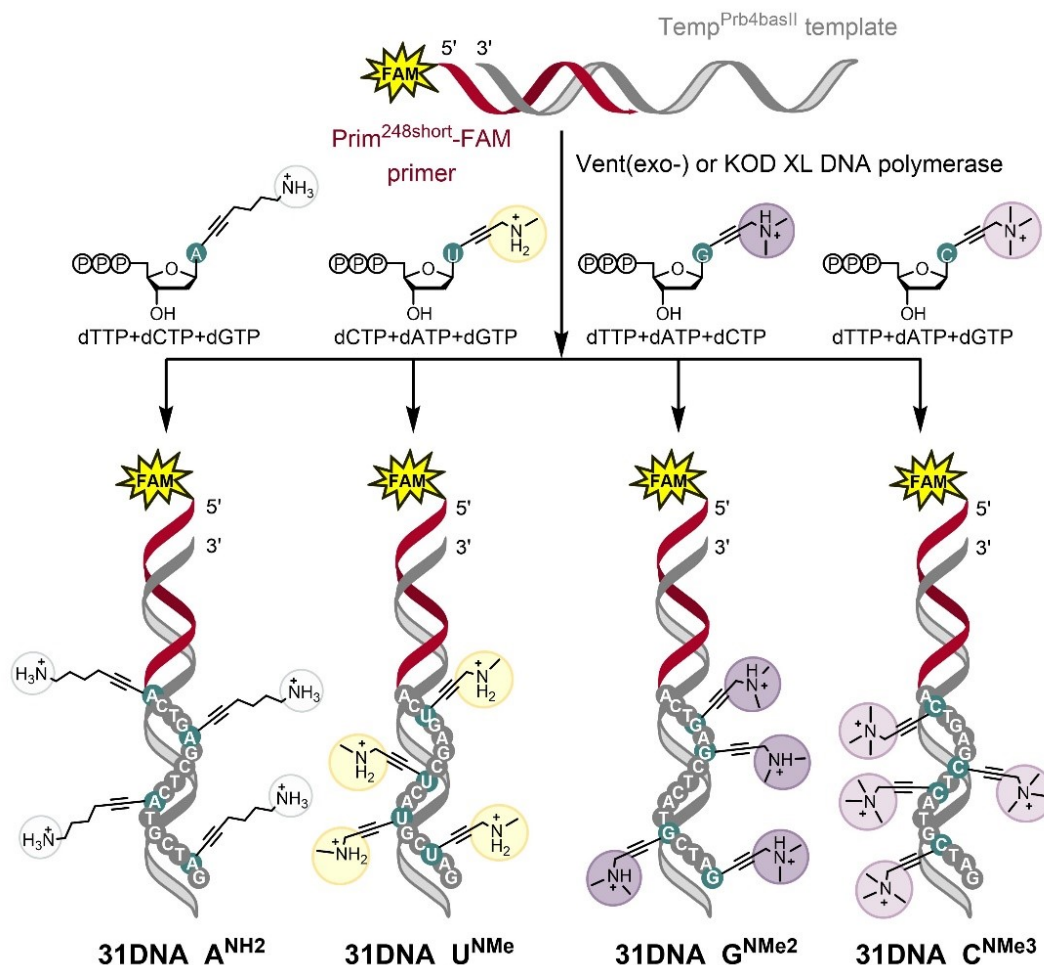


Figure 29. Enzymatic synthesis of 31-mer DNA duplexes (31DNA_N^R) containing four cationic modifications.

In the case of multiple nucleotide incorporation, **dA^{NH₂}TP** appeared to be the most active substrate for both KOD XL and Vent(exo–), requiring the same amount of polymerase as in the single nucleotide incorporation experiments (Table 13). In turn, **dU^{NMe}TP** was again shown to be more active than **dG^{NMe₂}TP** and **dC^{NMe₃}TP**, but less active than **dA^{NH₂}TP**. PEX reactions were analyzed by dPAGE and visualized using fluorescence imaging (Figure 30). The difference in mobility in this case is noticeable not only for **31DNA_A^{NH₂}** but for other modified PEX products as well. The pattern of the migration character seems to depend on the molecular mass of attached modifications – **31DNA_C^{NMe₃}** is heavier and moves slower than **31DNA_G^{NMe₂}**, which in turn moves slower than **31DNA_U^{NMe}**.

Table 13. Reaction condition specifications for multiple nucleotide incorporation of cationically modified **dN^RTPs**.

dN^RTP	KOD XL	Vent(exo-)
dA^{NH2}TP	0.01 U	0.05 U
dU^{NMe}TP	0.1 U	0.2 U
dG^{NMe2}TP	0.3 U	0.5 U
dC^{NMe3}TP	0.3 U	0.5 U

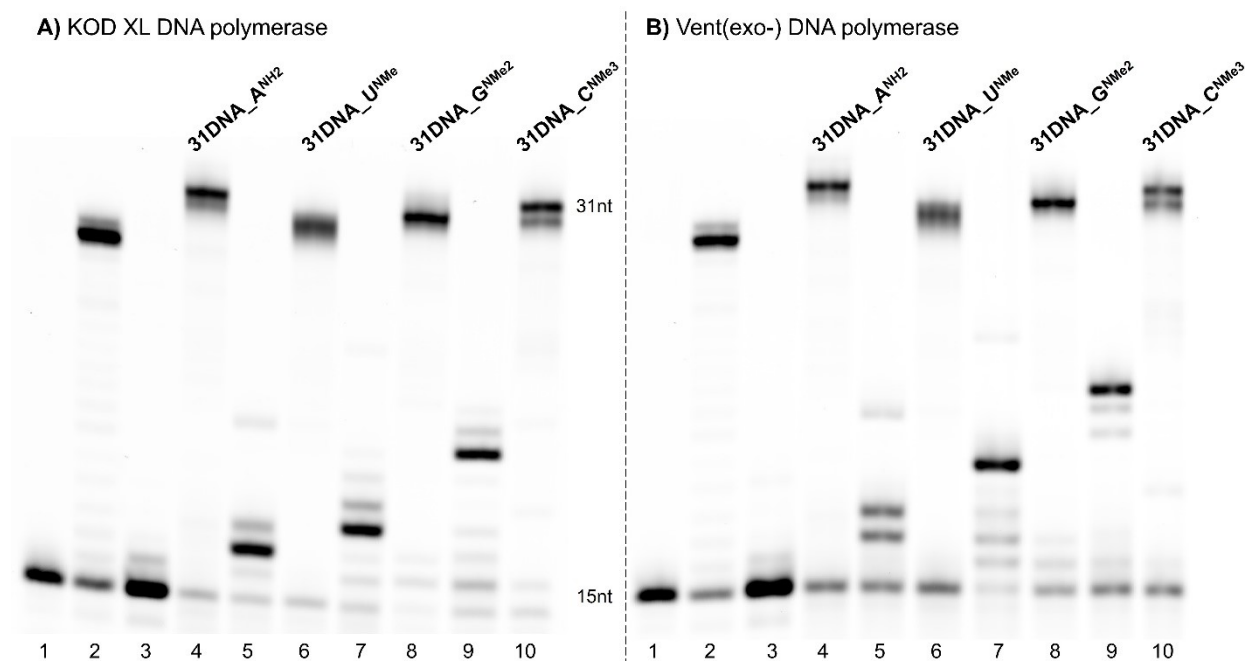


Figure 30. Denaturing PAGE analysis of PEX reactions with one cationic **dN^RTP** using template Temp^{Prb4basII} and: A) KOD XL DNA polymerase, B) Vent(exo-) DNA polymerase: (lane 1) primer Prim^{248short}-FAM; (lane 2) positive control with all natural dNTPs; PEX reactions with **dA^{NH2}TP** (lane 4), **dU^{NMe}TP** (lane 6), **dG^{NMe2}TP** (lane 8), **dC^{NMe3}TP** (lane 10); (lanes 3, 5, 7, and 9) negative controls missing natural or modified dNTP of interest.

Cationically modified **19DNA_{N^R}** were directly subjected to UPLC-MS analysis without a template separation step, whereas for **31DNA_{N^R}**, the templates were removed according to the procedure described in Section 3.1.2 and modified **31ON_{N^R}** (Table 11) were further characterized by UPLC-MS (Table 32 in ES). During the mass analysis of **31DNA_{C^{NMe3}}**, the residue of a different oligonucleotide was detected. Moreover, the formation of an additional faster-migrating band is visible in Figure 30 (lane 10). According to UPLC-MS results (Figure 53 in ES), this band can correspond to a product of one dTMP misincorporation instead of **dC^{NMe3}MP**. The template Temp^{Prb4basII} codes for CTC region (Table 1 in Section 3.1.2), which

requires the incorporation of two **dC^{NMe3}MP** molecules in close proximity to each other. Apparently, it is problematic for this modification, leading to partial dTMP misincorporation. This limitation of **dC^{NMe3}TP** as a substrate for DNA polymerase will be further discussed in the Section 3.2.3.

3.2.3. Enzymatic synthesis of zwitterionic DNA by PEX

After showing that each cationically modified **dN^RTP** is suitable for incorporation into DNA, their various combinations were further tested in PEX (Figure 31A). This time, KOD XL DNA polymerase was chosen to continue with. Using fluorescent primer Prim^{248short}-FAM and 31-mer template Temp^{Prb4basII} (Table 14), different combinations of two and three cationically modified **dN^RTPs** (complemented with the remaining natural dNTPs) were applied in PEX (for reaction specifications see Method C2 and Table 29 in Section 5.2.4 in ES). In all cases, the formation of full-length products was observed (Figure 31B; sequences are specified in Table 15). To maximize the product formation, the reaction mixtures were incubated at 72 °C for 30 min, instead of 60 °C as in all previous cases. This change in reaction conditions led to higher activity of KOD XL DNA polymerase, which partially digested the residual primer.

Next, PEX was performed with a full set of four cationically modified nucleotides, resulting in hypermodified **31DNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}** (Figure 32A, Table 16), which was characterized by MALDI-TOF analysis (Table 32 and Figure 56 in ES). This DNA was named ‘*zwitterionic*’ due to its unusual zwitterionic nature, where the positive charge of cationic modifications counterbalances the anionic phosphate DNA backbone. The dPAGE analysis (Figure 32B) demonstrated that the reaction proceeded even with longer templates (43, 61, and 98-mer; Table 14) giving zwitterionic hypermodified PEX products containing 16-73 modified nucleotides in a row (Table 16). The reaction conditions for most of the cases remained the same, except for the PEX reaction with 98-mer template Temp^{NK98}, which required a higher amount of DNA polymerase and MgSO₄ addition (for details see Section 5.2.5, Method D5 in ES).

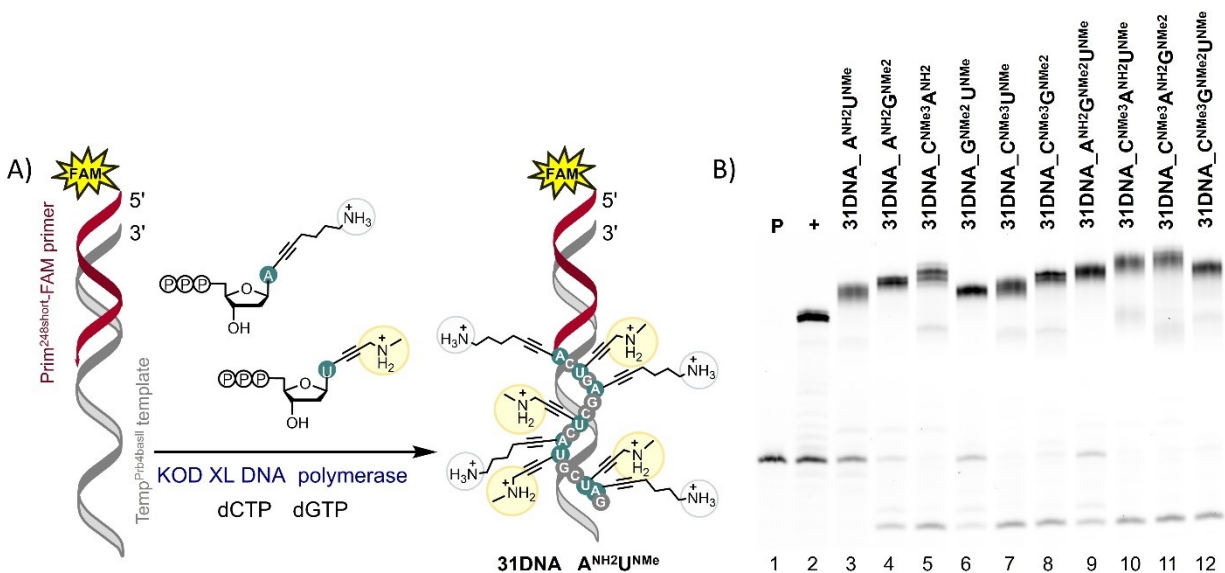


Figure 31. A) An example of PEX reaction with a combination of cationically modified **dN^RTTPs** (in this case **dA^{NH2}TP** and **dU^{NMe}TP**); B) Denaturing PAGE analysis of PEX reactions with 31-mer template Temp^{Prb4basII} and combinations of **dN^RTTPs**: (lanes 3-8) two **dN^RTTPs**; (lanes 9-12) three **dN^RTTPs**. Lane 1 is primer Prim^{248short}-FAM, lane 2 is positive control with all natural dNTPs.

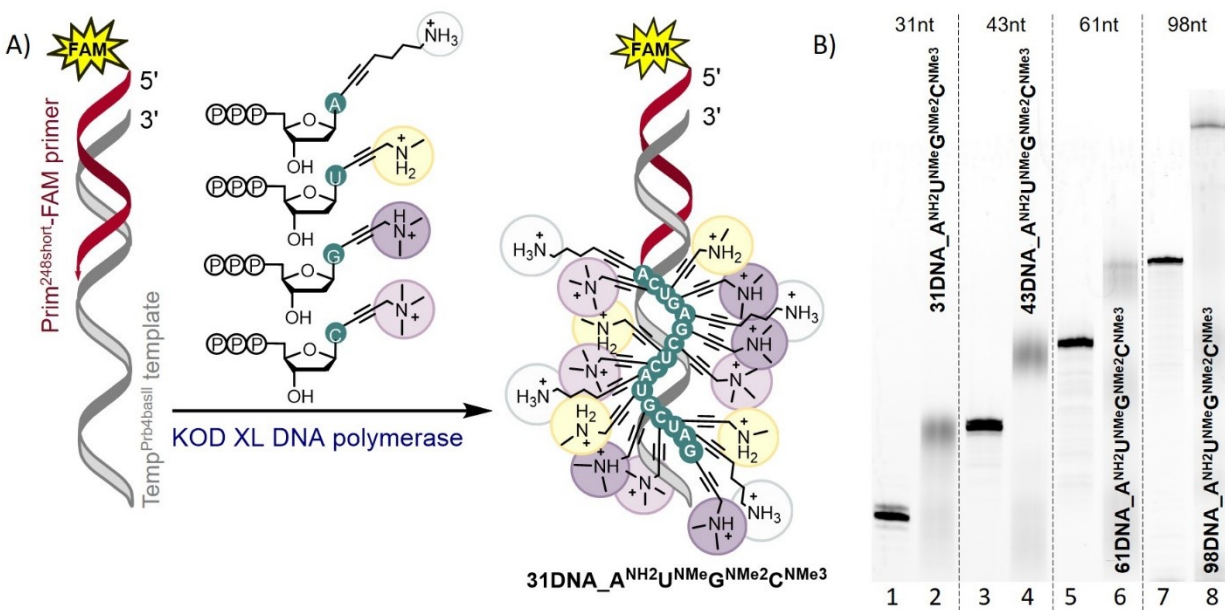


Figure 32. A) PEX reaction using all four cationically modified **dN^RTTPs**; B) Denaturing PAGE analysis of PEX with the set of four cationically modified **dN^RTTPs** and 31-mer template Temp^{Prb4basII} (lane 2), 43-mer template Temp^{MO43} (lane 4), 61-mer template Temp^{MO61} (lane 6), 98-mer template Temp^{NK98} (lane 8); lanes 1, 3, 5, and 7: positive controls with all natural dNTPs.

As seen in Figure 32B, the zwitterionic hypermodified PEX products migrate significantly slower in the electric field than the natural analogs of the same size. It is understandable given that zwitterionic DNA is overall less charged and has a higher mass. The smearing of the bands can be explained by secondary structures or higher aggregate formation that cannot be resolved under the conditions applied in standard denaturing PAGE electrophoretic analysis, even using buffer with a higher pH (for details see Figure 52 in ES).

Interestingly, PEX with previously used 98-mer template Temp^{FVL-A} did not proceed using the full set of cationically modified **dN^RTPs**. It appears that primer extension with **dC^{NMe3}TP** is sequence-dependent and terminates when a template encodes for two consecutive **dC^{NMe3}MP**. As it is shown in Figure 33, while other cationic **dN^RTPs** can be incorporated into DNA using Temp^{FVL-A} without any issues, PEX with **dC^{NMe3}TP** does not produce a full-length product (Figure 33, lane 6). Based on these observations, a new 98-mer template Temp^{NK98} was designed in a way to ensure that the resulting modified ON contains at least one nucleotide between **dC^{NMe3}MPs** (Table 14).

Table 14. Primers and templates used in PEX reactions with cationic **dN^RTPs**.

ON name	Length (nt)	Sequence (5' → 3')
Prim ^{248short} -FAM ^a	15	CATGGGCGGCATGGG
Prim ^{LT25TH} -FAM ^a	25	CAAGGACAAAATACCTGTATTCCTT
Prim ^{NK98pr1} -FAM ^a	25	CGATACGAACGTATGCTAGTTCTGT
Temp ^{Prb4basII} ^b	31	CTAGCATGAGCTCAGT <u>CCCATGCCGCCCATG</u>
Temp ^{MO43}	43	CATGAGCTCAGTCTAGCATGAGCTCAGT <u>CCCATGCCGCCCATG</u>
Temp ^{MO61}	61	GACATCATGAGAGACATCGCCTAGCATGAGCTAGCATGAGCTCAGT <u>CCCATGCCGCCCATG</u>
Temp ^{FVL-A}	98	GACATCATGAGAGACATCGCCTCTGGGCTAATAGGACTACTTCTAATCTGTAAGAGCAGATCCCTGGACAGGCAAGGAATACAGGTATTTGTCCTTG
Temp ^{NK98}	98	GCAATCAGTACGAACTGACTGTCTGACTGTCTAGCATGACTGTACTGTCTGTACGAACGATCAGTCTGTTTCAGACAGAACTAGCATACGTTTCGTATCG

Note: in the templates the segments forming duplex with the primer are underlined; ^a 5'-(6-FAM)-labeled; ^b 5'-dual-biotinylated version of the template was used for magnetoseparation.



Figure 33. Denaturing PAGE analysis of PEX reactions using template Temp^{FVL-A} and dA^{NH2}TP (lane 3), dU^{NMe}TP (lane 4), dG^{NMe2}TP (lane 5), and dC^{NMe3}TP (lane 6). Lane 1 is primer Prim^{LT25TH}-FAM, lane 2 is positive control with all natural dNTPs.

Table 15. The list of 5'-(6-FAM)-labeled modified dsDNA obtained by PEX with combination of two and three cationic dN^RTPs.

Name	Sequence (5' → 3')
31DNA_C ^{NMe3} A ^{NH2}	<u>CATGGGCGGCATGGGA</u> ^{NH2} C ^{NMe3} TGA ^{NH2} GC ^{NMe3} TC ^{NMe3} A ^{NH2} TGC ^{NMe3} T A ^{NH2} G
31DNA_C ^{NMe3} G ^{NMe2}	<u>CATGGGCGGCATGGGAC</u> ^{NMe3} TG ^{NMe2} AG ^{NMe2} C ^{NMe3} TC ^{NMe3} ATG ^{NMe2} C ^{NMe3} TAG ^{NMe2}
31DNA_C ^{NMe3} U ^{NMe}	<u>CATGGGCGGCATGGGAC</u> ^{NMe3} U ^{NMe} GAGC ^{NMe3} U ^{NMe} C ^{NMe3} AU ^{NMe} GC ^{NMe3} U ^{NMe} AG
31DNA_A ^{NH2} G ^{NMe2}	<u>CATGGGCGGCATGGGA</u> ^{NH2} CTG ^{NMe2} A ^{NH2} G ^{NMe2} CTCA ^{NH2} TG ^{NMe2} CTA ^{NH2} G ^{NMe2}
31DNA_A ^{NH2} U ^{NMe}	<u>CATGGGCGGCATGGGA</u> ^{NH2} CU ^{NMe} GA ^{NH2} GCU ^{NMe} CA ^{NH2} U ^{NMe} GCU ^{NMe} A ^{NH2} G
31DNA_G ^{NMe2} U ^{NMe}	<u>CATGGGCGGCATGGGACU</u> ^{NMe} G ^{NMe2} AG ^{NMe2} CU ^{NMe} CAU ^{NMe} G ^{NMe2} CU ^{NMe} AG ^{NMe2}
31DNA_C ^{NMe3} A ^{NH2} G ^{NMe2}	<u>CATGGGCGGCATGGGA</u> ^{NH2} C ^{NMe3} TG ^{NMe2} A ^{NH2} G ^{NMe2} C ^{NMe3} TC ^{NMe3} A ^{NH2} T G ^{NMe2} C ^{NMe3} TA ^{NH2} G ^{NMe2}
31DNA_C ^{NMe3} A ^{NH2} U ^{NMe}	<u>CATGGGCGGCATGGGA</u> ^{NH2} C ^{NMe3} U ^{NMe} GA ^{NH2} GC ^{NMe3} U ^{NMe} C ^{NMe3} A ^{NH2} U ^{NMe} GC ^{NMe3} U ^{NMe} A ^{NH2} G
31DNA_C ^{NMe3} G ^{NMe2} U ^{NMe}	<u>CATGGGCGGCATGGGAC</u> ^{NMe3} U ^{NMe} G ^{NMe2} AG ^{NMe2} C ^{NMe3} U ^{NMe} C ^{NMe3} AU ^{NMe} G ^{NMe2} C ^{NMe3} U ^{NMe} AG ^{NMe2}
31DNA_A ^{NH2} G ^{NMe2} U ^{NMe}	<u>CATGGGCGGCATGGGA</u> ^{NH2} CU ^{NMe} G ^{NMe2} A ^{NH2} G ^{NMe2} CU ^{NMe} CA ^{NH2} U ^{NMe} G ^{NMe2} CU ^{NMe} A ^{NH2} G ^{NMe2}

Note: primer regions are underlined, a complementary strand is an unmodified template.

Table 16. The list of 5'-(6-FAM)-labeled zwitterionic dsDNA and ssONs.

Name	Sequence (5' → 3')
31DNA _A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3}	<u>CATGGGCGGCATGGGA</u> ^{NH2} C ^{NMe3} U ^{NMe} G ^{NMe2} A ^{NH2} G ^{NMe2} C ^{NMe3} U ^{NMe}
31ON _A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3}	<u>C^{NMe3}A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}U^{NMe}A^{NH2}G^{NMe2}</u>
43DNA _A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3}	<u>CATGGGCGGCATGGGA</u> ^{NH2} C ^{NMe3} U ^{NMe} G ^{NMe2} A ^{NH2} G ^{NMe2} C ^{NMe3} U ^{NMe} C ^{NMe3} A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3} U ^{NMe} A ^{NH2} G ^{NMe2} A ^{NH2} C ^{NMe3} U ^{NMe} G ^{NMe2} A ^{NH2} G ^{NMe2} C ^{NMe3} U ^{NMe} C ^{NMe3} A ^{NH2} U ^{NMe} G ^{NMe2}
61DNA _A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3}	<u>CATGGGCGGCATGGGA</u> ^{NH2} C ^{NMe3} U ^{NMe} G ^{NMe2} A ^{NH2} G ^{NMe2} C ^{NMe3} U ^{NMe} C ^{NMe3} A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3} U ^{NMe} A ^{NH2} G ^{NMe2} C ^{NMe3} U ^{NMe} C ^{NMe3} A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3} U ^{NMe} A ^{NH2} G ^{NMe2} G ^{NMe2} C ^{NMe3} G ^{NMe2} A ^{NH2} U ^{NMe} G ^{NMe2} U ^{NMe} C ^{NMe3} U ^{NMe} C ^{NMe3} U ^{NMe} C ^{NMe3} A ^{NH2} U ^{NMe} G ^{NMe2} A ^{NH2} U ^{NMe} G ^{NMe2} U ^{NMe} C ^{NMe3}
98DNA _A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3}	<u>CGATACGAACGTATGCTAGTTCTGTC</u> ^{NMe3} U ^{NMe} G ^{NMe} A ^{NH2} A ^{NH2} C ^{NMe3} A ^{NH2} G ^{NMe2} A ^{NH2} C ^{NMe3} U ^{NMe} G ^{NMe2} A ^{NH2} U ^{NMe} C ^{NMe3} G ^{NMe2} U ^{NMe} U ^{NMe} C ^{NMe3} G ^{NMe2} U ^{NMe} A ^{NH2} C ^{NMe3} A ^{NH2} G ^{NMe2} A ^{NH2} C ^{NMe3} A ^{NH2} G ^{NMe2} U ^{NMe} A ^{NH2} C ^{NMe3} A ^{NH2} G ^{NMe2} U ^{NMe} C ^{NMe3} A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3} U ^{NMe} A ^{NH2} G ^{NMe2} A ^{NH2} C ^{NMe3} A ^{NH2} G ^{NMe2} U ^{NMe} C ^{NMe3} A ^{NH2} G ^{NMe2} A ^{NH2} C ^{NMe3} A ^{NH2} G ^{NMe2} U ^{NMe} C ^{NMe3} A ^{NH2} G ^{NMe2} U ^{NMe} U ^{NMe} C ^{NMe3} G ^{NMe2} U ^{NMe} A ^{NH2} C ^{NMe3} U ^{NMe} G ^{NMe2} A ^{NH2} U ^{NMe} U ^{NMe} G ^{NMe2} C ^{NMe3}

Note: primer regions are underlined; DNA – a double-stranded hypermodified PEX product (a complementary strand is an unmodified template); ON – a single-stranded modified product.

3.2.4. Application of cationic dN^RTPs in PCR

After demonstrating that the modified cationic dN^RTPs can be incorporated into DNA by PEX, we moved forward with their application in PCR. Using the set-up designed in Section 3.1.4, each cationic dN^RTP was tested in PCR as a substrate for KOD XL DNA polymerase using a single-stranded 98-mer template Temp^{NK98}, 5'-Cy5-labeled forward primer Prim^{NK98pr2}-Cy5, 5'-(6-FAM)-labeled reverse primer Prim^{NK98pr1}-FAM (Table 17), and the other three natural dNTPs (Figure 34, lanes 2-5; for uncropped and unmodified gels, see Figure 58 in ES). To achieve better yields, standard composition of PCR mixtures was complemented with several additives, such as formamide, betaine, DMSO, tetramethyl ammonium chloride (TMAC), and MgSO₄. Formamide and TMAC are known to increase hybridization specificity and eliminate non-specific priming,⁸⁷ while DMSO and betaine reduce complex secondary DNA structures,⁸⁸ and Mg²⁺ ions enhance the enzymatic activity of DNA polymerase.⁸⁹ After thorough optimization of PCR experiments with each cationic dN^RTP, it was found that addition of formamide solution improves PCR with dA^{NH2}TP, Mg²⁺ ions positively impact PCR with dU^{NMe}TP, and addition of betaine increases yields of PCR products obtained using dG^{NMe2}TP and dC^{NMe3}TP (Table 34 in ES).

Similar impact of the additives was observed in PCR with combinations of cationic **dN^RTPs** (Tables 37 and 38 in ES).

The PCR experiments were analyzed by dPAGE, where the elongation of reverse primer Prim^{NK98pr1}-FAM and forward primer Prim^{NK98pr2}-Cy5 were visualized using FAM and Cy5 scans, respectively (Figure 34). Full-length PCR products could be obtained for most combinations of two **dN^RTPs** (Figure 34, lanes 6-11; for uncropped and unmodified gels, see Figure 60 in ES), although the yields are significantly lower compared to the cases with one cationic **dN^RTP**. Combinations of three cationic **dN^RTPs** in PCR (Figure 34, lanes 12-15; for uncropped and unmodified gels, see Figure 61 in ES) gave only trace amounts of the products, while the attempt to use all four cationic **dN^RTPs** (Figure 34, lane 16) resulted in no product formation. In the latter case, it was possible to obtain the product of reverse primer extension through aPCR (Figure 24A), which involves using an increased amount of template in the absence of the forward primer and provides only linear amplification (for details see Section 5.2.11, Method H2 in ES).

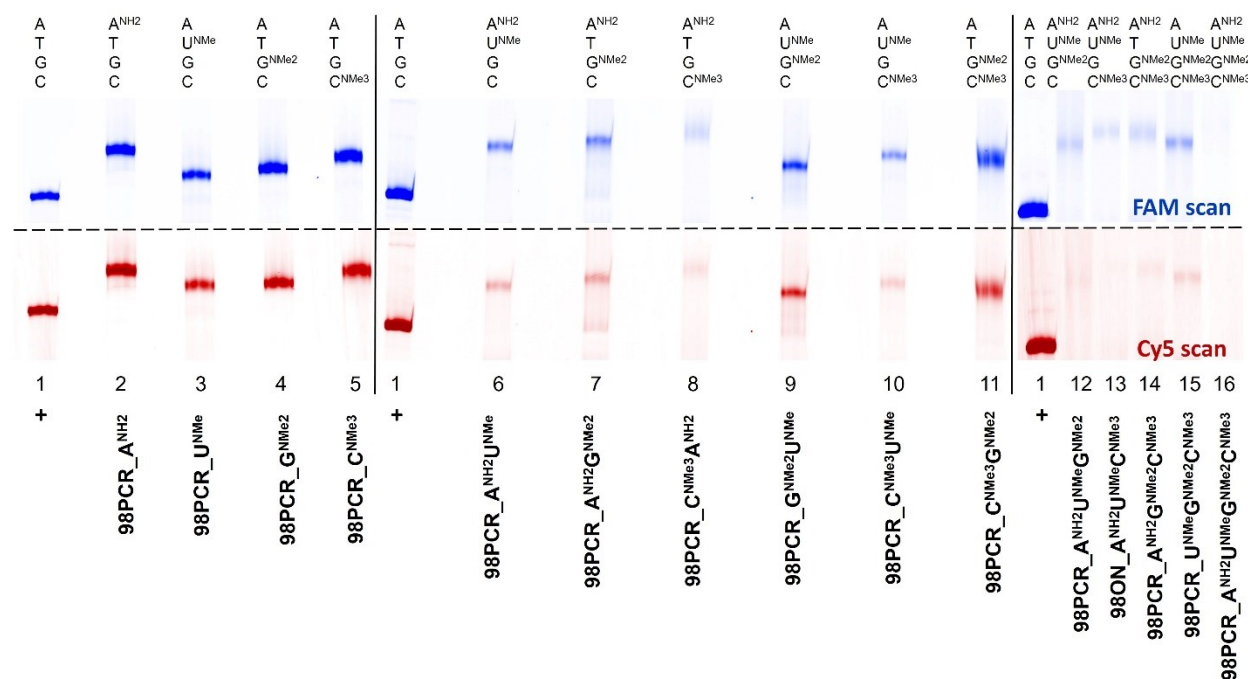


Figure 34. The part of denaturing PAGE displaying full-length PCR products obtained with one cationic **dN^RTP** (lanes 2-5) or their combinations (lanes 6-16) using 5'-(6-FAM)-labeled reverse primer Prim^{NK98pr1}-FAM, 5'-Cy5-labeled forward primer Prim^{NK98pr2}-Cy5, 98-mer template Temp^{NK98}, and KOD XL DNA polymerase; used **dN^RTPs** are specified above the gels. Lane 1 is positive control with all natural dNTPs.

Table 17. Primers and the template used in PCR experiments in Section 3.2.4.

ON name	Length (nt)	Sequence (5'→ 3')
Prim ^{NK98pr1} -FAM ^a	25	CGATACGAACGTATGCTAGTTCTGT
Prim ^{NK98pr2} -Cy5 ^b	25	GCAATCAGTACGAACTGACTGTCTG
Temp ^{NK98}	98	<u>GCAATCAGTACGAACTGACTGTCTGACTGTCTAGCATG</u> <u>ACTGTACTGTCTGTACGAACGATCAGTCTGTTACAGACAG</u> <u>AACTAGCATACGTTCTGATCG</u>

Note: in the template the segments forming duplex with the primer are underlined;

^a 5'-(6-FAM)-labeled, ^b 5'-Cy5-labeled.

Table 18. The list of obtained modified PCR products.

Name	Sequence (5'→ 3')
98PCR_A ^{NH2 a,b}	<u>CGATACGAACGTATGCTAGTTCTGTCTGA</u> ^{NH2} A ^{NH2} CA ^{NH2} GA ^{NH2} CT GA ^{NH2} TCGTTTCGT ^{NH2} CA ^{NH2} GA ^{NH2} CA ^{NH2} GTA ^{NH2} CA ^{NH2} GTCA ^{NH2} TGC TA ^{NH2} GA ^{NH2} CA ^{NH2} GTCA ^{NH2} GA ^{NH2} CA ^{NH2} GTCA ^{NH2} GTTTCGT ^{NH2} CTG A ^{NH2} TTGC
98PCR_U ^{NMe a,b}	<u>CGATACGAACGTATGCTAGTTCTGTCTU</u> ^{NMe} GAACAGACU ^{NMe} GA U ^{NMe} CGU ^{NMe} U ^{NMe} CGU ^{NMe} ACAGACAGU ^{NMe} ACAGU ^{NMe} CAU ^{NMe} GC U ^{NMe} AGACAGU ^{NMe} CAGACAGU ^{NMe} CAGU ^{NMe} U ^{NMe} CGU ^{NMe} ACU ^{NMe} G AU ^{NMe} U ^{NMe} GC
98PCR_G ^{NMe2 a,b}	<u>CGATACGAACGTATGCTAGTTCTGTCTG</u> ^{NMe2} AACAG ^{NMe2} ACT G ^{NMe2} ATCG ^{NMe2} TTTCG ^{NMe2} TACAG ^{NMe2} ACAG ^{NMe2} TACAG ^{NMe2} TCAT G ^{NMe2} CTAG ^{NMe2} ACAG ^{NMe2} TCAG ^{NMe2} ACAG ^{NMe2} TCAG ^{NMe2} TTTCG ^{NMe2} T ACTG ^{NMe2} ATTG ^{NMe2} C
98PCR_C ^{NMe3 a,b}	<u>CGATACGAACGTATGCTAGTTCTGTCT</u> ^{NMe3} TGAAC ^{NMe3} AGAC ^{NMe3} T GATC ^{NMe3} GTTC ^{NMe3} GTAC ^{NMe3} AGAC ^{NMe3} AGTAC ^{NMe3} AGTC ^{NMe3} ATG C ^{NMe3} TAGAC ^{NMe3} AGTC ^{NMe3} AGAC ^{NMe3} AGTC ^{NMe3} AGTTC ^{NMe3} GTA C ^{NMe3} TGATTGC ^{NMe3}
98PCR_C ^{NMe3 A^{NH2 a,b}}	<u>CGATACGAACGTATGCTAGTTCTGTCT</u> ^{NMe3} TGA ^{NH2} A ^{NH2} C ^{NMe3} A ^{NH2} G A ^{NH2} C ^{NMe3} TGA ^{NH2} TC ^{NMe3} GTTC ^{NMe3} GTA ^{NH2} C ^{NMe3} A ^{NH2} GA ^{NH2} C ^{NMe3} A ^{NH2} GTA ^{NH2} C ^{NMe3} A ^{NH2} GTC ^{NMe3} A ^{NH2} TGC ^{NMe3} TA ^{NH2} GA ^{NH2} C ^{NMe3} A ^{NH2} GT C ^{NMe3} A ^{NH2} GA ^{NH2} C ^{NMe3} A ^{NH2} GTC ^{NMe3} A ^{NH2} GTTC ^{NMe3} GTA ^{NH2} C ^{NMe3} TG A ^{NH2} TTGC ^{NMe3}
98PCR_C ^{NMe3 G^{NMe2 a,b}}	<u>CGATACGAACGTATGCTAGTTCTGTCT</u> ^{NMe3} TG ^{NMe} AAC ^{NMe3} AG ^{NMe2} A C ^{NMe3} TG ^{NMe2} ATC ^{NMe3} G ^{NMe2} TTTC ^{NMe3} G ^{NMe2} TAC ^{NMe3} AG ^{NMe2} AC ^{NMe3} A G ^{NMe2} TAC ^{NMe3} AG ^{NMe2} TC ^{NMe3} ATG ^{NMe2} C ^{NMe3} TAG ^{NMe2} AC ^{NMe3} AG ^{NMe2} T C ^{NMe3} AG ^{NMe2} AC ^{NMe3} AG ^{NMe2} TC ^{NMe3} AG ^{NMe2} TTTC ^{NMe3} G ^{NMe2} TAC ^{NMe3} T G ^{NMe2} ATTG ^{NMe2} C ^{NMe3}
98PCR_C ^{NMe3 U^{NMe a,b}}	<u>CGATACGAACGTATGCTAGTTCTGTCT</u> ^{NMe3} U ^{NMe} GAAC ^{NMe3} AGA C ^{NMe3} U ^{NMe} GAU ^{NMe} C ^{NMe3} GU ^{NMe} U ^{NMe} C ^{NMe3} GU ^{NMe} AC ^{NMe3} AGAC ^{NMe3} AG U ^{NMe} AC ^{NMe3} AGU ^{NMe} C ^{NMe3} AU ^{NMe} GC ^{NMe3} U ^{NMe} AGAC ^{NMe3} AGU ^{NMe} C ^{NMe3} AGAC ^{NMe3} AGU ^{NMe} C ^{NMe3} AGU ^{NMe} U ^{NMe} C ^{NMe3} GU ^{NMe} AC ^{NMe3} U ^{NMe} GAU ^{NMe} U ^{NMe} GC ^{NMe3}
98PCR_A ^{NH2 G^{NMe2 a,b}}	<u>CGATACGAACGTATGCTAGTTCTGTCTG</u> ^{NMe} A ^{NH2} A ^{NH2} CA ^{NH2} G ^{NMe2} A ^{NH2} CTG ^{NMe2} A ^{NH2} TCCG ^{NMe2} TTTCG ^{NMe2} TA ^{NH2} CA ^{NH2} G ^{NMe2} A ^{NH2} CA ^{NH2} G ^{NMe2} TA ^{NH2} CA ^{NH2} G ^{NMe2} TCA ^{NH2} TG ^{NMe2} CTA ^{NH2} G ^{NMe2} A ^{NH2} CA ^{NH2} G ^{NMe2} T

In conclusion, a full set of four modified **dN^RTPs** with various cationic groups was designed and synthesized by Sonogashira cross-coupling. All of them were shown to be good substrates for both KOD XL and Vent(exo-) DNA polymerases in PEX, where it was possible to obtain DNA strands containing one, two, three or even all four anionically modified nucleotides with up to 73 cationic modifications in a row. It was also discussed that the use of **dC^{NMe3}TP** in enzymatic DNA synthesis is restricted by template sequence. In PCR, the exponential amplification through extension of both forward and reverse primers proceeded with each modified nucleotide separately, while combinations of two or three cationic **dN^RTPs** gave either very low amplification or no PCR product. Using the set of all four cationically modified **dN^RTPs** in PCR did not lead to product formation. In this case, only linear amplification was observed.

3.3. Combination of charged and hydrophobic **dN^RTPs** in enzymatic DNA synthesis

Designing oligonucleotides modified with different types of functionalities allows for an increase in chemical potential of DNA, leading to greater variability in selection experiments towards different targets. For this reason, it is advantageous to be able to combine nucleotides displaying various types of modifications in enzymatic DNA synthesis. To synthesize such a *mixed* hypermodified DNA, PEX and PCR experiments were designed to introduce a set of four **dN^RTPs** bearing cationic, anionic, and hydrophobic groups. Alkyl-linked **dC^{EAlk}TP** and phenyl-linked **dU^{EPh}TP** (Figure 35) were chosen as nucleotides with hydrophobic moieties and were kindly provided by Dr. Marek Ondruš (the synthesis was previously described³⁷). **dG^{PA}TP** and **dA^{NH2}TP** were selected as representatives of anionic and cationic **dN^RTPs**, respectively, since both demonstrated good substrate activity in enzymatic DNA synthesis.

To obtain hypermodified DNA bearing different types of modifications, KOD XL DNA polymerase was used, as it was shown to incorporate all the modified **dN^RTPs**, including hydrophobic ones.³⁷ As in the cases of superanionic and zwitterionic DNA, 31-mer template Temp^{Prb4basII} (Table 14) was first used (Figure 36A), resulting in hypermodified **31DNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** (Table 19), which was further characterized by UPLC-MS after template separation (Table 32 and Figure 55 in ES). The dPAGE analysis (Figure 36B) showed

that the reaction proceeded as well with longer templates (43, 61, and 98-mer; Table 14) giving hypermodified PEX products containing 16-73 modified nucleotides with charged and hydrophobic groups (Table 19). As in the case of other hypermodified DNA (Figures 23B and 34B), the electrophoretic mobility of the hypermodified mixed PEX products differed from the natural analogs, but the bands were much less smeared.

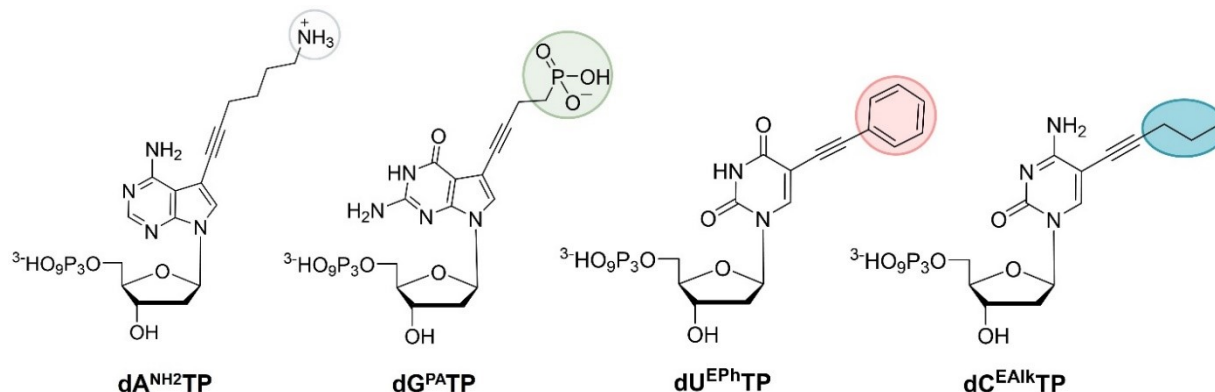


Figure 35. $\text{dN}^{\text{R}}\text{TPs}$ used for enzymatic synthesis of mixed DNA.

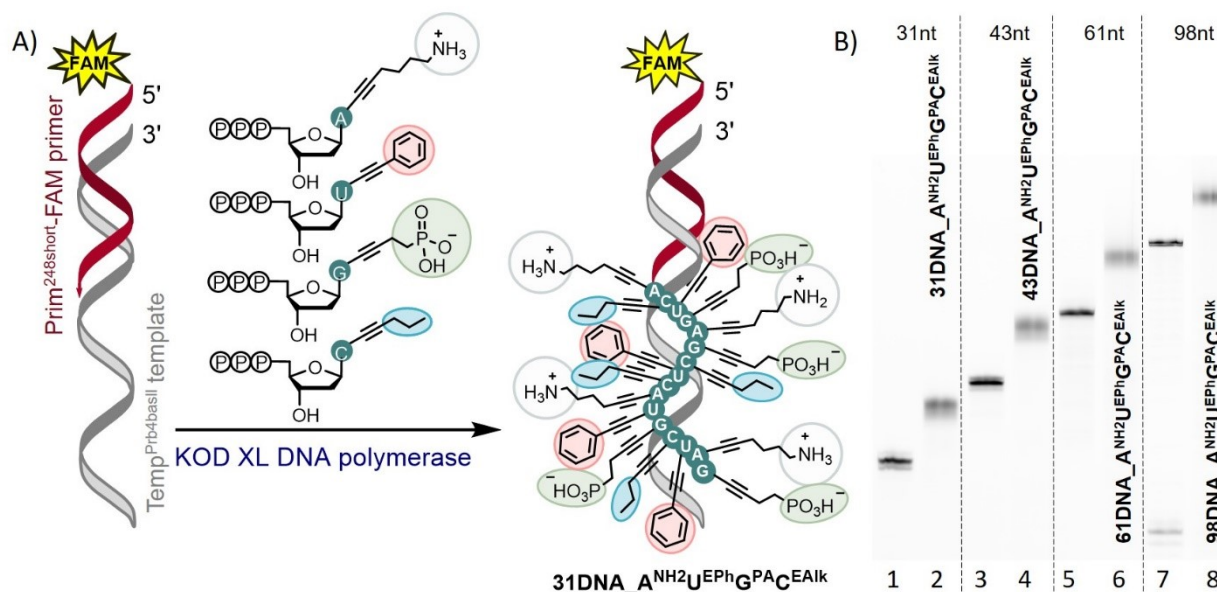


Figure 36. A) PEX reaction using four modified $\text{dN}^{\text{R}}\text{TPs}$ – $\text{dA}^{\text{NH}_2}\text{TP}$, $\text{dU}^{\text{EPH}}\text{TP}$, $\text{dG}^{\text{PA}}\text{TP}$, and $\text{dC}^{\text{EAlk}}\text{TP}$; B) Denaturing PAGE analysis of PEX with $\text{dA}^{\text{NH}_2}\text{TP}$, $\text{dU}^{\text{EPH}}\text{TP}$, $\text{dG}^{\text{PA}}\text{TP}$, $\text{dC}^{\text{EAlk}}\text{TP}$ and 31-mer template $\text{Temp}^{\text{Prb4basII}}$ (lane 2), 43-mer template $\text{Temp}^{\text{MO43}}$ (lane 4), 61-mer template $\text{Temp}^{\text{MO61}}$ (lane 6), 98-mer template $\text{Temp}^{\text{NK98}}$ (lane 8); lanes 1, 3, 5, and 7: positive controls with all natural dNTPs.

After obtaining hypermodified mixed DNA by PEX, the set of four **dN^RTPs** bearing cationic, anionic, and hydrophobic modifications was applied in PCR. Using KOD XL DNA polymerase, 5'-(6-FAM)-labeled reverse primer Prim^{NK98pr1}-FAM, 5'-Cy5-labeled forward primer Prim^{NK98pr2}-Cy5, 98-mer template Temp^{NK98} (Table 17), and 5% formamide, a full-length hypermodified product **98PCR_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** was successfully obtained (Table 19). As shown in Figure 37A (lane 3), both the reverse and forward primers were extended, indicating that the ON, hypermodified with several types of modifications, can serve as a template in enzymatic DNA synthesis of another hypermodified strand. Although partially extended sequences were also observed, full-length product **98PCR_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** was the first type of both-strand-hypermodified DNA obtained through PCR. The fully-modified **98ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}**, a FAM-labeled reverse primer extension product, was further synthesized by aPCR (Figure 37B).

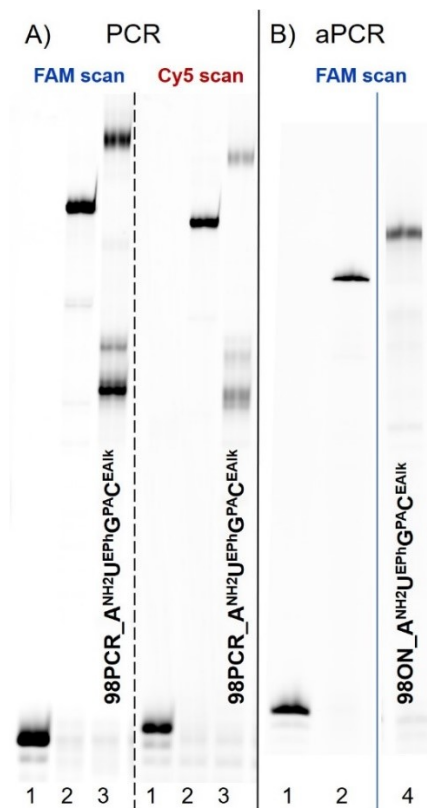


Figure 37. Denaturing PAGE analysis of A) PCR and B) aPCR experiments with **dA^{NH2}TP**, **dU^{EPh}TP**, **dG^{PA}TP**, and **dC^{EAlk}TP**: (lane 1) primer; (lane 2) positive control with all natural dNTPs; (lane 3) the product of exponential PCR; (lane 4) the product of asymmetric PCR. The blue line in Figure 37B indicates the merging site of two parts of the gel (an irrelevant dPAGE part was removed).

Table 19. The list of mixed hypermodified DNA.

Name	Sequence (5'→ 3')
31DNA _A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk} ^a	<u>CATGGGCGGCATGGGA</u> ^{NH2} C ^{EAlk} U ^{EPh} G ^{PA} A ^{NH2} G ^{PA} C ^{EAlk} U ^{EPh} C ^{EAlk}
31ON _A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk} ^a	A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk} U ^{EPh} A ^{NH2} G ^{PA}
43DNA _A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk} ^a	<u>CATGGGCGGCATGGGA</u> ^{NH2} C ^{EAlk} U ^{EPh} G ^{PA} A ^{NH2} G ^{PA} C ^{EAlk} U ^{EPh} C ^{EAlk} A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk} U ^{EPh} A ^{NH2} G ^{PA} A ^{NH2} C ^{EAlk} U ^{EPh} G ^{PA} A ^{NH2} G ^{PA} C ^{EAlk} U ^{EPh} C ^{EAlk} A ^{NH2} U ^{EPh} G ^{PA}
61DNA _A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk} ^a	<u>CATGGGCGGCATGGGA</u> ^{NH2} C ^{EAlk} U ^{EPh} G ^{PA} A ^{NH2} G ^{PA} C ^{EAlk} U ^{EPh} C ^{EAlk} A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk} U ^{EPh} A ^{NH2} G ^{PA} C ^{EAlk} U ^{EPh} C ^{EAlk} A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk} U ^{EPh} A ^{NH2} G ^{PA} G ^{PA} C ^{EAlk} G ^{PA} A ^{NH2} U ^{EPh} G ^{PA} U ^{EPh} C ^{EAlk} U ^{EPh} C ^{EAlk} U ^{EPh} C ^{EAlk} A ^{NH2} U ^{EPh} G ^{PA} A ^{NH2} U ^{EPh} G ^{PA} U ^{EPh} C ^{EAlk}
98DNA _A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk} ^a	<u>CGATACGAACGTATGCTAGTTCTGTC</u> ^{EAlk} U ^{EPh} G ^{PA} A ^{NH2} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} A ^{NH2} C ^{EAlk} U ^{EPh} G ^{PA} A ^{NH2} U ^{EPh} C ^{EAlk} G ^{PA} U ^{EPh} U ^{EPh} C ^{EAlk} G ^{PA} U ^{EPh} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} U ^{EPh} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} U ^{EPh} C ^{EAlk} A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk} U ^{EPh} A ^{NH2} G ^{PA} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} U ^{EPh} C ^{EAlk} A ^{NH2} G ^{PA} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} U ^{EPh} C ^{EAlk} A ^{NH2} G ^{PA} U ^{EPh} U ^{EPh} C ^{EAlk} G ^{PA} U ^{EPh} A ^{NH2} C ^{EAlk} U ^{EPh} G ^{PA} A ^{NH2} U ^{EPh} U ^{EPh} G ^{PA} C ^{EAlk}
98PCR _A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk} ^b	<u>CGATACGAACGTATGCTAGTTCTGTC</u> ^{EAlk} U ^{EPh} G ^{PA} A ^{NH2} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} A ^{NH2} C ^{EAlk} U ^{EPh} G ^{PA} A ^{NH2} U ^{EPh} C ^{EAlk} G ^{PA} U ^{EPh} U ^{EPh} C ^{EAlk} G ^{PA} U ^{EPh} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} U ^{EPh} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} U ^{EPh} C ^{EAlk} A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk} U ^{EPh} A ^{NH2} G ^{PA} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} U ^{EPh} C ^{EAlk} A ^{NH2} G ^{PA} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} U ^{EPh} C ^{EAlk} A ^{NH2} G ^{PA} U ^{EPh} U ^{EPh} C ^{EAlk} G ^{PA} U ^{EPh} A ^{NH2} C ^{EAlk} U ^{EPh} G ^{PA} A ^{NH2} U ^{EPh} U ^{EPh} G ^{PA} C ^{EAlk}

Note: primer regions are underlined; DNA – a double-stranded PEX product (a complementary strand is an unmodified template), PCR – a double-stranded PCR product with a complementary strand modified in the same manner using modifications under study (primer regions are not modified), ON – a single-stranded modified product; ^a 5'-(6-FAM)-labeled, ^b 5'-Cy5-labeled.

In conclusion, hypermodified DNA bearing cationic, anionic, and hydrophobic groups was obtained by PEX with up to 73 modifications in a row. Moreover, it was possible to create a duplex with both fully-modified strands by PCR, which was not the case for either superanionic or zwitterionic DNA.

3.4. Application of hypermodified ONs for sequencing

While short ONs can be identified by mass spectrometry, longer hypermodified strands require a different method of characterization. Moreover, to be used in the selection of functional NA, modified DNA need to be sequenceable. Previously, an approach for sequencing long hypermodified ONs was developed, ensuring that the modified strand, rather than the unmodified

template, is sequenced.³⁷ Here, the method is adapted for hypermodified PEX products (Figure 38) bearing anionic, cationic, or several types of modifications.

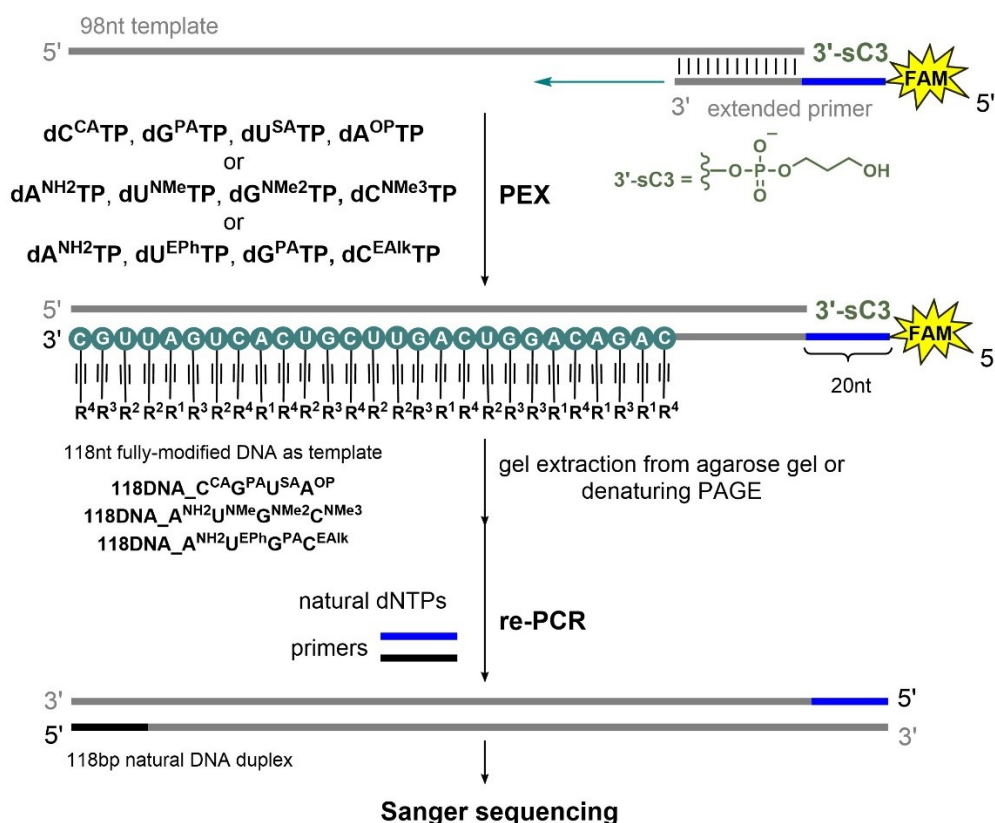


Figure 38. PEX with an extended primer and a set of four modified dN^RTPs followed by re-PCR with natural dNTPs to prepare dsDNA for Sanger sequencing.

First, PEX was performed using an appropriate set of modified dN^RTPs, KOD XL DNA polymerase, 5'-(6-FAM)-labeled primer extended with a 20-nucleotide flanking sequence, and a 98-mer template modified at 3'-end with a three-carbon spacer (sC3) (Table 20; for details see Section 5.2.14 in ES). The extended primer created a new 20nt primer region for subsequent re-PCR, while sC3-spacer prevented unwanted template extension during PEX (Table 21). The hypermodified PEX products were further extracted from either agarose gel or dPAGE. In the case of superanionic DNA, dPAGE was used to obtain hypermodified single-stranded 118ON_C^{CA}G^{PA}U^{SA}A^{OP} (Table 22; Figure 39, lane 2). However, due to severe smearing on dPAGE, zwitterionic 118DNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3} and mixed 118DNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}

were extracted as double strands along with unmodified templates from a native agarose gel (Table 22; Figure 39, lanes 6, 7).

Table 20. Specifications for reactions with hypermodified templates.

Modified template	dN ^R TPs	Synthesized by PEX using: extended primer 98-mer template	re-PCR	
			primers	DNA polymerase
118ON_C ^{CA} G ^{PA} U ^{SA} A ^{OP}	dU ^{SA} TP dC ^{CA} TP dA ^{OP} TP dG ^{PA} TP	Prim ^{Flank-LT25TH} -FAM Temp ^{FVL-A-sC3}	Prim ^{Flank} Prim ^{L20}	KOD XL
118ON_A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3}	dA ^{NH2} TP dU ^{NMe} TP dG ^{NMe2} TP dC ^{NMe3} TP	Prim ^{Flank-NK98pr1} -FAM Temp ^{NK98-sC3}	Prim ^{Flank} Prim ^{NK98pr2}	Pwo
118ON_A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk}	dA ^{NH2} TP dU ^{EPh} TP dG ^{PA} TP dC ^{EAlk} TP			

Table 21. The primer and templates used in re-PCR experiments in Section 3.4.

ON name	Length (nt)	Sequence (5' → 3')
Prim ^{Flank}	20	CATTCGGCTGCTCTTGATTT
Prim ^{Flank} -FAM ^a	20	CATTCGGCTGCTCTTGATTT
Prim ^{NK98pr2}	25	GCAATCAGTACGAACTGACTGTCTG
Prim ^{Flank-NK98pr1} -FAM ^a	45	CATTCGGCTGCTCTTGATTTTCGATACGAACGTATGC TAGTTCTGT
Temp ^{NK98-sC3} ^b	98	<u>GCAATCAGTACGAACTGACTGTCTGACTGTCTAGCA</u> TGACTGTACTGTCTGTACGAACGATCAGTCTGTTCA <u>GACAGAACTAGCATACGTTTCGTATCG</u>
Prim ^{L20}	20	GACATCATGAGAGACATCGC
Prim ^{L20} -FAM ^a	20	GACATCATGAGAGACATCGC
Prim ^{Flank-LT25TH} -FAM ^a	45	CATTCGGCTGCTCTTGATTTCAAGGACAAAATACCT GTATTCCTT
Temp ^{FVL-A-sC3} ^b	98	<u>GACATCATGAGAGACATCGCCTCTGGGCTAATAGG</u> ACTACTTCTAATCTGTAAGAGCAGATCCCTGGACAG GCAAGGAATACAGGTATTTTGTCTTG

Note: in the templates the segments forming duplex with the primer are underlined;
^a 5'-(6-FAM)-labeled, ^b 3'-sC3 (three carbon spacer).

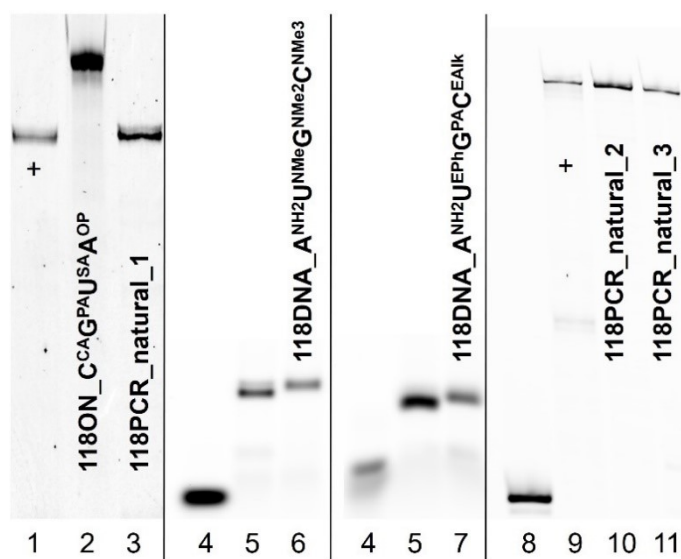


Figure 39. Denaturing PAGE analysis of PEX with an extended primer followed by re-PCR experiments with hypermodified templates: (lane 1) positive control obtained using natural dNTPs, Temp^{FVL-A}-sC3 and Prim^{Flank-LT25TH}-FAM; (lane 2) **118ON_C^{CA}G^{PA}U^{SA}A^{OP}** after gel extraction; (lane 3) **118PCR_natural_1** – re-PCR product of **118ON_C^{CA}G^{PA}U^{SA}A^{OP}**; (lane 4) primer Prim^{Flank-NK98pr1}-FAM; (lane 5) positive control obtained in PEX using natural dNTPs, Temp^{NK98}-sC3 and Prim^{Flank-NK98pr1}-FAM; (lane 6) **118DNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}**; (lane 7) **118DNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}**; (lane 8) primer Prim^{Flank}-FAM; (lane 9) positive control obtained in PCR using natural dNTPs, Temp^{NK98}-sC3, Prim^{Flank-NK98pr1}-FAM and Prim^{NK98pr2}; (lane 10) **118PCR_natural_2** – re-PCR product of **118ON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}**; (lane 11) **118PCR_natural_3** – re-PCR product of **118ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}**.

Once extracted, hypermodified 118-mer templates (Table 22) were introduced into re-PCR with natural dNTPs, followed by purification of the re-PCR products and their subsequent analysis by Sanger sequencing (Figure 65 in ES). KOD XL DNA polymerase was suitable for re-PCR using **118ON_C^{CA}G^{PA}U^{SA}A^{OP}** as a template, but not for zwitterionic **118ON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}** and mixed **118ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}**. As shown in Figures 40A and 40C, sequencing chromatograms of **118PCR_natural_2** and **118PCR_natural_3**, obtained by re-PCR with KOD XL, contain multiple overlapping peaks and misincorporations. The use of Pwo DNA polymerase allowed for accurate re-PCR of zwitterionic **118ON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}** (Figure 40B) and mixed **118ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** (Figure 40D; for more details see Figure 65).

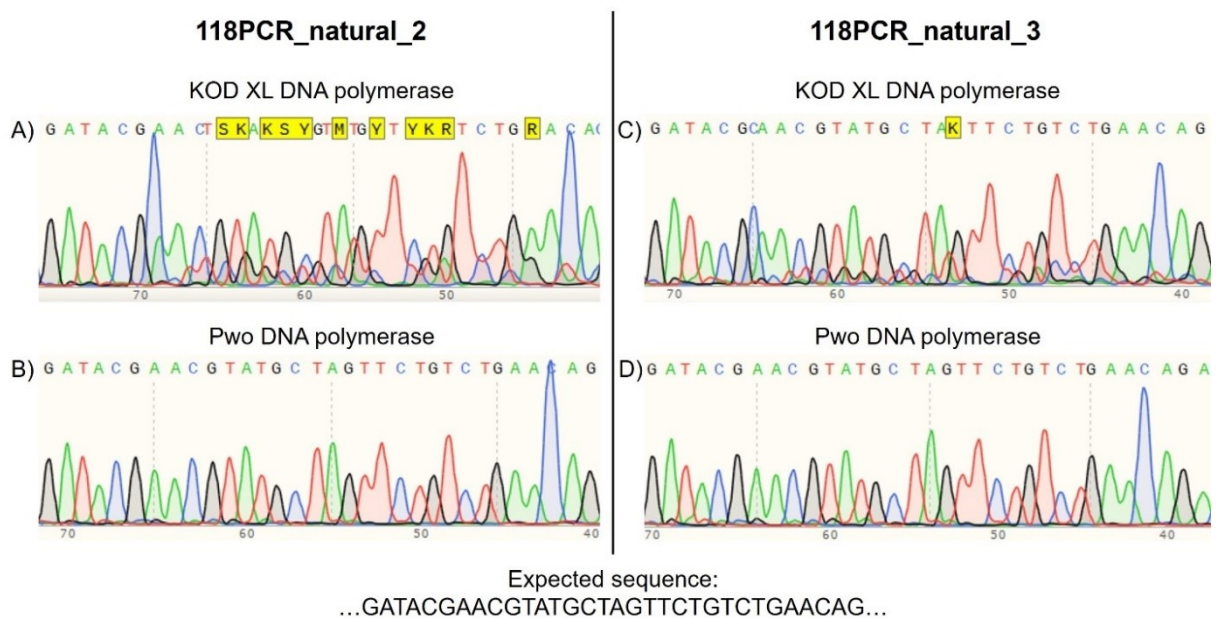


Figure 40. A region of sequencing chromatograms of 118PCR_natural_2 and 118PCR_natural_3, obtained by re-PCR of 118ON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3} and 118ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}, respectively, using: A, C) KOD XL DNA polymerase; B, D) Pwo DNA polymerase.

Table 22. The list of hypermodified templates and natural products of re-PCR.

Name	Sequence (5' → 3')
118ON_C ^{CA} G ^{PA} U ^{SA} A ^{OP a}	<u>CATTCGGCTGCTCTTGATTTCAAGGACAAAATACCTGTATT</u> <u>CCTTG^{PA}C^{CA}C^{CA}U^{SA}G^{PA}U^{SA}C^{CA}C^{CA}A^{OP}G^{PA}G^{PA}G^{PA}A^{OP}U^{SA}C^{CA}</u> <u>U^{SA}G^{PA}C^{CA}U^{SA}C^{CA}U^{SA}U^{SA}A^{OP}C^{CA}A^{OP}G^{PA}A^{OP}U^{SA}U^{SA}A^{OP}G^{PA}A^{OP}</u> <u>A^{OP}G^{PA}U^{SA}A^{OP}G^{PA}U^{SA}C^{CA}C^{CA}U^{SA}A^{OP}U^{SA}U^{SA}A^{OP}G^{PA}C^{CA}C^{CA}C^{CA}</u> <u>A^{OP}G^{PA}A^{OP}G^{PA}G^{PA}C^{CA}G^{PA}A^{OP}U^{SA}G^{PA}U^{SA}C^{CA}U^{SA}C^{CA}U^{SA}C^{CA}A^{OP}</u> <u>U^{SA}G^{PA}A^{OP}U^{SA}G^{PA}U^{SA}C^{CA}</u>
118ON_A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3 a} (118DNA_A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3 a})	<u>CATTCGGCTGCTCTTGATTTTCGATACGAACGTATGCTAGTT</u> <u>CTGTC^{NMe3}U^{NMe}G^{NMe}A^{NH2}A^{NH2}C^{NMe3}A^{NH2}G^{NMe2}A^{NH2}C^{NMe3}U^{NMe}</u> <u>G^{NMe2}A^{NH2}U^{NMe}C^{NMe3}G^{NMe2}U^{NMe}U^{NMe}C^{NMe3}G^{NMe2}U^{NMe}A^{NH2}C^{NMe3}</u> <u>A^{NH2}G^{NMe2}A^{NH2}C^{NMe3}A^{NH2}G^{NMe2}U^{NMe}A^{NH2}C^{NMe3}A^{NH2}G^{NMe2}U^{NMe}</u> <u>C^{NMe3}A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}U^{NMe}A^{NH2}G^{NMe2}A^{NH2}C^{NMe3}A^{NH2}G^{NMe2}</u> <u>U^{NMe}C^{NMe3}A^{NH2}G^{NMe2}A^{NH2}C^{NMe3}A^{NH2}G^{NMe2}U^{NMe}C^{NMe3}A^{NH2}G^{NMe2}</u> <u>U^{NMe}U^{NMe}C^{NMe3}G^{NMe2}U^{NMe}A^{NH2}C^{NMe3}U^{NMe}G^{NMe2}A^{NH2}U^{NMe}U^{NMe}</u> <u>G^{NMe2}C^{NMe3}</u>
118ON_A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk a} (118DNA_A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk a})	<u>CATTCGGCTGCTCTTGATTTTCGATACGAACGTATGCTAGTT</u> <u>CTGTC^{EAlk}U^{EPh}G^{PA}A^{NH2}A^{NH2}C^{EAlk}A^{NH2}G^{PA}A^{NH2}C^{EAlk}U^{EPh}G^{PA}A^{NH2}</u> <u>U^{EPh}C^{EAlk}G^{PA}U^{EPh}U^{EPh}C^{EAlk}G^{PA}U^{EPh}A^{NH2}C^{EAlk}A^{NH2}G^{PA}A^{NH2}C^{EAlk}</u> <u>A^{NH2}G^{PA}U^{EPh}A^{NH2}C^{EAlk}A^{NH2}G^{PA}U^{EPh}C^{EAlk}A^{NH2}U^{EPh}G^{PA}C^{EAlk}U^{EPh}</u> <u>A^{NH2}G^{PA}A^{NH2}C^{EAlk}A^{NH2}G^{PA}U^{EPh}C^{EAlk}A^{NH2}G^{PA}A^{NH2}C^{EAlk}A^{NH2}G^{PA}</u> <u>U^{EPh}C^{EAlk}A^{NH2}G^{PA}U^{EPh}U^{EPh}C^{EAlk}G^{PA}U^{EPh}A^{NH2}C^{EAlk}U^{EPh}G^{PA}A^{NH2}</u> <u>U^{EPh}U^{EPh}G^{PA}C^{EAlk}</u>

118PCR_natural_1	<u>CATTCGGCTGCTCTTGATTTCAAGGACAAAATACCTGTATT</u> <u>CCTTGCCTGTCCAGGGATCTGCTCTTACAGATTAGAAGTAG</u> <u>TCCTATTAGCCCAGAGGCGATGTCTCTCATGATGTC</u>
118PCR_natural_2	<u>CATTCGGCTGCTCTTGATTTTCGATACGAACGTATGCTAGTT</u>
118PCR_natural_3	<u>CTGTCTGAACAGACTGATCGTTCGTACAGACAGTACAGTCA</u> <u>TGCTAGACAGTCAGACAGTCAGTTCGTACTGATTGC</u>

Note: primer regions are underlined; ON – a single-stranded hypermodified product; DNA – a double-stranded hypermodified PEX product (a complementary strand is an unmodified template); PCR – an unmodified re-PCR product; ^a 5'-(6-FAM)-labeled.

In conclusion, all hypermodified ONs under study were shown to be efficient templates for replication with natural dNTPs, ensuring high fidelity and enabling re-PCR and further sequencing.

3.5. Biophysical studies of hypermodified DNA

After obtaining these unusual hypermodified DNA species, the next goal was to determine how the high density of charged modifications affects DNA stability and conformation. Previously, a significant duplex stabilization has been reported for hypermodified DNA containing all four alkyne-linked nucleobases^{37,30} due to the increased polarizability and π - π -stacking effect of the modified nucleobases compared to their natural counterpart. It is reasonable to expect that superanionic DNA will be less stable than its natural analog because of the additional repulsion effect of the modifications. On the contrary, introducing a high density of cationic modifications into DNA should promote duplex stabilization, which was previously shown with DNA bearing protonated basic aminopropyl or aminopropargyl groups.^{90,91}

Talking about the secondary structure of base-modified DNA, functional groups introduced at the C5 position of pyrimidines or C7 of 7-deazapurines were shown to not interfere with Watson-Crick base pairing¹⁸ and presumably do not disturb B-DNA helical structure. Although incorporation of cationic modifications into DNA was previously reported to only slightly bend B-DNA structure,⁹² a high density of modifications can induce bigger structural changes. To gain insights into the structure and stability of hypermodified DNA duplexes, UV and CD studies were performed, supported by MD simulations in ambiguous cases.

3.5.1. Samples preparation

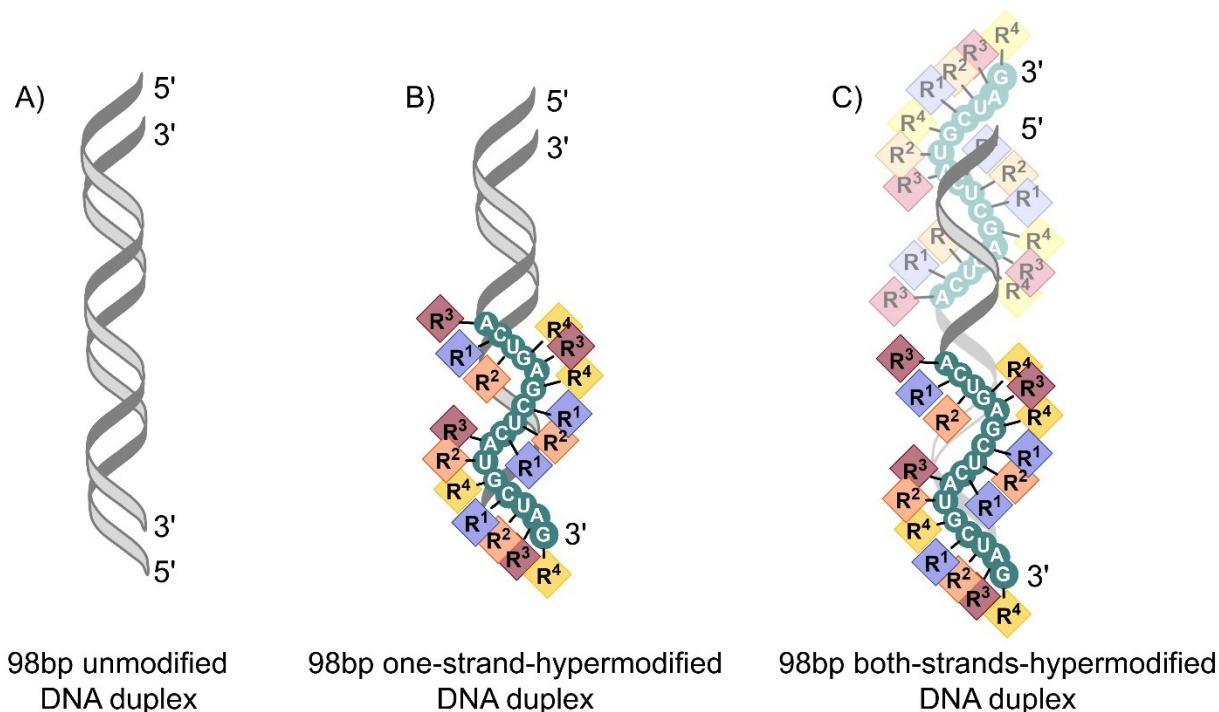


Figure 41. Samples for hybridization and CD spectroscopy studies.

For further biophysical studies, several DNA samples were prepared (Figure 41). Superanionic **98DNA_C^{CA}G^{PA}U^{SA}A^{OP}**, zwitterionic **98DNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}**, and mixed **98DNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** (Table 23, Figure 41B), containing one fully-modified strand, were obtained by semipreparative PEX (for details see Section 5.3.2 in ES), purified using Agencourt AMPure XP magnetic particles, concentrated, and dissolved in a buffer (10 mM Tris, 65 mM NaCl, 1 mM EDTA, pH 7.5). To prepare DNA samples with both hypermodified strands (Figure 41C), two complementary hypermodified ssONs were synthesized through PEX, followed by template separation and subsequent annealing (Figure 42).

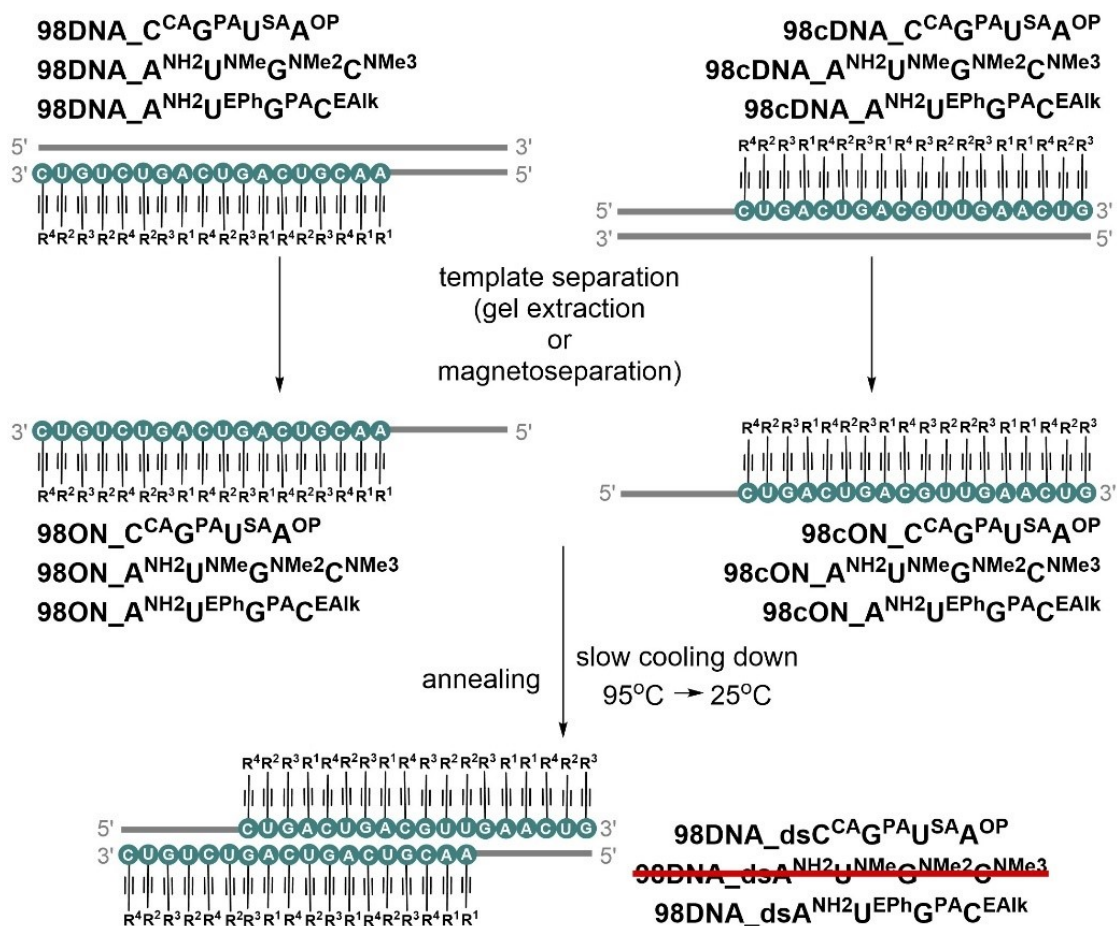


Figure 42. Preparation of the samples with both strands modified.

As a result, it was possible to anneal two complementary superanionic **98ON_CCA G^{PA} U^{SA} A^{OP}** and **98cON_CCA G^{PA} U^{SA} A^{OP}**, yielding in **98DNA_dsCCA G^{PA} U^{SA} A^{OP}**, hypermodified on both strands (Figure 43, lane 2). To overcome the mutual repulsion of superanionic ssONs, NaCl concentration in the buffer was increased from original 65 mM to 100 mM. Additionally, the cooling rate was set relatively low (0.8 °C/min) to promote more concerted annealing. **98DNA_dsA^{NH2} U^{EPh} G^{PA} C^{EAlk}** with both strands bearing anionic, cationic, and hydrophobic groups was obtained in a similar way (Figure 43, lane 5), except that no higher salt concentration was required.

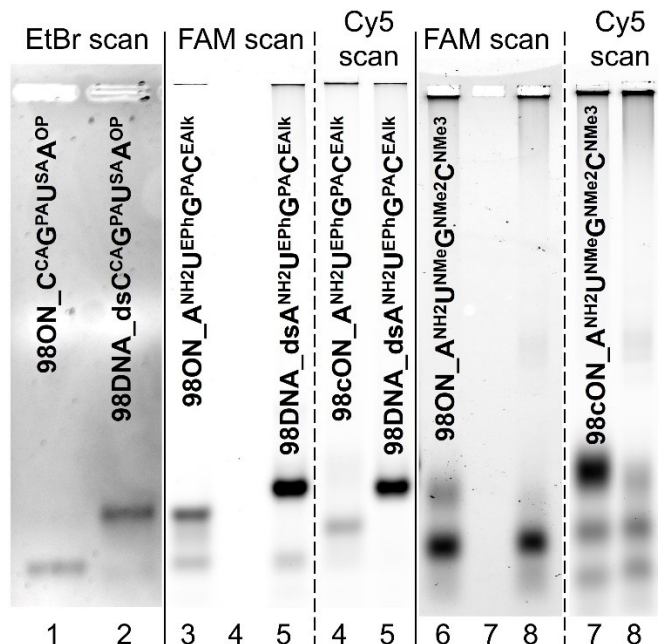


Figure 43. Native agarose gel analysis of annealing experiments, where lanes 1, 3, 4, 6, and 7 correspond to hypermodified ssONs, lanes 2 and 5 – to both-strands-hypermodified annealed dsDNA, and lane 8 – to an attempt of annealing $98\text{ON_A}^{\text{NH}_2}\text{U}^{\text{NMe}}\text{G}^{\text{NMe}_2}\text{C}^{\text{NMe}_3}$ and $98\text{cON_A}^{\text{NH}_2}\text{U}^{\text{NMe}}\text{G}^{\text{NMe}_2}\text{C}^{\text{NMe}_3}$.

However, attempts to anneal two zwitterionic ssONs, $98\text{ON_A}^{\text{NH}_2}\text{U}^{\text{NMe}}\text{G}^{\text{NMe}_2}\text{C}^{\text{NMe}_3}$ and $98\text{cON_A}^{\text{NH}_2}\text{U}^{\text{NMe}}\text{G}^{\text{NMe}_2}\text{C}^{\text{NMe}_3}$, were unsuccessful. Figure 43 shows scans of native agarose gels, comparing the mobility of hypermodified ssONs with that of double-stranded products of their annealing. It is clearly visible that the majority of $98\text{ON_A}^{\text{NH}_2}\text{U}^{\text{NMe}}\text{G}^{\text{NMe}_2}\text{C}^{\text{NMe}_3}$ and $98\text{cON_A}^{\text{NH}_2}\text{U}^{\text{NMe}}\text{G}^{\text{NMe}_2}\text{C}^{\text{NMe}_3}$ does not migrate and remains in the wells (Figure 43, lanes 6-8). It may be explained by the tendency of zwitterionic ssONs to partially form higher-order aggregates. To verify this hypothesis, confocal microscopy imaging of hypermodified ssONs water solutions was performed by Dr. Tomáš Kraus (Figure 44). The results show that all hypermodified ssONs under study tend to aggregate. Surprisingly, even superanionic $98\text{ON_C}^{\text{CA}}\text{G}^{\text{PA}}\text{U}^{\text{SA}}\text{A}^{\text{OP}}$ (Figure 44B) forms aggregates in water. However, their nature differs from aggregates of zwitterionic $98\text{ON_A}^{\text{NH}_2}\text{U}^{\text{NMe}}\text{G}^{\text{NMe}_2}\text{C}^{\text{NMe}_3}$ (Figure 44C) and mixed $98\text{ON_A}^{\text{NH}_2}\text{U}^{\text{EPH}}\text{G}^{\text{PA}}\text{C}^{\text{EAik}}$ (Figure 44D); they are rather unstable and continuously move, merge, and transform into each other (Figure 44E). Such lability of superanionic aggregates still allows for annealing reaction of $98\text{ON_C}^{\text{CA}}\text{G}^{\text{PA}}\text{U}^{\text{SA}}\text{A}^{\text{OP}}$ and $98\text{cON_C}^{\text{CA}}\text{G}^{\text{PA}}\text{U}^{\text{SA}}\text{A}^{\text{OP}}$, which do not exhibit unusual behavior on the gel.

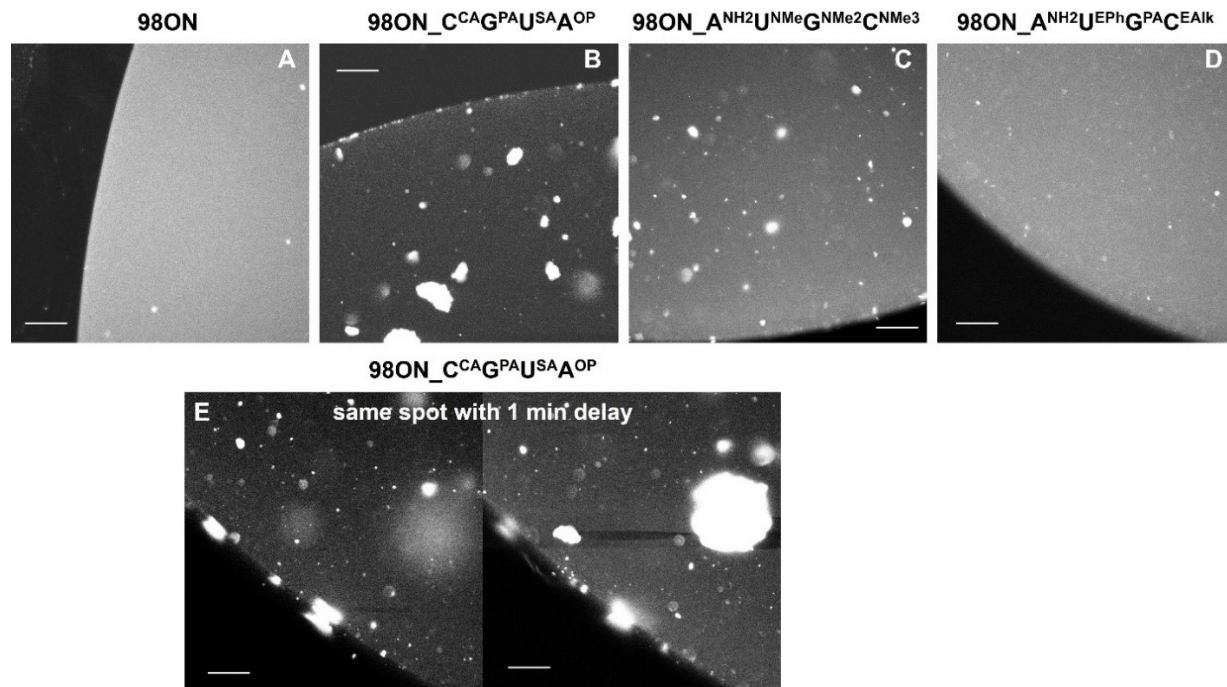


Figure 44. Confocal microscopy images of water solutions of single-stranded samples: (A) natural **98ON**, (B) superanionic **98ON_CCA G^{PA} U^{SA} A^{OP}**, (C) zwitterionic **98ON_A^{NH2} U^{NMe} G^{NMe2} C^{NMe3}**, (D) mixed **98ON_A^{NH2} U^{EPh} G^{PA} C^{EAlk}**. Figure 44E shows the behavior of superanionic aggregates in time. Scale bar is 50 μm .

The last two samples were unmodified duplexes **98DNA_1** and **98DNA_2** synthesized by PCR (Table 23). **98DNA_1** was required as a reference for **98DNA_CCA G^{PA} U^{SA} A^{OP}** and **98DNA_dsCCA G^{PA} U^{SA} A^{OP}**, as they were obtained using template Temp^{FVL-A} (Table 14), while **98DNA_2** was needed for comparison with **98DNA_A^{NH2} U^{NMe} G^{NMe2} C^{NMe3}**, **98DNA_A^{NH2} U^{EPh} G^{PA} C^{EAlk}**, and **98DNA_dsA^{NH2} U^{EPh} G^{PA} C^{EAlk}**, synthesized using template Temp^{NK98} (Table 14).

It is worth noting that in the cases of zwitterionic and mixed hypermodified DNA, 5'-(6-FAM)- and 5'-Cy5-labeled samples were used to optimize annealing experiments, making agarose gel analysis easier (Figure 43, lanes 3-8). Once the conditions were optimized, unlabeled samples were obtained and subjected to biophysical studies. Annealing experiments involving superanionic DNA were performed with unlabeled ssONs from the beginning. Since GelRed intercalator had to be added to agarose gel for proper migration of superanionic strands, labeling was unnecessary. The results were analyzed using a setup suitable for visualizing Ethidium Bromide (EtBr) (Figure 43, lanes 1,2).

Table 23. The list of samples used for biophysical studies.

Name	Sequence (5' → 3')
98ON_C ^{CA} G ^{PA} U ^{SA} A ^{OP}	<u>CAAGGACAAAATACCTGTATTCCTT</u> G ^{PA} C ^{CA} C ^{CA} U ^{SA} G ^{PA} U ^{SA} C ^{CA}
98DNA_C ^{CA} G ^{PA} U ^{SA} A ^{OP}	C ^{CA} A ^{OP} G ^{PA} G ^{PA} G ^{PA} A ^{OP} U ^{SA} C ^{CA} U ^{SA} G ^{PA} C ^{CA} U ^{SA} C ^{CA} U ^{SA} U ^{SA} A ^{OP} C ^{CA} A ^{OP}
98DNA_dsC ^{CA} G ^{PA} U ^{SA} A ^{OP}	G ^{PA} A ^{OP} U ^{SA} U ^{SA} A ^{OP} G ^{PA} A ^{OP} A ^{OP} G ^{PA} U ^{SA} A ^{OP} G ^{PA} U ^{SA} C ^{CA} C ^{CA} U ^{SA} A ^{OP} U ^{SA} U ^{SA} A ^{OP} G ^{PA} C ^{CA} C ^{CA} C ^{CA} A ^{OP} G ^{PA} A ^{OP} G ^{PA} G ^{PA} C ^{CA} G ^{PA} A ^{OP} U ^{SA} G ^{PA} U ^{SA} C ^{CA} U ^{SA} C ^{CA} U ^{SA} C ^{CA} A ^{OP} U ^{SA} G ^{PA} A ^{OP} U ^{SA} G ^{PA} U ^{SA} C ^{CA}
98cON_C ^{CA} G ^{PA} U ^{SA} A ^{OP}	<u>GACATCATGAGAGACATCGCC</u> C ^{CA} U ^{SA} C ^{CA} U ^{SA} G ^{PA} G ^{PA} G ^{PA} C ^{CA} U ^{SA}
98cDNA_C ^{CA} G ^{PA} U ^{SA} A ^{OP}	A ^{OP} A ^{OP} U ^{SA} A ^{OP} G ^{PA} G ^{PA} A ^{OP} C ^{CA} U ^{SA} A ^{OP} C ^{CA} U ^{SA} U ^{SA} C ^{CA} U ^{SA} A ^{OP} A ^{OP} U ^{SA} C ^{CA} U ^{SA} G ^{PA} U ^{SA} A ^{OP} A ^{OP} G ^{PA} A ^{OP} G ^{PA} C ^{CA} A ^{OP} G ^{PA} A ^{OP} U ^{SA} C ^{CA} C ^{CA} C ^{CA} U ^{SA} G ^{PA} G ^{PA} A ^{OP} C ^{CA} A ^{OP} G ^{PA} G ^{PA} C ^{CA} A ^{OP} A ^{OP} G ^{PA} G ^{PA} A ^{OP} A ^{OP} U ^{SA} A ^{OP} C ^{CA} A ^{OP} G ^{PA} G ^{PA} U ^{SA} A ^{OP} U ^{SA} U ^{SA} U ^{SA} U ^{SA} G ^{PA} U ^{SA} C ^{CA} C ^{CA} U ^{SA} U ^{SA} G ^{PA}
98ON_A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3}	<u>CGATACGAACGTATGCTAGTTCTGT</u> C ^{NMe3} U ^{NMe} G ^{NMe} A ^{NH2} A ^{NH2}
98DNA_A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3}	C ^{NMe3} A ^{NH2} G ^{NMe2} A ^{NH2} C ^{NMe3} U ^{NMe} G ^{NMe2} A ^{NH2} U ^{NMe} C ^{NMe3} G ^{NMe2} U ^{NMe} U ^{NMe} C ^{NMe3} G ^{NMe2} U ^{NMe} A ^{NH2} C ^{NMe3} A ^{NH2} G ^{NMe2} A ^{NH2} C ^{NMe3} A ^{NH2} G ^{NMe2} U ^{NMe} A ^{NH2} C ^{NMe3} A ^{NH2} G ^{NMe2} U ^{NMe} C ^{NMe3} A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3} U ^{NMe} A ^{NH2} G ^{NMe2} A ^{NH2} C ^{NMe3} A ^{NH2} G ^{NMe2} U ^{NMe} C ^{NMe3} A ^{NH2} G ^{NMe2} A ^{NH2} C ^{NMe3} A ^{NH2} G ^{NMe2} U ^{NMe} C ^{NMe3} A ^{NH2} G ^{NMe2} U ^{NMe} U ^{NMe} C ^{NMe3} G ^{NMe2} U ^{NMe} A ^{NH2} C ^{NMe3} U ^{NMe} G ^{NMe2} A ^{NH2} U ^{NMe} U ^{NMe} G ^{NMe2} C ^{NMe3}
98cON_A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3}	<u>GCAATCAGTACGA</u> ACTGACTGTCTGA ^{NH2} C ^{NMe3} U ^{NMe} G ^{NMe2} U ^{NMe}
98cDNA_A ^{NH2} U ^{NMe} G ^{NMe2} C ^{NMe3}	C ^{NMe3} U ^{NMe} A ^{NH2} G ^{NMe2} C ^{NMe3} A ^{NH2} U ^{NMe} G ^{NMe2} A ^{NH2} C ^{NMe3} U ^{NMe} G ^{NMe2} U ^{NMe} A ^{NH2} C ^{NMe3} U ^{NMe} G ^{NMe2} U ^{NMe} C ^{NMe3} U ^{NMe} G ^{NMe2} U ^{NMe} A ^{NH2} C ^{NMe3} G ^{NMe2} A ^{NH2} A ^{NH2} C ^{NMe3} G ^{NMe2} A ^{NH2} U ^{NMe} C ^{NMe3} A ^{NH2} G ^{NMe2} U ^{NMe} C ^{NMe3} U ^{NMe} G ^{NMe2} U ^{NMe} U ^{NMe} C ^{NMe3} A ^{NH2} G ^{NMe2} A ^{NH2} C ^{NMe3} A ^{NH2} G ^{NMe2} A ^{NH2} C ^{NMe3} U ^{NMe} A ^{NH2} G ^{NMe2} C ^{NMe3} A ^{NH2} U ^{NMe} A ^{NH2} C ^{NMe3} G ^{NMe2} U ^{NMe} U ^{NMe} C ^{NMe3} G ^{NMe2} U ^{NMe} A ^{NH2} U ^{NMe} C ^{NMe3} G ^{NMe2}
98ON_A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk}	<u>CGATACGAACGTATGCTAGTTCTGT</u> C ^{EAlk} U ^{EPh} G ^{PA} A ^{NH2} A ^{NH2} C ^{EAlk}
98DNA_A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk}	A ^{NH2} G ^{PA} A ^{NH2} C ^{EAlk} U ^{EPh} G ^{PA} A ^{NH2} U ^{EPh} C ^{EAlk} G ^{PA} U ^{EPh} U ^{EPh} C ^{EAlk} G ^{PA} U ^{EPh}
98DNA_dsA ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk}	A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} U ^{EPh} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} U ^{EPh} C ^{EAlk} A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk} U ^{EPh} A ^{NH2} G ^{PA} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} U ^{EPh} C ^{EAlk} A ^{NH2} G ^{PA} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} U ^{EPh} C ^{EAlk} A ^{NH2} G ^{PA} U ^{EPh} U ^{EPh} C ^{EAlk} G ^{PA} U ^{EPh} A ^{NH2} C ^{EAlk} U ^{EPh} G ^{PA} A ^{NH2} U ^{EPh} U ^{EPh} G ^{PA} C ^{EAlk}
98cON_A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk}	<u>GCAATCAGTACGA</u> ACTGACTGTCTGA ^{NH2} C ^{EAlk} U ^{EPh} G ^{PA} U ^{EPh} C ^{EAlk}
98cDNA_A ^{NH2} U ^{EPh} G ^{PA} C ^{EAlk}	U ^{EPh} A ^{NH2} G ^{PA} C ^{EAlk} A ^{NH2} U ^{EPh} G ^{PA} A ^{NH2} C ^{EAlk} U ^{EPh} G ^{PA} U ^{EPh} A ^{NH2} C ^{EAlk} U ^{EPh} G ^{PA} U ^{EPh} C ^{EAlk} U ^{EPh} G ^{PA} U ^{EPh} A ^{NH2} C ^{EAlk} G ^{PA} A ^{NH2} A ^{NH2} C ^{EAlk} G ^{PA} A ^{NH2} U ^{EPh} C ^{EAlk} A ^{NH2} G ^{PA} U ^{EPh} C ^{EAlk} U ^{EPh} G ^{PA} U ^{EPh} U ^{EPh} C ^{EAlk} A ^{NH2} G ^{PA} A ^{NH2} C ^{EAlk} A ^{NH2} G ^{PA} A ^{NH2} A ^{NH2} C ^{EAlk} U ^{EPh} A ^{NH2} G ^{PA} C ^{EAlk} A ^{NH2} U ^{EPh} A ^{NH2} C ^{EAlk} G ^{PA} U ^{EPh} U ^{EPh} C ^{EAlk} G ^{PA} U ^{EPh} A ^{NH2} U ^{EPh} C ^{EAlk} G ^{PA}
98DNA_1	<u>CAAGGACAAAATACCTGTATTCCTT</u> GCCTGTCCAGGGATCTGC TCTTACAGATTAGAAGTAGTCCTATTAGCCCAGAGGCGATGTC TCTCATGATGTC
98DNA_2	<u>CGATACGAACGTATGCTAGTTCTGT</u> CTGAACAGACTGATCGTT
98ON	CGTACAGACAGTACAGTCATGCTAGACAGTCAGACAGTCAGT TCGTA

Note: primer regions are underlined; ON – a single-stranded hypermodified or unmodified ON; DNA – a double-stranded hypermodified or unmodified DNA (a complementary strand is an unmodified template); DNA_ds – a double-stranded hypermodified DNA product of annealing (both strands are modified in the same manner).

3.5.2. DNA duplex stability studies using UV spectroscopy

To determine whether a high density of the studied modifications stabilizes or destabilizes hypermodified DNA duplexes, temperature-dependent measurements were performed with the samples, obtained in Section 3.5.1 (Table 23). As expected, the T_m of the samples displaying a high density of anionic modifications significantly decreased compared to their natural analog **98DNA_1**. In the case of **98DNA_C^{CA}G^{PA}U^{SA}A^{OP}**, containing one superanionic strand, T_m decreased from 78.6 °C (obtained for natural **98DNA_1**) to 65.5 °C. Meanwhile, the both-strand-superanionic **98DNA_dsC^{CA}G^{PA}U^{SA}A^{OP}** was even less stable, exhibiting a massive T_m value decrease of 33.4 °C (Figure 45, Table 24). Nevertheless, despite of decreased stability, all studied samples were shown to rehybridize repeatedly. In addition, the effect of divalent cations (Mg^{2+} , Mn^{2+} , Zn^{2+}) on T_m values of superanionic DNA have been studied. It was shown that Mg^{2+} can shield the negative charges more effectively than the other studied cations and increase the T_m of the duplexes (for details see Section 5.3.3 in ES).

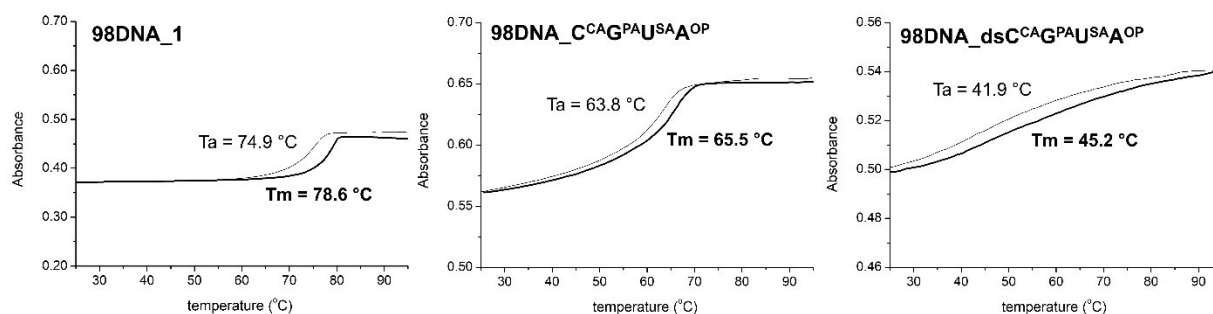


Figure 45. Melting curves with calculated melting (T_m) and annealing (T_a) temperatures of natural **98DNA_1** and superanionic duplexes **98DNA_C^{CA}G^{PA}U^{SA}A^{OP}** and **98DNA_dsC^{CA}G^{PA}U^{SA}A^{OP}** obtained from UV spectroscopy at 260 nm absorption (buffer composition: 10 mM Tris, 65 mM NaCl, 1 mM EDTA, pH 7.5; in the case of **98DNA_dsC^{CA}G^{PA}U^{SA}A^{OP}**: 10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5).

Regarding one-strand-zwitterionic **98DNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}**, T_m values increased from 82.9 °C (as observed for natural **98DNA_2**) to 88.5 °C, confirming duplex stabilization by a high density of cationic modifications (Figure 46). Interestingly, an increase in T_m was also observed in the case of mixed **98DNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** (T_m = 86.2 °C), despite the presence of anionic modifications in this duplex. The most plausible explanation is that the π - π -stacking effect of alkynyl linkers, nucleobases, and phenyl modifications complemented by the effect of cationic

groups, outweighed the destabilizing impact of the anionic modifications. The both-strand-hypermodified **98DNA_dsA^{NH2}U^{EPh}G^{PA}CEAlk** containing cationic, anionic, and hydrophobic modifications was destabilized by 2.2 °C compared to natural **98DNA_2** (Table 24) – an insignificant destabilization given the hypermodified nature of the duplex.

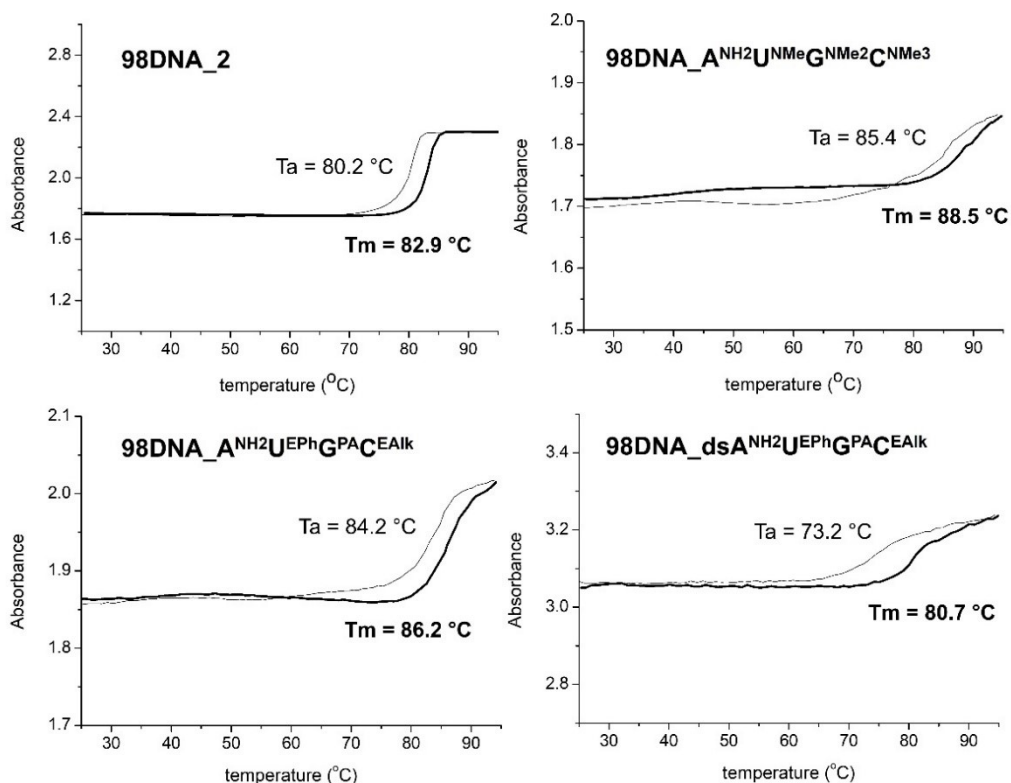


Figure 46. Melting curves with calculated T_m and T_a of natural **98DNA_2**, zwitterionic **98DNA_A^{NH2}UNMeGNMe2CNMe3**, and mixed duplexes **98DNA_A^{NH2}UEPhG^{PA}CEAlk** and **98DNA_dsA^{NH2}UEPhG^{PA}CEAlk** obtained from UV spectroscopy at 260 nm absorption (buffer composition: 10 mM Tris, 65 mM NaCl, 1 mM EDTA, pH 7.5).

Table 24. T_m and hysteresis of natural and modified DNA determined by UV spectroscopy.

DNA sample	T_m (°C)	Hysteresis = $T_m - T_a$ (°C)	ΔT_m with natural DNA	ΔT_m / modification
98DNA_1	78.6	3.7	—	—
98DNA_CCA^{PA}USA^{OP}	65.5	1.7	−13.1	−0.179
98DNA_dsCCA^{PA}USA^{OP}	45.2	3.28	−33.4	−0.221
98DNA_2	82.9	2.7	—	—
98DNA_A^{NH2}UNMeGNMe2CNMe3	88.5	3.1	+5.6	+0.077
98DNA_A^{NH2}UEPhG^{PA}CEAlk	86.2	2.0	+3.3	+0.049
98DNA_dsA^{NH2}UEPhG^{PA}CEAlk	80.7	7.5	−2.2	−0.015

3.5.3. Structural studies using CD spectroscopy and MD simulations

To get deeper understanding of the structure of hypermodified DNA, UV-vis absorption and CD spectroscopy studies were first performed on superanionic samples (Table 23) by Dr. Lucie Bednářová. The natural **98DNA_1** was characterized by the absorption maximum at ~260 nm and by conservative CD spectrum with maxima at ~244 nm (–) and ~277 nm (+), typical for B-form⁸ (Figure 47A, D). In the case of one-strand-superanionic **98DNA_C^{CA}G^{PA}U^{SA}A^{OP}**, a typical absorption maximum at ~260 nm is accompanied by an additional absorption band with lower intensity at ~306 nm (Figure 47E). The additional band might be caused by the increased conjugation of ethynyl-linked nucleotides, similar to the previously reported hydrophobic hypermodified alkyne-linked DNA.³⁷ CD spectrum of **98DNA_C^{CA}G^{PA}U^{SA}A^{OP}** was not significantly altered by modifications and showed a positive CD spectral band at ~274 nm (+), typical for B-type DNA, accompanied by a shoulder at ~282 nm (+) and by negative bands at ~229 nm (–), ~251 nm (–) and ~297 nm (–) (Figure 47B).

Curiously, the UV-vis absorption and CD spectra of both-strand-superanionic **98DNA_dsC^{CA}G^{PA}U^{SA}A^{OP}** were completely unlike those previously described. The UV-vis spectrum showed absorption maxima at ~243 nm and at ~279 nm and no maximum at ~260 nm (Figure 47F). The CD spectrum was characterized by the following spectral bands: 315 nm (+), 291 nm (–), 274 nm (+), 247 nm (+), and 229 nm (–) (Figure 47C). Although the observed spectra differed remarkably from those of typical B-DNA, conformational change of such heavily modified DNA cannot be confirmed based solely on CD spectroscopy results. In this case, the spectral signature of the DNA backbone is biased by spectral contribution of chromophores (such as alkynes conjugated to nucleobases as well as carboxylic and sulfonic groups). Their mutual orientation and arrangement with respect to the DNA backbone can cause changes in CD spectra.⁹³ Therefore, to accurately characterize conformational changes in superanionic DNA, the CD spectroscopy results were complemented with molecular dynamics (MD) simulations, performed by Miguel Riopedre Fernández.

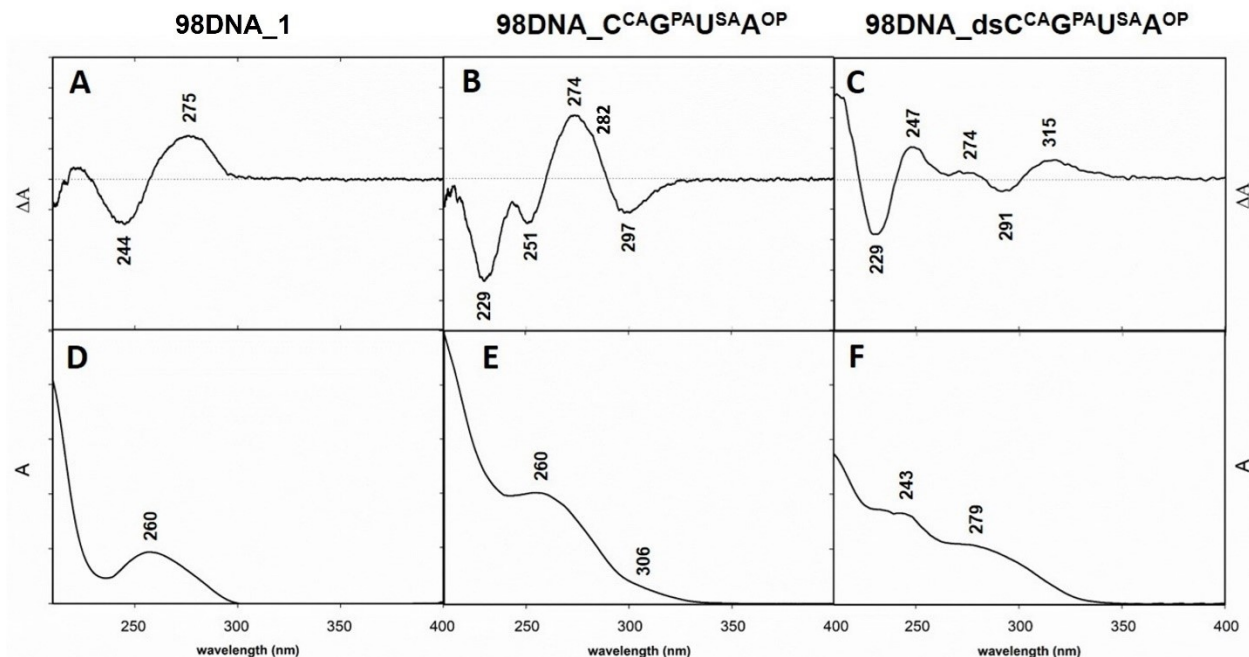


Figure 47. CD spectra (A, B, C) and UV absorption spectra (D, E, F) of natural **98DNA_1** and superanionic **98DNA_C^{CA}G^{PA}U^{SA}A^{OP}** and **98DNA_dsC^{CA}G^{PA}U^{SA}A^{OP}**.

MD simulations are a computational technique used to study molecular systems, predict their behavior, and validate experimental data by simulating atomic interactions according to physical principles. Firstly, the model of the desired system is built in its initial state, then the energy minimization is performed to remove unrealistic clashes between atoms. Followed by that, the system is equilibrated under set conditions (temperature, pressure, volume). Once it is done, the simulation is run, allowing for observation of the system's dynamic behavior in a fixed period of time.

Among other biomolecules, MD simulation can be applied to studies of DNA conformation.⁹⁴ In the case of modified DNA, the initial model with all modifications attached is fixed in the 'ideal' B-DNA conformation. Then, the simulation is run until the system reaches equilibrium with the potential energy minimized. The useful outputs for conformational studies are the time evolution of the root mean square deviation (RMSD) and the radius of gyration (Rg) values. RMSD is a standard measure of structural distance between a group of atoms. Calculating RMSD between two sets of atomic coordinates for two time points shows how much DNA conformation has changed with time. Rg value is a measure of the spread of a molecule around its center of mass.

The low Rg indicates a compact structure, the high Rg suggests an extended, unfolded structure. Both RMSD and Rg help to monitor conformational changes of modified DNA. Therefore, MD simulation results, supported by experimental data, allow for conclusions about whether the conformation of modified DNA differs from that of its natural analog.

Due to the limitations of the method, DNA models were built 12bp long. Three 12bp DNA duplexes were designed: a natural **12DNA**, the one-strand-superanionic **12DNA_C^{CA}G^{PA}U^{SA}A^{OP}**, and the both-strand-superanionic **12DNA_dsC^{CA}G^{PA}U^{SA}A^{OP}** (Table 25). In the case of one-strand-hypermodified **12DNA_C^{CA}G^{PA}U^{SA}A^{OP}**, RMSD and Rg with respect to a B-DNA reference structure do not differ statistically from the natural **12DNA** (Figure 48A, B), meaning that DNA with one superanionic strand preserves the B-DNA conformation. In contrast, the RMSD and Rg values for **12DNA_dsC^{CA}G^{PA}U^{SA}A^{OP}** are significantly higher indicating that the molecule adopts a different conformation than unmodified **12DNA**. Moreover, a helical structure is considerably loosened in **12DNA_dsC^{CA}G^{PA}U^{SA}A^{OP}**, as shown in Figure 48E. Hence, based on the combination of MD simulation results with experimental data (observed changes in CD spectra and low T_m), it can be concluded that the both-strand-superanionic **98DNA_dsC^{CA}G^{PA}U^{SA}A^{OP}** adopts a conformation different from B-form DNA while still preserving a loosened right-handed helix.

Table 25. The list of 12bp DNA duplexes designed for MD simulations.

Name	Sequence of the duplex
12DNA	5'-TCCAGGGATCTG-3' 3'-AGGTCCCTAGAC-5'
12DNA_C^{CA}G^{PA}U^{SA}A^{OP}	5'-U ^{SA} C ^{CA} C ^{CA} A ^{OP} G ^{PA} G ^{PA} G ^{PA} A ^{OP} U ^{SA} C ^{CA} U ^{SA} G ^{PA} -3' 3'-AGGTCCCTAGAC-5'
12DNA_dsC^{CA}G^{PA}U^{SA}A^{OP}	5'-U ^{SA} C ^{CA} C ^{CA} A ^{OP} G ^{PA} G ^{PA} G ^{PA} A ^{OP} U ^{SA} C ^{CA} U ^{SA} G ^{PA} -3' 3'-A ^{OP} G ^{PA} G ^{PA} U ^{SA} C ^{CA} C ^{CA} C ^{CA} U ^{SA} A ^{OP} G ^{PA} A ^{OP} C ^{CA} -5'

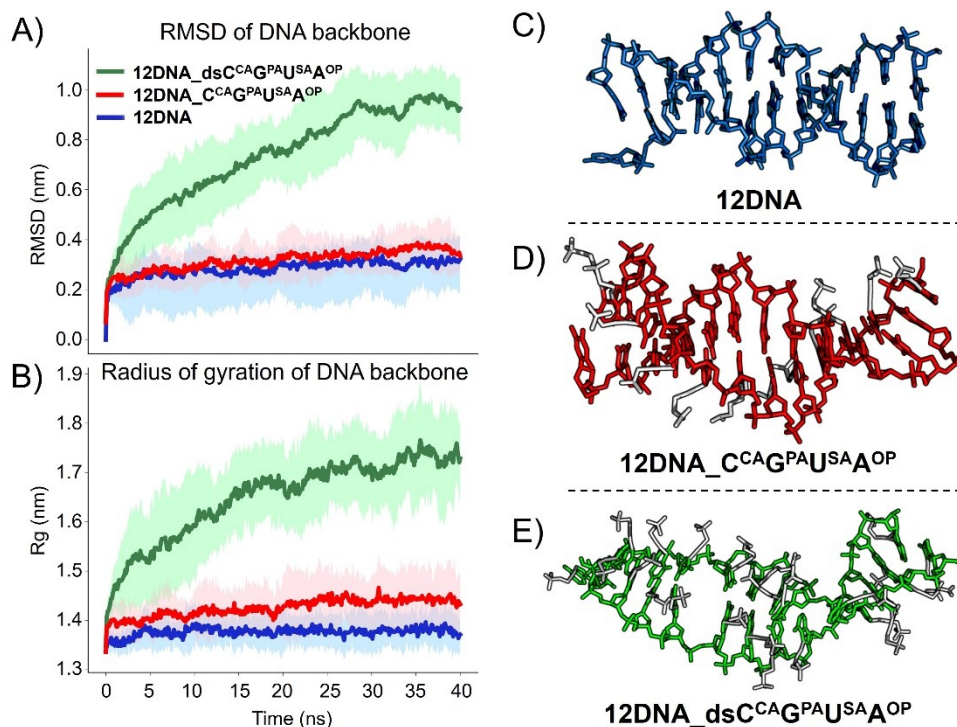


Figure 48. The time evolution of RMSD (A) and Rg (B) for **12DNA**, **12DNA_C^{CA}G^{PA}U^{SA}A^{OP}**, **12DNA_dsC^{CA}G^{PA}U^{SA}A^{OP}**; imaging of the built models in equilibrium (C-E): (C) **12DNA**; (D) **12DNA_C^{CA}G^{PA}U^{SA}A^{OP}**; (E) **12DNA_dsC^{CA}G^{PA}U^{SA}A^{OP}**.

Further, UV-vis absorption and CD spectroscopy studies were performed with zwitterionic and mixed hypermodified DNA. The natural **98DNA_2** was characterized by the absorption spectrum with maximum at ~257 nm and by conservative CD spectrum with maxima at ~247 nm (–) and ~279 nm (+), similar to the **98DNA_1** spectrum (Figure 49A, E). Introduction of the modifications into DNA duplexes caused spectral changes in both the absorption and CD spectra. The absorption spectra of the samples containing one hypermodified strand were characterized by a broader spectral band with a maximum at ~260 nm (257 nm for **98DNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}** and 259 nm for **98DNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}**) and a shoulder at ~305 nm (303 nm for **98DNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}** and 307 nm for **98DNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}**) (Figure 49F, G). CD spectra of **98DNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}** and **98DNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** showed a positive CD spectral band at ~273 nm (+) (Figure 49B, C). For **98DNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}**, this band was accompanied by negative spectral bands at ~299 nm (–), ~257 nm (–), ~226 nm (–), as well as a positive spectral band at ~244 nm (+). A similar spectral pattern was observed for **98DNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** with a positive band at ~273 nm (+) and negative bands at ~303 nm

(–), ~256 nm (–), and ~222 nm (–). These results suggest that one-strand-hypermodified duplexes still adopt B-DNA (or similar) conformation.

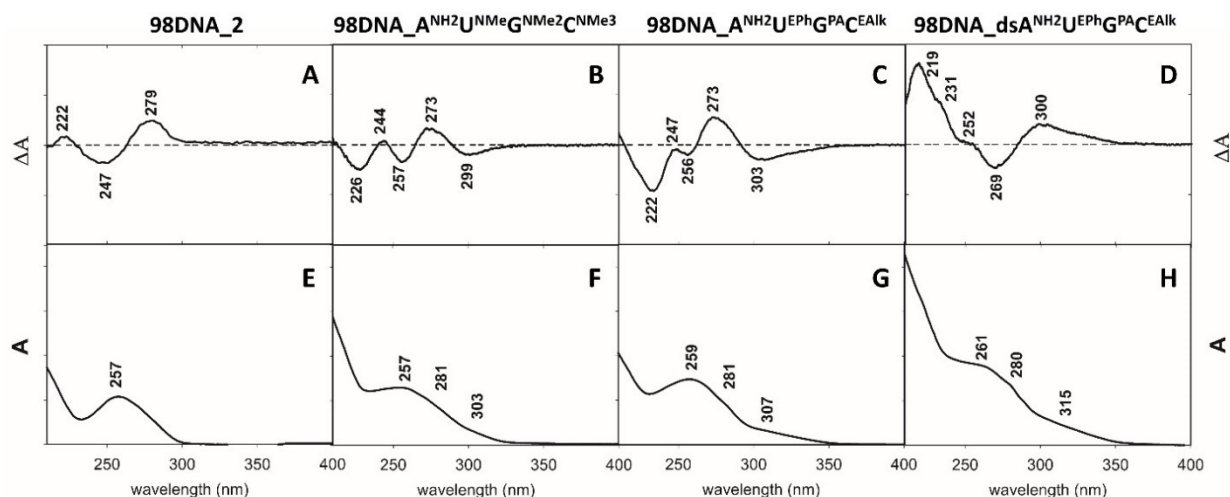


Figure 49. CD spectra (A–D) and UV absorption spectra (E–H) of natural **98DNA_2**, zwitterionic **98DNA_A^{NH2UNMeGNMe2CNMe3}**, and mixed **98DNA_A^{NH2UEPhG^{PAC}EAlk}** and **98DNA_dsA^{NH2UEPhG^{PAC}EAlk}**.

In the both-strands-hypermodified **98DNA_dsA^{NH2UEPhG^{PAC}EAlk}** bearing cationic, anionic, and hydrophobic groups, the UV-vis spectrum was quite similar to those of one-strand-hypermodified (Figure 5H), but on the other hand, the CD spectrum of **98DNA_dsA^{NH2UEPhG^{PAC}EAlk}** displayed significant differences. It was characterized by following spectral bands: 300 nm (+), 269 nm (–), 252 nm (+), 231 nm (+), and 219 nm (+). The CD spectral pattern of **98DNA_dsA^{NH2UEPhG^{PAC}EAlk}** also differed significantly from the spectra of both-strand-superanionic **98DNA_dsC^{CA}G^{PA}U^{SA}A^{OP}**, described previously. This may indicate some conformational changes, but any reliable conclusion about the possible structure cannot be drawn solely from changes in the CD spectra. Unfortunately, MD simulations were not performed in this case due to the higher complexity of the models and a lack of time.

In conclusion, the biophysical studies revealed that, as expected, a high density of anionic modifications in a DNA duplex leads to significant duplex destabilization due to the increased Coulombic repulsion between the polyanionic strands, while the incorporation of cationic modifications increases duplex stability. Even in the case of mixed hypermodified DNA, containing only one cationic modification among others, the stabilizing effect was significant.

Regarding the structural changes, all one-strand-hypermodified duplexes preserve the classical B-DNA form. The duplex composed of two superanionic strands was shown to adopt a different conformation with still right-handed but loosened helix. Based on CD spectroscopy results, DNA with both strands fully-modified with cationic, anionic, and hydrophobic modifications does not resemble the B-DNA patterns.

3.6. Resistance of hypermodified ONs to nuclease degradation

To investigate how the high density of functional groups affects DNA stability toward nuclease degradation, four 5'-(6-FAM)-labeled 31-mer ONs were prepared: natural **31ON**, superanionic **31ON_CCA^{G^{PA}}U^{SA}A^{OP}**, zwitterionic **31ON_A^{NH₂}U^{NMe}G^{NMe₂}C^{NMe₃}**, and mixed **31ON_A^{NH₂}U^{EPh}G^{PA}C^{EAlk}** bearing cationic, anionic, and hydrophobic modifications (Table 26; for details see Section 5.2.12 in ES).

Table 26. The list of 5'-(6-FAM)-labeled ssONs used in nuclease degradation experiments.

Name	Sequence (5' → 3')
31ON	<u>CATGGGCGGCATGGG</u> ACTGAGCTCATGCTAG
31ON_CCA^{G^{PA}}U^{SA}A^{OP}	<u>CATGGGCGGCATGGG</u> A ^{OP} C ^{CA} U ^{SA} G ^{PA} A ^{OP} G ^{PA} C ^{CA} U ^{SA} C ^{CA} A ^{OP} U ^{SA} G ^{PA} C ^{CA} U ^{SA} A ^{OP} G ^{PA}
31ON_A^{NH₂}U^{NMe}G^{NMe₂}C^{NMe₃}	<u>CATGGGCGGCATGGG</u> A ^{NH₂} C ^{NMe₃} U ^{NMe} G ^{NMe₂} A ^{NH₂} G ^{NMe₂} C ^{NMe₃} U ^{NMe} C ^{NMe₃} A ^{NH₂} U ^{NMe} G ^{NMe₂} C ^{NMe₃} U ^{NMe} A ^{NH₂} G ^{NMe₂}
31ON_A^{NH₂}U^{EPh}G^{PA}C^{EAlk}	<u>CATGGGCGGCATGGG</u> A ^{NH₂} C ^{EAlk} U ^{EPh} G ^{PA} A ^{NH₂} G ^{PA} C ^{EAlk} U ^{EPh} C ^{EAlk} A ^{NH₂} U ^{EPh} G ^{PA} C ^{EAlk} U ^{EPh} A ^{NH₂} G ^{PA}

Note: primer regions are underlined.

The obtained ONs were incubated with DNase I for 5, 30, and 60 minutes at 37 °C and the results were analyzed on dPAGE and visualized using fluorescence imaging (Figure 50). The average values of DNA recovery after incubation are given in Table 27. The results show that both anionic and cationic modifications improve ON stability toward DNase I. Furthermore, zwitterionic **31ON_A^{NH₂}U^{NMe}G^{NMe₂}C^{NMe₃}** exhibits higher stability compared to superanionic **31ON_CCA^{G^{PA}}U^{SA}A^{OP}**, suggesting that cationic modifications enhance ON resistance more effectively than the anionic ones (Table 27). The mixed **31ON_A^{NH₂}U^{EPh}G^{PA}C^{EAlk}** was still significantly more stable towards degradation than natural **31ON**, but less stable than superanionic and zwitterionic ONs.

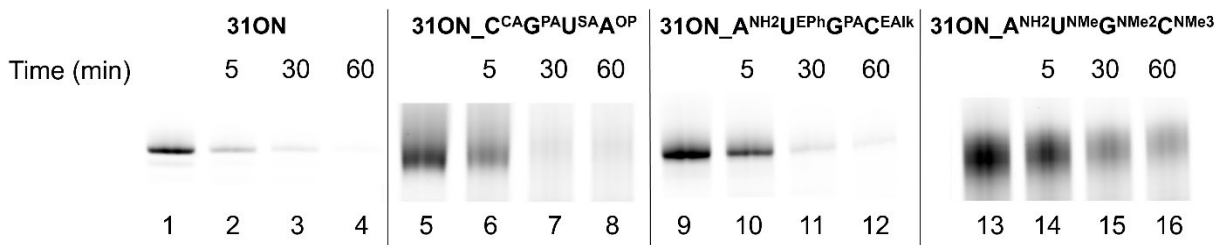


Figure 50. Denaturing PAGE analysis of the stability of natural **31ON** (lanes 2-4), superanionic **31ON_CCA G^{PA} U^{SA} A^{OP}** (lanes 6-8), mixed **31ON_A^{NH2} U^{EPH} G^{PA} C^{EAlk}** (lanes 10-12), and zwitterionic **31ON_A^{NH2} U^{NMe} G^{NMe2} C^{NMe3}** (lanes 14-16) in the presence of 0.01U of DNase I for 5 min (lanes 2, 6, 10, 14), 30 min (lanes 3, 7, 11, 15), and 60 min (lanes 4, 8, 12, 16). Lanes 1, 5, 9, and 13 were not incubated with DNase I.

Table 27. Recovery (expressed in percent) of natural and hypermodified ONs after incubation with 0.01 U of DNase I.

DNA sample	Incubation time with DNase I (0.01 U)		
	5 min	30 min	60 min
31ON	25±3%	6±2%	1%
31ON_CCA G^{PA} U^{SA} A^{OP}	60±5%	22±2%	15±4%
31ON_A^{NH2} U^{EPH} G^{PA} C^{EAlk}	56±7%	13±4%	6±2%
31ON_A^{NH2} U^{NMe} G^{NMe2} C^{NMe3}	79±1%	41±3%	24±6%

To further demonstrate enhanced stability of the hypermodified ONs in biological medium, the samples were incubated in human plasma for 17 hours (Figure 51; for details see Section 5.2.13 in ES). Zwitterionic **31ON_A^{NH2} U^{NMe} G^{NMe2} C^{NMe3}** and mixed **31ON_A^{NH2} U^{EPH} G^{PA} C^{EAlk}** demonstrated enhanced stability compared to natural **31ON**, while superanionic **31ON_CCA G^{PA} U^{SA} A^{OP}** supposedly formed aggregates with plasma content, which were trapped in the gel wells. Here, the recovery percentage was not calculated as the density of human plasma significantly differs from that of water, making quantitative comparison on dPAGE inadequate due to loading specificities.

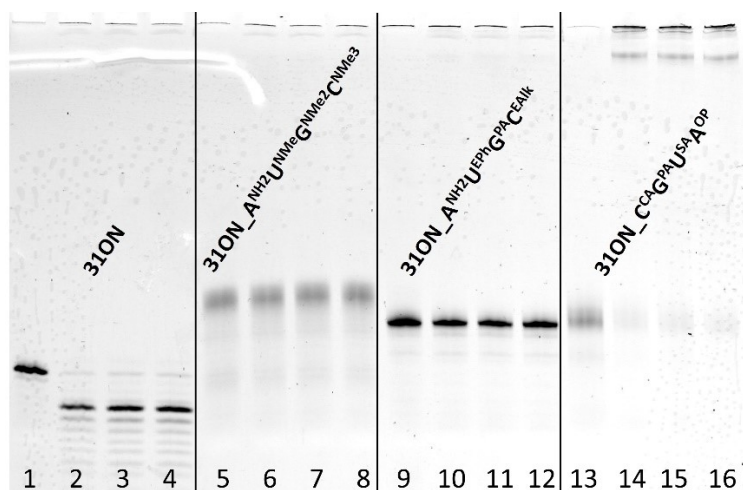


Figure 51. Denaturing PAGE analysis of natural **31ON** (lanes 2-4), zwitterionic **31ON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}** (lanes 6-8), mixed **31ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** (lanes 10-12), and superanionic **31ON_C^{CAG}PAU^{SA}A^{OP}** (lanes 14-16) incubated in human plasma for 17 hours (results shown in triplicates). Lanes 1, 5, 9, and 13 were not incubated in human plasma.

In conclusion, a high density of charged modifications was shown to protect DNA from nuclease degradation. Zwitterionic ON appeared to be the most resilient to DNase I, followed by superanionic ON. Mixed hypermodified ON was still more stable than the natural analog; however, it was the least stable of all hypermodified ONs, suggesting that hydrophobic modifications do not affect DNA stability against nuclease degradation as effectively as charged modifications.

4. CONCLUSION

Two sets of modified nucleotides displaying either anionic or cationic groups were synthesized using a direct and efficient methodology, requiring only two steps: triphosphorylation of iodosubstituted pyrimidines and 7-deazapurines, followed by Sonogashira cross-coupling with functionalized alkynes (for dNTP bearing a trimethylammonium group, order of these steps was reversed). This approach made it possible to avoid additional redundant protection of the functional groups and obtain the desired nucleoside triphosphates with moderate to good yields.

Modified nucleoside triphosphates were further tested in enzymatic DNA synthesis. All of them were shown to be efficient substrates for Vent(exo-) and KOD XL DNA polymerases in PEX experiments and were used for the synthesis of DNA containing one, two, three, or even four modified nucleotides. By replacing all four natural dNTPs with either a set of anionic or cationic dN^RTPs, two hypermodified DNA, where each nucleobase displayed a different functional group, were enzymatically synthesized. The DNA molecule bearing four anionic modifications – carboxylate, sulfonate, phosphonate, and phosphate groups – was named *superanionic* DNA, as it contained twice the number of negative charges compared to natural DNA. In turn, the hypermodified DNA displaying a full range of cationic functionalities – amino, methylamino, dimethylamino, and trimethylammonium groups – was referred to as *zwitterionic* DNA due to its zwitterionic character, where the anionic phosphate backbone is balanced by cationic substituents.

Overall, the activity of anionic and cationic dN^RTPs as substrates for DNA polymerase was comparable in PEX when incorporating a single modified nucleotide, complemented by remaining natural dNTPs. On the contrary, replacement of all four canonical nucleotides with cationic dN^RTPs proceeded much more difficult than with the set of anionic dN^RTPs. The synthesis of zwitterionic DNA appeared to be sequence-dependent due to the difficulties associated with the subsequent incorporation of dNTP modified with a trimethylammonium group. The reason might be connected to repulsive interactions of cationic groups and arginine residues in the active site of DNA polymerases. After carefully designing templates, zwitterionic DNA was obtained with 73 consecutive modifications, while superanionic DNA was synthesized sequence independently with up to 125 modifications in a row.

In PCR experiments, exponential amplification through the extension of both forward and reverse primers proceeded with each modified dN^RTPs separately. When comparing substrate activity, cationic dN^RTPs required more thorough optimization, including the use of additives, indicating that they are worse substrates than anionic dN^RTPs in PCR. Similar conclusions can be made after analyzing the results of PCR with various combinations of either anionic or cationic dN^RTPs – combinations of anionic dN^RTPs were better accepted by DNA polymerase than combinations of cationic ones. Applying all four dN^RTPs in PCR simultaneously did not yield any PCR product, whether using anionic or cationic nucleotides. In these cases, only linear amplification (aPCR) was observed.

PCR experiments with both sets of charged dN^RTPs indicated that neither superanionic nor zwitterionic DNA serves as a good template for enzymatic synthesis of another hypermodified strand. Yet, all hypermodified DNA were shown to be efficient templates for replication with natural dNTPs with high fidelity, allowing for re-PCR and further sequencing.

To demonstrate the compatibility of the charged nucleotides with hydrophobic ones, a combination of one cationic nucleotide displaying a primary amino group, one anionically modified nucleotide bearing a phosphonate group, and two previously reported alkyl- and phenyl-linked dN^RTPs was successfully applied in PEX, resulting in mixed hypermodified DNA with a wide range of functional groups. Interestingly, application of this combination of dN^RTPs in PCR yielded a full-length product with the extension of both primers, which was not observed for any other hypermodified DNA, including previously reported ones.

The results of hybridization studies demonstrated that incorporation of anionic modifications into DNA destabilizes the duplex, whereas cationic modifications enhance duplex stability. Comparing the effects, cationic modifications seem to have a greater impact than anionic ones, as the mixed hypermodified DNA containing one type of cationic modification along with the anionic one exhibits a significantly higher T_m than that of unmodified DNA. According to further biophysical studies, all duplexes with one hypermodified strand maintain the typical B-DNA form. However, when both strands are hypermodified, DNA appears to deviate from the B-conformation.

When comparing the resilience of hypermodified ONs against nuclease degradation, it was shown that a high density of charged groups increases ON stability in the presence of DNase I. Multiple cationic modifications were found to provide the greatest protection, followed by anionic modifications. Combined with hydrophobic groups in the mixed hypermodified DNA, charged modifications were less effective at protecting against degradation than in zwitterionic and superanionic ONs, but they still provided greater resistance to DNase I than natural DNA.

The concepts of *superanionic* and *zwitterionic* DNA illustrate two border cases of hypermodified DNA. Despite the increased Coulombic repulsion leading to decreased duplex stability, superanionic DNA is still able to rehybridize with complementary natural or another superanionic strand. Zwitterionic DNA, in turn, exhibits enhanced hybridization compared to natural DNA. Through these two types of hypermodified DNA, critical gaps in the field of enzymatic DNA functionalization were explored. And yet, from an application perspective, the ability to combine anionic, cationic, and hydrophobic modifications within a DNA molecule is the most valuable. It enables the creation of a vast library of peptide-like polymers with expanded chemical diversity. As an ongoing example, dC^{CA}TP, modified with a carboxylic group, is currently used in combination with dG^{NMe₂}TP, bearing a dimethylamino moiety, and two hydrophobic dN^RTPs to synthesize a hypermodified aptamer library toward various biomolecular targets. The accumulated knowledge about different types of hypermodified DNA will encourage and facilitate further research in the field of functional NA selection, including aptamers and DNazymes, the development or delivery of therapeutic oligonucleotides, the assembly of functionalized DNA nanostructures, and many other applications.

5. EXPERIMENTAL SECTION

5.1. Organic chemistry

5.1.1. General remarks – organic chemistry

All solvents and reagents were purchased from commercial suppliers and used as received. NMR spectra were recorded on Bruker Avance 400 MHz (400.0 MHz for ^1H , 162 MHz for ^{31}P , 100 MHz for ^{13}C) and Bruker Avance 500 MHz (500 MHz for ^1H , 125.7 MHz for ^{13}C , 202.3 for ^{31}P) spectrometers from sample solutions in D_2O and CD_3OD . Chemical shifts (in ppm, δ scale) were referenced as follows: D_2O (referenced to t-BuOH as an external reference; 1.25 ppm for ^1H NMR and 31.6 ppm for ^{13}C NMR). CD_3OD (referenced to solvent signal: 3.31 ppm for ^1H NMR and 49.00 ppm for ^{13}C NMR). ^{31}P chemical shifts were referenced to H_3PO_4 as an external reference. Chemical shifts are given in ppm (δ scale), coupling constants (J) in Hz. Low- and high-resolution mass spectra were measured on LTQ Orbitrap XL spectrometer (ESI ionization, Thermo Fisher Scientific). All mass spectra were acquired by the MS service at IOCB. Purification of the obtained compounds was performed using HPLC (Waters modular HPLC system) on columns Phenomenex Kinetex EVO C18 (Kinetex® 5 μm EVO C18 100 Å, AXIA Packed LC Column 250 x 21.2 mm) or Waters X-Bridge Shield RP18 (XBridge BEH Shield RP18 OBD Prep Column, 130Å, 5 μm , 19 x 150 mm) and columns packed with DEAE Sepharose Fast Flow ion-exchange resin (Cytiva). Buffer A (0.1 M TEAB in H_2O) and buffer B (0.1 M TEAB in 50% MeOH) were used for purification of nucleoside triphosphates on RP-HPLC columns. Reactions were monitored by thin layer chromatography (TLC) on TLC silica gel 60 F254 and TLC silica gel 60 RF-18 F254s (Merck Life Science) and detected by UV (254 nm) and by Advion Expression Compact Mass spectrometer connected with Plate Express® TLC Plate Reader using electrospray ionization (ESI). Reactions with nucleoside triphosphates were monitored by normal phase TLC using IPA/V (isopropyl alcohol / NH_4OH / H_2O , ratio 11/7/2) as mobile phase or reverse phase TLC using MeOH/ H_2O in ratio 1/1 as mobile phase.

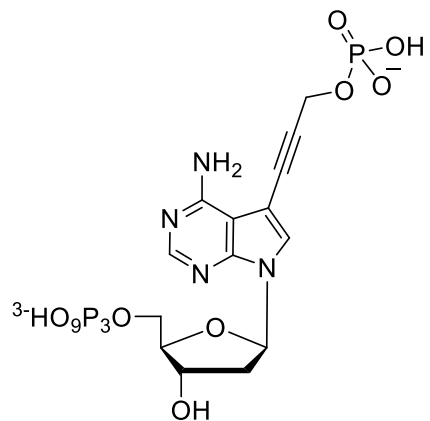
Chemicals were of analytical grade. 3-Dimethylamino-1-propyne, N-methylpropargylamine, were purchased from Merck Life Science. 5-Hexynoic acid, propargylamine, hex-5-yn-1-amine hydrochloride, 5-iodo-2'-deoxyuridine, and 5-iodo-2'-deoxycytidine were purchased from

Fluorochem Ltd. Synthesis and characterization data for 7-iodo-2'-deoxy-7-deazaadenosine 5'-*O*-triphosphate (dA^ITP),²⁰ 5-iodo-2'-deoxyuridine 5'-*O*-triphosphate (dU^ITP),⁸³ 7-iodo-2'-deoxy-7-deazaguanosine 5'-*O*-triphosphate (dG^ITP),⁸⁴ 5-iodo-2'-deoxycytidine 5'-*O*-triphosphate (dC^ITP),⁸² propargyltrimethylammonium iodide,⁹⁵ 3-butynyl-C-phosphonate,⁹⁶ propargyl sulfonate,⁹⁷ and propargyl phosphate⁹⁸ were reported previously.

5.1.2. Synthesis of modified nucleosides and nucleotides

Method A. 1:2 mixture of MeCN/H₂O (0.5 mL) followed by Et₃N (10 equiv.) were added through a septum to an argon-purged flask charged with a halogenated nucleoside triphosphate dN^ITP (N = U, A, G, C) (1 equiv.), a corresponding alkyne (1.5 equiv.) (Figure 14), CuI (10 mol%), PPh₃ (2 mol%) and [Pd(PPh₃)₂Cl₂] (5 mol%). The mixture was stirred for 1 h at 60 °C under argon atmosphere. Solvents were evaporated under vacuum. The product was purified by HPLC with linear gradient of 0.1 M TEAB (triethylammonium bicarbonate) in H₂O to 0.1 M TEAB in H₂O/MeOH (1:1) as eluent in 60 min followed by lyophilization to obtain a solid product.

7-(3-*O*-phosphate-prop-1-yn-1-yl)-2'-deoxy-7-deazaadenosine 5'-*O*-triphosphate (dA^{OP}TP)



The compound **dA^{OP}TP** was prepared from dA^ITP (75 mg, 0.122 mmol) by Method A as described above. The product was purified by reverse-phase HPLC with a linear gradient 10 to 100% of buffer B in buffer A and isolated as a colorless solid (36 mg, 0.057 mmol; 47% yield); ¹H NMR (500.0 MHz, D₂O): 2.50 (ddd, 1H, *J*_{gem} = 14.0 Hz, *J*_{2'a,1'} = 6.3 Hz, *J*_{2'a,3'} = 3.4 Hz, H-2'a); 2.68 (ddd, 1H, *J*_{gem} = 14.0 Hz, *J*_{2'b,1'} = 7.8 Hz, *J*_{2'b,3'} = 6.2 Hz, H-2'b); 4.17 (ddd, 1H, *J*_{gem} = 11.3 Hz, *J*_{5'a,P} = 5.6 Hz, *J*_{5'a,4'} = 4.1 Hz, H-5'a); 4.21 (ddd, 1H, *J*_{gem} = 11.3 Hz, *J*_{5'b,P} = 6.3 Hz, *J*_{5'b,4'} = 4.1 Hz, H-5'b); 4.26 (m, 1H; H-4'); 4.75 (dt, 1H, *J*_{3',2'b} = 6.3 Hz, *J*_{3',2'a} = *J*_{3',4'} = 3.2 Hz, H-3'); 4.79 (d, 2H, *J*_{CH2,P} = 10.6 Hz, C≡CCH₂); 6.62 (dd, 1H, *J*_{1',2'b} = 7.8 Hz, *J*_{1',2'a} = 6.2 Hz, H-1'); 7.82 (s, 1H, H-8); 8.24 (s, 1H, H-2).

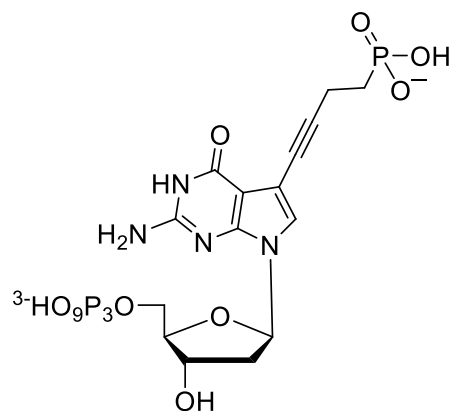
¹³C NMR (125.7 MHz, D₂O): 41.75 (CH₂-2'); 56.78 (d, *J*_{C,P} = 4.9 Hz, C≡CCH₂); 68.46 (d, *J*_{C,P} = 6.8 Hz, CH₂-5'); 73.99 (CH-3'); 80.33 (C≡CCH₂); 86.41 (CH-1'); 88.28 (d, *J*_{C,P} = 8.9 Hz,

CH-4'); 91.80 (d, $J_{C,P} = 6.2$ Hz, $C\equiv CCH_2$); 99.91 (C-7); 105.31 (C-5); 130.78 (CH-8); 150.33 (C-4); 150.54 (CH-2); 156.78 (C-6).

^{31}P NMR (202.4 MHz, D_2O): -22.57 (bt, 1P, $J_{\beta,\alpha} = J_{\beta,\gamma} = 19.8$ Hz, P_β); -10.65 (d, 1P, $J_{\alpha,\beta} = 19.9$ Hz, P_α); -10.19 (d, 1P, $J_{\gamma,\beta} = 19.7$ Hz, P_γ); 1.30 (s, 1P, $C\equiv CCH_2\text{OP}$).

HR MS (ESI⁻) for $\text{C}_{14}\text{H}_{19}\text{O}_{16}\text{N}_4\text{P}_4$ [$\text{M} - \text{H}$]⁻ calcd.: 622.97520 found: 622.97422.

7-(4-Phosphoryl-but-1-yn-1-yl)-2'-deoxy-7-deazaguanosine 5'-O-triphosphate (dG^{PA}TP)



The compound **dG^{PA}TP** was prepared from dG^ITP (46 mg, 0.073 mmol) by Method A as described above. The product was purified by reverse-phase HPLC with a linear gradient 10 to 100% of buffer B in buffer A and isolated as a colorless solid (20.5 mg, 0.032 mmol; 44% yield); ^1H NMR (500.0 MHz, D_2O): 1.89 – 1.98 (m, 2H, CH_2P); 2.38 (ddd, 1H, $J_{\text{gem}} = 14.0$ Hz, $J_{2'a,1'} = 6.2$ Hz, $J_{2'a,3'} = 3.1$ Hz, H-2'a); 2.62 – 2.68 (m, 2H, $C\equiv CCH_2$); 2.68 (ddd, 1H, $J_{\text{gem}} = 14.0$ Hz,

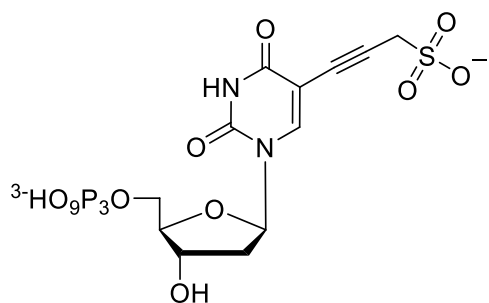
$J_{2'b,1'} = 8.2$ Hz, $J_{2'b,3'} = 6.3$ Hz, H-2'b); 4.11 - 4.17 (m, 2H, H-5'); 4.20 (tdd, 1H; $J_{4',5'} = 4.2$ Hz, $J_{4',3'} = 2.9$ Hz, $J_{4',P} = 1.0$ Hz, H-4'); 4.71 (dt, 1H, $J_{3',2'b} = 6.2$ Hz, $J_{3',2'a} = J_{3',4'} = 3.0$ Hz, H-3'); 6.39 (dd, 1H, $J_{1',2'b} = 8.2$ Hz, $J_{1',2'a} = 6.1$ Hz, H-1'); 7.27 (s, 1H, H-8).

^{13}C NMR (125.7 MHz, D_2O): 16.03 (d, $J_{C,P} = 2.8$ Hz, $C\equiv CCH_2$); 29.34 (d, $J_{C,P} = 132.0$ Hz, CH_2P); 39.97 (CH_2 -2'); 67.72 (d, $J_{C,P} = 5.8$ Hz, CH_2 -5'); 73.31 (CH-3'); 74.98 (d, $J_{C,P} = 1.7$ Hz, $C\equiv CCH_2$); 85.13 (CH-1'); 87.01 (d, $J_{C,P} = 9.0$ Hz, CH-4'); 94.18 (d, $J_{C,P} = 20.5$ Hz, $C\equiv CCH_2$); 101.55 (C-7); 102.21 (C-5); 124.89 (CH-8); 152.78 (C-4); 155.19 (C-2); 162.92 (C-6).

^{31}P NMR (202.4 MHz, D_2O): -22.66 (t, 1P, $J_{\beta,\alpha} = J_{\beta,\gamma} = 20.1$ Hz, P_β); -10.66 (d, 1P, $J_{\alpha,\beta} = 20.4$ Hz, P_α); -10.20 (d, 1P, $J_{\gamma,\beta} = 19.8$ Hz, P_γ); 23.75 (s, 1P, CH_2P).

HR MS (ESI⁻) for $\text{C}_{15}\text{H}_{18}\text{O}_{16}\text{N}_4\text{Na}_3\text{P}_4$ [$\text{M} + 3\text{Na} - 4\text{H}$]⁻ calcd.: 702.93668 found: 702.93555.

5-(3-Sulfo-prop-1-yn-1-yl)-2'-deoxyuridine 5'-O-triphosphate (dU^{SA}TP)



The compound **dU^{SA}TP** was prepared from dU^ITP (53 mg, 0.089 mmol) by Method A as described above.

The product was purified by reverse-phase HPLC with a linear gradient 10 to 100% of buffer B in buffer A and isolated as a colorless solid (44.3 mg, 0.076 mmol; 85% yield); ¹H NMR (500.0 MHz, D₂O): 2.40 (ddd, 1H,

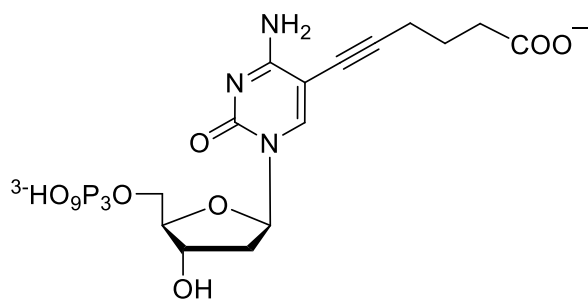
$J_{gem} = 14.2$ Hz, $J_{2'a,1'} = 6.6$ Hz, $J_{2'a,3'} = 4.5$ Hz, H-2'a); 2.43 (ddd, 1H, $J_{gem} = 14.2$ Hz, $J_{2'b,1'} = 7.1$ Hz, $J_{2'b,3'} = 6.0$ Hz, H-2'b); 4.07 (s, 2H, CH₂SO₃H); 4.15 - 4.23 (m, 3H; H-4',5'); 4.61 (ddd, 1H, $J_{3',2'b} = 5.9$ Hz, $J_{3',2'a} = 4.5$ Hz, $J_{3',4'} = 3.2$ Hz, H-3'); 6.25 (t, 1H, $J_{1',2'} = 6.8$ Hz, H-1'); 8.15 (s, 1H, H-6).

¹³C NMR (125.7 MHz, D₂O): 40.21 (CH₂-2'); 45.08 (CH₂SO₃H); 67.45 (d, $J_{C,P} = 5.6$ Hz, CH₂-5'); 72.62 (CH-3'); 77.38 (C≡CCH₂); 87.01 (C≡CCH₂); 87.38 (d, $J_{C,P} = 8.7$ Hz, CH-4'); 88.00 (CH-1'); 101.14 (C-5); 147.40 (CH-6); 152.52 (C-2); 166.66 (C-4).

³¹P NMR (202.4 MHz, D₂O): -22.63 (t, 1P, $J_{\beta,\alpha} = J_{\beta,\gamma} = 20.1$ Hz, P_β); -10.73 (d, 1P, $J_{\alpha,\beta} = 20.3$ Hz, P_α); -10.13 (d, 1P, $J_{\gamma,\beta} = 19.9$ Hz, P_γ).

HR MS (ESI⁻) for C₁₂H₁₆O₁₇N₂P₃S [M - H]⁻ calcd.: 584.93880 found: 584.93835.

5-(5-Carboxy-pent-1-yn-1-yl)-2'-deoxycytidine 5'-O-triphosphate (dC^{CA}TP)



The compound **dC^{CA}TP** was prepared from dC^ITP by (40 mg, 0.068 mmol) Method A as described above. The product was purified by reverse-phase HPLC with a linear gradient 0 to 100% of buffer B in buffer A and isolated as a colorless solid (14.5 mg, 0.025 mmol; 37%

yield); ¹H NMR (500.0 MHz, D₂O): 1.86 (pent, 2H, $J_{CH_2,CH_2} = 7.2$ Hz, CH₂CH₂CH₂COOH); 2.31 (dt, 1H, $J_{gem} = 14.1$ Hz, $J_{2'a,1'} = J_{2'a,3'} = 6.8$ Hz, H-2'a); 2.33 (t, 2H, $J_{CH_2,CH_2} = 7.3$ Hz, CH₂CH₂CH₂COOH); 2.43 (ddd, 1H, $J_{gem} = 14.1$ Hz, $J_{2'b,1'} = 6.3$ Hz, $J_{2'b,3'} = 3.9$ Hz, H-2'b); 2.50 (d, 2H, $J_{CH_2,CH_2} = 7.1$ Hz, CH₂CH₂CH₂COOH); 4.14 - 4.24 (m, 3H; H-4',5'); 4.60 (dt, 1H, $J_{3',2'a} = 6.4$ Hz, $J_{3',2'b} = J_{3',4'} = 3.5$ Hz, H-3'); 6.27 (t, 1H, $J_{1',2'} = 6.7$ Hz, H-1'); 8.03 (s, 1H, H-6).

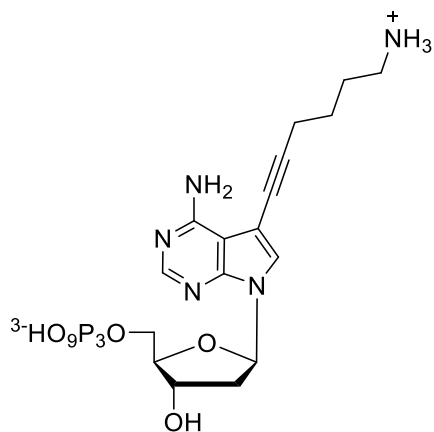
^{13}C NMR (125.7 MHz, D_2O): 20.71 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH}$); 26.56 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH}$); 38.65 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH}$); 40.94 ($\text{CH}_2\text{-}2'$); 67.33 (d, $J_{\text{C,P}} = 5.4$ Hz, $\text{CH}_2\text{-}5'$); 72.51 ($\text{CH}\text{-}3'$); 72.73 ($\text{C}\equiv\text{CCH}_2$); 87.33 (d, $J_{\text{C,P}} = 8.6$ Hz, $\text{CH}\text{-}4'$); 87.13 ($\text{CH}\text{-}1'$); 95.37 (C-5); 99.37 ($\text{C}\equiv\text{CCH}_2$); 145.71 ($\text{CH}\text{-}6$); 158.03 (C-2); 167.26 (C-4); 184.68 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH}$).

^{31}P NMR (202.4 MHz, D_2O): -22.57 (bt, 1P, $J_{\beta,\alpha} = J_{\beta,\gamma} = 19.5$ Hz, P_β); -10.70 (d, 1P, $J_{\alpha,\beta} = 20.0$ Hz, P_α); -9.76 (d, 1P, $J_{\gamma,\beta} = 19.0$ Hz, P_γ).

HR MS (ESI $^-$) for $\text{C}_{15}\text{H}_{21}\text{O}_{15}\text{N}_3\text{P}_3$ $[\text{M} - \text{H}]^-$ calcd.: 576.01910 found: 576.01801.

Method B. Anhydrous DMF followed by Et_3N (4.5 equiv.) were added through a septum to an argon-purged flask charged with a halogenated nucleoside triphosphate $\text{dN}^{\text{I}}\text{TP}$ ($\text{N} = \text{U}, \text{A}, \text{G}$) (1 equiv.), a corresponding alkyne (7.5 equiv.) (Figure 25), CuI (1.2 equiv.), and $\text{Pd}(\text{PPh}_3)_4$ (0.6 equiv.). The mixture was stirred at room temperature for 5 hours under argon atmosphere. After 5 hours, the solvent was evaporated under vacuum. The purification was done by reverse-phase HPLC with a linear gradient of MeOH in 0.1 M TEAB buffer (pH 7.6). In the case of $\text{dU}^{\text{NMe}}\text{TP}$, an additional purification using a Sepharose FF column with a linear gradient of 800 mM TEAB solution in H_2O (0–100%) was required.

7-(6-Aminohex-1-yn-1-yl)-2'-deoxy-7-deazaadenosine 5'-O-triphosphate ($\text{dA}^{\text{NH}_2}\text{TP}$)



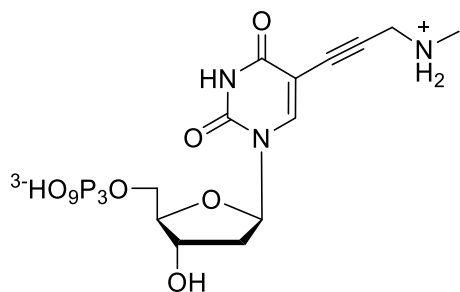
The compound $\text{dA}^{\text{NH}_2}\text{TP}$ was prepared from $\text{dA}^{\text{I}}\text{TP}$ (62.3 mg, 0.068 mmol) by Method B as described above. The product was purified by reverse-phase HPLC with a linear gradient 10 to 100% of buffer B in buffer A and isolated as a colorless solid (29.6 mg, 0.038 mmol; 55% yield); ^1H NMR (500.0 MHz, D_2O): 1.70 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$); 1.92 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$); 2.50 (ddd, 1H, $J_{\text{gem}} = 13.9$ Hz, $J_{2'a,1} = 6.4$ Hz, $J_{2'a,3'} = 3.5$ Hz, H-2'a); 2.56 (t, 2H, $J_{\text{CH}_2,\text{CH}_2} = 6.4$ Hz, $\text{C}\equiv\text{CCH}_2$); 2.62 (ddd, 1H, $J_{\text{gem}} = 13.9$ Hz, $J_{2'b,1'} = 7.4$ Hz, $J_{2'b,3'} = 6.0$ Hz, H-2'b); 3.08 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$); 4.17 (m, 1H, H-5'a); 4.20 – 4.27 (m, 2H, H-5'b,4'); 4.78 (m, 1H, H-3'); 6.59 (bt, 1H, $J_{1',2'b} = J_{1',2'a} = 6.9$ Hz, H-1'); 7.71 (s, 1H, H-8); 8.11 (s, 1H, H-2). ^{13}C NMR (125.7 MHz, D_2O): 20.38 ($\text{C}\equiv\text{CCH}_2$); 26.46 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$); 27.98 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$); 41.04 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$); 41.46 ($\text{CH}_2\text{-}2'$); 67.44 (d, $J_{\text{C,P}} = 5.7$ Hz, $\text{CH}_2\text{-}5'$);

73.24 (CH-3'); 75.35 (C≡CCH₂); 85.25 (CH-1'); 87.44 (d, $J_{C,P}$ = 9.2 Hz, CH-4'); 95.14 (C≡CCH₂); 99.17 (C-7); 104.36 (C-5); 128.22 (CH-8); 149.82 (C-4); 152.87 (CH-2); 158.39 (C-6).

³¹P NMR (202.4 MHz, D₂O): -21.89 (t, 1P, $J_{\beta,\alpha} = J_{\beta,\gamma} = 19.9$ Hz, P_β); -10.50 (d, 1P, $J_{\alpha,\beta} = 19.7$ Hz, P_α); -8.43 (d, 1P, $J_{\gamma,\beta} = 20.1$ Hz, P_γ).

HR MS (ESI⁻) for C₁₇H₂₅O₁₂N₅P₃ [M – H]⁻ calcd.: 584.07125 found: 584.07107.

5-(3-Methylaminoprop-1-yn-1-yl)-2'-deoxyuridine 5'-O-triphosphate (dU^{NMe}TP)



The compound **dU^{NMe}TP** was prepared from dU^ITP (30 mg, 0.033 mmol) by Method B as described above. The product was purified by reverse-phase HPLC with a linear gradient 0 to 100% of buffer B in buffer A. The appropriate fractions were collected, evaporated, and repurified by ion-exchange HPLC (0 to 100% of 800 mM TEAB in H₂O).

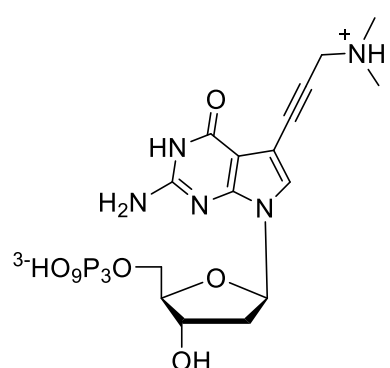
The pure product was isolated as a colorless solid (10.5 mg, 0.014 mmol; 43% yield); ¹H NMR (500.0 MHz, D₂O): 2.40 (dt, 1H, $J_{gem} = 14.1$ Hz, $J_{2'a,1'} = J_{2'a,3'} = 6.1$ Hz, H-2'a); 2.47 (ddd, 1H, $J_{gem} = 14.1$ Hz, $J_{2'a,1'} = 6.4$ Hz, $J_{2'a,3'} = 5.2$ Hz, H-2'b); 2.80 (s, 3H, CH₃NH); 4.04 and 4.09 (2×d, 2×1H, $J_{gem} = 16.6$ Hz, C≡CCH₂); 4.20 (m, 1H; H-4'); 4.26 (ddd, 1H, $J_{gem} = 11.9$ Hz, $J_{5'a,P} = 5.3$ Hz, $J_{5'a,4'} = 2.4$ Hz, 5'a); 4.31 (ddd, 1H, $J_{gem} = 11.9$ Hz, $J_{5'b,P} = 3.8$ Hz, $J_{5'b,4'} = 2.6$ Hz, 5'a); 4.67 (m, 1H, H-3'); 6.30 (t, 1H, $J_{1',2'a} = J_{1',2'b} = 6.1$ Hz, H-1'); 8.49 (s, 1H, H-6).

¹³C NMR (125.7 MHz, D₂O): 34.61 (CH₃NH); 40.60 (C≡CCH₂); 41.57 (CH₂-2'); 66.70 (d, $J_{C,P} = 5.5$ Hz, CH₂-5'); 71.48 (CH-3'); 81.24 (C≡CCH₂); 85.70 (C≡CCH₂); 87.80 (d, $J_{C,P} = 9.3$ Hz, CH-4'); 87.81 (CH-1'); 100.02 (C-5); 148.68 (CH-6); 152.63 (C-2); 166.53 (C-4).

³¹P NMR (202.4 MHz, D₂O): -21.65 (t, 1P, $J_{\beta,\alpha} = J_{\beta,\gamma} = 19.6$ Hz, P_β); -10.62 (d, 1P, $J_{\alpha,\beta} = 19.6$ Hz, P_α); -7.44 (bs, 1P, P_γ).

HR MS (ESI⁻) for C₁₃H₁₉O₁₄N₃P₃ [M – H]⁻ calcd.: 534.00798 found: 534.00848.

7-(3-Dimethylaminoprop-1-yn-1-yl)-2'-deoxy-7-deazaguanosine 5'-O-triphosphate (dG^{NMe2}TP)



The compound **dG^{NMe2}TP** was prepared from dG^ITP (44.8 mg, 0.048 mmol) by Method B as described above. The product was purified by reverse-phase HPLC with a linear gradient 10 to 100% of buffer B in buffer A and isolated as a colorless solid (10.3 mg, 0.013 mmol; 27% yield); ¹H NMR (500 MHz, D₂O): 2.43 (ddd, 1H, *J*_{gem} = 14.0 Hz, *J*_{2'a,1'} = 6.3 Hz, *J*_{2'a,3'} = 3.3 Hz, H-2'a); 2.63 (ddd, 1H, *J*_{gem} = 14.0 Hz, *J*_{2'b,1'} = 7.7 Hz, *J*_{2'b,3'} = 6.1 Hz, H-2'b);

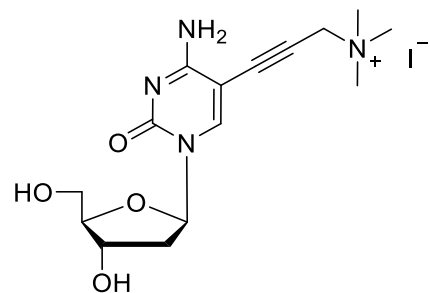
3.01 (s, 6H, (CH₃)₂N); 4.13 (dt, 1H, *J*_{gem} = 12.2 Hz, *J*_{5'a,P} = *J*_{5'a,4'} = 4.5 Hz, H-5'a); 4.18 – 4.25 (m, 2H, H-5'b,4'); 4.21 (s, 2H, C≡CCH₂); 6.46 (dd, 1H, *J*_{1',2'b} = 7.7 Hz, *J*_{1',2'a} = 6.3 Hz, H-1'); 7.61 (s, 1H, H-8). *The signal of hydrogen H-3' was overlapped by the signal of H₂O.*

¹³C NMR (125.7 MHz, D₂O): 40.92 (CH₂-2'); 44.18 ((CH₃)₂N); 50.06 (C≡CCH₂); 67.43 (d, *J*_{C,P} = 5.3 Hz, CH₂-5'); 73.31 (CH-3'); 81.41 and 84.93 (C≡CCH₂); 85.22 (CH-1'); 87.47 (d, *J*_{C,P} = 9.2 Hz, CH-4'); 99.29 (C-7); 101.89 (C-5); 127.52 (CH-8); 152.94 (C-4); 155.43 (C-2); 162.89 (C-6).

³¹P NMR (202.4 MHz, D₂O): -22.16 (t, 1P, *J*_{β,α} = *J*_{β,γ} = 19.7 Hz, P_β); -10.50 (d, 1P, *J*_{α,β} = 19.8 Hz, P_α); -7.90 (bs, 1P, P_γ).

HR MS (ESI⁻) for C₁₆H₂₃O₁₃N₅P₃ [M – H]⁻ calcd.: 586.05052 found: 586.05017.

5-(3-Trimethylaminoprop-1-yn-1-yl)-2'-deoxycytidine iodide (dC^{NMe3})



Anhydrous DMF followed by Et₃N (0.4 mL, 2.83 mmol) was added through a septum to an argon-purged flask charged with 5-iodo-2'-deoxycytidine dC^I (100 mg, 0.28 mmol), propargyltrimethylammonium iodide (446.2 mg, 1.98 mmol), CuI (43.2 mg, 0.23 mmol), TPPTS (96.61 mg, 0.17 mmol), and Pd(OAc)₂ (25.4 mg, 0.11 mmol) (Figure 26). The mixture

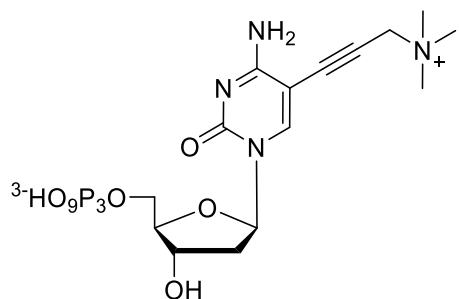
was stirred for 2.5 h at 60 °C under argon atmosphere. Then, the solvent was evaporated under vacuum. The reaction was monitored by normal phase TLC using DCM/MeOH in ratio 2/1 as mobile phase. The product was purified by reverse-phase HPLC with a linear gradient of MeOH and isolated as an orange-colored solid (50.8 mg, 0.11 mmol; 40% yield).

^1H NMR (500.0 MHz, D_2O): 2.30 (dt, 1H, $J_{\text{gem}} = 14.1$ Hz, $J_{2'a,1'} = J_{2'a,3'} = 6.4$ Hz, H-2'a); 2.50 (ddd, 1H, $J_{\text{gem}} = 14.1$ Hz, $J_{2'b,1'} = 6.5$ Hz, $J_{2'b,3'} = 4.7$ Hz, H-2'b); 3.26 (s, 9H, $(\text{CH}_3)_3\text{N}$); 3.78 (dd, 1H; $J_{\text{gem}} = 12.6$ Hz, $J_{5'a,4'} = 4.9$ Hz, H-5'a); 3.88 (dd, 1H; $J_{\text{gem}} = 12.6$ Hz, $J_{5'b,4'} = 3.3$ Hz, H-5'b); 4.08 (td, 1H, (dd, 1H; $J_{4',5'a} = J_{4',3'} = 4.7$ Hz, $J_{4',5'b} = 3.3$ Hz, H-4'); 4.43 (dt, 1H, $J_{3',2'} = 6.7$ Hz, $J_{3',2'} = J_{3',4'} = 4.5$ Hz, H-3'); 4.51 (s, 2H, $\text{C}\equiv\text{CCH}_2$); 6.20 (t, 1H, $J_{1',2'a} = J_{1',2'b} = 6.2$ Hz, H-1'); 8.37 (s, 1H, H-6).

^{13}C NMR (125.7 MHz, D_2O): 41.79 (CH_2 -2'); 54.71 ($(\text{CH}_3)_3\text{N}$); 59.11 ($\text{C}\equiv\text{CCH}_2$); 62.85 (CH_2 -5'); 71.85 (CH -3'); 84.51 and 84.97 ($\text{C}\equiv\text{CCH}_2$); 88.78 and 88.87 (CH -1',4'); 92.02 (C-5); 149.05 (CH -6); 157.83 (C-2); 166.92 (C-4).

HR MS (ESI⁺) for $\text{C}_{15}\text{H}_{23}\text{O}_4\text{N}_4$ $[\text{M} + \text{H}]^+$ calcd.: 323.17138 found: 323.17155.

5-(3-Trimethylaminoprop-1-yn-1-yl)-2'-deoxycytidine 5'-O-triphosphate ($\text{dC}^{\text{NMe}_3}\text{TP}$)



dC^{NMe_3} (50.8 mg, 0.11 mmol) was dried overnight on high vacuum and then dissolved in $\text{PO}(\text{OMe})_3$ (1 mL) in a sealed flask under argon atmosphere (Figure 26). The solution was cooled to 0 °C and POCl_3 (0.013 mL, 0.17 mmol) was added under constant stirring. The reaction was monitored by normal phase TLC using DCM/MeOH in ratio 7/1 as mobile

phase. After 45 min, a precooled solution of $(\text{NH}_4\text{Bu}_3)_2\text{H}_2\text{P}_2\text{O}_7$ (tributylammonium pyrophosphate) (309.5 mg, 0.56 mmol) in MeCN (3 mL) was added followed by Bu_3N (0.1 mL, 0.45 mmol), and the resulting mixture was stirred for another 1 hour at 0 °C. Then, the reaction was stopped with an excess of 2M TEAB solution, and the solvents were evaporated. The crude product was purified by reverse-phase HPLC with a linear gradient 0 to 100% of buffer B in buffer A. The appropriate fractions were collected, evaporated, and repurified by ion-exchange HPLC (0 to 100% of 800 mM TEAB in H_2O). The pure product was isolated as an orange-colored solid (5 mg, 0.007 mmol; 6% yield).

^1H NMR (500.0 MHz, D_2O): 2.33 (dt, 1H, $J_{\text{gem}} = 14.1$ Hz, $J_{2'a,1'} = J_{2'a,3'} = 6.4$ Hz, H-2'a); 2.48 (ddd, 1H, $J_{\text{gem}} = 14.1$ Hz, $J_{2'a,1'} = 6.3$ Hz, $J_{2'a,3'} = 4.0$ Hz, H-2'b); 3.27 (s, 9H, $(\text{CH}_3)_3\text{N}$); 4.20 - 4.30 (m, 3H; H-4',5'); 4.52 (s, 2H, $\text{C}\equiv\text{CCH}_2$); 4.64 (m, 1H, H-3'); 6.28 (t, 1H, $J_{1',2'a} = J_{1',2'b} = 6.4$ Hz, H-1'); 8.44 (s, 1H, H-6).

^{13}C NMR (125.7 MHz, D_2O): 41.95 ($\text{CH}_2\text{-}2'$); 54.90 ($(\text{CH}_3)_3\text{N}$); 59.16 ($\text{C}\equiv\text{CCH}_2$); 67.07 (d, $J_{\text{C,P}} = 5.5$ Hz, $\text{CH}_2\text{-}5'$); 72.33 ($\text{CH-}3'$); 84.42 ($\text{C}\equiv\text{CCH}_2$); 85.23 ($\text{C}\equiv\text{CCH}_2$); 87.96 (d, $J_{\text{C,P}} = 9.2$ Hz, $\text{CH-}4'$); 88.68 ($\text{CH-}1'$); 92.4 (C-5); 149.10 (CH-6); 157.97 (C-2); 166.94 (C-4).

^{31}P NMR (202.4 MHz, D_2O): -22.43 (t, 1P, $J_{\beta,\alpha} = J_{\beta,\gamma} = 20.1$ Hz, P_β); -11.02 (d, 1P, $J_{\alpha,\beta} = 20.1$ Hz, P_α); -9.09 (bs, 1P, P_γ).

HR MS (ESI $^-$) for $\text{C}_{15}\text{H}_{24}\text{O}_{13}\text{N}_4\text{P}_3$ [$\text{M} - 2\text{H}$] $^{2-}$ calcd.: 561.05472 found: 561.05527.

5-(2-Phenyl-1-ethyn-1-yl)-2'-deoxyuridine 5'-*O*-triphosphate (**dU^{EPh}TP**) and 5-(pent-1-yn-1-yl)-2'-deoxycytidine 5'-*O*-triphosphate (**dC^{EAlk}TP**) (Figure 35) were synthesized according to the described protocol.³⁷

5.2. Biochemistry

5.2.1. General remarks – biochemistry

Synthetic ONs were purchased from Eurofins Genomics (Germany), Generi Biotech (Czech Republic), Biomers (Germany), and GenScript Biotech (Netherlands). Natural nucleoside triphosphates (dATP, dGTP, dTTP, dCTP) were purchased from ThermoScientific. KOD XL and Pwo DNA polymerases and corresponding polymerase reaction buffers were supplied by Merck Life Science; Vent(exo $-$) DNA polymerase and a corresponding polymerase reaction buffer were purchased from New England Biolabs. DNase I and a corresponding polymerase reaction buffer were purchased from ThermoFisher Scientific. The human pooled plasma was purchased from Biowest. DNA recovery in experiments with DNase I was calculated using ImageJ analyzer. Milli-Q water was used for all experiments. Other chemicals were of analytical grade. Streptavidin magnetic particles (Roche) were purchased from Merck Life Science. ONs' purification was performed using QIAquick PCR Purification Kit (Qiagen), Agencourt AMPure XP magnetic particles (Beckman Coulter Life science - GE Healthcare), and mini Quick Spin Oligo Columns (Roche). Sanger sequencing was done by SeqMe (Czech Republic). Used kits for gel extraction Pur-A-Lyze Maxi Dialysis Kit (Sigma Aldrich) and Amicon Ultra-0.5 Centrifugal Filters were purchased from Merck Life Science. Enzymatic reactions and annealing reactions were performed in a thermal cycler Biometra Trio (Analytik Jena). To analyze samples under denaturing conditions (50 °C, 1X TBE, pH 8.5), either 12.5% or 20% dPAGE (acrylamide/bisacrylamide 19:1, 25%

urea) was used. PAGE stop solution contained: 95% [v/v] formamide, 0.5 mM EDTA, 0.025% [w/v] bromophenol blue and 0.025% [w/v] xylene cyanol FF, 0.025% [w/v] SDS in Milli-Q water. To analyze samples under native conditions (0.5X TBE buffer, 120 V, 75 min), 2% agarose gel (Serva) was used; 6X DNA Gel Loading Dye (60 mM EDTA, 10 mM Tris-HCl (pH 7.6), 60% glycerol, 0.03% bromophenol blue, 0.03% xylene cyanol FF, ThermoFisher Scientific). For annealing experiments with superanionic ssONs and dsDNA, GelRed intercalator (Biotium) was added into agarose gel. All gels were analyzed by fluorescence imaging using Amersham Typhoon (Cytiva). Samples were concentrated on CentriVap Vacuum Concentrator system (Labconco). The analysis of ONs was done by UHPLC-MS-ESI (Agilent 1290 Infinity II Bio LC System with DAD detector and mass spectrometer MSD XT). The analysis was carried out according to standard procedures using a system of mobile phases A [12.2 mM Et₃N (triethylamine), 300 mM HFIP (1,1,1,3,3,3-hexafluoro-2-propanol) in H₂O] and B (12.2 mM Et₃N, 300 mM HFIP in MeOH) using bioZen Oligo column 1.7 μ m 2.1 $\times$ 150 mm (Phenomenex). Deconvoluted MS spectra were obtained using UniDec⁷. The MALDI-TOF spectra were measured on a UltrafleXtreme MALDI-TOF/TOF (Bruker) mass spectrometer with a 1 kHz smart beam II laser. The matrix consisted of 3-hydroxypicolinic acid (HPA)/picolinic acid (PA)/ammonium tartrate in ratio 9:1:1.

5.2.2. PEX – Single incorporation (one modified dN^RTP)

The reaction mixture (10 μ L) contained one of 19-mer templates Temp^{Oligo1A} / Temp^{Oligo1T} / Temp^{Oligo1C} / Temp^{Oligo1G} (3 μ M, 0.75 μ L), primer Prim^{248short}-FAM (3 μ M, 0.5 μ L), dGTP (dTTP in the case of Temp^{Oligo1G}) (0.25 mM, 1 μ L), modified dN^RTP of study (dA^{OP}TP or dA^{NH₂}TP / dU^{SA}TP or dU^{NMe}TP / dC^{CA}TP or dC^{NMe₃}TP / dG^{PA}TP or dG^{NMe₂}TP) (2 mM, 1 μ L), either KOD XL or Vent(exo–) DNA polymerase (units specification in Tables 3 and 12) and a corresponding reaction buffer (10X, 1 μ L) as supplied by the manufacturer. The reaction mixture was incubated for 30 min at 60 °C, stopped by addition of PAGE stop solution (10 μ L) and denatured for 3 min at 95 °C. Samples were analyzed on 12.5% dPAGE and visualized using fluorescence imaging (Figures 16 and 28).

5.2.3. PEX – Multiple incorporation (one modified dN^RTP)

The reaction mixture (10 μ L) contained 31-mer template Temp^{Prb4basII} (3 μ M, 0.75 μ L), primer Prim^{248short}-FAM (3 μ M, 0.5 μ L), appropriate natural dNTPs (0.25 mM, 1 μ L each), modified dN^RTP of study (dA^{OP}TP or dA^{NH2}TP; dU^{SA}TP or dU^{NMe}TP; dC^{CA}TP or dC^{NMe3}TP; dG^{PA}TP or dG^{NMe2}TP) (2 mM, 1 μ L), either KOD XL or Vent(exo–) DNA polymerase (specifications in Tables 4 and 13) and a corresponding reaction buffer (10X, 1 μ L) as supplied by the manufacturer. The positive control contained either 0.3 U of KOD XL or 0.5 U of Vent(exo–) DNA polymerase and natural dNTPs (1 mM, 1 μ L). The reaction mixture was incubated for 30 min at 60 °C, stopped by addition of PAGE stop solution (10 μ L), and denatured for 3 min at 95 °C. Samples were analyzed on 12.5% dPAGE and visualized using fluorescence imaging (Figures 18 and 30).

5.2.4. PEX – Multiple incorporation (two and three modified dN^RTPs)

Method C1. The reaction mixture (10 μ L) contained 31-mer template Temp^{Prb4basII} (3 μ M, 0.75 μ L), primer Prim^{248short}-FAM (3 μ M, 0.5 μ L), appropriate natural dNTPs (0.25 mM, 1 μ L), combinations of modified dN^RTPs (dU^{SA}TP, dC^{CA}TP, dA^{OP}TP, dG^{PA}TP) (1 μ L, see specifications in Table 28), Vent(exo–) DNA polymerase (Table 28), and the enzyme reaction buffer (10X, 1 μ L) as supplied by the manufacturer. The reaction mixture was incubated for 30 min at 60 °C, stopped by addition of PAGE stop solution (10 μ L) and denatured for 3 min at 95 °C. Samples were analyzed on 12.5% dPAGE and visualized using fluorescence imaging (Figure 20B).

Table 28. Reaction condition specifications for multiple incorporation of combinations of anionic dN^RTPs.

Lanes in Figure 20B	Natural dNTPs	Vent(exo–)	dC ^{CA} TP	dG ^{PA} TP	dA ^{OP} TP	dU ^{SA} TP
2	dGTP, dTTP	0.5 U	0.25 mM	–	2 mM	–
3	dATP, dTTP	0.5 U	2 mM	2 mM	–	–
4	dATP, dGTP	0.5 U	2 mM	–	–	2 mM
5	dCTP, dTTP	0.5 U	–	2 mM	2 mM	–
6	dCTP, dGTP	1.5 U	–	–	2 mM	2 mM
7	dCTP, dATP	0.5 U	–	2 mM	–	2 mM
8	dTTP	0.5 U	0.25 mM	2 mM	2 mM	–
9	dGTP	0.5 U	0.25 mM	–	2 mM	2 mM
10	dATP	0.5 U	2 mM	2 mM	–	2 mM
11	dCTP	0.5 U	–	2 mM	2 mM	2 mM

Method C2. The reaction mixture (10 μ L) contained 31-mer template Temp^{Prb4basII} (3 μ M, 0.75 μ L), primer Prim^{248short}-FAM (3 μ M, 0.5 μ L), appropriate natural dNTPs (0.25 mM, 1 μ L), combinations of two and three modified dN^RTPs (dA^{NH₂}TP, dU^{NMe}TP, dG^{NMe₂}TP, dC^{NMe₃}TP) (1 μ L, see specifications in Table 29), KOD XL DNA polymerase (Table 29), and the enzyme reaction buffer (10X, 1 μ L) as supplied by the manufacturer. The reaction mixture was incubated for 30 min at 72 °C, stopped by addition of PAGE stop solution (10 μ L), and denatured for 5 min at 95 °C. Samples were analyzed on 20% dPAGE and visualized using fluorescence imaging (Figure 31B).

Table 29. Reaction condition specifications for multiple incorporation of combinations of cationic dN^RTPs.

Lanes in Figure 31B	Natural dNTPs	KOD XL	dA ^{NH₂} TP	dU ^{NMe} TP	dG ^{NMe₂} TP	dC ^{NMe₃} TP
3	dCTP, dGTP	0.01 U	0.25 mM	2 mM	–	–
4	dCTP, dTTP	0.1 U	0.25 mM	–	2 mM	–
5	dGTP, dTTP	0.2 U	0.25 mM	–	–	2 mM
6	dCTP, dATP	0.1 U	–	2 mM	2 mM	–
7	dATP, dGTP	0.7 U	–	2 mM	–	2 mM
8	dATP, dTTP	0.2 U	–	–	2 mM	2 mM
9	dCTP	0.1 U	0.25 mM	2 mM	2 mM	–
10	dGTP	0.5 U	0.25 mM	2 mM	–	2 mM
11	dTTP	0.6 U	0.25 mM	–	2 mM	2 mM
12	dATP	0.5 U	–	2 mM	2 mM	2 mM

5.2.5. PEX – Multiple incorporation of all four modified dN^RTPs

Method D1. The reaction mixture (10 μ L) contained 31-mer template Temp^{Prb4basII} (3 μ M, 0.75 μ L), primer Prim^{248short}-FAM (3 μ M, 0.5 μ L), dC^{CA}TP (0.25 mM, 1 μ L), dG^{PA}TP (2 mM, 1 μ L), dU^{SA}TP (2 mM, 1 μ L), dA^{OP}TP (2 mM, 1 μ L), Vent(exo–) DNA polymerase (1 U), and the enzyme reaction buffer (10X, 1 μ L) as supplied by the manufacturer. The reaction mixture was incubated for 30 min at 60 °C, stopped by addition of PAGE stop solution (10 μ L) and denatured for 5 min at 95 °C. Samples were analyzed by 12.5% dPAGE and visualized using fluorescence imaging (Figure 21B, lanes 1-2).

Method D2. The reaction mixture (10 μ L) contained one of two templates (Temp^{MO43} / Temp^{MO61}) (3 μ M, 0.75 μ L), primer Prim^{248short}-FAM (3 μ M, 0.5 μ L), dC^{CA}TP (0.5 mM, 1 μ L), dG^{PA}TP (2 mM, 1 μ L), dU^{SA}TP (2 mM, 1 μ L), dA^{OP}TP (4 mM, 1 μ L), Vent(exo–) DNA

polymerase (1 U), and the enzyme reaction buffer (10X, 1 μ L) as supplied by the manufacturer. Positive controls contained 1 U of Vent(exo-) DNA polymerase and natural dNTPs (1 mM, 1 μ L). The reaction mixture was incubated for 40 min at 60 °C, stopped by addition of PAGE stop solution (10 μ L) and denatured for 5 min at 95 °C. Samples were analyzed by 12.5% dPAGE and visualized using fluorescence imaging (Figure 21B, lanes 3-6).

Method D3. The reaction mixture (10 μ L) contained one of three templates (Temp^{FVL-A}/Temp^{MO120}/Temp^{MO150}) (3 μ M, 0.75 μ L), primer Prim^{LT25TH}-FAM (3 μ M, 0.5 μ L), **dC^{CA}TP** (1 mM, 1 μ L), **dG^{PA}TP** (2 mM, 1 μ L), **dU^{SA}TP** (2 mM, 1 μ L), **dA^{OP}TP** (4 mM, 1 μ L), Vent(exo-) DNA polymerase (1 U), and the enzyme reaction buffer (10X, 1 μ L) as supplied by the manufacturer. Positive controls contained 0.5 U of Vent(exo-) DNA polymerase and natural dNTPs (1 mM, 1 μ L). The reaction mixture was incubated for 40 min at 60 °C, stopped by addition of PAGE stop solution (10 μ L) and denatured for 5 min at 95 °C. Samples were analyzed by 12.5% dPAGE and visualized using fluorescence imaging (Figure 21B – lanes 7-12).

Method D4. The reaction mixture (10 μ L) contained one of three templates (31-mer Temp^{Prb4basII} / 43-mer Temp^{MO43}/ 61-mer Temp^{MO61}) (3 μ M, 0.75 μ L), primer Prim^{248short}-FAM (3 μ M, 0.5 μ L), **dA^{NH2}TP** (0.25 mM, 1 μ L), **dU^{NMe}TP** (2 mM, 1 μ L), **dG^{NMe2}TP** (2 mM, 1 μ L), **dC^{NMe3}TP** (2 mM, 1 μ L), KOD XL DNA polymerase (0.6 U), and the enzyme reaction buffer (10X, 1 μ L) as supplied by the manufacturer. Positive controls contained 0.3 U of KOD XL DNA polymerase and natural dNTPs (1 mM, 1 μ L). The reaction mixture was incubated for 30 min at 72 °C, stopped by addition of PAGE stop solution (10 μ L), and denatured for 5 min at 95 °C. Samples were analyzed on 20% dPAGE and visualized using fluorescence imaging (Figure 32B – lanes 1-6). In attempts to remove the smearing of the bands, samples were additionally analyzed on 20% dPAGE using 1X TBE buffer with pH 9.5 (Figure 52A – lanes 2-7), 10.5 (Figure 52B – lanes 2-7), and 11.9 (Figure 52C – lanes 2-7).

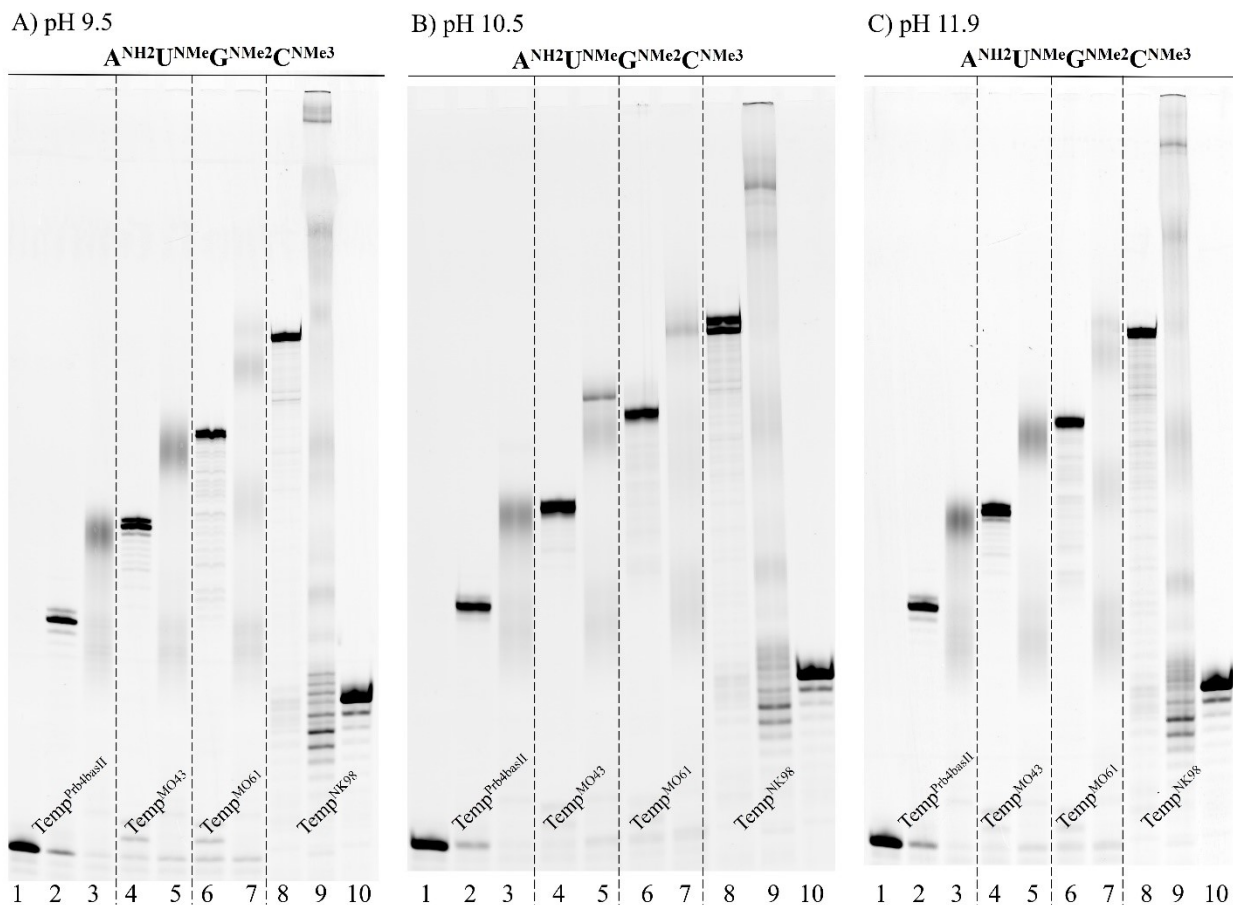


Figure 52. Denaturing PAGE analysis of PEX reactions using 1X TBE buffer with higher pH values: A) pH 9.5, B) pH 10.5, C) pH 11.9: (lane 1) primer Prim^{248short}-FAM; template Temp^{Prb4basII}: (lane 2) natural dNTPs; (lane 3) dA^{NH2}TP, dU^{NMe}TP, dG^{NMe2}TP, dC^{NMe3}TP; template Temp^{MO43}: (lane 4) natural dNTPs; (lane 5) dA^{NH2}TP, dU^{NMe}TP, dG^{NMe2}TP, dC^{NMe3}TP; template Temp^{MO61}: (lane 6) natural dNTPs; (lane 7) dA^{NH2}TP, dU^{NMe}TP, dG^{NMe2}TP, dC^{NMe3}TP; template Temp^{NK98}: (lane 8) natural dNTPs; (lane 9) dA^{NH2}TP, dU^{NMe}TP, dG^{NMe2}TP, dC^{NMe3}TP; (lane 10) primer Prim^{NK98pr1}-FAM.

Method D5. The reaction mixture (10 μ L) contained 98-mer template Temp^{NK98} (3 μ M, 0.75 μ L), primer Prim^{NK98pr1}-FAM (3 μ M, 0.5 μ L), dA^{NH2}TP (0.25 mM, 1 μ L), dU^{NMe}TP (2 mM, 1 μ L), dG^{NMe2}TP (2 mM, 1 μ L), dC^{NMe3}TP (2 mM, 1 μ L), MgSO₄ (25 mM, 0.6 μ L), KOD XL DNA polymerase (2 U), and the enzyme reaction buffer (10X, 1 μ L) as supplied by the manufacturer. Positive control contained 0.35 U of KOD XL DNA polymerase and natural dNTPs (1 mM, 1 μ L). The reaction mixture was incubated for 30 min at 72 $^{\circ}$ C, stopped by addition of PAGE stop solution (10 μ L), and denatured for 5 min at 95 $^{\circ}$ C. Samples were analyzed on 20% dPAGE and visualized using fluorescence imaging (Figure 32B – lanes 7-8). In attempts to remove

the smearing of the bands, samples were additionally analyzed on 20% dPAGE using 1X TBE buffer with pH 9.5 (Figure 52A – lanes 8-9), 10.5 (Figure 52B – lanes 8-9), and 11.9 (Figure 52C – lanes 8-9).

Method D6. The reaction mixture (10 μ L) contained one of three templates (31-mer Temp^{Prb4basII} / 43-mer Temp^{MO43}/ 61-mer Temp^{MO61}) (3 μ M, 0.75 μ L), primer Prim^{248short}-FAM (3 μ M, 0.5 μ L), **dA^{NH2}TP** (0.25 mM, 1 μ L), **dU^{EPh}TP** (2 mM, 1 μ L), **dG^{PA}TP** (2 mM, 1 μ L), **dC^{EAlk}TP** (2 mM, 1 μ L), KOD XL DNA polymerase (0.1 U), and the enzyme reaction buffer (10X, 1 μ L) as supplied by the manufacturer. Positive controls contained 0.3 U of KOD XL DNA polymerase and natural dNTPs (1 mM, 1 μ L). The reaction mixture was incubated for 30 min at 72 °C, stopped by addition of PAGE stop solution (10 μ L), and denatured for 5 min at 95 °C. Samples were analyzed on 20% dPAGE and visualized using fluorescence imaging (Figure 36B – lanes 1-6).

Method D7. The reaction mixture (10 μ L) contained 98-mer template Temp^{NK98} (3 μ M, 0.75 μ L), primer Prim^{NK98pr1}-FAM (3 μ M, 0.5 μ L), **dA^{NH2}TP** (0.25 mM, 1 μ L), **dU^{EPh}TP** (2 mM, 1 μ L), **dG^{PA}TP** (2 mM, 1 μ L), **dC^{EAlk}TP** (2 mM, 1 μ L), KOD XL DNA polymerase (0.2 U), and the enzyme reaction buffer (10X, 1 μ L) as supplied by the manufacturer. Positive control contained 0.35 U of KOD XL DNA polymerase and natural dNTPs (1 mM, 1 μ L). The reaction mixture was incubated for 30 min at 60 °C, stopped by addition of PAGE stop solution (10 μ L), and denatured for 5 min at 95 °C. Samples were analyzed on 20% dPAGE and visualized using fluorescence imaging (Figure 36B – lanes 7,8).

5.2.6. PEX with cationic dN^RTPs using template with nucleotide repetitions

The reaction mixture (10 μ L) contained 98-mer template Temp^{FVL-A} (3 μ M, 0.75 μ L), primer Prim^{LT25TH}-FAM (3 μ M, 0.5 μ L), appropriate natural dNTPs (0.25 mM, 1 μ L each), modified **dN^RTP** (**dA^{NH2}TP**, **dU^{NMe}TP**, **dG^{NMe2}TP**, or **dC^{NMe3}TP**) (2 mM, 1 μ L), KOD XL DNA polymerase (unit specification in Table 30), and a corresponding reaction buffer (10X, 1 μ L) as supplied by the manufacturer. The positive control contained 0.3 U of KOD XL DNA polymerase and natural dNTPs (1 mM, 1 μ L). The reaction mixture was incubated for 40 min at 60 °C, stopped

by addition of PAGE stop solution (10 μ L), and denatured for 5 min at 95 °C. Samples were analyzed on 12.5% dPAGE and visualized using fluorescence imaging (Figure 33).

Table 30. Reaction condition specifications for multiple incorporation of **dN^RTPs** using template Temp^{FVL-A}.

dN^RTP	KOD XL
dA^{NH2}TP	0.01 U
dU^{NMe}TP	0.2 U
dG^{NMe2}TP	0.5 U
dC^{NMe3}TP	0.6 U

5.2.7. Sample preparation for mass spectrometry analysis

Method E1. In order to obtain sufficient amounts of **19ON_G^{PA}**, **19ON_A^{OP}**, **19ON_U^{SA}**, **19ON_C^{CA}** and **31ON_C^{CA}** (for sequences see Table 2), corresponding PEX reactions (described in Sections 5.2.2 and 5.2.3) containing non-labeled primer Prim^{248short} and appropriate dual-biotinylated templates were ten times scaled up. The reactions were stopped by cooling to 8 °C. Joined fractions were purified using the DBStv magnetoseparation procedure (described further in this Section) followed by MALDI-TOF analysis (Table 32).

Method E2. In order to obtain sufficient amounts of **31ON_A^{OP}**, **31ON_U^{SA}**, **31ON_G^{PA}**, **31ON_C^{CA}G^{PA}U^{SA}A^{OP}** (for sequences see Tables 2 and 7), the reaction mixture (50 μ L) containing template Temp^{Prb4basII-bio} (100 μ M, 3.2 μ L), non-labeled primer Prim^{248short} (100 μ M, 2.13 μ L), appropriate natural dNTPs and modified **dN^RTPs** (4 mM, 5.2 μ L each), Vent(exo–) DNA polymerase (3 U; only in the case of **dG^{PA}TP** 2.6 U), and the enzyme reaction buffer (10X, 5 μ L) as supplied by the manufacturer was incubated for 40 min at 60 °C. The reaction was stopped by cooling to 8 °C. Products were purified using the DBStv magnetoseparation procedure followed by MALDI-TOF analysis (Table 32).

Method E3. In order to obtain sufficient amounts of **19DNA_A^{NH2}**, **19DNA_U^{NMe}**, **19DNA_G^{NMe2}**, and **19DNA_C^{NMe3}** (for sequences see Table 11), corresponding PEX reactions (described in Section 5.2.2) were ten times scaled up. The reactions were stopped by cooling to

8 °C, purified using QIAquick Nucleotide Removal Kit, concentrated to 10 µL, and analyzed by UHPLC-MS (for results see Table 32).

Method E4. In order to obtain sufficient amounts of **31ON_A^{NH2}**, **31ON_U^{NMe}**, **31ON_G^{NMe2}**, **31ON_C^{NMe3}**, **31ON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}**, and **31ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** (for sequences see Tables 11, 16, and 19), the reaction mixture (50 µL) containing dual-biotinylated template Temp^{Prb4basII}-bio, primer Prim^{248short}-FAM, appropriate natural dNTPs (4 mM, 2.5 µL) and modified **dN^RTPs** (4 mM, 2.5 µL; only in the case of **dC^{NMe3}TP** 6 mM), MgSO₄ (if needed, specified in Table 31), KOD XL DNA polymerase, and the enzyme reaction buffer (10X, 5 µL) as supplied by the manufacturer was incubated either at 60 or 72 °C (all conditions are specified in Table 31). The reaction was stopped by cooling to 8 °C. The products were purified using the DBStv magnetoseparation procedure followed by UHPLC-MS analysis (Table 32).

DBStv magnetoseparation procedure. Streptavidin particles (50 µL) were washed with binding buffer (3 × 200 µL; 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5). 50 µL of PEX solutions were mixed with binding buffer (200 µL) and incubated for 30 min at 15 °C and 1400 rpm. The magnetic beads were collected on a magnet (DynaMagTM-2, Invitrogen), and washed with washing buffer (3 × 200 µL; 10 mM Tris, 1 mM EDTA, 500 mM NaCl, pH 7.5) and water (3 × 200 µL). Then, water (50 µL) was added and the sample was denatured for 5 min at 900 rpm and 75 °C. The beads were collected on a magnet and the DNA containing solution was transferred into a clean vial.

Table 31. Reaction condition specifications for **Method E4**.

ON name	dN ^R TPs	Template	Primer	Reaction time	Temperature	KOD XL	MgSO ₄
31ON_A^{NH2}	dA^{NH2}TP	150 pmol	150 pmol	40 min	60 °C	0.5 U	–
31ON_U^{NMe}	dU^{NMe}TP					1.25 U	
31ON_G^{NMe2}	dG^{NMe2}TP					0.5 U	
31ON_C^{NMe3}	dC^{NMe3}TP	75 pmol	75 pmol	60 min	72 °C	2.5 U	1.5 mM
31ON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}	dA^{NH2}TP, dU^{NMe}TP, dG^{NMe2}TP, dC^{NMe3}TP					2.5 U	1.5 mM
31ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}	dA^{NH2}TP, dU^{EPh}TP, dG^{PA}TP, dC^{EAlk}TP			30 min		1 U	–

5.2.8. UHPLC-MS and MALDI-TOF measurements

Table 32. Overview of modified ssONs and their masses measured by MALDI-TOF and UHPLC-MS.

ON name	Mass calculated [Da]	Mass found [Da]	Δ [Da]
19ON_C^{CA}	6060.9	6062.1	1.2
19ON_G^{PA}	6046.9	6047.8	0.9
19ON_A^{OP}	6107.9	6108.3	0.4
19ON_U^{SA}	6069.9	6071.0	1.1
31ON_C^{CA}	10057.3	10058.4	1.1
31ON_G^{PA}	10141.3	10142.4	1.1
31ON_A^{OP}	10149.3	10150.5	1.2
31ON_U^{SA}	10033.3	10034.2	0.9
19ON_A^{NH2 a}	6605.9	6605	0.9
19ON_U^{NMe a}	6555.9	6555	0.9
19ON_G^{NMe2 a}	6532.9	6532	0.9
19ON_C^{NMe3 a}	6583.9	6582	1.9
31ON_A^{NH2 a}	10530.3	10530	0.3
31ON_U^{NMe a}	10366.3	10360	6.3
31ON_G^{NMe2 a}	10474.3	10470	4.3
31ON_C^{NMe3 a}	10538.3	10530	8.3
31ON_C^{CA}G^{PA}U^{SA}A^{OP}	11529.3	11533.0	3.7
31ON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3 a}	11446.3	11446.9	0.6
31ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk a}	11662.3	11660	2.3

Note: ^a 5'-(6-FAM)-labeled extended strand after PEX

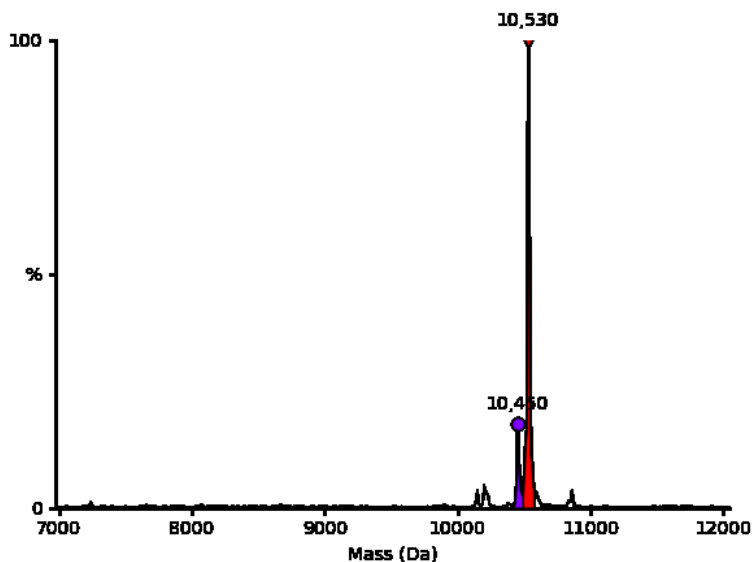


Figure 53. Deconvoluted MS spectrum of **31ON_C^{NMe3}**: calculated: 10538.3 Da; found: 10530 Da, $\Delta = 8.3$ Da; 10450 Da corresponds to a product of dTMP misincorporation instead of **dC^{NMe3}MP** (calculated: 10457 Da).

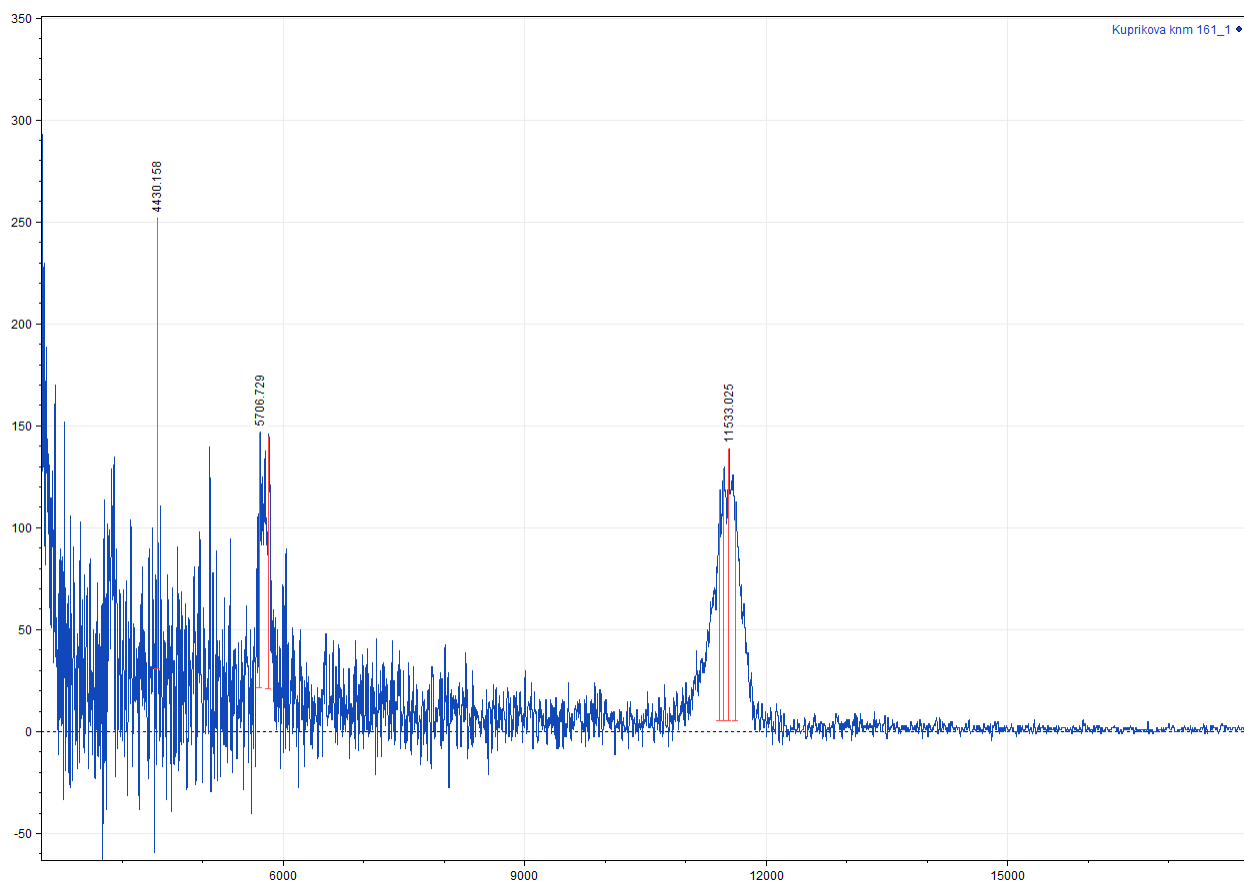


Figure 54. MALDI-TOF spectrum of **31ON_CCA^GPA^USA^AOP**: calculated: 11529.3 Da; found: 11533.0 Da; $\Delta = 3.7$ Da.

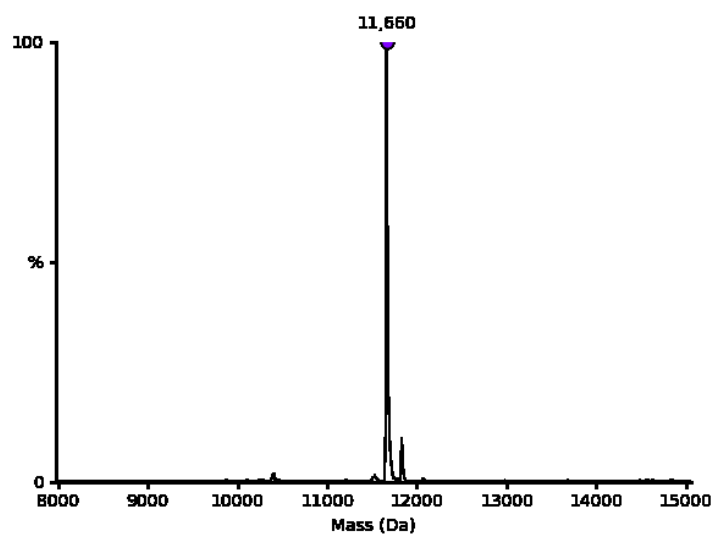


Figure 55. Deconvoluted MS spectrum of **31ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}**: calculated: 11662.3 Da; found: 11660 Da, $\Delta = 2.3$ Da.

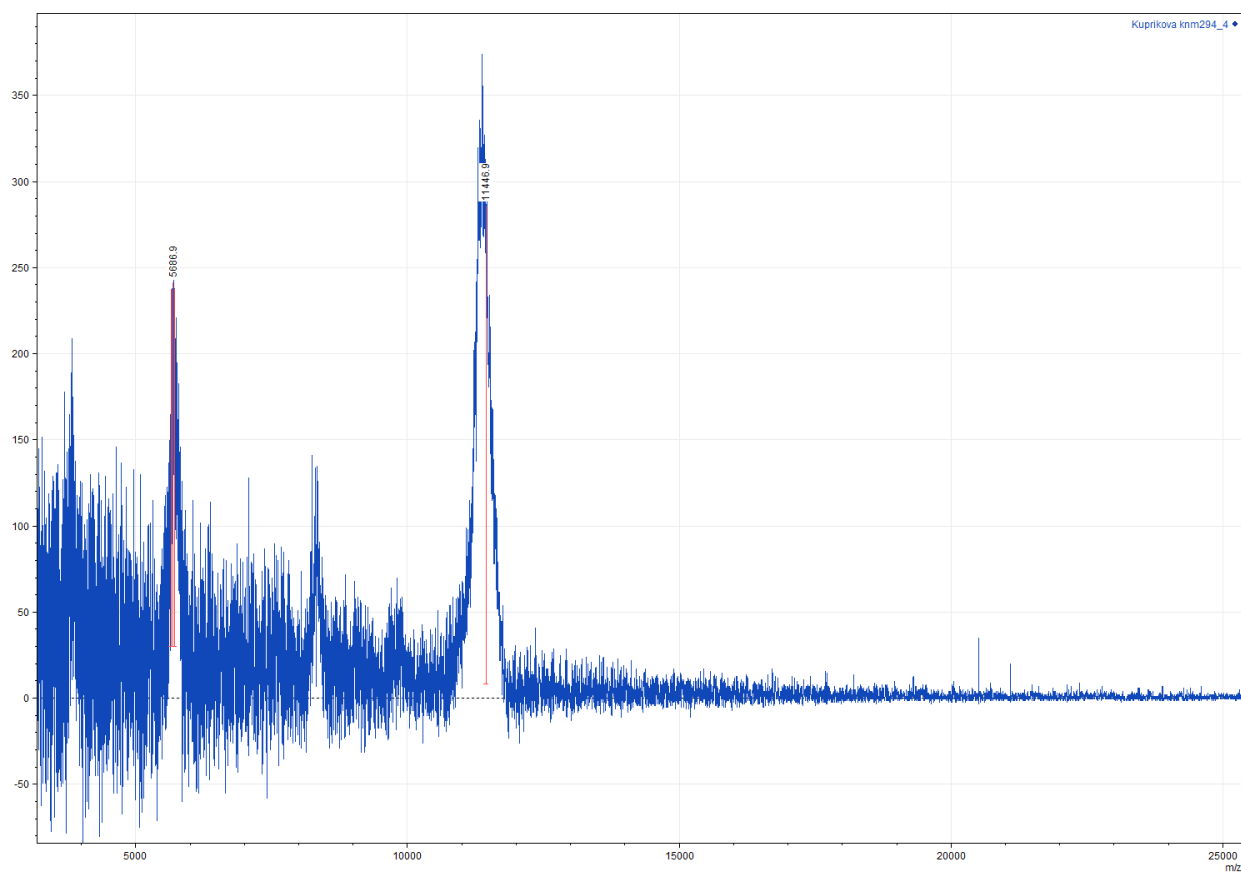


Figure 56. MALDI-TOF spectrum of $31\text{ON_A}^{\text{NH}_2}\text{UNMeG}^{\text{NMe}_2}\text{C}^{\text{NMe}_3}$: calculated: 11446.3 Da; found: 11446.9 Da; $\Delta = 0.6$ Da.

5.2.9. PCR – Multiple incorporation (one modified dN^RTP)

Method F1. The reaction mixture (10 μL) contained template Temp^{FVL-A} (0.5 μM , 0.5 μL), reverse primer Prim^{LT25TH}-FAM and forward primer Prim^{L20}-Cy5 (10 μM , 1 μL each), appropriate set of natural dNTPs (2 mM, 1 μL), one of anionic dN^RTPs (dU^{SA}TP, dC^{CA}TP, dA^{OP}TP, or dG^{PA}TP) (1 μL , conditions specified in Table 33), either Vent(exo–) or KOD XL DNA polymerase (Table 33) and a corresponding reaction buffer (10X, 1 μL) as supplied by the manufacturer. The positive control contained either 1 U of Vent(exo–) or 0.5 U of KOD XL DNA polymerase and natural dNTPs (2 mM, 2 μL). All reaction mixtures were under cycling protocol: 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 sec, 53 °C for 30 sec, and 72 °C for 1 min, followed by a final elongation step at 72 °C for 5 min. Samples were analyzed by 12.5% dPAGE

and visualized using fluorescence imaging (Figure 57A – PCR with KOD XL, Figure 57B – PCR with Vent(exo–)).

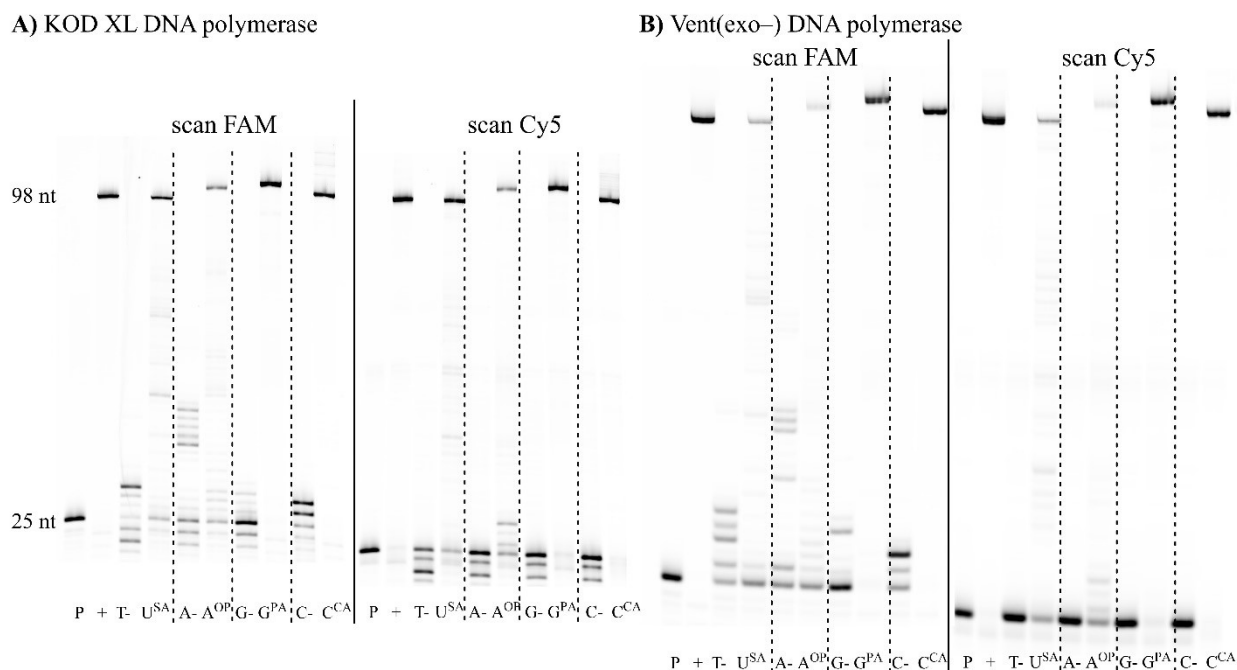


Figure 57. Denaturing PAGE analysis of PCR reactions with one anionic **dN^RTP** using 98-mer template Temp^{FVL-A} using: A) KOD XL DNA polymerase, B) Vent(exo–) DNA polymerase: (P) primer; (+) natural dNTPs; (T-) dCTP, dATP, dGTP; (U^{SA}) dCTP, dATP, dGTP, **dU^{SA}TP**; (A-) dCTP, dGTP, dTTP; (A^{OP}) dCTP, **dA^{OP}TP**, dGTP, dTTP; (G-) dCTP, dATP, dTTP; (G^{PA}) dCTP, dATP, **dG^{PA}TP**, dTTP; (C-) dATP, dGTP, dTTP; (C^{CA}) **dC^{CA}TP**, dATP, dGTP, dTTP.

Table 33. Reaction condition specifications for PCR (one anionic **dN^RTP**).

PCR product	dN^RTP , mM	KOD XL, U	Vent(exo–), U
98PCR_U^{SA}	dU^{SA}TP , 2 mM	2.5 U	5 U
98PCR_A^{OP}	dA^{OP}TP , 0.5 mM	1.25 U	5 U
98PCR_G^{PA}	dG^{PA}TP , 4 mM	1 U	2 U
98PCR_C^{CA}	dC^{CA}TP , 4 mM	0.5 U	1 U

Method F2. The reaction mixture (10 μ L) contained template Temp^{NK98} (0.5 μ M, 0.5 μ L), reverse primer Prim^{NK98pr1}-FAM and forward primer Prim^{NK98pr2}-Cy5 (10 μ M, 1 μ L each), appropriate set of natural dNTPs (2 mM, 1 μ L), one of cationic **dN^RTPs** (**dA^{NH2}TP**, **dU^{NMe}TP**,

dG^{NMe2}TP, or **dC^{NMe3}TP**) (1 μ L, conditions specified in Table 34), suitable additives, KOD XL DNA polymerase (Table 34), and a corresponding reaction buffer (10X, 1 μ L) as supplied by the manufacturer. The positive control contained 0.1 U of KOD XL DNA polymerase and natural dNTPs (2 mM, 2 μ L). All reaction mixtures were under cycling protocol: 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 53 °C for 30 sec, and 72 °C for 2 min, followed by a final elongation step at 72 °C for 5 min. Samples were analyzed on 12.5% dPAGE and visualized using fluorescence imaging (Figure 58).

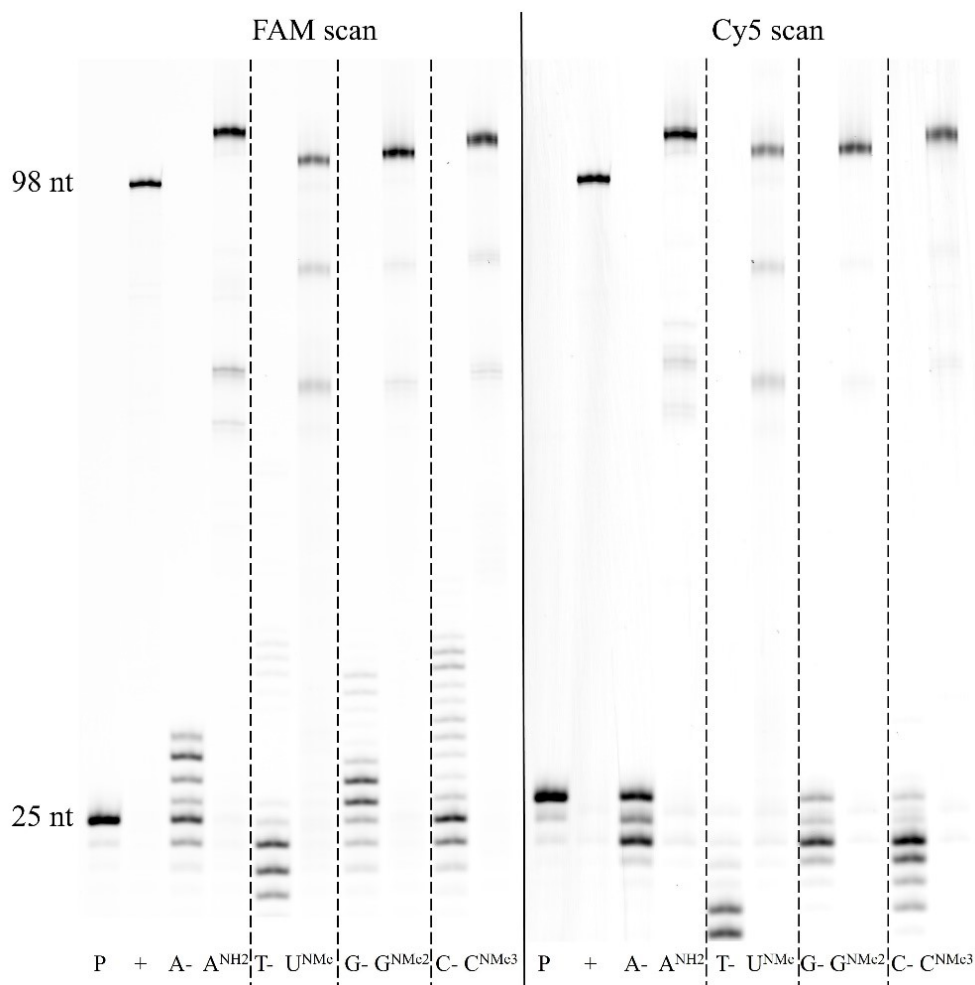


Figure 58. Denaturing PAGE gel analysis of PCR reactions with one cationic **dN^RTP** using 98-mer template Temp^{NK98} and KOD XL DNA polymerase: (P) primer; (+) natural dNTPs; (A-) dCTP, dGTP, dTTP; (A^{NH2}) dCTP, **dA^{NH2}TP**, dGTP, dTTP; (T-) dCTP, dATP, dGTP; (U^{NMe}) dCTP, dATP, dGTP, **dU^{NMe}TP**; (G-) dCTP, dATP, dTTP; (G^{NMe2}) dCTP, dATP, **dG^{NMe2}TP**, dTTP; (C-) dATP, dGTP, dTTP; (C^{NMe3}) **dC^{NMe3}TP**, dATP, dGTP, dTTP.

Table 34. Reaction condition specifications for PCR (one cationic **dN^RTP**).

PCR product	dN^RTP , mM	KOD XL, U	Additives
98PCR_A^{NH2}	dA^{NH2}TP , 2 mM	0.5 U	5% formamide
98PCR_U^{NMe}	dU^{NMe}TP , 4 mM	2 U	1.5 mM MgSO ₄
98PCR_G^{NMe2}	dG^{NMe2}TP , 2 mM	0.5 U	1 M betaine
98PCR_C^{NMe3}	dC^{NMe3}TP , 2 mM	1.5 U	1.5 M betaine

5.2.10. PCR – Multiple incorporation (combinations of modified **dN^RTPs)**

Method G1. The reaction mixture (10 μ L) contained template Temp^{FVL-A} (0.5 μ M, 0.5 μ L), reverse primer Prim^{LT25TH}-FAM and forward primer Prim^{L20}-Cy5 (10 μ M, 1 μ L each), an appropriate set of two natural dNTPs (2 mM, 1 μ L), combinations of two anionic **dN^RTPs** (**dU^{SA}TP**, **dC^{CA}TP**, **dA^{OP}TP**, **dG^{PA}TP**) (1 μ L each, conditions specified in Table 35), Vent(exo–) or KOD XL DNA polymerase (Table 35), and a corresponding reaction buffer (10X, 1 μ L) as supplied by the manufacturer. The positive control contained 0.5 U of KOD XL DNA polymerase and natural dNTPs (2 mM, 2 μ L). All reaction mixtures were under cycling protocol: 94 °C for 3 min, followed by 30 cycles at 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 6 min, followed by a final elongation step at 72 °C for 5 min. Samples were analyzed by 12.5% dPAGE and visualized using fluorescence imaging (Figure 59, lanes 2-5, 7, 8).

Table 35. Reaction condition specifications for PCR (two anionic **dN^RTPs**).

Lanes in Figure 59	dN^RTPs , mM	Polymerase, U	PCR product
2	dA^{OP}TP , 0.5 mM; dC^{CA}TP , 2 mM	KOD XL, 1.25 U	98PCR_A^{OP}C^{CA}
3	dG^{PA}TP , 4 mM; dC^{CA}TP , 4 mM	Vent(exo–), 2 U	98PCR_G^{PA}C^{CA}
4	dU^{SA}TP , 2 mM; dC^{CA}TP , 2 mM	KOD XL, 2.5 U	98PCR_U^{SA}C^{CA}
5	dA^{OP}TP , 0.5 mM; dG^{PA}TP , 1 mM	KOD XL, 1.25 U	98PCR_A^{OP}G^{PA}
7	dG^{PA}TP , 2 mM; dU^{SA}TP , 2 mM	KOD XL, 2.5 U	98PCR_G^{PA}U^{SA}
8	dU^{SA}TP , 1 mM; dA^{OP}TP , 0.5 mM	KOD XL, 2.5 U	98PCR_U^{SA}A^{OP}

Method G2. The reaction mixture (10 μ L) contained template Temp^{FVL-A} (0.5 μ M, 0.5 μ L), reverse primer Prim^{LT25TH}-FAM and forward primer Prim^{L20}-Cy5 (10 μ M, 1 μ L each), an appropriate natural dNTP (2 mM, 1 μ L), combinations of three anionic **dN^RTPs** (**dU^{SA}TP**, **dC^{CA}TP**, **dA^{OP}TP**, **dG^{PA}TP**) (1 μ L each, conditions specified in Table 36), KOD XL DNA

polymerase (2.5 U), and the enzyme reaction buffer (10X, 1 μ L) as supplied by the manufacturer. All reaction mixtures were under cycling protocol: 94 °C for 3 min, followed by 30 cycles at 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 6 min, followed by a final elongation step at 72 °C for 5 min. Samples were analyzed by 12.5% dPAGE and visualized using fluorescence imaging (Figure 59, lanes 6, 9-11).

Table 36. Reaction condition specifications for PCR (three anionic **dN^RTPs**).

Lanes in Figure 59	dN^RTPs , mM	PCR product
6	dC^{CA}TP , 2 mM; dG^{PA}TP , 4 mM; dA^{OP}TP , 0.5 mM	98PCR_CCA^{G^{PA}}AA^{OP}
9	dA^{OP}TP , 0.5 mM; dU^{SA}TP , 2 mM; dC^{CA}TP , 2 mM	98ON_AA^{OP}USAC^{CA}
10	dA^{OP}TP , 0.5 mM; dU^{SA}TP , 2 mM; dG^{PA}TP , 4 mM	98ON_AA^{OP}USAG^{PA}
11	dC^{CA}TP , 4 mM; dG^{PA}TP , 4 mM; dU^{SA}TP , 2 mM	98ON_CCA^{G^{PA}}U^{SA}

Method G3. The reaction mixture (10 μ L) contained template Temp^{FVL-A} (0.5 μ M, 0.5 μ L), reverse primer Prim^{LT25TH}-FAM and forward primer Prim^{L20}-Cy5 (10 μ M, 1 μ L each), **dU^{SA}TP** (2 mM, 1 μ L), **dC^{CA}TP** (2 mM, 1 μ L), **dA^{OP}TP** (0.5 mM, 1 μ L), and **dG^{PA}TP** (4 mM, 1 μ L), KOD XL DNA polymerase (2.5 U), and the enzyme reaction buffer (10X, 1 μ L) as supplied by the manufacturer. The reaction mixture was under cycling protocol: 94 °C for 3 min, followed by 30 cycles at 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 6 min, followed by a final elongation step at 72 °C for 5 min. The sample was analyzed by 12.5% dPAGE and visualized using fluorescence imaging (Figure 59, lane 12).

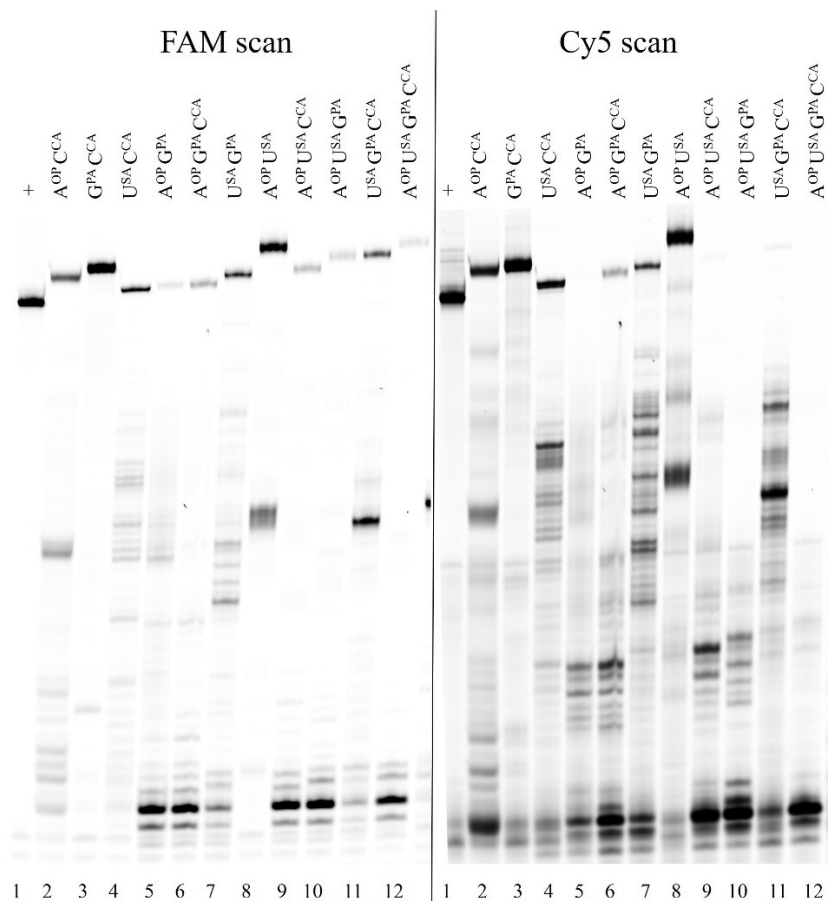


Figure 59. Denaturing PAGE analysis of PCR reactions with combinations of anionic **dN^RTPs** using 98-mer template Temp^{FVL-A} and KOD XL DNA or Vent(exo-) polymerase: (lane 1) natural dNTPs; (lane 2) **dC^{CA}TP**, **dA^{OP}TP**, dGTP, dTTP; (lane 3) **dC^{CA}TP**, dATP, **dG^{PA}TP**, dTTP; (lane 4) **dC^{CA}TP**, dATP, dGTP, **dU^{SA}TP**; (lane 5) dCTP, **dA^{OP}TP**, **dG^{PA}TP**, dTTP; (lane 6) **dC^{CA}TP**, **dG^{PA}TP**, **dA^{OP}TP**, dTTP; (lane 7) dCTP, dATP, **dG^{PA}TP**, **dU^{SA}TP**; (lane 8) dCTP, **dA^{OP}TP**, dGTP, **dU^{SA}TP**; (lane 9) **dA^{OP}TP**, **dU^{SA}TP**, **dC^{CA}TP**, dGTP; (lane 10) **dA^{OP}TP**, **dU^{SA}TP**, **dG^{PA}TP**, dCTP; (lane 11) **dC^{CA}TP**, **dG^{PA}TP**, **dU^{SA}TP**, dATP; (lane 12) **dC^{CA}TP**, **dG^{PA}TP**, **dU^{SA}TP**, **dA^{OP}TP**.

Method G4. The reaction mixture (10 μ L) contained template Temp^{NK98} (0.5 μ M, 0.5 μ L), reverse primer Prim^{NK98pr1}-FAM and forward primer Prim^{NK98pr2}-Cy5 (10 μ M, 1 μ L each), an appropriate set of two natural dNTPs (2 mM, 1 μ L), combinations of two cationic **dN^RTPs** (**dA^{NH2}TP**, **dU^{NMe}TP**, **dG^{NMe2}TP**, **dC^{NMe3}TP**) (2 mM, 1 μ L each), additives (specifications in Table 37), KOD XL DNA polymerase (specifications in Table 37), and a corresponding reaction buffer (10X, 1 μ L) as supplied by the manufacturer. The positive control contained 0.1 U of KOD XL DNA polymerase and natural dNTPs (2 mM, 1 μ L). All reaction mixtures were under cycling

protocol: 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 53 °C for 30 min, and 72 °C for either 2 or 5 min (elongation time is specified in Table 37), followed by a final elongation step at 72 °C for 5 min. Samples were analyzed on 12.5% dPAGE and visualized using fluorescence imaging (Figure 60).

Table 37. Reaction condition specifications for PCR (two cationic **dN^RTPs**).

Lanes in Figure 60	dN^RTPs	KOD XL, U	Additives	Elongation step, min	PCR product
5	dA^{NH2}TP, dU^{NMe}TP	1.5 U	5% formamide, 1.5 mM MgSO ₄	2	98PCR_A^{NH2}U^{NMe}
8	dA^{NH2}TP, dG^{NMe2}TP	1 U	1 M betaine	2	98PCR_A^{NH2}G^{NMe2}
11	dA^{NH2}TP, dC^{NMe3}TP	3 U	1.5 M betaine	5	98PCR_A^{NH2}C^{NMe3}
14	dU^{NMe}TP, dG^{NMe2}TP	1.5 U	–	2	98PCR_U^{NMe}G^{NMe2}
17	dU^{NMe}TP, dC^{NMe3}TP	1.5 U	1.5 M betaine, 1.5 mM MgSO ₄	5	98PCR_U^{NMe}C^{NMe3}
20	dG^{NMe2}TP, dC^{NMe3}TP	2 U	1.7 M betaine	5	98PCR_G^{NMe2}C^{NMe3}

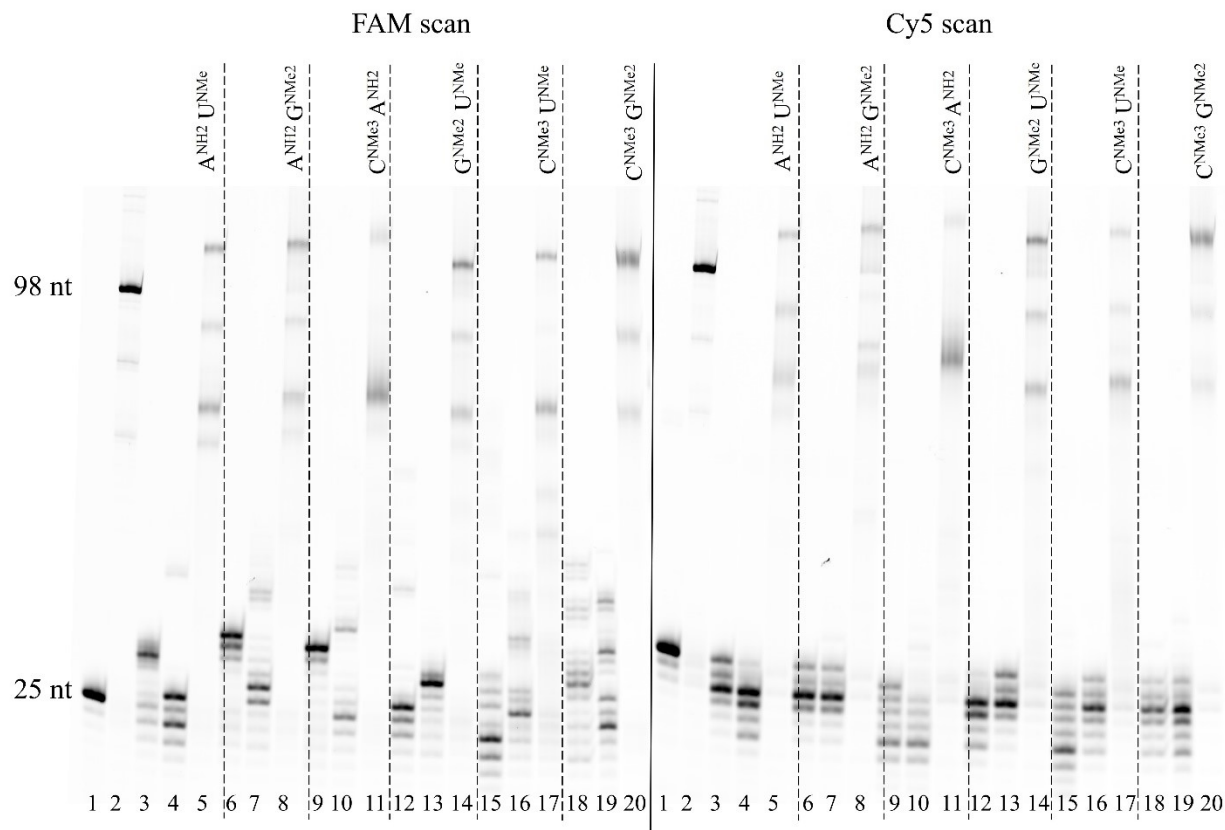


Figure 60. Denaturing PAGE analysis of PCR reactions with two modified **dN^RTPs** using 98-mer template Temp^{NK98} and KOD XL DNA polymerase: (lane 1) primer; (lane 2) natural dNTPs; (lane 3) dCTP, dGTP, **dU^{NMe}TP**; (lane 4) dCTP, **dA^{NH2}TP**, dGTP; (lane 5) dCTP, **dA^{NH2}TP**, dGTP, **dU^{NMe}TP**; (lane 6) dCTP, **dG^{NMe2}TP**, dTTP; (lane 7) dCTP, **dA^{NH2}TP**, dTTP; (lane 8) dCTP, **dA^{NH2}TP**, **dG^{NMe2}TP**, dTTP; (lane 9) **dC^{NMe3}TP**, dGTP, dTTP; (lane 10) **dA^{NH2}TP**, dGTP, dTTP; (lane 11) **dC^{NMe3}TP**, **dA^{NH2}TP**, dGTP, dTTP; (lane 12) dCTP, dATP, **dG^{NMe2}TP**; (lane 13) dCTP, dATP, **dU^{NMe}TP**; (lane 14) dCTP, dATP, **dG^{NMe2}TP**, **dU^{NMe}TP**; (lane 15) **dC^{NMe3}TP**, dATP, dGTP; (lane 16) dATP, dGTP, **dU^{NMe}TP**; (lane 17) **dC^{NMe3}TP**, dATP, dGTP, **dU^{NMe}TP**; (lane 18) **dC^{NMe3}TP**, dATP, dTTP; (lane 19) dATP, **dG^{NMe2}TP**, dTTP; (lane 20) **dC^{NMe3}TP**, dATP, **dG^{NMe2}TP**, dTTP.

Method G5. The reaction mixture (10 μ L) contained template Temp^{NK98} (0.5 μ M, 0.5 μ L), reverse primer Prim^{NK98pr1}-FAM and forward primer Prim^{NK98pr2}-Cy5 (10 μ M, 1 μ L each), an appropriate natural dNTP (2 mM, 1 μ L), combinations of three modified **dN^RTPs** (**dA^{NH2}TP**, **dU^{NMe}TP**, **dG^{NMe2}TP**, **dC^{NMe3}TP**) (2 mM, 1 μ L each), additives (specifications in Table 38), KOD XL DNA polymerase (specifications in Table 38) and a corresponding reaction buffer (10X, 1 μ L) as supplied by the manufacturer. The positive control contained 0.1 U of KOD XL DNA polymerase and natural dNTPs (2 mM, 1 μ L). All reaction mixtures were under cycling protocol:

94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 53 °C for 30 min, and 72 °C for 5 min, followed by a final elongation step at 72 °C for 5 min. Samples were analyzed on 12.5% dPAGE and visualized using fluorescence imaging (Figure 61 – lanes 3-6).

Method G6. The reaction mixture (10 μ L) contained template Temp^{NK98} (0.5 μ M, 0.5 μ L), reverse primer Prim^{NK98pr1}-FAM and forward primer Prim^{NK98pr2}-Cy5 (10 μ M, 1 μ L each), a set of four cationic **dN^RTPs** (**dA^{NH2}TP**, **dU^{NMe}TP**, **dG^{NMe2}TP**, **dC^{NMe3}TP**) (2 mM, 1 μ L each), betaine (5 M, 3 μ L), KOD XL DNA polymerase (3 U), and a corresponding reaction buffer (10X, 1 μ L) as supplied by the manufacturer. The reaction mixture was under cycling protocol: 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 53 °C for 30 min, and 72 °C for 5 min, followed by a final elongation step at 72 °C for 5 min. The sample was analyzed on 12.5% dPAGE and visualized using fluorescence imaging (Figure 61 – lane 7).

Table 38. Reaction condition specifications for PCR (three and four cationic **dN^RTPs**).

Lanes in Figure 61	dN^RTPs	KOD XL	Additives	PCR product
3	dA^{NH2}TP, dU^{NMe}TP, dG^{NMe2}TP	1.5 U	1 M betaine	98PCR_A^{NH2}U^{NMe}G^{NMe2}
4	dA^{NH2}TP, dU^{NMe}TP, dC^{NMe3}TP	3 U	1.5 mM MgSO ₄ , 1.5 M betaine	98ON_A^{NH2}U^{NMe}C^{NMe3}
5	dA^{NH2}TP, dG^{NMe2}TP, dC^{NMe3}TP	3 U	1.7 M betaine	98PCR_A^{NH2}G^{NMe2}C^{NMe3}
6	dU^{NMe}TP, dG^{NMe2}TP, dC^{NMe3}TP	2 U	1.7 M betaine	98PCR_U^{NMe}G^{NMe2}C^{NMe3}
7	dA^{NH2}TP, dU^{NMe}TP, dG^{NMe2}TP, dC^{NMe3}TP	3 U	1.5 M betaine	no product

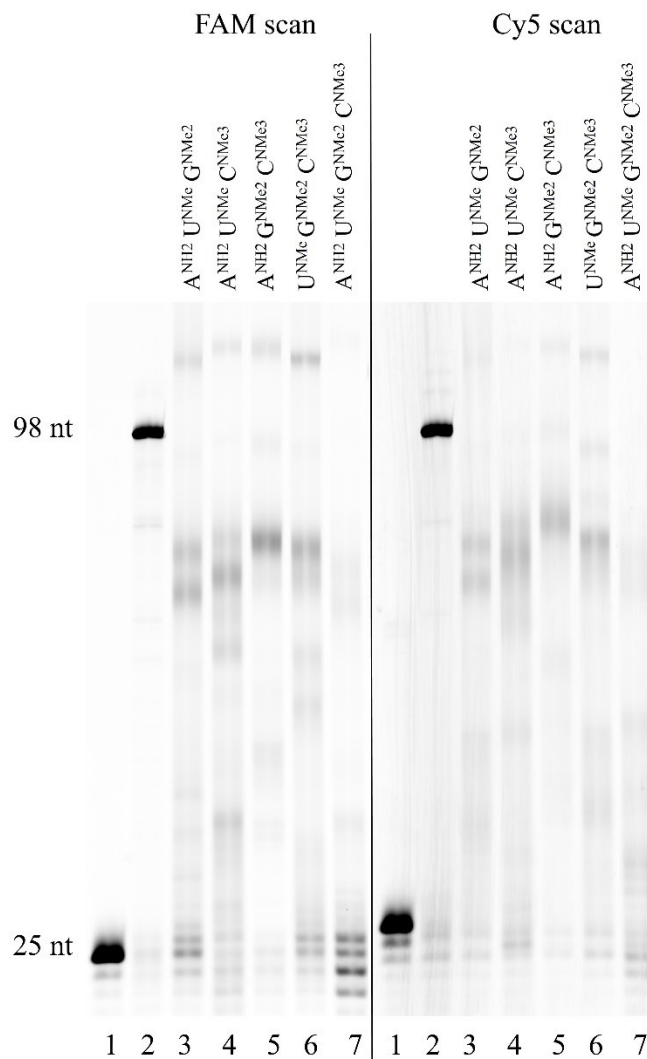


Figure 61. Denaturing PAGE analysis of PCR reactions with three and four modified **dN^RTPs** using 98-mer template Temp^{NK98} and KOD XL DNA polymerase: (lane 1) primer; (lane 2) natural dNTPs; (lane 3) **dA^{NH2}TP**, **dU^{NMe}TP**, **dG^{NMe2}TP**, **dCTP**; (lane 4) **dA^{NH2}TP**, **dU^{NMe}TP**, **dGTP**, **dC^{NMe3}TP**; (lane 5) **dA^{NH2}TP**, **dTTP**, **dG^{NMe2}TP**, **dC^{NMe3}TP**; (lane 6) **dATP**, **dU^{NMe}TP**, **dG^{NMe2}TP**, **dC^{NMe3}TP**; (lane 7) **dA^{NH2}TP**, **dU^{NMe}TP**, **dG^{NMe2}TP**, **dC^{NMe3}TP**.

Method G7. The reaction mixture (10 μ L) contained template Temp^{NK98} (0.5 μ M, 0.5 μ L), reverse primer Prim^{NK98pr1}-FAM and forward primer Prim^{NK98pr2}-Cy5 (10 μ M, 1 μ L each), a set of four modified **dN^RTPs** (**dA^{NH2}TP**, **dU^{EPh}TP**, **dG^{PA}TP**, **dC^{EAlk}TP**) (2 mM, 1 μ L each), 100% formamide (0.5 μ L), KOD XL DNA polymerase (0.5 U), and a corresponding reaction buffer (10X, 1 μ L) as supplied by the manufacturer. The reaction mixture was under cycling protocol: 94 $^{\circ}$ C for 5 min, followed by 30 cycles at 94 $^{\circ}$ C for 1 min, 53 $^{\circ}$ C for 30 min, and 72 $^{\circ}$ C

for 5 min, followed by a final elongation step at 72 °C for 5 min. The sample was analyzed on 12.5% dPAGE and visualized using fluorescence imaging (Figure 37A).

5.2.11. aPCR – Multiple incorporation (combinations of modified dN^RTPs)

Method H1. The reaction mixture (10 µL) contained template Temp^{FVL-A} (5 µM, 0.5 µL), primer Prim^{LT25TH}-FAM (10 µM, 1 µL), an appropriate natural dNTP (2 mM, 1 µL) or none in the case of four modifications, a set of anionic dN^RTPs (1 µL, conditions specified in Table 39), KOD XL DNA polymerase (2.5 U), and the enzyme reaction buffer (10X, 1 µL) as supplied by the manufacturer. The positive control contained 0.5 U of KOD XL DNA polymerase and all four natural dNTPs (2 mM, 2 µL). All reaction mixtures were under cycling protocol: 94 °C for 3 min, followed by 30 cycles at 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 6 min, followed by a final elongation step at 72 °C for 5 min. Samples were analyzed by 12.5% dPAGE and visualized using fluorescence imaging (Figure 62).

Table 39. Reaction condition specifications for aPCR using anionic dN^RTPs.

Lanes in Figure 62	dN ^R TPs, mM	aPCR product
3	dA ^{OP} TP, 0.5 mM; dU ^{SA} TP, 2 mM; dC ^{CA} TP, 2 mM	98ON_A ^{OP} U ^{SA} C ^{CA}
4	dA ^{OP} TP, 0.5 mM; dU ^{SA} TP, 2 mM; dG ^{PA} TP, 4 mM	98ON_A ^{OP} U ^{SA} G ^{PA}
5	dC ^{CA} TP, 4 mM; dG ^{PA} TP, 4 mM; dU ^{SA} TP, 2 mM	98ON_C ^{CA} G ^{PA} U ^{SA}
6	dC ^{CA} TP, 2 mM; dG ^{PA} TP, 4 mM; dU ^{SA} TP, 2 mM; dA ^{OP} TP, 0.5 mM	98ON_C ^{CA} G ^{PA} U ^{SA} A ^{OP}



Figure 62. Denaturing PAGE analysis of aPCR reactions with combinations of anionic **dN^RTPs** using 98-mer template Temp^{FVL-A} and KOD XL DNA polymerase: (lane 1) natural dNTPs; (lane 2) **dA^{OP}TP**, **dG^{PA}TP**, dCTP, dTTP; (lane 3) **dA^{OP}TP**, **dU^{SA}TP**, **dC^{CA}TP**, dGTP; (lane 4) **dA^{OP}TP**, **dU^{SA}TP**, **dG^{PA}TP**, dCTP; (lane 5) **dC^{CA}TP**, **dG^{PA}TP**, **dU^{SA}TP**, dATP; (lane 6) **dA^{OP}TP**, **dU^{SA}TP**, **dG^{PA}TP**, **dC^{CA}TP**.

Method H2. The reaction mixture (10 μ L) contained template Temp^{NK98} (5 μ M, 0.5 μ L), primer Prim^{NK98pr1}-FAM (10 μ M, 1 μ L), a set of modified **dN^RTPs** (2 mM, 1 μ L each) – **dA^{NH2}TP**, **dU^{NMe}TP**, **dG^{NMe2}TP**, **dC^{NMe3}TP** or **dA^{NH2}TP**, **dU^{EPh}TP**, **dG^{PA}TP**, **dC^{EAlk}TP**, additives (specifications in Table 40), KOD XL DNA polymerase (specifications in Table 40), and a corresponding reaction buffer (10X, 1 μ L) as supplied by the manufacturer. The positive control contained 0.1 U of KOD XL DNA polymerase and natural dNTPs (2 mM, 1 μ L). All reaction mixtures were under cycling protocol: 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min,

53 °C for 30 min, and 72 °C for 10 min, followed by a final elongation step at 72 °C for 5 min. Samples were analyzed on 12.5% PAGE and visualized using fluorescence imaging (Figure 63).

Table 40. Reaction condition specifications for aPCR using four modified **dN^RTPs**.

Lanes in Figure 63	dN ^R TPs	KOD XL	Additives	aPCR product
7	dA^{NH2}TP, dU^{NMe}TP, dG^{NMe2}TP, dC^{NMe3}TP	2 U	1.5 M betaine	98ON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}
12	dA^{NH2}TP, dU^{EPh}TP, dG^{PA}TP, dC^{EAlk}TP	0.5 U	5% formamide	98ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}

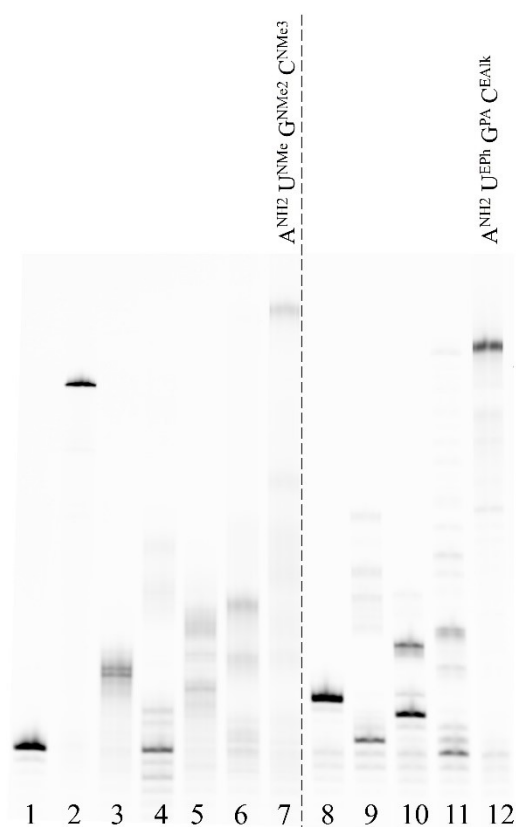


Figure 63. Denaturing PAGE analysis of aPCR reactions with four modified **dN^RTPs** using 98-mer template Temp^{NK98} and KOD XL DNA polymerase: (lane 1) primer Prim^{NK98pr1}-FAM; (lane 2) natural dNTPs; (lane 3) **dU^{NMe}TP, dG^{NMe2}TP, dC^{NMe3}TP**; (lane 4) **dA^{NH2}TP, dG^{NMe2}TP, dC^{NMe3}TP**; (lane 5) **dA^{NH2}TP, dU^{NMe}TP, dC^{NMe3}TP**; (lane 6) **dA^{NH2}TP, dU^{NMe}TP, dG^{NMe2}TP**; (lane 7) **dA^{NH2}TP, dU^{NMe}TP, dG^{NMe2}TP, dC^{NMe3}TP**; (lane 8) **dU^{EPh}TP, dG^{PA}TP, dC^{EAlk}TP**; (lane 9) **dA^{NH2}TP, dG^{PA}TP, dC^{EAlk}TP**; (lane 10) **dA^{NH2}TP, dU^{EPh}TP, dC^{EAlk}TP**; (lane 11) **dA^{NH2}TP, dU^{EPh}TP, dG^{PA}TP**; (lane 12) **dA^{NH2}TP, dU^{EPh}TP, dG^{PA}TP, dC^{EAlk}TP**.

5.2.12. Nuclease degradation experiments with DNase I

Four 5'-(6-FAM)-labeled 31-mer ssONs (**31ON**, **31ON_C^{CA}G^{PA}U^{SA}A^{OP}**, **31ON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}**, and **31ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}**) were prepared according to procedures described in Section 5.2.7 (for sequences see Table 26). Further, the ssONs (0.1 µg) were mixed with DNase I (0.01 U) and the supplied reaction buffer (10X, 1 µL) and further diluted to total volume of 10 µL with DNase-free water. The reaction mixtures were incubated at 37 °C for 5, 30, and 60 min and terminated by addition of 1 µL of 50 mM EDTA, followed by incubation at 70 °C for 10 min. The experiment was repeated three times. The results of **31ON** and **31ON_C^{CA}G^{PA}U^{SA}A^{OP}** digestion were analyzed by 12.5% dPAGE and the results of **31ON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}** and **31ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** digestion were analyzed by 20% dPAGE for a better band resolution. Samples were visualized using fluorescence imaging (Figure 64). The values of DNA recoveries in each experiment and the average values are calculated in Table 41.

Table 41. Recovery (expressed in percent) of natural and hypermodified DNA after incubation with 0.01 U of DNase I for a set of three experiments.

DNA title	Experiment No.	Incubation time with DNase I		
		5 min	30 min	60 min
31ON	1	22%	8%	1%
	2	26%	4%	1%
	3	28%	7%	2%
	average	25±3%	6±2%	1%
31ON_C^{CA}G^{PA}U^{SA}A^{OP}	1	61%	25%	12%
	2	65%	21%	20%
	3	54%	19%	12%
	average	60±5%	22±2%	15±4%
31ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}	1	47%	18%	8%
	2	59%	10%	5%
	3	63%	10%	4%
	average	56±7%	13±4%	6±2%
31ON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}	1	80%	39%	20%
	2	77%	46%	33%
	3	80%	39%	20%
	average	79±1%	41±3%	24±6%

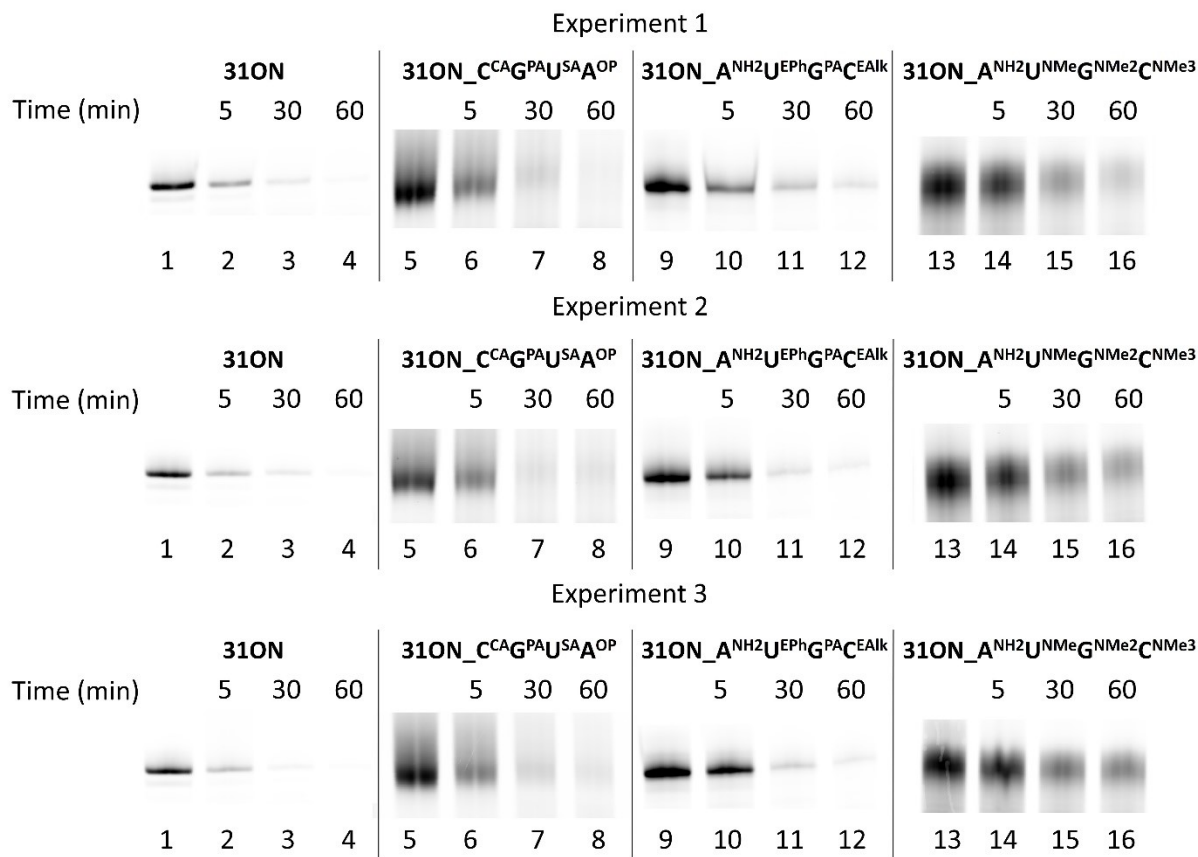


Figure 64. dPAGE analysis of stability of natural **31ON** (lanes 2-4), anionic **31ON_CCAAGPAUSAAOP** (lanes 6-8), mixed **31ON_ANH2UEPhGPAEAlk** (lanes 10-12), and cationic **31ON_ANH2UNMeGNMe2CNMe3** (lanes 14-16) in the presence of 0.01 U of DNase I for 5 min (lanes 2, 6, 10, 14), 30 min (lanes 3, 7, 11, 15), and 60 min (lanes 4, 8, 12, 16). Lanes 1, 5, 9, and 13 were not incubated with DNase I. Data presented for a set of three experiments (from top to bottom).

5.2.13. ONs stability experiments in human plasma

Four 5'-(6-FAM)-labeled 31-mer ssONs (**31ON**, **31ON_CCAAGPAUSAAOP**, **31ON_ANH2UNMeGNMe2CNMe3**, and **31ON_ANH2UEPhGPAEAlk**) were prepared according to procedures described in Section 5.2.7 (for sequences see Table 26). Further, the ssONs (0.1 µg) were mixed with 19 µL of human plasma and incubated at 37 °C for 17 h. Then, plasma was deactivated by incubation at 70 °C for 20 min. To analyze the liquid part of the samples, reaction mixtures were centrifuged for 3 min (13000 rpm), followed by separation of the supernatant from the sediment. The experiment was repeated three times. Prior loading on gel, samples were

20 times diluted with water and mixed with PAGE stop solution. The results were analyzed by 20% dPAGE and visualized using fluorescence imaging (Figure 51).

5.2.14. Preparation of hypermodified templates for further re-PCR

Synthesis of 118DNA_C^{CA}G^{PA}U^{SA}A^{OP}. The reaction mixture (10 μ L) containing template Temp^{FVL-A}-sC3 (100 μ M, 0.5 μ L) and primer Prim^{Flank-LT25TH}-FAM (100 μ M, 0.5 μ L), set of four anionic dN^RTPs (dU^{SA}TP, dC^{CA}TP, dA^{OP}TP, and dG^{PA}TP) (4 mM, 1.25 μ L each), KOD XL DNA polymerase (2.5 U), and the enzyme reaction buffer (10X, 1 μ L) as supplied by the manufacturer was incubated for 40 minutes at 60 °C. The reaction was stopped by cooling to 8 °C. Further, 118ON_C^{CA}G^{PA}U^{SA}A^{OP} (for sequence see Table 22) was obtained by separation from templates using gel extraction method (described further in this Section) and visualized using fluorescence imaging (Figure 39, lane 2).

Gel extraction method. An obtained PEX reaction product was mixed with the PAGE stop solution and loaded on 12.5% preparative dPAGE (1.5 mm thick) under denaturing conditions (1 h, 50 °C, 1X TBE buffer). After the run, a modified strand and a template were visualized by UV lamp, and the area containing modified product was cut out. In order to extract the product, Pur-A-Lyze Maxi Dialysis Kit was used. The cut-out piece of gel was placed into a prehydrated Pur-A-Lyze Maxi Dialysis Kit containing 3 mL of 1X TAE buffer. Further, the dialysis kit was placed into a horizontal electrophoretic system in a way that its membrane was in a perpendicular position to the current direction. Then, current was applied (45 min, 100 V) and the product was eluted from the piece of gel and trapped on the dialysis column membrane. To detach DNA molecules from the dialysis column membrane, the current was applied in the reverse direction (1 min, 100 V). The obtained DNA solution was desalted and concentrated using Amicon Ultra-0.5 Centrifugal Filters.

Synthesis of 118DNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}. The reaction mixture (10 μ L) containing template Temp^{NK98}-sC3 (3 μ M, 0.75 μ L), primer Prim^{Flank-NK98pr1}-FAM (3 μ M, 0.5 μ L), dA^{NH2}TP (0.25 mM, 1 μ L), dU^{NMe}TP (2 mM, 1 μ L), dG^{NMe2}TP (2 mM, 1 μ L), dC^{NMe3}TP (2 mM, 1 μ L), MgSO₄ (25 mM, 0.6 μ L), KOD XL DNA polymerase (2 U), and the enzyme reaction buffer (10X, 1 μ L) as supplied by the manufacturer was incubated for 60 min at 72 °C. The reaction was stopped

by cooling to 8 °C and the sample was analyzed by 2% agarose gel electrophoresis (60 min, 125 V) and visualized using fluorescence imaging (Figure 39, lane 6). The hypermodified-product-containing area was extracted using a plastic pipette tip. Further, the tip with gel fragments was soaked overnight in water, and then the extracted modified template was used for re-PCR (see Section 5.2.15).

Synthesis of 118DNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}. The reaction mixture (10 µL) containing template Temp^{NK98}-sC3 (3 µM, 0.75 µL), primer Prim^{Flank-NK98pr1}-FAM (3 µM, 0.5 µL), dA^{NH2}TP (0.25 mM, 1 µL), dU^{EPh}TP (2 mM, 1 µL), dG^{PA}TP (2 mM, 1 µL), dC^{EAlk}TP (2 mM, 1 µL), KOD XL DNA polymerase (0.2 U), and the enzyme reaction buffer (10X, 1 µL) as supplied by the manufacturer was incubated for 30 min at 60 °C. The reaction was stopped by cooling to 8 °C and the sample was analyzed by 2% agarose gel electrophoresis (60 min, 125 V) and visualized using fluorescence imaging (Figure 39, lane 7). The hypermodified-product-containing area was extracted using a plastic pipette tip. Further, the tip with gel fragments was soaked overnight in water, and then the extracted modified template was used for re-PCR (see Section 5.2.15).

5.2.15. Re-PCR of hypermodified templates using natural dNTPs

Method I1. The reaction mixture (20 µL) contained 118ON_C^{CA}G^{PA}U^{SA}A^{OP} as a template (0.5 µM, 1 µL) (synthesis described in Section 5.2.14), forward primer Prim^{Flank}-FAM and a reverse primer Prim^{L20} (10 µM, 2 µL each), all four natural dNTPs (1 mM, 4.5 µL), KOD XL DNA polymerase (1.25 U), and the enzyme reaction buffer (10X, 2 µL) as supplied by the manufacturer. The positive control contained 1.25 U of KOD XL DNA polymerase, template Temp^{FVL-A} (0.5 µM, 1 µL), primers Prim^{Flank-LT25TH}-FAM and Prim^{L20} (10 µM, 2 µL each), and natural dNTPs (1 mM, 4.5 µL). Reaction mixtures were under cycling protocol: 94 °C for 3 min, followed by 15 cycles at 94 °C for 30 sec, 50 °C for 1 min, and 72 °C for 1 min, followed by a final elongation step at 72 °C for 5 min. The obtained 118PCR_{natural_1} (for sequence see Table 22) was purified using Agencourt AMPure XP magnetic particles, analyzed by 12.5% dPAGE and visualized using fluorescence imaging (Figure 39, lane 3).

Method I2. The reaction mixture (20 µL) contained traces of 118DNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3} as a template (synthesis described in Section 5.2.14), forward primer Prim^{Flank}-FAM and reverse

primer Prim^{NK98pr2} (10 μ M, 2 μ L each), all four natural dNTPs (1 mM, 4.5 μ L), Pwo DNA polymerase (0.3 U), and the enzyme reaction buffer (10X, 2 μ L) as supplied by the manufacturer. The positive control contained 0.2 U of Pwo DNA polymerase, template Temp^{NK98} (0.5 μ M, 0.5 μ L), primers Prim^{Flank-NK98pr1}-FAM and Prim^{NK98pr2} (10 μ M, 2 μ L each), and natural dNTPs (1 mM, 4.5 μ L). The reaction mixture was under cycling protocol: 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 61 °C for 30 sec, and 72 °C for 1 min, followed by a final elongation step at 72 °C for 5 min. The obtained **118PCR_natural_2** (for sequence see Table 22) was analyzed by 12.5% dPAGE and visualized using fluorescence imaging (Figure 39, lane 10).

Method I3. The reaction mixture (20 μ L) contained traces of **118DNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** as a template (synthesis described in Section 5.2.14), forward primer Prim^{Flank}-FAM and reverse primer Prim^{NK98pr2} (10 μ M, 2 μ L each), all four natural dNTPs (1 mM, 4.5 μ L), MgSO₄ (25 mM, 1.2 μ L), Pwo DNA polymerase (0.3 U), and the enzyme reaction buffer (10X, 2 μ L) as supplied by the manufacturer. The reaction mixture was under cycling protocol: 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 54 °C for 30 sec, and 72 °C for 1 min, followed by a final elongation step at 72 °C for 5 min. The obtained **118PCR_natural_3** (for sequence see Table 22) was analyzed by 12.5% dPAGE and visualized using fluorescence imaging (Figure 39, lane 11).

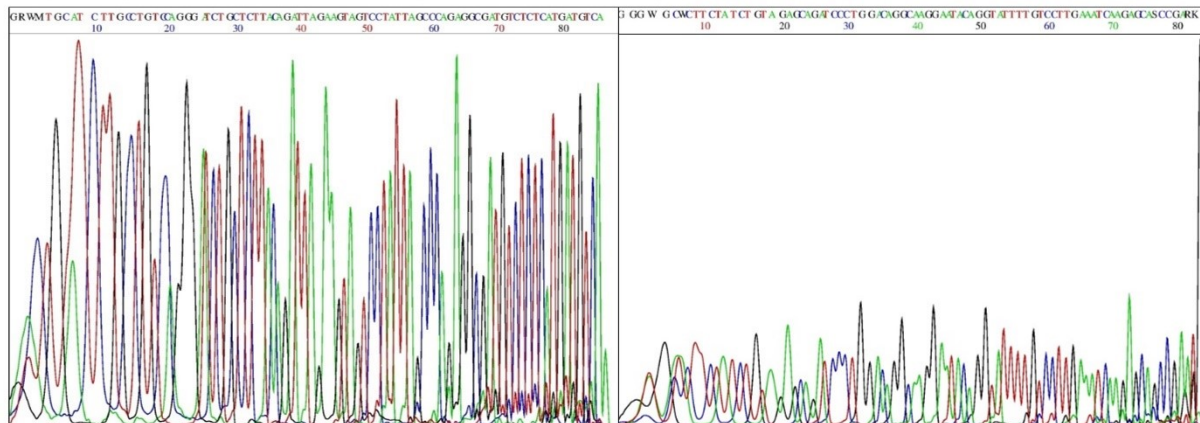
5.2.16. Sanger sequencing

In order to have dsDNA for Sanger sequencing, **118PCR_natural_1**, **118PCR_natural_2**, and **118PCR_natural_3** were obtained using either **118ON_C^{CA}G^{PA}U^{SA}A^{OP}**, **118DNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}** or **118DNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** as templates following procedures described in Section 5.2.15 with non-labeled primers. Samples were purified using QIAquick PCR Purification Kit according to the manufacturer's protocol. The resulting natural 118bp DNA samples (40 ng) were sent for Sanger sequencing using corresponding primers (5 μ L, 5 μ M) (Figure 65).

A) 118PCR_natural_1

Sequencing chromatogram with forward primer Prim^{Flank}

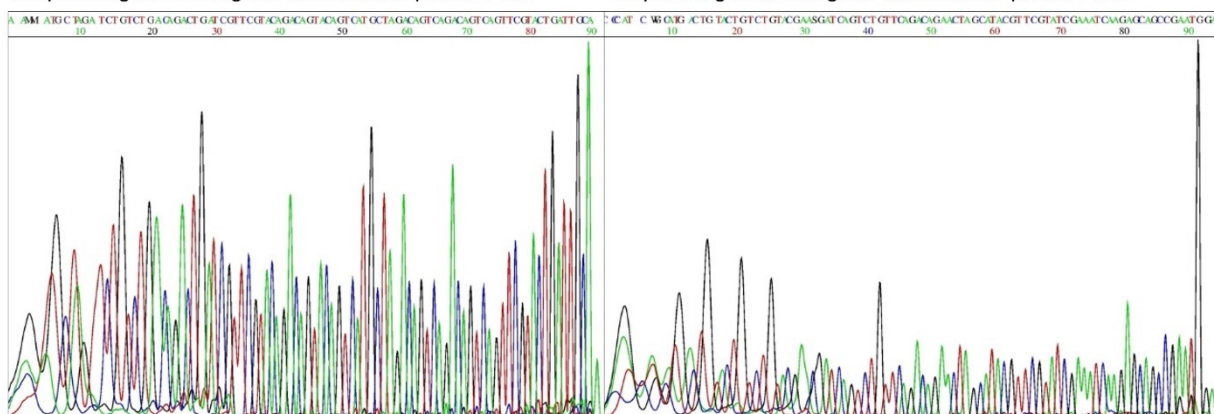
Sequencing chromatogram with reverse primer Prim^{L20}



B) 118PCR_natural_2

Sequencing chromatogram with forward primer Prim^{Flank}

Sequencing chromatogram with reverse primer Prim^{NK98pr2}



C) 118PCR_natural_3

Sequencing chromatogram with forward primer Prim^{Flank}

Sequencing chromatogram with reverse primer Prim^{NK98pr2}

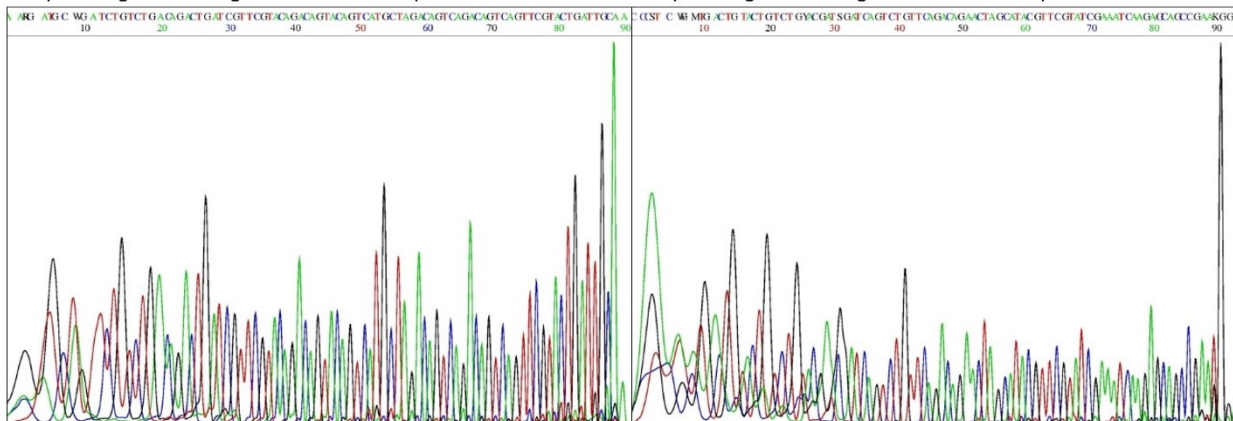


Figure 65. The results of Sanger sequencing of 118PCR_natural_1, 118PCR_natural_2, and 118PCR_natural_3.

5.3. CD spectroscopy and melting temperatures determination

5.3.1. General remarks

The CD measurements were performed on a Jasco-1500 spectropolarimeter (JASCO Inc. Easton, MD, USA) in spectral range from 200 nm to 400 nm in 1 mm rectangular quartz cell with following experimental setup: standard instrument sensitivity, 1 nm bandwidth, a scanning speed of 10 nm/min, a response time of 8 s and three accumulations. After baseline subtraction, the final data were recalculated on the concentration of nucleotide and expressed as molar differential extinction $\Delta\epsilon$ (cm⁻¹mol⁻¹).

Melting temperature measurements performed using UV-absorption were carried out on Cary 100 Bio UV/VIS Spectrophotometer with temperature controller (Varian). The spectra were recorded in 1 cm rectangular quartz cell, in temperature range 25 °C - 95 °C with temperature increment 1 °C/min under 260 nm detection and were obtained from three cycles (6 ramps in total). T_m values (in °C) were calculated using first negative derivative of intensity over temperature.

5.3.2. Preparation of the samples

Method J1: preparation of 98DNA_1. The reaction mixture (20 µL) contained template Temp^{FVL-A} (0.5 µM, 0.5 µL), reverse primer Prim^{LT25TH} and forward primer Prim^{L20} (10 µM, 4 µL each), natural dNTPs (3 mM, 1 µL), KOD XL DNA polymerase (1.25 U) and a corresponding reaction buffer (10X, 2 µL) as supplied by the manufacturer. The reaction mixture was under cycling protocol: 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 sec, 53 °C for 30 sec, and 72 °C for 1 min, followed by a final elongation step at 72 °C for 5 min. The reaction was repeated 8 times to obtain the sufficient DNA concentration for measurements. Joined fractions of **98DNA_1** were purified using QIAquick PCR Purification Kit, concentrated, diluted to 200 µL final volume with an annealing buffer of choice, and set for measurements.

Methos J2: preparation of 98DNA_2. The reaction mixture (20 µL) contained template Temp^{NK98} (0.5 µM, 0.5 µL), reverse primer Prim^{NK98pr1} and forward primer Prim^{NK98pr2} (10 µM, 6 µL each), natural dNTPs (2 mM, 2 µL), KOD XL DNA polymerase (0.6 U) and a corresponding reaction buffer (10X, 2 µL) as supplied by the manufacturer. The reaction mixture was under

cycling protocol: 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 53 °C for 30 sec, and 72 °C for 1 min, followed by a final elongation step at 72 °C for 5 min. The reaction was repeated 8 times to obtain sufficient DNA concentration for measurements. Joined fractions of **98DNA_2** were purified using QIAquick PCR Purification Kit, concentrated, diluted to 200 µL final volume with Tris-HCl buffer (10 mM, 1 mM EDTA, 65 mM NaCl, pH 7.5), and set for measurements.

Method J3: preparation of 98DNA_CCA^GPAU^{SA}A^{OP}. The reaction mixture (10 µL) containing template Temp^{FVL-A} (100 µM, 0.5 µL), primer Prim^{LT25TH} (100 µM, 0.5 µL), set of four anionic dN^RTPs (dU^{SA}TP, dC^{CA}TP, dA^{OP}TP, dG^{PA}TP) (4 mM, 1.25 µL each), KOD XL DNA polymerase (1.25 U), and the enzyme reaction buffer (10X, 1 µL) as supplied by the manufacturer was incubated for 40 min at 60 °C and stopped by cooling to 8 °C. The reaction was repeated 40 times to obtain the sufficient DNA concentration for measurements. Joined fractions of **98DNA_CCA^GPAU^{SA}A^{OP}** were purified using Agencourt AMPure XP magnetic particles, concentrated, diluted to 200 µL final volume with an annealing buffer of choice, and set for measurements.

Method J4: preparation of 98DNA_dsCCA^GPAU^{SA}A^{OP}. Oligonucleotides **98ON_CCA^GPAU^{SA}A^{OP}** and **98cON_CCA^GPAU^{SA}A^{OP}** (preparation described below) were annealed together in an annealing buffer of choice under following protocol: 95 °C for 5 min, followed by gradual cooling to 25 °C for 90 min. The annealed product was analyzed by agarose gel containing GelRed intercalator and visualized using fluorescence imaging (Figure 43, lanes 1,2). The sample was diluted to 200 µL final volume with an annealing buffer of choice and set for measurements.

Method J5: preparation of 98ON_CCA^GPAU^{SA}A^{OP} and 98cON_CCA^GPAU^{SA}A^{OP}. To obtain double-stranded products **98DNA_CCA^GPAU^{SA}A^{OP}** and **98cDNA_CCA^GPAU^{SA}A^{OP}**, PEX reaction, described in Method J3, was repeated 63 times (in the case of **98cDNA_CCA^GPAU^{SA}A^{OP}** template Temp^{FVL-A_comp} and primer Prim^{L20} were used) (for sequences see Table 42). Then modified strands were separated from the templates using gel extraction method (Section 5.2.14).

Method J6: preparation of 98DNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}. The reaction mixture (50 µL) containing template Temp^{NK98} (100 µM, 0.75 µL), primer Prim^{NK98pr1} (100 µM, 0.75 µL), set of

four modified **dN^RTPs** (**dA^{NH2}TP**, **dU^{NMe}TP**, **dG^{NMe2}TP**, **dC^{NMe3}TP**) (4 mM, 2.5 μ L; only in the case of **dC^{NMe3}TP** 6 mM), MgSO₄ (25 mM, 3 μ L), betaine (5 M, 15 μ L), KOD XL DNA polymerase (2.5 U), and the enzyme reaction buffer (10X, 5 μ L) as supplied by the manufacturer was incubated for 2 h at 72 °C. The reaction was stopped by cooling to 8 °C. The reaction was repeated 17 times to obtain sufficient DNA concentration for measurements. Joined fractions of **98DNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}** were purified using Agencourt AMPure XP magnetic particles, concentrated, diluted to 200 μ L final volume with Tris-HCl buffer (10 mM, 1 mM EDTA, 65 mM NaCl, pH 7.5), and set for measurements.

Method J7: preparation of 98DNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}. The reaction mixture (50 μ L) containing template Temp^{NK98} (100 μ M, 3 μ L), primer Prim^{NK98pr1} (100 μ M, 3 μ L), set of four modified **dN^RTPs** (**dA^{NH2}TP**, **dU^{EPh}TP**, **dG^{PA}TP**, **dC^{EAlk}TP**) (4 mM, 2.5 μ L), KOD XL DNA polymerase (2.5 U), and the enzyme reaction buffer (10X, 5 μ L) as supplied by the manufacturer was incubated for 1 h at 60 °C. The reaction was stopped by cooling to 8 °C. The reaction was repeated 3 times to obtain sufficient DNA concentration for measurements. Joined fractions of **98DNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** were purified using Agencourt AMPure XP magnetic particles, concentrated, diluted to 200 μ L final volume with Tris-HCl buffer (10 mM, 1 mM EDTA, 65 mM NaCl, pH 7.5), and set for measurements.

Method J8: preparation of 98DNA_dsA^{NH2}U^{EPh}G^{PA}C^{EAlk}. Non-labeled oligonucleotides **98ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** and **98cON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** (preparation described below) were annealed together in Tris-HCl buffer (10 mM, 1 mM EDTA, 65 mM NaCl, pH 7.5) under following protocol: 95 °C for 5 min, followed by gradual cooling to 25 °C for 90 min. To prove the suitability of the conditions, annealing was additionally performed with 5'-(6-FAM)-labeled **98ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** and 5'-Cy5-labeled **98cON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}**. The labeled double-stranded product was further analyzed by 2% agarose gel electrophoresis and visualized using fluorescence imaging (Figure 43, lanes 3-5). The non-labeled sample was diluted to 200 μ L final volume with the annealing buffer and set for measurements.

Method J9: preparation of 98ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk} and 98cON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}. To obtain double-stranded products **98DNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** and **98cDNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}**,

PEX reaction, described in Method J6, was repeated 8 times using dual-biotinylated templates (template Temp^{NK98}-bio and primer Prim^{NK98pr1} in the case of **98DNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}** and template Temp^{NK98_comp}-bio and primer Prim^{NK98pr2} in the case of **98cDNA_A^{NH2}U^{EPh}G^{PA}C^{EAlk}**) (Table 42). After that, the products were purified using Agencourt AMPure XP magnetic particles to remove shorter side-product ONs, and then modified strands were separated from the templates using Streptavidin magnetic particles according to DBStv magnetoseparation procedure (Section 5.2.8).

Method J10: preparation of 98DNA_dsA^{NH2}U^{NMe}G^{NMe2}C^{NMe3} (unsuccessful). The mixture of 5'-(6-FAM)-labeled **98ON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}** and 5'-Cy5-labeled **98cON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}** (preparation described below) in Tris-HCl buffer (10 mM, 1 mM EDTA, 65 mM NaCl, pH 7.5) was under the following protocol: 95 °C for 5 min, followed by gradual cooling to 25 °C for 90 min. The results were further analyzed by 2% agarose gel electrophoresis and visualized using fluorescence imaging (Figure 43, lanes 6-8).

Method J11: preparation of 98ON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3} and 98cON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}. To obtain double-stranded products **98DNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}** and **98cDNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}**, PEX reaction, described in Method J6, was performed using dual-biotinylated templates (template Temp^{NK98}-bio and primer Prim^{NK98pr1}-FAM in the case of **98DNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}** and template Temp^{NK98_comp}-bio and primer Prim^{NK98pr2}-Cy5 in the case of **98cDNA_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}**) (Table 42). After that, the products were purified using Agencourt AMPure XP magnetic particles to remove shorter side-product ONs, and then modified strands were separated from the templates using Streptavidin magnetic particles according to DBStv magnetoseparation procedure (Section 5.2.8).

Table 42. Additional templates used for CD samples preparation.

Name	Length (nt)	Sequence (5'→3') ^a
Temp ^{FVL-A_comp}	98	<u>CAAGGACAAAATACCTGTATTCTTGCCTGTCCAGGGAT</u> <u>CTGCTCTTACAGATTAGAAGTAGTCCTATTAGCCCAGAG</u> <u>GCGATGTCTCTCATGATGTC</u>
Temp ^{NK98_comp}	98	<u>CGATACGAACGTATGCTAGTTCTGTCTGAACAGACTGA</u> <u>TCGTTCGTACAGACAGTACAGTCATGCTAGACAGT</u> <u>CAG</u> <u>ACAGTCAGTTCGTA</u> CTGATTGC
Temp ^{NK98-bio} ^b	98	<u>GCAATCAGTACGA</u> ACTGACTGTCTGACTGTCTAGCATG ACTGTACTGTCTGTACGAACGATCAGTCTGTTCAG <u>ACA</u> <u>GAACTAGCATA</u> CGTTTCGTATCG
Temp ^{NK98_comp-bio} ^b	98	<u>CGATACGAACGTATGCTAGTTCTGTCTGAACAGACTGA</u> <u>TCGTTCGTACAGACAGTACAGTCATGCTAGACAGT</u> <u>CAG</u> <u>ACAGTCAGTTCGTA</u> CTGATTGC

5.3.3. The effect of divalent ions on T_m of superanionic DNA

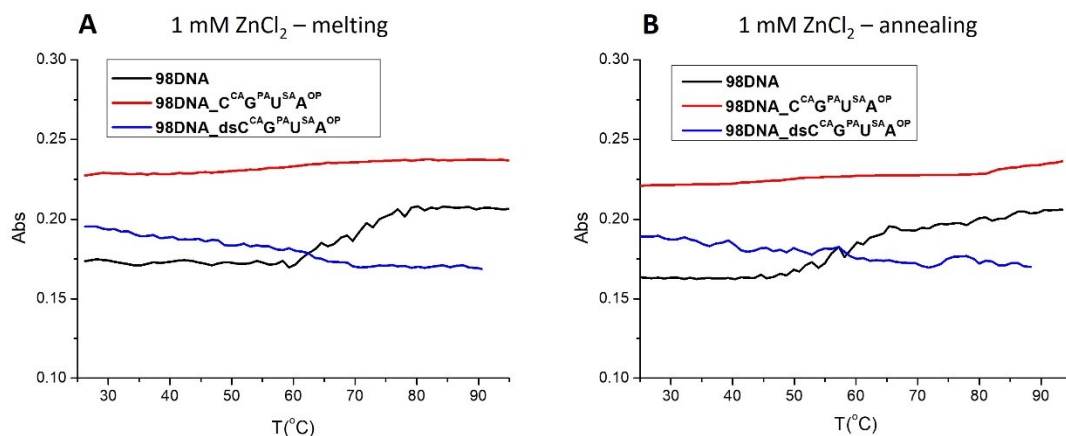


Figure 66. Melting (A) and annealing (B) curves of **98DNA_1**, **98DNA_C^{CAG}PAU^{SA}A^{OP}**, and **98DNA_dsC^{CAG}PAU^{SA}A^{OP}** in the presence of 1 mM ZnCl₂ (buffer composition: 10 mM Tris, 65 mM NaCl, 1 mM ZnCl₂, pH 7.5).

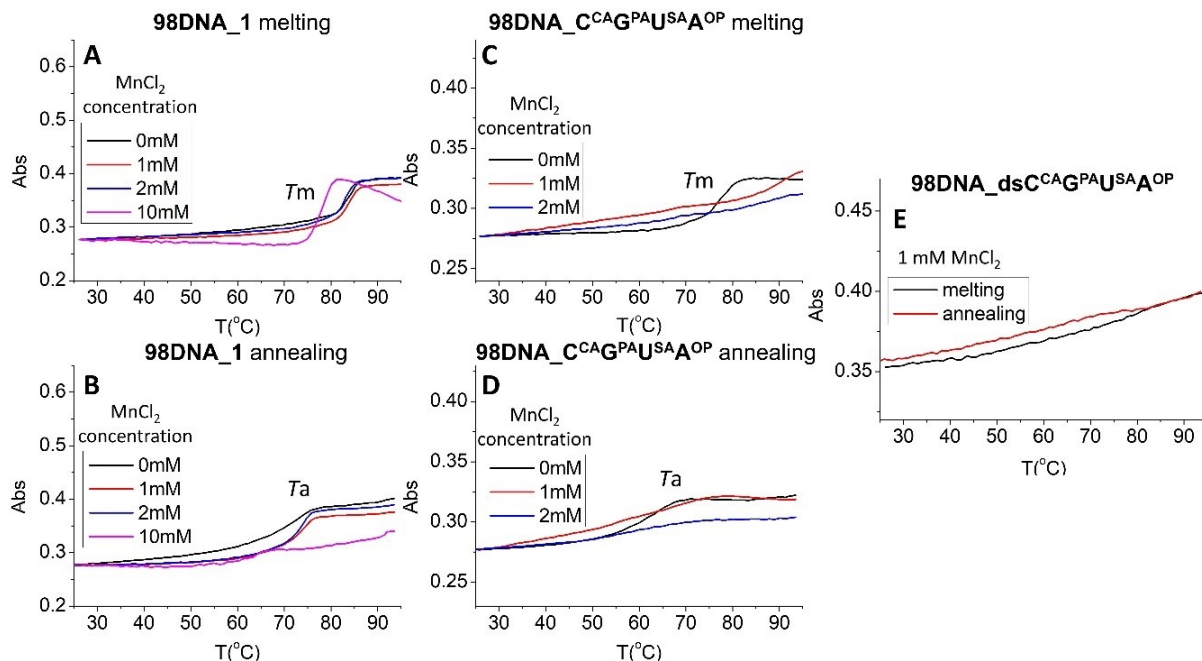


Figure 67. Melting (A, C) and annealing (B, D) curves of **98DNA_1**, **98DNA_CCAAGPAUSAAOP**, and **98DNA_dsCCAAGPAUSAAOP** (E) in the presence of MnCl_2 (buffer composition: 10 mM Tris, 65 mM NaCl, 1, 2 or 10 mM MnCl_2 , pH 7.5)

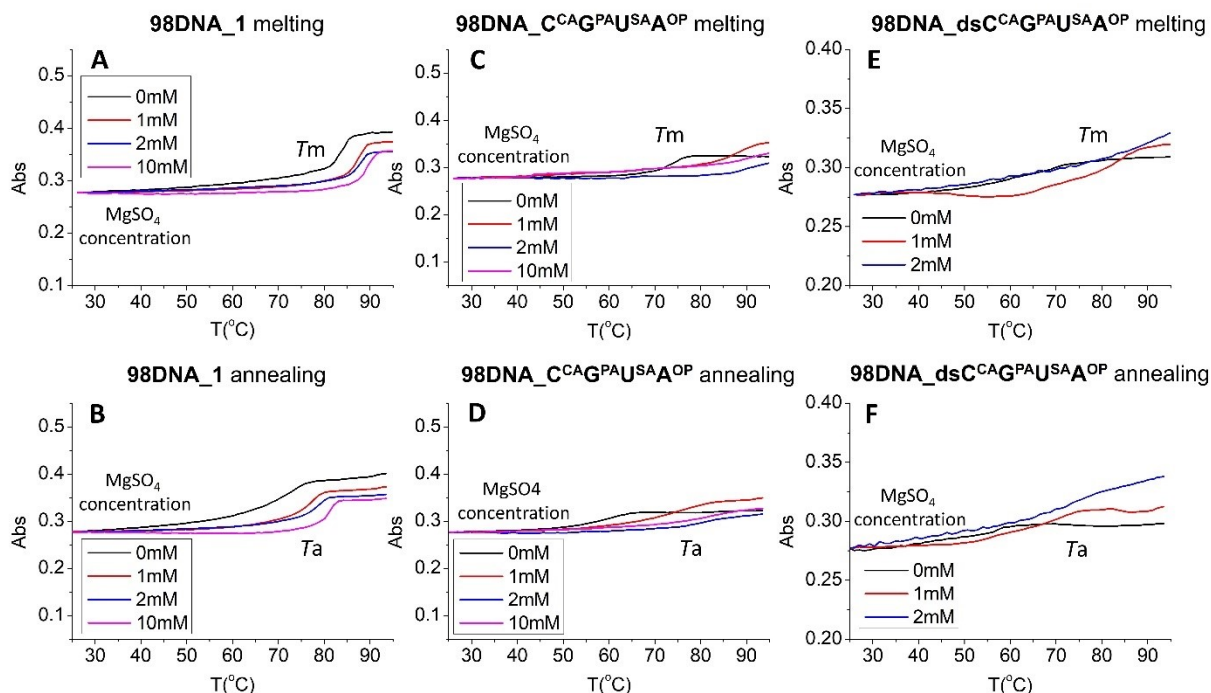


Figure 68. Melting (A, C, E) and annealing (B, D, F) curves of **98DNA_1**, **98DNA_CCAAGPAUSAAOP**, and **98DNA_dsCCAAGPAUSAAOP** in the presence of MgSO_4 (buffer composition: 10 mM Tris, 65 mM NaCl, 1, 2 or 10 mM MgSO_4 , pH 7.5-8.0).

Table 43. Melting (T_m) and annealing (T_a) temperatures of natural and modified DNA in presence of different divalent cations (Zn^{2+} , Mn^{2+} , Mg^{2+}) or in the absence of those determined by UV spectroscopy.

Divalent cation and its concentration		98DNA_1		98DNA_C ^{CA} G ^{PA} U ^{SA} A ^{OP}		98DNA_dsC ^{CA} G ^{PA} U ^{SA} A ^{OP}	
		T_m (°C)	T_a (°C)	T_m (°C)	T_a (°C)	T_m (°C)	T_a (°C)
–		84.1	73.8	73.1	60.1	65.7	55.3
Zn^{2+}	1 mM	61.8	55.2	–	–	–	–
Mn^{2+}	1 mM	84.2	74.4	85.7	52.1	–	–
	2 mM	83.1	73.8	85.2	53.4	–	–
	10 mM	78	64.2	–	–	–	–
Mg^{2+}	1 mM	87.4	77.2	87.6	74.3	84.1	71.4
	2 mM	88.1	78.8	89.7	81.2	85.2	70.3
	10 mM	89.6	80.8	90	82.2	–	–

5.4. Molecular Dynamics

5.4.1. Computational models

The native DNA dodecamer sequence, 5'-TCCAGGGATCTG-3', was built in Pymol and subsequently parameterized with the CHARMM36m forcefield⁹⁹ using CHARMM-GUI¹⁰⁰. Modifications to the DNA bases were incorporated using custom scripts based on the doGlycans tool¹⁰¹, and the corresponding parameters were obtained from CHARMM-GUI for individual modified bases.

The unmodified DNA dodecamer **12DNA**, **12DNA_C^{CA}G^{PA}U^{SA}A^{OP}** containing one strand fully-modified with anionic modifications, and **12DNA_dsC^{CA}G^{PA}U^{SA}A^{OP}** containing both strands fully-modified with anionic modifications were placed in a simulation box of volume 7 nm³ and solvated with 10738 TIP3P water molecules¹⁰². In all cases 100 mM NaCl were added to the simulation box, and additional Na⁺ ions were added accordingly to maintain electrostatic neutrality.

5.4.2. Simulation protocol

All systems were initially energy-minimized and equilibrated in the NPT ensemble while maintaining positional restraints on the DNA backbone. Afterwards, twenty 40 ns molecular dynamics simulations (MD) were conducted without restraints in the NPT ensemble to simulate the non-equilibrium unfolding event. Initial velocities were assigned randomly from a Maxwell-Boltzmann distribution at 298.15 K to ensure replica independence. The temperature was maintained at 298.15 K using the V-rescale thermostat¹⁰³ with a coupling time of 1 ps, while the pressure was maintained at 1 bar using the C-rescale barostat¹⁰⁴ with a coupling time of 5 ps. The cut-off for electrostatic interactions was set at 1.2 nm, and the long-range contribution was calculated using the PME method¹⁰⁵. The cut-off for Lennard-Jones interactions was also set at 1.2 nm, and was smoothly switched off between 1 to 1.2 nm using the force-based switching function¹⁰⁶. The SETTLE algorithm¹⁰⁷ was employed to constrain water molecule geometry, while the LINCS algorithm¹⁰⁸ was used to constrain all other covalent bonds involving hydrogens. All simulations were performed using the Gromacs software, version 2022.2¹⁰⁹.

5.4.3. Simulation analysis

MD trajectories were analyzed using both the MDAnalysis Python package¹¹⁰ and Gromacs analysis tools. The time evolution of the root mean squared deviation (RMSD) with respect to an equilibrated unmodified DNA reference was computed for all simulations, considering only backbone heavy atoms. Additionally, the radius of gyration was calculated for all systems. The average value and standard deviation for these two quantities in the 20 replicas of each system are reported in Figure 48. Starting coordinates, topologies, and trajectories, as well as all files needed to reproduce the simulations are available free of charge at <https://zenodo.org/record/7998610>.

5.5. Confocal microscopy

The confocal microscopy imaging measurements were performed on a confocal microscope ZEISS LSM 980. Water immersion objective C-Apochromat 40x/1.20W was used. The samples were irradiated with a laser beam being set at 488 nm, the detector (GaAsP – PMT) range was set

to 490 – 685 nm. Detector gain 845 V and detector offset 5 were set. The images were processed using ZEISS ZEN 3.2 software.

Preparation of single-stranded samples. Single-stranded **98ON**, **98ON_A^{NH2}U^{NMe}G^{NMe2}C^{NMe3}**, **98ON_A^{NH2}U^{EPh}G^{PA}C^{EAlk}**, and **98ON_C^{CA}G^{PA}U^{SA}A^{OP}** (for sequences see Table 23) were prepared from corresponding double-stranded PEX products (preparation described in Section 5.3.2), obtained with dual-biotinylated templates, using Streptavidin magnetic particles according to DBStv magnetoseparation procedure (Section 5.2.7). Further, single-stranded products were concentrated and diluted to 0.1 μ M solutions with Milli-Q water.

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