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Scope and Limitations of the Nicking Enzyme Amplification Reaction for the Synthesis of Base-Modified Oligonucleotides and Primers for PCR

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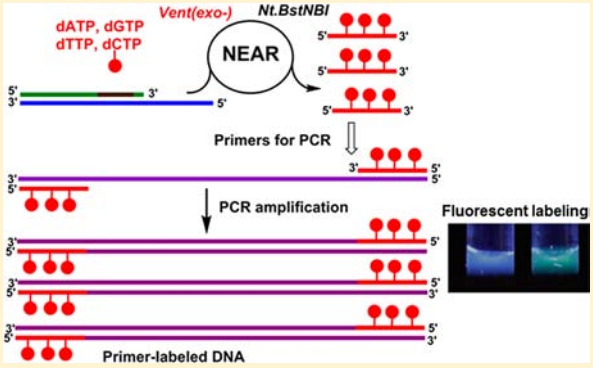
Supporting Information

ABSTRACT: Enzymatic synthesis of short (10−22 nt) base-modified oligonucleotides (ONs) was developed by nicking enzyme amplification reaction (NEAR) using Vent(exo-) polymerase, Nt.BstNBI nicking endonuclease, and a modified deoxyribonucleoside triphosphate (dNTP) derivative. The scope and limitations of the methodology in terms of different nucleobases, length, sequences, and modifications has been thoroughly studied. The methodology including isolation of the modified ONs was scaled up to nanomolar amounts and the modified ONs were successfully used as primers in primer extension and PCR. Two simple and efficient methods for fluorescent labeling of the PCR products were developed, based either on direct fluorescent labeling of primers or on NEAR synthesis of ethynylated primers, PCR, and final click labeling with fluorescent azides.



INTRODUCTION

Base-modified oligonucleotides (ONs) or DNA duplexes are of great importance in chemical biology and diagnostics. Apart from classical chemical synthesis on solid support, they can be efficiently prepared by polymerase incorporation of base-modified deoxyribonucleoside triphosphates (dNTPs).[1−4] This approach has been successfully applied for fluorescence,[5−8] redox,[9−14] spin,[15] barcode,[16] and reactive[17−23] labeling, as well as for the protection of DNA against cleavage[24−27] and for introduction of protein-like functional groups for catalytical applications.[28−33] The most useful protocols for the enzymatic incorporations of modified dNTPs include (i) PCR, which is typically used for the synthesis of long dsDNA with high density of modifications, and (ii) primer extension (PEX), which is suitable for the synthesis of medium-sized ONs (20−50 nt) bearing several modifications. PEX is often performed on biotinylated templates and combined with magnetoseparation on streptavidin[9] beads to produce ssONs. A typical scale for PEX is 1−100 pmol and any scale-up is problematic due to the high cost of the beads and capacity limits in separation columns for short ONs. The use of PEX for the synthesis of ONs shorter than 20 nts is problematic due to low denaturation temperatures of such duplexes.

Nicking enzyme amplification reaction (NEAR)[34] is an isothermal method for the amplification of short ON sequences. The methodology combines the use of a DNA polymerase and a nicking endonuclease and is based on the PEX of a longer primer in the presence of a template, cleavage of the extended primer by nicking endonuclease, and release of the resulting short ON due to insufficiently stable duplex under the elevated reaction temperature (55 °C). The primer is then regenerated and undergoes another round of PEX and nicking, etc., resulting in linear amplification of the short ON. There is also an exponential version of the method (EXPAR),[35−37] where the product also serves as a primer. The EXPAR is frequently used in diagnostics for the detection of RNA.[35−37] NEAR has never been used for the synthesis of any modified ONs until our recent preliminary communication[38] in which we reported the first application of NEAR methodology for the production of diverse cytosine-modified ONs. Here we report a full paper summarizing the scope and limitations of the methodology, as well as the use in the synthesis of labeled primers for PEX and PCR.

RESULTS AND DISCUSSION

Synthesis of New Modified dNTPs. A variety of previously known functionalized dNTPs was used in the experiments in order to evaluate the scope of the methodology. The preparation of three unknown compounds (**dG^{Fc}TP**, **dG^{FT}TP**, and **dU^{FT}TP**) is depicted in Scheme 1. **dG^{Fc}TP** was prepared by Sonogashira cross-coupling reaction of 7-iodo-7-deaza-dGTP (**dG^{I}TP**) with

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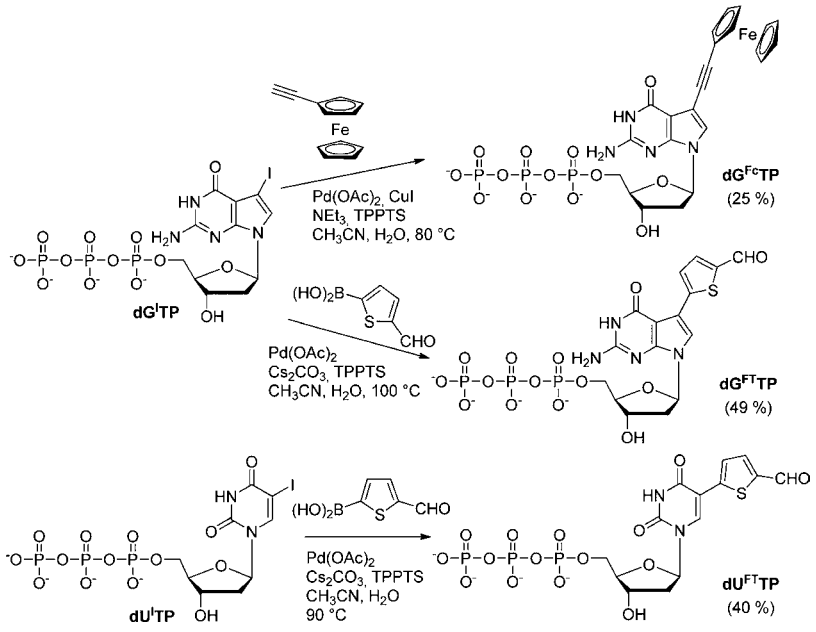
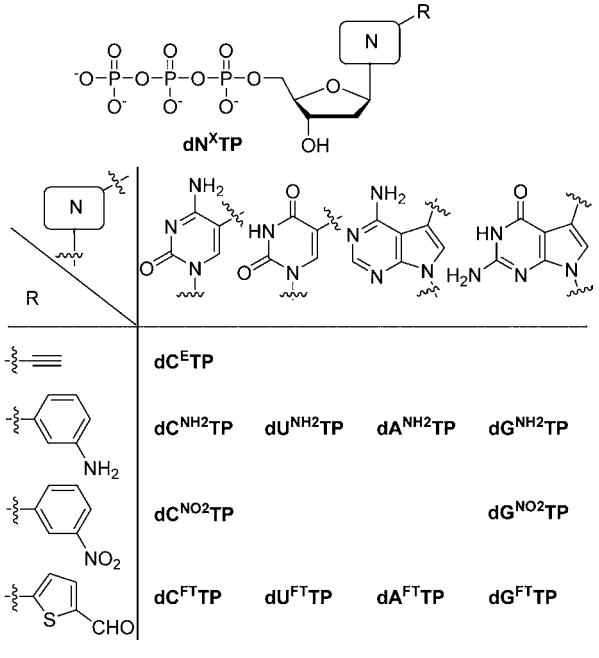
Scheme 1. Synthesis of Modified dNTPs



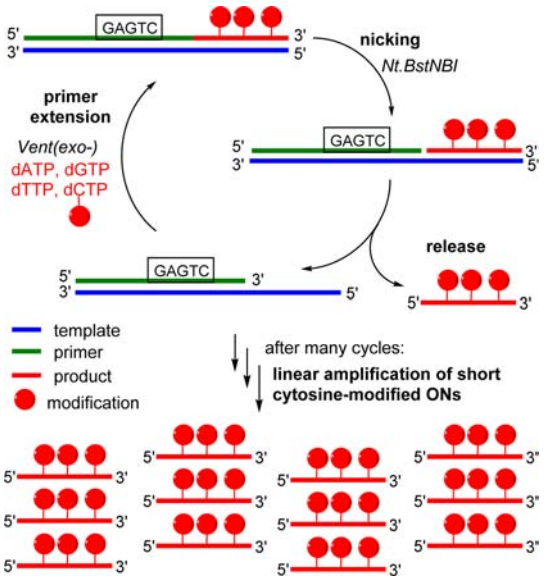
ethynylferrocene in moderate yield (25%). **dG^{FT}TP** and **dU^{FT}TP** were prepared in good yields by Suzuki cross-coupling reaction of 7-iodo-7-deaza-dGTP (**dG^{I}TP**) and 5-iodo-dUTP (**dU^{I}TP**), respectively, with 5-formylthiophene-2-boronic acid.

NEAR Optimization. The development of the preparative NEAR method was based on the pilot study on NEAR by Van Ness.[34] The use of the nicking enzyme Nt.BstNBI was adopted from the original protocol. This nickase was tested for the incorporation of a model modified nucleotide **dC^{NH2}TP** (Chart 1) in combination with various DNA polymerases (Vent(exo-), KOD XL, Deep Vent(exo-), Pwo, Phusion, and DyNAzyme) used in our previous papers[6−14] and known to tolerate substitution at position 5 of pyrimidine and 7 of 7-deazapurine dNTPs (Scheme 2). From these, Vent(exo-) gave the best

Chart 1. Structures of Modified dN^{X}TPs Used in the Study



Scheme 2. General Outline of the NEAR Synthesis of Short ssONs



results. From a series of kinetic experiments, a three-hour incubation was assessed as an optimal reaction time. Longer incubation led to a higher content of nondesired products, mainly the target ONs extended with an additional adenosine at the 5′ end. A slight excess (1.25-fold) of the modified dNTP over the natural dNTPs was used to facilitate its incorporation. Compared to our preliminary report,[38] we further lowered the amounts of dNTPs as well as DNA polymerase, thus making the whole procedure more cost-effective. The original and optimized conditions are compared and summarized in Supporting Information Table S2.

Next, we needed to develop a procedure for the isolation of the ON product from the crude reaction mixture. Neither phenol-chloroform extraction nor ethanol precipitation could be used. The modifications introduced on the nucleobases increase the lipophilicity of the product to such an extent that the ON is

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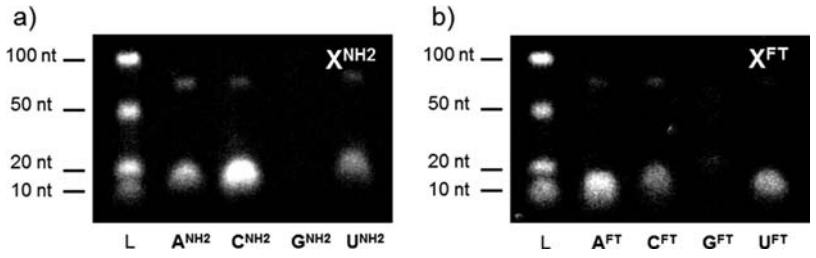

Figure 1. Incorporation of modified dNTPs in NEAR. (a) Aminophenyl-modified dNTPs; lane 1 (A^{NH2}): **$dA^{NH2}TP$**, dCTP, dGTP, dTTP; lane 2 (C^{NH2}): dATP, **$dC^{NH2}TP$**, dGTP, dTTP; lane 3 (G^{NH2}): dATP, dCTP, **$dG^{NH2}TP$**, dTTP; lane 4 (U^{NH2}): dATP, dCTP, dGTP, **$dU^{NH2}TP$**. (b) Formylthienyl-modified dNTPs; lane 1 (A^{FT}): **$dA^{FT}TP$**, dCTP, dGTP, dTTP; lane 2 (C^{FT}): dATP, **$dC^{FT}TP$**, dGTP, dTTP; lane 3 (G^{FT}): dATP, dCTP, **$dG^{FT}TP$**, dTTP; lane 4 (U^{FT}): dATP, dCTP, dGTP, **$dU^{FT}TP$**. **L** = DNA ladder; Template **Nick2**. Standard reaction conditions were used.

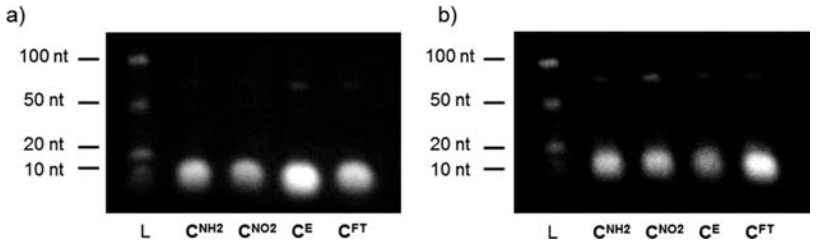

Figure 2. NEAR synthesis of cytidine-modified ONs. (a) 12-mer product (**ON2 C^X**) containing three C^X. (b) 16-mer product (**ON4 C^X**) containing four C^X. Lane 1 (+): natural dNTPs; lanes 2−5: **dC^XTP** (as indicated below each lane), dATP, dGTP, dTTP. **L** = DNA ladder. Standard reaction conditions were used.

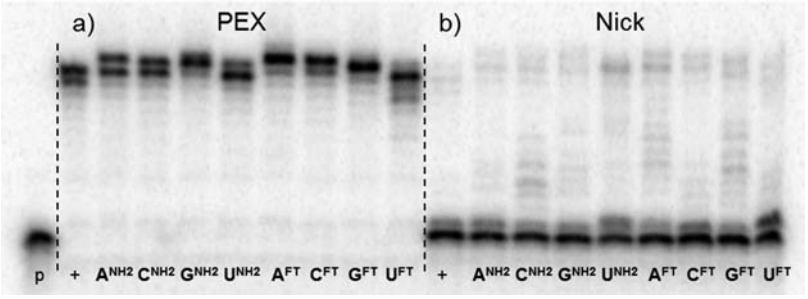

Figure 3. Denaturing PAGE analysis of PEX products (a) and products of PEX cleaved with Nt.BstNBI nicking enzyme (b). Template **Nick2**. Incubation at 55 °C, 15 min for PEX, 5 min for Nick. **p** = primer, + positive control (natural dNTPs).

extracted into the organic phase. Ethanol precipitation afforded only negligible yields and the precipitate was contaminated with unincorporated nucleoside triphosphates. Therefore, direct HPLC separation of a crude reaction mixture was attempted. Using ion-pair reversed-phase chromatography, the short modified ONs were successfully purified from unreacted triphosphates and separated from their shorter and longer byproduct homologues.

Scope of the NEAR Using Different Modified dNTPs. In order to study the scope of the NEAR in terms of a nucleobase and modification, a complete set of all four 3-aminophenyl- and 5-formyl-2-thienyl-modified dNTPs (**$dN^{NH2}TPs$**, **$dN^{FT}TPs$**) was tested as model substrates and used in combination with the three remaining natural dNTPs for the incorporation into DNA. DNA polymerase Vent(exo-) and nicking enzyme Nt.BstNBI were used with template **Nick2** and primer **PrimNick** to give a 12-mer product (Figure 1). In agreement with our previous results, NEAR proceeded best with modified cytidine triphosphates.[38] Modified uridine and 7-deazaadenosine triphosphates worked as well, but the yields were somewhat lower. In contrast, the use of modified 7-deazaguanosine triphosphates did not lead to any desired product. Several other modified **dG^XTPs** (ethynylferrocenyl **$dG^{Fc}TP$**, 3-nitrophenyl **$dG^{NO2}TP$**, 7-deaza **$dG^{7d}TP$**) were tested; however, the desired product was not observed in any case, even when using unmodified 7-deazaguanosine (see Supporting Information Figure S2). Only with G^{FT} were traces of the product observed and confirmed by MALDI-TOF analysis.

Since the 5-substituted cytidine dNTPs were proved to be the most efficient substrates for NEAR, four different modifications (aminophenyl, nitrophenyl, formylthienyl, and ethynyl) were chosen as representatives of various functional groups previously attached to DNA as redox, reactive, or protecting labels. The incorporation of these four **dC^XTPs** by NEAR was tested on two different templates, leading to 12-mer and 16-mer products, respectively (Figure 2). In all cases, the correct modified ONs were obtained (Figure 2).

To better understand why modified 7-deazaguanine nucleotides **dG^XTPs** are not substrates applicable in NEAR, we divided the standard NEAR reaction into two discrete steps, primer extension (PEX) and nicking, and studied these steps separately. A radiolabeled primer was used and the products were analyzed on a sequencing polyacrylamide gel (PAGE). In the first part of the experiment, a whole set of 8 modified dNTPs (**$dN^{FT}TP$** and **$dN^{NH2}TP$**, N = A, G, C, U) was used in PEX with Vent(exo-) polymerase. A combination of all four natural dNTPs was also

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Table 1. Sequences of Templates, Primer, and Products[a]

	sequence	product
template		
Nick1	5′-CAGACTGTTAcgagGACTCACTAGATCGG-3′	10-mer
Nick2	5′-TCGAGCAGTTCAcgagGACTCACTAGATCGG-3′	12-mer
Nick3	5′-TCAGACTGTAGACTcgagGACTCACTAGATCGG-3′	14-mer
Nick4	5′-TGCAGCATGTCAGCTAcgagGACTCACTAGATCGG-3′	16-mer
Nick5	5′-ACTGCAGCATGTCAGCTAcgagGACTCACTAGATCGG-3′	18-mer
Nick6	5′-GACTTGCAGCATGTCAGCTAcgagGACTCACTAGATCGG-3′	20-mer
Nick7	5′-CAGACTTGCAGCATGTCAGCTAcgagGACTCACTAGATCGG-3′	22-mer
Nick8	5′-ACTACGTCGAGACTTGCAGCATGTCAGCTAcgagGACTCACT AGATCGG-3′	30-mer
Nick9	5′-ACTGTGACCATGTACGTCGAGACTTGCAGCATGTCAGCTA cgagGACTCACTAGATCGG-3′	40-mer
primer		
PrimNick	5′-CCGATCTAGTGAGTCctcg-3′	
product		
ON1	5′-P-TAACAGTCTG-3′	10-mer
ON2	5′-P-TGAACTGCTCGA-3′	12-mer
ON3	5′-P-AGTCTACAGTCTGA-3′	14-mer
ON4	5′-P-TAGCTGACATGCTGCA-3′	16-mer
ON5	5′-P-TAGCTGACATGCTGCAGT-3′	18-mer
ON6	5′-P-TAGCTGACATGCTGCAAGTC-3′	20-mer
ON7	5′-P-TAGCTGACATGCTGCAAGTCTG-3′	22-mer
ON8	5′-P-TAGCTGACATGCTGCAAGTCTCGACGTAGT-3′	30-mer
ON9	5′-P-TAGCTGACATGCTGCAAGTCTCGACGTACATGGTCACAGT-3′	40-mer

[a]Lower case: spacer bases. Underlined: nicking enzyme recognition site. P: phosphate group.

used as a positive control. Next, the products of PEX were without further purification treated with nicking enzyme Nt.BstNBI. The products of both parts of the experiment were analyzed by PAGE (Figure 3). In all cases, the PEX proceeded very well to give fully extended products. Surprisingly, all the PEX products were cleaved by the nicking enzyme, even those with functionalized $\mathbf{G^X}$s. This result indicates that the activity of both the polymerase and the nicking enzyme is not inhibited by the presence of functionalized 7-deazaguanosine triphosphates or ONs and the current experiments did not give any explanation of the bad substrate activities of **$\mathbf{dG^X TP}$s** in NEAR amplification.

Along with the regenerated primer, some longer ONs can be observed on the gel after the treatment with the nicking enzyme (Figure 3b). These can be easily attributed to PEX products from the successive primer extension step. Since NEAR proceeds as a cyclic reaction, the primer regenerated by the nicking enzyme is immediately elongated by the present DNA polymerase to start a new reaction cycle.

Scope of the NEAR in Synthesis of ONs of Various Lengths. To evaluate the scope of the reaction with regard to the lengths of the products, 9 different templates designed for production of 8- to 40-mer products were tested in individual experiments (for sequences, see Table 1). In the pilot paper on NEAR, Van Ness et al. reported that the method is suitable for the preparation of 8−16-mer products.[34] However, our preliminary experiments revealed that even longer products can be obtained. Their formation can be assigned to strand displacement activity of the DNA polymerase, i.e., the ability to displace downstream DNA encountered during the synthesis. Although Vent(exo-) has only moderate strand displacement activity, it seemed to be sufficient for the displacement of the fully synthesized ON strands up to 22−30 nt.[39] We also attempted to use a polymerase with a stronger strand displacement activity (Bst DNA polymerase, large fragment); however, consistently with our previous results, NEAR did not proceed well when the DNA polymerase was changed.[38]

Analytical-scale reactions were performed on a 30 μL scale (3.75 pmol of the primer and template) with either only natural dNTPs or a mixture of a modified dNTP and three remaining natural dNTPs. The reactions were analyzed by agarose gel electrophoresis and MALDI-TOF analysis (Figure 4). According

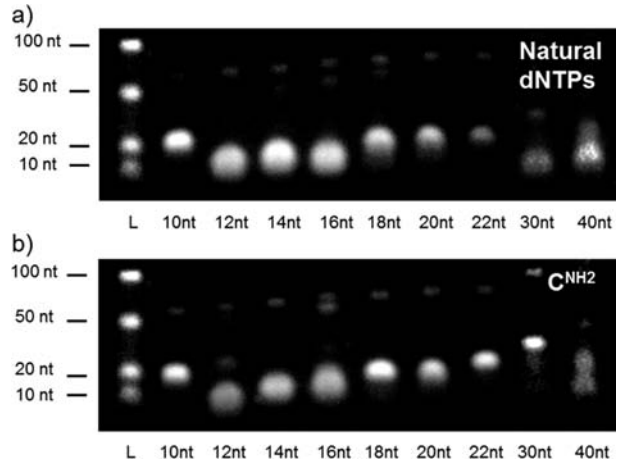

Figure 4. Agarose gel electrophoresis of NEAR products of different lengths. (a) Natural dNTPs; (b) incorporation of modified dCTP (dATP, **$\mathbf{dC^{NH2}TP}$**, dGTP, dTTP). **L** = DNA ladder. Standard reaction conditions were used.

to these analyses, the nicking enzyme reaction is applicable to the preparation of 10−22-mer ONs. An 8-mer product was not detected at all. A 30-mer product was detected in trace amounts in the experiment with all natural dNTPs and in somewhat higher concentrations in the experiment with modified dNTPs. Moreover, the product was accompanied by numerous shorter oligonucleotides. When trying to prepare a 40-mer product, only shorter ONs (mainly between 12 and 18 nt) were detected. Different reaction conditions had to be used when working with

natural dNTPs only and when incorporating a modified dNTP. While for the experiments with natural dNTPs the concentrations of Vent(exo-) and Nt.BstNBI were 0.075 U/μL and 0.9 U/μL, respectively, the experiments with **dN^{X}TP**s required higher amount of the DNA polymerase (0.1 U/μL) and lower amount of the nicking enzyme (0.6 U/μL), probably as a result of slower polymerase incorporation of modified dNTPs into DNA.[21] However, this applied only for shorter ONs (10−18 nt). For longer nonmodified ONs, the reactions gave higher yields and purer products when using the same reaction conditions as with modified dNTPs (see Supporting Information Figure S3).

When preparing shorter ON products, some longer ONs were always detected and isolated. These were mainly the desired products extended with an additional adenosine, but occasionally also with guanosine or thymidine at the 3′ end. The addition of an extra nucleobase can be considered as an example of template-free DNA synthesis. *Ab initio* DNA synthesis by thermophilic polymerases in the absence of any templating or priming DNA strands had first been reported by Clark[40,41] and Ogata.[42] This synthesis has been found to be accelerated in the presence of a restriction endonuclease[43] or nicking endonuclease[44] and has been attributed as the main source of nondesired background side-products in exponential NEAR experiments.[45]

Apart from working on an analytical scale, a series of semipreparative reactions (25-fold increase in all components) were tested. Although these reactions were conducted under the same conditions as the analytical ones, only the short ONs (10−14 nt) were obtained in pure form, whereas all the longer ONs were obtained with a mixture of shorter products (for chromatograms, see Supporting Information Figure S4). Further optimization of the reaction conditions did not lead to any improvement. The ONs were separated and purified by ion-pair reversed-phase HLPC, using a Waters XBridge OST C18 column. The isolated yields depended on the ON length, reaching almost 10 nmol for a 10-mer product and a still acceptable 2.5 nmol for a 22-mer product (Table 2). The yield of a 10-mer corresponds to over 100 successful cycles of the NEAR reaction.

Table 2. Yields of Semipreparative NEAR Reactions[a]

product	length	sequence	yield
ON1	10-mer	5′-P-TAACAGT**C**TG-3′	9.7 nmol
ON2	12-mer	5′-P-TGAACTG**C**TCGA-3′	7.3 nmol
ON3	14-mer	5′-P-AGT**C**TACAGT**C**TGA-3′	5.1 nmol
ON4	16-mer	5′-P-TAG**C**TGACATG**C**TG**C**A-3′	4.7 nmol
ON5	18-mer	5′-P-TAG**C**TGACATG**C**TG**C**AGT-3′	3.6 nmol
ON6	20-mer	5′-P-TAG**C**TGACATG**C**TG**C**AAGT**C**-3′	2.8 nmol
ON7	22-mer	5′-P-TAG**C**TGACATG**C**TG**C**AAGT**C**TG-3′	2.6 nmol

[a]Bold: position of modification in the product. P: phosphate group.

Sensitivity of the Nicking Enzyme toward Modifications within or Next to the Recognition Sequence. Recently, we have reported studies on the cleavage of DNA functionalized in the restriction site by type II restriction endonucleases.[24,25] While some restriction endonucleases tolerated at least small modifications on 7-deazaadenosines and uridines, no modifications were tolerated on cytidine derivatives. Since modified cytidines came out as the best substrates for NEAR, a question arose whether Nt.BstNBI would tolerate modifications within its recognition sequence or within the four-nucleotide spacer.

Four different templates were designed, leading to the incorporation of modified **C^X** next to the spacer (**Cleave1**), at the 3′ end of the spacer (**Cleave2**), within the spacer (**Cleave3**), and within the recognition sequence (**Cleave4**) (for sequences, see Table 3). For each template, an unsubstituted cytidine, a cytidine bearing a small modification (**C^E**), and a cytidine bearing a bulkier modification (**C^{NH2}**) were used. Generally, the nicking enzyme tolerated both modifications next to and within the four-base spacer. Surprisingly, it also tolerated a small modification (ethynyl) in the recognition sequence. However, when a larger modification (aminophenyl) was incorporated in the recognition sequence, no NEAR product was observed (Figure 5).

This result is crucial to understanding why the exponential mode of NEAR (EXPAR) cannot be applied for the synthesis of short oligonucleotides instead of the currently used linear mode. In EXPAR the primer has the same sequence as the product. Although the product itself is unstable in a duplex at the reaction temperature, it forms a transient duplex with the template and this transient duplex can act as a primer-template for the DNA polymerase. The primer is extended over the recognition site and four-base spacer, which further stabilizes the duplex. The polymerase-mediated extension then continues as in the linear mode (nicking beyond the four-base spacer and dissociation of the oligonucleotide to regenerate the primer-template duplex). The product of the linear reaction serves as a new primer, anneals to the template, which is present in a large excess, and creates a new primer-template. The use of modified nucleobases in EXPAR would be limited to small modifications since the incorporation of larger modifications into the recognition sequence prevents the nickase from cleavage. Furthermore, EXPAR starts to produce ssON product only when all the present template is turned into primer-template duplexes and when the kinetics turns to the linear mode.

Use of NEAR Products as Primers in PEX. The use of modified NEAR products as primers in PEX and PCR experiments represents a simple way to introduce modifications into the primer part of the target oligonucleotides. Such an approach would enable the preparation of oligonucleotides with modifications only in the primer part, fully modified oligonucleotides on one nucleobase, and even oligonucleotides with a different modification in the primer and product part on the same or different nucleobase (Scheme 3a,b).

We designed a simple PEX experiment with a 32-mer biotinylated template **Nick4PEX-bio** and 16-mer **ON4 C^X** primer (**X** = E, FT) (for sequences, see Table 4). Either natural dNTPs (positive control) or a combination of natural dNTPs with a modified dNTP (**dC^{X}TP** or **dA^{X}TP**) were used. After the PEX, the strands were separated using streptavidine-coated magnetic beads according to standard technique.[20] Unfortunately, besides the desired ssON product, part of the template was always released during the separation procedure, as proven from the agarose gel electrophoresis and MALDI-TOF analyses (Figure 6).

The primers were prepared by NEAR under standard reaction conditions and purified by HPLC. **ON4 C^{FT}** was obtained pure in 5.1 nmol yield, **ON4 C^E** (3.3 nmol) needed to be separated from a one-base longer product (3.8 nmol). An average melting temperature for a 16-mer primer is around 45 °C, the modifications can destabilize the duplex to some extent, and although most thermostable DNA polymerases, including KOD XL used in our experiments, work best at higher temperatures,

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Table 3. Sequences of Templates, Primers, and Products Used in the Experiments to Determine the Sensitivity of the Nicking Enzyme Toward Modifications within and next to the Recognition Sequence[a]

	primer	sequence
template		
Cleave1	PrimCleave1	5′-TCA**G**CA**G**TACT**G**ctacGACTCCCCATGCCGCCATCG-3′ modification next to the spacer
Cleave2	PrimCleave1	5′-TCA**G**CT**G**TA**G**CAgaccGACTCCCCATGCCGCCATCG-3′ modification in the 3′ end of the spacer
Cleave3	PrimCleave1	5′-TCA**G**CT**G**TA**G**CAcgacGACTCCCCATGCCGCCATCG-3′ modification within the spacer
Cleave4	PrimCleave2	5′-TC**G**ACT**G**AA**G**CTctacGACTCCCCATGCCGCCATCG-3′ modification within the recognition sequence
primers		
PrimCleave1	-	5′-CGATGGCGGCATGGGGAGTC-3′
PrimCleave2	-	5′-CGATGGCGGCATGGGGAGT-3′
products		
ONCleave1	-	5′-P-CAGTA**C**TG**C**TGA-3′
ONCleave2	-	5′-P-TG**C**TA**C**AG**C**TGA-3′
ONCleave3	-	5′-P-TG**C**TA**C**AG**C**TGA-3′
ONCleave4	-	5′-P-AG**C**TT**C**AGTCGA-3′

[a]Lower case: spacer bases. Underlined: nicking enzyme recognition site. Bold: position of the modification (in the complementary strand to the template). P: phosphate group.

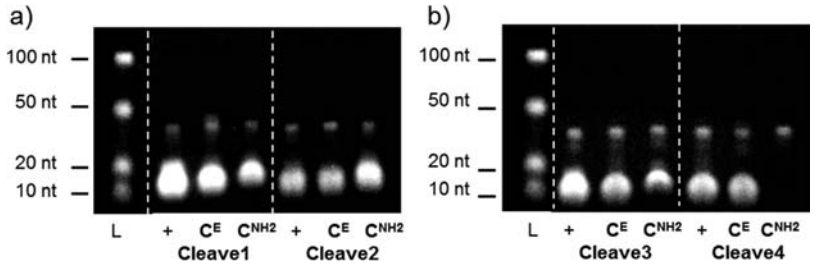


Figure 5. Agarose gel electrophoresis of NEAR products using templates **Cleave1−4**. **L** = DNA ladder; (+) positive control − incorporation of natural dNTPs; ($\mathbf{C^E}$) incorporation of $\mathbf{dC^ETP}$ (together with dATP, dGTP, dTTP; ($\mathbf{C^{NH2}}$) incorporation of $\mathbf{dC^{NH2}TP}$ (together with dATP, dGTP, dTTP). Standard reaction conditions were used.

after some optimization it turned out best to lower the PEX elongation temperature from commonly used 60 to 55 °C.[46]

Use of NEAR Products in PCR. Our next goal was to use NEAR products as primers in PCR (Scheme 3c,d). Both the forward and reverse primers were designed as 20-mers with 3 and 5 (modified) cytidines, respectively (for sequences, see Table 5). Their synthesis by NEAR encountered the same problem as the synthesis of primers for PEX—the reaction led to a mixture of the desired product and the product extended with an additional adenosine. Both products were separated by HPLC. The yields of the pure products were between 1.4 and 3.9 nmol (Table 6).

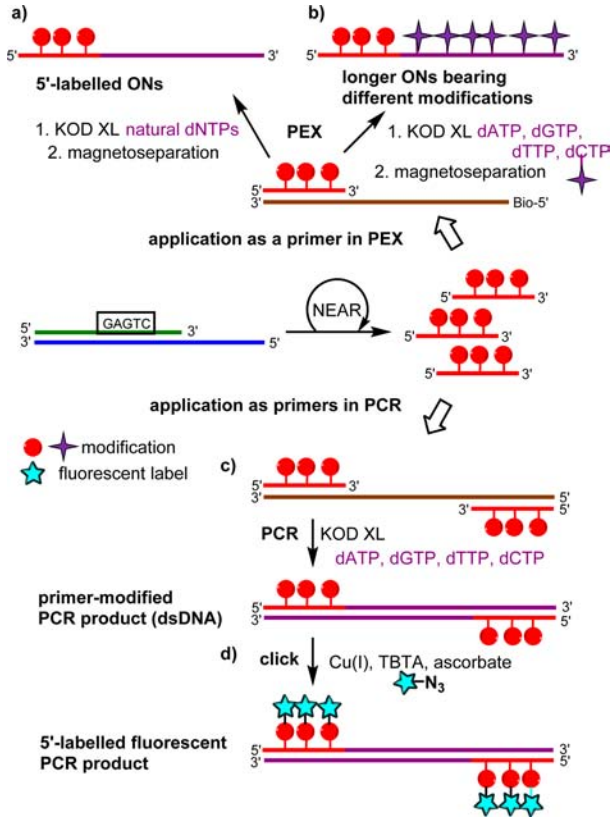
PCR with a 98-mer template proceeded smoothly without any additives. Cytidine-modified primers were used and the synthetized DNA contained either only natural dNTPs or cytidines bearing the same or different modification, or adenosines bearing a different modification, respectively (Figure 7).

Formylthienyl-Modified Primers for Fluorescent Labeling of PCR Products. In order to study the application potential for fluorescent labeling, the absorption and fluorescence emission spectra of all the formylthienyl-modified nucleoside triphosphates $\mathbf{dA^{FT}TP}$, $\mathbf{dC^{FT}TP}$, $\mathbf{dG^{FT}TP}$, and $\mathbf{dU^{FT}TP}$ were measured. While pyrimidine nucleobases $\mathbf{C^{FT}}$ and $\mathbf{U^{FT}}$ show quite strong fluorescence (with quantum yield 0.19 and 0.38, respectively), purine nucleobases $\mathbf{A^{FT}}$ and $\mathbf{G^{FT}}$ are almost nonfluorescent. The emission maxima of $\mathbf{A^{FT}}$, $\mathbf{C^{FT}}$, and $\mathbf{G^{FT}}$ are almost the same (459−462 nm), whereas the emission of $\mathbf{U^{FT}}$ is slightly blue-shifted, which is most likely caused by weaker conjugation. Next, we measured the fluorescence of $\mathbf{C^{FT}}$-labeled PCR primers (**ON PCR1** $\mathbf{C^{FT}}$ and **ON PCR2** $\mathbf{C^{FT}}$) and of the PCR product (**DNA primC**$^{\mathbf{FT}}$) (Figure 8, Table 7). All the samples showed quite strong fluorescence observable with the naked eye at a concentration of 0.4 μM. Thus the NEAR synthesis of $\mathbf{C^{FT}}$-containing ONs can be used for the preparation of primers useful in direct PCR synthesis of blue-fluorescent DNA (Figures 8d and 9a).

Indirect Fluorescent Labeling by Click Reactions of Ethynyl-Modified ONs. Our next objective was to evaluate whether the $\mathbf{C^E}$ incorporated in the primer part of the PCR product would be a suitable substrate for postsynthetic labeling based on CuAAC click chemistry.[47−54] We chose two commercial fluorescent azides as representatives of a large group of fluorescent labels used in the labeling of biomolecules, one derived from perylene (Prl, **1**) and the other from Lissamine rhodamine B (LRB, **2**).

The PCR reaction (Figure 9a) was carried out under the conditions described above, but the reaction scope was increased 10 times so that the reaction provided enough material for the subsequent CuAAC reaction. After the PCR, the reaction mixture was purified on QIAquick PCR purification kit to remove excess dNTPs and primers. The cycloaddition was performed under standard conditions, i.e., in aqueous DMSO/tBuOH, with excess of azide (**1** or **2**), Cu(I), and TBTA (Cu(I) stabilizing ligand).[48] However, using Cu(I), the presence of ascorbate as reducing agent proved to be significant to achieve the best results. The reaction was carried out for 3 h at 37 °C. The product was purified on Nucleospin Extract II columns and analyzed by agarose gel electrophoresis (Figure 9c). The absorption and fluorescence emission spectra of thus prepared fluorescent DNA were measured in water, with the DNA

Scheme 3. Use of NEAR Products as Primers in PEX[a]



[a](a) Using unmodified dNTPs or (b) using modified dNTPs, and in PCR (c) for the synthesis of primer-labelled DNA or (d) for post-synthetic click labelling.

concentrations 0.1−0.2 μM (Figure 9b). In this way, green or pink fluorescent labeling of the PCR products can be easily introduced to the primer part of the DNA.

CONCLUSIONS

NEAR was developed as a facile and efficient approach for the enzymatic synthesis of base-modified ssONs. Optimization of the procedure revealed Vent(exo-) as the best polymerase. The reaction is applicable for the synthesis of nanomolar amounts of short ONs (10−22 nt). The reaction works best for the incorporation of modified cytidines **dC^{X}TP**s and is also applicable for **dA^{X}TP**s and **dU^{X}TP**s, whereas it does not work for **dG^{X}TP**s. The nickase tolerates any modifications in the spacer but only a small (ethynyl) modification in the recognition sequence (which explains the inefficient exponential amplification in the EXPAR method). The NEAR was shown to be a very efficient procedure for the synthesis of cytosine-modified primers. These modified primers have been successfully used for PEX either with unmodified dNTPs (to form 5′-labeled ONs) or with modified dNTPs (to form ONs bearing different types of modifications in the primer stretch and in the extended part). The NEAR products also serve well as primers in PCR and were shown to be suitable for fluorescent labeling of PCR products. The use of blue-fluorescent **dC^{FT}TP** for NEAR furnished fluorescent primers, which directly gave blue-fluorescent DNA in PCR amplification. On the other hand, the use of **dC^{E}TP** in NEAR gave ethynyl-modified primers which were also used in PCR to produce ethynyl-modified DNA suitable for postsynthetic CuAAC labeling of the primer parts with fluorescent azides (or other suitable reactive azido-derivatives of choice). This indirect methodology can be easily applied in coding of different PCR products by different labels. The simplicity, efficiency, and versatility of the NEAR protocol in combination with the easy access to diverse base-modified dNTPs makes this methodology competitive in the synthesis of labeled primers to the chemical synthesis by phosphoramidite protocol—especially in the cases where a variety of different labels needs to be tested for the particular application since an unlimited variety of modified primers can be generated from the same template and primer using different modified **dN^{X}TP**s (large variety of which are commercially available).

EXPERIMENTAL SECTION

General Remarks. Synthesis and characterization data for 2′-deoxy-7-iodo-7-deazaguanosine 5′-*O*-triphosphate (**dG^{I}TP**),[55] 5-iodo-2′-deoxyuridine 5′-*O*-triphosphate (**dU^{I}TP**),[56] 7-(3-aminophenyl)-2′-deoxy-7-deazaadenosine 5′-*O*-triphosphate (**dA^{NH2}TP**),[10] 5-(3-aminophenyl)-2′-deoxycytidine 5′-*O*-triphosphate (**dC^{NH2}TP**),[10] 7-(3-aminophenyl)-2′-deoxy-7-deazaguanosine 5′-*O*-triphosphate (**dG^{NH2}TP**),[12] 5-(3-aminophenyl)-2′-deoxyuridine 5′-*O*-triphosphate (**dU^{NH2}TP**),[10] 2′-deoxy-7-(5-formylthiophene-2-yl)-7-deazaadenosine 5′-*O*-triphosphate (**dA^{FT}TP**),[20] 2′-deoxy-5-(5-formylthiophene-2-yl)cytidine 5′-*O*-triphosphate (**dC^{FT}TP**),[20] 2′-deoxy-7-(3-nitrophenyl)-cytidine 5′-*O*-triphosphate (**dC^{NO2}TP**),[10] 5-ethynyl-2′--deoxycytidine 5′-*O*-triphosphate (**dC^{E}TP**),[25] 2′-deoxy-7-(3-nitrophenyl)-7-deazaguanosine 5′-*O*-triphosphate (**dG^{NO2}TP**)[12] were reported previously. For the synthesis of 2′-deoxy-7-(ferrocene-1-yl-ethynyl)-7-deazaguanosine 5′-*O*-triphosphate (**dG^{Fc}TP**), 2′-deoxy-7-(5-formylthiophene-2-yl)-7-deazaguanosine 5′-*O*-triphosphate (**dG^{FT}TP**), and 2′-deoxy-5-(5-formylthiophene-2-yl)uridine 5′-*O*-triphosphate (**dU^{FT}TP**) see the Supporting Information.

Synthetic oligonucleotides (primer **PrimNick**; NEAR templates **Nick1**-**Nick9**, **NickPCR1**, **NickPCR2**; biotinylated PEX template **Nick4PEX-bio**; PCR template **TempPCR98**; ss ladder components **L10**-**L100**; for sequences see Table 8) were

Table 4. Sequences of the Template, Primer, and Product Used in NEAR and PEX Experiments[a]

NEAR		
template	**Nick4**	5′-TGCAGCATGTCAGCTA*cgag*<u>*GACTC*</u>*ACTAGATCGG*-3′
primer	**PrimNick**	5′-CCGATCTAGTGAGTCctcg-3′
PEX		
template	**Nick4 PEXbio**	5′-bio-ACCAGTTGTCAGAGCT*TGCAGCATGTCAGCTA*-3′
primer	**ON4 C^{X}**	5′-P-TAGCTGACATGCTGCA-3′
product	**ON4 PEX**	5′-P-TAGCTGACATGCTGCAAGCTCTGACAACTGGT-3′

[a]Italics: segments forming duplex with the primer. Lower case: spacer bases. Underlined: nicking enzyme recognition site. Bold: position of the modification in the primer. P: phosphate group. Bio: biotin.

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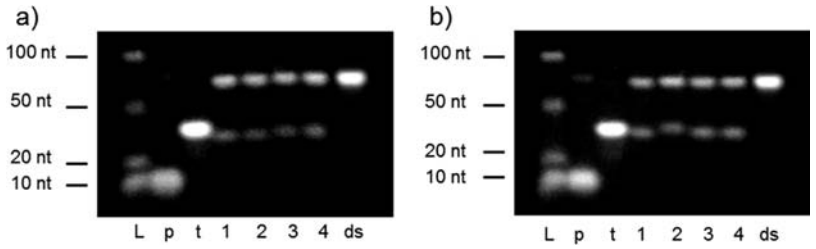

Figure 6. Agarose gel electrophoresis of PEX primer and products. **L** = DNA ladder; ***p*** = primer **ON4 C^X**; **t** = template **ON4 PEX-bio**. Lane 1: PEX product − positive control (natural dNTPs). Lane 2: PEX product − incorporation of the same modification on dCTP as is in the primer (**dC^{X}TP**, dATP, dGTP, dTTP). Lane 3: PEX product − incorporation of a different modification on dCTP (**dC^{Y}TP**, dATP, dGTP, dTTP). Lane 4: PEX product − incorporation of a different modification on dATP (**dA^{Y}TP**, dCTP, dGTP, dTTP). **ds** = double-stranded DNA (PEX product without strand separation). (a) **X** = E, **Y** = FT; (b) **X** = FT, **Y** = E.

Table 5. Sequences of the Templates and Primers Used in NEAR and PCR Experiments[a]

NEAR		
Template 1	**NickPCR1**	5′-AATACAGGTATTTTGTCCTT*cgag*<u>*GACTC*</u>*ACTAGATCGG*-3′
Template 2	**NickPCR2**	5′-GCGATGTCTCTCATGATGTC*cgag*<u>*GACTC*</u>*ACTAGATCGG*-3′
Primer	**PrimNick**	5′-CCGATCTAGTGAGTCctcg-3′
PCR		
Primer 1	**ON PCR1 C^X**	5′-P-AAGGA**C**AAAATA**CC**TGTATT-3′
Primer 2	**ON PCR2 C^X**	5′-P-GA**C**ATCATGAGAGA**C**ATC**GC**-3′
Template	**TempPCR98**	5′-*GACATCATGAGAGACATCGC*CCTCTGGGCTAATAGGACT ACTTCTAATCTGTAAGAGCAGATCCCTGGACAGGCAAGGA *ATACAGGTATTTTGTCCTT*G-3′

[a]Italics: segments forming duplex with the primer. Lower case: spacer bases. Underlined: nicking enzyme recognition site. Bold: position of the modification in the primer. P: phosphate group.

Table 6. Yields of PCR Primers Prepared by NEAR

	product	product + A
ON PCR1 C^E	2.3 nmol	0.9 nmol
ON PCR2 C^E	1.4 nmol	0.7 nmol
ON PCR1 C^{FT}	3.9 nmol	0.1 nmol
ON PCR2 C^{FT}	2.5 nmol	0.1 nmol

purchased from Sigma-Aldrich. Enzymes Vent(exo-) DNA polymerase and Nt.BstNBI as well as natural nucleoside triphosphates (dATP, dCTP, dGTP, dTTP) were purchased from New England Biolabs. KOD XL DNA polymerase was purchased from Merck. 7-Deaza-2′-deoxyguanosine was obtained from Jena Bioscience. Streptavidin Magnetic Particles were obtained from Roche. Acetonitrile for HPLC and TEAA buffer were purchased from Sigma-Aldrich. All solutions were prepared in Milli-Q water. Oligonucleotide samples were concentrated on CentriVap Vacuum Concentrator System (Labconco). Mass spectra of the prepared ONs were measured by MALDI-TOF, on UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany), with 1 kHz smartbeam II laser. UV spectra were measured on Varian CARY 100 Bio spectrophotometer and on NanoDrop1000 (ThermoScientific).

Preparation of Single-Stranded DNA Ladder. The ss ladder was prepared by mixing stock solutions of commercial ss oligonucleotides. The final concentrations were 40 μM L10, 2.0 μM L20, 0.75 μM L50, and 0.75 μM L100. The ss ladder (8 μL) was mixed with TrackIt Cyan/Orange loading buffer (1.6 μL) prior to loading on the gel.

NEAR General Procedure. The reaction mixture contained the template (0.125 μM), primer (0.125 μM), modified **dN^{X}TP** (156 μM), natural dNTPs (125 μM), 1× ThermoPol buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl/pH 8.8/, 0.1% Triton-X-100, and 2 mM $MgSO_4$), and 0.5× NEBuffer 3 (50 mM NaCl, 25 mM Tris-HCl/pH 7.9/, 5 mM $MgCl_2$, and 0.5 mM DTT). The amounts of enzymes depend on whether only natural dNTPs or modified **dN^{X}TP** together with the three remaining natural dNTPs were incorporated, being 0.075 U/μL Vent(exo-), 0.90 U/μL Nt.BstNBI and 0.10 U/μL Vent(exo-), 0.60 U/μL

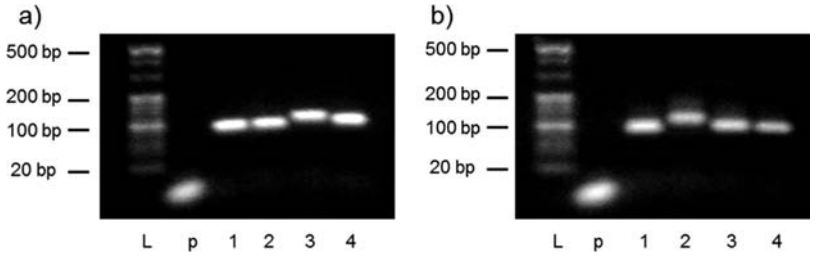

Figure 7. Agarose gel electrophoresis of PCR primer and products. **L** = DNA ladder; ***p*** = primer **ONPCR1 C^X**. Lane 1: positive control (natural dNTPs). Lane 2: incorporation of the same modification on dCTP as is in the primer (**dC^{X}TP**, dATP, dGTP, dTTP). Lane 3: incorporation of a different modification on dCTP (**dC^{Y}TP**, dATP, dGTP, dTTP). Lane 4: incorporation of a different modification on dATP (**dA^{Y}TP**, dCTP, dGTP, dTTP). (a) **X** = E, **Y** = FT; (b) **X** = FT, **Y** = E.

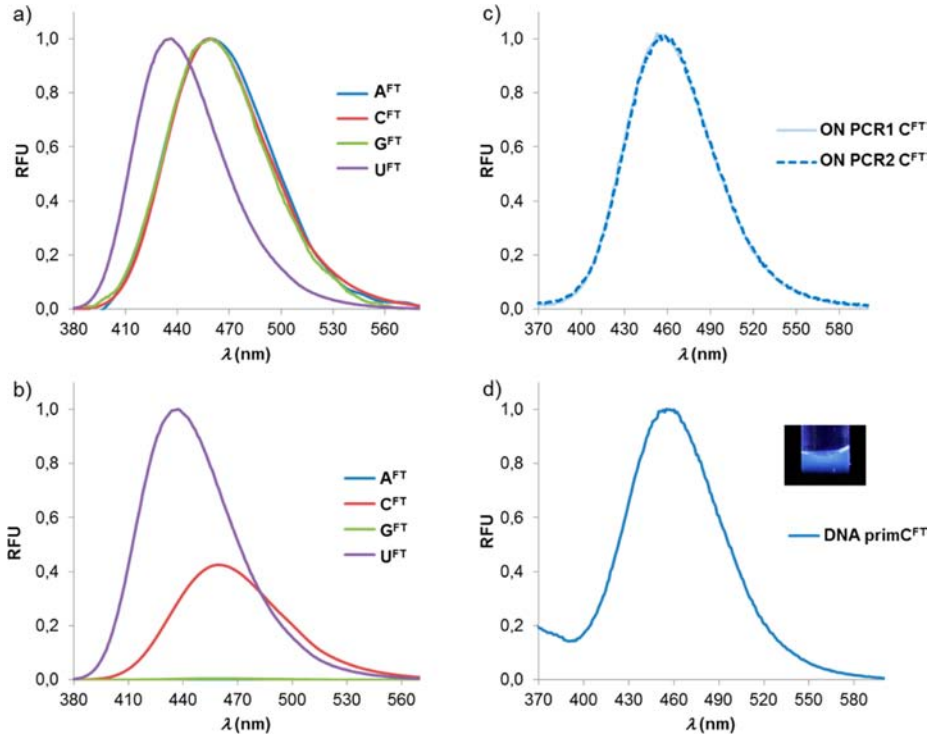

Figure 8. Photophysical properties of **dN^{FT}TPs**, ONs, and DNA. (a) Normalized emission spectra of **dN^{FT}TPs** (10 μM aqueous solutions) in water, **dA^{FT}TP**, **dC^{FT}TP**, **dG^{FT}TP**, **dU^{FT}TP**; (b) relative emission spectra of **dN^{FT}TPs** (10 μM aqueous solutions) in water; (c) emission spectra of PCR primers **ON PCR1 C^{FT}** and **ON PCR2 C^{FT}** (10 μM aqueous solutions); (d) emission spectrum of **DNA primC^{FT}**: PCR product with formylthienyl-modified primers **ON PCR1 C^{FT}** and **ON PCR2 C^{FT}** (0.4 μM aqueous solution). The spectra were measured by excitation at 330 nm.

Table 7. Photophysical Properties of the dN^{FT}TPs in Water

X^{FT}	λ_{abs} (nm)	ε (L mol^{-1} cm^{-1})	λ_{em} (nm)	Φ
A^{FT}	334	5200	459	0.001
C^{FT}	297	8900	462	0.19
G^{FT}	378	12100	460	0.002
U^{FT}	351	9100	435	0.38

Nt.BstNBI, respectively. The reaction mixture was incubated at 55 °C for 3 h. The reaction was stopped by cooling to 4 °C.

NEAR on Analytical Scale. The analytical reactions were performed according to the general procedure in a volume of 30 μL. The products were analyzed by agarose gel electrophoresis using 4% agarose gels stained with GelRed (Biotium). Samples were prepared by mixing 1.6 μL of TrackIt Cyan/Orange loading buffer (Invitrogen) and 8 μL of the reaction mixture or ss DNA ladder (*vide supra*). The gel was run for 70 min at 120 V and imaged using electronic dual wave transilluminator equipped with UltraCam 8gD digital imaging system (Ultra-Lum). For MALDI-TOF mass spectrometry analysis the excess nucleotides and buffer salts were removed by the filtration on Illustra MicroSpin G-25 columns (GE Healthcare).

NEAR on Semipreparative Scale. The semipreparative reactions were performed according to the general procedure in a volume of 750 μL. After the reaction was stopped, the solution was concentrated on a vacuum concentrator to approximately 150 μL. The viscous concentrate was injected on HPLC XBridge OST C_{18} Column (Waters; 2.5 μm particle size, 4.6 mm ×50 mm) and separated using a gradient of triethylammonium acetate (TEAA) and acetonitrile and flow rate 1 mL/min. Mobile phase A corresponds to 0.1 M TEAA in HPLC-grade water, mobile phase B to acetonitrile/0.1 M TEAA in HPLC-grade water 20/80 (v/v). The gradient started with 60% mobile phase A and 40% mobile phase B, going linearly to 80% mobile phase B in 53 min. The fractions containing the product were evaporated on a vacuum concentrator. The residue was diluted with known volume of water and a UV spectrum was measured. The concentration of the product was calculated from the absorbance at 260 nm and extinction coefficient obtained from an online calculator by IDT Biophysics.[57] The products were analyzed by MALDI-TOF mass spectrometry.

PEX using ON4 C^{X} as Primer. The reaction mixture (30 μL) contained KOD XL DNA polymerase (2.5 U/μL, 1.5 μL), dNTPs (either all natural or 3 natural and 1 modified, 4 mM, 1.5 μL), primer (**ON4 C^{E}** or **ON4 C^{FT}**, 10 μM, 6 μL), and 5′-biotinylated template (**Nick4PEX-bio**, 10 μM, 6 μL) in KOD XL reaction buffer (3 μL) supplied by the manufacturer. The reaction mixture was incubated for 30 min at 55 °C in a thermal cycler. The reaction was stopped by cooling to 4 °C.

Streptavidin Magnetic Particles (Roche, 20 μL) were washed with Binding buffer TEN_{100} (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5) (3 × 100 μL). The reaction mixture after PEX was diluted with the Binding buffer TEN_{100} (30 μL), the solution was added to the prewashed magnetic beads and incubated for 20 min at 18 °C and 1200 rpm. After the incubation, the magnetic beads were collected on a magnet (PureProteome Magnetic Stand, Merck) and the solution was discarded. The beads were washed successively with Wash buffer TEN_{1000} (10 mM Tris, 1 mM EDTA, 1 M NaCl, pH 7.5) (2 × 100 μL), and water (3 × 100 μL). Then water (20 μL) was added and the sample was denatured for 2 min at 55 °C and 900 rpm. The beads were collected on a magnet and the solution was transferred into a clean vial. The UV spectrum of the solution was measured on NanoDrop. The product was analyzed by MALDI-TOF mass spectrometry and on agarose gels.

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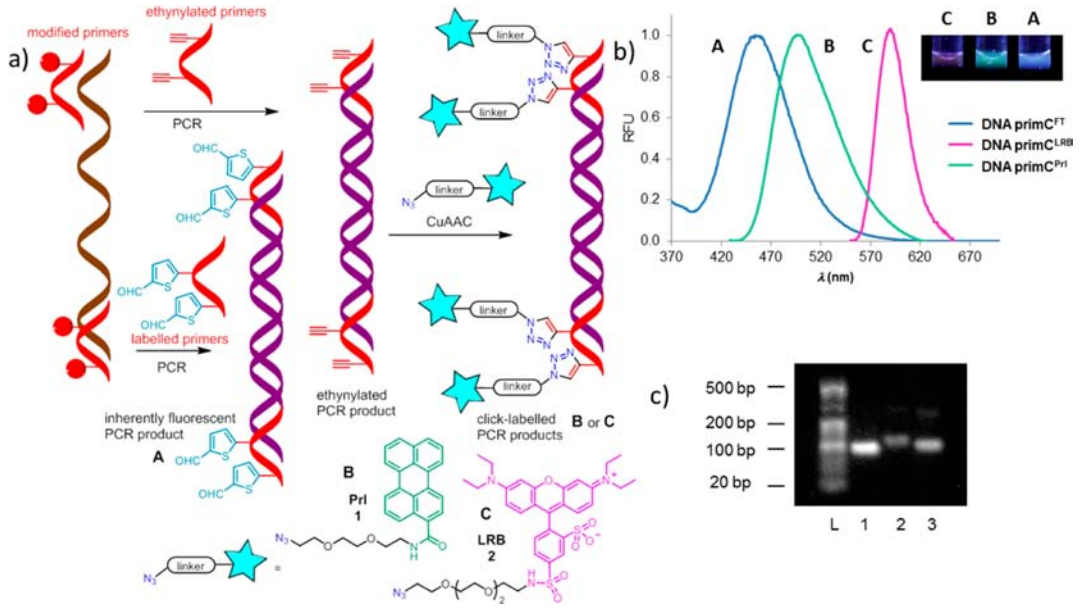

Figure 9. (a) Outline of fluorescent labeling of PCR products: direct fluorescent labeling of primers with formylthienyl, or post-PCR click reaction of ethynyl-modified PCR primers with fluorescent azides **1** or **2**. (b) Normalized emission spectra of fluorescently labeled DNA: **DNA primC^{FT}** (λ_{em} = 456 nm, excitation at 330 nm), **DNA primC^{Prl}** (λ_{em} = 495 nm, excitation at 390 nm), and **DNA primC^{LRB}** (λ_{em} = 590 nm, excitation at 510 nm). (c) Agarose gel electrophoresis of the products of CuAAC reaction on DNA modified with ethynyl groups in the primer part. **L** = DNA ladder. Lane 1: starting material, **DNA primC^{E}**. Lane 2: cycloaddition with Lissamine rhodamine B azide (**2**), **DNA primC^{LRB}**. Lane 3: cycloaddition with perylene azide (**1**), **DNA primC^{Prl}**.

Table 8. Sequences for All Commercial Oligonucleotides[a]

NEAR templates	
Nick1	5′-CAGACTGTTAcgagGACTCACTAGATCGG-3′
Nick2	5′-TCGAGCAGTTCAcgagGACTCACTAGATCGG-3′
Nick3	5′-TCAGACTGTAGACTcgagGACTCACTAGATCGG-3′
Nick4	5′-TGCAGCATGTCAGCTAcgagGACTCACTAGATCGG-3′
Nick5	5′-ACTGCAGCATGTCAGCTAcgagGACTCACTAGATCGG-3′
Nick6	5′-GACTTGCAGCATGTCAGCTAcgagGACTCACTAGATCGG-3′
Nick7	5′-CAGACTTGCAGCATGTCAGCTAcgagGACTCACTAGATCGG-3′
Nick8	5′-ACTACGTCGAGACTTGCAGCATGTCAGCTAcgagGACTCACT AGATCGG-3′
Nick9	5′-ACTGTGACCATGTACGTCGAGACTTGCAGCATGTCAGCTA cgagGACTCACTAGATCGG-3′
Cleave1	5′-TCAGCAGTACTGctacGACTCCCCATGCCGCCATCG-3′
Cleave2	5′-TCAGCTGTAGCAgaccGACTCCCCATGCCGCCATCG-3′
Cleave3	5′-TCAGCTGTAGCAcgacGACTCCCCATGCCGCCATCG-3′
Cleave4	5′-TCGACTGAAGCTctacGACTCCCCATGCCGCCATCG-3′
NickPCR1	5′-AATACAGGTATTTTGTCCTTcgagGACTCACTAGATCGG-3′
NickPCR2	5′-GCGATGTCTCTCATGATGTCcgagGACTCACTAGATCGG-3′
ss DNA ladder	
L10	5′-CAGTGACTAG-3′
L20	5′-CAGTGACTAGCTTACGGACT-3′
L50	5′-CAGTGCATGACTATCGGACCGTATGACTAGCTCAGGTATC CAGTGACTAG-3′
L100	5′-GACATCATGAGAGACATCGCCTCTGGGCTAATAGGACTACT TCTAATCTGTAAGAGCAGATCCCTGGACAGGCAAGGAATACA GGTATTTTGTCCTTG-3′
PEX and PCR templates	
Nick4PEX-bio	5′-bio-ACCAGTTGTCAGAGCTTGCAGCATGTCAGCTA-3′
TempPCR98	5′-GACATCATGAGAGACATCGCCTCTGGGCTAATAGGACT ACTTCTAATCTGTAAGAGCAGATCCCTGGACAGGCAAGGA ATACAGGTATTTTGTCCTTG-3′
primers	
PrimNick	5′-CCGATCTAGTGAGTCctcg-3′
PrimCleave1	5′-CGATGGCGGCATGGGGAGTC-3′
PrimCleave2	5′-CGATGGCGGCATGGGGAGT-3′

[a]Lower case: spacer bases. Underlined: nicking enzyme recognition site. bio: biotin.

Agarose gel electrophoresis was done on 4% agarose gels. Samples were prepared by mixing 1.6 μL of TrackIt Cyan/Orange loading buffer (Invitrogen) and 8 μL of the purified PEX product. As a reference, primer (10 μM, 8 μL) and template (10

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μM, 5 μL) were also loaded on the gel. The gel was run for 70 min at 120 V and imaged using electronic dual wave transilluminator equipped with UltraCam 8gD digital imaging system (Ultra-Lum).

PCR using ON PCR1 C^X and ON PCR4 C^X as Primers. The reaction mixture (10 μL) contained KOD XL DNA polymerase (2.5 U/μL, 0.5 μL), natural dNTPs (0.5 mM, 0.5 μL), functionalized dNTP (2 mM, 0.5 μL), primers (**ON PCR1 C^X** and **ON PCR4 C^X**, 10 μM, 1.0 μL), and a 98-mer template (**TempPCR98**, 1 μM, 0.25 μL,) in KOD XL reaction buffer (1.0 μL) supplied by the manufacturer. After the initial denaturation for 3 min at 94 °C, 30 PCR cycles were run under the following conditions: denaturation for 30 s at 94 °C, annealing for 1 min at 40 °C, extension for 1 min at 65 °C. These PCR process was terminated with a final extension step for 3 min at 65 °C. The PCR products were analyzed on a 2% agarose gel stained with GelRed. The samples (4 μL) were mixed with a loading buffer (0.8 μL). The gel was run for 75 min at 90 V and imaged using electronic dual wave transilluminator equipped with UltraCam 8gD digital imaging system (Ultra-Lum).

Click Reaction on PCR Product DNA primC^E. DNA **primC^E** obtained by PCR was purified on QIAquick PCR purification columns and eluted with Milli-Q water. The solution of Cu(I) catalyst (10 mM) was freshly prepared just before the reaction by mixing CuBr (1 μL, 100 mM in DMSO/tBuOH 3:1), TBTA ligand (2 μL, 100 mM in DMSO/tBuOH 3:1) and DMSO/tBuOH 3:1 (7 μL).

To the DNA solution (50 μL, 40 ng/μL) a solution of fluorescent azide **1** or **2** (62.50 μL, 10 mM in DMSO), sodium ascorbate (13 μL, 5 mM in water) and precomplexed Cu(I) (10 mM) were added. The sample was incubated for 3 h at 37 °C and 450 rpm. After the reaction, the crude mixture was purified on Nucleospin Extract II columns.

MALDI-TOF Experiments. The MALDI-TOF spectra were measured on UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany), with 1 kHz smartbeam II laser. The measurements were done in reflectron mode by dried droplet technique, with the mass range up to 30 kDa and resolution >25000. The matrix consisted of 3-hydroxypicolinic acid (HPA)/picolinic acid (PA)/ammonium tartrate in ratio 9/1/1. Matrix (1 μL) was applied on the target (ground steel) and dried down at room temperature. The sample (1 μL) was mixed with matrix (1 μL) and the mixture was added on top of the dried matrix preparation spot and dried down at room temperature.

Fluorescence Measurements. Samples were dissolved in Milli-Q water. The fluorescence measurements were performed on a spectrofluorometer with 220−850 nm range, xenon source, excitation and emission wavelength scans, spectral bandwidth 1−16 nm, PMT detector, scan rate 3−6000 nm/min, and Saya-Namioka grating monochromator. To evaluate the fluorescence quantum yield of a sample, a comparative method was applied, using a 10 mM solution of quinine sulfate in 0.1 M H_2SO_4 (in H_2O) as a standard (Φ = 0.54). The area of the emission spectrum was integrated using the instrumentation software, and the quantum yield was calculated according to the following equation:

$$\Phi_{F(SA)} = \Phi_{F(ST)}[F_{(SA)}/F_{(ST)}][A_{(ST)}/A_{(SA)}][n_{(SA)}/n_{(ST)}]^2$$

Here, $\Phi_{F(SA)}$ and $\Phi_{F(ST)}$ are the fluorescence quantum yields of the sample and the standard, respectively. The terms $F_{(SA)}$ and $F_{(ST)}$ are the integrated fluorescence intensities of the sample and the standard, respectively; $A_{(SA)}$ and $A_{(ST)}$ are the optical densities of the sample and the standard solution at the wavelength of excitation, respectively; and $n_{(SA)}$ and $n_{(ST)}$ are the values of the refractive index for the solvents used for the sample, respectively.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures and Tables, HPLC chromatograms, synthesis and characterization data for new **dN^{X}TPs**, copies of NMR, UV and MALDI spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Jager, S., Rasched, G., Kornreich-Leshem, H., Engeser, M., Thum, O., and Famulok, M. (2005) A versatile toolbox for variable DNA functionalization at high density. *J. Am. Chem. Soc. 127*, 15071−15082.

(2) Hocek, M., and Fojta, M. (2008) Cross-coupling reactions of nucleoside triphosphates followed by polymerase incorporation. Construction and applications of base-functionalized nucleic acids. *Org. Biomol. Chem. 6*, 2233−2241.

(3) Čapek, P., Cahová, H., Pohl, R., Hocek, M., Gloeckner, C., and Marx, A. (2007) An efficient method for the construction of functionalized DNA bearing amino acid groups through cross-coupling reactions of nucleoside triphosphates followed by primer extension or PCR. *Chem.—Eur. J. 13*, 6196−6203.

(4) Hollenstein, M. (2012) Nucleoside triphosphates - building blocks for the modification of nucleic acids. *Molecules 17*, 13569−13591.

(5) Thoresen, L. H., Jiao, G.-S., Haaland, W. C., Metzker, M. L., and Burgess, K. (2003) Rigid, conjugated, fluoresceinated thymidine triphosphates: syntheses and polymerase mediated incorporation into DNA analogues. *Chem.—Eur. J. 9*, 4603−4610.

(6) Riedl, J., Pohl, R., Rulíšek, L., and Hocek, M. (2012) Synthesis and photophysical properties of biaryl-substituted nucleos(t)ides. polymerase synthesis of DNA probes bearing solvatochromic and pH sensitive dual fluorescent and ^{19}F NMR labels. *J. Org. Chem. 77*, 1026−1044.

(7) Riedl, J., Pohl, R., Ernsting, N. P., Orság, P., Fojta, M., and Hocek, M. (2012) Labelling of nucleosides and oligonucleotides by solvatochromic 4-aminophthalimide fluorophore for studying DNA-protein interactions. *Chem. Sci. 3*, 2797−2806.

(8) Riedl, J., Ménová, P., Pohl, R., Orság, P., Fojta, M., and Hocek, M. (2012) GFP-like fluorophores as DNA labels for studying DNA-protein interactions. *J. Org. Chem. 77*, 8287−8293.

(9) Brázdilová, P., Vrábel, M., Pohl, R., Pivoňková, H., Havran, L., Hocek, M., and Fojta, M. (2007) Ferrocenylethynyl derivatives of nucleoside triphosphates: Synthesis, incorporation, electrochemistry, and bioanalytical applications. *Chem.—Eur. J. 13*, 9527−9533.

(10) Cahová, H., Havran, L., Brázdilová, P., Pivoňková, H., Pohl, R., Fojta, M., and Hocek, M. (2008) Aminophenyl- and nitrophenyl-labeled nucleoside triphosphates: Synthesis, enzymatic incorporation, and electrochemical detection. *Angew. Chem., Int. Ed. 47*, 2059−2062.

(11) Vrábel, M., Horáková, P., Pivoňková, H., Kalachova, L., Černocká, H., Cahová, H., Pohl, R., Šebest, P., Havran, L., Hocek, M., and Fojta, M. (2009) Base-modified DNA labeled by [Ru(bpy)(3)](2+) and [Os(bpy)(3)](2+) complexes: construction by polymerase incorpo-

ration of modified nucleoside triphosphates, electrochemical and luminescent properties, and applications. *Chem.—Eur. J. 15*, 1144−1154.
(12) Horáková, P., Macíčková-Cahová, H., Pivoňková, H., Špaček, J., Havran, L., Hocek, M., and Fojta, M. (2011) Tail-labelling of DNA probes using modified deoxynucleotide triphosphates and terminal deoxynucleotidyl tranferase. Application in electrochemical DNA hybridization and protein-DNA binding assays. *Org. Biomol. Chem. 9*, 1366−1371.
(13) Macíčková-Cahová, H., Pohl, R., Horáková, P., Havran, L., Špaček, J., Fojta, M., and Hocek, M. (2011) Alkylsulfanylphenyl derivatives of cytosine and 7-deazaadenine nucleosides, nucleotides and nucleoside triphosphates. Synthesis, polymerase incorporation to DNA and electrochemical study. *Chem.—Eur. J. 17*, 5833−5841.
(14) Balintová, J., Pohl, R., Horáková, P., Vidláková, P., Havran, L., Fojta, M., and Hocek, M. (2011) Anthraquinone as a redox label for DNA. Synthesis, enzymatic incorporation and electrochemistry of anthraquinone-modified of nucleosides, nucleotides and DNA. *Chem.—Eur. J. 17*, 14063−14073.
(15) Obeid, S., Yulikov, M., Jeschke, G., and Marx, A. (2008) Enzymatic synthesis of multiple spin-labeled DNA. *Angew. Chem., Int. Ed. 47*, 6782−6785.
(16) Baccaro, A., Steck, A. L., and Marx, A. (2012) Barcoded nucleotides. *Angew. Chem., Int. Ed. 51*, 254−257.
(17) Gramlich, P. M., Wirges, C. T., Gierlich, J., and Carell, T. (2008) Synthesis of modified DNA by PCR with alkyne-bearing purines followed by a click reaction. *Org. Lett. 10*, 249−251.
(18) Borsenberger, V., Kukwikila, M., and Howorka, S. (2009) Synthesis and enzymatic incorporation of modified deoxyuridine triphosphates. *Org. Biomol. Chem. 7*, 3826−3835.
(19) Wirges, C. T., Timper, J., Fischler, M., Sologubenko, A. S., Mayer, J., Simon, U., and Carell, T. (2009) Controlled nucleation of DNA metallization. *Angew. Chem., Int. Ed. 48*, 219−223.
(20) Raindlová, V., Pohl, R., Šanda, M., and Hocek, M. (2010) Direct polymerase synthesis of reactive aldehyde-functionalized DNA and its conjugation and staining with hydrazines. *Angew. Chem., Int. Ed. 49*, 1064−1066.
(21) Raindlová, V., Pohl, R., and Hocek, M. (2012) Synthesis of aldehyde-linked nucleotides and DNA and their bioconjugations with lysine and peptides through reductive amination. *Chem.—Eur. J. 18*, 4080−4087.
(22) Raindlová, V., Pohl, R., Klepetářová, B., Havran, L., Šimková, E., Horáková, P., Pivoňková, H., Fojta, M., and Hocek, M. (2012) Synthesis of hydrazone-modified nucleotides and their polymerase incorporation to DNA for redox labelling. *ChemPlusChem 77*, 652−662.
(23) Cheng, Y., Peng, H., Chen, W., Ni, N., Ke, B., Dai, C., and Wang, B. (2013) Rapid and specific post-synthesis modification of DNA through a biocompatible condensation of 1,2-aminothiols with 2-cyanobenzothiazole. *Chem.—Eur. J. 19*, 4036−4042.
(24) Macíčková-Cahová, H., and Hocek, M. (2009) Cleavage of adenine-modified functionalized DNA by type II restriction endonucleases. *Nucleic Acids Res. 37*, 7612−7622.
(25) Macíčková-Cahová, H., Pohl, R., and Hocek, M. (2011) Cleavage of functionalized DNA containing 5-modified pyrimidines by type II restriction endonucleases. *ChemBioChem 12*, 431−438.
(26) Kielkowski, P., Macíčková-Cahová, H., Pohl, R., and Hocek, M. (2011) Transient and switchable (triethylsilyl)ethynyl protection of DNA against cleavage by restriction endonucleases. *Angew. Chem., Int. Ed. 50*, 8727−8730.
(27) Kielkowski, P., Brock, N. L., Dickschat, J. S., and Hocek, M. (2013) Nucleobase protection strategy for gene cloning and expression. *ChemBioChem 14*, 801−804.
(28) Kuwahara, M., Nagashima, J., Hasegawa, M., Tamura, T., Kitagata, R., Hanawa, K., Hososhima, S., Katsmatsu, T., Ozaki, H., and Sawai, H. (2006) Systematic characterization of 2′-deoxynucleoside-5′-triphosphate analogs as substrates for DNA polymerase chain reaction and kinetic studies on enzymatic production of modified DNA. *Nucleic Acids Res. 34*, 5383−5394.
(29) Sidorov, A. V., Grasby, J. A., and Williams, D. M. (2004) Sequence-specific cleavage of RNA in the absence of divalent metal ions by a DNAzyme incorporating imidazolyl and amino functionalities. *Nucleic Acids Res. 32*, 1591−1601.
(30) Jäger, S., Rasched, G., Kornreich-Leshem, H., Engeser, M., Thum, O., and Famulok, M. (2005) A versatile toolbox for variable DNA functionalization at high density. *J. Am. Chem. Soc. 127*, 15071−15082.
(31) Hollenstein, M., Hipolito, C. J., Lam, C. H., and Perrin, D. M. (2009) A self-cleaving DNA enzyme modified with amines, guanidines and imidazoles operates independently of divalent metal cations (M^{2+}). *Nucleic Acids Res. 37*, 1638−1649.
(32) Hollenstein, M., Hipolito, C., Lam, C., Dietrich, D., and Perrin, D. M. (2008) A highly selective DNAzyme sensor for mercuric ions. *Angew. Chem., Int. Ed. 47*, 4346−4350.
(33) Hollenstein, M. (2012) Synthesis of deoxynucleoside triphosphates that include proline, urea, or sulfonamide groups and their polymerase incorporation into DNA. *Chem.—Eur. J. 18*, 13320−13330.
(34) Van Ness, J., Van Ness, L. K., and Galas, D. J. (2003) Isothermal reactions for the amplification of oligonucleotides. *Proc. Natl. Acad. Sci. U.S.A. 100*, 4504−4509.
(35) Tan, E., Wong, J., Nguyen, D., Zhang, Y., Erwin, B., Van Ness, L. K., Baker, S. M., Galas, D. J., and Niemz, A. (2005) Isothermal DNA amplification coupled with DNA nanosphere-based colorimetric detection. *Anal. Chem. 77*, 7984−7992.
(36) Tan, E., Erwin, B., Dames, S., Voelkerding, K., and Niemz, A. (2007) Isothermal DNA amplification with gold nanosphere-based visual colorimetric readout for Herpes simplex virus detection. *Clin. Chem. 53*, 2017−2020.
(37) Jia, H., Li, Z., Liu, C., and Cheng, Y. (2010) Ultrasensitive detection of microRNAs by exponential isothermal amplification. *Angew. Chem., Int. Ed. 49*, 5498−5501.
(38) Ménová, P., and Hocek, M. (2012) Preparation of short cytosine-modified oligonucleotides by nicking enzyme amplification reaction. *Chem. Commun. 48*, 6921−6923.
(39) Joneja, A., and Huang, X. (2011) Linear nicking endonuclease-mediated strand-displacement DNA amplification. *Anal. Biochem. 414*, 58−69.
(40) Clark, J. M., Joyce, C. M., and Beardsley, G. P. (1987) Novel blunt-end addition reactions catalyzed by DNA polymerase I of Escherichia coli. *J. Mol. Biol. 198*, 123−127.
(41) Clark, J. M. (1988) Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucleic Acids Res. 16*, 9677−9686.
(42) Ogata, N., and Miura, T. (1997) Genetic information 'created' by archaebacterial DNA polymerase. *Biochem. J. 324*, 667−671.
(43) Liang, X. G., Jensen, K., and Frank-Kamenetskii, M. D. (2004) Very efficient template/primer-independent DNA synthesis by thermophilic DNA polymerase in the presence of a thermophilic restriction endonuclease. *Biochemistry 43*, 13459−13466.
(44) Zyrina, N. V., Zheleznaya, L. A., Dvoretsky, E. V., Vasiliev, V. D., Chernov, A., and Matvienko, N. I. (2007) N.BspD6I DNA nickase strongly stimulates template-independent synthesis of non-palindromic repetitive DNA by Bst DNA polymerase. *Biol. Chem. 388*, 367−372.
(45) Tan, E., Erwin, B., Dames, S., Ferguson, T., Buechel, M., Irvine, B., Voelkerding, K., and Niemz, A. (2008) Specific versus nonspecific isothermal DNA amplification through thermophilic polymerase and nicking enzyme activities. *Biochemistry 47*, 9987−9999.
(46) Macíčková-Cahová, H., Vrábel, M., and Hocek, M. (2010) Cross-coupling modification of nucleoside triphosphates, PEX, and PCR construction of base-modified DNA. *Curr. Protoc. Chem Biol. 2*, 1−14.
(47) Gramlich, P. M. E., Wirges, C. T., Manetto, A., and Carell, T. (2008) Postsynthetic DNA modification through the copper-catalyzed azide-alkyne cycloaddition reaction. *Angew. Chem., Int. Ed. 47*, 8350−8358.
(48) Gierlich, J., Burley, G. A., Gramlich, P. M. E., Hammond, D. M., and Carell, T. (2006) Click chemistry as a reliable method for the high-density postsynthetic functionalization of alkyne-modified DNA. *Org. Lett. 8*, 3639−3642.

(49) Gutsmiedl, K., Fazio, D., and Carell, T. (2010) High-density DNA functionalization by a combination of Cu-catalyzed and Cu-free click chemistry. *Chem.—Eur. J. 16*, 6877−6883.
(50) Seela, F., and Sirivolu, V. R. (2007) Nucleosides and oligonucleotides with diynyl side chains: base pairing and functionalization of 2′-deoxyuridine derivatives by the CuI-catalyzed alkyne-azide "click" cycloaddition. *Helv. Chim. Acta 90*, 535−552.
(51) Seela, F., Sirivolu, V. R., and Chittepu, P. (2008) The modification of DNA with octadiynyl side chains: synthesis, base pairing and formation of fluorescent coumarin dye conjugates of four nucleobases by the alkyne−azide "click" reaction. *Bioconjugate Chem. 19*, 211−224.
(52) Chittepu, P., Sirivolu, V. R., and Seela, F. (2008) Nucleosides and oligonucleotides containing 1,2,3-triazole residues with nucleobase tethers: synthesis via the azide-alkyne "click" reaction. *Bioorg. Med. Chem. 16*, 8427−8439.
(53) El-Sagheerab, A. H., and Brown, T. (2010) Click chemistry with DNA. *Chem. Soc. Rev. 39*, 1388−1405.
(54) Wenge, U., Ehrenscwender, T., and Wagenknecht, H.-A. (2013) Synthesis of 2′-O-propargyl nucleoside triphosphates for enzymatic oligonucleotide preparation and ″click″ modification of DNA with nile red as fluorescent probe. *Bioconjugate Chem. 24*, 301−304.
(55) Ludwig, J. (1981) A new route to nucleoside 5′-triphosphates. *Acta Biochim. Biophys. Acad. Sci. Hung. 16*, 131−133.
(56) Kovacs, T., and Otvos, L. (1988) Simple synthesis of 5-vinyl and 5-ethynyl-2′-deoxyuridine-5′-triphosphates. *Tetrahedron Lett. 29*, 4525−4528.
(57) Tataurov, A. V., You, Y., and Owczarzy, R. (2008) Predicting ultraviolet spectrum of single stranded and double stranded deoxyribonucleic acids. *Biophys. Chem. 133*, 66−70.