Use of Base-Modified Duplex-Stabilizing Deoxynucleoside 5′-Triphosphates To Enhance the Hybridization Properties of Primers and Probes in Detection Polymerase Chain Reaction[†]

Igor V. Kutyavin*

18943 203rd Avenue Northeast, Woodinville, Washington 98077

Received September 17, 2008; Revised Manuscript Received November 3, 2008

ABSTRACT: Several base-modified duplex-stabilizing deoxyribonucleoside 5′-triphosphates (dNTPs) have been evaluated as agents for enhancing the hybridization properties of primers and probes in real-time polymerase chain reaction (PCR). It was shown that pyrimidines substituted at the 5-position with bromine or iodine atoms and methyl or propynyl groups are incorporated into PCR amplicons by Taq DNA polymerase as efficiently as natural dNTPs. The dNTP of 2-aminoadenosine was incorporated somewhat less efficiently than dATP but still supported PCR. Incorporation of these modified nucleotides into the amplified DNA represents a simple and inexpensive way to stabilize duplexes of primers and probes and is particularly effective in improving the amplification and detection of A/T-rich sequences. This technology permits the use of higher PCR annealing temperatures or alternatively a reduction in the length of the oligonucleotide components. Examples of successful application in TaqMan and Scorpion real-time detection assays are provided. Limits of the approach are identified and discussed. For example, application of the 5-bromo and 5-iodo derivatives may be limited to relatively G/C-rich DNA targets and, in particular, to those lacking long runs of adenylate and/or thymidylate. Simultaneous use of base-modified analogues of dATP and dTTP should be avoided in PCR due to "overstabilization" of the amplicon.

Structurally modified DNA and RNA have applications in bioengineering, nanotechnology, molecular biology, and medicine. Oftentimes, however, chemical synthesis of modified polynucleotides is difficult and inefficient. Moreover, certain modifications cannot be chemically introduced due to instability. Enzymatic synthesis is an alternative way to prepare DNA or RNA polymers. Relatively minor modifications at the α-phosphate moiety of deoxyribonucleoside 5′-triphosphates (dNTPs) are tolerated well by DNA polymerases (*1*). Examples include thio (*2*) and borano phosphate (*3, 4*) derivatives. DNA polymerases also tolerate certain base modifications in dNTPs as long as normal Watson−Crick base pairing is preserved. Successful synthesis of base-modified DNAs by polymerase chain reaction (PCR) has been reported for 5-substituted pyrimidine analogues (*5*−*9*) and various purine derivatives (*5, 10*−*14*). DNA polymerases are less able to accommodate modification of the sugar moiety in nucleotides (*15*). Many such modifications inhibit viral DNA polymerases, making these compounds interesting drug candidates for medicinal chemistry studies (*16, 17*).

Modified nucleotides have been successfully used in nucleic acid sequencing (*2, 4, 18*) and amplification (*19, 20*) and in the study of interactions of nucleic acids with proteins (*10*) and DNA binding drugs (*14*). Certain nucleotide modifications have been used to enhance the catalytic repertoire of nucleic acids (*5*−*8*) and to protect polynucleotides from exo- and endonucleases (*1*−*4, 10*). Deoxyuridine 5′-triphosphate (dUTP) is routinely substituted for thymidine 5′-triphosphate (dTTP) in PCR. DNA containing deoxyuridine is selectively cleaved by uracil glycosylase, and treatment of samples prior to PCR helps to prevent carryover from previously amplified samples (*21*).

In this study, modified dNTPs are used to enhance the hybridization properties of primers and probes in detection PCR. Instead of using duplex stabilizing agents like minor groove binders (*22, 23*), PNA or LNA linkages (*24*−*26*), or base analogues (*27, 28*) to improve the binding affinity of primers and probes, duplex-stabilizing base-modified dNTPs are used to modify the PCR amplicon. Such nucleotide analogues increase DNA duplex stability regardless of whether they are located in the primers and probes or in the amplified strands. This simple and straightforward strategy was found to be particularly effective in amplifying and detecting nucleic acids with elevated A/T content. Limits of the approach are identified and discussed.

MATERIALS AND METHODS

PCR Components. JumpStart Taq DNA polymerase, an antibody-inactivated hot start enzyme, and regular dNTPs were purchased from Sigma-Aldrich. Base-modified dNTPs (Figure 1) were obtained from TriLink Biotechnologies (San Diego, CA). Primers and FRET probes (TaqMan and Scorpion) were prepared by Cepheid (Sunnyvale, CA) using reagents from Glen Research (Sterling, VA) and as needed an internally prepared phosphoramidite of 2,6-diaminopurine

[†] This research project was funded by Cepheid, which also provided access to necessary facilities and instrumentation.

* To whom correspondence should be addressed. E-mail: perpetualgenomics@comcast.net. Phone: (425) 478-2645.

10.1021/bi8017784 CCC: $40.75
Published on Web 12/01/2008

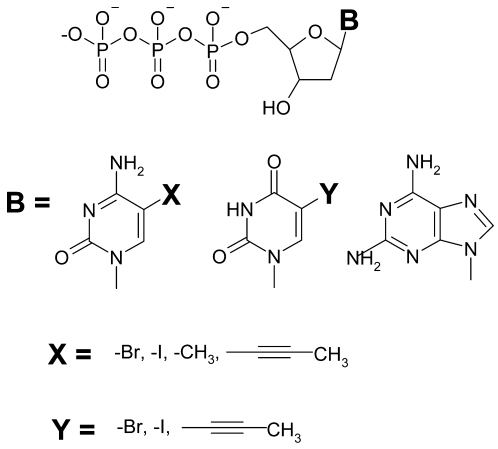

FIGURE 1: Structures of base-modified 2′-deoxynucleoside 5′-triphosphates used in this study. **B** denotes the modified bases. **X** and **Y** are 5-substituted halogen (iodine, **I**, and bromine, **Br**) and methyl (**me**) and propynyl (**Pr**) in pyrimidines, respectively.

(2-amA). Oligonucleotides (2 μM substocks) were stored refrigerated in 5 mM sodium cacodylate.

Physical Measurements and Calculations. Oligonucleotide extinction coefficients were calculated using CalcExt2.8 (Cepheid). Melting temperatures (T_m) of unmodified primers and probes were calculated using the "nearest-neighbor" approach (*29, 30*) for perfect-match duplexes at 200 nM with adjustment for the PCR buffer used in this study. The effect of dyes on duplex stability was disregarded in the calculation of TaqMan probe melting temperatures.

DNA Targets. A 96-mer synthetic oligodeoxynucleotide containing a portion of the β2-macroglobulin gene (Figure 2, GenBank accession number NM004048) and M13mp18 single-stranded viral DNA [250 μg/mL from New England Biolabs (Ipswich, MA), GenBank accession number M77815] were employed as targets for PCR. To prevent adsorption of target DNA to plastic tubes at subnanomolar concentrations, both targets were diluted in 100 nM $(dA)_{18}$ conjugated at the 3′-end with propanediol. Human genomic DNA (10 ng per 25 μL reaction mixture) was amplified prior to detection of a sequence flanking SNP IVS6+1143 A>T in the human CYP2E1 gene (NCBI SNP entry rs6413432).

Real-Time PCR. PCR mixtures were conducted in 25 μL volumes and contained forward and reverse PCR primers (200 nM each), TaqMan probe (200 nM), dNTPs (200 μM each), and JumpStart Taq DNA polymerase (Sigma) (0.04 unit/μL) in 50 mM KCl, 2 mM $MgCl_2$, and 20 mM Tris-HCl (pH 8.0). In this study, one or two base-modified dNTPs were completely substituted for the corresponding natural nucleotides. Partial substitutions were not investigated. Control PCR experiments that included only natural dNTPs were carried out for comparison. The identity and concentration of target DNAs are indicated in the figure legends. All real-time PCR experiments were performed on a SmartCycler (Cepheid). Background fluorescence was subtracted using instrument software, and the data (fluorescence vs PCR cycle) were transferred in Excel format (Microsoft Office) for further processing. Each real-time curve shown here represents an average of three to five independent experiments. The fluorescent curve threshold value (C_t) was determined as the cycle number at which the log of fluorescence reached 0.5. PCR was performed in two steps with the annealing and extension stages combined into a single step throughout the text termed "annealing". Unless otherwise noted, the reaction mixtures were incubated at 95 °C for 2 min to activate the antibody-blocked enzyme followed by 55 PCR cycles consisting of 10 s at 95 °C for denaturation and 45 s at a target specific temperature (t °C) for annealing and extension, i.e., (95 °C, 2 min) → (95 °C, 10 s → t °C, 45 s)$_{55}$.

RESULTS

Effect of Base-Modified dNTPs on Real-Time Detection of the β2-Macroglobulin Sequence (60% G/C-rich target). Certain modifications of nucleic acid bases are known to stabilize duplexes. For example, it has been well established that substitution of the C5 position in pyrimidines with small rigid, hydrophobic moieties such as methyl (*31*−*34*), propynyl (*35, 36*), or halogen (*37*−*40*) improves base stacking interactions and stabilizes duplexes. These modified bases, depicted in Figure 1, were used as dNTPs in this study. The dNTP of 2-aminoadenosine (2-amA) was the only purine derivative studied. Introduction of an additional amino group at the C2 heterocyclic atom leads to the formation of three hydrogen bonds with thymidine, and this strongly stabilizes duplexes (*41*−*47*).

Figure 2 shows the effect of base-modified dNTPs on real-time detection of the β2-macroglobulin target (panel A). One (C−F) or two (I) base-modified dNTPs were completely substituted for the respective natural dNTPs, and experiments in which the annealing temperature ranged from 65 to 75 °C were performed. A stabilizing effect was observed with each modified dNTP when compared with real-time PCR utilizing natural dNTPs (panel B). Analysis of the fluorescence curves indicated that the annealing temperature could be increased by 2−6 °C without loss of signal performance when base-modified dNTPs were present. The most profound stabilization effects were observed for d(5-meC)TP (∼2.5 °C, E), d(2-amA)TP (∼3 °C, F), and d(2-amA)TP with d(5-PrU)TP (∼6 °C, I). Although the degree of PCR stabilization was a function of the pairing strength of the respective analogues, it also depended on the base composition of the primers and probe. Hence, differences in the frequency of a modified base will alter the stability of short hybrids. The 22-mer TaqMan probe used for detection of β2-macroglobulin contained only one adenine, while the forward and reverse primers contain six and nine of these nucleotides, respectively. Therefore, the primers should be highly stabilized using d(5-BrU)TP or d(5-PrU)TP in PCR, whereas these same analogues should have little effect on the hybridization properties of the probe. This explains the relatively modest system stabilization achieved when using 5-substituted deoxyuridine dNTP analogues (panels C and D).

Incorporation of duplex-stabilizing base modifications into primers and probes positively affects PCR (*28*), and experiments shown in Figure 2G confirm this. Combining this approach with the use of d(2-amA)TP in real-time PCR stabilized the detection system by an extraordinary 8−10 °C (panel H). In particular, this allowed PCR to be conducted at 73−75 °C, a temperature range in which Taq polymerase is known to exhibit maximal activity (*48, 49*).

DNA amplification in the presence of duplex-stabilizing dNTP analogues leads to incorporation of the modified nucleotide into both DNA strands, and this may cause

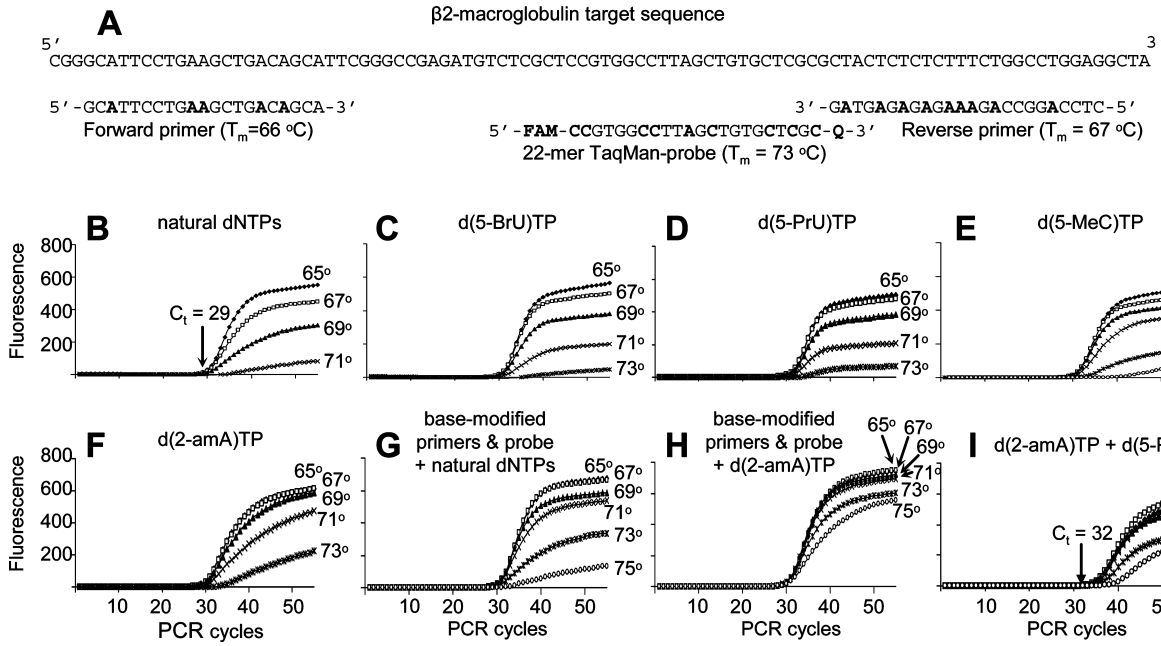

FIGURE 2: Real-time PCR detection assays for the β2-macroglobulin sequence using a TaqMan probe. Plots of fluorescence vs cycle number are shown for different combinations of dNTPs. Scheme A shows the 96-mer oligodeoxyribonucleotide used as a target (10^4 copies per reaction), the forward and reverse PCR primers, and the TaqMan probe. The primers and probe used were synthesized with standard bases or where highlighted contained modified bases. When base-modified oligonucleotides were used, the positions shown in bold were substituted with 2-aminoadenosine or 5-methylcytosine. FAM is 6-fluorescein, and Q is a Black Hole Quencher (BHQ1) from Biosearch Technologies. Panel B shows results of a set of experiments in which unmodified primers and TaqMan probe were employed with regular dNTPs. Panels C−F and I show results of analogous experiments in which one or two natural dNTPs were completely replaced by the indicated base-modified analogues. Base-modified primers and probes were used in panel G with natural dNTPs. In panel H, base-modified primers and probe were used with d(2-amA)TP substituted for dATP. As indicated for each real-time curve, the annealing temperature (t) was varied in the range of 65−75 °C using a PCR profile of (95 °C, 2 min) → (95 °C, 10 s → t °C, 45 s)$_{55}$.

overstabilization of the PCR product and incomplete separation of the strands during the denaturation step (*9*). This effect was observed as a several-cycle delay in the appearance of the real-time curve when both d(2-amA)TP and d(5-PrU)TP were present (Figure 2I) and when d(5-BrC)TP was substituted for dCTP (data not shown). However, in both cases, the threshold values were brought back to their normal position (28−29 cycles) by increasing the denaturation temperature from 95 to 96−97 °C (Figure 3).

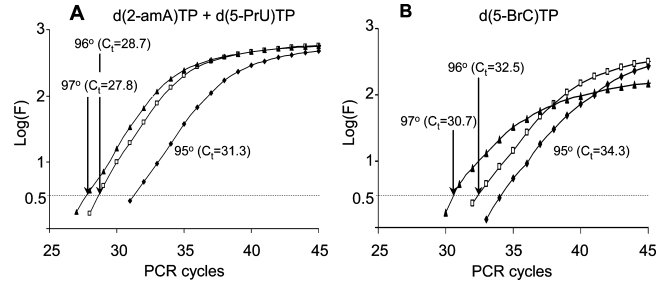

FIGURE 3: Increase of 1−2 °C in the PCR denaturation temperature which helps to eliminate two- and five-cycle delays found during amplification and detection of the β2-macroglobulin target sequence in the presence of d(2-amA)TP and d(5-PrU)TP (A) and d(5-BrC)TP (B). The target oligonucleotide, primers, and TaqMan probe (unmodified) used in these experiments are shown in Figure 2A. The real-time curves are plotted as log(fluorescence) vs PCR cycles. As indicated for each real-time curve, the denaturation temperature (t) was set to 95−97 °C using the PCR profile (95 °C, 2 min) → (t °C, 10 s → 67 °C, 45 s)$_{55}$. Fluorescence curve threshold values (C_t) are shown in parentheses and were determined as the cycle numbers at which the curves intercepted a value of 0.5 on the logarithm axis. Typical C_t values for this system design and target (10^4 copies per reaction) are 28−29 cycles.

Substrate Properties of Base-Modified dNTPs in PCR. Modified dNTPs commonly exhibit a reduced level of uptake by DNA polymerases (*10, 14*−*17*). Although certain modified dNTPs have been successfully used in PCR (*5*−*14, 21*), accurate and comprehensive studies of their effects on rate and yield of DNA synthesis are rare. Certain base-modified dNTPs used in PCR require the presence of the corresponding natural dNTP, i.e., fractional dNTP replacement (*10, 12, 13*). Moreover, in many cases, modified amplicons have been prepared using special reaction conditions, PCR additives (*5, 12*), or abnormal PCR profiles (*14*). For example, a 160 bp DNA fragment substituted with 2-aminoadenosine was obtained using a reaction profile in which the annealing temperature was as low as 37 °C (2 min) and the extension time was as long as 10 min at 72 °C (*14*). These conditions would be unacceptable for use in a detection PCR assay and imply that d(2-amA)TP might be a poor substrate for Taq polymerase.

Detection time is an important characteristic of all modern diagnostic assays. Challenging PCR by reducing the detection time is a way to study the substrate properties of dNTPs. Figure 4 summarizes experiments in which PCR efficiency (C_t value) was plotted as a function of annealing time for natural and base-modified dNTPs. Reducing the annealing time negatively impacts PCR yield by requiring more cycles to reach a detectable amplicon concentration (C_t). Plots of C_t values versus annealing time showed that the majority of base-modified dNTPs did not alter the efficiency of PCR when all natural dNTPs were used (Figure 4, white bars). Only d(2-amA)TP was found to have reduced substrate properties in PCR as indicated by a high threshold value at annealing times of <12−16 s. Although the cause is unclear,

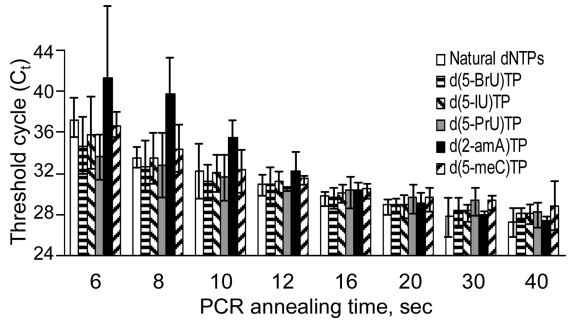

FIGURE 4: Effect of base-modified dNTPs on PCR efficiency. The fluorescence curve threshold for detection of the β2-macroglobulin sequence (10^4 copies per reaction) was determined as a function of annealing time for real-time PCR assays conducted in the presence of modified dNTPs. The primers and TaqMan probe are shown in Figure 2A. In these experiments, the indicated dNTP analogues were substituted for the respective natural dNTP in an otherwise standard reaction mixture (all natural dNTPs, white bars). The time (T) of the annealing step was reduced from 40 to 6 s using the PCR profile (95 °C, 2 min) → (95 °C, 10 s → 64 °C, T s)$_{55}$. Error bars indicate data ranges of 95% confidence for three to four independent reactions.

this base analogue has physical and chemical properties that are distinct from those of the other analogues studied. For example, compared to adenosine, addition of the second electron-donating NH_2 group in 2-amA increases electron density in the ring system, and this, in turn, reduces the acidity of hydrogen atoms participating in base pairing. The (2-amA)-T base pair is known to be less stable than the G-C base pair even though both base pairs have three hydrogen bonds (*41*−*46*). The electron-rich nucleus of 2-amA probably alters base stacking interactions as well. For example, a duplex destabilization effect has been reported for certain nearest bases (*50*).

Detection of A/T-Rich Sequences. Detection of A/T-rich sequences (>60%) is difficult due to the reduced pairing strength of primer and probes. The phage M13mp18 104 bp fragment shown in Figure 5A represents one of these sequences with 80% A/T content. Despite the use of a relatively low annealing temperature (56 °C), PCR did not give a fluorescent signal when using a 19-mer TaqMan probe (Figure 5B, natural dNTPs). However, replacement of either dTTP or dATP with the indicated analogues resulted in real-time curves with excellent signal change in the range of 300−600 fluorescence units (FU). The signal (600 FU) obtained using d(5-PrU)TP is probably attributable to the A/T composition of the probe (with nine adenines and six thymines). The d(2-amA)TP curve was found to be two cycles late in threshold value [C_t = 21 vs 19 for d(5-PrU)TP]. This was not caused by overstabilization of the amplicon since the PCR curve did not respond to a change in the denaturation temperature (data not shown). The reduced substrate properties of d(2-amA)TP (Figure 4) could be a contributing factor, but the most likely reason is low pairing strength of the reverse primer (T_m = 53 °C). Since this primer contains only two thymidines, it is not appreciably stabilized by d(2-amA)TP. On the other hand, it contains four G nucleotides and therefore was effectively stabilized by using d(5-meC)TP or d(5-PrC)TP in combination with d(2-amA)TP (Figure 5D). Use of these two dNTP analogues brought the threshold value back to the reference position (C_t = 19) yet improved signal performance. Similar results, i.e., signal enhancement, were obtained when d(5-PrU)TP was used in combination with d(5-meC)TP (Figure 5D).

Those researchers who study gene expression are least likely to experience difficulty in selecting a pair of PCR primers and an associated probe that function well in PCR. This is because the gene sequences are usually very long and provide ample opportunity for a suitable PCR design. In contrast, the assay designs for the detection of polymorphic loci are limited by location of the polymorphism of interest. Ideally, the PCR amplicon should be as short as possible. This is because PCR yield drops with length of the amplicon, reducing the overall performance of DNA detection assays. It becomes very challenging, if not impossible, to achieve a good detection design when the polymorphism is located in a region with an A/T content of >65−70%. The CYP2E1 gene belongs to a superfamily of cytochrome P450 genes that encode proteins participating in drug metabolism. Detection of polymorphic variations within these genes, including the A → T SNP shown in Figure 6A, is very important for pharmacogenomic studies. Although PCR primers can be made relatively short (18-mers) without loss of hybridization strength (T_m = 66 °C), this SNP is very difficult to detect because it is located in the middle of a 25-base A/T-rich stretch. FRET probes to this region are extremely unstable, and conventional approaches to stabilizing the duplex are unlikely to work. For example, the 21-mer TaqMan probe used in this study contained 18 2-amA and 5-PrU base modifications to provide a strong duplex stabilizing effect (*27, 28, 35, 36, 41*−*47*). Nonetheless, inclusion of this heavily modified FRET probe in PCRs did not result in a strong signal despite the use of a very low (56 °C) annealing temperature (Figure 6B). However, when this probe was used together with d(5-PrU)TP, the real-time signal response was almost tripled. Still better detection was obtained when d(2-amA)TP was used in place of (5-PrU)TP.

Amplicon Overstabilization. Amplification of extremely G/C-rich sequences (>80%) is as problematic as A/T-rich ones but for the opposite reason. The elevated stability of the dG-dC base pair commonly leads to overstabilization of the amplicon, requiring the use of duplex-destabilizing PCR additives (*51, 52*) or G/C-destabilizing dNTP analogues (*10*). Hybridization properties of primers and probes are usually not a problem in detecting DNA targets with ~50−80% G/C content, and therefore, use of duplex-stabilizing dCTP and dGTP analogues in these cases has little practical value. Although d(5-meC)TP was successfully used here in detecting a G/C-rich 89 bp sequence (Figure 2E), others have reported that overstabilization is a problem for longer amplicons with a G/C base content of 50−60% (*9*). Application of dCTP derivatives other than d(5-meC)TP may be even more problematic since the order of base pair stability is as follows: dC < 5-meC < 5-BrC < 5-IC = 5-PrC (*31, 33*−*35, 37, 39*). For example, in this study, PCR yield was reduced when using d(5-BrC)TP (Figure 3B). On the other hand, application of duplex-stabilizing analogues of dCTP and dGTP may be very useful in cases of A/T-rich targets. dCTP analogues can be effectively used in combination with dATP and dTTP analogues (Figure 5D) to provide system stabilization.

For obvious reasons, the main targets of this research were derivatives of dATP and dTTP. The results clearly show that such derivatives have an application in real-time PCR.

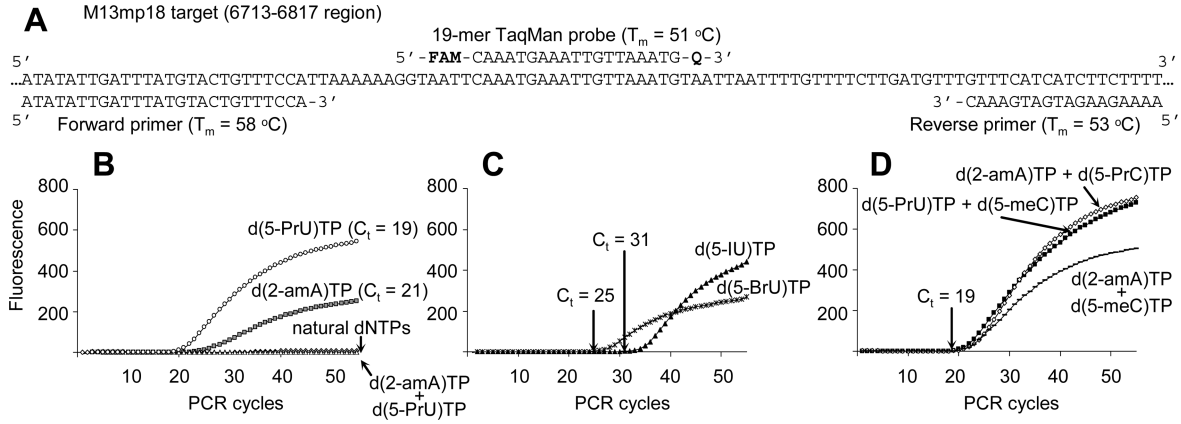

FIGURE 5: Real-time PCR detection of the M13mp18 sequence (10^6 copies per reaction) in the presence of base-modified duplex-stabilizing dNTPs. Scheme A shows the target sequence, the forward and reverse PCR primers, and the 19-mer TaqMan probe. The primers and probe contained standard bases. FAM is 6-fluorescein, and Q is a Black Hole Quencher (BHQ1) from Biosearch Technologies. One or two dNTP analogues were completely substituted for the respective natural dNTPs in an otherwise standard reaction using the PCR profile (95 °C, 2 min) → (95 °C, 10 s → 56 °C, 45 s)$_{55}$.

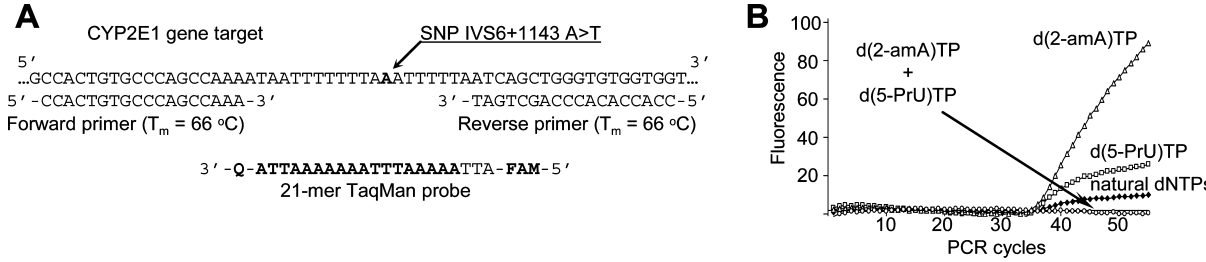

FIGURE 6: Real-time detection of the CYP2E1 sequence bracketing the A>T SNP IVS6+1143. Scheme A shows the target sequence in human genomic DNA (10 ng per 25 μL reaction mixture), the forward and reverse PCR primers, and the 21-mer TaqMan probe used in PCR. The probe contained 2-amA and 5-PrU bases at the positions in bold. dNTPs of these same bases were substituted for dATP and dTTP as indicated in panel B. The PCR profile was (95 °C, 2 min) → (95 °C, 10 s → 56 °C, 45 s)$_{55}$.

However, use of dATP and dTTP derivatives at the same time is limited. Nearest-neighbor thermodynamic parameters of these modified bases are unknown. Nevertheless, this study indicates that the d(2-amA)-d(5-PrU) base pair may be more stable than the dG-dC base pair. This hypothesis would explain why these modified dNTPs increased the C_t of the β2-macroglobulin amplicon [with 40% A/T (Figure 2I)] but abolished real-time detection of the CYP2E1 sequence [with 60% A/T (Figure 6B)] and the M13mp18 fragment [with 80% A/T (Figure 5B)]. While a modest increase in the denaturation temperature was helpful in resolving this issue for β2-macroglobulin detection (Figure 3A), this approach did not work for the two other A/T-rich targets shown in Figures 5 and 6.

Steric Clash in 5-Halogenated Pyrimidines. The study of 5-substituted dUTP derivatives led to yet another observation that may limit the applicability of 5-halogenated compounds in PCR. When the M13mp18 target system was being detected, stabilization was observed when dTTP was replaced with d(5-BrU)TP and d(5-IU)TP analogues (Figure 5C), and the overall signal strength was found to correlate with base pairing strength [i.e., d(5-BrU)TP < d(5-IU)TP = d(5-PrU)TP]. However, unlike the case with d(5-PrU)TP (Figure 5B), fluorescence thresholds for the 5-halogenated deoxyuridines (Figure 5C) were delayed, indicating a substantial reduction in PCR yield. Amplicon overstabilization was eliminated as a possible explanation on the basis of the insensitivity of C_t to increased denaturation temperature. The yield, however, did respond positively to an extension of the annealing time. For example, extending the annealing time from 45 s to 55 or 65 s at 58 °C reduced the C_t value by 2 or 6 cycles, respectively, over what was observed with d(5-IU)TP in Figure 5C. The same dUTP analogues were successfully used in detecting the β2-macroglobulin sequence (Figures 2 and 4), which has a low A/T content and randomly distributed thymines throughout the sequence. Unlike the β2-macroglobulin target, the M13mp18 target is very A/T-rich (80%) and includes several runs of thymine. When taken together, these results suggest that steric clash occurs when large halogen atoms are positioned next to each other in a DNA duplex due to runs of 5-substituted pyrimidine bases. The increase in C_t values, six cycles for d(5-BrU)TP and 12 cycles for d(5-IU)TP, correlates with the size of the halogen atoms. DNA extension across the hexa-adenylate run of the M13mp18 amplicon could be difficult since distortion of the DNA duplex by halogenated thymines might lead to duplex bending at this site. This abnormal structure would be expected to reduce the processivity of DNA synthesis.

DISCUSSION

DNA consists of only two base pairs, A-T and G-C. Nevertheless, the sequence and orientation of these base pairs are responsible for the structural and functional diversity of DNA. Base pair stability is a very important factor in defining diversity. However, as much as it is important for natural nucleic acids, the difference in duplex stability between A-T-rich and G-C-rich sequences poses a serious problem for detection of weakly pairing A/T-rich sequences (>50−60%) in real-time PCR assays. Several technologies have been

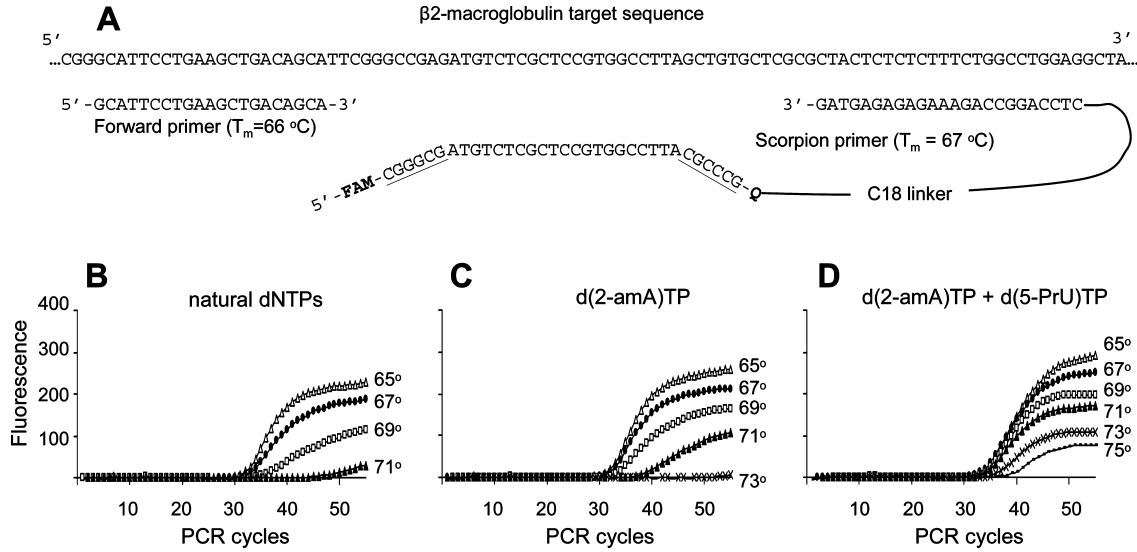

FIGURE 7: Scorpion detection assay conducted in the presence of base-modified dNTPs. Structures of a 96-mer oligodeoxyribonucleotide used as the target (10^4 copies per reaction mixture) and the PCR forward and reverse Scorpion primers are shown in scheme A. The primers were made from all natural nucleosides. A beacon-like FRET probe in the Scorpion is conjugated at its 3′-end to the 5′-end of a reverse PCR primer via an oligoglycol C18 linker (Glen Research). The complementary ends of the probe that participate in hairpin formation are underlined. FAM is 6-fluorescein, and Q is a Black Hole Quencher (BHQ1) from Biosearch Technologies. Panel B shows results of a set of control experiments in which all natural dNTPs were used in PCR. Panels C and D show results of experiments that are analogous to those of panel B but with dATP or dATP and dTTP completely replaced with d(2-amA)TP and d(5-PrU)TP. As indicated for each real-time curve, the annealing temperature (t) was varied in the range of 65−75 °C using the PCR profile (95 °C, 2 min) → (95 °C, 10 s → t °C, 45 s)$_{55}$. To avoid overstabilization of the amplicon (Figure 6A), the denaturation temperature in set D was elevated to 97 °C. All diagrams are shown using the same fluorescence scale. The real-time curves demonstrate that d(2-amA)TP and d(5-PrU)TP stabilized the detection system and in this respect are similar to the results obtained with classical TaqMan probes (Figures 2, 5, and 6).

developed to overcome this problem. The most notable examples are locked nucleic acids [LNA (*24, 25*)] and peptide nucleic acids [PNA (*26*)], duplex-stabilizing ligands such as minor groove binders [MGBs (*22, 23*)], and intercalators (*53, 54*) and nucleotide analogues. LNA (*55, 56*) and MGBs (*57*) are usually incorporated into probes since their use in primers can interfere with the function of DNA polymerases. Base modification of nucleotides is another effective way to enhance hybridization properties of primers and probes by allowing PCR at elevated temperatures (*28*). However, application of this strategy has not been reported since its original disclosure in 1996 (*28*). Difficulty in manufacturing and the relatively high cost have deterred the widespread use of base-modified primers and probes, especially those that contain 2-amA.

This study investigates an alternative way to enhance the hybridization properties of probes and primers in detection PCR by using base-modified duplex-stabilizing dNTP analogues (Figure 1). Several modified dNTPs, including those of 5-substituted pyrimidines and 2-aminoadenosine, are readily incorporated by Taq polymerase (Figure 4). Incorporation of these nucleotides into PCR products can enhance their pairing properties and stabilize hybrids formed with primers and probes in detection PCR (Figures 2, 5, and 6). This strategy is simple and straightforward but in the absence of predictive methods (see below) requires empirical optimization of primer and probe design and/or PCR conditions. Unlike conventional approaches which rely upon the use of modified primers and probes, significant hybrid stability is achieved with minimal additional cost since base-modified dNTPs are relatively inexpensive. This approach should be compatible with other duplex-stabilizing technologies. For example, in this study, extraordinary stabilization was observed when d(2-amA)TP was used in conjunction with 2-amA- and 5-meC-modified primers and probe (Figure 2H). Note, however, that the findings of this study relate to Taq DNA polymerase, the enzyme that is most commonly used in PCR. The response of other DNA polymerases, e.g., Pfu and Vent, to the base-modified dNTPs studied here may be different.

All known DNA and RNA detection techniques are based on the principle of complementarity, where an oligonucleotide forms a perfect match duplex with a predetermined site in a polynucleotide target. Detection of the probe−target duplex indicates the presence of the sequence in the reaction mixture. Stability of the hybrid is critical for detection (*58*), and its stabilization by the use of duplex-stabilizing dNTPs in PCR should enhance the performance of Molecular Beacon (*59*), Eclipse (*23*), and 3′ MGB-tailed TaqMan probes (*22*). Although many proof-of-principal results in this study were obtained using "classical" TaqMan probes (Figures 2, 5, and 6), duplex-stabilizing dNTPs were also found to be effective with Scorpion primers (*60*) (see Figure 7). Use of base-modified dNTPs is not limited to PCR and could be advantageously used in other amplification schemes performed at elevated temperatures such as nucleic acid sequence-based amplification (NASBA) (*61, 62*) and helicase-dependent amplification (HDA) (*63, 64*).

Duplex-stabilizing dNTPs facilitate the detection of sequences in real-time PCR, particularly those that are A/T-rich (Figures 5 and 6). Practical use of this strategy will require the availability of nearest-neighbor thermodynamic parameters of the modified base pairs. Programs augmented with this information would facilitate the design of optimized PCR assays that take advantage of modified dNTPs to enhance hybrid stability avoiding amplicon overstabilization (*65*). By predicting hybrid stability, such software would guide the selection of dNTPs analogues to be used with a

given sequence. The simplicity, low cost, and effectiveness of this technique indicate a high potential for routine use in high-throughput genomic studies.

ACKNOWLEDGMENT

I thank Cepheid's employees and managers, particularly John Bishop, David Persing, Humberto Reyes, Peter Daily, John Smith, Alexander Gall, and Ekaterina Viazovkina, for interest, technical help, and support. The assistance of Dr. Howard Gamper (Thomas Jefferson University, Philadelphia, PA) in revising the manuscript is gratefully acknowledged.

REFERENCES

1. Dobrikov, M. I., Sergueeva, Z. A., and Shaw, B. R. (2003) Incorporation of (α-P-borano)-2′,3′-dideoxycytidine 5′-triphosphate into DNA by drug-resistant MMLV reverse transcriptase and Taq polymerase. *Nucleosides, Nucleotides Nucleic Acids 22* (5−8), 1651–1655.
2. Ward, B., Snyder, L. M., Li, C., Song, K., Opper, K. E., Uder, S., and Hernan, R. (2005) Recombinant DNA processes using a dNTP mixture containing modified nucleotides. U.S. Patent 6,902,914.
3. Summers, J. S., and Shaw, B. R. (2001) Boranophosphates as mimics of natural phosphodiesters in DNA. *Curr. Med. Chem. 8*, 1147–1155.
4. Shaw, B. R., Porter, K. W., and Sergueev, D. (2004) Method of nucleic acid sequencing. U.S. Patent 6,808,897.
5. Jäger, S., and Famulok, M. (2004) Generation and enzymatic amplification of high-density functionalized DNA double strands. *Angew. Chem., Int. Ed. 43*, 3337–3340.
6. Lee, S. E., Sidorov, A., Gourlain, T., Mignet, N., Thorpe, S. J., Brazier, J. A., Dickman, M. J., Hornby, D. P., Grasby, J. A., and Williams, D. M. (2001) Enhancing the catalytic repertoire of nucleic acids: A systematic study of linker length and rigidity. *Nucleic Acids Res. 29*, 1565–1573.
7. Held, H. A., and Benner, S. A. (2002) Challenging artificial genetic systems: Thymidine analogs with 5-position sulfur functionality. *Nucleic Acids Res. 30*, 3857–3869.
8. Kuwahara, M., Hososhima, S., Takahata, Y., Kitagata, R., Shoji, A., Hanawa, K., Ozaki, A. N., Ozaki, H., and Sawai, H. (2003) Simultaneous incorporation of three different modified nucleotides during PCR. *Nucleic Acids Res. Suppl. 3*, 37–38.
9. Wong, K. K., and McClelland, M. (1991) PCR with 5-methyl-dCTP replacing dCTP. *Nucleic Acids Res. 19*, 1081–1085.
10. Seela, F., and Röling, A. (1992) 7-Deazapurine containing DNA: Efficiency of c7GdTP, c7AdTP and c7IdTP incorporation during PCR-amplification and protection from endodeoxyribonuclease hydrolysis. *Nucleic Acids Res. 20*, 55–61.
11. Gourlain, T., Sidorov, A., Mignet, N., Thorpe, S. J., Lee, S. E., Grasby, J. A., and Williams, D. M. (2001) Enhancing the catalytic repertoire of nucleic acids: A systematic study of linker length and rigidity. *Nucleic Acids Res. 29*, 1898–1905.
12. Dierick, H., Stul, M., De Kelver, W., Marynen, P., and Cassiman, J.-J. (1993) Incorporation of dITP or 7-deaza dGTP during PCR improves sequencing of the product. *Nucleic Acids Res. 21*, 4427–4428.
13. Auer, T., Sninsky, J. J., Gelfand, D. H., and Myers, T. W. (1996) Selective amplification of RNA utilizing the nucleotide analog dITP and *Thermus thermophilus* DNA polymerase. *Nucleic Acids Res. 24*, 5021–5026.
14. Bailly, C., and Waring, M. J. (1995) Transferring the purine 2-amino group from guanines to adenines in DNA changes the sequence-specific binding of antibiotics. *Nucleic Acids Res. 23*, 885–892.
15. Kempeneers, V., Renders, M., Froeyen, M., and Herdewijn, P. (2005) Investigation of the DNA-dependant cyclohexenyl nucleic acid-dependant DNA polymerization. *Nucleic Acids Res. 33*, 3828–3836.
16. Matthes, E., Reimer, K., von Janta-Lipinski, M., Meisel, H., and Lehmann, C. (1991) Comparative inhibition of hepatitis B virus DNA polymerase and cellular DNA polymerase by triphosphates of sugar-modified 5-methyldeoxycytidines and of other nucleoside analogs. *Antimicrob. Agents Chemother. 35*, 1254–1257.
17. Reid, R., Mar, E.-C., Huang, E.-S., and Topal, M. D. (1988) Insertion and extension of acyclic, dideoxy, and Ara nucleotides by Herpesviridae, Human α and Human β polymerases. *J. Biol. Chem. 263*, 3898–3904.
18. Porter, K. W., Briley, J. D., and Shaw, B. R. (1997) Direct PCR sequencing with boronated nucleotides. *Nucleic Acids Res. 25*, 1611–1617.
19. Walker, G. T., Little, M. C., and Nadeau, J. G. (1993) Nucleic acid target generation. U.S. Patent 5,270,184.
20. Walker, G. T., Linn, C. P., and Nadeau, J. G. (1996) DNA detection by strand displacement amplification and fluorescence polarization with signal enhancement using a DNA binding protein. *Nucleic Acids Res. 24*, 348–353.
21. Gelfand, D. H., Kwok, S. Y., and Sninsky, J. J. (1995) Reduction of non-specific amplification glycosylase using dUTP and DNA uracil. U.S. Patent 5,418,149.
22. Kutyavin, I. V., Afonina, I. A., Mills, A., Gorn, V. V., Lukhtanov, E. A., Belousov, E. S., Singer, M. J., Walburger, D. K., Lokhov, S. G., Gall, A. A., Dempcy, R., Reed, M. W., Meyer, R. B., and Hedgpeth, J. (2000) 3′-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Res. 28*, 655–661.
23. Afonina, I. A., Reed, M. W., Lusby, E., Shishkina, I. G., and Belousov, Y. S. (2002) Minor groove binder-conjugated DNA probes for quantitative DNA detection by hybridization-triggered fluorescence. *BioTechniques 32*, 940–949.
24. Goldenberg, O., Landt, O., Schumann, R. R., Gobel, U. B., and Hamann, L. (2005) Use of locked nucleic acid oligonucleotides as hybridization/FRET probes for quantitation of 16S rDNA by real-time PCR. *BioTechniques 38*, 29–32.
25. You, Y., Moreira, B. G., Behlke, M. A., and Owczarzy, R. (2006) Design of LNA probes that improve mismatch discrimination. *Nucleic Acids Res. 34*, e60.
26. Ortiz, E., Estrada, G., and Lizardi, P. M. (1998) PNA molecular beacons for rapid detection of PCR amplicons. *Mol. Cell. Probes 12*, 219–226.
27. Prosnyak, M. I., Veselovskaya, S. I., Myasnikov, V. A., Efremova, E. J., Potapov, V. K., Limborska, S. A., and Sverdlov, E. D. (1994) Substitution of 2-aminoadenine and 5-methylcytosine for adenine and cytosine in hybridization probes increases the sensitivity of DNA fingerprinting. *Genomics 21*, 490–494.
28. Lebedev, Y., Akopyans, N., Azhikina, T., Shevchenko, Y., Potapov, V., Stecenko, D., Berg, D., and Sverdlov, E. (1996) Oligonucleotides containing 2-aminoadenine and 5-methylcytosine are more effective as primers for PCR amplification than their nonmodified counterparts. *Genet. Anal. 13*, 15–21.
29. Breslauer, K. J., Frank, R., Blocker, H., and Marky, L. A. (1986) Predicting DNA duplex stability from the base sequence. *Proc. Natl. Acad. Sci. U.S.A. 83*, 3746–3750.
30. SantaLucia, J., Jr. (1998) A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. *Proc. Natl. Acad. Sci. U.S.A. 95*, 1460–1465.
31. Szer, W. (1965) Secondary structure of poly-5-methylcytidylic acid. *Biochem. Biophys. Res. Commun. 20*, 182–186.
32. Sagi, J., Brahms, S., Brahms, J., and Otvös, L. (1979) Effect of 5-alkyl substitution of uracil on the thermal stability of poly d(A-r^{5}U) copolymers. *Nucleic Acids Res. 6*, 2839–2848.
33. Butkus, V., Klimasauskas, S., Petrauskiene, L., Maneliene, Z., Janulaitis, A., Minchenkova, L. E., and Schyolkina, A. K. (1987) Synthesis and physical characterization of DNA fragments containing N4-methylcytosine and 5-methylcytosine. *Nucleic Acids Res. 15*, 8467–8478.
34. Uesugi, S., Miyashiro, H., Tomita, K.-I., and Ikehara, M. (1986) Synthesis and properties of d(ATACGCGTAT) and its derivatives containing one and two 5-methylcytosine residues. Effect of the methylation on deoxyribonucleic acid conformation. *Chem. Pharm. Bull. 34*, 51–60.
35. Petyuk, V., Serikov, R., Tolstikov, V., Potapov, V., Giege, R., Zenkova, M., and Vlassov, V. (2000) Invasion of strongly binding oligonucleotides into tRNA structure. *Nucleosides, Nucleotides Nucleic Acids 19*, 1145–1158.
36. Lacroix, L., and Mergny, J. L. (2000) Chemical modification of pyrimidine TFOs: Effect on i-motif and triple helix formation. *Arch. Biochem. Biophys. 381*, 153–163.
37. Radding, C. M., Josse, J., and Kornberg, A. (1962) Enzymatic synthesis of deoxyribonucleic acid. XII. A polymer of deoxyguanylate and deoxycytidylate. *J. Biol. Chem. 237*, 2869–2876.
38. Inman, R. B., and Baldwin, R. L. (1964) Helix-random coil transition in DNA homopolymer pairs. *J. Mol. Biol. 8*, 452–469.

39. Michelson, A. M., and Monny, C. (1967) Polynucleotide analogues. XII. Poly 5-bromocytidylic acid and poly 5-iodocytidylic acid. *Biochim. Biophys. Acta 149*, 88–98.
40. Howard, F. B., Frazier, J., and Miles, H. T. (1969) Interaction of poly-5-bromocytidylic acid with polyinosinic acid. A study of helix stability and spectroscopic properties. *J. Biol. Chem. 244*, 1291–1302.
41. Howard, F. B., Frazier, J., and Miles, H. T. (1966) A new polynucleotide complex stabilized by three interbase hydrogen bonds, poly-2-aminoadenylic acid + polyuridylic acid. *J. Biol. Chem. 241*, 4293–4295.
42. Scheit, K. H., and Rackwitz, H.-R. (1982) Synthesis and physicochemical properties of two analogs of poly(dA): Poly(2-aminopurine-9-β-D-deoxyribonucleotide) and poly 2-amino-deoxyadenylic acid. *Nucleic Acids Res. 10*, 4059–4069.
43. Gaffney, B. L., Marky, L. A., and Jones, R. A. (1982) The influence of the purine 2-amino group on DNA conformation and stability. Synthesis and conformational analysis of d[T(2-amino A)]$_3$. *Nucleic Acids Res. 10*, 4351–4361.
44. Gaffney, B. L., Marky, L. A., and Jones, R. A. (1984) The influence of the purine 2-amino group on DNA conformation and stability. II. Synthesis and physical characterization of d[CGT(2-NH_2)ACG], d[CCU(2-NH_2)ACG], and d[CGT(2-NH_2)AT(2-NH_2)ACG]. *Tetrahedron 40*, 3–13.
45. Howard, F. B., and Miles, H. T. (1984) 2NH_2A·T helices in the ribo- and deoxypolynucleotide series. Structural and energetic consequence of 2NH_2A substitution. *Biochemistry 23*, 6723–6732.
46. Chollet, A., and Kawashima, E. (1988) DNA containing the base analogue 2-aminoadenine: Preparation, use as hybridization probes and cleavage by restriction endonucleases. *Nucleic Acids Res. 16*, 305–317.
47. Potapov, V. K., Azhikina, T. L., Demin, V. V., Limborskaja, S. A., and Sverdlov, E. D. (1996) Modified oligonucleotides as a tool for DNA sequencing, fingerprinting and mapping. *Pure Appl. Chem. 68*, 1315–1320.
48. Takagi, M., Nishioka, M., Kakihara, H., Kitabayashi, M., Inoue, H., Kawakami, B., Oka, M., and Imanaka, T. (1997) Characterization of DNA polymerase from *Pyrococcus* sp. strain KOD1 and its application to PCR. *Appl. Environ. Microbiol. 63*, 4504–4510.
49. Innis, M. A., Myambo, K. B., Gelfand, D. H., and Brow, M. A. D. (1988) DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. *Proc. Natl. Acad. Sci. U.S.A. 85*, 9436–9440.
50. Cheong, C., Tinoco, I., and Chollet, A. (1988) Thermodynamic studies of base pairing involving 2,6-diaminopurine. *Nucleic Acids Res. 16*, 5115–5122.
51. Chakrabarti, R., and Schutt, C. E. (2001) The enhancement of PCR amplification by low molecular-weight sulfones. *Gene 274*, 293–298.
52. Chakrabarti, R., and Schutt, C. E. (2001) The enhancement of PCR amplification by low molecular weight amides. *Nucleic Acids Res. 29*, 2377–2381.
53. Asseline, V., Delarue, M., Laucelot, G., Toulme, F., Thuong, N. T., Montenay-Garestier, T., and Helene, C. (1984) Nucleic acid-binding with high affinity and base sequence specificity: Intercalating agents covalently linked to oligonucleotides. *Proc. Natl. Acad. Sci. U.S.A. 81*, 3297–3301.
54. Lokhov, S. G., Podyminogin, M. A., Sergeev, D. S., Silnikov, V. N., Kutyavin, I. V., Shishkin, G. V., and Zarytova, V. F. (1992) Synthesis and High Stability of Complementary Complexes of N-(2-Hydroxyethyl)phenazinium Derivatives of Oligonucleotides. *Bioconjugate Chem. 3*, 414–419.
55. Latorra, D., Arar, K., and Hurley, J. M. (2003) Design considerations and effects of LNA in PCR primers. *Mol. Cell. Probes 17*, 253–259.
56. Latorra, D., Campbell, K., Wolter, A., and Hurley, J. M. (2003) Enhanced allele-specific PCR discrimination in SNP genotyping using 3′ locked nucleic acid (LNA) primers. *Hum. Mutat. 22*, 79–85.
57. Afonina, I., Zivarts, M., Kutyavin, I., Lukhtanov, E., Gamper, H., and Meyer, R. B. (1997) Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. *Nucleic Acids Res. 25*, 2657–2660.
58. Didenko, V. V. (2001) DNA probes using fluorescence resonance energy transfer (FRET): Design and application. *BioTechniques 31*, 1106–1121.
59. Tyagi, S., and Kramer, F. R. (1996) Molecular beacons-probes that fluoresce upon hybridization. *Nat. Biotechnol. 14*, 303–308.
60. Whitcombe, D., Theaker, J., Guy, S. P., Brown, T., and Little, S. (1999) Detection of PCR products using self-probing amplicons and fluorescence. *Nat. Biotechnol. 17*, 804–807.
61. Oehlenschlager, F., Schwille, P., and Eigen, M. (1996) Detection of HIV-1 RNA by nucleic acid sequence-based amplification combined with fluorescence correlation spectroscopy. *Proc. Natl. Acad. Sci. U.S.A. 93*, 12811–12816.
62. Davey, C., and Malek, L. T. (2000) Nucleic acid amplification process. U.S. Patent 6,063,603.
63. Vincent, M., Xu, Y., and Kong, H. (2004) Helicase dependant isothermal DNA amplification. *EMBO Rep. 5*, 795–800.
64. An, L., Tang, W., Ranalli, T. A., Kim, H.-J., Wytiaz, J., and Kong, H. (2005) Characterization of a thermostable UvrD helicase and its participation in helicase dependant amplification. *J. Biol. Chem. 280*, 28952–28958.
65. Blake, R. D., Bizzaro, J. W., Blake, J. D., Day, G. R., Delcourt, S. G., Knowles, J., Marx, K. A., and SantaLucia, J., Jr. (1999) Statistical mechanical simulation of polymeric DNA melting with MELTSIM. *Bioinformatics 15*, 370–375.

BI8017784