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Increased Single-Nucleotide Discrimination in Allele-Specific Polymerase Chain Reactions through Primer Probes Bearing Nucleobase and 2′-Deoxyribose Modifications

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Abstract: The diagnosis of genetic dissimilarities between individuals is becoming increasingly important due to the discovery that these variations are related to complex phenotypes like the predisposition to certain diseases or compatibility with drugs. The most common among these sequence variations are single-nucleotide polymorphisms (SNPs). The availability of reliable and efficient methods for the interrogation of the respective genotypes is the basis for any progress along these lines. Many methods for the detection of nucleotide variations in genes exist, in which amplification of the target gene is required before analysis can take place. The allele-specific polymerase chain reaction (asPCR) combines target amplification and analysis in a single step. The principle of asPCR is based on the formation of matched or mismatched primer–target complexes. The most important parameter in asPCR is the discrimination of these matched or mismatched pairs. In recent publications we have shown that the reliability of SNP detection through asPCR is increased by employing chemically modified primer probes. In particular, primer probes that bear a polar 4′-*C*-methoxymethylene residue at the 3′ end have superior properties in discriminating single-nucleotide variations by PCR. Here we describe the synthesis of several primer probes that bear nucleobase modifications in addition to the 4′-*C*-methoxymethylenated 2′-deoxyriboses. We studied the effects of these alterations on single-nucleotide discrimination in allele-specific PCR promoted by a DNA polymerase and completed these results with single-nucleotide-incorporation kinetic studies. Moreover, we investigated thermal denaturing of the primer-probe–template complexes and recorded circular dichroism (CD) spectra for inspecting the thermodynamic and photophysical duplex behaviour of the introduced modifications. In short, we found that primer probes bearing a 4′-*C*-methoxymethylene residue at the 2′-deoxyribose moiety in combination with a thiolated thymidine moiety have synergistic effects and display significantly increased discrimination properties in asPCR.

Keywords: oligonucleotides · polymerase chain reaction · polymerases · thiothymidine

Introduction

Within the human genome comprising approximately 3 billion nucleobase pairs, individuals differ in approximately 0.1 percent of the nucleotide sequence.[1,2] The most common among these sequence variations are single-nucleotide polymorphisms (SNPs).[3–5] SNPs are defined as sites in which the less common variant has a frequency of at least 1% in a population. A direct linkage exists between some of these dissimilarities and certain diseases. Additionally, different effects of drugs on different patients can also be linked to SNPs.[3,5,6] Thus, considerable efforts have been focussed on finding new SNPs and elucidating connections between them and certain phenotypes. To date, almost two million SNPs have been discovered and characterised with a variety of methods. In a first step, methods are needed that identify unknown nucleotide variations; this is followed by an investigation of the medicinal relevance of the variations. After assessment of the exact sequence context, other methods are needed for high-throughput screening of populations in the search for known SNPs or to analyse individuals for SNP patterns. Obviously, in the latter case, methods are essential that allow time- and cost-effective verification of distinct nucleotide variations in daily laboratory practice.[7–16]

Most methods rely on amplification of the target gene before analysis can take place. Methods that enable amplification and analysis in a single step are desirable. In princi-

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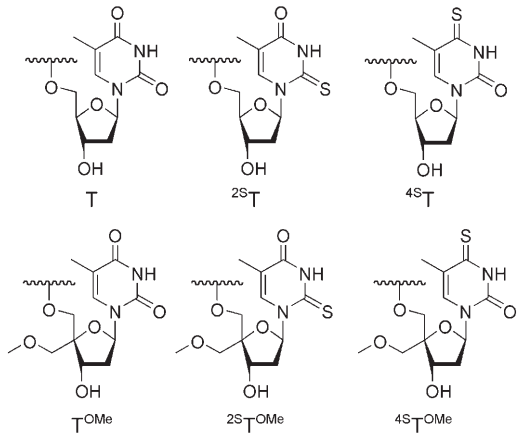
ple, allele-specific PCR (asPCR) comprises these features.[17–24] The concept of the asPCR is based on the principle that a DNA polymerase catalyses DNA synthesis from a matched 3′-primer terminus, while a mismatch due to hybridisation of the primer probe to a different sequence variant should obviate DNA synthesis and, thus, DNA amplification should be abolished. This conceptually simple and straightforward procedure is hampered somehow by the sequence dependency of the selectivity.[20–24] Thus, identification of the appropriate reaction conditions requires tedious optimisation. In earlier publications, we and others have shown an essential improvement in this method by employing chemically modified primer probes.[25–30] The employment of primer probes bearing a 4′-*C* modification at the 3′ end allowed the selectivity of asPCR to be increased significantly. These primer probes showed increased discrimination properties between matched and mismatched cases in the asPCR, as well as in primer extension. Additionally, these beneficial properties of the modified primer probes were independent of buffer conditions and applicable in different sequence contexts.[25–30] The combination of primer probes bearing 4′-*C*-methoxymethylene residues at the 3′ end together with the commercially available 3′–5′-exonuclease-deficient variant of a DNA polymerase from *Thermococcus litoralis* (Vent (exo$^-$) DNA polymerase) was found to exhibit superior performance in allele discrimination.[30] Encouraged by these findings, we set out to study the effects of some nucleobase modifications in conjunction with the previously described 4′-*C*-methoxymethylenated 2′-deoxyribose residues with regard to discrimination in the asPCR. In order to modify the size and hydrogen-bonding ability of the substrates, we substituted the carbonyl functionalities in thymidine for thiocarbonyl groups. Thiolated thymidines have an enlarged steric demand, due to the 0.45 Å longer double-bond length,[31] and additionally decreased H-bonding abilities compared to the native carbonyl groups. We found that primer probes bearing a 4′-*C*-methoxymethylene modification and thio modification placed at the 3′-terminal nucleotide displayed greatly increased discrimination properties in allele-specific PCR.

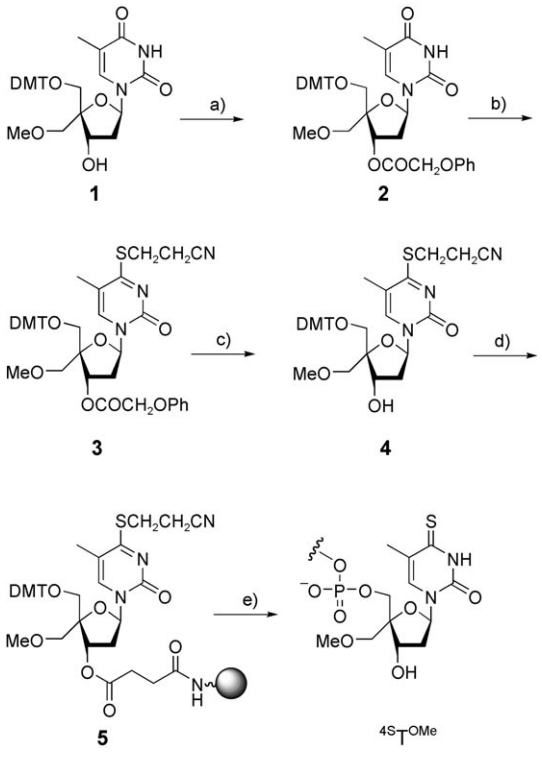
Results and Discussion

Synthesis of primer probes: To explore the effects of 2-S and 4-S substitutions in conjunction with 4′-C modification on the allele-discrimination in PCRs, we set out to synthesise the respective modified oligonucleotides.

The thiolated oligonucleotides ^{4S}T and ^{2S}T were synthesised by employing commercially available 5′-*O*-protected thiolated nucleosides that were coupled to a solid long-chain amino alkyl modified controlled pore glass (LCAA-CPG) support according to established protocols.[32] The solid supports were subsequently transferred into suitable cartridges and employed in standard automated DNA synthesis to yield the desired oligonucleotides ^{4S}T and ^{2S}T.



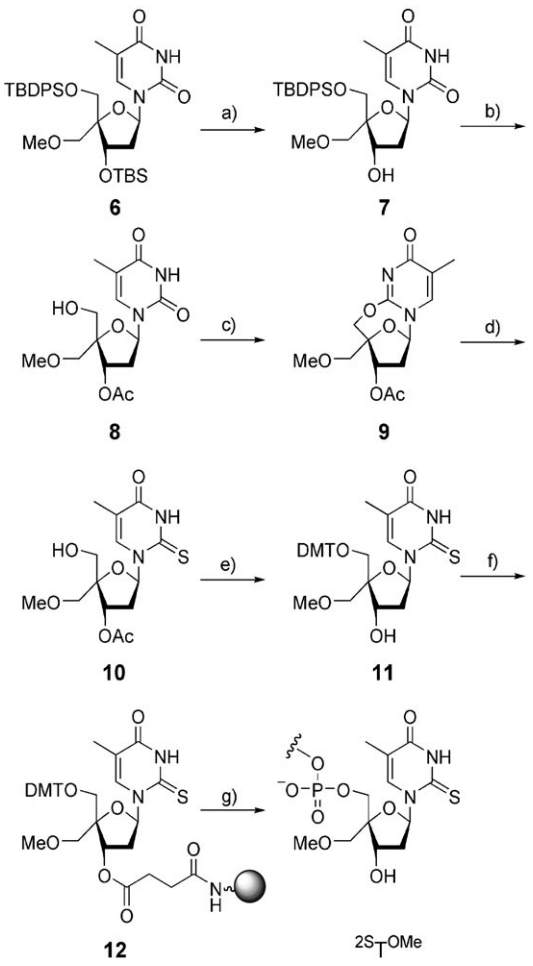
Next, we synthesised the 4-thio-4′-*C*-methoxymethylene thymidine bearing oligonucleotide $^{4S}T^{OMe}$. Our approach to the synthesis of the 4′-*C*-modified 4-thiothymidine building block makes use of the known 4′-*C*-methoxymethylene-modified thymidine **1**[30] as a starting point for further diversification (Scheme 1).



Scheme 1. a) $(PhOCH_2CO)_2O$, DMAP, pyridine, 95%; b) 1. TPSCl, DIPEA, DMAP, CH_2Cl_2; 2. $HSCH_2CH_2CN$, *N*-methylpyrolidine, 45%; c) aq NH_3, CH_3CN, 60%; d) EDC, DMAP, succinylated LCAA-CPG, pyridine; then 4-nitrophenol; then piperidine; then acetic anhydride/pyridine/THF (Cap A) and 1-methylimidazole/THF (Cap B); e) 1. oligonucleotide synthesis; 2. 25% NH_4OH, NaSH. DIPEA: *N*-ethyldiisopropylamine; DMAP: 4-(*N*,*N*-dimethylamino)pyridine; DMT: 4,4′-dimethoxytrityl; EDC: 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; LCAA-CPG: long-chain alkyl amine modified controlled pore glass; THF: tetrahydrofuran; TPSCl: triisopropylbenzenesulfonyl chloride.

Protection of the 3′-OH group of **1** was achieved with phenoxyacetic anhydride in very good yields.[33] Treatment of **2** with 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) and subsequently with 3-mercaptopropionitrile derived from freshly reduced 3,3′-dithiobis(propionitrile)[34] resulted in the 4-thiolated thymidine derivative **3**. Treatment with ammonia at room temperature resulted in the selective saponification of the 3′-*O*-phenoxyacetate without cleavage of the thioether. The resulting alcohol **4** was coupled to a solid support by using standard conditions to yield **5**, which was used for solid-support automated DNA synthesis.

For the synthesis of the 4′-*C*-methoxymethylen-2-thiothymidine containing oligonucleotide $^{2S}T^{OMe}$, we used **6** as the starting point. Compound **6** is an intermediate in the synthesis of **1** and is readily available in gram quantities (Scheme 2).[30] For further diversification we needed to establish a protocol to selectively cleave the 3′-*O*-*tert*-butyldimethylsilylether of **6** in the presence of a 5′-*O*-*tert*-butyldiphenylsilylether. We found that this can be achieved by treatment with $BF_3{\cdot}Et_2O$ in CH_2Cl_2 to afford **7** in very good yields. Further protection-group manipulations resulted in **8**. Conversion of **8** into the 5′-*O*-triflate and subsequent treatment with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) yielded the desired 2,5′-anhydrothymidine derivative **9** in good yields. It is noteworthy that other approaches that have been reported to yield the corresponding 4′-*C*-unmodified 2,5′-anhydrothymidines, for example, through the Mitsunobu reaction,[35] treatment of the 5′-iodothymidine derivative with silver acetate,[36] or ring closure via the 5′-*O*-tosylate[37] were not successful for the synthesis of the herein-depicted 4′-*C*-modified nucleoside. Opening of the ring system in **9** was achieved by treatment with H_2S in pyridine, a process resulting in moderate yields of **10**. Subsequently, protection-group manipulations yielded building block **11**, which was coupled to a solid support to form **12** for use in the automated synthesis of $^{2S}T^{OMe}$.



Scheme 2. a) $BF_3{\cdot}Et_2O$, CH_2Cl_2, 89%; b) 1. acetic anhydride, DMAP, pyridine; 2. TBAF, THF, 92%; c) 1. 2,6-lutidine, Tf_2O, DMF; 2. DBU, CH_3CN, 0°C–RT; 75% over both steps; d) H_2S, pyridine, 45°C, 28%; e) 1. DMTCl, DMAP, pyridine; 2. aq NH_3, MeOH; 40% over both steps; f) EDC, DMAP, succinylated LCAA-CPG, pyridine; then 4-nitrophenol; then piperidine; then acetic anhydride/pyridine/THF (Cap A) and 1-methylimidazole/THF (Cap B); g) 1. oligonucleotide synthesis; 2. 25% NH_4OH. DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene; DMF: *N*,*N*-dimethylformamide; TBAF: tetrabutylammonium fluoride; Tf_2O: trifluoroacetic anhydride.

Single-nucleotide discrimination in real-time PCR: For proof of principle experiments, we decided to use a PCR template (90-mer) within the human acid ceramidase and the Farber disease[38] sequence context. We conducted two reactions at a time in parallel. One PCR was conducted by employing a template bearing a deoxyadenosine (dA) residue opposite the respective 3′-terminal thymidine in the primer probe. In the other experiment, the same primer probe was combined with a template strand that had the same sequence apart from a dA to deoxyguanosine (dG) mutation opposite the 3′-terminal thymidine moiety. Both setups used the same unmodified reverse primer. In this study, we analyzed the PCRs by employing real-time double-stranded DNA detection through SybrGreen I fluorescence by using appropriate thermocycler equipment.[39] We determined the threshold-crossing point (C_t) as a measure for amplification efficiency. This parameter is defined as the point at which the reporter's fluorescence exceeds the background fluorescence significantly and crosses a threshold. The difference in the threshold-crossing points (ΔC_t) of canonical versus non-canonical primer–template amplification is a measure for single-nucleotide discrimination.

Amplification efficiency and the ability to discriminate against single-nucleotide mismatches varied with the modification employed (Figure 1, Table 1). Usage of the ^{2S}T probe introduced some degree of selectivity as compared to use of the unmodified probe. Strikingly, these effects were significantly enhanced when a 4′-*C*-methoxymethylene modification was present in conjunction with a 2-thiolation. $^{2S}T^{OMe}$ has superior properties when compared with the unmodified T, ^{2S}T and T^{OMe} probes. Different results were obtained when the effects of 4-thiolation on asPCRs were investigated. The ^{4S}T probe has no significant effect on the amplification selectivity or the efficiency. When $^{4S}T^{OMe}$ was used, the amplification efficiency decreased significantly, as indicated

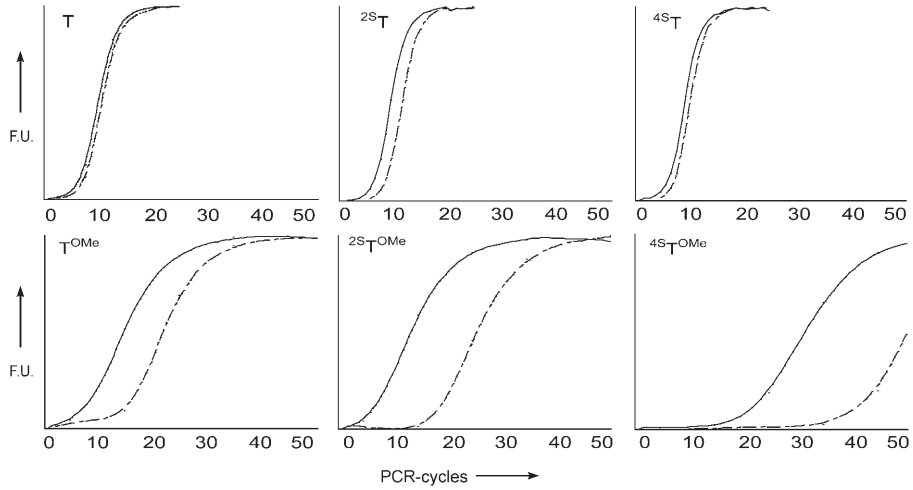


Figure 1. Results of real-time PCR experiments obtained by using primer probes bearing 4′-*C*-methoxymethylene residues at the 3′ end in combination with nucleobase thiomodifications. Results are shown with ^{2S}T, ^{4S}T, T^{OMe}, ^{2S}T^{OMe}, ^{4S}T^{OMe} or the unmodified primer T as indicated. PCR amplification in the presence of target template FarA (solid line) or FarG (dashed line). All experiments were conducted under the same conditions (see the Experimental Section).

Table 1. ΔC_t values by using base- and/or 4′-*C*-methoxymethylene-modified primer probes and DNA templates FarA or FarG.

5′-AGGAT/T* (Primer probe, 20-mer)[a] 3′-TCCT*N*TCCA (Template, 90-mer)[b]		
	C_t (*N*: A)	ΔC_t
T	5	0–0.5
^{2S}T	5	3
^{4S}T	5	1
T^{OMe}	6	9
^{2S}T^{OMe}	6	12
^{4S}T^{OMe}	20	19

[a] T*: modified thymidine ^{2S}T, ^{4S}T, T^{OMe}, ^{2S}T^{OMe} or ^{4S}T^{OMe}, as described above. [b] *N*: A or G for FarA or FarG, respectively.

by the C_t value of 20. Interestingly, this was combined with an unprecedentedly high ΔC_t value of 19 cycles! Agarose-gel analysis of the formed reaction products revealed that, when mismatched primer–template complexes containing the ^{4S}T^{OMe} probe were used, thermocycling resulted in amplification of non-specific PCR products (data not shown).

CD spectra and thermal-denaturing studies: To gain an insight into the origin of the observed effects, we conducted thermal-denaturing studies and recorded CD spectra. Duplexes between the respective primer strands (T, ^{2S}T, ^{4S}T, T^{OMe}, ^{2S}T^{OMe} and ^{4S}T^{OMe}) and 33-mer templates corresponding to matched (A–T/T*) and mismatched cases (G–T/T*) resulted in nearly superimposable CD spectra, a result indicating little, if any, dependence of the overall helix conformation on the presence of a matched or mismatched case and modifications at the nucleobase and/or 2′-deoxyribose (see Figure 2).

Next, thermal-denaturing studies were conducted in order to investigate the impact of chemical modification at the 3′-terminus on duplex stability. We found only small variations in melting points (Table 2). Interestingly, all duplexes containing modified strands have slightly increased melting points when compared to the native duplexes, in both matched and mismatched cases.

Thus, the influence of a single nucleotide at the primer terminus that has been modified at the 4′-*C*-deoxyribose and/or the nucleobase on the intrinsic formation of aberrant conformations or duplex stability is small.

Steady-state kinetics of single-nucleotide incorporation: To quantify the influence of the depicted chemical modifications

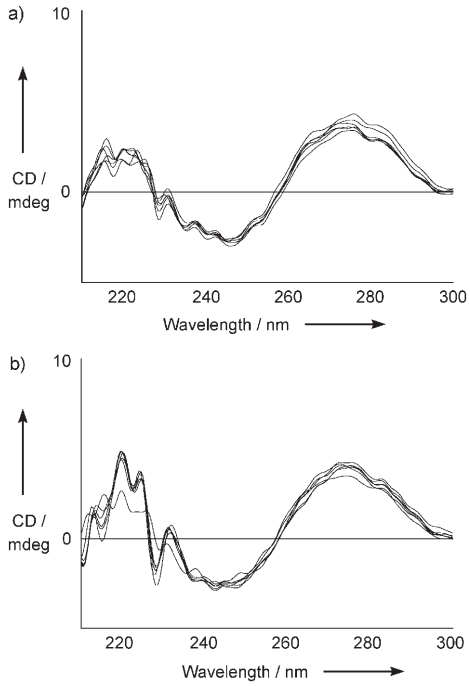


Figure 2. Circular dichroism spectra of native and modified oligonucleotides. Primer probes (20-mer) were hybridised with template (33-mer) in matched (A–T/T*) and mismatched (G–T/T*) cases. A) T match, T mismatch, ^{2S}T match, ^{2S}T mismatch, ^{4S}T match and ^{4S}T mismatch; B) T^{OMe} match, T^{OMe} mismatch, ^{2S}T^{OMe} match, ^{2S}T^{OMe} mismatch, ^{4S}T^{OMe} match and ^{4S}T^{OMe} mismatch. T*: modified thymidines ^{2S}T, ^{4S}T, T^{OMe}, ^{2S}T^{OMe} or ^{4S}T^{OMe} as described above.

on enzyme action, we investigated the steady-state extension efficiencies from the modified primer termini and determined the kinetic constants (k_{cat}: first order rate of catalysis; K_M: Michaelis constant; k_{cat}/K_M: incorporation efficiency) of single-nucleotide incorporation under single-completed-hit

Table 2. Thermal-denaturing experiments (melting temperature, T_m [°C], values shown) of primer probes bearing 2-*S*/4-*S*-thio modification and/or 4′-*C*-methoxymethylene-modification (^{2S}T, ^{4S}T, T^{OMe}, ^{2S}T^{OMe}, ^{4S}T^{OMe}) compared to those of unmodified primer probe (T) in matched (A–T/T*) and mismatched cases (G–T/T*). All experiments were conducted with the same double-stranded DNA and buffer concentrations.

5′-AGGAT/T* (Primer probe, 20-mer)[a] 3′-TCCTNTCCA (Template, 33-mer)[b]		
	matched (A–T/T*)	mismatched (G–T/T*)
T	65.5	64.5
^{2S}T	66.1	66.3
^{4S}T	67.5	67.4
T^{OMe}	65.5	66.0
^{2S}T^{OMe}	66.3	65.0
^{4S}T^{OMe}	67.0	66.1

[a] T*: modified thymidine ^{2S}T, ^{4S}T, T^{OMe}, ^{2S}T^{OMe} or ^{4S}T^{OMe}, as described above. [b] *N*: A or G for match or mismatch, respectively.

and steady-state conditions, as previously described[40] (see Table 3).

The best discrimination properties of the non-sugar-modified primer probes were shown by ^{2S}T, which is coherent with the results obtained in our real-time asPCR experiments. Recently, similar results were obtained for the incorporation of the respective thiolated thymidine triphosphates (TTPs).[41] The increased selectivity of the system comprising ^{2S}T is mainly achieved by a decreased steady-state k_{cat} value in the mismatched case, in comparison to that of the unmodified system. One can envision that DNA amplification from matched versus mismatched DNA complexes under PCR conditions (that is, 200 μM deoxynucleoside triphosphate (dNTP)) very much depends on the k_{cat} value of the proceeding nucleotide incorporation. In real-time PCR, the signal generation is dependent on the formation of double-stranded DNA. Thus, the reduced steady-state k_{cat} value of the ^{2S}T-comprising system in the mismatched case might well be the cause of the above-observed single-nucleotide discrimination ability in allele-specific PCR.

4′-*C*-modification has significant effects on the extension efficiency. The efficiency is greatly diminished due to a significantly increased K_M value (about 100-fold) and an approximately 5- to 10-fold decreased k_{cat} value. This is in line with earlier findings by us, although different enzymes and modifications were used.[42] However, when single-completed hit conditions were used (>10-fold excess primer/template over DNA polymerase concentration), no extension of mismatched primer termini was observed. As demonstrated by our results, these limitations can be overcome in PCRs in which standard dNTP concentrations are employed that are higher than the measured K_M values, thereby resulting in an efficient PCR when matched primer/templates are employed.

Table 3. Kinetic studies of single-nucleotide insertion. Single-nucleotide extension of matched (A–T/T*) and mismatched (G–T/T*) primer termini by Vent (exo$^-$) DNA polymerase. The incorporation of dATP was studied with the different primer probes ^{2S}T, ^{4S}T, T^{OMe}, ^{2S}T^{OMe} or ^{4S}T^{OMe} in comparison to that with the unmodified primer probe (T) in matched and mismatched cases with the respective template (33-mer).

	match (A–T/T*)[a]			mismatch (G–T/T*)[a]		
primer	K_M [μM]	k_{cat} [min^{-1}]	k_{cat}/K_M [min^{-1} μM^{-1}]	K_M [μM]	k_{cat} [min^{-1}]	k_{cat}/K_M [min^{-1} μM^{-1}]
T	0.23±0.02	1.11±0.1	4.8	51.8±6.0	0.48±0.05	0.009
^{2S}T	0.26±0.06	0.85±0.04	3.3	89.7±30	0.18±0.01	0.002
^{4S}T	0.26±0.08	1.09±0.21	4.2	33.7±9.2	0.48±0.03	0.014
T^{OMe}	33.6±4.2	0.14±0.02	0.004	n.a.	n.a.	n.a.
^{2S}T^{OMe}	30.2±1.3	0.21±0.02	0.007	n.a.	n.a.	n.a.
^{4S}T^{OMe}	26.5±2.4	0.10±0.007	0.004	n.a.	n.a.	n.a.

[a] T*: modified thymidine ^{2S}T, ^{4S}T, T^{OMe}, ^{2S}T^{OMe} or ^{4S}T^{OMe} as described above. n.a.: not accessible; no nucleotide insertions were observed when up to 8 nM of DNA polymerase, up to 1 h incubation time and up to 600 μM of dATP (higher dATP concentrations caused inhibition of the reaction) were applied.

Conclusion

Taken together, we show that primer probes that bear thiolated thymidines are able to increase single-nucleotide discrimination in allele-specific PCRs. These modifications, either at the 4′-C-deoxyribose or on the nucleobase of a single-modified nucleotide at the primer terminus, do not have any significant effect on duplex stability and the conformation of the respective primer–template complex. Therefore the characterised discrimination properties must result from the specific interaction between the DNA polymerase, the template–primer-probe duplex and the incoming dNTPs.

Nucleobase thiolation in conjunction with 4′-*C*-methoxymethylene modification at the 2′-deoxyribose exhibits the most pronounced effects. These compounds are readily available and can be incorporated into DNA strands by using standard oligonucleotide chemistry. This real-time PCR system supersedes recently discovered approaches[30] by us that use unmodified nucleobases. The described system with the ^{2S}T^{OMe} primer probe should be useful for the direct diagnosis of single-nucleotide variations within genes, such as single-nucleotide polymorphisms or point mutations, directly without the need for further time- and cost-intensive post-PCR analysis.

Experimental Section

General: All temperatures quoted are uncorrected. All reagents are commercially available and were used without further purification. Solvents were purchased over molecular sieves (Fluka) and used directly without further purification unless otherwise noted. 5′-*O*-(4,4′-Dimethoxytrityl)-(3-*N*/4-*O*-toluoyl)-2-*S*-thiothymidine and 5′-*O*-(4,4′-dimethoxytrityl)-4-*S*-(2-cyanoethyl)thiothymidine were purchased from Berry & Associates (USA). All reactions were conducted under exclusion of air and mois-

ture. NMR spectra were recorded on a Bruker DRX600 instrument, with the solvent peak as an internal standard. MALDI-TOF mass spectra were measured on a Bruker Biflex III instrument with 2,5-dihydroxybenzoic acid (DHB) as the matrix. ESI mass spectra were recorded on an Esquire 3000 plus instrument from Bruker. Oligonucleotide samples were dissolved in 2-propanol (20%) containing 0.5–1% triethylamine ($c = 1$–50 pmol μL^{-1}). Flash chromatography was performed over Merck silica gel 60 (230–400 mesh). MPLC was performed on Büchi apparatus, with silica gel 60M. Thin-layer chromatography was performed on Merck precoated plates (silica gel 60 F_{254}). UV/Vis analysis was performed with a Cary 100 Bio instrument from Varian. MS analysis of oligonucleotides was conducted by Metabion (Germany, Martinsried, MALDI-TOF) or by ESI.

5′-*O*-(4,4′-Dimethoxytrityl)-4′-*C*-methoxymethylen-3′-*O*-phenoxyacetylene thymidine (2): After co-evaporation and drying in vacuo, nucleoside **1** (45 mg, 0.085 mmol) was dissolved in dry pyridine (5 mL). Phenoxyacetic anhydride (29.2 mg, 0.1 mmol) was added and the reaction mixture was stirred for 5 h. Phenoxyacetic anhydride (10 mg, 0.035 mmol) and a catalytic amount of DMAP were then added and the solution was stirred at room temperature for 48 h. The reaction was stopped by addition of water (3 mL) and the solvent was removed in vacuo. The residue was dissolved in dichloromethane (5 mL, with 1% NEt_3). Sodium bicarbonate solution was added and the aqueous phase was extracted with dichloromethane (with 1% NEt_3). The combined organic phases were dried ($MgSO_4$) and concentrated in vacuo. The obtained residue was purified by flash column chromatography (silica gel, ethyl acetate/petroleum ether 2:1 with 1% NEt_3) and **2** was isolated as a colourless foam (60 mg, 0.08 mmol, 95%): $R_f = 0.57$ (ethyl acetate/petroleum ether 3:1 with 1% NEt_3); ^{1}H NMR (600 MHz, [D$_6$]acetone); $\delta = 1.48$ (s, 3H; 5-CH_3), 2.48 (ddd, $^2J = 14.2$, $^3J = 7.2$, 3.5 Hz, 1H; H-2′a), 2.61 (ddd, $^2J = 14.2$, $^3J = {}^3J =$ 7.2 Hz, 1H; H-2′b), 3.23 (s, 3H; OCH_3), 3.27 (d, $^2J = 9.6$ Hz, 1H; H-5′a), 3.4 (d, $^2J = 9.6$ Hz, 1H; 4′-C−CH_2a), 3.43 (d, $^2J = 9.6$ Hz, 1H; H-5′b), 3.50 (d, $^2J = 9.6$ Hz, 1H; 4′-C−CH_2b), 3.77 (s, 6H; OCH_3), 4.82 (d, $^2J =$ 16.6 Hz, 1H; PhO−CH_2a), 4.86 (d, $^2J = 16.6$ Hz, 1H; PhO−CH_2b), 5.79 (dd, $^3J = 3.5$, 7.2 Hz, 1H; H-3′), 6.32 (dd, $^3J = {}^3J = 7.2$ Hz, 1H; H-1′), 6.80–7.50 (m, 19H; Ar H, H-6), 9.90 ppm (brs, 1H; NH); ^{13}C NMR (150.9 MHz, [D$_6$]acetone): $\delta = 13.2$ (CH_3), 39.8 (C-2′), 56.5 (Ph−OCH_3), 60.5 (OCH_3), 66.7 (CH_2−OPh), 67.1 (C-5′), 74.3 (4′-C−CH_2), 76.4 (C-3′), 86.0 (C-1′), 88.4 (C-4′), 88.8 (Ar_3C), 112.2, 115.0, 116.4, 123.2, 128.8, 129.7, 130.0, 131.4, 132.03, 132.05, 137.3, 137.34, 137.5 (Ar C, C-5, C-6), 146.8 (C-2), 160.0, 160.76, 160.78 (C-4, Ar C), 169.8 ppm (OCO); MALDI-TOF MS (DHB matrix): m/z: 745.3 $[M+Na]^+$, 761.3 $[M+K]^+$.

5′-*O*-(4,4′-Dimethoxytrityl)-4′-*C*-methoxymethylen-3′-phenoxyacetylen-4-*S*-(2-cyanoethyl)thiothymidine (3): DIPEA (43 μL, 0.25 mmol), TPSCl (50 mg, 0.163 mmol) and DMAP (1.2 mg, 10 μmol) were added to a solution of **2** (56 mg, 0.078 mmol) in dichloromethane (5 mL). After the mixture had been stirred for 2 h, TPSCl (50 mg, 0.163 mmol) and DIPEA were again added and stirring was continued for 3 h. 3-Mercaptopropionitrile (43 μL, 0.543 mmol), derived from freshly reduced 3,3′-dithiobis-(propionitrile),[33] and *N*-methylpyrrolidine (10 μL, 0.094 mmol) were added to the reaction mixture. After stirring for 2 h, the reaction mixture was diluted with dichloromethane and washed with sodium bicarbonate solution and brine. The organic phase was evaporated in vacuo. Purification of the residue by flash column chromatography (silica gel, ethyl acetate/petroleum ether 1:3 with 1% NEt_3) gave **3** (28 mg, 0.035 mmol, 45%) as a yellow oil; $R_f = 0.41$ (ethyl acetate/petroleum ether 3:1 with 1% NEt_3); ^{1}H NMR (600 MHz, [D$_6$]acetone): $\delta = 1.69$ (s, 3H; CH_3-5), 2.48 (ddd, $^2J = 14.0$, $^3J = {}^3J = 7.1$ Hz, 1H; H-2′a), 2.69 (ddd, $^2J = 14.0$, $^3J =$ 3.9, 6.4 Hz, 1H; H-2′b), 2.93–3.00 (m, 2H; CH_2CN), 3.26 (s, 3H; OMe), 3.31 (d, $^2J = 10.0$ Hz, 1H; H-5′a), 3.40–3.50 (m, 4H; 4′-C−CH_2a, H-5′b, SCH_2), 3.53 (d, $^2J = 10.0$ Hz, 1H; 4′-C−CH_2b), 3.78 (s, 6H; OMe), 4.83 (d, $^2J = 16.4$ Hz, 1H; PhO−CH_2a), 4.87 (d, $^2J = 16.4$ Hz, 1H; PhO−CH_2b), 5.76 (dd, $^3J = 3.9$, 7.1 Hz, 1H; H-3′), 6.20 (dd, $^3J = {}^3J = 6.4$ Hz, 1H; H-1′), 6.8–7.8 ppm (m, 19H; Ar H, H-6); ^{13}C NMR (150.9 MHz, [D$_6$]acetone): $\delta = 14.7$ (CH_3), 19.2 (CH_2CN), 26.9 (SCH_2), 41.1 (C-2′), 56.5 (PhOCH_3), 60.5 (CH_2OCH_3), 66.5 (CH_2−OPh), 66.6 (C-5′), 74.0 (4′C−CH_2), 76.1 (C-3′), 88.3 (C-1′), 88.8 (C-4′), 89.5 (CAr_3), 112.5, 115.0, 116.4, 120.2, 123.2, 128.8, 129.8, 130.0, 130.3, 130.4, 131.4, 132.0, 133.6, 133.7, 137.2, 137.4, 140.4, 146.7, (Ar C, C5, C6), 154.1, 160.0, 160.8, 169.8, 177.6 ppm (C-2, C-4, Ar C, OCO); MALDI-TOF MS (DHB matrix): m/z: 815.0 $[M+Na]^+$, 830.9 $[M+K]^+$.

5′-*O*-(4,4′-Dimethoxytrityl)-4′-*C*-methoxymethylen-4-*S*-(2-cyanoethyl)-thiothymidine (4): Concentrated NH_4OH (450 μL, 25%) was added to a solution of nucleoside **3** (43.8 mg, 0.055 mmol) in acetonitrile (2 mL). After the reaction mixture had been stirred for 2 h, concentrated NH_4OH (140 μL, 25%) was again added. The reaction mixture was stirred for 10 h. Concentrated NH_4OH (45 μL, 25%) was again added and the mixture was stirred for further 2 h. The solvent was evaporated and the residue was purified by flash column chromatography (silica gel, ethyl acetate/petroleum ether 3:1 with 1% NEt_3). Nucleoside **4** was obtained as a colourless foam (21.4 mg, 0.033 mmol, 60%); $R_f = 0.14$ (ethyl acetate/petroleum ether 3:1 with 1% NEt_3); ^{1}H NMR (600 MHz, [D$_6$]acetone): $\delta = 1.64$ (d, $^4J = 1.0$ Hz, 3H; CH_3-5), 2.24 (ddd, $^2J = 13.8$, $^3J = {}^3J = 6.6$ Hz, 1H; H-2′a), 2.61 (ddd, $^2J = 13.8$, $^3J = 6.6$, 4.2 Hz, 1H; H-2′b), 2.95–3.00 (m, 2H; CH_2CN), 3.29 (d, $^2J = 10.0$ Hz, 1H; H-5′a), 3.31 (s, 3H; OCH_3), 3.40 (d, $^2J = 10.0$ Hz, 1H; H-5′b), 3.44 (m, 2H; SCH_2), 3.62 (d, $^2J = 10.0$ Hz, 1H; 4′-C−CH_2a), 3.67 (d, $^2J = 10.0$ Hz, 1H; 4′-C−CH_2b), 3.79 (s, 6H; OCH_3), 4.63 (dd, $^3J = {}^3J = 4.4$ Hz, 1H; H-3′), 6.21 (dd, $^3J = {}^3J = 6.6$ Hz, 1H; H-1′), 6.90–7.50 (m, 13H; Ar H), 7.85 ppm (d, $^4J =$ 1.0 Hz, 1H; H-6); ^{13}C NMR (150.9 MHz, [D$_6$]acetone): $\delta = 13.7$ (CH_3), 18.2 (CH_2CN), 25.9 (SCH_2), 42.9 (C-2′), 55.5 (Ar−OCH_3), 59.5 (OCH_3), 65.7 (C-5′), 72.5 (C-3′), 73.6 (4′C−CH_2), 87.2, 89.6 (CAr_3, C-4′), 87.5 (C-1′), 111.2, 114.0, 119.2, 127.7, 128.7, 129.0 (Ar C), 131.0 (C-6), 136.4, 136.6, 139.6, 145.9, 153.3, 159.7, 176.0 ppm (C-2, C-4, Ar C); MALDI-TOF MS (DHB matrix): m/z: 680.2 $[M+Na]^+$, 696.1 $[M+K]^+$.

5′-*O*-*tert*-Butyldiphenylsilyl-4′-*C*-methoxymethylene thymidine (7): Nucleoside **6** (20 mg, 0.031 mmol) was dissolved in dry dichloromethane (2 mL). $BF_3{\cdot}OEt_2$ (4.43 μL, 0.035 mmol) was added and the reaction mixture was stirred at room temperature for 4 h. After addition of saturated sodium bicarbonate solution, the mixture was extracted with dichloromethane. The organic phase was dried over $MgSO_4$. The solvent was removed in vacuo. The residue was purified by flash column chromatography (silica gel, ethyl acetate/petroleum ether 1:2) to give nucleoside **7** as a white foam (14.5 mg, 0.0276 mmol, 89%); $R_f = 0.23$ (ethyl acetate/petroleum ether 1:1); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 1.10$ (s, 9H; *t*Bu−Si), 1.56 (s, 3H; CH_3-5), 2.31 (ddd, $^2J = 13.4$, $^3J = 8.7$, 6.6 Hz, 1H; H-2′a), 2.42 (ddd, $^2J = 13.4$, $^3J = 5.6$, 2.0 Hz, 1H; H-2′b), 3.33 (s, 3H; OCH_3), 3.46 (d, $^2J = 9.5$ Hz, 1H; H-5′a), 3.61 (d, $^2J = 9.5$ Hz, 1H; H-5′b), 3.81 (d, $^2J =$ 11.0 Hz, 1H; 4′C−CH_2a), 3.85 (d, $^2J = 11.0$ Hz, 1H; 4′C−CH_2b), 4.6 (dd, $^3J = 2.0$, 6.6 Hz, 1H; H-3′), 6.47 (dd, $^3J = 5.6$, 8.7 Hz, 1H; H-1′), 7.37–7.70 (m, 11H; Ar H, H-6), 8.30 ppm (brs; NH); ^{13}C NMR (150.9 MHz, [D$_6$]acetone): $\delta = 12.0$ (CH_3), 19.4 (C−$(CH_3)_3$), 27.0 (C-($CH_3)_3$), 41.0 (C-2′), 59.5 (OMe), 66.7 (4′C−CH_2), 73.2 (C-5′), 73.9 (C-3′), 84.5 (C-1′), 88.0 (C-4′), 111.2 (C-5), 127.9, 128, 130.1, 130.2, 132.2, 132.9, 135.2, 135.3, 135.5 (Ar C, C-6), 150.1 (C-2), 163.4 ppm (C-4); MALDI-TOF MS (DHB matrix): m/z: 547.5 $[M+Na]^+$.

3′-*O*-Acetyl-4′-*C*-methoxymethylene thymidine (8): A mixture of nucleoside **7** (1.56 g, 2.98 mmol), acetic anhydride (700 μL, 0.76 g, 7.5 mmol) and DMAP (1.2 mg, 10 μmol) in dry pyridine (20 mL) was stirred overnight at room temperature. The solvent was removed in vacuo. The dried residue was dissolved in dry THF (20 mL) and TBAF solution (3.6 mL, 1 M in THF) was added. After the mixture had been stirred for 3.5 h, the solvent was removed in vacuo and the residue was purified by flash column chromatography (silica gel, ethyl acetate/petroleum ether 3:1–5:1). Compound **8** was obtained as a colourless residue (904 mg, 2.75 mmol, 92%); $R_f = 0.15$ (ethyl acetate/petroleum ether 2:1); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 1.90$ (d, $^4J = 1.2$ Hz, 3H; CH_3-5), 2.10 (s, 3H; OCH_3), 2.43 (ddd, $^2J = 14.0$, $^3J = 6.5$, 3.5 Hz, 1H; H-2′a), 2.46–2.51 (ddd, $^2J = 14.0$, $^3J = {}^3J = 7.0$ Hz, 1H; H-2′b), 3.35 (s, 3H; OCH_3), 3.47 (d, $^2J =$ 10.3 Hz, 1H; H-5′a), 3.48 (d, $^2J = 10.3$ Hz, 1H; H-5′b), 3.78 (d, $^2J =$ 12.0 Hz, 1H; 4′C−CH_2a), 3.82 (d, $^2J = 12.0$ Hz, 1H; 4′C−CH_2b), 5.55 (dd, $^3J = 3.5$, 7.0 Hz, 1H; H-3′), 6.26 (dd, $^3J = {}^3J = 6.5$ Hz, 1H, H-1′), 7.51 (d, $^4J = 1.2$ Hz, 1H; H-6), 8.97 ppm (brs, 1H; NH); ^{13}C NMR (150.9 MHz, $CDCl_3$): $\delta = 12.5$ (CH_3-5), 20.8 (OCH_3), 38.3 (C-2′), 59.6 (OCH_3), 64.6 (4′C−CH_2), 72.7 (C-5′), 73.5 (C-3′), 85.3 (C-1′), 87.3 (C-4′), 111.3 (C-5), 136.3 (C-6), 150.5 (C-2), 163.8 ($COCH_3$), 170.2 ppm (C-4); MALDI-TOF MS (DHB matrix): m/z: 351.0 $[M+Na]^+$.

2,5′-*O*-Cyclo-3′-*O*-acetyl-4′-*C*-methoxymethylene thymidine (9): 2,6-Lutidine (107 μL, 0.915 mmol) was added to a solution of nucleoside **8** (300 mg, 0.905 mmol) in dry dichloromethane (15 mL). The solution was cooled to −50 °C and a solution of trifluoroacetic anhydride (200 μL, 0.915 mmol) in dry dichloromethane (4 mL) was added dropwise. After addition, the reaction mixture was allowed to warm up to room temperature. A saturated solution of sodium bicarbonate was added and the mixture was extracted with dichloromethane. The organic solution was concentrated and the residue was purified with a short silica-gel column (ethyl acetate/petroleum ether 2:1). The crude triflate was dissolved in dry acetonitrile (20 mL) and cooled to 0 °C. DBU (152 μL, 0.112 mmol) was added dropwise. During stirring of the reaction mixture for 3 h at room temperature, a white solid precipitated. The reaction mixture was kept at −20 °C for 14 h. The precipitate was filtered off and washed with acetonitrile. The filtrate was concentrated in vacuo. The residue was purified by flash column chromatography (silica gel, dichloromethane with 5% methanol). Nucleoside **9** was obtained as a white foam (213 mg, 0.69 mmol, 75%); R_f=0.33 (dichloromethane with 10% methanol); ^{1}H NMR (600 MHz, $CDCl_3$): δ=1.92 (s, 3H; CH_3-5), 2.06 (s, 3H; OCH_3), 2.49 (ddd, ^2J=15.7, ^3J=8.2, ^3J<1 Hz, 1H; H-2′a), 2.80 (ddd, ^2J=15.7, ^3J=7.3, ^3J<1 Hz, 1H; H-2′b), 3.31 (s, 3H; OCH_3), 3.53 (d, ^2J=10.2 Hz, 1H; 4′C−CH_2a), 3.60 (d, ^2J=10.2 Hz, 1H; 4′C−CH_2b), 4.15 (d, ^2J=13.2 Hz, 1H; H-5′a), 4.53 (d, ^2J=13.2 Hz, 1H; H-5′b), 5.57 (dd, ^3J=7.3, ^3J<1 Hz, 1H; H-3′), 5.73 (dd, ^3J=8.2, ^3J<1 Hz, 1H; H-1′), 7.27 ppm (s, 1H; H-6); ^{13}C NMR (150.9 MHz, $CDCl_3$): δ=13.2 (CH_3-5), 20.7 ($COCH_3$), 42.1 (C-2′), 59.8 (OCH_3), 70.5 (4′-C−CH_2), 74.4 (C-3′), 76.9 (CH_2-5′), 88.7 (C-4′), 92.7 (C-1′), 120.3 (C-5), 138.9 (C-6), 158 (C-2), 170.8 ($COCH_3$), 172.6 ppm (C-4); MALDI-TOF MS (DHB matrix): *m*/*z*: 311.0 $[M+H]^+$, 333.0 $[M+Na]^+$, 349.0 $[M+K]^+$.

2-Thio-3′-*O*-acetyl-4′-*C*-methoxymethylene thymidine (10): Nucleoside **9** (118 mg, 0.38 mmol) was dissolved in pyridine (5 mL) in an autoclave. The solution was cooled to −68 °C and condensated H_2S (≈10 mL) was subsequently added. The autoclave was immediately closed and the reaction mixture was stirred at 45 °C for 12 days under pressure (23 bar). The autoclave was then slowly opened, the remaining H_2S in the mixture was purged with N_2 gas for 30 min and the solvent was removed in vacuo. The residue was purified by flash column chromatography (silica gel, dichloromethane with 2% methanol) to give nucleoside **10** as a colourless foam (37 mg, 0.107 mmol, 28%); R_f=0.42 (dichloromethane with 10% methanol); ^{1}H NMR (600 MHz, [D$_6$]acetone): δ=1.87 (d, ^4J=1.1 Hz, 3H; CH_3-5), 2.09 (s, 3H; OAc), 2.45 (ddd, ^2J=14.0, ^3J=^3J=7.0 Hz, 1H; H-2′a), 2.56 (ddd, ^3J=3.7, 6.5, ^2J=14.0 Hz, 1H; H-2′b), 3.33 (s, 3H; OMe), 3.51 (d, ^2J=9.9 Hz, 1H; 4′C−CH_2a), 3.53 (d, ^2J=9.9 Hz, 1H; 4′C−CH_2b), 3.84 (m, 2H; H-5′), 4.62 (dd, ^3J=^3J=4.8 Hz, 1H, HO-5′), 5.56 (dd, ^3J=3.7, ^3J=7.0 Hz, 1H, H-3′), 7.0 (dd, ^3J=^3J=6.7 Hz, 1H; H-1′), 8.19 (d, ^4J=1.1 Hz, 1H; H-6), 11.1 ppm (brs, 1H; NH); ^{13}C NMR (150.9 MHz, $CDCl_3$): δ=13.9 (CH_3-5), 21.8 ($OCOCH_3$), 40.6 (C-2′), 60.6 (OMe), 65.2 (C-5′), 74.1 (4′C−CH_2), 74.9 (C-3′), 89.3 (C-1′), 90.4 (C-4′), 117.5 (C-5), 138.4 (C-6), 162 (C-4), 171.2 ($OCOCH_3$), 176.9 ppm (C-2); MALDI-TOF MS (DHB matrix): *m*/*z*: 344.7.0 $[M+H]^+$, 366.7 $[M+Na]^+$.

2-Thio-5′-*O*-(4,4′-dimethoxytrityl)-4′-*C*-methoxymethylene thymidine (11): 4,4′-Dimethoxytritylchloride (33.3 mg, 0.11 mmol) and DMAP (1.2 mg, 10 μmol) were added to a solution of nucleoside **10** (26 mg, 0.0756 mmol) in dry pyridine (1 mL). After the reaction mixture had been stirred at room temperature for 15 h, MeOH (200 μL) was added. The solvent was removed in vacuo and the residue was dissolved in EtOH (0.5 mL), pyridine (0.5 mL) and concentrated NH_4OH (25%, 750 μL). After the reaction mixture had been stirred for 48 h, the solvent was removed and the residue was purified by MPLC (ethyl acetate/petroleum ether 1:3 with 1% NEt_3). Nucleoside **11** was obtained as a white foam (18.3 mg, 0.03 mmol, 40%); R_f=0.41 (ethyl acetate/petroleum ether 1:1 with 1% NEt_3); ^{1}H NMR (600 MHz, [D$_6$]acetone): δ=1.45 (d, ^4J=1.1 Hz, 3H; CH_3-5), 2.32 (ddd, ^2J=13.3, ^3J=^3J=6.5 Hz, 1H; H-2′a), 2.61 (ddd, ^2J=13.3, ^3J=4.5, 6.5 Hz, 1H; H-2′b), 3.30 (s, 3H; OMe), 3.36 (d, ^2J=10.1 Hz, 1H; 4′C−CH_2a), 3.43 (d, ^2J=10.1 Hz, 1H; 4′C−CH_2b), 3.58 (d, ^2J=10.0 Hz, 1H; H-5′a), 3.64 (d, ^2J=10.0 Hz, 1H; H-5′b), 3.80 (s, 6H; ArOMe), 4.37 (d, ^3J=4.7 Hz, 1H; HO-3′), 4.70 (m, 1H; H-3′), 6.92 (m, 4H; Ar), 7.01 (dd, ^3J=^3J=6.5 Hz, 1H; H-1′), 7.30 (m, 1H; Ar H), 7.33–7.38 (m, 6H; Ar H), 7.50 (m, 2H; Ar), 7.82 (d, ^4J=1.1 Hz, 1H; H-6), 11.1 ppm (brs, 1H; NH); ^{13}C NMR (150.9 MHz, $CDCl_3$): δ=12.4 (CH_3-5), 41.9 (C-2′), 55.5 (ArOMe), 59.5 (OMe), 65.9 (C-5′), 72.7(C-3′), 73.7 (4′-C−CH_2), 87.7, 89.6 (C-4′, CAr_3), 90.2 (C-1′), 114.0 (Ar C), 116.6 (C-5), 127.8, 128.8, 129.1, 131.1, 136.8 (Ar C), 136.4, 136.6, 145.8 (Ar C, C-6), 159.7, 159.8 (Ar C), 175.8 ppm (C-2); MALDI-TOF MS (DHB matrix): *m*/*z*: 627.3 $[M+Na]^+$, 643.4 $[M+K]^+$.

General procedure for coupling of 5′-*O*-protected nucleosides to succinylated LCAA-CPG: Compounds **1**, **4**, **11**, 5′-*O*-(4,4′-dimethoxytrityl)-(3-*N*/4-*O*-toluoyl)-2-*S*-thiothymidine and 5′-*O*-(4,4′-dimethoxytrityl)-4-*S*-(2-cyanoethyl)thiothymidine were coupled to succinylated LCAA-CPG by following standard protocols.[32] Briefly, succinylated LCAA-CPG, the respective nucleosides, DMAP (0.1 mmol of each per 1.0 g of CPG) and EDC (1.0 mmol per 1.0 g of CPG) were combined. Pyridine (10 mL per 1.0 g of CPG) and NEt_3 (80 μL per 1.0 g of CPG) were added and the reaction mixture was shaken under argon overnight. Afterwards, 4-nitrophenol (0.5 mmol per 1.0 g of CPG) was added and shaking was continued for an additional 24 h. Piperidine (5 mL per 1.0 g of CPG) was subsequently added and shaking was continued for 5 min. The beads were then filtered off and washed successively with pyridine, methanol and finally dichloromethane. After drying, the beads were suspended in equal amounts of acetic anhydride/pyridine/THF (Cap A) and 1-methylimidazole/THF (Cap B) capping reagents. After being shaken for 2 h, the beads were filtered off and intensively washed as described above. After drying, the loading was determined by trityl analysis of a small portion of the collected beads by known methods (loading range 12.7–44.8 μmol g^{-1}).[32]

Synthesis of modified oligonucleotides: The synthesis of oligonucleotides was carried out on a 0.2 μmol scale on an Applied Biosystems 392 DNA synthesiser with commercially available 2-(cyanoethyl)phosphoramidites. A standard method for 2-(cyanoethyl)phosphoramidites was used, with the exception that the coupling times for the synthesis from the solid-phase bound modified nucleotides were extended to 10 min. Yields for modified oligonucleotides are similar to those obtained for unmodified oligonucleotides. After synthesis (trityl off), the oligonucleotides were cleaved from the support by treatment with concentrated NH_4OH at room temperature for 24 h. For the primer probes bearing ^{4S}T and ^{4S}T^{OMe} at the 3′ end, NaSH hydrate (≈8–10 mg) was added into the concentrated NH_4OH. After removal of NH_4OH, the residue was purified by preparative gel electrophoresis on a 12% polyacrylamide gel containing 8 M urea. The DNA oligonucleotides were recovered by standard precipitation with ethanol in the presence of 0.3 M sodium acetate. The oligonucleotides were purified a second time by using HPLC with 0.1 M triethylammonium acetate buffer (pH 7). After removal of the solvent, the oligonucleotides were dissolved in water and quantified by absorption measurements at 260 nm. The total yields of purified oligonucleotides were in the range of 20–33%. The integrity of all modified oligonucleotides was confirmed by MALDI-TOF (Metabion GmbH) or ESI-MS. The presence of all thio modifications was also verified spectroscopically by their characteristic absorption bands at 335 and 277 nm, respectively, during HPLC purification.

Real-time PCR experiments: Real-time PCR was performed as described by using an iCycler system (BIORAD). In brief, the reactions were performed in an overall volume of 20 μL containing 400 pM of the respective templates, in the appropriate buffer provided by the supplier for Vent (exo$^-$) DNA polymerase (20 mM tris(hydroxymethyl)aminomethane-HCl (Tris-HCl; pH 8.8), 10 mM KCl, 10 mM $(NH_4)SO_4$, 2 mM $MgSO_4$, 0.1% Triton-X100). The final mixtures contained dNTPs (200 μM each of deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), and TTP), primers (0.5 μM each of the respective primer probe and reverse primer), 0.4 units Vent (exo$^-$) DNA polymerase (New England Biolabs; units defined by the supplier) and a 1/50000 aqueous dilution of a SybrGreen I 10000× solution in dimethylsulfoxide (DMSO; Molecular Probes). All PCR amplifications were performed by employing the following program: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 35 s and extension at 72 °C for 40 s. The presented results are from at least three repeated independent measurements of duplicates that originated from one master mix. Identical tem-

plate-target, primer-probe and reverse-primer DNA sequences were used to those employed in earlier studies for comparison. Sequences in the Farber disease[38] context: primer probes: 5′-d(CGT TGG TCC TGA AGG AGG AT*) with T*: T, ^{2S}T, ^{4S}T, T^{OMe}, ^{2S}T^{OMe} or ^{4S}T^{OMe}; reverse primer: 5′-d(CGC GCA GCA CGC GCC GCC GT); target template FarX: 5′-d(CCG TCA GCT GTG CCG TCG CGC AGC ACG CGC CGC CGT GGA CAG AGG ACT GCA GAA AAT CAA CCT *N*TC CTC CTT CAG GAC CAA CGT ACA GAG) where *N*: A, FarA; G, FarG.

DNA thermal-denaturation studies: Melting curves were recorded on a Cary 100 bio UV/Vis instrument with temperature controller. Data were obtained from three individual cooling/heating cycles. Melting temperatures (T_m values in °C) were obtained from the maximum of the first derivative of the melting curves (absorbance at 260 nm versus temperature). Measurements were conducted in the appropriate Vent (exo$^-$) DNA polymerase buffer (ThermoPol, New England BioLabs) without Triton-X100 (20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)SO_4$, 2 mM $MgSO_4$) and contained 4.75 μM duplex DNA. The mixtures were heated to 95 °C for 5 min and slowly cooled to room temperature prior to the melting curve measurements. A measurement of the buffer was conducted separately and subtracted from the spectra resulting from the sample. Template (33-mer): 5′-d(AAA TCA ACC T**A/G**T CCT CCT TCA GGA CCA ACG TAC)-3′; primer probe containing T, ^{2S}T, ^{4S}T, T^{OMe}, ^{2S}T^{OMe} or ^{4S}T^{OMe}: as above.

Circular dichroism spectra: CD spectra were recorded on a Jasco 720 instrument in ThermoPol buffer (New England Biolabs; 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)SO_4$, 2 mM $MgSO_4$, 0.1 % Triton-X100) at room temperature. The samples contained 4.75 μM duplex DNA. The mixtures were heated to 95 °C for 5 min and allowed to cool slowly to room temperature prior to measurements. A spectrum of the buffer was measured separately and subtracted from the spectra resulting from the samples. An average of 12 spectra were recorded in each experiment. The sequences were as described in the previous section.

Single-nucleotide incorporation studies: The reactions contained varying enzyme amounts (0.8–8 nM) and primer (150 nM)–template (300 nM) complex in ThermoPol buffer. Primer–template complexes were annealed and the reaction was initiated by addition of different concentrations of dATP in solution. After incubation for different times at 72 °C, reactions were quenched by addition of two reaction volumes of gel-loading buffer (80 % formamide, 20 mM ethylenediamine tetraacetate (EDTA)) and the product mixtures were analysed by 12 % denaturating PAGE. Incorporation quantities were measured by quantifying the intensity of each band produced by the DNA polymerase by using a Phosphorimager. From this quantification, the amount of incorporated nucleotide was calculated. The intensity of the background was subtracted from each band. The reaction conditions were adjusted for different reactions to allow 20 % or less of primer extension, thereby ensuring single-completed-hit conditions according to published procedures.[40] Steady-state K_M and k_{cat} values were obtained by fitting with the Hanes–Woolf equation. The presented results are from measurements that were repeated independently at least three times.

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