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A Primerless Molecular Diagnostic: phosphorothioated-terminal hairpin formation and self-priming extension (PS-THSP)

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Abstract

There are various ways that priming can occur in nucleic acid amplification reactions. While most reactions rely on a primer to initiate amplification, a mechanism for DNA amplification has been developed in which hairpin sequences at the 3' terminus of a single-stranded oligonucleotide fold on themselves to initiate priming. Unfortunately, this method is less useful for diagnostic applications because the self-folding efficiency is low and only works over a narrow range of reaction temperatures. In order to adapt this strategy for analytical applications we have developed a variant that we term phosphorothioated-terminal hairpin formation and self-priming extension (PS-THSP). In PS-THSP a phosphorothioate (PS) modification is incorporated into the DNA backbone, leading to a reduction in the thermal stability of dsDNA and increased self-folding of terminal hairpins. By optimizing the number of PS linkages that are included in the initial template, we greatly increased self-folding efficiency and the range of reaction temperatures, ultimately achieving a detection limit of 1 pM. This improved method was readily adapted to the detection of single nucleotide polymorphisms and to the detection of non-nucleic acid analytes, such as alkaline phosphatase, which was quantitatively detected at a limit of 0.05 mU/mL, approximately 10-fold better than commercial assays.

Keywords

PS-THSP; self-folding; isothermal amplification; phosphorothioate

Introduction

Molecular diagnostics is at root based on simple and robust molecular amplification reactions. While the polymerase chain reaction (PCR) has been the most extensively employed approach [1, 2], isothermal amplification methods may prove to be more tractable in many applications, including for point-of-care diagnostics, since they avoid the need for precise temperature cycling and complicated devices.

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Conflict of interest

The authors declare that they have no competing of interests.

Isothermal amplification methods span a range of approaches, including (but not limited to) nucleic acid sequence-based amplification (NASBA) [3], single primer isothermal amplification (SPIA) [4], isothermal chimeric primer-initiated amplification (ICAN) [5], strand displacement amplification (SDA) [6, 7], helicase-dependent amplification (HDA) [8], recombinase polymerase amplification (RPA) [9], isothermal target and signaling probe amplification (iTPA) [10], rolling circle amplification (RCA) [11, 12], loop-mediated amplification (LAMP) [13], and SmartAmp [14]. In the main these methods yield sensitive amplification similar to PCR by having mechanisms for strand invasion and / or primer binding that do not require temperature cycling.

In almost all of these reactions, though, the point is generally to amplify a natural template. Another option is to create a defined template that is amenable to amplification, and to detect analytes based on the presence or absence of this template. To this end, we have expanded a technique for the amplification of very short oligonucleotides (28 bp) that have defined hairpin structures at each end, termed terminal hairpin formation and self-priming (THSP) [15]. Like RCA and LAMP, THSP results in linear amplification and long repeated products. Unlike RCA, THSP generates a dsDNA rather than ssDNA product and importantly does not require template circularization. Unlike LAMP, which typically uses 4, 5, or 6 primers [13, 16, 17], depending on the amplicon and detection method, it requires no primers. It is perhaps one of the most simple and self-contained reactions available for molecular diagnostics, and therefore has great promise for developing point-of-care assays.

Unfortunately, the current version of THSP reaction is greatly limited because the self-folding efficiency is relatively low and the range of amplification reaction temperatures is quite narrow. We have now improved the self-folding efficiency and broadened the range of reaction temperatures by incorporating phosphorothioates (PS) into the nucleic acid substrate. Boczkowska and co-workers have carried out thermodynamic studies on the stability of duplexes formed between PS-modified ssDNA (all-Rp, all-Sp, and mixed Rp/Sp) and complementary phosphodiester (PO)-modified ssDNA [18]. They reported that any PS modifications substantially reduced the T_m of PS-PO dsDNA, allowing refolding and self-priming over a broader range of conditions [19]. The improved phosphorothioated-terminal hairpin formation and self-priming extension has been termed PS-THSP, and it represents the first example of applying the broad biochemical principle of phosphorothioate destabilization to strand invasion for isothermal amplification.

By developing design rules for dumbbell templates with phosphorothioate termini for efficient amplification we have now been able to apply PS-THSP to identifying SNPs and even to the sensitive detection of enzymes, such as alkaline phosphatase. Most importantly, though, since PS-THSP enables the facile amplification of short oligonucleotides it encourages very simple assay development via a very short list of reagents (oligonucleotide, polymerase, nucleotides). The PS-modified DNA should also display enhanced stability against degradation by various nucleases that may be present in biological samples (see Electronic Supplementary Material (ESM) Fig. S1) [20, 21].

Materials and methods

Reagents

All chemicals were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. All oligonucleotides were ordered from Integrated DNA Technology (IDT; Coralville, IA) and the sequences are summarized in Table S1. Bst 2.0 WarmStart DNA polymerase, Bst 2.0 DNA polymerase, dNTPs (10 mM each), and Taq DNA ligase were purchased from New England Biolabs (NEB; Ipswich, MA). Alkaline phosphatase (*E. coli* C75) was purchased from Clontech (Mountain View, CA) and EvaGreen dye was obtained from Biotium (Hayward, CA).

Phosphorothioated-terminal hairpin formation and self-priming (PS-THSP)

All basic PS-THSP reactions for end-point detection were performed as detailed here. Reactions were prepared with 1× isothermal amplification buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 50 mM KCl, 2 mM MgSO₄, 0.1% Tween-20, pH 8.8), dNTPs (0.25 mM each), and various concentrations of PS-THSP templates. Reactions were heated to 95 °C for 5 min and cooled at 0.1 °C/s to 37 °C for 5 min. After this, samples were incubated at 4 °C for 2 min, followed by the addition of 4 U of Bst 2.0 WarmStart DNA polymerase to produce a final volume of 20 µl (final concentration: 0.2 U/µl). Each PS-THSP reaction was then initiated at a specific temperature and continued for 1 h (on a T100™ thermal cycler; Bio-Rad; Hercules, CA). To measure fluorescence: 1 µl of 20× EvaGreen dye was added to each sample, incubated for 10 min at room temperature, and then 17 µl of the reaction was loaded on a 384-well plate. Fluorescence intensities were measured with an Infinite 200 PRO (Tecan) (Ex: 488 nm and Em: 532 nm).

Detection of single nucleotide polymorphisms (SNPs) with PS-THSP

Two steps were involved: ligation and PS-THSP. For ligation, 10 µl reactions were prepared with 1× Taq DNA ligase reaction buffer (20 mM Tris-HCl, 25 mM KAc, 10 mM MgAc, 1 mM NAD, 10 mM DTT, 0.1% triton X-100, pH 7.6), 10 nM mixed target (different ratios of wild-type/mutant), 20 nM oligonucleotide substrates, and 10 U of Taq DNA ligase (final concentration: 1 U/µl). The samples were heated at 95 °C for 5 min and cooled to 65 °C, followed by incubation at 65 °C for 20 min. To initiate the PS-THSP reaction 0.5 µl of 10 mM each dNTPs, 4 U of Bst 2.0 WarmStart DNA polymerase, and 9 µl of distilled water were added to the ligated samples and incubated at 65 °C for 1 h (final concentration of the wild-type/mutant mixed target: 5 nM).

Detection of alkaline phosphatase (AP) with PS-THSP

Two steps were involved: dephosphorylation and PS-THSP. For dephosphorylation, 10 µl reactions were prepared with 1× alkaline phosphatase buffer (50 mM Tris-HCl, 1 mM MgCl₂, pH 9.0) and varying concentrations of both alkaline phosphatase and template 1-4P. Reactions were incubated at 55 °C for 1 h. Following dephosphorylation, the PS-THSP reaction was initiated by adding 1 µl of 10× isothermal amplification (200 mM Tris-HCl, 100 mM (NH₄)₂SO₄, 500 mM KCl, 20 mM MgSO₄, 1% Tween-20, pH 8.8), 0.5 µl of each 10 mM dNTP, and 8 µl of distilled water. Next, the samples were heated at 95 °C for 5 min

and cooled at 0.1 °C/s to 37 °C for 5 min. Some 4 U of Bst 2.0 WarmStart DNA polymerase was added on ice to produce a final volume of 20 µl (final concentration: 0.2 U/µl). PS-THSP was carried out at 60 °C for 1 h.

Gel electrophoresis

A 10 µL aliquot of a PS-THSP reaction was mixed with 6× agarose gel loading buffer (NEB; Ipswich, MA) and subjected to electrophoretic analysis on a 2% agarose gel containing ethidium bromide. The amplification products were visualized using a UV transilluminator.

Results and discussion

Scheme for phosphorothioated-terminal hairpin formation and self-priming extension (PS-THSP)

The finding that short, dumbbell like DNA molecules could be amplified into longer concatemers without any additional primers [15] led us consider how the purported amplification mechanism could be engineered to be more efficient. Given that the hairpin D1*-L1*-D1 must refold during each cycle of amplification (Fig. 1), we hypothesized that phosphorothioate (PS) modifications within the hairpin region (D1*-L1-D1) would enable more efficient unfolding of the extended duplex, and hence better enable refolding of the hairpin and more efficient priming. Therefore, PS modifications and different sequences were introduced into a PS-THSP template similar to the one that had previously been published [15]: varying numbers of phosphorothioates were introduced into the hairpin D1*-L1-D1, and both AT-rich palindromic (D1*-L1-D1) and GC-rich (D1* and D1) sequences were examined.

In greater detail, we anticipated that the 3' end of the oligonucleotide (D2*) would be extended by a DNA polymerase, leading to the formation of an extended hairpin (i.e. the seeding state). In this extended hairpin the phosphorothioate-containing D1*-L1-D1 would form a duplex with its complementary strand (D1*-L1*-D1), but the D1*-L1*-D1 strand could potentially more readily dissociate and fold back on itself, leading to the next extension. This extension would read through both D2*-L2-D2 and D1-L1-D1*, thus placing D1*-L1-D1 and D1*-L1*-D1 sequences at each end of the molecule. Additional cycles of foldback and extension would result in concatemerization of the sequences D2-L2-D2* proximal to D1*-L1-D1 (with a short AA sequence in between). Over multiple cycles of extension and refolding, concatemeric dsDNA grows such that there is a repeating unit of D1*-(L1 or L1*)-D1-D2-(L2 or L2*)-D2* flanked by a D1*-L1-D1 (PS)/D1*-L1*-D1 (PO) heteroduplex at the base of the ever elongating hairpin. As with many isothermal amplification schemes, amplification of the concatemer is linear, rather than exponential.

Introduction of phosphorothioate modifications to improve THSP

Kato and co-workers previously found that the optimum temperature for the replication of THSP templates was generally quite narrow (within 1-2 °C of an optimum temperature). This limitation can be explained by examining the individual steps of the THSP mechanism. First, the D1*-L1*-D1 strand from the extended, duplex hairpin dissociates. The dissociated single strand then folds into the D1*-L1*-D1 hairpin and extension is initiated. The

competition between hybridization with the opposing strand (to form a duplex) and self-folding (to form a hairpin) relies on interactions that are very similar, and this of necessity means that there is only a narrow temperature range that distinguishes the two structures. In order to make the T_m difference between the extended hairpin (seeding state) and the folded hairpin (folded state) greater we introduced a chemical distinction between the two strands by incorporating phosphorothioates at the 5' end of the template, since it has been reported that the T_m of duplexes formed between PS-modified ssDNA (all-Rp, all-Sp and mixed Rp/Sp) and complementary phosphodiester (PO)-modified ssDNA is decreased compared with PO-DNA/PO-DNA duplexes [18]. The T_m values of PO-DNA/PO-DNA duplexes could be reduced by up to 9 °C by introducing PS modifications into one strand of a stem. Thus, if PS modifications are introduced into the upper strand (D1*-L1-D1) of the extended hairpin structure, the T_m of extended hairpin structure should be greatly reduced, while the T_m of D1*-L1*-D1 hairpin structure for self-folding will not change.

We designed THSP amplicons to test whether PS modifications would improve overall amplification. Previous work [15] emphasized that polymerization efficiency for THSP depended on the relative stabilities of the hairpins at both ends of the dumbbell-shaped template. The hairpin at 3' end should be stable in order to efficiently induce an initial extension, and we therefore included a stable hairpin (D2-L2-D2*) with a 8 base-pair stem (50% GC content). The hairpin at the 5' end (D1*-L1-D1) (Table S1) should be less stable, and was therefore designed to be the same as the best AT-rich palindromic sequence previously reported [15].

We then modified the 5' end (D1*-L1-D1) with different numbers of PS modifications (0, 5, 10 and 20). In order to confirm whether the PS modifications affected T_m of the extended hairpin, melting temperatures of the extended templates with different numbers of PS modifications were measured (ESM Fig. S2). As expected, the melting temperatures decreased from 70.5 °C to 65.5 °C as the number of PS modifications increased from 0 to 20. Because the 5' end serves as a competitor for extension in every round of concatemer formation, the same initial modifications impact the overall amplification.

PS-THSP reactions were then carried out at different temperatures from 54 °C to 72 °C. Unlike previous studies that used Vent (exo-) DNA polymerase, Bst 2.0 WarmStart DNA polymerase was used to allow more efficient amplification at lower temperatures. For detection, end-point fluorescence intensities were recorded after incubation with an EvaGreen intercalating dye. As expected, relatively low amplification with a narrow range of reaction temperatures from 66 °C to 70 °C was observed for the template 1-1 without PS modifications (Fig. 2a). In contrast, 5 or more PS modifications (template 1-2, template 1-3, and template 1-4) promoted the transient formation of the D1*-L1*-D1 hairpins and led to robust and efficient amplification. Moreover, the optimum temperature gradually shifted from 68 °C without phosphorothioate modification to 60 °C with 20 modifications (Fig. 2a), and as the number of PS modifications increased, the temperature range of amplification became broader. While some background fluorescence was observed, these signals resulted from binding of intercalating dyes to the aptamer that is the basis of the Bst 2.0 WarmStart DNA polymerase (ESM Fig. S3). The fact that the optimum temperature for amplification with 20 modifications (60 °C) was very near the calculated T_m for the folded hairpin

(60.7 °C) (ESM Fig. S2) suggests that the extensive introduction of phosphorothioates almost completely destabilized the end of the extended hairpin relative to the transient hairpin.

The effects of PS modification on amplification were further probed by gel-electrophoresis (Fig. 2b). No bands were observed for 0 and 5 phosphorothioate modifications, but concatemers were seen with 10PS of 20PS large size of bands were observed. This is consistent with amplification reported by intercalating dyes.

Clearly sequence can also impact the efficiency of amplification. However, because the difference in T_m between the termini of an extended duplex and the hairpin will always be similar, irrespective of sequence, it was anticipated that PS modifications should have a greater impact on amplification than changing sequence. To demonstrate the greater impact of PS modifications, a PS-THSP template was designed so that the 5' hairpin was more G:C rich (template 2). Since G:C base-pairs are less vulnerable to breathing than A:T base-pairs [22], this template amplified less well than template 1; there was almost no amplification (ESM Fig. S4). However, by introducing PS modifications, amplification signals appeared and a broadened range of reaction temperatures was observed (Fig. 3). Even though adopting an optimal A:T rich sequence can improve amplification, PS modifications remarkably improved amplification efficiency irrespective of sequence.

Quantitative measurement of PS-THSP

The amplification afforded by PS-THSP can be quantitated. The concentrations of template 1-4 were varied from 0 to 2 nM and end-point amplification was again monitored using the intercalating dye (EvaGreen). As little as 1 pM of template could be reliably detected (Fig. 4). There was an approximately linear relationship of template concentration to amplification product from 1 to 100 pM of input template (Fig. 4a and b).

This quantitative relationship suggested that it might be possible to carry out real-time monitoring of PS-THSP. We tested two different intercalating dyes, EvaGreen and SYTO 82, for their ability to detect amplification products. When EvaGreen was included in the amplification reaction the end-point fluorescence intensities at various temperatures from 54 °C to 72 °C were greatly reduced (ESM Fig. S5a). In addition, the optimum temperature for amplification increased from 60 °C to 68 °C, suggesting that the intercalating dye (EvaGreen) might have increased the melting temperature of the extended hairpin and therefore decreased the efficiency of self-folding. On the other hand, the SYTO 82 dye had less of an effect on T_m and amplification efficiency (ESM Fig. S5b). Therefore, the PS-THSP reaction could be monitored in real-time in the presence of SYTO 82, as shown in ESM Fig. S5c.

Detection of single nucleotide polymorphisms using PS-THSP

The advantages of PS-THSP are that it utilizes a very small substrate, the amplification reaction is extremely straightforward, and the accumulation of double-stranded concatemers can be readily monitored. These advantages may prove useful for the identification of genomic polymorphisms (SNPs) in either DNA or RNA samples. Template-directed ligation reactions have previously been used to detect SNPs, and ligated amplicons can potentially be

amplified in a variety of ways [12, 23–27]. Figure 5a shows our scheme for SNP detection using the PS-THSP reaction. The PS-THSP reaction occurs only after SNP-specific ligation.

As an example, we designed a split PS-THSP template and a complementary DNA ligation splint for a SNP in the nitric oxide synthase 1 adaptor protein gene (NOS1AP, rs12122048, A to G). Ligation substrate 1 has a PS-THSP domain 1 at its 5' end and a 16 complementary nucleotides (including the SNP recognition site) at its 3' end. Ligation substrate 2 has 20 complementary residues and a phosphate group for ligation at its 5' end and a PS-THSP domain 2 at its 3' end. The PS-THSP domain sequences (domain 1 and domain 2) of the ligation substrates had no internal complementarity to the target-binding domains (i.e. there were no predicted alternative stable secondary structures).

We assessed the specificity and sensitivity of our PS-THSP-mediated SNP detection method by preparing mixtures of mutant and wild-type templates (mutant:wild-type mole ratios of 0:5000, 5:4995, 50:4950, 500:4500 and 5000:0; constant total nucleic acid concentration was 5 nM). The PS-THSP substrates were incubated with Taq DNA ligase [28, 29] in the presence of the different template mixtures. Ligation and PS-THSP-based amplification were only seen when the mutant template was present. The presence of PS-THSP products was confirmed using gel electrophoresis as shown in ESM Fig. S6. The fluorescence intensity increased as the mole fraction of mutant template increased (Fig. 5b). Even a small change in the ratio (from 0:5000 to 5:4995) significantly increased fluorescence levels (two sample t-test p-value = 0.008). The PS-THSP/SNP detection method showed a limit of detection of 5 pM, which is about 20 times better than previously reported methods that relied on ligation and rolling circle amplification [30]. The impact of PS modifications for SNP detection was assessed using template 1 ligation substrates with and without PS modifications. As shown in ESM Fig. S7, in the absence of PS modifications (0PS) the signal increased very little, while in the presence of PS modifications (20PS) a substantial signal increase was observed, indicating that the PS modifications improved self-folding efficiency. The ligation method for SNP detection should be completely generalizable, since new target-specific domains can be easily designed and the same high efficiency PS-THSP domain sequences can be appended to these target-specific domains. Of course, the PS-THSP sequences of the ligation substrates should be carefully designed not to have internal complementarity with the target-binding domains.

Detection of alkaline phosphatase activity using PS-THSP

The same advantages of PS-THSP that proved useful for SNP detection (small substrate, simple amplification protocol, ready detection) will potentially also be useful for assays other than nucleic acid detection. However, this requires transduction mechanisms beyond binding and ligation to form the small PS-THSP amplicon. In this regard, we attempted to demonstrate PS-THSP as a detection method for alkaline phosphatase (AP). We designed and synthesized the substrate template 1-4P (see Table S1) with a phosphate group at its 3' terminus. As shown in Fig. 6a, in the absence of AP phosphorylated DNA cannot be extended by Bst 2.0 WarmStart DNA polymerase, but in the presence of AP a free 3' hydroxyl should be produced for extension and the PS-THSP reaction will be initiated, generating long concatenated dsDNA products. To confirm this, we prepared four samples:

template 1-4 (with a 3'-OH), template 1-4P (with a 3'-phosphate), each with and without AP (Fig. 6b). Template 1-4 (with a 3'-OH) was amplified into long concatemers with or without AP. Template 1-4P (with a 3'-phosphate) was amplified only in the presence of AP.

To quantify AP activity, four different concentrations of AP (0, 0.05, 0.5 and 5 mU/mL) were incubated with a 10 nM sample of template 1-4P. Again, PS-THSP reaction products were confirmed by gel electrophoresis (ESM Fig. S8). The limit of detection for AP was found to be 0.05 mU/mL, about 10 times better than current commercial assays.

Conclusion

In summary, primerless, isothermal amplification can now be robustly adapted to molecular diagnostic assays. We have greatly improved the initial terminal hairpin formation and self-priming extension (THSP) reaction by exploiting the biophysical observation that phosphorothioates destabilize duplexes. Terminal phosphorothioates enables the repetitive refolding of self-priming amplicons over a broad range of reaction temperatures from 54 °C to 66 °C (as opposed to the need for optimization within only a few degrees for a given THSP reaction). This is the first demonstration of the use of phosphorothioate destabilization for amplification reactions, and just as temperature (PCR, LAMP) and enzymes (helicases, recombinases) have been exploited for strand invasion, phosphorothioate inclusion may become a standard means of developing new amplification and detection mechanisms. The improved PS-THSP has a limit of detection around 1 pM. By coupling PS-THSP with template-directed ligation SNPs could be readily detected. Upon coupling with 3' end generation, PS-THSP could be used to detect enzyme activity. The latter strategy may prove to be generalizable, leading to the detection of specific nucleases that generate a given 3' end or of other enzymes, such as esterases, that act on synthetic, blocked substrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Biographies

Cheulhee Jung is currently a post-doctor in Prof. Andrew Ellington's laboratory of the University of Texas at Austin, USA. His major interests are in biological applications of enzyme-based and enzyme-free (catalytic nucleic acid circuits) isothermal amplification.

Andrew Ellington is the Fraser Professor of Biochemistry at the University of Texas at Austin. He is perhaps best known for the invention of the word 'aptamer,' but is now most involved in the development and evolution of artificial life, including nucleic acid operating systems that can function both in vitro and in vivo.

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Fig. 1.
Scheme of phosphorothioated-terminal hairpin formation and self-priming extension (PS-THSP).

Fig. 2.

Effects of phosphorothioate (PS) modification and temperature on PS-THSP. PS-THSP templates (100 pM) with different numbers of PS modifications (template 1-1: 0PS; template 1-2: 5PS; template 1-3: 10PS; template 1-4: 20PS) were amplified at different temperatures (from 54 °C to 72 °C) for 1 h. **(a)** Fluorescence intensities reflect the effects of varying PS modifications and temperatures on amplification of template 1. Optimal reaction temperatures are noted above the bars. **(b)** Visualizing PS-THSP reactions. The PS-THSP reactions were analysed after 1 hour at 60 °C by gel electrophoresis (M1: 50 bp DNA ladder; lane 1: template 1-1; lane 2: template 1-2; lane 3: template 1-3; lane 4: template 1-4; M2: 1 kb DNA ladder).

Fig. 3.

(a) Fluorescence ratios and (b) temperature ranges for template 1 and 2. AT-rich palindromic template 1 and GC-rich non-palindromic template 2 with different numbers of PS modifications underwent PS-THSP at 60 °C and 64 °C, respectively, for 1 h. (a) Fluorescence ratios for template 1 and template 2 were separately calculated by dividing the fluorescence intensity observed in the reaction with each PS template by the fluorescence intensity observed in the reaction with each template without PS modifications. (b) The temperature range is the range over which the amplification reaction still works. The signal produced by the reaction was determined between 54 °C to 72 °C. The maximal signal was identified, and any temperature at which 0.7 of the maximum signal was observed and where the signal was over at least 3,000 (a.u.) was deemed to be functional.

Three experiments	0 pM	1 pM	2 pM	5 pM	10 pM	20 pM	50 pM	100 pM	200 pM	500 pM	1000 pM	2000 pM
Average intensity	1408	1877	2095	2389	3505	4853	7522	12102	14441	16459	17336	17174
Standard deviation	131.2	28.1	128.5	91.9	164.3	186.8	365.6	562.7	1042.5	813.7	753.1	502.8

Fig. 4.

Standard curve for template 1-4 amplification. Different concentrations of template 1-4 (0, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 pM) underwent PS-THSP at 60 °C for 1 h.

(a) Fluorescence measured for specific template 1-4 concentrations ranging from 0 to 2000 pM. (b) Linear dependence of fluorescence intensities for template 1-4 concentrations ranging from 1 to 100 pM. This experiment was repeated three times in order to obtain the average and standard deviation fluorescence values for each template 1-4 concentration.

Fig. 5. Detection of single nucleotide polymorphisms (SNPs) using PS-THSP. **(a)** Scheme for SNP detection. **(b)** Quantitating SNP detection. The specificity and sensitivity of the SNP detection method were tested with 5000 pM (5 nM) mixed wild-type and SNP templates, and 10 nM of each ligation substrate. This experiment was repeated three times in order to obtain the average and standard deviation fluorescence values for each ratio. Based on these values, the limit of detection was estimated at about 5 pM.

Fig. 6.

Detection of alkaline phosphatase (AP) activity using PS-THSP. **(a)** Scheme for AP detection. **(b)** Trial assays. The AP detection method was initially tested with 100 nM PS-THSP template (Lane 1: template 1-4 without AP; Lane 2: template 1-4 with AP (10 mU/mL); Lane 3: template 1-4P without AP; Lane 4: template 1-4P with AP (10 mU/mL)). **(c)** Sensitivity of AP detection. Different concentrations of AP (0, 0.05, 0.5, and 5 mU/mL) were added to dephosphorylate the 3' end of template 1-4P (10 nM) before PS-THSP reactions were performed. This experiment was repeated three times in order to obtain the average and standard deviation fluorescence values for each AP concentration.