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Mismatch Extension of DNA Polymerases and High-Accuracy Single Nucleotide Polymorphism Diagnostics by Gold Nanoparticle-Improved Isothermal Amplification

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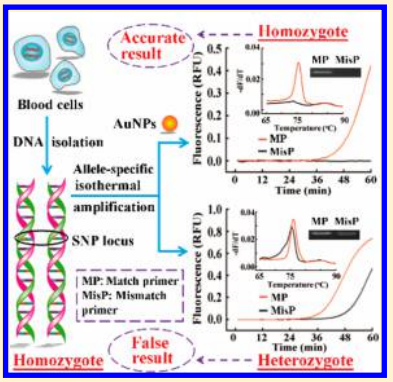
Supporting Information

ABSTRACT: Sequence mismatches may induce nonspecific extension reaction, causing false results for SNP diagnostics. Herein, we systematically investigated the impact of various 3′-terminal mismatches on isothermal amplification catalyzed by representative DNA polymerases. Despite their diverse efficiencies depending on types of mismatch and kinds of DNA polymerase, all 12 kinds of single 3′-terminal mismatches induced the extension reaction. Generally, only several mismatches (primer-template, C-C, G-A, A-G, and A-A) present an observable inhibitory effect on the amplification reaction, whereas other mismatches trigger amplified signals as high as those of Watson-Crick pairs. The related mechanism was deeply discussed, and a primer-design guideline for specific SNP analysis was summarized. Furthermore, we found that the addition of appropriate gold nanoparticles (AuNPs) can significantly inhibit mismatch extension and enhance the amplification specificity. Also the high-accuracy SNP analysis of human blood genomic DNA has been demonstrated by AuNPs-improved isothermal amplification, the result of which was verified by sequencing (the gold standard method for SNP assay). Collectively, this work provides mechanistic insight into mismatch behavior and achieves accurate SNP diagnostics, holding great potential for the application in molecular diagnostics and personalized medicine.



During the past 2 decades, isothermal nucleic acid amplification has been demonstrated as a very promising alternative to polymerase chain reaction (PCR).[1,2] These techniques such as helicase-dependent amplification (HDA) achieve fast and efficient signal amplification at a constant temperature without the precise thermal cycling process and greatly improve assay sensitivity even comparable with that of PCR.[3−6] Ascribed to these merits, they have been successfully employed for the detection of nucleic acids[7−10] and single nucleotide polymorphisms (SNPs),[11,12] showing great potential in biological research and diseases diagnostics.

Despite high sensitivity, amplification specificity is also critical for accurate analysis. For SNP genotyping,[12−18] effective differentiation between wild-type allele and mutant-type allele is challenged by mismatch extension. In 2007, Hayashizaki and co-workers achieved specific SNP detection of human genomic DNA.[12] In their method, a unique mismatch-binding protein was used to suppress the mismatch-induced amplification. Unfortunately, systematic investigation about the impact of all kinds of 3′-terminal mismatches on isothermal amplification has not been reported, and the underlying mechanism of mismatch extension is still unknown. Additionally, simple mismatch-suppression technologies are also urgently required as alternative tools. Recently, we and co-workers found that gold nanoparticle (AuNP) was contributive to the specific analysis of long-range haplotypes based on PCR-sequencing.[19] Inspired by the study, we speculate that AuNPs may well improve the specificity of isothermal amplification by inhibiting the mismatch extension.

Herein, we systematically evaluated the amplification efficiency of all 12 kinds of single 3′-terminal mismatches by representative DNA polymerases (Bst 2.0 DNA polymerase, Klenow Fragment exo$^-$, and Vent exo$^-$ DNA polymerase) in isothermal amplification. Also, the effect of mismatch position and number of mismatched bases were evaluated. We found that the mismatch amplification efficiency depended on not only types of mismatch but also kinds of DNA polymerase. Except several kinds (primer-template, C-C, A-G, G-A, and A-A), most can be efficiently extended and trigger comparable amplified signal with those of Watson-Crick pairs. Moreover, AuNPs was found to be helpful for the suppression of mismatch amplification. The potential mechanisms of these finding were discussed and summarized with a useful primer-

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design guideline for specific amplification. Also the highly accurate SNP assay of human blood genomic DNA is demonstrated by AuNPs-improved HDA without mismatch interference, results of which are in good agreement with those by sequencing. The corresponding illustration is presented in Figure 1.

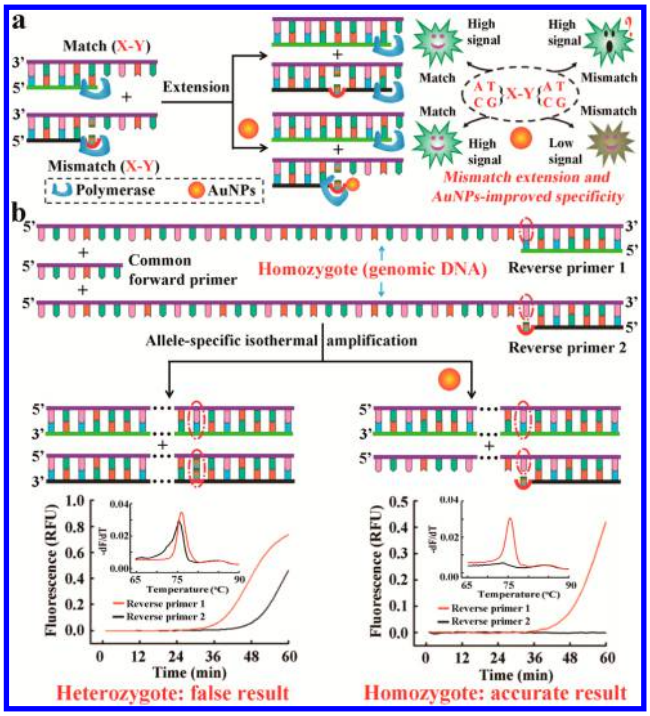

Figure 1. (a) Schematic illustration of mismatch extension reaction and AuNPs-improved specific reaction. "X-Y" represents the various 3′ terminal base pairs of primer-template duplex (X and Y can be base A, T, C, or G). The blue "right-hand" shape indicates the tertiary structure of polymerase. The green star and gray star each represent high and low fluorescence signal. (b) Schematic illustration of high-accuracy SNP diagnostics by AuNPs-improved allele-specific isothermal amplification. The red dotted circle indicates the SNP locus. Reverse primer 1 and Reverse primer 2 are each designed for wild-type allele or mutant-type allele. They are used for the exponential amplification of human genomic DNA with a common forward primer, respectively. A sequencing-confirmed homozygote sample is tested as homozygote by AuNPs-improved method (right, accurate result), whereas it is interpreted as a heterozygote in the absence of AuNPs due to mismatch amplification (left, false result).

EXPERIMENTAL SECTION

Materials and Reagents. Bst 2.0 DNA polymerase, Vent exo$^-$ DNA polymerase, Klenow Fragment exo$^-$, Nt.BbvCI and the corresponding buffers, and IsoAmp II Universal tHDA kit were obtained from New England Biolabs Ltd. (Beijing, China). Gold nanoparticles (AuNPs, 5 nm) were purchased from Sigma-Aldrich (St. Louis, MO). DNA marker was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Blood genomic DNA was isolated using the TIANamp Genomic DNA kit (Tiangen Biotech, Beijing, China), and the SNP genotyping was confirmed by sequencing (Shanghai Generay Biotech Co., Ltd.). All oligonucleotides were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China). The sequences are listed in Table S1.

Gel Electrophoresis Analysis. Extension reaction was carried out in 20 μL of 1× NEBuffer 4 containing 350 nM primer, 350 nM template, 0.25 units of Bst 2.0 DNA polymerase, and 0.2 mM dNTPs. After incubation of 30 min at 55 °C, the mixture was heated up to 80 °C for 20 min. Products of the extension reaction were analyzed by 3.5% agarose gel electrophoresis. The gel was imaged by the Syngene G:BOX Imaging System (Syngene System, Cambridge, U.K.).

Strand Displacement Amplification. For the real-time analysis using SYBR Green I as the fluorescence reporter, the reaction was performed in 20 μL of 1× NEBuffer 4 containing 5 nM primer, 150 nM template, 0.25× SYBR Green I, 0.25 units of Bst 2.0 DNA polymerase, 2.0 units of Nt.BbvCI, and 0.2 mM dNTPs. To utilize the molecular beacon (MB), the mixture contained 50 nM primer, 50 nM template, 250 nM MB, 0.25 units of Bst 2.0 DNA polymerase, 2.0 units of Nt.BbvCI, and 0.2 mM dNTPs in 20 μL of 1× NEBuffer 4. LightCycler 96 (Roche Applied Science, Mannheim, Germany) was used to record real-time fluorescence signal in this work.

Effect of AuNPs on Mismatch Amplification. To investigate the effect of AuNPs, the mixture contained 50 nM primer, 50 nM template, 250 nM MB, 0.25 units of Bst 2.0 DNA polymerase, 2.0 units of Nt.BbvCI, and 0.2 mM dNTPs with different AuNPs concentrations in 20 μL of 1× NEBuffer 4. It was incubated at 55 °C for 30 min before the measurement of fluorescence.

SNP Assay of Human Blood Genomic DNA by HDA. First, synthetic templates (Template Wt and Mt) were used as homozygous wild-type and mutant-type samples to evaluate the feasibility of highly accurate SNP analysis. Generally, the 25 μL reaction mixture contains 0.75 μL of IsoAmp Enzyme Mix, 1.7 μL of IsoAmp dNTP Solution, 2.5 μL of 10× annealing buffer II, 1 μL of 100 mM $MgSO_4$, 2 μL of 500 mM NaCl, 1 μL of 5× SYBR Green I, 1 μL of 5 μM FP primer, and 1 μL of 5 μM each RP primer in the absence or presence of 1 μL of 10 nM template. For the AuNPs-improved HDA, 3 μL of 90 nM AuNPs is added. The mixture is performed at 55 °C in LightCycler 96 for real-time fluorescence monitoring. Then, peripheral blood DNA of the candidates was tested by AuNPs-improved HDA to verify the clinical utility. Genomic DNA (150 ng) is first mixed with two primers (FP and RP), followed by a degeneration and annealing process (5 min at 95 °C and 10 min at 60 °C). Subsequently, other components are added and the whole reaction mixture is incubated at 65 °C in LightCycler 96 for real-time fluorescence monitoring. After that, these samples are consecutively analyzed and confirmed by melting curve and gel electrophoresis. The parameters for gel electrophoresis are mentioned above.

RESULTS AND DISCUSSION

Primer extension reaction was first carried out to investigate the mismatch behaviors. As depicted in Figure 2a, full-length extension products (46 bp) were obviously observed not only in Lane A-T (A-T Watson-Crick pair) but also in Lane A-A, A-C, and A-G (three mismatches). Then, the influence of these mismatches on strand displacement amplification (SDA), one representative isothermal amplification, was real-time monitored by using SYBR Green I as a reporter. All of them induced increased signals as the assay time prolongs (Figure S1). These results confirmed the mismatch extension and subsequent amplification catalyzed by Bst 2.0 DNA polymerase.

To more clearly present the differential impact of various 3′ mismatches on SDA (Figure S2), the molecular beacon (MB)

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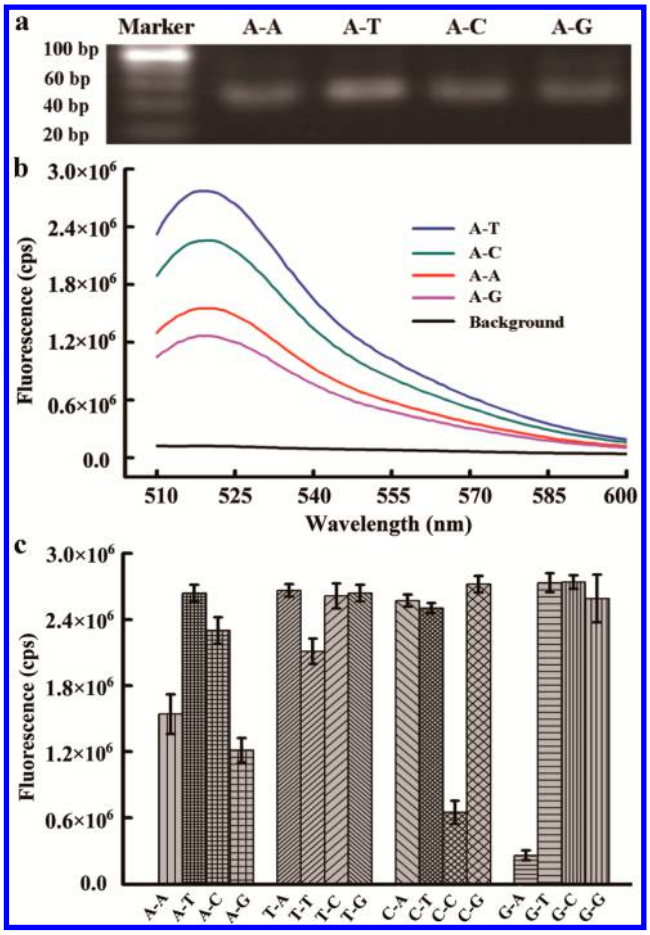

Figure 2. Analysis results of mismatch-induced extension and amplification. (a) Gel electrophoresis analysis of mismatch extension reaction by Bst 2.0 DNA polymerase. (b) Fluorescence spectra of mismatch-induced SDA using MB as reporter. Background indicates the blank experiment without primers. (c) Fluorescence response of all single 3′-end mismatches on Bst 2.0 DNA polymerase-catalyzed SDA.

was employed as a signal reporter owing to its higher signal-to-noise ratio than SYBR Green I. We found that A-C triggered a amplified signal almost as high as that of A-T, whereas the fluorescence intensities of A-A and A-G were much lower (Figure 2b). The kinetic analysis and corresponding melting curve also confirmed the consistent result (Figure S3). Also the position of the mismatched base showed no apparent impact on the amplified signal (Figure S4 and Table S5). However, increasing the number of mismatched base near 3′-end was found to dramatically inhibit the amplification reaction (Figure S5 and Table S5). For a comprehensive understanding, the effects of other single 3′-terminal mismatches were examined under the same conditions (Figure 2c). Detailed relative amplification efficiency (RAE) values of single 3′-terminal mismatches to a corresponding Watson-Crick pair were presented in Table S2. It can be seen that the values of all transition mismatches (A-C, C-A, G-T, and T-G) are higher than 87%, whereas those of transversion mismatches are at different levels.

Transition mismatches-induced efficient amplification is probably ascribed to their stable structure similar to those of Watson-Crick pairs.[20] Several crystallographic and thermodynamic studies have verified that G-T and A-C can form wobble pairs with two hydrogen bonds in diverse contexts, stability of which are comparable with an A-T perfect pair.[21−23] A similar mechanism may be used to explain the effective amplification of three pyrimidine-pyrimidine mismatches (T-T, C-T, and T-C).[24,25] The exception of another pyrimidine-pyrimidine mismatch C−C perhaps results from its relatively weak stability[26] and the disruption of the DNA polymerase active site.[27] It is well-known that the tertiary structures of DNA polymerases share a common "right-hand" shape with three featured subdomains including palm, thumb, and fingers (Figure 1a).[28] The polymerization catalytic site is located in the center of the palm subdomain. The primer-template duplex lies across the palm and is held by the thumb and fingers. The interactions between mismatches and polymerases may disrupt the active site of the polymerization reaction.[27] Such an explanation is potentially suitable for the inhibition of amplification by most purine-purine mismatches (A-G, G-A, and A-A) despite their hydrogen bonds (one in A-A, two in G-A and A-G).[21,25] The asymmetric impact of A-G and G-A may be caused by their different conformations in the active site.[27] Conversely, the efficient extension of another purine-purine mismatch, G-G, is probably owing to the stacking forces of the G residue with the penultimate base and the hydrogen bonded structure.[26,29] Overall, the variety of mismatch extension ability can be attributed to the consequence of numerous complex interactions (hydrogen bonding, base stacking, steric structures of enzyme and bases, etc.) rather than a single factor. It also depends on the kind of DNA polymerase. As shown in Figure 3a and Table S3, Klenow Fragment exo$^-$ can recognize a C-C mismatch to induce a high amplification signal, whereas the remarkable inhibition by C-C mismatch was observed in Vent exo$^-$ DNA polymerase (Figure 3b and Table S4).

The unexpected mismatch extension and followed amplification challenge isothermal amplification-based SNPs analysis.[12,30] Ascribed to the unique physical and chemical properties,[31,32] AuNPs have been widely used in biosensors.[33,34] Especially, AuNPs show selective binding to single-stranded DNA (e.g., primer) rather than double-stranded DNA (e.g., primer-template duplex).[33,35] Encouraged by these, we and co-workers have recently demonstrated the specific analysis of long-range haplotypes by PCR-sequencing utilizing AuNPs.[19] In this work, we hypothesize that AuNPs can also effectively inhibit the mismatch-induced isothermal amplification. As shown in Figure S7 and Table S6, amplified signal of all the mismatches were significantly reduced by the addition of appropriate AuNPs concentrations. In contrast, the fully correct primer-template pairs were minimally affected. The RAE values of mismatched pairs were effectively decreased with the increase of AuNPs concentration (Figure 4). Though the mechanism is not yet fully understood, we speculate that this specificity enhancement effect is attributed to the different binding affinities of AuNPs to mismatches and perfect pairs. This property resembles that of the single-stranded binding protein which largely minimizes primer-template mispairs during DNA replication *in vivo*.[36] It is worthy to point out that Tian et al.[6] recently demonstrated the AuNP-mediated inhibition of nonspecific exponential amplification caused by DNA-independent synthesis instead of 3′-terminal mismatches.

According to the diversiform RAE values, the mismatch primer could also be designed for specific SNP analysis besides using AuNPs. For example, to excellently distinguish T (mutant type) from C (wild type) in the C → T transition (reflecting the deamination of methylated cytosine residues *in vivo*), a primer with C rather than A or another base (T and G) at the

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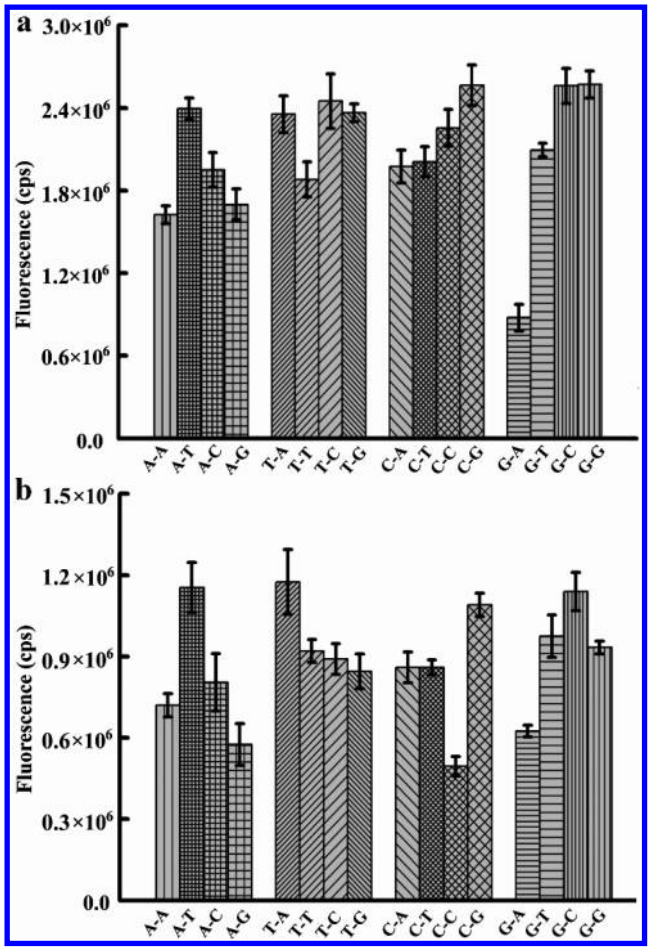

Figure 3. Fluorescence response of all single 3′-end mismatches on SDA catalyzed by Klenow Fragment exo⁻ DNA polymerase (a) and (b) Vent exo⁻ DNA polymerase. All the mismatches induce high amplification signals by both two DNA polymerases despite their diverse intensity values.

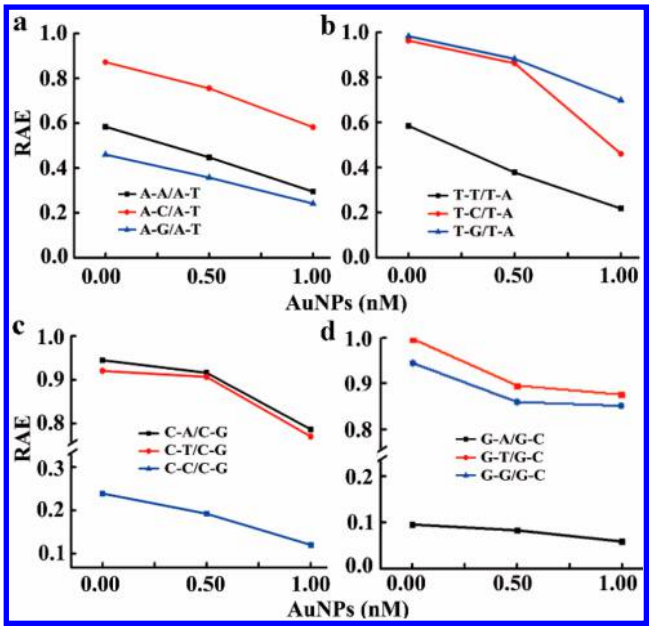

Figure 4. Effect of different AuNPs concentrations on mismatch-induced SDA catalyzed by Bst 2.0 DNA polymerase. MB is used as the reporter here.

3′-terminal should be used, owing to the most remarkable RAE difference between C-T and C-C. Generally, it should choose G-A, C-C, A-G, or A-A mismatch to inhibit the amplification of nontarget sequence and meanwhile utilize perfect matches or other mismatches to effectively amplify target template. Summarizing all of the possible mismatches, Table 1 shows

Table 1. Primer-Design Guideline and Prognosis for Specific SNP Analysis[a]

SNPs	prognosis	SNPs	prognosis
C → T	C-C, C-T (E)	T → C	T-T, T-C (P)
A → G	G-A, G-G (E)	G → A	A-G, A-A (P)
C → A	C-C, C-A (E)	A → C	G-A, G-C (E)
C → G	C-C, C-G (E)	G → C	A-G, A-C (G)
A → T	G-A, G-T (E)	T → A	T-T, T-A (P)
G → T	A-G, A-T (G)	T → G	T-T, T-G (P)

[a]Generally, SNPs can be mainly classified as either transitions (C → T or G → A) or transversions (C → G, A → T, C → A, or T → G) according to nucleotide substitution. C → T represents C to T mutation. Excellent (E), good (G), and poor (P) indicate the different discrimination levels of the target from interference. In C-T, C is the 3′-terminal base of the primer. Bst 2.0 DNA polymerase is used here.

the primer-design guideline for specific amplification with prognosis as a "rule of thumb". Despite these, a poor discrimination level between the mutant type and the wild type are still observed in some kinds of SNPs. To address the problem, an additional mismatch combined with the 3′-terminal mismatch should be adopted. We have demonstrated that a single mismatch of 1, 3, or 5 bases from the 3′-terminal of the primer shows no inhibition on the amplification reaction (Figure S4), while increasing the number of mismatched bases near the 3′-end leads to a significant inhibitory effect (Figure S5). Therefore, if necessary, the intentional introduction of a second mismatch can provide an excellent differentiation level. It is notable that viruses such as HIV replicate fast with low fidelity, leading to varied genomes among different patients.[37,38] Ineffective mismatch extension (e.g., C-C) may cause false negative results for virus detection. To maximize the detection of variant sequences, primer with T at the 3′-terminal is the best choice due to its efficient mismatch extension.

To demonstrate the high-accuracy SNP diagnostics by specific isothermal amplification using the aforementioned mismatch guideline and AuNPs-based methods, the SNP (G681A) of the CYP2C19 gene (CYP2C19*2), which is involved in metabolism of xenobiotics, was detected as the model analyte in the HDA-based sensing system (Figure 5a). As expected, the mismatch amplification in both homozygous wild-type and homozygous mutant-type (using synthetic templates) were observed (Figures S8a and S9). Fortunately, these nonspecific amplification were completely suppressed by mismatch primers designed according to the guideline in Table 1 (Figures S8b and S10). A similar result was also obtained by the addition of AuNPs (Figures S8c and S11) without the design of mismatch primers. Another SNP type (G → T) was also tested to validate the conclusions in Table 1 as a universal guideline despite the changing of the base pairs adjacent to the mutation site (Figure S12). We also evaluated the sensitivity and accuracy of the AuNPs-improved HDA for SNPs. Down to 4 pM, mutant DNA was detected (Figure S13A), and as low as 5% mutant DNA could be tested in mutant DNA and wild DNA mixtures (Figure S13B).

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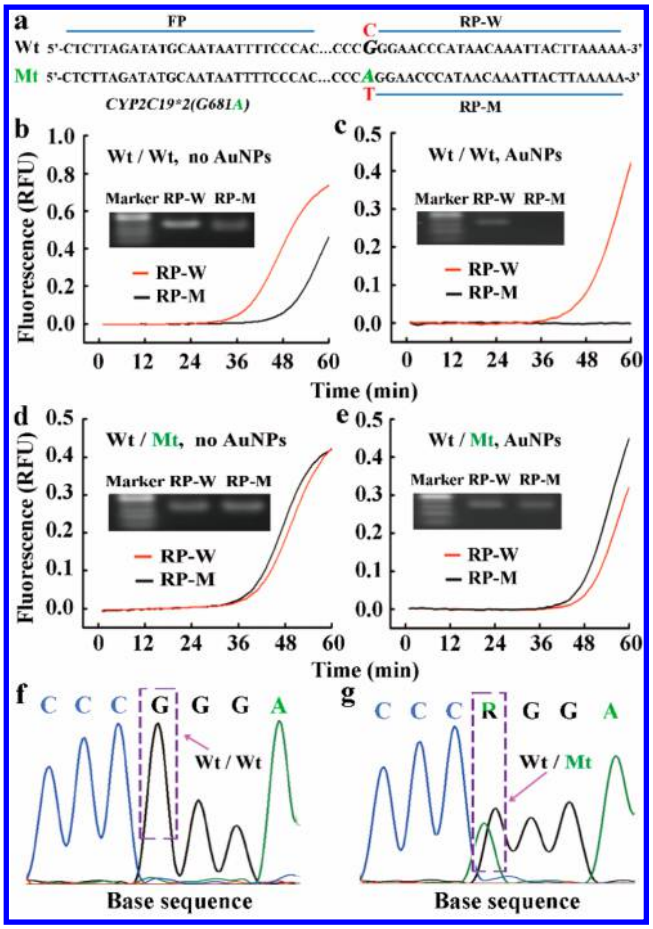

Figure 5. SNP diagnostics of CYP2C19*2. (a) Sequence alignment of CYP2C19 allele (wild-type, Wt; mutant-type, Mt). The forward primer (FP) and two reverse primers (RP-W for Wt and RP-M for Mt) are shown. (b–e) The effect of AuNPs-improved HDA on SNP detection using human blood specimens. Among them, parts b and d are obtained by HDA without AuNPs. (f, g) Sequencing maps of corresponding homozygous sample Wt/Wt and heterozygous sample Wt/Mt, respectively. The SNP locus is shown in the dashed box. Different peak colors represent types of bases (blue, C; green, A; black, G). Two sequencing peaks mean the presence of two alleles. The green and black letter R indicates base A and G.

To verify the clinical utility, human blood genomic DNA samples of candidates were tested by AuNPs-improved HDA. The false signal of mismatch in the absence of AuNPs (Figure 5b) was eliminated by AuNPs-improved method (Figure 5c). Fortunately, the specific amplification was always remarkable (Figure 5b–e). Corresponding melting curve analysis (Figure 6 and Figures S14–S17) also depicted the consistent results. Sequencing, the gold standard method for SNP detection, was used to verify the data for the human blood samples. As shown in Figure 5f,g, these results are of perfect concordance with those by AuNPs-improved HDA. Oppositely, the sequencing-verified homozygous wild-type sample was tested as a heterozygote (Figure 5b) by the method without AuNPs. The homozygous mutant-type, infrequent in an Asian population, was not found in our candidates. Theoretically, the proposed method can also output accurate result for this sample as that in Figure S8c (the synthetic template sample). It is worthy to point out that other isothermal amplification techniques such as recombinase polymerase amplification can also be used to achieve the SNP analysis similar to that of

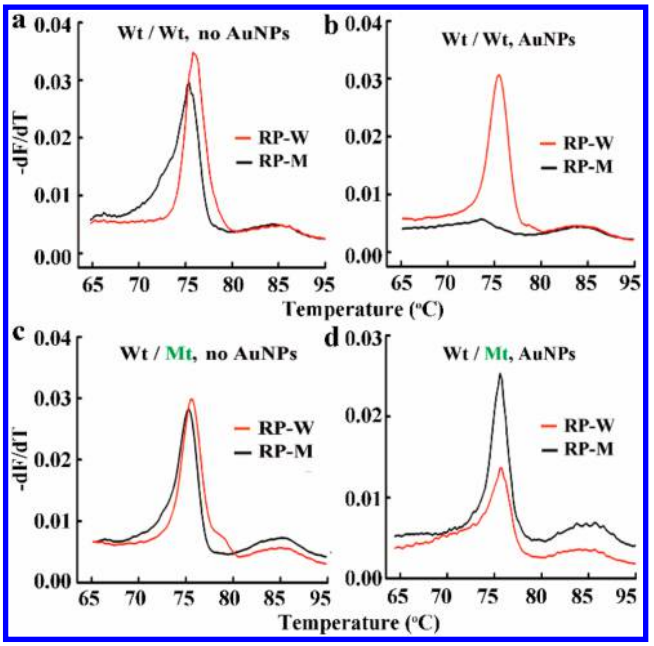

Figure 6. Melting curve analysis results of SNP diagnostics in Figure 5. The obtained T_m value is about 75 °C (peak value), which is in good agreement with the theoretical value of amplified product, demonstrating the generation of amplification reaction. It is worthy to point out that the fluctuation of the obtained T_m value is due to using SYBR Green I as a fluorescent dye which is of low saturation. The weak peaks at about 85 °C represent the existence of added genomic DNA.

HDA.[5,39] All these results indicate that AuNPs-improved isothermal amplification holds strong potential for the high-accuracy SNP analysis in clinical samples without mismatch interference.

CONCLUSION

In summary, it has been shown that single 3′-terminal mismatches can trigger extension and amplification reaction by DNA polymerases. Though their amplification efficiencies depend on both types of mismatch and kinds of DNA polymerase, most of them induce high amplified signals even comparable with those of perfect pairs. These findings reveal the interference on accurate SNPs analysis. Nevertheless, the diversity of mismatches extension will aid in designing primer-template pairs to reduce false results, and a useful primer-design guideline with prognosis for specific amplification has been summarized. Furthermore, we have demonstrated the AuNPs-mediated suppression of mismatch amplification. The probable mechanisms of these findings have been deeply discussed. Finally, we have achieved the high-accuracy SNP analysis of human blood genomic DNA without false results by AuNPs-improved isothermal amplification. Collectively, our studies provide mechanistic insight into mismatch behaviors and demonstrate specific amplification for high-accuracy SNPs analysis, providing a reliable sensing platform for disease diagnostics and personalized medicine.

ASSOCIATED CONTENT

Supporting Information

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Experimental procedures and analytical data (PDF)

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Notes

The authors declare no competing financial interest.

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