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SHORT COMMUNICATION

Isothermal cycling and cascade signal amplification strategy for ultrasensitive colorimetric detection of nucleic acids

Mingdi Xu · Yu He · Zhuangqiang Gao · Guonan Chen · Dianping Tang

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Abstract We have designed a novel isothermal cascade signal-amplification strategy for ultrasensitive colorimetric determination of nucleic acids. It is based on double-cycling amplification with formation of DNAzyme via a polymerase-induced strand-displacement reaction and nicking endonuclease-assisted recycling. The assay makes use of a hairpin DNA, a short primer, KF-polymerase, and nicking endonuclease. The presence of a target DNA triggers the strand-displacement and polymerization reaction with the formation of numerous DNAzyme molecules. Upon addition of H_2O_2 to the resulting mixture, the H_2O_2 reacts with 2,2′-azinobis (3-ethylbenzothiozoline)-6-sulfonate to form a colored product in the aid of DNAzyme, which is quantified by photometry at 415 nm. Under optimal conditions, the assay allows target DNA to be determined at concentration as low as 0.6 aM.

Keywords Nucleic acid · Colorimetric assay · Isothermal cycling signal amplification

Introduction

Nucleic acids have very important functions in biological systems, and are usually present in trace amounts in a cell extract. Enormous efforts have gone into development of experimental methods to determine the nucleotide sequence of biological DNA and RNA molecules [1]. Although northern blotting technology and microarrays are the most widely used approaches for nucleic acid quantification, their use is limited by their low sensitivity and specificity [2, 3]. Thus, ongoing efforts have been made worldwide by sophisticated signal-transduction method, e.g., electrochemistry, Raman spectroscopy, fluorescence, chemiluminescence, and colorimetric detection [4–7]. In these cases, polymerase chain reaction (PCR), rolling-cycle amplification, ligase chain reaction, loop-mediated isothermal amplification, catalytic silver deposition or stripping assays and enzyme-linked electrochemical assays were usually employed for the construction of sensing platforms [8–11]. Although these strategies are not free of limitations, they provide methodological improvement in the designing or labeling probes, modifying nucleotides, incorporating these methods for detecting minute amounts of DNA.

Hemin/G-quadruplex DNAzyme with peroxidase-like activity can exhibit the catalytic activity toward H_2O_2-mediated oxidation [12, 13]. This strategy has been applied widely for colorimetric and/or chemiluminescence detection of small molecules and metal ions [14–16]. Thermal cycling and isothermal cycling methods are usually used for the amplification of detectable signal [17, 18]. For the thermal cycling strategy, the increase in the product amount was achieved by repeated thermal cycling in an exponential way. Thus, thermal cycling methods are time-consuming, sometimes nonspecific, and limiting thermostable enzyme and laboratory setting [19]. In contrast, isothermal exponential amplification reaction is an outstanding project to enhance the sensitivity of nucleic acids in that the products of one reaction catalyze further reactions that create the same products [20]. It can synthesize (called triggers) short oligonucleotides by combining polymerase strand extension with single-strand nicking under the isothermal conditions within minutes [21]. Compared with other amplification methods, it has shown distinct advantages of ultra-high amplification efficiency (10^6-10^9-fold amplification) and rapid amplification kinetics [22, 23]. More importantly, such an isothermal exponential amplification does not require a thermal cycler, which greatly simplifies point-of-care

M. Xu · Y. He (✉) · Z. Gao · G. Chen · D. Tang (✉)
Institute of Nanomedicine and Nanobiosensing, Department of Chemistry, Fuzhou University, Fuzhou 350108, People's Republic of China
e-mail: heyu@fzu.edu.cn
e-mail: dianping.tang@fzu.edu.cn

diagnosis [24]. In most cases, it is cost effective, easy-to-use and more tolerant to inhibitory components from a crude sample compared with PCR, showing equivalent or high sensitivity and reliability in clinical diagnosis. Herein, we design a novel isothermal and cascade signal amplification method for colorimetric detection of specific nucleic acid at a ultra-low level through cycling amplification of target oligonucleotide with DNAzyme formation by coupling a DNA polymerase-induced strand-displacement reaction with a nicking endonuclease-assisted recycling reaction.

Experimental

All the oligonucleotides were purchased from Dingguo Biotechnol. Co., Ltd. (Beijing, China www.dingguo.com) and purified through high-performance liquid chromatography (HPLC). The sequences of oligonucleotides are given as follows:

Hairpin DNA: 5′-CCCAACCCGCCCTACCCAAAGTG C C T C **A G C A G C A A C A** *A T C T G G C A C G C G T T A C T C C* **TGTTGCTGCT**-3′ (*Note*: The underlined sequence was the sequence complementary to hemin-binding aptamer. The italic letters are the loop of the hairpin DNA, which are complementary to target DNA. The bold sequence represents the stem portion of hairpin DNA, which are completely complementary each other. The first 8 bases at 3′ end are the primer binding site).

Primer DNA: 5′-AGCAGCAA-3′

Target DNA (tDNA): 5′-*GGAGTAACGCGTGCCAGAT*-3′

Mismatched DNA: 5′-GG***C***GTAACGCGTGCCAGAT-3′

Deleted DNA: 5′-GGGTAACGCGTGCCAGAT-3′

Inserted DNA: 5′-GGA***G***GTAACGCGTGCCAGAT-3′

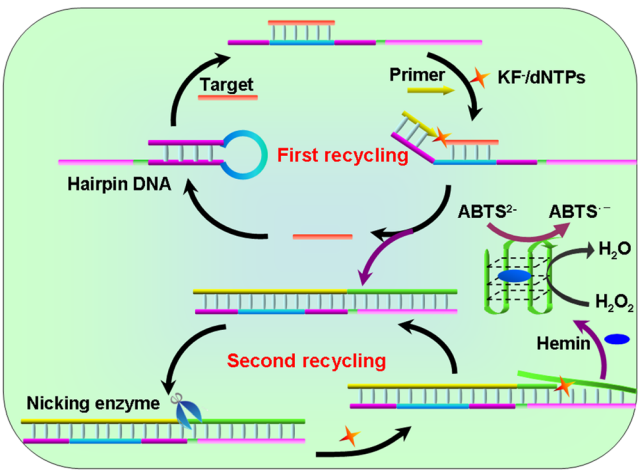
Polymerase klenow fragment exo− (KF−, exo-) (KF polymerase) and N.BbvCIA nicking endonuclease (NEase) were obtained from New England Biolabs (UK, www.neb.com). Deoxyribonucleotides (dNTP) were obtained from Dingguo Biotechnol. Co., Ltd. (Beijing, China, www.dingguo.com). 2,2′-azino-bis(3-ethylbenzothiozoline)-6-sulfonate ($ABTS^{2-}$) and hemin were purchased from Sigma-Aldrich (USA, www.signaaldrich.com). A hemin stock solution was prepared in dimethyl sulfoxide (DMSO) and stored in the dark at −20 °C. Reagents were purchased from commercial sources and used as received. Solvents were purified and degassed by standard procedures. Ultrapure water obtained from a Millipore water purification system (18 MΩ cm^{-1}, Milli-Q, Millipore, www.millipore.com) was used in all experiments.

The isothermal cycling amplification was carried out in a PCR tube as follows. Before use, hairpin DNA and primer DNA were heated to 88 °C for 10 min and allowed to cool naturally to room temperature. Following that, a volume of 25 μL mixture containing 1.0×10^{-6} M hairpin DNA, 1.0×10^{-6} M primer, NEB buffer (10 mM Tris-HCl, pH 7.9, 10 mM NaCl, 10 mM $MgCl_2$, and 1.0 mM dithiothreitol), KF polymerase (5 U), dNTP (1.25 mM), NEase (10 U) and various-concentration target DNA was initially incubated at 53 °C for 3 h, and the mixture was heated at 90 °C for 5 min in order to inactivate the enzymes. Afterwards, 500 nM of hemin was added into the above reaction mixture, and incubated for 1 h at room temperature, allowing the DNAzyme to properly fold to form the G-quadruplex/hemin complex.

Next, the mixture was injected to the ABTS-H_2O_2 system in pH 7.4 HEPES buffer (25 mM 2-[4-(2-hydroxyethyl)-1-piperazininyl]ethanesulfonic acid, 20 mM KCl, and 200 mM NaCl) containing H_2O_2 (4.4×10^{-5} M) and ABTS (1.82×10^{-4} M), and incubated for 450 s at room temperature. Finally, the obtained mixture was transferred to the 96 transparent microplates to monitor UV-vis absorption spectra. The absorbance was recorded at λ =415 nm on a Lab Microplate Reader (SH-1000, Corona Electric Co., Ltd., Japan, www.corona-el.co.jp). All measurements were conducted at room temperature. Analyses are always made in triplicate.

Results and discussion

In this work, the assay mainly contains signal amplification with double cycles, and achieves cascade linear amplification results (Scheme 1). The system consists of a hairpin DNA, a short primer, KF^- polymerase and nicking endonuclease (NEase). Initially, target DNA (tDNA) binds to a specifically designed molecular beacon, causing the opening of hairpin DNA. Upon addition of dNTP and polymerase klenow fragment exo- (KF-, exo-), the short primer can simultaneously carry out the strand-displacement and the polymerization reaction. The released target DNA can retrigger the hairpin DNA, thus resulting in the first isothermal cycling amplification. At the same time, the formed double-strand DNA can be also cleaved by using a specific N.BbvCIA nicking



Scheme 1 Schematic illustration of isothermal cycling amplification for ultrasensitive colorimetric detection of nucleic acid

endonuclease. In the presence of KF- polymerase, the cleaved strand can be implemented again the strand-displacement reaction, thereby leading to the second isothermal cycling amplification. Upon hemin introduction, the released DNA fragment can induce the formation of DNAzyme, which can oxidize the 2,2′-azino-bis (3-ethylbenzothiozoline)-6-sulfonic acid (ABTS) to the coloured product $ABTS^{\bullet-}$ with the aid of the added H_2O_2. Therefore, once initiated by tDNA, the polymerization, nicking, and displacement reactions are continuously repeated to produce a large number of fragments that prime the amplification template in a cyclic chain (exponential) reaction, and a large number of hemin-based G-quadrulex DNAzyme. By monitoring the visual colorimetric signal or the change in the absorbance, we can qualitatively or quantitatively evaluate the concentration of target DNA in the sample.

To realize our design, one precondition for the development of colorimetric assay system was whether target DNA could readily trigger the production of visible color, and the detectable signal really derived from the stimulation of target DNA. To verify this point, several control tests were carried out under the different conditions by using the colorimetric measurement (Fig. 1a) and UV-vis absorption spectroscopy (Fig. 1b) (500 aM tDNA used in this case). As seen from photograph '*c*' in Fig. 1a, a strong visible green color could be observed in the simultaneous presence of target DNA, polymerase and NEase. Moreover, the absorbance (curve '*c*' in Fig. 1b) was higher than those of other samples. Significantly, no visible color was appeared when target DNA was absent in the detection system (photograph '*d*'). The results by comparison of photograph '*c*' with photograph '*d*' preliminarily indicated that the visible color should be originated from the interaction of target DNA with other chemicals. Further, we also observed that the colors (i.e., colorless) of the assay system in the absence of NEase (photograph '*e*') or polymerase (photograph '*f*') were the same as those of dNTPs (photograph '*a*') and hemin (photograph '*b*') alone, respectively. The obtained results revealed that (i) the presence of target DNA in the assay system should be one sufficient and essential condition for the development of the colorimetric protocol, and (ii) KF polymerase and NEase should be simultaneously existed in the assay system even if target DNA was present.

As described above, the amplification of detectable signal was implemented through target-induced strand-displacement reaction and recycling reaction based on KF polymerase and NEase. To demonstrate this concern, we also used gel electrophoresis to characterize the reaction process (Fig. 1c). As seen from lane 1, the base number of hairpin DNA was in accordance with our design. Significantly, the mixture containing hairpin DNA, KF polymerase, NEase, primer and dNTPs in

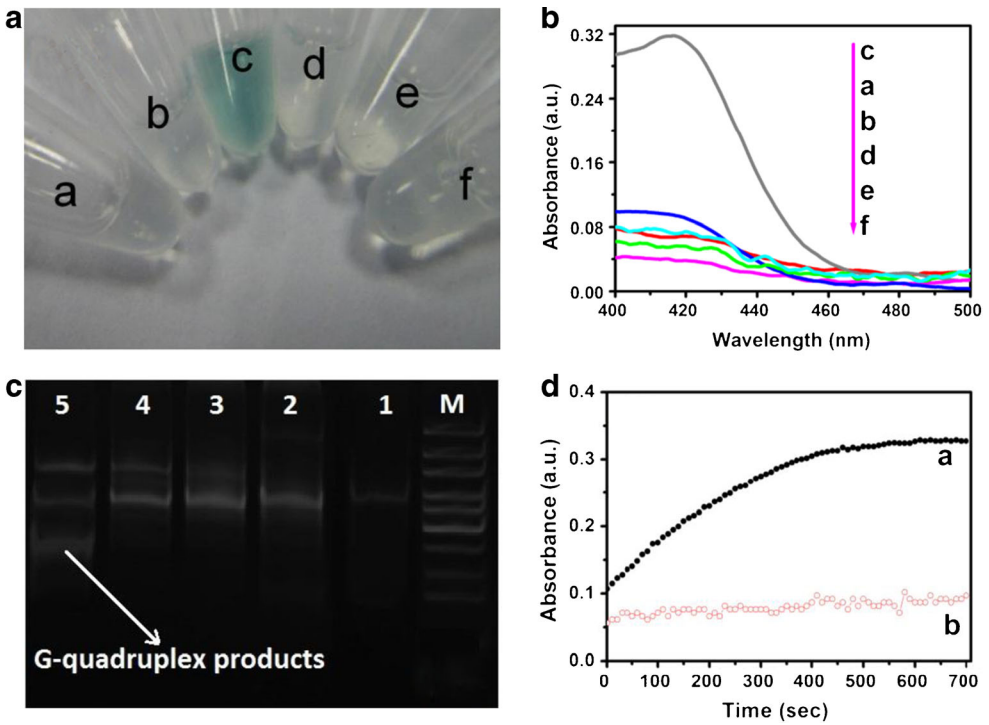


Fig. 1 **a** Photographs of (*a*) dNTP, (*b*) hemin, (*c*) hairpin DNA + tDNA + primer + polymerase + dNTP + NEase, (*d*) sample '*c*' in the absence of tDNA, (*e*) sample '*c*' in the absence of NEase, and (*f*) sample '*c*' in the absence of polymerase toward ABTS-H_2O_2 system. **b** The corresponding UV-vis absorption spectra of figure 'A' (Incubation: 60 min at RT; tDNA: 500 aM; polymerase: 5 U; NEase: 10 U). **c** Gel electrophoresis (*lane 1*: hairpin DNA, *lane 2*: sample '*5*' in the absence of tDNA, *lane 3*: sample '*5*' in the absence of KF polymerase, *lane 4*: sample '*5*' in the absence of NEase, *lane 5*: hairpin DNA + primer + polymerase + dNTP + NEase + tDNA) (Note: hairpin DNA: 1.0 μM; polymerase: 5 U; NEase: 10 U; tDNA: 1.0 μM). **d** Dynamic responses (absorbance vs. time) of the assay system in the (*a*) presence and (*b*) absence of 500 aM tDNA

the absence of target DNA did not cause the strand-displacement reaction and recycling reaction, since the obtained spot at lane 2 was almost the same as that of lane 1. Unfavorably, we did not observe the spot of the primer as a result of a few bases. To obtain the large-amount hemin/G-quardruplex-based DNAzyme, the hairpin DNA underwent two cycling processes with KF polymerase and NEase. The product of KF polymerase-induced strand-displacement reaction was used for the substrate of NEase-assisted recycling reaction. As shown from lane 3, the spots were slightly different from lane 1 (one strong spot and one weak spot). The weak spot at lane 3 might be ascribed to partial interaction between hairpin DNA and primer/target DNA in the absence of KF polymerase. Inspiringly, two strong spots at lane 4 were appeared in the absence of NEase. The result indicated that the polymerization chain reaction (i.e., strand-displacement reaction) could be successfully carried out in the presence of target DNA, primer, dNTP, hairpin probe and KF polymerase. The top spot was corresponded to the polymerization double-strand DNA, while the bottom one was from the hairpin DNA. Happily, three spots at lane 5 were acquired when NEase was injected into the sample '4'. The G-quardruplex could be achieved at the bottom of lane 5. Base on the results of gel electrophoresis, we could further make two concerns that (i) isothermal cycling amplification strategy could be executed with the aid of KF polymerase and ENase, and (ii) the designed system could be used for the detection of target DNA by KF polymerase-induced strand-displacement reaction and NEase-assisted cycling system.

To achieve an optimal analytical performance, we investigated the effect of various reaction times of the system (i.e., containing hairpin DNA, primer, dNTP, KF polymerase, NEase, hemin, and target DNA) with the ABTS-H_2O_2 on the absorbance (500 aM tDNA used in this case) (Fig. 1d). As shown from curve '*a*', the absorbance increased with the increasing reaction time, and tended to level off after 450 s. Longer reaction time did not cause the significant shift in the absorbance. In contrast, the absorbance of the assay system was not almost changed within the whole assay times in the absence of target DNA. To avoid possible error resulting from different additions of samples, the absorbance for each sample was recorded as the reaction proceeded from 5 s (after the addition of ABTS-H_2O_2) until equilibrium was reached ~450 s.

To further demonstrate the capability of our design, the assay system was used for the detection of target DNA. The absorbance relative to various-concentration tDNA was collected at 415 nm. As seen from the inset in Fig. 2a, the absorbance increased with the increase of tDNA concentration from 1.0 to 1,000 aM in the sample. Two linear relationships between the absorbance and target DNA were obtained within the range from 1.0 aM to 1,000 aM (i.e., 1.0 aM–10 aM and 10 aM– 1,000 aM). The linear regression equations were listed as follows: A (a.u.) $=0.0152\times C_{[tDNA]}+0.1039$ (aM, $R^2=0.9924$, $n=15$) for 1.0 aM–10 aM, and A (a.u.) $=0.0001\times C_{[tDNA]}+0.2721$ (aM, $R^2=0.9862$, $n=18$) for 10 aM–1,000 aM, respectively (Fig. 2a). The detection limit was 0.6 aM estimated at the $3s_{blank}$ criterion. The reason for such a low LOD might be the fact that the designed isothermal cycling amplification was readily carried out with the aid of KF polymerase and NEase when the amount of hairpin DNA was excessively higher than that of target DNA, thereby resulting in a strong change in the absorbance within the low-concentration range. To further clarify the advantages of the newly developed method, we compared the specific features of the colorimetric assay with other methods (Table 1).

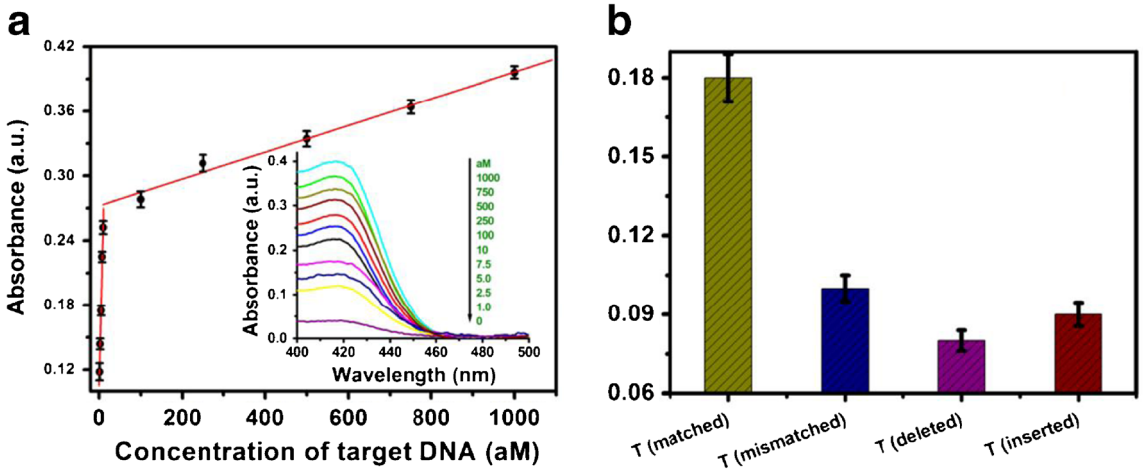


Fig. 2 **a** Calibration plots of the colorimetric assay system toward various-concentration target DNA (*Inset*: The corresponding UV-vis absorption spectra), and **b** absorbance intensity of the colorimetric assay for a matched target DNA, mismatched target DNA, a target DNA with a deleted nucleotide, and a target DNA with an inserted nucleotide (all 5 aM). Target sequence: GGXTAACGCGTGCCAG AT; T (matched): X=C; T (mismatched): X=A; T (deleted): no X; T (inserted): X=AG

Table 1 Figures of merits of comparable methods for signal amplification and detection of nucleic acid

Method (materials used)	Analytical range	LOD	Comments [a]	Ref.
Chemiluminescence assay (ssDNA1, hairpin DNA, Exo III, ssDNA2, hemin, luminol, H_2O_2)	0.01–1.0 pM	8 fM	Without labeling or modifying; homogeneous assay; avoiding the separation and washing steps, but requirement of chemiluminescence analyzer	[25]
Colorimetric assay (PCR products, CeO_2 nanoparticles, TMB, H_2O_2)	≤ 120 nM	–	Ultrafast; within a few minutes without the need of post-purification of PCR product, chemical modification of either the CeO_2 or DNA and tedious salt treatment.	[26]
Colorimetric assay (microplate, streptavidin, biotin-DNA, HRP, TMB, H_2O_2, H_2SO_4)	0.1–5 nM	0.08 nM	Without the labeling step and unafforable equipments; but requiring multistep incubations, washing, and separations.	[27]
Colorimetric assay (gold nanorod, FAM-labeled hairpin DNA)	1 pM–100 nM	0.26 pM	Label free; 10 nM by the naked eye; 0.26 pM for fluorescent assay	[28]
Fluorescent assay (NC membrane, sample pad, absorbent pad, FAM-labeled DNA, ROX-labeled DNA, capture DNA)	–	–	Assay within 30 min without the high-end or expensive machine; one single step; but detection with fluorescent instrument	[29]
Electrochemical assay (hairpin DNA, polymerase, dNTPS, streptavidin, nicking enzyme, ferrocene)	5 fM–10 pM	2.56 fM	Homogeneous assay; one-step detection; without separation and washing; but using of two enzymes and reaction complex	[30]
Colorimetric assay (hairpin DNA, primer DNA, KF polymerase, dNTP, NEase, hemin, ABTS, H_2O_2)	1.0 aM–1,000 aM	0.6 aM	Without any labels, any washing/separation steps, and sophisticate instruments; high specificity; low cost; pH 7.4	This work

[a] The comments mainly included pH effect, interfering, cost, applicability to specific problem, or competiveness (simplicity and instrumental effort) of the method

As seen from Table 1, the isothermal cycling and cascade signal amplification strategy should be comparable with the existing methods.

To investigate the specificity of the colorimetric assay toward target DNA, we used various oligonucleotides as matched, deleted and inserted targets (Fig. 2b). Only the matched DNA could cause the strand-displacement and cycling reaction, thus resulting in the strong change of the absorbance. The results indicated that our designed scheme was highly selective for completely complementary DNA.

In addition, our results in the buffer system, which was relatively pure in comparison with real physiological media, demonstrated that the developed colorimetric assay system had good selectivity and high sensitivity. To monitor the interfering effects of sample matrix components, we used a cell medium mixed with new-born cattle serum to investigate the feasibility of using the colorimetric assay scheme in biological samples. Three target DNA samples including 5 aM, 50 aM, and 500 aM were spiked in the cell medium, which were assayed by the developed method. The obtained results were 5.3 aM, 45.6 aM, and 532 aM at the mentioned-above analytes, respectively. The recoveries were 106 %, 91.2 % and 106.4 %, respectively, implying that the methodology was feasible for direct detection of target DNA in such a complex biological sample.

Conclusions

In summary, we have developed a convenient and feasible DNA colorimetric sensing system with high sensitivity and high selectivity based on the catalytic reaction of hemin/G-quadruplex –based DNAzyme by coupling with an isothermal cycling amplification strategy. Compared with the traditional DNA sensing strategy, the method is simple and sensitive. The signal can be easily readout by monitoring the absorbance of ABTS-H_2O_2 system with UV-vis absorption spectrometry, thereby representing a good isothermal signal amplification scheme. Meanwhile, the designed hairpin probe does not need any labels, and the assay does not require any washing/separation steps and sophisticate equipment. In addition, the methodology can be successfully executed under the isothermal condition, thus eliminating the requirement for thermal cycling during the procedure. Importantly, the outstanding sensitivity can make this approach a promising scheme for development of next-generation DNA sensing techniques.

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