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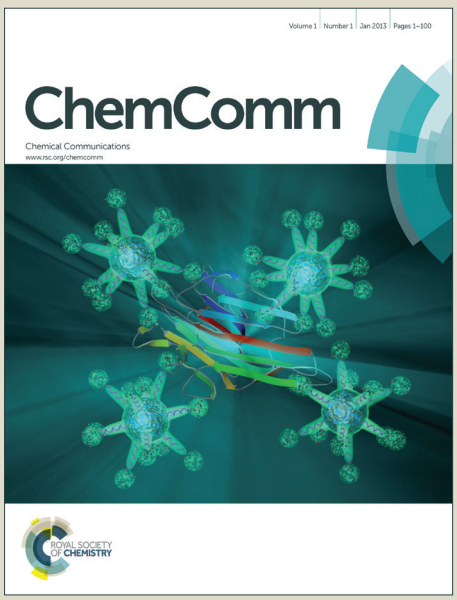

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Single primer-triggered isothermal amplification for double-stranded DNA detection

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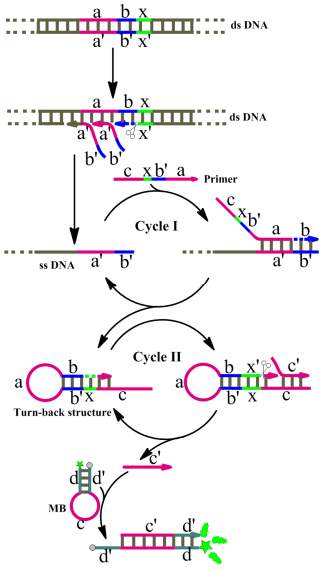
Here we have devised a new generation of isothermal double-stranded DNA (dsDNA) detection method, termed single primer-triggered isothermal amplification (SAMP). It is very simple only requiring one primer and a few copies of dsDNA in less than an hour are detectable with multiple signal amplification steps.

Nucleic acid detection routinely plays an important role in bioanalysis because it is often used as biomarkers in clinical diagnosis and bioassay. The analysis of nucleic acid is currently performed by amplifying the trace amounts of sequence to be detectable levels. PCR[1,2] and some derived technologies are widely used DNA exponential amplification techniques. However, some fundamental limitations make them be difficult to execute for point-of-care analysis[3,4]. For example, it needs electrically powered thermal cycling equipment for repeated heating and cooling process. In addition, PCR spurious priming usually is caused by non-specific hybridization at anneal process[5]. For these reasons, the design of non-PCR based approaches for DNA amplification is in continuous demand. Up to now, several types of isothermal nucleic acid amplification methods such as strand-displacement amplification (SDA)[6,7], rolling circle amplification (RCA)[8-10], loop-mediated isothermal amplification (LAMP)[11,12], and helicase-dependent amplification (HDA)[13] have been developed for "point-of-care" testing in clinical diagnosis[14-16], food safety[17,18], and environmental monitoring[19]. Although these methods offer several advantages over PCR in that they eliminate the need for an expensive thermocycler, there are different weaknesses to limit their uses in bioanalysis. For example, both SDA and RCA need to convert dsDNA target to ssDNA by an initial heat denaturation step[20,21], resulting in complex experimental procedures and the higher the feasibility of contamination. HDA circumvents the heat denaturation step by a DNA helicase, but shows lower amplification efficiency[22]. LAMP can achieve high amplification efficiency in a short time with high specificity, but it requires four specific primers so that their designs are complicated for users[23]. Moreover, most of reported isothermal amplification methods only work on single-stranded nucleic acid[24,25]. For these reasons, simple,sensitive, and true isothermal amplification methods are strong demand for dsDNA detection.

Herein, we present a new isothermal detection method for dsDNA by single primer-triggered isothermal amplification (SAMP). The method successfully uses nicking endonuclease exploited in amplification reaction to switch dsDNA target to ssDNA, without need of heating or extra chemicals. This makes SAMP technology be performed at one temperature from the beginning to the end of the reaction, realizing true isothermal detection. Especially, SAMP has a simple experimental scheme that only requires a single primer, the nicking endonuclease and DNA polymerase for the entire experimental process. Thus, the complicated primer design step has been omitted to greatly simplify experimental protocols and get rid of the inherent side reactions. On account of these, SAMP technology has a great potential for the development of simple portable detection devices for point-of-care testing.



Scheme. 1 The schematic illustration of SAMP for dsDNA detection. All base sequences of the target, primer and molecular beacon used are listed in Table S1in the ESI † .

As shown in the mechanism illustration of SAMP (Scheme. 1), the dsDNA target was first nicked by nicking enzyme, followed by polymerization and displacement reactions. Once initiated, the nicking, polymerization, and displacement reactions continuously cycled to generate ssDNA. As a result, the detection target of dsDNA was switched to this ssDNA at constant temperature, and this ssDNA was used as detection target for next steps. A primer bound to domain a′ of ssDNA and extended a few of bases (domain b) until 5′ end of ssDNA. The extended primer could dynamically dissociate from the ssDNA because its Tm value was close to the reaction temperature. The free ssDNA could bind to another primer and repeat above-described reaction process (designated Cycle I). After the extended primer was released, it could self-hybridize, due to extended domain b being complementary with itself b′ , which caused it switch conformations to become turn-back structure. Turn-back structure self-primered to extend, forming the nicking site (5′ -GAGTCNNNN$^{\downarrow}$N-3′) of *Nt.BstNBI*. After nicking, 3′ end of nicked strand triggered next cycle of displacement, nicking and polymerization reaction, designated Cycle II. During Cycle II, the displaced domain c′ was regenerated and repeatedly opened molecular beacons resulting in an increase of fluorescent signal. Moreover, the final products of c′ was no more the template for next amplification cycle reducing false positive pollution usually generated by other exponential amplification methods. In a word, SAMP employed a single primer as trigger to initiate a non-linear cascade signal amplification reaction with polymerase and nicking enzyme for dsDNA detection at constant temperature.

The nicking endonuclease was a key component in SAMP reaction. The generation of ssDNA target required the aid of nicking endonuclease. At present, nicking endonuclease usually recognizes a specific sequence of five, six, or seven bases. The nicking enzyme recognizing five bases as an example, every base of the recognition site may be A, G ,C or T. So all possibility of five-bases recognition site is 4^5,while the recognizing sequence of nicking endonuclease is two complementary sequences, thus, a recognition site appears one time every 512 bases ($4^5/2$) on genomic DNA in theory. Also, a variety of nicking enzymes currently have been reported. Therefore, it was not hard to find recognition sites of nicking enzyme on dsDNA target.

The design of primer was very crucial to the amplification efficiency of SAMP. Firstly, the primer could not self-hybridize to form turn-back structure in the absence of target. On the contrary, when the target was present, the primer could hybridize with ssDNA and extend with the polymerase. The extended primer could dynamically dissociate from ssDNA template at reaction temperature. Moreover, the dissociated primer self-hybridized and switched into turn-back structure to trigger Cycle II. Thus, the number of complementary bases and extended bases of primer need fine-tune to ensure high amplification efficiency of SAMP.

This design also indicated good target specificity. First, dsDNA target only could be switched to ssDNA in the presence of specific recognition sequence of nicking enzyme on the vicinity of dsDNA target, or it will be failed when any one base of recognition site was changed. Second, only well hybridizing with ssDNA target, the primer could be extended right bases and dynamically dissociated from ssDNA. Third, the extended bases must be complementary with domain b′ of primer, resulting in turn-back structure to form a unique site of nicking enzyme. Only so could Cycle II of displacement, nicking and polymerization be activated with the digestion of nicking enzyme. Therefore, these elements all together ensured high specificity for this method. We have verified the specificity of SAMP for differing from one or two bases in Supporting Information (Figure S1 in the ESI †).

The feasibility of SAMP was demonstrated by fluorescence curves and PAGE (Fig. S2 in the ESI †). The reaction was initiated by the specific sequence nucleotide of pBluescript II KS (+) plasmid DNA (Table S2 in the ESI †), and the following referred to as pBlu2KSP. No change of fluorescence signals were detected in the absence of any of polymerase, nicking enzyme, and primer. When the polymerase, nicking enzyme and primer all were simultaneously added to the SAMP system, the fluorescence signal was greatly increased (Fig. S2a in the ESI †). This implied that not a single one among them could be omitted for obtaining signal amplification. SAMP reactions were initiated by different concentrations of pBlu2KSP, incubated for 30 min at 55 ℃, then terminated by heating. The PAGE result of reaction products revealed that when all three elements existed, the expected reaction products correspondingly increased with the increase of pBlu2KSP concentration (Fig. S2b in the ESI †). Both of fluorescence curves and PAGE results indicated SAMP reaction could be performed as expected under experimental conditions.

Real-time fluorescence detection of SAMP was initiated by different amounts of pBlu2KSP target (Fig. 1a). The fluorescence signal was rapidly increased in a short time in the presence of a high concentration of pBlu2KSP. The fluorescence curves showed good regularity with the increase of amount of pBlu2KSP, demonstrating that SAMP could be used to detect changes of pBlu2KSP in the tested concentration range. Moreover, a few of pBlu2KSP copies in less than an hour could be amplified to be detectable and well distinguished from blank. The time corresponding to the maximum slope in the fluorescence curve as well as the point of inflection (POI) was linearly related to the negative logarithmic (lg) value of the target amount in the ranges from 10 amol to 0.01 zmol (Fig. 1b). The correlation equations was found to be POI= -34.5-4.3 lgA (mol) (A was the amount of pBlu2KSP, R^2=0.9958). So, the method showed a good dynamic range of 6 orders of magnitude.

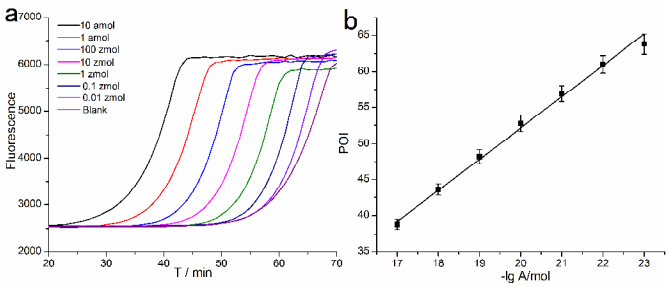


Fig.1 (a) The real-time fluorescence curves for achieving the sensitivity of SAMP. (b) The relationship between the POI values and the negative logarithmic values of the amount of pBlu2KSP plasmid DNA (Error bars showed mean standard deviation of three determinations).

To evaluate the anti-jamming of SAMP, 1 amol pBlu2KSP plasmid DNA mixed with 0-, 0.1-, 1-, 10-, 100-fold *Escherichia coli* genomic DNA was used to initiate SAMP reaction, respectively (Fig. S3 in the ESI†). Even if 100-fold *Escherichia coli* genomic DNA corresponding to 300 ng could not affect the amplification detection result of 1 amol pBlu2KSP DNA because of their real-time fluorescence curves fitting to that of the blank. As can be seen, SAMP is capable of specifically detect a unique DNA sequence in a complex nucleic acid extract. Such a good capability of anti-jamming is excellent for its practical application.

SAMP reaction possesses strong anti-jamming capability and the abilities of simplicity, which motivated us to explore this method

for simultaneous detection of multiple targets by using different molecular beacons. We firstly intended to simultaneously detect the target sequences of *Cyprinus carpio* and *Gallus gallus* by the same beacon in the same reaction (Fig. S4 in the ESI †). The fluorescence signal for two targets appeared earlier than that of any one separate target. This result indicated that multiple specific sequences in the same sample could be simultaneously detected by fluorescence signal superposition. This ability of SAMP makes it avoid tedious operation compared with detecting in turn multiple targets, and the detection accuracy is also greatly improved.

In comparison with fluorescence signal superposition of multiple targets, simultaneously individual detection of multiple targets with different molecular beacons is more useful for practical application. Therefore, we employed two molecular beacons MB1 and MB2 for specific sequences of *Cyprinus carpio* and *Gallus gallus*, which were labeled with FAM and HEX at the 5′ end, respectively. These two dyes avoided dye-to-dye energy transfer, which were individually excited at 495 and 538 nm, emitting at 520 nm and 555 nm, respectively. Probe mixture in the presence of different targets *Gallus gallus*, *Cyprinus carpio*, and mixture of both genomic DNA were detected by real-time fluorescence (Fig. 2). The target *Gallus gallus* led to the fluorescence signal of HEX, while minimal fluorescence signal of FAM. Similarly, the target *Cyprinus carpio* mainly led to the fluorescence signal of FAM (Fig. 2a, 2b). When both of targets were mixed, the fluorescence signals of FAM and HEX were simultaneously generated, and their fluorescence curves in probe mixture were close to that of each targets separately detected (Fig. 2c). Therefore, SAMP could individually detect multiple targets by multiple molecular beacons at the same time in the same sample. This was due to simple design of SAMP, decreasing the complexity of reaction and non-specific priming. The advantage of SAMP detecting multiple targets is very obvious, such as simultaneously qualitative detection of multiple pathogenic bacteria in clinical diagnosis. More importantly, detecting a new target by SAMP only requires a new primer, which resulting in a programmable molecular technology to significantly simplify experimental design. Therefore, this multiple DNA analysis provides a promising approach for challenging applications.

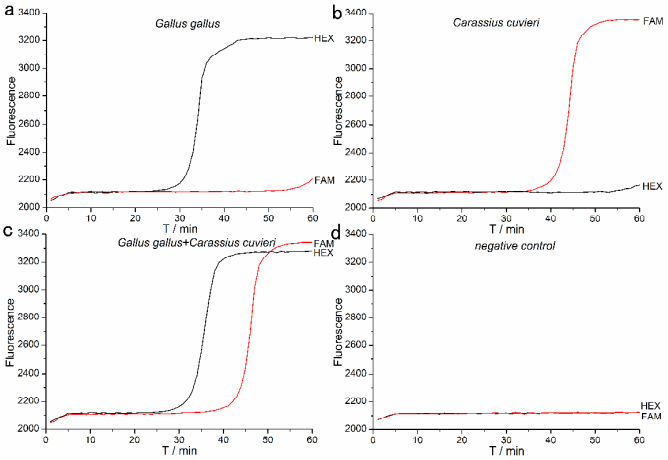


Fig.2 Multiple target assays for different molecular beacons. (a) 1 amol *Cyprinus carpio* genomic DNA was detected in the presence of 200 nM MB1 and MB2. (b) 1 amol *Gallus gallus* genomic DNA was detected in the presence of 200 nM MB1 and MB2. (c) 1 amol *Cyprinus carpio* genomic DNA plus 1 amol *Gallus gallus* genomic DNA was detected in the presence of 200 nM MB1 and MB2. (d) Negative control.

To further test the practical application of SAMP, the analysis of serially 10-fold diluted HBV DNA was performed using SAMP and classical RT-PCR method, respectively. SAMP could well detect 0.1×, 0.01×, and 0.001× HBV DNA sample (Fig. 3a). Compared with RT-PCR using SsoAdvanced™ SYBR Green Supermix (BioRad) (Fig. 3b), SMAP showed better regularity and higher fluorescence sensitivity. This means SAMP would be a useful tool for real sample detection.

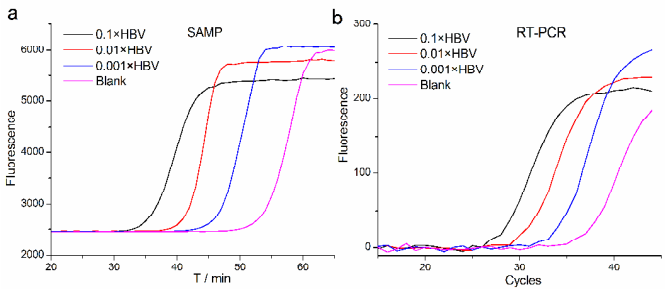


Fig.3 SAMP and RT-PCR detection of HBV DNA. The RT-PCR solution contained 1 μL serially diluted HBV DNA, specific primer set (200 nM each), and 5 μL of SsoAdvance™ SYBR Green Supermix in a final volume 10 μL. The RT-PCR was carried out as previously reported[26].

In this study, we have demonstrated a novel, simple, and true isothermal dsDNA detection method, named SAMP. SAMP can detect a few of DNA copies and this was comparable to PCR and LAMP. In addition to this, there were also the following three highlighted advantages: (i) SAMP successfully used nicking endonuclease exploited in next strand displacement amplification to produce ssDNA target, no need of denaturation of the target dsDNA, chemicals betaine or L-proline. It true realized one-pot isothermal amplification detection of dsDNA over existing isothermal DNA amplification methods; (ii) SAMP was very simple and easy to operate to new users, only requiring one primer, a nicking enzyme, and a polymerase. While other isothermal methods have complicated reaction mechanisms and experimental designs; (iii) SAMP had a high robustness. SAMP was not only anti-jamming and anti-pollution, but also could be used to detect multiple molecular targets in the same solution. This will offer great potential to multiple pathogens identification based on a DNA sequence in clinical diagnosis.

With its simplicity, sensitivity, and robustness, SAMP will be a useful platform of dsDNA detection, especially coupling with hand-held DNA detection devices to detect multiple targets of clinical, alimental, environmental, or biological samples.

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Notes and references

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