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Aligner mediated cleavage of nucleic acids for site-specific detection of single base mismatch

Wufan Pan[a,b], Dongdong Yu[c,**], Yazhou Qin[a], Wanghua Wu[a], Yuxiang Lu[a,b], Zhen Yuan[a], Jianguang Zhou[a,b,*]

[a] State Key Laboratory of Industrial Control Technology, Research Center for Analytical Instrumentation, College of Control Science and Engineering, Zhejiang University, 38 Zheda Road, Hangzhou, 310027, PR China
[b] Department of Chemistry, Zhejiang University, Hangzhou, 310027, PR China
[c] Hospital of Zhejiang University, Zhejiang University, Hangzhou, 310027, PR China

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ABSTRACT

Single base mismatch can always connect with various gene-related diseases, whose determination has aroused widespread interest. So far, various methods have been developed to determine the common base mismatch. However most of them are complex, time-consuming. Herein, we report a novel method, which only need one conventional endonuclease (NEase) and achieve site-specific cleavage in a programmable way, to detect single base mismatch, termed aligner-mediated cleavage-based single base mismatch discrimination (AMCMD). The DNA aligner (DA) is in a stem-loop structure, consistent with an incomplete recognition site of NEase on its stem and a 5′-side arm complementary to the target sequence (TS). Once TS contains matched base and hybridizes with DA, the complete recognition site of NEase is formed, and the TS will be cleavaged with fast speed, while converse is not. Based on it, the method can clearly distinguish mismatched and complementary bases. Without sample pre-processing, we were able to obtain and verify all the test result in about 30 min through the polyacrylamide gel electrophoresis analysis. This endows the proposed method with a simpler advantage. Then we combined AMCMD and EXPAR to create a new method for single base mismatch discrimination, the short sequence obtained by AMCMD as a target to trigger EXPAR, with a detection limit at 1pM level. Another process with human serum underlines that AMCMD is compatible with the complex biological sample, thus it has the potentials for practical applications.

1. Introduction

Determination of individual genotypes plays an important role in disease diagnosis, when DNA sequences containing mismatched bases may cause genetically related diseases like Alzheimer disease [1], Parkinson's disease [2], and cancers [3–5]. The most common genotype variation single nucleotide polymorphism (SNP), a single base variation in a given genetic location and widespread in the human genome [6,7], has aroused great research enthusiasm. Until now, many methods of detecting single base mismatch have been developed, such as direct DNA sequencing [8], electrochemical sensors [9–11], optical methods with gold nanoparticles [12–14] and graphene oxide nanosheets [15], and enzymatic methods [16–18]. However, the methods above have inevitable disadvantages, such as complex procedure, time-wasting and complex pre-treatment.

In addition, biosensor technology is attracting increasing attention for its good selectivity and sensitivity [19]. Polymerase chain reaction (PCR) [20–22] is applied for the detection of base mismatch, but the demand for specific thermal-cycling instrument limited its point-of-care test. Thus, great efforts have been made in developing a variety of isothermal amplification strategies, like Rolling Circle Amplification (RCA) [23], Strand Displacement Amplification (SDA) [24,25] and Exponential Amplification Reaction (EXPAR) [26,27]. However, these methods need ultra-clean work environment, expensive instrument and skilled experimenter. In summary, it is necessary to develop an affordable and easy-operate method for single base detection.

In this work, by taking advantage of enzymatic specificity and robustness, we developed a novel approach to discrimination single base

* Corresponding author. State Key Laboratory of Industrial Control Technology, Research Center for Analytical Instrumentation, College of Control Science and Engineering, Zhejiang University, 38 Zheda Road, Hangzhou, 310027, PR China.
** Corresponding author.
E-mail addresses: ddyu@zhu.edu.cn (D. Yu), jgzhou@zju.edu.cn (J. Zhou).

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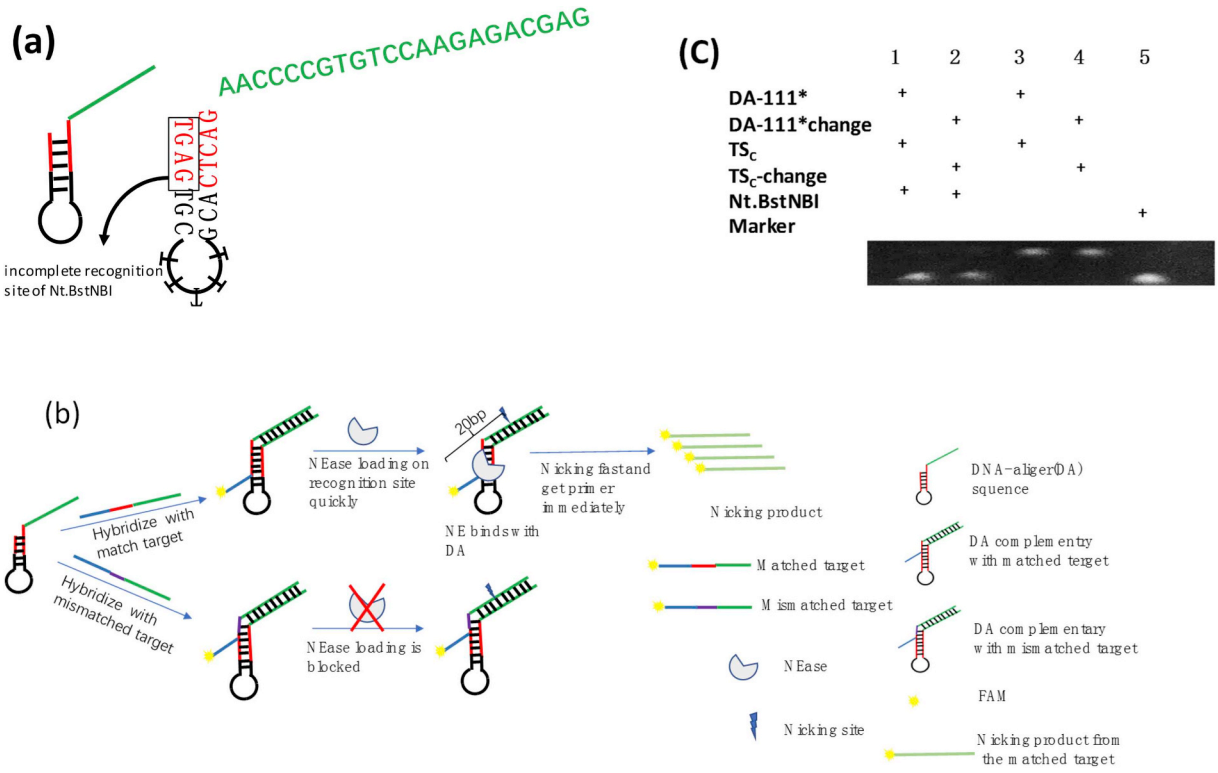

Fig. 1. (a) The structure of DA, contain the incomplete recognition site of Nt.BstNBI and a side arm complementary to target. (b) illustration of AMCMD, the recognition site of Nt.BstNBI will form when C base in TS, then Nt.BstNBI can bind to DA's stem and AMC will enable, while other base(G, T, A) in TS, AMC will fail for the incomplete recognition site to which Nt.BstNBI can't bind. (C) Polyacrylamide gel electrophoresis image of cleavage products. Line 1 and line 2 shows the cleave product with unchanged and simply modulated sequence of DA's side arm respectively, line 3 and line 4 show the uncleaved TS, line 5, as comparison, 20 nt marker. operation is consistent with above.

mismatch in a specific and programmable way by integrating an NEase and a hairpin shaped probe. The strategy is based on aligner-mediated cleavage (AMC) [28–30], sequence specific cleavage of nucleic acids, termed AMC-based single base mismatch discrimination (AMCMD). Based on Wu's work [29], we designed the DNA aligner (DA) in a stem-loop structure with an incomplete recognition site of NEase on its stem, remaining one to two matched bases on TS, and its 5′-side arm complementary to the TS. Once TS contains matched base and hybridizes with DA, the complete recognition site for NEase is formed, then AMC will enable and cleavage TS with fast speed. The main function of hairpin shaped DA is to align the enzyme with a specific locus of target and precision cleavage at the specific position. Thus, it can serve as a programmable nucleic acid "nicking" for site-specific cleavage of target. Besides, the cleavage result was verified by polyacrylamide gel electrophoresis and no DNA amplification experiments or additional expensive experimental instruments were required. Thus, the proposed AMCMD is characterized as specific, simple and programmable. Moreover, we combined AMCMD and EXPAR to create a new method for detection of single base mismatch, the short sequence obtained by AMCMD as a target to trigger EXPAR, and it has higher detection sensitivity.

2. Materials and methods

2.1. Materials and reagents

All Oligonucleotides purified by HPLC is provided by Sangon Biotech Co., Ltd. (Shanghai, China). The detailed sequences of these oligonucleotides are listed in Table S1. Endonuclease Nt.BstNBI (10,000 units/mL), Bst 2.0 WarmStart DNA polymerase (8000 units/mL), deoxyribonucleotide triphosphates (dNTPs, 10 mM), NEBuffer 3.1 (10×, 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 μg/mL BSA, pH 7.9) were purchased from New England Biolabs (Ipswich, MA). SYBR Green I (10,000×) was purchased from Invitrogen Life Technologies (Carlsbad, CA). 30% PAGE Pre-Solution (Acr/Bis, 29:1), 5×TBE Buffer (450 mM Tris, 450 mM Borate, 10 mM EDTA, pH 8.3), and TE Buffer (pH = 8.0) were purchased from Solar bio(Beijing, China), DNA grade water (DNase- and Protease-free) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Urea was from Sino pharm Chemical Reagent Co., Ltd. (Shanghai, China). The clinical serum samples of healthy person were obtained from the Hospital of Zhejiang University. All solutions were freshly prepared with DNA grade water.

2.2. Mismatch detection based on aligner-mediated cleavage (AMC)

2.2.1. Polyacrylamide gel electrophoresis

We prepared the solution with 0.1 μM DA, 0.1 μM target, 10U Nt. BstNBI and 1× NEBuffer 3.1, add water for a total volume of 25 μL.The solution was incubated at 55° for 2 h, then inactive at 95° for 5 min, the final product was store at minus 20° and spare. After that, 5 μL of each resultant was added to a 15% denaturing polyacrylamide gel having a urea concentration of 8 M, use 1×TBE (45 mM Tris, 45 mM borate, 1 mM EDTA, pH 8.3) as electrolyte, and the gel was run at 110 V constant voltage for 1 h at room temperature. The gel was analyzed with automatic gel imaging analysis system.

2.2.2. Real-time AMCMD

For real-time fluorescence detection, we divided the reaction mixture into two parts and prepared on ice. Part A consist of 0.1 μM DA, 0.1 μM template, 400 μM dNTPs, 6U Nt. BstNBI, 1×Buffer 3.1, and 2.5 μL target. Part B contained with 6U Nt. BstNBI, 0.8U, Bst 2.0 WarmStart, 0.5×SYBR Green I, 1×Buffer 3.1, and add water for a total volume of 25 μL each tube. Part A was first incubated at 55 °C for 5 min on a Bio-Rad CFX96 PCR system, then part B was added into A

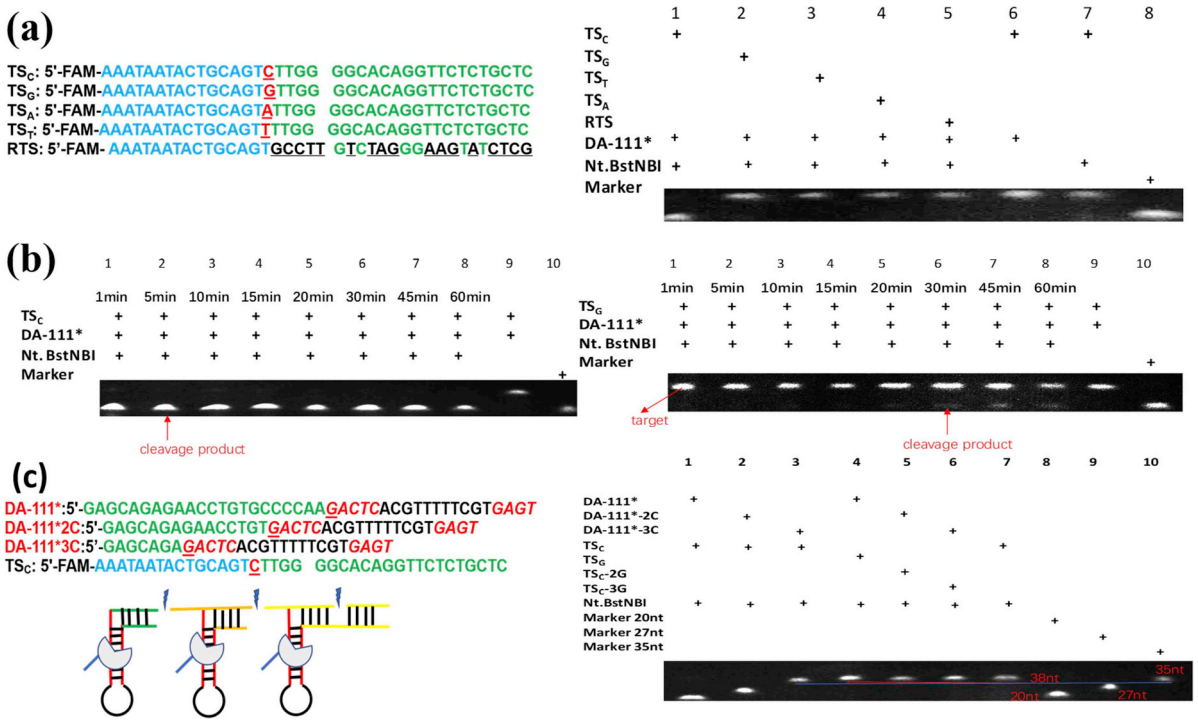

Fig. 2. (a) Polyacrylamide gel electrophoresis image of cleavage products. Line 1 shows a new and shorter band when base C in TS, line 2–4 represent the phenomenon when base G/T/A in TS, line 5 is the result for RTS, line 6–7 show the products when the absence of Nt.BstNBI/DA-111*, as comparison, line 8 is marker.(b) show the cleavage results after cutting for 1–60 min respectively, TS_C (left) and TS_G(right), line 10 is marker.(c) the scheme of programmable AMCMD, three DAs corresponding to various target base C at different sites of a FAM-labelled target strand(left), Polyacrylamide gel electrophoresis image of cleavage products. line 1–3 show three different bands of ~20, ~27 and ~35 nucleotides were respectively produced under the guidance of these Das, line 4–6 show the results when three targets are mismatched with the corresponding DA, line 7 shows the products when the absence of DA-111*, as comparison, line 8–10 20 nt, 27 nt, 35 nt markers.

immediately. Then the mixture was kept at 55° for 60 min and the real-time fluorescence was monitored at 30 s intervals.

2.3. Detection of nucleic acids spiked in human serum

In order to demonstrate the utility of AMCMD in complex biological matrix, it was performed in the presence of human serum. The serum was used, without dilution or other pretreatment, to dilute target sequence by tenfold in the series. The next.

3. Results and discussion

3.1. AMC-based single-base mismatch discrimination

Herein, we applied Nt.BstNBI as an example and detected C base mismatch successfully. The proposed AMCMD is illustrated in Fig. 1. DNA aligner (DA) as precision positioner to detect C base mismatch, is located in special hairpin structure, only with a 5′- side arm, and an incomplete recognition site of Nt.BstNBI in its stem (Fig. 1a), it contains four bases (GAGT) of Nt.BstNBI recognition site (GAGTC) in its 3′-side stem, and 5′-side arm is complementary to the target sequence (TS), while the remaining base C is in specific site of the target. When TS hybridizes with DA, base C in TS hybridize with the final base G in the sequence “CTCAG” to fulfil the complete recognition site of Nt.BstNBI, then enzyme loads on the stem and AMC will enable, nicking the TS to form a new and short band. On the contrary, when the TS is there with other base G, T or A, there is no band for the erroneous identification site affects Nt.BstNBI binding, and nicking is also blocked (Fig. 1b). Taking advantage of this, we can distinguish the match one from others and characterize results with polyacrylamide gel electrophoresis. Moreover, by simply modulating the sequence of DA's side arm, it is easy to recognize the C-mismatch at any target base (Fig. 1c). Thus, this method could be used to screen diseases caused by C base mismatches [31,32]. We further verified that AMCMD can be used to detect T base mismatches, as shown in Fig. S5.

3.2. Feasibility of AMCMD

To demonstrate the feasibility of AMCMD, four target sequences (TS_C, TS_G, TS_T, TS_A) which differ only in the target base, i.e., the complementary base C and the mismatched bases G, A and T were used. As showed in Fig. 2a, a new and shorter band can only be discovered in the case of TS_C, along with the disappearance of TS_C. This clearly indicates the cleavage of TS_C by AMC. However, TS_G ~ TS_A containing the mismatched target bases and random target sequence (RTS) cannot be cleaved by Nt.BstNBI and maintain an intact bands without new and shorter bands. This may be because the mismatched target base that results in an incomplete recognition site has great influence on the binding of Nt.BstNBI and the following cleavage. And the random sequence cannot be complementary to DA, fail to form the recognition site of Nt.BstNBI, thus blocks the binding and cleavage.

Then the cleavage rates of TS_C and TS_G by AMC were further compared. As depicted in Fig. 2b, TS_C was almost digested in less than 5 min, while TS_2 was only slightly cleaved by AMC even incubated for long as 30 min. This result confirms the ability of AMCMD to distinguish the matched one and the mismatched bases. In another experiment, the hybridization between the two bases “AG” in the sequence “CTCAG” of DA and the two continuous bases “TC” in TS also enabled AMCMD, while more continuous bases, such as “GTC” and “AGTC” in TS deprived the capability of AMCMD (Fig. S1). This endows AMCMD with a broader application for the identification of continuous two-bases mismatch like “VC”, “TD” or “VD” in the target sequence (V

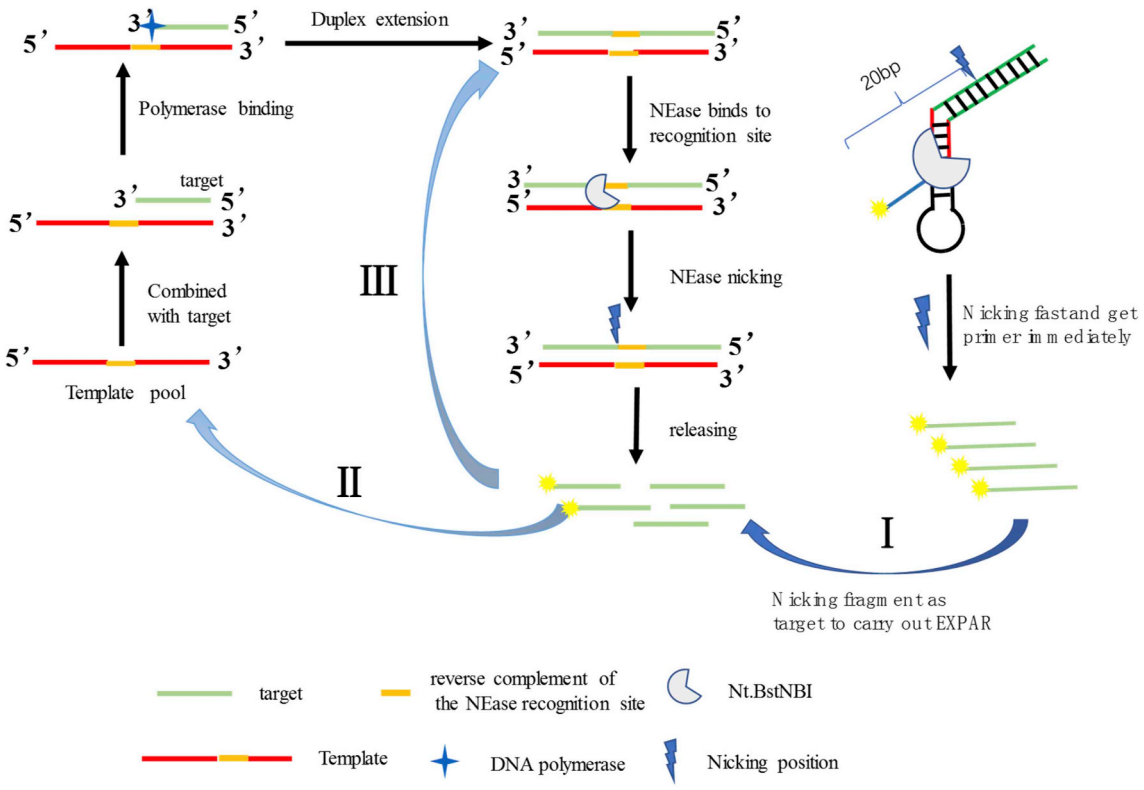

Fig. 3. Scheme of exponential amplification of reaction (EXPAR). The red fragment represents the sequence complement with the target sequence to be amplified, the green one. The amplification template, consists of two copies of the signal complement flanking the Nt. BstNBI recognition site, shown as in yellow. The target sequence is produced in the linear amplification cycle(II) for each amplification template created. When there is ample template, amplification can be performed in way, two amplification methods coexist and finally become exponentially amplification reaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

stands for the base A, C or G and D). Moreover, AMCMD functionality based on Nt.AlwI was also demonstrated, providing flexible choice for AMCMD (Fig. S2).

3.3. Programmable AMCMD

Our previous studies have proved that AMC was capable to cleave target sequence in a programmable manner, thus we believe that AMCMD will acquire the similar ability to identify the mismatch of the base C at any site. By designing three DAs corresponding to various target base C at different sites of a FAM-labelled target strand (Fig. 2c-left), the programmable cleavage pattern of AMCMD was researched. As the gel map shows (Fig. 2c-right), three different bands of ~20, ~27 and ~35 nucleotides were respectively produced under the guidance of these DAs. These results are consistent with our expectations and confirm that AMCMD is able to recognize the single-base mismatch of C at any site of target sequence by simply regulating the sequence of the DA's side arm. This endows AMCMD with notable universality.

3.4. Real-time AMCMD detection

To acquire AMC with higher sensitivity, we combined AMCMD with isothermal amplification reaction, termed a universal real-time AMCMD for identification and quantitative analysis of C-mismatch. By taking advantage of AMCMD's versatility, As showed in Fig. 3, the proposed real-time AMCMD mainly consists of two stages: cleavage initiation and exponential amplification. In cleavage initiation, AMC is firstly carried out to produce the cleaved TS (CTS) that leaves DA instantly for the greatly shortened hybridization length. Then CTS can serve as a trigger to initiate the following exponential amplification, *i.e.,* exponential amplification reaction (EXPAR). EXPAR is a simple, sensitive method, possessing a very high amplification efficiency (10^6–10^9-fold amplification within minutes), thus the real-time AMCMD also holds these features. Note that the 3′-end of DA should be blocked by a small molecule, like phosphate group, to prevent the undesired extension along the side arm, which will impede the hybridization between DA and TS.

3.5. Feasibility and sensibility of real-time AMCMD

The feasibility of real-time AMCMD was verified by conducting the amplification of five artificial sequences, *i.e.*, four TSs with C, G, A and T at the target site and a random sequence (RS). The four TSs all showed a sigmoidal fluorescence curve (Fig. 4) with a point of inflection (POI, defined as the time corresponding to the maximum slope) lower than RS and H_2O (as no-target control, NTC). In particular, the POI values of the mismatched TS_G, TS_A and TS_T were larger than those of complimentary TS_C and close to RS and NTC, indicating an excellent capability to discriminate single-base mismatch of real-time AMCMD. The sensitivity of real-time AMCMD was measured by monitoring the real-time fluorescence curves caused by various concentrations of TS_C. As we can see in Fig. S3, the POI values gradually decreased as the concentration of TS_C increased and 1 pM TS_C can be generally detected, which was comparable to some previous EXPAR methods.

3.6. Utility in complex biological matrix

To evaluate the utility of real-time AMCMD in complex biological matrix, it was carried out in human serum. To this end, 100 pM TS_C, TS_G, TS_A and TS_T were spiked into serum and subjected to real-time AMCMD, respectively. As showed in Fig. S4, the real-time fluorescence curves caused by TS were similar to that obtained by TS in buffer, also

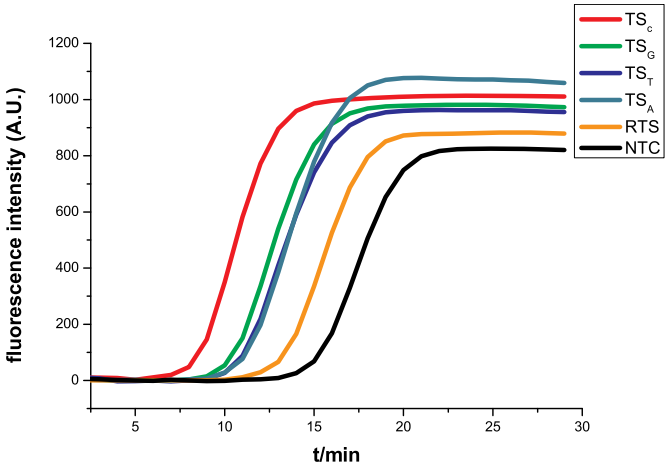

Fig. 4. Real-time fluorescence curves caused by TS_C, TS_G, TS_T, TS_A, RTS and NTC (no target control).

effectively discriminating the target base C and the mismatched G, A and T. Therefore, the proposed method has a good compatibility with real complex biological matrix.

4. Conclusion

In summary, a programmable site-specific cleavage method, only needs one endonuclease, has been developed to detection of single base mismatch. We have compared the performance of this technology with some reported methods, and the relevant data is shown in Table S1. We certified AMCMD acquires the ability to identify the mismatch of the base C at any site by simply modulating the sequence of DA's side arm. This kind ability makes it possible to discriminate C base mismatch in a programmable way. Not only the C base, in this paper, we proved that this method can also apply equally to other bases mismatch. We verified the cleavage result by using polyacrylamide gel electrophoresis analysis. which greatly simplifies the experimental process. What's more, combined with EXPAR, AMCMD can effectively improve sensitivity. For further study, we can modify the DNA sequence on stem, based on the recognition sequence of the selected NEase, to determine various single base mismatches and extend its application.

Conflicts of interest

There are no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2019.03.106.

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