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A label-free signal amplification assay for DNA detection based on exonuclease III and nucleic acid dye SYBR Green I

Aihua Zheng [a,b], Ming Luo [a], Dongshan Xiang [a], Xia Xiang [a], Xinghu Ji [a], Zhike He [a,*]

[a] Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, PR China

[b] School of Public Administration, Hubei University of Medicine, Shiyan 442000, PR China

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ABSTRACT

We have developed a new fluorescence method for specific single-stranded DNA sequences with exonuclease III (Exo III) and nucleic acid dye SYBR Green I. It is demonstrated by a reverse transcription oligonucleotide sequence (target DNA, 27 bases) of RNA fragment of human immunodeficiency virus (HIV) as a model system. In the absence of the target DNA, the hairpin-probe is in the stem-closed structure, the fluorescence of SYBR Green I is very strong. In the presence of the target DNA, the hairpin-probe hybridizes with the target DNA to form double-stranded structure with a blunt 3′-terminus. Thus, in the presence of Exo III, only the 3′-terminus of probe is subjected to digestion. Exo III catalyzes the stepwise removal of mononucleotides from this terminus, releasing the target DNA. The released target DNA then hybridizes with another probe, whence the cycle starts anew. The signal of SYBR Green I decreases greatly. This system provides a detection limit of 160 pM, which is comparable to the existing signal amplification methods that utilized Exo III as a signal amplification nuclease. Due to the unique property of Exo III, this method shows excellent detection selectivity for single-base discrimination. More importantly, superiors to other methods based on Exo III, these probes have the advantages of easier to design, synthesize, purify and thus are much cheaper and more applicable. This new approach could be widely applied to sensitive and selective nucleic acids detection.

1. Introduction

Highly sensitive and selective single-stranded DNA (ssDNA) sequences detection is of great demand in gene profiling, drug screening, clinical diagnostics and environmental analysis, food safety and a variety of biomedical studies [1–3]. Motivated by this demand, various techniques for specific ssDNA sequences have been developed, such as electrochemical [4], fluorescent [5], and chemiluminescent [6] methods. Fluorescence, a powerful and important analytical technique, has been proved of particular utility in this regard due to its inherent properties, which have motivated us to apply this technology and methodology in DNA assay alternative to conventional DNA detection methods [7–9].

Among available methods, signal amplification strategy based on nuclease, such as polymerase [10], FokI enzyme [11] and nicking endonuclease [12] have been employed to produce stronger optical signal in various DNA sensors. Polymerase-dependent amplification methods suffer from some drawbacks including complex handling procedures, false-positive signals, and vulnerability to contamination [10]. Despite their high sensitivity and selectivity, restriction endonuclease-dependent amplification approaches generally require a specific enzymatic recognition sequence in the target sequence which cannot be fulfilled in all systems [12]. To solve the above problems, it is important to find a universal nuclease alternative to polymerase and restriction endonuclease.

Exonuclease III (Exo III) specifically digests double-stranded DNA (dsDNA) from 3′-OH blunt or recessed end, while it exhibits less activity on ssDNA or 3′-protruding termini of dsDNA [13,14]. Compared with restriction endonuclease, Exo III does not require a specific recognition site, so cleavage occurs irrespective of the sequence present at the blunt terminus. This function was used to develop different Exo III-based amplified detection platforms that involved the biocatalytic recycling of the target DNA by Exo III [15–20]. For example, Zuo et al. have developed an Exo III-aided target recycling method for sensitive and selective amplified fluorescence DNA detection [15]. By this approach, they significantly improved the sensitivity of traditional, unamplified molecular beacons (by 3 orders of magnitude). However, this method relies on a double-labeled molecular beacon that is modified with a CAL Fluor Red 610 fluorophore at its 5′-terminus and a Black Hole Quencher at an internal position. This dye-modified oligonucleotide probe brings about complexity and high cost which may limit its practical use.

* Corresponding author. Tel.: +86 27 68756557; fax: +86 27 68754067.
E-mail address: zhkhe@whu.edu.cn (Z. He).

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Herein, we propose a label-free probe as one type of signaling probe to use in the Exo III-aided cyclic amplification detection method. In comparison with other Exo III-aided DNA detection platform, these probes have the advantages of easier to design, synthesize, purify and thus are much cheaper and more applicable. With these probes, a sensitive nucleic acids detection method was developed with the detection limit of less than 160 pM, which is comparable to the existing signal amplification methods that utilized Exo III as a signal amplification nuclease [17–20]. Due to the unique property of Exo III, this detection platform shows excellent detection selectivity for single-base discrimination. More importantly, superior to other methods based on nicking and FokI endonuclease, this target sequence-independent platform is generally applicable for DNA sensing. This new approach could be widely applied to sensitive and selective nucleic acids detection.

2. Material and methods

2.1. Chemical and material

Exonuclease III (Exo III) was purchased from Takara Biotechnology Co. Ltd. (Dalian, China) and used without further purification. Nucleic acid dye SYBR Green I is obtained from Shanghai Rui Ann biotechnology limited company (China), the original solution is a 10,000-fold concentrated solution prepared with anhydrous DMSO, the working solution is prepared by concentrated solution diluted 10,000-fold with water. The human serum sample was supplied by the Zhongnan Hospital of Wuhan University. The other chemical reagents are all of analytical reagent grades and purchased from Sigma Chemical Co., Ltd. (USA). Tris–HCl buffer solution (Tris) is prepared using 0.1 M Tris and 1 M HCl. All oligonucleotides with different sequences are synthesized and HPLC purified by Sangon Biotechnology Co., Ltd. (Shanghai, China) and were stored in 10 mM Tris–HCl buffer (1 mM EDTA, pH=8.0). The following are the sequences:

HIV probe (HP): 5/-TAT TTG GAA TGT TAT
TCC A AA TAT CTT CT-3/
Reverse transcription oligonucleotide sequence of RNA fragment of HIV (HT): 5/-AGA AGA TAT TTG GAA
TAA CAT GAC CTG-3/

Mismatched target sequences:

MT1: 5/-AGA AGA TAT TAG GAA TAA CAT GAC CTG-3/
MT3: 5/-AGA AGC TAT TAG GAA CAA CAT GAC CTG-3/

2.2. Apparatus

Fluorescence spectra data are collected with a RF-5301PC fluorescence spectrophotometer (Shimadzu, Tokyo, Japan) equipped with a 150 W xenon lamp (Ushio Inc., Japan), spectrometer slits are set for 10 nm band-pass. An incubator is used to control the temperature precisely. Ultrapure water is produced by a Millipore-Q Academic purification set (Millipore, Bedford, MA, USA). A pB-10 potentiometer (Sartorius) is used to measure pH of the solutions.

2.3. Detection of the target DNA

The Exo III-based signal amplification reaction was performed by mixing 50 μL of 1×10^{-7} M HP, 70 units of Exo III and varying concentrations of HT to a final volume of 500 μL in the 50 mM Tris–HCl buffer (0.1 M NaCl, 5 mM $MgCl_2$, pH 8.0), followed by incubation at 37 °C for 60 min. The reaction solution is cooled to room temperature, 35 μL SYBR Green I working solution is added and incubated for 60 min at room temperature, and then the fluorescence intensities of SYBR Green I are detected using synchronous scanning fluorescence spectrometry. The fixed wavelength difference ($\Delta\lambda$) of synchronous scanning fluorescence spectroscopy is set for 20 nm.

2.4. Comparison of the determination results in different fluids

The serum sample was spiked with 0.5, 1.5, and 2.5 nM HT to test the performance of the assay in complex matrixes. The Exo III-based signal amplification reaction is according to the procedure of detection of the target DNA mentioned above, and the fluorescence intensities of SYBR Green I are measured.

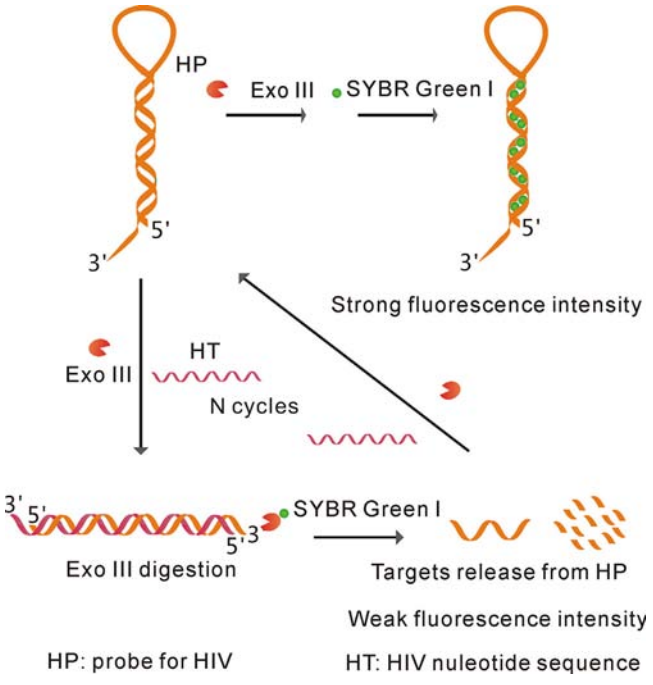
2.5. Single nucleotide polymorphism (SNP) analysis

20 μL of 5×10^{-8} M HT and the same amount of other mismatched strands are used to perform the Exo III-based signal amplification reaction according to the procedure of detection of the target DNA mentioned above, and the fluorescence intensities of SYBR Green I are measured.

3. Results and discussion

3.1. Principle of the platform

As shown in Scheme 1, this fluorescent detection system consists of Exo III and the hairpin-probe HP. In the absence of HT, the probe is in stem-loop structure. SYBR Green I binds to the stem of probe, the fluorescence intensity of the SYBR Green I will be very strong. In the presence of HT, the hairpin-probe HP hybridizes with HT to form double-stranded structure with a blunt 3′-terminus. It should be noted that the target only hybridizes partially with the probe, leaving a single-stranded overhang at its 3′-terminus that is resistant to the cleavage by Exo III because the resistance degree of Exo III on ssDNA at 3′-protruding terminus is dependent on the length of the extension, with extension 4-nt



Scheme 1. The detection principle for the target DNA.

or longer being essentially resistant to cleavage [14]. Thus, in the presence of Exo III, only the 3′-terminus of probe is subjected to digestion. Exo III catalyzes the stepwise removal of mononucleotides from this terminus, releasing HT. The released HT then hybridizes with another probe, whence the cycle starts anew. The signal of SYBR Green I significantly decreases. Thus, fluorescence quantitative detection for the target DNA can be realized through the fluorescence intensities decrease of SYBR Green I.

3.2. Effect of the concentration of SYBR Green I on the fluorescence intensity

We first investigate the effect of the concentration of SYBR Green I on the assay. As shown in Fig. 1, the change of SYBR Green I fluorescence intensity increases with the increasing the concentration of SYBR Green I in the range from 0 to 35 μL and then levels off in the range from 35 to 40 μL. Therefore, 35 μL of SYBR Green I is selected in this assay.

3.3. Effect of incubation temperature on the fluorescence intensity

In this experiment, the activity of Exo III is highly sensitive to incubation temperature that also influences the binding kinetics between the probe and the target DNA. Thus, as an essential factor of the experiment, the suitable incubation temperature is investigated in this study by detecting 1 nM target DNA at 4, 25, 37, and 45 °C. As shown in Fig. 2, the change of fluorescence intensity increases with the increasing incubation temperature in the range from 4 to 37 °C and then decreases in the range from 37 to 45 °C. Therefore, 37 °C is considered to be the optimum incubation temperature in this assay.

3.4. Effect of incubation time on the fluorescence intensity

We then investigate the influence of the incubation time for the performance of the assay upon analyzing HT at a concentration corresponding to 1 nM. As shown in Fig. 3, the change of fluorescence intensity is greatly increased with the increase of incubation time at the early stage, and reaches the maximum after 60 min. Therefore, the incubation time of 60 min is adopted for this assay.

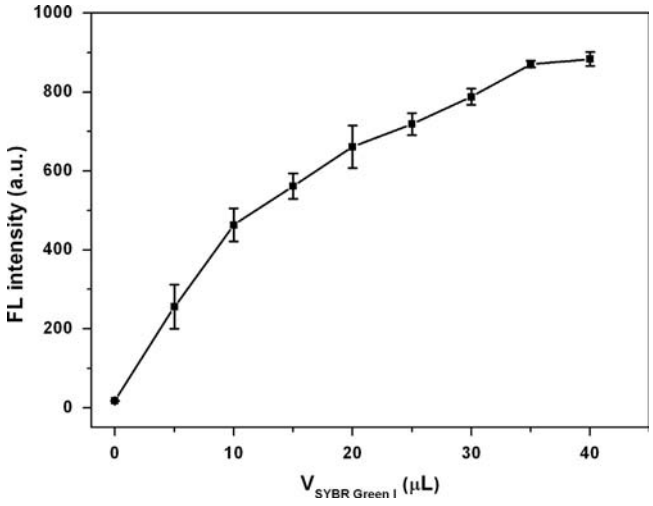


Fig. 1. Effect of the concentration of SYBR Green I on the fluorescence intensity. Concentration of HP: 10 nM. Concentration of HT: 1 nM.

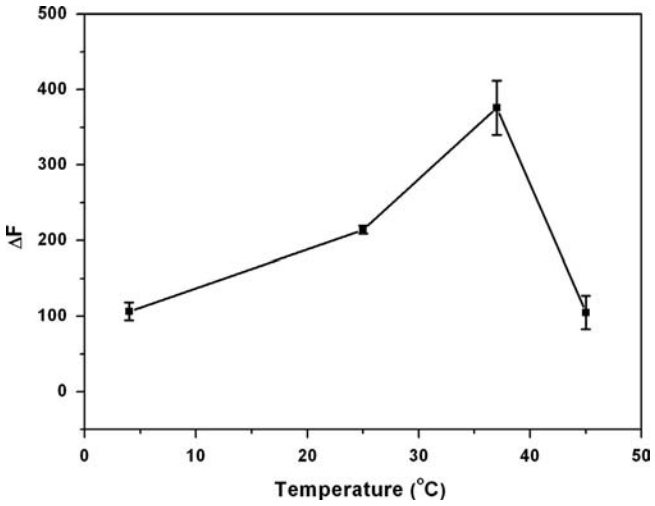


Fig. 2. Effect of incubation temperature on the fluorescence intensity. Concentration of HP: 10 nM. Concentration of HT: 1 nM.

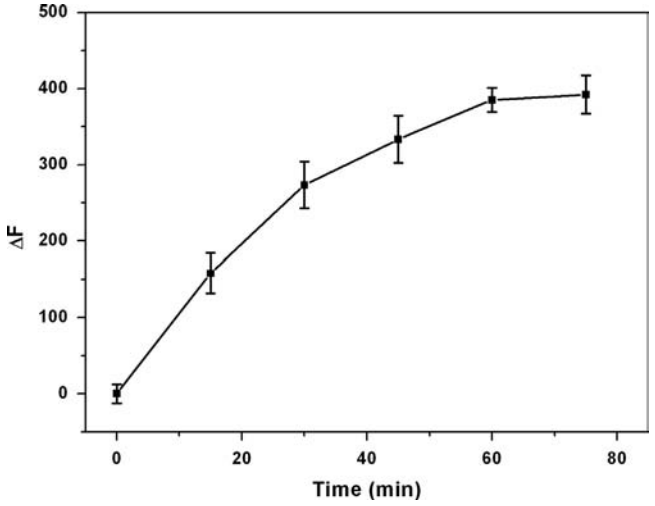


Fig. 3. Effect of incubation time on the fluorescence intensity. Concentration of HP: 10 nM. Concentration of HT: 1 nM.

3.5. Effect of ionic strength on the fluorescence intensity

In this experiment, the ionic strength influences the stabilization of dsDNA. We investigate the effect of ionic strength on the assay. As shown in Fig. 4, the change of fluorescence intensity increases with the increasing concentration of NaCl in the range from 0.05 to 0.1 M and then decreases in the range from 0.1 to 0.4 mM. Therefore, 0.1 M of NaCl is selected in this assay.

3.6. Effect of the amount of Exo III on the fluorescence intensity

To investigate the influence of the amount of Exo III used in this assay on DNA detection, the change of fluorescence intensity is measured by using 0, 30, 50, 70, and 90 units of Exo III. The blank sample is treated in the same way without HT. Fig. 5 depicts the influence of the amount of Exo III used in assay on the change of fluorescence intensity. The change of fluorescence intensity increases with the increasing amount of Exo III in the range from 0 to 70 units and then levels off in the range from 70 to 90 units. Therefore, 70 units of Exo III is selected in this assay.

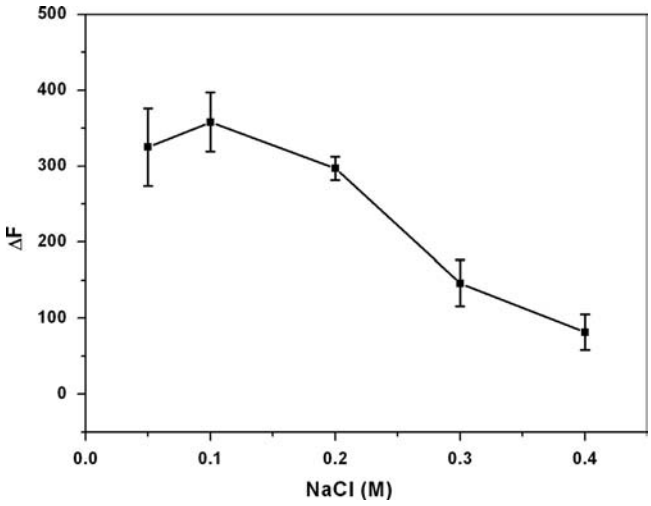


Fig. 4. Effect of ionic strength on the fluorescence intensity. Concentration of HP: 10 nM. Concentration of HT: 1 nM.

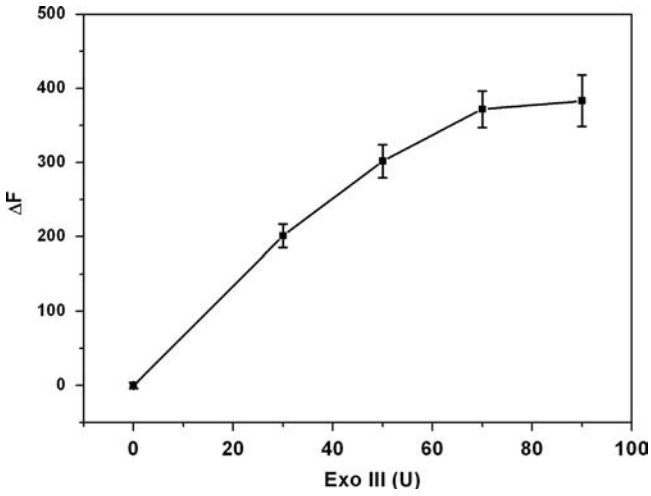


Fig. 5. Effect of the amount of Exo III on the fluorescence intensity. Concentration of HP: 10 nM. Concentration of HT: 1 nM.

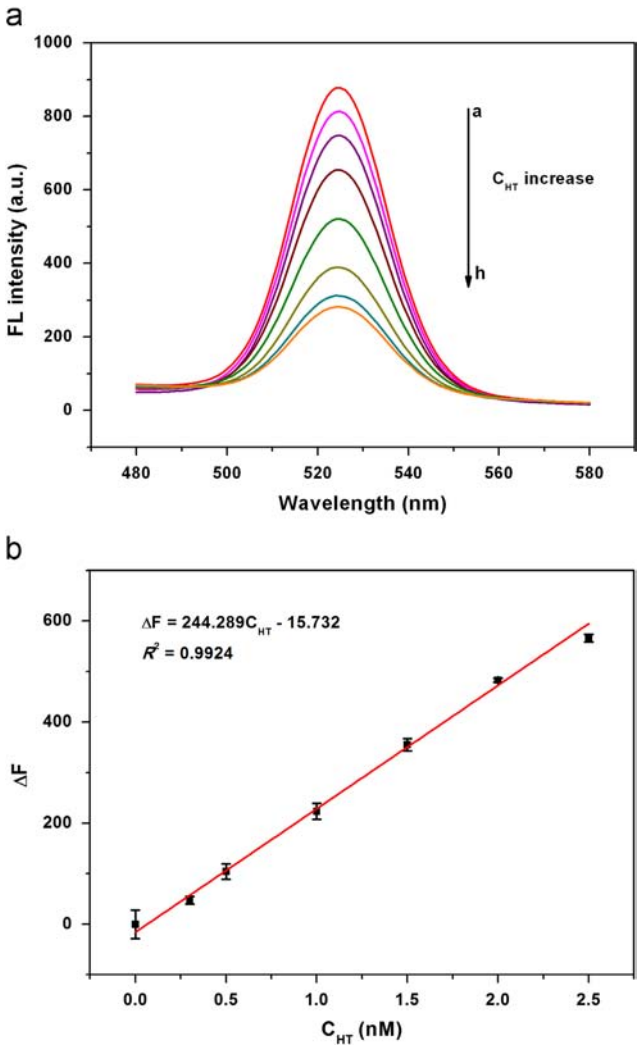


Fig. 6. Changes in the fluorescence spectra of the sensing system upon increasing the concentration of HT: 0, 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 (a→h, nM). Inset: The linear relationship between the change of fluorescence intensity (ΔF) of SYBR Green I and the concentration of the target DNA (C_{HT}). ($\Delta F = F_0 - F$, F_0 and F are the fluorescence intensities of SYBR Green I in the absence and the presence of the target DNA, respectively).

3.7. The linear correlation and the detection limit

Under the optimum conditions, the relationship between the change of fluorescence intensity of SYBR Green I (ΔF) and the concentration of target DNA (C_{HT}) is investigated. Fig. 6 shows synchronous scanning fluorescence spectrum in the presence of different C_{HT} and the linear relationship between ΔF and C_{HT}. A good linear relationship can be obtained under the optimal conditions. For the change of fluorescence intensity of SYBR Green I and the concentration of target DNA, the linear range is from 0.3 to 2.5 nM, and the fitted regression equation is $\Delta F = 244.289\ C_{HT} - 15.732$ ($R^2 = 0.9924$). The detection limit of HT was determined to be 160 pM based on a linear fitting and the noise level of 3σ (where σ is the standard deviation of a blank solution, $n = 11$). The detection limit is comparable to the existing signal amplification methods that utilized Exo III as a signal amplification nuclease [17,20]. In order to investigate the reproducibility, the change of fluorescence intensities of SYBR Green I in the presence of 2 nM HT was recorded. The relative standard deviation (*RSD*) for the change of fluorescence intensities with seven determinations is 3.90%, indicating excellent reproducibility of this method.

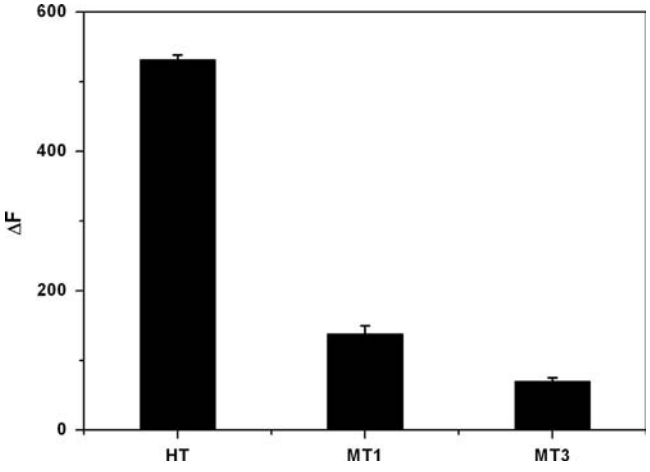


Fig. 7. The change of fluorescence intensities histogram of the sensing system including 2 nM of the complementary target HT, single-base mismatched target (MT1) and three-base mismatched target (MT3) hybridized with 10 nM HP. ($\Delta F = F_0 - F$, F_0 and F are the fluorescence intensities of SYBR Green I in the presence of 2 nM of HT, MT1 and MT3 hybridized with 10 nM HP.)

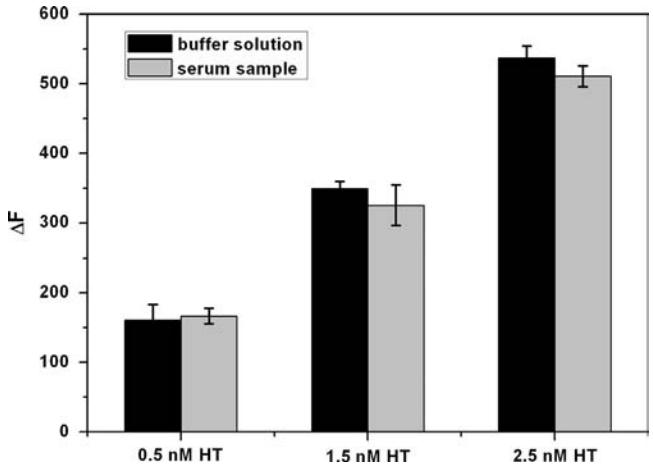


Fig. 8. Comparison of the determination results in the Tris–HCl buffer solution and the serum.

3.8. SNP analysis

In this study, we evaluate the specificity of this method. The assay is challenged with single-base mismatched target DNA (MT1) and three-mismatched target DNA (MT3). As shown in Fig. 7, the change of fluorescence intensity of MT1was about 25% of the value obtained upon addition of HT into mixtures. It is worthwhile to point out that the addition of MT3 only leads to very slight fluorescence change. The proposed method can provide an excellent capability in differentiating between perfectly matched and mismatched DNA targets. Therefore, this proposed method has a high selectivity, and it holds a great opportunity to allow SNP analysis.

3.9. Comparison of the determination results in different fluids

In order to examine the application of the proposed method in complex fluid, the target DNA is detected in the serum. The original serum is diluted 50 times by 50 mM Tris–HCl buffer (0.1 M NaCl, 5 mM $MgCl_2$, pH 8.0). And the determination results in the serum are compared with that in the Tris–HCl buffer solution. The results (Fig. 8) show the change of fluorescence intensity of SYBR Green I remains basically unchanged in different fluids at the same concentration of the target DNA. The results show that this assay has good performance in complex fluid.

4. Conclusion

In conclusion, we have developed a facile, label-free, nuclease-based amplified DNA detection platform based on Exo III and the nucleic acid dye SYBR Green I. The results indicate that this novel method can detect as low as 160 pM target DNA by simply mixing the probe, SYBR Green I, Exo III, and the complementary target DNA. Moreover, this detection platform is also selective to differentiate mismatched DNA, which makes it promising for biomedical applications. Critically, no sophisticated experimental techniques or any chemical modification of DNA is required. The assay can be accomplished by using a common spectrophotometer. It is simple in design, fast in operation and possesses high sensitivity and selectivity.

Acknowledgments

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