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Highly sensitive fluorescence quantitative detection of specific DNA sequences with molecular beacons and nucleic acid dye SYBR Green I

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ABSTRACT

A highly sensitive fluorescence method of quantitative detection for specific DNA sequence is developed based on molecular beacon (MB) and nucleic acid dye SYBR Green I by synchronous fluorescence analysis. It is demonstrated by an oligonucleotide sequence of wild-type HBV (target DNA) as a model system. In this strategy, the fluorophore of MB is designed to be 6-carboxyfluorescein group (FAM), and the maximum excitation wavelength and maximum emission wavelength are both very close to that of SYBR Green I. In the presence of targets DNA, the MBs hybridize with the targets DNA and form double-strand DNA (dsDNA), the fluorophore FAM is separated from the quencher BHQ-1, thus the fluorophore emit fluorescence. At the same time, SYBR Green I binds to dsDNA, the fluorescence intensity of SYBR Green I is significantly enhanced. When targets DNA are detected by synchronous fluorescence analysis, the fluorescence peaks of FAM and SYBR Green I overlap completely, so the fluorescence signal of system will be significantly enhanced. Thus, highly sensitive fluorescence quantitative detection for DNA can be realized. Under the optimum conditions, the total fluorescence intensity of FAM and SYBR Green I exhibits good linear dependence on concentration of targets DNA in the range from 2×10^{-11} to 2.5×10^{-9} M. The detection limit of target DNA is estimated to be 9×10^{-12} M (3σ). Compared with previously reported methods of detection DNA with MB, the proposed method can significantly enhance the detection sensitivity.

1. Introduction

Highly sensitive and selective DNA detection is vitally important in biochemistry, molecular biology, microbiology, genetics and molecular medicine. It is because many human genetic diseases are caused by genetic mutations, and DNA detection can provide useful diagnostic information for identification of disease [1–10]. In recent years, a lot of methods for DNA detection have been developed [11–16]. In these methods, MBs have been widely applied in various fields, for it has outstanding advantages including easy synthesis, simple operation, high selectivity, and real-time detection capability [14,17,18]. For example, Baptista et al. have developed a real-time monitoring method for RNA synthesis by Gold-nanobeacons [19]. Song et al. took advantage of gold nanoparticles labeled molecular beacons to develop a simultaneous detection method for multiple nucleic acids [8]. While MBs have been applied successfully in many fields, their utility for quantification in the practical application is very limited. And the low detection sensitivity of MB is one of the main reasons [14,18].

SYBR Green I is an unsymmetrical cyanine dye, it has no fluorescence when there is only single-stranded DNA (ssDNA). However, the fluorescence signals will be greatly enhanced when SYBR Green I bind to dsDNA. It has been reported that SYBR Green I exhibits an 800- to 1000-fold fluorescence enhancement upon binding to dsDNA [20]. And consequently, SYBR Green I has very high sensitivity in DNA detection. Unfortunately, SYBR Green I has no selectivity when it binds to dsDNA, the detection for DNA using SYBR Green I alone cannot markedly distinguish the perfectly complementary sequence from the single-base mismatched sequence [21–23].

In recent years, several methods to increase sensitivity by spectral combination were developed [23,24]. In order to improve the detection sensitivity of DNA based on the high selectivity of detection method, a highly sensitive and selective detection method for specific DNA sequence is developed by synchronous fluorescence analysis based on MB and nucleic acid dye SYBR Green I. This analytical method not only uses the good selectivity of MB, but also uses the high sensitivity of SYBR Green I. In this strategy, the fluorophore of MB is designed to be FAM. In the absence of targets DNA, the MBs are in the stem-closed form, the fluorophore FAM and the quencher BHQ-1 are close to each other, the fluorescence of FAM is quenched by BHQ-1. At this time, the interaction between SYBR Green I and MBs is too

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weak because the double-strand portion of MB is very short. So the fluorescence signals of FAM and SYBR Green I are both very low. In the presence of targets DNA, the MBs hybridize with the targets DNA and form dsDNA, the fluorophore FAM is separated from the quencher BHQ-1, and the fluorophore FAM emits fluorescence. At the same time, SYBR Green I binds to dsDNA, the fluorescence intensity of SYBR Green I is significantly enhanced. In this strategy, the maximum excitation wavelength and maximum emission wavelength of FAM and SYBR Green I are both very close. When targets DNA are detected by synchronous fluorescence analysis, the fluorescence peaks of FAM and SYBR Green I overlap completely, and the fluorescence signal of system will be significantly enhanced. Thus, highly sensitive fluorescence quantitative detection for DNA can be realized through the structure-induced fluorescence enhancement of MBs.

Compared with previously reported methods of detection DNA with MBs, this proposed method has three major advantages. First, the maximum excitation wavelength and maximum emission wavelength of FAM and SYBR Green I are both very close in this analytical system, the fluorescence peaks of two dyes overlap completely and the detection sensitivity of analytic method is greatly improved, and highly sensitive quantification detection for DNA is achieved. Second, this proposed method not only uses the high sensitivity of SYBR Green I to significantly enhance the detection sensitivity, but also uses the good specificity of MBs to guarantee the selectivity of the analytic method. Third, targets DNA are detected by synchronous fluorescence analysis, which avoids the interference of Raleigh light scattering signal to the fluorescence signal of dye and improves the detection sensitivity.

2. Material and methods

2.1. Apparatus and reagents

Fluorescence spectra are obtained by a RF-5301PC spectrophotometer (Shimadzu, Japan), spectrometer slits are set for 10 nm band-pass. The fixed wavelength difference ($\Delta\lambda$) of synchronous fluorescence analysis is set for 27 nm. A pB-10 potentiometer (Sartorius) is used to measure pH of solutions. A PCR system 2700 (Applied Biosystems) is used to amplify the target DNA sequences.

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 the concentrated solution to dilute 10,000-fold with water. The other chemical reagents are all analytical reagent grades and purchased from Sigma Chemical Co. (USA) and Sinopharm Chemical Reagent Co. (China). Ultra-pure water is produced by a Millipore-Q Academic purification set (Millipore, Bedford, MA, USA). The Brittom–Robinson buffer solution (BR, 0.04 M) is prepared using 0.04 M phosphoric acid, 0.04 M acetic acid, 0.04 M boric acid and 2 M sodium hydroxide; Phosphate buffer solution (PB, 0.2 M) is prepared using 0.2 M sodium dihydrogen phosphate anhydrous and 0.2 M disodium hydrogen phosphate; Boric acid-sodium borate buffer solution (B, 0.2 M) is prepared using 0.2 M Boric acid and 0.2 M sodium borate, Tris–HCl buffer solution (Tris, 0.1 M) is prepared using 0.1 M Tris and 0.1 M HCl. All the synthetic oligonucleotides are obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (China). The sequences are shown below:

The sequence of two primers:

Upstream primer (P1): 5′-CACCAAATGCCCCTATCTTA-3′
Downstream primer (P2): 5′-GTTTCCCACCTTATGAGTCC-3′
Molecular beacon probe (MB): 5′-FAM-$(CH_2)_6$-GCCGATCTT-CTGCGACGCGGCG GC-$(CH_2)_6$-BHQ-1-3′

Target oligonucleotide sequence of wild-type HBV (T): 5′-CCGCGTCGCAGAAGAT-3′
The perfectly complementary sequence of target DNA (cDNA): 5′-ATCTTCTGCGACGCGG-3′

Mismatched target sequences:

MT1: 5′-CCGCGTCGTAGAAGAT-3′
MT2: 5′-CCGCATCGCATAAGAT-3′
MT3: 5′-CCGAGTCGTAGACGAT-3′

2.2. Procedure for preparation of sample

A hybridization reaction is performed by mixing the MBs probe and the targets DNA. First, a certain amount of targets DNA is added into the appropriate concentration of MB probes and mix well, and then buffer solution is added into a final volume of 400 μL and incubated certain time at a certain temperature. In this experiment, the purpose of the incubation is to melt the stem of the MBs and accelerate the speed of hybridization reaction between MBs and targets DNA. Afterwards, the solution is cooled to room temperature, and then nucleic acid dye SYBR Green I is added. In a typical experiment, 100 μL of 1×10^{-8} M target DNA in hybridization buffer is added into 100 μL of 1×10^{-8} M MBs probe and mix well, and then buffer solution is added into a final volume of 400 μL and incubated for 3 min at 70 °C. The solution is cooled to room temperature, 100 μL working solution of SYBR Green I is added and incubated for 5 min at room temperature, and then the fluorescence signals are detected by synchronous scanning fluorescence spectroscopy.

2.3. Detection of target DNA

Detection of target DNA is performed by measuring the total fluorescence intensity of FAM and SYBR Green I using synchronous fluorescence analysis. The wavelength interval between the maximum excitation and emission wavelength of FAM is 28 nm, and that of SYBR Green I is 26 nm. In this method, the fixed wavelength difference ($\Delta\lambda$) of synchronous fluorescence analysis is set for 27 nm.

2.4. Analysis of real sample

The oligonucleotide sequence of real sample is obtained by asymmetric PCR. Asymmetric PCR is performed in reaction system of 50 μL containing two primers (the ratio of P1 to P2 is 40 to 1, and the sequences are shown in 2.1). 5 μL of DNA template from HBV-infected blood sample, 10 μL of 2.5 mM $MgCl_2$, 5 μL of PCR buffer, 4 μL of 2.5 mM dNTPs, 2 μL of 2 μM primer of P1, 1 μL of 0.1 μM primer of P2, 0.5 μL of enzyme, and ultra-pure water are added to a final volume of 50 μL. The DNA was amplified by 35 cycles of denaturation at 94 °C for 90 s, annealing at 55 °C for 45 s, and extension at 72 °C for 60 s. The PCR was stopped by holding the solution at 72 °C for 5 min. Followed, 100 μL of 1×10^{-8} M MBs probe is added, and then buffer solution is added into a final volume of 400 μL and incubated for 3 min at 70 °C. The solution is cooled to room temperature, 100 μL working solution of SYBR Green I is added and incubated for 5 min at room temperature, and then the fluorescence signals are detected by synchronous fluorescence analysis.

2.5. The specificity analysis of the detection method for DNA with MBs and SYBR Green I

2×10^{-9} M targets DNA and the same amount of other mismatched strands (MT1, MT2, MT3) are used to perform the

hybridization reactions according to the procedure of preparation of sample mentioned above, and the total fluorescence intensity of FAM and SYBR Green I is measured.

2.6. The specificity analysis of the detection method for DNA with individual SYBR Green I

100 μL of 1×10^{-8} M targets DNA and the same amount of other mismatched strands (MT1, MT2, MT3) in buffer solution are added separately into 100 μL of 1×10^{-8} M cDNA and mix well, and then buffer solution is added into a final volume of 400 μL and incubated for 5 min at room temperature. Followed, 100 μL working solution of SYBR Green I is added and incubated for 5 min, and then the fluorescence signals are detected by synchronous fluorescence analysis.

3. Results and discussions

3.1. The principle of detection

The detection principle for target DNA based on MBs and nucleic acid dye SYBR Green I is depicted in Fig. 1. SYBR Green I has no fluorescence when there is only ssDNA. However, the fluorescence signals will be greatly enhanced when SYBR Green I bind to dsDNA. SYBR Green I exhibits an 800- to 1000-fold fluorescence enhancement upon binding to dsDNA. In the absence of targets DNA, the MBs are in the stem-closed form, the fluorophore FAM and the quencher BHQ-1 are close to each other, the fluorescence of FAM is quenched by BHQ-1. At this time, the interaction between SYBR Green I and the MBs is very weak because the double-strand portion of MB is very short. So the fluorescence signals of FAM and SYBR Green I are both very low. In the presence of targets DNA, the MBs hybridize with the targets DNA and form dsDNA, the fluorophore FAM is separated from the quencher BHQ-1, and the fluorescence of FAM is recovered. At the same time, SYBR Green I bind to dsDNA, the fluorescence intensity of SYBR Green I is significantly enhanced. In this strategy, the maximum excitation wavelength of FAM is 497 nm, and that of SYBR Green I is 498 nm; the maximum emission wavelength of FAM is 525 nm, and that of SYBR Green I is 524 nm (Fig. 2). That is to say, the maximum excitation wavelength and maximum emission wavelength of FAM and SYBR Green I are both very close. When target DNA is detected by synchronous fluorescence analysis, the two fluorescence peaks of FAM and SYBR Green I can overlap completely, so the fluorescence signal is significantly enhanced. Thus, highly sensitive fluorescence detection for DNA can be realized through the structure-induced fluorescence enhancement of MBs. In this strategy, the enhancement of fluorescence signal of MBs is caused by the conformation changes of MBs resulting from hybridization with target DNA, and that of SYBR Green I is caused by SYBR Green I binding to dsDNA. Thereby, this analytical method still has very high selectivity.

3.2. The feasibility of analytical method

The feasibility of this analytical method is evaluated in this study. Fig. 2 shows the synchronous scanning fluorescence spectra of DNA sample and blank sample. In the absence of SYBR Green I, the fluorescence signals of blank sample (MBs) and sample (MBs+targets DNA) are both very weak (Fig. 2, curves a and b). The fluorescence signal of sample is approximately three times higher than that of background signal. In the presence of SYBR Green I, although the background signal (MBs+SYBR Green I) is increased, the increment of the sample signal (MBs+targets DNA+SYBR Green I) is greater than that of background signal. The fluorescence signal of sample is approximately five times higher than that of background signal (Fig. 2, curves c and d). This indicates that the introduction of SYBR Green I can significantly improved the signal-to-noise of analytical method. Compared with individual MBs, the detection sensitivity is significantly improved because the total fluorescence intensity of SYBR Green I and MBs is far larger than that of MBs. Fig. 2 shows that the total fluorescence signals of SYBR Green I and MB are approximately ten times higher than that of individual MB (Fig. 2, curves b and d). In addition, Fig. 2 also shows the two fluorescence peaks of FAM and SYBR Green I overlap completely and form a fluorescence peak. This indicates that the design is feasible.

3.3. Optimization of assay conditions

The parameters affecting the hybridization reaction and the fluorescence intensities of FAM and SYBR Green I are optimized. Buffer solution cannot only affect the hybridization reaction between MBs and targets DNA, but also directly affect the fluorescence intensities of FAM and SYBR Green I. In this experiment, four different buffer solutions (PB, BR, B, and Tris) are investigated, the result shows that the best buffer solution is Tris (Supporting information Fig. S1).

The pH of buffer solution can also affect the hybridization of MBs with targets DNA and combination of SYBR Green I with dsDNA. The result shows that the fluorescence intensity of system increased with the increasing pH of buffer solution from 7.4 to 8.6 and then decreased slowly (Supporting information Fig. S2). Therefore, pH 8.6 is selected in this study.

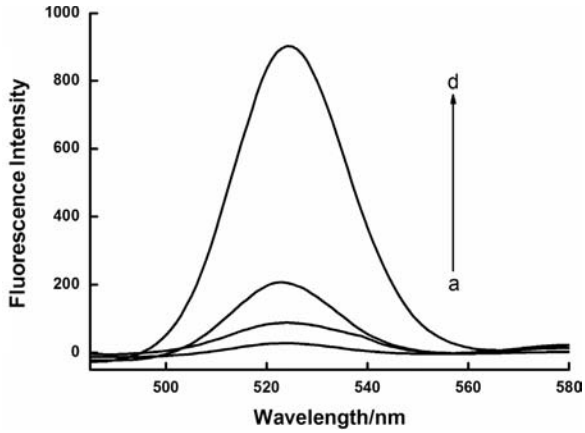

Fig. 2. Synchronous scanning fluorescence spectra of blank sample and sample. (a) 3×10^{-9} M MBs; (b) 3×10^{-9} M MBs+3×10^{-9} M targets DNA; (c) 3×10^{-9} M MBs+SYBR Green I; (d) 3×10^{-9} M MBs+3×10^{-9} M targets DNA+ SYBR Green I.

Fig. 1. The detection principle for target DNA.

In this experiment, the speed of hybridization reaction between MBs and targets DNA is very slow at room temperature due to the existence of the stem of MB. In order to accelerate the speed of hybridization reaction between MBs and targets DNA, the stem of MB is melted by heating before hybridization reaction. The result shows that the fluorescence intensity of system increased with increasing incubated temperature from 20 to 70 °C and then level off from 70 to 80 °C (Supporting information Fig. S3). The incubated temperature of 70 °C is selected in this study.

Incubated time is a major factor that influences the melt of the stem of MB. Therefore different incubated times can affect the fluorescence intensity of system. The result shows that the fluorescence intensity increased with increasing incubated time from 1 to 3 min and then decreased slowly from 3 to 9 min (Supporting information Fig. S4). Therefore, the incubated time of 3 min is selected in this study.

Cation can neutralize the charge of the DNA molecule and decreases the electrostatic repulsions of DNA molecules. So ionic strength of buffer solution can affect the rate of hybridization reaction between MBs and targets DNA, and further affect the fluorescence intensity of system. The result shows the fluorescence intensity of system increased with the increasing the concentration of NaCl from 0 to 20 mM and then decreased slowly from 20 to 100 mM (Supporting information Fig. S5). Thus, 20 mM of NaCl is selected in this study.

3.4. The linear correlation and the detection limit

Under the optimum conditions, the relationship between the fluorescence intensity (ΔI, $\Delta I = I - I_0$, where I_0 and I are fluorescence intensities in the absence and the presence of target DNA, respectively) of system and the concentration of targets DNA (C) is investigated (Fig. 3). Fig. 3 shows the fluorescence intensity of system increased with the increasing the concentration of targets DNA. The inset shows that fluorescence intensity of system exhibits good linear dependence on targets DNA concentration in the range from 2×10^{-11} to 2.5×10^{-9} M. The fitted regression equation is $\Delta I = 2.1101C + 22.5326$ with a correlation coefficient of 0.9945 (R^2). The detection limit (3σ, where σ is the standard deviation of a blank solution, $n = 11$) of target DNA is estimated to be 9×10^{-12} M. Seven parallel measurements of 2×10^{-10} M target DNA are used for estimating the precision and the relative standard deviation (RSD) is 3.83%. It demonstrates that the analytic method has a good precision and a low detection limit.

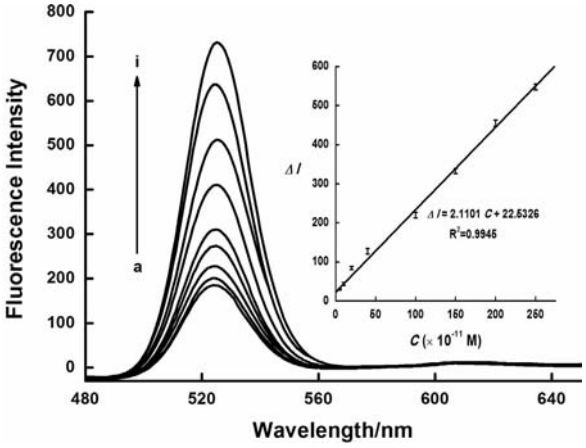

Fig. 3. The linear relationship between the fluorescence intensity of system and the concentration of targets DNA. Concentration of targets DNA: 0, 2, 8, 20, 40, 100, 150, 200, 250 (a→j, $\times 10^{-11}$ M).

3.5. The comparation of specificity of two detection methods

According to the specificity analysis procedure, we have investigated the specificity of two methods. The assay is challenged with different mismatched target DNA (T, MT1, MT2, MT3, and the DNA sequences are shown in 2.1). Fig. 4 shows that the detection method with MBs and SYBR Green I can markedly distinguish the perfectly complementary sequence from the single-base mismatched sequences. However, the detection method of DNA with individual SYBR Green I cannot markedly distinguish the perfectly complementary sequence from the single-base mismatched sequences (Fig. 5). This indicates that the detection method of DNA with MBs and SYBR Green I has a good selectivity, the detection method of DNA with SYBR Green I alone has a poor selectivity.

3.6. The analysis of real sample

In this study, the proposed method is applied to detect real sample based asymmetric PCR. According to the asymmetric PCR analysis procedure, we compared the results of asymmetric PCR with and without template DNA. The results are shown in Fig. 6. In the absence of template DNA, the fluorescence signal of system is weak (Fig. 6, curve c), this shows that there is no target DNA in amplification products. In the presence of template DNA (2×10^7 Copy/mL), the fluorescence signal of system is significantly enhanced (Fig. 6, curve d),

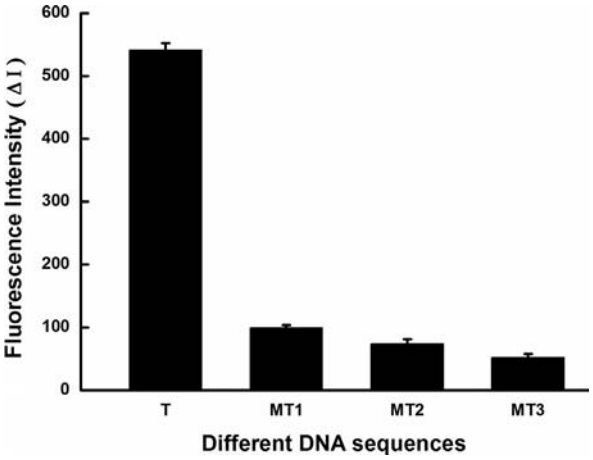

Fig. 4. The specificity analysis of the method with MBs and SYBR Green I. The concentration of MBs and different DNA sequences are all 2×10^{-9} M.

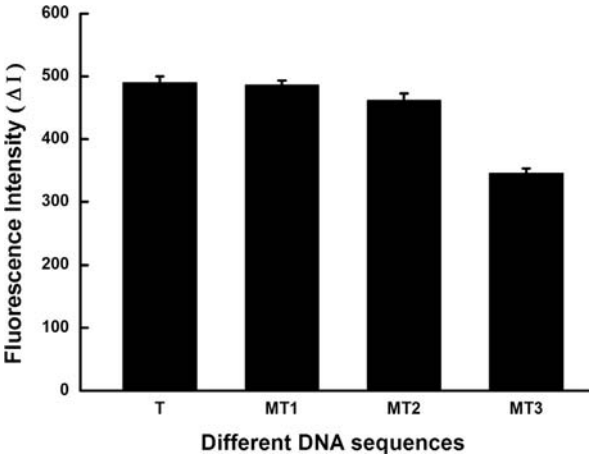

Fig. 5. The specificity analysis of the method with SYBR Green I alone. The concentration of cDNA and different DNA sequences are all 2×10^{-9} M.

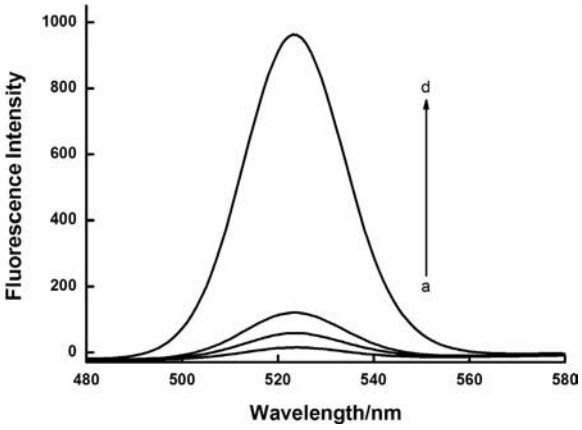

Fig. 6. The comparison of asymmetric PCR without (c) and with (a,b,d) template DNA (2×10^{7} Copy/mL). (a) SYBR Green I; (b) 3×10^{-9} M MBs; (c,d) 3×10^{-9} M MBs+SYBR Green I.

this indicated that there are a large number of targets DNA in amplification products. For amplification products of asymmetric PCR with template DNA, if only SYBR Green I is added, the fluorescence signal of system is very weak (Fig. 6, curve a), this shows the quantity of dsDNA is very limited in amplification products. If only MBs are added to the amplification products of asymmetric PCR with template DNA, the fluorescence signal of system is still very weak (Fig. 6, curve b). However, MBs and SYBR Green I are added simultaneously, the fluorescence signal of system is significantly enhanced (Fig. 6, curve d), this indicates that the introduction of SYBR Green I can significantly improved the detection sensitivity.

4. Conclusions

In conclusion, we presented a highly sensitive fluorescence method of quantitative detection for specific DNA sequence with MBs and nucleic acid dye SYBR Green I by synchronous fluorescence analysis. This proposed method not only uses the high sensitivity of SYBR Green I and significantly enhances the detection sensitivity, but also uses the specificity of MBs and guarantees the selectivity of the detection method. In addition, the proposed method can be applied to detect the real sample. In brief, the proposed method has a high sensitivity, low detection limit, good precision, and high accuracy and can markedly distinguish the perfectly complementary sequence from the single-base mismatched sequence.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.05.040.

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