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Hyperbranched rolling circle amplification (HRCA)-based fluorescence biosensor for ultrasensitive and specific detection of single-nucleotide polymorphism genotyping associated with the therapy of chronic hepatitis B virus infection

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ABSTRACT

Detection of specific genes related to drug action can provide scientific guidance for personalized medicine. Taking the detection of a single-nucleotide polymorphism (SNP) genotyping related to the chronic hepatitis B virus (HBV) therapy as an example, a novel biosensor with high sensitivity and selectivity was developed based on the hyperbranched rolling circle amplification (HRCA) in this work. The single-base mutant DNA (mutDNA) sequence can perfectly hybridize with the specially designed discrimination padlock probe and initiate the HRCA reaction. Subsequently, a great abundant of double-strand DNA sequences were released and a strong fluorescence signal can be detected after adding SYBR Green I. In particular, the enhanced fluorescence intensity exhibits a linear relationship with the logarithm of mutDNA concentration ranging from 0.1 nM to 40 nM with a low detection limit of 0.05 nM. However, when there was even a single base mismatch in the target DNA, the HRCA was suppressed and fluorescence response process could not occur, resulting in a high selectivity of this biosensor. Moreover, this detection strategy also performs well in human serums, demonstrating its potential application in detecting SNPs in real biological samples.

1. Introduction

Chronic hepatitis B virus (HBV), as one of the most common chronic infections, continues to be a major public health issue worldwide [1,2]. It is reported that about 2 billion people have been infected, and up to 400,000 people are supposed to suffer chronic HBV [3]. Fortunately, many antiviral drugs, such as adefovir dipivoxil, lamivudine, and famciclovir, are available for the potent antiviral treatments of chronic HBV [4]. However, the therapy for chronic HBV has been limited by the emergence of viral resistance, which is related to various drug resistance mutations derived from the single-nucleotide polymorphisms (SNPs) along with the widespread use of antiviral drugs [4]. Nowadays, the therapy of chronic hepatitis B infection greatly desires the personalized medicine, which is of significant importance for improving the efficacy and accuracy of the pharmacotherapy, reducing the side effects of the drug, as well as diminishing the treatment expense. Consequently, the highly sensitive and selective determination of SNPs associated with the drug action on chronic hepatitis B virus can contribute to providing the scientific guidance for individual patients' medication.

Single-nucleotide polymorphisms (SNPs) are reported to be the most frequent form of gene variant.[5] As SNPs are important genetic biomarkers, the detection of SNPs is essential for disease diagnostics and therapy, drug development, heredity-related risk assessment, research uses, and so on [5–7]. However, the combination of complex blood sample composition and trace level target put forwards high

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requirements for the sensitive and selective determination of SNPs. For the sake of improving the detection sensitivity and realizing the trace level determination, various signal amplification approaches have been employed to develop sensitive biosensors for SNPs detection [8–15]. Polymerase chain reaction (PCR), one of the most sophisticated amplification techniques, has been highly useful and effective, whereas its reliance on heating and cooling cycles hinders its widespread utility. On the contrary, the isothermal rolling circle amplification (RCA) [16] is much simpler and more convenient than PCR. However, the amplification efficiency of RCA is limited, and thus its sensitivity is less than that of PCR [17]. To realize ultrasensitive detection of targets, the hyperbranched rolling circle amplification (HRCA) with greatly higher efficiency (up to 10^9 fold) is evolved from RCA [18]. To date, HRCA has been successfully applied to develop highly sensitive biosensors for diverse targets [19–29]. Consequently, this HRCA technique shows great potential to serve as an ultrasensitive approach to detect various SNPs related to diverse diseases.

In the present study, a single-nucleotide mutation corresponding to the rtV173L mutation [4], which is related to the lamivudine and famciclovir drug resistance for chronic HBV therapy, was chosen as an assumptive target and accurately detected by a well-designed HRCA-based fluorescent sensor. In such a biosensor, the HRCA is triggered by introducing a second primer which is complementary to the RCA product, leading to a turn-by-turn cascade of primer extension and strand displacement. Hence, a strong fluorescence signal can be detected by embedding SYBR Green I into the resulting double-stranded DNA (ds-DNA) segments of the HRCA products. Our results reveal that this proposed fluorescent biosensor possesses high sensitivity and selectivity for SNPs, and thus offers an effective tool for SNPs detection in the bioanalysis and clinical medicine.

2. Experimental section

2.1. Reagents and apparatus

All of the designed DNA sequences were synthesized and purified by Sangon Inc. (Shanghai, China). The corresponding sequences are shown as follows:

Target (Wild type): 5′-CCTATGGGAGTGGGCCTCAGTC-3′

Target (Mutant): 5′-CCTATGGGATTGGGCCTCAGTC-3′

Padlock probe: 5′-P-**TCCCATAGG**TTCAACCAAAGGATAAAGAAAG GAAGA AGT**GACTGAGGCCCAC**-3′

Primer 1: 5′-TTCAACCAAAGGATA-3′

Primer 2: 5′-ACTTCTTCCTTTCTT-3′

Single-base-mismatched target (T1): 5′-CCTATGGGT**G**TGGGCCTCA GTC-3′

Single-base-mismatched target (T2): 5′-CCTATGGTA**G**TGGGCCTCA GTC-3′

Single-base-mismatched target (T3): 5′-CCTATCGGA**G**TGGGCCTCA GTC-3′

Single-base-mismatched target (T4):: 5′-CCTTTGGGA**G**TGGGCCTC AGTC-3′

Herein, the underlined letter G in the wild type gene indicates the mutated site, which is mutated into the underlined T in the mutant gene (mutDNA) corresponding to the rtV173L mutation [4]. In the single-base-mismatched targets T1-T4, the underlined letters correspond to the mutated bases at different sites as compared to the mutDNA target. In the padlock probe, the complementary region for the mutDNA was shown in bold, while the region with the same sequence as the HRCA primer 1 was marked by a wavy line and the binding region for the HRCA primer 2 was shown as the underlined portions. Escherichia coli (*E. coli*) DNA ligase set, including Escherichia coli DNA ligase, 10 ×Escherichia coli DNA ligase buffer, and 10 ×BSA (0.05%) were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Exonuclease I and III (Exo I and III) and corresponding buffer solutions were obtained from Thermo (Shanghai, China). The deoxynucleotide (dNTP) solution mixture and Bst DNA polymerase large fragment with corresponding buffer solutions were purchased from New England Biolabs (NEB). SYBR Green I was purchased from Xiamen Biovision Biotechnology Co. Ltd. (Xiamen, China).

The human serums of chronic HBV patients (confirmed by pathological examinations) were obtained from the First Affiliated Hospital of Fujian Medical University (Fujian, China). All of the other chemicals were obtained from Sigma Chemical Co., USA. Double distilled water (Milli-Q, Millipore, resistance 18.2-MΩ) was used throughout this work. DNA solutions were prepared by dissolving DNA into 50 mM pH 7.4 Tris buffer solutions.

2.2. Preparation of the HRCA-based fluorescent biosensor

Firstly, the mixture of target sequence of certain concentration and 1 μM padlock probe was incubated at 37 °C for 1 h. Then, the ligation reaction was initiated by adding 3 U *E. coli* DNA ligase, 0.05% BSA, and 0.024 nM nicotinamide adenine dinucleotide (NAD^+) into the above solution, and then the above mixture was further incubated at 37 °C for 1 h. Afterwards, the exonucleases (Exo I and III) were added and incubated at 37 °C for 2 h, following by the inactivation at 95 °C for 10 min. The HRCA reaction was eventually incubated at 63 °C for 60 min in the aforementioned solution with adding 40 nM primer 1, 40 nM primer 2, 9.6 U Bst DNA polymerase, and 0.2 mM dNTPs. Finally, the HRCA reaction products were incubated at 95 °C for 10 min to inactivate the Bst DNA polymerase, and then mingled with 4 mL SYBR Green I (20 ×), following by the incubation at room temperature for 15 min.

2.3. Fluorescence measurement

Fluorescence was measured at room temperature by using a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian Corporation, USA) at the excitation wavelength of 488 nm and emission spectra were obtained in the range from 500 to 600 nm. Both the excitation and emission slit widths were set to 10.0 nm. The fluorescence intensity at 518 nm was used for quantitative analysis.

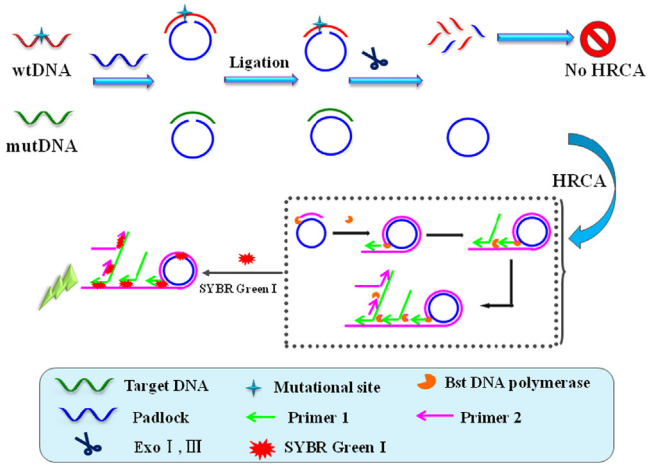
2.4. The preparation of human serum sample

Blood samples were collected in coagulant tube and centrifuged for 5 min at 3000 rpm after 1 h standing. The supernatant liquid, *i.e.*, serum was transferred and stored in the liquid nitrogen. Afterwards, 1 mL of serum was diluted to 10 mL by Tris buffer solutions. Finally, mutDNA of different concentrations were added into the diluted serum to prepare the artificial serum samples.

3. Results and discussion

3.1. Principle of the fluorescent biosensor

Scheme 1 clearly presented the principle of this proposed biosensor. The mutDNA sequence (one SNP related to the lamivudine and famciclovir drug resistance for chronic HBV therapy) can perfectly hybridize with the terminal regions of the designed padlock probe, leading to the approach of the 5′ and 3′ terminals of the padlock probe, and then yields a circular padlock probe with the help of *E. coli* DNA ligase. Under the shear action of Exo. I and III, only the padlock probes were remained in the solution. Afterwards, the HRCA reaction was initiated by adding the Bst DNA polymerase, primer 1, primer 2, and dNTPs to start the process of chain extension and strand displacement. To be specific, the Primer 2, which is complementary to part of circular padlock, is isothermally extended from its 3′ end by Bst DNA polymerase, generating a single-stranded DNA (ssDNA). Subsequently, the resulting ssDNA turns to be the template for the Primer 1 binding. Then, Primer 1 is extended by Bst DNA polymerase to displace the



Scheme 1. The principle of biosensor platform for SNPs detection based on the HRCA technique.

downstream growing DNA strands, which further provides more binding sites for the hybridization of Primer 2. Consequently, plentiful dsDNA and ssDNA with multifarious lengths are obtained. Finally, a strong fluorescence signal can be detected because the SYBR Green I can insert the groove of dsDNA with extremely high affinity [20,21]. In contrast, the DNA sequence of wild type (wtDNA) with only one base-difference cannot yield a circular padlock probe so that the HRCA will not be initiated; thereby, only a weak fluorescence signal can be detected. Herein, the fluorescence intensity shows a direct relationship with the logarithm of mutDNA concentration, and therefore the concentration of mutDNA sequences can be detected by the variation of fluorescent intensity.

3.2. Feasibility of the fluorescent biosensor

The feasibility of this biosensor for SNPs discrimination was firstly confirmed by comparing the spectra of the blank sample without mutDNA and the samples containing wtDNA and mutDNA, respectively. As can be observed from Fig. 1, the existence of target mutDNA causes intense fluorescence signal (curve a), suggesting that the HRCA process has been initiated and a mass of dsDNA were formed. In contrast, only weak signals were detected for the sample of wtDNA with single base difference (curve b) and the blank (curve c), indicating that the amplification reaction does not start in these samples. These results demonstrate that the HRCA can only be triggered by mutDNA, which can

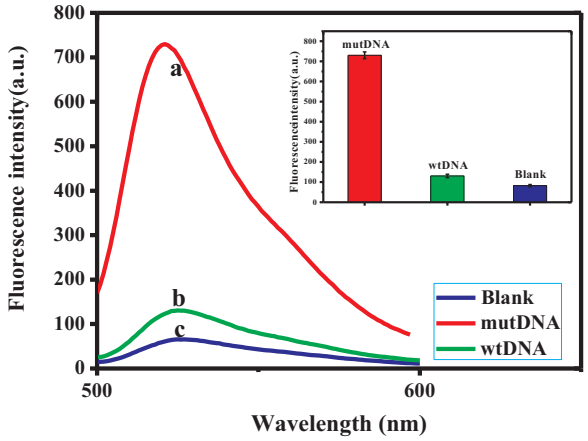


Fig. 1. Feasibility of the HRCA-based biosensor: the fluorescence spectra of 40 nM mutDNA (curve a), 40 nM wtDNA (curve b), blank without mutDNA and wtDNA (curve c).

generate numerous dsDNA and thus yield an unexceptionable ability of this biosensor for discriminating SNPs.

3.3. Optimization of the reaction conditions

To attain the best detecting performance of the proposed sensor, four crucial experimental variables, namely, the concentration of padlock probe, the dosage of *E.coli* ligase and Bst DNA polymerase, and the HRCA reaction time were respectively optimized and shown in Fig. 2. Padlock probe plays an important role in target DNA recycling process and HRCA process. From Fig. 2a, it is observed that the ΔF ($\Delta F = F - F_0$) intensity, where F and F_0 are the fluorescence intensity in the presence of mutDNA and wtDNA, respectively, gradually increases with the increase of padlock probe concentration and finally reaches a peak at 1.0 μM. Thus, the concentration of 1.0 μM has been used for padlock probe in the subsequent experiments.

In addition, the dosage of *E.coli* ligase was also crucially important for the HRCA process, and thereby the effect of *E.coli* ligase on the ΔF intensity was investigated. As can be seen from Fig. 2b, the ΔF intensity evidently increases with the augmentation of *E.coli* ligase from 0 to 3 U, and then decreases with adding more *E.coli* ligase, yielding a maximum ΔF intensity at 3 U. In fact, the decrease of ΔF intensity after 3 U is mainly attributed to the enhanced fluorescence intensity F_0 of wtDNA along with the addition of more *E.coli* ligase. Similarly, the superfluous dosage of Bst DNA polymerase involved in this system can also lead to enlarged background signal, so the effect of Bst DNA polymerase was investigated as well. As is expected, the ΔF intensity rapidly increases until 1U Bst DNA polymerase is added (see Fig. 2c). Afterwards, the ΔF gradually decreases when the dosage of Bst DNA polymerase increases from 1 and 15 U, which can also be attributed to the fact that the fluorescence intensity F_0 of wtDNA increases with the increasing dosage of Bst DNA polymerase. This suggests that 1 U Bst DNA polymerase was sufficient for this proposed biosensor. Therefore, the optimized dosages of 3 U for *E.coli* ligase and 1 U for Bst DNA polymerase were employed in this study.

Moreover, the HRCA reaction time is also considered to be crucial for the signal amplification process. Hence, the time-dependent ΔF intensity has been detected to monitor the HRCA reaction. As shown in Fig. 2d, the ΔF intensity gradually increases before 60 min, reflecting that the HRCA products are continuously generated at the beginning stage. Afterwards, the ΔF intensity reaches a constant value, indicating the saturation of HRCA products in the solution. Hence, 60 min has been chosen as the optimum time for HRCA reaction.

3.4. Calibration curve for target DNA determination

The fluorescence biosensor was utilized to quantify mutDNA by adding target DNA of different concentrations into the prepared reaction systems. Fig. 3a displayed the change of the fluorescence intensity with the different concentrations of mutDNA. Apparently, the peak intensity of fluorescence gradually increases along with the increasing concentration of mutDNA. This phenomenon is reasonable considering the fact that more mutDNA combines with enough padlock probes could bring forth more dsDNA in a certain period of time, which will lead to a higher fluorescence signal. Furthermore, an excellent linear relationship between the fluorescence intensity and the logarithm of mutDNA concentration ranging from 0.1 to 40 nM is obtained (Fig. 3B) as follows:

$$\Delta F = 247.74 \lg C_{\text{mutDNA}} + 278.32, \quad R^2 = 0.99934$$

where ΔF is the difference between the fluorescence intensities in the presence of mutDNA and wtDNA, C_{mutDNA} refers to the concentration of mutDNA, and R represents the regression coefficient. Herein, the limit of detection was estimated to be 0.05 nM by the defined protocol of $3\sigma_b/s$, where σ_b is the standard deviation of the blank samples, and s is the slope. Thanks to the high amplification efficiency of HRCA, the

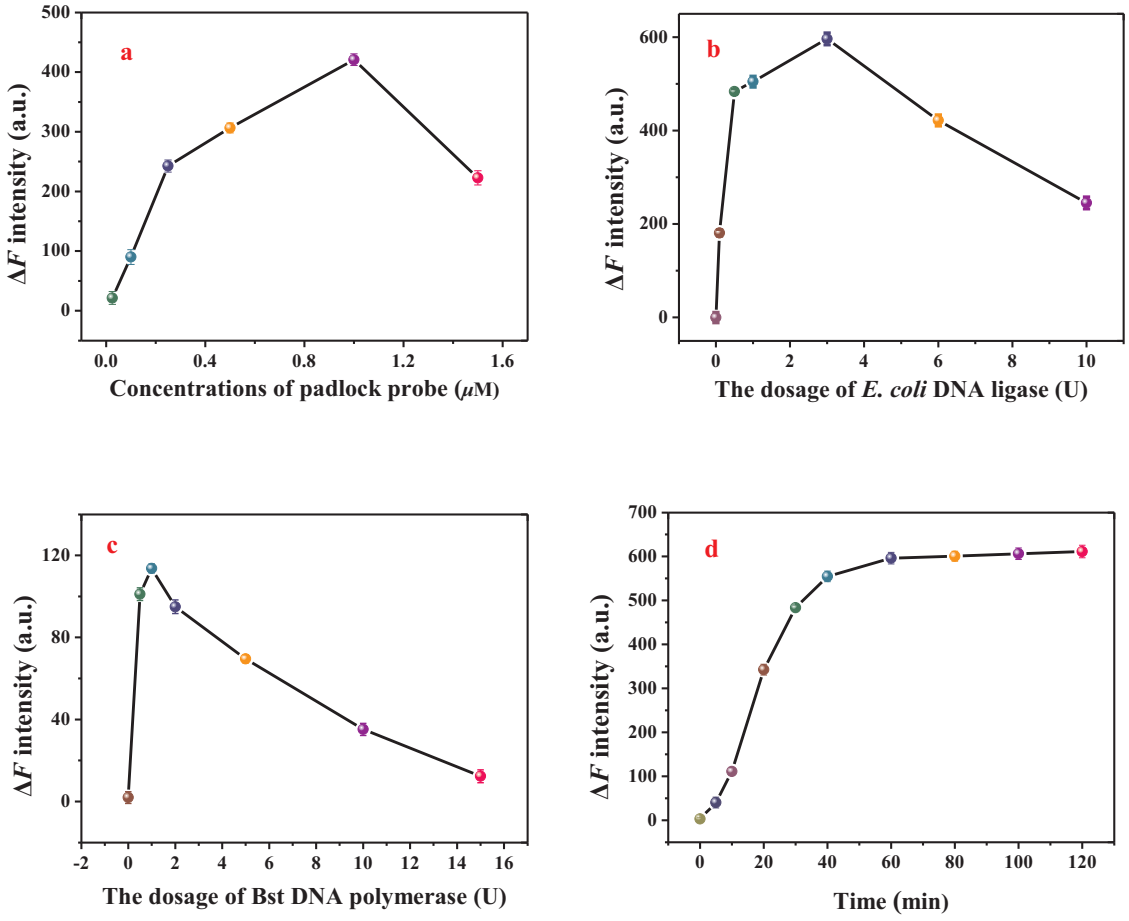


Fig. 2. Optimization of the reaction conditions with 40 nM mutDNA and wtDNA sequences: Effect of (a) the concentration of padlock probe, (b) the dosage of *E. coli* DNA ligase, (c) the dosage of Bst DNA polymerase, (d) HRCA reaction time on the Δ*F* intensity. The error bars represent the standard deviation of three repetitive measurements.

sensitivity of this developed biosensor was superior to those of earlier reported fluorescent methods for DNA sequence detection, such as polymerization-mediated strand-displacement amplification [30], magnetic enrichment methods [31], isothermal polymerization-based amplification system [32], and was also comparable with those of cascade DNA nanomachine-based signal amplification strategy [33], double-hairpin molecular beacon-based fluorescent method [34]. Apparently, this proposed fluorescent biosensor has an applicable linear range and adequate sensitivity, which can fully meet the requirement of clinical diagnosis for SNPs detection.

3.5. Selectivity and reproducibility of the proposed biosensor

High selectivity is another important criterion to assess an analytical method. Herein, the specificity was evaluated by the control hybridization experiments for different DNA sequences including the

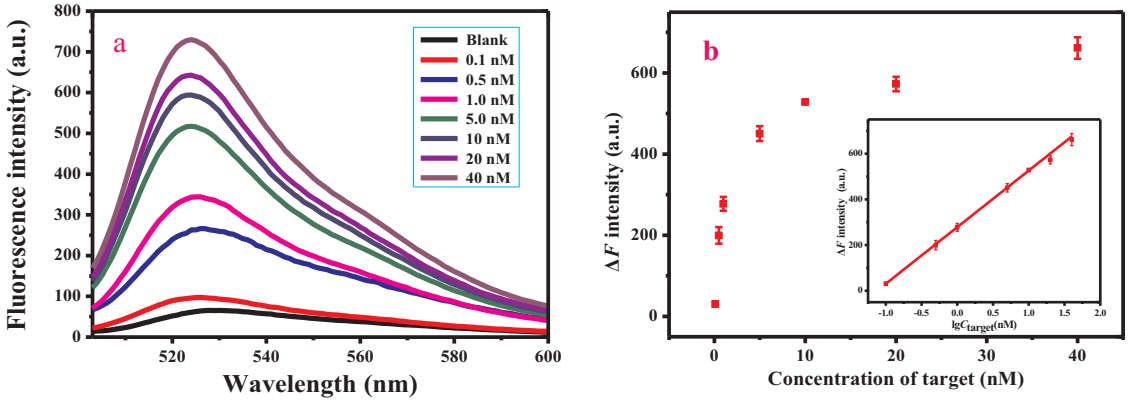


Fig. 3. (a) Fluorescence spectra at different concentrations of the target mutDNA sequence. (b) The relationship between Δ*F* and the DNA sequence concentration. Inset: The calibration curve between the Δ*F* and the logarithm of the DNA sequence concentration. The error bars show the standard deviation of three replicate determinations.

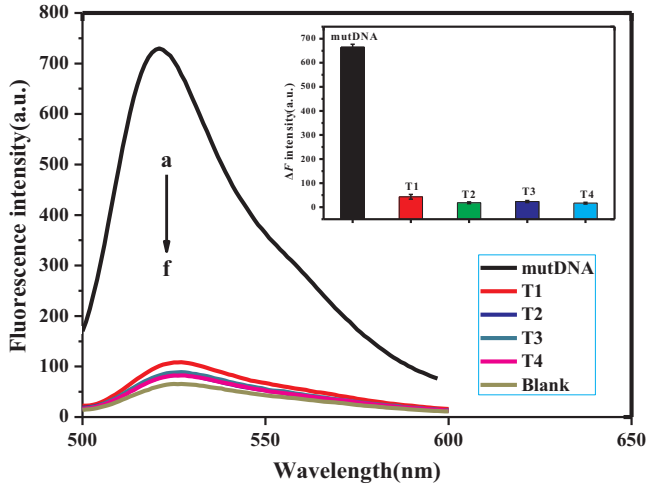


Fig. 4. Specificity of the HRCA-based assay detecting different targets: mutDNA (a), T1 (b), T2 (d), T3 (c), T4 (e), and no target DNA was added (f). The illustration is the Δ*F* obtained with different sequences: mutDNA, T1, T2, T3, and T4, whose concentrations are 40 nM.

target mutDNA sequence and single base mismatched DNA sequences (T1, T2, T3, and T4). Fig. 4 presents the detected fluorescence intensity after the addition of different targets, *i.e.*, mutDNA, T1, T2, T3, T4, and no target DNA. As shown in Fig. 4, the fluorescence intensity is obviously enhanced by adding the target mutDNA (curve a), whereas the fluorescence signals are sharply decreased for the single base mismatched DNA sequences (T1, T2, T3, and T4 indicated as curves b, d, c, and e, respectively), yielding similar intensities to the blank (curve f). As a result, from the inserted histogram, it can be observed that the Δ*F* values of T1, T2, T3, and T4 are much smaller than that of mutDNA, suggesting that the interfering signals of different SNPs can be negligible. The above results demonstrate the high selectivity of this proposed fluorescent biosensor for SNPs detection, which exhibits great potential to be used in actual sample analysis.

From the practical point of view, the reproducibility of the designed biosensor was another essential element for SNPs detection. To verify the reproducibility of this developed biosensor, five parallel experiments have been carried out with the mutDNA concentration of 40 nM. Our test results reveal that the relative standard deviation (RSD) is only 2.3%, suggesting the good repeatability of the proposed method for SNPs detection.

3.6. Determination of mutDNA in serum samples

For the sake of identifying the feasibility of this proposed strategy in detecting targets in complex biological samples, the mutDNA targets of different concentrations (5.0, 20, and 35 nM) were added in the diluted real serums and then detected by the developed biosensor. As shown in Table 1, it is clearly observed that this biosensor shows acceptable recoveries ranging from 94% to 106%, and RSDs ranging from 2.4% to 3.5%. Such results demonstrate that this proposed method can be effectively and reliably utilized to detect the SNPs in biomedical research and clinical analysis.

Table 1
Determination of mutDNA in serum samples (n = 5).

Added (nM)	Detected (nM)	Recovery (%)	RSD (%)
5.0	5.3	106	3.5
20	19	95	3.2
35	33	94	2.4

Abbreviations: RSD, relative standard deviation.

4. Conclusions

In summary, a simple, selective, and sensitive HRCA-based fluorescent biosensor has been successfully developed for detecting a SNP genotyping associated with the drug resistance for chronic HBV therapy. A pretty low limit of detection has been realized because of the high amplification efficiency of HRCA and high sensitivity of fluorescence detection. More strikingly, the mutant gene can be effectively distinguished from the wild-type one. As a low-level of the rtV173L mutation can be invariably found in the blood serums of patients for whom lamivudine and famciclovir treatment failed, the proposed biosensor can be used to determine if the lamivudine and famciclovir therapy is necessary for the chronic HBV patient. Moreover, it is highly expected that, based on a similar mechanism, this proposed strategy can also be expanded to detect a wide range of SNPs by designing suitable padlock probes, which will further promote the application of fluorescence detection in the clinical diagnosis.

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