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www.elsevier.com/locate/talanta

PII: S0039-9140(18)30502-2
DOI: https://doi.org/10.1016/j.talanta.2018.05.035
Reference: TAL18675

To appear in: *Talanta*

Received date: 18 December 2017
Revised date: 26 March 2018
Accepted date: 8 May 2018

Cite this article as: Lisha Wang, Huo Xu, Ying Han, Shuai Xiao, Sha Lv, Cong Wang, Nan Zhang, Zhengyong Wang, Yongqiong Tang, Hongbo Li, Jianxin Lyu and Zhifa Shen, Reverse strand-displacement amplification strategy for rapid detection of p53 gene, *Talanta,* https://doi.org/10.1016/j.talanta.2018.05.035

Reverse strand-displacement amplification strategy for rapid detection of p53 gene

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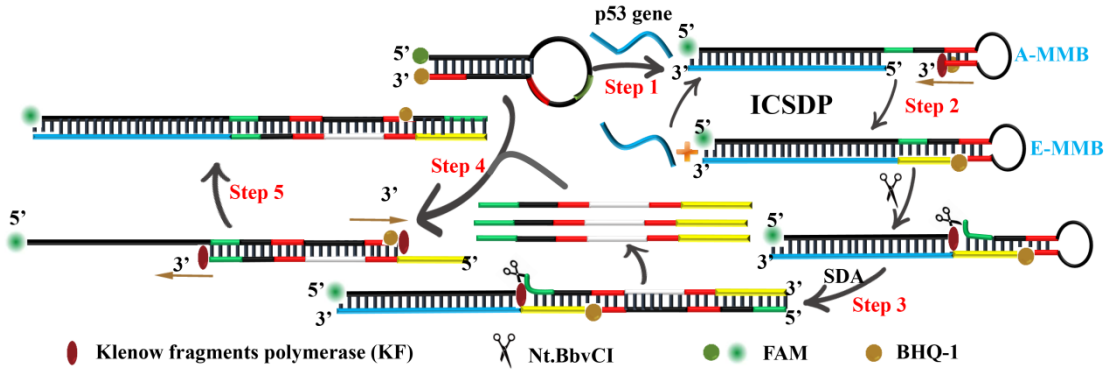
Abstract

The development of rapid approaches to detect prognostic markers is significant in reducing the morbidity and mortality of cancer. In this paper, we describe a rapid and specific biosensing platform for target DNA (p53 gene as a model) detection based on reverse strand displacement amplification (R-SDA). When the p53 gene is added, multifuctional molecular beacon (MMB) is unfolded via the hybridization with p53 gene. With the assist of Klenow fragment (KF) and Nt.BbvCI (the nicking endonuclease), p53 gene recycling could be initiated and considerable amount of complementary sequences for the MMBs (Nicked fragments, NFs) could be formed, generating enhanced fluorescence signal. Using this amplification strategy, the proposed biosensor displays the detection limit of 1 nM and a wide linear range from 1 to 100 nM, even if only one type of probe is involved. Notably, remarkable detection specificity for single-base mismatched target p53 gene is achieved. Moreover, the described biosensor also exhibited the stability in real biological samples (human serum). The rapid detection strategy can be performed less than 30 min without harsh reaction conditions or expensive nanoparticles. This biosensor

shows great potential for application in clinic assay, especially, for early cancer diagnosis.

Graphical abstract



***Key Words*:** Multifuctional molecular beacon (MMB); Reverse strand displacement amplification (R-SDA); tumor suppressor p53 gene

1. Introduction

The human cancer suppressor gene p53 gene plays a pivotal role in many cellular anti-tumor mechanisms and is known as “the guardian of genome”[1]. It is directly associated with transcriptional regulation and functions of some key proteins[2, 3]. The mutation of the p53 gene is one of the most common genetic alterations in about 50% of human cancers[2, 4-6]. For this reason, p53 gene has great promise as a diagnostic marker for monitoring and early screening of cancers. Accordingly, the development of sensitive and rapid strategies for the detection of p53 gene becomes significant in the field of molecular biology.

Over the past decades, various measuring techniques, including fluorescent[7-11], chemiluminescence[12], electrochemical (EC)[13-15] and colorimetric[16-19] methods have been reported for the gene detection. Among these techniques, fluorescent method has attracted much attention due to its simplicity, sensitivity, and rapidness for target analysis. Furthermore, in order to improve the sensitive detection, the effective biosensors for the enzymatic signal amplification continue to attract increasing research interest. The traditional approach polymerase chain reaction (PCR) exhibits some limitations such as temperature programmer, potential contamination and high cost[20, 21]. Meanwhile, strand displacement amplification (SDA)[22-24], isothermoal circular strand-displacement polymerization (ICSDP)[25, 26], rolling circle amplification (RCA)[27] and nanoparticles-based amplification[28], et al. have been employed in the sensing systems to improve the capacity of detecting the nucleic acids. Among them, SDA have gained more and more attention due to its excellent specificity and simplicity. Via combining a restriction enzyme wit DNA polymerase, SDA, a type of isothermal in vitro method for diagnostics, offers a

prominent signal amplification capability for the sensitive detection of nucleic acids, proteins, and small molecules[29-31]. ICSDP has also attracted researchers' attention because of its excellent property of signal amplification. The polymerization can be initiated by target DNA (trigger) hybridization with template DNA, followed by the replication-based displacement of the target DNA. Hence, one target DNA can be reused and produce numerous products that contributes the enhanced signal.

In the present study, we attempted to develop a simple and rapid biosensor to detect p53 gene specifically based on target-induced ICSDP and reverse SDA (R-SDA). The target p53 gene hybridizes with the multifunctional molecular beacon (MMB) and initiates the circular target-displacement process. At the same time, a double strand with the nicking site for Nt.BbvCI is formed. Subsequently, the double strand is cleaved and induces the new polymerization process, generating massive nicked fragments (NFs). The NFs can hybridize with another MMB, further amplifying the signal. The proposed strategy exhibits not only the high sensitivity and specificity, but also has an excellent performance in real human serum assays in about 30 min. Therefore, it seems to open up an exciting new avenue for early diagnosis of cancers.

2. Experimental section

2.1 Materials and reagents.

Oligonucleotides designed in this study were synthesized and purified by Shanghai Sangon Biological Engineering Technology. The corresponding sequences are displayed in **Table 1**. The sequence of MMB was designed with the help of DNA folding software (http://unafold.rna.albany.edu/). All DNA samples was prepared by dissolving in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and stored at 4 °C

refrigerator prior to use. The Klenow fragment (KF) polymerase (3′-5′ exo$^-$) (New England Biolabs, Inc., Ipswich, USA), Nt.BbvCI (New England Biolabs, Inc., Ipswich, USA), low DNA ladder (namely DNA marker) (New England Biolabs, Inc., Ipswich, USA), deoxyribonucleoside 5′-triphosphates (dNTPs) mixture (Dingguo Changsheng Biotechnology Co., Ltd, Beijing, China) and SYBR Green I (Dingguo Changsheng Biotechnology Co., Ltd, Beijing, China) were bought from agents. All chemicals were of analytical-reagent grade, and all aqueous solutions were prepared with double dis-tilled water (resistance =18.25 MΩ.cm), which was provided by a Kerton lab MINI water purification system (UK). Human serum sample was provided by First Affiliated Hospital of Wenzhou (Wenzhou, China). Hela cell lines were obtained from Cell Bank of Chinese Academy (Shanghai, China).

2.2 Instrument.

The fluorescence spectrum was collected by Hitachi F-7000 spectrophotometer (Hitachi, Ltd., Japan) controlled by FL Solution software and equipped with a Xenon lamp as the excitation source. The excitation wavelength of 492 nm was adopted, and the fluorescence emission spectrum was collected from 500 nm to 600 nm with integration time of 0.5 s at the scan rate of 240 nm/min. The slits for excitation and emission were both set at 5.0 nm, and the photomultiplier tube voltage was 600 V.

2.3 Gel Electrophoresis Analysis.

Gel electrophoresis was carried out on a 12% native poly-acrylamide gel electrophoresis (native-PAGE) in a 0.5×Tris-bo-rate-EDTA (TBE) buffer at 80 V for 90 min. The images were visualized with a ChemiDoc XRSþ imaging system (BIO-RAD, USA).

2.4 Fluorescent p53 gene assays.

Prior to experiments, 45.5 μL of target DNA and 1.5 μL of 10 μM MMB were heated at 90 °C for 5 min and then slowly cooled down to room temperature. Then, 1.5 μL of 10 mM dNTPs, 0.5 μL of 1% BSA, 0.5 μL of 2.5 U/μL KF (3′-5′ exo$^-$) and 0.5 μL of 5 U/μL Nt.BbvCI were successively added, and the final volume of resulting solution was 50 μL. After mixing thoroughly, the mixture was incubated at 37 °C for 20 min during which the enzymatic reaction proceeded in the dark. Subsequently, the reaction was terminated by heat inactivation at 80 °C for 20 min and slowly cooling to room temperature. Before collecting fluorescence spectrum, 150 μL of 1× NEBuffer 2 was added and thoroughly mixed. Unless otherwise specified, the experiments were conducted using the standard procedure depicted here. The fluorescence peaks at 519 nm were recorded and used to evaluate the performances of the proposed assay strategy.

3. Results and discussion

3.1 Mechanism of the proposed strategy.

In this work, we chose p53 gene as the target, and the signaling mechanism is elucidated in **Scheme 1.** A multi-functional MMB was rationally designed. The loop of MMB was designed for target recognition (black) and a nicking site for nicking endonuclease (green). After addition of a complementary DNA (p53 gene), activated MMB (A-MMB) structure is formed (Step 1), and the 3’ terminal fragment can act as the polymerization primer in the presence of KF polymerase (Step 2). As a result, an extended MMB (E-MMB) was formed, and the displaced and released target DNA (p53 gene) initiates the next hybridization. This is named isothermoal circular

strand-displacement polymerization (ICSDP). Because of the resultant E-MMB contains a complete nickase recognition site, the SDA reaction can be induced by the cooperation of KF polymerase and Nt.BbvCI. Specifically, Nt.BbvCI nicks E-MMB, and the 3' terminal in the newly-formed nick serves as the primer responsible for the polymerization on the downstream of nick. This leads to the opening of E-MMB's small hairpin and the nicking/polymerization/displacement occurs repeatedly, producing a large amount of NFs (Step 3). This process is arbitrarily called reverse SDA (R-SDA) because its polymerization occurs towards the opposite direction to ICSDP. These NFs can partly hybridize with new MMBs (Step 4). At the same time, the NFs could be acted as primers responsible for polymerization on MMBs (Step 5). Eventually, each target can cause an extremely amplified signal. In the absence of p53 gene, MMB can self-assemble into a large hairpin structure and inhibit all the subsequent processes. Thus, no obvious fluorescence change is detected. It is worthwhile to note that only one type of DNA probe, MMB, is involved and the total assay time for p53 gene detection is less than 30 min. On the basis of the newly-proposed signaling concept, reverse SDA, this biosensing system is called R-SDA platform despite ICSDP.

3.2 Feasibility of the proposed method for p53 gene detection.

To demonstrate the feasibility of the proposed method for p53 gene detection, a series of control experiments were performed based on the fluorescence spectra measurement and 12% native-PAGE analysis, and the results were exhibited in **Fig. 1**. As illustrated in **Fig. 1A**, the fluorescent intensity (curve b, MMB+p53 gene) is higher than that of curve a (MMB). The result shows that the hybridization between the MMB and p53 gene could lead to the conformation change of MMB. After adding

Klenow Fragment (KF), the fluorescence intensity could further increase (curve d), which indicates that the target p53 gene could be recycled by polymerization using the 3' terminal as primer. This results in 9.7 fold increase fluorescence compared with curve c. When a nicking endonuclease (Nt.BbvCI) coexists, a further increase in the fluorescent intensity is observed (curve f). The experimental results confirm the feasibility of this system for signal amplification.

The above-mentioned solutions were further verified by 12% native-PAGE analysis. As illustrated in **Fig. 1B**, one band below 50 bp observed in lane a, c, and e, should be MMB, suggesting no enzymatic reaction in the absence of target DNA. Upon the introduction of target p53 gene, a band at about 75 bp in lane b is detected. In the presence of p53 gene and KF, a very bright band is found in lane d between the position of MMB/p53 and MMB, indicating the generation of numerous dsDNA (extension of the MMB, E-MMB). In the presence of all reactants, a highest obvious band appears in lane f, indicating the expected amplification reactions are activated. These measured data verified the feasibility of the experiment.

3.3 Investigation of product composition.

To further show the DNA elements involved during enzymatic displacements and cleavages (lane f in fig.1), the electrophoresis analysis of intermediate product (NF)-mediated reactions using a 12% native-PAGE was performed. As shown in lane c of **Fig. 2**, two obvious new bands, which are at the same position as the lane d, appear compared with lane a and lane b, indicating the NF does be generated during enzymatic nicking and polymerization reactions and can induce the subsequent

reactions. The measured data demonstrate that the mechanism proposed for signal transduction is reasonable.

3.4 Assay performance.

Under the optimal conditions (**Fig. S1**), the sensitivity of the assay was investigated via evaluating the signal intensity of sensing system after adding a series of different concentrations of p53 gene into the reaction system. The fluorescence peak intensity increases with the increase in target p53 gene concentration (**Fig. 3A**). The inset reveals the fluorescence change could be also detected at low target concentration as low as 1 nM. Compared with the previous signal amplification strategies [32,33], the newly-proposed reverse strand-displacement amplification strategy shows lower detection limit.

Fig. 3B shows the calibration curve for quantitative analysis of p53 gene ranging from 1 to 200 nM. The linear regression equation is F=27.66+5.09C with a correlation coefficient of 0.9903.

The important point of the newly-described signaling strategy is that the single-base mismatched target fragment can be easily distinguished. The tumor suppressor gene p53 is one of the most frequently mutant genes in human cancers. Therefore, the ability to discriminate point mutations from the wild-type gene is one critical factor for a potential method system. In order to evaluate the detection specificity of the present detection system, the relative fluorescence intensity were

studied, where one (MT1), two (MT2), three (MT3), and four (MT4)-base mismatched target were involved besides perfectly complementary sequence (T). The measured data are seen in **Fig. 4**. If the fluorescence signal triggered by the wild-type p53 gene is defined as 100%, the relative fluorescence responses induced by MT1, MT2, MT3 and MT4 are not more than 4 %, demonstrating that only the perfectly complementary sequence can initiate the R-SDA signal. This might be attributed to the high hybridization selectivity of the hairpin structure probe toward target DNA[34].

3.5 Detection of p53 gene in complex biological matrix.

To investigate the potential application of the proposed sensing system in complex biological samples, samples containing different concentration of human serum (0, 1%, 5% and 10%) from First Affiliated Hospital of Wenzhou were spiked with 50 nM, 100 nM, and 200 nM p53 gene, and the response capability was evaluated after the fluorescence intensity at 519 nm were measured. As shown in **Fig. 5**, it was observed that the values of F/F_0 of different samples in the presence of target DNA at 200, 100 or 50 nM were well close to each other regardless of human serum concentration. We further measured the target p53 gene in 2.5% and 5% Hela cell lysate solution. As shown in Fig. S2, different concentrations of p53 gene in these cell lysate can be accurately determined. These suggest the developed method can be suitable for the target DNA detection in complex biological matrix.

4. Conclusion

In summary, a rapid, highly specific, and sensitive biosensing strategy based on combination of ICSDP with R-SDA was developed for p53 gene detection in this

study. The signal transduction of target binding event was based on isothermal enzymatic signal amplification and the target detection can be accomplished within 30 min. It is interesting to note that only one DNA probe, MMB, is involved in this sensing system. The biosensor not only shows a linear fluorescence response to the p53 gene concentration but also discriminates signal-base mismatched DNA from the wild-type target gene. Additionally, the target DNA in complex biological environment (the human serum) can be detected almost without compromising signal intensity, offering a promising sensing platform for early diagnosis and prognosis of cancer.

Acknowledgments

This work was supported by National Natural Science Foundation of China (NSFC) (grant NO: 21775024 and 21565015) and Independent Research Project of State Key Laboratory of Photocatalysis on Energy and Environment (NO. 2014CO1).

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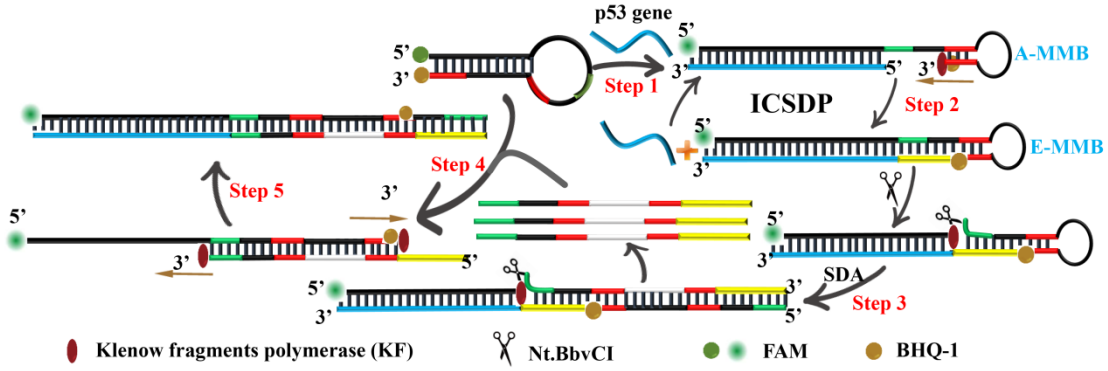

Scheme 1. Reverse strand-displacement amplification (R-SDA)-based detection of target DNA p53 gene. In the presence of target DNA p53 gene, MMB can bind to p53 gene, opening the large hairpin structure and forming a small hairpin at the end of 3'.

Thus, the isothermoal circular strand-displacement polymerization (ICSDP) is initiated by polymerase, displacing the hybridized target DNA. Upon addition of Nt. BbvCI, the reverse SDA reaction can be triggered, producing the nicked fragments (NFs) that in turn hybridize with other MMBs and are extended. Dual amplification of ICSDP and SDA offer the amplified fluorescence signal for highly sensitive detection of the p53 gene.

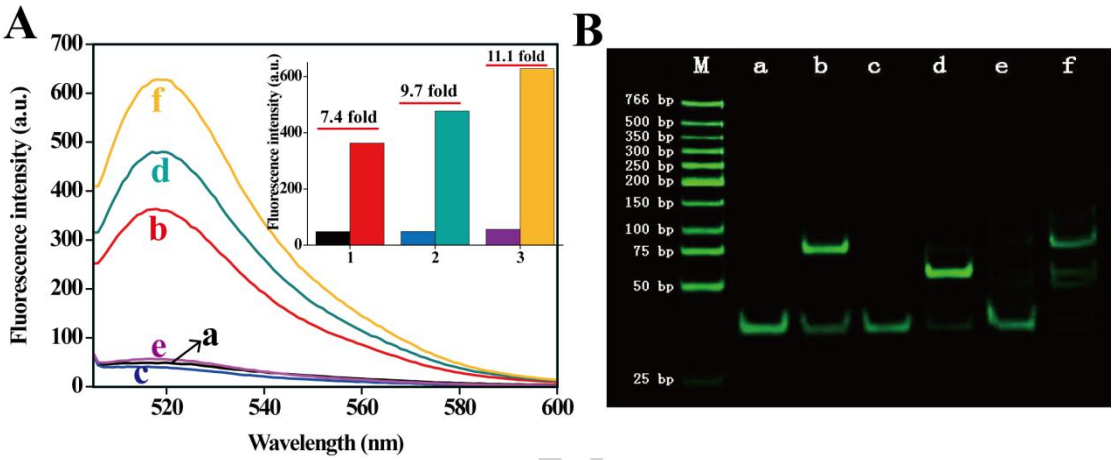

Fig. 1 Feasibility of the detection of tumor suppressor gene p53 on the basis of R-SDA. (**A**) Fluorescence emission spectra of seven systems consisting of different constituent components. (a) MMB; (b) MMB+p53 gene; (c) MMB+ Klenow fragment polymerase (KF); (d) MMB+p53 gene+KF; (e) MMB+KF+ Nt.BbvCI; (f) MMB+p53 gene +KF+ Nt.BbvCI. (**B**) The 12% native PAGE (nPAGE) analyses of the same

samples. The concentrations of MMB and target DNA p53 gene were 300 nM and 140 nM, respectively.

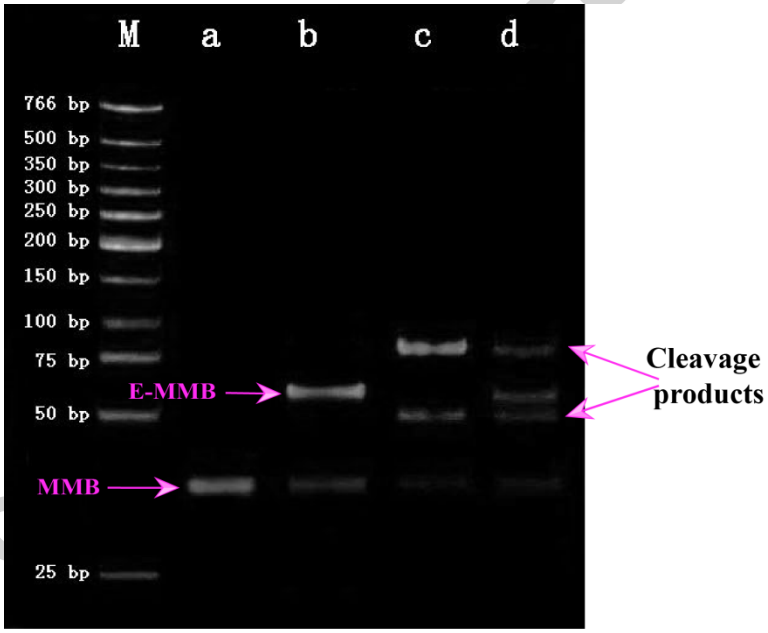

Fig. 2 The nPAGE (12%) analysis of the amplification products of R-SDA reaction. Lane a: MMB; Lane b: MMB+ p53+KF; Lane c: MMB+ nicked fragment+KF+

Nt.BbvCI; Lane d: MMB+p53+KF+ Nt.BbvCI. The concentrations of MMB, target DNA p53, nicked fragment, KF, Nt.BbvCI and dNTPs are 300 nM, 140 nM, 140 nM, 2.5 U, 5 U and 0.3 mM, respectively.

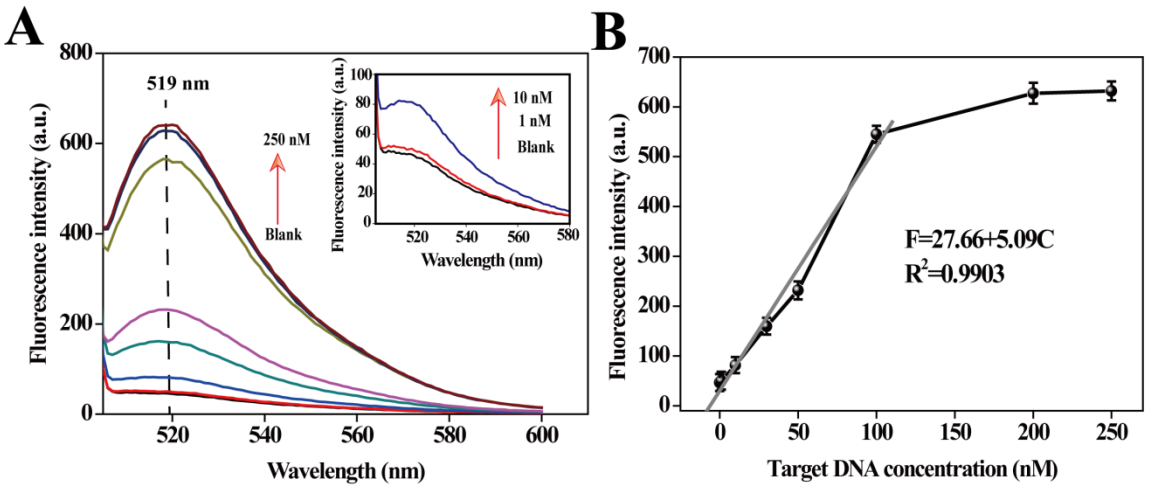

Fig. 3 (**A**) The fluorescence spectra of the R-SDA sensing system in the presence of different concentrations of p53 gene (from bottom to top: 0, 1, 10, 30, 50, 100, 200,

250 nM). Inset: the fluorescence spectra in the low target concentration range. (**B**) The dynamic relationship between target DNA concentration and fluorescence intensity. The linear response range of 1 to 100 nM is achieved. The regression equation could be expressed as F=27.66+5.09C with a correlation coefficient of 0.9903, where F and C indicate the peak intensity at 519 nm and target concentration, respectively. Error bars represent the standard deviation calculated from three independent experiments.

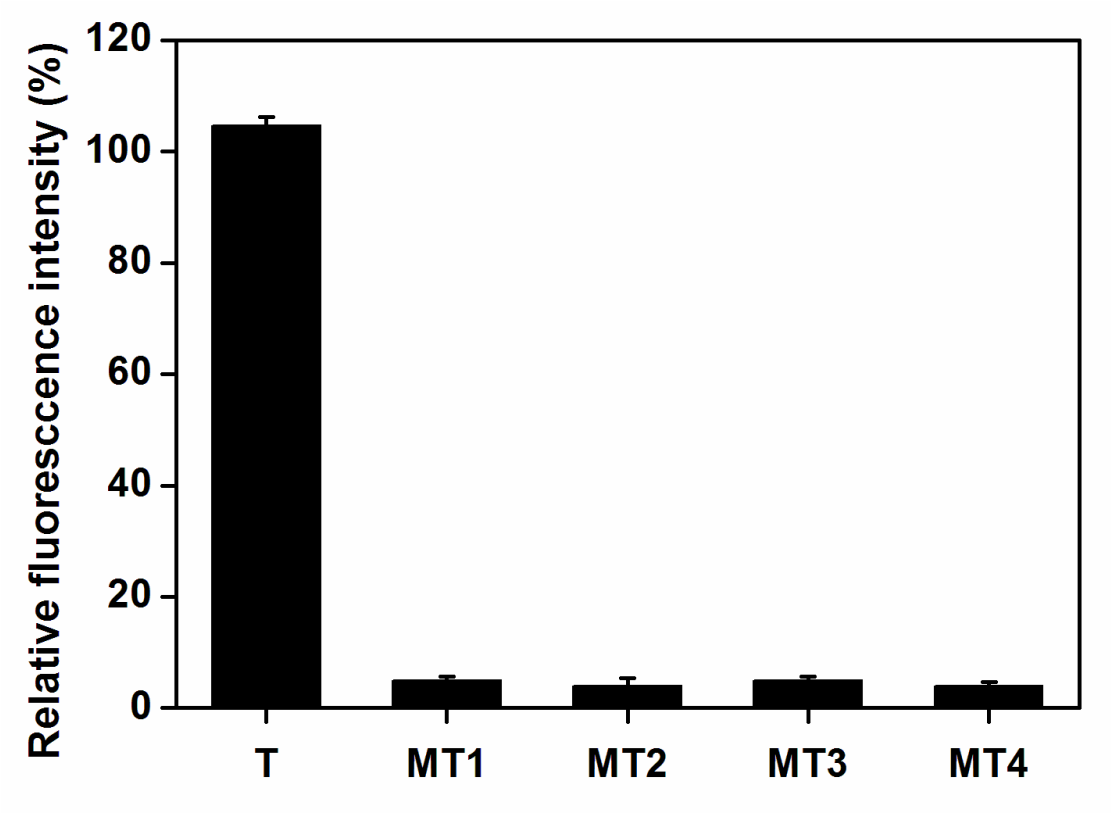

Fig. 4 The detection specificity of R-SDA system for p53 gene. Relative fluorescence intensity corresponding to four mutant target DNAs: single-base mismatched target (MT1), two-base mismatched target (MT2), three-base mismatched target (MT3) and four-base mismatched target (MT4). Herein, the optical signal induced by complementary p53 gene is defined as 100%. The concentrations of MMB and target

DNA are 300 nM and 140 nM, respectively. The error bars represent the standard deviation of three repetitive measurements.

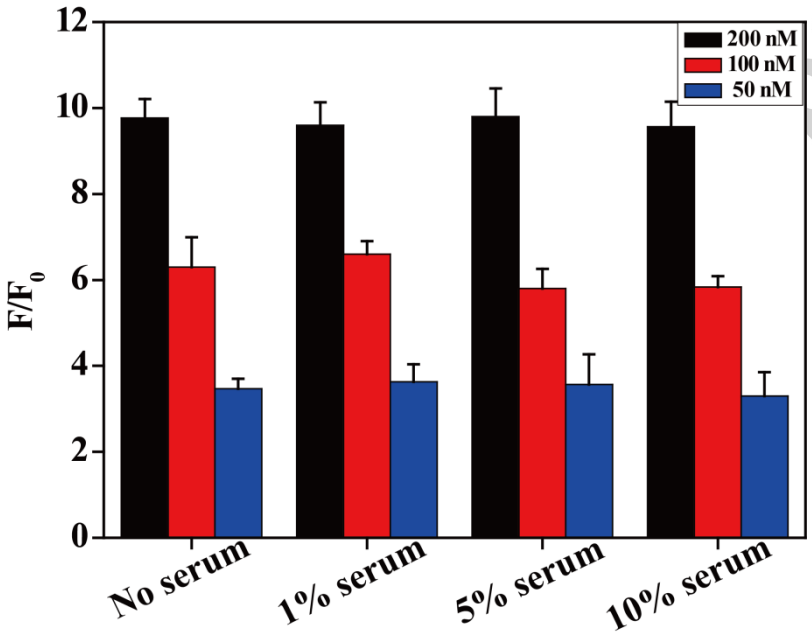

Fig. 5 Fluorescence ratio obtained from the samples containing different concentrations of human serum in the absence (F_0) and presence of p53 gene (F, 50, 100, 200 nM). The data shown here represent the average of three independent

experiments. The concentrations of MMB, KF, Nt. BbvCI and dNTPs are 300 nM, 2.5 U, 5 U and 0.3 mM, respectively.

Table 1. Oligonucleotide sequences designed in the current study[a]

Note	Sequence (5'-3')
Multifuction Molecular beacon (MMB)	(FAM)*ACAGGCACAAACACGCACC*TCAAAGC**CCTCAGC**TG TATACAGGCAttttt GGTGCGTGTTTGTGCCT(BHQ-1)GT
Nicked fragment (NF)	**TCAGC**TGTATACAGGCA CAAACACGCACCaaaaaTGCCTGT ATACA**GCTGA**

Target DNAs	WT	CA*GCTTTGAGGTGCGTGTTTGTGCCTGT*CCTG
	MT1	CA*GCTTTGAGGTGCaTGTTTGTGCCTGT*CCTG
	MT2	CA*GCTTTtAGGTGCaTGTTTGTGCCTGT*CCTG
	MT3	CA*GCTTTt AGGTGCaTGTTTGTtCCTGT*CCTG
	MT4	CA*GCTTTtAGtTGCaTGTTTGTtCCTGT*CCTG

[a]The MMB is able to fold into a hairpin structure via the self-hybridization of two boxed fragments. BHQ-1 and FAM were conjugated to the 3' and 5' ends, respectively. The italicized fragment was designed to hybridize with the italicized part of target DNA. The red underlined fragments of MMB are complementary to each other, while the short green bold segment, "**CCTCAGC**", is the half binding site of nicking endonuclease, Nt.BbvCI. The shadowed part of MMB is complementary to

the shadowed one in nicked fragment. The lowercase letters seen in the target DNAs indicate the point mutations.

Highlight

- We describe a rapid and specific biosensing platform for tumor-related target DNA detection based on reverse strand displacement amplification (R-SDA).
- The target detection can be accomplished within 30 min without harsh reaction conditions or expensive nanoparticles.
- Even if only one probe is involved, the proposed biosensor not only is able to quantify target DNA but also shows the high specificity toward target DNA of interest. Mutant target DNA can be easily discriminated.
- The sensing platform holds promising potential for application in biomedical research and early clinical diagnostics.