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Detection of p53 DNA using commercially available personal glucose meters based on rolling circle amplification coupled with nicking enzyme signal amplification

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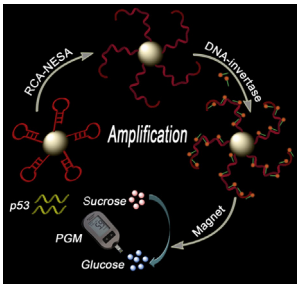
HIGHLIGHTS

- A portable and low-cost method for p53 DNA detection using the PGM was developed.
- The sensor combined recognition specificity of NESA and signal amplification of RCA.
- Single base mutation detection could be achieved by this electrochemical biosensor.
- The proposed method did not require sophisticated equipment or skilled professionals.
- The proposed biosensor might be a very promising strategy in point-of-care diagnosis.

GRAPHICAL ABSTRACT



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ABSTRACT

The development of cost-effective methods for early detection and identification of prognostic markers still remains a significant challenge to improve diagnosis and reduce the mortality of cancer. Herein, on the basis of rolling circle amplification (RCA) coupled with nicking endonuclease-assisted signal amplification (NESA), a simple, sensitive and portable biosensor was developed for the determination of p53 DNA by using the personal glucose meter (PGM) as readout. Initially, biotin-modified hairpin probe (HP) was immobilized onto streptavidin-coated magnetic beads (MBs). The target DNA hybridized with the loop region of the HP, which triggered target recycling process and produced the complementary sequences for the padlock probes. Next, the liberated complementary sequences hybridized with the padlock probes to form a circular template, inducing the subsequent RCA reaction and replicating a long tandem repeated sequences. Then, numerous DNA-invertase conjugation were tagged on the resulted RCA products on the surface of MBs. The DNA-invertase efficiently catalyzed the hydrolysis of sucrose to generate abundant glucose, leading to an amplified response of glucometer. By virtue of the multiple signal amplification strategy, the proposed biosensor toward p53 DNA could achieve a low detection limit of 0.36 pM with a linear calibration range from 0.5 to 10 pM and exhibited excellent sequence selectivity. In addition, the resulting biosensor was also applied to detect the p53 DNA sequence in spiked human serum samples with satisfactory results, which possessed enormous potential to be applied in clinical diagnostics and biomedical research.

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1. Introduction

The p53 gene is now well established as a tumor suppressor gene that in humans acts as “the guardian of the genome” [1]. The p53 tumor suppressor gene encodes p53 protein that binds to DNA and maintains the genomic stability by preventing genome mutation [2,3]. In addition, wild type p53 gene, which is the most frequently mutated gene in more than 50% of all human cancers, acts a pivotal part in cell cycle arrest, DNA repair, apoptosis, and tumor angiogenesis [4,5]. It's of great importance for the development of sensitive and selective method for the sequence-specific detection of the p53 gene and its mutations to the early diagnosis and prognosis of cancers.

Various measuring techniques have been employed for sensitive determination of the p53 gene. Amongst others, MALDI-TOF mass spectrometry is one of the powerful tools for p53 gene detection [6]. However, the expensive facility limits its further application. Up to the present, optical [7–9] and electrochemical [10–12] methods have been the useful analytical platforms for the p53 DNA detection. Despite the progress made so far, reliable and point-of-care testing (POCT) sensors are attracting increasing attention in clinical diagnostics due to the advantages of miniaturized devices and simple operation [13–15]. Considering the great potential of POCT technology, it is of considerable significance to develop a simple, portable, low-cost and quantitative method for the determination of p53 DNA which is easily available to use at home.

The personal glucose meter (PGM) have been recognized as one of the most widely used POCT devices due to its excellent performance in terms of portable pocket size, simple detection process, low cost, reliable quantitative ability [16–18]. Recently, commercial PGMs have been successfully used to quantitatively detect a series of non-glucose analytes, including metal ions, small biomolecules, nucleic acids and proteins, by establishing a functional relationship between target recognition and glucose generation [19–24]. Although promising, there is still a challenge to improve the sensitivity of PGM sensor for extra-laboratory testing with the POCT device.

Recently, a variety of isothermal amplification techniques have been employed to improve the sensitivity of nucleic acid analysis [25–27], including rolling circle amplification (RCA) [28], strand displacement amplification (SDA) [29,30], exonuclease-assisted signal amplification [31], and nicking endonuclease-assisted signal amplification (NESA) [32,33]. Specifically, NESA has rapidly become invaluable tools in single-base mismatch detection because of the high cleavage efficiency and specific recognition sites of nicking endonuclease [34,35]. Moreover, RCA is such a powerful isothermal DNA replication technique that has been widely used in sensitive biosensing [36–38].

In this paper, we propose a cascade signal amplification strategy coupled to the PGM as readout for portable, low cost, sensitive and high specific detection of p53 DNA sequence based on RCA coupled with NESA. The proposed biosensor combined the distinct recognition specificity of NESA and powerful signal amplification capability of RCA. Moreover, the proposed biosensor employed magnetic separation to collect target-dependent binding of DNA-invertase conjugate from the complex sample matrix, making this assay suitable for p53 DNA detection in biological samples. Therefore, the developed RCA coupled with NESA-based electrochemical biosensor might create a convenient and practical platform for the detection of p53 DNA without the use of sophisticated detection equipment or skilled professionals, and will be a very promising strategy in point-of-care diagnosis.

2. Experimental section

2.1. Materials and reagents

Streptavidin-modified magnetic beads (MBs, 1 μm average diameter), the nicking endonuclease (Nt.BstNBI), NEBuffer 3.1 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 μg/mL BSA, pH 7.9), T4 DNA ligase, and deoxynucleotide solution mixture (dNTPs) were purchased from New England Biolabs (Beijing, China). Adenosine triphosphate (ATP) was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Amicon Ultra-0.5 Centrifugal Filter Devices (Amicon Ultra 3 K device and 100 K device) were purchased from Merck Millipore Ltd. (Billerica, MA). Quant-iT™ PicoGreen® dsDNA reagent, and phi29 DNA polymerase were purchased from Thermo Fisher scientific. Grade VII invertase from baker's yeast (*S. cerevisiae*), and sulfosuccinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC) were obtained from Sigma-Aldrich (Shanghai, China). Tris(2-carboxyethyl)phosphine (TCEP) was purchased from innochem (Beijing, China). Sucrose was purchased from Alfa Aesar (Shanghai, China). All of the synthetic DNA sequences used in this study were obtained from Sangon Biotech (Shanghai, China). The sequences of the DNA are listed in Table 1. All reagents were purchased and used without further purification, deionized water (18.2 MΩ) obtained from a Milli-Q water purification system was utilized in all the experiments.

Buffer solutions used in this study: Buffer A: 0.1 M NaCl, 0.1 M sodium phosphate buffer solution, pH 7.4. Buffer B: 0.1 M NaCl, 0.1 M sodium phosphate buffer solution, pH 7.4, 0.05% Tween-20.

2.2. Apparatus

Fluorescence spectra were recorded using a RF-6000 (Shimadzu, Japan). UV–vis spectra were collected on a UV-2450 spectrophotometer (Shimadzu, Japan). The amplification reactions were performed on a Thermal Cycler GE 9612 (Bio-Gener, China). The glucose signal was recorded on a Roche ACCU-CHEK Performa personal glucose meter.

2.3. DNA-invertase conjugation

The conjugation procedure was similar to previous work with slight modifications [39,40]. In brief, 30 μL of 1 mM thiol-DNA in Millipore water, 2 μL of 1 M PBS buffer at pH 5.5, and 2 μL of 30 mM TCEP in Millipore water were mixed in a centrifuge tube and incubated in the dark at room temperature for 1 h to activate the thiol-modified DNA strands. After that, the excess TCEP was removed by Amicon-3 K using buffer A for 8 times. Meanwhile, 1 mg of sulfo-SMCC and 400 μL of 20 mg/mL invertase were added into buffer A. After vortexing, the solution was placed on a shaker for 1 h at room temperature. Note that the mixture was then centrifuged to remove excess insoluble sulfo-SMCC. The supernatant was washed 8 times by Amicon-100 K using buffer A. The above solution of TCEP-activated thiol-DNA and the purified solution of sulfo-SMCC-activated invertase were mixed and kept at room temperature for 48 h. To remove unreacted thiol-DNA, the solution was purified by Amicon-100 K using buffer A over eight wash cycles and kept at a 5 mg/mL calculated concentration at 4 °C for further use. The 20% native polyacrylamide gel electrophoresis (PAGE) analysis and the ultraviolet absorbance spectra were carried out to characterize the DNA-invertase conjugate synthesized.

Table 1
Oligonucleotide sequences designed in the current study.

Name	Detailed sequence information (from 5′ to 3′)	Discription
p53 DNA	TCA TCA CAC TGG AAG ACT C	Target DNA
HP	Biotin-TTT TTT TCC CGA TCC ATG AGT CTT CCA GTG TGA TGA GGA TCG GGA	Hairpin probe
Padlock probe	Phosphate-TGG ATC GGG ATA TCC TTT GGT TGA AAC TTC TTC CTT TCT TGG AAG ACT CA	Padlock probe
Thiol-DNA	HS-$(CH_2)_6$-GGA AGA CTC ATG GAT CGG GA	Thiol-DNA for invertase conjugation
T1	TCA TCA CAC TGG AAG CCT C	Single-base mismatched DNA
T2	TCA TCA CAC TGG AAG GAT C	Double-base mismatched DNA
T3	TCA TCA CAC TGG AAG GAC C	Three-base mismatched DNA
Tn	TAC GCG TAC ATC CTA GCT T	Non-complementary DNA

2.4. Immobilization of hairpin probe onto MBs

Hairpin probe (HP) was heated to 95 °C for 5 min and then allowed to cool to room temperature within 2 h before use. First, a portion of 200 μL streptavidin-modified MBs (1 μm average diameter, 4 mg/mL) were pre-washed three times by buffer B to remove the surfactants and then resuspended in 0.5 mL buffer A. Then, biotin modified HP was added into the above solution to achieve a final concentration of 1 μM, and incubated at room temperature for 1 h with vortexing to ensure the completion of the interaction between biotin and streptavidin. After that, the HP-conjugated MBs (HP-MBs) were separated from the reaction mixture by a magnet. Finally, the resulting HP-MBs were rinsed three times with buffer B and then resuspended in buffer A.

2.5. Nicking endonuclease signal amplification

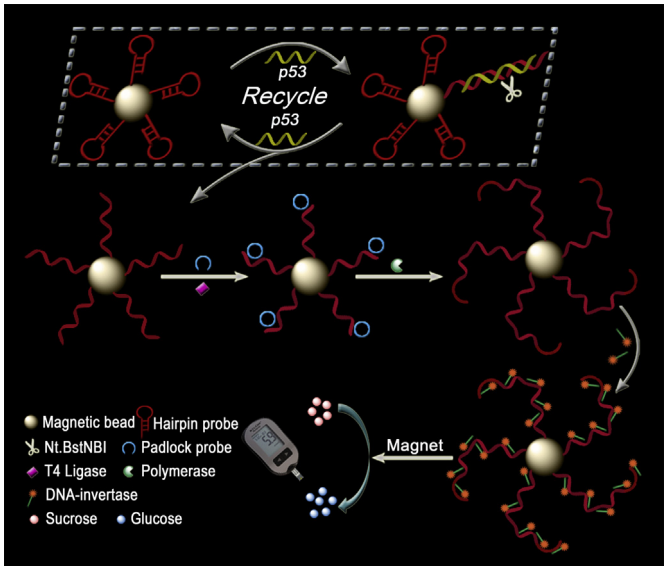
The 15 μL of obtained HP-MBs was reacted with 25 μL of p53 DNA with different concentrations. Upon incubation at 37 °C for 2 h, 10 μL of Nt.BstNBI reaction buffer (0.5 U/μL) was added, and incubated at 55 °C for 60 min. Then the HP-MBs were further separated from the mixture by a magnet, and rinsed with buffer B to remove unreacted target DNA.

2.6. Rolling circle amplification in vitro

The padlock probe ligation reaction was conducted by adding 4 μL 100 nM phosphorylated padlock probe, 4 μL of 10 × ligase reaction buffer, 2.5 μL of T4 DNA ligase (4 U/μL), 28.5 μL of H_2O, and 1 mM ATP into the achieved biosensor. First, before the ligase was added, the reaction mixture was heated at 95 °C for 3 min, and cooled slowly to room temperature. Then, the reaction mixture was incubated at 37 °C for 1 h. Afterward, the product of the ligation reaction was added to 10 μL of the RCA reaction mixture containing 5.5 μL of 10 × phi29 DNA polymerase reaction buffer, 0.5 μL of phi29 DNA polymerase (10 U/μL), and 4 μL of 10 mM dNTPs. Finally, the RCA process was carried out at 37 °C for 2 h and then terminated by incubation at 65 °C for 10 min.

2.7. Detection of p53 DNA with the PGM

100 μL of DNA-invertase conjugate was added into the biosensor, and the mixture was mixed on a roller for 1 h at room temperature, and then the solution was placed close to the magnet. After washing the HP-MBs residue by 5 times using buffer B, 50 μL of 1 M sucrose was added to the HP-MBs residue and then mixed on a roller 40 min at 55 °C. A portion of 5 μL of the final solution was measured through a commercially available PGM.



Scheme 1. Schematic illustration of p53 DNA sensing based on RCA coupled with NESA.

3. Results and discussion

3.1. p53 DNA sensing based on RCA coupled with NESA

Scheme 1 illustrated a three-step strategy for p53 DNA detection by using a PGM. The first step was a target-activated nicking endonuclease-assisted reaction. Hairpin probe containing padlock probe complementary sequence was designed to be complementary to p53 DNA to form the recognition sequence for the Nt.BstNBI enzyme. The Nt.BstNBI enzyme cleaved the unfolding duplex HP into two pieces and released the intact p53 DNA to participate in the next cycle of cleavage. The second step was to achieve signal transduction and amplification by the utilization of RCA coupled with NESA. The padlock probe was introduced and hybridized with the complementary sequence from the first step. The circular template was formed by specific ligation of the padlock probe under T4 DNA ligase. The RCA reaction was initiated by phi29 DNA polymerase. The third step was to achieve multiple signal amplification by enzymatic turnovers. Only in the presence of p53 DNA can the proposed biosensor be successful in making the DNA-invertase bound on the MBs. After magnetic separation of MBs, the supernatant containing the unbound DNA molecules and invertase were washed away, the bound DNA-invertase conjugates converted additional sucrose into glucose leading to establishment of quantitative p53 DNA detection by using any commercial glucose

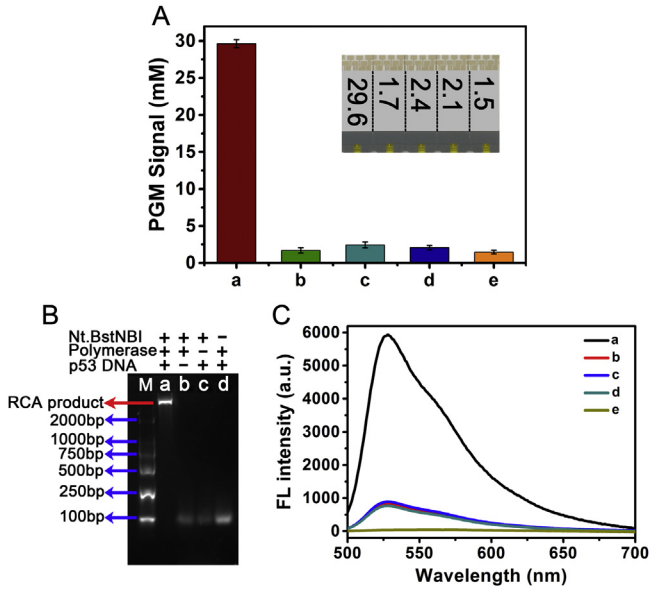


Fig. 1. (A) PGM analysis of the amplification products under different conditions: (a) p53 DNA + Nt.BstNBI + phi29 DNA polymerase; (b) phi29 DNA polymerase + Nt.BstNBI; (c) p53 DNA + Nt.BstNBI; (d) p53 DNA + phi29 DNA polymerase; (e) blank. Inset: the corresponding test strips and values. The error bars represented the standard deviation of three independent measurements. (B) Agarose gel (2%) electrophoresis analysis of the amplification products; the experimental conditions for lanes a–d were indicated above; Lane M: DNA size marker. (C) Fluorescence measurement of the amplification products in (A).

meter.

To confirm the reaction above, following experiments were performed: (1) in the presence of p53 DNA, Nt.BstNBI, and phi29 DNA polymerase (column a); (2) without p53 DNA (column b); (3) without phi29 DNA polymerase (column c); (4) without Nt.BstNBI (column d) and blank control experiment (column e). As shown in Fig. 1A, the PGM signal was enhanced (column a) when p53 DNA, Nt.BstNBI, and phi29 DNA polymerase were all presented. However, only weak PGM signals were observed (column b, c, and d), indicating that no cascade amplification reaction occurred at the other cases. Furthermore, the resulting amplification products were also recorded by conducting 2% agarose gel electrophoresis. As shown in Fig. 1B, the amplification products exhibited a wide bright band in the presence of p53 DNA, Nt.BstNBI, and phi29 DNA polymerase (lane a), which could be attributed to the large molecule weight of the RCA products. Comparing with lane a, no new bands were observed when only two reactants were present (lane b, c, and d) due to the lack of RCA reaction. Fig. 1C depicted the fluorescence spectral responses of the sensor with diluted PicoGreen® dsDNA reagent as the staining dye. It was observed that a strong fluorescence peak was obtained with the existence of p53 DNA, Nt.BstNBI, and phi29 DNA polymerase (curve a). Nevertheless, the fluorescence intensity for RCA amplicons remained low when the reaction was conducted with only two reactants (curve b, c, and d). These results confirmed that efficient RCA reaction could be triggered by the introduction of p53 DNA, Nt.BstNBI, and phi29 DNA polymerase and verified the feasibility of this electro-analytical platform for the determination of p53 DNA.

3.2. Optimization of experimental conditions

To achieve the optimal sensing performance, the experimental parameters were optimized in this study. Amongst others, target-dependent binding efficiency of DNA-invertase conjugate is a key

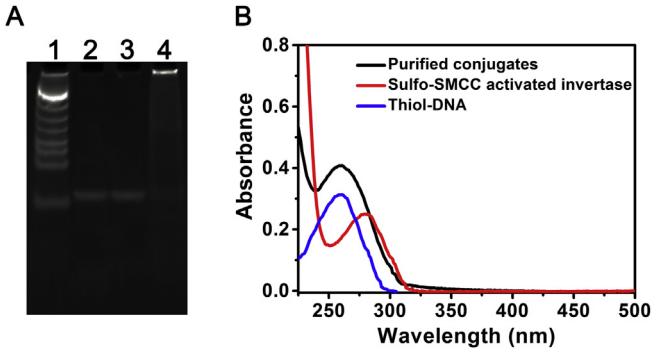


Fig. 2. (A) 20% native PAGE of the thiol-DNA and invertase affinity. 1: Marker; 2: pure thiol-DNA; 3: the mixture of thiol-DNA and invertase without linker; 4: the conjugant of thiol-DNA and invertase. (B) Absorption spectra of the conjugation products.

factor since it could convert sucrose into glucose through invertase-catalyzed hydrolysis reaction. In this method, magnetic separation was used to purify the DNA-invertase conjugates so as to make only DNA-invertase bound on the MBs. The conjugation products were loaded and run on a non-denaturing polyacrylamide gel causing separation of the DNA-invertase conjugates from the free DNA. As shown in Fig. 2A, a negligible difference between lane 2 and lane 3 was observed in terms of band positions, indicating that the thiol-DNA alone cannot be conjugated to the invertase without linker. The band stuck on the wells in lane 4 was because the higher molecular weight of conjugates. The UV–vis absorption spectra were conducted in Fig. 2B, thiol-DNA displayed a characteristic absorption peak at 260 nm (blue line), and 280 nm absorption peak was observed for invertase alone (red line). The ultraviolet absorbance spectra of the purified conjugates (black line) showed a maximum absorption peak at 260 nm and a mild shoulder around 280 nm, which derived from DNA and invertase in the conjugates respectively, indicating the successful formation of conjugates.

The RCA reaction is also a key factor as it directly affected the performance of PGM sensing for the targets. As Fig. 3A depicted, the PGM signal intensity increased gradually with the concentration of padlock probe from 10 nM to 100 nM, and then kept stable. Therefore, the padlock probe concentration of 100 nM was selected for this experiment. As showed in Fig. 3B, the effect of the concentration of phi29 DNA polymerase was checked. The PGM signal obviously increased with the increasing concentration of phi29 DNA polymerase and reached the maximum value at 5 U. Thus, 5 U was chosen as the optimized concentration of phi29 DNA polymerase. To enhance detection sensitivity, the effect of the concentration of dNTP substrates was also evaluated. According to Fig. 3C, the PGM readout increased with the increase of dNTP concentration and reached summit. As a result, 10 mM was chosen as the optimal dNTP concentration. Furthermore, the reaction time of RCA was crucial to the assay. As presented in Fig. 3D, the PGM readout rised rapidly with increasing RCA reaction time, and tended to remain constant after 2 h. Therefore, the RCA reaction time of 2 h was taken in the sensing performance.

The temperature affects the conversion of sucrose to glucose, which was also optimized in the range of 25–60 °C. As shown in Fig. 3E, 55 °C was chosen as the optimal temperature in the subsequent experiments. Another important factor influencing enzymatic catalysis reaction is the time. Fig. 3F showed the PGM signal changes at the different reaction time by using 0.1 mg/mL invertase as an example. The PGM signal increased rapidly for the first 40 min of incubation with sucrose, and then became saturated. Therefore, 40 min incubation time was chosen as the optimal reaction time for enzymatic reaction.

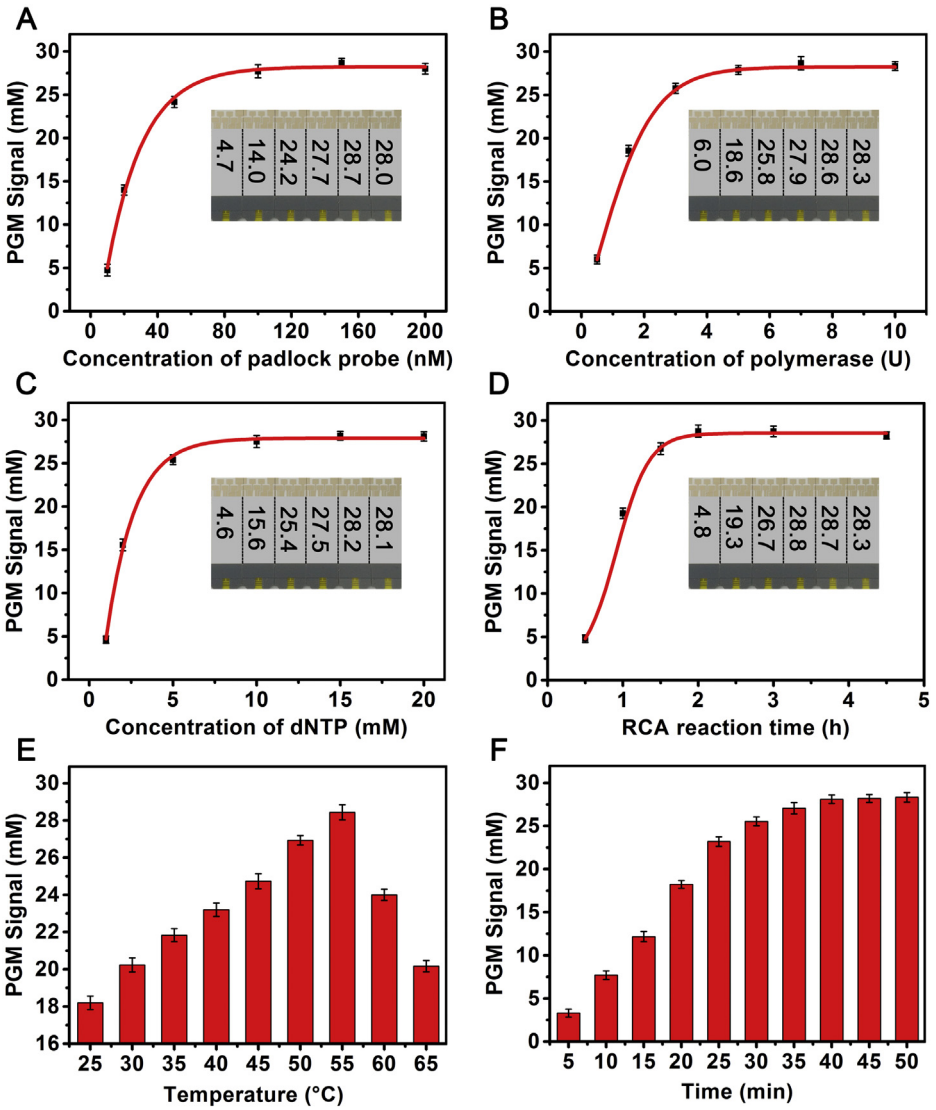


Fig. 3. Optimization of concentration of (A) padlock probe, (B) phi29 DNA polymerase, (C) dNTP, and (D) RCA reaction time. Inset: the corresponding test strips and values. (E) Temperature dependence of enzymatic reaction. (F) Time dependence of enzymatic reaction. The assays were carried out in the reaction buffer containing 1 nM p53 DNA. The error bars represented the standard deviation of three independent measurements.

3.3. Analytical performance

Under the optimal conditions, the linear relationship between the PGM signal and the p53 DNA concentration is ranged from 0.5 to 10 pM ($R^2 = 0.9944$), with a limit of detection of 0.36 pM, as shown in Fig. 4A. A comparison of the analytical performance with traditional methods using different equipment for DNA detection is given in Table S1. The proposed biosensor is comparable or even better than some of other previously reported methods. With the trend toward instrumentation miniaturization, the proposed cascade amplification strategy coupled to the PGM is more attractive and suitable for extra-laboratory testing as a POCT device due to its simple detecting procedures, cost-effective nature, and reliable quantitative ability. The method offers desirable linear range and relatively lower detection limit because of the cascade signal amplification. In addition, the specificity of amplification strategy was assessed by the control hybridization experiments for different DNA sequences. As shown in Fig. 4B, single-base mismatched DNA T1, double-base mismatched DNA T2, three-base mismatched DNA T3, and complete mismatched sequence DNA Tn were tested. The concentrations of Tn, T1, T2, and T3 were 10 times as many as that of target DNA (1 nM). As shown in Fig. 4B, PGM signals of T1, T2, T3, and Tn were similar to that of blank, indicating a desirable specificity for detection of p53 DNA assay. The result was due to the specific recognition site of Nt.BstNBI and only the target DNA sequence can trigger the Nt.BstNBI-assisted recycling amplification.

3.4. Sample analysis

To further investigate the capability of the proposed signal amplification strategy for the detection of p53 DNA in real samples, a series of different concentrations of p53 DNA were spiked in human serum (diluted 10 times with NEBuffer 3.1). As shown in Table 2, the recovery ranged from 95.0% to 108.0%, and the RSD ranged from 5.4% to 6.7%, which demonstrated that the other components of the serum did not interfere significantly in this method. The results illustrated that the proposed biosensor could be applied to detect p53 DNA in complex biological samples.

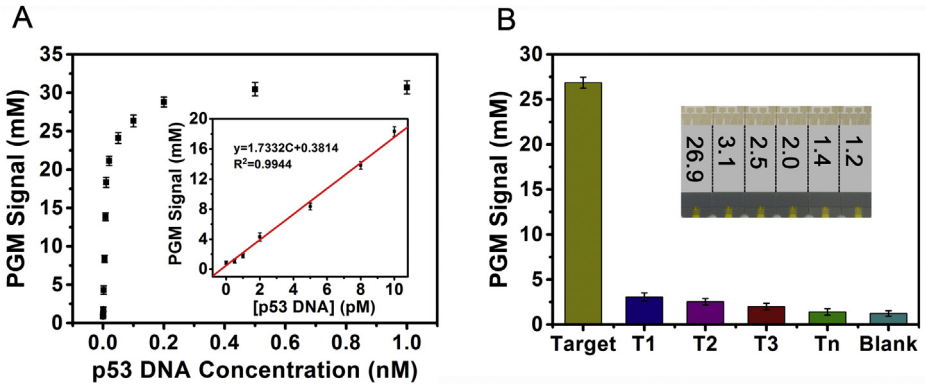


Fig. 4. Performance of p53 DNA detection in buffer using a PGM. (A) Concentration dependence of the PGM signal on the increasing concentration of p53 DNA, inset is the calibration curve of p53 DNA detection. (B) Selectivity of the proposed biosensor. Inset: the corresponding test strips and values. The assays were carried out in the reaction buffer containing 1 nM p53 DNA. The error bars represented the standard deviation of three independent measurements.

Table 2
Recovery results for the assay of p53 DNA in samples of human serum.

NOs.	Target DNA spiked (10^{-12} M)	Target DNA detected (10^{-12} M)	Recovery (%)	RSD% (n = 5)
1	10.0	10.8	108.0	5.4
2	8.0	7.6	95.0	5.7
3	5.0	4.8	96.0	6.7

4. Conclusion

In summary, a portable and low-cost method for quantitative detection of p53 DNA using a personal glucose meter was developed based on rolling circle amplification coupled with nicking enzyme signal amplification reaction. The p53 DNA triggered the Nt.BstNBI-assisted target recycling process, which continued to trigger another RCA reaction, resulting in multiple DNA-invertase conjugation bound to the surface of MBs. The DNA-invertase acted as a highly efficient signal amplifier to catalyze the hydrolysis of sucrose into glucose monitored by a glucometer. Compared with conventional strategy for p53 DNA sequence determination, the proposed biosensor is cost-effective, rapid and user-friendly without requiring professional assistance. With optimized detecting procedures, the proposed method exhibited a low detection limit and high specificity that was capable to detect p53 DNA in human serum, suggested a great potential application in clinical diagnosis as a point-of-care testing (POCT) tool. Therefore, this approach might offer a promising platform for nucleic acid detection using personal diagnosis devices.

Conflicts of interest

The authors declare no competing financial interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2019.01.061.

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