A highly sensitive and versatile fluorescent biosensor for pathogen nucleic acid detection based on toehold-mediated strand displacement initiated primer exchange reaction

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Method Article

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Abstract

Existing methods for pathogen nucleic acid detection, such as PCR, require complicated operation and expensive facility. Here, we report a simple and versatile strategy for highly sensitive detection of pathogen nucleic acid based on toehold-mediated strand displacement initiated primer exchange amplification (t-PER). In the presence of target, the blocked hairpin substrate would be released by toehold-mediated strand displacement, which triggered the primer exchange reaction amplification. Then, multiple long tandem-repeat single-strands generated by PER could open the molecular beacon to recover the fluorescence signal. Based on the target selection and conditions optimization, the developed platform could detect down to 18 fM of target in 1 hour of process time with high discrimination factor. It is also successful applied for direct detection of HPV from clinical cervical swab samples, the results were consistent with those obtained from RT-PCR. Moreover, the versatility and clinical feasibility of this method was further confirmed by measuring Epstein-Barr virus, Hepatitis B virus, and Ureaplasma Urealyticum from different clinical samples (serum samples and urine samples). This simple platform enables specific and sensitive detection of pathogen nucleic acid in a format that might hold great potential for point-of-care infection diagnosis.

1. Introduction

Pathogen nucleic acid detection play vita role in clinical diagnostics and infection management[1-3]. Compared to traditional pathogen culture, which requires long incubation time and specific operation, nucleic acid-based methods have been increasingly used for providing infection information[4-6]. Such as, human papilloma virus (HPV) gene detection is vital for cervical cancer screening[7-9]. More than 100 HPV subtypes are exited in the world, about 15 HPV subtypes are deemed as high pathogenic factor, which may cause cervical cancer[10, 11]. This disease progression is mainly linked to the specific HPV subtypes infection and continuous infection[12]. Point-of-care nucleic acid detection that can identify the HPV subtypes in clinical sample is of great important for disease monitoring and treatment[13].

At present, the traditional methods including real time polymerase chain reaction (RT-PCR), northern blotting, and microchip were developed for pathogen nucleic acids detection[14-17]. Nevertheless, some disadvantages of these methods, such as requirement of specialized equipment, tedious operation, and large scale, restricted their board practicability[18]. In this regard, various methods including electrochemistry, electrochemiluminescence, and fluorescence assay, have been developed[19-21]. Among these methods, the fluorescence-based method hold the greatest potential for pathogen nucleic acids detection because of its merits of high sensitivity, specificity, and dynamic monitoring[22]. In the past decades, many isothermal amplification-based fluorescence methods have been proposed for amplified detection pathogen nucleic acids[23]. Such as, loop-mediated isothermal amplification was used to detect the pathogen nucleic acids, which has improved sensitivity compared with other typical methods[24-26]. However, it requires professional personnel to handle and tedious sample preparation, and its probes design is complicated and specialized[27]. Hence, developing a simple platform with high sensitivity for pathogen nucleic acids detection remains urgent need.

Primer exchange amplification (PER) is an amplification strategy first developed by Yin's team, which obtains much attention because of its powerful signal amplification[28, 29]. PER exploits the sustaining amplification by hairpin DNA template and DNA polymerase, which generates multiple long DNA products for isothermal amplification[30, 31]. Compare to the other tedious isothermal amplification methods, PER can implement rapid and sensitive signal amplification only by simple hairpin template[32-34]. Afterwards, a new DNA nanotechnology based platform was proposed to promote the ongoing large-scale protein profiling in tissues by PER mechanism[35, 36]. These researches demonstrate the great potential of PER mechanism in clinical practices. Considering the above-mentioned features of PER mechanism via programmable automatic synthesis of tandem repeat strands, it holds great potential to develop PER-based method for pathogen nucleic acid detection.

In this work, we introduced a toehold-mediated strand displacement to initiate the PER amplification (t-PER). The toehold-mediated strand displacement exploits the ability of strand competition to improve the sequence specificity[37]. The inherent features of outstanding selectivity of toehold-mediated strand displacement and powerful signal amplification of PER would ensure high sensitivity and high specificity of the proposed platform. Based on the conditions optimization, the proposed t-PER assay can directly detect target down to the 18 fM in 1 hour with a higher discrimination factor when compared to the existing PER-based methods. This strategy could also distinguish multiple HPV subtypes with high specificity. Moreover, we successfully exhibit its versatility and clinical feasibility for detecting Epstein-Barr virus, Hepatitis B virus, and Ureaplasma Urealyticum (UU) from different clinical samples. Because of its versatility, low-cost, and simplicity, the t-PER assay can be easily integrated with POCT equipment, and as a potential complement to RT-PCR in clinical laboratories for pathogen nucleic acid detection.

2. Experimental Section

2.1. Materials and reagents

DNA oligonucleotides used for t-PER reaction were synthesized from Sangon Inc (Shanghai, China) (Table S1). Bst DNA Polymerase, Large Fragment (8000 U/μL), Magnesium Sulfate Solution (100 mM) and 10×ThermoPol Reaction Buffer were obtained from New England Biolabs, Inc (USA). dATP, dTTP and dCTP were purchased from Sangon Inc (Shanghai, China). 20 bp DNA Marker, 6×Loading Buffer and 4S Red Plus Nucleic Acid Stain were purchased from Takara (Dalian, China). Nucleic acid extraction kit and virus nucleic acid detection kit were acquired from Anpuli Bioengineering Co., Ltd (Xiamen, China). All chemical reagents were prepared with deionized water (≥ 18 MΩ, Milli-Q, Millipore).

2.2. t-PER protocol

The gate hairpin used for t-PER consisting of protector strand and blocked hairpin. All the probes were diluted to 10 μM by 1×TE Buffer and stored in 4℃ for further use. Firstly, all probes were denatured at 95℃ for 5 min and gradually reduced to room temperature before use. The protector strands and hairpins were mixed in a ratio of 1.2:1 (30 min). These probes were prepared for the subsequent t-PER reaction.

The reaction system was integrated two components including target-initiated strand displacement and PER amplification, which lead to one-pot t-PER assay. The components of target-initiated strand displacement included 500 nM pre-assembled gate hairpin, ThermoPol buffer and $MgSO_4$. Components of PER amplification included 10 μM primer, 2 units/μL Bst DNA Polymerase and 10 mM dATP/dTTP/dCTP mixture. In a typical assay, 12 μL components of target-initiated strand displacement was first mixed with 2 μL target DNA solution, and then supplemented with 6 μL components of PER amplification. The reaction mixture was then incubated at 37°C for 60 min for obtaining PER products. After that, molecular beacon (MB) (1 μM) was incubated with PER products for fluorescence measurement.

2.3. Polyacrylamide gel electrophoresis analysis

The 8% native polyacrylamide gel electrophoresis (PAGE) was used to confirm the procedure of t-PER. The process was carried out in 0.5×TBE buffer at 130 V for 50 min. After staining with 4S Red Plus, the gel was analyzed on UV Gel imaging system (Cambridge, UK).

2.4. Fluorescence measurements

Fluorescence measurements were performed on Luminescence Spectrometer (PerkinElmer LS-55, USA). The fluorescence assay was used to verify the displacement process between PER elongation products and MB. Firstly, the t-PER products were diluted by deionized water, and mixed with MB to a final volume of 60 μL. Then, the reaction mixture was incubated in the absence of light for 30 min following by fluorescence measurement (excitation wavelength: 488 nm, emission wavelength: 515nm).

2.5. DNA extraction

Clinical cervical samples were extracted by Nucleic acid extraction kit (Anpuli Bioengineering Co., Ltd, China). The Swab specimens were rinsed adequately by physiological saline, and the obtained solutions were transferred to new EP tube. After centrifuged at 13000 rpm for 5 min, the supernatant was discarded. Adding 200 μL Extract A into the tube and standing for 3 min. Then, 250 μL Extract B was added following by centrifugation (13000 rpm, 10 min). The supernatant was removed for subsequent 180 μL Extract C addition following by centrifugation (13000 rpm, 5 min). All the supernatant were removed and the remaining extractions were dried (60°C, 5 min). Finally, 40 μL TE buffer were added and the DNA samples can be used for subsequent analysis.

2.6 Preparation of clinical samples and ethical approval.

Cervical swab samples for HPV DNA detection, plasma samples for HBV and EB virus DNA detection, and urine samples for Ureaplasma urealyticum RNA detection were collected at the molecular biology laboratory, Nanfang Hospital of Southern Medical University, and the validation was executed by qRT-PCR. Cervical swab samples were collected with a separate sterile sampling tube, which contains 2 mL sterile normal saline. After collection, the samples were stored at − 80 ℃. Plasma samples were collected with anticoagulant tubes containing EDTA-2K anticoagulant. The collected samples were centrifuged at 3,500 rpm for 5 min, the supernatant was taken into centrifuge tube and stored at − 80 ℃. Urine samples

were collected with dry and sterile sample tubes. The laboratory testing, healthy donor testing, and on-site testing at Nanfang Hospital involved completely anonymized samples with no personal data or possible identification of individuals.

2.7. RT-PCR analysis

The RT-PCR assay was carried out on Light Cycler 480 II (Roche, USA). The RT-PCR reaction system included 10 μL component A (Tris, KCl, $MaCl_2$, dNTPs, and Taq enzyme), 8 μL component B (primers and signal probes) and 2 μL extracted nucleic acid. The thermal cycler protocols were as follows: 50°C (15 min), 95°C (10 min), 45 cycles of 95°C (10 s) and 58°C (40 s). Real-time quantitative analysis was performed in the real time PCR system (SLAN-96P).

2.8. Statistical analysis.

Data pre-processing (baseline subtractions and normalization) were performed using Origin 2018. Mean values and standard deviations were obtained from three identical tests. GraphPad Prism 9 was used for statistical analysis. The differences between groups were obtained by the unpaired two-tailed t test. (P values < 0.05 represents significant).

3. Results And Discussion

3.1. Principle of the t-PER-based pathogen nucleic acids biosensor

The principle of the proposed biosensor based on t-PER is shown in Scheme 1. The human papillomavirus (HPV) was chosen as the model. Two reactions are consisted in this system, including toehold mediated-PER amplification and signal out. The whole PER system includes hairpin with stop site, primer, and Bst DNA polymerase. The 3’ end of hairpin and protector strand were modified by inverted T base,which can inhibit the self-extended by DNA polymerase. The stop site is consisted of consecutive three G bases, which can prevent the polymerization in the presence of substrate (dATP, dCTP, and dTTP). At first, the hairpin was blocked by the protector strand, which leads to inactivation of DNA polymerase. In the addition of target, the blocked hairpin was released by toehold-mediated strand displacement. The primer was combined with the hairpin, and extended by the active DNA polymerase to the stop site, which is. Because of the breathe reaction and repeat sequence “a” design of hairpin stem, the extended primer would be set back to the initial binding site of hairpin, and the DNA polymerase works again. Then, a primer exchange reaction is completed. After multiple exchanges reacted, long massive DNA products were obtained. It would open plentiful molecular beacon (MB) to realize recovery of FAM fluorescence. The whole system was incubated at 37℃ in one pot. Thus, a simple and specific fluorescence platform was developed for HPV detection.

3.2. Characterization and feasibility of the proposed biosensor.

The polyacrylamide gel electrophoresis was first used to demonstrate the feasibility of the t-PER. As shown in Fig. 1a, single sequences (Lane 1: hairpin; Lane 2: protector) and double-strand products (Lane 3: target + protector; Lane 4: protector + hairpin) were synthesized without other heterochains. The protector could be hybridized with the target successfully by the toehold-mediated strand displacement (Lane 5). The PER was initiated to generate long single-strand products (Lane 6). In the presence of target DNA, the t-PER system could be realized as designed (Lane 8). Without the addition of target, no high molecular weight bands were obtained (Lane 7), showing an outstanding stability of this system.

The feasibility of this fluorescence platform based t-PER was further studied by measuring the fluorescence signals of recovered FAM. As described in Fig. 1b, when the hairpin and primer mixed with denatured DNA polymerase, the fluorescence intensity could hardly be observed (curve d). On the contrary, the PER was successful initiated by adding the activated DNA polymerase. Then, the multiple long DNA products obtained from PER would open the MB, which generate much higher fluorescence intensity (curve a). With the addition of protector, the PER will be blocked lead to weak fluorescence signal (curve c). In the presence of target DNA, the blocked PER can be triggered again (curve b), indicating that the target initiated PER amplification by toehold-mediated strand displacement. These results demonstrated that the t-PER could specificity recognize the target and generate an amplified fluorescence signal.

Most isothermal amplification for pathogen nucleic acids detection includes two steps (target specific recognition and signal amplification), which lead to the cumbersome operation[38]. To study the feasibility of the one-step reaction based t-PER, the fluorescence measurements of the one-step and two-step processes were adopted, respectively (Fig. 1c). The fluorescence signal of one-step process was 5.3% higher than that of two-step process, which proven that it is feasible to perform the toehold-mediated displacement and trigger PER amplification simultaneously, and also efficient for analytical performance. This may be due to the fact that when the target initiated the strand replacement and the PER system was separated for continuous reaction, there is relatively sufficient time for primer to generate the long single DNA strand, which tosome extent hampered the emergence of hairpin-protector-target terpolymer. While the concurrent occurrence of the two reactions made the target could bind with the protector-hairpin immediately. In addition, the fluorescence kinetic monitoring was used to further verify the viability of the one-step reaction. The results exhibited a time-dependent fluorescence intensities increase until a plateau from different target concentrations within 30 min (Fig. 1d). These above results demonstrate that the designed t-PER method could be adopted for the quantitative detection of pathogen nucleic acid in one-step.

3.2. Optimization of reaction conditions

To obtain optimal analytical performance of the proposed platform, several important parameters were optimized. Firstly, the concentration of DNA polymerase was investigated. As shown in Fig. 3a, the signal to noise ratio (S/B) achieved the perk value at a concentration of 0.2 U/μL DNA polymerase, thus the 0.2 U/μL was selected as optimal concentration of DNA polymerase for subsequent experiments. Next, the

length of primer probe from 8 to 16 nt was optimized. As depicted in Fig. 3b, the S/B increased as the increasing length of the primer up to 12 nt, the S/B decreased with the further increasing the primer length, demonstrating that increasing the length of primer not only improved the efficiency of t-PER amplification but also enhanced background signal. The effects of reaction time and temperature on the proposed platform were also studied. Figure 3c showed that the S/N reached the maximum at 60 min of incubating time, followed decline with the increasing time. As shown in Fig. 3d, the S/N reached the maximum at a temperature of 37°C. Thus, the incubating time of 60 min and reaction temperature of 37°C were chosen for the following test.

3.3. Analytical performance of the proposed platform.

The sensitivity of this method was studied first using synthesized target DNA with varying concentrations. As described in Fig. 4a, the results shown that the fluorescence intensity (FI) increased with the increasing of target DNA concentration (C_{target}) from 100 fM to 50 nM. There was a good linear correlation between the current signal and the logarithm of C_{target} in the range from 100 fM to 1 nM (Fig. 4b), fitted as $FI = 7.346\ lgC_{target\ (fM)} + 28.826$ with correlation coefficient of 0.999. The limit of detection (LOD) was 18 fM based on 3SD corresponding to the blank tests. Comparing with other fluorescent or colorimetric sensors based on isothermal amplification (Table S2), this method has moderate sensitivity. Notably, it allows detection to complete within 60 min in one step, without additional labeling or washing processes.

The specificity of this platform was evaluated by detecting multiple analogous sequences, including double-base mismatched DNA (DM), four-bases mismatched DNA (FM), random DNA (RS), five HPV subtypes (HPV 06, 18, 31, 33, and 58) and two human-associated viruses (HBV and EBV). As shown in Fig. 4c, the FI from the target DNA was 4 times high than that of DM and over 10 times higher than that of other analogous sequences, exhibiting high specificity of the developed platform. To further demonstrate the specificity of t-PER, we designed corresponding toehold-mediated strand replacement for specific distinguishing HPV subtypes (HPV 06, 18, 31, 33, and 58). As shown in Fig. 4d, compared to target HPV subtypes, the platform produced weak FI when responded to other HPV subtypes. The reproducibility of this biosensor was studied by measuring the samples contained 10 pM target DNA in six different batches. The variable coefficient of 2.8% was obtained, exhibiting a decent reproducibility of this platform.

3.3 Target selection in HPV whole genome.

Although some toe-hold based methods can be used in broad-spectrum pathogen detection, their clinical application is still limited. Some studies have pointed out that toehold-mediated recognition is restricted by the folding format of the gene region. The selection of target is important for the clinical application of toehold based methods[39, 40]. Therefore, we tested three specific target site using t-PER for HPV detection, including capsid protein coding gene (CPCG), L1 gene, and L2 gene (Fig. 2a). Before the performance analysis on this method for the whole genome, we adopted HPV DNA extracted from clinical

positive sample, which confirmed by RT-PCR (Ct value: 23.1). As illustrated in Fig. 2b-d, upon the target addition the t-PER reaction showed fluorescence signal enhancement, meaning that all of these three target genes could successfully work to transform the whole genome to the corresponding hairpin-primer template and further PER amplification. Notably, compared to the L1 gene (Fig. 2b) and L2 gene (Fig. 2c), the t-PER assay on the CPCG (Fig. 2d) showed better analytical performance (stronger fluorescence signal and higher signal to noise ratio (Fig. 2e)). The potential reason might be that the unexpected three-dimensional conformation and self-folding of the whole genome inhibits the correct binding of protector to corresponding hybrid site. To further explain the high efficiency of t-PER for CPCG, we carried out DNA structure analysis using Nupack[41]. By comparing the folded-binding sites with an additional 30 nt and 60 nt up and downstream, the L1 gene and L2 gene folded more compact than CPCG. And the whole hybridize site of L1 and L2 was in a structured region, whereas for the CPCG, the toehold region of the hybridize site was in a high probable unpaired region (Fig. S1). These results are consistent with the results of fluorescence measurement. The above results reveal that it is vital to choose a target sequence within the whole genome.

3.3 Clinical utility of the proposed platform

The clinical utility of this platform was studied by detecting HPV infections in clinical cervical swabs samples. As t-PER works through direct target binding, rather than via target amplification, we speculate that the t-PER could have reformative compatibility for clinical samples Fig. 5a. To achieve direct detection in clinical cervical swabs samples, we proposed a short heat lysis (95°C, 5 min) for released viral DNA (Fig. 5b) in five clinical positive samples. As shown in Figure S2, the FI obtained from heat lysis based t-PER was consistent with that from DNA extraction, the variable coefficient was under 2.5%. These results demonstrated that the viral DNA was released successfully, and its integrity was preserved. These may attribute to following reasons : 1) t-PER process through direct target replacement to initiate a polymerase. The polymerase-based elongation is hardly disrupted. 2) similar to biological functions, the design of t-PER has improved compatibility between enzymes and DNA strands. Then, we adopted the t-PER assay to determination DNA targets directly in these cervical swab samples (including 16 positive samples and 8 negative samples). It is worth mentioned that this platform distinguished all samples correctly. The results are consistent with those obtained from qPCR (Fig. 4g). Moreover, the signals of positive samples were significantly higher than those of negative samples (*$P < 0.005$, Student's *t* test), while Ct values of positive samples range from 22.3 to 32.03(Figure S3, Table S3).

3.5. The proposed platform used for other pathogen virus detection.

To better demonstrate the clinical utility, the developed platform was also used to detect HBV and EBV, which are vital biomarkers for hepatitis monitoring and nasopharyngeal carcinoma diagnosis, respectively. At first, the corresponding toehold-design and target region screening in genome was implemented (Fig. 5a and c). Considering that EBV LMP-2A gene and HBV S gene are widely adapted for

clinical application, the corresponding toehold-mediated strand placements were designed to identify these regions (Figure S4). As shown in Fig. 5C and D, this platform could detect all samples (including 10 positive samples and 5 negative samples) infected HBV and EBV with 100% concordance of the results obtained from qPCR. Significantly, the relative fluorescence signals of positive samples were highly consistent with the Ct values (Table S4). In order to further study the versatility of the proposed method, the RNA target of the Ureaplasma Urealyticum (UU) in 10 clinical samples (including 6 positive samples and 4 negative samples) were also successfully tested. These results also demonstrated that this method could be used for RNA target detection (Figure S5 and Table S5).

4. Conclusions

In summary, this work has exhibited a highly sensitive and versatile fluorescent biosensor for rapid and sensitive detection of pathogen nucleic acid using t-PER amplification. The outstanding selectivity of toehold-mediated strand displacement for identifying homologous sequence and powerful signal amplification of PER ensure the high specificity and high sensitivity of the proposed platform. Benefiting from this design principle, this platform possesses some innate features including one-step reaction, rapid detection, and is compatible with common microplate reader that is widely used in the clinical laboratories. Based the optimal target selection, this platform was also successfully used for detecting four human-associated pathogens (HPV, HBV, EBV, and UU) in clinical samples, and the results were consistent with the qPCR tests. Thus, this platform may provide a promising way to detect pathogen nucleic acid for clinical diagnosis and monitoring.

Declarations

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Authors' contributions

YZ, ZL, and WS conceived the idea and designed the experiments. GZ, XZ, and YW performed the experiments. BS and YX wrote and revised the manuscript. XY and LZ procured funding and supervised the research. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All experiments using human material were gained from Nanfang Hospital of Southern Medical University and approved by the medical ethics committee of the hospital.

Consent for publication

All authors agree to publication.

Competing interests

The authors declare no competing interests.

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Scheme

Scheme 1 is available in supplementary section.

Figures

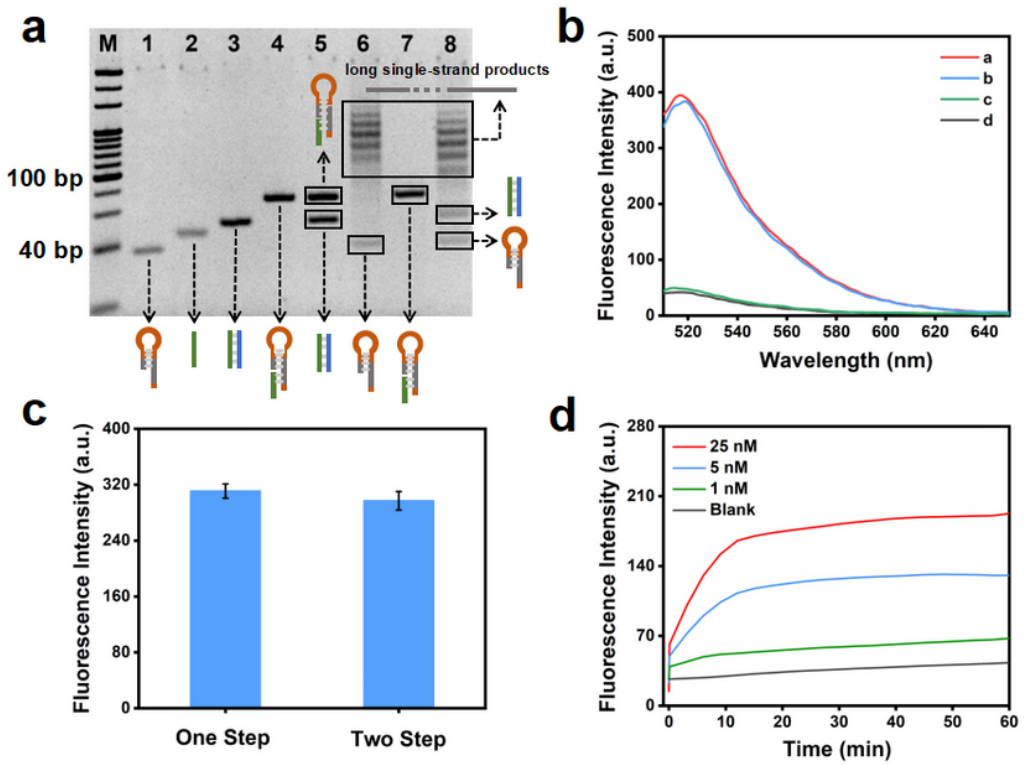

Figure 1

(a) Fluorescence spectra of different reaction solutions for target DNA detection. Each group contains DNA polymerase, dNTP and hairpin. (b) Analysis of PER products using 12% PAGE. Lane M: 20 bp DNA ladder marker; Lane 1: 500 nM hairpin; Lane 2: 500 nM protector; Lane 3: 500 nM target + 500 nM protector; Lane 4: 500 nM protector + 500 nM hairpin; Lane 5: 500 nM protector + 500 nM hairpin + 500 nM target; Lane 6: PER system; Lane 7: blocked PER system; Lane 8: blocked PER system + 500 nM target. (c) Fluorescence intensity measurement of two different reaction procedures: two-step and one-step. (d) Fluorescence kinetics monitoring conversion of different concentrations of target DNA.

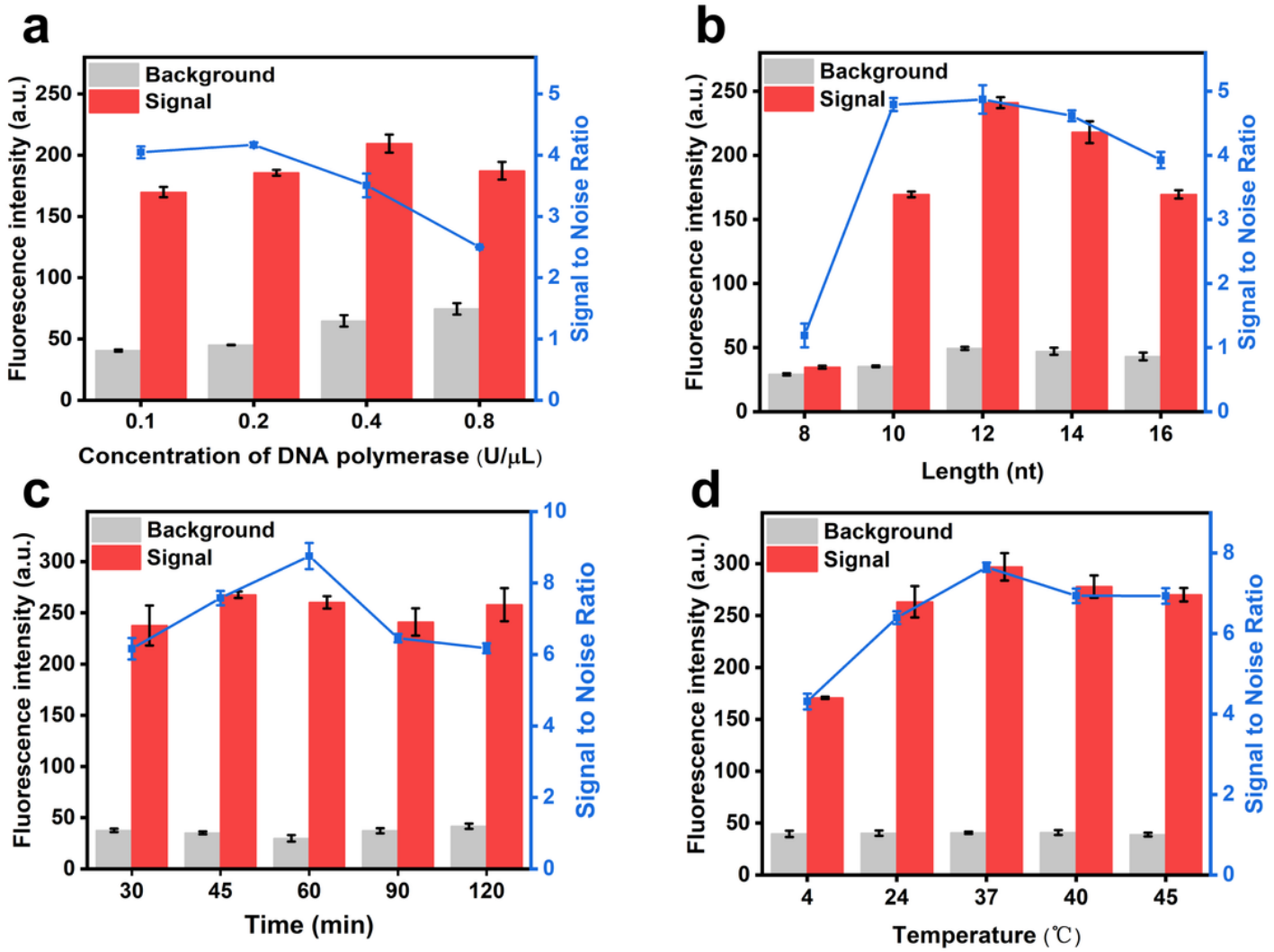

Figure 2

The optimizations of DNA polymerase concentration (a), the length of primer (b), incubating time (c) and reaction temperature (d).

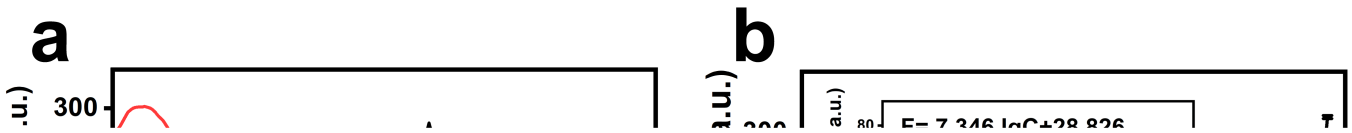

Figure 3

(a) The fluorescence spectra of the fluorescence biosensor with different concentrations of target DNA. (b) Changes in the fluorescence signals with different target concentrations. Inset: linear region of fluorescence signals of target DNA. (c) Fluorescence intensity respond to the target HPV 16, double-mismatched sequence (DM), four-mismatched sequence (FM), random sequence (RS), five HPV subtype, HBV, EBV and blank with a concentration of 50 nM. (d) Heat map performance of the orthogonal identification of six HPV subtype.

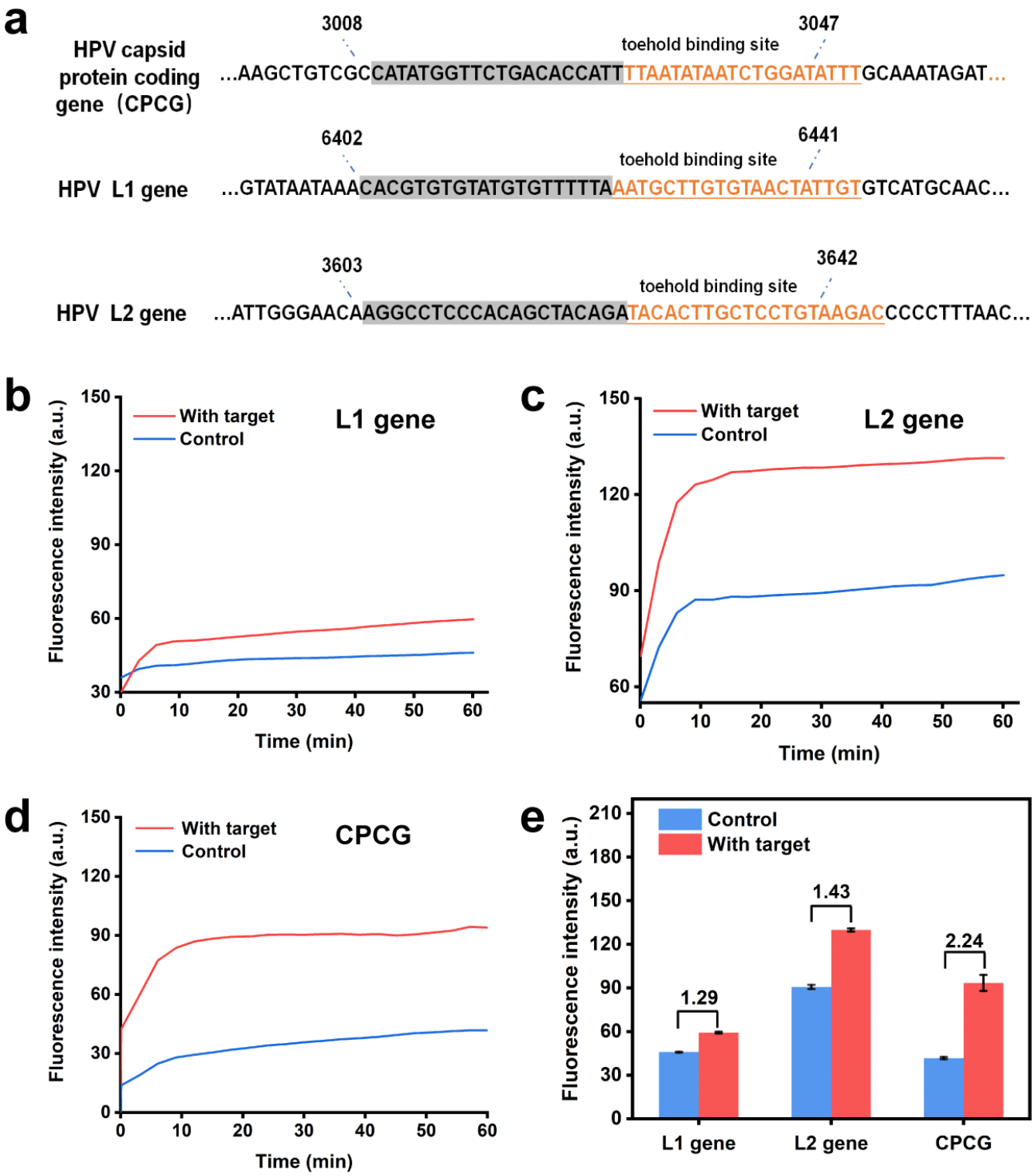

Figure 4

The selection of target sequence within the whole genome. (a) Location of the toehold-binding sites for both RdRP and N genes. Typical curves illustrating the fluorescence signal kinetics of the NISDA assay over time, designed for L1 (b), L2 (c), and CPCG (d) using t-PER assay. (e) The signal to noise ratio of the L1, L2, and CPCG using t-PER assay.

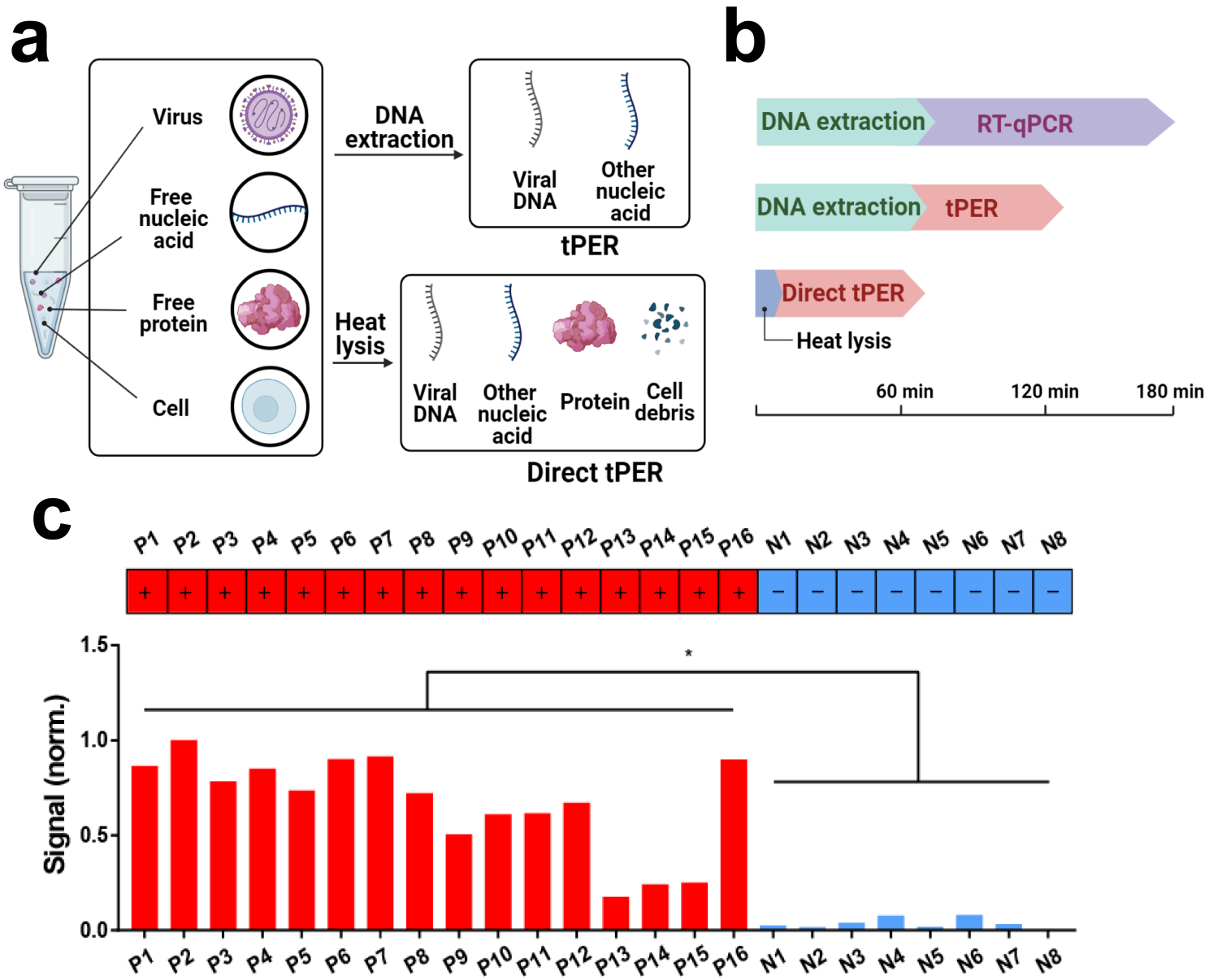

Figure 5

(a) T-PER could be used for analyzing extracted DNA and cervical swab lysates. (b) Comparison of reaction time for different methods. Typical method qPCR involves DNA extraction and time-consuming reaction time (≥180 min). The direct t-PER method can be completed within 60 min at constant temperature. (c) Measurement of HPV-16 virus in 24 patients (positive samples: P1-P16; negative samples: N1-N8). The clinical cervical swabs samples were confirmed by qPCR. (*P < 0.05, student's t test).

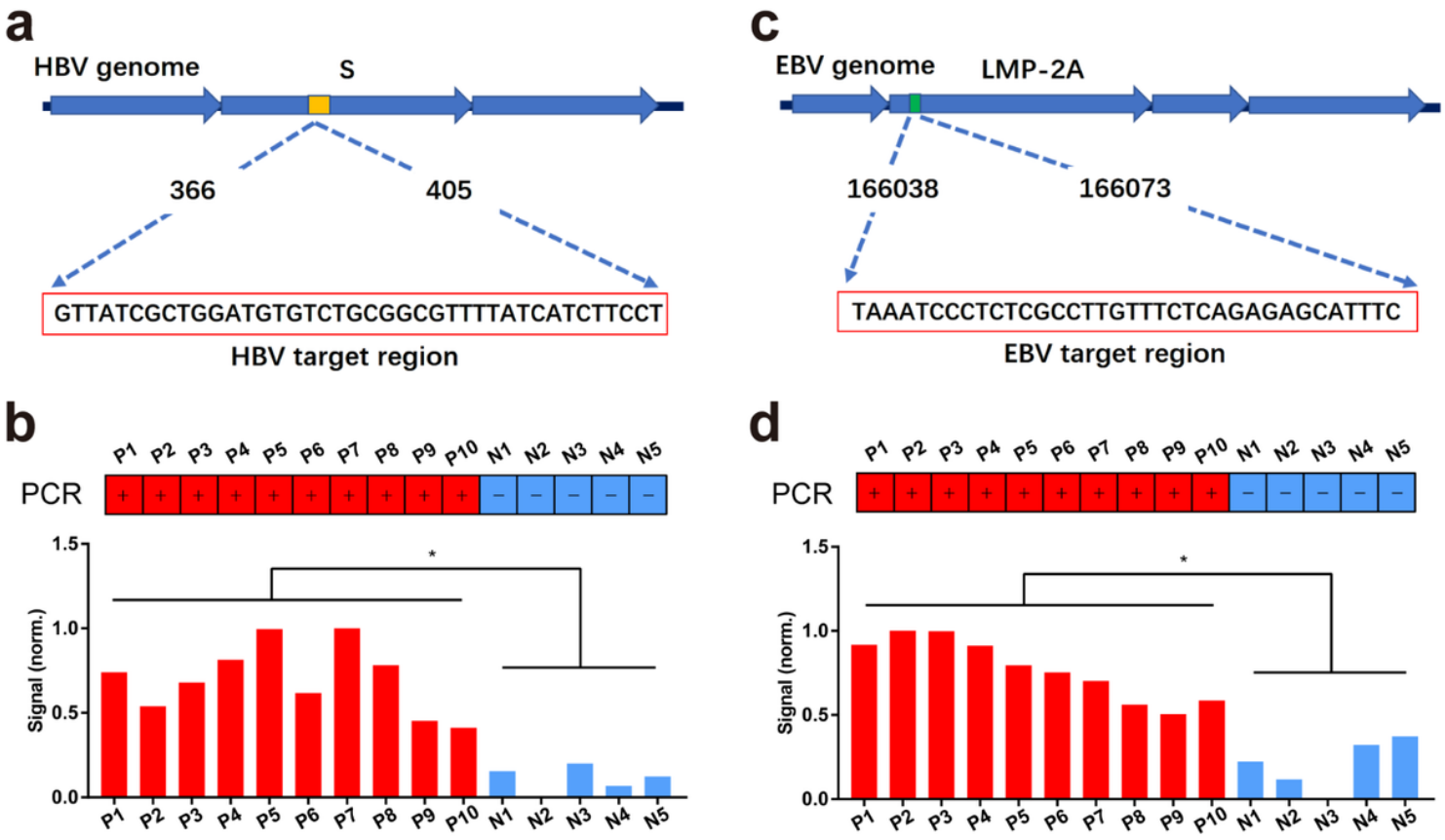

Figure 6

The proposed platform for two human-associated viruses detection. (a) Schematic of the HBV genome and corresponding target region. (b) Measurement of HPV-16 virus in 15 patients (positive samples: P1-P10; negative samples: N1-N5). The clinical serum samples were confirmed by qPCR. (c) Schematic of the EBV genome and corresponding target region. (d) Measurement of HPV-16 virus in 24 patients (positive samples: P1-P10; negative samples: N1-N5). The clinical serum samples were confirmed by qPCR. (*P < 0.05, student's t test).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 11.25Supportinginformation1.doc
- Scheme1.pdf