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SPECIAL: Phosphorothioate dNTP assisted RPA equipped with CRISPR/Cas12a amplifier enables high-specific nucleic acid testing

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ABSTRACT

Recombinase polymerase amplification (RPA) is one of the most widely used isothermal amplification methods and considered to be a promising tool for point-of-care testing (POCT) molecular diagnosis. However, RPA is prone to have nonspecific amplification occur, due to the poor recognition accuracy of polymerase and recombinase, which severely hindered its clinical application. It is important to improve the specificity of RPA further. Herein, we developed a novel nucleic acid testing method termed phosphorothioate dNTP (dNTPαS) assisted RPA (S-RPA) that employs dNTPαS as substrates to suppress nonspecific amplification effectively. We found that dNTPαS could improve the recognition accuracy of Bsu polymerase and recombinase, thereby enhancing their amplification specificity. Our S-RPA provided much higher specificity (approximately 40 % improvement compared to classical RPA), realizing detection target with single nucleotide mutation. Based on its outstanding performance, we further combined the S-RPA with CRISPR/Cas12a to achieve highly specific and sensitive fluorescence detection, namely S-RPA equipped with CRISPR/Cas12a amplifier (SPECIAL). Our SPECIAL was more sensitive (10-fold higher) than the classical RPA-CRISPR/Cas12a assay, offering 100 % agreement with the qPCR during clinical validation. In summary, a strategy based on dNTPαS was established to enhance the specificity of RPA, thereby improving its practicability and providing a potential POCT tool for molecular diagnosis.

1. Introduction

Nucleic acid testing based on isothermal amplification is considered to be one of the most promising tools for POCT molecular diagnosis due to its sensitivity, rapidity, robustness, and economy (Dong et al., 2022; Tian et al., 2020; Wei et al., 2023). Over the last two decades, a number of isothermal amplification technologies have been developed (Yan et al., 2014; Akhavan et al., 2012), such as RPA, loop-mediated isothermal amplification (LAMP), strand displacement amplification, exponential isothermal amplification reaction, and so on (Luo et al., 2021a, 2023; Zhang et al., 2024). For these methods, RPA is widely used in modern biological and clinical diagnosis (Daher et al., 2016). RPA adopts three proteins (i.e., recombinase, Bsu polymerase and single-stranded DNA binding protein) to realize exponential amplification (Del et al., 2019; Piepenburg et al., 2006). Although RPA is simple, fast, and sensitive, nonspecific amplification should not be ignored (Cui et al., 2022; Munawar et al., 2022).

In RPA, the recombinase forms a recombinase/primer filament, and

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the ssDNA primer invades the target dsDNA and initiates strand exchange, followed by dNTP incorporation and Bsu-mediated extension from the 3′ end of the primer, ultimately resulting in the exponential amplification of the target DNA (Del et al., 2019; Piepenburg et al., 2006). Consequently, the specificity of RPA is mainly dependent on the recognition accuracy of recombinase and Bsu polymerase. However, the accuracy of homologous recombination is not always satisfactory. Zhang et al. demonstrated that recombinase/primer filament can tolerate mismatches with a minimum of 18 (23 %) bases in an 80-mer DNA sequence (Zhang et al., 2001). In addition, Bsu polymerase is prone to trigger nonspecific amplification, leading to false positives. Emery et al. have shown that Bsu polymerase was capable of producing nonspecific nucleic acid in the presence of T7 RNAP, dNTPs and a single-stranded DNA (Emery et al., 2018). As a result, RPA tends to occur nonspecific amplification, consequently hampering its further development and clinical application (Cui et al., 2022; Munawar et al., 2022). Therefore, it is very important for RPA to increase its specificity to meet the strict requirements of clinical application.

Phosphorothioate triphosphates (dNTPαS) is a 5′-(α-P-Thio)-modified dNTP analogue (Eckstein et al., 2000). Mizrahi et al. demonstrated that dNTPαS can be well recognized by DNA polymerase as a substrate (Mizrahi et al., 1985). Studies have shown that dNTPαS can perturb DNA polymerases and decrease the rate of DNA polymerization, ultimately increasing the recognition accuracy of polymerase (Hu et al., 2020). Furthermore, research indicated that phosphorothioate substitution in dsDNA possesses the inherent decreased helix stability (Kellner et al., 2017). The stability of duplex is highly correlated with the amplification specificity (Luo et al., 2021b; Sipos et al., 2007; Tortajada et al., 2022). Specifically, the lower the stability of duplex is, the more favorable it is for minimizing nonspecific primer-combination and primer-extension, ultimately enhancing amplification specificity. Based on these understandings, we hypothesized that dNTPαS can improve the amplification specificity of RPA by increasing the recognition accuracy of Bsu polymerase and recombinase and reducing the nonspecific duplex stability between primers and nonspecific phosphorothioate-amplicons (Fig. 1A).

A promising alternative POCT tool is the clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) technology (Ahmed et al., 2021; Gootenberg et al., 2018; Qin et al., 2022). The origin of CRISPR/Cas technology was used for gene editing but has recently been repurposed in molecular diagnosis applications due to the finding of trans-cleavage of any available nonspecific single-stranded DNA (Cas12) or RNA (Cas13) (Chen et al., 2018; Gootenberg et al., 2017; Rabiee et al., 2022). It is an exciting technology with simple, fast, and robust performance. Herein, we developed a novel molecular diagnosis platform termed dNTPαS assisted RPA (S-RPA, Fig. 2A)) equipped with CRISPR/Cas12a amplifier (SPECIAL, Fig. 5A). SPECIAL consists of two processes: an isothermal amplification with specific product accumulation (S-RPA) and a CRISPR/Cas12a reaction for rapid target-specific signal magnification and output. With these unique properties, our SPECIAL is 40 % more specific and 10-fold more sensitive than the classical RPA-CRISPR/Cas12a assay. Due to the high-specific and ultrasensitive performance, SPECIAL has been successfully used for Hepatitis B virus (HBV, DNA target) detection, offering 100 % agreement with qPCR results.

2. Materials and methods

2.1. Materials

The reagents including DNA oligonucleotides, Plasmids, gRNA, dNTPs, SYBR Green, acrylamide/methylene acrylamide (29:1), polyacrylamide, acrylamide/bisacrylamide solution, 4SGelred nucleic acid dye, and 6 × DNA loading buffer were purchased from Shanghai Sangon Biotech (Shanghai, China). The dNTPαS was purchased from Zhongmeng Research Technology (Hubei, China). The RPA Isothermal Amplification Kit was purchased from Shenzhen Yi Zhi Biotechnology (Shenzhen, China). The LbCas12a protein was purchased from Meige Bio-technology (Guangzhou, China). The Bsu polymerase Kit was purchased from Xinhai Genetic Testing (Harbin, China).

2.2. Bsu polymerase-mediated primer extension reactions with dNTPαS

The Bsu polymerase-mediated primer extensions were performed in a 10 μL volume by using a Bsu polymerase Kit. The reaction mixture included 2 μM DNA primers, 2 μM DNA templates, Bsu polymerase (0.1X), Reaction Buffer (1X) and dNTPs (including canonical dNTPs and dNTPαS). The reaction mixtures were incubated at 37 °C for 30 min, and the reaction was terminated by heating at 75 °C for 20 min. Then the products were analyzed by non-denaturing polyacrylamide gel electrophoresis (PAGE).

2.3. Recombinase-mediated primer target recognition and binding reaction

The reaction was performed in a 20 μL volume by using RPA Isothermal Amplification Kit. The reaction mixture included 1 μM DNA primers, 1 μM ssDNA templates, Reaction buffer (1X), E-mix (1X), UvsX Recombinase (0.5X), and Starter (1X). All reagents except the DNA templates were prepared in a master mix, which was distributed into 0.2 mL reaction tubes. A total of 2 μL of DNA templates was added to the lid of the reaction tubes and lids were closed carefully. The tubes were instantaneously centrifuged, gently mixed and repeated three times. Final incubation at various temperature for 30 min, and the reaction was terminated by heating 65 °C for 10 min. Then the products were analyzed by non-denaturing polyacrylamide gel electrophoresis (PAGE). The template and primer sequences used are shown in Table S4.

2.4. RPA reaction and S-RPA reaction

The RPA reaction was performed in a 20 μL volume by using RPA Isothermal Amplification Kit. The reaction mixture included 0.5 μM of each primer, 0.36 mM of each dNTP, Reaction buffer (1X), E-mix (1X), P-mix (1X), Starter (1X) and 1 μL of the template. All reagents except the Starter were prepared in a master mix, which was distributed into 0.2 mL reaction tubes. A total of 2 μL of Starter (10X) was added to the lid of the reaction tubes and lids were closed carefully. The tubes were instantaneously centrifuged, gently mixed and repeated three times. Final incubation at 37 °C for 40 min. To address the nonspecific amplification of RPA, we attempted to add dNTPαS to the RPA reactions. We configured different proportions of dNTPαS and dNTP mixture as the amplification raw material.

2.5. PAGE analysis

The 12 % gel mixture (12 mL) was prepared with 4.8 mL acrylamide-bisacrylamide solution (29:1, 30 % w/v), 1.2 mL TBE (10X), 4.8 μL TEMED, 0.12 mL 10 % APS, and 5.875 mL ddH2O. Next, 4 μL of reaction products were mixed with 1 μL of loading buffer and loaded into the gel wells, followed by electrophoresis at 200 V for 45 min in TBE buffer (1X) at room temperature. After electrophoresis, the gel was stained with 4SGelred and visualized by the ChemiDoct XRS Imaging System (Bio-Rad Laboratories Co., Ltd, California, USA).

2.6. Melting curve analysis

The melting curve of the amplification product was analyzed by using the intercalating dye (SYBR Green) based on the fundamental thermodynamic property of DNA. Melting curve analysis was carried out on the SLAN-96 System by ramping the temperature from 50 °C to 95 °C at a rate of 0.03 °C/s. The melting curve was obtained from the continuous monitoring of fluorescence during temperature changes.

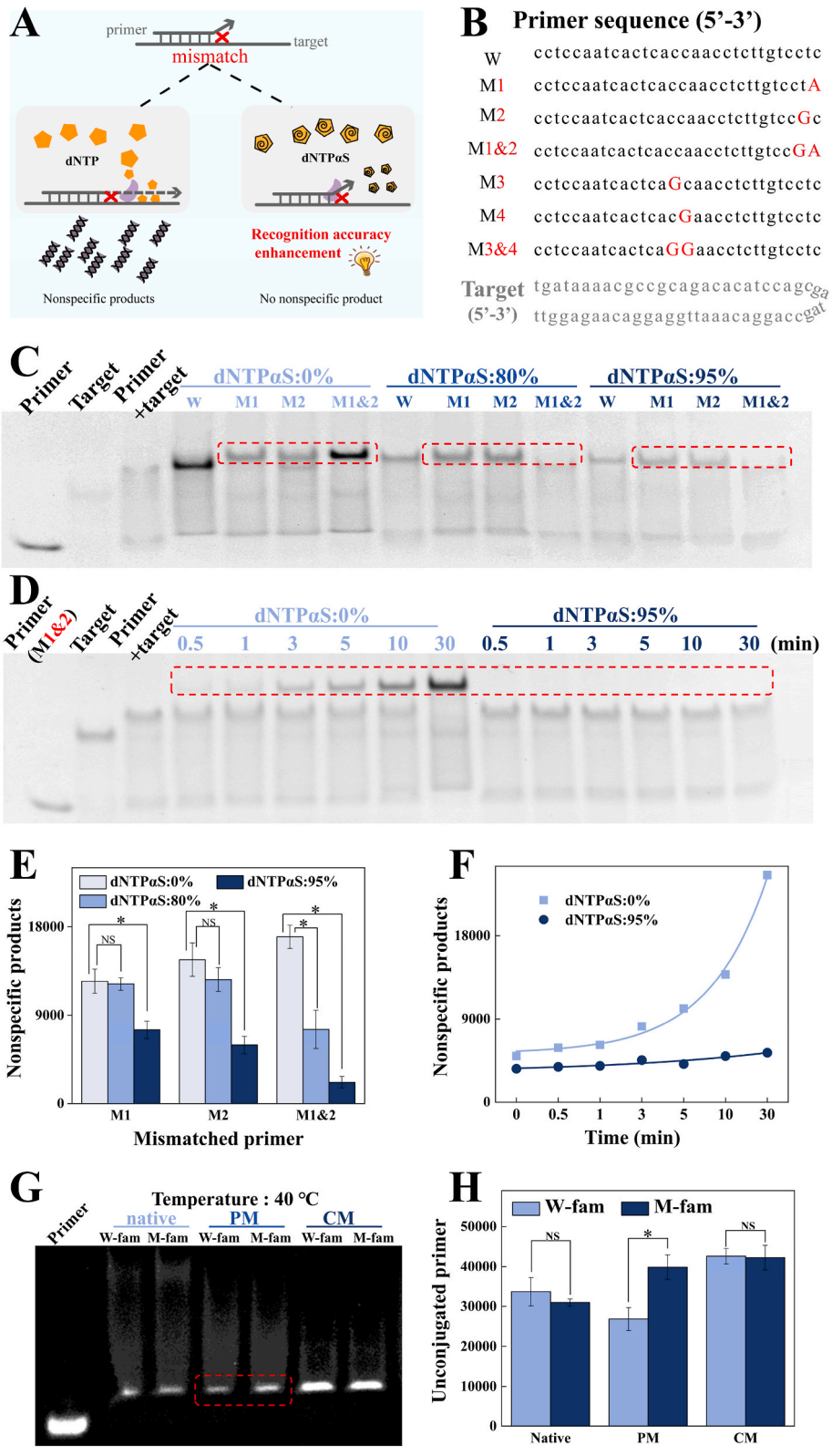


Fig. 1. dNTPαS enhances recognition accuracy of polymerase and recombinase. (A) The hypothesis of dNTPαS mediated recognition accuracy enhancement of Bsu polymerase. (B) Primer sequence for Bsu mediated extension. (C) Bsu mediated extension with different ratios of dNTPαS for 30 min reaction. The extension products were analyzed by PAGE. Red dashed boxes indicate nonspecific products. (D) Time course analysis for mismatched primer mediated extension. (E) Data from Fig. 1C based on gray value. (F) Data from Fig. 1D based on gray value. (G) Recombinase mediated recognition with different ssDNA template at 40 °C. Red dashed boxes indicate remaining primers. PM: partially phosphorothioate modification template; CM: completely phosphorothioate modification template. The ssDNA template and primer sequences used are shown in Table S4. (H) Data from Fig. 1G based on gray value. For C and G, each experiment was repeated three times, with one representative result shown. Error bars represent SD (n = 3). Statistical analysis was performed using a two-tailed *t*-test where NS refers to not significant with $p > 0.05$, and the asterisk ($^*p \leq 0.05$) denotes significant differences. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

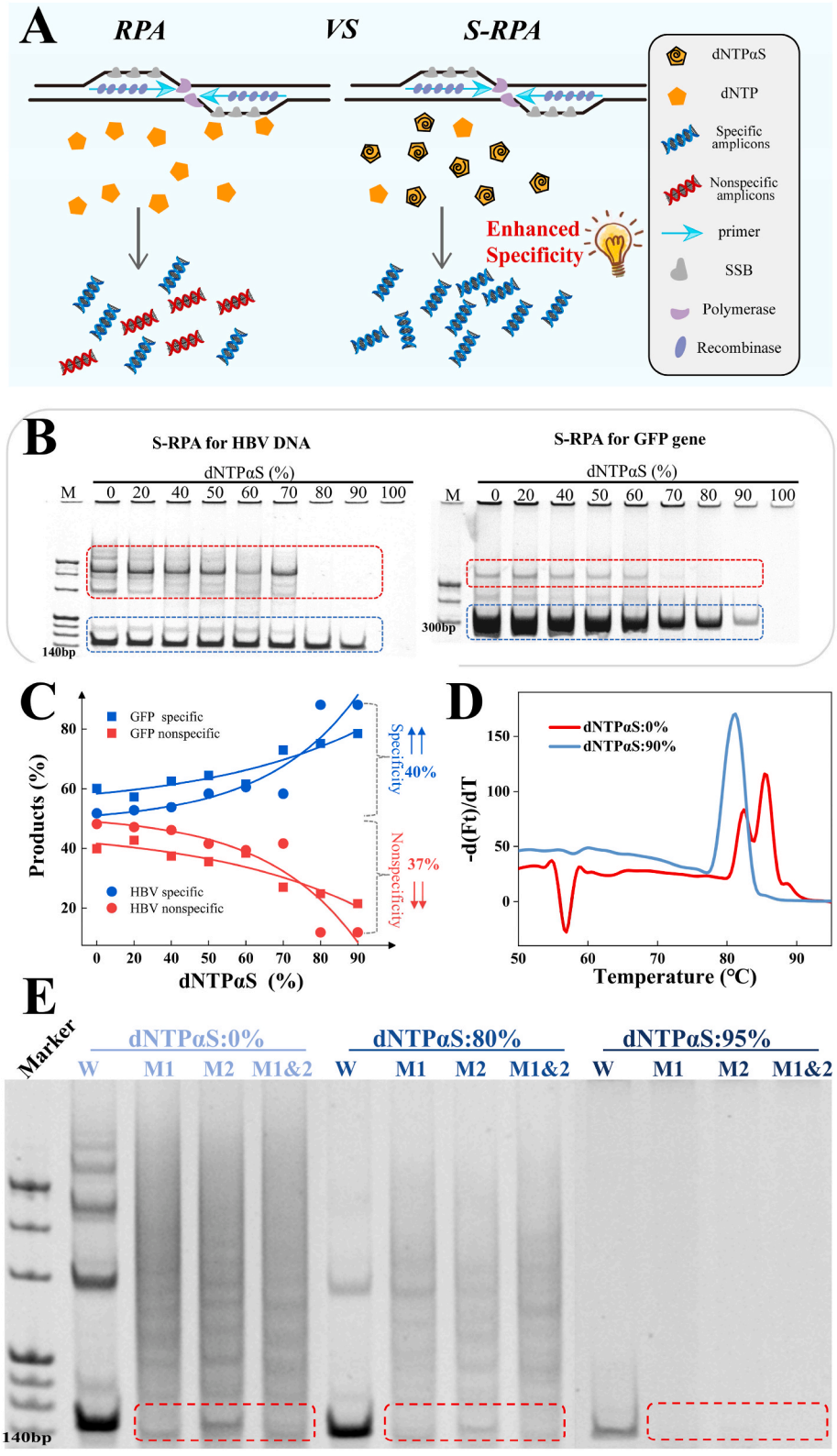


Fig. 2. Amplification specificity enhancement of S-RPA. (A) The hypothesis of dNTPαS mediated RPA specificity enhancement. (B) S-RPA for HBV DNA and GFP gene amplification. Red dashed boxes indicate nonspecific products and blue dashed boxes indicate specific products. (C) Data from Fig. 2B based on gray value. (D) Melting curve analysis of HBV gene amplificons. (E) Single base mismatch recognition of S-RPA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.7. Effect of thiophosphated DNA on CRISPR/Cas12a cis-cleavage

The cis-cleavage activity assay was performed in a 20 μl volume. For in cis-cleavage activity, LbCas12a and gRNA were incubated at room temperature for 30min in Reaction Buffer (1X) before being incubated with dsDNA at 37 °C. The reaction was terminated by heating at 65 °C for 10 min at various time points, and the products were visualized on a 12 % Tris-borate-EDTA (TBE) gel. The concentrations of LbCas12a and gRNA used were 100 nM, and the concentrations of dsDNA substrate were 300 nM. The percentages of substrates and products were quantified by Image J software. The cleavage efficiency at each time point was plotted as a function of time to calculate Kcleave values.

2.8. Mass spectrometry

Mass spectrometry was performed in a 20 μl volume containing 10 μM LbCas12a and 10 μM gRNA in Reaction Buffer (1X), and the concentration of dsDNA substrate activator used was 4 μM. First, LbCas12a and gRNA were incubated at room temperature for 30min in Reaction Buffer (1X) before being incubated with dsDNA at 37 °C. The reaction was terminated by heating at 65 °C for 10 min after 60 min of reaction at 37 °C. The product was then analyzed by mass spectrometry (Ionization spray voltage: 4.5 kv, atomization temperature: 350 °C, ion mode: negative ion mode). Mass spectrometry results were obtained from an LTQ liquid-mass ion Trap Mass Spectrometer (LTQ XL, Thermo Fisher Scientific, USA)

2.9. Effect of thiophosphated DNA on CRISPR/Cas12a trans-cleavage

The trans cleavage activity assay was performed in a 20 μl volume containing 100 nM LbCas12a, 100 nM gRNA and 100 nM ssDNA reporter in Reaction Buffer (1X), and the fluorescence signal was recorded using a Real-Time PCR Detection System (SLAN-96). The concentration of the dsDNA substrate used was 50 nM.

2.10. SPECIAL detection

The S-RPA product (4 μL) was mixed with 16 μL of the Cas12a reaction buffer (1X) containing 100 nM LbCas12a, 100 nM gRNA and 100 nM ssDNA reporter. Then, the signal was collected using a Real-Time PCR Detection System (SLAN-96) for up to 60 min at 37 °C.

2.11. Patients sample collection and qPCR assay

The clinical samples were approved by the Medical Ethics Committee of the Affiliated Hospital of North Sichuan Medical College (2024ER54-1), and the HBV samples were taken from the Affiliated Hospital of Nanchong North Sichuan Medical College. The Hepatitis B virus nucleic acid detection kit (Shanghai Kehua Biological Engineering, China) was used for qPCR assays (Registration certificate No.20233401120).

3. Results

1. dNTPαS enhances recognition accuracy of polymerase and recombinase

Based on our hypothesis, dNTPαS can increase the recognition accuracy of Bsu polymerase (Fig. 1A). To explore the dNTPαS inhibition of nonspecific extension at various pairing conditions of Bsu polymerase, we designed a ssDNA template and its matched and mismatched primers (Fig. 1B) and performed the extensions at various template primer combinations. PAGE analysis showed that all mismatched primers could generate a large amount of nonspecific products, both with 3′-end and intermediate mismatches (Fig. 1C and S1A). Excitingly, the mismatched primer-meditated nonspecific extension was gradually decreased with the addition of dNTPαS (Fig. 1C and E). Especially, when the primer has two mismatches at 3′-end (M1&2), its nonspecific extension was completely eliminated (Fig. 1D and F). These data collectively indicated that dNTPαS can increase the accuracy of Bsu polymerase in recognizing mismatched primers, and offer great potential to enhance the specificity of RPA.

Further, to explore the effect of thiophosphated DNA on the recognition accuracy of recombinase, we analyzed the binding efficiency between thiophosphated ssDNA template (equivalent to the amplicon of dNTPαS) and its primers labeled with FAM. Data showed that on the partially phosphorothioate modification template, the recombinase exhibited great mismatch discrimination ability under various temperature (with more unconjugated mismatch primers, Fig. 1G and H and S1). While, there was no difference in the amount of primer remaining for the native and completely phosphorothioate modification template. (Fig. 1G and H).

2. S-RPA with enhanced amplification specificity

To study the performance of RPA, we designed a series of RPA primer pairs for HBV gene on the basis of the typical rule of RPA primer (Table S1). PAGE analysis showed that only three primer pairs successfully amplified the target fragment (indicated by blue arrows) and accompanied by many nonspecific products (Fig. S2). These data confirmed that nonspecific amplification is prone to occur in RPA if the primers have not been rigorously refined, consistent with previous studies (Luo et al., 2019; Yi et al., 2021).

Due to the outstanding recognition accuracy of Bsu polymerase and recombinase in the presence of dNTPαS, we reasonably hypothesized that dNTPαS has the potential to enhance the specificity of RPA. Therefore, dNTPαS was employed as a substrate for RPA, namely S-RPA (Fig. 2A). To investigate its performance, S-RPA was used for HBV DNA and GFP gene detection. PAGE analysis showed that S-RPA provided significant specificity enhancement (Fig. 2B and C). Especially with 80–90 % of dNTPαS addition, the nonspecific products were obviously eliminated (about 37 % of decrease), whereas the specific products were significantly enhanced (approximate 40 % of increase). Further, the S-RPA for other two targets demonstrated identical performance (Fig. S2). Melting curve analysis showed that S-RPA products have a single peak, while classic RPA products have multiple peaks (Fig. 2D). These results collectively demonstrated the high specificity of S-RPA, thereby verifying our hypothesis.

Furthermore, the amplification specificity of S-RPA was evaluated by using the mismatched primer and HBV-plasmid template (pHBV). PAGE analysis showed that in the absence of dNTPαS, all mismatched primers unfortunately generated nonspecific products (Fig. 2E). The nonspecific amplification was gradually prevented with the addition of dNTPαS. Excitingly, it completely disappeared when the percentage of dNTPαS was 95 %, demonstrating that dNTPαS endows RPA with the ability of single base mismatch recognition.

3. Effect of thiophosphated DNA on CRISPR/Cas12a cis-cleavage.

CRISPR/Cas12a is one of the most widely used tools for signal amplification and output in molecular diagnosis (Ahmed et al., 2021; Gootenberg et al., 2018; Qin et al., 2022). We attempted to combine it with the high specific S-RPA for high performance nucleic acid assays. Due to the addition of dNTPαS, the S-RPA amplicon possesses the phosphorothioate bonds more or less(Fig. S4). To study the effect of phosphorothioated DNA, we performed the CRISPR/Cas12a mediated cis-cleavage by using native and thiophosphated DNAs (Fig. 3A). Data showed that the cis-cleavage was inhibited by thiophosphate modification. A time course analysis of cis-cleavage activity showed that the native DNA was completely cleaved after 150 min of reaction, whereas thiophosphated DNA could resist cis-cleavage for 180 min of incubation (with 50 % of the substrates remaining, Fig. 3B and C). Mass spectrometry provided the expected results that the thiophosphated dsDNA

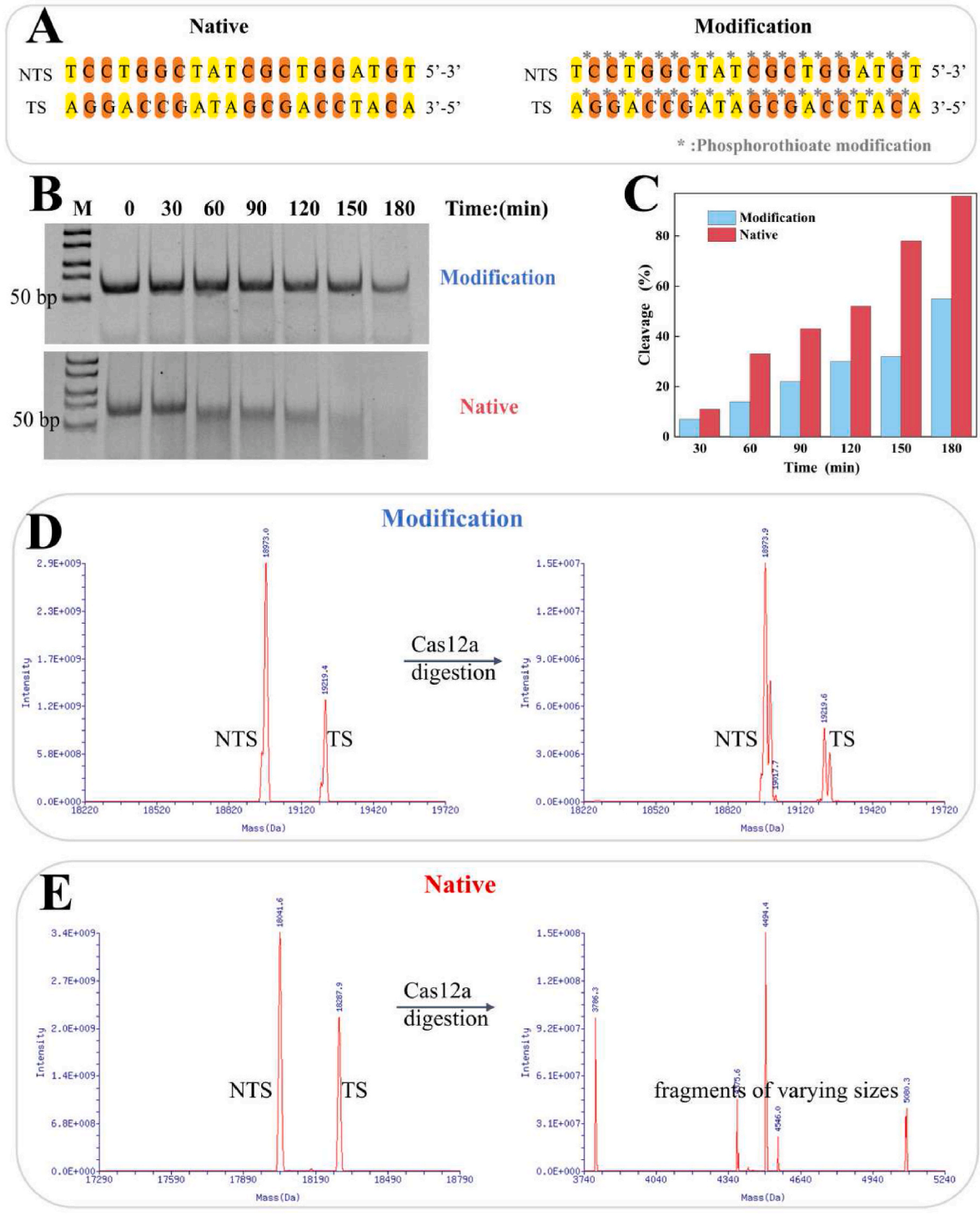
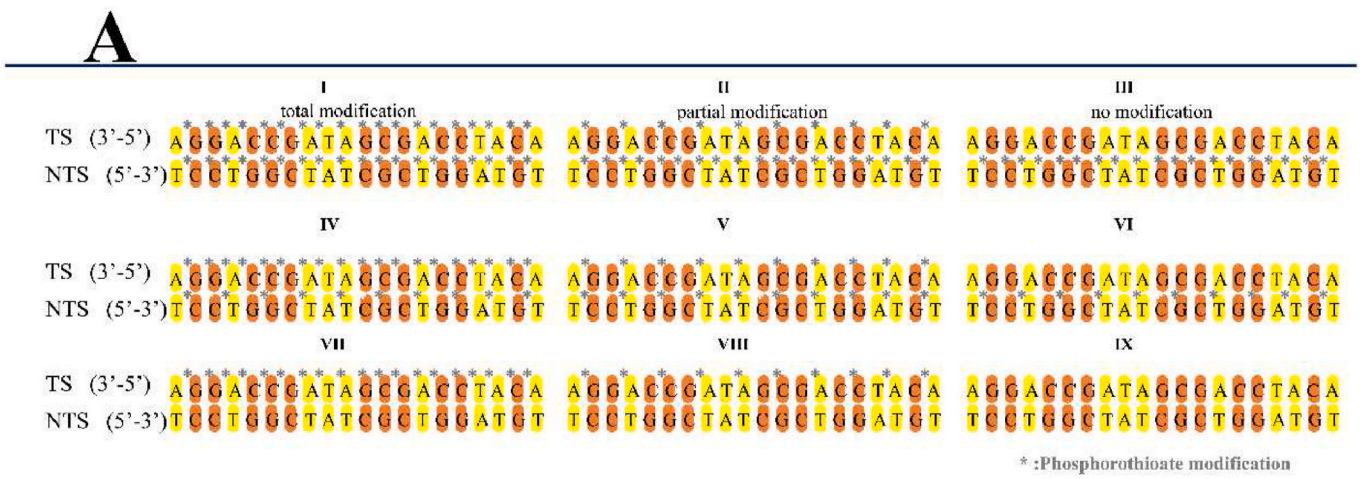


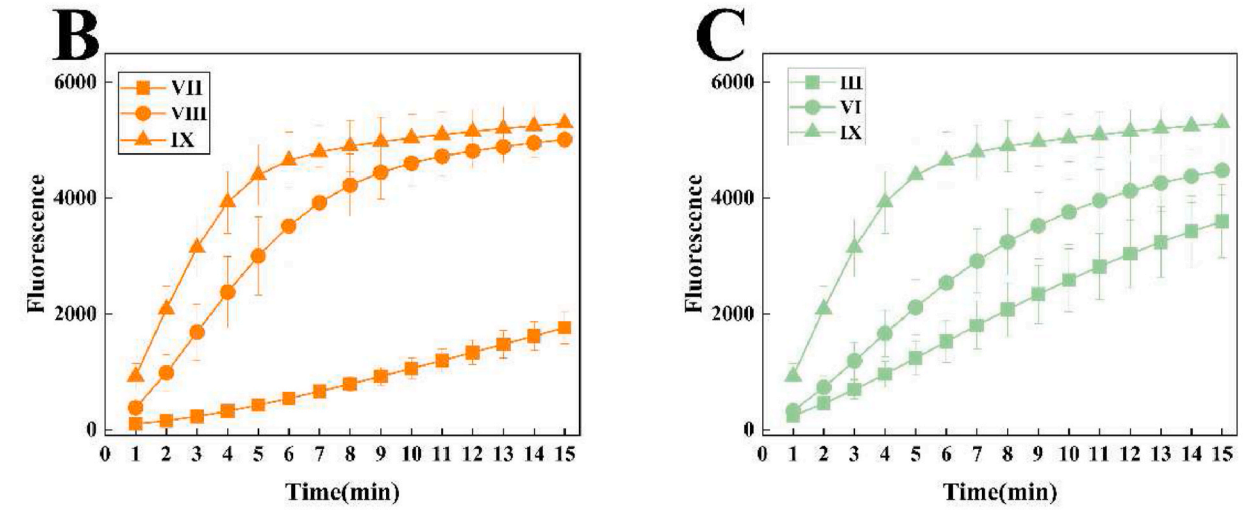
Fig. 3. Effect of thiophosphated DNA on CRISPR/Cas12a cis-cleavage. (A) Native and thiophosphated dsDNA sequence. Only the sequence complementary to gRNA is shown and refers to Table S4. TS represents the strand that is complementary with gRNA and NTS represents the strand that is complementary with TS. (B) Cis-cleavage of CRISPR/Cas12a for native and thiophosphated dsDNA. The gRNA/Cas12a complex was incubated with dsDNA at 37 °C for 0, 30, 60, 90, 120, 150 or 180 min and repeated three times, with one representative result shown. (C) Data from Fig. 3B based on gray value. (D) Mass spectrum results of thiophosphated dsDNA. (E) Mass spectrum results of native dsDNA.

had the same molecular weight before and after cleavage, whereas the native dsDNA was cleaved into fragments (Fig. 3D and E). These results indicate that phosphorothioate modification significantly attenuated the cis-cleavage of Cas12a.

4. Effect of thiophosphated DNA on CRISPR/Cas12a trans-cleavage.

To study the effect of phosphorothioated DNA on trans-cleavage, the fluorescence reporter based CRISPR/Cas12a trans-cleavage was performed (Fig. 4A). Real-time fluorescence showed that totally phosphorothioated TS significantly inhibited the trans-cleavage, whereas the TS with partially phosphorothioate modifications has almost identical trans-cleavage activity to the natural counterpart (Fig. 4B). Although the phosphorothioated NTS showed a similar tendency, its trans-cleavage





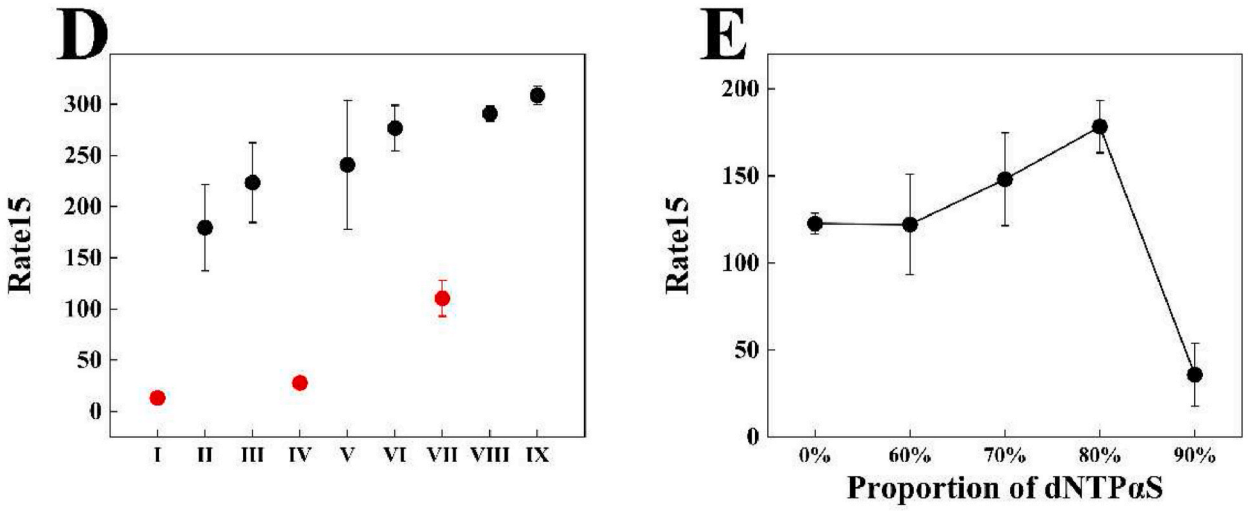


Fig. 4. Effect of thiophosphated DNA on CRISPR/Cas12a trans-cleavage. (A) Native and thiophosphated dsDNA sequence. (B) The Cas12a trans-cleavage with VII, VIII and IX in Fig. 4A. (C) The Cas12a trans-cleavage with III, VI and IX in Fig. 4A. (D) The Rate15 of natural or thiophosphated dsDNA in Fig. 4A. The Rate15 is defined as the total change of the fluorescence value, divided by the time duration of 15 min. The three red dots represent dsDNA composed of TS with totally phosphorothioate modification. (E) The Rate15 of the S-RPA product. Error bars represent SD (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

activity inhibition was attenuated (Fig. 4C and D). Further, when both strands were modified completely, the trans-cleavage activity was totally suppressed (Fig. S4). These data collectively demonstrated that it is reasonable and necessary to add dNTPαS proportionally in the amplification reaction. Further, the trans-cleavage of Cas12a for S-RPA amplicons gradually increased with the addition of dNTPαS (60 %–80 %, Fig. 4E), and we hypothesized that this was due to the increase in the amount of specific products even though the TS had partial

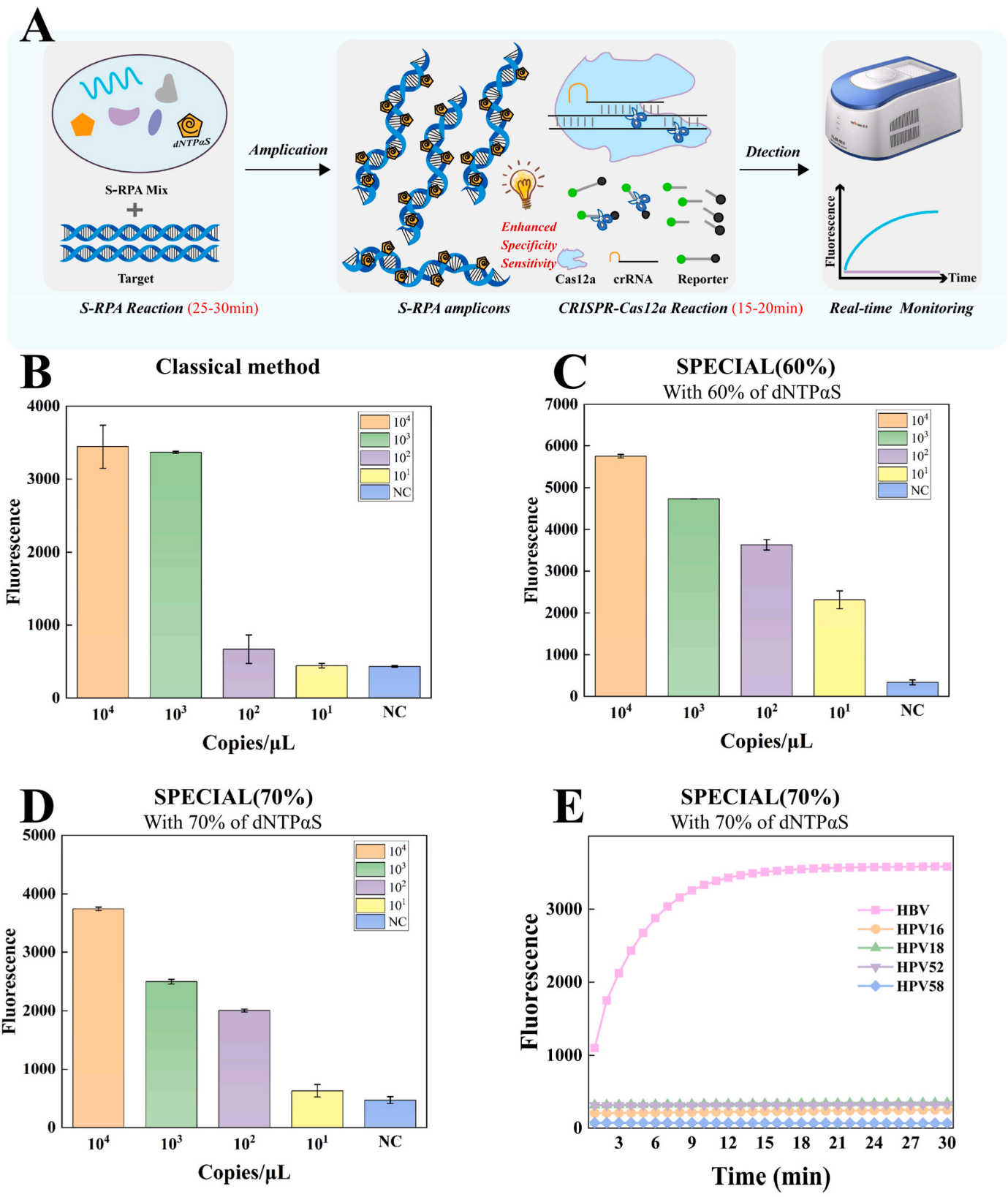


Fig. 5. Development of SPECIAL nucleic acid detection technology. (A) The workflow of SPECIAL. It takes a total of 40–50 min to complete all steps of SPECIAL. (B) Sensitivity of the classical RPA-coupled CRISPR/Cas12a detection system. (C) High sensitivity of the SPECIAL (dNTPαS 60 %). (D) High sensitivity of the SPECIAL (dNTPαS 70 %). (E) Specificity of SPECIAL (dNTPαS 70 %). The SPECIAL was performed with interfering plasmid with HPV16, HPV18, HPV52 or HPV58 fragment. Error bars represent SD (n = 3).

phosphorothioate modification. In conclusion, our results suggest that proper addition of dNTPαS during amplification could enhance RPA amplification specificity and Cas12a trans-cleavage activity simultaneously.

5. Development of SPECIAL nucleic acid detection technology

Enlightened by the enhancement of S-RPA specificity and Cas12a trans-cleavage activity, we further combined the S-RPA with CRISPR/Cas12a to achieve highly specific and sensitive nucleic acid detection, namely S-RPA equipped with CRISPR/Cas12a amplifier (SPECIAL, Fig. 5A). We compared the sensitivity of SPECIAL and the classical RPA-CRISPR/Cas12a assay using a DNA fragment of HBV. Since high specificity can facilitate sensitivity by reducing the consumption of primers and dNTPs and accumulating more specific products, SPECIAL (Fig. 5C and D and S6A). provided better sensitivity (10-fold) than the classical counterpart (Fig. 5B). However, when the dNTPαS addition was up to 90 %, the sensitivity of SPECIAL was comparable to that of the classical assay due to the reduction of Cas12a trans-cleavage activity caused by excessive thiophosphate modification (Fig. S6B). Further, the specificity of SPECIAL was evaluated by using interfering DNAs, including HPV16, HPV18, HPV52, and HPV58 DNA. The sequences of interfering DNAs are shown in Fig. S7. Data showed that SPECIAL could detect the target DNA exclusively (Fig. 5E).

6. SPECIAL for clinical sample detection

We evaluated the performance of SPECIAL in HBV-positive and -negative clinical samples (Fig. 6A), and compared its performance with qPCR. A total of 45 HBV-positive blood samples with a wide range of Ct values (18.0–34.9) and 35 HBV-negative samples were analyzed. SPECIAL had a sensitivity of 100 % and a specificity of 100 % (Fig. 6B–D). There were no false-positive or negative results, demonstrating great promise in clinical applications.

4. Discussion

In this study, we found that dNTPαS could improve the recognition accuracy of Bsu polymerase and recombinase, thereby enhancing their specificity of recombination and polymerization. Based on this understanding of the mechanism, we developed a novel nucleic acid testing method (S-RPA) and realized unprecedented enhancement of the amplification specificity of RPA. Based on the outstanding S-RPA system, we established a novel, simple, ultrasensitive and high-specific nucleic acid testing technology (SPECIAL), thereby providing a useful POCT tool for molecular diagnosis.

Previous studies have shown (Hu et al., 2020) that two major causes can jeopardize the specificity of DNA polymerization: (1) the mis-incorporation, which is the infidelity polymerization with mis-incorporated dNTPs; (2) the mis-priming, which is the nonspecific amplification from the mismatched primer/template extension. Our research showed that dNTPαS can increase the accuracy of Bsu polymerase in recognizing mismatched primers. We also found that the thiophosphate modification DNA template can improve the recognition ability of the recombinase for mismatched primers. Therefore, the primers no longer bind to nonspecific amplicons with thiophosphate

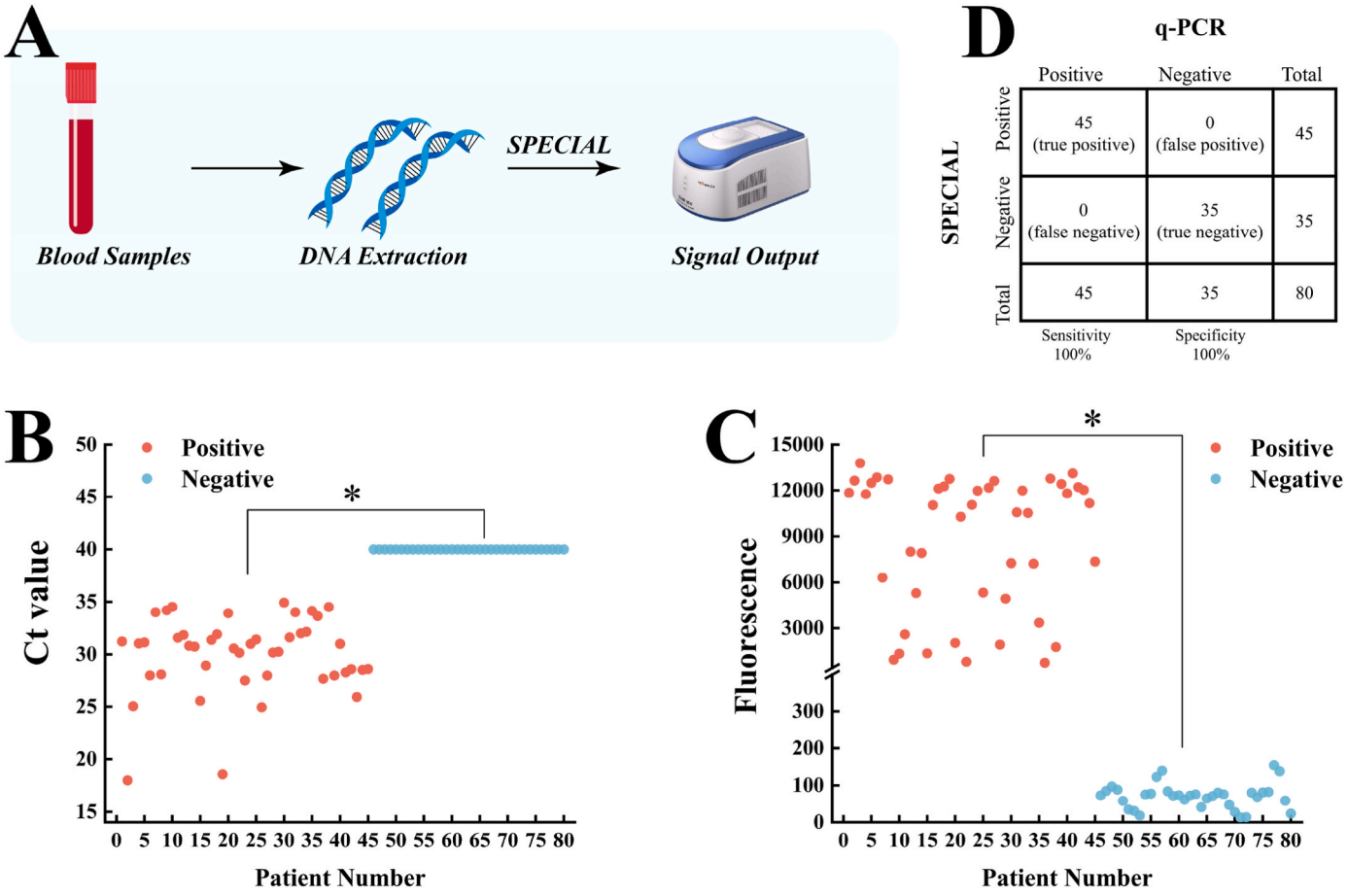


Fig. 6. SPECIAL for clinical sample detection (A) The workflow of detection process. (B) qPCR results for 80 blood samples. The Ct value of the negative is defined as 40. Ct: The cycle threshold. (C) SPECIAL results for 80 blood samples. The fluorescence readout was measured at 15 min at 37 °C. The cut-off fluorescence value (Fig. S8) was defined as Mean + 3SD of 3 negative control. (D) Concordance table between SPECIAL and qPCR.

modification. As a result, S-RPA possesses higher specificity than the classical one. Studies have found that dNTPαS decreases the rate of dNTPs polymerization so that the mismatched dNTPs have enough time to separate from the extension sites (Hu et al., 2020). Consequently, our S-RPA can improve the specificity in terms of minimizing the polymerization of incorrect dNTPs and reducing the binding and extension of incorrect templates to primers.

Our S-RPA has several advantages: (1) dNTPαS enhances the ability of Bsu to recognize single-base mismatches; (2) S-RPA significantly inhibited nonspecific amplification and increased the yield of specific products. And this specificity enhancement is highly correlated with the proportion of dNTPαS, which suggests that we can flexibly regulate the assay performance by varying the amount of dNTPαS. Our S-RPA is simple and effective, requiring only the addition of dNTPαS, and requiring neither precise nor specific primer design nor strict optimization of experimental conditions. The application of S-RPA on four targets has fully demonstrated its superiority and versatility. (3) S-RPA provided better sensitivity (10-fold higher) than the classical RPA-CRISPR/Cas12a assay. Based on the above outstanding advantages, it is reasonable to speculate that the dNTPαS assisted specificity enhancement is not only applicable for RPA, but also to other amplification methods (such as LAMP), and it would be interesting to explore the performance of other isothermal amplification techniques by using dNTPαS.

To take advantage of the S-RPA system, we developed a novel isothermal amplification technology, namely SPECIAL (Fig. 5A), which is realized by using the S-RPA for specific product accumulation and CRISPR/Cas12a for rapidly target-specific signal magnification and output. Thanks to the outstanding specificity and sensitivity of S-RPA and CRISPR/Cas12a, our SPECIAL assay is more sensitive as expected (a 10-fold increase). In addition, the SPECIAL has demonstrated excellent performance in the detection of clinical samples, offering 100 % agreement with the qPCR, which implies its robustness in practical applications.

Currently, most RPA-coupled CRISPR/Cas12a assays use a two-step process (amplification of the target sequence followed by CRISPR detection), with multiple liquid-handling steps increasing time of operation and risk of cross-contamination (Broughton et al., 2020; Patchsung et al., 2020). To avoid these disadvantages, isothermal amplification and CRISPR/Cas12a detection were integrated into a single step, creating methods such as SHINE and STOPCovid (Arizti et al., 2020; Joung et al., 2020). However, they are much less sensitive and require longer times (Arizti et al., 2020; Joung et al., 2020).It has been reported (Lu et al., 2022) that the cis-cleavage of CRISPR/Cas12a depletes the primers and amplification products, and therefore does not accumulate enough amplicons in a short period of time, which lengthens the reaction time and reduces the sensitivity. Therefore, it is crucial to reduce the cis-cleavage activity at the initial stage of the reaction. Notably, our results revealed that the thiophosphate modification DNA significantly reduced the cis-cleavage activity of CRISPR/Cas12a (Fig. 3B), and it will be interesting to see whether the development of S-RPA with CRISPR/Cas12a one-pot assay will exhibit higher speed and sensitivity. We attempted the one-pot method combining S-RPA and CRISPR/Cas12a, but its performance did not meet our expectations due to the incompatibility of the reaction buffers of the two systems, resulting in the inhibition of CRISPR/Cas12a activity. We are making effort to solve these problems, thereby providing the one-pot SPECIAL with the potential for superior performance in clinical applications.

In conclusion, we found that the recognition accuracy of Bsu polymerase and recombinase could be elevated by dNTPαS, thereby enhancing the specificity of RPA (S-RPA). By introducing CRISPR/Cas12a into S-RPA, we have developed a novel, simple, ultrasensitive and high-specific nucleic acid testing technology termed S-RPA equipped with CRISPR/Cas12a amplifier (SPECIAL), thereby providing a useful POCT tool for molecular diagnosis.

CRediT authorship contribution statement

Li Fang: Writing – review & editing. **Xin Yang:** Writing – review & editing, Writing – original draft. **Yong Li:** Writing – review & editing. **Chenglu Xue:** Validation. **Zhanggang Li:** Validation. **Huan Jiang:** Resources. **Xinxin Li:** Validation. **Shiyue Lu:** Validation. **Dongsheng Wang:** Writing – review & editing. **Hongfei He:** Writing – review & editing. **Zhen Huang:** Writing – review & editing. **Xiaolan Guo:** Writing – review & editing. **Guangcheng Luo:** Writing – review & editing.

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Declaration of competing interest

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2025.117421.

Data availability

Data will be made available on request.

References

Ahmed, M.Z., Badani, P., Reddy, R., Mishra, G., 2021. J. Mol. Diagn. 23 (11), 1433–1442.
Akhavan, O., Ghaderi, E., Rahighi, R., 2012. ACS Nano 6 (4), 2904–2916.
Arizti-Sanz, J., et al., 2020. Nat. Commun. 11, 5921.
Broughton, J.P., et al., 2020. Nat. Biotechnol. 38, 870–874.
Chen, J.S., Ma, E., Harrington, L.B., Da Costa, M., Tian, X., Palefsky, J.M., Doudna, J.A., 2018. Science 360 (6387), 436–439.
Cui, J.Q., Liu, F.X., Park, H., Chan, K.W., Leung, T., Tang, B.Z., Yao, S., 2022. Biosens. Bioelectron. 202, 114019.
Daher, R.K., Stewart, G., Boissinot, M., Bergeron, M.G., 2016. Clin. Chem. 62 (7), 947–958.
Del Val, E., Nasser, W., Abaïbou, H., Reverchon, S., 2019. Biochem. Soc. Trans. 47 (5), 1511–1531.
Dong, Y., Zhao, Y., Li, S., Wan, Z., Lu, R., Yang, X., Yu, G., Reboud, J., Cooper, J.M., Tian, Z., Zhang, C., 2022. ACS Sens. 7 (3), 730–739.
Eckstein, F., 2000. Antisense nucleic acid drug. Dev 10 (2), 117–121.
Emery, N.J., Majumder, S., Liu, A.P., 2018. Synth Syst Biotechnol. 3 (2), 130–134.
Gootenberg, J.S., Abudayyeh, O.O., Kellner, M.J., Joung, J., Collins, J.J., Zhang, F., 2018. Science 360 (6387), 439–444.
Gootenberg, J.S., Abudayyeh, O.O., Lee, J.W., Essletzbichler, P., Dy, A.J., Joung, J., Verdine, V., Donghia, N., Daringer, N.M., Freije, C.A., Myhrvold, C., Bhattacharyya, R.P., Livny, J., Regev, A., Koonin, E.V., Hung, D.T., Sabeti, P.C., Collins, J.J., Zhang, F., 2017. Science 356 (6336), 438–442.
Hu, B., Wang, Y., Sun, S., Luo, G., Zhang, S., Zhang, J., Chen, L., Huang, Z., 2020. Anal. Chem. 92 (24), 15872–15879.
Joung, J., et al., 2020. N. Engl. J. Med. 383, 1492–1494.
Kellner, S., DeMott, M.S., Cheng, C.P., Russell, B.S., Cao, B., You, D., Dedon, P.C., 2017. Nat. Chem. Biol. 13 (8), 888–894.
Lu, S., Tong, X., Han, Y., Zhang, K., Zhang, Y., Chen, Q., et al., 2022. Nat. Biomed. Eng. 6, 286–297.
Luo, G., He, H., Wang, D., Liu, S., Tian, S., Chen, M., Wang, Q., Zhao, C., Leng, Z., Hou, L., Guo, X., 2023. Clin. Chem. 69 (4), 363–373.
Luo, G., Yi, T., Wang, Q., Guo, B., Fang, L., Zhang, G., Guo, X., 2021a. Biosens. Bioelectron. 184, 113239.

Luo, G., Zhang, J., Yang, M., He, H., Huang, Z., 2021b. J. Mater. Chem. B 9, 5636–5644.
Luo, G.C., Yi, T.T., Jiang, B., Guo, X.L., Zhang, G.Y., 2019. Anal. Biochem. 575, 36–39.
Mizrahi, V., Henrie, R.N., Marlier, J.F., Johnson, K.A., Benkovic, S.J., 1985. Biochemistry 24 (15), 4010–4018.
Munawar, M.A., 2022. Expert Rev. Mol. Diagn 22 (7), 725–737.
Patchsung, M., et al., 2020. Nat. Biomed. Eng. 4, 1140–1149.
Piepenburg, O., Williams, C.H., Stemple, D.L., Armes, N.A., 2006. PLoS Biol. 4 (7), e204.
Qin, J., Wang, W., Gao, L., Yao, S.Q., 2022. Chem. Sci. 13 (10), 2857–2876.
Rabiee, N., Akhavan, O., Fatahi, Y., Ghadiri, A.M., Kiani, M., Makvandi, P., Rabiee, M., Nicknam, M.H., Saeb, M.R., Varma, R.S., Ashrafizadeh, M., Zare, E.N., Sharifi, E., Lima, E.C., 2022. Chemosphere 306, 135578.
Sipos, R., Székely, A.J., Palatinszky, M., Révész, S., Márialigeti, K., Nikolausz, M., 2007. FEMS Microbiol. Ecol. 60 (2), 341–350.
Tian, B., Minero, G.A.S., Fock, J., Dufva, M., Hansen, M.F., 2020. Nucleic Acids Res. 48, e30.
Tortajada-Genaro, L.A., 2022. Methods Mol. Biol. 2392, 35–51.
Wei, J., Wang, W., Yu, Q., Zhang, M., Xue, F., Fan, B., Zhang, T., Gao, Y., Li, J., Meng, X., Pang, B., 2023. Sensor. Actuator. B Chem. 390, 133950.
Yan, L., Zhou, J., Zheng, Y., Gamson, A.S., Roembke, B.T., Nakayama, S., Sintim, H.O., 2014. Mol. Biosyst. 10, 970–1003.
Yi, T.T., Zhang, H.Y., Liang, H., Gong, G.Z., Cai, Y., 2021. Biotechnol. Appl. Biochem. 68 (3), 469–475.
Zhang, J., Chen, M., Jiang, H., Sun, H., Ren, J., Yang, X., Liu, S., Wang, D., Huang, Z., Liu, J., Ma, D., Guo, X., Luo, G., 2024. Adv. Sci. 11 (28), e2403120.
Zhang, Z., Yoon, D., LaPorte, J.R., Chen, J., 2001. J. Mol. Biol. 309 (1), 29–43.