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Study on the detection of prostate cancer using MIRA-CRISPR/Cas12a technology

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Background: Prostate cancer (PCa) is the most diagnosed cancer among men globally and a leading cause of cancer-related mortality. However, current conventional prostate diagnostic methods fail to meet the growing demands of clinical practice in terms of speed, simplicity, sensitivity, and specificity. To address these limitations, we established a molecular detection system based on MIRA-CRISPR/Cas12a technology. Using reverse transcription-multienzyme isothermal rapid amplification (RT-MIRA) to amplify minute PCA3-specific fragments in samples, we employ CRISPR/Cas12a to detect the fluorescence signal released by these fragments, enabling the detection of trace PCA3 molecules in urine. *Methods*: PCA3 standard strain cultivation and total RNA extraction; establishment and optimization of the MIRA amplification system using primers designed for the PCA3 molecular marker; design of crRNA targeting optimal sites within the detection sequence, combined with CRISPR/Cas12a technology to establish the detection system; preliminary validation of the technology's sensitivity and specificity. *Results*: the MIRA-CRISPR/Cas12a technology was successfully established for visual detection of PCA3 molecules in prostate cancer. Primer and crRNA sequences within the reaction system were determined. The detection sensitivity for PCA3 molecules in urine reached 1×10^0 copies per μL with excellent specificity. *Conclusion*: the MIRA-CRISPR/Cas12a technology enables specific detection of PCA3 molecules in urine. This technique features high sensitivity, high specificity, visual results, and simple operation. It does not require specialized laboratory UV imaging equipment; results are visible to the naked eye under LED blue light. Following further optimization, it offers a feasible technical solution for rapid molecular screening of prostate cancer.

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1. Introduction

Prostate cancer (PCa) accounts for approximately 15% of all diagnosed cancers, making it the most diagnosed cancer in men worldwide and a leading cause of cancer-related mortality.[1–3] As part of the male reproductive system, the prostate plays a crucial role in male fertility. Certain benign and malignant prostate diseases not only impair male fertility but also pose a threat to men's health and life. Current clinical diagnostic criteria for prostate cancer typically rely on combined test results,[4] including serum prostate-specific antigen (PSA) testing (total PSA), digital rectal examination (DRE), and subsequent invasive needle biopsy.[1,5] That is, the differential diagnosis of prostate cancer primarily involves elevated serum prostate-specific antigen[6,7] and abnormal findings on digital rectal examination,[8] with final confirmation through prostate tissue biopsy.[3] However, both benign and malignant prostate conditions can elevate serum PSA levels, leading to false-positive results[9–12] and unnecessary biopsies for benign prostate lesions.[13] Furthermore, the poor stability and high detection costs of free PSA further limit its clinical utility. Additionally, needle biopsy is an invasive procedure, and unnecessary negative biopsies undoubtedly increase patient distress and infection risks.[14] Therefore, there is an urgent clinical need for novel molecular diagnostic technologies with enhanced specificity and suitability for large-scale screening to differentiate prostate cancer, thereby reducing unnecessary needle biopsies.

PCA3 is a long non-coding RNA (lncRNA) transcribed from the prostate cancer gene 3 located on chromosome 9q21-22.[2,15] It is a prostate cancer-specific gene expression product present in 95% of prostate cancer tissues,[15] significantly overexpressed in malignant cancer tissues,[16] and not detected in any other normal human tissues.[17] PCA3 does not encode a protein, making its mRNA the only testable molecular marker.[15] Furthermore, PCA3 exhibits high expression levels in the urine of prostate cancer patients,[3,11,18] and its high stability enables reliable detection in non-invasive urine samples.[19] This makes PCA3 a promising candidate for an ideal molecular biomarker in the clinical differential diagnosis of benign *versus* malignant prostate diseases.[20,21]

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In recent years, various novel isothermal amplification strategies have been developed, offering faster and more efficient alternatives for nucleic acid detection.[22] Examples include loop-mediated isothermal amplification (LAMP),[23,24] recombinase polymerase amplification (RPA),[25,26] and multi-enzyme isothermal rapid amplification (MIRA). These isothermal amplification technologies hold great promise in clinical testing and rapid diagnostics.[27,28] MIRA technology, independently developed in China, represents a multi-enzyme isothermal rapid amplification method distinct from those requiring high temperatures.[29] It is currently the only isothermal amplification technique capable of achieving effective amplification at reduced temperatures as low as 37 °C.[30] It primarily relies on the synergistic action of multiple functional proteins (helicase, recombinase, single-stranded DNA binding protein, DNA polymerase).[31,32] For RNA template amplification, it additionally depends on the collaboration of reverse transcriptase.[33,34]

Clustered regularly interspaced short palindromic repeats and their associated protein systems (CRISPR/Cas systems) represent an acquired immune system in the evolutionary history of bacteria and archaea, conferring resistance against exogenous genetic invasion.[35,36] In recent years, CRISPR technology has found extensive applications in the medical field as a research tool, for biotherapy, and *in vitro* molecular diagnostics (IVD).[37,38] In this study, we primarily leverage the recognition or "searching" function of CRISPR/Cas12a to detect amplified nucleic acids.[39,40] Therefore, we coupled the MIRA nucleic acid amplification technology with the CRISPR/Cas12a molecular detection system to achieve more sensitive, precise, and visual molecular diagnostics.

Technical principle: MIRA technology amplifies trace amounts of PCA3-specific fragments in urine.[41] The CRISPR/Cas12a system detects these fragments, releasing a fluorescent signal[42] to enhance the sensitivity of the result and achieve visualization. Reaction mechanism: at 37 °C, reverse transcriptase synthesizes cDNA strands using specific primers and template RNA. Assisted by chaperone proteins and single-stranded DNA-binding proteins, the recombinase forms a complex with the primer.[43] This complex performs a homology search and binds to the target homologous domain, simultaneously forming a D-loop region within this homologous area to initiate strand exchange.[44] As the recombinase dissociates from the complex, the polymerase binds to the 3′ end of the primer and initiates chain elongation, yielding abundant amplification products.[32,45] Upon recognizing specific target DNA (amplified products) guided by crRNA, Cas12a transitions to an enzyme-activated state,[46] capable of cleaving any non-target single-stranded DNA into fragments. At this stage, the fluorescent reporter probe pre-added to the system is cleaved by the *trans*-cleavage activity of the Cas12a protein.[42,47] The cleavage of the fluorescent reporter probe releases the fluorescent group, converting the target sequence information into a fluorescent signal. This technology offers high sensitivity, high specificity, visual results, simple operation, independence from specialized nucleic acid amplification laboratories, non-invasiveness, and ease of implementation. It provides a rapid diagnostic technique for molecular testing in prostate cancer.

2. Materials and reagents

RNA Thermocyclic Rapid Amplification (MIRA) Kit (Basic) – II was purchased from Anpu Future (Changzhou) Biotechnology Co., Ltd. LbCas12a (Cpf1) protein, 10× HOLMES buffer, and the T7 RNA polymerase-based *in vitro* RNA transcription kit was all purchased from Tolo Bay Biotechnology Co., Ltd. All sequences, including primers, crRNA, reporter probes, and recombinant plasmids, were synthesized by Sangon Biotech (Shanghai) Co., Ltd. A total of 3 urine samples were collected from the Affiliated Hospital of Chengdu University of Traditional Chinese Medicine, comprising three groups: the prostate cancer group, the prostatitis group, and the healthy control group (individuals from the health examination center). Urinary sediment was obtained *via* centrifugation. Subsequently, RNA templates were isolated using a magnetic bead-based extraction kit (Shanghai Tolo Biotech Co., Ltd), which ensures high purity and yield for downstream analysis.

3. Method

3.1 PCA3 standard strain cultivation and total RNA extraction

The target sequence of the PCA3 gene was submitted to Sangon Biotech (Shanghai) Co., Ltd for the construction of a standard strain containing the recombinant plasmid pUC57-PCA3. The strain was stored in 50% glycerol solution at −80 °C. Add 10 μL of glycerol stock to a 50 mL preservation tube containing 25 mL LB medium and 25 μL ampicillin. Seal the tube and incubate on a 37 °C, 200 rpm shaking incubator for 8 hours. Remove the tube, add 25 μL IPTG, reseal, and incubate a 37 °C, 200 rpm overnight.

Total RNA was extracted using the TRIzol™ method. Bacterial cultures collected after overnight incubation were centrifuged, the supernatant discarded, and the pellet transferred to an EP tube (1.5 mL). Add 1 mL TRIzol and let stand at room temperature for 5 minutes. Then add 200 μL chloroform, vigorously shake the tube for 15 seconds, let stand at room temperature for 2–3 minutes, and centrifuge at 12 000 g (2–8 °C) for 15 minutes. Transfer the upper aqueous layer to a fresh EP tube (1.5 mL). Add 500 μL isopropanol, let stand at room temperature for 10 minutes, then centrifuge at 12 000 g (2–8 °C) for 10 minutes. Discard the supernatant. Add 1 mL of 75% ethanol, vortex to mix, and centrifuge at 7500 g (2–8 °C) for 5 minutes. Discard the supernatant. Allow to dry at room temperature for 5–10 minutes, then dissolve in 30 μL of RNase-free water.

3.2 MIRA primer and crRNA design

Retrieve the complete transcript sequence of the PCA3 gene (AF103907, AY894120) from the National Center for Biotechnology Information (NCBI) database. Primers were designed using the PrimerQuest Tool (https://sg.idtdna.com/

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Primerquest/Home/Index) from IDT (Integrated DNA Technologies acquires Archer™), adhering to MIRA primer design requirements. Four pairs of MIRA primers meeting the criteria were selected (Table 1), all exhibiting BLAST query coverage exceeding 96%.

Design crRNA based on the conserved sequence of the PCA3 gene. Utilize the IDT Custom Alt-R™ CRISPR-Cas9 guide RNA tool to identify guide RNA sites within the amplified PCA3 fragment. Select a 20-bp target sequence upstream of the TTTV PAM site recognized by Cas12a.[40,48,49] After verifying specificity using the CRISPR-Cas9 gRNA Checker tool, two crRNAs were designed (Table 1). The self-designed Target Antisense Oligo was then synthesized by a biotechnology company. Subsequently, Cas12a crRNA will be transcribed *in vitro* using the independently designed Target Antisense Oligo as a template and the T7 promoter sequence as the transcription recognition site.

3.3 MIRA-CRISPR/Cas12a

3.3.1 Establishment and optimization of the MIRA amplification system. The target sequence of the PCA3 gene was submitted to Sangon Biotech (Shanghai) Co., Ltd for synthesis into a PCA3 gene standard plasmid. This plasmid was used as the detection template to prepare PCA3 gene standard samples to prepare a PCA3 gene standard. Fluorescent PCR (SYBR Green method) was employed to evaluate the amplification efficiency and product specificity of four primer pairs. Combining this with agarose gel electrophoresis (1.5%) analysis, the optimal MIRA primers were selected.

The MIRA reaction was conducted using the RNA Isothermal Rapid Amplification Kit (Anpu Future) in a final volume of 50 μL. The amplification system was prepared by mixing the multi-enzyme component buffer (buffer A), specific upstream and downstream primers (10 μM), and the RNA template, followed by the addition of the reaction activator (buffer B) to initiate the process. Isothermal amplification was performed at 42 °C for 15–20 minutes using a gene amplifier (TC-96/G/H(b)B, Bory).

Temperature optimization: under 20-minute amplification conditions, set four temperature gradients: 35 °C, 37 °C, 39 °C, and 41 °C. Screen for the optimal temperature based on MIRA product electrophoresis results.

Time optimization: at 37 °C, set four time gradients: 15 min, 17 min, 19 min, and 21 min. Select the optimal time based on the electrophoresis results of MIRA products.

3.3.2 Establishment and optimization of the CRISPR/Cas12a reaction system. The CRISPR/Cas12a assay was performed in a total volume of 20 μL using the Tolo Harbour Kit (LbCas12a (Cpf1) Nuclease). The reaction mixture consisted of HOLMES Buffer, crRNA, Cas12a nuclease, and a fluorogenic ssDNA reporter, which were incubated with the target DNA. Real-time fluorescence monitoring was conducted using a fluorescence quantitative PCR system (FQD-48A, Bio-Rad) at 37 °C. The signal was acquired every 30 seconds for a total of 40 cycles.

Two distinct crRNAs obtained *via in vitro* transcription were separately introduced into the CRISPR/Cas12a assay system. Deionized water served as the negative control. The optimal sequence was determined based on the final fluorescence intensity.

Using the optimized MIRA system product as a template, gradient optimization of the crRNA : LbCas12a Nuclease ratio (1 : 1 to 1 : 3) was performed in the CRISPR/Cas12a system. The optimal ratio was determined based on the final fluorescence intensity.

3.3.3 Establishment of the MIRA-CRISPR/Cas12a detection system. The MIRA-CRISPR one-pot reaction was performed in a total volume of 50 μL. Briefly, the LbCas12a-crRNA complex was formed by pre-incubating 1 μM LbCas12a nuclease and 1 μM crRNA in 10× HOLMES buffer at 37 °C for 10 min. The final reaction mixture contained MIRA polymerase buffer (buffer A), 10 μM of each primer (upstream and downstream), 10 μM ssDNA reporter, 5 μL of RNA template, and the pre-incubated Cas12a complex. The reaction was initiated by the addition of MIRA activator (buffer B). Fluorescence detection was carried out using a real-time PCR system (FQD-48A, Bio-Rad) at 37 °C for 42 cycles, with signals collected at 30-second intervals.

3.4 Sensitivity and specificity evaluation of MIRA-CRISPR/Cas12a

To evaluate the sensitivity of this technology, we performed sensitivity testing on the detection system using the PCA3 gene sequence constructed on the pUC57 vector as a standard template. A series of low-concentration gradients (1×10^5 to 1×10^0 copies per μL) of templates was amplified and subjected to

Table 1 Primer sequence used in this study

Gene	Primer	Sequence (5′–3′)	Product
AY894120	PCA3Primer1-F	CTCTCTCTTTGACCCATTATTTCAGACTTA	215 bp
	PCA3Primer1-R	GTGTAGCTGATGGTAGGAGTTAGATTTATG	
AY894120	PCA3Primer2-F	GGATTACAGATTTGAAATGAAGTCACAAAG	229 bp
	PCA3Primer2-R	GAGGGTTAGAAATATGAAATGATTTGGTTATG	
AF103907	PCA3Primer3-F	AATAAGTTGACTATCTTACTTCATGCAAAG	242 bp
	PCA3Primer3-R	GGATATTGTGATAGAGATAGAGAAATGAAGTA	
AF103907	PCA3Primer3-F	GGATTACAGATTTGAAATGAAGTCACAAAG	229 bp
	PCA3Primer3-R	GAGGGTTAGAAATATGAAATGATTTGGTTATG	
PCA3	crRNA1	UAAUUUCUACUAAGUGUAGAUUUGCAUGUCUUGUGAAGCCA	
PCA3	crRNA2	UAAUUUCUACUAAGUGUAGAUGGCAGCAGGGCCAGAAUCCU	

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fluorescence detection. Reactions were performed at 37 °C for 30 minutes, with results determined based on the final fluorescence signal. Using urine sediment samples from prostate cancer patients, prostatitis patients, and healthy individuals undergoing physical examinations, RNA templates were extracted using magnetic bead technology. A 1×10^4 copies per μL PCA3 standard template served as the positive control, while ddH_2O acted as the negative control for detection. This provided an initial evaluation of the technique's specificity.

3.5 Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). All statistical processing was conducted in SPSS Statistics 27, and graphical representations were created using OriginPro 2024. Significance levels were set as follows: ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$, all indicating statistically significant differences; ns denotes no significant difference ($p > 0.05$).

4. Results

4.1 Primer screening

Primers were selected based on fluorescence PCR results and the brightness, clarity, and specificity of target bands on 1.5% agarose gel electrophoresis. Primer 2 exhibited an S-shaped fluorescence curve, and its logarithmic phase curve after amplification showed good uniformity (Fig. 1b). Primer 2 effectively amplified products of uniform size without nonspecific amplification (Fig. 1c). Therefore, Primer2-F and Primer2-R were employed for the MIRA reaction, yielding a final target product of 229 bp.

4.2 Optimization of the MIRA system

Optimal parameters were determined based on 1.5% agarose gel electrophoresis band analysis. In the temperature optimization experiment, the brightest electrophoresis bands were observed at 37 °C amplification temperature (Fig. 1d). In the time optimization experiment, the brightest electrophoresis band was observed at an amplification time of 21 min (Fig. 1e). Therefore, 37 °C was determined as the optimal amplification temperature, and 21 min as the optimal amplification time.

4.3 Validation and detection of the CRISPR/Cas12a reaction system

Based on fluorescence intensity analysis of the fluorescence curve and UV fluorescence results, crRNA was selected. The standard group and negative control group for crRNA1 exhibited negligible fluorescence, while the standard group for crRNA2 showed high fluorescence intensity, and the negative control group displayed no fluorescence (Fig. 2a and b). Therefore, crRNA2 was employed in the CRISPR/Cas12a reaction system.

In the dose optimization experiment, the experimental results (Fig. 2c and d) indicate that the highest fluorescence intensity is achieved when the molar ratio of crRNA to LbCas12a Nuclease in the system is 1. The molar ratio of crRNA to LbCas12a Nuclease in the CRISPR system was ultimately determined to be 1.

4.4 Establishment and testing of the MIRA-CRISPR/Cas12a integrated detection system

The preliminary establishment of the MIRA-CRISPR/Cas12a integrated detection system yielded favorable results. The negative control showed no fluorescence, while the positive template demonstrated effective amplification with strong fluorescence intensity (Fig. 3a).

4.5 Sensitivity of MIRA-CRISPR/Cas12a

A low-concentration gradient (1×10^5 to 1×10^0 copies per μL) standard template was prepared for detection. The resulting fluorescence curve exhibited a gradient, with the 1×10^0 copies per μL sample showing a distinct fluorescence intensity compared to the negative control group. Preliminary confirmation indicates a minimum detection limit of 1×10^0 copies per μL for this assay's sensitivity metric. Subsequent experiments will employ digital PCR technology for comparative validation (Fig. 3b–d).

4.6 Specificity of MIRA-CRISPR/Cas12a

Using RNA extracted from urine sediment samples of prostate cancer cases, prostatitis cases, and healthy individuals undergoing physical examinations as templates, and employing a PCA3 standard at 1×10^4 copies per μL as the positive control, we conducted a preliminary evaluation of the specificity of the MIRA-CRISPR/Cas12a detection technology. Results showed positive outcomes for the positive control and one clinical prostate cancer sample, while all three prostatitis samples and one healthy control sample yielded negative results. This indicates good specificity of the method. Subsequent collection of sufficient case numbers will enable statistically significant validation (Fig. 3e).

5. Discussion

Prostate cancer is one of the most common malignant tumors in men, and its early diagnosis is crucial for improving patient prognosis. PCA3 expression is almost exclusively found in prostate tissue and shows significant enrichment in prostate cancer samples.[50] In recent years, combined detection technologies integrating isothermal amplification with the CRISPR/Cas system have emerged as a cutting-edge trend in biomedical sample analysis, demonstrating significant potential in addressing the challenge of detecting low-abundance nucleic acid targets.[51–53] Compared to traditional PCR and loop-mediated isothermal amplification (LAMP) technologies, MIRA technology has been validated to efficiently detect multiple pathogens—including hepatitis A virus (HAV), African horse sickness virus (AHSV), and bovine coronavirus—by leveraging its rapid initiation advantage under constant temperature conditions (37–42 °C).[33,34,54–56] However, when used solely for tumor marker detection, isothermal amplification often suffers from background signal interference due to non-

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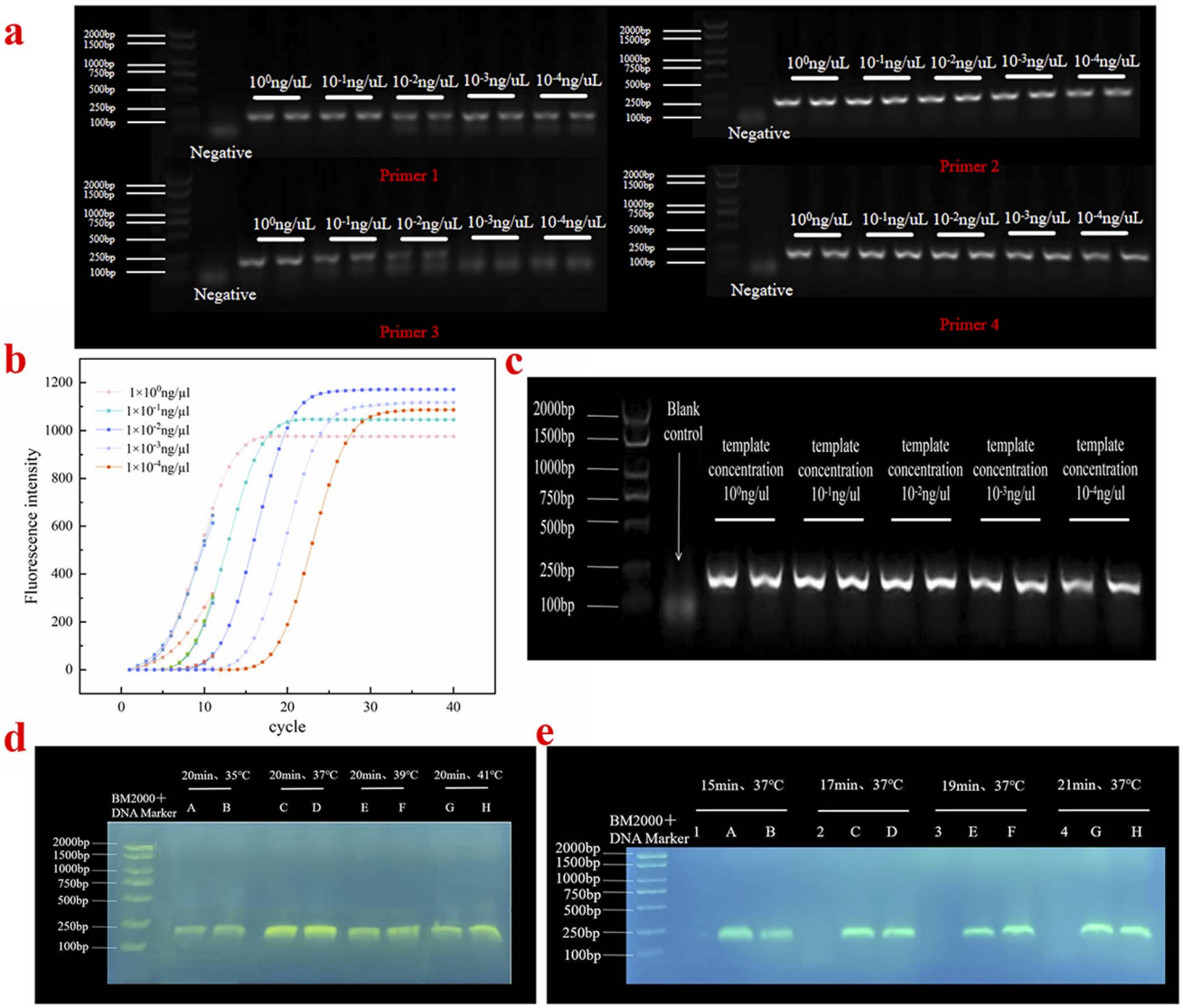


Fig. 1 Primer screening for the MIRA amplification system and optimization of the MIRA System. (a) Primer screening results. (b) Fluorescence curve of Primer 2 in fluorescent PCR. (c) Electrophoretic analysis of primer 2 PCR amplification products on a 1.5% agarose gel. (d) MIRA amplification products were analyzed by 1.5% agarose gel electrophoresis under constant time conditions with temperature gradients of 35 °C, 37 °C, 39 °C, and 41 °C. (e) Temperature held constant at 37, time gradient of 15 min, 17 min, 19 min, and 21 min; MIRA amplification products analyzed by 1.5% agarose gel electrophoresis.

specific amplification, making it difficult to meet the high specificity requirements demanded by cancer diagnostics. In the field of early cancer screening, the CRISPR/Cas12a system offers a novel approach to addressing the specificity challenges through its precise target recognition capability and highly efficient *trans*-cleavage activity.[57] Currently, the CRISPR/Cas12a system has been applied in research and validation for the early detection of various cancers, including breast cancer, lung cancer, and liver cancer. Simultaneously, it has played a significant role in detecting pathogens such as SARS-CoV-2, avian influenza virus, and African swine fever virus.[51,58] Furthermore, recent studies indicate that CRISPR/Cas12a-based detection methods,[59] such as aptamer-CRISPR-Cas12a,[60] CASMART,[61] CRISPR/Cas12a combined with nanoparticle-enhanced fluorescence detection,[62] and other technologies—have been applied for rapid, highly sensitive, and specific cancer screening. These advancements provide powerful tools for early cancer diagnosis.[58] The cascade system integrating isothermal amplification technology with CRISPR/Cas12a has achieved ultra-sensitive detection in liquid biopsies such as ctDNA and miRNA.[51,57,58,63,64] This dual mechanism of efficient amplification and specific recognition effectively reduces false-positive interference, overcomes the diagnostic limitations of traditional markers like PSA, and provides reliable evidence for early-stage precision cancer screening. Building upon this technological advancement, this study applies the MIRA-CRISPR/Cas12a system to non-invasive urinary detection of PCA3, a prostate cancer-specific biomarker. Although MIRA technology inherently lacks direct signal amplification capabilities and the CRISPR system exhibits limited sensitivity without

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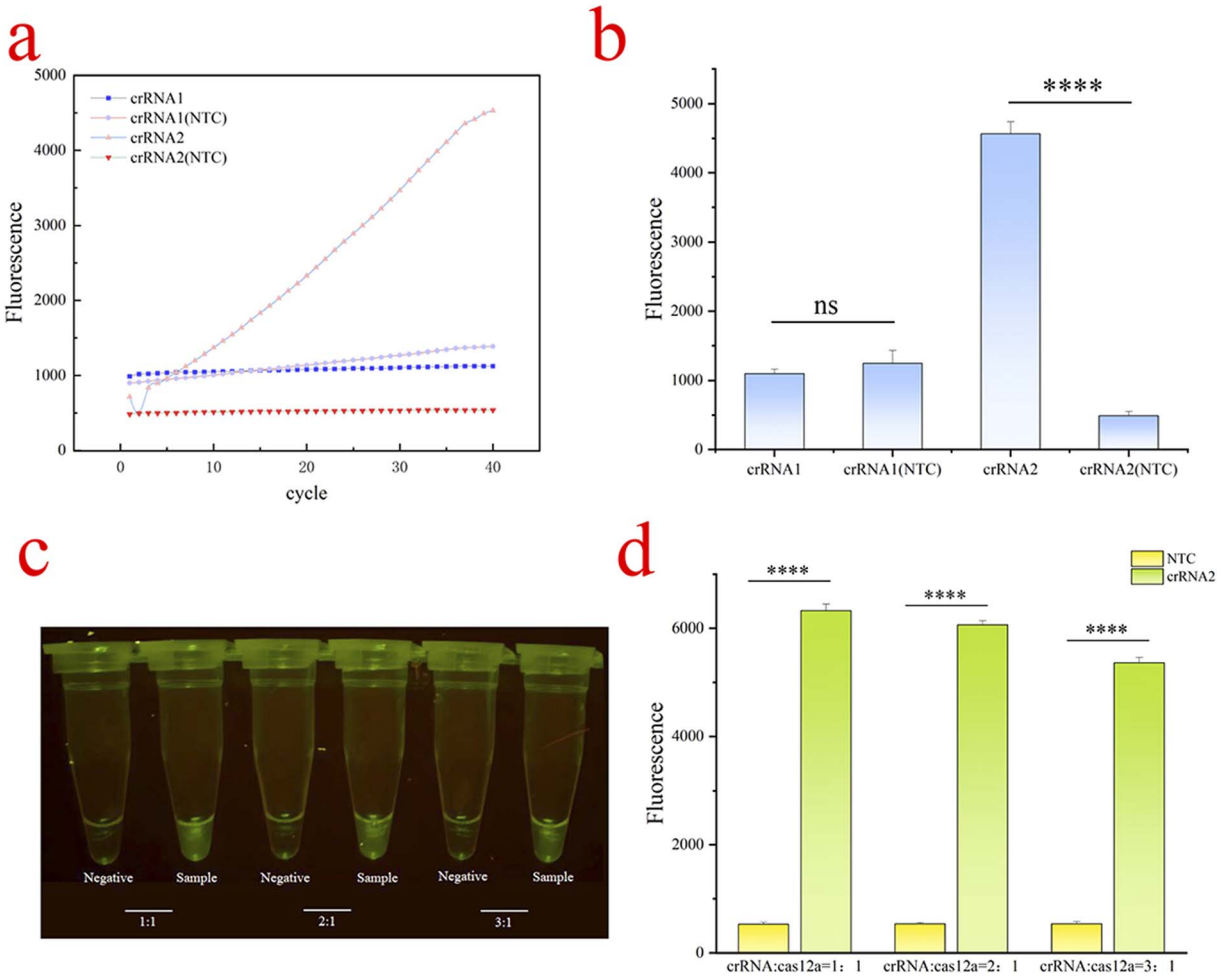


Fig. 2 Optimization of the CRISPR/Cas12a system (a) fluorescence curve plots of crRNA1 and crRNA2 CRISPR reactions. (b) Fluorescence intensity plots of crRNA1 and crRNA2 CRISPR reactions. (c) Visual detection of reaction products under blue light illumination. (d) Fluorescence intensity at molar concentration ratios of 1, 2, and 3 for crRNA/LbCas12a nuclease in the system.

preamplification, their integration achieves complementary advantages. Like the detection system developed by Yin *et al.* for high-risk human papillomavirus (HR-HPV).[52] The platform developed in this study enables detection within 30 minutes at 37 °C, achieves a sensitivity of 1×10^0 copies per μL, and allows for visual readout using simple blue light transilluminator. This not only addresses the clinical challenge of poor PSA screening specificity but also provides a new molecular diagnostic tool for large-scale early prostate cancer screening—one that is low-cost, non-invasive, and independent of expensive laboratory equipment.

Optimization results for the MIRA system indicate that maximum amplification efficiency is achieved at 37 °C after 21 minutes of reaction. This finding underscores MIRA's high efficiency as an isothermal amplification technology and its mild reaction conditions, consistent with the conclusions of Pengfei Heng *et al.*[32] Compared to traditional detection methods, the platform established in this study offers distinct advantages. Although qPCR remains the current clinical gold standard, its reliance on sophisticated thermal cyclers and time-consuming nature limit its application in point-of-care testing (POCT).[65] Meanwhile, common isothermal amplification techniques such as LAMP, though widely applied, exhibit slightly inferior timeliness due to their relatively high reaction temperatures (60–65 °C) and approximately one-hour reaction times.[66] In contrast, the MIRA system optimized in this study achieves faster detection at near-ambient temperatures, significantly enhancing testing efficiency. The performance of the CRISPR/Cas12a detection module is significantly regulated by the crRNA sequence and its molar ratio with the Cas12a enzyme.[67,68] In this study, the strong fluorescent signal exhibited by crRNA2 indicates that this specific recognition site possesses better target exposure and higher cleavage conversion efficiency. Meanwhile, a molar ratio of crRNA : Cas12a = 1 : 1 was demonstrated to be optimal, enabling this method to achieve a detection limit (LOD) at the single-copy level under this system. In clinical applications, the system exhibited exceptional specificity when processing samples from prostate cancer patients. This ultra-high specificity can be explained by the highly specific cleavage of Cas12a. As described by Mei Lin *et al.*, Cas12a-mediated *cis*-cleavage facilitates the rapid release of sticky DNA products distal to the PAM.[69] These products are

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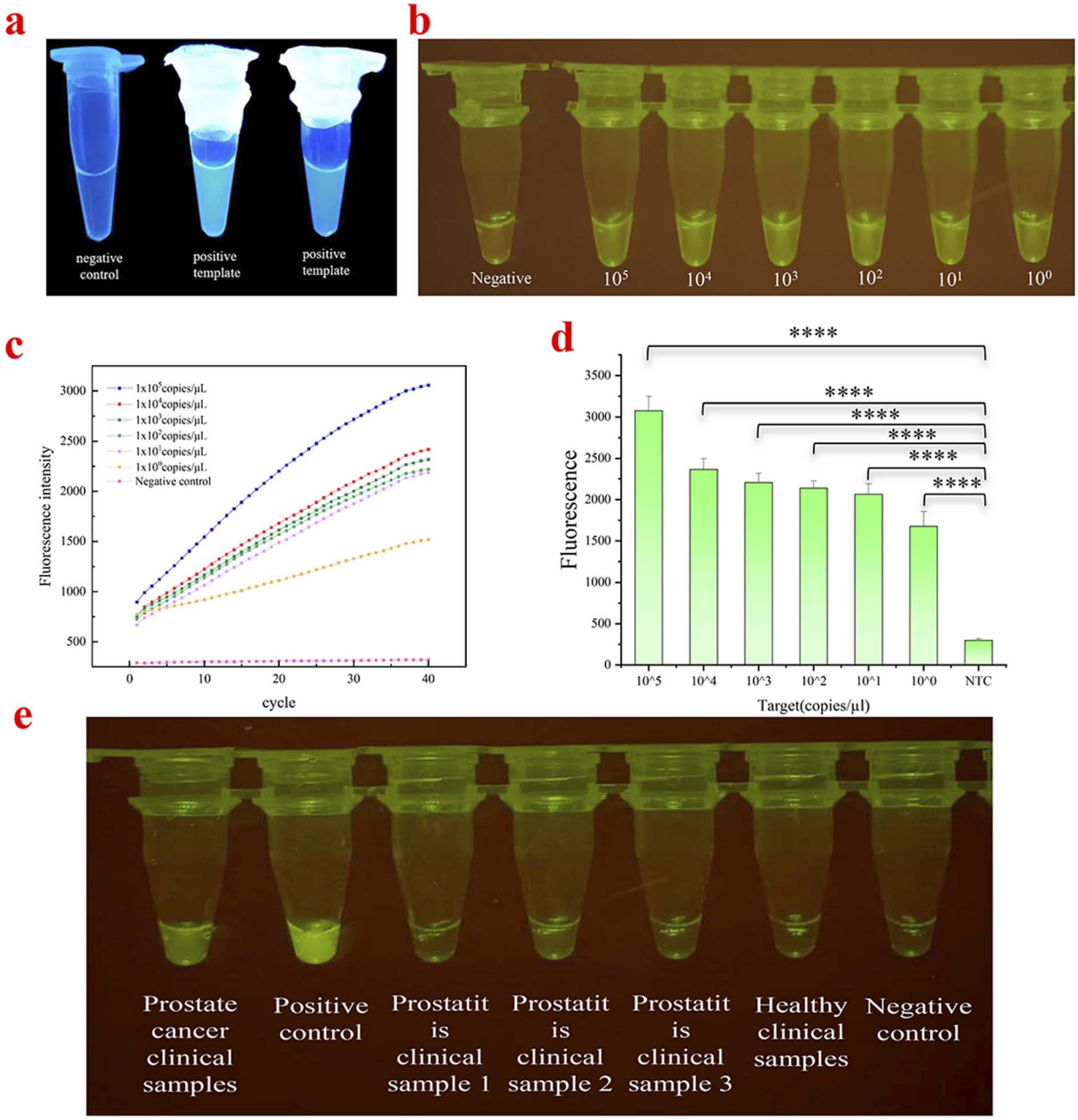


Fig. 3 Establishment, sensitivity, and specificity evaluation of the MIRA-CRISPR/Cas12a integrated detection system (a) preliminary establishment of the MIRA-CRISPR/Cas12a integrated detection system direct visual observation results under UV light. (b) Visual detection of reaction products under blue light illumination. (c) Fluorescence curves from integrated reactions with low-concentration gradient standards (1×10^5 to 1×10^0 copies per μL). (d) Fluorescence intensity plots from integrated reactions with low-concentration gradient standards (1×10^5 to 1×10^0 copies per μL). (e) Specificity testing.

subsequently captured through a highly selective "strand invasion hybridization" process, effectively circumventing non-specific interference.

The MIRA-CRISPR/Cas12a detection system based on the PCA3 molecular biomarker demonstrates significant application potential in rapid testing, primarily manifested in three aspects. First, this technology offers strong economic viability: it can rapidly adapt to detect various disease-related molecular markers by simply designing or replacing specific primers and crRNAs. Second, it substantially lowers the barrier to detection equipment: the system replaces traditional PCR thermal cycling with a constant–temperature reaction at 37 °C. It requires no expensive thermal cycler; only a simple thermostatic device and an LED blue light instrument for reading fluorescent signals, with results visible to the naked eye. Compared to traditional techniques like qPCR, this significantly reduces capital expenditure on equipment procurement and maintenance, making it more suitable for primary healthcare settings and resource-constrained testing scenarios. Finally, it demonstrates sample and reagent compatibility. Testing is based on non-invasive

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urine samples, which are easy to collect and highly acceptable to patients, avoiding the pain and infection risks associated with invasive procedures like needle punctures.

It should be noted that this study included only a limited number of clinical samples from early-stage prostate cancer, thus failing to evaluate the detection sensitivity of this method across different clinical stages. In subsequent research, we will expand the clinical sample size to further validate its diagnostic efficacy for prostate cancer at various stages. Although this reaction system has achieved preliminary integration of MIRA amplification and Cas12a detection within a single tube—eliminating the need for lid removal or complex operations while significantly reducing equipment dependency and contamination risks—issues such as buffer interference and template competitive inhibition persist between the MIRA amplification and CRISPR/Cas12a detection modules. These limitations constrain detection reproducibility. Furthermore, as noted in the study by Hongbo Liu *et al.*, the system's high sensitivity renders it susceptible to false positives triggered by aerosol contamination.[70] Future plans include optimizing ionic strength, designing compartmentalized reaction devices, or integrating microfluidic chip technology to achieve spatial isolation and automated sample dispensing, thereby reducing cross-contamination and interference. In summary, this platform offers high specificity and sensitivity, making it more suitable than traditional diagnostic methods reliant on prostate tissue biopsy for non-invasive urine testing. It demonstrates potential advantages of low equipment requirements, high speed, and high sensitivity.[71]

Ethical approval

This study was approved by the Medical Ethics Committee of the 416th Nuclear Industrial Hospital [No. YJ-2023-010].

Author contributions

Yuanyuan Chen, Daiyan Tang, Jin Zhan: conceptualization, data curation, methodology, writing – original draft. Yiyao Wang: data curation, validation. Zhen Song: methodology, supervision, conceptualization, supervision, funding acquisition, writing – review & editing. All authors have read and approved the final version of the manuscript.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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