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Development of a single-tube RPA/CRISPR-cas12a detection platform for monkeypox virus

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ABSTRACT

Monkeypox is a zoonotic disease caused by the monkeypox virus (MPXV), with outbreaks primarily occurring in West and Central Africa. The recent global MPXV outbreak underscores the urgent need for effective detection methods. Currently, qPCR is considered the gold standard for MPXV detection; however, it requires specialized personnel and costly equipment. This study introduces a CRISPR-Cas12a-based detection system targeting the MPXV A27L gene, achieving a detection limit as low as 10 aM. This system exhibits high specificity, with no cross-reactivity with other orthopoxviruses, and delivers results in under 40 min. To support point-of-care testing (POCT), we developed a lateral flow assay (LFA) strip for easy result visualization. The detection system was validated using six different clinical sample types, revealing that herpes fluid and saliva are the most suitable sources. The findings of this study align with qPCR results. Additionally, we lyophilized the RPA and CRISPR reagents to improve transport, storage, and field deployment. In conclusion, this study presents a reliable molecular diagnostic approach for early MPXV detection and point-of-care testing, contributing to epidemic prevention and control.

1. Introduction

Monkeypox is an infectious disease caused by the monkeypox virus (MPXV), a linear double-stranded DNA virus in the Orthopoxvirus genus, closely related to the variola virus(Ladnyj et al., 1972). MPXV was first isolated from monkeys in 1958 (Berthet et al., 2021). The first human case of MPXV was identified in 1970 in the Democratic Republic of the Congo, while the first U.S. case was reported in 2003, marking the virus's initial spread outside of Africa (Gul et al., 2022). The first case in mainland China was reported on September 16, 2022, followed by several hundred additional cases (X. Wang et al., 2024; Zhao et al., 2022). In August 2024, WHO Director-General Dr. Tedros Adhanom Ghebreyesus once again declared monkeypox a PHEIC. Therefore, proactive measures remain essential to curb the virus's spread.

Detecting MPXV nucleic acids is essential for monitoring outbreaks and diagnosing the disease. Currently, PCR is the most widely used technique for MPXV diagnosis and is considered the gold standard for detection due to its high sensitivity and specificity (Li et al., 2006). However, PCR methods require trained personnel and advanced laboratory equipment, such as thermal cyclers for DNA amplification, and involve a lengthy detection process. These requirements limit PCR's applicability in point-of-care testing (POCT) and resource-limited

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settings, hindering efforts to control the spread of the epidemic(Jiang et al., 2022; Sui et al., 2022). Consequently, there is an urgent need for rapid and ultrasensitive detection strategies that do not demand extensive expertise or complex equipment.

The clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) systems are adaptive immune mechanisms developed by archaea to protect bacteria from viruses and phages (Horvath and Barrangou, 2010). Since the initial application of the CRISPR/Cas9 system for gene editing in mammalian genomes, CRISPR/Cas technology has advanced rapidly in biotechnology, becoming essential for applications such as transcriptional regulation and genome editing (Feng et al., 2024; Konermann et al., 2015; Tang and Fu, 2018). Cas12a proteins are RNA-guided endonucleases; in the CRISPR-Cas12a detection system, complementary gRNA guides Cas12a to bind to and cleave dsDNA. Upon activation, Cas12a cuts ssDNA reporters carrying fluorophores (Xu et al., 2024; Yan et al., 2019).

Amplifying nucleic acids is essential for enhancing sensitivity in detection strategies. Isothermal amplification methods, such as recombinase polymerase amplification (RPA), can achieve analytical sensitivity comparable to PCR without requiring a thermal cycler(Craw and Balachandran, 2012; Zaghloul and El-Shahat, 2014). RPA's low operating temperature (37 °C–42 °C) and the availability of lyophilized reagents make it suitable for applications outside the laboratory. Recent reports have combined RPA with CRISPR-Cas12a for the detection of MPXV in samples(Ahamed et al., 2024; Chen et al., 2023; Li et al., 2023; Y. Wang et al., 2024; Yang et al., 2023; Yu et al., 2024; Zhao et al., 2023). However, due to potential interference between the RPA and CRISPR/Cas reactions, the RPA step is typically performed first, with only a small amount of the amplification product transferred to the CRISPR/Cas detection system. This process may lead to aerosol contamination in laboratory settings. Additionally, these studies either did not use or only used a limited number of positive clinical samples, lacking sufficient reliability for practical applications (Table S1).

Several studies have been published on the application of single-tube RPA-CRISPR/Cas12a methods for detecting various targets. Sun et al. placed CRISPR reagents in the tube cap and RPA reagents at the bottom of the tube(Sun et al., 2021). After the RPA reaction was completed, the two components were mixed by centrifugation, effectively improving the performance of single-tube detection. However, this approach involves relatively complex procedures and relies on centrifugation equipment, which makes it less suitable for point-of-care testing and high-throughput applications. Wang et al. developed a single-tube RPA-CRISPR/Cas12a detection strategy based on a sucrose-aided multiphase aqueous system, which spatially separates the RPA amplification and CRISPR/Cas12a cleavage reactions in a single tube using differences in density (Y. Wang et al., 2024). However, although the researchers addressed the incompatibility between the two reaction buffers, the rapid recognition of target sequences by the standard PAM (protospacer adjacent motifs) could still lead to premature cleavage by Cas12a, thereby affecting RPA amplification efficiency and introducing some uncertainty into the experiment. Lu et al. were the first to propose a single-tube RPA-CRISPR-Cas12a detection strategy based on suboptimal PAMs, replacing the standard PAM with suboptimal PAMs to decrease Cas12a′s binding affinity and reduce its interference with the amplification reaction (Lu et al., 2022). By minimizing substrate consumption in the early stage, this approach enhanced RPA amplification, providing sufficient amplification products for CRISPR detection. Additionally, Wang et al. identified the optimal suboptimal PAM for detecting Vibrio parahemolyticus (Wang et al., 2023).

In this study, we developed a single-tube RPA CRISPR/Cas12a approach for MPXV diagnosis, using primers and gRNA specifically targeting the A27L gene. We separately prepared freeze-dried spheres of RPA and CRISPR/Cas12a reagents, which only requires adding water for reconstitution. To improve the efficiency of the single-tube reaction, we employed a specially designed gRNA based on suboptimal PAM. Fluorescent and lateral flow assays (LFAs) were developed separately to meet various diagnostic needs. The sensitivity and specificity of these assays were evaluated, and their accuracy was validated using clinical samples. This method reduces detection time, lowers equipment costs, eliminates aerosol contamination, and removes the need for cold-chain transportation, enabling real-time long-distance transport. It is an innovative, practical, and scalable approach with promising applications in infectious disease prevention and control.

2. Materials and methods

2.1. Materials and reagents

EnGen® Lba (Lachnospiraceae bacteria) Cas12a (Cpf1) (cat-M0653T) was sourced from New England Biolabs. The RPA kit was acquired from Amp-Future Biotech Co., Ltd. Nuclease-free water was sourced from Takara Biomedical Technology Co., Ltd. LFA strips were obtained from Milenia Biotec Co., Ltd. The isothermal fluorescence detector was sourced from Hangzhou Allsheng Instruments Co., Ltd. PCR kits were acquired from Nanjing Vazyme Biotech Co., Ltd. The PCR thermal cycler was sourced from Thermo Fisher Scientific Co., Ltd. PEG was acquired from Biosharp Co., Ltd. Bovine serum albumin was sourced from Yeasen Biotechnology Co., Ltd. Mannitol, sucrose, and Tween 20 were acquired from Macklin Biochemical Technology Co., Ltd.

2.2. Preparation of plasmids

In this study, monkeypox virus DNA sequences were selected from the conserved region of the A27L gene (GenBank: OQ888844.1). Additionally, gene sequences from the vaccinia virus, variola virus, and cowpox virus were derived from a region of high similarity to the monkeypox gene (GenBank: MN369532.1; GenBank: LR800247.1; GenBank: MK035759.1). These sequences were synthesized and inserted into the pUC57 vector to construct specific recombinant plasmids (Table 1). The complete preparation process was carried out by Sangon Biotech Co., Ltd. All plasmid DNA contained the target gene fragment and was quantified. The DNA concentration was measured using the Nanodrop 2000 (Thermo Fisher Scientific).

2.3. Design of primers, gRNA, and ssDNA reporter

Monkeypox virus-specific RPA primers were designed based on the A27L sequence of the monkeypox virus (GenBank: OQ888844.1), adhering to established principles of RPA primer design. The designed RPA primers were validated and analyzed using the Basic Local Alignment Search Tool (BLAST) to minimize the potential for non-specific amplification. In this study, the target regions amplified by the RPA primers included the suboptimal PAM site, and the gRNA comprised a spacer sequence that matches the target sequence along with a scaffold sequence. FAM-BHQ-labeled ssDNA served as the reporter when the fluorescence signal was detected using the thermostatic fluorescence detector, while FAM-Biotin-labeled ssDNA was employed as the reporter for results observed using LFA strips. The sequences of the primers, gRNA, and ssDNA reporter are presented in Table 2. All primers and ssDNA reporter sequences were synthesized by Sangon Biotech Co., Ltd. The gRNAs were synthesized by Takara Bio-medical Technology Co., Ltd.

2.4. RPA reaction analysis and CRISPR-Cas12a system

For the RPA reaction, lyophilized RPA pellets were resuspended in 29.4 μL of buffer A and 14.1 μL of nuclease-free water. Subsequently, 2 μL of 10 μM RPA forward primer and 2 μL of 10 μM RPA reverse primer were added to create an RPA mixture, with slight modifications made following the manufacturer's guidelines. The CRISPR-Cas12a reaction mixture comprised LaCas12a (133 nM), gRNA (133 nM), FAM-ssDNA-BHQ1 reporter (933 nM), and 10 × NEBuffer 2.1, resulting in a total

Table 1
Target DNA sequence.

Target DNA	Sequences (5′-3′)
Monkeypox virus A27L gene	AGGATCTTCTAATGGATTGTATGGCTTGATAGCATCATCTTTATCATTATTAGGTGGGGGATGGACAACCTTAATTGGTTGGTCCTCCTTATCTCCTCCAGTAGCATGTG
Vaccinia virus A27L gene	AGGATCTTCTAATGGATTGTATGGCTTGATAGCATCATCTTTATCATTATTAGGGGGATGGACAACCTTAATTGGTTGGTCCTCATCTCCTCCAGTAGCGTGTG
Variola virus A27L gene	AGGATCTTCTAATGGATTGTATGGCTTGATAGCATCATCTTTATCATTATTAGGGGGATGGACAACCTTAATTGGTTGGTCCTCCTTATCTCCTCCAGTAGCATGTG
Cowpox virus A27L gene	AGGATCTTCTAATGGATTGTATGGCTTGATAGCATCATCTTTATCATTATTAGGGGGATGGACAACCTTAATTGGTTGGTCCTCCTTATCTCCTCCAGTAGCATGCG

Table 2
Oligonucleotide sequences.

Oligonucleotide	Sequences (5′-3′)
A27L gene RPA-F	AGGATCTTCTAATGGATTGTATGGCTTGAT
A27L gene RPA-R	CACATGCTACTGGAGGAGATAAGGAGGACC
A27L gene PCR-F	CTTCTAATGGATTGTATGGCT
A27L gene PCR-R	CACATGCTACTGGAGGAGAT
Canonical PAM gRNA	UAAUUUCUACUAAGUGUAGAUUCAUUAUUAGGUGGGGGAUG
Suboptimal PAM gRNA	UAAUUUCUACUAAGUGUAGAUGGUGGGGGAUGGACAACCUU
FAM-ssDNA-BHQ reporter	FAM-TTATT-BHQ1
FAM-ssDNA-Biotin reporter	FAM-TTATTATT-Biotin

volume of 8 μL. Eighteen microliters of RPA reagent were mixed with the CRISPR reagent to yield a final mixture.

2.5. Freeze dried procedure

Mannitol (12%), PEG (10%), bovine serum albumin (BSA) (2%), sucrose (15%), and Tween-20 (1.5%) were added to the RPA and CRISPR mixtures. The mixtures were inverted and briefly centrifuged before being dispensed into liquid nitrogen, where they condensed into spheres. The resulting spheres were subsequently collected after settling to the bottom of the nitrogen and transferred to vials pre-cooled with dry ice. The vials were then transferred to a freeze dryer. The process commenced with pre-freezing to protect and stabilize the proteins and other components within the sugar matrix, maintained for 3 h at −40 °C. This was followed by sublimation and vacuum drying for 5 h at 25 °C, subsequently for 10 h at 10 °C, and finally analytical drying for 3 h at 20 °C, followed by 5 h at 30 °C. The vacuum during these processes was sustained at 10 Pa. Upon completion of the freeze-drying process, nitrogen was introduced into the vials, and the box was sealed. Once the temperature and humidity of the environment met the specified requirements, the door of the freeze dryer was opened to retrieve the vials.

2.6. Single-tube RPA/CRISPR reaction and LFA analysis

The single-tube RPA/CRISPR platform integrates RPA with CRISPR/Cas12a detection in a total reaction volume of 30 μL. To re-dissolve the freeze-dried spheres, 26 μL of nuclease-free water was added, along with 2 μL of B buffer and DNA samples prior to the reaction. The centrifuge tube was placed in a thermostatic fluorescence detector at 37 °C to detect the fluorescence signal. The analytical reagents for the CRISPR system used in LFA analysis included LaCas12a (133 nM), gRNA (133 nM), Biotin-ssDNA-FAM reporter (800 nM), and 10 × NEBuffer 2.1. The other reaction procedures were the same as those for fluorescence detection. After the reaction, 1 μL of the product was added to 49 μL of PEG buffer, yielding a total volume of 50 μL, which was then introduced into the LFA strip and allowed to stand for 3–5 min to await the results.

2.7. Sensitivity and specificity analysis

The initial concentration of the MPXV plasmid was 10^5 aM, and the plasmid underwent gradient dilution to produce templates with varying concentrations (10^5, 10^4, 10^3, 10^2, 10^1, 10° aM). DNA from the vaccinia virus, variola virus and cowpox virus was utilized to assess the presence of cross-reactivity. We also designed several mixed viral samples containing monkeypox virus and other viruses to test the method's resistance to interference in the presence of a multiplex viral background, with results being read using fluorescence detection.

2.8. Clinical samples

A total of 30 clinical samples were collected from Shenzhen Third People's Hospital, encompassing various sample types, including urine, anal swabs, throat swabs, herpes fluid, saliva, and serum. Negative samples were obtained from healthy volunteers. The hospital's ethics committee granted approval, and the extracted DNA was stored at −80 °C. Results were compared with real-time fluorescence PCR findings from the hospital's laboratory.

2.9. Statistical analysis

In this study, all experiments were conducted with a minimum of three replicates. Graphs were generated and analyzed statistically using GraphPad Prism Version 10.0.3 (GraphPad Software, Inc.).

3. Results

3.1. Principle and target mapping of RPA/CRISPR diagnosis system

The principle of the single-tube RPA/CRISPR method is illustrated in Fig. 1A, which primarily involves RPA amplification and CRISPR/Cas12a-mediated cleavage. If the sample contains the target gene sequence, it binds to the corresponding primer to initiate the RPA reaction. In the CRISPR/Cas12a system, the gRNA localizes to the PAM site, specifically binds to the target fragment, cleaves the target amplicon, and activates Cas12a's auxiliary cleavage activity, which non-specifically cuts the reporter, generating a detectable signal. By using different reporters, the single-tube RPA/CRISPR reaction can either monitor fluorescence signals in real-time with an isothermal fluorescence detector or visualize signals through LFA strips.

We selected the conserved region of the A27L gene of MPXV as the target sequence for primer and gRNA design due to its 100% homology within MPXV and low homology with other Orthopoxviruses (Fig. 1B). A sequence comparison of this MPXV target region with corresponding sites of poxvirus, variola virus, and cowpox virus revealed that the sequences of these species differ by three bases in the gRNA PAM sequence required for Cas12a cleavage activity, along with slight nucleotide

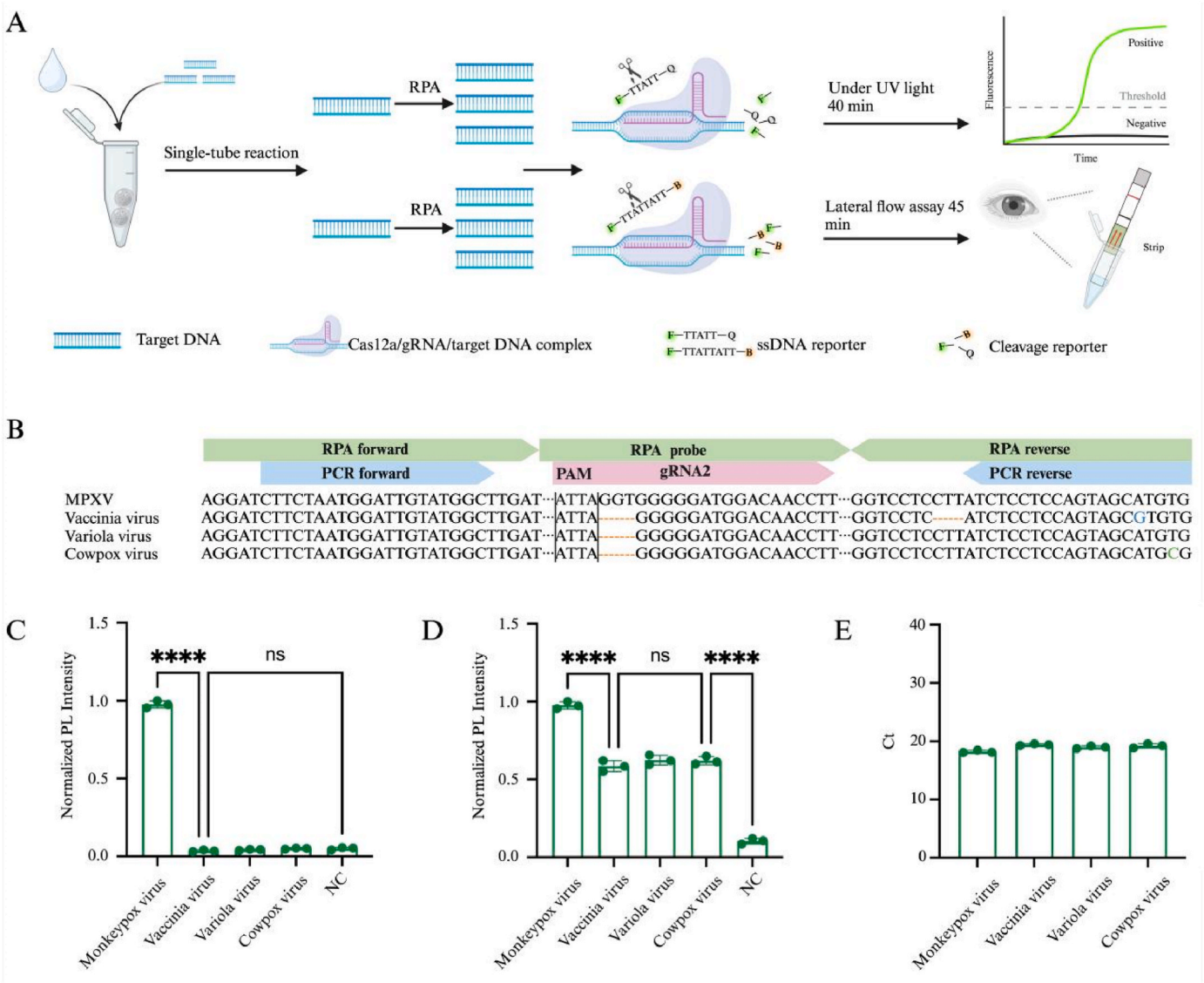


Fig. 1. The workflow of the MPXV single-tube RPA/CRISPR detection platform. (A) The products of MPXV genomic DNA amplification are recognized by gRNA and cleaved by Cas12a. This results in the cleavage of the corresponding FAM or biotin-labeled reporter. Consequently, results can be read using a fluorescent detector or LFA strip. (B) The single-tube RPA/CRISPR detection platform detects the locus of the MPXV A27L gene in comparison with other orthopoxviruses. (C) Detection of MPXV and other orthopoxviruses. (D) RPA and (E) PCR amplify each detection target. The CRISPR fluorescent signals were normalized, and the bar chart data represents the average value with standard deviation (SD) from three experimental replicates. (ns, $P > 0.05$; ****, $P < 0.0001$).

variations in the primer region used to generate the gRNA target, allowing clear differentiation from other Orthopoxviruses.

3.2. Establishment of the single-tube RPA/CRISPR using suboptimal PAM

We designed gRNA with a canonical PAM sequence targeting the MPXV A27L gene for RPA/CRISPR detection. When the assay was conducted using a two-step method, the limit of detection (LOD) was comparable to that of other molecular diagnostic methods(Li et al., 2010; Maksyutov et al., 2016; Shchelkunov et al., 2011). However, in the one-step assay, only a weak fluorescent signal was observed, even when a DNA target concentration of 10^3 aM was present (Fig. 2B and C). These results suggest that gRNA with a canonical PAM is unsuitable for RPA/CRISPR one-pot detection. Researchers found that Cas12a-mediated substrate binding and subsequent cis-cleavage can potentially interfere with RPA amplification. Experiments showed that DNA cleavage by Cas12a with a canonical PAM is completed within 30 s, while Cas12a with a suboptimal PAM binds more weakly to the DNA substrate, requiring 10–20 min to complete cleavage. This delay allows sufficient amplification products for detection (Lu et al., 2022). Therefore, optimizing PAM is essential for enhancing the Cas12a-mediated one-pot reaction (Fig. 2A). In addition to the canonical PAM sequence (TTTN), we also designed various suboptimal PAM sequences to achieve optimal results (Fig. S1) (Table S2). After screening, the gRNA with the best-performing suboptimal PAM (NTTN) was used in the RPA/CRISPR single-tube experiment, resulting in a significant sensitivity increase. A target DNA concentration of 10^2 aM also produced a clear fluorescence signal, indicating that suboptimal PAMs are more suitable for single-tube reactions (Fig. 2D and E).

3.3. Single-tube RPA/CRISPR assay optimization

The sensitivity of our proposed detection system for the MPXV A27L gene is influenced by various factors, prompting a systematic study to optimize its performance. We tested combinations of Cas12a and gRNA concentrations ranging from 33 to 266 nM and found that 133 nM for

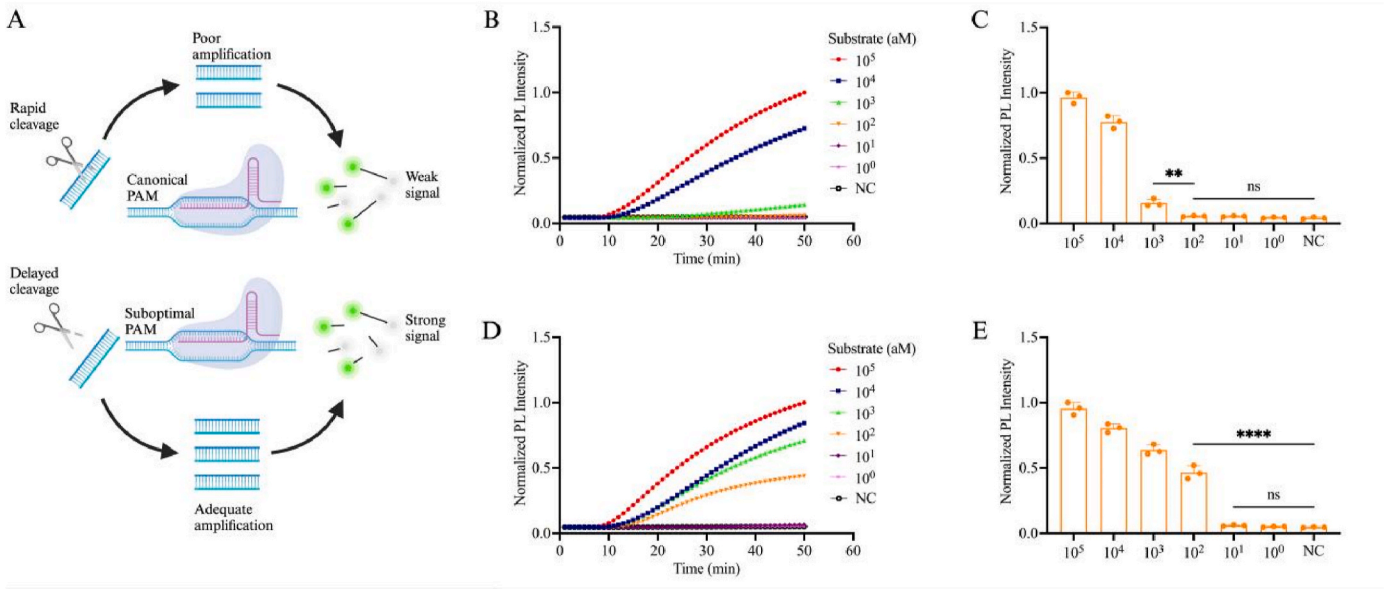


Fig. 2. Schematic diagram of amplification and cleavage in a single-tube reaction. (A) Using canonical PAM, the reaction is primarily focused on cleavage, resulting in a weaker signal; using suboptimal PAM enhances amplification, producing a stronger fluorescent signal. (B and C) Sensitivity of single-tube reactions mediated by canonical PAM. (D and E) Sensitivity of single-tube reactions mediated by suboptimal PAM. (ns, $P > 0.05$; **, $P < 0.01$; ****, $P < 0.0001$).

both yielded optimal signals. Higher concentrations did not improve results, and no visible signal was observed at 33 nM of Cas12a. Consequently, we selected 133 nM as the concentration for the ribonucleoprotein (RNP) complex (Fig. 3A). The CRISPR-mediated fluorescence signal increased with the concentration of the fluorescent reporter. We found that the fluorescence signal was optimal at a reporter concentration of 933 nM. When higher concentrations of the fluorescent reporter were used, no significant increase in fluorescence signal was observed. Therefore, we used 933 nM of the reporter in subsequent experiments (Fig. 3B). Fluorescent signals produced under incubation conditions ranging from 35 to 42 °C showed significant differences, with the most pronounced signals observed at 37 °C and 39 °C (Fig. 3C). NEBuffer provided optimal conditions for Cas12a activity, with tests comparing NEBuffer 1.1, NEBuffer 1, NEBuffer 2.1, NEBuffer 2, NEBuffer 3.1, NEBuffer 3, and an equal volume of nuclease-free water. The best performance was observed with buffer 2.1 (Fig. 3D).

Lyophilization technology focuses on developing freeze-drying protectants and processes, with the shelf life of lyophilized reagents depending on storage conditions. To determine the effective storage duration at different temperatures, we conducted stability experiments. After lyophilizing the RPA and CRISPR detection reagents, they were stored at 4 °C and room temperature for four weeks, with ongoing monitoring and comparison to non-freeze-dried reagents. The results showed that the fluorescence signal of the lyophilized RPA reagent began to decline after 2 weeks at room temperature and dropped significantly by the fourth week, falling below 50% of the signal from the non-freeze-dried reagent. However, at 4 °C, the fluorescence signal remained comparable to that of the non-freeze-dried reagent (Fig. 3E). After four weeks of storage at both room temperature and 4 °C, the fluorescence signal of the CRISPR reagent showed no significant differences compared to the non-lyophilized reagent (Fig. 3F). We then conducted single-tube detection and repeated stability experiments using the lyophilized RPA and CRISPR reagents. The results showed that under room temperature conditions, the fluorescence signal began to decline in the second week and decreased significantly by the fourth week, while at 4 °C, the fluorescence signal remained comparable to that of the non-freeze-dried reagent after four weeks (Fig. 3G and H). This suggests that after lyophilization, the reagents can be stored for up to 1 week at room temperature and for up to 4 weeks at 4 °C, significantly reducing transportation and storage requirements.

3.4. Sensitivity and specificity of the single-tube RPA/CRISPR assay

We further assessed the detection sensitivity by performing gradient dilutions of the MPXV A27L plasmid. After optimizing the system, a clear signal was detected even at a concentration of 10 aM (Fig. 4A and B). To evaluate the specificity of the single-tube RPA/CRISPR method, plasmids containing gene sequences from the corresponding regions of four orthopoxviruses were used as templates, with nuclease-free water serving as a negative control. Although the primers were designed to target a region highly conserved across the other three orthopoxviruses, the results showed that only MPXV was positive, while the others were negative, demonstrating high specificity for the MPXV A27L gene (Fig. 1C). Notably, when the same primers were used in the RPA assay, fluorescent signals were observed not only for MPXV but also for the other three orthopoxviruses (Fig. 1D). We then designed PCR primers for the PCR assay, which revealed no significant differences in Ct values among the viruses, making it impossible to distinguish MPXV from the other orthopoxviruses (Fig. 1E).

In mixed samples containing both monkeypox virus and other viruses, the RPA/CRISPR method was able to accurately detect the presence of monkeypox virus without cross-reactivity to other viruses. The signal for monkeypox virus was significantly higher than the background signals from the other viruses, with no interference observed (Fig. 4C and D). These results indicate that the RPA/CRISPR method has strong resistance to interference and can effectively detect monkeypox virus in complex viral mixtures.

3.5. LFA strip detection based on single-tube RPA/CRISPR reaction

The single-tube RPA/CRISPR reaction can be visualized using LFA strips, enabling flexible on-site assays, as illustrated in Fig. 5A. The sample binding zone contains rabbit anti-FAM antibodies conjugated with gold nanoparticles (NPs), which bind to the FAM moiety of the Biotin-ssDNA-FAM reporter. Anti-rabbit antibodies are embedded in the test line (T line) of the strip. If the target sequence is present, Cas12a cleaves the Biotin-ssDNA-FAM reporter, and the resulting complex of FAM and gold-labeled antibodies is captured by the anti-rabbit antibodies on the T line, producing a visible signal indicating the presence of the target. Additionally, the Biotin at the 3′ end binds to streptavidin on the control line (C line), resulting in color development. Since the C line

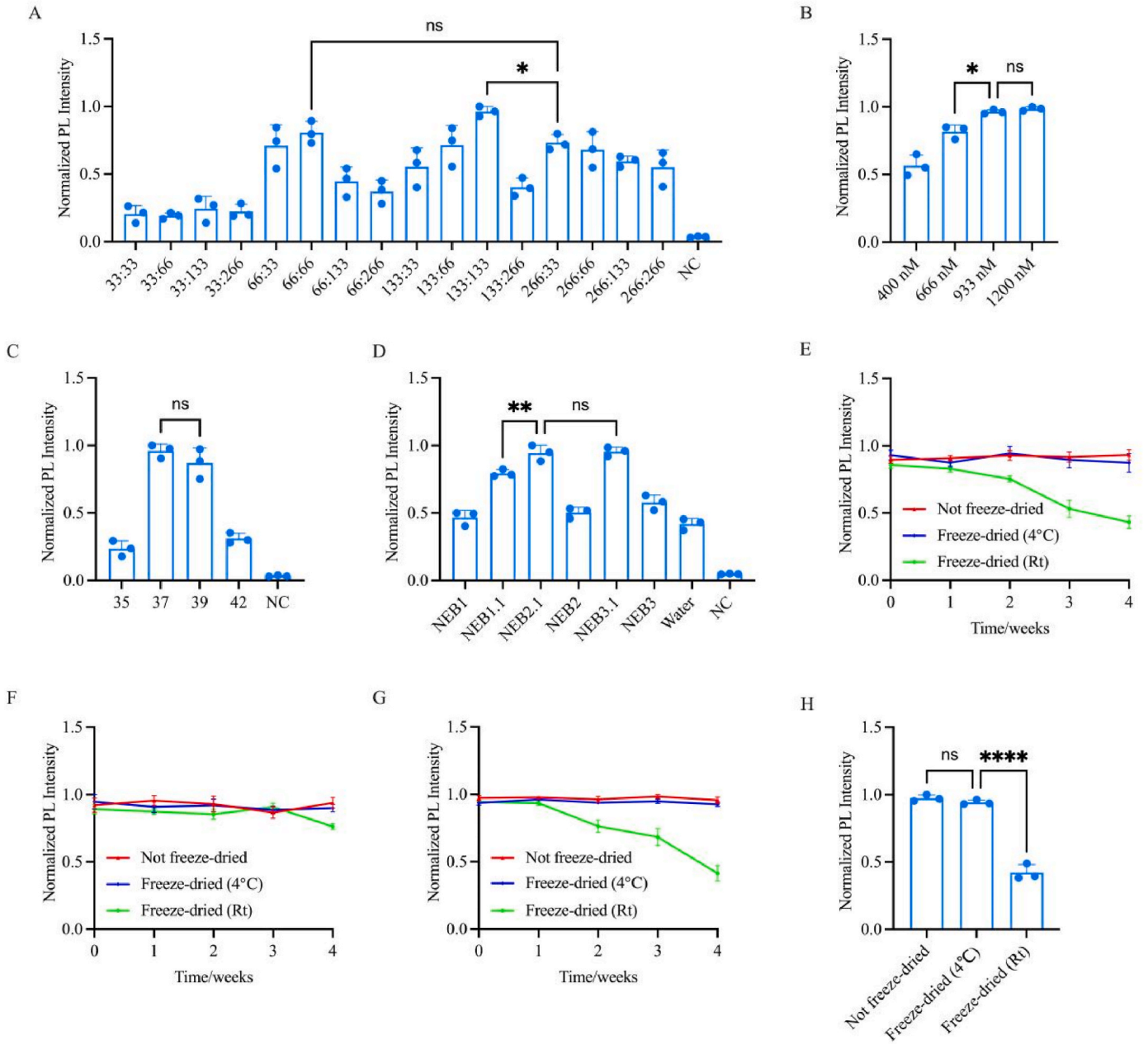


Fig. 3. MPXV single-tube RPA/CRISPR detection optimization. (A) Optimization of Cas12a and gRNA concentrations. (B) Exploration of the optimal concentration of FAM-labeled reporter in single-tube reactions. (C) Optimization of reaction temperature. (D) Investigation of the effects of different NEBuffer on the reaction. (E) Optimization of storage temperatures for RPA, (F) CRISPR, and (G) RPA/CRISPR freeze-dried spheres. (H) The terminal fluorescence bar chart of the optimization of storage temperatures for RPA/CRISPR freeze-dried spheres. (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$).

is located below the T line and the liquid flows from bottom to top, the C line does not serve as a standard quality control line; instead, it reflects the effectiveness of Cas12a cleavage. Specifically, when Cas12a's cutting efficiency is high, the C line color appears lighter or may even disappear. Conversely, when the cutting efficiency is low or no cleavage occurs, the color of the C line appears darker (Fig. 5B). To maximize sensitivity, we investigated the optimal quantity of reporters needed to eliminate the T lines (Fig. 5C). Through a series of experiments, we determined that the optimal concentration of the Biotin-ssDNA-FAM reporter for the CRISPR reaction is 800 nM. Additionally, we optimized the buffer by adding 5% PEG to effectively eliminate the T lines (Fig. 5D). In this study, the LOD of the LFA is defined as the lowest concentration of plasmid at which it can reliably distinguish between negative and positive samples. The results demonstrated an LOD of 10 aM for the LFA strips, with negative results when 10^4 aM concentrations of vaccinia virus plasmids were added (Fig. 5E).

3.6. Detection of clinical sample

To evaluate the accuracy and reliability of our single-tube RPA/CRISPR system for detecting MPXV infection in clinical samples, we tested 30 different types of positive clinical samples. A sample was considered positive if the average signal from the MPXV assay met or exceeded the negative control threshold plus three standard deviations.

We performed fluorescence and LFA strip detection on clinical samples using the single-tube RPA/CRISPR platform. The results showed that fluorescence signals were detected in anal swabs, throat swabs, herpes fluid, and saliva samples; however, some urine and serum

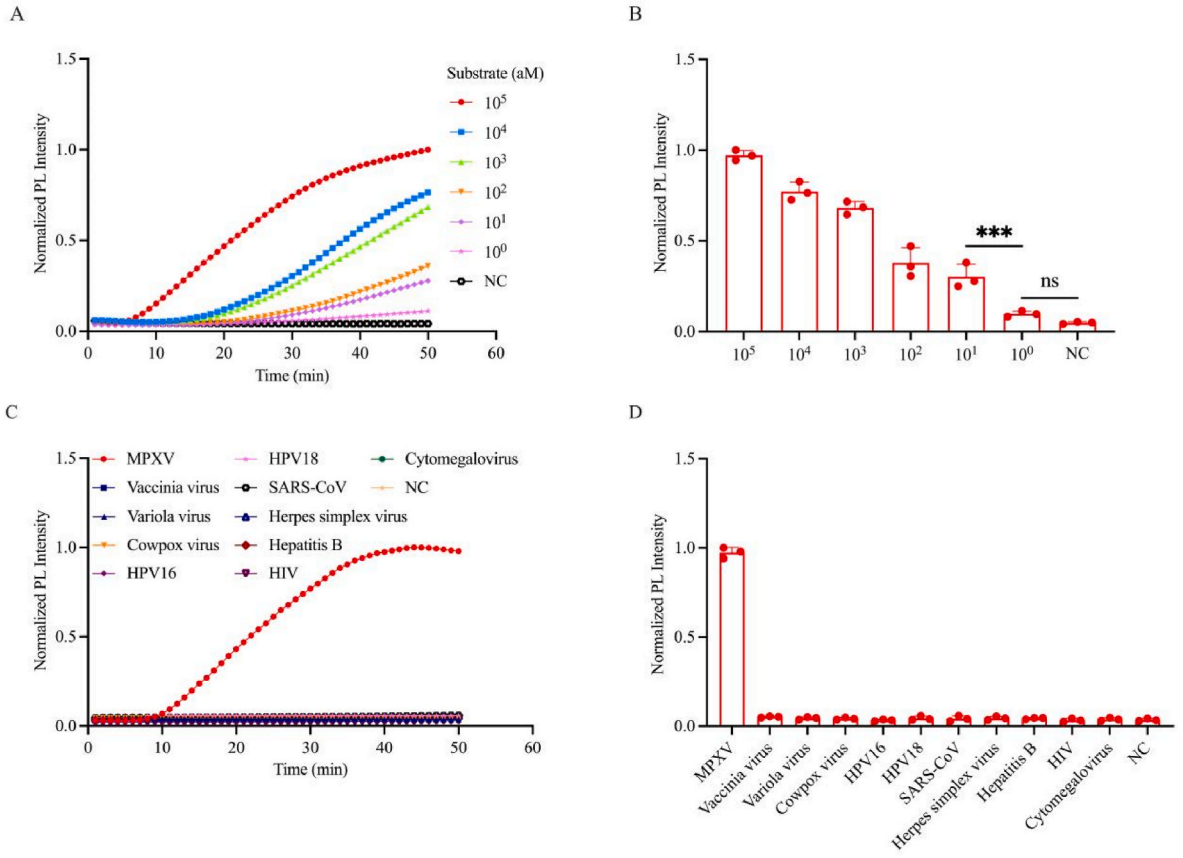


Fig. 4. Sensitivity and specificity of RPA/CRISPR single-tube detection. (A) Sensitivity curve of the experiment, with fluorescence curves of targets at different concentrations shown in different colors. (B) The terminal fluorescence bar chart data represents the average values and SD of three experimental repetitions. (C) Specificity evaluation curve for the mixed samples and (D) fluorescence bar chart. NC indicates that the assay template was replaced with water. (ns, $P > 0.05$; ***, $P < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

samples did not produce any fluorescence signals (Fig. 6A). When LFA strips were used, color development was observed in herpes fluid and saliva samples, while no bands appeared in urine samples, and some other sample types also showed no bands (Fig. 6B–G). Compared to PCR, the sensitivity of fluorescence detection for anal swabs, throat swabs, herpes fluid, and saliva samples was 100% (Tables S3–S8). The sensitivity of LFA strip detection for herpes fluid and saliva samples was also 100%, with no signals detected in negative samples, resulting in a specificity of 100% (Fig. 6H) (Tables S9–S14).

4. Discussion

The outbreak of monkeypox highlights the urgent need for a rapid and accurate detection system to support its prevention and control. According to WHO guidelines, qPCR is regarded as the gold standard for laboratory diagnosis of MPXV due to its speed, accuracy, and high sensitivity. However, the stringent requirements of qPCR, such as the need for specialized equipment and trained personnel, limit its applicability in resource-limited settings. Consequently, the development of a user-friendly, cost-effective detection platform that operates independently of large-scale instrumentation is critically important.

In recent years, significant advances have been made in single-tube CRISPR-Cas12a diagnostic technology. To improve the performance of single-tube detection, several researchers have explored suboptimal PAMs. In the study by Lu et al., which focused on suboptimal PAMs for SARS-CoV-2 (Lu et al., 2022), the researchers found that the optimal suboptimal PAM was "NTTN." In contrast, the study by Wang et al. (2023), which focused on suboptimal PAMs for Vibrio parahemolyticus, identified "TCTN" as the most effective PAM. These findings suggest that different optimal PAMs may exist under varying conditions for different target genes. In this study, we conducted a screening of suboptimal PAMs for the target gene of monkeypox virus. Based on this, we established a single-tube RPA-CRISPR/Cas12a detection system for monkeypox virus. After a series of optimizations, the LOD for both fluorescence and LFA strip detection reached a sensitivity of 10 aM. With real-time fluorescence detection, the reaction can be completed within 40 min. Using LFA strip detection, results can be visually observed within 5 min after the reaction is completed. Notably, despite only a few nucleobase differences between monkeypox virus and other orthopoxviruses in the target region, both monkeypox virus and other orthopoxviruses generated distinct signals when RPA and PCR were used to detect these regions. These findings demonstrate that the gRNA within the CRISPR system exhibits a high degree of specificity, allowing for precise recognition even with a single nucleobase difference.

We used this platform to analyze clinical samples from various sources, including urine, anal swabs, throat swabs, herpes fluid, saliva, and serum, and compared the results with qPCR-based laboratory diagnoses. The findings showed that when the sample types were saliva and herpes fluid, the platform exhibited high consistency with PCR, with no false positives and excellent specificity. Due to the low viral load in serum and urine samples (Guo et al., 2024; Veintimilla et al., 2022), some specimens were not detected (Tables S15–S16). Therefore, we suggested prioritizing herpes fluid and saliva for testing in practical applications to improve detection accuracy.

In summary, the platform we developed demonstrates exceptional sensitivity and specificity, making it highly suitable for the rapid detection of monkeypox virus. It meets several key characteristics of the ASSURED criteria—accuracy, specificity, sensitivity, ease of use, speed,

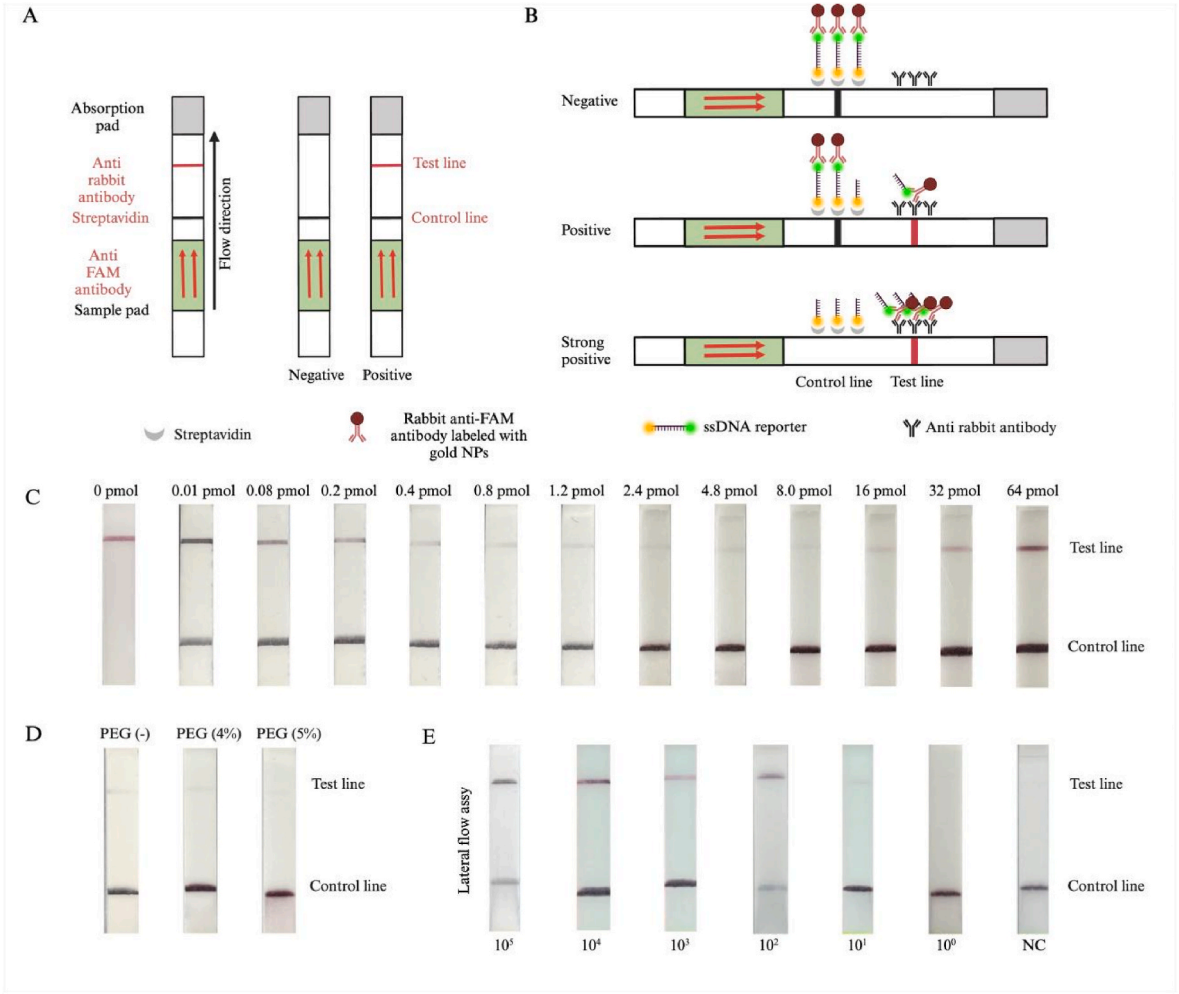


Fig. 5. Visualization of the results from LFA strips. (A) Structural diagram of the LFA strip and (B) workflow diagram. (C) Optimization of the concentration of biotin-labeled reporter. (D) Optimization of the buffer using PEG. (E) Results of the sensitivity analysis of the LFA strips, the NC refers to the vaccinia virus plasmid at a concentration of 10^4 aM.

durability, equipment-free operation, and accessibility for end users—-proposed by the WHO for POCT(Land et al., 2019). Compared to previously reported monkeypox detection studies, our method demonstrates high sensitivity and employs LFA detection in a one-step process. In contrast to previous studies that either did not incorporate or only utilized a limited number of positive clinical samples, we conducted a comprehensive comparison across six distinct types of clinical samples, validating the accuracy of our method for clinical applications. However, our study acknowledges several limitations. First, the presence of interference substances such as salts or proteins in serum and urine could hinder the binding of reporter and reduce the efficiency of the LFA strip reaction, leading to poor results. Further research is needed to identify and address these interfering substances to mitigate their impact. Additionally, since both RPA amplification and CRISPR signal release are conducted within the same reaction system. The efficiency of these reactions may be influenced by various factors, which makes precise quantitative detection unachievable. Finally, the viral nucleic acid extraction procedure remains relatively complex, we cannot entirely eliminate reliance on centrifuges, which poses challenges in processing large numbers of samples quickly.

To facilitate large-scale production and practical application, we stored the RPA and CRISPR/Cas12a reaction reagents as lyophilized spheres, simplifying both transportation and usage. Future research could explore further applications in microfluidics. By optimizing the design of primers and gRNA, we can extend this method to detect nucleic acids from various pathogens. During detection, only pathogen nucleic acid needs to be added, enabling on-site testing in non-traditional clinical settings, such as customs, outpatient clinics, and rural healthcare centers. This approach promotes true portability, significantly enhancing its applicability and ease of use.

5. Conclusion

This study successfully developed a single-tube RPA/CRISPR detection platform for monkeypox virus. Validation with clinical samples demonstrated that the platform achieves high sensitivity and specificity. The minimal equipment requirements for LFA strips make it particularly suitable for POCT, and the isothermal fluorescence detector offers the capability for real-time visualization and is less prone to interference. The detection methods can be flexibly selected based on specific application requirements. Additionally, we freeze-dried the detection reagents to facilitate storage and transport. This approach has the potential for integration into microfluidics and other biosensor platforms. Overall, the single-tube RPA/CRISPR detection platform offers an innovative technological solution for the rapid detection of pathogens, providing significant clinical application potential and paving the way for future industrial adoption.

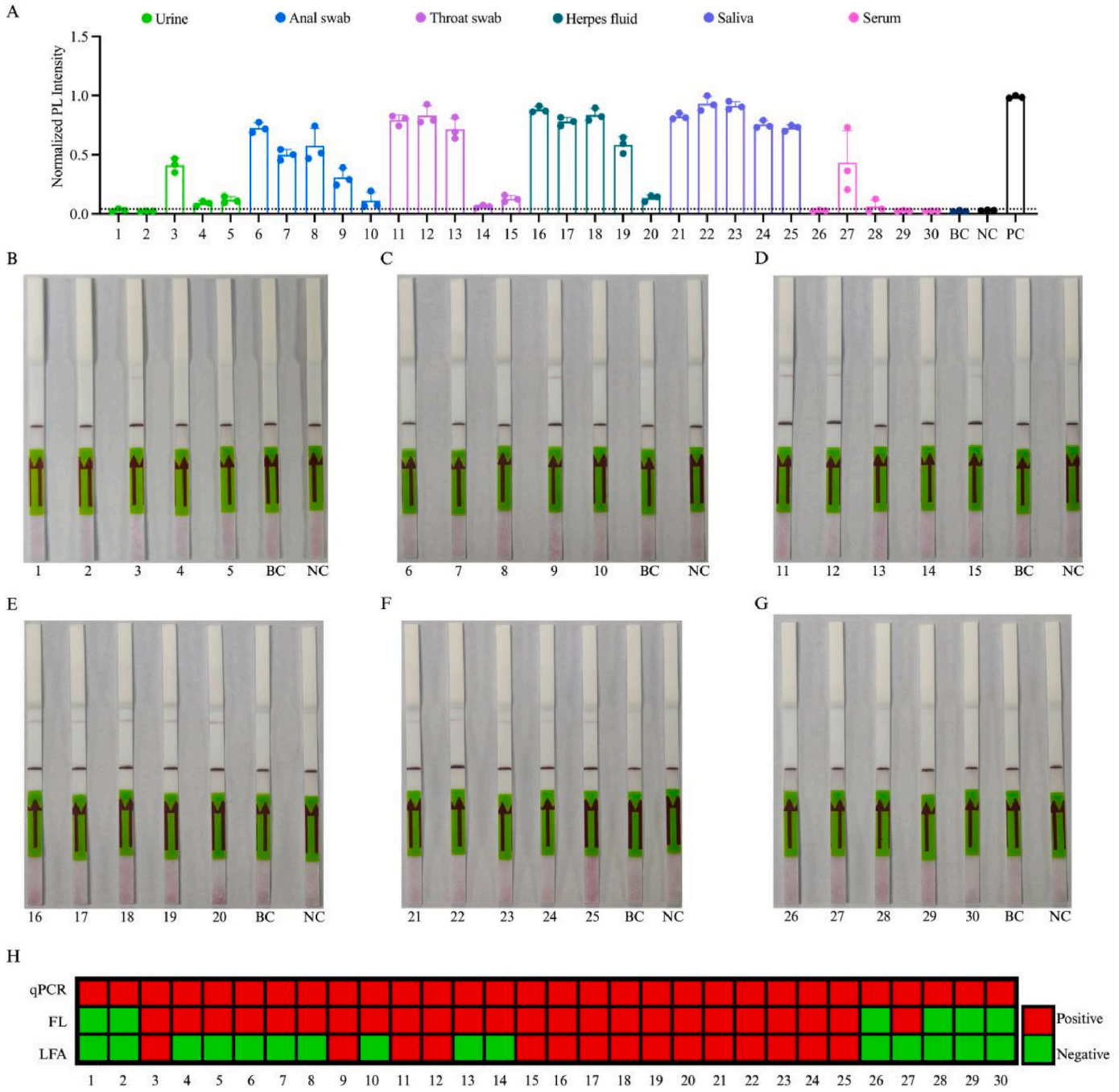


Fig. 6. Detection of MPXV clinical samples using single-tube RPA/CRISPR reactions. (A) Results of single-tube RPA/CRISPR fluorescence detection of samples from 30 MPXV-infected cases, along with blank (BC; nuclease-free water), negative (NC; nucleic acids from healthy volunteers), and positive (PC; 10^5 aM) controls, with the dashed line indicating the threshold for positive results and different sample types represented in various colors. The results describe the mean values from three experimental repetitions. (B) Results of LFA analysis for the urine, (C) anal swab, (D) throat swab, (E) herpes fluid, (F) saliva and (G) serum samples. (H) Comparison of detection results from clinical laboratory qPCR with those from single-tube RPA/CRISPR. Sample number 1–5: urine; sample number 6–10: anal swab; sample number 11–15: throat swab; sample number 16–20: herpes fluid; sample number 21–25: saliva; sample number 26–30: serum. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

CRediT authorship contribution statement

Shan Liu: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Yang Yang:** Resources, Methodology, Investigation, Funding acquisition, Data curation. **Xue Li:** Validation, Supervision, Software, Resources. **Jeong-Woo Choi:** Supervision. **Jinhong Guo:** Visualization, Validation, Supervision, Formal analysis, Conceptualization. **Hongzhi Luo:** Formal analysis, Data curation, Conceptualization. **Chenzhong Li:** Visualization, Validation, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2025.117221.

Data availability

Data will be made available on request.

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