




A cleavage-gated terminal exposure-driven CRISPR-RCA self-amplifying system for ultra-fast DNA detection

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ABSTRACT

The one-pot detection technology based on nucleic acid isothermal amplification combined with CRISPR has a significant advantage in on-site detection of infectious diseases. It is superior to quantitative polymerase chain reaction (qPCR) due to its lack of temperature variation and significantly faster reaction speed. Nevertheless, Cas proteins compete with amplification enzymes for reaction substrates, which results in the signal amplification effect being less than expected. To overcome this limitation, we have developed a single-tube self-amplifying system driven by cleavage-gated terminal exposure based on CRISPR and rolling circle amplification (AURORA), enabling ultra-fast and sensitive monkeypox virus (MPXV) detection. This method innovatively designs a dual-function probe (DF probe). In the presence of the target, the trans-cleavage activity of Cas12a is activated, only cleaving the single-stranded DNA to expose the 3' terminal of the DF probe. The cleaved DF probe hybridizes with the circular DNA template and is bound by phi29 DNA polymerase to initiate RCA. Here, we utilize the characteristics of Cas12a and phi29 DNA polymerase acting on substrates in different strand states to avoid substrate competition between the two enzymes in a single-tube reaction. This assay can achieve ultra-fast signal amplification of MPXV DNA within 8 min, with a limit of detection (LOD) of 88 aM (53 copies/μL). Combining the viral nucleic acid thermal lysis method, it is possible to achieve results from sample input to output in 10 min. The AURORA detection strategy was further used to detect MPXV in clinical samples (36 MPXV samples), and the results were completely consistent with qPCR. The AURORA system features ultra-fast and precise detection, providing a more efficient tool for the prevention and control of severe infectious diseases.

1. Introduction

Monkeypox virus (MPXV) is an enveloped double-stranded deoxyribonucleic acid virus from the orthopoxvirus genus in the Poxviridae

family (Mitja et al., 2023). It enters the human body through mucous membranes and broken skin. The transmission of MPXV occurs through contact with respiratory secretions, lesion exudate, blood, other bodily fluids of infected patients, or contaminated items (Huang et al., 2022;

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Thornhill et al., 2022). In recent years, MPXV has spread to 120 countries, infecting over 100,000 people (Rivers et al., 2024; Titanji et al., 2024). Serious MPXV infection can be fatal, with mortality rates reaching up to 10 % (Rosa et al., 2023). Furthermore, MPXV continues to mutate during human-to-human transmission, which may make the virus even more deadly (Isidro et al., 2022; O'Toole et al., 2023). Hence, the World Health Organization (WHO) has declared the monkeypox outbreak to be a public health emergency of international concern (PHEIC), recommending that infected individuals to isolate at home or in hospitals to curb the virus's spread (World Health Organization, 2024). Similar to the isolation protocols implemented during the severe acute respiratory syndrome coronavirus (SARS-CoV-2) pandemic, managing suspected cases consumes substantial social and medical resources (Ahamed et al., 2024; Zeng et al., 2024). Rapid, accurate, and sensitive diagnostic methods help identify infected patients and quickly release occupied resources.

The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas diagnostic system offers the advantages of high detection efficiency, excellent sensitivity, strong specificity and simple reaction conditions, with the potential to achieve ultra-fast detection of infectious pathogens (East-Seletsky et al., 2016; Huang et al., 2023; Li et al., 2018; Rabiee et al., 2022). Unfortunately, the detection limit of the CRISPR/Cas system is only at the pM level, which is far from meeting the demands of clinical testing (Chandrasekaran et al., 2022). By combining isothermal amplification and CRISPR, the one-pot assay improves reaction sensitivity and reduces aerosol contamination risks from amplification products, making it a prominent focus in current research (Liu et al., 2024; Shang et al., 2024; Zhang et al., 2025; Zhu et al., 2023). However, the target DNA as a reaction substrate was competed for binding by Cas proteins and isothermal amplification enzymes, resulting in impaired detection sensitivity (Chen et al., 2025; Hu et al., 2023; Wang et al., 2024; Yang et al., 2024). Although physical spatial separation or sucrose density gradients can be used to achieve phase separation between nucleic acid amplification and CRISPR cleavage reaction systems to overcome this effect, the detection still takes a long time (Kang et al., 2023; Sun et al., 2021; Wang et al., 2023; Wang et al., 2024). In addition, researchers have also designed crRNAs with suboptimal PAM sites to reduce the cleavage efficiency of Cas12a-crRNA complexes (RNP), making it easier to accumulate more amplicons during isothermal amplification and thus improving detection sensitivity (Lu et al., 2022). Whereas, the activity differences at different suboptimal PAM sites are significant, which limits the universality of the method. Hence, there remains an urgent requirement to develop faster and more universal detection technologies.

In this article, we innovatively construct a single-tube self-amplifying system driven by cleavage-gated terminal exposure based on CRISPR and rolling circle amplification (AURORA), enabling ultra-fast and sensitive detection of MPXV. We creatively designed a dual-function probe (DF probe). The trans-cleavage activity of activated Cas12a only acts on single-stranded DNA, thereby exposing the 3' terminal of the DF probe. The cleaved DF probe then constructs a primer-template complex through base pairing mediated by a circular DNA template. This process activates phi29 DNA polymerase, initiating rolling circle amplification (RCA). Since the RCA product contains Cas12a recognition sites, it can reactivate Cas12a to cleave the DF probe again, thereby forming a self-amplifying signal amplification system with positive feedback. Here, we utilize the properties of Cas12a and phi29 DNA polymerase acting on substrates in different strand states to address the substrate competition issue between the two enzymes in a co-reaction system, achieving efficient coordination of the two signal amplification systems in both temporal and spatial dimensions. In conclusion, AURORA as a dual-enzyme synergistic self-amplification system greatly enhances the efficiency of MPXV detection and can meet the requirements of ultra-fast detection speed and high sensitivity during epidemic prevention to control epidemics, especially in areas such as customs, train stations, hospitals.

2. Materials and methods

2.1. Materials and apparatus

The template sequences and crRNA sequences used in this study are listed in [Supplementary Table 1](#). Plasmids, crRNA, and DF probes were purchased from GenScript Biotech (Nanjing, China). Phi29 DNA Polymerase, SYBR Gold Nucleic Acid Gel Stain were purchased from Thermo Fisher Scientific (United States). T4 DNA Ligase, Exonuclease I, Exonuclease III, RNase Inhibitor (Murine) and Cas12a were purchased from New England Biolabs (United Kingdom). DNA Marker A (25–500 bp), dNTP, and DEPC treated water were purchased from Sangon Biotech (Shanghai, China). ChamQ Universal SYBR qPCR Mix was purchased from Vazyme Biotech (Nanjing, China). SYBR Green II nucleic acid dye was purchased from Solarbio LIFE SCIENCES (Beijing, China).

2.2. DNA sequences and plasmids

The target DNA was selected from the B6R gene of monkeypox virus, and a standard plasmid was constructed by inserting the sequence located at 165115-166068 in the MPXV genome (GenBank: ON563414.3) into pUC57. We constructed B6R homologous sequence plasmids from other human orthopoxviruses to validate the specificity of this system, including B7R of variola virus (GenBank: NC_001611.1), B4R of cowpox virus (GenBank: X94355.2) and B5R of vaccinia virus (GenBank: LT966077.1). The sequences are listed in [Supplementary Table 2](#).

2.3. T4 DNA ligation

The circular DNA template for RCA was prepared as follows: firstly, the phosphorylation-modified linear padlock (1.2 μ M), linker (1.2 μ M) was mixed, incubated at 85 °C for 5 min, and gradually cooled to room temperature. Later, the above products were mixed with 1 μ L of T4 DNA ligase (40 U/ μ L), 5 μ L of 10 \times T4 DNA ligase buffer (500 mM Tris-HCl, 100 mM MgCl₂, 10 mM ATP, 100 mM DTT), and DEPC-treated water in 50 μ L of water and incubated for 2 h at 25 °C, followed by inactivation for 15 min at 65 °C. Exonuclease I and Exonuclease III were used to digest the linear single strand that did not form the circular DNA. Then, the enzymes were heated at 80 °C for 20 min to inactivate them.

2.4. Validation and purification of circular DNA templates

15 % urea polyacrylamide gels (PAGE) were used to verify whether the circular DNA template was successfully prepared. Prepare a 15 % urea-PAGE gel, take 20 μ L of the above product and mix it with DNA/RNA Loading Buffer (2X, for Denaturing PAGE). Then, add the above mixture into the 15 % PAGE wells, and run at 120 V for 1 h in 0.5 \times TBE buffer. The gel was removed and immersed in 1 \times SYBR Gold Nucleic Acid Gel Stain for 15 min. The gel was exposed using a Bio-rad imager. When a clear bright band appears in lane 5, it indicates the successful preparation of the circular DNA template ([Fig. S1](#)). To purify the circular DNA template, the PAGE gel containing the circular DNA template was cut off using a disposable scalpel blade, and then the prepared circular DNA template was purified according to the classical gel DNA elution and extraction procedure. Finally, the concentration of the purified circular DNA templates was analyzed by NanoDrop spectrophotometer and then stored at –20 °C until further use.

2.5. Steps for Cas12a-only assay, two-step assay and three-step assay

The Cas12a-only assay consisted of 5 μ L of MPXV DNA, Cas12a (50 nM), crRNA (50 nM) and DF probe (500 nM), 2 μ L of 10 \times reaction buffer (330 mM Tris-HCl, 100 mM MgCl₂, 660 mM KCl, 1 % Tween 20, 10 mM DTT), RNase Inhibitor, and DEPC-treated water to form a 20 μ L reaction solution at 37 °C for 1 h, followed by inactivation at 65 °C for

10 min. Fluorescence emission measurements were recorded every minute at 635 nm using the ABI 7500 (Thermo, USA). In the two-step method, the above reaction products were mixed with circular DNA template (50 nM), 2 U phi29 DNA polymerase, dNTP (500 μ M), 0.5 μ L of 10 \times reaction buffer, and 2.5 μ L of 10 \times SYBR Green II, and 25 μ L of reaction solution was prepared with DEPC-treated water. The reaction was performed at 37 $^{\circ}$ C for 1 h, inactivated at 65 $^{\circ}$ C for 10 min, and the fluorescence emission measurements were recorded every minute at 518 nm using the ABI 7500. In the three-step method, Cas12a (50 nM), crRNA (50 nM) and DF probe (500 nM), 0.5 μ L of 10 \times reaction buffer and RNase Inhibitor were added to the reaction products of the two-step method, and DEPC-treated water was added to form a 30 μ L reaction system. The reaction was performed at 37 $^{\circ}$ C for 1 h and inactivated at 65 $^{\circ}$ C for 10 min. Fluorescence emission measurements were recorded every minute at 635 nm using the ABI 7500.

2.6. Overall protocol of AURORA for MPXV DNA detection

The complete detection system of the AURORA assay consisted of 5 μ L of MPXV DNA, circular DNA template (50 nM), 2 U of phi29 DNA polymerase, 2 μ L of 10 \times reaction buffer, Cas12a (50 nM), dNTP (500 μ M), crRNA (50 nM), DF probe (500 nM), and the RNase Inhibitor was configured into 20 μ L and incubated at 37 $^{\circ}$ C for 1 h. Fluorescence emission measurements were recorded every minute at 635 nm using an ABI 7500.

2.7. Clinical sample analysis

Clinical samples for this study were obtained from Jiangsu Provincial Center for Disease Control and Prevention, and complied with the Declaration of Helsinki (2024-691-01). Rash fluid swabs were collected in disposable virus preservation tubes and the samples were diluted 10-fold using lysis buffer and heated to 95 $^{\circ}$ C for 2 min. 5 μ L of lysed viral DNA was used for AURORA detection. The results were compared with

qPCR. For qPCR, 10 μ L of ChamQ Universal SYBR qPCR Mix ($2 \times$), 200 nM MPXV PCR forward and reverse primers were mixed with 5 μ L of lysed viral DNA, and the total volume was adjusted to 20 μ L by adding DEPC-treated water. The qPCR reaction was performed in the ABI 7500 according to the following protocol: pre-denaturation at 95 $^{\circ}$ C for 3 min, followed by 40 cycles of amplification at 95 $^{\circ}$ C for 10 s and 60 $^{\circ}$ C for 30 s.

3. Results and discussion

3.1. Design and principle of AURORA

AURORA is a single-tube self-amplification system driven by cleavage-gated terminal exposure based on CRISPR and RCA. The principle is shown in Fig. 1. We ingeniously designed a DF probe to construct a self-amplifying signal amplification system. DF probe consists of fluorescent and quenching groups, primer functional regions (RNA), and cleavage functional regions (DNA). Due to the high affinity of Cas12a's trans-cleavage activity for single-stranded DNA, the activated Cas12a can quickly cleave the DNA region of the DF probe, exposing the primer functional region on the probe. The cleaved DF probe activates phi29 DNA polymerase to initiate RCA after hybridizing with the circular DNA template through base complementary pairing. Since the circular DNA template consists of a primer-binding region and a Cas12a detection region, the RCA amplification product can also activate Cas12a. Therefore, the cleaved DF probe acts as an RCA primer, forming a positive feedback cycle of self-amplification between RCA and CRISPR-Cas, achieving ultra-fast signal amplification. When no target is present, Cas12a cannot trigger the cleavage gate due to the lack of trans-cleavage activity. Additionally, the full DF probe is unable to initiate RCA because it has no exposed 3' terminal. The traditional CRISPR-based isothermal amplification one-pot strategy faces the issue of substrate competition due to both the cis-cleavage reaction substrate of Cas12a and the amplification template of the amplification enzyme being the

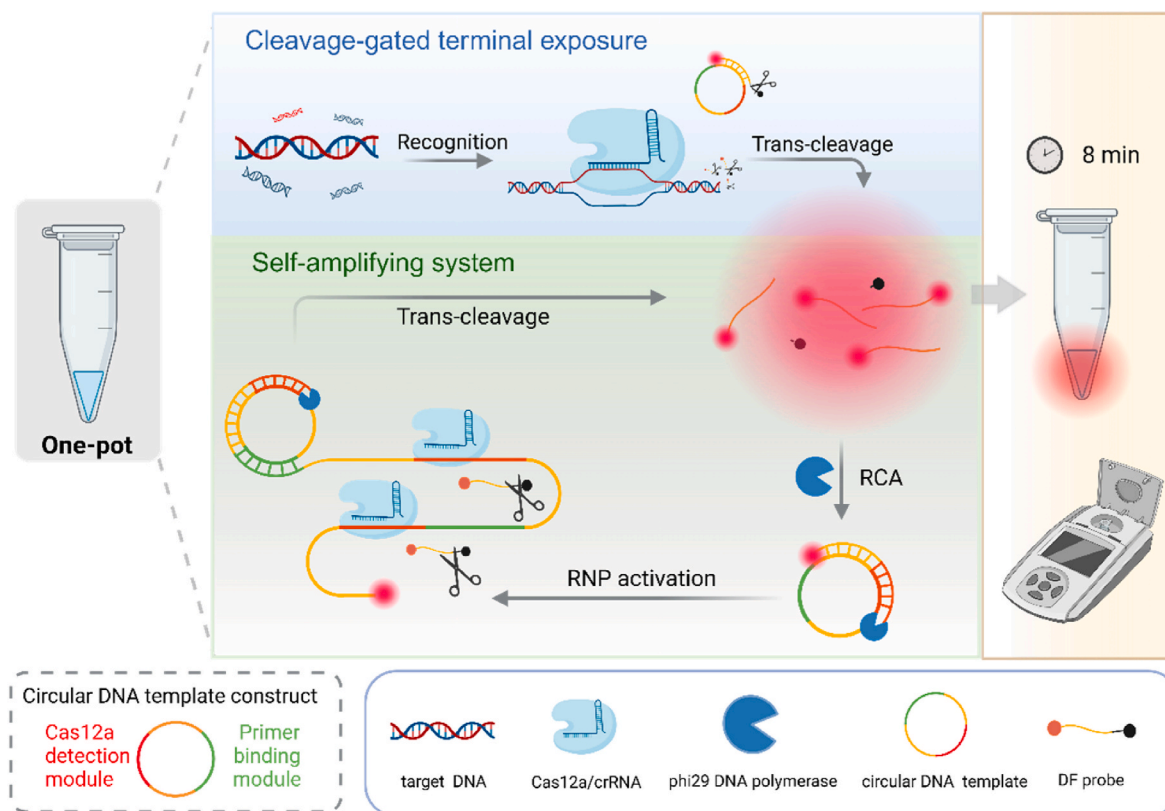


Fig. 1. Schematic diagram of the AURORA for diagnosing MPXV.

target DNA (Fig. S2). AURORA utilizes Cas12a and phi29 DNA polymerase to address the different substrate strand states, successfully avoiding substrate competition between the two enzymes, thereby achieving highly efficient spatiotemporal coordination between the two signal amplification systems.

3.2. Experimental validation of AURORA

AURORA is a one-pot self-amplification system created by combining RCA with the recognition and trans-cleavage activity of CRISPR-Cas12a. We verified the feasibility of AURORA through component removal experiments (Fig. 2A). As shown in Fig. 2B, Cas12a was activated to cut the DF probe when the target DNA was added to the one-pot reaction. This started the self-amplification reaction, leading to a rapid increase in the fluorescence signal, which reaches a plateau within 15 min. However, the absence of phi29 DNA polymerase failed to form a self-amplification cycle, and the fluorescence intensity only increased slightly (Fig. S3). Similarly, negligible fluorescence was observed when Cas12a, crRNA, or target DNA were omitted from the reaction (Fig. 2C). These results demonstrate that AURORA is feasible for detecting MPXV, and its efficient detection performance is dependent on the synergy between Cas12a and phi29 DNA polymerase. Fig. 2D and E illustrate the stepwise reaction process and AURORA workflow. Remarkably, AURORA does not require repeated lid opening and multiple sample transfers compared to stepwise reactions. In addition, we also compared the efficacy of detecting MPXV with the Cas12a-only assay, two-step assay, three-step assay, and AURORA (Fig. 2F–I). The Cas12a-only assay and two-step assay afford a high limit of detection (LOD) of only pM for MPXV. The three-step assay improved the sensitivity, achieving a

LOD of 686.35 fM. Notably, the AURORA was able to detect MPXV as low as 59.28 fM with unoptimized conditions (Fig. S4). In conclusion, the AURORA assay not only reduces the operation steps and the risk of contamination but also shortens the turnaround time and improves the sensitivity of the reaction.

3.3. The optimization for AURORA

To achieve optimal performance for the AURORA assay, it is crucial to optimize key factors, including Cas12a, crRNA, phi29 DNA polymerase, dNTP, circular DNA templates, and DF probe concentration. The DF probe is a key factor in the tandem use of Cas12a and phi29 DNA polymerase. It plays two important roles in the reaction: 1. reporting fluorescence; 2. serving as a primer to initiate self-amplification and generate a large number of amplicons after being cleaved. As shown in Fig. 3A, the detection performance of the DF probe is optimal at a concentration of 100 nM. As the probe concentration increases, although the positive signal rises significantly, the signal-to-noise ratio (S/N) is greatly reduced due to the non-specific responses caused by excessive probe. This may be related to the 3'-5' exonuclease activity of phi29 DNA polymerase. Excess probes increase the probability of collisions with the circular DNA template, causing the probes to be non-specifically exposed at the 3' terminal by the proofreading activity of phi29 DNA polymerase. Therefore, we chose a DF probe concentration of 100 nM for the subsequent experiments. Then, we optimized the concentration of Cas12a RNP. As shown in Fig. 3B, the S/N increased significantly with an increase in Cas12a RNP concentration. When the Cas12a RNP concentration was higher than 50 nM, the S/N did not continue to increase. This is because an excess of Cas12a RNP not only

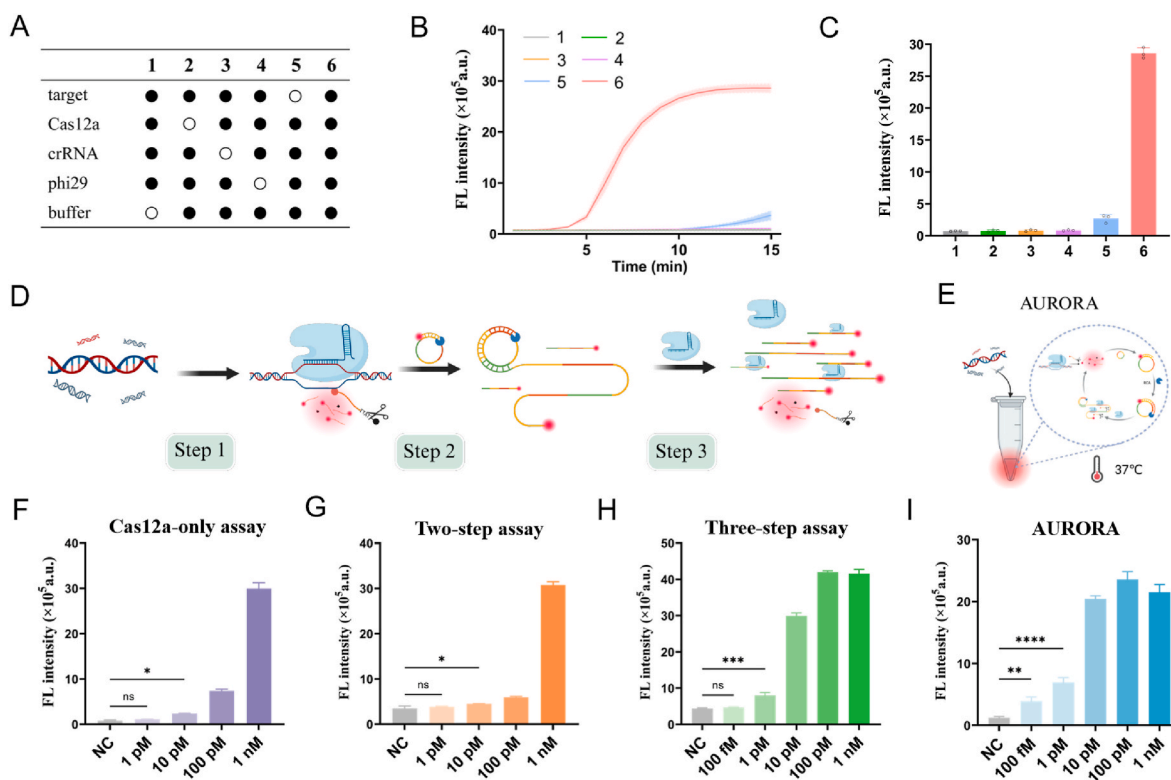


Fig. 2. Feasibility verification of AURORA for MPXV detection.

(A) Specific components under different experimental conditions. (B) Real-time fluorescence intensity for the constructed sensing system (AURORA) under different experimental conditions. (C) The endpoint fluorescence intensity of the AURORA by different experimental conditions (15 min). (D) Schematic diagram showing the workflow of the step-by-step reaction. Step 1, in vitro CRISPR/Cas12a cleavage; Step 2, rolling circle amplification; Step 3, in vitro CRISPR/Cas12a cleavage of amplification production. (E) Schematic diagram showing the workflow of AURORA. (F) Sensitivity of the Cas12a-only assay (Step 1 only). (G) Sensitivity of the two-step assay (includes Steps 1 and 2). (H) Sensitivity of the three-step assay (includes Steps 1, 2, and 3). (I) The fluorescence intensity of AURORA. (n = 3 technical replicates, error bars represent mean \pm s.d.).

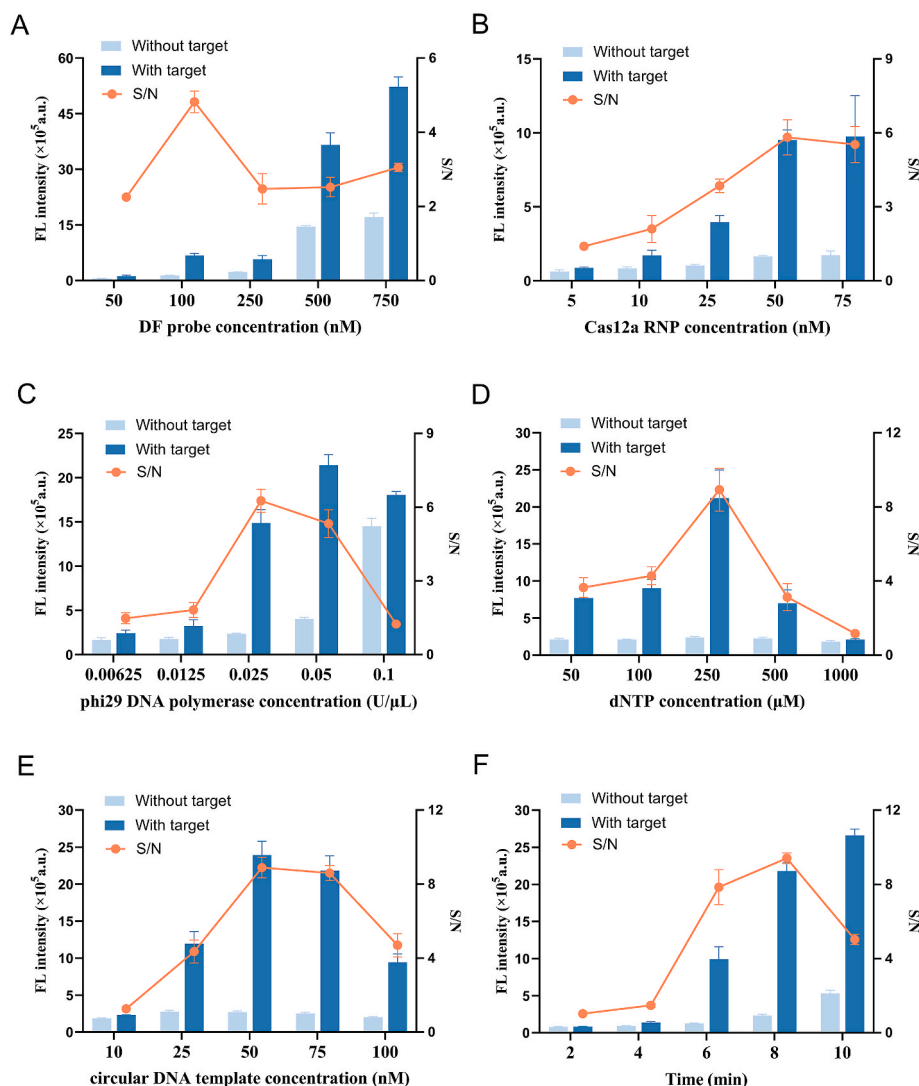


Fig. 3. The optimization for AURORA. (A) DF probe concentration. (B) Cas12a RNP concentration. (C) Phi29 DNA Polymerase concentration. (D) dNTP concentration. (E) Circular DNA template concentration. (F) Reaction time. (n = 3 technical replicates, error bars represent mean \pm s.d.).

rapidly cleaves probes and amplification products but also extensively cleaves the circular DNA template, thereby reducing amplification efficiency. Hence, we chose the concentration of Cas12a RNP as 50 nM for the subsequent experiments.

The AURORA depends on the synergistic action of Cas12a and phi29 DNA polymerase. With the increasing amount of phi29 DNA polymerase, the efficiency of RCA amplification gradually increases, achieving optimal detection performance at a phi29 DNA polymerase concentration of 0.025 U/ μ L (Fig. 3C). However, excessive polymerase concentrations (>0.025 U/ μ L) markedly elevated reaction non-specificity, likely due to hyperactive 3' -5' exonuclease proofreading activity. This overactivity triggered rapid nonspecific amplification during self-amplification cycles. In order to reduce the impact of non-specific amplification, we chose a concentration of 0.025 U/ μ L for phi29 DNA polymerase in subsequent experiments. Moreover, dNTP is an important component of RCA. According to Fig. 3D, AURORA achieved peak performance at 250 μ M dNTP. If there was too much dNTP, the reaction would be inhibited.

Circular DNA templates play an important role as amplification templates in the self-amplifying system, which significantly affects amplification efficiency. As shown in Fig. 3E, when the concentration of the circular DNA template is 50 nM, it exhibits high detection performance. Excessive circular DNA templates promote RCA efficiency,

generating abundant RCA products that compete with the DF probe for trans-cleavage by Cas12a. This leads to a decrease of the fluorescent signal. Furthermore, we explored the effects of temperature on the fluorescence intensity and found that 37 $^{\circ}$ C was the optimal temperature (Fig. S5). Finally, we optimized the reaction time of AURORA. Due to RCA being prone to non-specific amplification, the S/N begins to decline after 8 min as the reaction time extends. Therefore, the optimal detection performance was achieved at 8 min (Fig. 3F). In summary, the optimal reaction conditions for detecting MPXV with the AURORA system are 50 nM Cas12a, 0.025 U/ μ L phi29 DNA polymerase, 250 μ M dNTPs, 50 nM circular DNA template, and 100 nM DF probe, with incubation at 37 $^{\circ}$ C for 8 min.

3.4. Performance evaluation of the AURORA system

Finally, we evaluated the analytical performance of AURORA by using the optimal conditions for the detection of serial dilutions of the MPXV plasmid. As shown in Fig. 4A, as the concentration of MPXV decreased, the fluorescence intensity diminished meanwhile. The fluorescence intensity correlated logarithmically and linearly with the DNA concentration from 1 fM to 100 pM ($R^2 = 0.9452$). The linear regression equation was $Y = 3.458 \cdot \log(X) - 4.912$ (Fig. 4B). The LOD was calculated to be 88 aM (53 copies/ μ L) using the amplitude signal of the

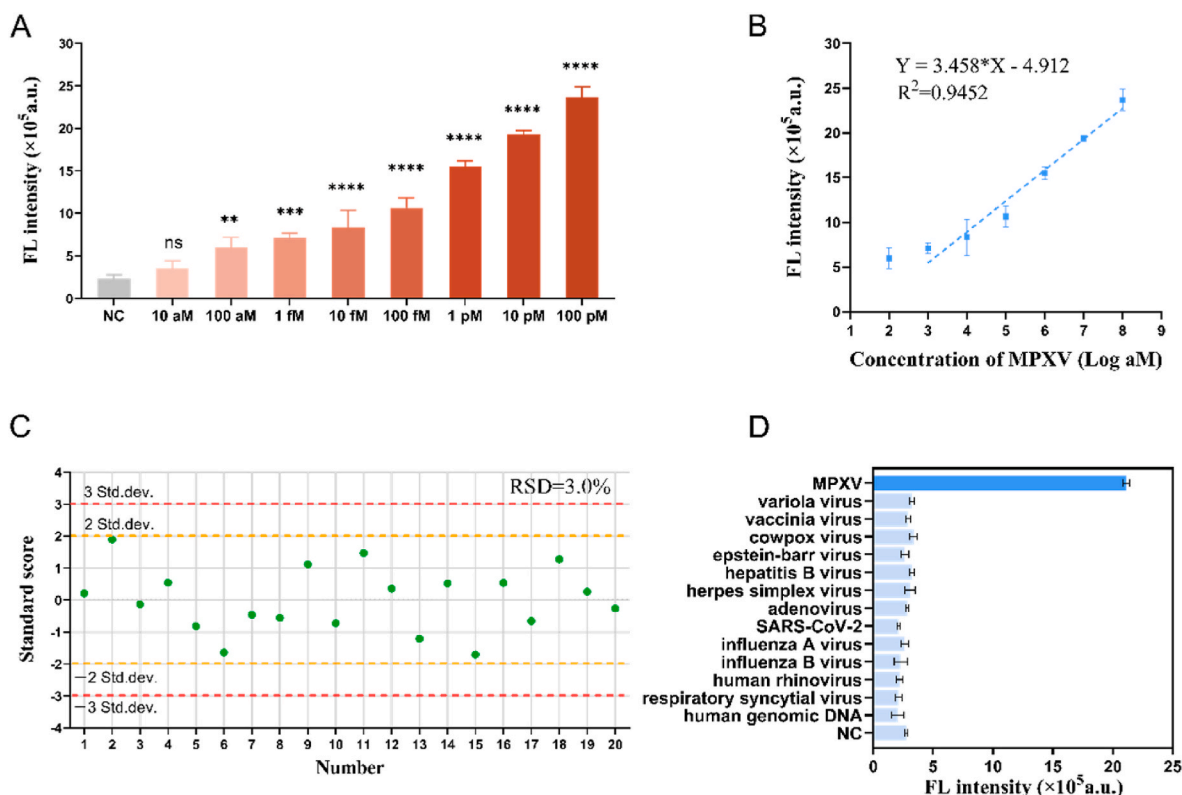


Fig. 4. Evaluation of the analytical performance of AURORA. (A) Sensitivity of AURORA for MPXV. (B) The linear relationship between fluorescence intensity and the concentration of target DNA. (C) The investigation of stability of the biosensor for target DNA of MPXV. (D) The investigation of specificity of the biosensor for target DNA. (n = 3 technical replicates, error bars represent mean \pm s.d.).

control plus a triple standard deviation. In addition, after 20 replicates of the experiment, the standard deviation of the AURORA detection system was as low as 3 %, indicating its excellent stability and reliability (Fig. 4C).

To evaluate the specificity of AURORA, we extended the MPXV assay to detect some non-targeted DNAs, including variola virus, vaccinia virus, cowpox virus and other common pathogens (Supplementary Table 3). As shown in Fig. 4D, only the MPXV sample generates a significant fluorescent signal, whereas the signals of non-targeted DNA samples are in agreement with those of the negative control. These results indicate that AURORA exhibits high detection specificity for DNA targets with different sequences.

3.5. Detection of MPXV in samples by AURORA

In order to investigate the practicality and reliability of the AURORA assay for analyzing real clinical samples, we tested the MPXV in 36 clinical skin swabs. In this study, the 36 samples were collected from Jiangsu Provincial Center for Disease Control and Prevention, including 18 MPXV patients and 18 healthy volunteers. The patient infection stages covered incubation period (n = 4), prodromal phase (n = 6), and cutaneous eruption phase (n = 8) (Supplementary Table 4). We detected MPXV in the samples using qPCR and defined samples with Ct values more than 35 and less than 35 as negative and positive, respectively. At the same time, we used AURORA to detect MPXV in clinical samples and collected fluorescence signal values at 8 min. As shown in Fig. 5A, the qPCR assay based on viral lysis takes nearly 1.5 h, while the AURORA strategy only takes 10 min. The results of AURORA were 100 % concordant with qPCR for all clinical samples (Fig. 5B–D). This means AURORA accurately identified MPXV in the samples with 100 % sensitivity and 100 % specificity (Fig. S6). As shown in Fig. 5E, there is a strong correlation between AURORA and qPCR detection of MPXV.

Researches have indicated that the viral load in skin swabs of monkeypox patients exceeds 172 copies/ μ L (Colavita et al., 2023; Palich et al., 2023), which is higher than the detection limit of AURORA. Therefore, the AURORA assay has excellent performance in the detection of clinical samples.

The recent outbreaks of infectious pathogens like MPXV have highlighted the critical need for rapid, sensitive, and accurate nucleic acid detection technologies (Dronina et al., 2021b; Erez et al., 2019; Li et al., 2010). Currently, qPCR is a commonly used laboratory method for detecting MPXV, featuring high sensitivity and a wide linear range (Fig. S7). CRISPR/Cas systems couple with various signal transduction platforms—including graphene field-effect transistors (FETs), surface plasmon resonance (SPR) biosensors, and surface-enhanced Raman scattering (SERS) biosensors—to convert nucleic acid recognition into electrical or Raman scattering signals, thereby enhancing the sensitivity of CRISPR-based one-pot detection strategies (Akhavan et al., 2012, 2014; Dronina et al., 2021a, 2022). In this study, we developed an ultra-rapid, ultra-sensitive CRISPR-based one-pot MPXV detection platform with excellent potential for field deployment. As shown in Supplementary Table 5, AURORA combines high detection sensitivity and short turnaround time in comparison to other single-tube CRISPR/Cas systems. Moreover, our reagents demonstrate excellent stability, maintaining strong detection performance even after 20 freeze-thaw cycles (Fig. S8A). In order to facilitate on-site use and transportation, we performed lyophilization on the reagent, but the signal of the lyophilized reagent is weaker compared to before lyophilization (Fig. S8B). In the future, we will optimize lyophilized reagents to make our work even more applicable for point-of-care testing (POCT).

4. Conclusion

In summary, this study proposed an innovative, ultra-sensitive, and

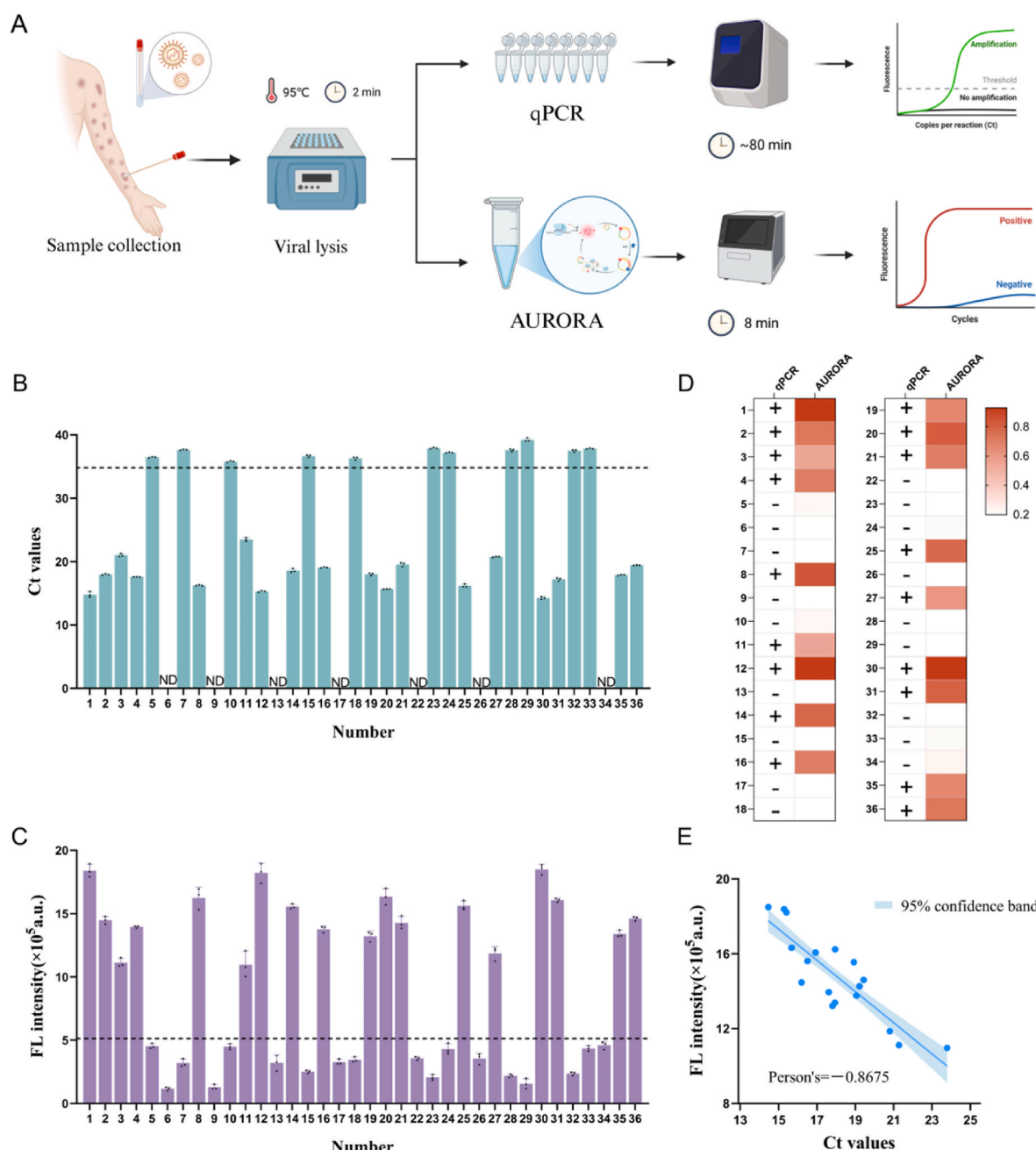


Fig. 5. Validation of the AURORA assay utilizing clinical MPXV specimens. (A) Diagram of the detection of MPXV in clinical samples using the AURORA system and qPCR. (B) Identification of MPXV in 36 clinical samples by qPCR. ND, not detected. (C) Identification of MPXV in 36 clinical samples by AURORA. (D) Heatmap showing comparison of MPXV assay results for 36 samples with qPCR and AURORA. The “+” and “-” respectively mean the qPCR positive and negative sample. The heatmap of AURORA represents normalized mean fluorescence values. (E) Correlation between qPCR and AURORA measurements of MPXV. Linear fits were performed on the data points to generate linear correlation curves. (n = 3 technical replicates, error bars represent mean \pm s.d.)

ultra-fast CRISPR-based diagnostic strategy called AURORA, enabling the detection of MPXV as low as 88 aM in less than 10 min. This reaction overcomes the issue of Cas12a competing with the amplification enzyme for substrates by constructing a self-amplifying system driven by cleavage-gated terminal exposure. Additionally, through a positive feedback self-amplification cycle, the detection efficiency is significantly improved, achieving efficient collaboration between two signal amplification systems in both spatial and temporal dimensions. Critically, AURORA has demonstrated 100 % sensitivity and specificity in clinical samples, offering excellent clinical feasibility and detection performance. With its ultra-fast and accurate detection, AURORA provides a more efficient tool for the prevention and control of severe infectious

diseases. We will keep working toward making AURORA-based molecular diagnostic sensors that combine sample preprocessing, detection, and result interpretation. This will allow for quick and immediate testing in places with few resources.

CRediT authorship contribution statement

Yiyue Jiang: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Jie Wu:** Visualization, Methodology, Investigation. **Xiayu Xiang:** Writing – original draft, Investigation. **Jiantao Wei:** Visualization, Investigation. **Kaihua Cheng:** Investigation. **Lunbiao**

Cui: Supervision, Resources. **Hongpan Xu:** Writing – review & editing, Supervision, Funding acquisition. **Zhiyang Li:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Availability of data and materials

The data sets generated or analyzed during this study are available from the corresponding author upon reasonable request.

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.2025.117857>.

Data availability

Data will be made available on request.

References

- Ahamed, M.A., Khalid, M.A.U., Dong, M., Politza, A.J., Zhang, Z., Kshirsagar, A., Liu, T., Guan, W., 2024. Sensitive and specific CRISPR-Cas12a assisted nanopore with RPA for Monkeypox detection. *Biosens. Bioelectron.* 246.
- Akhavan, O., Ghaderi, E., Hashemi, E., Rahighi, R., 2014. Ultra-sensitive detection of leukemia by graphene. *Nanoscale* 6 (24), 14810–14819.
- Akhavan, O., Ghaderi, E., Rahighi, R., 2012. Toward Single-DNA electrochemical biosensing by graphene nanowalls. *ACS Nano* 6 (4), 2904–2916.
- Chandrasekaran, S.S., Agrawal, S., Fantom, A., Jangid, A.R., Charrez, B., Escajeda, A.M., Son, S., McIntosh, R., Tran, H., Bhuiya, A., de León Derby, M.D., Switz, N.A., Armstrong, M., Harris, A.R., Prywes, N., Lukarska, M., Biering, S.B., Smock, D.C.J., Mok, A., Knott, G.J., Dang, Q., Van Dis, E., Dugan, E., Kim, S., Liu, T.Y., Hamilton, J. R., Lin-Shiao, E., Stahl, E.C., Tsuchida, C.A., Giannikopoulos, P., McElroy, M., McDevitt, S., Zur, A., Sylvain, I., Ciling, A., Zhu, M., Williams, C., Baldwin, A., Moehle, E.A., Kogut, K., Eskenazi, B., Harris, E., Stanley, S.A., Lareau, L.F., Tan, M. X., Fletcher, D.A., Doudna, J.A., Savage, D.F., Hsu, P.D., 2022. Rapid detection of SARS-CoV-2 RNA in saliva via Cas13. *Nat. Biomed. Eng.* 6 (8), 944–956.
- Chen, L., Hu, M., Zhou, X., 2025. Trends in developing one-pot CRISPR diagnostics strategies. *Trends Biotechnol.* 43 (1), 98–110.
- Colavita, F., Antinori, A., Nicastri, E., Focosi, D., Girardi, E., Vaia, F., Maggi, F., 2023. Monkeypox virus in human body sites and fluids: evidence for transmission. *Lancet Infect. Dis.* 23 (1), 6–8.
- Dronina, J., Bubniene, U.S., Ramanavicius, A., 2021a. The application of DNA polymerases and Cas9 as representative of DNA-modifying enzymes group in DNA sensor design. *Biosens. Bioelectron.* 175 (review).
- Dronina, J., Samukaite-Bubniene, U., Ramanavicius, A., 2021b. Advances and insights in the diagnosis of viral infections. *J. Nanobiotechnol.* 19 (1).
- Dronina, J., Samukaite-Bubniene, U., Ramanavicius, A., 2022. Towards application of CRISPR-Cas12a in the design of modern viral DNA detection tools. *J. Nanobiotechnol.* 20 (1) (Review).
- East-Seletsky, A., O'Connell, M.R., Knight, S.C., Burstein, D., Cate, J.H., Tjian, R., Doudna, J.A., 2016. Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection. *Nature* 538 (7624), 270–273.
- Erez, N., Achdout, H., Milrot, E., Schwartz, Y., Wiener-Well, Y., Paran, N., Politi, B., Tamir, H., Israeli, T., Weiss, S., Beth-Din, A., Shifman, O., Israeli, O., Yitzhaki, S., Shapira, S.C., Melamed, S., Schwartz, E., 2019. Diagnosis of imported Monkeypox, Israel, 2018. *Emerg. Infect. Dis.* 25 (5), 980–983.
- Hu, M., Liu, R., Qiu, Z., Cao, F., Tian, T., Lu, Y., Jiang, Y., Zhou, X., 2023. Light-start CRISPR-Cas12a reaction with caged crRNA enables rapid and sensitive nucleic acid detection. *Angew. Chem. Int. Ed.* 62 (23).
- Huang, D., Shen, P., Xu, C., Xu, Z., Cheng, D., Zhu, X., Fang, M., Wang, Z., Xu, Z., 2023. Dual nucleases-assisted cyclic amplification using polydopamine nanospheres-based biosensors for one-pot detection of microRNAs. *Biosens. Bioelectron.* 222.
- Huang, Y., Mu, L., Wang, W., 2022. Monkeypox: epidemiology, pathogenesis, treatment and prevention. *Signal Transduct. Targeted Ther.* 7 (1).
- Isidro, J., Borges, V., Pinto, M., Sobral, D., Santos, J.D., Nunes, A., Mixão, V., Ferreira, R., Santos, D., Duarte, S., Vieira, L., Borrego, M.J., Nuncio, S., de Carvalho, I.L., Pelerito, A., Cordeiro, R., Gomes, J.P., 2022. Phylogenomic characterization and signs of microevolution in the 2022 multi-country outbreak of monkeypox virus. *Nat. Med.* 28 (8), 1569–1572.
- Kang, Y., Wang, J., Zhang, W., Xu, Y., Xu, B., Qu, G., Yu, Y., Yan, B., Su, G., 2023. RNA extraction-free workflow integrated with a single-tube CRISPR-Cas-based colorimetric assay for rapid SARS-CoV-2 detection in different environmental matrices. *J. Hazard Mater.* 454.
- Li, S.Y., Cheng, Q.X., Liu, J.K., Nie, X.Q., Zhao, G.P., Wang, J., 2018. CRISPR-Cas12a has both cis- and trans-cleavage activities on single-stranded DNA. *Cell Res.* 28 (4), 491–493.
- Li, Y., Zhao, H., Wilkins, K., Hughes, C., Damon, I.K., 2010. Real-time PCR assays for the specific detection of monkeypox virus West African and Congo Basin strain DNA. *J. Virol. Methods* 169 (1), 223–227.
- Liu, P., Lin, Y., Zhuo, X., Zeng, J., Chen, B., Zou, Z., Liu, G., Xiong, E., Yang, R., 2024. Universal crRNA acylation strategy for robust photo-initiated one-pot CRISPR–Cas12a nucleic acid diagnostics. *Angew. Chem. Int. Ed.* 63 (23).
- Lu, S., Tong, X., Han, Y., Zhang, K., Zhang, Y., Chen, Q., Duan, J., Lei, X., Huang, M., Qiu, Y., Zhang, D.-Y., Zhou, X., Zhang, Y., Yin, H., 2022. Fast and sensitive detection of SARS-CoV-2 RNA using suboptimal protospacer adjacent motifs for Cas12a. *Nat. Biomed. Eng.* 6 (3), 286–297.
- Mitja, O., Ogoina, D., Titanji, B.K., Galvan, C., Muyembe, J.J., Marks, M., Orkin, C.M., 2023. Monkeypox. *Lancet* 401 (10370), 60–74.
- O'Toole, Á., Neher, R.A., Ndodo, N., Borges, V., Gannon, B., Gomes, J.P., Groves, N., King, D.J., Maloney, D., Lemey, P., Lewandowski, K., Loman, N., Myers, R., Omah, I. F., Suchard, M.A., Worobey, M., Chand, M., Ihekweazu, C., Ulaeto, D., Adetifa, I., Rambaut, A., 2023. APOBEC3 deaminase editing in mpox virus as evidence for sustained human transmission since at least 2016. *Science* 382 (6670), 595–600.
- Palich, R., Burrell, S., Monsel, G., Nouchi, A., Bleibtreu, A., Seang, S., Bérot, V., Brin, C., Gavaud, A., Wakim, Y., Godefroy, N., Fayçal, A., Tamzali, Y., Grunemwald, T., Ohayon, M., Todesco, E., Leducq, V., Marot, S., Calvez, V., Marcelin, A.-G., Pourcher, V., 2023. Viral loads in clinical samples of men with monkeypox virus infection: a French case series. *Lancet Infect. Dis.* 23 (1), 74–80.
- Rabiee, N., Akhavan, O., Fatahi, Y., Ghaderi, A.M., Kiani, M., Makvandi, P., Rabiee, M., Nicknam, M.H., Saeb, M.R., Varma, R.S., Ashrafzadeh, M., Zare, E.N., Sharifi, E., Lima, E.C., 2022. CaZnO-based nanoghosts for the detection of ssDNA, pCRISPR and recombinant SARS-CoV-2 spike antigen and targeted delivery of doxorubicin. *Chemosphere* 306.
- Rivers, C., Watson, C., Phelan, A.L., 2024. The resurgence of mpox in Africa. *JAMA* 332 (13), 1045–1046.
- Rosa, R.B., Ferreira de Castro, E., Vieira da Silva, M., Paiva Ferreira, D.C., Jardim, A.C.G., Santos, I.A., Marinho, M.d.S., Ferreira França, F.B., Pena, L.J., 2023. In vitro and in vivo models for monkeypox. *iScience* 26 (1).
- Shang, Y., Xing, G., Lin, J., Li, Y., Lin, Y., Chen, S., Lin, J.-M., 2024. Multiplex bacteria detection using one-pot CRISPR/Cas13a-based droplet microfluidics. *Biosens. Bioelectron.* 243.
- Sun, Y., Yu, L., Liu, C., Ye, S., Chen, W., Li, D., Huang, W., 2021. One-tube SARS-CoV-2 detection platform based on RT-RPA and CRISPR/Cas12a. *J. Transl. Med.* 19 (1).
- Thornhill, J.P., Barkati, S., Walmsley, S., Rockstroh, J., Antinori, A., Harrison, L.B., Palich, R., Nori, A., Reeves, I., Habibi, M.S., Apea, V., Boesecke, C., Vandekerckhove, L., Yakubovskiy, M., Sendagorta, E., Blanco, J.L., Florence, E., Moschese, D., Maltez, F.M., Goorhuis, A., Pourcher, V., Migaud, P., Noe, S., Pintado, C., Maggi, F., Hansen, A.-B.E., Hoffmann, C., Lezama, J.I., Mussini, C., Cattelan, A., Makofane, K., Tan, D., Nozza, S., Nemeth, J., Klein, M.B., Orkin, C.M., 2022. Monkeypox virus infection in humans across 16 countries — April–June 2022. *N. Engl. J. Med.* 387 (8), 679–691.
- Titanji, B.K., Hazra, A., Zucker, J., 2024. Mpox clinical presentation, diagnostic approaches, and treatment strategies. *JAMA* 332 (19).
- Wang, X., Deng, X., Zhang, Y., Dong, W., Rao, Q., Huang, Q., Tang, F., Shen, R., Xu, H., Jin, Z., Tang, Y., Du, D., 2024. A rapid and sensitive one-pot platform integrating fluorogenic RNA aptamers and CRISPR-Cas13a for visual detection of monkeypox virus. *Biosens. Bioelectron.* 257.
- Wang, X., Rao, Q., Lu, Z., Deng, X., Shen, R., Wang, R., Dong, W., Qi, X., Jin, Z., Tang, Y., Du, D., 2023. Rapid and sensitive Cas13a/Cas12a-based one-pot dual-target strategy to detect monkeypox virus and its co-infected viruses. *Sci. Bull.* 68 (24), 3142–3148.
- Wang, Y., Tang, Y., Chen, Y., Yu, G., Zhang, X., Yang, L., Zhao, C., Wang, P., Gao, S., Kibenge, F.S.B., Deng, R., Chen, W., Yang, S., 2024. Ultrasensitive one-pot detection of monkeypox virus with RPA and CRISPR in a sucrose-aided multiphase aqueous system. *Microbiol. Spectr.* 12 (1).
- World Health Organization, 2024. Mpox. <https://www.who.int/zh/news-room/fact-sheets/detail/mpox>.
- Yang, L., Chen, G., Wu, J., Wei, W., Peng, C., Ding, L., Chen, X., Xu, X., Wang, X., Xu, J., 2024. A PAM-free one-step asymmetric RPA and CRISPR/Cas12b combined assay (OAR-CRISPR) for rapid and ultrasensitive DNA detection. *Anal. Chem.* 96 (14), 5471–5477.

- Zeng, W., Chen, W., Liu, Y., Zhang, T., Zhai, C., Li, W., Wang, L., Zhang, C., Zeng, Q., Wang, F., Ma, L., 2024. Preamplification-free ultra-fast and ultra-sensitive point-of-care testing via LwaCas13a. *Biosens. Bioelectron.* 259.
- Zhang, Y., Su, R., Zhang, Z., Jiang, Y., Miao, Y., Zhou, S., Ji, M., Hsu, C.-W., Xu, H., Li, Z., Wang, G., 2025. An ultrasensitive one-pot Cas13a-based microfluidic assay for rapid multiplexed detection of microRNAs. *Biosens. Bioelectron.* 274.

- Zhu, Z., Guo, Y., Wang, C., Yang, Z., Li, R., Zeng, Z., Li, H., Zhang, D., Yang, L., 2023. An ultra-sensitive one-pot RNA-templated DNA ligation rolling circle amplification-assisted CRISPR/Cas12a detector assay for rapid detection of SARS-CoV-2. *Biosens. Bioelectron.* 228.