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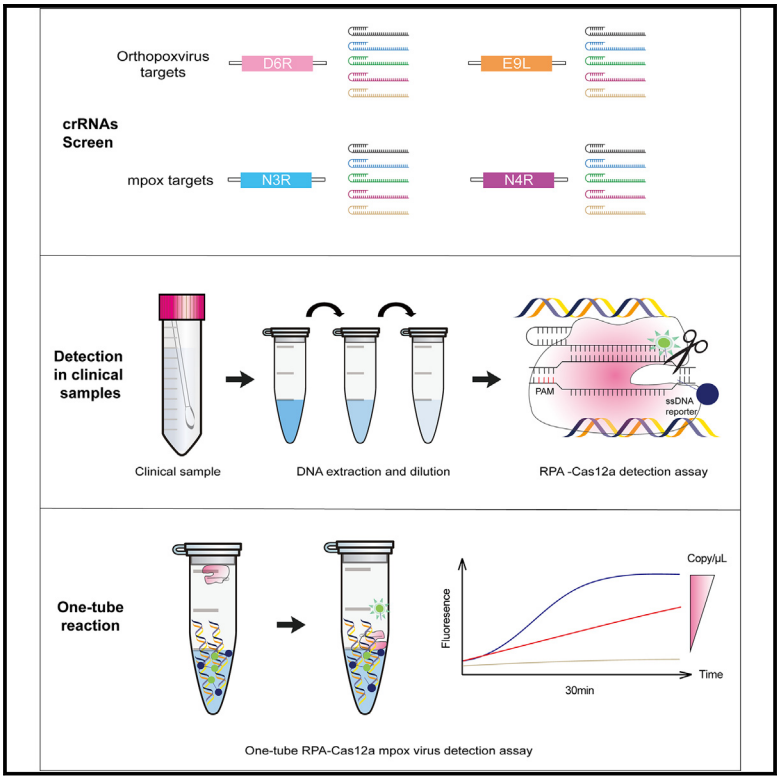
# Rapid and sensitive one-tube detection of mpox virus using RPA-coupled CRISPR-Cas12 assay

## Graphical abstract




## Highlights

- An RPA-Cas12a system is designed for detection of orthopox and mpox viruses
- The RPA-Cas12a detection system is highly sensitive, with an LOD of 1 copy DNA
- The one-tube RPA-Cas12a system is able to detect mpox virus within 30 min

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## In brief

Zhao et al. present an RPA-coupled CRISPR-Cas12a detection assay for the efficient and simultaneous detection of mpox virus and orthopoxvirus infections. The work also generates a rapid and sensitive assay for the detection of mpox in a tube and holds the promise of providing point-of-care diagnosis for the viral infection.

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# Rapid and sensitive one-tube detection of mpox virus using RPA-coupled CRISPR-Cas12 assay

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**MOTIVATION** The rise of mpox cases in many previously non-endemic countries in 2022 prompted the WHO to declare mpox as a public health emergency of international concern. Since it is difficult to detect mpox at the early stage of infection, a rapid and accurate diagnostic assay for the mpox virus is essential to control its spread and enable timely treatment. Here, we present an RPA-Cas12a ribonucleoprotein-based method for rapid and sensitive detection of mpox.

## SUMMARY

Mpox is caused by a zoonotic virus belonging to the *Orthopoxvirus* genus and the *Poxviridae* family. In this study, we develop a recombinase polymerase amplification (RPA)-coupled CRISPR-Cas12a detection assay for the mpox virus. We design and test a series of CRISPR-derived RNAs(crRNAs) targeting the conserved D6R and E9L genes for orthopoxvirus and the unique N3R and N4R genes for mpox viruses. D6R crRNA-1 exhibits the most robust activity in detecting orthopoxviruses, and N4R crRNA-2 is able to distinguish the mpox virus from other orthopoxviruses. The Cas12a/crRNA assay alone presents a detection limit of $10^8$ copies of viral DNA, whereas coupling RPA increases the detection limit to 1–10 copies. The one-tube RPA-Cas12a assay can, therefore, detect viral DNA as low as 1 copy within 30 min and holds the promise of providing point-of-care detection for mpox viral infection.

## INTRODUCTION

Mpox is a viral zoonotic disease caused by viruses belonging to the *Orthopoxvirus* genus, the *Poxviridae* family.[1] Mpox viruses are divided into two different genetic clades, the Central Africa clade and the West Africa clade, of which the West African clade is less virulent but more widely spread.[2] Mpox was originated in rodents such as squirrels, rats, and mice. Its name came from the finding of a smallpox-like disease in monkeys in 1958.[3] Mpox virus was transmitted into humans as a result of close contact with infected animals or contaminated inanimate objects.[4] Since the first human cases of mpox were reported in Zaire (Democratic Republic of Congo) in 1970, human mpox cases have been rising over the past five decades, especially in Congo and Nigeria.[5] Outside Africa, the first cases of human mpox emerged in 2003 in the US.[6] From 2018 to 2021, the UK, the US, Singapore, and Israel reported outbreaks of mpox associated with trips to Nigeria and animal-to-human transmission.[7] Since May 2022, many non-endemic regions have been reporting mpox outbreaks, which led to the declaration by the World Health Organization (WHO) of mpox as a public health emergency of international concern on July 23, 2022.[8] With the continuation of COVID-19, mpox poses a new and unique challenge for global health. There have been over 87,000 mpox cases (including 140 deaths) from 111 member states across all 6 WHO regions to date,[9] including one imported mpox case that was reported in mainland China.[10] Rapid and accurate diagnosis will be essential for the control and timely treatment of mpox, a valuable lesson learned from effective COVID-19 mitigation strategies.

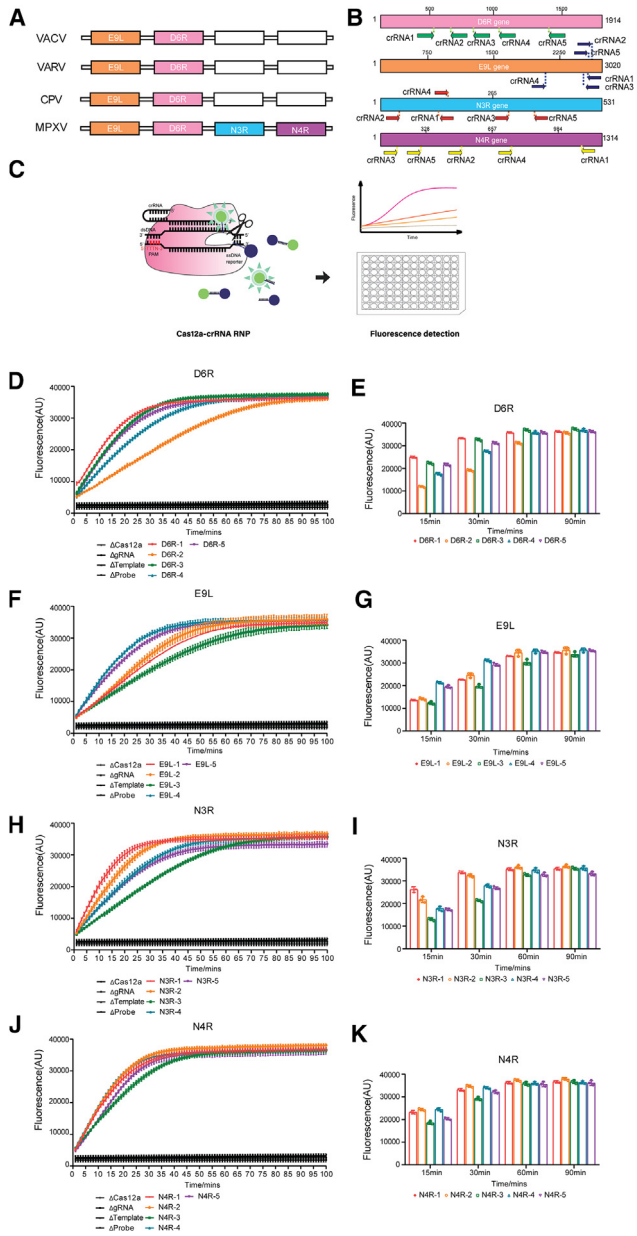


**Figure 1. Screening crRNAs for detection of orthopoxviruses and mpox viruses together with Cas12a**

(A) Illustration of D6R and E9L genes that are conserved among four orthopoxviruses and genes N3R and N4R that are unique for mpox viruses. VACV, vaccinia virus; VARV, variola virus; CPV, cowpox virus; MPXV, mpox virus.

(B) Depiction of the target sites of the designed crRNAs. All target genes are shown in the 5′ to 3′ direction.

(C) Illustration of Cas12a-crRNA ribonucleoprotein (RNP) complex binding target DNA, resulting a cleavage of target DNA with its RuvC domain. Upon Cas12a activation, quenched-fluorophore DNA reporters were cleaved, and fluorescence signals were detected.

(D, F, H, and J) Each crRNA was tested individually together with Cas12a against $10^{10}$ copies/reaction (final concentration: 0.05 nM) of each target plasmid DNA. Fluorescence signals were recorded every minute for 100 min. Background fluorescence in the absence of Cas12a, gRNA, template, or probe DNA is shown as ΔCas12a, ΔgRNA, Δtemplate, and Δprobe, respectively.

Much effort has been made to develop new diagnostics of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by leveraging novel technologies,[11] including the CRISPR-Cas-based SARS-CoV-2 diagnosis, which has the advantage of simplicity, convenient use, and high specificity.[12] The CRISPR-Cas system has been used for rapid detection of both DNA and RNA sequences.[13] To date, the four Cas enzymes Cas9, Cas12, Cas13, and Cas14, which are commonly used in nucleic acid detection, all belong to the class II system and are capable of recognizing and cleaving single-stranded (ss)DNA, double-stranded (ds)DNA, or ssRNA.[14] Cas12, Cas13, and Cas14 exhibit *trans*-cleavage activity, an ability to cut nucleic acids in a non-selective and collateral manner.[15] Cas12, together with its CRISPR-derived RNA (crRNA), recognizes ssDNA or dsDNA containing a PAM sequence and cleaves the DNA with its RuvC domain.[16] Cas12 executes robust and non-specific ssDNA cleavage while cleaving dsDNA in a sequence-specific manner, a property that has been widely used in many assays since its discovery.[17]

In the absence of nucleic acid amplification, the detection sensitivity of CRISPR-Cas varies between 0.01 and 1 nM and thus is not suitable for clinical diagnosis of pathogens.[18] PCR is routinely used for nucleic acid amplification but is often associated with relatively high costs, long durations, and the need for specialized equipment.[19] Therefore, isothermal amplification methods have been adopted in combination with CRISPR-Cas for diagnostic purposes such as the most frequently used recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP) reactions, which have been successfully integrated with CRISPR-Cas, leading to the development of a variety of CRISPR-Cas-based diagnosis platforms including SHERLOCK, DETECTR, HOLMES, and AIOD-CRISPR.[20,21]

The RPA method combines isothermal recombinase-driven primer targeting of template with strand-displacement DNA synthesis, which can occur at room temperature with minimal sample pretreatment.[22,23] Thus far, RPA-based diagnostics have been developed for the detection of different pathogens, including viruses,[24] fungi,[25] bacteria,[26] and parasites.[27] In this study, we combined RPA and CRISPR-Cas12a and developed a rapid and ultrasensitive diagnosis system for mpox virus detection. This system has optimized cRNAs that detect the viral gene that is conserved for all orthopoxviruses and the viral gene that is specific for mpox viruses. The entire reaction can be completed within 30 min at 37°C in one tube with a sensitivity of viral DNA detection under 10 copies. This platform thus has the potential for fast, accurate, and point-of-care diagnosis of mpox at low cost.

## RESULTS

### Design and test CRISPR-Cas12a crRNAs targeting mpox viruses

We designed two pools of crRNAs. One pool of crRNAs targets viral genes D6R and E9L that are conserved among

Data are presented as mean ± standard deviation (SD) of three technical replicates.

(E, G, I, and K) Shown are fluorescence signals at 15, 30, 60, and 90 min corresponding to (D), (F), (H), and (J).

See also Figures S1 and S2 and Table S1.

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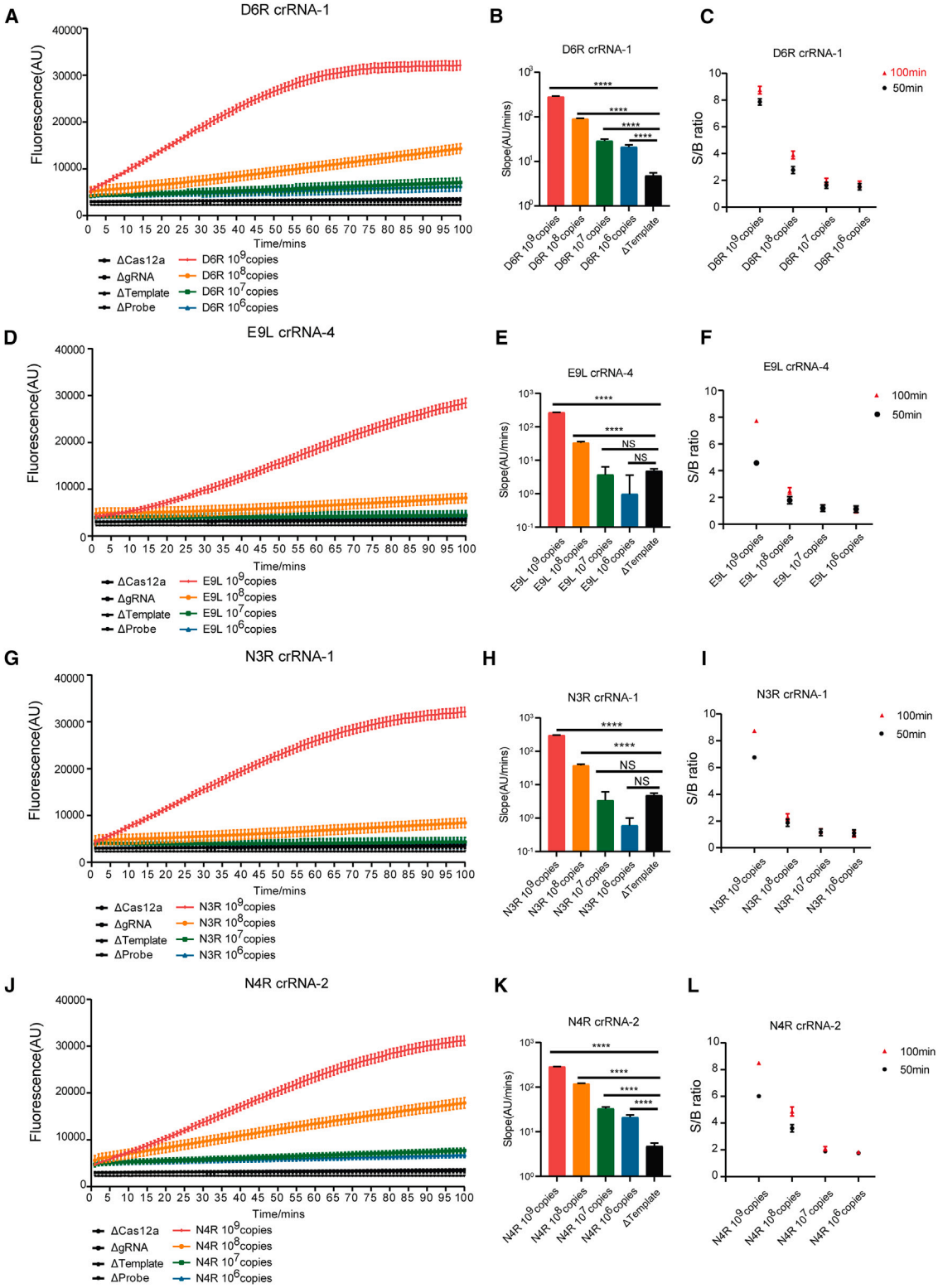



**Figure 2. The detection limit of Cas12a/crRNA for target plasmid DNA**

(A, D, G, and J) detection limits of crRNAs D6R-1, E9L-4, N3R-1, and N4R-2 were determined by testing 10-fold dilution of each target plasmid DNA ranging from $10^9$ to $10^6$ copies. Fluorescence signals over 100 min were recorded. Data are presented as mean ± SD of three technical replicates.

*(legend continued on next page)*

orthopoxviruses including mpox and thus can be used to detect all orthopoxviruses. The second pool of crRNAs target regions in the N3R and N4R genes that are nearly 100% conserved among multiple species of mpox viruses but exhibit low homology in other orthopoxviruses (Figures 1A and S1A–S1D).[28] The D6R and E9L genes were amplified from vaccinia virus and cloned into plasmid vectors. The N3R and N4R genes were synthesized. Five crRNAs were designed to target each viral gene (Figures 1B and S2A; Table S1). All crRNAs were blasted, and the sequence identities were calculated. crRNAs targeting N3R and N4R were highly conserved among mpox genomes, while some crRNAs targeting D6R and E9L showed one or two mismatches among orthopoxvirus genomes (Figure S1).

The detection assay takes advantage of the collateral cleavage of a quenched fluorescent DNA probe when AsCas12a/crRNA specifically recognizes its DNA target and is activated for DNA cleavage (Figure 1C).[29] We established this assay in a 96-well format and measured fluorescence signals for a duration of 100 min. At a 0.05 nM (1010 copies/reaction) final concentration of the target plasmid DNA, all five crRNAs targeting each viral gene showed reactivity profoundly higher than the negative control that contained Cas12a, crRNA, and reporter DNA, but not viral DNA target, with some crRNAs exhibiting stronger activity than others (Figures 1D–1K). The crRNAs D6R-1, E9L-4, N3R-1, and N4R-2 are selected for developing the diagnosis assay based on their strong and fast activation of the fluorescence signals and high conservation among orthopoxviruses or mpoxes.

### Cas12a/crRNA detects mpox viral DNA at 0.0005 nM

The minimal DNA concentrations for detectable Cas12a cleavage without amplification vary from 100 fM to 50 pM.[30] In order to determine the limit of detection (LOD) of Cas12a on mpox virus DNA, we performed Cas12a/crRNA cleavage assay with serially diluted target plasmid DNA. All four crRNAs, D6R-1, E9L-4, N3R-1, and N4R-2, generated significantly higher fluorescence signals than control at $10^9$ and $10^8$ copies of target DNA by 100 min of reactions (Figures 2A, 2D, 2G, and 2J). Since the linear rate of the fluorescence signals generated by the enzymatic activity of Cas12a should approximate Michaelis-Menten enzyme kinetics,[31] we also calculated the slopes by linear regression over 100 min within each group. Significantly higher than control signals were observed at $10^6$ and $10^7$ copies of target DNA for D6R-1 and N4R-2 crRNAs but not for E9L-4 and N3R-1 (Figures 2B, 2E, 2H, and 2K). To further confirm the LOD of these Cas12a/crRNAs, we calculated the signal-to-background ratio (S/B ratio) at 50 and 100 min (Figures 2C, 2F, 2I and 2L). We found that at $10^6$ and $10^7$ copies of target DNA in each group, the S/B ratios were lower than 2 and thus cannot be convincingly set as the LOD. Based on the S/B ratios at 100 min, we determine that the LOD of target DNA with D6R-1, E9L-4, N3R-1, and N4R-2 crRNAs is $10^8$ copies (final concentration: 0.0005 nM).

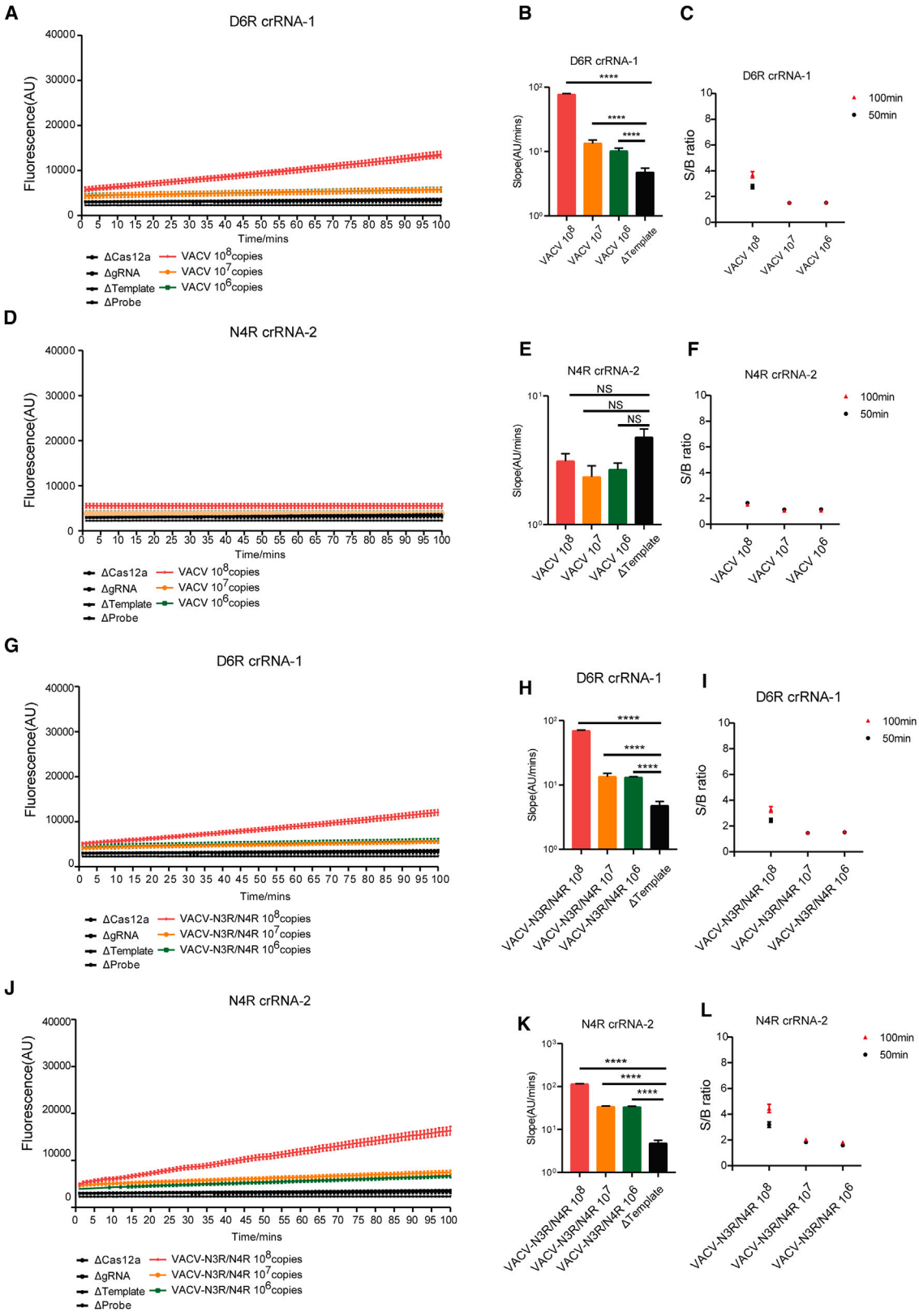
Orthopoxviruses are large, enveloped viruses containing linear, dsDNA genomes that can vary from 130 to 375 kb.[32] To evaluate the efficiency of the Cas12a/crRNA system in detecting these large DNA viruses, we used vaccinia virus (VACV) and a recombinant VACV (VACV-N3R/N4R), which contains mpox virus genes N3R and N4R as models (Figure S2B). The D6R-1 and N4R-2 crRNAs were used in detecting VACVs because of their high detection sensitivity as shown in Figure 2. Given the difficulty of preparing vaccinia viral DNA at a concentration of $10^9$ copies, we measured the LOD of Cas12a using vaccinia viral DNA at concentrations of $10^8$, $10^7$, and $10^6$ copies. When VACV DNA was tested as the target, the D6R-1 crRNA detected the viral genome at no lower than $10^8$ copies, while the N4R-2 crRNA did not detect VACV DNA since VACV does not have the target sequence of N4R-2 crRNA (Figures 3A–3F). Using VACV-N3R/N4R DNA as a template, both D6R-1 and N4R-2 crRNAs detected the VACV-N3R/N4R DNA with a detection limit of $10^8$ copies (Figures 3G–3L). These results demonstrate that the Cas12a/crRNA system is able to detect orthopoxviruses at a detection limit of $10^8$ copies (final concentration: 0.0005 nM) and that the D6R-1 and N4R-2 crRNAs together can distinguish mpox virus from other orthopoxviruses.

It has been reported that combination of crRNAs increases the sensitivity of Cas13a detection of SARS-CoV-2 RNA.[33] Similarly, multiple crRNAs render CRISPR-Cas12a to detect cytochrome *b* gene of meat adulteration at lower DNA copies.[34] We thus examined whether crRNA combination also enhances Cas12a detection of orthopoxvirus DNA. With plasmid DNA at $10^8$ copies, the fluorescence signals, the slopes of the detection, and the S/B ratios increased as a function of the number of crRNAs used in the DNA cleavage reactions, for either the D6R gene target or the N4R gene target (Figures S3A–S3C and S3G–S3I). However, even combinations of up to four crRNAs did not result in significantly higher than control DNA cleavage signals at the $10^7$ copies of target DNA (Figures S3D–S3F and S3J–S3L). The same observation was made with the VACV-N3R/N4R viral genome at $10^8$ copies when multiple crRNAs were added in the reactions (Figures S4A–S4C and S4G–S4I). It was encouraging to detect low, but significantly higher than control, fluorescence signals at $10^7$ copies of VACV-N3R/NR DNA (Figures S4D–S4F and S4J–S4L). We further investigated whether combination of crRNAs targeting different genes can also enhance Cas12a detection sensitivity. Indeed, combination of D6R-1 crRNA-1 and N4R-2 crRNA-2 increased the detection sensitivity of $10^8$ copies VACV-N3R/N4R viral DNA to a similar degree as the combination of N4R crRNA-2 and crRNA-4 (Figures S4M–S4O). Therefore, combination of multiple crRNAs markedly increases the sensitivity of the CRISPR-Cas12a system to detect orthopoxviruses.

(B, E, H, and K) Slopes of the curves over 100 min shown in (A), (D), (G), and (J) were calculated by performing simple linear regression to data merged from replicates and are shown as slope ±95% confidence interval. Slopes were compared to the Δtemplate alone control through an analysis of covariance (ANCOVA). NS, not significant; ****$p < 0.0001$.

(C, F, I, and L) Signal-to-background ratios (S/B ratios) at 50 (black) and 100 min (red) reaction times were calculated and are shown. Background fluorescence is the Δtemplate group. Data are presented as mean ± SD of three technical replicates.

See also Figure S3.

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### RPA increases the detection limit of Cas12a to under 10 copies of orthopoxvirus DNA

The detection limit of orthopoxvirus DNA by CRISPR-Cas12a is $10^8$ copies, which is not suitable for clinical diagnosis. To maximally increase the detection sensitivity by Cas12a, we designed primers that can be used to amplify the vaccinia viral DNA sequences containing the target of D6R-1 or N4R-2 crRNA. Sequence alignment analysis showed that these primers are highly conserved in orthopoxviruses (Figure S5). We tested viral DNA at concentrations of $10^2$ copies, 10 copies, and 1 copy. Viral DNA was first amplified with RPA using the designed primers at 37°C for 30 min, followed by incubation with the Cas12a/crRNA detection system for 100 min. Results showed that the VACV viral DNA was efficiently detected with the D6R crRNA-1 even at 1 copy (Figures 4A–4D). As expected, N4R crRNA-2 did not detect the VACV viral DNA at any concentration tested. The VACV-N3R/N4R viral DNA was detected with both D6R crRNA-1 and N4R crRNA-2 at 1 copy (Figures 4E–4H). Moreover, mpox virus (MPXV) was amplified, and viral DNA were extracted from MPXV-B.1-China-C-Tan-CQ01, which was isolated from the only mpox case imported from a European country to China on September 14, 2022.[35] MPXV DNA was diluted to $10^2$ copies, 10 copies, and 1 copy and determined by droplet digital PCR (Figure S7C). Both D6R crRNA-1 and N4R crRNA-2 are able to detect authentic MPXV DNA at 1 to 10 copies (Figures 5A–5D). These results demonstrate the extremely high sensitivity of the RPA-coupled Cas12a system in detecting orthopoxvirus at concentrations as low as 1–10 copies and its robust specificity in distinguishing MPXV from other orthopoxviruses.

To evaluate the potential utility of the RPA-Cas12a system in detecting orthopoxvirus in clinical samples, we first applied this detection system to test the total DNA of Vero cells that were infected with VACV or VACV-N3R/N4R at MOIs of 0.001, 0.0001, and 0.00001. The uninfected Vero cells served as mock controls. The number of viral DNA in the extracted total Vero cellular DNA was first determined by droplet digital PCR (ddPCR) (Figures S7A and S7B). The results showed that RPA-Cas12a system, when supplied with D6R crRNA-1, was able to detect both VACV and VACV-N3R/N4R from the infected cells (Figures S6A, S6B, S6E, and S6F), and N4R crRNA-2 allowed detection of VACV-N3R/N4R, but not VACV, in the infected cell DNA samples (Figures S6C, S6D, S6G, and S6H). The RPA-Cas12a system did not generate any positive fluorescence signals with samples of the uninfected Vero cells. We further evaluate the specimens of blister fluid swab, oropharyngeal swab, and nasopharyngeal swab from the only mpox case.[35] The results showed that both D6R crRNA-1 and N4R crRNA-2 were able to detect MPXV DNA in these clinical samples (Figures 5E–5H). To demonstrate the specificity for mpox, we next tested the designed crRNAs against negative controls including herpes simplex virus type 1/2, varicella-zoster virus, *Candida albicans*, *Staphylococcus epidermidis*, and *P. aeruginosa.* None of these pathogens generated positive signals, confirming that these crRNAs are highly specific and suitable for clinical sample detection (Figure S7D). Therefore, the RPA-Cas12a system is able to detect as low as 10 copies of orthopoxvirus DNA from infected cells (MOI: 0.00001) and clinical samples, which further supports their utility as a clinical diagnostic tool.

### The one-tube RPA-Cas12a system detects orthopoxvirus DNA at 10 copies within 30 min

Finally, we tested the integration of the amplification and detection steps into a one-tube, two-step system to simplify and shorten the entire procedure and minimize the risk of carry-over contamination or aerosol pollution.[36] We adopted the strategy of adding the amplification reagents to the bottom of the tube and Cas12a/crRNA reagents to the cap or wall of the tube as reported in other studies (Figure 6A).[37] RPA proceeded for 15 min, and then the Cas12a/crRNA reagents were spun down to start the detection reaction for 20 min. For both viral DNA and clinical swabs, fluorescence signals quickly rose and reached plateau between 15 and 20 min. VACV was readily detected using D6R crRNA-1, and VACV-N3R/N4R was detected with both D6R crRNA-1 and N4R crRNA-2 (Figures 6B–6E). MPXV and specimens' DNA were detected with both D6R crRNA-1 and N4R crRNA-2 (Figures 6F–6I). Therefore, this one-tube RPA-Cas12a system is able to detect orthopoxviruses and distinguish MPXVs from other orthopoxviruses within 30 min at 37°C with a detection limit of 10 copies viral DNA, thus holding great potential of being developed into a highly sensitive diagnostic tool for MPXV infection.

## DISCUSSION

There are four major species of orthopoxviruses that are pathogenic to humans, including variola virus, vaccinia virus, cowpox virus, and MPXV. Mpox has similar but much milder clinical manifestation compared with smallpox and has quickly spread from its original endemic regions in Africa to Europe and the US in recent years, thus now posing a greater threat to global health.[38] Molecule surveillance represents an effective tool to monitor the transmission and evolution of MPXVs and has primarily utilized the PCR method since 2006.[39] PCR is suitable for laboratory-based analysis but is not practical for mpox testing at home or in resource-limited settings. To meet this need, we have developed a one-tube RPA-Cas12a system that

**Figure 3. Detection limit of Cas12a/crRNA for viral genomic DNA**

(A, D, G, and J) Detection limits of crRNAs D6R-1 and N4R-2 were determined by testing 10-fold dilution of each target viral genome ranging from $10^8$ to $10^6$ copies. Fluorescence signals over 100 min were recorded. Results are presented as mean ± SD of three technical replicates. VACV, vaccinia virus; VACV-N3R/N4R, recombinant VACV containing mpox virus N3R and N4R genes.

(B, E, H, and K) Slopes of the curves over 100 min shown in (A), (D), (G), and (J) were calculated by performing simple linear regression to data merged from replicates and are shown as slope ±95% confidence interval. Slopes were compared to the Δtemplate alone control through an ANCOVA. NS, not significant; ****$p < 0.0001$.

(C, F, I, and L) S/B ratios at 50 (black) and 100 min (red) reaction times were calculated and are shown. Background fluorescence is the Δtemplate group. Data are presented as mean ± SD of three technical replicates.

See also Figure S4.

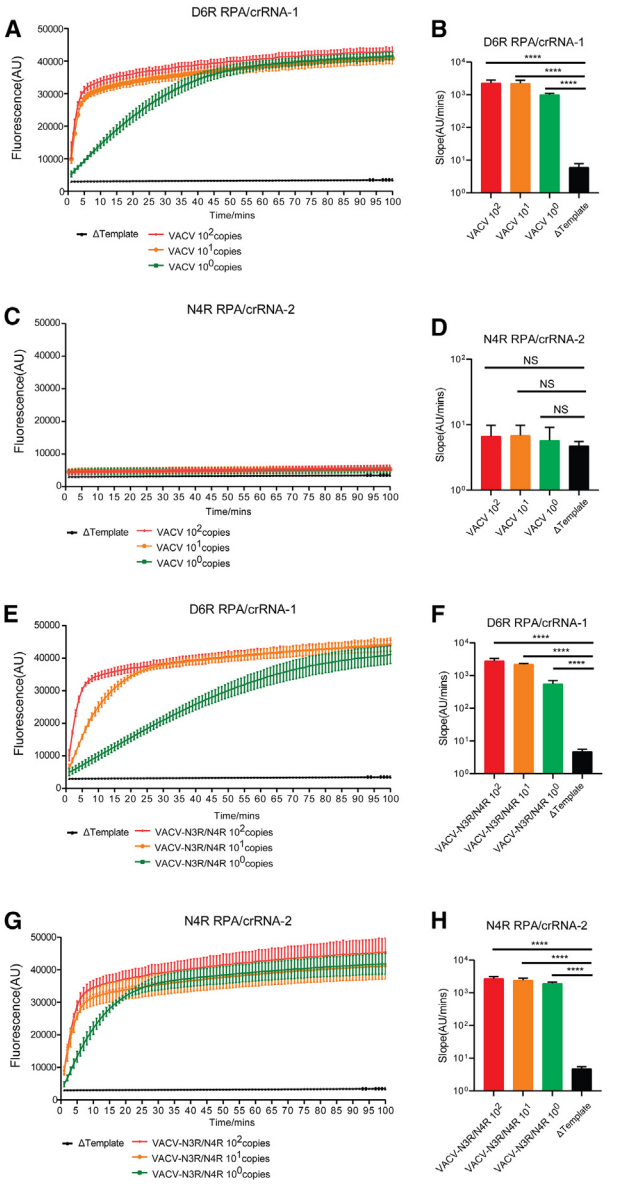


**Figure 4. Detection of viral genomic DNA with RPA-coupled Cas12a/crRNA**

(A, C, E, and G) VACV and VACV-N3R/N4R viral genomes were diluted to $10^2$ copies, $10^1$ copies, and 1 copy. Viral DNA samples were first amplified with RPA using both D6R primers and N4R primers and then added into the Cas12a/crRNA detection system. Fluorescence signals over 100 min were recorded. Background fluorescence is shown as Δtemplate. Data are presented as mean ± SD of three technical replicates.

(B, D, F, and H) Slopes of the curves over 20 min shown in (A), (C), (E), and (G) were calculated by performing simple linear regression to data merged from replicates and are shown as slope ±95% confidence interval. Slopes were compared to the Δtemplate alone control through an ANCOVA. NS, not significant; ****$p < 0.0001$. See also Figure S5.

can detect mpox infection within 30 min at 37°C and can distinguish MPXV from other orthopoxviruses.

The specificity of our RPA-Cas12 detection system is warranted by the carefully designed and vigorously tested crRNAs. One set of crRNAs recognizes the D6R and E9L genes that are conserved for all known orthopoxviruses, while the other set targets the N3R and N4R gene sequences that are unique for MPXVs. A combination of the selected D6R crRNA-1 and N4R crRNA-2 allows Cas12a to specifically detect MPXVs. This strategy can be applied to detect specific subtypes of MPXVs, including any new MPXV lineages to emerge, through properly designed and validated crRNAs that target the differential DNA sequences between MPXV subtypes.

The ultrasensitivity of RPA-Cas12a relies on the RPA step of the assay. Without RPA, the detection limit of Cas12a is $10^8$ copies viral DNA. Inclusion of RPA increases the detection sensitivity by several magnitudes to the range of 1–10 copies viral DNA. Both LAMP and RPA methods have been used to complement the CRISPR-Cas system in nucleic acid detection. One advantage of the LAMP method is its high sensitivity, as a set of four primer pairs are used to recognize multiple target sequences simultaneously.[40,41] However, LAMP requires 65°C to function. RPA can efficiently operate between 37°C and 42°C, and even at room temperature, which renders the use of RPA in field applications.[42,43] While RPA is suboptimal if the ambient temperature is below 10°C, this can be compensated for by longer reaction times.[44] It is thus optimistic that the RPA-Cas12a detection system can be deployed in at-home and field testing of MPXV infections.

The one-tube RPA-Cas12a system proceeds with two sequential reactions, the RPA and Cas12a cleavage. Different strategies have been reported to ensure this sequence of events. We have added amplification reagents to the bottom and Cas12a reagents to the cap of the tube so that these two reaction mixtures are temporarily separated. Different concentrations of sucrose solutions have been used to physically separate the RPA reaction system and the Cas12a reaction system in one tube.[36] A Cas12a-PB sensor composed of a chamber was also tested.[45] Finally, the one-tube SHERLOCK system has the RPA reagents directly mixed with the CRISPR reagents.[46] Testing these different methods will help optimize our one-tube RPA-Cas12a system for field testing of MPXV infection.

In the current RPA-Cas12a detection system, the readout is a fluorescence signal, which requires the use of a fluorescence plate reader. For home and field testing, visual readout is an asset of any successful diagnostic system. Different readout methods have been tested and reported with the CRISPR-Cas system, including visual colorimetric readout[29] and lateral flow-based readout.[47,48] In addition, point-of-care or field-deployable MPXV diagnostics will require rapid and easy sample preparation. A few methods of sample preparations have been reported for point-of care or field-deployable diagnostics, including one-step sample extraction with TNA-Cifer Reagent,[49] using dipstick extraction technology,[50] and disposable pen-like sensor systems,[51] which can be tested along with our mpox detection assay. Our RPA-Cas12a system can be optimized by incorporating the visual readout component and one-step sample preparations with the aim for field and home use.

In summary, we have successfully developed a one-tube RPA-Cas12a system that can detect and distinguish MPXV from other orthopoxviruses within 30 min at a minimum concentration of 1–10 copies viral DNA. Its ultrasensitivity, robust specificity, and short completion time support further development of this

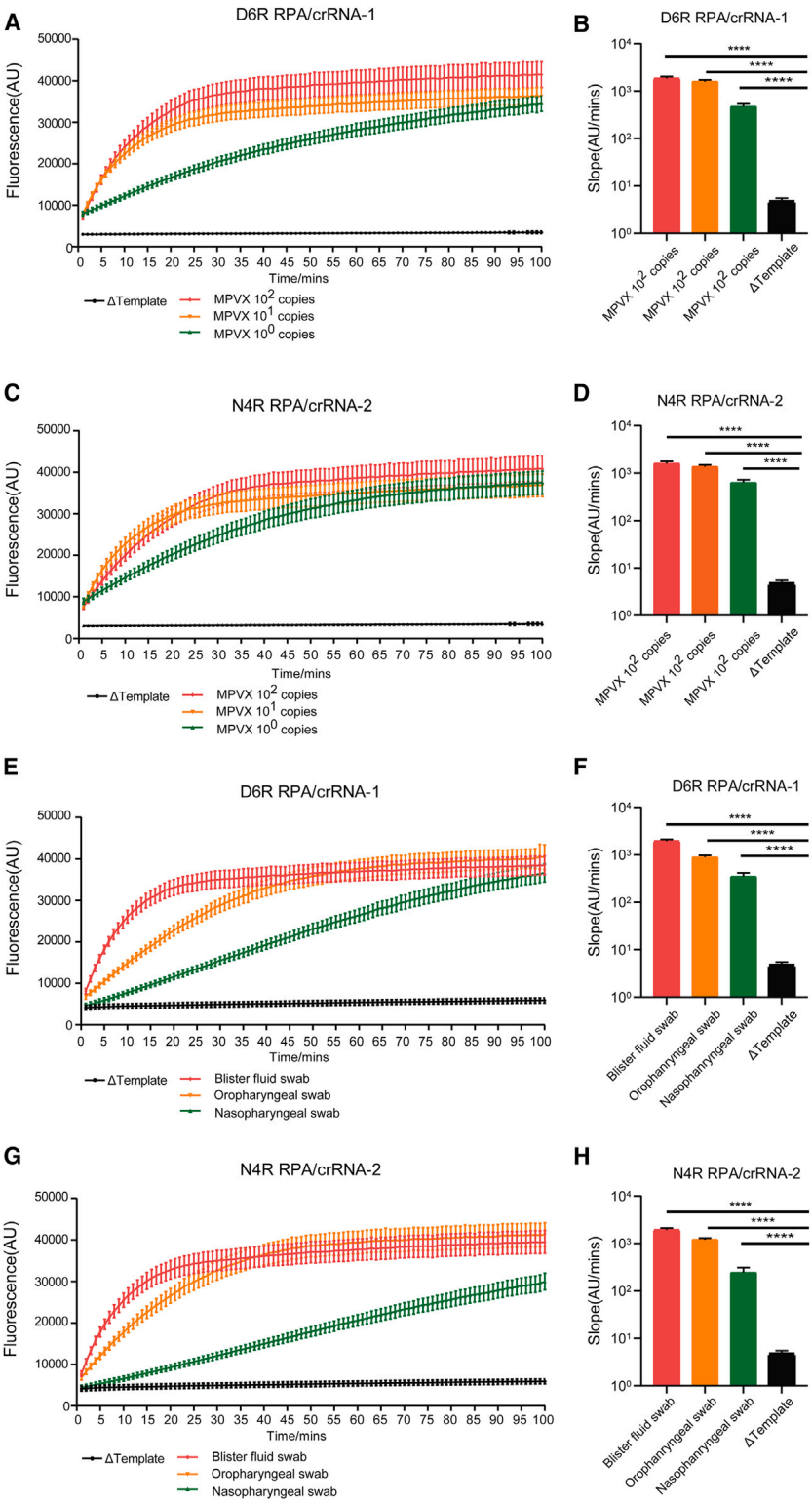


**Figure 5. Detection of authentic MPXV and clinical samples with RPA-Cas12a**

(A, C, E, and G) Viral DNA of MPXV and clinical samples was detected with the RPA-Cas12a system as shown in Figure 4. Fluorescence signals over 100 min are shown. Background fluorescence is shown as Δtemplate. Data are represented as mean ± SD of three technical replicates.

(B, D, F, and H) Slopes of the curves over 20 min shown in (A), (C), (E), and (G) were calculated by performing simple linear regression to data merged from replicates and are shown as slope ±95% confidence interval. Slopes were compared to the Δtemplate alone control through an ANCOVA. NS, not significant; ****$p < 0.0001$.

See also Figures S6 and S7.

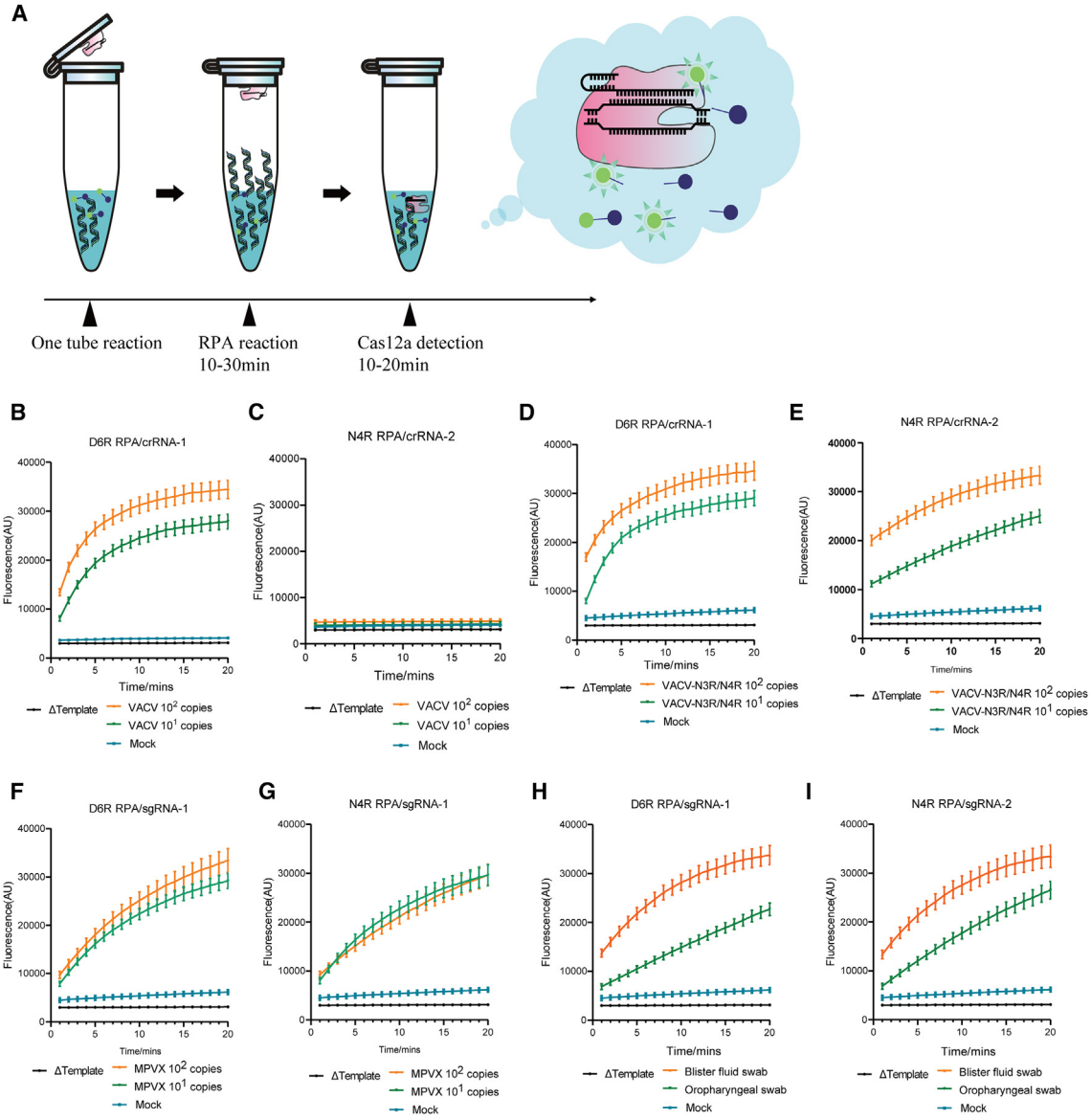


**Figure 6. Detection of viral DNA with the one-tube RPA-Cas12a system**

(A) Illustration of the one-tube assay. Cas12a/crRNA RNP was added to the cap of the tube, and the RPA reagent was added to the tube bottom. After 15 min incubation at 37°C for the RPA reaction to complete, Cas12a/crRNA RNP was centrifuged to the bottom and mixed with the RPA products. Fluorescence signals were recorded for 20 min.

(B–E) VACV and VACV-N3R/N4R viral DNA genomes were diluted to $10^2$ and $10^1$ copies/μL. RPA-Cas12a/crRNA reactions were performed as shown in (A). Viral DNA was amplified with RPA using both the D6R primers and the N4R primers. Data are presented as mean ± SD of three technical replicates.

(F–I) MPXV viral DNA genomes and clinical samples in Figure 5. RPA-Cas12a/crRNA reactions were performed as shown in (A). Viral DNA was amplified with RPA using both the D6R primers and the N4R primers. Data are presented as mean ± SD of three technical replicates.

detection system into a diagnostic tool for MPXV and potentially other pathogenic DNA viruses, which can be deployed in home-testing, field-screening, and resource-limited settings.

## Limitations of the study

The methods presented here allow for rapid and accurate diagnosis of MPXV under laboratory conditions. However, it has to be noted that sample preparation, visual readout, and temperature may interfere with the sensitivity of the detection system. Further development is needed for the deployment of field screening or home testing. Besides, clinical samples came from only one mpox case; the diversity of samples is insufficient.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Cell lines
  - Virus and DNA samples
- METHOD DETAILS
  - Plasmids construction
  - crRNAs and RPA primers design
  - Fluorescence Cas12a nuclease assay
  - Recombinase polymerase amplification (RPA)
  - One-tube RPA-Cas12a cleavage assay
  - Droplet digital PCR
- QUANTIFICATION AND STATISTICAL ANALYSIS

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.crmeth.2023.100620.

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## AUTHOR CONTRIBUTIONS

F.X., W.T., and F.G. conceived the project. F.Z., Y. Hu, B.H., and Z.F. performed the experiments and prepared the manuscript. L. Wei, Y.X., Y. Huang, S.M., Liming Wang, and Lingwa Wang performed the data curation and formal analysis. All authors contributed to the experimental design. B.A., J.F., C.L., W.T., and F.G. composed the manuscript. All authors reviewed the manuscript and discussed the work.

## DECLARATION OF INTERESTS

F.G., F.Z., Y. Hu, F.X., and S.M. are inventors of pending and issued patents on CRISPR-Cas12a detection of mpox.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Bacterial and virus strains | | |
| VACV Tiantan strain | this paper | JX489139.1 |
| MPVX | Wenjie Tan | MPVX-B.1-China-C-Tan-CQ01 |
| Chemicals, peptides, and recombinant proteins | | |
| Penicillin-Streptomycin (5,000 U/mL) | Thermo Fisher Scientific | Cat#15070063 |
| DMEM | Hyclone | Cat#SH30243.01 |
| FBS | Hyclone | Cat#SH30396.02 |
| EcoRI-HF | NEB | Cat#R3101S |
| BamHI-HF | NEB | Cat#R3136S |
| GenCRISPR™ Cas12a (Cpf1) Nuclease | GenScript | Cat#Z03502 |
| 10xNEBuffer2.1 | NEB | Cat# B6002S |
| Critical commercial assays | | |
| QIAamp DNA Mini Kit | Qiagen | Cat#51306 |
| TwistAmp® Basic kit | TwistDx | Cat#TABAS03KIT |
| QuantStudio 3D Digital PCR 20K Chip Kit v2 | Applied Biosystems | Cat#A26316 |
| Experimental models: Cell lines | | |
| Vero | ATCC | Cat# CRL-1586 |
| Oligonucleotides | | |
| ddPCR E9L-F | this paper | 5′-GAACATTTTTGGCAGAGAGAGCC-3′ |
| ddPCR E9L-R | this paper | 5′-CAACTCTTAGCCGAAGCGTATGAG-3′ |
| ddPCR Probe | this paper | FAM-ACGCTTCGGCTAAGAGTTGCACATCCA-TAMRA |
| crRNAs and RPA primers used in the study | this paper | Table S1 |
| Recombinant DNA | | |
| puc57-D6R | this paper | N/A |
| puc57-E9L | this paper | N/A |
| puc57-N3R | this paper | N/A |
| puc57-N4R | this paper | N/A |
| Software and algorithms | | |
| BIO-RAD CFX manager | Bio-RAD | 1845000 |
| GraphPad Prism 8.0 software | GraphPad Prism | N/A |

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Fei Guo (guofei@ipb.pumc.edu.cn)

#### Materials availability

This study did not generate new unique reagents. The detailed info of all materials and reagents used is given in the key resources table.

#### Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

## EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

### Cell lines

Monkey kidney epithelial cells (Vero, CRL-1586) were purchased from American Type Culture Collection (ATCC). Cells were cultured in DMEM supplemented with 10% FBS (Hyclone), 1% penicillin (100 U/ml) and streptomycin (100 μg/mL) (Thermo Fisher Scientific).

### Virus and DNA samples

Recombinant vaccinia virus (VACV-N3R/N4R) bearing mpox virus genes N3R and N4R were generated by homologous recombination as reported.[52] Briefly, mpox virus N3R and N4R sequences without ATG start codon were inserted into thymidine kinase (TK) locus of the VACV Tiantan strain by homologous recombination. Recombinant vaccinia virus was obtained with five rounds of plaque purification and the insertion of N3R and N4R into the viral genome was confirmed by PCR and DNA sequencing. VACV viruses and purified recombinant VACV-N3R/N4R viruses were amplified by infecting Vero cells, and viral titers were determined with plaque assay. To prepare DNA samples of infected cells, Vero cells were infected with VACV or VACV-N3R/N4R at different MOIs for 24 h.

MPXV were amplified by infecting Vero cells with the mpox virus MPXV-B.1-China-C-Tan-CQ01 that was isolated from the only mpox case in China. Standard negative controls were purchased from GeneWell Company (Shenzhen) and all DNA were extracted using QIAamp DNA mini kit (Qiagen), in accordance with the manufacturer's instructions.

## METHOD DETAILS

### Plasmids construction

Full-length D6R gene (nucleotide positions 100176 to 102089) and E9L gene (nucleotide positions 53110 to 56130) were amplified by PCR from vaccinia virus Tiantan strain DNA (GenBank accession number JX489139.1) and cloned into pUC57 vector usning EcoRI and BamHI restriction sites. Full-length N3R gene (positions 190389 to 190919) and N4R gene (positions 191033 to 192346) from mpox virus (GenBank accession number ON563414.3) were synthesized by Beijing Ruibio BioTech Co., LTD (Beijing, China) and cloned into pUC57 vector using the same restriction sites. DNA copy number was calculated using the following formula: DNA copy number per microliter = [(6.02x$10^{23}$) x (plasmid concentration, in nanograms per microliter) x $10^{-9}$]/[(fragment length, in nucleotides) x 660].

### crRNAs and RPA primers design

crRNAs of 23 nt were designed using CRISPR RGEN Tools (http://www.rgenome.net/cas-designer/). We confirmed the specificity of each crRNA to orthopoxviruses or mpox using NCBI BLAST. For each target gene, we selected the top five crRNAs based on the highest sequence identity to orthopoxviruses and the lowest sequence identity to human genes, to reduce the odds of off-target. The 20 nt crRNA stem sequence is: 5′-UAAUUUCUACUCUUGUAGAU-3'.

For RPA amplification, the amplicons were determined based on the target sequences of the most effective crRNAs of D6R and N4R, RPA primers were designed based on TwistAmp Assay Design Manual. We also confirmed the specificity of RPA primers using NCBI BLAST (Figure S5). See Table S1 for sequences of crRNAs and primers.

### Fluorescence Cas12a nuclease assay

The Fluorescence Cas12a detection assay was performed with 50 nM AsCas12a, 62.5 nM crRNA, plasmid DNA or viral DNA of different concentrations, and 500 nM quenched fluorescence reporter ssDNA. Briefly, the AsCas12a-crRNA RNP complexes were preassembled by incubating 1.5 μL of AsCas12a (667 nM) and 2.5 μL of crRNA (500 nM) for 30 min at 37°C, then added 1 μL of ssDNA-FQ reporter (10 μM), 2 μL of 10×reaction buffer, and 1 μL of plasmid DNA or viral DNA ($10^{9}$ to $10^{\circ}$ copies/μL) or 10 μL RPA amplification product. Ultrapure water was added to keep the total volume of 20 μL. For assays containing more than one crRNA simultaneously, the RNP complexes were separately assembled and then combined in the reaction at half (in 2 RNP combinations) or one-third (in 3 RNP combinations) or one-fourth (in 4 RNP combinations) the volume to keep the total or combined concentration of RNP constant. Reactions proceeded for 100 min at 37°C on BIO-RAD CFX96 Real-Time System. The fluorescence signals were measured every 1 min (λex: 485nM; λem: 535nM).

### Recombinase polymerase amplification (RPA)

RPA reaction was conducted using TwistAmp Basic kit (TwistDx). In brief, 1.2 μL of primer RPA-F (10 μM), 1.2 μL of primer RPA-R (10 μM), 15 μL of RPA reaction buffer, 5.35 μL of ultrapure water, 1.25 μL of magnesium acetate (280 mM, MgOAc), and 1 μL of the template DNA were mixed and incubated in BIO-RAD CFX96 Real-Time System at a constant temperature of 37°C for 15 to 30 min.

### One-tube RPA-Cas12a cleavage assay

The 30 μL mixture containing AsCas12a (667 nM), 2.5 μL of crRNA (500 nM) and 3.5 μL of 10×NEBuffer2.1 of 1.5 μL of AsCas12a (667 nM), 2.5 μL of crRNA (500 nM) and 3.5 μL of 10×NEBuffer2.1 (NEB, #B6002S) was added on the tube cap. The RPA mixture containing 0.56 μL MgOAc (280 mM), 1.5 μL of primer RPA-F (10 μM), 1.5 μL of primer RPA-R (10 μM), and 14.75 μL of RPA reaction

buffer was added at the tube bottom. After RPA reaction at 37°C for 15 to 30 min, the mix on the tube cap was centrifuged into the reaction solution and reaction continued at 37°C for 20min in BIO-RAD CFX96 Real-Time System.

### Droplet digital PCR

The viral DNA was quantified by droplet-digital PCR (ddPCR) using QuantStudio 3D Digital PCR System (Applied Biosystems) following QuantStudio 3D Digital PCR System USER GUIDE. The ddPCR reaction system contained target-specific forward and reverse primers, FAM-labeled probe, master mix, template and ddH2O. The final concentration of primers used for E9L were 1μM. Probe (E9L: FAM-ACGCTTCGGCTAAGAGTTGCACATCCA-TAMRA) was synthesized by Tsingke Biotechnology Co., Ltd. (Beijing, China) and the final concentration was 0.325 μM. Procedure of the ddPCR were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s. The ddPCR primers are E9L-F (5′-GAACATTTTTGGCAGAGAGAGCC-3′) and E9L-R (5′-CAACTCT TAGCCGAAGCGTATGAG-3′).

## QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed with GraphPad Prism 8.0 software (GraphPad). Unless otherwise indicated, results are presented as mean ± standard deviation (SD), and represent data from at least three independent experiments. The slope of the Cas12a reaction is calculated with simple linear regression of raw data from time 0 for the set duration. Statistical significance was analyzed using unpaired t test. Significance is indicated by asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; NS, not significant.