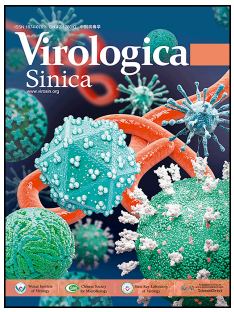
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Specific and visual detection of EBOV based on a one-pot RT-RAA-CRISPR/Cas12a assay

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Specific and visual detection of EBOV based on a one-pot RT-RAA-CRISPR/Cas12a assay

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Highlights

1. The one-pot RT-RAA-CRISPR/Cas12a assay for EBOV has single-copy resolution within 40 min.
2. This assay targeting the *VP40* gene has high specificity for EBOV and no cross-reaction with SUDV.
3. This one-pot assay is easy to operate and can effectively avoid aerosol contamination.

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Dear Editor,

The frequent emergence of life-threatening infectious diseases has posed a constant global threat in recent decades. Since the first identification of Ebola Virus Disease (EVD) in the Democratic Republic of Congo (then Zaire) in 1976, there have been approximately 40 outbreaks. About 30 of these were caused by the Ebola virus (EBOV), resulting in roughly 34,000 reported cases and 15,000 deaths. The overall case fatality rate for Ebola is estimated at 44%, with EBOV being the most prevalent of the virus species within the genus *Ebolavirus* (WHO; Jacob et al., 2020). Early and rapid diagnosis of these diseases is important to treat infected individuals and to control pandemics (Chams et al., 2020). Evidence suggests that the survival rate for patients with EBOV infection reaches 90% when the disease is diagnosed and treated early in the course of infection, compared to only 30% for those treated at a later stage (Mulangu et al., 2019).

Reverse transcription polymerase chain reaction (RT-PCR), encompassing both conventional and real-time approaches, is the primary method for the laboratory diagnosis of EVD. Of these approaches, the GeneXpert® Ebola assay stands out as a widely used commercial kit (Kaushik et al., 2016). However, RT-PCR has several limitations, including the need for time-consuming thermal cycling on specific instruments, and for skilled laboratory technicians (Broadhurst et al., 2016). Conventional RT-PCR testing therefore requires mobile laboratories when deployed in the field, posing significant challenges in conflict zones (Mukadi-Bamuleka et al., 2023). Developing a safe, rapid, and effective detection strategy for this high-biosecurity-level pathogen in resource-limited settings remains a formidable challenge (Broadhurst et al., 2016). Hence, there is an urgent need for development of a diagnostic method that can be implemented without reliance on complex instrumentation, thereby reducing the operational burden on testing personnel.

Accordingly, we have developed a simple, rapid and specific one-pot assay for the detection of EBOV *VP40* gene using an all-in-one RT-RAA-CRISPR/Cas12a method. All components for reverse transcript recombinase-aided amplification (RT-RAA) and CRISPR-based methods can be integrated into a single reaction system. This allows for the transfer and complete mixing of amplification products without opening the lid. Ultimately, this assay is capable of detecting 3.6 copies/μL linearized plasmid within 40 min.

First, the target is amplified using RT-RAA, and the amplified product is then specifically recognized by Cas12a and activated for trans-cleavage. This enables the cleavage of the single-

stranded fluorescent probe present in the system. Results are interpreted based on the presence or absence of a fluorescent signal. Traditional two-step methods often cause aerosol contamination when the reaction tube is opened. Furthermore, Cas12a can weaken RAA amplification efficiency by cutting system components. To address these issues, we designed a novel closure device with a tube-sleeve-tube structure. The RT-RAA system is prepared following standard protocols, and the RT-RAA reaction mixture is placed at the bottom of a 1.5 mL eppendorf tube. The Cas12a reaction mixture is then loaded into an uncapped PCR tube. After adding the sample to be examined into the RT-RAA system, the PCR tube is inserted into a 1.5 mL eppendorf tube, sealed and put into a thermal cycler or water bath at 39 °C for 20 min for amplification. After amplification, the CRISPR/Cas12a reaction mixture and RT-RAA reaction mixture are mixed by centrifugation and incubated at 37 °C for 20 min. Finally, the reaction tube is placed under ultraviolet excitation light to observe the occurrence of green fluorescence (Fig. 1A).

We first expressed and purified the Cas12a protein with a SUMO tag and confirmed that it exhibits good cleavage activity (Supplementary Fig. S1). To identify a highly specific and broadly conserved CRISPR/Cas12a target across EBOV strains, we compared 35 EBOV sequences from the GenBank database, representing diverse geographical regions and time periods, using MAFFT version 7 for multiple sequence alignment. Based on the restriction of the PAM site (TTTV) we then selected three different highly conserved sequences of 20–23 bp in the EBOV *VP40* gene as targets. Details of the EBOV strains are provided in Supplementary Fig. S2A. Additionally, the sequences of the target regions in EBOV were found to be distinct from those of other viruses, including Bundibugyo virus (BDBV), Taï Forest virus (TAFV), Reston virus (RESTV), Bombali virus (BOMV), Sudan virus (SUDV), Marburg virus (MARV), Dengue virus 2 (DENV2) and Nipah virus (NiV), demonstrating that the target regions in EBOV were distinct from those in the other viruses, and indicating that the reaction would have good specificity (Supplementary Fig. S2A). The corresponding crRNAs were designed according to the conserved targets. All three crRNAs were able to specifically recognize the target sequence, but were found to exhibit different degrees of fluorescence intensity. All three crRNAs produced strong fluorescence signals within 10 min and reached their maximum fluorescence signals within 20 min. crRNA2 not only generated the highest fluorescence signals across all time points but also reached the detection threshold in the shortest time (Supplementary Fig. S2B). Therefore, crRNA2 was selected for subsequent related experiments.

To ensure that the CRISPR/Cas12a reaction system had optimal cleavage efficiency, we optimized the reaction components related to the CRISPR/Cas12a system. First, we evaluated the effect of the concentration of crRNA. We found that within a certain range, increasing the crRNA concentration also increased the Cas12a cutting efficiency. The optimal reaction efficiency and the strongest fluorescence signal were observed at a concentration of 100 nM, but at concentrations greater than 100 nM, the efficiency declined (Fig. 1B).

Next, we optimized the Cas12a concentration. We found that the effect of Cas12a was less pronounced than that of crRNA when adjusted within a certain concentration range. However, similar to crRNA, the efficiency increased with the protein concentration, and the efficiency was optimal at a protein concentration of 100 nM, after which the efficiency enhancement was insignificant with further increases in protein concentration (Fig. 1C). Finally, to examine the effect of reaction buffers on the cleavage activity, we established four distinct CRISPR/Cas12a reaction systems with different reaction buffers. We found that the effect of different buffer compositions on the efficiency of Cas12a cleavage was pronounced, with the optimal reaction efficiency seen in the Bio-lifesci buffer, which might be attributed to its higher magnesium ion concentration and optimal pH (Supplementary Fig. S2C).

To mitigate the risk of false negatives caused by workspace contamination during the opening of the RT-RAA reaction, we developed a one-pot assay combining the RT-RAA amplification and the CRISPR/Cas12a reaction. In this assay, the RT-RAA amplification system and CRISPR/Cas12a system were preloaded into two separate reaction tubes, and the samples to be detected were added to the RT-RAA system for amplification for 20 min. After amplification, the CRISPR/Cas12a and RT-RAA systems were combined via brief centrifugation, and the CRISPR/Cas12a was used for the specific identification of the amplified target. After 10 to 20 min of CRISPR/Cas12a reaction, the presence of fluorescent signals was assessed visually under UV light.

After optimizing the CRISPR/Cas12a system, we next focused on developing a one-pot assay. We designed three sets of RT-RAA primers on both sides of the optimal crRNA2, and found that the primer combinations of Target2-F and Target2-R1 demonstrated superior amplification efficiency and specificity. Subsequently, we optimized the reaction temperature within the typical RT-RAA range (37-42 °C) to maximize the overall assay performance. The assay detected 3.6 copies/μL linearized plasmid within 40 min at both 39 °C and 42 °C (Fig.1D), but Cas12a activity was affected by the RT-RAA reaction temperature. We therefore optimized the reaction volume ratios at 39 °C. To determine the

optimal volume ratio that minimizes this inhibitory effect, we compared several combinations. We found that when both systems were adjusted to 25 μL, the assay achieved a limit of detection of 3.6 copies/μL for the linearized plasmid within 40 min, while the other two groups detected only 3.6×10^1 copies/μL linearized plasmid (Fig. 1E). To determine if extending the RT-RAA amplification time could improve assay sensitivity, we tested various time points. While longer amplification times resulted in stronger fluorescence signals, they did not improve the limit of detection, which remained at 3.6 copies/μL (Fig. 1F).

The sensitivity of the one-pot assay was evaluated using a serial dilution series of the pCAGGS-EBOV-linearized plasmid, with concentrations from ranging from 3.6×10^4 to 10^0 copies/μL. Amplification was performed according to the previously optimized conditions for the RT-RAA amplification and CRISPR/Cas12a reactions. The results showed that the assay could detect 3.6 copies/μL linearized plasmid (Fig. 1G).

Due to the high mortality rate of EBOV relative to other ebolaviruses and the fact that early infection does not have specific symptoms, it is extremely important to differentiate EBOV from other hemorrhagic fever viruses in a timely manner. In order to verify the specificity of the RT-RAA-CRISPR/Cas12a assay, we selected six viral RNA samples to assess assay specificity. To verify the specificity of the RT-RAA-CRISPR/Cas12a assay, we assessed it against six viral RNA samples, including different subtypes of ebolaviruses (EBOV, BDBV, SUDV, TAFV) and other related viruses with similar clinical symptoms (DENV2 and NiV). To ensure the reliability of the specificity results, we set up a positive control using the pCAGGS-EBOV-*VP40* linearized plasmid as a template and a negative control using nucleic acid-free water. The results showed that only the positive control and the EBOV RNA sample yielded a positive fluorescence signal, while no signal was observed in any of the remaining groups, including the negative control (Fig. 1H).

To validate the applicability of the one-pot RT-RAA-CRISPR/Cas12a assay to real samples and to assess the clinical detection capability, we extracted high-purity (OD_{260}/OD_{280} = 1.8~2.0) total RNA from HEK 293T cells. Varying concentrations of EBOV RNA were spiked into the total RNA, and these samples were used to evaluate the one-pot RT-RAA-CRISPR/Cas12a assay. Based on the sensitivity results, 40 min was chosen as the reaction time for the one-pot RT-RAA-CRISPR/Cas12a assay to avoid false negatives. Fluorescence image acquisition was performed at the end of the reaction and was analyzed using ImageJ, and 57.1 % (40/70) of the EBOV-positive samples tested positive (Fig.

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1I). At the same time, a real-time RT-PCR method was performed to assess the same spiked RNA samples targeting the EBOV *N* gene, with *Ct* values between 13 and 31 for positive samples. Of these, 57.1% (40/70) of the EBOV-positive samples tested positive (Fig. 1J). The real-time RT-PCR method corroborated the results of our RT-RAA-CRISPR/Cas12a assay, identifying the same number of positive samples. The real-time RT-PCR has been recommended by the WHO as the standard diagnostic method for EBOV. Therefore, we selected real-time RT-PCR as the reference method for comparison. The results showed that the two methods had good consistency.

Most current isothermal amplification technologies for EBOV, as well as existing one-pot methods, are limited to a detection limit of 10^1 copies/μL (Bonney et al., 2020, Chen et al., 2020, Li et al., 2022.). Furthermore, many of these one-pot approaches suffer from relatively long detection times, even where they achieve consistent sensitivity (Xiong et al., 2022). However, the method described here achieves a detection limit of 3.6 copies/μL linearized plasmid. A major advantage of this method is its high specificity. RT-RAA, due to its low amplification temperature and longer primer sequences, is highly prone to non-specific amplification. In contrast, this method further enhances specificity by performing gRNA-specific recognition of the PAM site after RT-RAA amplification. This method minimizes the reaction volume of RT-RAA and Cas12a, significantly reducing the amount of RT-RAA reagents used. The detection time of this method is slightly longer than that of the pure RT-RAA detection method, but the entire process is completed within 40 min, making it suitable for home testing or field deployment.

While placing the Cas12a reaction system in a PCR tube enables one-pot detection by simplifying the setup, it introduces a risk of premature mixing with the RAA system if the tube is accidentally inverted. This can cause false-negative results, but can be avoided by carefully ensuring the liquid does not spill before centrifugation and mixing. Furthermore, visualizing low-copy samples can be difficult due to their weak fluorescence signals, which requires a dark environment. Therefore, we are working on a new device. This device will integrate a light source and a filter into a single, compact unit to provide the necessary dark environment for on-site visualization.

In conclusion, we have developed an efficient, reliable, and visual one-pot RT-RAA-CRISPR/Cas12a assay. This method effectively addresses the aerosol contamination and operational complexities commonly associated with RT-RAA amplification. The assay can complete detection within 40 min with a sensitivity of a single copy and shows excellent concordance with the gold-

175 standard real-time RT-PCR for both positive and negative results. Therefore, it holds great promise for
176 meeting the on-site detection needs for EBOV RNA. We expect that this technique will be used for the
177 detection of EBOV and can be expanded to the diagnosis of other RNA/DNA viral infections.

178 **Footnotes**

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183 the work reported in this paper.

184 All the data generated during the current study are included in the manuscript. Supplementary data to
185 this article can be found online at XXX.
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Figure legends

Fig. 1 Visual and specific detection of EBOV using a one-pot RT-RAA-CRISPR/Cas12a assay. A Schematic of the one-pot RT-RAA-CRISPR/Cas12a assay. **B** The one-pot RT-RAA-CRISPR/Cas12a method was performed using different concentrations of crRNA (the Cas12a concentration was fixed at 100 nM). The left panel shows real-time fluorescence kinetics (mean ± s.d., n = 3), and the right panel shows endpoint fluorescence intensity. **C** The one-pot RT-RAA-CRISPR/Cas12a method was performed using different concentrations of Cas12a (crRNA concentration was fixed at 100 nM). The left panel shows real-time fluorescence kinetics, and the right panel shows endpoint fluorescence intensity. Error bars represent the means ± s.d. from three replicates (n = 3). **D** Screening for the optimal temperature for RT-RAA amplification. Templates at different concentrations were amplified at different temperatures. **E** Optimization of the volume ratios of RT-RAA and CRISPR/Cas12a reactions system for different concentrations of template. **F** Optimization of RT-RAA amplification time and RT-RAA amplification of different concentrations of template at different amplification times. Visual inspection was performed at the end of the reaction for comparison, and the pixel intensities of the green light channel were collected and analyzed from images taken with a smartphone **(A–C)**. Error bars represent the means ± s.d. from three replicates (n = 3). **G** Determination of the detection limit of the one-pot RT-RAA-CRISPR/Cas12a method for the detection of EBOV. Values represent mean ± s.e.m. from three replicates (Ordinary one-way ANOVA; ****, $P < 0.0001$). **H** The specificity of the one-pot RT-RAA-CRISPR/Cas12a method to EBOV RNA using other Ebola viruses (SUDV, BDBV, TAFV) and other virulent viruses (DENV2, NIV) was analyzed in the positive control (PC) group with 3.6×10^5 copies/μL of pCAGGS-EBOV-*VP40* plasmid. **I** Heat map and scatter plot showing the detection of EBOV in 70 spiked RNA samples using the one-pot RT-RAA-CRISPR/Cas12a method. Signal values in the heat map are the normalized fluorescence of each sample in the detection channels. **(J)** Heatmap and scatter plot showing the *Ct* values of EBOV in 70 spiked RNA samples as assessed using real-time RT-PCR. The *Ct* values of all positive samples were below 31, and the *Ct* values of all negative samples were exceeded 31. Each reaction tube was captured and converted into JPG format through the mobile phone. Then, the RGB pixel values in the image were extracted using the ImageJ software.

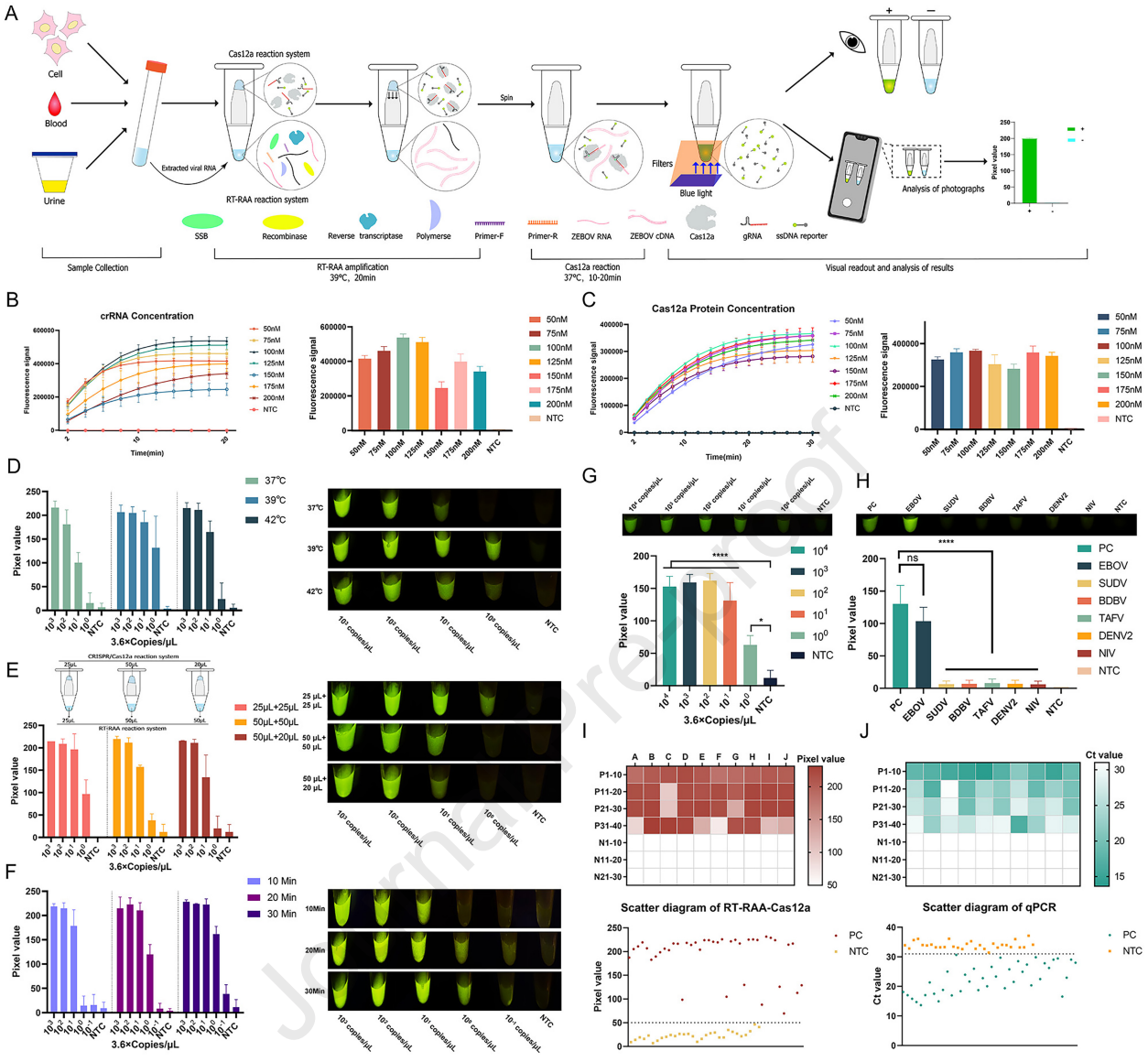
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Supplementary Figures

Supplementary Fig. S1 Purification and activity validation of Cas12a protein. **A** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified Cas12a protein. **B** Investigation of whether the Cas12a system will work using fluorescence imaging of the endpoints after 40 min of incubation with the reaction components missing in the system. Cas12a can exert cleavage activity only when all the various components are present. The caveat is that Cas12a and crRNA form a binary complex to recognize the target DNA to cut the fluorescent probe, and Mg^{2+} in the buffer can greatly catalyze the reaction to proceed. Therefore, Cas12a activity was inactive in the absence of Buffer and no detection fluorescence was emitted in lane 4. **C** Sodium dodecyl sulfate-polyacrylamide gels of purified Cas12a protein were analyzed using thin-layer scanning to determine the purity of purified Cas12a protein.

Supplementary Fig. S2 Screening of crRNA and buffer for the one-pot RT-RAA-CRISPR/Cas12a assay. **A** Sequence comparison of 35 EBOV strains and sequence comparison of EBOV with different viruses. **B** Screening of crRNAs for the detection of the one-pot RT-RAA-CRISPR/Cas12a assay. The crRNA1, crRNA2 and crRNA3 experimental groups are shown from left to right. Each experiment was performed in triplicate, and each line represents the data from a single replicate. **C** Buffer optimization for the one-pot RT-RAA-CRISPR/Cas12a assay. The figure on the left shows the four buffers and components used for the one-pot RT-RAA-CRISPR/Cas12a assay. On the right is a comparison of the endpoint fluorescence signal intensities of the one-pot RT-RAA-CRISPR/Cas12a assay using the four different buffers. NTC, non-target control reaction. Error bars represent the means ± s.d. from three replicates (n = 3).

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