Rapid and Fully Microfluidic Ebola Virus Detection with CRISPR-Cas13a

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Supporting Information

ABSTRACT: Highly infectious illness caused by pathogens is endemic especially in developing nations where there is limited laboratory infrastructure and trained personnel. Rapid point-of-care (POC) serological assays with minimal sample manipulation and low cost are desired in clinical practice. In this study, we report an automated POC system for Ebola RNA detection with RNA-guided RNA endonuclease Cas13a, utilizing its collateral RNA degradation after its activation. After automated microfluidic mixing and hybridization, nonspecific cleavage products of Cas13a are immediately measured by a custom integrated fluorometer which is small in size and convenient for in-field diagnosis. Within 5 min, a detection limit of 20 pfu/mL (5.45 × 10^7 copies/mL) of purified Ebola RNA is achieved. This isothermal and fully solution-based diagnostic method is rapid, amplification-free, simple, and sensitive, thus establishing a key technology toward a useful POC diagnostic platform.

KEYWORDS: CRISPR, Ebola, microfluidics, amplification-free, fluorescence, point-of-care

RNA detection with high sensitivity and single-base specificity has important implications for medical healthcare.1,2 The 2014 Ebola outbreak infected over 28,000 people and caused over 11,000 deaths.3,4 The epidemic was initially misdiagnosed, and almost three months were needed to transport and analyze the blood samples in Europe to confirm the disease as Ebola.5 The lack of a reliable, inexpensive, and sensitive diagnostic platform in low resource settings highlights the urgent need for a powerful POC detection system that allows self-diagnosis in infected communities.6

The current gold standard for infectious disease detection at POC relies on a lab-in-a-box polymerase-chain reaction (PCR) platform known as GeneXpert.7 PCR can generate up to billions of DNA copies by amplifying a single copy or a few copies of a DNA segment.8 Even though PCR demonstrates excellent sensitivity, it has several drawbacks such as expensive reagents and instrumentation, sophisticated operation, and inverse transcription for RNA detection.9 Conversely, an immunosorbent-based technique known as OraQuick has been developed to detect the presence of viruses in blood samples.10 However, the immunosorbent-based technique exhibits poor sensitivity and cannot be used for early diagnosis when the viral load is lower than the detection limit.11 Recombinase polymerase amplification (RPA) at room temperature is an alternative to traditional PCR where heating cycles are accurately controlled for template denaturation and reannealing.12 However, there are concerns that deviations from the manufacturer’s protocol and/or storage conditions in low resource settings could influence its performance. In addition,
RPA amplification relies upon viscous crowding agents for optimal nucleic acid amplification, and RPA reagents show reduced sensitivity only after >3 weeks at 45 °C.\textsuperscript{13}

We previously developed an automated microfluidic device combined with a sensitive liquid-core antiresonant reflecting optical waveguide (ARROW) biosensor chip for Ebola RNA detection.\textsuperscript{14} The automated microfluidic device purified and concentrated Ebola RNA molecules, and the purified RNA targets were pumped to the ARROW chip for individually labeled RNA molecule detection. To improve the scalability and selectivity, we recently developed a microfluidic multiplexer capable of analyzing 80 samples in parallel. We also developed a protocol based on a sequence-specific barcode fluorescence reporter and a photocleavable capture probe, enabling solid phase extraction of Ebola RNA from blood.\textsuperscript{15} Fluorescence detection was performed by using a parabolic mirror-based fluorometer. However, this protocol is time-consuming and complicated because it requires solid phase capture and release using magnetic beads. The previous fluorometer was not customized to integrate with microfluidic systems and required manual transportation of purified targets to the detection system. Thus, additional innovation is needed to produce a simple, rapid, sensitive, and integrated solution phase POC diagnosis.

In this study, we present an integrated and fully solution-based POC detection system using an RNA endonuclease of the clustered regularly interspaced short palindromic repeats (CRISPR) bacterial adaptive immune system.\textsuperscript{16,17} CRISPR-associated (Cas) genes are located next to CRISPR sequences, and the CRISPR/Cas system is used to degrade foreign nucleic acids. The expression was induced with 0.5 mM IPTG at an OD\textsubscript{600} of 0.6, and cells were cooled to 18 °C for protein expression for 16 h. Cells were subsequently collected by centrifugation and ruptured by sonication in lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM DTT, pH 8.0) supplemented with 1 tablet of protease inhibitors, lysozyme, and benzonase after sonication. Lysate was cleared by centrifugation, and the supernatant was filtered and applied to StreptTactin Sepharose for 1 h with rotation. The SUMO tag was then digested with SUMO protease and confirmed by SDS PAGE. The cleaved Cas13a was further purified through cation exchange and gel filtration chromatography. Protein was aliquoted and flash frozen by liquid nitrogen and stored at ~80 °C. Details of the Cas13a purification are available in Supporting Information Figure S1.

**MATERIALS AND METHODS**

**LwCas13a Protein Purification.** PC013 plasmid that expresses Cas13a from *Leptotrichia wadei* was ordered from Addgene (#90097). The amplified plasmids were transformed into Rosetta(DE3) pLysin competent cells for protein expression. The purification procedures followed the protocol described previously.\textsuperscript{22} Briefly, protein expression was induced with 0.5 mM IPTG at an OD\textsubscript{600} of 0.6, and cells were cooled to 18 °C for protein expression for 16 h. Cells were subsequently collected by centrifugation and ruptured by sonication in lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM DTT, pH 8.0) supplemented with 1 tablet of protease inhibitors, lysozyme, and benzonase after sonication. Lysate was cleared by centrifugation, and the supernatant was filtered and applied to StreptTactin Sepharose for 1 h with rotation. The SUMO tag was then digested with SUMO protease and confirmed by SDS PAGE. The cleaved Cas13a was further purified through cation exchange and gel filtration chromatography. Protein was aliquoted and flash frozen by liquid nitrogen and stored at ~80 °C. Details of the Cas13a preparation are available in Supporting Information Figure S1.

**CRISPR RNA and RNA Target Sequences.** CRISPR RNA and synthetic target RNAs were obtained from Integrated DNA Technologies (IDT, Coralville, IA, United States) with HPLC purification. The concentrations were determined by UV absorption, and the RNA pellets were serially diluted by DEPC-treated ultrapure water before use in experiments.\textsuperscript{19,20}

To avoid target amplification and improve the detection sensitivity, we combined an automated and multiplexing CRISPR microfluidic chip with a custom-designed benchtop fluorometer for rapid and low volume (~10 μL) Ebola virus detection. The microfluidic chip is mounted on the fluorometer for in situ detection. Exploiting the collateral cleavage of CRISPR technology, we were able to detect the presence of total Ebola RNA with a detection limit of ~20 pfu/mL (5.45 × 10\textsuperscript{5} copies/mL). The entire detection procedure can be accomplished within 5 min and does not require solid phase extraction. The low volume consumption makes the system suitable for finger-prick tests, where the blood sample volume is limited.\textsuperscript{21} With this integrated system, clinical diagnosis for any viral RNA can be achieved by programming the spacer sequence of crRNA complementary with different RNA targets.
transferred to a new tube and incubated with isopropanol and GlycoBlue Coprecipitant (Thermo Scientific). Samples were centrifuged, and the resulting pellet of precipitated RNA was briefly washed with ethanol. The pellet was air-dried and resuspended in 50 µL of RNase-free water and stored below −65 °C until use.

Reverse transcription polymerase chain reaction (RT-PCR) was used to determine the viral RNA concentrations.23 Primers and probe were designed to detect a region of the glycoprotein gene. The assay was run on an Applied Biosystems 7500 Real Time PCR Instrument: 50 °C for 15 min (1 cycle); 95 °C for 5 min (1 cycle); 95 °C for 1 s and 60 °C for 35 s (45 cycles); and 40 °C for 60 s (1 cycle).

The measured Ebola and MARV concentrations are 5.45 × 1011 copies/mL, respectively. The sequences of the primers and probes are: F 5’- TTT TCA ATC CTC AAC CGT AAG GC-3’; R 5’- CAG TCC GGT CCC AGA ATG TG-3’; 5’- p 6FAM - CAT GTG CCG CCC CAT CGC TGC - TAMRA-3’.

**RESULTS**

The design of the automated CRISPR microfluidic chip is shown in Figures 2a and 2b. The microfluidic device can run 24 assays in parallel, as controlled by eight pneumatic inlets. The Cas13a–crRNA complex was pumped to the detection reservoir from the reagent inlet. Next, Ebola RNA of various concentrations were pumped via microvalves into the detection reservoir, where the Ebola RNA hybridizes with Cas13-crRNA. One of the wells was dedicated to the scrambled RNA control for background subtraction. An example of closed and open states of a microvalve. (d) Design of a benchtop fluorometer system integrated with a microfluidic device for in situ virus sensing. (e) Photograph of chip and detection system.

**Figure 2.** (a) Design of the automated CRISPR microfluidic chip for Ebola virus detection. (b) Blow up of design of the fluidic layer. Ebola target RNA is pumped into the detection reservoir and reacted with Cas13a–crRNA. (c) Open (left) and closed (right) states of a microvalve. (d) Design of a benchtop fluorometer system integrated with a microfluidic device for in situ virus sensing. (e) Photograph of chip and detection system.

On-Chip RNA Cleavage Detection. On-chip RNA cleavage assays were performed by automatically pumping 20 nM purified LwCas13a, 10 nM CRISPR RNA, 12.5 nM quenched fluorescent RNA reporter (RNase Alert v2, Thermo Scientific), 1 µL of murine RNase inhibitor (New England Biolabs), and 50 ng of background total human RNA (purified from HeLa culture) into the detection reservoir (total volume ~20 µL). Then, Ebola RNA target (10 µL) at various concentrations was pumped to the detection reservoir for hybridization. After 5 min incubation at 37 °C, the microfluidic device was aligned with the custom designed fluorometer for in situ detection. The laser power was reduced to 3 W/cm² to reduce sample photobleaching.

Off-Chip RNA Cleavage Assays. Off-chip experiments were performed by incubating 50 µL of 20 nM purified LwCas13a, 10 nM CRISPR RNA, 12.5 nM quenched fluorescent RNA reporter (RNase Alert v2, Thermo Scientific), 1 µL of murine RNase inhibitor (New England Biolabs), 50 ng of background total human RNA (purified from HeLa cells), and varying amounts of input Ebola RNA oligos in RNase-free water and stored below 80 °C until use.

To form the pneumatic layer, polydimethylsiloxane (PDMS) was coated on the SU-8 mold to a thickness of 400 µm. To form the fluidic layer, 8 mm-thick PDMS was casted on the SU-8 mold, holes for pneumatic control were punched on the pneumatic layer. The fabrication method for the microfluidic device for in situ virus sensing.
integrated after background subtraction (Figure 3b), showing a linear dependence (Pearson’s $R = 0.96$) on the concentration of the target RNA. The linear correlation between fluorescent intensity and virus concentration indicated that this system is able to quantitatively measure virus RNA concentration.

Next, we performed an on-chip RNA cleavage assays by pumping Cas13a−crRNA with 12.5 nM quenched fluorescent RNA reporter to the detection reservoir (signal integration time: 1s). Upon addition of 10 μL of Ebola RNA (Figure 3c), we observed linear increase of the fluorescence signal increases linearly with target concentration from 1 pM to 10 nM (Figure 3d). The linear correlation (Pearson’s $R = 0.96$) between fluorescent intensity and virus concentration further demonstrates our ability to measure virus RNA concentration. By increasing the signal integration time to 3s, a detection limit of $\sim 50$ fM is achieved for Ebola RNA oligo (Supporting Information Figure S2).

Finally, we introduced 10 μL of Ebola total RNA into the detection reservoir and detected the changes in fluorescence signal with an integration time of 3 s (Figure 4). The fluorescence signal linearly increases from 20 to $2 \times 10^2$ pfu/mL, spanning 3 orders of magnitude (slope = 0.16 ± 0.008). In comparison, the fluorescence signal of the negative control sample (MARV) showed modest increase over concentrations ranging from 20 to $2 \times 10^6$ pfu/mL (all of the individual data points are shown in Supporting Table 1). There is a significant

Figure 3. (a) Off-chip uncorrected emission curve versus various Ebola RNA oligo concentrations. (b) Fluorescence intensity versus Ebola RNA oligo concentrations showing linear dependence at the low picomolar level (off-chip). (c) On-chip uncorrected emission curve versus various Ebola RNA oligo concentrations. (d) Fluorescence intensity versus Ebola RNA oligo concentrations showing linear dependence at the low picomolar level (on-chip). Pearson’s $R$ values are presented for the linear fits. Error bars are the standard deviation of the mean, and each experiment was repeated three times.

Figure 4. Detection of Ebola virus with integrated microfluidic chip and fluorescence detection system. Normalized curve showing the relationship between fluorescence intensity and the target concentrations on a logarithmic scale. A limit of detection of $\sim 20$ pfu/mL ($5.45 \times 10^7$ copies/mL) is achieved with 5 min incubation time at 37 °C. Pearson’s $R$ values are ~1 for both linear fittings. The slopes are distinguishable to be 0.16 (±0.008) and 0.05 (±0.006) for the positive (black) and negative (red) measurements, respectively. Error bars are the standard deviation of the mean, and each experiment was repeated three times.
difference in the scores for Ebola (positive) and MARV (negative) results (two-tailed t test, \( p = 0.03004 \)). Thus, our integrated diagnostic platform is capable of sensing Ebola with a detection limit of \( \sim 20 \) pfu/mL (5.45 \( \times \) 10\(^7\) copies/mL).

### DISCUSSION

In this study, we developed a microfluidic platform for target specific and sensitive detection of pathogens. The method uses Cas13a as one of the CRISPR-Cas systems to generate fluorescent reporter RNAs resulting from the nonspecific cleavage of quencher, when it is bound to a target viral RNA at extremely low concentrations.\(^{27,28}\) The microfluidic platform has several advantages over existing methods that require complicated solid phase extraction processes. For example, in microarrays, the capture efficiency is generally low due to the limited surface area and binding sites.\(^ {29,30}\) In comparison, magnetic beads offer a high surface area and higher capture efficiency,\(^ {31,32}\) but additional steps such as active mixing are required to prevent settling and sticking of the beads in the microchannels.\(^ {14}\) In addition, because autofluorescence of beads reduces the detection limit,\(^ {33,34}\) the captured nucleic acid must be released from the beads for detection.\(^ {35}\) These strategies increase the complexity of POC detection.

Our CRISPR-based detection system eliminates extra steps in sample preparation. Without target amplification, a single RNA target activated by CRISPR-Cas13a can cleave \( \sim 10,000 \) fluorescence probes at physiological temperature, resulting in a 4 orders of magnitude amplification of the signal. In the absence of target RNA, the reaction mixture does not generate high background, indicating that the system is very robust and specific. We notice deviation from the linear fit at low concentrations (1 pM, Figures 3b and 3d) and an increase in fluorescence signal for negative control samples (Figure 4). These may be due to the off-target activity of Cas13a. In future studies, the linearity of the detection method can in principle be increased by engineering high-fidelity Cas13a mutants to reduce off-targeting.

Recently, a SHERLOCK method has been developed combining the isothermal target amplification and collateral cleavage of CRISPR-Cas13a. This system can detect attomolar level ZIKA RNA in body fluids.\(^ {39}\) However, for viruses with a single RNA target, the system requires a reverse transcriptase process followed by a recombinase polymerase amplification (RPA). Even though RPA can further extend the detection limit, it is complicated, and the enzymatic activity can be unstable, which is not desirable for POC diagnostics.\(^ {6}\) We achieved a detection limit of 20 pfu/mL (5.45 \( \times \) 10\(^7\) copies/mL) for Ebola total RNA without target amplification by detecting fluorescence signal using a sensitive parabolic mirror-based fluorometer. Future studies are required to test whether the method would be effective for samples from early symptomatic patients in the field. The integrated fluorometer and microfluidic system only cost \( \sim $3,300 \) USD. The system is compact, portable, easy to align, and does not require expertise in optics for field operation. Thus, our method significantly simplifies the sample preparation for self-diagnosis.

An ideal POC test should have a low cost, be highly multiplexed, and require as little patient sample as possible. Compared to PCR, this isothermal CRISPR detection occurs completely in solution without expensive temperature control systems or thermal cyclers. Because no diffusion barrier is present in the solution, the reaction is rapid: requiring only 5 min, it is approximately 25 times faster than a PCR analysis.\(^ {35}\) The reagents, including LwCas13a, CrRNA, RNA reporter, RNase inhibitor, and total human RNA, cost only \( \sim $1 \) USD/assay. Disposable PDMS chip and the associated parts cost \( \sim $5 \) USD/assay. Thus, the total cost for detection is \( \sim $6 \) USD/assay. It has been reported that Cas13a–CrRNA complex is stable and can be lyophilized and then rehydrated for POC detection.\(^ {19}\) Leveraging an automated and miniaturized microfluidic system, our system can be loaded with different virus RNA samples and is capable of screening 24 subjects in parallel practically. Because each measurement only takes \( \sim 1 \) min, our system can screen 24 samples within 30 min. The on-chip protocol only requires 10 \( \mu \)L purified total RNA for each reaction; thus, it is compatible with finger-prick tests. Finger-prick tests are easy to perform and have been widely used for self-diagnosis.\(^ {36,37}\) When working in POC settings, total RNA can be rapidly purified from 50 \( \mu \)L blood samples by using an off-chip commercial kit, and then the RNA is introduced into the microfluidic system for automatic Ebola detection. Future work can be done to integrate RNA extraction and detection on the same chip. Combined with the rapid and sensitive CRISPR system, the overall detection time is \( \sim 15 \) min starting from the raw blood sample obtained by finger-prick tests.

The new, sensitive parabolic mirror-based fluorometer is customized to integrate with the automated CRISPR microfluidic chip for in situ detection. Our fluorometer has a sensitivity comparable with that of confocal microscopes and is considerably smaller in size and lighter in weight.\(^ {15,38}\) Compared to microscope images, which require experts to interpret, the integrated fluorescence signal from the fluorometer is easy to read and user-friendly. The fluorescent signal can be calibrated with titration curve for unknown virus concentration measurements and virus detection.

### CONCLUSIONS

In this work, we developed an automated microfluidic system and sensitive fluorometer coupled with a fully solution-based CRISPR assay for Ebola RNA sensing. This amplification-free diagnostic platform is small in size, low cost, and exhibits excellent sensitivity and specificity. Furthermore, this all-solution-phase diagnosis protocol is rapid and simple without using complicated solid-phase extraction and purification. All of these advantages satisfy the requirements for POC detection of Ebola and other infectious diseases in low resource locations.

### ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssens.9b00239.

Supporting Figure 1: protocol of LwCas13a purification and the SDS page gel; Supporting Figure 2: on-chip low concentration RNA oligo cleavage detection; Supporting Table 1: data points of Figure 4 (PDF)

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