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Rapid and Fully Microfluidic Ebola Virus Detection with CRISPR-Cas13a

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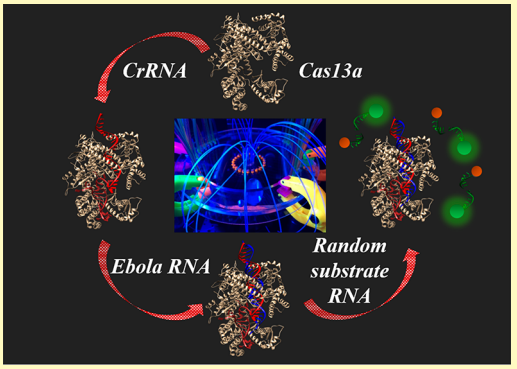
Supporting Information

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 immunoassay-based technique known as OraQuick has been developed to detect the presence of viruses in blood samples.[10] However, the immunoassay-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,

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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.[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.[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.[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.[16,17] CRISPR-associated (Cas) genes are located next to CRISPR sequences, and the CRISPR/Cas system is used to degrade foreign nucleic acids entering the host cell. Of the 93 C*as* genes, Cas13a has been characterized as an RNA-guided RNA editing enzyme that binds to a 57-nt-long single stranded CRISPR RNA (crRNA), and the 28 nt at the 3′ terminal is the spacer region for target binding.[18] Remarkably, binding of Cas13a-cRNA to target RNA induces a conformational change that activates nonspecific RNA degradation. The cleavage of the target and surrounding nonspecific RNA occurs simultaneously, and on average, Cas13a cleaves ∼10^4 nonspecific RNA within cleavage of a target RNA (Figure 1).[18] Collateral cleavage of Cas13a dramatically increases the sensitivity of RNA detection. Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) based on Cas13a detection has been used to detect the Zika and Dengue viruses, and the enzyme is readily reconstituted on paper for field applications.[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 × 10^7 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.[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.

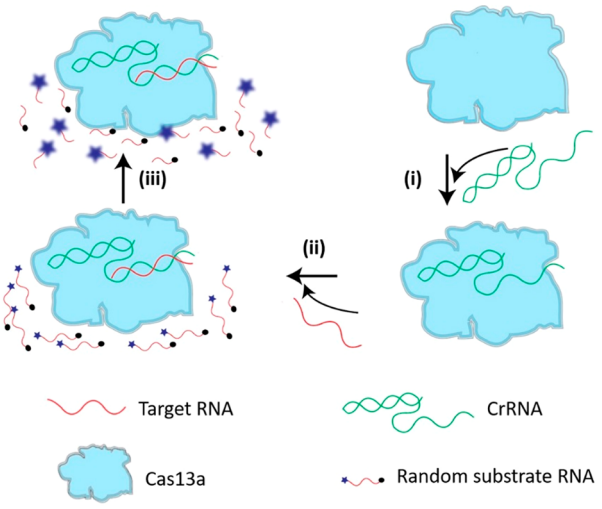

Figure 1. Schematic of CRISPR-Cas13a-based sensing mechanism: (i) purified Cas13a binds with CRISPR RNA. (ii) The Cas13a-CRISPR RNA complex hybridizes with Ebola target RNA. (iii) The Cas13a-CRISPR RNA−Ebola RNA complex cleaves random RNA strands and releases fluorophores into the solution.

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) pLysis competent cells for protein expression. The purification procedures followed the protocol described previously.[22] Briefly, protein expression was induced with 0.5 mM IPTG at an OD_{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 filtrated and applied to StrepTactin 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 with DEPC-treated ultrapure water before use in experiments.

CRISPR RNA. 5′GGGGAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACGUGGCGUCAUCUCCAGCCUUAUCAAUG.

Target RNA. 5′GAACAUUGAUAAGGCUGGAGAUGACGCCACAACAGCUUUGUGAGCUAUUUUCCAUUCAAAAACACUGGGGGCAUCCUGUGCUACAUAGUGAAACAGCAAU 3′.

Ebola and Marburg RNA. The following virus stocks were used to obtain Ebola and Marburg RNA: Ebola virus *Homo sapiens*-tc/COD/1995/Kikwit-95106210 (species *Zaire ebolavirus*) passage 3 on Vero E6 cells, with a titer of 2 × 10^5 pfu/mL and Marburg virus *Homo sapiens*-tc/AGO/2005/Angola-0501379 (species *Marburg marburgvirus*), passage 3 on Vero E6 cells, with a titer of 4 × 10^6 pfu/mL. Virus stocks were previously titered using a neutral red and agarose plaque assay following standard procedures. Stocks were removed from liquid nitrogen storage and inactivated with TRIzol LS reagent (Thermo Scientific) prior to removal from biosafety level 4. RNA extraction was then performed following the manufacturer instructions. Briefly, samples were incubated at room temperature with chloroform and centrifuged for 15 min at 4 °C, and then the aqueous phase was

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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.[23,24] 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). A single fluorescence read was taken at the end of each 60 °C step. The measured Ebola and MARV concentrations are 5.45×10^{11} and 1.38×10^{11} 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′.

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 nuclease assay buffer (40 mM Tris-HCl, 60 mM NaCl, 6 mM $MgCl_2$, pH 7.3). The molecular weight of LwCas13a protein (126 kDa) was verified by a denaturing gel and 45 μM protein was obtained at greater than 95% purity. Reactions were allowed to proceed for 5−20 min at 37 °C followed by evaluation with a fluorometer (JASCO FP-750).

Automated CRISPR Microfluidic Chip Fabrication and Operation. The fabrication method for the microfluidic chip was reported previously. SU-8 molds (~80 μm thickness) were created by standard photolithography.[25,26] To form the pneumatic layer, polydimethylsiloxane (PDMS) was coated on the SU-8 mold to a thickness of ~300 μm by spin-coating and baking. After peeling away from the SU-8 mold, holes for pneumatic control were punched on the pneumatic layer. To form the fluidic layer, 8 mm-thick PDMS was casted on the SU-8 mold followed by baking at 80 °C. Next, the two PDMS layers were bonded together by oxygen plasma activation and peeled away from the fluidic SU-8 mold. Finally, the reaction reservoir and the Ebola RNA inlet were created by punching holes through the bonded PDMS layers. The microvalves and micropumps were operated by a solenoid valve and a custom-designed LabVIEW program for the open-close transition of micropumps.

Integrated Benchtop Fluorometer. A continuous wave laser at 488 nm (Sapphire 488 LP, Coherent) was used to directly excite each sample solution in the reservoir of the microfluidic chip. The individual reservoir was aligned by adjusting the three-axis translation stage without the sample transfer step. To reduce photodamage, the laser power was reduced to 3 W/cm^2 by a variable neutral-density filter, and the beam was slightly defocused to have ~500 μm diameter spot by f-200 mm plano-convex lens. The laser power/area was ~3 W/cm^2 at the microfluidic chip position. Fluorescence was collected by an off-axis parabolic (OAP) mirror with a centered hole for passing the excitation beam (1′′ diameter, 50 mm reflected focal length, ~2 mm center hole diameter, Thorlabs). Scattered light was eliminated by a 488 nm notch filter (NF488-15, ~6 optical density, 15 nm fwhm, Thorlabs, Inc.). The fluorescence was focused into an optical fiber (M93L01, Thorlabs) on an XYZ translation stage connected to a mini USB spectrometer (USB 2000+, Ocean Optics). Data measurement and analysis were performed by utilizing Spectra Suite (Ocean Optics) and Origin Pro (OriginLab) software. The fluorescence signal was integrated with wavelengths from 510 to 550 nm.

On-Chip RNA Cleavage Detection. On-chip RNA cleavage assays we performed by automatically pumping 20 nM purified LwCas13a, 10 nM crRNA, 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/cm^2 to reduce sample photobleaching.

RESULTS

The design of the automated CRISPR microfluidic chip is shown in Figures 2a and 2b. The microfluidic device can run

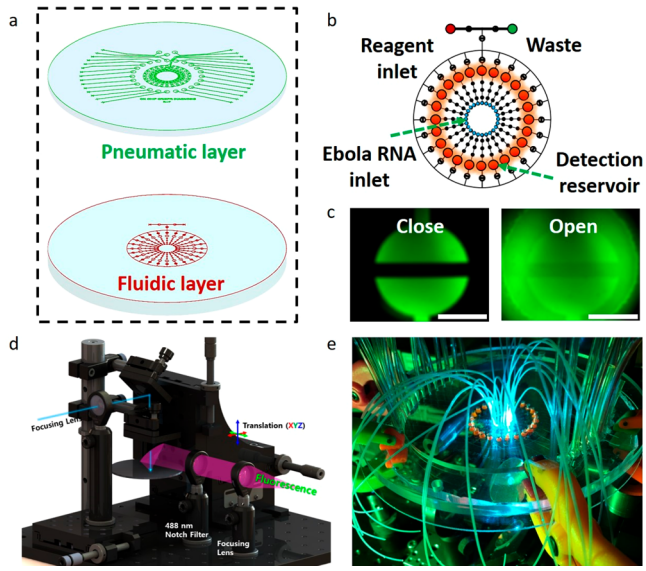

Figure 2. (a) Design of the automated CRISPR microfluidic chip for Ebola virus detection. (b) Blow up of design of the fluidic layer. Ebola target RNA is pumped into the detection reservoir and reacted with Cas13a−crRNA. (c) Open (left) and closed (right) states of a microvalve. (d) Design of a benchtop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

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 under a fluorescence microscope is presented in Figure 2c. In the closed state, the lifting gate halts the reagent flow. By opening the gate, reagents flow through the microvalve and fill the projection area below the pneumatic chamber. The microfluidic chip is integrated with the custom designed fluorometer. The XYZ translation stage was used to move between detection reservoirs for rapid in situ fluorescence detection (Figures 2d and 2e).

To explore the CRISPR-Cas13a cleavage mechanism, we incubated Cas13a and crRNA that targets Ebola virus RNA with 12.5 nM quenched fluorescence RNA reporter and varying amount of input Ebola RNA oligos (Figure 3a). Without adding Ebola RNA oligos, the background (0 pM) is comparable with pure buffer background, which indicates limited nonspecific RNA degradation. Conversely, adding 1−10 pM Ebola RNA oligo into the mixture increases the fluorescence signal. The signal is saturated at 10 nM RNA target. The fluorescence signal of each concentration was

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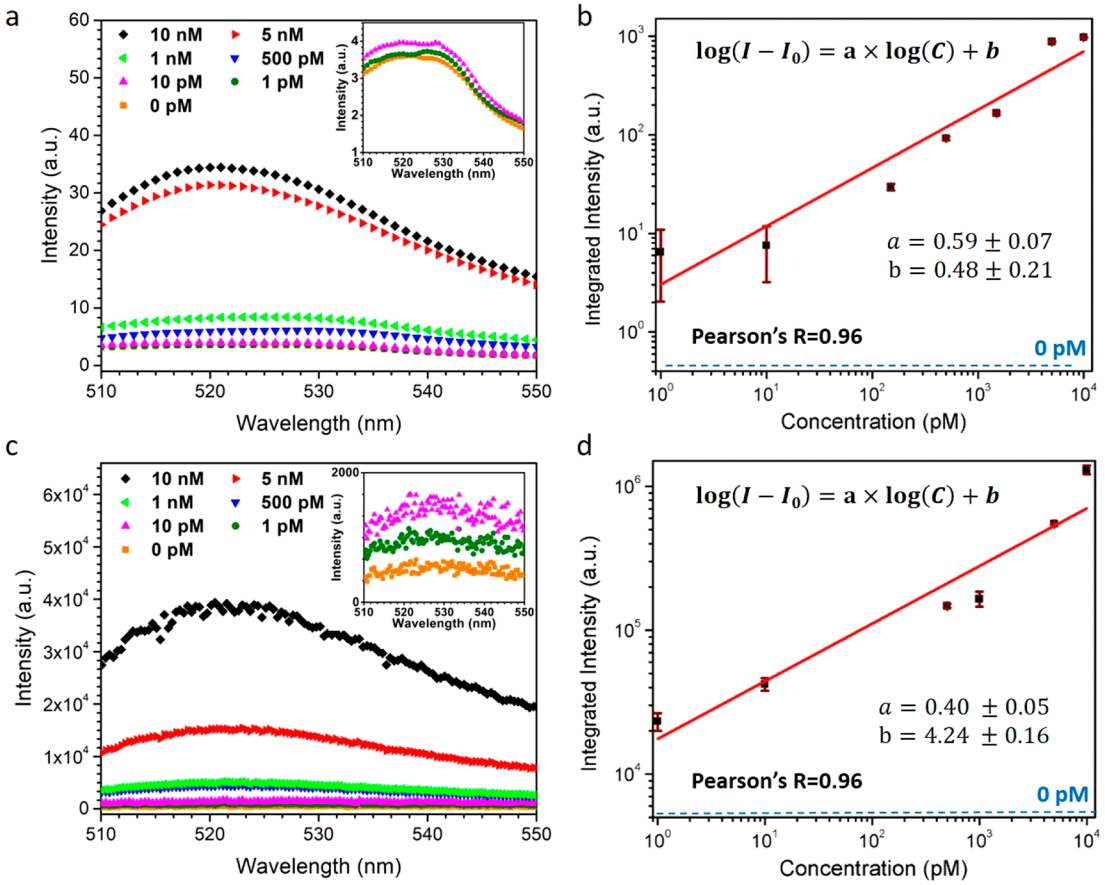

Figure 3. (a) Off-chip uncorrected emission curve versus various Ebola RNA oligo concentrations. (b) Fluorescence intensity versus Ebola RNA oligo concentrations showing linear dependence at the low picomolar level (off-chip). (c) On-chip uncorrected emission curve versus various Ebola RNA oligo concentrations. (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.

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 ~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 2×10^4 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 2×10^4 pfu/mL (all of the individual data points are shown in Supporting Table 1). There is a significant

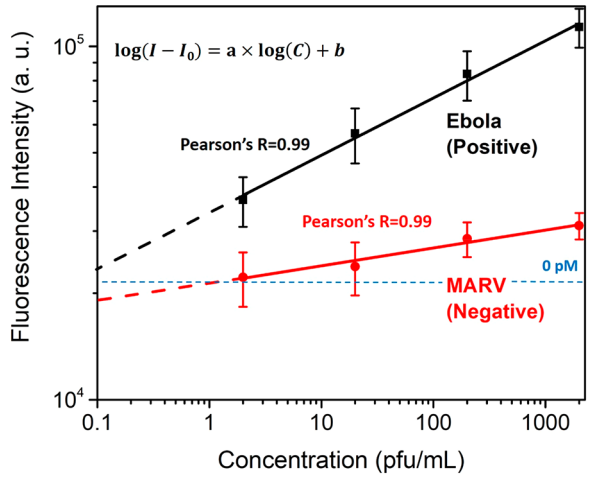

Figure 4. Detection of Ebola virus with integrated microfluidic chip and fluorescence detection system. Normalized curve showing the relationship between fluorescence intensity and the target concentrations on a logarithmic scale. A limit of detection of ~20 pfu/mL (5.45×10^7 copies/mL) is achieved with 5 min incubation time at 37 °C. Pearson's *R* values are ~1 for the 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.

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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 ~20 pfu/mL (5.45×10^7 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.[15] 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 ~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.[19] 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 (5.45×10^7 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 ~$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 ~$1 USD/assay. Disposable PDMS chip and the associated parts cost ~$5 USD/assay. Thus, the total cost for detection is ~$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 ~1 min, our system can screen 24 samples within 30 min. The on-chip protocol only requires 10 μL 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 μL 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 ~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/acssensors.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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Author Contributions

△P.Q. and M.P. contributed equally.

Notes

The authors declare no competing financial interest.

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