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Instrument-Free Point-of-Care Molecular Detection of Zika Virus

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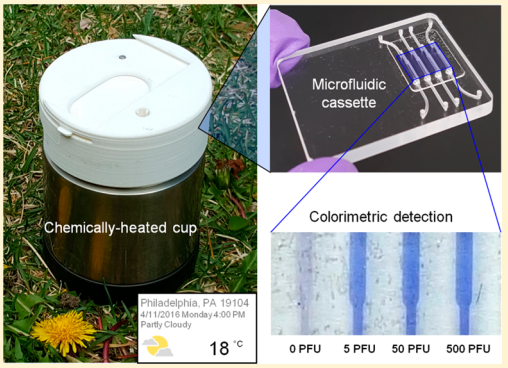
Supporting Information

ABSTRACT: The recent outbreak of Zika virus (ZIKV) infection in the Americas and its devastating impact on fetal development have prompted the World Health Organization (WHO) to declare the ZIKV pandemic as a Public Health Emergency of International Concern. Rapid and reliable diagnostics for ZIKV are vital because ZIKV-infected individuals display no symptoms or nonspecific symptoms similar to other viral infections. Because immunoassays lack adequate sensitivity and selectivity and are unable to identify active state of infection, molecular diagnostics are an effective means to detect ZIKV soon after infection and throughout pregnancy. We report on a highly sensitive reverse-transcription loop-mediated, isothermal amplification (RT-LAMP) assay for rapid detection of ZIKV and its implementation in a simple, easy-to-use, inexpensive, point-of-care (POC) disposable cassette that carries out all the unit operations from sample introduction to detection. For thermal control of the cassette, we use a chemically heated cup without a need for electrical power. Amplification products are detected with leuco crystal violet (LCV) dye by eye without a need for instrumentation. We demonstrated the utility of our POC diagnostic system by detecting ZIKV in oral samples with sensitivity of 5 plaque-forming units (PFU) in less than 40 min. Our system is particularly suitable for resource-poor settings, where centralized laboratory facilities, funds, and trained personnel are in short supply, and for use in doctors' offices, clinics, and at home.



Zika virus (ZIKV) is an *Aedes* mosquito-borne flavivirus that emerged in Brazil in 2015 and has rapidly spread throughout tropical and subtropical Americas.[1−4] In addition to transmission via infected mosquito bite, the virus can be transmitted from an infected woman to her unborn baby, through sexual contact, and via blood transfusion. ZIKV is a major health concern because it has been linked to Guillain-Barré syndrome (GBS),[5] congenital microcephaly, and other severe neurological defects in newborns of mothers, who were infected with ZIKV while pregnant.[6−8] Because ZIKV-infected individuals are asymptomatic or present symptoms common to many other febrile illnesses,[4] rapid and reliable diagnostic tools for ZIKV are vital. Such tools are also important (i) for risk management throughout pregnancy; (ii) to track the effects of ZIKV on fetal development; (iii) to monitor treatment and vaccine efficacy; (iv) to track the spread of the infection and support control and eradication efforts; and (v) to ensure the safety of the blood supply.

Although lateral-flow immunochromatographic assays[9−11] for ZIKV are rapid, simple, inexpensive, and instrument-free, they often suffer from low sensitivity and specificity. This is especially true in the case of ZIKV infection because antibodies to ZIKV cross-react with other highly homologous flaviviruses (including dengue), which may lead to nonspecific test results.[11] RT-PCR diagnostics for ZIKV is highly specific and sensitive, and considered the gold standard for ZIKV detection.[4,12−14] Conventional PCR amplification requires, however, extensive sample preparation, sophisticated and expensive equipment, centralized laboratory facilities, and trained personnel, all of which are in short supply in resource-poor settings. Point-of-care (POC) molecular diagnostics may alleviate this bottleneck due to constrained resources, and improve the quality of health care. Recently, Pardee et al. have reported a low-cost molecular diagnostic method for the detection of the Zika virus by combining nucleic-acid-sequence-based amplification (NASBA) with biosensors.[15] However, it has still some limitations for POC diagnostic applications, such as relatively long detection time (~3 h), low sensitivity, and relatively tedious operation steps.

To facilitate inexpensive enzymatic amplification with minimal or no instrumentation, we utilized reverse-transcription loop-mediated amplification (RT-LAMP).[16,17] We examined bioinformatic data to identify highly conserved regions of the ZIKV genome and designed six specific primers for the Zika lineage that is prevalent in the Americas. To enable POC molecular diagnostics, we used a custom-made disposable

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microfluidic cassette[17] that combines viral nucleic acid capture, concentration, and purification; isothermal amplification; and detection. For isothermal amplification, our cassette is combined with a custom-made electricity-free cup that generates heat with an exothermic reaction and regulates temperature with a phase change material.[18] To eliminate the need for fluorescence excitation and detection, we employed a colorless leuco crystal violet (LCV) that undergoes a color change to violet in the presence of amplicon dsDNA.[19]

ZIKV can be detected in blood, oral fluid (saliva), urine, and semen.[20−23] Our method can operate with any of these body fluids. In this paper, we use, however, oral fluid samples. Recent studies indicate that ZIKV is more frequently detectable in saliva and at higher concentrations than in blood.[24] Saliva samples were routinely used during the French Polynesian ZIKV outbreak.[24] Saliva is also an attractive sampling medium because it can be collected noninvasively;[25−28] furthermore, sample collection is possible when blood is difficult to collect, such as from young children and neonates, in remote places lacking medical facilities, and in community settings for purposes of surveillance and epidemiology.

EXPERIMENTAL SECTION

RT-LAMP Primer Design. To design specific RT-LAMP primers for the ZIKV strains prevailing in the Americas, we aligned and analyzed complete genome sequences of isolates from Brazil (15 strains), Mexico (2 strains), Suriname (1 strain), and Columbia (1 strain) (Table S1 and Figure S1 of Supporting Information (SI)). These sequences were compared with sequences of other flaviviruses (Figure S1 of SI) using DNAMAN software.[29] The envelope protein coding region (Figure S2 of SI) was selected to design ZIKV RT-LAMP primers due to its high homology among ZIKV isolates and high divergence from the other flaviviruses examined. The six RT-LAMP primer-set was designed with the PrimerExplorer V4 software available from Eiken Chemical Co. Ltd. A BLAST search of the GenBank nucleotide database was carried out for the selected primers' sequences to verify specificity. The RT-LAMP sequences were synthesized by a commercial vendor (IDT, Coralville, IA) and documented in Table 1 together with the concentrations that we used in the reaction mixture. Our FIP primer consists of a mixture of two primers, differing in a single base pair, denoted with the letter R. This mixture of two FIP primers is used to maintain high amplification efficiency of the various ZIKV strains prevailing in the Americas.

Table 1. Sequences and Concentrations of ZIKV RT- LAMP Primers

primer name	sequence (5′ to 3′)	concentration (μM)
F3	CAGTTCACACGGCCCTTG	0.2
B3	TGTACCTCCACTGTGACTGT	0.2
FIP	GGCGACATTTCAAGTGGCCAGA-GAGCTCTR*GAGGCTGAGA	1.6
BIP	AGGGCGTGTCATACTCCTTGTG-AGTGTTTCAGCCGGGATCT	1.6
loop F	CCTTCCCTTTGCACCATCCA	0.8
loop B	TACCGCAGCGTTCACATTCA	0.8
*R = A, G		

Target Virus. Zika virus (mex 2−81) was obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA, Dr. Robert Tesh, Director)[30] and propagated in the mosquito cell line C6/36 cells. We determined the viable viral concentration (PFU/mL) with a plaque assay on Vero cells.

Benchtop RT-LAMP Amplification and RT-qPCR. Zika viral RNA was extracted with Qiagen Viral RNA mini kit (QIAGEN, Valencia, CA), following manufacturer's recommendations. In addition to the primers (Table 1) and template, the RT-LAMP reaction mixture (15 μL) included: 9 μL of OptiGene Isothermal Master Mix ISO-100 (OptiGene, U.K.), 2 U of AMV reverse transcriptase (Invitrogen, Carlsbad, CA), and 0.5 μL of EvaGreen fluorescent dye (Biotium, Hayward, CA). Amplification was carried out and monitored with Peltier Thermal Cycler PTC-200 (Bio-Rad DNA Engine, Hercules, CA) at 63 °C. Fluorescence emission intensity data were collected once every minute for 40 min. For comparison with standard laboratory procedures, benchtop RT-PCR was carried out as described in SI.

Saliva Samples Spiked with ZIKV. To demonstrate our POC RT-LAMP assay and to test possible interference from agents in saliva, we collected whole mouth saliva (WMS) from healthy, consenting, adult volunteers (Figure 1A). Then, the ZIKV was spiked in the saliva at various concentrations. For safety, the ZIKVs were first inactivated by mixing the medium laden with ZIKVs with a binding/lysis buffer (AVL buffer) (QIAamp Viral RNA Mini Kit) in a biosafety facility, followed by addition of ethanol to the lysate according to the Qiagen protocol.

ZIKV-Processing on the Microfluidic Cassette. Each disposable cassette contains four independent, multifunctional, isothermal, amplification reactors (Figure 1B). Each reactor features, at its entry port, a flow-through Qiagen porous silica membrane (QIAamp Viral RNA Mini Kit) that serves as a solid binding phase for nucleic acid immobilization.[17,31,32]

A 65-μL sample, consisting of ZIKV lysate with various ZIKV concentrations, was filtered through the isolation membrane of each reactor. The nucleic acids bound to the silica membrane, and the filtrate was discharged to waste. Subsequent to sample introduction, 100 μL of Qiagen ethanol-based wash buffer 1 (AW1) was pipetted through the membrane to remove any amplification inhibitors. Then, the silica membrane was washed with 200 μL of Qiagen ethanol-based wash buffer 2 (AW2), followed by air-drying with a disposable syringe for 30 s.

For colorimetric detection, a LCV dye containing 0.5 mM crystal violet (CV), 30 mM sodium sulfate, and 5 mM β-cyclodextrin was prepared.[19] The RT-LAMP reaction was carried out with a Loopamp RNA amplification kit (Eiken Chemical Co., Ltd., Japan). The 25-μL reaction master mixture, consisting of 1.6 μM FIP and BIP, 0.8 μM LF and LB, 0.2 μM F3 and B3; 1 μL enzyme; and 5.5 μL LCV dye, was injected into each reaction chamber through the inlet port and the immobilization membrane. The cassette was then inserted into the chemically heated cup for isothermal amplification.

Electricity-Free POC Processor. As an inexpensive, optionally disposable processor, we used a simple, thermally insulated portable cup heated by an exothermic reaction for chip-based isothermal amplification. Figure 1C,D show, respectively, a schematic depiction and a photo of the chemically heated cup.[18] Briefly, the cup consists of a thermos cup body (Thermos L.L.C), a microfluidic chip holder, and a 3D printed cup lid. The thermos cup body can be alternatively made with Styrofoam for further cost reduction. A pack of Mg−Fe alloy, (Innotech Products Ltd., U.S.A., costing ~ $0.15 per charge) served as the heat source and was placed in a drawer in

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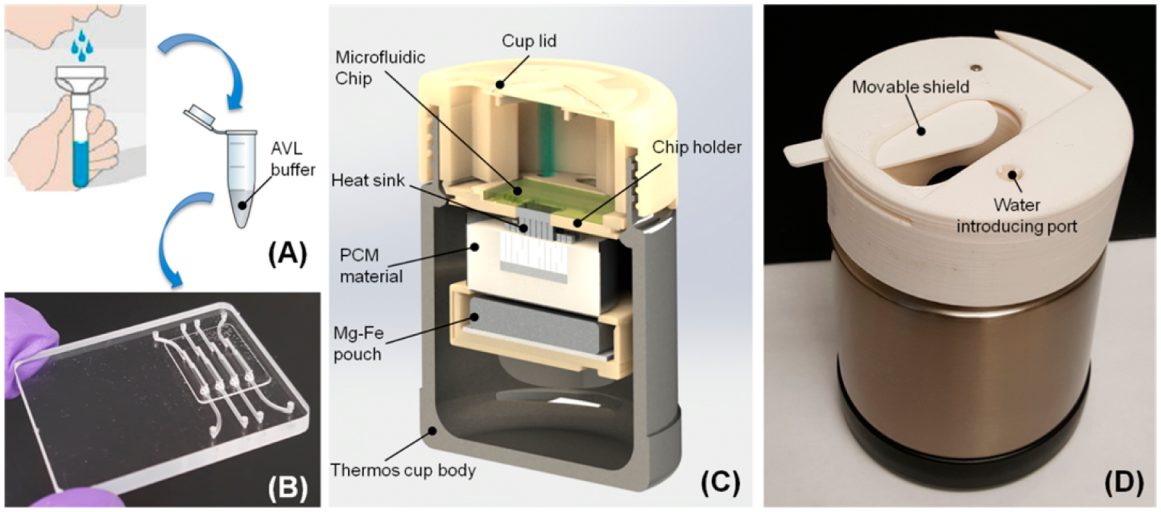

Figure 1. (A) Schematic of saliva sample preparation. Saliva samples are first collected in a saliva collection tube and then lysed in Qiagen binding/lysis (AVL) buffer. (B) The lysed sample is filtered through the isolation membrane of our microfluidic cassette for nucleic acid extraction. (C) Exploded view of the chemically heated cup. The cup consists of a thermos cup body, a 3D-printed cup lid, a chip holder, PCM material, heat sink and single-use Mg−Fe alloy pack heat source. (D) A photograph of the chemically heated cup for point of care molecular diagnostics of ZIKV.

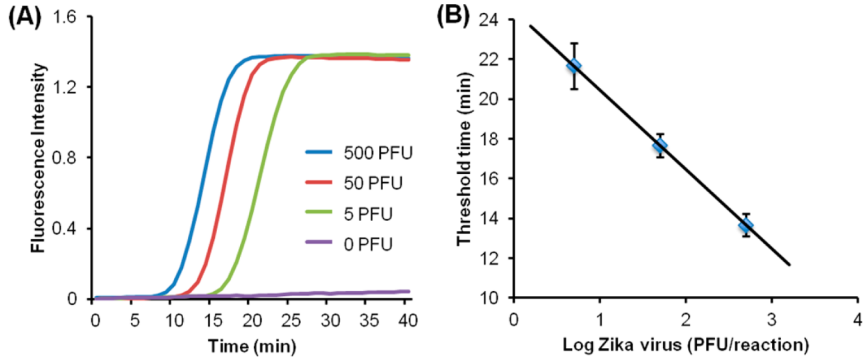

Figure 2. (A) RT-LAMP amplification curves in the presence of 500, 50, 5, and 0 PFU of ZIKV per reaction. (B) The threshold time t_T (in minutes) as a function of the PFU (n = 3).

the cup lid (Figure 1C). Tap water was introduced into the drawer through a port in the cup lid (Figure 1D) to interact with the Mg−Fe alloy to produce heat. To isolate the amplification reactors from variable ambient conditions, we used a phase change material (PCM) that melts at 68 °C (PureTemp 68, Entropy Solutions Inc., Plymouth, MN) to regulate the temperature. An aluminum heat sink embedded in the PCM enhanced heat transfer from the PCM to the cassette. After the desired incubation time (typically, 40 min), the detection results were directly observed by eye and/or recorded with a cell phone.

RESULTS AND DISCUSSION

RT-LAMP Assay. RT-PCR is considered the gold standard for ZIKV detection.[24] PCR requires, however, thermal cycling and considerable operator skills, which in turn require centralized laboratories, expensive equipment, and highly trained personnel—all of which are in short supply in resource-poor settings. To avoid the complications and cost of thermal cycling, we selected to use isothermal enzymatic amplification method. The ability to carry out the amplification at a fixed temperature, without thermal cycling, greatly reduces instrumentation complexity and cost compared to PCR. We use here RT-LAMP assay for molecular detection of ZIKV. Because LAMP typically employs a set of six primers that recognize eight different locations along the target sequence, its specificity is better than that of PCR, wherein the target is recognized only at two locations. LAMP is also highly efficient, producing a very large number of amplicons, which simplifies detection and reduces processing time compared to PCR. Since we did not find any reports of RT-LAMP primer designs for ZIKV, we first designed and verified such primers.

According to a recent report,[33] patients infected in Surinam and Brazil in 2015 are closely related to the strain that circulated in French Polynesia in 2013, with more than 99.7% and 99.9% of nucleotide and amino acid identity, respectively. This suggests that well-designed primers will provide efficient enzymatic amplification for most, if not all, ZIKV strains in the Americas. We aligned the sequences of 19 ZIKV strains form the Americas and identified a highly conserved region of 218 nucleotides (nt) in the envelope protein coding region with 99.74% nucleotide identity across the known strains of the virus (Figure S2 in SI). This region is also highly divergent from other common flaviviruses (Figure S1 in SI). With the aid of the PrimerExplorer V4 software (Eiken Chemical Co. Ltd.), we designed the primer set documented in Table 1. We then used this primer set to amplify various concentrations of ZIKV RNA.

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Figure 2A depicts normalized fluorescence emission intensity as a function of time during the amplification process of 0 (negative control), 5, 50, and 500 PFU of ZIKV on the benchtop. The fluorescent intensity of the negative control (no target) remains nearly level throughout the entire incubation time, indicating negligible amplicon formation, if any, of primer-dimers. In the presence of ZIKV, the emission intensity increased from the baseline to the saturation level as the incubation time increased. The higher the target concentration, the earlier the intensity curve increased above the baseline. The results clearly indicate that 5 PFU of ZIKV can be readily detected. To quantify the amplification process, we define the threshold time t_T. This is the time required for the amplification curve to reach half its saturation value. Figure 2B depicts the threshold time t_T (min) as a function of the log of the ZIKV PFU. In the range from 5 PFU to 500 PFU, the threshold time t_T decreases nearly linearly as a function of log (PFU). The experiment indicates that by monitoring the threshold time t_T, it is possible to quantify the target. The experimental data was also remarkably reproducible. The error bars in Figure 2B represent one standard deviation. For comparison, we also carried out a real-time RT-PCR assay and obtained a similar detection limit (Table S2 and Figure S3 in SI) to that of our benchtop RT-LAMP.

Instrument-Free Point-of-Care Molecular Diagnostic System. Because the LAMP reaction is robust and performs efficiently over a range of temperature (i.e., 60−65 °C), high precision temperature control is not essential. Our POC molecular diagnostic system consists of: (i) a disposable microfluidic cassette and (ii) a chemically heated cup. Our custom-made microfluidic cassette (Figure 1B) consists of four independent multifunctional amplification reactors. Here, we used these reactors to amplify saliva samples with various Zika virus concentrations to demonstrate the utility of our technology platform. In applications, the reactor array can be used to provide positive and negative controls and to codetect multiple targets such as ZIKV and dengue viruses. Each reactor is equipped with a nucleic acid isolation, purification, and concentration membrane. When the lysed sample is introduced into the reactor, the nucleic acids bind to the membrane. The membrane allows us to use sample volumes that far exceed the amplification reactor's volume, which enables the test to achieve high sensitivity.

To provide the incubation temperature without reliance on electric power, we use our simple, inexpensive chemically heated cup.[18] The commercially available flameless ration heater (FRH) is our heat source.[34] Heat is produced when the magnesium−iron alloy interacts with water. To maintain the temperature of the amplification reactors at the desired value, we use a PCM with a melting temperature of 68 °C. When the exothermic reaction produces more energy than needed to bring the PCM to 68 °C, the excess energy is consumed as latent heat and the PCM remains at its phase transition temperature of 68 °C as long as two phases (solid and liquid) coexist. Due to thermal resistance, the amplification chambers' temperature is about 5 °C lower than the phase transition temperature. When the ambient temperature ranges from 12 to 35 °C, a single Mg−Fe packet is sufficient to maintain the temperature within accepted tolerances for over 1 h[18] at the cost of ∼ $0.15 per cassette. The cup can be reused once the FRH is replaced. Alternatively, the thermos cup body can be replaced with Styrofoam (∼$ 0.1−0.5 per processor) for single use. Based on what we pay for reagents[35] and materials, we estimate the cost of the ZIKV test at approximately $2.

Zika Virus Detection in Saliva at the Point of Care. To demonstrate the utility of our system for ZIKV detection, we spiked saliva samples with varying concentration of ZIKV. One of the objectives of this experiment is to verify that substances present in saliva do not interfere with RT-LAMP. We use colorimetric detection to enable us to determine test results by eye for instrument-free detection. To this end, we included LCV dye with the reaction mixture. The colorless LCV is converted into violet CV in presence of dsDNA.[24] Thus, successful amplification is detected by a change of color.

We carried out our experiments outdoors (at ambient temperature of 18 °C) with Zika virus spiked in saliva (Figure S4 in SI). Saliva samples containing 0 (negative control), 5, 50, and 500 PFU of ZIKV were pipetted into the various reactors in our microfluidic cassette. The cassette was then inserted into the chemically heated cup, and the exothermic reaction was triggered by introducing water into the cup. The cassette was removed from the chemically heated cup after 40 min for visual detection. Figure 3A,B are, respectively, optical photographs of the cassette before and after RT-LAMP. Prior to incubation, all reactors are nearly colorless due to the absence of dsDNA (Figure 3A). After the RT-LAMP reaction, reactor 1 with no target (negative control) remains nearly colorless, indicating the absence of dsDNA and the lack of amplification. In contrast, reactors 2, 3, and 4 display violet color, indicating the presence of ds DNA and successful amplification (Figure 3B). The color change in our experients is more significant compared with previously reported metallochromic indicators[36−38] such as calcein, hydroxynaphthol blue (HNB). The intensity of the color appears independent of the number of amplification templates, likely because the amplification reactions have saturated. As an added advantage, the cassette remains sealed during detection, which prevents contamination of the test area by amplicons and avoiding false-positive results in subsequent tests as it is often encountered with open systems such as when gel electrophoresis or lateral flow strips are used for detection of amplification products.[39]

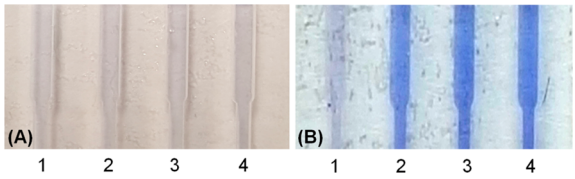

Figure 3. Photographs of the isothermal amplification reactors (A) before and (B) after 40 min incubation in the chemically heated cup. Leuco crystal violet dye is used as an amplification indicator. Amplification reactors 1, 2, 3, and 4 contain 0, 5, 50, and 500 PFU of ZIKV.

Figure 3B clearly indicates that as few as 5 PFU of ZIKV can be detected in saliva sample with our simple, inexpensive point of care diagnostic system. In this work, we relied on end point detection. The fluorescence intensity change of the dye can, however, be monitored in real time with a cellphone camera as we have previously done with intercalating fluorescent dye.[18] Real-time monitoring would yield a threshold time which can be correlated with the number of templates in the sample, as shown in Figure 2.

To evaluate the sensitivity of our POC diagnostic system, we compared its qualitative performance with that of a benchtop

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Anal. Chem. 2016, 88, 7289−7294

RT-LAMP, amplifying in each case known concentrations of ZIKV. Table 2 summarizes the number of positive experiments

Table 2. ZIKV RT-LAMP Assay with Benchtop Equipment and with Our POC Diagnostic System[a]

samples	benchtop tests	on-chip tests
500 PFU/reaction	3/3	5/5
50 PFU/reaction	3/3	5/5
5 PFU/reaction	3/3	5/5
1 PFU/reaction	2/3	3/5
0 PFU/reaction	0/3	0/5

[a]The table documents the number of positive results normalized with the number of tests.

normalized with the total number of tests in each category. In each case, we have successfully detected as few as 5 PFU ZIKV per sample.

Typical viremia of a symptomatic patient infected with ZIKV ranges from 10^3 PFU/mL to 10^6 PFU/mL.[40] The typical sample volume processed by our device is 50−100 μL. This corresponds to a detection limit better than 50−100 PFU/mL, which is more than sufficient to detect viremia in patients infected with ZIKV. Because the sample volume that can be processed with our device is decoupled from the reaction volume, we can increase device sensitivity even further by increasing the sample volume.

CONCLUSIONS AND OUTLOOK

We designed and verified a set of new RT-LAMP primers for ZIKV, targeting the highly conserved sequence of the envelope protein-coding region. This RT-LAMP assay facilitates rapid and sensitive benchtop detection of ZIKV. The sensitivity of the RT-LAMP assay is comparable to that of RT-PCR. Our in-silico design of the LAMP primers suggests high-efficiency amplification ZIKV templates and lack cross-interaction (false positives) with any of the sequences in the GenBank database. In this paper, we have verified that our primers are, indeed, highly efficient. The experimental verification of the absence of cross-reactivity with any other pathogens is deferred to future work.

More importantly, the RT-LAMP assay can be readily adapted for use at the point of care. To demonstrate the applicability of our assay for point of care detection, we used our microfluidic POC diagnostic system to detect ZIKV in saliva samples spiked with ZIKV. Our disposable, inexpensive microfluidic cassette contains four independent amplification reactors, each equipped with a silica membrane at its inlet for nucleic acid isolation, purification, and concentration. This design has the advantage of decoupling the sample volume from the reaction volume, enabling one to use relatively high sample volumes and achieve high sensitivity. Nucleic acids immobilized on the membrane serve as templates in an enzymatic isothermal amplification process without a need for an elution step, reducing the number of operations and simplifying flow control. The cassette is combined with an inexpensive processor composed of a chemically heated cup for electricity-free operation. The cup generates heat for the isothermal amplification process by an exothermic chemical reaction. The RT-LAMP products are detected by eye with a LCV dye that changes from colorless to violet in the presence of dsDNA.

We tested the utility of our POC diagnostic system with raw saliva samples spiked with various concentrations of the ZIKV. Our experiments indicate that our electricity-free point of care diagnostic system detects ZIKV in saliva with the sensitivity of less than 5 PFU of ZIKV per sample within 40 min. Our POC system is comparable to that of the benchtop assay without a need for laboratory facilities, expensive equipment, and skilled personnel.

Further improvements of our POC device will include the storage of lyophilized LAMP/RT-LAMP reagents in the enzymatic amplification reactor.[41] The prestored reagents will be protected by paraffin to avoid premature hydration during nucleic acid extraction. When the amplification chamber is heated to its operating temperature, the paraffin will melt and move out of the way and the amplification reagents will get hydrated just in time.

Although in this paper, we demonstrated only qualitative detection of ZIKV, we anticipate that our method can be expanded to include quantification. Indeed, in a previous work[18] with a different target, we used intercalating fluorescent dye to identify the presence of dsDNA. Our chemically heated cup interfaced with a smartphone. The smartphone flashlight excited the fluorescent dye and the phone camera recorded the fluorescence emission during the amplification process. A custom-made smartphone application will be developed to convert the images into emission intensity, analyze the data, and determine threshold times of the amplification curves, which is then used to estimate template concentration. The smartphone can be programmed not only for image processing and data analysis and reporting but also for communications and GPS recording, which can be useful in tracking the migration of the vector. Other extensions of our work can include multiplexing for concurrent detection of mosquito-borne pathogens such as dengue[42] and chikungunya.[43]

In summary, our paper describes a simple, inexpensive, and sensitive RT-LAMP assay and an inexpensive instrument-free POC diagnostic system for rapid nucleic acid−based molecular diagnostics at the point of care. This convenient, inexpensive, portable assay strategy has the potential to meet urgent needs in endemic regions with stressed resources.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b01632.

Section S1: RT-LAMP primer design; Section S2: Description of the RT-PCR assay protocol; Section S3: Field detection of Zika virus with chemically heated cup (PDF)

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Notes

The authors declare no competing financial interest.

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