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Lab-on-a-Chip Zika Detection With Reverse Transcription Loop-Mediated Isothermal Amplification–Based Assay for Point-of-Care Settings

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• Context.—Zika virus (ZIKV) infection, primarily transmitted by mosquitoes, causes various neurologic disorders. To differentiate ZIKV from other arboviruses, such as dengue, chikungunya, and yellow fever viruses, a highly specific, sensitive, and automated detection system is needed for point-of-care (POC) settings.

Objective.—To detect ZIKV at POC settings, we have developed a fully automated lab-on-a-chip microfluidic platform for rapid disease detection by using reverse transcription loop–mediated isothermal amplification.

Design.—The developed setup consists of a microfluidic chip, a platform for magnetic actuation, and a heater along with the sensor to precisely control the temperature for the target amplification. The platform accurately controls the movement of the magnetic beads that enable the isolation and purification of the target nucleotides adhered to their surface for the amplification and disease detection on the microfluidic chip.

Results.—Within 40 minutes, change in color due to the presence of ZIKV amplicons was visually observed with the spiked plasma samples in the end point analysis. Also, we have accurately and specifically identified ZIKV in a small number of de-identified clinical samples.

Conclusions.—All-inclusive, the developed fully automated POC ZIKV diagnostic chip is rapid, simple, easy-to-use, inexpensive, and suitable for the areas where facilities are limited.

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There are many diseases spread by mosquitoes, including Zika, dengue, yellow fever, and chikungunya infections.1,2 Zika virus (ZIKV) infection is associated with neurologic complications, such as Guillain–Barre syndrome, meningoencephalitis, acute myelitis in adults, and microcephaly in infants.3 According to the Centers for Disease Control and Prevention, from 2015 to 2018, a total of 5304 ZIKV cases were reported in the United States, and during the same time period 36 522 ZIKV cases were observed in US territories.4 The main mode of ZIKV transmission is mosquitoes; the other transmission modes include sexual transmission, infants born to mothers with established ZIKV infection, breast milk, saliva, blood transfusion, and needlestick.5–7 The symptoms of ZIKV are closely associated with those of other mosquito-borne vector infections, such as dengue, yellow fever, and chikungunya virus, which include headache, rash, fever, and joint pain. All of these viruses share similar symptoms, making it difficult to detect ZIKV in the patient.8,9 This demands a need for a highly specific and sensitive ZIKV test that can detect the infection quickly. There are several diagnostic methods developed for ZIKV detection, such as lateral flow assays,9 enzyme-linked immunosorbent assay,10 immunoglobulin M (IgM) antibody capture enzyme-linked immunosorbent assay,11,12 and reverse transcriptase–polymerase chain reaction (RT-PCR).13,14 RT-PCR is a conventional ZIKV-specific method that remains the gold standard for disease detection from the patient sample. However, RT-PCR is time-consuming and requires trained personnel as well as expensive equipment, such as a thermocycler. This poses a challenging issue for people living in areas where medical facilities are minimal and access to laboratory services is difficult. Additionally, pure RNA isolated from blood, urine, or plasma is used for the detection of the virus; impurities from the raw sample can inhibit the reaction and can also give false-negative results.6,15 Nearly 2 decades ago, the loop-mediated isothermal amplification (LAMP) method was reported and was capable of amplifying DNA and RNA at an isothermal temperature.16 It is a quick, robust, and specific method that amplifies the target at a fixed temperature that usually ranges between 65°C to 74°C with the help of a 4 to 6 set of primers. LAMP eradicates the requirement of different temperature cycling6 making it the better amplification method over PCR for the low-cost point-of-care (POC) diagnostics. For disease detection, treatment validation, and outcome, POC diagnostics plays

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an important role in expediting the detection of the disease in resource-constrained areas.27 The Zika virus detection chip we report in this manuscript will provide a practical approach for a simple, reliable, and cost-efficient platform that provides high sensitivity and specificity without the access of trained technicians, expensive equipment, or special facilities including electricity.

Previously, significant efforts have been made for the development of POC Zika diagnostics.18–20 Nevertheless, all of these developed methods have several limitations, such as complex chip assembly, manhandled processing steps, air-drying membrane before introducing the LAMP reagents, and equipment (eg, smartphone) required for result interpretation. Furthermore, the colorimetric detection method in at least 1 of the studies demonstrated false-positive results due to nonspecific binding.6 To address these limitations of current Zika diagnostics we have developed an automated lab-on-a-chip microfluidic platform that detects Zika virus from human blood plasma with high specificity and sensitivity, eliminating human intervention at any step after sample loading. The newly designed microfluidic chip described in this article is planned according to the approach that unifies multiple steps on the same platform. Once the target sample is introduced into the inlet chamber (chamber 1), the developed automated platform will motorize the target isolation, purification, and amplification for the disease detection on the chip. Change of color upon the presence of the Zika virus target in the amplification chamber (chamber 4) is observed due to the colorimetric properties of leucocrystal violet (LCV) dye.21–23 The on-chip results from this automated assay revealed its sensitivity by showing positive results with the plasma having a minimum clinical range of target (10^2 plaque-forming units [PFUs] per milliliter) found in a Zika-infected patient. Moreover, the developed portable microfluidic chip also provides a rapid diagnostic assay that is easy to use and affordable. The developed system is a real-sample-in-answer-out assay, which is fully automated and does not require any user intervention after the sample input.

MATERIALS AND METHODS

Zika Target and Zika Reverse Transcription Loop-Mediated Isothermal Amplification Primer Design

In this investigation, RNA sequences of 50 different Zika virus samples isolated from blood, urine, breast milk, serum, plasma, and fetal brain were obtained from the National Center for Biotechnology Information24 to design Zika-specific reverse transcription LAMP (RT-LAMP) primers. These strains were mainly from North and South America, and the detailed information (such as accession number, host, isolation source, country, and collection date) of these sequences is presented in Supplemental Table 1. These genomic sequences were aligned in the multiple sequence alignment tool Clustal Omega.25 Supplemental Figure 1 represents the picture of sequences aligned in Clustal W. The most conserved region, which was the 429 bp from the GenBank accession number KX702400, was chosen as the target sequence to design Zika-specific RT-LAMP primers. The RT-LAMP primers were designed with the freely available online software Primer Explorer V5 from Eiken Chemical Co Ltd. Aimed at the preliminary testing of the primers, the conserved target sequence was synthesized in the form of DNA (based on conserved Zika RNA sequence). The primers and conserved target sequence were synthesized by the commercial vendor Integrated DNA Technologies (Coralville, Iowa). The designed Zika virus primers are documented in Table 1, along with their concentrations.

| Primer Name | Sequence 5’ to 3’ | Concentration, μM |
|-------------|-------------------|-------------------|
| F3          | CTGGTTGGTTCACAAG  | 0.2               |
| B3          | CAGACGTTCCACGCAAG| 0.2               |
| FIP         | GTTGGTACCGTGTCCTT  | 1.6               |
| BIP         | CTGGTAGAGTGCAAGCGGA-TGCTCCTCTTGTACCTCCCTA | 1.6 |
| LF          | CCCAGGCTGCCAAGGTAT | 0.4               |
| LB          | CATGGCCGAAAGGCAACTGTC | 0.4               |

Off-Chip Benchtop Testing

Benchtop LAMP Amplification.—For the amplification of the nucleotide target with the designed primers, the LavaLAMP RNA Master Mix kit from Lucigen (Middleton, Wisconsin) was used. The amplification reaction was carried out with different Zika virus target concentrations (1 to 10^8 DNA copies per reaction) to assess the sensitivity. Simultaneously the negative control reactions, to examine the nontarget amplification, included primers plus Master Mix (PM), and to check the specificity, human immunodeficiency virus (HIV) target reactions were also carried out with Zika virus primers. Each LAMP reaction had a total volume of 25 μL, and the setup included Master Mix (12.5 μL), green fluorescent dye (1 μL), Zika RT-LAMP primers (2.5 μL), target Zika/HIV (1 μL), and nanopure water (8 μL). For the isothermal amplification, the Agilent AriaMix Real-Time PCR system (thermocycler; Santa Clara, California) was used at 68°C for 40 minutes for the result analysis. Real-time amplification data were collected with the help of the fluorescence signal at 520 nm. The benchtop LAMP-amplified products were further confirmed with 1% agarose gel electrophoresis where the resultant amplicons were stained with 6X Orange loading dye (Thermo Scientific) before being loaded into the wells, and to estimate the size of the amplicons, a 1-kbp size DNA ladder was used.

For visual testing, we prepared LCV by adding 5 mM β-cyclodextrin in 20 mL of Nanopure water, followed by the addition of 0.5 mM crystal violet, which turned the solution to a dark violet color. The colorless LCV solution was obtained by adding 30 mM sodium sulfite.19,21 The 25-μL LAMP reaction setup included Master Mix (12.5 μL), LCV (5 μL), Zika RT-LAMP primers (2.5 μL), target Zika/HIV DNA (1 μL), and water (4 μL). The amplification reaction was carried at 68°C for 40 minutes in the thermocycler.

Zika Spiked Human Blood Plasma Sample and Clinical Zika Sample Testing.—To characterize the sensitivity and specificity of the primers with the human blood plasma, we used synthetic Zika DNA for the initial validation. To simulate the real samples, we collected multiple de-identified human plasma samples (from the healthy patient) and spiked them with the various concentrations of the Zika virus target. The viremia found in an infected human ranges from 10^2 to 10^6 PFU/mL.19,26,27 Considering a Zika virus particle contains a single RNA copy, we spiked the plasma samples ranging from 10^2 to 10^6 DNA copies/mL with Zika virus target and 1 plasma sample spiked with 10^6 DNA copies/mL of HIV target.
for negative control. The spiked target nucleotides were isolated from 100 µL of plasma with magnetic beads (1-µm size) from Dynabeads SILANE viral Nucleic Acid (NA) kit (Invitrogen, Carlsbad, California). After the isolation and purification of the magnetic beads, all the DNA (from host and ZIKV) was collected in 50 µL of elution buffer followed by the LAMP amplification in a thermocycler at 68°C. Figure 1, A, illustrates the manual processing of the blood plasma with the laboratory settings. LAMP reaction setup consists of Master Mix (12.5 µL), green fluorescent dye (1 µL), and ZIKV RT-LAMP primers (2.5 µL). To increase the sensitivity, the volume of the target was increased from 1 to 10 µL, making the final volume of the reaction 26 µL. The fluorescence data of the isothermal amplification at 68°C for 40 minutes were collected for the end point investigation, followed by 1% gel electrophoresis to further validate the amplification.

To verify the specificity of the designed primers and viral NA kit with the clinical virus samples, we tested them against cultured and clinical ZIKV samples. The 5 tested virus samples included 2 ZIKV cultured samples, 2 ZIKV plasma samples, and 1 dengue (type 3) plasma sample collected from infected patients. These samples were labeled as Zika 3, Zika 4, Zika 118, Zika 119, and 545-dengue, respectively. Table 2 describes the details about these samples, such as the number of days of sample collection from the patient after the first sign of symptom, the quantification of viremia of the cultured samples, and the commercial source from which these clinical samples were obtained. Extraction and purification of the nucleic acid from these samples were performed in a biologic safety cabinet by using a Dynabeads SILANE viral NA kit. A total of 100 µL of each viral sample was used in the beginning, and all the nucleic acid content was eluted in 50 µL of elution buffer for the benchtop isothermal amplification at 68°C for 40 cycles (1 cycle = 1 minute). The RT-LAMP reaction setup included Master Mix (12.5 µL), green fluorescent dye (1 µL), ZIKV RT-LAMP primers (2.5 µL), elution buffer containing target (5 µL), and Nanopure water (4 µL).

To verify the results the amplification product was further subjected to 1% gel electrophoresis.

### Microfluidic Chip, Automated Platform, and On-Chip Testing

**Microfluidic Chip Assembly.**—The microfluidic chip (70 × 75 mm) consists of 3 layers made up of poly(methyl methacrylate). The top and bottom layers are 750 µm thick, and the middle well layer is 1.5 mm thick (Figure 1, B). These layers are attached with double-sided adhesive tape. The layout of the individual layer (for both poly(methyl methacrylate) and double-sided adhesive) was patterned in AutoCAD, and CO2 laser cutter was used to cut these layers, as previously reported.28–32 Each designed microfluidic chip consists of 4 independent diamond-shaped aqueous chambers—1 inlet chamber (chamber 1), 2 washing buffer chambers (chambers 2 and 3), and 1 amplification chamber (chamber 4)—3 elliptical-shaped valving chambers containing mineral oil (viscosity- 15 cSt), and 1 unconnected oval-shaped sensor chamber. Figure 1, C, is a colored representation of the chip filled with the buffers and reagents, and Figure 2 represents the overall layout and the approach of the developed microfluidic chip, indicating the task of each designed chamber. The dimension of aqueous chambers and valving chambers of the microfluidic chip is illustrated in Supplemental Figure 2.

**Magnetic Actuation.**—The magnetic actuation platform used in this research work was designed and optimized previously in our lab.28 The movement of magnetic beads is executed by the small magnets (5-mm–diameter neodymium), which are enclosed with a stepper motor, and it can move bidirectionally. The magnetic actuation is coordinated by an Arduino, and the magnetic beads’ movement from one chamber to another as well as the incubation time in each chamber was controlled by a gcode scripted in python.

| Clinical Patient Samples | Disease | Source | Amount | No. of Days of Sample Collection After the First Sign of Symptoms | Commercial Source | PCR Results |
|--------------------------|---------|--------|--------|---------------------------------------------------------------|-------------------|-------------|
| D0000202545 (545-dengue) | Dengue  | Plasma | —      | 1                                                             | Boca Biolistic    | Type 3      |
| NR-51118 (Zika 118)      | Zika    | Plasma | —      | 7                                                             | BEI Resources     | NA          |
| NR-51119 (Zika 119)      | Zika    | Plasma | —      | 7                                                             | BEI Resources     | NA          |

| Cultured Samples         | Amount | No. of Days of Sample Collection After the First Sign of Symptoms | Commercial Source | PCR Results |
|--------------------------|--------|---------------------------------------------------------------|-------------------|-------------|
| M P T3 (Zika 3)          | 5 × 10³ PFU/mL | 5                                                             | BEI Resources     | NA          |
| M P T4 (Zika 4)          | 5 × 10³ PFU/mL | —                                                             | Boca Biolistic    | Type 3      |

Abbreviations: NA, not available; PCR, polymerase chain reaction; PFU, plague-forming unit.

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**Table 2. Information of the Clinical Virus Sample Used for the Validation of Designed Zika Virus Primers**
Surface Heater Controller Setup.—To enable on-chip heating capabilities, we developed an automated Arduino-based temperature controller to strictly control the temperature of the reagents enclosed in chamber 4 on this microfluidic chip. The sensor chamber (contains sensor) and chamber 4 were filled with the same reagents, and 1 surface heater was attached on top of both chambers. Figure 3 represents the assembly of the automated setup. The k-type thermocouple sensor reads the actual temperature of the reagent in real time by interfacing with the MAX6675 module (Maxim Integrated, San Jose, California). The Arduino shield controls the 1.98-W, 2 × 2 cm, rectangular-shaped ultrathin nano carbon flexible heater (TSA(C) 0200020eR12.6, Pelonis Tech., Exton, Pennsylvania) by controlling the MOSFET gate signals (Supplemental Figure 3 is the circuit schematic illustration for the heater). The Arduino powers the heater (5V); therefore, no external power source was required. The “on” red LED visualizes the heater operation by lighting the LED when it is heating the reagents in the sensor chamber, and it is turned “off” when the feedback temperature is higher than the set temperature. The sensor controls the temperature of the chamber 4, which was set at 70°C ± 2°C.

Automated Platform Integration and On-Chip Sample Processing.—The automated platform consists of disposable microfluidic chip, magnetic actuation platform, surface heater, and a laptop to regulate the automatic magnetic actuation and control the heater’s temperature. The human plasma samples from a healthy individual were spiked with the different concentrations of ZIKV target (10, 10², 10³, 10⁴, and 10⁸ target copies/mL) relevant to the clinical range of the patients (10² to 10⁸ PFU/mL) to load in chamber 1. The negative controls were 10⁸ copies/mL HIV target spiked in human plasma and unbiased plasma sample collected from a healthy person. For the on-chip testing, chamber 4 was filled with LavaLAMP RNA Master Mix, ZIKV-specific primers, elution buffer, and LCV for the colorimetric detection. The LAMP reaction reagents for chamber 4 were formulated before running the experimentation. The pipette inlets of chamber 4 and sensor chamber were sealed with epoxy glue (Loctite epoxy) to avoid evaporation of the Master Mix, which dried within 5 minutes. The first step (plasma sample for chamber 1) was carried out in an Eppendorf tube (0.5-mL tubes) to ensure accuracy. In the Eppendorf tube, 20 μL of protease K was added to 100 μL of plasma, followed by the addition of 95 μL of lysis and binding buffer. The tube was incubated for 2 to 3 minutes.
Abbreviation: LCV, leucocrystal violet.

at room temperature, after which 45 μL of isopropanol and 15 μL of magnetic beads were added and incubated on the rocker for about 5 to 7 minutes. While the sample was incubating on the rocker, with the pipette through pipette inlets the chip was loaded with buffer 1 (1:1, buffer 1 to Nanopure water) in chamber 2 and buffer 2 in chamber 3. Subsequently loading these chambers, the mineral oil was added to the valving chambers, and in the end the plasma sample mixed with buffers and magnetic beads was introduced into the chamber 1.

Immediately after the chip loading, the surface heater was attached on top of chamber 4 and sensor chamber (epoxy glue is dried by now). The heater was turned “on” 3 minutes before the start of magnetic actuation and once the target is eluted in chamber 4, magnetic beads were moved back to chamber 3. The heater was kept “on” for an additional 30 minutes for the LAMP amplification process and after the isothermal incubation, the results were visually observed. The volume of each chamber and incubation time of the magnetic beads is presented in Table 3.

### RESULTS AND DISCUSSION

As a member of the Flaviviridae family, ZIKV symptoms overlap with dengue and chikungunya infections. For this reason, there is an unmet need for designing highly specific ZIKV primers that can differentiate the ZIKV infection from other arboviruses. In this paper we have identified a highly conserved target sequence by aligning sequences of 50 different ZIKV strains. The conserved section of the genome encodes for “partial envelope protein” in ZIKV and is used to design the novel ZIKV-specific primers in the presented work. The conserved target sequence was BLAST searched against other flaviviruses, and the results showed high diversity with other flaviviruses and excessive homology to ZIKV. Additionally, to check the unambiguity of the primers for ZIKV, another BLAST search was carried in the GenBank nucleotide database that revealed that the primers are specific to the ZIKV genome.

### Benchtop LAMP Amplification Results

Isothermal amplification and less sensitivity of LAMP toward common amplification inhibitors make it a better choice for health care infrastructure in resource-limited areas and also overcome the main limitations (cost, sensitivity, etc) of the current gold standard PCR technique. However, the inadequacy of using several primers in the LAMP leads to the primer-dimer formation (nontarget amplification), which can result in false positives. The reaction carried out with the designed ZIKV primers and Master Mix to eliminate the nontarget amplification indicated no sign of amplification. The collected fluorescent data showed no change in fluorescence intensity during the period which stipulates that designed primers would bind exclusively to the ZIKV target. Simultaneously, the primers amplified the synthetic ZIKV target, and an abrupt increase in the fluorescence value was seen in the reactions holding different ZIKV concentrations. The lowest limit of detection observed was 10^5 DNA copies per reaction, and the time to result (TTR) for the amplification reaction carrying 10^5, 10^6, 10^7, and 10^8 copies per reaction fell anywhere between 5 and 15 minutes. At the same time, no increase in the fluorescent signal was observed in the negative control reaction carrying HIV target; this disclosed the primer’s specificity toward the ZIKV target. Figure 4, A, represents the amplification plot of the benchtop experiment for specificity, sensitivity, and the primer dimer formation evaluation. In Supplemental Figure 4, we observed the good linear fit curve with R^2 = 0.8852; the higher the concentration of the target in the reaction, the lesser the TTR. In the LAMP reaction, the real-time fluorescent signals are not always exponential because during the amplification process the DNA segments are extended, and simultaneously short segments are also produced. The 1% gel electrophoresis further confirmed these LAMP off-chip results (Figure 4, B). The long, sharp bands of the ZIKV amplicons stained with loading dye in the wells could be seen exhibiting amplification confirmation, and no band formation was spotted in HIV, PM, and water wells, validating the specificity of these primers.

Furthermore, we examined a visual detection method that eliminated the need for fluorescence excitation equipment, making the platform more user-friendly. Leucocrystal violet is generally colorless; however, in the presence of double-stranded DNA the LCV interrelates with the major grooves of the double-stranded DNA, thereby converting LCV into crystal violet. This imparts a violet color to the solution for colorimetric visual observation. In Figure 5, after the isothermal amplification the reactions that exhibited a dark crystal violet coloration show that the ZIKV target amplified. On the other hand, the faint blue coloration in tubes illustrates that the LAMP reaction did not proceed, and no amplification occurred. Notably, the target detection sensitivity remained as before, that is, 10^5 DNA copies per reaction. Further, the original RGB image was analyzed using ImageJ, and the intensity was calculated by converting RGB pixels to gray value. Supplemental Figure 5 corresponds to the gray intensity value of the resultant products. The amplification reaction is saturated; therefore, the intensity of the color was independent of the initial target concentration. The defined threshold value (175 arbitrary units [a.u.]) clearly depicts the difference between the negative reactions (W, PM, HIV, and 10 copies per reaction) and the positive reactions (10^5, 10^6, 10^7, and 10^8 copies per reaction). This test indicates that the naked-eye test is accurate and amplified the specific ZIKV target in LAMP reaction.

### Results of Spiked Samples and Clinical Samples

To present a proof-of-concept of our designed assay, we spiked the synthetic target into human plasma samples with
a clinical viremia range (10^2 to 10^7 copies/mL). The target DNA was isolated from the human plasma with the abovementioned viral NA kit followed by the LAMP. The low pH of the buffers facilitates target binding to the magnetic beads, and a high pH of elution buffer at a high temperature of 70°C releases the target into the elution buffer. The isothermal fluorescent data obtained from the thermocycler (Figure 6, A) showed a rise in fluorescent signals with the reactions containing the ZIKV target extract from the human plasma. The lowest limit of detection observed was 10^2 copies/mL. There was neither an increase in fluorescence intensity of the HIV target (extracted from plasma) nor primer-dimer formation observed during the entire time of incubation. The results indicate that the viral NA kit is highly capable of isolating the target from plasma with excellent capture efficiency, and the designed LAMP primers are very efficient in amplifying the low quantity of ZIKV. Moreover, this also validates that the buffers used from the kit remove the unwanted components from the plasma that could possibly interfere with the LAMP. Supplemental Figure 6 represents the linear trendline with R^2 = 0.9366, certifying the efficiency of the LAMP reactions carrying different concentrations of targets isolated from plasma samples. We successfully verified the results with 1% agarose gel electrophoresis (Figure 6, B) in which the sharp bands correspond to ZIKA amplification in the wells containing the ZIKV target.

All of the flaviviruses contain single positive-strand RNA, but the RNA-based detection methods come with the challenge of RNA degradation during the extraction process. Therefore, we tested the viral NA kit and the designed primers against the clinical virus samples. The real-time fluorescent data obtained from the RT-LAMP reaction (Figure 7, A) showed a clear amplification trend with the Zika 3, Zika 4, Zika 118, and Zika 119 (ZIKV sample). Zika 3–cultured sample showed an early sign of amplification at TTR 7 minutes. Zika 4, Zika 118, and Zika 119 samples showed a rise in the signal after 15 minutes of incubation; however, the saturated fluorescent amplification signals were achieved within 30 minutes. The viral quantification of the clinical plasma sample was unknown; however, amplification signal validates the detection sensitivity of the assay in terms of “yes-or-no” at different TTRs. The RT-LAMP exponential curve is often unprecise, and therefore difference in the amplification efficacy and TTR were observed in the cultured samples. Furthermore, no rise in fluorescent signals with 545-dengue (dengue sample) and PM reactions was observed that eradicated the risks of cross-reactivity with these novel designed primers. These results were verified by 1% gel electrophoresis, where the band formation illustrated no inhibitory effect of viral NA kit.
Figure 6. A, Amplification curve of the Zika virus (ZIKV) spiked plasma samples (10^2 to 10^7 copies/mL) pertinent to clinical range. B, The 1% gel electrophoresis results of the loop-mediated isothermal amplification products of plasma samples spiked with ZIKV. Abbreviations: HIV, human immunodeficiency virus; L, DNA ladder; PM, primer plus Master Mix; W, water.

Figure 7. A, Reverse transcription loop-mediated isothermal amplification plot for the 2 cultured and 2 clinical plasma Zika virus and 1 clinical plasma dengue samples. B, Validation of the clinical sample amplification results with 1% gel electrophoresis (lane L is 1-kbp DNA ladder used as size indicator). Abbreviations: PM, primer plus Master Mix; W, water.
and primers with the ZIKV samples, and the dengue sample did not show any sign of amplification, further verifying the primer’s specificity toward ZIKV (Figure 7, B). The experimental results of clinical virus samples in this project proved that the developed assay and reagents used are compatible not only with DNA but also with RNA by maintaining its integrity for virus detection. These experiments validated the capability of the designed primers to specifically amplify ZIKV RNA targets.

Microfluidic Chip and Automated Platform

To enable POC testing, we designed a microfluidic chip containing 4 different chambers for different processes involved in LAMP-based detection. The chip was optimized to retain the liquid inside the channels, avoid mixing of the reagents, and assist movement of magnetic beads for the POC testing. The hydrophobic interaction in the aqueous chambers holds the fluid and the curvature of valving chamber provides less turbulence, which facilitates the easy flow of the magnetic beads. The chamber 4 is carefully separated from other chambers to prevent the precipitation formation of the buffers due to the heating effect. The top layer contains 2 pipette inlets (0.4-mm diameter) above each chamber: 1 inlet to discharge of the fluid into the chip and 1 inlet to liberate the air out of the chamber.

The optimal temperature for LAMP amplification ranges from 68°C to 74°C; therefore, to maintain the ideal on-chip amplification conditions, the temperature was chosen to be 2°C higher than the off-chip experimental temperature used in the thermocycler. Additionally, the elution of nucleic acid content from the magnetic beads takes place at 70°C, and thus the heater was turned “on” 3 minutes before the start of magnetic actuation to ensure the chamber acquired the required temperature by the time the beads reached chamber 4 to elute the target. The temperature display (Supplemental Figure 7) shows the measured feedback every 2 seconds. The graph illustrates that after approximately 4 minutes the chamber reagents gain the set temperature of 70°C, which is optimal for the elution of the target. The feedback temperature control system efficiently controls the temperature for all of the phases, which supports accurate target elution and amplification for the ZIKV detection. Additionally, to avoid interference with colorimetric results, the magnetic beads are moved back to chamber 3 after the target elution.

On-Chip Spiked Plasma Results

To demonstrate the capability of visual detection of ZIKV using our developed system, we conducted the automated microfluidic platform test in the laboratory. Figure 8, A, is an image of chamber 4 (amplification chamber) filled with LAMP reaction reagents before the microfluidic automated chip run. The spiked ZIKV plasma samples containing $10^8$, $10^9$, $10^3$, and $10^2$ target copies/mL showed positive results of the target intensification (Figure 8, B, C, D, and E, respectively) by changing the color of the reagents in the chamber 4. The presence of double-stranded DNA changed the color to violet, which showed the successful amplification of the ZIKV target. The results revealed that the device has optimal settings for the target isolation and purification. The lowest clinical viremia range of a ZIKV-infected patient is $10^2$ PFU/mL, and this setup achieves the sensitivity of $10^2$ copies/mL for target detection. At the same time, the off-chip negative control HIV-spiked sample assay did not change color in chamber 4 (Figure 8, F), which implied that the assay was highly specific to the ZIKV target. Similarly, on-chip negative control unbiased plasma results (Figure 8, G) also eliminated the odds of the false-positive outcome by not showing the color change, showing no primer dimer formation. Furthermore, for the sensitivity test with an automated microfluidic platform, we lowered the target copies to 10 ZIKV copies/mL in the plasma. No change in the color of chamber 4 demonstrated negative results (Figure 8, H), indicating the setup was unable to detect the ZIKV target with such a low target concentration in the plasma. The plasma sample volume used for this setup was 100 μL for each run. If we increase the initial plasma sample volume, the sensitivity of the chip can be further increased if required. The multistep target extraction process, which is unified on the platform, has proven to avoid contamination for the downstream amplification process. The magnetic beads are compatible with the LAMP reaction reagents, and therefore the elution and amplification can take place in the same chamber, making this microfluidic chip less complex. The successful on-chip amplification of the ZIKV target from the plasma sample is attributed to several factors, such as the use of small-scale volume of the buffers and reagents for the operations, capture efficiency of the magnetic beads, and compatibility of the magnetic beads with the LAMP reagents/polymerase. Moreover, the visual and noninhibitory effect of the dye provided a high on-chip sensitivity. Furthermore, the gray intensity plot (Supplemental Figure 8) of on-chip positive amplification reaction ($10^6$, $10^7$, $10^3$, and $10^2$ target copies/mL) showed a value significantly lower than 175 a.u. (threshold value), and negative amplification reactions showed a value higher than the threshold. The on-chip and off-chip gray intensity values follow the same trend, hence validating the accuracy and specificity of the primers, reagents, and the colorimetric dye on the microfluidic chip.

Supplemental Video 1 demonstrates the execution sequence of the developed microfluidic platform. The video, in the beginning, labeled as “before,” demonstrates the chip loaded with all the reagents and buffers right before the automated run. Next is a “time-lapse” magnetic actuation run that displays target isolation, washing, elution, and amplification. The magnetic bead movement from one chamber to another and oscillation in each chamber are directed by small magnets situated in the platform. After the beads eluted the target into chamber 4 (amplification...
occur while performing benchtop assays. The inaccuracies the developed setup can be battery operated and may have a superior to the existing conventional methods. The developed assay would also eliminate the cost setback, making it competitive with the assay are optimized to provide highly specific and qualitative results without any human involvement after sample loading. The experimental confirmation of the test against negative controls, including dengue, HIV, and unbiased plasma from healthy humans verifies the specificity of the primers toward ZIKV, which addresses one of the main issues of false-positive (cross-reactivity) results faced in POC diagnostics. With this automated microfluidic platform, we were able to achieve sensitivity with the lowest clinical range (found in the infected patient) sample, having 2 copies/mL of ZIKV target, within 40 minutes, making it a rapid ZIKV screening POC diagnostic method. This automated setup would facilitate accurate ZIKV diagnosis with low cost and high sensitivity, providing an excellent opportunity for field application and eliminating the requirement of benchtop examination in resource-limited areas.

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CONCLUSIONS

We have developed an automated microfluidic chip–based LAMP assay that combines the isolation, purification, and amplification steps on the same platform and enables the visual detection of the ZIKV from human plasma within 40 minutes. The entire ZIKV diagnosis procedure is executed inside a uniquely designed, inexpensive, disposable microfluidic chip. All the components and reagents associated with the assay are optimized to provide highly specific and qualitative results without any human involvement after sample loading. The experimental confirmation of the test against negative controls, including dengue, HIV, and unbiased plasma from healthy humans verifies the specificity of the primers toward ZIKV, which addresses one of the main issues of false-positive (cross-reactivity) results faced in POC diagnostics. With this automated microfluidic platform, we were able to achieve sensitivity with the lowest clinical range (found in the infected patient) sample, having 2 copies/mL of ZIKV target, within 40 minutes, making it a rapid ZIKV screening POC diagnostic method. This automated setup would facilitate accurate ZIKV diagnosis with low cost and high sensitivity, providing an excellent opportunity for field application and eliminating the requirement of benchtop examination in resource-limited areas.

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