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Mayaro virus detection by integrating sample preparation with isothermal amplification in portable devices

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Abstract

Mayaro virus (MAYV) is an emerging mosquito-borne alphavirus that causes clinical symptoms similar to those caused by Chikungunya virus (CHIKV), Dengue virus (DENV), and Zika virus (ZIKV). To differentiate MAYV from these viruses diagnostically, we have developed a portable device that integrates sample preparation with real-time, reverse-transcription, loop-mediated isothermal amplification (rRT-LAMP). First, we designed a rRT-LAMP assay targeting MAYV's *non-structural protein* (NS1) gene and determined the limit of detection of at least 10 viral genome equivalents per reaction. The assay was specific for MAYV, without cross-reactions with CHIKV, DENV, or ZIKV. The rRT-LAMP assay was integrated with a sample preparation device (SPD) wherein virus lysis and RNA enrichment/purification were carried out on the spot, without requiring pipetting, while subsequent real-time amplification device (RAD) enables virus detection at the point of care (POC). The functions of our platform were demonstrated using purified MAYV RNA or blood samples containing viable viruses. We have used the devices for detection of MAYV in as short as 13 min, with limit of detection to as low as 10 GEs/reaction.

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Keywords

Mayaro virus; rRT-LAMP; Point of care; Valve; Real-time amplification

Introduction

Mayaro virus (MAYV), genus *Alphavirus*, is an RNA virus that is transmitted by mosquitoes [1–3]. Human infections with MAYV may result in Mayaro fever, which is an acute febrile illness that is often accompanied by joint and muscle pain. The clinical presentation and course of MAYV is similar to that seen with another alphavirus, Chikungunya virus (CHIKV); there are also similarities with the clinical presentation seen with arboviruses, Dengue viruses 1 to 4 (DENV-1 to DENV-4), and Zika virus (ZIKV), making it difficult to differentiate MAYV infection clinically from these other viruses [4–6]. MAYV has been identified as an emerging virus in the Americas, highlighting the need for development of effective detection methods for diagnostic evaluations [1, 7]. This challenge is particularly true in regions where the aforementioned viruses are endemic, resulting in the requirement of specific diagnosis at the point of care (POC) for clinical management and development of appropriate public health interventions.

The standard techniques for detection of MAYV infection include serological tests such as enzyme-linked immunosorbent assay (ELISA) [4, 6, 8–10] and molecular tests such as reverse transcription polymerase chain reactions (RT-PCR) [6, 8, 11–13]. Traditional serological tests are of limited value for detection of acute infections (due to time required to produce antibodies in the blood of patients), and there are potential issues with cross reactivity with other closely related viruses. Acute diagnosis of MAYV infections generally requires RT-PCR, but standard RT-PCR techniques can be time-consuming and require skilled personal and costly equipment. As a result, they are typically carried out in laboratories, making them impractical for diagnostics at the POC, and are not affordable in clinical settings with limited resources, such as those regions where MAYV infections are usually encountered. Antigen-test strips based on lateral flow assays, such as those commonly used for coronavirus disease 2019 (COVID-19), influenza, and other virus' diagnostic tests, are not yet available for MAYV. They also tend to be less sensitive than molecular tests.

Loop-mediated isothermal amplification (LAMP) has proven to be a rapid and sensitive diagnostic technique for the detection of various pathogens [14–19]. Compared with PCR, LAMP requires simpler thermal management due to the isothermal condition, is faster (~ 30 min. versus 2 h for PCR) and has similar sensitivity. LAMP requires 4–6 primers that target 6–8 distinct sequences, providing high specificity. For RNA viruses such as MAYV, reverse transcription LAMP (RT-LAMP) can be used. RT-LAMP has been demonstrated to detect a wide range of RNA viruses including CHIKV [20–22], DENV-1 to DENV-4 [20–24], Ebola virus [25], human immunodeficiency virus (HIV) [26, 27], influenza virus [28–30], severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [28, 29, 31–38], and ZIKV [20–22, 39–41]. A few commercially available instruments based on LAMP or RT-LAMP

also exist, including NINA from PATH, BioRanger from Diagenetix, and Lucira Check It from Lucira Health [42–44].

A few real-time LAMP-based POC platforms have been reported in the literature. Although they are well-designed, sample preparation was not included in their detection systems, either using RNA/DNA purified in laboratory or directly adding a sample to the reaction mixture. It has been shown that LAMP assay without sample preparation reduced the detection sensitivity [31] and increased the number of false negatives [45]. We have aimed to integrate sample preparation with real-time RT-LAMP (rRT-LAMP) for virus detection at POC.

In this work, we report the development of a highly sensitive, specific, and rapid rRT-LAMP assay for the detection of MAYV. Additionally, we have developed POC platforms for carrying out the rRT-LAMP assay.

To achieve this, we designed a sample preparation device that performs virus lysis and RNA enrichment/purification without requiring pipetting. This device follows similar concepts to our previously developed device [40] and is capable of processing blood samples at the POC. Note that the previous device was done with colorimetric detection only, which was carried out at the end of 30-min amplification. The colorimetric detection gives only binary results: the presence or absence of viruses of interest. The real-time POC detection platform, integrated with the sample preparation device, enables the quantitative detection of MAYV (i.e., virus burden) at the POC.

We have assessed the sensitivity and specificity of the rRT-LAMP assay and demonstrated the utility of our platforms using purified MAYV RNA, plasma containing virus, or blood samples containing virus. Our results indicate that the rRT-LAMP assay is specific and highly sensitive, and the POC platform has the potential to be used in the field, including resource-limited regions.

Materials and methods

Cell culture for MAYV

MAYV strain Guyane was obtained from BEI Resources (Manassas, VA; Catalog. no. NR-49911) and propagated in Vero E6 cells, which were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cell culture took place at 37 °C and 5% CO₂ using advanced Dulbecco's essential medium (aDMEM) and 10% fetal bovine serum (FBS, Thermo Fisher Scientific) as previously described [46].

After about 80% of the cells displayed virus-induced cytopathic effects, virus RNA was extracted from spent cell culture medium using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions [47].

rRT-LAMP mix

A solution of 25 µL of mixture was used for each RT-LAMP assay. The 25 µL of mixture contains 2.5 µL of 10X isothermal amplification buffer (ISO), 8 U Bst 2.0 WarmStart[®] DNA

polymerase, 7.5 U WarmStart[®] RTx reverse transcriptase (RTx), 2.5 μ L of 10X concentrated primer mix (PMX), 1.4 mM deoxynucleotide triphosphate (dNTPs), and 6 mM MgSO₄. To eliminate possible carryover contamination, 0.5 units of antarctic thermolabile uracil-DNA glycosylase (UDG) and 0.7 mM of deoxyuridine triphosphate (dUTP) were also added to the mixture [48, 49]. Nuclease-free water was then added to fill the 25 μ L volume. The nuclease-free water and dNTPs were purchased from Thermo Fisher Scientific (MA, USA). All other reagents were obtained from New England Biolabs (NEB, Ipswich, MA, USA). The final volume of each component for preparation of 25 μ L RT-LAMP reaction mixture is tabulated in Electronic Supplementary Material Table S2. When real-time detection was used, an appropriate dye (either SYBR Green I or SYTO9) was added as detailed below.

rRT-LAMP using a commercial cyclor

We used a QuantStudio-3 real-time PCR (qPCR) instrument to study the effects of the primer concentrations on the incubation time and to determine the detection limit of our rRT-LAMP assay. rRT-LAMP assays were carried out by adding 1 μ L of purified RNA and 0.5 μ L of 10X concentrate SYBR Green I nucleic acid gel stain in dimethyl sulfoxide (Thermo Fisher Scientific) to the 25 μ L RT-LAMP reaction mixture. A no-template control (NTC) was included as a negative control in each test. The reactions were carried out at 62.5 °C for 60 min. The fluorescence signals obtained by the PCR machine were analyzed through the QuantStudio-3 (Thermo Fisher Scientific). We carried out three replicates for each RNA concentration, and the results presented in this work are the average of the three replicates.

POC sample preparation device

The sample preparation device (SPD) is an improved version of our previously developed device for the detection of ZIKV [40]. As shown in Electronic Supplementary Material Figure S1, SPD consists of buffer, mixing, detection, and drain units. The buffer and mixing units were 3D printed from polylactic acid (PLA). The detection unit is a paper-based amplification device consisting of a polycarbonate well layer and a laminated paper pad. The laminated paper pad is a 4-mm Whatman chromatography paper (Fisher Scientific, Pittsburgh, PA, USA) that is sandwiched between two 75- μ m-thick polyester lamination films. Chromatography paper was chosen in the device because it showed a lower limit of detection than FTA card and glass-fiber paper [50]. The SPD is developed to enable RNA purification and enrichment at the POC without the need for laboratory equipment. The RNA enrichment process in the previous device relied on the wicking effects of chromatography paper in the detection unit, which was adequate for processing low viscous samples such as saliva and urine. However, it took long time to process a viscous blood sample. To improve the device, we have developed a drain unit made of polydimethylsiloxane (PDMS), including a 1.9 cm \times 1.9 cm chamber for the detection unit and a 6 mm drain well in the center connected to a 1-mm drain channel (Figure S1). This unit allows for the use of external forces such as a syringe to connect with the drain channel to discharge both the lysed sample and buffers quickly when needed. The size of the chamber was optimized to ensure PDMS's intrinsic sealing capability without the use of any sealant during the integration of the detection unit into the drain unit. The new design significantly reduced the sample preparation time to as low as 7 min (5-min incubation for

lysis and 2-min flushing of all reagents in the buffer unit). A comparison between previously reported device (named VLEAD [40]) and SPD is presented in Electronic Supplementary Material Table S1.

Real-time amplification device

As shown in Fig. 1 and Figure S2, our real-time amplification device (RAD) for rRT-LAMP at POC is composed of a portable stand, digital microscope (AM4117MT-G2FBW, Dino-Lite, USA), a temperature controller, a heater, and two 9-V rechargeable batteries. The stand was 3D printed from PLA, with a base containing three chambers for the heater, batteries, and temperature controller. Three caps were also 3D-printed for covering the three chambers. The cap for the heater chamber was designed to contain a 2×2 cm² cavity, which properly fits the detection unit in Figure S1 (which is also described later). In addition, two 5-mm holes were created in the cap, enabling real-time detection of the rRT-LAMP reactions (one hole for the sample and the other for the negative control).

The digital microscope possesses two bandpass filters which allow only desired excitation and emission wavelength to be transmitted through. The peak excitation wavelength is 465 nm while the emission band is 510–545 nm, as shown in Figure S3b. These wavelengths are compatible with the excitation/emission wavelengths of SYTO9 (Figure S3a), which was used as fluorescent dye in our real-time detection platform. We replaced SYBR Green with SYTO9 in the RAD since SYTO9 has far less inhibitory effects on rRT-LAMP than SYBR Green [51]. We found that 4 μ M of SYTO9 is optimal for obtaining highest fluorescence intensity without inhibition on rRT-LAMP.

The heater used for rRT-LAMP is a positive temperature coefficient (PTC) heater (Bolsen Tech, USA), which can be powered by batteries, a laptop, or a smartphone. A commercially available, low-cost temperature controller was used to switch the PTC heater on or off. The controller's thermostat sensor was attached to the PTC heater, and the sensor temperature was calibrated to set the rRT-LAMP reaction at 62.5 °C. We characterized the heating system by placing a T-type thermocouple in the rRT-LAMP well, and the resultant temperature profile is shown in Figure S4. The results illustrate that the rRT-LAMP temperature was maintained between 62.5 and 63.1 °C, which is within the standard temperature range (60–65 °C) recommended for LAMP. The batteries can last for about 3 h, sufficient for at least 5 experiments. The total weight of RAD is 983 g, which is portable.

Results and discussion

POC testing

Detection can be performed at the end of the amplification reactions by direct visual observation of color change, which is known as endpoint detection. The endpoint detection is suitable for POC applications when the test can be performed for a specific, predetermined time, without the need for an expensive and complex detector. This method provides a binary result (“yes” or “no”), representing the presence or absence of a target virus, without any quantitative information. Alternatively, real-time detection can be employed to continuously monitor and analyze RT-LAMP, enabling dynamic observation

of the RT-LAMP results. For positive samples, signals can be detected way before the predetermined time used for the endpoint detection, resulting in a much shorter analysis time. Additionally, this analysis time can be used to infer quantitative information about the amount of virus present in the sample tested because the less the amount of virus, the longer the amplification time required to have detectable signals.

We have employed the SPD for either endpoint detection or real-time detection of MAYV at the POC. To perform real-time detection of MAYV, we have integrated the SPD with RAD for sample preparation and real-time amplification. The SPD was designed for sample preparation and RNA enrichment/purification at POC. After RNA purification on the paper pad of the detection unit, the detection unit of the SPD was separated from mixing unit and then placed in the cavity designed in the cap of the heater chamber in the RAD shown in Fig. 1 and Figure S2. The digital microscope in Fig. 1 was programmed to acquire fluorescence images every minute. The light-emitting diode (LED) in the microscope was programmed to turn on for 5 s and off for 55 s to avoid possible photobleaching. Two Python-based apps (one compatible with the computer operating system while the other with the smartphone operating system) were developed for measuring fluorescence intensity and displaying the data in real time.

Figure 2 shows the process flow of our portable pathogen detection platform. For the detection of MAYV, a sample (e.g., virus, plasma, or blood) is introduced to the lysis buffer in the first reservoir of the buffer unit (Fig. 2a). By sliding the mixing unit along the buffer unit (detailed in Figure S1), the pin in the mixing unit pushes up the ball at the bottom of the first reservoir when they are aligned (Figure S1b and Figure S1c), discharging the solution in the reservoir from the buffer unit to the mixing unit. This ball-based valve design was inspired by a ballpoint pen, in which ink is dispensed onto paper when the metal ball at the tip is pressed while writing. Further sliding of the mixing unit along the buffer unit discharges the binding buffer in the second reservoir, resulting in retention of viral RNA on the paper pad when the solution in the mixing unit flows through the detection unit. The discharge process is sped up by pulling the plunger of the syringe that is connected to the drain unit (Figure S1). The enriched RNA on the paper pad is then purified after two wash buffers in the third and fourth reservoirs are discharged in sequence by further sliding the mixing unit. After RNA purification, the detection unit is taken apart from SPD, followed by adding 25 μ L rRT-LAMP mixture (Fig. 2b). Afterwards, the detection unit is placed in the cavity inside the cap of the heater chamber (while the temperature controller and the PTC heater have been switched on about 5 min earlier). The microscope is then positioned on top of the rRT-LAMP reaction well and takes pictures with 1-min intervals (Fig. 2c). The Python-coded software converts the images from the microscope into a graph. The sample preparation using SPD and rRT-LAMP using RAD are shown in the Electronic Supplementary Material (Video 1).

In some situations when binary detection is adequate, we performed isothermal amplification using endpoint detection. In this case, 25 μ L RT-LAMP mixture is added into the detection unit, which is placed in a battery-powered coffee mug as illustrated in Figure S5. The mug functions as an isothermal water bath incubator, enabling RT-LAMP at

62.5 °C for 30 min. The colorimetric detection is carried out at the end using SYBR Green I dye; color change is observed by naked eye or recorded by a smartphone [40].

rRT-LAMP assay

To design LAMP primers, we obtained the genome sequence of MAYV strain MAYLC from French Guiana from National Center for Biotechnology Information (NCBI, accession no. DQ001069). This virus strain was chosen as its genomic sequence has high identity with MAYV strains from Haiti and Venezuela that we have recently analyzed [46, 52] and thus serves as a contemporary reference strain for the type of MAYV we have tested in this work. Primer Explorer V5 program (<https://primerexplorer.jp>) was used to design the specific primers for MAYV under default control parameters. Two sets of rRT-LAMP primers were selected, targeting the conserved NS1 region of MAYV (Figure S6). Each primer set consists of forward inner primer (FIP), backward inner primer (BIP), forward loop primer (FLP), backward loop primer (BLP), forward external primer (F3), and backward external primer (B3), and their sequences are listed in Table 1 [14, 15].

Both primer set#1 and primer set#2 were checked for possible primer dimerization using Multiple Primer Analyzer from Thermo Fisher Scientific, and we found no problematic dimer formation among primers. Possible hairpin structure formation was analyzed using OligoAnalyzer from Integrated DNA Technologies (IDT); no hairpin structures were found to exist at 62.5 °C, the reaction temperature that was used for rRT-LAMP in this work.

First, we employed the qPCR instrument to study the rRT-LAMP conditions such as assay time. We also used the qPCR instrument to compare primer set #1 with primer set #2 in Table 1 in terms of rRT-LAMP assay performance. In addition, we studied the effects of the primer concentrations on the assay performance since they are known to affect LAMP assays [53–55]. We compared the primer concentrations recommended by the LAMP kit manufacturer, New England Biolabs (NEB), with the primer concentrations at half the recommended concentrations, referred to as “1/2NEB,” based on our experience and the literature [28]. The concentrations of all primers used under these two conditions are detailed in Table S3. rRT-LAMP assays were carried out by using 1 µL RNA at concentrations from 5×10^3 to 5×10^6 GEs/µl. Figure S7 shows rRT-LAMP curves using primer set #1 and #2. In either primer sets, inferior assay performance was obtained at NEB than at 1/2NEB. For example, for primer set #1, all four concentrations of viral RNA tested were detectable at 1/2NEB, whereas only two highest concentrations (5×10^6 GEs/µl and 5×10^5 GEs/µl) were detectable at NEB. In addition, although all four concentrations were detected using both primer sets under the 1/2NEB condition, the detection time with primer set #1 (threshold time of 8–14 min.) was shorter than primer set #2 (threshold time of 18–26 min.). Therefore, we chose primer set #1 and 1/2NEB primer concentrations for all subsequent experiments.

Assay sensitivity and specificity

We investigated the limit of detection of the rRT-LAMP assay for MAYV detection. First, we used the qPCR machine to determine the required amplification time for low amount of viral RNA. Figure 3a shows real-time amplification curves of viral RNA ranging from 5

$\times 10^6$ to 50 GE μ L. The results showed that all samples containing various concentrations of RNA reached a plateau within 30 min, indicating that 30 min RT-LAMP reaction is sufficient if we use the endpoint detection. The calibration curve between the threshold time (reported by the qPCR machine) and viral RNA concentration (Fig. 3b) indicates the feasibility of semiquantitative MAYV detection.

By fixing the amplification time at 30 min and not using the qPCR machine as an optical detector, we explored the colorimetric detection at the end. This method provides “yes (the presence of MAYV)” or “no (the absence of MAYV)” results observed with the naked eye. For this endpoint detection, 0.5 μ L of 10000X concentrate SYBR Green I was added to the mixture after the reaction. We first performed the endpoint detection of serial dilutions of the MAYV RNA, ranging from 5 to 5×10^6 , to compare with the real-time detection results shown in Fig. 3a and determine the visual limit of detection of the assay. The results shown in Figure S8 confirmed that the visual limit of detection of the 30-min RT-LAMP is between 50 and 5 GE μ L. To further narrow down the limit of detection of 30 min assay, we performed the endpoint detection in tube using 15, 10, and 5 GE μ L of MAYV RNA. Ten experiments at each RNA concentration were conducted to ensure reproducibility of the assay and the results shown in Fig. 3c. The results in Fig. 3c show that all samples containing 10 GE μ L and 15 GE μ L of MAYV RNA were detected, whereas only six out of 10 samples with 5 GE μ L were detected, indicating that visual limit of detection of our RT-LAMP assay is at least 10 GE μ L of MAYV RNA (i.e., 1 μ L of 10 GE μ L). This limit of detection is at least comparable to, if not better than, the limit of detection of 8.2 copies/ μ L with 5 μ L of MAYV RNA (i.e., 41 copies total) using rRT-PCR [56].

To study the specificity of the RT-LAMP assay for MAYV detection, we tested it against four different mosquito-borne RNA viruses: CHIKV, two subtypes of DENV: DENV-1 and DENV-4, and ZIKV. Before experiments, we employed the basic local alignment search tool (BLAST) from NCBI to evaluate the similarities of the primer sequences designed for MAYV with the genomic sequences of the other four viruses. No similarities between the MAYV primers and sequences of other viruses were found. Isothermal amplifications were subsequently performed using the MAYV primers, and each of the other viruses while the endpoint detection was used. For each test, 1 μ L of 10^6 GE μ L virus RNA was tested per 25- μ L RT-LAMP reaction. As shown in Fig. 3d, the RT-LAMP assay generated positive signals only for MAYV RNA, remaining negative for RNA of the other four viruses, their mixture, and the no-template control (NTC). The results indicate that there is no cross-reactivity between the MAYV RT-LAMP assays for the four mosquito-borne viruses tested.

MAYV endpoint detection using SPD

Our platform, which utilizes molecular amplification for the detection of MAYV, offers improved sensitivity and specificity compared to other LAMP-based POC platforms. This is due to the ability to process larger sample volumes (50 to 200 μ L) than typical microfluidic platforms which can only handle a few microliters or less [57]. This leads to a lower detection limit, as more virus RNA can be enriched onto the detection unit's paper pad. Additionally, compared to those devices that benefit from the use of direct sample load,

our detection system enables sample preparation and RNA purification at POC. It has been shown that LAMP assay without sample preparation reduced the detection sensitivity [31] and increased the number of false negatives [45].

A number of reports have documented erroneous outcomes in blood tests employing the LAMP method [58–62]. The LAMP technique has shown to generate false positive outcomes in turbidity assays due to the presence of inhibitors in the anticoagulant-containing blood samples [63] and protein precipitation [63, 64]. Unlike other POC LAMP-based devices developed for blood analysis in which the lysed blood is directly added to either RT-LAMP or LAMP reaction mixture [22, 65], our POC device eliminates all blood components by washing them out using washing buffers, as described above. This approach potentially eliminates false positive results stemming from blood components.

To carry out POC testing, we first employed SPD (Figure S1) for sample preparation and used the MAYV RT-LAMP for the endpoint detection. Prior to the test, for 1 volume (e.g., 50 μL) of the sample, SPD was pre-loaded with 4 volume of lysis buffer (e.g., 200 μL), 4 volume of binding buffer (molecular biology grade ethanol), 4 volume of washing buffer 1 (AW1, QIAGEN), and 4 volume washing buffer 2 (AW2, QIAGEN), followed by sealing reservoirs using a thermoplastic film. The lysis buffer was made by mixing 1 volume of AVL (AVL, QIAGEN) and 2 volume of red blood cell lysis buffer (2.5 mM KHCO_3 , 37.5 mM NH_4Cl , and 0.025 mM EDTA) (1 \times RBC lysis buffer, Thermo Fisher Scientific). Note that AVL was used for lysing viruses [28, 50], and 1 \times RBC lysis buffer was used for lysing red blood cells [66]. The operation of the SPD started with introducing 50 μL of MAYV sample. After a 5 min of incubation for sample lysis, the binding step was carried out by the sliding mechanism and ball-based valving incorporated in SPD as explained above. Washing steps were then performed in sequence by sliding between the buffer and mixing units. Enriched and purified RNA in the detection unit was subsequently subjected to 25 μL RT-LAMP and placed in a battery-powered coffee mug, for the endpoint detection. For the safety of the operator, virus samples were processed in a BSL-2+ (biosafety level 2 enhanced) laboratory. The device, syringe, and others in contact with the virus sample were disposed of as biohazard waste.

In order to assess the detection limit of our devices for sample-to-result testing at POC, we created whole blood samples by adding viable MAYV virus to healthy whole blood that were obtained from Innovative Research (Novi, MI, USA). The cultured virus used for spiking had a titer of 6×10^4 GE μL . We then prepared tenfold serial dilutions of this spiked sample using healthy blood to evaluate the device's performance. The SPD operation began by introducing 50 μL of the blood samples, followed by the SPD operation described above. As depicted in Fig. 4, we were able to detect MAYV from 6 GE μL (300 GE μL /reaction), but no signals were observed for the 0.6 GE μL (30 GE μL /reaction) and the negative blood control (NB, blood samples with no MAYV added). There were no false positives with the five negative blood samples tested. To ensure the robustness of our platform, ten replicates at two lowest MAYV amount were tested. As shown in Figure S9, all samples containing 6 GE μL (300 GE μL /reaction) were detected, whereas only five samples with 0.6 GE μL (30 GE μL /reaction) were detected, indicating that our POC system can detect at least 300 GE μL /reaction in 37 min (7 min for sample preparation using SPD and 30 min for

RT-LAMP reaction) when the endpoint detection is used. The detection limit of the device for blood samples spiked with cultured MAYV (viable viruses) is lower than the in-tube assay using purified MAYV RNA. The possible reasons include (1) some RNA molecules might be lost during the processing; (2) inhibitory substances in blood samples might not be completely removed, thus having adverse effects on RT-LAMP assays; or (3) combination of both.

To evaluate our detection platform for detection of MAYV in clinical samples, we tested three patient plasma samples from either Haiti or Venezuela. These samples were collected according to protocols approved by US and country-specific Institutional Review Boards (IRB), as described previously [67]. One patient from Haiti was infected with MAYV and one from Venezuela had DENV-1 and MAYV infections [67]. The third plasma sample was from a Haitian patient that has tested free of mosquito-borne viruses and served as a negative control sample. The analyst performing the work was initially blinded as to the identity of these samples. RT-LAMP assays were first performed in tubes, with three replicate tests for each sample. Positive MAYV signals resulted for the Haiti and Venezuela samples, but no signals were generated for the negative-control plasma samples and the NTC samples (Figure S10). We then performed the RT-LAMP assay for the detection of MAYV in these clinical samples using SPD by following the procedure explained above. Three replicates were carried out for each sample. Images of the detection unit for each sample are shown in Fig. 5a and b. The RT-LAMP results were also confirmed by gel electrophoresis, shown in Fig. 5c. These results demonstrated that our RT-LAMP assay with SPD could detect MAYV at POC. The test results agree with those obtained by RT-LAMP assay in tubes (Fig. S10) and those obtained by standard RT-PCR [67].

Real-time detection of MAYV

We developed a POC platform for rRT-LAMP, which was designed to assess the MAYV viral load at POC by combining the SPD (Figure S1) with RAD (Fig. 1). We first used four MAYV RNA samples containing 5×10^6 , 5×10^4 , 5×10^2 , and 50 GEs to evaluate the RAD. Each RNA sample was enriched using SPD, and the detection unit was then prepared for rRT-LAMP and placed in the heater chamber in Fig. 1. The amplification curves obtained from the flashlight-shaped digital microscope via the Python-based app are shown in Fig. 6a. The threshold time of each rRT-LAMP assay was calculated by the app, following the same definition as in real-time PCR (the time when the fluorescence signal reached 10 times the standard deviation of the baseline signals). We plotted the threshold time with the MAYV RNA amount, and a linear relationship between them was obtained as shown in Fig. 6b. This result indicates that our RAD system has the potential to provide quantitative information associated with MAYV virus load. We also performed rRT-LAMP assays of the same RNA samples using the commercial real-time PCR machine, and the threshold times were given by the software associated with the PCR machine. The results of amplification curves and the calibration curves obtained from the PCR machine are shown in Figure S11; they are comparable to that in Fig. 6, indicating that our POC platform is capable of providing with quantitative analysis of MAYV. The copy number of a gene of interest can be calculated from the threshold time using a standard curve [68]. This capability is crucial when/where the viral load of the virus in a patient needs to be monitored for clinical management and

for taking appropriate measures to prevent virus transmission. Figure 6 also depicts that the assay time was reduced to as low as 6 min if a positive signal was detected using the RAD. In other words, the time from the sample to result can be as short as 13 min (7 min for sample preparation using SPD and 6 min for rRT-LAMP reaction using RAD).

To further evaluate our RAD for POC testing, we used whole blood samples spiked with cultured MAYV virus. Two virus loads of 6000 and 600 GE/μL were tested. Figure 7 shows the amplification curve generated by the app, showing the fluorescence intensity as a function of time for different concentrations of MAYV. These results indicate that our platform can be used to obtain the MAYV viral load in whole blood samples at POC.

To evaluate the feasibility of RAD for quantitative detection of other pathogens, we performed the real-time detection of ZIKV, DENV-4, and SARS-CoV-2 using RAD, and the amplification curves were shown in Figure S12. The results demonstrated that our detection system can also detect other pathogens.

In our current real-time detection platform, the detection unit consists of two reaction wells: one for processing a sample and the other for the negative control (NTC). However, it is possible to accommodate multiple samples by adjusting the microscope to have a larger field of view.

The cost of a POC test plays a crucial role in its potential commercialization. Each SPD costs \$1.3, while the over-all cost of reagents and the RT-LAMP mixture amounts to \$4.45. Therefore, the total cost for a POC test using endpoint detection is \$5.75, which is lower than other POC devices in the literature [42, 44, 69].

For real-time detection, however, there is a one-time cost of RAD at \$1003. Although the RAD cost is higher than other real-time platforms reported in the literature [36, 43, 65, 70], our system is reusable, capable of extending for other functions. Note that our real-time detection platform is still more than one order of magnitude cheaper than those commercially available real-time PCR machines.

Conclusion

One of the main barriers for preventing and reducing virus-associated disease outbreaks, such as MAYV infection, is the lack of adequate tools for diagnosis, especially those providing fast and reliable detection at POC. Rapid testing is very crucial for clinical management and transmission reduction because the clinical symptoms of MAYV infection overlap with that of other mosquitoes-borne viruses such as CHIKV, DENV, and ZIKV.

LAMP is ideal for POC testing since it is an isothermal amplification technique, enabling it to be carried out using a constant heat source such as a heat block, water bath, and chemical reaction heat [71]. This advantage, as well as its faster reactions (30 min of endpoint detection versus 2 h of PCR) and similar sensitivity, represents the key advantages of LAMP over the PCR-based methods.

Our team developed a rRT-LAMP assay for detecting MAYV and determined that its visual limit of detection was at least 10 GEs/reaction using MAYV RNA. The assay exhibited no cross-reactivity with CHIKV, DENV, or ZIKV. To enable detection of MAYV at POC, we incorporated the assay with SPD capable of detecting at least 300 GEs/reaction of MAYV in a blood sample. Additionally, we developed a portable RAD capable of quantitatively detecting MAYV and demonstrated its capacity for POC quantitative analysis of the virus. The results showed that the integrated devices have the potential for addressing challenges associated with infectious disease outbreaks caused by MAYV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Biographies



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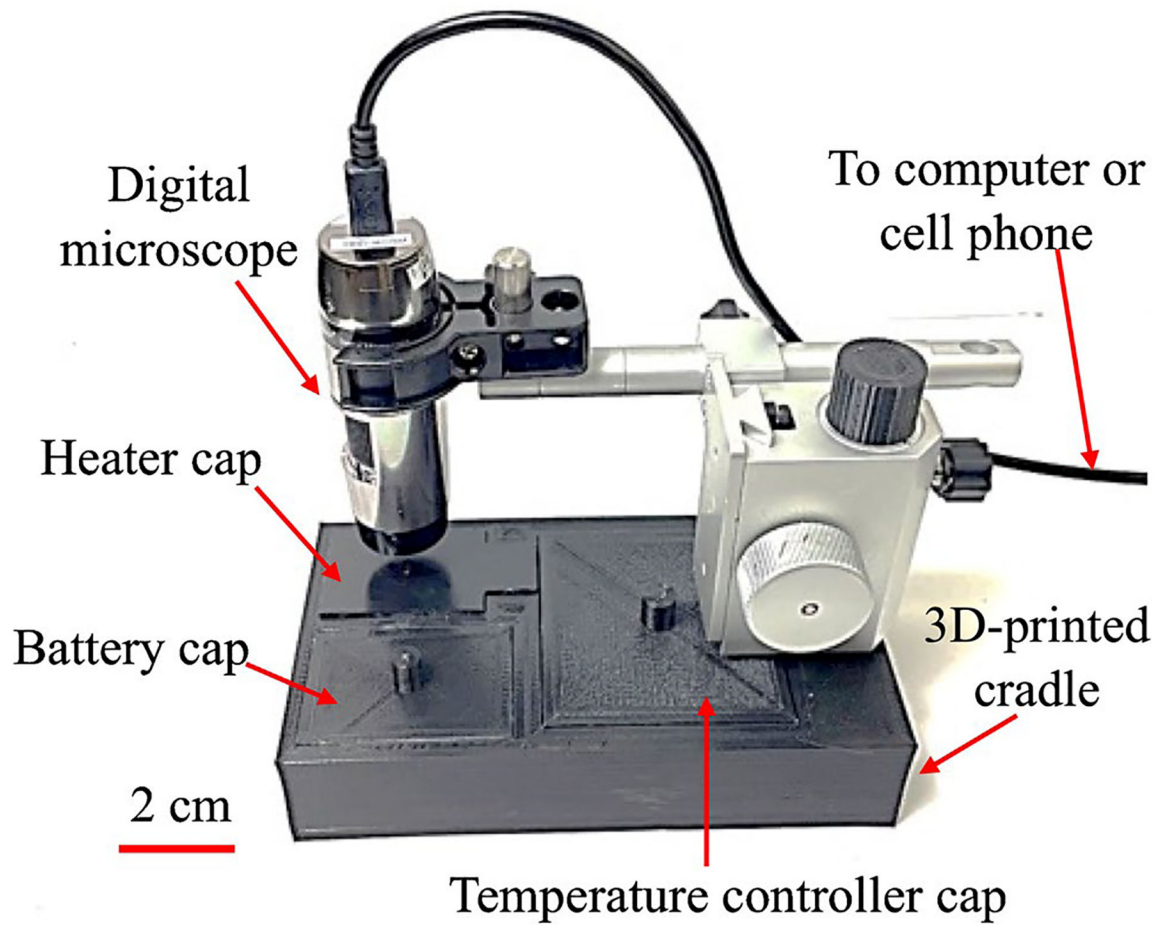


Fig. 1. Picture of a real-time amplification device (RAD) consisting of a flashlight-shaped microscope and 3D-printed chambers for a heater, battery, and integrated electrical circuit for temperature control. A scale bar of 2 cm is shown on the bottom left

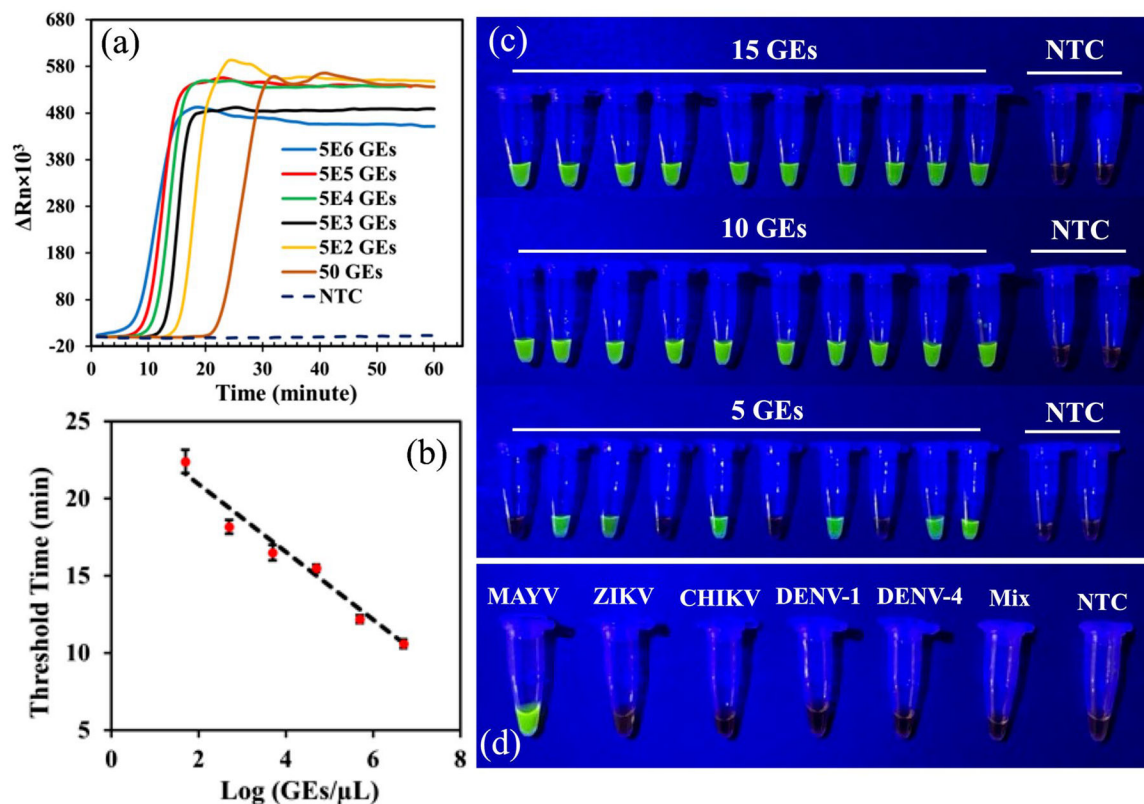


Fig. 3.
a. rRT-LAMP assay of 5×10^6 to 50 GEs MAYV RNA per reaction. All plots are an average of three replicates. **b.** Calibration curve between threshold time and MAYV concentration. **c.** RT-LAMP endpoint detection of 15, 10, and 5 GEs of MAYV RNA. The endpoint detection was carried out after 30-min RT-LAMP reactions. NTC stands for no-template control. **d.** No cross-reactivity was observed for MAYV-targeted RT-LAMP assay when it was carried out against ZIKV, CHIKV, DENV-1, DENV-4, and a mixture containing these four viruses.

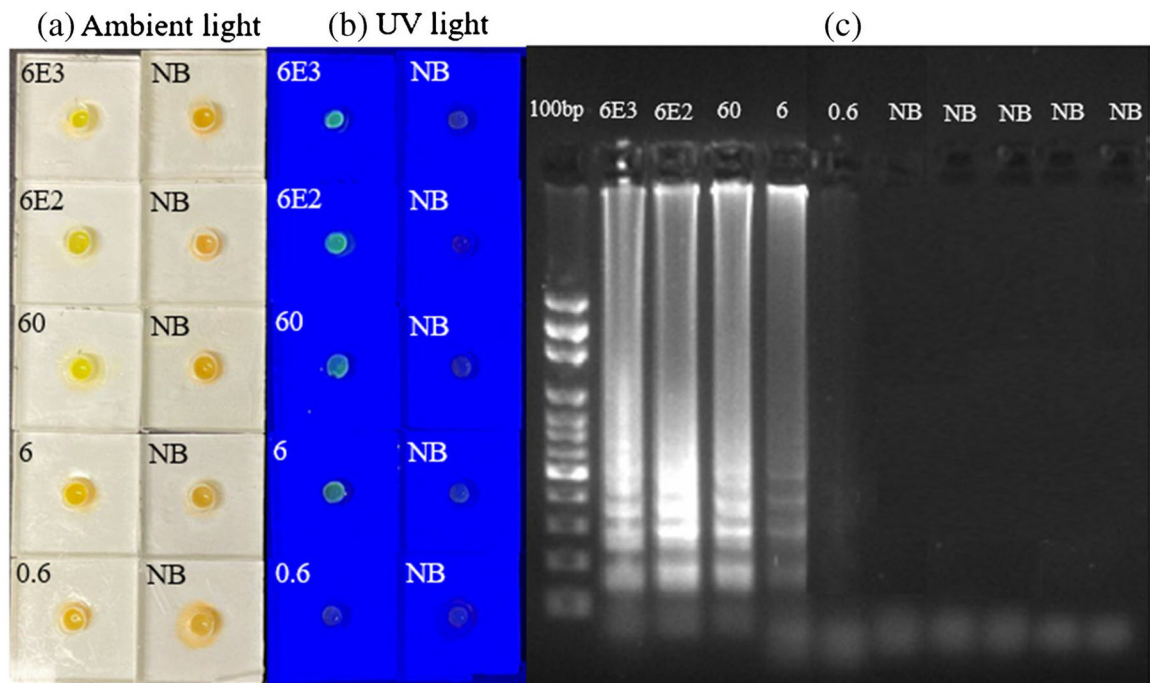


Fig. 4. Endpoint detection of MAYV in whole blood samples using SPD. **a.** Pictures of the detection units under ambient light after RT-LAMP assay of whole blood samples spiked with cultured MAYV (viable virus) and blood samples without MAYV as negative controls (NB). **b.** Pictures of the same detection units in (a), illuminated by a blue LED flashlight. **c.** Gel electrophoresis image of the amplicons from each device in (a). The first lane is a DNA ladder while other lanes are for samples in (a)

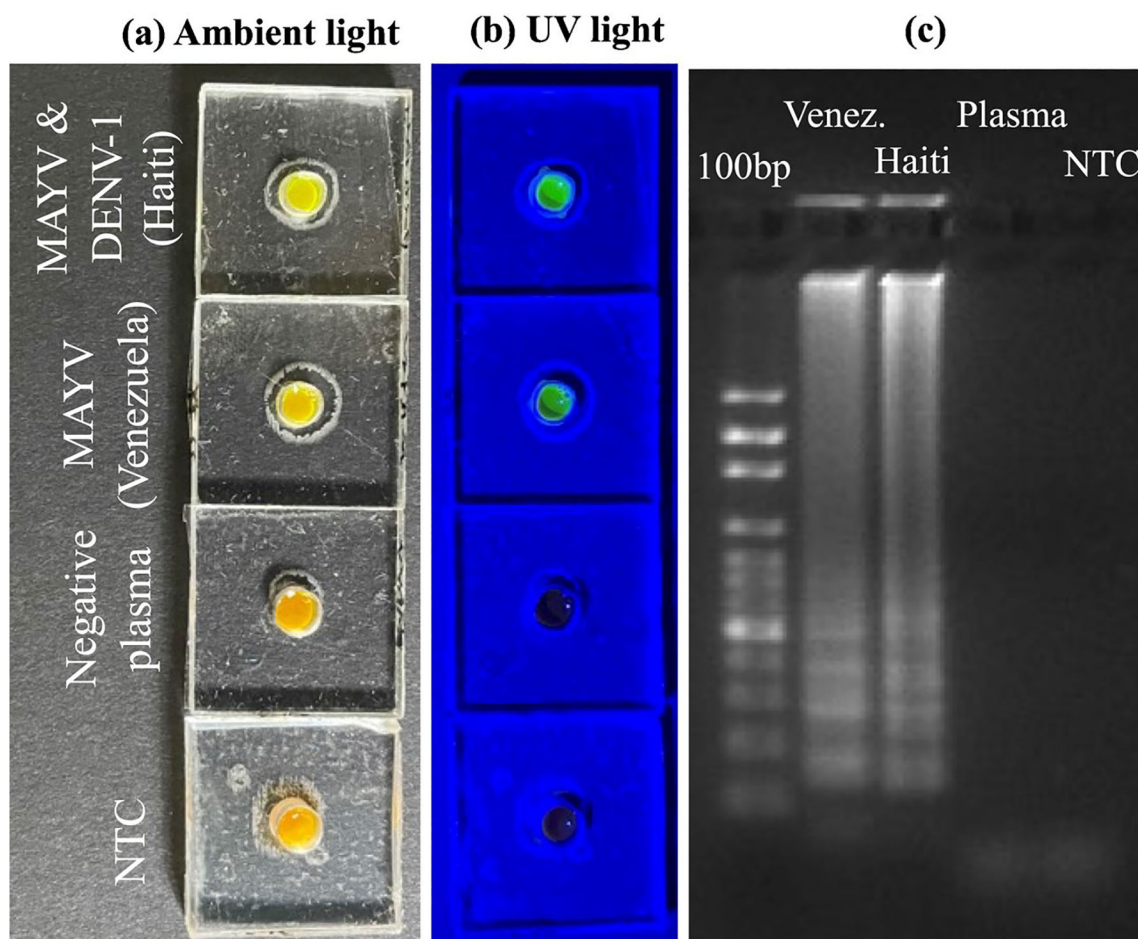


Fig. 5. Detection of MAYV in clinical samples using SPD. **a.** Pictures of the detection units under ambient light after RT-LAMP assay of three clinical samples containing MAYV, MAYV and DENV-1, and free of viruses, respectively, as indicated on the left. The NTC stands for no-template control. **b.** Pictures of the same detection units in (a), illuminated by a blue LED flashlight. **c.** Gel electrophoresis image of the amplicons from each device. The first lane is a DNA ladder while other lanes are for clinical samples and NTC

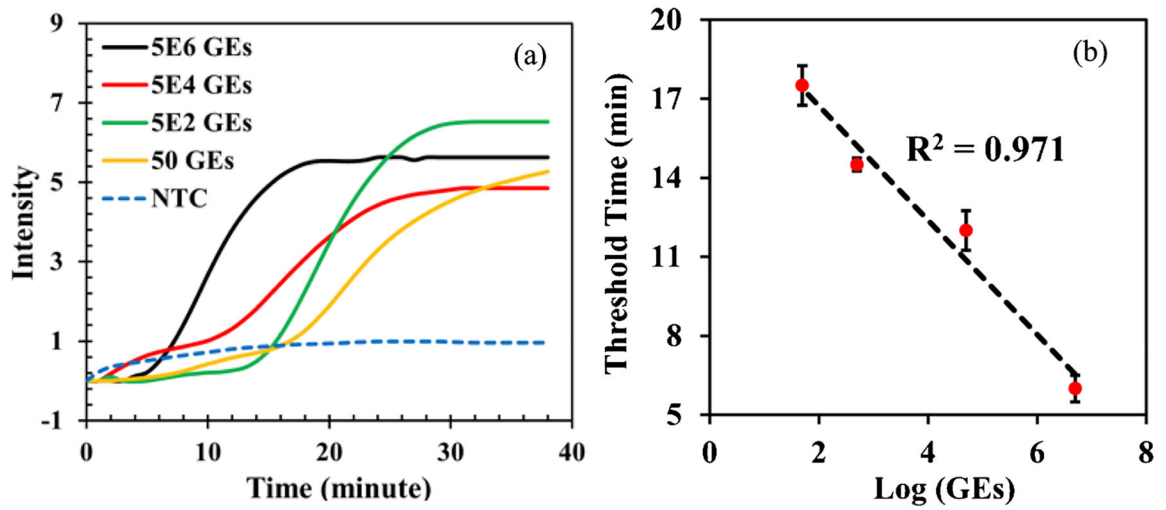


Fig. 6.

a. Real-time amplification curves obtained from RAD in Fig. 1. MAYV RNA concentrations of 5×10^6 , 5×10^4 , 5×10^2 , and 50 GEs were used in the rRT-LAMP assay. NTC stands for no-template control. **b.** Calibration curve of the rRT-LAMP assay between the threshold time and RNA amount of MAYV.

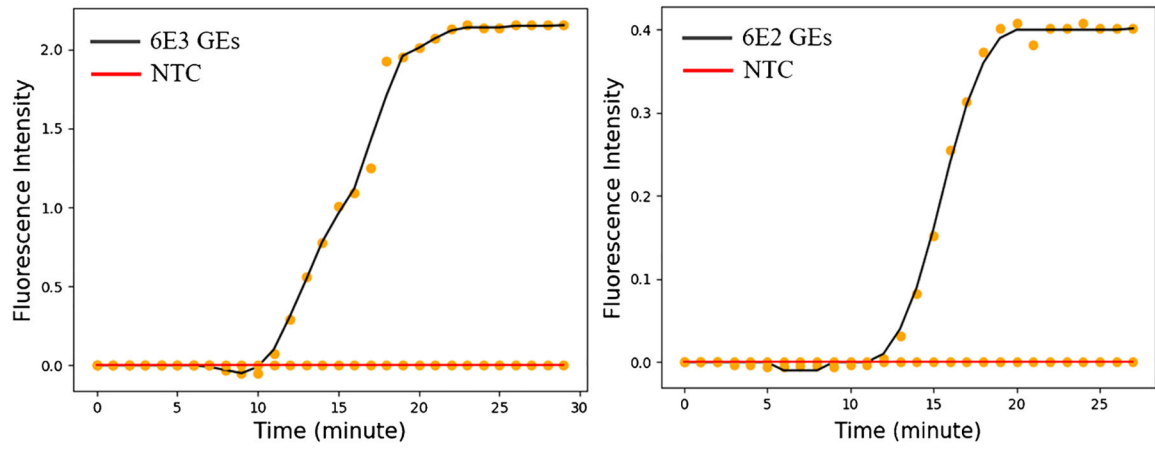


Fig. 7.

Amplification curves obtained from the RAD in Fig. 1. Two whole blood samples spiked with two different amounts of MAYV were tested. One replicate was carried out for each concentration. NTC stands for no-template control

Table 1

Sequences of the rRT-LAMP primers used for detection of MAYV RNA

Primer set #1 sequences (5'→3')		Primer set #2 sequence (5'→3')	
F3	ACTCATCCTGGATATCGGCA	F3	CGGACATTTGCCTTCACAC
B3	ACTCATTGTCCGGGGTCG	B3	GGCCAGTTGGTTGCATAT
FIP	AGACGCTCTGGGTCCTCAGCAATGATGTCTGAGCACACGT	FIP	TCGGTGCGTGAACTGCATAGACATCAGACATGCAGGACTCCA
BIP	AGCCAAGGCATCAGGTGAAGTACTGACTGCAGGTCGTCTA	BIP	GGAGTACGCACAGCGTACTGGCCCCGGCCATTGTATCGA
FLP	CATTGGGCACACACAATGGT	FLP	TGATAGACTGCCACCTCAGC
BLP	CGTTGACAGAAATATTGCAGCAAAG	BLP	ATTGGGTTTCGACACTACCCC

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