A Direct from Blood/Plasma Reverse Transcription–Polymerase Chain Reaction for Dengue Virus Detection in Point-of-Care Settings

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Abstract. Infection with dengue virus (DENV) is widespread across tropical regions and can result in severe disease. Early diagnosis is important both for patient management and to differentiate infections that present with similar symptoms, such as malaria, chikungunya, and Zika. Rapid diagnostic tests that are used presently for point-of-care detection of DENV antigens lack the sensitivity of molecular diagnostics that detect viral RNA. However, no molecular diagnostic test for DENV is available for use in field settings. In this study, we developed and validated a reverse transcription–polymerase chain reaction (RT-PCR) for the detection of DENV adapted for use in field settings. Reverse transcription–polymerase chain reaction was performed directly from plasma samples without RNA extraction. The assay detected all four serotypes of DENV spiked into blood or plasma. Our RT-PCR does not cross-react with pathogens that cause symptoms that overlap with dengue infection. The test performed equally well in a conventional laboratory qPCR instrument and a small, low-cost portable instrument that can be used in a field setting. The lower limit of detection for the assay was 1×10^4 genome copy equivalents/mL in blood. Finally, we validated our test using 126 archived patient samples. The sensitivity of our RT-PCR was 76.7% (95% CI: 65.8–87.9%) on the conventional instrument, and 78.3% (95% CI: 65.8–87.9%) on the field instrument, when compared with the RealStar Dengue RT-PCR Kit 2.0. The molecular test described here is user-friendly, low-cost, and can be used in regions with limited laboratory capabilities.

INTRODUCTION

Dengue is caused by dengue virus (DENV), a member of the family *Flaviviridae* and genus *Flavivirus*. Dengue virus is a single-stranded, positive strand, enveloped virus comprising four antigenically distinct serotypes, DENV-1 to DENV-4.¹ It is estimated that there are ~390 million infections per year, of which 96 million infections present with clinical or subclinical symptoms.² Approximately 70% of reported infections are in Asia.²

Dengue virus infection can result in asymptomatic infection or cause clinical manifestations in the form of acute febrile illness, which can then lead to severe dengue.^{3,4} Secondary DENV infection (when an individual is sequentially infected with a different serotype) is usually more severe than a primary infection.⁵ Presently, there are no Food and Drug Administration-approved drugs for the treatment of dengue,³ and the only licensed vaccine, Dengvaxia, is recommended only for people who have had a previous DENV infection.^{6,7}

The diagnosis of DENV infection is important to identify potential cases of hemorrhagic fever that can lead to death. In addition, early identification and diagnosis of dengue can help public health agencies enact community and vector control measures. Clinical diagnosis of DENV infection is not accurate as patients present with fever and other symptoms that are also observed with malaria, typhoid, typhus, Zika, and chikungunya. Traditionally, DENV infection was diagnosed based on virus isolation. However, this laborious

and time-consuming method has gradually been replaced by reverse transcription–polymerase chain reaction (RT-PCR) to detect DENV RNA isolated from patient samples or with enzyme-linked immunosorbent assays to detect viral antigen.^{8,9} The CDC DENV 1–4 Real-Time RT-PCR assay is widely used by public health laboratories for detection and serotype identification of DENV. The test consists of a pan-DENV singleplex reaction with a lower limit of detection of 1×10^2 genome copy equivalents (GCE)/mL, and a multiplex reaction to identify serotypes with a lower limit of detection of 1×10^3 to 1×10^4 GCE/mL.^{10,11} Although there are multiple pan-DENV kits that are commercially available, their sensitivities vary significantly. For example, one study reported that the Simplexa™ Dengue Kit (Focus Diagnostics, Cypress, CA) had a sensitivity of 85.2% (95% CI: 79.7–90.7), the RealStar Dengue RT-PCR Kit 2.0 (Altona Diagnostics, Hamburg, Germany) had a sensitivity of 83.2% (95% CI: 77.6–89.1) and Geno-Sen's Dengue 1–4 Real-Time RT-PCR Kit (Genome Diagnostics, Pvt., New Delhi, India) had a sensitivity of 93.2% (95% CI: 89.3–97.1) relative to hemi-nested RT-PCR.¹²

Reverse transcription–polymerase chain reaction methods require relatively expensive instruments, trained personnel, and well-equipped laboratories. In communities with no access to suitably equipped laboratories, patients may be diagnosed using rapid diagnostic tests (RDTs) based on immunoassays that detect the DENV nonstructural protein 1 (NS1), and/or IgG and IgM. Rapid diagnostic tests are typically performed using serum or plasma samples and, thus, still require some laboratory equipment to process patient samples. Although RDTs are affordable, rapid, and accurate, certain limitations affect their broad utility as a DENV diagnostic. For example, the purported sensitivities and specificities of the NS1 RDTs vary based on the study and country of use. Meta-analysis of

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the diagnostic accuracy of the NS1 RDTs reported a sensitivity of 67% (95% CI: 59–74%) to 71% (95% CI: 61–79%) and a specificity of 99% (95% CI: 98–100%).^{13,14} This may be explained by a poorer sensitivity for DENV-2 and DENV-4, compared with DENV-1.¹⁵ Importantly, the sensitivity is reduced for diagnosis of secondary infections.^{15,16} Furthermore, IgM and IgG RDTs suffer from cross-reactivity with other flaviviruses, thus reducing test specificity.¹⁷

The limited performance of RDTs compared with RT-PCR highlights the need for molecular tests that can be used in low-resource settings. Here, we describe an RT-PCR that detects DENV directly from a small volume of blood or plasma and can be conducted using a portable PCR instrument.

MATERIALS AND METHODS

Cell culture, virus, and parasite cultures. African green monkey kidney (Vero) cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA) and were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Waltham, MA) supplemented with 100 U/mL penicillin and streptomycin, 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Gibco), 2 mM glutamine (Gibco), and 10% heat-inactivated fetal bovine serum (Gibco) at 37°C in 5% CO₂. The four serotypes of DENV, DENV-1 (Hawaii strain), DENV-2 (New Guinea C), DENV-3 (H87) and DENV-4 (H241), and Zika virus were obtained from the Public Health Agency of Canada. Mayaro virus (15537 strain) was obtained from the ATCC. The virus stocks were generated in Vero cells, aliquoted, and stored at –80°C until use. Viral titres were determined by plaque assay in Vero cells.¹⁸ All viruses were handled according to Biosafety level 2 containment procedures. *Plasmodium falciparum* 3D7 parasites were cultured in human blood as described previously and purified after synchronization with sorbitol.¹⁹

Virus stock quantification. Previous studies suggested that there are more *Flavivirus* genomes compared with the number of infectious particles.^{20,21} As the RT-PCR detects genomic RNA regardless of infectivity, we quantified the DENV viral stocks in terms of genome equivalents based on in vitro transcribed RNA as the standard.²² The gBlock templates for the 3'-untranslated regions of each DENV serotype were synthesized with the T7 promoter at the 3' end of the sequence (Integrated DNA Technologies, Coralville, IA). The target RNA was transcribed with T7 RNA polymerase using the MEGAscript™ T7 Transcription Kit, according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). The resulting RNA was quantified by spectrophotometry and expressed as GCE/mL. A standard curve was generated with the in vitro transcribed RNA serially diluted from 1×10^{11} GCE/mL to 1×10^5 GCE/mL. Viral stocks (1 µL) were tested in triplicate against this standard curve to quantify the concentration of viral RNA in GCE/mL. The samples were tested in the RT-PCR with the same conditions used for plasma samples, described below.

Patient samples. A blood sample from a traveler infected with *Plasmodium vivax* was obtained from the Public Health Laboratories (ProvLab), Alberta and used for the specificity analysis. For the panel, deidentified plasma samples were used from a separate study on dengue in the Philippines. Ethical approval was obtained from the Institutional Review Board of Chong Hua Hospital (Cebu City, Philippines), the

Research Ethics Committee of the University of Toronto (Toronto, Canada), and the Human Research Ethics Board of the University of Alberta (Pro00002383) (Alberta, Canada). Patients who presented to a single outpatient clinic, the Lebumfacil-Santa Ana Medical Center, Toledo, Cebu, Philippines, between September 2015 and February 2017, were recruited. Patients were included if they were aged between 1 and 26 years and the treating physician made a presumptive clinical diagnosis of dengue. A venous plasma sample (ethylenediaminetetraacetic acid anticoagulant) was collected and tested using the SD BIOLINE Dengue Duo NS1 Ag + Ab Combo (Abbott, Abbott Park, IL). In cases of acute dengue, this RDT had a reported sensitivity of 59% and specificity of 80% when compared with RT-PCR.¹⁶ A panel consisting of 91 RDT-positive (DENV NS1 or IgM detected) and 35 RDT-negative (neither DENV NS1 nor IgM detected) samples were selected and subsequently tested by RT-PCR in a blinded fashion.

RNA extraction. RNA was extracted from 250 µL of patient plasma using the QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD), according to the manufacturer's instructions. The extracted RNA was resuspended in 50 µL of elution buffer.

Reverse transcription–polymerase chain reaction. The following pan-DENV primers were used for the study: forward primer 5'-TTGAGTAAACYRTGCTGCCTGTAGCTC-3' and reverse primer 5'-GAGACAGCAGG ATCTCTGGTCTYTC-3', which were described previously.²³ These primers target a 253-nt sequence within the highly conserved region in the 3'-untranslated regions of the DENV genome.²³ Reverse transcription–polymerase chain reaction was performed using the Direct Blood RT-PCR Kit (VitaNavi Technology, Manchester, MO). The direct from blood RT-PCR was performed in a 25-µL reaction volume consisting of 1X blood RT-PCR buffer, 1.0 µL of the RT polymerase mix, 40X SYBR Green (Thermo Fisher Scientific), 1.6 U/µL of the Ribolock RNase Inhibitor (Thermo Fisher Scientific), and primers at a final concentration of 200 nM. Blood was added at 10% of the final reaction volume and was either added as uninfected blood (as a “no template control,” NTC) or blood spiked with cultured virus. The direct from plasma RT-PCR was performed in a 25-µL reaction consisting of 1X blood RT-PCR buffer, 0.7 µL of the RT polymerase mix, 4X SYBR Green (Thermo Fisher Scientific), 1.6 U/µL of the Ribolock RNase Inhibitor (Thermo Fisher Scientific), and primers at a final concentration of 200 nM. Plasma was added at 10% of the final volume of the reaction, and was either added as uninfected plasma controls, plasma spiked with cultured virus, or plasma from patient samples.

The RT-PCR reaction was performed in a conventional qPCR instrument and a portable qPCR instrument. For the conventional instrument, reactions were run in 200-µL low-profile PCR 8-strip tubes (Bio-Rad Laboratories, Inc., Hercules, CA) on a Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). The RT-PCR thermocycling program was as follows: reverse transcription at 55°C for 30 minutes, denaturation for 3 minutes at 95°C, 40 cycles of: 95°C for 20 seconds, 60°C for 30 seconds, and 70°C for 30 seconds, followed by a final extension for 2 minutes at 70°C, and melt curve analysis from 65°C to 95°C. For the portable instrument, we used the Open qPCR Thermocycler (Chai Biotechnologies, Inc., Santa Clara, CA).

FIGURE 1. Amplification of dengue virus (DENV) directly from blood. (A) Real-time reverse transcription–polymerase chain reaction amplification and (B) melt curve analysis of blood samples spiked with cultured DENV (DENV-1, DENV-2, DENV-3, or DENV-4) or uninfected blood (no template control [NTC]). The experiment was performed in triplicate on the Bio-Rad CFX Connect polymerase chain reaction machine with the threshold set at 100. This figure appears in color at www.ajtmh.org.

The RT-PCR thermocycling program was as follows: reverse transcription at 55°C for 30 minutes, denaturation for 3 minutes at 95°C, 45 cycles of: 95°C for 20 seconds, 60°C for 30 seconds, and 70°C for 30 seconds, followed by a final extension for 2 minutes at 70°C, and melt curve analysis according to the default settings. Samples with a melt temperature $\geq 82^{\circ}\text{C}$ were defined as DENV positive.

The RealStar Dengue PCR Kit 2.0 (Altona Diagnostics) served as the RT-PCR reference test. This kit does not

discriminate the four serotypes. The RT-PCR reaction was performed according to the manufacturer's instructions. The RealStar Dengue RT-PCR Kit 1.0 (Altona Diagnostics) was used to determine the serotypes of the samples that were positive for viral RNA. The serotype RT-PCR was performed according to the manufacturer's instructions.

Statistical analysis. The sensitivity and specificity of diagnostic tests with 95% CIs were calculated using MedCalc 16.4 (MedCalc, Ostend, Belgium). Comparison of test performance characteristics (between platforms, between PCR) used the RealStar Dengue PCR Kit 2.0 as the gold standard to classify samples as DENV positive or DENV negative. The sensitivity and specificity of the two assays were compared using the McNemar test for positive and negative samples, respectively.²⁴

RESULTS

Detection of DENV RNA directly from blood. The direct from blood/plasma RT-PCR reaction amplified RNA from virus of each of the four DENV serotypes spiked into blood (Figure 1). During the standardization process, we observed late amplification of the NTC controls (uninfected blood); however, these products had a melt temperature distinct from the blood that was spiked with DENV. Sequencing of the PCR product which had a melt temperature of $78^{\circ}\text{C} \pm 2^{\circ}\text{C}$ did not identify a specific DNA sequence. In contrast, PCR products amplified from blood spiked with DENV had a melt temperature of $86^{\circ}\text{C} \pm 4^{\circ}\text{C}$. Based on these observations, samples with a melt peak $\geq 82^{\circ}\text{C}$ were defined as positive for DENV RNA.

Analytical specificity and sensitivity. The specificity of the direct from blood test was evaluated by spiking uninfected blood with Zika virus, Mayaro virus, and *P. falciparum* parasites, and with a *P. vivax* patient sample (Table 1). These pathogens co-circulate with DENV and cause similar symptoms. No cross-reactivity with these other parasites or viruses was observed. We did observe an amplification curve with Mayaro virus, but the product melted at a temperature below 82°C (data not shown).

The limit of detection (LoD) was defined as the lowest dilution in which DENV was detected in all replicates with 100% reproducibility. The LoD of the assay was determined with 1×10^8 GCE/mL DENV (whole virus) spiked into uninfected blood and serially diluted 10-fold from 1×10^8 to 1×10^2 GCE/mL with five replicates of each dilution (Figure 2). The LoD was based on a melt curve $\geq 82^{\circ}\text{C}$; at lower dilutions, this peak was often a minor peak in the melt curve analysis. For all serotypes, the LoD was 1×10^4 GCE/mL of DENV in spiked blood.

TABLE 1
Specificity of the direct from blood DENV RT-PCR against clinically relevant pathogens

Pathogen (whole organisms)	Sample type	Concentration	Melt curve $\geq 82^{\circ}\text{C}$ (no. of replicates)
DENV-1	Spiked in blood	1×10^6 (GCE/mL)	3/3
DENV-2	Spiked in blood	1×10^6 (GCE/mL)	3/3
DENV-3	Spiked in blood	1×10^6 (GCE/mL)	3/3
DENV-4	Spiked in blood	1×10^6 (GCE/mL)	3/3
Zika virus	Spiked in blood	1×10^5 (PFU/mL)	0/3
Mayaro virus	Spiked in blood	1×10^5 (PFU/mL)	0/3
<i>Plasmodium falciparum</i>	Spiked in blood	1×10^7 (parasites/mL)	0/3
<i>Plasmodium vivax</i>	Clinical sample	2×10^5 (parasites/mL)	0/3

DENV = dengue virus; GCE = genome copy equivalent; RT-PCR = reverse transcription–polymerase chain reaction.

FIGURE 2. Limit of detection of dengue virus (DENV) serotypes detected directly from blood. The limit of detection (LoD) was determined using blood spiked with cultured DENV (DENV-1, DENV-2, DENV-3, or DENV-4). The samples were serially diluted 10-fold. Uninfected blood was used as the no template control. Samples with a melt peak $\geq 82^\circ\text{C}$ were considered positive. The LoDs are representative of five independent experiments. This figure appears in color at www.ajtmh.org.

Performance of the DENV assay on a portable real-time PCR instrument. The use of a portable PCR platform makes it possible to perform molecular diagnostics in field settings. We optimized the assay for the Open qPCR Thermocycler, an instrument that is easy to use and relatively low cost ($\sim \$4,299$ USD). All four serotypes of DENV were amplified from virus spiked into blood, with similar melt temperatures as the Bio-Rad CFX Connect (Table 2). The LoD on the portable PCR platform was 1×10^4 GCE/mL.

Detection of DENV directly from plasma. Samples collected from patients for epidemiological studies of dengue are often collected as plasma rather than whole blood. Thus, we tested whether DENV RNA could also be detected directly from plasma. To optimize this reaction, we reduced the amount of SYBR Green dye from 40X to 4X and reduced the amount of enzyme from 1.0 to 0.7 μL . We detected all four DENV serotypes in plasma spiked with virus (Figure 3). We consistently detected 1×10^4 GCE/mL for DENV-1 and DENV-2 virus spiked into plasma (data not shown). Similar results

were obtained on both the Bio-Rad CFX Connect and the Open qPCR Thermocycler.

Evaluation of the direct from plasma RT-PCR with patient samples. We next validated the direct from plasma RT-PCR with 126 archived plasma samples collected from participants of a study on dengue in the Philippines. Direct from plasma RT-PCR reactions were run in parallel on the Bio-Rad CFX

TABLE 2
DENV RT-PCR performed on the Open qPCR Thermocycler

Sample*	Mean melt temperature	Melt curve $\geq 82^\circ\text{C}$ (no. of replicates)
DENV-1	$88.5^\circ\text{C} \pm 5.1^\circ\text{C}$	5/5
DENV-2	$88.0^\circ\text{C} \pm 3.2^\circ\text{C}$	5/5
DENV-3	$86.4^\circ\text{C} \pm 1.7^\circ\text{C}$	5/5
DENV-4	$87.3^\circ\text{C} \pm 2.2^\circ\text{C}$	5/5
NTC†	$73.9^\circ\text{C} \pm 2.5^\circ\text{C}$	0/5

DENV = dengue virus; NTC = no template control; RT-PCR = reverse transcription-polymerase chain reaction.

* All viruses were spiked into blood.

† Blood only.

FIGURE 3. Amplification of dengue virus (DENV) directly from plasma. (A) Real-time reverse transcription–polymerase chain reaction amplification and (B) melt curve analysis of plasma samples spiked with cultured DENV (DENV-1, DENV-2, DENV-3, or DENV-4) or uninfected blood (no template control [NTC]). The experiment was performed in triplicate on the Bio-Rad CFX Connect polymerase chain reaction machine with the threshold set at 1,000. This figure appears in color at www.ajtmh.org.

Connect and the Open qPCR Thermocycler. Fifty samples were positive for DENV RNA on the Bio-Rad CFX Connect, and 53 were positive on the Open qPCR Thermocycler. At the same time, RNA was extracted from all samples and tested using the RealStar Dengue RT-PCR Kit 2.0 as the gold standard test. With this kit, 60 samples were positive for DENV RNA. The results from these three experiments are presented in Table 3. Compared with the RealStar Dengue PCR Kit 2.0, the sensitivity of our direct from plasma RT-PCR on the Bio-Rad CFX Connect was 76.7% (95% CI: 65.8–87.9%) and the specificity was 93.9% (95% CI: 85.2–98.3%) (Table 3). We obtained similar results with the Open qPCR Thermocycler; the

sensitivity was 78.3% (95% CI: 65.8–87.9%) and specificity was 90.9% (95% CI: 81.3–96.6%) (Table 4). Differences in sensitivity and specificity between PCR platforms (Bio-Rad CFX versus Open qPCR) were not statistically significant ($P > 0.99$ and $P = 0.62$, respectively).

Compared with the RealStar Dengue PCR Kit 2.0, the direct from plasma RT-PCR failed to detect DENV in 13 positive samples. We analyzed the serotypes in the panel using the RealStar Dengue RT-PCR Kit 1.0 to determine whether the test had a lower sensitivity for a particular serotype and found that of the 60 positive samples, four were DENV-1, 11 were DENV-2, 34 were DENV-3, nine were DENV-4, and the serotype could not be determined for two samples. Of the 13 discordant samples, there was one sample infected with DENV-1, two with DENV-2, seven with DENV-3, and three with DENV-4. We also checked whether the discordant samples had lower viremias. The average cycle threshold value for the discordant samples was 34 in the RealStar Dengue RT-PCR Kit 2.0, which could represent a viremia below the limit of detection of our assay. We next analyzed the results based on the timing of sample collection after the onset of symptoms. All the samples were collected between days 0 and 11 after the onset of symptoms. From day 0 to day 4, the direct from plasma RT-PCR identified 51 positive samples, whereas the RealStar Dengue RT-PCR Kit 2.0 identified 55 positive samples. After day 5, only one sample was positive in the direct from plasma RT-PCR, whereas five samples were positive with the RealStar Dengue RT-PCR Kit 2.0.

We next compared the test performance characteristics of the direct from plasma RT-PCR to NS1 detection by RDT. No statistically significant differences in test sensitivity were observed between RT-PCR on either platform versus NS1 detection: 77% versus 85% ($P = 0.23$) for the Bio-Rad CFX and 78% versus 85% ($P = 0.34$) for the Open qPCR Thermocycler. On the other hand, specificity of the PCR was statistically superior to NS1 detection on both platforms: 94% versus 76% ($P = 0.0033$) for the Bio-Rad CFX and 91% versus 76% ($P = 0.024$) for the Open qPCR Thermocycler.

DISCUSSION

In this article, we describe a robust, field-ready test that can detect DENV directly from blood or plasma, offering a crucial advantage for a field diagnostic. It eliminates the need for highly trained staff, costly equipment, and laboratory infrastructure. Importantly, this test detects DENV without the need for RNA extraction, a step that poses a major challenge given the unstable nature of the RNA molecule.^{25,26} Furthermore, the ability of our direct from blood/plasma RT-PCR to detect viral RNA from blood or plasma supports the use of this test with the two most common sample types collected for DENV screening.

TABLE 3
Evaluation of the direct from plasma RT-PCR using clinical samples on the Bio-Rad CFX Connect

Test		RealStar Dengue PCR Kit 2.0		Sensitivity (95% CI)	Specificity (95% CI)
		Positive (n)	Negative (n)		
Direct from plasma RT-PCR	Positive (n)	46	4	76.7% (65.8–87.9%)	93.9% (85.2–98.3%)
	Negative (n)	14	62		

RT-PCR = reverse transcription–polymerase chain reaction.

TABLE 4
Evaluation of the direct from plasma RT-PCR using clinical samples on the Open qPCR Thermocycler

Test		RealStar Dengue PCR Kit 2.0		Sensitivity (95% CI)	Specificity (95% CI)
		Positive (n)	Negative (n)		
Direct from plasma RT-PCR	Positive (n)	47	6	78.3% (65.8–87.9%)	90.9% (81.3–96.6%)
	Negative (n)	13	60		

RT-PCR = reverse transcription–polymerase chain reaction.

The direct from blood/plasma RT-PCR described here can detect all four DENV serotypes which is important as multiple serotypes of DENV can circulate in an area. In addition, the direct from blood/plasma RT-PCR has robust specificity that was not interfered with by the presence of other pathogens that cause clinical symptoms similar to dengue. The method described here can detect DENV RNA as low as 10^4 GCE/mL in blood. This limit of detection is approximately a log higher than what was previously reported for the CDC pan-DENV test.¹¹ According to the product insert, the limit of detection for the RealStar Dengue RT-PCR Kit 2.0 is 4.7×10^3 GCE/mL for DENV-1 and 0.7×10^3 GCE/mL for DENV-4. The difference in sensitivity of our test and the commercial kit may explain the 13 false-negative results in our analysis of the panel. Given that the viremia in patients presenting with DENV is generally on the order of $\sim 10^4$ plaque-forming unit equivalents/mL,²⁷ the trade-off between sensitivity and accessibility in low-resource environments remains favorable. Our test performance was comparable with the RealStar Dengue RT-PCR Kit 2.0 during the first 4 days of symptom onset, consistent with the period when DENV viremia in blood or plasma is expected to be highest.²⁸

There is an urgent need for new DENV diagnostic tests in hyperendemic regions, particularly in low-resource settings that lack the infrastructure to perform WHO-recommended laboratory diagnosis.²⁹ One other method, the pan-DENV POKIT assay, was developed as a point-of-care RT-PCR for DENV and validated with archived patient samples.^{30,31} However, this test requires nucleic acid extraction. Our direct from blood/plasma RT-PCR coupled with the use of the portable Open qPCR Thermocycler offers a simple method for the detection of DENV RNA directly from patient samples. A specific, inexpensive (< \$4 USD), and easy to perform DENV test can be used for clinical management and surveillance and would allow for early intervention to prevent or control epidemics. It can also be used to distinguish DENV infection from other pathogens that cause fever (such as malaria, typhus, chikungunya, Mayaro virus, and Zika virus).^{4,32–34} We previously developed a molecular test for direct detection of malaria parasites from blood using the Open qPCR platform.^{35,36} Combining the tests for DENV and malaria on a single diagnostic platform would allow for rapid differentiation of common infectious etiologies in regions where both diseases coexist.

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