Video Article

Reverse Transcription-Loop-mediated Isothermal Amplification (RT-LAMP) Assay for Zika Virus and Housekeeping Genes in Urine, Serum, and Mosquito Samples

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Abstract

Infection with Zika virus (ZIKV) can be asymptomatic in adults, however, infection during pregnancy can lead to miscarriage and severe neurological birth defects. The goal of this protocol is to quickly detect ZIKV in both human and mosquito samples. The current gold standard for ZIKV detection is quantitative reverse transcription PCR (qRT-PCR); reverse transcription loop-mediated isothermal amplification (RT-LAMP) may allow for a more efficient and low-cost testing without the need for expensive equipment. In this study, RT-LAMP is used for ZIKV detection in various biological samples within 30 min, without first isolating the RNA from the sample. This technique is demonstrated using ZIKV infected patient urine and serum, and infected mosquito samples. 18S ribosomal ribonucleic acid and actin are used as controls in human and mosquito samples, respectively.

Video Link

The video component of this article can be found at https://www.jove.com/video/58436/

Introduction

In 2015, Zika virus (ZIKV) gained prominent global attention as an infectious disease of concern because infection during pregnancy was linked to miscarriage, stillbirth, severe neurological birth defects including microcephaly, as well as other congenital birth defects[1]. In rare cases, ZIKV has been associated with Guillain-Barré syndrome. ZIKV is primarily transmitted by *Aedes* mosquitoes; however, it can also be spread through sexual contact[1]. Given that the infection with ZIKV is asymptomatic in most people or presents with mild flu-like symptoms that overlap with the symptoms of infection of other arboviruses[1], there was a need for improved methods for rapid and cost-effective detection of ZIKV to screen both people as well as local mosquito populations.

Quantitative reverse transcription PCR (qRT-PCR) is a reliable assay for ZIKV detection; however, this technique requires expensive specialized equipment, trained personnel, and RNA isolation from the sample of interest. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a one-step nucleic acid amplification method based on PCR technology that only requires one incubation temperature due to the use of a thermophilic DNA polymerase with strand displacement properties. This circumvents the need for a thermocycler and decreases the length of time needed to complete the assay. Additional advantages of RT-LAMP include its high specificity and sensitivity, robustness at a range of pH levels and temperatures, and resistance to many PCR inhibitors[2]. It has a relatively low cost and reagents are stable at room temperature. Given these characteristics, RT-LAMP can be deployed in a laboratory or in the field. As such, LAMP reactions have been developed to detect a range of pathogens and other types of infection[3,4,5]. The goal of the RT-LAMP protocol described in this paper is to detect ZIKV without RNA isolation in human serum and urine samples as well as in a single infected mosquito within 30 min through RT-LAMP. This method can be used to replace qRT-PCR as it is a sensitive, rapid diagnostic tool that works quickly and in settings outside of the laboratory.

Protocol

All methods described here have been approved by the Institutional Review Board (IRB) of Beaumont Health. All experiments were performed in accordance with relevant guidelines and regulations.

CAUTION: All potentially infectious materials should be handled according to Biosafety Level 2 standards including the use of personal protective equipment. Any procedures which may produce aerosol should be performed in a biosafety cabinet. Additionally, work with live mosquitoes should be performed in the appropriate Arthropod Containment Level 1-3 facility. Given the association of ZIKV infection with

congenital abnormalities, women who are pregnant, trying to conceive, or the partners of these women should significantly minimize their laboratory exposure to ZIKV. Transport of ZIKV samples is classified in the United States as Category B Biological Substances in accordance with Department of Transportation Hazardous Materials Regulations (49 CFR Part 171-180) and therefore shipping samples should adhere to those guidelines. Import into the United States of ZIKV biospecimens requires a Centers for Disease Control and Prevention (CDC) Import Permit. Import of any arthropods that could serve as a vector for ZIKV transmission, even if they are not infected, requires a United States Department of Agriculture (USDA) permit. This information is current at the time of publication. Up-to-date recommendations can be found at https://www.cdc.gov/zika/laboratories/lab-safety.html.

NOTE: RT-LAMP reactions are prone to higher rates of false positive reactions, so precautions should be taken in experimental planning. All set-up and execution of RT-LAMP reactions should use designated pipettes and filter tips. Ideally, a lateral work flow should be established. If possible, analysis and imaging (Section 5) should occur in a separate enclosed room to prevent contamination. Opening of test tubes containing RT-LAMP products should be kept to a minimum.

1. RT-LAMP Primer Preparation

1. Reconstitute each lyophilized RT-LAMP primer in molecular grade water to a final concentration of 100 µM (**Table 1**). Briefly vortex the primer solution to ensure a homogenous solution and briefly spin down the solution at maximum speed in a table top centrifuge to collect all primer solution.
2. Prepare a 10x RT-LAMP Primer Mix of the FIP, BIP, F3, B3, LF, and LB primers using the volumes in **Table 2**. Briefly vortex the primer solution to ensure a homogenous solution, and briefly spin down the solution at maximum speed in a table top centrifuge to avoid any loss.
   NOTE: The RT-LAMP primer set for *Ae. aegypti* actin (AEDAE) does not contain a LB primer (loop primers are not always necessary for RT-LAMP reactions). In this case, replace the LB primer volume with molecular grade water.
3. Store the primers at -20 °C between uses and avoid free-thaw cycles.

2. Sample Preparation

1. For human urine samples: Use either fresh urine, frozen urine, or urine in preservative. For fresh or frozen sample: immediately spin down the sample after the collection for 10 min at 700 x *g*, and use the supernatant for analysis or freeze at -80 °C for future use. Thaw frozen samples on ice before use.
2. For human serum samples: Use either fresh or frozen serum samples.
3. For ZIKV infected cell lines: Use either conditioned media or cell lysates.
   NOTE: Cell-free conditioned media from Ae. albopictus C6/36 cells 8 days post-infection can be used as positive controls for ZIKV RT-LAMP reactions.
4. For *Ae. aegypti* mosquitoes: Use either whole fresh or frozen mosquito carcasses. Thaw frozen mosquitoes on ice. Prepare a crude mosquito lysate by placing an individual mosquito in 100 µL of phosphate-buffered saline (PBS) and homogenize by crushing the mosquito 10 times with a P10 pipet tip (**Figure 1**). Briefly centrifuge the crude lysate to pellet any debris. Use the supernatant for downstream RT-LAMP analysis.
   Note: Positive controls can be generated in a laboratory setting by infecting female mosquitoes using intrathoracic microinjection with approximately 10^3 genome equivalents of virus in a volume of 200 nL and harvesting the mosquito 5 days post-infection.

3. Prepare RT-LAMP Master Mix

1. Prepare a RT-LAMP master mix reaction on ice for each primer set to be used by using volume guide in **Table 3**.
   NOTE: The use of thermolabile Uracil DNA Glycosylase (UDG) assists in preventing false positives.
2. Vortex briefly to ensure that all samples are well mixed, then spin down briefly to prevent volume loss.

4. RT-LAMP Assay

1. **Pipet 23.0 µL of the RT-LAMP master mix per reaction into a 200 µL PCR tube. Add 2.0 µL of sample (urine, serum, or crude mosquito lysate supernatant as described in Step 2). This will bring the total volume to 25.0 µL per RT-LAMP reaction. For the negative control, use molecular grade water. Include a positive control.**
   NOTE: A PCR standard or virus stocks from supernatant of infected cell lines can be used as a positive control.
   1. Optional: Include a specificity control reaction of a related arbovirus such as dengue virus (DENV). Prepare the sample as outlined in Step 2 according to sample type. Add 2.0 µL of the specificity control to 23.0 µL of the RT-LAMP master mix into a 200 µL PCR tube.
2. Heat the samples at 61 °C for 30 min using a heat block, water bath, or thermocycler.
3. Heat deactivate the polymerase by heating to 80 °C for 10 min.

5. RT-LAMP Analysis

NOTE: Perform RT-LAMP analysis in a separate, enclosed space.

1. **After the incubation, access RT-LAMP reactions visually by looking for the presence of a color change.**
   1. Dilute the fluorescent nucleic acid dye 1:10 in TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) buffer.
      CAUTION: Any nucleic acid stain binds to nucleic acids and therefore is a carcinogen. Wear gloves when handling.
   2. To 12 µL of the RT-LAMP reaction, add 2 µL of fluorescent nucleic acid dye dilution.
      NOTE: Negative reactions will be orange in color and positive reactions will be yellow/green in color.

3. Optional: Take pictures of RT-LAMP reactions on a white background using a camera.

2. Place the samples under 302 nm UV light to confirm the presence of RT-LAMP products by fluorescence. Take picture of the result using a camera.
   NOTE: Positive RT-LAMP reactions will have a fluorescent output.
   CAUTION: Wear UV protective eye googles or face shield when working with UV light.
3. **Optional: Confirm the presence of RT-LAMP products by performing gel electrophoresis on the samples.**
   1. Pour a 2% agarose gel in 1x TAE buffer with a nucleic acid stain for visualization.
      CAUTION: Any nucleic acid stain binds to nucleic acids and therefore is a carcinogen. Wear gloves when handling.
   2. Add 5 µL of a DNA ladder in the first lane to compare the molecular weights.
   3. For each RT-LAMP reaction, mix 13 µL of RT-LAMP reaction mixture with 2 µL of DNA loading dye. Load the 15 µL mixture containing the DNA loading dye.
   4. Run the gel at 90 V for 90 min or until the bands are separated and image with 302 nm UV light.
      NOTE: Positive RT-LAMP reactions will have a laddering pattern. Negative RT-LAMP reactions should not contain any bands.

6. Disposal

1. Dispose of RT-LAMP reactions in double sealed bags. Do not autoclave as this may aerosolize the RT-LAMP products, leading to false positive reactions in the future.

Representative Results

RT-LAMP reactions can be analyzed using three different methods. First, with the addition of a fluorescent nucleic acid dye, positive reactions will be yellow/green in color where negative reactions will appear orange in color to the naked eye. Second, the addition of the fluorescent nucleic acid dye to RT-LAMP reaction results in a fluorescent signal when the samples are excited by UV light. Negative reactions will not have a detectable fluorescent signal over any background fluorescence that may be present in small amounts in the negative control. Lastly, RT-LAMP reactions can be run out on an agarose gel. Positive RT-LAMP reactions will have a banding pattern, whereas negative reactions will have no DNA bands. Examples of this using all three of these analysis methods are demonstrated in **Figure 2** and **3**. None of these samples had RNA isolated prior to RT-LAMP. **Figure 1** demonstrates the crushing of a whole mosquito in PBS, which is sufficient for the use in the RT-LAMP reaction. In **Figure 2**, the specificity of the ZIKV RT-LAMP reaction is demonstrated: only the ZIKV molecular control, not the DENV molecular control or negative control, has a positive RT-LAMP reaction. Furthermore, ZIKV is detected in both the urine and serum of a patient with known ZIKV infection, but not in the asymptomatic control patient without ZIKV infection (**Figure 2A**). In **Figure 2B**, the samples are tested for human 18s rRNA using RT-LAMP. Only the samples from the patients (urine or serum), but not the molecular controls or the negative control, are positive for 18s rRNA. Therefore, 18s rRNA RT-LAMP can be used as a quality control for RT-LAMP reactions for the samples from human patients. Mosquito samples can be similarly tested for ZIKV or the RT-LAMP quality control, AEDAE (**Figure 3**). In this example, the positive molecular control for ZIKV as well as the mosquito infected with ZIKV are positive for ZIKV. The negative control, the molecular control for DENV, the mock infected mosquito (mosquito microinjected with cell culture media containing no virus), and the DENV infected mosquito are all negative for ZIKV (**Figure 3A**). However, all the mosquitoes are positive for AEDAE by RT-LAMP (**Figure 3B**).

When possible, all three analytical methods should be used to confirm a positive or negative reaction as sometimes the fluorescent signal can be weak and some individuals may be color blind, making the visual color change difficult to determine. In cases where the negative control is positive, the entire set of samples tested is invalid as contamination and therefore false positives cannot be ruled out. In cases where the positive control is negative, the entire set of samples is invalid as the RT-LAMP reaction may not have worked. This is a qualitative read out, however, higher copies of virus will result in an increase in RT-LAMP products that can be appreciated by fluorescent intensity or by the intensity of the DNA banding pattern by gel electrophoresis.

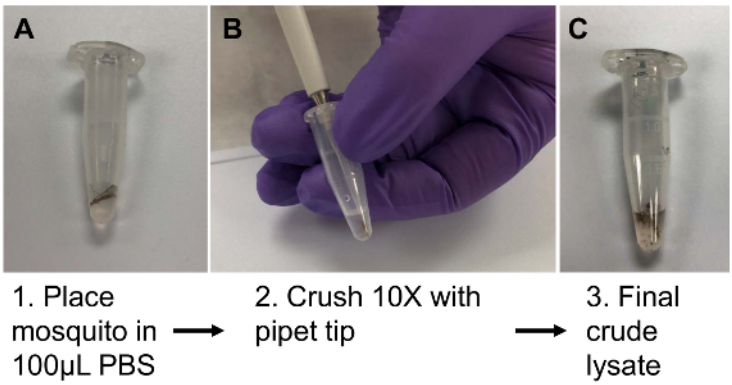

Figure 1: Preparation of mosquito crude lysate. (**A**) Place a mosquito into 100 µL of PBS in a microcentrifuge tube. (**B**) Using a P10 pipet tip, crush the mosquito against the sides of the tube 10 times. (**C**) This produces a crude lysate that should be spun down briefly in a table top centrifuge to pellet debris. Only the supernatant should be used for RT-LAMP reaction. Please click here to view a larger version of this figure.

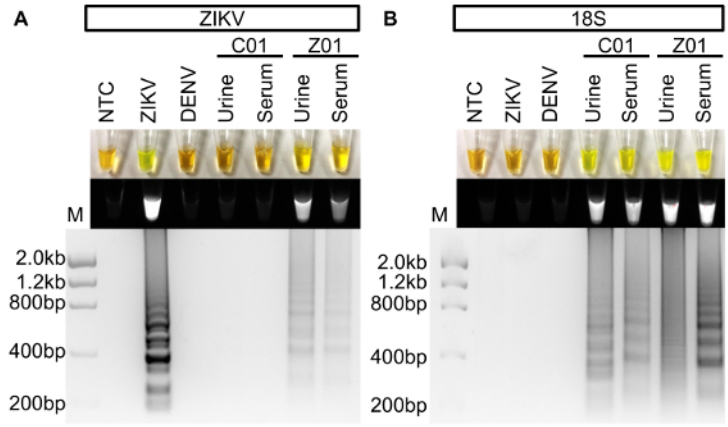

Figure 2: ZIKV and 18S rRNA RT-LAMP in patient urine and serum samples. Urine or serum samples from an asymptomatic control patient (C01) or ZIKV infected patient (Z01) were subjected to a ZIKV (**A**) or 18S rRNA (**B**) specific RT-LAMP reactions. RT-LAMP reactions were visualized after the addition of a fluorescent nucleic acid dye by eye (upper panel), green fluorescence (middle panel), or gel electrophoresis (bottom panel). Lane M: DNA Mass Marker; NTC: No template control (negative control); ZIKV: ZIKV PCR Standard positive control; DENV: DENV positive control. Please click here to view a larger version of this figure.

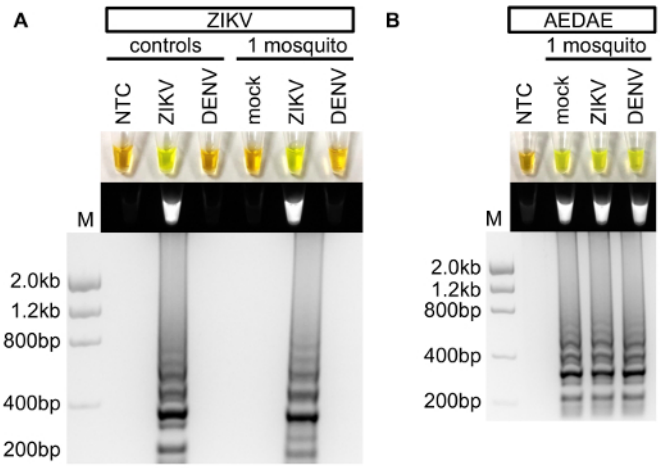

Figure 3: ZIKV and AEDAE RT-LAMP in *Ae. aegypti*. Single *Ae. aegypti* infected with mock control, ZIKV, or DENV were subjected to ZIKV (**A**) or AEDAE (**B**) specific RT-LAMP reactions. RT-LAMP reactions were visualized after the addition of a fluorescent nucleic acid dye by eye (upper panel), green fluorescence (middle panel), or gel electrophoresis (bottom panel). Lane M: DNA Mass Marker; NTC: No template control (negative control); ZIKV: ZIKV PCR Standard positive control; DENV: DENV positive control. Please click here to view a larger version of this figure.

Target	Primer	Sequence (5'-3')
Homo sapien 18S rRNA	18SrRNA-FIP* 18SrRNA-BIP* 18SrRNA-F3 18SrRNA-B3 18SrRNA-LF 18SrRNA-LB	TGGCCTCAGTTCCGAAAACCAACCTGGATACCGCAGCTAGG GGCATTCGTATTGCGCCGCTGGCAAATGCTTTCGCTCTG GTTCAAAGCAGGCCCGAG CCTCCGACTTTCGTTCTTGA AGAACCGCGGTCCTATTCCATTATT ATTCCTTGGACCGGCGCAAG
ZIKV	ZIKV-FIP* ZIKV-BIP* ZIKV-F3 ZIKV-B3 ZIKV-LF ZIKV-LB	AACCTGAGGGCATGTGCAAACCGCGGTCAGTGGAGATGACT CACAGGAGTGGAAACCCTCGACTGAAGTGGTGGGAGCAGAA GCAGAGCAATGGATGGGATA CCCATCCTTGAGGTACAGCT TCGATTGGCTTCACAACGC GGAGCAATTGGGAAGAAGTCC
Ae. aegypti actin	Ae21-FIP* Ae21-BIP* Ae21-LF Ae21-F3 Ae21-B3	TGCTTGGTCCCTGCCTGGGAGACAGCCCACCAGAACGA GAGACGAGAACGGCCCAGCGGGTCTGGTGTGTGTCTTTG ACCGCAAGGCCAAGAACCG CGGCGCCACCACAAGA TCGTGCCTGTGTTTGTCG
*Primers should be purified by rapid high-performance liquid chromatography (HPLC). For all other primers, standard desalting conditions are sufficient.		

Table 1: Primer sequences for RT-LAMP reactions. Primer sequences are originally described in previous publication[6].

Volume (µL)	Primer	Stock Concentration (µM)	Final Concentration (µM)
80	Forward Inner Primer (FIP)	100	16
80	Backward Inner Primer (BIP)	100	16
10	Forward Outer Primer (F3)	100	2
10	Backward Outer Primer (B3)	100	2
20	Loop Forward (LF)	100	4
20	Loop Backward (LB)	100	4
280	molecular grade water	-	-
500	**Total**		

Table 2: Preparation of 10x RT-LAMP Primer Mix. The RT-LAMP primer set for *Ae. aegypti actin* does not contain a BF primer; replace this volume with water.

1x Volume (µL)	Component	Final Conc. For 25 µL Volume
2.5	10x Isothermic Amplification Buffer	1x
1.8	100 mM dU/A/T/C/GTPs	1.4 mM
2.0	100 mM MgSO4	6 mM + 2 mM in buffer = 8 mM [4-10 mM range]
2.5	10x Primers	1.6 µM FIP/BIP, 0.2 µM F3/B3, 0.4 µM FL/BL
1.0	thermophilic DNA polymerase with strand displacement (8,000 U/mL)	8 U
0.5	Reverse Transcriptase (15,000 U/mL)	7.5 U
0.5	UDG (1,000 U/mL)	0.5 U
12.3	molecular grade water	-
23.0	**Total**	

Table 3: Preparation of RT-LAMP Master Mix.

Discussion

The ZIKV RT-LAMP assay described in this paper works using both human and mosquito samples[6]. The limit of detection was approximately 1 genome equivalent[6], which should be sufficient since the typical viral load of a symptomatic ZIKV infected patient is 10^3 to 10^6 PFU/mL[7]. Additionally, this method can detect ZIKV in samples without first isolating RNA and without virus amplification in cell culture. This significantly decreases the time, cost, and health risks of this assay compared to qRT-PCR.

Urine samples used at the volume ratio indicated in this protocol should allow RT-LAMP reactions to occur without an RNA isolation. While one could theoretically increase the amount of sample used in an RT-LAMP reaction, care should be exercised when this sample is urine. Urine contains multiple chemicals that could inhibit PCR reactions; urea in the urine is known to degrade polymerases[8] and using a urine/RT-LAMP reaction ratio that is too high will prevent the RT-LAMP reaction. If a low amount of virus is expected, it would be better to perform an RNA isolation using a kit specific for urine. Similarly, serum can contain PCR inhibitors that may inhibit RT-LAMP when serum is used at a higher volume[8].

Included in this protocol is the inclusion of quality controls for RT-LAMP reactions, akin to a loading control in western blotting protocols. If a clinical sample is negative for the RT-LAMP quality control, then a negative reaction for the ZIKV RT-LAMP may be a false negative as the sample may contain a high amount of PCR inhibitors or low amounts of total RNA. It is not necessary to isolate RNA from the sample prior to the RT-LAMP assay. The representative RT-LAMP results in this protocol were obtained without RNA isolation. However, RNA isolation may improve the detection in samples with low virus load or that may contain high levels of PCR inhibitors. For most sample types including serum, an RNA isolation kit can be used following the manufacturer's suggested protocol. For urine samples, an RNA isolation kit specific for urine samples should be used. For mosquito samples, it is recommended to lyse the mosquito by running the mosquito in 100 µL of PBS through a shredding column for 2 min at maximum speed or douncing before RNA isolation. RNA can be eluted in 30 µL of elution buffer and stored at -80 °C until use.

RNA isolation is then suggested to remove the endogenous PCR inhibitors or concentrate the RNA prior to RT-LAMP. If the RT-LAMP quality control is still negative, then qRT-PCR should be used for ZIKV detection.

The RT-LAMP primers in this protocol have been confirmed to work at a range of temperatures (57–65 °C) and incubation times (8-60 min), as well as using unconventional heating sources (*e.g.*, flameless ration heater, water bath) outside standard laboratory conditions. All the temperatures tested gave comparable results. Based on the detection by visual color change alone, the optimal time for the detection of ZIKV RT-LAMP products was 30 min. However, the detection by UV light excitation or banding patterns on gels were positive with as little as 8 min total incubation time. Some RT-LAMP protocols include DMSO, but in this protocol, it can result in the inhibition of the RT-LAMP reaction. Using a thermophilic DNA polymerase that can be set up at room temperature may have advantages over wild-type thermophilic DNA polymerase including faster amplification signals and have increased stability at room temperature[9,10]. Ethidium bromide can be used instead for nucleic acid visualization in 2% agarose gels, however, other fluorescent nucleic acid dyes for agarose gels have lower mutagenic potential making them a safer choice. However, appropriate safety precautions such as wearing gloves should still be used.

A limitation of this protocol is that it is a qualitative and not a quantitative technique, however, relative quantification can be done[6]. Furthermore, RT-LAMP primers are highly specific and were designed against a highly conserved sequence of the nonstructural protein 5 of ZIKV that is found in 16 strains and verified to work in at least 5 strains[6]. It is possible that extensive mutations to ZIKV in the wild may not be detectable by these primers in the future. Other groups have also developed new technologies for the detection of ZIKV that may be of interest[11,12,13,14,15,16,17].

In summary, this protocol provides a fast, simple, and cost-effective method for ZIKV in a variety of sample types that does not require specialized equipment. This provides a new diagnostic tool for ZIKV detection in which RNA does not have to be isolated before the RT-LAMP reaction.

Disclosures

LEL and MBC have intellectual property on Zika virus diagnosis methods. The remaining authors have no competing interests.

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