Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - Author Produced Video
Manuscript Number:	JoVE61025R3
Full Title:	Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) Assay for the Specific and Rapid Detection of Tilapia Lake Virus
Section/Category:	JoVE Immunology and Infection
Keywords:	tilapia lake virus; diagnosis; tilapia; RT-LAMP; RT-qPCR; screening tool; TiLV
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)

TITLE

Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) Assay for the Specific and Rapid Detection of Tilapia Lake Virus

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KEYWORDS:

tilapia lake virus, diagnosis, tilapia, RT-LAMP, RT-qPCR, screening tool

SUMMARY:

We present an RT-LAMP assay for the detection of TiLV in tilapia fish using simple instruments over a relatively short period of time compared to conventional RT-PCR techniques. This protocol may help control the epidemic spread of TiLVD, especially in developing countries.

ABSTRACT:

Tilapia lake virus disease (TiLVD), an emerging viral disease in tilapia caused by the tilapia lake virus (TiLV), is a persistent challenge in the aquaculture industry that has resulted in the mass morbidity and mortality of tilapia in many parts of the world. An effective, rapid, and accurate diagnostic assay for TiLV infection is therefore necessary to detect the initial infection and to prevent the spread of the disease in aquaculture farming. In this study, a highly sensitive and practical reverse transcription loop-mediated isothermal amplification (RT-LAMP) method is presented to detect tilapia lake virus in fish tissue. A comparison of the RT-qPCR and RT-LAMP assays of infected samples revealed positive results in 63 (100%) and 51 (80.95%) samples,

respectively. Moreover, an analysis of uninfected samples showed that all 63 uninfected tissues yielded negative results for both the RT-qPCR and RT-LAMP assays. The cross-reactivity with five pathogens in tilapia was evaluated using RT-LAMP, and all the tests showed negative results. Both the liver and mucus samples obtained from infected fish showed comparable results using the RT-LAMP method, suggesting that mucus can be used in RT-LAMP as a nonlethal assay to avoid killing fish. In conclusion, the results demonstrated that the presented RT-LAMP assay provides an effective method for TiLV detection in tilapia tissue within 1 h. The method is therefore recommended as a screening tool on farms for the rapid diagnosis of TiLV.

INTRODUCTION:

Tilapia lake virus disease (TiLVD) is a viral disease in tilapia (*Oreochromis* spp.) that reportedly causes tilapia deaths in many regions of the world, including Asia, South Africa, and South America[1,2]. The disease was first recognized during the mass mortality of tilapia in 2009 in Israel, where the number of wild tilapia in Lake Kinneret plummeted dramatically from 257 to 8 tons per year[2]. The disease is caused by the tilapia lake virus (TiLV), which has been assigned to the family *Amnoonviridae* as a new genus *Tilapinevirus* and a new species *Tilapia tilapinevirus*[3]. Genetic characterization of TiLV showed that the virus is a novel enveloped, negative-sense, single-stranded RNA virus that has 10 segments encoding 10 proteins[1,2,4]. Various species of tilapia in the genus *Sarotherodon*, *Oreochromis*, and *Tilapine* and other warm water fish (e.g., giant gourami (*Osphronemus goramy*)) have been shown to be susceptible to TiLV[2,5]. Currently, this virus continues to spread globally, possibly through the movement of infected live fish[6,7], while the risk of viral transmission via frozen tilapia or its product is limited[8]. Substantial mortality due to TiLV infection has the potential to have a significantly detrimental economic impact on the tilapia industry. For example, the economic impact of summer mortality syndrome in Egypt associated with TiLV infection was calculated to be US$100 million[9]. Accordingly, it is important to develop a rapid and proper diagnostic method to facilitate the control of this disease in fish farms.

Until now, the diagnosis of TiLVD has been based on molecular assays, viral isolation, and histopathology. Different PCR protocols and primers have been developed for TiLV diagnosis[10,11]. For instance, a SYBR green-based reverse transcription quantitative PCR (RT-qPCR) method with the sensitivity to detect as few as two copies/µL of the virus has been developed and validated for TiLV detection[10]. Other PCR methods for TiLV detection include TaqMan quantitative PCR[11], RT-PCR[2], nested RT-PCR[12], and semi-nested RT-PCR[13]. However, these methods require sophisticated laboratory equipment and relatively extended periods of time to yield results due to the complexity of the reactions, which makes them unsuitable for field application.

The loop-mediated isothermal amplification (LAMP) assay is a rapid, simple, and practical for-field application[14,15]. The technique employs the principle of a strand displacement reaction, while the amplification reaction runs under isothermal conditions without a sophisticated and expensive thermal cycler[14,15]. Consequently, amplified LAMP products or RT-LAMP products are analyzed in ladder-like bands using agarose gel electrophoresis with a fluorescent stain for either the safe visualization of DNA or RNA[14] or observation with the naked eye for the presence of turbidity or a white precipitate[16-18]. For these reasons, this technique has been used for the on-

site detection of different fish pathogens[17-27]. The purpose of this study was to establish a rapid, sensitive, and accurate RT-LAMP assay for TiLV detection. The RT-LAMP assay offers screening for TiLV in fish samples within 30 min. The technique may be applied for the diagnosis and surveillance of TiLVD.

PROTOCOL:

This experiment, which involved the use of animal tissue, was approved by the Institutional Animal Care and Use Committee of Kasetsart University, Bangkok, Thailand (protocol number ACKU61-VET-009).

1. Tissue collection

1.1. Euthanize tilapia fish using an overdose of clove oil (i.e., more than 3 mL/L). Tricaine methanesulfonate can be used as an alternative to clove oil.

1.2. Use sterile mayo scissors and forceps to cut open the abdomen of the postmortem tilapia and excise approximately 30–50 mg of liver tissue, or using a microscope cover glass, collect 100 µL of mucus by scraping the fish skin layer longitudinally (from anterior to posterior) into a 1.5 mL microcentrifuge tube.

1.3. Proceed to the RNA extraction step immediately or keep the collected sample at −80 °C until the experiment. As intact RNA is more sensitive than DNA, use RNase-free materials and reagents during the RNA extraction.

2. RNA extraction

2.1. To extract the RNA from the liver tissue, add 30–50 mg of the tilapia tissue to a 1.5 mL microcentrifuge tube containing 600–1,000 µL of guanidinium-acid-phenol extraction reagent (**Table of Materials**), and pulverize the sample until homogenous using a hand-held tissue homogenizer. The tissue samples require approximately 10% of the guanidinium-acid-phenol extraction reagent. For the mucus samples, use only 300 µL of the guanidinium-acid-phenol extraction reagent (3:1).

CAUTION: The guanidinium-acid-phenol extraction reagent is toxic; hence, handling must be undertaken with care. Protective equipment, such as safety glasses, a laboratory gown, and safety gloves, must be worn.

2.2. Centrifuge the homogenized sample at 10,000 x *g* for 30 s at room temperature, and transfer the supernatant into a new sterile 1.5 mL microcentrifuge tube.

2.3. Add an equal volume of 95% ethanol to the tube and mix well.

2.4. Transfer the solution into a spin column (**Table of Materials**) placed in a collection tube and centrifuge at 10,000 x *g* for 30 s at room temperature. Discard the flow-through, and transfer the spin column to a new collection tube.

2.5. Add 400 µL of RNA Pre-Wash reagent (**Table of Materials**) to the column and centrifuge at 10,000 x *g* for 30 s at room temperature before discarding the flow-through. Repeat this step one more time.

NOTE: To prepare the RNA Pre-Wash, add 10 mL of 95% ethanol to 40 mL of RNA Pre-Wash concentrate.

2.6. Add 700 µL of RNA Wash Buffer (**Table of Materials**) to the column and centrifuge at 10,000 x *g* for 2 min at room temperature. Transfer the column to a sterile 1.5 mL microcentrifuge tube.

NOTE: To prepare RNA Wash Buffer, add 52 mL of 95% ethanol to 12 mL of RNA Wash Buffer concentrate.

2.7. Elute the RNA sample captured in the spin column matrix with 100 µL of nuclease-free water and centrifuge at 10,000 x *g* for 30 s at room temperature.

2.8. Measure the concentration of the extracted RNA using a microvolume spectrophotometer and dilute the RNA to a desired concentration using nuclease-free water.

NOTE: The qualified absorbance value of OD260/OD280 ranges from 1.6 to 1.9, indicating the acceptable RNA purity for the RT-LAMP assay.

2.9. Proceed to the next step immediately, or keep the sample at −80°C until use.

3. Primer design

3.1. Use Primer Explorer version 4 to design the specific primers for the RT-LAMP. Go to the website, https://primerexplorer.jp/e/, and click on the PrimerExplorer V4 button.

3.2. Select the file containing the sequences of segment 3 of TiLV in FASTA format and then click the **Primer Design** button.

NOTE: Retrieve the sequences in FASTA format from GenBank accession number KX631923, which represents tilapia lake virus TV1 segment 3[1].

3.3. To design the primers, input the following basic parameters:

- A GC content of 40%–60%
- An amplicon of ≥280 base pairs (bp)
- A similar melting temperature (Tm) among primers with a maximum difference of 5°C

NOTE: The primers must not complement each other.

3.3.1. Avoid sequence regions prone to forming secondary structures.

3.3.2. Select the dimer primer analysis with a minimum of −3.5 for an optimal design for the largest ΔG, and select the ends of the primers with a maximum of −4 for an optimal design for the smaller ΔG.

3.4. Click the **Generate** button.

NOTE: After the software finishes processing the input data, the primer results will be shown (**Figure 1**).

4. RT-LAMP assay

4.1. Prepare an RT-LAMP master mix containing 2.5 µL of 1x SD II reaction buffer, 3 µL of 6 mM $MgSO_4$, 1.4 µL of 1.4 mM dNTP set, 4 µL of 0.8 M betaine, 1.3 µL of 0.052 mM calcein mixture, 1 µL of 1.6 µM TiLV-F3 primer, 1 µL of 1.6 µM TiLV-B3 primer, 1 µL of 0.2 µM TiLV-BIP primer, 1 µL of 0.2 µM TiLV-FIP primer, 1 µL of 0.3 U Bst DNA polymerase, 1 µL of 0.1 U AMV reverse transcriptase, and 3.8 µL of nuclease-free water per reaction.

NOTE: Prepare excess master mix comprising at least 10% of the total reaction volume.

4.2. Dispense 22 µL of the RT-LAMP master mix into a sterile 1.5 microcentrifuge tube.

4.3. Add 3 µL of the extracted RNA to the reaction tube, and mix the sample by vortexing. For the negative control, use distilled water instead of RNA materials.

4.4. Incubate the reaction at 65 °C for 60 min, followed by 80 °C for 10 min to terminate the reaction.

4.5. After incubation, visually observe the colorimetric changes with the naked eye. A positive result will appear as a fluorescent green color.

5. Agarose gel electrophoresis

5.1. Prepare a 1.5% w/v agarose gel by suspending 0.6 g of agarose powder in 40 mL of 1x TAE buffer. Melt the mixture by heating it in a microwave for 3–5 min until the agarose is completely dissolved, and swirl to mix.

5.2. Allow the agarose to cool down until the temperature reaches 65 °C. Pour 40 mL of the agarose solution onto a gel tray and add a comb to the gel mold.

5.3. Allow the agarose gel to set at room temperature for 20–30 min until it has completely solidified. Then remove the comb and place the gel in the gel tank.

5.4. Add a running buffer to cover the surface of the gel in the gel tank.

5.5. Add 10 µL of the RT-LAMP sample and 2 µL of 6x gel loading buffer to each well. Add 5 µL of a 1 kb DNA ladder to the reference lane.

5.6. Plug in the lid attached to the cathode and the anode connected to a power supply. Turn on the power supply, set it to a constant 100 V, and run for 40 min.

5.7. After completing the gel separation, remove the gel from the gel tray. Then stain the drained gel using ethidium bromide (EtBr) at a concentration of 10 mg/mL for 7 min and restain it in distilled water for 5 min.

CAUTION: EtBr is toxic and considered a carcinogen; therefore, be careful when using this agent.

5.8. Expose the gel to UV light using a gel documentation system where bands appear, and take a picture of the gel.

6. Complementary DNA (cDNA) synthesis

6.1. Prepare a cDNA synthesis master mix containing 2 µL of 5x RT buffer, 0.5 µL of primer mix, 0.5 µL of RT enzyme, and 2 µL of nuclease-free water per reaction.

NOTE: Prepare excess master mix comprising 1x the reaction due to potential loss during pipetting.

6.2. Dispense 5 µL of the master mix into a sterile 1.5 mL microcentrifuge tube.

6.3. Add 100 ng of the diluted extracted RNA (obtained from step 2.8) to the reaction tube, mix and spin down to move all the mixture to the bottom of the vessel.

6.4. Incubate the reactions at 42 °C for 60 min, followed by 98 °C for 5 min in a PCR machine. Store the cDNA at −20 °C until use.

7. RT-qPCR

7.1. Prepare a TiLV qPCR master mix containing 0.3 µL of 10 µM forward primer, 0.3 µL of 10 reverse primer, 5 µL of 2x SYBR Green DNA polymerase, and 0.4 µL of nuclease-free water per reaction. Use the following primers and controls:
Forward primer (TiLV-112F): 5'-CTGAGCTAAAGAGGCAATATGGATT-3'
Reverse primer (TiLV-112R): 5'-CGTGCGTACTCGTTCAGTATAAGTTCT-3'

7.2. Dispense 6 µL of the TiLV qPCR master mix into each well of the qPCR strip compatible with the real-time thermal cycler used.

7.3. Add 4 µL of the cDNA template, negative control (nuclease-free water), positive control (10 pg/µL), and pTiLV (plasmid)[10] or serially diluted TiLV plasmid to the well, and mix each sample by flicking. Conduct each sample in triplicate.

7.4. Place the qPCR reactions into the programmed real-time thermal cycler. Set the qPCR program to perform the incubation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s before the melting curve step in which the temperature needs to increase from 65 °C to 95 °C at 0.5 °C/5 s increments.

7.5. Conduct the data analysis by evaluating the amplification and melting curves, and then compute the number of TiLV copies using the standard curve obtained from the data using the serially diluted plasmids.

REPRESENTATIVE RESULTS:

In this study, an RT-LAMP assay was developed to detect TiLV infection in tilapia. The tested samples were collected from 14 farms located in different parts of Thailand between 2015 and 2016. The infected and uninfected fish were primarily grouped based on physical diagnoses and the appearances of symptomatic TiLVD. TiLV infection was subsequently confirmed using RT-PCR after the collection process. Agarose gel electrophoresis and the detection of a luminescent green color were selected as the evaluation methods of the LAMP amplicons (**Figure 2**). The liver and mucus of the infected and uninfected tilapia fish were characterized by the clinical appearance of TiLV disease symptoms, including skin erosion, skin redness, exophthalmos, and abdominal swelling. Previous reports have demonstrated the use of liver samples in molecular diagnostic assays to determine the presence of TiLV[35]. Alternatively, mucus may be beneficial in the assay as it may help avoid killing the animals. The results showed a ladder-like DNA band pattern and a fluorescent green color in the infected liver and mucus samples of infected fish (**Figure 2**), while no DNA band and the yellow color of calcein were observed in the RT-LAMP mixtures in uninfected animal tissues (**Figure 2**). Interestingly, a TiLV-infected tilapia sample collected from a farm in Malaysia was also diagnosed as positive using this RT-LAMP assay; however, variations in the PCR product's size compared to the samples collected from local Thai farms were observed (**Figure 2**).

To verify the sensitivity and specificity of the RT-LAMP assay, a total of 63 TiLV-infected tissues and 63 uninfected tissues were analyzed using both the RT-LAMP and RT-qPCR assays (**Table 1**). A comparison of the RT-qPCR and RT-LAMP assays of the infected samples revealed a positive result in 63 (100%) and 51 (80.95%) of the samples, respectively. Moreover, an analysis of the uninfected samples showed that all 63 uninfected tissues yielded negative results using both the RT-qPCR and RT-LAMP assays (**Table 1**). This analysis demonstrated the reliability of the RT-LAMP assay for primary TiLV detection. To test the specificity of the RT-LAMP assay, tissue from fish infected with other pathogenic bacteria and viruses, including *Streptococcus agalactiae*, *Francisella noatunensis*, *Flavobacterium columnare*, *Aeromonas hydrophila*, and Iridovirus were

used as templates for the RT-LAMP and RT-qPCR analyses. Both the RT-LAMP and RT-qPCR primers yielded negative results with no colorimetric changes and no fluorescent signals in any of the tested samples (**Table 2**). Additionally, the sensitivity of the RT-LAMP assay was assessed using a serial 10-fold dilution of the RNA templates extracted from TiLV-infected fish. Comparatively, the RT-qPCR assay was more sensitive than the RT-LAMP assay as the RT-qPCR assay had a detection limit of 10^{-8} while the RT-LAMP method required a 10^{-7}-fold dilution to detect the TiLV genome (**Table 2**).

Figure 1. The nucleotide sequences of the six RT-LAMP primers used in this study that were specific to the detection of TiLV. The position of each primer was aligned on segment 3 of the TiLV genome (accession number KX631923).

Figure 2. Analysis of the RT-LAMP amplicons obtained from the TiLV-infected and uninfected samples (A) in 1.5% agarose gel electrophoresis and (B) by fluorescent visualization. M = 1 kb DNA ladder, 1–2 = RNA from the livers of TiLV-infected fish, 3–4 = the cDNA of TiLV-infected fish, 5–6 = RNA from the mucus of TiLV-infected fish, 7–8 = cell lines of TiLV-infected fish, 9 = RNA from the livers of TiLV-infected fish (Malaysia), 10 = RNA from the livers of non-TiLV-infected fish, 11 = the cDNA of non-TiLV-infected fish, 12 = RNA from the mucus of non-TiLV-infected fish, 13 = no template control

Table 1. Verification of RT-LAMP for TiLV detection in infected and uninfected fish samples using RT-qPCR and RT-LAMP

Table 2. Specificity and sensitivity of the RT-LAMP assay compared with RT-qPCR. For the specificity evaluation, RNA obtained from fish tissue infected with other bacteria or viruses, including *Streptococcus agalactiae* (S.a.), *Francisella noatunensis* (F.n.), *Flavobacterium columnare* (F.c.), *Aeromonas hydrophila* (A.h.), and Iridovirus (I.v.), was used as templates for RT-qPCR and RT-LAMP. For the sensitivity evaluation, RNA obtained from TiLV-infected fish was 10-fold serially diluted from 100 ng to 1 fg and used as templates for RT-qPCR and RT-LAMP. The + and – signs mean positive and negative results, respectively.

DISCUSSION:

The aquaculture industry is continuously threatened by viral infections that cause substantial economic losses[9,23,28]. For instance, the emerging TiLV poses a major threat to tilapia-producing countries in many parts of the world[1,6,9]. Until now, there have been no specific therapeutics available to prevent TiLVD. While the development of a vaccine is ongoing, an efficient vaccine will require substantial time before it is available for commercial purposes. Given these circumstances, strict biosecurity measurements, such as the application of disinfectants, are necessary in fish farms to prevent the spread of TiLVD[29,30]. Currently, one of the most efficient control measures to reduce TiLV transmission is the screening of juvenile fish and adults for the presence or absence of TiLV[31]. For screening purposes, the diagnostic tool needs to be rapid, sensitive, and specific so that it can assist in eliminating infected populations and prevent the further spread of disease. However, the current molecular assays for TiLV are difficult to implement on-site. In the first instance, they require skillful researchers and expensive

equipment. Second, it may take several days to obtain the laboratory results, which makes it difficult to control and prevent the spread of disease promptly[15,16].

To overcome these problems, Notomi et al.[14] established a novel nucleic acid-based amplification assay called loop-mediated isothermal amplification (LAMP) in 2000. The LAMP reaction has since been successfully applied to detect various fish viruses[16-26,32-34]. In this study, we developed an RT-LAMP protocol to detect TiLV in tilapia fish samples. Although the sensitivity of the RT-LAMP method is 10 times less efficient than that of the RT-qPCR, the RT-LAMP assay can detect the presence of a TiLV RNA genome as low as 100 fg, which is sufficient for TiLV detection in clinically diseased fish[34]. Notably, the RT-LAMP assay yields a result within 60 min and requires only a simple water bath or heat block instruments[34], while the RT-qPCR assay takes more time and requires more expensive real-time PCR equipment for the analysis[15,34]. Moreover, the end product of the RT-LAMP was observed through a change in color of the fluorescent dye from light yellow to fluorescent green, making it visible to the naked eye without any requirement for sophisticated equipment[34]. These advantages make RT-LAMP suitable for field diagnosis. Furthermore, the study findings suggested that both liver and mucus can be used for TiLV detection using RT-LAMP. Similar to previous studies, a nonlethal sample using mucus allowed the diagnosis of TiLV without killing fish or valuable broodstock[35]. Recently, an RT-LAMP assay was developed to detect Chinese and Thai isolates of TiLV nucleotide sequences in segment 1 (S1 region) using a set of six primers[36]. The present study demonstrated that the developed RT-LAMP assay diagnosed a false-negative signal of TiLV infection at 27.78% compared to RT-PCR when using the same primer set. On further comparison, the false-negative result was relatively less at 19.05% when detecting segment 3 of TiLV compared to RT-PCR. When comparing the efficiency of the viral detection method with other reports, we were able to detect TiLV infection at 80.95%, while other works detected the virus at 72.22%[36] and 82.89%[37]. Altogether, it may be hypothesized that the different components of the RT-LAMP assay, for example, the different primer sets and the different targeted segments of the TiLV genome are important factors influencing the validity of the assay.

Although the RT-LAMP assay is a powerful tool for disease screening and has several demonstrable benefits, this study was not without limitations. One of the critical points for the RT-LAMP assay was the design of an appropriate primer set comprising four to six primers. To promote the formation of the stem-loop structures of the PCR products, the appropriate lengths of the targeted genes or nucleotide sequences needed to be longer than about 500 bp, while the targeted genes of the RT-PCR assay had to be relatively short, in the range of 50–150 bp[14,38,39].

In conclusion, the developed RT-LAMP assay is rapid, cost-effective, sensitive, and specific for TiLV detection. The analysis can be completed within 1 h compared with 4–5 h for the RT-qPCR assay. Notably, the RT-LAMP assay is practical for field conditions as positive results can be observed with the naked eye without requiring the use of sophisticated equipment.

ACKNOWLEDGMENTS:
The project is financially funded by Thailand Research Fund (TRF) grant number RDG6050078 and the Center for Advanced Studies for Agriculture and Food, Institute for Advanced Studies,

Kasetsart University, Bangkok, Thailand under the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Ministry of Education, Thailand. The research is supported in part by the Graduate Program Scholarship from the Graduate School, Kasetsart University. The authors would like to thank Dr. Kwanrawee Sirikanchana for the narrative speaking of the video and Piyawatchara Sikarin for editing the video.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

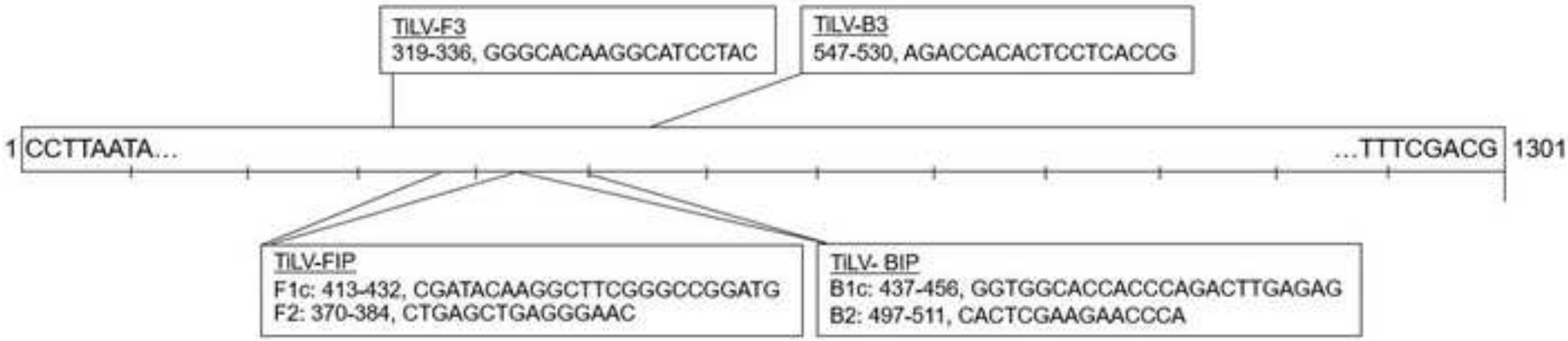


Figure 2

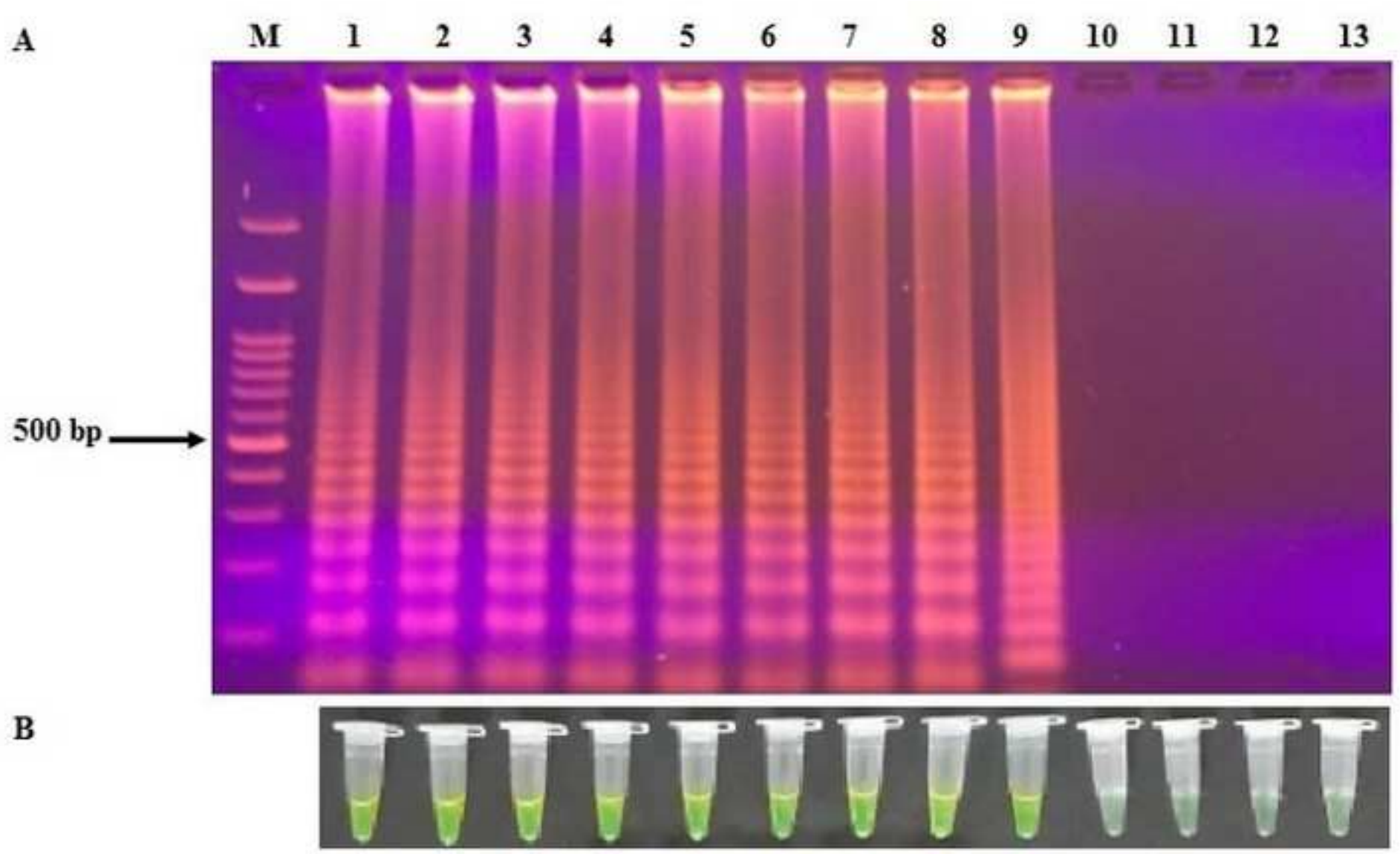


Table 1

Types of samples	Numbers of samples	Detection validity (%)	
		RT-qPCR	RT-LAMP
Infected samples	63	100.00 (63/63)	80.95 (51/63)
Uninfected samples	63	0 (0/63)	0 (0/63)

Table 2

		S.a.		F.n.		F.c.	
Specificity	qPCR	LAMP	qPCR	LAMP	qPCR	LAMP
	–	–	–	–	–	–
Sensitivity	method/ dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
	qPCR	+	+	+	+	+
	LAMP	+	+	+	+	+

A.h.		I.v.	
qPCR	LAMP	qPCR	LAMP
–	–	–	–
10^{-6}	10^{-7}	10^{-8}	10^{-9}
+	+	+	–
+	+	–	–

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_R1.xlsx

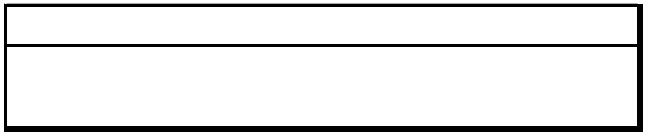
Name of Material/Equipment	Company	Catalog Number
Tissue collection:		
Clove oil	Better Pharma	N/A
Tricaine methanesulfonate	Sigma-Aldrich	E10521
RNA extraction:		
Acid guanidinium-phenol based reagent (TRIzol reagent)	ThermoFisher Scientific Corp.	15596026
Acid guanidinium-phenol based reagent (GENEzol reagent)	Geneaid	GZR100
Direct-zol RNA Kit:	Zymo Research	R2071
- Direct-zol RNA PreWash		
- RNA Wash Buffer		
- DNase/RNase-free water		
- Zymo-spin IIICG columns		
- Collection Tubes		
RT-LAMP:		
1x SD II reaction buffer	Biotechrabbit	BR1101301
Magnesium sulfate ($MgSO_4$)	Sigma-Aldrich	7487-88-9
dNTP set	Bioline	BIO-39053
Betaine	Sigma-Aldrich	B2629
Calcein mixture	Merck	1461-15-0
Bst DNA polymerase	Biotechrabbit	BR1101301
AMV reverse transcriptase	Promega	M510A
Nuclease-free water	Invitrogen	10320995
Elite dry bath incubator, single unit	Major Science	EL-01-220
Gel electrophoresis:		
Agarose	Vivantis Technologies	PC0701-500G
Tris-borate-EDTA (TBE) buffer	Sigma-Aldrich	SRE0062
Tris-acetic-EDTA (TAE) buffer:		
- Tris	Vivantis Technologies	PR0612-1KG
- Acetic acid (glacial), EMSURE	Merck Millipore	1000632500

- Disodium Ethylenediaminetetraacetate dihydrate (EDTA), Vetec	Sigma-Aldrich	V800170-500G
Neogreen	NeoScience Co., Ltd.	GR107
DNA gel loading dye (6X)	ThermoFisher Scientific Corp.	R0611
DNA ladder and markers	Vivantis Technologies	PC701-100G
Mini Ready Sub-Cell GT (Horizontal electrophoresis system)	Bio-Rad	1704487
PowerPac HC power supply	Bio-Rad	1645052
Gel Doc EZ System	Bio-Rad	1708270
UV sample tray	Bio-Rad	1708271
NαBI imager	Neogene Science	
cDNA synthesis:		
ReverTra Ace qPCR RT Kit	Toyobo	FSQ-101
Viva cDNA Synthesis Kit	Vivantis Technologies	cDSK01
NanoDrop2000 (microvolume spectrophotometer)	ThermoFisher Scientific Corp.	ND-2000
T100 Thermal Cycler	Bio-Rad	1861096
RT-qPCR:		
iTaq Universal SYBR Green Supermix	Bio-Rad	1725120
Nuclease-free water, sterile water	MultiCell	809-115-CL
8-tube PCR strips, white	Bio-Rad	TLS0851
Flat PCR tube 8-cap strips, optical	Bio-Rad	TCS0803
CFX96 Touch Thermal Cycler	Bio-Rad	1855196
General equipment and materials:		
Mayo scissors		N/A
Forceps		N/A
Vortex Genie 2 (vortex mixer)	Scientific Industries	
Microcentrifuge LM-60	LioFuge	CM610
Corning LSE mini microcentrifuge	Corning	6765
Pipettes	Rainin	Pipete-Lite XLS
QSP filtered pipette tips	Quality Scientific Plastics	TF series

Corning Isotip filtered tips	Merck	CLS series
Nuclease-free 1.5 mL microcentrifuge tubes, NEST	Wuxi NEST Biotechnology	615601

Comments/Description
An alternative option to clove oil

An alternative option for cDNA synthesis



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April 10, 2020

Dear Editor, *Journal of Visualized Experiments*

We greatly appreciate all of the feedback and suggestions given to improve our revised manuscript. Following the editor suggestions, the paper and video have been amended. Please find attached the revised version of the manuscript. The revised video was uploaded on the dropbox link provided.

Please let me know if you have any additional questions or comments regarding the manuscript and video. I am looking forward to hearing from you,

Sincerely,

Win Surachetpong (On behalf of all authors)

Kasetsart University, 50 Ngam Wong Wan Road, Ladyao,
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Editorial comments: R2

Changes to be made by the author(s) regarding the manuscript:
1. Please employ professional copyediting services. The language in the manuscript is not publication grade as there are many awkward phrasings throughout.

Answer: The revised manuscript has been corrected by professional English editing services. A confirmation receipt has been attached with the rebuttal letter.

2. Additional comments are in the attached manuscript.
Answer: Thanks for the valuable suggestions. All points have been considered and revised (highlight in yellow) as suggested in lines 216–218, 220–221, 223, and 235 (Page 8).

3. Please do not abbreviate journal titles.
Answer: Corrections have been made. (page 14–18).

Changes to be made by the author(s) regarding the video:
1. Audio Quality:
@00:08–00:53 (Intro) & @07:04–07:40 Please reduce the audio volume in the introduction and conclusion statements with Dr. Surachetpong by -6 dB.
The narration is in stereo, which is causing disorientation as a listener. Make sure the narration track is mono in the edit timeline. Convert or set the narration track to MONO audio.

Answer: The audio volume in the introduction and conclusion is reduced by -6 dB as suggested. The narration is checked by the technical person and confirm as MONO audio.

2. Editing & Pacing:
@01:41 Please convert this jump-cut into a dissolve.

Answer: Additional dissolve effect is included at @01:41.

3. Screen Capture Segments:
@03:20 Start here already in the website, do not show a Google page prior to the actual tool.
@03:22 Please show the URL of the website here. It is mentioned in the narration and would make sense to put it on-screen now.

Answer: We remove the google page from @03:20 and include URL of the website from @03.22 to @03.50

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Please upload a revised high-resolution video here:
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Kind regards,
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