Chapter 14

Comparative Analysis of Zika Virus Detection by RT-qPCR, RT-LAMP, and RT-RPA

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Abstract

Molecular detection of Zika virus (ZIKV) is a key element of outbreak management. Multiple PCR and isothermal ZIKV assays targeting different ZIKV sequences have been published. In this study, we compared a qRT-PCR, 2 RT-LAMP assays (based on different primer design approaches), and an RT-RPA for the detection of African and Asian/American lineages of ZIKV isolates from human, mosquito, and monkey. Results showed that RT-LAMP detected 100% of samples with a time threshold (Tt) of 18.01 ± 11.71 min while qRT-PCR detected 88.88% of samples with a Tt of 58.30 ± 16.58 min and RT-RPA 50% of samples with a Tt of 3.70 ± 0.44 min.

Key words Diagnostics, Zika virus, Isothermal amplification, RT-LAMP, RT-PCR, RT-RPA

1 Introduction

Zika virus (ZIKV) is a mosquito-borne flavivirus belonging to the *Flaviviridae* family and Spondweni serocomplex. It is of public health concern worldwide and was first isolated in 1947 in Uganda from a febrile sentinel rhesus monkey during a Yellow fever virus (YFV) study [1]. ZIKV natural transmission cycle involves mainly mosquitoes of the *Aedes* genus and monkeys [2–6], while human infections are accidental and generally asymptomatic. However, clinical pictures of human infection of Zika fever range from a febrile syndrome associated to fever, headache, arthralgia, myalgia, conjunctivitis, and cutaneous rash to severe neurological symptoms such as Guillain-Barré syndrome and microcephaly in newborns [7–10]. Overall, sexual intercourse, perinatal infection, and blood transfusion have been reported as potential routes of direct transmission of ZIKV among humans [11–13]. Early diagnostics then is

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crucial for prognosis, management of patients, and implementation of public health measures.

During the past decade, significant progress has been made in ZIKV diagnostic methods as alternatives to real-time quantitative PCR [14, 15]. Among them are loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA), both of which have demonstrated high sensitivity and specificity [16–19].

1.1 Principle of qPCR

Real-time quantitative PCR reliably detects and measures the amount of DNA template generated exponentially. The real-time quantitative PCR uses a set of forward and reverse primers and a TaqMan fluorogenic non-extendable probe located between positions of primers [14]. The oligonucleotide probe is dual labeled with a fluorescent reporter dye attached to its 5′ end and a quencher dye at its 3′ terminus. Without any reaction, fluorescence resonance energy transfer (FRET) occurs in the intact probe and the fluorescence emission of the reporter dye is absorbed by the quenching dye. During PCR reaction, the internal probe anneals to the specific target sequence downstream from one of the primer sites and is degraded by the 5′ nuclease activity of the Taq polymerase enzyme (originating from the bacteria *Thermus aquaticus*) during the extension phase of the PCR. The cleavage of the probe occurs only if the target sequence is present and then separates the reporter and quencher dyes [15]. The fluorescence emitted by the reporter is measured in a real-time PCR device.

The reaction results in a quantitative relationship between the amount of starting target sequence and the amount of PCR product accumulated at any particular cycle by adding a standard curve generated using serial tenfold dilutions of an in vitro synthetic nucleic acid [15].

1.2 Principle of LAMP

LAMP is an isothermal nucleic acid amplification method discovered in 2000 [20]. The reaction utilizes four primers to cover six gene regions for amplification that occurs through repetition of two types of elongation reactions. Elongation takes place at the loop regions that serve as template self-elongation from the loop structure at the 3′-terminal and subsequent binding and elongation of new primers to the loop region.

In this reaction, pairs of inner and outer primers are used. Each of the inner primers possesses a sequence complementary to one chain of the amplification region at the 3′-terminal and are identical to the inner region of the same chain at the 5′-terminal. The elongation reactions are sequentially repeated by DNA polymerase-mediated strand-displacement synthesis using the aforementioned stem loop regions as a stage. This method operates on the fundamental principle of the production of a large quantity of DNA amplification products with a mutually complementary

sequence and an alternating, repeated structure [21] (a detailed description of the LAMP mechanism and an animation can be found here: http://loopamp.eiken.co.jp/e/index.html).

1.3 Principle of RPA

Recombinase polymerase amplification (RPA) is a proprietary isothermal molecular and detection technology with portable instrumentation currently used in many diagnostic fields, particularly in clinical settings [22, 23]. Besides the bacterial recombinase enzymes used for annealing primers to homologous sequences in a DNA template and extension by isothermal (39–42 °C) strand displacement amplification via the polymerase activity, a typical RPA platform has been used in concert with a reverse transcriptase and a fluorescent probe system for real-time detection of nucleic acids [24]. Once the probe hybridizes to its target sequence, the abasic site is recognized and cleaved by exonuclease III. The smaller downstream probe section carrying the quencher is released, and fluorescence develops proportionally to the RPA-mediated amplification. Real-time detection can then be performed by using TwistAmp exo probes carrying internal fluorophore and quencher linked to thymine bases and separated by an abasic site mimic (tetrahydrofuran) localized approximately 15 nucleotides (nt) upstream from the 3′ end of the probe (45–55 nt) [25] (a detailed description of LAMP mechanism can be found here: https://www.twistdx.co.uk/).

The purpose of the present chapter is to compare the methods established for the detection of Zika virus including qRT-PCR [14], 2 RT-LAMP assay (*see* Chapter 13 of this book series), and RT-RPA [16]. First, we describe the techniques, sensitivity, specificity, and clinical validation approaches for the three assays; and second, we compare their performance on a set of ZIKV isolates from the African and Asian/American lineages.

2 Materials

2.1 Primers and Probes Sequences

1. Primers and probes sequences for qRT-PCR, RT-RPA, and RT-LAMP are, respectively, developed by [14, 16], and by Lopez-Jimena et al. (*see* Chapter 13 of this book series). They are listed in Table 1.

2.2 Zika Virus Isolates

1. Table 2 contains ZIKV strains, obtained from mosquitoes, humans, and other mammals, isolated in Uganda, Ivory Coast, Senegal, and Brazil, and provided by the WHO Collaborating Center for Reference and Research on Arboviruses (CRORA) at Institut Pasteur de Dakar.
2. Use 500 μL of 10^7 plaque-forming unit (pfu)/mL of each ZIKV strain for virus culture.

Table 1
qRT-PCR, RT-LAMP, and RT-RPA Primers and probes sequences

Study	Design approach	Clusters/ Sets	Location (nt)	Oligo name	Oligo sequence (5′–3′)
Lopez-Jimena et al. (in press)	Phylogeny, PCA and LAVA	1	5469–5776	1F3	AGGCCCACTTCACAGATCCCTCA
				1B3	TCTGCTGAGCTGTATGACCCGT
				1FIP	TCCATAATTGGTGAGTTGGAGTCCGGAGGGTTGAGA TGGGCGAGGC
				1BIP	AGTCCCAGAGAGAGCCTGGAGCAGCCTTTG TCAGACAAGCTGCGA
				1LF	GCGTCACGGGTTCCTGGTGG
				1LB	CAGGCTTTGATTGGGTGACGGA
		2	4808–5044	2F3	AGGAGCCGCACTGAGGAGCG
				2B3	CCTGCAGGGTAGTCCAGAGCA
				2FIP	CGCTGAGTCCATCCCAAGCTGCGGGAAGACTTGATCCA TACTGGGGG
				2BIP	TGGCCGTACCTCCCGGAGAGTGCTCCGATGTCCCCGTCC T
				2LF	TCCAAGGCCCACAGTATGACACCA
				2LB	GGCCAGAAACATTCAGACCCTGCC
		3	7379–7614	3F3	CCAGCCGTCCAACATGCGGT
				3B3	GCTGCCTGCAAACCTGGGATCA
				3FIP	AGTCCCATGCATAAAATGGCATCCCACTACTCCCTGA TGGCGATGGC
				3BIP	GGAGTCCCGCTGCTAATGATGGGTGCCACAAGCAGAA TGATGGCCA

			3LF	GCCCATGCCAAACAGCACTCC
			3LB	ACTCACAATTAACACCCCTGACCCT
Primer explorer V4	1		1F3	TTGGGGAAAGCTGTGCAG
			1B3	GTTCAGGCCCCAGATTRAAG
			1FIP	TGGCTTCTTCCGTGCCATGGCAGGAGAAGCTGGGAAACC
			1BIP	CTGCCTGTGAGCCCCTCAGACCACCTTCTTTTCCCATCC T
			1LF1	TCTCGGCCTGACTATAGGCT
			1LF2	TATCGGCCTGACTATGAGCT
			1LB1	GGACACTGAGTCAAAAAACCCC
	2		2F3	TGGAATAGGGTGTGGATTGAAG
			2B3	GCGTGGACAAGTAATCCATGT
			2FIP	GCCGCACCATAGGTCTTCTCTCTACAAAACACCGG TTACAAAGTG
			2BIP	CACTAATAGGGCACAGACCACGCTCACCTATGATCCG TCGCA
			2LF	TCCCAGGTAGGGAATATCTGTC
			2LB	AACATCAAGGATACCGTCAACATG
Abd El Wahed et al. (2017)			FP	TCTCTTGGAGTGCTPGTGATTCTACTCATGGT
			RP	GCTTGGCCAGGTCACTCATTGAAAATCCTC
			exo-probe	CCAGCACTGCCATTGA(BHQl-dT)(THF)(FAM-dT) GCTYATDATGATCTTTGTGGTCATTCTCTTC-P
Faye et al. (2013)		9352–9373	Reverse primer	TCCRCTCCCYCTYTGGTCTTG
		9271–9297	Forward primer	AARTACACATACCARAACAAAGTG GT
		9304–9320	Probe	FAM-CTYAGACCAGCTGAAR-BBQ

Table 2
Zika virus isolate origins

Sample number	Sample name	Sample origin
1	MR766	Monkey (Uganda 1947)
2	T3F	Human (Brazil 2015)
3	HD78788	Human (Senegal 1991)
4	ArD30332	Monkey (Senegal 1979)
5	ARA27091	Mosquito (Côte d'ivoire 1991)
6	226191	Mosquito (Senegal 2011)
7	220990	Mosquito (Senegal 2011)
8	221154	Mosquito (Senegal 2011)
9	ARA975-99	*Aedes aegypti* (Côte d'Ivoire 1999)
10	NS2a	Synthetic NS2a fragment
11	Neg	H_2O

2.3 Growth Medium for Virus Culture

1. Growth medium consists of Leibovitz 15 (L-15) supplemented with 5% fetal bovine serum (FBS), 10% tryptose phosphate, and antibiotics (1000 U/mL penicillin, 10,000 μg/mL streptomycin, and 0.25 μg/mL amphotericin B).
2. Culture monolayer cell lines of *Aedes albopictus* clone C6/36 (ATCC CRL-1660) in 80 cm^2 flasks containing 20 mL of growth medium.

2.4 RNA Extraction

1. RNA extraction kit.

2.5 One-Step Real-Time RT-LAMP

1. 1 M magnesium sulfate ($MgSO_4$) solution in molecular-grade water.
2. Prepare 0.1 M $MgSO_4$: mix 0.1 mL 1 M $MgSO_4$ and 0.9 mL molecular-grade water.
3. 25% polyethylene glycol (PEG) solution in molecular-grade water.
4. Fluorochrome dye (FD).
5. *Bst* 2.0 DNA polymerase (8 U/μL).
6. Transcriptor reverse transcriptase (20 U/μL).
7. RT-LAMP reaction mix consists of 1× RM trehalose (Mast Diagnostica), 6 mM $MgSO_4$, 5% PEG, 1 μL FD.

2.6 One-Step Real-Time RT-RPA

1. 2.1 μL of each 420 nM RPA primers in molecular-grade water.
2. 0.6 μL of 120 nM RPA exo-RT-probe (TwistDx) in molecular-grade water.

3. 4 μL of 14 mM Mg acetate in molecular-grade water.
4. 29.5 μL of 4× RPA rehydration buffer.

2.7 One-Step Real-Time RT-PCR

1. 10 μL of each 10 μM primer and 10 μM probe in molecular-grade water.
2. 0.2 μL of kit enzyme mixture (including reverse transcriptase RT and Taq polymerase).

3 Methods

3.1 Virus Culture

1. Prepare viral stocks by inoculating 500 μL of 10^7 pfu/mL of ZIKV viral strains listed in Table 2 into *Aedes albopictus* clone C6/36 cell monolayer cultured in 80 cm^2 flask containing 20 mL of growth medium (*see* **Note 1**).
2. Four days after inoculation, confirm viral infection by an indirect immunofluorescence assay (IFA) using specific hyperimmune mouse ascitic fluid, as described previously [26].
3. Collect culture supernatants for virus isolation and/or viral RNA extraction.

3.2 RNA Extraction

1. Extract viral RNA from the samples listed in Subheading 2.2 with a viral RNA extraction kit, according to the manufacturer's instructions (*see* **Note 2**).
2. Keep viral RNA samples on ice whilst in use and store them at −80 °C (*see* **Note 3**).

3.3 ZIKV qRT-PCR Primer Design

1. Use the ClustalW program [27] with ZIKV sequences from Africa (GenBank accession number, KF38304-KF383114), from Malaysia (NC_012532), and from Micronesia (EU545988) to conduct an in silico analysis.
2. Identify a stretch of conserved nucleotides in the NS5 protein as a target region for the primers.
3. Design ZIKV primers and a short locked nucleic acids (LNA) probe sequence (16 nt) labeled using a 6-carboxyfluorescein dye (FAM) at the 5′-end as reporter and a 6-carboxytetramethylrhodamin dye (TAMRA) at the 3′-end as quencher [28].

3.4 One-Step ZIKV qRT-PCR

1. Set up a real-time thermal cycler with FAM channel, as follows: reverse transcription for 10 min at 50 °C, initial denaturation for 30 s at 95 °C, followed by 40 amplification cycles consisting of denaturation for 15 s at 95 °C and annealing/extension for 60 s at 60 °C.

2. Use the RT-PCR primers and probe described in (Subheading 2.1, Table 1) to carry out the qRT-PCR assay as previously published [14].
3. Use ZIKV RNA sample MR766 as a qualitative positive amplification control template.
4. Place the RT-PCR reactions in the real-time thermal cycler and start the program, as indicated in **step 1**.

3.5 ZIKV qRT-PCR Performance Evaluation

1. Generate an in vitro transcribed RNA of 1083 bp encompassing the target region as previously described [14].
2. Calculate sensitivity and specificity using standard statistical formulas for diagnostics performance evaluation [29].
3. Validate the clinical performance of ZIKV qRT-PCR on mosquito and serum samples as previously described [14].
4. This assay exhibits an analytical specificity of 100% and detection limits of 32 RNA molecules ($R^2 = 0.9987$) and 0.05 pfu in growth medium or synthetic normal human plasma.
5. In addition, the ZIKV qRT-PCR assay detects ZIKV covering a wide geographic region in Africa and Asia in less than 3 h.
6. Intra-assay and inter-assay coefficient variations of the mean Ct values range from 1.19–1.61% and 1.23–2.72%, respectively, when using synthetic RNA.
7. This highly reproducible and repeatable assay shows also a high sensitivity on ZIKV strains from field-caught mosquitoes, ZIKV stocks spiked in growth medium, or synthetic normal human plasma with a cut-off Ct value of 36.24 [14].

3.6 ZIKV RT-LAMP Primer Design

1. Design primer sets using two approaches: (1) Open source approach, based on a combination of sequence diversity clustering (phylogeny and principal component analysis) and LAVA algorithm [30–32], and (2) a standard software for LAMP primer design (Primer Explorer V4). The final selection of primer sets is included in Table 1.
2. A detailed description of the RT-LAMP primer design, following two different approaches, is described in Chapter 13 of this book series.

3.7 ZIKV One-Step Real-Time RT-LAMP

1. Set up a fluorimeter or real-time thermal cycler with FAM channel as follows: 64 °C for 45 min and melt curve analysis from 98 to 80 °C (0.05 °C/s) at the end of the reaction.
2. Reconstitute RT-LAMP primers listed in Table 1 with nuclease-free water at 100 μM.
3. Incubate these on ice for 20 min, vortex, and briefly spin down before their use.

4. Set up RT-LAMP reactions (final volume of 25 μL) by combining: 17.5 μL RT-LAMP reaction mix (Subheading 2.5, **item 7**), 5 μL or 4 μL of appropriate primer sets, respectively, for phylogeny/PCA/LAVA and Primer Explorer V4 approaches (Subheading 3.6, Table 1).
5. Adjust final primer concentration for each primer set as follows: 100 nM F3, 100 nM B3, 800 nM FIP, 800 nM BIP, 400 nM LF, 400 nM LB.
6. Then, add 1 μL *Bst* 2.0 DNA polymerase (8 U/μL) and 0.5 μL transcriptor reverse transcriptase (20 U/μL). Finally, add 1 μL template (ZIKV RNA or H_2O as negative control) (*see* **Note 4**).
7. Place the RT-LAMP reactions in the fluorimeter or real-time thermal cycler and start the program, as indicated in **step 1**.

3.8 ZIKV RT-LAMP Assay Performance Evaluation

1. Both RT-LAMP protocols detect as few as 10^2 molecules per reaction, although this amount is only obtained in four of eight repetitions with the following mean ± SD times: 35.3 ± 1.5 min (phylogeny, PCA, and LAVA design) and 37.4 ± 7.8 min (Primer Explorer V4 design).
2. The assays are 100% reproducible when adding 10^3 molecules per reaction. Considering eight independent reactions per protocol developed, the probit analysis reveals that the limit of detection at 95% probability for both developed RT-LAMP assays is 181 molecules.
3. The RT-LAMP assays detect the two positive RNA samples of the ZIKV EQA panels provided by INSTAND e.V.
4. In relation to the EVD-LabNet qualification panel [33], different results are observed when comparing the two RT-LAMP assays. None of the negative samples included in the panel is amplified by the assay designed by phylogeny, PCA, and LAVA; however, a plasma sample containing a medium concentration (~10^5 ZIKV RNA copies/mL) of the ZIKV Asian lineage is only amplified in 1/2 replicates. Primers designed with the second approach (Primer Explorer V4) additionally detects the low concentration (~10^3 ZIKV RNA copies/mL) of the ZIKV Asian lineage (1/2 replicates) but also produces non-specific amplification of RNA extracted from a negative plasma sample (1/2 replicates). Samples containing a high concentration (~10^9 ZIKV RNA copies/mL) of ZIKV are successfully amplified by the two RT-LAMP assays.
5. A second evaluation of the RT-LAMP assays was performed on nine different ZIKV strains from the Institut Pasteur de Dakar biobank, isolated from C6/36 cell culture. Eight samples from the African and Asian lineages were successfully detected by

both assays while only the RT-LAMP assay designed by phylogeny, PCA, and LAVA was able to detect the ninth strain (sample 226191) (Table 2).

6. Chapter 13 of this book series contains a more detailed description of the results included in this section.

3.9 ZIKV RT-RPA Primer Design

1. This ZIKV RT-RPA primers and probe target the NS2A region conserved among all ZIKV lineages (nt 3572–3713, GenBank: LC002520.1).
2. The exo-probe is designed with an arrangement of quencher (black hole quencher 1 [BHQ-1]), internal abasic site mimic (tetrahydrofuran spacer [THF]), fluorophore (6-carboxyfluorescein [FAM]), and block elongation (phosphate [P]) (*see* Table 1 for sequence details) [16].

3.10 ZIKV One-Step Real-Time RT-RPA

1. Set up an isothermal cycler at 42 °C for 20 min with a fluorescence detection in the FAM channel (excitation 470 nm and detection 520 nm).
2. Use the RT-RPA primers and probe described in (Subheading 2.1, Table 1) and prepare the reaction mix to carry out the RT-RPA assay as previously published [16] (*see* **Note 5**).
3. Use ZIKV RNA sample MR766 as a qualitative positive amplification control template.
4. Place the RT-RPA reactions in isothermal cycler and start the program, as indicated in **step 1**.
5. After the run is completed, use a combined threshold and signal slope analysis confirmed by second derivative to interpret the signal [34].

3.11 ZIKV RT-RPA Performance Evaluation

1. Evaluate the analytical sensitivity of the ZIKV RT-RPA using a ZIKV NS1/NS2 molecular RNA standard as previously published [16].
2. The RT-RPA is portable and rapid (3–15 min) and detects as few as 21 RNA molecules per reaction.
3. Evaluate cross-reactivity of the ZIKV RT-RPA with other flaviviruses, alphaviruses, and arboviruses provided by the European Network for Diagnostics of Imported Viral Diseases (ENIVD) [16].
4. The assay is highly specific as no amplification is observed with other flaviviruses, alphaviruses, and arboviruses.
5. The clinical performance of the assay was evaluated on acute-phase (2–10 days from the onset of symptoms) urine samples collected from suspected cases at the Municipal Hospital of Tuparetama, Pernambuco, Brazil.

6. Compared to qRT-PCR [16], this RT-RPA assay shows a diagnostic sensitivity of 92% on urine specimens with a cut-off Ct value of 39 while the diagnostic specificity was 100%.
7. The RT-RPA assay identifies African (GenBank: AY632535) and Brazilian strains (Instituto Evandro Chagas, Belém, Brazil) down to 65 and 35 RNA genome equivalents, respectively, using tenfold serial dilutions from virus culture supernatant.
8. A detailed description of the ZIKV RT-RPA performance can be found in [16].

3.12 Time Threshold Comparison of ZIKV qRT-PCR, RT-LAMP, and RT-RPA

1. To compare the different assays, use nine ZIKV strains belonging to the African and Asian/American lineages and selected from Institut Pasteur de Dakar biobank and isolated in C6/36 cells as previously described [26] (Subheading 2.2, Table 2).
2. Perform qRT-PCR, RT-LAMP, and RT-RPA on the viral RNA extracted in Subheading 3.2, as well as on a ZIKV NS2a in vitro RNA, as previously published [14, 16].
3. Of the two RT-LAMP assays designed to cover all ZIKV strains, include in this study only data given by the assay with the best performance in this study (*see* **Note 6**).
4. Contrary to RT-RPA and RT-LAMP where test outcomes are given in minutes, cycle numbers for qRT-PCR are converted into equivalent time threshold (Tt) to allow a comparison of the different assays on ZIKV detection (*see* **Note 7**).
5. Calculate the Tt mean of each assay for samples 1–10 including only positive results using a Kruskal-Wallis Chi-squared method (*see* Table 3).
6. Tt means comparison reveals, with a p-value of 6.8E−05, that RT-RPA is $\simeq$5 times faster than the RT-LAMP assays, which were $\simeq$3 times faster than the qRT-PCR (Table 3 and Fig. 1).
7. qRT-PCR values range from 40.59 to 91.36 min with a mean of 58.30 ± 16.58 min.
8. RT-LAMP values range from 8.36 to 37.5 min with a mean of 18.01 ± 11.71 min.
9. RT-RPA values range from 3.5 to 4.5 min with a mean of 3.7 ± 0.44 min.
10. All three assays detected samples 1–4, which are, respectively, a monkey isolate of the African lineage, a human isolate of the Asia/American lineage, a human isolate of the African lineage, and a monkey isolate of the african lineage.
11. Samples 5, 6, 8, and 9 (mosquito isolates, African lineage) are only detected by qRT-PCR and RT-LAMP.
12. Sample 7 (mosquito isolate, African lineage) is only detected by RT-LAMP.

Table 3
Time threshold analysis for qRT-PCR, RT-LAMP, and RT-RPA

	Mean	SD	Range
qRT-PCR	58.30	16.58	[40.59–91.36]
RT-LAMP	18.01	11.71	[8.36–37.5]
RT-RPA	3.70	0.44	[3.5–4.5]

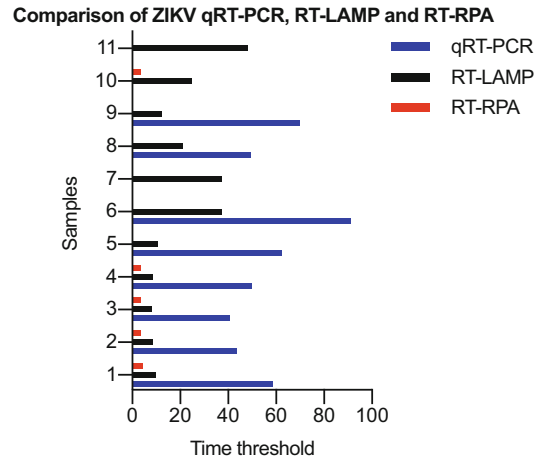

Fig. 1 Comparison of ZIKV qRT-PCR, RT-LAMP, and RT-RPA

13. Sample 7 is not picked up by the qRT-PCR assay which was reported to efficiently detect African ZIKV isolates from mosquitoes [14]. We believe that the negative result might be due to sequence variation in sample 7, which is isolated from an atypical vector (i.e., *Culex perfuscus*).
14. On the other hand, further investigations are needed to understand why the RT-RPA assay failed to detect samples 5–9 in single replicate.
15. Sample 10 (NS2a fragment) is successfully detected by RT-LAMP and RT-RPA and not by qRT-PCR, which only targets the NS5 region.
16. The Tt cut-off for RT-LAMP is set to 45 min (*see* Chapter 13 of this book series), and therefore, none of the assays give false-positive results with H_2O (sample 11).
17. In this study, RT-LAMP proves itself to be more suitable for ZIKV detection since it detects all samples in a short turnaround time.

18. However, although LAMP offers the advantage of being cheaper than RPA, primer design requires extensive bio-informatic ability if using phylogeny, PCA, and LAVA.
19. The combination of the two RT-LAMP assays covering whole ZIKV genomes (first approach—phylogeny, PCA, and LAVA) and 3′-UTR (second approach—Primer Explorer V4) has a net advantage in terms of sensitivity over the single qRT-PCR and RT-RPA assays targeting NS5 and NS1/NS2, respectively.

4 Notes

1. Virus culture in C6/36 cell line is done in an incubator at 28 °C.
2. Precaution should be taken while extracting RNA in order to avoid cross-contamination. It is recommended that RNA extraction and RT-PCR mix preparation are physically separated.
3. Aliquot RNA samples for single use only to avoid freeze-thaw cycles.
4. Before adding the *Bst* 2.0 DNA polymerase, transcriptor reverse transcriptase, and template, reaction mixes are incubated at 95 °C for 5 min to melt any possible primer multimers and cooled immediately on ice for 5 min.
5. Prepare the RT-RPA reaction mixture and then add RNA without magnesium, which instantaneously triggers the reaction. Add magnesium on the tube cap cavity and gently close the tube. Briefly centrifuge and vortex the tube and put it directly into the isothermal device to proceed with fluorescence signal reading.
6. The two RT-LAMP assays have different performances on the ZIKV isolates in our study. We noticed one assay can be positive for a specific isolate and the second being negative or Tt being significantly different. For that reason, only positive results and best Tt of the combined RT-LAMP assays were included in this study.
7. To convert qRT-PCR Ct values into minutes, note the equivalent time for each Ct value and then add the reverse transcription total time, which is not displayed in the fluorescence graph.

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