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#### Calvert

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## (54) RAPID DETECTION OF ZIKA VIRUS BY REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

## (71) Applicant: The U.S.A., as represented by the Secretary, Department of Health and

Human Services, Bethesda, MD (US)

#### (72) Inventor: Amanda E. Calvert, Mead, CO (US)

# (73) Assignee: The United States of America, as Represened by the Secretary, Department of Health and Human Services, Bethesda, MD (US)

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  C12Q 2525/301; C12Q 2531/119; C12Q

See application file for complete search history.

2600/16

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Primary Examiner — David C Thomas (74) Attorney, Agent, or Firm — Klarquist Sprakman, LLP

#### (57) ABSTRACT

A highly sensitive and specific reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay to detect ZIKV nucleic acid in biological samples is described. The disclosed assay is capable of detecting as few as one RNA copy per  $\mu$ l and can be performed in a clinical or field setting with minimal equipment and technological expertise. Oligonucleotide primers and kits for detecting ZIKV nucleic acid are also described.

#### 20 Claims, 2 Drawing Sheets

#### Specification includes a Sequence Listing.

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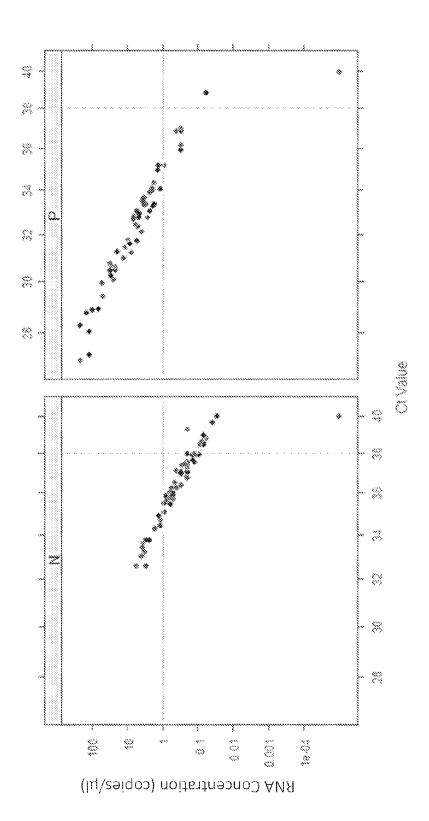
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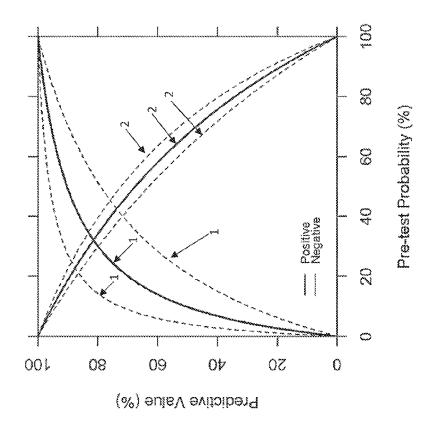
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#### RAPID DETECTION OF ZIKA VIRUS BY REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL **AMPLIFICATION**

#### CROSS REFERENCE TO RELATED APPLICATIONS

This application is the U.S. National Stage of International Application No. PCT/US2018/029738, filed Apr. 27, 10 2018, published in English under PCT Article 21(2), which claims the benefit of U.S. Provisional Application No. 62/500,866, filed May 3, 2017, which is herein incorporated by reference in its entirety.

#### **FIELD**

This disclosure concerns a rapid and sensitive point-ofcare (POC) diagnostic assay for the detection of Zika virus nucleic acid in biological samples.

#### BACKGROUND

Zika virus (ZIKV) is a mosquito-borne virus from the genus Flavivirus in the family Flaviviridae. Other notable 25 viruses in this genus are dengue 1-4 viruses (DENV1-4), yellow fever virus (YFV), West Nile virus (WNV) and Japanese encephalitis virus (JEV). ZIKV was first isolated in 1947 from the blood of a febrile sentinel rhesus monkey during a yellow fever study in the Zika forest Uganda (Dick 30 et al., Trans R Soc Trop Med Hyg 46:509-520, 1952). Until recently, ZIKV was only known to cause a mild febrile illness with very few cases reported since its discovery. In April 2007, ZIKV caused an epidemic of disease in Yap State. Federated States of Micronesia (Lanciotti et al., 35 ZIKV nucleic acid in biological samples. The disclosed Emerg Infect Dis 14:1232-1239, 2008), after which the virus spread across the South Pacific for the next 8 years before being identified as the causative agent of an outbreak of disease in March 2015 in Bahia, Brazil (Campos et al., Emerg Infect Dis 21:1885-1886, 2015; Zanluca et al., Mem 40 Inst Oswaldo Cru. 110:569-572, 2015; Petersen et al., N Engl J Med 374:1552-1563, 2016). Since this time, ZIKV has been shown to be transmitted sexually, from mother to fetus, and possibly through blood transfusions (Calvet et al., Lancet Infect Dis 16:653-660, 2016; Calvet et al., Curr Opin 45 Infect Dis 29:459-466, 2016; D'Ortenzio et al., N Engl J Med 374:2195-2198, 2016: Foy et al., Emerg Infect Dis 17:880-882, 2011; Musso et al., Euro Surveill 19, 2014; Venturi et al., Euro Surveill 21, 2016). It has also been shown as the causative agent of microcephaly and other 50 congenital brain abnormalities in infants born to mothers infected during pregnancy (Campos et al., Emerg Infect Dis 21:1885-1886, 2015; Petersen et al., N Engi J Med 374: 1552-1563, 2016: Heukelbach et al., Lancet 388:846-847, 2016: Rasmussen et al., N Engl J Med 374:1981-1987, 55 2016), as well as triggering cases of Guillain-Barre syndrome in infected patients (Brasil et al., Lancer 387:1482, 2016; Fauci et al., N Engl J Med 374:601-604, 2016).

Laboratory diagnosis of ZIKV relies on analysis of urine or serum for the detection of viral RNA within the first 10 60 days after symptom onset, or the analysis of serum for IgM by ELISA after the acute phase of disease. Due to the cross-reactivity of flaviviruses during secondary infections, a definitive diagnosis based on serology results is not possible (Lanciotti et al., Emerg Infect Dis 14:1232-1239, 65 2008: Petersen et al., N Engl J Med 374:1552-1563, 2016). Some data suggests that ZIKV RNA may be detected longer

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in urine than from serum (Gourinat et al., Emerg Infect Dis 21:84-86, 2015; Korhonen et al., Euro Surveill 21, 2016; Kutsuna et al., Euro Surveill 19, 2014; Roze et al., Euro Surveill 21, 2016). Since the only way ZIKV infections can be definitively diagnosed is through the detection of ZIKV RNA during the acute phase of infection, a rapid diagnostic assay that can be performed throughout pregnancy in a clinical setting is vital for prenatal care of women living in areas of possible transmission of the virus.

Loop-mediated isothermal amplification (LAMP) developed by Notomi et al. (Nucleic Acids Res 28:E63, 2000) is a technique for the amplification of nucleic acid utilizing 4-6 primers designed to amplify the gene target through creation of stem-loop structures that aid in synthesizing new DNA by the polymerase. Synthesis of DNA occurs rapidly at a constant temperature, unlike PCR which requires specialized equipment for temperature cycling. LAMP is also highly specific, since several primers are used to amplify a specific nucleic acid sequence. Reverse-transcription LAMP (RT-LAMP) assays have previously been developed for the <sup>20</sup> rapid detection of several mosquito-borne viruses in humans and mosquito pools including DENV, WNV, YFV, chikungunya virus (CHIKV), Rift Valley fever virus (RVFV). St. Louis encephalitis virus (SLEV) and Western equine encephalitis virus (Li et al., Virol J 8:360, 2011; Parida et al., Clin Microbiol 43:2895-2903, 2005; Parida et al., Clin Microbiol 45:351-357, 2007; Parida et al., Clin Microbiol 44:4172-4178, 2006; Peyrefitte et al., Clin Microbiol 46:3653-3659, 2008; Teoh et al., BMC Infect Dis 13:387, 2013; Toriniwa and Komiya, Microbiol Immunol 50:379-387, 2006; Wheeler et al., PLoS One 2016; 11:e0147962).

#### **SUMMARY**

Disclosed herein are methods and kits for detection of methods and kits allow for the rapid, sensitive and specific diagnosis of ZIKV infection in POC settings.

Provided herein is a method for detecting ZIKV nucleic acid in a biological sample. In some embodiments, the method includes subjecting the sample to a reverse transcription loop-mediated isothermal amplification (RT-LAMP) reaction using a set of primers specific for ZIKV nucleic acid to produce a ZIKV nucleic acid amplification product; and detecting the ZIKV nucleic acid amplification product. In particular embodiments, the RT-LAMP reaction includes a pH sensitive indicator dye, such as a dye detectable in visible light.

Also provided herein are kits for detecting ZIKV nucleic acid in a biological sample. In some embodiments, the kit includes a set of primers specific for ZIKV nucleic acid, such as primers that can be used in an RT-LAMP assay for detection of ZIKV nucleic acid. In some examples, the kit further includes one or more of buffer, reverse transcriptase and DNA polymerase.

Further provided herein are oligonucleotide primers for detection of ZIKV nucleic acid. In some embodiments, the primers have a sequence at least 85% identical to any one of SEO ID NOs: 1-18.

The foregoing and other features and advantages will become more apparent from the following detailed description of several embodiments, which proceeds with reference to the accompanying figures.

#### BRIEF DESCRIPTION OF THE FIGURES

FIG. 1: Evaluation of ZIKV RT-LAMP assay with diagnostic specimens. Negative (N; left) ZIKV RT-LAMP results

and positive (P; right) ZIKV RT-LAMP results were separated and plotted out based on Ct value (x-axis) and RNA concentration (y-axis) from the corresponding RT-PCR. Each diamond represents one urine or serum sample. The solid horizontal line represents the 1 RNA copy/pi cutoff value. The dashed vertical line represents the Ct value 38.0. All Ct values >38.0 are considered negative in the RT-PCR.

FIG. 2: Predictive values for the ZIKV RT-LAMP assay based on pre-test probability percentages of ZIKV infection. Positive (1—solid line) and negative (2—solid line) predictive values (%), and 95% confidence intervals (positive: 1—dashed lines, negative: 2—dashed lines), plotted against pre-test probability (%) for the RT-LAMP assay using all diagnostic samples combined.

#### SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is submitted as an ASCII text file, created on Sep. 13, 2019, 25 4.66 KB, which is incorporated by reference herein. In the accompanying sequence listing:

SEQ ID NOs: 1-18 are primer sequences for RT-LAMP. SEQ ID NOs: 19-21 are primer and probe sequences for qRT-PCR.

SEQ ID NO: 22 is a T7 promoter sequence.

#### DETAILED DESCRIPTION

#### I. Abbreviations

CHIKV chikungunya virus

CI confidence interval

CT cycle threshold

DENV dengue virus

JEV Japanese encephalitis virus

LAMP loop-mediated isothermal amplification

LOD limit of detection

MM master mix

NS non-structural

POC point-of-care

RT reverse transcriptase

RT-LAMP reverse transcription loop-mediated isothermal amplification

RVFV Rift Valley fever virus

SLEV St. Louis encephalitis virus

WNV West Nile virus

YFV yellow fever virus

ZIKV Zika virus

#### II. Terms and Methods

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, Genes 60 V, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew et al. (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive 65 Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

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In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:

Amplification: Increasing the number of copies of a nucleic acid molecule, such as a gene or fragment of a gene, for example at least a portion of ZIKV nucleic acid molecule. The products of an amplification reaction are called amplification products. An example of in vitro amplification is the polymerase chain reaction (PCR), in which a sample (such as a biological sample from a subject) is contacted with a pair of oligonucleotide primers, under conditions that allow for hybridization of the primers to a nucleic acid molecule in the sample. The primers are extended under suitable conditions, dissociated from the template, and then 15 re-annealed, extended, and dissociated to amplify the number of copies of the nucleic acid molecule. Other examples of in vitro amplification techniques include real-time PCR, quantitative real-time PCR (qPCR), reverse transcription PCR (RT-PCR), quantitative RT-PCR (qRT-PCR), loopmediated isothermal amplification (LAMP; see Notomi et al., Nucl. Acids Res. 28:e63, 2000); reverse-transcription LAMP (RT-LAMP); strand displacement amplification (see U.S. Pat. No. 5,744,311); transcription-mediated amplification (U.S. Pat. No. 5,399,491) transcription-free isothermal amplification (see U.S. Pat. No. 6,033,881); repair chain reaction amplification (see WO 90/01069); ligase chain reaction amplification (see U.S. Pat. No. 5,686,272); gap filling ligase chain reaction amplification (see U.S. Pat. No. 5,427,930); coupled ligase detection and PCR (see U.S. Pat. No. 6,027,889); and NASBATM RNA transcription-free amplification (see U.S. Pat. No. 6,025,134).

Animal: Living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term mammal includes both human and non-human mammals. Similarly, the term "subject" includes both human and veterinary subjects, for example, humans, non-human primates, dogs, cats, horses, and cows.

Biological sample: A sample obtained from a subject (such as a human or veterinary subject). Biological samples, include, for example, fluid, cell and/or tissue samples. In some embodiments herein, the biological sample is a fluid sample. Fluid sample include, but are not limited to, serum, urine, blood, plasma, feces, saliva, cerebral spinal fluid (CSF) and bronchoalveolar lavage (BAL) fluid.

45 Contacting: Placement in direct physical association; includes both in solid and liquid form. "Contacting" is often used interchangeably with "exposed." For example, contacting can occur in vitro with one or more primers and/or probes and a biological sample (such as a sample including 50 nucleic acids) in solution.

Control: A reference standard, for example a positive control or negative control. A positive control is known to provide a positive test result. A negative control is known to provide a negative test result. However, the reference standard can be a theoretical or computed result, for example a result obtained in a population.

Detectable label: A compound or composition that is conjugated (e.g., covalently linked) directly or indirectly to another molecule (such as a nucleic acid molecule) to facilitate detection of that molecule. Specific non-limiting examples of labels include fluorescent and fluorogenic moieties (e.g., fluorophores), chromogenic moieties, haptens (such as biotin, digoxigenin, and fluorescein), affinity tags, and radioactive isotopes (such as <sup>32</sup>P, <sup>33</sup>P, <sup>35</sup>S, and <sup>125</sup>I). The label can be directly detectable (e.g., optically detectable) or indirectly detectable (for example, via interaction with one or more additional molecules that are in turn detectable).

Methods for labeling nucleic acids, and guidance in the choice of labels useful for various purposes, are discussed, e.g., in Green and Sambrook, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Fourth Edition, 2012, and Ausubel et al., *Short Protocols in 5 Molecular Biology, Current Protocols*, Fifth Edition, 2002.

Fluorescent indicator dye: A fluorescent compound that responds to changes in environmental conditions (such as pH or metal ion concentration) by changes in fluorescence properties. In some examples, fluorescence of a fluorescent indicator dye is increased by a change in pH. The fluorescent indicator dye can be detected by any suitable method, including visually (e.g., under ambient or ultraviolet light) or using instrumentation for detection of fluorescence (such as a fluorimeter or real-time PCR system). Exemplary fluo- 15 rescent indicator dyes include, for example, calcein, hydroxynaphthol blue, Mag-Fura-2 and Magnesium Green (Life Technologies, Grand Island, N.Y.) and Fluo-2 Mg, Fura-2 Mg. Indo-1 Mg, and Asante Magnesium Green (TEF Labs. Austin, Tex.). In some examples, fluorescence from 20 the fluorescent indicator dyes useful in the methods disclosed herein is visibly detectable (for example, by eye, such as a colorimetric reagent), while in other examples, the fluorescence is detectable using an instrument, such as a fluorimeter or real-time PCR platform.

Isolated: An "isolated" or "purified" biological component (such as a nucleic acid, peptide, protein, protein complex, or virus-like particle) has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs, that is, other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids, peptides and proteins that have been "isolated" or "purified" thus include nucleic acids and proteins purified by standard purification methods. The term also embraces 35 nucleic acids, peptides and proteins prepared by recombinant expression in a host cell, as well as chemically synthesized nucleic acids or proteins. The term "isolated" or "purified" does not require absolute purity; rather, it is intended as a relative term. Thus, for example, an isolated 40 biological component is one in which the biological component is more enriched than the biological component is in its natural environment within a cell, or other production vessel. Preferably, a preparation is purified such that the biological component represents at least 50%, such as at 45 least 70%, at least 90%, at least 95%, or greater, of the total biological component content of the preparation.

Isothermal amplification: Nucleic acid amplification that is not dependent on significant changes in temperature (in contrast to PCR, for example). Thus, it is carried out 50 substantially at about the same single temperature. In some examples, isothermal amplification is substantially isothermal, for example, may include small variations in temperature, such as changes in temperature of no more than about 1-2° C. during the amplification reaction. In one example, 55 isothermal amplification is carried out at about 65° C.

Loop-mediated isothermal amplification (LAMP): A method for amplifying nucleic acid. The method is a single-step amplification reaction utilizing a DNA polymerase with strand displacement activity (e.g., Notomi et al., *Nucl. Acids.* 60 *Res.* 28:E63, 2000; Nagamine et al., *Mol. Cell. Probes* 16:223-229, 2002; Mori et al., *J. Biochem. Biophys. Methods* 59:145-157, 2004). At least four primers, which are specific for eight regions within a target nucleic acid sequence, are typically used for LAMP; however, in some 65 examples, two primers may be used for LAMP. The primers include a forward outer primer (F3), a backward outer

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primer (B3), a forward inner primer (FIP), and a backward inner primer (BIP). A forward loop primer (Loop F or LF), and/or a backward loop primer (Loop B or LB) can also be included in some embodiments. The amplification reaction produces a stem-loop DNA with inverted repeats of the target nucleic acid sequence. To amplify RNA sequences using LAMP, reverse transcriptase (RT) is added to the reaction. This variation is referred to as RT-LAMP. In contrast to PCR, LAMP and RT-LAMP are carried out at a constant temperature and do not require a thermal cycler.

pH-sensitive indicator dye: A dye that undergoes a color change as pH increases or decreases. In the context of the present disclosure, pH-sensitive indicator dyes can be used to detect nucleic acid amplification products. When a DNA polymerase incorporates a deoxynucleoside triphosphate into a nascent DNA, the released by-products include a pyrophosphate moiety and a hydrogen ion. Thus, as nucleic acid amplification products increase in a reaction, the pH of the solution decreases, resulting in a color change of the pH-sensitive dye (see Tanner et al. *BioTechniques* 58:59-68, 2015). Exemplary pH-sensitive indicator dyes include, but are not limited to, phenol red, cresol red, neutral red, m-cresol purple, bromocresol purple, naphtholphthalein, thymol blue and naphtolphthalein.

Primer: Primers are short nucleic acids, generally DNA oligonucleotides 10 nucleotides or more in length (such as 10-60, 15-50, 20-40, 20-50, 25-50, or 30-60 nucleotides in length). Primers may be annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, and then extended along the target DNA strand by a DNA polymerase enzyme. Primer pairs or sets of primers (such as 2, 3, 4, 5, 6, or more primers) can be used for amplification of a target nucleic acid, e.g., by PCR, LAMP. RT-LAMP, or other nucleic acid amplification methods known in the art.

Probe: A probe typically comprises an isolated nucleic acid (for example, at least 10 or more nucleotides in length, such as 10-60, 15-50, 20-40, 20-50, 25-50, or 30-60 nucleotides in length) with an attached detectable label or reporter molecule. Exemplary labels include radioactive isotopes, ligands, haptens, chemiluminescent agents, fluorescent molecules (e.g., fluorophores), and enzymes. Methods for labeling oligonucleotides and guidance in the choice of labels appropriate for various purposes are discussed, e.g., in Green and Sambrook, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Fourth Edition, 2012, and Ausubel et al., *Short Protocols in Molecular Biology*, Current Protocols, Fifth Edition, 2002.

Recombinant nucleic add: A nucleic acid molecule that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of nucleotide sequence. This artificial combination is accomplished by chemical synthesis or by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques such as those described in Sambrook and Russell, in Molecular Cloning: A Laboratory Manual, 3<sup>rd</sup> Ed., Cold Spring Harbor Laboratory Press (2001). The term "recombinant" includes nucleic acids that have been altered solely by addition, substitution, or deletion of a portion of a natural nucleic acid molecule. A recombinant nucleic acid also includes a heterologous nucleic acid that is inserted in a vector. A "heterologous nucleic acid" refers to a nucleic acid that originates from a different genetic source or species.

Sequence identity/similarity: The identity/similarity between two or more nucleic acid sequences, or two or more amino acid sequences, is expressed in terms of the identity

or similarity between the sequences. Sequence identity can be measured in terms of percentage identity; the higher the percentage, the more identical the sequences are. Sequence similarity can be measured in terms of percentage similarity (which takes into account conservative amino acid substitutions); the higher the percentage, the more similar the sequences are. Homologs or orthologs of nucleic acid or amino acid sequences possess a relatively high degree of sequence identity/similarity when aligned using standard methods.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, Adv. Appl. Math. 2:482, 1981; Needleman & Wunsch, J. Mol. Biol. 48:443, 1970; Pearson & Lipman, Proc. Natl. Acad. Sci. 15 USA 85:2444, 1988; Higgins & Sharp, Gene, 73:237-44, 1988; Higgins & Sharp, CABIOS 5:151-3, 1989; Corpet et al., Nuc. Acids Res. 16:10881-90, 1988; Huang et al. Computer Appls, in the Biosciences 8, 155-65, 1992: and Pearson et al., Meth. Mol. Bio. 24:307-31, 1994. Altschul et al., J. 20 Mol. Biol. 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., *J. Mol. Biol.* 215:403-10, 1990) is available 25 from several sources, including the National Center for Biological Information (NCBI, National Library of Medicine, Building 38A, Room 8N805, Bethesda, Md. 20894) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. 30 Additional information can be found at the NCBI web site.

BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. If the two compared sequences share homology, then the designated output file will present those regions of homology as aligned sequences. If the two compared sequences do not share homology, then the designated output file will not present aligned sequences.

Serum: The fluid portion of the blood that separates out from clotted blood. Serum contains many proteins, including antibodies, but does not contain clotting factors.

Subject: Living multi-cellular vertebrate organisms, a category that includes both human and non-human animals, such as non-human mammals (such as mice, rats, rabbits, sheep, horses, cows, and non-human primates).

Zika virus (ZIKV): A member of the virus family Flaviviridae and the genus Flavivirus. Other members of this genus include dengue virus, yellow fever virus, Japanese encephalitis virus. West Nile virus and Spondweni virus. ZIKV is spread by the daytime-active mosquitoes Aedes 50 aegypti and A. albopictus. This virus was first isolated from a Rhesus macague from the Zika Forest of Uganda in 1947. Since the 1950s, ZIKV has been known to occur within a narrow equatorial belt from Africa to Asia. The virus spread eastward across the Pacific Ocean in 2013-2014, resulting in 55 ZIKV outbreaks in Oceania to French Polynesia, New Caledonia, the Cook Islands, and Faster Island. In 2015, 71 KV spread to Mexico, Central America, the Caribbean and South America, where ZIKV has reached pandemic levels. Infection by ZIKV generally causes either no symptoms or 60 mild symptoms, including mild headache, maculopapular rash, fever, malaise, conjunctivitis and joint pain. ZIKV causes symptoms in about 20% of infected individuals, and no deaths from the virus have yet been reported. However, ZIKV infection has been linked to the birth of microcephalic 65 infants following maternal infection, as well an increase in cases of GBS. Reports have also indicated that ZIKV has the

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potential for human blood-borne and sexual transmission. ZIKV has also been found in human saliva and breastmilk.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms "a." "an," and "the" include plural referents unless context clearly indicates otherwise. "Comprising A or B" means including A, or B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

#### III. Introduction

Zika virus (ZIKV) has emerged as a major global public health concern due to its link as the causative agent of human birth defects. Its rapid expansion into the Western Hemisphere as well as the discovery of new modes of transmission of the virus, including sexual transmission, from mother to fetus, and possibly through blood transfusions, has only increased the need for a rapid and expansive public health response to this unprecedented epidemic. A non-invasive and rapid ZIKV diagnostic assay that can be performed in a clinical setting throughout pregnancy is vital for prenatal care of women living in areas of the world where exposure to the virus is possible. To meet this need, disclosed herein is the development of a highly sensitive and specific reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay to detect ZIKV RNA in biological samples, including urine and serum samples. As little as one RNA copy per µl were detected by RT-LAMP, and the assay was shown to be highly specific for ZIKV RNA when tested against a panel of urine samples spiked with arboviruses that may circulate in the same geographic regions as ZIKV. The ZIKV assay described herein offers a fast, reliable, sensitive and specific assay for the detection of ZIKV from biological samples that can be performed in a clinical or field setting with minimal equipment and technological expertise.

#### IV. RT-LAMP Assay for Detection of ZIKV RNA

Disclosed herein is a method for the detection of ZIKV nucleic acid in biological samples, such as biological fluid samples, using reverse transcription loop-mediated isothermal amplification (RT-LAMP). The disclosed ZIKV detection assay provides a rapid, sensitive and specific means for diagnosing ZIKV infection in susceptible subjects.

Provided herein is a method for detecting ZIKV RNA in a biological sample by subjecting the sample to an RT-LAMP reaction using a set of primers specific for ZIKV nucleic acid to produce a ZIKV nucleic acid amplification product; and detecting the ZIKV nucleic acid amplification product. In some embodiments, the set of primers includes (a) six primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to TGGTTCCACGACATTCCATT

(F3 1-1; SEQ ID NO: 1); CATTTCAAGTGGCCAGAGGA (B3 1-1; SEQ ID NO: 2); GGCATGTGCGTCCTT-GAACTCTGACACCGGAACTCCACACT (FIP 1-1; SEQ NO: 3); AGAAGGAGCAGTT-CACACGGCCCCTTTGCACCATCCATCTC (BIP 1-1; 5 SEQ ID NO: 4); ACCAGTGCTTCTTGTGTTCC (LF 1-1; SEQ ID NO: 5); and CCTTGCTGGAGCTCTGGAG (LB 1-1; SEQ ID NO: 6); (b) six primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to 10 GATCTTGGATGTGGCAGAGG (F3 5-5; SEQ ID NO: 7); TGCTTCTTCCACTCAGGAC (B3 5-5; SEQ ID NO: 8) CGGCTTCTCATGACCACiGCGTACTACGTCGCCAC-CATC (FIP 5-5; SEQ ID NO: 9); TCCGTCT-TAAGAGTTGGGGTGGATGTCACACAGCAACGTGTC (BIP 5-5; SEQ ID NO: 10); CCTTTCACTTCTT-GAACTTTGCG (LF 5-5; SEQ ID NO: 11); and CGTCTTT-CATATGGCGGCTG (LB 5-5; SEQ ID NO: 12); or (c) six primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 20 99% identical to AATGAGTGACCTGGCTAAGC (F3 2-5; SEQ ID NO: 13); AAAAGACACGAGGCCAAGG (B3 2-5; SEQ ID NO: 14); CAGCGCCAGATGAGCTA-CATCTTTGATGGGTGCCACCTC (FIP 2-5; SEQ ID NO: AGCGGCATTCAAAGTCAGACCATT- 25 CACGGGGTGTCCAATT (BIP 2-5; SEQ ID NO: 16); CCTCCATGTTGCATTTCCGC (LF 2-5; SEQ ID NO: 17); and GCGTTGCTGGTATCTTCATCT (LB 2-5; SEQ ID NO: 18).

In some examples, the set of primers includes six primers 30 each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to TGGTTCCACGACATTCCATT (F3 1-1; SEQ ID NO: 1); CATTTCAAGTGGCCAGAGGA (B3 1-1; SEQ ID NO: 2); GGCATGTGCGTCCTGAACTCTGACACCG- 35 GAACTCCACACT (FIP 1-1; SEQ ID NO: 3); AGAAGGAGCAGTTCACACGGCCCCTTGCACCATC-(BIP 1-1; SEQ ID ACCAGTGCTTCTTTGTGTTCC (LF 1-1; SEQ ID NO: 5); and CCTTGCTGGAGCTCTGGAG (LB 1-1; SEQ ID NO: 40 6); and six additional primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to GATCTTG-GATGTGGCAGAGG (F3 5-5; SEQ ID NO: TGCTTCTTCCACTTCAGGAC (B3 5-5; SEQ ID NO: 8); 45 CGGGTTCTTCATGACCAGGGCGTTACTACGTCGC-CACCATC (FIP 5-5; SEO ID NO: 9); TCCGTCT-TAAGAGTGGGTGGATGTCACACAGCAACGTGTC (BIP 5-5; SEQ ID NO: 10); CCTTCACTTCTT-GAACTGCG (LF 5-5; SEQ ID NO: 11); and CGTCRTF- 50 CATATGCCCTG (LB 5-5; SEQ ID NO: 12).

In other examples, the set of primers includes six primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to TGGTTCCACGACATTCCATT (F3 1-1; SEQ 55 ID NO: 1); CATTTCAAGTGGCCAGAGGA (B3 1-1; SEQ GGCATGTGCGTCCTT-2); GAACTCTGACACCGGAACTCCAC T (FIP 1-1; SEQ ID AGAAGGAGCAGTT-3); CACACGGCCCCTTTGCACCATCCATCTC (BIP 1-1; 60 SEQ ID NO: 4); ACCAGTGCTTCTTGTTGTTCC (LF 1-1; SEQ ID NO: 5); and CCTTGCTGGAGCTCTGGAG (LB 1-1; SEQ ID NO: 6); and six additional primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical 65 to AATGAGTGACCTGGCTAAGC (F3 2-5; SEQ ID NO: 13); AAAAGACACGAGGCCCAAGG (B3 2-5; SEQ ID

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NO: 14); CAGCGCCAGATGAGCTACATGTFT-GATGGGTGCCACCTTC (FIP 2-5; SEQ ID NO: 15); AGCGGCATTCAAAGTCAGACCATTT-

CACGGGGTGTCCAATT (BIP 2-5; SEQ ID NO: 16); CCTCCAGTGTTCATTCCGC (LF 2-5; SEQ ID NO: 17); and GCGTTCGCTCGOTATCTTCATCTT (LB 2-5; SEQ ID NO: 18).

In other examples, the set of primers includes six primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to GATCTTGGATGTGGCAGAGG (F3 5-5; SEQ ID NO: 7); TGCTTCTTCCACTTCAGGAC (B3 5-5; SEQ ID NO: 8); CGGGTTCTTCATGACCAGGGCGTTATACGTCGCCACCATC (FIP 5-5; SEQ ID NO: 9); TCCGTGTAAGAGTGGOGTGGATGT-

CACACAGCAACGTGTC (BIP 5-5; SEQ ID NO: 10); CCTGTCACTTCTTGAACTTGCG (LF 5-5; SEQ ID NO: 11); and CGTCTTTCATATGGCGGCTG (LB 5-5; SEQ ID NO: 12); and six additional primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to AATGAGTGACCTOOCTAAGC (F3 2-5; SEQ ID NO: 13); AAAAGACACGAGGCCAAGG (B3 2-5; SEQ ID NO: 14); CAGCGCCAGTGACCACTTTTTTGATGGTGCCACCTTC (FIP 2-5; SEQ ID NO: 15); AGCGCCATTCAAAGTCAAGACCATT-

CACGGGGTGTCCAAT (BIP 2-5; SEQ ID NO: 16); CCTCCAGTGTCATTCCGC (LF 2-5; SEQ ID NO: 17); and GCGTTGCTGGTATCTTCATCATCT (LB 2-5; SEQ ID NO: 18).

In yet other examples, the set of primers includes six primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to TGGTTCCACGACATTCCAT (F3 1-1; SEQ ID NO: 1); CATTCAAGTGGCCAGAGGA (B3 1-1; SEQ ID NO: 2); GGCATGTGCGTCCT-GAACTCTGACACCCOiAACTCCACACT (FIP 1-1; SEQ NO: AGAAGGAGCAGTT-CACACGGCCCCTTGCACCACCATCATCTC (BIP 1-1; SEQ ID NO: 4); ACCAGTGCTTCTTTGTTGTTCC (LF 1-1; SEQ ID NO: 5); and CCTTGCTGGAGCTCTGGAG (LB 1-1; SEQ ID NO: 6); six additional primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to GATCTTGGATGTGGCAGAGG (F3 5-5; SEQ ID NO: 7); TGCTTCTTCCACTTCAGGAC (B3 5-5; SEQ ID NO: 8); CGGGTTCTTCATGACCAGGGCGTTAC-TACGTCGCCACCATC (FIP 5-5; SEQ ID NO: 9); TCCGTCTTAAGAGTGGGGTGGATGT-

CACACAGCAACGTGTC (BIP 5-5; SEQ ID NO: 10); CCTTTCACTTCTTGAACTGCG (LF 5-5; SEQ ID NO: 11); and CGTCTTCATATGGCGGCTG (LB 5-5; SEQ ID NO: 12); and six additional primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to AATGAGTGACCTGCTCTAAGC (F3 2-5; SEQ ID NO: 13); AAAAGACACGAGGCCAAGG (B3 2-5; SEQ ID NO: 14); CAGCGCCAGATGAGCTACATCTTTTGATGGGTGCCACCTTC (FIP 2-5; SEQ ID NO: 15); AGCGGCATTCAAAGTCAGACCATT-

CACGGGGTGTCCAATT (BIP 2-5; SEQ ID NO: 16); CCTCCAGTGTTCATTTCCGC (LF 2-5; SEQ ID NO: 17); and GCGTTGCTGGTATCTTTCATCTT (LB 2-5; SEQ ID NO: 18).

In specific examples, the set of primers includes (a) six primers each respectively having a sequence comprising or consisting of TGGTTCCACGACATTCCATT (F3 1-1; SEQ

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ID NO: 1); CATTCAAGTGGCCAGAGGA (B3 1-1; SEQ NO: GGCATGTGCGTCCT-2); GAACTCTGACACCCGOAACTCCACACT (FTP 1-1; **SEO** ID NO: 3): AGAAGGAGCAGT-CACACGGCCCCTGCACCATCCATCTC (BIP 1-1; SEQ 5 ID NO: 4): ACCAGTGCTCTTGTGTTCC (LF 1-1: SEO ID NO: 5); and CCTTGCTGGAGCTCTGGAG (LB 1-1; SEQ ID NO: 6); (b) six primers each respectively having a sequence comprising or consisting of GATCTG-GATGTGGCAGAGG (F3 5-5; SEQ ID NO: 7): TGCTTCTTCCACTTCAGGAC (B3 5-5; SEQ ID NO: 8); CGGGTTCTTCATGACCAGGGCGTTACTACGTCGC-CACCATC (FIP 5-5; SEQ ID NO: 9); TCCGTCT-TAAGAGTGGGTGGATGTCACACAGCAACGTGTC (BIP 5-5; SEQ ID NO: 10); CCTTCACTCTT-GAACTTGCG (LF 5-5; SEQ ID NO: 11); and CGTCTT-CATATGGCGGCTG (LB 5-5; SEQ ID NO: 12); or (c) six primers each respectively having a sequence comprising or consisting of AATGAGTGACCTGGCTAAGC (F3 2-5; 20 SEQ ID NO: 13); AAAAGACACGAGGCCAAGG (B3 2-5; SEQ ID NO: 14); CAGCGCCAGATGAGCTA-CATCTTTTGATGGGTGCCACCTTC (FIP 2-5; SEQ ID AGCGGCATTCAAAGTCAGACCATT-NO: 15): CACGGGGTGTCCAAT (BIP 2-5; SEQ ID NO: 16); 25 CCTCCAGTGTTCATTCCGC (LF 2-5; SEQ ID NO: 17); and GCGTTGTGGTATCTTTCATCT (LB 2-5; SEQ ID

In other specific examples, the set of primers includes six primers each respectively having a sequence comprising or consisting of TGGTTCCACGACATTCCATT (F3 1-1; SEQ ID NO: 1); CATTTCAAGTGGCCAGAGGA (B3 1-1; SEQ GGCATGTGCGTCCTT-NO: 2); GAACTCTGACACCGGAACTCCACT (FIP 1-1; SEQ ID AGAAGGAGCAGTT-3); CACACGGCCCCTTTGCACCATCCATCTC (BIP 1-1; SEQ ID NO: 4); ACCAGTGCTTCTTGTTGTTCC (LF 1-1; SEQ ID NO: 5); and CCTTGCTGGAGCTCTGGAG (LB respectively having a sequence comprising or consisting of GATCTTGGATGTGGCAGAGG (F3 5-5; SEQ ID NO: 7); TCCTCTTCCACTCAGGAC (B3 5-5; SEQ ID NO: 8); CGGGTCTCATGACCAGGGCGTTACTACGTCGCCAC-CATC (FIP 5-5; SEQ ID NO: 9); TCCGTCT- 45 TAAGAGTGGGGTGGATGTCACACAGCAACGTGTC(BIP 5-5; SEO ID NO: 10); CCTTTCACTTCTT-GAACTTGCG (LF 5-5; SEQ ID NO: 11); and CGTCTT-CATATGGCGGCTG (LB 5-5; SEQ ID NO: 12).

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primers each respectively having a sequence comprising or consisting of TGGTTCCACGACATTCCAT (F3 1-1; SEQ ID NO: 1); CATTTCAAGTGGCCAGAGGA (B3 1-1; SEQ ID NO: 2); GGCATGTGCGTCCTT-GAACTCTGACACCGGAACTCCACTAC (FIP 1-1; SEQ 55 NO: 3): AGAAGGAGCAGTT-CACACGGCCCCTTTGCACCATCCATCTC (BIP 1-1; SEQ ID NO: 4); ACCAGTGCTTCTGGTTGTTCC (LF 1-1; SEQ ID NO: 5); and CCTTCCTGGAGCTCTGGAG (LB 1-1; SEQ ID NO: 6); and six additional primers each 60 respectively having a sequence comprising or consisting of AATGAGTGACCTGGCTAAGC (F3 2-5; SEQ ID NO: 13); AAAAGACACGAGGCCAAGG (B3 2-5; SEQ ID CAGCGCCAGATGAGCTACATCTTT-NO: 14); GATGGGTGCCACCTTC (FIP 2-5; SEQ ID NO: 15); 65 AGCGGCATTCAAAGTCAGACCATFT-CACGGGGTGTCCAATT (BIP 2-5; SEQ ID NO: 16);

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CCTCCAGTGTTCATTTCCGC (LF 2-5; SEQ ID NO: 17); and GCGTTGCTGGTATCTTTCATCTT (LB 2-5; SEQ ID

In other specific examples, the set of primers includes six primers each respectively having a sequence comprising or consisting of GATCTTGGATGTGGCAGAGG (F3 5-5; SEQ ID NO: 7); TGCTTCTTCCACTTCAGGAC (B3 5-5; SEQ ID NO: 8): CGGGTTCTTCATGACCAGGGCGT-TACTACGTCGCCACCATC (FIP 5-5; SEQ ID NO: 9): TCCGTCTTAAGAGTGCGGGTGGATGT-CACACAGCAACGTGTC (BIP 5-5; SEQ ID NO: 10); CCTTCACTTCTTGAACTTGCG (LF 5-5; SEQ ID NO: 11); and CGTCTTCATATGCCGOCTG (LB 5-5; SEQ ID NO: 12); and six additional primers each respectively having a sequence comprising or consisting of AAT-GAGTGACCTGGCTAAGC (F3 2-5; SEQ ID NO: 13); AAAAGACACGAGGCCAAGG (B3 2-5; SEQ ID NO: CAGCGCCAGATGAGCTACATCTTT-

AGCGGCATTCAAAGTCAGACCATT-CACGGGGTGTCCAATI (BIP 2-5; SEQ ID NO: 16); CCTCCAGTGTTCATTTCCGC (LF 2-5; SEQ ID NO: 17); and GCGTTGCTGGTATCTCATCTT (LB 2-5; SEQ ID NO: 18).

GATGGGTGCCACCTTC (FIP 2-5; SEQ ID NO: 15);

In one specific non-limiting example, the set of primers includes six primers each respectively having a sequence comprising or consisting of TGGTTCCACGACATTC-CATT (F3 1-1; SEQ ID NO: 1); CATTT-CAAGTGGCCAGAGGA (B3 1-1; SEQ ID NO: 2); GGCATGTGCGTCCTGAACTCTGACACCGAACTC-CACACT (FIP 1-1; SEQ ID NO: 3); AGAAGGAGCAGT-CACACGGCCCCFTGCACCATCCATCIC (BIP 1-1; SEQ ID NO: 4): ACCAGTGCTTCTTTGTGTCC (LF 1-1; SEQ ID NO: 5); and CCTTGCTGGAGCTCTGGAG (LB 1-1; SEQ ID NO: 6); six additional primers each respectively having a sequence comprising or consisting of GATCTTG-GATGTGGCAGAGG (F3 5-5; SEQ ID NO: 7); TGCTCTTCCACTTCAGGAC (B3 5-5; SEQ ID NO: 8); CGGGCTTCATGACCAGGGCGGTTACTACGTCGC-1-1; SEQ ID NO: 6); and six additional primers each 40 CACCATC (FIP 5-5; SEQ ID NO: 9); TCCGTCT-TAAGAGTGGGTGGATGTCACACAGCAACGTGTC (BIP 5-5; SEQ ID NO: 10); CCTTTCACTTCTT-GAACTTTGCG (LF 5-5; SEQ ID NO: 11); and CGTCTTT-CATATGGCGGCTG (LB 5-5; SEQ ID NO: 12); and six additional primers each respectively having a sequence of comprising consisting GAGTGACCTGGCTAAGC (F3 2-5; SEO ID NO: 13); AAAAGACACGAGGCCAAGG (B3 2-5; SEQ ID NO: 14): CAGCGCCAGATGAGCTACATCTTTT-In other specific examples, the set of primers includes six 50 GATGGGTGCCACCTTC (FIP 2-5; SEQ ID NO: 15); AGCGGCATTCAAAGTCAGACCATT-CACGGGGTGTCCAAT (BIP 2-5; SEQ ID NO: 16); CCTCCAGTGTTCATTTCCGC (LF 2-5; SEQ ID NO: 17); and GCGTTGCTGGTATCTTTCATCTT (LB 2-5; SEQ ID

> In some embodiments, the RT-LAMP reaction is performed at about 60° C. to about 68° C., such as about 63° C. to about 67° C., or about 64° C. to about 66° C. In some examples, the RT-LAMP reaction is performed at about 60° C., about 61° C. about 62° C. about 63° C., about 64° C., about 65° C., about 66° C., about 67° C. or about 68° C. In specific examples, the RT-LAMP reaction is performed at 65° C.

> In some embodiments, the RT-LAMP reaction is allowed to proceed for about 15 to about 45 minutes, such as about 20 minutes to about 40 minutes, or about 25 minutes to about 35 minutes. In some examples, the RT-LAMP reaction is

allowed to proceed for about 30 minutes. In some examples, the RT-LAMP reaction is allowed to proceed for no more than 20 minutes, no more than 25 minutes, no more than 30 minutes, no more than 35 minutes, no more than 40 minutes or no more than 45 minutes.

In some embodiments, the RT-LAMP reaction includes a pH-sensitive indicator dye. In some examples, the pH-sensitive indicator dye is a colored dye detectable in visible light. In particular examples, the colored dye comprises cresol red, phenol red, m-cresol purple, bromocresol purple, 10 neutral red, naphtholphthalein, thymol blue or naphtolphthalein. In other examples, the pH-sensitive indicator dye is a fluorescent indicator dye. In particular examples, the fluorescent dye comprises 2',7-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein, 5(6)-carboxy-2',7'-dichlorofluorescein, 15 5(6)-carboxyfluorescein, 3,6-diacetoxyphthalonitrile, 6,8-dihydroxy-1,3-pyrenedisulfonic acid, or 5-(and-6)-carboxyl seminaphthorhodafluor.

In some embodiments, detecting the ZIKV nucleic acid amplification product comprises detecting a visible color 20 change. In other embodiments, detecting the ZIKV nucleic acid amplification product comprises detecting fluorescence. In other embodiments, detecting the ZIKV nucleic acid amplification product comprises measuring turbidity.

In some embodiments, the biological sample is a biological fluid sample. In some examples, the biological fluid sample comprises serum, urine, blood, plasma, feces, saliva and/or CSF. In specific examples, the biological sample comprises serum or urine.

The DNA polymerase used in the RT-LAMP reaction can 30 be any enzyme appropriate for the assay, which can be selected by one of skill in the art. In some embodiments, the RT-LAMP reaction includes a DNA polymerase with high strand displacement activity. In some examples, the DNA polymerase is a DNA polymerase long fragment (LF) of a 35 thermophilic bacteria such as *Bacillus stearothermophilus* (Bst). *Bacillus Smithii* (Bsm), *Geobacillus* sp. M (GspM) or *Thermodesulfatator indicus* (Tin), an engineered variant therefrom or a Taq DNA polymerase variant. In some examples, the DNA polymerase is Bst LF DNA polymerase, 40 GspM LF DNA polymerase, GspSSD LF DNA polymerase, Tin exo-LF DNA polymerase or SD DNA polymerase (see, e.g., WO 2016/189490).

The reverse transcriptase used in the RT-LAMP reaction can be any RT enzyme appropriate for the assay, which can 45 be selected by one of skill in the art. In some embodiments, the RT is from avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (MMLV).

RT-LAMP reactions generally result in the synthesis of large amounts of target nucleic acid, allowing for detection 50 of target nucleic acid from relatively small sample volumes. Furthermore, the large amount of amplification product allows for detection visually or by simple detectors. Although the use of a pH-sensitive indicator dye is exemplified herein, other means for detection of amplification 55 product can be used with the disclosed methods. For example, any one of the following methods can be used to detect RT-LAMP amplification products: (i) fluorescence, using DNA intercalating dyes, fluorescent molecular beacon probes or a fluorescence metal indicator such as calcein; (ii) 60 colorimetry, using a colored indicator for alkaline metal ions, such as hydroxy naphthol blue (Goto et al., BioTechniques 46(3): 167-172, 2009) or pH indicators (Tanner et al., BioTechniques 58(2):59-68, 2015); (iii) turbidity, as the RT-LAMP reaction produces large amounts of magnesium 65 pyrophosphate (a white precipitate) and dsDNA, which allow visual inspection of results using a turbidimeter (Mori

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et al., Biochem Biophys Res Commun, 289(1):150-154, 2001); (iv) electrochemically, using a pH meter for direct measurement of released hydrogen ions during the RT-LAMP procedure (Xie et al., Chem Comnum 50(100): 15932-15935, 2014), or using integrated electrodes for measuring decreases in current resulting from increasing binding of electrochemically-active DNA-binding redox reporters, such as Methylene Blue, to RT-LAMP reaction products (Xie et al., Biosens Bioelectron 55:324-329, 2014); (v) enzyme-linked immunosorbent assays (ELISA) or lateral flow immunoassays based on the use of specific probes (Tsai et al., J Virol Methods 181(1): 117-124, 2012; Ravan and Yazdanparast, Anal Biochem 439(2): 102-108, 2013); (vi) bioluminescence, through bioluminescent output of the coupled conversion of inorganic pyrophosphate produced stoichiometrically during nucleic acid synthesis to ATP by the enzyme ATP sulfurylase (Gandelman et al., PLoS ONE 5(11):e14155, 2010).

#### V. Kits for Detection of ZIKA Nucleic Acid

Also provided herein are kits for detecting ZIKV nucleic acid in a biological sample. The kits include a set of oligonucleotide primers specific for ZIKV nucleic acid. In some embodiments, the set of primers includes (a) six primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to TGGTTCCACGACATTCCATT (F3 1-1; SEQ ID NO: 1); CATTTCAAGTGGCCAGAGGA (B3 1-1; SEO ID NO: 2); GGCATGTGCGTCCTT-GAACTCTGACACCGGAACTCCACACT (FIP 1-1; SEQ ID NO: AGAAGGAGCAGTT-3); CACACGGCCCCTTGCACCATCCATCTC (BIP 1-1; SEQ ID NO: 4); ACCAGTGCTTCTTTGTTGTTCC (LF 1-1; SEQ ID NO: 5); and CCTGCTGGAGCTCTGGAG (LB 1-1; SEQ ID NO: 6); (b) six primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to GATCTTGGATGTGGCAGAGG (F3 5-5; SEQ ID NO: 7); TGCTTCTTCCACTTCAGGAC (B3 5-5; SEQ ID NO: 8); CGGGTTCTTCATGACCAGGGCGTTACTACGTCGC-CACCATC (FIP 5-5; SEQ ID NO: 9); TCCGTCT-TAAGAGTGGGGTCGATGTCACACAGCAACGTGTC (BIP 5-5; SEQ ID NO: 10); CCTTTCAGTFTCT-GAACTTGCG (LF 5-5; SEQ ID NO: 11); and CGTCTT-CATATGGCGGCTG (LB 5-5; SEQ ID NO: 12); or (c) six primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to AATGAGTGACCTGGCTAAGC (F3 2-5; SEQ ID NO: 13); AAAAGACACGAGGCCAAGG (B3 2-5; SEQ ID NO: 14); CAGCGCCAGATGAGCTA-CATCTTTGATGGGTGCCACCTTC (FIP 2-5; SEQ ID NO: AGCGGCATCAAAGTCAGACCATT-CACGGGGTGTCCAATT (BIP 2-5; SEQ ID NO: 16); CCTCCAGTGTTCATTTCCGC (LF 2-5; SEQ ID NO: 17); and GCGTFGCTGGTATCTTTCATCTT (LB 2-5; SEQ ID

In some examples, the set of primers includes six primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to TCGTTCCACGACATTCCATT (F3 1-1; SEQ ID NO: 1); CATTTCAAGTGGCCAGAGGA (B3 1-1; SEQ ID NO: 2); GGCATGTOCGTCCTGAACTCTGACACCGGAACTCCACACT (FIP 1-1; SEQ ID NO: 3); AGAAGGAGCAGTTCACACGGCCCTTGCACCATCCATCTC (BIP 1-1; SEQ ID NO: 4); ACCAGTGCTTCTTFTGTTGTTCC (LF 1-1; SEQ ID NO:

NO: 18).

5); and CCTTGCTGGAGCTCTGGAG (LB 1-1; SEQ ID NO: 6); and six additional primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to GATCTG-GATGTGGCAGAGG (F3 5-5; SEQ ID NO: 7); 5 TGCTTCTCCACTCAGGAC (B3 5-5; SEQ ID NO: 8); CGGGTTCTTCATACCAGGGCTTACTACGTCGCCAC-CATC (FIP 5-5; SEQ ID NO: 9); TCCGTCT-TAAGAGTGGGTGGATGTCACACAGCAACGTGTC (BIP 5-5; SEQ ID NO: 10); CCTTCACTTCTT- 10 GAACTTTGCG (LF 5-5; SEQ ID NO: 11); and CGTCTT-CATATGGCGGCTG (LB 5-5; SEQ ID NO: 12).

In other examples, the set of primers includes six primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% 15 identical to TGGTTCCACGACATTCCATT (F3 1-1; SEQ ID NO: 1); CATTTCAAGTGGCCAGAGGA (B3 1-1; SEQ ID NO: 2); GGCATGTOCGTCCTT-GAACTCTGACACCGGAACTCCACACT (FIP 1-1; SEQ AGAAGGAGCAGTT- 20 NO: 3); CACACGGCCCCTTGCACCATCCATCTC (BIP 1-1; SEQ ID NO: 4): ACCAGTGCTTCTTGTTGTTCC (LF 1-1; SEQ ID NO: 5); and CCTTGCTGGAGCTCTGGAG (LB 1-1; SEQ ID NO: 6); and six additional primers each respectively having a sequence at least 90%, at least 95%, at least 96%, 25 at least 97%, at least 98% or at least 99% identical to AATGAGTGACCTGCCTTAAGC (F3 2-5; SEQ ID NO: 13); AAAAGACACGAGGCCAAGCG (B3 2-5; SEQ ID 14); CAGCGCCAGATGAGCTACATCTTT-GATGGGTGCCACCTTC (FIP 2-5; SEQ ID NO: 15); 30 AGCGGCATTCAAAGTCAGACCATTT-

CACGGGGTGTCCAATT (BIP 2-5; SEQ ID NO: 16); CCTCCAGTGTTCATTTCCGC (LF 2-5; SEQ ID NO: 17); and GCGTTGCTGGTATCTTCATCTT (LB 2-5; SEQ ID NO: 18).

In other examples, the set of primers includes six primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to GATCTTGGATGTGGCAGAGG (F3 5-5; SEQ ID NO: 7); TGCTTCTTCCACTTCAGGAC (B3 5-5; SEQ 40 ID NO: 8); CGGGTTCTTCATGACCAGGGCGTTAC-TACGTCGCCACCATC (FIP 5-5; SEQ ID NO: 9); TCCGTCTTAAGAGTGGGGTOOATOT-

CACACAGCAACGTGTC (BIP 5-5; SEQ ID NO: 10); CCTTCACTCTTGAACTTTTGCG (LF 5-5; SEQ ID NO: 45 1); and CGTCTTCATATGGCGGCTG (LB 5-5; SEQ ID NO: 12); and six additional primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to AAT-GAGTGACCTGGCTAAGC (F3 2-5; SEQ ID NO: 13); 50 AAAAGACACGAGGCCAAGG (B3 2-5; SEQ ID NO: 14); CAGCGCCAGATGAGCTACATCTTGATGGGTGC-CACCTTC (FP 2-5; SEQ ID NO: 15); AGCGGCATT-CAAAGTCAGACCATCACGGGGTGTCCAATC 2-5; SEQ ID NO: 16); CCTCCAGTGTTCATTTCCGC (LF 55 2-5; SEQ ID NO: 17); and GCGTTGCTGGTATCTT-CATCTT (LB 2-5; SEQ ID NO: 18).

In yet other examples, the set of primers includes six primers each respectively having a sequence at least 90%, at 99% identical to TGGTTCCACGACATTCCATT (F3 1-1; SEQ ID NO: 1); CATTTCAAGTGGCCAGAGGA (B3 1-1; GGCATGTGCGTCCTT-SEQ IDNO: 2); GAACTCTGACACCGGAACTCCACACT (FIP 1-1; SEQ NO: AGAAGGAGCAGTT- 65 3): CACACGGCCCCTTTGCACCATCCATCTC (BIP 1-1; SEQ ID NO: 4); ACCAGTGCTTCTTTGTGTTCC (LF 1-1;

SEQ ID NO: 5); and CCTTGCTGGAGCTCTGGAG (LB 1-1; SEQ ID NO: 6); six additional primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to GATCTTGGATGTGGCAGAGG (F3 5-5; SEQ ID NO: 7); TGCTTCTCCACTCAGGAC (B3 5-5; SEQ ID NO: 8); CGGGTTCTTCATGACCAGGGCGTTACTACGTCGC-CACCATC (FTP 5-5; SEQ ID NO: 9); TCCGTCT-TAAGAGTGGGGTGGATGTCACACAGCAACGTGTC (BIP 5-5; SEQ ID NO: 10); CCTTTCACTTCTT-GAACTTFGCG (LF 5-5; SEQ ID NO: 11); and CGTCTT-CATATGGCGGCTG (LB 5-5; SEQ ID NO: 12); and six additional primers each respectively having a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least or at least 99% identical to GAGTGACCTGGCTAAGC (F3 2-5; SEQ ID NO: 13); AAAAGACACGAGGCCAAGG (B3 2-5; SEQ ID NO: 14); CAGCGCCAGATGAGCTACATCTTT-GATGGGTGCCACCTC (FIP 2-5; SEQ ID NO: 15); AGCGGCATTCAAAGTCAGACCATT-CACGGGGTGTCCAAT (BIP 2-5; SEQ ID NO: 16); CCTCCAGTGTTCATTCCGC (LF 2-5; SEQ ID NO: 17); and GCGTTGCTGGTATCTTTCATCTT (LB 2-5; SEQ ID

In specific examples, the set of primers includes (a) six primers each respectively having a sequence comprising or consisting of TGGTTCCACGACATTCCATT (F3 1-1; SEQ ID NO: 1): CATTCAAGTGGCCAGAGGA (B3 1-1; SEQ ID NO: GGCATGTOCGTCCTT-2): GAACTCTGACACCGGAACTCCACT (FP 1-1; SEQ ID NO: 3); AGAAGGAGCAGTCACACGGCCCCFTGCAC-CATCCATCIC (BIP 1-1; SEQ ID NO: ACCAGTGCTTCTTGTGTTCC (LF 1-1; SEQ ID NO: 5); and CCTTGCTGGAGCTCTGGAG (LB 1-1; SEQ ID NO: 35 6); (b) six primers each respectively having a sequence comprising consisting of GATCTTG-GATGTGGCAGAGG (F3 5-5; SEQ ID NO: 7); TGCTCTTCCACTTCAGGAC (B3 5-5; SEQ ID NO: 8); CGGGTTCTCATGACCAGGGCGTTATACGTCGCCAC-CATC (FIP 5-5; SEQ ID NO: 9); TCCGTCT-TAAGAGTGGGGTGGATGTCACACAGCAACGTGTC (BIP 5-5; SEQ ID NO: 10); CCTTTCACTTCTT-GAACTTTGCG (LF 5-5; SEQ ID NO: 11); and CGTCTT-CATATGGCGGCTG (LB 5-5; SEQ ID NO: 12); or (c) six primers each respectively having a sequence comprising or consisting of AATGAGTGACCTGGCTAAGC. (F3 2-5; SEQ ID NO: 13); AAAAGACACGAGGCCAAGG (B3 2-5; SEQ ID NO: 14); CAGCGCCAGATGAGCTA-CATCTTTGATGGGTGCCACCTTC (FIP 2-5; SEQ ID NO: 15); AGCGGCATTCAAAGTCAGACCATT-CACGGGGTGTCCAATT (BIP 2-5; SEQ ID NO: 16); CCTCCAGTGTTCATTTCCGC (LF 2-5; SEQ ID NO: 17); and GCGTTGCTGGTATCTTTCATCTT (LB 2-5; SEQ ID NO: 18).

In other specific examples, the set of primers includes six primers each respectively having a sequence comprising or consisting of TGGTTCCACGACATCCATT (F3 1-1; SEQ ID NO: 1); CATTCAAGTGGCCAGAGGA (B3 1-1; SEQ IDNO: GGCATGTGCGTCCTT-2); least 95%, at least 96%, at least 97%, at least 98% or at least 60 GAACTCTGACACGGAACTCCACACT (FIP 1-1; SEQ 3): AGAAGGAGCAGTT-CACACGGCCCCTTTGCACCATCCATCTC (BIP SEQ ID NO: 4); ACCAGTGCTTCTTTGTTCC (LF 1-1; SEQ ID NO: 5); and CCTPGCTGGAGCTCTGGAG (LB 1-1; SEQ ID NO: 6); and six additional primers each respectively having a sequence comprising or consisting of GATCTTGGATGTCAGAGG (F3 5-5; SEQ ID NO: 7);

TGCTTCTTCCACTTCAGGAC (B3 5-5; SEQ ID NO: 8); CGGGTTCTTCATGACCAGGGCGTTACTACGTCGC-CACCATC (FIP 5-5; SEQ ID NO: 9); TCCGTCT-TAAGAGTGGGGTGGATGTCACACAGCAACGTGTC (BIP 5-5; SEQ ID NO: 10); CCTTCACTTCTT-5 GAACTTTTGCG (LF 5-5; SEQ ID NO: 11); and CGTCTT-CATATGGCGGCTG (LB 5-5; SEQ ID NO: 12).

In other specific examples, the set of primers includes six primers each respectively having a sequence comprising or consisting of TGGTTCCACGACATTCCATT (F3 1-1; SEQ 10 ID NO: 1); CATTTCAAGTGGCCAGAGGA (B3 1-1; SEQ ID NO: 2); GGCATGTGCGTCCTGAACTCTGACACCG-GAACTCCACACT (FIP 1-1; SEQ ID NO: AGAAGGAGCAGTTCACACGGCCCCTTGCACCATC-(BIP 1-1: SEQ ID NO: ACCAGTGCTTCTTGTTGTGCC (LF 1-1; SEQ ID NO: 5); and CCTTGCTGGAGCTCGGAG (LB 1-1; SEQ ID NO: 6); and six additional primers each respectively having sequence comprising or consisting of AAT-GAGTGACCTGGCTAAGC (F3 2-5; SEQ ID NO: 13); 20 AAAAGACACGAGGCCAAGG (B3 2-5; SEQ ID NO: CAGCGCCAGATGAGCTACATCTTT-GATGGGTGCCACCTC (FIP 2-5; SEQ ID NO: 15); AGCGGCATTCAAAGTCAGACCATT-CACGGGGTGTCCAAT (BIP 2-5; SEQ ID NO: 16); 25 CCTCCAGTGTTCATTCCGC (LF 2-5; SEQ ID NO: 17); and GCGTTGCTGGTATCTTTCATCTT (LB 2-5; SEQ ID

primers each respectively having a sequence comprising or 30 consisting of GATCTTGGATGTGGCAGAGG (F3 5-5; SEQ ID NO: 7); TGCTTCTTCCACTTCAGGAC (B3 5-5; SEQ ID NO: 8); CGGGTTCTTCATGACCAGGGCGTTACTACGTCGCCACCATC (FIP 5-5; SEQ ID NO: 9); TCCGTCTTAAGAGTGGGGTGGATGTCACACACAGCAACGTGTC (BIP 5-5; SEQ ID NO: 10); CCTTTCACTTCTTGAACTTTCG (LF 5-5; SEQ ID NO: 11); and CGTCTTCATATGGCGGCTG (LB 5-5; SEQ ID NO: 12); and six additional primers each respectively having a sequence comprising or consisting of AAT-40 GAGTGACCTGGCTAAGC (F3 2-5; SEQ ID NO: 13);

In other specific examples, the set of primers includes six

NO: 18).

AAAAGACACGAGGCCAAGG (B3 2-5; SEQ ID NO: 14): CAGCGCCAGATGAGCTACATCTTT-GATGGGTGCCACCTTC (FIP 2-5; SEQ ID NO: 15); AGCGGCATTCAAGTCAGACCATTCACGGGTGTC-CAATT (BIP 2-5; SEQ ID NO: 16); CCTCCAGTGTTCATTCCGC (LF 2-5; SEQ ID NO: 17); and GCGTCCTGGTATCTTCATCTT (LB 2-5; SEQ ID NO: 18).

In one specific non-limiting example, the set of primers 50 includes six primers each respectively having a sequence comprising or consisting of TGGTTCCACGACATCCAT-(F3 1-1; SEQ ID NO: 1); CAAGTGGCCAGAGGA (B3 1-1; SEQ ID NO: 2); GGCATGTGCGTCCTFGAACTCTGACACCGGAACTC- 55 CACACT (FIP 1-1; SEQ ID NO: 3); AGAAGGAGCAGTT-CACACGGCCCCTTGCACCATCCATCTC (BIP 1-1; SEQ ID NO: 4); ACCAGTGCTCTTGTTGTCC (LF 1-1; SEQ ID NO: 5); and CCTTGCTGGAGCTCTGGAG (LB 1-1; SEQ ID NO: 6); six additional primers each respectively having 60 a sequence comprising or consisting of GATCTTG-GATGTGGCAGAGG (F3 5-5; SEQ ID NO: TGCTCTTCCACTTCAGGAC (B3 5-5; SEQ ID NO: 8); CGGGTTCTTCATGACCAGGGCGTTACTACGTCGC-CACCATC (PIP 5-5; SEQ ID NO: 9); TCCGTCT- 65 TAAGAGTGGGTGGATGTCACACAGCAACGTGTC (BIP 5-5; SEQ ID NO: 10); CCTTCACTTCTT-

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GAACTTGCG (LF 5-5; SEQ ID NO: 11); and CGTCTT-CATATOCGCICTG (LB 5-5; SEQ ID NO: 12); and six additional primers each respectively having a sequence comprising or consisting of AAT-GAGTGACCTGGCTAAGC (F3 2-5; SEQ ID NO: 13); AAAAGACACGAGGCCAAGG (B3 2-5; SEQ ID NO: 14): CAGCGCCAGATGAGCTACATCTTTT-GATGGGTGCCACCTTC (FIP 2-5; SEQ ID NO: 15); AGCGGCATTCAAAGTCAGACCATT-CACGGGGTGTCCATTCCACTGCATTCCCC (FE 2-5; SEQ ID NO: 16); CGTGCAACTGCTTCATTCCCC (FE 2-5; SEQ ID NO: 17);

CACGGGGTGTCCAATT (BIP 2-5; SEQ ID NO: 16); CCTCCAGTGTTCATTCCGC (LF 2-5; SEQ ID NO: 17); and GCGTTGCTGGTATCTTCATCTT (LB 2-5; SEQ ID NO: 18).

In some embodiments, the kit further includes one or more components for performing an RT-LAMP reaction. In some examples, the kit includes buffer, reverse transcriptase, DNA polymerase, a pH-sensitive indicator dye, dNTPs, master mix, or any combination thereof.

In the disclosed kits, one or more of the oligonucleotide primers (such as one or more of SEQ ID NOs: 1-18), or one or more sets of oligonucleotide primers (such as SEQ ID NOs: 1-6; SEQ ID NOs: 7-12; and/or SEQ ID NOs: 13-18), are provided in one or more containers or in one or more individual wells of a multi-well plate or card. Nucleic acid primers may be provided suspended in an aqueous solution or as a freeze-dried or lyophilized powder, for instance. The container(s) in which the nucleic acid(s) are supplied can be any conventional container that is capable of holding the supplied form, for instance, microfuge tubes, multi-well plates, ampoules, or bottles. The kits can include either labeled or unlabeled nucleic acid primers for use in amplification and/or detection of ZIKV nucleic acids, such as by RT-LAMP.

One or more positive and/or negative control primers and/or nucleic acids also may be supplied in the kit Exemplary negative controls include non-ZIKV nucleic acids (such nucleic acids from other flaviviruses). Exemplary positive controls include purified ZIKV nucleic acid or a vector or plasmid including the ZIKV target sequence. One of skill in the art can select suitable positive and negative controls for the assays disclosed herein.

In some examples, one or more primers (such as one or more sets of primers), are provided in pre-measured single use amounts in individual, typically disposable, tubes, wells, plates, cards, or equivalent containers. In some examples, a set of primers (such as each of SEQ ID NOs: 1-6, each of SEQ ID NOs: 7-12 or each of SEQ ID NOs: 13-18) is included in a single container. In this example, the sample to be tested for the presence of the target nucleic acids can be added to the individual container(s) and amplification and/or detection can be carried out directly. The kit may also include additional reagents for the detection and/or amplification of ZIKV nucleic acids, such as buffer(s), nucleotides (such as dNTPs), enzymes (such as DNA polymerase and/or reverse transcriptase), or other suitable reagents. The additional reagents may be in separate container(s) from the one or more primers or may be included in the same container as the primer(s).

In some examples, the kit includes one or more compounds for detecting an amplification product, such as pH-sensitive indicator dye, a DNA intercalator (e.g., propidium iodide, SYBR green or PICOGREEN fluorescent dyes) a chromogenic or colorimetric reagent (such as hydroxynaphthol blue), or a fluorescent indicator (such as calcein).

#### VI. Oligonucleotide Primers

Primers (such as isolated nucleic acid primers) suitable for use in the disclosed methods and kits are described

herein. In some examples, the primers are suitable for detection of ZIKV nucleic acids by RT-LAMP, such as the assay described herein. RT-LAMP primer sets typically include a forward outer primer (F3), a backward outer primer (B3), a forward inner primer (HP), a backward inner primer (BIP), a forward loop primer (Lop F or LF), and/or a backward loop primer (Lop B or LB). At least some of the RT-LAMP primers are non-naturally occurring nucleic acid molecules, for example, the primers have sequences that do not occur in nature. In particular, the FIP and BIP primers are composed of non-contiguous nucleic acid sequences, and include a portion complementary to a first strand of a double-strand nucleic acid and another portion complement to a second strand of a double-stranded nucleic acid (e.g., reverse complement of a first strand sequence).

In some embodiments, the disclosed primers are between 10 and 60 nucleotides in length (for example 15-50, 20-50, 30-60, or 25-40 nucleotides in length). In some examples, the primers are 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 29, 30, 31, 32, 32, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 nucleotides in length and are capable of hybridizing to ZIKV nucleic acid molecules. In some examples, the primers are at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 nucleotides in length. In other examples, the primers may be no more than 20, 25, 30, 35, 40, 45, 50, 55, or 60 nucleotides in length.

In some examples, the disclosed primers include RT-LAMP primers for amplification of ZIKV nucleic acid. The primers include nucleic acid sequences with at least 85% sequence identity (for example, at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity) to any one of the following:

TGGTTCCACGACATTCCATT	(F3 11; SEQ ID NO: 1)
CATTTCAAGTGGCCAGAGGA	(B3 1-1; SEQ ID NO: 2)
GGCATGTGCGTCCTTGAACTCTGA	(FIP 1-1; SEQ ID NO: 3) CACCGGAACTCCACACT
AGAAGGAGCAGTTCACACGGCCCC	(BIP 1-1; SEQ ID NO: 4)
ACCAGTGCTTCTTTGTTGTTCC	(LF 1-1; SEQ ID NO: 5)
CCTTGCTGGAGCTCTGGAG	(LB 1-1; SEQ ID NO: 6)
GATCTTGGATGTGGCAGAGG	(F3 5-5; SEQ ID NO: 7)
TGCTTCTTCCACTTCAGGAC	(B3 5-5; SEQ ID NO: 8)
CGGGTTCTTCATGACCAGGGCGTT	(FIP 5-5; SEQ ID NO: 9)
TCCGTCTTAAGAGTGGGGTGGATG	(BIP 5-5; SEQ ID NO: 10) FCACACAGCAACGTGTC
CCTTTCACTTCTTGAACTTTGCG	(LF 5-5; SEQ ID NO; 11)
CGTCTTTCATATGGCGGCTG	(LB 5-5; SEQ ID NO: 12)
AATGAGTGACCTGGCTAAGC	(F3 2-5; SEQ 1D NO: 13)

#### -continued

AAAAGACACGAGGCCAAGG	(B3	2-5;	SEQ	ID :	NO :	14)
CAGCGCCAGATGAGCTACAT		2-5; GGGTG	~			15)
AGCGGCATTCAAAGTCAGAC		2-5; GGGT	~		NO:	16)
CCTCCAGTGTTCATTTCCGC	(LF	2-5;	SEQ	ID :	NO:	17)
GCGTTGCTGGTATCTTTCAT		2-5;	SEQ	ID :	NO :	18)

In some examples, at least one of the primers includes a detectable label such as a fluorophore, radiolabel, hapten (such as biotin), or chromogen. In some examples, a detectable label is attached (e.g., covalently or non-covalently attached) to an oligonucleotide. The attachment may be to any portion of the oligonucleotide, including to a base, sugar, phosphate backbone, or 5' or 3' end of the oligonucleotide. The label may be directly attached to the oligonucleotide or indirectly attached, for example through a linker molecule. In particular examples, an RT-LAMP primer (e.g., one of SEQ ID NOs: 1-18) includes a fluorophore at the 5' or 3' end. In some examples, the fluorophore is HEX, FAM, TET, fluorescein, fluorescein isothiocyanate (FITC), or QFITC (XRITC). One of skill in the art can select additional suitable fluorophores (see. e.g., *The Molecular Probes Handbook*, Life Technologies, 11<sup>th</sup> Edition, 2010).

Although exemplary primer sequences are provided herein, the primer sequences can be varied slightly by moving the primer a few nucleotides upstream or downstream from the nucleotide positions that they hybridize to on the target nucleic molecule acid, provided that the probe and/or primer is still specific for the target nucleic acid sequence. For example, variations of the primers disclosed as SEQ ID NOs: 1-18 can be made by "sliding" the probes or primers a few nucleotides 5' or 3' from their positions, and such variations will still be specific for the respective target nucleic acid sequence.

Also provided by the present disclosure are primers that include variations to the nucleotide sequences shown in any of SEQ ID NOs: 1-18, as long as such variations permit detection of the target nucleic acid molecule. For example, a primer can have at least 85%% sequence identity such as at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to a nucleic acid including the sequence shown in any of SEQ ID NOs: 1-18. In such examples, the number of nucleotides does not change, but the nucleic acid sequence shown in any of SEQ ID NOs: 1-18 can vary at a few nucleotides, such as changes at 1, 2, 3, 4, 5, or 6 nucleotides.

The present application also provides primers that are slightly longer or shorter than the nucleotide sequences shown in any of SEQ ID NOs: 1-18, as long as such deletions or additions permit amplification and/or detection of the desired target nucleic acid molecule. For example, a primer can include a few nucleotide deletions or additions at the 5'- or 3'-end of the primers shown in any of SEQ ID NOs: 1-18, such as addition or deletion of 1, 2, 3, 4, 5, or 6 nucleotides from the 5'- or 3'-end, or combinations thereof (such as a deletion from one end and an addition to the other end). In such examples, the number of nucleotides changes.

Also provided are primers that are degenerate at one or more positions (such as 1, 2, 3, 4, 5, or more positions), for example, a primer that includes a mixture of nucleotides

(such as 2, 3, or 4 nucleotides) at a specified position in the primer. In other examples, the primers disclosed herein include one or more synthetic (e.g., non-naturally occurring) bases or alternative bases (such as inosine). In other examples, the primers disclosed herein include one or more 5 modified nucleotides or nucleic acid analogues, such as one or more locked nucleic acids (see, e.g., U.S. Pat. No. 6,794,499), an altered sugar moiety, an inter-sugar linkage, a non-naturally occurring nucleotide linkage, a phosphorothioate oligodeoxynucleotide, a peptide nucleic acid (PNA), 10 or one or more superbases (Nanogen, Inc., Bothell, Wash.).

The nucleic acid primers disclosed herein can be supplied in the form of a kit, for example, for use in the detection or amplification of ZIKV nucleic acid.

The following examples are provided to illustrate certain 15 particular features and/or embodiments. These examples should not be construed to limit the disclosure to the particular features or embodiments described.

#### **EXAMPLES**

#### Example 1: Materials and Methods

This example describes the materials and experimental procedures used for the studies described in Example 2. Viruses

The following viruses utilized in this study were obtained from the Diagnostic and Reference Laboratory, Arboviral Diseases Branch, DVBD (Fort Collins, Colo.), or the Dengue Branch, DVBD (San Juan. Puerto Rico). ZIKV, strain 30 PRVABC59, was isolated from a human infected in Puerto Rico in 2015. DENV1, strain R99142, was isolated from a traveler who visited Guatemala in 2013. DENV2, strain PR65-98, was isolated from a human in 1998 in Puerto Rico. DENV3, strain 100345, was isolated from a traveler who

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visited Nicaragua in 2014. DENV4, strain CAREC 08-10822, was isolated from a human specimen from St. Vincent, US Virgin Islands in 2008. CHIKV, strain 103268b, was isolated from a traveler who visited Bolivia in 2015. WNV, strain NY99-35262-11, was isolated from a flamingo at the Bronx zoo, New York, N.Y, in 1999. SLEV, Strain MSI-7, was isolated from a house sparrow in Indianola, Miss., in 1975.

#### Diagnostic Specimens

A total of 178 acute diagnostic specimens (urine: 84, serum: 94) randomly selected from patients with a ZIKV infection tested at the Centers for Disease Control and Prevention (CDC) by the Trioplex assay for detection of ZIKV, DENV1-4 and CHIKV were included in the study. A total of 68 diagnostic specimens (urine: 27, serum: 41) from patients testing negative for ZIKV infection were also included. Diagnostic specimens were de-identified for this study so that no link could be made between patient identification and assay result.

#### RT-LAMP Primer Design

ZIKV-specific RT-LAMP primers were designed using the nucleotide sequence of strain PRVABC59 (GenBank accession no. KU501215.1) and PrimerExplorer V5 software. Fifteen different primer sets were designed, and three primer sets (1-1, 2-5 and 5-5) with the most promising initial amplifications were evaluated in the RT-LAMP assay (Table 1). Primer set 1-1 amplifies the region in the ZIKV genome from nucleotides 1626 to 1849 included in the envelope (E) gene. Primer set 2-5 amplifies the region in the ZIKV genome between nucleotides 3682 to 3873 in the non-structural 2a (NS2a) gene. Primer set 5-5 amplifies the region in the ZIKV genome from nucleotides 7901 to 8143 included in the non-structural 5 (NS5) gene.

TABLE 1

	Primers and probes used in RT-LAMP and qRT	-PCR
Primer	Sequence	SEQ ID NO:
F3 1-1	TGGTTCCACGACATTCCATT	1
B3 1-1	CATTTCAAGTGGCCAGAGGA	2
FIP 1-1	GGCATGTGCGTCCTTGAACTCTGACACCGGAACTCCACACT	3
BIP 1-1	AGAAGGAGCAGTTCACACGGCCCCTTTGCACCATCCATCTC	4
LF 1-1	ACCAGTGCTTCTTTGTTGTTCC	5
LB 1-1	CCTTGCTGGAGCTCTGGAG	6
F3 5-5	GATCTTGGATGTGGCAGAGG	7
B3 5-5	TGCTTCTTCCACTTCAGGAC	8
FIP 5-5	CGGGTTCTTCATGACCAGGGCGTTACTACGTCGCCACCATC	9
BIP 5-5	${\tt TCCGTCTTAAGAGTGGGGTGGATGTCACACAGCAACGTGTC}$	10
LF 5-5	CCTTTCACTTCTTGAACTTTGCG	11
LB 5-5	CGTCTTTCATATGGCGGCTG	12
F3 2-5	AATGAGTGACCTGGCTAAGC	13
B3 2-5	AAAAGACACGAGGCCAAGG	14
FIP 2-5	${\tt CAGCGCCAGATGAGCTACATCTTTTTGATGGGTGCCACCTTC}$	15
BIP 2-5	AGCGGCATTCAAAGTCAGACCATTTCACGGGGTGTCCAATT	16

Primer

LF 2-5

LB 2-5

Q1-1F

Q1-1R

01-1P

TABLE 1-continued							
Primers and probes used in RT-LAMP	and qRT-PCR						
Sequence	SEQ ID NO:						
CCTCCAGTGTTCATTTCCGC 17							
GCGTTGCTGGTATCTTTCATCTT	18						
GGAACTCCACACTGGAACAA	19						
AACCACGACAGTTTGCCTTT	20						

RNA Extraction and In Vitro Transcribed RNA Controls

[6FAM]AGAGTTCAAGGACGCACATGCCA[BHQ1a-Q]

Viral RNA from samples was extracted from virus supernatant with a QIAmp Viral RNA kit (Qiagen) following the manufacturer's protocols. To prepare in vitro transcribed RNA from the gene region amplified by the 1-1 RT-LAMP primer set for a copy number control, the ZIKV consensus primers (F3 1-1 and B3 1-1) with the T promoter sequence (TAATACGACTCACTATAGGGAGA; SEQ ID NO: 22) added to the 5' end of the F3 1-1 primer were used to amplify 25 a 224 bp segment of cDNA (Table 1). The same size segment of RNA was transcribed from the cDNA using the mMessage mMachine kit (Life Technologies) according to the manufacturer's protocol. RNA was quantified using the RNA Analysis screen tape on the Agilent 4200 Tapestation, 30 and RNA copy numbers/pi were calculated based on spectrophotometry readings.

Viral RNA from 300 d of specimens from spiked serum and urine panels was extracted using the QIAmp Viral RNA kit and eluted in 60 µl of AVE buffer (Qiagen). For diag- 35 nostic specimens, viral RNA was extracted from 300 µl of sample using the MagMAX Pathogen RNA/DNA sample preparation system (ThermoFisher Scientific) according to the manufacturer's protocol for low-cell-content samples and eluted in 90 µl of AVE buffer.

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

ZIKV-specific RNA extracted from samples were analyzed in triplicate by qRT-PCR using the RNA standard described above. A 5 µl aliquot of each purified RNA sample 45 was added to master mix from Quantifast Pathogen RT-PCR kit containing primers and probe designed using the region amplified by the 1-1 RT-LAMP primer set described above. To each reaction, 0.25 μl of 100 μM forward primer Q1-1F, 0.25 μl of 100 μM reverse primer Q1-1R, and 0.15 μl of 25 50 μM probe Q1-1P were added (Table 1). The reactions were analyzed on a BioRad CFX96 instrument under the following conditions: 50° C. for 20 minutes, 95° C. for 15 minutes, followed by 45 cycles of 95° C. for 15 seconds, and 60° C. for 30 seconds with continuous fluorescence data collection. 55 Average C, values were calculated based on triplicate wells. If no amplification occurred, a value of 40 was assigned to the well.

#### RT-LAMP Assay

RT-LAMP reactions were carried out in duplicate in a 25 60 μl volume containing 1.6 μM each of inner primers FIP and BIP, 0.2 μM each of outer primers F3 and B3, 0.4 μM each of loop primers FL and BL, and either 12.5 µl of 2× Colorimetric LAMP Master Mix (2x; Cat. No. M1800, New England Biolabs) and 10 μl of RNA template, or 5.0 μl of a 65 custom-made 5× Colorimetric LAMP Master Mix and 17.5 μl of RNA template (Tanner et al. Bio Techniques 58:59-68,

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2015; Poole et al., PLoS One 12(2):e0169011, 2017). Colorimetric LAMP Master Mix (2x) is a low-Tris reaction buffer containing 16.0 mM MgSO<sub>4</sub>, Bst 2.0 WARMSTART<sup>TM</sup> DNA Polymerase. WARMSTART<sup>TM</sup> RTx reverse transcriptase and phenol red for pH detection. LAMP Reactions were incubated at 65° C. between 25 and 40 minutes in a heat block before results were recorded.

#### Statistical Analysis

Estimates of sensitivity, specificity and (diagnostic) likelihood ratios positive and negative, and accuracy of the ZIKV RT-LAMP assay were calculated based on the results from 110 positive and 68 negative samples by RT-PCR (Pepe, "The statistical evaluation of medical tests for classification and prediction," Oxford; New York: Oxford University Press; 2003, xvi, 302 pages). Wilson's score 95% binomial confidence intervals (CI) were computed for sensitivity and specificity, and score CIs were computed for the likelihood ratios and predictive values (Miettinen et al., Star Med 4(2):213-226, 1985). Estimates of positive and negative predictive values were computed using the estimated likelihood ratios over the full range of pre-test probabilities of ZIKV infection, and these values were displayed graphically. Differences in RT-LAMP positivity by specimen type 40 were evaluated using Fisher's exact test (mid-p). Estimated Limit of Detection Analysis

At each master mix/primer set combination, the recorded RNA concentrations represent a limit of detection for the individual well measurements, while the algorithm determining positivity of a sample-2 of 3 replicate wells positivegives a limit of detection (LOD) for the assay as implemented. The expected LOD(s) of the assay are estimated from the reported experiment. The data suggest that the LOD may vary by master mix and primer set, although because the available information limits the ability to model these experimental combinations simultaneously, the LODs are estimated separately.

Since the RNA concentration/dilution sequences differed for two different batches of sample, observations were recorded as one of batch 1 concentrations (1.06, 2.39, 3.74, 4.46, 13.18, 27.10, 345.05 RNA copies/µl) and batch 2 concentrations (0.47, 0.93, 1.67, 3.40, 6.00, 12.00, 116.00 copies/µl) for individual replicate measurements of RNA concentration. This mixing of concentrations and unequal dilution stepping made standard methods for dilution assays not readily applicable. Depending on the master mix/primer set combination, either 6 or 9 replicates were performed. These replicates were grouped in triplicates and the assay algorithm of identifying the second smallest value of the three as the final response for the triplicate set was used (if the smallest two were equal, this value was used), and this yielded either 2 or 3 observations of assay LOD per master

is the quantity that is used to characterize samples.

mix/primer set combination. The distribution of this final response from the assay algorithm is of interest when making inference regarding the LOD for the assay, since this

The distribution of the assay LOD may be derived from 5 the distribution of the individual replicates by noting that the value of the second-largest observation is the second order statistic of three observations for independent replicates grouped into threes. A model for the distribution of the individual LOD replicates thus readily yields the required distribution for the assay LOD using standard results from the distribution theory of order statistics. Noting that individual replicate responses are reasonably modeled as continuous, positive, skewed right, and interval censored, these values were modeled using standard survival analysis meth- 15 ods assuming either a Weibull (when at least 4 unique values were available) or an exponential (fewer than 4 unique values available); recall the exponential distribution is a special case of the Weibull. Resulting shape and scale estimates therefore provide a model fit for the individual 20 LOD replicates. Denote by  $F(x; r,\lambda)$  the cumulative distribution function (CDF) of the Weibull distribution with shape parameter r and scale parameter  $\lambda$ ; r is fixed at 1 for the exponential distribution. The resulting CDF of the distribution of the assay LOD is, therefore,  $F_{2,3}(x;r,\lambda)=F(x;r,\lambda)=F(x;$  25  $(r,\lambda)^2[3-2F(x; r,\lambda)]$ , where the subscript 2;3 indicates the second order statistic of a sample of size three.

Implementing the survival analysis approach described provides maximum likelihood estimates  $\hat{r}$  and  $\hat{\lambda}$  in the case of the Weibull, and  $\hat{\lambda}$  for the exponential, and these may be 30 used directly in the expression for  $F_{x,\nu}(x;r, \lambda)$ , giving an estimate of the distribution for the assay LOD. The expected assay LOD is thus the mean of this distribution, which must be computed numerically using the probability density function derived from  $F_{2;3}(x; r, \lambda)$  and numerical integration. <sup>35</sup> Confidence intervals for the mean assay LOD may theoretically be computed using asymptotic results, but with few observations here, instead bootstrap resampling was used (Davidson and Hinkley, "Bootstrap methods and their appli-University Press; 1997). Briefly, the 6 or 9 individual replicates were resampled with replacement 10,000 times, and for each of these resample datasets, maximum likelihood estimates  $\hat{r}$  and  $\hat{\lambda}$  were computed and the mean of  $F_{2,3}(x;\hat{r},\hat{\lambda})$  calculated. The average of these 10,000 bootstrap 45 resample values of the mean assay LOD are the final estimates for the assay LOD, while the empirical 2.5th and 97.5th quantile points of the 10,000 bootstrap resample values provide a 95% confidence interval (CI) for the mean assay LOD. Estimates of the mean assay LOD (95% CI) are 50 reported in Table 2.

Example 2: RT-LAMP Assay for Detection of ZIKV RNA

This example describes optimization and characterization of an RT-LAMP assay to detect ZIKV in urine and serum samples. The studies described in this example report results obtained from primer sets 1-1, 2-5 and 5-5.

Optimization and Amplification Efficiency of ZIKV RT- 60 LAMP Assay

Viral RNA extracted from ZIKV-infected Vero cell supernatant was used as a template to determine optimal reaction conditions for the RT-LAMP assay. RNA copies/ $\mu l$  were quantitated using qRT-PCR and determined to be 4.8×10<sup>6</sup> RNA copies/µl. Varying dilutions of viral RNA from 100 to 0.625 RNA copy/μl were made and tested in the RT-LAMP

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assay using primer sets 1-1, 2-5, 5-5 and combinations of primer sets  $(1-\frac{1}{2}-5, 1-\frac{1}{5}-5, 2-\frac{5}{5}-5, \text{ and } 1-\frac{1}{2}-\frac{5}{5}-5)$  at 65° C. Reaction results were recorded between 20 to 40 minutes in 5 minute intervals. Detection of nucleic acid amplification was determined visually with a color change from pink to yellow indicating amplification of nucleic acid. The reaction was considered complete once the non-template control (NTC) wells began to change color. The duration of this color change occurred between 30 and 40 minutes and was dependent on each individual primer set Results were recorded at the 5-minute time interval in which all NTC wells were still negative for the primer set evaluated. Reactions were considered positive when 2 of the 3 wells displayed a color change. Two or three independent tests conducted in triplicate were used to calculate the expected limits of detection for each primer set. Primer sets were tested with both the 2×LAMP master mix (MM) commercially available (New England Biolabs) and a 5XMM which allowed for an additional 7.5 µl of RNA to be added to the reaction.

A decrease in the limit of detection was observed for all primer sets in the reaction using 5XMM and subsequent higher volumes of viral RNA with one exception (primer set 5-5). Primer sets 1-1 and  $1-\frac{1}{2}-\frac{5}{5}-5$  had the greatest decrease in the expected limit of detection from 19.3 and 8.5 RNA copies/μl, respectively, using the 2XMM and 10 μl of RNA in the reaction to 7.2 and 2.1 RNA copies/d, respectively, in the reaction containing 5XMM concentration and 17.5 µl of RNA. Primer set 1-1/s-5 also had a notable decrease in the expected limit of detection from 10.0 RNA copies/µl with 2XMM to 5.4 RNA copies/µl with 5XMM. Relatively similar values were recorded for primer set 5-5 (6.2 RNA copies/µl and 6.6 RNA copies/µl with 2XMM and 5XMM, respectively) and primer set 2-5 (3.9 and 3.4 RNA copies/µl with 2XMM and 5XMM, respectively). Using the 5XMM, primer sets  $1-\frac{1}{2}-5$  and  $1-\frac{1}{2}-5/5-5$  were found to have the lowest expected limit of detection in the assay at 2.1 RNA copies/µl (Table 2). Three primer sets (2-5, 1-1/2-5 and 1-1/2-5/5-5) were subsequently tested for their ability to cation," Cambridge; New York, N.Y., USA: Cambridge 40 detect viral RNA in a panel of virus-spiked human serum specimens.

TABLE 2 Estimates (95% CI) of mean ZIKV RT-LAMP assay limit of detection for each primer set using 2X and 5X master mix (MM)

Primer set	2XMM	5XMM
1-1	19.3 <sup>a</sup> (9.5, 38.8) <sup>b</sup>	7.2 (3.9, 11.8)
2-5	3.9 (2.9, 5.0)	3.4 (2.5, 4.2)
5-5	6.2 (3.7, 9.7)	6.6 (3.6, 10.8)
1-1/2-5	4.7 (3.5, 5.8)	2.1 (1.4, 2.7)
1-1/5-5	10.0 (6.6, 13.1)	5.4 (3.4, 9.5)
2-5/5-5	4.3 (3.0, 5.5)	2.8 (2.3, 3.2)
1-1/2-5/5-5	8.5 (4.6, 15.4)	2.1 (1.4, 2.9)

55 <sup>a</sup>RNA copies/µl. The mean of the expected limit of detection was determined using ZIKV viral RNA with quantitated RNA copies measured by qRT-PCR using the gene region amplified by primer set 1-1. Reactions were incubated at 65° C. for 20-40 minutes before results were recorded.
by 5% confidence interval

#### Sensitivity of ZIKV RT-LAMP assay

To evaluate the sensitivity of the ZIKV RT-LAMP assay compared to qRT-PCR, viral RNA was extracted from 300 μl of sample from a virus-spiked serum panel and tested with primer sets 2-5,  $1-\frac{1}{2}$ -5 and  $1-\frac{1}{2}$ -5/5-5. The RNA in each sample was quantitated by qRT-PCR and ranged from 2.8 to 1476 RNA copies/pi. Ct values were also recorded. A positive result in the qRT-PCR is any average Ct value under 38.0 from triplicate wells while a negative test result is any

of 40.0 was assigned if no amplification occurred in one of the triplicate wells. Ct values in the serum panel for samples containing ZIKV RNA ranged from 27.6 to 36.3 (Table 3). All primer sets reacted similarly when tested with the 5 virus-spiked serum samples. All primer sets were able to detect viral RNA in samples containing more than 50 RNA copies/µl. Only 1-1/2-5/5-5 was not able to detect viral RNA in the sample (#9) containing 12.5 RNA copies/µl. None of

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TABLE 4-continued

Sensitivity of RT-LAMP with ZIKV urine panel					
	RNA	Ct	RT-	-LAMP <sup>c</sup>	
Sample	copies/µla	value <sup>b</sup>	2-5	1-1/2-5	
7	7047	23.1	+	+	
8	0	39.1	_	_	

A panel consisting of a variety of diagnostic specimens including whole blood, serum, and urine with and without nucleic acid preservative was also evaluated in the RT-LAMP assay with primer set 1-1/2-5 to determine the sensitivity and specificity of the assay compared to qRT-PCR 20 results. The sensitivity of the qRT-PCR described here was validated in a separate experiment with quantitated viral RNA and displayed an equivalent sensitivity compared to the CDC's single-plex real-time RT-PCR assay. RNA was extracted from 300 µl of sample and eluted in 100 µl AVE buffer. Results from the CDC's single-plex real-time RT-PCR assay for the detection of ZIKV using 20 µl of RNA and the ZIKV RT-LAMP assay using 17.5 µl of RNA were compared. RNA concentrations using the qRT-PCR along with the Ct values for this PCR were included in the analysis for each sample (Table 5). The ZIKV RT-LAMP assay was comparable to the CDC's single-plex qRT-PCR when tested with the specimen panel with only two false negative samples recorded (#2, 3.3 RNA copies/µl and #8 9.6 RNA copies/µl). The same RNA used in the single-plex real-time RT-PCR was used for the LAMP assay and the PCR for quantitation, but had been freeze-thawed between uses possibly resulting in degradation of the RNA sample which may account for the drop in Ct values between the two PCR tests for all samples and the negative reactions in the ZIKV RT-LAMP for samples #2 and #8. Overall, when compared to the single-plex real-time RT-PCR the RT-LAMP assay had a sensitivity of 80% (95% CI 49.0±94.3%) and specificity of 100% (95% CI 75.7±100%). While the sample size was low, these results provide confidence that the ZIKV RT-LAMP assay should perform well with a large set of diagnostic specimens when compared to the qRT-PCR assay.

TABLE 3

negative samples included in the panel with any primer set

(Table 3).

No false positive results were detected for any of the 15

the primer sets were able to detect viral RNA in sample (#1) containing 2.8 RNA copies/µl. This is not surprising since the limit of detection of the assay was determine to be in the range of 2.7 and 3.9 RNA copies/l using diluted viral RNA.

		11 1151	JL 3			_
	Sensitivity of	RT-LAMP	with ZII	KV serum pa	anel	_ :
	RNA	Ct		RT-LA	$MP^c$	_
Sample	copies/µla	${\rm value}^b$	2-5	1-1/2-5	1-1/2-5/5-5	
1	2.8	36.3	_	_	_	•
2	0	>40.0	_	_	_	
3	164	30.6	+	+	+	
4	0	>40.0	-	_	_	
5	104	31.3	+	+	+	
6	55	32.1	+	+	+	
7	1476	27.6	+	+	+	
8	0	>40.0	_	_	_	
9	12.5	34.2	+	+	_	
10	544	29.0	+	+	+	
11	368	29.5	+	+	+	
12	0	38.9	-	-	-	

The assay was also evaluated using a panel of urine samples spiked with varying concentrations of ZIKV RNA. ZIKV RNA concentrations in the panel ranged from 1.2 to 7047 RNA copies/l and Ct values ranged from 23.1 to 35.5 for samples containing ZIKV RNA (Table 4). Similar results 45 to the serum panel were obtained for the urine panel. When the assay was performed using primer sets 2-5 and 1-1/2-5, all samples containing ZIKV RNA except the samples with the least amount of RNA (sample #4, 1.2 RNA copies/µl and sample #2, 12 RNA copies/µl) were detected (Table 4). No false positive results were detected for any of the negative samples included in the panel with either primer set (Table 4).

TABLE 4

	RNA	Ct	RT	-LAMP <sup>c</sup>
Sample	copies/µla	value <sup>b</sup>	2-5	1-1/2-5
1	316	27.5	+	+
2	12	33.7	_	-
3	44	30.3	+	+
4	1.2	35.5	_	_
5	0	41.4	_	_
6	0	39.9	_	_

TABLE 5

Performance of RT-LAMP with a proficiency panel of diagnostic specimens					
_	Sample	Ct value <sup>a</sup>	Ct value 1-1 <sup>b</sup>	RNA copies/ $\mu$ l $^c$	RT-LAMP <sup>d</sup>
	1	>40.0	>40.0	0	_
	2	36.4	37.4	3.3	_
,	3	>40.0	>40.0	0	_
	4	29.7	33.3	47.8	+
	5	38.0	>40.0	0	_
	6	30.5	32.7	71.2	+
	7	>40.0	>40.0	0	_
`	8	32.5	35.4	9.6	_
)	9	31.3	36.7	11.1	+
	10	32.3	34.5	18.5	+
	11	>40.0	>40.0	0	_
	12	32.4	36.5	5.2	+
	13	38.1	>40.0	0	_
	14	>40.0	>40.0	0	_
5	15	>40.0	>40.0	0	_
	16	30.5	33.7	35.1	+

<sup>&</sup>lt;sup>a</sup>RNA copies/μl in each sample were evaluated in qRT-PCR using RNA transcript made within the gene region amplified by primer set 1-1.

<sup>b</sup>Average Ct values of the quantitated urine panel based on primers from gene region amplified by primer set 1-1 (5 μl RNA/reaction).

<sup>c</sup>Sensitivity of RT-LAMP was evaluated with each primer set using 5X MM. Reactions were incubated at 65° C. for 20-40 minutes before results were recorded as positive (+) or negative (-).

<sup>&</sup>quot;RNA copies/μl in each sample were evaluated in qRT-PCR using in vitro transcribed RNA encompassing the gene region amplified by primer set 1-1.

\*Average Ct values of the quantitated serum panel based on primers from gene region amplified by primer set 1-1 (5 μl RNA/reaction).

\*sensitivity of RT-LAMP was evaluated with each primer set using 5X MM. Reactions were incubated at 65° C. for 20-40 minutes before results were recorded as positive (+)

TABLE 5-continued

	Performance of RT-LAMP with a proficiency panel of diagnostic specimens						
Sample	Ct value <sup>a</sup>	Ct value 1-1 <sup>b</sup>	RNA copies/ $\mu$ l $^c$	RT-LAMP <sup>d</sup>			
17	>40.0	>40.0	0	-			
18	31.9	35.3	10.3	+			
19	38.3	>40.0	0	-			
20	28.9	32.0	124.6	+			
21	>40.0	>40.0	0	_			
22	>40.0	>40.0	0	_			

positive was 2.5 (95% CI 0.8-7.4), and the likelihood ratio negative was 0.9 (95% CI 0.8-1.0). When diagnostic specimens had RNA concentrations >1.0 copy/µl (Ct values ranging 35.20 to 24.23) the ZIKV RT-LAMP assay was able to detect 54 out of 67 positive samples, a sensitivity of 80.6% (95% CI 69.6-89.3%); there were no RT-PCR negative samples, so specificity and the likelihood ratios could not be estimated. These results are summarized in FIG. 1 which separates negative and positive RT-LAMP results and plots them based on the Ct value from the real-time RT-PCR and RNA concentration. When all diagnostic samples were included in the statistical analysis, the assay had a sensitivity of 54.5% (95% CI 45.2-63.5%), specificity of 94.1% (95% CI 85.8-97.7%), likelihood ratio positive of 9.3 (95% CI 3.8-23.9) and likelihood ratio negative of 0.5 (95% CI 0.4-0.6). The overall accuracy of the ZIKV RT-LAMP assay was 69.7% (95% CI 62.6-75.9%) (Table 6).

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TABLE 6

Diagnostic performance of RT-LAMP based on RNA concentration					
RNA copies/µl	Sensitivity (%)	Specificity (%)	Likelihood Ratio Positive	Likelihood Ratio Negative	Accuracy (%)
<1.0 >1.0	14.0 (5.4-28.5)* 80.6 (69.6-89.3)	94.1 (85.8-97.7) N/A**	2.5 (0.8-7.4) N/A**	0.9 (0.8-1.0) N/A**	63.1 (53.8-71.5) 80.6 (69.6-88.3)
total	55.4 (45.2-63.5)	94.1 (85.8-97.7)	9.3 (3.8-23.9)	0.5 (0.4-0.6)	69.7 (62.6-75.9)

<sup>\*95%</sup> confidence interval:

#### Specificity of ZIKV RT-LAMP Assay

In order to estimate the specificity of the assay to detect only ZIKV RNA, a panel of urine samples spiked with 35 varying concentrations of arboviruses including DENV1  $(1.1 \log_{10} PFU/\mu I)$ , DENV2  $(4.9 \log_{10} PFU/\mu I)$ , DENV3 (0.2 $\log_{10} PFU/\mu l$ ). DENV4 (0.3  $\log_{10} PFU/\mu l$ ). WNV (3.6  $\log_{10} l$ PFU/ $\mu$ l) SLEV (4.1  $\log_{10}$  PFU/ $\mu$ l), and CHIKV (0.1 PFU/ $\mu$ l) were tested. RNA was extracted from 300 µl of spiked urine 40 and tested in the assay using primer set 1-1/2-5 under the same conditions as previously described. The ZIKV RT-LAMP assay was shown to be highly specific for ZIKV since none of the spiked urine samples with other arboviruses were positive in the assay. Additionally, A panel of diagnostic specimens which tested positive in the CDC's diagnostic Trio-plex RT-PCR assay for DEN V (n=10) with Ct values ranging from 15.77 to 31.92 or CHIKV (n=10) with Ct values ranging from 18.47 to 24.23, were negative when 50 tested in the ZIKV RT-LAMP assay.

Evaluation of RT-LAMP Assay for Clinical Diagnosis of ZIKV

A total of 178 diagnostic specimens (94 serum specimens and 84 urine specimens) were collected for testing in the ZIKV RT-LAMP assay. Of these, 110 were positive for ZIKV by qRT-PCR (53 ZIKV-positive serum samples, 57 ZIKV-positive urine samples), and 68 were negative by qRT-PCR (41 ZIKV negative serum samples, 27 ZIKV negative urine samples). When diagnostic specimens had RNA concentrations <1.0 copy/µl (Ct values ranging from 35.09 to 40.00), the ZIKV RT-LAMP assay was only able to detect 6 of 43 positive samples, a sensitivity rate of only 14.0% (95% CI 6.6-27.3%), while 64 of 68 RT-PCR negative samples were RT-LAMP negative, for a specificity of 94.1% (95% CI 85.8-97.7%) (FIG. 1). The likelihood ratio

FIG. 2 shows the predictive values (and 95% CIs) for the ZIKV RT-LAMP assay based on pre-test probability percentages of ZIKV infection. When the pre-test probability of ZIKV infection in the population was plotted against the positive and negative predictive values of the ZIKV RT-LAMP assay, the test is estimated to maximize both predictive values simultaneous when the pre-test probability of disease is approximately 30% (FIG. 2). No statistical difference in RT-LAMP positivity by specimen type were detected in either PCR positive (Fisher's exact mid-p=0.9) or PCR negative (Fisher's exact mid-p=0.7) samples. Diagnostic Applications

Described herein is the development of RT-LAMP for the rapid detection of ZIKV in urine and serum. The ZIKV RT-LAMP assay demonstrated a level of detection similar to RT-PCR when the Ct value was below 35.2 corresponding to an RNA concentration of 21.0 RNA copies/µl, resulting in an accuracy rate of 80.6%. When the analysis included samples with an RNA concentration of <1.0 RNA copies/µl, the accuracy of the diagnostic test dropped to 69.7%. The ZIKV RT-LAMP assay also demonstrated a high degree of specificity with only a 2.2% false positive rate calculated from negative diagnostic specimens tested in the assay; the false negative rate was 27.5%.

These results indicate that the ZIKV RT-LAMP assay can be used as a rapid point-of-care test for clinical screening of suspected ZIKV infections or routine testing for ZIKV infections in asymptomatic pregnant women. RT-LAMP offers an easy to use, convenient and cost-effective alternative to laboratory-based testing, particularly in field or clinical settings where the rapidity and convenience of screening diagnostic samples may outweigh the need for definitive diagnoses. In this study, the median value of RNA concentration from specimens detected by qRT-PCR was 1.92 RNA copies/μl (a median Ct value of 34.09). These

<sup>&</sup>quot;Average Ct values based on the CDC RT-PCR single-plex assay using freshly extracted RNA run in duplicate wells for the detection of ZIKV."
"Average Ct values of the qRT-PCR after freeze-thawing of RNA based on primers from gene region amplified by primer set 1-1 (5 µl RNA/extraction).

"RNA copies/µl in each sample were evaluated in qRT-PCR after freeze-thawing of RNA using RNA transcript made within the gene region amplified by primer set 1-1. (2-5 and 5X MM. Reactions were incubated at 65° C. for 20-40 minutes before results were recorded as positive (+)

<sup>\*\*</sup>no samples were RT-PCR negative

specimens and values for qRT-PCR are typical of diagnostic specimens tested for ZIKV, and it is believed this study accurately demonstrates the level of sensitivity that can be obtained with RT-LAMP.

Using the rapid point-of-care RT-LAMP assay disclosed herein can greatly enhance prenatal care of women living in areas where ZIKV may circulate. By utilizing this diagnostic screening tool for the detection of ZIKV infections in asymptomatic pregnant women throughout pregnancy more

32 information will be available to health care providers and patients during critical times of care.

In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated embodiments are only preferred examples of the invention and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

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The invention claimed is:

- 1. A method for detecting Zika virus (ZIKV) RNA in a biological sample, comprising:
  - (i) subjecting the sample to a reverse transcription loop-mediated isothermal amplification (RT-LAMP) reaction using a set of primers specific for ZIKV nucleic acid to produce a ZIKV nucleic acid amplification product, wherein the set of primers comprises six primers each respectively having a sequence at least 90% identical to SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17 and SEQ ID NO: 18; and
  - (ii) detecting the ZIKV nucleic acid amplification product, thereby detecting ZIKV RNA in the biological sample.
- 2. The method of claim 1, wherein the set of primers comprises:
  - (a) six primers each respectively having a sequence consisting of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17 and SEQ ID NO: 18;
  - (b) six primers each respectively having a sequence 60 consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6: and
  - (c) six primers each respectively having a sequence consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID 65 NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO: 12.

- 3. The method of claim 1, wherein the set of primers further comprises:
- six primers each respectively having a sequence at east 90% identical to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6; and/or
- six primers each respectively having a sequence at east 90% identical to SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO: 12.
- 4. The method of claim 1, wherein the RT-LAMP reaction is performed at about  $65^{\circ}$  C.
- 5. The method of claim 1, wherein the RT-LAMP reaction includes a pH-sensitive indicator dye.
- **6**. The method of claim **5**, wherein the pH-sensitive indicator dye is a colored dye detectable in visible light.
- 7. The method of claim 6, wherein the pH-sensitive indicator dye comprises cresol red, phenol red, m-cresol purple, bromocresol purple, neutral red, naphtholphthalein, thymol blue or naphtolphthalein.
- **8**. The method of claim **5**, wherein the pH-sensitive indicator dye is a fluorescent indicator dye.
- 9. The method of claim 8, wherein the fluorescent dye comprises 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein, 5(6)-carboxy-2', 7'-dichlorofluorescein, 5(6)-carboxyfluorescein, 3,6-diacetoxyphthalonitrile, 6,8-dihydroxy-1,3-pyrenedisulfonic acid, or 5-(and-6)-carboxyl seminaphthorhodafluor.
- 10. The method of claim 1, wherein detecting the ZIKV nucleic acid amplification product comprises detecting a visible color change.

- 11. The method of claim 1, wherein detecting the ZIKV nucleic acid amplification product comprises detecting fluorescence
- 12. The method of claim 1, wherein the biological sample is a biological fluid sample.
- 13. The method of claim 12, wherein the biological fluid sample comprises a urine or serum sample.
- **14**. A kit for detecting Zika virus (ZIKV) RNA in a biological sample, comprising a set of oligonucleotide primers, wherein the set of primers comprises six primers each respectively having a sequence at least 90% identical to SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17 and SEQ ID NO: 18.
  - 15. The kit of claim 14, further comprising:
  - six primers each respectively having a sequence at least 90% identical to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6; or
  - six primers each respectively having a sequence at least 90% identical to SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO: 12.
  - 16. The kit of claim 14, further comprising:
  - six primers each respectively having a sequence at least 90% identical to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6; and

- six primers each respectively having a sequence at least 90% identical to SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO: 12.
- 17. The kit of claim 14, wherein the set of primers comprises six primers each respectively having a sequence consisting of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17 and SEQ ID NO: 18.
- 18. The kit of claim 17, wherein the set of primers further to comprises:
  - six primers each respectively having a sequence consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6; or six primers each respectively having a sequence consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO: 12.
  - 19. The kit of claim 17, wherein the set of primers further comprises:
  - six primers each respectively having a sequence consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6; and six primers each respectively having a sequence consisting of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO: 12.
  - 20. The kit of claim 14, further comprising buffer, reverse transcriptase, DNA polymerase, or any combination thereof.

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