

A Simple Visual Detection Method of Human Zika Virus Using Reverse Transcription Loop Mediated Isothermal Amplification

Yang Li^{1,2}, Zhenzhou Wan³, Yi Zhou^{2,4}, Xia Jin⁵, Haifeng Shi^{1*} and Chiyu Zhang^{2*}

¹School of Food and Biological Engineering, Jiangsu University, Zhenjiang, Jiangsu, China

²Pathogen Diagnostic Center, CAS Key Laboratory of Molecular Virology and Immunology, Institut Pasteur of Shanghai, Chinese Academy of Science, Shanghai, China

³Medical Laboratory of Taizhou Fourth People's Hospital, Taizhou, Jiangsu 225300, China

⁴School of Life Sciences, East China Normal University, Shanghai 200241, China

⁵Viral Disease and Vaccine Translational Research Unit, CAS Key Laboratory of Molecular Virology & Immunology, Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China

Corresponding authors: Chiyu Zhang, PhD, Professor, Pathogen Diagnostic Center, CAS Key Laboratory of Molecular Virology and Immunology, Institut Pasteur of Shanghai, Chinese Academy of Sciences, Yueyang Road 320, Shanghai 200031, China, Tel: 8615800781776; E-mail: zhangcy1999@ips.ac.cn

Haifeng Shi, PhD, Professor, School of Food and Biological Engineering, Jiangsu University, 301 Xuefu Road, Jingkou District, Zhenjiang, 212013, People's Republic of China, E-mail: shihf@ujs.edu.cn

Received date: November 09, 2017; **Accepted date:** November 22, 2017; **Published date:** November 27, 2017

Copyright: ©2018 Li Y, et al. This is an open-access article distributed under the terms of the creative commons attribution license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abstract

Zika virus (ZIKV) is an emerging mosquito-borne pathogen, and belongs to family *Flaviviridae*, genus *Flavivirus*. ZIKV infection was associated with microcephaly and male infertility. The surveillance of ZIKA infection is important for human reproductive health, and the control of the virus spread. Development of a fast and sensitive assay for ZIKV detection is urgently needed for China and other Southeast Asian countries. Here, we developed a one-step, single-tube reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for ZIKV detection. The assay has a limit of detection (LOD) of 88 copies of Zika RNA per 25 μ l reaction, and can be completed within 20 min when the template input is more than the LOD. Furthermore, it exhibits high specificity and good reproducibility. Importantly, the result can be visualized by colorimetric change when HNB or calcein is used. To evaluate the viability of the assay, a comparison between the RT-LAMP assay and a more conventional RT-qPCR was performed using 20 cell culture supernatants and three urine samples. The ZIKV RT-LAMP showed perfect agreement in detection with the RT-qPCR. These results demonstrate that the newly developed ZIKV RT-LAMP is simple, rapid and well suited for ZIKV surveillance, especially in a resource-limited regions.

Keywords: ZIKV; RT-LAMP; Visual detection; HNB; Calcein

Introduction

Zika virus (ZIKV) is a mosquito-borne *flavivirus* [1]. It was first identified from a rhesus monkey in the Zika forest, Uganda in 1947, and then in mosquitoes (*Aedes africanus*) in the same forest in 1948 [2]. Until 1954, the first human case of ZIKV infection was discovered in Nigeria [3]. For half a century, ZIKV was mainly circulating among *Aedes* mosquitos, although sporadic infections occurred in humans and primates (rhesus monkey) [4]. The first large outbreak of ZIKV occurred in Yap Island in the Federated States of Micronesia in 2007 [5]. ZIKV infection often causes asymptomatic or mildly symptomatic in most cases and the syndromes include mild fever, arthralgia, myalgia, headache, retroorbital pain, conjunctivitis and cutaneous maculopapular rash [6]. From 2015 to 2016, a major outbreak of ZIKV occurred in Brazil and other countries in Americas, and caused more than four million human infections [7]. ZIKV infection in pregnant women during the first trimester was associated with a significant increase in microcephaly in the newborns [8]. Furthermore, new reports showed that ZIKV infection also results in testis damage and may cause male infertility [9,10]. In December 2015, the World Health Organization (WHO) added ZIKV to the second tier of three diseases ranked as serious, and issued travel warnings to ZIKV epidemic areas.

The potential for more widespread of ZIKV infection is real because the viral vector, *Aedes aegypti*, is prevalent in Southeast Asia. This poses a new potential public threat for Southeast Asian countries. Therefore, the development of a fast and sensitive assay for the surveillance of ZIKA infection is urgently needed for the prevention and control of ZIKV transmission in China, and other countries.

Loop-mediated isothermal amplification (LAMP) is a simpler and faster pathogen diagnostic method compared to other methods [11], and was widely used in the detection of various pathogens (e.g. HIV, respiratory syncytial virus (RSV), adenovirus, enterovirus, etc.) [12-15]. LAMP employs two to three pairs of primers to efficiently amplify target sequences under isothermal condition (62-65°C in general) within 1 h. Compared to other conventional methods (e.g. PCR), LAMP has higher sensitivity [16]. LAMP produces a typical ladder like pattern of DNA fragments ranging from approximately 200 bp to several kilobase pairs. The products of LAMP can be detected by agarose gel or visual inspection of fluorescent signal emitted by fluorescence dyes (e.g. SYBR green I, calcein etc.) under ultraviolet (UV) light, or color change when using hydroxynaphthol blue (HNB), phenol red, neutral red, cresol red or m-cresol purple [17-19]. Furthermore, the amplification of LAMP can be measured using a real-time fluorescence-detecting PCR instrument using SYBR green I or SYTO 9 as fluorescence dyes. Because of the isothermal amplification, LAMP is a good choice as a point-of care test (POCT)

for diagnosis of various pathogens, especially in resource-limited regions.

To develop a RT-LAMP method for detecting human ZIKV, we downloaded all available ZIKV genomic sequences from GenBank as of February 28, 2016. A LAMP primer set for ZIKV detection was designed using the open access Primer Explorer V.5 software tool (<http://primerexplorer.jp/>). The primers were located in NS5 gene

region of human ZIKV (7621 to 7813 nt in GD01 KU740184.1), one of the most conserved regions of ZIKV genome (Figure 1). To prepare the Zika RNA standard, a 238 nt plus-strand ZIKV genomic fragment (nucleotide covering 7595 to 7832 nt in GD01 KU740184.1) with T7 promoter at 5'-end was synthesized. The RNA standard was obtained by *in vitro* transcription and was quantitated by Nanodrop 2000C (Thermo, USA).

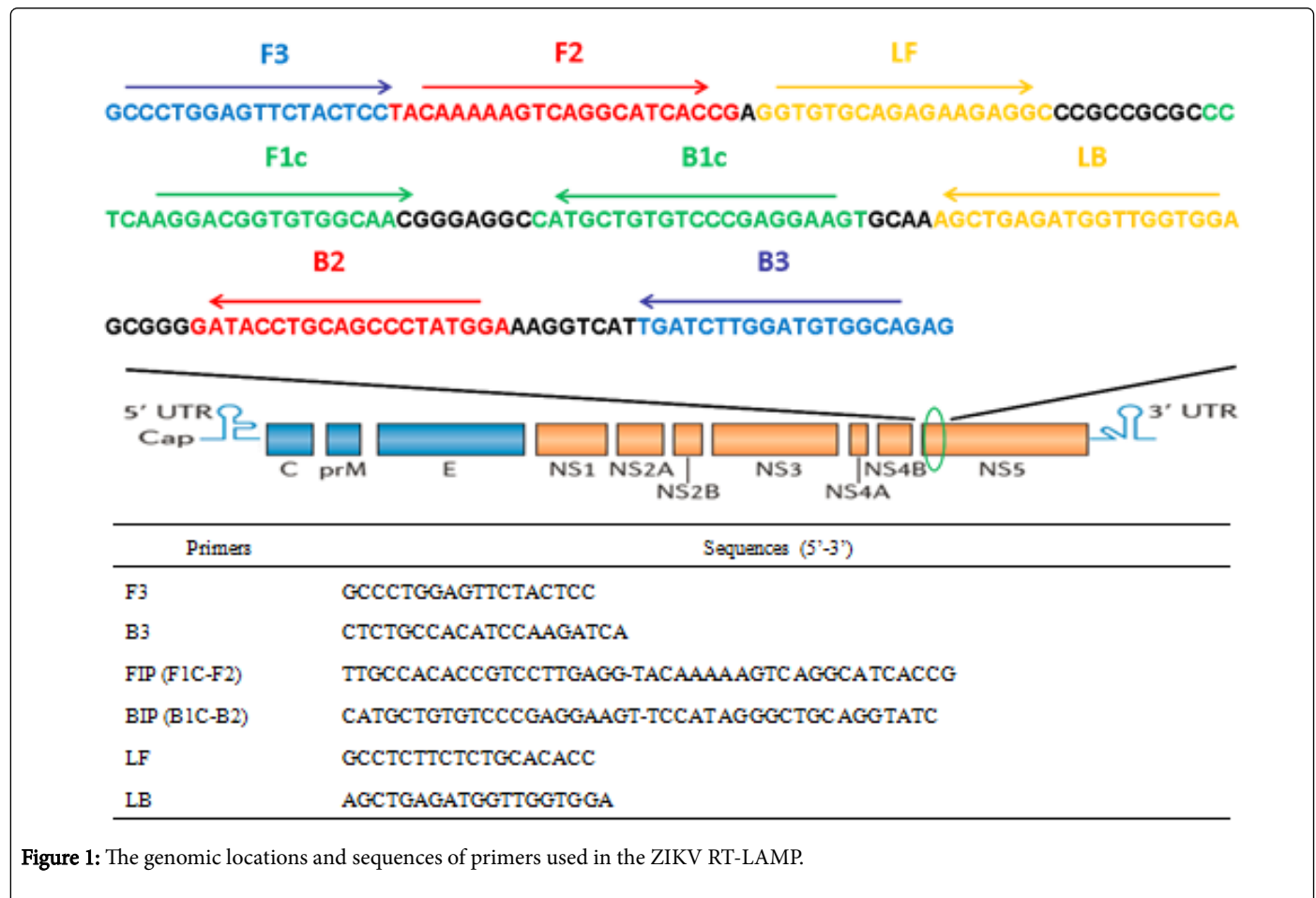


Figure 1: The genomic locations and sequences of primers used in the ZIKV RT-LAMP.

The RT-LAMP reaction system was set up and optimized according to our previous study [13]. Bst 2.0 DNA Polymerase and Quant RTase (Tiangen, China) were used in the RT-LAMP reaction. The optimal 25 μ l reaction mix contains 2.5 μ l 10 \times isothermopol buffer, 4 mM MgSO₄ (Biolabs, UK), 1.6 mM dNTPs (Roche, Switzerland), 1.6 μ M each of FIP and BIP primers, 0.2 μ M each of F3 and B3 primers, 0.8 μ M each of LF and LB primers, 8 units of Bst 2.0 DNA polymerase (Biolabs, UK) and 0.5 μ l of Quant RTase (Tiangen, China). For the real-time monitoring, 0.4 μ M SYTO 9 (Life, USA) was added and the reaction was detected using Roche LightCycle 96. For the colorimetric measurement, 120 μ M HNB (Sigma-Aldrich, USA) or 50 μ M/1 mM Calcein/MnCl₂ (Sigma-Aldrich, USA) was added with 6 mM MgSO₄ in place of 4 mM MgSO₄. The reaction was performed at 62°C for 60 min.

To evaluate the sensitivity of detection of the ZIKV RT-LAMP assay, a 10-fold serial dilution of ZIKV RNA standard ranging from 10⁴ to

10⁰ copies per μ l was prepared. We firstly performed a SYTO 9-based real-time detection. The result showed that the RT-LAMP can detect as few as 50 copies of Zika RNA per 25 μ l reaction. Almost all amplification curves of various template input reached plateau within 20 min, showing a rapid amplification (Figure 2a). The product was separated by gel electrophoresis (Figure 2b). Similar sensitivity of detection was obtained when either HNB (Figure 2c) or calcein (Figures 2d and 2e) was used for visual inspection. When the HNB was used, the reactions with the negative control and 5 copies ZIKV RNA input showed violet, while the reaction with 50 to 5 \times 10⁴ copies of ZIKV RNA input showed azure color which indicates a positive amplification result. In the reaction with calcein, the positive reaction with 50 to 5 \times 10⁴ copies of ZIKV RNA input showed yellow-green and bright green fluorescence under daylight and UV light, respectively, while there was orange and no fluorescence for negative control.

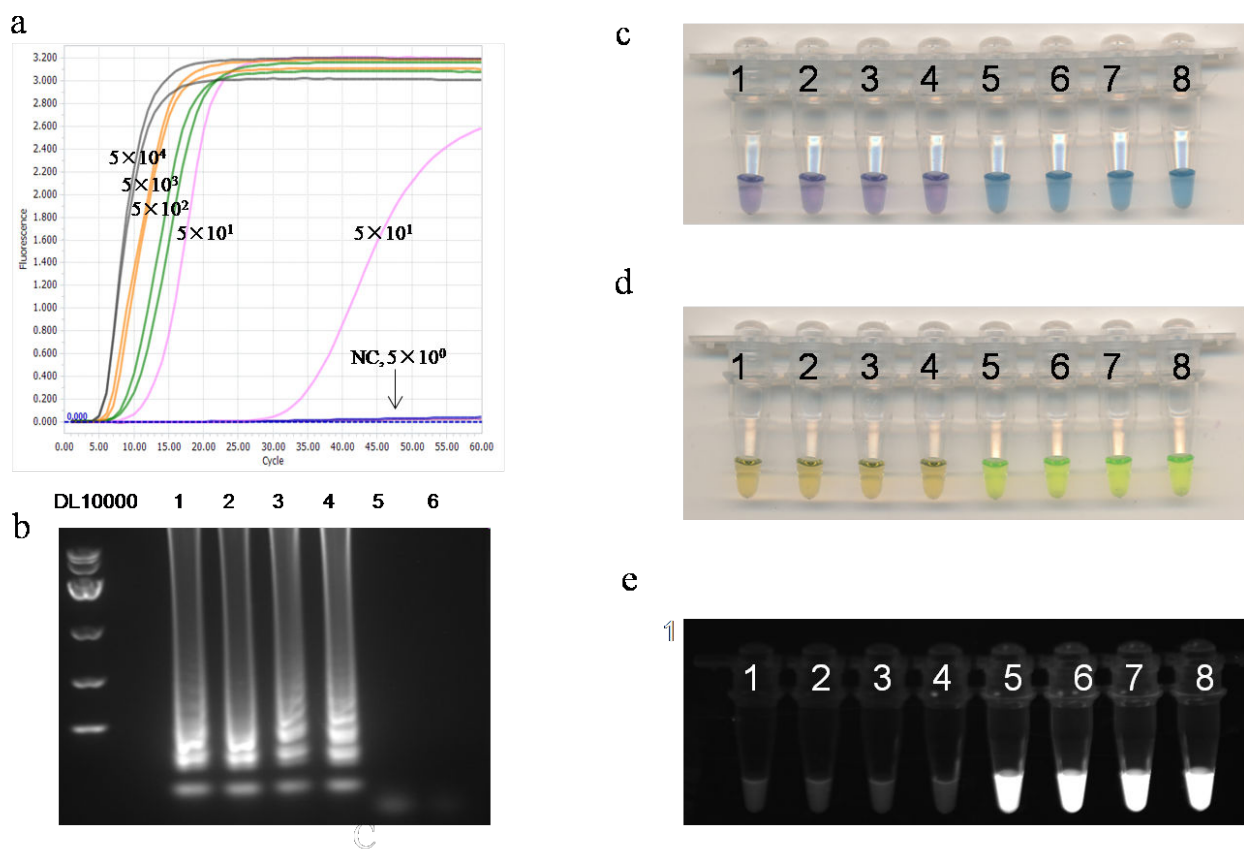


Figure 2: The detection sensitivity of the ZIKV RT-LAMP assay. The sensitivity of detection was determined by 10-fold serial dilutions of the ZIKV RNA standard from 5×10^0 to 5×10^4 copies per reaction. (a) SYTO 9-based RT-LAMP assay (b) Agarose gel electrophoresis of LAMP products. Lanes 1-6: 5×10^4 , 5×10^3 , 5×10^2 , 5×10^1 , 5×10^0 copies of ZIKV RNA standard per reaction and negative control (NC), respectively. (c) HNB-based RT-LAMP assay. (d) Calcein-based RT-LAMP assay under daylight. (e) Calcein-based RT-LAMP assay under UV light Tubes 1-3: negative control (NC); Tubes 4-8: 5×10^0 , 5×10^1 , 5×10^2 , 5×10^3 , 5×10^4 copies of ZIKV RNA standard per reaction, respectively.

We further determined the limit of detection (LOD) of the novel RT-LAMP assay using template input of 100, 50, 10 and 5 copies per 25 μ l reaction (Supplementary Table S1). Each dilution of the ZIKV standard was tested with 10 replicates. The LOD was defined as a 95% probability of obtaining a positive ZIKV RNA result [20], and was determined with probit regression analysis using the SPSS 19.0 software. When the template input of was 100 copies of ZIKV RNA per 25 μ l reaction, all 10 replicates (100%) showed positive results (Supplementary Table S1). When 50, 10, and 5 copies ZIKV RNA were used in the 25 μ l reaction, 8, 5 and 2 positive results were observed, respectively. Based on these results, the LOD of the novel ZIKV RT-LAMP assay was calculated as 88 copies per 25 μ l reaction with a 95% confidence interval of 40-806 copies per 25 μ l reaction ($p \leq 0.05$). The LOD of the Zika RT-LAMP was comparable with a previously reported two real-time RT-qPCR method with a sensitivity of detection of 25 and 100 [21].

To determine the specificity of RT-LAMP for ZIKV detection, 14 standard virus strains, including dengue virus serotypes 1-4, RSV A and B, Influenza A and B, parainfluenza virus (PIV) 3, human conaovirus (HCoV)-OC43, HCoV-229E, enterovirus EV68, rhinovirus

and adenovirus were tested together with 5×10 to 5×10^4 copies of ZIKV RNA standard using the SYTO 9-based real-time PCR assay. The results showed that the amplification curves of ZIKV RNA reached plateau within 20 min, while the amplification curves of dengue viruses reached the same level after 45 min and no amplification curve was observed for other viruses (Figure 3). The appearance of amplification for dengue viruses after 45 min might be due to their sequence homology at the nucleotide level because both ZIKV and dengue virus belong to *flaviviridae* [22].

The reproducibility of the RT-LAMP assay was assessed using 5×10^4 copies of ZIKV RNA standard per reaction with the SYTO 9-based real time assay. Intra-assay reproducibility was evaluated by comparing the Threshold cycle (Ct, min) values obtained from three replicates of 5×10^4 copies ZIKV RNA standard per reaction in the same experiment, and inter-assay reproducibility was assessed by comparing the Ct values obtained from four individual experiments performed at four separate days. The results showed that the coefficient of variation (CV) of intra-reproducibility of the RT-LAMP ranged from 7.91% to 13.41% and the CV of inter-reproducibility was 14.28% (Table S1). The CVs of both intra-and inter-reproducibility were within an acceptable range,

indicating that the RT-LAMP assay for ZIKV detection has a relatively high reproducibility.

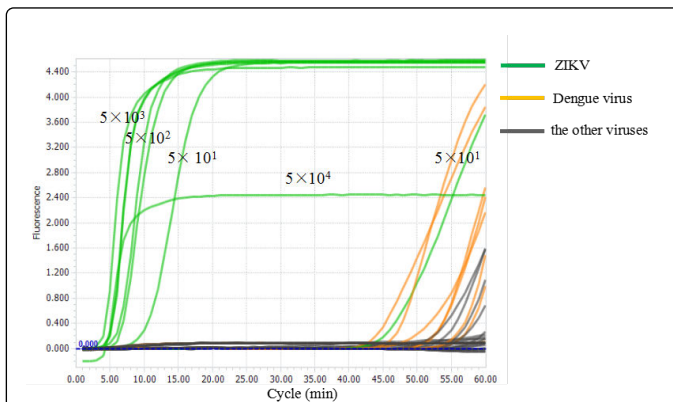


Figure 3: The specificity of the Zika RT-LAMP assay. The specificity of the assay was determined using a panel of 14 virus strains, including dengue virus serotypes 1-4, RSV A and B, Influenza A and B, PIV 3, OC43, 229E, EV68, rhinovirus and adenovirus.

To further evaluate the performance of RT-LAMP assay for ZIKV, we compared it with a RT-quantitative PCR (RT-qPCR) using three urine samples under the patients' consent (obtained from a father and his two daughters who have returned home from Zika endemic foreign countries) and twenty culture supernatants from ZIKV infected C6/36 cells (provided by Prof. Dimitri Lavillette at Institut Pasteur of Shanghai, Chinese Academy of Science). RNA was extracted from 140 μ l of urine or supernatant using the QIAamp Viral RNA Mini Kit (Qiagen, Venlo, Netherlands). Among 20 supernatants, ten were detected as ZIKV positive with viral copy numbers of 1,735-511,200 by the RT-qPCR. All these positive samples were also detected as positive by the RT-LAMP assay. All negative samples by RT-qPCR were negative by the RT-LAMP assay regardless of using SYTO-9 as real-time monitoring, or HNB and calcein for colorimetric changes (Supplementary Table S2 and Figure S1). For the three urine samples, one was detected as weak positive by both RT-qPCR (17 copies/reaction) and RT-LAMP (Supplementary Figure S2). These results demonstrated complete agreement between the RT-qPCR and RT-LAMP assay regardless of culture supernatants and urine samples were used. Despite only a limited number of clinical samples were used, these results support further development of the RT-LAMP assay as a practical tool for conveniently detect ZIKV infection.

In summary, we developed a new RT-LAMP assay for fast and accurate detection of ZIKV. The reaction can be completed within 20 min with a high specificity. Validation using twenty cell culture supernatants and three urine samples confirmed a similar performance between the new RT-LAMP and the RT-qPCR assays. These results suggest that the new RT-LAMP could be developed into a simple and rapid diagnostic kit suitable for ZIKV detection. Such a kit will be especially useful in resource-limited regions.

Conflict of Interests

The authors have declared no conflict of interests

Acknowledgements

We thank Prof. Dimitri Lavillette and Dr. Ke Xu at Institut Pasteur of Shanghai, Chinese Academy of Science for providing the culture supernatants of ZIKV. This work was supported by grants from the China National Mega-projects for Infectious Diseases (2017ZX10101001-005-001) to CZ and National Key Research Program Project Grant (2016YFC1201000) to XJ.

References

1. Kuno G, Chang GJ, Tsuchiya KR, Karabatsos N, Cropp CB (1998) Phylogeny of the genus *Flavivirus*. *J Virol* 72: 73-83.
2. Dick GW, Kitchen SF, Haddock AJ (1952) Zika virus. I. Isolations and serological specificity. *Trans R Soc Trop Med Hyg* 46: 509-520.
3. Simpson DI (1964) Zika Virus Infection in Man. *Trans R Soc Trop Med Hyg* 58: 335-338.
4. Haddock AJ, Williams MC, Woodall JP, Simpson DI, Goma LK (1964) Twelve Isolations of Zika Virus from *Aedes* (*Stegomyia*) *Africanus* (*Theobald*) Taken in and above a Uganda Forest. *Bull World Health Organ* 31: 57-69.
5. Duffy MR, Chen TH, Hancock WT, Powers AM, Kool JL, et al. (2009) Zika virus outbreak on Yap Island, Federated States of Micronesia. *N Engl J Med* 360: 2536-2543.
6. Mlakar J, Korva M, Tul N, Popovic M, Poljsak-Prijatelj M, et al. (2016) Zika Virus Associated with Microcephaly. *N Engl J Med* 374: 951-958.
7. Li XF, Han JF, Shi PY, Qin CF (2016) Zika virus: a new threat from mosquitoes. *Sci China Life Sci* 59: 440-442.
8. Ventura CV, Maia M, Bravo-Filho V, Gois AL, Belfort R Jr (2016) Zika virus in Brazil and macular atrophy in a child with microcephaly. *Lancet* 387: 228.
9. Ma W, Li S, Ma S, Jia L, Zhang F, et al. (2016) Zika Virus Causes Testis Damage and Leads to Male Infertility in Mice. *Cell* 167: 1511-1524.
10. Govero J, Esakky P, Scheaffer SM, Fernandez E, Drury A, et al. (2016) Zika virus infection damages the testes in mice. *Nature* 540: 438-442.
11. Yan L, Zhou J, Zheng Y, Gamson AS, Roembke BT, et al. (2014) Isothermal amplified detection of DNA and RNA. *Mol Biosyst* 10: 970-1003.
12. Sun Y, Zhao LQ, Zhao M, Zhu RA, Deng J, et al. (2014) Four DNA extraction methods used in loop-mediated isothermal amplification for rapid adenovirus detection. *J Virol Methods* 204: 49-52.
13. Mu YL, Zeng JW, Chen QQ, Liu J, Wang LL, et al. (2014) New method for the visual detection of human respiratory syncytial virus using reverse transcription loop-mediated amplification. *J Virol Methods* 206: 84-88.
14. Curtis KA, Rudolph DL, Owen SM (2008) Rapid detection of HIV-1 by reverse-transcription, loop-mediated isothermal amplification (RT-LAMP). *J Virol Methods* 151: 264-270.
15. Nie K, Zhang Y, Luo L, Yang MJ, Hu XM, et al. (2011) Visual detection of human enterovirus 71 subgenotype C4 and Coxsackievirus A16 by reverse transcription loop-mediated isothermal amplification with the hydroxynaphthol blue dye. *J Virol Methods* 175: 283-286.
16. Parida M, Posadas G, Inoue S, Hasebe F, Morita K (2004) Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile virus. *J Clin Microbiol* 42: 257-263.
17. Tomita N, Mori Y, Kanda H, Notomi T (2008) Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat Protoc* 3: 877-882.
18. Fischbach J, Xander NC, Frohme M, Glokler JF (2015) Shining a light on LAMP assays-A comparison of LAMP visualization methods including the novel use of berberine. *Biotechniques* 58: 189-194.
19. Tanner NA, Zhang YH, Evans TC (2015) Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes. *Biotechniques* 58: 59-68.
20. Anderson DJ (1989) Determination of the Lower Limit of Detection. *Clin Chem* 35: 2152-2153.

21. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, et al. (2008) Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. *Emerg Infect Dis* 14: 1232-1239.
22. Wang DG, Brewster JD, Paul M, Tomasula PM (2015) Two Methods for Increased Specificity and Sensitivity in Loop-Mediated Isothermal Amplification. *Molecules* 20: 6048-6059.