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# Accepted Manuscript

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**AN INNOVATIVE MULTIPLEXED AND FLEXIBLE MOLECULAR APPROACH FOR THE DIFFERENTIAL DETECTION OF ARBOVIRUSES**

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Short running head: Multiplex identification of arboviruses

## Abstract

Nucleic acid testing during the preseroconversion viremic phase is required to differentially diagnose arboviral infections. The continuing emergence of arboviruses such as Zika (ZIKV), dengue (DENV), and chikungunya (CHIKV) viruses necessitates the development of a flexible diagnostic approach. Similar clinical signs and the priority to protect pregnant women from ZIKV infection indicate that the differential diagnosis of arboviruses is essential for effective patient management, clinical care, and epidemiological surveillance. Here, we describe an innovative diagnostic approach combining generic reverse transcription-PCR amplification and identification by hybridization to specific probes. Original tetrathiolated probes were designed for the robust, sensitive, and specific detection of amplified arboviral genomes. The limit of detection using cultured and quantified stocks of whole viruses was 1 TCID<sub>50</sub>/mL for DENV-1, DENV-3, and CHIKV and 10 TCID<sub>50</sub>/mL for DENV-2, DENV-4, and ZIKV. The assay showed 100% specificity with no false positives. The approach was evaluated using 179 human samples previously tested as positive for the presence of ZIKV, DENV, or CHIKV genomes. Accordingly, the diagnostic sensitivity for ZIKV, DENV, and CHIKV was 87.88% (58/66), 96.67% (58/60), and 94.34% (50/53), respectively. This methodology could be easily adapted to include additional molecular targets. Moreover, this approach may also be adapted to develop highly specific, sensitive, and easy to handle platforms dedicated to the multiplex screening and identification of emerging viruses.

## Introduction

Zika (ZIKV), Dengue (DENV), and Chikungunya (CHIKV) arboviruses are mainly transmitted to humans via bites from several species of *Aedes* mosquitoes, including *Aedes aegypti* and *Aedes albopictus*.<sup>1-3</sup>

ZIKV is phylogenetically related to other flaviviruses of the *Flaviviridae* family such as DENV, West Nile virus (WNV), Yellow Fever virus (YFV), and Usutu virus (USUV).<sup>1, 4</sup> Whereas, CHIKV belongs to the alphavirus genus of the *Togaviridae* family.<sup>5</sup> Due to the similarity in clinical symptoms and the co-circulation of ZIKV, DENV, and CHIKV in endemic areas, the differential diagnosis of these infections represents a key challenge.<sup>1</sup>

Infections by ZIKV, DENV, and CHIKV are diagnosed by virus isolation, molecular amplification of viral genomes or by immunoassays to detect viral antigens or virus-specific IgM or IgG antibodies.<sup>1, 6-9</sup> The molecular detection of arboviruses in serum or plasma provides a definitive diagnosis, but this is often only possible within 10 days post infection due to the short viremia period.<sup>4</sup> The detection of virus-specific IgM or IgG antibodies can be challenging, in particular following ZIKV and DENV infections, as cross reactivity can occur due to the high degree of structural and sequence homology between ZIKV and other flaviviruses.<sup>2, 10, 11</sup> In addition, the antibody response to a previous infecting flavivirus can be more vigorous than the response to the current infection.<sup>2</sup> Therefore, the presence of ZIKV or DENV IgM antibodies provides evidence of a recent flavivirus infection, but additional confirmatory testing using a plaque-reduction neutralization test (PRNT) is required to differentiate between ZIKV and DENV.<sup>12, 13</sup> However, PRNTs are performed by only a small number of laboratories worldwide, are labor-intensive, and cross-reactivity has been observed in patients with a secondary flavivirus infection.<sup>1</sup> Finally, a validated test that can reliably differentiate previous flavivirus infection from current ZIKV infection is clearly required to achieve early diagnosis.<sup>1, 12, 14, 15</sup>

We developed an innovative and flexible multiplex molecular approach for the differential diagnosis of ZIKV, DENV, and CHIKV based on proven technologies combining pan-flavivirus and CHIKV classical reverse transcription-PCR amplifications, avoiding real-time PCR instruments, and identification of

amplicons after hybridization to specific molecular probes. We previously developed original tetrathiolated probes, based on a patented chemistry, for hepatitis C (HCV) genotyping which were compatible with optical or electrochemical detection methods.<sup>16</sup> In the current study, tetrathiolated probes were designed to specifically hybridize to ZIKV, DENV, and CHIKV genomes. The diagnostic specificity of the assay was evaluated using plasma samples from blood donors that tested negative for any infectious agents. Moreover, reference viral reagents were used to determine the analytical sensitivity of this assay. Finally, 179 biological samples were tested from DENV-, ZIKV-, or CHIKV-infected patients to evaluate the ability of the multiplex approach to discriminate between these arboviruses in clinical samples.

## Materials and Methods

### Viruses

ZIKV Asian lineage (French Polynesia 2013 strain), DENV (DENV-1: Djibouti 2000 strain 1588; DENV-2: Martinique 1998 strain 703; DENV-3: Martinique 2001 strain 2023; DENV-4: Indonesia 1998 strain 812), and CHIKV (Reunion Island 2005 strain 6368) viruses were provided as reference material by the National Surveillance Center of Arboviruses in Marseille, France. They were supplied as frozen vials consisting of 10-fold serial dilutions of supernatants from infected cell cultures ranging from  $10^6$  TCID<sub>50</sub>/mL to  $10^{-3}$  TCID<sub>50</sub>/mL. The tissue culture infectious dose 50 (TCID<sub>50</sub>) is the quantity of virus in a specified suspension volume that will infect 50% of inoculated culture cells.

### Biological samples

Plasma samples from 42 blood donors with no history of contact with arboviruses and that tested negative for human immunodeficiency virus (HIV), HCV, and hepatitis B virus (HBV) by the Etablissement Français du Sang (EFS) in Montpellier (France) were used as negative controls.

A total of 179 biological samples (serum/plasma/urine) were used to evaluate the diagnostic potential of the multiplex approach. An initial set of 30, 17, and 10 samples were analyzed from patients infected with ZIKV, DENV, and CHIKV, respectively. These patients were symptomatic imported cases with acute infections that were diagnosed at the University Hospital, Montpellier, using commercial molecular assays (RealStar DENV, CHIKV and ZIKV; Altona, Hamburg, Germany). A second panel of 20 ZIKV and eight CHIKV clinical samples from asymptomatic blood donors were collected in Martinique (local cases) and tested at EFS Marseille using a commercial assay (RealStar ZIKV; Altona). These samples were coded for blind testing before shipment. A set of samples from 16 and 30 patients infected with ZIKV and CHIKV, respectively (local cases), were collected and tested at the Institut Pasteur de Nouvelle-Calédonie using an in-house real-time PCR method before shipment. The National Surveillance Center of Arboviruses of Marseille (France) provided a set of 43 and five plasma samples from patients infected with DENV and CHIKV, respectively. These DENV plasma samples were previously tested using an in-house real-time PCR method and typed using a sequencing method (Supplemental Tables S1, S2, and S3).

### Viral nucleic acid extraction

Viral nucleic acid extraction was performed using the MagNA Pure Compact automated system with the MagNA Pure Compact Nucleic Acid Isolation Kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The input volume of supernatants from infected cell cultures or biological samples was 200  $\mu$ L and the elution volume was 50  $\mu$ L. The purified viral nucleic acids were aliquoted and stored at -80 °C until their use.

### Primer and probe design

For pan-flavivirus amplification the MAMD/cFD2 primer pair was used, targeting the flavivirus *NS5* gene.<sup>17</sup> CHIKV primers were designed to target the *NSP1* gene and were adapted from a previous

publication.<sup>18</sup> The forward primers were tagged with biotin to generate biotinylated amplicons (Table 1).

Nucleotide sequences of each ZIKV, DENV genotype, and CHIKV were retrieved from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). Nucleotide sequences of the *NS5* gene from 16 strains of ZIKV and 53 strains of DENV (seven strains DENV- 1, 13 strains DENV- 2, 18 strains DENV- 3, 15 strains DENV- 4) were aligned (Table 2). Nucleotide sequences of the *NSP1* gene from 48 strains of CHIKV were also aligned (Table 2). All sequences were aligned using Clustal W version 2 sequence alignment software.<sup>19</sup> Phylogenetic analyses were performed using Mega Vs. 6.0 software.<sup>20</sup> After identification of the most conserved regions, polythiolated 15 mer-probes were designed to specifically detect amplicons after hybridization. Polythiolated probes were synthesized for ZIKV, all DENV serotypes (DENV Gen), and CHIKV (Table 1). They were synthesized according to a patented method (Patent WO 2013150122) on a 1  $\mu$ mol-scale using a DNA synthesizer as described previously.<sup>16</sup> Basically, starting from commercially available solid supports the oligonucleotides were elongated by the phosphoramidite method on a DNA synthesizer using a standard cycle with a 30 seconds coupling time. Then the thiol phosphoramidite was coupled four times at the 5'end of the sequence using a 60 seconds coupling time. For the deprotection a first treatment with 10% of dry piperidine acetonitrile was applied for 15 minutes and then with concentrated ammonia (32%) at 55 °C for 5 hours. After evaporation the crude tetrathiolated oligonucleotides were used without purification.

### One-Step reverse transcription-PCR amplification

Reverse transcription-PCR was performed using 5  $\mu$ L of extracted viral RNAs mixed with 3  $\mu$ L of Forward primer (10  $\mu$ M) and 0.3  $\mu$ L of Reverse primer (10  $\mu$ M) in a final volume of 50  $\mu$ L using the One-Step RT-PCR kit (Qiagen, Valencia, CA). The reverse transcription-PCR conditions consisted of a 30 minutes reverse transcription step at 50 °C and a 15 minutes Taq polymerase activation step at 95 °C, followed by an initial denaturation at 95 °C for 5 minutes, then 40 cycles of 95 °C for 40 seconds (denaturation), 56 °C for 40 seconds (annealing), and 72 °C for one minute (extension), followed by a



final extension step of 72°C for 10 minutes. The PCR procedures were performed using a Mastercycler Gradient (Eppendorf, Hamburg, Germany). The total run time for this protocol was 2 hours and 45 minutes. The PCR products were diluted 1:10 in hybridization buffer (6X SSPE, 5X Denhardt) for direct hybridization to tetrathiolated probes or stored at 4 °C until their use.

## Molecular detection on microplates

Tetrathiolated probes were grafted on to maleimide-activated 96-well microplates at a concentration of 0.04 nmol/well (Thermo Fischer Scientific, Waltham, MA). After a blocking step with 10 µg/mL cysteine-HCl (Thermo Fischer Scientific), the microplates can be used immediately or stored for up to 6 months at -20 °C. In each assay biotinylated amplicons were tested in duplicate and were incubated in the microplate for 2 hours at 37 °C. After washing steps (5X SSPE, 0.1% SDS preheated at 50 °C) and incubation at room-temperature for 30 minutes with 100 ng/mL of DELFIA europium-labeled streptavidin (Perkin-Elmer, Waltham, MA), molecular hybridization events were detected by time-resolved fluorescence using a microplate reader (Victor Instrument, Perkin-Elmer). Synthetic 15-mer DNA oligonucleotides biotinylated at their 5'-end (Eurogentec, Angers, France) and which were strictly complementary to each probe were used at 1000 pM and 10 pM concentrations as positive controls. Plasma samples from blood donors that tested negative for virological markers were used as negative controls. The cut-off value was established by determining the mean OD<sub>610</sub> of amplified product from DNA extracted from the negative control plasma samples tested in duplicate. A positive result was determined as a mean OD<sub>610</sub> value above the mean OD<sub>610</sub> of the negative control samples (the cut-off value) plus three times the standard deviation.

## Statistical analysis

The data in Tables 3 and 4 represent the results obtained for each biological sample tested in duplicate. The 95% confidence intervals (CI) for a proportion were calculated according to the

method described by Robert Newcombe, derived from a procedure outlined by E. B. Wilson in 1927.<sup>21, 22</sup>

## Results

Nucleic acids were extracted from infected cell cultures or biological samples and pan-flavivirus and CHIKV reverse transcription-PCR amplifications were performed in parallel for each sample. Amplification using the pan-flavivirus primers produced a 263-bp fragment of the *NS5* region, whereas the CHIKV primers were designed to amplify a 100-bp fragment of the *NSP1* gene (Table 1). The biotinylated amplicons were then hybridized to tetrathiolated probes grafted on to microplates. The fluorescence was recorded after addition of a streptavidin-europium conjugate, using a simple microplate reader (Figure 1).

### Tetrathiolated probe design

After alignment of 69 viral strains belonging to the four DENV types and the ZIKV species, well-conserved regions were identified which allowed a generic DENV probe (all types, DENV- Gen) and a specific ZIKV probe to be designed. Alignment of 48 CHIKV strains allowed a specific CHIKV probe to be designed (Tables 1, 2).

### Analytical sensitivity

The limit of detection (LoD) of our assay was determined using reference panels consisting of serial dilutions of supernatants from infected cell cultures. Ten replicates of each dilution of ZIKV, all four serotypes of DENV and CHIKV were amplified and then tested for hybridization to ZIKV, DENV-Gen, and CHIKV probes. The LoD was defined as the amount of virus that gives a 95% detection rate. Sensitivities of 1 TCID<sub>50</sub>/mL were obtained for DENV serotypes 1 and 3 and for CHIKV, and sensitivities of 10 TCID<sub>50</sub>/mL were obtained for ZIKV and DENV serotypes 2 and 4 (Table 3).

## Specificity of the assay

To evaluate the diagnostic specificity of the assay, 42 deidentified plasma samples from blood donors that tested negative for virological markers were separately extracted, then amplified by reverse transcription-PCR using pan-flavivirus and CHIKV primers before hybridization to ZIKV (Supplemental Figure S1), DENV-Gen (Supplemental Figure S2), and CHIKV tetrathiolated probes (Supplemental Figure S3). The molecular assay showed 100% diagnostic specificity, as none of the 42 samples analyzed produced positive results (Table 4). The analytical specificity of the three probes (ZIKV, DENV-Gen, and CHIKV) was tested in duplicate on  $10^6$  TCID<sub>50</sub>/mL dilutions of the reference panels. No false positive signals were observed between amplicons and non-specific probes (Supplemental Figure S4).

## Molecular detection in clinical samples

The ability of the multiplex molecular approach to discriminate arboviruses in deidentified samples was evaluated using plasma samples from patients with acute infections previously confirmed by real-time PCR methods (Supplemental Tables S1, S2, and S3). The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold (ie, exceeds background level). The highest Ct values indicate very low viral loads. A total of 66, 60, and 53 local and imported cases of ZIKV, DENV, and CHIKV were tested, respectively. After pan-flavivirus and CHIKV reverse transcription-PCR amplification, each amplicon was tested in duplicate for hybridization to ZIKV, DENV-Gen, and CHIKV probes. Our assay detected 58 of the 66 ZIKV cases (87.88% diagnostic sensitivity; 95% CI, 77.86% to 93.73%), 58 of the 60 DENV cases (96.67% diagnostic sensitivity; 95% CI, 88.64% to 99.08%) and 50 of the 53 CHIKV cases (94.34% diagnostic sensitivity; 95% CI, 84.63% to 98.06%). The discrepant results observed between this approach and the reference real-time reverse transcription-PCR methods correspond essentially to samples with

low viral loads (Supplemental Tables S1, S2, and S3). Due to insufficient sample volume, it was not possible to retest the discrepant samples. The ability of this test to correctly differentiate infected and uninfected samples is indicated by accuracy values of 92.59%, 98.07%, and 96.93% for ZIKV, DENV-Gen, and CHIKV probes, respectively (Table 4).

## Discussion

The ZIKV epidemic continues to impact the global community and represents a long-term public health challenge.<sup>1, 2, 13</sup> More worryingly, severe neurological complications have been observed, in particular cases of microcephaly and brain-related disorders in newborns resulting from ZIKV infection in pregnant women and cases of Guillain-Barré syndrome in infected adults.<sup>23-25</sup> In addition, confirmation of the transmission of ZIKV through sexual contact and blood transfusion enhances the need for specific screening.<sup>26, 27</sup> Arboviruses have overlapping geographical distributions and can cause symptoms also associated with more common infections. Physicians attending to travelers with particular symptoms often neglect those infections that are transmitted by arthropods or test for inappropriate arboviruses.<sup>1, 28</sup> The development of multiplex molecular tests to discriminate ZIKV infection from other arboviral diseases, including dengue fever, is essential not only for the management of pregnant women and patients, but also for the epidemiological surveillance of these infections and the epidemic alert.<sup>2, 12, 13</sup> Scientists estimate that more than 2.5 billion individuals live in areas of Africa and the Asia-Pacific region where the presence of competent mosquito vectors and suitable climatic conditions may support local transmission of ZIKV.<sup>29, 30</sup> The long-term outlook with regard to the current arboviral outbreaks linked to ZIKV, DENV, or CHIKV is uncertain, but what has already been clearly identified is the need for more flexible and multiplex approaches for diagnosis and epidemiological surveillance.<sup>25, 28, 31</sup>

In this context, the development of innovative diagnostic platforms that can simultaneously test for multiple pathogens during the acute phase of infection is a priority, not only for the differential

diagnosis of arboviruses but also to face the ongoing emergence of pathogens. Real-time reverse transcription-PCR assays for the multiplex detection of arboviruses have been published.<sup>32-34</sup> However, the ability to analyze multiple viral targets using real-time PCR assays is limited by the number of optical channels in the real-time PCR platform. Hence, an original approach combining an OpenArray nanofluidic real-time PCR platform for the spatial multiplexing of assays and Taqman chemistry for target identification was published by Grigorenko et al for the simultaneous detection of multiple pathogens.<sup>35, 36</sup> Even if improvements to sensitivity and analytical performance for plasma or whole blood samples spiked with cultured pathogens are ongoing, this approach represents a proof of concept for the multiplexing of blood donor screening and clinical diagnostics.<sup>35</sup> A novel assay combining SYBR Green–based reverse transcription-PCR with low density DNA microarray has also recently been published.<sup>37</sup> Finally, multiplex tests for the molecular diagnosis of blood-borne pathogens are limited in number, and experts agree that direct determination of the sequence of the infecting pathogen should be the future universal method.<sup>38</sup> Alternatively, affordable and portable devices for simple sample-to-answer molecular diagnostics represent a suitable strategy for resource-limited settings.

In this study, we developed a novel assay combining reverse transcription-PCR amplifications and identification of amplicons after hybridization to original tetrathiolated probes in a microplate format. The analytical sensitivity of the multiplex assay was evaluated by testing 10 replicates of each dilution of cultured ZIKV, DENV, and CHIKV. The results demonstrate that this approach can detect viral titers as low as 1 or 10 TCID<sub>50</sub>/mL. A specificity of 100% was achieved with no false positive signals detected for negative plasma samples from 42 blood donors. WThis multiplex approach is able to detect ZIKV, DENV, and CHIKV in 179 biological samples with a diagnostic sensitivity of 87.88%, 96.67%, and 94.34%, respectively. All the samples had been previously tested using real-time PCR methods before shipment to our laboratory, and the samples not detected by this approach correspond essentially to samples with low viral loads. Additional freeze-thaw cycles performed after reception of the shipped samples may also have altered the quality of the clinical

samples. In the future, any clinical samples with discrepant results will be retested and possibly sequenced. Finally, this assay provides pathogen-specific molecular information that may be used for more detailed discrimination of detected viral strains. For instance, in areas where DENV and ZIKV co-circulate, the risk of severe disease is highest within a narrow range of preexisting anti-DENV antibody titers, and an accurate molecular diagnosis of DENV is important for effective case management.<sup>10, 11</sup> This methodology can be easily adapted to include additional molecular targets so to improve the surveillance and prevention of arboviral infections. These results represent a proof of concept that validates the use of original tetrathiolated probes to detect amplified nucleic acids from clinical samples with a high level of specificity and sensitivity. Compared to classical microarray developments previously published<sup>37, 38</sup>, this process avoids the use of sophisticated real-time PCR instruments, does not require amplified genomes to be purified before their hybridization on to probes, and only requires a simple microplate reader for direct detection. The optical detection method was developed in this microplate-based assay is simple and provides an alternative to identification of viral genome by next-generation sequencing (NGS) which requires complex bioinformatics analysis and high-level expertise.

Finally, the accurate and rapid differential diagnosis of ZIKV, DENV, and CHIKV infections is crucial for surveillance, clinical management of patients or returned travelers presenting with an acute febrile illness and diagnosis of ZIKV in pregnant women. The possibility that tetrathiolated probes could be easily grafted on to surfaces other than microplates, such as electrodes or magnetic microparticles, opens the way to develop new multiplexed and cost-effective diagnostics that may be adapted either to high-throughput platforms or to portable point-of-care systems.

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## Figure legend

**Figure 1** Microplate-based molecular approach. The molecular approach consists of three main steps: grafting of tetrathiolated probes on to maleimide-activated microplates (1), hybridization of amplified and biotinylated viral genomes to complementary probes (2), and optical detection by time resolved fluorescence (3).

**Table 1. Primers and probes for amplification and detection.**

Name	Function	Sequence	Target gene	Amplicon size (bp)	Specificity
<b>Target amplification (reverse transcription-PCR)</b>					
<b>Pan-Flavivirus</b> (pan-Flavi)	Forward primer (MAMD)	5'-Biot - AACATGATGGGAAARAGRGARAA-3'	NS5	263	Zika (ZIKV) Asia, Africa
	Reverse primer (cFD2)	5'-GTGTCCCAGCCGGGTGTCATCAGC-3'			Dengue (DENV) all serotypes (1, 2, 3, 4)
<b>Chikungunya</b> (CHIKV)	Forward primer	5'-Biot - ACCATGCYAATGCTAGAGCGTTCTCGCA-3'	NSP1	100	CHIKV
	Reverse primer	5'-TGGCGCACTRCCTATRTCCAGGAT-3'			
<b>Virus identification</b>					
ZIKV	Tetrathiolated probe	5'-CAAATCCCCTTGYT-3'	ZIKV asia		
DENV-Gen	Tetrathiolated probe	5'-TCCTTCYACTCCRCT-3'	Dengue (DENV) all serotypes (1, 2, 3, 4)		
CHIKV	Tetrathiolated probe	5'-ATTCCTGCTCTATT-3'	CHIKV		

ZIKV, zika virus; DENV, dengue virus; CHIKV, chikungunya virus; Biot, biotin; Y, C or T; R, A or G.

**Table 2. Viral nucleotide sequences used for alignment and probe design.**

<b>Virus</b>	<b>Nb of sequences</b>	<b>Gene</b>	<b>Genbank accession number*</b>
<b>ZIKV</b>	16	NS5	KF268949, KF268948, KF268950, HQ234500, KF383116, LC002520, KF383119 KF383115, KF383118, KF383117, KF993678, HQ234499, JN860885, KF258813 EU545988, KJ77679
<b>DENV-1</b>	7	NS5	FJ639683, EU482480, AY726549, FJ882517, GU131762, FJ882552, GU131982
<b>DENV-2</b>	13	NS5	AF169683, AF169682, HM582116, DQ181803, GU131896, EU073981, AF169687 AF169686, HM582117, EU056812, AY744150, GQ398257, HM582110
<b>DENV-3</b>	18	NS5	GU131951, AY770511, GQ199870, GQ199888, FJ024465, FJ744740, GU189648 EU660409, FJ744730, AY676351, DQ109310, AY676350, AY744677, HM631854 AB214880, EF629370, DQ109400, GQ868593
<b>DENV-4</b>	15	NS5	GQ199882, EF457906, GQ868582, GQ868584, AF326573, AY762085, AY618989 AY618993, EU854301, AY618990, AY858049, AY618992, FJ882582, GQ398256 AF289029
<b>CHIKV</b>	48	NSP1	HM045786, HM045785, HM045816, HM045815, AY726732, HM045819, HM045804 HM045820, HM045818, HM045817, EF012359, FJ807899, FJ445427, FJ445432 GU199353, FJ000068, GU199351, JQ067624, HM045793, HM045823, HM045784 HM045822, AF369024, HM045811, EF027139, HM045809, HM045806, HM045792 HM045795, HM045810, HM045788, HM045813, EF027140, HM045787, HM045791 EU703759, EU703760, EU703761, EU703762, FJ807897, HM045789, HM045790 HM045796, HM045797, HM045802, HM045808, HM045814, L37661

\*<https://www.ncbi.nlm.nih.gov/genbank/>

**Table 3. Analytical sensitivity.**

	TCID <sub>50</sub> /mL	Replicates n	Positive	Rate
<b>ZIKV</b>	0.1	10	1	10%
	1	10	3	30%
	<b>10</b>	<b>10</b>	<b>10</b>	<b>100%</b>
<b>DENV-1</b>	0.1	10	1	10%
	<b>1</b>	<b>10</b>	<b>10</b>	<b>100%</b>
	10	10	10	100%
<b>DENV-2</b>	0.1	10	8	80%
	1	10	9	90%
	<b>10</b>	<b>10</b>	<b>10</b>	<b>100%</b>
<b>DENV-3</b>	0.1	10	4	40%
	<b>1</b>	<b>10</b>	<b>10</b>	<b>100%</b>
	10	10	10	100%
<b>DENV-4</b>	0.1	10	1	10%
	1	10	9	90%
	<b>10</b>	<b>10</b>	<b>10</b>	<b>100%</b>
<b>CHIKV</b>	0.01	10	6	60%
	0.1	10	9	90%
	<b>1</b>	<b>10</b>	<b>10</b>	<b>100%</b>

The limit of detection (LoD), indicated in bold, was defined as the amount of virus that gives a minimal 95% detection rate.

ZIKV, zika virus; DENV, dengue virus; CHIKV, chikungunya virus; TCID<sub>50</sub>/mL, Tissue Culture Infectious Dose 50.

**Table 4. Molecular detection in clinical samples.**

Sample Type (All origins)	No. of samples	No. of samples correctly detected	Diagnostic sensitivity % (95% CI)*	Diagnostic specificity % (95% CI)†	Accuracy % ‡
ZIKV	66	58	87.88 (77.86-93.73)		92.59
DENV	60	58	96.67 (88.64-99.08)		98.07
CHIKV	53	50	94.34 (84.63-98.06)		96.93
Healthy (Blood donors)	42	0		100 (91.62-100)	

Detailed results can be found in Supplemental Tables S1 to S3.

\*[number of positive samples/ (number of positive samples + number of false-negative samples)] x 100.

† [number of negative samples/ (number of negative samples + number of false-positive samples)] x 100.

‡ [(number of negative samples + number of positive samples)/ (number of negative samples + number of positive samples + number of false-negative samples + number of false-positive samples)] x 100.

ZIKV, zika virus; DENV, dengue virus; CHIKV, chikungunya virus; CI, confidence interval.

**Figure 1** Microplate-based molecular approach