Zika Virus Infection Diagnostics Landscape

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http://www.idc-dx.org/resources/zika-virus-diagnostics-landscape
**Background and History**

Because of a recent and ongoing epidemic, Zika virus (ZIKV) infection is of worldwide concern. ZIKV infection has generally been considered an unimportant public health problem with only sporadic cases in humans reported in the literature until 2006 (1). However, with an epidemic in Brazil in 2015-2016, which is estimated at between 440,000 and 1.3 million cases, some of which have been associated with fetal microcephaly and other neurological disorders, ZIKV infection is now considered by the World Health Organization (WHO) to be a Public Health Emergency of International Concern (2,3). As of early October 2016, active ZIKV transmission was taking place in 50 countries and territories in the Americas, 8 countries and territories in the Oceania/Pacific Islands, 1 country in Africa and 1 country in Asia.¹

ZIKV was first identified in 1947 in a sentinel rhesus monkey that was part of a jungle Yellow Fever research program in the Zika Forest of Uganda (4). However, no incidence of disease in humans was reported at that time, and it wasn’t until the mid-1960’s that a scientist identified and detailed his own infection with ZIKV (5) (Tilak). ZIKV was subsequently isolated from humans for the first time in 1969 in Nigeria during population studies (6).

Subsequently, there were some reports of ZIKV infection in humans, but they were rare. Then, in 2007, an outbreak of ZIKV infection occurred on Yap Island in Micronesia, with a reported 185 probable cases, marking its first occurrence outside of Africa and Asia (6) (Chang). Despite this outbreak, as late as 2008 in a review of ZIKV, Lanciotti et al stated that ZIKV was “rarely associated with human disease” (7).

Following the outbreak of ZIKV in Micronesia, outbreaks also occurred in 2013 – 2014 in New Caledonia, French Polynesia, the Cook Islands, Easter Island, Vanuatu, and Samoa, with approximately 120,000 people infected by 2014 (8,9,10). Although ZIKV generally caused only mild disease in humans, the outbreak in French Polynesia revealed the possible neurological complications of the virus (i.e., Guillain-Barré syndrome and meningoencephalitis) (11). In March 2015, Brazil confirmed the presence of ZIKV in the country based on samples from an outbreak in Bahia (5), and with the epidemic there, a rise in the cases of infants born with microcephaly (8). Since then, ZIKV transmission has been found throughout Central and South America, with 31 of these countries reporting autochthonous transmission (8,11).

A timeline and potential pathways of major global ZIKV outbreaks are illustrated below (6).

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¹ This data is updated regularly and can be found at: [http://www.cdc.gov/zika/geo/active-countries.html](http://www.cdc.gov/zika/geo/active-countries.html)
In addition, several cases of ZIKV infection have been reported as a result of returning travelers to countries with ongoing transmission of ZIKV. These include a traveler to Guatemala and El Salvador who returned to Switzerland (12) (Ginier), a traveler to Thailand who returned to Japan (13), and a traveler to Thailand who returned to Canada (14). These cases have drawn attention to the possible importation and autochthonous transmission of ZIKV to non-tropical areas via international travel. Concerns about the spread of ZIKV are on the rise.

**ZIKV Transmission**

Like the dengue virus (DENV) and chikungunya virus (CHIKV), ZIKV is an arbovirus (an arthropod borne virus) that is transmitted to humans by mosquitoes, primarily the *Aedes aegypti* mosquito, but also by the *Aedes albopictus*, or Asian tiger mosquito (15,16). *A. Aegypti* mosquitoes live in tropical, subtropical, and some temperate climates. The *A. albopictus* mosquito, also called the Asian tiger mosquito, originated in Southeast Asia, and while found primarily in tropical and subtropical climates, it can live in cooler climates than the *A. Aegypti* mosquito (16).

Further, additional mosquitoes have been found to be susceptible to ZIKV. For example, a study in Senegal found that in addition to *A. aegypti* and *A. albopictus*, the *Aedes unilineatus* and *Aedes vittatus* mosquitoes were also susceptible to ZIKV (17). In 2011, mosquito pools were collected in Senegal and tested for ZIKV; in addition to *A. Aegypti*, *A. unilineatus* and *A. vittatus*, *Aedes fusciger*, *Aedes luteocephalus*, *Aedes dalzielli*, *Aedes hirsutus*, *Aedes taylori* and *Aedes metallicus* mosquitoes were positive for ZIKV (6,18,19). Musso et al have commented that the adaptation of ZIKV to an urban or peri-urban cycle involving these mosquitoes as vectors and humans as amplification hosts, “should be of great concern to public health officials” (20).
In addition to transmission of ZIKV to humans via mosquitoes, there are also reports of direct human-to-human transmission. These include perinatal transmission in the case of two mothers passing ZIKV onto their newborns during a ZIKV outbreak in French Polynesia (19,21), as well as the possibility of blood transfusion-related transmission (22). Further studies are needed to assess these forms of transmission of ZIKV.

There has also been reported sexual transmission of ZIKV. For example, one case involved the reported sexual transmission of ZIKV in the U.S. to the wife of a patient who had been infected in Senegal. Although ZIKV was confirmed by serologic testing in both husband and wife, the husband’s semen was not tested (19,23). Another report indicated the presence of replicative ZIKV and a high ZIKV RNA load in both the semen and urine of a patient during an outbreak in Tahiti in 2013; however, no molecular testing was done on the sera collected from the patient (24). Finally, in January 2016, after notification from a local health care provider, an investigation by Dallas County Health and Human Services identified a case of sexual transmission of ZIKV between a man who had recently travelled to Venezuela, an area of active transmission, and his male partner, who had not travelled, which indicates that ZIKV can be transmitted via anal as well as vaginal sex (25).

**ZIKV Biology**

ZIKV belongs to the *Flaviviridae* family and the *Flavivirus* genus, which consists of some 39 different mosquito-borne viruses, including DENV, as well as yellow fever, West Nile and Japanese encephalitis viruses (6,19). ZIKV is a single strand, positive RNA virus with a genomic size of about 11 kilo-base pair (kb) that is closely related to the Spondweni virus (6,26). Phylogenetic analyses have revealed three lineages of ZIKV: two African lineages and one Asian lineage, although exact lineages are still in dispute (7,9).

As illustrated below, the single open reading frame sequence of ZIKV’s RNA genome encodes a polyprotein containing 3 components: a capsid (C), membrane (M), and premembrane portion (P) (6). There are also an envelope (E) protein and 7 non-structural proteins (NS1, NS2a, ND2b, NS3, NS4a, NS4b and NS5 (6, 27).
Clinical Manifestations of ZIKV Infection

Less than 20% of ZIKV infections are symptomatic (29). When symptomatic, ZIKV infection is generally mild and self-limiting (19). Symptoms are not specific, but include: acute onset of low-grade fever, maculopapular pruritic rash, arthralgia (generally affecting the small joints of the hands and feet), and conjunctivitis; clinical illness is considered consistent with ZIKV infection if two or more of these symptoms are present (30,31,32).

There is currently no vaccine to prevent ZIKV infection.² Like DENV and CHIKV infections, there is no specific treatment for ZIKV infection, and care generally consists of management of symptoms with non-salicylate analgesics and anti-pyretics (32).

Because of its non-specific symptoms, ZIKV infection is often confused with DENV and CHIKV infections. However, there are differences in the symptoms among these arboviral infections (6). For example, fever in DENV or CHIKV infection is generally higher than in ZIKV infection, and DENV- and CHIKV-infected patients generally present with more intense pains – severe muscle pain and headache in the case of DENV infection, and intense joint pain affecting the hands, feet and back in the case of CHIKV infection (6,33). Further, CHIKV infection is not generally associated with conjunctivitis, which is one of the symptoms of both ZIKV and DENV infections (33). However, there have been no studies comparing

² In the U.S., the National Institute of Allergy and Infectious Diseases is working to develop multiple vaccine candidates. The current status of this work can be found at: https://www.niaid.nih.gov/topics/zika/researchapproach/Pages/vaccineResearch.aspx.
the clinical and laboratory findings for patients with ZIKV, DENV and CHIKV infections in a defined cohort (34).

Unlike DENV infection, there are generally no long-term sequellae associated with ZIKV infection, excluding fetal losses and any complications in babies born to infected mothers (6). However, there is a possible link between ZIKV and Guillan-Barré syndrome (GBS), an auto-immune disease, as there is with DENV (11,19,35). For example, during the ZIKV outbreak in French Polynesia, the incidence rate of GBS was about 20 times higher than expected given the size of the population and the previously-established incidence there (1-2 per 100,000 population per year) (11). There were also reported cases of GBS in an outbreak of ZIKV in northeast Brazil in 2015 (36). Table 1 below describes the symptoms, clinical management, sequelae and vectors for ZIKV fever compared to DENV and CHIKV infections.

In addition, in 2015, there was an increased number of newborns in Pernamcuo in Northeast Brazil with microcephaly and neurological disorders; it has been suggested that these disorders may be associated with a spike in reported cases of ZIKV infection in the same region at about the same time (37) (Schuler). Other congenital infections, which may be associated with ZIKV exposure, have also been found. These include bilateral macular and perimacular lesions and optic nerve abnormalities (38,39,40). At least eight other Brazilian states have reported increases in microcephaly as well (41). Nonetheless, the association of microcephaly with ZIKV is still controversial and additional research is needed to prove a causal relationship between ZIKV and serious neurological disorders, including microcephaly (19).

Evidence of a causal relationship is mounting. For example, Cugola et al have demonstrated that what they refer to as the Brazilian strain of ZIKV (ZIKV\textsuperscript{BR}) infects mouse fetuses, causing intrauterine growth restriction, including signs of microcephaly. The authors also found that ZIKV infects human cortical progenitor cells \textit{in vitro}, leading to an increase in cell death. They say that their results indicate that ZIKV crosses the placenta and causes microcephaly by targeting those cortical progenitor cells, inducing cell death by apoptosis and autophagy, and impairing neurodevelopment (42).
<table>
<thead>
<tr>
<th></th>
<th><strong>DENV</strong></th>
<th><strong>ZIKV</strong></th>
<th><strong>CHIKV</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Signs and symptoms</td>
<td>Maculopapular rash, myalgia and arthralgia, conjunctivitis</td>
<td>Maculopapular rash, myalgia and arthralgia, conjunctivitis</td>
<td>Intense/incapacitating arthralgia affecting hands, feet, knees and back, but not conjunctivitis</td>
</tr>
<tr>
<td>Incubation period</td>
<td>About 4–7 days</td>
<td>Unknown, but may be similar to DENV and CHIKV</td>
<td>About 2–4 days (43) (Pialoux)</td>
</tr>
<tr>
<td>Duration of symptoms</td>
<td>Usually 2–7 days, but may lead to DHF or DSS</td>
<td>Usually 2–7 days; self-limiting</td>
<td>Usually less than 7 days; but severe joint pain may persist for months</td>
</tr>
<tr>
<td>Clinical management</td>
<td>No specific treatment. Close assessment of hydration, bleeding status and signs of organ failure or respiratory distress</td>
<td>No specific treatment. Management of symptoms with non-salicylate analgesics and anti-pyretics. Non-steroid anti-inflammatory drugs (NSAIDs) acceptable if DENV ruled out.</td>
<td>No specific treatment. Can be severe, requiring hospitalization (44) (Weaver). Management of symptoms with non-salicylate analgesics and NSAIDS.</td>
</tr>
<tr>
<td>Long-term effects and sequelae</td>
<td>Long-term effects documented for up to 2 years following illness. Link to Guillain-Barré, encephalitis and other neuro syndromes, autoimmune disease</td>
<td>No long-term effects known. Possible link to Guillain-Barré, encephalitis, other neuro syndromes, autoimmune diseases, especially if previously infected with DENV</td>
<td>Long-term effects documented. Severe CHIKV can manifest as encephalopathy and encephalitis, myocarditis, hepatitis and multiorgan failure. Severe disease more likely in young children (44) (Weaver).</td>
</tr>
<tr>
<td>Primary vectors</td>
<td><em>A. Aegypti, A. albopictus</em></td>
<td><em>A. Aegypti, A. albopictus</em> and multiple other <em>Aedes</em> spp</td>
<td><em>A. Aegypti, A. albopictus</em></td>
</tr>
</tbody>
</table>

Table 1 Symptoms, Clinical Management, Sequelae and Vectors of DENV, ZIKV and CHIKV Infections. Adapted from Kelser (32).
Diagnosis of ZIKV

ZIKV infection is challenging to diagnose because it shares symptoms, geographic distribution and vectors with DENV and CHIKV infections. As a result, despite some differentiating symptoms among the three, clinical manifestations and relevant epidemiologic exposure alone are not reliable indicators of ZIKV infection (32). However, it is important to distinguish ZIKV infection both from CHIKV infection, and particularly from DENV infection, due to the need to monitor patients for the onset of severe illness from the latter (32,45). Furthermore, routine laboratory testing, including complete blood count and routine blood chemistries, have not shown distinct patterns for ZIKV-infected patients, and in fact, are normal in most cases (34,45). Abnormal findings may include leukopenia, thrombocytopenia and elevation of liver transaminases, but where they occur, they are typically mild (14,46-49).

Further complicating this picture is the fact that there is no “gold standard” in vitro diagnostic (IVD) for the confirmation of ZIKV infection (19). In addition, serologic assays are unreliable because antibodies among flaviviruses, like ZIKV and DENV, are often cross-reactive (6,19,32,34) making it difficult, if not impossible, to distinguish between and among them, and to date, there are no available antigen-based assays to detect ZIKV infection (50). Other than serological assays, diagnosis of acute ZIKV infection generally relies upon the detection of ZIKV RNA in a variety of specimens, including serum, plasma, urine, saliva and amniotic fluid (50).

The literature related to diagnostic tests for ZIKV Infection, including performance, is sparse. Below is a summary of the available tests for diagnosing ZIKV infection, which include virus isolation, viral nucleic acid detection and serological testing.

Virus Isolation

While it is possible to use culture-based methods to isolate ZIKV, the methods are laborious and impractical, especially in resource-limited settings. ZIKV can be cultured from intracerebral mouse inoculation, which is the reference method, or from several cell lines, including MOS61 (Aedes pseudoscutellaris) and African green monkey (Vero) (4,51). However, because of the complexity of the process, such methods are only used in research laboratories and are not available for clinical use (34).

Molecular Testing – RNA Detection

Nucleic acid amplification (NAT) methods, specifically reverse transcriptase-polymerase chain reaction (RT-PCR) tests for detection and identification of ZIKV and other flaviviruses, were established by 1994 (52). The first primers were derived from nucleotide sequences from the NS5 gene or from the 3` non-coding region, but by 2008, an RT-PCR protocol using sequences encoding the E protein region had been developed (53).

Today, RT PCR, specifically real-time RT-PCR assays (rRT-PCR), are the laboratory diagnostics of choice for detection of acute ZIKV infection. A number of these assays have been developed and are summarized in Table 2. Virtually all of these are in-house/home brew assays and are available for research use only.
<table>
<thead>
<tr>
<th>Reference or Source</th>
<th>Year</th>
<th>RT-PCR type</th>
<th>Target*</th>
<th>ZIKV lineage analytical</th>
<th>ZIKV lineage field</th>
<th>#Human patients</th>
<th>Sample types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lanciotti et al (7)</td>
<td>2008</td>
<td>One step, real time; hydrolysis probe</td>
<td>prM</td>
<td>Asian, African</td>
<td>Asian</td>
<td>&gt;200 (combined set)</td>
<td>Serum, urine, amniotic fluid</td>
</tr>
<tr>
<td>Lanciotti et al (7)</td>
<td>2008</td>
<td>One step real time; hydrolysis probe</td>
<td>E</td>
<td>Asian, African</td>
<td>Asian</td>
<td>&gt;200 (combined set)</td>
<td>Serum, urine, amniotic fluid</td>
</tr>
<tr>
<td>Faye et al (53)</td>
<td>2008</td>
<td>One step, conventional</td>
<td>E</td>
<td>African</td>
<td>Asian</td>
<td>&gt;15</td>
<td>Serum</td>
</tr>
<tr>
<td>Balm et al. (54)</td>
<td>2012</td>
<td>One step, conventional</td>
<td>NS5</td>
<td>African</td>
<td>Asian</td>
<td>88 (none tested positive for ZIKV)</td>
<td>Plasma</td>
</tr>
<tr>
<td>Faye et al (55)</td>
<td>2013</td>
<td>One step, real time, LNA probe</td>
<td>NS5</td>
<td>Asian, African</td>
<td>African</td>
<td>0 (only validated in monkeys)</td>
<td>Serum</td>
</tr>
<tr>
<td>Tappe (56)</td>
<td>2014</td>
<td>One step, real time; hydrolysis probe</td>
<td>NS3</td>
<td>Asian</td>
<td>Asian</td>
<td>5</td>
<td>Serum</td>
</tr>
<tr>
<td>Pyke et al (57)</td>
<td>2014</td>
<td>One step, real time</td>
<td>E</td>
<td>Asian</td>
<td>Asian</td>
<td>1</td>
<td>Serum</td>
</tr>
<tr>
<td>Pyke et al (57)</td>
<td>2014</td>
<td>One step, real time</td>
<td>NS1</td>
<td>Asian</td>
<td>Asian</td>
<td>1</td>
<td>Serum</td>
</tr>
</tbody>
</table>

Table 2. Overview of RT-RCR assays for the detection of ZIKV infection. Adapted from Charrel (3) and Waggoner (34).

*prM, precursor membrane; E, envelope; NS5, nonstructural protein 5; NS3, nonstructural protein 3; NS1, non-structural protein 1.
Of the RT-PCR assays listed in Table 1, the most widely-evaluated assay is one developed by the United States Centers for Disease Control and Prevention (CDC) using a sequence derived from the 2007 Yap Islands outbreak (7). The CDC assay is a combined assay made up of two one-step rRT-PCR reactions targeting the prM and E genes, respectively. Interpretation of the test involves evaluation of both reactions. A sample is positive only if both reactions demonstrate amplification with crossing threshold (C_T) values of <38.5 cycles. A result is considered equivocal if a sample is amplified in only one of the two reactions or if the C_T value in either reaction was >38.5 cycles. The lower limits of detection (LoD) were estimated as 100 copies and 25 copies for the prM and E gene targets, respectively (7).

Of 157 serum samples tested from the Yap Islands epidemic using the CDC rRT-PCR assay, 10.8% (17/157) were positive, 6.4% (10/157) were equivocal, and 82.8% (130/157) were negative (7). Quantitation of the Yap epidemic specimens using the assay also revealed relatively low levels of viremia (7).

Of the positive samples in the Yap Islands study, 88.2% (15/17) were drawn within the first 3 days of the onset of illness, which may indicate that the duration of detectable ZIKV RNA in serum is relatively short once the patient exhibits clinical symptoms (34). Similar findings were observed during the 2013-2014 ZIKV outbreak in French Polynesia, where on average patients were serum positive by the CDC rRT-PCR assay for 3.5 days (34).

The CDC ZIKV rRT-PCR assay has also been evaluated on several different specimen types, including on saliva, where ZIKV RNA was detected more frequently than in serum, although the mean day of illness seen was not significantly different between the two specimens (50). Of the 182 paired specimens tested from the 2013-2014 French Polynesia outbreak, 28.6% (52/182) were positive in serum and saliva, 19.2% (35/182) were positive in saliva only, and 8.8% in serum only (50). Waggoner et al indicate that the results of this study suggest that optimal diagnosis of acute ZIKV infection “may require testing of multiple specimen types” (34).

Similarly, the CDC ZIKV rRT-PCR assay has been used to test serial urine and serum samples, which were collected from six patients infected during the 2013-2014 New Caledonia outbreak (58). The results showed that ZIKV RNA was detected in urine 7 or more days after becoming undetectable in serum, confirming results from previous studies with regard to DENV and West Nile virus (58,59,60).

Finally, the CDC rRT-PCR was also analyzed on amniotic fluid in two cases of fetal microcephaly (41). Although both patients had negative test results in blood, they had positive results in amniotic fluid using the CDC rRT-PCR assay (41).

The additional RT-PCR methods described in Table 1 have also been evaluated, although the literature is limited. For example, Faye et al developed a one tube, conventional RT-PCR protocol using sequences encoding the E gene (53). Using ZIKV and flavivirus isolates supplied by the WHO Collaborating Center for Arboviruses and Viral Hemorrhagic Fever (CRORA) at Institut Pasteur in Dakar, the LoD was determined to be 7/7 plaque forming units (pfu)/reaction in both human serum and Leibovitz 15 medium (L-15), corresponding to a titer of 337/pfu/ml for both of the ZIKV isolates. The method was determined to be 100% specific (as it detected none of 19 other flaviviruses tested, including 3 strains of DENV) (53). This assay was used to detect ZIKV RNA specimens from Camacari (61) and Natal (48) during the Brazil outbreak.
Subsequent assays have targeted the NS5 gene because it tends to be the most well conserved among flaviviruses (54). Using sequences encoding the NS5 gene, Balm et al developed a one-step, RT-PCR with an internal control (IC) that was tested on 88 archived samples of DENV-negative and CHIKV-negative sera from patients who had presented to the National University Hospital, Singapore. Although the assay was specific for detection of ZIKV infection, none of the 88 samples tested was positive for ZIKV RNA (54). Therefore, the clinical sensitivity of the assay is unknown.

In a second study from the Institut Pasteur in Dakar, Faye et al developed and evaluated a one-step, rRT-PCR assay for ZIKV targeting the NS5 gene (55). The assay could be performed in 3 hours and was found to be highly sensitive for 37 ZIKV strains covering both Africa and Asia; it also exhibited high specificity (55). However, this assay was tested only in field-caught mosquitoes, and to date, has not been tested in humans (6).

Other rRT-PCR assays have been developed targeting the NS3 gene (56) and the E and NS1 genes (57). The assay targeting the NS3 gene was developed in Germany and was used to evaluate returning travelers (56,62,63). The assays targeting the E and NS1 genes were developed in Australia; although the assays were both positive in tests of a serum specimen, they were only tested on one person (57). There are no published analytical and clinical validations of these assays (34).

Recently, commercial RT-PCR assays for ZIKV have become available (3). These include the RealStar® Zika Virus RT-PCR Kit 1.0 (altona Diagnostics GmbH), Sentosa® SA ZIKV RT-PCR Test (Vela Diagnostics U.S., Inc. U.S.), Genesig® Easy Kit (Primerdesign™ Ltd., UK), Zika Virus – Single Check (Genekam Biotechnology AG, Germany), and FTD Zika virus (Fast Track Diagnostics, Luxembourg), all of which are CE-IVD marked. Other than one study on the performance of the RealStar® Zika Virus test (64), no peer reviewed, published reviews of any of these assays were found in the literature.

The WHO has been active in identifying and evaluating ZIKV assays. In March 2016, the WHO published a document on the ZIKV diagnostic product pipeline (65), and subsequently, the WHO started an Emergency Use Assessment and Listing (EUAL) procedure for ZIKV IVD submission and approval (http://www.who.int/diagnostics_laboratory/eual-zika-virus/zika/en/); the EUAL procedure was discontinued in November 2016. However, products for which application had been made to the WHO EUAL for approval will be processed by the WHO; these products are listed in this report as being the EUAL pipeline. Before its discontinuation, two products had completed the WHO process, the RealStar® Zika Virus RT-PCR Kit 1.0 (altona Diagnostics GmbH, Germany) and the AccuPower® ZIKV (DENV, CHIKV) Multiplex Real-Time RT-PCR Kit (Bioneer, Republic of Korea). Both of these kits are also CE-IVD marked. No peer reviewed, published reviews of the AccuPower® ZIKA (DENV, CHIKV) Multiplex Real-Time RT PCR test were found in the literature.

In addition, the U.S. Food and Drug Administration (FDA) has issued an Emergency Use Authorization (EUA) to permit the use of certain IVDs for the diagnosis of ZIKV infection (http://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm#zika). Currently approved molecular kits include those listed in Table 3 below. For each of these approved assays, testing is to be conducted only on specimens from individuals that meet the CDC ZIKV clinical and/or epidemiological criteria for testing (available at http://www.cdc.gov/zika/hc-providers/index.html) by laboratories in the U.S. that are certified under the Clinical Laboratory Improvement Amendments of
1988 (CLIA) to perform high complexity tests, or by similarly-qualified laboratories outside of the U.S. As a result, access to such assays is limited, especially in resource-limited settings.

<table>
<thead>
<tr>
<th>Product</th>
<th>EUA</th>
<th>EUAL</th>
<th>CE-IVD</th>
<th>Review</th>
</tr>
</thead>
<tbody>
<tr>
<td>RealStar® Zika Virus RT-PCR Kit 1.0 (altona Diagnostics GmbH, Germany)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Zika ELITE MGB® Kit U.S. (ELITechGroup Solutions, U.S.)</td>
<td>✓</td>
<td></td>
<td></td>
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<tr>
<td>Abbott RealTime Zika (Abbott Molecular Inc., U.S.)</td>
<td>✓</td>
<td></td>
<td>No</td>
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<tr>
<td>Zika Virus RNA Qualitative Real-Time RT-PCR (Focus Diagnostics, Inc., a subsidiary of Quest Diagnostics, U.S.)</td>
<td>✓</td>
<td></td>
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<tr>
<td>Aptima® Zika Virus Assay (Hologic, Inc., U.S.)</td>
<td>✓</td>
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<td>Zika Virus Real-time RT-PCR Test (Viracor-IBT Laboratories, Inc., U.S.)</td>
<td>✓</td>
<td></td>
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<tr>
<td>VERSANT® Zika RNA 1.0 Assay (kPCR) Kit (Siemens Healthcare Diagnostics Inc., U.S.),</td>
<td>✓</td>
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<tr>
<td>xMAP® MultiFLEX™ Zika RNA Assay (Luminex Corporation, U.S.)</td>
<td>✓</td>
<td></td>
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<tr>
<td>Sentosa® SA ZIKV RT-PCR Test (Vela Diagnostics U.S., Inc., U.S.)</td>
<td>✓</td>
<td>✓</td>
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<td>Zika Virus Detection by RT-PCR Test (ARUP Laboratories, U.S.)</td>
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<td>Gene-RADAR® Zika Virus Test (Nanobiosym Diagnostics, Inc., U.S.)</td>
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<td>Zika Virus Real Time RT-PCR Kit (Liferiver/Shanghai ZJ Biotech Co. (China)</td>
<td>Pipeline</td>
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<td>Genesig® Easy Kit (Primerdesign™ Ltd., UK)</td>
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<td>Zika Virus – Single Check (Genekam Biotechnology AG, Germany)</td>
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<td>FTD Zika Virus (Fast Track Diagnostics, Luxembourg)</td>
<td>✓</td>
<td></td>
<td>No</td>
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</table>

Table 3. Commercially Available Molecular Assays for Detection of ZIKV Infection

The assays listed in Table 3 are described in more detail below. The information on these assays is drawn from product inserts and other materials available on each company’s website.

**EUA-approved Molecular Assays**

**RealStar® Zika Virus RT-PCR Kit U.S. (altona Diagnostics GmbH, Germany)**

The RealStar® Zika Virus RT-PCR Kit 1.0, which is CE-IVD marked, is an *in vitro* diagnostic test, based on RT-PCR technology, for the qualitative detection of ZIKV-specific RNA. The assay includes a heterologous amplification system (IC). The product manual does not specify the primer and probe sequences in the kit.

The test consists of three processes in a single tube: (a) reverse transcription of target RNA to cDNA; (b) PCR amplification of target cDNA and IC; and (c) simultaneous detection of PCR amplicons by fluorescent dye-labelled probes. The Real Star® Zika Virus RT-PCR Kit 1.0 was developed and validated to be used with a number of real-time PCR instruments. These include: (a) Mx 3005P™ QOCR system (Stratagene, U.S.); (b) VERSANT® kPCR Molecular system AD (Siemens, U.S.); (c) ABI Prism® 7500 SDS and 7500 Fast SDS (Thermo Fisher Scientific, U.S.); (d) LightCycler® 480 Instrument II (Roche Molecular Diagnostics, U.S.); (e) Rotor-Gene® 6000 (Corbett Research); (f) Rotor-Gene® Q 5/6 plex Platform
(Qiagen, Germany); and (g) CFX96™ Real-Time System and CFX96™ Deep Well Real-Time System (Bio-Rad, France).

The company reports that the analytical sensitivity of the RealStar® Zika Virus RT-PCR Kit 1.0 determined by Probit analysis is 0.61 copies/µl eluate [95% confidence interval (CI): 0.39 – 1.27 copies/µl]. The analytical specificity of the kit, determined by testing a panel of genomic RNA extracted from non-Zika virus alphaviruses and other pathogens, was 100% as it exhibited no cross-reaction with any of the specified organisms, which included CHIKV and all 4 strains of DENV.

Further, one peer-reviewed, published performance evaluation of the RealStar® Zika Virus RT-PCR Kit 1.0 was found in the literature. L’Huillier et al verified the assay on patients in Ontario, Canada, by comparing it to the CDC-designed dual target ZIKV virus rRT-PCR as the reference assay. They found that the test had very good sensitivity (91%) and specificity (97%) compared to the reference assay (64).

**Zika ELITe MGB® Kit U.S. (ELITechGroup Solutions, U.S.)**

The Zika ELITe MGB® Kit U.S. is an rRt-PCR assay for the qualitative detection of ZIKV in plasma samples. The test uses a primer set and a single, uniquely labeled probe to amplify and detect the NS3 protein encoding gene of ZIKV.

The Zika ELITe MGB® Kit U.S. is performed using the ELITe InGenius™ instrument (pictured below) or other authorized instruments. The ELITe InGenius™ instrument automates nucleic acid extraction, amplification, and detection and provides operator guided step-by-step touch screen interface.

![Figure 3. ELITe InGenius™ Instrument](image)

The ELITe InGenius™ uses a cassette-based format to improve accuracy and control reagent consumption. The overall time to result from extraction to result analysis is approximately 2.5 hours, with hands-on time of less than 2 minutes per sample. The system permits 1 to 12 samples to be processed in parallel tracks, offering the possibility to mix any kind of sample matrices and use diverse thermal profiles simultaneously, including different PCR chemistries within the same run.

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3 Probit analysis is a type of regression used to analyze binomial response variables. It transforms the number of positive tests at various dilutions and concentrations to a straight line that can then be analyzed by regression either through least squares or maximum likelihood.
The ELITe InGenius™ also provides bi-direction laboratory information system (LIS) connectivity. Remote access for service and diagnostics is also available.

There are no peer-reviewed, published studies available on the assay.

**Abbott RealTime RT-PCR ZIKA Kit (Abbott Molecular, Inc. (U.S.))**

The Abbott RealTime RT-PCR ZIKA Kit is an RT-PCR assay for the qualitative detection of RNA from ZIKV in serum, EDTA plasma, whole blood (EDTA) and urine. Specimens are tested using the m2000sp (pictured below top) for automated specimen processing and the m2000rt (pictured below bottom) for amplification and detection.

![Image of Abbott m2000sp and m2000rt instruments](image)

**Figure 4. Abbott m2000sp (above left) and m2000rt (below right) instruments**

The m2000sp is an automated, high-throughput sample processing system with a maximum batch size of 96 samples per run. When combined with Abbott m2000rt, the amplification and detection instrument, the system can provide automation from bar coded laboratory tube through patient result.

The Abbott m2000rt is the amplification and detection platform for use with the m2000sp instrument described above. It is a high-performance system, but is relatively compact, weighing just over 75 lbs. The m2000rt can run 96 samples at a time in about three hours of cycling time (not including time for sample preparation). The system will run both quantitative and qualitative analyses and offers key
validity parameters such as maxRatio. Like other laboratory-based molecular testing systems, the operator must have a thorough knowledge of the applications run on the instrument (and on the sample preparation instrument) and must follow good laboratory practices when operating them.

The company reports that the Abbott RealTime ZIKA assay has a limit of detection of 30 copies per mL in serum, 40 copies per mL in plasma and urine, and 120 copies per mL in whole blood and demonstrates high specificity against challenges from closely related infectious agents. There are, however, no peer-reviewed, published studies available on the assay.

**Zika Virus RNA Qualitative Real-Time RT-PCR (Focus Diagnostics, Inc., a subsidiary of Quest Diagnostics, U.S.)**

The Zika Virus RNA Qualitative Real-Time PCR test is an rRT-PCR assay intended for the qualitative detection of RNA from ZIKV in human serum or urine (collected alongside a patient-matched serum specimen). The assay utilizes 5’ nuclease technology (also known as Taqman technology) and is composed of two primary steps: (i) extraction of total nucleic acide from patient specimens using the Roche MagNA Pure Instrument and (ii) real-time amplification and detection of two separate 77 nucleotide sequences within the “M” and “E” regions of the ZIKV genome using an Applied Biosystem 7500 Real-Time PCR System (pictured below). An internal control is included with each specimen to verify nucleic acid extraction and amplification.

![Applied Biosystems 7500 Real-Time PCR System](image)

**Figure 5. Applied Biosystems 7500 Real-Time PCR System**

The Applied Biosystems 7500 Real-Time PCR System is a 96-well, five color platform that uses fluorescence-based PCR reagents to provide: (i) quantitative detection of nucleic acid sequences using real-time analysis; and (ii) qualitative detection of nucleic acid sequences using end-point and dissociation-curve analysis. Regardless of the run type, a data collection or read on the 7500 instrument consists of three phases:

- **Excitation** – The instrument illuminates all wells of the reaction plate within the instrument, exciting the fluorophores in each reaction.
• **Emission** – The instrument optics collect the residual fluorescence emitted from the wells of the reaction plate. The resulting image collected by the device consists only of light that corresponds to the range of emission wavelengths.

• **Collection** – The instrument assembles a digital representation of the residual fluorescence collected over a fixed time interval. The 7500 software stores the raw fluorescence image for analysis.

After a run, the 7500 software uses region of interest, optical, dye, and background calibrations to determine the location and intensity of the fluorescence in each read, the dye associated with each fluorescent signal, and the significance of the signals.

There are no peer-reviewed, published studies of the Zika Virus RNA Qualitative Real-Time RT-PCR assay.

**Aptima® Zika Virus Assay (Hologic, Inc.)**

The Aptima® Zika Virus Assay from Hologic, Inc. is a transcription-mediated amplification (TMA) test intended for the qualitative detection of RNA from ZIKV in serum or plasma from individuals meeting the CDC ZIKV clinical criteria (i.e., clinical signs and symptoms associated with ZIKV infection) and/or CDC ZIKV epidemiological criteria (e.g., history of travel to, or residence in, a geographic region with active ZIKV transmission at the time of travel, or other epidemiologic criteria for which ZIKV testing may be indicated). The assay is designed to be run on Hologic’s Panther® system (pictured below) for automated specimen processing, amplification and detection.

![Hologic Panther® System](image)

**Figure 6. Hologic Panther® System**

The Aptima® Zika Virus assay consists of three main steps, which take place in a single tube on the Panther system: sample preparation, ZIKV RNA target amplification by TMA, and detection of the amplification products (amplicon) by the Hybridization Protection Assay (HPA). The assay incorporates an IC to monitor nucleic acid capture, amplification, and detection, as well as operator or instrument error.
During sample preparation, RNA is isolated from specimens using target capture, and the specimen is treated with a detergent to solubilize the viral envelope, denature proteins, and release viral genomic RNA. So called capture oligonucleotides that are homologous to highly conserved regions of ZIKV are hybridized to the ZIKV RNA target, if present, in the test specimen. The hybridized target is then captured onto magnetic microparticles that are separated from the specimen in a magnetic field. Wash steps are utilized to remove extraneous components from the reaction tube. Both magnetic separation and wash steps are performed with a target capture system.

Target amplification takes place via TMA, which is a transcription-based NAT method that utilizes two enzymes, MMLV reverse transcriptase and T7 RNA polymerase. The reverse transcriptase is used to generate a DNA copy (containing a promoter sequence for T7 RNA polymerase) of the target RNA sequence. The T7 RNA polymerase produces multiple copies of RNA amplicon from the DNA copy template. The Aptima Zika Virus assay utilizes the TMA method to amplify regions of the ZIKV RNA.

HPA, using single-stranded nucleic acid probes with chemiluminescent labels that are complementary to the amplicon, achieves detection. The labeled nucleic acid probes hybridize specifically to the amplicon. The Selection Reagent differentiates between hybridized and unhybridized probes by inactivating the label on unhybridized probes. During the detection step, the chemiluminescent signal produced by the hybridized probe is measured in a luminometer and is reported as Relative Light Units (RLU).

IC is added to each test specimen and assay calibrators via the working Target Capture Reagent. The IC in the Aptima® Zika Virus assay controls for specimen processing, amplification and detection steps.

Based on donor plasma from a Brazilian patient from 2015 of 5.9 copies/mL at 95% detection probability, the company reports a LoD of the assay. The LoD for an in vitro transcript in the Aptma® Zika Virus assay was determined to be 13.4 copies/mL at 95% detection probability. The cross-reactivity of the assay was evaluated against DENV, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, HIV 1/2, Parvovirus B19 and West Nile virus. The Aptima® Zika Virus assay results were negative for all samples.

In conclusion, there are an increasing number of NAT-based assays available commercially, most of which are for research use only at this time. All of these assays are complex, requiring sophisticated laboratories and well-trained laboratory technicians to be performed successfully. Qualified testing laboratories are limited. For example, in the United States, testing is only performed at the CDC Arboviral Diagnostic Laboratory and at some state and regional health departments (6). In other regions, the Pan American Health Organization and the WHO perform laboratory testing for ZIKV. Realistically, these NAT-based assays in their present iterations are not well-suited for use in resource-limited settings. In addition, evaluations studies on these assays are required to demonstrate the performance (analytical sensitivity and specificity) of these assays in clinical specimens.

Despite the current drawbacks of NAT-based assays for ZIKV infection, it is generally recommended that for individuals presenting within 7 days of onset of symptoms (i.e., during the acute phase of infection), testing for ZIKV should include an rRT-PCR test for ZIKV viral RNA (66-69). The test can be conducted on whole blood, serum, plasma, urine, saliva, and amniotic fluid specimens (34) (Wagoner). It has been shown that the highest concentrations of viral RNA are found in saliva during the early acute phase of the infection, but that viral RNA may be detectable for a longer period in urine (34,58). Nonetheless, until there is further evidence, the WHO recommends that whole blood, serum collected in a dry tube and/or urine be used for rRT-PCR testing for ZIKV infection (69).
While a positive rRT-PCR result in a patient suspected of ZIKV confirms the presence of ZIKV, a negative rRT-PCR does not definitely rule out the presence of ZIKV (7,70,71). Accordingly, a secondary antibody test is required to confirm the diagnosis.

**Zika Virus Real-time RT-PCR (Viracor –IBT Laboratories)**

The Zika Virus Real-time RT-PCR from Viracor IBT-Laboratories is an RT-PCR assay for the qualitative detection of RNA from ZIKV in human plasma, serum or urine (collected alongside a patient matched serum or plasma specimen). The test procedure involves the extraction of ZIKV nucleic acid from the specimen, followed by combined reverse transcription of viral RNA and PCR amplification using rRT-PCR methods. No particular required PCR equipment is specified by the company. An internal control is added to ensure that extraction was performed correctly and that the RT-PCR reaction was not inhibited.

The company indicates that the ZIKV RT-PCR assay is not cross-reactive with other viruses in the *Flaviviridae* family, including DENV, or with other viruses known to cause similar clinical manifestations, like CHIKV. No published performance studies are available, however.

As indicated previously, the test has not been cleared or approved for diagnostic use in the U.S., but has been authorized by the FDA under an EUA for use by authorized laboratories.

**VERSANT® Zika RNA 1.0 Assay (kPCR) Kit (Siemens Healthcare Diagnostics, Inc., U.S.)**

The VERSANT® Zika RNA 1.0 Assay (kPCR) Kit is a real-time PCR-based assay intended for the qualitative detection of RNA from ZIKV in serum, EDTA plasma and urine (collected alongside a patient-matched serum or plasma specimen).

To perform the VERSANT® Zika RNA 1.0 Assay (kPCR) Kit, samples are first extracted to isolate ZIKV RNA. Nucleic acids are isolated and purified from serum, plasma or urine using the Siemens automated system, the VERSANT® kPCR Sample Prep (SP) (also referred to as VERSANT® kPCR Molecular System SP) with VERSANT® MiPLX Software Solution and the VERSANT® Sample Preparation 1.0 Reagents. The VERSANT® Sample Preparation 1.0 Reagents use a nucleic acid isolation technology based on iron oxide beads coated with a nanolayer of silica. Magnetic silica technology is a nonspecific capture method and is target independent; it captures any DNA or RNA present in the sample. VERSANT® sample preparation technology employs a classic method of disrupting the cells in chaotropic high salt conditions to release nucleic acids, as well as to protect them from cellular nucleases. The nucleic acids are captured on silica coated beads. Using a magnetic field, the beads are separated and washed to remove proteins, nucleases, and other cellular impurities. The nucleic acids are then eluted in a small volume of elution buffer and ready for subsequent analysis.

Manual extraction of samples may be performed using the QIAamp viral RNA Mini Kit (QIAGEN). Purified RNA is added to a PCR plate containing Zika Enzyme Mix and Zika Primer/Probe Mix, and the wells are sealed. The Zika Enzyme Mix contains dNTPs, reference dye, and enzymes for nucleic acid amplification. The assay targets two regions of the ZIKV genome. The Zika Primer/Probe Mix contains synthetic DNA primers and probes and the Zika Internal Control. An assay IC sequence is included in the kit, and is used as a sample extraction control. The probes include fluorescent dyes and quenchers as modifiers. The dual-labeled probes specifically detect the presence of ZIKV and Internal Control amplicons during amplification. In their native state, the probes adopt a folded structure, positioning the quencher next to
the fluorescent dye. In this condition, most of the fluorescence of the dye is absorbed by the neighboring quencher, minimizing the emitted fluorescence. When amplicons are generated, fluorescent dye-labeled probes uncoil as they hybridize to the amplicons, separating the fluorescent dye from the quencher, thereby increasing the observed fluorescence. During the extension step, the hybridized probe is cleaved through the exonuclease activity of the polymerase. When free in solution, increased fluorescence is continuously detectable. The increased fluorescence of both cleaved and bound probes correlates with the amount of amplicons generated, and is proportional to the amount of ZIKV RNA in the sample.

The VERSANT® Zika RNA 1.0 Assay can be performed using any of the following validated RT-PCR systems: QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, U.S.); Applied Biosystems® 7500 Fast Dx Real-Time PCR Instrument (Thermo Fisher Scientific, U.S.); and CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, France).

The tentative LoD of the test is 0.05 TCID$_{50}$/mL or 721 copies/mL. Additional performance data is available at: http://www.fda.gov/downloads/MedicalDevices/Safety/EmergencySituations/UCM514170.pdf.

No peer-reviewed, published evaluations of the performance of the VERSANT® Zika RNA 1.0 Assay (kPCR) Kit are available.

**xMAP® MultiFLEX™ Zika RNA Assay (Luminex Corporation, U.S.)**

The xMAP® MultiFLEX™ Zika RNA Assay is a RT-PCR test intended for the qualitative detection of RNA from ZIKV in serum, plasma, or urine (collected alongside a patient-matched serum or plasma specimen) as well as an IC. The assay incorporates multiplex RT-PCR in conjunction with the Luminex® xMAP® Technology for target detection, which technology uses the principles of fluidics, fluorescent-dyed microspheres, and digital signal processing to enable detection of up to 50 analytes per sample on a MAGPIX instrument and up to 100 analytes per sample on a Luminex® 200™ instrument.

![Figure 7. Luminex MAGPIX instrument (left) and Luminex 200 (right).](image)

The xMAP® MultiFLEX™ Zika RNA Assay uses proprietary RT-PCR primers and probes in a panel designed for selective amplification of 6 distinct genomic regions of ZIKV. The company indicates that amplifying the 6 distinct genomic regions allow for improved detection of the genetic variation of ZIKV. The panel also includes an IC: MS2 (bacteriophage RNA extraction control).

The xMAP® MultiFLEX™ Zika RNA Assay workflow is pictured below.
Following the extraction of samples using a validated automated extraction method/system, extracted total nucleic acid is then simultaneously amplified using target-specific primers and probes for RT-PCR followed by amplicon hybridization to probe-coupled microspheres. Detection of the targets of interest is then carried out with a Luminex® instrument after adding streptavidin-conjugated R-Phycoerythrin (SAPE).

The company reports the LoD of the xMAP® MultiFLEX™ assay as 687 copies/mL. Additional performance data for the assay can be found at: http://www.fda.gov/downloads/MedicalDevices/Safety/EmergencySituations/UCM515137.pdf.

No peer-reviewed, published evaluations of the xMAP® MultiFLEX™ Zika RNA assay are available.

**Sentosa® SA ZIKV RT-PCR Test (Vela Diagnostics U.S., Inc., U.S.)**

The Sentosa® SA ZIKV RT-PCR Test is an rRT-PCR assay intended for the qualitative detection of RNA from ZIKV in human serum, EDTA plasma and urine (collected alongside a patient-matched serum or plasma specimen). The test is configured to run on the automated Sentosa SX101 real-time PCR workflow on the Sentosa SA201 rRT-PCR cycler (ABI 7500 Fast Dx), along with the Sentosa SX Virus Total Nucleic Acid Kit v2.0. Time to result is approximately 3 hours.

The company reports a limit of detection of $3 \times 10^3$ copies/mL for the assay, and reports analytical performance with 82 ZIKV strains detected and no cross reactivity with 44 pathogens (including DENV and CHIKV). However, no peer-reviewed, published evaluations for the Sentosa® SA ZIKV RT-PCR Test are available.
Zika Virus Detection by RT-PCR Test (ARUP Laboratories, U.S.)

The ARUP Laboratories Zika Virus Detection by RT-PCR is a rRT-PCR test intended for the qualitative detection of RNA from ZIKV in serum, EDTA plasma and urine (paired with a patient-matched serum or plasma sample). At the present time, the assay is intended for use only by trained laboratory personnel at ARUP Laboratories in Salt Lake City, Utah. Therefore, access to the test is extremely limited.

Following patient specimen collection, nucleic acids are isolated and purified from serum, plasma, or urine using the Chemagic MSM I extraction platform (Perkin Elmer, U.S.) using a protocol for total nucleic acid extraction. The purified nucleic acid is reverse transcribed and amplified using a 2X custom Multiplex 1-step RT-qPCR master mix (Quantabio, U.S.) with thermal cycling and detection on the QuantStudio™ 12 Flex real-time PCR instrument (Thermo-Fisher). In the process, the probe anneals to a specific target sequence located between the forward and reverse primers generating a fluorescent signal that is measured during the annealing phase of the PCR cycle. With each cycle, additional amplicon is produced, increasing the amount of annealed probe and subsequent fluorescence signal. Fluorescence intensity is monitored at each PCR cycle by the QuantStudio™ 12 Flex (pictured below).

Figure 10. QuantStudio™ 12 Flex RT-PCR Instrument (Thermo-Fisher)

There are no peer-reviewed, published studies of the ARUP Laboratories Zika Virus Detection by RT-PCR assay.
Gene-RADAR® Zika Test (Nanobiosym Diagnostics, Inc., U.S.)

The Gene-RADAR® Zika Virus Test is an rRT-PCR based assay intended for the qualitative detection of RNA from ZIKV in serum samples. Samples are extracted from patient serum samples using the QIAmp® Viral RNA Mini Kit (Qiagen) and amplified and detected on the Gene-RADAR® Platform (pictured below).

![Gene-RADAR® Platform](image)

**Figure 11. Gene-RADAR® Platform**

The company describes the Gene-RADAR® as a portable nanotechnology platform, which is a fully integrated, chip-based system about the size of an iPad, that can recognize the genetic “fingerprint” (DNA or RNA signature) of any disease, using small quantities of specimen (50 µl of serum in the case of the ZIKV test). There is no further information on the test principle.

There are no available peer-reviewed, published evaluations of the Gene-RADAR® Zika Test.

**Additional Molecular Assays**

**Zika Virus Real Time RT-PCR Kit (Liferiver™/Shanghai ZJ Bio-Tech Co., China)**

The Zika Virus Real Time RT-PCR Kit from Liferiver™ can be used for the detection of ZIKV infection in serum or plasma. The assay is designed to be used with the LightCycler 2.0 real time PCR system from Roche Diagnostics GmbH (Germany), pictured below. Like other kits described here, the ZIKV kit from Liferiver is based on the 5’UTR and separates the reporter dye from the quencher dye only when the probe hybridizes to the target DNA. The fluorescent signal generated by the cleaved reporter dye is monitored in real-time by the PCR detection system.
The Liferiver ZIKV kit contains a specific, ready-to-use assay for the detection of ZIKV using RT-PCR in the LightCycler system. The reaction is done in a one-step, real time RT PCR process. The first step is a reverse transcription, during which the ZIKV RNA is transcribed into cDNA. Subsequently, a thermostable DNA polymerase is used to amplify the specific gene fragments by means of PCR. Fluorescence is emitted and measured by the optical unit of the real time system during the PCR.

The company notes that the sensitivity of the test depends on the sample type, sample volume, elution volume, nucleic acid extraction methods and other factors. No performance data are given or published.

**Genesig® Kits for ZIKV (Primerdesign™ Ltd., UK)**

The Genesig® range of products for Primerdesign™ includes three assays for ZIKV, the Easy, Standard and Advanced kits, all of which are for research use only and are not licensed for diagnostic procedures. Each of the Standard and Advanced kits runs on equipment that must be supplied by the user, including a real-time PCR instrument and an RNA extraction kit, for which the company recommends the use of the genesig® Easy DNA/RNA Extraction kit. Genesig provides the primer and probe mix, which uses the TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to ZIKV cDNA. The same reaction mixture, which consists of a DNA probe labeled with a 5`-dye and a 3` quencher, includes a fluorogenic probe. During PCR amplification, the probe is cleaved and the reporter dye and the quencher are separated. The resulting increase in fluorescence can be detected on a range of real-time PCR platforms.

Although the genesig assays will work using a two-step approach, the company recommends the use of a one-step qRT-PCR approach, which combines the reverse transcription and real-time PCR reaction in a simple, closed-tube protocol. This not only saves hands-on time, it also reduces errors. In addition, the sensitivity of the one-step protocol is also greater than a two-step protocol. No sensitivity and specificity data are provided by the company on these assays.

Finally, the genesig® Easy kit is designed to be used on the genesig® q16 qPCR instrument, pictured below.
The instrument, which weighs about 2 kg (4.4 pounds) and measures 160 mm (6.3 inches) in height and 120 mm (4.7 inches) in diameter, operates from a PC or Mac computer, via the Internet, or can be run on a standalone basis using a USB drive. The device has 16 wells and uses 20µl reaction volume. The company indicates that the software interface between the instrument and the computer is very simple. Data analysis is automated and there are no moving parts in the device. The cost of the device is about $6,800.

The company offers the genesig® Lab-In-A-Box kit, which contains all of the pipettes, tips and racks needed to use the Easy Kit. Along with the q16 instrument and the genesig® East DNA/RNA Extraction Kit, the Lab-In-A Box kit must be procured by the user in addition to the assay. No further information on the principle of the Easy Kit or the q16 instrument was found. No performance data are available.

**Zika Virus – Single Check FR325 (Genekam Biotechnology AG)**

Although the Zika Virus – Single Check FR325 assay from Genekam Biotechnology AG for use with RT-PCR instruments is CE-IVD marked, there is very little information available publicly about the kit. It can be used on blood, plasma and serum specimens; its use on urine specimens is under investigation. The company indicates that the assay's sensitivity is 99.5% with an LoD of 6.53 genome equivalent. The company also reports analytical specificity of the assay of 99.9%. No published performance data is available or published.

**FTD Zika Virus (Fast Track Diagnostics Ltd., Luxembourg)**

FTD Zika Virus is an IVD for the qualitative detection of viral nucleic acid as an aid in the detection of ZIKV infections. The viral RNA is transcribed into cDNA using a specific primer mediated reverse transcription step followed immediately by polymerase chain reaction in the same tube. The presence of specific pathogen sequences in the reaction is detected by an increase in fluorescence observed from the relevant dual-labeled probe, and is reported as a C<sub>T</sub> by the RT thermocycler. The assay uses Streptococcus equi (Sequi) as an extraction control - the IC - which is introduced into each sample and the negative control at the lysis buffer stage of the extraction process. The product manual does not specify the primer and probe sequences in the kit.

FTD kits are suited for use with the Applied Biosystems® 7500/7500Fast (Thermo Fisher Scientific, U.S.), CFX96™ (Bio-Rad, France), LightCycler®480 (Roche) and Rotor-Gene 3000, 6000, Q (Qiagen, Germany) and SmartCycler® (Cepheid; U.S., in combination with Life Science software 2.0d). The assay has been
fully validated on an Applied Biosystems® 7500 with Fast-track mastermix and with the NucliSENS® easyMag® (bioMérieux, France).

Fast Track reports sensitivity of the FTD Zika virus kit as 86.7% with an average LoD of 735 cps/ml (range 374.31-1.5e3 cps/ml) using a 1:50,000,000 dilution of extracted RNA. The company reports specificity of the FTD assay as 100%. Twenty-one negative plasma samples and 20 negative urine samples were tested and did not generate any positive signals with FTD ZIKV. In addition, the company tested 84 clinical samples containing bacteria, parasites and viruses with FTD ZIKV, including several non-ZIKV flaviviruses, of which DENV was one. There was no cross reactivity with the other pathogens.

Immunoaassays for ZIKV Infection

For individuals with suspected ZIKV infection who test negative using an rRT-PCR assay or for individuals presenting 7 or more days following the onset of symptoms, antibody testing, namely immunoglobulin type M (IgM) or immunoglobulin type G (IgG) and neutralizing antibody testing, can be used to detect recent ZIKV infection (7,70). An IgM enzyme-linked immunosorbent assay (ELISA) was developed at the CDC Arboviral Diagnostic and Reference Laboratory to detect ZIKV infection using samples from the Yap Island outbreak in 2007/2008 (7). IgM was detectable as early as 3 days after the appearance of symptoms of ZIKA infection, but the assay was cross-reactive with other flaviviruses. In general, IgM tests can be unreliable because of cross-reactivity with other flaviviruses. This is particularly problematic in individuals who have previously been infected with, or vaccinated against, a related flavivirus, particularly DENV (7, 29, 72). The ability for ZIKV testing to discriminate against related infections, especially DENV fever, is important for the clinical management of patients. For example, pregnant women who test positive for ZIKV infection should be managed for possible adverse outcomes associated with pregnancy, including fetal microcephaly, while individuals who test positive for DENV fever should be managed to reduce the risk of mortality from acute DENV infection.

Antibody assays for the diagnosis of ZIKV infection include ELISAs and immunofluorescence assays (IFAs) that detect IgM antibodies using viral lysate, cell culture supernatant or recombinant proteins (68). In addition, neutralization assays such as plaque-reduction neutralization tests (PRNT) may be used. Generally speaking, although PRNT assays have greater specificity, because of the availability of reagents for assays to detect IgM antibodies, IgM ELISAs are more frequently used. When using IgM detection assays, wherever possible, paired serum specimens should be obtained at least 2 – 3 weeks apart, with the first specimen collected during the first 5 days of symptom onset (68). This, of course, can be difficult, especially in resource-limited settings.

Commercial IgM antibody tests for ZIKV are just becoming available. The FDA has granted an EUA for the Zika MAC-ELISA (CDC, U.S.), the ZIKV Detect™ IgM Capture ELISA (InBios International, Inc., U.S.) and the LIAISON® XL Zika Capture IgM Assay (DiaSorin S.p.A., Italy); none of the assays has yet been approved via the WHO EUAL process. In addition, a number of other ELISAs are available, some of which are CE-IVD marked, and are listed in Table 4 below. There are also three commercially-available RDTs for the detection of ZIKV infection: DPP Zika IgM/IgG Assay (Chembio Diagnostics, Inc., U.S.), STANDARD Q Zika IgM/IgG Test (SD Biosensor, Republic of Korea), and TELL ME FAST Zika Virus IgG/IgM Antibody Rapid Test (Biocan, Canada). Like their molecular counterparts, these tests have not been extensively validated. They are summarized in Table 4 and are further described below.
### Table 4. Commercially Available Immunoassays for Detection of ZIKV Infection

<table>
<thead>
<tr>
<th>Product</th>
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<th>EUAL</th>
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<td><strong>ELISAs</strong></td>
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<tr>
<td>Zika MAC-ELISA (CDC, U.S.)</td>
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<td>ZIKV Detect™ IgM Capture ELISA (InBios International, Inc. U.S.)</td>
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<td>NovaLisa® Zika Virus IgM μ-capture ELISA (NovaTec Immunodagnostica GmbH, Germany)</td>
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<td>Pipeline</td>
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<td>Anti-Zika Virus ELISA (IgM or IgG) (EUROIMMUN, Germany)</td>
<td></td>
<td>Pipeline</td>
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</tr>
<tr>
<td>ZIKV IgG ELISA kit (DIA.PRO Diagnostic Bioprobes Srl, Italy)</td>
<td></td>
<td>Pipeline</td>
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<td></td>
</tr>
<tr>
<td>ZIKV IgM ELISA kit (DIA.PRO Diagnostic Bioprobes Srl, Italy)</td>
<td></td>
<td>Pipeline</td>
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<td></td>
</tr>
<tr>
<td>STANDARD E Zika IgM ELISA (SD Biosensor Inc., South Korea)</td>
<td></td>
<td>Pipeline</td>
<td>No</td>
<td></td>
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<tr>
<td><strong>RDTs</strong></td>
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<tr>
<td>DPP® Zika IgM/IgG Assay (Chembio Diagnostic Systems, Inc., U.S.)</td>
<td></td>
<td>Pipeline</td>
<td>✓ (RUO)</td>
<td>No</td>
</tr>
<tr>
<td>STANDARD Q Zika IgM/IgG Test (RDT) (SD Biosensor Inc., South Korea)</td>
<td></td>
<td>Pipeline</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>TELL ME FAST Zika Virus IgG/IgM Antibody Rapid Test (Biocan, Canada)</td>
<td>✓</td>
<td></td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

#### EUA ELISA Assays

**Zika MAC-ELISA (CDC, U.S.)**

The CDC Zika MAC-ELISA is intended for the qualitative detection of ZIKV IgM antibodies in human sera or cerebrospinal fluid that is submitted alongside a patient-matched serum specimen. The principle of the assay is as follows. Anti-IgM (the capture antibody) is coated on 96-well plates. This is followed sequentially by adding the patient's serum, then known non-infectious viral antigen. The presence of antigen is detected by using enzyme-conjugated anti-ZIKV antibody. A colorimetric result is generated by the interaction of the enzyme and a chromogenic substrate. This colorimetric change is detected by a spectrophotometer (ELISA reader).


**ZIKV Detect™ IgM Capture ELISA (InBios International, Inc., U.S.)**

The ZIKV Detect™ IgM Capture ELISA is intended for the presumptive detection of ZIKV IgM antibodies in human sera. The assay targets the ZIKV envelope glycoproteins. Polystyrene microtiter wells are pre-coated with polyclonal capture antibodies against human IgM. Positive Control, Negative Control, and unknown test samples are diluted into a sample dilution buffer and then added to the ELISA plate in appropriate locations. After incubation and washing, a subsequent Ready-To-Use (RTU) ZIKV antigen (Zika Ag), a Cross-reactive Control Antigen (CCA) and a Normal Cell Antigen (NCA) are added separately to each corresponding well. After a subsequent incubation step and wash step, an enzyme conjugate
solution comprising horseradish peroxidase-labeled monoclonal anti-flavivirus antibody is added to each well. After washing, wells are incubated with a tetramethylbenzidine (TMB) substrate. An acidic Stop Solution is then added and the degree of enzymatic turnover is determined by the absorbance (optical density) measurement at 450 nanometers. If human IgM antibodies targeting the ZIKV envelope glycoproteins are present, a complex is formed consisting of the IgM, antigen, and conjugate. If IgM antibodies targeting the ZIKV envelope glycoproteins are not present, then the antigen and conjugate are washed away.


LIAISON® XL Zika Capture IgM Assay (DiaSorin S.p.A., Italy)

DiaSorin has developed a Zika capture assay, the LIAISON® XL, for the qualitative detection of IgM antibodies to ZIKV in 25 µl of human serum. The assay uses recombinant ZIKV NS1 antigen to assure good specificity and avoid cross reaction with other arboviruses. The company reports that samples collected from 58 symptomatic patients, including 15 pregnant women, initially PCR positive for ZIKV, were confirmed positive by the LIAISON® XL Zika Capture IgM assay by the first sample collected following 8 days post symptom onset – i.e., sensitivity of the assay was 100%. The company also reports 100% specificity for the assay with respect to DENV, CHIKV, West Nile Virus and Yellow Fever virus post-immunization.

The LIAISON® XL Zika Capture IgM Assay is only available for use on the LIAISON® XL platform. The LIAISON® XL, pictured below, utilizes chemiluminescence technology with magnetic microparticles; all of the components are ready to use; infectious disease assays include calibrators.
operator. The throughput of the system is up to 180 tests per hour, with a time to first result of 17 minutes.

Most tests do not require daily maintenance; the instrument notifies the user when maintenance is due and monitors the scheduled needs automatically. The LIAISON® XL allows for interface with the operator by touch screen monitor embedded in the platform. The system software is connectable to LIS and offers a service remote access.

There are no published, peer-reviewed studies of the LIAISON® XL Zika Capture IgM Assay.

Other ELISA Assays

**NovoLisa® Zika Virus IgM µ-capture ELISA (NovaTec Immunodiagnostica GmbH, Germany)**

The NovoLisa® Zika Virus IgM µ-capture ELISA is intended for the qualitative detection of IgM antibodies to ZIKV in human serum or plasma. The qualitative immunoenzymatic determination of specific IgM-class antibodies is based on the ELISA µ-capture technique.

The principle of the assay is as follows. Microplates are coated with anti-human IgM-class antibodies to bind the corresponding antibodies of the sample. After washing the wells to remove all of the unbound sample material, horseradish peroxidase (HRP)-labelled ZIKV antigen is added. This antigen-conjugate binds to the captured specific IgM antibodies. In a second washing step, unbound conjugate is removed. The immune complex formed by the bound conjugate is visualized by adding Tetramethylbenzidine (TMB) substrate, which gives a blue reaction product. The intensity of this product is proportional to the amount of specific IgM antibodies in the specimen. Sulphuric acid is added to stop the reaction. This produces a yellow endpoint color. Absorbance at 450/620 nanometers (nm) is read using an ELISA microwell plate reader.

The company claims that the diagnostic specificity of the NovoLisa® Zika Virus IgM µ-capture assay is 98.2%, while the sensitivity is said to be 100%. However, there is no published, peer-reviewed data on the product.

**Anti-Zika Virus ELISA (EUROIMMUN, Germany)**

EUROIMMUN has developed the Anti-Zika Virus ELISA assay, which is now CE-IVD marked. The assay provides fully automated antibody detection using microplates coated with a recombinant protein from ZIKV. Per the company, because this coating is a highly specific antigen, it avoids cross-reactivity and other flaviviruses, and in early studies of well characterized sera, has demonstrated no-cross reactivity with flaviruses, including DENV, West Nile and Japanese encephalitis. Larger studies are underway to evaluate the specificity of the Anti-ZIKV ELISA test, but no published performance data are available.

**ZIKV IgG ELISA kit and ZIKV IgM ELISA kit (DIA.PRO Diagnostic Bioprobes Srl, Italy)**

DIA.PRO Diagnostic Bioprobes Srl has developed two ELISA kits for ZIKV – the ZIKV IgG ELISA kit and the ZIKV IgM ELISA kit. There is no specific information publicly available about either of these assays.

**STANDARD E Zika IgM ELISA (SD Biosensor Inc., South Korea)**

The STANDARD E Zika IgM ELISA from BD Biosensor is a direct sandwich ELISA for the qualitative detection of specific IgM to ZIKV in serum and plasma. The company indicates that it is easy to use and
reports a specificity of 100% (60/60). There is no additional information publicly available about the assay.

**RDTs for ZIKV Detection**

**DPP® Zika IgM/IgG Assay (Chembio Diagnostics, Inc., U.S.)**

The DPP® Zika IgM/IgG Assay from Chembio Diagnostics has developed a RDT using Chembio’s patented dual path platform (DPP®) technology. It also includes a digital reader, the DPP® Micro Reader. The assay detects antibodies from a 10 µl fingerstick sample (serum, plasma, fingerstick or venipuncture whole blood) and provides a semi-quantitative result in 20 minutes. The test cartridge and reader, pictured below, were recently CE-IVD marked, paving the way for commercialization and sale in 17 European countries, as well as a majority of the Caribbean countries, including Puerto Rico. The test has also been approved by Brazil’s Health Regulatory Agency.

![DPP® Zika IgM/IgG Test and reader](image)

Chembio reports good specificity for the test with specimens presumed to be negative for ZIKV, and also reports virtually no IgM cross-reactivity with other arboviruses, including DENV, CHIKV, Yellow Fever, and West Nile. The company also reports excellent agreement between the DPP® Zika IgM/IgG Test and laboratory EIA tests: Overall IgM agreement is reported as 94.7% and overall IgG agreement as 91.7%.

There is currently no peer-reviewed, published data available performance data for the DPP Zika IgM/IgG Assay.

**STANDARD Q Zika IgM/IgG Test (SD Biosensor Inc., Republic of Korea)**

SD Biosensor Inc. has developed the STANDARD Q Zika IgM/IgG test. It is a screening test using immunochromatography. The assay can detect ZIKV infection in serum, plasma and whole blood with results within 15 minutes. The company indicates that the test is easy to use and read, can be stored at room temperature, and requires only 10 µl of sample.

Although there are no peer reviewed, published data available on the performance of the STANDARD Q Zika IgM/IgG test, the company reports sensitivity of 98.0% and specificity of 100%.
TELL ME FAST Zika Virus IgG/IgM Antibody Rapid Test (Biocan, Canada)

Biocan has developed the TELL ME FAST Zika Virus IgG/IgM Antibody Rapid Test, pictured below, which is a qualitative lateral flow immunoassay for the simultaneous detection and differentiation of IgG and IgM antibodies to ZIKV in human serum, plasma or whole blood. The company indicates it can deliver test results in 10 minutes and can be performed using human finger stick blood.

Figure 16. TELL ME FAST Zika Virus IgG/IgM Antibody Rapid Test

There are no published performance data available on the TELL ME FAST Zika Virus IgG/IgM Antibody Rapid Test.

In conclusion, to date, there are only a few commercially available ZIKV immunoassays, all of which are designed to detect antibodies. In the U.S., ZIKV antibody assays are available only through the CDC and other public health laboratories. Given the association of ZIKV with Guillain-Barré and fetal microcephaly, improved accessibility to quality-assured ZIKV-specific serological tests is required for effective clinical management. All commercially available ZIKV antibody tests, whether laboratory-based or targeted for use at the point-of-care, and any in the pipeline will require systematic clinical evaluation of test specificity, particularly in populations with a high prevalence of other flaviviruses, especially DENV.

Recent Developments and Next Generation Diagnostic Technologies for ZIKV Infection

Because ZIKV infection is currently considered a public health emergency, many parties have been trying to develop more effective and accessible diagnostic tests for the infection. Although there are several new tools in development, including a new laboratory-based assay from Baylor College of Medicine (U.S.) and a rapid ZIKV test from MD Biosciences laboratory in St. Paul, Minnesota (U.S.) (73), very little information is available about these assays. However, an interesting next generation test has been developed by a team of researchers at seven universities in the U.S. Through synthetic biology, the team
has developed a prototype for a simple, inexpensive ZIKV test using RNA sensors embedded in tiny discs of paper, which turn from yellow to purple in the presence of ZIKV. The team has also developed a prototype reader. Both are pictured below (74).

Figure 17. Prototype ZIKV test and reader.

The instrument is a NAT-based device that relies on two recently developed technologies. The first are programmable sensors called toehold switches that can be used to bind and sense virtually any RNA sequence (75). The second technology is a freeze-dried, paper-based, cell-free protein expression platform that allows for the deployment of the toehold switch sensors outside of a research laboratory by providing a sterile and abiotic method for the storage and distribution of genetic circuits at room temperature (76).

In order to increase the sensitivity of the assay, which was inadequate in the first generation prototype, the team incorporated an isothermal RNA amplification technique, NASBA, into the workflow. The assay also includes a new molecular tool to differentiate between two ZIKV strains. The tool is a CRISPR/Cas9-based gene-editing technique that recognizes the strain of ZIKV that is prevalent in the Americas, but not the African strain (74).
The prototype was able to achieve low femtomolar detection of ZIKV from the plasma of an infected monkey, but it has not been tested in human specimens (74). Time to results is approximately 3 hours. The device is expected to be portable, and the team predicts a price per test of about $1.00 (74). Although test results are readable by the naked eye, the portable, handheld reader can also be used, and is expected to cost about $250.

The team is currently refining the prototype to include isothermal amplification, among other improvements. In addition, the team is building a new reader that can incubate reactions on a chip.

Another interesting molecular assay has been developed by Song et al (77). The assay utilizes reverse-transcription loop-mediated amplification (RT-LAMP) and six specific primers for the ZIKV lineage that is prevalent in the Americas. The platform consists of a disposable microfluidic cassette that combines (i) viral nucleic acid capture, concentration, and purification; (ii) isothermal amplification; and (iii) detection. It also includes a custom-made, electricity-free cup that generates heat with an exothermic reaction and regulates temperature with a phase change material. Amplification products are visually detected with leuco crystal violet (LCV) dye; no instrumentation is required (77).

![Image of the POC RT-LAMP system showing (A) saliva sample preparation; (B) sample being filtered through the isolation membrane of the microfluidic cassette for nucleic acid extraction; (C) expanded view of chemically heated cup, which consists of a thermos cup body, a 3D-printed cup lid, a chip holder, PCM material, heat sink and a single-use Mg-Fe alloy pack heat source; and (D) photograph of the chemically heated cup. Images reproduced from Song et al (77).](image_url)

Song et al were able to demonstrate the utility of the system by detecting ZIKV in oral samples; sensitivity was 5 pfu in less than 40 minutes.

**Multiplex Diagnostic Assays for Simultaneous Detection and Differentiation of DENV, ZIKV and CHIKV Infections**

Driven in large part by the recent and ongoing outbreaks of ZIKV infection, several polyvalent assays to diagnose and differentiate DENV, ZIKV and CHIKV infections have been developed or are in development. One of these, the Trioplex Real-time RT-PCR Assay (Trioplex rRT-PCR) (Thermo Fisher
Scientific, U.S.), has received an EUA from the FDA. There are also several other commercially available molecular tests. A multiplexed qPCR assay (the ZCD assay) has been developed by scientists from Stanford University, Sustainable Sciences Institute (Nicaragua), Nicaragua Ministry of Health, and University of California, Berkeley, but it is not commercially available.

In addition to these molecular assays, EUROIMMUN has developed a polyvalent immunoassay, the IIFT Arbovirus Fever Mosaic test, and MIKROGEN Diagnostik has developed the recomLine Tropical Fever IgG and IgM assays. In addition, Biocan Diagnostics has developed a multiplex RDT for the simultaneous detection of DENV, ZIKV and CHIKV. Chembio and bioLytical, among others, are also developing polyvalent RDTs.

These molecular tests and immunoassays are summarized in Table 5 below. While there is limited information available on these assays, to the extent it is available, these assays are described below. The information is taken from company-published sources.

<table>
<thead>
<tr>
<th>Product</th>
<th>EUA</th>
<th>EUAL</th>
<th>CE-IVD</th>
<th>Review</th>
</tr>
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<tr>
<td>Trioplex Real-time Rt-PCR (Thermo Fisher Scientific, U.S.)</td>
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<td></td>
<td>✓</td>
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<td>AccuPower® ZIKV (DENV, CHIKV) Multiplex Real-Time RT-PCR Kit (Bioneer, Republic of Korean)</td>
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<td>✓</td>
<td>No</td>
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<tr>
<td>VIASURE Zika, Dengue &amp; Chikungunya Real Time PCR Detection Kit (Certest Biotec, Spain)</td>
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<td>FTD Zika/Dengue/Chik assay (Fast-Track Diagnostics, Malta)</td>
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<td>DiaPlexQ™ ZCD Virus Detection Kit (SolGent Co., Ltd., Republic of Korea)</td>
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<td>Pipeline</td>
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<td></td>
</tr>
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<td>recomLine Tropical Fever IgG; recomLineTropical Fever IgM (MIKROGEN Diagnostik, Germany)</td>
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<tr>
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</tr>
</tbody>
</table>

Table 5. Commercially Available Multiplex Assays to Detect DENV, ZIKV and CHIKV Infections

Multiplex Molecular Assays

**Trioplex Real-time RT-PCR (Thermo Fisher Scientific, U.S.)**

The Trioplex Real-time RT-PCR is intended for the qualitative detection and differentiation of RNA from ZIKV, DENV and CHIKV in human sera or cerebrospinal fluid (each collected alongside a patient-matched serum specimen), and for the qualitative detection of ZIKV virus RNA in urine and amniotic fluid (collected alongside a patient-matched serum specimen). Serum, however, is the preferred specimen. Testing is limited pursuant to the EUA of the FDA, including that testing must be conducted at qualified laboratories designated by the CDC.
The Trioplex Real-time PCR is a lyophilized, one-step real-time PCR assay. It includes primers and dual-labeled hydrolysis (TaqMan®) probes. A reverse transcription step produces cDNA from RNA present in the sample. The probe binds to the target DNA between two unlabeled PCR primers. For the DENV-specific probe, the signal from the fluorescent dye (FAM) on the 5’ end is quenched by Black Hole Quencher dye (BHQ-1) on its 3’ end. For the CHIKV-specific probe, the signal from the fluorescent dye (HEX) on the 5’ end is quenched by BHQ-1 on its 3’ end. And, for the ZIKV-specific probe, the signal from the fluorescent dye (Texas Red [TxRd]) on the 5’ end is quenched by BHQ-2 on its 3’ end. During PCR, Taq polymerase extends the unlabeled primers using the template strand as a guide, and when it reaches the probe, it cleaves the probe separating the dye from the quencher allowing it to fluoresce. The RT PCR instrument detects this fluorescence from the unquenched dye. With each PCR cycle, more probes are cleaved, resulting in an increase in fluorescence that is proportional to the amount of target nucleic acid present.

The assay is optimized for use on the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, pictured below, and it can also be performed on the 7500, 7500 Fast, and other QuantStudio™ systems (also from Applied Biosystems™, U.S.). For viral RNA purification, the company recommends use of the MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems™).

The company indicates that the Trioplex rRT-PCR assay is easy to use, environmentally friendly, and requires no cold-chain management; it may be shipped and stored at room temperature.

The Trioplex rRT-PCR has not been extensively tested with clinical specimens. No published, peer-reviewed studies are currently available.

**AccuPower® ZIKV (DENV, CHIKV) Multiplex Real-Time RT-PCR Kit (Bioneer, Republic of Korea)**

Bioneer has developed the AccuPower® ZIKV (DENV, CHIKV) Multiplex Real-Time RT-PCR Kit for the simultaneous detection of ZIKV, DENV and CHIKV in human serum and plasma samples through rRT-PCR using the company’s ExiStation™ Universal Molecular Diagnostic System and its Dual-HotStart™ technology (a modified form of PCR that avoids a non-specific amplification of DNA by inactivating the tag polymerase at lower temperatures) to enhance sensitivity and specificity. The AccuPower® ZIKV (DENV, CHIKV) Multiplex Real-Time RT-PCR Kit contains all of the necessary components in a RT-PCR master mix (enzymes, buffer and dNTPs) aliquoted in each tube. The test is CE-IVD marked.
The ExiStation™ Universal Molecular Diagnostic System is a semi-automated real-time qPCR based molecular diagnostic system. ExiStation™ is a workflow platform consisting of three ExiPrep™ 16 Dx nucleic acid extraction instruments and one Exicycler™ 96 real-time quantitative thermal cycler (pictured below).

ExiStation™ is able to process on average 36 samples per hour, and is able to handle up to 6 different types of clinical samples within a single run. The system also contains a refrigeration function to preserve the nucleic acids and diagnostic reagents. ExiStation™ is a pipetting-free system that mixes the extracted nucleic acids with the diagnostic reagents automatically, increasing convenience, practicality and reproducibility.

There is no publicly-published performance data on the AccuPower® ZIKV (DENV, CHIKV) Multiplex Real-Time RT-PCR Kit.

Figure 20. Workflow for the ExiStation™ Universal Molecular Diagnostic System.

VIASURE Zika, Dengue & Chikungunya Real Time PCR Detection Kit (Certest Biotec, Spain)

The VIASURE Zika, Dengue & Chikungunya Real Time PCR Detection Kit (Certest Biotec, Spain) is designed for the specific detection and differentiation of ZIKV, DENV and/or CHIKV in samples, including blood, serum, plasma and urine. The detection is done in a one-step RT format where the reverse transcription and the subsequent amplification of specific target sequences occur in the same reaction well. The isolated RNA target is transcribed, generating complementary DNA by reverse transcriptase,
which is followed by the amplification of a conserved region of the envelope gene (ZIKV), 3’ non-coding region (DENV) and NSP1 gene (CHIKV), using specific primers and a fluorescent-labelleled probe.

The VIASURE Zika, Dengue & Chikungunya RT PCR Detection Kit is based on 5’ exonuclease activity of DNA polymerase. During DNA amplification, this enzyme cleaves the probe bound to the complementary DNA sequence, separating the quencher dye from the reporter. This reaction generates an increase in the fluorescent signal that is proportional to the quantity of the target template. This fluorescence can be measured on RT PCR instrument platforms. The assay can be run on a large number of such platforms, including the 7500 Fast Real-Time PCR System and the QuantStudio™ 5 Real-Time PCR System, both from Applied Biosystems, the LightCycler® 96 and 480 Real-Time PCR Systems from Roche (U.S.), and the Rotor-Gene® Q from Qiagen (Switzerland).

The company reports that the VIASURE Zika, Dengue & Chikungunya Real Time PCR Detection Kit has a limit of detection of ≥10 RNA copies per reaction for ZIKV, DENV and CHIKV. The specificity of the assays were confirmed by testing a panel consisting of 10 different microorganisms representing the most common arboviruses. The company reports no cross-reactivity of any of these viruses against St. Louis Encephalitis virus strain 17D, West Nile virus strain H160/99, West Nile virus Heja, West Nile virus Ug37 and Yellow Fever virus strain 17D.

No published, peer-reviewed studies of the VIASURE Zika, Dengue & Chikungunya Real Time PCR Detection Kit were found.

FTD Zika/Dengue/Chik assay (Fast-Track Diagnostics, Malta)

The FTD Zika/Dengue/Chik assay (Fast-Track Diagnostics, Malta) is a two tube, multiplex rRT-PCR assay for detection of ZIKV, DENV and CHIKV (and an IC) using TaqMan® technology. The test is CE-IVD marked and can be used with extracted nucleic acid from human serum/plasma or urine. For DENV and CHIKV, whole blood specimens can also be used.

For extraction, a number of different platforms can be used. These include: NucliSENS® easyMAG® (bioMérieux); QIAamp Mini Elute virus Spin or QIAamp viral RNA kit - manual or in combination with QIAcuble (Qiagen); QIASymphony SP in combination with DSP Virus/Pathogen and the DNA Mini kit (Qiagen); RTP® Pathogen kit (STRATEC); DuplicaPrep (EuroClone®); or Maxwell® 16 Tissue LEV Total (stool samples) & Maxwell® 16 Buccal Swab LEV (Promega).

For amplification, the following platforms can be used: Applied Biosystems® 7500/7500 Fast (Applied Biosystems®); Rotor-Gene 3000/6000/Q (Qiagen); CFX96®/DX® with CFX software (Bio-Rad); LightCycler® 480 (Roche); and Smartcycler® with Life Science software 2.0d (Cepheid).

The FTD Zika/Dengue/Chik assay is fully validated with Fast-Track mastermix (Fast-Track Diagnostics and AgPath ID™ One-Step RT-PCR kit (Life Technologies™)).

There are no peer reviewed, published evaluations of the FTD Zika/Dengue/Chik assay.

DiaPlexQ™ ZCD Virus Detection Kit (SolGent Co., Ltd., Republic of Korea)

The DiaPlexQ™ ZCD Virus Detection Kit (SolGent Co., Ltd., Republic of Korea) is CE-IVD marked for research use. The kit is designed to simultaneously detect ZIKV, DENV and CHIKV in RNA isolated from
whole blood or serum using single-tube rRT-PCR technology, including hot start technology. The time to result is approximately 150 minutes.

The DiaPlexQ™ ZCD Virus Detection Kit is compatible with two PCR instruments: the CFX96™ Real-Time PCR System (Bio-Rad) and the Applied Biosystems™ 7500/7500 Fast Real-Time PCR System (Applied Biosystems).

There are no peer reviewed, published evaluations of the DiaPlexQ™ ZCD Virus Detection Kit.

In addition to the commercially available tests described above, the following molecular assay is in development.

**ZCD Assay**

Waggoner et al have described a ZIKV rRT-PCR assay that was designed to be run in multiplex with published assays for pan-DENV and CHIKV detection (78,79,80). The authors subsequently evaluated the assay by testing samples from people with suspected cases in Nicaragua.

The ZCD assay is not commercially available. However, it performed well in laboratory testing on the ABI7500 instrument (Applied Biosystems, U.S.) using genomic RNA. The 95% lower limit of detection (LoD) for each target, in copies per mL of eluate (5 mL added to each ZCD reaction) were: ZIKV, 7.8; CHIKV, 13.2; DENV-1, 11.7; DENV-2, 13.5; DENV-3, 4.1; DENV-4, 10.5. Similarly, the assay was highly specific, with no amplification found for: West Nile, Japanese encephalitis, tickborne encephalitis, yellow fever, Saint Louis encephalitis, o’nyong-nyong, Semliki Forest, Mayaro, Ross River, Getah, Barmah Fores, and Unas viruses (80).

Two hundred and sixteen serum samples collected from patients in Nicaragua with suspected ZIKV, CHIKV and/or DENV infections were tested using the ZCD assay and the pan-DENV-CHIKV rRT-PCR, which is a validated duplex assay containing the DENV and CHIKV primers and probes used in the ZCD assay (79). A total of 173 samples were positive for DENV alone (n = 25), CHIKV alone (n = 110), or both (n = 38). Per the authors, the ZCD assay and pan-DENV-CHIKV rRT-PCR showed very good agreement for DENV detection (k = 0.907) and good agreement for CHIKV detection (k = 0.662).

A case of ZIKV infection was detected with the ZCD assay during the testing described above. Subsequently, 133 consecutive samples were tested by using both the ZCD assay and a comparator ZIKV rRT-PCR targeting the capsid gene (81). The ZCD assay performed better than the comparator assay (80). Of the 56 ZIKV-positive samples in the ZCD assay, 39 were positive for ZIKV only, and 17 showed evidence of co-infections: ZIKV-DENV (n = 3); ZIKV-CHIKV (n = 10); ZIKV-CHIKV-DENV (n = 4) (80).

The authors conclude that the single-reaction multiplex ZCD assay successfully detected and differentiated ZIKV, CHIKV and DENV and could be used to streamline molecular test laboratory workflow and decrease the cost of testing while improving detection of these three infections (80).
Multiplex Immunoassays

IIFT Arbovirus Fever Mosaic 2 (IgG or IgM) (EUROIMMUN AG, Germany)

EUROIMMUN has developed an indirect immunofluorescence assay (IIFT), the IIFT Arbovirus Fever Mosaic 2 for the determination of specific antibodies against each of DENV (1-4), ZIKV and CHIKV in human serum or plasma. Similar to its IIFT assay for CHIV infection, BIOCHIP technology is used.

In the Arbovirus Fever Mosaic 2 assay, the ZIKV substrate is incubated in parallel with substrates for CHIKV and DENV serotypes 1 to 4. This BIOCHIP combination can help in differential diagnostic delimitation of each of these three virus infections. Due to the use of whole virus particles, cross-reactivity between flavivirus antibodies such as DENV and ZIKV virus can occur, however.

No further information is available on the assay.

recomLine Tropical Fever IgG, recomLine Tropical Fever IgM (MIKROGEN Diagnostik, Germany)

MIKROGEN Diagnostik has developed the recomLine Tropical Fever IgG, IgM test, which is a line immunoassay. It allows specific recombinant antigens for DENV, ZIKV and CHIKV viruses to be localized individually onto one test strip, thus enabling the simultaneous capture and detection of various antibodies against multiple species. The assay includes the following recombinantly produced antigens: DENV (NS-1 and E-protein), ZIKV (NS-1 and E protein) and CHIKV (E-1 protein).

The recomLine Tropical Fever IgG, IgM assay requires a serum or plasma sample collected ≥4 – 6 days after the onset of symptoms, and 1 – 2 week follow-up samples. Analysis of test strips can be done visually or computer-assisted using recomScan software. Visual reading of the test strips requires an analysis of the intensity of bands on the test strip. The company reports that there is minimal cross-reaction with other genetically related viruses in primary flavivirus infections, but acknowledges that there could be more cross reactivity in secondary flavivirus infections.

No peer-reviewed, published evaluations of the assay are available; the company reports limited data in its product insert.
Multiplex Rapid Diagnostic Tests

STANDARD Q Zika/Dengue Trio (SD Biosensor, Republic of Korea)

SD Biosensor has developed the STANDARD Q Zika/Dengue Trio test (pictured below), which is an immunochromatographic assay for the detection of IgM and IgG antibodies specific to DENV and NS1 antigen as well as ZIKA virus specific IgM and IgG antibodies in human serum, plasma and whole blood.

![STANDARD Q Zika/Dengue Trio Test](image)

**Figure 21. STANDARD Q Zika/Dengue Trio Test**

Per the company, screening test results can be obtained within 15 minutes with high sensitivity and specificity. As an RDT, the test is easy to use, read and interpret. Tests can be stored at temperatures between 1-40°C and have a shelf life of 24 months.

With the exception of Zika IgM, for which the company reports 33.7% sensitivity, the company reports strong sensitivity and specificity for each of the other test components DENV NS1 Ag, DENV IgM, DENV IgG, and ZIKV IgG. However, no peer-reviewed, published studies are available on the assay.4

TELL ME FAST Dengue, Chikungunya & Zika Virus Combo test (Biocan Diagnostics Inc., Canada)

Biocan Diagnostics has developed the TELL ME FAST Dengue, Chikungunya & Zika combo test. It is a rapid, lateral flow, qualitative test in cassette format (pictured below) for the simultaneous detection of IgG and IgM antibodies and NS1 Antigen to DENV and IgG & IgM antibodies to CHIKV and ZIKV in human serum, plasma or whole blood. It is a screening test for the presumptive distinction between a primary, secondary and an early DENV, CHIKV and ZIKV infection. However, results should be confirmed with other assays.

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4 This data can be found on the BD Biosensor website at: [http://en.sdbiosensor.com/xe/product/2530](http://en.sdbiosensor.com/xe/product/2530).
Figure 22. Biocan ZIK/CHIK/DENG Ab-Ag Test Cassette

The principle of each component test in the cartridge is similar. The ZIKV IgG/IgM component of the test, which is a qualitative test, consists of a pink colored conjugate pad containing recombinant (ZIKV NS1 protein and envelope protein) common antigens conjugated with colloid gold and rabbit IgG-gold conjugates, a nitrocellulose membrane strip containing two test bands (T1 and T2 bands) and a control band (C band). The T1 and T2 bands are pre-coated with monoclonal anti-human IgM and IgG, and the C band is pre-coated with goat anti-rabbit IgG. When test specimen (human serum, plasma or whole blood) is dispensed into the sample well of the test cassette, the specimen migrates via capillary action across the cassette. If present in the specimen, ZIKV IgM antibodies will bind to the ZIKV conjugates. The immunocomplex is then captured on the test membrane by the pre-coated anti-human IgM antibody, forming a burgundy-colored T1 band, indicating a ZIKV IgM positive test result. ZIKV IgG antibodies, if present in the specimen, will also bind to the ZIKV conjugates. The absence of any test band lines (T1 and T2) suggest a negative result. The test contains an IC (C band), which should exhibit a burgundy-colored band regardless of the color development of the test bands. Otherwise, the test result is invalid.

Similarly, in the DENV IgA/IgG/IgM test, a specimen (human serum, plasma or whole blood) is dispensed into the sample well along with sample buffer. The Gold antigen conjugate in the test binds to anti-DENV IgG and IgM antibodies in the specimen sample, which in turn, binds with anti-human IgG and anti-human IgM coated on the test membrane. As the test sample moves via capillary action across the membrane, the anti-human antibodies on the membrane bind the IgG or IgM antigen complex at the relevant IgG and/or IgM test lines, causing pale or dark pink likes to form at the IgG or IgM region of the test membrane. The intensity of the lines vary depending upon the amount of antibody present in the sample. The appearance of a pink line in a specific test region (IgG or IgM) should be considered as
positive for that particular antibody type. The test also includes an IC (C band), which should exhibit a burgundy-colored band regardless of the color development in the test bands. Otherwise, the test result is invalid.

In the DENV NS1 qualitative antigen test on the multiple cassette, a specimen (human serum, plasma or whole blood) is dispensed into the sample well, and Gold antibody conjugate then binds to the DENV antigen in the specimen, which in turn binds with anti-DENV NS1 coated on the test membrane. As the reagent moves across the membrane, the DENV NS1 antibody on the membrane binds to the antibody-antigen complex causing pale or dark pink lines to form at the test line region of the test membrane. The intensity of the lines will vary depending upon the amount of antigen present in the sample. The appearance of a pink line in the test region is considered a positive result. The test also includes an IC (C band), which should exhibit a burgundy-colored band regardless of the color development in the test bands. Otherwise, the test result is invalid.

Finally, in the CHIKV component of the test, which is also qualitative, specimen (human serum or plasma) is dispensed with sample buffer into the sample well, and the Gold antigen conjugate binds to anti-CHIKV IgG and IgM antibodies in the sample. As the reagent moves across the test membrane, this complex then binds with anti-human IgG and anti-human IgM coated on the test membrane as two separate lines in the test region. The anti-human antibodies on the membrane will bind the IgG or IgM antigen complex at the relevant IgG and/or IgM test lines causing pale or dark pink lines to form at the IgG or IgM region of the test membrane. While the intensity of the lines may vary, the appearance of a pink line in a specific test region (IgG or IgM) is considered as positive for that particular antibody. The test also includes an IC (C band), which should exhibit a burgundy-colored band regardless of the color development in the test bands. Otherwise, the test result is invalid.

There are no peer reviewed, published evaluations of the TELL ME FAST Dengue, Chikungunya & Zika combo test.

**Multiplex RDT Assays in the Pipeline**

In addition to the commercially available multiplex immunoassays described above, Chembio and bioLytical Laboratories are each developing polyvalent RDTs.

**DPP® Zika/Chikungunya/Dengue IgM/IgG Combination Assay (Chembio Diagnostic Systems, U.S.)**

Chembio Diagnostic Systems has been awarded grants from the Paul G. Allen Family Foundation and the U.S. Government to develop a combination Zika/Chikungunya/Dengue IgM/IgG assay using its patented dual path platform (DPP®) technology. Here, the DPP® platform has been expanded to include two test strips in a single cassette. A single sample of fingerstick blood is used to detect IgM antibodies and IgG antibodies against each of ZIKV, DENV and CHIKV infections. Results are obtained in a few seconds using
a small, portable reflectance reader. By differentiating IgM and IgG results, information on disease phase is possible.

The company has evaluated the assay and has found strong sensitivity and specificity, but no peer reviewed, published evaluations are currently available.

**INSTI Zika (Arbovirus) Total Antibody Test (bioLytical Laboratories, Canada)**

BioLytical Laboratories is developing a novel, 60 second POC assay for the simultaneous, qualitative detection of IgG and IgM antibodies to current and past ZIKV, DENV and CHIKV infection in human EDTA blood, finger stick blood, serum or plasma. The test is manual and visually read.

The company reports that the current combination of antigens in the assay produces overall sensitivity of 96.7% with anti-ZIKV, -DENV, or –CHIV positive specimens and a specificity of 100% against West Nile Virus and other potentially interfering disease conditions. Work is continuing to optimize INSTI performance in the populations of intended use.

No peer-reviewed, published evaluations are currently available with respect to the assay.

**Conclusion**

There is an urgent need for quality-assured IVDs for ZIKV infection. The need is especially acute in Brazil and the Americas, but other countries are affected as well. The EUAL program of the WHO and the EUA program of the FDA are helpful in this regard, and developers are responding positively to these programs. However, access to testing is still very limited, especially in resource-limited settings where ZIKV infection is most prevalent.

Moreover, the most reliable diagnostic assay for detection of ZIKV infection is the RT-PCR assay from the CDC. However, this assay, like other RT-PCR and rRT PCR assays, is only useful for the detection of acute ZIKV infection when viremia is present. On the flip side, there are a few available serological tests for ZIKV IgM and IgG, but their usefulness is confounded by high cross-reactivity with other flaviviruses, in particular DENV. PRNT assays are not always able to resolve this cross reactivity. Yet the ability to distinguish among ZIKV, DENV and CHIKV infections is critical for purposes of patient management.

Very few ZIKV assays, whether molecular or serological, have been adequately and independently evaluated using clinical specimens. For many assays described in this report, there is no performance data available publicly, much less peer-reviewed, published studies. These studies are badly needed.

Multiplex assays to diagnose and differentiate DENV from ZIKV and CHIKV have been developed, and additional assays are in development, including tests in RDT format. These will be a valuable addition to the diagnostic tools for these viruses, especially in endemic settings. However, studies of these multiplex assays are lacking. There are no published, peer-reviewed evaluations of any of them. These are badly needed.

Finally, for use in resource-limited settings, assays that can be used at or near the point of patient care will provide access to testing that laboratory-based testing cannot. Development of such assays for ZIKV infection needs to be encouraged.
References


