Rx ONLY

ZIKV *Detect*™ 2.0 IgM Capture ELISA Instructions for Use

For Use Under an Emergency Use Authorization Only

PURPOSE

This document describes the use of an IgM antibody capture enzyme linked immunosorbent assay (MAC-ELISA) for the presumptive detection of antibodies to Zika virus in individuals meeting Centers for Disease Control and Prevention (CDC) clinical and/or epidemiological criteria for Zika virus testing.

INTENDED USE

The ZIKV *Detect*™ 2.0 IgM Capture ELISA is intended for the presumptive detection of Zika virus IgM antibodies in human sera collected from individuals meeting CDC Zika virus clinical criteria (e.g., a history of clinical signs and symptoms associated with Zika virus infection) and/or CDC Zika virus epidemiological criteria (e.g., history of residence in or travel to a geographic region with active Zika transmission at the time of travel, or other epidemiological criteria for which Zika virus testing may be indicated). The assay is intended for use in laboratories in the United States that are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, to perform high complexity tests, or by similarly qualified non-U.S. laboratories, consistent with the latest CDC guideline for the diagnosis of Zika virus infection.

Assay results are for the presumptive detection of IgM antibodies to Zika virus (ZIKV). Reactive results are not definitive for the diagnosis of Zika virus infection. False positive results are possible in patients with a history of infection with other Flaviviruses. Confirmation of the presence of anti-Zika IgM antibodies in presumptive positive specimens requires additional testing according to the latest CDC guideline for the diagnosis of Zika virus infection. Within the United States and its territories, laboratories are required to report presumptive positive results to the appropriate public health authorities.

Results of this test cannot be used as the sole basis of patient management decisions and must be combined with clinical observations, patient history, epidemiological information, and other laboratory evidences. Zika IgM levels over the course of illness are not well characterized. IgM levels are variable, may be detectable near day four post onset of symptoms and persist up to approximately 12 weeks following initial infection.

Negative results do not preclude the possibility of Zika virus infection, past or present. Negative results may be seen in specimens collected before day four post onset of symptoms or after the window of detectable IgM closes.

The ZIKV *Detect*™ 2.0 IgM Capture ELISA is intended for use by trained laboratory personnel who are proficient in performing and interpreting immunoassays.

The ZIKV *Detect*™ 2.0 IgM Capture ELISA is only for use under the FDA's Emergency Use Authorization.

PROTOCOL USE LIMITATIONS

The ZIKV *Detect*™ 2.0 IgM Capture ELISA described here has not been extensively tested with clinical specimens. Modifications of these assays (i.e., use of platforms or chemistries other than those described) are not permitted.

SUMMARY AND EXPLANATION OF THE TEST

Zika virus disease (Zika) is a disease caused by Zika virus that is spread to people primarily through the bite of an infected mosquito from the *Aedes* genus, mainly *Aedes aegypti* in tropical regions. This is the same mosquito that transmits dengue, chikungunya, and yellow fever. Common symptoms of Zika are fever, rash, joint pain, and conjunctivitis (red eyes). The illness is usually mild with symptoms lasting for several days to a week after being bitten by an infected mosquito. The Zika virus was first discovered in 1947 and is named after the Zika forest in Uganda. In 1952, the first human cases of Zika were detected and since then, outbreaks of Zika have been reported in tropical Africa, Southeast Asia, and the Pacific Islands. Zika outbreaks have probably occurred in many locations but remain unrecognized because the symptoms are similar to many other diseases such as dengue and chikungunya. In May 2015, the Pan American Health Organization (PAHO) issued an alert regarding the first confirmed Zika virus infection in Brazil and on Feb. 1, 2016, the World Health Organization (WHO) declared Zika virus a public health emergency of international concern (PHEIC). Local transmission has been reported in many other countries and territories. Of major concern is the effect the Zika virus may have on pregnant women. Infection with Zika virus during pregnancy

has been linked to congenital microcephaly and other brain defects in fetuses and infants. Sexual transmission of Zika virus is also of great concern and cases of individuals contracting the disease from their partners in the United States have been reported.

The ZIKV *Detect*™ 2.0 IgM Capture ELISA tests for IgM antibodies in human serum.

PRINCIPLE OF THE TEST

The ZIKV *Detect*™ 2.0 IgM Capture ELISA is an enzyme linked capture immunoassay for the detection of human IgM antibodies targeting 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 (see Example Plate Layout). 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 incubation and washing, a Ready-To-Use secondary antibody solution is added to each well. After a subsequent incubation and wash steps, an enzyme conjugate solution comprising horseradish peroxidase-labeled anti-mouse 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, secondary antibody, and conjugate. If IgM antibodies targeting the ZIKV envelope glycoproteins are not present, then the antigen, antibody, and conjugate are washed away.

The analysis of the results incorporates both the raw OD_{450} values and the ratios that compare the reactivity of a specimen with a given antigen in order to properly categorize the sample.

MATERIALS SUPPLIED

Warning: Do not use any reagents where damage to the packaging has occurred.

The ZIKV *Detect*™ 2.0 IgM Capture ELISA contains sufficient reagents for one plate of 96 wells (12 x 8 strips) for human IgM targeting Zika virus. This is sufficient for testing a maximum of 28 unknown samples for human IgM, with controls included in duplicate.

Below is a list of the kit contents.

1. **Coated Microtiter Test Strips for IgM (1 plate containing twelve 1x8 strips for human IgM):** ELISA plate strip holder with 96 (12x8 strips) polystyrene microtiter wells pre-coated with capture antibodies specific for human IgM. Store at 2-8°C until expiry.
2. **ZIKV IgM Negative Control (1x50µL):** The negative control aids in verifying the validity of the kit. Store at 2-8°C until expiry. Centrifuge briefly prior to use to sediment any precipitate.
3. **ZIKV IgM Positive Control (1x50µL):** The positive control aids in verifying the validity of the kit. Store at 2-8°C until expiry. Centrifuge briefly prior to use to sediment any precipitate.
4. **ZIKV Sample Dilution Buffer (1x25mL):** This buffer solution is used for diluting all serum samples and controls prior to testing in the ELISA. Store at 2-8°C until expiry.
5. **Ready-To-Use ZIKV Recombinant Antigen for IgM (1x3mL):** This vial contains ready-to-use (RTU) ZIKV antigen (Zika Ag) that comprises the Zika envelope glycoproteins. Store at 2-8°C until expiry.
6. **Cross-reactive Control Antigen for ZIKV IgM (1x3mL):** This vial contains a cross-reactive control antigen (CCA) cocktail. This is used to aid in the interpretation of the ELISA results. Store at 2-8°C until expiry.
7. **Normal Cell Antigen for ZIKV IgM (1x3mL):** This vial contains a normal control antigen (NCA). This is used to aid in the interpretation of the ELISA results. Store at 2-8°C until expiry.
8. **Ready-To-Use Secondary Antibody (1x9mL):** This vial contains secondary antibodies targeting the flavivirus antigens. Store at 2-8°C until expiry.
9. **100X Conjugate for ZIKV IgM (1x150µL):** This vial contains horseradish peroxidase-labeled anti-mouse antibody. Mix well prior to use. The 100X Conjugate is added to the Conjugate Diluent before use. Store the undiluted 100X conjugate at 2-8°C until expiry.
10. **Conjugate Diluent for ZIKV (1x9mL):** This solution is used to dilute the 100X conjugate before adding to the ELISA plate. Store at 2-8°C until expiry.
11. **10X Wash Buffer (1x120mL):** One bottle of 10X concentrated Wash Buffer is used as directed in Test Procedure. Store at 2-8°C until expiry.
12. **Liquid TMB Substrate (1x12mL):** Chromogenic substrate that reacts to horseradish peroxidase to generate the optical signal measured by the ELISA spectrophotometer. The substrate is light sensitive. Store at 2-8°C until expiry.
13. **Stop Solution (1x9mL):** Is used to terminate the reaction as directed in the Test Procedure. Store at 2-8°C until expiry.

Caution: strong acid, wear protective gloves, mask and safety glasses. Dispose of all the materials according to safety rules and regulations.

MATERIALS AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

- ELISA Spectrophotometer capable of absorbance measurement at 450 nm
- Biological or High-Grade Water
- Vacuum Pump
- Plate Washer
- 37°C Incubator without CO_2 supply or humidification
- 1-10 µL Single-Channel Pipettors, 50-200 µL Single-and Multi-Channel Pipettors.
- Filtered Pipette tips - recommended to reduce cross contamination
- Polypropylene tubes
- Adhesive plate cover or plastic plate cover
- Timer
- Vortex

WARNING AND PRECAUTIONS

FOR *IN VITRO* DIAGNOSTIC USE under the Emergency Use Authorization only. A thorough understanding of the instructions for use is necessary for successful use of the product. Reliable results will only be obtained by using precise laboratory techniques and accurately following these instructions for use.

Note: In the case of specimens originating from regions with a known West Nile virus outbreak, an FDA-cleared West Nile virus IgM assay should be run in parallel with the ZIKV *Detect*™ 2.0 IgM Capture ELISA.

SAFETY PRECAUTIONS

It is recommended that laboratories perform a risk assessment when conducting new tests and safety precautions should be based on the laboratory's risk assessment. If infection with chikungunya virus may be possible, then personnel should recognize that chikungunya virus produces high levels of viremia and serum from suspected chikungunya virus cases should be treated as potentially infectious even for serological procedures. Please review CDC guidance for state and local public health laboratories: http://www.cdc.gov/zika/state-labs/index.html

See the Biosafety in Microbiological and Biomedical Laboratories (BMBL) for additional biosafety information about these viruses and laboratory biosafety practices.

This procedure should be performed under laboratory safety conditions that take into consideration the potential infectious nature of the serum specimens involved. At a minimum, following heat inactivation, it is recommended that these procedures be performed using BSL-2 facilities and BSL-3 practices. To ensure safety of laboratory personnel, perform all sample manipulations within a Class II (or higher) Biological Safety Laboratory (BSL).

- All human source materials used in the preparation of controls have been either heat-inactivated or tested negative for antibodies to HIV 1&2, Hepatitis C and Hepatitis B surface antigen. However, no test method can ensure 100% efficiency. Therefore, all human controls and antigen should be handled as potentially infectious material. The Centers for Disease Control and Prevention and the National Institutes of Health recommend that potentially infectious agents be handled at Biosafety Level 2.
- Wear protective clothing, eye protection, and disposable gloves while performing the assay. Wash hands thoroughly afterwards.
- Do not eat, drink, smoke, or apply cosmetics where immunodiagnostic materials are being handled.
- Do not pipette by mouth.

TECHNICAL PRECAUTIONS

- This test must be performed on serum only. The use of whole blood, plasma or other specimen matrix has not been validated.
- Do not mix various lots of any kit component within an individual assay.
- All reagents must be equilibrated to room temperature (20-25°C) before commencing the assay. The assay will be affected by temperature changes.
- Avoid repeated freezing and thawing of the serum specimens to be evaluated.

- While diluting the controls and test sera in sample dilution buffer for use in ELISA testing, it is critical that a **new pipette tip** be used for each sample to avoid cross contamination. Take care to ensure the shaft of the pipette does not come into contact with the sample and/or sample dilution buffer. Filter pipette tips are recommended to further reduce the chance of contamination.
- **All reagents are susceptible to contamination,** thus, it is advisable to dispense reagents directly from bottles using clean pipettes or by carefully pouring. Pipettes should be used **only once** to avoid contamination of the components.
- Unused microwells must be resealed immediately and stored in the presence of desiccant. Failure to do so may cause erroneous results with those unused microwells.
- Do not use any component beyond the expiration date shown on its label.
- Avoid exposure of the reagents to excessive heat or direct sunlight during storage and incubation.
- Do not use a humidified incubator or a water bath for 37°C incubation steps. Doing so may lead to erroneous results.
- Some reagents may form a slight precipitate, mix gently before use.
- Incomplete washing will adversely affect the outcome and assay performance.
- To minimize potential assay drift due to variation in the substrate incubation time, care should be taken to add the stop solution into the wells in the same order and speed used to add the TMB solution.
- Avoid microbial contamination of reagents.
- Cover working area with disposable absorbent paper.

WARNING:
POTENTIALLY BIOHAZARDOUS MATERIAL

This kit contains reagents made with human serum or plasma. The serum or plasma used has been heat inactivated unless otherwise stated. Handle all sera and kits used as if they contain infectious agents. Observe established precautions against microbiological hazards while performing all procedures and follow the standard procedures for proper disposal of specimens.

SPECIMEN COLLECTION AND PREPARATION

- Only serum should be used for this assay, and the usual precautions for venipuncture should be observed. Blood obtained by venipuncture should be allowed to clot at room temperature (20-25°C) for 30 to 60 minutes and then centrifuged according to the Clinical and Laboratory Standards Institute recommendations (CLSI Approved Guideline – Procedures for the Handling and Processing of Blood Specimens for Common Laboratory Tests).
- Testing should be performed as soon as possible after collection. Do not leave sera at room temperature for prolonged periods. Separated serum should remain at 20-25°C for no longer than 8 hours. If assays are not completed within 8 hours, serum should be refrigerated at 2-8°C. If assays are not completed within 48 hours, or the separated serum is to be stored beyond 48 hours, serum should be frozen at or below -20°C.
- Avoid repeated freezing and thawing of samples since this can cause analyte deterioration. Frost-free freezers are not suitable for sample storage.
- Frozen samples should be thawed to room temperature and mixed thoroughly by gentle swirling or inversion prior to use. Always quick spin before use.
- If sera are to be shipped, they should be packed in compliance with Federal Regulations covering transportation of infectious agents.
- Do not use sera if any indication of microbial growth is observed.

TEST PROCEDURE

CAUTION: *The test procedure must be strictly followed. Any deviations from the procedure may produce erroneous results. Bring all kit reagents and specimens to room temperature (20-25°C) before use. Thoroughly mix the reagents and samples before use by gentle inversion. For long-term storage, serum samples should not be repeatedly thawed and frozen more than three times. Sera should be further aliquoted in a smaller volume and stored at -20°C or colder.*

PREPARATION OF REAGENTS

- *Preparation of 1X Wash Buffer:*

Dilute the 10X Wash Buffer to 1X using Biological or High-Grade Water. To prepare a 1X wash buffer solution, mix 120 mL 10X Wash Buffer with 1080 mL distilled (or deionized) water and rinse out any crystals. Swirl until well mixed and all crystals are dissolved. After diluting to 1X, store at room temperature for up to 6 months. Check for contamination prior to use. Discard if contamination is suspected.

- *Microtiter Strip Wells:*

Select the number of coated wells required for the assay. The remaining unused wells should be placed back into the pouch quickly, sealed, and stored at 2-8°C until ready to use or expiration.

- *Preparation of Conjugate Solution:*

Add 90 µL of 100X Conjugate for ZIKV IgM directly to the 9 mL bottle of Conjugate Diluent for ZIKV (1 part : 100 parts). Mix by inverting solution several times. Please note that smaller volumes of the 100X Conjugate may be diluted into the corresponding volume of Conjugate Diluent (1 part: 100 parts). The Conjugate Solution should be prepared fresh with every assay that is performed. Undiluted 100X Conjugate for ZIKV IgM that is stored at 2-8°C is stable for the duration of the kit shelf life.

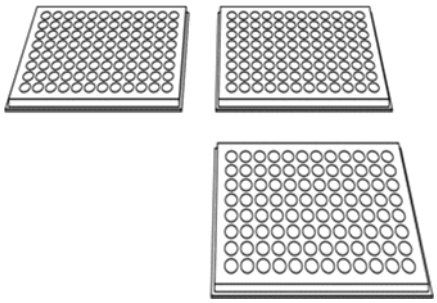
ASSAY PROCEDURE

1. Positive and negative controls must be assayed in duplicate with the Zika Ag, CCA and NCA portions of assay. Unknown serum samples to be tested are assayed singly and must be assayed with the Zika Ag, CCA, and NCA. See the Example Plate Layout at the end of these instructions for use.
2. Mark the microtiter strips to be used.
3. Using a new pipette tip each time, dilute test sera and controls to 1/100 using the provided Sample Dilution Buffer. Take care to avoid contamination due to aerosols or contamination of the pipette. Use small polypropylene tubes for these dilutions and at least 4 µL of sera and positive and negative controls. Place the full volume of Sample Dilution Buffer into the polypropylene tube first and then add the sera and controls. For example: place 396 µL of ZIKV Sample Dilution Buffer into a tube and add 4 µL of serum sample to make a 1/100 dilution. Do not use a repeat pipettor at any point during the sample dilution process. Make sure the specimen is thoroughly and evenly mixed into the sample dilution buffer. This may be done by either vortexing/inverting the dilution tube or by pipetting up and down at least 8 times using > 100 µL mixing volumes. If the dilution tube is vortexed, briefly spin the tube in a centrifuge to ensure no liquid aerosolizes once the tube is opened.
4. Apply 50 µL per well of 1/100 diluted test sera, ZIKV IgM Negative Control, and ZIKV IgM Positive Control to the plate by single or multi-channel pipettor as appropriate. Apply specimens at the appropriate locations on the plate, taking care to avoid bubbles. Cover the plate with an adhesive plate cover or with a plastic plate cover just on the well opening surface, so the bottom of the plate is not covered.
5. Incubate the plate at 37°C (± 2ºC) for **1 hour** (± 5 minutes) in an incubator.

***Note**: Do not stack plates on top of each other. They should be spread out as a single layer. This is very important for even temperature distribution. Do not use CO_2 or other gas incubators. Do not place plates in contact with any wet substances such as wet paper towels*

INCORRECT METHOD

CORRECT METHOD



6. After the incubation, wash the plate 6 times with an automatic plate washer using 1X wash buffer. Use 300 µL per well in each wash cycle.
7. Add 50µL per well of Zika Ag, 50µL per well of CCA and 50µL per well of NCA by multi-channel pipettor into the appropriate wells. Please see the **Example Plate Layout** for a sample method of sample placement and antigen addition.
    a. Cover the plate with an adhesive plate cover or with a plastic plate cover just on the well opening surface. The bottom of the plate should not be covered (see step 4).
    b. Incubate the plate at 37°C (± 2ºC) for **1 hour** (± 5 minutes) in an incubator (see step 5).
    c. After the incubation, wash the plate 6 times with an automatic plate washer using 1X wash buffer. Use 300 µL per well in each wash cycle.
8. Add 50µL per well of Ready-To-Use Secondary Antibody solution into all wells using a multi-channel pipettor.
    a. Cover the plate with an adhesive plate cover or with a plastic plate cover just on the well opening surface. The bottom of the plate should not be covered (see step 4).
    b. Incubate the plate at 37°C (± 2ºC) for **30 minutes** (± 2 minutes) in an incubator (see step 5).
    c. After the incubation, wash the plate 6 times with an automatic plate washer using 1X wash buffer. Use 300 µL per well in each wash cycle.
9. Prepare a fresh volume of Conjugate Solution (see Preparation of Reagents section) by diluting the appropriate volumes of 100X Enzyme Conjugate into the Conjugate Diluent (1 part : 100 parts).
10. Add 50 µL per well of Conjugate Solution into all wells by multi-channel pipettor.
    a. Cover the plate with parafilm or with plastic plate cover just on the well opening surface. The bottom of the plate should not be covered (see step 4).
    b. Incubate the plate at 37ºC (± 2ºC) for **30 minutes** (± 2 minutes) in an incubator (see step 5).
    c. After the incubation, wash the plate 6 times with an automatic plate washer using 1X wash buffer. Use 300 µL per well in each wash cycle.
11. Add 75 µL/well of Liquid TMB substrate into all wells using a multi-channel pipettor.
12. Incubate the plate at room temperature (20-25°C) in a dark place (or container) for **20 minutes** (± 30 seconds) **without any cover on the plate.**
13. After the incubation, add 50µL/well of Stop solution into all wells by multi-channel pipettor and incubate at room temperature for a minimum of 1 minute without a cover on the plate, and then proceed with reading the optical density. Optical densities must be read within 30 minutes of stop solution addition, as optical densities may begin to change over an extended period of time.
14. After the incubation, read the **RAW** OD 450 nm (optical density at 450 nm) value with a microplate reader. **Do NOT subtract or normalize for any blank values or wells. Do NOT use a reference wavelength**. This may result in low CCA and NCA values and incorrect ISR values.

******Please make sure the microplate reader does NOT subtract or normalize for any blank values or wells.*** ***

QUALITY CONTROL AND EXAMPLE

The control material to be used with the ZIKV *Detect*™ 2.0 IgM Capture ELISA test includes positive and negative control samples. Positive and negative controls must be run in duplicate on each plate tested. Acceptable Zika Immune Status Ratio (Zika ISR) values for these controls are shown below. The negative and positive controls are intended to monitor for substantial reagent failure. In addition, the negative control provides information for the acceptable raw OD limits for potentially Zika positive specimens. The test is invalid and must be repeated if either of the controls do not meet the specifications. If the test is invalid, patient results cannot be reported. Quality Control (QC) requirements must be performed in conformance with local, state, and/or federal regulations or accreditation requirements and the user's own laboratory's standard QC procedures. It is recommended that the user refer to CLSI C24-A and 42 CFR 493.1256 for guidance on appropriate Quality Control practices.

The raw materials used in the positive and negative controls are purchased through various commercial sera vendors. However, these sera are processed and titrated by InBios International for each ZIKV *Detect*™ 2.0 IgM Capture ELISA kit lot. Users must use the controls provided by InBios to validate all runs.

The results below are given strictly for guidance purposes only. Analysis is applicable when using RAW spectrophotometric readings only and where automatic subtraction of water or reagent blanks is not employed.

Calculation of the Negative Control: Calculate the average ZIKV IgM Negative Control values with Zika Ag, CCA, and NCA:

ZIKV IgM Negative Control Example

	OD_{450}		
	Zika Ag	CCA	NCA
Replicate 1	0.076	0.065	0.054
Replicate 2	0.071	0.073	0.068
Sum	0.147	0.138	0.122

Average Zika Ag = 0.147 ÷ 2 = 0.0735
Average CCA = 0.138 ÷ 2 = 0.069
Average NCA = 0.122 ÷ 2 = 0.061

Use the average values to perform the following calculations:

Calculate the Zika Ag/CCA Ratio (Zika ISR) ≡ Zika Ag ÷ CCA:

0.0735 ÷ 0.069 = 1.065

Calculate the CCA/NCA Ratio ≡ CCA ÷ NCA:

0.069 ÷ 0.061 = 1.131

Calculation of the Positive Control: Calculate ZIKV IgM Positive Control values with Zika Ag, CCA and NCA:

ZIKV IgM Positive Control Example

	OD_{450}		
	Zika Ag	CCA	NCA
Replicate 1	1.121	0.160	0.121
Replicate 2	1.205	0.152	0.105
Sum	2.326	0.312	0.226

Average Zika Ag = 2.326 ÷ 2 = 1.163
Average CCA = 0.312 ÷ 2 = 0.156
Average NCA = 0.226 ÷ 2 = 0.113

Use the average values to perform the following calculations:

Calculate the Zika Ag/CCA Ratio (Zika ISR) ≡ Zika Ag ÷ CCA:

1.163 ÷ 0.156 = 7.455

Calculation of the Threshold Zika Ag OD_{450}: Calculate a raw OD_{450} threshold. Samples must have Zika Ag OD_{450} values equal to or greater than this threshold in order to be considered positive for Zika IgM antibodies.

Threshold Zika Ag OD_{450} Example

The Threshold Zika Ag OD_{450} is equal to the average Zika Ag OD_{450} obtained with the Negative Control sample + 0.130.

That is,

Threshold Zika Ag OD_{450} = 0.130 + average Zika Ag OD_{450} of the Negative Control

In the example from above, the average Zika Ag OD450 for the Negative Control = 0.0735.

Therefore, in this case:

Threshold Zika Ag OD_{450} = 0.130 + 0.0735 = 0.2035

QC CRITERIA

The values in the table below must be obtained in order to report results of the assay. Non-fulfillment of these criteria is an indication of deterioration of reagents or an error in the test procedure and the assay must be repeated.

Factor (For Assay Verification)	Tolerance
Average Negative Control OD_{450} with Zika Antigen, CCA, and NCA	< 0.120
Average Negative Control OD_{450} with Zika Antigen	> 0.030
Average Positive Control OD_{450} in Zika Antigen	> 0.500
Positive Control Zika Immune Status Ratio (Zika ISR)	≥ 4.00
Negative Control Zika Immune Status Ratio (Zika ISR)	< 2.00
Negative Control CCA/NCA Ratio	< 2.00

Note the strict requirement that the average Negative Control OD_{450} with the Zika Antigen must be between 0.030 – 0.120. Due to this acceptance range for the Negative Control, this indicates that the *Threshold* Zika Ag OD_{450} must range from 0.160 – 0.250 (*Threshold Zika Ag OD_{450} = 0.13 + Zika Ag OD_{450} for the Negative Control).*

INTERPRETATION OF RESULTS

The ZIKV *Detect*™ 2.0 IgM Capture ELISA kit provides critical controls in order to aid in the discrimination between those specimens that have IgM antibodies to Zika virus and those specimens that may have IgM antibodies targeting a related flavivirus.

The ZIKV *Detect*™ 2.0 IgM Capture ELISA classifies a sample into three possible categories:

1) Reactive for Zika IgM Antibodies
2) Reactive for Other Flavivirus IgM Antibodies
3) Negative

For clarity, we provide definitions of relevant terms below:

DEFINITIONS

Zika Ag OD_{450}: This is the raw OD_{450} value obtained with a specimen using the Zika Antigen.

CCA OD_{450}: This is the raw OD_{450} value obtained with a specimen using the Cross-reactive Control Antigen (CCA).

NCA OD_{450}: This is the raw OD_{450} value obtained with a specimen using the Normal Cell Antigen (NCA).

Zika ISR: This is the ratio of the Zika Ag OD_{450} to the CCA OD_{450}. That is, Zika ISR = Zika Ag OD_{450} ÷ CCA OD_{450}.

CCA/NCA ratio: This is the ratio of the CCA OD_{450} to the NCA OD_{450}. That is, CCA OD_{450} ÷ NCA OD_{450}.

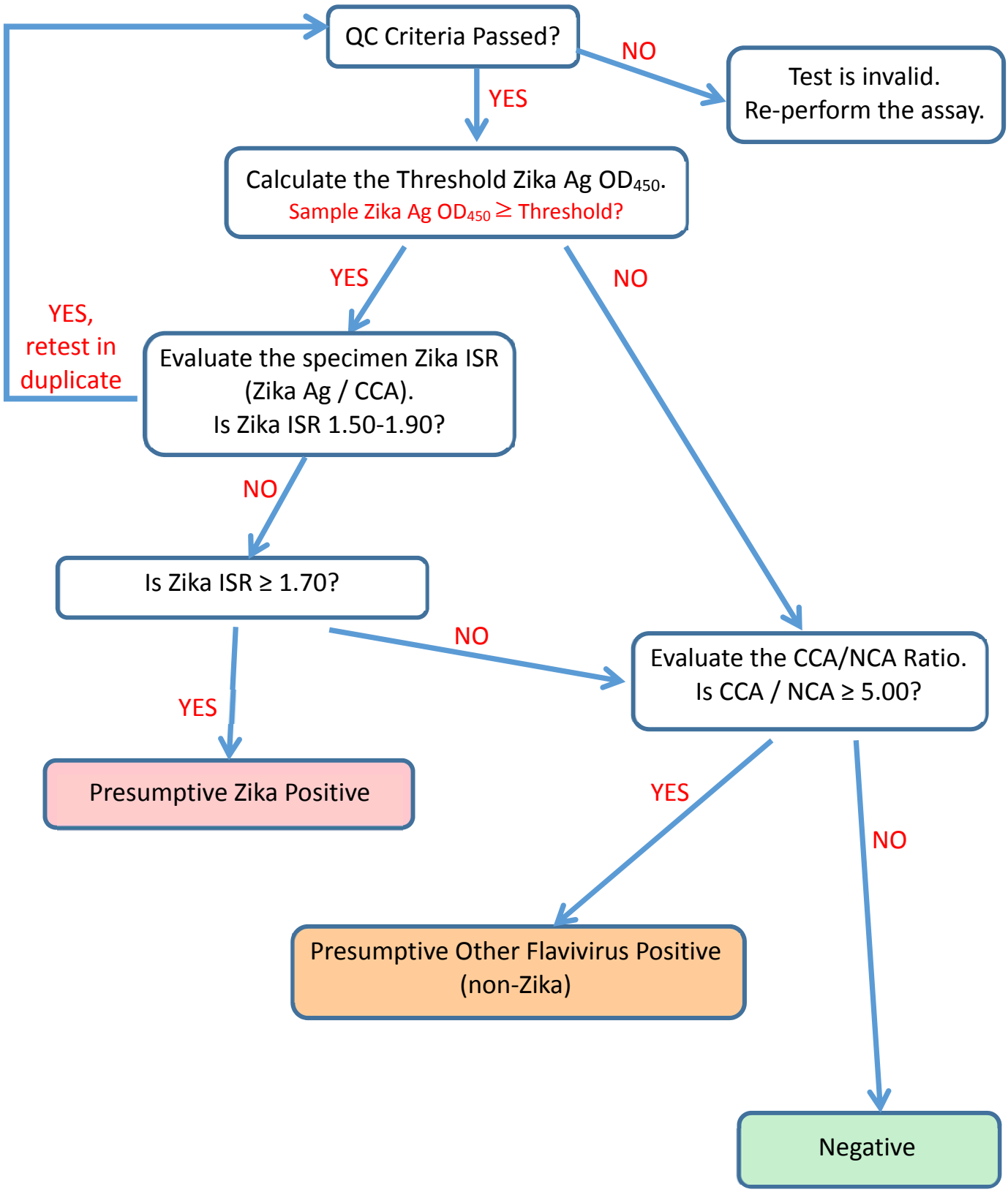
Threshold Zika Ag OD_{450}: This is equal to 0.130 + the average OD_{450} value of the Negative Control with the Zika Antigen.

Properly interpreting specimen data includes the following steps:

(1) Ensure that the QC Criteria are met.
(2) Determine the Threshold Zika Ag OD_{450}.
(3) Calculate the Zika ISR value and CCA / NCA ratio for each specimen.
(4) Determine if the specimen requires duplicate repeat testing: If the Zika antigen raw OD_{450} value ≥ Threshold OD_{450} AND 1.50 ≤ Zika ISR ≤ 1.90, then the sample must be re-tested in duplicate. The average re-test value (OD, ISR and ratio) should then be considered the final value and the rest of the analysis can be followed.
(5) If the specimen has a Zika ISR value ≥ 1.70 **AND** the specimen Zika Ag OD_{450} is ≥ *Threshold Zika Ag OD_{450},* then the specimen is considered **Presumptive Zika Positive** and the interpretation is completed for this specimen.
(6) Otherwise (if the specimen is NOT Presumptive Zika Positive), evaluate the CCA / NCA ratio. If the CCA / NCA ratio is ≥ 5.00, then the specimen is considered **Presumptive Other Flavivirus Positive (non-Zika)** and the interpretation is completed for this specimen.
(7) Otherwise (if the specimen is NOT Presumptive Zika Positive and NOT Presumptive Other Flavivirus Positive (non-Zika)), the specimen is considered **Negative**.

QUICK REFERENCE GUIDE FOR SAMPLE INTERPRETATION

START HERE



Zika Interpretation Table

Result Interpretation and Follow-up Testing	
Final Interpretation*	**Follow-up Testing**
Presumptive Zika Positive	The result should be confirmed by the latest CDC testing algorithms**.
Presumptive Other Flavivirus Positive (non-Zika)	The result should be confirmed by the latest CDC testing algorithms**. The result should be confirmed with FDA-cleared Dengue, West Nile virus or other appropriate IgM devices.
Negative	None#

*All Zika virus IgM detected and Flavivirus IgM detected results are presumptive positive results.
** For information regarding Zika testing algorithms, please refer to CDC guidance for state and local public health laboratories: https://www.cdc.gov/zika/laboratories/index.html
# Negative results with specimens collected before 8 days after onset of symptoms should be repeated with a later bleed taken at least 7 days from the first specimen. In addition, in the case of pregnant women please follow the latest *Interim Guidance for Health Care Providers Caring for Pregnant Women with Possible Zika Virus* regarding clinical management of negative results (https://www.cdc.gov/zika/hc-providers/index.html).

For additional clarity, we provide three example specimens with sample data for evaluation:

	Zika Ag OD_{450}	CCA OD_{450}	NCA OD_{450}
Positive Control – Replicate #1	1.640	0.165	0.075
Positive Control – Replicate #2	1.584	0.184	0.081
Negative Control – Replicate #1	0.081	0.064	0.062
Negative Control – Replicate #2	0.071	0.066	0.069
Sample #1	1.379	0.085	0.062
Sample #2	0.120	0.946	0.049
Sample #3	0.114	0.099	0.108

Step 1: *Evaluate the QC Criteria*

Evaluating the QC Criteria, we find the following:

Factor (For Assay Verification)	Tolerance	Calculated Value	Criteria met? (Yes/No)
Average Negative Control OD_{450} with Zika Antigen, CCA and NCA	< 0.120	0.076 / 0.065 / 0.0655	Yes
Average Negative Control OD_{450} with Zika Antigen	> 0.030	0.076	Yes
Average Positive Control OD_{450} in Zika Antigen	> 0.500	1.612	Yes
Positive Control Zika Immune Status Ratio (Zika ISR)	≥ 4.00	9.24 (1.612÷0.1745)	Yes
Negative Control Zika Immune Status Ratio (Zika ISR)	< 2.00	1.17 (0.076÷0.065)	Yes
Negative Control CCA/NCA Ratio	< 2.00	0.992 (0.065÷0.0655)	Yes

The criteria were met, so we may continue the analysis.

Step 2: *Determine the Threshold Zika Ag OD_{450}*

We next calculate the Threshold Zika Ag OD_{450} = 0.130 + Average Negative Control OD_{450} with the Zika Antigen. That is **Threshold Zika Ag OD_{450}** = 0.130 + 0.076 = **0.206.**

Step 3: *Calculate the Zika ISR and CCA / NCA ratio*

The Zika ISR and CCA ÷ NCA ratios are calculated and shown below.

	Zika Ag OD_{450}	CCA OD_{450}	NCA OD_{450}	Zika ISR	CCA / NCA
Average Positive Control	1.612	0.1745	0.078	9.24	2.24
Average Negative Control	0.076	0.065	0.0655	1.17	0.99
Sample #1	1.379	0.085	0.062	16.22	1.37
Sample #2	0.120	0.946	0.049	0.13	19.31
Sample #3	0.114	0.099	0.108	1.15	0.92

Step 4: *Determine if the specimen requires duplicate repeat testing*

None of the samples meet both criteria that Zika antigen raw OD_{450} value ≥ Threshold OD_{450} AND 1.50 ≤ Zika ISR ≤ 1.90. Hence, no samples require duplicate repeat testing.

Step 5: *Categorize the Presumptive Zika Positive specimens*

Any specimen with a raw Zika Ag OD_{450} ≥ Threshold Zika Ag OD_{450} AND a Zika ISR ≥ 1.70 is considered Presumptive Zika Positive.

	Zika Ag OD_{450}	CCA OD_{450}	NCA OD_{450}	Zika ISR	CCA / NCA	Presumptive Zika Positive?
Average Positive Control	1.612	0.1745	0.078	9.24	2.24	YES
Average Negative Control	0.076	0.065	0.0655	1.17	0.99	NO
Sample #1	1.379	0.085	0.062	16.22	1.37	YES
Sample #2	0.120	0.946	0.049	0.13	19.31	NO
Sample #3	0.114	0.099	0.108	1.15	0.92	NO

Step 6: *Categorize the Presumptive Other Flavivirus (non-Zika) specimens*

We then evaluate all of the remaining specimens that are not categorized as Zika positive. If a (non-Zika) specimen has a CCA ÷ NCA ≥ 5.00, then the sample is considered Presumptive Other Flavivirus Positive (non-Zika).

	Zika Ag OD_{450}	CCA OD_{450}	NCA OD_{450}	Zika ISR	CCA / NCA	Presumptive Zika Positive?	Presumptive Other Flavivirus (non-Zika)?
Average Positive Control	1.612	0.1745	0.078	9.24	2.24	YES	N/A
Average Negative Control	0.076	0.065	0.0655	1.17	0.99	NO	NO
Sample #1	1.379	0.085	0.062	16.22	1.37	YES	N/A
Sample #2	0.120	0.946	0.049	0.13	19.31	NO	YES
Sample #3	0.114	0.099	0.108	1.15	0.92	NO	NO

Step 7: *Categorize the Negative specimens*

If a specimen is not categorized as Presumptive Zika Positive or as Presumptive Other Flavivirus (non-Zika), then the sample should be considered Negative. All specimens are now categorized and can be interpreted appropriately.

	Zika Ag OD_{450}	CCA OD_{450}	NCA OD_{450}	Zika ISR	CCA / NCA	Interpretation
Average Positive Control	1.612	0.1745	0.078	9.24	2.24	Presumptive Zika
Average Negative Control	0.076	0.065	0.0655	1.17	0.99	Negative
Sample #1	1.379	0.085	0.062	16.22	1.37	Presumptive Zika
Sample #2	0.120	0.946	0.049	0.13	19.31	Presumptive Other Flavivirus
Sample #3	0.114	0.099	0.108	1.15	0.92	Negative

LIMITATIONS

- This assay is for *in vitro* diagnostic use under FDA Emergency Use Authorization only and is limited to laboratories in the United States that are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. 263a, to perform high complexity tests, or by similarly qualified non-U.S. laboratories.
- All reactive samples must be confirmed by using the latest CDC guideline for diagnosis of Zika virus infection. Review the latest information on diagnosis of Zika virus disease at the CDC website: http://www.cdc.gov/zika/laboratories/lab-guidance.html.
- The presence of false positive and false negative results must be considered.
- Assay performance characteristics have not been established for visual result determination.
- Assay performance characteristics have not been established for matrices other than serum.
- Results from immunosuppressed patients must be interpreted with caution.
- Infection with Babesia may result in false positives.
- This test can cross-react with Yellow Fever Virus.
- High HAMA levels (>80 ng/mL) may result in false negatives.
- Elevated hemoglobin concentrations >20mg/mL may interfere with OD readings and hence result in false results; therefore hemolyzed samples should not be tested.
- Assay results should be interpreted by a trained professional only in the context of other laboratory findings, patient's history, and clinical signs and symptoms.

CONDITIONS FOR AUTHORIZATION FOR THE LABORATORY

The ZIKV *Detect*™ 2.0 IgM Capture ELISA Letter of Authorization, along with the authorized Fact Sheet for Healthcare Providers, the authorized Fact Sheet for Patients and authorized labeling are available on the FDA website: https://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm. Use of the ZIKV *Detect*™ 2.0 IgM Capture ELISA assay must follow the procedures outlined in these manufacturer's Instructions for Use and the conditions of authorization outlined in the Letter of Authorization. Deviations from the procedures outlined are not permitted under the Emergency Use Authorization. To assist clinical laboratories running the ZIKV *Detect*™ 2.0 IgM Capture ELISA assay, the relevant Conditions of Authorization are listed verbatim below.

- Authorized laboratories will include with reports of the results of the ZIKV *Detect*™ IgM Capture ELISA[1] the authorized Fact Sheet for Health Care Providers, the authorized Fact Sheet for Pregnant Women, and the authorized Fact Sheet for Patients, and any additional ZIKV *Detect*™ IgM Capture ELISA Fact Sheets for Health Care Providers, Pregnant Women[2], and Patients that OCET/OCS/OC and DMD/OIR/CDRH may authorize. Under exigent circumstances, other appropriate methods for disseminating these Fact Sheets may be used, which may include mass media.
- Authorized laboratories will perform the ZIKV *Detect*™ IgM Capture ELISA on serum or with other authorized specimen types.
- Within the United States and its territories, authorized laboratories will report all presumptive Zika positive, possible Zika positive[3], and presumptive other flavivirus positive (non-Zika) results to InBios.
- Authorized laboratories will have a process in place to assure that, for presumptive Zika positive, possible Zika positive[3], and presumptive other flavivirus positive (non-Zika) results, additional testing (as described in the Instructions for Use document) is performed and/or test results for other patient-matched specimens, using the latest CDC guideline for the diagnosis of Zika virus infection, are considered.
- Authorized laboratories will have a process in place for reporting test results to health care providers and relevant public health authorities, as appropriate.[4]
- Authorized laboratories will collect information on the performance of the assay and report to InBios any suspected occurrence of false negative results and significant deviations from the established performance characteristics of which they become aware.
- All laboratory personnel using the assay should be appropriately trained in performing and interpreting immunoassays techniques, use appropriate laboratory and personal protective equipment when handling this kit, and use the test in accordance with the authorized labeling.
- InBios, its authorized distributor(s), and authorized laboratories will ensure that any records associated with this EUA are maintained until notified by FDA. Such records will be made available to FDA for inspection upon request.

[1]Please note, subsequent to the original Letter of Authorization the name of the assay was updated from ZIKV *Detect*™ IgM Capture ELISA to ZIKV *Detect*™ 2.0 IgM Capture ELISA as of May 18, 2018.
[2]Please note, subsequent to the original Letter of Authorization the Pregnant Women and Patient Fact Sheets were combined into one Patient Fact Sheet as of March 27, 2017.
[3]Please note, subsequent to the original Letter of Authorization, as of May 18, 2018, the result interpretation was updated to report either Presumptive Zika Positive, Presumptive Other Flavivirus Positive (non-Zika) or Negative.
[4]For questions related to reporting Zika test results to relevant public health authorities, it is recommended that InBios and authorized laboratories consult with the applicable, country, state or territory health department(s) and/or CDC. According to CDC, Zika is a nationally notifiable condition. http://www.cdc.gov/zika/.

PERFORMANCE CHARACTERISTICS

Clinical evaluation to estimate sensitivity and specificity:

Sensitivity was estimated using archived Zika IgM positive specimens, including a limited number of samples from pregnant women. Specificity was estimated using a total of 70 non-endemic negative serum samples and 30 endemic negative specimens. 95% confidence intervals were calculated using the Wilson Score method.

		Sample status		
		Zika Positive	Negative	Total
InBios ZIKV *Detect*™ 2.0 IgM Capture ELISA	Zika Positive	55	0	55
	Other Flavivirus	1	0	1
	Negative	1	100	101
	Total	57	100	157

Positive Percent Agreement (for Zika IgM):	96.5% (55/57, 95% C.I. 88.1%-99.0%)
Negative Percent Agreement:	100.0% (100/100, 95% C.I. 96.3%-100%)

Cross-reactivity:

Cross-reactivity was evaluated by testing specimens from patients with confirmed IgM antibodies to other microorganisms which could potentially cause false positive results. This list is composed of organisms whose infection produces symptoms similar to those observed at the onset of Zika virus infection and also viral strains which have a significant likelihood to result in cross-reactivity due to genetic similarity with Zika virus. We have also included organisms/strains which are likely to be observed in the currently affected and endemic area (i.e., Brazil and South America) since these organisms/strains will be an important part of the differential diagnosis of Zika virus infection.

Category	Disease/ Infectious agent Positive Sera	# of samples	# Zika Reactive	# Other Flavivirus Reactive	# Non-Reactive	% False "Zika Reactive"	% False "Other Flavivirus Reactive"
Other disease present (IgM Positive)	Anti-Chikungunya virus	18	1	0	17	5.5%	0%
	Anti-Eastern equine encephalitis virus (EEEV)	3	0	0	3	0%	0%
	Anti-Cytomegalovirus (CMV)	7	0	0	7	0%	0%
	Anti-Epstein Barr Virus (EBV) –CA	6	0	0	6	0%	0%
	Anti-Parvovirus B19	5	0	0	5	0%	0%
	Anti-Varicella zoster virus	10	0	0	10	0%	0%
	Anti-nuclear Antibodies (ANA)	10	0	0	10	0%	0%
	Rheumatoid Factor	7	0	0	7	0%	0%
	HAMA (human anti-mouse antibody)	9	0	0	9	0%	0%
	Anti-Malaria/anti-*plasmodium falciparum*	10	0	0	10	0%	0%
	Anti-Hepatitis (C) virus	10	0	0	10	0%	0%
	Anti-Hepatitis (B) virus	16	0	0	16	0%	0%
	Anti-Hepatitis (A) virus	3	0	0	3	0%	0%
	Anti-HIV	5	0	0	5	0%	0%
	Anti-babesia	15	3	0	12	20.0%	0%
	Anti-Lyme	5	0	0	5	0%	0%
	Anti-toxoplasmosis	6	0	0	6	0%	0%
Flavivirus Specimens (non-Zika, IgM Positive)	Anti-Dengue virus	134	0	133	1	0%	0%
	Anti-West Nile Virus	64	0	62	2	0%	0%
	Anti-Japanese Encephalitis	1	0	0	1	0%	0%
	Anti-Saint Louis encephalitis (SLE)	10	0	1	9	0%	0%
Immunization to flavivirus	Yellow fever virus (YFV) post-immunization	25	6	0	19	24.0%*	0%
	Dengue virus post-immunization	20	0	0	20	0%	0%

*Of the 6 YFV vaccination samples that tested zika reactive on ZIKV *Detect*™ 2.0 IgM Capture ELISA, 2 (ARSFA 23S-3 and ARSFA 19P-5) were Zika positive and 2 (ARSFA 11P-3 and ARSFA 2S-4) were Zika suspect on CDC Zika MAC-ELISA. These 6 were among 16 samples collected from YFV immunized subjects in Colombia in 2016, where there was a possibility of exposure to Zika virus. 9/9 YFV immunized subjects from US tested negative on ZIKV *Detect*™ 2.0 IgM Capture ELISA.

Interference testing: Potentially interfering substances commonly occurring in serum were evaluated with the ZIKV *Detect*™ 2.0 IgM Capture ELISA. Interfering substances included conjugated and unconjugated bilirubin (0.4 mg/mL), hemoglobin (20 mg/mL), albumin (60 mg/mL), cholesterol (5 mg/mL), triglycerides (30 mg/mL), HAMA (~800 and ~80 ng/mL), and rheumatoid factor (2060 IU/mL). These interfering substances were spiked into low reactive (n=3) and normal human serum samples (n=3) to evaluate their impact on assay performance. Of the interfering substances tested, only very high levels of HAMA seemed to have a deleterious effect by decreasing Zika Ag reactivity, resulting in false negative results with the panel tested. At the lower HAMA concentration tested, no interference was observed.

Interfering Substance	Concentration Tested	Effect on Low Reactive Specimens	Effect on Negative Specimens
Bilirubin unconjugated	0.4 mg/mL	None observed (0/3)	None observed (0/3)
Bilirubin conjugated	0.4 mg/mL	None observed (0/3)	None observed (0/3)
Hemoglobin	20 mg/mL	None observed (0/3)	None observed (0/3)
Human Serum Albumin	60 mg/mL	None observed (0/3)	None observed (0/3)
Cholesterol	5 mg/mL	None observed (0/3)	None observed (0/3)
Intralipids (triglycerides)	30 mg/mL	None observed (0/3)	None observed (0/3)
HAMA	798.7 ng/mL	*Interference observed (3/3)*	None observed (0/3)
	79.9 ng/mL	None observed (0/3)	None observed (0/3)
RF	2060 IU/mL	None observed (0/3)	None observed (0/3)

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Example Plate Layout

An example plate layout is shown below which indicates a method for screening 28 specimens against Zika Ag, CCA and NCA.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Positive Control**	**Positive Control**	Sample #13	Sample #21	**Positive Control**	**Positive Control**	Sample #13	Sample #21	**Positive Control**	**Positive Control**	Sample #13	Sample #21
B	**Negative Control**	**Negative Control**	Sample #14	Sample #22	**Negative Control**	**Negative Control**	Sample #14	Sample #22	**Negative Control**	**Negative Control**	Sample #14	Sample #22
C	Sample #1	Sample #7	Sample #15	Sample #23	Sample #1	Sample #7	Sample #15	Sample #23	Sample #1	Sample #7	Sample #15	Sample #23
D	Sample #2	Sample #8	Sample #16	Sample #24	Sample #2	Sample #8	Sample #16	Sample #24	Sample #2	Sample #8	Sample #16	Sample #24
E	Sample #3	Sample #9	Sample #17	Sample #25	Sample #3	Sample #9	Sample #17	Sample #25	Sample #3	Sample #9	Sample #17	Sample #25
F	Sample #4	Sample #10	Sample #18	Sample #26	Sample #4	Sample #10	Sample #18	Sample #26	Sample #4	Sample #10	Sample #18	Sample #26
G	Sample #5	Sample #11	Sample #19	Sample #27	Sample #5	Sample #11	Sample #19	Sample #27	Sample #5	Sample #11	Sample #19	Sample #27
H	Sample #6	Sample #12	Sample #20	Sample #28	Sample #6	Sample #12	Sample #20	Sample #28	Sample #6	Sample #12	Sample #20	Sample #28
	Ready to Use ZIKV Antigen (Zika Ag)				Cross-reactive Control Antigen (CCA)				Normal Cell Antigen (NCA)			