Zika MAC-ELISA -

Centers for Disease Control and Prevention

For Use Under an Emergency Use Authorization Only

Instructions for Use

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Introduction

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 persons meeting Centers for Disease Control and Prevention (CDC) clinical and/or epidemiological criteria for Zika virus testing.

This test is only intended for use as described in the CDC Zika diagnostic testing guidance and under the Food and Drug Administration's (FDA) Emergency Use Authorization (EUA). Please refer to the CDC website for current laboratory guidance: http://www.cdc.gov/zika/state-labs/index.html

INTENDED USE

The CDC Zika MAC-ELISA is intended for the qualitative detection of Zika virus IgM antibodies in human sera or cerebrospinal fluid (CSF) that is submitted alongside a patient-matched serum specimen, 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., recent history of travel to geographic regions during a period of active Zika virus transmissions at the time of travel, or other epidemiologic criteria for which Zika virus testing may be indicated as part of a public health response). The assay is intended for use in qualified laboratories designated by the CDC, as a part of a multi-test algorithm.

Assay results are for the presumptive identification of IgM antibodies to Zika virus. Positive and equivocal results are not definitive for 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 equivocal or presumptive positive specimens requires additional testing by CDC, or by qualified laboratories designated by CDC in consultation with CDC, using the CDC-issued algorithm. Laboratories are required to report positive results to the appropriate public health authorities. Within the United States and its territories, equivocal and presumptive positive results must be reported to CDC by qualified laboratories designated by CDC.

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, but generally are positive starting near day four post onset of symptoms and continuing for 12 or more 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 Zika MAC-ELISA is intended for use by trained laboratory personnel who are proficient in performing and interpreting immunoassays in qualified laboratories designated by the CDC. The Zika MAC-ELISA is only for use under the FDA's EUA.

PROTOCOL USE LIMITATIONS

The MAC-ELISA assay 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) is not permitted. These assays should not be further distributed without the explicit consent of the CDC.

ASSAY PRINCIPLE

Assays that detect viral specific immunoglobulin M (IgM) are advantageous because they detect antibodies produced during the first few days after onset of clinical symptoms in a primary infection, obviating the need for convalescent-phase specimens in many cases. IgM capture is the optimum approach to IgM detection because it is simple, sensitive, and applicable to serum and cerebrospinal fluid (CSF) samples from a variety of animal species (*e.g.* human, equine, avian).

IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) provides a useful alternative to immunofluorescence for documentation of a serologic response. ELISA is less subjective than immunofluorescence and large numbers of samples can be processed. 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-viral 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).

Specimens

ACCEPTABLE SPECIMENS

- Acute and convalescent human serum
  NOTE: Serum should be collected in a serum separator tube. Tube should be centrifuged and serum decanted prior to shipment to avoid hemolysis.
- Cerebrospinal fluid (CSF) specimens
  CSF may only be tested when submitted alongside a patient-matched serum specimen

SPECIMEN HANDLING AND STORAGE

Store all diagnostic specimens at 2-8° C prior to testing, and ≤ -20° C after all anticipated testing has been completed. Avoid repeated freeze-thaw cycles.

Patient samples should be heat inactivated for 30 minutes in a 56° C water bath. If a possibility exists that chikungunya virus may be in the sample, inactivation should be extended to 2 hours.

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 laboratorians 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 cabinet (BSC).

Equipment and Consumables

DISCLAIMER: Names of vendors or manufacturers are provided as examples of suitable product sources. Use of trade names is for identification purposes only and does not constitute endorsement by CDC or the Department of Health and Human Services.

MATERIALS PROVIDED BY CDC

NOTE: These materials will be provided by CDC, Ft. Collins, CO. To request these reagents, please email Dr. Barbara Johnson at bfj9@cdc.gov

- **Normal Vero E6 Antigen (CDC catalog #AV0001);** Lyophilized normal antigen
- **Zika Vero E6 Tissue Culture Antigen (CDC catalog #AV0002 or AV0003;** these are antigens produced from different Zika strains; only one is required for the assay); Lyophilized Zika antigen (inactivated) prepared for use in Zika IgM ELISA.
- **Flavivirus IgM positive control (CDC catalog #AV0004):** Chimeric monoclonal antibody specific for Flavivirus; lyophilized.

Positive and negative assay controls should be run concurrently with all test samples.

MATERIALS REQUIRED BUT NOT PROVIDED

NOTE: for materials requiring dilution/titration, see **Formulations** below.

- Detecting antibody conjugate: Horseradish peroxidase conjugated monoclonal antibody 6B6C-1. Available from:
  - Hennessy Research, catalog #DC153-100 or
  - InBios (either item is acceptable),
    - Item 500510: 6B6C-1/HRP Conjugate (Undiluted), 50 uL

    - Item 500510D: 6B6C-1/HRP Conjugate (1/100 Diluted from Stock), 1mL
- Goat anti-human IgM (Kirkegaard and Perry Laboratories, catalog #01-10-03)
- Deionized water
- Hydrochloric acid (to adjust pH of coating buffer)
- Sodium carbonate (Na_2CO_3); (available from multiple commercial sources, e.g. Sigma, Thermo Fisher, etc.)
- Sodium bicarbonate ($NaHCO_3$); (available from multiple commercial sources, e.g. Sigma, Thermo Fisher, etc.)
- Phosphate buffered saline (PBS); (available from multiple commercial sources, e.g. Sigma, Thermo Fisher, etc.)
- Tween 20 (available from multiple commercial sources, e.g. Sigma, Thermo Fisher, etc.)
- Nonfat dry milk (available from multiple commercial sources, e.g. Sigma, Thermo Fisher, etc.)
- Sulfuric acid (H_2SO_4); (available from multiple commercial sources, e.g. Sigma, Thermo Fisher)
- Immulon II HB flat-bottomed 96 well plates, Dynatech Technologies catalog #3455 (available from multiple commercial sources, e.g. Sigma, Thermo Fisher, etc.)
  NOTE: This is the only 96 well plate approved for this assay.
- Enhanced K-Blue TMB substrate (3,3', 5, 5' tetramethylbenzidine base; Neogen Corp, catalog # 308175
- Normal human sera—tested negative for Zika virus antibodies

EQUIPMENT AND CONSUMABLES

- Microplate washer
- Microplate reader with 450 nm filter
- Biosafety cabinet (BSC)
- Incubator set at 37° C
- Single and multi-channel pipettors (100 µL and/or 200 µL Single Channel, 100 µL and/or 200 µL 12 channel)
- Pipet tips for listed pipettors
- Reagent reservoirs
- Timer
- Reagent mixing bottles; sterile 1L glass bottles; Gibco or alternate vendor
- Microfuge tubes to dilute patient serum; purchase sterile or autoclave and cool before use; Corning or alternate vendor
- Weigh boats for measuring dry chemical components, chemical resistant
- Permanent marker

Formulations

NOTE: Dilutions given are a starting point for titration. Laboratories must determine the optimum dilution for their individual laboratory. See additional information in Assay Standardization on page 13.

- Coating buffer: Carbonate/bicarbonate buffer, pH 9.6
  1.59 g Na_2CO_3 + 2.93 g $NaHCO_3$ diluted in 1L water.
- Wash buffer: Phosphate buffered saline (PBS); 0.05% Tween 20, pH 7.2.
  PBS is available in powdered form from multiple commercial sources
- Blocking buffer: PBS/ 5% milk/ 0.5% Tween 20
- Stop solution: 1 N H_2SO_4
- Detecting antibody conjugate: Conjugate can be diluted up to 1:5000 in blocking buffer
- Flavivirus IgM positive control: Flavivirus IgM positive control diluted up to 1:3000 in wash buffer
- Zika Vero E6 antigen: diluted up to 1:160 in wash buffer
- Normal Vero E6 antigen: diluted up to 1:160 in wash buffer
- Goat anti-human IgM: diluted 1:2000 in coating buffer (titration may be required)
- Patient serum: diluted 1:400 in wash buffer (no titration required)
- Negative control: Normal human sera diluted to 1:400 (no titration required)
    - New lots of normal human sera should be tested using this protocol as if they were experimental. If the OD on viral antigen is NOT 2X greater than the OD on normal antigen, it may be presumed to be negative.

Quality Control

GENERAL CONSIDERATIONS

- Personnel must be familiar with the protocol and instruments used.
- Wear clean, previously unworn, disposable gowns and new, powder-free gloves during assay reagent setup and handling. Change gloves whenever you suspect they may be contaminated.
- Store all reagents at appropriate temperatures (see product inserts). Do not use reagents beyond their expiration dates.
- Keep reagent tubes capped as much as possible.
- Use aerosol barrier (filter) pipette tips only.
- Empty all trash daily.

ASSAY CONTROLS

Assay controls should be run concurrently with all test samples.

Antibody controls:

- Positive control: Flavivirus IgM Positive Control
- Negative control: normal human serum.

Background Determination:

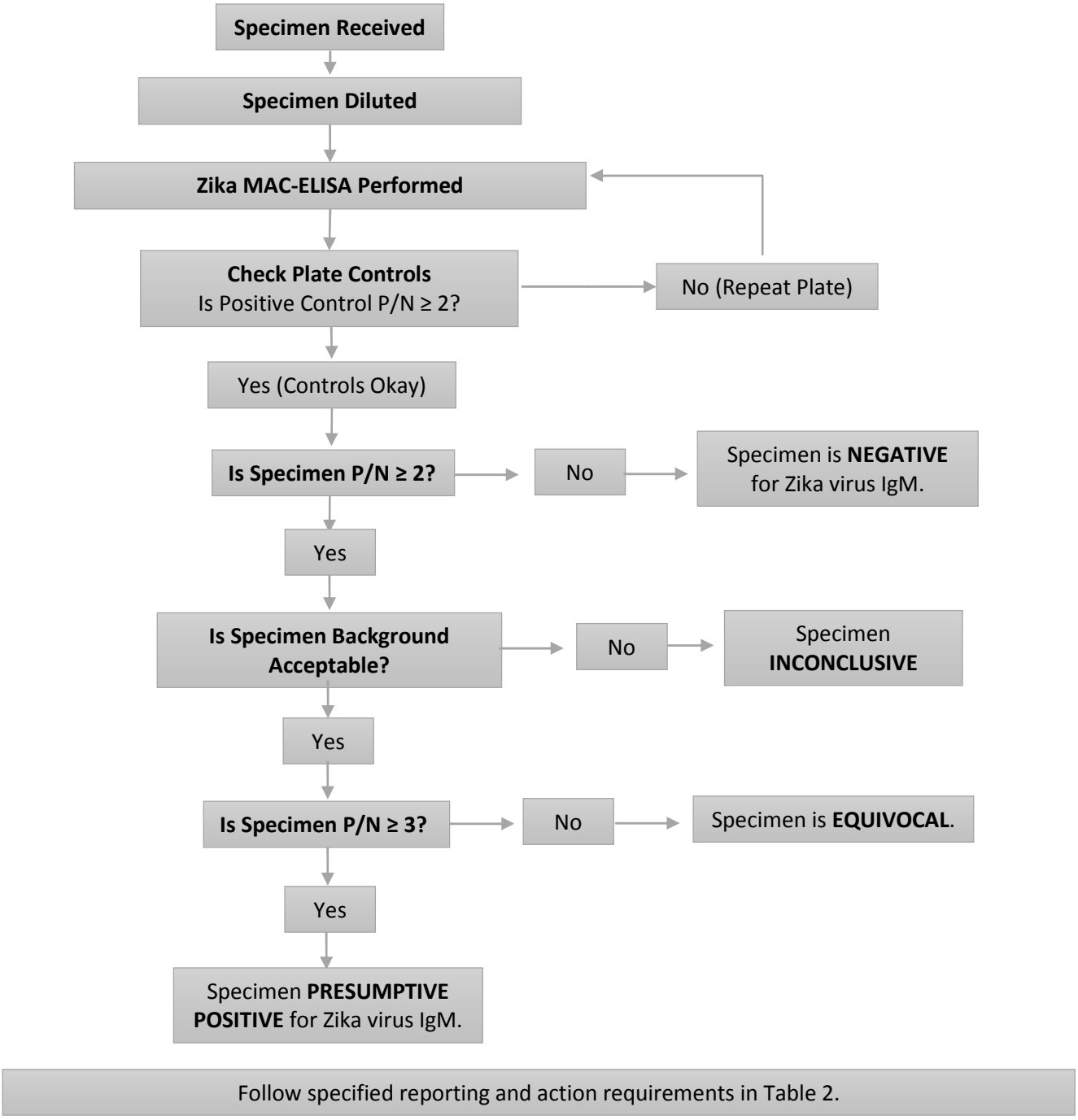
- Specimen reacted with Normal Vero E6 antigen (to measure background signal generated by the specimen).

Table 1: Overview of positive and negative controls

Calculation	Ratio	Result
Positive Control P/N	$\frac{\text{Mean OD of the positive control serum reacted with Zika Vero E6 antigen (P)}}{\text{Mean OD of the negative control serum reacted with Zika Vero E6 antigen (N)}}$	< 2 Plate IS NOT Valid
		≥ 2 Plate IS Valid
Specimen Background P/N (for specimens with Specimen P/N ≥2 See Figure 1)	$\frac{\text{Mean OD of the specimen reacted with Zika Vero E6 antigen (P)}}{\text{Mean OD of the specimen reacted with Normal Vero E6 antigen}}$	< 2 Specimen is Inconclusive
		≥ 2 Sample can be interpreted according to Testing Algorithm

Testing Algorithm

Figure 1: Summary of Test Results Interpretation



Follow specified reporting and action requirements in Table 2.

Zika MAC-ELISA Assay

NOTES REGARDING THE ELISA PROCEDURE:

- Plates can be coated and kept at 2-8° C for up to a week. (See Step 2: Coating the Plates, below).
- Undiluted control sera can be stored at 2-8° C for up to 2 weeks.
- Reconstituted, undiluted viral and Normal Vero E6 antigens can be stored at ≤ -20° C for an undefined period of time.
- Test and control sera can be diluted to the working dilutions and refrigerated one day prior to use.
- Antigens and conjugate *must be diluted to the working dilutions immediately prior to use.*

NOTE: THE FOLLOWING PROCEDURE INCLUDES INFORMATION ON QUALITY CONTROL AND INTERPRETATION. EACH SERUM SPECIMEN IS TESTED IN TRIPLICATE ON BOTH VIRAL AND NORMAL VERO E6 ANTIGENS. EIGHT (8) TEST SPECIMENS CAN BE ANALYZED PER PLATE. DUE TO LIMITED VOLUME, CSF SPECIMENS ARE USUALLY TESTED ONLY SINGLY.

1. PREPARING THE PLATE:
   Determine the number of ELISA plates needed. Using a fine-tipped permanent marker, number and label the 96-well plates. Identify the location of each clinical specimen (S1-S8) by using a corresponding template (see Fig. 2). *To keep timing of reagent addition consistent, process plates in the order that they are numbered during all steps of the procedure.* Plates should be kept in an enclosed, humidified environment during all incubation times with the exception of the coating step. A large Ziploc-type bag containing a moist paper towel works well for this purpose.
2. COATING THE PLATES:
   - Dilute goat anti-human IgM 1:2000 in coating buffer, pH 9.6.
   - Coat the inner 60 wells of the 96 well plate with 75 µL per well of diluted goat anti-human IgM. Leave outer rows/columns empty (see Fig. 2).
   - Incubate at **2-8°C overnight**. Plates should remain at 2-8°C until needed for testing, up to one week.
3. BLOCKING THE PLATES:
   - After overnight incubation, dump out the coating antibody.
   - Blot plates on paper towels or other absorbent material.
   - Block plates with 200 µL blocking buffer per well.
   - Incubate at **room temperature for 30 minutes**.
4. WASHING THE PLATES:
   - Wash wells 5X with wash buffer by using an automatic plate washer.
   - Wells should be filled to the top each cycle (i.e. 300 µL).
5. ADDITION OF SAMPLE/CONTROLS:
   - Dilute patient's serum 1:400 in wash buffer.

- Add 50 µL per well of the diluted patient's serum (S) to a block of 6 wells or undiluted CSF to two wells only. CSF will be tested singly against the viral and Normal Vero E6 antigens.
  NOTE: CSF can be diluted to a maximum of 1:5 in wash buffer if necessary to obtain enough volume for test.
- Add 50 µL Flavivirus IgM positive control (Ref) diluted in wash buffer according to a previously determined titration.
- Dilute negative human serum control (N) 1:400 in wash buffer.
- Add 50 µL diluted (1:400 in wash buffer) negative human serum control (N) to a block of 6 wells.
- Incubate plates for **1 hour at 37° C** in a humidified chamber.

6. WASHING THE PLATES:
   - Wash wells 5X with wash buffer by using an automatic plate washer.
   - Wells should be filled to the top each cycle.
7. ADDITION OF ANTIGEN:
   - Dilute **Zika** Vero E6 antigen in wash buffer according to a previously determined titration.
   - Dilute **Normal** Vero E6 antigen in wash buffer to the same concentration as the **Zika** Vero E6 antigen.
   - Add 50 µL per well of diluted **Zika** Vero E6 antigen to the left three wells of each serum block (see Fig 2).
   - Add 50 µL per well of diluted **Normal** Vero E6 antigen to the right three wells of each block (see Fig. 2).
   - Incubate plates **overnight at 2-8°C** in a humidified chamber.
8. WASHING THE PLATES:
   - Wash wells 5X with wash buffer by using an automatic plate washer.
   - Wells should be filled to the top each cycle.
9. ADDITION OF CONJUGATE:
   - Dilute horseradish peroxidase-conjugated monoclonal antibody in blocking buffer according to a previously determined titration.
   - Add 50 µL per well of diluted horseradish peroxidase-conjugated monoclonal antibody
   - Incubate plates for **1 hour at 37° C** in a humidified chamber.
10. Turn on plate reader to warm up
11. Remove TMB-ELISA from refrigerator.
12. WASHING THE PLATES:
    - Wash wells 5X **twice** with wash buffer by using an automatic plate washer.
    - Turn the plates 180^{o} in the washer after the first series of 5 cycles. This promotes consistent results.
    - Wells should be filled to the top each cycle.
13. ADDITION OF SUBSTRATE:
    - With the plate at room temperature (20-25°C), add 75 µL per well of TMB substrate to all wells.

- Immediately cover plates to block out light. Incubate at room temperature for 10 minutes.
- A blue color will develop in antibody-positive wells.

14. ADDITION OF STOP SOLUTION:
    - Add 50 µL per well of stop solution to all wells, including the outer rows of wells on the plate

      NOTE: The plate reader should be set to zero itself on some of these wells
    - The wells that were blue will now change to a yellow color.
    - Allow plates to sit at room temperature for 1 minute.
    - Read plates in microtiter plate reader by using a 450 nm filter.

	1	2	3	4	5	6	7	8	9	10	11	12
A	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY
B	EMPTY	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	EMPTY
C	EMPTY	Viral Antigen S1	Normal Antigen	Viral Antigen S3	Normal Antigen	Viral Antigen S5	Normal Antigen	Viral Antigen S7	Normal Antigen	Viral Antigen REF	Normal Antigen	EMPTY
D	EMPTY	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	EMPTY
E	EMPTY	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	EMPTY
F	EMPTY	Viral Antigen S2	Normal Antigen	Viral Antigen S4	Normal Antigen	Viral Antigen S6	Normal Antigen	Viral Antigen S8	Normal Antigen	Viral Antigen N	Normal Antigen	EMPTY
G	EMPTY	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	EMPTY
H	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY

Figure 2: Plate layout for 8 samples and controls

ASSAY STANDARDIZATION

The MAC-ELISA should be standardized and validated prior to use in the laboratory and re-standardization is required periodically. This should occur when new lot numbers of reagents are introduced, and at the very least, once a year. It is recommended that the mean optical density of the positive control serum reacted with the Zika Vero E6 antigen be set to approx. 1.0. The normal control serum reacted with the Zika Vero E6 antigen should be < 0.2 (this varies). The standardization of reagents is normally achieved via titration, always comparing the optical densities of the reagents when reacted on viral and Normal Vero E6 antigen. Standardization and re-standardization may be confirmed by testing verification panels.

Interpreting Test Results

TEST VALIDITY DETERMINATION

Before the results can be calculated for each clinical specimen, the test must be determined to be **valid**. For a test to be valid, the following ratio must be greater than or equal to 2.0. **This is the P/N of the positive control.**

$$\frac{\text{Mean OD of the Flavivirus IgM Positive Control reacted with Zika Vero E6 antigen (P)}}{\text{Mean OD of the normal human serum reacted with Zika Vero E6 antigen (N)}}$$

Test validity must be determined for each plate. Results for clinical specimens may only be determined if the test is valid. If the test is not valid, then that plate must be repeated. If the P/N for the positive control still fails after a repeat, then one or more of the reagent or test parameters was likely in error, and troubleshooting should be performed.

DETERMINATION OF SPECIMEN P/N

To determine whether the clinical specimens (S1-S8) contain IgM to the Zika virus (which would indicate recent infections with that virus) the following must be calculated:

$$\frac{\text{Mean OD of the test specimen reacted with Zika Vero E6 antigen (P)}}{\text{Mean OD of the normal human serum reacted with Zika Vero E6 antigen (N)}}$$

This is the P/N of the test specimen.

All specimens for which Specimen P/N is < 2, report as **negative**. No further analysis is required. See Table 2 below.

SPECIMEN BACKGROUND EVALUATION

For each specimen with a Specimen P/N ≥ 2, determine whether non-specific background is being generated.

The value of P (mean of the test specimen reacted with Zika Vero E6 antigen) for the test specimen must be greater than or equal to twice (2X) the mean OD of the test specimen reacted with Normal Vero E6 antigen. If this requirement is not met, non-specific background is being generated, and the result

MUST be reported as **inconclusive**. Inconclusive specimens should be retested. If repeat testing also yields inconclusive results, forward specimen for further analysis and/or request collection of additional serum for analysis. If requirement is met, proceed with specimen result interpretation.

ANALYSIS OF POSITIVE AND EQUIVOCAL RESULTS

All test specimen P/N values greater than or equal to 3.0 should be reported as presumptive IgM-positive (see table below), as long as they meet the requirements listed above. In the event that an early acute CSF or serum is negative by this test, a convalescent serum specimen must be requested and tested before that patient is reported as negative for serological evidence of recent viral infection. Without testing of a convalescent specimen, a negative result may reflect testing of an acute-phase specimen obtained before antibody has risen to detectable levels.

P/N values that lie between 2.0 and 3.0 should be considered **equivocal**. Further tests should be performed to determine the status of these specimens (see Table 2 below).

It should be stressed that the P/N value for a specimen at the screening dilution of 1:400 is not an indication of absolute antibody concentration, i.e., the P/N value is not quantitative.

Table 2: Zika MAC-ELISA Results Interpretation

Test Specimen P/N	Interpretation	Report	Action
< 2	Negative	No evidence of recent Zika virus infection detected.	Report results. If an early acute specimen, refer to interpretation instructions above.
2 ≤ P/N < 3	Equivocal	Zika MAC-ELISA results were equivocal for the presence of anti-Zika virus antibodies.	Send report to CDC along with the specimen for confirmatory testing.
≥ 3	Presumptive Positive	Serological evidence of possible recent Zika virus infection identified. Additional testing required.	Send report to CDC along with the specimen for confirmatory testing.

All positive results should be reported to CDC via ArboNET.

For information regarding Zika testing algorithm, please refer to CDC guidance for state and local public health laboratories: http://www.cdc.gov/zika/state-labs/index.html

For specimen referral instructions, please refer to:

http://www.cdc.gov/zika/hc-providers/diagnostic.html

Assay Limitations

Interpretation of Zika MAC-ELISA results must account for the possibility of false-negative and false-positive results. False-negative results can arise from:

- Specimen collection conducted before IgM has reached detectable levels (typically around 4 days post-onset of symptoms).
- Specimen collection conducted after IgM levels have decreased below detectable levels (typically around 12 weeks post-onset of symptoms).
- Failure to follow the authorized assay procedures.

The most common cause of false-positive results is cross reactivity with IgM specific for other flaviviruses such as dengue virus. Only limited evaluation of cross-reactivity with flaviviruses or arboviruses has been conducted. No evaluation of cross-reactivity with Rheumatoid Factor has been conducted. Clinical data indicate cross-reactivity with anti-dengue virus antibodies is likely. Follow-up testing is necessary to rule-out a false-positive result. Confirmation of the presence of anti-Zika IgM requires testing by CDC or a CDC-designated laboratory. The gold-standard method for confirmation of the presence of anti-Zika antibodies is the plaque reduction neutralization test (PRNT).

All Zika testing must be conducted following the CDC-issued Zika laboratory guidance and testing algorithms: http://www.cdc.gov/zika/state-labs/index.html.

Negative results do not preclude infection with Zika virus and should not be used as the sole basis of a patient treatment/management decision. All results should be interpreted by a trained professional in conjunction with review of the patient's history and clinical signs and symptoms.

This assay is for *in vitro* diagnostic use under FDA Emergency Use Authorization only and is limited to qualified laboratories designated by CDC.

All specimens should be handled as if infectious. Proper biosafety precautions, including personal protective equipment, must be used when handling specimen materials.

Proper collection, storage and transport of specimens are essential for correct results.

Performance has only been established with the specimen types listed in the Intended Use. Other specimen types are not acceptable for use with this assay.

Performance Characteristics

Cross Reactivity

Flavivirus Cross-reactivity

Banked known sera from CDC's repository were selected to evaluate the cross-reactivity of the Zika MAC-ELISA. No cross-reactivity was observed between the Zika MAC-ELISA and flaviviruses other than dengue.

Table 3: Flavivirus Cross-reactivity Summary

Flavivirus	Specimen description	Specimens Tested	Negative by Zika MAC-ELISA
WNV	Sera from confirmed West Nile virus cases.	4	4 (100%)
SLE	Sera from confirmed St. Louis Encephalitis cases	1	1 (100%)
YFV	Sera from individuals vaccinated against yellow fever virus	4	4 (100%)
JEV	Sera from individuals vaccinated against Japanese encephalitis virus	2*	2 (100%)

*Two sera within the YF vaccine recipient pool came from individuals who had also received the JE vaccine, thus these are a subset of the specimens in the row above.

Dengue virus was not included in the cross-reactivity evaluation. Clinical testing data presented below demonstrates significant cross-reactivity of the Zika MAC-ELISA to anti-dengue virus IgM antibodies.

Non-flavivirus Cross-reactivity

No experimental study was carried out with the Zika MAC-ELISA to determine cross-reactivity with IgM against non-flaviviruses. However, the literature (Martin, et al., 2000) indicates that only minimal cross-reactivity is expected with IgM against alphaviruses and bunyaviruses.

Arboviruses were originally delineated into three groups based on significant serological differences as characterized with early, crude serological techniques. These delineations remain: Group A viruses are now alphaviruses; Group B are now flaviviruses; Group C are now bunyaviruses. As serological methods have evolved, the serological distinctions that originally defined the groups mean that cross reactivity between groups in immunoassays is not expected.

Clinical Performance

Performance with U. S. Specimens Submitted to CDC, Ft. Collins, 2015 to Present

In the period from January 2015 to February 13, 2016, 167 sera and 2 CSF specimens were tested by both the CDC Zika MAC-ELISA and by the CDC Zika PRNT assay.

Summary of Clinical Performance with Sera

Of the 167 sera test records, a subset were paired specimens from serial bleeds. From serial bleeds, only the first IgM positive or equivocal bleed were included. If both serial bleeds were negative, only the first one was included. The resulting data set used in this analysis is 161 sera. A summary of results for these sera is presented in Table 5. Forty-four of these testing records indicated they are from pregnant women. A summary of data for the subset of sera from pregnant women is presented in Table 6.

Table 5: Data for Sera Submitted to CDC Ft. Collins for Testing 2015-present

		PRNT Results			
		Zika	flavivirus	dengue	negative
Zika MAC-ELISA	positive	45	16	23	9
	equivocal	1	0	9	13
	negative	0	0	6	39

Positive percent agreement (PRNT definitive Zika positives only): 45/46 = 97.8% (95% CI: 88.7% - 99.6%)
Negative percent agreement: 45/99 = 45.5% (95% CI: 36.0% - 55.3%)

Table 6: Data for Sera from Pregnant Women Submitted to CDC Ft. Collins for Testing 2015-present

		PRNT Results			
		Zika	flavivirus	dengue	negative
Zika MAC-ELISA	positive	3	2	1	3
	equivocal	0	0	2	8
	negative	0	0	1	24

Positive percent agreement (PRNT definitive Zika positives only): 3/3 = 100% (95% CI: 43.9% – 100%)
Negative percent agreement: 25/39 = 64.1% (95% CI: 48.4% - 77.3%)

Summary of CSF Data

Both CSF specimens tested by CDC yielded positive results for Zika virus infection by both Zika MAC-ELISA and by PRNT. The CSF results agreed with the paired serum testing.

Evaluation of Performance with Primary and Secondary Zika Infections, Yap State, Micronesia, 2007

The CDC Zika MAC-ELISA was included in a battery of CDC MAC-ELISA and PRNT flavivirus immunological methods for the evaluation of paired serum specimens from 11 Zika virus cases identified in a Zika outbreak in Yap State, Micronesia in 2007 (Lanciotti, et al., 2008). At the time of this outbreak, the Zika MAC-ELISA employed sucrose-acetone extracted suckling mouse brain antigen. Four of the 11 cases are primary flavivirus infections, while seven are probable secondary flavivirus infections.

Of the paired sera collected and evaluated in the publication, all but one patient has at least one serum specimen within our claimed window of ≥ 4 days post onset of symptoms and < 12 weeks post onset of symptoms. For each of the remaining 10 cases, the earliest collected serum specimen within our claimed window is included in our analysis.

Zika MAC-ELISA results for these specimens are compared to their Plaque Reduction Neutralization Test (PRNT) results, the gold standard for flavivirus immunological testing.

Table 4: Summary of Zika MAC-ELISA and Flavivirus PRNT Results for earliest within-window specimens for primary and secondary Zika infections in Yap State, Micronesia, 2007

	Case	Specimen	Days Post Onset	Zika MAC-ELISA	PRNT (7 flaviviruses)
Primary Infections	822	822a	5	23.2	Zika
	830	830b	21	16.3	Zika
	849	849b	18	18.2	Zika
	862	862a	6	25.4	Zika
Probable Secondary Infections	817	817b	19	8.1	flavivirus
	833	833b	19	3.1	Zika
	844	844b	16	12.7	dengue
	955	955b	14	10.9	flavivirus
	968	No specimens within claimed window			
	839	839b	20	17.2	Zika
	847	847a	5	0.94	yellow fever

		PRNT Results (7 flaviviruses)			
		Zika	flavivirus	dengue	yellow fever
Zika MAC-ELISA	Positive	6	2	1	0
	Negative	0	0	0	1

Primary infections:

All 4 cases identified as primary infections yielded positive Zika MAC-ELISA results for their initial within-window serum specimen. These 4 specimens also yielded positive results by PRNT for Zika virus infection.

Secondary Infections (probable):

Of the six cases with within-window serum specimens, five yielded positive results for the earliest within-window serum specimens by Zika MAC-ELISA. Two of these (833b and 839b) yielded clearly positive results for Zika virus infection by PRNT. Two other specimens (817b and 955b) yielded greater PRNT results for Zika virus than for any of the other flaviviruses tested. However, these results were not 4-fold higher than all other results, thus were interpreted a Flavivirus positive by PRNT. The remaining specimen yielded positive results for Zika MAC-ELISA and 4-fold higher PRNT results for Dengue than for Zika, the only specimen for which the PRNT did not agree with the Zika MAC-ELISA.

Specimen 847a, a day 5 specimen, was negative by Zika MAC-ELISA and was positive for Yellow Fever Virus by PRNT. No neutralization effect was observed for Zika by PRNT. Thus the MAC-ELISA and PRNT results for this specimen are in agreement.

Contact

Questions or comments about this procedure may be directed to the Laboratory Response Network (LRN) helpdesk: LRN@cdc.gov.

References

Johnson, AJ, Martin, DA, Karabatsos, N and Roehrig, JT. *Detection of anti-arboviral immunoglobulin G by using a monoclonal antibody-based capture enzyme-linked immunosorbent assay*. J. Clinical Microbiology, 38:1827-1831, 2000.

Koneman EW, Allen SD, Janda WM, Schreckenberger PC, and Winn Jr. WC , (Eds). *Diagnosis of Infections caused by Viruses, Chlamydia, Rickettsia,* Diagnostic Microbiology, 4th Edition, JB Lippicott Co: 956-1074, 1992.

Lanciotti, RS, O.L. Kosoy, J.J. Laven, J.O. Velez, A.J. Lambert, A.J. Johnson, S.M. Stanfield, and M.R. Duffy. *Genetic and Serologic Properties of Zika Virus Associated with an Epidemic, Yap State, Micronesia, 2007.* Emerg Infect Dis. 2008 (Aug); 14(8): 1232-1239.

Monath, TP, Nystrom, RR, Bailey, RE, Calisher, CH, and Muth, DJ:
Immunoglobulin M antibody capture enzyme-linked immunosorbent assay for diagnosis of St. Louis encephalitis. Journal of Clinical Microbiology 20:784-790, 1984.

Martin, DA., Muth, DA., Brown, T., Karabatsos, N., and Roehrig, JT.
Standardization of immunoglobulin M capture enzyme-linked immunosorbent assays (MAC-ELISA) for routine diagnosis of arboviral infections. Journal of Clinical Microbiology 38:1823-1826, 2000.

Tsai, TH: *Arboviruses*, In Rose NR, Marcario EC, Fahey JL, Friedman H, and Penn GM, (Eds):
Manual of Clinical Laboratory Immunology, 4th Edition, American Society for Microbiology: 606-618, 1976.