BIOGENIX
INC. PVT. LTD.

IVD

# Dengue IgM Ab Elisa Kit

- 96-well ELISA kit for the qualitative detection of elevated anti-dengue viruses (DEN1,2,3,4) IgM in human serum or plasma

## INTENDED USE

The Dengue IgM Ab ELISA Kit is a solid-phase enzyme-linked immunosorbent assay for the qualitative detection of IgM anti-dengue viruses (DEN1, 2, 3, 4) in human serum or plasma. It is intended for professional use only as an aid in the diagnosis of acute infection with dengue viruses.

## INTRODUCTION

Dengue virus is an enveloped, single-stranded, positive-sense RNA virus that belongs to the *Flaviviridae* family and can be classified into four distinct serotypes (DEN1, 2, 3, 4). The virus is transmitted by daytime-biting mosquitoes, principally *Aedes aegypti* and *Aedes albopictus*. Currently, more than 2.5 billion people living in tropical and subtropical areas of Asia, Africa, Australia, and the Americas are at risk for dengue infection. An estimated 67-136 million cases of dengue fever and 20,000 deaths occur annually on a worldwide basis.

Serological detection is a common method for the diagnosis of infection with dengue virus. During a primary infection, IgM anti-dengue virus starts to appear approximately 4-6 days after the onset of fever, peaks after approximately two weeks, and remains in circulation for about 2-3 months. IgG anti-dengue virus levels increase slowly, peak around 14-21 days, and then decrease to low levels, persisting for life[6].

During secondary infection, IgM antibodies increase simultaneously with, or following the IgG secondary antibodies, which is a strong and rapid response, and can be detected as early as three days after the onset of symptoms. IgM antibody levels occur, in general, at a lower titer than that of IgG. Importantly, during secondary infection, IgM antibody levels are significantly lower in comparison with primary infection[6].

The Dengue IgM Ab ELISA Kit uses recombinant dengue virus antigens to specifically detect IgM antibodies for all four dengue virus serotypes (DEN1, 2, 3 and 4).

## TEST PRINCIPLE

The Dengue IgM Ab ELISA Kit is a solid-phase enzyme-linked immunosorbent assay based on the principle of the capture immunoassay methodology for the detection of IgM anti-dengue virus in human serum or plasma.

The Dengue IgM Ab ELISA Kit is composed of two key components:
1) Solid microwells pre-coated with mouse monoclonal anti-human IgM antibody.
2) Conjugate working solution composed of dengue antigen and HRP-anti-dengue conjugates.

During the assay, the test specimen is first incubated in the coated microwell. IgM anti-dengue, if present in the specimen, binds to the anti-human IgM antibodies coated on the microwell surface, and any unbound specimen is then removed by a wash step. During a second incubation with HRP-anti-dengue conjugate working solution, the IgM anti-dengue absorbed on the surface of microwell binds to the conjugate through dengue antigen, forming a conjugate complex. Unbound conjugates are then removed by washing. After addition of the TMB substrate, the presence of the conjugate complex is shown by development of a blue color resulting from a reaction between the enzyme and substrate. This reaction is then quenched by addition of the Stop Solution, and the absorbance value for each microwell is determined using a spectrophotometer at 450/620-690 nm.

## MATERIALS AND REAGENTS

**Materials and reagents provided with the kit**

| Item | Description | Quantity |
|---|---|---|
| 1 | Anti-human IgM Coated Microwells | 8 wells x 12 strips |
| 2 | Lyophilized Dengue Antigen | 3 vials |
| 3 | HRP-anti-dengue Conjugates (100X Concentrate) | 0.2 mL |
| 4 | Enzyme Diluent | 15 mL |
| 5 | Dengue IgM Positive Control | 0.2 mL |
| 6 | Dengue IgM Negative Control | 0.2 mL |
| 7 | Sample Diluent | 2x30 mL |
| 8 | Wash Buffer (30X Concentrate) | 25 mL |
| 9 | TMB Substrate A | 6 mL |
| 10 | TMB Substrate B | 6 mL |
| 11 | Stop Solution | 12 mL |
| 12 | Product Insert | 1 |

**Materials and reagents required but not provided in the kit**

1. Pipette capable of delivering 5 µL, 50 µL and 100 µL volumes with a precision better than 98.5%
2. Microplate reader with a bandwidth of 10 nm or less and an optical density range of 0-3 OD or greater at 450 nm wavelength is acceptable
3. Absorbent paper for blotting the microwells
4. Timer
5. Distilled or de-ionized water

## STORAGE AND STABILITY

All reagents except HRP-anti-dengue Conjugates and concentrated Wash Buffer are ready to use as supplied. Store all components at 2-8°C. Do not freeze. Ensure that the reagents are brought to room temperature before opening. Return all reagents requiring storage at 2-8°C to refrigeration immediately after use. Re-seal the unused microwells in provided Ziploc bag with desiccant. All reagents are stable through the expiration date printed on the label if not opened. Once opened, the kit is stable for 8 weeks at 2-8°C, or until the labeled expiration date, whichever is earlier.

## SPECIMEN COLLECTION AND PREPARATION

1. Serum or plasma should be prepared from a whole blood specimen obtained by acceptable venipuncture technique.
2. This Test is designed for use with serum or plasma specimens without additives only.
3. If a specimen is not tested immediately, refrigerate at 2-8°C for up to 3 days. For storage longer than 3 days, the specimen should be frozen at -20°C. Avoid multiple freeze-thaw cycles. If a specimen is to be shipped, pack in compliance with federal regulations covering the transportation of etiologic agents.
4. Specimens containing precipitates may give inconsistent test results. Clarify such specimens by centrifugation prior to performing the assay.
5. Do not use specimens demonstrating gross lipemia, gross hemolysis or turbidity. Do not use specimens containing sodium azide.

## PREPARATION OF THE REAGENTS PRIOR TO ASSAYING

1. Bring all reagents and controls to room temperature (20-25°C).
2. Determine the volumes of conjugate working solution and wash buffer required for the assay. Each strip of microwells requires 900 µL of conjugate working solution and 60 mL of working wash buffer.
3. **Preparation of reconstituted dengue antigen:**

3.1 Tap the dengue antigen vial on the bench to collect all lyophilized antigen powder. Transfer 2.0 mL Enzyme Diluent to each vial of dengue antigen. Each vial of antigen is enough for 4 strips of microwells.

3.2 Swirl the vial to dissolve the antigen completely. Incubate on the bench for exactly 10 minutes.

3.3 The reconstituted antigen is stable at 2-8˚C up to 3 weeks. Seal the cap with parafilm to minimize evaporation and place the vial immediately at 2-8˚C.

| Preparation of reconstituted dengue antigen preparation | | |
|---|---|---|
| 3.1 | Tap the vial and add Enzyme Diluent  | 2.0 mL |
| 3.2 | Swirl the vial and incubate  | 10 minutes |
| 3.3 | Seal and store  | 2-8°C, up to 3 weeks |

4. **Preparation of conjugate working solution:**
Working conjugate should be prepared at least 30 minutes prior to use.

4.1 In a separate tube, mix reconstituted antigen, enzyme diluent and 100x conjugate concentrate as shown in the following table. Incubate the three mixed reagents, also termed “conjugate working solution” at room temperature (20-25°C) for exactly 60 minutes.

| | 1 strip | 2 strips | 3 strips | 4 strips |
|---|---|---|---|---|
| Reconstituted antigen | 450 µL | 900 µL | 1,350 µL | 1,800 µL |
| Enzyme diluent | 450 µL | 900 µL | 1,350 µL | 1,800 µL |
| 100x conjugate | 9 µL | 18 µL | 27 µL | 36 µL |

4.2 **The conjugate working solution should be stored at 2-8°C for up to 4 hours after one hour incubation at room temperature if not used immediately**. Discard unused conjugate working solution after 4 hours.

| Preparation of conjugate working solution | | |
|---|---|---|
| 4.1 | Mix and incubate (reconstituted dengue antigen, enzyme diluent and 100x conjugate)  | 20-25°C 60 minutes |
| 4.2 | Seal and store  | 2-8°C Up to 4 hours |

5. **Preparation of dengue IgM controls and specimens:**
Dilute the Dengue IgM Negative Control, Positive Control and patient specimens 100 fold with Sample Diluent: e.g. to 5 µL of control or specimen, add 500 µL Sample Diluent.

6. **Preparation of working Wash Buffer:**
If precipitants are visible, warm the Wash Buffer to 37˚C. Dilute concentrated Wash Buffer 30 fold with distilled or de-ionized water as follows:

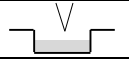
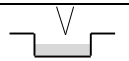
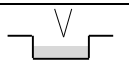
| Plate | DI water | Wash buffer (30X) | Final volume |
|---|---|---|---|
| 1 strip | 58 mL | 2 mL | 60 mL |
| 2 strips | 116 mL | 4 mL | 120 mL |
| 3 strips | 174 mL | 6 mL | 180 mL |
| 4 strips | 232 mL | 8 mL | 240 mL |

The diluted wash buffer can be stored at 2-8˚C for up to 3 days.

7. Mix each reagent before adding it to the test wells.
8. Determine the number of microwells needed and mark the ELISA working sheet with the appropriate information. Run positive and negative controls in duplicate to ensure accuracy.

## ASSAY PROCEDURE

1. Remove the desired number of strips and secure them in the microwell frame. Reseal un-used strips in Ziploc bag.
2. Add specimens according to the designation on the ELISA Working Sheet:
2. 1 Blank well: Do not add specimens or control.
2. 2 Control wells: Add 100 µL diluted Dengue IgM Positive Control and 100 µL diluted Dengue IgM Negative Control into the designated control wells.
2. 3 Test wells: Add 100 µL diluted patient specimen into each designated test well.
2. 4 Gently rock the plate for 20 seconds, and then cover the plate with the sealer.
3. Incubate the plate at 37°C for 20 minutes.
4. Wash to remove unbounded materials:
**Manual washing:** Carefully remove the incubation mixture by disposing of solution into a waste container. Fill each well with 350 µL diluted wash buffer and rock gently for 20-30 seconds. Discard the wash solution completely. Repeat 4 more times. After completing the last wash step, tap the plate on absorbent paper to remove residual liquid.
**Automated washing:** Automatic plate washer must be calibrated to ensure efficient washing. Fill each well with 350 µL diluted wash buffer and soak for 20-30 seconds. Aspirate all wells completely. Repeat 4 more times.
5. Add 100 µL prepared conjugate working solution into each well except the blank well. Gently rock the microwells for 20 seconds to ensure thorough mixing.
6. Cover the wells and incubate at 37°C for 60 minutes.
7. Wash the plate 5 times as described in step 4.
8. Add 50 µL TMB Substrate A and 50 µL TMB Substrate B into each well, including the Blank Well. Gently rock the microwells for 20 seconds to ensure thorough mixing.
9. Incubate at room temperature (20-25°C) in the dark for 15 minutes.
10. Stop the reaction by adding 100 µL Stop Solution to each well, including the Blank Well. Gently rock for 20 seconds. Pipette the Stop Solution in the same sequence as substrate addition. **It is important to make sure that all the blue color completely changes to the color yellow.**
11. Set the microplate reader wavelength at 450 nm. Measure the absorbance (OD) of each well against the blank well within 30 minutes after adding Stop Solution. A filter of 620-690 nm can be used as a reference wavelength to optimize the assay result.

| Flow chart of assay procedure | | | |
|---|---|---|---|
| 1. | Secure strips in microwell frame |  | Number of strips |
| 2. | Add diluted controls or specimens. Gently rock |  | 100 µL 20 seconds |
| 3. | Incubate |  | 37°C, 20 minutes |
| 4. | Wash: manual or automatic |  | 5 times |
| 5. | Add conjugate working solution. Gently rock |  | 100 µL 20 seconds |
| 6. | Incubate |  | 37°C, 60 minutes |
| 7. | Wash: manual or automatic |  | 5 times |
| 8. | Add TMB Substrate A and B. Gently rock |  | 50 µL + 50 µL 20 seconds |
| 9. | Incubate in dark |  | RT (20-25°C) 15 minutes |
| 10. | Add Stop Solution. Gently rock |  | 100 µL, 20 seconds |
| 11. | Read result |  | 450/620-690 nm within 30 minutes |

## INTERPRETATION OF RESULTS

**A. Set up the cut-off value**

The cut-off value = 0.26 + N

N: Mean OD of the negative control. Use N=0.04 for calculation of the cut-off value if the mean OD is less than 0.04.

**B. Calculation of specimen OD ratio**

Calculate an OD ratio for each specimen by dividing its OD value by the cut-off value as follows:

$$\text{Specimen OD ratio} = \frac{\text{Specimen OD}}{\text{Cut-off Value}}$$

**C. Assay Validation**

The mean OD value of the positive controls should be ≥ 0.80

The mean OD value of the negative controls should be ≤ 0.13

Check the assay procedure including incubation time and temperature and repeat assay if above conditions are not met.

**D. Interpretation of the results**

| | ***Specimen OD ratio*** |
|---|---|
| Negative | < 1.0 |
| Positive | ≥ 1.0 |

1. A negative result indicates that there is no detectable IgM anti-dengue antibody in the specimen.
2. Results just below the cut-off value (lower than 10% of the cut-off value) should be interpreted with caution (it is advisable to re-test in duplicate the corresponding specimens when it is applicable).
3. Specimens with OD ratio ≥ 1.0 are initially considered to be positive by the Dengue IgM ELISA Kit. They should be retested in duplicate before a final interpretation is made.

   If after re-testing of a specimen, the absorbance value of the 2 duplicates are less than the cut-off value, the initial result is non-repeatable and the specimen is considered to be negative with the Dengue IgM ELISA Kit.

   Non-repeatable reactions are often caused by:
   - Inadequate microwell washing,
   - Contamination of negative specimens by serum or plasma with a high antibody titer,
   - Contamination of the substrate solution by oxidizing agents (bleach, metal ions, etc.),
   - Contamination of the Stop Solution.

   If after retesting the absorbance of one of the duplicates is equal to or greater than the cut-off value, the initial result is repeatable and the specimen is considered to be positive with the Dengue IgM ELISA Kit, subject to the limitations of the procedure, described below.

## PERFORMANCE CHARACTERISTICS

**1. Accuracy of Detection**

A total of 491 patient specimens were collected from susceptible subjects and tested by Dengue IgM ELISA Kit and by a commercial leading brand EIA. Comparison for all subjects is shown in the following table:

| | Dengue IgM Ab ELISA | | |
|---|---|---|---|
| **Reference EIA** | **Positive** | **Negative** | **Total** |
| **Positive** | 158 | 16 | 174 |
| **Negative** | 13 | 304 | 317 |
| **Total** | 171 | 320 | 491 |

Relative Sensitivity: 90.8% (95% Confidence Interval = 85.5 – 94.4%)
Relative Specificity: 95.9% (93.1 – 97.7%)
Overall Agreement: 94.1% (91.6 – 95.9%)

**2. Precision**

a. Intra-assay Precision was determined by assaying 20 replicates of three patient specimens.

| Sample | N | OD | SD | CV |
|---|---|---|---|---|
| Negative | 20 | 0.198 | 0.009 | 4.29% |
| High Positive | 20 | 1.421 | 0.058 | 4.09% |
| Low Positive | 20 | 0.728 | 0.047 | 6.52% |

b. Inter-assay Precision was determined by assaying three patient specimens in 10 separate runs. Data were analyzed by ANOVA (analysis of variance).

| Specimen | Runs | OD | SD | CV |
|---|---|---|---|---|
| Negative | 10 | 0.164 | 0.013 | 7.91% |
| High Positive | 10 | 1.120 | 0.057 | 5.09% |
| Low Positive | 10 | 0.433 | 0.038 | 8.78% |

**3. Cross-Reactivity**

No false positive Dengue IgM Ab ELISA test results were observed on 2-10 positive specimens from each of the following disease states or special conditions, respectively:

| | | | | |
|---|---|---|---|---|
| CHIK | HCV | HBsAg | HIV | *H. pylori* |
| Malaria | Syphilis | ANA | HAMA | RF (up to 8,400 IU/mL) |

**4. Interference**

Common substances (such as pain and fever medication and blood components) may affect the performance of the Dengue IgM Ab ELISA Kit. Interference was studied by spiking these substances into 3 dengue IgM clinical specimens: negative, low positive and high positive. The results demonstrate that at the concentrations tested, the substances studied do not affect the performance of the Dengue IgM ELISA Kit.

List of potentially interfering substances and concentrations tested:

| | | | |
|---|---|---|---|
| 1. Salicylic acid | 4.34 mmol/L | 5. Glucose | 55 mmol/L |
| 2. Sodium citrate | 1.3 % | 6. Heparin | 3,000 U/L |
| 3. Creatinine | 442 µmol/L | 7. Bilirubin | 10 mg/dL |
| 4. EDTA | 3.4 µmol/L | | |

## WARNING AND PRECAUTIONS

**For *In Vitro* Diagnostic Use**

1. This package insert must be read completely before performing the test. Failure to follow the insert may lead to inaccurate test results.
2. Bring all reagents to room temperature (20-25°C) before use.
3. Do not use expired test kits.
4. Do not use the components from any other type of test kit as a substitute for the components in this kit.
5. Do not use hemolyzed blood specimens for testing.
6. Do not ingest the reagents. Avoid contact with eyes, skin and mouth. Wear protective clothing and disposable gloves while handling the kit reagents and clinical specimens. Wash hands thoroughly after performing the test.
7. Do not smoke, drink or eat in areas where specimens or kit reagents are being handled.
8. The human serum materials used for the preparation of controls have been tested and found non-reactive with antibodies to HIV-1 & 2, HCV, and HBsAg. However, users of this test should follow the US CDC Universal Precautions for prevention of transmission of HIV, HBV and other blood-borne pathogens.
9. Dispose of all specimens and materials used to perform the test as bio-hazardous waste.
10. At the beginning of each incubation, and after the addition of Stop Solution, gently rock the microwells for 20 seconds to ensure thorough mixing and even distribution. Avoid the formation of air bubbles which results in inaccurate absorbance values. Avoid splashing liquid while rocking or shaking the wells.
11. Do not allow the microwells to dry between the end of the washing operation and the reagent distribution.
12. The enzyme-substrate reaction is very sensitive to metal ions. Do not allow any metal element to come into contact with the conjugate or substrate solution.
13. The enzyme-substrate is temperature dependent. Ensure that the room temperature for TMB incubation falls between 20-25˚C.
14. The TMB Substrate must be colorless. The appearance of color indicates that the reagent cannot be used and must be replaced. The TMB Substrate B must be stored in the dark.
15. Use a new distribution tip for each specimen and working step. Never use the specimen container to distribute dispense conjugate and TMB Substrate.
16. **The wash procedure is critical. Wells must be aspirated completely before adding the wash buffer or liquid reagents. Automatic washer must be validated with Dengue IgM Ab ELISA Kit prior to use. Insufficient washing will result in poor precision and falsely elevated absorbance values.**
17. **Microplate reader must be calibrated per manufacturer's instruction to ensure accurate determination of absorbance. Non-calibrated readers often lead to invalid test results.**
18. Avoid exposure of the wells to strong light during color development.

## LIMITATION OF THE TEST

1. The Assay Procedure and the Interpretation of Results must be followed closely when testing for the presence of antibodies to dengue virus in serum or plasma from individual subjects. Failure to follow the procedure may give inaccurate results.
2. The Dengue IgM Ab ELISA Kit is limited to the qualitative detection of IgM antibodies to dengue virus in human serum or plasma.
3. The Dengue IgM Ab ELISA Kit cannot be used to differentiate if the infection is primary or secondary. However, it has been proposed that antibody ratios (IgG/IgM) can be used to distinguish between primary and secondary dengue infections[5].
4. Serological cross reactivity with other flaviviruses is common (e.g., Japanese encephalitis, West Nile, yellow fever, etc.), therefore, it is possible that patients infected with these viruses may show some level of the reactivity with this test.
5. A negative result for an individual subject indicates absence of detectable dengue virus antibodies. However, a negative test result does not preclude the possibility of exposure to or infection with dengue virus.
6. A negative result can occur if the titer of the dengue IgM antibodies present in the specimen is below the detection limit of the assay, or the IgM antibodies that are detected are not present during the stage of disease in which a specimen is collected.
7. Infection may progress rapidly. If the symptoms persist, while the result from the Dengue IgM ELISA Kit is negative, it is recommended to test with an alternative test method.
8. Some specimens containing unusually high titers of heterophile antibodies or rheumatoid factor may affect the expected results.
9. Any interpretation or use of this test result must also rely on other clinical findings as well as on the professional judgment of health care providers.

## REFERENCES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