



West Nile *Detect*[™] IgM Capture ELISA
For In Vitro Diagnostic Use

INTENDED USE

The West Nile *Detect*[™] IgM Capture ELISA is designed for the qualitative detection of IgM antibodies to West Nile recombinant antigens (WNRA) in human serum. This test is intended for use for the presumptive clinical laboratory diagnosis of West Nile virus infection in patients with clinical symptoms consistent with meningoencephalitis. Positive results must be confirmed by Plaque Reduction Neutralization Test (PRNT), or by using the current CDC guidelines for diagnosis of this disease.

Assay performance characteristics have not been established for testing cord blood, neonate, prenatal screening, general population screening without symptoms of meningoencephalitis or automated instruments. This assay is not FDA cleared or approved for testing blood or plasma donors.

Caution: IgM assay cross-reactivity has been noted with some West Nile IgM assays testing specimens containing antibody to enteroviruses. Reactive results reported from children must contain a caution statement regarding possible cross-reactivity with enteroviruses.

SUMMARY AND EXPLANATION OF THE TEST

Exposure to West Nile Virus causes a disease with a number of symptoms including encephalitis. West Nile virus (WNV) is a potentially neuroinvasive agent that causes asymptomatic infection and fevers in humans. Human and animal infections were not documented in the Western Hemisphere until the 1999 outbreak in the New York City metropolitan area (1,2). Since then, the disease has spread across the United States. In 2003, WNV activity occurred in 46 states and caused illness in over 9,800 people. Most WNV infected humans usually have no symptoms. A small proportion develops mild symptoms that include fever, headache, body aches, skin rash and swollen lymph glands. Clinically, WNV fever in humans is a self-limited acute febrile illness accompanied by headache, myalgia, polyarthropathy, rash, gastrointestinal symptoms, and lymphadenopathy (3, 4). Less than 1% of infected people develop more severe illness that includes meningitis or encephalitis (5, 6). The West Nile *Detect*[™] IgM Capture ELISA employs a recombinant WNV antigen called WNRA, which can be used as a rapid serological marker for WNV infection. The WNRA protein is a

recombinant antigen, which consists of a stretch of sequences from two WNV antigens (encoded by prM-E gene).

PRINCIPLE OF THE TEST

The West Nile *Detect*[™] IgM Capture ELISA consists of one enzymatically amplified "two-step" sandwich-type immunoassay. In this assay, controls and unknown serum samples are incubated in microtiter wells which have been coated with anti-human IgM antibodies. The serum samples may be directly mixed with sample dilution buffer for WN IgM, then added in the wells. This is followed by incubation with the West Nile Virus derived recombinant WNRA protein and a control preparation (NCA) separately. After one hour incubation and washing, the wells are treated with a WNRA-specific antibody labeled with the enzyme horseradish peroxidase (HRP). After a second incubation and washing step, the wells are incubated with the tetramethylbenzidine (TMB) substrate. An acidic stopping solution is then added and the degree of enzymatic turnover of the substrate is determined by absorbance measurement at 450 nanometers. Above a certain threshold, the ratio of the absorbance of the WNRA and the control wells presumptively determines whether antibodies to WNV are present. A set of positive and negative controls is provided in order to monitor the integrity of the kit components.

MATERIALS SUPPLIED

The West Nile *Detect*[™] IgM Capture ELISA Kit contains sufficient reagents for one plate of 96 wells (12 x 8 strips) each.

Warning: Do not use any reagents where damage to the packaging has occurred. The kit contains the following reagents:

- 1. Coated Microtiter Strips for Human IgM**
Cat. No. 500613
96 polystyrene microtiter wells (12x8 strips). Coated with goat anti-human IgM antibody in each well. Ready to use. Stable at 2-8°C until the expiration date.
- 2. Sample Dilution Buffer for WN IgM**
Cat. No. 500117
One bottle (25 ml). Ready to use. Phosphate Buffered Saline (pH7.2-7.6) with Tween 20, preservative (0.01% thimerosal) and additives. Use for the dilution of test samples, positive and negative controls. Stable at -70° C until the expiration date.
Note: For quick thaw, place the bottle containing Sample Dilution Buffer in a container with clean water maintained at room temperature (just immerse up to the height of the content).

Following complete thaw, take out the bottle, remove excess water on exterior with clean paper towels. *If any precipitate is seen after the thaw process, vortex the tube very well to obtain a homogeneous solution before use.*

3. WN Human IgM Positive Control
Cat. No. 500118

One vial (50µl). Heat-inactivated positive serum containing 0.01% thimerosal. The WN IgM positive control will aid in monitoring the integrity of the kit as well. Stable at -70°C until the expiration date. Before use, quickly centrifuge the vials so that contents can be collected at the bottom.

Note: For long-term storage, serum should be further aliquoted in smaller volumes and stored at -70°C.

4. WN Human IgM Negative Control
Cat. No. 500119

One vial (50µl). Heat-inactivated negative serum. The negative control will aid in monitoring the integrity of the kit as well. It is stable at -70°C until the expiration date. Before use, quickly centrifuge the vials so that contents can be collected at the bottom.

Note: For long-term storage, serum should be aliquoted in smaller volumes and stored at -70°C.

5. West Nile Antigen (WNRA) for IgM
Cat. No. 500120

One tube (5 ml). Ready to use. Contains Tween 20, preservative (0.002-0.005% Thimerosal), antibiotics (0.0025-0.004% G418 sulphate), WNV antigens (non-infectious WNV PrM and E antigens) and additives. Stable at -70°C until the expiration date.

Caution: The WNRA should not be repeatedly frozen and thawed. It should be aliquoted in a smaller volume and frozen at -70°C.

6. Ready to Use Normal Cell Antigen (NCA) for WN IgM
Cat. No. 500121

One tube (5 ml). Ready to use. Contains Tween 20, preservative (0.002-0.005% Thimerosal), culture supernatant of COS-1 cell line and additives. Stable at -70° C until the expiration date.

Caution: To avoid repetitive freezing and thawing, the sample should be aliquoted and stored at -70°C.

7. Ready to Use Enzyme Conjugate-HRP for WN IgM
Cat. No. 500220

One bottle (9 ml). Ready to use. Contains 6B6C-1 mAb (to WN virus E protein antigen) conjugated with horseradish peroxidase in phosphate buffered saline (pH7.2-7.6) with Tween 20, preservative (0.01% thimerosal) and additives. Stable at 2-8°C until the expiration date.

8. 10X Wash Buffer
Cat. No. 500100

One bottle (120 ml). 10X concentrate of phosphate buffered saline with Tween 20, pH6.7-7.1. Stable 2-8°C until the expiration date.

Note: See Preparation of Reagents in Test procedure section to prepare 1X Wash Buffer.

9. EnWash
Cat. No. 500114

One bottle (20ml). Ready to use. Phosphate buffered saline with Tween 20, pH 7.2-7.6. Stable at 2-8°C until the expiration date.

10. Liquid TMB Substrate
Cat. No. 500104

One bottle (12ml). Ready to use. Contains 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide in a citric-acid citrate buffer (pH3.3-3.8). Stable at 2-8°C until the expiration date.

Note: The substrate should always be stored in the light-protected bottle provided.

11. Stop Solution
Cat. No. 500658

One bottle (9ml). Ready to use. 1N Sulfuric Acid. Used to stop the reaction. Stable at 2-8°C until the expiration date.

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

MATERIALS REQUIRED BUT NOT SUPPLIED

- ELISA Spectrophotometer
- Measurement at 450 nm
- Biological or High-Grade Water
- Vacuum Pump
- 37°C incubator without CO₂ supply (do not use a humidified chamber).
- Plate washer
- Polypropylene tubes
- Multipipettors and tips
- Parafilm
- Timer
- Vortex

PRECAUTIONS

FOR *IN VITRO* DIAGNOSTIC USE

LAB PRECAUTIONS

- ◆ 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.
- ◆ Cover working area with disposable absorbent paper.
- ◆ Using humidified chambers instead of incubators for the 37°C incubation step may invalidate the results of the test.

TEST SERA PRECAUTIONS

- ◆ All human source materials used in the preparation of controls have tested negative for antibodies to Human Immunodeficiency virus 1 & 2 (HIV 1&2), Hepatitis C (HCV) as well as Hepatitis B surface antigen. However no test method can ensure 100% efficiency, thus, all human controls and antigen should be handled as potentially infectious materials. The Centers for Disease Control and Prevention and the National Institutes of Health recommend that potentially infectious agents be handled at the Biosafety Level 2.
- ◆ This test must be performed on serum only. The use of whole blood, plasma or other specimen matrix has not been established.
- ◆ Icteric or lipaemic sera, or sera exhibiting hemolysis or microbial growth must not be used.
- ◆ Do not heat-inactivate sera.

REAGENT PRECAUTIONS

- ◆ Avoid exposure of the reagents to excessive heat or direct sunlight during storage and incubation
- ◆ Do not use any component beyond the expiration date shown on its label.
- ◆ Do not mix lots of any kit component within an individual assay microtiter plate.
- ◆ Some reagents may form a slight precipitate, mix gently before use.
- ◆ Unused microwells must be resealed immediately and stored in the presence of desiccant. Failure to do this may cause erroneous results.
- ◆ The ready-to-use conjugate is very susceptible to bacterial contamination, which will affect performance. Keep lid on when not in use and dispense with sterile pipets.
- ◆ Substrate System:
 - (a) Avoid prolonged exposure to direct light.
 - (b) TMB is susceptible to contamination from metal ions, do not allow the substrate system to come into contact with metal surfaces
 - (c) Avoid contamination of TMB with conjugate.

- (d) Some detergents may interfere with the performance of the TMB thus, it is imperative to accurately follow plate washing directions.
- (e) The TMB may have a faint blue color. This will not affect the activity of the substrate or the results of the assay.

TESTING PRECAUTIONS

- ◆ 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 cycles of the reagents supplied in the kit and of specimens.
- ◆ **To avoid sample contamination, a fresh pipettor tip must be used to dispense each control and test sera.**
- ◆ Dispense reagents directly from bottles using clean pipette tips or sterile pipets.
- ◆ Incomplete washing will adversely affect the outcome and assay precision.
- ◆ To minimize potential assay drift due to variation in the substrate incubation time, care should be taken to add the stopping solution into the wells in the same order and speed used to add the TMB solution.
- ◆ Do not subtract blank well readings.

WARNING: POTENTIAL 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.

CHEMICAL HAZARD:

Safety Data Sheets (SDS) are available for all components of this kit. SDS sheets are available through our website or it can be sent upon request. Review all appropriate SDS before performing this assay. Avoid all contact between hands and eyes or mucous membranes during testing. If contact does occur, consult the applicable SDS for appropriate treatment.

SPECIMEN COLLECTION AND PREPARATION

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 (CLSI)⁷ (GP44: Procedures for the Handling and Processing of Blood Specimens for Common Laboratory Tests; Approved Guideline).

- Only Human serum can be used with this assay. Whole blood or plasma cannot be used.

- Remove serum from the clot of red cells as soon as possible to avoid hemolysis.
- Testing should be performed as soon as possible after collection. Do not leave sera at room temperature for prolonged periods.
- Serum should be used and the usual precautions for venipuncture should be observed. The samples may be stored at 2-8°C for up to 48 hours or frozen at -70°C or lower for up to 30 days. To maintain long-term longevity of the serum, store at -70°C. Avoid repeated freezing and thawing of samples.
- Do not use hemolyzed or lipemic samples.
- Frozen samples should be thawed to room temperature and mixed thoroughly by gentle swirling or inversion prior to use.
- If sera are shipped, pack in compliance with Federal Regulations covering transportation of infectious agents.

and stored at 2-8°C until ready to use or expiration.

Assay Procedure:

1. Positive, negative, and unknown serum to be tested should be assayed in duplicate. Refer to flow chart at the end of this section for illustration of this procedure. Twenty-two test specimens can be tested on one 96 well plate.
2. Mark the microtitration strips to be used.
3. Dilute test sera and the controls to 1/100 using the provided Sample Dilution Buffer for WN IgM (SD). (You may use small polypropylene tubes, multi-well untreated plastic strips, or plates for these dilutions and use at least 4 µL of unknown serum samples, positive, and negative controls. For example, 4 µL serum plus 396 µL of Sample Dilution Buffer for WN IgM to make 1/100 dilution.)
4. Apply the 50 µL/well of 1/100 diluted test sera, and controls to the plate by appropriate pipette. An exemplary arrangement for twenty-two test serum samples in duplicate is shown below. Note: samples and controls are to be assayed with WNRA and NCA.

TEST PROCEDURE

CAUTION: The test procedure must be adhered to. Any deviations from the procedure may produce erroneous results.

This kit has not been optimized by InBios for use with any particular automated ELISA processing system. Use with an automated ELISA processing system will require proper validation to ensure results are equivalent to the expectations described in this package insert. Modifications to the protocol of these systems and/or different volumes of reagents may be required.

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.

Note: For long-term storage, all serum, including the experimental, cannot be repeatedly thawed and frozen. Sera should be further aliquoted in a smaller volume and stored at -70°C.

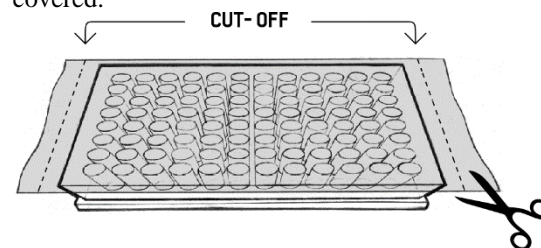
Preparation of Reagents:

- Preparation of 1X Wash Buffer
Dilute the 10X Wash Buffer to 1X using Biological or High-Grade Water. The bottle contains 120ml of 10X Wash Buffer. Mix 120ml of 10X Wash Buffer with 1080 ml of biological or High-Grade Water to make 1X Wash Buffer. After diluting to 1X, store at room temperature for a maximum of four months.
Note: Discard the 1X Wash Buffer if you see any microbial growth.
- Microtitration Wells
Select the number of coated wells required for the assay. The remaining unused wells should be covered and immediately returned to the foil pouch with desiccant on top of the plate, resealed

Example for sera application

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|----------|-----|-----|-----|-----|------|------|------|------|------|------|------|
| A | IgM Neg. | S#1 | S#3 | S#5 | S#7 | S#9 | S#11 | S#13 | S#15 | S#17 | S#19 | S#21 |
| B | IgM Neg. | S#1 | S#3 | S#5 | S#7 | S#9 | S#11 | S#13 | S#15 | S#17 | S#19 | S#21 |
| C | IgM Pos. | S#2 | S#4 | S#6 | S#8 | S#10 | S#12 | S#14 | S#16 | S#18 | S#20 | S#22 |
| D | IgM Pos. | S#2 | S#4 | S#6 | S#8 | S#10 | S#12 | S#14 | S#16 | S#18 | S#20 | S#22 |
| E | IgM Pos. | S#2 | S#4 | S#6 | S#8 | S#10 | S#12 | S#14 | S#16 | S#18 | S#20 | S#22 |
| F | IgM Pos. | S#2 | S#4 | S#6 | S#8 | S#10 | S#12 | S#14 | S#16 | S#18 | S#20 | S#22 |
| G | IgM Neg. | S#1 | S#3 | S#5 | S#7 | S#9 | S#11 | S#13 | S#15 | S#17 | S#19 | S#21 |
| H | IgM Neg. | S#1 | S#3 | S#5 | S#7 | S#9 | S#11 | S#13 | S#15 | S#17 | S#19 | S#21 |

5. Cover the plate with parafilm or plate cover just on the well opening surface so the bottom of the plate is not covered.

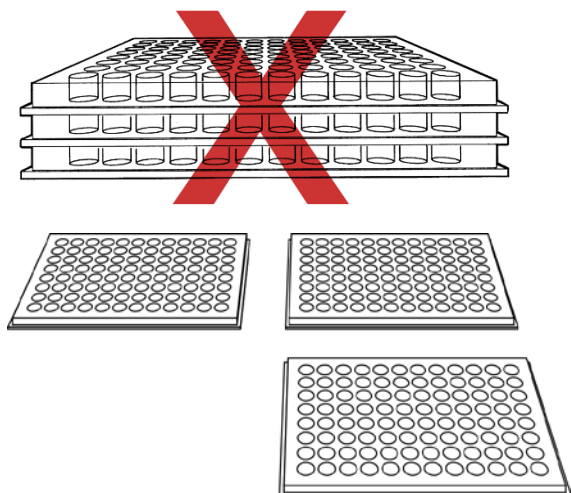


Note: This is to make sure the temperature distribution is

evenly spread out in all wells from bottom and sides; once the top is sealed to block evaporation, any extra parafilm should be cut off.

6. Incubate the plate at 37°C for 1 hour in a non-humidified 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 CO₂, or any other gases used for tissue culture work.



CORRECT METHOD

7. After the incubation, wash the plate 6 times with automatic plate washer using 1X Wash buffer. Use 300µl/well in each wash cycle.

8. Add 50µl per well of WNRA into row A-D and 50µl/well of NCA into row E-H by multi-pipette. An exemplary application for WNRA and NCA is shown below.

| Example for WN Antigens Application | | | | | | | | | | | | |
|-------------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA |
| B | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA |
| C | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA |
| D | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA | WN RA |
| E | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA |
| F | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA |
| G | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA |
| H | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA | NCA |

9. Cover the plate with parafilm just on the well opening surface, so the bottom of the plate is not

covered. (as described in step 5)

10. Incubate the plate at 37°C for 1 hour in a non-humidified incubator.

11. After the incubation, wash the plate 6 times with automatic plate washer using 1X Wash buffer. Use 300µl/well in each wash cycle.

12. Add 50µl/well of ready to use Enzyme-HRP Conjugate into all wells by multi-pipetter.

13. Cover the plate with parafilm just on the well opening surface, so the bottom of the plate is not covered. (as described in step 5)

14. Incubate the plate at 37°C for 1 hour in an a non-humidified incubator.

15. After the incubation, wash the plate 6 times with automatic plate washer using 1X wash buffer. Use 300µl/well in each wash cycle.

16. Add 150µl/well of *En*Wash into all wells by a multi-channel pipette.

17. Incubate the plate at room temperature (20-25°C) for 5 minutes. Do not cover the plate.

18. Wash the plate 6 times with automatic plate washer using 1X wash buffer. Use 300µl/well in each wash cycle.

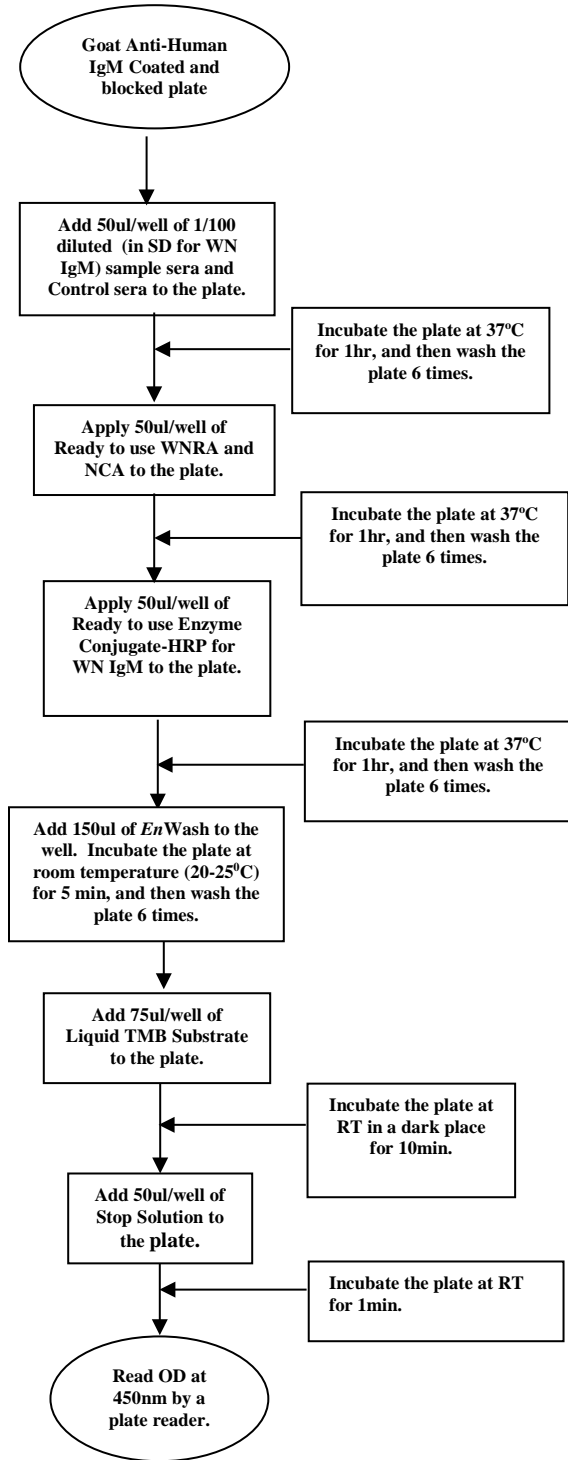
19. Add 75µl/well of Liquid TMB substrate into all wells by multi-channel pipetter.

20. Place and incubate the plate at room temperature (20-25°C) in a dark place (or container) for 10 minutes without any cover on the plate.

21. After the incubation, add 50µl/well of Stop Solution into all wells by multi-channel pipetter and incubate at room temperature (20-25°C) for 1 minute. Do not cover the plate.

22. After the incubation, read the OD 450 value with a Microplate reader.

FOR ACCURATE RESULTS, DO NOT SUBTRACT BLANK READINGS.



QUALITY CONTROL

Each kit contains positive and negative control sera. Acceptable Immune Status Ratio (ISR) values for these controls are found on the specification table below. The negative and positive controls are intended to monitor for substantial reagent failure. The positive control will not ensure precision at the assay cutoff. The test is invalid and must be repeated if the ISR value of either of the controls does not meet the specifications. If the test is invalid, patient results cannot be reported. Quality control requirements must be performed in conformance with local, state, and/or federal regulations or accreditation requirements and your laboratory’s standard Quality Control procedures. It is recommended that the user refer to CLSI C24 and 42 CFR 493.1256 for guidance on appropriate QC practices. The results below are given strictly for guidance purposes only and are applicable solely for spectrophotometric readings. Note: Do not subtract the “blank” from the OD readings.

Calculation of the Negative Control: Calculate the mean Negative Control values with WNRA and with the Control antigen:

Example: Negative Control (NC)

| | OD | |
|-------|-------|-------|
| | WNRA | NCA |
| No. 1 | 0.085 | 0.076 |
| No. 2 | 0.075 | 0.060 |
| Total | 0.160 | 0.136 |

Averages (WNRA) = $0.160 \div 2 = 0.080$

(NCA) = $0.136 \div 2 = 0.068$

Compute the WNRA/NCA ratio: $0.080 \div 0.068 = 1.18$

Any Negative Control WNRA/NCA ratio greater than 4.47 indicates that the test is invalid and the assay must be repeated.

Calculation of the Positive Control: Calculate Positive Control values with WNRA and with the NCA.

Example: Positive Control (PC)

| | OD | |
|-------|-------|-------|
| | WNRA | NCA |
| No. 1 | 0.935 | 0.090 |
| No. 2 | 0.955 | 0.078 |
| Total | 1.890 | 0.168 |

Averages (WNRA) = $1.890 \div 2 = 0.945$

(NCA) = $0.168 \div 2 = 0.084$

Calculate the WNRA/NCA ratio: $0.945 \div 0.084 = 11.3$

Any Positive Control WNRA/NCA ratio less than 5.66 indicates that the test is invalid and the assay must be repeated.

The results in the table below must be obtained in order that the results of the assay may be reported. 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 | Tolerance |
|---------------------------------------|-----------|
| Mean Negative Control (NC) OD in WNRA | < 0.300 |
| Mean Positive Control (PC) OD in WNRA | > 0.500 |
| PC Immune Status Ratio (ISR) | > 5.66 |
| NC Immune Status Ratio (ISR) | < 4.47 |

CALCULATIONS

Calculation of the Immune Status Ratio (ISR): Compute the average of the sample replicates with the WNRA, the sample replicates with the NCA, and then calculate the WNRA/NCA ratio (ISR) for all unknown samples the same way calculations were performed for the positive and negative controls.

Calculation of the ISR values for the test sera: Calculate the mean serum ISR values:

Example: Test Serum #1

| | OD | |
|-------|-------|-------|
| | WNRA | NCA |
| No. 1 | 0.195 | 0.066 |
| No. 2 | 0.205 | 0.070 |
| Total | 0.400 | 0.136 |

Averages (WNRA) = $0.400 / 2 = 0.200$

(NCA) = $0.136 / 2 = 0.068$

Compute the WNRA/NCA ratio: $0.200 / 0.068 = 2.94$

Any serum WNRA/NCA ratio less than 4.47 indicates no detectable IgM antibody is present. See interpretation of results section.

Example: Test Serum #2

| | OD | |
|-------|-------|-------|
| | WNRA | NCA |
| No. 1 | 0.695 | 0.085 |
| No. 2 | 0.725 | 0.100 |
| Total | 1.420 | 0.185 |

Averages (WNRA) = $1.42 / 2 = 0.71$

(NCA) = $0.185 / 2 = 0.0925$

Compute the WNRA/NCA ratio: $0.710 / 0.0925 = 7.68$

Any serum WNRA/NCA ratio greater than 5.66 indicates presence of detectable WN IgM antibody. See interpretation of results section.

Example: Test Serum #3

| | OD | |
|-------|-------|-------|
| | WNRA | NCA |
| No. 1 | 0.310 | 0.065 |
| No. 2 | 0.295 | 0.070 |
| Total | 0.605 | 0.135 |

Averages (WNRA) = $0.605 / 2 = 0.3025$

(NCA) = $0.135 / 2 = 0.0675$

Compute the WNRA/NCA ratio: $0.3025 / 0.0675 = 4.48$

Any serum WNRA/NCA ratio greater than 4.47 but less than 5.66 is an equivocal result and no conclusion can be made. See interpretation of results section.

Selection of the Cut-off: The cut-off was selected using sera from an endemic population in the United States. The 282 samples consisted of 163 positive samples and 119 negative samples characterized by the CDC IgM Antibody Capture ELISA. The cut-off was determined by two-graph receiver operating characteristic (TG-ROC) analysis.

Interpretation of Results: The West Nile *Detect*TM IgM Capture ELISA determines the presence of IgM antibodies to WN virus in serum of patients with clinical symptoms of meningoencephalitis. A positive result is indicative of presumptive WNV meningoencephalitis. The table below shows how the results should be interpreted.

| ISR | Result | Interpretation |
|-----------|-----------|--|
| < 4.47 | Negative | No detectable IgM antibody, individual does not appear to be infected with WN virus. The result does not rule out WN virus infection. An additional sample should be tested within 7-14 days if early infection is suspected. Other WN virus assays should be performed to rule out acute infection. Refer to the current CDC guidelines for diagnosis of this disease ¹ . |
| 4.47-5.66 | Equivocal | Equivocal samples should be repeated in duplicate. If both duplicates are above or below the cut-off the specimens may be reported as positive or negative respectively. Samples that remain equivocal after repeat testing should be reported, that WN virus IgM antibody cannot be determined, and repeated by an alternative method or another sample should be collected. |
| > 5.66 | Positive | Presence of detectable IgM antibody, presumptive infection with WN virus. The result must be confirmed by plaque reduction neutralization test (PRNT), or alternatively, consult the current CDC guidelines for diagnosis of this disease ¹ . A positive IgM result may not indicate a recent infection. IgM antibodies to WN virus have been shown to persist for more than 500 days⁶. Serological cross-reactivity across the flavivirus group is common (i.e. between Saint Louis encephalitis; dengue serotypes 1, 2, 3 and 4; Murray Valley |

| | |
|--|--|
| | encephalitis; Japanese encephalitis; and yellow fever viruses). Additionally, cross-reactivity has been noted with some West Nile virus assays to enteroviruses. Reactive results reported for children must contain a caution statement regarding possible cross-reactivity with enterovirus. These diseases must be excluded before confirmation of diagnosis. |
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The following is a recommended method for reporting the results obtained: "The following results were obtained with the InBios West Nile *Detect*TM IgM Capture ELISA. Values obtained with other assays may not be used interchangeably. The magnitude of the measured result, above the cut-off, is not indicative of the total amount of antibody present." The result should be reported as positive, negative or equivocal, and not as a numeric value. The reported results should contain an appropriate interpretation.

EXPECTED VALUES

West Nile virus infection is generally recognized by the presence of IgM antibodies within one week from the beginning of symptoms. Detectable levels of IgM may be low in early infection. Two hundred samples were prospectively collected from Florida, Texas and Pennsylvania during March 2004. The distribution of females was 50% (100/200) and males were 50% (100/200). The data in Table 1 illustrates the prevalence of IgM antibodies in different age groups when using the West Nile *Detect*TM IgM Capture ELISA Test.

Of the 200 normal sera, one was positive and one was equivocal. The latter specimen was repeated in duplicate and remained equivocal on the West Nile *Detect*TM IgM Capture ELISA Test. The positive and equivocal sera were from Pennsylvania. Of the 200 sera, 66 were from Pennsylvania, resulting in a 3.0% prevalence (2/66) in Pennsylvania.

Table 1

| Age | Total | Equivocal | Positive | Prevalence |
|-------|-------|-----------|----------|------------|
| 10-20 | 12 | 0 | 0 | 0.0% |
| 21-30 | 68 | 1 | 0 | 1.5% |
| 31-40 | 63 | 0 | 0 | 0.0% |
| 41-50 | 47 | 0 | 1 | 2.1% |
| 51-60 | 10 | 0 | 0 | 0.0% |
| Total | 200 | 1 | 1 | 1.0% |

LIMITATIONS

- All positive WN IgM Capture ELISA test results are presumptive and require confirmation by Plaque Reduction Neutralization Test or by using the latest CDC guideline for diagnosis of this disease.
- Testing should only be performed on patients with clinical symptoms of meningoencephalitis. This test is not intended for screening the general population.

The positive predictive value depends on the likelihood of the virus being present.

- Serological cross-reactivity across the flavivirus group is common (i.e. between St. Louis encephalitis, Eastern Equine, Dengue 1, 2, 3, and 4; Murray Valley encephalitis, Japanese encephalitis and yellow fever viruses). These diseases must be excluded before confirmation of diagnosis.
- IgM antibodies may persist for more than 500 days in up to 60% of cases. Positive results should be interpreted in the context of clinical and other laboratory findings and may not indicate active West Nile virus induced disease.
- Assay results should be interpreted only in the context of other laboratory findings and the total clinical status of the patient.
- The reagents supplied in this kit are optimized to measure WNRA reactive antibody levels in serum.
- The assay performance characteristics have not been established for visual result determination.
- Results from immunosuppressed patients must be interpreted with caution.
- Generally primary responders exhibit mainly monotypic antibody responses, However, during successive infections the antibody response broadens to include heterotypic reactivity to other flaviviruses in the same or different antigenic groups⁵.

PERFORMANCE CHARACTERISTICS

Clinical Sensitivity and Specificity:

Table 1 – Study Site 1

A clinical laboratory located in the midwestern U.S. tested 50 retrospective samples with clinically and laboratory confirmed cases of WNV (n=50) or undetermined flavivirus (positive for both WNV and SLE; n=2). The samples were suspected to have come from patients that had exhibited signs or symptoms of WN but specific clinical information could not be confirmed. In addition, 125 retrospective sequential endemic samples were tested. The sera were sequentially submitted to the laboratory, archived, and masked. Two were confirmed with undetermined flavivirus or WNV by PRNT.

| Clinical Category | Positive | Negative | Equivocal | Total |
|-------------------|----------|----------|-----------|-------|
| PRNT Positive | 50 | 2 | 0 | 52 |
| Negative | 1 | 121 | 1 | 123 |
| Total | 51 | 123 | 1 | 175 |

WN Virus Positive:

Serological Sensitivity = 50/52 = 96.2%

95% Confidence Interval: 87.0 – 98.9%

WN Virus Negative:
 Serological Specificity = 121/123 = 98.4%
 95% Confidence Interval: 94.3 - 99.6%

Table 2 – Study Site 2

A State Department of Health laboratory located in the midwestern U.S. tested 88 retrospective samples clinically and laboratory confirmed cases of WNV and/or SLE and confirmed by PRNT. Seven patient samples were suspected of having either viral encephalitis or viral meningitis. The remaining patient samples had signs or symptoms of WN fever and headache. In addition, 130 retrospective, sequential endemic samples were tested. The sera were sequentially submitted to the laboratory, archived, and masked. Fourteen (14) were confirmed with SLE and/or WNV by PRNT.

| Clinical Category | Positive | Negative | Equivocal | Total |
|-------------------|----------|----------|-----------|-------|
| PRNT Positive | 99 | 2 | 1 | 102 |
| Negative | 1 | 115 | 0 | 116 |
| Total | 100 | 117 | 1 | 218 |

Note: Some of these specimens may have been previously tested for WNV antibodies before being sent to this laboratory for confirmation by PRNT. The possible bias effect on the performance of this assay is unknown.

WN Virus Positive:
 Clinical Sensitivity = 99/102 = 97.1%
 95% Confidence Interval: 91.7 – 99.0%
 WN Virus Negative:
 Clinical Specificity = 115/116 = 99.1%
 95% Confidence Interval: 95.3 - 99.9%

Table 3 – Study Site 3

A State Department of Health laboratory located in southeastern U.S. tested 150 retrospective samples clinically and laboratory confirmed cases of WNV by PRNT. In addition, 150 retrospective, sequential endemic samples were tested. The sera were sequentially submitted to the laboratory, archived, and masked. Twenty-three (23) were confirmed with SLE and/or WNV by CDC ELISA.

| Clinical Category | Positive | Negative | Equivocal | Total |
|-------------------|----------|----------|-----------|-------|
| PRNT Positive | 172 | 1 | 0 | 173 |
| Negative | 0 | 127 | 0 | 127 |
| Total | 172 | 128 | 0 | 300 |

WN Virus Positive:
 Serological Sensitivity = 172/173 = 99.4%
 95% Confidence Interval: 96.8 – 99.9%
 WN Virus Negative:
 Serological Specificity = 127/127 = 100.0%
 95% Confidence Interval: 97.1 - 100%

Table 4 - Study Site 4

A State Department of Health laboratory located in northeastern U.S. tested 210 retrospective, sequential endemic samples with the West Nile *Detect*TM IgM Capture ELISA and with the CDC MAC-ELISA. The sera were sequentially submitted to the laboratory, archived, and masked. None of the samples gave a positive result with both tests.

| Clinical Category | Positive | Negative | Equivocal | Total |
|------------------------|----------|----------|-----------|-------|
| CDC MAC-ELISA Positive | 0 | 0 | 0 | 0 |
| Negative | 0 | 210 | 0 | 210 |
| Total | 0 | 210 | 0 | 210 |

Negative Presumptive Agreement

210/210 = 100.0%

95% Confidence Interval: 98.2 – 100.0%

Freeze Thaw Study: Eight serum samples were exposed to five freeze-thaw cycles and assayed for IgM antibodies to West Nile recombinant antigen after each cycle. All five serum samples that were positive in the untreated version of the assay were positive after all five freeze-thaw cycles. One sample that was negative but close to being equivocal in the beginning became equivocal at cycles 1, 3, 4 and 5, but not at cycle 2. It is recommended that serum samples for use in the West Nile *Detect*TM IgM Capture ELISA Test not be frozen and thawed for more than five times.

IgM Specificity Study: Thirteen serum samples, including a positive and a negative control, were treated with dithiothreitol and assayed for IgM antibodies to West Nile recombinant antigen. All nine WNV infected serum samples that were positive in the untreated version of the assay were negative in the dithiothreitol treated version. No substantial effect was seen in the IgG assay. This study indicates that the West Nile *Detect*TM IgM Capture ELISA assay is specific for IgM.

Interference Study

Four potentially interfering substances commonly occurring in serum were tested for their effect on the West Nile *Detect*TM IgM Capture ELISA test. Pools of two West Nile positive sera and one West Nile negative serum were used. The four potentially interfering substances were Bilirubin (1& 2 mg/dL), Triglycerides (500 and 3000 mg/dL), Hemoglobin (1600 and 16,000 mg/dL) and Cholesterol (300 and 500 mg/dL) The results of this study indicate that West Nile low positive results with high levels of triglycerides may increase ISR values of WNRA reactive sera. The other interfering substances did not exhibit any deleterious effect on the sensitivity and specificity of the assay.

Reproducibility Study:

The reproducibility of the West Nile *Detect*TM IgM Capture ELISA was evaluated at three sites. One site was InBios International. Ten serum specimens using clinical specimens diluted into an analyte-negative matrix was used. The ten serum specimens (not including positive and negative controls) included specimens that were below the cutoff values (negative samples) and above the cutoff value (positive and weak positive or borderline samples). The serum dilutions selected also ensured that the analyte concentration in the specimens represented a clinically relevant range. The results were analyzed by an outside statistician and are shown in the table below.

Reproducibility results from three sites after deleting 2 outlying data points from Site #3 are shown below.

| Sample ID | n | Mean | Intra-Assay | | Between Day | | Between Lab | | Total | |
|-----------|----|-------|-------------|------|-------------|-------|-------------|-------|-------|-------|
| | | | *S.D. | %CV | *S.D. | %CV | *S.D. | %CV | *S.D. | %CV |
| 1 | 27 | 1.18 | 0.07 | 5.8% | 0.14 | 11.4% | 0.29 | 24.5% | 0.33 | 27.6% |
| 2 | 27 | 9.39 | 1.04 | % | 3.10 | 33.1% | 1.46 | 15.5% | 3.58 | 38.2% |
| 3 | 27 | 17.98 | 1.50 | 8.3% | 4.00 | 22.2% | 4.18 | 23.3% | 5.98 | 33.2% |
| 4 | 27 | 6.48 | 1.04 | % | 1.98 | 30.5% | 2.47 | 38.2% | 3.33 | 51.4% |
| 5 | 26 | 21.07 | 2.54 | % | 5.53 | 26.3% | 7.56 | 35.9% | 9.70 | 46.1% |
| 6 | 27 | 7.92 | 0.74 | 9.4% | 2.33 | 29.5% | 3.46 | 43.7% | 4.24 | 53.5% |
| 7 | 26 | 12.75 | 1.46 | % | 2.21 | 17.3% | 3.85 | 30.2% | 4.67 | 36.6% |
| 8 | 27 | 5.94 | 0.64 | % | 1.62 | 27.3% | 1.87 | 31.5% | 2.56 | 43.1% |
| 9 | 27 | 24.81 | 2.53 | % | 8.86 | 35.7% | 4.23 | 17.0% | 10.14 | 40.9% |
| 10 | 27 | 1.14 | 0.07 | 5.8% | 0.04 | 3.2% | 0.16 | 14.3% | 0.18 | 15.8% |

All values are calculated as WNRA/NCA ratios

SD = Standard Deviation

%CV = % Coefficient of Variation

26*: 1 statistically outlying (>5.5 x Standard Deviation of previous run) data point was removed.

Cross-reactivity Study:

Two hundred and seventy-one sera that tested positive for other potentially cross-reactive pathogens were tested with the West Nile *Detect*TM IgM Capture ELISA test to determine the potential for cross-reactivity. The table below summarizes the results of this study.

| Disease | Number of Samples | West Nile <i>Detect</i> TM IgM ELISA | | Total of Positive and Equivocal |
|-----------------------------|-------------------|---|----------|---------------------------------|
| | | Equivocal | Positive | |
| Eastern Equine encephalitis | 17 | 0 | 0 | 0/17 |
| Japanese encephalitis | 2 | 0 | 0 | 0/2 |
| Saint Louis encephalitis | 32 | 1 | 16 | 17/32 |
| La Crosse virus | 6 | 0 | 0 | 0/6 |
| Dengue virus | 7 | 0 | 2 | 2/7 |
| Epstein-Barr virus | 15 | 0 | 0 | 0/15 |
| Hepatitis A virus | 10 | 0 | 0 | 0/10 |
| Hepatitis B virus | 49 | 0 | 0 | 0/49 |
| Hepatitis C virus | 30 | 0 | 0 | 0/30 |

| Disease | Number of Samples | West Nile <i>Detect</i> TM IgM ELISA | | Total of Positive and Equivocal |
|------------------------------|-------------------|---|-----------|---------------------------------|
| | | Equivocal | Positive | |
| Herpes simplex virus | 32 | 0 | 0 | 0/32 |
| California Encephalitis (CE) | 1 | 0 | 0 | 0/1 |
| HIV | 20 | 0 | 0 | 0/20 |
| Syphilis | 5 | 0 | 0 | 0/5 |
| Cytomegalovirus | 12 | 0 | 0 | 0/12 |
| Varicella zoster virus | 10 | 0 | 0 | 0/10 |
| Coxsackievirus B 1-6 | 1 | 0 | 0 | 0/1 |
| Echovirus 16 | 1 | 0 | 0 | 0/1 |
| Measles | 1 | 0 | 0 | 0/1 |
| Mumps | 1 | 0 | 0 | 0/1 |
| Polio Blend | 1 | 0 | 0 | 0/1 |
| Legionnaires' disease | 3 | 0 | 0 | 0/3 |
| Rheumatoid factor | 5 | 0 | 0 | 0/5 |
| Anti-nuclear antibody | 10 | 0 | 0 | 0/10 |
| Total | 271 | 1 | 18 | 19/271 |

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TROUBLESHOOTING

| Problem | Possible Cause | Possible Resolution |
|---|---|---|
| High Absorbances for WNRA/NCA (Incubator temperature must be verified with a source independent of incubator external display) | Incorrect component used | Do not combine controls or reagents between different lots of the ELISA kits. |
| | Samples incorrectly diluted/Wrong samples used | Sera should be diluted 1:100 in kit's sample dilution buffer. This kit has been validated for use with sera samples only. |
| | Cross contamination of wells | A new tip must be used for every test or control sera. |
| | Incomplete washing of wells | Wells must be completely filled and emptied 6 times during each wash cycle. |
| | Incubation times too long | Incubation times vary, please refer to the "Test Procedure" section for correct times. |
| | Conjugate contamination with TMB | It is recommended to use a new pipette/ pipette tip each time to dispense conjugate and TMB. |
| | Incorrect wavelength filter | The optical density readings must be read with only a 450nm filter. There must not be any background subtraction. |
| Low Absorbances for WNRA/NCA (Incubator temperature must be verified with a source independent of incubator external display) | Samples incorrectly diluted | Sera should be diluted 1:100 in kit's sample dilution buffer. |
| | Kit expiration date and storage | Verify that the kit is not expired and that components were properly stored |
| | Incorrect component used | Do not combine controls or reagents between different lots of the ELISA kits. |
| | Component temperatures | All kit components must be equilibrated at room temperature for optimal performance. |
| | Incubation times too short | Incubation times vary, please refer to the "Test Procedure" section for correct times. |
| | Incubation temperature too low | Verify that incubators are calibrated and that the temperatures are monitored. |
| | WNRA/NCA contamination | The antigens are very susceptible to contamination. It is recommended to use a new pipet/pipette tip each time to dispense antigens. Keep the lid on the antigens unless in use. When possible, dispense antigens in a clean laminar flow hood or biological safety cabinet. |
| | Conjugate contamination | The conjugate is very susceptible to contamination. It is recommended to use a new pipet/pipette tip each time to dispense conjugate. Keep the lid on the conjugate unless in use. When possible, dispense conjugate in a clean laminar flow hood or biological safety cabinet. |
| | TMB contamination with Stop solution | It is recommended to use a new pipet/ pipette tip each time to dispense TMB and stop solution. |
| | Use of reagents in the wrong sequence, or omission of step(s) | Check the "Test Procedure" section and component labels prior to use. |
| Incorrect wavelength filter | The optical density readings must be read with only a 450nm filter. There must not be any background subtraction. | |