West Nile Virus Antibody Test Kit, ELISA

INTENDED USE

The West Nile Virus Antibody Test Kit, ELISA is an ELISA assay system for the detection of IgM antibodies in equine serum to WNV-derived recombinant antigen (WNRA) (1-3).

SUMMARY AND EXPLANATION OF THE TEST

Exposure to West Nile Virus causes a disease with a number of symptoms including encephalitis (5-8). West Nile Virus is becoming widespread and has been detected in horses from 48 states. The West Nile Virus Antibody Test Kit, ELISA employs a recombinant antigen called WNRA, (West Nile Noninfectious Recombinant Antigen), which consists of peptides from two WNV antigens.

PRINCIPLE OF THE TEST

The West Nile Virus Antibody Test Kit, ELISA consists of an enzymatically amplified "two-step" sandwich-type immunoassay. This assay is for antibodies of the IgM class.

Standards, controls and unknown serum samples are incubated in microtiter wells which have been coated with anti-equine IgM antibodies. The serum samples are directly mixed with Sample Dilution Buffer for sample dilution and then applied to the wells. This is followed by overnight incubation with either West Nile Virus-derived recombinant WNRA protein or a control preparation. After 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 NCA (Normal Control Antigen) wells indicates whether antibodies to WNV are present. A set of positive and negative samples is provided as internal controls in order to monitor the integrity of the kit components.

MATERIALS SUPPLIED

The West Nile Virus Antibody Test Kit, ELISA contains sufficient reagents to test 44 equine serum samples and the positive and negative controls in duplicate.

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

The kit contains the following reagents:

1. Coated Microtiter Strips: 96 polystyrene microtiter wells coated with antibody to equine IgM in each well. Store at 2-8°C until ready to use.
2. Sample Dilution Buffer: One bottle, 25 mL, for ELISA plate wells in IgM assay. Store at -65±5°C until ready to use.
3. WN Equine IgM Negative Control: One vial containing 50 µL of heat-inactivated negative serum. Store at -65±5°C until ready to use. Centrifuge the vial briefly before use to collect the content at the bottom. Note: The serum should not be frozen and thawed repeatedly.
4. WN Equine IgM Positive Control: One vial containing 50 µL of heat-inactivated positive serum. Store at -65±5°C until ready to use. Centrifuge the vial briefly before use to collect the content at the bottom. Note: The serum should not be frozen and thawed frequently.
5. Ready to Use WN Antigen (WNRA): One tube, 3 mL of Ready-to-use WNRA solution. Store at -65±5°C until ready to use.
6. Ready to Use Normal Cell Antigen (NCA): One tube, 3 mL of Ready-to-use control solution. Store at -65±5°C until ready to use.
7. Ready to Use Enzyme Conjugate-HRP for WN IgM: One bottle, 6 mL of a pre-diluted conjugate to be used as is in the procedure below. Store at 2-8°C until ready to use.
8. 10X Wash Buffer: One bottle, 120 mL of Wash Buffer to be used in all the washing steps of this procedure. Store 10X Wash Buffer at 2-8°C until ready to use.
9. EnWash: One bottle, 20 mL of EnWash to be used in between the washing steps after the addition of enzyme conjugate-HRP of this procedure. Store at 2-8°C until ready to use.
10. Liquid TMB Substrate: One bottle, 9 mL of liquid substrate to be used in this procedure. Store at 2-8°C until ready to use. The substrate should be kept in a light-protected bottle at all times as provided.
11. Stop Solution: One bottle, 6 mL to be used to stop the reaction. Store at 2-8°C until ready to use. Caution: strong acid, wear protective gloves, mask and safety glasses. Dispose of all the materials according to safety rules and regulations.

MATERIALS REQUIRED BUT NOT SUPPLIED

- Microtiter plate reader capable of absorbance measurement at 450 nm
- Biological or high-grade water
- Microtiter plate washer
- Vacuum pump
- 37°C incubator without CO₂ supply or humidification
- Microliter pipettes
- Parafilm or similar plate covers

PRECAUTIONS

- A thorough understanding of this package insert is necessary for successful use of the product. Reliable results will only be obtained by using precise laboratory techniques and accurately following the package insert.
- Do not mix various lots of any kit component within an individual assay.
- Do not use any component beyond the expiration date shown on its label.
- Avoid exposure of the reagents to excessive heat or direct sunlight during storage and incubation.
- Some reagents may form a slight precipitate, mix gently before use.
- 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.
- Avoid microbial contamination of reagents, especially of the Ready to Use Conjugate-HRP. Avoid contamination of the TMB Substrate Solution with the Enzyme Conjugate-HRP.
- 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 pipet by mouth.
- Use a clean disposable pipettor tip for each reagent.
- Standard, Control or specimen.
- Cover working area with disposable absorbent paper.

**WARNING: POTENTIAL BIOHAZARDOUS MATERIAL**
This kit may contain reagents made with equine 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:**
Material Safety Data Sheets (MSDS) are available for all components of this kit. Review all appropriate MSDS before performing this assay. Avoid all contact between hands and eyes or mucous membranes during testing. If contact does occur, consult the applicable MSDS for appropriate treatment.

### SPECIMEN COLLECTION AND PREPARATION

- Equine serum must be used with this assay.
- 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 20-25°C for periods exceeding 4 hours.
- The samples may be stored at 2-8°C for up to 7 days, or frozen at -20°C or lower for up to 30 days. To maintain long-term longevity of the serum, store at -65±5°C. Avoid repeated freezing and thawing of samples.
- Do not use hemolyzed or lipemic samples.
- Frozen samples should be thawed to 20-25°C and mixed thoroughly by gentle swirling or inversion prior to use.
- If sera are to be shipped, they should be packed in compliance with Federal Regulations covering transportation of infectious agents.

### TEST PROCEDURE

**CAUTION:** The test procedure must be adhered to. Any deviations from the procedure may produce erroneous results. Bring all kit reagents and specimens to room temperature (20-25°C) before use. Thoroughly mix the reagents and samples before use by gentle inversion. NOTE: For long-term storage, serum samples should not be repeatedly thawed and frozen more than 5 times. Sera should be aliquoted into smaller volumes if repeated use is anticipated.

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 the package insert. Modifications to the protocol of these systems and/or different volumes of reagents may be required.

### Preparation of Reagents:

- Preparation of 1X Wash Buffer
  - Dilute the 10X Wash Buffer to 1X using Biological or High-Grade Water. The bottle contains 120 mL of 10X Wash Buffer. Mix 120 mL of 10X Wash Buffer with 1080 mL of biological or High-Grade Water to make 1X Wash Buffer. After diluting to 1X, store at 20-25°C for a maximum of six 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 and stored at 2-8°C until ready to use or expiration.

### Assay Procedure:

1. Controls, and test sera are to be assayed both in the WNRA and the NCA wells. Forty-four test specimens can be tested on one 96 well plate. The negative and positive controls must be tested in duplicate.
2. Mark the microtitration strips to be used.
3. Dilute test sera and the controls to 1/100 using the provided Sample Dilution Buffer. Use small polypropylene tubes or multi-well untreated plastic strips for these dilutions. 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 duplicate wells on the plate using an appropriate pipettor. An example for forty-four test serum samples is shown below.

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<th>Example for sera Application</th>
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**Date:** 2015/11/01
Example for WN Antigens Application

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<td>S#33</td>
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</table>

9. Cover the plate (as described in step 5).

10. Incubate the plate at 2-8°C for 16-20 hours.

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

12. Add 50 µL/well of Ready to Use Enzyme Conjugate-HRP into all wells by multi-pipettor.

13. Cover the plate (as described in step 5).

14. Incubate the plate at 37±2°C for 1 hour ±5 minutes in a non-humidified chamber.

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

16. Add 150 µL/well of EnWash into all wells using a multi-channel pipettor.

17. Incubate the plate at room temperature (20-25°C) for 5±1 minutes. Do not cover the plate.

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

19. Add 75 µL/well of Liquid TMB substrate into all wells using multi-channel pipettor.

20. Incubate the uncovered plate at room temperature (20-25°C) in darkness for 10 minutes ±30 seconds.

21. After the incubation, add 50 µL per well of Stop Solution into all wells using a multi-channel pipettor and incubate at room temperature (20-25°C) for 1 minute (and no more than 3 minutes). Do not cover the plate.

22. Read the OD 450nm value with a Microplate reader.

**NOTE:** The microplate reader should not subtract or normalize for any blank valued or wells.
Applicable for spectrophotometric readings that are not background subtracted. Do not use another wavelength such as 650nm to subtract 450nm water or reagent background values.

Interpretation of Results:

The Immune Status Ratio (ISR) is calculated as follows:

Compute the WNRA/NCA ratio (ISR) for the negative and positive controls and each sample by dividing the optical density (OD) for the WNRA antigen by the OD value for the NCA.

Calculate the Negative Control values with WNRA and NCA:
Example: Negative Control (NC) OD
WNRA  NCA
Optical densities: 0.130 0.118

Calculate the WNRA/NCA ratio: 0.130 ÷ 0.118 = 1.10

Calculate the Positive Control values with the WNRA and NCA.
Example: Positive Control (PC) OD
WNRA  NCA
Optical densities: 1.235 0.095

Calculate the WNRA/NCA ratio: 1.235 ÷ 0.095 = 13.0

The results in the table below must be obtained for a valid assay. Should the controls fail to meet any validity requirement, the assay must be repeated.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Tolerance</th>
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<tbody>
<tr>
<td>Negative Control Reading</td>
<td>&lt; 0.150</td>
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<tr>
<td>(NC)</td>
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</tr>
<tr>
<td>Positive Control (PC)</td>
<td>≥ 1.000</td>
</tr>
<tr>
<td>Reading</td>
<td></td>
</tr>
<tr>
<td>PC Immune Status Ratio</td>
<td>&gt; 10.0</td>
</tr>
<tr>
<td>(ISR)</td>
<td></td>
</tr>
<tr>
<td>NC Immune Status Ratio</td>
<td>&lt; 2.0</td>
</tr>
</tbody>
</table>

Interpretation of the results:

1. Samples with ISR ≥3.0 are considered to be “Positive”. Any “Positive” sample must be repeated to verify the result.
2. Samples with ISR ≤2.0 are considered to be “Negative”.
3. Samples with ISR <3.0 but >2.0 are considered to be “Indeterminate” but possible positives, and should be repeated in triplicate or more.
4. ISR >2.0 arising from low optical densities in both the WNRA and control wells must be considered potential false positives.

Exclusion Criteria:

Example #1 Low optical density samples:
Serum Sample OD
WNRA  NCA
Optical densities: 0.030 0.013

Calculate the WNRA/NCA ratio: 0.030 ÷ 0.013 = 2.31

While the ISR is >2.0, this sample must be considered a potential false positive, due to the low optical densities. This can occur when the plate reader is used in a mode that subtracts for the reagent blanks. Do not use the plate reader in this mode.

REFERENCES

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