INTENDED USE
The Focus Diagnostics’ Dengue NS1 Antigen DxSelect™ assay is intended for the early detection of dengue virus NS1 antigen in human serum. This test may aid in the early diagnosis of dengue virus in human serum prior to the presence of IgM or IgG antibodies. The assay is not intended to screen blood or blood components. The assay is for professional use only.

SUMMARY AND EXPLANATION OF TEST
Dengue fever (DF) is an acute, self-limiting, viral disease that is characterized by bi-phasic fever, headache, body pains, rash, lymphadenopathy, and prostration. In its most severe form, dengue hemorrhagic fever (DHF), infected patients will experience severe fever and renal failure leading to the often fatal dengue shock syndrome (DSS).1 It is estimated that approximately 2 billion people are at risk for DF worldwide, and that over 1 million people per year are infected.2 This, combined with the hundreds of thousands of cases of DSS, make dengue one of the most important arbovirus diseases in the world.2

Dengue virus (DV) is a flavivirus and is closely related to the yellow fever virus, Japanese encephalitis virus and other group B arboviruses. Members of this group possess single stranded RNA which is surrounded by an icosahedral nucleocapsid surrounded by a 10 nm lipid envelope.3 There are four strains of dengue virus, each serologically distinct. Infection with one strain does not protect the host from infection by the others. In fact, a report suggests DHF and DSS occurs most commonly in individuals that have been infected previously by another strain.4 The presence of circulating, non-neutralizing, cross-reactive DV antibody may act as an immune infection enhancement factor.5 However, non-neutralizing, cross-reactive antibodies against other non-DV flaviviruses are not associated with immune infection enhancement.4

Dengue fever epidemics have been reported regularly throughout the world. The largest epidemics have occurred in the southern United States (1922 affecting over 1 million people), Australia (1925 and 1942), Greece (1927) and Japan (1942-1945).2 Peruvian and CDC officials have reported a major DF outbreak that occurred between March to July 1990.5 This epidemic centered around Iquitos, Peru, involving DV types 1 and 4, is the first laboratory confirmation of indigenous transmission of dengue in Peru.5

Dengue virus can be transmitted wherever the mosquito vectors, Aedes aegypti and Aedes albopictus, are found. A. aegypti is primarily localized to the tropical and subtropical Americas and is indigenous to the southern part of the United States.2 The primary vector for DF in Asia is A. albopictus. This mosquito has recently established itself in the United States as far north as central Illinois; however, DV transmission has not been associated with it to date.3

Dengue NS1 (non-structural) protein is a highly-conserved glycoprotein. It is believed to play a role in viral RNA replication. It is strongly immunogenic eliciting antibodies with complement fixing activity. NS1 antigen can be detected in sera during acute dengue infection. It is found from the first day and up to 9 days after onset of fever in sample of primary or secondary dengue infected patients.

TEST PRINCIPLE
The Dengue NS1 Antigen DxSelect™ assay is a highly sensitive assay. It uses one enzymatically amplified “two-step” sandwich-type immunoassay to detect low levels of NS1 in serum.

In the Dengue NS1 Antigen DxSelect™ assay, controls and unknown serum samples are diluted in sample dilution buffer containing secondary antibody, and incubated in microtiteration wells. These wells have been coated with NS1 antibody. NS1 antigens present in the samples are then, “sandwiched” between the capture and secondary antibodies. The presence of NS1 antigen is confirmed by the colorimetric response obtained using an enzyme-conjugate-HRP and liquid TMB substrate. Once the reaction is stopped, using an acidic solution, the enzymatic turnover of the substrate is determined by absorbance measurement at 450 nanometers. Sample optical density readings are compared with reference cut-off OD readings to determine presence of NS1 antigens.

Note: A set of negative, positive and cut-off controls are provided as internal controls in order to monitor the integrity of the kit components.
**MATERIALS SUPPLIED**
The Dengue NS1 Antigen DxSelect™ kit contains sufficient reagents for one plate of 96 wells (12 x 8 strips) each. All unopened materials are stable at 2 to 8°C until the expiration date stated on the reagent label.

**Dengue NS1 Coated Microtiter Strips, 96 Wells**
12 eight-well polystyrene microwell strips on a frame. Each well is coated with Anti-Dengue NS1 antibody.

**Dengue NS1 Coated Microtiter Strips, 96 Wells**

**Dengue NS1 Negative Control, 300µL**
One vial of human serum. Centrifuge briefly prior to use to sediment any precipitate.

**Dengue NS1 Positive Control, 300µL**
One vial of recombinant NS1. Centrifuge briefly prior to use to sediment any precipitate.

**Dengue NS1 Cut-Off Control, 300µL**
One vial of recombinant NS1. Centrifuge briefly prior to use to sediment any precipitate.

**Dengue NS1 Sample Diluent , 15mL**
One bottle of solution with Dengue NS1 antibody. Contains Proclin (0.02-0.03%) as a preservative.

**100x Dengue NS1 Conjugate, 150µL**
One bottle of horse radish peroxidase-labeled antibody. Mix well prior to use. Store at 2 to 8°C.

**Dengue NS1 Conjugate Diluent, 12mL**
This contains the diluent solution for the 100x Conjugate. Contains Thimerosal (0.01%) as a preservative. The 100x conjugate is diluted directly into this solution. After diluting 100X Conjugate into this solution, the now ready-to-use conjugate may be stored for 2 weeks at 2 to 8°C.

**10 X Wash Buffer, 120 mL**
One bottle of Wash Buffer to be used as directed in Test Procedure.

**Liquid TMB Substrate, 12mL**
One bottle of tetramethyl benzidine (TMB) and hydrogen peroxide in buffer. To be used as directed in Test Procedure.

**Stop Solution, 6mL**
One bottle of sulfuric acid to be used to terminate the reaction as directed in Test Procedure.

**WARNINGS AND PRECAUTIONS**

1. Kits and kit reagents are stable through the end of the month indicated by the label expiration date when stored at 2 to 8°C.
2. Store reagents at 2 to 8°C.
3. Do not use test kit or reagents beyond their expiration dates.
4. Do not mix various lots of any kit component within an individual assay.
5. Do not use any component beyond the expiration date shown on its label.
6. After initial use, return each component to refrigerator (2 to 8°C) and store for one month or until expiration date, whichever comes first with exception of the Conjugate Solution. This solution may be stored for up to 2 weeks if stored at 2 to 8°C. After 2 weeks, this conjugate solution should be discarded and no longer used in this assay.
7. Do not pour back used reagents into the original containers.
8. Do not use contaminated reagents.
9. Store reagents in their original containers.
10. Do not substitute or mix reagents from different kit lots or from other manufacturers.

**MATERIALS REQUIRED, BUT NOT SUPPLIED**

1. ELISA Spectrophotometer capable of absorbance measurement at 450 nm
2. Biological or High-Grade Water
3. Vacuum Pump
4. Automatic Plate Washer
5. 37°C Incubator
7. Polypropylene tubes or 96 well dilution plates
8. Parafilm
9. Timer
10. Vortex

**SHELF LIFE AND HANDLING**

1. Kits and kit reagents are stable through the end of the month indicated by the label expiration date when stored at 2 to 8°C.
2. Store reagents at 2 to 8°C.
3. Do not use test kit or reagents beyond their expiration dates.
4. Do not expose reagents to strong light during storage or incubation.
5. Allow reagents to warm to 20 to 25°C before use.
6. After initial use, return each component to refrigerator (2 to 8°C) and store for one month or until expiration date, whichever comes first with exception of the Conjugate Solution. This solution may be stored for up to 2 weeks if stored at 2 to 8°C. After 2 weeks, this conjugate solution should be discarded and no longer used in this assay.
7. Do not pour back used reagents into the original containers.
8. Do not use contaminated reagents.
9. Store reagents in their original containers.
10. Do not substitute or mix reagents from different kit lots or from other manufacturers.
14. 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
1. Human serum must be used with this assay. Reagents have not been optimized, or tested with whole blood or plasma so they cannot be tested directly.
2. Remove serum from the clot of red cells as soon as possible to avoid hemolysis.
3. Testing should be performed as soon as possible after collection. Do not leave sera at room temperature for prolonged periods.
4. Serum should be used and the usual precautions for venipuncture should be observed. The samples may be stored at 2 to 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 -70°C. Avoid repeated freezing and thawing of samples.
5. Frozen samples should be thawed to room temperature and mixed thoroughly by gentle swirling or inversion prior to use. Always quick spin before use.
6. Do not use sera if any indication of growth is observed.

TEST PROCEDURE
Bring all kit reagents and specimens to room temperature (~25°C) before use. Thoroughly mix the reagents and samples before use by gentle inversion.

Preparation of Reagents
Preparation of 1X Wash Buffer: Dilute the 10X Wash Buffer to 1X using Biological or High-Grade Water. To prepare a 1X wash buffer solution, mix 120 ml 10X wash buffer with 1080 ml distilled (or deionized water). Mix thoroughly to ensure that any precipitate is dissolved and that the solution is uniform. Once diluted to 1X, the solution can be stored at room temperature for up to 6 months. Check for contamination prior to use. Discard if contamination is suspected.

Microtitration Wells: Select the number of coated wells required for the assay. The remaining unused wells should be repackaged immediately with the supplied desiccant and stored at 2 to 8°C until ready to use or expiration.

Preparation of Conjugate Solution: Add 120 µl of 100x Conjugate for Dengue NS1 ELISA directly to the 12 ml bottle of Conjugate Diluent for Dengue NS1 (1 part : 100 parts). Mix by inverting solution several times. This solution may be stored for up to 2 weeks if stored at 2 to 8°C. After 2 weeks, this conjugate solution should be discarded and no longer used in this assay.

Test Procedure
1. Positive, negative, and cut-off controls should be assayed in duplicate (and run each time assay is performed on every plate). Unknown serum samples may be tested in singlet. (However, it is recommended to run samples in duplicate until the operator is familiar with the assay.) Ninety test specimens can be tested in singlet on each plate.
2. Using a single channel or multichannel pipettor, aliquot 50 µl of Sample Diluent for Dengue NS1 ELISA into each of the required wells.
3. Add 50 µl of each undiluted sera (test samples and control samples) directly to the center of the wells containing the Sample Diluent. Rock the plate gently from side to side 5 times.
4. Cover the top of the plate with parafilm and remove excess.

Note: This is to make sure the temperature distribution is evenly spread out in all wells from bottom and sides; any extra parafilm can be cut off once the top is sealed to block evaporation.

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 other gases. Do not place plates in contact with any wet substances such as wet paper towels etc.

5. Incubate the plate at 37°C for 1 hour in an incubator.
6. After the incubation, wash the plate 6 times with an automatic plate washer using 1x Wash buffer. Use 300 µl per well in each wash cycle.
7. Prepare the Conjugate Solution (120 µl of 100x Conjugate : 12 ml of Conjugate Diluent) and add 100 µL/well of this Conjugate Solution into all wells using a multi-channel pipettor. Discard the remaining Conjugate Solution or store for up to 2 weeks at 2 to 8°C.
8. Cover the plate with parafilm, as shown above, and incubate at 37°C for 30 minutes in an incubator.
9. After the incubation, wash the plate 6 times with the automatic plate washer using 3x Wash buffer.
10. Add 100 µL per well of Liquid TMB substrate into all wells using a multi-channel pipettor.
11. Incubate the plate in the dark, at room temperature for 20 minutes.
12. Add 50 µL per well of Stop Solution into all wells using a multi-channel pipettor and let the plate stand, uncovered at room temperature for 1 minute.
13. Read the optical density at 450nm (OD450) value with a Microplate reader. DO NOT SUBTRACT OR NORMALIZE ANY BLANK VALUES OR WELLS.
14. Record the raw OD450 and evaluate the sample status as indicated in the Quality Control section.

QUALITY CONTROL
Each kit contains positive, negative and cut-off control samples. An acceptable Discrimination Capacity (RPC/NC) must be obtained to ensure assay validity. 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 (RPC/NC) value is too low or if the control samples do not meet the specifications. If the test is invalid, the results cannot be used. 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-A3 and 42 CFR 493.1256 for guidance on appropriate QC practices. The results below are given strictly for guidance purposes only and applicable for spectrophotometric readings only.

First, calculate the RPC/NC as shown in the example.

| Example |
| Calculate the mean Negative Control (NC): |
| Example: Negative Control OD |
| No 1 | 0.108 |
| No 2 | 0.084 |
| Total | 0.192 |
| Average of Negative Control = 0.192 ÷ 2 = 0.096 |

| Example: Positive Control OD |
| No 1 | 1.112 |
| No 2 | 1.089 |
| Total | 2.201 |
| Average of Positive Control = 2.201 ÷ 2 = 1.101 |

Calculate the ratio (RPC/NC) between Positive and Negative Control:
Example: (RPC/NC) = 1.101 ÷ 0.096 = 11.47

Quality Control Requirements
Next, ensure that the quality control requirements, listed in the table below, are fulfilled.

<table>
<thead>
<tr>
<th>Control</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Sample</td>
<td>OD ≥ 0.500</td>
</tr>
<tr>
<td>Negative Sample</td>
<td>OD &lt; 0.200</td>
</tr>
<tr>
<td>Cut-Off Sample</td>
<td>OD &gt; Negative Sample</td>
</tr>
</tbody>
</table>

R_{PC/NC} ≥ 8.00

Summary:
The results on the table above must be obtained for the assay to be considered valid. 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.
ASSAY CALCULATIONS
The status of the unknown sample is determined by first calculating the cut-off of the assay, followed by calculating the ratio of the optical density (OD₄₅₀) divided by the cut-off.

Calculation of Cut-off: The cut-off is calculated based on the average OD values obtained with the cut-off control sample.

Example

Calculate Mean Cut-off Control:
Example: Cut-off Control OD
| No 1 | 0.152 |
| No 2 | 0.189 |
| Total | 0.341 |

Mean of Cut-off Control = 0.341 / 2 = 0.171

Example Cut-off Value: 0.171

Note: It is recommended to verify cut-off using sera from geographically relevant population.

Calculate Index Value: The value is calculated from the ratio of the optical density (OD) obtained with the test sample divided by the calculated Cut-Off Value.

Calculate Index Value for each sample:
Example: Test Sample OD

Test Sample OD 0.431

Test Sample Index Value = OD of Test Sample / Cut-off Value

Test Sample Index Value = 0.431 / 0.171 = 2.52

Calculation of Cut-off: Endemic control sera were not used for the cut-off calculation. It is recommended to verify cut-off using sera from geographically relevant population.

Interpretation of Test Results: OD values ≥ cut-off (Index values ≥ 1.00) will be considered positive for the presence of circulating NS1 antigen. Those sera with OD values close to cut-off (1.10 > Index values > 0.90) should be repeated in duplicate to verify sample status.

LIMITATIONS
1. For in vitro diagnostic use.
2. For Export Use Only.
3. Since this is an indirect screening method, the presence of false positive and negative results must be considered.
4. All reactive samples must be evaluated by a confirmatory test.
5. The reagents supplied in this kit are optimized to measure Dengue NS1 levels in serum specimens.
6. Serological cross-reactivity across the flavivirus group is common. Certain sera from patients infected with Japanese Encephalitis, West Nile, and/or Saint Louis viruses may give false positive results. Therefore any Dengue positive sera must be confirmed with other tests.
7. The assay performance characteristics have not been established for visual result determination.
8. Results from immunosuppressed patients must be interpreted with caution.
9. Assay results should be interpreted only in the context of other laboratory findings and the total clinical status of the patient.
PERFORMANCE CHARACTERISTICS

Positive Percent Agreement (PPA)
An external site assessed positive percent agreement by assaying 34 well characterized serum samples. Each of 34 serum samples was RT-PCR positive. The positive percent agreement (PPA) of Dengue NS1 Antigen DxSelect™ assay compared to RT-PCR is 88.2% (30/34) with 95% CI: 73.4 - 95.3%.

Negative Percent Agreement (NPA)
An internal site assessed negative percent agreement by assaying 53 well characterized serum samples during pre-clinical phase. Each of 53 serum samples was RT-PCR negative. The negative percent agreement (NPA) of Dengue NS1 Antigen DxSelect™ assay compared to RT-PCR is 100.0% (53/53) with 95% CI: 93.2 -100.0%.

Reproducibility
The reproducibility study was performed by 2 different individuals at 3 different sites (total 6 operators) on 5 consecutive days. Operators tested the same panel of samples in triplicate using the same lot of Dengue NS1 Antigen DxSelect™ ELISA kit. The reproducibility of the assay was assessed using a sample panel consisting of four levels of serum samples and the controls.

The data is also presented for both the raw OD values obtained, as well as the Index values (Index Values – ratio of sample OD to the cut-off control OD value).

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>N</th>
<th>Mean</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Between Sites</td>
<td>Between Days</td>
<td>Between Operator/Run</td>
<td>Within Assay</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>%CV</td>
<td>SD</td>
<td>%CV</td>
<td>SD</td>
<td>%CV</td>
<td>SD</td>
</tr>
<tr>
<td>Sample #1</td>
<td>0.00</td>
<td>0.00</td>
<td>0.02</td>
<td>3.3</td>
<td>0.05</td>
<td>8.0</td>
<td>0.03</td>
</tr>
<tr>
<td>Sample #2</td>
<td>0.01</td>
<td>3.80</td>
<td>0.01</td>
<td>3.00</td>
<td>0.02</td>
<td>7.10</td>
<td>0.02</td>
</tr>
<tr>
<td>Sample #3</td>
<td>0.02</td>
<td>7.80</td>
<td>0.00</td>
<td>0.00</td>
<td>0.02</td>
<td>9.00</td>
<td>0.01</td>
</tr>
<tr>
<td>Sample #4</td>
<td>0.01</td>
<td>9.40</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>12.20</td>
<td>0.00</td>
</tr>
<tr>
<td>(+) Control</td>
<td>0.15</td>
<td>8.90</td>
<td>0.06</td>
<td>3.70</td>
<td>0.14</td>
<td>8.30</td>
<td>0.09</td>
</tr>
<tr>
<td>(-) Control</td>
<td>0.01</td>
<td>11.10</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>10.10</td>
<td>0.01</td>
</tr>
<tr>
<td>Cut Off Control</td>
<td>0.01</td>
<td>10.30</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>9.40</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Limit of Detection
The Limit of Blank and Limit of Detection were effectively measured using recombinant NS1 spiked into normal human serum (NHS). The limit of detection was determined to be 5.2pg/ml of recombinant NS1 diluted into NHS. However, it should be noted that the Cut-Off sample is above this limit of detection so it should not be expected that end-users would be able to detect 5.2pg/ml of NS1 in human samples and obtain OD values above the cut-off sample.
REFERENCES