Instructions for Use

Dengue Virus NS1 Ag ELISA

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REF **EIA-6090**

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(01/18/2019)

Please use only the valid version of the Instructions for Use provided with the kit.
Verwenden Sie nur die jeweils gültige, im Testkit enthaltene, Gebrauchsanweisung.
Si prega di usare la versione valida delle istruzioni per l'uso a disposizione con il kit.
Por favor, se usa solo la version valida de la metodico técnico incluido aqui en el kit.
Utilisez seulement la version valide des Instructions d'utilisation fournies avec le kit.

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For In Vitro Diagnostic Use

1 INTENDED USE

The Dengue Virus NS1 Ag ELISA is for the early detection of Dengue virus (DENV) NS1 antigen in human serum. This test is for the presumptive clinical laboratory diagnosis of Dengue virus infection. This assay is intended for use in patients with clinical symptoms consistent with either dengue fever or dengue hemorrhagic fever. Samples collected from patients within seven (7) days after the onset of clinical symptoms should be evaluated with this assay (day 0 – day 7). Negative results obtained with this test do not preclude the diagnosis of dengue and should not be used as the sole basis for treatment or other patient management decision.

This assay is not FDA cleared or approved for testing blood or plasma donors.

2 SUMMARY AND EXPLANATION OF THE TEST

Dengue Fever (DF) is an acute viral disease of man, which is transmitted by *Aedes aegypti* mosquitoes. DF is characterized clinically by biphasic fever, rash and hematopoietic depression, and by constitutional symptoms such as malaise, arthralgia, myalgia and headache (1). Infrequently, more severe disease is seen, manifested by hemorrhagic fever which may progress to lethal shock (2, 3). It is endemic in the tropics and subtropics, worldwide, where an estimated 100,000,000 cases occur annually (4). It has been estimated that about 50 to 100 million cases of DF occur every year with about 250,000 to 500,000 cases of Dengue Hemorrhagic Fever (DHF). During 2002, more than 30 Latin American countries reported over 10,000,000 DF cases with large number of DHF cases. This has been followed by extensive epidemics of DHF in several parts of India during 2003 through 2005. In the Americas, the reported incidence has more than tripled from 1996 to 2002. The incidence of Dengue outbreak has been reported in Hawaii (5), and in Laredo, Texas. Dengue NS1 (non-structural) protein is a secreted protein and is believed to play a role in viral RNA replication. NS1 is strongly immunogenic, eliciting antibodies with complement fixing activity. NS1 antigen can be detected in circulating blood during acute Dengue infection (6) (7). The Dengue Virus NS1 Ag ELISA can detect NS1 antigen in serum samples within 1 to 2 days following infection.

3 PRINCIPLE OF THE TEST

The Dengue Virus NS1 Ag ELISA is an enzymatically amplified "two- step" sandwich-type immunoassay to detect low levels of NS1 in serum. In this assay, controls and unknown serum samples are diluted in sample dilution buffer, containing secondary antibody, and incubated in microtitration wells. These wells have been coated with a highly effective NS1 antibody and then blocked. 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 antibody-HRP conjugate and liquid 3, 3’, 5, 5’-tetramethylbenzidine (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. The values obtained for the kit controls serve as guidelines as to determining if a sample contains NS1 antigen.

4 MATERIALS SUPPLIED

The Dengue Virus NS1 Ag 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:

Dengue Virus NS1 Ag ELISA supplied materials:

1. **Coated Microtiter Strips for Dengue Virus NS1 Ag ELISA:**
   ELISA strip holder in a ziplock foil pouch with desiccant, containing 96 polystyrene microtiter wells coated with anti-NS1 antibody in each well.
   Stable at 2 °C - 8 °C until the expiration date.
2. **Negative Control for Dengue Virus NS1 Ag ELISA:**
   One vial, 300 µL containing heat-inactivated negative control serum.
   The negative control will aid in verifying the validity of the kit.
   Stable at 2 °C - 8 °C until the expiration date of the kit.
3. **Positive Control for Dengue Virus NS1 Ag ELISA:**
   One vial, 300 µL containing recombinant NS1 in a phosphate-based buffer with 0.05% Proclin-300.
   The positive control will aid in verifying the validity of the kit.
   Stable at 2 °C - 8 °C until the expiration date.
4. **Cut-off Control for Dengue Virus NS1 Ag ELISA:**
   One vial, 300 µL containing recombinant NS1 in a phosphate-based buffer with 0.05% Proclin-300.
   The Cut-off Control will aid in determining the cut-off value for the ELISA.
   Stable at 2 °C - 8 °C until the expiration date.
5. **Sample Dilution Buffer for Dengue Virus NS1 Ag ELISA:**
   One bottle, 15mL, containing the secondary antibody in a Tris-based buffer with 0.02%-0.05% Proclin-300.
   Stable at 2 °C - 8 °C until the expiration date.
6. **100X Conjugate for Dengue Virus NS1 Ag ELISA:**
   One vial, 150µL, containing horseradish peroxidase-labeled polyclonal antibody in a Tris-based buffer with 0.03% - 0.05% Proclin-300.
   Stable at 2 °C - 8 °C until the expiration date.
7. **Conjugate Diluent for Dengue Virus NS1 Ag ELISA:**
   One bottle, 12mL. This contains the diluent solution for the 100X Conjugate in a Tris-based buffer with 0.01% Thimerosal 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 up to 2 weeks at 2 °C - 8 °C before it should be discarded.
   Stable at 2 °C - 8 °C until the expiration date.
8. **10X Wash Buffer:**
   One bottle, 120 mL. 10X concentrated phosphate buffered saline with Tween 20 (pH 6.8 – pH 7.0).
   Stable at 2 °C - 8 °C until the expiration date.
9. **Liquid TMB Substrate:**
   One bottle, 12 mL, ready to use. Contains 3, 3', 5, 5'-tetramethylbenzidine (TMB) and hydrogen peroxide in a citric acid-citrate buffer (pH 3.3 - pH 3.8).
   Stable at 2 °C - 8 °C until the expiration date.
   Note: The substrate should always be stored in the light- protected bottle provided.
10. **Stop Solution:**
    One bottle, 6 mL, read to use 1 N Sulfuric Acid. Used to stop the reaction.
    Stable at 2 °C - 8 °C until the expiration date.
    Warning: Strong acid. Wear protective gloves, mask and safety glasses. Dispose all materials according to all applicable safety rules and regulations.

5 MATERIALS AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

- ELISA spectrophotometer capable of absorbance measurement at 450 nm
- Biological or high-grade water
- Appropriately sized beakers and stir bars
- Vacuum pump
- Automatic plate washer
- 37 °C incubator *without* CO_2 supply
- 1 µL - 10 µL single-channel pipettors, 50 µL - 200 µL single- and multi- channel pipettors
- Polypropylene tubes or 96 well dilution plates
- Parafilm or plastic plate cover
- Timer
- Vortex

6 WARNINGS AND PRECAUTIONS

FOR *IN VITRO* DIAGNOSTIC USE.

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.

6.1 SAFETY PRECAUTIONS

- All human source materials used in the preparation of the negative control have tested negative for antibodies to HIV 1&2, Hepatitis C and Hepatitis B surface antigen. However, no test method can ensure 100% efficiency. Therefore, all human controls and antigen should be handled as potentially infectious material. 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.
- Dispose of hazardous or biologically contaminated materials according to the practices of your institution. Discard all materials in a safe and acceptable manner and in compliance with prevailing regulatory requirements.
- Wear protective clothing, eye protection and disposable gloves while performing the assay. Wash hands thoroughly afterwards.
- Do not eat, drink, smoke, or apply cosmetics in the laboratory where immunodiagnostic materials are being handled.
- Do not pipette by mouth.

6.2 TECHNICAL PRECAUTIONS

- This test must be performed on **human serum only**.
  The use of whole blood, plasma or other specimen matrices has not been validated.
- Do not mix various lots of any kit component within an individual assay.
- **Do not heat inactivate test sera.**
- All reagents must be equilibrated to room temperature (20 °C to 25 °C) before commencing the assay. The assay will be affected by temperature changes.
- Avoid repeated freezing and thawing of the serum specimens to be evaluated.
- Dispense reagents directly from bottles using clean pipette tips. Transferring reagents may result in contamination.
- Unused microtiter wells must be resealed immediately in the ziplock foil pouch with the desiccant provided. Failure to do so may cause erroneous results with those unused microwells.
- 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 performance.
- To minimize potential assay drift due to variation in the substrate incubation time, care should be taken to add the stop solution into the wells in the same order and speed used to add the TMB solution.
- Avoid microbial contamination of reagents.
- Avoid contamination of the TMB Substrate Solution with the Conjugate Solution. The TMB Substrate Solution should be clear in color; a blue color change prior to use may indicate contamination has occurred.
- Use a clean disposable pipette tip for each reagent, standard, control or specimen.
- Cover working area with disposable absorbent paper.

7 WARNING

7.1 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.

7.2 CHEMICAL HAZARD

Safety Data Sheets (SDSs) are available for all components of this kit. Review all appropriate SDSs 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.

8 SPECIMEN COLLECTION AND PREPARATION

- **Only human serum should be used for this assay**, and the usual precautions for venipuncture should be observed.

  Blood obtained by venipuncture should be allowed to clot at room temperature (20 °C - 25 °C) for 30 to 60 minutes and then centrifuged according to the Clinical and Laboratory Standards Institute (CLSI Approved Guideline – Procedures for the Handling and Processing of Blood Specimens for Common Laboratory Tests; GP44).
- Testing should be performed as soon as possible after collection.
  Do not leave sera at room temperature for prolonged periods. Separated serum should remain at 20 °C - 25 °C for no longer than 8 hours.
  If assays are not completed within 8 hours, serum should be refrigerated at 2 °C - 8 °C.
  If assays are not completed within 48 hours, or the separated serum is to be stored beyond 48 hours, serum should be frozen at or below -20°C.
- Avoid repeated freezing and thawing of samples more than four times as this can cause analyte deterioration.
  Frost-free freezers are not suitable for sample storage.
- Frozen samples should be thawed to room temperature and mixed thoroughly by gentle swirling or inversion prior to use. Always quick spin before use.
- If sera are to be shipped, they should be packed in compliance with Federal Regulations covering transportation of infectious agents.
- Do not use sera if any indication of microbial growth is observed.

9 TEST PROCEDURE

CAUTION: *The test procedure must be strictly followed. Any deviations from the procedure may produce erroneous results. Bring all reagents and specimens to room temperature (~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 four times. Sera should be further divided into small aliquots and stored at -20 °C or below.*

This kit has not been optimized by DRG for use with a specific 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.

9.1 Preparation of Reagents

9.1.1 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.

Properly label the 1X wash buffer solution and carefully note the expiration date on the label. Check for contamination prior to use. Discard if contamination is suspected.

9.1.2 Microtiter Strip 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 °C - 8 °C until ready to use or expiration.

9.1.3 Preparation of Conjugate Solution

Add 120 µL of 100x Conjugate for Dengue Virus NS1 Ag ELISA directly to the 12 mL bottle of Conjugate Diluent for Dengue NS1 (1 part : 100 parts).

Alternatively, use a clean pipette to remove the required volume of Conjugate Diluent and add the necessary volume of 100x Conjugate for Dengue Virus NS1 Ag ELISA into a clean polypropylene test tube in order to maintain the 1:100 ratio.

Mix by inverting the solution several times.

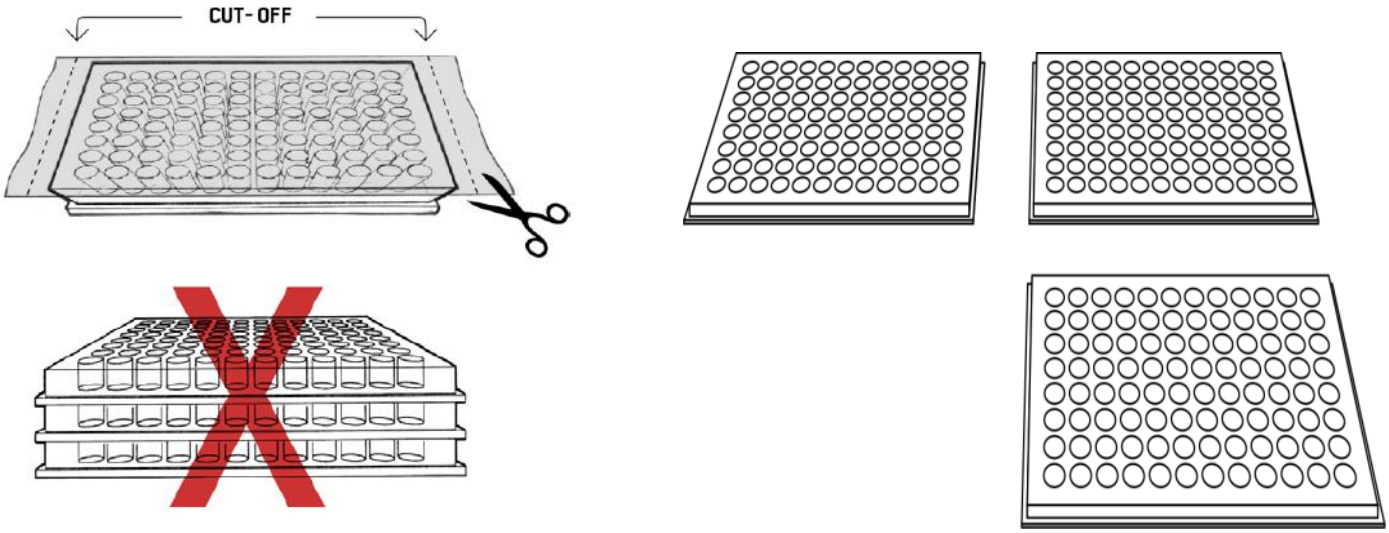
This solution may be stored for up to 2 weeks if stored at 2 °C - 8 °C.

After 2 weeks, this conjugate solution should be discarded and no longer used in this assay.

9.2 Assay Procedure

1. Positive, negative, and cut-off controls should be assayed in duplicate (and run on every plate, each time an assay is performed). 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.)
   Up to ninety test specimens can be tested in singlet with an entire plate.
   Immediately place any unused ELISA plate wells back into the original foil packaging with the provided desiccant, properly seal, and store at 2 °C - 8 °C.
2. Using a single channel or multichannel pipettor, aliquot **50 µL of Sample Dilution Buffer** for the Dengue Virus NS1 Ag 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 Dilution Buffer. Use a clean disposable pipette tip for each control or test sample. Gently rock the plate by hand from side to side 5 times to ensure the samples are well mixed.
4. Cover the top of the plate with parafilm (or plastic plate cover) and remove any excess parafilm from the edges of the plate.

***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.*



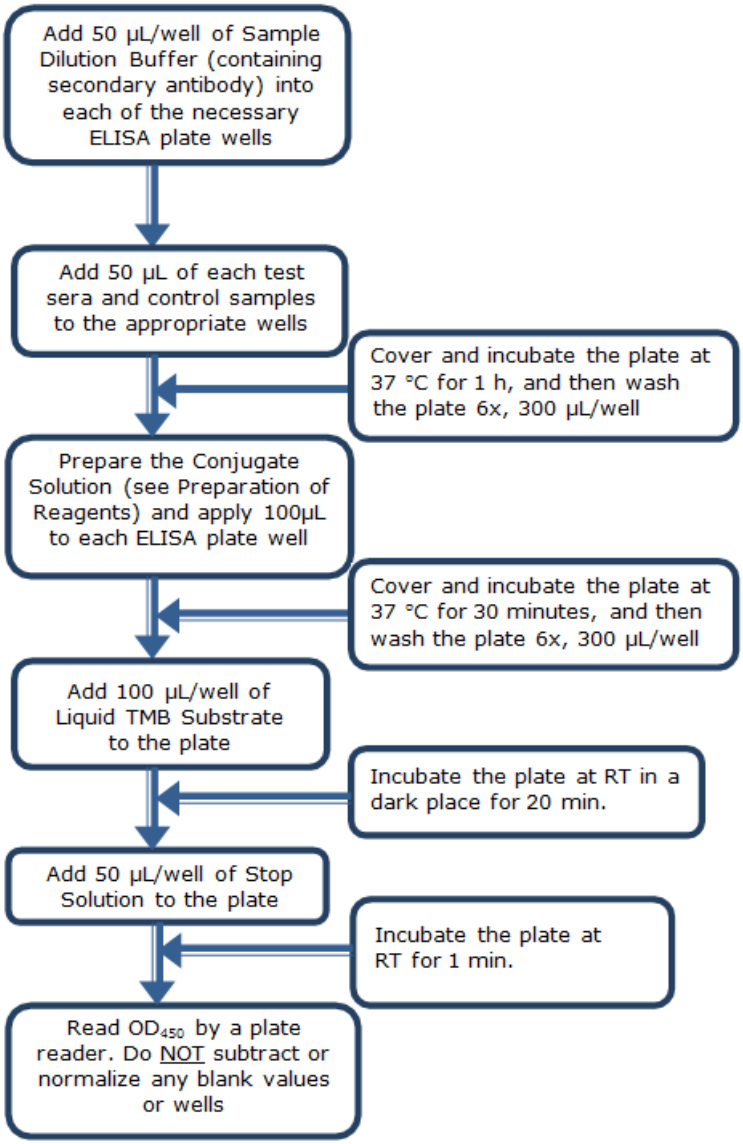
CORRECT METHOD

***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 CO_2 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 <u>1 hour</u>** 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. *<u>Prepare the Conjugate Solution (120 µL of 100X Conjugate: 12 mL of Conjugate Diluent)</u>* and add **100 µL** per 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 °C - 8 °C.
8. Cover the plate with parafilm or plastic plate cover, as shown above, and incubate **at 37 °C for <u>30 minutes</u>** in an incubator.
9. After the incubation, **wash** the plate **6 times** with the automatic plate washer using 1X Wash Buffer.

10. Add **100 µL** per well of **Liquid TMB substrate** into all wells using a multi-channel pipettor.
11. Incubate the plate, uncovered at **room temperature <u>in the dark</u>, for 20 minutes**.
12. Add **50 µL** per well of **Stop Solution** into all appropriate wells using a multi-channel pipettor.
    Make sure to add the Stop Solution in the same order and at approximately the same speed at which the TMB was applied. (Note: As the TMB substrate produces an enzymatic reaction with the HRP-conjugate, it is critical this incubation time point is followed as closely as possible).
    Let the plate stand, uncovered at room temperature, for 1 minute.
13. **Read** the optical density at 450 nm (OD450) 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 and Interpretations of Results sections.

Flow Chart



10 QUALITY CONTROL

Each kit contains positive, negative and cut-off controls. 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 (QC) 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 applicable for spectrophotometric readings only.

First, calculate the mean (average) negative, positive and cut-off control raw OD450 values as shown in the following examples.

Example 1: Dengue NS1 Negative Control

	OD_{450}
Replicate 1	0.108
Replicate 2	0.084
Sum	0.192

Average Negative Control = 0.192 ÷ 2 = 0.096

Example 2: Dengue NS1 Positive Control

	OD_{450}
Replicate 1	1.205
Replicate 2	1.311
Sum	2.516

Average Positive Control = 2.516 ÷ 2 = 1.258

Example 3: Dengue NS1 Cut-off Control

	OD_{450}
Replicate 1	0.146
Replicate 2	0.128
Sum	0.274

Average Cut-off Control = 0.274 ÷ 2 = 0.137

Next, calculate the **ratio** between the positive and negative controls as shown in the following example.

Example 4: Calculate the Ratio of Positive to Negative Control (RPC/NC)

$\mathbf{R_{PC/NC}}$ = Avg. Positive Control ÷ Avg. Negative Control

$\mathbf{R_{PC/NC}}$ = 1.258 ÷ 0.096 = 13.10

Finally, verify that the quality control requirements, listed in the table below, are fulfilled.

Quality Control Requirements

Control	***Requirement***
Positive Control	OD ≥ 0.5
Negative Control	OD < 0.2
Cut-Off Control	OD > Negative Control
$R_{PC/NC}$	≥ 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.

11 INTERPRETATION OF RESULTS

The assay cut-off value was determined by testing one hundred forty-three (143) dengue-positive and thirty-seven (37) dengue-negative by RT-PCR with the Dengue Virus NS1 Ag ELISA. The raw OD values and sample status were used for generating the ROC curves. The optimal ISR cut-off was defined so that equal weight was given to both specificity and sensitivity. A rudimentary bootstrap method was applied to minimize bias by any potential outliers in the sample set.

The status of the unknown sample is determined by first calculating the cut-off of the assay (shown above in Example 3), followed by calculating the ratio of the optical density (OD_{450}) divided by the cut-off.

Calculate Immune Status Ratio (ISR):

The immune status ratio (ISR) is calculated from the ratio of the optical density (OD) obtained with the test sample divided by the calculated Cut-Off Value. Calculate the ISR for each test sample. If unknown samples were tested in duplicate, then calculate the average optical density (OD_{450}) before dividing by cut-off to determine ISR.

Example 5: Calculate the ISR for a Sample

	OD $_{450}$
Unknown Sample #1	0.431

ISR Value = Raw OD_{450} ÷ Cut-Off Value

ISR Value = 0.431 ÷ 0.137 = 3.15

SAMPLE INTERPRETATION CHART

ISR Value	Results	Interpretation
≥ 1	Positive	Presence of detectable Dengue NS1 antigen. The results should be confirmed by RT-PCR or a serological assay. Refer to the latest CDC guidelines for diagnosis of this disease.
0.9 - 1.1	Retest	If tested in singlet, those sera with OD values close to the cut-off (0.9 < ISR < 1.1) should be repeated in duplicate along with controls to verify the sample status. If the average ISR value from the repeat duplicate testing is ≥ *1*, the sample should be considered positive for Dengue NS1 antigen. If the average ISR value from the duplicate testing is < *1*, the sample should be considered negative for Dengue NS1 antigen.
< 1	Negative	No detectable Dengue NS1 antigen and the individual does not appear to be recently infected with Dengue virus. The result does not rule out the possibility of Dengue virus infection. The sample should be tested with RT-PCR or other serological assays if paired (acute / convalescent) samples are available.

12 LIMITATIONS

- All reactive samples must be confirmed by PCR or a serological assay. Review the latest information on diagnosis at the CDC website: http:www.cdc.gov/dengue/clinicalLab/laboratory.html.
- Cross-reactivity with malaria and syphilis has not been evaluated with this assay.
- The assay performance characteristics have not been established for visual result determination.
- The assay performance characteristics have not been established for matrices other than serum.
- Assay performance characteristics have not been established for testing cord blood, for testing neonates, for prenatal screening, or for general population screening.
- False negatives may arise from patients co-infected with hepatitis B or with HIV.
- Samples containing high levels of triglycerides or samples that are hemolyzed should be avoided for analysis with this assay.
- HAMA does not show the false positive results, but may reduce ISR values for positive samples.
- Results from immunosuppressed patients must be interpreted with caution.
- Assay results should be interpreted only in the context of other laboratory findings and the total clinical status of the patient.

13 EXPECTED VALUES

Endemic Population

Serum samples from 505 patients displaying signs and symptoms characteristic of dengue infections were prospectively collected in Colombia, Argentina, and Sri Lanka. These samples were collected within 7 days (inclusive) after the onset of clinical symptoms. The reactivities of the Dengue Virus NS1 Ag ELISA with this endemic population are shown in Table 1, below.

Table 1: Expected results from an endemic region with individuals displaying symptoms for Dengue

			Dengue Virus NS1 Ag ELISA Results		
Age (years)	**# Male**	**# Female**	**Non-reactive**	**Reactive**	**% Reactive**
0 - 10	68	60	109	19	14.8%
11 - 20	45	37	50	32	39.0%
21 - 30	36	38	48	26	35.1%
31 - 40	47	42	51	38	42.7%
41 - 50	22	28	26	24	48.0%
51 - 60	14	31	26	19	42.2%
61 - 70	9	16	15	10	40.0%
71 - 80	4	5	9	0	0.0%
81 - 90	1	1	1	1	50.0%
91 - 100	0	1	1	0	0.0%
Total	246	259	336	169	33.5%

14 PERFORMANCE CHARACTERISTICS

14.1 Clinical Studies

1. Prospective Study in the Endemic Area

Prospectively collected archived serum samples from 505 patients from Argentina (65), Colombia (229), and Sri Lanka (211 subjects) were tested. Samples were collected from individuals within 7 days of onset of signs and symptoms of Dengue infection. A repeat sample was collected after 7- 20 days from each patient for a possible Dengue Virus IgG testing. Samples were evaluated with the Dengue Virus NS1 Ag ELISA and compared to results by a composite reference method. The samples were tested by a validated RT-PCR followed by sequencing, or an FDA-cleared Dengue IgM ELISA or a validated Dengue IgG ELISA. The IgG was considered positive for dengue fever if a there was a 16-fold increase in IgG titer between the first and the second blood draw. A sample was considered positive by the reference assay if any one of the three tests were positive. The results are presented below.

Table 2: Prospective Patient Study in Endemic Area

		DENV Reference Method Result (RT-PCR or IgM or IgG ELISAs)		
		Positive	**Negative**	**Total**
Dengue Virus NS1 Ag ELISA	**Positive**	162	7	169
	Negative	25[(1)]	311	336
	Total	187	318	505
PPA; 95% CI		86.6% (162/187); (95% CI: 80.9% - 91.2%)		
NPA; 95% CI		97.8% (311/318); (95% CI: 95.5% - 99.1%)		

[(1)] Of the 25 false negatives by the Dengue Virus NS1 Ag ELISA, 13 samples were found to be positive by the RT-PCR, 4 samples were found to be positive by DENV IgM ELISA, and 8 samples were found to be positive by DENV IgG ELISA.

2. Prospective Study in U.S.

Archived serum samples from 193 patients, who presented with clinical symptoms associated with similar dengue virus, were evaluated with the Dengue Virus NS1 Ag ELISA at a reference laboratory in Florida, United States. The samples were tested with a RT-PCR as a comparator. The results are presented below.

Table 3: Prospective Patient Study in U.S.

		RT-PCR		
		Positive	**Negative**	**Total**
Dengue Virus NS1 Ag ELISA	**Positive**	0	1	1
	Negative	1	191	192
	Total	1	192	193
NPA; 95% CI		99.5% (191/192); (95% C.I.: 97.1% - 100%)		

14.2 Analytical Reactivity Study

The analytical reactivity was performed to determine the limits of detection (LODs) of Dengue Virus NS1 Ag ELISA when tested with native NS1 from viral culture supernatants representing all four serotypes of dengue virus. Dilutions were tested in replicates of 20, and the lowest concentrations at which ≥95% of replicates tested positive were considered the LOD for each serotype.

The LOD of Dengue Virus NS1 Ag ELISA ranges from 82 - 611 pfu/mL, depending upon DENV serotype, as shown in Table 4 below.

Table 4: Limits of Detection for all dengue serotypes

DENV serotype	Average ISR	Native NS1 LOD (pfu/mL)
1	1.32	109.8
2	1.25	271.5
3	1.20	81.6
4	1.27	610.8

14.3 Reproducibility Study

The reproducibility study of the Dengue Virus NS1 Ag ELISA kit was performed at 3 different sites by 2 different individuals (6 operators total) on 5 separate days. All samples (including controls) were run in triplicate. Four serum specimens (Panels 1-4) using clinical specimens diluted in an analyte-negative matrix, plus a positive control, a negative control and cut-off control, were used. The four serum specimens included a positive specimen, two weakly positive specimens and a negative specimen.

The ISR total precision %CV varied from 3.7 - 11.9%, depending on the sample. The results are shown in the following table.

Table 5: Reproducibility of the Dengue Virus NS1 Ag ELISA

			Intra-Assay (within- run)		Day-to-Day		Operator-to-Operator		Site-to-Site		Total	
Sample ID	**n**	**Mean ISR**	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**
Panel #1	90	5.01	0.24	4.7	0.48	9.64	0.44	8.71	0.37	7.47	0.54	10.73
Panel #2	90	2.93	0.15	5.08	0.2	6.71	0.18	6.2	0.16	5.62	0.25	8.42
Panel #3	90	1.91	0.1	5.15	0.11	5.61	0.08	4.1	0.05	2.58	0.15	7.61
Panel #4	90	0.88	0.04	4.58	0.06	7.09	0.04	4.83	0.04	4.06	0.07	8.44
(+) Control	90	14.33	0.79	5.54	0.88	6.12	0.63	4.37	0.27	1.87	1.18	8.25
(-) Control	90	0.54	0.04	7.66	0.05	9.04	0.04	6.71	0.02	4.54	0.06	11.85
Cut-off control	90	1	0.05	4.54	0	0	0	0.07	0	0.04	0.04	3.71

Note: SD = Standard Deviation. CV = Coefficient of Variation.

14.4 Cross-Reactivity Study

Fifty-eight (58) antigen or viral load positive samples that tested positive for other potentially cross-reactive pathogens were evaluated with the Dengue Virus NS1 Ag ELISA in order to determine potential cross-reactivity.

West Nile virus samples ranged 100 - 43,000 copies/mL by nucleic acid testing; hepatitis B virus (HBV) levels ranged 500 - 5,000,000,000 by chemiluminescent immunoassay; hepatitis C virus (HCV) viral loads ranged 3.9×10^6 – 3.8×10^8 by nucleic acid testing; human immunodeficiency virus (HIV) viral loads ranged 3.8×10^3 – 4.4×10^6 by nucleic acid testing and signal/cutoff ratios ranged 8.55 - 16.87 in enzyme immunoassay (EIA); SLE samples were all >1:100 by IFA.

Table 6: Dengue Virus NS1 Ag ELISA cross-reactivity with positive clinical specimens

Disease	# Samples	# Positive	# Negative
West Nile Virus	18	0	18
HBV	10	0	10
HCV	10	0	10
HIV (Viral Load +)	10	0	10
HIV EIA Positive	5	0	5
Systemic Lupus Erythematosus (SLE)	5	0	5
Total	58	0	58

In addition, forty-two (42) contrived samples that consisted of cultured micro-organisms spiked into normal human sera were also evaluated with the Dengue Virus NS1 Ag ELISA.
Clinically relevant levels of viruses at 105 pfu/mL and bacteria at 106 cfu/mL were tested.
VZV was tested at only $5\times10^{3.43}$ U/mL, due to low concentration of stock obtained.
For EBV, both 107 and 108 copies/mL (as determined by quantitative RT-PCR) of EBV were tested in this study.

Table 7: Dengue Virus NS1 Ag ELISA cross-reactivity with spiked samples

Disease	# Samples	# Positive	# Negative
West Nile Virus	3	0	3
Japanese Encephalitis Virus	3	0	3
Zika Virus	3	0	3
Yellow Fever Virus	3	0	3
Chikungunya Virus	3	0	3
HSV-1 and -2	6	0	6
Rubella	3	0	3
EBV	6	0	6
CMV	3	0	3
VZV	3	0	3
Leptospira	3	0	3
Borrelia burgdorferi (Lyme)	3	0	3
Total	42	0	42

14.5 Microbial Interference Study

Samples were evaluated to assess microbial interference in the presence of dengue virus. Fifty-eight viral load positive samples that tested positive for other potentially cross-reactive pathogens were evaluated, as well as forty-two contrived samples that consisted of cultured micro-organisms diluted into normal human sera. Samples were then spiked with cultured dengue virus (ISR 2.0-3.2) just prior to testing with Dengue Virus NS1 Ag ELISA. The tables below summarize the results of this study.

Table 8: Dengue Virus NS1 Ag ELISA microbial interference with positive clinical specimens

Disease	# Samples	# Positive	# Negative
West Nile Virus	18	18	0
HBV	10	7	3
HCV	10	10	0
HIV (Viral Load +)	10	8	2
HIV EIA Positive	5	5	0
Systemic Lupus Erythematosus (SLE)	5	5	0
Total	58	53	5

Table 9: Dengue Virus NS1 Ag ELISA microbial interference with spiked samples

Disease	# Samples	# Positive	# Negative
West Nile Virus	3	3	0
Japanese Encephalitis Virus	3	3	0
Zika Virus	3	3	0
Yellow Fever Virus	3	3	0
Chikungunya Virus	3	3	0
HSV-1 and -2	6	6	0
Rubella	3	3	0
EBV	6	6	0
CMV	3	3	0
VZV	3	3	0
Leptospira	3	3	0
Borrelia burgdorferi (Lyme)	3	3	0
Total	42	42	0

14.6 Interfering Substances Study

Interference by endogenous substances in the Dengue Virus NS1 Ag ELISA test was evaluated using a panel of four simulated clinical specimens (a strongly positive specimen, two weakly positive specimens and a negative specimen). Interfering substances at the levels indicated were tested as described in CLSI EP07-A2. There was no inhibition at the following concentrations of interferents that were tested.

Table 10: Endogenous Interfering Substances Study

Interferent	Concentrations Tested
Bilirubin	0.02 mg/mL
Triglycerides	30 mg/mL
Hemoglobin	0.16 mg/mL
Cholesterol	5 mg/mL
HAMA	46 ng/mL

15 REFERENCES / LITERATURE

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16 TROUBLESHOOTING

Problem	Possible Cause	Possible Resolution
High Absorbances	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 1:2 in kit's sample dilution buffer (i.e. 50 µL sample plus 50 µL 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.
Low Absorbances	Samples incorrectly diluted	Sera should be diluted 1:2 in kit's sample dilution buffer (i.e. 50 µL sample plus 50 µL 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.
	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.
	Diluted conjugate stored too long	Prepare only the volume of diluted conjugate necessary. Diluted conjugate should not be stored longer than 2 weeks at 2 °C – 8 °C. Undiluted conjugate can be stored at 2 °C – 8 °C until expiration listed on vial.
	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.
Common Equipment Issues	Plate washing too vigorous	Check and ensure correct pressure in automatic wash system. Pipette wash buffer gently if washes are done manually.
	Wells not washed equally/thoroughly	Check that all parts of the automatic wash system are unobstructed. Wash wells as recommended.
	Automatic wash system errors	Use only calibrated equipment. Operate automatic wash system per manufacturer's recommendations.
	Pipetting errors	Use calibrated pipettes and proper pipetting technique.
	Incorrect wavelength filter	The optical density readings must be read with only a 450 nm filter. There must not be any background subtraction.

SYMBOLS USED

Symbol	English	Deutsch	Italiano	Español	Français
CE	European Conformity	CE-Konformitäts-kennzeichnung	Conformità europea	Conformidad europea	Conformité normes européennes
	Consult instructions for use	Gebrauchsanweisung beachten	Consultare le istruzioni per l'uso	Consulte las instrucciones de uso	Consulter les instructions d'utilisation
IVD	*In vitro* diagnostic medical device	*In-vitro*-Diagnostikum	Dispositivo medico-diagnostico in vitro	Producto sanitario para diagnóstico In vitro	Dispositif médical de diagnostic in vitro
REF	Catalogue number	Katalognummer	Numero di Catalogo	Nûmero de catálogo	Référence de catalogue
LOT	Batch code	Chargenbezeichnung	Codice del lotto	Codigo de lote	Numéro de lot
	Contains sufficient for <n> tests	Ausreichend für <n> Prüfungen	Contenuto sufficiente per "n" saggi	Contenido suficiente para <n> ensayos	Contenu suffisant pour "n" tests
	Temperature limit	Temperaturgrenzwerte	Temperatura di conservazione	Temperatura de conservacion	Température de conservation
	Use-by date	Verwendbar bis	Utilizzare prima del	Establa hasta	Utiliser jusque
	Manufacturer	Hersteller	Fabbricante	Fabricante	Fabricant
	Distributor *	Vertriebspartner *	Distributore	Distribuidor	Distributeur
	Date of manufacture	Herstellungsdatum	Data di produzione	Fecha de fabricación	Date de production
	Biological risks	Biologische Risiken	Rischi biologici	Riesgos biológicos	Risques biologiques
	Caution	Achtung	Attenzione	Precaución	Attention
UDI	Unique device Identifier	eindeutige Produktidentifizierung			
RUO	For research use only	Nur für Forschungszwecke	Solo a scopo di ricerca	Sólo para uso en investigación	Seulement dans le cadre de recherches
Content	Content	Inhalt	Contenuto	Contenido	Contenu
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantità	Volumen/Número	Volume/Quantité