DENGUE NS1 Ag MICROLISA

Microwell ELISA Test for the Detection of Dengue NS1 Antigen in Human Serum/Plasma

CE

1. INTRODUCTION

Dengue virus is a flavivirus found largely in areas of the tropic and sub-tropics. There are four distinct but antigenically related serotypes of dengue viruses, and transmission is by mosquito, prinicipally Aedes aegypti and Aedes albopictus.

The mosquito-borne dengue viruses (serotype 1-4) cause dengue fever, a severe flu-like illness. The disease is prevalent in third world tropical regions and spreading to sub-tropical developed countries - including the United States. WHO estimates that 50-80 million cases of dengue fever occur worldwide each year, including a potentially deadly form of the disease called dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). Primary infection with dengue virus results in a self-limiting disease characterized by mild to high fever lasting 3 to 7 days, severe headache with pain behind the eyes, muscle and joint pain, rash and vomiting. Secondry infection is the more common form of the disease in many parts of Southeast Asia and South America. This form of the disease is more serious and can result in DHF and DSS. The major clinical symptoms can include high fever, haemorrhagic evets, and circulatory failure, and the fatality rate can be as high as 40%. Early diagnosis of DSS is particularly important, as patients may die within 12 to 24 hours if appropriate treatment is not administered.

Primary dengue virus infection is characterized by elevations in specific NS1 antigen levels 0 to 9 days after the onset of symptoms; this generally persists upto 15 days. Earlier diagnosis of Dengue reduces risk of complication such as DHF or DSS, especially in countries where dengue is endemic.

2. INTENDED USE

DENGUE NS1 Ag MICROLISA is designed for *in vitro* qualitative detection of Dengue NS1 antigen in human serum or plasma and is used as a screening test for testing of collected blood samples suspected for DENGUE. The kit detects all four subtypes; DEN1, DEN2, DEN3 & DEN4 of Dengue Virus.

3. PRINCIPLE

DENGUE NS1 Ag MICROLISA is a solid phase enzyme linked immunosorbent assay (ELISA) based on the "Direct Sandwich" principle. The microwells are coated with Anti-dengue NS1antibodies with high reactivity for Dengue NS1 Ag. The samples are added in the wells followed by addition of enzyme conjugate (monoclonal anti-dengue NS1 antibodies linked to Horseradish Peroxidase (HRPO)). A sandwich complex is formed in the well wherein dengue NS1 (from serum sample) is "trapped" or "sandwiched" between the antibody and antibody HRPO conjugate. Unbound conjugate is then washed off with wash buffer. The amount of bound peroxidase is proportional to the concentration of dengue NS1 antigen present in the sample. Upon addition of the substrate buffer and chromogen, a blue colour develops. The intensity of developed blue colour is proportional to the concentration of dengue NS1 antigen in sample. To limit the enzyme-substrate reaction, stop solution is added and a yellow colour develops which is finally read at 450nm spectrophotometrically.

4. DESCRIPTION OF SYMBOLS USED

The following are graphical symbols used in or found on J. Mitra diagnostic products and packing. These symbols are the most common ones appearing on medical devices and their packing. They are explained in more detail in the British and European Standard EN ISO 15223-1:2016.

Manufactured By		*In vitro* diagnostic medical device	IVD
No. of tests		See Instruction for use	
Lot Number Batch Number	LOT	Temperature Limitation	
Manufacturing Date		Caution See instruction for use	
Expiry Date		Catalogue Number	REF
Do not use if package is damaged		Authorized Representative in the European Community	EC REP
Keep away from sunlight			

5. KIT PRESENTATION

- 48 Test Pack
- 96 Test Pack

6. KIT & ITS COMPONENTS

COMPONENT	DESCRIPTION	48 Tests	96 Tests
Microwells	**Microwells** coated with anti-Dengue NS1 antibodies packed in a sealed pouch with desiccant.	1 Plate (48 wells)	1 Plate (96 wells)
Diluent	Buffer containing protein stablizers & antimicrobial agents as preservative and to be used for Sample & Conjugate dilution.	(10 ml)	(20 ml)
Enzyme Conjugate Concentrate (50X)	Containing Monoclonal Anti-Dengue NS1 linked to horseradish peroxidase with protein stabilizers.	(0.3 ml)	(0.5 ml)
Wash Buffer Concentrate (25X)	Concentrated Phosphate buffer with surfactant.	(35 ml)	(50 ml)
TMB Substrate	TMB, to be diluted with TMB Diluent before use.	(10 ml)	(15 ml)
TMB Diluent	Buffer solution containing H_2O_2 with preservative.	(10 ml)	(15 ml)
Control - ⚠	Normal human serum negative for Dengue NS1 antigen with preservative.	(0.8 ml)	(1 ml)
Control + ⚠	Recombinant Dengue NS1 antigen, with preservative.	(0.8 ml)	(1 ml)
Calibrator	Recombinant Dengue NS1 antigen, with preservative.	(1 ml)	(2 ml)
Stop Solution	Ready to use, 1N H_2SO_4.	(6 ml)	(15 ml)
Plate Sealers	Adhesive sheets to cover the microwells during incubation.	2 Nos.	4 Nos.

7. STORAGE AND STABILITY

Store the kit & its components at 2-8°C. Expiry date on the kit indicates the date beyond which kit should not be used.

8. ADDITIONAL MATERIAL AND INSTRUMENTS REQUIRED

- Micropipettes and microtips
- Elisa reader
- Distilled or deionized water
- Graduated Cylinders, for reagent dilution
- Sodium hypochlorite solution
- Paper towels or absorbent tissue
- Timer
- Elisa washer
- Incubator 37⁰C
- Vials to store the diluted reagent
- Disposable gloves

9. SPECIMEN COLLECTION & HANDLING

1. Only human serum or plasma samples should be used for the test. While preparing serum samples, remove the serum from the clot as soon as possible to avoid hemolysis. Fresh serum/plasma samples are preferred.

2. Specimens should be free of microbial contamination and may be stored at 2-8⁰C for one week, or frozen at -20⁰C or lower. Avoid repeated freezing and thawing.

3. Do not use heat inactivated samples as their use may give false results. Hemolyzed and Icteric hyperlipemic samples may give erroneous results.

4. Do not use Sodium Azide as preservative because it inactivates Horseradish peroxidase.

10. WARNING & PRECAUTION

⚠ **CAUTION:** THIS KIT CONTAINS MATERIALS OF HUMAN ORIGIN. NO TEST METHOD CAN OFFER COMPLETE ASSURANCE THAT HUMAN BLOOD PRODUCTS WILL NOT TRANSMIT INFECTION. NEGATIVE CONTROL, POSITIVE CONTROL & ALL THE SAMPLES TO BE TESTED SHOULD BE HANDLED AS THOUGH CAPABLE OF TRANSMITTING INFECTION.

1. The use of disposable gloves and proper biohazardous clothing is STRONGLY RECOMMENDED while running the test.

2. In case there is a cut or wound in hand, DO NOT PERFORM THE TEST.

3. Do not smoke, drink or eat in areas where specimens or kit reagents are being handled.

4. Tests are for *in vitro* diagnostic use only and should be run by competent person only.

5. Do not pipette by mouth.

6. All materials used in the assay and samples should be decontaminated in 5% sodium hypochlorite solution for 30-60 min. before disposal or by autoclaving at 121°C at 15psi for 60 min. Do not autoclave materials or solution containing sodium hypochlorite. They should be disposed off in accordance with established safety procedures.

7. Wash hands thoroughly with soap or any suitable detergent, after the use of the kit. Consult a physician immediately in case of accident or contact with eyes, in the event that contaminated material are ingested or come in contact with skin puncture or wounds.

8. Spills should be decontaminated promptly with Sodium Hypochlorite or any other suitable disinfectant.

9. Stop solution contains sulfuric acid. If sulfuric acid comes in contact with the skin, wash thoroughly with water. In case of contact with eyes, flush with excess of water.

11. SPECIMEN PROCESSING
(A) FROZEN SAMPLE
Dengue NS1 Ag Microlisa test is best used with fresh samples that have not been frozen and thawed. However most frozen samples will perform well if the procedure suggested below is followed.

Allow the frozen sample to thaw in a vertical position in the rack. Do not shake the sample. This allows particles to settle to the bottom. If a centrifuge is available, the sample should be centrifuged. (10,000 rpm for 15 min.)

(B) TRANSPORTATION
If the specimen is to be transported, it should be packed in compliance with the current Government regulations regarding transport of aetiologic agents.

12. PRECAUTIONS FOR USE
Optimal assay performance requires strict adherence to the assay procedure described in the manual.

1. Do not use kit components beyond the expiration date, which is printed on the kit.

2. Avoid microbial contamination of reagents. The use of sterile disposable tips is recommended while removing aliquots from reagent bottles.

3. Prepare working substrate solution just 10 minutes prior to adding in the wells.

4. If blue colour or white particles appears in working substrate solution then do not use it. Take fresh containers and tips and prepare it again.

5. Use separate tips for TMB substrate and TMB diluent.

6. Do not allow microwells to dry once the assay has started.

7. Ensure that the microwell strips are levelled in the strip holder. Before reading, wipe the bottom of the microwell strips carefully with soft, absorbent tissue to remove any moisture.

8. If available, a microwell reader which contains a reference filter with settings at 620 or 630 nm should be used. Use of a reference filter minimises interference due to microwells that are opaque, scratched or irregular. However, if a reference filter is unavailable, the absorbance may be read at 450 nm without a reference filter.

9. Distilled or deionised water must be used for wash buffer preparation.

10. Bring all the reagents to room temperature (20-30°C) before use.

11. Do not combine reagents from different batches, as they are optimized for individual batch to give best results.

12. Due to interchange of caps the reagents may get contaminated. Care should be taken while handling the reagents to avoid any sort of contamination.

13. Run negative control, positive control and calibrator in each assay.

14. Use freshly collected, clean serum/ plasma samples for assay. Try to avoid Haemolyzed/ turbid/ lipemic serum or plasma samples.

15. Use a separate tip for each sample and then discard it as biohazardous waste.

16. Thorough washing of the wells is critical to the performance of the assay.

17. Avoid strong light exposure during the assay.

13. PRELIMINARY PREPARATIONS
● Pre-warm the incubator to + 37°C.

● Bring foil pack to room temperature (20-30°C) before opening to prevent condensation on the microwell strips.

a. Break-off the required number of strips needed for the assay and place in the strip holder. Take the strip holder with the required number of strips, taking into account that, one negative control, one positive control and three calibrator should be included in the run while opening the fresh kit. However for one or two strips one negative control, one positive control and two calibrator should be included in each subsequent runs.

b. Unused wells should be stored at 2-8°C with desiccant in an aluminium pouch with clamp & rod. Microwells are stable for 30 days at 2-8°C from the date of opening of sealed pouch, when stored with desicant along with clamp & rod.

Caution: Handle microwell strip with care. Do not touch the bottom exterior surface of the wells.

● **Preparation of Working Wash Buffer:**
a) Check the buffer concentrate for the presence of salt crystals. If crystals are present in the solution, resolubilize by warming at 37°C until all crystals dissolve.

b) Prepare at least 25ml. (1ml. concentrated buffer with 24 ml. water) of buffer for each strip used. Mix well before use.

c) Mix 20 ml. of 25X wash buffer concentrate with 480ml. of distilled or deionized water. Wash buffer is stable for 2 months when stored at 2-8°C.

● **Preparation of Working Conjugate:**
Dilute conjugate concentrate 1:50 in Diluent. **Do not store working conjugate.** Prepare a fresh dilution for each assay in a clean glass vessel. Determine the quantity of working conjugate solution to be prepared from the table below. Mix solution thoroughly before use.

No. of Strips	1	2	3	4	5	6	7	8	9	10	11	12
No. of Wells	8	16	24	32	40	48	56	64	72	80	88	96
Enzyme Conjugate Concentrate (μl)	20	40	60	80	100	120	140	160	180	200	220	240
Diluent (ml)	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0

● **Preparation of working substrate solution :**
Mix TMB substrate and TMB Diluent in 1:1 ratio to prepare working substrate.

Do not store working substrate. Prepare a fresh dilution for each assay in a clean plastic/glass vessel. Determine the quantity of working substrate solution to be prepared from table. Mix solution thoroughly before use.

No. of Strips	1	2	3	4	5	6	7	8	9	10	11	12
No. of Wells	8	16	24	32	40	48	56	64	72	80	88	96
TMB Susbstrate (ml)	0.8	1.6	2.4	3.2	4.0	4.8	5.6	6.4	7.2	8.0	8.8	9.6
TMB Diluent (ml)	0.8	1.6	2.4	3.2	4.0	4.8	5.6	6.4	7.2	8.0	8.8	9.6

15. PROCEDURAL NOTES:
1. Material should not be used after the expiry date shown on the labels. Components and test specimen should be at room temperature (20-25°C) before testing begins. Return the reagents to 2-8°C after use.

2 . All reagents must be mixed well before use.

3. To avoid contamination, do not touch the top or bottom of strips or edge of wells.

4. All pipetting steps should be performed with utmost care and accuracy. Cross contamination between reagents and samples will invalidate results.

5. Prevent evaporation during sample incubation by covering the strips with sealer; remove sealer before washing.

6. Routine maintenance of wash system is strongly recommended to prevent carry over from highly reactive specimens to non reactive specimens.

16. TEST PROCEDURE
Once the assay has started, complete the procedure without interruption. All the reagents should be dispensed in the centre of the well and the tip of the pipette should not touch the wall of the microwell.

Fit the stripholder with the required number of Anti-Dengue NS1 antibody coated strips. The sequence of the procedure must be carefully followed. Arrange the assay control wells in a horizontal or vertical configuration. Configuration is dependent upon reader software.

1. Add 50 μl Diluent in all the wells.
2. Add 50 μl Negative Control in A-1well.
3. Add 50 μl Calibrator in B-1, C-1 & D-1 well.
4. Add 50 μl Positive Control in E-1 well.
5. Add 50 μl sample in F-1 well onwards.
6. Add 100 μl of working Conjugate Solution in each well.
7. Ensure thorough mixing of controls, samples to be tested & working conjugate to get reproducible results.
8. Apply cover seal.
9. Incubate at 37ºC $\pm$ 1ºC for 90 min. $\pm$ 1min.
10. While the samples and working Conjugate are incubating, prepare working Wash Solution as specified in preparation of reagents.
11. Take out the plate from the incubator after the incubation time is over and, wash the wells 6 times with working Wash Solution.
12. Add 150 μl of working substrate solution in each well.
13. Incubate at room temperature (20-30ºC) for 30 min. in dark.
14. Add 100 μl of stop solution.
15. Read absorbance at 450 nm. within 30 minutes in ELISA READER. (Bichromatic absorbance measurement with a reference wavelength 600-650 nm is recommended when available).

SUMMARY OF PROCEDURE		
Add controls, calibrator and samples		50 μl Diluent 50 μl controls, calibrator and samples
Prepare working conjugate		No of Strips 1 2 3 4 5 6 7 8 9 10 11 12 Enz. conc. (μl) 20 40 60 80 100 120 140 160 180 200 220 240 Diluent (ml.) 1 2 3 4 5 6 7 8 9 10 11 12
Add Conjugate		100 μl
Cover the plate & incubate		90 mins. at 37ºC
Wash		6 Cycles
Prepare Chromogenic Substrate		No of Strips 1 2 3 4 5 6 7 8 9 10 11 12 TMB Substrate (ml) 0.8 1.6 2.4 3.2 4.0 4.8 5.6 6.4 7.2 8.0 8.8 9.6 TMB Diluent (ml.) 0.8 1.6 2.4 3.2 4.0 4.8 5.6 6.4 7.2 8.0 8.8 9.6
Add Substrate		150 μl
Incubate in dark		30 mins. at Room Temp.
Add Stop Solution		100 μl
Read Results		450 nm./630 nm.

17. CALCULATION OF RESULTS
TEST VALIDITY:
Ensure the following is within specified acceptance criteria
i) NC O.D. must be < 0.3. If it is not so, the run is invalid and must be repeated.
ii) PC O.D. must be > 1.0. If it is not so, the run is invalid and must be repeated.
iii) Mean Calibrator O.D. must be $\geq$ 0.35. If it is not so, the run is invalid and must be repeated.
iv) Cut off value must be $\geq$ 1.5 x NC O.D. If it is not so, the run is invalid and must be repeated.
v) Ratio of PC O.D. / cut off must be > 1.1. If it is not so, the run is invalid and must be repeated.

Imp. Note: The calibration factor detail is batch specific and stamped on back page of Instruction manual.

a. Cut off value = mean O.D. of calibrator x calibration factor
b. Calculation of sample O.D. ratio : Calculate sample O.D. ratio as follows:

$$\text{Sample O.D. ratio} = \frac{\text{Sample O.D.}}{\text{Cut off Value}}$$

c. Calculation of Dengue NS1 Ag units : Calculate by multiplying the sample O.D. ratio by 10.
   Dengue NS1 Ag units = sample O.D. ratio x 10.
   e.g.: Mean O.D. of calibrator = 0.75
   Calibration factor = 0.7
   Cut off value = 0.75 x 0.7 = 0.525
   e.g.: sample absorbance (O.D.) = 0.925
   Cut off value = 0.525
   Sample O.D. ratio = 0.925 / 0.525 = 1.761
   Dengue NS1 Ag units = 1.761 x 10 = 17.61

18. INTERPRETATION OF RESULTS
a. If the Dengue NS1 Ag Units is < 9 then interpret the sample as Negative for Dengue NS1 Antigen.
b. If the Dengue NS1 Ag Units is between 9 - 11 then interpret the sample as Equivocal for Dengue NS1 Antigen.
c. If the Dengue NS1 Ag Units is > 11 then interpret the sample as Positive for Dengue NS1 Antigen.

19. PERFORMANCE CHARACTERISTICS
Sensitivity with RT-PCR confirmed positive samples
The sensitivity of Dengue NS1 Ag Microlisa kit has been evaluated on 58 retrospective serum samples from patients with dengue infection confirmed by Dengue RT-PCR. The samples were from the four different dengue serotypes (DEN 1, DEN 2, DEN 3 & DEN 4).

The sensitivity of the Dengue NS1 Ag Microlisa kit when compared with Dengue RT- PCR is as follows:

Serotype	Dengue RT PCR Positive Samples	Result of Dengue NS1 Ag Microlisa	Sensitivity of Dengue NS1 Ag Microlisa
Den 1	25	25	100%
Den 2	8	7	87.5%
Den 3	22	22	100%
Den 4	3	3	100%

The overall sensitivity of Dengue NS1 Ag Microlisa kit on the avove panel was found to be 98.28%.

The kit has also been evaluated in-house with the known panel of fresh as well as frozen Dengue NS1 antigen positive of all 4 dengue serotypes and Negative samples. The samples included cross-reacting samples; Epstein-Barr virus, Malaria, Rheumatoid factor, Leptospirosis, Japanese encephalitis, yellow fever and West Nile viruses. Following are the result of evaluation:

No. of Samples	Status	Dengue NS1 Ag Microlisa	
		Positive	Negative
200	Dengue NS1 Ag Positive	199	1
2050	Dengue Negative	0	2050

Sensitivity : 99.5%   **Specificity** : 100%

Precision : Within-run and between-run precisions have been determined by testing 10 replicates of five samples: 1 negative and 4 dengue NS1 Ag positive; 2 weak positive, 1 medium positive & 1 strong positive. The C.V.(%) of negative, weak positive, medium positive & strong positive values were within 10%.

20. LIMITATION OF THE TEST
1. The test should be used for detection of NS1 Ag in serum or plasma only and not in other body fluids.
2. **This is only a screening test** and will only indicate the presence or absence of Dengue NS1 antigen in the specimen. All reactive samples should be confirmed by confirmatory test. Therefore for a definitive diagnosis, the patients clinical history, symptomatology as well as serological data should be considered. The results should be reported only after complying with the above procedure.
3. False positive results can be obtained due to cross reaction with Murray Valley and encephalitis, Japanese encephalitis, yellow fever and West Nile viruses. This occurs in less then 1% of the sample tested.

21. LIMITED EXPRESSED WARRANTY DISCLAIMER

The manufacturer limits the warranty to the test kit, as much as that the test kit will function as an *in vitro* diagnostic assay within the limitations and specifications as described in the product instruction-manual, when used strictly in accordance with the instructions contained therein. The manufacturer disclaims any warranty expressed or implied including such expressed or implied warranty with respect to merchantability, fitness for use or implied utility for any purpose. The manufacture's liability is limited to either replacement of the product or refund of the purchase price of the product and in no case liable to for claim of any kind for an amount greater than the purchase price of the goods in respect of which damages are likely to be claimed.

The manufacturer shall not be liable to the purchaser or third parties for any injury, damage or economic loss, howsoever caused by the product in the use or in the application there of.

22. REFERENCES

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23. TROUBLE SHOOTING CHART

PROBLEM	POSSIBLE CAUSE	SOLUTION
1. Controls and calibrator out of validation limit	a) Incorrect temperature timing or pipetting	Check procedure & repeat assay
	b) Improper preparation of reagents, error of dilution, improper mixing of reagents.	Check procedure & repeat assay
	c) Cross contamination of controls & calibrator	Pipette carefully and do not interchange caps. Repeat assay
	d) Incorrect reading filter or readings without reference filter.	Check the filter used. It should be 450nm. If no reference filter is used absorbance will increase.
	e) Interference in the optical pathway	Check the reader. Clean or dry the bottom of micro wells, check for bubbles & repeat the readings.
	f) Used components from different lots.	Do not use components from different lots as they are adjusted for each batch released.
	g) Expired Reagents.	Check the kit expiry date. Use the kit with-in shelf life.
2. No colour or light colour developed at the end of assay	a) Any one reagent has been added in wrong sequence.	Check procedure and repeat assay.
	b) Inactivated conjugate, wrong dilution used, improper conservation.	Check for contamination, recheck procedure.
	c) Microplate inactivated, due to improper conservation	Keep unused strips in aluminium pouch and seal with clamp & rod with dessicant pouch inside.
	d) Inactivated substrate, improper conservation or preparation	Use freshly prepared substrate solution. Recheck procedure, repeat assay.

PROBLEM	POSSIBLE CAUSE	SOLUTION
3. Too much colour in all wells of the plate	a) Contaminated substrate use of same container for preparing & dispensing substrate & conjugate.	Check substrate (TMB Diluent) it should be colourless. If blue in colour then discard and use acid washed or disposable container.
	b) Contaminated or improper dilution of reagents.	Check for contamination, check dilutions.
	c) Contaminated washing solution (1X).	Check the container and quality of water used for dilution.
	d) Over incubation of substrate and delay in addition of stop solution.	Repeat assay.
	e) Insufficient washing.	Check wash device, fill the
	i) Washing not consistent	well close to the top.
	ii) Filling volume not sufficient.	After washing, blot the
	iii) Insufficient no. of wash cycles.	microwells on absorbent tissue.
	iv) Contaminated wash device	
	f) Use of wash buffer from other manufacturer.	Use only Dengue NS1 Ag Microlisa wash buffer.
4. Poor reproducibility	a) Washing problems.	
	b) Uncalibrated pipettes or tips not well fitted, improper pipetting.	Use only calibrated pipettes with well fitted tips & pipette carefully without bubbling.
	c) Reagent & sera not at room temperature or not well mixed before use.	Equilibrate reagents to room temperature and mix thoroughly before use.
	d) Too long time for addition of samples or reagents, Inconsistency in time intervals	Develop consistent and uniform technique.
	e) Interference in optical pathway due to Air bubbles.	Refer 1(e).
5. False Positive	Beside 3a, b, c, d, e, incorrect interpretation and calculation of final results	Check the calculation part given in the insert and correctly interpret.
6. False Negative/ low O.D. for PC, calibrator and positive sample	a) Sample used was having sodium azide as preservative.	Do not add azide in samples.
	b Inadequate addition of substrate/conjugate solution.	Recheck the test procedure and reagent volume.
	c) Kit expired, reagent of different kit used.	Check the expiry of the kit before use.
	d) White particles in working substrate solution.	Discard the substrate and prepare the working substrate again in fresh tube.

For *in vitro* diagnostic use only, not for medicinal use