LEPTO IgM MICROLISA

Microwell ELISA Test for the Qualitative Detection of Leptospira Specific IgM antibodies in Human Serum/Plasma

1. INTRODUCTION

Leptospirosis is a world-wide occuring zoonotic disease, caused by infection with pathogenic spirochetes of the genus Leptospira. Although traditionally considered as an occupational risk among persons exposed to contaminated water or infected animal urine, leptospirosis is becoming recognised as a common cause of febrile illness in tropical environmments world-wide. The organism enters the human body through cuts or abrasions on the skin or through intact mucosa of the mouth, nose or conjunctiva. The clinical manifestations of leptospirosis range from a mild catarrh like fever, chills, nausea, muscle aches to icteric disease such as Weil's syndrome, which are charaterized by renal failure, liver impairments & haemorrhages and have a high mortality rate. As clinical symptoms & signs of this infection resemble those of many other infectious diseases including Viral haemorrhage fever and Dengue fever, clinical findings need to be confirmed by laboratory diagnostic techniques.

2. INTENDED USE

Lepto IgM Microlisa is designed for in vitro qualitative detection of Leptospira IgM antibodies in human serum or plasma and is used as a screening test for testing of collected blood samples suspected for Leptospirosis.

3. PRINCIPLE

Lepto IgM Microlisa test is an enzyme immunoassay based on "Indirect ELISA".

Recombinant proteins mixture of various molecular weight representing immunodominant epitopes are coated onto microtiter wells. Specimens and controls are added to the microtiter wells and incubated.

IgM antibodies to Leptospira if present in the specimen, will bind to the specific leptospira antigens absorbed onto the surface of the wells. The plate is then washed to remove unbound material. Horseradish peroxidase (HRP) conjugated Anti-Human IgM is added to each well. This conjugate will bind LEPTOSPIRA antigen-antibody complex present. Finally substrate solution containing chromogen and hydrogen peroxide is added to the wells and incubated. A blue colour will develop in proportion to the amount of LEPTOSPIRA antibodies present in the specimen. The colour reaction is stopped by a stop solution. The enzyme substrate reaction is read by EIA reader for absorbance at a wavelength of 450 nm. If the sample does not contain LEPTOSPIRA antibodies then enzyme conjugate will not bind and the solution in the wells will be either colourless or only a faint background colour develops.

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 BS EN 15223-1:2012.

	Manufactured By	IVD	*In vitro* diagnostic medical device
Σ	No. of tests		See Instruction for use
LOT	Lot Number Batch Number	2°C 8°C	Temperature Limitation
	Manufacturing Date		Caution See instruction for use
	Expiry Date	REF	Catalogue Number

5. KIT PRESENTATION

- 48 Test Pack

6. KIT & ITS COMPONENTS

1 Plate (Microwells)	Microwells coated with LEPTOSPIRA antigens packed in a pouch provided with desiccant.
Sample Diluent	Buffer containing protein stablizers and antimicrobial agents as preservative.
Enzyme Conjugate Concentrate (50X)	Anti-human IgM labelled with horseradish peroxidase with protein stablizers
Conjugate Diluent	Buffer containing stabilizers.
Wash Buffer Concentrate (25X)	PBS with surfactant. Dilute 1:25 with distilled water before use.
Rf Absorbent	Buffer containing anti-human IgG antibodies with preservatives.
TMB Substrate	TMB solution
TMB Diluent	Buffer solution containing H_2O_2 with preservative
Control —	Ready to use, Normal human serum negative for Lepto Antibodies
Control +	Ready to use, Non infections synthetic control, Positive for Leptospira IgM antibodies and contains sodium azide as preservative.
Stop Solution	Ready to use, 1N sulfuric acid
Plate Sealers	Adhesive sheets to cover the microwells during incubation.

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 37^{0}C
- Vials to store the diluted reagent
- Disposable gloves

9. SPECIMEN COLLECTION & HANDLING

1. Human serum or plasma samples should be used for the test. While preparing serum samples, remove the serum form 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 2-8^{0}C for one week, or frozen at -20^{0}C or lower. Avoid repeated freezing and thawing.
3. Do not use heat inactivated samples as their use may given 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. SPECIMEN PROCESSING

(A) FROZEN SAMPLE

Lepto IgM 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. (5000 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.

11. CAUTION

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.
5. All the samples to be tested should be handled as though capable of transmitting infection.
6. Wash hands thoroughly with soap or any suitable detergent, after the use of the kit. In case of needle prick or other skin puncture or wounds, wash the hands with excess of water and soap.
7. Controls contain Sodium Azide as a preservative. If these material are to be disposed

off through a sink or other common plumbing systems, flush with generous amounts of water to prevent accumulation of potentially explosive compounds.

8. All materials used in the assay and samples should be disposed off in the manner that will inactivate bacteria.

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. 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.
4. Take care while preparing working substrate solution as vials of TMB substrate & diluent are of same size.
5. Prepare working substrate solution just 10 minutes prior to adding in the wells.
6. 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.
7. Use separate tips for TMB substrate and TMB diluent.
8. Do not allow microwells to dry once the assay has started.
9. 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.
10. 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.
11. Distilled or deionised water must be used for wash buffer preparation.
12. Bring all the reagents to room temperature (20-30ºC) before use.
13. Do not combine reagents form different batches, as they are optimized for individual batch to give best results.
14. Due to interchange of caps the reagents may get contaminated. Care should be taken while handling the reagents to avoid contamination of any sort.
15. Run negative and positive controls in each assay.
16. Use freshly collected, clean serum samples for assay. Try to avoid Haemolyzed turbid, lipemic serum or plasma samples.
17. Use a separate tip for each sample and then discard it as biohazardous waste.
18. Thorough washing of the wells is critical to the performance of the assay.
19. Avoid strong light exposure during the assay.

13. PREPARATION OF REAGENTS

Prepare the following reagents before or during assay procedures. Reagents and samples should be at room temperature (20-30ºC) before beginning the assay and can remain at room temperature during testing. Return reagents to 2-8ºC after use. All containers used for preparation of reagents must be cleaned throughly and rinsed with distilled or deionized water. Prewarm the incubator to 37ºC.

i) LEPTOSPIRA Antigen strip

Bring foil pack to room temperature (20-30ºC) 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 well holder. Take the strip holder with the required number of strips, taking into account that two negative & one positive control should be included in the run while opening the fresh kit. However for one or two strips, one each of negative and positive control and for more strips two negative and one positive control should be included in each subsequent runs.

b. Unused wells should be stored at 2-8ºC, with dessicant in an aluminium pouch with clamp & rod.

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

ii) 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 37ºC 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 480 ml. of distilled or deionized water. Wash buffer is stable for 2 months when stored at 2-8ºC.

iii) Preparation of Working Conjugate:

Dilute conjugate concentrate 1:50 in conjugate 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
Conjugate Diluent in (ml)	1	2	3	4	5	6	7	8	9	10	11	12

iv) 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)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
TMB Diluent (ml)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0

14. REAGENT PREPARATION

Reagent	Preparation	Stability of opened/ diluted reagents (+2ºC to +8ºC)
1. Lepto Antigen coated Microwells	Ready to use	30 days
2. Sample Diluent	Ready to use	
3. Rf Absorbent	Ready to use	
4.a Negative Control	Ready to use	
4.b Positive Control	Ready to use	
5. Washing Solution	Dilute 1: 25 (1+24) with distilled water	2 months.
6. Conjugate Diluent	Ready to use for the dilution of Enzyme Conjugate	
7. Enzyme Conjugate Concentrate (50X)	Diluent 1:50 in conjugate Diluent	4 hours
8. TMB Substrate	Dilute 1:1 in TMB Diluent just before use	Discard unused solution. A deep blue colour present in the substrate solution indicates that the solution has been contaminated and must be discarded.
9. TMB Diluent	for the dilution of TMB Substrate	
10. Stop Solution	Ready to use	

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 (20-30ºC) before testing begins. Return the reagents to 2-8ºC 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 Lepto Antigen 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.

16.1 TREATMENT OF SAMPLES

a. Mark the tubes carefully for the proper identification of the samples.
b. Treat serum/ plasma samples to be tested, with Rf absorbent in 1:11 dilution in separate tubes (10 μl serum/plasma samples + 100 μl Rf absorbent). Use a separate tip for each sample and then discard as biohazardous waste.
c. Ensure thorough mixing of the sample with Rf Absorbent.
d. Incubate at Room Temperature for 10 minutes.
e. Use 25 μl of this treated sample for the ELISA procedure.

16.2 ELISA Procedure

1. Add 125 μl Negative Control in A-1 & B-1 well.
2. Add 125 μl Positive Control in C-1 well.
3. Add 100 μl of sample diluent in each well, starting from D-1 followed by addition of 25μl of treated sample from Step 16.1(e).
4. Apply cover seal.
5. Incubate at 37°C $\pm$ 1°C for 30 min. $\pm$ 1min.
6. While the samples are incubating, prepare working Wash Solution and working Conjugate as specified in preparation of reagents.
7. Take out the plate from the incubator after the incubation time is over and, wash the wells 5 times with working Wash Solution.
8. Add 100 μl of working Conjugate Solution in each well.
9. Apply cover seal.
10. Incubate at 37°C $\pm$ 1°C for 30 min $\pm$ 1min.
11. Aspirate and wash as described in step no. 7.
12. Add 100 μl of working substrate solution in each well.
13. Incubate at room temperature (20-30°C) for 30 min. in dark.
14. Add 50 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

Samples treatment		Sample 10 μl — Rf Absorbent 100 μl
Add controls*		125 μl
Add sample Diluent		100 μl
Add Treated Sample		25 μl
Cover the plate & incubate		30 mins. at 37°C
Wash		5 Cycles
Prepare Working conjugate		No of Strips: 1 2 3 4 5 6 7 8 9 10 11 12 Enz. conc. (ml): 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		30 mins. at 37°C
Wash		5 Cycles
Prepare Working Substrate		No of Strips: 1 2 3 4 5 6 7 8 9 10 11 12 TMB Substrate (ml): 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 TMB Diluent (ml.): 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0
Add Substrate		100 μl
Incubate in dark		30 mins. at Room Temp.
Add Stop Solution		50 μl
Read Results		450 nm./630 nm.

* Controls are Ready to use

TEST VALIDITY:

Ensure the following is within specified acceptance criteria

i) Blank must be < 0.100 in case of differential filter being used. In case differential filter is not available in the reader the blank value may go higher.

ii) NC or NC$\bar{x}$ O.D. must be < 0.3. If it is not so, the run is invalid and must be repeated.

iii) PC O.D. must be > 1.1. If it is not so, the run is invalid and must be repeated.

17. CALCULATION OF RESULTS

a. Cut off value = NC$\bar{x}$ + 0.500

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 Lepto IgM units : Calculate by multiplying the sample O.D. ratio by 10.

Lepto IgM units = sample O.D. ratio x 10.

e.g. : sample absorbance (O.D.) = 1.132

Cut off value = 0.653

Sample O.D. ratio = 1.132 / 0.653 = 1.733

Lepto IgM units = 1.733 x 10 = 17.3

18. INTERPRETATION OF RESULTS

a. If the Lepto IgM units is < 9 then interpret the sample as Negative for Lepto IgM antibodies.
b. If the Lepto IgM units is between 9 - 11 then interpret the sample as Equivocal for Lepto IgM antibodies.
c. If the Lepto IgM units is > 11 then interpret the sample as Positive for Lepto IgM antibodies.

19. PERFORMANCE CHARACTERISTICS

An elaborated study has been done on Lepto IgM Microlisa to determine its performance. The performance of the test was evaluated in-house with a known panel of fresh as well as frozen lepto IgM Negative & Positive Serum / Plasma samples. The panel included cross-reacting samples : Epstein-BARR virus, Influenza A & B, Brucella and Dengue Virus . The results obtained are as follows:

No. of Samples	Status	Lepto IgM Microlisa		
		Positive	Equivocal	Negative
265	Lepto Positive	264	0	1
2650	Lepto Negative	2	2	2648

Sensitivity : 99.62% **Specificity : 99.92%**

Precision : Within-run and between-run precisions have been determined by testing 10 replicates of three specimens : a negative, a low positive and a strong positive. The C.V.(%) of negative, low positive and strong positive values were within 10% of the time.

20. LIMITATION OF THE TEST

1. The test should be used for detection of IgM antibodies of Leptospira in human serum / plasma.
2. This is only a screening test and will only indicate the presence or absence of LEPTOSPIRA antibodies 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 Epstein-BARR virus, Influenza A & B, Brucella, Dengue Virus. 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. Control 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	Pipette carefully and do not interchange caps. Repeat assay
	d) Incorrect reading filter or readings without blanking the reader.	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 sealable plastic bag, very well closed with the dessicant pouch inside
	d) Inactivated substrate, improper conservation or preparation	Use freshly prepared substrate solution Recheck procedure, repeat assay
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 acidwashed 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. i) Washing not consistent	Check wash device, fill the 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 solution from other manufacturer.	Use only Lepto IgM Microlisa wash solution.
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 & positive sample	a) Inadequate addition of substrate/conjugate solution.	Recheck the test procedure and reagent volume.
	b) Kit expired, reagent of different kit used.	Check the expiry of the kit before use.
	c) 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

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