SCRUB TYPHUS IgM MICROLISA

Microwell ELISA Test for the Qualitative Detection of Scrub Typhus IgM antibodies in Human Serum/Plasma

1. INTRODUCTION

Scrub Typhus, or tsutsugamushi fever, is a zoonotic disease that is accidentally transmitted to humans. The infection is found in South East Asia and western Pacific islands. The causative organism, Orientia tsutsugamushi, belongs to family Rickettsiaceae is transmitted to humans by the bite of a larval trobiculid mite or chigger. A cigratte burn-like sore, called an eschar, sometimes develops at the site of infection. Swollen lymph glands also are common. The bite from an infected chigger may by followed by a systemic illness ranging in severity from inapparent to fatal. Many scrub typhus cases go undiagnosed, particularly those in which an eschar cannot be found. Most common symptoms are fever, headache, body ache and sometime rashes. As very few health facilities have accessible accurate diagnostic tests, the diagnosis of scrub fever must be based on clinical features. However, this is difficult because the clinical symptoms and signs are similar to those of many other febrile diseases, such as murine typhus, leptospirosis, and dengue virus infection. The diagnosis of scrub typhus infection has relied on the detection of O. tsutsugamushi antibodies during the acute phase of the disease.

2. INTENDED USE

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

3. PRINCIPLE

Scrub Typhus IgM Microlisa test is an enzyme immunoassay based on “Indirect ELISA”.

Recombinant protein representing immunodominant epitopes of Scrub Typhus are coated onto microtiter wells. Specimens and controls are added to the microtiter wells and incubated.

IgM antibodies to Scrub Typhus if present in the specimen, will bind to the specific Scrub Typhus antigen 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 Scrub Typhus antigen-antibody complex present. Finally substrate solution containing chromogen and hydrogen peroxide is added to the wells and incubated. A blue color will develop in proportion to the amount of Scrub Typhus IgM antibodies present in the specimen. The color reaction is stopped by a stop solution. The enzyme substrate reaction is read by EIA reader for absorbance at a wavelength of 450 nm along with 630 nm (reference filter). If the sample does not contain Scrub Typhus IgM antibodies then enzyme conjugate will not bind and the solution in the wells will be either colorless or only a faint background color 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 European Standard EN ISO 15223-1:2016.

	Manufactured By		*In vitro* diagnostic medical device
	No. of tests		See Instruction for use
	Lot Number Batch Number		Temperature Limitation
	Manufacturing Date		Caution See instruction for use
	Expiry Date		Catalogue Number
	Do not use if package is damaged		Keep away from sunlight
	Keep Dry		

5. KIT PRESENTATION

- 96 Test Pack

6. KIT & ITS COMPONENTS

Scrub typhus antigen coated (Microwells)	Microwells coated with Scrub Typhus antigens packed in a pouch provided with desiccant.
Sample Diluent	Buffer containing protein stablizers and antimicrobial agents as preservative.
Enzyme Conjugate	Anti-human IgM labelled with horseradish peroxidase with protein stablizers. Ready to use
Wash Buffer Concentrate (25X)	PBS with surfactant. Dilute 1:25 with distilled water before use.
TMB Substrate	TMB solution
TMB Diluent	Buffer solution containing H_2O_2 with preservative
Control —	Ready to use, Normal human serum negative for Scrub Typhus Antibodies
Control +	Ready to use, Non infections, Positive serum for Scrub Typhus 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
- Paper towels or absorbent tissue
- Timer
- Elisa washer
- Incubator 37°C
- Disinfectant solution
- 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°C for one week, or frozen at -20°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

Scrub Typhus 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. Do not pipette by mouth.
5. Tests are for in vitro diagnostic use only and should be run by competent person only.
6. All the samples to be tested should be handled as though capable of transmitting infection.
7. All materials used in the assay and samples should be decontaminated in suitable disinfectant solution for 30-60 min. before disposal. They should be disposed

off in accordance with established biosafety guidelines for handling & disposal of potentially infective material.

8. Spills should be decontaminated promptly with Sodium Hypochlorite or any other suitable disinfectant.
9. 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.
10. Controls contain Sodium Azide as a preservative. If these materials 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.
11. ELISA Reader & micropipettes used in testing should be calibrated at regular interval to ensure accurate results.

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. 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.
11. Distilled or deionised water must be used for wash buffer preparation.
12. Bring all the reagents to room temperature (20-30^{0}C) 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/ plasma 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) Scrub Typhus 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 a aluminium pouch with clamp & rod. Microwells are stable for 30 days at 2-8ºC 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.

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 37^{0}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 480 ml. of distilled or deionized water. Wash buffer is stable for 2 months when stored at 2-8°C.

iii) Preparation of working substrate solution :

Mix TMB substrate and TMB Diluent in 1:1 ratio to prepare working substrate.

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 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

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. Discard unused solution. A deep blue color present in the substrate solution indicates that the solution has been contaminated and must be discarded.

14. 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 (20-30^{0}C) before testing begins. Return the reagents to 2-8^{0}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.

15. SAMPLE PREPARATION

TUBE DILUTION: Mark the tubes carefully for the proper identification of the samples. Dilute the serum samples to be tested, with sample diluent 1:101 in separate tubes (0.5 ml. sample diluent + 5 µl serum samples). Use a separate tip for each sample and then discard as biohazards waste.

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 Scrub Typhus 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.

1) Add 100 µl Negative Control in A-1 & B-1 well.
2) Add 100 µl Positive Control in C-1 well.
3) Add 100 µl of each diluted sample with 1:100 dilution in sample diluent, in each well starting from D-1 well. (Refer TUBE DILUTION).
4) Apply cover seal.
5) Incubate at 37ºC $\pm$ 1ºC for 30 min. $\pm$ 1min.
6) While the samples are incubating, prepare working Wash Solution 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 Enzyme Conjugate Solution in each well.

9) Apply cover seal.

10) Incubate at 37ºC ± 1ºC for 30 min ± 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 (20-30ºC) for 15 min. in dark.

14) Add 100 µl of Stop Solution.

15) Read absorbance at 450 nm and 630 nm (reference filter) within 30 minutes in ELISA READER.

SUMMARY OF PROCEDURE

Dilute Serum samples		Sample 5 µl — Sample Diluent 0.5 ml
Add diluted samples & controls*		100 µl
Cover the plate & incubate		30 mins. at 37ºC
Wash		5 Cycles
Add Enzyme Conjugate		100 µl
Cover the plate & incubate		30 mins. at 37ºC
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		15 mins. at Room Temp.
Add Stop Solution		100 µl
Read Results		450 nm./630 nm.

* Controls are Ready to use

TEST VALIDITY:

Ensure the following is within specified acceptance criteria

i) NC or NC$\bar{x}$ O.D. must be < 0.15. If it is not so, the run is invalid and must be repeated.

ii) 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$\bar{x}$ + 0.350

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

Scrub Typhus IgM units = sample O.D. ratio x 10.

e.g. : sample absorbance (O.D.) = 1.316

Cut off value = 0.403

Sample O.D. ratio = 1.316 / 0.403 = 3.265

Scrub Typhus IgM units = 3.265 x 10 = 32.655

18. INTERPRETATION OF RESULTS

a. If the Scrub Typhus IgM units is < 9 then interpret the sample as Negative for Scrub Typhus IgM antibodies.

b. If the Scrub Typhus IgM units is between 9 - 11 then interpret the sample as Equivocal for Scrub Typhus IgM antibodies.

c. If the Scrub Typhus IgM units is > 11 then interpret the sample as Positive for Scrub Typhus IgM antibodies.

19. PERFORMANCE CHARACTERISTICS

In-House Evaluation:

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

No. of Samples	Status	Scrub Typhus IgM Microlisa		
		Positive	Equivocal	Negative
20	Scrub Typhus Positive	20	0	0
70	Scrub Typhus Negative	1	0	69

Sensitivity : 100% **Specificity : 98.58%**

External Evaluation:

The performance of the test kit has also been evaluated with clinical specimen confirmed scrub typhus positive (28), negative (64) and cross-reacting (2 each of leptospira and typhoid positive) by a licensed commercially available ELISA test kit from a referral government institute and the results are as follows:

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

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 15%.

20. LIMITATION OF THE TEST

1. The test should be used for detection of IgM antibodies of Scrub Typhus in human serum / plasma.
2. This is only a screening test and will only indicate the presence or absence of Scrub Typhus 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, Brucella, Dengue Virus. This occurs in less than 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

1) Kelly, D.J., A.K Rucgardsm H, Tenebajm D, Strickman, G. A. Dasch. 2001. The present and past challenge of rickettsial disease to military medicine and public health. Clin Infect Dis 2002;34(suppl 4):s145-s169.

2) Weddle, J.R., T.C. Chan, K. Thompson, H. Paxton, D.J. Kelly, G. Dasch, and D. Strickman 1995. Effectiveness of a dot-blot immunoassay of anti-Rickettsia tsutsugamushi antibodies for serologic analysis of scrub typhus. Am. J. Trop. Med. Hyg. 53:43-46, 12.

3) Kelly, D.J., D. Marana, C. Stover, E. Oaks, and M. Carl. 1990. Detection of Rickettsia tsutsugamushi by gene amplification using polymerase chain reaction techniques. Ann. N.Y. Acad. Sci. 590:564-571.

4) Tamura, A., H. Sakurami, K. Takahashi, and M. Oyanagi. 1985 Analysis of polypeptide composition and antigenic components of Rickettsia tsutsugamushi by polyacrylamide gel electroporesis and immunoblotting. Infect. Immum. 48:671-675

5) Ohashi, N., A. Tamura, M. Ohta, and K. Hayashi. 1989. Purification and partial characterization of a type-specific antigen of Rickettsia tsutsugamushi. Infect. Immun. 57-1427-1431, 20. Eisemann, C. S., and J.V. Osterman. 1981.

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) Use of non calibrated micropipette and/or ELISA Reader	Calibrate micropipette and ELISA Reader at defined interval.
	h) 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 Scrub Typhus 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.
	d) Uncalibrated pipettes, improper pipetting.	Use only calibrated pipettes with well fitted tips & pipette carefully without bubbling.

in vitro diagnostic Reagent, not for medicinal use

VER-01 R-00

J. Mitra & Co. Pvt. Ltd.
A 180-181, Okhla Ind. Area, Ph-1, New Delhi-110 020, INDIA
Ph: +91-11-47130300, 47130500, 26818971-73
e-mail: jmitra@jmitra.co.in Internet: www.jmitra.co.in

MNEST074
Rev. Date: Mar.-2020