MALARIA *Ag* MICROLISA

Microwell ELISA Test for detecting infection with plasmodium species
(P. falciparum/P. vivax/ P. malariae/ P. ovale) based on pLDH Antigen in Human Blood

SUMMARY AND EXPLANATION OF THE TEST

Malaria is a serious, sometimes fatal, parasitic disease characterized by fever, chills, and anaemia and is caused by a parasite that is transmitted from one human to another by the bite of infected Anopheles mosquitoes. There are four kinds of malaria parasite that can infect human: Plasmodium falciparum, P. vivax, P. ovale and P. malariae. The disease now occurs in more than 90 countries worldwide, and it is estimated that there are over 500 million clinical cases and 2.7 million malaria-caused deaths per year. At present, malaria is diagnosed microscopically by looking for the parasites in a drop of blood. Malaria Ag Microlisa is a microlwell ELISA test for the diagnosis of infection with all four Plasmodium Species (P. falciparum/P. vivax/ P. malariae/ P. ovale) in human whole blood. Malaria Ag Microlisa is specially designed to exluded infected blood to prevent transfusion aquired malaria and is used to monitoring of anti-malarial theraphy.

INTENDED USE

Malaria Ag Microlisa is an in-vitro qualitative enzyme immunoassay for detecting infection with plasmodium species (P. falciparum/P. vivax/ P. malariae/ P. ovale) based on pLDH antigen in human blood. It is intended for screening of blood donors or other individuals at risk for malaria infection and for clinical diagnostic testing.

3. PRINCIPLE OF THE TEST

Malaria Ag Microlisa test is an enzyme immunoassay based on sandwich ELISA.
Anti-pLDH antibodies has been coated onto the wells of the microwell plate provided. Blood sample including controls are pipetted into these wells. During the first incubation, the plasmodium species pLDH antigen from samples and coated antibody is react and after washing the enzyme conjugate (anti-pLDH antibody HRPO) is added after incubation and washing to remove all the unbounded enzyme conjugate. A substrate solution which acts on the bound enzyme is added to induce a colured reaction product. The intensity of this coloured product is directly proportional to the concentration of pLDH present in the samples.

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	IVD	*In vitro* diagnostic medical device
Σ	No. of tests		See Instruction for use
LOT	Lot Number Batch Number	8°C 2°C	Temperature Limitation
	Manufacturing Date		Caution See instruction for use
	Expiry Date	REF	Catalogue Number
	Do not use if package is damaged		Keep away from sunlight

5. PACK SIZE

- 96 Tests

6. COMPONENTS IN EACH MALARIA Ag MICROLISA KIT

Store all components at 2-8°C when not in use. Expiry date on the kit indicates that beyond which the kit should not be used.

Malaria Ag Microlisa Strip Plates	12 Strips (12 X 8 wells) Breakway microwells coated with anti-pLDH antibody packed in a pouch with dessicant.
Sample Diluent	1 Bottle (15 ml.) Buffer containing protein stabilizers and antimicrobial agents as preservative.
Enzyme Conjugate Concentrate (51x)	1 Vial (0.3 ml.) Anti-pLDH antibody conjugated with horseradish peroxidase with protein stabilizers and preservatives.
Conjugate Diluent	1 Bottle (15 ml.) Buffer containing protein stabilizers and preservatives.
Wash Buffer Concentrate (25x)	1 Bottle (50 ml.) PBS with surfactant. Dilute 1:25 with distilled water before use.
TMB Substrate	1 Bottle (10 ml.) To be diluted with TMB diluent before use.
TMB Diluent	1 Bottle (10 ml.) Buffer solution containing H_2O_2 with preservative
Control — ⚠	1 Vial (1.0 ml.) Ready to use, normal bovine serum albumin negative for Malaria, contains sodium azide as preservative.
Control + ⚠	1 Vial (1.0 ml.) Ready to use, pLDH antigen positive for Malaria, contains sodium azide as preservative.
Stop Solution	1 Bottle (15 ml.) Ready to use, 1N sulfuric acid.
Plate Sealers	Adhesive backed sheets for sealing microwell plate/strips.

7. ADDITIONAL MATERIAL AND INSTRUMENTS REQUIRED

- Micropipettes and microtips.
- Elisa reader
- Distilled or deionized water
- Graduated Cylinders, for reagent dilution
- Disposable gloves
- Glassware
- Timer
- Elisa washer
- Incubator 37°C
- Vortex Mixer
- Paper towels or absorbent tissue

8. SPECIMEN COLLECTION & PREPARATION

1. Collect the whole blood in a clean container (containing EDTA, citrate or heparin) by venipuncture. Fresh anticoagulated whole blood samples are preferred for testing as they perform best when tested immediately after collection. If samples are not immediately tested, they should be stored at 2-8°C for not more than 3 days, otherwise false / erroneous results may be obtained
2. Specimens should be free of microbial contamination and may be stored at 2-8°C for 24 hrs. Sample should not be frozen.

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. Sample Diluent and controls contains 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. In addition, consult the manual guideline "Safety Management No. CDC-22", Decontamination of Laboratory Sink Drains to remove Azide salts" (Centre for Disease Control, Atlanta, Georgia, April 30, 1976).
10. Follow standard biosafety guidelines for handling & disposal of potentially infective material.

11. 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. Bring all the reagents & samples to room temperature (20-30ºC) before use.
3. Do not combine reagents from different batches, as they are optimised for individual batch to give best results.
4. Avoid microbial contamination of reagents. The use of sterile disposable tips is recommended while removing aliquots from reagent bottles.
5. Due to interchange of caps the reagents may get contaminated. Care should be taken while handling the reagents to avoid contamination of any sort.
6. Use freshly collected, clean blood samples for assay.
7. Use a separate tip for each sample and then discard it as biohazardous waste.
8. All pipetting steps should be performed with utmost care and accuracy. Cross contamination between reagents and samples will invalidate results.
9. Do not allow microwells to dry once the assay has started.
10. Run negative and positive controls in each assay to evaluate validity of the kit.
11. Incubation time should not vary by more than $\pm$ 2 min.
12. Prevent evaporation during sample incubation by covering the strips with strip sealer. Remove sealer before washing.
13. Distilled or deionised water must be used for wash buffer preparation.
14. Thorough washing of the wells is critical to the performance of the assay. Overflowing of reagents or washing to adjacent wells must be prevented during washing, which may lead to incorrect results due to carry over effect.
15. Take care while preparing working substrate solution as Bottle of TMB Substrate & Diluent are of same size.
16. Prepare working substrate solution just 10 minutes prior to adding in the wells.
17. If blue colour or white particles appear in working substrate solution then do not use it. Take fresh containers and tips and prepare it again.
18. Use separate tips for TMB Substrate and TMB diluent.
19. Avoid strong light exposure during the assay.
20. 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.
21. 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.
22. In case of any doubt the run should be repeated.

12. 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 thoroughly and rinsed with distilled or deionized water. Prewarm the incubator at 37ºC.

1. Malaria Ag Microlisa Strip:

Bring plate pouch 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 three negative & two positive controls should be included in the run while opening the fresh kit. However for one or two strips, two negative and one positive control and for more strips at least three negative and two positive controls 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.

2. Preparation of 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 50 ml. (2ml. concentrated buffer with 48 ml. water) of buffer for each microlisa strip used. Mix well before use.

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

3. Preparation of Working Conjugate:

Dilute Enzyme conjugate concentrate 1:51 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 (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

Note: In case any precipitate is found in conjugate diluent, it should be allowed to settle and the supernatant can be used for the test. The precipitate does not interfere with the working of the kit.

4. 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

13. WASH PROCEDURE:

1. Incomplete washing will adversely affect the test outcome.
2. Aspirate the well contents completely into a waste container. Then fill the wells completely with wash buffer avoiding overflow of buffer from one well to another and allow to soak (approx. 30 seconds). Aspirate completely and repeat the wash and soak procedure 5 additional times for a total of 6 washes.
3. Automated washer if used should be well adjusted to fill each well completely without over filling or under filling.
4. Tap upside down on absorbent sheet till no droplets appear on the sheet, taking care not to dislodge the wells.

14. 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 Malaria Ag Microlisa strips. The sequence of the procedure must be carefully followed. From well A-1 arrange all controls in a horizontal or vertical configuration. Configuration is dependent upon reader software.

1. Add 100 μl sample diluent in each well.
2. Add 25μl Negative Control in each well no. A-1, B-1 & C-1 respectively.
3. Add 25μl Positive Control in D-1 & E-1 wells.
4. Add 25 μl blood sample in each well starting from F-1.
5. Apply cover seal.
6. Incubate at 37ºC $\pm$ 2ºC for 60 min. $\pm$ 2 min.

7. While the samples are incubating, prepare Working Wash Solution and Working Conjugate as specified in Preparation of Reagents.
8. Take out the plate form the incubator after the incubation time is over and, wash the wells 6 times with Working Wash solution according to the wash procedure given in the previous section (wash procedure).
9. Add 100 μl of Working Conjugate Solution in each well.
10. Apply cover seal.
11. Incubate at 37^{0}C $\pm$ 2^{0}C for 30 min. $\pm$ 2 min.
12. Aspirate and wash as described in step no. 8.
13. Add 100 μl of working substrate solution in each well.
14. Incubate at room temperature (20 - 30^{0}C) for 30 min. in dark.
15. Add 100 μl of stop solution.
16. Read absorbance at 450 nm at 30 minutes in ELISA READER. (Bichromatic absorbance measurement with a reference wavelength 600 - 650 nm is recommended when available).

15. SUMMARY OF PROCEDURE

Add Sample Diluent		100 μl
Add control & Blood samples		25 μl
Cover the plate & incubate		60 mins. at 37^{o}C
Wash		6 Cycles
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		30 mins. at 37^{o}C
Wash		6 Cycles
Prepare working TMB 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		100 μl
Read Results		450 nm./630 nm.

16. CALCULATION OF RESULTS

Abbreviations

NC - Absorbance of the Negative Control

NC$\bar{x}$ - Mean Negative Control

PC - Absorbance of the Positive Control

PC$\bar{x}$ - Mean Positive Control

TEST VALIDITY:

Negative Control Acceptance Criteria:

NC must be $\leq$ 0.150. If it is not so, the run is invalid and must be repeated.

Positive Control Acceptance Criteria:

1. PC must be $\geq$ 1.00

CUT OFF VALUE

Cut-off value can be determined by using the following formula:

Cut-off Value = NC$\bar{x}$ + 0.100

Where NC$\bar{x}$ is mean absorbance (O.D) of Negative Control.

e.g. 0.041 +0.100 = 0.141

17. INTERPRETATION OF RESULTS

1. Test specimens with absorbance value less than the cut off value are non-reactive and may be considered as negative for plasmodium species pLDH antigen and interpret results as malaria negative.
2. Test specimens with absorbance value greater than or equal to the cut off value are reactive for pLDH antigen by Malaria Ag Microlisa and interpret results as malaria positive.

Note: Test specimens with absorbance value within 10% below the cut off should be considered suspect for the presence of antigen, should be retested in duplicate.

3. Specimens with absorbance value equal to or greater than the cut off value are considered initially reactive by the criteria of Malaia Ag Microlisa. Original specimen should be retested in duplicate.
4. If both duplicate retest sample absorbance value is less than cut off value, the specimen is considered non reactive.
5. If any one of the duplicates retest sample absorbance value is equal to or greater than the cut off or both duplicate retest value are equal to or greater than the cut off, the specimen is considered reactive by the criteria of Malaria Ag Microlisa. Further confirmation by other EIA assays is recommended.
6. Specimens which are not repeatedly reactive, may have shown colour due to:

a) Carry over of a highly reactive sample due to contamination of pipette tips.

b) Substrate contamination

c) Inadequate wash or aspiration during wash procedure.

18. LIMITATIONS OF THE ASSAY

1. Malaria Ag Microlisa test kit is designed for testing plasmodium species pLDH antigen in human blood. Other body fluids and pooled samples are not recommended in this assay. Any result derived from the test of any other body fluid or from test of pooled serum/plasma may not be interpreted correctly based on the current criteria. **This is only a screening test.** All samples detected reactive must be confirmed by using other EIA test, microscopy or PCR. Therefore for a definitive diagnosis, the patient's clinical history, symptomatology as well as serological data should be considered. The results should be reported only after complying with the above procedure.

2. **This is only a screening test.** The results of the test are to be interpreted within the epidemiological, clinical and therapeutic context. When it seems indicated, the parasitological techniques of reference should be considered (microscopic examination of the thick smear and thin blood films).

3. The performance of Malaria Ag Microlisa has not been evaluated with P. malarie and P. ovale positive samples.

19. PERFORMANCE CHARACTERISTICS

The **Malaria Ag Microlisa** test kit has been evaluated in-house with malaria positive and negative clinical whole blood samples and compared with microscopic examination. The evaluation also included cross-reacting samples; Dengue, Rheumatoid factor, Leptospira, HIV, HCV, HBV, M. tuberculosis, Syphilis, Brucella, Scrub typhus positive samples. The results obtained are as follows:

Sample	Total no. of samples tested	Malaria Ag Microlisa		Sensitivity (%)	Specificity (%)
		Positive	Negative		
Malaria Negative	100	0	100	-	100
P. falciparum Positive	10	10	0	100	-
P. vivax Positive	30	30	0	100	-

Analytical Sensitivity: The Malaria Ag Microlisa test can detect parasitemia level of $\geq$20 parasites per μl of blood for both P. falciparum (pLDH) and P. vivax (pLDH).

Analytical Specificity: There was no significant interference in the test results of Malaria Ag Microlisa when potentially interferring samples; high bilirubin (38 mg/dl), high Hb (18 mg/dl), High triglycerides (975 mg/dl), high albumin (20 mg/dl) were added to the test specimen with much higher level than in normal blood.

Precision :

Intra-Assay: Repeatibility has been determined by testing 10 replicates of two weak (1 each of P.f & P.v) positive and two strong positive (1 each of P.f & P.v) and 2 negative samples run on the same lot. The C.V (%) for all samples is < 10%.

Inter-Assay: Reproducibility has been determined by testing 10 replicates of two weak (1 each of P.f & P.v) positive and two strong positive (1 each of P.f & P.v) and 2 negative samples in 10 different run on different day. The C.V (%) for all samples is <15%.

20. 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.

21. REFERENCES

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22. 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	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 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 solution from other manufacturer.	Use only Malaria Ag 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.	1e.
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 and 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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