PROBLEM	POSSIBLE CAUSE	SOLUTION
	f) Working substrate not protected from light	Incubate the plate in dark after addition of substrate.
	g) Improper preparation of working wash buffer (dilution error), improper mixing of reagents.	Check procedure & repeat assay
4. Poor reproducibility	a) Washing problems.	Check all 8 ports/ manifold for uniform flow of wash buffer. If there are blockage, clean the ports.
	b) Uncalibrated pipettes or tips not well fitted, improper pipetting/ dispensing.	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 calibrator, controls, samples or reagents, Inconsistency in time intervals.	Develop consistent and uniform technique.
	e) Interference in optical pathway due to Air bubbles.	Clean or dry the bottom of microwells, check for bubbles and repeat the readings.
5. False Positive	a) Beside 3a, b, c, d, e & f	Check the calculation
	incorrect interpretation and calculation of final results	part given in the insert and correctly interpret.
	b) High incubator temperature, incorrect timing or pipetting	Check incubator temperature, procedure & repeat assay.
	c) Use of turbid/ lipaemic or hemolyzed sample.	Centrifuge the sample at 5000 rpm for 30 minutes and re-run the test with clear sample.
6. False Negative/ low O.D. of Calibrator, Positive control / positive sample	a) Inadequate addition of substrate/conjugate solution.	Follow the procedure meticulously & repeat assay.
	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.
	e) Deterioration of Enzyme conjugate, TMB Substrate/ TMB Diluent.	Check storage of reagents. They shall be stored at 2-8°C.
	f) Stop solution is added before 30 minutes. Reaction terminated before 30 minutes.	Follow the test procedure meticulously.
	g) O.D. taken at incorrect wavelength.	Read O.D. values at 450 nm and 630 nm.
	h) Incorrect incubator temperature, timing or pipetting	Check incubator temperature, procedure & repeat assay.
	i) Kit deterioration	Check storage of kit and it should be stored at 2-8°C.
	j) Sample deterioration due to improper storage and / or microbial contamination.	Store the sample at 2-8°C / -20°C as recommended in the specimen collection & handling.

in vitro diagnostic Reagent, not for medicinal use

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Syphi Microlisa

Microwell ELISA Test for the Detection of Antibodies to Treponema pallidum in Human Serum/Plasma

1. SUMMARY AND EXPLANATION OF THE TEST

Syphilis is a venereal disease caused by infection with the spirochetal bacterium Treponema pallidum(TP). Multiple clinical stages and long periods of latent, asymptomatic infection are characteristic of syphilis. Primary syphilis is defined by the presence of a chancre at the site of incoculation. The antibodies response to the TP bacterium can be detected within 4 to 7 days after the chancre appears. The infection remains detectable until the patient receives adequate treatment. Clinical diagnostic issues realted to syphilis antibodies in human blood by immunoassay. Serological tests (non-treponemal specifc & treponemal specific) are currently the primary method for syphilis diagnosis and management Non-treponemal test (VDRL, RPR, etc.) are generally used for screening and treponemal test (TPHA,FTA-ABS,etc.) are used as confirmatory tests because they detect the presence of antibodies specific to Treponema pallidum .

2. INTENDED USE

Syphi Microlisa is an in-vitro qualitative enzyme immunoassay for the detection of antibodies to Treponema pallidum (TP) in human serum or plasma. It is intended for screening of blood donors and for clinical diagnostic testing.

3. PRINCIPLE

Syphi Microlisa test is an enzyme immunoassay based on **sandwich ELISA.**

Syphilis antigen are coated onto microtitre wells. Specimens and controls are added to the microtitre wells and incubated. Antibodies to syphilis if present in the specimen will binds the specific antigens absorbed onto the surface of the wells. The plate is then washed to remove unbound material. Horseradish peroxidase (HRP) conjugated syphilis antigen added to each well. This conjugate will bind syphilis antigen-antibody complex present. Finally substrate solution containing chromogen and hydrogen peroxide is added to wells and incubated. A blue colour will develop in proportion to the syphilis 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 450/630nm . If the sample does not contain syphilis antibodies then enzyme conjugate will not bind and the solution in the wells will be either colorless 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 European Standard EN ISO 15223-1:2021.

	Manufactured By	IVD	In vitro diagnostic medical device
Σ	No. of tests		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
	Keep away from sunlight		Do not use if package is damaged
BIO	Contains biological Material of Human Origin	BIO	Contains biological Material of Animal Origin
IN	Country of Manufacture		Keep Dry

5. KIT PRESENTATION

- 96 Tests

6. KIT & ITS COMPONENTS

Microwells	Microwells coated with Syphilis antigens packed in a pouch provided with desiccant.
Sample Diluent	Buffer containing protein stablizers and antimicrobial agents as preservative.
Enzyme Conjugate	Syphilis antigen labeled to horseradish peroxidase with protein stablizers. Ready to use
Wash Buffer Concentrate (25x)	PBS with surfactant. Dilute 1:25 with distilled water before use.
TMB Diluent	Buffer solution containing H_2O_2 with preservative
TMB Substrate	To be diluted with TMB diluent before use.
Control —	Ready to use, normal human serum negative for syphilis.
Control +	Ready to use, inactivated and diluted human serum; positive for syphilis antibodies and contains sodium azide as preservative.
Stop Solution	Ready to use, 1N sulfuric acid.
Plate Sealers	Adhesive backed sheets for sealing microwell plate/strips.

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 preparation
- Paper towels or absorbent tissue
- Timer
- Elisa washer
- Incubator 37°C
- Disposable gloves
- Disinfectant Solution

9. SPECIMEN COLLECTION & PREPARATION

1. 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 haemolysis. 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. Haemolyzed and Icteric hyperlipemic samples may give erroneous results.

10. SPECIMEN PROCESSING

(A) FROZEN SAMPLE

Syphi 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 5 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. 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. 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 instruction for use.

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. Mark the test specimen with patient's name or identification number. Improper identification may lead to wrong result reporting.
5. Take care while preparing working substrate solution as vials of TMB Substrate & TMB Diluent are of same size.

6. Prepare working substrate solution just 10 minutes prior to adding in the wells.
7. 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.
8. Use separate tips for TMB substrate and TMB diluent.
9. Do not allow microwells to dry once the assay has started.
10. 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.
11. 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.
12. Distilled or deionised water must be used for wash buffer preparation.
13. Bring all the reagents to room temperature (20-300C) before use.
14. Do not combine reagents from different batches, as they are optimized for individual batch to give best results.
15. Due to interchange of caps the reagents may get contaminated. Care should be taken while handling the reagents to avoid contamination of any sort.
16. Run calibrator, negative and positive controls in each assay.
17. Use freshly collected, clean serum samples for assay. Try to avoid Haemolyzed turbid, lipemic serum or plasma samples.
18. Use a separate tip for each sample and then discard it as biohazardous waste.
19. Thorough washing of the wells is critical to the performance of the assay.
20. 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.

1. Syphi Microlisa 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 one each of negative & positive control & three calibrator should be included in the run while opening the fresh kit. However for one or two strips, one each of negative, positive control & two calibrator and for more strips one each of negative and positive control & three calibrator should be included in each subsequent runs.

b. Unused wells should be stored at 2-8ºC with desiccant in an 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 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. **Working wash buffer is stable for 2 months when stored at 2-8ºC.**

3) 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 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

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.

14. 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 (300 - 350 μl) 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.
4. Tap upside down on absorbant sheet till no droplets appear on the sheet, taking care not to dislodge the wells.

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 center of the well and the tip of the pipette should not touch the wall of the microwell.

Fit the strip holder with the required number of syphilis 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 50μl sample diluent in each well.
2. Add 50μl Negative Control in each well no A-1, B1 & C1 respectively.
3. Add 50μl Positive Control in D-1 well.
4. Add 50μl samples in each well starting from E-1.
5. Apply plate sealer.
6. Incubate at 37ºC + 1ºC for 30 minutes + 2 minutes
7. While the samples are incubating, prepare working Wash Buffer as specified in preparation of reagents.
8. Take out the plate from the incubator after the incubation time is over and, wash the wells with working Wash Solution. Washing can be performed either with ELISA WASHER or manually as as specified in washing procedure (Heading: 14).
9. Add 100μl of Enzyme Conjugate in each well.
10. Apply plate sealer.
11. Incubate at 37ºC + 1ºC for 30 minutes + 2 minutes
12. Aspirate and wash as described in Wash Procedure.
13. Add 100μl of working substrate solution in each well.
14. Incubate at room temperature (20-30ºC) for 30 minutes in dark.
15. Add 100μl of stop solution.
16. Read absorbance at 450 and 630 nm(reference filter) Within 30 minutes in ELISA READER.

17. SUMMARY OF PROCEDURE

Add Sample Diluent		Sample Diluent 50μl
Add controls and samples		50μl
Cover the plate & incubate		30 mins. at 37ºC
Wash		6 Cycles
Add Enzyme Conjugate		100μl
Cover the plate & incubate		30 mins. at 37ºC
Wash		6 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		100μl
Cover the plate & incubate		30 mins. at Room Temp.
Add Stop Solution		100μl
Read Results		450 nm./630 nm.

18. CALCULATION OF RESULTS

TEST VALIDITY:

Negative Control Acceptance Criteria:

NC or NC$\bar{x}$ O.D. must be < 0.100. If it is not so, the run is invalid and must be repeated.

Positive Control Acceptance Criteria:

PC O.D. must be > 1.5. If it is not so, the run is invalid and must be repeated.

Abbreviations

NC - Absorbance of the Negative Control

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

PC - Absorbance of the Positive Control

Calculation of results:

a. Cut off value (COV) = NCx + 0.2

b. Calculation of sample O.D./ Cut off ratio = Calculate sample O.D / Cut off ratio as follows:

$$\text{Sample O.D/ cut off (S/CO) ratio} = \frac{\text{Sample O.D}}{\text{Cut off Value}}$$

e.g. : sample absorbance (O.D) = 1.868

Cut off value = 0.223

S/CO ratio = 1.868 / 0.223 = 8.37

19. INTERPRETATION OF RESULTS

1. Test specimens with sample O.D. less than COV and/or S/CO ratio value less than 1.0 are non-reactive and shall be considered as negative for syphilis antibodies.
2. Test specimens with sample O.D. more than COV and/or S/CO ratio value greater than or equal to 1.0 are reactive for syphilis antibodies by syphi Microlisa.

Note: Test specimens with S/CO ratio value between 0.9 to 1.1 should be considered suspect for the presence of syphilis antibodies and should be retested in duplicate.

3. Specimens with S/CO ratio between 0.9 to 1.1 are considered initially reactive by the criteria of syphi Microlisa. Original specimen should be retested in duplicate.
4. If both duplicate retest S/CO ratio value is less than 1.0, the specimen is considered non reactive.
5. If any one of the duplicates retest S/CO ratio value is equal to or greater than 1.0 or both duplicate retest value are equal to or greater than 1.0, the specimen is considered reactive by the criteria of Syphi Microlisa. Further confirmation by other EIA assays or confirmation assays shall be done.

20. PERFORMANCE CHARACTERISTICS

In-House Evaluation:

An elaborate study has been done on Syphi Microlisa to determine its performance.the performance of the test evaluated with fresh as well as frozen samples and compare with known negative and positive serum /plasma samples. The specificity is checked with a panel of 286 negative samples and 10 cross-reacting samples with other diseases; HIV, HCV, Dengue, Chikungunya, Leptospira positive the results obtained are as follows:

No. of Samples	Status	Syphi Microlisa		
		Positive	Equivocal	Negative
50	Syphilis Positive	50	0	0
286	Syphilis Negative	0	0	286
10	Cross-reactivity	0	0	10

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

Precision: Within-run and between-run precisions have been determined by testing 10 replicates of five specimens : a weak positive, a medium positive and a strong positive and two negative. The C.V.(%) of positive values were within 15%.

21. LIMITATIONS OF THE ASSAY

1. Syphi Microlisa assay is designed for testing syphilis antibodies in human serum and plasma. Other body fluids and pooled samples are not recommended in this assay.
2. Any result derived from the test of pooled serum/plasma may not be interpreted correctly based on the current criteria.
3. Syphi Microlisa testing alone cannot be used to diagnose syphilis even if antibodies against syphilis are present in human serum or plasma.
4. A negative test result at any time does not preclude the possibility of exposure to, or infection with syphilis.
5. **This is only a screening test.** All samples detected reactive must be confirmed by using other EIA assays/ confirmatory assays.

22. 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 for use, 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.

23. REFERENCES

1. Brif.f.vener. Dis. (1975) 51, 227
2. Experimental and therapeutic medicine 14: 4729-4736,2017
3. Doi: 10.1002/IJGO.13154 (ELISA versus VDRL and Rapid Plasma regain test (RPR) for screening of syphilis in pregnant women)

24. TROUBLE SHOOTING CHART

PROBLEM	POSSIBLE CAUSE	SOLUTION
1. No colour developed at the end of assay	a) Any one reagent has been added in wrong sequence.	Follow the procedure meticulously & repeat assay.
	b) Inactivated enzyme conjugate due to improper storage	Check storage of enzyme conjugate conc. at 2-8ºC and it should be free of any contamination.
	c) Microplate inactivated, due to improper storage	Keep unused strips in aluminium poly pouch with the dessicant pouch inside and properly closed with clamp & rod provided with the kit.
	d) Inactivated substrate, improper storage or preparation	Use freshly prepared substrate solution. Recheck procedure, repeat assay
	e) Omission of any step in test procedure	Follow the procedure meticulously & repeat assay.
	f) Incorrect (low) incubator temperature, timing or pipetting	Check incubator temperature, procedure & repeat assay
	g) Improper preparation of wash buffer, error of dilution, improper mixing of reagents.	Check procedure & repeat assay
	h) Kit deterioration	Check storage of the kit and should be store at 2-8ºC.
2. High O.D. value of Negative control	a) Plate not stopped after 20 minutes of additing stop solution	Follow the procedure meticulously & repeat assay.
	b) Same microtip used for calibrator, positive and negative controls	Change micropipette tips while addition of negative/ positive control or calibrator
	c) Nonspecific attachment/ binding of other reagent	If plates get scratches/ aberrations during washing, non specific proteins may bind while addition of next step.
3. Too much colour in all wells of the plate (high background)	a) Contaminated substrate	Check substrate (TMB Diluent) it should be colourless. If blue in colour then discard and use clean disposable container.
	b) Contaminated washing solution (1X). Poor quality of water used for diluting wash buffer conc.	Check the container and quality of water used for dilution. Use of distilled water is preferred.
	c) Over incubation of substrate and delay in addition of stop solution.	Follow the procedure meticulously.
	d) Insufficient washing. i) Washing not consistent due to blockage of probes	Check wash device and clean probes of manifold. fill the well close to the top.
	ii) Filling volume not sufficient.	After washing, blot the microwells on absorbent
	iii) Insufficient no. of wash cycles.	tissue. Follow wash protocol meticulously
	iv) Contaminated wash device	
	e) Use of wash solution from other manufacturer.	Use only Syphi Microlisa wash solution.