COVID 19 (IgM + IgG + IgA) MICROLISA

Microwell ELISA Test for the Qualitative Detection of Covid-19 (SARS-COV-2) IgM, IgG & IgA antibodies in Human Serum/Plasma

1. INTRODUCTION

Coronaviruses are important human and animal pathogens. At the end of 2019, a novel coronavirus was identified as the cause of a cluster of pneumonia cases in Wuhan, a city in the Hubei Province of China. It rapidly spread, resulting in an epidemic throughout China, followed by an increasing number of cases in other countries throughout the world. In February 2020, the World Health Organization designated the disease COVID-19, which stands for coronavirus disease 2019. The virus that causes COVID-19 is designated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); previously, it was referred to as 2019-nCoV.

SARS-CoV-2 belongs to the family of coronaviruses which are capable of causing illnesses ranging from the common cold to more severe diseases. Common signs of infection include respiratory symptoms, fever, cough, shortness of breath and breathing difficulties. In more severe cases, infection can cause pneumonia, severe acute respiratory syndrome, kidney failure and even death. Most patients recover without special treatment, however few patients like Older people and those with underlying medical problems, like high blood pressure, heart problems or diabetes, are more likely to develop serious illness become seriously ill and develop difficulty breathing.

Human-to-human transmission of the virus occur primarily via respiratory droplets from coughs and sneezes within a range of about 6 feet. Currently, the laboratory method for detecting SARS-CoV-2 infection is RT-PCR. However, this method requires sophisticated equipment and highly trained laboratory technicians. Moreover, viral load decreases rapidly 9 or 10 days after onset of symptoms. During the acute phase of infection, the titer of IgM & IgA antibodies to SARS-CoV-2 rises rapidly and IgA antibodies peaks around 22 days while IgM antibodies peaks around 14 days. The IgG antibodies to Covid 19 appear around 14-15 days and may persist for 6 months or more.

The detection of total antibodies (IgM, IgG & IgA) to SARS-Covid-2 virus provides the improved sensitivity and better tool for screening and serosurveillance of covid-19 infection. Signal to noise ratio of RT-PCR confirmed Covid positive samples is high in double antigen sandwich ELISA as compared to Covid IgM or Covid IgG differential ELISA tests kit.

2. INTENDED USE

Covid 19 (IgM + IgG + IgA) Microlisa is designed for in vitro qualitative detection of Covid IgM, IgG & IgA antibodies in human serum or plasma and is used as a screening test for testing of collected blood samples suspected for Covid-19 infection.

3. PRINCIPLE

Covid 19 (IgM + IgG + IgA) Microlisa test is an enzyme immunoassay based on "Double Antigen Sandwich ELISA".

Covid 19 antigens are coated onto microtiter wells. Specimens and controls are added to the microtiter wells and incubated.

Covid antibodies (IgM, IgG and/or IgA) if present in the specimen, will bind to the Covid-19 antigens absorbed onto the surface of the wells. The plate is then washed to remove unbound material. Horseradish peroxidase (HRP) conjugated Covid-19 antigens is added to each well. This conjugate will bind to covid antigen-antibody complex present in the microwells. 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 Covid 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 Covid 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 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	2°C 8°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
	Keep Dry		

5. KIT PRESENTATION

- 96 Test Pack

6. KIT & ITS COMPONENTS

Microwells	Microwells coated with Covid 19 antigens packed in a pouch provided with desiccant.
Sample Diluent	Buffer containing protein stabilizers and antimicrobial agents as preservative.
Enzyme Conjugate	Covid-19 antigen labelled with horseradish peroxidase with protein stabilizers. 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 Covid Antibodies
Control +	Ready to use, Non infections, Positive serum for Covid 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

Covid 19 (IgM + IgG + IgA) 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 (20-30^{0}C) before beginning the assay and can remain at room temperature during testing. Return reagents to 2-8^{0}C after use. All containers used for preparation of reagents must be cleaned throughly and rinsed with distilled or deionized water. Prewarm the incubator to 37^{0}C.

i) Covid 19 Antigens coated strip

Bring foil pack to room temperature (20-30^{0}C) before opening to prevent condensation on the microwell strips.

a. Break-off the required number of strips needed for the assay and place in the 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 2-8^{0}C, with dessicant in an aluminium pouch with clamp & rod. Microwells are stable for 30 days at 2-8^{0}C from the date of opening of sealed pouch, when stored with desicant along with clamp & rod.**

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

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. REAGENT STABILITY

Reagent	Preparation	Stability of opened/ diluted reagents (+2°C to +8°C)
1. Covid-19 antigens coated Microwells	Ready to use	30 days
2. Enzyme Conjugate	Ready to use	30 days
3. Washing Solution	Dilute 1: 25 (1+24) with distilled water	2 months.
4. TMB Substrate & TMB Diluent	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.

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 (20-30^{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.

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 Covid 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 50 μl sample diluent in each well.
2. Add 50μl Negative Control in each well no. A-1 & B-1 respectively.
3. Add 50μl Positive Control in C-1 wells.
4. Add 50 μl sample in each well starting from D-1.
5. Apply cover seal.
6. Incubate at 37^{0}C $\pm$ 2^{0}C for 30 min. $\pm$ 2 min.
7. While the samples are incubating, prepare working Wash Solution as specified in preparation of reagents.
8. Take out the plate from the incubator after the incubation time is over and, wash the wells 5 times with working Wash Solution.
9. Add 100 μl of Enzyme Conjugate Solution in each well.
10. Apply cover seal.
11. Incubate at 37^{0}C $\pm$ 1^{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 and 630 nm (reference filter) within 30 minutes in ELISA READER.

SUMMARY OF PROCEDURE

Add Sample Diuent		50 μl
Add samples & controls		50 μl
Cover the plate & incubate		30 mins. at 37^{0}C
Wash		5 Cycles
Add Enzyme Conjugate		100 μl
Cover the plate & incubate		30 mins. at 37^{0}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		100 μl
Read Results		450 nm./630 nm.

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$\bar{x}$ + 0.2

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 Covid antibodies units : Calculate by multiplying the sample O.D. ratio by 10.

Covid antibodies units = sample O.D. ratio x 10.

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

Cut off value = 0.220

Sample O.D. ratio = 1.316 / 0.220 = 5.981

Covid antibodies units = 5.981 x 10 = 59.81

18. INTERPRETATION OF RESULTS

a. If Covid antibodies unit is < 9 then interpret the sample as Negative for Covid antibodies.

b. If Covid antibodies unit is between 9 - 11 then interpret the sample as Equivocal for Covid antibodies.

c. If Covid antibodies unit is > 11 then interpret the sample as Positive for Covid antibodies.

19. PERFORMANCE CHARACTERISTICS

a) In-House Evaluation

The performance of Covid 19 (IgM + IgG + IgA) Microlisa has been evaluated in-house with the panel of 345 clinical serum/ plasma samples. The sensitivity was checked with 60 positive samples of patients with confirmed COV-SARS-2 infection. The specificity is checked with a panel of 263 negative samples and 22 cross-reacting samples with other diseases; HIV, HCV, HBV, Dengue, Chikungunya, Leptospira, Malaria, Typhi, Rheumatoid factor positive, CRP positive and ASO positive samples. The results obtained are as follows:

No. of Samples	Status	Covid 19 (IgM + IgG + IgA) Microlisa		
		Positive	Equivocal	Negative
60	Covid Positive	59	0	1
263	Covid Negative	0	0	263
22	Cross-reacting	0	0	22

Sensitivity : 96.72% **Specificity : 100%**

b) External Evaluation

The performance of 3 batches of Covid 19 (IgM + IgG + IgA) Microlisa has also been validated by ICMR, NARI, Pune. The results obtained are as follows:

Sensitivity : 94.04% **Specificity : 100%**

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

20. LIMITATION OF THE TEST

1. The test is for in vitro diagnostic use only.
2. This test detects the presence of antibodies to Covid-19 in the specimen and should not be used as the sole criteria for the diagnosis of Covid-19 infection.
3. As with all diagnostic tests, all results must be correlated with other clinical findings. If the test result is negative and clinical symptoms persist, additional follow-up testing using other clinical methods is recommended. A negative result at any time does not preclude the possibility of an early infection of Covid-19 infection.
4. This is only a screening test. Therefore, other more specific test like PCR, alternative diagnosis method must be used in order to obtain a confirmation of Covid-19 infection.

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. 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 conjugate, improper storage	Check storage of enzyme conjugate and it should be free of any contamination and used within 30 days after opening the vial.
	c) Microplate inactivated, due to improper storage	Keep unused strips in aluminium poly pouch with the dessicant pouch inside and proerly closed with clamp & rod.
	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 temperature timing or pipetting	Check procedure & repeat assay
	g) Improper preparation of reagents, error of dilution, improper mixing of reagents.	Check procedure & repeat assay
2. High O.D. value of Negative control	a) Plate not stopped after 30 minutes of additing stop solution	Follow the procedure meticulously & repeat assay.
	b) Same microtip used for Positive and negative controls	Change micropipette tips while addition of negative/ positive control
	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 glass 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	Check wash device, 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 Covid-19 (IgM+IgG +IgA) Microlisa wash solution.
	f) Working substrate not protected from light	Incubate the plate in dark after addition of substrate.
4. Poor reproducibility	a) Washing problems.	Check all 8 ports/ manifold for uniform flow of wash buffer. If there are blockage, clen 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) 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	Beside 3a, b, c, d, e & f incorrect interpretation and calculation of final results	Check the calculation part given in the insert and correctly interpret.
6. False Negative/ low O.D. of 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	Check storage of Enzyme conjugate. It shall be stored at 2-8°C and used within 30 days after opening the vial.
	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.

in vitro diagnostic Reagent, not for medicinal use

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MW/ECC/078
Rev. Date: May.-21