COVID 19 NEUTRALIZING ANTIBODY MICROLISA

Microwell ELISA Test for the Semi-Quantitative Detection of Covid-19 (SARS-COV-2) neutralizing antibodies in Human Serum/Plasma

1. INTRODUCTION

SARS-CoV-2 belongs to the family of coronavirus which are capable of causing illnesses ranging from the common cold to more severe diseases. Common sings of infection include respiratory symptoms, fever, cough, shortness of breath breathing difficulties. In more service case, infection can cause pneumonia, severe acute respiratory syndrome kidney failure and even death. Most patients recover without special treatment, however few patients like other 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 occurs 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 to SARS-CoV-2 rises rapidly and peaks around 2-3 weeks after the infection. SARS-CoV-2 specific IgG antibodies appear shortly after IgM and persist for 6 months. The development of antibodies against SARS-CoV-2 virus provide protection for future infection and remain in circulating system for months to years after infection and will bind strogly to pathogens to block cellular infiltration and replication that is why named as neutralizing antibody. The detection of neutralizing antibodies to SARS-Covid-2 virus provides the future immunity from virus.

2. INTENDED USE

Covid-19 neutralizing Antibody Microlisa is designed for in vitro semi-quantitative detection of neutralizing antibodies developed against SARS-CoV-2 in human serum/ plasma that prevent the interaction between receptor binding domain viral spike glycoprotein (RBD) and cell surface receptor angiotensin converting enzyme-2 (ACE2).

3. PRINCIPLE

Covid-19 neutralizing Antibody Microlisa test is an enzyme immunoassay based on "Blocking ELISA".

Specimen and controls are pre-incubated with HRPO conjugated recombinant SARS-CoV-2 RBD protein in tube, and added to the microtiter wells coated with recombinant hACE2 proetin, incubated and then washed to remove the unbound HRP-RBD –neutralizing Antibodies complex. Finally substrate solution containing chromogenic and hydrogen peroxide is added to the wells and incubated. A blue 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.

Antibodies against SARS-CoV2 if present in the specimen, will bind to recombinant SARS-CoV-2 RBD protein and block the protein-protein interaction between HRPO conjugated RBD and cell receptor protein hACE2 .The absorbance of the sample is inversely proportional to the titre of neutralizing antibodies against SARS-CoV-2.

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


No. of tests


Lot Number
Batch Number


Manufacturing
Date


Expiry Date


Do not use if package
is damaged


Keep Dry


In vitro diagnostic
medical device


See Instruction
for use


Temperature
Limitation


Caution
See instruction for use


Catalogue Number


Keep away from sunlight

5. KIT PRESENTATION

- 96 Test Pack

6. KIT & ITS COMPONENTS

Microwells	Microwells coated with recombinant hACE2 protein packed in a pouch provided with desiccant.
Sample Diluent	Buffer containing protein stabilizers and antimicrobial agents as preservative.
Conjugate Diluent	Buffer containing protein stabilizers and antimicrobial agents as preservative.
Enzyme Conjugate Concentrate (50X)	Recombinant SARS-CoV-2 RBD labeled with horseradish peroxidase with protein stabilizers.
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 —	Normal human serum negative for Covid Antibodies and contains thimmerosal as preservative.
Control +	Non infectious, inactivated human positive serum for Covid antibodies and contains thimmerosal 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^{0}C
- Disinfectant solution
- Disposable gloves

9. SPECIMEN COLLECTION & HANDLING

1. Human serum or plasma samples (EDTA anti-coagulated) should be used for the test. While preparing serum samples, remove the serum form the clot as soon as possible to avoid hemolysis. Fresh serum/plasma samples are preferred.
2. Specimens should be free of microbial contamination and may be stored at 2-8^{0}C for one week, or frozen at -20^{0}C or lower. Avoid repeated freezing and thawing.
3. Do not use heat inactivated samples as their use may given false results. Hemolyzed and Icteric hyperlipemic samples may give erroneous results.
4. Do not use Sodium Azide as preservative because it inactivates Horseradish peroxidase.

10. SPECIMEN PROCESSING

(A) FROZEN SAMPLE

Covid-19 neutralizing Antibody 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. 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) Recombinant hACE2 protein 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 three negative & one positive control should be included in the run while opening the fresh kit. However for one or two 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 Conjugate solution:

Dilute conjugate concentrate 1:50 in conjugate diluent. **Do not store working conjugate.** Prepare a fresh dilution for each assay in a clean glass vessel. Determine the quantity of working conjugate solution to be prepared from the table below. Mix solution thoroughly before use.

No. of Strips	1	2	3	4	5	6	7	8	9	10	11	12
No. of Wells	8	16	24	32	40	48	56	64	72	80	88	96
Enzyme Conjugate Concentrate (μl.)	20	40	60	80	100	120	140	160	180	200	220	240
Conjugate Diluent (ml)	1	2	3	4	5	6	7	8	9	10	11	12

iv) Preparation of working substrate solution :

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

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)	0.8	1.6	2.4	3.2	4.0	4.8	5.6	6.4	7.2	8.0	8.8	9.6
TMB Diluent (ml)	0.8	1.6	2.4	3.2	4.0	4.8	5.6	6.4	7.2	8.0	8.8	9.6

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. 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 stripholder with the required number of recombinant ACE2 protein 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.

15.1 Sample Preparation:

Dilute sample, negative control and positive control in a volume ratio of 1+9 with sample diluent i.e. 20μl sample/ control +180μl sample diluent in a clean glass/ plastic test tube. **Use separate tube for each sample/ control**.

15.2 Neutralization Reaction

1) Prepare working enzyme conjugate solution as specified in reagent preparation step 13 (iii).
2) Take 120 μl diluted sample/ control from step 15.1 in clean/ fresh test tube and add 60 μl working conjugate solution in each tube.
3) Mix each tubes properly and incubate at RT for 30 minutes $\pm$ 2 minutes.

15.3 ELISA Procedure (Recombinant hACE2 Protein coated strip)

1) Add 150 µl neutralized Negative Control in each well no A-1, B1and C1 respectively.
2) Add 150 µl neutralized Positive Control in D-1 well.
3) Add 150 µl neutralized samples in each well starting from E-1.
4) Apply plate sealer and incubate at 37ºC ± 1ºC for 30 minutes ± 2 minutes.
5) During the incubation of samples & conjugate mixture, prepare working Wash Solution as specified in preparation of reagents.
6) Take out the plate from the incubator after the incubation time is over and, wash the wells 5 times with working Wash Solution.
7) Add 150 µl of working substrate solution in each well.
8) Incubate at room temperature (20-30ºC) for 15 minutes in dark.
9) Add 100 µl of stop solution.
10) Read absorbance at 450 nm. and 630 nm. within 15 minutes in ELISA READER.

SUMMARY OF PROCEDURE		
Prapare Sample/ Control Dilution in Sample Diluent		20 µl Sample + 180 µl Sample Diluent
Prepare working Enzyme Conjugate		No of Strips 1 2 3 4 5 6 7 8 9 10 11 12 Enzyme Conjugate Conc. (µl) 20 40 60 80 100 120 140 160 180 200 220 240 Conjugate Diluent (ml.) 1 2 3 4 5 6 7 8 9 10 11 12
Mix diluted Sample/ control & workking Enzyme Conjugate		120 µl sample/ control + 60 µl working enzyme conjugate
Cover & incubate		30 minutes at R.T. (20-30ºC)
Add neutralize sample/ control & working Enzyme Conjugate mixture		150 µl
Cover the plate & incubate		30 minutes at 37ºC ± 1ºC
Wash		5 Cycles
Prepare Working Substrate		No of Strips 1 2 3 4 5 6 7 8 9 10 11 12 TMB Substrate (ml) 0.8 1.6 2.4 3.2 4.0 4.8 5.6 6.4 7.2 8.0 8.8 9.6 TMB Diluent (ml.) 0.8 1.6 2.4 3.2 4.0 4.8 5.6 6.4 7.2 8.0 8.8 9.6
Add Substrate		150 µl
Incubate in dark		15 minutes at Room Temp. (20 - 30ºC)
Add Stop Solution		100 µl
Read Results		450 nm./630 nm.

TEST VALIDITY:

Ensure the following is within specified acceptance criteria

i) NC or NCx O.D. must be > 1.0. If it is not so, the run is invalid and must be repeated.

ii) PC O.D. must be < 0.3. If it is not so, the run is invalid and must be repeated.

16. CALCULATION OF RESULTS

The negative control value can be used for calculation of inhibition rate of each sample.

a) $\%\text{Inhibition} = \left(1 - \frac{\text{Sample O.D.}}{\text{Negative Control O.D.}}\right) \times 100\%$

b) Calculation of % inhibition rate:

e.g. Sample absorbance (O.D.) = 1.502

Negative control $\overline{NCx} = 2.520$

$$\%\text{Inhibition} = \left(1 - \frac{1.502}{2.520}\right) \times 100$$

Inhibition rate = 40.4%

17. INTERPRETATION OF RESULTS

Sample	%inhibition	Result	Interpretation
Covid 19 neutralizing Antibody	≥30%	Positive	SARS-CoV-2 Neutralizing Antibody present
	<30%	Negative	SARS-CoV-2 neutralizing Antibody absent

18. PERFORMANCE CHARACTERISTICS

a) In-House Evaluation

The performance of Covid-19 neutralizing Antibody Microlisa has been evaluated in-house with the panel of 5 clinical serum samples. The Inhibition rate was checked with 5 positive serum samples of patients with confirmed covid 19 and 2 healty control sera (EL-10 & EL-11) in comparison with gen script SARS-CoV-2 SURROGATE VIRUS NEUTRALIZATION test kit. The comparison result as follows:

Covid-19 serum sample	Dilution of sample	Gen Script % Inhibition Rate	Interpretation	J.mitra% Inhibition Rate	Interpretation
RD/NE/1	Neat	82	Positive	86	Positive
	1:5	55	Positive	60	Positive
	1:10	32	Positive	39	Positive
	1:20	15	Negative	18	Negative
RD/NE/2	Neat	81	Positive	86	Positive
	1:5	57	Positive	60	Positive
	1:10	40	Positive	43	Positive
	1:20	22	Negative	25	Negative
RD/NE/3	Neat	54	Positive	59	Positive
	1:5	38	Positive	40	Positive
	1:10	25	Negative	27	Negative
	1:20	12	Negative	16	Negative
RD/NE/4	Neat	81	Positive	77	Positive
	1:5	52	Positive	48	Positive
	1:10	37	Positive	33	Positive
	1:20	22	Negative	18	Negative
RD/NE/5	Neat	75	Positive	75	Positive
	1:5	47	Positive	46	Positive
	1:10	32	Positive	31	Positive
	1:20	15	Negative	12	Negative
EL-10	Neat	15	Negative	16	Negative
EL-11	Neat	18	Negative	17	Negative

b) External Evaluation

The performance of Covid-19 neutralizing Antibody Microlisa has been evaluated by ICMR approved Lab /Institute for COVID-19 with the Positive (Vaccinated persons) and negative samples (not vaccinated). The Inhibition rate was checked for all negative and positive samples. The result obtained of 3 different lots are as follows:

Lot Details	No. of Positive samples	No. of Negative samples	Sensitivity	Specificity
LOT 1	66	16	96.99%	100%
LOT 2	47	9	95.7%	100%
LOT 3	60	8	95.0 %	100%

Precision : Within-run and between-run precisions have been determined by testing 5 replicates of five specimens : two negative and three (high, medium & weak) covid antibody positive. The C.V.(%) of negative and weak positive values were within 15%.

19. LIMITATION OF THE TEST

1. The test is for in vitro diagnostic use only.
2. The test should be used for detection of neutralizing antibodies to Covid-19 in human serum/ plasma only.
3. Follow the test procedure and instructions given in instruction manual strictly to obtain accurate result.
4. As with all diagnostic test, all results must be correlated with other clinical findings.The test results can be negative if the titre of the neutralizing Antibodies is below the detection level of the kit.
5. A negative result does not preclude the possibility of less sensitivity.If the sympotms persists. It is recommended to collect and test sample after few days.

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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3. NCCLS. 1997. National Committee for clinical laboratory Standard, Internal quality.

22. 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 samples, 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 15 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 reagents.
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 Neutralizing antibodies 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, 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) Interference in optical pathway due to Air bubbles.	Clean or dry the bottom of microwells, check for bubbles and repeat the readings.
5. 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 Conc.	Check storage of Enzyme Conjugate Conc. It shall be stored at 2-8°C.
	f) Stop Solution is added before 15 minutes. Reaction terminated before 15 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

VER-01 R-00

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