COVID-19 ImmunoRank™ Neutralization MICRO-ELISA

Test for the determination of SARS-CoV-2 neutralizing antibodies in human plasma or serum

REF	Antibodies Detected	Ig Class	Platform	Format
S2500	SARS-CoV-2 Neutralizing Antibodies	All	SARS-CoV-2 Receptor Coated ELISA Strips	96 Wells

IVD

For Prescription Use Only
For In Vitro Diagnostic (IVD) Use Rx Only

NAME

COVID-19 ImmunoRank™ Neutralization MICRO-ELISA

INTENDED USE

The COVID-19 ImmunoRank™ Neutralization MICRO-ELISA, is a test for the qualitative detection of SARS-CoV-2 neutralizing antibodies in sodium citrate plasma or serum. This assay is intended for detection of circulating SARS-CoV-2 neutralizing antibodies of all Ig classes and to identify individuals who have mounted an adaptive immune response to SARS-CoV-2 indicating recent or prior infection. At this time, it is unknown for how long antibodies persist following infection and if the presence of neutralizing antibodies confers protective immunity. The COVID-19 ImmunoRank™ Neutralization MICRO-ELISA test should not be used to diagnose acute SARS-CoV-2 infection. Use of this test is limited to laboratories certified under the Clinical Laboratory Improvement Amendments (CLIA) of 1988, 42 U.S.C. §263a, that meet the requirements to perform high complexity tests. The sensitivity of the COVID-19 Neutralization™ MICRO-ELISA assay early after infection is unknown. Negative results do not preclude acute SARS-CoV-2 infection. If acute infection is suspected, direct testing for SARS-CoV-2 is necessary for confirmation. False positive results from the COVID-19 Neutralization™ MICRO-ELISA assay may occur due to cross-reactivity from pre-existing antibodies or other possible causes. **The COVID-19 ImmunoRank™ Neutralization MICRO-ELISA assay has completed Section IV.D notification process under FDA's "Policy for Coronavirus Disease – 2019 Test During the Public Health Emergency (Revised)" and has not been reviewed by FDA.**

SUMMARY AND EXPLANATION OF TEST

According to the U.S. Department of Health and Human Services/Centers for Disease Control and Prevention (CDC), Chinese authorities identified an outbreak caused by a novel—or new—coronavirus termed SARS-CoV-2. The virus can cause mild to severe respiratory illness; known as Coronavirus Disease 2019 (COVID-19) formerly called 2019-nCoV.[1] The outbreak began in Wuhan, Hubei Province, China and has spread to a growing number of countries worldwide including the United States. On March 11, 2020, the World Health Organization declared COVID-19 a pandemic. SARS-CoV-2 is different from six other, previously identified human coronaviruses, including those that have caused previous outbreaks of Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS). Additional information on coronaviruses is available on the U.S. Centers for Disease Control and Prevention coronavirus website.

The COVID-19 ImmunoRank™ Neutralization MICRO-ELISA, is a qualitative test for detection of circulating SARS-CoV-2 neutralizing antibodies. The assay is designed to detect antibodies of all Ig classes in human plasma or serum that bind to the SARS-CoV-2 receptor binding domain (RBD) and are capable of blocking binding of the RBD to angiotensin-converting enzyme 2 (ACE2), the viral entry receptor on the surface of target cells. The SARS-CoV-2 Spike (S) protein consists of the S1 and S2 domains.[2] The SARS-CoV-2 S1 domain contains the receptor-binding domain (RBD) that can specifically bind to angiotensin-converting enzyme 2 (ACE2) receptors on target cells.[2] SARS-CoV-2 is closely related to the SARS virus, which was first identified in 2002-2003.[3] In-depth analysis has identified the SARS-CoV-2 RBD as being essential for ACE2 binding.[3] Both SARS-CoV and SARS-CoV-2 utilize the ACE2 cell surface receptor to gain entry into cells, with SARS-CoV-2 binding with higher affinity.[4] Vaccine and therapeutic development are targeting portions of the spike protein, including the RBD portion.[5]

BIOLOGICAL PRINCIPLES OF THE TEST

The COVID-19 ImmunoRank™ Neutralization MICRO-ELISA, is a qualitative test designed for detection of circulating SARS-CoV-2 neutralizing antibodies of all immunoglobulin classes in serum or sodium citrate plasma capable of binding to the RBD and neutralizing or blocking the interaction between the RBD and the ACE2 receptor. The test is a solid phase enzyme-linked immunosorbent assay (ELISA) using a chromogenic enzyme substrate as an indicator. The recombinant SARS CoV-2 viral entry receptor protein, human angiotensin-converting enzyme 2 (ACE2), is immobilized to polystyrene wells of the test microplate (solid phase). In a separate mixing and incubation microplate, the soluble recombinant SARS-CoV-2 receptor binding domain (RBD) protein conjugated to horseradish peroxidase (HRP) is added to each well. Then the controls and diluted test specimens are also added to the wells of the incubation plate as shown in figure 1 below. During the plate shaking and incubation period, antibodies with binding specificity to the RBD region of the SARS-CoV-2 spike protein, if present in test specimens and controls, will bind to the RBD-horseradish peroxidase conjugate. After the incubation period, the controls and test specimens are transferred from the wells of the incubation plate to the wells of the test plate containing immobilized recombinant human ACE2 receptor and allowed to incubate. After the incubation period, the wells are washed to remove unbound sample matrix and an enzyme substrate-chromogen (hydrogen peroxide, H_2O_2, and tetramethylbenzidine, TMB) is added to each well and incubated, resulting in the development of a blue color. The intensity of the blue color is indirectly proportional to the concentration of the SARS-CoV-2 neutralizing antibodies in the test specimens. An assay stop solution is added at the 15 minute mark after addition of enzyme substrate-chromogen and the color intensity is read in an absorbance plate reader at wavelength 450 nm. Positive and negative quality controls are provided to ensure the integrity of the test. An algorithm is provided to calculate the sample neutralization index expressed as percent neutralization (SNI%) for each unknown test specimen.

REAGENTS AND MATERIALS SUPPLIED

⚠ **CAUTION:** Do not use any reagents where damage to the packaging has occurred.

COMPONENTS IN EACH COVID-19 NEUTRALIZATION MICRO-ELISA TEST KIT FOR DETECTION OF CIRCULATING SARS-CoV-2 NEUTRLIZING ANTIBODIES

Volumes listed in the table below indicate the volume per bottle in the kit.

REF	Reagent/Component	Amount	Storage	Symbol
S2500-1	1 round bottom Incubation Plate 96 well polystyrene plate. Plate is provided in a clear sealed pouch.	1 X 96 Wells	Not a reagent but supplied with the test kit.	INCUBATION PLATE

REF	Reagent/Component	Amount	Storage	Symbol
S2500-2	1 Microplate 96-well polystyrene strips coated with recombinant human angiotensin-converting enzyme 2 (ACE2). Wells are provided in a sealed foil pack with silica gel desiccant.	12 X 8	2° - 8°C until expiration date.	MICROPLATE
S2500-3	1 bottle Positive Control (1X) Buffered protein solution containing a human recombinant SARS-CoV-2 anti RBD neutralizing antibody (human IgG_1) and ProClin-300 as a preservative. The Positive Control is used to monitor the integrity of the test and to determine the Sample Neutralization Index (SNI%) of the test specimen.	300 (µl)	2° - 8°C until expiration date.	CONTROL +
S2500-4	1 bottle Negative Control (1X) Buffered protein solution containing ProClin-300 as a preservative. The Negative Control is used to monitor the integrity of the test.	300 (µl)	2° - 8°C until expiration date.	CONTROL -
S2500-5	1 bottle Calibrator Control (1X) Buffered protein solution containing a human recombinant SARS-CoV-2 anti RBD neutralizing antibody (human IgG_1) and ProClin-300 as a preservative. The Calibrator Control is used to monitor the integrity of the test.	300 (µl)	2° - 8°C until expiration date.	CALIBRATOR CONTROL
S2500-6	1 bottle SAMPLE DILUENT (1X) Buffered protein solution containing ProClin-300 as a preservative.	15 (ml)	2° - 8°C until expiration date.	SAMPLE DILUENT
S2500-7	1 bottle RBD-ENZYME CONJUGATE (100X) Buffered protein solution containing recombinant SARS-CoV-2 receptor binding domain (RBD) conjugated to horseradish peroxidase containing ProClin-300 as a preservative.	80 (µl)	2° - 8°C until expiration date.	RBD-ENZYME CONJUGATE

REF	Reagent/Component	Amount	Storage	Symbol
S2500-8	1 bottle Conjugate Diluent (1X) Buffered protein solution containing ProClin-300 as a preservative. This diluent is used to dilute the 100 X RBD Enzyme Conjugate. The 100 X RBD Enzyme Conjugate should only be diluted into the Conjugate Diluent immediately prior to running the assay. Unused diluted conjugate should be discarded.	10 (ml)	2° - 8°C until expiration date.	CONJUGATE DILUENT
S2500-9	1 bottle WASH BUFFER (20X) Phosphate buffered saline 20X concentrate solution containing a surfactant and ProClin-300 as a preservative.	25 (ml)	2° - 8°C until expiration date.	WASH BUFFER
S2500-10	1 bottle SUBSTRATE-CHROMOGEN Buffered hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB) solution.	12 (ml)	2° - 8°C until expiration date. Protect from light.	SUBSTRATE CHROMOGEN
S2500-11	1 bottle STOP SOLUTION 1.0 N sulfuric acid (H_2SO_4).	10 (ml)	2° - 8°C until expiration date.	STOP SOLUTION
S2500-12	2 Adhesive Plate Sealers	2 X 1 Ea.	Non Reagent but supplied with the test kit.	PLATE SEALER

CONTROLS AND CALIBRATOR

The positive control and calibrator control contain a recombinant human monoclonal antibody (human IgG_1). The vH and vL variable chains were sequenced from plasma B-cells of COVID-19 survivors.

ADDITIONAL MATERIALS REQUIRED

- Boxes of Pipette Tips - 0.1 ml, 0.2 ml and 1.0 ml
- Calibrated pipettes capable of accurately delivering volumes from 4 µl – 1.0 ml
- Disposable tubes for making dilutions (micro-centrifuge tubes or borosilicate glass test tubes).
- Precision micro-titer absorbance plate reader wavelength of 450 nm.
- Distilled or deionized water
- Lab timer
- 37°C Incubator without CO_2 supply

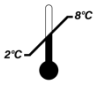
WARNINGS AND PRECAUTIONS

- For *In Vitro* diagnostic use only. For prescription use only.
- The COVID-19 ImmunoRank™ Neutralization MICRO-ELISA assay has completed Section IV.D notification process under FDA's "Policy for Coronavirus Disease – 2019 Test During the Public Health Emergency (Revised)" and has not been reviewed by FDA.
- A thorough understanding of this package insert is necessary for the successful use of the product. Reliable results will only be obtained by using precise laboratory techniques and following the package insert.
- This test has been authorized only for the detection of SARS-CoV-2 neutralizing antibodies, not for any other viruses or pathogens.
- Laboratories within the United States and its territories are required to report all positive results to the appropriate public health authorities.
- Do not use kit components past the expiration date printed on the outside of the kit box.
- All patient specimens should be considered potentially infectious and handled in accordance with good laboratory procedure.
- Wear suitable protective attire, gloves, eye/face protection when handling any of the components of this kit.
- Sample collection and handling procedures require specific training and guidance and should only be performed by properly trained healthcare professionals.
- Dispose of containers and unused contents in accordance with Federal, State, and Local regulatory requirements.
- Follow package insert instructions precisely to obtain accurate test results.
- Some Reagents contain thimerosal; avoid contact with skin.
- Avoid contact with SUBSTRATE-CHROMOGEN (tetramethylbenzidine) solution. It is harmful if inhaled or absorbed through skin (may cause irritation).
- Do not interchange reagents between test kits with different lot numbers.
- Specimen and reagents should be allowed to come to room temperature and mixed thoroughly by gentle inversion or swirling before assay is run.
- Azide inhibits this enzyme reaction. Avoid the use of samples or commercial controls that contain sodium azide.

SAFETY PRECAUSTIONS

CAUTION: This product requires the handling of human specimens. It is recommended that all human-sourced materials and all consumables contaminated with potentially infectious materials be considered potentially infectious and handled in accordance with the OSHA Standard on Blood borne Pathogens. Bio-safety Level 2 or other appropriate regional, national, and institutional biosafety practices should be used for materials that contain, are suspected of containing, or are contaminated with infectious agents.[10-13]

KIT STORAGE AND STABILITY



UNOPENED: This test kit must be stored at 2 – 8°C upon receipt. For the expiration date of the kit refer to the label on the kit box. All components are stable until this expiration date.

OPENED: Once opened, the kit reagents are stable when stored at 2 – 8°C. Refer to label on the kit box for expiration date.

INDICATION OF INSTABILITY OR DETERIORATION: Deterioration of kit reagents may be indicated when a quality control value is out of the specified range. Associated test results are invalid, and samples must be retested with a new kit where the control values are within the specified ranges.

SPECIMEN COLLECTION AND PREPARATION

SPECIMEN TYPES The following specimen types listed below may be used with this assay.

Specimen Types	Collection Tubes
Serum	Serum
Plasma (Sodium Citrate)	Sodium Citrate

- Each Laboratory must follow their own internal procedures to establish the use of additional collection tubes.
- This test has not been validated for performance characteristics using cadaveric specimens or the use of bodily fluids other than human serum or plasma.
- Collection tubes containing anticoagulants may have a dilution effect resulting in a lower Cutoff Ratio.
- Heat inactivation at 56° C for thirty (30) minutes has been shown to have no effect on test results.

SPECIMEN CONDITIONS

- Do not use:
  - Pooled specimens
  - Grossly hemolyzed specimens
  - Specimens with obvious microbial contamination
  - Specimens with fungal growth

Blood obtained by venipuncture should be allowed to clot at room temperature (18 - 30°C) for 30 to 60 minutes and then centrifuged according to the Clinical and Laboratory Standards Institute (CLSI Approved Guideline – Procedures for the Handling and Processing of Blood Specimens for Common Laboratory Tests; GP44). Testing of serum or plasma should be performed as soon as possible after collection and processing. Do not leave specimens at room temperature for prolonged periods. Separated serum should remain at 18 - 30°C for no longer than 8 hours. If assays are not completed within 8 hours, serum should be refrigerated at 2 - 8°C. If assays are not completed within 7 days, or the serum or plasma is to be stored beyond 7 days, the test specimens should be frozen at or below -20°C. Avoid repeated freezing and thawing of samples more than four times as this can cause antibody deterioration. Frost-free freezers are not suitable for sample storage. Frozen samples should be thawed to room temperature and mixed thoroughly by gentle swirling or inversion prior to use. If specimens are to be shipped, they should be packed in compliance with Federal Regulations covering transportation of infectious agents.

SPECIMEN STORAGE

Specimen Types	Storage Temperature	Storage Time
Serum or Plasma	18 - 30°C	8 hours
Serum or Plasma	2 - 8°C	7 Days
Serum or Plasma	≤ - 20°C	> 7 Days

SPECIMEN SHIPPING If specimens are to be shipped, they should be packed in compliance with Local, State and Federal Regulations covering transportation of clinical specimens and infectious agents.

SPECIMEN CONDITIONS PRIOR TO ANALYSIS

- Follow blood tube manufacturer's processing instructions for test specimens. Do not use gravity separation.
- Recentrifuge specimens if fibrin, red blood cells, or other particulate matter are observed.

PREPARATION OF FROZEN SPECIMENS PRIOR TO ANALYSIS

- Allow frozen samples to come to room temperature 18 - 30°C before processing.
- Thoroughly mix thawed specimens by gentle vortexing on low speed or inversion 10 times.
- Recentrifuge thawed and mixed specimens.

PREPARATION OF TEST SPECIMEN

Prepare the specimen by diluting 1:10 in Sample Diluent. Example: Dilute 10 µl of serum or plasma into 90 µl of Sample Diluent and vortex at low speed or invert tube 10 times.

TEST PROCEDURE

REAGENT PREPARATION

- Upon removal of reagents from refrigerator, allow all reagents to stand for 30 minutes to reach room temperature 18 – 30°C before use. Vortex reagents on low speed or invert 10 times to mix.
- **Microplate** Allow the 96-well polystyrene microplate strips coated with recombinant human angiotensin-converting enzyme 2 (ACE2) and sealed in a foil pack with silica gel desiccant to come to room temperature 18 – 30°C before opening. Once the microplate strips have been opened, if not completely used, reseal with desiccant in the foil pouch and store at 2 - 8°C in a dry place.
- 1 bottle 300 µl **Calibrator Control** (1X) Preparation: Mix by vortexing at low speed or invert the tube 10 times. RTU
- 1 bottle 300 µl **Positive Control** (1X) Preparation: Mix by vortexing at low speed or invert the tube 10 times. RTU
- 1 bottle 300 µl **Negative Control** (1X) Preparation: Mix by vortexing at low speed or invert the tube 10 times. RTU
- 1 bottle 15 ml **SAMPLE DILUENT** (1X) Preparation: Mix by vortexing at low speed or invert the tube 10 times. RTU
- 1 bottle 80 µl **RBD-ENZYME CONJUGATE** (100X) Preparation: Just prior to running a full 96 well incubation plate, dilute 70 µl of RBD-Enzyme Antibody Conjugate into 7 ml of Conjugate Diluent (1X).
- 1 bottle 10.0 ml **Conjugate Diluent** (1X) Preparation: Ready to use. RTU
- 1 bottle 25 ml **WASH BUFFER CONCENTRATE** (20X) Preparation: If crystals appear during storage at 2 - 8°C, warm the concentrate by placing at 37°C with occasional mixing until clear. Dilute full bottle of Wash Buffer Concentrate (20X) with 475 ml of deionized water to a final volume of 500 ml and mix well before use.
- 1 bottle 12 ml **SUBSTRATE-CHROMOGEN** Preparation: Ready to use. RTU
- 1 bottle 10 ml **STOP SOLUTION** 1.0 N sulfuric acid (H_2SO_4.) Preparation: Ready to use. RTU

⚠ **CAUTION:** Strong Acid. Wear protective gloves, mask and safety glasses. Dispose of all materials according to all applicable safety rules and regulations.

ASSAY PROCEDURE

⚠ **CAUTION:** The test procedure must be followed as written. Any deviations from this procedure may produce erroneous results.

1. Allow all kit reagents to stand for 30 minutes to reach room temperature 18 – 30°C and gently mix each vial by vortexing on low speed or inverting 10 times.

2. The Positive Control, Negative Control and the Calibrator Control must be assayed in duplicate on the 96 well Microplate each time the test is performed. Up to ninety (90) test specimens may be run in singlicate on each full plate.

3. Add **60 µl** of the diluted RBD-ENZYME CONJUGATE into each well of the Incubation Plate corresponding to a control or test to be run. (See Figure 1 below.)

4. Pipette **60 µl** of the 1X **Positive Control, Negative Control** and **Calibrator Control** into the individual microwells of the Incubation Plate in duplicate as shown in Figure 1 below.

5. Pipette **60 µl** of the diluted Test Specimens into the corresponding individual microwells of the Incubation Plate in singlicate as shown in Figure 1 below.

6. Cover the Incubation Plate with an adhesive plate sealer and incubate on a microplate shaker set at 300 rpm for **30 +/- 1 minute** at room temperature **18 – 30°C**.

7. Place sufficient microplate strip wells containing immobilized recombinant human ACE2 in a strip holder to run all assay controls in duplicate and test specimens in singlicate. (Figure 2 below represents a suggested Test Plate layout for the Positive Control, Negative Control, Calibrator Control and each individual Test Specimen.)

8. After the 30 minute incubation period, add **100 µl** of the Positive Control, Negative Control and Calibrator Control from the Incubation Plate in duplicate to the test plate as shown in Figure 2 below.

9. After the 30 minute incubation period, add **100 µl** of the unknown test samples from the Incubation Plate in singlicate to the Test Plate as shown in Figure 2 below.

10. Cover each Test Plate with an adhesive plate sealer and incubate without shaking for **30 minutes +/- 1 minute** at **+37 °C ± 1 °C** in an incubator without carbon dioxide. For manual processing of microplate wells, cover the finished test plate with an adhesive protective plate sealer and start incubation. When using automated microplate processors, for incubation, follow the recommendations of the instrument manufacturer.

⚠ **Caution:** Do not stack plates on top of each other. They should be spread out as a single layer for even temperature distribution.

11. Plate Washing: Remove the protective adhesive strip and aspirate each well and wash, repeating the process for a total of **four washes**. Wash by filling each well with **300 µl** of 1X **Wash Buffer** using a manual squirt bottle, manifold dispenser, or autowasher leaving the 1X Wash Buffer in each well for **30 – 60 seconds.** Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper toweling.

12. Add **100 µl** of **Substrate Chromogen** to each well. Incubate for **15 +/- 1 minutes** at room temperature (18 – 30°C) protected from direct light.

13. Immediately upon concluding the **15 +/- 1** minute incubation, add **50 µL of Stop Solution** to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

14. Immediately after adding stop solution, at the **15 +/- 1 minute mark**, read the absorbance from the color intensity of each well at 450 nm. Prior to measuring, carefully shake the microplate to ensure a homogeneous distribution of the solution in the wells.

⚠ **Caution:** The plate should be read at **15 minute +/- 1** minute. If not read within this time period, results may not be accurate. The test must be repeated.

Figure 1: COVID-19 ImmunoRank™ MICRO-ELISA Round Bottom Incubation Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	S3	S11	S19	S27	S35	S43	S51	S59	S67	S75	S83
B	PC	S4	S12	S20	S28	S36	S44	S52	S60	S68	S76	S84
C	NC	S5	S13	S21	S29	S37	S45	S53	S61	S69	S77	S85
D	NC	S6	S14	S22	S30	S38	S46	S54	S62	S70	S78	S86
E	CC	S7	S15	S23	S31	S39	S47	S55	S63	S71	S79	S87
F	CC	S8	S16	S24	S32	S40	S48	S56	S64	S72	S80	S88
G	S1	S9	S17	S25	S33	S41	S49	S57	S65	S73	S81	S89
H	S2	S10	S18	S26	S34	S42	S50	S58	S66	S74	S82	S90

PC = Positive Control CC = Calibrator Control
NC = Negative Control S = Sample

Figure 2: COVID-19 ImmunoRank™ MICRO-ELISA Test Plate For Detection of Neutralizing Antibodies

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	S3	S11	S19	S27	S35	S43	S51	S59	S67	S75	S83
B	PC	S4	S12	S20	S28	S36	S44	S52	S60	S68	S76	S84
C	NC	S5	S13	S21	S29	S37	S45	S53	S61	S69	S77	S85
D	NC	S6	S14	S22	S30	S38	S46	S54	S62	S70	S78	S86
E	CC	S7	S15	S23	S31	S39	S47	S55	S63	S71	S79	S87
F	CC	S8	S16	S24	S32	S40	S48	S56	S64	S72	S80	S88
G	S1	S9	S17	S25	S33	S41	S49	S57	S65	S73	S81	S89
H	S2	S10	S18	S26	S34	S42	S50	S58	S66	S74	S82	S90

PC = Positive Control CC = Calibrator Control
NC = Negative Control S = Sample

QUALITY CONTROL

Each kit contains one Positive Control, one Negative Control and a Calibrator Control. The controls and calibrator control are intended to monitor the integrity of the test and detect substantial reagent failure. The test is invalid and must be repeated if the controls or calibrator samples do not meet the specifications listed in this procedure. If the test is invalid, the results cannot be used. Quality Control (QC) requirements must be performed in conformance with local, state, and/or federal regulations or accreditation requirements and your laboratory's standard Quality Control procedures. It is recommended that the user refer to CLSI C24 and 42 CFR 493.1256 for guidance on appropriate QC practices. The results below are given strictly for guidance purposes only and applicable for spectrophotometric readings only. (See Examples Below.)

Example 1: Negative Control COVID-19 ImmunoRank™ Neutralization MICRO-ELISA Test for Detection of Neutralizing Antibodies

Negative Control	$OD_{450\ nm}$
Replicate 1	2.8
Replicate 2	2.9
Negative Control Sum	5.7
Average Negative Control (Sum/2)	2.85

Example 2: Positive Control COVID-19 ImmunoRank™ Neutralization MICRO-ELISA Test for Detection of Neutralizing Antibodies

The Positive Control percent neutralization was determined for each replicate using the following equation.
% Neutralization = 1 – (Positive Control $OD_{450\ nm}$/Negative Control $OD_{450\ nm}$) X 100

Positive Control	%Neutralization
Replicate 1	95%
Replicate 2	93%
Positive Control Sum	188%
Average Positive Control (Sum/2)	94%

Example 3: Calibrator Control COVID-19 ImmunoRank™ Neutralization MICRO-ELISA Test for Detection of Neutralizing Antibodies

The Calibrator Control percent neutralization was determined for each replicate using the following equation.
% Neutralization = 1 – (Calibrator Control $OD_{450\ nm}$/Negative Control $OD_{450\ nm}$) X 100

Calibrator Control	% Neutralization
Replicate 1	49%
Replicate 2	47%
Calibrator Control Sum	96%
Average Calibrator Control (Sum/2)	48%

Quality Control Requirements for COVID-19 ImmunoRank™ Neutralization MICRO-ELISA
Test for Detection of Neutralizing Antibodies

Assay Control	Requirement $OD_{450\ nm}$
Negative Control	OD ≥ 2.0
Assay Control	**Requirement % Neutralization**
Positive Control	90% ≤ % Neutralization ≤ 100%
Calibrator Control	30% ≤ % Neutralization ≤ 65%

Quality Control: The results on the table above must be obtained for the assay to be considered valid. Non-fulfillment of these Quality Control criteria are an indication of deterioration of reagents or an error in the test procedure and the assay must be repeated.

INTERPRETATION OF RESULTS

The assay's Positive Control value and Calibration Control value are generated using a recombinant human monoclonal antibody (human IgG_1) sequenced from a plasma B-cell originating from a COVID-19 survivor and shown to be capable of neutralizing In vivo and In vitro, the SARS-CoV-2 virus. This recombinant monoclonal antibody blocks the binding of the recombinant SARS-CoV-2 receptor binding domain (RBD) protein to recombinant human angiotensin-converting enzyme 2 (ACE2), the viral entry receptor on the surface of target cells.

The COVID-19 ImmunoRank™ Neutralization MICRO-ELISA, is a test to qualitatively determine the presence of SARS-CoV-2 neutralizing antibodies in unknown test samples. The Sample Neutralization Index (SNI) expressed as percent neutralization (SNI%) for each test sample is determined using the following algorithm. Because this test procedure uses an algorithm that compares unknown test samples to a known high neutralizing positive control with a SNI% of less than 100%, SNI% values for test samples may be calculated as a negative percent or a positive percent. An unknown test sample with a SNI% value < 20% including Negative SNI% values indicate a negative test result. The COVID-19 ImmunoRank™ Neutralization MICRO-ELISA negative cutoff of SNI% < 20% was determined by measuring the mean SNI% of five hundred and thirty one (531) negative plasma samples collected prior to December 1, 2019 (Pre-COVID-19 outbreak) plus 4 times the standard deviation.

SNI% = [1 – (Sample $OD_{450\ nm}$ / Negative Control $OD_{450\ nm}$)]/[1 – (Positive Control $OD_{450\ nm}$ / Negative Control $OD_{450\ nm}$)] X100

SNI%	Test Result	Interpretation
20% ≤ SNI%	Positive	Indicates detection of SARS-CoV-2 neutralizing antibodies in the test sample. No further testing required.
20% > SNI%	Negative	Indicates the test sample has a non-detectable level of SARS-CoV-2 neutralizing antibodies. No further testing required.

LIMITATIONS OF TEST PROCEDURE

- IVD For In vitro Diagnostic Use.
- Positive results may be due to past or present infection with non-SARS-CoV-2 coronavirus strains such as coronavirus HKU1, NL63, OC43 or 229E.
- Not for the screening of donated blood.
- Results from this antibody test should not be used as the sole basis to diagnose or exclude SARS-CoV-2 infection or to inform infection status.

- This test is for clinical laboratory use only. It is not for home use.
- Specimens from patients who have received preparations of mouse monoclonal antibodies for diagnosis or therapy may contain human anti-mouse antibodies (HAMA). Such specimens may show either falsely elevated or depressed values when tested with assay kits such as SARS-CoV-2 IgG that employ mouse monoclonal antibodies.[7, 8]
- Heterophilic antibodies in human serum can react with reagent immunoglobulins, interfering with *in vitro* immunoassays. Patients routinely exposed to animals or to animal serum products can be prone to this interference, and anomalous values may be observed.[9]
- Rheumatoid factor (RF) in human serum can react with reagent immunoglobulins, interfering with *in vitro* immunoassays.[9]

PERFORMANCE CHARACTERISTICS

Specimens

Both natural and contrived specimens were used to validate the COVID-19 ImmunoRank™ Neutralization MICRO-ELISA Test. When contrived samples were used, they were made by spiking negative human plasma collected prior to December 1, 2019 with a cocktail of human recombinant monoclonal antibodies (human IgG_1), all of which were sequenced from plasma B-cells isolated from COVID-19 survivors and screened for specificity to the recombinant SARS-CoV-2 receptor binding domain (RBD) protein.

PRECISION

Laboratory Precision

Studies on laboratory precision and assay repeatability were carried out according to CLSI guideline EP05-A3.[6] Samples (reactivity distributed over the entire measurement range) were measured. The precision is given as standard deviation (SD) and coefficient of variation (CV). Testing was performed with one lot of a COVID-19 ImmunoRank™ Neutralization MICRO-ELISA kit containing Positive Control, Negative Control and a contrived Positive Plasma Panel. The two controls and one human plasma panel were assayed in triplicates at two separate times per day and on 5 different days. The contrived human plasma panel near cutoff was assayed in triplicate at two separate times per day and on three different days. Results are presented in the following table.

Sample	n	COVID-19 ImmunoRank™ Neutralization MICRO-ELISA Kit				
		Mean SNI%	Repeatability Within-Assay		Within-Laboratory*	
			SD	%CV	SD	%CV
Positive Control	30	94%	0.005	0.6%	0.009	0.9%
Negative Control	30	0%	0.025	N/A[a]	0.030	N/A[a]
Contrived Positive Plasma Panel	30	44.9%	0.014	3.1%	0.058	13.0%
Contrived Positive Plasma Panel Near Cutoff	21	28.1%	0.025	8.9%	0.033	11.8%

*Includes repeatability within-assay, between assays and between day variability.
[a] Not Applicable

Study for Determination of LoB, LoD and LoQ

Limit of Blank (LoB), Limit of Detection (LoD) and Limit of Quantitation (LoQ) studies were performed based on guidance from the CLSI EP17 using the COVID-19 ImmunoRank™ Neutralization MICRO-ELISA assay to support the measuring interval of this neutralization assay.[16] Each study was performed in triplicate using two reagent lots (including 2 calibrator lots) assayed twice per day on three days. The level of blank (LoB) is defined as the highest apparent analyte concentration expected to be found when replicates of a blank sample containing no analyte are tested. The LoB was determined by testing five (5) negative plasma samples collected prior to December 1, 2019. LoB = mean $_{blank}$ + 1.645(SD_{blank}). The LoD is the lowest analyte concentration likely to be reliably distinguished from the LoB and at which detection is feasible. The LoD is determined by utilizing both the measured LoB and test replicates of five (5) samples contrived with a low concentration of analytes. LoD = LoB + 1.645 (SD $_{low\ concentration\ sample}$). The LoQ is the lowest concentration at which the analytes can not only be reliably detected but at which some predefined goals for bias and imprecision are met. The LoQ should have a %CV $\leq$ 20%. The contrived samples for determining LoD and LoQ were prepared by diluting positive patient samples with known negative samples collected prior to December 1, 2019 (Pre-Outbreak). The following table contains the LoB, LoD and LoQ for the COVID-19 ImmunoRank™ Neutralization MICRO-ELISA assay. Negative SNI% values are reported as zero.

Limit of Blank (LoB) Limit of Detection (LoD) and Limit of Quantitation (LoQ)

Description of Sample	n	Mean	SD	%CV	LoB	LoD	LoQ
Negative Plasma (Pre-COVID-19 Outbreak)	60	0%	0%	0%	0%		
Contrived Low Level Plasma A	60	7.0%	3.0%	43.0%		4.9%	
Contrived Low Level Plasma B	60	13.8%	2.7%	19.5%			13.8%

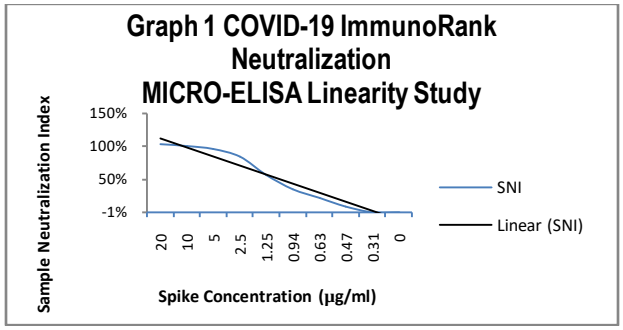
Linearity

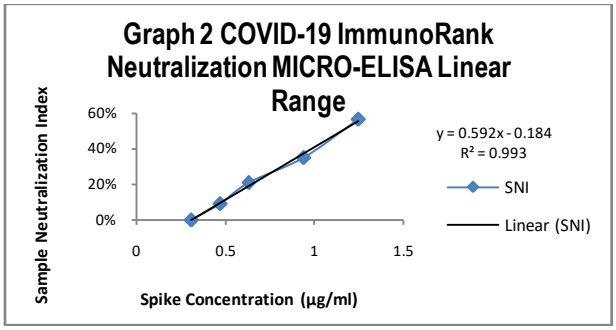
Plasma samples collected prior to December 1, 2019 (pre-COVID19 outbreak) which also tested negative for SARS CoV-2 antibodies were spiked with decreasing concentrations of a cocktail of human recombinant monoclonal antibodies, all of which were sequenced from plasma B-cells isolated from COVID-19 survivors and screened for specificity to the recombinant SARS-CoV-2 receptor binding domain (RBD) protein. The recombinant human monoclonal antibodies included in these contrived samples have been shown to neutralize the SARS-CoV-2 virus in PRNT or FRNT live virus neutralization assays. The positive predictive values (PPA) and 95% confidence interval (CI) were calculated. Results are presented in the table below.

SARS-CoV-2 Antibody Concentration	20.0 μg/ml	10.0 μg/ml	5.0 μg/ml	2.5 μg/ml	1.25 μg/ml	0.94 μg/ml	0.63 μg/ml	0.47 μg/ml	0.31 μg/ml	0 μg/ml
n	10	10	5	5	5	5	5	5	5	5
Average SNI%	102.1%	99.9%	91.6%	83.9%	56.8%	34.8%	21.0%	8.8%	0%	0%
POS.	10	10	5	5	5	5	5	0	0	0
NEG.	0	0	0	0	0	0	0	5	5	5
PPA	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
*95% C.I.	72% - 100%	72% - 100%	57% - 100%	57% - 100%	57% - 100%	57% - 100%	57% - 100%	57% - 100%	57% - 100%	57% - 100%

*95% Confidence Interval is calculated using the Wilson Method[14]

The graphs below were constructed using the same data points from the above linearity table. Graph 1 is the full sigmoid curve and Graph 2 is a focus on five (5) data points from the linear portion of the curve.





Clinical Agreement Study

To estimate the positive percent agreement (PPA) and negative percent agreement (NPA) between the COVID-19 ImmunoRank™ Neutralization MICRO-ELISA assay with the comparator plaque reduction neutralization test (PRNT), one hundred and seventeen (117) total specimens were collected retrospectively consisting of a study specific cohort of seventy five (75) negative plasma samples from apparently healthy donors collected prior to December 1, 2019, thirty two (32) positive specimens collected from subjects who tested positive for SARS-CoV-2 by an EUA approved polymerase chain reaction (PCR) test and ten (10) specimens containing potentially cross-reactive antibodies to HIV. Each specimen was tested using the COVID-19 ImmunoRank™ Neutralization MICRO-ELISA and then submitted blinded and randomized for testing using the comparator PRNT assay conducted at an accredited Regional Biocontainment Laboratory sponsored by the National Institute of Allergy and Infectious Diseases. The positive percent agreement (PPA), negative percent agreement (NPA) and 95% confidence interval (CI) were calculated. The results from both assays are presented in the tables below.

Positive Percent Agreement Using $PRNT_{50}$ Titers as Comparator Assay

Positive Specimens	n	Plaque Reduction Neutralization Test (PRNT)		COVID-19 ImmunoRank™ Neutralization MICRO-ELISA Test			
		POS.	NEG.	POS.	NEG.	PPA	*95% CI
RT-PCR Positive Plasma and Serum	32	32	0	32	0	100%	89.28% - 100%

*95% Confidence Interval is calculated using the Wilson Method[14]

Negative Percent Agreement Using $PRNT_{50}$ Titers as Comparator Assay

Negative Specimens	n	Plaque Reduction Neutralization Test (PRNT)		COVID-19 ImmunoRank™ Neutralization MICRO-ELISA Test			
		POS.	NEG.	POS.	NEG.	NPA	*95% CI
Plasma Before 12/1/19	75	2	73	0	75	97.3%	90.78% - 99.26%

*95% Confidence Interval is calculated using the Wilson Method[14]

Potentially Cross-Reactive Specimens Containing HIV Antibodies Using the $PRNT_{50}$ Comparator Assay

HIV Specimens	n	Plaque Reduction Neutralization Test (PRNT)		COVID-19 ImmunoRank™ Neutralization MICRO-ELISA Test			
		POS.	NEG.	POS.	NEG.	NPA	*95% CI
Specimens with Potentially Cross-Reactive HIV Antibodies	10	0	10	0	10	100%	72.25% - 100%

*95% Confidence Interval is calculated using the Wilson Method[14]

Negative Agreement

To evaluate the negative percent agreement of the COVID-19 ImmunoRank™ Neutralization MICRO-ELISA Test for presumed negative samples, five hundred and thirty one samples from unselected apparently healthy US plasma donors collected prior to December 1, 2019 were tested. The negative percent agreement (NPA) and 95% confidence interval (CI) were calculated. The results are presented in the following table.

Plasma Panel Before December 1, 2019	n	COVID-19 ImmunoRank™ Neutralization MICRO-ELISA Kit			
		POS.	NEG.	NPA	95%CI*
Plasma Donors (USA)	531	4	527	99.3%	98.10% - 99.71%

*95% Confidence Interval is calculated using the Wilson Method[14]

Sample Matrix Comparison

The COVID-19 ImmunoRank™ Neutralization MICRO-ELISA has been validated for use with human serum and sodium citrate plasma. Human serum and plasma was tested using fifteen (15) seronegative sample pairs spiked with a cocktail of SARS-CoV-2 neutralizing recombinant human monoclonal antibodies (IgG1) sequenced from plasma B-cells of COVID-19 survivors and screened for specificity to the SARS-CoV-2 receptor binding domain (RBD) protein. The contrived antibody cocktail consisted of four recombinant antibodies spiked at moderate and low concentrations with seronegative plasma and serum. All samples, spiked and seronegative, were run in duplicate. The Passing-Bablok regression was performed for the comparison of plasma to serum. The results from the assays using sample pairs spiked with recombinant human monoclonal antibodies are presented in the following tables:

Spiked with Recombiant Human Monoclonal Antibodies

	Serum
n	**45**
Sample Neutralization Index Range - Plasma	**0% - 94%**
Sample Neutralization Index Range - Serum	**0% - 76%**
Regression Equation (*x* = plasma, *y* = serum)	**0.701*x* – 0.0061**
95% C.I. of Intercept	**-0.038 – 0.026**
95% C.I. of Slope	**0.652 – 0.750**
Coefficient of Determination (R^2)	**0.9506**

Sample Type	COVID-19 ImmunoRank™ Neutralization MICRO-ELISA Kit								
	Low Positive			Mid Positive			Seronegative		
	NEG	POS	Percent Agreement	NEG	POS	Percent Agreement	NEG	POS	Percent Agreement
Plasma (Sodium Citrate)	0	15	100%	0	15	100%	15	0	100%
Serum	0	15	100%	0	15	100%	15	0	100%

Additionally, the COVID-19 ImmunoRank™ Neutralization MICRO-ELISA has been validated for use with human serum and sodium citrate plasma using fifteen (15) seronegative sample pairs spiked at moderate and low levels with antibody positive natural specimens (plasma). All samples, spiked and seronegative, were run in duplicate. The results of the assays are presented in the following tables:

Spiked with Natural Specimens

	Serum
n	45
Sample Neutralization Index Range - Plasma	0% - 53%
Sample Neutralization Index Range - Serum	0% - 43%
Regression Equation (*x* = plasma, *y* = serum)	0.7743*x* – 0.0155
95% C.I. of Intercept	-0.031 – 0.0004
95% C.I. of Slope	0.7271 – 0.8216
Coefficient of Determination (R^2)	0.9621

Sample Type	COVID-19 ImmunoRank™ Neutralization MICRO-ELISA Kit								
	Low Positive			Mid Positive			Seronegative		
	NEG	POS	Percent Agreement	NEG	POS	Percent Agreement	NEG	POS	Percent Agreement
Plasma (Sodium Citrate)	0	15	100%	0	15	100%	15	0	100%
Serum	0	15	100%	0	15	100%	15	0	100%

Heat Inactivation Study

The COVID-19 ImmunoRank™ Neutralization assay has been evaluated for use with heat inactivated specimens. Aliquots of positive plasma samples were assayed after heat inactivation at 56° C for 30 minutes and compared with aliquots from the same plasma samples that were not heat inactivated. A total of six (6) samples, three (3) natural and three (3) contrived representing high, medium and near the cutoff were run in triplicate. Negative plasma samples were collected prior to December 1, 2019 (Pre-Outbreak). The data are summarized in the following table.

Description of Sample	n	Not Heat Inactivated			Heat Inactivated		
		Mean	SD	%CV	Mean	SD	%CV
Negative Plasma	6	0	0	0	0	0	0
Low SNI% Plasma	6	38.1%	2.0%	5.1%	37.4%	2.3%	6.1%
Medium SNI% Plasma	6	55.8%	1.1%	2.0%	57.0%	1.3%	2.3%
High SNI% Plasma	6	93.7%	0.2%	0.2%	91.6%	0.5%	0.5%

INTERFERENCE

Potentially Interfering Endogenous Substances

A study was performed based on guidance from CLSI EP07, 3rd edition.[15] Potentially interfering substance were tested at or above physiological levels to determine whether they could cause false positives or false negatives using the COVID-19 ImmunoRank™ Neutralization MICRO-ELISA assay. Each Interfering substance was tested at 2 levels (high negative and low positive) in replicates of ten (10) using a seronegative plasma sample spiked with a cocktail of four (4) recombinant human IgG_1 neutralizing monoclonal antibodies sequenced from plasma B-cells of COVID-19 survivors and screened to be specific to the SARS-CoV-2 receptor binding domain (RBD) protein. The study showed no interference from these endogenous substances.

Potential Interfering Endogenous Substances	Concentration of Potentially Interfering Endogenous Substance
Unconjugated Bilirubin	40 mg/dL
Conjugated Bilirubin	40 mg/dL
Hemoglobin	1000 mg/dL
Triglycerides	2000 mg/dL
Cholesterol	40 mg/dL
Albumen	15 g/dL

Analytical Specificity
Potentially Cross Reacting Antibodies

The COVID-19 ImmunoRank™ Neutralization assay has been evaluated for potentially cross-reacting antibodies. A total of sixty three (63) specimens from 12 different categories were tested. Sixty three (63) samples tested negative and zero samples tested positive. The data are summarized in the following table.

Cross Reactivity Panel	n	COVID-19 ImmunoRank™ Neutralization MICRO-ELISA Kit	
		NEG.	Negative (%)
HCoV-NL63 Infection	5	5	100%
HCoV-229E Infection	5	5	100%
HCoV-OC43 Infection	5	5	100%
HCoV-HKU1 Infection	5	5	100%
Anti Influenza A	5	5	100%
Anti Influenza B	5	5	100%
Anti HCV IgG	5	5	100%

Cross Reactivity Panel	n	COVID-19 ImmunoRank™ Neutralization MICRO-ELISA Kit	
		NEG.	Negative (%)
Anti HBV IgG	5	5	100%
Anti Haemophilus Influenzae IgG	5	5	100%
Anti Respiratory Syncytial Virus IgG	5	5	100%
ANA	3	3	100%
Anti HIV	10	10	100%

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SYMBOLS KEY

ISO 15223 SYMBOLS		OTHER SYMBOLS	
	Caution	INCUBATION PLATE	Incubation Plate
	Consult instructions for use	MICROPLATE	Microplate Strips
	Manufacturer	CONTROL +	Positive Control
	Temperature limitations	CONTROL -	Negative Control
	Use by/Expiration date	CALIBRATOR CONTROL	Calibrator Control
	Total number of IVD tests that can be performed with the IVD medical device	SAMPLE DILUENT	Sample Diluent
LOT	Lot Number	RBD-ENZYME CONJUGATE	RBD-Enzyme Conjugate
REF	Product Number	CONJUGATE DILUENT	Conjugate Diluent
IVD	*In Vitro* Diagnostic Medical Devices	WASH BUFFER	Wash Buffer
		SUBSTRATE CHROMOGEN	Substrate Chromogen
		STOP SOLUTION	Stop Solution
		PLATE SEALER	Plate Sealer
		RTU	Ready to Use

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