Results Interpretation

Table 3. Algorithm used for calculating qualitative determination of human IgG against each of the three SARS-CoV-2 proteins

#	Ratio of the sample Test sample to Average of duplicate wells of Calibrator			Interpretation
	RBD-CoV-2	NP-CoV-2	RBD-NP	
1	> 1.2	> 1.2	> 1.2	Positive
2	> 0.9 and < 1.2	> 0.9 and < 1.2	> 0.9 and < 1.2	Equivocal
3	> 0.9 and < 1.2	> 1.2	> 1.2	Equivocal
4	> 1.2	> 0.9 and < 1.2	> 1.2	Equivocal
5	> 1.2	> 1.2	> 0.9 and < 1.2	Equivocal
6	> 1.2	> 0.9 and < 1.2	> 0.9 and < 1.2	Equivocal
7	> 0.9 and < 1.2	> 1.2	> 0.9 and < 1.2	Equivocal
8	> 0.9 and < 1.2	> 0.9 and < 1.2	> 1.2	Equivocal
9	< 0.9	< 0.9	< 0.9	Negative
10	< 0.9	> 0.9 and < 1.2	> 0.9 and < 1.2	Negative
11	> 0.9 and < 1.2	< 0.9	> 0.9 and < 1.2	Negative
12	> 0.9 and < 1.2	> 0.9 and < 1.2	< 0.9	Negative

Positive - SARS-CoV-2 specific IgG antibodies were detected in the sample

Equivocal - Indeterminate result as the antibodies may be at the limit of detection of the test or the response observed may be due to cross reaction.

Negative - SARS-CoV2 specific IgG antibodies were NOT detected in the sample

TETRACORE INC.
9901 BELWARD CAMPUS DRIVE SUITE 300
ROCKVILLE, MD 20850, USA.
www.tetracore.com
Phone: 240-268-5400
FAX: 240-268-1107

INSTRUCTIONS FOR USE

Product Catalog # TC-5053-090

FlexImmArray™ 7-Plex SARS-CoV-2 Human IgG Antibody Test (RUO)

Read this IFU completely before using this product.
Follow the instructions carefully while performing the test to assure accuracy of the results.
Research Use Only (RUO), and not for use in diagnostics procedures.

Intended Use:

This test is intended for detection of Human IgG antibodies to SARS-CoV-2 in unknown human serum or plasma samples.

Introduction:

The SARS-CoV-2 serology test is a blood-based test to determine SARS CoV-2 specific IgG antibodies among human subjects who may have been exposed or infected by SARS-CoV-2. The SARS CoV-2 antibody test can also be used to detect specific human IgG antibodies in people who were infected but remained asymptomatic or have recovered. In addition, antibody responses to SARS-CoV-2 antigens may also be detected in individuals who have been immunized with COVID 19 vaccines.

Principle of the Test:

This 7-Plex microsphere array includes three immobilized SARS-CoV-2 recombinant antigens on three unique microsphere regions for detecting IgG antibodies to the virus. Diluted (1:400) serum or plasma samples are incubated with the 7-Plex microsphere mixture in 96-well plate wells for the antigen-antibody reaction to occur. The antigen-specific antibodies get immobilized on the antigen-coupled microspheres, and unbound material is washed away after the incubation. The antigen-captured human IgG antibodies are then detected by addition of Fluorescent Anti-human IgG-phycoerythrin (AHIgG-PE) reporter conjugate to each well. After a final wash to remove the unbound reporter conjugate, the microspheres are resuspended in the buffer and analyzed using either Luminex® LX 200™ or MAGPIX® or FLEXMAP 3D® instruments. In addition to the three antigen targets used in the multiplex assay, four built-in internal control microsphere regions are also incorporated in every single test. The first control measures the fluorescent intensity measured by Luminex instrument and assures the quality of fluorescence measurement. The second control measures the non-specific binding that may arise from the sample. The third control measures the addition of the human sample in the assay for the measurement of Human IgG antibody. The fourth control monitors for the addition of fluorescent reporter in the assay thus assuring the assay reagent is added correctly. In addition, this test also includes three external ready-to-use sample controls for assessing the assay performance and to help in results interpretation. The Positive Control shows positive reactivity to all 3 antigen targets. The Calibrator helps to set the positive threshold

for the multiplex assay for each of the three antigens. The Negative Control shows signal near background for the three antigens.

Limitations of the Procedure:

- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- The Human IgG antibody response becomes detectable in convalescent samples after about 2 weeks of infection. Acute samples taken within 7 days post onset of symptoms may be negative for IgG.
- At this time research is limited on how long IgG response may persist following infection and may provide protective immunity. Immunocompromised subjects with COVID 19 disease may have a delayed IgG response. Enough information is not available currently to understand assay performance in such samples.

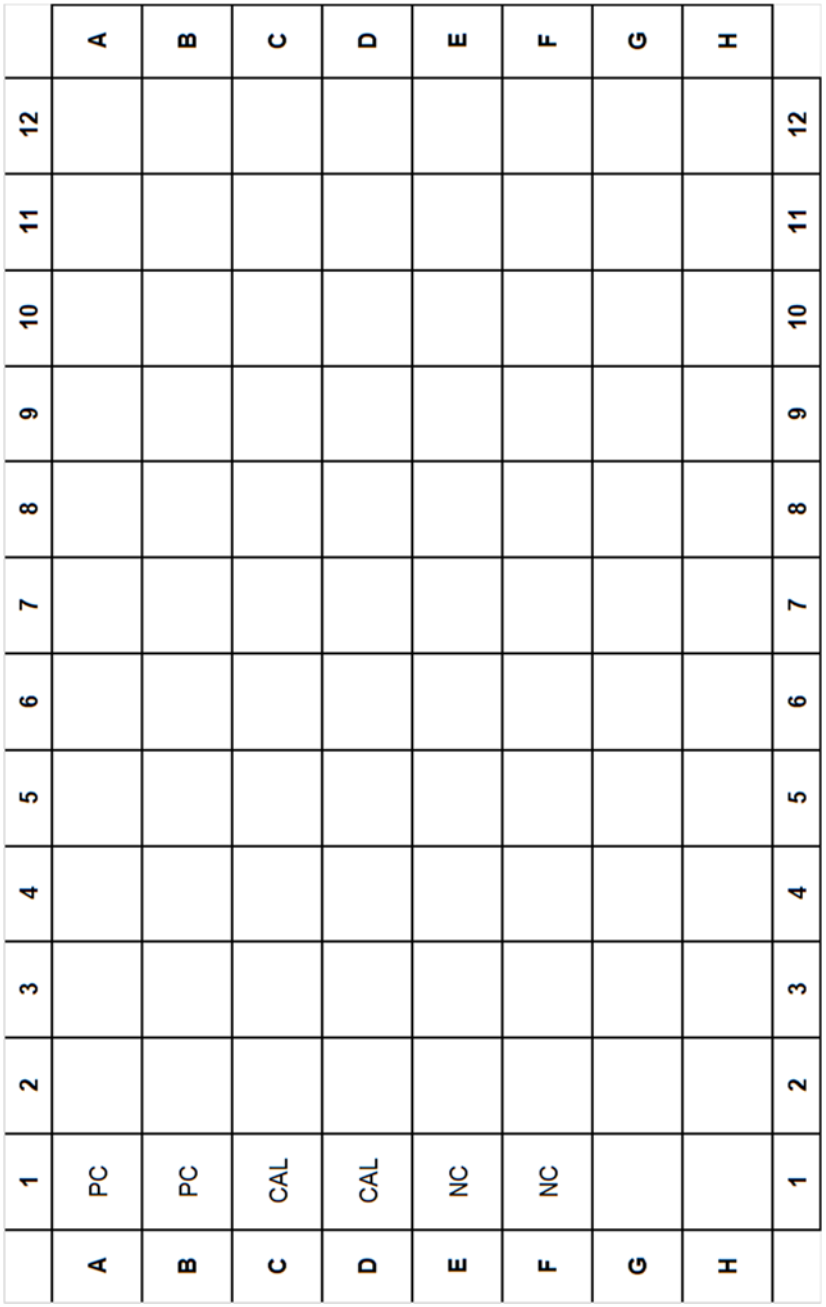
Table 1. Materials Included & Storage Conditions

#	PART	Description	Storage
1	Premixed 7-Plex Microsphere mix**	1 Vial of 1.2 mL of ready to use magnetic microsphere mix	2-8°C within the expiration date
2	5X Sample Dilution Buffer	1 Vial of 12 mL 5X Sample dilution buffer (SDB)	2-8°C within the expiration date
3	Fluorescence Reporter	1 Vial of 3 mL ready to use anti-Human IgG-PE conjugate	2-8°C within the expiration date
4	20X Assay Wash Buffer	2 Vials with 15 mL each of 20X concentrated assay wash buffer. May develop crystals when stored at 2-8°C	1 Month after dilution at room temperature
5	Human IgG Positive Control	1 Vial of 0.2 mL ready to use external assay Positive Control for positive IgG antibodies to three SARS-Cov-2 antigens	2-8°C within the expiration date
6	Human IgG assay Calibrator	1 Vial of 0.2 mL ready to use external assay Calibrator for IgG antibodies to three SARS-Cov-2 antigens	2-8°C within the expiration date
7	Human IgG Negative Control	1 Vial of 0.2 mL ready to use external assay Negative Control for IgG antibodies to three SARS-Cov-2 antigens	2-8°C within the expiration date
5	Sample dilution plates	2 non-binding 96 well plates for dilution of samples	
6	Assay Plate	1 Flat and micro-clear bottom 96-well microplate used for assay performance	
7	Plate sealers	5 Adhesive foils	

***** Table 2 on page 4 provides the description of 7-Plex microsphere mixture.***

Precautions:

All human samples are presumed hazardous and must be handled according to laboratory safety guidelines at your institution. Wear appropriate PPE and wash hands thoroughly after handling.



Assay Procedure

1) Write down the sample information in 96-well plate layout given in Page 7.
2) Vortex the 7-Plex microsphere mixture for about 20 seconds.
3) Add 10 µL of well mixed microsphere mix to each well of the plate.
4) Transfer 50 µL of 1:400 diluted serum or plasma samples to the assay plate as per the plate format.
5) Add 50 µL of the ready-to-use Positive Control, Calibrator and Negative Control in the designated wells as per the plate layout.
6) Cover the plate and incubate at room temperature in dark for 20 minutes while shaking at 800 rpm.
7) Wash the assay plate four times with 200 µL 1X Assay Wash Buffer.
8) Add 25 µL Fluorescence Reporter PE-Anti Human IgG to appropriate wells of assay plate.
9) Cover the plate with plate seal and incubate at room temperature for 20 minutes while shaking at 800 rpm.
10) Wash the assay plate four times with 200 µL 1X Assay Wash Buffer.
11) Add 150 µL 1X Assay Wash Buffer to appropriate wells of assay plate.
12) Cover the plate and re-suspend for 10 minutes at 800 rpm shaking.
13) Place the assay plate in correct orientation and read within 60 minutes in the Luminex Analyzer.

Results Calculation

All the results are normalized based on the median fluorescent intensity (MFI) of each of the viral antigen coupled microspheres of the Calibrator tested in every plate along with the test samples. The MFI signal ratios of the test sample to the mean of the calibrator on each of the three antigens are used to qualitatively determine the IgG response in the test sample. Ratio of each of the antigen-coupled microsphere sets are calculated as follows:

$$Ratio = \frac{Observed\ MFI\ value\ of\ the\ Test\ sample\ for\ the\ SARS\ CoV\text{-}2\ antigen}{Average\ of\ duplicate\ MFI\ values\ of\ the\ Calibrator\ for\ SARS\ CoV\text{-}2\ antigen}$$

Twelve different combinations of the ratios of three SARS-CoV-2 antigens are shown in Table 3. The sample is considered to have SARS-CoV-2 specific IgG antibodies only when the ratios of all three antigens are > 1.2. Three possible results can interpreted based on each of the combination and are described below.

Positive - SARS-CoV-2 specific IgG antibodies were detected in the sample

Equivocal - Indeterminate result as the antibodies may be at the limit of detection of the test or the response observed may be due to cross reaction.

Negative - SARS-CoV2 specific IgG antibodies were NOT detected in the sample

Material Required but Not Included:

Multichannel pipettes and appropriate tips
Deionized or filtered water
Pipettes in common laboratory sizes viz; P10, P20, P100, P1000
Serological pipettes of 10, 15, 25 mL sizes and pipette aid

Equipment Required, but not Provided:

Automated or manual plate washer for magnetic microspheres
Tabletop centrifuge
Plate shaker
Luminex FLEXMAP 3D, or Luminex 200 system, or MAGPIX instrument

Instrument Settings and Preparation

1) Ensure that Luminex analyzer being used is appropriately maintained per Luminex recommendations in the respective instrument user manual or as per your laboratory guidelines.
2) Assign the microsphere region for each of the antigen and control microspheres in the protocol as per Table 2.
3) Set the protocol parameters as 50 events /region
4) Sample size: 50 µL
5) Data type: Median Fluorescent Intensity (MFI)
6) Power up the Luminex instrument and run "Daily Fluidics Prep (Luminex)" routine in the Maintenance Tab. Also calibrate the machine using appropriate command routine. Please check the System Status and Cal/Ver buttons are Green at the lower left-hand corner of the screen.
7) Select the specific 7-Plex protocol and set up a new batch for bead testing.
8) Vortex 7-Plex microsphere mixture thoroughly for about 20 seconds and add 10 µL of 7-Plex microsphere mixture into two wells of assay microtiter plate and add 140 µL of 1X wash buffer (See Reagent Preparation section, Page 4).
9) Start the 7-Plex-protocol and read the plate.
10) Open the run results csv file and review the "Data type-counts" for the number in each microsphere region. If the count of each bead region is $\geq$ 50 in FLEXMAP 3D and Luminex 200 instruments, $\geq$ 100 in MAGPIX instrument, then the test is considered "Pass". Once the 7-Plex microsphere mixture test passes proceed further with the testing.

Table 2: Microsphere regions and targets

#	Microsphere coupling functionality	Antigen Identification	Microsphere Region
1	SARS-CoV-2 Receptor Binding Domain	RBD-CoV-2	25
2	SARS-CoV-2 Nucleocapsid Protein	NP-CoV-2	28
3	SARS-CoV-2 RBD-NP Hybrid	RBD-NP	36
4	IC (Instrument Control)	IC	47
5	NC (Non-specific binding Control)	NC	54
6	ScG (Human IgG Sample Control)	ScG	52
7	FC (Fluorescent Reporter Control)	FC	53

Reagent Preparation

1) **Wash Buffer** – If crystals have formed in the concentrated wash buffer, warm to room temperature and mix gently until the crystals have completely dissolved. Add 15 mL of 20 X wash buffer to distilled water to make 300 mL of wash buffer.
2) **Sample Dilution Buffer**: Prepare required amount of 1X Sample Dilution Buffer from the stock **5X Sample Dilution Buffer** (SDB) by 5-fold dilution. (e.g., 1 mL 5X SDB + 4mL of distilled or deionized water)
3) **Controls**: 0.2 mL each of three external controls, 1) Positive Control, 2) Calibrator, and 3) Negative Control, are provided in the kit in ready to use format.
   a) Positive controls give a positive signal for all three antigens.
   b) Calibrator helps to set the positive threshold for each of the three antigens
   c) Negative Control shows the signal near background on all the microspheres These external controls are included in the kit for use and to monitor the functionality of each target microsphere in this 7-Plex kit.
   d) While testing every plate, it is recommended that duplicate wells are used for each of the three controls provided in the kit.
   e) Optional position of the ready-to-use controls in the assay plate for testing:
   Wells A1& B1 – Positive Control (PC)
   Wells C1 & D1 – Calibrator (CAL)
   Wells E1 & F1 – Negative Control (NC)

Sample Collection and Storage

1) Blood (Serum or plasma samples) should be collected safely by following Institutional Biosafety protocols.
2) Plasma samples using dipotassium EDTA and sodium citrate as anticoagulants have been validated for use in this test.
3) Aliquot the serum or plasma samples in convenient volumes and tubes; these aliquots may be stored at 2-8°C for up to a week for testing within that period. If testing is delayed, we recommend freezing the sample ≤ –20°C as per your laboratory guidelines. Avoid repeated freezing and thawing of samples.

Sample Preparation

We recommend inactivation of serum and plasma samples in a water bath at 56°C for 30 minutes to one hour depending upon the laboratory safety procedures. Inactivation for longer than an hour and temperature higher than 56°C has not been validated and may affect the assay performance.

1) Following inactivation, bring the samples to room temperature.
2) Centrifuge inactivated human serum or plasma samples at room temperature for 2 minutes at 10,000 rpm using a tabletop centrifuge to bring the entire sample to the bottom of the tube.
3) Label the two microtiter plates as Sample dilution Plate 1 and Sample dilution Plate 2 respectively.
4) Designate the sample locations in Sample dilution Plate 1 and 2 based on the Assay plate layout to be tested.
5) Each plate will be used to make 1 to 20 times dilution of the serum or plasma sample. Add 190 µL of 1X Sample Dilution Buffer to each well of the two serum dilution plates. (Do not use wells A1 to F1 as those wells must be reserved for external assay controls in the Assay Plate)
6) For 1:20 dilution, add 10 µL of serum / plasma sample from the tubes to assigned wells in Sample dilution Plate 1, mix the sample by pipetting up and down with the buffer.
7) For 1:400 final dilution of the sample, take 10 µL of 1:20 diluted sample from Sample dilution Plate 1 and add to the corresponding wells in the Sample dilution Plate 2. Mix the samples by pipetting up and down in Plate 2.

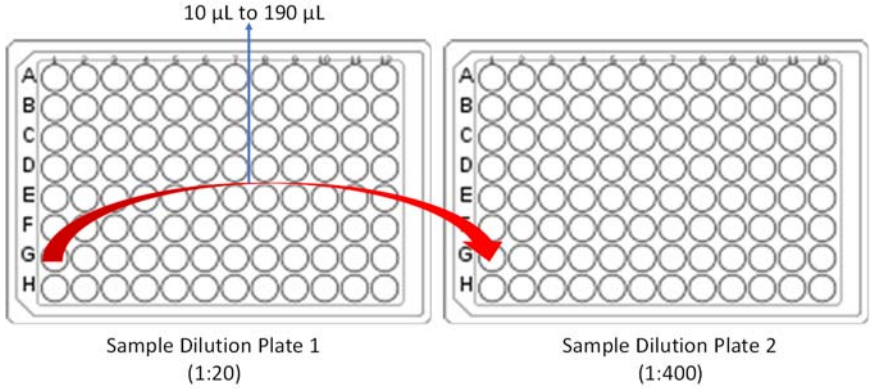


Figure 1. Dilution of the test sample 1:20 in the first step; in the second step another 1:20 to get a final dilution of 1:400 of the sample

8) Samples are now ready for testing at 1:400 dilutions for addition to the appropriate wells of the Assay plate.