



Enabling Legendary Discovery™

LEGENDplex™

Multi-Analyte Flow Assay Kit

Cat. No. 741126 SARS-CoV-2 Neut. Ab Assay (1-plex) with Filter Plate

Cat. No. 741127 SARS-CoV-2 Neut. Ab Assay (1-plex) with V-Bottom Plate

Please read the entire manual before running the assay.

BioLegend.com

It is highly recommended that this manual be read in its entirety before using this product. Do not use this kit beyond the expiration date.

For Research Purposes Only. Not for use in diagnostic or therapeutic procedures. Purchase does not include or carry the right to resell or transfer this product either as a stand-alone product or as a component of another product. Any use of this product other than the permitted use without the express written authorization of BioLegend is strictly prohibited.

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Chapter 1: KIT DESCRIPTION

Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a single stranded RNA virus that belongs to a family of viruses known as coronaviruses. SARS-CoV-2 infection, known as COVID-19, was declared a pandemic in 2020 and among other symptoms leads to respiratory infection, pulmonary failure which can be fatal. SARS-CoV-2 is structurally composed of 4 main proteins (spike glycoprotein, envelope glycoprotein, membrane glycoprotein and nucleocapsid protein) and several accessory proteins. The coronavirus spike (S) glycoprotein is a class I viral fusion protein on the outer envelope of the virion that plays a critical role in viral infection by recognizing host cell receptors and mediating fusion of the viral and cellular membranes. The S glycoprotein is synthesized as a precursor protein consisting of ~1,300 amino acids (aa) that is then cleaved into an amino (N)-terminal S1 subunit (~700 aa) and a carboxyl (C)-terminal S2 subunit (~600 aa). The S1 subunit contains a receptor-binding domain (RBD) that can specifically bind to angiotensin-converting enzyme 2 (ACE2), the receptor on target cells. The binding of RBD domain to ACE2 triggers a fusion process that leads to virus entry into the host cell. Neutralization antibodies in the circulatory system that can bind to the RBD region, block its the binding to ACE2 and prevent virus entry into host cells.

This assay is for research use only.

Principle of the Assay

BioLegend's LEGENDplex™ SARS-CoV-2 Neut. Ab Assay (1-plex) immunoassay is a single plex beads-based assay that uses the same basic principle as competitive immunoassay. Beads are conjugated with ACE2 protein. Biotinylated S1-Fc chimera and anti-human S1 recombinant antibody serve as the detection and neutralization antibodies, respectively. These are added to all wells prior to the addition of the ACE2 capture beads and will compete for binding to ACE2, producing a competitive assay. Streptavidin-phycoerythrin (SA-PE) is subsequently added, which will bind to the biotinylated S1-Fc detection, providing fluorescent signal intensity in proportion to the amount of bound analyte. The concentration of a particular analyte is determined using a standard curve generated in the same assay. The competition between the detection and the standard neutralization antibody will form an inverted standard curve. Due to the nature of the assay, the standard curve will have the highest signal at the lowest standard neutralization antibody concentration and the signal will decrease as the standard neutralization antibody concentration increases.

Beads Usage

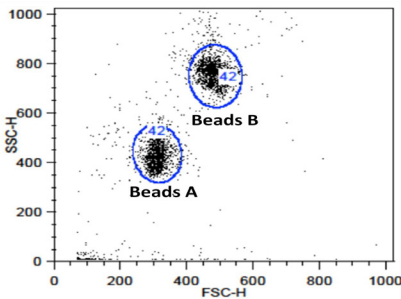
The LEGENDplex™ beads-based assay usually includes two sets of beads. Each set has a unique size that can be identified on flow cytometer based on their forward scatter (FSC) and side scatter (SSC) profiles (Beads A and Beads B,

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Figure 1). Each bead set can be further resolved based on their internal fluorescence intensities. The internal dye can be detected using FL3, FL4, or APC channel, depending on the type of flow cytometer used. The smaller Beads A consists of 6 bead populations and the larger Beads B consists of 7 bead populations (Figure 2-3).

The SARS-Cov-2 Neut. Ab Assay uses only one of the 13 bead populations (B3) distinguished by size and internal fluorescent dye.

Figure 1. Beads Differentiated by Size



Beads A = smaller beads

Beads B = larger beads

Figure 2. Beads A Classification by FL4

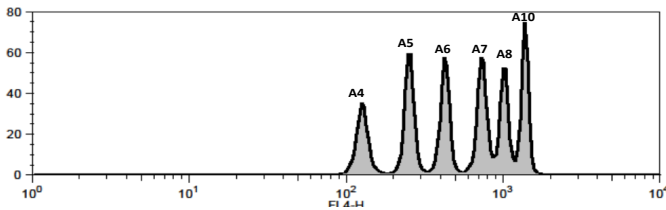
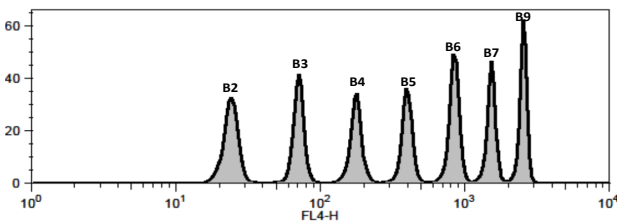


Figure 3. Beads B Classification by FL4



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Table 1. Beads ID and Target Information*

Target	Bead ID	Top Standard Concentrations
SARS-CoV-2 Neutralizing Antibody	B3	Note: The top standard concentrations of the analyte in this Assay may be subject to change from lot to lot (please visit biolegend.com/en-us/legendplex to download a lot-specific certificate of analysis).

*Bead ID is used to associate a bead population to a particular analyte when using the LEGENDplex™ data analysis software program. For further information regarding the use of the program please visit biolegend.com/en-us/legendplex.

Storage Information

Recommended storage for all original kit components is between 2°C and 8°C. DO NOT FREEZE Beads, Detection Antibodies or SA-PE.

- Once the standards have been sufficiently reconstituted, immediately transfer contents into polypropylene vials. DO NOT STORE RECONSTITUTED STANDARDS IN GLASS VIALS.
- Upon reconstitution, leftover top standard should be stored at ≤-70°C for use within one month. Avoid multiple (>2) freeze-thaw cycles. Discard any leftover diluted standards.

Materials Supplied

The LEGENDplex™ kit contains reagents for 100 tests, listed in the table below. When assayed in duplicate, this is enough for an 8-point standard curve and 40 samples.

Kit Components	Quantity	Volume	Part #
Setup Beads 1: FITC Beads	1 vial	1 mL	77840
Setup Beads 2: PE Beads	1 vial	1 mL	77842
Setup Beads 3: Raw Beads	1 vial	2 mL	77844
SARS-CoV-2 Neut. Ab Assay Beads B3, 1X	1 bottle	3.5 mL	750002257
SARS-CoV-2 Neut. Ab Assay Detection	1 vial	Lyophilized	750002258
SARS-CoV-2 Neut. Ab Assay Standard	1 vial	Lyophilized	750002259
LEGENDplex™ SA-PE	1 bottle	3.5 mL	77743
LEGENDplex™ Assay Buffer	1 bottle	25 mL	77562
LEGENDplex™ Wash Buffer, 20X	1 bottle	25 mL	77564
Filter Plate*** or V-bottom Plate****	1 plate		76187*** or 76883****
Plate Sealers	4 sheets		78101

Either a filter plate* or a V-bottom plate will be provided in each kit.**

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Materials to be Provided by the End-User

- A flow cytometer equipped with two lasers (e.g., a 488 nm blue laser or 532 nm green laser and a 633-635 nm red laser) capable of distinguishing 575 nm and 660 nm or a flow cytometer equipped with one laser (e.g., 488 nm blue laser) capable of distinguishing 575 nm and 670 nm.

Partial list of compatible flow cytometers:

Flow Cytometer	Reporter Channel	Reporter Emission	Classification Channel	Channel Emission	Compensation needed?
BD FACSCalibur™	FL2	575 nm	FL4	660 nm	No*
BD Accuri™ C6	FL2	585 nm	FL4	675 nm	No*
BD FACSCanto™, BD FACSCanto™ II	PE	575 nm	APC	660 nm	No*
BD™ LSR, LSR II BD LSRFortessa™	PE	575 nm	APC	660 nm	No*
Gallios™	PE	575 nm	APC	660 nm	No*
CytoFLEX	PE	585 nm	APC	660 nm	No*
NovoCyte	PE	572 nm	APC	660 nm	No*
Attune™ NxT	PE	574 nm	APC	670 nm	No*

***Compensation is not required for the specified flow cytometers when set up properly.**

For setting up various flow cytometers, please visit: www.biolegend.com/legendplex and click on the **Instrument Setup** tab.

- Multichannel pipettes capable of dispensing 5 µL to 200 µL
- Reagent reservoirs for multichannel pipette
- Polypropylene microfuge tubes (1.5 mL)
- Micro FACS tubes, 1.1 mL (if the flow cytometer does not contain an autosampler)
- Laboratory vortex mixer
- Sonicator bath (e.g., Branson Ultrasonic Cleaner model #B200, or equivalent)
- Aluminum foil
- Absorbent pads or paper towels
- Plate shaker (e.g., Lab-Line Instruments model #4625, or equivalent)
- Tabletop centrifuges (e.g., Eppendorf centrifuge 5415 C, or equivalent)
- 1.1 mL polypropylene micro FACS tubes, in 96-tube rack (e.g., National Scientific Supply Co, catalog # TN0946-01R, or equivalent).

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If the assay is performed in a filter plate;

- A vacuum filtration unit (Millipore MultiScreen® HTS Vacuum Manifold, cat # MSVMHTS00 or equivalent). Instructions on how to use the vacuum manifold can be found at the supplier's website.
- A vacuum source (mini vacuum pump or line vacuum, e.g., Millipore Vacuum Pump, catalog # WP6111560, or equivalent).
- If needed, additional Filter plate can be ordered from BioLegend (Cat# 740377 or 740378).

If the assay is performed in a V-bottom plate;

- Centrifuge with a swinging bucket adaptor for microtiter plates (e.g., Beckman Coulter Allegra™ 6R Centrifuge with MICROPLUS CARRIER adaptor for GH3.8 and JS4.3 Rotors).
- If needed, additional V-bottom plate can be ordered from BioLegend (Cat# 740379).

Precautions

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Center for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium azide has been added to some reagents as a preservative. Although the concentrations are low, sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up.
- Do not mix or substitute reagents from different kits or lots. Reagents from different manufacturers should not be used with this kit.
- Do not use this kit beyond its expiration date.
- SA-PE and Beads are light-sensitive. Minimize light exposure.

Chapter 2: ASSAY PREPARATION

Sample Collection and Handling

Preparation of Serum Samples:

- Allow the blood to clot for at least 30 minutes and centrifuge for 20 minutes at 1,000 x *g*.
- Remove serum and assay immediately or aliquot and store samples at ≤-20°C. Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended that samples be thawed completely, mixed and centrifuged to remove particulates prior to use.

Preparation of Plasma Samples:

- Plasma collection should be collected using an anti-coagulant (e.g., EDTA). Centrifuge for 20 minutes at 1,000 x *g* within 30 minutes of blood collection.
- Remove plasma and assay immediately, or aliquot and store samples at ≤-20°C. Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended that samples be thawed completely, mixed well and centrifuged to remove particulates.

Reagent Preparation

Preparation of Beads

Sonicate the beads bottle for 1 minute in a sonicator bath and then vortex for 30 seconds prior to use. If no sonicator bath is available, increase the vortexing time to 1 minute to completely resuspend the beads.

Preparation of Wash Buffer

- Bring the 20X Wash Buffer to room temperature and mix to bring all salts into solution.
- Dilute 25 mL of 20X Wash Buffer with 475 mL deionized water. Store unused portions between 2°C and 8°C for up to one month.

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Preparation of Detection

- Prior to use, reconstitute the lyophilized SARS-CoV-2 Neut. Ab Assay (1-plex) Detection with 250 μ L of Assay Buffer.
- Mix and allow the vial to sit at room temperature for 15 minutes, and then transfer the detection to an appropriately labeled polypropylene microcentrifuge tube. Take 250 μ L of Assay Buffer to rinse the vial thoroughly and transfer the liquid to the tube. Add 2.5 mL of Assay Buffer. The final volume of detection is 3 mL.

Standard Preparation

1. Prior to use, reconstitute the lyophilized SARS-CoV-2 Neut. Ab Assay (1-plex) Standard with 250 μ L LEGENDplex™ Assay Buffer.
2. Mix and allow the vial to sit at room temperature for 15 minutes, and then transfer the standard to an appropriately labeled polypropylene microcentrifuge tube. This will be used as the top standard C7.

Note: The top standard concentrations of analytes in this panel were set at various concentrations, but may be subject to change from lot to lot (see lot-specific Certificate of Analysis provided in the kit box for details).

3. Label 6 polypropylene microcentrifuge tubes as C6, C5, C4, C3, C2 and C1, respectively.
4. Add 75 μ L of Assay Buffer to each of the six tubes. Prepare 1:4 dilution of the top standard by transferring 25 μ L of the top standard C7 to the C6 tube and mix well. This will be the C6 standard.
5. In the same manner, perform serial 1:4 dilutions to obtain C5, C4, C3, C2 and C1 standards (see the table below using the top standard at 10,000 ng/mL as an example). Assay Buffer will be used as the 0 ng/mL standard (C0).

Tube/Standard ID	Serial Dilution	Assay Buffer to add (μ L)	Standard to add	Final Conc. (ng/mL)
C7	--	--	--	100,000
C6	1:4	75	25 μ L of C7	25,000
C5	1:16	75	25 μ L of C6	6,250
C4	1:64	75	25 μ L of C5	1562.5
C3	1:256	75	25 μ L of C4	390.1
C2	1:1024	75	25 μ L of C3	97.7
C1	1:4096	75	25 μ L of C2	24.4
C0	--	75	--	0

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Sample Dilution

For serum and plasma samples, follow dilution recommendations below:
Serum or plasma samples must be diluted 100-fold with Assay Buffer as described in the table below.

Sample	Dilution	Final Dilution Fold
Serum, Plasma	2 μ L + 198 μ L (Assay Buffer)	100

Chapter 3: ASSAY PROCEDURE

The LEGENDplex™ assay can be performed in a filter plate, or in a V-bottom plate.

- The in-filter plate assay procedure requires a vacuum filtration unit for washing (see **Materials to be Provided by the End-User, page 6**).
- If the in-filter plate assay procedure is not possible or if you prefer, the assay can be performed in a V-bottom plate.

Performing the Assay Using a Filter Plate

- Allow all reagents to warm to room temperature (20-25°C) before use.
 - Set the filter plate on an inverted plate cover at all times during assay setup and incubation steps, so that the bottom of the plate does not touch any surface. Touching a surface may cause leakage.
 - Keep the plate upright during the entire assay procedure, including the washing steps, to avoid losing beads.
 - The plate should be placed in the dark or wrapped with aluminum foil for all incubation steps.
 - **Standards and samples should be run in duplicate and arranged on the plate in a vertical configuration convenient for data acquisition and analysis (as shown in attached PLATE MAP, page 24). Be sure to load standards in the first two columns. If an automation device is used for reading, the orientation and reading sequence should be carefully planned.**
1. Pre-wet the plate by adding 100 µL of LEGENDplex™ 1X Wash Buffer to each well and let it sit for 1 minute at room temperature. To remove the excess volume, place the plate on the vacuum manifold and apply vacuum. Do not exceed 10" Hg of vacuum. Vacuum until wells are drained (5-10 seconds). Blot excess Wash Buffer from the bottom of the plate by pressing the plate on a stack of clean paper towels. Place the plate on top of the inverted plate cover.

For measuring serum or plasma samples, load the plate as shown in the table below (in the order from left to right):

	Assay Buffer	Standard	Sample*	Detection [#]
Standard Wells	25 µL	25 µL	---	25 µL
Sample wells	25 µL	---	25 µL	25 µL

*See **Sample Dilution on page 10**

[#] See **Detection Preparation on page 8**

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2. Vortex mixed beads bottle for 30 seconds. Add 25 μL of beads to each well. The volume should be 100 μL in each well after beads addition. (Note: During addition of the beads, shake mixed beads bottle intermittently to avoid bead settling).
3. Seal the plate with a plate sealer. **To avoid plate leaking, do not apply positive pressure to the sealer when sealing the plate.** Wrap the entire plate, including the inverted plate cover, with aluminum foil. Place the plate on a plate shaker, secure it with a rubber band and shake at approximate 500 rpm for 2 hours at room temperature.
4. **Do not vacuum!** Add 25 μL of SA-PE to each well directly.
5. Seal the plate with a fresh plate sealer. Wrap the entire plate, including the inverted plate cover, with aluminum foil. Place the plate on a plate shaker and shake at approximate 500 rpm for 30 minutes at room temperature.
6. **Do not invert the plate!** Place the plate on the vacuum manifold and apply vacuum as before in Step 1. Add 200 μL of 1X Wash Buffer to each well. Remove Wash Buffer by vacuum filtration. Blot excess Wash Buffer from the bottom of the plate with an absorbent pad or paper towels. Repeat this washing step once more.
7. Add 150 μL of 1X Wash Buffer to each well. Resuspend the beads on a plate shaker for 1 minute.
8. Read samples on a flow cytometer, preferably within the same day of the assay (Note: Prolonged sample storage can lead to reduced signal).

If the flow cytometer is equipped with an autosampler, read the plate directly using the autosampler. **Please be sure to program the autosampler to resuspend beads in the well immediately before taking samples. The probe height may need to be adjusted when using an autosampler.**

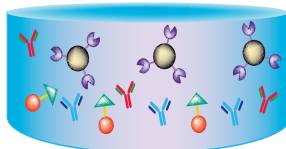
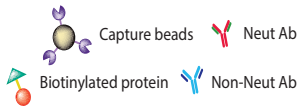
If an autosampler is not available, the samples can be transferred from the filter plate to micro FACS (or FACS) tubes and read manually.

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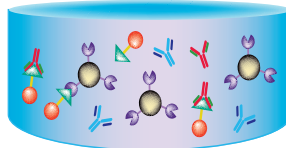
Assay Procedure Summary for Filter Plate

Add 100 μL 1X Wash Buffer to filter plate wells

Vacuum to remove excess buffer



Incubate 2 hours, RT, shaking



Without washing, add 25 μL SA-PE
Incubate 30 min, RT, shaking

Wash 2 times using vacuum filtration unit
Add 150 μL of 1x Wash Buffer
Read on a flow cytometer

(Refer to Assay Procedure)

Add to the plate:
25 μL Assay Buffer to all wells
25 μL diluted standard to standard wells or, 25 μL diluted sample to sample wells
25 μL diluted detection to all wells.
25 μL of beads (1X) to all wells

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Performing the Assay Using a V-bottom Plate

- Allow all reagents to warm to room temperature (20-25°C) before use.
- Keep the plate upright during the entire assay procedure, including the washing steps, to avoid losing beads.
- The plate should be placed in the dark or wrapped with aluminum foil for all incubation steps.
- Standards and samples should be run in duplicate and arranged on the plate in a vertical configuration convenient for data acquisition and analysis (as shown in attached PLATE MAP, page 24). Be sure to load standards in the first two columns. If an automation device is used for reading, the orientation and reading sequence should be carefully planned.

For measuring serum or plasma samples, load the plate as shown in the table below (in the order from left to right):

	Assay Buffer	Standard	Sample*	Detection [#]
Standard Wells	25 µL	25 µL	---	25 µL
Sample wells	25 µL	---	25 µL	25 µL

*See **Sample Dilution on page 10**

[#]See **Detection Preparation on page 8**

9. Vortex beads (1X) for 30 seconds. Add 25 µL of beads to each well. The total volume should be 100 µL in each well after beads addition. (Note: During beads addition, shake mixed beads bottle intermittently to avoid bead settling).
10. Seal the plate with a plate sealer. Cover the entire plate with aluminum foil to protect the plate from light. Shake at 800 rpm on a plate shaker for 2 hours at room temperature (**Depending on the shaker, the speed may need to be adjusted. The optimal speed is one that is high enough to keep beads in suspension during incubation, but not too high that it may cause sample to spill from the wells**).
11. **Do not wash the plate!** Add 25 µL of SA-PE to each well directly.
12. Seal the plate with a new plate sealer. Wrap the entire plate with aluminum foil and shake the plate on a plate shaker at approximate 800 rpm for 30 minutes at room temperature.
13. Centrifuge the plate at 1050 rpm (~250 g) for 5 minutes, using a swinging bucket rotor (G.H 3.8) with microplate adaptor (Please refer to **Materials to be Provided by the End-User, page 6**). Do not use excessive centrifugation speed as it may make it harder to resuspend beads in later steps. **Make sure the timer of the centrifuge works properly and standby to make sure the centrifuge reaches preset speed.**
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14. Immediately after centrifugation, dump the supernatant into a biohazard waste container by quickly inverting and flicking the plate **in one continuous and forceful motion**. The beads pellet may or may not be visible after dumping the supernatant. Loss of beads should not be a concern as the beads will stay in the tip of the well nicely. Blot the plate on a stack of clean paper towel and drain the remaining liquid from the well as much as possible. Be careful not to disturb the bead pellet.

Alternatively, removal of the supernatant may be completed using a multichannel pipette set at 100 μL . Try to remove as much liquid as possible without removing any beads. Be sure to change pipette tips between each row or column.

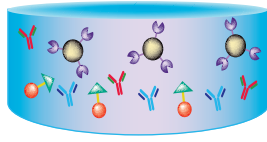
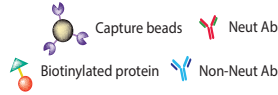
15. Wash the plate by dispensing 200 μL of 1X Wash Buffer into each well and incubate for one minute. Repeat step 13 and 14 above. A second wash is optional, but may help reduce background.
16. Add 150 μL of 1X Wash Buffer to each well. Resuspend the beads by pipetting.
17. Read samples on a flow cytometer, preferably within the same day of the assay (Note: Prolonged sample storage can lead to reduced signal).

If the flow cytometer is equipped with an autosampler, the samples can be read directly. **Please be sure to program the autosampler to resuspend beads in the well immediately before taking samples. The probe height may need to be adjusted when using an autosampler.**

If an autosampler is not available, the samples can be transferred from the plate to micro FACS (or FACS) tubes and read manually.

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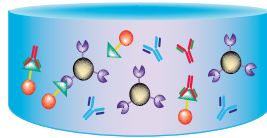
Assay Procedure Summary for V-bottom Plate



Incubate 2 hours, RT, shaking

(Refer to Assay Procedure)

- Add to the plate:
- 25µL Assay Buffer to all wells
- 25µL diluted standard to standard wells or, 25µL diluted sample to sample wells
- 25µL diluted detection to all wells
- 25µL of beads (1X) to all wells



Without washing, add 25 µL SA-PE
Incubate 30 min, RT, shaking

- Spin down beads, remove supernatant
- Wash 1 time
- Add 150 µL of 1x Wash Buffer
- Read on a flow cytometer

Chapter 4: FLOW CYTOMETER SETUP

In order to generate reliable data, the flow cytometer must be set up properly before data acquisition.

The setup instructions have been removed from this manual and uploaded onto our website to save paper.

To access the setup instructions, please visit: www.biolegend.com/legendplex and click on the **Instrument Setup** tab.

Chapter 5: DATA ACQUISITION AND ANALYSIS

Data Acquisition

1. Before reading samples, make sure that the flow cytometer is set up properly.
2. Create a new template or open an existing template (for details on how to create a cytometer-specific template, please refer to the Flow Cytometer Setup Guide).
3. Vortex each sample for 5 seconds before analysis.
4. Set the flow rate to low. Set the number of beads to be acquired to about 300 per analyte (e.g., acquire 1,200 beads for a 4-plex assay or 3,000 beads for a 10-plex assay). Do not set to acquire total events as samples may contain large amounts of debris. Instead, create a large gate to include both Beads A and Beads B (gate A+B) and set to acquire the number of events in gate A + B. This will exclude majority of the debris.

Note: Do not acquire too few or too many beads. Too few beads acquired may result in high CVs and too many beads acquired may result in slow data analysis later.

5. Read samples.

When reading samples, set the flow cytometer to setup mode first and wait until bead population is stabilized before recording or switching to acquisition mode.

To simplify data analysis using the LEGENDplex™ Data Analysis Software, read samples in the same order as shown on the PLATE MAP attached at the end of the manual. For an in-plate assay, read column by column (A1, B1, C1...A2, B2, C2...).

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When naming data files, try to use simple names with a consecutive numbering for easy data analysis (e.g. for standards, C0.001, C0.002, C1.003, C1.004, C2.005, C2.006, C3.007, C3.008, ... C7.015, C7.016; for samples, S1.017, S1.018, S2.019, S2.020, S3.021, S3.022...).

Store all FCS files in the same folder for each assay. If running multiple assays, create a separate folder for each assay.

6. Proceed to data analysis using LEGENDplex™ Data Analysis Software when data acquisition is completed.

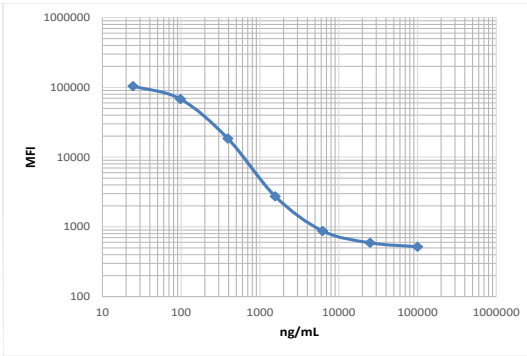
Data Analysis

- The FCS file generated on a flow cytometer should be analyzed using Bio-Legend's LEGENDplex™ Data Analysis Software. The LEGENDplex™ Data Analysis Software can be downloaded for free here: www.biolegend.com/legendplex.

Chapter 6: ASSAY CHARACTERIZATION

Representative Standard Curve

This standard curve was generated using the LEGENDplex™ SARS-CoV-2 Neut. Ab Assay for demonstration purposes only. A standard curve must be run with each assay.



Assay Sensitivity

The assay sensitivity or minimum detectable concentration (MDC) is the theoretical limit of detection calculated using the LEGENDplex™ Data Analysis Software by applying a 5-parameter curve fitting algorithm. The sensitivity determined based on LLOD values.

Analyte	Sensitivity (ng/mL) (N = 12)
Neut. Ab	7.5+5.28

Cross-Reactivity

Anti-SARS-CoV-2 S Protein S1 Recombinant Antibody (Clone# AM006415, Cat# 938602) was tested individually at the indicated concentrations below using the LEGENDplex™ SARS-CoV-2 Neut. Ab Assay, with negligible cross-reactivity observed for non-neutralizing antibody.

Analyte	Conc. (ng/mL)
Anti-SARS-CoV-2 S Protein S1	10000

LEGENDplex™ SARS-CoV-2 Neut. Ab Assay (1-plex)

Accuracy (Spike Recovery)

For spike recovery in cell culture medium, RPMI or DMEM with 10% FBS were spiked with target proteins at three different levels within the assay range. For spike recovery in serum and plasma, samples were first diluted 50-fold* with Assay Buffer and spiked with target proteins at three different levels within the assay range.

The spiked samples were then assayed, and the measured concentrations were compared with the expected values.

Analyte	% of Spike Recovery		
	Tissue Culture Supernatant (N= 2)	Serum (N = 8)	Plasma (N = 24)
Neut. Ab	90%	90%*	91%*

Linearity of Dilution

Serum and plasma samples were initially diluted 50-fold in Assay Buffer and then spiked with target proteins with known concentrations in the assay range. The spiked samples were then serially diluted 2, 4, and 8-fold with Assay Buffer and assayed.*Refer to the table on the next page.

The measured concentrations of serially diluted samples were then compared with the concentration of the lowest dilution based on serial dilution factor used.

Analyte	% Linearity		
	Tissue Culture Supernatant (N= 2)	Serum (N = 4)	Plasma (N = 24)
Neut. Ab	98%	97%*	99%*

Intra-Assay Precision

Two samples with different concentrations of each target protein were analyzed in one assay with 16 replicates per sample. The intra-assay precision is shown below.

Analyte	Sample	Mean (ng/mL)	STDEV	%CV
Neut. Ab	Sample 1	8013	608	7.59%
	Sample 2	208	15	7.28%

Inter-Assay Precision

Two samples with different concentrations of each target protein were analyzed in ten independent assays with four replicates per sample. The inter-assay precision is shown below.

Analyte	Sample	Mean (ng/mL)	STDEV	%CV
Neut. Ab	Sample 1	1420	170	12.1%
	Sample 2	5921	510	8.6%

Biological Samples

The values in this section are provided for reference only. The assays provided in this kit are intended for research use only.

Human serum, citrated plasma, EDTA plasma and Heparin plasma samples from matched sets collected from 20 healthy human donors prior to the COVID-19 pandemic were tested for endogenous levels of the target antibodies. Pre-existing SARS-CoV-2 reactive antibodies have been reported at low levels in healthy donor cohorts¹, inconclusive data may reflect this phenomenon and can be verified with clinical information. Concentrations reported between the minimum detectable concentration and C1 (24 ng/mL) were considered inconclusive and excluded from % detectable. Users may modify this cutoff. The concentrations after multiplying by the dilution factor are shown below (in ng/mL).

Sample type	Serum	Citrate Plasma	EDTA Plasma	Heparin Plasma
# of samples	20	20	20	20
Median	0	1500	1000	2000
Minimum	ND	ND	ND	ND
Maximum	0	2000	3000	3000
% Detected	0	0	10%	5%

¹K.W. Ng *et al.*, *Science* 10.1126/science.abe1107 (2020)

TROUBLESHOOTING

Problem	Possible Cause	Solution
Bead population shifting upward or downward during acquisition	The strong PE signal from high concentration samples or standards may spill over to classification Channel (e.g., FL3/FL4/APC) and mess up the bead separation.	Optimize instrument settings using Kit Setup Beads, and make appropriate compensation between channels.
Filter plate will not vacuum or some wells clogged	Vacuum pressure is insufficient or vacuum manifold does not seal properly.	Increase vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds. Clean the vacuum manifold and make sure no debris on the manifold. Press down the plate on the manifold to make a good seal.
	Samples have insoluble particles or sample is too viscous (e.g., serum and plasma samples)	<p>Centrifuge samples just prior to assay setup and use supernatant. If high lipid content is present, remove lipid layer after centrifugation. Sample may need dilution if too viscous.</p> <p>If some wells are still clogged during washing, try the following:</p> <ol style="list-style-type: none"> 1). Add buffer to all the wells, pipette up and down the clogged wells and vacuum again. 2). Use a piece of clean wipe, wipe the under side of the clogged wells and vacuum again. 3). Take a thin needle (e.g., insulin needle), while holding the plate upward, poke the little hole under each of the clogged wells and vacuum again. Do not poke too hard or too deep as it may damage the filter and cause leaking.
	Filter plate was used without pre-wet.	Pre-wet plate with wash buffer before running the assay.

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Insufficient bead count or slow reading	Beads inappropriately prepared	Sonicate bead vials and vortex just prior to addition. Agitate mixed beads intermittently in reservoir while pipetting this into the plate.
	Samples cause beads aggregation due to particulate matter or viscosity.	Centrifuge samples just prior to assay setup and use supernatant. If high lipid content is present, remove lipid layer after centrifugation. Sample may need dilution if too viscous.
	Beads were lost during washing for in-tube assay	Make sure beads are spun down by visually check the pellet (beads are in light blue or blue color). Be very careful when removing supernatant during washing.
	Probe might be partially clogged.	Sample probe may need to be cleaned, or if needed, probe should be removed and sonicated.
Plate leaked	Vacuum pressure set too high	Adjust vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds. Do not exceed 10" Hg of vacuum.
	Plate set directly on table or absorbent towels during incubations or reagent additions	Set plate on plate holder or raised edge so bottom of filter is not touching any surface.
	Liquid present on the underside of the plate after vacuum	After washing, press down plate firmly on a stack of clean paper towels to dry the underside of the plate.
	Pipette touching and damaged plate filter during additions.	Pipette to the side of wells.
High Background	Background wells were contaminated	Avoid cross-well contamination by changing tips between pipetting when performing the assay using a multichannel pipette.
	Insufficient washes	The background may be due to non-specific binding of SA-PE. Increase number of washes.
Debris (FSC/SSC) during sample acquisition	Debris or platelet may exist in sample solution.	Centrifuge samples before analyzing samples. Remove platelet as much as possible.

PLATE MAP (for in-plate assay)

	1	2	3	4	5	6	7	8	9	10	11	12
A	C0	C4	Sample1	Sample5	Sample 9	Sample 13	Sample 17	Sample 21	Sample 25	Sample 29	Sample 33	Sample 37
B	C0	C4	Sample1	Sample5	Sample 9	Sample 13	Sample 17	Sample 21	Sample 25	Sample 29	Sample 33	Sample 37
C	C1	C5	Sample2	Sample6	Sample 10	Sample 14	Sample 18	Sample 22	Sample 26	Sample 30	Sample 34	Sample 38
D	C1	C5	Sample2	Sample6	Sample 10	Sample 14	Sample 18	Sample 22	Sample 26	Sample 30	Sample 34	Sample 38
E	C2	C6	Sample3	Sample7	Sample 11	Sample 15	Sample 19	Sample 23	Sample 27	Sample 31	Sample 35	Sample 39
F	C2	C6	Sample3	Sample7	Sample 11	Sample 15	Sample 19	Sample 23	Sample 27	Sample 31	Sample 35	Sample 39
G	C3	C7	Sample4	Sample8	Sample 12	Sample 16	Sample 20	Sample 24	Sample 28	Sample 32	Sample 36	Sample 40
H	C3	C7	Sample4	Sample8	Sample 12	Sample 16	Sample 20	Sample 24	Sample 28	Sample 32	Sample 36	Sample 40



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