
COVID-19 Immune monitoring

LegendPlex Multi-Analyte Flow Cytometry assay

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Notes: This SOP is based on Biolegend's LegendPlex assay protocols for the use of the Anti-Viral Response panel (Cat No: 740390) and Human Th panels (Cat No: 740722).

	<i>Print name</i>	<i>Signature</i>	<i>Date</i>
Authorised by			

1. Preparations

General:

- Allow all reagents to warm to room temperature (20-25°C) before use.
- Defrost one vial of plasma/patient (100ul)
- When using frozen samples, it is recommended that samples are thawed completely, mixed and centrifuged to remove particulates prior to use.
- Prepare a bucket with tissues soaked in disinfectant P3 and place it in a hood where liquid waste will be disposed of.
- Plate shaker should be kept in the TC hood
- All work and disposing of supernatants during wash steps should be done in the TC hood.
- The plate should be placed in the dark or wrapped with aluminum foil for all incubation steps.
- Run standards in duplicate. Run 1 well/sample. Arranged on the plate in a vertical configuration convenient for data acquisition and analysis (example PLATE MAP below). 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.
- **Sample Dilution:** Serum samples are diluted 2-fold with Assay Buffer before being tested (e.g. dilute 40 µL of sample with 40 µL of Assay Buffer). If further sample dilution is desired, dilution should be done with Matrix B to ensure accurate measurement. Adding serum samples without dilution will result in low assay accuracy.

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

LEGENDplex™ Human Th Cytokine Panel

Reagent Preparations:

Preparation of Antibody-Immobilized Beads

- If pre-mixed beads are provided in the kit:

Sonicate pre-mixed 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.

- If individual beads (13X) are provided in the kit:

The individual beads (13X) should be mixed with each other and diluted to 1X final concentration with Assay Buffer prior to use. To mix the beads, follow the steps below (a 5-plex subpanel is used as an example):

1. Sonicate each bead vial for 1 minute in a sonicator bath and then vortex for 30 seconds to completely resuspend the beads.
2. Calculate the amount of mixed and diluted beads needed for the assay. Prepare extra to compensate for pipetting loss. Each reaction needs 25 μL of mixed and diluted beads. For 50 reactions, prepare 1.5 mL of mixed beads. For 100 reactions, prepare 3 mL of mixed beads.
3. To make 1.5 ml of 5-plex 1X diluted beads, transfer 115 μL of each of the 5 individual beads (13X) to a fresh tube (total bead volume = 575 μL) and add 925 μL of Assay Buffer to make the final volume of 1.5 mL.

Note: To save beads and run more samples per kit, prepare bead mix for half the samples number as described above and dilute that two times with Assay buffer.

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.

Preparation of Matrix B

- Add 5.0 mL LEGENDplex™ Assay Buffer to the bottle containing lyophilized Matrix B. Allow at least 15 minutes for complete reconstitution. Vortex to mix well. Leftover reconstituted Matrix B should be stored at $\leq -70^\circ\text{C}$ for up to one month.

Standard Preparation

1. Prior to use, reconstitute the lyophilized Human Th Panel Standard Cocktail with 250 μL Assay Buffer.
2. Mix and allow the vial to sit at room temperature for 10 minutes, and then transfer the standard to an appropriately labelled polypropylene microcentrifuge tube. This will be used

as the top standard C7. Note: The top standard concentrations of analytes in this panel was 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 μL of Assay Buffer to each of the six tubes. Prepare 1:4 dilution of the top standard by transferring 25 μL 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 pg/mL as an example). Assay Buffer will be used as the 0 pg/mL standard (C0).

Tube/Standard ID	Serial Dilution	Assay Buffer to add (μL)	Standard to add	Final Conc. (pg/mL)
C7	--	--	--	10,000
C6	1:4	75	25 μL of C7	2,500
C5	1:16	75	25 μL of C6	625
C4	1:64	75	25 μL of C5	156.3
C3	1:256	75	25 μL of C4	39.1
C2	1:1024	75	25 μL of C3	9.8
C1	1:4096	75	25 μL of C2	2.4
C0	--	75	--	0

2. Protocol

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

	Assay Buffer	Matrix B	Standard	Sample*
Standard Wells	---	25 μ L	25 μ L	---
Sample wells	25 μ L	---	---	25 μ L

*See Sample Dilution

2. Vortex mixed beads for 30 seconds. Add 25 μ L of mixed beads (diluted 1:2 in assay buffer) to each well. The total volume should be 75 μ L in each well after beads addition. (Note: During beads addition, shake mixed beads bottle intermittently to avoid bead settling).

3. Seal the plate and put a lid on it. Cover the entire plate with aluminium foil to protect the plate from light. Shake at 600 rpm on a plate shaker for 1.5 hours at room temperature.

4. Centrifuge the plate at 1050 rpm (~250 g) for 5 minutes, using buckets with lids if available. **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 pre-set speed.

5. Immediately after centrifugation, in a TC hood, remove the supernatant using a multichannel pipette set at 75ul and disposing of the liquid in a bucket containing disinfectant P3 steril. Try to remove as much liquid as possible without removing any beads. Be sure to change pipette tips between each row or column.

Note: In subsequent wash steps dump the supernatant into a bucket which is placed in a TC hood and contains paper towels soaked in disinfectant P3 steril by quickly inverting and flicking the plate in one continuous and forceful motion. Do not worry about losing beads even if the pellet is not visible. 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. Dispose of the paper towel in the bucket. Be careful not to disturb the bead pellet. After each wash, re-apply dry towel in the bucket to avoid splashing in the subsequent flicking steps. Alternatively, removal of the supernatant may be completed using a multichannel pipette set at 75 μ L and disposing of the liquid in a bucket containing disinfectant P3 steril in the TC hood.

6. Wash the plate by dispensing 200 μ L of 1X Wash Buffer into each well and incubate for 1 minute. Repeat step 4 and 5 above. A second wash is optional, but may help reduce background.

7. Add 25 μ L of Detection Antibodies (diluted 1:2 in assay buffer) to each well.

8. Seal the plate and put a lid on it. Cover the entire plate with aluminum foil to protect the plate from light. Shake at 600 rpm on a plate shaker for 45min at room temperature.

9. Do not wash the plate! Add 25 μ L of SA-PE (diluted 1:2 in assay buffer) to each well directly.

10. Seal the plate and put a lid on it. Wrap the entire plate with aluminium foil and shake the plate on a plate shaker at approximate 600 rpm for 30 minutes at room temperature.

11. Repeat step 4, and 5.

12. Wash the plate by dispensing 200 μ L of 1X Wash Buffer into each well and incubate for one minute. Repeat step 4 and 5 above. This washing step is optional but helps to reduce the background.

13. Add 200 μ L of 1X Wash Buffer to each well. Resuspend the beads by pipetting.

14. 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.

Note: After finishing work, clean the inside and outside of TC hoods and centrifuge with 70% ethanol. Place in the TC hoods all plastics and waste that have been used during the experiment and turn on the UV programme. Dispose of all waste on the following day.

3. Flow Cytometer Setup

For the BD FACS machines running FACSDiva™, use the PE channel for reporter and the APC channel for beads classification. In general, there is no need for compensation between these channels if the machine is set up properly following the setup procedure described below.

This setup procedure is required under the following situations:

- You are running the LEGENDplex kit for the first time.
- It has been over a month since the procedure was last performed.
- Your flow cytometer has been serviced since you last performed this procedure.

This setup process is not needed if you have run this experiment before and have access to a saved experiment template (The settings will be saved in the final step of this setup procedure and any settings saved can be imported to a new experiment).

1. Start up the Instrument

2. Obtain a Template for Data Acquisition

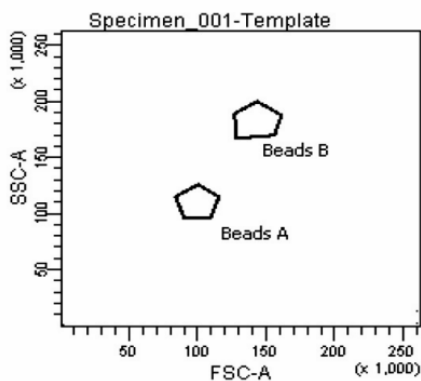
If a template is not yet available, create a new template by following the instructions in step 2. After a template is created, save the file in D:\BDExport\Templates\Experiment. Do not change the name of the Templates folder.

If you have already created a template for the flow cytometer, open that template and proceed to Step 3. To open an existing template, select Experiment > New Experiment. A list of templates saved in D:\BDExport\Templates\ Experiment will pop up. Select the desired template from the list.

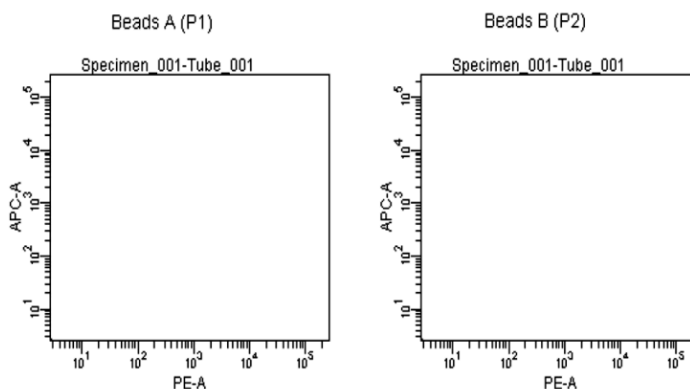
To create a new template, follow the instructions below:

2.1 From the BD FACSDiva™ software, go to Experiment > New Experiment.

2.2 In the global worksheet, open the worksheet. Create a dot plot with FSC (forward scatter) for X-axis and SSC (side scatter) for Y-axis. Be sure to set FSC and SSC to linear mode. Create two gates and label them Beads A and Beads B (as shown below).



2.3 Create two dot plots with PE for X-axis, APC for Y-axis (shown below), gated on Beads A (left panel below) and Beads B (right panel below), respectively. Create one dot plot with FITC for X-axis, APC for Y-axis, gated on Beads A and Beads B (graph not shown). The plots should all be in log mode.



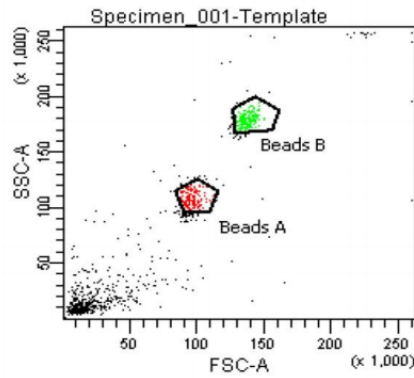
2.4 Save the document as “LEGENDplex Template for FACS Diva in D:\BDExport\Templates\Experiment and proceed to the next step of setup.

3. Set up PMT Voltages

The Setup Beads 3: Raw Beads are used to set up the PMT voltage of the classification channel APC, reporter channel PE, and FITC channel. The Setup Beads 1: FITC Beads and 2: PE Beads are not needed for this setup because no compensation is required if the setup procedure described here is closely followed.

Follow the instructions below for setting up the PMT settings:

- 3.1** Vortex the vial of Raw Beads for 30 seconds to resuspend the beads.
- 3.2** Transfer 400 µL of the Raw Beads to a fresh FACS tube.
- 3.3** Set the flow cytometer flow rate to low. Run the Raw Beads. Adjust the settings for FSC and SSC so that both bead populations are visible (shown below). Pause and restart acquisition frequently during the setup procedure to refresh the beads populations after adjusting settings.

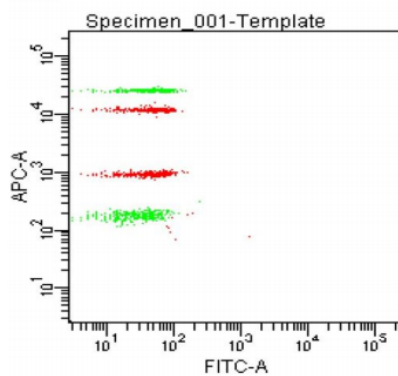


3.4 Continue adjusting the settings so that Beads A and Beads B are well separated and the FSC and SSC readings are >50 (x1000).

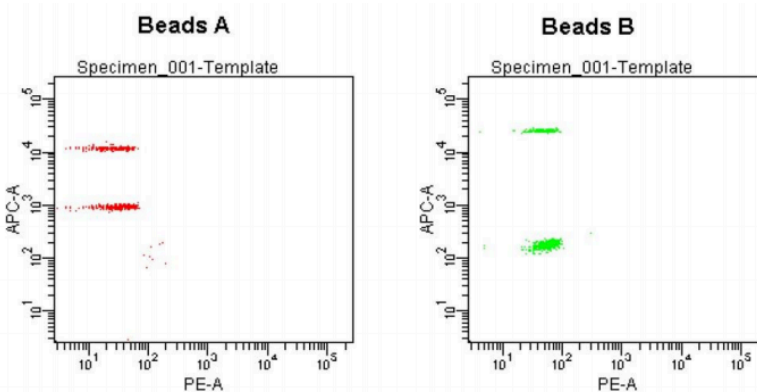
3.5 Move the gates for Beads A and Beads B so that the smaller beads fall into Beads A gate and the larger beads fall into Beads B gate (as shown above).

3.6 Adjust the FITC setting so that the FITC signal for the majority of beads is between 1×10^1 and 1×10^2 (as shown below).

Beads B A+ Beads B



3.7 Adjust the PE setting so that the PE signal for the majority of beads is between 1×10^1 and 1×10^2 (as shown below).



3.8 Adjust the APC settings so that the the APC fluorescence intensities of all bead populations are between 1×10^2 and 5×10^4 (as shown above).

3.9 Save the document again for future use. To save your assay-specific settings, in the browser, right-click Cytometer Settings and select Save to Catalog. Name the file,

and then click OK. To import the saved setting for a new experiment, right-click on cytometer settings and select import settings.

3.10 The flow cytometer is now ready for sample analysis.

4. Data Acquisition

1. Before reading samples, make sure that the flow cytometer is set up properly.
2. Open an existing template
3. Vortex each sample for 5 seconds before analysis. If plate reading – set well resuspension before each sample acquisition – sample flow rate 1, mixing volume 75µl, mixing speed 200µl/sec, number of mixes 2, wash volume 200µl.
4. Set the flow rate to low. Set the number of beads to be acquired to about 300 per analyte (e.g., acquire 2,400 beads for a 8-plex assay or 4000 beads for a 13-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. For an in-plate assay, read column by column (A1, B1, C1...A2, B2, C2...).

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...)