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LEGENDplex™

Multi-Analyte Flow Assay Kit

Cat. No. 741186, Human CD8/NK Panel (13-plex) with Filter Plate V02

Cat. No. 741187, Human CD8/NK Panel (13-plex) with V-bottom Plate V02

Please read the entire manual before running the assay.

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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.

Table of Contents Page

Chapter 1: KIT DESCRIPTION

Introduction

CD8 cytotoxic lymphocytes and Natural Killer (NK) cells play important roles in the host defense. $CD8^+$ T and NK cells produce cytokines and utilize Fas/FasL, granzymes, perforin and granulysin to eradicate tumor cells, virus-infected cells and intracellular pathogens. Accurate measurement of the expression of these biomarkers is critical for in-depth understanding of the immune responses and many disease processes.

The LEGENDplex™ Human CD8/NK Panel V02 is a bead-based multiplex assay, utilizing fluorescence–encoded beads suitable for use on various flow cytometers. This panel allows simultaneous quantification of 13 human proteins, including IL-2, 4, 6, 10, 17A, IFN-γ, TNF-α, soluble Fas, soluble FasL, granzyme A, granzyme B, perforin and granulysin. This panel provides high sensitivities and broad dynamic range. The panel has been validated for use on serum, plasma and cell culture supernatant samples.

The Human CD8/NK Panel V02 is designed to allow flexible customization. For mix and match within the panel, please visit **www.biolegend.com/legendplex.**

This assay is for research use only.

Principle of the Assay

BioLegend's LEGENDplex™ assays are bead-based immunoassays using the same basic principle as sandwich immunoassays.

Beads are differentiated by size and internal fluorescence intensities. Each bead is conjugated with a specific antibody on its surface and serves as the capture bead for that particular analyte. When a selected panel of capture beads is mixed and incubated with a sample containing target analytes specific to the capture antibodies, each analyte will bind to its specific capture beads. After washing, a biotinylated detection antibody cocktail is added, and each detection antibody in the cocktail will bind to its specific analyte bound on the capture beads, thus forming capture bead-analyte-detection antibody sandwiches. Streptavidin-phycoerythrin (SA-PE) is subsequently added, which will bind to the biotinylated detection antibodies, providing fluorescent signal intensities in proportion to the amount of bound analytes.

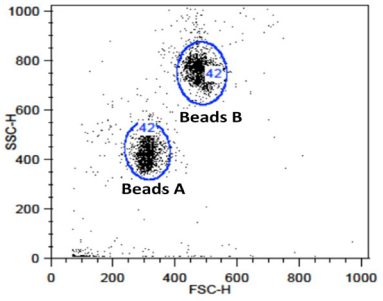
Since the beads are differentiated by size and internal fluorescence intensity on a flow cytometer, analyte-specific populations can be segregated and PE fluorescent signal quantified. The concentration of a particular analyte is determined using a standard curve generated in the same assay.

Beads Usage

The Human CD8/NK Panel V02 uses two sets of beads. Each set has a unique size that can be identified based on their forward scatter (FSC) and side scatter (SSC) profiles (Beads A and Beads B, 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 7 bead populations and the larger Beads B consists of 7 bead populations (Figure 2-3).

Using a total of 13 bead populations distinguished by size and internal fluorescent dye, the Human CD8/NK Panel V02 allows simultaneous detection of 13 proteins in a single sample. Each analyte is associated with a particular bead set as indicated in Table 1.

Figure 1. Beads Differentiated by Size



Beads A = smaller beads

Beads B = larger beads

Figure 2. Beads A Classification by FL4

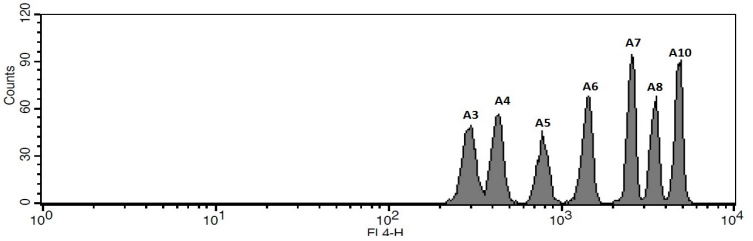
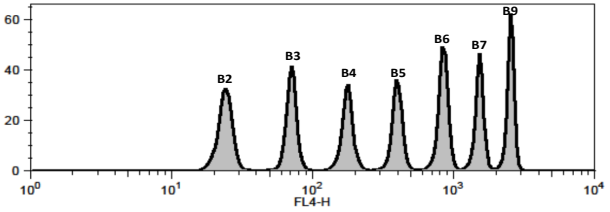


Figure 3. Beads B Classification by FL4



For Beads usage in various panels, please refer to Table 1 below:

Table 1. Beads ID and Target information

Target	Bead ID	Top Standard Concentrations (ng/mL)
IL-17A	A3	The top standard concentration of each target may vary and may subject to change from lot to lot. Please refer to the lot-specific Certificate of Analysis for this information
IL-2	A4	
IL-4	A5	
IL-10	A6	
IL-6	A7	
TNF-α	A10	
sFas	B2	
sFasL	B3	
IFN-γ	B4	
Granzyme A	B5	
Granzyme B	B6	
Perforin	B7	
Granulysin	B9	

*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 reconstituted, immediately transfer contents into polypropylene vials. DO NOT STORE RECONSTITUTED STANDARDS IN GLASS VIALS.
- Upon reconstitution, leftover standard and Matrix D4 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™ panel 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: PE Beads	1 vial	1 mL	77842
Setup Beads: Raw Beads	1 vial	1.8 mL	77844
Human CD8/NK Panel Premixed Beads V02	1 bottle	3.3 mL	750002605
Human CD8/NK Panel Detection Antibodies V02	1 bottle	3.3 mL	750002607
Human CD8/NK Panel Standard Cocktail, Lyophilized V02	1 vial	lyophilized	750002609
LEGENDplex™ SA-PE	1 bottle	3.3 mL	77743
LEGENDplex™ Matrix D4, Lyophilized	1 vial	lyophilized	750002695
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

*For kit with filter plate. **For kit with V-bottom plate. Only one plate is provided for 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	Channel Emission	Classification Channel	Channel Emission	Compensation needed?
BD FACSCalibur™ (single laser)	FL2	575 nm	FL3	670 nm	Yes
BD FACSCalibur™ (dual laser)	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	575-585 nm	APC	660 nm	No*
BD FACSAria™	PE	575 nm	APC	660 nm	No*
Beckman Coulter-CytoFLEX	PE	585 nm	APC	660 nm	No*
Gallios	PE	575 nm	APC	660 nm	No*
NovoCyte	PE	572 nm	APC	660 nm	No*

***Compensation is not required for the specified flow cytometers when set up properly, but is recommended for consistent results.**

For setting up the flow cytometers, please follow the **Flow Cytometer Setup** guide in this manual or visit: **www.biolegend.com/legendplex.**

- Multichannel pipettes capable of dispensing 5 µL to 200 µL
- Reagent reservoirs for multichannel pipette
- Polypropylene microfuge tubes (1.5 mL)
- 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).

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 (optional);

- 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, 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.
- Matrix D4 for LEGENDplex™ kits contains components of human origin and should be handled as potentially hazardous. The raw material has been screened for infectious diseases and is negative for HIV, HBV and HCV using FDA-approved test methods.
- 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 10 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 are thawed completely, mixed and centrifuged to remove particulates prior to use.

Preparation of Plasma Samples:

- Plasma collection using EDTA as an anti-coagulant is recommended. Centrifuge for 10 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 are thawed completely, mixed well and centrifuged to remove particulates.

Preparation of Tissue Culture Supernatant:

- Centrifuge the sample to remove debris and assay immediately. If not possible, aliquot and store samples at ≤-20°C. Avoid multiple (>2) freeze/thaw cycles.

Reagent Preparation

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.

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 D4 (for Serum or Plasma Samples Only)

- Add 5.0 mL LEGENDplex™ Assay Buffer to the bottle containing lyophilized Matrix D4. Allow at least 15 minutes for complete reconstitution. Vortex to mix well. Leftover reconstituted Matrix D4 should be stored at ≤-70°C for up to one month.

Standard Preparation

1. Prior to use, reconstitute the lyophilized Human CD8/NK Panel Standard Cocktail V02 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 labeled polypropylene microfuge 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 (please visit biolegend.com/en-us/legendplex to download a lot-specific certificate of analysis).

3. Label 6 polypropylene microfuge 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 10ng/mL of top standard concentration 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

Sample Dilution

- Serum or plasma samples must be diluted 2-fold with Assay Buffer before being tested (e.g. dilute 50 µL of sample with 50 µL of Assay Buffer).

  If further sample dilution is desired, dilution should be done with Matrix D4 to ensure accurate measurement.

  Adding serum or plasma samples without dilution will result in low assay accuracy and possibly, clogging of the filter plate.

- For cell culture supernatant samples, the levels of analyte can vary greatly from sample to sample. While the samples can be tested without dilutions, a preliminary experiment may be required to determine the appropriate dilution factor.

  If sample dilution is desired, dilution should be done with corresponding fresh cell culture medium or Assay Buffer to ensure accurate measurement.

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 7)**.
- If the in-filter plate assay procedure is not possible or if you prefer, the assay can be performed in a V-bottom plate. If needed, V-bottom plate can be ordered from BioLegend (Cat# 740379).

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 33). 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 cell culture supernatant samples, load the plate as shown in the table below (in the order from left to right):

	Assay Buffer	Matrix D4	Standard	Sample*
Standard Wells	25 µL	---	25 µL	---
Sample wells	25 µL	---	---	25 µL

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

	Assay Buffer	Matrix D4	Standard	Sample*
Standard Wells	---	25 µL	25 µL	---
Sample wells	25 µL	---	---	25 µL

*See **Sample Dilution**

2. Vortex mixed beads bottle for 30 seconds. Add 25 µL of mixed beads to each well. The volume should be 75 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 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.

5. Add 25 µL of Detection Antibodies to each well.

6. 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 approximately 500 rpm for 1 hour at room temperature.

7. **Do not vacuum!** Add 25 µL of SA-PE to each well directly.

8. 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.

9. Repeat step 4 above.

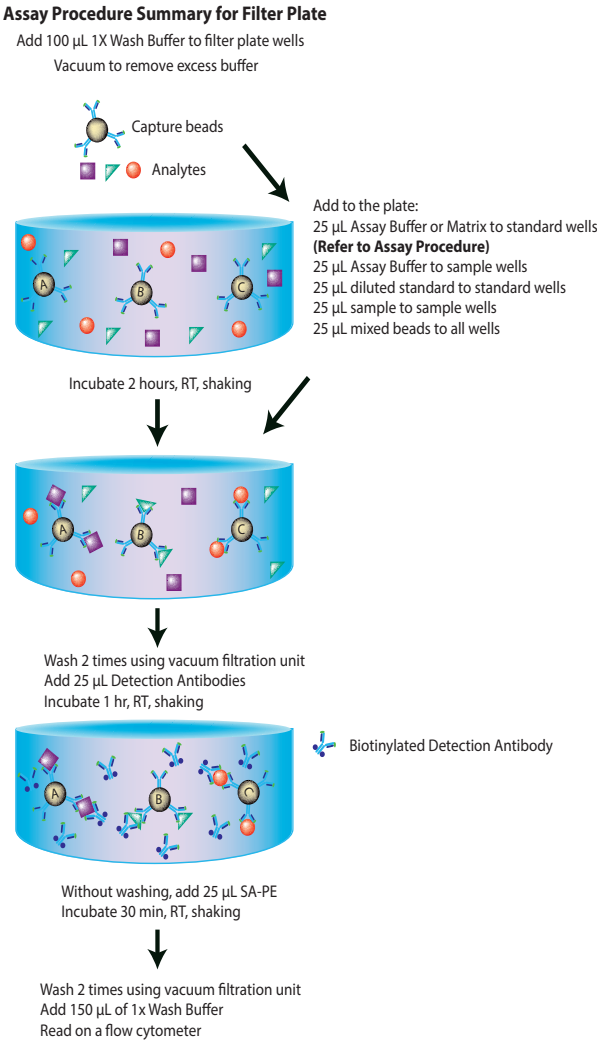
10. Add 150 µL of 1X Wash Buffer to each well. Resuspend the beads on a plate shaker for 1 minute.

11. 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.



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 33). 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. **For measuring cell culture supernatant samples,** load the plate as shown in the table below (in the order from left to right):

	Assay Buffer	Matrix D4	Standard	Sample*
Standard Wells	25 µL	---	25 µL	---
Sample wells	25 µL	---	---	25 µL

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

	Assay Buffer	Matrix D4	Standard	Sample*
Standard Wells	---	25 µL	25 µL	---
Sample wells	25 µL	---	---	25 µL

*See **Sample Dilution**

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

3. 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 so it causes spill from the wells)**.

4. 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 7).** 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.

5. Immediately after centrifugation, dump the supernatant into a sink 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. Be careful not to disturb the bead pellet.

   Alternatively, removal of the supernatant may be completed using a multichannel pipette set at 75 µL. Try to remove as much liquid as possible without removing any beads. Be sure to change pipette tips between each row or column.

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

7. Add 25 µL of Detection Antibodies to each well.

8. Seal the plate with a new plate sealer. Cover the entire plate with aluminum foil to protect the plate from light. Shake at 800 rpm on a plate shaker for 1 hour at room temperature.

9. **Do not wash the plate!** Add 25 µL of SA-PE to each well directly.

10. 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.

11. Repeat step 4, and 5.

12. Wash the plate by dispensing 200 µL 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.

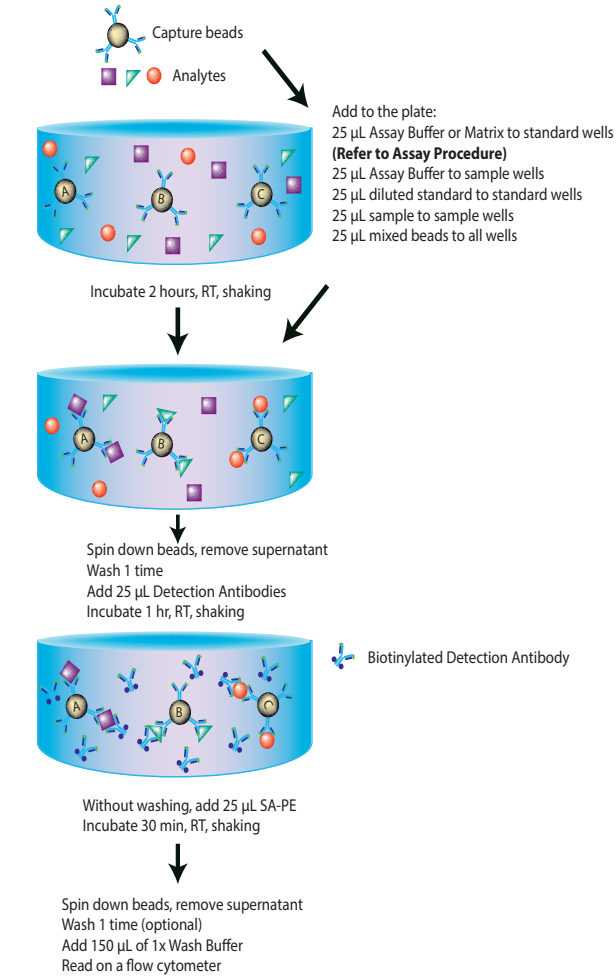
13. Add 150 µL of 1X Wash Buffer to each well. Resuspend the beads by pipetting.

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.

Assay Procedure Summary for V-bottom Plate



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 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 exlude 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 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 or RACK MAP attached at the end of the manual. For an in-plate assay, read column by column (A1, B1, C1...A2, B2, C2...). For an in-tube assay, read row by row (A1, A2, A3,...B1, B2, B3...).

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.

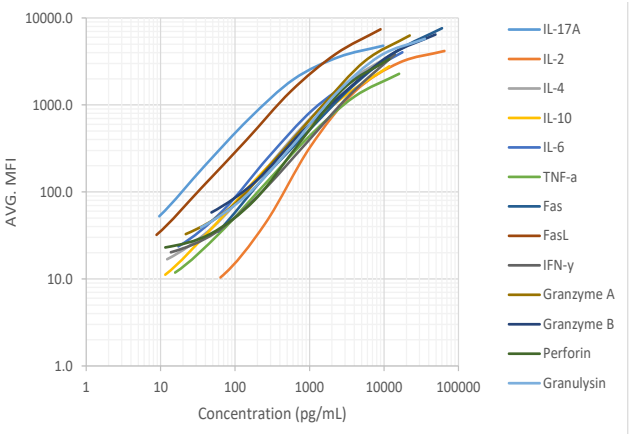
Data Analysis

The assay FCS files should be analyzed using BioLegend's LEGENDplex™ data analysis software. The program is offered free of charge with the purchase of any LEGENDplex™ assay. For further information regarding acccess to, and use of the program please visit biolegend.com/en-us/legendplex.

Chapter 6: ASSAY CHARACTERIZATION

Standard Curve

This standard curve was generated using the LEGENDplex™ Human CD8/NK Panel V02 for demonstration purpose only. A standard curve must be run with each assay.



Assay Sensitivity

The limit of detection (LOD) is the theoretical limit of detection calculated using the LEGENDplex™ Data Analysis Software by applying a 5-parameter curve fitting algorithm. Assay sensitivity presented here is LOD ± 2 STDEV.

Analyte	LOD in Cell Culture Medium (pg/mL)	LOD in Serum (pg/mL)
Human IL-17A	0.41 ± 0.28	0.57 ± 0.80
Human IL-2	13.41 ± 21.52	8.94 ± 10.59
Human IL-4	1.51 ± 2.01	1.91 ± 3.19
Human IL-10	0.78 ± 0.72	0.81 ± 0.71
Human IL-6	1.97 ± 2.62	1.81 ± 2.21
Human TNF-α	1.13 ± 0.94	1.58 ± 2.17
Human sFas	2.19 ± 1.57	2.17 ± 1.07
Human sFasL	0.56 ± 0.55	1.33 ± 2.67
Human IFN-γ	3.15 ± 4.71	7.33 ± 10.50
Human Granzyme A	2.14 ± 2.59	2.37 ± 2.66

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Human Granzyme B	2.82 ± 3.40	4.17 ± 3.81
Human Perforin	3.08 ± 4.31	3.30 ± 5.13
Human Granulysin	3.18 ± 5.57	11.47 ± 26.44

Cross-Reactivity

The following recombinant proteins were tested at 50 ng/mL using the LEGENDplex™ Human CD8/NK Panel V02. No or negligible non-specific cross-reactivity was observed

ENA-78 (CXCL5)	GRO-α	IL-6	IL-17A	IP-10 (CXCL10)	RANTES (CCL5)
Eotaxin (CCL11)	IFN-α	IL-8 (CXCL8)	IL-17F	I-TAC (CXCL11)	TARC (CCL17)
sFas	IFN-γ	IL-9	IL-18	MCP-1 (CCL2)	TNF-α
sFasL	IL-1α	IL-10	IL-21	MIG (CXCL9)	TSLP
GM-CSF	IL-1β	IL-11	IL-22	MIP-1α (CCL3)	
Granulysin	IL-2	IL-12p70	IL-23	MIP-1β (CCL4)	
Granzyme A	IL-4	IL-13	IL-27	MIP-3α (CCL20)	
Granzyme B	IL-5	IL-15	IL-33	Perforin	

Accuracy (Spike Recovery)

For spike recovery in cell culture medium, RPMI or DMEM with 10% FCS was first diluted two-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.

For spike recovery in serum (n=10) and plasma (n=10), samples were first diluted two-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 Recovery in Cell Culture Medium	% of Recovery in Serum	% of Recovery in Plasma
Human IL-17A	73%	64%	64%
Human IL-2	121%	105%	65%
Human IL-4	93%	56%	48%
Human IL-10	100%	85%	65%
Human IL-6	62%	90%	63%
Human TNF-α	98%	82%	41%
Human sFas	92%	94%	50%
Human sFasL	96%	86%	47%
Human IFN-γ	107%	117%	68%
Human Granzyme A	86%	183%	146%
Human Granzyme B	95%	132%	87%
Human Perforin	76%	103%	56%
Human Granulysin	94%	144%	178%

Linearity of Dilution

For spike linearity in cell culture medium, RPMI or DMEM with 10% FCS was first diluted two-fold with Assay Buffer and spiked with a known concentration of target proteins. The spiked samples were serially diluted 1:2, 1:4, 1:8 with Assay Buffer and assayed. The measured concentrations of serially diluted samples were compared with that of the spiked samples.

For testing linearity in serum (n=10) and plasma (n=10), samples were first diluted two-fold with Assay Buffer and spiked with a known concentration of target proteins. The spiked samples were serially diluted 1:2, 1:4, 1:8 with Matrix D4 and assayed. The measured concentrations of serially diluted samples were compared with that of the spiked samples.

Analyte	Linearity in Cell Culture Medium	Linearity in Serum	Linearity in Plasma
Human IL-17A	89%	176%	148%
Human IL-2	115%	104%	113%

Human IL-4	93%	116%	98%
Human IL-10	99%	116%	120%
Human IL-6	84%	118%	126%
Human TNF-α	97%	122%	149%
Human sFas	95%	138%	119%
Human sFasL	91%	117%	122%
Human IFN-γ	108%	100%	115%
Human Granzyme A	90%	123%	120%
Human Granzyme B	98%	111%	194%
Human Perforin	87%	144%	174%
Human Granulysin	85%	147%	112%

Intra-Assay Precision

Two samples with different concentrations of target proteins were analyzed in one assay with 16 replicates for each sample. The intra-assay precision was calculated as below.

Analyte	Sample	Mean (pg/mL)	STDEV	%CV
Human IL-17A	Sample 1	43.9	1.5	3%
	Sample 2	158.6	3.8	2%
Human IL-2	Sample 1	311.6	6.6	2%
	Sample 2	1253.7	15.0	1%
Human IL-4	Sample 1	31.0	1.4	5%
	Sample 2	118.4	3.4	3%
Human IL-10	Sample 1	57.0	1.8	3%
	Sample 2	219.3	4.2	2%
Human IL-6	Sample 1	51.4	2.3	4%
	Sample 2	232.9	7.5	3%
Human TNF-α	Sample 1	68.4	2.3	3%
	Sample 2	279.0	6.4	2%
Human sFas	Sample 1	258.2	6.9	3%
	Sample 2	1025.6	32.2	3%

Human sFasL	Sample 1	32.7	1.2	4%
	Sample 2	113.0	3.9	3%
Human IFN-γ	Sample 1	45.7	3.3	7%
	Sample 2	160.3	5.7	4%
Human Granzyme A	Sample 1	85.4	3.9	5%
	Sample 2	252.7	7.9	3%
Human Granzyme B	Sample 1	175.7	8.8	5%
	Sample 2	380.9	8.2	2%
Human Perforin	Sample 1	46.9	2.4	5%
	Sample 2	200.6	7.0	3%
Human Granulysin	Sample 1	79.1	4.2	5%
	Sample 2	179.7	5.1	3%

Inter-Assay Precision

Two samples with different concentrations of target proteins were analyzed in three independent assays with 3 replicates for each sample. The inter-assay precision was calculated as below.

Analyte	Sample	Mean (pg/mL)	STDEV	%CV
Human IL-17A	Sample 1	43.31	6.22	14%
	Sample 2	154.77	23.46	15%
Human IL-2	Sample 1	270.27	31.05	11%
	Sample 2	1009.40	88.60	9%
Human IL-4	Sample 1	42.56	3.83	9%
	Sample 2	135.41	9.83	7%
Human IL-10	Sample 1	61.29	4.77	8%
	Sample 2	227.94	18.70	8%
Human IL-6	Sample 1	83.63	9.15	11%
	Sample 2	362.07	33.32	9%
Human TNF-α	Sample 1	61.64	5.68	9%
	Sample 2	210.09	14.17	7%

Human sFas	Sample 1	313.11	30.20	10%
	Sample 2	1268.88	110.04	9%
Human sFasL	Sample 1	43.79	2.76	6%
	Sample 2	138.92	9.31	7%
Human IFN-γ	Sample 1	60.94	7.07	12%
	Sample 2	184.60	24.13	13%
Human Granzyme A	Sample 1	118.53	11.25	9%
	Sample 2	363.10	48.53	13%
Human Granzyme B	Sample 1	259.02	28.06	11%
	Sample 2	628.06	90.79	14%
Human Perforin	Sample 1	54.68	3.90	7%
	Sample 2	212.96	15.54	7%
Human Granulysin	Sample 1	103.09	7.28	7%
	Sample 2	211.49	24.70	12%

Biological Samples

Serum and Plasma (Samples are not paired)

Normal human serum samples (n=16) were tested for endogenous levels of the proteins. The concentrations measured are shown below:

Analyte	Range (pg/ml)	% of Detectable	Mean (pg/mL)
Human IL-17A	ND - 6.57	36%	3.26
Human IL-2	ND - 443.3	21%	191.5
Human IL-4	ND - 316.8	86%	50.6
Human IL-10	ND - 148.5	36%	33.4
Human IL-6	ND - 151.5	57%	21.5
Human TNF-α	ND - 4.9	64%	3.5
Human sFas	25.5 - 663.9	100%	663.9
Human sFasL	8.4 - 92.4	100%	51.4
Human IFN-γ	ND - 1202.0	43%	515.0
Human Granzyme A	22.1 - 3528.1	86%	857.7
Human Granzyme B	ND - 439.3	57%	93.6

Human Perforin	756.7 - 1965.9	100%	1367.4
Human Granulysin	463.0 - 2294.4	100%	1304.1

ND = Non-detectable

Normal human plasma samples (n=16) were tested for endogenous levels of proteins. The concentrations measured are shown below:

Analyte	Range (pg/mL)	% of Detectable	Mean (pg/mL)
Human IL-17A	ND - 12.7	32%	4.0
Human IL-2	ND - 305.6	44%	116.6
Human IL-4	ND - 201.9	65%	38.8
Human IL-10	ND - 115.4	88%	10.0
Human IL-6	ND - 117.2	38%	17.6
Human TNF-α	ND - 217.2	56%	37.3
Human sFas	2.40 - 492.4	100%	46.6
Human sFasL	1.31 - 61.0	100%	18.4
Human IFN-γ	ND - 370.8	41%	135.4
Human Granzyme A	ND - 10624.7	74%	688.4
Human Granzyme B	ND - 767.9	59%	122.5
Human Perforin	376.5 - 1189.0	100%	730.5
Human Granulysin	107.9 - 2895.7	100%	915.9

ND = Non-detectable

Cell Culture Supernatant

Human PBMCs (1 x 10^6 cells/mL) were cultured under various conditions (LPS, 100 ng/mL; CD3, 1 µg/mL plate-coated; CD28, 1 µg/mL soluble; PMA, 20 ng/mL; Ionomycin (I), 500 ng/mL; IFN-γ, 100 ng/mL). Supernatants were collected after 3 days and assayed with the LEGENDplex™ Human CD8/NK Panel V02. The results (all in pg/mL) are summarized below.

Analyte	Control Day 3	CD3 + CD28 Day 3	PMA + I Day 3	LPS Day 3	IFN-γ + LPS Day 3
IL-17A	3.6	428.7	87.1	ND	ND
IL-2	130.5	6830.0	25852.3	ND	ND
IL-4	1.0	6.1	4.5	ND	ND
IL-10	60.9	2606.6	28.2	49.4	14.2
IL-6	20.5	19229.8	11245.4	93.3	191.3
TNF-α	ND	25906.3	1129.9	4.5	1.4
sFas	3.5	8.4	3.5	5.5	26.7
sFasL	28.7	990.2	97.3	ND	ND
IFN-γ	173.7	62213.2	12972.5	ND	30787.7
Granzyme A	170.5	2967.9	516.2	5.4	2.3
Granzyme B	189.4	22776.0	10940.4	13.5	ND
Perforin	330.7	3589.0	811.5	ND	7.0
Granulysin	956.3	4483.8	729.9	38.2	25.7

ND = Non-detectable

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)	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. If some wells are still clogged during washing, try the following: 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.

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 under side 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.

Variation between duplicate samples	Beads aggregation	Sonicate and vortex the Beads prior to use.
	Multichannel pipette may not be calibrated or inconsistent pipetting	Calibrate Pipette. Ensure good pipetting practice. Prime pipette before use may help.
	Plate washing was not uniform	Make sure all reagents are vacuumed out completely in all wash steps.
	Samples may contain particulate matters.	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.
Low or poor standard curve signal	The standard was incorrectly reconstituted, stored or diluted	Follow the protocol to reconstitute, store and dilute standard. Double check your calculation.
	Wrong or short incubation time	Ensure the time of all incubations was appropriate.
Signals too high, standard curves saturated	PMT value for FL2/PE set too high	Make sure the PMT setting for the reporter channel is appropriate
	Plate incubation time was too long	Use shorter incubation time.
Sample readings are out of range	Samples contain no or below detectable levels of analyte	Make sure the experiment to generate the samples worked. Use proper positive controls.
	Samples concentrations higher than highest standard point.	Dilute samples and analyze again.
	Standard curve was saturated at higher end of curve.	Make sure the PMT setting for the reporter channel is appropriate. Use shorter incubation time if incubation time was too long
Missed beads populations during reading, or distribution is unequal	Sample may cause some beads to aggregate.	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 populations are not mixed properly	Make sure all bead populations are mixed. and in similar numbers.

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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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