BioLegend®

LEGENDplex™

Multi-Analyte Flow Assay Kit

Human Tumor-Associated Macrophage Panel

Mix and Match Subpanel

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.

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Chapter 1: KIT DESCRIPTION

Introduction

Macrophages are produced by differentiation of monocytes in response to an infection or tissue damage. They play important roles in the immune response and in the maintenance of tissue homeostasis, such as recognize, engulf, destroy, and clear foreign or harmful substances including pathogens, dying or dead cells, cellular debris and tumor cells. Many tumors recruit monocytes from circulation and transform them into an immunosuppressive subset called Tumor-Associated Macrophages (TAMs). TAMs are highly plastic and can alter their phenotype between M1 pro-inflammatory and M2 anti-inflammatory according to their location and surrounding cytokine milieu in the tumor microenvironment. M1 macrophages are considered to be functioning in the recognition and destruction of cancer cells whereas M2 macrophages are thought to promote proliferation, angiogenesis, and metastasis of cancer cells. Therefore, the phenotypes of TAMs and the soluble factors produced by them influence various aspects of cancer progression and patient prognosis.

The LEGENDplex™ Human Tumor-Associated Macrophage Panel is a bead-based multiplex assay, using fluorescence-encoded beads suitable for use on various flow cytometers. This panel allows simultaneous quantification of 13 key targets involved in the interplay of macrophages with the tumor microphage environment, including MMP-9, CXCL-9, CCL18, CD163, MMP-2, Galectin-3, CCL20 (MIP-3α), VEGF, M-CSF, PDGF-BB, G-CSF, CCL22 (MDC), and YKL-40. This assay panel provides higher sensitivity and broader dynamic range than traditional ELISA methods. The panel has been validated for use on cell culture supernatant, serum, and plasma samples.

The LEGENDplex™ Human Tumor-Associated Macrophage Panel is configured as shown below depending on sample types and suggested dilutions:

Catalog No.	Plex Size	Targets	Recommended Sample Type	Recommended Dilution Factor
741447 741448	13-plex	MMP-9, CXCL-9, CCL18, CD163, MMP-2, Galectin-3, CCL20 (MIP-3α), VEGF, M-CSF, PDGF-BB, G-CSF, CCL22 (MDC), YKL-40.	Tissue Culture	Varies
741449 741450	8-plex	CXCL-9, MMP-2, Galectin-3, CCL20 (MIP-3α), VEGF, M-CSF, G-CSF, CCL22 (MDC),	Serum, Plasma	1:4
741451 741452	5-plex	MMP-9,CCL18, CD163, PDGF-BB, YKL-40.	Serum, Plasma	1:100

The LEGENDplex™ Human Tumor-Associated Macrophage Panel is designed to allow flexible customization within the panel. For mix and match within the panel, please visit **https://www.biolegend.com/en-us/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 set is conjugated with a specific antibody on its surface and serves as the capture beads 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.

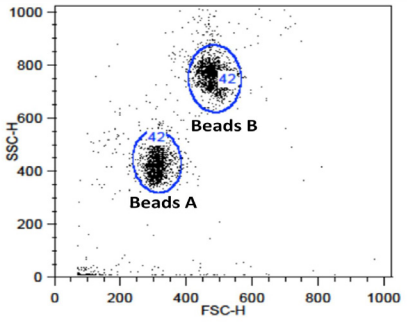
Since the beads are differentiated by size and internal fluorescence intensity on a flow cytometer, analyte-specific populations can be segregated and quantified by the PE fluorescent signal. The concentration of a particular analyte is determined by a standard curve generated in the same assay.

Beads Usage

The Human Tumor-Associated Macrophage Panel 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, 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. Each set of beads consists of 7 bead populations (Figure 2-3).

Using a total of 13 bead populations out of 14 distinguished by size and internal fluorescent dye, the Human Tumor-Associated Macrophage Panel allows simultaneous detection of 13 targets in one sample test. Each analyte is associated with a particular bead set as indicated (Figures 2-3 and Table 1).

Figure 1. Beads Differentiated by Size



Beads A = smaller beads

Beads B = larger beads

Figure 2. Beads A Classification by FL4

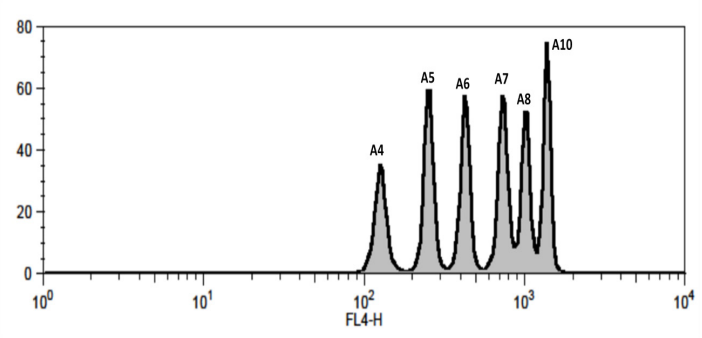
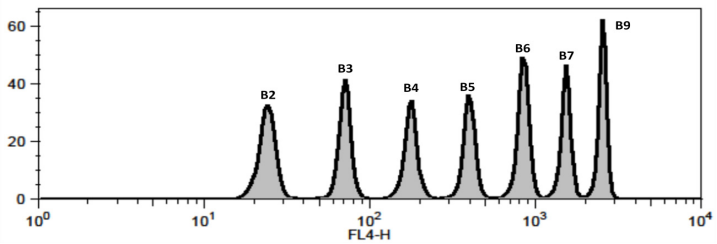


Figure 3. Beads B Classification by FL4



LEGENDplex™ Human Tumor-Associated Macrophage Panel Mix and Match

For Beads usage in the panel, please refer to Table 1 below:

Table 1. Beads ID, Panel Specific Target Selection and Target information

Target	Bead ID*	Top Standard Concentrations
MMP-9	A3	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).
CXCL-9	A4	
CCL18	A5	
CD163	A6	
MMP-2	A7	
Galectin-3	A10	
CCL20 (MIP-3α)	B2	
VEGF	B3	
M-CSF	B4	
PDGF-BB	B5	
G-CSF	B6	
CCL22 (MDC)	B7	
YKL-40	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 should be stored at 4 °C or on ice for use within four hours. Also, leftover standard can be stored at ≤-70°C for use with one month. Avoid multiple (>2) freeze-thaw cycles. Discard any leftover diluted standards.
- Upon reconstitution, Matrix C should be stored at ≤-70°C for use within one month. Avoid multiple (>2) freeze-thaw cycles.

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 #
Capture Beads * (see tables below for more information)	Varies	Varies	Varies*
Human Tumor-Associated Macrophage Panel Detection Antibodies	1 bottle	3.3 mL	741453
Human Tumor-Associated Macrophage Panel Standard	1 vial	Lyophilized	741454
LEGENDplex™ Buffer Set S	1		741226
Filter Plate* or V-bottom Plate**	1 plate		76187** or 76883***

For kit with filter plate. * For kit with V-bottom plate. Only one plate is provided for each kit.

*Capture beads for Mix and Match Subpanels:

Kit Components	Quantity	Volume	Cat #
Human MMP-9 Capture Bead A3, 13X	1 Vial	270 µL	741455
Human CXCL-9 Capture Bead A4, 13X	1 Vial	270 µL	741456
Human CCL18 Capture Bead A5, 13X	1 Vial	270 µL	741457
Human CD163 Capture Bead A6, 13X	1 Vial	270 µL	741458
Human MMP-2 Capture Bead A7, 13X	1 Vial	270 µL	741459
Human Galectin-3 Capture Bead A10, 13X	1 Vial	270 µL	741460
Human CCL20 (MIP-3α) Capture Bead B2, 13X	1 Vial	270 µL	741461
Human VEGF Capture Bead B3, 13X	1 Vial	270 µL	741462
Human M-CSF Capture Bead B4, 13X	1 Vial	270 µL	741463
Human PDGF-BB Capture Bead B5, 13X	1 Vial	270 µL	741464
Human G-CSF Capture Bead B6, 13X	1 Vial	270 µL	741465
Human CCL22 (MDC) Capture Bead B7, 13X	1 Vial	270 µL	741466
Human YKL-40 Capture Bead B9, 13X	1 Vial	270 µL	741467

LEGENDplex™ Buffer Set S (Cat#: 741226):

Kit Components	Quantity	Volume	Cat #
Setup Beads: PE Beads	1 vial	1 mL	77842
Setup Beads: Raw Beads	1 vial	1.8 mL	77844
LEGENDplex™ SA-PE	1 bottle	3.3 mL	77743
LEGENDplex™ Matrix C	1 vial	Lyophilized	76077
LEGENDplex™ Assay Buffer	1 bottle	25 mL	77562
Lyophilized Standard Reconstitution buffer	1 bottle	5 mL	750002725
LEGENDplex™ Wash Buffer, 20X	1 bottle	25 mL	77564
Plate Sealers	4 sheets		78101

No plate is included in Buffer Set S. Plate need to be ordered separately. Please order the correct type of plate based on the preferred assay protocol (Cat# 741447 for Filter Plate and Cat# 741448 for V-bottom Plate).

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 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 the above 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 plates can be ordered from BioLegend (Cat# 740377 or 740378).

If the assay is run 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 plates 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 C for LEGENDplex™ kits contains components of animal origin and should be handled as potentially hazardous.
- 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 Pre-mixed Beads are light-sensitive. Minimize light exposure

Chapter 2: ASSAY PREPARATION

Sample Collection and Handling

Preparation of Serum Samples (Recommended over plasma 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:

- 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 Cell 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:

1. The individual subpanels beads (13X) need to be combined with one-another and diluted with Assay Buffer to create a 1X working solution of beads prior to use.

2. Sonicate each bead vial for 1 minute in a sonicator bath and then vortex for 30 seconds to completely resuspend the beads.

3. Calculate and prepare a 1X beads working solution based on the desired number of reactions and plex-size of your assay (i.e. the number of individual bead vials) following the steps described below.

A. Total volume (µL) = 30 x (number of reactions)

B. Volume needed from each 13X beads vial (µL) = 2.3 x (number of reactions)

C. Assay Buffer needed (µL) = A – B x (number of individual beads vials to be mixed)

Note: calculations for total volume include a 20% excess to account for any loss during pipetting

Example: to prepare 50 reactions for a 5-plex assay

A. Total volume (µL) = 30 x 50 = 1500 µL
B. Volume per beads vial needed (µL) = 2.3 x 50 = 115 µL
C. Assay Buffer needed (µL) = A – B x (number of individual beads vials) =1500 – (115 x 5) = 925 µL

Combine 115 µL of each beads vial (5 vials) with 925 µL of assay buffer to get the desired final volume of 1500 µL of 1X working solution of 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 C (for 8-plex panel Serum or Plasma Samples Only)

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

Standard Preparation

1. Prior to use, reconstitute a vial of the lyophilized Human Tumor-Associated Macrophage Panel Standard Cocktail with **500 µL** Lyophilized Standard Reconstitution 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 the top standard at 10,000 ng/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

Sample Dilution

For cell culture supernatant, serum and plasma samples, follow panel specific dilution recommendations below:

- **For Human Tumor-Associated Macrophage Panel (13-plex):**

  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 for samples.

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

- **For Human Tumor-Associated Macrophage Panel (8-plex):**

  Serum or plasma samples must be diluted 4-fold with Assay Buffer as described in the table below:

Sample	Dilution (1:4)	Final Dilution Fold
Serum, Plasma	50 µL + 150 µL (Assay Buffer)	4

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

- **For Human Tumor-Associated Macrophage Panel (5-plex):**

  Serum or plasma samples must be diluted 100-fold with Assay Buffer as described in the table below:

Sample	Dilution (1:100)	Final Dilution Fold
Serum, Plasma	2 µL + 198 µL (Assay Buffer)	100

If further dilution is desired, dilution should be done with Assay Buffer to ensure accurate measurement.

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

Chapter 3: ASSAY PROCEDURE

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

- The in-filter assay procedure requires a vacuum filtration unit for washing (see **Materials to be Provided by the End-User, page 9)**.
- If the 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 37). 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 in 13-plex, load the plate as shown in the table below (in the order from left to right):

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

   For measuring serum or plasma samples in 8-plex, load the plate as shown in the table below (in the order from left to right):

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

For measuring serum or plasma samples in 5-plex, load the plate as shown in the table below (in the order from left to right):

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

***See Sample Dilution on page 14**

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 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).

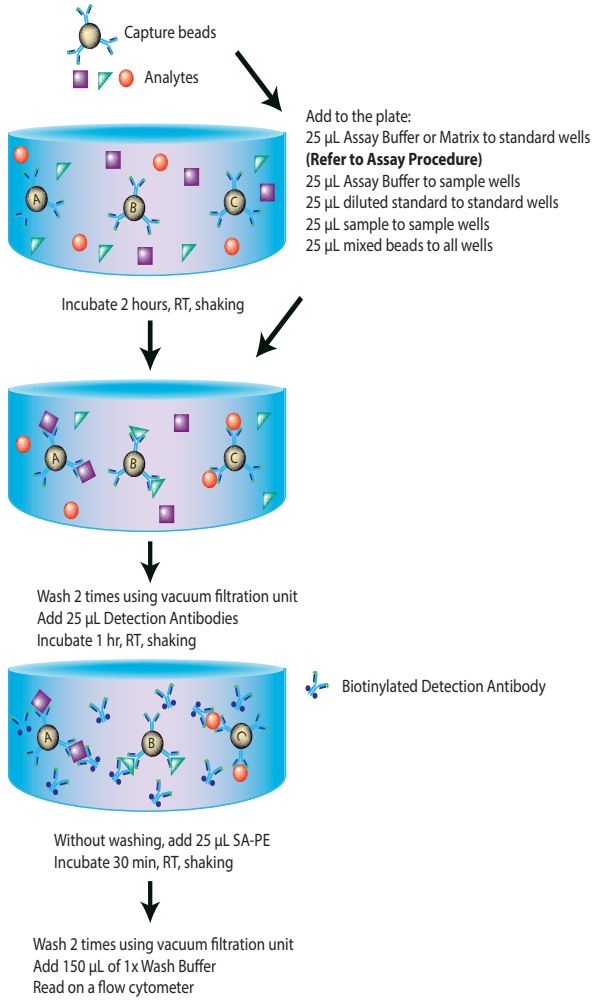
    If the flow cytometer is equipped with an autosampler, read the plate directly using the autosampler. **Please be sure to program the autosampler to resuspend beads in the well immediately before taking samples. The probe height may need to be adjusted when using an autosampler**.

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

Assay Procedure Summary for Filter Plate

Add 100 µL 1X Wash Buffer to filter plate wells

Vacuum to remove excess buffer



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 37). 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 in 13-plex,** load the plate as shown in the table below (in the order from left to right):

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

For measuring serum or plasma samples in 8-plex, load the plate as shown in the table below (in the order from left to right):

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

For measuring serum or plasma samples in 5-plex, load the plate as shown in the table below (in the order from left to right):

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

***See Sample Dilution on page 14**

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 that it may cause sample to spill from the wells)**.

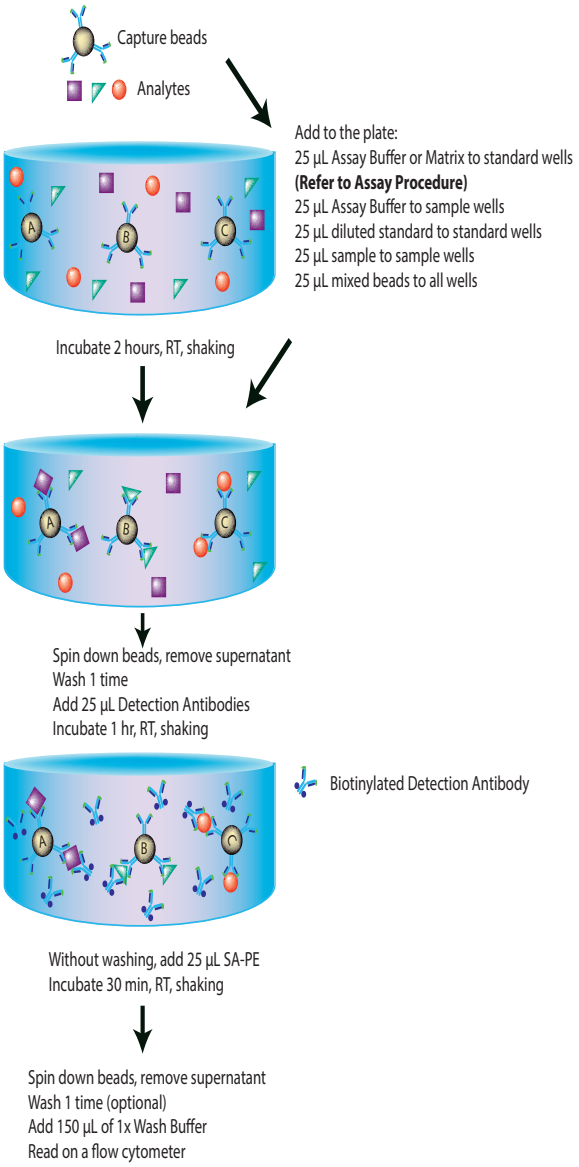
4. Centrifuge the plate at 1050 rpm (~250 g) for 5 minutes, using a swinging bucket rotor (G.H 3.8) with microplate adaptor (Please refer to **Materials to be Provided by the End-User, page 9).** Do not use excessive centrifugation speed as it may make it harder to resuspend beads in later steps. **Make 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 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 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. (This washing step is optional but helps to reduce the background.) 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.
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 for various flow cytometers are available on our website biolegend.com

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 900 beads for a 3-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...).

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.

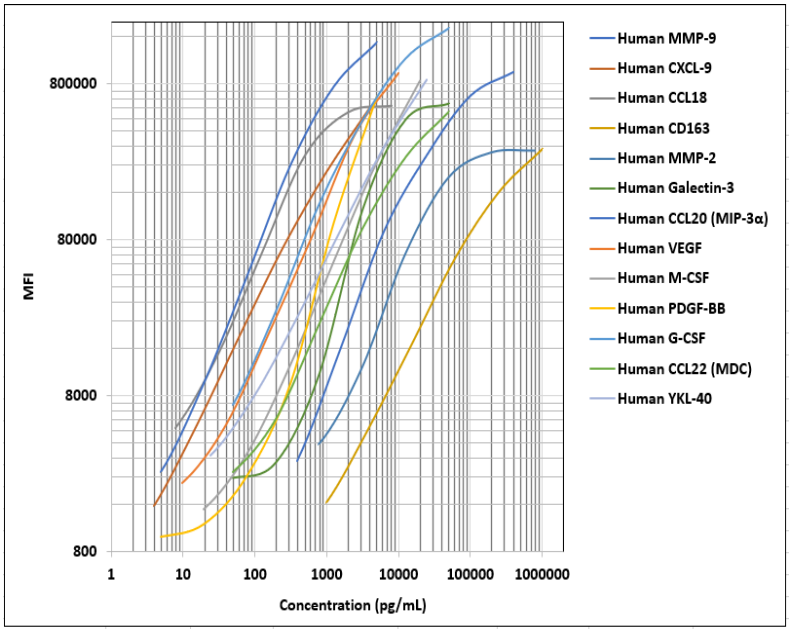
Data Analysis

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

Representative Standard Curve

This standard curve was generated using the LEGENDplex™ Human Tumor-Associated Macrophage Panel for demonstration purpose only. A standard curve must be run with each assay.



Assay Sensitivity

The assay sensitivity 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 ≤Mean LOD + 2xSTDEV LOD.

Analyte	LOD in Assay Buffer (pg/mL)	LOD in Matrix (pg/mL)
Human MMP-9	39.4	N/A
Human CXCL-9	0.6	1.5
Human CCL18	0.6	N/A
Human CD163	130.5	N/A
Human MMP-2	191.4	1516.3

Human Galectin-3	119.4	187.2
Human CCL20 (MIP-3α)	0.6	3.1
Human VEGF	2.8	7.9
Human M-CSF	3.2	9.1
Human PDGF-BB	11.5	N/A
Human G-CSF	5.5	19.3
Human CCL22 (MDC)	26.6	48.1
Human YKL-40	4.8	N/A

Cross-Reactivity

The following human recombinant proteins were tested at 50 ng/mL using the LEGENDplex™ Human Tumor-Associated Macrophage Panel. No or negligible non-specific cross-reactivity was observed for all the targets except for Human CXCL-9 protein, there is a 1% cross-reactivity with Human MMP-2, and 1% cross-reactivity with Human PDGF-BB. Also, there is a 2% cross-reactivity of YKL-40 protein with Human Galectin-3.

MMP-9	CCL20 (MIP-3α)	YKL-40	IL-5	CXCL8 (IL-8)	CXCL1 (GROα)
CXCL-9	VEGF	CXCL5	GM-CSF	CXCL10 (IP-10)	CXCL11 (I-TAC)
CCL18	M-CSF	IL-10	IFN-γ	CCL11 (Eotaxin)	CCL4 (MIP-1β)
CD163	PDGF-BB	EGF	IL-1β	CCL17 (TARC)	MCP-1
MMP-2	G-CSF	TNF-a	IL-6	CCL5 (RANTES)	
Galectin-3	CCL22 (MDC)	IL-2	TGF-β1	CCL3 (MIP-1α)	

Accuracy (Spike Recovery)

For spike recovery in cell culture medium, RPMI or DMEM with 10% FCS was 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=8) and plasma (n=24), samples were first diluted 4-fold or 100-fold (see Sample Dilution on page 14) 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 MMP-9	96%	84%	80%*
Human CXCL-9	88%	148%	168%
Human CCL18	90%	152%	148%
Human CD163	87%	112%	93%
Human MMP-2	89%	54%	52%
Human Galectin-3	86%	130%	111%
Human CCL20 (MIP-3α)	86%	58%	65%
Human VEGF	95%	50%	88%
Human M-CSF	90%	100%	103%
Human PDGF-BB	67%	111%	86%
Human G-CSF	89%	107%	102%
Human CCL22 (MDC)	87%	122%	108%
Human YKL-40	87%	101%	85%

***Note:** Human Plasma samples were spiked with MMP-9/NGAL complex at three different levels within the assay range.

Linearity of Dilution

For spike linearity in cell culture medium, RPMI or DMEM with 10% FCS was 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=8) and plasma (n=24), samples were first diluted 4-fold or 100-fold (see Sample Dilution on page 14) 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 C (for 4-fold diluted samples) or with Assay buffer (for 100-fold diluted samples) 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 MMP-9	80%	148%	114%
Human CXCL-9	128%	59%	94%
Human CCL18	79%	108%	93%
Human CD163	120%	111%	115%
Human MMP-2	86%	77%	82%
Human Galectin-3	88%	61%	75%
Human CCL20 (MIP-3α)	94%	93%	93%
Human VEGF	98%	93%	95%
Human M-CSF	129%	84%	107%
Human PDGF-BB	119%	103%	111%
Human G-CSF	107%	78%	92%
Human CCL22 (MDC)	68%	138%	103%
Human YKL-40	97%	111%	104%

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 MMP-9	QC1	3,199	100	3%
	QC2	15,921	872	5%
Human CXCL9	QC1	7	1	14%
	QC2	43	4	10%
Human CCL18	QC1	67	3	4%
	QC2	355	20	6%
Human CD163	QC1	10,616	481	5%
	QC2	51,119	3,676	7%
Human MMP-2	QC1	10,535	446	4%
	QC2	47,327	2,012	4%
Human Galectin-3	QC1	597	38	6%
	QC2	2,523	105	4%
Human CCL20 (MIP-3α)	QC1	22	2	8%
	QC2	125	8	7%
Human VEGF	QC1	121	12	10%
	QC2	538	34	6%
Human M-CSF	QC1	229	13	6%
	QC2	1,020	62	6%
Human PDGF-BB	QC1	36	2	5%
	QC2	215	15	7%
Human G-CSF	QC1	522	17	3%
	QC2	2,420	141	6%
Human CCL22 (MDC)	QC1	325	30	9%
	QC2	1,755	121	7%
Human YKL-40	QC1	273	11	4%
	QC2	1,231	55	4%

Inter-Assay Precision

Two samples with different concentrations of target proteins were analyzed in ten independent assays. The inter-assay precision was calculated as below.

Analyte	Sample	Mean (pg/mL)	STDEV	%CV
Human MMP-9	QC1	3,347	528	16%
	QC2	15,174	2,377	16%
Human CXCL9	QC1	13	3	22%
	QC2	68	11	16%
Human CCL18	QC1	78	8	11%
	QC2	388	36	9%
Human CD163	QC1	13,868	1,245	9%
	QC2	57,725	5,651	10%
Human MMP-2	QC1	10,477	984	9%
	QC2	45,485	3,389	7%
Human Galectin-3	QC1	610	121	20%
	QC2	2,678	453	17%
Human CCL20 (MIP-3α)	QC1	25	4	18%
	QC2	133	25	19%
Human VEGF	QC1	137	12	9%
	QC2	588	45	8%
Human M-CSF	QC1	270	23	9%
	QC2	1,158	87	7%
Human PDGF-BB	QC1	38	6	17%
	QC2	224	21	9%
Human G-CSF	QC1	629	53	8%
	QC2	2,577	193	7%
Human CCL22 (MDC)	QC1	386	47	12%
	QC2	1,995	207	10%
Human YKL-40	QC1	306	55	18%
	QC2	1,356	290	21%

Biological Samples

Serum

Normal human serum samples (n=8) were tested for endogenous levels of Human Tumor-Associated Macrophage Panel targets. The concentrations measured are shown below:

Analyte	Range (pg/mL)	% of Detectable	Mean of detectable (pg/mL)
Human MMP-9	97,720-1,016,850	100%	484,406
Human CXCL-9	15-75	100%	40
Human CCL18	8,781-51,933	100%	26,834
Human CD163	287,300-963,600	100%	463,500
Human MMP-2	49,041-115,928	100%	76,177
Human Galectin-3	5,489-11,779	100%	8,556
Human CCL20 (MIP-3α)	8-27	100%	18
Human VEGF	123-362	100%	218
Human M-CSF	123-208	100%	161
Human PDGF-BB	1,032-23,375	100%	7,674
Human G-CSF	28-52	100%	40
Human CCL22 (MDC)	1,189-1,519	100%	1,338
Human YKL-40	10,387-54,061	100%	22,873

Plasma

Normal human plasma samples (n=24) were tested for endogenous levels of Human Tumor-Associated Macrophage Panel targets. The concentrations measured are shown below:

Analyte	Range (pg/mL)	% of Detectable	Mean of detectable (pg/mL)
Human MMP-9	68,470-869,990	100%	280,264
Human CXCL-9	16-175	100%	67
Human CCL18	14,137-167,145	100%	42,674

Human CD163	289,300-941,400	100%	582,917
Human MMP-2	25,243-87,816	100%	65,107
Human Galectin-3	2,510-10,366	100%	7,029
Human CCL20 (MIP-3α)	8-193	100%	39
Human VEGF	35-425	100%	121
Human M-CSF	100-1,148	100%	255
Human PDGF-BB	553-8,283	92%	3,127
Human G-CSF	18-855	100%	137
Human CCL22 (MDC)	586-3,106	100%	1,320
Human YKL-40	10,027-64,155	100%	27,611

Cell Culture Supernatant

Human PBMCs (1 x 10^6 cells/mL) were cultured unstimulated and stimulated. The stimulation conditions were PHA (5 µg/mL); Poly I:C (50 ng/mL); LPS (100 ng/mL); anti-CD3 (10 µg/mL plate-coated); anti-CD28 (2 µg/mL soluble). Supernatants were collected after 3 days of culturing and assayed with the LEGENDplex™ Human Tumor-Associated Macrophage Panel The results (all in pg/mL) are summarized below.

Analyte	PBMC Unstim-ulated	PBMC PHA	PBMC Poly IC	PBMC LPS	PBMC CD3 +CD28
Human MMP-9	54,333	228,912	56,307	87,209	79,355
Human CXCL-9	0.38	274	ND	ND	0.36
Human CCL18	13	59	8	11	8
Human CD163	409	1,872	319	259	185
Human MMP-2	ND	661	ND	ND	ND
Human Galectin-3	1,554	1,293	1,366	612	167
Human CCL20 (MIP-3α)	0.75	103	0.26	41	0.56
Human VEGF	10	2	4	ND	3
Human M-CSF	3	24	2	2	11
Human PDGF-BB	93	79	133	56	50
Human G-CSF	6	4,592	ND	886	ND

Human CCL22 (MDC)	91	3,878	71	701	1,998
Human YKL-40	858	3,554	495	1,188	2,852

ND = Not Detectable

Various human cancer cell lines including HaCat, THP-1,and HepG2 were cultured and assayed with LEGENDplex™ Human Tumor-Associated Macrophage Panel. The results (all in pg/mL) are summarized below.

Analyte	HaCat	THP-1	HepG2
Human MMP-9	413	79,910	47
Human CXCL-9	ND	1.3	ND
Human CCL18	ND	11	0.71
Human CD163	55	653	94
Human MMP-2	588	1,694	457
Human Galectin-3	385	274	466
Human CCL20 (MIP-3α)	0.25	28	4,526
Human VEGF	26	418	2,273
Human M-CSF	2	4	307
Human PDGF-BB	5	35	5
Human G-CSF	ND	4	17
Human CCL22 (MDC)	ND	65	14
Human YKL-40	ND	ND	14

ND = Not 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.

Notes

Notes

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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