BioLegend®

Enabling Legendary Discovery™

LEGENDplex™

Multi-Analyte Flow Assay Kit

Mouse Macrophage/Microglia
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.

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

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Chapter 1: KIT DESCRIPTION

Introduction

Macrophages are produced by differentiation of monocyte in response to an infection or tissue damage. Their primary function is to recognize, engulf, and destroy target cells including pathogens, dying or dead cells, and cellular debris. Like dendritic cells, macrophages are also professional antigen presenting cells that play a crucial role in initiating an immune response. Macrophages secrete an array of cytokines which aid in host defense, tissue repair, and immunoregulation. Macrophages can be divided into multiple subtypes based on different functions. Inflammation-encouraging M1 macrophages produce proinflammatory cytokines, such as CXCL1 (KC), IL-18, IL-23, IL-12p70, IL-6, TNF-α, IL-12p40, and IL-1β. Anti-inflammatory and tissue-repairing M2 macrophages decrease immune reactions and promote tissue repair by releasing a different set of factors, such as Free Active TGF-β1, CCL22 (MDC), IL-10, IL-6, G-CSF, and CCL17 (TARC).

Due to their important roles in immune responses, macrophages are critically involved in a variety of inflammatory disorders, such as sepsis-related multiple organ dysfunction/failure, microbial infection, acute injuries, neurodegenerative disorders, cancer, cardiovascular, and autoimmune diseases. Measuring the mediators of proinflammation and anti-inflammation produced by macrophages may help not only in understanding the fundamental functions of macrophages, but also in finding the mechanisms of various pathological processes.

The LEGENDplex™ Mouse Macrophage/Microglia Panel (13-plex) is a bead-based multiplex assay panel, using fluorescence-encoded beads suitable for use on various flow cytometers. It allows for simultaneous quantification of 13 key targets involved in macrophage functions such as CXCL1 (KC), Free Active TGF-β1, IL-18, IL-23, CCL22 (MDC), IL-10, IL-12p70, IL-6, TNF-α, G-CSF, CCL17 (TARC), IL-12p40, and IL-1β. This assay panel provides higher detection 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™ Mouse Macrophage/Microglia Panel (13-plex) is designed to allow flexible customization within the panel. It can also be divided into subpanels such as:

LEGENDplex™ Mouse M1 Macrophage Panel (8-plex)
LEGENDplex™ Mouse M2 Macrophage Panel (6-plex)

Please visit **www.biolegend.com/legendplex** for more information on how to mix and match within the panel.

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, 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.

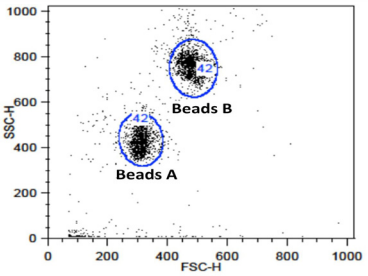
Since the beads are differentiated by size and internal fluorescence intensity on a flow cytometer, analyte-specific populations can be segregated and PE fluorescent signal quantified. The concentration of a particular analyte is determined using a standard curve generated in the same assay.

Beads Usage

The Mouse Macrophage/Microglia Panel 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 5 bead populations (A4, A5, A6, A7, A8, A10) and the larger Beads B consists of 5 bead populations (B2, B3, B4, B5, B6, B7, B9) (Figure 2-3).

Using a total of 13 bead populations distinguished by size and internal fluorescent dye, the Mouse Macrophage/Microglia Panel allows simultaneous detection of 13 proteins in a single sample. 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

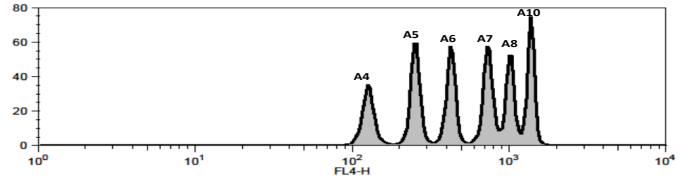
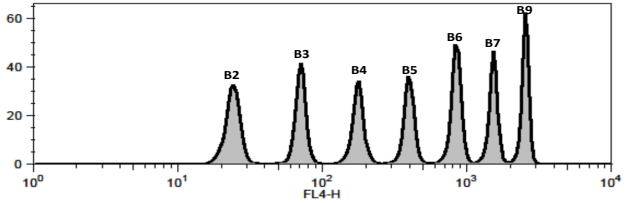


Figure 3. Beads B Classification by FL4



For Beads usage in the full panel as well as sample specific subpanels, please refer to Table 1 below.

Table 1. Panel Targets and Bead ID*

Target	Bead ID	Macrophage/ Microglia Panel (13-plex) Cat. # 740845 or 740846	M1 Macrophage Panel (8-plex) Cat. # 740847 or 740848	M2 Macrophage Panel (6-plex) Cat. # 740849 or 740850	Top Standard Concentrations
CXCL1 (KC)	A4	√	√		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.
Free Active TGF-β1	A5	√		√	
IL-18	A6	√	√		
IL-23	A7	√	√		
CCL22 (MDC)	A8	√		√	
IL-10	A10	√		√	
IL-12p70	B2	√	√		
IL-6	B3	√	√	√	
TNF-α	B4	√	√		
G-CSF	B5	√		√	
CCL17 (TARC)	B6	√		√	
IL-12p40	B7	√	√		
IL-1β	B9	√	√		

*Bead ID is used to associate a bead population to a particular analyte in the LEGENDplex™ Data Analysis Software. The association of analyte and bead ID will be defined during the gating step of the data analysis.

When entering analyte and bead ID infomation during the gating step, always enter in the sequential order of the bead ID (e.g, A4, A5, A6...B2, B4, B5...). Please refer to the LEGENDplex™ Data Analysis Software User Guide and Online Help for details (**www.biolegend.com/legendplex**).

Storage Information

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

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

Materials Supplied

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

Kit Components	Quantity	Volume	Part #
Capture Beads* (see tables below for more information)	**Varies**	**Varies**	**Varies***
Mouse Macrophage/Microglia Panel Detection Antibodies	**1 bottle**	**3.5 mL**	**Varies***
Mouse Macrophage/Microglia Panel Standard	**1 vial**	**Lyophilized**	**Varies***
LEGENDplex™ Buffer Set B	1 bottle	3.5 mL	740373
Filter Plate** or V-bottom Plate***	1 plate		76187** or 76883***
Mouse Macrophage/Microglia Mix and Match Subpanel Manual	1		750000418

** 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.#
LEGENDplex™ Mouse CXCL1 (KC) Capture Bead A4, 13X	1 vial	270 µL	740853
LEGENDplex™ Mouse Free Active TGF-β1 Capture Bead A5, 13X	1 vial	270 µL	740854
LEGENDplex™ Mouse IL-18 Capture Bead A6, 13X	1 vial	270 µL	740855
LEGENDplex™ Mouse IL-23 Capture Bead A7, 13X	1 vial	270 µL	740856
LEGENDplex™ Mouse CCL22 (MDC) Capture Bead A8, 13X	1 vial	270 µL	740857
LEGENDplex™ Mouse IL-10 Capture Bead A10, 13X	1 vial	270 µL	740858
LEGENDplex™ Mouse IL-12p70 Capture Bead B2, 13X	1 vial	270 µL	740859
LEGENDplex™ Mouse IL-6 Capture Bead B3, 13X	1 vial	270 µL	740860
LEGENDplex™ Mouse TNF-α Capture Bead B4, 13X	1 vial	270 µL	740861
LEGENDplex™ Mouse G-CSF Capture Bead B5, 13X	1 vial	270 µL	740862
LEGENDplex™ Mouse CCL17 (TARC) Capture Bead B6, 13X	1 vial	270 µL	740863
LEGENDplex™ Mouse IL-12p40 Capture Bead B7, 13X	1 vial	270 µL	740864
LEGENDplex™ Mouse IL-1β Capture Bead B9, 13X	1 vial	270 µL	740865
LEGENDplex™ Mouse Macrophage/Microglia Panel Detection Antibodies	1 bottle	3.5 mL	740851
LEGENDplex™ Mouse Macrophage/Microglia Panel Standard	1 vial	Lyophi-lized	740852

*** Please refer to Beads ID and Panel-Specific Target Selection table (Table 1), to see which capture beads are included in each panel.**

LEGENDplex™ Buffer Set B (Cat#: 740373)

Components	Quantity	Volume	Part #
Setup Beads 1: FITC Beads	1 vial	1 mL	77840
Setup Beads 2: PE Beads	1 vial	1 mL	77842
Setup Beads 3: Raw Beads	1 vial	2 mL	77844
LEGENDplex™ SA-PE	1 bottle	3.5 mL	77743
LEGENDplex™ Matrix C, Lyophilized	1 vial	lyophilized	76077
LEGENDplex™ Assay Buffer	1 bottle	25 mL	77562
LEGENDplex™ Wash Buffer, 20X	1 bottle	25 mL	77564
Data Analysis Software Dongle	1		21217
Plate Sealers	4 sheets		78101

No plate is included in Buffer Set B. Plate need to be ordered separately. Please order the correct type of plate based on the preferred assay protocol (Cat# 740377 or 740378 for Filter Plate and Cat# 740379 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 Channel Emission	Beads Classification Channel	Classification Channel Emission	Compensation needed?
BD FACSCalibur™	FL2	575 nm	FL4	660 nm	No*
BD Accuri™ C6	FL2	585 nm	FL4	675 nm	No*
BD FACSCanto™, BD FACSCanto™ II	PE	575 nm	APC	660 nm	No*
BD™ LSR, LSR II BD LSRFortessa™	PE	575 nm	APC	660 nm	No*
Gallios™	PE	575 nm	APC	660 nm	No*
CytoFLEX	PE	585 nm	APC	660 nm	No*
NovoCyte	PE	572 nm	APC	660 nm	No*
Attune™ NxT	PE	574 nm	APC	670 nm	No*
NovoCyte	PE	572 nm	APC	660 nm	No*
Guava® easyCyte	YLW	583 nm	RED2	661 nm	No*
Sony SH800	PE	585 nm	APC	665 nm	No*

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

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

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

- 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 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, 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.
- Do not mix or substitute reagents from different kits or lots. Reagents from different manufacturers should not be used with this kit.
- Do not use this kit beyond its expiration date.
- SA-PE and beads are light-sensitive. Minimize light exposure.

Chapter 2: ASSAY PREPARATION

Sample Collection and Handling

Preparation of Serum Samples:

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

Preparation of Plasma Samples:

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

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.

- **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 (an 8-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 8-plex 1X diluted beads, transfer 115 µL of each of the 8 individual beads (13X) to a fresh tube (total bead volume = 920 µL) and add 580 µL of Assay Buffer to make the final volume of 1.5 mL.

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 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 the lyophilized Mouse Macrophage/Microglia Panel Standard 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 microcentrifuge tube. This will be used as the top standard C7.

   Note: The top standard concentrations of analytes in this panel were set at various concentrations, but may be subject to change from lot to lot (see lot-specific Certificate of Analysis provided in the kit box for details).
3. Label 6 polypropylene microcentrifuge tubes as C6, C5, C4, C3, C2 and C1, respectively.
4. Add 75 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	156.25
C3	1:256	75	25 µL of C4	39.01
C2	1:1024	75	25 µL of C3	9.77
C1	1:4096	75	25 µL of C2	2.44
C0	--	75	--	0

Sample Dilution

- For tissue culture supernatant samples, the levels of analyte can vary greatly from sample to sample. To test tissue culture supernatant samples, a preliminary experiment may be required to determine the appropriate dilution factor. If further dilution is desired, dilution should be done with corresponding fresh cell culture medium or Assay Buffer as a diluent to ensure accurate measurement.

  Serum or plasma samples must be diluted 2-fold with Assay Buffer as described in the table below.

Sample	Dilution (1:2)	Final Dilution Fold
Serum, Plasma	50 µL + 50 µL (Assay Buffer)	2

If further dilution is desired, dilution should be done with Matrix C 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 Filter plate assay procedure is recommended due to its good sample to sample consistency, assay robustness and ease of handling. This procedure requires a vacuum filtration unit for washing (see **Materials to be Provided by the End-User, page 8)**. If you have performed bead-based multiplex assays before, your lab may already have the vacuum filtration unit set up.
- 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 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.

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

	Culture Media or Assay Buffer	Assay Buffer	Standard	Sample*
Standard Wells	25 µL	---	25 µL	---
Sample wells	---	25 µL	---	25 µL

For measuring serum or plasma samples, 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

*See **Sample Dilution on page 12**

3. 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).

4. 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.

5. **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.

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

7. 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.

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

9. 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.

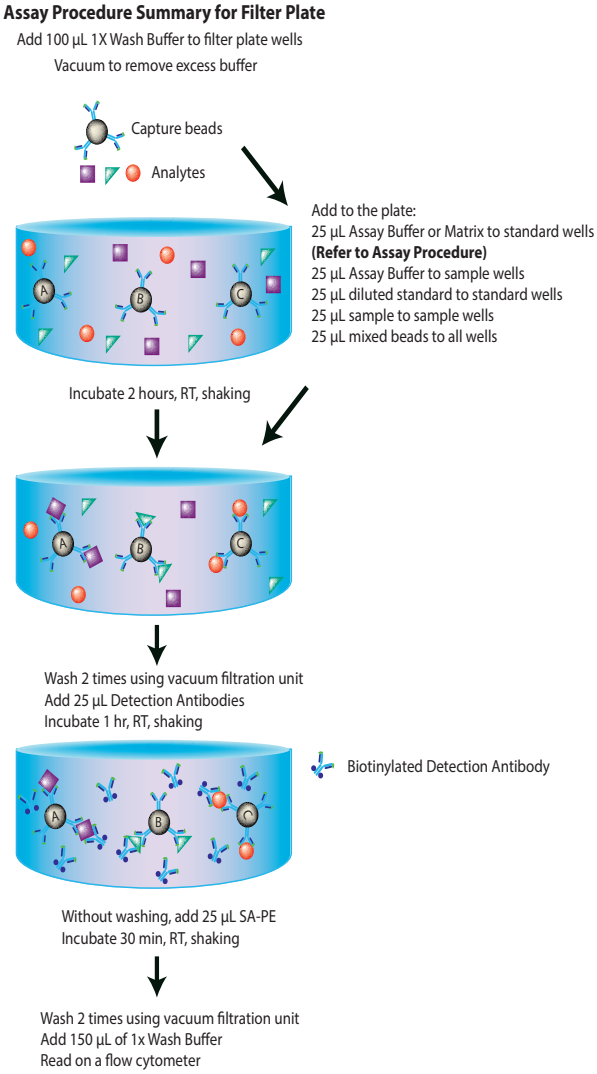
10. Repeat step 5 above.

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

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

	Culture Media or Assay Buffer	Assay Buffer	Standard	Sample*
Standard Wells	25 µL	---	25 µL	---
Sample wells	---	25 µL	---	25 µL

For measuring serum or plasma samples, 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

*See **Sample Dilution on page 12**

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 8).** 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.

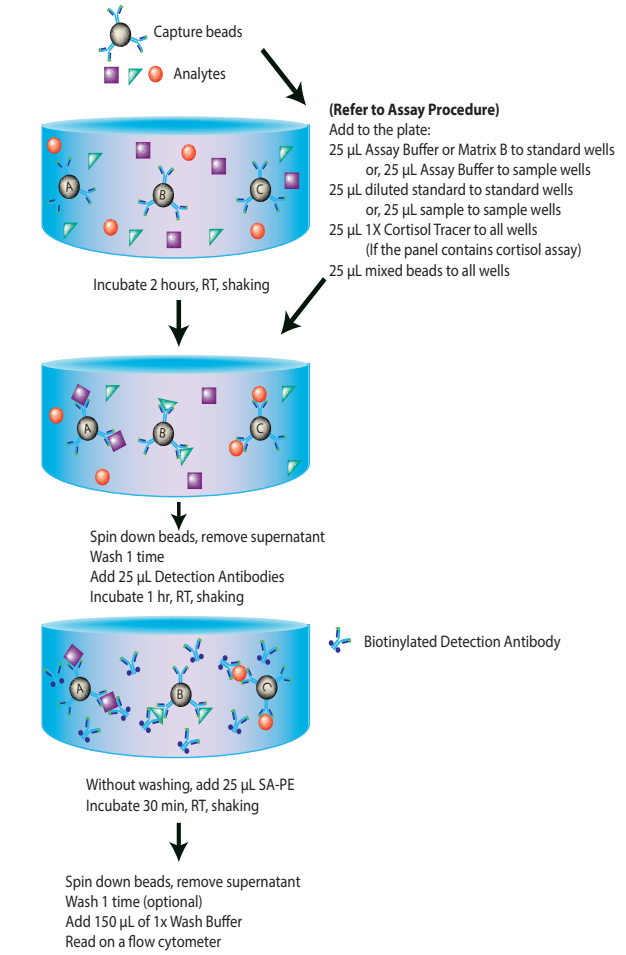
13. Add 150 µL of 1X Wash Buffer to each well. Resuspend the beads by pipetting.

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 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 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.

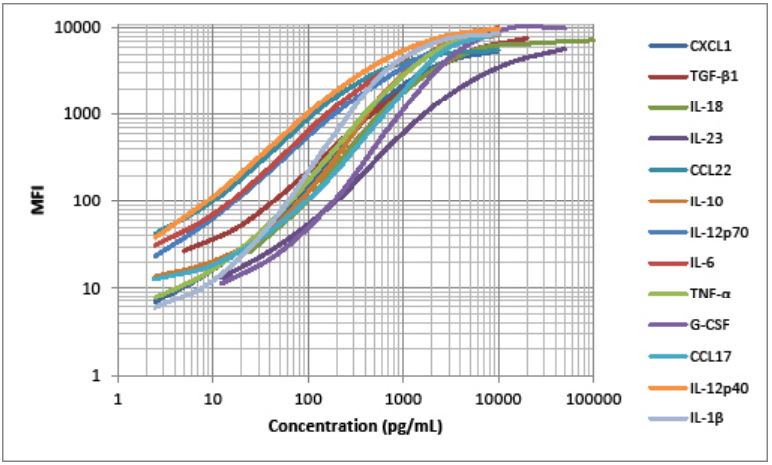
Data Analysis

- The FCS file generated on a flow cytometer should be analyzed using BioLegend's LEGENDplex™ Data Analysis Software. The LEGENDplex™ Data Analysis Software can be downloaded for free here: **www.biolegend.com/legendplex.**
- For PC users, install the software on a PC running Windows 7 or Windows 8 and use it in conjunction with the Data Analysis Software Dongle included in this kit. The dongle has a license key stored in it and is needed to run the software. To use the dongle, simply plug it in the USB port of the computer on which the data analysis software is installed, prior to launching the software.
- For Mac users, install on a Mac OS X version 10.7 (Lion) or later and you will be promoted to request a software license key after the software installation.
- Follow the LEGENDplex™ Data Analysis Software User Guide and Online Help to use the software (**www.bioLegend.com/legendplex; or press F1 for online help at any step of the data analysis**).

Chapter 6: ASSAY CHARACTERIZATION

Representative Standard Curve

This standard curve was generated using the LEGENDplex™ Mouse Macrophage/Microglia Panel for demonstration purposes only. A standard curve must be run with each assay.



Assay Sensitivity

The assay sensitivity or minimum detectable concentration (MDC) is the theoretical limit of detection calculated using the LEGENDplex™ Data Analysis Software by applying a 5-parameter curve fitting algorithm. Assay sensitivity presented here is MDC + 2 STDEV.

Analyte	Sensitivity in Cell Culture Media (pg/mL) (N = 12)	Sensitivity in Serum (pg/mL) (N = 12)
Mouse CXCL1 (KC)	0.57 + 0.62	0.59 + 0.54
Mouse Free Active TGF-β1	2.30 + 2.04	1.81 + 2.26
Mouse IL-18	14.78 + 14.12	9.94 + 9.50
Mouse IL-23	3.80 + 4.98	3.41 + 3.85
Mouse CCL22 (MDC)	1.00 + 1.32	0.92 + 1.00
Mouse IL-10	1.26 + 1.02	1.03 + 0.76
Mouse IL-12p70	0.85 + 1.13	0.71 + 1.05

Mouse IL-6	1.15 + 1.41	1.16 + 1.24
Mouse TNF-α	0.69 + 0.78	0.91 + 0.66
Mouse G-CSF	5.71 + 5.74	5.95 + 5.71
Mouse CCL17 (TARC)	1.35 + 1.11	1.30 + 0.90
Mouse IL-12p40	1.02 + 1.48	0.81 + 1.27
Mouse IL-1β	0.77 + 0.73	0.91 + 0.91

Cross-Reactivity

The following recombinant proteins were tested individually at 50 ng/mL. The IL-12p40 assay detects the p40 subunit of IL-23 and IL-12p70. No or negligible cross-reactivity was found for all other analytes.

Mouse Proteins					
CXCL1 (KC)	TGF-β1	IL-18	IL-23	CCL22 (MDC)	IL-10
IL-12p70	IL-6	TNF-α	G-CSF	CCL17 (TARC)	IL-12p40
IL-1β	CXCL2 (MIP-2)	CXCL3 (DCIP-1)	CXCL4 (PF-4)	CXCL5 (LIX)	CXCL7 (NAP-2)
CXCL9 (MIG)	CXCL10 (IP-10)	CXCL11 (ITAC)	CXCL12 (SDF-1)	CXCL13 (BLC)	CXCL14 (BRAK)
CXCL17 (VCC-1)	CCL1 (I-309)	CCL8 (MCP-2)	CCL9 (MIG-1γ)	CCL11 (Eotaxin)	CCL12 (MCP-5)
CCL19 (MIP-3β)	CCL20 (MIP-3α)	CCL21	CCL24 (Eotaxin-2)	CCL25 (TECK)	CCL28 (MEC)
CX3CL1 (Fractalkine)	IL-11	IL-13	IL-15	IL-17A	IL-17F
IL-1α	IL-2	IL-21	IL-22	IL-25	IL-27
IL-3	IL-33	IL-34	IL-4	IL-5	IL-7
GM-CSF	M-CSF	FGF-Basic	IFN-γ	IFN-β1	IGF-II

Human Proteins					
CXCL1 (GRO-α)	IL-18	IL-23	CCL22 (MDC)	IL-10	IL-12p70
IL-6	TNF-α	G-CSF	CCL17	IL-1β	

Accuracy (Spike Recovery)

For spike recovery in tissue culture media, serum, and plasma, target proteins with known concentrations in the assay range were spiked into sample. The spiked samples were then assayed, and the measured concentrations were compared with the expected values.

Analyte	% of Recovery in Tissue Culture Media (N= 2)	% of Recovery in Serum (N = 4)	% of Recovery in Plasma (N = 8)
Mouse CXCL1 (KC)	95%	93%	76%
Mouse Free Active TGF-β1	99%	115%	80%
Mouse IL-18	97%	54%	55%
Mouse IL-23	93%	110%	111%
Mouse CCL22 (MDC)	91%	127%	119%
Mouse IL-10	99%	75%	69%
Mouse IL-12p70	94%	86%	87%
Mouse IL-6	90%	95%	91%
Mouse TNF-α	93%	83%	88%
Mouse G-CSF	90%	48%	54%
Mouse CCL17 (TARC)	93%	104%	81%
Mouse IL-12p40	95%	78%	69%
Mouse IL-1β	101%	90%	89%

Linearity of Dilution

For testing linearity of diution, serum and plasma samples were first diluted two-fold with Assay Buffer and spiked with target proteins at known concentrations. The spiked samples were then serially diluted 1:2, 1:4, 1:8 with Matrix C and assayed. For tissue culture media samples, neat samples were spiked with target proteins at known concentrations. The spiked samples were then serially diluted 1:2, 1:4, 1:8 with tissue culture media and assayed. The measured concentrations of serially diluted samples were then compared with that of the two-fold diluted and neat samples, respectively.

Analyte	Linearity of Dilution (Tissue Culture Media) (N= 2)	Linearity of Dilution (Serum) (N = 4)	Linearity of Dilution (Plasma) (N = 8)
Mouse CXCL1 (KC)	104%	111%	135%
Mouse Free Active TGF-β1	119%	103%	147%
Mouse IL-18	114%	132%	134%
Mouse IL-23	105%	77%	84%
Mouse CCL22 (MDC)	107%	117%	147%
Mouse IL-10	99%	112%	139%
Mouse IL-12p70	100%	116%	118%
Mouse IL-6	101%	122%	144%
Mouse TNF-α	109%	115%	125%
Mouse G-CSF	106%	149%	152%
Mouse CCL17 (TARC)	99%	115%	145%
Mouse IL-12p40	112%	149%	147%
Mouse IL-1β	106%	109%	118%

Intra-Assay Precision

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

Analyte	Sample	Mean (pg/mL)	STDEV	%CV
Mouse CXCL1 (KC)	Sample 1	32.83	1.17	4%
	Sample 2	123.75	3.12	3%
Mouse Free Active TGF-β1	Sample 1	57.12	3.14	6%
	Sample 2	275.06	15.00	5%
Mouse IL-18	Sample 1	193.87	7.51	4%
	Sample 2	1019.60	35.78	4%

Mouse IL-23	Sample 1	126.86	5.69	4%
	Sample 2	641.33	35.74	6%
Mouse CCL22 (MDC)	Sample 1	37.28	1.85	5%
	Sample 2	126.52	5.94	5%
Mouse IL-10	Sample 1	26.96	0.75	3%
	Sample 2	117.13	3.87	3%
Mouse IL-12p70	Sample 1	23.73	0.97	4%
	Sample 2	109.72	4.88	4%
Mouse IL-6	Sample 1	29.81	1.61	5%
	Sample 2	150.01	7.12	5%
Mouse TNF-α	Sample 1	24.78	0.71	3%
	Sample 2	107.53	3.39	3%
Mouse G-CSF	Sample 1	183.83	8.58	5%
	Sample 2	866.14	27.19	3%
Mouse CCL17 (TARC)	Sample 1	38.25	1.44	4%
	Sample 2	149.28	5.11	3%
Mouse IL-12p40	Sample 1	22.36	0.96	4%
	Sample 2	96.86	4.57	5%
Mouse IL-1β	Sample 1	29.93	0.86	3%
	Sample 2	129.21	4.59	4%

Inter-Assay Precision

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

Analyte	Sample	Mean (pg/mL)	STDEV	%CV
Mouse CXCL1 (KC)	Sample 1	44.43	7.48	17%
	Sample 2	149.14	19.26	13%
Mouse Free Active TGF-β1	Sample 1	104.94	15.42	15%
	Sample 2	399.08	50.75	13%
Mouse IL-18	Sample 1	250.69	29.95	12%
	Sample 2	1162.68	172.95	15%

Mouse IL-23	Sample 1	220.86	36.43	16%
	Sample 2	883.86	128.81	15%
Mouse CCL22 (MDC)	Sample 1	66.03	10.49	16%
	Sample 2	181.72	23.44	13%
Mouse IL-10	Sample 1	41.73	8.06	19%
	Sample 2	150.05	20.05	13%
Mouse IL-12p70	Sample 1	35.19	5.29	15%
	Sample 2	139.07	14.70	11%
Mouse IL-6	Sample 1	55.35	9.61	17%
	Sample 2	190.39	25.40	13%
Mouse TNF-α	Sample 1	39.05	5.45	14%
	Sample 2	146.59	16.09	11%
Mouse G-CSF	Sample 1	252.96	58.63	23%
	Sample 2	951.27	156.80	16%
Mouse CCL17 (TARC)	Sample 1	57.98	12.53	22%
	Sample 2	200.44	27.35	14%
Mouse IL-12p40	Sample 1	35.67	5.75	16%
	Sample 2	128.81	12.33	10%
Mouse IL-1β	Sample 1	43.80	6.65	15%
	Sample 2	165.21	19.69	12%

Biological Samples

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

Serum

Pooled normal serum samples from 4 different strains were tested for endogenous levels using the LEGENDplex™ Mouse Macrophage/Microglia Panel. The concentrations measured are shown below.

Analyte	C57BL/6	BALB/c	Swiss Webster	CD-1
Mouse CXCL1 (KC)	332.84	259.50	175.00	106.96
Mouse Free Active TGF-β1	847.92	1119.64	2028.78	700.03

Mouse IL-18	268.43	113.69	232.60	157.36
Mouse IL-23	29.98	30.89	86.11	22.94
Mouse CCL22 (MDC)	266.67	219.69	542.19	275.33
Mouse IL-10	8.39	7.27	15.26	4.96
Mouse IL-12p70	ND	2.12	2.38	ND
Mouse IL-6	44.15	166.35	32.15	12.54
Mouse TNF-α	55.93	37.30	41.88	37.76
Mouse G-CSF	1061.38	1670.96	121.47	183.37
Mouse CCL17 (TARC)	255.82	114.09	145.20	103.10
Mouse IL-12p40	203.11	204.88	329.47	175.03
Mouse IL-1β	19.97	ND	1.62	ND

ND = Non-detectable

Plasma

Pooled mouse plasma samples (citrate and EDTA plasma) representing 4 different strains were tested for endogenous levels using the LEGENDplex™ Mouse Macrophage/Microglia Panel. The concentrations measured are shown below.

Analyte	C57BL/6	BALB/c	Swiss Webster	CD-1
Mouse CXCL1 (KC)	90.15	139.61	82.37	61.08
Mouse Free Active TGF-β1	74.35	76.04	149.49	128.13
Mouse IL-18	287.20	105.53	146.51	71.83
Mouse IL-23	21.86	53.35	31.77	15.89
Mouse CCL22 (MDC)	140.98	153.71	256.79	163.12
Mouse IL-10	7.17	4.66	13.18	5.80
Mouse IL-12p70	0.76	ND	ND	ND
Mouse IL-6	26.05	37.50	10.18	5.81

Mouse TNF-α	39.81	27.72	23.81	16.77
Mouse G-CSF	416.20	778.49	211.58	206.43
Mouse CCL17 (TARC)	57.61	52.22	51.66	26.28
Mouse IL-12p40	231.08	107.17	118.03	108.97
Mouse IL-1β	5.57	ND	4.49	ND

ND = Non-detectable

Cell Culture Supernatant

Mouse RAW 264.7 macrophage cells (1x10^6 cells/mL) were cultured under various conditions (LPS, 1 µg/mL; GM-CSF, 100 ng/mL; IFN-γ, 100 ng/ml). Supernatants were collected after 72 hours and assayed with the LEGENDplex™ Mouse Macrophage/Microglia Panel. The results (all in pg/mL) are summarized below.

Analyte	Control	LPS	LPS + GM-CSF	LPS + IFN-γ
Mouse CXCL1 (KC)	0.57	40.15	33.54	34.70
Mouse Free Active TGF-β1	19.86	8.98	2.51	2.48
Mouse IL-18	ND	ND	3.70	ND
Mouse IL-23	ND	7.68	14.25	9.54
Mouse CCL22 (MDC)	7.12	765.74	1996.50	705.81
Mouse IL-10	1.70	11.11	16.44	19.36
Mouse IL-12p70	0.89	7.07	14.17	7.39
Mouse IL-6	ND	7009.00	9621.00	3363.20
Mouse TNF-α	78.26	2089.13	3803.50	1772.19
Mouse G-CSF	5.95	12569.50	25134.00	12572.70
Mouse CCL17 (TARC)	1.38	45.32	137.17	40.14
Mouse IL-12p40	ND	9.44	13.88	8.82
Mouse IL-1β	1.39	8.86	26.74	8.15

ND = Non-detectable

Mouse splenocyte cells (1x10^6 cells/mL) were cultured under various conditions (LPS, 1 µg/mL; IFN-γ, 30 ng/ml). Supernatants were collected after 72 hours and assayed with the LEGENDplex™ Mouse Macrophage/Microglia Panel. The results (all in pg/mL) are summarized below.

Analyte	Control	LPS	LPS + IFN-γ
Mouse CXCL1 (KC)	1.25	73.49	74.42
Mouse Free Active TGF-β1	5.64	5.79	7.95
Mouse IL-18	ND	ND	22.47
Mouse IL-23	ND	5.51	4.13
Mouse CCL22 (MDC)	38.59	210.26	130.03
Mouse IL-10	1.17	35.28	38.92
Mouse IL-12p70	ND	ND	1.68
Mouse IL-6	ND	40.52	288.64
Mouse TNF-α	1.48	79.44	209.30
Mouse G-CSF	ND	18.50	34.45
Mouse CCL17 (TARC)	1.16	1.84	1.90
Mouse IL-12p40	3.48	4.98	19.83
Mouse IL-1β	0.90	1.16	3.76

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.

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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