Enabling Legendary Discovery™

LEGENDplex™

Mouse Cytokine Panel 2
Mix and Match Subpanel

Please read the entire manual before running the assay.

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

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Chapter 1: KIT DESCRIPTION

Introduction

Cytokines are small soluble glycoproteins that act as extracellular signaling molecuels and meidate communications between cells. They regulate immunity, inflammation and hematopoiesis as well as cell growth, migration, development and differentiation. Cytokines act in large tightly regulated networks where both the production and action of one cytokine is affected by the behavior of other cytokines. The accurate measurement of the expression profile of cytokines in a sample is critical for in-depth understanding of disease progression and immune responses.

The LEGENDplex™ Mouse Cytokine Panel 2 is a multiplex bead-based assay, using fluorescence–encoded beads suitable for use on various flow cytometers. This panel allows simultaneous quantification of 13 mouse cytokines, including IL-1α, IL-1β, IL-3, IL-7, IL-11, IL-12p40, IL-12p70, IL-23, IL-27, IL-33, IFN-β, GM-CSF and TSLP. This assay panel provides higher detection sensitivity and broader dynamic range than traditional ELISA methods. The panel has been validated for use on erum, plasma and cell culture supernatant samples.

The Mouse Cytokine Panel 2 is designed to allow flexible customization within the panel.For mix and match within the panel, please visit **www.biolegend.com/legendplex.**

This assay is for research use only.

Principle of the Assay

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

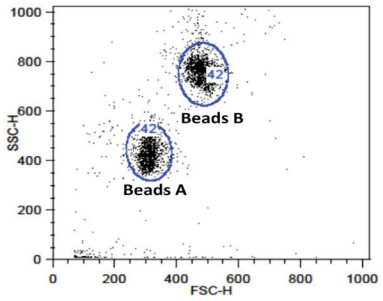
Beads are differentiated by size and internal fluorescence intensities. Each bead set is conjugated with a specific antibody on its surface and serves as the capture beads for that particular analyte. When a selected panel of capture beads is mixed and incubated with a sample containing target analytes 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 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 Cytokine Panel 2 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 6 bead populations and the larger Beads B consists of 7 bead populations (Figure 2-3).

Using a total of 13 bead populations distinguished by size and internal fluorescent dye, the Mouse Cytokine Panel 2 allows simultaneous detection of 13 cytokines 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

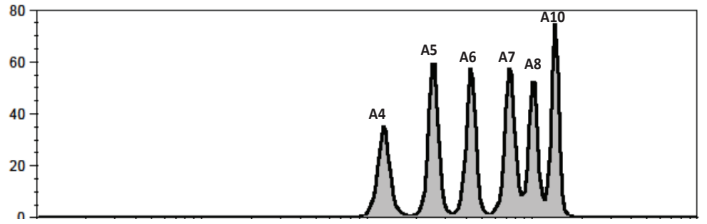
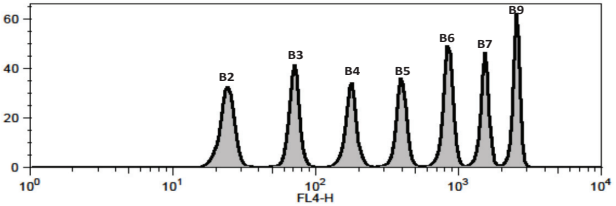


Figure 3. Beads B Classification by FL4



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

Table 1. Beads ID*, Top Standard Concentrations and Panel-Specific Target Selection

Target	Bead ID	Top Standard Concentrations (ng/mL)	Cytokine Panel 2 Cat. No. 740134	Mix & Match
IL-1α	A4	10	√	
IL-1β	A5	10	√	
IL-3	A6	10	√	
IL-12p40	A7	10	√	
IL-12p70	A8	10	√	
IL-23	A10	50	√	
IL-7	B2	50	√	
IL-11	B3	50	√	
IL-27	B4	50	√	
IL-33	B5	50	√	
IFN-β	B6	50	√	
GM-CSF	B7	10	√	
TSLP	B9	50	√	

*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, B3, B4...). 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 reconstituted, immediately transfer contents into polypropylene vials. DO NOT STORE RECONSTITUTED STANDARDS IN GLASS VIALS.
- Upon reconstitution, leftover standard and Matrix C 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.

For the Mix and Match Subpanels, individual beads are provided at 13X concentration. The Buffer Set contains Setup Beads, all Buffers, Plate Sealers, Matrix, SA-PE and Data Analysis Software Dongle. A manual is provided for each Mix and Match subpanel.

Kit Components	Quantity	Volume	Cat/ Part #
Capture Beads* (see tables below for more information)	**varies**	**varies**	**varies**
LEGENDplex™ Mouse Cytokine Panel 2 Detection Antibodies	1 bottle	3.5 mL	740149
LEGENDplex™ Mouse Cytokine Panel 2 Standard	1 vial	lyophilized	740372
LEGENDplex™ Buffer Set B	1		740373
Filter Plate* or V-bottom Plate**	1 Plate		740377*or 740379**
Mouse Cytokine Panel 2 Mix and Match Custom Subpanel Manual	1		76189

* For kit with filter plate. ** For kit with V-bottom plate.

Capture beads for Mix and Match Subpanels*:

Bead Name	Quantity	Volume	Cat #
LEGENDplex™ Mouse IL-1α Capture Bead A4, 13X	1 vial	270 µL	740135
LEGENDplex™ Mouse IL-1β Capture Bead A5, 13X	1 vial	270 µL	740136
LEGENDplex™ Mouse IL-3 Capture Bead A6, 13X	1 vial	270 µL	740137
LEGENDplex™ Mouse IL-12 (p40) Capture Bead A7, 13X	1 vial	270 µL	740138
LEGENDplex™ Mouse IL-12 (p70) Capture Bead A8, 13X	1 vial	270 µL	740139
LEGENDplex™ Mouse IL-23 Capture Bead A10, 13X	1 vial	270 µL	740140
LEGENDplex™ Mouse IL-7 Capture Bead B2, 13X	1 vial	270 µL	740141
LEGENDplex™ Mouse IL-11 Capture Bead B3, 13X	1 vial	270 µL	740142
LEGENDplex™ Mouse IL-27 Capture Bead B4, 13X	1 vial	270 µL	740143
LEGENDplex™ Mouse IL-33 Capture Bead B5, 13X	1 vial	270 µL	740144
LEGENDplex™ Mouse IFN-β Capture Bead B6, 13X	1 vial	270 µL	740145
LEGENDplex™ Mouse GM-CSF Capture Bead B7, 13X	1 vial	270 µL	740146
LEGENDplex™ Mouse TSLP Capture Bead B9, 13X	1 vial	270 µL	740147

*** 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)

Component	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	Channel Emission	Classification Channel	Channel Emission	Compensation needed?
BD FACSCalibur™ (single laser)	FL2	575 nm	FL3	670 nm	Yes
BD FACSCalibur™ (dual laser)	FL2	575 nm	FL4	660 nm	No*
BD FACSArray™	Yellow	575 nm	Red	660 nm	No*
BD FACSCanto™ BD FACSCanto™ II	PE	575 nm	APC	660 nm	No*
BD™ LSR, LSR II BD LSRFortessa™	PE	575-585 nm	APC	660 nm	No*
BD FACSAria™	PE	575 nm	APC	660 nm	No*

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

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

For flow cytometers not listed here, the end-user needs to set up the machine following similar guidelines. Please refer to **Setup Procedure for Other Flow Cytometers** section in Chapter 4.

- 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)

If the assay is run using the filter plate provided (recommended),

- A vacuum filtration unit (Millipore MultiScreen ® HTS Vacuum Manifold, catalog # MSVMHTS00 or equivalent). Instructions on how to use the vacuum manifold can be found 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 the assay is run in microtubes, V- or U-bottom 96-well plate (optional),

- 1.1 mL polypropylene micro FACS tubes, in 96-tube rack (e.g., National Scientific Supply Co, catalog # TN0946-01R, or equivalent).
- Centrifuge with a swinging bucket adaptor for microtiter plates or micro-tube racks (e.g., Beckman Coulter Allegra™ 6R Centrifuge with MICROPLUS CARRIER adaptor for GH3.8 and JS4.3 Rotors) .
- 96-well, V- or U-bottom, clear polypropylene microplate (low protein binding, e.g., greiner bio-one, Catalog # 651201 or equivalent).

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

Preparation of Plasma Samples:

- Plasma collection using EDTA as an anti-coagulant is recommended. Centrifuge for 10 minutes at 1,000 x *g* within 30 minutes of blood collection.
- Remove plasma and assay immediately, or aliquot and store samples at ≤-20°C. Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended that samples are thawed completely, mixed well and centrifuged to remove particulates.

Preparation of Tissue Culture Supernatant:

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

Reagent Preparation

Preparation of Antibody-Immobilized Beads

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

1. Sonicate the beads vials for 1 minute in a sonicator bath and then vortex for 30 seconds prior to use.
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 96 reactions, prepare 3 mL of mixed beads.
3. To make 1.5 ml of 5-plex 1X diluted beads, transfer 115 µL of each of

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the 5 individual beads (13X) to a fresh tube (total bead volume = 575 µL) and add 925 µL of Assay Buffer to make the final volume of 1.5 mL.

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 Cytokine Panel 2 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 microfuge tube. This will be used as the top standard C7.

   Note: Analytes in this panel have different top standard concentrations (see Table 1, page 5 for deteails).

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**). Assay Buffer will be used as the 0 pg/mL standard (C0).

Mouse Cytokine Panel 2 Mix and Match Subpanel

Tube/ Standard ID	Serial Dilution	Assay Buffer to add (µL)	Standard to add	Final Conc. (pg/mL) *	Final Conc. (pg/mL) **
C7	--	--	--	10,000	50,000
C6	1:4	75	25 µL of C7	2,500	12,500
C5	1:16	75	25 µL of C6	625	3,125
C4	1:64	75	25 µL of C5	156.3	781.3
C3	1:256	75	25 µL of C4	39.1	195.3
C2	1:1024	75	25 µL of C3	9.8	48.8
C1	1:4096	75	25 µL of C2	2.4	12.2
C0	--	75	--	0	0

***Top standard concentration of IL-1α, IL-1β, IL-3, IL-12p40 , GM-CSF, IL-12p70, is 10 ng/mL; **Top standard concentration of IL-7, IL-11, IL-23, IL-27, IL-33, IFN-β, and TSLP is 50 ng/mL.**

Dilution of Samples

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

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

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

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

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

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Chapter 3: ASSAY PROCEDURE

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

- The in-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 a Luminex®-based multiplex assay before, your lab should already have the vacuum filtration unit set up.

- If the in-filter plate assay procedure is not possible or if you prefer, the assay can be performed in microtubes or in a V/U-bottom microplate. For in-tube assay, we recommend using micro FACS tubes (see **Materials to be Provided by the End-User, page 8).**

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). 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:

   - Add 25 µL of Assay Buffer to all wells.
   - Add 25 µL of each standard to the standard wells.

- Add 25 µL of each sample to the sample wells (See **Sample Dilution**)

For measuring serum or plasma samples:

- Add 25 µL of Matrix C to the standard wells.
- Add 25 µL of Assay Buffer to the sample wells.
- Add 25 µL of each standard to the standard wells.
- Add 25 µL of each diluted serum or plasma sample to the sample wells (See **Sample Dilution**).

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

3. Seal the plate with a plate sealer. **To avoid plate leaking, do not apply positive pressure to the sealer when sealing the plate**. Wrap the entire plate, including the inverted plate cover, with aluminum foil. Place the plate on a plate shaker, secure it 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.

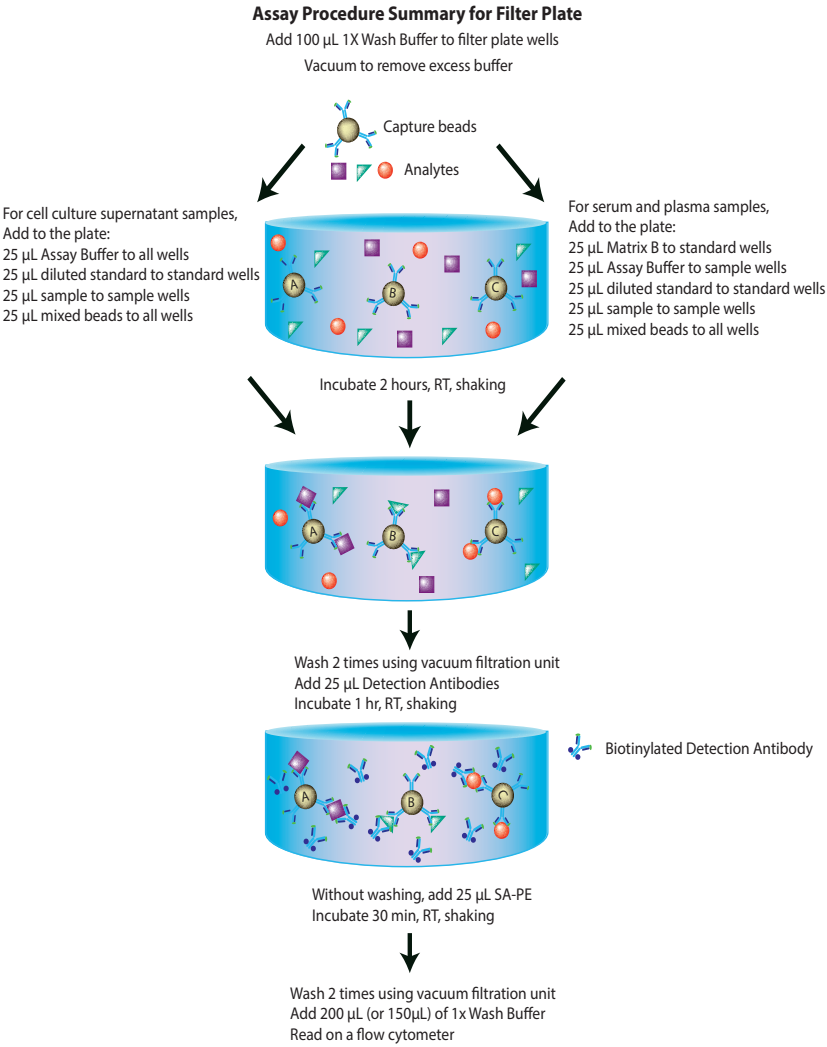
10. Add 200 µL of 1X Wash Buffer to each well (**or 150 µL if plate is to be read with an autoampler. Higher volume may result in leaking of the filter plate during reading. Be sure to set the sample volume to be analyzed to 70 uL or less so that sample can be read again if needed**). Resuspend the beads 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. **The probe height may need to be adjusted when using an autosampler**.

If an autosampler is not available, the samples need to be transferred from the filter plate to FACS tubes and read manually. In this case, the sample volume may need to be increased from 200 µL to 300 µL by adding extra 100 µL of 1X Wash Buffer to each tube to avoid sample running dry when read on a flow cytometer.



Performing the Assay Using Microtubes, a 96-Well V- or U-bottom Microplate

- Allow all reagents to warm to room temperature (20-25°C) before use.
- Determine the number of assays to run. **Standards and samples should be run in duplicate and arranged in the order convenient for data acquisition and analysis (as shown below). If an automation device is used for reading, the orientation and reading sequence should be carefully planned.**

  If using tubes, label all tubes and arrange tubes on a rack (as shown in attached RACK MAP, assuming using micro FACS tubes and a 96-microtube rack, e.g., National Scientific Supply Co, catalog #: TN0946-01R). A 96-microtube rack is recommended because it allows pipetting with a multichannel pipette.

  If using a microplate, arrange the standard and samples according to the the PLATE MAP attached.

1. **For measuring cell culture supernatant samples:**
   - Add 25 µL of Assay Buffer to all tubes/wells.
   - Add 25 µL of each standard to the standard tubes/wells .
   - Add 25 µL of each sample to the sample tubes/wells (See **Sample Dilution**).
   - Add 25 µL of mixed beads to all tubes/wells.
   - Add 25 µL Detection Antibodies to all tubes/wells.

   For measuring serum or plasma samples:
   - Add 25 µL of Matrix C to the standard tubes/wells.
   - Add 25 µL of Assay Buffer to sample tubes/wells.
   - Add 25 µL of each standard to standard tubes/wells.
   - Add 25 µL of each diluted serum or plasma sample to sample tubes/wells (See **Sample Dilution**).
   - Add 25 µL of mixed beads to all tubes/wells.
   - Add 25 µL Detection Antibodies to all tubes/wells.

   Note: The volume should be 100 µL in each tube/well. Shake beads bottle intermittently during the addition to avoid bead settling.

2. Cover the entire rack/plate with aluminum foil to protect the tubes/plate from light. Shake (approximate 1,000 rpm for in-tube assay and 600 rpm for in-plate assay) on a plate shaker for 2 hours at room temperature.
3. Without washing the tubes/plate, add 25 µL SA-PE to each tube/well.
4. Cover the entire rack/plate with aluminum foil to protect the tubes/plate

from light. Shake (approximate 1,000 rpm for in-tube assay and 600 rpm for in-plate assay) on a plate shaker for 30 minutes at room temperature.

5. Centrifuge the tubes/plate at 1,000 x *g* for 5 minutes, using a swinging bucket rotor with microplate adaptor (Please refer to **Materials to be Provided by the End-User, page 8).**
6. Remove the supernatant using a multichannel pipette. Be careful not to remove beads, but to remove as much liquid as possible.
7. Add 200 µL of 1X Wash Buffer to all tubes/wells. Resuspend the beads by vortexing (for in-tube assay) or shaking on a plate shaker for 1 minute (for in-plate assay). Centrifuge the tubes/plate at 1,000 x *g* for 5 minutes, using a swinging bucket rotor with microplate adaptor. Remove the supernatant.
8. Add 200 µL of 1X Wash Buffer to each tube/well (**or 150 µL if plate is to be read with an autoampler. Higher volume may result in leaking of the filter plate during reading. Be sure to set the sample volume to be analyzed to 70 uL or less so that sample can be read again if needed**). Resuspend the beads by vortexing (for in-tube assay) or pippeting (for in-plate assay).
9. 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 either directly (for in-plate assay) or after being transferred to a 96-well plate ((for in-tube assay). **The probe height may need to be adjusted when using an autosampler**.

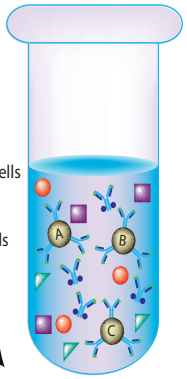
   If an autosampler is not available, the samples can be read directly in FACS tubes or after being transferred from microplate to micro FACS tubes. In this case, the sample volume may need to be increased from 200 µL to 300 µL by adding extra 100 µL of 1X Wash Buffer to each tube, to avoid sample running dry when analyzed on a flow cytometer.

Assay Procedure Summary for Tubes (or V-Bottom Plate)

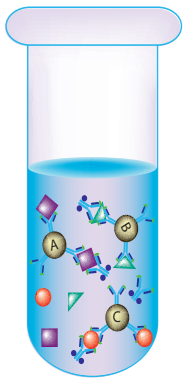
Arrange the number of tubes needed on a rack (or set up the plate)

For cell culture supernatant samples,
Add to the tubes/wells:
25 µL Assay Buffer to all tubes/wells
25 µL diluted standard to standard tubes/wells
25 µL sample to sample tubes/wells
25 µL mixed beads to all tubes/wells
25 µL Detection Antibodies to all tubes/wells

For serum and plasma samples,
Add to the tubes/well:
25 µL Matrix B to standard tubes/wells
25 µL Assay Buffer to sample tubes/wells
25 µL diluted standard to standard tubes/wells
25 µL sample to sample tubes/wells
25 µL mixed beads to all tubes/wells
25 µL Detection Antibodies to all tubes/wells



Incubate 2 hours, RT, shaking



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

Spin down beads
Wash one time
Add 200 µL (or 150µL) of 1x Wash Buffer
Read on a flow cytometer

Chapter 4: FLOW CYTOMETER SETUP

In order to generate reliable data, the flow cytometer must be set up properly before data acquisition. The following sections will address machine setup for the flow cytometers listed below.

List of Flow Cytometers and Possible Configurations

Flow Cytometer	Reporter Channel	Channel Emission	Classification Channel	Channel Emission	Compensation needed?
BD FACSCalibur™ (single laser)	FL2	575 nm	FL3	670 nm	Yes
BD FACSCalibur™ (dual laser)	FL2	575 nm	FL4	660 nm	No*
BD FACSCanto™, BD FACSCanto™ II	PE	575 nm	APC	660 nm	No*
BD™ LSR, LSRII, BD LSRFortessa™	PE	575-585 nm	APC	660 nm	No*
BD FACSAria™	PE	575 nm	APC	660 nm	No*

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

For flow cytometers not listed here, the end-user needs to set up the machine following similar guidelines. Please refer to **Setup Procedure for Other Flow Cytometers** section in this chapter.

The setup process typically includes the following steps. Please see the detailed setup procedure that follows, regarding your specific instrument.

1). Start up the instrument following the manufacturer's recommendations.

2). Create a template for data acquisition using your instrument's data acquisition software. A template is a document or worksheet with density plots that allows the user to perform machine setup and data acquisition.

3). Set up the PMT voltages of each channel to be used for data acquisition using the Setup Beads provided in the kit.

4). Determine whether compensation is needed based on the configuration of your system as shown in Table 1. If compensation is needed, perform compensation using the Setup Beads provided in the kit.

Setup Procedure for FACSCalibur™ with Dual Lasers

For a dual laser FACSCalibur™, use FL2 for reporter and FL4 for beads classification. In general, there is no need for compensation between these channels if the machine is set up properly, following the setup procedure described below.

1. Start up the Instrument

Perform instrument startup and verification check following the manufacturer's recommendations.

2. Obtain a Template for Data Acquisition

A template for FACSCalibur™ is a document with density plot that allows the user to perform machine setup and data acquisition.

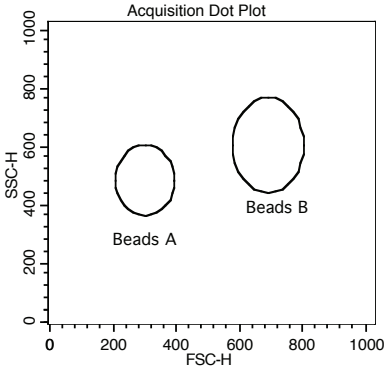
If you have already created a template for the flow cytometer, open that template and proceed to Step 3.

If a template is not yet available, create a new template by following the instructions below:

2.1 From the BD CellQuest data acquisition software, go to File →new document.

2.2 Create a dot plot with FSC (forward scatter) for X-axis and SSC (side scatter) for Y-axis. **Be sure to set FSC and SSC to linear mode**. Create two gates and label them Beads A and Beads B (Figure 4).

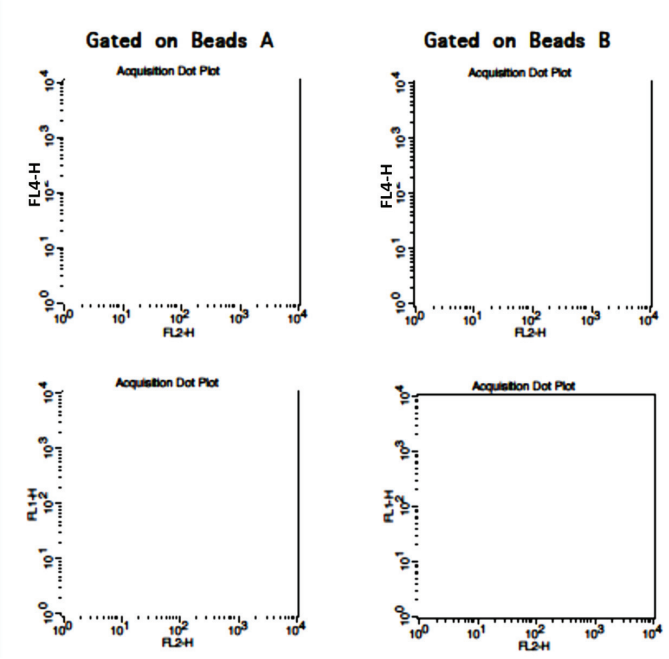
Figure 4.



2.3 Create 4 fluorescent dot plots as shown below (Figure 5) with FL2 for X-axis, FL4 or FL1 for Y-axis. For the fluorescent dot plots, gate on Beads A (dot plots on the left panel below) and Beads B (dot plots on the right panel below). **The dot plots should be in log mode**.

2.4 Save the new document as “LEGENDplex Template for FACSCalibur Dual Laser” and proceed to the next step of the setup.

Figure 5.



3. Set up the PMT Voltages

The Setup Beads 3: Raw Beads are used to set up the PMT voltage of the classification channel (FL4 /APC) and channel FL1. The Setup Beads 2: PE Beads are used to set up the PMT voltage of the reporter channel (FL2/PE) The Setup Beads 1: FITC Beads are not needed for this setup.

Follow the instructions below for setting up the PMT settings:

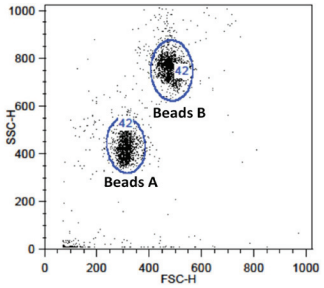
3.1 Vortex the vial of the Raw Beads for 30 seconds to resuspend the beads.

3.2 Transfer 400 µL of the Raw Beads to a fresh FACS tube.

3.3 Set the flow cytometer flow rate to low. In setup mode, run the Raw Beads. Adjust the settings for FSC and SSC so that both bead populations are visible (Figure 6).

Pause and restart acquisition frequently during the setup procedure to refresh the bead populations after adjusting settings.

Figure 6.



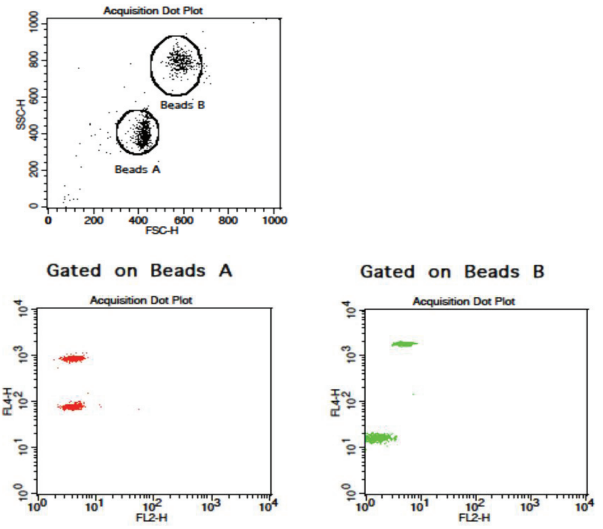
3.4 Continue adjusting the settings so that Beads A and Beads B are well separated and the FSC and SSC readings are >200.

3.5 Move the gates for Beads A and Beads B so that the smaller beads fall into Beads A gate and the larger beads fall into Beads B gate (Figure 6).

3.6 Adjust the FL1 setting so that the FL1 signals for all beads are between $1x10^0$ and $1x10^1$ (Note: This step is not required, but is recommended).

3.7 Adjust the FL4 setting so that the FL4 signals for all beads are between $1x10^1$ and $5x10^3$ (Figure 7).

Figure 7.



3.8 Vortex the vial of Setup Beads 2: PE Beads for 30 seconds to resuspend the beads.

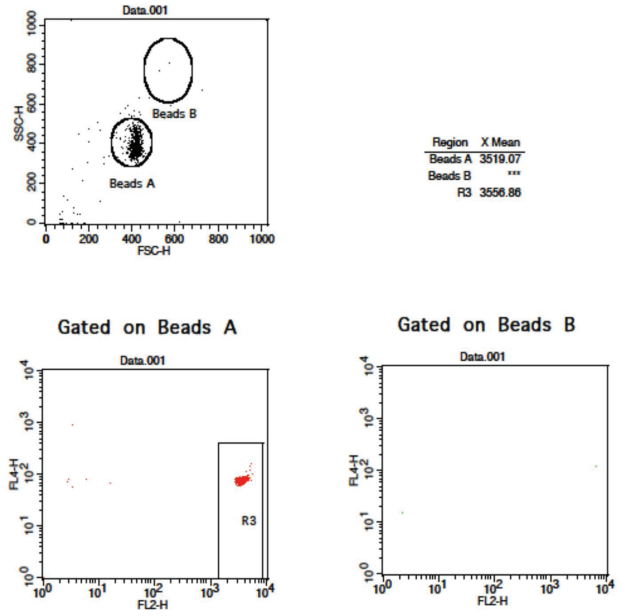
3.9 Transfer 400 µL of the PE Beads to a fresh FACS tube.

3.10 Replace the Raw Beads tube from the flow cytometer with the PE beads tube.

3.11 Set the flow cytometer flow rate to low. In setup mode, run the PE Beads. Note: PE beads are only of small size, falling in the Beads A gate (Figure 8).

3.12 Adjust the FL2 setting so that the median fluorescence intensity of the PE beads falls between the lot-specific range labeled on the PE Beads vial (Figure 8, gate R3).

Figure 8.



3.13 Save the document again for future use.

3.14 The flow cytometer is now ready for sample acquisition.

Setup Procedure for FACSCalibur™ with a Single Laser

For a single laser FACSCalibur™, use FL2 for reporter and FL3 for beads classification. Compensation is needed to properly set up the instrument.

1. Start up the Instrument

Perform instrument startup and verification check following the manufacturer's recommendations.

2. Obtain a Template for Data Acquisition

A template for FACSCalibur™ is a document with density plot that allows the user to perform machine setup and data acquisition.

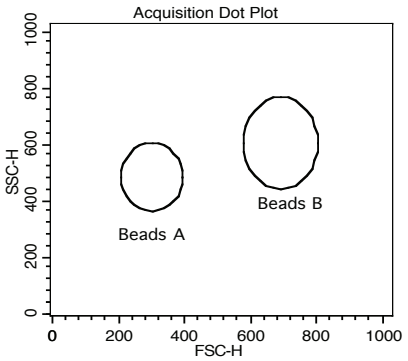
If you have already created a template for the flow cytometer, open that template and proceed to Step 3.

If a template is not yet available, create a new template by following the instructions below:

2.1 From the BD CellQuest data acquisition software, go to File → new document.

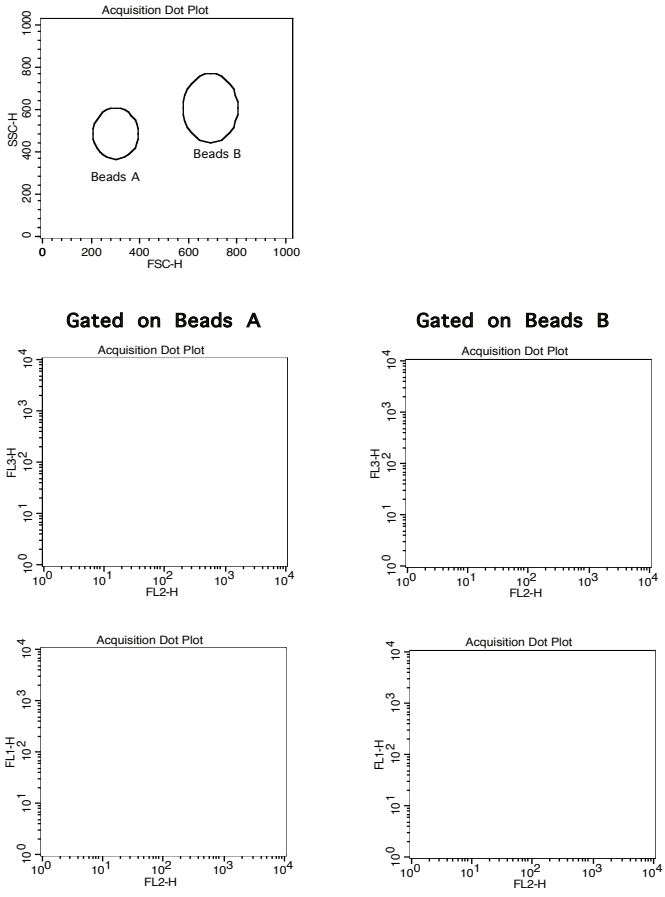
2.2 Create a dot plot with FSC (forward scatter) for X-axis and SSC (side scatter) for Y-axis. **Be sure to set FSC and SSC to linear mode**. Create two gates and label them Beads A and Beads B (Figure 9).

Figure 9.



2.3 Create 4 fluorescent dot plots as shown below (Figure 10) with FL2 for X-axis, and FL3 or FL1 for Y-axis. For the fluorescent dot plots, gate on Beads A (dot plots on the left panel below) and Beads B (dot plots on the right panel below). **The dot plots should be in log mode.**

Figure 10.



2.4 Set all compensations to zero.

2.5 Save the new document as “LEGENDplex Template for FACSCalibur Single Laser” and proceed to the next step of the setup.

3. Set up PMT Voltages and Compensation

The Setup Beads 3: Raw Beads are used to set up the PMT voltage of the classification channel FL3 and channel FL1. The Setup Beads 2: PE Beads are used to set up the PMT voltage of the reporter channel FL2. All three setup beads are needed for setting up compensation.

Follow the instructions below for setting up the PMT settings and compensation:

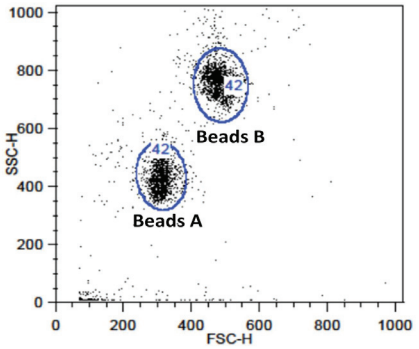
3.1 Vortex the vial of the Raw Beads for 30 seconds to resuspend the beads.

3.2 Transfer 400 µL of the Raw Beads to a fresh FACS tube.

3.3 Set the flow cytometer flow rate to low. In setup mode, run the Raw Beads. Adjust the settings for FSC and SSC so that both beads populations are visible (Figure 11).

Pause and restart acquisition frequently during the setup procedure to refresh the bead populations, after adjusting settings.

Figure 11.

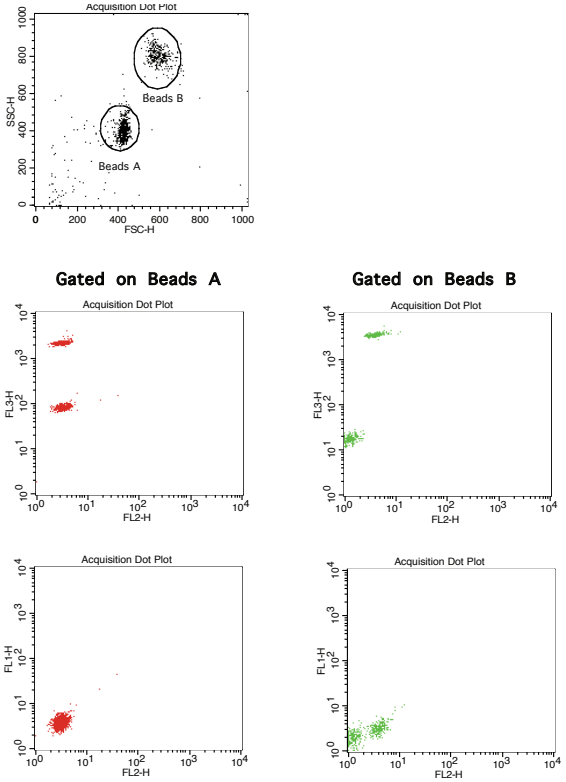


3.4 Continue adjusting the settings so that Beads A and Beads B are well separated and the FSC and SSC readings are >200.

3.5 Move the gates for Beads A and Beads B so that the smaller beads fall into Beads A gate and the larger beads fall into Beads B gate (Figure 11).

3.6 Adjust the FL1 setting so that the FL1 signals for all beads are between $1x10^0$ and $1x10^1$ (Figure 12).

Figure 12.



3.7 Adjust the FL2 settings so that the FL2 signals for all beads are between $1x10^0$ and $1x10^1$ (Figure 12).

3.8 Adjust the FL3 setting so that the FL3 signals for all beads are between $1x10^1$ and 5×10^3 (Figure 12).

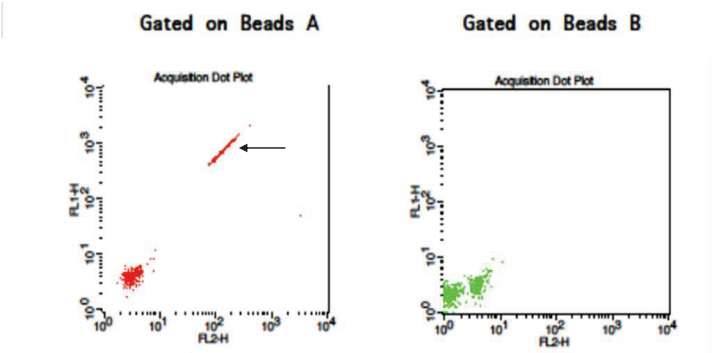
3.9 Vortex the vial of the Setup Beads 1: FITC Beads for 30 seconds to resuspend the beads.

3.10 Transfer 200 µL of the FITC Beads to a fresh FACS tube. Add 200 µL of Raw Beads and mix well (this will generate FITC-positive and FITC-negative populations of beads and is needed for proper compensation).

3.11 In setup mode, run the mixed FITC and Raw Beads.

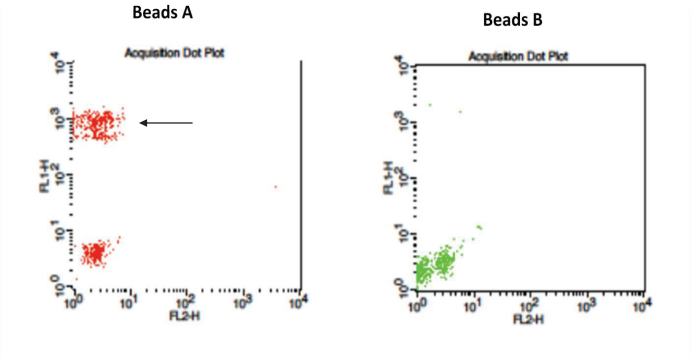
3.12 On the FL1 vs FL2 dot plot (Figure 13), the beads will display as FITC-negative and FITC-positive populations (indicated by an arrow). Note: FITC beads are only of small size, falling in the Beads A gate.

Figure 13.



3.13 Adjust the FL2-%FL1 compensation setting (e.g., FL2-FL1 = 20%) so that the FITC-negative and FITC-positive populations have similar mean FL2 fluorescence intensities (Figure 14).

Figure 14.



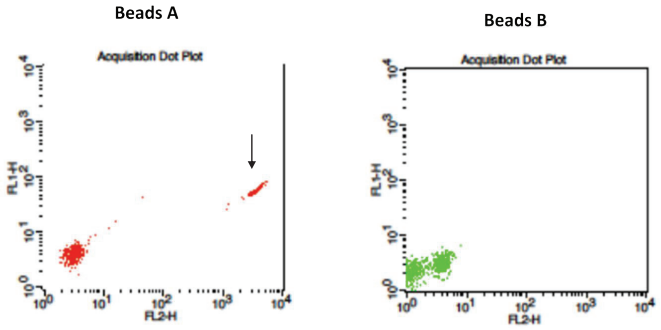
3.14 Vortex the vial of PE Beads for 30 seconds to resuspend the beads.

3.15 Transfer 200 µL of the PE Beads to a fresh FACS tube. Add 200 µL of Raw Beads and mix well (This will generate PE-positive and PE-negative populations of beads and is needed for proper compensation).

3.16 In setup mode, run the mixed PE and Raw Beads.

3.17 On the FL1 vs FL2 dot plot (Figure 15), the beads will display as PE-negative and PE-positive populations (indicated by an arrow). Note: PE beads are only of small size, falling in the Beads A gate.

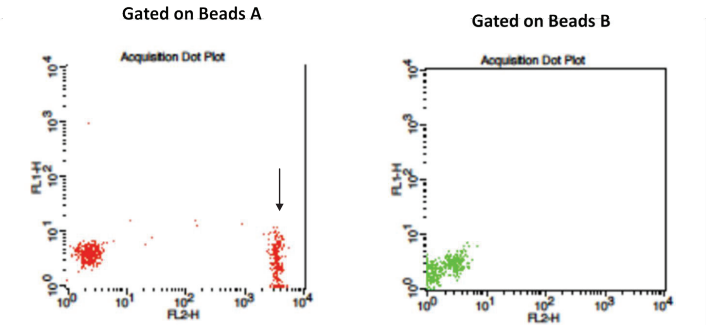
Figure 15.



3.18 Adjust the FL2 setting so that the median fluorescence intensity of the PE beads falls between the lot-specific range labeled on the PE Beads vial (Figure 16).

3.19 Adjust the FL1-%FL2 compensation setting (e.g., FL1-FL2 = 1.5%) so that the PE-negative and PE-positive populations have similar mean FL1 fluorescence intensities (Figure 16).

Figure 16.



3.20 On the FL3 vs FL2 dot plot (Figure 17), the beads will display as PE-negative and PE-positive populations (indicated by an arrow).

3.21 Adjust the FL3-%FL2 compensation setting (e.g., FL3-%FL2= 40%) so that the FL3-low population and the PE-positive population have similar median FL3 signal (Figure 18).

Figure 17.

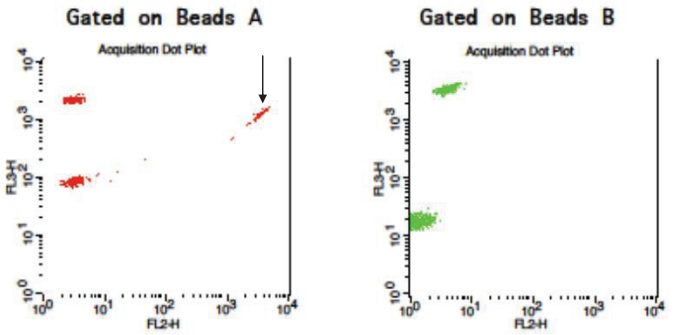
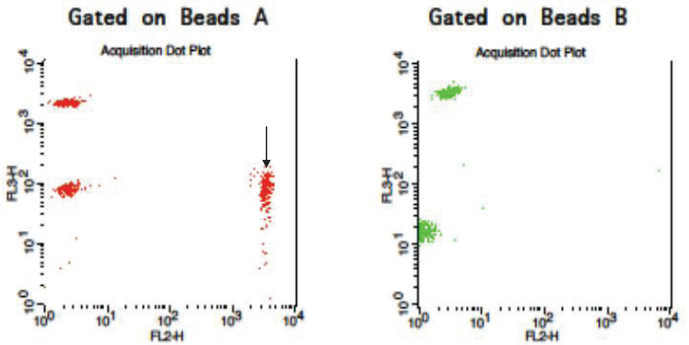


Figure 18.



3.22 Save the document again for future use.

3.23 The flow cytometer is now compensated and ready for sample acquisition.

Setup Procedure for BD FACSAria™, FACSCanto™ and LSR Series

This part of the guide applies to BD digital flow cytometers using FACSDiva™ software version 6.0 and above.

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

This setup procedure is required under the following situations:

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

This setup process is not needed if you have run this experiment before and have access to a saved experiment template (The settings will be saved in the final step of this setup procedure and any settings saved can be imported to a new experiment. Please refer to Step 2 and Step 3.9 below).

1. Start up the Instrument

Perform instrument startup and verification check following the manufacturer's recommendations.

2. Obtain a Template for Data Acquisition

A template for FACSDiva™ is a worksheet with density plots that allows the user to perform machine setup and data acquisition.

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

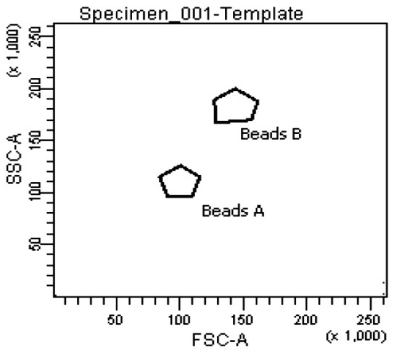
If you have already created a template for the flow cytometer, open that template and proceed to Step 3. To open an existing template, select Experiment> New Experiment. A list of templates saved in D:\BDExport\Templates\ Experiment will pop up. Select the desired template from the list.

To create a new template, follow the instructions below:

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

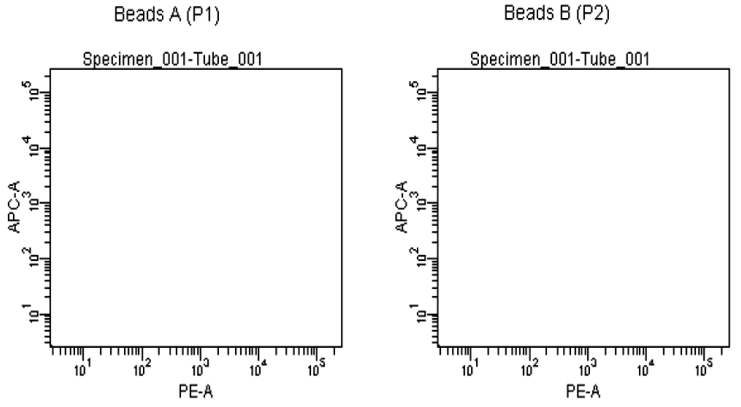
2.2 In the global worksheet, open the worksheet. Create a dot plot with FSC (forward scatter) for X-axis and SSC (side scatter) for Y-axis. **Be sure to set FSC and SSC to linear mode.** Create two gates and label them Beads A and Beads B (Figure 19).

Figure 19.



2.3 Create two dot plots with PE for X-axis, APC for Y-axis (Figure 20), gated on Beads A (left panel below) and Beads B (right panel below), respectively. Create one dot plot with FITC for X-axis APC for Y-axis, gated on Beads A and Beads B (graph not shown). **The plots should all be in log mode**.

Figure 20.



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

3. Set up PMT Voltages

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

Follow the instructions below for setting up the PMT settings:

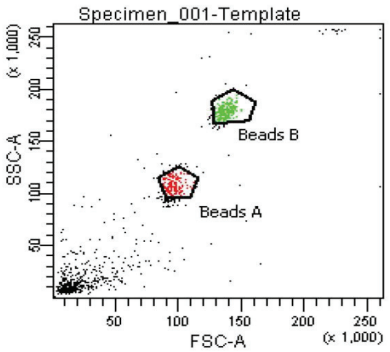
3.1 Vortex the vial of Raw Beads for 30 seconds to resuspend the beads.

3.2 Transfer 400 µL of the Raw Beads to a fresh FACS tube.

3.3 Set the flow cytometer flow rate to low. Run the Raw Beads. Adjust the settings for FSC and SSC so that both bead populations are visible (Figure 21).

Pause and restart acquisition frequently during the setup procedure to refresh the beads populations after adjusting settings.

Figure 21.

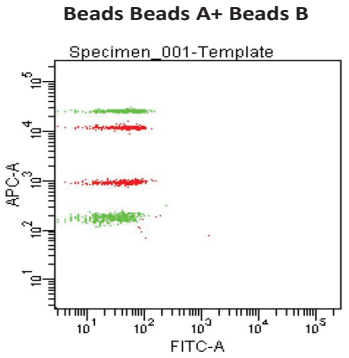


3.4 Continue adjusting the settings so that Beads A and Beads B are well separated and the FSC and SSC readings are >50 (x1000).

3.5 Move the gates for Beads A and Beads B so that the smaller beads fall into Beads A gate and the larger beads fall into Beads B gate (Figure 21).

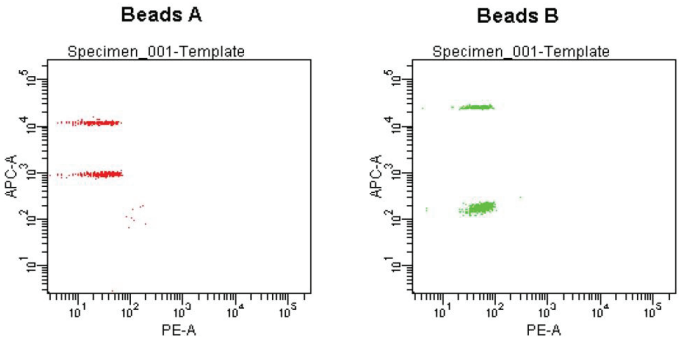
3.6 Adjust the FITC setting so that the FITC signal for the majority of beads is between $1x10^1$ and $1x10^2$ (Figure 22).

Figure 22.



3.7 Adjust the PE setting so that the PE signal for the majority of beads is between $1x10^1$ and $1x10^2$ (Figure 23).

Figure 23.



3.8 Adjust the APC settings so that the the APC fluorescence intensities of all bead populations are between $1x10^2$ and 5×10^4 (Figure 23).

3.9 Save the document again for future use.

To save your assay-specific settings, in the browser, right-click Cytometer Settings and select Save to **Catalog**. Name the file, and then click OK. To import the saved setting for a new experiment, right click on cytometer settings and select import settings.

3.10 The flow cytometer is now ready for sample acquisition.

Setup Procedure for Other Flow Cytometers

For flow cytometers not addressed above, the setup procedure will differ from one to another. It is very important for the end-user to set up the machine following similar guidelines. **In this case, machine compensation between the reporter and beads classification channels is strongly recommended.**

The flow cytometer setup instructions are also posted on our website: **www.biolegend.com/legendplex.** New flow cytometer setup instructions will be added to on our website when they become available.

Chapter 5: DATA ACQUISITION AND ANALYSIS

Data Acquisition

1. Before reading samples, make sure that the flow cytometer is set up properly. For flow cytometer setup, please follow the Flow Cytometer Setup guide in this manual or visit: **www.biolegend.com/legendplex**.

2. Create a new template or open an existing template (for details on how to create a cytometer-specific template, please refer to the Flow Cytometer Setup Guide).

3. Vortex each sample for 5 seconds before analysis.

4. Set the flow rate to low. Set the number of beads to be acquired to about 300 per analyte (e.g., acquire 1,500 beads for a 5-plex panel). Do not acquire too many events (e.g. >10,000 total events).

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

5. Read samples.

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

   To simplify data analysis using the LEGENDplex™ Data Analysis Software, read samples in the same order as shown on the PLATE MAP or RACK MAP attached at the end of the manual. For an in-plate assay, read column by column (A1, B1, C1...A2, B2, C2...). For an in-tube assay, read row by row (A1, A2, A3,...B1, B2, B3...).

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

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

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

Data Analysis

- The FCS file generated on a flow cytometer should be analyzed using BioLegend's LEGENDplex™ Data Analysis Software or other compatible data analysis software. The LEGENDplex™ Data Analysis Software can be downloaded here: **www.biolegend.com/legendplex.**

- After downloading, install it 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.

- The Dongle has a fixed number of points. Each analysis will consume a certain number of points from the dongle. The number of points consumed depends on assay plex size and number of samples. After the points on a dongle are consumed, a new dongle will be needed to run more data analysis. Although the software will continue to be functional, the data will not be saved until a new dongle is available. A new dongle is provided in each LEGENDplex™ kit. A saved analysis can always be re-analyzed using the software regardless of dongle status.

- 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: FREQUENTLY ASKED QUESTIONS

Q. **What is the difference between LEGENDplex™ and Luminex® Assays?**

BioLegend's LEGENDplex™ assays and Luminex®-based multiplex assays are both bead-based immunoassays using the same basic principle of sandwich immunoassays. Both systems use fluorescence-coded beads to achieve multiplexing. The major difference is how the data is acquired. LEGENDplex™ assays use common lab flow cytometers and their respective software for data acquisition, whereas Luminex®-based assays use dedicated machines and software for data acquisition. Therefore, one of the advantages of LEGENDplex™ assays is that they can be run on common flow cytometers and no specialized machine is needed.

Q. **Can I use LEGENDplex™ kits on Luminex® machines?**

No. LEGENDplex™ kits should be used on a regular flow cytometer and the data can be analyzed using the user-friendly data analysis software available for download at www.biolegend.com/legendplex.

Q. **What are the compatible flow cytometers for the LEGENDplex™ assays?**

In general, LEGENDplex™ assays can be used on most common flow cytometers, such as:

BD FACSCalibur™
BD FACSCanto™
BD FACSCanto™ II
BD ™ LSR I
BD ™ LSR II
BD LSRFortessa™
BD FACSAria™
BD FACSArray™

Please refer to the MATERIALS TO BE PROVIDED BY END-USER section for details on the channel configurations of the cytometer.

For flow cytometers not listed above, the end user needs to make sure that the machine is set up properly before use (refer to **Setup Procedure for Other Flow Cytometers** section in Chapter 4). **In this case, machine compensation between the reporter and beads classification channels is strongly recommended**.

The FCS or Listmode files from the following instruments are compatible with the LEGENDplex™ Data Analysis Software.

Vendor	Instrument Model	Vendor	Instrument Model
BD	FACScan™	Sony	iCyt Eclipse™
	FACSCalibur™	Life Technologies	Attune®
	FACSCanto™ II	Miltenyi Biotec	MACSQuant®
	LSRFortessa™	Partec	PAS
	LSR II	Stratedigm	S1400
	FACSAria™	Beckman Coulter	CyAn™ ADP
	FACSAria™ II		FC 500
	Accuri™ C6		Gallios™
ORFLO	Moxi Flow™		MoFlo® Astrios™
CyteK	DxP10™		MoFlo® XDP

Q. **What is the right procedure for running the assay?**

1. Perform the assay as instructed in this kit manual.
2. Determine the type of flow cytometer you have and perform machine setup as instructed on Flow Cytometer Setup guide in this manual or visit: **www.biolegend.com/legendplex**. Open an existing or create a machine-specific template for data acquisition.
3. Analyze the samples on the flow cytometer and save the FCS files in a new folder.
4. Install the LEGENDplex™ Data Analysis Software along with the software dongle on a PC with operating system Windows 7 or Windows 8 (32 bit or 64 bit). Make sure to install the correct version (32 bit or 64 bit version), depending on your computer's operating system.
5. Transfer the folder containing the FCS files of your experiment to the computer where the data analysis software is installed.
6. Perform data analysis as instructed in the Data Analysis Software User Guide.

Q. When do I need to do machine compensation?

If a flow cytometer equipped with a single laser is used for both reporter (FL2/PE) and classification (FL3), then compensation is absolutely required to compensate the signal spill over between the two channels, especially from FL2 to FL3. Please use the Setup Beads provided and follow the **FLOW CYTOMETER SETUP** guide in this manual.

If a flow cytometer is listed in the compatible flow cytometer list in this manual and is equipped with two lasers, one for reporter (FL2/PE) and the other for classification (FL4/APC), then compensation is not required if the **FLOW CYTOMETER SETUP** guide in this manual is closely followed. **However, compensation is recommended for consistent results.**

For other flow cytometers not listed in the compatible flow cytometer list, the end-user needs to set up the machine following similar guidelines (refer to manufacturer's manual for proper instrument setup). **In this case, machine compensation between the reporter and beads classification channels is strongly recommended**.

Q. Is there a special software required for data analysis?

The flow cytometer raw data files (FCS 2.0, 3.0) can be analyzed using the LEGENDplex™ Data Analysis Software or other equivalent analysis software. Download the LEGENDplex™ Data Analysis Software for free here: **www.biolegend.com/legendplex**. A software dongle with license key is provided in the kit.

For data acquisition, no special software is needed. Just use the data acquisition software that comes with the flow cytometer, as long as the data generated is in FCS format, which meets FCS convention 2.0, 3.0 and 3.1.

Q. Can I select to measure only some analytes within a panel?

Yes. The targets within a panel are fully customizable. The customer can select any combination of targets within a panel and order a customized product. Please use our website target selection tool for ordering customized product (**www.biolegend.com/legendplex**).

Q. Can I select to measure analytes across panels?

Cross-panel customization is also possible, as long as the total plex size is no more than 13 and there is no overlapping beads region among targets selected. For cross-panel customization, contact CST@biolegend.com.

Q. I have run out of a component from the kit. Can I use a similar component from a different kit?

The LEGENDplex™ Beads, Standards, Detection Antibodies, Matrix C, and SA-PE are lot-specific and must be used in combination with each other. Do not mix these components from different kits or lots.

Other components, such as Assay Buffer, Wash Buffer, Plate, and Plate Sealer are not lot-specific and can therefore be exchanged between different kits and lots.

Q. My standard curve is not linear; can I use the non-linear part of the standard curve during analysis?

Yes. It is possible to use the non-linear part of the standard curve for calculating the results. The LEGENDplex™ Data Analysis Software uses a five-parameter curve fitting algorithm, which determines the minimum and maximum detection concentrations of each target and reports them. If sample concentrations fall above the maximum detectable concentration, the sample will have to be diluted and reanalyzed. If sample concentrations fall below the minimum detection concentration, it is considered non-detectable by the particular assay.

Q. During data acquisition, why do the bead populations (defined by FSC and SSC) sometimes appear to be dispersed or shifted?

This is usually caused by a fast flow rate or a sudden change in flow rate. There are three flow-through settings: low, medium, and high. If you are using a low flow rate and then change to medium and high flow rate, the sudden change of flow rate may sometimes result in a dispersed or shifted bead population. Therefore, it is not recommended to change flow rate during data acquisition. The best way is to run the sample in setup mode using an ideal flow rate, and once the population is stable, then change it to acquisition mode.

Q. Does the LEGENDplex™ Data Analysis Software run on Apple Macintosh?

Not yet. The current version of the software has to run on a PC with operating system Windows 7 or Windows 8 (32 bit or 64 bit). If your flow cytometer is connected to a Mac, after data acquisition, the entire folder containing the data (FCS files) should be transferred to a PC and analyzed.

Q. Why does each kit include a software dongle?

The dongle allows you to use the data analysis software for data analysis. and each dongle contains 1 million data points which is sufficient for analyzing more than 750 plates.

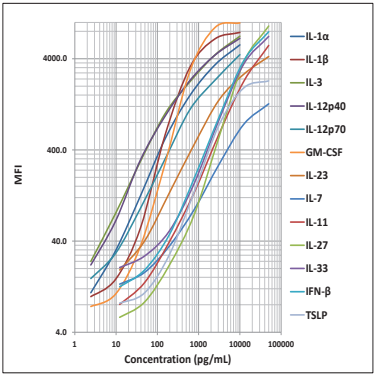
Q. **I ran out of points on my dongle. Can I get a new one separately? Or, can I use a dongle from one kit for another kit?**

Dongle is not sold separately. Contact BioLegend if you need a new dongle. The dongle can be used across kits.

Chapter 7: ASSAY CHARACTERIZATION

Representative Standard Curve

This standard curve was generated using the LEGENDplex™ Mouse Cytokine Panel 2 for demonstration purpose 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.

Analyte	MDC in Cell Culture Medium (pg/mL)	MDC in Serum (pg/mL)
Mouse IL-1α	1.0	0.9
Mouse IL-1β	1.9	2.5
Mouse IL-3	0.9	0.6
Mouse IL-12p40	0.8	0.8
Mouse IL-12p70	0.9	0.9
Mouse IL-23	4.5	3.5
Mouse IL-7	6.0	4.9
Mouse IL-11	3.3	5.6
Mouse IL-27	8.7	10.0
Mouse IL-33	3.5	6.5

Mouse IFN-β	3.8	6.3
Mouse GM-CSF	2.7	2.5
Mouse TSLP	10.6	11.4

Cross-Reactivity

The following recombinant proteins were tested at 50 ng/mL using the LEGENDplex™ Mouse Cytokine Panel 2. IL-12p40 pair can also detect the p40 subunit in IL-12p70 and IL-23. No or negligible cross-reactivity was found for all other analytes.

IL-1α	IL-1β	IL-3	IL-7	IL-11	IL-12p40	IL-12p70
IFN-β	IL-18	IL-23	IL-27	IL-33	IL-15	TSLP
IL-2	IL-4	IL-5	IL-6	IL-9	IL-10	IL-13
IL-17A	IL-17F	IL-21	IL-22	IFN-γ	TNF-α	MIP-3α
RANTES	Eotaxin	TARC	MCP-1	MDC	MIG	MIP-1α
MIP-1β	KC	LIX	BLC	IP-10	GM-CSF	TSLP

Accuracy (Spike Recovery)

For spike recovery in cell culture medium, target proteins with known concentrations were spiked into cell culture medium (RPMI and DMEM with 10% FBS) 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, a sample with known high concentrations of target proteins was spiked into unknown serum samples (n=16). 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
Mouse IL-1α	94%	83%	81%
Mouse IL-1β	97%	91%	90%
Mouse IL-3	88%	79%	80%
Mouse IL-12p40	107%	61%	55%

Mouse IL-12p70	97%	64%	79%
Mouse IL-23	106%	112%	112%
Mouse IL-7	100%	112%	104%
Mouse IL-11	111%	96%	105%
Mouse IL-27	102%	79%	79%
Mouse IL-33	157%	120%	103%
Mouse IFN-β	109%	95%	102%
Mouse GM-CSF	97%	77%	67%
Mouse TSLP	97%	98%	77%

Linearity of Dilution

For testing linearity of dilution, serum samples (n=16) were first diluted two-fold with Assay Buffer, then serially diluted 1:2, 1:4, 1:8 with Matrix C and assayed. The measured concentrations of serially diluted samples were then compared with that of the two-fold diluted samples.

Analyte	Linearity in Cell Culture Medium	Linearity in Serum	Linearity in Plasma
Mouse IL-1α	102%	99%	113%
Mouse IL-1β	98%	96%	99%
Mouse IL-3	107%	117%	118%
Mouse IL-12p40	90%	134%	121%
Mouse IL-12p70	96%	92%	112%
Mouse IL-23	86%	79%	83%
Mouse IL-7	87%	86%	113%
Mouse IL-11	86%	108%	108%
Mouse IL-27	97%	106%	113%
Mouse IL-33	63%	89%	102%
Mouse IFN-β	85%	95%	98%
Mouse GM-CSF	93%	99%	106%
Mouse TSLP	96%	105%	115%

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
Mouse IL-1α	Sample 1	159.4	5.3	3%
	Sample 2	646.4	34.1	5%
Mouse IL-1β	Sample 1	159.7	7.7	5%
	Sample 2	600.4	24.1	4%
Mouse IL-3	Sample 1	154.0	6.9	5%
	Sample 2	626.1	24.9	4%
Mouse IL-12p40	Sample 1	160.8	8.7	5%
	Sample 2	631.9	29.7	5%
Mouse IL-12p70	Sample 1	158.8	9.3	6%
	Sample 2	648.1	34.8	5%
Mouse IL-23	Sample 1	151.1	7.4	5%
	Sample 2	612.5	38.1	6%
Mouse IL-7	Sample 1	161.8	13.1	8%
	Sample 2	589.6	45.2	8%
Mouse IL-11	Sample 1	158.3	8.0	5%
	Sample 2	589.2	19.3	3%
Mouse IL-27	Sample 1	167.6	9.1	5%
	Sample 2	585.8	23.3	4%
Mouse IL-33	Sample 1	157.2	13.7	9%
	Sample 2	568.6	36.5	6%
Mouse IFN-β	Sample 1	163.6	13.5	8%
	Sample 2	600.9	36.8	6%
Mouse GM-CSF	Sample 1	160.0	6.9	4%
	Sample 2	596.9	25.4	4%
Mouse TSLP	Sample 1	162.7	5.6	3%
	Sample 2	607.5	27.1	4%

Inter-Assay Precision

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

Analyte	Sample	Mean (pg/mL)	STDEV	%CV
Mouse IL-1α	Sample 1	158.2	8.2	5%
	Sample 2	642.7	49.1	8%
Mouse IL-1β	Sample 1	155.1	9.0	6%
	Sample 2	602.9	34.7	6%
Mouse IL-3	Sample 1	155.8	11.3	7%
	Sample 2	605.1	59.2	10%
Mouse IL-12p40	Sample 1	156.3	11.2	7%
	Sample 2	609.3	46.6	8%
Mouse IL-12p70	Sample 1	159.8	12.5	8%
	Sample 2	645.5	47.0	7%
Mouse IL-23	Sample 1	151.8	9.5	6%
	Sample 2	606.4	54.7	9%
Mouse IL-7	Sample 1	152.7	15.1	10%
	Sample 2	589.1	50.5	9%
Mouse IL-11	Sample 1	152.6	10.5	7%
	Sample 2	590.3	26.1	4%
Mouse IL-27	Sample 1	158.2	12.6	8%
	Sample 2	595.9	30.2	5%
Mouse IL-33	Sample 1	149.6	14.1	9%
	Sample 2	585.2	51.4	9%
Mouse IFN-β	Sample 1	155.7	14.7	9%
	Sample 2	613.8	46.8	8%
Mouse GM-CSF	Sample 1	157.9	9.6	6%
	Sample 2	632.1	53.2	8%
Mouse TSLP	Sample 1	158.4	8.9	6%
	Sample 2	633.8	42.8	7%

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Biological Samples

Serum

Pooled normal mouse serum samples representing 4 different strains were tested for endogenous levels of the cytokines. The concentrations measured are shown below:

Analyte	BALB/c	CD-1	C57BL/6	Swiss Webster
Mouse IL-1α	9.5	10.1	12.0	29.2
Mouse IL-1β	ND	9.6	3.8	43.8
Mouse IL-3	1.5	1.4	2.1	15.1
Mouse IL-12p40	73.8	53.4	95.2	50.2
Mouse IL-12p70	ND	ND	ND	38.1
Mouse IL-23	21.2	29.7	3.9	1736.4
Mouse IL-7	15.6	13.8	9.0	15.0
Mouse IL-11	277.4	112.2	183.3	2511.7
Mouse IL-27	215.3	421.5	281.4	753.4
Mouse IL-33	5.9	7.3	ND	11.3
Mouse IFN-β	15.3	29.8	13.8	392.1
Mouse GM-CSF	10.7	4.9	12.6	97.2
Mouse TSLP	58.5	96.7	54.2	539.3

ND = Non-detectable

Plasma

Pooled normal mouse serum samples representing 4 different strains were tested for endogenous levels of the cytokines. The concentrations measured are shown below:

Analyte	BALB/c	CD-1	C57BL/6	Swiss Webster
Mouse IL-1α	12.3	10.8	10.8	12.2
Mouse IL-1β	8.0	11.1	4.3	18.4
Mouse IL-3	5.7	7.8	3.9	6.6
Mouse IL-12p40	71.4	59.3	94.5	56.0
Mouse IL-12p70	3.1	5.9	1.0	6.8
Mouse IL-23	224.2	178.8	33.4	25.7

Mouse IL-7	18.2	72.1	41.2	51.2
Mouse IL-11	219.2	354.7	71.2	423.8
Mouse IL-27	328.0	598.2	197.9	317.3
Mouse IL-33	32.3	29.6	22.3	1.3
Mouse IFN-β	25.3	68.7	19.1	60.3
Mouse GM-CSF	28.3	14.2	14.9	38.7
Mouse TSLP	128.9	112.2	67.9	172.2

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 Cytokine Panel 2 kit. The results (all in pg/mL) are summarized below.

Analyte	Control	LPS	LPS + GM-CSF	LPS + IFN-γ
Mouse IL-1α	0.9	2383.2	4057.6	464.8
Mouse IL-1β	ND	34.7	56.9	10.0
Mouse IL-3	ND	ND	ND	ND
Mouse IL-12p40	ND	45.6	45.9	88.0
Mouse IL-12p70	ND	ND	ND	ND
Mouse IL-23	ND	ND	4.0	ND
Mouse IL-7	11.8	17.0	17.9	22.3
Mouse IL-11	6.1	29.1	42.9	15.3
Mouse IL-27	ND	36084.9	20566.4	58024.1
Mouse IL-33	ND	16.7	10.2	8.4
Mouse IFN-β	5.1	195.3	174.3	1210.1
Mouse GM-CSF	ND	603.6	5926.5	4.2
Mouse TSLP	ND	29.7	31.1	ND

ND = Non-detectable

Mouse splenocyte cells (1x10^6 cells/mL) were cultured under various conditions (LPS, 1 µg/mL; IFN-γ, 100 ng/mL; CD3, 1 µg/mL plate-coated; CD28, 1 µg/mL soluble). Supernatants were collected after 72 hours and assayed with the LEGENDplex™ Mouse Cytokine Panel 2 kit. The results (all in pg/

mL) are summarized below.

Analyte	Control	LPS	LPS + IFN-γ	CD3 + CD28
Mouse IL-1α	1.1	10.1	28.4	10.1
Mouse IL-1β	2.4	3.2	2.9	3.5
Mouse IL-3	ND	0.8	1.1	2179.3
Mouse IL-12p40	7.6	10.2	30.9	6.2
Mouse IL-12p70	ND	ND	2.8	ND
Mouse IL-23	ND	ND	ND	ND
Mouse IL-7	8.5	7.5	9.0	10.8
Mouse IL-11	7.2	5.1	6.3	9.4
Mouse IL-27	ND	ND	ND	ND
Mouse IL-33	5.4	ND	ND	4.8
Mouse IFN-β	7.2	33.7	17.5	14.4
Mouse GM-CSF	ND	ND	4.7	1392.0
Mouse TSLP	ND	ND	ND	ND

ND = Non-detectable

Lungs from one BALB/c mouse were chopped into 1-2 mm pieces and seeded into RPMI, 10% fetal bovine serum, 10 µg/mL Concanavalin A. The heart from the same mouse was chopped into 1-2 mm pieces and seeded into RPMI with 10% fetal bovine serum. The cell culture supernatant was removed after three days and assayed with the LEGENDplex™ Mouse Cytokine Panel 2 kit. The results (all in pg/mL) are summarized below.

Analyte	Heart	Lungs
Mouse IL-1α	1.1	143.4
Mouse IL-1β	3.2	7.8
Mouse IL-3	ND	35.5
Mouse IL-12p40	1.7	59.7
Mouse IL-12p70	ND	1.1
Mouse IL-23	4.1	10.5
Mouse IL-7	14.0	18.2
Mouse IL-11	399.7	9864.9

Mouse Cytokine Panel 2 Mix and Match Subpanel

Mouse IL-27	16.2	59.0
Mouse IL-33	34.4	597.8
Mouse IFN-β	13.0	105.6
Mouse GM-CSF	460.7	1271.8
Mouse TSLP	141.1	248.7

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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

RACK MAP (for in-tube assay)

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

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