BioLegend®

Enabling Legendary Discovery™

LEGENDplex™

Multi-Analyte Flow Assay Kit

Cat. No. 740283, Rat Th Cytokine Panel (13-plex) with Filter plate
Cat. No. 740402, Rat Th Cytokine Panel (13-plex) with V-bottom Plate

Cat. No. 740284, Rat Th1 Panel (6-plex) with Filter plate
Cat. No. 740403, Rat Th1 Panel (6-plex) with V-bottom Plate
Cat. No. 740285, Rat Th2 Panel (7-plex) with Filter plate
Cat. No. 740404, Rat Th2 Panel (7-plex) with V-bottom Plate
Cat. No. 740286, Rat Th1/Th2 Panel (9-plex) with Filter plate
Cat. No. 740405, Rat Th1/Th2 Panel (9-plex) with V-bottom Plate
Cat. No. 740287, Rat Th17 Panel (8-plex) with Filter plate
Cat. No. 740406, Rat Th17 Panel (8-plex) with V-bottom Plate
Cat. No. 740288, Rat Th9 Panel (3-plex) with Filter plate
Cat. No. 740407, Rat Th9 Panel (3-plex) with V-bottom Plate
Cat. No. 740289, Rat Th22 Panel (5-plex) with Filter plate
Cat. No. 740408, Rat Th22 Panel (5-plex) with V-bottom Plate
Cat. No. 740290, Rat Tfh Panel (5-plex) with Filter plate
Cat. No. 740409, Rat Tfh Panel (5-plex) with V-bottom Plate
Cat. No. 740291, Rat ThGM Panel (4-plex) with Filter plate
Cat. No. 740410, Rat ThGM Panel (4-plex) with V-bottom Plate

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.

Table of Contents Page

Chapter 1: KIT DESCRIPTION 3
Introduction 3
Principle of the Assay 3
Beads Usage 4
Storage Information 5
Materials Supplied 6
Materials to be Provided by the End-User 8
Precautions 9
Chapter 2: ASSAY PREPARATION 10
Sample Collection and Handling 10
Reagents Preparation 10
Standard Preparation 11
Sample Dilution 12
Chapter 3: ASSAY PROCEDURE 13
Performing the Assay Using a Filter Plate 13
Performing the Assay Using a V-bottom Plate 16
Chapter 4: FLOW CYTOMETER SETUP 19
Setup Procedure for FACSCalibur™ with Dual Laser 20
Setup Procedure for FACSCalibur™ with a Single Laser 24
Setup Procedure for BD FACSAria™, FACSCanto™ and LSR Series 31
Setup Procedure for Other Flow Cytometers 35
Chapter 5: DATA ACQUISITION AND ANALYSIS 36
Data Acquisition 36
Data Analysis 37

Chapter 6: FREQUENTLY ASKED QUESTIONS..... 38

Chapter 7: ASSAY CHARACTERIZATION..... 42

Standard Curve..... 42

Assay Sensitivity..... 42

Cross-Reactivity..... 43

Accuracy..... 43

Linearity of Dilution..... 44

Intra-Assay Precision..... 45

Inter-Assay Precision..... 46

Biological Samples..... 47

TROUBLESHOOTING..... 49

PLATE MAP..... 53

Chapter 1: KIT DESCRIPTION

Introduction

Upon antigen presentation by APCs (Antigen Presenting Cells), naive $CD4^+$ T cells can differentiate into one of several lineages of T helper (Th) cells, including Th1, Th2, Th17, Th9, Tfh, Th22, and ThGM as defined by their patterns of cytokine production and functions. T helper cells play critical roles in mediating adaptive immunity to a variety of pathogens. They secrete cytokines to stimulate various effector cells, such as cytotoxic T cells, B cells and macrophages. Accurate measurement of Th cytokine expression is critical to understand the nature of the immune response and many disease processes.

The LEGENDplex™ Rat Th Cytokine Panel is a bead-based multiplex assay panel, using fluorescence–encoded beads suitable for use on various flow cytometers. This panel allows simultaneous quantification of 13 rat cytokines, including IL-2, 4, 5, 6, 9, 10, 13, 17A, 17F, 22, GM-CSF, IFN-γ and TNF-α, which are collectively secreted by Th1, Th2, Th9, Th17, Th22, ThGM, and Tfh cells. 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 Rat Th Cytokine Panel is designed to allow flexible customization within the panel. It can also be divided into cell type-specific subpanels. Please refer to the target selection table for panel-specific target information (Table 1). For mix and match within the panel, please visit **www.biolegend.com/legendplex.**

This assay is for research use only.

Principle of the Assay

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

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

Since the beads are differentiated by size and internal fluorescence intensity

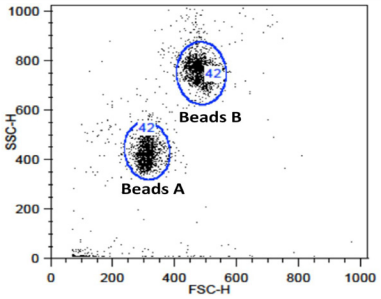
on a flow cytometer, analyte-specific populations can be segregated and PE fluorescent signal quantified. The concentration of a particular analyte is determined using a standard curve generated in the same assay.

Beads Usage

The Rat Th Cytokine 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 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 Rat Th Cytokine Panel allows simultaneous detection of 13 analytes in a single sample. Each analyte is associated with a particular bead set as indicated in Table 1.

Figure 1. Beads Differentiated by Size



Beads A = smaller beads

Beads B = larger beads

Figure 2. Beads A Classification by FL4

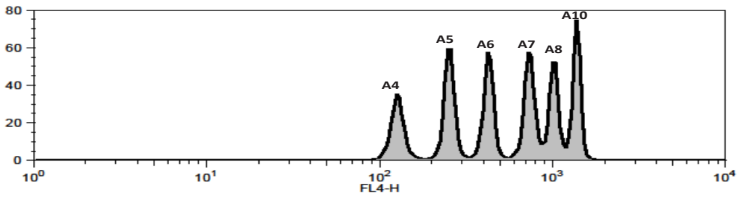
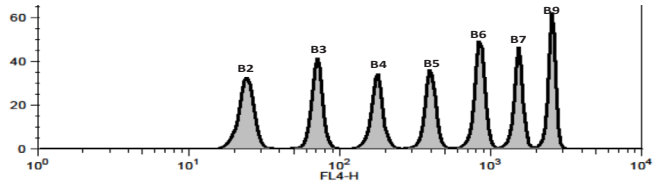


Figure 3. Beads B Classification by FL4



Tel: 858-768-5800

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

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

Target	Bead ID	Top Standard Conc. (ng/mL)	Th Cat.No. 740283* & 740402**	Th1 Cat.No. 740284* & 740403**	Th2 Cat.No. 740285* & 740404**	Th17 Cat.No. 740287* & 740405**	Th1/Th2 Cat. No. 740286* & 740406**	Th9 Cat.No. 740288* & 740407**	Th22 Cat.No. 740289* & 740408**	ThGM Cat.No. 740291* & 740410**	Tfh Cat. No. 740290* & 740409**
IL-10	A4	20	√	√	√	√	√	√	√	√	√
IFN-γ	A5	20	√	√		√	√			√	√
IL-5	A6	20	√		√		√				
IL-2	A7	20	√	√			√			√	
TNF-α	A8	20	√	√	√	√	√	√	√		√
GM-CSF	A10	20	√	√	√	√	√			√	
IL-4	B2	10	√		√		√				√
IL-17F	B3	20	√			√					
IL-9	B4	20	√					√			
IL-17A	B5	10	√			√					
IL-13	B6	10	√		√		√		√		
IL-22	B7	10	√			√			√		
IL-6	B9	20	√	√	√	√	√		√		√

* Cat# for kit with Filter plate. **Cat# for kit with V-bottom Plate

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

Kit Components	Quantity	Volume	Part #
Setup Beads 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
Capture Beads* (see tables below for more information)	**varies**	**varies**	**varies**
Rat Th Cytokine Panel Detection Antibodies*	**1 bottle**	**3.5 mL**	**varies**
Rat Th Cytokine Panel Standard Cocktail, Lyophilized*	**1 vial**	**lyophilized**	**varies**
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
Filter Plate** or V-bottom Plate***	1 plate		76187** or 76883***
Plate Sealers	4 sheets		78101
Data Analysis Software Dongle	1		21217
Rat Th Cytokine Panel Manual	1		76800

* For full panel, premixed beads are provided ready-to-use. For subpanels, individual beads are provided at 13X concentration. For Standard and Detection Antibodies, full panel and subpanels used different part/cat. numbers (See tables below for details).

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

Tel: 858-768-5800

For Rat Th Cytokine Panel (Full Panel):

Kit Components	Quantity	Volume	Part #
Rat Th Cytokine Panel Premixed Beads	1 bottle	3.5 mL	76789
Rat Th Cytokine Panel Detection Antibodies	1 bottle	3.5 mL	76518
Rat Th Cytokine Panel Standard Cocktail, Lyophilized	1 vial	lyophilized	76520

For Rat Th Cytokine Subpanels:

Kit Components	Quantity	Volume	Cat#
Rat IL-10 Capture Bead A4, 13X	1	270 µL	740252
Rat IFN-γ Capture Bead A5	1	270 µL	740253
Rat IL-5 Capture Bead A6, 13X	1	270 µL	740294
Rat IL-2 Capture Bead A7, 13X	1	270 µL	740295
Rat TNF-α Capture Bead A8, 13X	1	270 µL	740256
Rat GM-CSF Capture Bead A10, 13X	1	270 µL	740257
Rat IL-4 Capture Bead B2, 13X	1	270 µL	740297
Rat IL-17F Capture Bead B3, 13X	1	270 µL	740298
Rat IL-9 Capture Bead B4, 13X	1	270 µL	740299
Rat IL-17A Capture Bead B5, 13X	1	270 µL	740261
Rat IL-13 Capture Bead B6, 13X	1	270 µL	740300
Rat IL-22 Capture Bead B7, 13X	1	270 µL	740301
Rat IL-6 Capture Bead B9, 13X	1	270 µL	740264
Rat Th Panel Detection Antibodies	1	3.5 mL	740292
Rat Th Panel Standard	1	lyophilized	740293

Please refer to **Beads ID and Panel-Specific Target Selection table (Table 1, page 5),** to see which capture beads are included in each panel

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 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)

- 1.1 mL polypropylene micro FACS tubes, in 96-tube rack (e.g., National Scientific Supply Co, cat # 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 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) .

Precautions

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Center for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium azide has been added to some reagents as a preservative. Although the concentrations are low, sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up.
- Matrix C for LEGENDplex™ kits contains components of animal origin and should be handled as potentially hazardous.
- Do not mix or substitute reagents from different kits or lots. Reagents from different manufacturers should not be used with this kit.
- Do not use this kit beyond its expiration date.
- SA-PE and 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 important that samples are thawed completely, mixed well and centrifuged to remove particulates. Particulates in samples may interfere with the assay.

Preparation of Tissue Culture Supernatant:

- Centrifuge the sample to remove debris and assay immediately, or 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 and diluted to 1X with Assay Buffer prior to use. To mix the beads, follow the steps below:

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 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 10 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 Rat Th Cytokine Panel Standard Cocktail with 250 µL Assay Buffer.
2. Mix and allow the vial to sit at room temperature for 10 minutes, and then transfer the standard to an appropriately labeled polypropylene microfuge tube. This will be used as the top standard C7.

   Note: Analytes in this panel have different top standard concentartions (See Table 1, page 5 for details).

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).

Tube/ Standard ID	Serial Dilution	Assay Buffer to add (µL)	Standard to add	Final Conc. (pg/mL)*	Final Conc. (pg/mL)**
C7	--	--	--	10,000	20,000
C6	1:4	75	25 µL of C7	2,500	5,000
C5	1:16	75	25 µL of C6	625	1,250
C4	1:64	75	25 µL of C5	156.3	312.5
C3	1:256	75	25 µL of C4	39.1	78.1
C2	1:1024	75	25 µL of C3	9.8	19.5
C1	1:4096	75	25 µL of C2	2.4	4.9
C0	--	75	--	0	0

*Top Standard concentration of IL-4, IL-13, IL-17A and IL-22 is 10 ng/mL;

**Top standard concentration of IL-2, IL-5, IL-6, IL-9, IL-10, IL-17F, GM-CSF, IFN-γ and TNF-α is 20 ng/mL

Sample Dilution

- Serum or plasma samples must be diluted 4-fold with Assay Buffer before being tested (e.g. dilute 25 µL of sample with 75 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.

Chapter 3: ASSAY PROCEDURE

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

- The in-filter plate assay procedure 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 in-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 53). Be sure to load standards in the first two columns.

1. Pre-wet the plate by adding 100 µL of 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 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. **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 new 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 new plate sealer. Wrap the entire plate, including the inverted plate cover, with aluminum foil. Place the plate on a plate shaker and shake at approximate 500 rpm for 30 minutes at room temperature.

9. Repeat step 4 above.

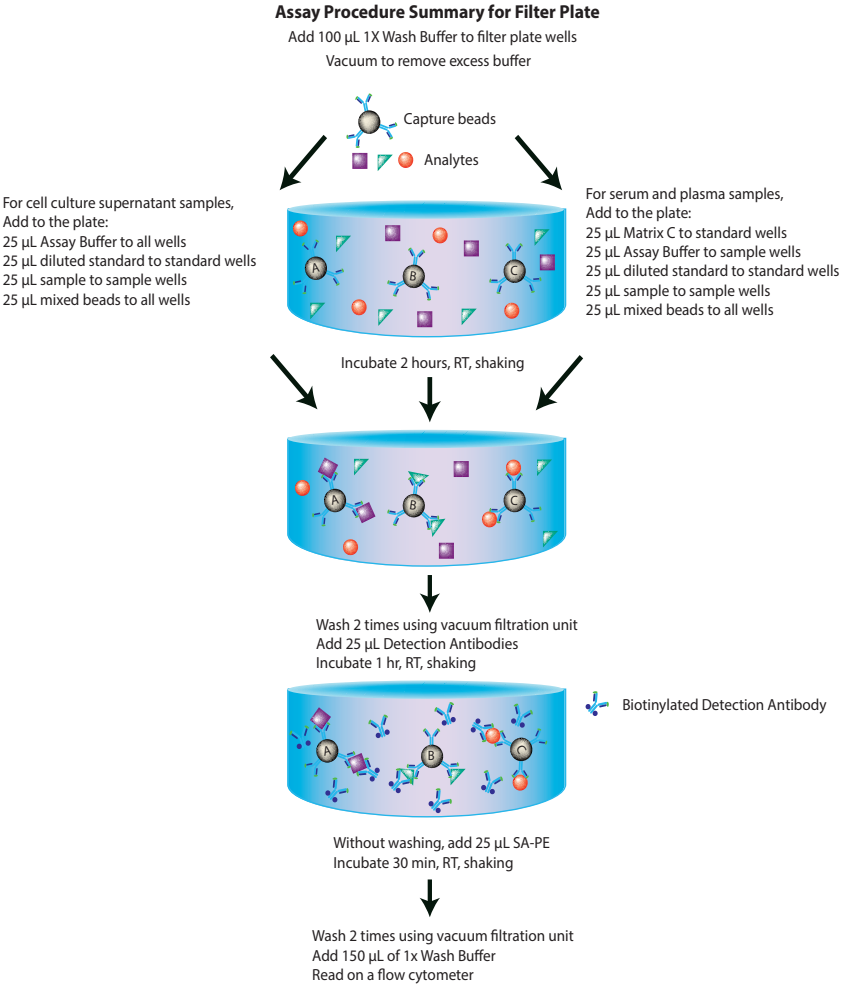
10. Add 150 µL of 1X Wash Buffer to each well. Resuspend the beads on a plate shaker for 1 minute.

11. Read samples on a flow cytometer, preferably within the same day of the assay (Note: Prolonged sample storage can lead to reduced signal).

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

probe height may need to be adjusted when using an autosampler.

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



Performing the Assay Using a V-bottom Plate

- Allow all reagents to warm to room temperature (20-25°C) before use.
- Keep the plate upright during the entire assay procedure, including the washing steps, to avoid losing beads.
- The plate should be placed in the dark or wrapped with aluminum foil for all incubation steps.
- Standards and samples should be run in duplicate and arranged on the plate in a vertical configuration convenient for data acquisition and analysis (as shown in attached PLATE MAP, page 53). Be sure to load standards in the first two columns.

1. **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 sample wells.
    - Add 25 µL of each standard to standard wells.
    - Add 25 µL of each diluted serum or plasma sample to sample wells (See **Sample Dilution**).

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

3. Seal the plate with a plate sealer. Cover the entire plate with aluminum foil to protect the plate from light. Shake at 800 rpm on a plate shaker for 2 hours at room temperature **(Depending on the shaker, the speed may need to be adjusted. The optimal speed is one that is high enough to keep beads in suspension during incubation, but not too high so it causes spill from the wells)**.

4. Centrifuge the plate at 1050 rpm (~250 g) for 5 minutes, using a swinging bucket rotor (G.H 3.8) with microplate adaptor (Please refer to **Materials to be Provided by the End-User, page 9).** Do not exceed centrifugation speed as it can affect beads resuspension in later steps.

5. Immediately after centrifugation, dump the supernatant into the sink by quickly inverting and flicking the plate. Gently blot the plate only once on a stack of clean paper towel. Be careful not to disturb the bead pellet.

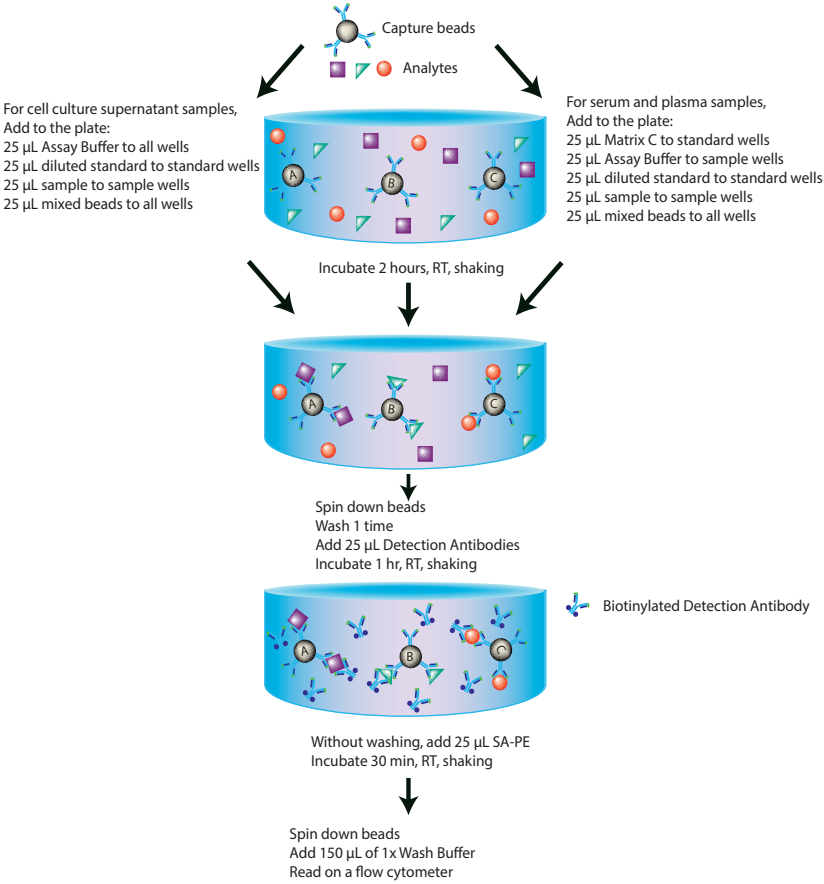
Alternatively. removal of the supernatant may be completed using a multichannel pipette set at 75 µL. Try to remove as much liquid as possible without removing any beads. Be sure to change pipette tips between each row or column.

6. Wash the plate once by dispensing 200 µL of washing buffer into each well. Shake the plate at 800 rpm for 1 minute and repeat step 4 and 5.
7. Add 25 µL 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. Add 150 µL of 1X Wash Buffer to each well. Resuspend the beads by pipetting.
13. 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 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.

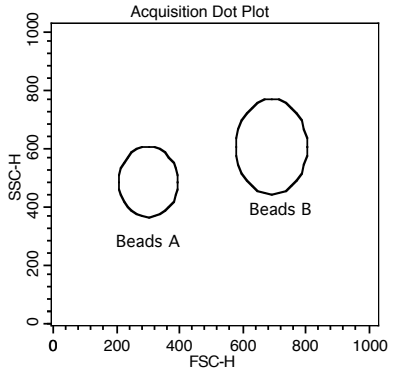
If you have already created a template for the flow cytometer, open that template and proceed to Step 3.

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).

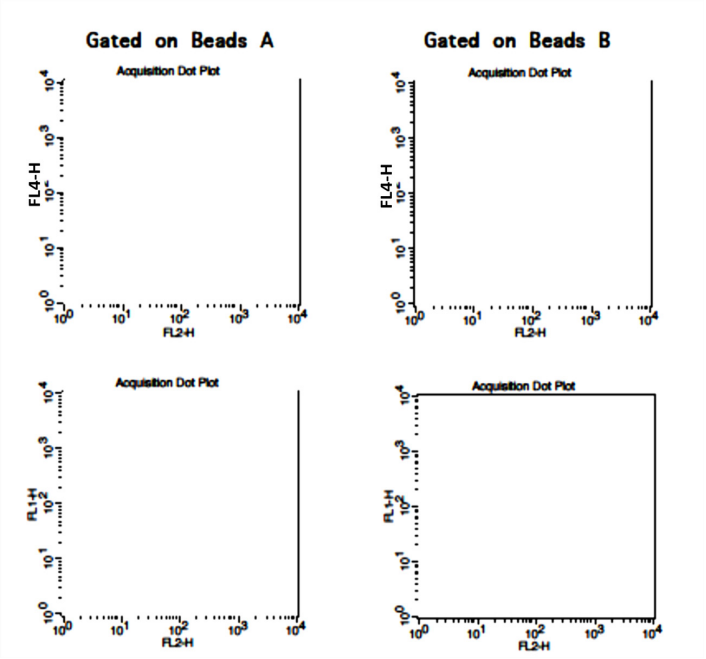
Figure 4.



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:

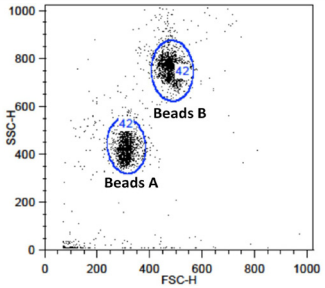
3.1 Vortex the vial of the Raw Beads for 30 seconds to resuspend the beads.

3.2 ransfer 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.



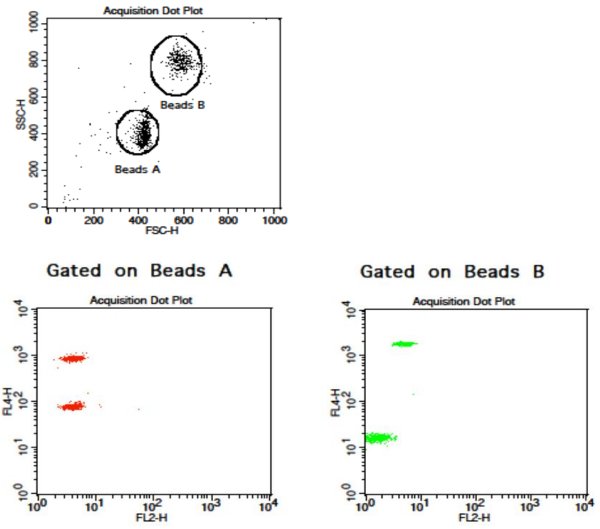
3.4 Continue adjusting the settings so that Beads A and Beads B are well separated and the FSC and SSC readings are >200.

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.

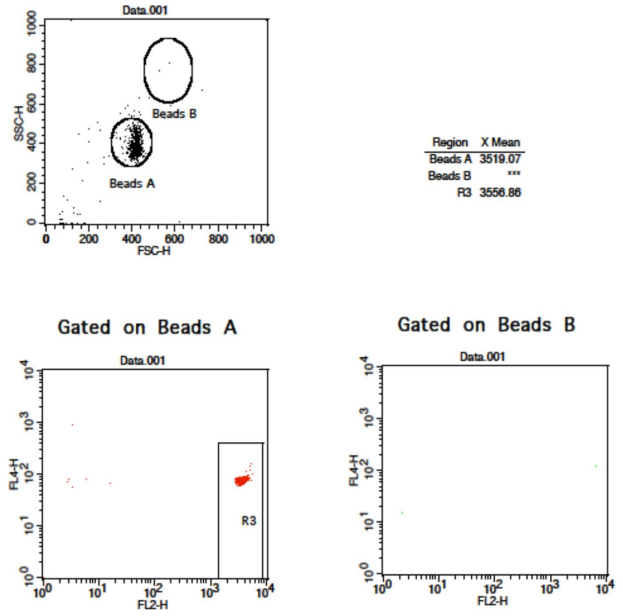
3.9 Transfer 400 µL of the PE Beads to a fresh FACS tube.

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.

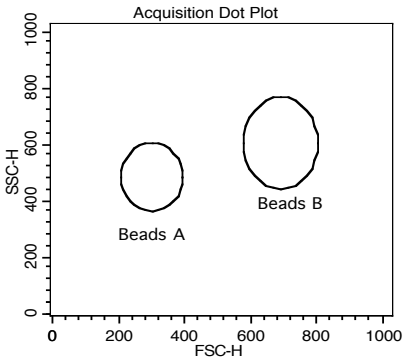
If you have already created a template for the flow cytometer, open that template and proceed to Step 3.

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.

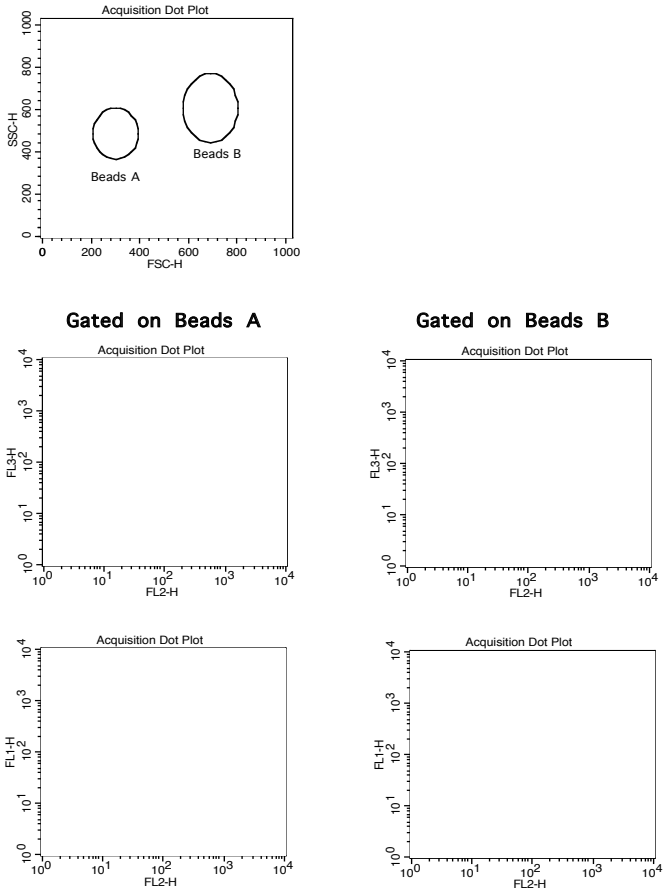
2.2 Create a dot plot with FSC (forward scatter) for X-axis and SSC (side scatter) for Y-axis. **Be sure to set FSC and SSC to linear mode**. Create two gates and label them Beads A and Beads B (Figure 9).

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:

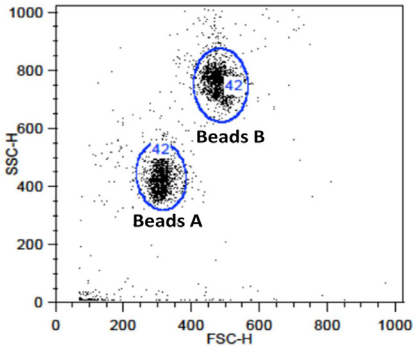
3.1 Vortex the vial of the Raw Beads for 30 seconds to resuspend the beads.

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.



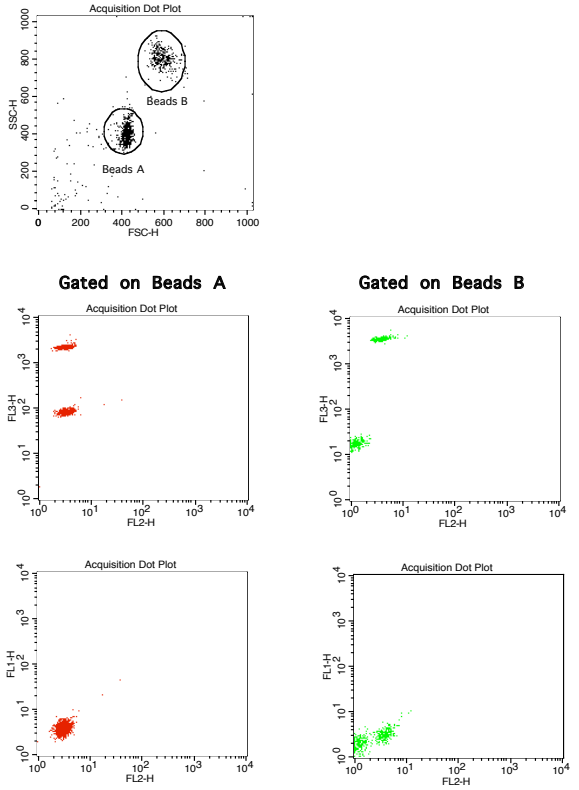
3.4 Continue adjusting the settings so that Beads A and Beads B are well separated and the FSC and SSC readings are >200.

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 be tween $1x10^0$ and $1x10^1$ (Figure 12).

Tel: 858-768-5800

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).

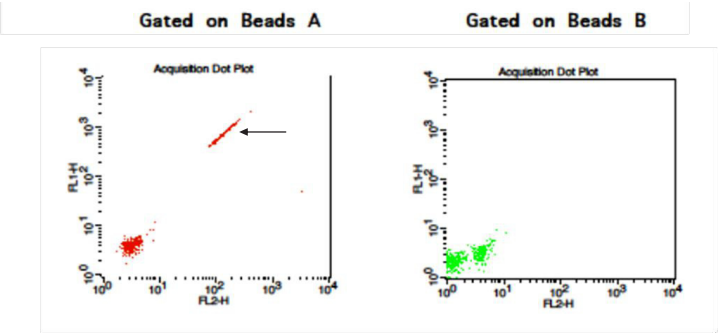
3.9 Vortex the vial of the Setup Beads 1: FITC Beads for 30 seconds to resuspend the beads.

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.

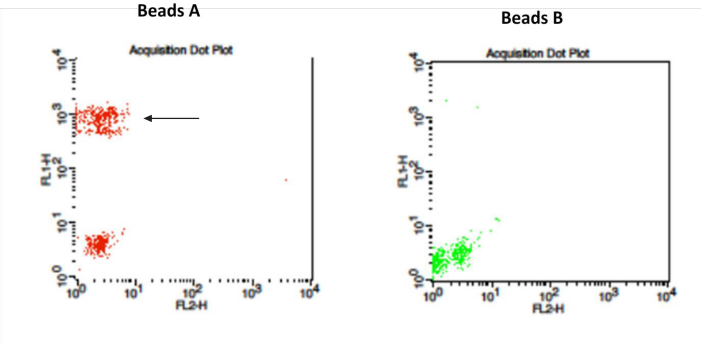
3.12 On the FL1 vs FL2 dot plot (Figure 13), the beads will display as FITC-negative and FITC-positive populations (indicated by an arrow). Note: FITC beads are only of small size, falling in the Beads A gate.

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 median FL2 fluorescence intensities (Figure 14).

Figure 14.



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

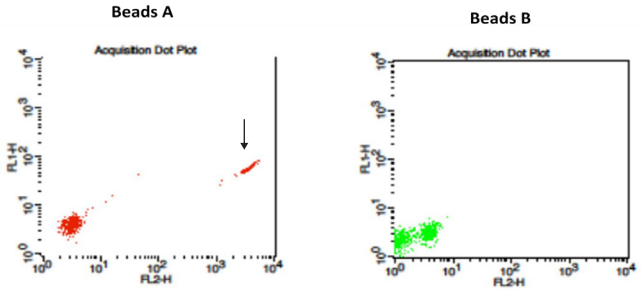
3.15 Transfer 200 µL of the PE Beads to a fresh FACS tube. Add 200 µL of Raw Beads and mix well (This will generate PE-positive and PE-nega tive populations of beads and is needed for proper compensation).

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.

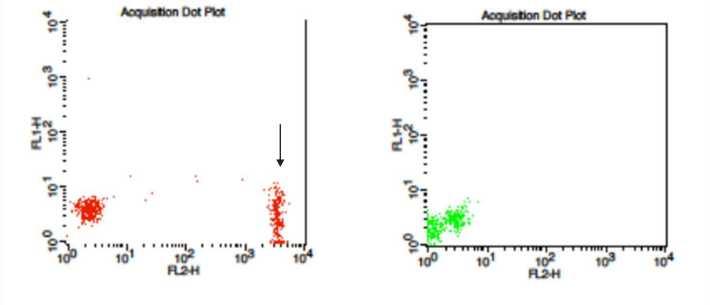
Figure 15.



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 median 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.

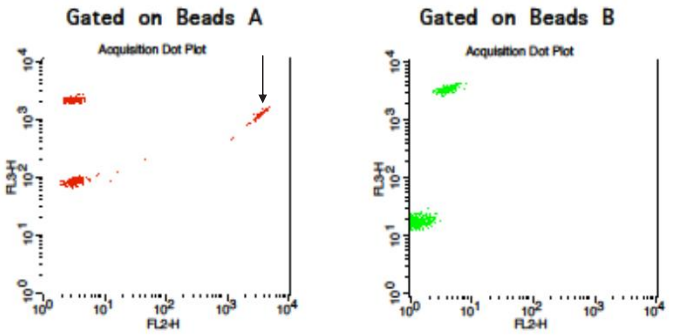
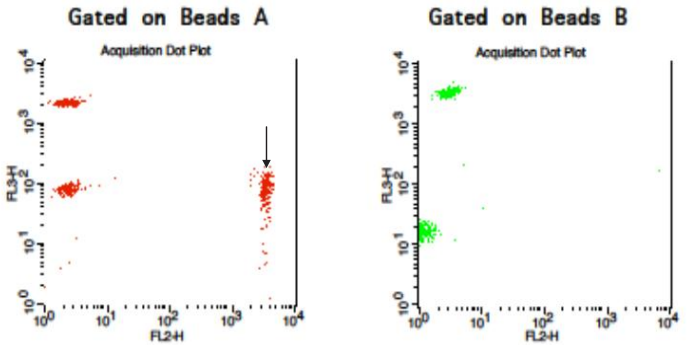


Figure 18.



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 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.

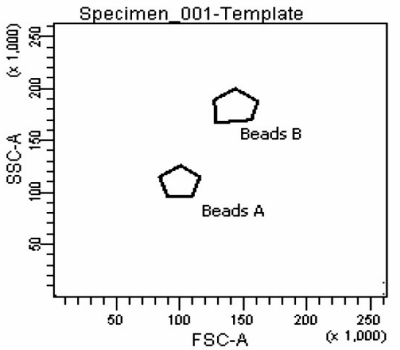
If you have already created a template for the flow cytometer, open that template and proceed to Step 3. To open an existing template, select Experiment> New Experiment. A list of templates saved in D:\BDExport\Templates\ Experiment will pop up. Select the desired template from the list.

To create a new template, follow the instructions below:

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

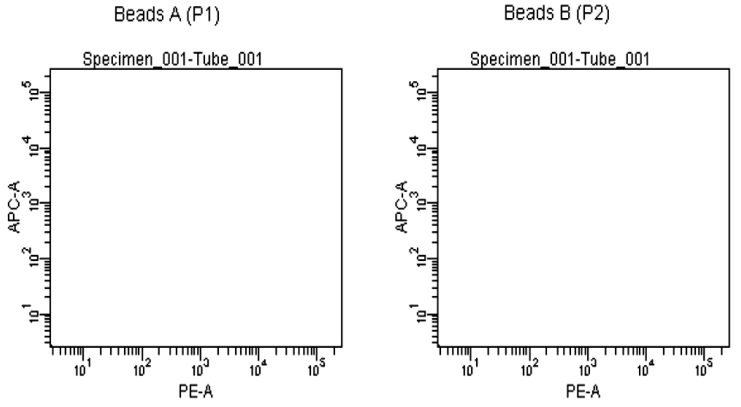
2.2 In the global worksheet, open the worksheet. Create a dot plot with FSC (forward scatter) for X-axis and SSC (side scatter) for Y-axis. **Be sure to set FSC and SSC to linear mode.** Create two gates and label them Beads A and Beads B (Figure 19).

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). 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.

Tel: 858-768-5800

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:

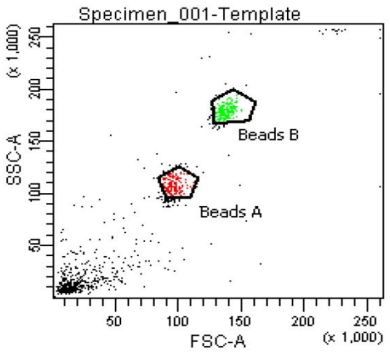
3.1 Vortex the vial of Raw Beads for 30 seconds to resuspend the beads.

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.

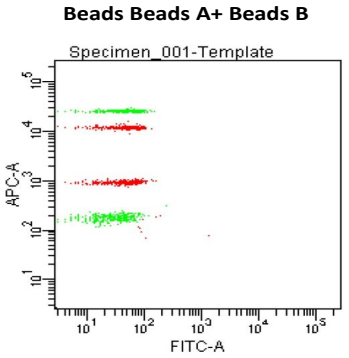


3.4 Continue adjusting the settings so that Beads A and Beads B are well separated and the FSC and SSC readings are >50 (x1000).

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).

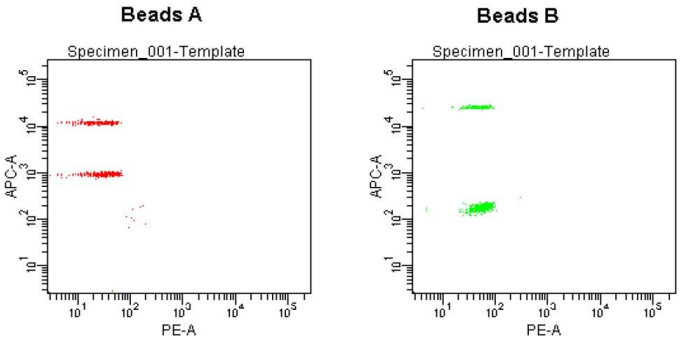
3.6 Adjust the FITC setting so that the FITC signal for the majority of beads is between $1x10^1$ and $1x10^2$ (Figure 22).

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 4,000 beads for a 13-plex panel). Do not acquire too many beads (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 can 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.** Please alsways use the most recent version of the software. If not sure, download the most recent version from BioLegend website.
- After downloading, install it on a PC 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. For Mac version of the software, please contact BioLgend.
- 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.

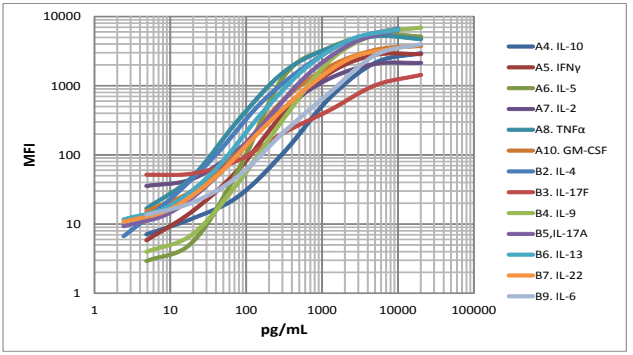
Q. **I ran out of points on my dongle. Can I get a new one separately? Or, can I use a dongle from one kit for another kit?**

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

Chapter 7: ASSAY CHARACTERIZATION

Standard Curve

This standard curve was generated using the LEGENDplex™ Rat Th Cytokine Panel 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)
IL-2	6.2	6.8
IL-4	1.2	1.0
IL-5	5.7	5.3
IL-6	5.6	3.0
IL-9	3.1	2.8
IL-10	4.5	3.3
IL-13	1.8	1.5
IL-17A	2.0	1.7
IL-17F	10.8	10.7
IL-22	3.4	1.4
GM-CSF	5.1	5.1
TNF-α	3.6	3.4
IFN-γ	3.1	4.0

Cross-Reactivity

The following rat recombinant proteins were tested at 50 ng/mL using the LEGENDplex™ Rat Th Cytokine Panel. No or negligible cross-reactivity was found.

CCL20	EPO	IGF-1	IL-3	SCF
VEGF164	CXCL1	TPO	EGF	M-CSF
IL-2	IL-4	IL-5	IL-6	IL-9
IL-10	IL-13	IL-17A	IL-17F	IL-22
GM-CSF	TNF-α	IFN-γ	IL-1a	IL-1b
IL-12p70	IL-18	IL-33	MCP-1	KC

Accuracy (Spike Recovery)

For spike recovery in cell culture medium, DMEM with 10% FCS were spiked with target proteins at three different levels within the assay range. The spiked samples were then assayed, and the measured concentrations were compared with the expected values.

For spike recovery in serum and plasma, four pooled rat serum samples from four different strains and six pooled rat plasma samples from two different strains were first diluted four-fold with Assay Buffer and spiked with target proteins at three different levels within the assay range. The spiked samples were then assayed, and the measured concentrations were compared with the expected values.

Analyte	% of Recovery in DMEM	% of Recovery in Serum	% of Recovery in Plasma
IL-2	73%	44%	52%
IL-4	91%	89%	85%
IL-5	95%	93%	92%
IL-6	65%	38%	49%
IL-9	128%	42%	46%
IL-10	106%	75%	86%
IL-13	94%	40%	39%
IL-17A	80%	66%	57%
IL-17F	61%	32%	45%
IL-22	78%	40%	44%

GM-CSF	94%	85%	89%
TNF-α	60%	77%	80%
IFN-γ	125%	172%	150%

Linearity of Dilution

For linearity in cell culture medium, 2 cell culture supernatant samples from stimulated rat splenocytes were serially diluted 1:2, 1:4, 1:8 with Assay Buffer and assayed. The measured concentrations of serially diluted samples were compared with that of the neat samples.

For testing linearity in serum and plasma, four pooled rat serum samples from four different strains and six pooled rat plasma samples from two different strains were first diluted four-fold with Assay Buffer and spiked with a known concentration of target proteins. The spiked samples were serially diluted 1:2, 1:4, 1:8 with Matrix C and assayed. The measured concentrations of serially diluted samples were compared with that of the spiked samples.

Analyte	Linearity in Cell Culture Medium	Linearity in Serum	Linearity in Plasma
IL-2	107%	135%	125%
IL-4	107%	101%	101%
IL-5	88%	96%	95%
IL-6	91%	126%	112%
IL-9	n/a	167%	143%
IL-10	108%	113%	113%
IL-13	77%	121%	126%
IL-17A	107%	99%	98%
IL-17F	119%	156%	100%
IL-22	142%	128%	112%
GM-CSF	113%	104%	109%
TNF-α	85%	118%	127%
IFN-γ	89%	93%	97%

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	4%
IL-2	Sample 1	1073.5	183.4	17%
	Sample 2	83.8	7.7	9%
IL-4	Sample 1	1272.6	146.8	12%
	Sample 2	84.8	6.6	8%
IL-5	Sample 1	1209.5	163.4	14%
	Sample 2	84.2	5.4	6%
IL-6	Sample 1	1057.3	143.1	14%
	Sample 2	72.1	5.0	7%
IL-9	Sample 1	1299.0	102.7	8%
	Sample 2	97.7	7.6	8%
IL-10	Sample 1	1042.9	108.7	10%
	Sample 2	83.29	7.12	9%
IL-13	Sample 1	1236.1	114.2	9%
	Sample 2	89.0	5.5	6%
IL-17A	Sample 1	1291.1	110.3	9%
	Sample 2	92.9	6.29	9%
IL-17F	Sample 1	993.8	148.3	15%
	Sample 2	64.8	15.7	24%
IL-22	Sample 1	1306.3	152.1	12%
	Sample 2	91.5	9.4	10%
GM-CSF	Sample 1	1070.7	154.9	14%
	Sample 2	75.7	6.4	9%
TNF-α	Sample 1	961.7	226.7	24%
	Sample 2	74.3	4.1	6%
IFN-γ	Sample 1	1355.0	139.7	10%
	Sample 2	95.1	6.8	7%

Inter-Assay Precision

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

Analyte	Sample	Mean (pg/mL)	STDEV	%CV
IL-2	Sample 1	1280.8	223.5	17%
	Sample 2	81.1	7	9%
IL-4	Sample 1	1255.4	79.5	6%
	Sample 2	79.6	13.6	9%
IL-5	Sample 1	1299.0	145.3	11%
	Sample 2	78.5	5.6	7%
IL-6	Sample 1	1245.4	166.2	13%
	Sample 2	78.6	11.4	14%
IL-9	Sample 1	1375.7	165.7	12%
	Sample 2	86.3	8.8	10%
IL-10	Sample 1	1166.3	171.3	15%
	Sample 2	77.19	12.25	16%
IL-13	Sample 1	1250.2	62.0	5%
	Sample 2	82.3	6.8	8%
IL-17A	Sample 1	1311.3	129.8	10%
	Sample 2	85.6	7.9	9%
IL-17F	Sample 1	1212.8	249.5	21%
	Sample 2	63.8	8.7	14%
IL-22	Sample 1	1351.1	104.8	8%
	Sample 2	86.9	7.3	8%
GM-CSF	Sample 1	1254.5	154.6	12%
	Sample 2	78.3	8.1	10%
TNF-α	Sample 1	1178.2	177.5	15%
	Sample 2	72.7	7.4	10%
IFN-γ	Sample 1	1375.5	130.2	9%
	Sample 2	88.3	6.9	8%

Tel: 858-768-5800

Biological Samples

Serum and Plasma

Four pooled rat serum samples from four different strains were tested for endogenous levels of the proteins. The concentrations measured are shown below.

Analyte	Range (pg/ml)	% of Detectable	Mean (pg/mL)
IL-2	ND	0%	ND
IL-4	ND-23.7	50%	11.0
IL-5	ND	0%	ND
IL-6	ND-73.6	25%	18.4
IL-9	ND-30.1	25%	7.5
IL-10	ND-22.4	75%	14.5
IL-13	ND-83.8	50%	31.4
IL-17A	ND-30.6	25%	7.7
IL-17F	ND	0%	ND
IL-22	ND-17.6	25%	4.4
GM-CSF	ND-20.7	25%	5.2
TNF-α	ND-14.0	6%	3.5
IFN-γ	10.7-356.2	100%	110.4

ND = Non-detectable

Six pooled rat plasma samples from two different stanins were tested for endogenous levels of proteins. The concentrations measured are shown below.

Analyte	Range (pg/mL)	% of Detectable	Mean (pg/mL)
IL-2	ND-128.6	17%	21.4
IL-4	ND-12.2	50%	3.7
IL-5	ND	0%	ND
IL-6	ND-435.8	50%	75.4
IL-9	ND-98.1	17%	65.4
IL-10	ND-22.4	50%	6.7
IL-13	ND-39.9	17%	8.6

IL-17A	ND	0%	ND
IL-17F	ND-1278.0	17%	213.0
IL-22	ND-476.8	33%	81.4
GM-CSF	ND-84.0	33%	26.1
TNF-α	ND-20.4	17%	3.4
IFN-γ	10.7-37.8	100%	19.0

ND = Non-detectable

Cell Culture Supernatant

Rat splencytes (1 x 10^6 cells/mL) were cultured under various conditions (control; CD3, 5 µg/mL plate-coated + CD28, 2 µg/mL soluble; LPS, 1 µg/mL; PMA 50 ng/mL; Ionomycin, 500 ng/mL). Supernatants were collected after 96 hours and assayed with the LEGENDplex™ Rat Th Cytokine Panel. The results (all in pg/mL) are summarized below.

Analyte	Control	CD3 + CD28	LPS	PMA + Ionomycin
IL-2	5	80	17	>20,000
IL-4	ND	6	ND	6
IL-5	14.3	44.4	15.2	18.8
IL-6	36	294	2,924	311
IL-9	ND	8	ND	9
IL-10	187	1,110	362	201
IL-13	4	55	6	212
IL-17A	14	>10,000	336	>10,000
IL-17F	ND	118	22	79
IL-22	7	1,461	140	2,693
GM-CSF	12	863	27	1,148
TNF-α	6	169	229	293
IFN-γ	6	>20,000	85	7541

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.

Tel: 858-768-5800

PLATE MAP (for in-plate assay)

	1	2	3	4	5	6	7	8	9	10	11	12
A	**C0**	**C4**	Sample1	Sample5	Sample 9	Sample 13	Sample 17	Sample 21	Sample 25	Sample 29	Sample 33	Sample 37
B	**C0**	**C4**	Sample1	Sample5	Sample 9	Sample 13	Sample 17	Sample 21	Sample 25	Sample 29	Sample 33	Sample 37
C	**C1**	**C5**	Sample2	Sample6	Sample 10	Sample 14	Sample 18	Sample 22	Sample 26	Sample 30	Sample 34	Sample 38
D	**C1**	**C5**	Sample2	Sample6	Sample 10	Sample 14	Sample 18	Sample 22	Sample 26	Sample 30	Sample 34	Sample 38
E	**C2**	**C6**	Sample3	Sample7	Sample 11	Sample 15	Sample 19	Sample 23	Sample 27	Sample 31	Sample 35	Sample 39
F	**C2**	**C6**	Sample3	Sample7	Sample 11	Sample 15	Sample 19	Sample 23	Sample 27	Sample 31	Sample 35	Sample 39
G	**C3**	**C7**	Sample4	Sample8	Sample 12	Sample 16	Sample 20	Sample 24	Sample 28	Sample 32	Sample 36	Sample 40
H	**C3**	**C7**	Sample4	Sample8	Sample 12	Sample 16	Sample 20	Sample 24	Sample 28	Sample 32	Sample 36	Sample 40

LEGENDplex™ Kits are manufactured by **BioLegend**
9727 Pacific Heights Blvd.
San Diego, CA 92121
Tel: 1.858.768.5800
Tel: US & Canada Toll-Free: 1.877.Bio-Legend (1.877.246.5343)
Fax: 1.877.455.9587
Email: info@biolegend.com
biolegend.com

For a complete list of world-wide BioLegend offices and distributors, please visit our website at: biolegend.com

76800_V01