Enabling Legendary Discovery™

LEGENDplex™

Multi-Analyte Flow Assay Kit

Cat. No. 740818, Mouse B cell Panel (13-plex) w/FP
Cat. No. 740819, Mouse B cell Panel (13-plex) w/VBP

Cat. No. 740826, Mouse B cell Panel - S/P (12-plex) w/FP
Cat. No. 740827, Mouse B cell Panel - S/P (12-plex) w/VBP
Cat. No. 740982, Mouse B cell Panel - S/P (1-plex) w/FP
Cat. No. 740983, Mouse B cell Panel - S/P (1-plex) w/VBP
Cat. No. 740824, Mouse B Effector 1 (Be1) Panel (3-plex) w/FP
Cat. No. 740825, Mouse B Effector 1 (Be1) Panel (3-plex) w/VBP
Cat. No. 740822, Mouse B Effector 2 (Be2) Panel (5-plex) w/FP
Cat. No. 740823, Mouse B Effector 2 (Be2) Panel (5-plex) w/VBP
Cat. No. 740820, Mouse B Effector 1/2 (Be1/2) Panel (8-plex) w/FP
Cat. No. 740821, Mouse B Effector 1/2 (Be1/2) Panel (8-plex) w/VBP
Cat. No. 740828, Mouse Regulatory B cell Panel (2-plex) w/FP
Cat. No. 740829, Mouse Regulatory B cell Panel (2-plex) w/VBP

Please read the entire manual before running the assay.

BioLegend.com

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

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Chapter 1: KIT DESCRIPTION

Introduction

B cells, also known as B lymphocytes, are a type of white blood cells that play key roles in adaptive immune responses. B cells are classified as professional antigen presenting cells (APCs) due to their ability to present antigens on the cell surface. They function in the humoral immunity component of the adaptive immune system by secreting antibodies. In addition to antibody production, B cells also secrete an array of cytokines that mediate Th1- and Th2-like immune responses. These cytokines are produced by regulatory B cells (e.g., IL-10, TGF-β1) and B effector cells, namely Be1 (e.g., TNF-α, IFN-γ, and IL-12p70) and Be2 cells (e.g., IL-2, IL-4, IL-6, TNF-α, IL-13). Other cytokines associated with activation and survival of B cells, such as BAFF, BCMA, and sCD40L, are also important targets in B cell related processes. Dysregulation of B cell functions is often related to autoimmune diseases, such as multiple sclerosis, systemic lupus erythematosus, type 1 diabetes, and rheumatoid arthritis. Therefore, simultaneous quantification of these cytokines will be important in understanding B cell functions and B cell-related diseases.

The LEGENDplex™ Mouse B cell Panel (13-plex) is a bead-based multiplex assay panel, using fluorescence-encoded beads suitable for use on various flow cytometers. This panel focuses on 13 key biomarkers involved in B cell function, activation, and survival. It allows for simultaneous quantification of 13 mouse targets such as IL-4, IL-6, IL-12p70, IL-17A, IL-2, TNF-α, Free Active TGF-β1, IL-13, IFN-γ, BAFF, BCMA, sCD40L, and IL-10. This assay panel provides higher detection sensitivity and broader dynamic range than traditional ELISA methods. The panel has been validated for use on cell culture supernatant, serum, and plasma samples.

The LEGENDplex™ Mouse B cell Panel is configured as shown below depending on sample types and required dilutions:

Catalog No.	Plex Size	Targets	Recommended Sample Type	Recommended Dilution Factor
740818 740819	13-plex	IL-4, IL-6, IL-12p70, IL-17A, IL-2, TNF-α, Free Active TGF-β1, IL-13, IFN-γ, BAFF, BCMA, sCD40L, IL-10	Tissue culture*	Varies
740826 740827	12-plex	IL-4, IL-6, IL-12p70, IL-17A, IL-2, TNF-α, Free Active TGF-β1, IL-13, IFN-γ, BCMA, sCD40L, IL-10	Serum, Plasma	2
			Tissue culture	Varies
740982 740983	1-plex	BAFF	Serum, Plasma	25
			Tissue culture	Varies

740824 740825	3-plex	IL-12p70, TNF-α, IFN-γ	Serum, Plasma	2
			Tissue culture	Varies
740822 740823	5-plex	IL-4, IL-6, IL-2, TNF-α, IL-13	Serum, Plasma	2
			Tissue culture	Varies
740820 740821	8-plex	IL-4, IL-6, IL-12p70, IL-17A, IL-2, TNF-α, IL-13, IFN-γ	Serum, Plasma	2
			Tissue culture	Varies
740828 740829	2-plex	Free Active TGF-β1, IL-10	Serum, Plasma	2
			Tissue culture	Varies

***Note: Serum and plasma are not the recommended sample type. Refer to Sample Dilution section in the manual for details.**

The LEGENDplex™ Mouse B cell Panel is designed to allow flexible customization within the panel. Please visit www.biolegend.com/legendplex for more information on how to mix and match within the panel. This assay is for research use only.

Principle of the Assay

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

Beads are differentiated by size and internal fluorescence intensities. Each bead set is conjugated with a specific antibody on its surface and serves as the capture beads for that 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.

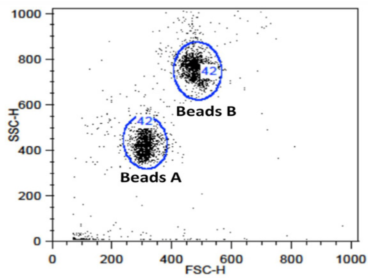
Since the beads are differentiated by size and internal fluorescence intensity on a flow cytometer, analyte-specific populations can be segregated and PE fluorescent signal quantified. The concentration of a particular analyte is determined using a standard curve generated in the same assay.

Bead Usage

The LEGENDplex™ bead-based assay system 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 13 bead populations distinguished by size and internal fluorescent dye, the Mouse B cell Panel allows simultaneous detection of 13 analytes 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

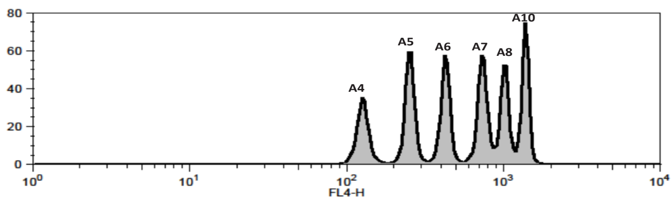
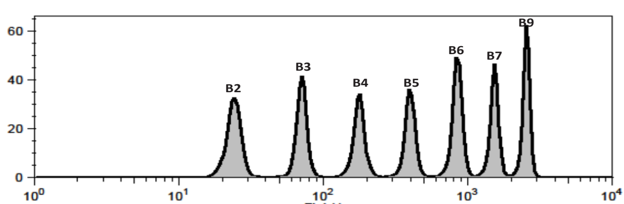


Figure 3. Beads B Classification by FL4



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

Table 1. Panel Targets and Bead ID

Target	Bead ID	B cell Panel (13-plex) Cat No. 740818, 740819	B cell Panel - S/P (12-plex) Cat. No. 740826, 740827	B cell Panel - S/P (1-plex) Cat. No. 740982, 740983	Be1 Panel (3-plex) Cat. No. 740824, 740825	Be2 Panel (5-plex) Cat. No. 740822, 740823	Be1/2 Panel (8-plex) Cat. No. 740820, 740821	Regulatory B cell Panel (2-plex) Cat. No. 740828, 740829	Top Standard Concentrations
IL-4	A4	√	√			√	√		The top standard concentration of each target may vary and may subject to change from lot to lot. Please refer to the lot-specific Certificate of Analysis for this information
IL-6	A5	√	√			√	√		
IL-12p70	A6	√	√		√		√		
IL-17A	A7	√	√				√		
IL-2	A8	√	√			√	√		
TNF-α	A10	√	√		√	√	√		
Free Active TGF-β1	B2	√	√					√	
IL-13	B3	√	√			√	√		
IFN-γ	B4	√	√		√		√		
BAFF	B5	√		√					
BCMA	B6	√	√						
sCD40L	B7	√	√						
IL-10	B9	√	√					√	

*Bead ID is used to associate a bead population to a particular analyte when using the LEGENDplex™ data analysis software program. For further information regarding the use of the program please visit biolegend.com/en-us/legendplex

Storage Information

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

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

Materials Supplied

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

Kit Components	Quantity	Volume	Part #
Setup Beads: PE Beads	1 vial	1 mL	77842
Setup Beads: Raw Beads	1 vial	1.8 mL	77844
Capture Beads* (see tables below for more information)	**varies**	**varies**	**varies***
Mouse B cell Panel Detection Antibodies	1 bottle	3.3 mL	**varies***
Mouse B cell Panel Standard Cocktail, Lyophilized	1 vial	lyophilized	**varies***
LEGENDplex^{TM} Matrix C1, Lyophilized	1 vial	lyophilized	750004013
LEGENDplex^{TM} SA-PE	1 bottle	3.3 mL	77743
LEGENDplex^{TM} Assay Buffer	1 bottle	25 mL	77562
LEGENDplex^{TM} Wash Buffer, 20X	1 bottle	25 mL	77564
Filter Plate**or V-bottom Plate***	1 plate		76187** or 76883***
Plate Sealers	4 sheets		78101

* For 13-plex, premixed beads are provided ready-to-use. For 12-plex, 1-plex, 3-plex, 5-plex, 8-plex, and 2-plex, individual beads are provided at 13X concentration. For Standard and Detection Antibodies: 13-plex uses part numbers and 12-plex, 1-plex, 3-plex, 5-plex, 8-plex, and 2-plex use catalog 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.

For Mouse B cell Panel (13-plex):

Kit Components	Quantity	Volume	Part #
Mouse B cell Panel Premixed Beads (13-plex)	1 bottle	3.3 mL	750000421
Mouse B cell Panel Detection Antibodies	1 bottle	3.3 mL	750000439
Mouse B cell Panel Standard Cocktail, Lyophilized	1 vial	lyophi-lized	750000441

For Mouse B cell Panel - S/P (12-plex):

Kit Components	Quantity	Volume	Part #
LEGENDplex™ Mouse IL-4 Capture Bead A4, 13X	1 vial	270 µL	740832
LEGENDplex™ Mouse IL-6 Capture Bead A5, 13X	1 vial	270 µL	740833
LEGENDplex™ Mouse IL-12p70 Capture Bead A6, 13X	1 vial	270 µL	740834
LEGENDplex™ Mouse IL-17A Capture Bead A7, 13X	1 vial	270 µL	740835
LEGENDplex™ Mouse IL-2 Capture Bead A8, 13X	1 vial	270 µL	740836
LEGENDplex™ Mouse TNF-α Capture Bead A10, 13X	1 vial	270 µL	740837
LEGENDplex™ Mouse Free Active TGF-β1 Capture Bead B2, 13X	1 vial	270 µL	740838
LEGENDplex™ Mouse IL-13 Capture Bead B3, 13X	1 vial	270 µL	740839
LEGENDplex™ Mouse IFN-γ Capture Bead B4, 13X	1 vial	270 µL	740840
LEGENDplex™ Mouse BCMA Capture Bead B6, 13X	1 vial	270 µL	740842
LEGENDplex™ Mouse sCD40L Capture Bead B7, 13X	1 vial	270 µL	740843
LEGENDplex™ Mouse IL-10 Capture Bead B9, 13X	1 vial	270 µL	740844
LEGENDplex™ Mouse B cell Panel Detection Antibodies	1 bottle	3.3 mL	740830
LEGENDplex™ Mouse B cell Panel Standard	1 vial	lyophi-lized	740831

For Mouse B cell Panel - S/P (1-plex):

Kit Components	Quantity	Volume	Part #
LEGENDplex™ Mouse BAFF Capture Bead B5, 13X	1 vial	270 µL	740841
LEGENDplex™ Mouse B cell Panel Detection Antibodies	1 bottle	3.3 mL	740830
LEGENDplex™ Mouse B cell Panel Standard	1 vial	lyophi-lized	740831

For Mouse B Effector 1 (Be1) Panel (3-plex):

Kit Components	Quantity	Volume	Part #
LEGENDplex™ Mouse IL-12p70 Capture Bead A6, 13X	1 vial	270 µL	740834
LEGENDplex™ Mouse TNF-α Capture Bead A10, 13X	1 vial	270 µL	740837
LEGENDplex™ Mouse IFN-γ Capture Bead B4, 13X	1 vial	270 µL	740840
LEGENDplex™ Mouse B cell Panel Detection Antibodies	1 bottle	3.3 mL	740830
LEGENDplex™ Mouse B cell Panel Standard	1 vial	lyophi-lized	740831

For Mouse B Effector 2 (Be2) Panel (5-plex):

Kit Components	Quantity	Volume	Part #
LEGENDplex™ Mouse IL-4 Capture Bead A4, 13X	1 vial	270 µL	740832
LEGENDplex™ Mouse IL-6 Capture Bead A5, 13X	1 vial	270 µL	740833
LEGENDplex™ Mouse IL-2 Capture Bead A8, 13X	1 vial	270 µL	740836
LEGENDplex™ Mouse TNF-α Capture Bead A10, 13X	1 vial	270 µL	740837
LEGENDplex™ Mouse IL-13 Capture Bead B3, 13X	1 vial	270 µL	740839
LEGENDplex™ Mouse B cell Panel Detection Antibodies	1 bottle	3.3 mL	740830
LEGENDplex™ Mouse B cell Panel Standard	1 vial	lyophi-lized	740831

For Mouse B Effector 1/2 (Be1/2) Panel (8-plex):

Kit Components	Quantity	Volume	Part #
LEGENDplex™ Mouse IL-4 Capture Bead A4, 13X	1 vial	270 µL	740832
LEGENDplex™ Mouse IL-6 Capture Bead A5, 13X	1 vial	270 µL	740833
LEGENDplex™ Mouse IL-12p70 Capture Bead A6, 13X	1 vial	270 µL	740834
LEGENDplex™ Mouse IL-17A Capture Bead A7, 13X	1 vial	270 µL	740835
LEGENDplex™ Mouse IL-2 Capture Bead A8, 13X	1 vial	270 µL	740836
LEGENDplex™ Mouse TNF-α Capture Bead A10, 13X	1 vial	270 µL	740837
LEGENDplex™ Mouse IL-13 Capture Bead B3, 13X	1 vial	270 µL	740839
LEGENDplex™ Mouse IFN-γ Capture Bead B4, 13X	1 vial	270 µL	740840
LEGENDplex™ Mouse B cell Panel Detection Antibodies	1 bottle	3.3 mL	740830
LEGENDplex™ Mouse B cell Panel Standard	1 vial	lyophi-lized	740831

For Mouse Regulatory B cell Panel (2-plex):

Kit Components	Quantity	Volume	Part #
LEGENDplex™ Mouse Free Active TGF-β1 Capture Bead B2, 13X	1 vial	270 µL	740838
LEGENDplex™ Mouse IL-10 Capture Bead B9, 13X	1 vial	270 µL	740844
LEGENDplex™ Mouse B cell Panel Detection Antibodies	1 bottle	3.3 mL	740830
LEGENDplex™ Mouse B cell Panel Standard	1 vial	lyophi-lized	740831

Please refer to Beads ID and Panel-Specific Target Selection table (Table 1), 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	Reporter Channel Emission	Beads Classification Channel	Classification Channel Emission	Compensation needed?
BD FACSCalibur™	**FL2**	**575 nm**	**FL4**	**660 nm**	**No***
BD Accuri™ C6	**FL2**	**585 nm**	**FL4**	**675 nm**	**No***
BD FACSCanto™, BD FACSCanto™ II	**PE**	**575 nm**	**APC**	**660 nm**	**No***
BD™ LSR, LSR II BD LSRFortessa™	**PE**	**575 nm**	**APC**	**660 nm**	**No***
Gallios™	**PE**	**575 nm**	**APC**	**660 nm**	**No***
CytoFLEX	**PE**	**585 nm**	**APC**	**660 nm**	**No***
NovoCyte	**PE**	**572 nm**	**APC**	**660 nm**	**No***
Attune™ NxT	**PE**	**574 nm**	**APC**	**670 nm**	**No***

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

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

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

- Sonicator bath (e.g., Branson Ultrasonic Cleaner model #B200, or equivalent)
- Aluminum foil
- Absorbent pads or paper towels
- Plate shaker (e.g., Lab-Line Instruments model #4625, or equivalent)
- Tabletop centrifuges (e.g., Eppendorf centrifuge 5415 C, or equivalent)
- 1.1 mL polypropylene micro FACS tubes, in 96-tube rack (e.g., National Scientific Supply Co, catalog # TN0946-01R, or equivalent).

If the assay is performed in a filter plate;

- A vacuum filtration unit (Millipore MultiScreen ® HTS Vacuum Manifold, cat # MSVMHTS00 or equivalent). Instructions on how to use the vacuum manifold can be found at the supplier's website.
- A vacuum source (mini vacuum pump or line vacuum, e.g., Millipore Vacuum Pump, catalog # WP6111560, or equivalent)
- If needed, additional Filter plate can be ordered from BioLegend (Cat# 740377 or 740378)

If the assay is performed in a V-bottom plate;

- Centrifuge with a swinging bucket adaptor for microtiter plates (e.g., Beckman Coulter Allegra™ 6R Centrifuge with MICROPLUS CARRIER adaptor for GH3.8 and JS4.3 Rotors).
- If needed, additional V-bottom plate can be ordered from BioLegend (Cat# 740379)

Precautions

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Center for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium azide has been added to some reagents as a preservative. Although the concentrations are low, sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up.
- Matrix C1 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 20 minutes at 1,000 x *g*.
- Remove serum and assay immediately or aliquot and store samples at ≤-20°C. Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended that samples are thawed completely, mixed and centrifuged to remove particulates prior to use.

Preparation of Plasma Samples:

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

Preparation of Tissue Culture Supernatant:

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

Reagent Preparation

Preparation of Antibody-Immobilized Beads

- **If pre-mixed beads are provided in the kit:**

  Sonicate pre-mixed beads bottle for 1 minute in a sonicator bath and then vortex for 30 seconds prior to use. If no sonicator bath is available, increase the vortexing time to 1 minute to completely resuspend the beads.

- **If individual beads (13X) are provided in the kit:**

  The individual beads (13X) should be diluted to 1X final concentration with Assay Buffer prior to use.

  1. Sonicate the beads vials for 1 minute in a sonicator bath and then vortex for 30 seconds to completely resuspend the beads.

2. Calculate the amount of diluted beads needed for the assay. Prepare extra to compensate for pipetting loss. Each reaction needs 25 µL of diluted beads. For 50 reactions, prepare 1.5 mL of mixed beads. For 100 reactions, prepare 3 mL of mixed beads.

3. To make 1.5 mL of 10-plex 1X diluted beads, transfer 115 µL of each of the 10 individual beads (13X) to a fresh tube (total bead volume = 1150 µL) and add 350 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 C1 (for Serum or Plasma Samples Only)

- Add 5.0 mL DI water to the bottle containing lyophilized Matrix C1. Allow at least 15 minutes for complete reconstitution. Vortex to mix well. Leftover reconstituted Matrix C1 should be stored at ≤-70°C for up to one month.

Standard Preparation

1. Prior to use, reconstitute the lyophilized Mouse B cell Panel Standard Cocktail, with 250 µL of Assay Buffer.
2. Mix and allow the vial to sit at room temperature for 15 minutes, and then transfer the standard to an appropriately labeled polypropylene microfuge tube. This will be used as the top standard C7.

   Note: The top standard concentrations of analytes in this panel were set at various concentrations, but may be subject to change from lot to lot (please visit biolegend.com/en-us/legendplex to download a lot-specific certificate of analysis).
3. Label 6 polypropylene microfuge tubes as C6, C5, C4, C3, C2 and C1, respectively.
4. Add 75 µL of Assay Buffer to each of the six tubes. Prepare 1:4 dilution of the top standard by transferring 25 µL of the top standard C7 to the C6 tube and mix well. This will be the C6 standard.
5. In the same manner, perform serial 1:4 dilutions to obtain C5, C4, C3, C2 and C1 standards (**see the table below using the top standard at 10,000 pg/mL as an example**). Assay Buffer will be used as the 0 pg/mL standard (C0).

Tube/Standard ID	Serial Dilution	Assay Buffer to add (µL)	Standard to add	Final Conc. (pg/mL)
C7	--	--	--	10,000
C6	1:4	75	25 µL of C7	2,500
C5	1:16	75	25 µL of C6	625
C4	1:64	75	25 µL of C5	156.25
C3	1:256	75	25 µL of C4	39.06
C2	1:1024	75	25 µL of C3	9.76
C1	1:4096	75	25 µL of C2	2.44
C0	--	75	--	0

Sample Dilution

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.

For serum and plasma samples, follow panel specific dilution recommendations below.

- **For Mouse B cell Panel (13-plex);**

  Tissue culture samples are the recommended sample type. Follow the cell culture supernatant dilution guidelines mentioned above.

  Serum and plasma are not the recommended sample type for the 13-plex assay due to differences in recommended dilution factors for certain targets. If measuring serum or plasma samples using the 13-plex assay is desired, two sets of sample dilutions and two standard curves will be needed; this limits the total number of testable samples per kit. For example, in order to measure serum or plasma samples using 13-plex, the targets listed in 1-plex require samples to be diluted 25-fold and the assay to be performed using a standard curve in Assay Buffer. Similarly, targets listed in 12-plex require samples to be diluted 2-fold and the assay to be performed using standard curve in Matrix C1.

- **For Mouse B cell Panel - S/P (12-plex), Mouse B Effector 1 (Be1) Panel (3-plex), Mouse B Effector 2 (Be2) Panel (5-plex), Mouse B Effector 1/2 (Be1/2) Panel (8-plex), and Mouse Regulatory B cell Panel (2-plex);**

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

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

  If further sample dilution is desired, dilution should be done with Matrix C1 to ensure accurate measurement.

- **For Mouse B cell Panel - S/P (1-plex);**

  Serum or plasma samples must be diluted 25-fold with Assay Buffer as described in the table below.

Sample	Dilution (1:25)	Final dilution fold
Serum, Plasma	4 µL + 96 µL Assay Buffer	25

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

Chapter 3: ASSAY PROCEDURE

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

- The in-filter plate assay procedure requires a vacuum filtration unit for washing (see **Materials to be Provided by the End-User, page 10)**. 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.
- Always set the filter plate on an inverted plate cover 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 41). Be sure to load standards in the first two columns. If an automation device is used for reading, the orientation and reading sequence should be carefully planned.**

1. Pre-wet the plate by adding 100 µL of LEGENDplex™ 1X Wash Buffer to each well and let it sit for 1 minute at room temperature. To remove the excess volume, place the plate on the vacuum manifold and apply vacuum. Do not exceed 10” Hg of vacuum. Vacuum until wells are drained (5-10 seconds). Blot excess Wash Buffer from the bottom of the plate by pressing the plate on a stack of clean paper towels. Place the plate on top of the inverted plate cover.

   For measuring cell culture supernatant samples in 13-plex, 12-plex, 1-plex, 3-plex, 5-plex, 8-plex and 2-plex; and serum or plasma samples in 1-plex, load the plate as shown in the table below (in the order from left to right):

	Assay Buffer	Matrix C1	Standard	Sample*
Standard Wells	25 µL	---	25 µL	---
Sample wells	25 µL	---	---	25 µL

For measuring serum or plasma samples in 12-plex, 3-plex, 5-plex, 8-plex, and 2-plex, load the plate as shown in the table below (in the order from left to right):

	Assay Buffer	Matrix C1	Standard	Sample*
Standard Wells	---	25 µL	25 µL	---
Sample wells	25 µL	---	---	25 µL

*See **Sample Dilution**

For measuring serum or plasma samples using 13-plex;

In general, this is not recommended due to differences in recommended dilution factors for certain targets. If measuring serum or plasma samples using the 13-plex assay is desired, two sets of sample dilutions and two standard curves will be needed; this limits the total number of testable samples per kit. For example, to measure serum or plasma samples using 13-plex, the targets listed in 1-plex require samples to be diluted 25-fold and the assay to be performed using a standard curve in Assay Buffer (follow the plate loading order illustrated in the upper table). Similarly, targets listed in 12-plex require samples to be diluted 2-fold and the assay to be performed using standard curve in Matrix C1 (follow the plate loading order illustrated in the lower table).

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

3. Seal the plate with a plate sealer. **To avoid plate leaking, do not apply positive pressure to the sealer when sealing the plate**. Wrap the entire plate, including the inverted plate cover, with aluminum foil. Place the plate on a plate shaker, secure it and shake at approximate 500 rpm for 2 hours at room temperature.

4. **Do not invert the plate!** Place the plate on the vacuum manifold and apply vacuum as before in Step 1. Add 200 µL of 1X Wash Buffer to each well. Remove Wash Buffer by vacuum filtration. Blot excess Wash Buffer from the bottom of the plate with an absorbent pad or paper towels. Repeat this washing step once more.

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

6. Seal the plate with a fresh plate sealer. Wrap the entire plate, including the inverted plate cover, with aluminum foil. Place the plate on a plate shaker and shake at approximately 500 rpm for 1 hour at room temperature.

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

8. Seal the plate with a fresh plate sealer. Wrap the entire plate, including the inverted plate cover, with aluminum foil. Place the plate on a plate shaker and shake at approximate 500 rpm for 30 minutes at room temperature.

9. Repeat step 4 above.

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

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

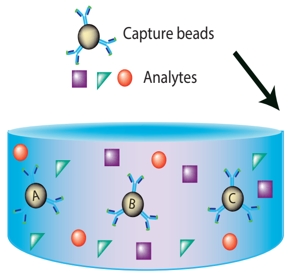
    If the flow cytometer is equipped with an autosampler, read the plate directly using the autosampler. **Please be sure to program the autosampler to resuspend beads in the well immediately before taking samples. The probe height may need to be adjusted when using an autosampler**.

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

Assay Procedure Summary for Filter Plate

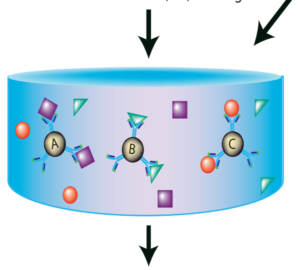
Add 100 µL 1X Wash Buffer to filter plate wells

Vacuum to remove excess buffer

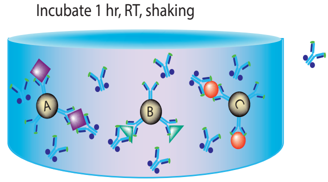


Add to the plate:
25 µL Assay Buffer or Matrix to standard wells
(Refer to Assay Procedure)
25 µL Assay Buffer to sample wells
25 µL diluted standard to standard wells
25 µL sample to sample wells
25 µL mixed beads to all wells

Incubate 2 hours, RT, shaking



Wash 2 times using vacuum filtration unit
Add 25 µL Detection Antibodies
Incubate 1 hr, RT, shaking



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

Wash 2 times using vacuum filtration unit
Add 150 µL of 1x Wash Buffer
Read on a flow cytometer

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 41). Be sure to load standards in the first two columns. If an automation device is used for reading, the orientation and reading sequence should be carefully planned.

1. **For measuring cell culture supernatant samples in 13-plex, 12-plex, 1-plex, 3-plex, 5-plex, 8-plex and 2-plex; and serum or plasma samples in 1-plex,** load the plate as shown in the table below (in the order from left to right):

	Assay Buffer	Matrix C1	Standard	Sample*
Standard Wells	25 µL	---	25 µL	---
Sample wells	25 µL	---	---	25 µL

For measuring serum or plasma samples in 12-plex, 3-plex, 5-plex, 8-plex, and 2-plex, load the plate as shown in the table below (in the order from left to right):

	Assay Buffer	Matrix C1	Standard	Sample*
Standard Wells	---	25 µL	25 µL	---
Sample wells	25 µL	---	---	25 µL

*See **Sample Dilution**

For measuring serum or plasma samples using 13-plex;

In general, this is not recommended due to differences in recommended dilution factors for certain targets. If measuring serum or plasma samples using the 13-plex assay is desired, two sets of sample dilutions and two standard curves will be needed; this limits the total number of testable samples per kit. For example, to measure serum or plasma samples using 13-plex, the targets listed in 1-plex require samples to be diluted 25-fold and the assay to be performed using a standard curve in Assay Buffer (follow the plate loading order illustrated in the upper table). Similarly, targets listed in 12-plex require samples to be diluted 2-fold and the assay to be performed using standard curve in Matrix C1 (follow the plate loading order illustrated in the lower table).

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 10).** Do not use excessive centrifugation speed as it may make it harder to resuspend beads in later steps. **Make sure that the timer of the centrifuge works properly and standby to make sure the centrifuge reaches preset speed.**

5. Immediately after centrifugation, dump the supernatant into a sink by quickly inverting and flicking the plate **in one continuous and forceful motion**. Do not worry about losing beads even if the pellet is not visible. The beads will stay in the tip of the well nicely. Blot the plate on a stack of clean paper towel and drain the remaining liquid from the well as much as possible. Be careful not to disturb the bead pellet.

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

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

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

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

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

10. Seal the plate with a new plate sealer. Wrap the entire plate with aluminum foil and shake the plate on a plate shaker at approximate 800 rpm for 30 minutes at room temperature.

11. Repeat step 4 and 5.

12. Wash the plate by dispensing 200 µL of 1X Wash Buffer into each well and

incubate for one minute. Repeat step 4 and 5 above. This washing step is optional, but it helps to reduce the background.

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

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

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

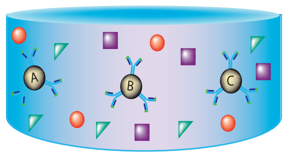
    If an autosampler is not available, the samples can be transferred from the plate to micro FACS (or FACS) tubes and read manually.

Assay Procedure Summary for V-bottom Plate

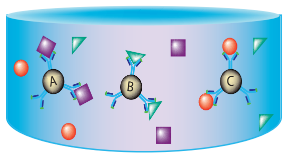
Capture beads

Analytes

Add to the plate:
25 µL Assay Buffer or Matrix to standard wells
(Refer to Assay Procedure)
25 µL Assay Buffer to sample wells
25 µL diluted standard to standard wells
25 µL sample to sample wells
25 µL mixed beads to all wells

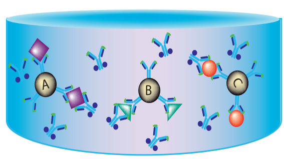


Incubate 2 hours, RT, shaking



Spin down beads, remove supernatant
Wash 1 time
Add 25 µL Detection Antibodies
Incubate 1 hr, RT, shaking

Biotinylated Detection Antibody



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

Spin down beads, remove supernatant
Wash 1 time (optional)
Add 150 µL 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 setup instructions have been removed from this manual and uploaded onto our website to save paper.

To access the setup instructions, please visit: **www.biolegend.com/legendplex** and click on the **Instrument Setup** tab.

Chapter 5: DATA ACQUISITION AND ANALYSIS

Data Acquisition

1. Before reading samples, make sure that the flow cytometer is set up properly.

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

3. Vortex each sample for 5 seconds before analysis.

4. Set the flow rate to low. Set the number of beads to be acquired to about 300 per analyte. Do not set to acquire total events as samples may contain large amounts of debris. Instead, create a large gate to include both Beads A and Beads B (gate A+B) and set to acquire the number of events in gate A + B. This will exlude majority of the debris.

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

5. Read samples.

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

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

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

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

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

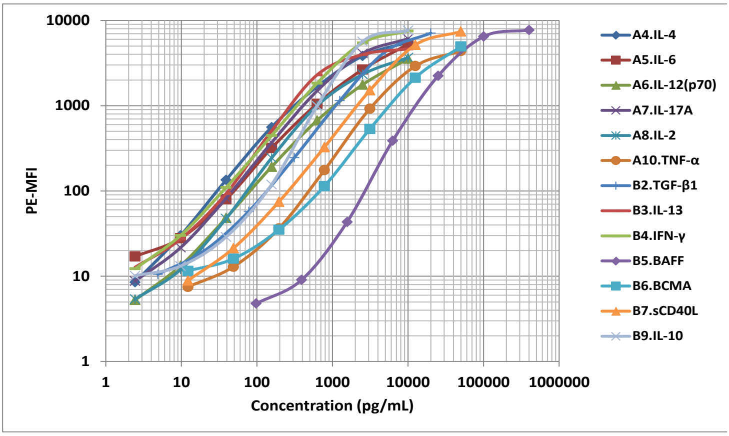
Data Analysis

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

Chapter 6: ASSAY CHARACTERIZATION

Representative Standard Curve

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



Assay Sensitivity

The assay sensitivity is the theoretical limit of detection calculated usingthe LEGENDplexTM Data Analysis Software by applying a 5-paramater curve fitting algorithm. Assay Sensitivity presented here is ≤Mean LOD + 2xSTDEV LOD.

Analyte	LOD in Assay Buffer (pg/mL) (n=10)	LOD in Matrix (pg/mL) (n=10)
Mouse IL-4	1.52	1.58
Mouse IL-6	2.00	2.60
Mouse IL-12p(70)	1.00	1.10
Mouse IL-17A	1.52	0.86
Mouse IL-2	1.20	3.12
Mouse TNF-α	2.40	2.22
Mouse Free Active TGF-β1	5.50	14.50
Mouse IL-13	1.22	1.90
Mouse IFN-γ	1.57	2.10
Mouse BAFF	90.10	165.36
Mouse BCMA	15.60	39.80
Mouse sCD40L	2.80	3.90
Mouse IL-10	3.10	5.80

Cross-Reactivity

Cross-reactivity was tested in the panel using the LEGENDplex™ Mouse B cell Panel. The following recombinant proteins were tested at respective concentrations. No or negligible cross-reactivity was found for all the tested analytes.

Recombinant Protein	Species Reactivity	Concentration Tested (ng/mL)
APRIL	Mouse	500
APRIL	Human	100
BAFF	Mouse	4000
BAFF	Human	50
BCMA	Mouse	500
CCL11 (Eotaxin)	Mouse	10

CCL17 (TARC)	Mouse	10
CCL2 (MCP-1)	Mouse	10
CCL20 (MIP-3α)	Mouse	10
CCL22 (MDC)	Mouse	10
CCL3 (MIP-1α)	Mouse	10
CCL4 (MIP-1β)	Mouse	10
CCL5 (RANTES)	Mouse	10
CXCL1 (KC)	Mouse	10
CXCL10 (IP-10)	Mouse	10
CXCL12 (SDF-1)	Mouse	250
CXCL13 (BLC)	Mouse	10
CXCL5 (LIX)	Mouse	10
CXCL9 (MIG)	Mouse	10
EPO	Mouse	25
G-CSF	Mouse	50
GM-CSF	Mouse	10
IFN-α	Mouse	10
IFN-β	Mouse	50
IFN-γ	Mouse	100
IFN-γ	Human	10
IL-10	Mouse	100
IL-10	Human	10
IL-11	Mouse	50
IL-12(p70)	Mouse	100
IL-12(p70)	Human	10
IL-13	Mouse	100
IL-13	Human	10
IL-15	Mouse	50
IL-17A	Mouse	100
IL-17A	Human	10
IL-17F	Mouse	10
IL-18	Mouse	100
IL-1α	Mouse	10
IL-1β	Mouse	10

IL-2	Mouse	100
IL-2	Human	50
IL-21	Mouse	10
IL-22	Mouse	10
IL-23	Mouse	50
IL-27	Mouse	50
IL-3	Mouse	10
IL-33	Mouse	50
IL-34	Mouse	50
IL-4	Mouse	100
IL-4	Human	10
IL-5	Mouse	10
IL-6	Mouse	100
IL-6	Human	10
IL-7	Mouse	50
IL-9	Mouse	2.5
LIF	Mouse	10
M-CSF	Mouse	10
sCD40L	Mouse	200
sCD40L	Human	200
SCF	Mouse	50
TACI	Mouse	500
TGF-β1	Mouse	200
TNF-α	Mouse	100
TNF-α	Human	10
TNF-β	Human	50
TPO	Mouse	10
TSLP	Mouse	50

Accuracy (Spike Recovery)

For spike recovery in serum and plasma, target proteins with known concentrations were spiked into mouse serum and plasma 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 Serum (N = 8)	% of Recovery in Plasma (N = 12)
Mouse IL-4	89%	92%
Mouse IL-6	65%	100%
Mouse IL-12(p70)	101%	91%
Mouse IL-17A	126%	115%
Mouse IL-2	137%	136%
Mouse TNF-α	83%	103%
Mouse Free Active TGF-β1	92%	69%
Mouse IL-13	40%	47%
Mouse IFN-γ	84%	85%
Mouse BAFF	102%	84%
Mouse BCMA	109%	120%
Mouse sCD40L	91%	89%
Mouse IL-10	86%	89%

Linearity of Dilution

For testing linearity of dilution, serum and plasma samples were first diluted two-fold with Assay Buffer and spiked with target proteins at known concentrations. The spiked samples were then serially diluted 1:2, 1:4, 1:8 with Matrix C1 and assayed. For mouse BAFF, serum and plasma samples were first diluted 25-fold with Assay Buffer and then serially diluted 1:2, 1:4, 1:8 with Assay Buffer and assayed. The measured concentrations of serially diluted samples were then compared with that of the two-fold diluted samples and neat samples, respectively.

Analyte	Linearity of Dilution (Serum) (N = 8)	Linearity of Dilution (Plasma) (N = 12)
Mouse IL-4	114%	110%
Mouse IL-6	95%	91%
Mouse IL-12(p70)	112%	122%
Mouse IL-17A	101%	98%
Mouse IL-2	92%	90%
Mouse TNF-α	107%	101%
Mouse Free Active TGF-β1	124%	129%
Mouse IL-13	184%	173%

LEGENDplex™ Mouse B cell Panel

Mouse IFN-γ	126%	121%
Mouse BAFF	109%	100%
Mouse BCMA	76%	83%
Mouse sCD40L	120%	119%
Mouse IL-10	135%	131%

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-4	Sample 1	31.74	1.47	5%
	Sample 2	133.36	8.36	6%
Mouse IL-6	Sample 1	55.33	3.96	7%
	Sample 2	216.87	9.92	5%
Mouse IL-12(p70)	Sample 1	29.72	1.85	6%
	Sample 2	118.30	7.31	6%
Mouse IL-17A	Sample 1	51.84	2.82	5%
	Sample 2	190.11	9.77	5%
Mouse IL-2	Sample 1	23.80	1.12	5%
	Sample 2	95.34	5.19	5%
Mouse TNF-α	Sample 1	44.78	2.32	5%
	Sample 2	177.89	6.30	4%
Mouse Free Active TGF-β1	Sample 1	113.77	9.45	8%
	Sample 2	405.45	17.72	4%
Mouse IL-13	Sample 1	48.56	3.26	7%
	Sample 2	184.92	8.94	5%
Mouse IFN-γ	Sample 1	48.07	2.18	5%
	Sample 2	164.24	10.77	7%
Mouse BAFF	Sample 1	2004.11	93.22	5%
	Sample 2	6427.91	198.61	3%
Mouse BCMA	Sample 1	238.55	19.15	8%
	Sample 2	914.89	42.09	5%
Mouse sCD40L	Sample 1	98.17	7.22	7%
	Sample 2	351.19	14.60	4%
Mouse IL-10	Sample 1	60.90	5.34	9%
	Sample 2	200.50	8.45	4%

Inter-Assay Precision

Two samples with different concentrations of target proteins were analyzed in four independent assays with 4 replicates for each sample. The inter-assay precision was calculated as below.

Analyte	Sample	Mean (pg/mL)	STDEV	%CV
Mouse IL-4	Sample 1	32.65	2.37	7%
	Sample 2	137.19	11.18	8%
Mouse IL-6	Sample 1	53.36	6.5	12%
	Sample 2	208.45	25.02	12%
Mouse IL-12(p70)	Sample 1	29.29	2.16	7%
	Sample 2	116.92	16.70	14%
Mouse IL-17A	Sample 1	50.74	5.72	11%
	Sample 2	185.43	18.87	10%
Mouse IL-2	Sample 1	23.52	2.88	12%
	Sample 2	94.00	11.72	12%
Mouse TNF-α	Sample 1	43.69	5.10	12%
	Sample 2	171.39	19.30	11%
Mouse Free Active TGF-β1	Sample 1	107.86	12.97	12%
	Sample 2	397.50	36.75	9%
Mouse IL-13	Sample 1	47.57	6.08	13%
	Sample 2	179.85	20.68	12%
Mouse IFN-γ	Sample 1	45.33	6.18	14%
	Sample 2	164.68	25.37	15%
Mouse BAFF	Sample 1	1975.57	196.56	10%
	Sample 2	6347.13	657.66	10%
Mouse BCMA	Sample 1	242.73	32.21	13%
	Sample 2	919.76	112.15	12%
Mouse sCD40L	Sample 1	95.95	8.63	9%
	Sample 2	349.89	31.10	9%
Mouse IL-10	Sample 1	55.84	8.90	16%
	Sample 2	190.26	20.16	11%

Biological Samples

Serum

Pooled normal serum samples from 4 different strains were tested for endogenous levels using the LEGENDplex™ Mouse B cell Panel. The concentrations measured (in pg/mL) are shown below.

Analyte	C57BL/6	BALB/c	Swiss Webster	CD-1
Mouse IL-4	4.3	ND	ND	ND
Mouse IL-6	66.7	198.48	23.8	15.3
Mouse IL-12(p70)	5.1	1.83	1.1	1.5
Mouse IL-17A	45.6	19.95	4.1	13.8
Mouse IL-2	12.9	2.49	ND	2.2
Mouse TNF-α	183.0	42.82	32.4	36.0
Mouse Free Active TGF-β1	139.1	443.18	808.2	216.8
Mouse IL-13	4.7	3.9	2.5	4.7
Mouse IFN-γ	91.9	48.96	35.6	58.6
Mouse BAFF	67478.5	62365.75	107992.3	46603.8
Mouse BCMA	5256.1	1737.84	294.5	4384.6
Mouse sCD40L	17.2	3.34	ND	5.2
Mouse IL-10	12.5	5.43	16.1	11.8

ND = Non-detectable

Plasma

Pooled mouse EDTA plasma samples from 4 different strains were tested for endogenous levels using the LEGENDplex™ Mouse B cell Panel. The concentrations measured (in pg/mL) are shown below.

Analyte	C57BL/6	BALB/c	Swiss Webster	CD-1
Mouse IL-4	2.06	ND	ND	2.94
Mouse IL-6	72.49	38.24	14.8	21.39
Mouse IL-12(p70)	3.92	1.73	1.37	1.84
Mouse IL-17A	36.44	52.95	4.24	15.05
Mouse IL-2	7.53	3.39	3.68	8.4
Mouse TNF-α	73.62	27.78	92.14	40.98
Mouse Free Active TGF-β1	65.06	47.23	423.41	594.5
Mouse IL-13	7.21	ND	ND	16.96
Mouse IFN-γ	69.18	46.67	68.67	32.89
Mouse BAFF	35128.00	77484.75	145741.50	35027.25
Mouse BCMA	3499.17	1613.50	1224.71	2478.54
Mouse sCD40L	4.73	3.5	5.15	7.66
Mouse IL-10	14.33	6.78	19.14	11.7

ND = Non-detectable

Pooled mouse citrate plasma samples from 4 different strains were tested for endogenous levels using the LEGENDplex™ Mouse B cell Panel. The concentrations measured (in pg/mL) are shown below.

Analyte	C57BL/6	BALB/c	Swiss Webster	CD-1
Mouse IL-4	1.6	ND	ND	ND
Mouse IL-6	16.00	81.38	12.70	10.00
Mouse IL-12(p70)	1.8	2.08	1.4	1.0
Mouse IL-17A	14.3	54.46	2.9	4.9
Mouse IL-2	1.8	3.55	ND	ND
Mouse TNF-α	33.1	33.54	25.1	12.4
Mouse Free Active TGF-β1	56.5	ND	53.4	ND
Mouse IL-13	10.9	ND	ND	ND
Mouse IFN-γ	7.4	43.08	28.9	45.5
Mouse BAFF	58593.50	22845.00	55748.30	35803.00
Mouse BCMA	1857.50	2064.55	183.60	1262.00
Mouse sCD40L	4.50	ND	4.50	3.10
Mouse IL-10	5.70	4.16	12.80	8.30

ND = Non-detectable

Pooled mouse heparin plasma samples from 4 different strains were tested for endogenous levels using the LEGENDplex™ Mouse B cell Panel. The concentrations measured (in pg/mL) are shown below.

Analyte	C57BL/6	BALB/c	Swiss Webster	CD-1
Mouse IL-4	4.48	ND	ND	ND
Mouse IL-6	97.47	217.33	14.95	87.17
Mouse IL-12(p70)	3.88	2.31	1.29	2.33
Mouse IL-17A	43.46	24.85	2.15	26.37
Mouse IL-2	7.98	2.91	ND	2.66
Mouse TNF-α	165.00	30.26	21.27	41.03
Mouse Free Active TGF-β1	ND	ND	50.07	ND
Mouse IL-13	15.62	1.88	ND	3.86
Mouse IFN-γ	40.50	69.10	22.57	29.24
Mouse BAFF	101806.50	34995.50	76843.75	49466.00
Mouse BCMA	9792.81	6353.58	125.97	4799.89
Mouse sCD40L	8.86	3.04	3.98	3.14
Mouse IL-10	8.87	4.64	7.94	13.87

ND = Non-detectable

Cell culture supernatant

Mouse splenocytes (1 x 10^6 cell/mL) were cultured under various conditions (unstimulated; LPS, 100 ng/mL; PMA, 20 ng/mL and Ionomycin, 500 ng/mL; and PHA, 10 µg/mL). Supernatants were collected after 48 hours and assayed with LEGENDplex™ Mouse B cell Panel. sCD40L was measured at 1.45 pg/mL; 134.85 pg/mL; 50.66 pg/mL; and 178.57 pg/mL for the respective conditions.

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 underside 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 underside 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

Enabling Legendary Discovery™

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