# BioLegend®

### Enabling Legendary Discovery™

# LEGENDplex™
## Multi-Analyte Flow Assay Kit

### Human Inflammation Panel 2
### Mix and Match Subpanel

**Please read the entire manual before running the assay.**

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

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# Chapter 1:   KIT DESCRIPTION

## Introduction

Inflammation is associated with a variety of human diseases ranging from cardiovascular, pulmonary, neurodegenerative, and autoimmune diseases. A large number of soluble receptors, growth factors, cytokines, chemokines and other factors possess pro-inflammatory and/or anti-inflammatory properties and are important players in immune-regulation. They are pivotal in inflammatory processes and serve as promising inflammatory disease biomarkers.

The LEGENDplex™ Human Inflammation Panel 2 (13-plex) is a bead-based multiplex assay panel, using fluorescence-encoded beads suitable for use on various flow cytometers. It allows for simultaneous quantification of 13 key targets involved in inflammation and immune response such as TGF-β1, PAI-1, sTREM-1, PTX3, sCD40L, sCD25 (IL-2Ra), CXCL12 (SDF-1), sST2, sTNF-RI, sTNF-RII, sRAGE, CX3CL1 (Fractalkine), and sCD130 (gp130). 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™ Human Inflammation Panel 2 is configured as shown below depending on sample types and required dilutions:

| Catalog No. | Plex Size | Targets | Recommended Sample Type | Recommended Dilution Factor |
|---|---|---|---|---|
| 740775 740776 | 13-plex | Free Active TGF-β1, PAI-1, sTREM-1, PTX3, sCD40L, sCD25 (IL-2Ra), CXCL12 (SDF-1), sST2, sTNF-RI, sTNF-RII, sRAGE, CX3CL1 (Fractalkine), sCD130 (gp130) | Tissue culture | Varies |
| 740883 740884 | 11-plex | Free Active TGF-β1, sTREM-1, PTX3, sCD40L, sCD25 (IL-2Ra), CXCL12 (SDF-1), sST2, sTNF-RI, sTNF-RII, sRAGE, CX3CL1 (Fractalkine) | Serum, Plasma | 2 |
| 740885 740886 | 2-plex | PAI-1, sCD130 (gp130) | Serum, Plasma | 20 |

The LEGENDplex™ Human Inflammation Panel 2 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 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 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 Human Inflammation Panel 2 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
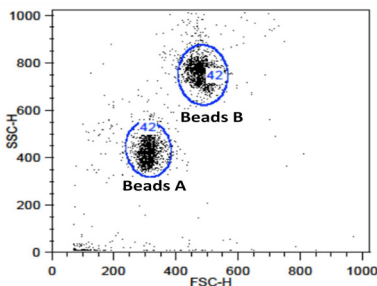


Beads A = smaller beads

Beads B = larger beads

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## Figure 2. Beads A Classification by FL4
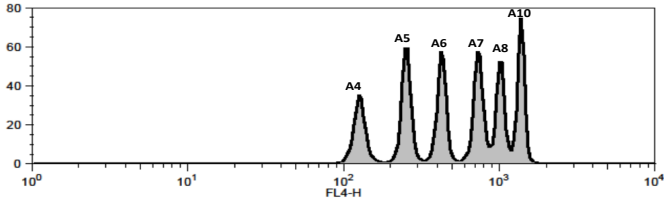


## Figure 3. Beads B Classification by FL4
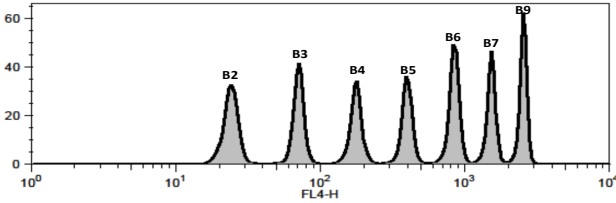


For Beads usage in the panel, please refer to Table 1 below:

## Table 1. Panel Targets and Bead ID*

| Target | Bead ID | Inflammation Panel 2 - TC (13-plex) Cat No. 740775 or 740776 | Inflammation Panel 2 - S/P (11-plex) Cat. No. 740883 or 740884 | Inflammation Panel 2 - S/P (2-plex) Cat. No. 740885 or 740886 | Top Standard Concentrations |
|---|---|---|---|---|---|
| Free Active TGF-β1 | A4 | √ | √ | | 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 ). |
| PAI-1 | A5 | √ | | √ | |
| sTREM-1 | A6 | √ | √ | | |
| PTX3 | A7 | √ | √ | | |
| sCD40L | A8 | √ | √ | | |
| sCD25 (IL-2Ra) | A10 | √ | √ | | |
| CXCL12 (SDF-1) | B2 | √ | √ | | |
| sST2 | B3 | √ | √ | | |
| sTNF-RI | B4 | √ | √ | | |
| sTNF-RII | B5 | √ | √ | | |
| sRAGE | B6 | √ | √ | | |
| CX3CL1 (Fractalkine) | B7 | √ | √ | | |
| sCD130 (gp130) | 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™ kit contains reagents for 100 tests, listed in the table below. When assayed in duplicate, this is enough for an 8-point standard curve and 40 samples.

For the Mix and Match Subpanels, individual beads are provided at 13X concentration. The Buffer Set contains Setup Beads, all Buffers, Plate Sealers, Matrix, and SA-PE.

| Kit Components | Quantity | Volume | Cat. # |
|---|---|---|---|
| **Capture Beads\* (see tables below for more information)** | **varies** | **varies** | **varies** |
| LEGENDplex™ Human Inflammation Panel 2 Detection Antibodies | 1 bottle | 3.5 mL | 740777 |
| LEGENDplex™ Human Inflammation Panel 2 Standard | 1 vial | lyophilized | 740778 |
| LEGENDplex™ Buffer Set H1 | 1 | | 741206 |
| Filter Plate\*\* or V-bottom Plate\*\*\* | 1 Plate | | 740377\*\*or 740379\*\*\* |

\*\* For assay with filter plate. \*\*\* For assay with V-bottom plate

**Capture beads for Mix and Match Subpanels\*:**

| Bead Name | Quantity | Volume | Cat. # |
|---|---|---|---|
| LEGENDplex™ Human Free Active TGF-β1 Capture Bead A4, 13X | 1 vial | 270 µL | 740779 |
| LEGENDplex™ Human PAI-1 Capture Bead A5, 13X | 1 vial | 270 µL | 740780 |

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| LEGENDplex™ Human sTREM-1 Capture Bead A6, 13X | 1 vial | 270 µL | 740781 |
|---|---|---|---|
| LEGENDplex™ Human PTX3 Capture Bead A7, 13X | 1 vial | 270 µL | 740782 |
| LEGENDplex™ Human sCD40L Capture Bead A8, 13X | 1 vial | 270 µL | 740783 |
| LEGENDplex™ Human sCD25 (IL-2Ra) Capture Bead A10, 13X | 1 vial | 270 µL | 740784 |
| LEGENDplex™ Human CXCL12 (SDF-1) Capture Bead B2, 13X | 1 vial | 270 µL | 740785 |
| LEGENDplex™ Human sST2 Capture Bead B3, 13X | 1 vial | 270 µL | 740786 |
| LEGENDplex™ Human sTNF-RI Capture Bead B4, 13X | 1 vial | 270 µL | 740787 |
| LEGENDplex™ Human sTNF-RII Capture Bead B5, 13X | 1 vial | 270 µL | 740788 |
| LEGENDplex™ Human sRAGE Capture Bead B6, 13X | 1 vial | 270 µL | 740789 |
| LEGENDplex™ Human CX3CL1 (Fractalkine) Capture Bead B7, 13X | 1 vial | 270 µL | 740790 |
| LEGENDplex™ Human sCD130 (gp130) Capture Bead B9, 13X | 1 vial | 270 µL | 740791 |

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

**LEGENDplex™ Buffer Set H1 (Cat#: 741206)**

| Components | Quan-tity | Volume | Part # |
|---|---|---|---|
| Setup Beads 1: FITC Beads | 1 vial | 1 mL | 77840 |
| Setup Beads 2: PE Beads | 1 vial | 1 mL | 77842 |
| Setup Beads 3: Raw Beads | 1 vial | 2 mL | 77844 |
| LEGENDplex™ SA-PE | 1 bottle | 3.5 mL | 77743 |
| LEGENDplex™ Matrix A1, Lyophilized | 1 vial | lyophilized | 750000601 |
| Lyophilized Standard Reconstitution Buffer | 1 vial | 1 mL | 75241 |
| LEGENDplex™ Assay Buffer | 1 bottle | 25 mL | 77562 |
| LEGENDplex™ Wash Buffer, 20X | 1 bottle | 25 mL | 77564 |
| Plate Sealers | 4 sheets | | 78101 |

**No plate is included in Buffer Set H1. Plate need to be ordered separately. Please order the correct type of plate based on the preferred assay protocol**

**(Cat# 740377 or 740378 for Filter Plate and Cat# 740379 for V-bottom Plate)**

## Materials to be Provided by the End-User

- A flow cytometer equipped with two lasers (e.g., a 488 nm blue laser or 532 nm green laser and a 633-635 nm red laser) capable of distinguishing 575 nm and 660 nm or a flow cytometer equipped with one laser (e.g., 488 nm blue laser) capable of distinguishing 575 nm and 670 nm.

**Partial list of compatible flow cytometers:**

| Flow Cytometer | Reporter Channel | Reporter Channel Emission | Beads Classification Channel | Classification Channel Emission | Compensation needed? |
|---|---|---|---|---|---|
| BD FACSCalibur™ | FL2 | 575 nm | FL4 | 660 nm | No* |
| BD Accuri™ C6 | FL2 | 585 nm | FL4 | 675 nm | No* |
| BD FACSCanto™, BD FACSCanto™ II | PE | 575 nm | APC | 660 nm | No* |
| BD™ LSR, LSR II BD LSRFortessa™ | PE | 575 nm | APC | 660 nm | No* |
| Gallios™ | PE | 575 nm | APC | 660 nm | No* |
| CytoFLEX | PE | 585 nm | APC | 660 nm | No* |
| NovoCyte | PE | 572 nm | APC | 660 nm | No* |
| Attune™ NxT | PE | 574 nm | APC | 670 nm | No* |

**\*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 A1 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**

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.

**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.

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**Preparation of Matrix A1 (for Serum or Plasma Samples Only)**

- Add 5.0 mL LEGENDplex™ Assay Buffer to the bottle containing lyophilized Matrix A1. Allow at least 15 minutes for complete reconstitution. Vortex to mix well. Leftover reconstituted Matrix A1 should be stored at ≤-70°C for up to one month.

## Standard Preparation

1. Prior to use, reconstitute the lyophilized Human Inflammation Panel 2 Standard Cocktail, with 250 µL Lyophilized Standard Reconstitution 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 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 Human Inflammation Panel 2 - TC (13-plex);**

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

- **For Human Inflammation Panel 2 - S/P (11-plex);**

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

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

- **For Human Inflammation Panel 2 - S/P (2-plex);**

  Serum or plasma samples must be diluted 20-fold with Assay Buffer as described in the table below.

| Sample | Dilution (1:20) | Final dilution fold |
|--------|-----------------|---------------------|
| Serum, Plasma | 5 µL + 95 µL Assay Buffer | 20 |

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

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

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

   **For measuring cell culture supernatant samples in 13-plex, 11-plex, and 2-plex; and serum or plasma samples in 2plex,** load the plate as shown in the table below (in the order from left to right):

| | Assay Buffer | Matrix A1 | Standard | Sample* |
|---|---|---|---|---|
| Standard Wells | 25 µL | --- | 25 µL | --- |

| | | | | |
|---|---|---|---|---|
| Sample wells | 25 µL | --- | --- | 25 µL |

**For measuring serum or plasma samples in 11-plex,** load the plate as shown in the table below (in the order from left to right):

| | Assay Buffer | Matrix A1 | Standard | Sample* |
|---|---|---|---|---|
| Standard Wells | --- | 25 µL | 25 µL | --- |
| Sample wells | 25 µL | --- | --- | 25 µL |

*See **Sample Dilution**

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

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

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

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

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

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

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

9. Repeat step 4 above.

10. Add 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).

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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

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

Wash 2 times using vacuum filtration unit
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

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

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## Performing the Assay Using a V-bottom Plate

- Allow all reagents to warm to room temperature (20-25°C) before use.
- Keep the plate upright during the entire assay procedure, including the washing steps, to avoid losing beads.
- The plate should be placed in the dark or wrapped with aluminum foil for all incubation steps.
- Standards and samples should be run in duplicate and arranged on the plate in a vertical configuration convenient for data acquisition and analysis (as shown in attached PLATE MAP, page 37). Be sure to load standards in the first two columns. If an automation device is used for reading, the orientation and reading sequence should be carefully planned.

1. **For measuring cell culture supernatant samples in 13-plex, 11-plex, and 2-plex; and serum or plasma samples in 2plex,** load the plate as shown in the table below (in the order from left to right):

| | Assay Buffer | Matrix A1 | Standard | Sample* |
|---|---|---|---|---|
| Standard Wells | 25 μL | --- | 25 μL | --- |
| Sample wells | 25 μL | --- | --- | 25 μL |

**For measuring serum or plasma samples in 11-plex,** load the plate as shown in the table below (in the order from left to right):

| | Assay Buffer | Matrix A1 | Standard | Sample* |
|---|---|---|---|---|
| Standard Wells | --- | 25 μL | 25 μL | --- |
| Sample wells | 25 μL | --- | --- | 25 μL |

*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 8).** Do not use excessive centrifugation

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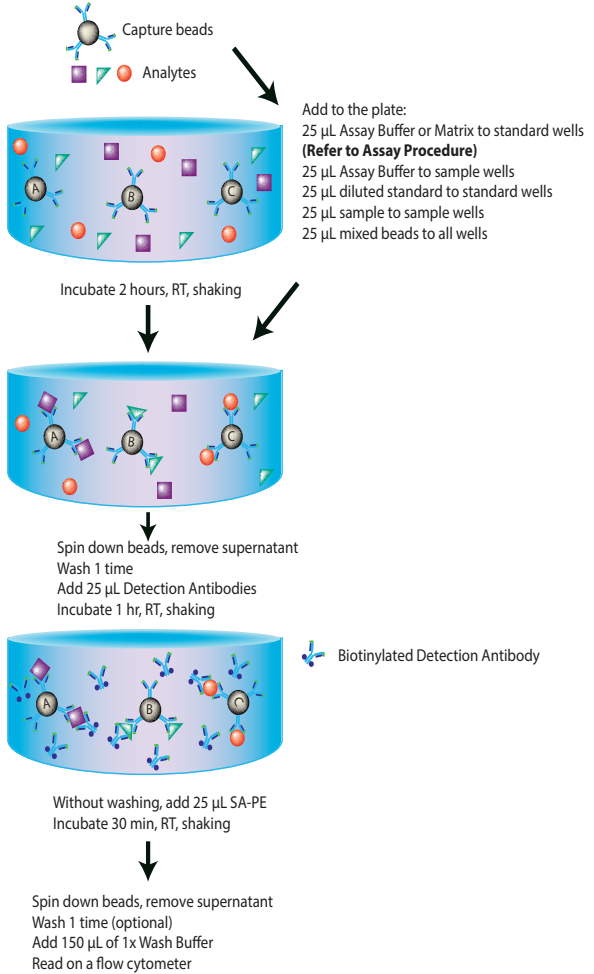
    Alternatively, removal of the supernatant may be completed using a multichannel pipette set at 75 µL. Try to remove as much liquid as possible without removing any beads. Be sure to change pipette tips between each row or column.

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

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

# Chapter 6: ASSAY CHARACTERIZATION

## Representative Standard Curve

This standard curve was generated using the LEGENDplex™ Human Inflammation Panel 2 for demonstration purposes only. A standard curve must be run with each assay.
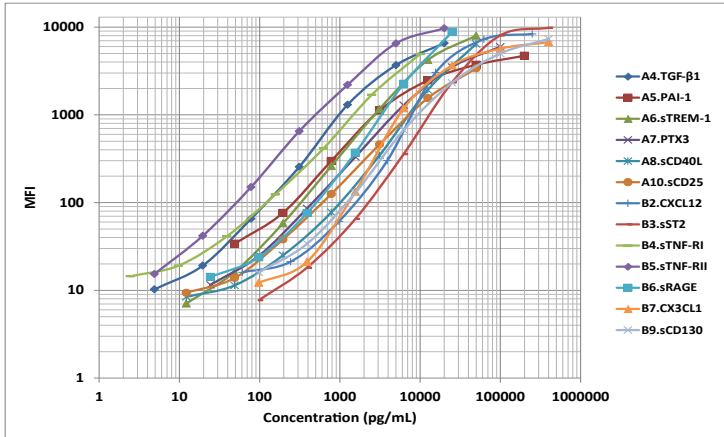


## Assay Sensitivity

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

| Analyte | Sensitivity in Serum (pg/mL) (N=12) | Sensitivity in Cell Culture Medium (pg/mL) (N=12) |
|---|---|---|
| Human Free Active TGF-β1 | 3.00 + 4.40 | 1.45 + 0.78 |
| Human PAI-1 | 18.64 + 25.00 | 11.8 + 17.43 |
| Human sTREM-1 | 5.71 + 5.23 | 3.35 + 2.59 |
| Human PTX3 | 9.00 + 12.06 | 4.87 + 4.06 |
| Human sCD40L | 5.57 + 5.79 | 3.75 + 2.20 |
| Human sCD25 (IL-2Ra) | 4.97 + 3.83 | 3.51 + 2.24 |
| Human CXCL12 (SDF-1) | 31.21 + 30.94 | 32.17 + 21.50 |
| Human sST2 | 53.69 + 34.65 | 24.20 + 27.51 |
| Human sTNF-RI | 1.19 + 1.25 | 0.96 + 0.63 |

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| | | |
|---|---|---|
| Human sTNF-RII | 1.75 + 2.24 | 1.19 + 0.5 |
| Human sRAGE | 9.58 + 14.31 | 5.18 + 3.31 |
| Human CX3CL1 (Fractalkine) | 49.92 + 39.85 | 46.34 + 20.93 |
| Human sCD130 (gp130) | 34.67 + 35.60 | 23.17 + 20.37 |

## Cross-Reactivity

Crossreactivity was tested in the panel using the LEGENDplex™ Human Inflammation Panel 2. The following recombinant proteins were tested at 50 ng/mL concentration. Human CXCL12 (SDF-1) assay detects both Human SDF-1α and Human SDF-1β and cross-reacts with Mouse SDF-1α and Mouse SDF-1β. No or negligible cross-reactivity was found for all the other tested analytes.

| | | | | | |
|---|---|---|---|---|---|
| hBDNF | hβ-NGF | hCCL17 (TARC) | hCCL2 (MCP-1) | hCD255 (TWEAK) | hCX3CL1 |
| hCXCL1 (GRO-α) | hCXCL10 (IP-10) | hCXCL11 (ITAC) | hCXCL12 (SDF-1α) | hCXCL12 (SDF-1β) | hGDNF |
| hGITRL (TNFSF18) | hHMGB-1 | hIL-2 | hIL-33 | hIL-6 | hPAI-1 |
| hPTX3 | hsCD130 (gp130) | hsCD25 (IL-2Ra) | hsCD40L (CD154) | hsRAGE | hsST2 (IL-1RL1) |
| hsTNF-RI (CD120a) | hsTNF-RII (CD120b) | hs-TREM-1 | hTGF-α | hTGF-β1 | hTGF-β2 |
| hTGF-β3 | hTSG-6 | hTNF-α | hTREM-2 | hTSLP | hVEGF-165 |
| mCD25 (IL-2Ra) | mCX3CL1 | mCXCL12 (SDF-1α) | mCXCL12 (SDF-1β) | rCX3CL1 | |

## Accuracy (Spike Recovery)

For spike recovery in serum and plasma, target proteins with known concentrations were spiked into human 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) |
|---|---|---|
| Human Free Active TGF-β1 | 73% | 76% |
| Human PAI-1 | 40% | 45% |
| Human sTREM-1 | 101% | 99% |
| Human PTX3 | 113% | 114% |

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| | | |
|---|---|---|
| Human sCD40L | 126% | 96% |
| Human sCD25 (IL-2Ra) | 92% | 89% |
| Human CXCL12 (SDF-1) | 146% | 60% |
| Human sST2 | 61% | 52% |
| Human sTNF-RI | 70% | 103% |
| Human sTNF-RII | 78% | 92% |
| Human sRAGE | 110% | 115% |
| Human CX3CL1 (Fractalkine) | 82% | 95% |
| Human sCD130 (gp130) | 59% | 51% |

## 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 A1 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 = 10) | Linearity of Dilution (Plasma) (N = 10) |
|---|---|---|
| Human Free Active TGF-β1 | 198% | 112% |
| Human PAI-1 | 120% | 142% |
| Human sTREM-1 | 121% | 101% |
| Human PTX3 | 101% | 81% |
| Human sCD40L | 105% | 112% |
| Human sCD25 (IL-2Ra) | 127% | 119% |
| Human CXCL12 (SDF-1) | 153% | 206% |
| Human sST2 | 84% | 177% |
| Human sTNF-RI | 99% | 79% |
| Human sTNF-RII | 153% | 109% |
| Human sRAGE | 112% | 89% |
| Human CX3CL1 (Fractalkine) | 138% | 112% |
| Human sCD130 (gp130) | 151% | 119% |

## Intra-Assay Precision

Two samples with different concentrations of target proteins were analyzed in one assay with 16 replicates for each sample. The intra-assay precision was calculated as below.

| Analyte | Sample | Mean (pg/mL) | STDEV | %CV |
|---|---|---|---|---|
| Human Free Active TGF-β1 | Sample 1 | 105.68 | 4.55 | 4% |
| | Sample 2 | 415.15 | 12.48 | 3% |
| Human PAI-1 | Sample 1 | 711.13 | 40.37 | 6% |
| | Sample 2 | 2346.57 | 82.63 | 4% |
| Human sTREM-1 | Sample 1 | 233.44 | 15.56 | 7% |
| | Sample 2 | 794.63 | 38.17 | 5% |
| Human PTX3 | Sample 1 | 486.57 | 16.51 | 3% |
| | Sample 2 | 1481.54 | 44.99 | 3% |
| Human sCD40L | Sample 1 | 310.28 | 17.61 | 6% |
| | Sample 2 | 688.48 | 26.20 | 4% |
| Human sCD25 (IL-2Ra) | Sample 1 | 296.59 | 19.36 | 7% |
| | Sample 2 | 977.36 | 37.09 | 4% |
| Human CXCL12 (SDF-1) | Sample 1 | 1944.69 | 96.96 | 5% |
| | Sample 2 | 6378.50 | 203.77 | 3% |
| Human sST2 | Sample 1 | 1833.76 | 83.73 | 5% |
| | Sample 2 | 5576.06 | 338.75 | 6% |
| Human sTNF-RI | Sample 1 | 47.73 | 2.46 | 5% |
| | Sample 2 | 163.85 | 5.52 | 3% |
| Human sTNF-RII | Sample 1 | 100.12 | 4.60 | 5% |
| | Sample 2 | 392.49 | 12.83 | 3% |
| Human sRAGE | Sample 1 | 551.80 | 18.48 | 3% |
| | Sample 2 | 1072.54 | 33.29 | 3% |
| Human CX3CL1 (Fractalkine) | Sample 1 | 1225.94 | 29.30 | 2% |
| | Sample 2 | 4465.08 | 85.64 | 2% |
| Human sCD130 (gp130) | Sample 1 | 1331.39 | 72.14 | 5% |
| | Sample 2 | 4717.66 | 184.73 | 4% |

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## 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 |
|---|---|---|---|---|
| Human Free Active TGF-β1 | Sample 1 | 95.84 | 11.15 | 12% |
| | Sample 2 | 397.37 | 24.62 | 6% |
| Human PAI-1 | Sample 1 | 604.49 | 89.83 | 15% |
| | Sample 2 | 2484.58 | 84.13 | 3% |
| Human sTREM-1 | Sample 1 | 216.70 | 27.57 | 13% |
| | Sample 2 | 814.98 | 51.63 | 6% |
| Human PTX3 | Sample 1 | 413.66 | 66.97 | 16% |
| | Sample 2 | 1632.27 | 163.34 | 10% |
| Human sCD40L | Sample 1 | 92.44 | 12.81 | 14% |
| | Sample 2 | 419.53 | 43.08 | 10% |
| Human sCD25 (IL-2Ra) | Sample 1 | 194.60 | 18.80 | 10% |
| | Sample 2 | 737.03 | 23.46 | 3% |
| Human CXCL12 (SDF-1) | Sample 1 | 1270.30 | 274.13 | 22% |
| | Sample 2 | 4955.19 | 605.05 | 12% |
| Human sST2 | Sample 1 | 1708.64 | 257.88 | 15% |
| | Sample 2 | 5402.63 | 450.76 | 8% |
| Human sTNF-RI | Sample 1 | 42.17 | 8.04 | 19% |
| | Sample 2 | 156.53 | 8.17 | 5% |
| Human sTNF-RII | Sample 1 | 95.79 | 9.70 | 10% |
| | Sample 2 | 394.04 | 14.31 | 4% |
| Human sRAGE | Sample 1 | 362.78 | 75.03 | 21% |
| | Sample 2 | 1223.59 | 221.23 | 18% |
| Human CX3CL1 (Fractalkine) | Sample 1 | 1023.66 | 109.06 | 11% |
| | Sample 2 | 4038.74 | 592.21 | 15% |
| Human sCD130 (gp130) | Sample 1 | 1072.59 | 182.83 | 17% |
| | Sample 2 | 4621.18 | 162.01 | 4% |

## Biological Samples

### Serum and Plasma  (Samples are paired)

Normal human serum samples (n=20) were tested for endogenous levels using the LEGENDplex™ Human Inflammation Panel 2. The concentrations measured are shown below.

| Analyte | Range (pg/mL) | % of Detectable | Median (pg/mL) |
|---|---|---|---|
| Human Free Active TGF-β1 | 147.91 - 755.81 | 100% | 357.71 |
| Human PAI-1 | 20844.73 - 70261.01 | 100% | 40786.09 |
| Human sTREM-1 | 13.72 - 405.50 | 100% | 23.52 |
| Human PTX3 | 1481.51 - 13191.53 | 100% | 3649.58 |
| Human sCD40L | 96.32 - 64726.71 | 100% | 11674.88 |
| Human sCD25 (IL-2Ra) | 697.11 - 8147.02 | 100% | 2924.17 |
| Human CXCL12 (SDF-1) | 429.68 - 13133.67 | 100% | 1491.51 |
| Human sST2 | 144.96 - 1238.30 | 100% | 257.73 |
| Human sTNF-RI | 1047.00 - 7580.09 | 100% | 2315.39 |
| Human sTNF-RII | 92.32 - 1583.42 | 100% | 178.37 |
| Human sRAGE | 28.62 - 979.42 | 100% | 559.42 |
| Human CX3CL1 (Fractalkine) | 166.04 - 3929.54 | 100% | 365.73 |
| Human sCD130 (gp130) | 26523.51 - 273477.49 | 100% | 128545.11 |

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Normal human plasma samples (n=20) were tested for endogenous levels using the LEGENDplex™ Human Inflammation Panel 2. The concentrations measured are shown below.

| Analyte | Range (pg/mL) | % of Detectable | Median (pg/mL) |
|---|---|---|---|
| Human Free Active TGF-β1 | 5.62 - 99.22 | 70% | 32.75 |
| Human PAI-1 | 13040.63 - 37241.00 | 100% | 24865.20 |
| Human sTREM-1 | 12.56 - 767.29 | 100% | 26.17 |
| Human PTX3 | 1391.34 - 13074.98 | 100% | 3467.38 |
| Human sCD40L | 52.92 - 11803.88 | 100% | 2229.96 |
| Human sCD25 (IL-2Ra) | 56.76 - 6420.98 | 100% | 1969.55 |
| Human CXCL12 (SDF-1) | 60.60 - 1457.14 | 85% | 509.59 |
| Human sST2 | 134.87 - 1886.17 | 100% | 349.01 |
| Human sTNF-RI | 1172.55 - 4535.55 | 100% | 2309.55 |
| Human sTNF-RII | 89.24 - 845.22 | 100% | 226.72 |
| Human sRAGE | 9.78 - 4238.15 | 100% | 559.58 |
| Human CX3CL1 (Fractalkine) | 217.83 - 5103.02 | 100% | 383.54 |
| Human sCD130 (gp130) | 3366.63 - 193432.42 | 100% | 89500.05 |

**Rheumatoid Arthritis Patient Samples**

Rheumatoid arthritis patient serum samples (n=14) were purchased from a commercial source and tested for endogenous levels using LEGENDplex™ Human Inflammation Panel 2. The concentrations measured are shown below.

| Analyte | Range (pg/mL) | % of Detectable | Median (pg/mL) |
|---|---|---|---|
| Human Free Active TGF-β1 | ND - 194.27 | 50% | 105.16 |
| Human PAI-1 | 28259.36 - 80733.81 | 100% | 39157.96 |
| Human sTREM-1 | 13.72 - 692.68 | 100% | 31.86 |
| Human PTX3 | 1484.74 - 9706.58 | 100% | 4475.85 |
| Human sCD40L | 1068.80 - 24798.94 | 100% | 7365.60 |
| Human sCD25 (IL-2Ra) | 1617.73 - 78371.4 | 100% | 9729.11 |
| Human CXCL12 (SDF-1) | 910.97 - 5910.69 | 100% | 2512.86 |
| Human sST2 | 104.37 - 1276.14 | 100% | 306.12 |
| Human sTNF-RI | 1654.49 - 14442.12 | 100% | 2906.47 |
| Human sTNF-RII | 71.56 - 6520.72 | 100% | 585.11 |
| Human sRAGE | 27.21 - 1929.58 | 100% | 525.41 |
| Human CX3CL1 (Fractalkine) | 154.45 - 6061.49 | 100% | 218.38 |
| Human sCD130 (gp130) | 15926.52 - 294866.33 | 100% | 140797.06 |

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Rheumatoid arthritis patient synovial fluid samples (n=7) were purchased from a commercial source and tested for endogenous levels using LEGENDplex™ Human Inflammation Panel 2. The concentrations measured are shown below.

| Analyte | Range (pg/mL) | % of Detectable | Median (pg/mL) |
|---|---|---|---|
| Human Free Active TGF-β1 | ND | 0% | N/A |
| Human PAI-1 | 5052.90 - 62116.39 | 100% | 54848.95 |
| Human sTREM-1 | 61.15 - 3737.75 | 100% | 2879.97 |
| Human PTX3 | 239.74 - 1154.32 | 100% | 341.65 |
| Human sCD40L | 10.30 - 697.39 | 100% | 42.63 |
| Human sCD25 (IL-2Ra) | 944.39 - 6709.25 | 100% | 5144.01 |
| Human CXCL12 (SDF-1) | ND - 922.17 | 71% | 102.69 |
| Human sST2 | ND - 147.04 | 57% | 137.71 |
| Human sTNF-RI | 3911.50 - 15717.67 | 100% | 11802.03 |
| Human sTNF-RII | 232.95 - 2260.83 | 100% | 2147.90 |
| Human sRAGE | 20.06 - 233.92 | 100% | 91.31 |
| Human CX3CL1 (Fractalkine) | 132.42 - 881.12 | 100% | 175.01 |
| Human sCD130 (gp130) | 29094.12 - 89934.06 | 100% | 80272.41 |

## Congestive Heart Failure Patient Samples

Congestive Heart Failure patient plasma samples (n=10) were purchased from a commercial source and tested for endogenous levels using LEGENDplex™ Human Inflammation Panel 2. The concentrations measured are shown below.

| Analyte | Range (pg/mL) | % of Detectable | Median (pg/mL) |
|---|---|---|---|
| Human Free Active TGF-β1 | ND | 0% | N/A |
| Human PAI-1 | 12509.74 - 40711.04 | 100% | 16584.95 |
| Human sTREM-1 | 13.88 - 88.78 | 100% | 33.80 |
| Human PTX3 | 4509.48 - 39528.28 | 100% | 7362.55 |
| Human sCD40L | 218.81 - 43840.64 | 100% | 870.95 |
| Human sCD25 (IL-2Ra) | 1392.65 - 11034.68 | 100% | 5269.10 |
| Human CXCL12 (SDF-1) | 861.65 - 2433.39 | 100% | 1373.00 |
| Human sST2 | 779.37 - 6289.40 | 100% | 2087.52 |
| Human sTNF-RI | 1486.42 - 9728.54 | 100% | 4493.44 |
| Human sTNF-RII | 214.48 - 1046.94 | 100% | 388.76 |
| Human sRAGE | ND - 2264.19 | 90% | 246.38 |
| Human CX3CL1 (Fractalkine) | 161.05 - 485.04 | 100% | 273.79 |
| Human sCD130 (gp130) | 98598.66 - 365199.39 | 100% | 121874.03 |

## Cell Culture Supernatant

Human PBMCs (1 x 10$^6$ cells/mL) were cultured unstimulated and stimulated. The stimulation conditions were PMA (20 ng/mL) and Ionomycin (500 ng/mL); and CD3 (1 µg/mL; plate coated) and CD28 (1 µg/mL; soluble). Supernatants were collected after 24 hours of culturing and assayed with LEGENDplex™ Human Inflammation Panel 2. The results (all in pg/mL) are summarized below.

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| Analyte | PBMC Control | PBMC + PMA + Ionomycin | PBMC + CD3 + CD28 |
|---|---|---|---|
| Human Free Active TGF-β1 | 76.41 | 114.48 | 123.47 |
| Human PAI-1 | 5836.27 | 8729.38 | 8285.76 |
| Human sTREM-1 | 14.52 | 9.12 | 50.78 |
| Human PTX3 | 32.39 | 29.46 | 27.49 |
| Human sCD40L | 307.94 | 2053.22 | 4389.46 |
| Human sCD25 (IL-2Ra) | 338.23 | 39603.73 | 105894.18 |
| Human CXCL12 (SDF-1) | 122.74 | 202.32 | 395.21 |
| Human sST2 | 56.63 | 59.67 | 95.10 |
| Human sTNF-RI | 100.43 | 252.20 | 391.70 |
| Human sTNF-RII | 84.02 | 105.10 | 358.63 |
| Human sRAGE | 9.78 | 8.76 | 20.90 |
| Human CX3CL1 (Fractalkine) | 82.40 | 69.05 | 77.99 |
| Human sCD130 (gp130) | 380.78 | 892.18 | 876.20 |

Human umbilical vein endothelial cells (HUVECs) were cultured unstimulated and stimulated (1 µg/mL LPS; and 50 ng/mL IFN-γ with 200 ng/mL TNF-α) for 24 hours. Supernatants were collected after 24 hours of culturing and assayed with LEGENDplex™ Human Inflammation Panel 2. The results (all in pg/mL) are summarized below.

| Analyte | HUVEC Control | HUVEC + LPS | HUVEC + IFN-γ + TNF-α |
|---|---|---|---|
| Human Free Active TGF-β1 | 69.18 | 48.55 | 18.11 |
| Human PAI-1 | 52849.54 | 64610.70 | 215187.20 |
| Human sTREM-1 | 3.62 | 3.77 | 4.96 |
| Human PTX3 | 5482.52 | 7577.79 | 11690.70 |
| Human sCD40L | 6.89 | 7.70 | 10.72 |
| Human sCD25 (IL-2Ra) | 6.40 | 7.43 | 10.88 |
| Human CXCL12 (SDF-1) | ND | 70.49 | 572.07 |
| Human sST2 | 566.46 | 589.51 | 653.19 |

| | | | |
|---|---|---|---|
| Human sTNF-RI | 450.11 | 491.37 | 217.92 |
| Human sTNF-RII | 18.39 | 16.80 | 34.82 |
| Human sRAGE | 21.05 | 19.96 | 51.17 |
| Human CX3CL1 (Fractalkine) | 158.83 | 150.52 | 5572.84 |
| Human sCD130 (gp130) | 1375.60 | 1613.17 | 3088.76 |

U937 cells were cultured unstimulated and stimulated (100 ng/mL LPS; 100 ng/mL LPS and 20 ng/mL IFN-γ; and 100 ng/mL LPS with 20 ng/mL IFN-γ and 20 ng/mL TNF-α) for 48 hours. Supernatants were collected after 48 hours of culturing and assayed with LEGENDplex™ Human Inflammation Panel 2. The results (all in pg/mL) are summarized below.

| Analyte | U937 Control | U937 + LPS | U937 + LPS + IFN-γ | U937 + LPS + IFN-γ + TNF-α |
|---|---|---|---|---|
| Human Free Active TGF-β1 | 31.05 | 29.92 | 8.58 | 5.73 |
| Human PAI-1 | 2387.76 | 7029.28 | 2942.31 | 4627.30 |
| Human sTREM-1 | 14.05 | 925.41 | 439.06 | 439.92 |
| Human PTX3 | 17.26 | 342.05 | 57.09 | 46.88 |
| Human sCD40L | 7.22 | 8.88 | 6.64 | 5.86 |
| Human sCD25 (IL-2Ra) | ND | 316.90 | 794.15 | 2354.45 |
| Human CXCL12 (SDF-1) | 91.34 | 107.02 | 133.31 | 138.92 |
| Human sST2 | ND | 57.63 | ND | ND |
| Human sTNF-RI | 436.04 | 543.76 | 492.21 | 332.52 |
| Human sTNF-RII | 221.00 | 1227.42 | 1075.70 | 1192.29 |
| Human sRAGE | 6.74 | 21.39 | 10.28 | 11.20 |
| Human CX3CL1 (Fractalkine) | 80.03 | 77.97 | 64.47 | 69.00 |
| Human sCD130 (gp130) | 33.80 | 51.58 | 54.71 | 43.90 |

## 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.<br><br>If some wells are still clogged during washing, try the following:<br><br>1). Add buffer to all the wells, pipette up and down the clogged wells and vacuum again.<br><br>2). Use a piece of clean wipe, wipe the under side of the clogged wells and vacuum again.<br><br>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. |

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## PLATE MAP (for in-plate assay)

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

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