Enabling Legendary Discovery™

LEGENDplex™

Multi-Analyte Flow Assay Kit

Human Vascular Inflammation Panel 1
Mix and Match Subpanel

Please read the entire manual before running the assay.

BioLegend.com

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

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Tel: 858-768-5800

Chapter 1: KIT DESCRIPTION

Introduction

Alterations in the levels of vascular inflammation proteins have been associated with cardiovascular disease, cancer, rheumatoid arthritis, kidney function, bacterial or viral infection, and liver failure. Accurate quantification of proteinaceous biomarkers involved in these inflammatory and immune disorders is an area of ongoing biomedical research, and important to further understanding these disease states.

The Human Vascular Inflammation Panel 1 is a multiplex bead-based assay panel, using fluorescence–encoded beads suitable for use on various flow cytometers. This panel allows simultaneous quantification of 13 human proteins, including Myoglobin, Calprotectin (MRP8/14), Lipocalin A (NGAL), C-Reactive Protein (CRP), MMP-2, Osteopontin (OPN), Myeloperoxidase (MPO), Serum Amyloid A (SAA), IGFBP-4, ICAM-1 (CD54), VCAM-1 (CD106), MMP-9, and Cystatin C. This assay panel provides higher detection sensitivities and broader dynamic ranges than traditional ELISA methods. The panel has been validated for use with serum (S), plasma (P), urine (U) and tissue culture supernatants (TC).

The LEGENDplex™ Human Vascular Inflammation Panel 1 is designed to allow flexible customization within the panel. It can also be divided into sub-panels based on biological sample type and suggested dilution factor, listed below:

LEGENDplex™ Human Vascular Inflammation Panel 1-TC (13-plex)
LEGENDplex™ Human Vascular Inflammation Panel 1-S/P (12-plex)
LEGENDplex™ Human Vascular Inflammation Panel 1-CRP (1-plex)
LEGENDplex™ Human Vascular Inflammation Panel 1-U (10-plex)
LEGENDplex™ Human Vascular Inflammation Panel 1-U (3-plex)

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

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.

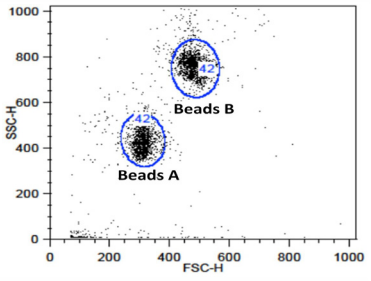
Since the beads are differentiated by size and internal fluorescence intensity on a flow cytometer, analyte-specific populations can be segregated and PE fluorescent signal quantified. The concentration of a particular analyte is determined using a standard curve generated in the same assay.

Beads Usage

The Human Vascular Inflammation Panel 1 uses two sets of beads. Each set has a unique size that can be identified based on their forward scatter (FSC) and side scatter (SSC) profiles (Beads A and Beads B, Figure 1). Each bead set can be further resolved based on their internal fluorescence intensities. The internal dye can be detected using FL3, FL4, or APC channel, depending on the type of flow cytometer used. The smaller Beads A consists of 6 bead populations and the larger Beads B consists of 7 bead populations (Figure 2-3).

Using a total of 13 bead populations distinguished by size and internal fluorescent dye, the Human Vascular Inflammation Panel 1 allows simultaneous detection of 13 proteins in a single sample. Each analyte is associated with a particular bead set as indicated (Figures 2-3 and Table 1).

Figure 1. Beads Differentiated by Size



Beads A = smaller beads

Beads B = larger beads

Figure 2. Beads A Classification by FL4

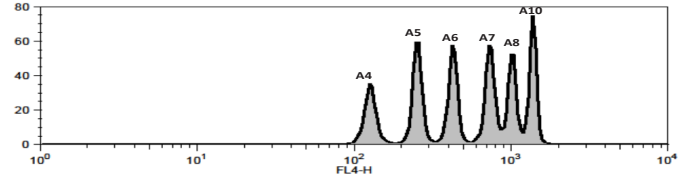
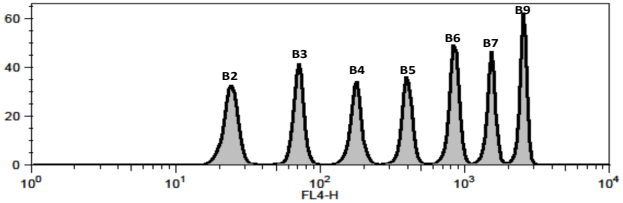


Figure 3. Beads B Classification by FL4



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

Table 1. Panel Targets and Bead ID*

Target	Bead ID	Vasc. Inflam. Panel 1-TC (13-plex) Cat. No. 740551 or 740552	Vasc. Inflam. Panel 1-S/P (12-plex) Cat. No. 740589 or 740590	Vasc. Inflam. Panel 1-CRP (1-plex) Cat. No. 740591 or 740592	Vasc. Inflam. Panel 1-U (10-plex) Cat. No. 740593 or 740594	Vasc. Inflam. Panel 1-U (3-plex) Cat. No. 740595 or 740596	Top Standard Concentrations
Myoglobin	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).
MRP8/14	A5	√	√			√	
NGAL	A6	√	√		√		
CRP	A7	√		√	√		
MMP-2	A8	√	√		√		
OPN	A10	√	√			√	
MPO	B2	√	√		√		
SAA	B3	√	√		√		
IGFBP-4	B4	√	√		√		
ICAM-1	B5	√	√		√		
VCAM-1	B6	√	√		√		
MMP-9	B7	√	√		√		
Cystatin C	B9	√	√			√	
Sample Type		Tissue Culture Supernatant	Serum, Plasma	Serum, Plasma	Urine	Urine	
Dilution Factor**		as desired	1:100	1:1,000	Neat	1:100	

*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.

**Dilution Factor provided is suggested. If further sample dilution is desired, dilution should be done based on sample type. See page 12 for details.

Storage Information

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

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

Materials Supplied

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

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**
Human Vascular Inflammation Panel 1 Detection Antibodies	1 bottle	3.3 mL	740553
Human Vascular Inflammation Panel 1 Standard	1 vial	lyophilized	740554
LEGENDplex™ Buffer Set G	1		740539
Filter Plate* or V-bottom Plate**	1 plate		740377* or 740379**

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

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Capture Beads for Mix and Match Subpanels*

Kit Components	Quantity	Volume	Cat. #
LEGENDplex™ Human Myoglobin Capture Bead A4, 13X	1 vial	270 µL	740555
LEGENDplex™ Human MRP8/14 Capture Bead A5, 13X	1 vial	270 µL	740556
LEGENDplex™ Human NGAL Capture Bead A6, 13X	1 vial	270 µL	740557
LEGENDplex™ Human CRP Capture Bead A7, 13X	1 vial	270 µL	740558
LEGENDplex™ Human MMP-2 Capture Bead A8, 13X	1 vial	270 µL	740559
LEGENDplex™ Human OPN Capture Bead A10, 13X	1 vial	270 µL	740560
LEGENDplex™ Human MPO Capture Bead B2, 13X	1 vial	270 µL	740561
LEGENDplex™ Human SAA Capture Bead B3, 13X	1 vial	270 µL	740562
LEGENDplex™ Human IGFBP-4 Capture Bead B4, 13X	1 vial	270 µL	740476
LEGENDplex™ Human ICAM-1 Capture Bead B5, 13X	1 vial	270 µL	740563
LEGENDplex™ Human VCAM-1 Capture Bead B6, 13X	1 vial	270 µL	740564
LEGENDplex™ Human MMP-9 Capture Bead B7, 13X	1 vial	270 µL	740565
LEGENDplex™ Human Cystatin C Capture Bead B9, 13X	1 vial	270 µL	740566

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

LEGENDplex™ Buffer Set G (Cat#: 740539)

Components	Quantity	Volume	Part #
Setup Beads: PE Beads	1 vial	1 mL	77842
Setup Beads: Raw Beads	1 vial	1.8 mL	77844
LEGENDplex™ SA-PE	1 bottle	3.3 mL	77743
LEGENDplex™ Assay Buffer	1 bottle	25 mL	77562
Lyophilized Standard Reconstitution Buffer	1 vial	1 mL	75241
LEGENDplex™ Wash Buffer, 20X	1 bottle	25 mL	77564
Plate Sealers	4 sheets		78101

No plate is included in Buffer Set G. Plate needs to be ordered separately. Please order the correct type of plate based on the preferred assay protocol (Cat# 740377 or 740378 for Filter Plate and Cat# 740379 for V-bottom Plate).

Materials to be Provided by the End-User

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

Partial list of compatible flow cytometers:

Flow Cytometer	Reporter Channel	Channel Emission	Classification Channel	Channel Emission	Compensation needed?
BD FACSCalibur™ (single laser)	FL2	575 nm	FL3	670 nm	Yes
BD FACSCalibur™ (dual laser)	FL2	575 nm	FL4	660 nm	No*
BD FACSArray™	Yellow	575 nm	Red	660 nm	No*
BD 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	575-585 nm	APC	660 nm	No*
BD FACSAria™	PE	575 nm	APC	660 nm	No*
Beckman Coulter-CytoFLEX	PE	585 nm	APC	660 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)

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 plates 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 plates can be ordered from BioLegend (Cat# 740379).

Precautions

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Center for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium azide has been added to some reagents as a preservative. Although the concentrations are low, sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up.
- Do not mix or substitute reagents from different kits or lots. Reagents from different manufacturers should not be used with this kit.
- Do not use this kit beyond its expiration date.
- SA-PE and beads are light-sensitive. Minimize light exposure.

Chapter 2: ASSAY PREPARATION

Sample Collection and Handling

Preparation of Serum Samples:

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

Preparation of Plasma Samples:

- Plasma collection using Heparin as an anti-coagulant 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 be thawed completely, mixed well and centrifuged to remove particulates.

Preparation of Urine Samples:

- Aseptically collect the first urine of the day (mid-stream) directly into a sterile container. Centrifuge to remove particulate matter.
- Remove urine supernatant and assay immediately, or aliquot and store samples at ≤-20°C. Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended that samples be thawed completely, mixed well and centrifuged to remove particulates.

Preparation of Tissue Culture Supernatant:

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

Reagent Preparation

Preparation of Antibody-Immobilized Beads

The individual beads (13X) should be mixed with each other and diluted to 1X final concentration with Assay Buffer prior to use. To mix the beads, follow the steps below (a 3-plex subpanel is used as an example):

1. Sonicate each bead vial for 1 minute in a sonicator bath and then vortex for 30 seconds to completely resuspend the beads.
2. Calculate the amount of mixed and diluted beads needed for the assay. Prepare extra to compensate for pipetting loss. Each reaction needs 25 µL of mixed and diluted beads. For 50 reactions, prepare 1.5 mL of mixed beads. For 100 reactions, prepare 3 mL of mixed beads.
3. To make 1.5 ml of 3-plex 1X diluted beads, transfer 115 µL of each of the 3 individual beads (13X) to a fresh tube (total bead volume = 345 µL) and add 1,155 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.

Standard Preparation

1. Prior to use, reconstitute the lyophilized Human Vascular Inflammation Panel 1 Standard with 250 µL Lyophilized Standard Reconstitution Buffer.
2. Mix and allow the vial to sit at room temperature for 10 minutes, and then transfer the standard to an appropriately labeled polypropylene microcentrifuge tube. This will be used as the top standard C7.

   Note: The top standard concentrations of analytes in this panel were set at various concentrations, but may be subject to change from lot to lot (please visit biolegend.com/en-us/legendplex to download a lot-specific certificate of analysis).
3. Label 6 polypropylene microcentrifuge tubes as C6, C5, C4, C3, C2 and C1, respectively.
4. Add 75 µL of Assay Buffer to each of the six tubes. Prepare 1:4 dilution of the top standard by transferring 25 µL of the top standard C7 to the C6 tube and mix well. This will be the C6 standard.
5. In the same manner, perform serial 1:4 dilutions to obtain C5, C4, C3, C2 and C1 standards (**see the table below using the top standard at 100 ng/mL as an example**). Assay Buffer will be used as the 0 ng/mL standard (C0).

Tube/Standard ID	Serial Dilution	Assay Buffer to add (µL)	Standard to add	Final Conc. (ng/mL)
C7	--	--	--	100
C6	1:4	75	25 µL of C7	25
C5	1:16	75	25 µL of C6	6.25
C4	1:64	75	25 µL of C5	1.563
C3	1:256	75	25 µL of C4	0.391
C2	1:1024	75	25 µL of C3	0.098
C1	1:4096	75	25 µL of C2	0.024
C0	--	75	--	0

Sample Dilution

- For tissue culture supernatant samples, the levels of analyte can vary greatly from sample to sample. To test tissue culture supernatant samples with the LEGENDplex™ Human Vascular Inflammation Panel 1-TC (13-plex) kit, a preliminary experiment may be required to determine the appropriate dilution factor. If further dilution is desired, dilution should be done with Assay Buffer to ensure accurate measurement.

- Serum or plasma samples must be diluted with Assay Buffer before being tested based on selected subpanel. For the LEGENDplex™ Human Vascular Inflammation Panel 1-S/P (12-plex), use a 1:100 dilution (e.g., 2 µL of sample in 198 µL of Assay Buffer). For the LEGENDplex™ Human Vascular Inflammation Panel 1-CRP (1-plex), use a 2-step dilution. First, prepare a 1:100 dilution of sample with 2 µL of sample in 198 µL of Assay Buffer, then prepare a 1:10 dilution of the 1:100 diluted sample (e.g., 10 µL 1:100 diluted sample in 90 µL of Assay Buffer). If further dilution is desired, dilution should be done with Assay Buffer to ensure accurate measurement.

- For urine samples, preparation is based on selected subpanel (e.g. no dilution for the LEGENDplex™ Human Vascular Inflammation Panel 1-U (10-plex), and 1:100 dilution with 2 µL of sample in 198 µL of Assay Buffer for the LEGENDplex™ Human Vascular Inflammation Panel 1-U (3-plex)). If further dilution is desired, dilution should be done with Assay Buffer to ensure accurate measurement.

Panel	Vasc. Inflam. Panel 1-TC (13-plex)	Vasc. Inflam. Panel 1-S/P (12-plex)	Vasc. Inflam. Panel 1-CRP (1-plex)	Vasc. Inflam. Panel 1-U (10-plex)	Vasc. Inflam. Panel 1-U (3-plex)
Cat. No.	740551 or 740552	740589 or 740590	740591 or 740592	740593 or 740594	740595 or 740596
Sample Type	TC	S, P	S, P	U	U
Dilution Factor	as desired	1:100	1:1000	Neat	1:100

Chapter 3: ASSAY PROCEDURE

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

- The Filter plate assay procedure requires a vacuum filtration unit for washing (see **Materials to be Provided by the End-User, page 7)**. If you have performed bead-based multiplex assays before, your lab may already have the vacuum filtration unit set up.

- If the Filter plate assay procedure is not possible or if you prefer, the assay

can be performed in a V-bottom plate.

Performing the Assay Using a Filter Plate

- Allow all reagents to warm to room temperature (20-25°C) before use.
- Set the filter plate on an inverted plate cover at all times during assay setup and incubation steps, so that the bottom of the plate does not touch any surface. Touching a surface may cause leakage.
- Keep the plate upright during the entire assay procedure, including the washing steps, to avoid losing beads.
- The plate should be placed in the dark or wrapped with aluminum foil for all incubation steps.
- **Standards and samples should be run in duplicate and arranged on the plate in a vertical configuration convenient for data acquisition and analysis (as shown in attached PLATE MAP, page 33). Be sure to load standards in the first two columns. If an automation device is used for reading, the orientation and reading sequence should be carefully planned.**

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

   For measuring samples, load the plate as shown in the table below (in the order from left to right):

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

   *See **Sample Dilution on page 12**

2. Vortex mixed beads 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 with a rubber band 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.

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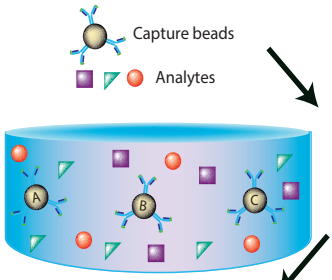
Assay Procedure Summary for Filter Plate

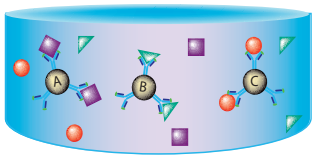
Add 100 µL 1X Wash Buffer to filter plate wells

Vacuum to remove excess buffer

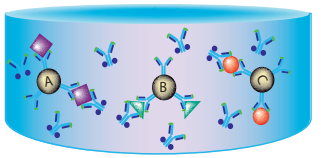


Add to the plate:
25 µL Assay Buffer to all wells
25 µL diluted standard to standard wells
or 25 µL sample to sample wells
25 µL pre-mixed beads to all wells

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

Performing the Assay Using a V-bottom Plate

- Allow all reagents to warm to room temperature (20-25°C) before use.
- Keep the plate upright during the entire assay procedure, including the washing steps, to avoid losing beads.
- The plate should be placed in the dark or wrapped with aluminum foil for all incubation steps.
- Standards and samples should be run in duplicate and arranged on the plate in a vertical configuration convenient for data acquisition and analysis (as shown in attached PLATE MAP, page 33). Be sure to load standards in the first two columns. If an automation device is used for reading, the orientation and reading sequence should be carefully planned.

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

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

*See **Sample Dilution on page 12**

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

3. Seal the plate with a plate sealer. Cover the entire plate with aluminum foil to protect the plate from light. Shake at 800 rpm on a plate shaker for 2 hours at room temperature **(Depending on the shaker, the speed may need to be adjusted. The optimal speed is one that is high enough to keep beads in suspension during incubation, but not too high 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 7).** Do not use excessive centrifugation speed as it may make it harder to resuspend beads in later steps. **Make sure the timer of the centrifuge works properly and standby to make sure the centrifuge reaches preset speed.**

5. Immediately after centrifugation, dump the supernatant into a 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.

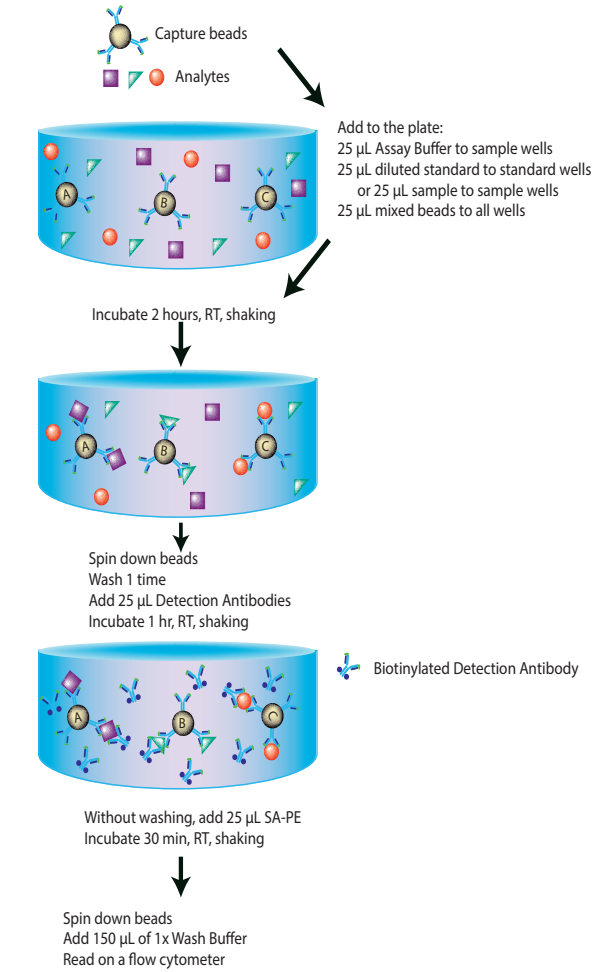
Alternatively, removal of the supernatant may be completed using a multichannel pipette set at 75 µL. Try to remove as much liquid as possible without removing any beads. Be sure to change pipette tips between each row or column.

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 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



Chapter 4: FLOW CYTOMETER SETUP

In order to generate reliable data, the flow cytometer must be set up properly before data acquisition.

The setup instructions have been removed from this manual and uploaded onto our website to save paper.

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

Chapter 5: DATA ACQUISITION AND ANALYSIS

Data Acquisition

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

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

3. Vortex each sample for 5 seconds before analysis.

4. Set the flow rate to low. Set the number of beads to be acquired to about 300 per analyte (e.g., acquire 2,400 beads for a 8-plex assay or 3,000 beads for a 13-plex assay). Do not set to acquire total events as samples may contain large amounts of debris. Instead, create a large gate to include both Beads A and Beads B (gate A+B) and set to acquire the number of events in gate A + B. This will exclude majority of the debris.

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

5. Read samples.

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

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

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

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

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

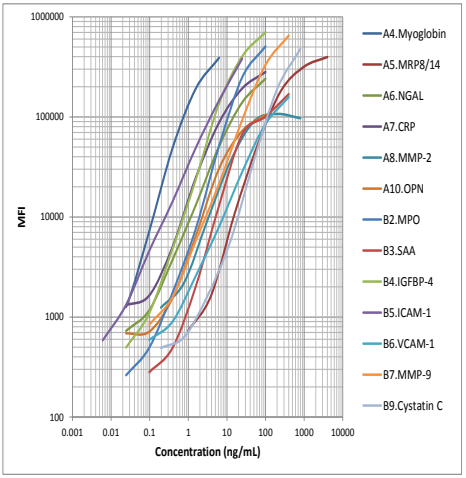
Data Analysis

- The 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 access 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 Vascular Inflammation Panel 1 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 using the 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)
Myoglobin	37.4
MRP8/14	2000.0
NGAL	32.8
CRP	45.7
MMP-2	273.0
OPN	104.0
MPO	63.2

SAA	52.5
IGFBP-4	12.2
ICAM-1	7.4
VCAM-1	55.7
MMP-9	120.0
Cystatin C	258.7

Cross-Reactivity

Target proteins were tested individually at indicated concentrations below using the LEGENDplex™ Human Vascular Inflammation Panel 1, with negligible cross-reactivity observed for non-intended targets.

Analyte	Conc. (ng/mL)	Analyte	Conc. (ng/mL)
Myoglobin	250	SAA**	4,000
MRP8/14	40,000	IGFBP-4	1,000
NGAL*	1,000	ICAM-1	250
CRP	1,000	VCAM-1	4,000
MMP-2	8,000	MMP-9*	4,000
OPN	1,000	Cystatin C	8,000
MPO	1,000		

*Refer to specificity of NGAL and MMP-9 on page 23

**SAA2 and SAA4 were both tested at 400 ng/mL, and were non-detectable.

Additional recombinant proteins were tested individually at 50 ng/mL. No or negligible cross-reactivity was found for the human, mouse, and rat proteins in the table below.

Human			Mouse		Rat
MMP-1	MMP-14	IGFBP-5	MMP-2	CCL1	IGFI
MMP-2	MMP-16	IGFBP-6	MMP-3	CCL2	MIF
MMP-3	TIMP-1	IGFBP-7	MMP-9	CCL7	
MMP-7	TIMP-2	IGF-1	NGAL	CCL9	
MMP-8	TIMP-3	IGF-II	IGFBP-1	CCL11	
MMP-9	TIMP-4	MRP8	IGFBP-2	CCL12	
MMP-10	IGFBP-1	MRP14	IGFBP-4	CCL17	
MMP-11	IGFBP-2	IL-21	IGFBP-5	CCL19	
MMP-12	IGFBP-3	IL-6	IGFBP-6	CCL5	
MMP-13	IGFBP-4	RANKL	IGFII	CD74	

The specificity of NGAL and MMP-9 was determined with the following human proteins in the table below.

Sample	Reactivity with NGAL	Reactivity with MMP-9
MMP-9 monomer	No significant reactivity	Strong reactivity
MMP-9 dimer	No significant reactivity	Strong reactivity
MMP-9-NGAL complex	Weak reactivity	Strong reactivity
MMP-9-NGAL TIMP-1 complex	Weak reactivity	Strong reactivity
NGAL free	Strong reactivity	No reactivity
MMP-9 free	No reactivity	Strong reactivity

Accuracy (Spike Recovery)

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

Analyte	% of Spike Recovery	
	Tissue Culture Supernatant	Urine
Myoglobin	88%	86%
MRP8/14	148%	N/A*
NGAL	85%	89%
CRP	59%	59%
MMP-2	68%	53%
OPN	73%	N/A*
MPO	85%	78%
SAA	61%	53%
IGFBP-4	89%	51%
ICAM-1	85%	79%
VCAM-1	83%	98%
MMP-9	142%	68%
Cystatin C	95%	N/A*

*N/A = Not applicable due to high endogenous level of the target in urine.

Linearity of Dilution

- Serum and plasma samples (n=5 each) were first diluted 100-fold and 1000-fold with Assay Buffer, then serially diluted 1:2, 1:4, 1:8 with Assay Buffer and assayed with the LEGENDplex™ Human Vascular Inflammation Panel 1-S/P (12-plex) kit and 1-CRP (1-plex) kit, respectively.

- Urine samples (n=2) were first diluted 32-fold with Assay Buffer, then serially diluted 1:2, 1:4 with Assay Buffer and assayed with the LEGENDplex™ Human Vascular Inflammation Panel 1-U (3-plex) kit.

- Urine samples (n=3) were spiked* with target proteins with known concentrations in the assay range, then serially diluted 1:2, 1:4, 1:8 with Assay Buffer and assayed with the LEGENDplex™ Human Vascular Inflammation Panel 1-U (10-plex) kit.

The measured concentrations of serially diluted samples were then compared with the concentration of the lowest dilution based on serial dilution factor used.

Analyte	Serum	Heparin Plasma	EDTA Plasma	Citrate Plasma	Urine
Myoglobin	83%	76%	73%	71%	95%*
MRP8/14	77%	75%	111%	85%	103%
NGAL	110%	105%	95%	96%	105%*
CRP	95%	130%	78%	143%	112%*
MMP-2	76%	81%	93%	73%	77%*
OPN	80%	74%	84%	80%	108%
MPO	84%	68%	121%	103%	101%*
SAA	79%	73%	92%	70%	124%*
IGFBP-4	79%	76%	69%	70%	95%*
ICAM-1	97%	93%	100%	94%	133%*
VCAM-1	63%	63%	50%	49%	120%*
MMP-9	133%	74%	88%	92%	114%*
Cystatin C	74%	68%	72%	92%	106%

*Data is based on results from spike linearity testing.

Intra-Assay Precision

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

Analyte	Sample	Mean (ng/mL)	STDEV	%CV
Myoglobin	Sample 1	0.1	0.01	7%
	Sample 2	0.4	0.04	9%
MRP8/14	Sample 1	18.7	0.97	5%
	Sample 2	67.0	6.69	10%
NGAL	Sample 1	0.5	0.03	7%
	Sample 2	1.7	0.17	10%
CRP	Sample 1	0.1	0.01	9%
	Sample 2	1.0	0.12	13%
MMP-2	Sample 1	2.2	0.16	8%
	Sample 2	10.3	1.00	10%
OPN	Sample 1	0.5	0.03	6%
	Sample 2	1.7	0.15	9%
MPO	Sample 1	0.5	0.04	8%
	Sample 2	1.8	0.18	10%
SAA	Sample 1	0.9	0.08	8%
	Sample 2	3.3	0.28	8%
IGFBP-4	Sample 1	0.4	0.03	9%
	Sample 2	1.4	0.17	11%
ICAM-1	Sample 1	0.1	0.01	7%
	Sample 2	0.5	0.05	11%
VCAM-1	Sample 1	1.8	0.15	8%
	Sample 2	6.9	0.76	11%
MMP-9	Sample 1	1.6	0.13	8%
	Sample 2	6.8	0.70	10%
Cystatin C	Sample 1	2.7	0.15	5%
	Sample 2	11.9	1.15	10%

Inter-Assay Precision

Two samples with different concentrations of each target protein were analyzed in four independent assays with 4 replicates per sample. The inter-assay precision is shown below.

Analyte	Sample	Mean (ng/mL)	STDEV	%CV
Myoglobin	Sample 1	0.1	0.01	10%
	Sample 2	0.5	0.03	7%
MRP8/14	Sample 1	18.7	2.04	11%
	Sample 2	80.4	9.18	11%
NGAL	Sample 1	0.5	0.05	11%
	Sample 2	2.1	0.19	9%
CRP	Sample 1	0.4	0.12	34%
	Sample 2	1.7	0.26	15%
MMP-2	Sample 1	2.6	0.28	11%
	Sample 2	14.5	2.30	16%
OPN	Sample 1	0.5	0.05	11%
	Sample 2	1.9	0.11	6%
MPO	Sample 1	0.5	0.09	18%
	Sample 2	2.0	0.24	12%
SAA	Sample 1	0.9	0.26	30%
	Sample 2	4.0	0.53	13%
IGFBP-4	Sample 1	0.4	0.12	27%
	Sample 2	1.9	0.34	18%
ICAM-1	Sample 1	0.1	0.01	11%
	Sample 2	0.5	0.07	14%
VCAM-1	Sample 1	1.8	0.19	11%
	Sample 2	7.6	0.89	12%
MMP-9	Sample 1	1.7	0.25	14%
	Sample 2	7.5	0.82	11%
Cystatin C	Sample 1	3.3	0.47	14%
	Sample 2	13.7	1.63	12%

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Biological Samples

Serum, Plasma, and Urine Samples

Normal human serum and plasma samples from 15 donors, and normal urine samples from 8 donors were tested for endogenous levels of the target proteins with corresponding panels and dilution factors (see table under **Sample Dilution on page 12**). The range and mean concentrations adjusted for dilution factor used are shown below (in ng/mL).

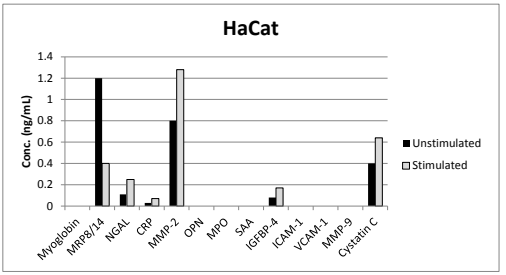
Analyte	Serum (n=15)	Heparin plasma (n=15)	EDTA plasma (n=15)	Citrate plasma (n=15)	Urine (n=8)
Myoglobin	18-3,851.5	17.5-1,763.5	17.5-1,439.5	12.5-999.5	0.01-0.28
	297.1	158.2	133.9	97.0	0.1
MRP8/14	1,280-53,680	1,160-15,120	280-1,960	360-1,960	2.4-6,694.4
	11,402.7	4,050.7	808.0	1,114.7	913.3
NGAL	138-841	75-282	48-105	42-145	0.4-22.8
	311.9	132.3	71.7	67.3	5.9
CRP	79-456	69-406	60-426	53-397	0.02-0.8
	279.3	278.9	272.8	246.4	0.2
MMP-2	72-176	72-264	32-128	56-128	0.02-0.3
	106.7	112.5	68.3	77.3	0.1
OPN	13,971.0	15-54	19-60	18,203.00	52.4-177.2
	13.7	29.5	30.5	21.2	95.4
MPO	26-225	7-67	4-20	3-42	0.02-0.41
	91.3	28.8	8.3	8.9	0.2
SAA	112-4,664	116-4,528	108-22,676	80-4,976	0.08-1.8
	1,105.7	994.0	2,374.7	1,002.9	0.8
IGFBP-4	147-276	146-257	170-339	119-199	0.02-2.8
	218.9	206.1	223.8	157.9	0.6
ICAM-1	0.75-2.25	0.75-4.25	0.75-2.5	0.5-1.75	0.003-0.7
	1.15	1.32	1.18	0.95	0.1
VCAM-1	384-640	388-672	356-632	228-532	0.04-1.9
	481.6	494.9	465.3	355.2	0.8
MMP-9	164-1,460	24-348	12-476	8-252	0.04-0.8
	737.1	170.4	149.3	80.8	0.3
Cystatin C	136-346	136-312	144-360	104-312	3.7-125.9
	239.5	219.7	217.6	177.6	39.7

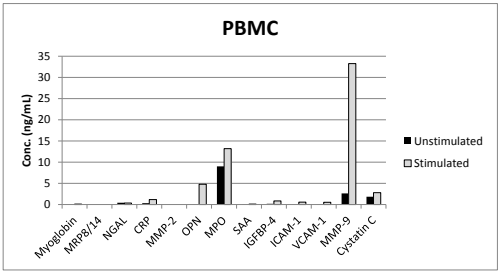
Tissue Culture Supernatant

Human PBMCs, HaCat, and K562 cells (1 x 10^6 cells/mL) were cultured under various stimulation conditions listed below, using unstimulated cells as a control. HaCat supernatants were collected 9 hours after stimulation, and PBMCs and K562 supernatants on day 3, and assayed with the LEGENDplex™ Human Vascular Inflammation Panel 1-TC kit (13-plex).

Human Cell Line	Stimulator	Concentration
HaCat	TNFα	100 ng/mL
	IL-8	100 ng/mL
	LPS	1 µg/mL
K562	$CoCl_2$	100 µM
PBMCs	CD3	1 µg/mL
	CD28	1 µg/mL
	IL-2	20 ng/mL

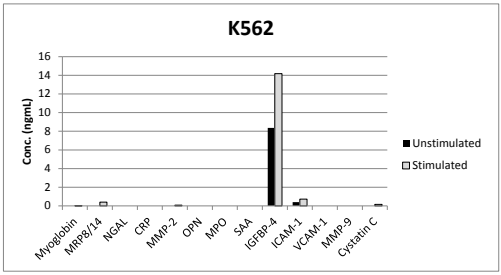
The results are summarized below.





Additional tissue culture supernatants were collected from different cell lines in the table below at the indicated timepoints. and tested with the LEGENDplex™ Human Vascular Inflammation Panel 1-TC kit (13-plex). Matched culture media was tested as a control with non-detectable (ND) levels (data not shown). The results of the tissue culture supernatants are summarized in ng/mL.

Analyte	THP-1	MDA-MB-231	A549	HUVEC	HepG2	MIA PaCa-2	HT29	DAUDI	NB4	U87-MG	NCCIT
	Day 3	Day 4	Day 3	Day 3	Day 4	Day 4	Day 3	Day 3	Day 4	Day 4	Day 3
Myoglobin	ND	0.35	ND	ND	ND	0.01	ND	ND	0.51	ND	ND
MRP8/14	96.80	ND	5.20	ND	ND	ND	0.40	1.20	22.00	ND	ND
NGAL	ND	2.03	0.02	ND	0.02	ND	5.02	ND	2.71	ND	ND
CRP	0.03	ND	0.08	0.03	0.04	ND	ND	ND	0.22	0.08	ND
MMP-2	2.00	0.16	ND	18.40	2.88	ND	ND	ND	12.00	ND	0.56
OPN	0.02	ND	0.33	ND	10.92	ND	ND	0.16	7.32	3.91	30.70
MPO	0.08	ND	ND	ND	ND	ND	ND	ND	22.42	ND	ND
SAA	ND	ND	ND	ND	ND	ND	ND	ND	3.00	ND	ND
IGFBP-4	5.61	126.77	1.36	0.10	13.77	5.18	10.80	ND	8.90	0.01	6.27
ICAM-1	0.01	0.05	ND	ND	0.22	ND	ND	ND	0.34	ND	ND
VCAM-1	ND	ND	ND	ND	ND	ND	ND	ND	0.40	ND	ND
MMP-9	86.96	ND	ND	ND	ND	ND	ND	ND	136.52	ND	ND
Cystatin C	4.24	3.12	ND	ND	68.40	ND	1.44	ND	3.36	ND	1.60



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.

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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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