BioLegend®

Enabling Legendary Discovery™

LEGENDplex™
Multi-Analyte Flow Assay Kit

Human Proinflammatory Chemokine Panel 2
Mix and Match Subpanel

Please read the entire manual before running the assay.

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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Human Proinflammatory Chemokine Panel 2 Mix and Match Subpanel

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Chapter 1:   KIT DESCRIPTION

Introduction

Chemotactic cytokines or chemokines play pivotal roles in various processes such as immune surveillance, organ development, angiogenesis, and immune responses. Expression profiling of chemokines, especially those involved in inflammation and immune disorders, is important in achieving a deeper understanding of disease states.

The Human Proinflammatory Chemokine Panel 2 is a multiplex bead-based assay panel, using fluorescence–encoded beads suitable for use on various flow cytometers. This panel allows simultaneous quantification of 12 human chemokines, including CCL1 (I-309), CCL13 (MCP-4), CCL22 (MDC), CCL7 (MCP-3), CCL24 (Eotaxin-2), CCL19 (ELC), CXCL13 (BLC), CCL8 (MCP-2), CX3CL1 (Fractalkine), CXCL12 (SDF-1), CXCL2(GRO-β) and CCL18 (PARC). This assay panel provides higher detection sensitivities and broader dynamic ranges than traditional ELISA methods. The panel has been validated by detecting expected changes in biological samples.

The LEGENDplex™ Human Proinflammatory Chemokine Panel 2 (12-plex) is designed to allow flexible customization within the panel. It can also be divided into subpanels. The table below shows the panel configuration and sample diltuion requirement.

Cat #	Plex Size	Targets	Sample Type	Dilution Factor
741157, 741158	12-plex	CCL1, CCL13, CCL22, CCL7, CCL24, CCL19, CXCL13, CCL8, CX3CL1, CXCL12, CXCL2, CCL18	Tissue Culture	Varies
741182, 741183	11-plex	CCL1, CCL13, CCL22, CCL7, CCL24, CCL19, CXCL13, CCL8, CX3CL1, CXCL12, CXCL2	Tissue Culture	Varies
			Serum/Plasma	2x
741184, 741185	1-plex	CCL18	Tissue Culture	Varies
			Serum Plasma	50x

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

Using a total of 12 bead populations distinguished by size and internal fluorescent dye, the Human Proinflammatory Chemokine Panel 2 allows simultaneous detection of 12 chemokines 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
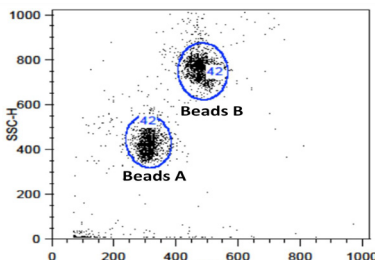
Beads A = smaller beads

Beads B = larger beads

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Figure 2. Beads A Classification by FL4
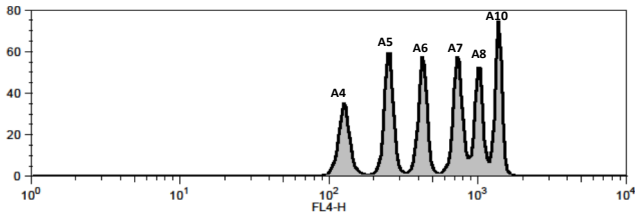
Figure 3. Beads B Classification by FL4
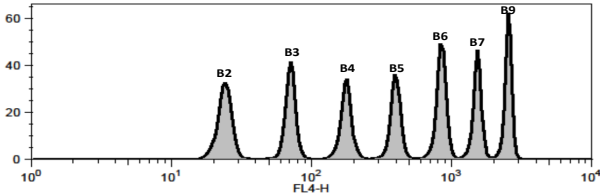
For Beads usage in the panel, please refer to Table 1 below:

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

Target	Bead ID	(12-Plex) No. 741157, 741158	(11-Plex) No. 741182, 741183	(1-Plex) No. 741184, 741185	Top Standard Concentration
CCL1	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).
CCL13	A5	√	√		
CCL22	A6	√	√		
CCL7	A7	√	√		
CCL24	A10	√	√		
CCL19	B2	√	√		
CXCL13	B3	√	√		
CCL8	B4	√	√		
CX3CL1	B5	√	√		
CXCL12	B6	√	√		
CXCL2	B7	√	√		
CCL18	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 and Matrix A 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™ HU Proinflam. Chemokine Panel 2 Detection Abs	1 bottle	3.5 mL	741159
LEGENDplex™ HU Proinflam. Chemokine Panel 2 Standard	1 vial	lyophilized	741160
LEGENDplex™ Buffer Set H	1		740620
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*:

Bead Name	Quantity	Volume	Cat#
LEGENDplex™ Human CCL1 (I-309) Capture Bead A4, 13X	1 vial	270 µL	741161
LEGENDplex™ Human CCL13 (MCP-4) Capture Bead A5, 13X	1 vial	270 µL	741162
LEGENDplex™ Human CCL22 (MDC) Capture Bead A6, 13X	1 vial	270 µL	741163
LEGENDplex™ Human CCL7 (MCP-3) Capture Bead A7, 13X	1 vial	270 µL	741164
LEGENDplex™ Human CCL24 (Eotaxin-2) Capture Bead A10, 13X	1 vial	270 µL	741165
LEGENDplex™ Human CCL19 (ELC) Capture Bead B2, 13X	1 vial	270 µL	741166
LEGENDplex™ Human CXCL13 (BLC) Capture Bead B3, 13X	1 vial	270 µL	741167
LEGENDplex™ Human CCL8 (MCP-2) Capture Bead B4, 13X	1 vial	270 µL	741168
LEGENDplex™ Human CX3CL1 (Fractalkine) Capture Bead B5, 13X	1 vial	270 µL	741169
LEGENDplex™ Human CXCL12 (SDF-1) Capture Bead B6, 13X	1 vial	270 µL	741170
LEGENDplex™ Human CXCL2 (GRO-β) Capture Bead B7, 13X	1 vial	270 µL	741171
LEGENDplex™ Human CCL18 (PARC) Capture Bead B9, 13X*	1 vial	270 µL	741172

*For serum or plasma samples, mixing and matching of CCL18 (PARC) with other targets is not recommended due to high dilution requirement. A single plex is recommended for measuring CCL18 (PARC) in serum or plasma samples

LEGENDplex™ Buffer Set H (Cat#: 740620)

Components	Quantity	Volume	Part #
Setup Beads 1: FITC Beads	1 vial	1 mL	77840
Setup Beads 2: PE Beads	1 vial	1 mL	77842
Setup Beads 3: Raw Beads	1 vial	2 mL	77844
LEGENDplex™ SA-PE	1 bottle	3.5 mL	77743
LEGENDplex™ Matrix A, Lyophilized	1 vial	lyophilized	75306
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 H. Plate need to be ordered separately. Please order the correct type of plate based on the preferred assay protocol (Cat# 740377 or 740378 for Filter Plate and Cat# 740379 for V-bottom Plate)

Materials to be Provided by the End-User

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

Partial list of compatible flow cytometers:

Flow Cytometer	Reporter Channel	Channel Emission	Classification Channel	Channel Emission	Compensation needed?
BD FACSCalibur™ (single laser)	FL2	575 nm	FL3	670 nm	Yes
BD FACSCalibur™ (dual laser)	FL2	575 nm	FL4	660 nm	No*
BD 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*
Gallios	PE	575 nm	APC	660 nm	No*
NovoCyte	PE	572 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)

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- 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 A for LEGENDplex™ kits contains components of animal origin and should be handled as potentially hazardous.

- Do not mix or substitute reagents from different kits or lots. Reagents from different manufacturers should not be used with this kit.

- Do not use this kit beyond its expiration date.

- SA-PE and Beads are light-sensitive. Minimize light exposure.

Chapter 2: ASSAY PREPARATION

Sample Collection and Handling

Preparation of Serum Samples:

- Allow the blood to clot for at least 30 minutes and centrifuge for 10 minutes at 1,000 x *g*.

- Remove serum and assay immediately or aliquot and store samples at ≤-20°C. Avoid multiple (>2) freeze/thaw cycles.

- When using frozen samples, it is recommended that samples are thawed completely, mixed and centrifuged to remove particulates prior to use.

Preparation of Plasma Samples:

- Centrifuge for 10 minutes at 1,000 x *g* within 30 minutes of blood collection.

- Remove plasma and assay immediately, or aliquot and store samples at ≤-20°C. Avoid multiple (>2) freeze/thaw cycles.

- When using frozen samples, it is recommended that samples are thawed completely, mixed well and centrifuged to remove particulates.

Preparation of Tissue Culture Supernatant:

- Centrifuge the sample to remove debris and assay immediately. If not 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 5-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 5-plex 1X diluted beads, transfer 115 µL of each of the 5 individual beads (13X) to a fresh tube (total bead volume = 575 µL) and add 925 µL of Assay Buffer to make the final volume of 1.5 mL.

Preparation of Wash Buffer

- Bring the 20X Wash Buffer to room temperature and mix to bring all salts into solution.
- Dilute 25 mL of 20X Wash Buffer with 475 mL deionized water. Store unused portions between 2°C and 8°C for up to one month.

Preparation of Matrix A (for Serum or Plasma Samples Only)

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

Standard Preparation

1. Prior to use, reconstitute the lyophilized Human Proinflammatory Chemokine Panel 2 Standard Cocktail with 250 µL 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 microfuge tube. This will be used as the top standard C7.

   Note: The top standard concentrations of analytes in this panel were set at various concentrations, but may be subject to change from lot to lot (please visit biolegend.com/en-us/legendplex to download a lot-specific certificate of analysis).

3. Label 6 polypropylene microfuge tubes as C6, C5, C4, C3, C2 and C1, respectively.

4. Add 100 µL of Assay Buffer to each of the six tubes. Prepare 1:3 dilution of the top standard by transferring 50 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 1:3dilutions to obtain C5, C4, C3, C2 and C1 standards (**see the table below using 10ng/mL of top standard 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	1:3	100	50 μL of C7	3333.3
C5	1:9	100	50 μL of C6	1111.1
C4	1:27	100	50 μL of C5	370.4
C3	1:81	100	50 μL of C4	123.5
C2	1:243	100	50 μL of C3	41.2
C1	1:729	100	50 μL of C2	13.7
C0	--	100	--	0

Sample Dilution

- For measuring serum or plasma samples for all targets except CCL18 (PARC) the samples need to be diluted 2-fold with Assay Buffer before being tested (e.g. dilute 50 µL of sample with 50 µL of Assay Buffer). If furthe sample dilution is needed, the samples should be diuted with Matrix A provided in the kit.

- For measuring serum or plasma samples uisng the 1-plex CCL18 (PARC) kit, a 50-fold dilution using Assay Buffer is recommended due to the high con-centration of CCL18 (PARC) in samples. If furthe sample dilution is needed, the samples should be diuted with Assay Buffer provided in the kit.

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

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

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

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Chapter 3:   ASSAY PROCEDURE

The LEGENDplex™ assay can be performed in a filter plate, 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 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 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 cell culture supernatant samples, load the plate as shown in the table below (in the order from left to right):

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

*Note: Fresh cell culture media is preferred instead of assay buffer in the standard wells if available.

For measuring serum/plasma samples for all targets except CCL18 (PARC), load the plate as shown in the table below (in the order from left to right):

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

For measuring serum/plasma samples for CCL18 (PARC), load the plate as shown in the table below (in the order from left to right):

	Assay Buffer	Matrix A	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 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

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shaker for 1 minute.

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

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

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

Assay Procedure Summary for Filter Plate

Add 100 μL 1X Wash Buffer to filter plate wells
Vacuum to remove excess buffer

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

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 cell culture supernatant samples,** load the plate as shown in the table below (in the order from left to right):

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

*Note: Fresh cell culture media is preferred instead of assay buffer in the standard wells if available.

For measuring serum/plasma samples for all targets except CCL18 (PARC), load the plate as shown in the table below (in the order from left to right):

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

For measuring serum/plasma samples for CCL18 (PARC), load the plate as shown in the table below (in the order from left to right):

	Assay Buffer	Matrix A	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

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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 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

Capture beads

Analytes

Add to the plate:
25 µL Assay Buffer or Matrix to standard well
(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 (e.g., acquire 2,400 beads for a 8-plex assay or 4000 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 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,

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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.
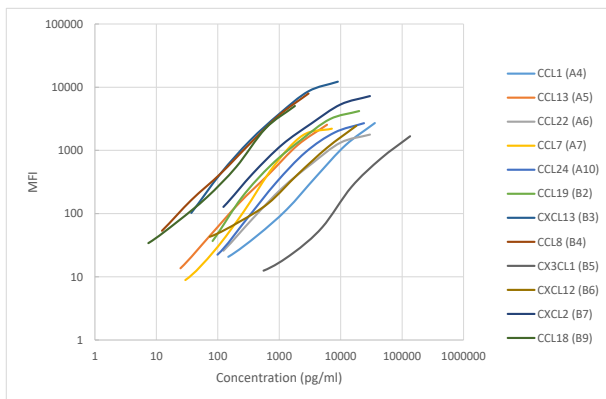
Data Analysis

• 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.**

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Chapter 6:   ASSAY CHARACTERIZATION

Representative Standard Curve

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

Assay Sensitivity

The lower limity of detection (LOD) 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 LOD + 2 STDEV.

Analyte	MDC in Cell Culture Medium (pg/mL)	MDC in Serum (pg/mL)
Human CCL1	20.2+26.8	54.8+91.1
Human CCL13	3.2+2.2	4.0+2.1
Human CCL22	7.6+5.9	13.1+13.1
Huamn CCL7	6.5+8.2	9.2+7.7
Human CCL24	13.0+18.5	27.7+28.2
Human CCL19	5.1+3.5	5.8+4.1
Human CXCL13	1.5+0.5	2.1+1.5
Human CCL8	0.7+0.5	0.7+0.65
Human CX3CL1	46.8+87.5	126.0+189.3
Human CXCL12	14.9+16.0	19.9+40.3

Human CXCL2	16.3+13.4	14.8+14.0
Human CCL18	0.5+0.6	0.6+0.6

Cross-Reactivity

The following human recombinant proteins were tested at 50 ng/mL using the LEGENDplex™ Human Proinflammatory Chemokine Panel 2. CXCL1 showed a crossreactivity of 2% for CX3CL1 No or negligible cross-reactivity was found between all other analytes.

CCL1	CCL2	CCL3	CCL4	CCL5	CCL7
CCL8	CCL11	CCL13	CCl17	CCL18	CCL19
CCL20	CCL22	CCL24	CXCL1	CXCL2	CXCL5
CXCL8	CXCL9	CXCL10	CXCL11	CXCL12	CXCL13
CX3CL1					

Accuracy (Spike Recovery)

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

For spike recovery in serum, a sample with known high concentrations of target proteins was spiked into unknown serum samples. The spiked samples were then assayed, and the measured concentrations were compared with expected values.

Analyte	% of Recovery in Cell Culture Medium	% of Recovery in Serum
Human CCL1	103.9%	51.3%
Human CCL13	107.0%	78.3%
Human CCL22	103.6%	155.9%
Human CCL7	100.9%	65.8%
Human CCL24	102.3%	50.0%
Human CCL19	104.7%	84.0%
Human CXCL13	102.7%	87.3%

Human CCL8	102.9%	105.4%
Human CX3CL1	101.7%	89.0%
Human CXCL12	101.1%	141.5%
Human CXCL2	101.2%	60.8%
Human CCL18	102.9%	135.5%

Linearity of Dilution

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

Analyte	Linearity of Dilution	Analyte	Linearity of Dilution
Human CCL1	124.2%	Human CXCL13	110.9%
Human CCL13	125.2%	Human CCL8	110.0%
Human CCL22	88.5%	Human CX3CL1	123.2%
Human CCL7	125.0%	Human CXCL12	87.0%
Human CCL24	143.2%	Human CXCL2	143.5%
Human CCL19	112.2%	Human CCL18	73.3%

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 CCL1	Sample 1	953.5	53.5	5.6%
	Sample 2	3153.3	254.0	8.1%
Human CCL13	Sample 1	175.6	16.7	9.5%
	Sample 2	504.2	29.8	5.9%
Human CCL22	Sample 1	792.6	72.2	9.1%
	Sample 2	2578.6	181.3	7.0%

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Human CCL7	Sample 1	200.6	17.3	8.6%
	Sample 2	637.9	49.6	7.8%
Human CCL24	Sample 1	651.9	26.1	4.0%
	Sample 2	2124.5	125.3	5.9%
Human CCL19	Sample 1	525.1	52.4	10.0%
	Sample 2	1953.2	188	9.6%
Human CXCL13	Sample 1	226.3	11.8	5.2%
	Sample 2	738.4	41.5	5.6%
Human CCL8	Sample 1	87.0	7.1	8.2
	Sample 2	267.1	15.6	5.8%
Human CX3CL1	Sample 1	3545.8	267.8	7.6%
	Sample 2	11072.1	458.5	4.1%
Human CXCL12	Sample 1	358.5	24.3	6.8%
	Sample 2	1322.1	58.7	4.4%
Human CXCL2	Sample 1	879.3	56.4	6.4%
	Sample 2	2786.4	154.5	5.5%
Human CCL18	Sample 1	47.5	4.0	8.4%
	Sample 2	152.2	9.7	6.4%

Inter-Assay Precision

Two samples with different concentrations of target proteins were analyzed in ten independent assays by five different people. The inter-assay precision was calculated as below.

Analyte	Sample	Mean (pg/mL)	STDEV	%CV
Human CCL1	Sample 1	1146.9	183.5	16.0%
	Sample 2	3548.5	387.6	10.9%
Human CCL13	Sample 1	206.2	23.8	11.5%
	Sample 2	572.3	53.4	9.3%
Human CCL22	Sample 1	937.4	120.3	12.8%
	Sample 2	2842.8	337.9	11.9%
Human CCL7	Sample 1	238.8	28.6	12.0%
	Sample 2	717.7	65.1	9.1%

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Human CCL24	Sample 1	783.7	89.9	11.5%
	Sample 2	2399.1	174.9	7.3%
Human CCL19	Sample 1	623.2	78.9	12.7%
	Sample 2	2086.7	258.4	12.4%
Human CXCL13	Sample 1	274.0	29.9	10.9%
	Sample 2	846.8	85.6	10.1%
Human CCL8	Sample 1	104.1	11.3	10.9%
	Sample 2	209.7	25.6	8.8%
Human CX3CL1	Sample 1	4401.5	558.8	12.7%
	Sample 2	13263.4	1167.8	8.8%
Human CXCL12	Sample 1	482.7	70.7	14.9%
	Sample 2	1630.8	151.2	9.6%
Human CXCL2	Sample 1	1021.2	119.3	11.7%
	Sample 2	2943.4	192.6	6.5%
Human CCL18	Sample 1	57.8	7.7	13.2%
	Sample 2	172.0	18.2	10.6%

Biological Samples

Serum and Plasma

Normal human serum samples (n=20) were tested for endogenous levels of the chemokines. The concentrations measured are shown below:

Analyte	Range (pg/ml)	No. of Detectable	% of Detectable	Mean (pg/mL)
Human CCL1	ND-398	14	70%	216
Human CCL13	42-113	20	100%	80
Human CCL22	789-1904	20	100%	1266
Human CCL7	ND-43	12	60%	21
Human CCL24	29-186	20	100%	80
Human CCL19	21-77	20	100%	46
Human CXCL13	19-41	20	100%	30
Human CCL8	10-22	20	100%	16
Human CX3CL1	74-1397	12	60%	381
Human CXCL12	168-2245	20	100%	653
Human CXCL2	53-1186	20	100%	388

Human I-TAC	2539-3736	20	100%	3096

ND = Non-detectable

Normal human plasma samples (n=40) were tested for endogenous levels of chemokines. The concentrations measured are shown below:

Analyte	Range (pg/mL)	No. of Detectable	% of Detectable	Mean (pg/mL)
Human CCL1	ND-1466	16	40%	224
Human CCL13	8-380	40	100%	34
Human CCL22	207-1020	40	100%	481
Human CCL7	ND-137	24	60%	21
Human CCL24	17-157	32	80%	53
Human CCL19	10-100	40	100%	33
Human CXCL13	7-84	40	100%	26
Human CCL8	2-30	40	100%	8
Human CX3CL1	ND-5229	16	40%	944
Human CXCL12	ND-2256	32	80%	439
Human CXCL2	25-619	40	100%	145
Human CCL18	379-2449	40	100%	1628

ND = Non-detectable

Cell Culture Supernatant

Human PBMC (1 x 10^6 cells/mL) were cultured under various conditions (LPS, 1 ug/mL; LPS,1 ug/mL; IFN-y, 100 ng/mL; CD3, 1 µg/mL plate-coated; CD28, 1 µg/mL soluble; R848, 2 ug/ml; PMA, 20 ng/ml; PMA, 20ng/ml, Ionomycin, 500 ng/ml; Poly I:C 50 ug/ml). Supernatants were collected after 3 days and assayed with the LEGENDplex™ Human Proinflammatory Chemokine Panel 2 kit. The results (all in pg/mL) are summarized below.

Analyte	Control	LPS	LPS + IFN-y	CD3 + CD28	R848
Human CCL1	ND	25	ND	1739	ND
Human CCL13	72	8	7	803	10
Human CCL22	1614	5732	1005	>340622	1454
Human CCL7	7	686	886	996	>16339

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Human CCL24	98	46	29	14371	31
Human CCL19	18	319	414	30	740
Human CXCL13	24	1906	200	817	3069
Human CCL8	96	101	5646	6604	7756
Human CX3CL1	80	996	932	1353	1089
Human CXCL12	17	13	32	38	37
Human CXCL2	61	16816	2598	1847	3088
Human CCL18	252	851	153	3247	952

Analyte	PHA	PMA + Iono	PMA	Poly I:C
Human CCL1	23	ND	1393	132
Human CCL13	77	13	10	139
Human CCL22	43901	>340622	>340622	32354
Human CCL7	715	361	4073	4621
Human CCL24	292	2445	1473	39
Human CCL19	67	17	<17	730
Human CXCL13	3527	14	379	241
Human CCL8	5691	478	900	7124
Human CX3CL1	1112	834	504	273
Human CXCL12	32	13	14	29
Human CXCL2	3951	3414	11624	101
Human CCL18	1673	109	27	175

TROUBLESHOOTING

Problem	Possible Cause	Solution
Bead population shifting upward or downward during acquisition	The strong PE signal from high concentration samples or standards may spill over to classification Channel (e.g., FL3/FL4/APC) and mess up the bead separation.	Optimize instrument settings using Kit Setup Beads, and make appropriate compensation between channels.
Filter plate will not vacuum or some wells clogged	Vacuum pressure is insufficient or vacuum manifold does not seal properly.	Increase vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds. Clean the vacuum manifold and make sure no debris on the manifold. Press down the plate on the manifold to make a good seal.
	Samples have insoluble particles or sample is too viscous (e.g., serum and plasma samples)	Centrifuge samples just prior to assay setup and use supernatant. If high lipid content is present, remove lipid layer after centrifugation. Sample may need dilution if too viscous. If some wells are still clogged during washing, try the following: 1). Add buffer to all the wells, pipette up and down the clogged wells and vacuum again. 2). Use a piece of clean wipe, wipe the under side of the clogged wells and vacuum again. 3). Take a thin needle (e.g., insulin needle), while holding the plate upward, poke the little hole under each of the clogged wells and vacuum again. Do not poke too hard or too deep as it may damage the filter and cause leaking.
	Filter plate was used without pre-wet.	Pre-wet plate with wash buffer before running the assay.

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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 be-tween duplicate samples	Beads aggregation	Sonicate and vortex the Beads prior to use.
	Multichannel pipette may not be calibrated or inconsistent pipet-ting	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 in-correctly reconstituted, stored or diluted	Follow the protocol to reconstitute, store and dilute standard. Double check your calculation.
	Wrong or short incuba-tion time	Ensure the time of all incubations was appropriate.
Signals too high, standard curves satu-rated	PMT value for FL2/PE set too high	Make sure the PMT setting for the re-porter channel is appropriate
	Plate incubation time was too long	Use shorter incubation time.
Sample read-ings 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 re-porter 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 ag-gregate.	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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