BioLegend®

Enabling Legendary Discovery™

LEGENDplex™

Multi-Analyte Flow Assay Kit

Rat Th Cytokine Panel V02
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

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

The LEGENDplex™ Rat Th Cytokine Panel V02 is a bead-based multiplex assay panel, using fluorescence–encoded beads suitable for use on various flow cytometers. This panel allows simultaneous quantification of 13 rat cytokines, including IL-2, IL-4, IL-5, IL-6,IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-22, GM-CSF, IFN-γ and TNF-α, which are collectively secreted by Th1, Th2, Th9, Th17, Th22, ThGM, and Tfh cells. This assay panel provides higher detection sensitivity and broader dynamic range than traditional ELISA method. The panel has been validated for use on cell culture supernatant, serum and plasma samples.

The Rat Th Cytokine Panel V02 is designed to allow flexible customization within the panel. Please visit **www.biolegend.com/legendplex** for more information on how to mix and match within the panel.

This assay is for research use only.

Principle of the Assay

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

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

Since the beads are differentiated by size and internal fluorescence intensity on a flow cytometer, analyte-specific populations can be segregated and PE

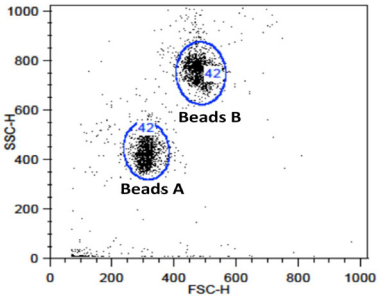
fluorescent signal quantified. The concentration of a particular analyte is determined using a standard curve generated in the same assay.

Beads Usage

The Rat Th Cytokine Panel V02 uses two sets of beads. Each set has a unique size that can be identified based on their forward scatter (FSC) and side scatter (SSC) profiles (Beads A and Beads B, Figure 1). Each bead set can be further resolved based on their internal fluorescence intensities. The internal dye can be detected using FL3, FL4, or APC channel, depending on the type of flow cytometer used. The smaller Beads A consists of 6 bead populations and the larger Beads B consists of 7 bead populations (Figure 2-3).

Using a total of 13 bead populations distinguished by size and internal fluorescent dye, the Rat Th Cytokine Panel V02 allows simultaneous detection of 13 analytes in a single sample. Each analyte is associated with a particular bead set as indicated in Table 1.

Figure 1. Beads Differentiated by Size



Beads A = smaller beads

Beads B = larger beads

Figure 2. Beads A Classification by FL4

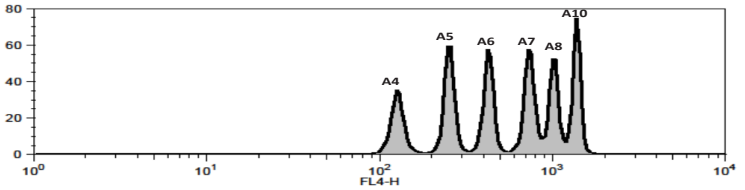
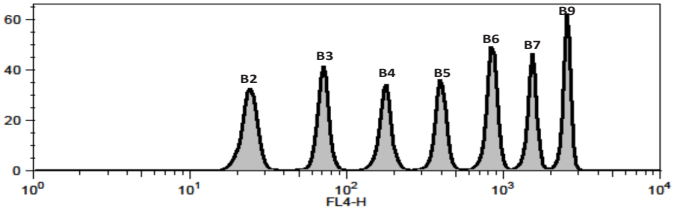


Figure 3. Beads B Classification by FL4



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

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

Target	Bead ID	Rat Th Cytokine Panel V02 Cat# 741219 or 741220	Top Standard Concentrations
IL-10	A4	√	The top standard concentration of each target may vary and may be subject to change from lot to lot. Please refer to the lot-specific Certificate of Analysis for this information.
IFN-γ	A5	√	
IL-5	A6	√	
IL-2	A7	√	
TNF-α	A8	√	
GM-CSF	A10	√	
IL-4	B2	√	
IL-17F	B3	√	
IL-9	B4	√	
IL-17A	B5	√	
IL-13	B6	√	
IL-22	B7	√	
IL-6	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.
- For Matrix C storage, the leftover should be stored at ≤-70°C upon reconstitution for use within one month. For optimal assay performance, it's not recommended to store the leftover top standards due to some proteins storage stability. If it's inevitable to rerun the assay, it's acceptable to freeze the reconstituted top standards at ≤-70°C immediately for use within one week. Avoid multiple (>2) freeze-thaw cycles. Discard any leftover diluted standards.

Materials Supplied

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

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**
Rat Th Cytokine Panel Detection Antibodies V02	1 bottle	3.5 mL	741221
Rat Th Cytokine Panel Standard A, Lyophilized	1 vial	lyophilized	741222
Rat Th Cytokine Panel Standard B, Lyophilized	1 vial	lyophilized	741223
LEGENDplex™ Buffer Set S	1		741226
Filter Plate* or V-bottom Plate**	1 plate		76187*or 76883**

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

Capture beads for Mix and Match Subpanels:

Beads Name	Quantity	Volume	Cat#
Rat IL-10 Capture Bead A4, 13X	1	270 µL	741225
Rat IFN-γ Capture Bead A5, 13X	1	270 µL	741263
Rat IL-5 Capture Bead A6, 13X	1	270 µL	740294
Rat IL-2 Capture Bead A7, 13X	1	270 µL	740295
Rat TNF-α Capture Bead A8, 13X	1	270 µL	741264
Rat GM-CSF Capture Bead A10, 13X	1	270 µL	741267
Rat IL-4 Capture Bead B2, 13X	1	270 µL	740297
Rat IL-17F Capture Bead B3, 13X	1	270 µL	740298
Rat IL-9 Capture Bead B4, 13X	1	270 µL	741224
Rat IL-17A Capture Bead B5, 13X	1	270 µL	741266
Rat IL-13 Capture Bead B6, 13X	1	270 µL	740300
Rat IL-22 Capture Bead B7, 13X	1	270 µL	740301
Rat IL-6 Capture Bead B9, 13X	1	270 µL	741265

LEGENDplex™ Buffer Set S (Cat#: 741226)

Component	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 C, Lyophilized	1 vial	lyophilized	76077
LEGENDplex™ Assay Buffer	1 bottle	25 mL	77562
Lyophilized Standard Reconstitution buffer	1 bottle	5 mL	750002725
LEGENDplex™ Wash Buffer, 20X	1 bottle	25 mL	77564
Plate Sealers	4 sheets		78101

No plate is included in the Buffer Set. Plate need to be ordered separately. Please order the correct type of plate based on the preferred assay protocol (Cat# 741219 for Filter Plate and Cat# 741220 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)

- 1.1 mL polypropylene micro FACS tubes, in 96-tube rack (e.g., National Scientific Supply Co, cat # TN0946-01R, or equivalent)

If the assay is performed in a filter plate;

- 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 C for LEGENDplex™ kits contains components of animal origin and should be handled as potentially hazardous.
- Do not mix or substitute reagents from different kits or lots. Reagents from different manufacturers should not be used with this kit.
- Do not use this kit beyond its expiration date.
- SA-PE and Beads are light-sensitive. Minimize light exposure.

Chapter 2: ASSAY PREPARATION

Sample Collection and Handling

Preparation of Serum Samples:

- Allow the blood to clot for at least 30 minutes and centrifuge for 10 minutes at 1,000 x *g*.
- Remove serum and assay immediately or aliquot and store samples at ≤-20°C. Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended that samples are thawed completely, mixed and centrifuged to remove particulates prior to use.

Preparation of Plasma Samples:

- Plasma collection should be collected using an anti-coagulant (e.g., EDTA, Heparin, Citrate). 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 Cell Culture Supernatant:

- Centrifuge the sample to remove debris and assay immediately, or aliquot and store samples at ≤-20°C. Avoid multiple (>2) freeze/thaw cycles.

Reagent Preparation

Preparation of Antibody-Immobilized Beads

1. The individual beads (13X) need to be combined with one another and diluted with Assay Buffer to create a 1X working solution of beads prior to use.

2. Sonicate each bead vial for 1 minute in a sonicator bath and then vortex for 30 seconds to completely resuspend the beads.

3. Calculate and prepare a 1X beads working solution based on the desired number of reactions and plex-size of your assay (i.e. the number of individual bead vials) following the steps described below.

   A. Total volume (µL) = 30 x (number of reactions)

   B. Volume needed from each 13X beads vial (µL) = 2.3 x (number of reactions)

C. Assay Buffer needed (µL) = A – B x (number of individual beads vials to be mixed)

Note: calculations for total volume include a 20% excess to account for any loss during pipetting

Example: to prepare 50 reactions for a 5-plex assay

A. Total volume (µL) = 30 x 50 = 1500 µL

B. Volume per beads vial needed (µL) = 2.3 x 50 = 115 µL

C. Assay Buffer needed (µL) = A – B x (number of individual beads vials) =1500 – (115 x 5) = 925 µL

Combine 115 µL of each beads vial (5 vials) with 925 µL of assay buffer to get the desired final volume of 1500 µL of 1X working solution of beads.

Preparation of Wash Buffer

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

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

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

Standard Preparation

1. Prior to use, reconstitute the lyophilized Rat Th Cytokine Panel Standard A with 2.5 mL of the Lyophilized Standard Reconstitution Buffer.
2. Mix and allow the vial to sit at room temperature for 10 minutes.
3. Reconstitute the lyophilized Rat Th Cytokine Panel Standard B with 250 µL of the reconstituted Rat Th Cytokine Panel Standard A
4. Mix and allow the vial to sit at room temperature for 10 minutes, then transfer the reconstituted **Standard B only** 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).

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

6. 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.
7. In the same manner, perform serial 1:4 dilutions to obtain C5, C4, C3, C2 and C1 standards (**see the table below using 10ng/mL of top standard concentration as an example**). Assay Buffer will be used as the 0 pg/mL standard (C0).

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

Sample Dilution

- In general, serum or plasma samples need to be diluted 4-fold with Assay Buffer before testing (e.g. dilute 25 µL of sample with 75 µL of Assay Buffer).

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

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

- For cell culture supernatant samples, the levels of analyte can vary greatly from sample to sample. While the 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.

Chapter 3: ASSAY PROCEDURE

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

- The in-filter plate assay procedure requires a vacuum filtration unit for washing (see **Materials to be Provided by the End-User, page 8)**. If you have performed bead-based multiplex assays before, your lab may already have the vacuum filtration unit set up.
- If the in-filter plate assay procedure is not possible or if you prefer, the assay can be performed in a V-bottom plate.

Performing the Assay Using a Filter Plate

- Allow all reagents to warm to room temperature (20-25°C) before use.
- Set the filter plate on an inverted plate cover at all times during assay setup and incubation steps, so that the bottom of the plate does not touch any surface. Touching a surface may cause leakage.
- Keep the plate upright during the entire assay procedure, including the washing steps, to avoid losing beads.
- The plate should be placed in the dark or wrapped with aluminum foil for all incubation steps.
- **Standards and samples should be run in duplicate and arranged on the plate in a vertical configuration convenient for data acquisition and analysis (as shown in attached PLATE MAP, page 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 C	Standard	Sample*
Standard Wells	25 µL	---	25 µL	---
Sample Wells	25 µL	---	---	25 µL

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

	Assay Buffer	Matrix C	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.

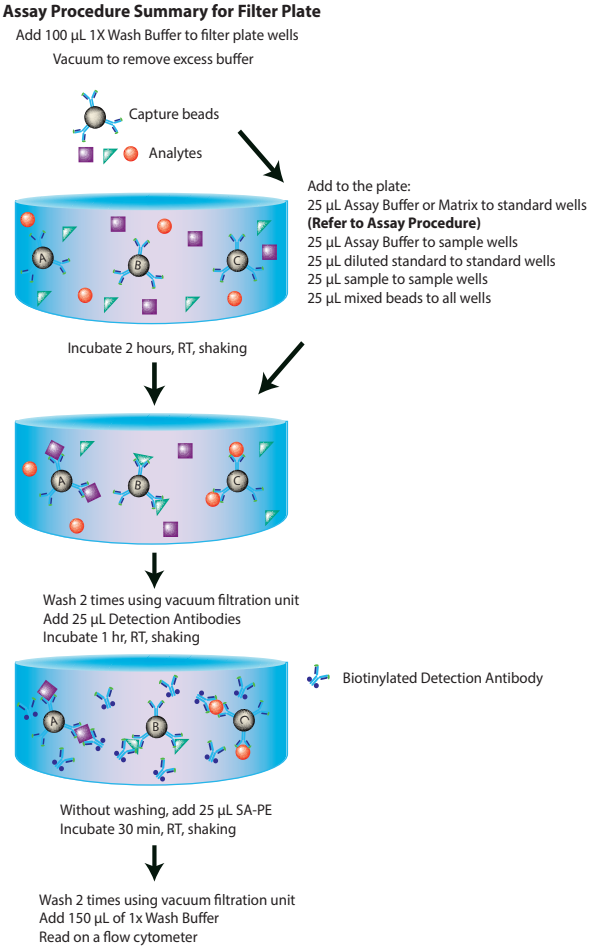
10. Add 150 µL of 1X Wash Buffer to each well. Resuspend the beads on a plate shaker for 1 minute.

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

    If the flow cytometer is equipped with an autosampler, read the plate directly using the autosampler. **Please be sure to program the autosampler**

to resuspend beads in the well immediately before taking samples. The probe height may need to be adjusted when using an autosampler.

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



Performing the Assay Using a V-bottom Plate

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

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

	Assay Buffer	Matrix C	Standard	Sample*
Standard Wells	---	25 µL	25 µL	---
Sample Wells	25 µL	---	---	25 µL

*See **Sample Dilution**

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

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

4. Centrifuge the plate at 1050 rpm (~250 g) for 5 minutes, using a swinging bucket rotor (G.H 3.8) with microplate adaptor (Please refer to **Materials to be Provided by the End-User, page 8).** Do not use excessive centrifugation 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.

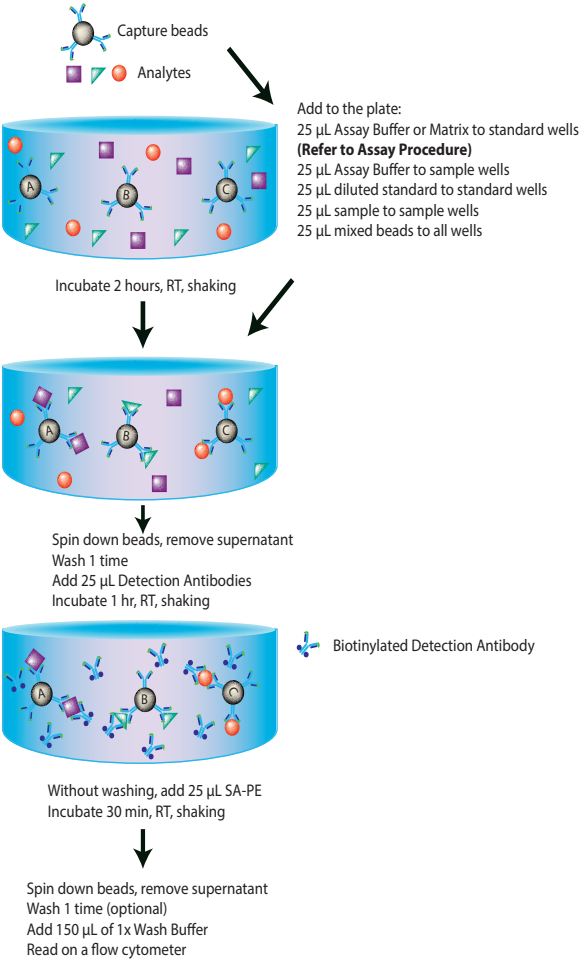
13. Add 150 µL of 1X Wash Buffer to each well. Resuspend the beads by pipetting.

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 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, B1, C1...A2, B2, C2...).

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

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

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

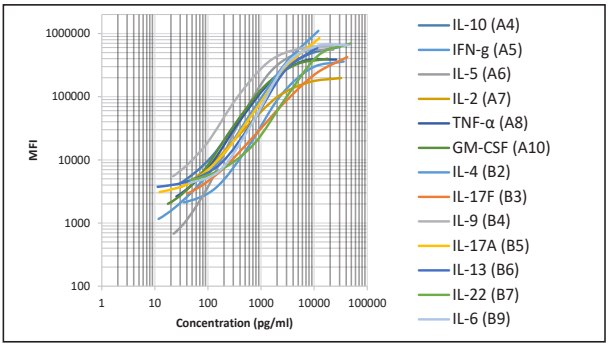
Data Analysis

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

Chapter 6: ASSAY CHARACTERIZATION

Standard Curve

This standard curve was generated using the LEGENDplex™ Rat Th Cytokine Panel V02 for demonstration purpose only. A standard curve must be run with each assay.



Assay Sensitivity

The assay sensitivity or Limit of Detection (LOD) is the theoretical limit of detection calculated using the LEGENDplex™ Data Analysis Software by applying a 5-parameter curve fitting algorithm.

Analyte	LOD in Assay Buffer (pg/mL)	LOD in Matrix (pg/mL)
IL-2	11.2	12.6
IL-4	30.0	1.5
IL-5	19.8	21.9
IL-6	7.0	34.3
IL-9	7.8	5.4
IL-10	16.1	15.1
IL-13	2.0	10.5
IL-17A	9.3	5.0
IL-17F	8.0	6.3
IL-22	4.1	2.6
GM-CSF	9.8	19.6
TNF-α	3.3	25.8
IFN-γ	15.0	25.2

Cross-Reactivity

The following rat recombinant proteins were tested at 50 ng/mL using the LEGENDplex™ Rat Th Cytokine Panel V02. No or negligible cross-reactivity was found.

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

Accuracy (Spike Recovery)

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

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

Analyte	% of Recovery in DMEM	% of Recovery in Serum	% of Recovery in Plasma
IL-2	61%	110%	62%
IL-4	62%	99%	68%
IL-5	77%	113%	66%
IL-6	63%	102%	87%
IL-9	56%	149%	72%
IL-10	55%	102%	85%
IL-13	53%	75%	59%
IL-17A	77%	109%	77%
IL-17F	63%	150%	70%

IL-22	68%	91%	79%
GM-CSF	61%	94%	102%
TNF-α	55%	82%	53%
IFN-γ	55%	68%	55%

Linearity of Dilution

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

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

Analyte	Linearity in Cell Culture Medium	Linearity in Serum	Linearity in Plasma
IL-2	106%	101%	96%
IL-4	125%	121%	106%
IL-5	99%	89%	95%
IL-6	101%	78%	73%
IL-9	110%	101%	89%
IL-10	92%	53%	51%
IL-13	91%	93%	85%
IL-17A	85%	77%	64%
IL-17F	97%	94%	88%
IL-22	92%	112%	87%
GM-CSF	103%	118%	114%
TNF-α	100%	95%	91%
IFN-γ	121%	133%	107%

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%
IL-2	Sample 1	915.4	83.3	9%
	Sample 2	4055.7	600.0	15%
IL-4	Sample 1	144.7	10.1	7%
	Sample 2	625.6	78.9	13%
IL-5	Sample 1	595.4	57.7	10%
	Sample 2	1848.5	192.3	10%
IL-6	Sample 1	963.2	113.0	12%
	Sample 2	3708.4	322.3	9%
IL-9	Sample 1	259.8	29.6	11%
	Sample 2	1153.4	159.4	14%
IL-10	Sample 1	93.9	7.7	8%
	Sample 2	570.8	46.9	8%
IL-13	Sample 1	183.7	18.8	10%
	Sample 2	862.9	90.9	11%
IL-17A	Sample 1	97.5	13.4	14%
	Sample 2	393.7	49.7	13%
IL-17F	Sample 1	399.4	45.0	11%
	Sample 2	1724.4	143.9	8%
IL-22	Sample 1	249.4	25.7	10%
	Sample 2	972.7	126.6	13%
GM-CSF	Sample 1	427.9	41.4	10%
	Sample 2	1795.6	183.7	10%
TNF-α	Sample 1	432.4	26.2	6%
	Sample 2	1837.2	253.2	14%
IFN-γ	Sample 1	616.4	38.7	6%
	Sample 2	2422.6	241.5	10%

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Inter-Assay Precision

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

Analyte	Sample	Mean (pg/mL)	STDEV	%CV
IL-2	Sample 1	1191.1	212.8	18%
	Sample 2	4466.6	896.0	20%
IL-4	Sample 1	177.2	32.3	18%
	Sample 2	654.8	70.9	11%
IL-5	Sample 1	553.3	87.7	16%
	Sample 2	2049.3	169.0	8%
IL-6	Sample 1	1076.6	205.3	19%
	Sample 2	4297.8	474.1	11%
IL-9	Sample 1	314.3	58.8	19%
	Sample 2	1310.5	191.4	15%
IL-10	Sample 1	121.3	34.0	28%
	Sample 2	610.5	115.8	19%
IL-13	Sample 1	262.7	46.8	18%
	Sample 2	1131.9	102.4	9%
IL-17A	Sample 1	125.0	23.8	19%
	Sample 2	477.4	46.1	10%
IL-17F	Sample 1	503.8	91.8	18%
	Sample 2	1989.5	187.1	9%
IL-22	Sample 1	318.6	78.0	24%
	Sample 2	1158.7	140.7	12%
GM-CSF	Sample 1	524.0	83.5	16%
	Sample 2	2177.7	269.2	12%
TNF-α	Sample 1	529.3	68.2	13%
	Sample 2	1958.6	309.9	16%
IFN-γ	Sample 1	764.2	126.4	17%
	Sample 2	2809.3	231.6	8%

Biological Samples

Serum and Plasma

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

Analyte	Range (pg/ml)	% of Detectable	Mean (pg/mL)
IL-2	ND	0%	ND
IL-4	1.0-2.4	67%	1.7
IL-5	ND	0%	ND
IL-6	7.1-15.0	67%	11.0
IL-9	ND	0%	ND
IL-10	ND	0%	ND
IL-13	7.4-21.5	67%	14.4
IL-17A	0.9-6.1	100%	3.3
IL-17F	ND	0%	ND
IL-22	ND-2.3	33%	2.3
GM-CSF	ND-5.1	33%	5.1
TNF-α	2.5-2.8	67%	2.6
IFN-γ	ND-35.0	33%	35.0

ND = Non-detectable

Two pooled rat plasma samples (using EDTA, Heparin, Citrate coagulant of each) from two different strains were tested for endogenous levels of proteins. The concentrations measured are shown below.

Analyte	Range (pg/mL)	% of Detectable	Mean (pg/mL)
IL-2	ND-21.2	17%	21.2
IL-4	0.9-6.4	33%	3.6
IL-5	ND-6.5	17%	6.5
IL-6	7.2-47.0	67%	18.8
IL-9	2.7-3.5	33%	3.1
IL-10	ND	0%	ND
IL-13	5.9-47.1	33%	26.5

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IL-17A	1.0-4.4	100%	2.3
IL-17F	5.7-41.8	83%	15.4
IL-22	2.0-12.5	83%	4.3
GM-CSF	ND	0%	ND
TNF-α	2.9-3.3	67%	3.0
IFN-γ	ND-8.7	17%	8.7

ND = Non-detectable

Cell Culture Supernatant

Rat splenocytes (1 x 10^6 cells/mL) were cultured under various conditions (LPS, 1 µg/mL; PMA 20 ng/mL; Ionomycin, 500 ng/mL, PHA, 5µg/ mL). Th2 culture was prepared as below: rat splenocytes were incubated with plate-coated anti-CD3 (1 µg/mL) in the presence of anti CD28 (1µg/mL). In the same culture, anti-IFN-g (10 µg/mL), IL-4 (40 ng/mL) and IL-2 (10 ng/mL) were added to promote Th2 culture condition. Supernatants were collected after 96 hours and assayed with the LEGENDplex™ Rat Th Cytokine Panel V02. The results (all in pg/mL) are summarized below.

Analyte	Control	LPS	PHA	PMA+ Ionomycin	Th2 Stim+ CD3+CD28
IL-2	<7.2	25.5	10.2	2949.1	2510.6
IL-4	<3.3	<3.3	<3.3	<3.3	>10000.0
IL-5	<19.6	<19.6	20.7	23.0	1055.0
IL-6	159.8	765.2	93.4	57.2	1198.0
IL-9	<1.3	<1.3	13.9	10.5	15.3
IL-10	40.8	112.6	43.9	8.4	991.7
IL-13	<7.9	<7.9	<7.9	62.5	701.6
IL-17A	22.9	83.1	<2.4	53.4	2194.7
IL-17F	6.8	21.0	9.4	715.1	261.1
IL-22	22.4	142.5	8.0	1611.9	324.8
GM-CSF	<30.4	53.8	<30.4	1080.8	786.8
TNF-α	4.9	52.4	<0.8	147.0	87.8
IFN-γ	<36.3	61.0	<36.3	3704.9	1833.6

*IL-4 cytokine was added to the Th2 polarization culture

TROUBLESHOOTING

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

Insufficient bead count or slow reading	Beads inappropriately prepared	Sonicate bead vials and vortex just prior to addition. Agitate mixed beads intermittently in reservoir while pipetting this into the plate.
	Samples cause beads aggregation due to particulate matter or viscosity.	Centrifuge samples just prior to assay setup and use supernatant. If high lipid content is present, remove lipid layer after centrifugation. Sample may need dilution if too viscous.
	Beads were lost during washing for in-tube assay	Make sure beads are spun down by visually check the pellet (beads are in light blue or blue color). Be very careful when removing supernatant during washing.
	Probe might be partially clogged.	Sample probe may need to be cleaned, or if needed, probe should be removed and sonicated.
Plate leaked	Vacuum pressure set too high	Adjust vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds. Do not exceed 10" Hg of vacuum.
	Plate set directly on table or absorbent towels during incubations or reagent additions	Set plate on plate holder or raised edge so bottom of filter is not touching any surface.
	Liquid present on the under side of the plate after vacuum	After washing, press down plate firmly on a stack of clean paper towels to dry the underside of the plate.
	Pipette touching and damaged plate filter during additions.	Pipette to the side of wells.
High Background	Background wells were contaminated	Avoid cross-well contamination by changing tips between pipetting when performing the assay using a multichannel pipette.
	Insufficient washes	The background may be due to non-specific binding of SA-PE. Increase number of washes.
Debris (FSC/SSC) during sample acquisition	Debris or platelet may exist in sample solution.	Centrifuge samples before analyzing samples. Remove platelet as much as possible.

Variation between duplicate samples	Beads aggregation	Sonicate and vortex the Beads prior to use.
	Multichannel pipette may not be calibrated or inconsistent pipetting	Calibrate Pipette. Ensure good pipetting practice. Prime pipette before use may help.
	Plate washing was not uniform	Make sure all reagents are vacuumed out completely in all wash steps.
	Samples may contain particulate matters.	Centrifuge samples just prior to assay setup and use supernatant. If high lipid content is present, remove lipid layer after centrifugation. Sample may need dilution if too viscous.
Low or poor standard curve signal	The standard was incorrectly reconstituted, stored or diluted	Follow the protocol to reconstitute, store and dilute standard. Double check your calculation.
	Wrong or short incubation time	Ensure the time of all incubations was appropriate.
Signals too high, standard curves saturated	PMT value for FL2/PE set too high	Make sure the PMT setting for the reporter channel is appropriate
	Plate incubation time was too long	Use shorter incubation time.
Sample readings are out of range	Samples contain no or below detectable levels of analyte	Make sure the experiment to generate the samples worked. Use proper positive controls.
	Samples concentrations higher than highest standard point.	Dilute samples and analyze again.
	Standard curve was saturated at higher end of curve.	Make sure the PMT setting for the reporter channel is appropriate. Use shorter incubation time if incubation time was too long
Missed beads populations during reading, or distribution is unequal	Sample may cause some beads to aggregate.	Centrifuge samples just prior to assay setup and use supernatant. If high lipid content is present, remove lipid layer after centrifugation. Sample may need dilution if too viscous.
	Beads populations are not mixed properly	Make sure all bead populations are mixed. and in similar numbers.

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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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