BioLegend®

The path to legendary discovery™

LEGENDplex™

Multi-Analyte Flow Assay Kit

Cat. No. 740196, Human Adipokine Panel (13-plex) w/FP

Cat. No. 741062, Human Adipokine Panel (13-plex) w/VbP

For Cell Culture and Urine Samples

Please read the entire manual before running the assay.

BioLegend.com

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

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Chapter 1: KIT DESCRIPTION

Introduction

Adipose tissue (AT) plays an active role in regulating metabolism and energy homeostasis. AT expresses and secretes numerous soluble factors, including adipokines, cytokines, chemokines and hormones. Altered levels of these proteins have been associated with a variety of metabolic diseases and inflammatory-autoimmune conditions. The accurate measurement of these adipose tissue hormones and cytokines is critical for a better understanding of disease progression and immune responses.

The LEGENDplex™ Human Adipokine Panel is a bead-based multiplex assay, using fluorescence–encoded beads suitable for use on various flow cytometers. This panel allows simultaneous quantification of 13 human adipokines and cytokines: Adiponectin, Adipsin, RBP4, MCP-1, IL-1β , IP-10, IL-10, IL-8, Leptin, IL-6, IFN-γ, Resistin and TNF-α. This panel provides higher sensitivity and broader dynamic range than traditional ELISA methods. The panel has been validated for use on urine and cell culture supernatant samples. The panel is not suitable for serum or plasma samples due to different sample dilution requirements for different analytes.

The Human Adipokine Panel is designed to allow flexible customization within the panel. For mix and match within the panel, please visit **https://www.biolegend.com/en-us/legendplex.**

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.

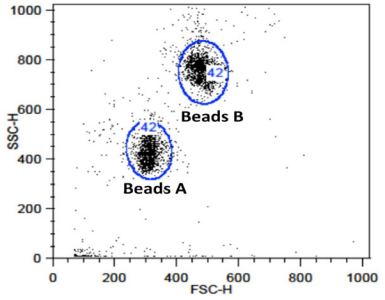
Since the beads are differentiated by size and internal fluorescence intensity on a flow cytometer, analyte-specific populations can be segregated and quantified by the PE fluorescent signal. The concentration of a particular analyte is detemined by a standard curve generated in the same assay.

Beads Usage

The Human Adipokine Panel includes two sets of beads. Each set has a unique size that can be identified on flow cytometer 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 Adipokine Panel allows simultaneous detection of 13 adipokines and cytokines in one sample test. 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

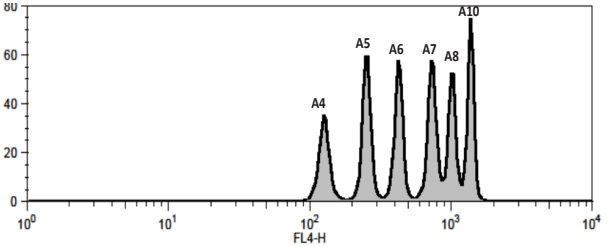
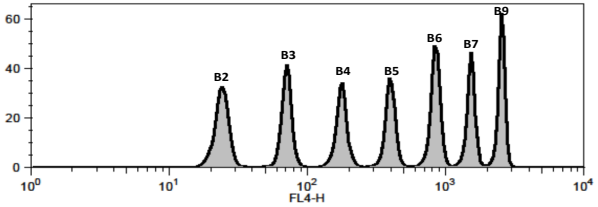


Figure 3. Beads B Classification by FL4



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

Table 1. Beads ID and Target Information

Target	Bead ID	Human Adipokine Panel Cat. No. 740196	Top Standard Concentrations (ng/mL)
Human Adiponectin	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).
Human Adipsin	A5	√	
Human RBP4	A6	√	
Human MCP-1	A7	√	
Human IL-1β	A8	√	
Human IP-10	A10	√	
Human IL-10	B2	√	
Human IL-8	B3	√	
Human Leptin	B4	√	
Human IL-6	B5	√	
Human IFN-γ	B6	√	
Human Resistin	B7	√	
Human TNF-α	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 Pre-mixed 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 and leftover standard should be stored at ≤-70°C for use within one month. Avoid multiple (>2) freeze-thaw cycles. Discard any leftover diluted standards.

Materials Supplied

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

Kit 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
Human Adipokine Panel Premixed Beads	1 bottle	3.5 mL	76320
Human Adipokine Panel Detection Antibodies	1 bottle	3.5 mL	76334
Human Adipokine Panel Standard Cocktail, Lyophilized	1 vial	lyophilized	76348
LEGENDplex™ SA-PE	1 bottle	3.5 mL	77743
LEGENDplex™ Assay Buffer	1 bottle	25 mL	77562
LEGENDplex™ Wash Buffer, 20X	1 bottle	25 mL	77564
Filter Plate** or V-bottom Plate***	1 plate		76187** or 76883***
Plate Sealers	4 sheets		78101

** For kit with filter plate. *** For kit with V-bottom plate. Only one plate is provided for each kit.

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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 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*

***Compensation is not required for the specified flow cytometers when set up properly, but is recommended for consistent results.**

For setting up the above flow cytometers, please follow the **Flow Cytometer Setup** guide in this manual or visit: **www.biolegend.com/legendplex.**

For flow cytometers not listed here, the end-user needs to set up the machine following similar guidelines. Please refer to **Setup Procedure for Other Flow Cytometers** section in Chapter 4.

- Multichannel pipettes capable of dispensing 5 µL to 200 µL
- Reagent reservoirs for multichannel pipette
- Polypropylene microfuge tubes (1.5 mL)
- Laboratory vortex mixer
- Sonicator bath (e.g., Branson Ultrasonic Cleaner model #B200, or equivalent)
- Aluminum foil
- Absorbent pads or paper towels
- Plate shaker (e.g., Lab-Line Instruments model #4625, or equivalent)
- Tabletop centrifuges (e.g., Eppendorf centrifuge 5415 C, or equivalent)

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

If the assay is performed in a filter plate

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

If the assay is performed in a V-bottom plate

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

• Precautions

- 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 Pre-mixed Beads are light-sensitive. Minimize light exposure.

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Chapter 2: ASSAY PREPARATION

Sample Collection and Handling

Preparation of Tissue Culture Supernatant or Urine Samples:

- 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

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

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 Adipokine Panel Standard Cocktail with 250 µL Assay 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 75 µL of Assay Buffer to each of the six tubes. Prepare 1:4 dilution of the top standard by transferring 25 µL of the top standard C7 to the C6 tube and mix well. This will be the C6 standard.

5. In the same manner, perform serial 1:4 dilutions to obtain C5, C4, C3, C2 and C1 standards (**see the table below using the top standard at 10,000 pg/mL as an example**). Assay Buffer will be used as the 0 pg/mL standard (C0).

Tube	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

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

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

- Urine samples must be diluted two-fold with Assay Buffer before testing (e.g. dilute 50 µL of sample with 50 µL of Assay Buffer).

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Chapter 3: ASSAY PROCEDURE

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

- The in-filter plate assay procedure irequires a vacuum filtration unit for washing (see **Materials to be Provided by the end-user, Page 7)**.
- If the in-filter plate assay procedure is not possible, or if you prefer, the assay can be performed in a V-bottom microplate**.**

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 32). 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 and urine samples:

   - Add 25 µL of Assay Buffer to all wells.
   - Add 25 µL of each standard to the standard wells.
   - Add 25 µL of each sample to the sample wells (See **Dilution of Samples**)

2. Vortex the pre-mixed beads b ottle for 30 seconds. Add 25 µL of the pre-mixed beads to each well. The volume should be 75 µL in each well after

beads addition. (Note: During addition of the beads, shake the pre-mixed beads bottle intermittently to avoid bead settling).

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

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

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

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

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

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

9. Repeat step 4 above.

10. Add 200 µL of 1X Wash Buffer to each well (**or 150 µL if plate is to be read with an autosampler. Higher volume may result in leaking of the filter plate during reading. Be sure to set the sample volume to be analyzed to 70 uL or less so that sample can be read again if needed**). 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).

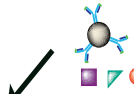
    If the flow cytometer is equipped with an autosampler, read the plate directly using the autosampler. **The probe height may need to be adjusted when using an autosampler**.

    If an autosampler is not available, the samples need to be transferred from the filter plate to FACS tubes and read manually. In this case, the sample volume may need to be increased from 200 µL to 300 µL by adding extra 100 µL of 1X Wash Buffer to each tube to avoid sample running dry when read on a flow cytometer.

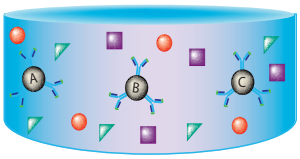
Assay Procedure Summary for Filter Plate

Add 100 µL 1X Wash Buffer to filter plate wells

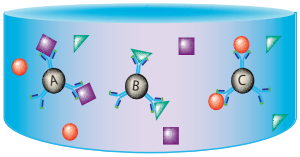
Vacuum to remove excess buffer



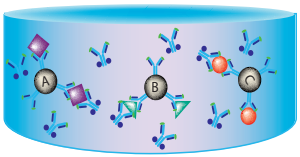
For cell culture supernatant/urine samples,
Add to the plate:
25 µL Assay Buffer to all wells
25 µL diluted standard to standard wells
25 µL sample to sample wells
25 µL pre-mixed beads to all wells



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 200 µL (or 150µL) 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 32). 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 and urine samples:**
   - Add 25 µL of Assay Buffer to all wells.
   - Add 25 µL of each standard to the standard wells.
   - Add 25 µL of each sample to the sample wells (See **Dilution of Samples**).
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.

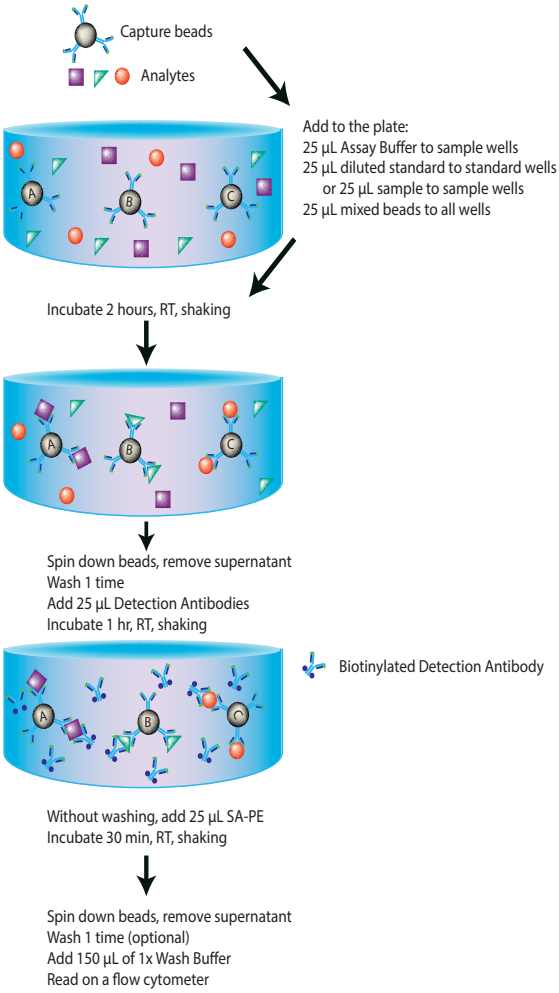
Alternatively, removal of the supernatant may be completed using a multichannel pipette set at 75 µL. Try to remove as much liquid as possible without removing any beads. Be sure to change pipette tips between each row or column.

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 it helps to reduce the background.
13. Add 150 µL of 1X Wash Buffer to each well. Resuspend the beads by pipetting.
14. Read samples on a flow cytometer, preferably within the same day of the assay (Note: Prolonged sample storage can lead to reduced signal).

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

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

Assay Procedure Summary for V-bottom Plate



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.

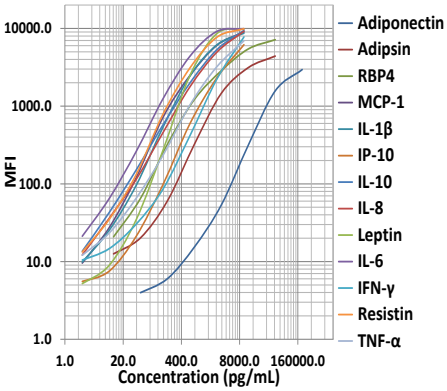
Data Analysis

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 7: ASSAY CHARACTERIZATION

Representative Standard Curve

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



Assay Sensitivity

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

Analyte	MDC (pg/mL)
Human Adiponectin	41.4
Human Adipsin	5.4
Human RBP4	6.8
Human MCP-1	0.6
Human IL-1β	0.7
Human IP-10	1.5
Human IL-10	1.0

Human IL-8	0.9
Human Leptin	1.6
Human IL-6	2.1
Human IFN-γ	1.7
Human Resistin	1.4
Human TNF-α	0.8

Cross-Reactivity

The following recombinant proteins were tested at 50 ng/mL using the LEGENDplex™ Human Adipokine Panel. No or negligible cross-reactivity was found for all the tested analytes.

RANTES	IL-1α	IL-33	TARC	MIP-3α
IL-5	IL-23	IL-2	I-TAC	IL-22
IL-9	IL-12p40	CCL11	IL-27	Groα
IL-17F	IL-12p70	MIP-1α	IL-17A	TSLP
IL-4	IL-15	MIP-1β	IL-11	MIG
IL-21	IL-18	ENA-78	IL-13	TSLP

Accuracy (Spike Recovery)

For spike recovery in cell culture medium (CCM), RPMI or DMEM with 10% FCS was first diluted two-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.

For spike recovery in urine, samples (n=4) were first diluted two-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	Recovery in CCM	Recovery in Urine
Human Adiponectin	94%	108%

Human Adipsin	93%	99%
Human RBP4	78%	NA
Human MCP-1	103%	99%
Human IL-1β	99%	77%
Human IP-10	76%	134%
Human IL-10	98%	98%
Human IL-8	104%	102%
Human Leptin	91%	85%
Human IL-6	97%	97%
Human IFN-γ	87%	156%
Human Resistin	94%	106%
Human TNF-α	94%	83%

NA= Not Applicable due to high endogenous levels

Linearity of Dilution

For spike linearity in CCM, RPMI or DMEM with 10% FCS was first diluted two-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 assay buffer and assayed. The measured concentrations of serially diluted samples were compared with that of the spiked samples.

For sprike linearity in urine, samples (n=4) were diluted two-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 Assay Buffer and assayed. The measured concentrations of serially diluted samples were compared with that of the spiked samples.

Analyte	Linearity in CCM	Linearity in Urine
Human Adiponectin	96%	100%
Human Adipsin	95%	96%
Human RBP4	131%	101%
Human MCP-1	95%	113%

Human IL-1β	87%	108%
Human IP-10	119%	88%
Human IL-10	86%	100%
Human IL-8	76%	96%
Human Leptin	100%	95%
Human IL-6	93%	110%
Human IFN-γ	101%	87%
Human Resistin	102%	105%
Human TNF-α	94%	121%

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 Adiponectin	Sample 1	36.0	2.5	7%
	Sample 2	575.1	24.3	4%
Human Adipsin	Sample 1	41.2	2.4	6%
	Sample 2	678.9	44.8	7%
Human RBP4	Sample 1	39.2	1.7	4%
	Sample 2	593.2	31.4	5%
Human MCP-1	Sample 1	35.2	2.3	7%
	Sample 2	646.3	31.3	5%
Human IL-1β	Sample 1	35.1	2.5	7%
	Sample 2	650.4	31.4	5%
Human IP-10	Sample 1	37.2	2.8	7%
	Sample 2	712.7	62.7	9%
Human IL-10	Sample 1	36.6	2.1	6%
	Sample 2	665.0	39.8	6%
Human IL-8	Sample 1	34.8	2.1	6%
	Sample 2	694.0	56.4	8%

Human Leptin	Sample 1	32.3	2.1	6%
	Sample 2	613.1	32.3	5%
Human IL-6	Sample 1	36.7	2.0	5%
	Sample 2	625.4	53.1	8%
Human IFN-γ	Sample 1	38.7	2.6	7%
	Sample 2	687.7	59.3	9%
Human Resistin	Sample 1	36.1	2.4	7%
	Sample 2	664.4	48.3	7%
Human TNF-α	Sample 1	36.8	2.6	7%
	Sample 2	707.9	57.4	8%

Inter-Assay Precision

Two samples with different concentrations of target proteins were analyzed in three independent assays with 3 replicates for each sample. The inter-assay precision was calculated as below.

Analyte	Sample	Mean (pg/mL)	STDEV	%CV
Human Adiponectin	Sample 1	37.3	3.9	11%
	Sample 2	538.0	40.7	8%
Human Adipsin	Sample 1	36.5	2.2	6%
	Sample 2	601.0	67.5	11%
Human RBP4	Sample 1	37.3	2.0	5%
	Sample 2	530.4	49.9	9%
Human MCP-1	Sample 1	36.8	2.2	6%
	Sample 2	569.8	61.2	11%
Human IL-1β	Sample 1	36.2	2.3	6%
	Sample 2	582.0	55.9	10%
Human IP-10	Sample 1	37.4	3.0	8%
	Sample 2	592.8	89.9	15%
Human IL-10	Sample 1	36.8	2.2	6%
	Sample 2	587.3	64.2	11%

Human IL-8	Sample 1	37.3	3.2	8%
	Sample 2	596.4	80.3	13%
Human Leptin	Sample 1	34.6	2.7	8%
	Sample 2	562.9	42.7	8%
Human IL-6	Sample 1	37.0	2.2	6%
	Sample 2	541.9	75.5	14%
Human IFN-γ	Sample 1	38.1	3.7	10%
	Sample 2	589.4	79.9	14%
Human Resistin	Sample 1	36.4	1.9	5%
	Sample 2	569.6	71.7	13%
Human TNF-α	Sample 1	37.8	2.2	6%
	Sample 2	608.1	86.6	14%

Biological Samples

Urine

Normal human urine samples (n=16) were tested for endogenous levels of adipokines and cytokines. The concentrations measured are shown below:

Analyte	Range (pg/mL)	% of Detectable	Mean of detectable (pg/mL)
Human Adiponectin	381 - 5755	100%	1873
Human Adipsin	330 - 2886	100%	1106
Human RBP4	20,590 - 181,113	100%	74,976
Human MCP-1	94 - 662	100%	232
Human IL-1β	2.4 - 11.7	100%	7.3
Human IP-10	48 - 151	100%	96
Human IL-10	0.5 - 7.7	100%	2.5
Human IL-8	3.4 - 176	100%	29.8
Human Leptin	5.2 - 46.2	100%	18.7
Human IL-6	2.2 - 15.9	100%	7.6
Human IFN-γ	ND - 72.4	94%	18.9

Human Resistin	359 - 7344	100%	1923
Human TNF-α	0.7 - 9.7	100%	2.5

ND = Not Detectable

Cell Culture Supernatant

Culture medium of human pre-adipocytes and adipocytes from a commercial source were measured for hormone concentrations. Human subcutaneous preadipocytes were obtained from adipose tissue. Culture medium was prepared from confluent preadipocytes or 2-3 week old differentiated adipocytes after 24 hrs incubation. The results (all in pg/mL) are summarized below.

Analyte	pre-adipocyte	Normal Adipocyte	Diabetic Adipocyte
Human Adiponectin	ND	44,156	388,451
Human Adipsin	10.8	8,092	32,382
Human RBP4	ND	6,215	31,566
Human MCP-1	71.7	409.3	607.8
Human IL-1β	1.5	2.6	5.4
Human IP-10	4.5	11.4	24.6
Human IL-10	ND	ND	5.21
Human IL-8	1106	30.8	14.1
Human Leptin	3.9	63.9	64.7
Human IL-6	71.8	21.3	11.4
Human IFN-γ	3.87	18.5	69.2
Human Resistin	ND	ND	ND
Human TNF-α	2.3	4.8	11.1

ND = Not Detectable

Human PBMC (1x10^6 cells/mL) were cultured under various conditions (PHA, 10 µg/mL; PMA, 50 ng/mL; LPS, 50 ng/mL). Supernatants were collected after 1 and 4 days. All samples were diluted two-fold with Assay Buffer and assayed with the LEGENDplex™ Human Adipokine Panel. The results (all in pg/mL) are summarized below.

Analyte	Control 1 Day	PHA 1 Day	PMA 1 Day	LPS 1 Day
Human Adiponectin	130.4	178.2	228.8	192.8
Human Adipsin	1,935	2,036	821.9	2,023
Human RBP4	329.8	410.1	525.6	420.4
Human MCP-1	18,553	23,352	14,551	10,023
Human IL-1β	8.6	671.7	4318.4	933.5
Human IP-10	810.9	5415.1	433.1	514.5
Human IL-10	29.1	8395.4	9.5	8155.6
Human IL-8	>23,272	>23,272	>23,272	>23,272
Human Leptin	8.6	8.6	10.2	14.7
Human IL-6	174.6	11,139	231.3	11,139
Human IFN-γ	6.0	943.8	298.3	26.7
Human Resistin	194.6	220.4	229.9	373.2
Human TNF-α	2.5	211.2	2,621	67.4

Analyte	Control 4 Day	PHA 4 Day	PMA 4 Day	LPS 4 Day
Human Adiponectin	405	211.6	215	382.8
Human Adipsin	13,567	5,293	1,466	5,433
Human RBP4	465.8	509.8	831.1	575
Human MCP-1	24,987	8,812	24,454	2,146
Human IL-1β	5.8	295.8	2,251	353.9
Human IP-10	6,176	5,354	543.3	849.5
Human IL-10	99.7	3,869	24.5	3,570
Human IL-8	>23,272	>23,272	>23,272	>23,272
Human Leptin	85.7	12.4	13.6	41.8
Human IL-6	203.7	11,139	2,975	11,139
Human IFN-γ	113.7	551.4	272.5	119
Human Resistin	230.1	513.5	291.3	4,021
Human TNF-α	8.7	84.3	2,809	38.2

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 the pre-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 may 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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