BioLegend®

Enabling Legendary Discovery™

LEGENDplex™

Cat. No. 740453

Human Apolipoprotein (Apo) Panel (11-plex) with Filter Plate

Cat. No. 740454

Human Apolipoprotein (Apo) Panel (11-plex) with V-bottom Plate

Please read the entire manual before running the assay.

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

For Research Purposes Only. Not for use in diagnostic or therapeutic procedures. Purchase does not include or carry the right to resell or transfer this product either as a stand-alone product or as a component of another product. Any use of this product other than the permitted use without the express written authorization of BioLegend is strictly prohibited.

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Tel: 858-768-5800

Chapter 1: KIT DESCRIPTION

Introduction

Apolipoproteins (Apo) are proteins that bind to lipids to form lipoproteins, whose main function is to transport lipids. Apolipoproteins are important in maintaining the structural integrity and solubility of lipoproteins and play important roles in lipoprotein receptor recognition and the regulation of certain enzymes in lipoprotein metabolism. There are six major classes of Apo: A, B, C, D, E and H. The Apo system is complex and the balance between Apo proteins is important. Disturbance of the balance may be associated with diseases such as Alzheimer's Diease, Hyperlipoproteinemia, heart disease, atherosclerosis, diabetes, and cancer.

The Human Apo panel is a multiplex bead-based assay panel, using fluorescence–encoded beads suitable for use on various flow cytometers. This panel allows simultaneous quantification of 11 human Apo proteins, including Apo AI, AII, B100, CII, CIII, D, E, E4, H, J and M. This assay panel provides broader dynamic ranges than traditional ELISA methods. The panel has been validated for use with serum, plasma and cell culture supernatant samples.

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

This assay is for research use only.

Principle of the Assay

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

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

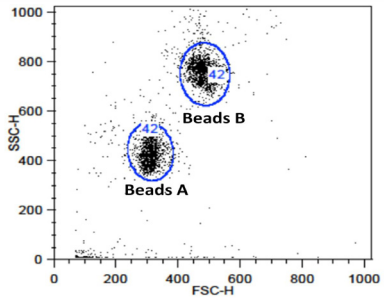
Since the beads are differentiated by size and internal fluorescence intensity on a flow cytometer, analyte-specific populations can be segregated and PE fluorescent signal quantified. The concentration of a particular analyte is determined using a standard curve generated in the same assay.

Beads Usage

The Human Apo Panel 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) consist of 6 (shown in Figure 2) bead populations and 5 (A4, A5, A6, A7 and A10) are used. The larger Beads (B) consist of 7 (shown in Figure 3) bead populations and 6 (B3, B4, B5, B6, B7 and B9) are used for this panel.

Using a total of 11 bead populations distinguished by size and internal fluorescent dye, the Human Apo Panel allows simultaneous detection of 11 Apo proteins in a single sample. Each analyte is associated with a particular bead set as indicated (Figures 2-3 and Table 1).

Figure 1. Beads Differentiated by Size



Beads A = smaller beads

Beads B = larger beads

Figure 2. Beads A Classification by FL4

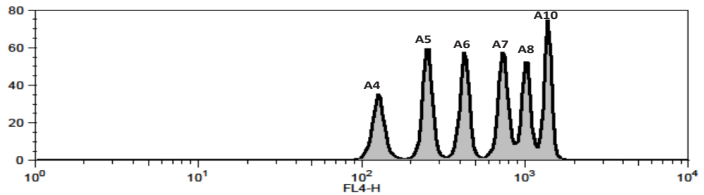
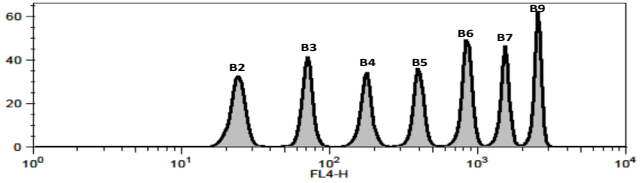


Figure 3. Beads B Classification by FL4



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

Table 1. Panel Targets and Bead ID*

Target	Bead ID	Top Standard Concentrations
Apo AI	A4	The top standard concentration of each target may vary and may subject to change from lot to lot. Please refer to the lot-specific Certificate of Analysis for this information.
Apo AII	A5	
Apo B100	A6	
Apo CII	A7	
Apo CIII	A10	
Apo D	B3	
Apo E	B4	
Apo E4	B5	
Apo H	B6	
Apo J	B7	
Apo M	B9	

***Bead ID is used to associate a bead population to a particular analyte in the LEGENDplex™ Data Analysis Software. The association of analyte and bead ID will be defined during the gating step in the data analysis.

When entering analyte and bead ID infomation during the gating step, always enter in the sequential order of the bead ID (e.g, A4, A5, A6...B3, B4...). Please refer to the LEGENDplex™ Data Analysis Software User Guide and Online Help for details (**www.biolegend.com/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, 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 Apo Panel Premixed Beads	1 bottle	3.5 mL	78946
Human Apo Panel Detection Antibodies	1 bottle	3.5 mL	78944
Human Apo Panel Standard Cocktail, Lyophilized	1 vial	lyophilized	78948
LEGENDplex™ SA-PE	1 bottle	3.5 mL	77743
LEGENDplex™ Assay Buffer	2 bottles	25 mL each	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
Data Analysis Software Dongle	1		21217
Human Apo Panel Manual	1		78949

* For assay with filter plate. ** For assay with 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 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*
Beckman Coulter-CytoFLEX	PE	585 nm	APC	660 nm	No*

***Compensation is not required for the specified flow cytometers when set up properly.**

For setting up various flow cytometers, please visit: **www.biolegend.com/legendplex** and click on the **Instrument Setup** tab.

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

If the assay is performed in a filter plate (recommended):

- 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 (optional):

- 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.
- 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 using either EDTA, Heparin, or Citrate as an anti-coagulant are acceptable sample types to be tested with this kit. 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

Sonicate 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 Apo 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 (100, 500, 1000, 4000 ng/mL) depending on the analyte, but may subject to change from lot to lot (see lot-specific Certificate of Analysis for details).

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 1000 ng/mL as an example**). Assay Buffer will be used as the 0 ng/mL standard (C0).

Tube/Standard ID	Serial Dilution	Assay Buffer to add (µL)	Standard to add	Final Conc. (ng/mL)
C7	--	--	--	1000
C6	1:4	75	25 µL of C7	250
C5	1:16	75	25 µL of C6	62.5
C4	1:64	75	25 µL of C5	15.6
C3	1:256	75	25 µL of C4	3.91
C2	1:1024	75	25 µL of C3	0.98
C1	1:4096	75	25 µL of C2	0.24
C0	--	75	--	0

Sample Dilution

- Serum or plasma samples should be diluted at least 4000-fold or 8000-fold with Assay Buffer as described in the table below.

Sample	1st dilution (1:40 or 1:80)	2nd dilution (1:100)	Final dilution fold
Serum	2.5 µL + 97.5 µL	2 µL 1st + 198 µL	4,000
Plasma	2.5 µL + 197.5 µL		8,000

- 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.

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 is recommended due to its good sample-to-sample consistency, assay robustness and ease of handling. This procedure requires a vacuum filtration unit for washing (see **Materials to be Provided by the End-User, page 7)**. If you have performed bead-based multiplex assays before, your lab may already have the vacuum filtration unit set up.

- If the 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 29). 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.**

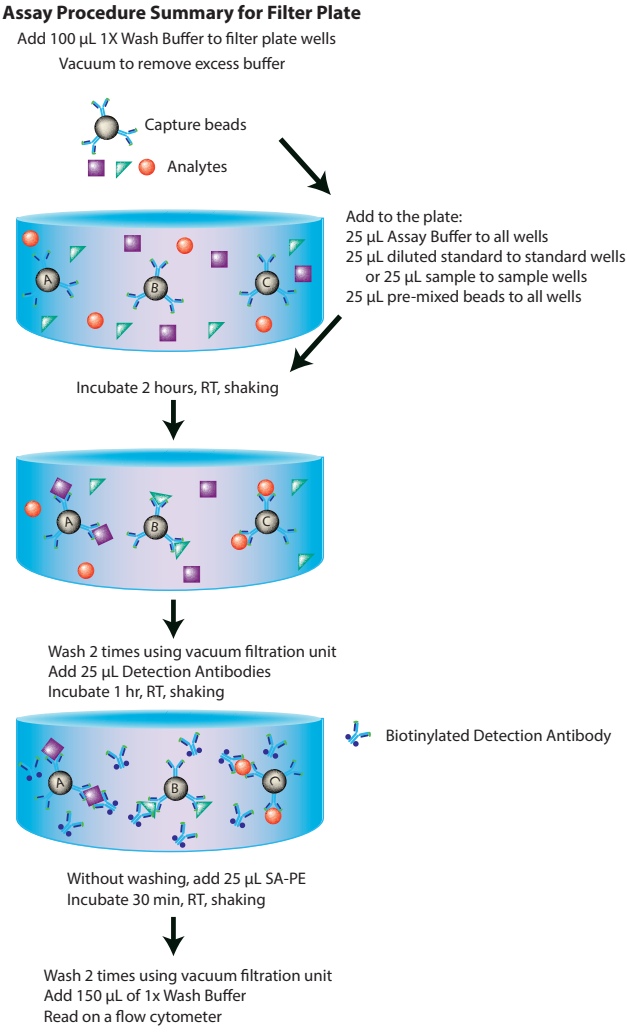
1. Pre-wet the plate by adding 100 µL of LEGENDplex™ 1X Wash Buffer to each well and let it sit for 1 minute at room temperature. To remove the excess volume, place the plate on the vacuum manifold and apply vacuum. Do not exceed 10" Hg of vacuum. Vacuum until wells are drained (5-10 seconds). Blot excess Wash Buffer from the bottom of the plate with an absorbent pad or paper towels. Place the plate on top of the inverted plate cover.
2. Load all the wells including standards and samples wells with 25 µL of Assay Buffer.
3. Load 25 µL of either prepared standards to corresponding standard wells or diluted samples to sample wells.
4. 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).
5. 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.
6. **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.
7. Add 25 µL of Detection Antibodies to each well.
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 approximately 500 rpm for 1 hour at room temperature.
9. **Do not vacuum!** Add 25 µL of SA-PE to each well directly.
10. 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.
11. Repeat step 6 above.
12. Add 150 µL of 1X Wash Buffer to each well. Resuspend the beads on a

plate shaker for 1 minute.

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

1. Load all the wells including standards and samples wells with 25 µL of Assay Buffer.
2. Load 25 µL of either prepared standards to corresponding standard wells or diluted samples to sample wells.
3. 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).
4. 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)**.
5. 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.**
6. Immediately after centrifugation, dump the supernatant into a sink by quickly inverting and flicking the plate **in one continuous and forcefull 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

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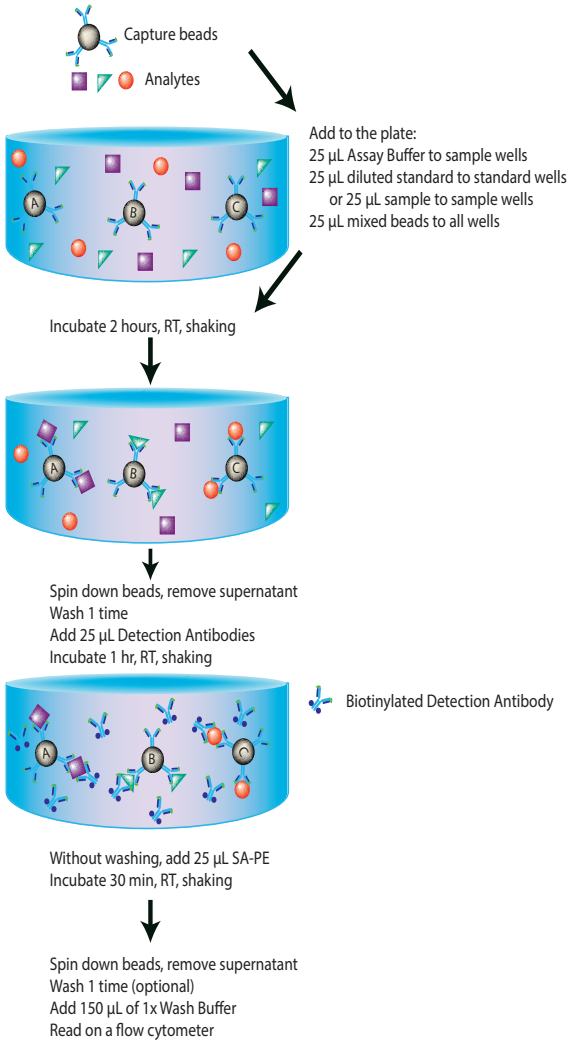
without removing any beads. Be sure to change pipette tips between each row or column.

7. Wash the plate by dispensing 200 µL of washing buffer into each well and incubate for one minute. Repeat step 5 and 6 above. A second wash is optional , but may help reduce background.
8. Add 25 µL Detection Antibodies to each well.
9. 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.
10. **Do not wash the plate!** Add 25 µL of SA-PE to each well directly.
11. 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.
12. Repeat step 5 and 6 above.
13. Wash the plate by dispensing 200 µL of washing buffer into each well and incubate for one minute. Repeat step 5 and 6 above. This final wash is optional, it may help reduce background.
14. Add 150 µL of 1X Wash Buffer to each well. Resuspend the beads by pipetting.
15. 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 accurate 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 3300 beads for a 11-plex assay). Do not set to acquire total events as samples may contain large amounts of debris. Instead, create a large gate to include both Beads A and Beads B (gate A+B) and set to acquire the number of events in gate A + B. This will exclude majority of 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.

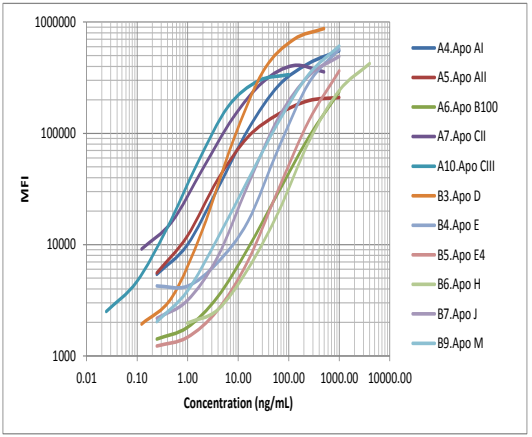
Data Analysis

- The FCS file generated on a flow cytometer should be analyzed using BioLegend's LEGENDplex™ Data Analysis Software. The LEGENDplex™ Data Analysis Software can be downloaded here: **www.biolegend.com/legendplex.**
- For PC users, install the software on a PC running Windows 7 or Windows 8 and use it in conjunction with the Data Analysis Software Dongle included in this kit. The dongle has a license key stored in it and is needed to run the software. To use the dongle, simply plug it in the USB port of the computer on which the data analysis software is installed, prior to launching the software.
- For Mac users, install on a Mac running Mac OS X version 10.7 (Lion) and late and you will be promoted to request a software license key after the software installation.
- Follow the LEGENDplex™ Data Analysis Software User Guide and Online Help to use the software (**www.bioLegend.com/legendplex; or press F1 for online help at any step of the data analysis**).

Chapter 6: ASSAY CHARACTERIZATION

Representative Standard Curve

This standard curve was generated using the LEGENDplex™ Human Apo-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 (ng/mL)
Human Apo AI	0.2
Human Apo AII	0.3
Human Apo B100	1.0
Human Apo CII	0.3
Human Apo CIII	0.7
Human Apo D	0.3
Human Apo E	1.4
Human Apo E4	0.1
Human Apo H	0.3
Human Apo J	0.2
Human Apo M	0.2

Cross-Reactivity

The following human purified or recombinant (PCSK9, CCL8, and CCL9) proteins were tested individually at 100 ng/mL using the LEGENDplex™ Human Apo Panel. There was negligible cross-reactivity found for a majority of proteins tested with the exceptions of Apo CII, Apo CIII and Apo E. Apo CII and Apo CIII shared about 3% cross-reactivity with each other. Apo E showed broad cross-reactivity to Apo B100 (19.3%), Apo H (17.6%), Apo M (4.7%), Apo AII (2.2%), Apo D (2.0%), and Apo J (1.6%). The observed cross-reactivities may be due, at least partially, to the purity of the proteins used.

Apo AI	Apo AII	Apo B100	Apo CII	Apo CIII	Apo D	Apo E
Apo H	Apo J	Apo M	PCSK9	CCL8	CCL19	

Linearity of Dilution

For testing linearity of dilution, serum (n=5) and plasma samples (EDTA, Heparin, Citrate, n=5 each) were first diluted 4000-fold and 8000-fold with Assay Buffer, respectively, then serially diluted 1:2, 1:4, 1:8 with Assay Buffer and assayed. The measured concentrations of serially diluted samples were then compared with the concentration of 4000-fold diluted serum or 8000-fold diluted plasma samples based on serial dilution factor used.

Analyte	Serum	EDTA plasma	Heparin Plasma	Citrate plasma
Human Apo AI	106%	89%	106%	69%
Human Apo AII	82%	105%	106%	108%
Human Apo B100	116%	104%	111%	125%
Human Apo CII	73%	96%	98%	113%
Human Apo CIII	72%	77%	83%	93%
Human Apo D	77%	80%	76%	69%
Human Apo E	75%	118%	75%	104%
Human Apo E4	77%	83%	71%	81%
Human Apo H	72%	84%	71%	77%
Human Apo J	77%	78%	77%	62%
Human Apo M	80%	87%	82%	86%

Intra-Assay Precision

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

Analyte	Sample	Mean (ng/mL)	STDEV	%CV
Human Apo AI	Sample 1	38.0	1.9	5%
	Sample 2	10.5	0.4	4%
Human Apo AII	Sample 1	56.2	8.6	15%
	Sample 2	17.8	1.5	8%
Human Apo B100	Sample 1	756.4	130.2	17%
	Sample 2	200.3	13.9	7%
Human Apo CII	Sample 1	45.7	2.4	5%
	Sample 2	10.3	0.9	9%
Human Apo CIII	Sample 1	444.4	111.9	25%
	Sample 2	59.4	7.6	13%
Human Apo D	Sample 1	27.5	2.1	7%
	Sample 2	5.4	0.3	6%
Human Apo E	Sample 1	3.2	0.1	3%
	Sample 2	1.6	0.2	12%
Human Apo E4	Sample 1	2.0	0.2	12%
	Sample 2	0.8	0.04	5%
Human Apo H	Sample 1	12.5	0.6	5%
	Sample 2	2.5	0.1	6%
Human Apo J	Sample 1	10.3	0.8	8%
	Sample 2	1.8	0.2	11%
Human Apo M	Sample 1	11.6	1.1	10%
	Sample 2	2.3	0.2	9%

Inter-Assay Precision

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

Analyte	Sample	Mean (ng/mL)	STDEV	%CV
Human Apo AI	Sample 1	38.1	0.5	1%
	Sample 2	10.3	0.3	2%
Human Apo AII	Sample 1	60.1	15.4	26%
	Sample 2	19.3	1.8	9%
Human Apo B100	Sample 1	845.0	95.4	11%
	Sample 2	236.6	35.6	15%
Human Apo CII	Sample 1	43.5	9.5	22%
	Sample 2	8.8	1.5	17%
Human Apo CIII	Sample 1	420.1	45.9	11%
	Sample 2	61.0	2.2	4%
Human Apo D	Sample 1	27.5	0.3	1%
	Sample 2	5.4	0.22	4%
Human Apo E	Sample 1	1.5	0.1	8%
	Sample 2	ND	ND	ND
Human Apo E4	Sample 1	1.9	0.1	7%
	Sample 2	ND	ND	ND
Human Apo H	Sample 1	12.6	0.3	2%
	Sample 2	2.6	0.1	5%
Human Apo J	Sample 1	10.5	0.2	2%
	Sample 2	1.8	0.2	9%
Human Apo M	Sample 1	11.8	0.2	2%
	Sample 2	2.5	0.2	8%

ND = Non-detectable

Biological Samples

Serum and Plasma

Normal human serum and plasma samples from 20 different donors were tested for endogenous levels of human Apo proteins. The range and mean concentrations (ug/mL) measured are shown below.

Analyte	Serum (n=20)	EDTA plasma (n=20)	Heparin plasma (n=20)	Citrate plasma (n=20)
Human Apo AI	63 - 13383	201 - 10668	1627 - 6042	77 - 5012
	3055	4194	4185	2051
Human Apo AII	108 - 1761	305 - 1237	507 - 1841	391 - 1548
	662	777	1146	882
Human Apo B100	2525 - 5751	2652 - 9203	1202 - 8080	1970 - 5363
	4270	4277	3591	3413
Human Apo CII	15 - 379	62 - 759	33 - 402	21 - 186
	188	183	120	71
Human Apo CIII	16 - 513	46 - 1022	49 - 643	26 - 180
	154	222	196	88
Human Apo D	29 - 127	35 - 149	23 - 121	13 - 115
	71	76	64	46
Human Apo E	4 - 180	8 - 246	34 - 268	8 - 104
	69	102	98	47
Human Apo E4	1 - 152	1 - 206	20 - 96	2 - 66
	43	59	51	28
Human Apo H	26 - 2037	60 - 1372	174 - 1332	72 - 754
	934	817	660	394
Human Apo J	1 - 102	2 - 70	5 - 117	1 - 51
	14	23	51	15
Human Apo M	56 - 204	57 - 176	51 - 257	49 - 186
	122	119	129	102

Cell Culture Supernatant

Culture media of normal human pre-adipocytes and adipocytes and diabetic adipocytes, obtained from a commercial source, were measured for human Apo proteins' concentrations. The results (in ng/mL) are summarized below.

Analyte	Pre-adipocytes	Normal adipocytes	Diabetic adipocytes
Human Apo AI	ND	ND	2.4
Human Apo AII	ND	0.4	6.3
Human Apo B100	ND	2.1	38.6
Human Apo CII	ND	ND	0.6
Human Apo CIII	ND	0.2	4.1
Human Apo D	ND	11.7	9.5
Human Apo E	ND	47.7	260.8
Human Apo E4	ND	8.7	45.7
Human Apo H	ND	4.4	43.5
Human Apo J	ND	0.6	5.0
Human Apo M	ND	1.0	15.9

ND = Non-detectable

Human hepatocellular carcinoma cell line Hep G2 cells were cultured with EMEM/10% FBS culture media for 3 days. Cell Culture supernatant was collected on day 4 and assayed with the LEGENDplex™ Human Apo Panel kit. The results are shown below.

Analyte	Concentration (ng/mL)
Human Apo AI	26.8
Human Apo AII	26.9
Human Apo B100	1794.6
Human Apo CII	9.7
Human Apo CIII	2.8
Human Apo D	0.8
Human Apo E	964.6
Human Apo E4	1.8

Human Apo H	650.0
Human Apo J	3.0
Human Apo M	117.0

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.

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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LEGEND MAX™ Kits are manufactured by **BioLegend Inc.**
9727 Pacific Heights Blvd.
San Diego, CA 92121
Tel: 1.858.768.5800
Tel: US & Canada Toll-Free: 1.877.Bio-Legend (1.877.246.5343)
Fax: 1.877.455.9587
Email: info@biolegend.com
biolegend.com

For a complete list of world-wide BioLegend offices and distributors, please visit our website at: biolegend.com

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