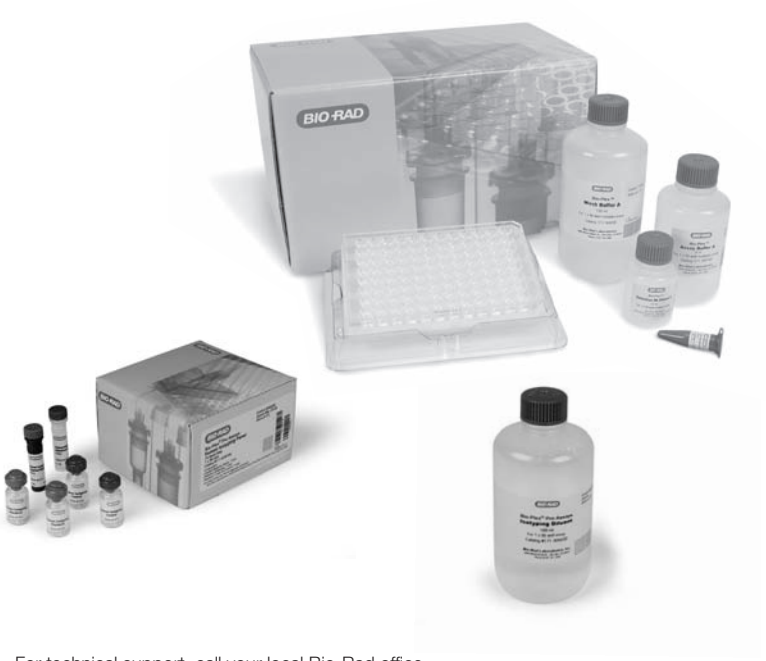
Bio-Plex Pro™ Assays
Immunoglobulin Isotyping
Instruction Manual



For technical support, call your local Bio-Rad office,
or in the US, call 1-800-4BIORAD (1-800-424-6723).

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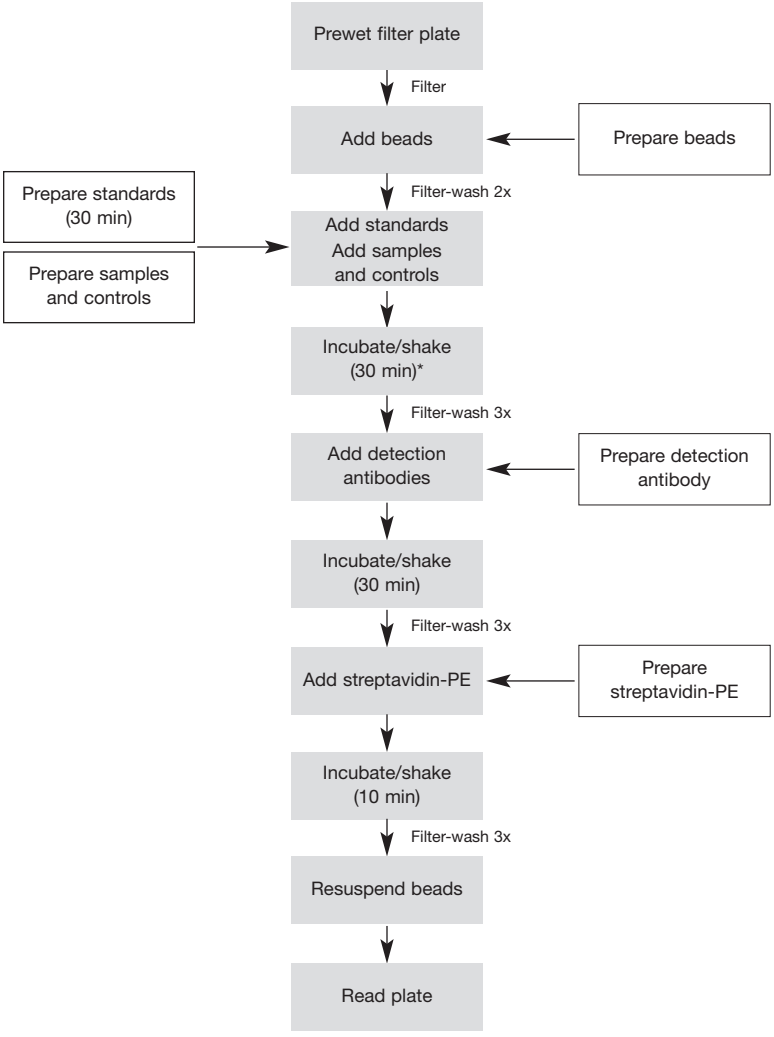
IMPORTANT NOTE: Bio-Plex Manager™ software version 4.1 or higher is required to use this kit.

Bio-Plex Isotyping Assay Workflow



Section 1
Introduction

Bio-Plex Pro™ immunoglobulin isotyping assays enable you to measure multiple immunoglobulin isotypes in only 10 µl of sample. A detailed profile of the immune response to infection, disease, vaccination, or drug therapy can now be obtained in a single 3-hour experiment. Bio-Plex Pro isotyping assays are multiplex bead-based assays designed to quantitate multiple immunoglobulin isotypes in diverse matrices, including serum, plasma, and tissue culture supernatants. These assays are optimized for the Bio-Plex suspension array system, which utilizes xMAP detection technology. For a brief overview of the assay protocol, see the Bio-Plex isotyping assay workflow above. The Bio-Plex suspension array system, which incorporates novel technology using color-coded beads, allows the simultaneous detection of up to 100 analytes in a single well of a 96-well microplate. The advantages over traditional methods of immunoglobulin isotyping include the ability to create a complete quantitative immunoglobulin class and subclass profile from limited sample, reduced sample preparation and assay time, and increased throughput. The use of magnetic beads offers the possibility for automated assay preparation. For a current listing of Bio-Plex assays, panels, and reagents, visit us on the Web at **www.bio-rad.com/bio-plex/**

Advantages of Magnetic Bead-Based Assays

Products in the Bio-Plex Pro family of assays are on magnetic polystyrene beads. These beads provide a choice in the method of assay preparation—standard or magnet-based. The standard workflow for Bio-Plex assay preparation requires multiple manual wash steps in which the 96-well filter plate is placed on a vacuum manifold to draw the liquid through the bottom of the filter plate. In contrast, magnet-based assay preparation permits liquid removal from the top of the well and thus does not require a filter plate or vacuum manifold. As a result, either an automated plate washer or a completely automated assay preparation system can be used, thus reducing hands-on time.

Section 2
Principle

The Bio-Plex® suspension array system is built around three core technologies. The first is the family of fluorescently dyed microspheres (beads) to which biomolecules are bound. The second is a flow cytometer with two lasers and associated optics to measure biochemical reactions that occur on the surface of the microspheres. The third is a high-speed digital signal processor that efficiently manages the fluorescent output. The Bio-Plex suspension array system in conjunction with the Bio-Plex Pro™ assays employs patented multiplexing technology that uses up to 25 magnetic polystyrene color-coded bead sets, each of which can be conjugated with a specific reactant. Each reactant is specific for a different target molecule. Bio-Plex isotyping assays are designed in a capture sandwich immunoassay format. Antibody specifically directed against the immunoglobulin isotype of interest is covalently coupled to color-coded 8 µm diameter magnetic polystyrene beads. The antibody-coupled beads are allowed to react with a sample containing an unknown amount of immunoglobulin isotypes, or with a standard or control solution containing a known amount of immunoglobulin isotypes. After performing a series of washes to remove unbound protein, a biotinylated detection antibody specific for a different epitope on the immunoglobulin class or subclass is added to the beads. The result is the formation of a sandwich of antibodies around the specific immunoglobulin isotype. The reaction mixture is detected by the addition of streptavidin-phycoerythrin (streptavidin-PE), which binds to the biotinylated detection antibodies. The constituents of each well are drawn up into the flow-based Bio-Plex suspension array system, which identifies and quantitates each specific reaction based on bead color and fluorescence. The magnitude of the reaction is measured using fluorescently labeled reporter molecules associated with each target protein. Unknown isotype concentrations are automatically calculated by Bio-Plex Manager™ software using a standard curve derived from a recombinant isotype standard. By using colored beads as the solid phase instead of a coated well, up to 25 differently colored beads can be mixed and used for quantitating up to 25 different analytes simultaneously.

Section 3
Bio-Plex® Reagent Kit Product Description

Isotype testing requires the Bio-Plex Pro™ isotyping reagent kit to run the multiplex panel.

Components of Bio-Plex Isotyping Reagent Kit	171-A3001M 1 x 96-Well Format	171-A3002M 10 x 96-Well Format
Bio-Plex assay buffer Store at 4°C. Do not freeze.	1 x 75 ml	1 x 750 ml
Bio-Plex wash buffer Store at 4°C. Do not freeze.	1 x 150 ml	2 x 750 ml
Bio-Plex detection antibody diluent Store at 4°C. Do not freeze.	1 x 15 ml	1 x 150 ml
Streptavidin-PE (100x) Store at 4°C. Do not freeze.	1 vial	1 vial
Sterile filter plate (96-well) with cover and tray	1 plate	10 plates
Sealing tape	1 pack of 4	10 packs of 4 (40)
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Storage and Stability

Kit components should be stored at 4ºC. Keep the streptavidin-PE in the dark. Do not freeze. All components are guaranteed for 6 months from the date of purchase when stored as specified in this manual.

Section 4
Materials Required or Recommended but Not Supplied

Required Materials: Isotyping Multiplex Panel

Isotype testing requires the Bio-Plex Pro™ isotyping reagent kit and a multiplex panel.

- Anti-isotype conjugated beads (25x concentration)
- Isotyping detection antibody (10x concentration)
- Isotyping standard (2 vials, lyophilized)
- Isotyping control (2 vials, lyophilized)

Please visit the Bio-Plex web site at **www.bio-rad.com/bio-plex/** for our latest list of assays and panels.

Recommended Materials: Isotyping Diluent

	Catalog #
Bio-Plex Isotyping Diluent	**171-305030 (1 x 96) 180 ml** **171-305031 (10 x 96) 1,800 ml**

Required Materials: Instrument and Accessories

In addition to the reagents and kits listed above, the following materials are required to run Bio-Plex assays or panels. For optimal results, we recommend the use of these specific items:

	Catalog #
Bio-Plex 200 Suspension Array System or Luminex System*	**171-000201**
Bio-Plex 200 Suspension Array System With High-Throughput Fluidics	**171-000205**
Bio-Plex Validation Kit Includes optics validation, classify validation, reporter validation, and fluidics validation bead set for approximately 50 validation routines using Bio-Plex Manager and MCV plate	**171-203001** **(for Bio-Plex Manager™ 4.1)**
Bio-Plex Calibration Kit	**171-203060**
Microplate Shakers IKA MTS 2/4 shaker for 2 or 4 microplates or Barnstead/Lab-Line Model 4625 plate shaker (or equivalent, capable of 300–1,100 rpm)	**IKA MTS 2/4 digital microtiter** **(IKA catalog #3208000)** **Model 4625** **(VWR catalog #57019-600)**

* Bio-Plex Manager 4.1 or higher must be used with the isotyping assays. Please select the 25-bead map. See Section 7 for details.

MultiScreen Resist Vacuum Manifold, available through Millipore, or **Aurum™ Vacuum Manifold**, available through Bio-Rad Warning: The use of filter plate manifolds other than the ones specified may result in filter plate leakage. See Vacuum Calibration Procedure in Section 6 for instructions specific to this assay.	**(Millipore catalog #MSVMHTS00)** **732-6470**
Vortexer VWR brand vortex mixer Scientific Instruments Vortex-Genie 2 mixer	**(VWR catalog #58816-121)** **(VWR catalog #58815-234)**
Sterilized Reagent Reservoirs Costar 50 ml reagent reservoir, available through Bio-Rad	**224-4872**
Other Pipets and pipet tips, sterile distilled water, aluminum foil, absorbent paper towels, and 1.5 ml or 2 ml microcentrifuge tubes	

Note Regarding Bio-Plex Pro Isotyping Assay

Bio-Plex Pro assays contain magnetic polystyrene beads that enable automated assay preparation, but do not require it. The standard protocol using a filter plate and vaccum manifold can be used as with standard polystyrene beads. For advice on automating magnetic bead panels, please contact Bio-Rad Technical Support at 1-800-4BIORAD or your local Bio-Rad office. Bio-Plex Pro assays will only work on Bio-Plex or Luminex instruments using Bio-Plex Manager software version 4.1 or higher.

Section 5
Sample Preparation and Premixed Standard Dilution

Bio-Plex® isotyping assays are designed to quantitate classes and subclasses of immunoglobulins in diverse matrices including serum, plasma, and tissue culture supernatants. For optimal recovery and sensitivity, it is important to properly prepare samples and standard curve dilutions. This section provides instructions for preparing sample and standard curve dilutions in the three different matrices.

Sample Preparation

Serum Samples (Bio-Plex Isotyping Diluent Is Recommended)

Allow the whole blood samples to clot for 1–2 hr at 37°C. Alternatively, use a serum separator tube and allow the blood samples to clot for 30 min. Centrifuge at 1,000 x g at 4°C. Collect the serum and assay immediately or freeze at –20°C. Avoid repeated freezing and thawing.

Prepare the thawed serum samples for analysis by diluting the sample 10,000-fold. A two-step serial dilution is recommended. First, dilute 10 µl of the serum in 990 µl of Bio-Plex Pro™ isotying diluent (ordered separately) for a 1:100 dilution. Second, dilute 10 µl of the 1:100 serum in 990 µl of Isotyping diluent for another 1:100 dilution. This gives you a final 1:10,000 dilution.

Extremely lipemic samples may be filtered through a 0.22 µm filter to prevent clogging. Please remember to use the Wash Between Plates command after every plate run to reduce the possibility of clogging the Bio-Plex instrument.

Reconstitute and dilute the Bio-Plex isotyping standard in the isotyping diluent.

Plasma Samples

Sodium citrate tubes are recommended; EDTA tubes are acceptable, but sodium citrate yields less clumping. Centrifuge at 1,000 x g at 4°C for 10 min. Collect the supernatant and filter through a sterile 0.22 µm filter. Collect the plasma and assay immediately or freeze at –20°C. Avoid

repeated freezing and thawing.

Prepare the thawed plasma samples for analysis by diluting the sample 1:10,000-fold. A two-step serial dilution is recommended. First dilute 10 µl of the serum in 990 µl of Isotyping diluent (ordered separately) for a 1:100 dilution. Dilute 10 µl of the 1:100 serum in 990 µl of Isotyping diluent for another 1:100 dilution. This gives you a final 1:10,000 dilution.

Please remember to use the Wash Between Plates command after every plate run to reduce the possibility of clogging the Bio-Plex instrument.

Reconstitute and dilute the Bio-Plex isotyping standard in the isotyping diluent.

Warning: Hemolyzed samples may not be suitable for Bio-Plex isotyping assays.

Cell Culture Samples

Keep all samples on ice until ready for use. Culture medium is recommended if dilution is required. Serum-free culture medium should contain carrier protein (such as BSA) at a concentration of at least 0.5%. Aliquot and store the samples at –70°C and avoid repeated freezing and thawing. Reconstitute and dilute the isotyping standard in the same medium or matrix in which cells are prepared. Be sure to include all medium components (such as FBS) as appropriate. To minimize error due to lot-to-lot variation of culture media, use the same lot of culture medium that was used to prepare the cells.

Premixed Standard Dilution

Reconstituting the Standards and Control

The isotyping standard should be reconstituted in the same matrix as that tested. For example, tissue culture samples grown in serum-supplemented RPMI should be reconstituted in serum-supplemented RPMI. Serum-free culture medium and saline solutions such as PBS should contain carrier protein (e.g., BSA) at a concentration of at least 0.5%. For serum and plasma samples, use Bio-Plex isotyping diluent (ordered separately). Refer to Section 4 for ordering information.

Two tubes of lyophilized isotyping standards and two tubes of lyophilized isotyping controls are provided in each 1 x 96-well isotyping kit. However, only one tube of each is required per 96-well plate. The insert provided with the isotyping kit lists the contents and the concentration values upon dilution for each isotype target. Each target has a different concentration range that will be entered into the Bio-Plex Manager™ software.

Making the Master Standard Stock

Do not store reconstituted multiplex standard stock for reuse. Reconstituted standard must be kept on ice and is stable for up to 12 hr.

1. Gently tap the glass vial containing the lyophilized standard on a solid surface to ensure the pellet is at the bottom. Reconstitute 1 tube of the lyophilized isotyping standard with 500 µl of the appropriate matrix (refer to Sample Preparation in this section). Do not use assay buffer to dilute standards.

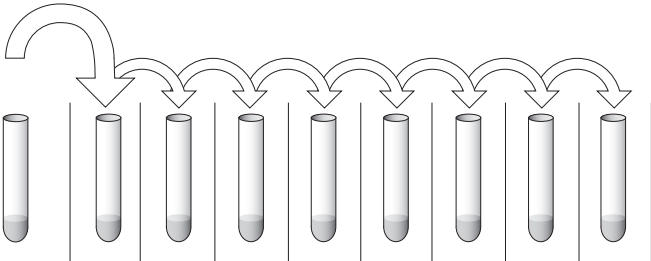
2. Gently vortex 1–3 sec and incubate on ice for 30 min. Refer to the product insert for the value of Standard I for each analyte.

Preparing Serial Dilutions of the Isotyping Standard

1. Label a set of 1.5 ml Eppendorf tubes with the concentrations shown in one of the isotyping standard curve charts. Pipet the appropriate volume of isotyping diluent or tissue culture medium into the tubes (see figure below).

Quick tip: The concentrations specified for the standard dilution set have been selected for optimized curve fitting using the 5-parameter logistic (5PL) or 4-parameter logistic (4PL) regression in Bio-Plex Manager software. Results generated using dilution points other than those listed in this manual have not been optimized.

Note: Dilute the isotyping standard in the same matrix as tested. Do not use assay buffer to dilute standards. Keep all tubes on ice throughout this procedure until ready for use.



Master standard stock

Stock (µl)		50	50	50	50	50	50	50
Standard diluent or medium (µl)		150	150	150	150	150	150	150
Concentration (ng/ml)*	**Std 1**	**Std 2**	**Std 3**	**Std 4**	**Std 5**	**Std 6**	**Std 7**	**Std 8**

* Each standard is a 4-fold dilution of the preceding one.

2. The reconstituted stock vial is Standard 1. Prepare 4-fold serial dilutions for standards 2–8.

3. Continue making serial dilutions of the standard as shown. After making each dilution, vortex gently and change the pipet tip after every transfer.

Quick-tip: Running at least two 0 pg/ml blanks is strongly recommended. The 0 pg/ml points should be formatted as "blanks", not as points in the curve, when using Bio-Plex Manager software. The "blank" wells are also useful for troubleshooting and determining LOD (Limit of Detection).

Making the Master Control Stock

Do not store reconstituted multiplex control stock for reuse. Reconstituted isotyping control must be kept on ice and is stable for up to 12 hr only.

1. Gently tap the glass vial containing the lyophilized control on a solid surface to ensure the pellet is at the bottom. Reconstitute 1 tube of the lyophilized Isotyping control with 1,000 µl of the appropriate matrix (Refer to Sample Preparation in this section).

2. Gently vortex 1-3 sec and incubate on ice for 30 min. Refer to the product insert for the range of the control for each analyte.

Section 6
Assay Procedure

Use these instructions for premixed Bio-Plex Pro™ isotyping assays. All the necessary components are provided premixed for ease of use. Prepare the Bio-Plex Pro standard dilution set, the Bio-Plex® isotyping control dilution, the Bio-Plex Pro bead stock (premixed, single vial), and the Bio-Plex detection antibody (premixed, single vial). Calibrate the vacuum manifold as specified in the Vacuum Calibration Procedure below.

Vacuum Calibration Procedure

Prior to performing any Bio-Plex assay, the vacuum apparatus must be calibrated to ensure an optimal bead yield. The procedure is provided here for reference. Please refer to Vacuum Manifold Setup in Section 3.9 of the Bio-Plex suspension array system hardware instruction manual for complete instructions for the manifold setup and validation.

1. Place a standard 96-well flat-bottom microplate (not a filter plate) on the vacuum apparatus.
2. Turn on the lab vacuum to maximum level and press down on the plate until a vacuum is established (typically 20–30" Hg).
3. Adjust the vacuum pressure using the gross and fine control valves on the unit. The pressure should be set to 1–2" Hg.
4. Once the vacuum is set correctly, remove the flat-bottom plate. Check the vacuum periodically, as house vacuum systems can fluctuate. Ensure that all wells are exposed to vacuum, as excess liquid can lead to less precise results. As a general guideline, 100 µl of liquid should take approximately 2 sec to completely clear the well.

Multiplex Assay Procedure

Prepare the samples and isotyping standard dilutions as directed in the previous sections. Reconstitute the isotyping control as directed in previous sections. Make sure the standards and samples have equilibrated to room temperature before adding them to the beads. Turn on the Bio-Plex system at least 30 min prior to reading a plate (see System Preparation in Section 7).

1. Bring all buffers and diluents to room temperature prior to use. Avoid bubbles when pipetting. Prepare multiplex bead working solution from 25x beads. Protect the beads from light as much as possible (for example, cover the bead tubes with aluminum foil). Keep all tubes on ice until ready for use.

Wells	25x Stock Beads (µl)	Bio-Plex Assay Buffer (µl)	Total Volume (µl)
96	240	5,760	6,000
48	120	2,880	3,000
32	80	1,920	2,000
24	60	1,440	1,500

   a. Calculate the total number of wells on a 96-well filter plate that will be used in this assay. Include the wells required for the test samples, controls, and the wells used for the isotyping standard dilution set. As a precaution, always factor in at least two extra wells for every eight wells required. Testing each sample in duplicate is recommended. For your convenience, a table for determining bead and assay buffer volumes is provided:

   b. Vortex the anti-isotype conjugated beads (25x) at medium speed for 30 sec.

   c. Prepare the conjugated beads using the volumes in the chart above or by calculating the volumes using the following formula: each well requires 2 µl of anti-isotype conjugated beads (25x) adjusted to a final volume of 50 µl using Bio-Plex assay buffer; multiply the “per well” volume by the total number of wells to calculate the multiplex bead working solution. Multiplying calculations by 1.25 to create 25% excess is recommended.

2. Prewet the desired number of wells of a 96-well filter plate with **100 µl** of **Bio-Plex assay buffer**. If fewer than 96 wells will be used, mark the plate to identify the unused wells for later use and cover the unused wells with sealing tape. Place the prewetted filter plate on a calibrated filter plate vacuum manifold. Remove the buffer by vacuum filtration. Dry the bottom of the filter plate thoroughly with a clean paper towel (preferably lint-free).

3. Vortex the **multiplex bead working solution** for 15–20 sec at medium speed and pipet **50 µl** into each well. Remove the buffer by vacuum filtration.

4. Dispense **100 µl** of **Bio-Plex wash buffer** to each well. Remove the buffer by vacuum filtration. Repeat this step. Blot the bottom of the filter plate once with a clean paper towel (preferably lint-free) to prevent cross-contamination. Place the filter plate on the plastic plate holder included with the kit.

5. Gently vortex each diluted standard and sample tube for 3–5 sec. Gently vortex the control and add 50 µl per well (no further dilution is required). Pipet **50 µl** of **diluted standard or sample** per well. Change the pipet tip after every volume transfer. Cover the entire filter plate with the plate sealing tape provided. Place the filter plate onto a microplate shaker, and then cover with aluminum foil. Slowly increase the shaker speed to 1,100 rpm, maintain for the first 30 sec of incubation, then reduce speed to 300 rpm and incubate at room temperature for 30 min.

6. At the end of the first incubation, place the plate on a flat surface and slowly remove the sealing tape. Be careful not to tip the plate or splash material from one well into another. Remove the buffer by vacuum filtration.

7. Wash 3 times with **100 µl** of **Bio-Plex wash buffer**. Remove the buffer by vacuum filtration after every wash. Blot the bottom of the filter plate with a clean paper towel (preferably lint-free) after every wash to prevent cross-contamination. Place the filter plate on the plastic plate holder included with the kit.

8. Prepare detection antibody solution. Note: Working detection antibody solution can be made 10 min before use.

Important: Store plate in dark while preparing solution.

a. Perform a 30 sec quick-spin centrifugation of the detection antibody vial prior to pipetting to collect the entire volume at the bottom of the vial.

b. Dilute the detection antibody to a 1x concentration using detection antibody diluent. For convenience, the following dilution tables are provided for the Bio-Plex detection antibody.

c. The 1x detection antibody is stable for up to 4 hr when stored in the dark at room temperature.

Note: Perform a 30 sec quick-spin centrifugation of the detection antibody vial before pipetting to collect the entire volume at the bottom of the vial.

Detection Antibody (10x)

Wells	10x Stock Detection Antibody (µl)	Detection Antibody Diluent A (µl)	Total Volume (µl)
96	300	2,700	3,000
48	150	1,350	1,500
32	100	900	1,000
24	75	675	750

d. Alternatively, the following formula can be applied to make up the detection antibody:

Each well requires 2.5 µl of detection antibody (10x stock) adjusted to a final volume of 25 µl using detection antibody diluent. Multiply these volumes by the number of wells required to prepare the Bio-Plex detection antibody stock. Multiplying calculations by 1.25 to create 25% excess is recommended.

9. Vortex the **Bio-Plex detection antibody** working solution gently and add **25 µl** to each well. Cover the entire filter plate with a new sheet of sealing tape (provided). Place the filter plate and plastic plate holder onto a microplate shaker, then cover it with aluminum foil.

Slowly increase the shaker speed to 1,100 rpm, maintain 1,100 rpm for the first 30 sec of incubation, and reduce to 300 rpm for 30 min. Incubate at room temperature. At the end of the 30 min incubation, remove the plate from the shaker and discard the sealing tape. Remove the buffer by vacuum filtration.

10. Wash 3 times with **100 µl** of **Bio-Plex wash buffer**. Remove the buffer by vacuum filtration after every wash. Blot the bottom of the filter plate with a clean paper towel (preferably lint-free) after each wash. Place the filter plate on the plastic plate holder included with the kit.

11. Prepare streptavidin-PE. Note: Streptavidin-PE can be made 10 min before use.

    Important: Store plate in dark while preparing solution.

    a. Perform a 30 sec quick-spin centrifugation of the streptavidin-PE vial before pipetting to collect the entire volume at the bottom of the vial.

    b. Dilute the streptavidin-PE (100x) to a 1x concentration with Bio-Plex assay buffer. Store in the dark after preparation. For convenience, the following dilution table is provided for the Bio-Plex streptavidin-PE dilution.

    c. The 1x streptavidin-PE is stable for up to 4 hr when stored in the dark at room temperature.

Wells	Streptavidin-PE (100x) (µl)	Bio-Plex Assay Buffer (µl)	Total Volume (µl)
96	60	5,940	6,000
48	30	2,970	3,000
32	20	1,980	2,000
24	15	1,485	1,500

d. Alternatively, the following formula can be applied to make up the streptavidin-PE:

   Dilute the streptavidin-PE (100x) to a 1x concentration with Bio-Plex assay buffer. Each well requires 0.5 µl of streptavidin-PE (100x) adjusted to a final volume of 50 µl using Bio-Plex assay buffer. The total volume of 1x streptavidin-PE required is based on the number of wells used; allow 50 µl per well. Multiplying calculations by 1.25 to create 25% excess is recommended.

12. Vortex the **1x streptavidin-PE** vigorously and add **50 µl** to each well. Cover the filter plate with a new sheet of sealing tape. Place the filter plate on a microplate shaker, and then cover it with aluminum foil. Slowly increase the shaker speed to 1,100 rpm, maintain for the first 30 sec of incubation, and reduce to 300 rpm. Incubation is 10 min at room temperature. At the end of the 10 min incubation, remove the plate from the shaker and discard the sealing tape. Remove the buffer by vacuum filtration.

13. Wash 3 times with **100 µl** of **Bio-Plex wash buffer**. Remove the buffer by vacuum filtration after every wash. Blot the bottom of the filter plate with a clean paper towel after each wash. Place the filter plate on the plastic plate holder included with the kit.

14. Resuspend the beads in each well with **125 µl** of **Bio-Plex assay buffer**. Cover the filter plate with a new sheet of sealing tape (provided). Place the filter plate and plastic plate holder on a microplate shaker, and shake the filter plate at room temperature at 1,100 rpm for 30 sec immediately before reading the plate on the Bio-Plex system. Remove the sealing tape before reading.

Section 7
Bio-Plex® Suspension Array System Operation

System Preparation

Recommendations for reading the Bio-Plex Pro™ isotyping assay on the Bio-Plex suspension array system are listed below. Alternatively, refer to the Bio-Plex Manager™ software user guide. Bio-Plex Pro isotyping assays require the low PMT setting.

1. Turn on the Bio-Plex array reader and microplate platform (and HTF system if present). Allow the system to warm up for 30 min.

Note: If the system is left idle for 4 hr, the lasers will automatically turn off. Another 30 min warm-up period will be required prior to reading an assay. Select Warm up from the tool bar and wait for the optics to reach operational temperature.

2. Select Start up from the toolbar and follow the instructions shown on the screen to prepare the reader to read an assay.

Note: Empty the waste and fill the sheath fluid bottle before starting. If the waste is overfilled, the fluidics system may back up and the assay signal may be lost. The sheath reservoir contains enough fluid for approximately two 96-well plates. If the sheath fluid level falls below the Sheath output tubing on the bottle, Bio-Plex Manager will pause the assay reading until the bottle is refilled.

Selecting the Low RP1 Target Value Using CAL2

1. Select Calibrate from the toolbar and follow the instructions shown on the screen to calibrate the reader. Daily calibration is recommended before reading the first assay.

2. Use the RP1 Low target value for CAL2 calibration. Enter the Low RP1 target value listed on the CAL2 calibration bottle label.

Preparing the Protocol

1. Select Step 1: Describe Protocol — enter any relevant information about your assay.
2. Select Step 2: Select Analytes — select the analytes in your assay.
3. Select Step 3: Format Plate — format all the wells that contain samples.

Note: The plate must be formatted and the analytes selected prior to reading a sample. The standard concentrations can be added before or after the plate has been read.

4. Select Step 4: Enter standards information — enter the concentrations for the standards. The 0 pg/ml multiplex standard dilution point is intended as a negative control to estimate the contribution of the background to the relative signal of the assay. This sample is not necessary for the generation of a standard curve and should be formatted as "blank" using Bio-Plex Manager software. Format the remaining wells that contain samples in them as unknown samples.
5. Select Step 7: Run Protocol — select 25 region under bead map in the Advanced Settings window and a 50 µl sample size.

Reading the Plate

1. Visually inspect the plate and ensure that corresponding assay wells are filled with buffer prior to placing the plate on the Bio-Plex microplate platform.
2. Shake the assay plate at 900 rpm for 30 sec immediately before starting the run. Failure to do so will result in an increased read time due to settling of the beads. Remove the sealing tape and any plate cover before placing the plate on the Bio-Plex microplate platform.
3. Select START in the Run Protocol dialog to initiate the assay read process.
4. If reading more than one plate, empty the waste and refill the sheath containers after each plate is run (see note in Step 2 of System Preparation at the beginning of this section). Select Wash Between

Plates  from the toolbar and follow the instructions shown on the screen to perform fluidics maintenance. Repeat steps 1–4.

5. When all the assay runs are complete, select Shut Down  from the toolbar and follow the instructions shown on the screen to prepare the reader for nonoperation.

Section 8
Data Analysis

Bio-Plex Manager™ software contains features that simplify the process of multiplex isotyping assay data analysis including determination of assay precision, selection of an appropriate curve fitting routine, and determination of the goodness of fit of the regression algorithm. For more details about data analysis features, see the Bio-Plex Manager software user guide.

For reference, several useful concepts relevant to analysis of immunoassay results derived from a standard curve are defined below.

Precision — The ability of a measurement to be consistently reproduced. Precision is represented by the coefficient of variation (CV) in Bio-Plex® assays and is shown for replicate samples in the CV% column of the report table. A CV% <10% indicates a good level of precision.

Outlier — A value that is perceived to be invalid compared to other replicate values. Outliers may be eliminated in Bio-Plex assays by clicking on the check box in the outlier column in the report table.

4PL, 5PL — The terms 4PL and 5PL refer to four-parameter or five-parameter logistic regression algorithms. These regressions are commonly used in immunoassays, including Bio-Plex assays, and provide a larger range of quantitation than standard linear regression analysis.

Goodness of fit — A practical method for measuring the goodness of the fit of a regression is known as “backfit” of standards or “backcalculation” of standards. Once a regression equation is derived, the fluorescence intensity (FI) values of the standards are treated as unknowns and the concentration of each standard is calculated. A ratio of the calculated value to the expected value of this standard is determined. A ratio between 70 and 130% for each of the standards indicates a good fit. The “Conc in Range” column in the report tables displays only the values for samples that are within the valid range of the standard curve. Data for all samples is displayed in the “Obs Conc” column.

Section 9
Troubleshooting Guide

This troubleshooting guide addresses problems that may be encountered with the Bio-Plex Pro™ isotyping assay. If you experience any of the problems listed below, review the possible causes and solutions provided. This will assist you in resolving problems directly related to how the assay steps should be performed. Poor assay performance may also be due to the Bio-Plex® array reader. To eliminate this possibility, we highly recommend use of the Bio-Plex validation kit. This kit will validate all the key functions of the array reader and assist the user in determining whether or not the array reader is functioning properly.

Possible Causes	Possible Solutions
Filter Plate Leakage	
Vacuum setting too high	This could tear the filter. Confirm that the vacuum pressure is set as specified in the vacuum calibration procedure section. Also refer to the Vacuum Manifold Setup in Section 3.9 of the Bio-Plex suspension array system hardware instruction manual. Use the recommended filter plate vacuum apparatus.
Filter plate incubated at an angle	Be sure to set the plate on a flat and level surface when incubating.
High Coefficient of Variation (CV)	
Standards and samples were not kept on ice during preparation	Prepare standards and samples on ice prior to transferring to the filter plate.
Bottom of filter plate not dry	Dry the bottom of the filter plate with absorbent paper towel (preferably lint-free) to prevent cross-contamination.

Possible Causes	Possible Solutions
Plate sealer was reused	This could cause contamination. Use a new sheet of plate sealer for each incubation.
Pipetting technique	Pipet carefully and slowly when adding standards, samples, detection antibodies, and streptavidin-PE, especially when using a multichannel pipet. Use a calibrated pipet. Change pipet tip after every volume transfer.
Contamination with Bio-Plex wash buffer A during wash steps	During the wash steps, be careful not to splash Bio-Plex wash buffer A from one well to another. Be sure that the wells are filtered completely and that no residual volume remains. Also, be sure that the microplate shaker setting is not too high. Reduce the microplate shaker speed to minimize splashing.
Low Bead Count	
Miscalculation of bead dilution	Check your calculations and be careful to add the correct volumes.
Beads clumped in multiplex bead stock tube	Vortex for 15–20 sec at medium speed before aliquoting beads.
Vacuum setting too high	This could tear the filter. Check the vacuum pressure and use the recommended setting. Use the recommended filter plate vacuum apparatus.

Possible Causes	Possible Solutions
Vacuum on for too long when aspirating buffer from wells	Do not apply vacuum to the filter plate for longer than 10 sec after the buffer is completely drained from each well.
Added too much Bio-Plex assay buffer A before reading plate	Be sure to resuspend the beads in each well with the correct volume of Bio-Plex assay buffer A prior to reading the plate.
Beads exposed to too much light	Always store beads in the dark. Be sure to incubate plate in the dark. Prolonged exposure to light may affect some bead regions more than others.
Did not shake filter plate enough before incubation steps and prior to reading	Shake the filter plate at 1,100 rpm for 30 sec before incubation steps and immediately before reading the plate.
Reader is clogged	Refer to the troubleshooting guide in the Bio-Plex hardware instruction manual.
Low Signal or Poor Sensitivity	
Standards and samples were not kept on ice during preparation	Be sure to prepare standards and samples on ice prior to transferring to the filter plate.
Standards reconstituted incorrectly	Follow the isotyping standard instructions carefully.
Detection antibody or streptavidin-PE diluted incorrectly	Check your calculations and be careful to add the correct volumes.

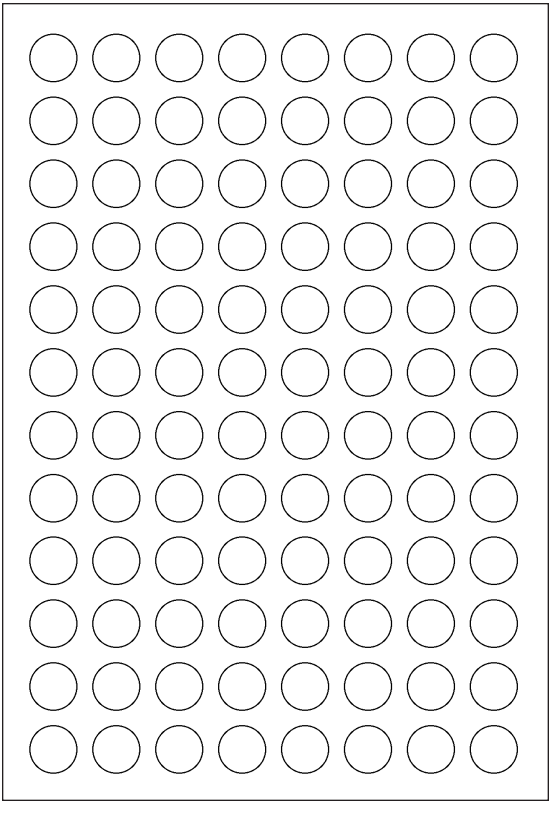
Possible Causes	Possible Solutions
Expired beads, standards, detection antibody, or streptavidin-PE were used	Use new or unexpired components.
Did not shake filter plate enough before incubation steps and prior to reading	Shake the filter plate at 1,100 rpm for 30 sec before incubation steps and immediately before reading the plate.
Did not shake filter plate during incubation steps	Shake the filter plate as specified in the incubation step instructions. Be sure to follow the recommended incubation times.
No Beads Seen in Bead Map	
25 bead map not selected prior to run	Repeat the assay. Rereading the plate is also possible. See instructions in the Cytokine Assay Instruction Manual.
High Background Signal	
Incorrect buffer was used (for example, assay buffer A used to dilute standards)	Use sample matrix or serum standard diluent to dilute isotype standards.
Expired Bio-Plex reagents were used	Check that reagents have not expired. Use new or unexpired components.
Spiked “0 pg/ml” wells by mistake	Be careful when spiking standards. Do not add any antigens in the 0 (blank) point.
Streptavidin-PE incubated too long	Follow the procedure incubation time.

Possible Causes	Possible Solutions
Filter plate sat at room temperature too long before reading	If the plate will not be read immediately, place it on the tray provided, cover with aluminum foil, and store at 4°C.
Poor Recovery	
Expired Bio-Plex reagents were used	Check that reagents have not expired. Use new or unexpired components.
Incorrect amounts of components were added	Check your calculations and be careful to add the correct volumes.
Samples and standards not loaded at the same time	Samples must be loaded at the same time as the standards.
Microplate shaker set to an incorrect speed	Check the microplate shaker speed and use the recommended setting. Setting the speed too high may cause splashing and contamination. Use the recommended plate shaker.
Pipetting technique	Pipet carefully and slowly when adding standards, samples, controls, detection antibodies, and streptavidin-PE, especially when using a multichannel pipet. Use a calibrated pipet. Change pipet tip after every volume transfer.

Section 10
Safety Considerations

Eye protection and gloves are recommended while using this product. Consult the MSDS for additional information.

Section 11
Bio-Plex Pro™ Isotyping Assay Template and Dilution Worksheet



# of required wells _____

# of extra wells _____ (2 wells for every 8 required wells)

_____ total number of wells for dilution calculations

Bead Dilution

50 µl/well

2 µl of anti-isotype bead (25x) stock solution/well

_____ x 2 µl = _____ anti-isotype bead (25x) stock solution

_____ x 48 µl = _____ **Bio-Plex® assay buffer A**

_____ x 50 µl = _____ total volume

Detection Antibody Dilution

Note: The degree to which the stock detection antibody solution needs to be diluted depends on the level of multiplexing of the premixed panel that is being used. Detection antibodies for premixed panels containing 2 to 9 target analytes are supplied in a 50x stock solution. Detection antibodies for premixed panels containing more than 9 target analytes are supplied in a 25x stock solution.

25 µl/well

2.5 µl of detection antibody (**10x**) stock solution/well

_____ x 2.5 µl = _____ detection antibody (10x) stock solution

_____ x 22.5 µl = _____ **Bio-Plex detection antibody diluent A**

_____ x 25 µl = _____ total volume

Streptavidin-PE Dilution

50 µl/well

0.5 µl of streptavidin-PE (**100x**) stock solution/well

_____ x 0.5 µl = _____ streptavidin-PE (100x) stock solution

_____ x 49.5 µl = _____ **Bio-Plex assay buffer A**

_____ x 50 µl = _____ total volume

Section 12
Legal Notices

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By purchasing this kit, which contains fluorescently labeled microsphere beads authorized by Luminex, you, the customer, acquire the right under Luminex's patent rights* to use this kit or any portion of this kit, including without limitation the microsphere beads contained herein, only with Luminex's laser-based fluorescent analytical test instrumentation known under the name of Luminex 100, for example as marketed by Bio-Rad Laboratories, Inc. in the Bio-Plex system.

* Including, but not limited to US patents 5,981,180; 6,046,807; 6,057,107

Bio-Rad Laboratories, Inc.

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Web site *www.bio-rad.com* ***USA*** *800 4BIORAD* ***Australia*** *02 9914 2800*
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Taiwan *886 2 2578 7189/2578 7241* ***United Kingdom*** *020 8328 2000*

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