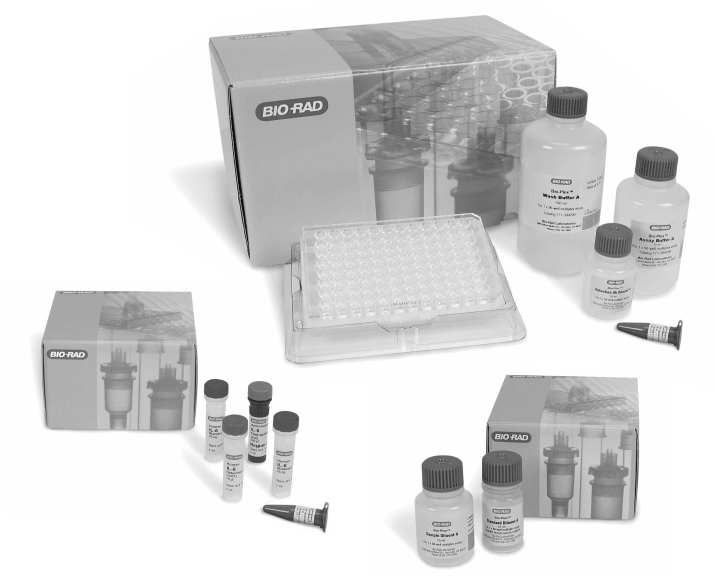
Bio-Plex™
Cytokine Assay
Instruction Manual



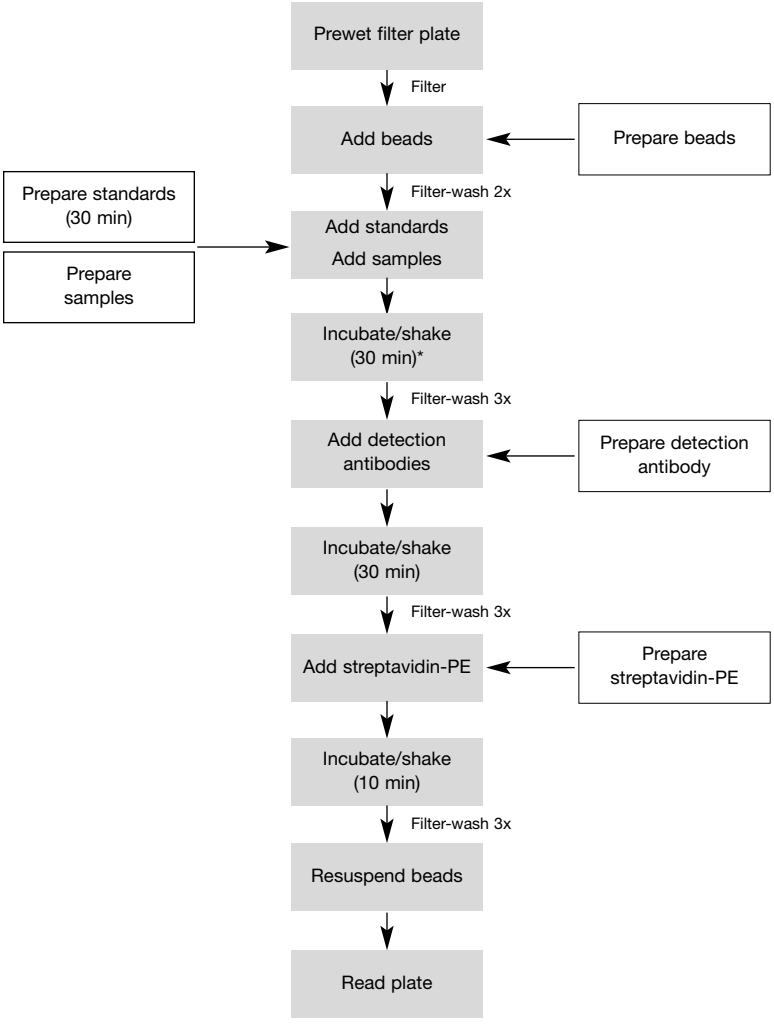
For technical service, call your local Bio-Rad office,
or in the US, call 1-800-4BIORAD (1-800-424-6723).

For research use only. Not for diagnostic procedures.

BIO-RAD

Table of Contents

Bio-Plex Cytokine Assay Workflow



* For the human Th1/Th2 magnetic panel, the incubation time is 60 min.

Section 1
Introduction

Cytokines are important cell signaling proteins, mediating a wide range of physiological responses, including immunity, inflammation, and hematopoiesis. They are also associated with a spectrum of diseases ranging from tumor growth to infections to Parkinson's disease. Cytokines are typically measured either by bioassay or immunoassay. Both techniques are time consuming and can facilitate the analysis of only a single cytokine at a time. The Bio-Plex suspension array system, which incorporates novel technology using color-coded beads, permits the simultaneous detection of up to 100 cytokines in a single well of a 96-well microplate.

Bio-Plex cytokine assays are multiplex bead-based assays designed to quantitate multiple cytokines in diverse matrices, including serum samples, plasma samples, and tissue culture supernatants. For a brief overview of the protocol, see the Bio-Plex Cytokine Assay Workflow. The 96-well microplate-format Bio-Plex assays are optimized for the Bio-Plex suspension array system, which utilizes xMAP detection technology. By multiplexing, it is possible to quantitate the level of multiple cytokines in a single well in just 3 hr, using as little as 12.5 µl of serum or 50 µl of tissue culture sample. The advantages over traditional immunoassays that analyze only a single cytokine at a time include the ability to create a complete cytokine profile from limited sample, reduce sample preparation time, and increase throughput. For a current listing of Bio-Plex cytokine assays, panels, and reagents, visit us on the Web at **www.bio-rad.com/BioPlexSystem/**

Available As Premixed or Unmixed Multiplex Assays

Bio-Rad offers both mixed-to-order panels for multiplex cytokine assays (panels include premixed beads, detection antibody, and standard) and singleplex configurations. Premixed multiplex panels test for the presence of a predetermined set of cytokines in a single sample. All the necessary panel components are provided premixed for ease of use. Procedures for this configuration are provided in Section 6, Assay Procedure for Premixed Multiplex Panels and Singleplex Assays.

Bio-Plex technology allows end users to combine multiplex or singleplex reagents. By choosing among a series of available singleplex cytokine assays, components can be combined to create a tailored multiplex assay. The singleplex configuration provides maximum flexibility, enabling end users to choose the cytokines that they wish to combine to meet their specific analysis needs. Procedures for mixing assays are provided in Section 7, Mixing Multiplex Assays: Bead, Standard, and Detection Antibody Preparation.

For research use only. Not for diagnostic procedures.

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 employs patented multiplexing technology that uses up to 100 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 cytokine assays are designed in a capture sandwich immunoassay format. Antibody specifically directed against the cytokine of interest is covalently coupled to color-coded 5.6 µm polystyrene beads. The antibody-coupled beads are allowed to react with a sample containing an unknown amount of cytokine, or with a standard solution containing a known amount of cytokine. After performing a series of washes to remove unbound protein, a biotinylated detection antibody specific for a different epitope on the cytokine is added to the beads. The result is the formation of a sandwich of antibodies around the cytokine. 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 cytokine concentrations are automatically calculated by Bio-Plex Manager™ software using a standard curve derived from a recombinant cytokine standard. By using colored beads as the solid phase instead of a coated well, up to 100 differently colored beads can be mixed and used for quantitating up to 100 different analytes simultaneously.

Section 3
Product Description

Cytokine testing requires the Bio-Plex cytokine reagent kit to run any singleplex assay, any multiplex panel, or any x-Plex™ custom panel. If serum or plasma samples are to be tested, Bio-Rad recommends species-specific diluent kits for optimum recovery (refer to Section 4).

The Bio-Plex cytokine reagent kit contains the following components:	**171-304000 1 x 96-Well Format**	**171-304001 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)
Cytokine instruction manual	1	1

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: Cytokine Singleplex Assay or Multiplex Panel

Cytokine testing requires the Bio-Plex cytokine reagent kit and either a singleplex assay, a multiplex panel, or an x-Plex multiplex panel. If serum or plasma samples are to be tested, Bio-Rad recommends species-specific diluent kits for optimum recovery.

The Bio-Plex cytokine assays and panels contain the following components:
Anti-cytokine conjugated beads (25x concentration)
Cytokine detection antibody (check vial for concentration prior to dilution and/or mixing)
Cytokine standard (2 vials, lyophilized)

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

Recommended Materials: Serum Diluent

If serum or plasma samples are to be tested, Bio-Rad recommends these species-specific diluent kits for optimum recovery:

	Catalog #
Bio-Plex Human Serum Diluent Kit	**171-305000 (1 x 96)** **171-305001 (10 x 96)**
Bio-Plex human serum sample diluent	15 ml/150 ml
Bio-Plex human serum standard diluent	10 ml/100 ml
Bio-Plex Mouse Serum Diluent Kit	**171-305004 (1 x 96)** **171-305005 (10 x 96)**
Bio-Plex mouse serum sample diluent	15 ml/150 ml
Bio-Plex mouse serum standard diluent	10 ml/100 ml
Bio-Plex Rat Serum Diluent Kit	**171-305008 (1 x 96)**
Bio-Plex rat serum sample diluent	15 ml
Bio-Plex rat serum standard diluent	10 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 4.0)**
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)**

* See p. 13 for directions for using Bio-Plex cytokine assays on the Luminex 100 or 200 system.

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 7 for instructions specific to this assay.	**(Millipore catalog #MAVM0960R)** **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 Magnetic Bead Cytokine Assay Panels

For magnetic bead panels, filter plates can be used as with polystyrene beads. For advice on atuomating magnetic bead panels, please contact Technical Support. Bio-Plex magnetic bead panels will only work on Bio-Plex or Luminex instruments using Bio-Plex Manager software version 4.1 or greater.

Section 5
Sample Preparation and Premixed Standard Dilution

Bio-Plex cytokine assays are designed to quantitate multiple cytokines in diverse matrices including serum samples, plasma samples, 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. For sample preparations not mentioned here (including tissue, branchoaleovar lavage, cerebrospinal fluid, and others), consult the publications listed in Bio-Rad bulletin 5297, available for download at **discover.bio-rad.com**.

Sample Preparation

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 cytokine 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.

Lavage Samples

Keep all samples on ice until ready for use. If dilution is required, use the lavage wash buffer that was used to collect the sample. Reconstitute and dilute the cytokine standard in the same lot of lavage wash buffer that was used to collect the sample. Add carrier protein (such as BSA) at a concentration of at least 0.5%.

Sputum and Other Biological Fluids

Keep all samples on ice until ready for use. If dilution is required, use a buffer that is similar to the sample. Reconstitute and dilute the cytokine standard using a buffer that is as similar to the sample as possible. Add carrier protein (such as BSA) at a concentration of at least 0.5%.

Serum Samples (Bio-Plex Serum Diluent Kit 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 1 volume of the serum sample with 3 volumes of the appropriate species-specific Bio-Plex sample diluent. For human serum samples, use Bio-Plex human serum sample diluent. Likewise, for mouse serum samples use Bio-Plex mouse serum sample diluent. For rat serum samples, use Bio-Plex rat serum sample diluent.

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 cytokine standard in the appropriate Bio-Plex species-specific serum standard 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 1 volume of the plasma sample with 3 volumes of the appropriate species-specific Bio-Plex sample diluent.

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 cytokine standard in the appropriate Bio-Plex species-specific standard diluent.

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

Premixed Standard Dilution

Reconstituting the Cytokine Standard

The cytokine 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 samples, use Bio-Plex serum standard diluent (ordered separately in the Bio-Plex human, mouse, or rat serum diluent kit). Refer to Section 4 for ordering information.

Two tubes of lyophilized cytokine standard are provided in each 1 x 96-well Bio-Plex cytokine assay or panel. However, only one of the tubes is required per 96-well plate. The insert provided with the cytokine assay lists the contents of the cytokine standard and the values for each standard. If you are mixing cytokine standards, please refer to Section 7, Mixing Multiplex Assays: Bead, Standard, and Detection Antibody Preparation instead of using the procedure below.

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 only.

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 cytokine standard with 500 µl of the appropriate matrix (refer to Sample Preparation in this section). Do not use assay buffer to dilute standards.

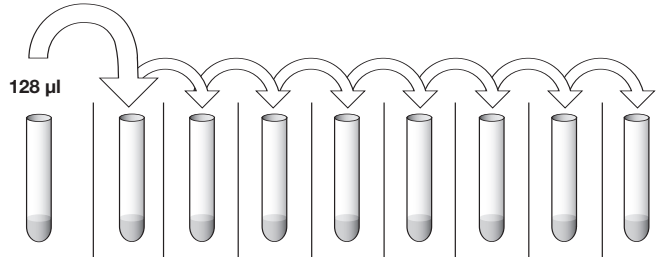
2. Gently vortex 1–3 sec and incubate on ice for 30 min. Refer to the product insert for the value of Standard I for each analyte. **If no insert is provided, use 32,000 pg/ml as the concentration of Standard I, when running at the Bio-Plex standard PMT setting.**

Preparing Serial Dilutions of the Cytokine Standard

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

Quick tip: The cytokine 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.

Low PMT Setting for Broad Range Standard Curve (Calibrate Bio-Plex system with CAL2 low RP1 target value)



Master standard stock								
Stock (µl)	128	50	50	50	50	50	50	50
Standard diluent or medium (µl)	72	150	150	150	150	150	150	150
Concentration (pg/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.

Note: Dilute the cytokine 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.

2. Add 128 µl of the multiplex master stock to a single 1.5 ml tube containing 72 µl of the appropriate serum standard diluent or tissue culture medium. Vortex gently.

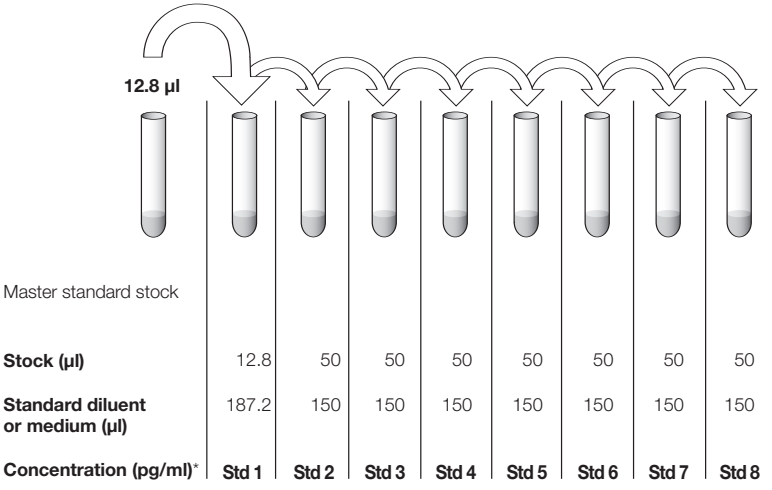
3. Continue making serial dilutions of the standard as shown. After making each dilution, vortex gently and change the pipet tip after every transfer.

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.

Optional (Narrow Range) Curve

If the concentrations are expected to be in the range 10–1,000 pg/ml, such as in serum, then use the high PMT setting (see below). This procedure will prepare enough standard to run each dilution in duplicate. It is recommended to run a low PMT setting standard curve first.

High PMT Setting for Narrow Range Standard Curve (Calibrate Bio-Plex system with CAL2 high RP1 target value)



Stock (µl)	12.8	50	50	50	50	50	50	50
Standard diluent or medium (µl)	187.2	150	150	150	150	150	150	150
Concentration (pg/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.

Information for Running Bio-Plex Cytokine Assays on the Luminex 100 or 200 Instrument

When running the Bio-Plex low PMT standard curve, do not change the Luminex settings. Calibrate with the Luminex CAL2 settings. Set gates according to Luminex procedure.

When running the Bio-Plex high PMT standard curve, calibrate using High RP1 (high PMT) calibration for CAL2. When using Luminex calibration beads, notice that the High RP1 value (high PMT) is not printed on the vial. The equation below provides the conversion factor to calculate the High RP1 (high PMT) value when using Luminex calibration beads.

Luminex RP1 x 4.55 = Bio-Plex High RP1 (high PMT).

Set gates according to Luminex procedure.

Doublet discriminator (DD) gates are automatically set by Bio-Plex Manager software in the Bio-Plex instrument. For the Luminex instrument, the DD gates should be set according to Luminex procedure.

Section 6
Assay Procedure for Premixed Multiplex Panels and Singleplex Assays

Use these instructions for premixed Bio-Plex cytokine panels and singleplex assays that are designed for the analysis of a predetermined set of cytokines. If you intend to mix beads from different panels or assays, refer to Section 7, Mixing Multiplex Assays: Bead, Standard, and Detection Antibody Preparation.

All the necessary components are provided premixed for ease of use. Prepare the Bio-Plex standard dilution set (premixed, single vial), the Bio-Plex bead stock (premixed, single vial), and the Bio-Plex detection antibody. 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 (for Premixed Assays)

Prepare the samples and cytokine standard dilutions as directed in the previous sections. Turn on the Bio-Plex system at least 30 min prior to reading a plate (see System Preparation in Section 8).

Bring all buffers and diluents to room temperature prior to use. Avoid bubbles when pipetting.

1. 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.

   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 and the wells used for the cytokine 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:

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

   b. Vortex the anti-cytokine 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-cytokine 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 flick the bottom of each diluted standard and sample tube 3–5 times. 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. If using magnetic bead cytokine assays, incubate for 60 min at room temperature..

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.

Important: Bio-Plex detection antibody concentrations are not all the same. Always check the detection antibody concentration on the vial label before diluting.

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

Detection Antibody (25x)

Wells	25x Stock Detection Antibody (µl)	Detection Antibody Diluent A (µl)	Total Volume (µl)
96	120	2,880	3,000
48	60	1,440	1,500
32	40	960	1,000
24	30	720	750

Detection Antibody (50x)

Wells	50x Stock Detection Antibody (µl)	Detection Antibody Diluent A (µl)	Total Volume (µl)
96	60	2,940	3,000
48	30	1,470	1,500
32	20	980	1,000
24	15	735	750

Detection Antibody (100x)

Wells	100x Stock Detection Antibody (µl)	Detection Antibody Diluent A (µl)	Total Volume (µl)
96	30	2,970	3,000
48	15	1,485	1,500
32	10	990	1,000
24	7.5	742.5	750

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.

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

Each well requires 0.5 µl of detection antibody (assuming 50x) 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. 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
Mixing Multiplex Assays: Bead, Standard, and Detection Antibody Preparation

Mixing assay beads and detection antibody can expand cytokine panels and singleplex assays. For example, conjugated beads and detection antibody from the Bio-Plex human G-CSF assay can be combined with the Bio-Plex human 8-Plex A panel to create a 9-plex panel. By choosing among available singleplex cytokine assays and following this procedure, these assay components can be combined to create a tailored multiplex assay.

Prepare the Bio-Plex cytokine standard dilutions, combining standards if necessary. Refer to the assay kit insert to determine which cytokines are present in the cytokine standard. If all analytes to be assayed are already present in the standard, mixing is not necessary. Prepare the Bio-Plex conjugated beads (combine conjugated beads) and the Bio-Plex detection antibody (combine detection antibody).

The procedure to run assays that are manually mixed is the same as that for panels that are premixed. The only difference is that the conjugated beads and the detection antibody must be mixed manually prior to use. The cytokine standard may not require mixing if it contains all the analytes to be tested.

Reconstituting the Cytokine Standard

The insert provided with the cytokine assay lists the contents of the cytokine standard. In most cases, all cytokines to be tested are already included in the cytokine standard. Prior to performing this procedure, check the contents of the cytokine standard by referring to the assay insert.

Please review Section 5, Sample Preparation and Premixed Standard Dilution, prior to making dilutions for the standard curve. The cytokine standard should be diluted in the same matrix as tested. For example, tissue culture samples grown in serum-supplemented RPMI should be diluted in serum-supplemented RPMI. For serum samples, use Bio-Plex serum standard diluent (ordered separately in the Bio-Plex human, mouse, or rat serum diluent kit). Refer to Section 4 for ordering information.

Preparing Master Standard for Mixing With Other Cytokine Standards

Duplicate vials of lyophilized cytokine standard are provided in each 1 x 96-well Bio-Plex cytokine assay or panel. However, only one vial is required per 96-well plate.

Do not store reconstituted multiplex standard stock for reuse. Reconstituted standard must be kept on ice and is stable for up to 6 hr only.

1. Reconstitute each lyophilized cytokine standard with 50 µl of the same matrix as samples. Do not use assay buffer to dilute standards.

2. Gently vortex 1–3 sec and incubate on ice for 30 min. This produces a multiplex cytokine standard.

3. Add 24 µl from each of the master standards stock into a single 1.5 ml tube containing 150 µl of the appropriate standard diluent or tissue culture medium. Adjust to a final volume of 375 µl. Vortex gently. This master mix will serve as Standard I in the low PMT setting standard curve.

Mixing Cytokine Standards

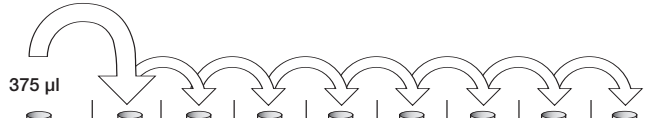
1. Label a set of 1.5 ml Eppendorf tubes with the concentrations shown in one of the cytokine standard curve charts below. Pipet the appropriate volume of standard diluent or tissue culture medium into the tubes.

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

2. Continue making serial dilutions of the standard as shown in the following chart. After making each dilution, vortex gently and change the pipet tip after every volume 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.

Low PMT Setting Cytokine Standard Curve (Calibrate Bio-Plex system with CAL2 low RP1 target value)



Master standard stock								
Stock (µl)	375	50	50	50	50	50	50	50
Standard diluent or medium (µl)	0	150	150	150	150	150	150	150
Concentration (pg/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.

Mixing Conjugated Beads

This procedure is for preparing a multiplex bead working solution from 25x beads. Protect the beads from light as much as possible (cover the bead tubes with aluminum foil). Keep all tubes on ice until ready for use.

1. 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 and the wells used for the multiplex 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, the following table is provided as an example for mixing two sets of conjugated beads:

Mixing Two Sets of Conjugated Beads

Wells	25x Stock Beads (µl), for Example, G-CSF	25x Stock Beads (µl), for Example, 8-plex	Bio-Plex Assay Buffer (µl)	Total Volume (µl)
96	240	240	5,520	6,000
48	120	120	2,760	3,000
32	80	80	1,840	2,000
24	60	60	1,380	1,500

2. Vortex each vial of anti-cytokine conjugated beads (25x) at medium speed for 15–20 sec. Use equal volumes of each conjugated bead when mixing. Total volume should always be the same regardless of how many beads are combined, as in the example above.

3. Prepare the multiplex bead stock using the volumes in the chart above or by calculating using the formula below:

   Each well requires 2 µl of anti-cytokine conjugated beads (25x) adjusted to a final volume of 50 µl using Bio-Plex cytokine assay buffer. Multiply the per well volumes by the total number of wells to calculate the multiplex bead working solution. Multiplying calculations by 1.25 to create 25% excess is recommended.

Detection Antibody Preparation

1. 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.
2. Dilute the detection antibody to a 1x concentration using detection antibody diluent. For added convenience, dilution tables are provided as examples for mixing Bio-Plex detection antibody.
3. The 1x detection antibody is stable for up to 4 hr when stored in the dark at room temperature.

Important: Bio-Plex detection antibody concentrations are not all the same. Always check the detection antibody concentration on the vial label prior to dilution.

Mixing 100x With 10x Detection Antibody

Wells	100x Stock Detection Antibody (µl)	10x Stock Detection Antibody (µl)	Detection Antibody Diluent A (µl)	Total Volume (µl)
96	30	300	2,670	3,000
48	15	150	1,335	1,500
32	10	100	890	1,000
24	7.5	75	667.5	750

Mixing 10x With 10x Detection Antibody

Wells	10x Stock Detection Antibody (µl)	10x Stock Detection Antibody (µl)	Detection Antibody Diluent A (µl)	Total Volume (µl)
96	300	300	2,400	3,000
48	150	150	1,200	1,500
32	100	100	800	1,000
24	75	75	600	750

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.

Alternatively, the following formula can be applied to make up the detection antibody: each well requires 0.5 µl of detection antibody (assuming 50x) 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.

Streptavidin-PE Preparation

Streptavidin-PE preparation is the same for premixed and manually mixed assays. Please refer to Streptavidin-PE Preparation in Section 6 for instructions.

Section 8
Bio-Plex Suspension Array System Operation

System Preparation

Recommendations for reading the Bio-Plex cytokine assay on the Bio-Plex suspension array system are listed below. Alternatively, refer to the Bio-Plex Manager software user guide.

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 tool bar 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 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 High or Low RP1 target value using CAL2

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

2. If you have prepared the low PMT setting standards set, use the RP1 Low target value for CAL2 calibration. If you have set up the high PMT setting standards set, use the RP1 High target value for CAL2 calibration. Both the High and Low RP1 target values are 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 100 beads per region 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 1,100 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 tool bar and follow the instructions shown on the screen to prepare the reader for nonoperation.

Rereading a Plate

It is possible to analyze a well (sample) a second time using the Rerun/Recovery mode of Bio-Plex Manager software. To reread a well or the entire plate, remove the buffer by vacuum filtration and resuspend the beads in each well with 125 µl of Bio-Plex assay buffer. Cover the filter plate with a new sheet of the 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. Perform Reading the Plate steps 4–6 above.

Important: If you reread a well in Rerun/Recovery mode, any previous data for that well will be overwritten. Make sure that only the well(s) to be reread are checked off.

Section 9
Data Analysis

Bio-Plex Manager software contains features that simplify the process of multiplex cytokine 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 details about the data analysis features of Bio-Plex Manager, see the Bio-Plex Manager 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 variartion (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 10
Troubleshooting Guide

This troubleshooting guide addresses problems that may be encountered with the Bio-Plex cytokine 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 Set Up in Section 3.9 of the Bio-Plex suspension array system hardware instruction manual. Use the recommended filter plate vacuum apparatus.
Filter plate incubated overnight 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 aliquotting 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 cytokine 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.
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 cytokine 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.
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.

Possible Causes	Possible Solutions
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, detection antibodies, and streptavidin-PE, especially when using a multichannel pipet. Use a calibrated pipet. Change pipet tip after every volume transfer.

Section 11
Safety Considerations

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

Section 12
Publications Citing the Bio-Plex Cytokine Assay

For the most current list of publications, please download bulletin 5297 from the Bio-Rad web site, **discover.bio-rad.com**.

Brandt k, et al., Interleukin-21 inhibits dendritic cell activation and maturation, Blood 102, 4090–4098 (2003)

Cui X, et al., Lethality during continuous anthrax lethal toxin infusion is associated with circulatory shock but not inflammatory cytokine or nitric oxide release in rats, Am J Physiol Regul Integr Comp Physiol 286, R699–R709 (2004)

Cummings KL and Tarleton RL, Inducible nitric oxide synthase is not essential for control of *Trypanosoma cruzi* infection in mice, Infect Immun 72, 4081–4089 (2004)

Dixit VD, et al., Ghrelin inhibits leptin- and activation-induced proinflammatory cytokine expression by human monocytes and T cells, J Clin Invest 114, 57–66 (2004)

Eko FO, et al., A novel recombinant multisubunit vaccine against *Chlamydia*, J Immunol 173, 3375–3382 (2004)

Eriksson AM, et al., The cholera toxin-derived CTA1-DD vaccine adjuvant administered intranasally does not cause inflammation or accumulate in the nervous tissues, J Immunol 173, 3310–3319 (2004)

Foster-Cuevas M, et al., Human herpesvirus 8 K14 protein mimics CD200 in down-regulating macrophage activation through CD200 receptor, J Virol 78, 7667–7676 (2004)

Galindo CL, et al., Microarray analysis of *Aeromonas hydrophila* cytotoxic enterotoxin-treated murine primary macrophages, Infect Immun 72, 5439–5445 (2004)

Hartmann E, et al., Identification and functional analysis of tumor-infiltrating plasmacytoid dendritic cells in head and neck cancer, Cancer Res 63, 6478–6487 (2003)

Hausl C, et al., Preventing restimulation of memory B cells in hemophilia A: a potential new strategy for the treatment of antibody-dependent immune disorders, Blood 104, 115–122 (2004)

Iwata A, et al., Over-expression of Bcl-2 provides protection in septic mice by a trans effect, J Immunol 171, 3136–3141 (2003)

Kaschina E, et al., Genetic kininogen deficiency contributes to aortic aneurysm formation but not to atherosclerosis, Physiol Genomics 19, 41–49 (2004)

Koh KR, et al., Immunomodulatory derivative of thalidomide (ImiD CC-4047) induces a shift in lineage commitment by suppressing erythropoiesis and promoting myelopoiesis,

Blood (2004) Aug. 3 [e-pub ahead of print, 2004-03-0828]

Latham KA, et al., Estradiol treatment redirects the isotype of the autoantibody response and prevents the development of autoimmune arthritis, J Immunol 171, 5820–5827 (2003)

Lentzsch S, et al., Macrophage inflammatory protein 1-alpha (MIP-1 alpha) triggers migration and signaling cascades mediating survival and proliferation in multiple myeloma (MM) cells, Blood 101, 3568–3573 (2003)

Mekala DJ and Geiger TL, Immunotherapy of autoimmune encephalomyelitis with redirected CD4+CD25+ T lymphocytes, Blood 105, 2090–2092 (2004)

Murphey ED, et al., Gamma interferon does not enhance clearance of *Pseudomonas aeruginosa* but does amplify a proinflammatory response in a murine model of postseptic immunosuppression, Infect Immun 72, 6892–6901 (2004)

Oku H, et al., Role of IL-18 in pathogenesis of endometriosis, Hum Reprod 19, 709–714 (2004)

Rutella S, et al., Role for granulocyte colony-stimulating factor in the generation of human T regulatory type 1 cells, Blood 100, 2562–2571 (2002)

Singh UP et al., Inhibition of IFN-gamma-inducible protein-10 abrogates colitis in IL-10-/- mice, J Immunol 171, 1401–1406 (2003)

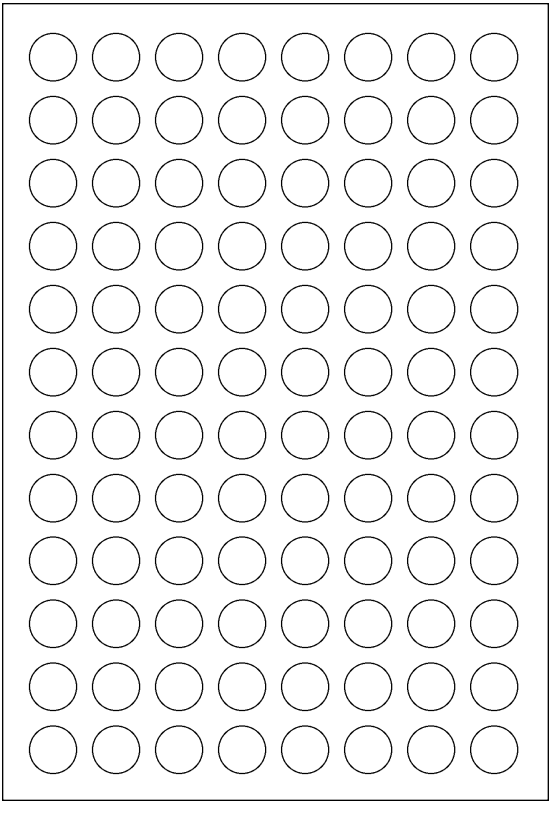
Smed-Sorensen A, et al., HIV-1-infected dendritic cells up-regulate cell surface markers but fail to produce IL-12 p70 in response to CD40 ligand stimulation, Blood 104, 2810–2817 (2004)

Takabe W, et al., Lysophosphatidylcholine enhances cytokine production of endothelial cells via induction of L-type amino acid transporter 1 and cell surface antigen 4F2, Arterioscler Thromb Vasc Biol 24, 1640–1645 (2004)

van Rijn RS, et al., A new xenograft model for graft-versus-host disease by intravenous transfer of human peripheral blood mononuclear cells in RAG2-/- gammac-/- double-mutant mice, Blood 102, 2522–2531 (2003)

Yang R, et al., Papillomavirus-like particles stimulate murine bone marrow-derived dendritic cells to produce alpha interferon and Th1 immune responses via MyD88, J Virol 78, 11152–11160 (2004)

Section 13
Bio-Plex Multiplex Cytokine 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-cytokine bead (25x) stock solution/well

_____ x 2 µl = _____ anti-cytokine 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

0.5 µl of detection antibody (**50x**) stock solution/well

_____ x 0.5 µl = _____ detection antibody (50x) stock solution

_____ x 24.5 µl = _____ **Bio-Plex detection antibody diluent A**

_____ x 25 µl = _____ total volume

or

25 µl/well

1 µl of detection antibody (**25x**) stock solution/well

_____ x 1 µl = _____ detection antibody (25x) stock solution

_____ x 24 µl = _____ **Bio-Plex detection antibody diluent A**

_____ x 25 µl = _____ total volume

or

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 15
Legal Notices

Costar is a trademark of Coming Costar Corporation. Eppendorf is a trademark of Eppendorf-Netheler-Hinz GmbH. Luminex 100 and xMAP are trademarks of Luminex Corporation. Multiscreen is a trademark of Millipore Corporation. Vortex-Genie is a trademark of Scientific Industries, Inc.

Leukemia Inhibitory Factor Antigen (LIF) is provided under an agreement between Chemicon International, Inc. and Bio-Rad and the manufacture, use, sale or import of this product may be subject to one or more of U.S. Patents and corresponding foreign equivalents. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product for research use only by the buyer, where such research does not involve medical, diagnostic, or any other clinical testing, analysis or screening services or providing clinical information or clinical analysis in return for compensation on a per-test basis. The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research.

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.

Life Science Group

Web site *www.bio-rad.com* ***USA*** *800 4BIORAD* ***Australia*** *02 9914 2800*
Austria *01 877 89 01* ***Belgium*** *09 385 55 11* ***Brazil*** *55 21 3237 9400*
Canada *905 712 2771* ***China*** *86 21 6426 0808*
Czech Republic *420 241 430 532* ***Denmark*** *44 52 10 00*
Finland *09 804 22 00* ***France*** *01 47 95 69 65* ***Germany*** *089 318 84 0*
Greece *30 210 777 4396* ***Hong Kong*** *852 2789 3300*
Hungary *36 1 455 8800* ***India*** *91 124 4029300/5013478* ***Israel*** *03 963 6050*
Italy *39 02 216091* ***Japan*** *03 5811 6270* ***Korea*** *82 2 3473 4460*
Mexico *55 5200 05 20* ***The Netherlands*** *0318 540666*
New Zealand *64 9415 2280* ***Norway*** *23 38 41 30* ***Poland*** *48 22 331 99 99*
Portugal *351 21 472 7700* ***Russia*** *7 095 721 14 04*
Singapore *65 6415 3188* ***South Africa*** *27 0861 246 723*
Spain *34 91 590 5200* ***Sweden*** *08 555 12700* ***Switzerland*** *061 717 95 55*
Taiwan *886 2 2578 7189/2578 7241* ***United Kingdom*** *020 8328 2000*

4110004 US/EG Rev F *06-0143 0305 Sig 1205*