

# Bio-Plex Pro™ Cytokine, Chemokine, and Growth Factor Assays

## Instruction Manual

For technical support, call your local Bio-Rad office, or in the U.S., call 1-800-424-6723.  
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**BIO-RAD**

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# Introduction

Cytokines, chemokines, and growth factors are a diverse group of cell signaling proteins expressed and secreted by virtually all cell types, including cells of endothelial, epithelial, and immune origin. These proteins interact with specific receptors on target cells to mediate important physiological responses such as growth, immunity, inflammation, and hematopoiesis. Dysregulation of expression is associated with pathological conditions ranging from cancer and diabetes to infection and autoimmune disease.

Bio-Plex Pro™ assays enable researchers to quantify multiple protein biomarkers in a single well of a 96-well plate in 3–4 hours. These robust immunoassays require as little as 12.5 µl serum or plasma or 50 µl cell culture supernatant or other biological fluid. The use of magnetic (MagPlex) beads allows researchers to automate wash steps on a Bio-Plex Pro (or similar) wash station. Magnetic separation offers greater convenience and reproducibility compared to vacuum filtration.

For more information please visit [www.bio-rad.com/bio-plex](http://www.bio-rad.com/bio-plex).

# Principle

## Technology

The Bio-Plex® multiplex system is built upon the three core elements of xMAP technology:

- Fluorescently dyed microspheres (also called beads), each with a distinct color code or spectral address to permit discrimination of individual tests within a multiplex suspension. This allows simultaneous detection of up to 500 different types of molecules in a single well of the 96-well microplate on the Bio-Plex® 3D system, up to 100 different types of molecules on the Bio-Plex® 200 system, and up to 50 different types of molecules on the Bio-Plex® MAGPIX™ system
- On the Bio-Plex 200 and Bio-Plex 3D systems, a dedicated flow cytometer with two lasers and associated optics to measure the different molecules bound to the surface of the beads. In the Bio-Plex MAGPIX, the entire sample load volume is injected into a chamber where the beads are imaged using LED and CCD technology
- A high-speed digital signal processor that efficiently manages the fluorescence data

## Assay Format

Bio-Plex Pro™ assays are essentially immunoassays formatted on magnetic beads. The assay principle is similar to that of a sandwich ELISA (Figure 1). Capture antibodies directed against the desired biomarker are covalently coupled to the beads. Coupled beads react with the sample containing the biomarker of interest. After a series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin-phycoerythrin (SA-PE) conjugate. Phycoerythrin serves as a fluorescent indicator or reporter.

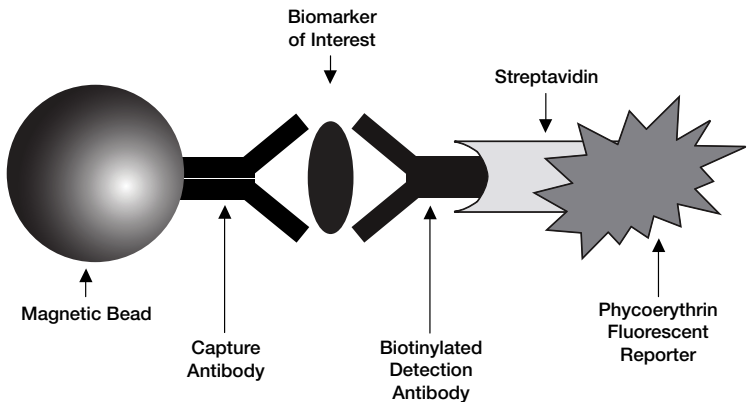


Fig. 1. Bio-Plex sandwich immunoassay.

### Data Acquisition and Analysis

Data from the reactions are acquired using a Bio-Plex system or similar Luminex-based reader. When a multiplex assay suspension is drawn into the Bio-Plex 200 reader, for example, a red (635 nm) laser illuminates the fluorescent dyes within each bead to provide bead classification and thus assay identification. At the same time, a green (532 nm) laser excites PE to generate a reporter signal, which is detected by a photomultiplier tube (PMT). A high-speed digital processor manages data output, and Bio-Plex Manager™ software presents data as median fluorescence intensity (MFI) as well as concentration (pg/ml). The concentration of analyte bound to each bead is proportional to the MFI of reporter signal.

Using Bio-Plex Data Pro™ software, data from multiple instrument runs can be combined into a single project for easy data management, quick visualization of results, and simple statistical analysis.

# Kit Contents and Storage

## Reagents Supplied

Bio-Plex Pro™ cytokine assays are offered in a convenient kit format that includes assay, reagent, and diluent components in a single box (Table 1).

**Table 1. Contents of Bio-Plex Pro cytokine, chemokine, and growth factor assays.\***

<b>Component</b>	<b>1 x 96-Well Format</b>	<b>10 x 96-Well Format</b>
Standard diluent*	10 ml	100 ml
Sample diluent*	40 ml	80 ml
Assay buffer	50 ml	500 ml
Wash buffer	200 ml	1.5 L
Detection antibody diluent	5 ml	50 ml
Streptavidin-PE (100x)	1 tube	1 tube
Filter plate and/or flat bottom plate (96-well)	1 plate	10 plates
Sealing tape	1 pack of 4	10 packs of 4
Assay Quick Guide	1 booklet	1 booklet
Standard	1 vial	10 vials
<b>Human and Mouse Cytokine (Group I and II)</b>		
Coupled magnetic beads (10x)	1 tube	1 tube
Detection antibodies (10x)	1 tube	1 tube
<b>Mouse Cytokine (Group III) and Rat Cytokine (Group I)</b>		
Coupled magnetic beads (20x)	1 tube	1 tube
Detection antibodies (20x)	1 tube	1 tube

\* Bio-Plex Pro high dilution reagent kit, 1 x 96-well, contains 70 ml serum-based diluent in lieu of standard diluent and sample diluent.

## Storage and Stability

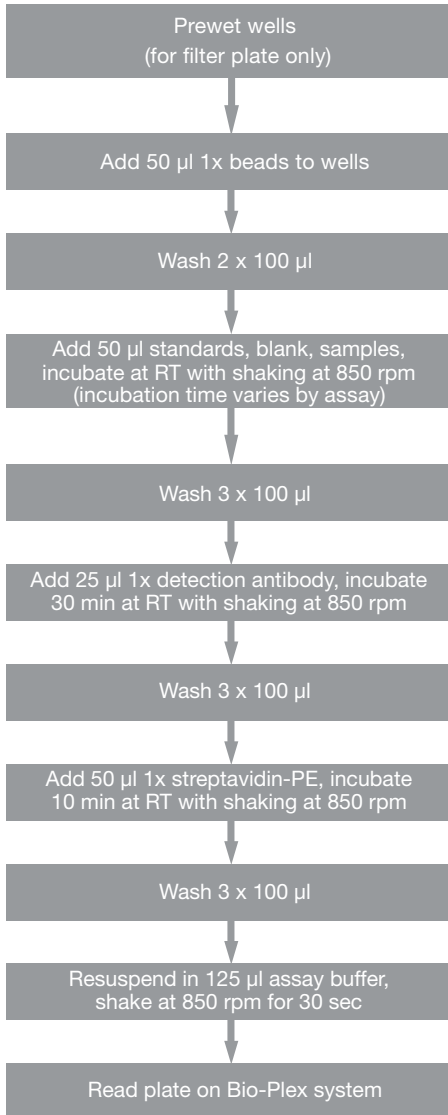
Kit contents should be stored at 4°C and never frozen. Coupled magnetic beads and streptavidin-PE should be stored in the dark. All components are guaranteed for a minimum of six months from the date of purchase when stored as specified.

**Table 2. Recommended materials.**

<b>Item</b>	<b>Ordering Information</b>
<b>Bio-Plex Pro Assays Quick Guide 4</b>	Bulletin #10024985 (download at <a href="http://www.bio-rad.com/bio-plex">www.bio-rad.com/bio-plex</a> )
<b>Bio-Plex® 200 system or Luminex system with HTF</b>	Bio-Rad catalog #171-000205
<b>Bio-Plex validation kit</b> Run the validation kit monthly to ensure optimal performance of fluidics and optics systems	Bio-Rad catalog #171-203001
<b>Bio-Plex calibration kit</b> Run the calibration kit daily to standardize fluorescence signal	Bio-Rad catalog #171-203060
<b>Bio-Plex Pro wash station</b> For use with magnetic bead-based assays only	Bio-Rad catalog #300-34376
<b>Bio-Plex Pro II wash station</b> For use with both polystyrene (nonmagnetic) and magnetic bead-based assays	Bio-Rad catalog #300-34377
<b>Bio-Plex handheld magnetic washer</b> For use with magnetic bead-based assays only	Bio-Rad catalog #170-20100
<b>Bio-Plex Pro flat bottom plates (40 x 96-well)</b> For magnetic separation on the Bio-Plex Pro wash station	Bio-Rad catalog #171-025001
<b>Microtiter plate shaker</b> 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 catalog #320-8000  VWR catalog #57019-600
<b>Bio-Rad® Aurum™ vacuum manifold</b> For vacuum filtration	Bio-Rad catalog #732-6470
<b>BR-2000 vortexer</b>	Bio-Rad catalog #166-0610
<b>Reagent reservoirs, 25 ml</b> For capture beads and detection antibodies	VistaLab catalog #3054-1002 or VistaLab catalog #3054-1004
<b>Reagent reservoir, 50 ml (for reagents and buffers)</b>	VistaLab catalog #3054-1006
<b>Pall Life Science Acrodisc:</b> 25 mm PF syringe filter (0.8/0.2 µm Supor membrane)	Pall Life Sciences catalog #4187
<b>Filter plate, 1 x 96-well, with clear plastic lid and tray</b>	Bio-Rad catalog #171-304502
<b>Titertube® micro test tube</b>	Bio-Rad catalog #223-9390

**Other:** 15 ml polypropylene tubes for reagent dilutions, calibrated pipets, pipet tips, sterile distilled water, aluminum foil, absorbent paper towels, 1.5 or 2 ml microcentrifuge tubes, and standard flat bottom microplate (for calibrating vacuum manifold).

# Assay Workflow





# Important Considerations

## Instruments and Software

The cytokine assays described in this manual are compatible with all currently available Luminex-based life science research instruments. Assays can be read and analyzed with either Bio-Plex Manager™ software or Luminex xPonent software.

## Assay Procedures

Pay close attention to vortexing, shaking, and incubation times and to Bio-Plex® reader PMT (RP1) setting, as these have been optimized specifically for each assay panel.

## Assay Quick Guide

Each assay kit includes a printed Bio-Plex Pro™ Assay Quick Guide (bulletin #10024985), which can be used to prepare and run a full 1 x 96-well assay plate. Users can also download a copy at [www.bio-rad.com/bio-plex](http://www.bio-rad.com/bio-plex).

## Bead Regions

Bead regions for all analytes are listed in the Read Plate section.

## Multiplexing Compatibility

For human and mouse, the maximum number of singleplex diabetes and cytokine analytes that may be mixed is limited by the 10x cytokine antibody stock concentrations as shown in the table below.

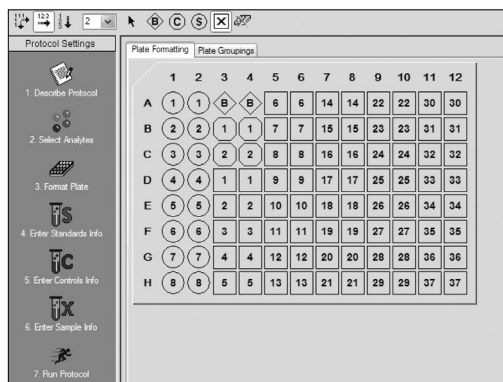
**Table 3. Maximum number of singleplex cytokine and diabetes analytes that may be multiplexed.**

Human, mouse, and rat diabetes Mouse cytokine (group III) analytes (20x)	0	2	4	6	8	10
Human and mouse cytokine (groups I, II) analytes (10x)	10	9	8	7	6	5





# 1. Plan Plate Layout

Prior to running the assay, determine the total number of wells in the experiment using the Plate Layout Template on page 40 or the Plate Formatting tab in Bio-Plex Manager™. A suggested plate layout is shown in Figure 2, with all conditions in duplicate.

1. Assign standards to columns 1 and 2, with the highest concentration in row A and the lowest concentration in row H.
2. Assign the blank to wells A3 and A4. The blank should consist of your chosen standard diluent. Note that Bio-Plex Manager automatically subtracts the blank (B) MFI value from all other assay wells.
3. User-defined controls are assigned to wells in columns 3 and 4.
4. The remainder of the plate is available for samples.
5. Once the total number of wells is known, you can calculate the required volumes of beads, detection antibody, and streptavidin-PE. Use Tables 10–14, 18–22, and 23, respectively, or the Calculation Worksheet on page 41.



## Legend

-  Standard
-  Blank
-  Samples
-  Controls



**Fig. 2. Suggested plate layout.** For detailed instructions on plate formatting in Bio-Plex Manager, see section 8.

## 2. Prepare Instrument


Start up and calibrate the Bio-Plex® 100/200 or similar system with Bio-Plex Manager™ software prior to setting up the assay. The calibration kit should be run daily or before each use of the instrument to standardize the fluorescent signal. To prepare either a Bio-Plex 3D or Bio-Plex® MAGPIX™ reader, consult its respective user manual.


The validation kit should be run monthly to ensure performance of fluidics and optics systems. Refer to either the software manual or online Help for directions on how to conduct validation.

### Start Up System (Bio-Plex 100, 200, or Similar)

1. Empty the waste bottle and fill the sheath fluid bottle before starting if high throughput fluidics (HTF) are not present. This will prevent fluidic system backup and potential data loss.
2. Turn on the reader, XY platform, and HTF (if included). Allow the system to warm up for 30 min (if not already done).
3. Select Start up  and follow the instructions. If the system is idle for 4 hr without acquiring data, the lasers will automatically turn off. To reset the 4-hr countdown, select Warm up  and wait for the lasers/ optics to reach operational temperature.

### Calibrate System

1. Select Calibrate  and confirm that the default values for CAL1 and CAL2 are the same as the values printed on the bottle of Bio-Plex calibration beads. Use the Bio-Plex system low RP1 target value even if assays will be run at high RP1.
2. Select OK and follow the software prompts for step-by-step instructions for CAL1 and CAL2 calibration.

**Note:** In Bio-Plex Manager version 6.1 and higher, startup, warm up, and calibration can be performed together by selecting the “Start up and calibrate” icon. 

### 3. Prepare Wash Method

Bio-Plex Pro™ assays are compatible with both magnetic separation and vacuum filtration methods. However, for best results, we recommend performing the assays in a flat bottom plate with magnetic separation.

**Table 4. Summary of compatible wash stations and plate types.**

<b>Wash Method</b>	<b>Wash Station</b>	<b>Assay Plate</b>
Magnetic separation	Bio-Plex Pro Bio-Plex Pro II (use MAG programs) Bio-Plex® handheld magnetic washer	Flat bottom plate
Vacuum filtration	Bio-Plex Pro II (use VAC programs) Vacuum manifold (manual)	Filter plate

#### **Setting up the Bio-Plex Pro or Bio-Plex Pro II Wash Station**

The wash station does not require calibration; however, it should be primed before use. For more information, refer to the Bio-Plex Pro and Pro II wash station quick guide (bulletin #5826).

1. Install the appropriate plate carrier on the wash station.
2. Use the prime procedure to prime channel 1 with wash buffer.

#### **Setting Up the Bio-Plex Handheld Magnetic Washer**

Place an empty flat bottom plate on the magnetic washer by sliding it under the retaining clips. Push the clips inward to secure the plate. Make sure the plate is held securely. If needed, the clips can be adjusted for height and tension. For detailed instructions, refer to the user guide (bulletin #10023087).

#### **Setting up a Vacuum Manifold**

Calibrate the vacuum manifold by placing a standard 96-well flat bottom plate on the unit and adjusting the pressure to -1 to -3" Hg. In general, 100 µl liquid should take 3–4 sec to clear the well. For more detailed instructions, refer to bulletin #10005042.

## 4. Prepare Standards

### General Instructions

- **It is essential to reconstitute and dilute standards exactly as described in this section.** Incorrect preparation may lead to low signal, high background, or inconsistent measurements from plate to plate
- The peel-off label provided with the standards lists the concentration of the most concentrated dilution point, S1. Enter this information into Bio-Plex Manager™ software as instructed in Section 8
- For users who wish to mix assays from different panels, such as diabetes assays with group I cytokines, guidance is provided here for mixing 2 different lyophilized standards. Bead regions were chosen to avoid overlap whenever possible. However, performance of multiplexes containing assays from different groups have not been extensively validated. Therefore, users must confirm that the assay performance is still fit for purpose

### Selecting a Diluent for Standards

Refer to Table 5 for recommended diluents based on different sample types.

As a general rule, reconstitute and dilute standards in a diluent similar to the final sample type or sample matrix.

**Table 5. Summary of recommended diluents for standards.**

<b>Sample Type</b>	<b>Diluent for Standards</b>	<b>Add BSA</b>
Serum and plasma	Standard diluent	None
Culture media, with serum	Culture media	None
Culture media, serum-free	Culture media	To 0.5% final
Lavage, sputum, other fluids	Bio-Plex® sample diluent	To 0.5% final*
Lysate	Bio-Plex sample diluent	To 0.5% final*

\* At least 0.5% final w/v BSA is recommended to stabilize analytes and reduce absorption to labware.

## RP1 (PMT) Setting for Standard Curves

The Bio-Plex 200 and 3D systems have two RP1 (PMT or photomultiplier tube) setting options, while the Bio-Plex® MAGPIX™ has no PMT and therefore no PMT setting options. Instead, MAGPIX uses default instrument settings similar to low PMT on the Bio-Plex 200 (Table 6).

**Table 6. Overview of PMT setting options on Bio-Plex systems.**

<b>Instrument</b>	<b>RP1 (PMT)</b>
Bio-Plex 100, 200*	Low, high
Bio-Plex 3D*	Standard, enhanced
Bio-Plex MAGPIX*	N/A, use default instrument settings

\* Or similar Luminex-based system.

The Bio-Plex Pro™ human and mouse cytokine assays were developed on the low PMT setting using the Bio-Plex 200 system, while the rat cytokine assays were developed on the high PMT setting. Protocols using alternative standard dilution series or different PMT settings should be validated by the end user, for example when mixing the cytokine assays with diabetes assays (Table 7 and Table 24).

**Table 7. Settings for optimal sensitivity on the Bio-Plex 200 system\*.**

<b>Assay</b>	<b>Low RP1 (PMT)</b>	<b>High RP1 (PMT)</b>
Human cytokines (group I, II)	•	User validation required*
Mouse cytokines (group I, II, III)	•	User validation required*
Rat cytokines (group I)	User validation required*	•
<b>Cross-panel mixing</b>	<b>Low RP1 (PMT), broad range cytokine curve</b>	<b>High RP1 (PMT), narrow range cytokine curve</b>
Human cytokines + diabetes		•
Mouse cytokines + diabetes	User validation required*	•
Rat cytokines + diabetes		•

\* Contact Bio-Rad technical support for the most up to date recommendations on PMT settings and cross-panel multiplexing compatibility.

## Reconstitute a Single Vial of Standards

This procedure prepares enough material to run each dilution in duplicate.

1. Gently tap the vial containing the lyophilized standard.

2. Add **500 µl** of the appropriate diluent (see Table 5). Do not use assay buffer to reconstitute the standards.
3. Gently vortex the reconstituted standard for **5 sec** then incubate on ice for **30 min**. Be consistent with the incubation time in every assay to ensure best results.
4. During the incubation period, prepare the samples as instructed in the Prepare Samples section.

## Prepare Standard Dilution Series From a Single Antigen Vial

The following procedure produces an eight-point standard curve with a fourfold dilution between each point. Pipet carefully using calibrated pipets and use new pipet tips for every volume transfer.

1. Label nine 1.5 ml polypropylene tubes S1 through S8 and Blank.
2. Add the specified volume of standard diluent to each tube (Figures 3 and 4).
3. **Vortex** the reconstituted standards gently for **5 sec** before removing any volume. Add **128 µl** into the S1 tube containing **72 µl** of standard diluent. **Vortex** at medium speed for **5 sec**, then use a new pipet tip to transfer **50 µl** from S1 tube to S2 tube. **Vortex**.
4. Continue with 1:4 (fourfold) serial dilutions from tube S2 to S8 as shown in Figure 3. Use reconstituted and diluted standards immediately. Do not freeze for future use.

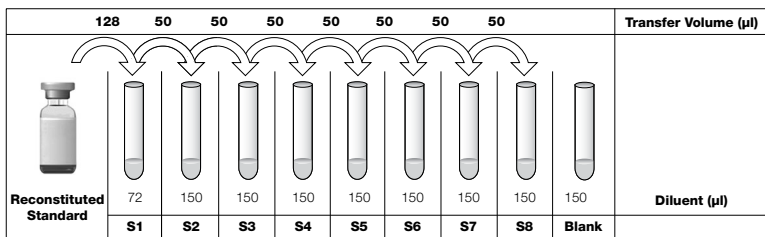


Fig. 3. Preparing a fourfold dilution series of cytokine standards.

## Reconstituting Standards for Cross-Panel Plexing

Follow these directions when mixing human or mouse cytokine and diabetes assays. Note that rat diabetes and cytokine standards are premixed into one standard vial. Therefore, no extra mixing is required.

Two mixing scenarios are provided in Figures 4 and 5 for detection at high and low PMT respectively. One results in a narrow range cytokine standard curve for detection at high RP1 (PMT); the other gives a broad range cytokine standard curve for detection at low RP1 (PMT).

1. Gently tap both vials of lyophilized cytokine and diabetes standards.
2. **For high PMT** setting/narrow range cytokine standard curve, add **500  $\mu$ l** of the appropriate standard diluent to each vial. **For low PMT** setting/broad range cytokine standard curve, add **250  $\mu$ l** of diluent to each vial. Do not use assay buffer or sample diluent to reconstitute the standards.
3. **Gently vortex** the reconstituted standards for **5 sec** then incubate **on ice for 30 min**. Be consistent with the incubation time in every assay to ensure best results.
4. During the incubation period, prepare the samples as instructed in the Prepare Samples step.

## Preparing Serial Dilutions

Pipet carefully with a calibrated pipet, and use new pipet tips for every volume transfer.

1. Label nine 1.5 ml polypropylene tubes S1 through S8 and Blank.
2. **For high PMT/narrow range** cytokine standard curve, add **59.2  $\mu$ l** of standard diluent to the S1 tube. **For low PMT/broad range** cytokine standard curve, add **72  $\mu$ l** of diluent to S1 (Figures 4 and 5).
3. **For high PMT/narrow range** cytokine standard curve, add **12.8  $\mu$ l** of the reconstituted cytokine standard and **128  $\mu$ l** of the reconstituted diabetes standard to the S1 tube for a total volume of 200  $\mu$ l (Figure 4). **Vortex** for **5 sec**.



- Add **150  $\mu\text{l}$**  of standard diluent to the remaining tubes, as shown in Figure 4.

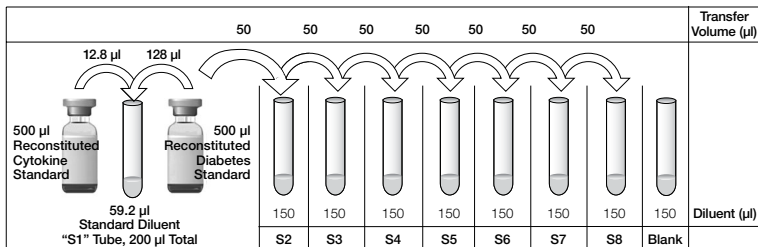


Fig. 4. S1 mixture and fourfold dilution series of diabetes and cytokine standards for detection at high PMT. Produces a narrow range cytokine standard curve.

- For low PMT/broad range cytokine standard curve, add **64  $\mu\text{l}$**  of each standard to the S1 tube, total volume 200  $\mu\text{l}$  (Figure 5). **Vortex for 5 sec.**
- Use a new pipet tip to transfer **50  $\mu\text{l}$**  from the S1 tube to the S2 tube containing standard diluent. **Vortex for 5 sec.**
- Continue with 1:4 (fourfold) serial dilutions from tube S2 to S8 as shown in Figure 5, vortexing after each volume transfer.

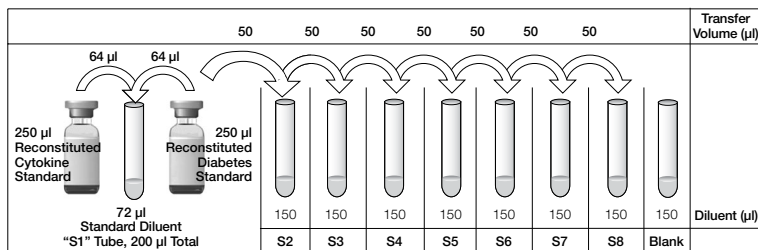


Fig. 5. S1 mixture and fourfold dilution series of cytokine and diabetes standards for detection at low PMT. Produces a broad range cytokine standard curve.

## 5. Prepare Samples

General guidelines for preparing different sample types are provided here. For more information, contact Bio-Rad Technical Support.

- Once thawed, keep samples on ice. Prepare dilutions just prior to the start of the assay and equilibrate to room temperature before use
- Do not freeze diluted samples

**Table 8. Summary of recommended sample diluents and dilution factors.**

Assay	Serum and Plasma		Culture Sup and Other Fluids		Cell and Tissue Lysates	
	Dilution	Diluent	Dilution	Diluent	Dilution	Diluent
Human, mouse, and rat cytokines	1:4	Bio-Plex® sample diluent	User optimized	Cell culture medium or buffer similar to sample**	User optimized (at least 1:2 of lysates at 200–900 ug/ml protein)	Bio-Plex sample diluent**
Human ICAM-1/VCAM-1	1:100	Bio-Plex sample, standard diluent*	User optimized			
Mouse ICAM-1	1:200	Bio-Plex serum-based diluent*	User optimized			

\* Requires a two-step dilution scheme as described on pages 18–19. Due to differences in dilution factors, it is not possible to multiplex ICAM-1 or VCAM-1 with other assays.

\*\* If samples are serum-free, add BSA to at least 0.5% final w/v to stabilize analytes and reduce adsorption to labware.

### Sample Preparation

#### Serum and Plasma

EDTA or citrate is preferred as an anticoagulant. Heparin-treated plasma, while compatible with Bio-Plex Pro™ assays, may absorb certain soluble proteins of interest. Avoid using hemolyzed samples as this may lead to false positive results.

1. Draw whole blood into collection tubes containing anticoagulant. Invert tubes several times to mix.

2. For serum, allow blood to clot at room temperature for **30 to 45 min**. For plasma, proceed directly to the centrifugation steps.
3. Perform centrifugation at 1,000 x g for **15 min** at 4°C and transfer the serum or plasma to a clean polypropylene tube.
4. To completely remove platelets and precipitates, centrifuge again at 10,000 x g for **10 min** at 4°C.
5. Dilute samples fourfold (1:4) by adding 1 volume of sample to 3 volumes of Bio-Plex sample diluent (for example, **40 µl** sample + **120 µl** sample diluent).
6. Assay samples immediately or aliquot into single-use tubes and store at -70°C. Avoid repeated freeze-thaw cycles.

### **Cell Culture Supernatant**

1. Collect supernatants and centrifuge at 1,000 x g for **15 min** at 4°C. For cell lines cultured in serum-free culture media, collect samples and add BSA as a carrier protein to a final concentration of at least 0.5% to stabilize protein analytes and to prevent adsorption to labware.
2. Transfer to a clean polypropylene tube. If cellular debris or precipitates are present, centrifuge again at 10,000 x g for **10 min** at 4°C.
3. We recommend testing undiluted samples first. If levels are anticipated to be high, samples can be further diluted in culture medium. Rarely would samples need to be diluted greater than 1:10.
4. Assay immediately or store samples in single-use aliquots at -70°C. Avoid repeated freeze-thaw cycles.

### **Lavage, Sputum, and Other Biological Fluid Samples**

Keep all samples on ice until ready for use. The appropriate sample dilution factor should be optimized by the user.

1. If required, dilute the sample in Bio-Plex sample diluent with BSA added to a final concentration of 0.5%.
2. Centrifugation at 10,000 x g for **10 min** at 4°C may be required to clarify the sample.

## Lysates

The Bio-Plex cell lysis kit is required for lysate preparation (available separately, catalog #171-304011 and #171-304012). Refer to bulletin #5297 for a list of published articles on cytokine analysis in tissue samples.

1. Prepare the cell or tissue lysates according to the instructions provided with the Bio-Plex cell lysis kit. The protease inhibitors factor I and factor II are included in the kit. PMSF needs to be added to lysis buffer at a final concentration of 2 mM. The lysates should be free of particulate matter.
2. Determine the total protein concentration of the lysate. It may be necessary to test lyse your samples with different volumes of lysing solution to obtain the specified protein concentration range.
3. Dilute at least 1:2 in sample diluent + 0.5% BSA, to a final protein concentration of 200–900 µg/ml. For analytes with high expression, a lysate protein concentration as low as 50 µg/ml may be sufficient.  
**Note:** For optimum antibody binding during sample incubation, it is important to dilute lysates as much as possible to reduce the detergent concentration.
4. If the lysate is not tested immediately, store at –20°C to –70°C. Avoid repeated freeze-thaw cycles.

## Sample Dilution for ICAM-1 and VCAM-1

ICAM-1 and VCAM-1 are typically found at high concentrations in the blood; therefore, higher sample dilutions are required to achieve measurable concentrations within the standard curve range.

**For human ICAM-1 and VCAM-1** assays, dilute serum or plasma **1:100**.

For example:

- First dilution (1:4)— 10 µl sample + 30 µl Bio-Plex sample diluent
- Second dilution (1:25)— 5 µl from the first dilution + 120 µl Bio-Plex standard diluent

**For the mouse ICAM-1** assay, dilute serum or plasma **1:200** in Bio-Plex **serum-based diluent**, which is included in the Bio-Plex Pro high dilution reagent kit, catalog #171-304080M.

For example:

- First dilution (1:10)— 10 µl sample + 90 µl diluent
- Second dilution (1:20)— 10 µl from the first dilution + 190 µl diluent

## 6. Prepare Coupled Beads

Instructions are provided for diluting the coupled beads to a 1x concentration. When mixing cytokine and diabetes assays, keep in mind the stock concentrations of coupled beads as listed in Table 9.

**Table 9. Stock concentration of coupled beads.**

<b>Assay</b>	<b>Stock Concentration of Coupled Beads</b>
Human and mouse cytokines (groups I, II)	10x
Rat cytokines (group I)	20x
Mouse cytokines (group III)	20x
Human, mouse, and rat diabetes	20x

**Note:** When using 10-pack reagents, ensure that only the required volumes of coupled beads, detection antibodies, streptavidin-PE, and buffers have been removed from the tubes or bottles. For example, transfer a one-time volume of assay buffer, sufficient to perform all steps of the assay procedure (that is, prewetting the filter plate, diluting coupled beads, diluting streptavidin-PE, and resuspending the beads) into a 50 ml reservoir.

1. Use Tables 10–14 to calculate the volume of coupled beads and assay buffer needed.
2. Add the required volume of Bio-Plex assay buffer to a 15 ml polypropylene tube.

3. **Vortex** the stock coupled beads at medium speed for **30 sec**. Carefully open the cap and pipet any liquid trapped in the cap back into the tube. This is important to ensure maximum bead recovery. **Do not centrifuge the vial**; doing so will cause the beads to pellet.
4. Dilute coupled beads to 1x by pipetting the required volume into the 15 ml tube. **Vortex**.  
Each well of the assay plate requires either 2.5  $\mu\text{l}$  (20x stock) or 5.0  $\mu\text{l}$  (10x stock) adjusted to a final volume of 50  $\mu\text{l}$  in assay buffer.
5. Protect the beads from light with aluminum foil. Equilibrate to room temperature prior to use.  
**Note:** To minimize volume loss, use a 200–300  $\mu\text{l}$  capacity pipet to remove beads from the stock tube. If necessary, perform the volume transfer in two steps. Do not use a 1,000  $\mu\text{l}$  capacity pipet and/or wide bore pipet tip.

**Preparing 1x coupled beads from 10x stock (includes 20% excess volume)**

**Table 10. Premixed panel or one singleplex assay.**

# of Wells	10x Beads, $\mu\text{l}$	Assay Buffer, $\mu\text{l}$	Total Volume, $\mu\text{l}$
96	575	5,175	5,750
48	288	2,587	2,875

**Table 11. Mixing two singleplex assays or a premixed panel + singleplex assay.**

# of Wells	10x Beads, $\mu\text{l}$ Singleplex #1	10x Beads, $\mu\text{l}$ Singleplex #2	Assay Buffer, $\mu\text{l}$	Total Volume, $\mu\text{l}$
96	575	575	4,600	5,750
48	288	288	2,300	2,876

Preparing 1x coupled beads from 20x stock (includes 20% excess volume)

Table 12. Premixed panel or one singleplex assay.

# of Wells	20x Beads, $\mu$ l	Assay Buffer, $\mu$ l	Total Volume, $\mu$ l
96	288	5,472	5,760
48	144	2,736	2,880

Table 13. Mixing two singleplex assays or a premixed panel + singleplex assay.

# of Wells	20x Beads, $\mu$ l Singleplex #1	20x Beads, $\mu$ l Singleplex #2	Assay Buffer, $\mu$ l	Total Volume, $\mu$ l
96	288	288	5,184	5,760
48	144	144	2,592	2,880

Table 14. Preparing 1x beads from two stocks at different concentrations. Mixing human insulin (20x) with human IL-6 (10x) is one example\*.

# of Wells	20x Beads, $\mu$ l Diabetes	10x Beads, $\mu$ l Cytokines	Assay Buffer, $\mu$ l	Total Volume, $\mu$ l
96	288	576	4,896	5,760
48	144	288	2,448	2,880

\* Due to differences in dilution factors, it is not possible to multiplex adiponectin, adipsin, VCAM-1, or ICAM-1 with other diabetes or cytokine assays.

## 7. Run Assay

### Considerations

- Bring all assay components and samples to room temperature before use
- Use calibrated pipets and pipet carefully, avoiding bubbles. Use new pipet tips for every volume transfer
- Pay close attention to vortexing, shaking, and incubation instructions. Deviation from the protocol may result in low assay signal and assay variability
- Assay incubations are carried out in the dark on a shaker at **850 ± 50 rpm**. Cover the plate with sealing tape and protect from light with aluminum foil

**Table 15. Summary of wash options and protocols.** After each assay step, select the appropriate Bio-Plex Pro™ wash station program or perform the appropriate manual wash step as summarized below.

Assay Step	Bio-Plex Pro or Pro II Wash Station	Bio-Plex Pro II Wash Station	Handheld Magnet or Vacuum Manifold
	Magnetic Program	Vacuum Program	Manual Wash Steps
Add beads to plate	MAG x2	VAC x2	2 x 100 µl
Sample incubation	MAG x3	VAC x3	3 x 100 µl
Detection Ab incubation	MAG x3	VAC x3	3 x 100 µl
SA-PE incubation	MAG x3	VAC x3	3 x 100 µl

### Considerations when Using a Vacuum Manifold

- After each incubation, place the filter plate on a calibrated vacuum apparatus and remove the liquid by vacuum filtration
- To wash, add 100 µl wash buffer to each well and remove the liquid as before. Ensure that all wells are exposed to the vacuum
- Thoroughly blot the bottom of the filter plate with a clean paper towel between each vacuum step to prevent cross contamination
- Place the assay plate on the plastic plate holder/tray as needed
- Before each incubation, gently cover the plate with a new sheet of sealing tape. Avoid pressing down over the wells to prevent leaking from the bottom



## Add Coupled Beads, Standards, and Samples

1. Cover unused wells with sealing tape.
2. Prewet the filter plate. Skip this step if using a flat-bottom plate.  
Prewet the wells using 100  $\mu$ l assay buffer and remove the liquid by vacuum filtration. Dry the bottom of the filter plate thoroughly by blotting on a clean paper towel.
3. **Vortex** the diluted (1x) coupled beads for **30 sec** at medium speed. Pour the diluted coupled beads into a reagent reservoir and transfer 50  $\mu$ l to each well of the assay plate.  
**Tip:** A multichannel pipet is highly recommended for ease of use and efficiency.
4. **Wash the plate two times** with **100  $\mu$ l** Bio-Plex<sup>®</sup> wash buffer using the wash method of choice.
5. **Gently vortex** the diluted standards, blanks, samples, and controls (if applicable) for **5 sec**. Transfer **50  $\mu$ l** to each well of the assay plate, changing the pipet tip after every volume transfer.
6. **Cover** plate with a new sheet of sealing tape and protect from light with aluminum foil. **Incubate** on shaker at **850  $\pm$  50 rpm** at room temperature (RT). See Table 16 for incubation time.  
**Note:** 850 rpm provides equivalent performance to shaker settings recommended in previous manuals (1,100 rpm for 30 sec, 300 rpm for incubation).

Table 16. Sample incubation times.

Assay	Incubation Time
Bio-Plex Pro human cytokine (group I and II)	30 min
Bio-Plex Pro mouse cytokine (group I and II)	30 min
Bio-Plex Pro mouse cytokine (group III)	1 hr
Bio-Plex Pro rat cytokine (group I)	1 hr

**Note:** Be consistent with this incubation time for optimal assay performance and reproducibility.

## Prepare and Add Detection Antibodies

Instructions are provided for diluting the detection antibodies to a 1x concentration. When mixing diabetes and cytokine assays, keep in mind the stock concentrations of detection antibodies as shown below.

Table 17. Stock concentration of detection antibodies.

Assay	Stock Concentration of Detection Antibodies
Human and mouse cytokines (groups I, II)	10x
Mouse cytokines (group III)	20x
Rat cytokines (group I)	20x
Human, mouse, and rat diabetes	20x

1. While the samples are incubating, use Tables 18–22 or the Calculation Worksheet on page 41 to calculate the volume of detection antibodies and detection antibody diluent needed. Detection antibodies should be prepared **10 min before use**.
2. Add the required volume of Bio-Plex detection antibody diluent to a 15 ml polypropylene tube.
3. **Vortex** the stock detection antibodies for **15–20 sec** at medium speed, then perform a **30 sec** spin to collect the entire volume at the bottom of the tube.
4. Dilute detection antibodies to 1x by pipetting the required volume into the 15 ml tube.

Each well of the assay requires either 1.25  $\mu\text{l}$  (20x stock) or 2.5  $\mu\text{l}$  (10x stock) adjusted to a final volume of 25  $\mu\text{l}$  in detection antibody diluent.

Preparing 1x detection antibodies from 10x stock (includes 25% excess volume)

Table 18. Premixed panel or one singleplex assay.

# of Wells	10x Detection Antibodies, $\mu\text{l}$	Detection Antibody Diluent, $\mu\text{l}$	Total Volume, $\mu\text{l}$
96	300	2,700	3,000
48	150	1,350	1,500

Table 19. Mixing two singleplex assays or a premixed panel + singleplex assay.

# of Wells	10x Detection Antibodies, $\mu$ l Singleplex #1	10x Detection Antibodies, $\mu$ l Singleplex #2	Detection Antibody Diluent, $\mu$ l	Total Volume, $\mu$ l
96	300	300	2,400	3,000
48	150	150	1,200	1,500

Preparing 1x detection antibodies from 20x stock (includes 25% excess volume)

Table 20. Premixed panel or one singleplex assay.

# of Wells	20x Detection Antibodies, $\mu$ l	Detection Antibody Diluent, $\mu$ l	Total Volume, $\mu$ l
96	150	2,850	3,000
48	75	1,425	1,500

Table 21. Mixing two singleplex assays or a premixed panel + singleplex assay.

# of Wells	20x Detection Antibodies, $\mu$ l Singleplex #1	20x Detection Antibodies, $\mu$ l Singleplex #2	Detection Antibody Diluent, $\mu$ l	Total Volume, $\mu$ l
96	150	150	2,700	3,000
48	75	75	1,350	1,500

Table 22. Preparing 1x detection antibodies from two stocks at different concentrations.

Mixing human insulin (20x) with human IL-6 (10x) is one example.\*

# of Wells	20x Detection Antibodies, $\mu$ l Diabetes	10x Detection Antibodies, $\mu$ l Cytokines	Detection Antibody Diluent, $\mu$ l	Total Volume, $\mu$ l
96	150	300	2,550	3,000
48	75	150	1,275	1,500

\* Due to differences in dilution factors, it is not possible to multiplex adiponectin, adipisin, VCAM-1, or ICAM-1 with other diabetes or cytokine assays.

- After incubating the beads, samples, standards, and blank, slowly remove and discard the sealing tape.
- Wash the plate three times** with **100  $\mu$ l** wash buffer.
- Vortex** the diluted (1x) detection antibodies gently for **5 sec**. Pour into a reagent reservoir and transfer **25  $\mu$ l** to each well using a multichannel pipet.

8. **Cover** plate with a new sheet of sealing tape and protect from light with aluminum foil. **Incubate** on shaker at **850 ± 50 rpm** for **30 min** at room temperature.

## Prepare and Add Streptavidin-PE (SA-PE)

1. While the detection antibodies are incubating, use Table 23 or the Calculation Worksheet on page 41 to calculate the volume of SA-PE (100x) and assay buffer needed. Streptavidin-PE should be prepared **10 min before use**.
2. Add the required volume of assay buffer to a 15 ml polypropylene tube.
3. **Vortex** the 100x SA-PE for **5 sec** at medium speed. Perform a 30 sec spin to collect the entire volume at the bottom of the tube.
4. Dilute SA-PE to 1x by pipetting the required volume into the 15 ml tube. **Vortex** and protect from light until ready to use.

Each well of the assay requires 0.5 µl (100x stock) adjusted to a final volume of 50 µl in assay buffer.

**Table 23. Preparing 1x SA-PE from 100x stock (includes 25% excess volume).**

# of Wells	100x SA-PE, µl	Assay Buffer, µl	Total Volume, µl
96	60	5,940	6,000
48	30	2,970	3,000

5. After the detection antibody incubation, slowly remove and discard the sealing tape.
6. **Wash the plate three times** with **100 µl** wash buffer.
7. **Vortex** the diluted (1x) SA-PE at medium speed for **5 sec**. Pour into a reagent reservoir and transfer **50 µl** to each well using a multichannel pipet.

8. **Cover** plate with a new sheet of sealing tape and protect from light with aluminum foil. **Incubate** on shaker at **850 ± 50 rpm** for **10 min** at room temperature.
9. After the streptavidin-PE incubation step, slowly remove and discard the sealing tape.
10. **Wash the plate three times** with **100 µl** wash buffer.
11. To resuspend beads for plate reading, add **125 µl** of assay buffer to each well. Cover the plate with a new sheet of sealing tape. Shake at room temperature at **850 ± 50 rpm** for **30 sec**, and slowly remove the sealing tape. Ensure that the plate cover has been removed before placing the plate on the reader.
12. Remove the sealing tape and read the plate using the settings below. Refer to section 8 for details.

**Note:** Reading at alternative PMT settings on the Bio-Plex 100, 200, or Bio-Plex 3D (for example when mixing diabetes assays with cytokine assays) requires validation by the end user to ensure that results meet the user's acceptance criteria.

**Table 24. Read the plate using the appropriate instrument settings.**

Bio-Plex Pro Assay	RP1 (PMT) Setting		
	Bio-Plex 100/200*	Bio-Plex 3D*	Bio-Plex® MAGPIX™*
Human cytokines (group I, II)	Low		N/A, Use default settings
Mouse cytokines (group I, II, III)	Low	Standard	
Rat cytokines (group I)	High		
<b>Cross-Panel Mixing</b>			
Human cytokines + diabetes			
Mouse cytokines + diabetes	High	Standard	
Rat cytokines + diabetes			
<b>Other Instrument Settings</b>			
DD gates	5,000–25,000	Select MagPlex beads	
Bead events	50	50	

\* Or similar Luminex-based system.

## 8. Read Plate

Bio-Plex Manager™ software is recommended for all Bio-Plex Pro™ assay data acquisition and analysis. Instructions for Luminex xPONENT software are also included. For instructions using other xMAP system software packages, contact Bio-Rad Technical Support or your regional Bio-Rad field applications specialist.


### **Prepare Protocol in Bio-Plex Manager Software v 6.0 and Higher**

The protocol should be prepared in advance so that the plate is read as soon as the experiment is complete.

A protocol file specifies the analytes used in the reading, the plate wells to be read, sample information, the values of standards and controls, and instrument settings.

Bio-Plex Manager software contains protocols for most Bio-Plex® assays. Choose from available protocols or create a new protocol. To create a new protocol, select **File**, then **New** from the main menu. Locate and follow the steps under **Protocol Settings**.

1. Click **Describe Protocol** and enter information about the assay (optional).

2. Click **Select Analytes** and create a new panel. Visually confirm the selected analytes and proceed to step 3.
  - a. Click the **Add Panel** button  in the Select Analytes toolbar. Enter a new panel name. Select **Bio-Plex Pro Assay Magnetic** from the assay pull-down menu. If using Bio-Plex Manager version 5.0 or lower, select **MagPlex** from the assay pull-down menu.
  - b. Click the **Add** button. Enter the bead region number and name for the first analyte. Click **Add Continue** to repeat for each analyte in the assay.

For reference, bead regions are shown in Table 25.
  - c. Click the **Add** button when the last analyte has been added and click **OK** to save the new panel.
  - d. Highlight analytes from the Available list (left) and move to the Selected list (right) using the Add button. To move all analytes at once, simply click the **Add All** button.
  - e. If some of the analytes need to be removed from the Selected list, highlight them and select **Remove**. If desired, it is possible to rename the panel by clicking on **Rename Panel** and entering a new panel name.

**Note:** Do not use preset panels found in Bio-Plex Manager software version 5.0 or earlier as the bead regions are not up to date.

Table 25. Bead regions for Bio-Plex Pro cytokine assays.

Human Cytokines				Mouse Cytokines				Rat Cytokines		
Group I	Bead Region	Group II	Bead Region	Group I	Bead Region	Group II	Bead Region	Group I	Bead Region	
IL-1 $\beta$	39	IL-1 $\alpha$	63	IL-1 $\alpha$	53	IL-15	42	IL-1 $\alpha$	21	
IL-1ra	25	IL-2R $\alpha$	13	IL-1 $\beta$	19	IL-18*	20	IL-1 $\beta$	28	
IL-2	38	IL-3	64	IL-2	36	Basic FGF	25	IL-2	22	
IL-4	52	IL-12p40**	28	IL-3	18	LIF	45	IL-4	33	
IL-5	33	IL-16	27	IL-4	39	M-CSF	26	IL-5	52	
IL-6	19	IL-18*	42	IL-5	52	MIG	44	IL-6	56	
IL-7	74	CTACK	72	IL-6	38	MIP-2	27	IL-7	38	
IL-8	54	GRO- $\alpha$	61	IL-9	33	PDGF-BB	35	IL-10	19	
IL-9	77	HGF	62	IL-10	56	VEGF	47	IL-12p40**	76	
IL-10	56	IFN- $\alpha$ 2	20	IL-12p40	76	Group III		IL-12p70	78	
IL-12p70	75	LIF	29	IL-12p70	78				IL-13	15
IL-13	51	MCP-3	26	IL-13	37				IL-17A	72
IL-15	73	M-CSF	67	IL-17	72	CD40L	30	IL-18*	20	
IL-17	76	MIF	35	Eotaxin	74	IL-17F	28	EPO	14	
Eotaxin	43	MIG	14	G-CSF	54	IL-21	14	G-CSF	54	
Basic FGF	44	$\beta$ -NGF	46	GM-CSF	73	IL-22	15	GM-CSF	37	
G-CSF	57	SCF	65	IFN- $\gamma$	34	IL-23p19	61	GRO/KC	57	
GM-CSF	34	SCGF- $\beta$	78	KC	57	IL-31	29	IFN- $\gamma$	34	
IFN- $\gamma$	21	SDF-1 $\alpha$	22	MCP-1 (MCAF)	51	IL-33	13	M-CSF	26	
IP-10	48	TNF- $\beta$	30	MIP-1 $\alpha$	77	MIP-3 $\alpha$	12	MCP-1	51	
MCP-1	53	TRAIL	66	MIP-1 $\beta$	75	Group III Singleplexes		MIP-1 $\alpha$	77	
MIP-1 $\alpha$	55	Group II Singleplexes		RANTES	55				MIP-2***	27
MIP-1 $\beta$	18				TNF- $\alpha$		21			MIP-3 $\alpha$
PDGF-BB	47								RANTES	55
RANTES	37	ICAM-1	12			ICAM-1	22	TNF- $\alpha$	43	
TNF- $\alpha$	36	V-CAM1	15			IL-25 (IL-17E)	67	VEGF	47	
VEGF	45					IL-27p28	43			

\* IL-18 is not available as a singleplex assay but only as part of an assay panel.

\*\* IL-12p40 cannot be multiplexed with IL-12p70.

\*\*\* MIP-2 cannot be multiplexed with GRO/KC.

3. Click **Format Plate** and format the plate according to the plate layout created in Section 1 (Plan Plate Layout). To modify the plate layout, follow the steps below (see Figure 6).

- a. Select the **Plate Formatting** tab.
- b. Select the standards icon **(S)** and drag the cursor over all the wells that contain standards. Repeat this process for blanks **(B)**, controls **(C)**, and samples **(X)**.



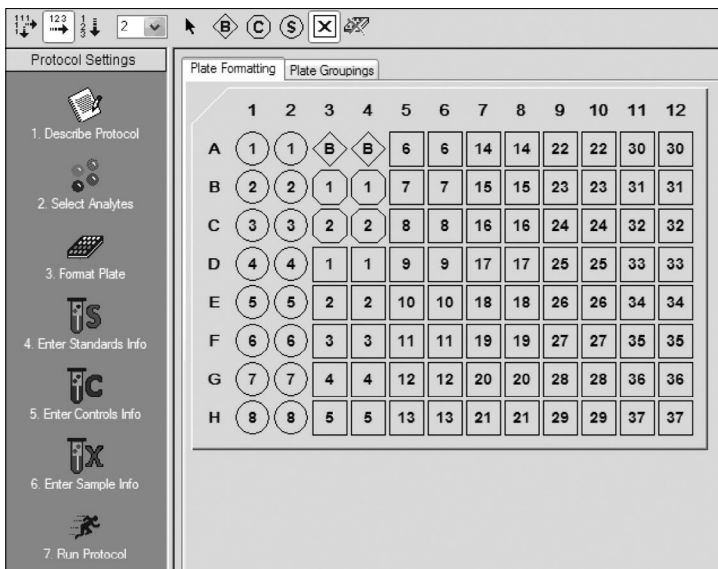



Fig. 6. Plate formatting.

4. Click **Enter Standards Info** in the Protocol Settings bar.
  - a. Enter the highest concentration of each analyte in the top row (labeled S1) of the table. S1 concentration information is included on the peel-off sticker provided with each vial of standards.
  - b. Enter a dilution factor of 4 and click **Calculate**. The concentrations for each standard point will be populated for all analytes in the table.
  - c. Optional: enter the lot number of the vial of standards into the **Standard Lot** box and click **Save**.
5. Click **Enter Controls Info**, and for user-defined controls, select an analyte from the dropdown menu, then enter a description and concentration. Repeat for each additional analyte in the assay.
6. Click **Enter Sample Info** and enter sample information and the appropriate dilution factor.

7. Click **Run Protocol** and confirm that the assay settings are correct.
  - a. Refer to Table 24 for the recommended RP1 (PMT) setting. Protocols using alternative PMT settings should be validated by the end user, for example when mixing diabetes assays with cytokine assays.
  - b. Confirm data acquisition is set to **50 beads per region**. In Advanced Settings, confirm that the bead map is set to **100 region**, the sample size is set to **50 µl**, and the DD gates are set to **5,000 (Low)** and **25,000 (High)**. In Bio-Plex Manager software versions 4.0, 4.1, and 4.1.1, check **Override Gates** and set the DD gate values as indicated.
  - c. Select **Start**, name and save the .rbx file, and begin data acquisition. The Run Protocol pop-up screen will appear. Click **Eject/Retract** to eject the plate carrier.

## Acquire Data

1. Shake the assay plate at **850 ± 50 rpm** for **30 sec** and visually inspect the plate to ensure that the assay wells are filled with buffer. Slowly remove the sealing tape and any plate cover before placing the plate on the plate carrier.
2. Click **Run Protocol** — on the pop-up screen, select **Load Plate** and click **OK** to start acquiring data.
3. Use the **Wash Between Plates**  command after every plate run to reduce the possibility of clogging the instrument.
4. If acquiring data from more than one plate, empty the waste bottle and refill the sheath bottle after each plate (if HTF are not present). Select **Wash Between Plates** and follow the instructions. Then repeat the Prepare Protocol and Acquire Data instructions.
5. When data acquisition is complete, select Shut Down  and follow the instructions.

## Reacquire Data

It is possible to acquire data from a well or plate a second time using the **Rerun/Recovery** mode located below Start in the Run Protocol step. Any previous data will be overwritten.

1. Check the wells from which data will be reacquired.
2. Remove the buffer with the wash method of choice.
3. Add **100  $\mu$ l** assay buffer to each well. Cover the filter plate with a new sheet of sealing tape. Shake the plate at **850  $\pm$  50 rpm** for **30 sec**. Slowly remove the sealing tape before placing the plate on the plate reader.
4. Repeat the **Acquire Data** steps to reacquire data. The data acquired should be similar to those acquired initially; however, the acquisition time will be extended because the wells have fewer beads.

## Data Analysis: Removing Outliers

Outliers are identified as standard data points that do not meet accuracy or precision requirements and should be considered invalid when performing curve fitting. As such, they should be removed to generate a more realistic and accurate standard curve. This may result in an extended assay working range and allow quantitation of samples that might otherwise be considered out of range (OOR).

In Bio-Plex Manager software version 6.0 and higher, outliers can be automatically removed by selecting the **Optimize** button in the Standard Curve window. In Bio-Plex Manager software 6.0 and earlier versions, outliers also can be manually selected in the Report Table. Visit online Help to learn more about the standard curve optimizer feature and how outliers are determined.

## Previous Versions of Bio-Plex Manager Software

For instructions on using previous versions of Bio-Plex manager software, please contact Bio-Rad Technical Support.

## Luminex xPONENT Software

Although guidelines are provided here, consult the xPONENT software manual for more details. Perform a system initialization with Luminex's calibration and performance verification kit, as directed by Luminex. Select Batches to set up the protocol and follow the information under Settings.

**Note:** The instrument settings described below apply to Luminex 100/200 and FLEXMAP 3D or Bio-Plex® 3D instruments. For the Bio-Plex® MAGPIX™ reader, use the default instrument settings.

1. Select MagPlex as the bead type for magnetic beads, which automatically sets the DD gates.
2. Volume = 50 µl.
3. Refer to Table 24 to select the appropriate PMT setting for your instrument.
4. Plate name: 96-well plate.
5. Analysis type: Quantitative; 5PL Curve Fit.
6. Number of standards: 8.

Select **Analytes** to set up the panel.

1. Enter **pg/ml** in the Units field.
2. Enter **50** in the Count field.
3. Select the bead region and enter the analyte name.
4. Click **Apply all** for Units and Count.

Select **Stds and Ctrls**.

1. Enter standard concentrations, lot number, dilution factor, and other information as applicable.

After the assay is complete, select **Results**, then select **Saved Batches**.

# Troubleshooting Guide

This troubleshooting guide addresses problems that may be encountered with Bio-Plex Pro™ assays. If you experience any of the problems listed below, review the possible causes and solutions provided. Poor assay performance may also be due to the Bio-Plex® suspension array reader. To eliminate this possibility, use the validation kit to assist in determining if the array reader is functioning properly.

## Possible Causes

### High Inter-Assay CV

Standards were not reconstituted consistently between assays

Reconstituted standards and diluted samples were not stored properly

Bottom of filter plate not dry

## Possible Solutions

Incubate the reconstituted standards for 30 min on ice. Always be consistent with the incubation time and temperature.

Reconstituted standards and diluted samples should be prepared on ice as instructed. Prior to plating, the reconstituted standards and diluted samples should be equilibrated to room temperature.

Dry the bottom of the filter plate with absorbent paper towel (preferably lint-free) to prevent cross-well contamination.

## Possible Causes

### High Intra-Assay CV

Improper pipetting technique

Reagents and assay components not equilibrated to room temperature prior to pipetting

Contamination with wash buffer during wash steps

Slow pipetting of samples and reagents across the plate

Bio-Plex wash station: insufficient washing due to clogged pins

## Possible Solutions

Pipet carefully 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.

All reagents and assay components should be equilibrated to room temperature prior to pipetting.

During the wash steps, be careful not to splash wash buffer from one well to another. Be sure that the wells are filtered completely and that no residual volume remains. Ensure that the microplate shaker setting is not too high. Reduce the microplate shaker speed to minimize splashing.

Sample pipetting across the entire plate should take less than 4 min. Reagent pipetting across the entire plate should take less than 1 min.

Clean dispensing pins with the thicker of the two cleaning needles provided with washer. Perform regular rinses to minimize salt buildup.

## **Possible Causes**

### **Low Bead Count**

Miscalculation of bead dilution

Beads clumped in multiplex bead stock tube

Vacuum on for too long when aspirating buffer from wells

Filter plate not shaken enough before incubation steps and prior to reading

Reader is clogged

### **Low Signal or Poor Sensitivity**

Standards reconstituted incorrectly

Detection antibody or streptavidin-PE diluted incorrectly

## **Possible Solutions**

Check your calculations and be careful to add the correct volumes.

Vortex for 30 sec at medium speed before aliquoting beads.

Do not apply vacuum to the filter plate for longer than 10 sec after the buffer is completely drained from each well.

Shake the filter plate at  $850 \pm 50$  rpm for 30 sec before incubation steps and immediately before reading the plate.

Refer to the troubleshooting guide in the Bio-Plex system hardware instruction manual (bulletin #10005042).

Follow the standard preparation instructions carefully.

Check your calculations and be careful to add the correct volumes.

## **Possible Causes**

### **High Background Signal**

Incorrect buffer was used  
(for example, assay buffer used  
to dilute standards)

Accidentally spiked blank wells

Detection antibodies or  
streptavidin-PE incubated  
too long

### **Poor Recovery**

Expired Bio-Plex reagents  
were used

Incorrect amounts of components  
were added

Microplate shaker set to an  
incorrect speed

## **Possible Solutions**

Use standard diluent or diluent  
similar to final sample matrix to dilute  
standards.

Do not add any antigens to the  
blank wells.

Follow the procedure incubation  
time precisely.

Check that reagents have not  
expired. Use new or nonexpired  
components.

Check your calculations and be  
careful to add the correct volumes.

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.



## Possible Causes

### Poor Recovery

Improper pipetting technique

### Impact of Sample Matrix

Negative MFI values in samples or standards

Poor precision in serum and plasma sample measurements

## Possible Solutions

Pipet carefully 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.

If samples contain little or no analyte, negative values observed may be due to statistical variation. If assay drift is suspected, retest the samples by positioning them next to the standards. If contamination of standards is suspected, check the standard replicate value and be careful when adding samples to the wells. Matrix effects could also produce negative sample values.

Bio-Plex Manager™ software automatically subtracts the blank (B) FI value from all other assay wells. While this has no impact on observed concentrations of samples within the assay working range, it may result in a negative FI value if the blank's FI value is greater than either the standard or sample value. If this is undesirable, then assign wells as a sample (X) or control (C) in the protocol or results file.

Check if any interfering components such as heparin-based anticoagulant, additives, or gel from separators were introduced into the samples. Avoid using hemolyzed and heavily lipemic samples. Remove visible particulate in samples by centrifugation. Avoid multiple freeze-thaw cycles of samples.

# Plate Layout Template

<b>12</b>								
<b>11</b>								
<b>10</b>								
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<b>2</b>								
<b>1</b>								
	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	<b>E</b>	<b>F</b>	<b>G</b>	<b>H</b>

# Calculation Worksheet

If using either a **premixed panel or one singleplex assay** with 20x stocks of beads and detection antibodies, follow these directions. If using 10x stocks, divide by 10 instead of 20 in steps 1d and 2d.

Plan the plate layout and enter the number of wells to be used in the assay: \_\_\_\_\_  
1

## 1. Determine the volume of 1x coupled beads needed.

- Each well requires 50  $\mu\text{l}$  of coupled beads (1x): \_\_\_\_\_<sub>1</sub> x 50  $\mu\text{l}$  = \_\_\_\_\_<sub>2</sub>  $\mu\text{l}$
- Include 20% excess to ensure enough volume: \_\_\_\_\_<sub>2</sub>  $\mu\text{l}$  x 0.20 = \_\_\_\_\_<sub>3</sub>  $\mu\text{l}$
- Total volume of 1x coupled beads: \_\_\_\_\_<sub>2</sub>  $\mu\text{l}$  + \_\_\_\_\_<sub>3</sub>  $\mu\text{l}$  = \_\_\_\_\_<sub>4</sub>  $\mu\text{l}$
- Volume of **20x coupled beads** required: \_\_\_\_\_<sub>4</sub>  $\mu\text{l}$  / 20 = \_\_\_\_\_<sub>5</sub>  $\mu\text{l}$
- Volume of **assay buffer** required: \_\_\_\_\_<sub>4</sub>  $\mu\text{l}$  - \_\_\_\_\_<sub>5</sub>  $\mu\text{l}$  = \_\_\_\_\_<sub>6</sub>

## 2. Determine the volume of 1x detection antibody needed.

- Each well requires 25  $\mu\text{l}$  detection antibodies (1x): \_\_\_\_\_<sub>1</sub> x 25  $\mu\text{l}$  = \_\_\_\_\_<sub>7</sub>  $\mu\text{l}$
- Include 25% excess to ensure enough volume: \_\_\_\_\_<sub>7</sub>  $\mu\text{l}$  x 0.25 = \_\_\_\_\_<sub>8</sub>  $\mu\text{l}$
- Total volume of 1x detection antibodies: \_\_\_\_\_<sub>7</sub>  $\mu\text{l}$  + \_\_\_\_\_<sub>8</sub>  $\mu\text{l}$  = \_\_\_\_\_<sub>9</sub>  $\mu\text{l}$
- Volume of **20x detection antibodies** required: \_\_\_\_\_<sub>9</sub>  $\mu\text{l}$  / 20 = \_\_\_\_\_<sub>10</sub>  $\mu\text{l}$
- Volume of **detection antibody diluent** required: \_\_\_\_\_<sub>9</sub>  $\mu\text{l}$  - \_\_\_\_\_<sub>10</sub>  $\mu\text{l}$  = \_\_\_\_\_<sub>11</sub>  $\mu\text{l}$

## 3. Determine the volume of 1x streptavidin-PE needed.

- Each well requires 50  $\mu\text{l}$  streptavidin-PE (1x): \_\_\_\_\_<sub>1</sub> x 50  $\mu\text{l}$  = \_\_\_\_\_<sub>12</sub>  $\mu\text{l}$
- Include 25% excess to ensure enough volume: \_\_\_\_\_<sub>12</sub>  $\mu\text{l}$  x 0.25 = \_\_\_\_\_<sub>13</sub>  $\mu\text{l}$
- Total volume of 1x streptavidin-PE: \_\_\_\_\_<sub>12</sub>  $\mu\text{l}$  + \_\_\_\_\_<sub>13</sub>  $\mu\text{l}$  = \_\_\_\_\_<sub>14</sub>  $\mu\text{l}$
- Volume of **100x streptavidin-PE** required: \_\_\_\_\_<sub>14</sub>  $\mu\text{l}$  / 100 = \_\_\_\_\_<sub>15</sub>  $\mu\text{l}$
- Volume of **assay buffer** required: \_\_\_\_\_<sub>14</sub>  $\mu\text{l}$  - \_\_\_\_\_<sub>15</sub>  $\mu\text{l}$  = \_\_\_\_\_<sub>16</sub>  $\mu\text{l}$

If **mixing singleplex assays** with 20x stocks of beads and detection antibodies, follow these directions. If using 10x stocks, divide by 10 instead of 20 in steps 1e and 2e. Enter the number of wells to be used in the assay: \_\_\_\_\_  
1

**1. Determine the volume of 1x coupled beads needed.**

- a. Each well requires 50 µl coupled beads (1x): \_\_\_\_\_ x 50 µl = \_\_\_\_\_ µl  
1 2
- b. Include 20% excess to ensure enough volume: \_\_\_\_\_ µl x 0.20 = \_\_\_\_\_ µl  
2 3
- c. Total volume of 1x coupled beads: \_\_\_\_\_ µl + \_\_\_\_\_ µl = \_\_\_\_\_ µl  
2 3 4
- d. Enter the number of singleplex sets (or analytes) tubes that will be multiplexed = \_\_\_\_\_  
5
- e. Volume of **20x coupled beads** required from **each stock tube**:  
\_\_\_\_\_ µl / 20 = \_\_\_\_\_ µl  
4 6
- f. Total volume of combined bead stocks: \_\_\_\_\_ x \_\_\_\_\_ µl = \_\_\_\_\_ µl  
5 6 7
- g. Volume of **assay buffer** required: \_\_\_\_\_ µl - \_\_\_\_\_ µl = \_\_\_\_\_ µl  
4 7 8

**2. Determine the volume of 1x detection antibody needed.**

- a. Each well requires 25 µl detection antibodies (1x): \_\_\_\_\_ x 25 µl = \_\_\_\_\_ µl  
1 9
- b. Include 25% excess to ensure enough volume: \_\_\_\_\_ µl x 0.25 = \_\_\_\_\_ µl  
9 10
- c. Total volume of 1x detection antibodies: \_\_\_\_\_ µl + \_\_\_\_\_ µl = \_\_\_\_\_ µl  
9 10 11
- d. Enter the number of singleplex sets (or analytes) that will be multiplexed = \_\_\_\_\_  
5
- e. Volume of **20x detection antibodies** required from **each stock tube**:  
\_\_\_\_\_ µl / 20 = \_\_\_\_\_ µl  
11 12
- f. Total volume of combined detection antibody stock: \_\_\_\_\_ µl x \_\_\_\_\_ = \_\_\_\_\_ µl  
12 5 13
- g. Volume of **detection antibody diluent** required: \_\_\_\_\_ µl - \_\_\_\_\_ µl = \_\_\_\_\_ µl  
11 13 14

**3. Determine the volume of 1x streptavidin-PE needed.**

- a. Each well requires 50 µl streptavidin-PE (1x): \_\_\_\_\_ x 50 µl = \_\_\_\_\_ µl  
1 15
- b. Include 25% excess to ensure enough volume: \_\_\_\_\_ µl x 0.25 = \_\_\_\_\_ µl  
15 16
- c. Total volume of 1x streptavidin-PE: \_\_\_\_\_ µl + \_\_\_\_\_ µl = \_\_\_\_\_ µl  
15 16 17
- d. Volume of **100x streptavidin-PE** required: \_\_\_\_\_ µl / 100 = \_\_\_\_\_ µl  
17 18
- e. Volume of **assay buffer** required: \_\_\_\_\_ µl - \_\_\_\_\_ µl = \_\_\_\_\_ µl  
17 18 19

If **mixing diabetes assays** (20x bead and detection antibody stocks) **with cytokine assays** (10x stocks), follow these directions.

**Note:** Refer to Table 26 for the maximum number of diabetes and cytokine assays that may be multiplexed. Mixing across panels is not applicable to NHP diabetes assays.

**Table 26. Maximum number of singleplex diabetes and cytokine analytes that may be multiplexed.**

<b>Human, mouse, and rat diabetes Mouse cytokine (group III) analytes (20x)</b>	0	2	4	6	8	10
<b>Human and mouse cytokine (groups I, II) analytes (10x)</b>	10	9	8	7	6	5

Enter the number of wells to be used in the assay: \_\_\_\_\_  
1

Enter the number of diabetes tubes (either singleplex or multiplex) that will be mixed: \_\_\_\_\_  
2

Enter the number of cytokine tubes (either singleplex or multiplex) that will be mixed: \_\_\_\_\_  
3

### 1. Determine the volume of 1x diabetes and cytokine coupled beads needed.

a) Each well requires 50  $\mu\text{l}$  of coupled beads (1x): \_\_\_\_\_ x 50  $\mu\text{l}$  = \_\_\_\_\_  $\mu\text{l}$   
1 4

b) Include 20% excess to ensure enough volume: \_\_\_\_\_  $\mu\text{l}$  x 0.20 = \_\_\_\_\_  $\mu\text{l}$   
4 5

c) Total volume of 1x coupled beads: \_\_\_\_\_  $\mu\text{l}$  + \_\_\_\_\_  $\mu\text{l}$  = \_\_\_\_\_  $\mu\text{l}$   
4 5 6

d) **Volume of 20x beads** required from each diabetes tube(s): \_\_\_\_\_  $\mu\text{l}$  / 20 = \_\_\_\_\_  $\mu\text{l}$   
6 7

e) **Volume of 10x beads** required from each cytokine tube(s): \_\_\_\_\_  $\mu\text{l}$  / 10 = \_\_\_\_\_  $\mu\text{l}$   
6 8

f) Total volume of diabetes bead stock required: \_\_\_\_\_  $\mu\text{l}$  x \_\_\_\_\_ = \_\_\_\_\_  $\mu\text{l}$   
7 2 9

g) Total volume of cytokine bead stock required: \_\_\_\_\_  $\mu\text{l}$  x \_\_\_\_\_ = \_\_\_\_\_  $\mu\text{l}$   
8 3 10

h) Total volume of combined bead stocks required: \_\_\_\_\_  $\mu\text{l}$  + \_\_\_\_\_ = \_\_\_\_\_  $\mu\text{l}$   
9 10 11

i) Volume of **assay buffer** required: \_\_\_\_\_  $\mu\text{l}$  - \_\_\_\_\_  $\mu\text{l}$  = \_\_\_\_\_  
6 11 12

## 2. Determine the volume of 1x diabetes and cytokine detection antibodies needed.

a) Each well requires 25  $\mu\text{l}$  of detection antibodies (1x):  $\frac{\quad}{1} \times 25 \mu\text{l} = \frac{\quad}{13} \mu\text{l}$

b) Include 25% excess to ensure enough volume:  $\frac{\quad}{13} \mu\text{l} \times 0.25 = \frac{\quad}{14} \mu\text{l}$

c) Total volume of 1x detection antibodies:  $\frac{\quad}{13} \mu\text{l} + \frac{\quad}{14} \mu\text{l} = \frac{\quad}{15} \mu\text{l}$

d) **Volume of 20x detection antibodies** required from each diabetes tube(s):

$$\frac{\quad}{15} \mu\text{l} / 20 = \frac{\quad}{16} \mu\text{l}$$

e) **Volume of 10x detection antibodies** required from each cytokine tube(s):

$$\frac{\quad}{15} \mu\text{l} / 10 = \frac{\quad}{17} \mu\text{l}$$

f) Total volume of diabetes detection antibodies stock required:  $\frac{\quad}{16} \mu\text{l} \times \frac{\quad}{2} = \frac{\quad}{18} \mu\text{l}$

g) Total volume of cytokine detection antibodies stock required:  $\frac{\quad}{17} \mu\text{l} \times \frac{\quad}{3} = \frac{\quad}{19} \mu\text{l}$

h) Total volume of combined detection antibodies required:

$$\frac{\quad}{18} \mu\text{l} + \frac{\quad}{19} = \frac{\quad}{20} \mu\text{l}$$

i) Volume of **detection antibody diluent** required:  $\frac{\quad}{15} \mu\text{l} - \frac{\quad}{20} \mu\text{l} = \frac{\quad}{21} \mu\text{l}$

## 3. Determine the volume of 1x streptavidin-PE needed.

a) Each well requires 50  $\mu\text{l}$  of streptavidin-PE (1x):  $\frac{\quad}{1} \times 50 \mu\text{l} = \frac{\quad}{15} \mu\text{l}$

b) Include 25% excess to ensure enough volume:  $\frac{\quad}{15} \mu\text{l} \times 0.25 = \frac{\quad}{16} \mu\text{l}$

c) Total volume of 1x streptavidin-PE:  $\frac{\quad}{15} \mu\text{l} + \frac{\quad}{16} \mu\text{l} = \frac{\quad}{17} \mu\text{l}$

d) Volume of **100x streptavidin-PE** required:  $\frac{\quad}{17} \mu\text{l} / 100 = \frac{\quad}{18} \mu\text{l}$

e) Volume of **assay buffer** required:  $\frac{\quad}{17} \mu\text{l} - \frac{\quad}{18} \mu\text{l} = \frac{\quad}{19} \mu\text{l}$

# Safety Considerations

Eye protection and gloves are recommended when using these products. Consult the MSDS for additional information. The Bio-Plex Pro™ assays contain components of animal origin. This material should be handled as if capable of transmitting infectious agents. Use universal precautions. These components should be handled at Biosafety Level 2 containment (U.S. government publication: Biosafety in Microbiological and Biomedical Laboratories (CDC, 1999)).

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# Ordering Information

Detailed ordering information can be found at [www.bio-rad.com/bio-plex](http://www.bio-rad.com/bio-plex).

Premixed All-In-One Multiplex Assays	Catalog #
Bio-Plex Pro Human Cytokine 8-Plex Panel, 1 x 96	M50-000007A
Bio-Plex Pro Human Cytokine 17-Plex Panel, 1 x 96	M50-00031YV
Bio-Plex Pro Human Cytokine 21-Plex Panel, 1 x 96	MF0-005KMII
Bio-Plex Pro Human Cytokine 27-Plex Panel, 1 x 96	M50-0KCAFOY
Bio-Plex Pro Human Cytokine Th1/Th2 Panel, 1 x 96	M50-00005L3
Bio-Plex Pro Mouse Cytokine 8-Plex Panel, 1 x 96	M60-000007A
Bio-Plex Pro Mouse Cytokine 9-Plex Panel, 1 x 96	MD0-00000EL
Bio-Plex Pro Mouse Cytokine 23-Plex Panel, 1 x 96	M60-009RDPD
Bio-Plex Pro Mouse Cytokine Th1/Th2 Panel, 1 x 96	M60-00003J7
Bio-Plex Pro Mouse Th17 Cytokine Panel A 6-Plex, 1 x 96	M60-00007NY
Bio-Plex Pro Mouse Th17 Cytokine Panel B 8-Plex, 1 x 96	171-FA001M
Bio-Plex Pro Mouse Cytokine Th1 Panel, 1 x 96	L60-00004C6
Bio-Plex Pro Mouse Cytokine Th2 Panel, 1 x 96	L60-000UKVT
Bio-Plex Pro Rat Th1/Th2 Panel, 1 x 96	171-K1002M
Bio-Plex Pro Rat 24-Plex Panel, 1 x 96	171-K1001M

## Bio-Plex® x-Plex™ Assays (We Mix)

Premium custom assay service using the Bio-Plex Assay Builder, [www.bio-rad.com/bio-plex/assaybuilder](http://www.bio-rad.com/bio-plex/assaybuilder), to select analytes and plate type of interest. Assays are supplied as premixed coupled beads and detection antibodies in the all-in-one kit format.

## Bio-Plex Express Assays (You Mix)

Fast and economical custom assay service using the Bio-Plex Assay Builder, [www.bio-rad.com/bio-plex/assaybuilder](http://www.bio-rad.com/bio-plex/assaybuilder), to select analytes and plate type of interest. Assays are supplied as individual sets of coupled beads and detection antibodies in the all-in-one kit format.

## Singleplex Sets and Individual Components

A host of singleplex sets and individual assay components are available. For more information, refer to bulletin #5507 or go to [www.bio-rad.com/bio-plex](http://www.bio-rad.com/bio-plex).





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