NEW magnetic protocol and software requirements!

Bio-Plex Pro™ Assays

Cytokine, Chemokine, and Growth Factors Instruction Manual

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

Cytokines, chemokines, and growth factors are 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. These molecules are typically measured either by bioassay or immunoassay. Both techniques are time consuming and can facilitate the analysis of only a single target at a time. The Bio-Plex® 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 Pro™ cytokine, chemokine, and growth factor assays are magnetic bead-based multiplex assays designed to measure multiple cytokines, chemokines, and growth factors in diverse matrices like serum, plasma, and tissue culture supernatants. The multiplexing feature makes it possible to quantitate the level of multiple proteins in a single well, in just 3 hrs, using as little as 12.5 µl of serum or plasma, or 50 µl of tissue culture supernatants.

As one of the most recent additions to the Bio-Plex system, these assays incorporate magnetic beads into their design. The magnetic beads allow for the option of using magnetic separation during wash steps instead of vacuum filtration. Magnetic separation allows for greater automation without significant alterations to the standard Bio-Plex assay protocol.

Bio-Plex Manager™ software is recommended for Bio-Plex Pro cytokine, chemokine, and growth factor assays. For instructions using other xMAP system software packages, contact Bio-Rad technical support or your Bio-Rad field application specialist.

For a current listing of Bio-Plex cytokine, chemokines, and growth factor assays, visit us on the Web at www.bio-rad.com/bio-plex/.

Section 2
Principle

Technology

The Bio-Plex® system is built around three core technologies. The first is a novel technology that uses up to 100 unique fluorescently dyed beads (xMAP technology) that permit the simultaneous detection of up to 100 different types of molecules in a single well of a 96-well microplate. The second is a dedicated flow cytometer with two lasers and associated optics to measure the different molecules bound to the surface of the beads. The third is a high-speed digital signal processor that efficiently manages the fluorescent data.

Assay Format

The principle behind the 96-well plate-formatted, bead-based assays is similar to a capture sandwich immunoassay. An antibody directed against the desired cytokine, chemokine, or growth factor target is covalently coupled to internally dyed beads. The coupled beads are allowed to react with a sample containing the target biomolecules. After a series of washes to remove unbound protein, a biotinylated detection antibody specific to an epitope different from that of the capture antibody is added to the reaction. This results in the formation of a sandwich of antibodies around the cytokine, chemokine, or growth factor target. A streptavidin-phycoerythrin (streptavidin-PE) reporter complex is then added to bind to the biotinylated detection antibodies on the bead surface.

Data Acquisition and Analysis

Data from the reaction are acquired using the Bio-Plex system (or Luminex system), a dual-laser, flow-based microplate reader system. The contents of the well are drawn up into the reader. The lasers and associated optics detect the internal fluorescence of the individual dyed beads as well as the fluorescent reporter signal on the bead surface. This identifies each assay and reports the level of target protein in the sample. Intensity of fluorescence detected on the beads indicates the relative quantity of target molecules in the tested samples. A high-speed digital processor efficiently manages the data output, which is further analyzed and presented as fluorescence intensity (FI) and target concentration on Bio-Plex Manager™ software.

Assay Workflow

Section 3
Product Description

Bio-Plex Pro™ cytokine, chemokine, and growth factor assays are offered in a convenient kit format that includes assay, reagent, and diluent components all in a single box. If magnetic separation is being used, flat bottom plates are sold separately. Instructions for this kit may be used for the detection of the following:

Premixed Multiplex Assays

Description	Catalog #
Bio-Plex Pro Human Cytokine 8-plex Assay, 1 x 96-well	M50000007A
Bio-Plex Pro Human Cytokine TH1/TH2 Assay, 1 x 96-well	M5000005L3
Bio-Plex Pro Human Cytokine 17-plex Assay, 1 x 96-well	M5000031YV
Bio-Plex Pro Human Cytokine 27-plex Assay, 1 x 96-well	M500KCAF0Y
Bio-Plex Pro Human Cytokine 21-plex Assay, 1 x 96-well	MF0005KMII
Bio-Plex Pro Mouse Cytokine 8-plex Assay, 1 x 96-well	M60000007A
Bio-Plex Pro Mouse Cytokine TH1/TH2 Assay, 1 x 96-well	M6000003J7
Bio-Plex Pro Mouse Cytokine 23-plex Assay, 1 x 96-well	M60009RDPD
Bio-Plex Pro Mouse Cytokine Group 9-plex Assay, 1 x 96-well	MD000000EL

Off-the-Shelf Singleplex Assays

The following are catalog numbers required for ordering singleplex assays:

Bio-Plex Pro Cytokine Reagent Kits	Catalog #
1 x 96-well	171-304070
10 x 96-well	171-304071
Bio-Plex Human Cytokine Standards	**Catalog #**
Human group I, 1 vial of 27 targets	171-D50001
Human group I, 50 vials of 27 targets	171-D10501
Human group II, 1 vial of 23 targets	171-D60001
Human group II, 50 vials of 23 targets	171-D10502
Bio-Plex Mouse Cytokine Standards	**Catalog #**
Mouse group I, 1 vial of 23 targets	171-I50001
Mouse group I, 50 vials of 23 targets	171-I10501
Mouse group II, 1 vial of 9 targets	171-I60001
Mouse group II, 50 vials of 9 targets	171-I10502

Bio-Plex Pro Human Cytokine Assays			
Group I	Catalog #	Group II	Catalog #
IL-1	171-B5001M	IL-1	171-B6001M
IL-1ra	171-B5002M	IL-2R	171-B6002M
IL-2	171-B5003M	IL-3	171-B6003M
IL-4	171-B5004M	IL-12 (p40)	171-B6004M
IL-5	171-B5005M	IL-16	171-B6005M
IL-6	171-B5006M	IL-18*	N/A
IL-7	171-B5007M	CTACK	171-B6006M
IL-8	171-B5008M	GRO-	171-B6007M
IL-9	171-B5009M	HGF	171-B6008M
IL-10	171-B5010M	ICAM-1	171-B6009M
IL-12 (p70)	171-B5011M	IFN- 2	171-B6010M
IL-13	171-B5012M	LIF	171-B6011M
IL-15	171-B5013M	MCP-3	171-B6012M
IL-17	171-B5014M	M-CSF	171-B6013M
Basic FGF	171-B5016M	MIF	171-B6014M
Eotaxin	171-B5015M	MIG	171-B6015M
G-CSF	171-B5017M	-NGF	171-B6016M
GM-CSF	171-B5018M	SCF	171-B6017M
IFN-	171-B5019M	SCGF-	171-B6018M
IP-10	171-B5020M	SDF-1	171-B6019M
MCP-1 (MCAF)	171-B5021M	TNF-	171-B6020M
MIP-1	171-B5022M	TRAIL	171-B6021M
MIP-1	171-B5023M	VCAM-1	171-B6022M
PDGF-BB	171-B5024M		
RANTES	171-B5025M		
TNF-	171-B5026M		
VEGF	171-B5027M		

Bio-Plex Pro Mouse Cytokine Assays			
Group I	Catalog #	Group II	Catalog #
IL-1	171-G5001M	IL-15	171-G6001M
IL-1	171-G5002M	IL-18*	N/A
IL-2	171-G5003M	Basic FGF	171-G6002M
IL-3	171-G5004M	LIF	171-G6003M
IL-4	171-G5005M	M-CSF	171-G6004M
IL-5	171-G5006M	MIG	171-G6005M
IL-6	171-G5007M	MIP-2	171-G6006M
IL-9	171-G5008M	PDGF-BB	171-G6007M
IL-10	171-G5009M	VEGF	171-G6008M
IL-12 (p40)	171-G5010M		
IL-12 (p70)	171-G5011M		
IL-13	171-G5012M		
IL-17	171-G5013M		
Eotaxin	171-G5014M		
G-CSF	171-G5015M		
GM-CSF	171-G5016M		
IFN-	171-G5017M		
KC	171-G5018M		
MCP-1 (MCAF)	171-G5019M		
MIP-1	171-G5020M		
MIP-1	171-G5021M		
RANTES	171-G5022M		
TNF-	171-G5023M		

**IL-18 is not available as singleplex assays.*

Bio-Plex Express Assays

Visit Bio-Rad's Bio-Plex Express assay service at www.bio-rad.com/bio-plex/x-plex to select from available Bio-Plex cytokine, chemokine, and growth factor targets and mix your own multiplex assay.

Bio-Plex x-Plex Assays

Visit Bio-Rad's x-Plex blending service at www.bio-rad.com/bio-plex/x-plex to configure QC tested custom-mixed multiplex assays.

Required Materials

Bio-Plex Pro cytokine, chemokine, and growth factor assays contain the following components all in one kit:

Components	1 x 96-Well Format	10 x 96-Well Format
Coupled magnetic beads (10x)	600 µl	6,000 µl
Detection antibodies (10x)	320 µl	3,200 µl
Standard (additional vials sold separately)	1 vial	10 vials
Universal standard diluent	10 ml	100 ml
Universal sample diluent	8 ml	80 ml
Assay buffer	50 ml	500 ml
Wash buffer	130 ml	1,300 ml
Detection antibody diluent	5 ml	50 ml
Streptavidin-PE (100x)	1 vial	1 vial
Filter plate (96-well)	1 plate	10 plates
Sealing tape	1 pack of 4	5 packs of 4
Instruction Manual	1	1
Tube holder	1	1

Sold separately are the Bio-Plex Pro flat bottom plates, required for magnetic separation on the Bio-Plex Pro wash stations. Order Bio-Rad catalog #171-025001.

Storage and Stability

Kit components 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 up to 6 months from the date of purchase when stored as specified in this manual.

Section 4
Recommended Materials

Items	Ordering Information
Bio-Plex® or Luminex system	Bio-Rad catalog# 171-000205
Bio-Plex or Luminex system (includes Bio-Plex Pro wash station II)	Bio-Rad catalog #171-0HPCP1
Bio-Plex validation kit	Bio-Rad catalog #171-203001
Bio-Plex calibration kit	Bio-Rad catalog #171-203060
Bio-Plex Pro wash station	Bio-Rad catalog #30034376
Bio-Plex Pro II wash station	Bio-Rad catalog #30034377
Bio-Plex Pro flat bottom plates (40, 1 x 96-well plates)	Bio-Rad catalog #171-025001
Microtiter plate shaker 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 #3208000 VWR catalog #57019-600
Bio-Rad Aurum™ vacuum manifold IMPORTANT: The use of filter plate manifolds other than the one specified may result in diminished assay performance; see Section 8 for instructions specific to this assay	Bio-Rad catalog #732-6470
Vortexer VWR mini-vortexer Scientific Instruments Vortex-Genie 2 mixer	VWR catalog #58816-121 VWR catalog #58815-234
Reagent reservoirs, 25 ml (for capture beads and detection antibodies)	VistaLab catalog #3054-2002 VistaLab catalog #3054-1004
Reagent reservoir, 50 ml (for reagents and buffers)	VistaLab catalog #3054-1006
Pall Life Science Acrodisc: 25 mm PF syringe filter (0.8/0.2 μm Supor membrane)	Pall catalog #PN4187
Other materials: Pipets and pipet tips, sterile distilled water, aluminium foil, absorbent paper towels, 1.5 or 2 ml microcentrifuge tubes, and standard flat bottom microplate (for calibrating vacuum manifold).	

Section 5
Bead Regions

Each bead has been assigned a number (bead region) from 1 to 100 according to the ratio of the two dyes contained. For correct data acquisition using the Bio-Plex® (or Luminex) system and associated software, use the chart below to identify the bead region for the targets in your Bio-Plex assay.

NOTE: DO NOT use preset panels found in Bio-Plex Manager version 5.0 or lower. For details, see Section 9 (Data Acquisition). Bead regions for Bio-Plex Pro cytokine, chemokine and growth factor assays are not identical to existing preset panels.

Human Cytokines				Mouse Cytokines			
Group I	Bead Region	Group II	Bead Region	Group I	Bead Region	Group II	Bead Region
IL-1β	39 NEW	IL-1α	63	IL-1α	53	IL-15	42
IL-1ra	25	IL-2Rα	13 NEW	IL-1β	19	IL-18	20
IL-2	38	IL-3	64	IL-2	36	Basic FGF	25
IL-4	52	IL-12 (p40)	28	IL-3	18	LIF	45
IL-5	33	IL-16	27	IL-4	39 NEW	M-CSF	26
IL-6	19	IL-18	42	IL-5	52	MIG	44
IL-7	74	CTACK	72	IL-6	38	MIP-2	27 NEW
IL-8	54	GRO-α	61	IL-9	33	PDGF-BB	35
IL-9	77	HGF	62	IL-10	56	VEGF	47
IL-10	56	ICAM-1	12 NEW	IL-12 (p40)	76		
IL-12 (p70)	75	IFN-α2	20	IL-12 (p70)	78 NEW		
IL-13	51	LIF	29	IL-13	37		
IL-15	73	MCP-3	26	IL-17	72		
IL-17	76	M-CSF	67	Eotaxin	74		
Eotaxin	43	MIF	35	G-CSF	54		
Basic FGF	44	MIG	14 NEW	GM-CSF	73		
G-CSF	57	β-NGF	46	IFN-γ	34		
GM-CSF	34	SCF	65	KC	57		
IFN-γ	21	SCGF-β	78	MCP-1 (MCAF)	51		
IP-10	48 NEW	SDF-1α	22 NEW	MIP-1α	77		
(MCAF)	53	TNF-β	30 NEW	MIP-1β	75		
MIP-1α	55	TRAIL	66	RANTES	55		
MIP-1β	18	VCAM-1	15 NEW	TNF-α	21		
PDGF-BB	47						
RANTES	37						
TNF-α	36						
VEGF	45						

Section 6
Sample Preparation

This section provides instructions for preparing samples derived from serum, plasma, and tissue culture supernatant. For sample preparations not mentioned here, consult the publications listed in Bio-Rad bulletin 5297, available for download at discover.bio-rad.com. Additional information can also be found in Section 10 (Troubleshooting Guide).

Serum and Plasma Samples

NOTE: For plasma samples, both EDTA plasma and citrate plasma are acceptable. Heparin plasma may absorb certain cytokines and is therefore not preferred. Avoid using hemolyzed samples.

1. To prepare serum, allow blood to clot at room temperature for 30 min to 1 hr. For plasma, centrifuge immediately after collection in tubes containing anticoagulant. Centrifuge serum or plasma samples at 13,200 rpm for 10 min at 4°C to clear the samples of precipitate. Alternatively, carefully filter the samples with a 0.8/0.22 µm dual filter to prevent instrument clogging. Assay immediately or aliquot and store samples in single use aliquots at –70°C. Avoid repeated freeze/thaw cycles.

2. Dilute 1 volume of sample with 3 volumes of sample diluent. Keep the samples on ice until ready for use.

   NOTE: Physiological levels of VCAM-1 and ICAM-1 are typically found at much higher concentrations. Sample dilutions of 1:100 are frequently required to achieve concentrations in the measurable range of the standard curve. Dilute serum 1:50 or 1:100 as follows: 1) dilute serum 1:4 in sample diluent, and 2) dilute further 1:25 using standard diluent.

Tissue Culture Supernatant Samples

1. Collect and process the tissue culture supernatant samples and assay immediately or store samples in single use aliquots at –70°C. Centrifuge samples at 13,200 rpm for 10 min at 4°C to clear the samples of precipitate. Avoid repeated freeze/thaw.

2. If required, dilute the culture supernatant with culture medium. For cell lines cultured in serum-free culture medium, collect samples, centrifuge as above, and add a carrier protein (such as BSA) at a final concentration of at least 0.5%. Keep the samples on ice until ready for use.

Tissue Lysates

Refer to bulletin 5297 for a list of published articles on cytokine analysis in tissue samples.

Lavage, Sputum, and Other Biological Fluid Samples

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%.

Section 7
Standard Preparation

One vial of lot-specific lyophilized standards is required for each Bio-Plex Pro™ cytokine, chemokine, and growth factor assay. By choosing among available assays, mixing standards may be necessary. This procedure will prepare enough standard to run each diluted standard point in duplicate.

Prior to performing this procedure, verify the contents of the cytokine standard by referring to the lot-specific peel-off sicker provided with the standard. This also lists the concentrations of the reconstituted standards, which are required for **Data Acquisition** (Section 9).

Reconstitute Standards

1. Gently tap the glass vial containing the lyophilized standard on a solid surface to ensure the pellet is at the bottom.

2. Reconstitute lyophilized standard:

   Premixed Standards

   1. Reconstitute one vial of lyophilized standard with 500 µl of the same matrix as samples. Do not use assay buffer to dilute standards.

   2. Gently vortex reconstituted standard 1–3 sec and incubate on ice for 30 min. Be **consistent** with the incubation time for optimal assay performance and reproducibility.

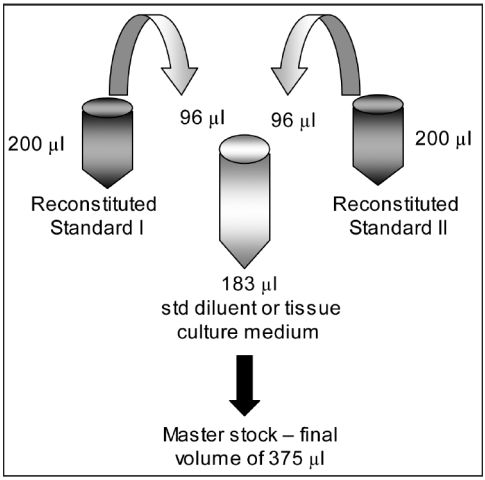
   Preparation of Master Stock From Two Premixed Standards

   1. Refer to diagram on the following page to reconstitute each vial of lyophilized standard with 200 µl of the same matrix as samples. Do not use assay buffer to dilute standards.

   2. Gently vortex reconstituted standard 1–3 sec and incubate on ice for 30 min. **Be consistent** with the incubation time for optimal assay performance.

3. Add 96 µl from each of the reconstituted standards into a single 1.5 ml tube containing 183 µl of the standard diluent. This will become the **master stock** and will be at a final volume of 375 µl. This will serve as Standard I in the standard Photomultiplier Tube (PMT) setting standard curve.

4. Gently vortex for 1–3 seconds.



Prepare Standard Dilution Series

The cytokine, chemokine, and growth factor concentrations specified for the eight-point standard dilution set have been selected for optimized curve fitting using the five-parameter logistic (5PL) or four-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.

1. Label a set of 1.5 ml Eppendorf tubes as shown in the diagrams on the following page.

2. Use the provided serum standard diluent, and pipet the appropriate volume of standard diluent into the tubes (refer to the diagrams on the following page). Alternatively use culture medium for culture samples.

   NOTE: 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 and prepare serial dilutions accordingly (see following page). It is recommended to run a low PMT setting standard curve first.

3. Transfer the respective volume of reconstituted standard:

   Premixed Standards
   Add 128 µl of reconstituted standard to the first 1.5 ml tube with 72 µl of standard diluent or appropriate matrix. This is identified as S1 in the diagram on the following page.

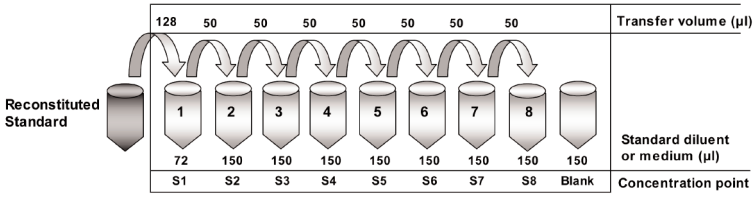
   Master Stock (Two Cytokine Standards)
   Add 375 µl of reconstituted standard to the first 1.5 ml tube with 0 µl of standard diluent or appropriate matrix. This is identified as S1 in the diagram on the following page.

4. Continue making serial dilutions of the standard as shown. After making each dilution, vortex gently for 1–3 sec and change the pipet tip after every transfer.

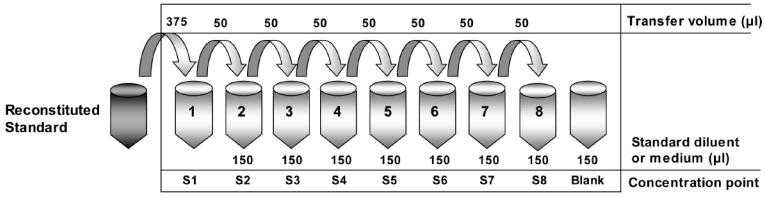
   NOTE: Running an additional two 0 pg/ml blanks is strongly recommended. The blank wells are useful for troubleshooting and determining limit of detection (LOD). Use 50 µl of the appropriate standard diluent as the blank sample. The 0 pg/ml points should be formatted as blanks, not as points in the curve, when using Bio-Plex Manager software. Formatting the wells as blanks automatically subtracts the background mean fluorescence intensity (MFI) values from the reading and may result in negative MFI values in some wells. If negative MFI values are undesirable, format the 0 pg/ml wells as background controls.

5. Keep the standards on ice until ready for use. Reconstituted standards should be used immediately and not frozen for future use.

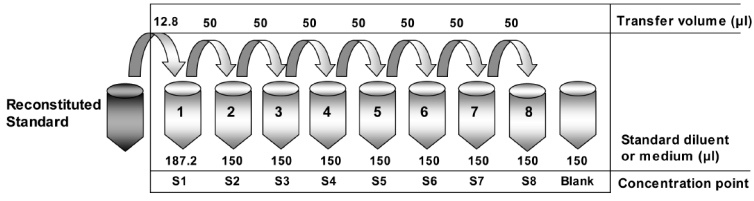
Low PMT – Standard Dilution Series for Premixed Standards



Low PMT – Standard Dilution Series for Master Stock (Two Cytokine Standards)



High PMT – Standard Dilution Series for Premixed Standards



Section 8
Assay Instructions

The following instructions apply to Bio-Plex Pro™ cytokine, chemokine, and growth factor assays in a premixed multiplex, singleplex, Bio-Plex Express, and Bio-Plex x-plex assay formats. The assay protocol to run assays that are manually mixed is the same as that for assays that are premixed, or in a singleplex format. The only difference is the additional step of manually mixing the coupled beads and detection antibodies prior to use.

8.1 Assay Plate and Wash Format

Assays can be prepared in 96-well filter plates for vacuum based washing, or Bio-Plex Pro flat bottom plates for magnetic based washing. The wash steps can be performed with the filter plates using a manual vacuum manifold, or the vacuum feature of the Pro II Wash Station. Magnetic washing can be performed using the magnetic settings of the Pro, or Pro II Wash Station.

8.2 Plan Experiment

For calculating coupled beads and detection dilutions, see tables on pages 18 and 21.

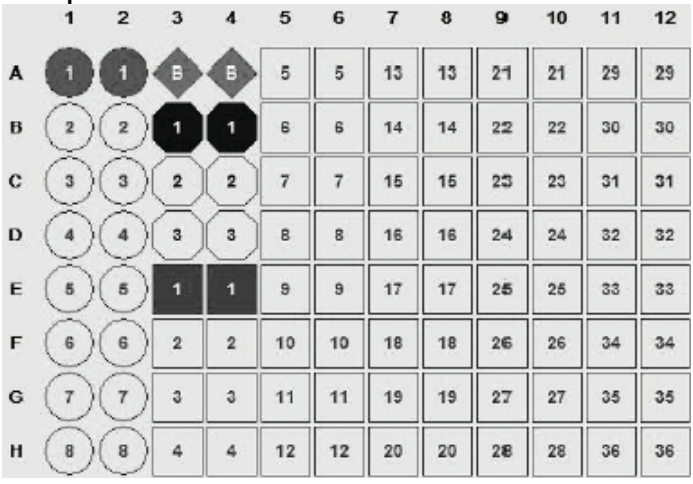
1. Bring all buffers to room temperature. Avoid bubbles when pipetting.

   NOTE: To ensure adequate volume to run 10 plates using 10x96 reagent kits, remove only the required amount from the bottles for each assay run. For example, transfer a one-time volume of assay buffer into a 50 ml reservoir, sufficient to perform all steps of the assay procedure (i.e. prewetting the filter plate, diluting 10x coupled beads, diluting 100x streptavidin-PE, and resuspending the beads).

2. Assign wells of a 96-well plate to be used for each standard, control, and sample (see the example on the following page).

3. Determine the total number of wells that will be used in the assay and design the plate layout. Notice that the template in Section 12 can be used as a reference.

Example Plate



8.3 Calibrate Vacuum Manifold (if appropriate)

Prior to performing any Bio-Plex assay, the vacuum apparatus must be calibrated to ensure optimal bead yield. For more detailed instructions refer to the Bio-Plex® suspension array system hardware instruction manual.

NOTE: Calibration for Bio-Plex Pro™ washers is not required.

1. Place a standard flat-bottom microplate (not a filter plate) on the vacuum apparatus.
2. Turn on the vacuum source 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 control valves on the unit. The pressure should be set to 1–3" 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 residual fluid can lead to imprecise results. As a general guideline, 100 µl of liquid should take approximately 3–4 sec to completely clear the well.

8.4 Prepare Coupled Magnetic Beads

Protect the beads from light. Keep all tubes on ice until ready for use.

1. Quick-spin the coupled beads (10x). Carefully open the cap and gently resuspend the coupled beads with a pipet before removing desired volume.

2. Prepare coupled beads (1x) in assay buffer. Each well requires 5 µl of coupled beads (10x) adjusted to a final volume of 50 µl using assay buffer. For mixing different bead sets, use equal volumes of each coupled bead. The final volume should always be the same regardless of how many beads are combined. Refer to the example bead calculations below, which include 20% excess in coupled beads to compensate for transfer loss.

Example Coupled Magnetic Bead Calculations for Premixed or Singleplex Assays (1x)

# of Wells	10x Beads (µl)	Assay Buffer (µl)	Total Volume (µl)
96	575.0	5,175	5,750
88	527.1	4,744	5,271
80	479.2	4,312	4,792
72	431.2	3,881	4,312
64	383.3	3,450	3,833
56	335.4	3,019	3,354
48	287.5	2,587	2,875

Example Coupled Magnetic Bead Calculations for Mixing Coupled Beads (1x from10x stocks)

# of Wells	10x Beads (µl) G-CSF, for example	10x Beads (µl) 8-plex, for example	Assay Buffer (µl)	Total Volume (µl)
96	575.0	575.0	4,600	5,750
88	527.1	527.1	4,217	5,271
80	479.2	479.2	3,833	4,792
72	431.2	431.2	3,450	4,312
64	383.3	383.3	3,067	3,833
56	335.4	335.4	2,683	3,354
48	287.5	287.5	2,300	2,875

8.5 Assay Procedure

Assay Key – The terms below are repeated throughout the assay procedure. Refer to these detailed instructions when **wash** and **incubate on shaker** are shown in bold.

Step	Detailed Directions
Wash	**Vacuum Manifold** Place the filter plate on a calibrated vacuum apparatus and remove the buffer by vacuum filtration. Add 100 µl of wash buffer to each well and remove the liquid as before. Repeat as specified. Thoroughly blot the bottom of the filter plate with a clean paper towel between each vacuum step to prevent cross contamination and plate leakage. Place the filter plate on the plastic plate holder. **Bio-Plex Pro Wash Station** For preloaded magnetic programs with the Bio-Plex Pro flat bottom plates, use MAG X2 for two cycles of initial bead wash and MAG X3 for three cycles of washing following three incubation steps. Always rinse the instrument after use. Prime the instrument without liquid to empty all liquid from the system before removing the manifold. **Bio-Plex Pro II Wash Station** For preloaded magnetic programs with the Bio-Plex Pro flat bottom plates use MAG X2 for two cycles of initial bead wash and MAG X3 for three cycles of washing folllowing three incubation steps. For preloaded vacuum programs using filter plates, use VAC X2 for two cycles of initial bead wash and VAC X3 for three cycles of wash following three incubation steps. Always rinse the instrument after use. Prime the instrument without liquid to empty all liquid from the system before removing the manifold.
Incubate on shaker	Gently cover the filter plate with a new sheet of sealing tape and press down on edges to prevent liquid escaping from the bottom of the wells. Place the filter plate on a microplate shaker and then cover with aluminum foil. Shake the filter plate at room temperature at 1,100 rpm for 30 sec, and then at 300 rpm for the specified incubation time.

1. Equilibrate the diluted standards, samples, and controls at room temperature for 20 min prior to use.

2. Prewet (skip this step if using a flat bottom plate).

   a. **Vacuum Manifold**
   Prewet the wells of a 96-well filter plate using 100 µl of assay buffer and remove the liquid by vacuum filtration. Dry the bottom of the filter plate thoroughly by blotting on a clean paper towel. If fewer than 96 wells are required mark the plate to identify the unused wells for later use and cover the unused wells with sealing tape prior to vacuum filtration.

   b. **Vacuum With Pro II Wash Station**
   Prewet the wells of a 96-well filter plate using 125 µl of assay buffer and remove the liquid following the PREVAC program on the Pro II Wash Station.

3. Vortex the coupled beads (1x) for 30 sec at medium speed. Add 50 µl to each well.

4. **Wash** twice with the vacuum manifold or Bio-Plex Pro wash stations (VAC 2X or MAG 2X). See page 19 for detailed instructions.

5. Gently vortex the diluted standards, controls, and samples for 1–3 sec. Add 50 µl of standard, control, or sample to each well, changing the pipet tip after every volume transfer. **Incubate on shaker** at room temperature for 30 min (see page 19 for detailed instructions).

6. While the samples are incubating, vortex the detection antibody (10x) for 15–20 sec at medium speed. Always perform a 30 sec spin to collect the entire volume at the bottom of the vial.

7. Prepare detection antibodies (1x) using detection antibody diluent. Each well requires 2.5 µl of detection antibodies (10x) adjusted to a final volume of 25 µl with detection antibody diluent. Refer to the example detection antibody calculations on the following page, which include 25% excess in detection antibodies to compensate for transfer loss.

Example Detection Antibody Calculations for Premixed or Singleplex Assays

# of Wells	10x Detection Antibody (µl)	Detection Antibody Diluent (µl)	Total Volume (µl)
96	300	2,700	3,000
88	275	2,475	2,750
80	250	2,250	2,500
72	225	2,025	2,250
64	200	1,800	2,000
56	175	1,575	1,750
48	150	1,350	1,500

Example Detection Antibody Calculations for Mixing Detection Antibodies

# of Wells	10x Detection Antibody (µl) G-CSF, for example	10x Detection Antibody (µl) 8-plex, for example	Detection Antibody Diluent (µl)	Total Volume (µl)
96	300	300	2,400	3,000
88	275	275	2,200	2,750
80	250	250	2,000	2,500
72	225	225	1,800	2,250
64	200	200	1,600	2,000
56	175	175	1,400	1,750
48	150	150	1,200	1,500

8. After incubating the samples, slowly remove and discard the sealing tape.

9. **Wash** three times with the vacuum manifold or Bio-Plex Pro wash stations (VAC 3X or MAG 3X). See page 19 for detailed instructions.

10. Vortex the detection antibodies gently for 1–3 sec and add 25 µl to each well. **Incubate on shaker** at room temperature for 30 min (see page 19 for detailed instructions).

11. While the detection antibodies are incubating, vortex the streptavidin-PE (100x) for 15–20 sec at medium speed. Always perform a 30 sec spin to collect the entire volume at the bottom of the vial.

12. Prepare streptavidin-PE (1x) using assay buffer. Each well requires 0.5 µl of streptavidin-PE (100x) adjusted to a final volume of 50 µl with assay buffer. Refer to the example streptavidin-PE calculation below, which includes 25% excess in streptavidin-PE to compensate for transfer loss.

Example Streptavidin-PE Calculations

# of Wells	100x Streptavidin-PE (µl)	Assay Buffer (µl)	Total Volume (µl)
96	60.0	5,940	6,000
88	55.0	5,445	5,500
80	50.0	4,950	5,000
72	45.0	4,455	4,500
64	40.0	3,960	4,000
56	35.0	3,465	3,500
48	30.0	2,970	3,000

13. After the detection antibody incubation, slowly remove and discard the sealing tape.
14. **Wash** three times with the vacuum manifold or Bio-Plex Pro wash stations (VAC 3X or MAG 3X). See page 19 for detailed instructions.
15. Vortex the streptavidin-PE (1x) vigorously for 3–5 sec and add 50 µl to each well. **Incubate on shaker** at room temperature for 10 min (see page 19 for detailed instructions).

    NOTE: It is important to **be consistent** with this incubation step to reduce inter-assay variation.
16. After the streptavidin-PE incubation, slowly remove and discard the sealing tape.
17. **Wash** three times with the vacuum manifold or Bio-Plex Pro wash stations (VAC 3X or MAG 3X). See page 19 for detailed instructions.
18. Add 125 µl of assay buffer to each well. Cover the plate with a new sheet of sealing tape. Shake the plate at room temperature at 1,100 rpm for 30 sec and remove the sealing tape before reading the plate.

Section 9
Data Acquisition

Bio-Plex Manager™ software is recommended for Bio-Plex Pro™ cytokine, chemokine, and growth factor assays. To minimize protocol setup, lot-specific protocols for Bio-Plex Manager version 4.0 and higher are available for download at www.biorad.com/bio-plex/standards.

For instructions using other xMAP system software packages, contact Bio-Rad technical support or your regional Bio-Rad field applications specialist.

Prepare System

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 hours without acquiring data, the lasers will automatically turn off. To reset the 4-hour countdown, select Warm up  and wait for the optics to reach operational temperature.

Calibrate With Low RP1 Target Value

Daily calibration is recommended before acquiring data. Calibrate using Bio-Plex® calibration beads and target values. Bio-Plex cytokine, chemokine, and growth factor assays are run using the low RP1 target value on the Cal 2 bottle.

Bio-Plex Manager Software version 5.0 and Higher

This software version requires calibration only with the low RP1 target value.

1. Select Calibrate  and confirm that the default values for CAL1 and CAL2 are the same as the values on the Bio-Plex calibration bead labels. Use the Bio-Plex low RP1 target value.

2. Select OK and follow the instructions for CAL1 and CAL2 calibration.

Bio-Plex Manager Software version 4.0, 4.1, and 4.1.1

These software versions require calibration at either low or high RP1 target values. Recalibration is necessary between assays requiring different RP1 target values. Daily calibration is recommended before acquiring data.

1. Select Calibrate  and confirm that the default values for CAL1 and CAL2 are the same as the values on the Bio-Plex calibration bead labels. Use the appropriate Bio-Plex low RP1 target value.

2. Select OK and follow the instructions for CAL1 and CAL2 calibration.

Prepare Protocol

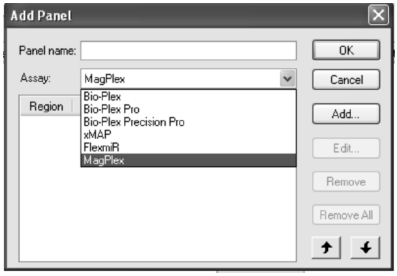
1. Open the lot-specific assay protocols (available for download at www.biorad.com/bio-plex/standards) or create a new protocol by selecting File, then New from the main menu. Locate the steps at the left of the protocol menu.

2. Select Step 1 (Describe Protocol) and enter information about the assay.

3. Select Step 2 (Select Analytes) and create a new panel. **Do not** use preset panels found in the Bio-Plex Manager software, version 5.0 or lower. If using a downloaded assay protocol from the Bio-Plex website, analytes will already be entered. Confirm the selected analytes and proceed to #4.

   a) Click the Add Panel button  in the Select Analytes toolbar.

   b) Enter a name for the new panel in the top field. If using Bio-Plex Manager version 5.0 (or higher), select the **MagPlex** assay type from the pull-down menu. Then click Add to add analytes.



c) Enter the bead region number of the first analyte in the Region field and the analyte name in the Name field.

NOTE: The bead region number must be correct for proper detection of analytes. Refer to Section 5 (Bead Regions) to confirm the bead region for each analyte in the assay is correct.

d) Click Add Continue to add the analyte to the panel and continue adding more analytes. When you have entered your last analyte, click the Add button to add it to the list.

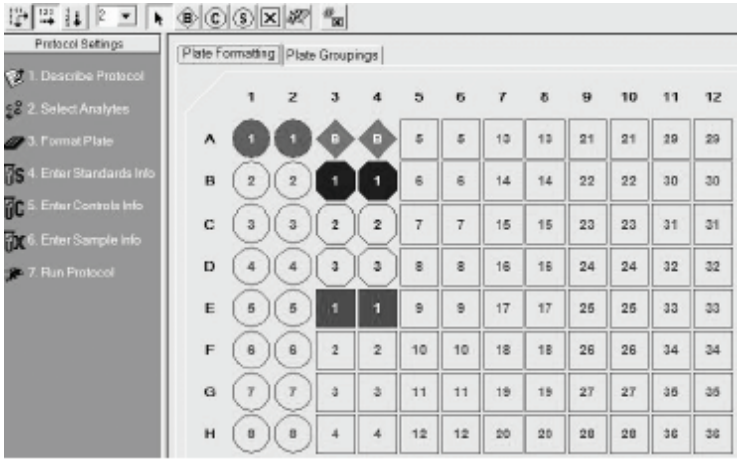
e) When you are finished creating the panel, click OK to save your changes and return to the Select Analytes window.

f) After selecting the panel, move analytes to the Selected view by choosing from analytes in the Available view using the Add button or Add All button. Note that the analytes will already be entered in the Selected view when using a downloaded protocol.

4. Select Step 3 (Format Plate) and click on the Plate Formatting tab. Select the number of replicates and the auto fill direction. Then click the (S) icon and drag the cursor over all the wells that contain standards. Then click on the ◈B◈ icon and drag the cursor over the wells that contain blanks. Repeat with (C) and [X] to identify all the wells that contain controls and samples.

NOTE: If the protocol was downloaded from at www.biorad.com/bio-plex/standards, the standards will already be added to the plate format. Make any necessary changes to the plate layout to match your plate requirements.

Plate Formatting Example



5. Select Step 4 (Enter Standards Info) to enter standards information. Skip to the next step if using a downloaded protocol from the web site; this information will already be entered.

   NOTE: If running the assay at high PMT setting, divide the standard values provided on the lot-specific peel-off sticker by ten.

   a) Deselect the box labeled “same concentration values for all analytes.”

   b) Select the first analyte from the pull-down cell.

   c) Click on the Enter Automatically button and then select the most concentrated value (typically S1).

   d) Enter the value for the highest concentration. The information is included on the product insert provided with the lot-specific standards.

   e) Enter the dilution factor (usually 4) and select Calculate. The concentrations for each replicate point of the standard will be populated for the selected analyte.

   f) Repeat steps 6b through 6e for each analyte in the assay.

6. Select Step 6 (Enter Sample Info) and enter sample information. Remember to enter information for the appropriate dilution factor.

7. Select Step 7 (Run Protocol) and run protocol for Low or High PMT setting, as appropriate.

   NOTE: To run the assay on High PMT for Bio-Plex Manager software version 5.0, check the box to run high RP1 target.

Acquire Data

1. Shake the assay plate at 1,100 rpm for 30 sec immediately before acquiring data.

2. Visually inspect the plate and ensure that the assay wells are filled with buffer prior to placing the plate in the Bio-Plex microplate plaform.

3. Slowly remove the sealing tape and any plate cover before placing the plate in the reader.

4. Select Step 7 (Run Protocol) and refer to the details in the appropriate section below.

   Bio-Plex Manager Software Version 5.0

   a) Change data acquisition for **50 beads per region**.

   b) In Advanced Settings, confirm that the bead map is set to **100 region**.

   c) In Advanced Settings, confirm that the sample size is set to **50 µl**.

   d) In Advanced Settings, confirm that the DD gate values are set to **5,000 (low)** and **25,000 (high)**.

   e) Select Start, save the .rbx file, and begin data acquisition.

   Bio-Plex Manager Software Version 4.0, 4.1, and 4.1.1

   a) Change data acquisition for **50 beads per region**.

   b) In Advanced Settings, confirm that the sample size is set to **50 µl**.

   c) In Advanced Settings, check the Override Gates and set the DD gate value to **5,000 (low)** and **25,000 (high)**.

   d) Select Start, save the .rbx file, and begin data acquisition.

5. If acquiring data from more than one plate, empty the waste bottle and refill the sheath bottle after each plate (if HTF not present). Select Wash Between Plates  and follow the instructions for fluidics maintenance. Then repeat the **Prepare Protocol** and **Acquire Data** steps.

   NOTE: Use the Wash Between Plates command after every plate run to reduce the possibility of clogging the instrument.

6. 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 Step 7 (Run Protocol).

1. Check the wells where data will be acquired a second time. Any previous data will be overwritten.

2. Remove the buffer by manual vacuum filtration or using the Bio-Plex Pro wash stations. Add 100 µl of assay buffer to each well. Cover the filter plate with a new sheet of sealing tape.

3. Repeat **Acquire Data** steps 1–6 to acquire data a second time. The data acquired should be similar to the data acquired initially; however, the data acquisition time will be extended since fewer beads are present in each well.

Section 10
Troubleshooting Guide

This troubleshooting guide addresses problems that may be encountered with Bio-Plex Pro™ cytokine, chemokine, and growth factor assays. 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® suspension array reader. To eliminate this possibility, the Bio-Plex validation kit is highly recommended. This kit will validate all the key functions of the array reader and assist in determining whether or not the array reader is functioning properly.

Possible Causes	Possible Solutions
High Inter-Assay CV	
Standards were not reconstituted consistently between assays	Incubate the reconstituted standards for 30 min on ice. Always be consistent with the incubation time and temperature.
Reconstituted standards and diluted samples were not stored properly	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.

Possible Causes	Possible Solutions
High Intra-Assay CV	
Bottom of filter plate not dry	Dry the bottom of the filter plate with absorbent paper towel (preferably lint-free) to prevent cross-contamination.
Pipetting technique	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.
Reagents and assay components were not equilibrated to room temperature prior to pipetting	All reagents and assay components should be equilibrated to room temperature prior to pipetting.
Contamination with wash buffer during wash steps	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. Also, be sure that the microplate shaker setting is not too high. Reduce the microplate shaker speed to minimize splashing.
Slow pipetting samples and reagents across the plate	Sample pipetting across the entire plate should take less than 4 min. Reagent pipetting across the entire plate should take less than 1 min.

Possible Causes	Possible Solutions
Low Bead Count	
Miscalculation of bead dilution	Check your calculations and be careful to add the correct volumes.
Beads clumped in multiplex bead stock tube	Vortex for 15–20 sec at medium speed before aliquoting beads.
Vacuum 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.
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 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
High Background Signal	
Incorrect buffer was used (for example, assay buffer used to dilute standards)	Use sample matrix or serum standard diluent to dilute cytokine standards.
Spiked “0 pg/ml” wells by mistake	Be careful when spiking standards. Do not add any antigens in the 0 (blank) point.
Detection antibodies or streptavidin-PE incubated too long	Follow the procedure incubation time.
Poor Recovery	
Expired Bio-Plex reagents were used	Check that reagents have not expired. Use new or nonexpired components.
Incorrect amounts of components were added	Check your calculations and be careful to add the correct volumes.
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.

Possible Causes	Possible Solutions
Pipetting technique	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.
Instrument Clogs	Use the Wash Between Plates command after every plate run to reduce the possibility of clogging the BioPlex instrument. For lipemic samples run Sanitize after plate read is complete.
Other Troubleshooting Tips	
Sample preparation - lipemic serum/plasma samples	Lipemic serum/plasma samples have not been validated with Bio-Plex Pro cytokine, chemokine, and growth factor assays. For lipemic serum/plasma, clear the fat by treating samples with LipoClear (www.Staspin.com). Dilute samples appropriately with standard diluent. To determine if treatment with Lipoclear interferes with the assay, test samples with and without spike controls.

Possible Causes	Possible Solutions
Sample preparation - tissue lysates	Refer to bulletin 5297 (list of published articles). Protein extraction methods (sonication, homogenization) will affect protein content of lysate and differences in lysis buffer composition may impact antigen-antibody binding and the detection of cytokines. Bio-Rad's cell lysis buffer (cat # 171-304011) has been used for cytokine analysis. General guideline: prepare tissue lysate with concentrated total proteins. Dilute lysate with PBS+0.5% BSA to bring protein concentration down to 1-2 mg/ml. Alternatively, try cytokine analysis on lysates diluted serially down from a starting concentration of 10mg/ml. For lysates prepared from cell culture samples, add 75 ul lysis buffer to each well in a 96-well plate. For 10 cm plates with confluent cells, scrape cells using a rubber policeman, spin cells down at 4ºC, remove supernatant, and lyse in 1–2 ml lysis buffer. To dilute the lysate use lysis buffer minus detergent. Lysis buffers containing nonionic detergents (1% v/v) are preferable to ionic detergents. Avoid SDS and reducing agents in lysis buffer. Add protease inhibitors just before use. For dilution of standards use as similar a matrix to that used to prepare samples. In order to determine if there is interference due to the matrix, run a spike control.

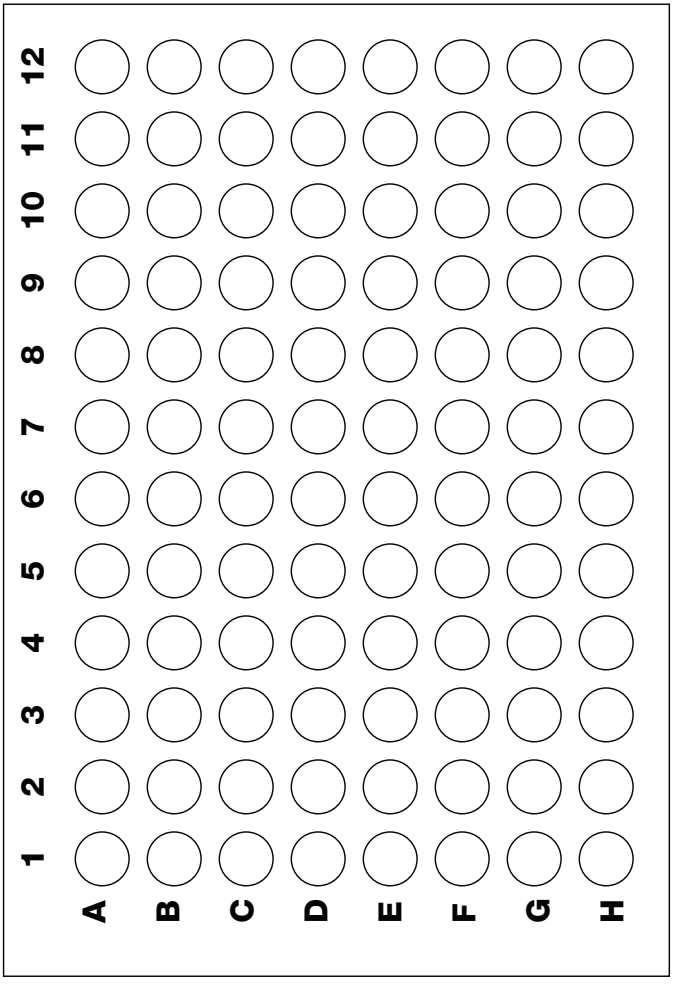
Section 11
Safety Considerations

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

The Bio-Plex Pro™ cytokine, chemokine, and growth factor assays contain components of animal origin. This material should be handled as if capable of transmitting infectious agents. Please use universal precautions. These components should be handled at Biosafety Level 2 containment (US Government publication: Biosafety in Microbiological and Biomedical Laboratories (CDC, 1999)).

Section 12
Plate Layout Templates

(Pull out and copy for repeat use)



Section 13
Legal Notices

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By purchasing this kit, which contains fluorescent 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 patent 5,981,180; 6,046,807; 6,057,107.

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