

Bio-Plex Pro™ Mouse Chemokine Assays

Instruction Manual

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Not for diagnostic procedures.



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Section 1

Description

Cytokines and chemokines are a host of extracellular mediators and regulators within a signaling network between cells and are key modulators of inflammation, participating in acute and chronic conditions via a complex network of interactions. The Bio-Plex Pro™ mouse chemokine assays are designed for the quantification of a panel of 34 cytokines, chemokines, and growth factors in serum, plasma, culture supernatant, and many other sample types. Table 1 shows the assays in the panel and their respective bead regions.

Table 1. Bioplex Pro mouse chemokine assays and bead regions.

Assay	Bead Region	Assay	Bead Region	Assay	Bead Region
BCA-1/CXCL13	28	IL-6	38	MIP-1β/CCL4	75
CTACK/CCL27	62	IL-10	56	MIP-2/CXCL2	27
ENA-78/CXCL5	20	IL-16	29	MIP-3α/CCL20	12
Eotaxin/CCL11	74	IP-10/CXCL10	35	MIP-3β/CCL19	64
Eotaxin-2/CCL24	73	I-TAC/CXCL11	37	RANTES/CCL5	55
Fractalkine/CX3CL1	25	KC/CXCL1	57	SCYB16/CXCL16	65
GM-CSF	22	MCP-1/CCL2	51	SDF-1α/CXCL12	67
I-309/CCL1	26	MCP-2/CCL8*	42	TARC/CCL17	63
IFN-γ	34	MCP-3/CCL7	46	TECK/CCL25	78
IL-1β	19	MCP-5/CCL12	48	TNF-α	21
IL-2	36	MDC/CCL22	52		
IL-4	39	MIP-1α/CCL3	77		

* MCP-2/CCL8 is sold as a singleplex assay.

By compiling profiles of a series of cytokine and chemokine markers, the assay panel provides more data from each sample, saving both money and time. These multiplex assays are highly suitable for researchers working with small rodent models to study multiple biomarkers using small sample volumes (10 µl per well). The assays can be run in a significantly shorter time (~2 hr) than conventional enzyme-linked immunosorbent assay (ELISA) techniques.

Visit bio-rad.com/bio-plex for more information.

Section 2

Principle

Technology

There are three core elements of the Bio-Plex[®] multiplex system:

- Fluorescently dyed magnetic microspheres (also called beads), each with a distinct color code or spectral address to permit discrimination of individual tests in a multiplex suspension. This allows simultaneous detection of up to 500 different molecules in a single well of a 96-well microplate on the Bio-Plex 3D suspension array system, up to 100 different molecules on the Bio-Plex 200 system, and up to 50 different molecules on the Bio-Plex[®] MAGPIX[™] multiplex reader
- A dedicated plate reader. The Bio-Plex 200 and Bio-Plex 3D systems are flow cytometry–based instruments with 2 lasers and associated optics to measure the different molecules bound to the surface of the beads
- A high-speed digital signal processor that efficiently manages the fluorescence data

Assay Format

Bio-Plex Pro[™] assays are sandwich 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.

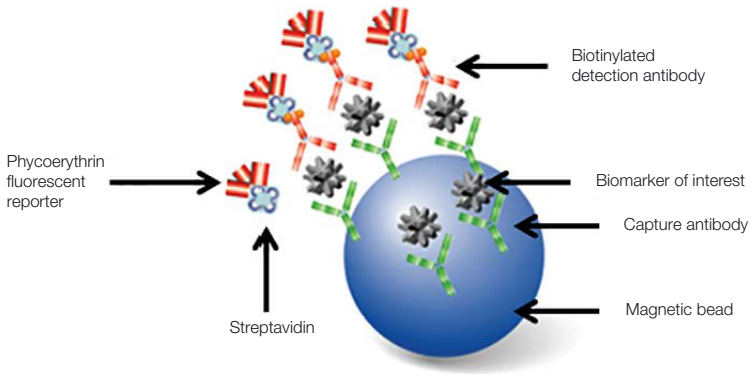


Fig. 1. Bio-Plex sandwich immunoassay.

Data Acquisition and Analysis

Data from the reactions are acquired using a Bio-Plex system or similar Luminex-based reader. For example, when a multiplex bead suspension is drawn into the Bio-Plex 200 reader, 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.

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

Section 3

Kit Contents and Storage

Reagents Supplied

Bio-Plex Pro™ mouse chemokine assays are available in a convenient all-in-one kit format that includes assay reagents, diluents, and buffers in a single box (Table 2).

Table 2. Contents of 1 x 96-well kit.

Component	Quantity
Coupled magnetic beads (10x)	1 tube
Detection antibodies (10x)	1 tube
Standards	1 vial
Quality control*	1 vial
Sample diluent	1 bottle
Detection antibody diluent HP	1 bottle
Standard diluent	1 bottle
Assay buffer	1 bottle
Wash buffer (10x)	1 bottle
Streptavidin-PE (100x)	1 tube
Assay plate (96-well flat bottom plate)	1 plate
Sealing tape	1 pack of 4
Assay quick guide	1 booklet
Product data sheet	1 sheet

* Provided with the premixed panel only.

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 6 months from the date of purchase when stored as specified.

Section 4

Recommended Materials

For recommended materials, see Table 3.

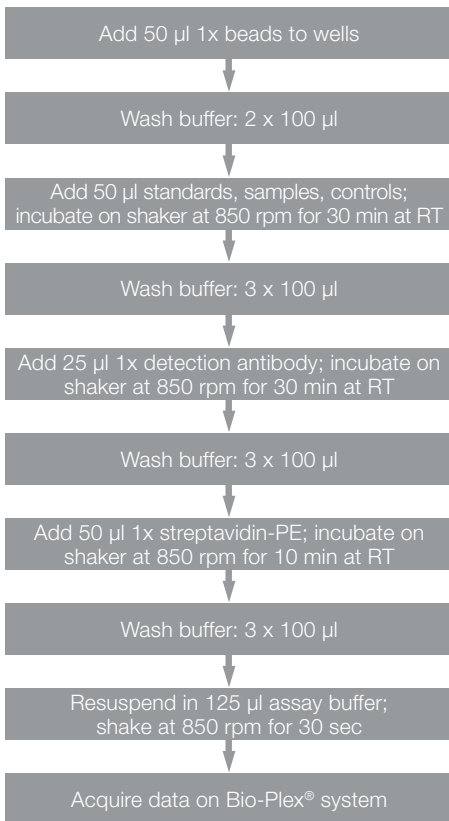
Table 3. Recommended materials.

Item	Ordering Information
Bio-Plex Pro™ mouse chemokine assay quick guide	Document #10000061997 (download at bio-rad.com/bio-plex)
Bio-Plex® validation kit 4.0 Note: Run the validation kit monthly to ensure optimal performance of fluidics and optics systems	Bio-Rad catalog #171203001
Bio-Plex calibration kit Note: Run the calibration kit daily to standardize the fluorescence signal	Bio-Rad catalog #171203060
Bio-Plex Pro wash station For use with magnetic bead-based assays only	Bio-Rad catalog #30034376
Bio-Plex handheld magnetic washer For use with magnetic bead-based assays only	Bio-Rad catalog #171020100
Bio-Plex Pro flat bottom plates (40 x 96-well) For magnetic separation on the Bio-Plex Pro wash station	Bio-Rad catalog #171025001
Titertube® micro test tubes For preparing replicate standards, samples, and controls prior to loading the plate	Bio-Rad catalog #2239390
Microtiter plate shaker IKA MTS 2/4 shaker for 2 or 4 microplates or Barnstead/Lab-Line titer plate shaker Model 4625 (or equivalent capable of 300–1,100 rpm)	IKA catalog #3208000 VWR catalog #57019-600
BR-2000 vortexer	Bio-Rad catalog #1660610
Reagent reservoirs, 25 ml For capture beads and detection antibodies	VistaLab catalog #3054-1002 or VistaLab catalog #3054-1004
Reagent reservoir, 50 ml For reagents and buffers	VistaLab catalog #3054-1006
Acrodisc PF syringe (25 mm) with Supor membrane (0.8/0.2 µm), sterile	Pall Corporation catalog #4187

Other: 5 or 15 ml polypropylene tubes for reagent dilutions, calibrated pipets, pipet tips, sterile distilled water, absorbent paper towels, and 1.5 or 2 ml microcentrifuge tubes.

Section 5

Assay Workflow



Note: Once thawed, keep samples on ice. Prepare dilutions just prior to the start of the assay and equilibrate to room temperature before use.

Section 6

Important Considerations

Instruments and Software

The Bio-Plex Pro™ 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 (see 9. Read Plate in section 7).

Assay Procedures

Please pay close attention to specific assay reagents and incubation times as they have been specifically optimized for each assay panel. Do not mix analytes between different Bio-Plex® panels or reagent kits. This kit uses Bio-Plex Pro reagent kit 5. Bead regions for all analytes are listed in Table 1.

Assay Quick Guide

Each assay kit comes complete with a printed Bio-Plex Pro assay quick guide (document #10000061997), which can be used to prepare and run a full 1 x 96-well assay plate. Users can also download a copy at bio-rad.com/bio-plex.

Section 7

Detailed Instructions

The following pages provide detailed instructions for each step of the assay procedure, including preparation, running the assay, and reading the plate with Bio-Plex Manager™ and Luminex xPONENT software.

1. Plan Plate Layout

Determine the total number of wells in the experiment using the plate layout template on page 32 or the Plate Formatting tab in Bio-Plex Manager software. A suggested plate layout is shown in Figure 2, with all conditions in duplicate.

Note: For detailed instructions about plate formatting in Bio-Plex Manager software, see 9. Read Plate in this section.

1. Assign the standards to columns 1 and 2, with the highest concentration in row A and the lowest concentration in row G.
2. Assign the blank to wells H1 and H2. The blank should consist of the chosen standard diluent. Note that Bio-Plex Manager software automatically subtracts the blank (B) median fluorescence intensity value from all other assay wells.
3. Assign the kit control to wells A3 and A4.
4. The remainder of the plate is available for samples.
5. Calculate the required volumes of beads, detection antibody, and streptavidin-PE using Tables 7–8, 10–11, and 12, respectively.

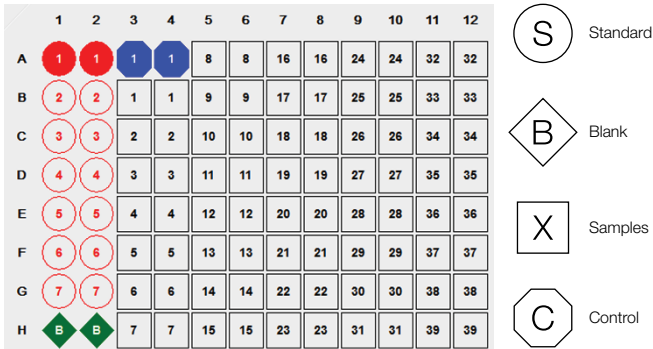


Fig. 2. Suggested plate layout.

2. Prepare Instrument

These directions are specific for the Bio-Plex® 100/200 reader. To prepare either a Bio-Plex 3D reader or Bio-Plex® MAGPIX™ multiplex reader, consult their respective user manuals.



Note: While the instrument is warming up, bring the 10x wash buffer, assay buffer, and diluents to room temperature. Keep other items on ice until needed. Also, begin to thaw frozen samples.

Start up and calibrate the Bio-Plex 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 fluorescence signal. For instructions on using other xMAP system software packages, contact Bio-Rad Technical Support.


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


Starting Up the System (Bio-Plex 100, 200, or similar)

1. Empty the waste bottle and fill the sheath fluid bottle before starting if the high-throughput fluidics (HTF) unit is not used. This will prevent fluidics 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.
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.

Calibrating the 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.
2. Select **OK** and follow the software prompts and step-by-step instructions for CAL1 and CAL2 calibration.

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

3. Prepare Wash Method

Compatible wash stations and plates are listed in Table 4.

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

Wash Method	Wash Station	Assay Plate
Magnetic separation	Bio-Plex Pro™ Bio-Plex handheld magnetic washer	Flat bottom plate

Setting Up the Bio-Plex Pro Wash Station

The wash station should be primed before use. For more information, refer to the Bio-Plex Pro 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 1x 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 (document #10023087).

4. Prepare Wash Buffer

1. Bring the 10x stock solution to room temperature.
2. If crystals are present, ensure that they are completely dissolved. Mix the 10x stock solution by inversion before preparing the 1x wash buffer.
3. To prepare 1x wash buffer, dilute 1 part 10x stock solution with 9 parts deionized water.

5. Prepare Samples

General guidelines for preparing different sample types are provided here. For more information, consult the publications listed in Bio-Rad bulletin #5297, available for download at bio-rad.com, or contact Bio-Rad Technical Support.

- Once thawed, keep samples on ice. Prepare the sample dilutions just prior to the start of the assay. Do not freeze diluted samples
- Prepare the sample dilutions in microcentrifuge tubes. Refer to Table 5 for recommended sample diluents and dilutions. Alternatively, if a multichannel pipet will be used to load the plate, aliquot the required volumes into Titer tube[®] micro test tubes

Table 5. Summary of recommended sample diluents and dilution factors.

Sample Type	Dilution Factor	Diluent
Serum and plasma	1:5 dilution	Sample diluent
Fluids	User defined	Diluent + 0.5% bovine serum albumin (BSA)

Serum and Plasma

Note: If using plasma, ethylenediaminetetraacetic acid (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.

1. Draw whole blood into collection tubes containing anticoagulant. Invert tubes several times to mix.
2. For serum, allow the 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. Alternatively, filter the samples with a 0.8/0.2 µm dual filter to prevent clogging.
5. It is recommended to dilute samples fivefold (for example, **30 µl** sample + **120 µl** sample diluent).
6. Assay samples immediately. Avoid repeated sample freeze-thaw cycles.

Cell Culture Supernatant

1. Collect supernatants by pelleting cells 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. It is recommended to test undiluted samples first. If high levels of analyte are expected, samples can be further diluted with an appropriate medium.
4. Assay immediately. Avoid repeated sample freeze-thaw cycles.

Cell and Tissue Lysates

The Bio-Plex cell lysis kit (catalog #171304011) is required for preparing lysates derived from cell culture and tissue samples. Just before use, prepare an adequate volume of cell lysis buffer by adding factor 1 and factor 2.

- Prepare 500 mM phenylmethylsulfonyl fluoride (PMSF) by dissolving 0.436 g PMSF in 5 ml dimethyl sulfoxide (DMSO). Store as aliquots at -20°C . Add PMSF to the cell lysis buffer at a final concentration of 2 mM
- Reconstitute cell lysis factor QG to 100x with 250 μl of distilled water and vortex to mix. Add the reconstituted factor to the cell lysis buffer to a final 1x working concentration

Adherent Cells

1. Once cultured cells are ready, aspirate the culture medium and quickly rinse the cells with ice-cold cell signaling cell wash buffer (bottle with the blue cap). The volume of buffer required is the same as the volume of aspirated cell culture medium. Keep the cells on ice during all steps when possible.
2. Completely remove the buffer before lysing the cells.
3. Immediately add the cell lysis buffer to the cells. The amount of lysing solution needed depends on the cell density in the culture vessel (for example, add 1.5–2 ml of lysis buffer to a 10 cm dish that is ~80% confluent).

Note: It may be necessary to lyse the samples with different volumes of cell lysis solution to obtain the specified protein concentration range.

4. Scrape the cells with a cell scraper, collect cell suspension into an appropriately sized tube, and gently rock for 20 min at 4°C .

5. Perform either of the following steps to remove insoluble cellular particulates:
 - Centrifuge the cell lysate solution at 4,500 x g for 20 min at 4°C, and then filter the lysate using a 0.45 µm syringe filter
 - If the lysate volume is not adequate for filtration, centrifuge the lysate at 15,000 x g for 10 min at 4°C using a benchtop microcentrifuge
6. Collect the filtered lysate or supernatant after centrifugation.
7. Measure the protein concentration using Bio-Rad's DC™ protein assay reagents package (catalog #5000116) and, if needed, adjust protein concentration with cell lysis buffer containing PMSF and cell lysis factor QG.
8. The suggested working protein concentration range for these assays is 200–900 µg/ml (10–45 µg per assay well).
9. Store the aliquoted lysates at –70°C until ready to use.

Suspension Cells

1. Collect the cell suspension and pellet the cells by spinning at 1,000 x g for 5 min at 4°C.
2. Aspirate off cell culture medium completely.
3. Wash the cells by resuspending them in ice-cold cell wash buffer (bottle with the blue cap).
4. Centrifuge the cells at 1,000 x g for 5 min at 4°C.
5. Completely remove the buffer.
6. Immediately add the proper volume of cell lysis buffer and gently rock for 20 min at 4°C.
7. Remove insoluble cellular particulates as described in Adherent Cells step 5.
8. Follow Adherent Cells steps 6–9.

Preparation of Tissue Lysate with Mini Disposable Pestle Grinder

1. Prepare an adequate volume of lysing solution. For 50 ml of lysing solution, add 200 μ l of factor 1 and 100 μ l of factor 2 to 49.5 ml of cell lysis buffer. Vortex gently to mix and set aside on ice. Then add 200 μ l of 500 mM PMSF. Label the buffer as total lysis buffer (TLB).
2. Use a sterile disposable scalpel to transfer roughly 25–35 mg of tissue sample into a sterile 1.5 ml microcentrifuge tube.
3. Add 100 μ l of TLB to the tube and grind the tissue 10–15 times until no large pieces remain.
4. Add 650 μ l of TLB to the tube and mix gently. Close the cap and store the tube on ice for 10 min.
5. Centrifuge the tube at 4°C for 10 min at 10,000 g.
6. Carefully transfer the clear supernatant to a prechilled 1.5 ml microcentrifuge tube.
7. Label the supernatant as total lysate.
8. Determine the protein concentration. Prepare aliquots and store them at –80°C for single use.

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.
2. Centrifuge the diluted sample at 10,000 x g for 10 min at 4°C to clarify the sample.

6. Prepare Standards and Controls

General Instructions

It is essential to prepare standards and quality controls (if included) exactly as described in this section. Incorrect preparation may lead to low signal or variable measurements.

The product data sheet provided with the standards lists the most concentrated point on the standard curve (S1). Enter this information into Bio-Plex Manager software as instructed in 9. Read Plate (see step 4 in Preparing the Protocol in Bio-Plex Manager Software Version 6.0 and Higher section).

Using the Quality Control (optional)

A single vial of control is provided only with the premixed panels. Its use is intended for monitoring the day-to-day quality of assay results.

Selecting a Diluent for Standards and Controls

Refer to Table 6 for recommended diluents based on different sample types.

In order to meet the lot-specific control ranges provided in the product data sheet, both the standards and controls should be reconstituted using the Bio-Plex standard diluent provided in the kit. If reconstituting in a different diluent, users will need to establish/validate their own control ranges or acceptance criteria.

Table 6. Summary of recommended diluents for standards and controls.

Sample Type	Diluent for Standards and Controls*	Addition of BSA
Serum and plasma	Standard diluent	None
Culture media, with serum	Culture medium	None
Culture media, serum free	Culture medium	To 0.5% final**
Lavage, sputum, other fluids	Sample diluent	To 0.5% final**

*If using diluents other than standard diluent, users must establish their own control ranges.

**At least 0.5% final BSA is recommended to stabilize analytes and reduce adsorption to labware.

Reconstituting the Standards and Quality Controls

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

1. Gently tap the vial containing the lyophilized standards on a solid surface to ensure the pellet is at the bottom of the vial.
2. Reconstitute a single vial of standards with 250 μ l of standard diluent (default diluent).

Optional: at the same time, reconstitute the control vial with 250 μ l of standard diluent. The control does not require further dilution.

3. Vortex the reconstituted standards and control at medium speed for 5 sec, then incubate on ice for 30 min. It is important that reconstitution of standards and control is started and ended at the same time. Be consistent with this incubation time to ensure optimal assay performance and reproducibility.

Preparing the Standard Dilution Series

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

1. Label seven 1.5 ml polypropylene tubes S2 through S7 and blank. Alternatively, using Tiertube micro test tubes may prove to be more convenient if a multichannel pipet will be used to load the plate.
2. Add 150 μ l of standard diluent to tubes S2–S7 (Figure 3).
3. Vortex the reconstituted standards at medium speed for 5 sec before removing any volume. Transfer the entire amount of 250 μ l to a tube labeled with S1. Transfer 50 μ l of S1 to the S2 tube already containing 150 μ l standard diluent. Vortex for 5 sec.
4. Use a new pipet tip to transfer 50 μ l from the S2 tube to the S3 tube. Vortex for 5 sec.
5. Continue with 1:4 (fourfold) serial dilutions as shown in Figure 3.

- Use reconstituted and diluted standards and controls immediately. Do not freeze for future use.

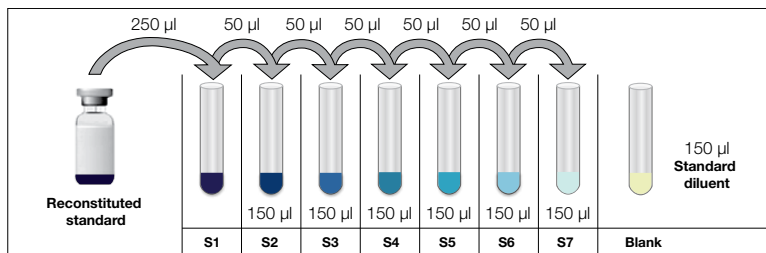


Fig. 3. Preparing a fourfold dilution series with a single reconstituted standard.

Note: For samples with very low endogenous analytes, preparing an additional standard point to extend the bottom end of the standard curve may help to improve sample detectability.

7. Prepare Coupled Beads

- Use Tables 7 and 8 to calculate the volume of coupled beads and assay buffer needed to prepare a 1x stock.
- Add the required volume of Bio-Plex assay buffer to a 15 ml polypropylene tube.
- Vortex the 10x stock of 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.
- Dilute coupled beads to 1x by pipetting the required volume into the 15 ml conical centrifuge tube. Vortex the tube.
- Protect the beads from light with aluminum foil.

Note: To minimize volume loss, use a 200–300 µl capacity pipet to remove beads from the 10x stock tube. If necessary, perform the volume transfer in two steps. Avoid using a 1,000 µl capacity pipet and/or wide bore pipet tip.

Preparing 1x Coupled Beads from 10x Stock (includes 20% excess volume)

Table 7. Premixed panel or one singleplex assay.

Number of Wells	10x Beads, μ l	Assay Buffer, μ l	Total Volume, μ l
96	570	5,130	5,700

Table 8. Mixing singleplex assays.

Number of Wells	Singleplex #1		Singleplex #2	
	10x Beads, μ l	10x Beads, μ l	Assay Buffer, μ l	Total Volume, μ l
96	570	570	4,560	5,700

8. Run Assay

Considerations

- Bring all assay components and samples to room temperature (RT) before use
- Use calibrated pipets and pipet carefully, avoiding bubbles
- Pay close attention to vortexing, shaking, and incubation instructions; deviating from the protocol may result in assay variability
- Assay incubations are carried out on a shaker at 850 ± 50 rpm at RT. Cover the plate with sealing tape
- After each assay step, select the appropriate Bio-Plex Pro wash station program or perform the appropriate manual wash step noted in Table 9

Table 9. Summary of wash options and protocols.

Assay Step	Bio-Plex Pro Wash Station	Handheld Magnet
	Magnetic Program	Manual Wash Steps
Add beads to plate	MAG x2	2 x 100 μ l
Sample incubation	MAG x3	3 x 100 μ l
Detection antibody incubation		
SA-PE incubation		

Adding Coupled Beads, Samples, Standards, Blank, and Controls

1. Cover the unused wells of the assay plate with sealing tape.
2. Vortex the diluted (1x) beads for 30 sec at medium speed. Pour into a reagent reservoir and transfer 50 μ l to each well of the assay plate.

Tip: A multichannel pipet is highly recommended for ease of use and efficiency.

3. Wash the plate two times with 100 μ l Bio-Plex wash buffer per well using the wash method of choice.
4. Vortex the diluted samples, standards, blank, and controls at medium speed for 5 sec. Transfer 50 μ l of each to the appropriate well of the assay plate, changing the pipet tip after every volume transfer.
5. Cover the plate with a new sheet of sealing tape. Incubate on shaker at 850 ± 50 rpm for 30 min at RT.

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

Preparing and Adding Detection Antibodies

1. While the samples are incubating, use Tables 10 and 11 to calculate the volume of detection antibodies and detection antibody diluent HP needed to prepare a 1x stock. Detection antibodies should be prepared 10 min before use.
2. Add the required volume of detection antibody diluent HP to a 5 ml polypropylene tube.
3. Vortex the 10x stock of 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 5 ml tube. Vortex the tube.

Table 10. Premixed panel or one singleplex assay.

Number of Wells	10x Detection Antibodies, μl	Detection Antibody Diluent HP, μl	Total Volume, μl
96	300	2,700	3,000

Table 11. Mixing singleplex assays.

Number of Wells	Singleplex #1	Singleplex #2	Detection Antibody Diluent HP, μl	Total Volume, μl
	10x Detection Antibodies, μl	10x Detection Antibodies, μl		
96	300	300	2,400	3,000

- After incubating the beads, samples, standards, blank, and controls, slowly remove and discard the sealing tape.
- Wash the plate three times with 100 μl wash buffer per well.
- Vortex the diluted (1x) detection antibodies at medium speed for 5 sec. Transfer into a reagent reservoir and dispense 25 μl to each well of the assay plate using a multichannel pipet.
- Cover the plate with a new sheet of sealing tape. Incubate on shaker at 850 ± 50 rpm for 30 min at RT.

Preparing and Adding Streptavidin-PE (SA-PE)

- While detection antibodies are incubating, use Table 12 to calculate the volume of SA-PE and assay buffer needed to prepare a 1x stock. SA-PE should be prepared 10 min before use.
- Add the required volume of assay buffer to a 15 ml polypropylene tube.
- Vortex the 100x stock of SA-PE for 5 sec at medium speed. Perform a 30 sec spin to collect the entire volume at the bottom of the tube.
- Dilute SA-PE to 1x by pipetting the required volume into the 15 ml tube. Vortex and protect from light until ready to use.

Table 12. Preparation of 1x SA-PE from 100x stock.

Number of Wells	100x SA-PE, μl	Assay Buffer, μl	Total Volume, μl
96	60	5,940	6,000

5. After detection antibody incubation, slowly remove and discard the sealing tape.
6. Wash the plate three times with 100 μ l of wash buffer per well.
7. Vortex the diluted (1x) SA-PE at medium speed for 5 sec. Pour into a reagent reservoir and transfer 50 μ l to each well using a multichannel pipet.
8. Cover the plate with a new sheet of sealing tape. Incubate on shaker at 850 \pm 50 rpm for 10 min at RT.
9. After the streptavidin-PE incubation step, slowly remove and discard the sealing tape.
10. Wash the plate three times with 100 μ l of wash buffer per well.
11. To resuspend beads for plate reading, add 125 μ l assay buffer to each well. Cover the plate with a new sheet of sealing tape. Shake at room temperature at 850 \pm 50 rpm for 30 sec. Slowly remove and discard the sealing tape before placing the plate on the reader.
12. Refer to Table 13 for instrument settings.

Table 13. Appropriate instrument settings for reading the plate.

Instrument	RP1 (PMT)	DD Gates	Bead Events
Bio-Plex 100, 200*	Low	5,000 (low), 25,000 (high)	50
Bio-Plex 3D*	Standard	Select MagPlex beads	50
Bio-Plex MAGPIX	N/A, use default instrument settings		

* Or similar Luminex-based system.

9. 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.

Preparing the Protocol in Bio-Plex Manager Software Version 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 in the assay, the plate wells to be read, sample information, the values of standards and controls, and instrument settings.

Bio-Plex Manager software version 6.0 and higher contain 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 **Add Panel**  in the Select Analytes toolbar. Enter a new panel name. Select **Bio-Plex Pro Assay Magnetic** from the assay dropdown list. If using Bio-Plex Manager software version 5.0 or lower, select **MagPlex** from the assay dropdown list.
 - b. Click **Add**. Enter the bead region number and name for the first analyte. Click **Add Continue** to repeat for each analyte in the assay. Refer to the bead regions in parentheses () listed on the peel-off label provided with the standards. For reference, bead regions are shown in Table 1.
 - c. Click **Add** 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, click **Add All**.
 - e. If some of the analytes need to be removed from the Selected list, highlight them and select **Remove**. It is possible to rename the panel by clicking **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 because the bead regions are not up to date.

3. Click **Format Plate** and format the plate according to the plate layout created in 1. Plan Plate Layout in this section. To modify the plate layout, follow the steps below (see Figure 4).
 - a. Select **Plate Formatting**.
 - 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). Note that Bio-Plex Manager software automatically subtracts the blank median fluorescence intensity value from all other assay wells.
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 with each vial of standards.



	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	8	8	16	16	24	24	32	32
B	2	2	1	1	9	9	17	17	25	25	33	33
C	3	3	2	2	10	10	18	18	26	26	34	34
D	4	4	3	3	11	11	19	19	27	27	35	35
E	5	5	4	4	12	12	20	20	28	28	36	36
F	6	6	5	5	13	13	21	21	29	29	37	37
G	7	7	6	6	14	14	22	22	30	30	38	38
H	B	B	7	7	15	15	23	23	31	31	39	39

Fig. 4. Plate formatting.

- 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**.
 - a. For user-specified controls, select an analyte from the dropdown menu, then enter a description and concentration. Repeat for each additional analyte in the assay.
 - a. For only the quality controls supplied with the premixed panel, format the appropriate wells as controls, entering descriptions but leaving the concentrations blank. Alternatively, the quality controls can be formatted as samples with clear descriptions such as "quality control." In any case, the expected control ranges provided are not entered into Bio-Plex Manager software version 6.1 and earlier.
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.
 - b. Refer to Table 13 for the recommended RP1 (PMT) setting. Protocols using alternative PMT settings should be validated by the end user.
 - c. Confirm that 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 μ l, and the doublet discriminator (DD) gates are set to 5,000 (Low) and 25,000 (High). 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.

Acquiring the 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 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. Select **Wash Between Plates**  after every plate run to reduce the possibility of clogging the instrument.
4. If data are acquired 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.

Reacquiring the 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. Aspirate the buffer with the wash method of choice, but do not perform the wash step.
3. Add 100 μ l of assay buffer to each well. Cover the plate with a new sheet of sealing tape. Shake the plate at 850 ± 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

Controls

If the quality controls were run in the assay plate, open the results (.rbx) file, click **Report Table**, and locate the control wells. Visually compare the observed concentrations of the control in the Report Table against the lot-specific control ranges shown in the product data sheet.

Note: Expected control ranges are provided for reference and should be used as general guidelines. Actual results may vary for some operators. If the controls do not fall within the expected ranges, please refer to the troubleshooting section for possible causes and solutions.

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. They should be removed to generate a more accurate standard curve.

In Bio-Plex Manager software version 6.0 and higher, outliers can be automatically removed by selecting **Optimize** in the Standard Curve window. In Bio-Plex Manager software 5.0 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 multiplex 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 μ l.
3. Refer to Table 12 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: 7.

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**.

Section 8

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 determine whether the array reader is functioning properly.

Possible Causes	Possible Solutions
High Inter-Assay Coefficient of Variation (CV)	
Standards and controls 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, controls, 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
High Intra-Assay CV	
Improper pipetting technique	Pipet carefully when adding standards, controls, 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 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 to monitor residual volume after each wash cycle. Ensure that the microplate shaker setting is not too high. Reduce the microplate shaker speed to minimize splashing
Slow pipetting of 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
Bio-Plex wash station: insufficient washing due to clogged pins	Clean dispensing pins with the thicker of the two cleaning needles provided with washer. Perform regular rinses to minimize salt buildup
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 30 sec at medium speed before aliquoting beads
Assay plate not shaken enough during incubation steps and prior to reading	Shake the plate at 850 ± 50 rpm during incubation steps and for 30 sec immediately before reading the plate
Reader is clogged	Refer to the troubleshooting guide in the Bio-Plex 200 system hardware instruction manual (document #10005042)
Incorrect needle height of the reader	Adjust the needle height to coincide with the plate type provided in the kit
Low Signal or Poor Sensitivity	
Standards reconstituted incorrectly	Follow the standard preparation instructions carefully
Detection antibody or streptavidin-PE diluted incorrectly	Check your calculations and be careful to add the correct volumes
High Background Signal	
Incorrect buffer was used (for example, assay buffer used to dilute standards)	Use standard diluent to dilute standards and as a reagent blank
Accidentally spiked blank wells	Do not add any antigens to the blank wells
Detection antibodies or streptavidin-PE incubated too long	Follow the procedure incubation time precisely

Possible Causes	Possible Solutions
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. Setting the speed too low may cause low assay signal and false plateau or saturation at the high end of standard curves
Quality controls do not fall within expected ranges	Make sure that the control vial is reconstituted at the same time as standards and in the same standard diluent. Incubate for precisely 30 min
Improper 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
Impact of Sample Matrix	
Poor precision in serum and plasma sample measurements	Check if any interfering components, 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

Section 9

Plate Layout Template

12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								
	A	B	C	D	E	F	G	H

Section 10

Safety Considerations

Eye protection and gloves are recommended when using these products. Consult the safety data sheet for additional information. Bio-Plex Pro™ assays contain components of animal origin. These assays should be handled as if capable of transmitting infectious agents. Use universal precautions. These components should be handled at Biosafety Level 2 containment as defined by the U.S. government publication, *Biosafety in Microbiological and Biomedical Laboratories* (U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, 1999).

Section 11

Ordering Information

Catalog # Description

Premixed Multiplex Assay Panel

12002231 **Bio-Plex Pro™ Mouse Chemokine Panel**, 33-plex, 1 x 96-well plate

Singleplex Set

12002247 **Bio-Plex Pro Mouse Chemokine Panel**, MCP-2/CCL8, 1 x 96-well plate

Other Components and Accessories

12002798 **Bio-Plex Pro Reagent Kit V**, 1 x 96-well plate

12002796 **Bio-Plex Pro Mouse Chemokine Standards**, 1 x 96-well plate

Bio-Plex Manager™ software, wash buffer, Bio-Plex Pro flat bottom plates, and streptavidin-PE are also available individually.

Go to bio-rad.com/bio-plex for detailed ordering information.

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