

Bio-Plex Manager™ software
recommended

Bio-Plex Pro™ Assays

Acute Phase

Instruction Manual



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

For research use only. Not for diagnostic procedures.

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Section 1

Introduction

Acute phase proteins are a class of proteins that have altered levels in response to inflammation and are thus relevant biomarkers in many disease processes. Typically, initial release of cytokines at the site of damage, most notably IL-1, IL-6, IL-8 and TNF- α , triggers the liver to release a number of acute phase proteins into the bloodstream. In turn, the increased levels of these proteins stimulate the release of more cytokines, presumably to initiate processes to prevent further tissue damage. Positive acute phase proteins—those that increase in response to tissue damage—are associated with infection, physical trauma, cardiac injury, diabetes, cancer, and other diseases.

Bio-Plex Pro™ assays are bead-based multiplex assays that allow the measurement of nine positive acute phase biomarkers in diverse matrices, including serum, plasma, and tissue culture supernatant. The biomarkers selected are circulating proteins that are known to be present in elevated levels during the acute phase response. The multiplexing feature makes it possible to quantify the levels of multiple target proteins simultaneously in a single well of a 96-well microplate in just 3 hours with as little as 5 μ l of sample.

As one of the most recent additions to the Bio-Plex family of products, Bio-Plex Pro assays incorporate magnetic beads into their design. The magnetic beads allow the use of an assay protocol similar to nonmagnetic Bio-Plex assays, with the option of using magnetic separation during wash steps instead of vacuum filtration. The magnetic washing option also enables automated assay preparation.

Bio-Plex Manager™ software is recommended for Bio-Plex Pro acute phase assays. Instructions for Luminex xPONENT software are also provided. For instructions using other xMAP system software packages, contact Bio-Rad Technical Support or your Bio-Rad Field Applications Scientist.

For a current listing of Bio-Plex Pro acute phase assays, visit us on the Web at **www.bio-rad.com/bio-plex/**

Section 2

Principle

Technology

The Bio-Plex[®] suspension array 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 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 output.

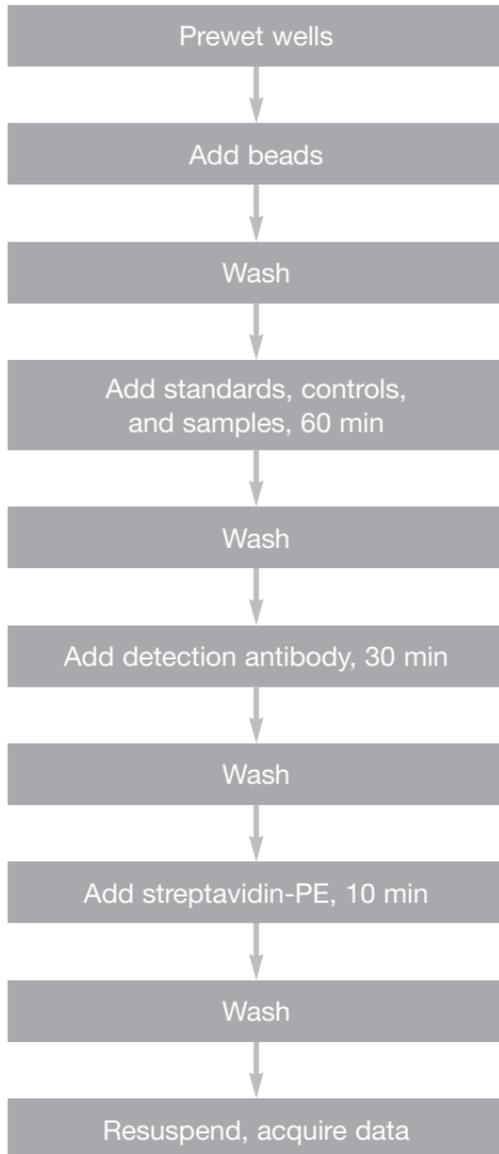
Assay Format

The principle of these 96-well plate-formatted, bead-based assays is similar to a capture sandwich immunoassay. An antibody directed against the desired target protein is covalently coupled to internally dyed beads. The coupled beads are allowed to react with a sample containing the target protein. After a series of washes to remove unbound protein, a biotinylated detection antibody specific for a different epitope is added to the reaction. The result is the formation of a sandwich of antibodies around the target protein. Streptavidin-phycoerythrin (streptavidin-PE) is then added to bind to the biotinylated detection antibodies on the bead surface for fluorescence-based detection.

Data Acquisition and Analysis

Data from the reaction are then acquired using the Bio-Plex suspension array system, 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 signal on the bead surface that corresponds to the amount of detected target protein. Intensity of fluorescence detected on the beads indicates the relative quantity of targeted molecules. A high-speed digital processor efficiently manages the data output, which is further analyzed and presented as fluorescence intensity on Bio-Plex Manager[™] software.

Assay Workflow



Section 3

Bio-Plex[®] Acute Phase Reagent Kit

Product Description

Bio-Plex Pro[™] acute phase assays require the use of the Bio-Plex acute phase reagent kit.

Component of the Bio-Plex Acute Phase Reagent Kit	Catalog # 171-304050 1 x 96-Well Format	Catalog # 171-304051 10 x 96-Well Format
Bio-Plex assay buffer Store at 4°C. Do not freeze.	1 x 75 ml	1 x 750 ml
Bio-Plex wash buffer Store at 4°C. Do not freeze.	1 x 150 ml	2 x 750 ml
Bio-Plex detection antibody diluent Store at 4°C. Do not freeze.	1 x 15 ml	1 x 150 ml
Streptavidin-PE (100x) Store at 4°C. Do not freeze.	1 vial	1 vial
Sterile filter plate (96-well) with cover and tray	1 plate	10 plates
Sealing tape	1 pack of 4	10 packs of 4 (40)
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Storage and Stability

Kit components should be stored at 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

Required Materials

The Bio-Plex Pro™ acute phase assay panel is divided into two separate multiplex assay kits based on the sample dilution required for optimal performance.

- Each assay requires one Bio Plex® acute phase reagent kit.
- The Bio-Plex acute phase diluent kit (see next section) is recommended for serum and plasma samples. One diluent kit contains sufficient volumes for performing a 5-plex assay and a 4-plex assay **or** two 5-plex assays **or** two 4-plex assays, as long as dilutions are performed as instructed.

The Bio Plex Pro acute phase assay kits contain the following components:

Multiplex assays

- Antibody-conjugated beads (25x concentration)
- Detection antibody (10x concentration)
- Acute phase standard (2 vials/lyophilized)
- Acute phase control (2 vials/lyophilized)

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

Bio-Plex Manager™ software version 3.0 and Luminex IS Software Users

Contact Bio-Rad Technical Support or your Bio-Rad Field Applications Scientist. Additional supplies are required to run the acute phase assays using these systems.

Section 5

Recommended or Optional Materials

Item	Catalog #
Bio-Plex [®] Acute Phase Diluent Kit Serum-based diluent Serum-free diluent	171-305050 (1x96) (10 ml) (300 ml)
Bio-Plex Pro [™] Human Acute Phase Standards Optional additional standards sold separately	171-D40002 (2 vials, lyophilized) 171-D00006 (50 vials, lyophilized)
Item	Ordering Information
Bio-Plex 200 System with HTF (or Luminex System)	Bio-Rad catalog #171-000205
Bio-Plex Validation Kit 4.0	Bio-Rad catalog #171-203001
Bio-Plex Calibration Kit	Bio-Rad catalog #171-203060
Microtiter Plate Shaker IKA-Schuttler MTS-4 shaker for 4 microplates or Lab-Line Model 4625 plate shaker (or equivalent, capable of 300-1,100 rpm)	IKA catalog #3208000 VWR catalog #57019-600
Filter Plate Vacuum Apparatus 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

Item (continued)	Ordering Information
Reagent Reservoir Corning, Inc. Costar 50 ml reagent reservoir 4870	Bio-Rad catalog #224-4872
1.2 ml Tubes in 96 Rack For performing sample dilutions in 96-well format	VWR catalog #82006-698 (tubes in rack) VWR catalog #83009-678 (refill tubes)
Other Materials Pipets and pipet tips, sterile distilled water, aluminum foil, absorbent paper towels, 1.5 ml microcentrifuge tubes, 15 ml culture tubes	

Section 6

Sample Preparation

This section provides instructions for preparing samples derived from serum, plasma, and culture supernatant. For sample preparations not mentioned here, consult the publications listed in Bio-Rad bulletin 5297, available for download at www.discover.bio-rad.com

Serum and Plasma Samples

Collect and process the serum or plasma samples and assay immediately or freeze at -20°C . Avoid repeated freeze-thaw cycles. EDTA is the recommended anticoagulant for preparing plasma samples. Extremely lipemic samples may be filtered with a $0.22\ \mu\text{m}$ filter to prevent clogging. Hemolyzed samples are not suitable for Bio-Plex Pro™ acute phase assays.

1. After collecting blood samples, immediately add appropriate inhibitors according to manufacturer's instructions if necessary, invert tube several times to mix.
2. Prior to assay setup, centrifuge the samples at 13,200 rpm for 10 min at 4°C to clear the samples of precipitate. Alternatively, carefully filter the samples with a $0.22\ \mu\text{m}$ filter to prevent instrument clogging. Keep samples on ice until ready for use.
3. Refer to the steps and the table below for sample dilution instructions for preparing the 5-plex assay, the 4-plex assay, or for preparing samples for the 4-plex and 5-plex assays at the same time.

5-plex assay: Dilute $5\ \mu\text{l}$ of sample in $495\ \mu\text{l}$ of serum free diluent. Mix by vortexing gently or pipetting. This results in a final 1:100 sample dilution.

4-plex assay: First dilute $5\ \mu\text{l}$ of sample in $495\ \mu\text{l}$ of serum-free diluent to create a 1:100 dilution. Mix gently. Next, dilute $5\ \mu\text{l}$ of this mixture into an additional $495\ \mu\text{l}$ of serum-free diluent. Mix gently. This results in a final dilution of 1:10,000.

5-plex and 4-plex assays For the 5-plex assay, dilute 5 μ l of sample in 495 μ l of serum-free diluent. Mix by vortexing gently or pipetting. This results in a final 1:100 sample dilution. For the 4-plex assay, dilute 5 μ l of the 1:100 mixture into an additional 495 μ l of serum-free diluent. Mix gently. This results in a final sample dilution of 1:10,000.

Panel	Step	Dilution	Sample Volume	Serum-free Diluent Volume	Total Volume
5-plex	1	100-fold	5 μ l sample	495 μ l	500 μ l
4-plex	1	100-fold	5 μ l sample	495 μ l	500 μ l
	2	10,000-fold	5 μ l of 1:100	495 μ l	500 μ l

NOTE: Additional dilutions may be required in order for samples with higher target concentrations to be within assay range.

Culture Supernatant Samples

1. Collect and process the culture supernatant samples and assay immediately or freeze at -20°C . Avoid repeated freeze-thaw cycles.
2. If required, dilute the culture supernatant with culture medium. Serum-free culture medium should contain carrier protein (such as BSA) at a concentration of at least 0.5%. Keep the samples on ice until ready for use.

Section 7

Standard Preparation

Two vials of lyophilized acute phase standards are provided in each multiplex Bio-Plex Pro™ acute phase assay kit and one vial is provided in each singleplex assay kit. Only one vial is required per 96-well plate. The product insert provided with the assay lists the concentration of the most concentrated dilution point of the standard curve. This procedure will prepare enough standard to run each dilution in duplicate.

Reconstitute Standards

1. Reconstitute one 5-plex standard for every 5-plex assay and one 4-plex standard for every 4-plex assay. Gently tap the glass vial containing the lyophilized acute phase standards on a solid surface to ensure that the pellet is on the bottom.
2. Refer to the following steps for preparation of diluents for the 5-plex and 4-plex standards and controls. Use the serum-based diluent and serum-free diluent provided in the Bio-Plex® acute phase diluent kit (catalog# 171-305050) for serum and plasma samples. For tissue culture samples, use tissue culture medium. Do not use assay buffer to dilute standards and controls.

5-plex standard/control diluent: Dilute 1 ml of serum-based diluent in 24 ml of serum-free diluent. Vortex 10 sec. Label this mixture "5-plex standard/control diluent".

4-plex standard/control diluent: The serum-free diluent is used directly for diluting 4-plex standards and controls. Using a sterile pipette, transfer 25 ml into a clean container labeled "4-plex standard/control diluent".

3. Reconstitute 1 standard vial per assay with 0.5 ml of the appropriate diluent as described in Step 2. The mixtures are identified as S0 in the Standard Dilution Series diagrams on page 11.

- Gently vortex 1-3 sec and incubate on ice for 60 min. For optimal assay performance, be consistent with the incubation time.
- Save the standard/control diluents for preparation of the controls (Section 8).

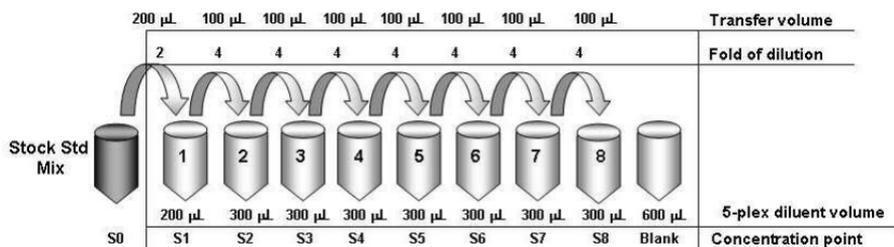
Prepare Standard Dilution Series

The standard concentrations specified for the 8-point standard dilution set have been selected for optimized curve fitting using the 5-parameter logistic (5PL) or 4-parameter logistic (4PL) regression in Bio-Plex Manager™ software. Results generated using dilution points other than those listed in this manual have not been optimized.

- Label a set of 1.5 ml Eppendorf tubes as shown in the Standard Dilution Series diagram on the next page.
- Pipet the appropriate volume of standard diluent into the tubes (see diagram below).
- Refer to the steps and diagrams below for preparation of the standard dilution series for the 5-plex and 4-plex assays.

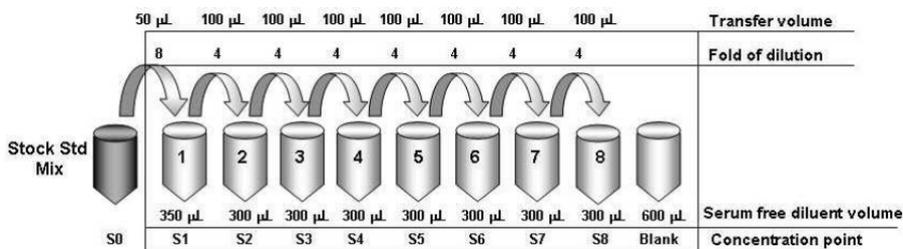
5-plex standard dilution series: Add 200 μl of reconstituted 5-plex standard to the first 1.5 ml tube containing 200 μl 5-plex standard diluent. Vortex gently. This mixture is identified as S1 in the diagram below and in the standard and control values product insert provided with the assay. Continue making serial dilutions of the standards as shown in the diagram. Before making each dilution, vortex gently and change the pipet tip.

5-plex Standard Dilution Series



4-plex standard dilution series: Add 50 μl of reconstituted 4-plex standard to the first 1.5 ml tube containing 350 μl 4-plex standard diluent. Vortex gently. This mixture is identified as S1 in the diagram below and in the standard and control values product insert provided with the assay. Continue making serial dilutions of the standards as shown in the diagram. Before making each dilution, vortex gently and change the pipet tip.

4-plex Standard Dilution Series



Note: The addition of duplicate 0 pg/ml blanks is strongly recommended. 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 0 pg/ml wells as blanks results in an automatic subtraction of the background Median Fluorescence Intensity (MFI) values from all readings in a run. This might result in negative MFI values in some wells. If this is undesirable, the 0 pg/ml wells can be formatted as background controls. The 0 pg/ml background control wells are also useful for troubleshooting and determining the limit of detection (LOD).

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

Section 8

Control Preparation

Two vials of lyophilized acute phase controls are provided in each multiplex Bio-Plex Pro™ acute phase assay kit. This section provides instructions on how to reconstitute the lyophilized control. The product insert provided with the assay lists the concentration ranges of the prepared controls.

Prepare Acute Phase Controls

To ensure optimal assay performance, the acute phase controls should be prepared in a manner consistent with that used to prepare the acute phase standards.

1. Gently tap the glass vial containing the lyophilized acute phase control on a solid surface to ensure the pellet is at the bottom.
2. Refer to the steps below for reconstituting the 5-plex and 4-plex controls. Use the standard/control diluent prepared in the previous section (Section 7, step 2). Do not use assay buffer to reconstitute the controls.

5-plex control: Reconstitute one vial of lyophilized control with 1 ml of 5-plex standard/control diluent. This solution will be used directly in the assay; no additional dilutions are required.

4-plex control: Reconstitute one vial of lyophilized control with 2 ml of 4-plex standard/control diluent. This solution will be used directly in the assay; no additional dilutions are required.

3. Gently vortex 1-3 sec and incubate on ice for 60 min. For optimal assay performance, be consistent with the incubation time.
4. Expected concentration ranges of the prepared controls are provided in the standard and control values product insert.

Section 9

Assay Instructions

The following instructions apply to both the 5-plex and 4-plex assays. All of the necessary components are provided premixed for ease of use.

Plan Experiment

1. Assign which wells of a 96-well plate will be used for each standard, control, and sample (see the example plate layout below).
2. Determine the total number of wells that will be used in the assay. Include a 25% excess (or add 2 wells for every 8 wells used) to ensure that enough diluted coupled beads, detection antibodies, and streptavidin-PE are prepared.

Example Plate

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

Prepare Coupled Magnetic Beads

Protect the beads from light by covering the tubes with aluminum foil. Keep all tubes on ice until ready to use.

1. Vortex the coupled beads (25x) at medium speed for 30 sec.
2. Prepare a sufficient volume of coupled beads (1x) using assay buffer. Each well requires 2 μl of coupled beads (25x) adjusted to a final volume of 50 μl with assay buffer (refer to the example below).

Example Bead Calculations

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

Calibrate Vacuum Apparatus

The vacuum apparatus must be calibrated at the beginning of the assay to ensure an optimal bead yield. For more detailed instructions, refer to the Bio-Plex[®] Suspension Array System hardware instruction manual.

1. Prewet all the wells of a 96-well filter plate with 100 μl of assay buffer.
2. Place the filter plate on the vacuum apparatus and turn on the vacuum to the maximum level.
3. Press on the filter plate and note the time required to remove the buffer from the wells by vacuum filtration. The evacuation time should be 2–5 sec.

If the evacuation time is <2 sec, the pressure is too high. Open the vacuum control valve slightly and repeat steps 1–3.

If the evacuation time is >5 sec, the pressure is too low. Close the vacuum control valve slightly and repeat steps 1–3.

Assay Procedure

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

The following terms are repeated throughout the assay procedure. Refer to these detailed instructions when wash, incubate, and vacuum-filter are shown in bold.

	Detailed Directions
Wash	<p>Add 100μL of wash buffer to each well. Place the filter plate on a calibrated vacuum apparatus and remove the buffer by vacuum filtration. Blot the bottom of the filter plate with a clean paper towel. Repeat as specified.</p> <p>Tip: Thoroughly blot the bottom of the filter plate with a clean paper towel to prevent cross-contamination and plate leakage.</p>
Incubate	<p>Gently cover the filter plate with a new sheet of sealing tape. 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, then at 300 rpm for the specified incubation time.</p> <p>Tip: Apply sealing tape gently on the filter plate (e.g. press down only on edges) to prevent positive pressure inside the wells that could lead to plate leaking during shaking. To avoid splashing of samples that may lead to cross-well contamination, slowly ramp up the shaker to the maximum speed before incubation. Cover plate with aluminum foil to prevent photobleaching.</p>
Vacuum-filter	<p>Place the filter plate on a calibrated vacuum apparatus and remove the buffer by vacuum filtration. Blot the bottom of the filter plate with a clean paper towel.</p> <p>Tip: Visually examine each well to ensure that buffers are completely drained from each well.</p>

1. Equilibrate the diluted standards, samples, and controls at room temperature for 20 min prior to use.
2. Prewet and block the desired number of wells in a 96-well filter plate with 100 μ l of assay buffer and **vacuum-filter**. 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.
3. Vortex the coupled magnetic beads (1x) for 15–20 sec at medium speed. Add 50 μ l to each well and immediately **vacuum-filter**.
4. **Wash** twice.
5. Gently vortex the diluted standards, controls, and samples for 1–3 sec. Add 50 μ l of standard, control, or sample to each well, changing the pipet tip after every volume transfer. **Incubate** for 1 hr.

- While the samples are incubating, perform a 30 sec quick-spin centrifugation of the detection antibody (10x for multiplex assays, 100x for singleplex assays) prior to pipetting to collect the entire required volume at the bottom of the vial.
- Prepare a sufficient volume of detection antibodies (1x) using detection antibody diluent. Refer to the example tables below.

NOTE: The detection antibody stock concentration is 10x in the multiplex assays and 100x in the singleplex assays. Always check the detection antibody concentration on the vial label before diluting.

Example Detection Antibody Calculations (10x stock)

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

Example Detection Antibody Calculations (100x stock)

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

- After incubating the samples, slowly remove and discard the sealing tape, then **vacuum-filter**.
- Wash** 3 times.
- Vortex the detection antibodies gently and add 25 µl to each well. **Incubate** for 30 min.

11. While the detection antibodies are incubating, perform a 30 sec quick-spin centrifugation of the streptavidin-PE (100x) prior to pipetting to collect the entire volume at the bottom of the vial.
12. Prepare a sufficient volume of streptavidin-PE (1x) using assay buffer. Each well requires 0.5 μ l of streptavidin-PE (100x) adjusted to a final volume of 50 μ l with assay buffer (refer to the example below).

Example Streptavidin-PE Calculations

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

13. After the detection antibody incubation, slowly remove and discard the sealing tape, then **vacuum-filter**.
14. **Wash** 3 times.
15. Vortex the streptavidin-PE (1x) vigorously and add 50 μ l to each well. **Incubate** for 10 min.
16. After the streptavidin-PE incubation, slowly remove and discard the sealing tape, then **vacuum-filter**.
17. **Wash** 3 times.
18. Add 125 μ l of assay buffer to each well. Cover the filter plate with a new sheet of sealing tape. Shake the filter plate at room temperature at 900 rpm for 30 sec and remove the sealing tape before reading the plate.

Section 10

Data Acquisition

Bio-Plex Manager™ software is recommended for Bio-Plex Pro™ acute phase assays. Refer to the details in the appropriate section below for your xMAP system software package. Instructions for Luminex xPONENT software are also provided.

For instructions using other xMAP system software packages, contact Bio-Rad Technical Support or your Bio-Rad Field Applications Scientist.

NOTE: Bio-Plex Pro acute phase assays are analyzed using the low RP1 target value (low PMT). Be sure to follow the instructions for your software version on how to calibrate and analyze data.

NOTE: To minimize protocol setup, lot-specific Bio-Plex Pro acute phase assay protocols for Bio-Plex Manager version 4.0 and higher are available for download at www.biorad.com/bio-plex/standards

Bio-Plex Manager Software Version 5.0

Prepare System

1. Empty the waste bottle and make sure that there is sufficient sheath fluid in the bottle or cube before proceeding.
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 to prepare the reader to acquire data. If the system is idle for 4 hr 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

Calibrate using Bio-Plex calibration beads and low RP1 target values on the CAL2 bottle. This software version requires calibration only with the low

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 CAL 2 calibration.

Prepare Protocol

Lot-specific assay panel protocols are available for download at www.bio-rad.com/bio-plex/standards

If the desired protocol is not available for download, create a new protocol:

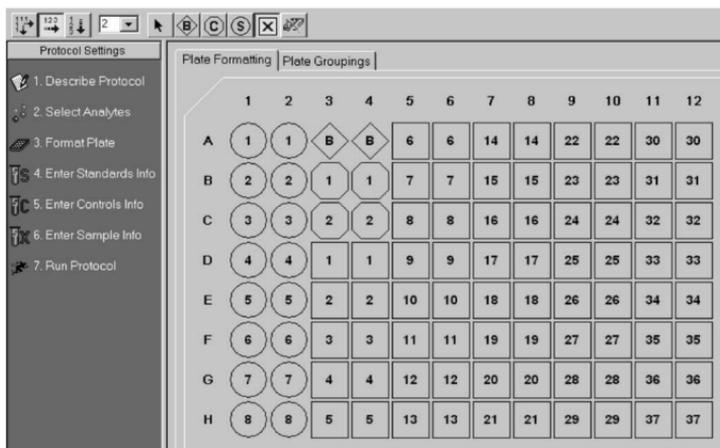
1. Open a new protocol by selecting File, then New from the main menu. Locate the steps at the left of the protocol menu.
2. Select Step 1 (Describe Protocol) and enter information about the assay.
3. Select Step 2 (Select Analytes) and choose the appropriate acute phase panel, from the assay pull-down menu. If the panel is not listed, create a new panel and select the “Bio-Plex Pro” assay type from the assay pull-down menu. See the Bio-Plex Manager software instruction manual for details.

NOTE: It is important to select the appropriate assay type in the assay pull-down menu to ensure that data is acquired using the correct bead map and gate settings.

4. Choose the analytes to be selected for the assay from the available list of targets. Note the targets will already be entered in the selected view when using a downloaded protocol. In future runs, the selected panel can be copied into the “available” view.
5. Select Step 3 (Format Plate) and click on the Plate Formatting tab. Select the number of replicates, the autofill direction, and click on the  icon. Drag the cursor over all the wells that contain standards. Repeat by replacing the  with , , and  to identify all the wells that contain blanks, controls, and samples, respectively.

NOTE: If the preset protocol was downloaded from the web site, 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



6. Select Step 4 (Enter Standards Info) to enter standards information. Note that this information will already be entered when using a download protocol. Otherwise:
 - a) Deselect the box labeled “Same concentration values for all analytes”. If analyzing a 5-plex assay, also deselect the box labeled “Same units for all analytes”.
 - b) Select the first analyte from the pull-down cell.
 - c) Click on the Enter Automatically button and then select the highest concentration value (typically S1).
 - d) Enter the value for the highest concentration. The information is included on the product insert provided with the assay.
 - e) Enter the dilution factor (typically 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.

7. Select Step 5 (Enter Controls Info) to enter controls information. This is where the concentration of the user-specified controls is entered into the protocol.
 - a) If necessary, deselect the box for same concentration values for all analytes.
 - b) Select an analyte from the pull-down cell.
 - c) Enter the description and dilution information for each user-specified control.
 - d) Repeat steps 7b and 7c for each additional analyte in the assay.
8. Select Step 6 (Enter Sample Info) and enter sample information. Remember to enter the appropriate dilution factor information.
9. Select Step 7 (Run Protocol). Then:
 - a) Choose **100 beads per region**.
 - b) In Advanced Settings, set the bead map to **25 region**.
 - c) In Advanced Settings, set the sample size to **50 μ l**.
 - d) In Advanced Settings, confirm that the DD gate is set to **5,000** (low) and **32,000** (high).
 - e) Save the protocol (.pbx) file.

Acquire Data

1. Shake the assay plate at 900 rpm for 30 sec immediately before acquiring data. Failure to do so will result in increased read time due to bead settling.
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 platform.
3. Slowly remove the sealing tape and any plate cover before placing the plate in the reader.
4. Select the appropriate protocol (.pbx) file.
5. Select Start and save the report (.rbx) file.
6. Begin data acquisition.

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

7. 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). For details, refer to the Bio-Plex Manager instruction manual.

Bio-Plex Manager Software Version 4.1 and 4.1.1

Prepare System

1. Empty the waste bottle and make sure that there is sufficient sheath fluid in the bottle or cube before proceeding.
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 to prepare the reader to acquire data. If the system is idle for 4 hr 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

Calibrate using Bio-Plex calibration beads and the **low RP1 target** on the CAL2 bottle. This software version requires the user to calibrate either on the low or high RP1 target value. 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 Bio-Plex **low RP1** target value.
2. Select OK and follow the instructions for CAL1 and CAL2 calibration.

Prepare Protocol

1. Open a new protocol by selecting File, then New from the main menu. Locate the steps at the left of the protocol menu.

NOTE: To minimize protocol setup, previously prepared lot-specific Bio-Plex Pro acute phase assay protocols in BPM 4.0 are available for download at www.biorad.com/bio-plex/standards. The user will need to select the 25 region bead map in Step 7 (Run Protocol).

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

- Select Step 2 (Select Analytes) and choose the appropriate acute phase panel name, if available in the pull-down menu. If the panel name is not available, create a new panel. See the Bio-Plex Manager software instruction manual for details.

Choose the analytes to be selected from the available list of targets. Note that the targets will already be entered in the selected view when using a downloaded protocol. The selected panel cannot be copied into the Available view and pull-down list with this version of software, though it can be copied in Bio-Plex Manager version 5.0.

- Select Step 3 (Format Plate) and click on the Plate Formatting tab. Click on and drag the cursor over all the wells that contain standards. Then click on and drag the cursor over the wells that contain blanks. Repeat to identify all the wells that contain controls and to identify all the wells that contain samples.

Plate Formatting Example

The screenshot shows the Bio-Plex Manager software interface. On the left is a 'Protocol Settings' sidebar with steps 1 through 7. The main window is titled 'Plate Formatting' and 'Plate Groupings'. The 'Plate Formatting' tab is active, showing a 12x8 grid of wells. The wells are labeled with letters A-H and numbers 1-12. The grid contains various shapes and numbers representing different well types: circles for standards, hexagons for blanks, and diamonds for controls. The numbers in the wells represent the values for each well type.

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

5. Select Step 4 (Enter Standards Info) to enter standards information. Note that this information will already be entered with the preset download protocol.
 - a) Select each analyte individually from the pull-down cell. Deselect the box labeled "Same concentration values for all analytes". If analyzing a 5-plex assay, also deselect the box labeled "Same units for all analytes".
 - b) Select the Enter Automatically option and then select the most concentrated value as S1.
 - c) Enter the concentration of S1 from the product insert provided with the assay.
 - d) Enter the dilution factor as 4 and select Calculate. The standards information will be populated for the selected analyte.
 - e) Repeat steps 5a through 5d for each analyte in the assay.
6. Select Step 5 (Enter Controls Info) to enter controls information. This is where the concentration of the user-specified controls is entered into the protocol.
 - a) Select each analyte individually from the pull-down cell. Deselect the box for same concentration values for all analytes.
 - b) Enter the description and dilution information for each user-specified control.
 - c) Repeat steps 6a and 6b for each analyte in the assay.
7. Select Step 6 (Enter Sample Info) and enter sample information.

Acquire Data

1. Shake the assay plate at 900 rpm for 30 sec immediately before acquiring data. Failure to do so will result in increased data acquisition time due to bead settling.
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 platform.
3. Slowly remove the sealing tape and any plate cover before placing the plate in the reader.
4. Select Step 7 (Run Protocol):
 - a) Choose **100 beads per region**.
 - b) In Advanced Settings, set the bead map to **25 region**.
 - c) In Advanced Settings, set the sample size to **50 µl**.
 - d) In Advanced Settings, confirm that the DD gate are set to **5,000** (low) and **32,000** (high).

NOTE: These settings are already selected in preset downloaded protocols.
 - e) Select Start and save the protocol (.pbx) file. Then follow the instructions for 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 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.

Require 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). For details, refer to the Bio-Plex Manager instruction manual.

Bio-Plex Manager Software Version 4.0

Bio-Plex Manager version 4.0 does **not** include the 25-bead map. Hence, the 100-bead map is used. Follow the instructions above for Bio-Plex Manager version 4.1 and 4.1.1 for preparing and calibrating the system. Be careful to calibrate on Low RP1 and in Step 7 (Run Protocol) under Advanced Settings, set the **DD gates to 5,000 (low) and 32,000 (high)**.

Acquire Data

1. Shake the assay plate at 1,100 rpm for 30 sec immediately before acquiring data. Failure to do so will result in increased data acquisition time due to bead settling.
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 platform.
3. Slowly remove the sealing tape and any plate cover before placing the plate in the reader.
4. Select Step 7 (Run Protocol):
 - a) Choose **100 beads per region**.
 - b) In Advanced Settings, set the Bead Map to **100 region**.
 - c) In Advanced Settings, set the sample size to **50 μ l**.
 - d) In Advanced Settings, confirm that the DD gates are set to **5,000 (low) and 32,000 (high)**.

NOTE: These settings are already selected in preset downloaded protocols. If creating a new protocol, manually change the default setting to reset the gates to 5,000 (low) and 32,000 (high).

- e) Select Start and save the report (.rbx) file. Then follow the instructions for 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 fluidic 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). For details, refer to the Bio-Plex Manager instruction manual.

Luminex xPONENT software

Luminex xPONENT software does not include the 25-bead map. Hence, the 100-bead map is used. Refer to the detailed instructions in the xPONENT software manual.

1. Calibrate with Luminex **MagPlex** Classification Calibration Microspheres and Luminex xMAP Reporter Calibration Microspheres (sold by Luminex).
2. Verify with xMAP **MagPlex** Classification Control Microspheres and xMAP Control Microspheres (sold by Luminex).
3. Create the acute phase panel protocol. Make sure to include the correct bead region numbers and target names.
4. The 100-region map should be selected.
5. Set the gate settings at **10,000** (low) to **22,500** (high).

Section 11

Troubleshooting Guide

This troubleshooting guide addresses problems that may be encountered with Bio-Plex Pro™ acute phase 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 array reader. To eliminate this possibility, we highly recommend use of the Bio-Plex validation kit. This kit will validate all the key functions of the array reader and assist the user in determining whether or not the array reader is functioning properly.

Possible Causes

High Inter-Assay %CV

Standards were not reconstituted consistently

Reconstituted standards and diluted samples were not stored properly

High Intra-Assay %CV

Bottom of filter plate not dry

Possible Solutions

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

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

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

Possible Causes

Pipetting technique

Reagents and assay components were not equilibrated to room temperature prior to plating

Contamination with wash buffer during wash steps

Slow pipeting samples and reagents across the plate

Possible Solutions

Pipet carefully and slowly when adding standards, samples, detection antibodies, and streptavidin-PE, especially when using a multichannel pipet. Use a calibrated pipet. Change pipet tip after every volume transfer.

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

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.

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

Possible Causes

Low Bead Count

Miscalculation of bead dilution

Beads clumped in multiplex bead stock tube

Vacuum on for too long when aspirating buffer from wells

Did not shake filter plate enough before incubation steps and prior to reading

Reader is clogged

Low Signal or Poor Sensitivity

Standards reconstituted incorrectly

Detection antibody or streptavidin-PE diluted incorrectly

Possible Solutions

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

Vortex for 15–20 sec at medium speed before aliquoting beads.

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

Shake the filter plate at 1,100 rpm for 30 sec before incubation steps and immediately before reading the plate.

Refer to the troubleshooting guide in the Bio-Plex hardware instruction manual.

Follow the standard preparation instructions carefully.

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

Possible Causes

High Background Signal

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

Spiked 0 pg/ml wells by mistake

Streptavidin-PE incubated too long

Poor Recovery

Expired Bio-Plex reagents were used

Incorrect amounts of components were added

Microplate shaker set to an incorrect speed

Pipetting technique

Possible Solutions

Use sample matrix or serum-based diluent to dilute standards.

Be careful when spiking standards. Do not add any antigens in the 0 pg/ml (blank) point.

Follow the procedure incubation time.

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

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

Check the microplate shaker speed and use the recommended setting. Setting the speed too high may cause splashing and contamination. Use the recommended plate shaker.

Pipet carefully and slowly when adding standards, samples, detection antibodies, and streptavidin-PE, especially when using a multichannel pipet. Use a calibrated pipet. Change pipet tip after every volume transfer.

Section 12

Safety Considerations

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

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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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