Bio-Plex Pro™ Human TIMP Assays

Instruction Manual

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Section 1
Introduction

Bio-Plex Pro™ TIMP Assays

The matrix metalloproteinases (MMPs) are a family of zinc proteases with essential roles in breaking down components of the extracellular matrix (ECM). The MMPs are inhibited by specific endogenous tissue inhibitors of metalloproteinases (TIMPs), which are a family of four protease inhibitors: TIMP-1, TIMP-2, TIMP-3, and TIMP-4. The balance between MMP and TIMP levels is crucial for the timely degradation of ECM.

MMPs and TIMPs play important roles in tissue remodeling associated with various physiological and pathological processes such as inflammation, apoptosis, morphogenesis, angiogenesis, tumor invasion,and metastasis. The new MMP and TIMP panels will expand the Bio-Plex Pro Assay menu in research areas including cancer, autoimmune disease, wound healing, and cardiovascular disease.

Advantages of Magnetic Bead–Based Assays

Products in the Bio-Plex Pro family of assays are on magnetic polystyrene beads. These beads provide a choice in the method of assay preparation — standard or magnet-based. The standard workflow for Bio-Plex® Assay preparation requires multiple wash steps in which the 96-well filter plate is placed on a vacuum manifold to draw the wash liquid through the bottom of the filter plate. In contrast, magnet-based assay preparation permits liquid removal from the top of the well and thus does not require a filter plate or vacuum manifold. As a result, either an automated or manual washer can be used. Bio-Rad offers both solutions with the automated Bio-Plex Pro wash station and the manual Bio-Plex handheld magnetic washer. Magnetic separation offers greater convenience and reproducibility compared to vacuum filtration.

Please visit **bio-rad.com/bio-plex** for a current listing of Bio-Plex Assays, Panels, and Reagents.

Section 2
Principle

Technology

The Bio-Plex® Suspension Array System is built upon the three core elements of xMAP technology:

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

Assay Format

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

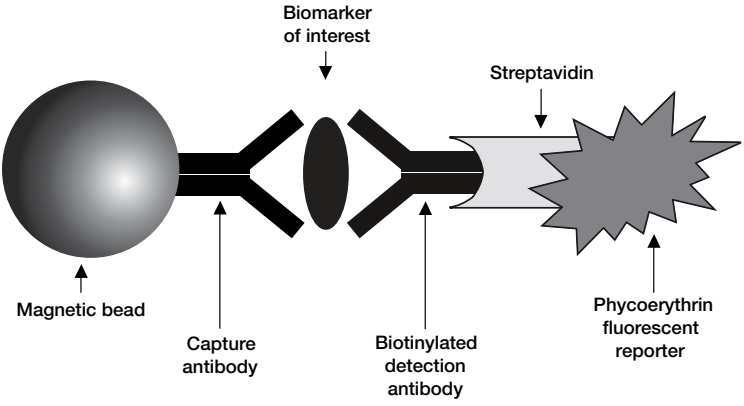


Fig. 1. Bio-Plex sandwich immunoassay.

Data Acquisition and Analysis

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

Section 3
Kit Contents and Storage

Reagents Supplied

Bio-Plex Pro™ TIMP Assays are available in a convenient all-in-one kit format that includes assay, reagent, and diluent components in a single box.

Table 1. Contents of 1 x 96-well kits.

Component	Quantity**
Diluent HD	1 bottle, 180 ml
Assay buffer	1 bottle, 50 ml
Wash buffer (10x)	1 bottle, 60 ml
Detection antibody diluent HB	1 bottle, 5 ml
SA-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
Coupled magnetic beads (20x)	1 tube
Detection antibodies (20x)	1 tube
Standard	1 vial
Control	1 vial

* Volumes shown are approximate.

Storage and Stability

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

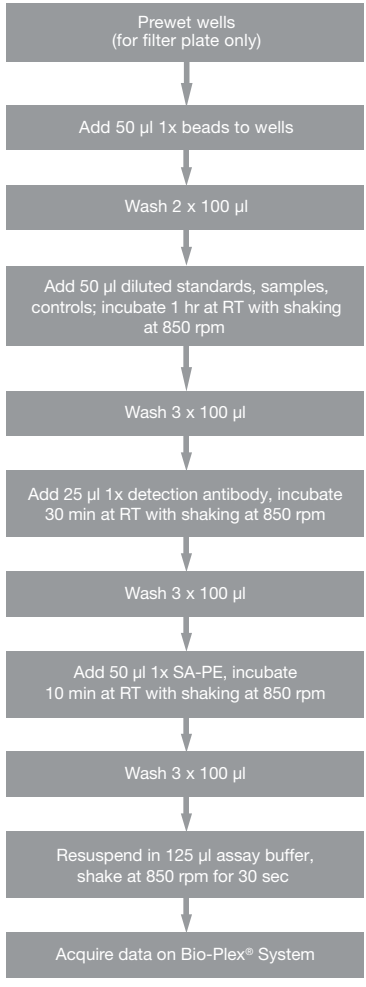
Section 4
Recommended Materials

Table 2. Recommended materials.

Item	Ordering Information
Bio-Plex® 200 System or Luminex System with HTF	Bio-Rad catalog #171000205
Bio-Plex Validation Kit 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 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 #17020100
Bio-Plex Pro Flat Bottom Plates (forty 96-well plates) 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 Model 4625 Plate Shaker (or equivalent capable of 300–1,100 rpm)	IKA catalog #320-8000 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 25 mm PF Syringe Filter (0.8/0.2 µm Supor membrane)	Pall Life Sciences catalog #4187
Filter plate, 1 x 96 with clear plastic lid and tray	Bio-Rad catalog #171304502

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

Section 5
Assay Workflow



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 (section 9).

Assay Procedures

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

Assay Quick Guide

Each assay kit includes a printed Bio-Plex Pro Assay Quick Guide (bulletin #10041638), which can be used to prepare and run a full 1 x 96-well assay plate. Users can also go to **bio-rad.com/bio-plex** to download a copy.

Bead Regions

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

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

Prior to running the assay, determine the total number of wells in the experiment using the Plate Layout Template on page 33 or the Plate Formatting tab in Bio-Plex Manager Software. A suggested plate layout is shown in Figure 2, with all conditions in duplicate.

1. Assign standards to columns 1 and 2, with the highest concentration in row A and the lowest concentration in row H.
2. Assign the blank to wells A3 and A4. The blank should consist of your chosen diluent HD or a diluent similar to your final sample type or matrix. Note that Bio-Plex Manager automatically subtracts the blank (B) MFI value from all other assay wells.
3. Controls, either user-specified or the controls supplied, are assigned to wells in columns 3 and 4.
4. The remainder of the plate is available for samples. Once the total number of wells to be used is known, calculate the required volumes of beads, detection antibody, and SA-PE needed. Use Tables 6, 8, and 9, respectively, or the Calculation Worksheet on page 33.

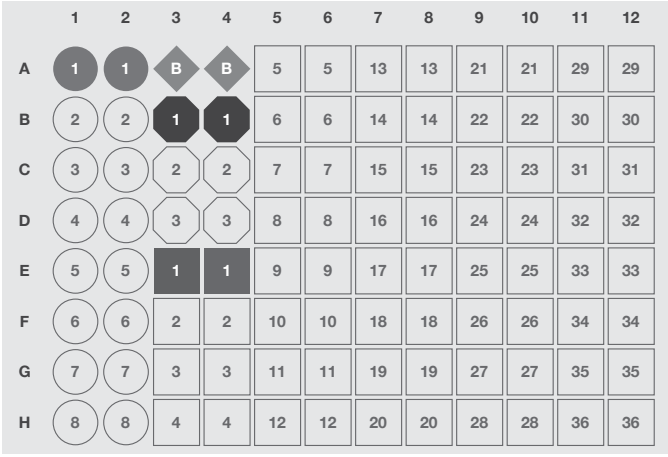


Fig. 2. Suggested plate layout. For detailed instructions on plate formatting in Bio-Plex Manager Software, see the Read Plate section.

2. Prepare Instrument

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

Note: While the instrument is warming up, bring the 10x wash buffer, assay buffer, and diluent HD 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 fluorescent 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 a validation.

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

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

Calibrate System

1. Select **Calibrate**  and confirm that the default values for CAL1 and CAL2 are the same as the values printed on the bottle of Bio-Plex calibration beads. Use the Bio-Plex System low RP1 target value.
2. Select **OK** and follow the software prompts for step-by-step instructions for CAL1 and CAL2 calibration.

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

3. Prepare Wash Method

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

Table 3. 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
Vacuum filtration	Vacuum manifold (manual)	Filter plate

Setting Up the Bio-Plex Pro Wash Station

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

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

Setting Up the Bio-Plex Handheld Magnetic Washer

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

Setting Up a Vacuum Manifold

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

4. Prepare Wash Buffer

1. Bring the 10x stock solution to room temperature.
2. If crystals exist, 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 Standards and Controls

General Instructions

- It is essential to prepare standards and controls exactly as described in this section. Incorrect preparation may lead to low signal or variable measurements from plate to plate
- The product data sheet provided lists the most concentrated point on the standard curve (S1). Enter the values and units into Bio-Plex Manager Software as instructed in section 9

Using the Controls (Optional)

One vial of controls is included. Its intended use is for monitoring the day-to-day quality of assay results.

Selecting a Diluent for Standards and Controls

Refer to Table 4 for recommended diluents based on different sample types. In order to meet the lot-specific control ranges provided on the product data sheet, both the standards and controls should be reconstituted in Bio-Plex Diluent HD. If reconstituting in a different diluent, users will need to establish validate their own control ranges or acceptance criteria.

Table 4. Summary of recommended diluents for standards and controls.

Sample Type	Diluent for Standards and Controls*	Add BSA
Serum and plasma	Diluent HD	None
Culture media, with serum	Culture media	None
Culture media, serum-free	Culture media	To 0.5% final

* If using diluents other than the diluent HD provided, then users must establish their own control ranges.

Reconstitute Standards and Controls

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

Note: The appearance of the lyophilized standards or controls may vary from a white pellet to clear crystals. Regardless of appearance, the vials have passed QC specifications and will perform accordingly.

1. Gently tap the vial of lyophilized standards on a solid surface to ensure that all material is at the bottom of the vial.
2. Reconstitute the vial of standards with **781 µl** of the appropriate diluent. Optional: at the same time, reconstitute the vial of controls with **250 µl** of the appropriate diluent as summarized in Table 4. **Controls do not require further dilution.** If using diluents other than the diluent HD provided, then users must establish their own control ranges.

3. Gently **vortex** the reconstituted standards and controls for **5 sec**, then incubate **on ice for 30 min**. It is important that reconstitution of standards and controls is started and ended at the same time. Be consistent with this incubation time to ensure optimal assay performance and reproducibility.
4. During the incubation period, prepare the samples as instructed in the prepare Samples section.

Prepare the Standard Dilution Series

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

1. Label eight 1.5 ml microcentrifuge tubes S2 through S8 and Blank. Alternatively, using Titertube® Micro Test Tubes may be more convenient if a multichannel pipet will be used to load the plate.
2. Add **150 µl** of the appropriate diluent to tubes S2–S8 and Blank. (Figure 3).
3. **Vortex** reconstituted standards at medium speed for **5 sec** before removing any volume. Transfer **75 µl** to the S2 tube containing the chosen standard diluent. **Vortex** for **5 sec**.
4. Use a new pipet tip to transfer **75 µl** from the S2 tube to the S3 tube. **Vortex** for **5 sec**.
5. Continue with 1:3 (threefold) serial dilutions as shown in Figure 3.
6. Use reconstituted and diluted standards and controls immediately. Do not freeze for future use.

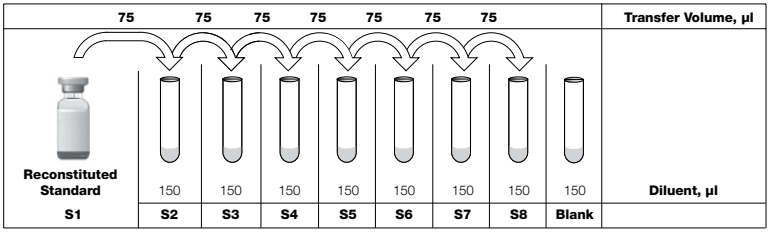


Fig. 3. Preparing a threefold dilution series with a single reconstituted standard.

6. Prepare Samples

These assays are designed to quantitate classes and subclasses of immunoglobulins in serum, plasma, and cell culture media. For optimal recovery and sensitivity, it is important to properly prepare samples.

- Once thawed, keep samples on ice. Prepare dilutions just prior to the start of the assay and equilibrate to room temperature before use
- Prepare sample dilutions in 1.5 or 2 ml microcentrifuge tubes. If a multichannel pipet will be used to load the plate, aliquot the required volumes into Titertube® Micro Test Tubes
- Do not freeze diluted samples

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

Sample Type	Dilution Factor	Diluent
Serum and plasma	TIMP: 1:50 dilution	Diluent HD
Fluids	TIMP: 1:4 and 1:40	Diluent + 0.5% BSA w/v

* TIMP-3 is not intended for serum and plasma samples.

Sample Preparation

Serum and Plasma

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

1. Draw whole blood into collection tubes containing anticoagulant. Invert tubes several times to mix.
2. For serum, allow blood to clot at room temperature for **30 to 45 min**. For plasma, proceed directly to the centrifugation steps.
3. Perform centrifugation at 1,000 x g for **15 min** at 4°C and transfer the serum or plasma to a clean polypropylene tube.
4. To completely remove platelets and precipitates, centrifuge again at 10,000 x g for **10 min** at 4°C. Alternatively, filter the samples with a 0.8/0.2 µm dual filter to prevent clogging.

5. Dilute 5 µl sample with 245 µl diluent HD for a **1:50 dilution**. **Vortex** for **5 sec**.
6. Assay samples immediately or aliquot into single-use tubes and store at –70°C. Avoid repeated freeze-thaw cycles.

Cell Culture Supernatant

1. Collect supernatants and centrifuge at 1,000 x g for **15 min** at 4°C. For cell lines cultured in serum-free culture media, collect samples and add BSA as a carrier protein to a final concentration of 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. Reconstitute and dilute the standard in the same medium or matrix in which cells are prepared. Be sure to include all medium components (such as **FBS**) as appropriate. To minimize error due to lot-to-lot variation of culture media, use the same lot of culture medium that was used to prepare the cells.
4. Assay immediately or store samples in single-use aliquots at –70°C. Avoid repeated freeze-thaw cycles.

7. Prepare Coupled Beads

1. Use Table 6 or the Calculation Worksheet on page 33 to calculate the volume of coupled beads and assay buffer needed.
2. Add the required volume of Bio-Plex Assay Buffer to a 15 ml polypropylene tube.
3. **Vortex** the 20x 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.
4. Dilute coupled beads to 1x by pipetting the required volume into the 15 ml tube. **Vortex**.

   Each well of the assay requires 2.5 µl of the 20x stock adjusted to a final volume of 50 µl in assay buffer.
5. Protect the beads from light with aluminum foil. Equilibrate to room temperature prior to use.

Note: To minimize volume loss, use a 200–300 µl capacity pipet to remove beads from the 20x stock tube. If necessary, perform the volume transfer in two steps. Do not use a 1,000 µl capacity pipet and/or wide bore pipet tip.

Table 6. Preparing 1x coupled beads from 20x stock (includes 20% excess volume).

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

8. Run Assay

Considerations

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

Table 7. Summary of wash options and incubations. After each assay step, select the appropriate Bio-Plex Pro Wash Station program or perform the appropriate manual wash step as summarized below.

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

Considerations When Using a Vacuum Manifold

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

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

1. Cover unused wells of the assay plate with sealing tape.
2. Prewet the filter plate. Skip this step if using a flat bottom plate.

   Prewet the wells with **100 µl** assay buffer and remove the liquid by vacuum filtration. Dry the bottom of the filter plate thoroughly by blotting on a clean paper towel.
3. **Vortex** the diluted (1x) beads for **30 sec** at medium speed. Pour into a reagent reservoir and transfer **50 µl** to each well of the assay plate.

   Tip: A multichannel pipet is highly recommended for ease of use and efficiency.
4. **Wash the plate two times** with **100 µl** Bio-Plex Wash Buffer according to your wash method of choice.
5. **Vortex** the diluted samples, standards, blank, and controls for **5 sec**. Transfer **50 µl** of each to the appropriate well of the assay plate, changing the pipet tip after every volume transfer.
6. **Cover** with a new sheet of sealing tape and **incubate** in the dark for **1 hr** at room temperature with shaking at **850 ± 50 rpm**.

Prepare and Add Detection Antibodies

1. While the samples are incubating use Table 8 or the Calculation Worksheet on page 33 to calculate the volume of detection antibodies and Bio-Plex Detection Antibody Diluent needed. Detection antibodies should be prepared **10 min before use**.
2. Add the required volume of Bio-Plex Detection Antibody Diluent to a 15 ml polypropylene tube.
3. Vortex the 20x stock of detection antibodies for 15 sec at medium speed, then perform a 30 sec spin to collect the entire volume at the bottom of the tube.
4. Dilute detection antibodies to 1x by pipetting the required volume into the 15 ml tube. **Vortex**.

   Each well of the assay requires 1.25 µl of the 20x stock adjusted to a final volume of 25 µl in detection antibody diluent.

Table 8. Preparing 1x detection antibodies from 20x stock (includes 25% excess volume).

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

5. After incubating the beads, samples, standards, blank, and controls, carefully remove and discard the sealing tape.
6. **Wash the plate three times** with **100 µl** wash buffer according to your wash method of choice.
7. Vortex the diluted (1x) detection antibodies gently for 5 sec. Pour into a reagent reservoir and transfer 25 µl to each well of the assay plate using a multichannel pipet.
8. **Cover** the plate with a new sheet of sealing tape. **Incubate** in the dark on shaker at **850 ± 50 rpm** for **30 min** at room temperature.

Prepare and Add Streptavidin-PE (SA-PE)

1. While detection antibodies are incubating, use Table 9 or the Calculation Worksheet on page 33 to calculate the volume of SA-PE and assay buffer needed. SA-PE should be prepared **10 min before use**.
2. Add the required volume of assay buffer to a 15 ml polypropylene tube.
3. **Vortex** the 100x 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 vial.
4. Dilute SA-PE to 1x by pipetting the required volume into the 15 ml tube. **Vortex** and protect from light until ready to use.

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

Table 9. Preparing 1x SA-PE from 100x stock (includes 25% excess volume).

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

5. After detection antibody incubation, carefully remove and discard the sealing tape.
6. **Wash the plate three times** with **100 µl** of wash buffer according to your wash method of choice.
7. **Vortex** the diluted (1x) SA-PE at medium speed for 5 sec. Pour into a reagent reservoir and transfer **50 µl** to each well using a multichannel pipet.
8. **Cover** the plate with a new sheet of sealing tape. **Incubate** in the dark on shaker at **850 ± 50 rpm** for **30 min** at room temperature.
9. After the SA-PE incubation step, carefully remove and discard the sealing tape.
10. **Wash the plate three times** with **100 µl** of wash buffer according to your wash method of choice.

11. To resuspend beads for plate reading, add **125 µl** assay buffer to each well. Cover the plate with a new sheet of sealing tape. Shake at room temperature at **850 ± 50 rpm** for **30 sec** and carefully remove the sealing tape. Ensure that the plate cover has been removed before placing the plate on the reader.

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.

Prepare 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 versions 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** in the Protocol Settings bar 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 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.

   For reference, bead regions are shown in Table 10.

Table 10. Bead regions for Bio-Plex Pro TIMP Assays.

Analyte	Bead Region	Analyte	Bead Region
TIMP-1	21	TIMP-3	52
TIMP-2	64	TIMP-4	35

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, simply click **Add All**.

e. If some of the analytes need to be removed from the Selected list, highlight them and select **Remove**. If desired, it is possible to rename the panel by clicking **Rename Panel** and entering a new panel name.

3. Click **Format Plate** and format according to the plate template created in section 1 (Plan Plate Layout). To modify the plate layout, follow the steps below (see Figure 4).

   a. Select the **Plate Formatting** tab.

   b. Select the standards icon (S) and drag the cursor over all the wells that contain standards. Repeat this process for Blanks ◇B◇, Controls (C), and Samples [X]. Note that Bio-Plex Manager automatically subtracts the blank MFI value from all other assay wells.

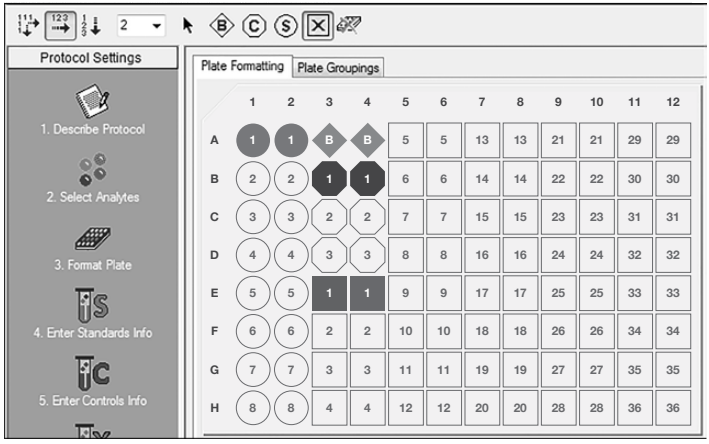


Fig. 4. Plate formatting.

4. Click **Enter Standards** Info in the Protocol Settings bar.
   a. Enter the highest concentration and units of each analyte in the top row (labeled S1) of the table. S1 concentration information is listed in the product data sheet.
   b. Enter a dilution factor of 3 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.
   b. For the vial of controls supplied, format the appropriate wells as controls and enter descriptions, but leave the concentrations blank. Alternatively, the controls can be formatted as samples with clear descriptions, such as “control.” In any case, **the expected control ranges provided on the product data sheet 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 settings follow Table 11.

Table 11. Read the plate using the appropriate instrument settings.

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 instruments settings		

* A similar Luminex-based system may be used.

   a. Confirm that data acquisition is set to **50 beads per region**.
   b. In Bio-Plex Manager Software prior to 6.1, go to Advanced Settings and confirm that the bead map is set to **100 region**, the sample size is set to **50 µl**, and the doublet discriminator (DD) gates are set to **5,000 (Low) and 25,000 (High)**. In Bio-Plex Manager Software versions 4.0, 4.1, 4.1.1, and 5.0, check **Override Gates** and set the DD gate values as indicated.
   c. Select **Start**, name and save the .rbx file, and begin data acquisition. The Run Protocol pop-up screen will appear. Click **Eject/Retract** to eject the plate carrier.

Acquire Data

1. Shake the assay plate at **850 ± 50 rpm** for **30 sec** and visually inspect the plate to ensure that the assay wells are filled with buffer. Carefully remove the sealing tape and any plate cover before placing the plate on the plate carrier.
2. Run Protocol — on the pop-up screen, select **Load Plate** and click **OK** to start acquiring data.
3. Use the **Wash Between Plates**  command after every plate run to reduce the possibility of clogging the instrument.

4. If acquiring data from more than one plate, empty the waste bottle and refill the sheath bottle after each plate (if HTF are not present). Select **Wash Between Plates** and follow the instructions. Then repeat the Prepare Protocol and Acquire Data instructions.
5. When data acquisition is complete, select **Shut Down**  and follow the instructions.

Reacquire Data

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

1. Check the wells from which data will be reacquired.
2. Aspirate the buffer with the wash method of choice, but do not perform wash step.
3. Add **100 µl** of assay buffer to each well. Cover the plate with a new sheet of sealing tape.
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 controls were run in the assay plate, open the results (.rbx) file, click **Report Table**, and locate the control wells. Compare the observed concentrations against the lot-specific control ranges 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. As such, they should be removed to generate a more realistic and accurate standard curve. This may result in an extended assay working range and allow quantitation of samples that might otherwise be considered out of range.

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 6.0 and earlier versions, outliers 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.

In Depth Data Analysis

To better understand your data's biological significance, consider using Bio-Plex Data Pro™ Software, which makes data management and comparison easier. This software offers various graphing options and automatic calculations with simple, conservative statistical calculations.

Contact your local Bio-Rad representative for more information.

Luminex xPONENT Software

Luminex xPONENT software may be used to analyze Bio-Plex Assays. 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 or Bio-Plex 3D Instruments. For the Bio-Plex MAGPIX Reader, use the default instrument settings.

1. Select MagPlex as the bead type for magnetic beads. This automatically sets the DD gates.
2. Volume = 50 µl.
3. Refer to Table 11 to select the appropriate PMT setting for your instrument.
4. Plate name: 96-well plate.
5. Analysis type: Quantitative, 5PL Curve Fit.
6. Number of standards: 8.

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

1. Enter **ng/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 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.
Bottom of filter plate not dry	Dry the bottom of the filter plate with absorbent paper towel (preferably lint-free) to prevent cross-well contamination.

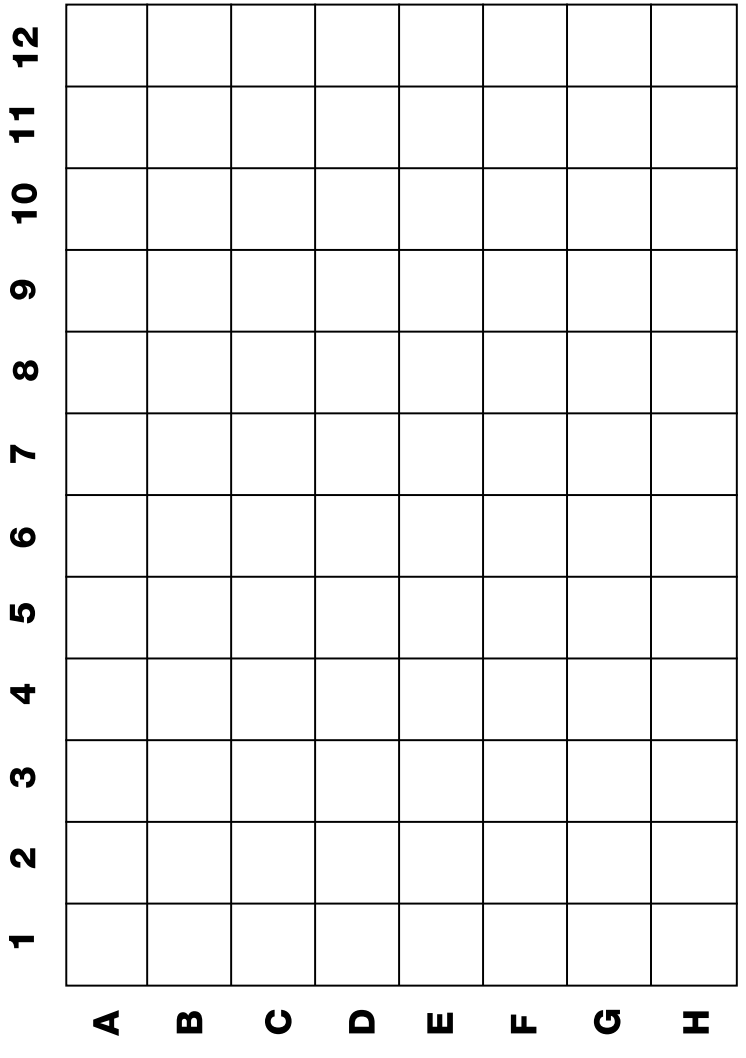
Possible Causes	Possible Solutions
High Intra-Assay CV	
Improper pipetting technique	Pipet carefully when adding standards, controls, samples, detection antibodies, and SA-PE, especially when using a multichannel pipet. Use a calibrated pipet. Change pipet tip after every volume transfer.
Reagents and 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 that the wells are filtered completely and that no residual volume remains. Ensure that the microplate shaker setting is not too high. Reduce the microplate shaker speed to minimize splashing.
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
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.
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.
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 (bulletin #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 instructions carefully.
Detection antibody or SA-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 diluent HD to dilute standards.
Accidentally spiked blank wells	Do not add any antigens to the blank wells.
Detection antibodies or SA-PE incubated too long	Follow the procedure incubation time precisely.
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.
High end saturation of the standard curve	Make sure that correct shaker speed and incubation times are used. Remove S1 from data analysis if needed.
Controls do not fall within expected ranges	Make sure that the vial of controls is reconstituted at the same time as standards and in the same diluent. Incubate for precisely 30 min.
Improper pipetting technique	Pipet carefully when adding tandards, samples, detection antibodies, and SA-PE, especially when using a multichannel pipet. Use a calibrated pipet. Change pipet tip after every volume transfer.

Possible Causes	Possible Solutions
Impact of Sample Matrix	
Negative MFI values in samples or standards	If samples contain little or no analyte, negative values observed may be due to statistical variation. If assay drift is suspected, retest the samples by positioning them next to the standards. If contamination of standards is suspected, check the standard replicate value and be careful when adding samples to the wells. Matrix effects could also produce negative sample values. Bio-Plex Manager™ Software automatically subtracts the blank (B) FI value from all other assay wells. While this has no impact on observed concentrations of samples within the assay working range, it may result in a negative FI value if the blank's FI value is greater than either the standard or the sample value. If this is undesirable, then reformat the blank wells as sample (X) or control (C) in the protocol or results file.
Poor precision in serum and plasma sample measurements	Check if any interfering components such as heparin-based anticoagulant, additives, or gel from separators were introduced into the samples. Avoid using hemolyzed and heavily lipemic samples. Remove visible particulate in samples by centrifugation. Avoid multiple freeze-thaw cycles of samples.

Section 9
Plate Layout Template



Section 10
Calculation Worksheet

If using either a premixed panel or one singleplex assay, follow these directions.

Plan the plate layout and enter the number of wells to be used in the assay:_____

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

a. Each well requires 50 µl of coupled beads (1x):
_____ x 50 µl = _____ µl

b. Include 20% excess to ensure enough volume:
_____ µl x 0.20 = _____ µl

c. Total volume of 1x coupled beads:
_____ µl + _____ µl = _____ µl

d. Volume of **20x coupled beads** required:
_____ µl/20 = _____ µl

e. Volume of **assay buffer** required:
_____ µl – _____ µl = _____ µl

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

a. Each well requires 25 µl detection antibodies (1x):
_____ x 25 µl = _____ µl

b. Include 25% excess to ensure enough volume:
_____ µl x 0.25 = _____ µl

c. Total volume of 1x detection antibodies:
_____ µl + _____ µl = _____ µl

d. Volume of **20x detection antibodies** required:
_____ µl/20 = _____ µl

e. Volume of **detection antibody diluent** required:
_____ µl – _____ µl = _____ µl

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

a. Each well requires 50 µl SA-PE (1x):
_____ x 50 µl = _____ µl

b. Include 25% excess to ensure enough volume:
_____ µl x 0.25 = _____ µl

c. Total volume of 1x SA-PE:
_____ µl + _____ µl = _____ µl

d. Volume of **100x SA-PE** required:
_____ µl/100 = _____ µl

e. Volume of **assay buffer** required:
_____ µl – _____ µl = _____ µl

Section 11
Safety Considerations

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

Section 12
Legal Notices

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Section 13
Ordering Information

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

Catalog #	Description
Premixed All-In-One Multiplex Kit	
Premixed multiplex kit includes coupled magnetic beads, detection antibodies, standards, 1-level controls, detection antibody diluent HB, diluent HD (for use with samples, standards, and controls), assay buffer, wash buffer, SA-PE, 96-well flat bottom plate, sealing tape, assay quick guide, and product data sheet.	
171AM002M	**Bio-Plex Pro Human TIMP Panel**, 4-plex, 1 x 96-well, for the detection of TIMP-1, TIMP-2, TIMP-3, TIMP-4
Accessories	
171025001	**Bio-Plex Pro Flat Bottom Plates**, 40 x 96-well plates
171304500	**Bio-Plex Wash Buffer**, 1.5 L
171304502	**Filter plate**, pkg of 1, 96-well plate with clear plastic lid and tray, for Bio-Plex Assays using the vacuum wash method, sealing tape not included
171020100	**Bio-Plex Handheld Magnetic Washer**, includes magnetic washer and adjustment hex tools for use in manual wash steps for all Bio-Plex Magnetic Assays

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Web site *bio-rad.com* ***USA*** *1 800 424 6723* ***Australia*** *61 2 9914 2800*
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Poland *48 22 331 99 99* ***Portugal*** *351 21 472 7700*
Russia *7 495 721 14 04* ***Singapore*** *65 6415 3188*
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Sweden *46 08 555 12700* ***Switzerland*** *41 026 674 55 05*
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