
Bio-Plex Pro[™] Diabetes Assays

Instruction Manual

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

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Introduction

Bio-Plex Pro™ diabetes assays are magnetic bead-based multiplex assays designed to measure multiple diabetes-related biomarkers in a minimal volume of matrix such as serum, plasma, tissue culture supernatant, or other biological fluids. The biomarkers chosen for these assays are circulating proteins involved in the regulation of glucose metabolism.

These multiplex assays are configured for the detection of various metabolic markers in human, mouse, and rat samples. The multiplex assays for human matrices were tested and found to be cross-reactive to at least four common non-human primate (NHP) species, including rhesus, cynomolgus, baboon, and marmoset. The degree of cross-reactivity was profiled according to the ability of each assay to detect these metabolic markers in the sera and mitogen-stimulated peripheral blood mononuclear cell (PBMC) culture supernatant of these animals.

The high level of gene homology across the major NHP species may allow the NHP diabetes assays to be used in other species, such as chimpanzees, African green monkey, and pigtail macaques. However, assay performance has not been specifically evaluated in these animals.

Cross-Reactivity with Non-Human Primates

	Rhesus	Cynomolgus	Baboon	Marmoset	Chimpanzee	African green monkey	Pigtail macaque
Tested	•	•	•	•			
Not tested					•	•	•

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

For more information please visit www.bio-rad.com/bio-plex.

Principle

Technology

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

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

Assay Format

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

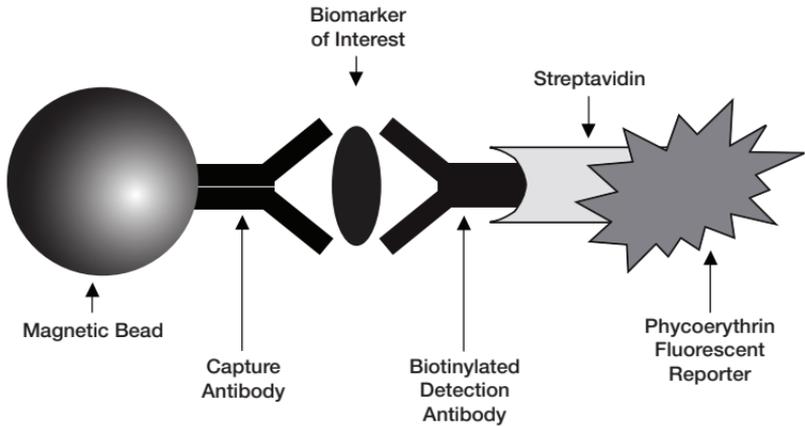


Fig. 1. Bio-Plex sandwich immunoassay.

Data Acquisition and Analysis

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

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

Kit Contents and Storage

Reagents Supplied

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

Table 1. Contents of Bio-Plex Pro diabetes assays.*

Component	1 x 96-Well Format	10 x 96-Well Format
Standard diluent (not included in adiponectin/adipsin assays)	10 ml	100 ml
Sample diluent (not included in adiponectin/adipsin assays)	40 ml	80 ml
Serum-based diluent (adiponectin/adipsin assays only)	70 ml	N/A
Assay buffer	50 ml	500 ml
Wash buffer	200 ml	1.5 L
Detection antibody diluent	5 ml	50 ml
Streptavidin-PE (100x)	1 tube	1 tube
Filter and/or flat bottom plate (96-well)	1 plate	10 plates
Sealing tape	1 pack of 4	10 packs of 4
Assay Quick Guide		1 booklet
Coupled magnetic beads (20x)	1 tube	1 tube
Detection antibodies (20x)	1 tube	1 tube
Standard	1 vial	10 vials

* Volumes shown are approximate. Quantities in Bio-Plex® Express assays will vary.

Storage and Stability

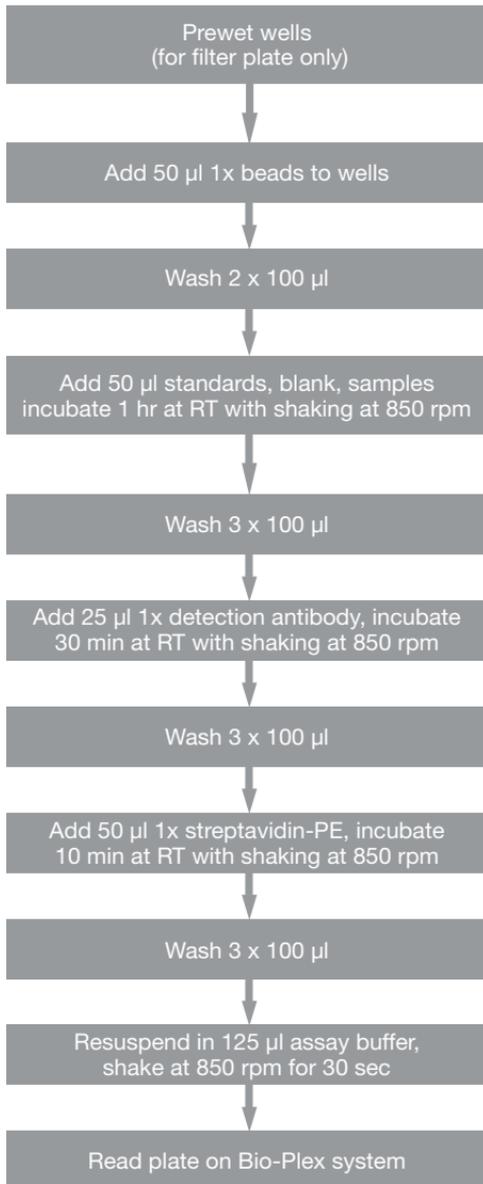
Kit contents should be stored at 4°C and never frozen. Coupled magnetic beads and streptavidin-PE should be stored in the dark. All components are guaranteed for a minimum of six months from the date of purchase when stored as specified.

Table 2. Recommended materials.

Item	Ordering Information
Bio-Plex Pro Assays Quick Guide 2	Bulletin #10024973 (download at www.bio-rad.com/bio-plex)
Bio-Plex 200 system or Luminex system with HTF	Bio-Rad catalog #171-000205
Bio-Plex validation kit Run the validation kit monthly to ensure optimal performance of fluidics and optics systems	Bio-Rad catalog #171-203001
Bio-Plex calibration kit Run the calibration kit daily to standardize fluorescence signal	Bio-Rad catalog #171-203060
Bio-Plex Pro wash station For use with magnetic bead-based assays only	Bio-Rad catalog #300-34376
Bio-Plex Pro II wash station For use with both polystyrene (nonmagnetic) and magnetic bead-based assays	Bio-Rad catalog #300-34377
Bio-Plex handheld magnetic washer For use with magnetic bead-based assays only	Bio-Rad catalog #170-20100
Bio-Plex Pro flat bottom plates, 40 x 96-well For magnetic separation on the Bio-Plex Pro wash station	Bio-Rad catalog #171-025001
Microtiter plate shaker IKA MTS 2/4 shaker for 2 or 4 microplates or Barnstead/Lab-Line Model 4625 plate shaker (or equivalent capable of 300–1,100 rpm)	IKA catalog #320-8000 VWR catalog #57019-600
Bio-Rad® Aurum™ vacuum manifold For vacuum filtration	Bio-Rad catalog #732-6470
BR-2000 vortexer	Bio-Rad catalog #166-0610
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
Pall Life Science Acrodisc, 25 mm PF syringe filter (0.8/0.2 µm Supor membrane)	Pall Life Sciences catalog #4187
Filter plate, 1 x 96-well, clear plastic lid and tray	Bio-Rad catalog #171-304502
Titertube® Micro test tubes For preparing replicate standards, samples, and controls prior to loading the plate	Bio-Rad catalog #223-9390

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

Assay Workflow



Important Considerations

Instruments and Software

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

Assay Procedures

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

Assay Quick Guide

Each assay kit includes a printed Bio-Plex Pro Assay Quick Guide (bulletin #10024973), which can be used to prepare and run a full 1 x 96-well assay plate. Users can also download a copy at www.bio-rad.com/bio-plex.

Bead Regions

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

Multiplexing Compatibility

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

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

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

1. Plan Plate Layout

Prior to running the assay, determine the total number of wells in the experiment using the Plate Layout Template on page 41 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 standard diluent and be processed in the same manner as sample and standard wells. Note that Bio-Plex Manager automatically subtracts the blank (B) MFI value from all other assay wells.
3. User-defined controls are assigned to wells in columns 3 and 4.
4. The remainder of the plate is available for samples.
5. Once the total number of wells is known, calculate the required volumes of beads, detection antibody, and streptavidin-PE needed. Use Tables 9–11, 14–16, and 17, respectively, or the Calculation Worksheet on page 43.

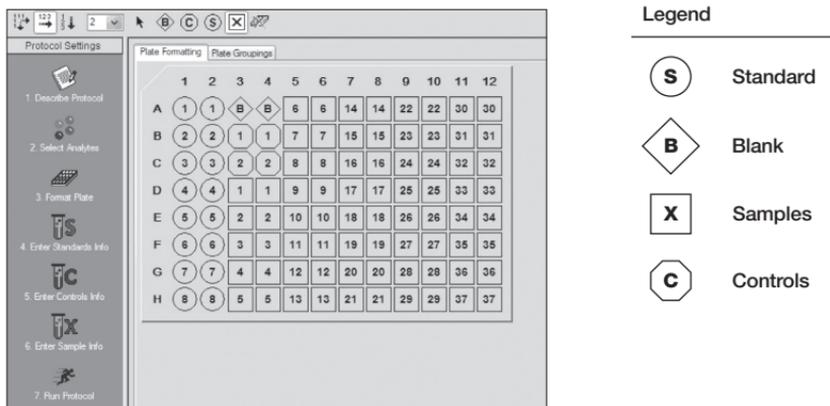


Fig. 2. Suggested plate layout. For detailed instructions on plate formatting in Bio-Plex Manager, see Section 8.

2. Prepare Instrument

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

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

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

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

Calibrate System

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

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

3. Prepare Wash Method

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

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

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

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

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

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

Setting Up the Bio-Plex Handheld Magnetic Washer

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

Setting up a Vacuum Manifold

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

4. Prepare Standards

General Instructions

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

Selecting a Diluent for Standards

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

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

Table 5. Summary of recommended diluents for standards.

Sample Type	Diluent for Standards	Add BSA
Serum and plasma	Standard diluent	None
Culture media, with serum	Culture media	None
Culture media, serum-free	Culture media	To 0.5% final
For Adiponectin and/or Adipsin Assays		
Serum and plasma	Serum-based diluent	None

RP1 (PMT) Setting for Standard Curves

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

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

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

* Or similar Luminex-based system.

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

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

Assay	Low RP1 (PMT)	High RP1 (PMT)
Human/NHP* diabetes		•
Mouse diabetes	User validation required*	•
Rat diabetes		•
Cross-panel mixing	Low RP1 (PMT), broad range cytokine curve	High RP1 (PMT), narrow range cytokine curve
Human diabetes + cytokines		•
Mouse diabetes + cytokines	User validation required*	•
Rat diabetes + cytokines		•

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

Reconstitute a Single Vial of Diabetes Standards

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

1. Gently tap the vial containing the lyophilized standard.
2. Add **500 µl** of the appropriate standard diluent. Do not use assay buffer or sample diluent to reconstitute the standards.

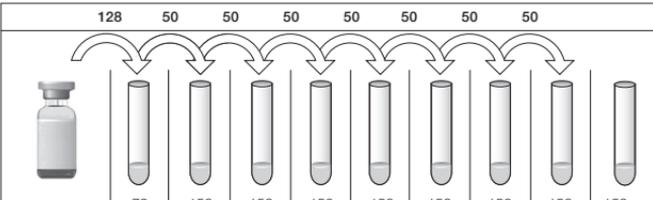
- Gently **vortex** the reconstituted standard for **5 sec** then incubate on ice for **30 min**. Be consistent with the incubation time in every assay to ensure best results.
- During the incubation period, prepare the samples as instructed in the Prepare Samples section.

Prepare Diabetes Standard Dilution Series from a Single Antigen Vial

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

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

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

	128	50	50	50	50	50	50	50	50	Transfer Volume, μ l
	72	150	150	150	150	150	150	150	150	
Reconstituted Diabetes Standard	S1	S2	S3	S4	S5	S6	S7	S8	Blank	Diluent, μ l

Please skip this section if not mixing diabetes with cytokine assays.

Reconstituting Standards for Cross-Panel Plexing

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

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

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

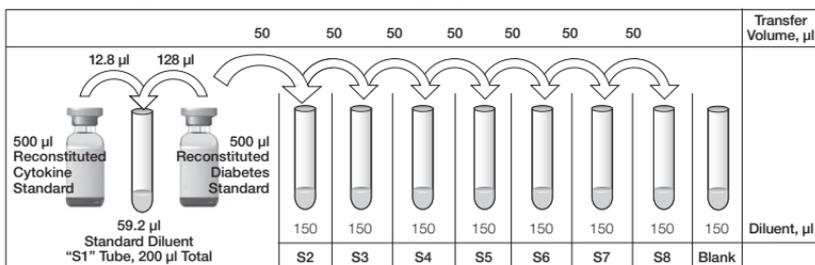
Preparing Serial Dilutions

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

1. Label nine 1.5 ml polypropylene tubes S1 through S8 and Blank.
2. **For high PMT**/narrow range cytokine standard curve, add **59.2 μ l** of standard diluent to the S1 tube. **For low PMT**/broad range cytokine standard curve, add **72 μ l** of standard diluent to S1 (Figures 4 and 5).

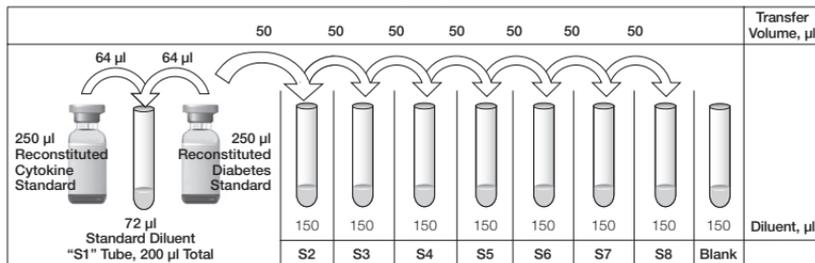
3. **For high PMT/narrow range cytokine standard curve**, add **12.8 μ l** of the reconstituted cytokine standard and **128 μ l** of the reconstituted diabetes standard to the S1 tube for a total volume of 200 μ l (Figure 4). **Vortex** at medium speed for **5 sec**.
4. Add **150 μ l** of standard diluent to the remaining tubes, as shown in Figure 4.

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



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

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



5. Prepare Samples

General guidelines on preparing samples derived from serum, plasma, and tissue culture supernatant are provided here, including the use of protease inhibitors with plasma 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 polypropylene microcentrifuge tubes. If a multichannel pipet will be used to load the plate, then aliquot the required volumes into Titer tube[®] micro test tubes.
- Do not freeze diluted samples

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

Sample Type	Diluent	Add BSA	Sample Dilution
Serum and plasma	Sample diluent	None	Fourfold (1:4)
Culture media, with serum	Culture media	None	User optimized
Culture media, serum-free	Culture media	To 0.5% final	User optimized
For Adiponectin Assay			
Serum and plasma	Serum-based diluent	None	Human (1:400) Mouse (1:1600) NHP (1:1600)
For Adipsin Assay			
Serum and plasma	Serum-based diluent	None	Human (1:400)

Note: certain sample types may require a different dilution factor.

Protease Inhibitors

In general, diabetes biomarkers are detectable in EDTA-treated plasma. Freezing plasma immediately after preparation and keeping samples frozen until use should provide adequate protection from degradation.

However, users may choose to add protease inhibitors as a precautionary measure.

Note: Protease inhibitors are recommended for use with plasma samples only, not with serum.

Either protease inhibitors may be added to samples at the time of blood collection (see protocol in the Appendix) or blood samples may be collected directly into a BD P800 collection tube (Becton-Dickinson, catalog #366420 or #366421).

Note: BD P800 tubes are not designed for use with mouse samples; therefore protease inhibitors, if required, should be added at the point of blood collection.

Plasma

K₂EDTA-treated plasma is acceptable as long as the sample is immediately frozen upon collection. Avoid using heparin-treated plasma as it may absorb certain soluble proteins. Avoid using hemolyzed samples as this may lead to false positive results.

1. Draw whole blood into collection tubes containing anticoagulant.
2. If desired, add protease inhibitors (see protocol in the Appendix).
3. Invert tubes several times to mix with either the anticoagulant or the protease inhibitors.
4. Perform centrifugation at 1,000 x g for 15 min at 4°C and transfer the plasma to a clean polypropylene tube.
5. 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 instrument clogging.

6. For assays other than adiponectin and adipisin, dilute plasma fourfold (1:4) by adding 1 volume of sample to 3 volumes of Bio-Plex sample diluent (for example: 40 μ l sample + 120 μ l sample diluent).
7. Assay samples immediately or aliquot into single-use tubes and store at -70°C . Avoid repeated freeze/thaw cycles.

Serum

1. To prepare serum, allow blood to clot at room temperature for 30 to 45 min.
2. Perform centrifugation at 1,000 x g for 15 min at 4°C and transfer the serum to a clean polypropylene tube.
3. To completely remove platelets and precipitates, centrifuge again at 10,000 x g for 10 min at 4°C . Alternatively, carefully filter the samples with a 0.8/0.2 μm dual filter to prevent instrument clogging.
4. Dilute and handle samples as described in steps 6 and 7 above.

Tissue 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%. This is done 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. If high levels of analyte are expected, samples can be further diluted in culture media. Supplement serum-free media with 0.5% BSA final.
4. Assay samples immediately or aliquot and store at -70°C .

Lavage, Sputum, and Other Biological Fluid Samples

Keep all samples on ice until ready for use.

1. If dilution is required, use Bio-Plex sample diluent with 0.5% BSA final.
2. Centrifugation at 10,000 x g for 10 min at 4°C may be needed to clarify the sample.

Sample Dilution for Adiponectin and Adipsin Assays

Note: Physiological levels of adiponectin and adipsin are typically found at high concentrations; therefore, higher sample dilutions are required to achieve measurable concentrations within the standard curve.

1. For **human** adiponectin and adipsin assays, dilute serum or plasma 1:400 in serum-based diluent with two 1:20 serial dilutions
 - First dilution: 10 µl sample + 190 µl serum-based diluent
 - Second dilution: 10 µl from the first dilution + 190 µl serum-based diluent
2. For **mouse** and **NHP** adiponectin assays, dilute serum or plasma 1:1,600 in serum-based diluent with two 1:40 serial dilutions
 - First dilution: 10 µl sample + 390 µl serum-based diluent
 - Second dilution: 10 µl from the first dilution + 390 µl serum-based diluent

6. Prepare Coupled Beads

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

Table 9. Stock concentration of coupled beads.

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

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

1. Use Tables 10–12 to calculate the volume of coupled beads and assay buffer needed.
2. Add the required volume of Bio-Plex assay buffer to a 15 ml polypropylene tube.
3. **Vortex** the stock coupled beads at medium speed for **30 sec**. Carefully open the cap and pipet any liquid trapped in the cap back into the tube. This is important to ensure maximum bead recovery. **Do not centrifuge the vial**; doing so will cause the beads to pellet.
4. Dilute coupled beads to 1x by pipetting the required volume into the 15 ml tube. **Vortex**.

Each well of the assay plate requires either 2.5 μ l (20x stock) or 5.0 μ l (10x stock) adjusted to a final volume of 50 μ l in assay buffer.

5. Protect the beads from light with aluminum foil. Equilibrate to room temperature prior to use.

Table 10 summarizes volumes required for preparing 1x beads from a single 20x stock. Table 11 summarizes volumes required for preparing 1x beads from a mix of two 20x stocks; volumes required for preparing beads of one diabetes (20x) assay and one cytokine (10x) assay (for example, human insulin and human IL-6) are listed in Table 12.

Note: To minimize volume loss, use a 200–300 µl capacity pipet to remove beads from the 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.

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

Table 10. Premixed panel or one singleplex assay.

# of Wells	20x Beads, µl	Assay Buffer, µl	Total Volume, µl
96	288	5,472	5,760

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

# of Wells	20x beads, µl Singleplex #1	20x beads, µl Singleplex #2	Assay Buffer, µl	Total Volume, µl
96	288	288	5,184	5,760

Table 12. Preparing 1x beads from two stocks at different concentrations. Mixing human insulin (20x) with human IL-6 (10x) is one example*.

# of Wells	20x Beads, µl Diabetes	10x Beads, µl Cytokines	Assay Buffer, µl	Total Volume, µl
96	288	576	4,896	5,760

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

7. Run Assay

Considerations

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

Table 13. Summary of wash options and protocols. After each assay step, select the appropriate Bio-Plex Pro™ wash station program or perform the appropriate manual wash step as summarized below.

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

Considerations When Using a Vacuum Manifold

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

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

1. Cover unused wells with sealing tape.
2. Prewet the filter plate. Skip this step if using a flat bottom plate.
 - a) Prewet the wells with 100 μ l of assay buffer and remove the liquid by vacuum filtration. Dry the bottom of the filter plate thoroughly by blotting on a clean paper towel.
3. **Vortex** the diluted (1x) coupled beads for **30 sec** at medium speed. Pour the diluted coupled beads into a reagent reservoir and transfer 50 μ l to each well of the assay plate.

Tip: A multichannel pipet is highly recommended for ease of use and efficiency.
4. **Wash the plate two times** with **100 μ l** Bio-Plex wash buffer using the wash method of choice.
5. **Gently vortex** the diluted standards, blanks, samples, and controls (if applicable) for **5 sec**. Transfer **50 μ l** to each well of the assay plate, changing the pipet tip after every volume transfer
6. **Cover** plate with a new sheet of sealing tape and protect from light with aluminum foil. **Incubate** on shaker at **850 \pm 50 rpm** for **1 hr** at room temperature (RT).

Note: 850 rpm provides equivalent performance to previously recommended shaker settings (1,100 rpm for 30 sec, 300 rpm for incubation).

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

Prepare and Add Detection Antibodies

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

Table 14. Stock concentration of detection antibodies.

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

1. While the samples are incubating, use Tables 15–17 to calculate the volume of detection antibodies and detection antibody diluent needed. Detection antibodies should be prepared **10 min before use**.
2. Add the required volume of Bio-Plex detection antibody diluent to a 15 ml polypropylene tube.
3. **Vortex** the stock detection antibodies for **15–20 sec** at medium speed, then perform a **30 sec** spin to collect the entire volume at the bottom of the tube.
4. Dilute detection antibodies to 1x by pipetting the required volume into the 15 ml tube.

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

For reference, Table 15 summarizes volumes required for preparing 1x detection antibodies from a single 20x stock.

Table 16 summarizes volumes required for preparing 1x detection antibodies from a mix of two 20x stocks; volumes required for preparing detection antibodies of one diabetes (20x) assay and one cytokine (10x) assay (for example, human insulin and human IL-6) are listed in Table 17.

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

Table 15. Premixed panel or one singleplex assay.

# of Wells	20x Detection Antibodies, μ l	Detection Antibody Diluent, μ l	Total Volume, μ l
96	150	2,850	3,000

Table 16. Mixing two singleplex assays or a premixed panel + singleplex assay.

# of Wells	20x Detection Antibodies, μ l Singleplex #1	20x Detection Antibodies, μ l Singleplex #2	Detection Antibody Diluent, μ l	Total Volume, μ l
96	150	150	2,700	3,000

Table 17. Preparing 1x detection antibodies from two stocks at different concentrations. Mixing human insulin (20x) with human IL-6 (10x) is one example.*

# of Wells	20x Detection Antibodies, μ l Diabetes	10x Detection Antibodies, μ l Cytokines	Detection Antibody Diluent, μ l	Total Volume, μ l
96	150	300	2,550	3,000

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

5. After incubating the beads, samples, standards, and blank, slowly remove and discard the sealing tape.

6. **Wash the plate three times** with **100 μ l** wash buffer.
7. **Vortex** the diluted (1x) detection antibodies gently for **5 sec**.
Pour into a reagent reservoir and transfer **25 μ l** to each well of the assay plate using a multichannel pipet.
8. **Cover** plate with sealing tape and protect from light with aluminum foil.
Incubate on shaker at **850 \pm 50 rpm** for **30 min** at room temperature.

Prepare and Add Streptavidin-PE (SA-PE)

1. While the detection antibodies are incubating, use Table 18 to calculate the volume of SA-PE (100x) and assay buffer needed. Streptavidin-PE should be prepared **10 min before use**.
2. Add the required volume of assay buffer to a 15 ml polypropylene tube.
3. **Vortex** the 100x SA-PE for **5 sec** at medium speed.
Perform a **30 sec** spin to collect the entire volume at the bottom of the 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 μ l (100x stock) adjusted to a final volume of 50 μ l in assay buffer.

Table 18 shows an example calculation.

Table 18. Preparing 1x SA-PE from 100x stock (includes 25% excess volume).

# of Wells	100x SA-PE, μ l	Assay Buffer, μ l	Total Volume, μ l
96	60	5,940	6,000

5. After the detection antibody incubation, slowly remove and discard the sealing tape.
6. **Wash the plate three times** with **100 µl** wash buffer.
7. **Vortex** the diluted (1x) SA-PE at medium speed for **5 sec**. Pour into a reagent reservoir and transfer **50 µl** to each well using a multichannel pipet.
8. **Cover** plate with sealing tape and protect from light with aluminum foil. **Incubate** on shaker at **850 ± 50 rpm** for **10 min** at room temperature.
9. After the streptavidin-PE incubation step, slowly remove and discard the sealing tape.
10. **Wash the plate three times** with **100 µl** wash buffer.
11. To resuspend beads for plate reading, add **125 µl** of assay buffer to each well. Cover the plate with a new sheet of sealing tape. Shake the plate at room temperature at **850 ± 50 rpm** for **30 sec**, and slowly remove the sealing tape. Ensure that the plate cover has been removed before placing the plate on the reader.
12. Remove the sealing tape and read the plate using the settings below.
Note: Reading at alternative PMT settings on the Bio-Plex 100, 200, or Bio-Plex 3D (for example when mixing diabetes assays with cytokine assays) requires validation by the end user to ensure that results meet the user's acceptance criteria.

Table 19. Read the plate using the appropriate instrument settings.

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

* Or similar Luminex-based system.

8. Read Plate

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

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

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

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

Bio-Plex Manager software version 6.0 contains protocols for most Bio-Plex® assays. Choose from available protocols or create a new protocol. To create a new protocol, select **File**, then **New** from the main menu. Locate and follow the steps under **Protocol Settings**.

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

For reference, bead regions are shown in Table 20.

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

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

Table 20. Bead regions for available Bio-Plex Pro diabetes assays.

Human/NHP Diabetes		Mouse Diabetes		Rat Diabetes	
Analyte	Bead Region	Analyte	Bead Region	Analyte	Bead Region
Adiponectin	64	Adiponectin	29	Ghrelin	64
Adipsin	35	Ghrelin	64	GLP-1	62
C-Peptide	72	GIP	46	Glucagon	63
Ghrelin	26	GLP-1	62	Leptin	65
GIP	14	Glucagon	63	PAI-1	61
GL P-1	27	Insulin	66		
Glucagon	15	Leptin	65		
Insulin	12	PAI-1	48		
Leptin	78	Resistin	30		
PAI-1	61				
Resistin	65				
Visfatin	22				

Table 21. Bead regions for compatible Bio-Plex Pro cytokine assays. Assays shown have bead regions that are non-overlapping with diabetes assays.

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

* Mouse group III cytokines have not been tested for cross reactivity with diabetes or other panels.

3. Click **Format Plate** and format the plate according to the plate layout created in Section 1 (Plan Plate Layout). To modify the plate layout, follow the steps below (see Figure 6).
 - a. Select the **Plate Formatting** tab.
 - b. Select the standards icon  and drag the cursor over all the wells that contain standards. Repeat this process for blanks , controls , and samples .

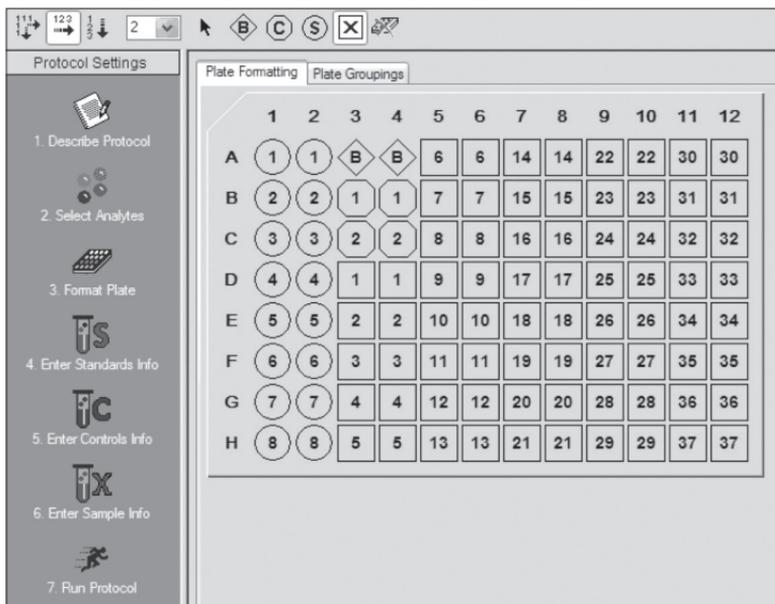


Fig. 6. Plate formatting.

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

7. Click **Run Protocol** and confirm that the assay settings are correct.
 - a. The Bio-Plex Pro diabetes assays were developed on the high RP1 (high PMT) setting using the Bio-Plex 200 system. Protocols using alternative PMT settings should be validated by the end user, for example when mixing diabetes assays with cytokine assays.
 - b. Confirm data acquisition is set to **50 beads per region**. In Advanced Settings, confirm that the bead map is set to **100 region**, the sample size is set to **50 μ l**, and the DD gates are set to **5,000 (Low)** and **25,000 (High)**. In Bio-Plex Manager software versions 4.0, 4.1, and 4.1.1, check **Override Gates** and set the DD gate values as indicated.
 - c. Select **Start**, name and save the .rbx file, and begin data acquisition. The Run Protocol pop-up screen will appear. Click **Eject/Retract** to eject the plate carrier.

Acquire Data

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

Reacquire Data

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

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

Data Analysis: Removing Outliers

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

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

Previous Versions of Bio-Plex Manager Software

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

Luminex xPONENT Software

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

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

1. Select MagPlex as the bead type for magnetic beads, which automatically sets the DD gates.
2. Volume = 50 µl.
3. Refer to Table 19 to select the appropriate PMT setting for your instrument.
4. Plate name: 96-well plate.
5. Analysis type: Quantitative; 5PL Curve Fit.
6. Number of standards: 8.

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

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

Select **Stds and Ctrls**.

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

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

Troubleshooting Guide

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

Possible Causes

High Inter-Assay CV

Standards were not reconstituted consistently between assays

Reconstituted standards and diluted samples were not stored properly

Bottom of filter plate not dry

Possible Solutions

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

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

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

Possible Causes

High Intra-Assay CV

Improper pipetting technique

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

Contamination with wash buffer during wash steps

Slow pipetting of samples and reagents across the plate

Bio-Plex Wash Station: insufficient washing due to clogged pins

Possible Solutions

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

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

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

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

Clean dispensing pins with the thicker of the 2 cleaning needles provided with washer. Perform regular rinses to minimize salt build up.

Possible Causes

Low Bead Count

Miscalculation of bead dilution

Beads clumped in multiplex bead stock tube

Vacuum on for too long when aspirating buffer from wells

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

Reader is clogged

Low Signal or Poor Sensitivity

Standards reconstituted incorrectly

Detection antibody or streptavidin-PE diluted incorrectly

Possible Solutions

Check your calculations and be careful to add the correct volumes

Vortex for 30 sec at medium speed before aliquoting beads.

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

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

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

Follow the standard preparation instructions carefully.

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

Possible Causes

High Background Signal

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

Accidentally spiked blank wells

Detection antibodies or streptavidin-PE incubated too long

Poor Recovery

Expired Bio-Plex reagents were used

Incorrect amounts of components were added

Microplate shaker set to an incorrect speed

Possible Solutions

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

Do not add any antigens to the blank wells.

Follow the procedure incubation time precisely.

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

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

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

Possible Causes

Poor Recovery

Improper pipetting technique

Impact of Sample Matrix

Negative MFI values in samples or standards

Poor precision in serum and plasma sample measurements

Possible Solutions

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

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

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

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

Appendix: Protease Inhibitors

Refer to the recommended protocol below for preparing the inhibitors.

Materials

- DDP-IV Inhibitor (Sigma Aldrich, K4264, Molecular Weight: 370.24):
Store at 4°C
- Aprotinin (Sigma Aldrich, A3428, 3–8 TIU/mg or 3,900–10,400 KIU/mg):
Store at 4°C

Preparation of Protease Inhibitors Stock Solution

1. Preparation of 10 mM DDP-IV inhibitor solution
 - a) Weigh 18.5 mg of DDP-IV inhibitor.
 - b) Dissolve completely in 5 ml of 0.9% NaCl.
 - c) Aliquot and store at –20°C. Avoid repeated freeze/thaw cycles.
2. Preparation of 1.3% Aprotinin
 - a) Weigh 100 mg Aprotinin.
 - b) Dissolve completely in 7.5 ml of 0.9% NaCl.
 - c) Aliquot and store at –20°C. Avoid repeated freeze/thaw cycles.

Blood Sample Preparation

1. Collect whole blood in plasma collection tubes.
2. Add 10 μ l of 10 mM DDP-IV inhibitor per 1 ml of whole blood for a final concentration of 100 μ M.
3. Add 10 μ l of 1.3% Aprotinin per 1 ml of whole blood for a final amount of >500 KIU per 1 ml of whole blood.
4. Invert the tubes several times to mix the protease inhibitors with blood.

Plate Layout Template

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

Calculation Worksheet

If using either a **premixed panel** or **one singleplex assay** with 20x stocks of beads and detection antibodies, follow these directions.

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

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

- Each well requires 50 μl of coupled beads (1x): _____ x 50 μl = _____ μl
1 2
- Include 20% excess to ensure enough volume: _____ μl x 0.20 = _____ μl
2 3
- Total volume of 1x coupled beads: _____ μl + _____ μl = _____ μl
2 3 4
- Volume of **20x coupled beads** required: _____ μl / 20 = _____ μl
4 5
- Volume of **assay buffer** required: _____ μl - _____ μl = _____ μl
4 5 6

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

- Each well requires 25 μl detection antibodies (1x): _____ x 25 μl = _____ μl
1 7
- Include 25% excess to ensure enough volume: _____ μl x 0.25 = _____ μl
7 8
- Total volume of 1x detection antibodies: _____ μl + _____ μl = _____ μl
7 8 9
- Volume of **20x detection antibodies** required: _____ μl / 20 = _____ μl
9 10
- Volume of **detection antibody diluent** required: _____ μl - _____ μl = _____ μl
9 10 11

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

- Each well requires 50 μl streptavidin-PE (1x): _____ x 50 μl = _____ μl
1 10
- Include 25% excess to ensure enough volume: _____ μl x 0.25 = _____ μl
10 11
- Total volume of 1x streptavidin-PE: _____ μl + _____ μl = _____ μl
10 11 12
- Volume of **100x streptavidin-PE** required: _____ μl / 100 = _____ μl
12 13
- Volume of **assay buffer** required: _____ μl - _____ μl = _____ μl
12 13 14

If **mixing singleplex assays** with 20x stocks of beads and detection antibodies, follow these directions. Enter the number of wells to be used in the assay: _____
1

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

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

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

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

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

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

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

Note: Refer to Table 20 for the maximum number of diabetes and cytokine assays that may be multiplexed. Mixing across panels is not applicable to NHP diabetes assays.

Table 20. Maximum number of singleplex diabetes and cytokine analytes that may be multiplexed.

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

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

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

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

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

a) Each well requires 50 μl of coupled beads (1x): _____ x 50 μl = _____ μl
1 4

b) Include 20% excess to ensure enough volume: _____ μl x 0.20 = _____ μl
4 5

c) Total volume of 1x coupled beads: _____ μl + _____ μl = _____ μl
4 5 6

d) **Volume of 20x beads** required from each diabetes tube(s): _____ μl / 20 = _____ μl
6 7

e) **Volume of 10x beads** required from each cytokines tube(s): _____ μl / 10 = _____ μl
6 8

f) Total volume of diabetes bead stock required: _____ μl x _____ = _____ μl
7 2 9

g) Total volume of cytokine bead stock required: _____ μl x _____ = _____ μl
8 3 10

h) Total volume of combined bead stocks required: _____ μl + _____ = _____ μl
9 10 11

i) Volume of **assay buffer** required: _____ μl - _____ μl = _____ μl
6 11 12

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

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

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

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

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

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

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

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

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

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

h) Total volume of combined detection antibodies required:

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

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

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

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

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

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

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

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

Safety Considerations

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

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Ordering Information

Premixed All-in-One Multiplex Assays

Description	Catalog #
Bio-Plex Pro™ Human Diabetes 10-plex Assay, 1 x 96-well	171-A7001M
Bio-Plex Pro Mouse Diabetes 8-plex Assay, 1 x 96-well	171-F7001M
Bio-Plex Pro NHP Diabetes 11-plex Assay, 1 x 96-well	171-W7001M

Bio-Plex® Express Assays (You Mix)

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

Singleplex Sets and Individual Components

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



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