Bio-Plex Pro Rat 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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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 rat samples. 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.

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.

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

Principle

Technology

The Bio-Plex Multiplex Immunoassay 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 2 lasers and associated optics to measure the different molecules bound to the surface of the beads
- A high-speed digital signal processor that efficiently manages the fluorescence data

Assay Format

Bio-Plex Pro Assays are 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.

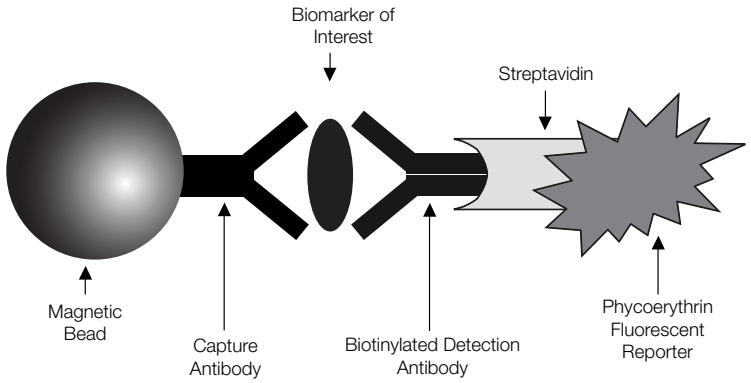


Fig. 1. Bio-Plex sandwich immunoassay.

Data Acquisition and Analysis

Data from the reactions are acquired using a Bio-Plex System or similar Luminex 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 a 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 Required

Bio-Plex Pro Rat Diabetes Assays are offered in singleplex sets. These sets can be mixed into a multiplex kit by the user. Singeplex kits require the purchase of Bio-Plex Pro Reagent Kit V (catalog #12002798) and Bio-Plex Pro Rat Standards (#171NZ0001).

Storage and Stability

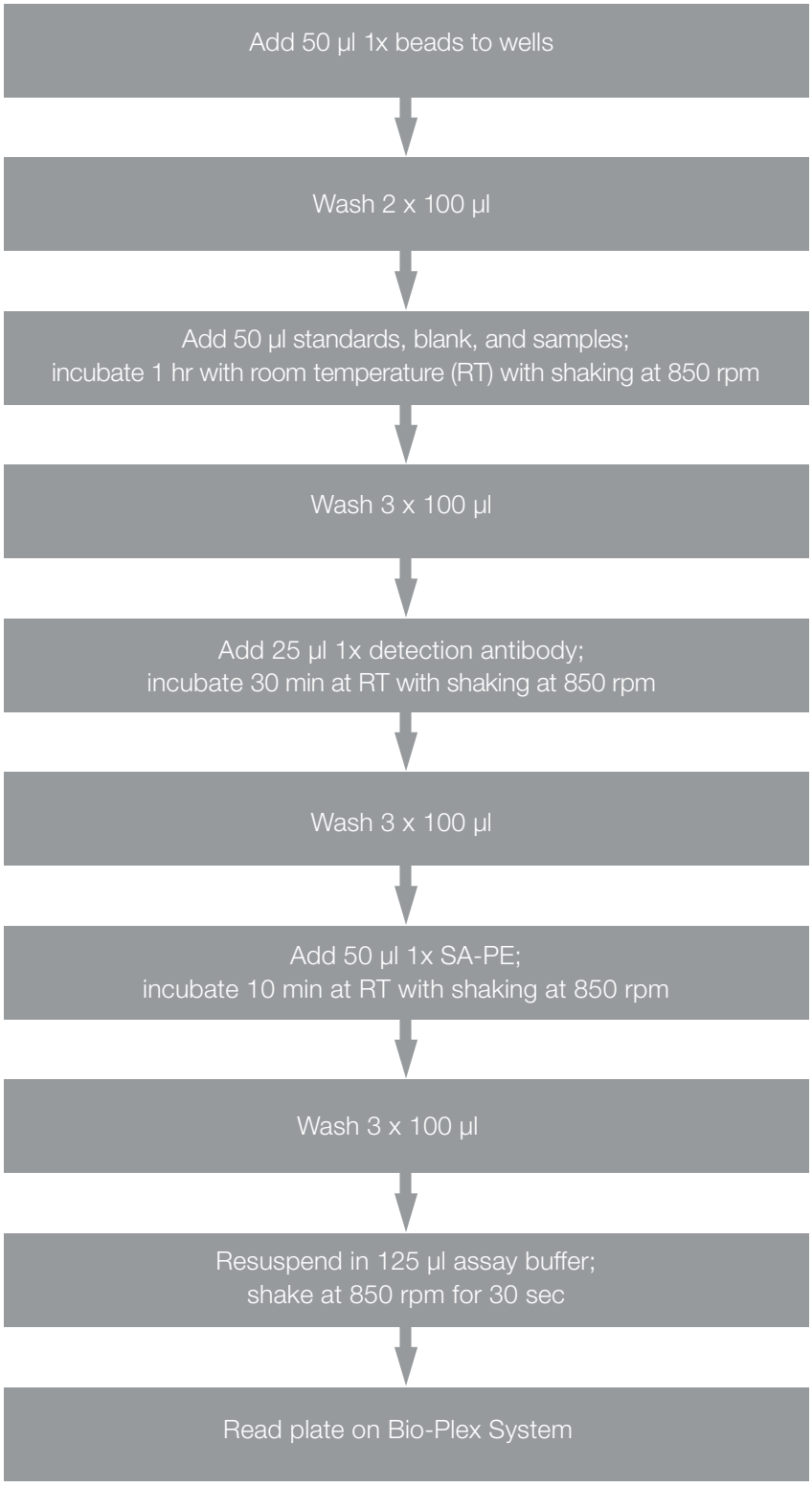
Kit contents should be stored at 4°C and never frozen. Coupled magnetic beads and SA-PE should be stored in the dark. All components are guaranteed for a minimum of 6 months from the date of purchase when stored as specified. Recommended materials are included in Table 1.

Table 1. Recommended materials.*

Item	Ordering Information
Bio-Plex Pro Rat Diabetes Assays Quick Guide	Bulletin #10000101541 (download at bio-rad.com/bio-plex)
Bio-Plex 200 System or Luminex System with HTF	Bio-Rad catalog #171000205
Bio-Plex Validation Kit Run the validation kit monthly to ensure optimal performance of fluidics and optics systems	Bio-Rad catalog #171203001
Bio-Plex Calibration Kit 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, 40 x 96-well For magnetic separation on the Bio-Plex Pro Wash Station	Bio-Rad catalog #171025001
Microtiter plate shaker IKA MTS 2/4 Shaker for 2 or 4 microplates or Barnstead/Lab-Line Model 4625 Plate Shaker (or equivalent capable of 300–1,100 rpm)	IKA catalog #3208000 or VWR catalog #57019600
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
Pall Life Science Acrodisc Syringe Filter, 25 mm PF (0.8/0.2 µm Supor Membrane)	Pall Life Sciences catalog #4187
Titertube Micro Test Tubes For preparing replicate standards, samples, and controls prior to loading the plate	Bio-Rad catalog #2239390

* **Other materials:** 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.

Assay Workflow



Important Considerations

Instruments and Software

The diabetes assays described in this manual are compatible with all currently available Luminex 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 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 Rat Diabetes Assays Quick Guide (bulletin #10000101541), which can be used to prepare and run a full 1 x 96-well assay plate. Users can also download a copy at bio-rad.com/bio-plex.

Bead Regions

Bead regions for all analytes are listed in the Read Plate section (9. Read Plate).

Detailed Instructions

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 21 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 SA-PE needed. Use the Calculation Worksheet on page 22.

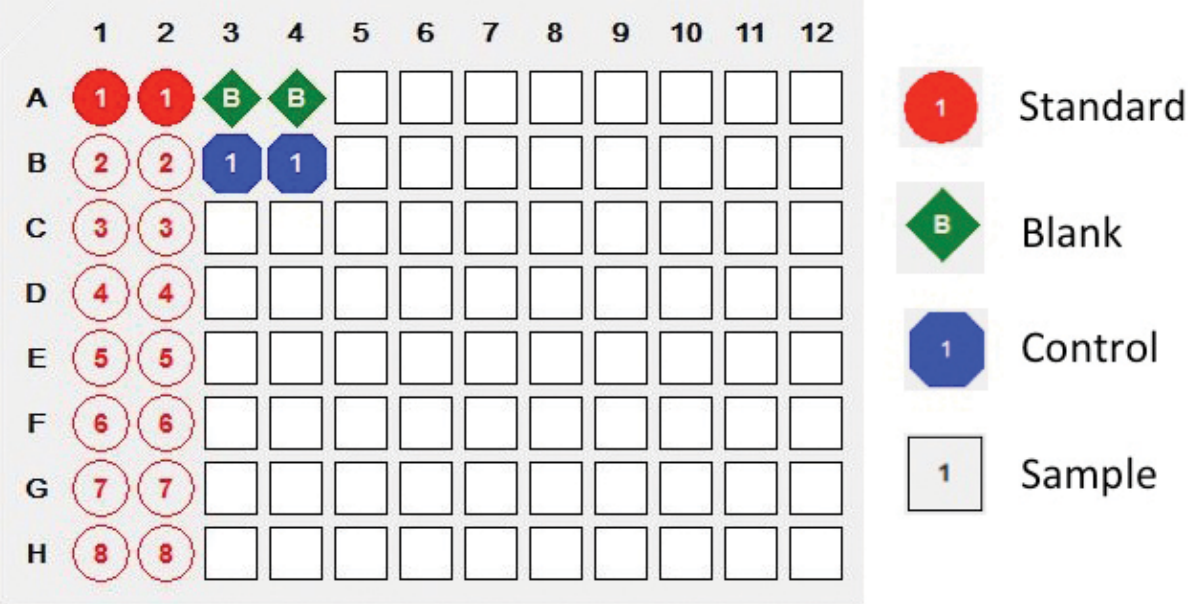


Fig. 2. Suggested plate layout. For detailed instructions on plate formatting for Bio-Plex Manager Software, see section 9. Read Plate.

2. Prepare Instrument

Start up and calibrate the Bio-Plex 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 a MAGPIX Reader, consult its 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 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.
4. 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, warmup, and calibration can be performed together by selecting the Start up and calibrate icon.

3. Prepare Wash Method

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).

4. 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 (Table 2)
- 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 Titertube Micro Test Tubes
- Do not freeze diluted samples

Table 2. Summary of recommended sample diluents and dilution factors.

Sample Type	Diluent	Add Bovine Serum Albumin (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

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. Protease inhibitors may be added to samples at the time of blood collection (see protocol in the appendix).

Plasma

K_2EDTA-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.

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. Dilute plasma fourfold (1:4) by adding 1 volume of sample to 3 volumes of Bio-Plex Sample Diluent (for example: 40 µl sample + 120 µl 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.

5. 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

Selecting a Diluent for Standards

Refer to Table 3 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 3. 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

RP1 (PMT) Setting for Standard Curves

The Bio-Plex 200 and 3D Systems have two RP1 (PMT or photomultiplier tube) setting options (Table 4).

Table 4. Overview of PMT setting options for Bio-Plex Systems.

Instrument	RP1 (PMT)
Bio-Plex 200*	Low, high
Bio-Plex 3D*	Standard, enhanced

***** Or similar Luminex Systems. For optimal results, we recommend setting the Bio-Plex 200 to “Run at high RP1 target” and the Bio-Plex 3D to “Enhanced” RP1. 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.
3. Gently **vortex** the reconstituted standard for **5 sec**, and then **incubate** on ice for **30 min**. Be consistent with the incubation time in every assay to ensure best results.

Prepare Diabetes Standard Dilution Series from a Single Standards 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.

1. Label nine 1.5 ml polypropylene tubes S1 through S8 and Blank.
2. Add the specified volume of standard diluent to each tube (Figure 3).
3. **Vortex** the reconstituted standards gently for **5 sec** before removing any volume. Add **128 µl** into the S1 tube containing **72 µl** of standard diluent. **Vortex** at medium speed for **5 sec**, then use a new pipet tip to transfer **50 µl** from S1 tube to S2 tube.
4. 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.

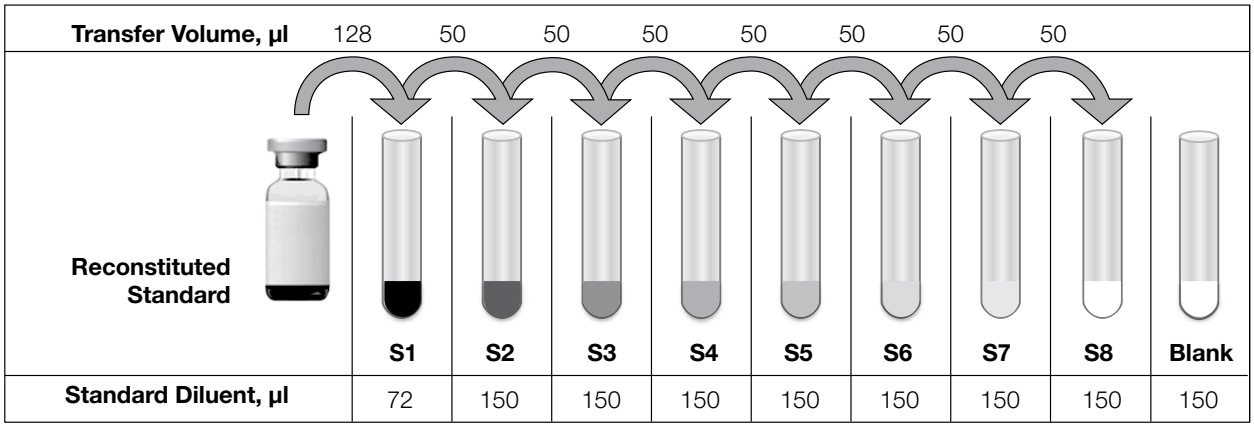


Fig. 3. Preparing a fourfold dilution series of diabetes standards.

6. Prepare Coupled Beads

Instructions are provided for diluting the 20x coupled beads to a 1x concentration.

Note: When using 10-pack reagents, ensure that only the required volumes of coupled beads, detection antibodies, SA-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 diluting coupled beads, diluting SA-PE, and resuspending the beads) into a 50 ml reservoir.

1. Table 5 shows 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 µl (20x stock) or 5.0 µl (10x 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.

Table 5 summarizes volumes required for preparing 1x beads from a single 20x stock.

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.

Table 5. Premixed panel or one singleplex assay. Preparing 1x coupled beads from 20x stock (includes 20% excess volume).

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

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

7. Prepare 1x Wash Buffer

Mix 10x stock by inversion to ensure all salts are in solution, then dilute 1 part 10x wash buffer (60 ml) with 9 parts dH_2O (540 ml).

8. 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 on a shaker at **850 ± 50 rpm**. Cover the plate with sealing tape and protect from light with aluminum foil
- Table 6 summarizes wash options and protocols

Table 6. 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.

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

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

1. Cover unused wells with sealing tape.
2. **Vortex** the diluted (1x) coupled beads for **30 sec** at medium speed. Pour the diluted coupled beads 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.
3. **Wash the plate two times** with **100 µl** 1x Bio-Plex Wash Buffer using the wash method of choice.
4. **Gently vortex** the diluted standards, blanks, samples, and controls (if applicable) for **5 sec**. Transfer **50 µl** to each well of the assay plate, changing the pipet tip after every volume transfer.
5. **Cover** plate with a new sheet of sealing tape and protect from light with aluminum foil. **Incubate** on shaker at **850 ± 50 rpm** for **1 hr** at room temperature (RT).

   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 (20x) to a 1x concentration.

1. While the samples are incubating, 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 150 ml of 20x detection antibody into 2,850 ml of detection antibody diluent in a 15 ml tube.
5. After incubating the beads, samples, standards, and blank, slowly remove and discard the sealing tape.
6. **Wash the plate three times** with **100 µl** 1x wash buffer.
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** plate with sealing tape and protect from light with aluminum foil. **Incubate** on shaker at **850 ± 50 rpm** for **30 min** at room temperature.

Prepare and Add Streptavidin-PE (SA-PE)

1. While the detection antibodies are incubating, use Table 7 to calculate the volume of SA-PE (100x) 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 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 (100x stock) adjusted to a final volume of 50 µl in assay buffer.

Table 7 shows an example calculation.

Table 7. Preparing 1x SA-PE from 100x stock (includes 25% excess volume).

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

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

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 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 ± 50 rpm** for **10 min** at room temperature.
9. After the SA-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 then slowly remove the sealing tape.
12. Ensure that the plate cover has been removed before placing the plate on the reader. Read the plate using the settings in Table 8.

    Note: Reading at alternative PMT settings on the Bio-Plex 200 or Bio-Plex 3D System requires validation by the end user to ensure that results meet the user's acceptance criteria.

Table 8. Read the plate using the appropriate instrument settings.

Instrument	RPI (PMT)	DD Gates	Bead Events
Bio-Plex 200*	High	5,000 (low), 25,000 (high)	50
Bio-Plex 3D*	Standard	Select MagPlex beads	50

* Or similar Luminex System.

9. Read Plate

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

Prepare Protocol in Bio-Plex Manager Software v6.0 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 **Add Panel** in the Select Analytes toolbar. Enter a new panel name. Select **Bio-Plex Pro Assay Magnetic** from the assay dropdown menu. If using Bio-Plex Manager version 5.0 or lower, select **MagPlex** from the assay dropdown menu.
   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 9.
   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.

      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 9. Bead regions for available Bio-Plex Pro Rat Diabetes Assays.

Analyte	Bead Region
Ghrelin	64
GLP-1	62
Glucagon	63
Leptin	65
PAI-1	61

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 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].

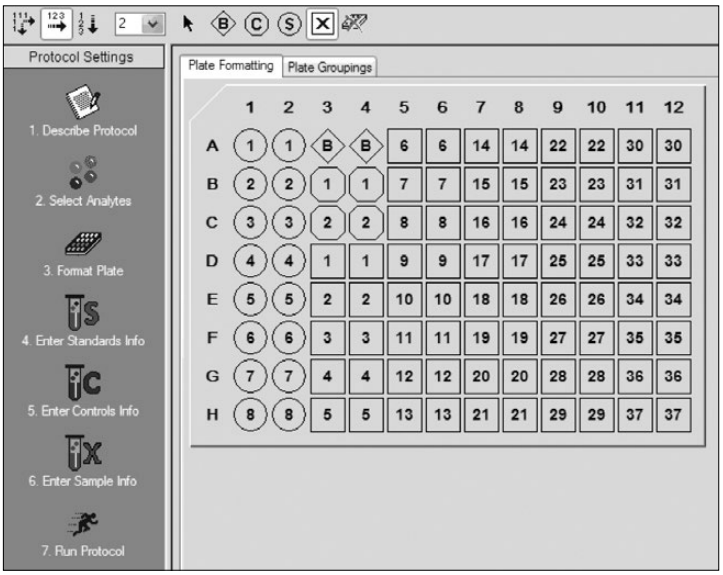


Fig. 4. Plate formatting.

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 µl**, 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 ± 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 µl** assay buffer to each well. Cover the filter plate with a new sheet of sealing tape. Shake the plate at **850 ± 50 rpm** for **30 sec**. Slowly remove the sealing tape before placing the plate on the plate reader.

4. Repeat the **Acquire Data** steps to reacquire data. The data acquired should be similar to those acquired initially; however, the acquisition time will be extended because the wells will 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.

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 whether the array reader is functioning properly.

Possible Causes	Possible Solutions
High Inter-Assay CV	
Standards were not reconstituted consistently between assays	Incubate the reconstituted standards for 30 min on ice. Always be consistent with the incubation time and temperature.
Reconstituted standards and diluted samples were not stored properly	Reconstituted standards and diluted samples should be prepared on ice as instructed. Prior to plating, the reconstituted standards and diluted samples should be equilibrated to room temperature.
High Intra-Assay CV	
Improper pipetting technique	Pipet carefully when adding standards, 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.
Bio-Plex Wash Station: insufficient washing due to clogged pins	Clean dispensing pins with the thicker of the two cleaning needles provided with washer. Perform regular rinses to minimize salt buildup.
Low Bead Count	
Miscalculation of bead dilution	Check your calculations and be careful to add the correct volumes.
Beads clumped in multiplex bead stock tube	Vortex for 30 sec at medium speed before aliquoting beads.
Reader is clogged	Refer to the troubleshooting guide in the Bio-Plex 200 System Hardware Instruction Manual (bulletin #10005042).

Possible Causes	Possible Solutions
Low Signal or Poor Sensitivity	
Standards reconstituted incorrectly	Follow the standard preparation instructions carefully.
Detection antibody or SA-PE diluted incorrectly	Check your calculations and be careful to add the correct volumes.
High Background Signal	
Incorrect buffer was used (for example, assay buffer used to dilute standards)	Use standard diluent or diluent similar to final sample matrix 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.
Improper pipetting technique	Pipet carefully when adding standards, samples, detection antibodies, and SA-PE, especially when using a multichannel pipet. Use a calibrated pipet. Change pipet tip after every volume transfer.
Impact of Sample Matrix	
Poor precision in serum and plasma sample measurements	Check whether 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

- DPP IV inhibitor (Sigma-Aldrich, Inc., K4264, molecular weight: 370.24): store at 4ºC
- Aprotinin (Sigma-Aldrich, A3428, 3–8 TIU/mg or 3,900–10,400 KIU/mg): store at 4ºC

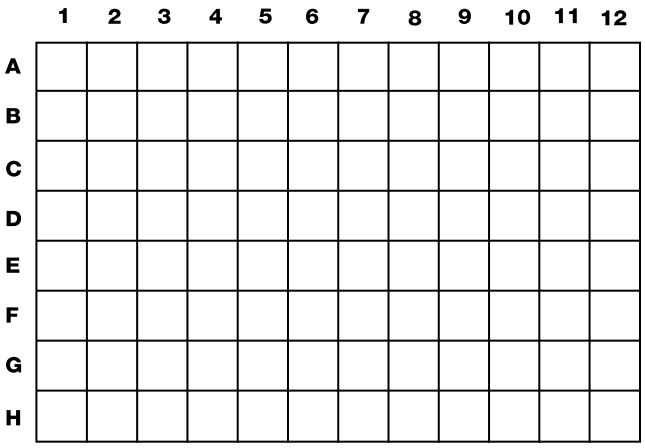
Preparation of Protease Inhibitors Stock Solution

1. Preparation of 10 mM DPP IV inhibitor solution:
   a. Weigh 18.5 mg of DPP IV inhibitor.
   b. Dissolve completely in 5 ml of 0.9% NaCl.
   c. Aliquot and store at –20ºC. 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 –20ºC. Avoid repeated freeze/thaw cycles.

Blood Sample Preparation

1. Collect whole blood in plasma collection tubes.
2. Add 10 µl of 10 mM DPP IV inhibitor per 1 ml of whole blood for a final concentration of 100 µM.
3. Add 10 µl 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



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.

a. Each well requires 50 µl of coupled beads (1x): _____ (1) x 50 µl = _____ (2) µl

b. Include 20% excess to ensure enough volume: _____ (2) µl x 0.20 = _____ (3) µl

c. Total volume of 1x coupled beads: _____ (2) µl + _____ (3) µl = _____ (4) µl

d. Volume of 20x coupled beads required: _____ (4) µl/20 = _____ (5) µl

e. Volume of assay buffer required: _____ (4) µl – _____ (5) µl = _____ (6) µl

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

a. Each well requires 25 µl detection antibodies (1x): _____ (1) x 25 µl = _____ (7) µl

b. Include 25% excess to ensure enough volume: _____ (7) µl x 0.25 = _____ (8) µl

c. Total volume of 1x detection antibodies: _____ (7) µl + _____ (8) µl = _____ (9) µl

d. Volume of 20x detection antibodies required: _____ (9) µl/20 = _____ (10) µl

e. Volume of detection antibody diluent required: _____ (9) µl – _____ (10) µl = _____ (11) µl

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

a. Each well requires 50 µl SA-PE (1x): _____ (1) x 50 µl = _____ (10) µl

b. Include 25% excess to ensure enough volume: _____ (10) µl x 0.25 = _____ (11) µl

c. Total volume of 1x SA-PE: _____ (10) µl + _____ (11) µl = _____ (12) µl

d. Volume of 100x SA-PE required: _____ (12) µl/100 = _____ (13) µl

e. Volume of assay buffer required: _____ (12) µl – _____ (13) µl = _____ (14) µl

Safety Considerations

Eye protection and gloves are recommended when using these products.

Consult the safety data sheet (SDS) 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 according to the U.S. Department of Health and Human Services publication, Biosafety in Microbiological and Biomedical Laboratories, 5th Edition (Centers for Disease Control and Prevention No. 21-1112, 2009).

Ordering Information

Catalog #	Description
171L7001M	**Bio-Plex Pro Rat Diabetes Ghrelin Set**
171L7003M	**Bio-Plex Pro Rat Diabetes GLP-1 Set**
171L7004M	**Bio-Plex Pro Rat Diabetes Glucagon Set**
171L7006M	**Bio-Plex Pro Rat Diabetes Leptin Set**
171L7007M	**Bio-Plex Pro Rat Diabetes PAI-1 Set**

Bio-Plex Express Assays (you mix)

Bio-Rad provides a fast and economical custom assay service using the Bio-Plex Assay Builder, **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.

Visit **bio-rad.com/bio-plexprodiabetes** for more information.

Bio-Rad Laboratories, Inc.

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