

Bio-Plex Pro[™] RBM Apoptosis 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

Apoptosis refers to a genetically controlled process by which cells die following a programmed physiological state or pathological condition. The balance between pro- and anti-apoptotic processes is delicate, and dysregulation is observed in a wide range of pathological conditions (Rutledge 2002). Excessive apoptosis is implicated in neurodegenerative and autoimmune diseases, myocardial infarction, stroke, and viral infection. In contrast, insufficient apoptosis is linked to almost every known human cancer.

Cell death activation is thought to be regulated by the Bcl-2 family of proteins, which consists of both pro-apoptotic and anti-apoptotic members. At least 17 human proteins with Bcl-2 homology have already been identified. Although much has been studied about Bcl-2 family-mediated apoptosis, there is clearly more to discover, specifically the mechanism by which heterodimerization regulates both pro- and anti-apoptotic proteins (Rutledge 2002).

Apoptosis is induced by at least two distinct signaling pathways. The **extrinsic pathway** is triggered by cell signaling through death receptors such as Fas, followed by downstream activation of caspase-8 and caspase-3. The **intrinsic pathway** is triggered by cytotoxic stress, which leads to translocation of pro-apoptotic Bcl-2 proteins such as Bax and Bak to the mitochondrial membrane. This causes release of cytochrome C into the cytosol, where it promotes apoptosome formation and final activation of caspase-3.

Bio-Plex Pro™ RBM Apoptosis Assays

The Bio-Plex Pro RBM apoptosis assays enable researchers to quantify multiple intracellular proteins involved in the commitment, onset, and induction of apoptosis by the **intrinsic pathway**. Compatible sample types include cell lysates and tissue homogenates.

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.

References

Rutledge SE et al. (2002). A view to a kill: ligands for Bcl-2 family proteins. *Current Opinion in Chemical Biology* (6), 479-485.

Principle

Technology

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

- Fluorescently dyed magnetic 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 molecules in a single well of a 96-well microplate on the Bio-Plex® 3D system, up to 100 different molecules on the Bio-Plex® 200 system, and up to 50 different molecules on the Bio-Plex® MAGPIX™ system
- A dedicated plate reader. The Bio-Plex 200 and Bio-Plex 3D systems are flow cytometry–based instruments with two lasers and associated optics to measure the different molecules bound to the surface of the beads. In the Bio-Plex MAGPIX system, the 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

The Bio-Plex Pro™ RBM apoptosis 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.

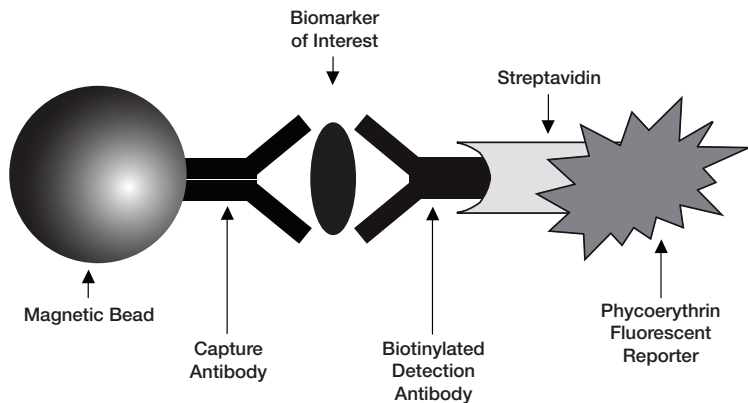


Fig. 1. Bio-Plex sandwich immunoassay.

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. The concentration of analyte bound to each bead is proportional to the MFI of reporter signal.

Kit Contents and Storage

The Bio-Plex Pro™ RBM apoptosis assays are available in a convenient kit format that includes assay, reagent, and diluent components in a single box (Table 1). All other recommended materials are listed in Table 2.

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

Component	Quantity	Volume	Volume after Reconstitution or Dilution
Capture beads (1x)	1 tube	1.4 ml	
Detection antibodies	1 vial	Lyophilized	4.8 ml
Standards mix	1 vial	Lyophilized	150 µl
Control 1 (high)	1 vial	Lyophilized	100 µl
Control 2 (low)	1 vial	Lyophilized	100 µl
Blocking buffer	1 vial	Lyophilized	1.5 ml
Standard diluent	1 vial	Lyophilized	1.0 ml
Assay buffer (10x)	1 bottle	60 ml	600 ml
Lysate dilution buffer (LDB)*	1 bottle	100 ml	
Cytosolic extraction buffer (CEB)*	1 bottle	60 ml	
Streptavidin-PE (10x)	1 tube	250 µl	2.5 ml
Assay plate (96-well flat bottom)	1 plate		
Plate seals	1 pack of 3		
Assay quick guide	1 sheet		
Product data sheet	1 sheet		

* Protease inhibitors not included.

Storage and Stability

Kit contents should be stored at 2–8°C and never frozen. Coupled magnetic capture 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® 200 system or Luminex system with HTF	Bio-Rad catalog #171-000205
Bio-Plex validation kit Note: Run the validation kit monthly to ensure optimal performance of fluidics and optics systems	Bio-Rad catalog #171-203001
Bio-Plex calibration kit Note: 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
Titertube® micro test tubes For preparing replicate standards, samples, and controls prior to loading the plate	Bio-Rad catalog #223-9390
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 WWR catalog #57019-600
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 with clear plastic lid and tray	Bio-Rad catalog #171-304502

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

Reconstitute lyophilized reagents, dilute assay buffer to 1x, prepare standards and samples

Add 10 μ l blocking buffer to all wells

Add 30 μ l standards, blank, samples, and controls to appropriate wells

Add 10 μ l 1x capture beads per well.
Incubate at 850 ± 50 rpm for 1 hr at RT

Wash 3x with 100 μ l assay buffer (1x)

Add 40 μ l reconstituted detection antibodies.
Incubate at 850 ± 50 rpm for 1 hr at RT

Do not aspirate after incubation

Add 20 μ l 1x streptavidin-PE.
Incubate at 850 ± 50 rpm for 30 min at RT

Wash 3x with 100 μ l assay buffer (1x)

Resuspend beads in 100 μ l assay buffer (1x).
Shake at 850 ± 50 rpm for 30 sec

Read plate on Bio-Plex system

Important Considerations

Instruments and Software

The 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 comes complete with a printed Bio-Plex Pro RBM Apoptosis Assays Quick Guide (bulletin #10033632), which can be used to set up and prepare a full 1 x 96-well assay plate. Users can also download a copy at www.bio-rad.com/bio-plex.

Bead Regions and Multiplexing Compatibility

- Bead regions for all analytes are listed in the Read Plate section
- Do not mix analytes between different apoptosis panels, or with other Bio-Plex assay panels or reagents

Detailed Instructions

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

1. Plan Plate Layout

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

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 standard diluent alone and be processed in the same manner as sample and standard wells. Bio-Plex Manager™ software automatically subtracts the assay blank (B) MFI value from all other assay wells.
3. User-specific controls, as well as the quality controls supplied in the kits, are assigned to wells in columns 3 and 4.
4. The remainder of the plate is available for samples.

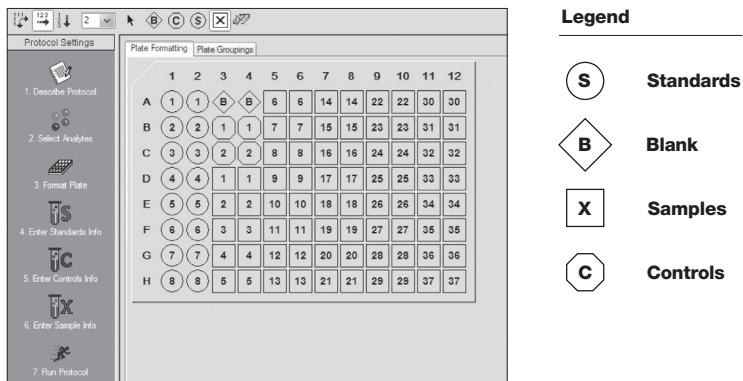


Fig. 2. Suggested plate layout. For detailed instructions on plate formatting in Bio-Plex Manager, see section Read Plate.

2. Prepare Instrument



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

Start up and calibrate the Bio-Plex® system with Bio-Plex Manager™ software prior to setting up the assay. The calibration kit should be run daily or before each use of the instrument to standardize the fluorescent signal. For instructions on using other xMAP system software packages, contact Bio-Rad Technical Support.


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


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

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

3. Prepare Wash Method

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

Table 3. Summary of compatible wash stations and plate types.

Wash Method	Wash Station	Assay Plate
Magnetic separation	Bio-Plex Pro Bio-Plex 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 should be primed before use. For more information, refer to the Bio-Plex Pro Wash Stations Quick Guide (bulletin #5826).

1. Install the appropriate plate carrier on the wash station.
2. Use the prime procedure to prime channel 1 with 1x assay 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, $100\ \mu\text{l}$ liquid should take 3–4 sec to clear the well. For more detailed instructions, refer to bulletin #10005042.

4. Prepare Reagents

1. Reconstitute the following lyophilized reagents in dH_2O before use according to the table below.

Table 4. Reagent volume.

Reagent	dH_2O Volume
Standards mix	$150\ \mu\text{l}$
Control 1	$100\ \mu\text{l}$
Control 2	$100\ \mu\text{l}$
Blocking buffer	$1.5\ \text{ml}$
Standard diluent	$1.0\ \text{ml}$
Detection antibodies	$4.8\ \text{ml}$

- a. Allow vial to sit at room temperature for a minimum of **5 min**, not to exceed **30 min**.
- b. Mix by **vortexing** at a medium setting.

2. Bring the **10x** assay buffer to room temperature (RT).
 - a. Mix by inversion to ensure all salts are in solution.
 - b. Prepare **1x** assay buffer — dilute **1 part 10x** assay buffer (60 ml) with **9 parts of dH₂O** (540 ml).

Test Sample Preparation

Thaw and dilute samples within **1 hr** of use. Remove any particulates by centrifugation or filtration. Avoid multiple freeze and thaw cycles.

Dilution of Standard (1:3 Serial Dilution)

This preparation provides sufficient volume to run duplicate standard dilution curves. Ensure that each new standard is mixed well by vortexing before proceeding to the next dilution. Change tips between each dilution.

Note: The product data sheet in each kit lists the most concentrated point on the standard curve (S1). Enter the values and units into Bio-Plex Manager™ software as instructed in the Read Plate section.

1. Label 8 polypropylene tubes **S1** through **S8**. Label an additional tube **Blank**.
2. Transfer the reconstituted standard into the tube labeled “**S1**.”
3. Add the appropriate amount of the standard diluent into the labeled tubes according to Figure 3 (this will be sufficient for duplicate standard curves and blanks).
4. Prepare working standards (**S2–S8**) by 1:3 (threefold) serial dilution. Transfer the appropriate volume of standard into each of the labeled tubes containing standard diluent as outlined above.
5. **Vortex** each standard at a medium setting for **5 sec** before proceeding with the next serial dilution. Change pipet tip at each dilution step.

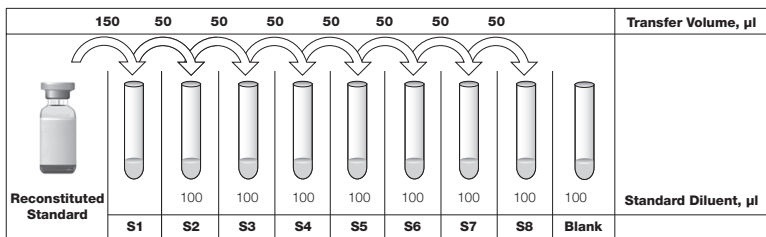


Fig. 3. Preparing a threefold dilution series with a single reconstituted standard.

5. Prepare Samples

The kit has sufficient reagents to run 19 fractionated samples (cytosolic and nuclear + mitochondrial fractions) in duplicate or 38 total homogenates in duplicate.

The following reagents are recommended for the preparation of fractionated samples and total homogenate:

- **Protease inhibitors** — complete, Mini; Roche Diagnostics GmbH (cat #11 836 153 001), or equivalent
- **Refrigerated centrifuge**
- **Tubes**
 - a. 1.5 ml conical microcentrifuge tubes, USA Scientific (cat #1415-2500)
 - b. 2 ml screw cap tubes, USA Scientific (cat #1420-8700)
- **Homogenizer**
 - a. **Pestle grinder** — Kontes grinder (cat #KT749540-0000), pestle (cat #KT749520-0090), and 1.5 ml Kontes grinder snap cap tube (cat #KT749520-0090), or equivalent

- b. **Homogenizer** — Bio-Gen Pro200 (cat #01-01200) with a 5 mm generator (cat #02-05075) or equivalent.
- c. **Dounce homogenizer** — Fisher (cat #06-434) using tight pestle, or equivalent.

Preparation of Cytosolic and Nuclear + Mitochondrial (N + M) Fractions

Cytosolic Fraction

1. Prepare the cytosolic extraction buffer (CEB) and lysate dilution buffer (LDB) by adding 1 tablet each of the protease inhibitors listed above to 10 ml of each buffer. Once the tablets are in solution, store the buffers at 4°C for up to 1 week or at -20°C for up to 3 months.
2. Pre-chill all tubes and keep samples on ice during sample preparation. CEB additions and homogenization of samples are summarized in Table 5.

Table 5. Summary of homogenization methods for cytosolic and Nuclear + Mitochondrial fractions.

Sample Type	Homogenization Method	Cytosolic Extraction Buffer (CEB) Additions
Tissue: 20–30 mg (750 µl total volume)	Homogenizer Pestle grinder	Add 100 µl, mince, add 650 µl, and homogenize Add 100 µl, grind, and add 650 µl
Tissue: <20 mg (ratio of 30 µl CEB to 1 mg of tissue)	Homogenizer Pestle grinder	Add 100 µl, mince, add remaining, and homogenize Add 100 µl, grind, and add remaining
Tissue: fine needle aspirate (350 µl total volume)	Homogenizer Pestle grinder	Add 100 µl, mince, add 250 µl, and homogenize Add 100 µl, grind, and add 250 µl
Cultured cells: 5 x 10 ⁶ to 2 x 10 ⁷ (750 µl total volume)	Dounce homogenizer	Resuspend in 750 µl, incubate 45–60 min on ice, and dounce with ~40 passes (will depend on cell line)

3. Homogenize the samples using the following guidelines (depending on sample type, additional time may be needed to ensure the sample is completely homogenized).
 - a. **Tissue sample with homogenizer** — add 100 µl CEB to the 2 ml screw cap tube containing tissue and mince the tissue 10–15x using micro scissors. Add the remaining volume of CEB and

homogenize the minced tissue for approximately 5 sec on medium power. Homogenized tissue samples should be free of large tissue fragments. If large fragments are visible in the sample, homogenize for an additional 5 sec. Any large fragments remaining after the additional 5 sec homogenization step should be removed before proceeding. Incubate processed samples on ice for 10 min. After incubation, transfer the sample to a prechilled 1.5 ml microcentrifuge tube.

- b. **Tissue sample with pestle grinder** — add 100 μ l CEB to the 1.5 ml pestle tube containing tissue sample. Grind the tissue for approximately 10 sec or until no large pieces remain. Then add the remaining volume of CEB, and remove any large fragments that did not homogenize. Incubate processed samples on ice for 10 min.

c. **Cultured Cells**

- i. Collect the cells (5×10^6 to 2×10^7) by centrifugation at 4°C for 5 min at 500 x g
- ii. Wash the cells in 1–2 ml of ice-cold PBS. Centrifuge at 4°C for 5 min at 500 x g. Remove supernatant.
- iii. Freeze the pellet at –80°C for a minimum of 15 min. Remove and place on ice.
- iv. Add 750 μ l CEB to the pellet, resuspend the cells by pipetting up and down 10–15x, and vortex for 5 sec.
- v. Incubate the suspension on ice for **45–60** min.
- vi. Homogenize on ice using approximately 40 passes with a Dounce homogenizer.

Note: The number of passes will depend on the cell line and may need to be adjusted by the end user for effective lysis.

- vii. Transfer the homogenate to a 1.5 ml microcentrifuge tube.

4. Centrifuge the homogenized sample at 4°C for 10 min at 10,000 x g.
5. Carefully remove the supernatant with a pipet and transfer to a pre-chilled 1.5 ml microcentrifuge tube. Measure the volume of cytosolic fraction removed, add an equal amount of LDB, and vortex. Label as cytosolic fraction.

6. Aliquot samples at appropriate volumes into labeled tubes and store at -80°C . Prepare an aliquot for protein determination. Cytosolic fractions may be stored on ice while the nuclear + mitochondrial fractions are prepared.

Nuclear + Mitochondrial Fraction

7. Wash the pellet from the cytosolic preparation by resuspending in $750\ \mu\text{l}$ of CEB and centrifuge at 4°C for 10 min at $10,000 \times g$.
8. Aspirate and discard the supernatant from the tube, being careful not to disturb the pellet.
9. Resuspend the pellet in LDB. The volume of LDB needed will be the same as the total volume of CEB used in Table 5. Be sure to completely disrupt the pellet by pipet mixing. Vortex for approximately 10 sec and incubate sample on ice for 15 min, vortexing for 10 sec at each 5 min interval.
10. Centrifuge the samples at 4°C for 10 min at $10,000 \times g$.
11. Aspirate the supernatant and transfer to a 1.5 ml snap cap tube labeled "N + M Fraction". Be sure not to disturb the pellet when aspirating the supernatant. Discard the pellet. Aliquot samples at appropriate volumes into labeled tubes, store at -80°C , and prepare an aliquot for protein determination.
12. Determine the protein concentration of the samples using the Bio-Rad protein assay reagent (Bio-Rad cat #500-0006) or equivalent and diluting samples 1:20 in PBS.
13. For sample analysis, bring samples to final concentrations of $250\ \mu\text{g}/\text{ml}$ for Apoptosis Panel 1 and $500\ \mu\text{g}/\text{ml}$ for Apoptosis Panels 2 and 3 by diluting with LDB. Additional dilutions may be required depending on sample.

Preparation of Total Extracts

1. Prepare the LDB by adding 1 tablet each of the protease inhibitors listed above to 10 ml of buffer. Once the tablets are in solution, store the buffer at 4°C for up to 1 week or at -20°C for up to 3 months.

- Prechill all tubes and keep samples on ice during sample preparation. LDB additions and homogenization of samples are summarized in Table 6.

Table 6. Summary of homogenization methods for total extracts.

Sample Type	Homogenization	
	Method	Lysate Dilution Buffer (LDB) Additions
Tissue sample: 20–30 mg (750 µl total volume)	Homogenizer Pestle grinder	Add 100 µl, mince, add 650 µl, and homogenize Add 100 µl, grind, and add 650 µl
Tissue sample: <20 mg (ratio of 30 µl LDB to 1 mg of tissue)	Homogenizer Pestle grinder	Add 100 µl, mince, add remaining, and homogenize Add 100 µl, grind, and add remaining
Tissue sample: fine needle aspirate (350 µl total volume)	Homogenizer Pestle grinder	Add 100 µl, mince, add 250 µl, and homogenize Add 100 µl, grind, and add 250 µl
Cultured cells: 5×10^6 to 2×10^7 (750 µl total volume)	Dounce homogenizer	Resuspend in 750 µl, incubate 30 min on ice, and dounce with ~40 passes (depends on cell line)

- Homogenize the samples using the following guidelines (depending on sample type, additional time may be needed to ensure the sample is completely homogenized).
 - Tissue sample with homogenizer** — add 100 µl LDB to the 2 ml screw cap tube containing tissue and mince the tissue 10–15x using micro scissors. Add the remaining volume of LDB and homogenize the minced tissue for approximately 5 sec on medium power. Homogenized tissue samples should be free of large tissue fragments. If large fragments are visible in the sample, homogenize for an additional 5 sec. Any large fragments remaining after the additional 5 sec homogenization step should be removed before proceeding. Incubate processed samples on ice for 10 min. After incubation, transfer the sample to a prechilled 1.5 ml microcentrifuge tube.
 - Tissue sample with pestle grinder** — add 100 µl LDB to the 1.5 ml pestle tube containing tissue sample. Grind the tissue for approximately 10 sec or until no large pieces remain. Then add the remaining volume of LDB, and remove any large tissue fragments that did not homogenize. Incubate the processed samples on ice for 10 min.

c. Cultured Cells

- i. Collect the cells (5×10^6 to 2×10^7) by centrifugation at 4°C for 5 min at 500 x g. Discard supernatant.
 - ii. Wash the cells in 1–2 ml of ice-cold PBS. Centrifuge at 4°C for 5 min at 500 x g. Discard supernatant.
 - iii. Add 750 μl LDB to the pellet, resuspend the cells by pipetting up and down 10–15x, and vortex for 5 sec.
 - iv. Incubate the suspension on ice for 30 min.
 - v. Homogenize using with a Dounce homogenizer.
 - vi. Transfer the homogenate to a 1.5 ml microcentrifuge tube.
4. Centrifuge the homogenized tissue at 4°C for 10 min at 10,000 x g.
 5. Carefully remove the supernatant with a pipet and transfer to a prechilled 1.5 ml microcentrifuge tube. Label as total extract.
 6. Aliquot samples at appropriate volumes into labeled tubes and store at -80°C . Prepare an aliquot for protein determination.
 7. For sample analysis, bring samples to final concentrations of 250 $\mu\text{g/ml}$ for Bio-Plex Pro RBM Apoptosis Panel 1 and 500 $\mu\text{g/ml}$ for Apoptosis Panels 2 and 3 by diluting with LDB. Additional dilutions may be required depending on sample.

6. Run the 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 protocol may result in low assay signal and assay variability
- Assay incubations are carried out in the dark on a shaker at **850 \pm 50 rpm**. Cover the plate with a plate seal and protect from light with aluminum foil

Table 7. 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
Sample incubation	MAG x3	VAC x3	3 x 100 μ l
SA-PE incubation			

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 μ l 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 plate seal. Avoid pressing down on the wells to prevent leaking from the bottom

Assay Protocol: Dispensing of Reagents

1. Add **10 μ l** of blocker to all wells of the plate.
2. Add **30 μ l** of the standard, control, or sample to the appropriate well of the plate.
3. **Vortex** the capture beads at medium speed for **10–20 sec**. Add **10 μ l** of the beads to all wells of the plate.
4. Cover plate with plate seal and protect from light with aluminum foil. Incubate on shaker at **850 \pm 50 rpm** for **1 hr** at RT.
5. Wash the plate three times with **100 μ l** 1x assay buffer.
6. **Vortex** the reconstituted detection antibodies at medium speed for **10–20 sec**. Add **40 μ l** to each well.

7. Cover and incubate at **850 ± 50 rpm**, as in Step 4, for **1 hr** at RT. **Do not aspirate after incubation.**
8. Prepare the required dilution of streptavidin-PE (SA-PE) as outlined in Table 8.

Note: Volumes in the table are for an entire 96-well plate. Smaller volumes can be prepared, provided that dilution ratios are maintained.

Table 8. SA-PE dilution.

SA-PE Dilution	Volume of SA-PE, μl	Volume of 1x Assay Buffer, μl	Total Volume, μl
1:10	225	2,025	2,250

9. Add **20 μ l** of diluted SA-PE to the required plate wells.
10. Cover and incubate at **850 ± 50 rpm**, as in Step 4, for **30 min** at RT.
11. Wash the plate three times with **100 μ l** 1x assay buffer.
12. After the final wash, resuspend the beads in **100 μ l** assay buffer. Cover plate as in Step 4 and shake the plate at **850 ± 50 rpm** for **30 sec**.
13. Remove the plate seal and read plate at low PMT (Bio-Plex[®] 200), standard PMT (Bio-Plex 3D), or default settings (Bio-Plex[®] MAGPIX[™]).

7. 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 on using other xMAP system software packages, contact Bio-Rad Technical Support or your regional Bio-Rad field applications specialist.

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

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

A protocol file specifies the analytes used in the reading, the plate wells to be read, sample information, the values of standards and controls, and instrument settings. Protocols may be obtained from within Bio-Plex Manager software version 6.1 or created from the File menu.

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.
 - 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. Refer to the bead regions in parentheses () listed on the peel-off label provided with the standards.

For reference, bead regions for the individual assays are listed in Table 8.

- 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 on **Rename Panel** and entering a new panel name.

Table 9. Bead regions for Bio-Plex Pro RBM apoptosis assays.

Panel 1	Bak (74)	Bax (27)	Lamin B (14)	Smac (19)	
Panel 2	Bad (73)	Bcl-2/Bax (42)	Bcl-xL (22)	Bim (12)	Mcl-1 (18)
Panel 3	Active caspase (57)	Bcl-xL/Bak (47)	Mcl-1/Bak (54)	Survivin (20)	

3. Click **Format Plate** and format the plate according to the plate layout template (located at the back of the manual) created for the assay. 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).

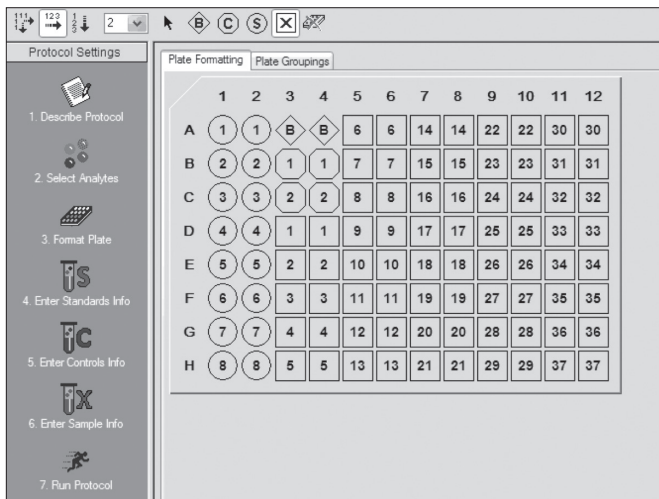


Fig. 4. Plate formatting.

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 listed in the product data sheet.
 - b. Enter a dilution factor of 3 and click **Calculate**. The concentrations for each standard point will be populated for all analytes in the table.
 - c. Optional: enter the lot number of the vial of standards into the **Standard Lot** box and click **Save**.

5. Click **Enter Controls Info**.
 - a. For user-specified controls, select an analyte from the dropdown menu, then enter a description and concentration. Repeat for each additional analyte in the assay.
 - b. For the kit controls supplied, format the appropriate wells as controls and enter descriptions, but leave the concentrations blank. Alternatively, the controls can be formatted as samples with clear descriptions such as “quality control.” In any case, the expected control ranges provided on the product data sheet are not entered into Bio-Plex Manager software version 6.1 and earlier.

6. Click **Enter Sample Info** — enter sample information and the appropriate dilution factor.

7. Click **Run Protocol** — confirm that the assay settings follow Table 10.

Table 10. Read the plate using the appropriate instrument settings.



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

* A similar Luminex-based system may be used.

- a. Confirm that data acquisition is set to **50 beads per region**.
- b. In Bio-Plex Manager software prior to 6.1, go to Advanced Settings, 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, 4.1.1, and 5.0, check **Override Gates** and set the DD gate values as indicated.

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.

Data Analysis

Quality Controls

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

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

Removing Outliers

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

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

Note: The instrument settings described below apply to Luminex 100/200 and FLEXMAP 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 μ l
3. Low PMT (Standard PMT).
4. Plate name: 96-well plate.
5. Analysis type: Quantitative; 5PL Curve Fit.
6. Number of standards: 8.

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

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

Select **Stds and Ctrl.**

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™ RBM 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 validate all the key functions of the array reader and to assist in determining whether or not 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 for the recommended time period. Always be consistent with the incubation time and temperature.

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 buffer during wash steps

Slow pipetting of samples and reagents across the plate

Low Bead Count

Beads clumped in multiplex bead stock tube

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 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.

Vortex for 30 sec at medium speed before aliquoting beads.

Possible Causes

Low Bead Count

Vacuum on for too long when aspirating buffer from wells

Reader is clogged

Low Signal or Poor Sensitivity

Standards reconstituted incorrectly

Detection antibody or streptavidin-PE prepared incorrectly

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

Possible Solutions

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

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.

Use standard diluent to dilute standards.

Do not add any antigens to the blank wells.

Follow the procedure incubation time precisely.

Possible Causes

Poor Recovery

Expired Bio-Plex reagents were used

Incorrect amounts of components were added

Microplate shaker set to an incorrect speed

High end saturation of the standard curve

Controls do not fall within expected ranges

Improper pipetting technique

Possible Solutions

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.

Make sure that correct shaker speed and incubation times are used. Remove S1 for data analysis if needed.

Make sure that the vial of controls is reconstituted at the same time as standards and in the correct diluent. Incubate for times indicated.

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.

Possible Causes

Impact of Sample Matrix

Negative MFI values in samples

Possible Solutions

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) MFI 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 MFI value if the blank's MFI value is greater than either the standard or the sample value. If this is undesirable, then reformat the blank wells as sample (X) or control (C) in the protocol or results file.

Plate Layout Template

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

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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The Bio-Plex suspension array system includes fluorescently labeled microspheres and instrumentation licensed to Bio-Rad Laboratories, Inc. by the Luminex Corporation.

Ordering Information

Detailed ordering information can be found at www.bio-rad.com/bio-plex.

Catalog #	Premixed 1 x 96-Well All-In-One Multiplex Kit
	Includes premixed magnetic capture beads, premixed detection antibodies, standards mix, 2-level controls, blocking buffer, standard diluent, lysate dilution buffer (LDB), cytosolic extraction buffer (CEB), 10x assay buffer, 10x streptavidin-PE, 96-well flat bottom plate, plate seals, and instructions.
171-WAR1CK	Bio-Plex Pro RBM Apoptosis Panel 1 , 1 x 96
171-WAR2CK	Bio-Plex Pro RBM Apoptosis Panel 2 , 1 x 96
171-WAR3CK	Bio-Plex Pro RBM Apoptosis Panel 3 , 1 x 96
Catalog #	Wash Stations and Accessories
300-34376	Bio-Plex Pro Wash Station , includes magnetic plate carrier, waste bottle, 2 buffer bottles
300-34377	Bio-Plex Pro II Wash Station , includes magnetic plate carrier, vacuum manifold plate carrier, waste bottle, 2 buffer bottles
171-020100	Bio-Plex Handheld Magnetic Washer , includes magnetic washer and adjustment hex tools for use in manual wash steps for all Bio-Plex magnetic assays
171-025001	Bio-Plex Pro Flat Bottom Plates , 40 x 96-well plates
171-304500	Bio-Plex Wash Buffer , 1.5 L
171-304502	Filter plate , pkg of 1, 96-well plate with clear plastic lid and tray, for Bio-Plex assays using the vacuum wash method, sealing tape not included
Catalog #	Software
171-001510	Bio-Plex Data Pro™ with Bio-Plex Manager Software (5 seats) , for multi-experiment analysis and advanced data visualization
171-001513	Bio-Plex Data Pro Software (5 seats) , for multi-experiment analysis and advanced data visualization
171-001523	Bio-Plex Data Pro Plus Software , contains all the features of Bio-Plex Data Pro software with added visualization, sharing, and analysis functionality
171-STND01	Bio-Plex Manager Software (1 seat) , for instrument data evaluation and optimization

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