Bio-Plex Pro™ Cell Signaling 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™ Cell Signaling Assays

Cell signaling is a complex process through which cells receive and respond to stimuli from the surrounding environment. For example, circulating cytokines and chemokines elicit a response from lymphocytes by binding to cell surface receptors and activating intracellular phosphoprotein signaling cascades. This turns on and off specific genes in the nucleus — thus regulating protein expression, cell growth, proliferation, motility, and survival (Chang and Karin 2001). Aberrant signaling can lead to serious pathologies including cancer, autoimmune diseases, cardiovascular disease, and neurological disorders. Understanding which cell types and signaling pathways are involved in a disease allows researchers to develop more precisely targeted therapies with better efficacy and safety.

Bio-Plex Pro cell signaling assays are magnetic bead-based immunoassays for the detection of intracellular phosphoproteins and total target proteins in cell and tissue lysates. The assays are available as singleplex sets, which researchers can combine on their own to make a multiplex assay, or as premixed, all-in-one, multiplex kits. Phosphoprotein detection and total target detection are carried out in separate wells of a 96-well assay plate, with just 1–10 µg of sample per well.

The assays have been optimized for exceptional sensitivity, high specificity and improved performance over western blotting. The use of magnetic (MagPlex) beads allows automation of wash steps on a Bio-Plex Pro or similar wash station, which greatly simplifies assay processing and improves assay precision.

For a complete list of all Bio-Plex Pro cell signaling targets, please visit **www.bio-rad.com/bio-plex**.

References

Chang L and Karin M (2001). Mammalian MAP kinase signalling cascades. Nature 410, 37–40.

Principle

Technology

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

- Fluorescently dyed microspheres (also called beads), each with a distinct color code or spectral address to permit discrimination of individual tests within a multiplex suspension. This allows simultaneous detection of 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
- 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

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, which serves as a fluorescent indicator or reporter.

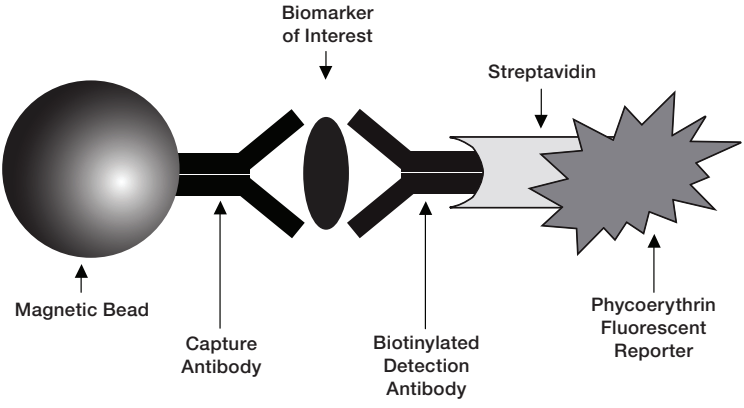


Fig. 1. Bio-Plex sandwich immunoassay.

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). The relative concentration of analyte bound to each bead is proportional to the MFI of the 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 Components and Storage

Table 1. Components required for a complete 1 x 96-well cell signaling assay. Note that custom x-Plex™ assays include a premixed (multiplex) set of beads and detection antibodies in an all-in-one kit.

Component	Quantity***
Singleplex* or Multiplex Set	
Coupled magnetic beads (20x)	1 tube
Detection antibodies (20x)	1 tube
Cell Signaling Reagent Kit (catalog #171-304006M)	
Cell wash buffer	1 bottle (50 ml)
Cell lysis buffer	1 bottle (50 ml)
Cell lysis factor QG	1 vial
Wash buffer	1 bottle (330 ml)
Detection antibody diluent	1 bottle (10 ml)
Resuspension buffer	1 bottle (40 ml)
Streptavidin-PE (100x)	1 tube
Flat bottom plate	1 plate
Sealing tape	1 pack of 4
Assay Quick Guide	1 booklet
Bio-Rad Cell Lysate Controls (optional)**	
Positive control (treated or untreated)	50 µg per vial
Negative control (phosphatase treated)	50 µg per vial

* Users can mix compatible singleplex sets to create their own multiplex assays.

** Refer to Table 3 to identify the appropriate controls for your phosphoprotein or total target of interest.

*** Volumes shown are approximate.

Storage and Stability

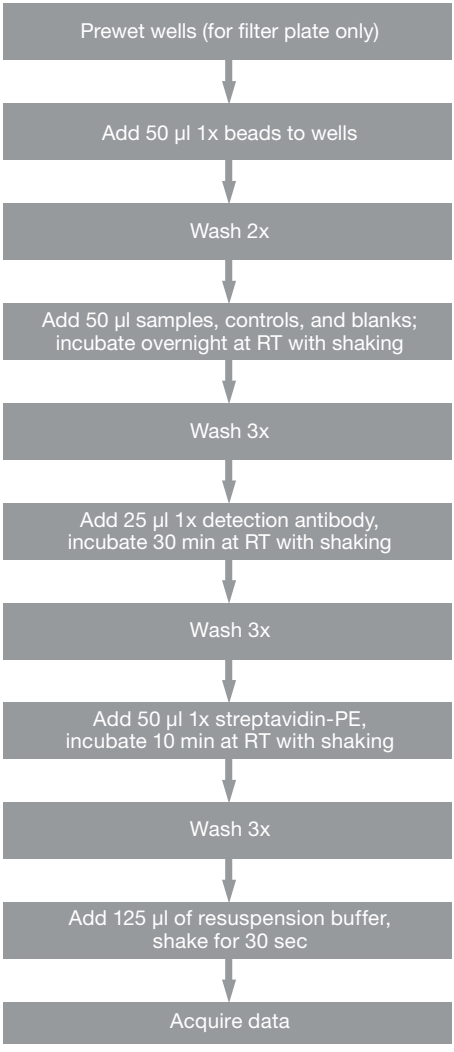
Kit contents should be stored at 4°C and never frozen. Coupled magnetic beads and streptavidin-PE should be stored in the dark. All components are guaranteed for a minimum of six months from the date of purchase when stored as specified.

Table 2. Recommended materials.

Item	Ordering Information
Bio-Plex Pro™ Assays Quick Guide 3	Bulletin #10024930 (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 monthly	Bio-Rad catalog #171-203001
Bio-Plex calibration kit Run 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 handheld magnetic washer For magnetic bead–based assays only	Bio-Rad catalog #171-020100
Bio-Plex Pro flat bottom plates (forty 96-well plates) 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
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: 32 mm PF syringe filter (0.45 µm Supor membrane)	Pall Life Sciences catalog #4654
***DC*™ protein assay kit II**	Bio-Rad catalog #500-0112
Phenylmethylsulfonyl fluoride (PMSF)	Sigma catalog #P7626
Dimethyl sulfoxide (DMSO)	Sigma catalog #D2650
Kontes tissue grinder	VWR catalog #KT885000-0002

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

Assay Workflow



Important Considerations

Assay Procedures

- Please pay close attention to vortexing, shaking, and incubation times and to Bio-Plex® reader PMT (RP1) setting, as these have been optimized specifically for the cell signaling assays
- For optimal performance, use only reagents specific for Bio-Plex Pro™ cell signaling assays. Reagents in other Bio-Plex assay panels have not been validated for use in the cell signaling assays
- Do not reuse diluted (1x) coupled beads, detection antibodies, or streptavidin-PE
- Wash as outlined in Table 5. Incomplete washes may cause assay variation
- If the data are not acquired immediately, the assay plate may be stored at 4°C for up to 24 hr protected from light

Assay Quick Guide

Each assay kit comes complete with a printed Bio-Plex Pro Assay Quick Guide (bulletin 10024930), 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 and Multiplexing Compatibility

- Bead regions for all analytes are listed in Table 12
- Compatible singleplex assays may be mixed to create a multiplex assay
- **Do not** mix phosphoprotein assays with corresponding total target assays (for example, phospho-Akt and total Akt)

Instruments and Software

The Bio-Plex Pro cell signaling assays described in this manual are compatible with all currently available Luminex-based life science research instruments. Assays can be read and analyzed with either Bio-Plex Manager™ software or Luminex xPONENT software (Section 5, Read the Plate, under Detailed Instructions).

Detailed Instructions

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

1. Prepare the Samples

Considerations

- The degree of phosphorylation of a given analyte is highly dependent on the cell type and cell stimulation or treatment conditions
- Cell lines may vary in their signaling responses to the same stimulation
- The suggested final protein concentration range in the assay is 3–200 µg/ml (0.15–10 µg per assay well) except for PI3K p85 (Tyr^{458}) which is 31–1,000 µg/ml (1.6–50 µg/well)
- Optimization of cell lysate concentration may be needed based on target protein expression levels
- Cell lysate should be clear of particulate matter before use

Cell Lysates

The Bio-Plex Pro™ cell signaling reagent kit (catalog #171-304006M) is required for preparing lysates derived from cell culture and tissue samples. Just before use, prepare an adequate volume of cell lysis buffer by adding PMSF and cell lysis factor QG.

- Prepare 500 mM PMSF by dissolving 0.436 g PMSF in 5 ml DMSO. (Store as aliquots at –20°C). Add PMSF to the cell lysis buffer at a final concentration of 2 mM
- Reconstitute cell lysis factor QG to 100x with 250 µl of diH_2O and vortex to mix. Add the reconstituted factor to the cell lysis buffer to a final 1x working concentration

Adherent Cells

1. Stop the treatment reaction by aspirating the culture medium and quickly rinsing the cells with ice-cold cell signaling **cell wash buffer** (bottle with the **blue cap**). The volume of buffer required is the same as the volume of aspirated cell culture medium. Keep the cells on ice during all steps when possible.
2. Completely remove the buffer before lysing the cells.
3. Immediately add the cell lysis buffer to the cells. The amount of lysing solution needed depends on the cell density in the culture vessel (for example, add 1.5–2 ml of lysis buffer to a 10 cm dish that is ~80% confluent).

Note: It may be necessary to lyse the samples with different volumes of cell lysis solution to obtain the specified protein concentration range.

4. Scrape the cells with a cell scraper, collect cell suspension into an appropriately sized tube and gently rock for 20 min at 4°C.
5. Perform either of the following to remove insoluble cellular particulates:
   - Centrifuge the cell lysate solution at 4,500 x g for 20 min at 4°C, and then filter the lysate using a 0.45 µm syringe filter
   - If the lysate volume is not adequate for filtration, centrifuge the lysate at 15,000 x g for 10 min at 4°C using a benchtop microcentrifuge
6. Collect the filtered lysate or supernatant after centrifugation.
7. Measure protein concentration using Bio-Rad's *DC*™ protein assay kit and if needed, adjust protein concentration with cell lysis buffer containing PMSF and cell lysis factor QG.
8. The suggested working protein concentration range for Bio-Plex® cell signaling assays is 3–200 µg/ml (0.15–10 µg per assay well).
9. Store the aliquoted lysates at –70°C until ready to use.

Suspension Cells

1. Collect cell suspension and pellet the cells by spinning at 1,000 x g for 5 min at 4°C.
2. Aspirate off cell culture medium completely.
3. Wash by resuspending the cells with ice-cold **cell wash buffer** (bottle with the **blue cap**).
4. Centrifuge the cells at 1,000 x g for 5 min at 4ºC.
5. Completely remove the buffer.
6. Immediately add the proper volume of cell lysis buffer and gently rock for 20 min at 4°C.
7. Remove insoluble cellular particulates as described in Adherent Cells step 5 above.
8. Follow Adherent Cells steps 6–9 above.

Tissue Samples

1. Cut the tissue into small pieces (~3 x 3 mm) for ease of handling and blood removal. If necessary, wash the tissue with ice-cold **cell wash buffer** (bottle with the **blue cap**) to completely remove all blood. Then transfer the tissue to a 2 ml tissue grinder.
2. Add an adequate volume of cell lysis solution and grind the tissue sample on ice using approximately 20 strokes.

Note: It may be necessary to lyse the samples with different volumes of cell lysis solution to obtain the specified protein concentration range.

3. Transfer the ground tissue to a clean microcentrifuge tube and freeze sample at –70°C. Freezing and thawing samples helps increase cell lysis effects.
4. Thaw the sample and sonicate on ice (for example, with a Sonifier 450: Duty Cycle = 40, Output = 1, Pulse Sonicating = 18x).
5. Remove insoluble cellular matter as in Adherent Cells step 5 above.
6. Follow Adherent Cells steps 6–9 above.

Bio-Rad Cell Lysate Controls

The positive and negative cell lysate controls are used for qualitative verification of assay performance. Refer to Table 3 to select the appropriate controls for your phosphoprotein or total target of interest.

Table 3. Selection guide for Bio-Rad cell lysate controls.

Phosphoprotein of Interest		Lysate Control	Catalog #
Akt (Ser^{473}) Akt (Thr^{308}) Erk1/2 (Thr^{202}/Tyr^{204}, Thr^{185}/Tyr^{187})	GSK-3α/β (Ser^{21}/Ser^{9}) MEK1 (Ser^{217}/Ser^{221})	EGF-treated HEK-293	171-YZ0001
ATF-2 (Thr^{71}) c-Jun (Ser^{63}) CREB (Ser^{133})	JNK (Thr^{183}/Tyr^{185}) p38 MAPK (Thr^{180}/Tyr^{182}) p53 (Ser^{15})	UV-treated HEK-293	171-YZ0009
Btk (Tyr^{223}) Lyn (Tyr^{507})	PI3K p85 (Tyr^{458}) Syk (Tyr^{352})	H_2O_2-treated Ramos	171-YZ0011
c-Abl (Tyr^{245})		Untreated K-562	171-YZT003
EGFR (Tyr^{1068})	EGFR (Tyr^{1173})	EGF-treated HeLa	171-YZ0002
HER-2 (Tyr^{1248}) HSP27 (Ser^{78})	p90 RSK (Ser^{380}) S6 ribosomal protein (Ser^{235}/Ser^{236})	EGF-treated SK-BR-3	171-YZ0003
IGF-1R (Tyr^{1131})	IR-β (Tyr^{1146})	IGF-1-treated HEK-293	171-YZ0005
IκB-α (Ser^{32}/Ser^{36}) NF-κB p65 (Ser^{536})	Smad2 (Ser^{465}/Ser^{467})	TNF-α–treated HeLa	171-YZ0008
p70 S6 Kinase (Thr^{421}/Ser^{424})	p70 S6 Kinase (Thr^{389})	NGF-β–treated PC12	171-YZ0006
BAD (Ser^{136}) IRS-1 (Ser^{636}/Ser^{639}) mTOR (Ser^{2448})	PDGFR-α (Tyr^{754}) PDGFR-β (Tyr^{751}) PTEN (Ser^{380})	PDGF-treated NIH3T3	171-YZ0007
Stat1 (Tyr^{701}) Stat3 (Ser^{727})	Stat3 (Tyr^{705})	IFNα-treated HeLa	171-YZ0004
Src (Tyr^{416})		Src-transfected NIH3T3	171-YZ0013
VEGFR-2 (Tyr^{1175})		VEGF-treated HUVEC	171-YZ0010
ZAP70 (Tyr^{319})		H_2O_2-treated Jurkat	171-YZ0012
Negative control for all phosphoprotein assays		Phosphatase-treated HeLa	171-YZB001

continues

Table 3. Selection guide for Bio-Rad cell lysate controls (continued).

Total Target		Lysate Control	Catalog #
Total Akt Total Erk 1/2 Total GSK-3β Total IκB-α Total JNK Total MEK1	Total mTOR Total p38 MAPK Total p70 S6 Kinase Total PTEN Total Smad2 Total IGF-1R	Untreated HeLa	171-YZT002
Total Btk		H_2O_2-treated Ramos	171-YZ0011
Total c-Jun Total CREB		Untreated HEK-293	171-YZT001
Total HER-2		EGF-treated SK-BR-3	171-YZ0003
Total Src		Src-transfected NIH3T3	171-YZ0013
Total ZAP-70		H_2O_2-treated Jurkat	171-YZ0012
Negative control for all total target assays		Detection antibody diluent	
House Keeping Protein			
Human GAPDH	β-Actin	Untreated HeLa	171-YZT002
Negative control for all total target assays		Detection antibody diluent	

2. Plan the Plate Layout

Prior to running the assay, determine the total number of wells in the experiment using the Plate Layout Template on page 30 or the Plate Formatting tab in Bio-Plex Manager. A suggested plate layout is shown in Figure 2, with all conditions in duplicate. Please note that the Bio-Plex 200 instrument reads the plate vertically.

1. Assign blank, negative control, positive control, and samples accordingly.

   Note: When designating blank using 

, Bio-Plex Manager software will automatically subtract the blank MFI value from all other assay wells.

2. Once the total number of wells is known, see Tables 6–9 or the Calculation Worksheet on pages 32–33 to determine the required volumes of beads, detection antibodies, and streptavidin-PE to use. Note that 20–25% excess volume is included in the calculations to compensate for transfer loss.

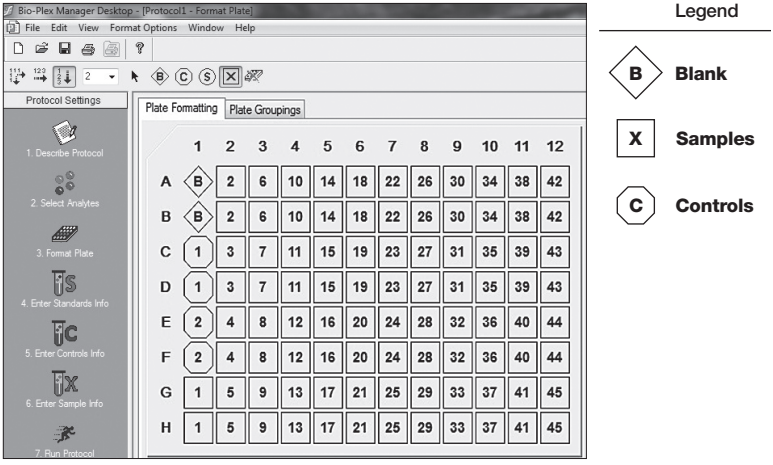


Fig. 2. Suggested plate layout. For detailed instructions on plate formatting in Bio-Plex Manager, see Section 5, Read the Plate.

3. Prepare the Wash Method

The cell signaling 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 handheld magnetic washer	Flat bottom plate
Vacuum filtration	Vacuum manifold (manual)	Filter plate

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. For detailed instructions, refer to the user guide (bulletin 10023087).

Setting up the Bio-Plex Pro

The wash station should be primed before use. See bulletin 5826.

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

Note:

- Before using the Bio-Plex Pro wash station, make sure to define/edit a program with the correct settings for cell signaling assays (Table 5)
- Existing cytokine assay programs: MAGx2, MAGx3, VACx2, and VACx3 should **not** be used

  Refer to the wash station instruction manual (bulletin 10013125), or contact Bio-Rad Technical Support for more information on defining, editing, or importing wash station programs.

Table 5. Summary of wash steps and settings. After each assay incubation step, perform the appropriate wash step as shown below.

Bio-Plex Pro Wash Station/Handheld Magnet/Vacuum Manifold*			
Wash Program Settings and Manual Wash Steps			
Assay Step	**# of Washes**	**Volume, µl**	**Magnetic Soak, min**
1. Add beads to plate	2	200	1
2. Sample incubation	3	200	1
3. Detection Ab incubation	3	200	1
4. SA-PE incubation	3	200	1

* No magnetic soak for vacuum filtration.

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 between –1 and –3" Hg. In general, 200 µl liquid should take 5–6 sec to clear the well. For detailed instructions, refer to bulletin 10005042.

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 200 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

4. Run the Assay

Considerations

- Bring all assay components and samples to room temperature before use
- Use calibrated pipets and pipet carefully, avoiding bubbles
- Assay incubations are carried out in the dark. Cover the plate with aluminum foil or otherwise protect from extended exposure to light

DAY 1

Prepare Samples and Controls

1. Thaw sample lysates and keep on ice (see Section 1, Prepare the Samples, for lysate preparation).
2. Reconstitute lyophilized cell lysate control with 250 µl of diH_2O, **vortex** for 5 sec to mix, and incubate at room temperature for 20 min. Protein concentration is now 200 µg/ml. Unused lysate can be stored at –20°C for 3 months.
3. Centrifuge all samples and lysate controls at 15,000 x g for 10 min at 4°C before dispensing to wells.

Prepare and Add Coupled Beads and Samples

4. Use Tables 6 and 7 or the Calculation Worksheet on pages 32–33 as a reference to calculate the volume of coupled beads and wash buffer needed.
5. Add the required volume of wash buffer to an appropriately-sized polypropylene tube. This will be used to dilute beads to 1x.
6. **Vortex** the 20x stock of coupled beads at mid 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.

7. Dilute coupled beads to 1x by pipetting the required volume into the tube containing wash buffer. **Vortex**.

   Each well of the assay requires 2.5 µl of the 20x stock adjusted to a final volume of 50 µl in wash buffer.

Note: To minimize volume loss, use a 200–300 µl capacity pipet to remove beads from the 20x stock tube. If necessary, perform the volume transfer in 2 steps. **Do not** use a 1,000 µl capacity pipet and/or wide bore pipet tip.

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

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

Table 6. Premixed panel or one singleplex assay.

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

Table 7. Mixing two singleplex assays.

# of Wells	20x Beads, µl Singleplex #1	20x Beads, µl Singleplex #2	Wash Buffer, µl	Total Volume, µl
96	288	288	5,184	5,760
48	144	144	2,592	2,880

9. Cover unused wells of the assay plate with sealing tape.
10. Prewet the filter plate. Skip this step if using a flat bottom plate.
    a. Prewet the wells with 200 µl wash buffer and remove the liquid by vacuum filtration. Dry the bottom of the filter plate thoroughly by blotting on a clean paper towel.
11. **Vortex** the diluted (1x) beads for 15 sec at medium speed. Transfer **50 µl** to each well of the assay plate.
12. **Wash the plate two times** with **200 µl** wash buffer according to your method of choice.

13. Gather the samples, Bio-Rad cell lysate controls, and blank. Use detection antibody diluent as the blank. Transfer **50 µl** of each sample or blank to the appropriate wells of the plate.

14. Cover with a new sheet of sealing tape and **incubate** in the dark **overnight (15–18 hr)** at room temperature with shaking.

Note: Fully resuspend the beads/sample mixture by vigorously shaking at **900–1,100 rpm** for **30 sec**. Slowly ramp up to speed to avoid splashing. Then turn down to **300–450 rpm** for the specified incubation time.

DAY 2

Prepare Instrument and Wash Method

15. Start up, warm up, and calibrate the Bio-Plex system as described in Section 5, Read the Plate. This may take up to 30 min.

16. Meanwhile bring all buffers and diluents to room temperature.

17. Prepare the wash method as described in Section 3, Prepare the Wash.

Prepare and Add Detection Antibodies

18. Use Tables 8 and 9 or the Calculation Worksheet on pages 32–33 to calculate the volume of detection antibodies and Bio-Plex detection antibody diluent needed. Detection antibodies should be prepared **10 min before use**.

19. Add the required volume of Bio-Plex detection antibody diluent to an appropriately sized polypropylene tube.

20. **Vortex** the 20x stock of detection antibodies for **15–20 sec** at medium speed, then perform a quick spin to collect the entire volume at the bottom of the tube.

21. Dilute detection antibodies to 1x by pipetting the required volume into the tube containing detection antibody diluent. **Vortex**.

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

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

Table 8. Premixed panel or one singleplex assay.

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

Table 9. Mixing two singleplex assays.

# of Wells	20x Detection Antibodies, µl Singleplex #1	20x Detection Antibodies, µl Singleplex #2	Detection Antibody Diluent, µl	Total Volume, µl
96	150	150	2,700	3,000
48	75	75	1,350	1,500

22. After the overnight incubation, slowly remove and discard the sealing tape.

23. **Wash the plate three times** with **200 µl** wash buffer according to your method of choice.

24. **Vortex** the diluted (1x) detection antibodies gently for **5 sec** and transfer **25 µl** to each well of the assay plate.

25. **Cover** with a new sheet of sealing tape and **incubate** in the dark for **30 min** at room temperature with shaking. Fully resuspend the beads/detection antibody mixture by shaking at **900–1,100 rpm** for **30 sec**. Then turn down to **300–450 rpm** for the specified incubation time.

Prepare and Add Streptavidin-PE (SA-PE)

26. While detection antibodies are incubating, use Table 10 or the Calculation Worksheet on pages 32–33 to calculate the volume of SA-PE and detection antibody diluent needed. SA-PE should be prepared **10 min before use**.

27. Add the required volume of detection antibody diluent to an appropriately sized polypropylene tube. This will be used to dilute SA-PE to 1x.

28. **Vortex** the 100x stock of SA-PE for **5 sec** at medium speed. Perform a quick spin to collect the entire volume at the bottom of the vial.

29. Dilute SA-PE to 1x by pipetting the required volume into the tube containing detection antibody diluent. **Vortex** and protect from light until ready to use.

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

Table 10. Preparing 1x SA-PE from 100x stock (includes 25% excess volume).

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

30. After detection antibody incubation, slowly remove and discard the sealing tape.

31. **Wash the plate three times** with **200 µl** wash buffer according to your method of choice.

32. **Vortex** the diluted (1x) SA-PE at medium speed for **5 sec** and transfer **50 µl** to each well of the assay plate.

33. **Cover** with a new sheet of sealing tape and **incubate** in the dark for **10 min** at room temperature with shaking. Fully resuspend the beads/SA-PE mixture by shaking at **900–1,100 rpm** for **30 sec**. Then turn down to **300–450 rpm** for the specified incubation time.

34. After the streptavidin-PE incubation step, slowly remove and discard the sealing tape.

35. **Wash the plate three times** with **200 µl** wash buffer according to your method of choice.

36. To resuspend beads for plate reading, add **125 µl** resuspension buffer to each well. Cover the plate with a new sheet of sealing tape and shake at **900–1,100 rpm** for **30 sec**.

36. Slowly remove the sealing tape and place the plate on the reader to acquire data.

Table 11. 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*	Enhanced	Select MagPlex beads	50
Bio-Plex MAGPIX*	N/A, use default instrument settings		

* Or similar Luminex-based system.

5. Read the 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 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. For instructions on using other xMAP system software packages, contact Bio-Rad Technical Support.

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

4. 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 the assays will be run at high RP1. Bio-Plex Manager version 6.1 and higher will automatically calibrate at both high and low RP1 settings although only the low RP1 value option is listed under CAL2.

5. 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. 

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

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

A protocol file specifies the analytes in the assay, the plate wells to be read, sample information, and instrument settings.

Bio-Plex Manager software versions 6.0 and higher contain protocols for most Bio-Plex assays. Choose from available protocols or create a new

protocol. To create a new protocol, select **File**, then **New** from the main menu. Locate and follow the steps under **Protocol Settings**.

6. Click **Describe Protocol** and enter information about the assay (optional).

7. Click **Select Analytes** and create a new panel. Visually confirm the selected analytes and proceed to step 8.

    a. Click the **Add Panel** button in the Select Analytes toolbar. Enter a new panel name. Select **Bio-Plex Pro Assay (Magnetic)** or **MagPlex Beads (Magnetic)** from the assay dropdown list. If using Bio-Plex Manager version 5.0 or lower, select **MagPlex** from the assay dropdown list.

    b. Click 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. Refer to the bead regions listed in Table 12.

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

8. Click **Format Plate**, and format the plate according to the plate layout created in Section 2, Plan the Plate Layout. To modify the plate layout, follow the steps below (see Figure 3).

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

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

Table 12. Bead regions for cell signaling assays.

Phosphoprotein Targets	Bead Region	Phosphoprotein Targets	Bead Region
Akt (Ser^{473})	75	mTOR (Ser^{2448})	46
Akt (Thr^{308})	75	NF-κB p65 (Ser^{536})	37
ATF-2 (Thr^{71})	20	p38 MAPK (Thr^{180}/Tyr^{182})	36
BAD (Ser^{136})	26	p53 (Ser^{15})	53
Btk (Tyr^{223})	39	p70 S6 Kinase (Thr^{389})	55
c-Abl (Tyr^{245})	45	p70 S6 Kinase (Thr^{421}/Ser^{424})	55
c-Jun (Ser^{63})	56	p90 RSK (Ser^{380})	35
CREB (Ser^{133})	19	PDGFR-α (Tyr^{754})	28
EGFR (Tyr^{1068})	44	PDGFR-β (Tyr^{751})	57
EGFR (Tyr^{1173})	44	PI3K p85 (Tyr^{458})	54
Erk1/2 (Thr^{202}/Tyr^{204}, Thr^{185}/Tyr^{187})	38	PTEN (Ser^{380})	22
GSK-3α/β (Ser^{21}/Ser^{9})	18	S6 ribosomal protein (Ser^{235}/Ser^{236})	74
HER-2 (Tyr^{1248})	30	Smad2 (Ser^{465}/Ser^{467})	14
HSP27 (Ser^{78})	51	Src (Tyr^{416})	42
IGF-IR (Tyr^{1131})	43	Stat1 (Tyr^{701})	61
IR-b (Tyr^{1146})	43	Stat3 (Ser^{727})	52
IRS-1 (Ser^{636}/Ser^{639})	76	Stat3 (Tyr^{705})	52
IκB-α (Ser^{32}/Ser^{36})	67	Syk (Tyr^{352})	65
JNK (Thr^{183}/Tyr^{185})	34	VEGFR-2 (Tyr^{1175})	29
Lyn (Tyr^{507})	33	ZAP-70 (Tyr^{319})	64
MEK1 (Ser^{217}/Ser^{221})	27		

Total Targets	Bead Region	Total Targets	Bead Region
Akt	75	JNK	34
Btk	39	MEK1	27
c-Jun	56	mTOR	46
CREB	19	p38 MAPK	36
Erk 1/2	38	p70 S6 Kinase	55
GSK3β	18	PTEN	22
HER-2	30	Smad2	14
IGF-1R	43	Src	42
IκBα	67	ZAP-70	64

Housekeeping Proteins	Bead Region	Housekeeping Proteins	Bead Region
Human GAPDH	21	β-Actin	47

9. Click **Enter Controls Info**, and 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.

   For Bio-Rad cell lysate controls, format the appropriate wells as controls, enter descriptions, but leave the concentrations blank. Alternatively, both blanks and controls can be formatted as samples with clear descriptions.

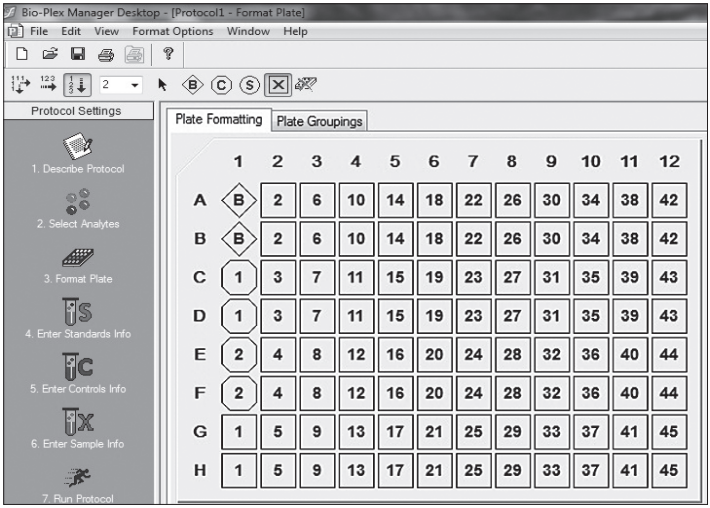


Fig. 3. Plate formatting.

10. Click **Enter Sample Info** and enter sample information and the appropriate dilution factor if any.

11. Click **Run Protocol** and confirm that the assay settings are correct.

    a. Refer to Table 11 for the recommended RP1 (PMT) setting. Protocols using alternative settings should be validated by the end user.

    b. Confirm that 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 doublet discriminator (DD) gates are set to **5,000 (Low)** and **25,000 (High)**. In Bio-Plex Manager software versions 4.0, 4.1, 4.1.1, and 5.0, check **Override Gates** and set the DD gate values as indicated.

    c. Select **Start**, name and save the .rbx file, and begin data acquisition. The Run Protocol pop-up screen will appear. Click **Eject/Retract** to eject the plate carrier.

Acquire Data

1. Shake the assay plate at **900–1,100 rpm** for **30 sec**, and visually inspect the plate to ensure that the assay wells are filled with buffer. Slowly remove the sealing tape before placing the plate on the plate carrier.

2. Click **Run Protocol**, and 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 is 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 unless the second run is saved under a different file name.

1. Check the wells from which data will be reacquired.

2. Aspirate the buffer with the wash method of choice, but do not perform wash step.

3. Add **100 µl** of resuspension buffer to each well. Cover the plate with a new sheet of sealing tape and shake plate at **900–1,100 rpm** for **30 sec**.

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.

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. This automatically sets the DD gates.
2. Volume = 50 µl.
3. Refer to Table 11 to select the appropriate PMT setting for your instrument.
4. Plate name: 96-well plate.
5. Analysis type: Qualitative.

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

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

Select **Stds and Ctrls**.

1. Enter descriptions 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. Suboptimal assay performance may also be due to the Bio-Plex® suspension array reader. To eliminate this possibility, use the Bio-Plex validation kit to assist in determining if the array reader is functioning properly.

Table 13. Troubleshooting guide.

Problem	Possible Causes	Possible Solutions
Low signals for experimental samples	Sample protein concentration too low or too high	Verify sample protein concentration is within assay range. Optimization of protein concentration may be needed based on targeted protein expression level
	Poor cell lysate quality	Prepare fresh lysate accordingly
Low signals for experimental samples and Bio-Rad cell lysate controls	Incorrect dilution of detection antibody or streptavidin-PE	Check the calculations and be careful to add the correct volumes
	Expired Bio-Plex reagents were used	Check that reagents have not expired. Use new or non-expired components
	Incorrect incubation temperature	Incubations should be at room temperature (20–22°C)
	Insufficient incubation time	Adhere to the recommended incubation time
	Beads lost	Use recommended magnetic washer settings with correct magnet soaking time

continues

Table 13. Troubleshooting guide (continued).

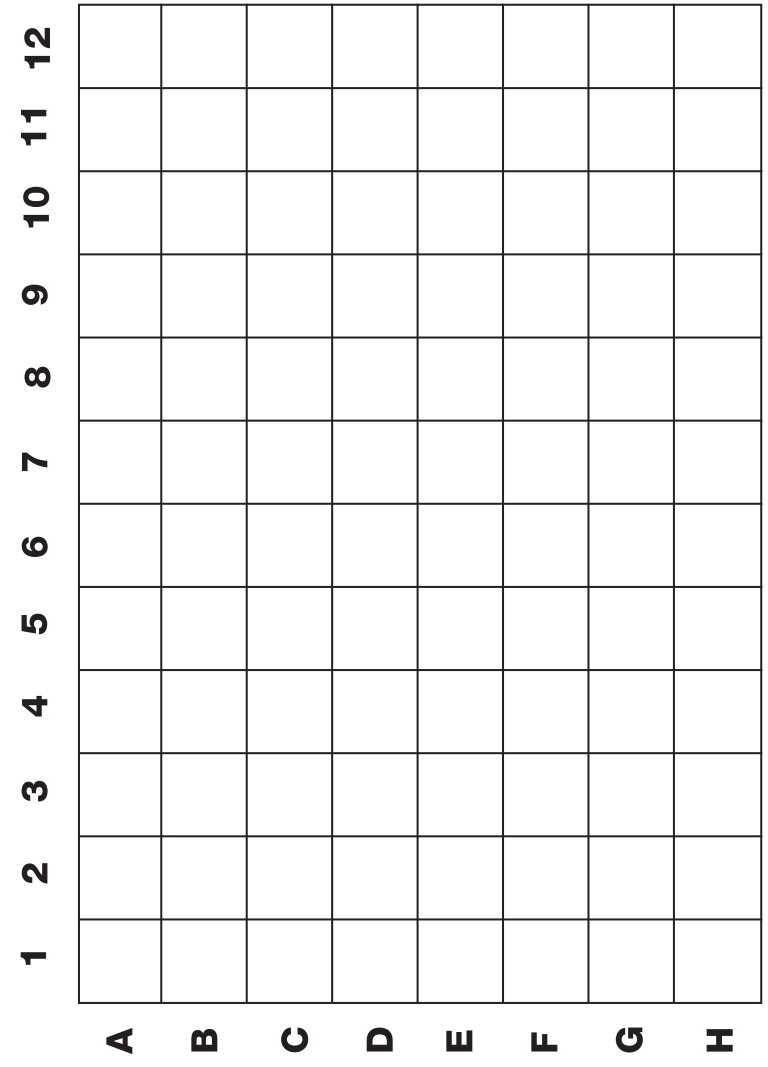
Problem	Possible Causes	Possible Solutions
Low bead count	Cell debris in lysate not cleared	Centrifuge at 15,000 x g for 10 min at 4°C to remove cellular debris
	Assay plate not shaken enough prior to reading	Shake plate at 900–1,100 rpm for 30 sec before data acquisition
	Clogged reader	Refer to the troubleshooting guide in the Bio-Plex system hardware instruction manual (bulletin 10005042)
	Miscalculation of bead dilution	Check the calculations and be careful to add the correct volumes
	Clumping of stock beads in vials	Vortex the stock vial at different angles for 30 sec at medium speed before aliquoting beads
	Vacuum setting too high during suction of assay plate	Adjust pressure to –1 to –3" Hg. Generally, 100 µl liquid should take 3–4 sec to clear from the well
	Reader needle height incorrect	Adjust the needle height to coincide with the plate type provided in the kit
	Beads lost	Use recommended magnetic washer settings with correct soaking time

continues

Table 13. Troubleshooting guide (continued).

Problem	Possible Causes	Possible Solutions
High background	Prolonged incubation of detection antibodies and/or streptavidin-PE	Follow the procedure incubation time precisely
	Wash steps performed incorrectly or insufficient washing volume	Perform washes as described in the assay instructions
High assay CV	Bottom of filter plate not dry	Dry the bottom of the filter plate with absorbent paper towel (preferably lint-free) to prevent cross-well contamination
	Contamination with wash buffer during wash steps	Be careful not to splash wash buffer from well to well. Filter wells completely to remove residual buffer if using filter plate. Reduce microplate shaking speed to minimize splashing
	Cell debris in lysate not cleared	Centrifuge at 15,000 x g for 10 min at 4°C to remove cellular debris
	Shaking speed too high during assay incubation	Fully resuspend bead mixture at 900–1,100 rpm for 30 sec, then turn down to 300–450 rpm for the specified incubation time
	Reagents and assay components not equilibrated to room temperature	Bring all reagents and assay components to room temperature prior to dispensing

Plate Layout Template



Calculation Worksheet

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

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** stock: _____ (4) µl/20 = _____ (5) µl

e. Volume of **wash 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 streptavidin-PE needed.

a. Each well requires 50 µl streptavidin-PE (1x): _____ (1) x 50 µl = _____ (12) µl

b. Include 25% excess to ensure enough volume: _____ (12) µl x 0.25 = _____ (13) µl

c. Total volume of 100x streptavidin-PE: _____ (12) µl + _____ (13) µl = _____ (14) µl

d. Volume of **100x streptavidin-PE** required: _____ (14) µl/100 = _____ (15) µl

e. Volume of **detection antibody diluent required**: _____ (14) µl – _____ (15) µl = _____ (16) µl

If **mixing two or more singleplex assays**, follow these instructions.

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) 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 bead stock required: _____ x _____ µl = _____ µl
5 6 7

g. Volume of **wash 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 100x streptavidin-PE: _____ µl + _____ µl = _____ µl
15 16 17

d. Volume of **100x streptavidin-PE** required: _____ µl/100 = _____ µl
17 18

e. Volume of **detection antibody diluent required**: _____ µl – _____ µl = _____ µl
17 18 19

Safety Considerations

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

Human source material. Treat as potentially infectious.
The lysates provided with Bio-Plex Pro™ cell signaling assays contain components of human origin. The components are known to contain an agent that requires handling at Biosafety Level 2 containment as defined by U.S. government publication, *Biosafety in Microbiological and Biomedical Laboratories* (Centers for Disease Control 1999). These agents have been associated with human disease. These components have not been screened for hepatitis B, human immunodeficiency viruses, or other adventitious agents. Handle Bio-Plex® phosphoprotein positive and negative controls as potentially biohazardous material under at least Biosafety Level 2 containment.

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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.

CST antibodies developed and validated for Bio-Plex cell signaling, phosphoprotein, and total target assays.

Ordering Information

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

Catalog #	Description
Individual Components and Accessories	
Various	**Bio-Plex Pro™ cell signaling singleplex sets**, 1 x 96-well
171-304006M	**Bio-Plex Pro cell signaling reagent kit**, 1 x 96-well
Various	**Bio-Rad cell lysate controls**, pkg of 1 vial
171-304515	**Bio-Plex Pro cell signaling wash buffer**, for 1 x 96-well assay, 330 ml
171-304502	**Filter plate**, 1 x 96-well with clear plastic lid and tray
Bio-Plex® x-Plex™ Assays (We Mix)	
Create a premium custom assay using the online Bio-Plex Assay Builder.	
Go to **www.bio-rad.com/bio-plex/assaybuilder** to select analytes of interest. Assays are supplied as premixed coupled beads and detection antibodies in the all-in-one kit format. Bio-Rad cell lysate controls are included.	

Bio-Rad
Laboratories, Inc.

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Web site *www.bio-rad.com* ***USA*** *800 424 6723* ***Australia*** *61 2 9914 2800*
Austria *01 877 89 01* ***Belgium*** *09 385 55 11* ***Brazil*** *55 11 5044 5699*
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New Zealand *64 9 415 2280* ***Norway*** *23 38 41 30*
Poland *48 22 331 99 99* ***Portugal*** *351 21 472 7700*
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