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Now Includes Protocols for Bio-Plex Phospho-Histone H3 Lysate Preparation!

Bio-Plex™ Phoshoprotein Detection

Instruction Manual

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Section 1
Introduction

Bio-Plex phosphoprotein assays and Bio-Plex total target assays are bead-based multiplex assays (Luminex xMAP technology) that detect the phosphorylation of proteins in lysates derived from cell culture or tissue samples. These 96-well plate-format assays allow profiling of the specific phosphorylation state of up to 100 different proteins using as few as two wells and as little as 25 µl of lysate per well. The Bio-Plex total target assay reports the abundance of the target protein in one well, while the Bio-Plex phosphoprotein assay reports the level of phosphorylation of that protein in a separate well. These instructions apply to both assays. For a current list of all Bio-Plex phosphoproteins and total target assays, visit **www.bio-rad.com/products/phosphoproteins**.

Available as Singleplex or Premixed Multiplex Assays
Bio-Plex phosphoprotein and total target assays are available as singleplex or premixed multiplex assays. Singleplex assays are designed to be flexible. They can be used individually to test for a single phosphoprotein at a time, or they can be combined to create a multiplex assay to test for a specific set of phosphoproteins in a single sample. Premixed multiplex assays are the more convenient format for repeat testing of a specific set of phosphoproteins. Both the coupled beads and the detection antibodies are premixed and quality tested at Bio-Rad. These assays are only available through Bio-Rad's online x-Plex assay service (**www.bio-rad.com/bio-plex/x-plex/**).

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Section 2
Principle

The principle of these 96-well plate-format, bead-based assays is similar to a capture sandwich immunoassay. An antibody directed against the desired target protein is covalently coupled to internally dyed beads. The coupled beads are allowed to react with a lysate sample containing target protein. After a series of washes to remove unbound protein, a biotinylated detection antibody specific for a different epitope is added to the reaction. The result is the formation of a sandwich of antibodies around the target protein. Streptavidin-phycoerythrin (streptavidin-PE) is then added to bind to the biotinylated detection antibodies on the bead surface.

Data from the reaction are then acquired using the Bio-Plex suspension array system (or Luminex 100 system), a dual-laser, flow-based microplate reader system. The contents of the well are drawn up into the reader. The lasers and associated optics detect the internal fluorescence of the individual dyed beads as well as the fluorescent signal on the bead surface. This identifies each assay and reports the level of target protein in the well. Intensity of fluorescence detected on the beads indicate the relative quantity of targeted proteins. A high-speed digital processor efficiently manages the data output, which is further analyzed and presented as fluorescence intensity on Bio-Plex Manager™ Software, the accompanying software package. If specific wells are identified for comparison, the ratio of fluorescence intensity between those wells is automatically calculated.

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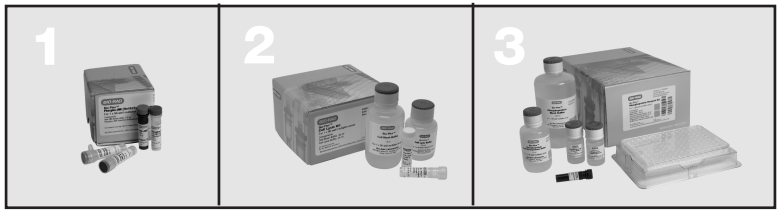
Bio-Plex phosphoprotein and total target assays feature CST antibodies, exclusively developed for Bio-Rad.

Section 3
Required and Recommended Materials

Required Materials

The following are required for phosphoprotein detection: 1) Bio-Plex phosphoprotein or total target assays to test for specific target proteins in lysate samples, 2) a Bio-Plex cell lysis kit to optimally lyse cell culture or tissue samples, and 3) a Bio-Plex phosphoprotein detection reagent kit in order to prepare the assays and acquire data on the instrument.



Item	Ordering Information
Bio-Plex Phosphoprotein Assays	Current list of assays can be found at www.bio-rad.com/products/phosphoproteins/
Bio-Plex Total Target Assays	Current list of assays can be found at www.bio-rad.com/products/phosphoproteins/
Bio-Plex Cell Lysis Kit*	Bio-Rad catalog #171-304011 (One 96-Well Assay) Bio-Rad catalog #171-304012 (Ten 96-Well Assays)
Bio-Plex Phosphoprotein Detection Reagent Kit*	Bio-Rad catalog #171-304004 (One 96-Well Assay) Bio-Rad catalog #171-304005 (Ten 96-Well Assays)
Other Materials Pipets and pipet tips, sterile distilled water, aluminum foil, absorbent paper towels, 1.5 ml microcentrifuge tubes, 15 ml culture tubes	

*Buffers contained in this kit have not been optimized for the use with Bio-Plex cytokine assays.

Recommended Materials

For optimal results, the use of the items listed below is recommended.

Item	Ordering Information
Bio-Plex Suspension Array System (or Luminex System)	Bio-Rad catalog #171-000205 (includes HTF)
Bio-Plex Validation Kit	Bio-Rad catalog #171-203001
Bio-Plex Calibration Kit	Bio-Rad catalog #171-203060
***DC* Protein Assay Kit I**	Bio-Rad catalog #500-0111
Microtiter Plate Shaker IKA-Schuttler MTS-4 shaker for 4 microplates or Lab-Line Model 4625 Plate Shaker (or equivalent, capable of 300–1,100 rpm)	IKA catalog #3208000
Filter Plate Vacuum Apparatus Millipore MultiScreen vacuum manifold or Bio-Rad Aurum™ vacuum manifold **IMPORTANT**: The use of filter plate manifolds other than the one specified may result in diminished assay performance; see section 6 for instructions specific to this assay	Millipore catalog #MAVM0960R Bio-Rad catalog #732-6470
Vortexer VWR brand mini-vortexer	VWR catalog #58816-121
Reagent Reservoir Corning, Inc. Costar 50 ml reagent reservoir 4870	Bio-Rad catalog #224-4872
Phenylmethylsulfonyl Fluoride (PMSF)	Sigma catalog #P7626
Dimethyl Sulfoxide (DMSO)	Sigma catalog #D2650
Tissue Grinder Kontes	VWR catalog #KT885000-0002

Section 4
Storage

Store the individual components as specified. Note that lysates are shipped and stored separately from the coupled beads and detection antibodies. Factors are shipped and stored separately from the cell wash and cell lysis buffers.

Component	Storage
Bio-Plex phosphoprotein and total target assays	
Coupled beads (50x)	Store at 4ºC in the dark. Do not freeze.
Detection antibodies (25x)	Store at 4ºC.
Lysates (protein concentration = 200 µg/ml)	Store at –20ºC. Lysates can be frozen and thawed up to 5 times.
Bio-Plex cell lysis kit	
Cell wash buffer	Store at 4ºC.
Cell lysis buffer	Store at 4ºC.
Cell lysis buffer, factor 1 (250x)	Store at –20ºC. Factors can be frozen and thawed up to 5 times.
Cell lysis buffer, factor 2 (500x)	Store at –20ºC. Factors can be frozen and thawed up to 5 times.
Bio-Plex phosphoprotein detection reagent kit	
Assay buffer	Store at 4ºC.
Wash buffer	Store at 4ºC.
Detection antibody diluent	Store at 4ºC.
Resuspension buffer	Store at 4ºC.
Streptavidin-PE (100x)	Store at 4ºC in the dark.

Section 5
Lysate Preparation

This section provides instructions for preparing lysates derived from cell culture and tissure samples. For optimal recovery and sensitivity with the phospho-Histone H3 assay, refer to suggested protocol for lysate preparation.

1. Rinse the samples with *cell wash buffer* as follows:

   Adherent Cells — Stop the treatment reaction by aspirating the culture medium and quickly rinsing the cells with ice-cold cell wash buffer. The volume of cell wash buffer required is the same as the volume of aspirated cell culture medium. Keep the cells on ice.

   Suspension Cells — Stop the treatment reaction by adding ice-cold wash buffer to the cells. The volume of cell wash buffer required is twice that of the culture medium. Centrifuge the cells at 1,000 rpm for 5 min at 4ºC. Aspirate the supernatant.

   Tissue Samples — Rinse the tissue sample with cell wash buffer once. Cut the tissue into 3 x 3 mm pieces and transfer them to a 2 ml tissue grinder.

2. Prepare 500 mM PMSF by dissolving 0.436 g PMSF in 5 ml DMSO. Store as 0.5 ml aliquots at –20ºC. Aliquots can be frozen and thawed up to 5 times.

Lysing Solution Volume Guide

Culture vessel	Culture medium volume	Lysing solution volume	Notes
96-well plate	100 µl/well	75 µl/well	Grow cells to 80–85% confluence Recommend leaving external wells empty due to edge effect
10 cm culture dish	10 ml	2–3 ml	Grow cells to 80–90% confluence

3. Prepare an adequate volume of lysing solution (refer to the table on the left). For 10 ml of lysing solution, add 40 µl of *factor 1* and 20 µl of *factor 2* to 9.9 ml of *cell lysis buffer*. Vortex gently to mix and set aside on ice. Then add 40 µl of 500 mM PMSF.

4. Lyse the samples:

   Adherent and Suspension Cells

   a) Immediately add the lysing solution to the cells. The amount of lysing solution needed depends on the cell concentration in the culture vessel (see table on the left).

   b) Agitate the cells as follows:

      Culture Plate — For suspension cells, place the plate on ice and pipet the contents of the wells up and down 5 times. For adherent cells, scrape the cells with a cell scraper. For both, agitate the plate on a microplate shaker at 300 rpm for 20 min at 4ºC.

      Other Culture Vessel — Transfer the cell lysate to a centrifuge tube and rotate for 20 min at 4ºC.

      HINT: Freeze-thawing the lysate once using dry ice or a –20ºC freezer may increase the extent of the lysis. Alternatively, briefly sonicate (eg., with a Sonifier 450 as follows: Duty cycle = 40, Output = 1, Pulse sonicating = 18 times).

   c) Centrifuge the samples at 4,500 g for 20 min at 4ºC.

   Tissue Samples

   a) Immediately add 500 µl of lysing solution to the tissue grinder and grind the tissue sample on ice using about 20 strokes.

   b) Transfer the ground tissue to a clean microcentrifuge tube and freeze the sample at –70ºC.

   c) Thaw the samples, then sonicate on ice as suggested above.

   d) Centrifuge the samples at 4,500 g for 4 min.

5. Collect the supernatant without disturbing the pellet.

6. Determine the lysate protein concentration. The protein concentration should be 200–900 µg/ml. It may be necessary to test-lyse your samples with different volumes of lysing solution to obtain the specified protein concentration range.

7. Add an equal volume of *assay buffer* to the lysate.

8. If the lysate is not tested immediately, store at –20ºC. The lysate is stable for up to 5 freeze-thaw cycles.

Suggested protocol for lysate preparation of Histone H3 assay:

1. Follow steps 1–3 above.

4. Lyse the samples:

   a) Immediately add the lysing solution to the cells. The amount of lysing solution needed depends on the cell concentration in the culture vessel (see table on the left).

   b) Briefly sonicate (e.g., with a Sonifer 450 as follows: Duty cycle = 40, Output = 1, Pulse sonicating = two 10 min pulses with a 1 min break in between).

   c) Agitate the cells. Transfer the cell lysate to a centrifuge tube and rotate for 20 min at 4ºC.

   d) Centrifuge the samples at 4,500 g for 20 min at 4ºC.

5. Collect the supernatant without disturbing the pellet.

6. Determine the lysate protein concentration. The protein concentration should be 200–900 µg/ml. It may be necessary to test-lyse your samples with different volumes of lysing solution to obtain the specified protein concentration range.

7. Add equal volume of assay buffer to the lysate.

8. Freeze (overnight) at -20ºC and thaw before testing.

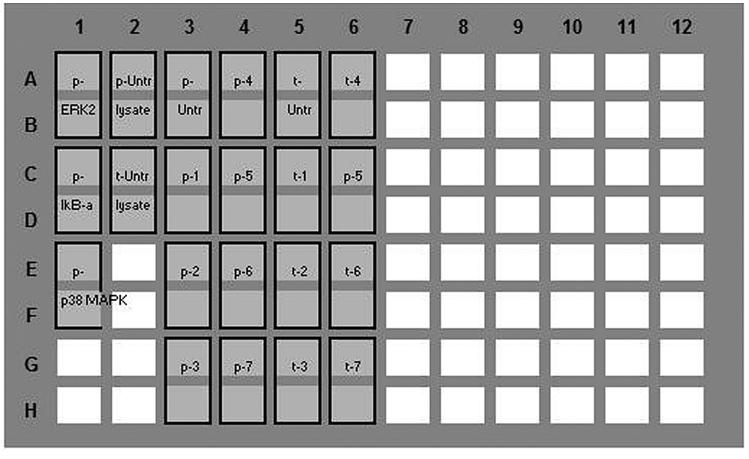
Section 6
Assay Instructions

The following instructions apply to Bio-Plex phosphoprotein and total target singleplex, custom-premixed, and mutliplex assays. Do not mix phosphoprotein assays with its corresponding total target assays (e.g. phospho-Akt and total Akt).

Plan Experiment

1. Assign which wells of a 96-well plate will be used for each lysate (see the example below). Keep in mind that the instrument reads wells down the plate and not across. Consider assigning the wells vertically. A pullout worksheet has been provided in this manual that may be used as a reference during the different assay steps.

Example Plate



2. Determine the total number of wells that will be used in the assay. Include a 25% excess (or add 2 wells for every 8 wells used) to ensure that enough diluted coupled beads, detection antibodies, and streptavidin-PE are prepared. Record these numbers on the worksheet since they will be referenced throughout the assay.

Thaw Lysates

1. Retrieve the experiment lysates and lysates shipped with the assays from –20ºC storage. These lysates were prepared using the protocol in section 5 and contain 50% assay buffer.

   NOTE: Refer to the table provided with the lysate packaging to identify which lysates shipped with the assays (visit www.bio-rad.com/products/phosphoproteins/ to download the PDF). Select the treated and untreated lysates from the table, which is used to determine the assay performance of each Bio-Plex phosphoprotein and total target assay. These should not be considered as references. Instead, activation signals and ratios should be based on experimental control lysates. For determining total protein concentrations, consider Bio-Rad's *DC* protein assay kit (Bio-Rad catalog #500-0112).

2. Thaw the lysates at room temperature and then place them on ice.

3. If necessary, it is possible to further dilute the lysates. Lysing solution freshly prepared (as specified in section 5) and assay buffer are required. Use a 1:1 mixture of lysing solution and *assay buffer* to further dilute the lysate.

Prepare Coupled Beads

Protect the beads from light by covering the tubes with aluminum foil. Keep all tubes on ice until ready to use. Coupled beads must be mixed manually prior to use when combining singleplex or premixed assays.

1. Vortex the coupled beads (50x) at medium speed for 5 sec.

2. Prepare a sufficient volume of coupled beads (1x) using *wash buffer*. When preparing a multiplex assay, use equal volumes of each bead (see sample below). Each well requires 1 µl of coupled beads (50x) for each target adjusted to a final volume of 50 µl (refer to the table below). These calculations can be done on the worksheet.

Volume of Coupled Beads (50x) in Each Well

Assay	Volume
Singleplex	1 µl from each singleplex
Premixed mulitplex	1 µl from each premixed multiplex
Singleplex and premixed multiplex	1 µl from each singleplex and 1 µl from each premixed multiplex

Example Coupled Bead Calculations

Assay Format	Singleplex and Premixed	Multiplex
Number of wells	Used for assay: **18** wells Include 25% excess: **18** wells x 1.25% = *23* Total number of wells = ***23 wells***	Used for assay: **24** wells Include 25% excess: **24** wells x 1.25% = *30* Total number of wells = ***30 wells***
Volume of coupled beads (50x)	Premixed assays: 3-plex = ***23 µl*** (ERK2, IkB-a, p38)	Singleplex assays: ERK2 = 30 µl IkB-a = 30 µl p38 = 30 µl **Total = *90 µl***
Volume of coupled beads (1x)	**23 wells** x 50 µl = *1150 µl*	**30 wells** x 50 µl = *1500 µl*
Volume of wash buffer	**1150 µl** – **23 µl** = *1127 µl*	**1500 µl** – **90 µl** = *1410 µl*

Calibrate Vacuum Apparatus

The vacuum apparatus must be calibrated at the beginning of the assay to ensure an optimal bead yield. For more detailed instructions, refer to the Bio-Plex suspension array system hardware instruction manual.

1. Prewet all the wells of a 96-well filter plate with 100 µl of wash buffer.

2. Place the filter plate on the vacuum apparatus and turn on the vacuum to the maximum level.

3. Press on the filter plate and note the time required to remove the buffer from the wells by vacuum filtration. The evacuation time should be 2–5 sec.

   If the evacuation time is <2 sec, the pressure is too high. Open the vacuum control valve slightly and repeat steps 1–3.

   If the evacuation time is >5 sec, the pressure is too low. Close the vacuum control valve slightly and repeat steps 1–3.

Assay Key

The following terms are repeated throughout the assay procedure. Refer to these detailed instructions when **wash**, **rinse**, **incubate**, and **vacuum-filter** are shown in bold.

Term	Detailed Directions
Wash	Add 100 µl of *wash buffer* to each well. Place the filter plate on a calibrated vacuum apparatus and remove the buffer by vacuum filtration. Blot the bottom of the filter plate with a clean paper towel. Repeat as specified.
Rinse	Add 100 µl of *resuspension buffer* to each well. Place the filter plate on a calibrated vacuum apparatus and remove the buffer by vacuum filtration. Blot the bottom of the filter plate with a clean paper towel. Repeat as specified.
Incubate	Cover the filter plate with a new sheet of sealing tape. Place the filter plate on a microplate shaker and then cover with aluminum foil. Shake the filter plate at room temperature at 1,100 rpm for 30 sec, then at 300 rpm for the specified incubation time.
Vacuum -filter	Place the filter plate on a calibrated vacuum apparatus and remove the buffer by vacuum filtration. Blot the bottom of the filter plate with a clean paper towel.

Assay Procedure

Bring all buffers to room temperature. Avoid bubbles when pipetting.

1. **Wash** the desired number of wells in a 96-well filter plate. If fewer than 96 wells are required, cover the unused wells with sealing tape for later use.

2. Vortex the coupled beads (1x) for 5 sec at medium speed. Add 50 µl to each well and immediately **vacuum-filter**.

3. **Wash** twice.

4. Vortex the thawed lysates gently for 3 sec. Add 50 µl of lysate to each well, changing the pipet tip after every volume transfer. **Incubate** for 15–18 hr (or overnight).

Volume of Detection Antibodies (25x) in Each Well

Assay	Volume
Singleplex	1 µl from each singleplex
Premixed mulitplex	1 µl from each premixed multiplex
Singleplex and premixed multiplex	1 µl from each singleplex and 1 µl from each premixed multiplex

Example Detection Antibody Calculations

Assay Format	Singleplex and Premixed	Multiplex
Number of wells	Used for assay: **18** wells Include 25% excess: **18** wells x 1.25% = *23* Total number of wells = ***23 wells***	Used for assay: **24** wells Include 25% excess: **24** wells x 1.25% = *30* Total number of wells = ***30 wells***
Volume of detection antibodies (25x)	Premixed assays: 3-plex = ***23 µl*** (ERK2, IkB-a, p38)	Singleplex assays: ERK2 = 30 µl IkB-a = 30 µl p38 = 30 µl **Total = *90 µl***
Volume of detection antibodies (1x)	**23 wells** x 25 µl = *575 µl*	**30 wells** x 25 µl = *750 µl*
Volume of detection antibody diluent	**575 µl** – **23 µl** = *552 µl*	**750 µl** – **90 µl** = *660 µl*

5. The next day, prepare a sufficient volume of detection antibodies (1x) using *detection antibody diluent* (see the example on the previous page). Each well requires 1µl of detection antibodies (25x) for each target adjusted to a final volume of 25 µl (refer to the table on the previous page).

6. After the incubation, slowly remove and discard the sealing tape, then **vacuum-filter**.

7. **Wash** 3 times.

8. Vortex the detection antibodies gently and add 25 µl to each well, changing the pipet tip after every volume transfer. **Incubate** for 30 min.

9. After the incubation, slowly remove and discard the sealing tape, then **vacuum-filter**.

10. **Wash** 3 times.

11. Keep the plate in the dark and prepare a sufficient volume of streptavidin-PE (1x) using *wash buffer* (see example below). Each well requires 0.5 µl of streptavidin-PE (100x) adjusted to a final volume of 50 µl. Store in the dark after preparation.

Example Streptavidin-PE Calculations

Assay	Phosphoprotein and Total Target
Total number of wells	30 + 23 = **53**
Volume of SA-PE (100x)	53 x 0.5 µl = **26.5 µl**
Volume of SA-PE (1x)	53 x 50 µl = **2650 µl**
Volume of wash buffer	2650 µl – 26.5 µl = **2623.5 µl**

12. Vortex the diluted streptavidin-PE vigorously and add 50 µl to each well. **Incubate** for 10 min.

13. After the incubation, slowly remove and discard the sealing tape, then **vacuum-filter**.

14. **Rinse** 3 times.

15. Add 125 µl of *resuspension buffer* to each well. **Incubate** for 30 sec. If the data is not acquired immediately, the assay may be stored in the dark at 4ºC for up to 24 hrs.

Section 7
Data Acquisition

Recommendations for acquiring data using the Bio-Plex suspension array system are listed below. Alternatively, refer to the Bio-Plex Manager software user guide or the instructions provided with the Luminex 100 instrument.

Prepare System

1. Empty the waste bottle and fill the sheath fluid bottle before starting. This will prevent fluidic system backup and potential data loss.

2. Turn on the reader and microplate platform (and HTF if present). Allow the system to warm up for 30 min.

3. Select Startup  and follow the instructions to prepare the reader to acquire data. If the system is idle for 4 hr the lasers will automatically turn off and a 30 min warm-up period will again be required prior to acquiring data. Select Warm up  and wait for the optics to reach operational temperature.

Calibrate With High RP1 Target Value

Calibrate using Bio-Plex calibration beads and target values. Daily calibration is recommended before acquiring data.

1. Select Calibrate  and confirm that the default values for CAL1 and CAL2 are the same as the values on the Bio-Plex calibration bead labels. Use the Bio-Plex High RP1 target value for CAL2 calibration for Bio-Plex phosphoprotein and total target assays.

   NOTE: When acquiring data for Bio-Plex phosphoprotein or total target assays with a Luminex 100, Luminex Data Collector software, and Luminex calibration beads, it is necessary to convert the Luminex CAL2 calibration bead RP1 target value using the following equation:

   Bio-Plex High RP1 target value = (Luminex RP1 target value) x 4.55

   Add the new target value to the Luminex software by selecting Calibrate, then New under the Reporter Channel in the Start Calibration dialog. Enter the new target value and save it as a new lot. Then calibrate using the new RP1 target value.

2. Select OK and follow the instructions for CAL1 and CAL 2 calibration.

Prepare Protocol

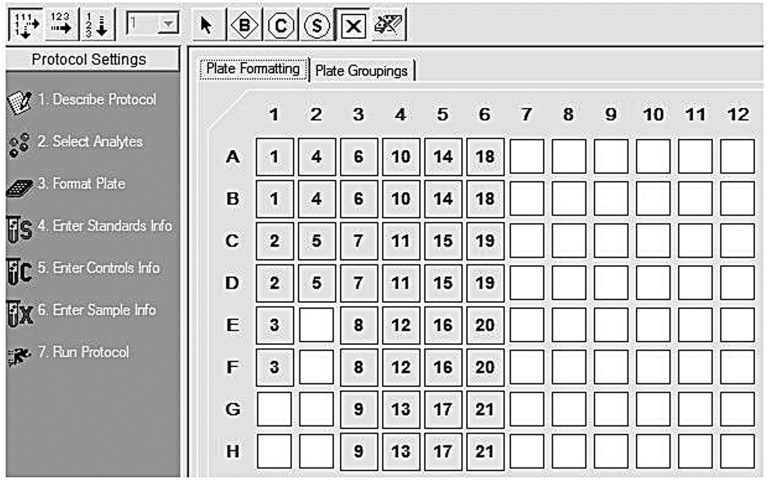
1. Open a new protocol by selecting File, then New from the main menu. Locate the steps at the left of the protocol menu.

2. Select Step 1 (Describe Protocol) and enter information about the assay.

3. Select Step 2 (Select Analytes) and select the panel for Phosphoproteins or Total Targets. Choose the target proteins for all the assays on the plate. If both phosphoprotein and total target assays are run on the same plate, two separate protocols must be entered.

4. Select Step 3 (Format Plate) and click on the Plate Formatting tab. Click on ✕ and drag the cursor over all the wells that contain lysates.

Plate Formatting Example



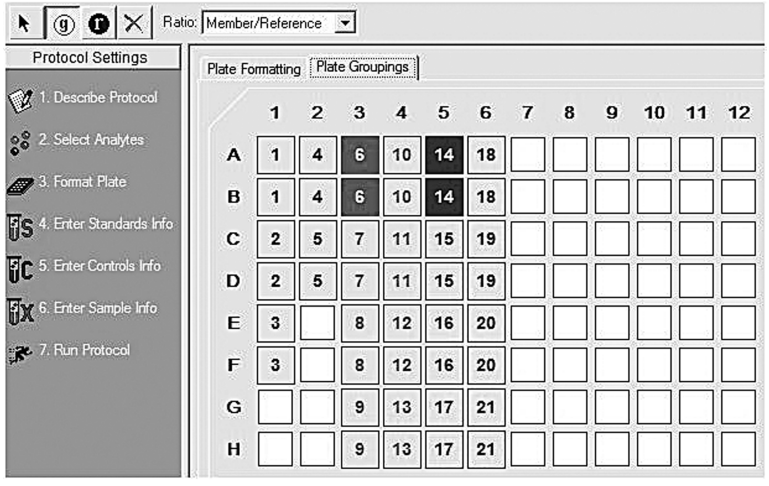
5. Then select the Plate Groupings tab to display the plate grouping tools.

   a) Select Group ⓖ and drag the cursor across all the wells to define each assay group (exclude the ones that contain lysates provided with the assays).

   b) The first well of the group is automatically assigned as the Reference well and the remaining wells in the group are Member wells. To change the Reference well, select Reference ⓡ and click on the new Reference well.

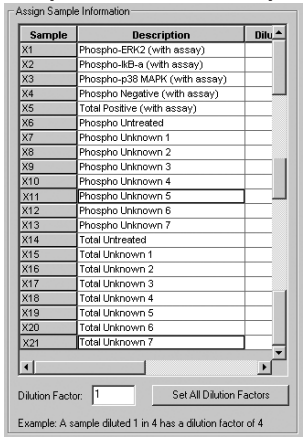
   c) Select Member/Reference or Reference/Member from the Ratio pull-down list. When selecting the more common Member/Reference option, the ratio of fluorescence intensity of each well will be calculated against the fluorescence intensity of the Reference well. This value will be reported as a ratio in the results file.

Plate Grouping Example



6. Select Step 6 (Enter Sample Info) and enter sample information. This is the location where the wells are identified as containing either Bio-Plex phosphoprotein or total target assays (see the example on the right).

Sample Information Example



Acquire Data

1. Shake the assay plate at 1,100 rpm for 30 sec immediately before acquiring data. Failure to do so will result in increased data acquisition time due to bead settling.

2. Check that the filter plate is flat. While pressing on one end of the plate, observe the distance that the opposite end of the plate is raised off a flat surface. If the distance is >1 mm, transfer all contents to a flat-bottom 96-well plate or another filter plate.

3. Visually inspect the plate and ensure that the assay wells are filled with buffer prior to placing the plate in the Bio-Plex microplate platform.

4. Slowly remove the sealing tape and any plate cover before placing the plate in the reader.

5. Select Step 7 (Run Protocol):

   a) Specify data acquisition for **25 beads per region**.

   b) In Advanced Settings, confirm that the default DD gate values are set to 4335 (low) and 10000 (high).

      NOTE: When using a Luminex 100 instrument, set the gates according to the Luminex procedure located in the manual.

   c) Select Start and save the .rbx file. Then follow the instructions for data acquisition.

6. If acquiring data from more than one plate, empty the waste bottle and refill the sheath bottle after each plate. Select Wash Between Plates and follow the instructions for fluidics maintenance. Then repeat the **Prepare Protocol** and **Acquire Data** steps.

7. 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 Step 7 (Run Protocol).

1. Check the wells where data will be acquired a second time. Any previous data will be overwritten.

2. Remove the buffer by vacuum filtration and add 125 µl of resuspension buffer to each well. Cover the filter plate with a new sheet of sealing tape.

3. Repeat **Acquire Data** steps 1–6 to acquire data a second time. The data acquired should be similar to the data acquired initially; however, the data acquisition time will be extended since fewer beads are present in each well.

Section 8
Troubleshooting

This troubleshooting section addresses problems that may be encountered with Bio-Plex phosphoprotein or total target assays. If the problems listed below are encountered, review the possible causes and solutions provided. This will assist in resolving problems directly related to the assay. Use the Bio-Plex validation kit to validate all the key functions of the array reader and assist in determining whether or not the array reader is functioning properly.

Possible Causes	Possible Solutions
Filter Plate Leakage	
Vacuum setting too high	This could result in tearing of the filter. Confirm that the vacuum pressure is set as specified in the vacuum calibration procedure section. Also refer to the Vacuum Manifold Setup in the Bio-Plex suspension array system hardware instruction manual. Use the recommended filter plate vacuum apparatus
Low Signal (Good Signal From Lysates Provided with the Assays but Weak or No Signal From Experiment Lysates)	
Protein concentration in lysate too low or too high	Verify the protein concentration in the cell lysate samples. Adjust the amount of lysing solution used in the lysate preparation to achieve an optimal protein concentration of 200–900 µg/ml prior to adding an equal part of assay buffer
Low Signal (Weak or No Signal From Lysates Provided with the Assays and Experiment Lysates)	
Detection antibody and/or streptavidin-PE diluted incorrectly	Check the calculations and be careful to add the correct volumes for dilution
Expired beads, detection antibody, and/or streptavidin-PE used	Use new or unexpired components
Incorrect incubation temperature used during incubation steps	Incubations should be at room temperature (20–22ºC)
Incubation time insufficient	Adhere to the recommended incubation times
Low Bead Count	
Cell debris in lysate not cleared	Remove the cellular debris by centrifugation at 4,500 g for 20 min at 4ºC. Avoid disturbing the pellet while collecting the supernatant
Resuspension buffer not used after streptavidin-PE incubation	Rinse the beads 3 times with resuspension buffer after the streptavidin-PE incubation step
Vacuum setting too high	This results in bead loss. Calibrate the vacuum apparatus as specified
Filter plate not shaken enough before each incubation step and prior to data acquisition	Shake the filter plate at 1,100 rpm for 30 sec before each incubation and immediately before acquiring data
Reader clogged	Refer to the troubleshooting guide in the Bio-Plex hardware instruction manual
High Coefficient of Variation (CV)	
Plate sealer reused	This could result in contamination. Use a new sheet of sealing tape for each incubation
Buffer not completely filtered from wells	Be sure that the wells are filtered completely and that no residual volume remains

Possible Causes	Possible Solutions
Contamination with wash buffer during wash steps	During the wash steps, do not splash wash buffer from one well to another. Filter the wells completely so that no residual volume remains. Also, be sure that the microplate shaker setting is not too high. Reduce the microplate shaker speed to minimize splashing
Microplate shaker set to an incorrect speed	Check the microplate shaker speed and use the recommended setting. Setting the speed too high may cause splashing and contamination. Use the recommended plate shaker
Cellular debris not cleared from lysate	Be sure to remove cellular debris by centrifugation as directed. Avoid disturbing the pellet while collecting the supernatant
Bead resuspension buffer not used after streptavidin-PE incubation	Wash 3 times with bead resuspension buffer after streptavidin-PE incubation as described in the assay instructions
High Background Signal (From Both Lysates Provided with the Assays and Experiment Lysates)	
Vacuum pressure too low, resulting in residue in wells	Use recommended filter plate vacuum apparatus with proper vacuum pressure setting
Wash steps performed incorrectly	Perform washes as described in the assay instructions
Streptavidin-PE incubation step too long	Check suggested incubation times for appropriate steps of the assay. Follow the suggested time for incubation

Section 9
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 phosphoprotein and total target assay contain components of human origin. The components are known to contain an agent that requires handling at Biosafety Level 2 containment [US Government Publication: Biosafety in Microbiological and Biomedical Laboratories (CDC, 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.

Section 10
References

Fulton R, McDade R, Smith P, Kienker L, and Kettman J Jr, Advanced multiplexed analysis with the FlowMetrix system, Clin Chem 43, 1749–1756 (1997)

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

For a complete list of publications using Bio-Plex Phosphoprotein Detection Assays, refer to bulletin 5394.