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4110012 Rev B 0203

Bio-Plex™ *Amine Coupling Kit*

Instruction Manual

For technical service, call your local Bio-Rad office, or in the US, call 1-800-424-6723

For research use only. Not for diagnostic procedures.

BIO-RAD

Table of Contents

Section 1
Introduction

The Bio-Plex™ amine coupling kit provides the buffers necessary to covalently couple 6–150 kD proteins to 5.5 µm dyed carboxylated polystyrene beads in under 5 hr. This is achieved via carbodiimide reactions involving the protein primary amino groups and the carboxyl functional groups bound on the surface of polystyrene beads. The covalent attachment is permanent, leaving no unbound protein after cleanup, even after months of storage. The contents of this kit are sufficient for 30 coupling reactions, with each coupling reaction requiring 1.25×10^6 carboxylated polystyrene beads (1x scale). For larger scale coupling reactions, the volume of the buffers used in each step can be proportionally increased to a maximum 10x scale. The protein-coupled beads can then be used in multiplex protein-protein binding studies or in the development of multiplex assays that can be analyzed with the Bio-Plex protein array system. The bead yield per coupling reaction is approximately 80%, or enough protein-coupled beads for two 96-well microtiter plates using 5,000 beads per well.

Single Source Solution

Bio-Rad now offers the Bio-Plex amine coupling kit and Bio-Plex COOH (carboxylated) beads for up to 100 different regions as part of the expanded Bio-Plex product line. These coupling accessories complement the Bio-Plex cytokine assays and phosphoprotein assays as well as the associated reagent, diluent, and cell lysis kits. Make Bio-Rad the source for all your protein multiplexing needs. For a current listing of Bio-Plex products, visit us on the web at **www.bio-rad.com/bioplexsystem/**.

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Section 2
Principle

Protein Preparation

This coupling procedure can be used to covalently couple water-soluble proteins ranging in size from 6–150 kD via carboxyl groups on the surface of polystyrene beads. The protein sample must be free of sodium azide, BSA, glycine, Tris or amine-containing additives and must be suspended in PBS, pH 7.4. Optimal protein-coupling conditions must be established by determining how much protein will be required for the coupling reaction to later promote optimal binding between the bound protein and its complementary ligand in the protein assay. The table below provides examples of optimal amounts per coupling reaction for four different proteins. Note that using the highest level of protein for the coupling reaction will not necessarily yield an optimal assay. The ultimate test is the functional assay for each coupled protein.

Example: Optimal amount of protein for one coupling reaction

Protein	MW (kD)	Mass (µg)
Insulin	6	5
Human IL-10	18.6	2
Erk	44	11
Mouse IgG	150	9

Protein Coupling

The coupling procedure involves a two-step carbodiimide reaction. The carboxyl groups on the surface of the polystyrene beads must first be activated with a carbodiimide derivative prior to coupling the protein. EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) reacts with carboxyl groups on the surface of the polystyrene beads to form an active O-acylisourea intermediate. This unstable intermediate is stabilized in aqueous solutions using S-NHS (*N*-hydroxysulfosuccinimide). EDC couples S-NHS to the carboxyl group resulting in an S-NHS-activated site. Both the carbodiimide's O-acylisourea intermediate and the S-NHS-ester formed are amine-reactive; however, an S-NSH-ester has much greater stability at physiological pH. These intermediates then react with primary amines of proteins to form amide bonds. The intermediate is hydrolyzed if it fails to react with an amine and the carboxyl is regenerated, releasing an N-unsubstituted urea. This reaction occurs fairly rapidly and reaches completion within a few minutes.

Protein Coupling Validation

Once the coupling reaction has been completed, the protein-coupled beads are enumerated and the efficiency of the protein coupling reaction is validated. In this procedure, the protein-coupled beads are reacted with a phycoerythrin (PE)-labeled antibody that binds to the coupled protein, which is then analyzed using the Bio-Plex™ protein array system. This procedure may be performed by reacting the beads with a PE-labeled antibody; alternatively, a reaction using a biotinylated antibody followed by streptavidin-PE may be used. The intensity of the fluorescent signal of this reaction is directly proportional to the amount of protein on the surface of the beads. A successful coupling typically yields a fluorescent intensity signal greater than 2,000 MFI. The protein coupling validation procedure provides a rapid relative assessment of the amount of protein coupled to the beads; however, this procedure does not verify the functionality of the protein.

Section 3
Product Description

The following components are required to covalently couple proteins to carboxylated polystyrene beads:

Bio-Plex™ Amine Coupling Kit
Bead wash buffer
Bead activation buffer
PBS, pH 7.4
Blocking buffer
Storage buffer
Staining buffer
Coupling reaction tubes

Bio-Plex COOH (Carboxylated) Beads
COOH Bead 24, 1.25 x 10^7 beads/ml, 1 ml
COOH Bead 25, 1.25 x 10^7 beads/ml, 1 ml
COOH Bead 26, 1.25 x 10^7 beads/ml, 1 ml
COOH Bead 27, 1.25 x 10^7 beads/ml, 1 ml
COOH Bead 28, 1.25 x 10^7 beads/ml, 1 ml
COOH Bead 42, 1.25 x 10^7 beads/ml, 1 ml
COOH Bead 43, 1.25 x 10^7 beads/ml, 1 ml
COOH Bead 44, 1.25 x 10^7 beads/ml, 1 ml
COOH Bead 45, 1.25 x 10^7 beads/ml, 1 ml
COOH Bead 46, 1.25 x 10^7 beads/ml, 1 ml

Section 4
Storage and Stability

Protect the photosensitive COOH beads from light by storing them in the resealable foil bags provided. All kit components should be stored at 4°C. Do not freeze.

All components are guaranteed for 6 months from the date of purchase when stored as specified in this manual.

Section 5
Materials Required but Not Supplied

For optimal results we strongly recommend the use of the following:

Bio-Plex™ Protein Array System
Bio-Rad catalog #171-000001, 171-000003, or 171-000005

Bio-Plex Protein Array System Accessories
Bio-Plex validation kit, Bio-Rad catalog #171-203000
Bio-Plex calibration kit, Bio-Rad catalog #171-203060

Vortexer
VWR brand mini-vortexer, VWR catalog #58816-121
Vortexer adaptors, VWR catalog #58816-130

Centrifuge
Microcentrifuge model 5415C, VWR catalog #20901-051

Tissue Culture Rotator
Glas-Col 120v, Glas-Col catalog #RD4512

Sonicator
Branson ultrasonic cleaner model 1510-DTH, VWR catalog #21812-175

Cell Counter
Coulter Z2 particle counter and size analyzer, Beckman Coulter Model Z2, catalog #9914558, or a hemacytometer

Protein Assay
Bio-Rad *DC*™ protein assay kit I (bovine gamma globulin standard), Bio-Rad catalog #500-0111
Bio-Rad *DC* protein assay kit II (BSA standard), Bio-Rad catalog #500-0112

Buffer Exchange Columns
Micro Bio-Spin™ 6 Tris chromatography columns, Bio-Rad catalog #732-6221, 732-6222, or 732-6225

Chemicals
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), Pierce catalog #22980
N-hydroxysulfosuccinimide (S-NHS), Pierce catalog #24510

Reagent Reservoir
Corning, Inc., catalog #224-4872

Other
Antibody specific for coupled protein (labeled with R-phycoerythrin or biotin), streptavidin-R-PE, pipets and pipet tips, aluminum foil, small weigh boats, microcentrifuge tubes, 96-well flat-bottom plates

Section 6
Protein Preparation

Bring all the buffers to room temperature prior to their use. We recommend setting up as many as ten protein coupling reactions at one time using various amounts of protein in order to determine the optimal coupling conditions for your protein.

1. a) If your protein sample is not free of sodium azide, BSA, glycine, Tris or amine-containing additives, proceed to step 2 and follow the buffer exchange procedure using Micro Bio-Spin™ 6 Tris chromatography columns. Alternatively, dialyze overnight against PBS, pH 7.4.

   OR

   b) If your protein sample is already suspended in PBS, pH 7.4, and is free of sodium azide, BSA, glycine, Tris or amine-containing additives, determine the protein concentration with the Bio-Rad *DC*™ protein assay kit or any other protein assay of choice and proceed to step 9.

2. Obtain one Micro Bio-Spin 6 Tris chromatography column for each different protein requiring buffer exchange with PBS, pH 7.4. The exclusion limit for the Bio-Gel® P-6 gel contained in the columns is 6,000 Da.

   NOTE: Up to 20% of the protein can be lost during this buffer exchange procedure. For further instructions, refer to the manual provided with the columns. The manual can also be found on the Bio-Rad web site (**discover.bio-rad.com**).

3. Invert the column sharply several times to resuspend the settled gel and remove any bubbles. Snap off the tip and place the column in a 2 ml microcentrifuge tube (included with the chromatography columns).

4. Centrifuge the column for 2 min in a microcentrifuge at 1,000 x g to remove the remaining packing buffer. Discard the buffer.

5. Apply 500 µl Bio-Plex PBS, pH 7.4 to the top of the column and centrifuge at 1,000 x g for 2 min. Discard the buffer from the collection tube.

6. Repeat step 5 four additional times. On the last wash step, centrifuge for 4 min.

7. Place the column in a clean and labeled 1.5 or 2 ml microcentrifuge tube. Carefully apply the protein sample (20–75 µl) directly to the center of the column.

   NOTE: Application of more or less than the recommended sample volume may decrease column performance.

8. Centrifuge the column for 5 min at 1,000 x g. The purified protein sample is now in PBS, pH 7.4. Place the sample on ice and calculate the amount of protein recovered using a Bio-Rad *DC* protein assay kit or any other protein assay of choice.

   NOTE: One coupling reaction requires 5-12 µg protein depending on the protein used. If you are coupling for the first time, you may wish to prepare multiple coupling reactions varying the amount of protein used to determine the optimal coupling conditions for your protein.

Section 7
Protein Coupling

Bring all the buffers to room temperature prior to their use. Protect the beads from light as much as possible by covering the tubes with aluminum foil during the procedure. Remove EDC and S-NHS from the –20°C freezer and store them in a desiccator at room temperature for approximately 1 hr prior to their use. The bottles of EDC and S-NHS should be discarded after five uses.

Bead Activation

1. Select the COOH beads (1.25 x 10^7 beads/ml) for the protein coupling reaction. Vortex the beads for 30 sec at a speed of 6–7 on the recommended vortexer, then sonicate the beads by bath sonication for 30 sec.

   NOTE: It is important that the COOH beads are completely resuspended as single monodisperse particles. Aggregated beads may be detected visually as large clumps throughout the suspension. If you see aggregates in your suspension, sonicate the beads by bath sonication for a maximum of 30 sec and check again for aggregates. Repeat the sonication until you no longer see aggregates in your sample.

2. For a 1x scale coupling reaction, transfer 100 µl of monodisperse COOH beads (1.25 x 10^6 beads) to one of the coupling reaction tubes provided with the kit. Centrifuge the beads at 14,000 x g for 4 min. Carefully remove and discard the supernatant from the bead pellet.

   NOTE: The bead pellet will be difficult to view. Mark the coupling reaction tube to identify where the pellet will be located. To avoid disturbing the bead pellet, tilt the tube forward with the pellet on the opposite side of the inner tube wall. Remove the supernatant along the front of the tube wall.

3. Add 100 µl of bead wash buffer. Vortex for 10 sec, then sonicate for 10 sec. Centrifuge the beads at 14,000 x g for 4 min, then carefully remove and discard the supernatant.

4. Resuspend the bead pellet in 80 µl of bead activation buffer. Vortex the beads for 30 sec, then sonicate the beads by bath sonication for 30 sec.

5. Prepare EDC (50 mg/ml) and S-NHS (50 mg/ml) in bead activation buffer immediately prior to its use. Add 10 µl of the freshly made 50 mg/ml EDC closely followed by 10 µl of the freshly made 50 mg/ml S-NHS to the beads. Vortex at high speed for 30 sec. Cover the coupling reaction tube with aluminum foil and agitate the beads with a rotator at room temperature for 20 min.

   NOTE: It is essential that the beads remain in suspension during this activation step.

6. Add 150 µl of PBS, pH 7.4, and vortex the activated beads at high speed for 10 sec. Centrifuge the activated beads at 14,000 x g for 4 min, then carefully remove and discard the supernatant. Repeat this step.

7. Resuspend the activated beads with 100 µl of PBS, pH 7.4. Vortex the activated beads at medium speed for 30 sec and sonicate the activated beads by bath sonication for 15 sec.

Protein Coupling

1. Add the protein sample (5–12 µg) from step 8 in Section 6 to the activated beads. Adjust the final volume to 500 µl with PBS, pH 7.4. Cover the microcentrifuge tube with aluminum foil and agitate the beads with a rotator at room temperature for 2 hr. Alternatively, agitate the beads with a rotator at 4°C overnight.

   NOTE: It is essential that the beads remain in suspension during this conjugation step.

2. Centrifuge the coupled beads at 14,000 x g for 4 min, then carefully remove and discard the supernatant.

3. Wash the beads with 500 µl of PBS, pH 7.4. Centrifuge at 14,000 x g for 4 min, then carefully remove and discard the supernatant. Do not sonicate.

4. Resuspend the coupled beads with 250 µl of blocking buffer. Vortex the beads at medium speed for 15 sec. Cover the microcentrifuge tube with aluminum foil and agitate the beads with a rotator at room temperature for 30 min.

5. Centrifuge the coupled beads at 14,000 x g for 4 min, then carefully remove and discard the supernatant.

6. Wash the coupled beads with 500 µl storage buffer. Centrifuge the coupled beads at 16,000 x g for 6 min, then carefully remove and discard the supernatant.

7. Resuspend the coupled beads in 150 µl of storage buffer or an alternate storage buffer to complement the protein assay. Determine the bead concentration using a Coulter Z2 counter or a hemocytometer. Store the coupled beads at 4°C covered with aluminum foil. Proceed to Section 8 to validate the efficiency of your coupling reaction.

   NOTE: Coupled beads are stable for one year if stored at 4°C, protected from light.

Section 8
Protein Coupling Validation

This validation method is based on the detection of the coupled protein with labeled antibodies. This procedure describes two validation methods, using either a PE-conjugated antibody or a biotinylated antibody followed by streptavidin-PE.

NOTE: If you have coupled an antibody to your beads, be sure that the antibody used in this procedure is specific for the host species of your coupled antibody. For example, if you have coupled a mouse anti-human antibody, your PE-labeled antibody should be directed against the mouse antibody (i.e. goat anti-mouse or rabbit anti-mouse). If you do not use the correct species of antibody, it will appear that your coupling reaction was not successful.

1. Label two microcentrifuge tubes for each bead coupled, one as the negative control and one as the test.

2. Vortex the coupled beads at medium speed for 15 sec. Add approximately 10,000 coupled beads to each of the two tubes.

3. a) When using a PE-conjugated antibody, dilute the PE labeled antibody to 1 µg/ml with staining buffer. Add 50 µl of the 1 µg/ml diluted PE-labeled antibody to the tube labeled “test”. Do not add antibody to the negative control tube. Cover the tubes with aluminum foil and agitate the beads with a rotator at room temperature for 30 min.

   OR

   b) When using a biotinylated antibody followed by streptavidin-PE, dilute the biotinylated antibody to 2 µg/ml with staining buffer. Add 50 µl of staining buffer to the negative control tube. Add 50 µl of the diluted biotinylated antibody to the tube labeled “test”. Do not add antibody to the negative control tube. Cover the tubes with aluminum foil and agitate the beads with a rotator at room temperature for 30 min.
   Centrifuge the tubes at 14,000 x g for 4 min. Carefully remove and discard the supernatant. Dilute the streptavidin-PE to 2 µg/ml with staining buffer. Add 50 µl of the diluted streptavidin-PE to the tube labeled “test” only. Add 50 µl of staining buffer to the negative control tube. Do not add streptavidin-PE to the negative control tube. Cover the tubes with aluminum foil and incubate at room temperature for 10 min without rotation.

4. Centrifuge the tubes at 14,000 x g for 4 min. Carefully remove and discard the supernatant without disturbing the pellet.

5. Resuspend the pellet in 125 µl of storage buffer. Vortex the beads at medium speed for 15 sec. Transfer each 125 µl sample to a single well of a flat-bottom 96-well plate. Proceed to Section 9 to read your coupled beads.

Section 9
Bio-Plex™ Protein Array System Operation

System Preparation
Recommendations for reading your coupled beads on the Bio-Plex protein array system are listed below. Alternatively, refer to the Bio-Plex Manager™ software user guide.

1. Turn on the Bio-Plex array reader and microplate platform (and high throughput fluidics system if present). Allow the system to warm up for 30 min.

   NOTE: If the system is left idle for 4 hr, the lasers will automatically turn off. A 30 min warm-up period will again be required prior to reading an assay. Select Warm up from the tool bar and wait for the optics to reach operational temperature.

2. Select Start up from the tool bar and follow the instructions shown on the screen to prepare the reader.

   NOTE: If the waste is overfilled, the fluidics system may back up and the assay signal will be lost. The sheath reservoir contains enough fluid for approximately two 96-well plates. If the sheath fluid level falls below the "Sheath" output tubing on the bottle, the assay read cannot be completed.

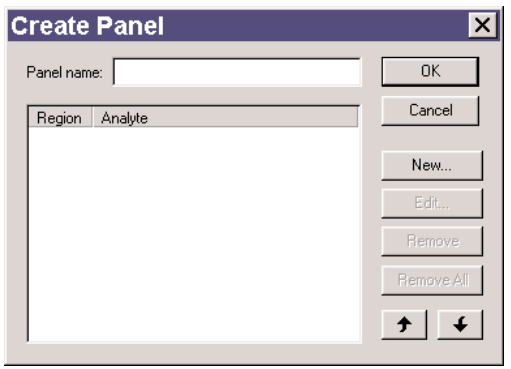
3. Select Calibrate from the toolbar and follow the instructions on the screen. When entering the target values be sure to use the Low RP1 target value for the CAL2 calibration. Daily calibration is recommended prior to reading the first assay.

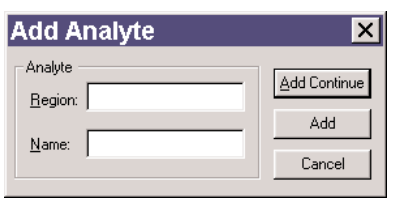
   NOTE: Be sure to calibrate the reader using the low RP1 target value printed on the label of the CAL2 beads. The high RP1 target value will give a falsely elevated signal.

4. Open a new protocol by selecting File then New from the main menu. Locate the Protocol Settings steps on the left side of the protocol view.

5. Select Step 1 and enter any relevant information about your coupling.

6. Select Step 2 Select Analytes. Select Create New Panel in the toolbar. A Create Panel dialog will appear. Enter a name for your panel and select New. Enter the region number of your analyte and the name of your protein in the designated fields. Select OK when you have entered your information. This panel will be saved for future use. You may also add your analyte to an existing panel by selecting Edit Panel

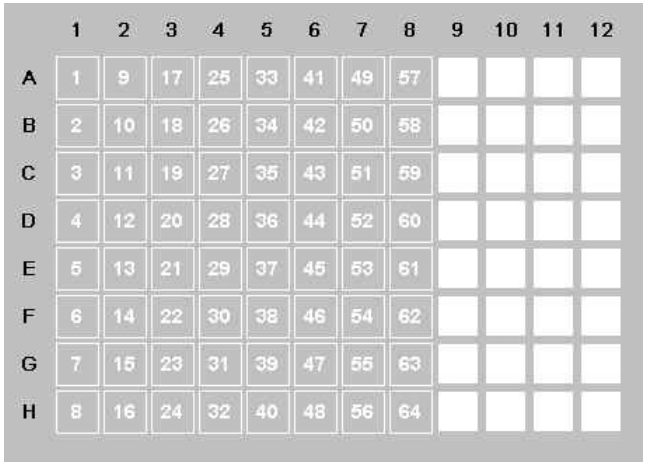




7. Select Step 3 Format Plate and format the wells that contain samples as unknown samples, as shown on page 16.

   NOTE: The plate must be formatted and analytes must be selected prior to reading a sample.

8. Select Step 6 Enter Sample Info to enter sample information. You may wish to specify which samples are negative controls and which are test samples in this step.



9. Select Step 7 Run Protocol and select 100 beads per region with a sample size of 50 µl.

Reading Coupled Beads

1. Make sure you have emptied the waste bottle of the Bio-Plex system. Ensure that there is sufficient sheath present in the sheath cube prior to proceeding.
2. Shake the plate at 1,100 rpm for 30 sec immediately before starting the run.
3. Remove sealing tape and any plate cover before placing the plate on the Bio-Plex system.
4. Select Start in the Run Protocol dialog to initiate the run.
5. Once the reading is complete and you wish to turn off the system, select Shut Down from the toolbar and follow the instructions to shut the reader down.

Data Analysis

The negative control tube should be less than 100 MFI. A successful coupling is indicated by an MFI value greater than 2,000.

NOTE: Be sure that you have read the tubes after calibrating using the low RP1 target value. Failure to do so will give you falsely elevated results.

Section 10
Troubleshooting Guide

This troubleshooting guide addresses problems that may be encountered during a protein coupling reaction. If you experience any of the problems listed below, review the possible causes and solutions provided.

Possible Causes	Possible Solutions
Low MFI signal in validation procedure (below 2,000)	
EDC and S-NHS may have expired	Use fresh EDC and S-NHS for each conjugation
Purified protein was not used immediately or was not kept on ice	Keep the protein on ice during the conjugation procedure
S-NHS was not added to the beads immediately after adding EDC	Add S-NHS to the beads immediately after adding EDC
Wrong antibody was used in the validation procedure	Ensure that the correct species-specific antibody is being used for validation (if you coupled an antibody)
Greater than 30% bead loss during conjugation	
Poor pipetting technique	Remove 50 µl of buffer at a time from the bead pellet to minimize bead pellet disturbance
Bead count too high to be counted by Coulter Z2 counter	
Beads are too concentrated	Add 100% more storage buffer
Low MFI signal in assay	
Conjugation failed	Check validation procedure again and repeat the conjugation if necessary
Problem with protein integrity	Repeat conjugation with new lot of protein

Conjugated beads have higher background signal in assay than in previous conjugation

Blocking step was skipped following the conjugation	Include the blocking buffer step
Coupling incubation time too long	Use a consistent incubation time during the coupling step

Section 11
Safety Considerations

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

Section 12
Ordering Information

Note that each Bio-Plex™ amine coupling kit contains enough reagents for 30 coupling reactions and each 1 ml tube of Bio-Plex COOH beads contains enough beads for ten coupling reactions. One coupling kit and three 1 ml tubes of beads will produce enough protein-coupled beads for approximately sixty 96-well microtiter plates using 5,000 beads per well.

Bio-Plex Assay Service

Bio-Rad offers a Bio-Plex assay service for multiplexing existing Bio-Plex assays. For more information on this Bio-Plex assay service, call your local Bio-Rad office, or in the US, call 1-800-424-6723.

Catalog #	Description
Coupling Kits	
171-406001	Bio-Plex Amine Coupling Kit, 30 reactions, includes 4 ml bead wash buffer, 16 ml bead activation buffer, 135 ml PBS, pH 7.4, 10 ml blocking buffer, 25 ml storage buffer, 105 ml staining buffer, 40 coupling reaction tubes
Beads	
171-506024	Bio-Plex COOH Bead 24, 1.25 x 10^7 beads/ml, 1 ml
171-506025	Bio-Plex COOH Bead 25, 1.25 x 10^7 beads/ml, 1 ml
171-506026	Bio-Plex COOH Bead 26, 1.25 x 10^7 beads/ml, 1 ml
171-506027	Bio-Plex COOH Bead 27, 1.25 x 10^7 beads/ml, 1 ml
171-506028	Bio-Plex COOH Bead 28, 1.25 x 10^7 beads/ml, 1 ml
171-506042	Bio-Plex COOH Bead 42, 1.25 x 10^7 beads/ml, 1 ml
171-506043	Bio-Plex COOH Bead 43, 1.25 x 10^7 beads/ml, 1 ml
171-506044	Bio-Plex COOH Bead 44, 1.25 x 10^7 beads/ml, 1 ml
171-506045	Bio-Plex COOH Bead 45, 1.25 x 10^7 beads/ml, 1 ml
171-506046	Bio-Plex COOH Bead 46, 1.25 x 10^7 beads/ml, 1 ml

NOTE: There are a total of 100 different Bio-Plex COOH beads available. To order any of the remaining 90 Bio-Plex COOH beads not listed here, contact your local Bio-Rad sales representative.

Reagent Kits

171-304000	Bio-Plex Cytokine Reagent Kit, 1 x 96-well, includes 75 ml assay buffer, 150 ml wash buffer, 15 ml detection antibody diluent, 100 µl streptavidin-PE (100x), 96-well sterile filter plate, sealing tape, instructions
171-304004	Bio-Plex Phosphoprotein Reagent Kit, 1 x 96-well, includes 10 ml assay buffer, 150 ml wash buffer, 10 ml detection antibody diluent, 100 µl streptavidin-PE (100x), 96-well filter plate, sealing tape, instructions

Sample Preparation Kits

171-305000	Bio-Plex Human Serum Diluent Kit, 1 x 96-well, includes 15 ml sample diluent, 10 ml standard diluent
171-305004	Bio-Plex Mouse Serum Diluent Kit, 1 x 96-well, includes 15 ml sample diluent, 10 ml standard diluent
171-304011	Bio-Plex Cell Lysis Kit, 1 x 96-well, includes 150 ml cell wash buffer, 25 ml cell lysis buffer, 100 µl factor 1 (250x), 50 µl factor 2 (500x)

Section 13
References

Carson R and Vignali D, Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay, J Immunol Methods 227, 41–52 (1999)

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

Kettman J, Davies T, Chandler D, Oliver K, Fulton R, Classification and properties of 64 multiplexed microsphere sets, Cytometry 33, 234–243 (1998)

Vignali D, Multiplexed particle-based flow cytometric assays, J Immunol Methods 243, 243–255 (2000)