Bio-Plex Amine Coupling Kit

Instruction Manual

For research use only. Not for diagnostic procedures.

BIO-RAD

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Section 1
Description

Bio-Plex Pro Magnetic COOH Beads and Bio-Plex COOH Beads (nonmagnetic) are carboxylated beads that enable users to build their own multiplex assays for use with either magnetic- or vacuum-based wash stations. These beads are identical to MagPlex Microspheres, developed by Luminex Corporation, and are internally labeled with two fluorescent dyes for bead identification. The beads come in a 1 ml volume with 1.25 x 10^7 beads/ml. The Bio-Plex Amine Coupling Kit (Bio-Rad Laboratories, Inc., catalog #171406001) provides a complete set of reagents for coupling proteins to the beads. This instruction manual describes the coupling procedures for protein analytes.

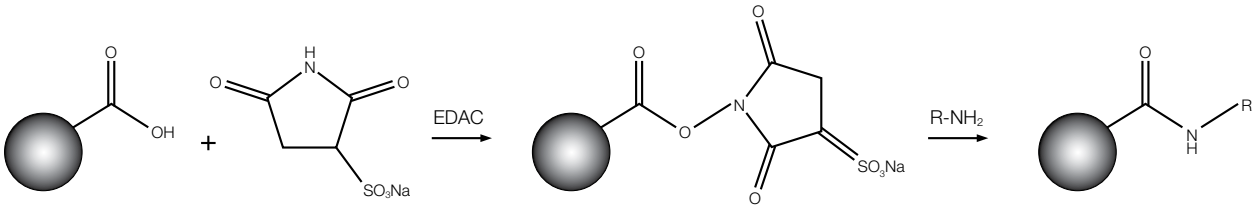
The Bio-Plex Amine Coupling Kit contains all of the buffers necessary to covalently couple 6–150 kD proteins to 6.5 µm Bio-Plex Pro Magnetic COOH Beads (magnetic, carboxylated) or 5.5 µm Bio-Plex COOH Beads (nonmagnetic, carboxylated) in under three hours. Coupling is achieved via carbodiimide reactions involving the primary amino groups on the protein and the carboxyl functional groups on the bead surface. The covalent attachment is permanent, leaving no unbound protein after cleanup, even after months of storage. The contents of the kit are sufficient for 30 coupling reactions. Each coupling reaction requires 1.25 x 10^6 carboxylated 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 Multiplex Immunoassay System. The bead yield per coupling reaction is approximately 80%, providing enough protein-coupled beads for four 96-well microtiter plates, assuming 2,500 beads per well.

The coupling accessories for multiplex assay development complement the Bio-Plex Assay products and associated kits. Visit **bio-rad.com/Bio-Plex** for a current listing of Bio-Plex products.

Section 2
Principle

Amine 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. EDAC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) reacts with carboxyl groups on the bead surface to form an active O-acylisourea intermediate. This intermediate forms a more stable ester using S-NHS (N-hydroxysulfosuccinimide). The ester reacts with the primary amines (NH_2 groups) of proteins.



S-NHS Active Ester

A number of buffers can be used successfully in this coupling reaction. As no buffer is ideal for every ligand, the protocols provided in this manual do not contain recommendations for specific buffers. Generally, the pH at which a coupling reaction occurs should be compatible with the solubility of the ligand of interest. Phosphate buffered saline (PBS) and 2-(N-morpholino)ethanesulfonic acid (MES) buffers are two popular choices. PBS buffer is provided in the kit.

Protein Preparation

The coupling procedure can be used to covalently couple water-soluble proteins ranging in size from 6–150 kD via carboxyl groups on the bead surface. The protein sample must be free of sodium azide, bovine serum albumin (BSA), glycine, Tris, or amine-containing additives and must be suspended in PBS, pH 7.4 (included with kit). Optimal protein coupling conditions must be established empirically. First, determine how much protein will be required for the coupling reaction to promote optimal binding between the bound protein and its complementary ligand in the protein assay.

Table 1 provides examples of optimal amounts per coupling reaction for four proteins of different molecular weights (MW). Note that these values are guidelines and should be empirically validated for each protein, using a functional assay.

Table 1. Optimal amount of protein for one coupling reaction.

Protein	MW, kD	Mass, µg
Insulin	6	5
IL-10	18.6	2
Erk	44	11
IgG	150	9

Erk, extracellular signal-regulated kinase; IgG, immunoglobulin G; IL-10, interleukin-10; MW, molecular weight.

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 combined with a phycoerythrin (PE)-labeled antibody that binds to the coupled protein. The binding of the antibody to the protein-coupled beads is then confirmed using the Bio-Plex Multiplex Immunoassay 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. The protein coupling validation procedure provides a rapid assessment of the relative amount of protein coupled to the beads; however, this procedure does not verify the functionality of the protein.

Section 3
Kit Contents and Storage

Table 2. Contents of the Bio-Plex Amine Coupling Kit.

Components	Quantity
Bio-Plex Blocking Buffer	1 bottle (10 ml)
Bio-Plex Bead Activation Buffer	1 bottle (85 ml)
Bio-Plex Storage Buffer	1 bottle (25 ml)
Bio-Plex Staining Buffer	1 bottle (105 ml)
Bio-Plex PBS Buffer, pH 7.4	1 bottle (135 ml)
Bio-Plex Bead Wash Buffer	1 bottle (4 ml)
Coupling Reaction Tubes	40 tubes

Note: When using this kit with the Bio-Plex Pro Human SARS-CoV-2 Neutralization Antibody Custom Assay Developer Kit (#17007632), substitute the included Storage Buffer with Bio-Plex Pro Serology Beads Storage Buffer (#12016837).

Storage and Stability

Kit contents should be stored at 4°C and never frozen. All components are guaranteed for a minimum of 6 months from the date of purchase when stored as specified.

Section 4
Required Materials

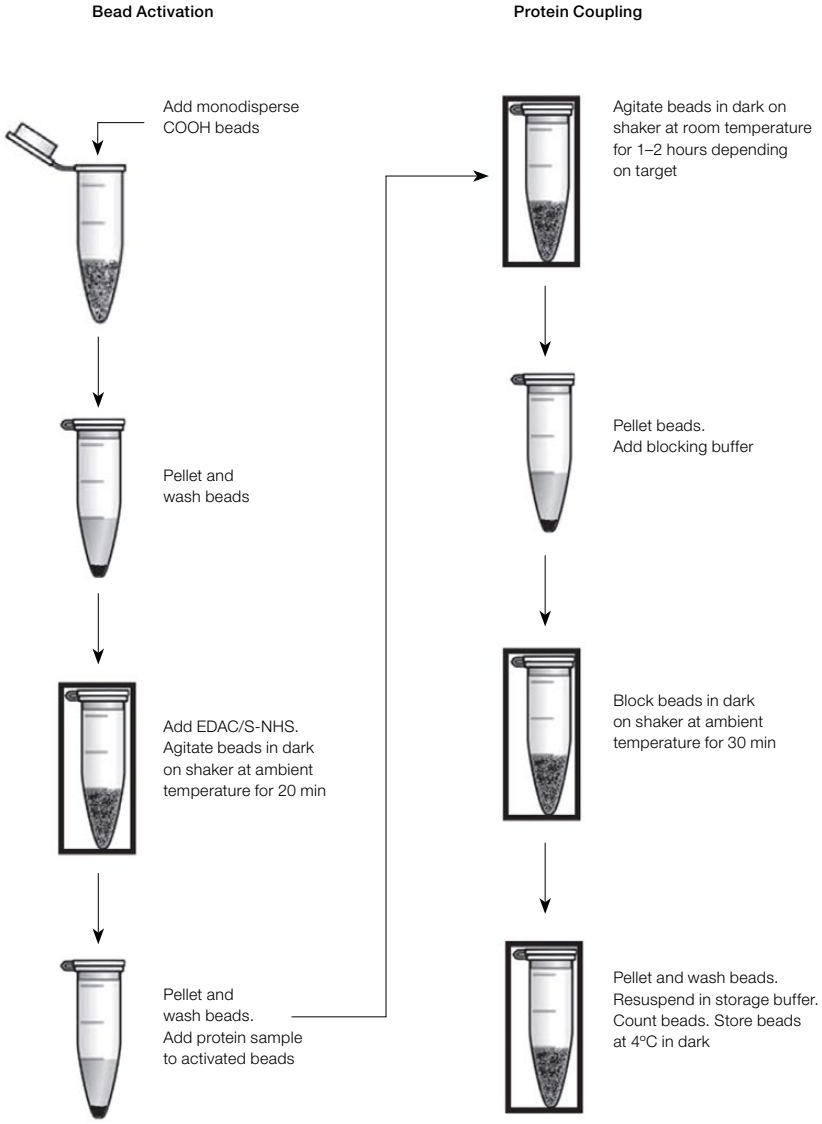
Table 3 provides a list of materials that are required to fully carry out the procedures in this manual. In many cases, the listed products can be substituted with equivalent products.

Table 3. Required materials.

Item	Ordering Information
Bio-Plex System For coupling validation	Visit **bio-rad.com/Bio-Plex** for ordering information.
Bio-Plex COOH Beads or Bio-Plex Pro Magnetic COOH Beads	Visit **bio-rad.com/Bio-Plex** for ordering information.
Bio-Plex Validation Kit 4.0 **Note:** Run the validation kit monthly to ensure optimal performance of fluidics and optics systems	Bio-Rad catalog #171203001
Bio-Plex Calibration Kit **Note:** Run the calibration kit daily to standardize the fluorescence signal	Bio-Rad catalog #171203060
Bio-Plex Pro Wash Station For use with magnetic bead–based assays only	Bio-Rad catalog #30034376
16-Tube SureBeads Magnetic Rack For magnetic bead separation in 1.5 ml tubes	Bio-Rad catalog #1614916
LifeSep 96F Separator For magnetic bead separation in 96-well plates	Dexter Magnetic Technologies catalog #2501008
BR-2000 Vortexer	Bio-Rad catalog #1660610
Model 16K Microcentrifuge	Bio-Rad catalog #1660602
MTS 2/4 Digital Microtiter Shaker For 2 or 4 microtiter plates	IKA catalog #3208000
Branson Ultrasonic Cleaner Model 1510R-DTH	VWR catalog #21812-175
TC20 Automated Cell Counter	Bio-Rad catalog #1450102
Protein Assays *DC* Protein Assay Kit I, 450 assays, bovine γ-globulin standard *DC* Protein Assay Kit II, 450 assays, BSA standard	 Bio-Rad catalog #5000111 Bio-Rad catalog #5000112
Buffer Exchange Columns Micro Bio-Spin P-6 Gel Columns, Tris Buffer, pkg of 25 (MW limit: 6,000) Micro Bio-Spin P-30 Gel Columns, Tris Buffer, pkg of 25 (MW limit: 40,000)	 Bio-Rad catalog #7326221 Bio-Rad catalog #7326223
Chemicals EDAC S-NHS	 Bio-Rad catalog #1530990 Thermo Fisher Scientific Inc., catalog #24510
Sterilized Reagent Reservoirs, 200 reservoirs	Bio-Rad catalog #2244872
Other: antibody specific for coupled protein (labeled with PE or biotin), streptavidin-PE, calibrated pipets, pipet tips, sterile distilled water, weigh boats, absorbent paper towels, aluminum foil, 96-well flat bottom plates, 15 ml polypropylene tubes for large-scale coupling	

Section 5
Assay Workflow

General Protein Coupling Workflow



Section 6
Protein Preparation

Bring all buffers to room temperature prior to 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.

If your protein sample is already suspended in PBS, pH 7.4, and does not contain sodium azide, BSA, glycine, Tris, or amine-containing additives, determine the protein concentration with the Bio-Rad *DC* Protein Assay Kit I or II or any other protein assay of choice and skip to protein coupling (section 7). If your protein sample contains sodium azide, BSA, glycine, Tris, or amine-containing additives, follow the buffer exchange procedure using Micro Bio-Spin Columns filled with Bio-Gel P-6 or P-30 Gel. Alternatively, dialyze your samples overnight in PBS, pH 7.4.

Buffer Exchange Protocol

1. Use one Micro Bio-Spin P-6 or P-30 Gel Column for each different protein requiring buffer exchange with PBS, pH 7.4. The exclusion limit for Bio-Gel P-6 Gel is 6,000 Da, whereas the exclusion limit for Bio-Gel P-30 is 40,000 Da.

   Note: Up to 20% of the protein can be lost during this buffer exchange procedure. Refer to the Micro Bio-Spin Chromatography Columns manual (#4006051) for more details.

2. 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).

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

4. Apply 500 µl 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.

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

6. Place the column in a clean and labeled 1.5 or 2 ml microcentrifuge tube. Carefully apply the protein sample (30–100 µl) directly to the center of the column.

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

7. 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 I or II or any other protein assay of choice.

   Note: One coupling reaction requires 5–12 µg of 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 use. Protect the beads from light by covering the tubes with aluminum foil during the procedure.

Remove EDAC 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 EDAC and S-NHS should be discarded after five uses.

Sample Protocol Using Bio-Plex Pro Magnetic COOH Beads

Notes

- Beads must be completely protected from light throughout this procedure
- The stock bead concentration is 1.25 x 10^7 beads/ml
- 1x scale = 1.25 x 10^6 beads
- Volume stock beads required (ml) = ([1.25 x 10^6] x [scale]) + (1.25 x 10^7 bead/ml)

Procedure

1. Vortex the stock uncoupled beads at speed 7 for 30 sec, then sonicate for 15 sec.
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.
3. Place the tube into a magnetic separator and allow separation to occur for 30–60 seconds.
4. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the beads.
5. Remove the tube from the magnetic separator and resuspend the beads in 100 µl bead wash buffer by vortexing on a medium setting for approximately 30 seconds.
6. Place the tube into a magnetic separator and allow separation to occur for 30–60 seconds.
7. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the beads.
8. Remove the tube from the magnetic separator and resuspend the washed beads in 80 µl of bead activation buffer by vortexing on a medium setting for approximately 30 seconds.
9. Add 10 µl of 50 mg/ml S-NHS (prepared in bead activation buffer immediately prior to its use) to the beads and mix gently by vortexing on a medium setting.
10. Add 10 µl of 50 mg/ml EDAC (prepared in bead activation buffer immediately prior to its use) to the beads and mix gently by vortexing on a medium setting.
11. Cover the coupling reaction tube with aluminum foil and agitate the beads on a shaker at 850 ± 50 rpm for 20 min at room temperature.

12. Add 150 µl of PBS, pH 7.4, and vortex at medium speed for 10 sec.
13. Place the tube into a magnetic separator and allow separation to occur for 30–60 seconds.
14. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the beads.
15. Repeat steps 12–14.
16. Resuspend the activated beads with 100 µl of PBS, pH 7.4.
17. Vortex the activated beads at medium speed for 30 sec.
18. Add 5–12 µg of protein prepared in section 6 to the activated beads.
19. Bring total volume to 500 µl with PBS, pH 7.4.
20. Mix coupling reaction by vortexing on a medium setting.
21. Incubate at room temperature for 1–2 hours (incubation time should be optimized by end user and may vary depending on the protein coupled) on a shaker set to 850 ± 50 rpm.
22. Place the tube into a magnetic separator and allow separation to occur for 30–60 seconds.
23. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the beads.
24. Remove the tube from the magnetic separator and resuspend the coupled beads in 500 µl of PBS, pH 7.4.
25. Vortex the beads at medium speed for 15 sec.
26. Place the tube into a magnetic separator and allow separation to occur for 30–60 seconds.
27. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the beads.
28. Resuspend the coupled beads with 250 µl of blocking buffer.
29. Vortex the beads at medium speed for 15 sec.
30. Cover the coupling reaction tube with aluminum foil and agitate the beads on a shaker set to 850 ± 50 rpm for 30 min at room temperature.
31. Place the tube into a magnetic separator and allow separation to occur for 30–60 seconds.
32. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the beads.
33. Remove the tube from the magnetic separator and resuspend the beads in 500 µl of storage buffer by vortexing on a medium setting for 20 sec.

    Note: For the Bio-Plex Pro Human SARS-CoV-2 Neutralization Antibody Developer Kit, use the Bio-Plex Pro Serology Beads Storage Buffer instead.
34. Place the tube into a magnetic separator and allow separation to occur for 30–60 seconds..
35. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the beads.

36. Remove the tube from the magnetic separator and resuspend the coupled and washed beads in 150 µl of storage buffer by vortexing for 15 sec on a medium setting.

    Note: For the Bio-Plex Pro Human SARS-CoV-2 Neutralization Antibody Custom Assay Developer Kit, use the Bio-Plex Pro Serology Beads Storage Buffer instead.

37. Determine the bead concentration using a TC20 Automated Cell Counter or a hemocytometer.

38. Store coupled beads refrigerated at 2–8°C in the dark.

Sample Protocol Using Bio-Plex COOH Beads (nonmagnetic)

Notes

- Beads must be completely protected from light throughout this procedure
- The stock bead concentration is 1.25 x 10^7 beads/ml
- 1x scale = 1.25 x 10^6 beads
- Volume stock beads required (ml) = ([1.25 x 10^6] x [scale]) + (1.25 x 10^7 bead/ml)

Procedure

1. Vortex the stock uncoupled beads at speed 7 for 30 sec, then sonicate for 15 sec.
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.
3. Centrifuge the beads at 14,000 x g for 4 min.
4. Carefully remove and discard the supernatant from the bead pellet.
5. Resuspend the beads in 100 µl of bead wash buffer by vortexing for approximately 30 seconds.
6. Centrifuge the beads at 14,000 x g for 4 min.
7. Carefully remove and discard the supernatant from the bead pellet.
8. Resuspend the washed beads in 80 µl of bead activation buffer by vortexinig for approximately 30 seconds.
9. Add 10 µl of 50 mg/ml S-NHS (prepared in bead activation buffer immediately prior to its use) to the beads and mix gently by vortexing.
10. Add 10 µl of 50 mg/ml EDAC (prepared in bead activation buffer immediately prior to its use) to the beads and mix gently by vortexing.
11. Cover the coupling reaction tube with aluminum foil and agitate the beads on a shaker for 20 min at room temperature.
12. Add 150 µl of PBS, pH 7.4, and vortex the activated beads at high speed for 10 sec.
13. Centrifuge the beads at 14,000 x g for 4 min.
14. Carefully remove and discard the supernatant from the bead pellet.

15. Repeat steps 12–14.
16. Resuspend the activated beads with 100 µl of PBS, pH 7.4.
17. Vortex the activated beads at medium speed for 30 sec.
18. Add 5–12 µg of protein prepared in section 6 to the activated beads.
19. Bring total volume to 500 µl with PBS, pH 7.4.
20. Mix coupling reaction by vortexing.
21. Incubate at room temperature between 1–2 hours (incubation time should be optimized by end user and may vary depending on the protein coupled) on a shaker set to 850 ± 50 rpm.
22. Centrifuge the beads at 14,000 x g for 4 min.
23. Carefully remove and discard the supernatant from the bead pellet.
24. Resuspend the coupled beads in 500 µl of PBS, pH 7.4.
25. Centrifuge the beads at 14,000 x g for 4 min.
26. Carefully remove and discard the supernatant from the bead pellet.
27. Resuspend the coupled beads with 250 µl of blocking buffer.
28. Vortex the beads at medium speed for 15 sec.
29. Cover the coupling reaction tube with aluminum foil and agitate the beads on a shaker for 30 min at room temperature.
30. Centrifuge the beads at 14,000 x g for 4 min.
31. Carefully remove and discard the supernatant from the bead pellet.
32. Resuspend the beads in 500 µl of storage buffer by vortexing for 20 sec.

    Note: For the Bio-Plex Pro Human SARS-CoV-2 Neutralization Antibody Custom Assay Developer Kit, use the Bio-Plex Pro Serology Beads Storage Buffer instead.
33. Centrifuge the beads at 14,000 x g for 6 min.
34. Carefully remove and discard the supernatant from the bead pellet.
35. Resuspend the coupled and washed beads in 150 µl of storage buffer.

    Note: For the Bio-Plex Pro Human SARS-CoV-2 Neutralization Antibody Custom Assay Developer Kit, use the Bio-Plex Pro Serology Beads Storage Buffer instead.
36. Determine the bead concentration using a TC20 Automated Cell Counter or a hemocytometer.
37. Store coupled beads refrigerated at 2–8°C in the dark.

Section 8
Validation of Coupling

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 an antibody is coupled to the beads, ensure that the antibody used in this procedure is specific for the host species of your coupled antibody. If you have coupled a mouse anti-human antibody, your PE-labeled antibody should be directed against the mouse antibody (for example, goat anti-mouse or rabbit anti-mouse).

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 5,000 coupled beads to each of the two tubes.
3. If using a biotinylated antibody, skip to step 4. If 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." Add 50 µl of staining buffer to negative control tubes. Do not add antibody to the negative control tube. Cover the tubes with aluminum foil and agitate the beads with a shaker at room temperature for 30 min. Skip to step 6.
4. 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 shaker at room temperature for 30 min.
5. 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". 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.
6. Centrifuge tubes at 14,000 x g for 4 min. Carefully remove and discard the supernatant.
7. Resuspend the pellet in 125 µl of storage buffer.

   Note: For the Bio-Plex Pro Human SARS-CoV-2 Neutralization Antibody Custom Assay Developer Kit, use the Bio-Plex Pro Serology Beads Storage Buffer instead.
8. 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 and read the plate on the Bio-Plex System.

Section 9
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.

Problem and Possible Causes	Possible Solutions
Low MFI signal in validation procedure	
EDAC and S-NHS may have expired	Use fresh EDAC and S-NHS for each conjugation
Purified protein was not used immediately during the conjugation procedure 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 EDAC	Add S-NHS to the beads immediately after adding EDAC
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
Inconsistent bead count values	
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 a 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

Bio-Rad Technical Support

The Bio-Rad Technical Support department in the U.S. is open Monday through Friday, 5:00 AM to 5:00 PM, Pacific time.

Phone: 1-800-424-6723, option 2
Email: support@bio-rad.com (U.S. and Canada only)

For technical assistance outside the U.S. and Canada, contact your local technical support office or click the Contact Us link at **bio-rad.com**.

Section 10
Safety Considerations

Eye protection and gloves are recommended while using this product. Consult the relevant Safety Data Sheet for additional information.

Visit bio-rad.com/Bio-Plex for more information.

Bio-Rad Laboratories, Inc.

Life Science Group

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