Bio-Plex Pro™ Magnetic COOH Beads
Bio-Plex® COOH Beads
Amine Coupling Kit

Instruction Manual

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

For research use only. Not for diagnostic procedures.

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Section 1
Introduction

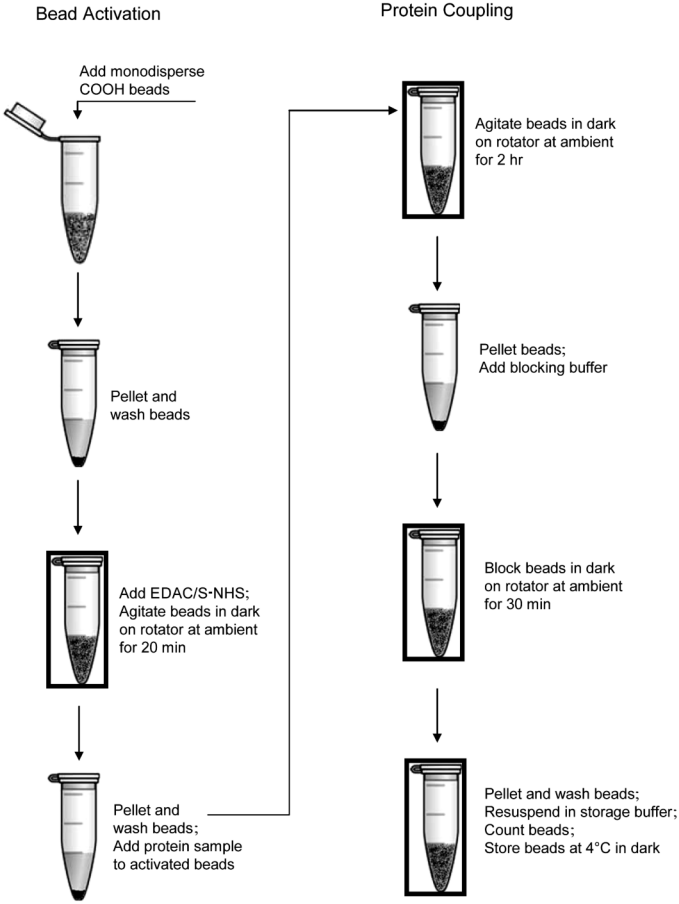
The Bio-Plex Pro Magnetic COOH Beads and Bio-Plex COOH Beads are suited for coupling to proteins or nucleic acids. Bio-Plex Pro Magnetic Beads are identical to MagPlex® Microspheres, and Bio-Plex COOH Beads are identical to Microplex Microspheres, both developed by Luminex corporation. This instruction manual describes the coupling procedures.

The Bio-Plex Amine Coupling Kit (catalog #171-406001) 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 1.25×10^6 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 suspension array system. The bead yield per coupling reaction is approximately 80%, enough protein-coupled beads for four 96-well microtiter plates using 2,500 beads per well.

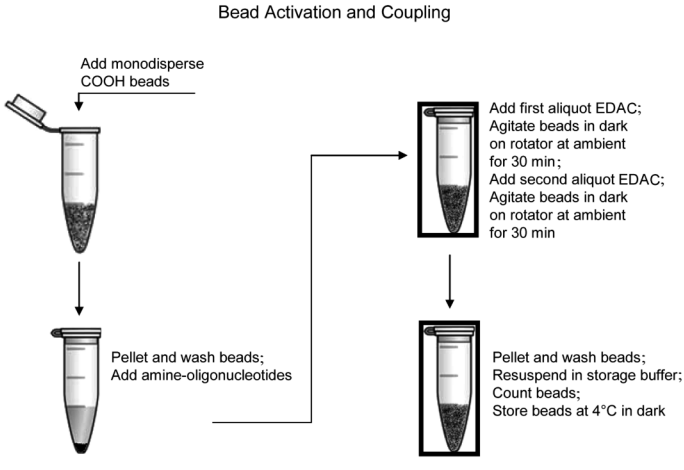
For nucleic acid oligonucleotide coupling, refer to this manual for ordering information and buffer recipes. The amine coupling kit contains buffers used for coupling proteins only.

The coupling accessories for multiplex assay development complement the Bio-Plex assay products and associated kits. For a current listing of Bio-Plex products,visit us on the Web at www.bio-rad.com/bio-plex.

General Protein Coupling Workflow



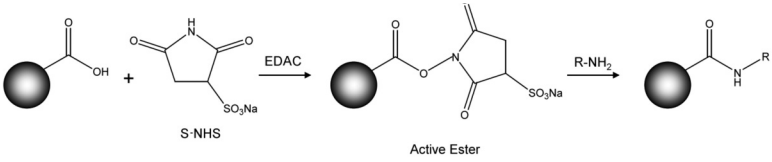
General Oligonucleotide Coupling Workflow



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 surface of the beads 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 or amine-modified oligonucleotides to form a covalent bond (amide linkages).



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 to the solubility of the ligand of interest. PBS and MES buffers are two popular choices mentioned in this manual. PBS buffer is provided in the kit. MES buffer can be prepared according to the formulation provided in Section 8 for the coupling of oligonucleotides.

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 the 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. 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.

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 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 suspension 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. 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
Product Description

Bio-Plex Pro Magnetic COOH Beads

For magnetic or vacuum separation methods
All regions are at a concentration of 1.25 x 10^7 beads/mL

MC10026-01 Bio-Plex Pro Magnetic COOH Beads, Region 26, 1 ml
MC10027-01 Bio-Plex Pro Magnetic COOH Beads, Region 27, 1 ml
MC10028-01 Bio-Plex Pro Magnetic COOH Beads, Region 28, 1 ml
MC10029-01 Bio-Plex Pro Magnetic COOH Beads, Region 29, 1 ml
MC10034-01 Bio-Plex Pro Magnetic COOH Beads, Region 34, 1 ml
MC10035-01 Bio-Plex Pro Magnetic COOH Beads, Region 35, 1 ml
MC10036-01 Bio-Plex Pro Magnetic COOH Beads, Region 36, 1 ml
MC10037-01 Bio-Plex Pro Magnetic COOH Beads, Region 37, 1 ml
MC10043-01 Bio-Plex Pro Magnetic COOH Beads, Region 43, 1 ml
MC10044-01 Bio-Plex Pro Magnetic COOH Beads, Region 44, 1 ml
MC10045-01 Bio-Plex Pro Magnetic COOH Beads, Region 45, 1 ml
MC10046-01 Bio-Plex Pro Magnetic COOH Beads, Region 46, 1 ml
MC10049-01 Bio-Plex Pro Magnetic COOH Beads, Region 49, 1 ml
MC10052-01 Bio-Plex Pro Magnetic COOH Beads, Region 52, 1 ml
MC10053-01 Bio-Plex Pro Magnetic COOH Beads, Region 53, 1 ml
MC10054-01 Bio-Plex Pro Magnetic COOH Beads, Region 54, 1 ml
MC10055-01 Bio-Plex Pro Magnetic COOH Beads, Region 55, 1 ml
MC10062-01 Bio-Plex Pro Magnetic COOH Beads, Region 62,1 ml
MC10063-01 Bio-Plex Pro Magnetic COOH Beads, Region 63, 1 ml
MC10064-01 Bio-Plex Pro Magnetic COOH Beads, Region 64, 1 ml
MC10065-01 Bio-Plex Pro Magnetic COOH Beads, Region 65, 1 ml

Additional bead regions and larger volumes are available with longer delivery times. Call your local Bio-Rad Technical Service center for more information.
NOTE: The following bead regions are not compatible with the Bio-Plex 100/200 or Luminex 100/200 systems: 1–5, 10, 11, 16, 17, 23, 24, 31, 32, 40, 41, 50, 60, 71, 92, and all regions over 100.

Bio-Plex COOH Beads

For vacuum separation methods only.
All regions are at a concentration of 1.25 x 10^7 beads/mL

171-506011 Bio-Plex COOH Beads, Region 11, 1 ml
171-506016 Bio-Plex COOH Beads, Region 16, 1 ml
171-506018 Bio-Plex COOH Beads, Region 28, 1 ml
171-506020 Bio-Plex COOH Beads, Region 20, 1 ml
171-506024 Bio-Plex COOH Beads, Region 24, 1 ml
171-506025 Bio-Plex COOH Beads, Region 25, 1 ml
171-506026 Bio-Plex COOH Beads, Region 26, 1 ml
171-506027 Bio-Plex COOH Beads, Region 27, 1 ml
171-506028 Bio-Plex COOH Beads, Region 28, 1 ml
171-506030 Bio-Plex COOH Beads, Region 30, 1 ml
171-506031 Bio-Plex COOH Beads, Region 31, 1 ml
171-506033 Bio-Plex COOH Beads, Region 33, 1 ml
171-506035 Bio-Plex COOH Beads, Region 35, 1 ml
171-506038 Bio-Plex COOH Beads, Region 38, 1 ml
171-506042 Bio-Plex COOH Beads, Region 42, 1 ml
171-506043 Bio-Plex COOH Beads, Region 43, 1 ml
171-506044 Bio-Plex COOH Beads, Region 44, 1 ml
171-506045 Bio-Plex COOH Beads, Region 45, 1 ml
171-506046 Bio-Plex COOH Beads, Region 46, 1 ml
171-506050 Bio-Plex COOH Beads, Region 50, 1 ml
171-506051 Bio-Plex COOH Beads, Region 51, 1 ml
171-506052 Bio-Plex COOH Beads, Region 53, 1 ml
171-506053 Bio-Plex COOH Beads, Region 53, 1 ml
171-506056 Bio-Plex COOH Beads, Region 56, 1 ml
171-506066 Bio-Plex COOH Beads, Region 66, 1 ml

Additional bead regions and larger volumes are available with longer delivery times. Call your local Bio-Rad Technical Service center for more information.

Required Reagents for Protein Coupling

Bio-Plex Amine Coupling Kit (catalog #171-406001)

Bead wash buffer
Bead activation buffer
PBS, pH 7.4
Blocking buffer
Storage buffer
Staining buffer
Coupling reaction tubes

Required Reagents for Oligonucleotide Coupling

See Section 8 for buffer recipes

Other Required Reagents

EDAC coupling reagent, 5g (catalog #153-0990)

Section 4
Storage and Stability

Protect the photosensitive 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
Recommended Materials

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

Bio-Plex Suspension Array System
See bulletin 5405 or go to **www.bio-rad.com/bio-plex** for ordering information

Bio-Plex Suspension Array System Accessories
Bio-Plex validation kit, Bio-Rad catalog #171-203000
Bio-Plex calibration kit, Bio-Rad catalog #171-203060
Bio-Plex Pro wash station (for magnetic washing only), Bio-Rad catalog #300-34376
Bio-Plex Pro II wash station (for magnetic or vacuum washing), Bio-Rad catalog #300-34377
Bio-Rad Aurum vacuum manifold (alternative for vacuum washing), Bio-Rad catalog #732-6470

Magnetic separator for 1.5 mL tubes
Dynal MPC-S magnetic bead separator, Dynal Biotech catalog #120-20D
Dynal MPC-S magnetic bead separator for 1.5 mL tubes, Dynal Biotech (cat#120-20D)
Dynal MPC-S magnetic bead separator for 15 mL tubes, Dynal Biotech (cat#120-01D)

Magnetic Separators for Round-Bottom Plates
96-well plate magnet, PerkinElmer catalog #5083175
LifeSep 96F, Dexter Magnetic Technologies catalog #2501008

Vortexer
VWR vortexer mini, VWR catalog #58816-121

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

Microtiter Plate Shaker
MTS 2/4 shaker for 2 or 4 microplates, IKA catalog #3208000

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

Cell Counter
Z2 Coulter Counter, Beckman Coulter Model Z2, catalog #6605700

Protein Assay
Microcentrifuge model 5415C, VWR catalog #20901-051

Microtiter Plate Shaker
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 (EDAC), Pierce catalog #22980 N-hydroxysulfosuccinimide (S-NHS), Pierce catalog #24510

Reagent Reservoir
VistaLab catalog #3054-2002
VistaLab catalog #3054-1004

Other Materials
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, 15 ml conical tubes (for large scale coupling).

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.

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.

2. Use 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 website (**www.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 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 (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.

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 use. Protect the beads from light as much as possible by covering the tubes with aluminum foil during the procedure.

Remove EDAC and S-NHS from the –20ºC 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.

7.1 Sample Protocol Using Bio-Plex Pro Magnetic COOH Beads

Notes:

a. Beads must be completely protected from light throughout this procedure.
b. The stock bead concentration is 1.25×10^7 beads/ml.
c. 1x scale = 1.25×10^6 beads.
d. Volume stock beads required (ml) = $[(1.25 \times 10^6) \times (\text{scale})] \div [1.25 \times 107 \text{ bead/ml}]$

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 (1.25×10^6 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 to 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 vortex for approximately 30 seconds.

6. Place the tube into a magnetic separator and allow separation to occur for 30 to 60 sec.
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 vortex for approximately 30 sec.
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 vortex.
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 vortex.
11. Cover the coupling reaction tube with aluminum foil and agitate the beads on a shaker (or rotator) 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. Place the tube into a magnetic separator and allow separation to occur for 30 to 60 sec.
14. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the beads.
15. Repeat steps 12 to 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) 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 vortex.
21. Incubate for 2 h at room temperature with mixing on a shaker or rotor.

22. Place the tube into a magnetic separator and allow separation to occur for 30 to 60 sec.
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. Place the tube into a magnetic separator and allow separation to occur for 30 to 60 sec.
26. Resuspend the coupled beads with 250 µL of blocking buffer.
27. Vortex the beads at medium speed for 15 sec.
28. Cover the coupling reaction tube with aluminum foil and agitate the beads on a shaker (or rotator) for 30 min at room temperature.
29. Place the tube into a magnetic separator and allow separation to occur for 30 to 60 sec.
30. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the beads.
31. Remove the tube from the magnetic separator and resuspend the beads in 500 µL of storage buffer by vortex 20 sec.
32. Place the tube into a magnetic separator and allow separation to occur for 30 to 60 sec.
33. Remove the tube from the magnetic separator and resuspend the coupled and washed beads in 150 µL of storage buffer.
34. Determine the bead concentration using a Coulter Z2 counter or a hemocytometer.
35. Store coupled beads refrigerated at 2–8°C in the dark.

7.2 Sample Protocol Using Bio-Plex COOH Beads

Notes:

a. Beads must be completely protected from light throughout this procedure.
b. The stock bead concentration is 1.25×10^7 beads/ml.
c. 1x scale = 1.25×10^6 beads.
d. Volume stock beads required (ml) = $[(1.25 \times 10^6) \times (\text{scale})] \div [1.25 \times 10^7 \text{ bead/ml}]$

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 (1.25×10^6 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 bead wash buffer by vortex for approximately 30 sec.
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 vortex for approximately 30 sec.
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 vortex.
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 vortex.
11. Cover the coupling reaction tube with aluminum foil and agitate the beads on a shaker (or rotator) 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 to 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) 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 vortex.
21. Incubate for 2 h at room temperature with mixing on a shaker or rotor.
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 (or rotator) for 30 minutes 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 vortex 20 sec.
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.
36. Determine the bead concentration using a Coulter Z2 counter or a hemocytometer.
37. Store coupled beads refrigerated at 2–8°C in the dark.

Section 8
Oligonucleotide Buffers

Buffers	Reagent	Catalog Number	Final Concentration	Amount
0.1 MES, pH 4.5 (Coupling Buffer)	MES (2[N-Morpholino] ethanesulfonic acid)	Sigma M2933	0.1 M	4.88 g
	5 N NaOH	Fisher SS256-500	----	~ 5 drops
	Filter Sterilize and store at 4°C			
0.02% Tween-20 (Wash Buffer I)	TWEEN® 20 (Polyoxyethylenesorbitan monolaurate)	Sigma P9416	0.02%	50 µL
	Filter Sterilize and store at ambient			
0.1% SDS (Wash Buffer II)	SDS (Sodium lauryl sulfate) 10% solution	Sigma L4522	0.10%	2.5 mL
	Filter Sterilize and store at ambient			
TE, pH 8.0 (Sample Diluent)	Tris-EDTA Buffer, pH 8.0, 100X	Sigma T9285	1X	2.5 mL
	Filter Sterilize and store at ambient			
1.5 X TMAC Hybridization Solution (Microsphere Diluent)	5 M TMAC	Sigma T3411	4.5 M	225 mL
	20% Sarkosyl solution	Sigma L7414	0.15%	1.88 mL
	1 M Tris-HCl, pH 8.0	Sigma T3038	75 mM	18.75 mL
	0.5 M EDTA, pH 8.0	Invitrogen 15575-020	4 mM	3.0 mL
	Store at ambient			
1 X TMAC Hybridization Solution (Detection Buffer)	5 M TMAC	Sigma T3411	3 M	3.0 mL
	20% Sarkosyl solution	Sigma L7414	0.10%	1.25 mL
	1 M Tris-HCl, pH 8.0	Sigma T3038	50 mM	12.5 mL
	0.5 M EDTA, pH 8.0	Invitrogen 15575-020	4 mM	2 mL
	H_20	----	----	84.25 mL
	Store at ambient			
6X SSPET (Stringent Wash Buffer)	SSPE, 20X (Phosphate buffer, pH 7.4, sodium chloride, EDTA)	Sigma S2015	6X Concentrate	75 mL
	Triton® X-100	Sigma T9284	0.005%-0.01%	12.5-25 µL
	Filter Sterilize and store at ambient			

Section 9
Coupling of Amine-Modified Oligonucleotides

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

9.1 Sample Protocol Using Bio-Plex Pro Magnetic COOH Beads

Notes:

a. Beads must be completely protected from light throughout this procedure.
b. The stock bead concentration is 1.25×10^7 beads / ml.
c. 1x scale = 1.25×10^6 beads.
d. Volume stock beads required (ml) = $[(1.25 \times 10^6) \times (\text{scale})] \div [1.25 \times 10^7 \text{ bead/ml}]$
e. Capture oligo concentration should be optimized for the specific reagents in use. Typically, optimal coupling is achieved using 0.2 to 1 nmole per scale.

Procedure:

1. Bring a fresh aliquot of –20°C desiccated EDAC powder to room temperature.
2. Resuspend the amine-substituted oligonucleotide (probe or capture oligo) to 1 mM (1 nmole/µl) in dH_2O.
3. Resuspend the stock vial of beads by vortexing at speed 7, followed by sonication for 15 sec.
4. Transfer desired volume of the stock beads (based on the scale) to an appropriately sized tube. For 1–25× scale, use a 1.5 ml siliconized clear microcentrifuge tube.

5. Place the tube into a magnetic separator and allow separation to occur for 30–60 sec.
6. With the tube still positioned in the magnetic separator, carefully remove the supernatant without disturbing the beads.
7. Remove the tube from the magnetic separator and resuspend the beads in 50 µl of 0.1 M MES, pH 4.5, by vortex and sonication for approximately 20 sec.
8. Prepare a 1:10 dilution of the 1 mM capture oligo in dH_2O (0.1 nmole/µl).
9. Add 2 µl (0.2 nmole) of the 1:10 diluted capture oligo to the resuspended beads and mix by vortexing.
10. Prepare a fresh solution of 10 mg/ml EDAC in dH_2O.
11. Add 2.5 µl of fresh 10 mg/ml EDAC to the beads (25 µg or ≅ [0.5 µg/µl]final) and mix by vortexing.
12. Incubate for 30 min at room temperature in the dark.
13. Use a fresh aliquot of EDAC powder to prepare a second fresh solution of 10 mg/ml EDAC in dH_2O.
14. Add 2.5 µl of fresh 10 mg/ml EDAC to the beads and mix by vortexing.
15. Incubate for 30 min at room temperature in the dark.
16. Add 1.0 ml of 0.02% Tween-20 to the coupled beads (use the same volume for 1–25x scale).
17. Place the tube into a magnetic separator and allow separation to occur for 30–60 sec.
18. With the tube still positioned in the magnetic separator, carefully remove the supernatant without disturbing the beads.
19. Remove the tube from the magnetic separator and resuspend the coupled beads in 1.0 ml of 0.1% SDS by vortex (use the same volume for 1–25x scale).

20. Place the tube into a magnetic separator and allow separation to occur for 30–60 sec.

21. With the tube still positioned in the magnetic separator, carefully remove the supernatant without disturbing the bead pellet.

22. Remove the tube from the magnetic separator and resuspend the coupled beads in 100 µl of TE, pH 8.0, by vortex and sonication for approximately 20 sec.

23. Enumerate the coupled beads by hemacytometer:

    a. Dilute the resuspended coupled beads 1:100 in dH_2O.

    b. Mix thoroughly by vortex.

    c. Transfer 10 µl to the hemacytometer.

    d. Count the beads within the 4 large corners of the hemacytometer grid.

    e. Beads/µl = (sum of beads in 4 large corners) x 2.5 x 100 (dilution factor).

    f. Note: maximum is 50,000 beads/µl.

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

9.2 Sample Protocol Using Bio-Plex COOH Beads

Notes:

a. Beads must be completely protected from light throughout this procedure.
b. The stock bead concentration is 1.25×10^7 beads /ml.
c. 1x scale = 1.25×10^6 beads.
d. Volume stock beads required (ml) = $[(1.25 \times 10^6) \times (\text{scale})] \div [1.25 \times 10^7 \text{ bead/ml}]$

Procedure:

1. Bring a fresh aliquot of –20°C desiccated EDAC powder to room temperature.
2. Resuspend the amine-substituted oligonucleotide (probe or capture oligo) to 1 mM (1 nmole/µl) in dH_2O.
3. Resuspend the stock vial of beads by vortexing at speed 7, followed by sonication for 15 sec.
4. Transfer desired volume of the stock beads (based on the scale) to an appropriately sized tube. For 1–25× scale, use a 1.5 ml siliconized clear microcentrifuge tube.
5. Pellet the beads by microcentrifugation at ≥ 8000 x g for 1–2 min.
6. Carefully remove the supernatant without disturbing the beads.
7. Resuspend the beads in 50 µl of 0.1 M MES, pH 4.5, by vortex and sonication for approximately 20 sec.
8. Prepare a 1:10 dilution of the 1 mM capture oligo in dH_2O (0.1 nmole/µl).
9. Add 2 µl (0.2 nmole) of the 1:10 diluted capture oligo to the resuspended beads and mix by vortexing.
10. Prepare a fresh solution of 10 mg/ml EDAC in dH_2O.
11. Add 2.5 µl of fresh 10 mg/ml EDAC to the beads (25 µg or ≅ [0.5 µg/µl]final) and mix by vortexing.
12. Incubate for 30 min at room temperature in the dark.

13. Use a fresh aliquot of EDAC powder to prepare a second fresh solution of 10 mg/ml EDAC in dH_2O.
14. Add 2.5 µl of fresh 10 mg/ml EDAC to the beads and mix by vortexing.
15. Incubate for 30 min at room temperature in the dark.
16. Add 1.0 ml of 0.02% Tween-20 to the coupled beads (use the same volume for 1–25x scale).
17. Pellet the beads by microcentrifugation at ≥ 8000 x g for 1–2 min.
18. Carefully remove the supernatant without disturbing the beads.
19. Resuspend the coupled beads in 1.0 ml of 0.1% SDS by vortex (use the same volume for 1–25x scale).
20. Pellet the beads by microcentrifugation at ≥ 8000 x g for 1–2 min.
21. Carefully remove the supernatant without disturbing the bead pellet.
22. Resuspend the coupled beads in 100 µl of TE, pH 8.0, by vortex and sonication for approximately 20 sec.
23. Enumerate the coupled beads by hemacytometer:
    a. Dilute the resuspended coupled beads 1:100 in dH_2O.
    b. Mix thoroughly by vortex.
    c. Transfer 10 µl to the hemacytometer.
    d. Count the beads within the 4 large corners of the hemacytometer grid.
    e. Beads/µl = (sum of beads in 4 large corners) x 2.5 x 100 (dilution factor).
    f. Note: maximum is 50,000 beads/µl.
24. Store coupled beads refrigerated at 2–8°C in the dark.

Section 10
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. 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).

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. 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 shaker or 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.

4. 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 only 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.

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 11 to read the coupled beads.

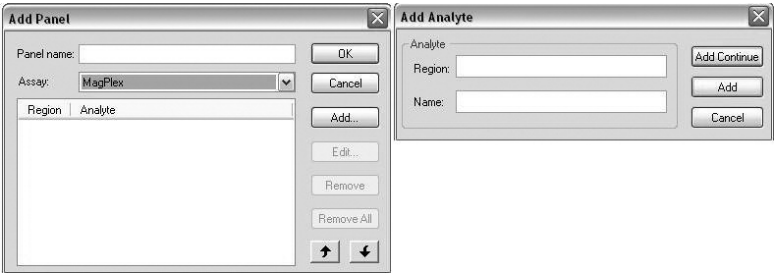
Section 11
Bio-Plex Protein Array System Operation

System Preparation

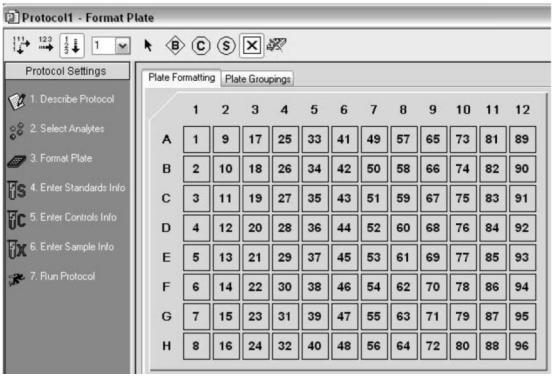
Recommendations for reading the coupled beads on the Bio-Plex suspension array system are listed below. Alternatively, refer to the Bio-Plex Manager™ software user guide.

1. Turn on the Bio-Plex array reader and microplate platform (and high-through put 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 reservior 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 reading 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.
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 the coupling.

6. Select Step 2 Select Analytes. Select Add Panel in the toolbar. An Add Panel dialog box will appear. Enter a name for the panel. In Bio- Plex Manager v5.0 and above, select the MagPlex bead type from the Assay Type pull-down menu for magnetic assays, or select the Bio-Plex bead type for non-magnetic assays. Enter the region number and name of your analyte in the designated fields." Please remove "and the name of your protein Select OK when you have entered your information. This panel will be saved for future use. You may also add an analyte to an existing panel by selecting Edit Panel.



7. Select Step 3 to format the plate layout.
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 50 beads per region with a sample size of 50 µl.
10. If analyzing the assay at the high PMT setting, ensure that this setting is selected prior to the run.

Analyzing Coupled Beads

1. Ensure the presence of sufficient sheath fluid and empty the waste bottle.
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. When data acquisition is complete, select Shut Down from the toolbar and follow the instructions to shut the reader down.

Section 12
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. Contact Luminex Tech Support for technical issues related to coupling of oligonucleotides (source: http://www.luminexcorp.com/support/tech_tips.html).

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
Wrong antibody was used in the validation procedure	Ensure that the correct species-specific antibody is being used for validation (if an antibody is coupled)

Possible Causes	Possible Solutions
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

Section 13
Safety Considerations

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

Section 14
References

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Section 15
Legal Notices

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