Luminex®
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xMAP® Antibody Coupling Kit Package Insert

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RUO For Research Use Only. Not for use in diagnostic procedures.

89-00002-00-319 Rev F
04/2021

Symbols Glossary

You will encounter these symbols throughout this manual. They represent warnings, conditions, identifications, instructions, and regulatory agencies.

Symbol	Meaning	Symbol	Meaning
	Catalog(ue) Number.		Batch Code.
	For Research Use Only. Not for use in diagnostic procedures.		Use-by date.
	Manufacturer.		Temperature Limit.
	Consult instructions for use.		May cause an allergic skin reaction. Harmful to aquatic life.
	Caution.		Contains Sufficient for <n> Tests.

Luminex Technical Support

Contact Luminex Technical Support by telephone in the U.S. and Canada by calling: 1-877-785-2323
Contact outside the U.S. and Canada by calling: +1 512-381-4397
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Additional information is available on the Luminex website. Search on the desired topic, navigate through menus. Also, review the website's FAQ section. Enter *http://www.luminexcorp.com* in your browser's address field.

This manual can be updated periodically. To ensure that you have a current version, contact Technical Support.

Description

The xMAP® Antibody Coupling (AbC) Kit contains all of the reagents necessary to covalently couple antibodies to Luminex® MagPlex® microspheres (beads) in approximately three hours. This kit can also be used to couple other proteins to Luminex microspheres, but due to the large diversity in protein composition, coupling performance with other proteins is not guaranteed. For more information on protein coupling please visit *http://www.luminexcorp.com/support*.

Coupling is achieved through carbodiimide reactions involving the primary amino groups on the antibody, or protein of choice, and the carboxyl functional groups on the microsphere surface.

This kit is configured for a one-time use and contains enough reagent to couple as few as $2.5x10^6$ or as many as $50x10^6$ microspheres. The kit can be used to perform as many as 10 individual coupling reactions at scales of $2.5x10^6$ to $5x10^6$ microspheres per reaction; or as many as 4 reactions at scales of up to $12.5x10^6$ microspheres per reaction.

Scale (beads/rxn)	Number of Reactions
$5x10^6$ or less	10
Up to $12x10^6$	4

NOTE: Luminex strongly recommends that the EDC reagent be discarded after one use.

NOTE: Each coupling reaction requires a minimum of $2.5x10^6$ microspheres.

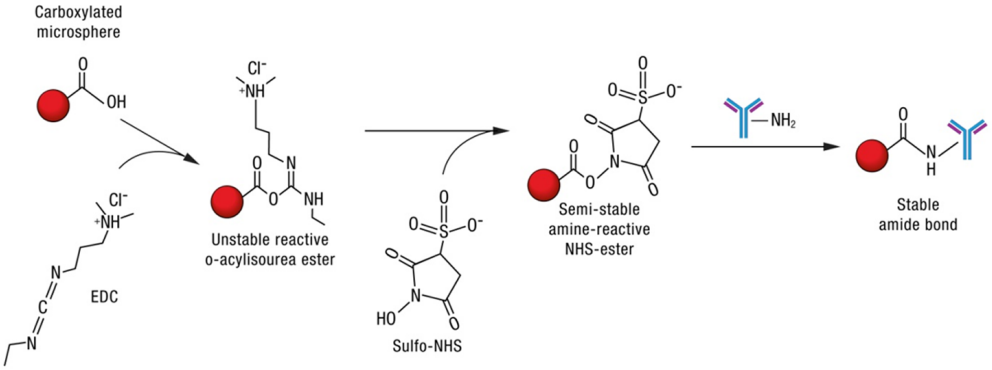
The coupled microspheres can then be used with a Luminex xMAP instrument to develop monoplex or multiplex assays.

This kit is ideal for use in two modes:

- Coupling optimization and assay optimization: For users developing a new assay, the kit will allow them to perform up to 10 small scale coupling reactions to test multiple concentrations of antibody with multiple bead regions.
- Small-to-medium scale assay manufacturing: For users who have already developed and optimized an assay, the kit will allow them to couple up to 50 million beads at once to meet their routine usage needs.

With Luminex MagPlex microspheres, if the kit protocol is performed carefully, the percent recovery of the coupling reaction is typically 90% or greater; enough for more than 45 96-well plates (@ 2,500 beads/well) for every $12.5x10^6$ microspheres coupled.

Principles of the Procedure



NOTE: The diagram above illustrates the chemical reaction taking place during coupling and is not intended to be a literal representation of the order in which reagents are added to the reaction.

The coupling procedure involves a two-step carbodiimide reaction. The carboxyl groups on the surface of the polystyrene microspheres must first be activated with a carbodiimide derivative prior to coupling the antibody. EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) reacts with the carboxyl groups on the surface of the microspheres to form an active O-acylisourea intermediate. This intermediate forms a more stable ester using Sulfo-NHS (*N*-hydroxysulfosuccinimide). The ester reacts with the primary amines (NH2 groups) of antibodies to form a covalent bond (amide linkage). In the protocol described in this package insert, the Sulfo-NHS is added to the reaction prior to the addition of EDC to maximize efficiency of the reaction. The presence of Sulfo-NHS in the mixture at the time of the EDC addition is critical due to the limited stability of the EDC microsphere conjugate. The reaction with the carboxylated microsphere does not begin until EDC is added to the mixture.

Protein Considerations

This kit includes a specially formulated Activation Buffer (pH 6.0) which is compatible with most antibodies.

The protein to be coupled must be free of sodium azide, bovine serum albumin (BSA), glycine, tris(hydroxymethyl)aminomethane (Tris), glycerol, or amine-containing additives and shall be suspended in phosphate buffered saline (PBS), pH 7.4.

A number of buffers can be used successfully in this coupling reaction. Generally, the pH at which a coupling reaction occurs shall be compatible with the solubility of the protein of interest. This shall be considered when coupling different proteins.

Additionally, coupling efficiency will vary depending on a variety of factors, including type of antibody, quantity of antibody, quantity of microspheres, etc. As such, Luminex recommends that several quantities of antibody input be tested to optimize the coupling reaction and functionality in the final assay. If coupling for the first time, 2 µg to 5 µg of antibody per $1x10^6$ microspheres is a good starting input.

The amount of antibody to use in the coupling reaction depends on the quantity of coupled antibody necessary to promote optimal binding of the desired target molecule.

Materials Provided

The xMAP® Antibody Coupling Kit, 40-50016, includes:

Component	Part Number	Volume/Mass	Quantity
EDC Reagent	10-40144 (ThermoSci #77149)	10 mg	1
Sulfo-NHS	11-25168	250 µL	1
Activation Buffer (green cap)	11-25171	45 mL	1
Wash Buffer	11-25167	30 mL	1
Microcentrifuge Tubes	11-00277	1.5 mL	10
Disposable Transfer Pipettes	11-00321	Varies per lot	20

NOTE: The EDC reagent is a Thermo Scientific® product and is manufactured for Luminex® for use in this kit. For questions regarding the use of this product with this kit, please contact *Luminex Technical Support*.

Materials Required But Not Provided

Equipment

- Luminex® xMAP® instrument: MAGPIX®, Luminex 100/200™, or FLEXMAP 3D®
- Luminex xPONENT® software
- Luminex MagPlex® microspheres (Cat# MC1XXXX-01, -04, and MC10XXX-ID)

  NOTE: Visit *http://www.luminexcorp.com/Products/ReagentsMicrospheres/MAGPLEX-MICROSPHERES* for a complete list of MagPlex microspheres and guidelines for selecting the right regions for your instrument.
- Luminex Performance Calibration and Verification Kits
- xMAP Sheath Fluid PLUS (Luminex 100/200 and FLEXMAP 3D) or MAGPIX Drive Fluid PLUS
- Magnetic Separator
- Tube Rotisserie or Rotator
- Water-bath sonicator
- Vortex mixer
- Plate shaker
- Pipettor

Optional Equipment

- xMAP® Antibody Coupling Kit Quick Guide (89-30000-00-361) (Available at *http://www.luminexcorp.com/downloads*.)
- Luminex® Tube Magnetic Separator (Cat# CN-0288-01)
- Luminex Magnetic Plate Separator (Cat# CN-0269-01)
- Microcentrifuge

Warnings and Precautions

1. For Research Use Only. Not for use in diagnostic procedures.
2. Safety Data Sheets (SDS) are available by contacting Luminex Corporation or visiting our website at *www.luminexcorp.com*.
3. Wear appropriate personal protective equipment (PPE), including a lab coat and disposable gloves, when performing procedures. Fresh clean gloves must be worn in each area and must be changed before leaving that area. Wash your hands thoroughly after performing the test.
4. Follow your institution's safety procedures for working with chemicals and handling biological samples.
5. Adhere to standard laboratory safety practices when handling hazardous, toxic, or flammable reagents and chemicals. Consult the package insert for the assay you are running and the Safety Data Sheet (SDS) for more information. Contact *Luminex Technical Support* when in doubt about compatibility of cleaning and decontamination agents or materials.
6. Train personnel who use, maintain, or clean the instrument in standard laboratory safety practices and follow those practices when handling the instrument.
7. Do not pipette by mouth.
8. Handle waste disposal in accordance with accepted medical practice and applicable regulations. If spillage occurs immediately disinfect following appropriate laboratory procedures.
9. Protect photosensitive microspheres from light at all times.
10. The xMAP® Antibody Coupling Kit Sulfo-NHS Solution and xMAP Antibody Coupling Kit Activation Buffer may cause an allergic skin reaction. Harmful to aquatic life.
11. EDC causes severe eye irritation. Causes respiratory tract and skin irritation.

Reagent Storage and Stability

Protect photosensitive microspheres from light at all times. Store all kit components at 2°C to 8°C. Store the EDC reagent, in its original packaging, at -20°C to ensure the longest shelf-life possible. Do not freeze the other components.

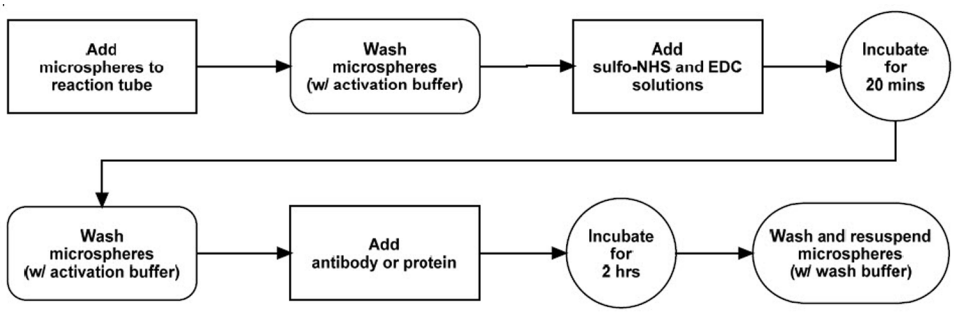
NOTE: Due to the instability of the EDC reagent in solution, store the solution in its original sealed packaging until needed and discard after one use. Never store or reuse reconstituted EDC.

All components are guaranteed up to the expiration date found on the label; when stored in their original packaging and as specified in this package insert.

The stability of coupled microspheres is dependent upon several factors; including the protein stability and composition, aseptic processing conditions, presence of preservatives, storage buffer, storage temperature conditions, etc. However, stability studies have shown that antibody coupled microspheres, stored in appropriate storage buffer, are stable over a period of 18 months.

Procedure

General Workflow



Couple Antibodies to Microspheres

The following is the detailed step-by-step process for coupling antibodies (or similar proteins) to carboxylated magnetic microspheres. An abridged quick reference guide can be found online at *http://www.luminexcorp.com/downloads*.

NOTE: Protect photosensitive microspheres from light at all times.

1. Remove kit and all reagents from the refrigerator and allow them to equilibrate to room temperature for 20 to 30 minutes.
2. While the reagents equilibrate to room temperature, calculate and note the required volumes for each reaction being performed.
3. Resuspend the stock microspheres.
    - If using a 1 mL stock microsphere vial, vortex the stock microsphere vial for 10 seconds and then sonicate for 10 seconds to disperse the microspheres. Alternatively, rotate the microsphere vials on a rotator for 15 minutes.
    - If using a 4 mL stock microsphere vial, rotate the vial for 15 minutes at 15 to 30 rpm.

4. Dispense the desired amount of microspheres from the stock vial into one of the microcentrifuge tubes ("reaction tube") provided with the kit.

   Example Calculation:

   If coupling 5 million microspheres, with microsphere stock concentration of 12.5×10^6 microspheres:

$$\text{Volume of stock needed} = \frac{(\#\text{ of beads to couple})}{(\text{conc of stock vial})}$$

$$\text{Volume of stock needed} = \frac{(5 \times 10^6 \text{ beads})}{\left(12.5 \times 10^6 \text{ beads}/\text{mL}\right)}$$

$$\text{Volume of stock needed} = 0.4\text{mL or } 400\ \mu\text{L}$$

   NOTE: This kit is designed for a maximum of 12.5×10^6 microspheres per reaction tube.

5. Wash the microspheres.
   a. Place the reaction tube with microspheres into the magnetic separator for 1 to 2 minutes.

      NOTE: If performing multiple reactions simultaneously, a microcentrifuge may be used, in place of a magnetic separator, to pellet the microspheres during wash steps. Beads can be pelleted by microcentrifugation at ≥ 8000 x g for 1 to 2 minutes.

   b. With the reaction tube still positioned in the magnetic separator, remove the supernatant with the transfer pipette.

      NOTE: For your convenience, 20 disposable transfer pipettes have been included for the removal of supernatant during the various wash steps in this protocol. If using one pipette for the removal of Activation Buffer and one for the removal of Wash Buffer, the kit has enough to perform up to 10 individual reactions. (i.e., 2/rxn). Take special care to avoid cross contamination, when performing multiple reactions simultaneously.

   c. Add 500 µL of Activation Buffer into the reaction tube.
   d. Vortex the reaction tube for 10 seconds and then sonicate for 10 seconds to disperse the microspheres.
6. Wash the microspheres again, for a total of two washes.
7. Place the reaction tube with microspheres into the magnetic separator for 1 to 2 minutes and, with the reaction tube still positioned in the magnetic separator, remove the supernatant with a transfer pipette.
8. Add Activation Buffer into the reaction tube.

   - If coupling more than 5×10^6 microspheres, add 400 µL of Activation Buffer into the reaction tube.
   - If coupling 5×10^6 microspheres or less, add 480 µL of Activation Buffer into the reaction tube.

9. Vortex the reaction tube for 10 seconds and then sonicate for 10 seconds to disperse the microspheres.
10. Vortex the provided Sulfo-NHS tube for a minimum of 10 seconds.
11. Add Sulfo-NHS solution to the reaction tube.

    - If coupling more than 5×10^6 microspheres, add 50 µL of the Sulfo-NHS solution into the reaction tube.
    - If coupling 5×10^6 microspheres or less, add 10 µL of the Sulfo-NHS solution into the reaction tube.

12. Add 250 µL of Activation Buffer into the 10 mg vial of EDC. Invert the EDC vial and then vortex the vial for 10 to 12 seconds to dissolve the EDC.

    NOTE: EDC will begin to degrade once exposed to moisture in the atmosphere and the Activation Buffer. Once opened, prepare the EDC solution and use quickly. If performing multiple reactions, be sure to prepare all of them prior to this step so that the EDC solution can be quickly added to all of the reaction tubes immediately after dissolution. The EDC solution must be made fresh for each coupling event and discard the excess.

13. Add EDC Solution to the reaction tube.

    - If coupling more than $5x10^6$ microspheres, add 50 µL of the EDC solution into the reaction tube.
    - If coupling $5x10^6$ microspheres or less, add 10 µL of the EDC solution into the reaction tube.

14. Vortex the reaction tube for a minimum of 10 seconds.
15. Protect photosensitive microspheres from light and rotate on rotator for 20 minutes. (Rotation speed should be ~15 to 30 rpm.)
16. Wash the microspheres.
    a. Place the reaction tube with microspheres into the magnetic separator for 1 to 2 minutes.
    b. With the reaction tube still positioned in the magnetic separator, remove the supernatant with a transfer pipette.
    c. Add 500 µL of Activation Buffer into the reaction tube.
    d. Vortex the reaction tube for 10 seconds and then sonicate for 10 seconds to disperse the microspheres.
17. Wash the microspheres two more times, for a total of three washes.
18. Place the reaction tube with microspheres into the magnetic separator for 1 to 2 minutes and, with the tube still positioned in the magnetic separator, remove the supernatant with a transfer pipette.
19. Calculate volume of antibody to be used in the reaction.

    - Typically, a range of 2 to 5 µg of antibody per $1x10^6$ microspheres is a good starting point, if performing the coupling reaction for the first time.

      Example Calculation:

      If coupling 5 million microspheres, with an antibody at a concentration of 5 mg/mL, and using the suggested starting point of 5 µg of antibody per $1x10^6$ microspheres:

$$\text{Volume of Ab needed} = \frac{(\#\text{ of beads to couple})(\text{Desired Ab conc})}{\text{Stock Ab conc}}$$

$$\text{Volume of Ab needed} = \frac{(5\times10^6\text{beads})\left(\frac{5\mu g}{1\times10^6\text{beads}}\right)}{5\,^{mg}/_{mL}}$$

$$\text{Volume of Ab needed} = 5\mu L$$

20. Calculate the volume of Activation Buffer needed for the reaction.
    - If coupling more than $5x10^6$ microspheres, subtract the calculated volume of antibody from 1000 µL.
    - If coupling $5x10^6$ microspheres or less, subtract the calculated volume of antibody from 500 µL.

      Example Calculation:

      If 5 µL of the stock antibody is needed (as calculated in the example above) for the coupling of 5 million microspheres:

      Volume of Activation Buffer needed = (1000 µL or 500 µL) - (Volume of Ab needed)

      Volume of Activation Buffer needed = 500 µL - 5 µL

      Volume of Activation Buffer needed = 495 µL
21. Add the calculated volume of Activation Buffer to the reaction tube.
22. Add the calculated volume of antibody to the reaction tube.
23. Vortex the reaction tube for a minimum of 10 seconds.
24. Protect photosensitive microspheres from light and rotate on a rotator for 2 hours. (Rotation speed should be ~15 to 30 rpm.)
25. Wash the microspheres.
    a. Place the reaction tube with microspheres into the magnetic separator for 1 to 2 minutes.
    b. With the reaction tube still positioned in the magnetic separator, remove the supernatant with a transfer pipette.
    c. Add 500 µL of Wash Buffer into the reaction tube.
    d. Vortex the reaction tube for 10 seconds and then sonicate for 10 seconds to disperse the microspheres.
26. Wash the microspheres two more times, for a total of three washes.
27. Place the reaction tube with microspheres into the magnetic separator for 1 to 2 minutes and, with the tube still positioned in the magnetic separator, remove the supernatant with a transfer pipette.
28. Add 1 mL of Wash Buffer into the reaction tube.

    NOTE: The Wash Buffer is used as a storage buffer after completing the coupling reaction.
29. Vortex the reaction tube for 10 seconds and then sonicate for 10 seconds to disperse the microspheres.
30. Protect photosensitive microspheres from light and store at 2°C to 8°C until needed.

    NOTE: For optimal performance, allow the coupled microspheres to block overnight before first use.

Coupling Assessment

Once the coupling reaction has been completed, enumerate the coupled microspheres and assess the efficiency of the coupling reaction.

Microsphere Enumeration

Although, the protocol described in this package insert will typically yield over a 90% recovery, Luminex recommends that the user count the number of microspheres recovered after each coupling reaction with the use of a cell counter or hemacytometer. Please refer to the cell counter or hemacytometer's users manual for instructions.

Coupling Confirmation

Luminex strongly recommends to assess coupling efficiency before proceeding to assay development. The coupled microspheres can be reacted with a phycoerythrin (PE)-labeled target or antibody that binds to the coupled protein. Alternatively, the target or antibody may be biotinylated, then labeled with PE. Analyze this complex on a Luminex® xMAP® instrument. The fluorescent signal intensity of this reaction is directly proportional to the amount of protein on the surface of the microspheres. This process provides a rapid assessment of the relative amount of protein coupled to the microspheres; however, this does not necessarily verify the functionality of the protein. The ultimate test is the functional assay of the coupled protein.

Confirm Sample Coupling Protocol

1. Select the appropriate antibody-coupled microsphere set or sets.
2. Resuspend the microspheres by vortex and sonication for approximately 20 seconds.
3. Prepare a working microsphere solution by diluting the coupled microsphere stocks to a final concentration of 50 beads/µL in PBS-1% BSA.

   NOTE: Prepare up to four different microsphere sets in the same mixture (in multiplex), provided each set is a different microsphere region and the various antibodies coupled to those regions can be detected with the same detection antibody.

   NOTE: At least 1 mL of the microsphere solution is required for each reaction.

   NOTE: Either PBS-1% BSA or PBS-BN (PBS, 1% BSA, 0.05% Azide, pH 7.4) may be used as Assay Buffer.

4. Prepare a solution of phycoerythrin-labeled anti-species IgG detection antibody at 4 µg/mL in PBS-1% BSA. Prepare a 1:2 dilution series of that detection antibody solution to a concentration of 0.0625 µg/mL as shown in the following table.

Dilution Tube	Volume of PBS-1% BSA	Volume of Detection Antibody	Concentration
1:1	-	-	4 µg/mL
1:2	500 µL	500 µL from Tube 1:1	2 µg/mL
1:4	500 µL	500 µL from Tube 1:2	1 µg/mL
1:8	500 µL	500 µL from Tube 1:4	0.5 µg/mL
1:16	500 µL	500 µL from Tube 1:8	0.25 µg/mL
1:32	500 µL	500 µL from Tube 1:16	0.125 µg/mL
1:64	500 µL	500 µL from Tube 1:32	0.0625 µg/mL

   NOTE: For optimal results, use Costar® round-bottom 96-well plates and Luminex® Magnetic Plate Separator with MagPlex® microspheres.

5. Aliquot 50 µL of the working microsphere solution previously prepared into 2 entire columns of wells of the plate (duplicate sets of 8 wells, 16 wells total).
6. Add 50 µL of PBS-1% BSA, as a blank sample, into the wells in Row A containing the microsphere solution.

7. Add 50 µL of each of the diluted detection antibody solutions previously prepared into the appropriate wells of the plate (as shown in the plate layout below).

Table 1. Example Plate Layout using Columns 1 and 2

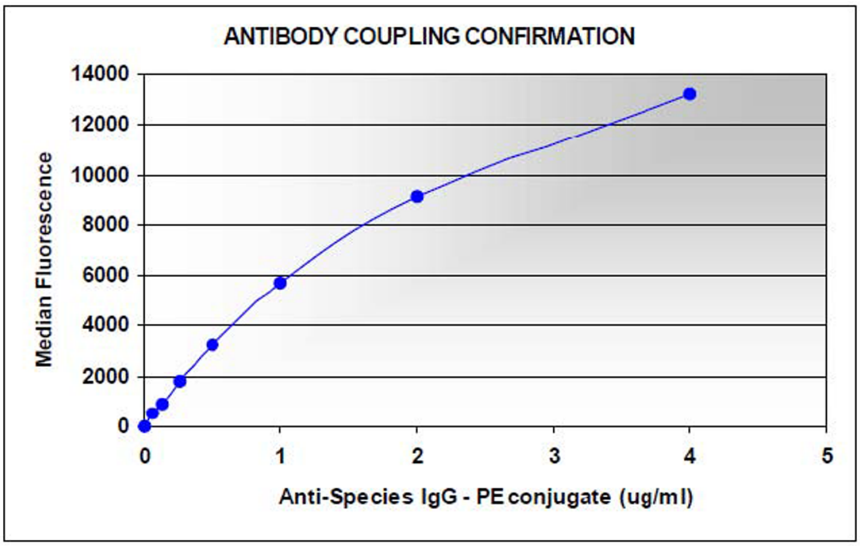
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank										
B	1:64	1:64										
C	1:32	1:32										
D	1:16	1:16										
E	1:8	1:8										
F	1:4	1:4										
G	1:2	1:2										
H	1:1	1:1										

8. Mix the reactions gently by pipetting up and down several times with a pipettor.
9. Cover the plate and incubate for 30 minutes at room temperature on a plate shaker.
10. Clip the plate in place on the Luminex Magnetic Plate Separator and rapidly and forcefully invert over a biohazard receptacle to evacuate the liquid from the wells.

    NOTE: For information on the MagPlex Manual Wash Method, please visit: *http://www.luminexcorp.com/Products/ReagentsMicrospheres/MAGPLEX-MICROSPHERES*.

11. Wash each well with 100 µL of PBS-1% BSA by gently pipetting up and down several times with a pipettor, and remove the liquid by using the procedure described above.
12. Wash each well again and remove the liquid.
13. Resuspend the microspheres in 100 µL of PBS-1% BSA by gently pipetting up and down several times with a pipettor.

14. Analyze 50 µL to 75 µL on the Luminex instrument according to the user manual. An example of typical results is shown below.



Troubleshooting

This troubleshooting guide may be helpful in solving problems that may arise. For more information, contact *Luminex Technical Support*.

Problem	Possible Cause(s)	Recommendations
Low Bead Count	Uncoupled microspheres tend to stick to the inner surfaces of some tubes	Make sure to use tubes supplied in this kit only.
	Microspheres were lost during washes	Use the recommended magnetic separator to perform all washes. Refer to the Materials Required but not Provided. Use the transfer pipette to remove all supernatant during washes. The microsphere pellets are loose and using a pipettor could disturb the bead pellet.
	Incorrect sample probe height adjustment	Adjust the sample probe height according to the Luminex® 200™, FLEXMAP 3D®, or MAGPIX® user manuals.
	Incorrect protocol set-up on the Luminex® instrument	Make sure correct microsphere regions are selected based on your particular custom designed assay.

Problem	Possible Cause(s)	Recommendations
Low/No signals	The reagents may not have been stored correctly	Store all reagents as recommended.
	EDC may not have been stored correctly or rehydrated long before use	Leave EDC in the pouch it is shipped in until ready to use. Use EDC immediately in the coupling procedure after rehydration.
	The stock protein concentration is possibly low	Make sure the protein concentration is correct and the recommended amount is coupled to the microspheres.
	The stock protein suspension possibly contains foreign proteins, azide, glycine, Tris or some primary amines	Dialyze the protein of interest to remove the competing substances.
	The reaction volumes and/or mixing method may be incorrect	The final reaction volume for the 2 hr incubation is critical for successful coupling. Refer to the Coupling Protocol. End-to-end mixing during the 2 hr incubation is very important. Please use a rotator.
	The anti-species detection antibody may not be correct	Make sure the anti-species detection antibody is correct, for example, if you are using a mouse monoclonal to couple to the microspheres, use an anti-mouse detection antibody. Make sure the anti-species detection antibody has the appropriate label on it, either biotin or phycoerythrin.