



xMAP[®] Antibody Coupling Kit User Manual

Product	Catalog Number
xMAP [®] Antibody Coupling Kit	40-50016

For Research Use Only
Not For Use In Diagnostic Procedures

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Rev B

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Safety



The following informational notes and cautions are used in this manual.

NOTE: This message is used to provide general helpful information.

IMPORTANT: This message advises users that failure to take action or avoid a certain action can result in data loss or the reporting of incorrect data.

You may encounter the symbols in the table below, within this manual. These represent warnings, conditions, identifications, and important information.

Table 1: Symbols

Symbol	Meaning
	General Warning, Caution, Risk of Danger
	Manufacturer
	Decision Point

Material Safety Data Sheets (MSDS's) are available upon request. Visit <http://www.luminexcorp.com/support> for more information.



CAUTION: xMAP® Antibody Coupling Kit Sulfo-NHS Solution MAY BE HARMFUL IF ABSORBED THROUGH SKIN OR IF SWALLOWED. MAY CAUSE EYE AND SKIN IRRITATION.



CAUTION: xMAP® Antibody Coupling Kit Activation Buffer MAY BE HARMFUL IF ABSORBED THROUGH SKIN OR IF SWALLOWED.



CAUTION: EDC causes severe eye irritation. Causes respiratory tract and skin irritation.

Introduction

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 2.5×10^6 or as many as 50×10^6 microspheres. The kit can be used to perform as many as ten individual coupling reactions at scales of 2.5×10^6 to 5×10^6 microspheres per reaction**; or as many as four reactions at scales of up to 12.5×10^6 microspheres per reaction.

Scale (beads/rxn)	Number of Reactions
5×10^6 or less**	10
Up to 12.5×10^6	4

IMPORTANT:

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

** Each coupling reaction requires a minimum of 2.5×10^6 microspheres.

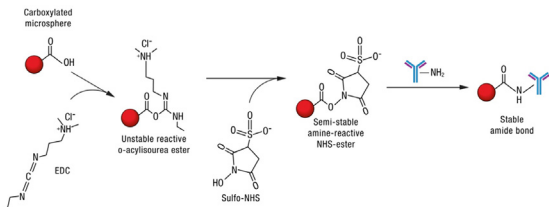
The coupled microspheres can then be used with a Luminex xMAP instrument to develop monoplex or multiplex assays.

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 forty-five 96-well plates (@ 2,500 beads/well) for every 12.5×10^6 microspheres coupled.

Method



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 (NH_2 groups) of antibodies to form a covalent bond (amide linkage). In the protocol described in this manual, 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 suitable for 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 should 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 should be compatible with the solubility of the protein of interest. This should 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, it is recommended 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, 2-5 μ g of antibody per 1x10⁶ microspheres is generally a good starting input.

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

Kit Contents

The following items are included in this kit:

Component	Part Number	Volume/Mass	Quantity
EDC Reagent	11-40144 (ThermoSci #77149)	10 mg	1
Sulfo-NHS	11-25169	250 μ L	1
Activation Buffer (green cap)	11-25171	45 mL	1
Wash Buffer	11-25167	30 mL	1
1.5 mL tubes	11-00277	n/a	10
Disposable pipettes	11-00321	n/a	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.

Storage and Stability

Photosensitive microspheres should be protected from light at all times. All kit components are to be stored at 2-8°C. The EDC reagent, in its original packaging, may be stored at -20°C to ensure the longest shelf-life possible. All other components should never be frozen.

IMPORTANT: Due to the instability of the EDC reagent in solution, it should always be stored in its original sealed packaging until needed and discarded after one use. Reconstituted EDC should never be stored and reused.

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

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.

Necessary Materials and Equipment

There are various materials and pieces of equipment that are required for microsphere coupling which are **not included with this kit.**

Required Equipment

- Luminex xMAP instrument: MAGPIX, LX100/200, or FLEXMAP 3D
- Luminex xPONENT software
- Luminex MagPlex Microspheres (Cat# MC1XXXX-01, -04, & MC10XXX-ID)

NOTE: Visit <http://www.luminexcorp.com/Products/ReagentsMicrospheres/MAG-PLEX-MICROSPHERES> for a complete list of MagPlex[®] microspheres and guidelines for selecting the right regions for your instrument.

- Luminex Instrument Performance Calibration and Verification Kits
- Luminex Instrument Sheath Fluid (LX100/200 & FLEXMAP 3D) or Drive Fluid (MAGPIX)
- Magnetic Separator

Other Necessary Equipment

- Tube Rotisserie or Rotator
- Water-bath sonicator

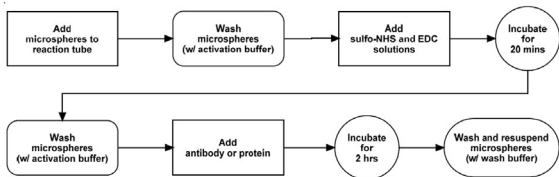
- Vortexer
- Pipettor

Optional Equipment

- Luminex Tube Magnetic Separator (Cat# CN-0288-01)
- Microcentrifuge
- Luminex Plate Magnetic Separator (Cat# CN-0269-01)

Instructions

General Workflow



Coupling Protocol

The following is the detailed step-by-step process for coupling antibodies (or similar proteins) to carboxylated magnetic microspheres.

IMPORTANT: Protect photosensitive microspheres from light whenever possible throughout this entire procedure.

1. Remove kit and all reagents from the refrigerator and allow them to equilibrate to room temperature for 20-30 minutes.
2. While the reagents equilibrate to room temperature, calculate and note the required volumes for Decision Point steps 4, 8, 11, 13, 19, and 20; for each reaction being performed.
3. Resuspend the stock microspheres.
 - If using a 1mL stock microsphere vial, vortex the stock microsphere vial for 10 seconds and then sonicate for 10 seconds to disperse the microspheres. Alternatively, the microsphere vials can be rotated on a rotator for 15 minutes.
 - If using a 4mL stock microsphere vial, rotate the vial for 15 minutes at 15-30 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:

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

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

Volume of stock needed = 0.4mL or 400 μ L

IMPORTANT: 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 1-2 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 $\geq 8000 \times g$ for 1-2 minutes.

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

NOTE: For your convenience, twenty 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 should have enough to perform up to ten individual reactions. (i.e. 2/rxn) Special care should be taken to avoid cross contamination, when performing multiple reactions simultaneously.

C. Add 500 μ L 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. Repeat Step 5 once for a total of two washes.

7. Place the reaction tube with microspheres into the magnetic separator for 1-2 minutes and, with the 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 10-12 seconds to dissolve the EDC.

IMPORTANT: EDC will begin to degrade once exposed to moisture in the atmosphere and the Activation Buffer. Once opened the EDC solution must be prepared and used 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 the excess should be discarded.

13. Add EDC Solution to reaction tube.
- If coupling more than 5×10^6 microspheres, add 50 μL of the EDC solution into the reaction tube.
 - If coupling 5×10^6 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 microspheres from light and incubate at room temperature for 20 ± 1 minutes and vortex the microspheres again, for 10 seconds, halfway through the incubation period. Alternatively, this incubation step can be done on a rotator at 15-30 rpm for 20 minutes, without vortexing.

16. Wash the microspheres.
- Place the reaction tube with microspheres into the magnetic separator for 1-2 minutes.
 - With the tube still positioned in the magnetic separator, remove the supernatant with a transfer pipette.
 - Add 500 μL of Activation Buffer into the reaction tube.
 - Vortex the reaction tube for 10 seconds and then sonicate for 10 seconds to disperse the microspheres.
17. Repeat Step 16 twice, for a total of three washes.
18. Place the reaction tube with microspheres into the magnetic separator for 1-2 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 2-5 μg of antibody per 1×10^6 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 1×10^6 microspheres

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

$$\text{Volume of Ab needed} = \frac{(5 \times 10^6 \text{ beads}) \left(\frac{5 \mu\text{g}}{1 \times 10^6 \text{ beads}} \right)}{5 \text{ mg/mL}}$$

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



20. Calculate the volume of Activation Buffer needed for the reaction.
 - If coupling more than 5×10^6 microspheres, subtract the volume of antibody calculated in Step 19 from 1000 μL .
 - If coupling 5×10^6 microspheres or less, subtract the volume of antibody calculated in Step 19 from 500 μL .

Example Calculation:

If 5 μL of the stock antibody is needed (as calculated in the example in Step 19) 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 appropriate volume of Activation Buffer, calculated in Step 20, to the reaction tube.
22. Add the appropriate volume of antibody, calculated in Step 19, to the reaction tube.
23. Vortex the reaction tube for a minimum of 10 seconds.
24. Protect microspheres from light and rotate on rotator for 2 hours. (Rotation speed should be $\sim 15\text{-}30$ rpm)
25. Wash the microspheres.
 - A. Place the reaction tube with microspheres into the magnetic separator for 1-2 minutes.
 - B. With the 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. Repeat Step 25 twice, for a total of three washes.
27. Place the reaction tube with microspheres into the magnetic separator for 1-2 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 from light and store at 2-8 $^{\circ}\text{C}$ 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, the coupled microspheres can be enumerated and the efficiency of the coupling reaction assessed.

Microsphere Enumeration

Although, the protocol described in this manual will typically yield over a 90% recovery, it is recommended that the user count the number of microspheres actually recovered after each coupling reaction. This can be done with the use of a cell counter or hemacytometer. Please refer to the cell counter or hemacytometer's users manual for appropriate instructions for doing so.

Coupling Confirmation

It is strongly recommended 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. This complex can then be analyzed on a Luminex xMAP instrument. The intensity of the fluorescent signal 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.

Sample Coupling Confirmation 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/ μ L in PBS-1% BSA.

NOTE: Up to 4 different microsphere sets may be prepared 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 1mL 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 microsphere solution prepared in Step 3 into two 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 prepared in Step 4 into the appropriate wells of the plate (as shown in the plate layout below).

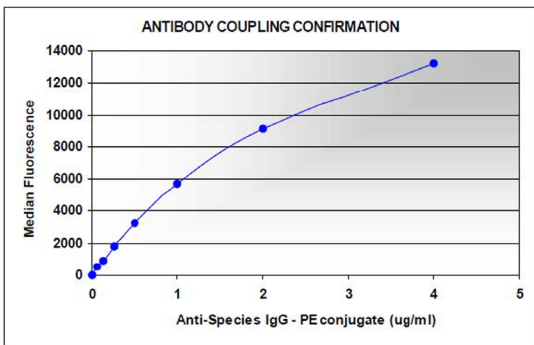
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank										
B	1:64	1:64										
C	1:32	1:32										
D	1:16	1:16										
E	1:8	1:8										
F	1:4	1:4										
G	1:2	1:2										
H	1:1	1:1										

Example plate layout using columns 1 & 2

- Mix the reactions gently by pipetting up and down several times with a pipettor.
- Cover the plate and incubate for 30 minutes at room temperature on a plate shaker.
- 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>

- Wash each well with 100 μ L of PBS-1% BSA by gently pipetting up and down several times with a pipettor, and remove the liquid by using the procedure described in the previous step.
- Repeat the previous wash step once.
- Resuspend the microspheres in 100 μ L of PBS-1% BSA by gently pipetting up and down several times with a pipettor.
- Analyze 50-75 μ L on the Luminex analyzer according to the system 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, call Technical Support toll free at 1-877-785-BEAD (2323). Outside of U.S. and Canada, call +1 512-381-4397. Send e-mail inquiries to: support@luminexcorp.com.

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. See Recommended Material list. 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 probe height adjustment	Adjust probe height according to the Luminex [®] 200 [™] , FLEXMAP 3D [®] or MAGPIX [®] user manual.
	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	EDC must remain in the pouch it is shipped in until ready to use. EDC must be used 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 protocol. End-to-end mixing during the 2 hr incubation is very important. Please use an optical 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.

