

## MICROSPHERE-BASED MULTIPLEX IMMUNOASSAYS: DEVELOPMENT AND APPLICATIONS USING LUMINEX® XMAP® TECHNOLOGY

**Sherry A. Dunbar** (sdunbar@luminexcorp.com)

**Michaela R. Hoffmeyer**

The ability to test for multiple analytes simultaneously, termed multiplexing, affords several advantages over traditional single analyte testing and has become a preferred testing method in many research and diagnostic laboratories. Using differentially dyed, functionalized microspheres, Luminex® xMAP® Technology and platforms allow users to quickly detect and quantify analytes in multiplex from the same sample, at lower cost than single analyte testing and with excellent assay performance. Standard chemistries used for covalently coating the xMAP microspheres allow for a broad range of biomolecules to be assayed, including proteins, nucleic acids, polysaccharides, and phospholipids. The rapid adoption of the bead-based multiplex analysis platform is evidenced by more than 9,000 instruments placed globally and a broad menu of assay content available from Luminex and its partners.

In addition to the many commercial assays available for use with the xMAP platform, the open architecture of xMAP Technology allows users to quickly develop, optimize, and validate custom assays for their application of choice. More than 14,000 peer-reviewed publications describe the use and/or development of xMAP-based assays for a variety of research and diagnostic applications, emphasizing the versatility of the technology (Luminex Corporation, 2010a). In this chapter, we describe immunoassay applications of xMAP Technology, including assay development, optimization, and sample protocols for a variety of immunoassay formats, and provide information on the commercially available instrumentation, immunoassay menu, and selected references for each.

### XMAP TECHNOLOGY

The configuration of an xMAP assay consists of a suspension array where specific capture moieties are covalently coupled to the surfaces of internally dyed microspheres. Binding reactions take place on the surface of the microspheres which together make up a 100- to 500-membered array, depending on which Luminex analyzer is utilized. Analyte binding is detected by the use of a spectrally compatible reporter fluorophore, preferably phycoerythrin (PE) due to its high quantum yield. Microspheres are then interrogated within a Luminex analyzer to identify the microsphere region, as determined by internal fluorescence, and

quantitate the surface analyte binding, as detected by PE emission. This approach offers several advantages over traditional two-dimensional platforms, including shorter incubation times due to favorable near-liquid-phase binding kinetics, enhanced reproducibility, excellent sensitivity and specificity, reduced sample requirement, and multiplexed analysis capability (Nolan and Mandy, 2001; Nolan and Sklar, 2002; Kellar and Iannone, 2002; Kellar, 2003).

### XMAP MICROSPHERES

A key component of xMAP Technology is the polystyrene microspheres that are internally dyed with precise amounts of two or three spectrally distinct fluorochromes. Each type of multi-analyte microsphere is identical in size but differs in the quantities of the internal classification dyes. The dyes have similar excitation requirements but unique emission profiles, which provide unique spectral characteristics within individual microsphere regions or sets and allow each set to be specifically differentiated from all others in a multiplex. Each microsphere set can be covalently linked to a unique capture reagent, and since the microspheres can be distinguished by their spectral address and each address is associated with a specific analyte, the microspheres can be combined in a single reaction to measure multiple analytes simultaneously. A single uniform reporter fluorochrome quantifies the binding events that have occurred and the microsphere internal fluorescence allows deconvolution of the multiplexed data.

Several types of xMAP microspheres have been developed to suit the needs of various applications and are commercially available for development of immunoassay applications. The first generation of xMAP microspheres is differentiated based on the fluorescence profile of two internal dyes, providing a 100-membered array of spectrally distinct microsphere sets. Addition of a third dye has allowed expansion of the array to 500 members (Fig. 1). The original MicroPlex® Microspheres are 5.6µm diameter polystyrene microspheres, functionalized with carboxyl groups for covalent attachment of assay capture molecules, and available in 500 different color sets. LumAvidin® Microspheres contain a surface layer of avidin for binding biotinylated capture reagents. This attachment chemistry can be advantageous when working with small molecules

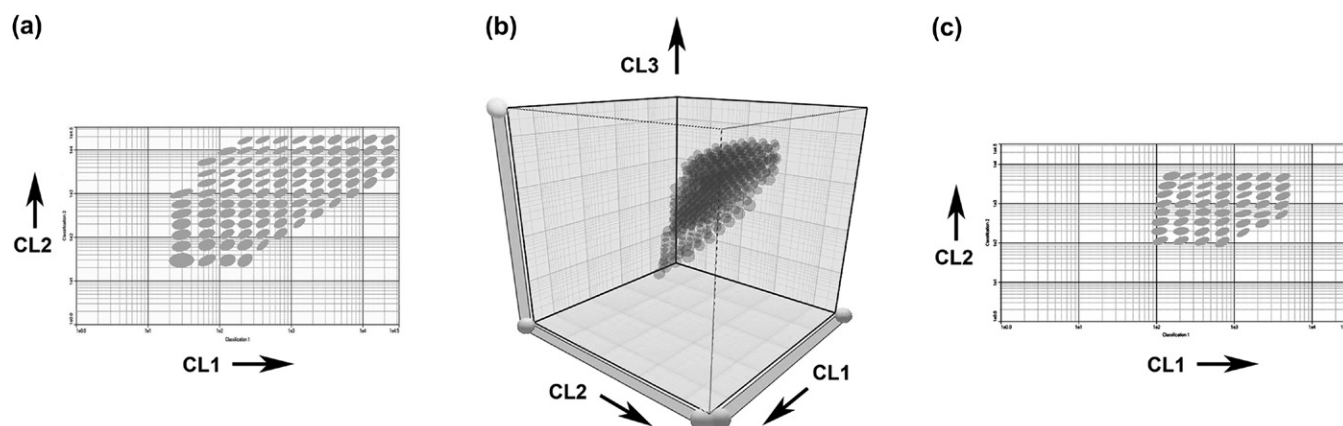


**FIGURE 1** Luminex bead maps. (a) Two-dimensional representation of the 100-plex bead map with Classification 1 dye (CL1) on the x-axis and Classification 2 dye (CL2) on the y-axis. Gray ovals indicate the positions of the 100 microsphere regions (color sets). (b) Three-dimensional representation of the 500-plex bead map with CL2 on the x-axis, Classification 3 dye (CL3) on the y-axis, and CL1 on the z-axis. Gray ovals show the positions of the 500 microsphere regions. The software allows the user to zoom into specific areas (slices) of the bead map for a more detailed view. (c) A 50 region subset of the 100-plex map is available on the MAGPIX® instrument.

or peptides, which may exhibit better performance when adequately spaced from the microsphere surface. However, use of LumAvidin® Microspheres prevents the use of an avidin–biotin reporter as the assay detection system. SeroMAP™ Microspheres were specifically developed to address high backgrounds that have been reported to occur in some serological assays. SeroMAP™ Microspheres are 5.6 µm carboxylated microspheres available in 100 different color sets. They are formulated to reduce nonspecific binding and perform well in serological applications (Waterboer *et al.*, 2006). MagPlex® Microspheres are 6.5 µm superparamagnetic microspheres functionalized with surface carboxyl groups and are available in 500 different color sets. MagPlex Microspheres can be magnetically separated from solution, allowing easy automation and facilitating washing steps. Similar in formulation to SeroMAP™, MagPlex Microspheres exhibit high performance with low nonspecific binding and reduced backgrounds in serological applications (Luminex Corporation, 2008a). Protocols for the use of MagPlex Microspheres with commercial magnetic plate washers have been developed and handheld magnets for manual MagPlex Microsphere washing are also available from Luminex and other vendors (Luminex Corporation, 2007b, 2010b).

## LUMINEX ANALYZERS

The principles of flow cytometry form the basis of two Luminex instruments, the Luminex 100/200™ and the FLEXMAP 3D® (Fig. 2). In these systems, a sample probe aspirates the completed microsphere-containing reactions from the wells of a 96- or 384-well assay plate and the sample is hydrodynamically focused into a rapidly flowing fluid stream. As the stream passes through an imaging cuvette, each microsphere is individually interrogated by two lasers. A 635 nm, 10 mW red diode laser excites the internal dyes within each microsphere and emission from the internal dyes is detected through the use of two avalanche photodiodes. Side scatter is used to gate for single microsphere events, thus gating out air and microsphere

aggregates. A 532 nm, 13 mW yttrium aluminum garnet laser excites the surface captured reporter fluorophore (PE), and the reporter fluorescence emission is detected by a photomultiplier tube (PMT). High-speed digital signal processing deconvolutes the internal dye emission into the region (assay) identity and quantitates the reporter signal that was bound to the microsphere surface. Multiple readings are taken per microsphere set, lending statistical validity and robustness to the data. Reporter channel readings are taken of the surrounding assay fluid and subtracted from assay results using a specific background subtraction algorithm. Typically, a minimum of 50 or 100 microspheres are read per region per well and the result is reported as the median fluorescent intensity (MFI). The use of the median statistic guards against the effects of physical carryover well-to-well, providing that a sufficient minimum number of microspheres per region per sample is collected.

The Luminex 100/200 analyzer is well established in both diagnostic and research settings, with more than fifty 510(k) cleared kits available and numerous end user laboratory-developed tests. Up to 100 different analytes can be analyzed on the Luminex 100/200 using MicroPlex Microspheres or up to 80 using MagPlex Microspheres. This robust and flexible system can be used for a variety of applications including immunoassays, genotyping, gene expression, enzymatic analysis, and infectious disease detection. Because a sample of just a few microliters can yield results for up to 100 analytes, this technology is excellent for rare samples or samples of limited volume. Assays show excellent concordance to predicates such as enzyme-linked immunosorbent assay (ELISA) and allow quantitation over 3–4 logs of dynamic range. The intuitive xPONENT software facilitates assay set-up, plate reading, and data analysis for both custom assays and commercial assay kits. A protocol is created for each specific assay and can be reused each time a new batch is processed. The 21 CFR Part 11 compatible upgrade offers multilevel user management, full audit trail, electronic records, and electronic signatures. The system is also approved for *in vitro* diagnostics (IVD) use.

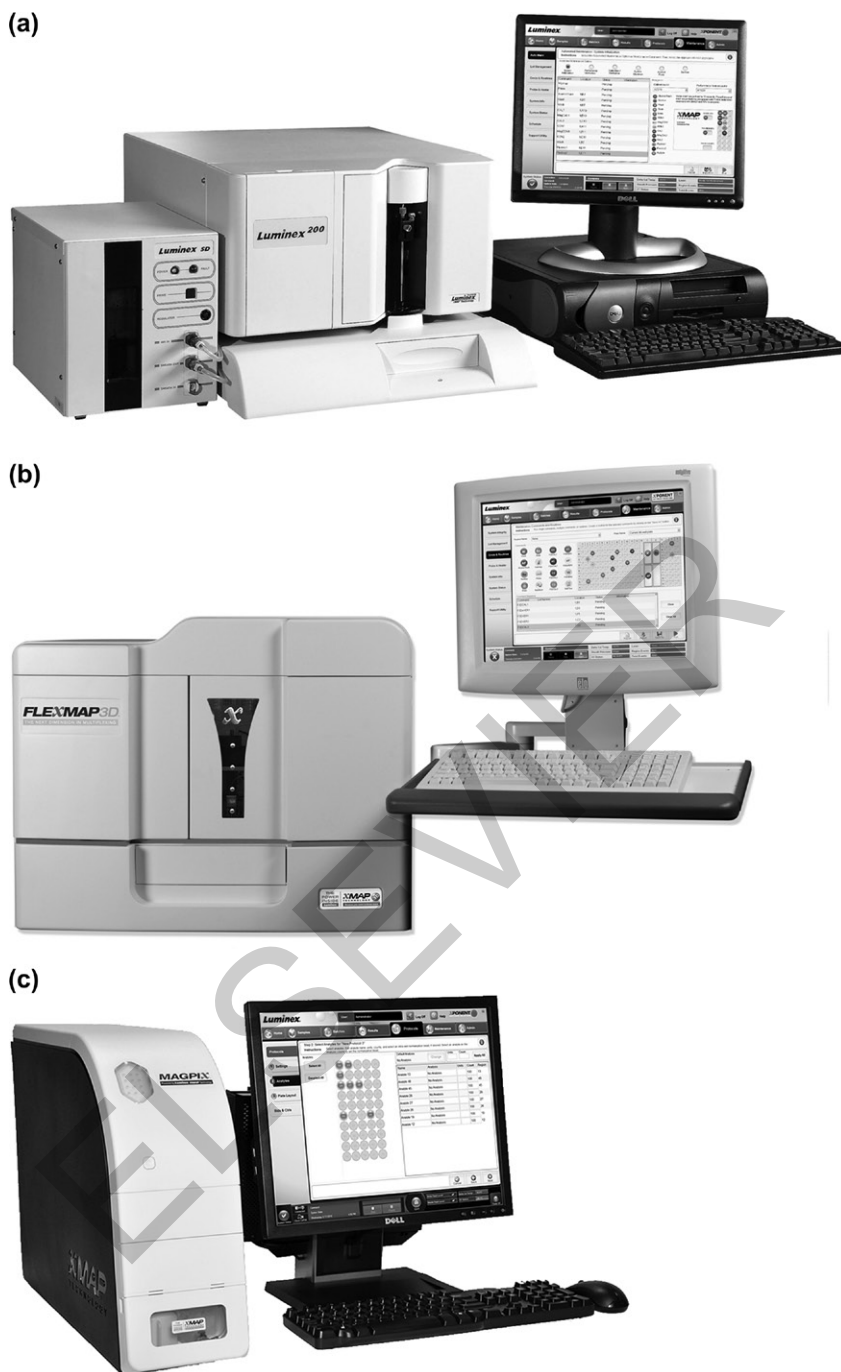


**FIGURE 2** Luminex analyzers. (a) The Luminex® 200™ total system includes the Luminex 200 flow analyzer, the Luminex XYP™ plate handling platform, the Luminex SD™ sheath fluid delivery system, xPONENT® software, and computer. (b) The FLEXMAP 3D™ system includes analyzer, plate handling, and fluid delivery systems integrated within a single unit. Also included are xPONENT® software and computer with an articulating arm to house the monitor and keyboard. (c) The MAGPIX® is a compact system based on CCD imaging technology. xPONENT® software provides stream-lined start-up and shutdown protocols and minimal maintenance requirements.

The FLEXMAP 3D system is the second flow-based analysis platform developed by Luminex and offers enhanced sensitivity, extended dynamic range, increased multiplexing of up to 500 analytes, and increased throughput. Additionally, this instrument was designed to easily interface with automated liquid handlers as well as Laboratory Information Management System (LIMS) systems and is compatible with both 96-well and 384-well plates. The system is also equipped with self-adjusting sample

probe height and a piercing function, allowing analysis of sealed plates. Throughput is enhanced through a dual syringe system, which allows plates to be analyzed two to three times faster than on the Luminex 200 instrument. A 96-well plate can be read in approximately 20 min while a 384-well plate can be analyzed in about 75 min. The FLEXMAP 3D also includes walkaway routines for calibration and regular maintenance functions. Due to enhanced dynamic range of up to 4.5 logs, analytes of

vastly different levels that previously needed to be split between wells can now be multiplexed into the same assay. This combination of enhanced features and capabilities makes the FLEXMAP 3D ideal for quick assay development and high-throughput discovery applications.

A third system, MAGPIX®, utilizes a flow cell and CCD-based optics with magnetic microspheres for multiplexing up to 50 tests in a single reaction volume (Fig. 2c). In MAGPIX, the reacted magnetic microspheres are sent through a flow cell into an imaging chamber where a magnetic actuator pulls the beads out of suspension and holds them in place for optical analysis. Red LEDs (630 nm) excite the fluorescent dyes contained within the microspheres, and green LEDs (515–521 nm) excite the reporter fluorochrome bound to the bead surface. A CCD imager identifies the bead region and quantifies the bound reporter. xPONENT for MAGPIX operates the system and can be used with commercial kits or user-developed assays. With a lower cost and a compact size (requiring only 64.8 cm bench space), MAGPIX provides an affordable multiplexing solution ideal for the low- to medium-throughput laboratory and remote laboratory testing sites.

## ASSAY DEVELOPMENT

xMAP Technology provides a flexible, open platform, allowing users to construct their own custom assays as well as use commercial assay kits or custom assays from Luminex and its partners. The Support section of the Luminex website provides sample protocols, recommendations, and various information to assist users in the assay

development process (Luminex Corporation, 2011d). Courses on xMAP assay development, both live and web-based, are also available from Luminex. Various publications provide detailed descriptions of assay development for specific xMAP applications, but a general workflow and recommendations for immunoassay development are described below.

Assay development begins with the acquisition of necessary reagents (antibodies, analytes, standards/controls, and matrix). The reagent to be coupled should be purified of extraneous primary amines (carrier proteins, Tris, etc.), if needed. The microspheres are conjugated with the capture reagent (e.g., antibody, protein, etc.), and uncoupled reactant is removed. Microsphere coupling reactions should be confirmed in the assay buffer system using an appropriate detection reagent (e.g., anti-mouse IgG for microspheres coupled with mouse monoclonal antibody). The background should be assessed in the target matrix to evaluate nonspecific binding and cross-reactivity, and positive control reagents similar to the sample should be used to assess positive binding reactions, sensitivity, and specificity.

Generally, reagents used for immunoassays on other, single-plex platforms will perform well in xMAP assays, provided they have sufficient sensitivity and specificity to be multiplexed. Further, standard immunoassay buffers can typically be used for xMAP immunoassays with the exception that amine-containing buffers, such as Tris, should not be used for microsphere coupling. Table 1 lists various buffers that are used for microsphere coupling and xMAP immunoassays (Luminex Corporation, 2007a). Refer to Luminex's website for a comprehensive lists of reagents, materials, and equipment that have been tested and found

**TABLE 1** xMAP Immunoassay Buffers

| Buffer                                               | Composition                                       | Use(s)                                             | Source      | Notes                                                                                  |
|------------------------------------------------------|---------------------------------------------------|----------------------------------------------------|-------------|----------------------------------------------------------------------------------------|
| Activation buffer <sup>1</sup>                       | 0.1 M NaH <sub>2</sub> PO <sub>4</sub> , pH 6.2   | Microsphere activation buffer for protein coupling | Sigma S3139 | Adjust to pH 6.2 with 5 N NaOH<br>Filter sterilize<br>Store at 4°C                     |
| Coupling buffer <sup>2</sup>                         | 50 mM MES, pH 5.0                                 | Microsphere-protein coupling buffer                | Sigma M2933 | Adjust to pH 5.0 with 5 N NaOH<br>Filter sterilize<br>Store at 4°C<br>Can also be used |
| Phosphate buffered saline (PBS), pH 7.4 <sup>3</sup> | 138 mM NaCl, 2.7 mM KCl, pH 7.4                   | Alternate microsphere-protein coupling buffer      | Sigma P3813 | Filter sterilize<br>Store at 4°C                                                       |
| PS-T wash buffer                                     | PBS, 0.05% Tween-20, pH 7.4                       | Microsphere wash buffer                            | Sigma P3563 | Filter sterilize<br>Store at 4°C                                                       |
| PBS-BN buffer <sup>4</sup>                           | PBS, 1% BSA, 0.05% sodium azide                   | Microsphere blocking/storage buffer                | Sigma P3688 | Filter sterilize                                                                       |
| PBS-TBN buffer <sup>4,5</sup>                        | PBS, 0.1% BSA, 0.02% Tween-20, 0.05% sodium azide | Microsphere blocking/storage buffer                | Sigma S8032 | Store at 4°C                                                                           |
|                                                      |                                                   | Microsphere wash buffer                            | Sigma P3813 | Filter sterilize                                                                       |
|                                                      |                                                   | Assay buffer                                       | Sigma A7888 | Store at 4°C                                                                           |
| PBS-BSA Buffer                                       | PBS, 1% BSA, pH 7.4                               | Assay buffer                                       | Sigma P9416 |                                                                                        |
|                                                      |                                                   |                                                    | Sigma S8032 |                                                                                        |
|                                                      |                                                   |                                                    | Sigma P3688 | Filter sterilize<br>Store at 4°C                                                       |

<sup>1</sup>Activation can be performed in 50 mM MES, pH 6.0–6.2, with similar results.

<sup>2</sup>Coupling can be performed in 100 mM MES, pH 6.0, with similar results. For some proteins, better solubility and better coupling may be achieved at a higher coupling pH.

<sup>3</sup>Alternative coupling buffer for proteins that do not couple well at pH 5–6.

<sup>4</sup>Also used as assay buffer.

<sup>5</sup>Also used as wash buffer.

to be compatible with xMAP Technology (Luminex Corporation, 2011b).

## MICROSPHERE COUPLING

Primary amines on antibodies or other proteins can be covalently linked to the carboxyl groups on the surface of the microspheres using a standard two-step carbodiimide coupling procedure (Luminex Corporation, 2007e,f). Some common additives to proteins can interfere with the coupling reaction, including amine-containing compounds such as Tris, BSA, or azide, and some detergents, glycerol, urea, and imidazole may also interfere. These compounds should be removed from the protein preparation by dialysis or desalting if possible. In some cases where interfering substances cannot be removed, such as detergents or urea, a sufficient dilution of the protein to be coupled can be performed to improve coupling efficiency. The carbodiimide coupling reaction is most efficient at low pH (i.e., pH 5–6); however, it may be preferred to maintain the protein at the pH of its storage buffer to ensure stability and functional conformation. Monoclonal antibodies should be used for capturing the analyte to the microsphere surface to achieve best sensitivity and specificity. If a polyclonal antibody is used for capture, it should be monospecific and affinity-purified. The optimal amount of capture reagent may vary depending on the reagent used and should be titrated, but generally, 3–5 µg of antibody per 1 million microspheres performs well.

An example protocol for two-step carbodiimide protein coupling to 5 million MagPlex Microspheres is outlined below. The same protocol can also be used with MicroPlex or SeroMAP Microspheres with the exception that centrifugation (at  $\geq 8000g$  for 1–2 min), instead of magnetic separation, must be used for washing steps. Table 2 lists magnetic separators and 96-well plates suitable for use with MagPlex microspheres during coupling and assay wash steps (Luminex Corporation, 2007b). Coupled microsphere stability depends on the stability of the coupled protein but when properly stored, coupled microspheres are usually stable for more than 1 year (Luminex Corporation, 2008b).

## Sample Protocol for Two-Step Carbodiimide Coupling of Protein to MagPlex Microspheres

1. Resuspend the stock uncoupled microsphere suspension according to the instructions described in the Product Information Sheet provided with your microspheres.
2. Transfer  $5.0 \times 10^6$  of the stock microspheres to a USA Scientific (1415–2500) or Eppendorf Protein LoBind (022431081) microcentrifuge tube.
3. Place the tube into a magnetic separator and allow separation to occur for 30–60 s.
4. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the microspheres.
5. Remove the tube from the magnetic separator and resuspend the microspheres in 100 µL dH<sub>2</sub>O by vortex and sonication for approximately 20 s.
6. Place the tube into a magnetic separator and allow separation to occur for 30–60 s.
7. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the microspheres.
8. Remove the tube from the magnetic separator and resuspend the washed microspheres in 80 µL Activation Buffer (100 mM monobasic sodium phosphate, pH 6.2) by vortex and sonication for approximately 20 s.
9. Add 10 µL of 50 mg/mL Sulfo-NHS (diluted in dH<sub>2</sub>O or Coupling Buffer) to the microspheres and mix gently by vortex.
10. Add 10 µL of 50 mg/mL EDC (diluted in dH<sub>2</sub>O or Coupling Buffer) to the microspheres and mix gently by vortex.
11. Incubate for 20 min at room temperature with gentle mixing by vortex at 10 min intervals.
12. Place the tube into a magnetic separator and allow separation to occur for 30–60 s.
13. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the microspheres.

**TABLE 2** Magnetic Separators for MagPlex Microspheres

| Product                                                    | Use      | Source                                      | Compatible Tube/Plate Types                                                                                                                |
|------------------------------------------------------------|----------|---------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|
| Dynal MPC®-S magnetic particle concentrator                | Coupling | Life Technologies, #A13346                  | 1.5 mL, co-polymer microcentrifuge tubes (USA Scientific, #1415-2500):                                                                     |
| Luminex magnetic plate separator                           | Assays   | Luminex Corporation, #CN-0269-01            | 96-well, round-bottom polystyrene solid plates (Costar, #3789 or #3792):                                                                   |
| LifeSep™ 96F magnetic separation unit                      | Assays   | Dexter Magnetic Technologies, Inc. #2501008 | 96-well, round-bottom polystyrene solid plates (Costar, #3789 or #3792):                                                                   |
| Ambion® 96-well magnetic ring stand                        | Assays   | Life Technologies, #AM10050                 | 96-well, round-bottom polystyrene solid plates (Costar, #3789 or #3792):                                                                   |
| DynaMag™-96 side skirted (previously named Dynal MPC®-96S) | Assays   | Life Technologies, #120-27                  | 96-well, round-bottom polystyrene solid plates (Costar, #3789 or #3792):<br>96-well, Thermowell P polycarbonate PCR plates (Costar, #6509) |
| 96-well plate magnet                                       | Assays   | PerkinElmer (Customer Care) 5083175         | 96-well, round-bottom polystyrene solid plates (Costar, #3789 or #3792):<br>96-well, Thermowell P polycarbonate PCR plates (Costar, #6509) |

14. Remove the tube from the magnetic separator and resuspend the microspheres in 250  $\mu$ L of Coupling Buffer (50 mM MES, pH 5.0) by vortex and sonication for approximately 20 s.
15. Repeat steps 12 and 13. This is a total of two washes with Coupling Buffer.
16. Remove the tube from the magnetic separator and resuspend the activated and washed microspheres in 100  $\mu$ L of Coupling Buffer by vortex and sonication for approximately 20 s.
17. Add protein to the resuspended microspheres. The optimal amount of protein should be determined by titration in the range of 1–25  $\mu$ g per million microspheres; generally 3–5  $\mu$ g/million microspheres perform well.
18. Bring total volume to 500  $\mu$ L with Coupling Buffer.
19. Mix coupling reaction by vortex.
20. Incubate for 2 h with mixing (by rotation) at room temperature.
21. Place the tube into a magnetic separator and allow separation to occur for 30–60 s.
22. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the microspheres.
23. Remove the tube from the magnetic separator and resuspend the coupled microspheres in 500  $\mu$ L of PBS-T Wash Buffer or PBS-TBN Buffer by vortex and sonication for approximately 20 s. Use PBS-TBN for this step if performing optional step 24.
24. Incubate for 30 min with mixing (by rotation) at room temperature (note: optional—perform this step when using the microspheres the same day.)
25. Place the tube into a magnetic separator and allow separation to occur for 30–60 s.
26. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the microspheres.
27. Remove the tube from the magnetic separator and resuspend the microspheres in 1 mL of Blocking/Storage Buffer (PBS-BN or PBS-TBN) by vortex and sonication for approximately 20 s.
28. Repeat steps 25 and 26. This is a total of two washes with 1 mL of Blocking/Storage Buffer.
29. Remove the tube from the magnetic separator and resuspend the coupled and washed microspheres in 250–1000  $\mu$ L of Blocking/Storage Buffer.
30. Store coupled microspheres refrigerated at 2–8 °C in the dark.

### Technical notes

- A. Microspheres should be protected from prolonged exposure to light throughout this procedure.
- B. See Table 1 for information on buffers.
- C. See Table 2 for information on magnetic separators.

Optimal coupling conditions, such as the amount of antigen or antibody to utilize in the coupling reaction, are generally determined in small-scale coupling reactions using 2.5–5 million microspheres. Coupling can then be

scaled up or down as needed; however, the ratio of capture reagent per million microspheres should be maintained. The volume of activation buffer, amount of EDC and Sulfo-NHS used, and the total coupling reaction volume may change depending on scale and the size of the tube used for the coupling reaction (Luminex Corporation, 2007h). General recommendations for coupling from 2.5–200 million microsphere are as follows: (1) The volume for activation is 100  $\mu$ L for 2.5–12.5 million microspheres and 500  $\mu$ L for 50–200 million microspheres; (2) Sulfo-NHS and EDC are at 0.5 mg each (10  $\mu$ L of 50 mg/mL) for 2.5–5 million microspheres, 2.5 mg each (50  $\mu$ L of 50 mg/mL) for 10–50 million microspheres, and 5 mg each (100  $\mu$ L of 50 mg/mL) for 100–200 million microspheres; (3) the coupling volume is 0.5 mL for 2.5–5 million microspheres, 1 mL for 10–12.5 million microspheres, and 2 mL for 50–200 million microspheres; and (4) 0.5–1 mL coupling reactions are performed in 1.5 mL microcentrifuge tubes and 2 mL coupling reactions are performed in 4 mL microcentrifuge tubes or 15 mL polypropylene centrifuge tubes.

Uncoupled microspheres tend to be sticky and will adhere to the walls of most centrifuge tubes. Co-polymer microcentrifuge tubes from USA Scientific (catalog # 1415–2500) and Eppendorf Protein LoBind (catalog # 022431081) have been found to provide optimal microsphere recovery post-coupling. Optimal coupling is achieved when coupling reactions are incubated with end-over-end mixing on a rotator (VWR, catalog # 56264–302). Larger scale coupling reactions performed in 15 mL polypropylene conical tubes can be placed at an angle on an orbital microtiter plate shaker set to 300 rpm.

Other chemistries have been used for coupling proteins and polysaccharides to the microspheres. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium (DMTMM) has been used as a one-step alternative to EDC and Sulfo-NHS for coupling primary amines to carboxylated microspheres. DMTMM is more stable than EDC or NHS and has been shown to be a robust method for microsphere coupling (Schlottmann *et al.*, 2006). Other coupling chemistries, including the use of hydrazide and maleimide, may also be used for covalent attachment of proteins to the microsphere surface.

Peptides, phospholipids, and other small molecules can be directly coupled to the microsphere surface (Komatsu *et al.*, 2004; Shichijo *et al.*, 2004) but may be more efficiently accomplished through modification of the small molecule or the microsphere to provide adequate spacing from the microsphere surface. This can be accomplished through the use of a linker or carrier protein attached to the small molecule, which can then be coupled to the microsphere surface using the standard two-step carbodiimide chemistry. If the small molecule is available in a biotinylated form, it can be bound to LumAvidin Microspheres where the avidin provides spacing from the microsphere surface (Iannone *et al.*, 2001; Drummond *et al.*, 2008; Gu *et al.*, 2008). A sample protocol for binding of biotinylated molecules to LumAvidin Microspheres is described below (Luminex Corporation, 2007c). However, with this approach a biotin–streptavidin system cannot be used for reporter

labeling and an alternative reporter labeling method, such as a direct conjugation of PE to the detection reagent, would be necessary.

### Sample Protocol for Binding Biotin-Conjugated Molecules to LumAvidin Microspheres

1. Resuspend the stock LumAvidin microsphere suspension according to the instructions described in the Product Information Sheet provided with your microspheres.
2. Transfer  $1.0 \times 10^5$  of the stock microspheres to a USA Scientific (1415–2500) or Eppendorf Protein LoBind (022431081) microcentrifuge tube.
3. Pellet the stock microspheres by microcentrifugation at  $\geq 8000g$  for 1–2 min.
4. Remove the supernatant and resuspend the pelleted microspheres in 250  $\mu$ L of PBS–BSA by vortex and sonication for approximately 20 s.
5. Dilute the biotin-conjugated molecule in PBS–BSA. The optimal concentration should be determined by titration in the 4–4000 nM range.
6. Add 250  $\mu$ L of the biotin-conjugated molecule solution to the microsphere suspension and mix immediately by vortex.
7. Incubate for 30 min with mixing (by rotation) at room temperature.
8. Pellet the bound microspheres by microcentrifugation at  $\geq 8000g$  for 1–2 min.
9. Remove the supernatant and resuspend the pelleted microspheres in 500  $\mu$ L of Blocking/Storage Buffer (PBS–BN or PBS–TBN) by vortex.
10. Pellet the bound microspheres by microcentrifugation at  $\geq 8000g$  for 1–2 min.
11. Repeat steps 9 and 10. This is a total of two washes with Blocking/Storage Buffer.
12. Remove the supernatant and resuspend the microspheres in 250–1000  $\mu$ L Blocking/Storage Buffer by vortex and sonication for approximately 20 s.
13. Store the bound LumAvidin microspheres refrigerated at 2–8°C in the dark.

#### Technical notes

- A. Microspheres should be protected from prolonged exposure to light throughout this procedure.
- B. See Table 1 for information on buffers.

Coupling a functionalized linker to the microsphere surface is another approach used for attaching small molecules. These crosslinking reagents can vary in functional group and act as spacers to position the small molecule away from the microsphere surface. Adipic acid dihydrazide (ADH) provides a 10-atom spacer with a hydrazide for coupling to carboxyl groups, and 4-(4-*N*-maleimido-phenyl)butyric acid hydrazide hydrochloride (MPBH) provides an eight-atom spacer for coupling to sulfhydryl groups. Sample protocols for modification of carboxylated microspheres with ADH and MPBH are described below. The capture moieties can then be coupled to the modified microspheres in one step using EDC.

### Sample Protocol for ADH Modification of Carboxylated Microspheres

1. Resuspend the stock microsphere suspension according to the instructions described in the Product Information Sheet provided with your microspheres.
2. Remove an aliquot of  $25 \times 10^6$  of microspheres and pellet by centrifugation at  $\geq 4000g$  for 2 min.
3. Remove the supernatant and resuspend the pelleted microspheres in 1 mL of 0.1 M MES, pH 6.0, by vortex and sonication for approximately 20 s.
4. Transfer the resuspended microspheres to a USA Scientific (1415–2500) or Eppendorf Protein LoBind (022431081) microcentrifuge tube and pellet the microspheres by microcentrifugation at  $\geq 8000g$  for 1–2 min.
5. Remove the supernatant and resuspend the microspheres in 1 mL of 35 mg/mL ADH (diluted in 0.1 M MES, pH 6.0) by vortex.
6. Add 200  $\mu$ L of 200 mg/mL EDC (prepared immediately before use in 0.1 M MES, pH 6.0) and mix by vortex.
7. Incubate for 1 h with mixing (by rotation) at room temperature.
8. Pellet the microspheres by microcentrifugation at  $\geq 8000g$  for 1–2 min.
9. Remove the supernatant and resuspend the pelleted microspheres in 1 mL of 0.1 M MES, pH 4.5, by vortex.
10. Pellet the microspheres by microcentrifugation at 8000  $g$  for 1–2 min.
11. Repeat steps 9 and 10 twice. This is a total of three washes with 1 mL of 0.1 M MES, pH 4.5.
12. Resuspend the ADH-modified microspheres in 1 mL of 0.1 M MES, pH 4.5, and store refrigerated at 2–8°C in the dark.

#### Technical notes

- A. Microspheres should be protected from prolonged exposure to light throughout this procedure.

### Sample Protocol for MPBH Modification of Carboxylated Microspheres

1. Resuspend the stock microsphere suspension according to the instructions described in the Product Information Sheet provided with your microspheres.
2. Remove an aliquot of  $25 \times 10^6$  of microspheres and pellet by centrifugation at  $\geq 4000g$  for 2 min.
3. Remove the supernatant and resuspend the pelleted microspheres in 1 mL of 0.1 M MES, pH 6.0, by vortex and sonication for approximately 20 s.
4. Transfer the resuspended microspheres to a USA Scientific (1415–2500) or Eppendorf Protein LoBind (022431081) microcentrifuge tube and pellet the microspheres by microcentrifugation at  $\geq 8000g$  for 1–2 min.
5. Remove the supernatant.
6. Dissolve MPBH at 80 mM (28.3 mg/mL) with DMSO.

7. Dilute dissolved MPBH to 16 mM (5.7 mg/mL) with 0.1 M MES, pH 6.0.
8. Resuspend the microspheres in 250  $\mu$ L of diluted MPBH by vortex.
9. Add 100  $\mu$ L of 20 mg/mL EDC (prepared immediately before use in 0.1 M MES, pH 6.0) and mix by vortex.
10. Incubate for 1 h with mixing (by rotation) at room temperature.
11. Add 1 mL of 0.1 M MES, pH 4.5, and mix by vortex.
12. Pellet the microspheres by microcentrifugation at  $\geq 8000g$  for 1–2 min.
13. Remove the supernatant and resuspend the pelleted microspheres in 1 mL of 0.1 M MES, pH 4.5, by vortex.
14. Pellet the bound microspheres by microcentrifugation at  $\geq 8000g$  for 1–2 min.
15. Repeat steps 9 and 10. This is a total of two washes with 1 mL of 0.1 M MES, pH 4.5.
16. Resuspend the MPBH-modified microspheres in 1 mL of 0.1 M MES, pH 4.5, and store refrigerated at 2–8°C in the dark.

### Technical notes

- A. Microspheres should be protected from prolonged exposure to light throughout this procedure.

Polysaccharide antigens have also been conjugated to carboxylated microspheres through modification of the antigen or microsphere surface. For example, poly-L-lysine modification with cyanuric chloride crosslinking has been used with standard EDC-mediated chemistries to attach pneumococcal serotype polysaccharides to the microsphere surface carboxyl groups (Pickering *et al.*, 2002). Sodium periodate oxidized pneumococcal polysaccharides have also been successfully bound to ADH-functionalized microspheres (Biagini *et al.*, 2003). DMTMM chemistry has also been employed to covalently couple pneumococcal polysaccharides to carboxylated microspheres (Schlottmann *et al.*, 2006).

## COUPLING CONFIRMATION

Once capture reagent is coupled to the microsphere surface, verification of efficient covalent attachment of the capture reagent should be performed (Luminex Corporation, 2006c). Confirmation for antibody coupling can be performed by titration with a PE-labeled anti-species antibody or in the case of antigen-coupled microspheres, titration with a labeled detection antibody specific for the antigen. As an example, a protocol for verifying antibody coupling is provided below. This example employs the use of filter plates with vacuum filtration for wash steps; however, magnetic separation can be used for wash steps with MagPlex microspheres. When using vacuum filtration, it is important to not vacuum the filter to dryness which can cause the microspheres to stick to the membrane. Microspheres can be resuspended by gently pipetting up and down several times to ensure the microspheres have been dislodged from the membrane. A dose response increase in MFI should be observed as concentration of labeled detection antibody

increases. In general, an antibody coupling should yield at least 10,000 MFI at saturation for optimal use in immunoassays (Luminex Corporation, 2006g).

## Sample Protocol for Confirmation of Antibody Coupling

1. Select the appropriate antibody-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for approximately 20 s.
3. Prepare a Working Microsphere Mixture by diluting the coupled microsphere stocks to a final concentration of 100 microspheres of each set/ $\mu$ L in Assay Buffer. 50  $\mu$ L of Working Microsphere Mixture is required for each reaction.
4. Prepare two-fold serial dilutions of PE-labeled anti-species IgG detection antibody from 4 to 0.0625  $\mu$ g/mL in Assay Buffer. 50  $\mu$ L of diluted detection antibody is required for each reaction.
5. Pre-wet a 1.2  $\mu$ m filter plate (Millipore, MABVN1250) with 100  $\mu$ L/well of Assay Buffer and aspirate by vacuum manifold.
6. Aliquot 50  $\mu$ L of the Working Microsphere Mixture into the appropriate wells of the filter plate.
7. Add 50  $\mu$ L of the diluted detection antibody into the appropriate wells of the filter plate.
8. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
9. Cover the filter plate and incubate for 30 min at room temperature on a plate shaker.
10. Aspirate the supernatant by vacuum manifold.
11. Wash each well twice with 100  $\mu$ L of Assay Buffer and aspirate by vacuum manifold.
12. Resuspend the microspheres in 100  $\mu$ L of Assay Buffer by gently pipetting up and down five times with a multi-channel pipettor.
13. Analyze 50–75  $\mu$ L on the Luminex analyzer according to the system manual.

### Technical notes

- A. Microspheres should be protected from prolonged exposure to light throughout this procedure.
- B. See Table 1 for information on buffers.

## ASSAY OPTIMIZATION

After confirmation of successful coupling, the best performing reagents are selected for further testing with standard or control materials. These are often recombinant proteins or known positive and negative samples, which should be prepared in the appropriate sample matrix to match the composition of the test samples as closely as possible. One advantage of multiplexing is that it can facilitate the screening of candidate capture and detection reagents. For example, several different potential capture antibodies for a particular analyte can each be coupled to a different microsphere set and then tested in multiplex with the individual candidate detection antibodies and analytes, allowing for rapid identification of the best-performing capture and detection antibody pair for a particular analyte.

Both polyclonal and monoclonal antibodies can be used for detection, but monoclonal antibodies should be specific for a different epitope than the capture antibody or can be used if they are directed to a repeating epitope on the analyte. Detection antibodies are typically biotinylated to use with streptavidin-R-phycoerythrin (SAPE) as the reporter but detection antibodies may also be directly conjugated to PE, which eliminates the need for a separate reporter labeling step in the assay protocol. Once optimal antibody pairs are identified for each analyte, the multiplexed coupled microsphere sets should be tested with each individual analyte and individual detection antibody to evaluate performance and determine specificity. If cross-reactivity between antibodies for different analytes is observed, it may be necessary to replace the cross-reacting capture or detection antibody with another that was identified during the initial antibody screening.

Next, the multiplexed microspheres are analyzed with individual analytes and multiplexed detection antibodies to determine sensitivity and detect interference between the various detection antibodies. The optimal detection antibody concentration will vary with the specific reagent and should be determined by titration (e.g., two-fold serial dilution from 4 to 1  $\mu\text{g/mL}$ ), but generally, 2–4  $\mu\text{g/mL}$  is adequate. Detection antibody concentration may need to be increased in multiplex as compared to the concentration used in single-plex due to interactions between various detection antibodies. In general, as the level of multiplexing increases, the amount needed for each detection antibody may also increase. In unwashed assay formats, detection antibody concentrations may need to be increased by up to as much as five-fold to compensate for excess unbound analyte in the supernatant. Typically, reporter fluorophore (SAPE) concentration should be one and one-half to two times the concentration of detection antibody. Concentrations above 4  $\mu\text{g/mL}$  of SAPE may require a post-labeling wash step to reduce backgrounds, and final concentrations above 8  $\mu\text{g/mL}$  may interfere with the background subtraction performed by the analyzer.

Finally, the fully multiplexed assay is performed to determine sensitivity and interference when all analytes and reagents are present in the reaction. Multiplexed assay development can be an iterative process, requiring further optimization as complex interactions between assay components are observed. Assay conditions, such as buffer system, blocking agents, sample volume and dilution, total reaction volume, number of microspheres per reaction (2000–5000 per region per well), concentration of capture reagent for coupling, detection antibody and reporter concentration, assay format (washed *vs.* unwashed), and incubation times, are optimized to provide best results according to the specific assay requirements, and the performance is evaluated and validated with known samples. Concentrated biological samples and samples of a highly complex nature, such as serum, plasma, or tissue lysates, should be diluted at least 1:5 to prevent interference or microsphere agglutination from matrix effects. Any reagents that show interference, cross-reactivity, or poor performance should be replaced.

Optimization of assay performance and meeting requirements for sensitivity, dynamic range, ease of use, and time to result should be kept in mind when developing the multiplexed assay. To improve sensitivity or increase signal,

several signal amplification techniques can be explored, including adjustment of the PMT setting on Luminex 100/200 and FLEXMAP 3D instruments, vendor and type of SAPE reporter, dendrimers, rolling circle amplification, and incorporation of additional reporter labeling steps. Reducing the volume of the initial incubation with microspheres and sample and/or increasing the initial incubation time may improve the kinetics for analyte binding, thus improving sensitivity. Though seemingly paradoxical, improved sensitivity can be sometimes accomplished by decreasing the amount of capture reagent coupled to the microspheres. While this may result in saturation at lower analyte concentrations and lower the maximum achievable signal, it may improve linearity at low concentrations, thus improving the limit of detection (Fig. 3a). Antibodies with higher affinity can also improve sensitivity, both as capture and detection reagents. Higher signals and extended dynamic range at high analyte concentrations can be achieved by increasing the amount of capture reagent coupled to the microspheres (Fig. 3a). Both high sensitivity and broad dynamic range can sometimes be achieved through coupling capture antibodies of different affinities to different microsphere color sets and combining them to create a multiplexed standard curve (Fig. 3b). The same effect can be accomplished by coupling different concentrations of the same capture reagent to different microsphere color sets.

Conversion of a washed assay to an unwashed assay format can reduce hands-on time as well as decrease total assay time. To convert to an unwashed format, sample volume may be decreased and/or detection antibody and SAPE concentrations are increased to compensate for higher concentrations of unbound analyte and detection antibody present in the reaction. Increasing the volume of the detection antibody used as compared to the washed format assay introduces more detection antibody and dilutes the sample prior to analysis, which may overcome matrix effects or issues caused by interfering substances. In some cases, a final post-labeling wash step may be included to reduce background signals and improve overall assay performance and sensitivity.

## IMMUNOASSAY FORMATS

### IMMUNOMETRIC

The flexibility of xMAP Technology allows various assay formats to be used with the system, and common immunoassay formats used with other platforms have been easily adapted for use with Luminex analyzers. The immunometric (or capture sandwich) immunoassay, commonly used in ELISA, can be similarly used with the xMAP platform but differing in the use of a fixed and stable fluorescent reporter as opposed to an enzymatically-derived reporter system. Microspheres covalently coated with specific capture antibodies for various analytes are multiplexed and incubated with the sample (Fig. 4). The various analytes bind to the corresponding specific microsphere set and are then detected by addition and binding of analyte-specific detection antibodies, typically labeled with biotin and SAPE. Assays can be washed between sequential reagent additions, or unwashed, depending on the

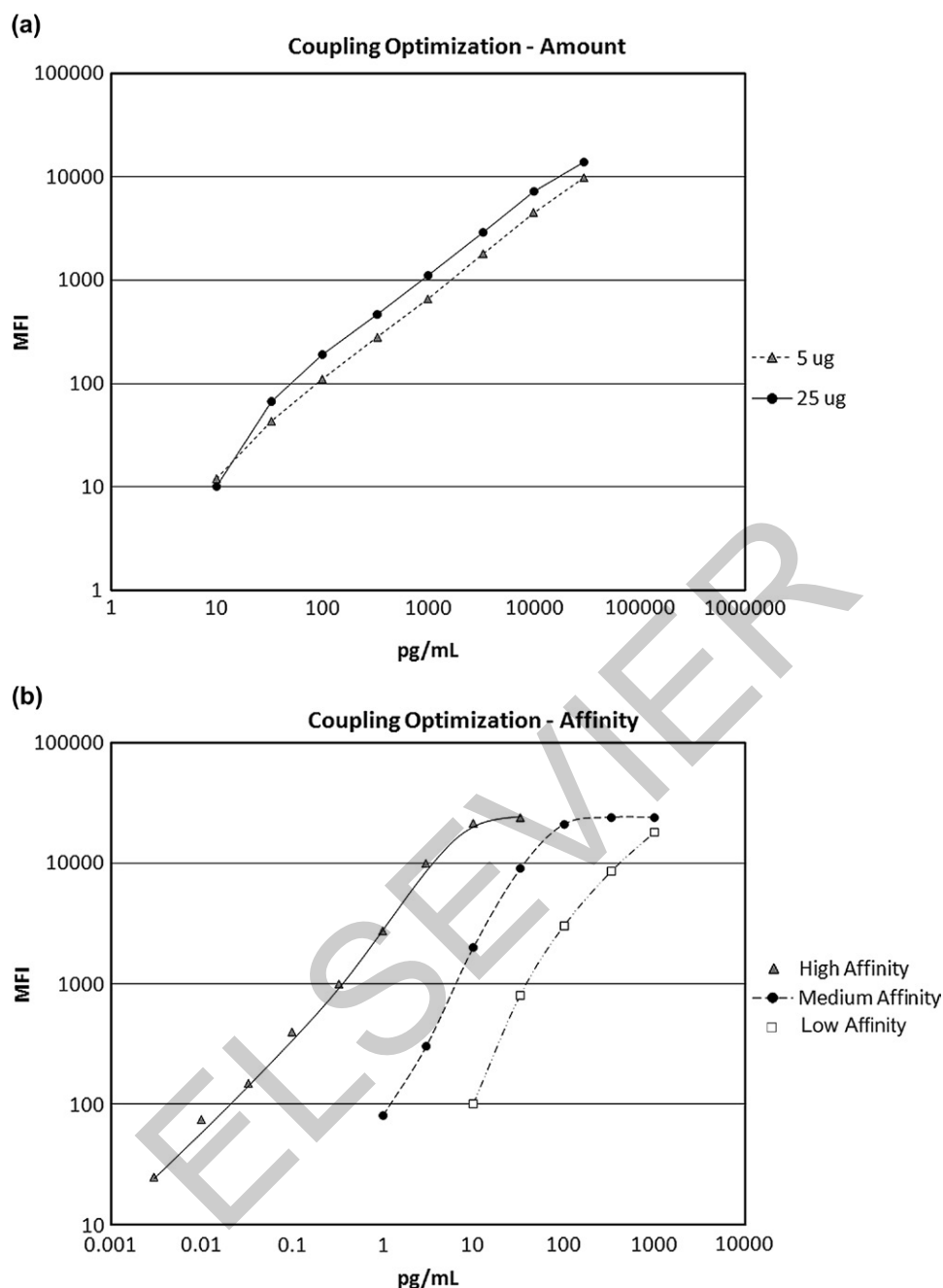


**FIGURE 3** Effect of microsphere coupling on assay performance. (a) Coupling less capture antibody leads to lower MFI but better sensitivity, whereas coupling more antibody yields higher MFI and extends dynamic range. (b) A multiplexed standard curve using capture antibodies of different affinities coupled to different microsphere sets provides both high sensitivity and broad dynamic range.

specific assay performance and time requirements and the nature of the sample matrix and reagents used. Washed assays can be performed manually or automated with microtiter plate washers, using either vacuum filtration or magnetic separation (with MagPlex Microspheres). Several sample protocols for immunometric (sandwich) assays using MicroPlex or MagPlex Microspheres are available (Luminex Corporation, 2006e,f, 2007g). As a representative example, protocols for a washed immunometric assay (using MagPlex Microspheres) and a no-wash immunometric assay are described.

### Sample Protocol for Immunometric (Sandwich) Immunoassay Using MagPlex Microspheres

1. Select the appropriate antibody-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for approximately 20 s.
3. Prepare a Working Microsphere Mixture by diluting the coupled microsphere stocks to a final concentration of 100 microspheres of each set/ $\mu$ L in

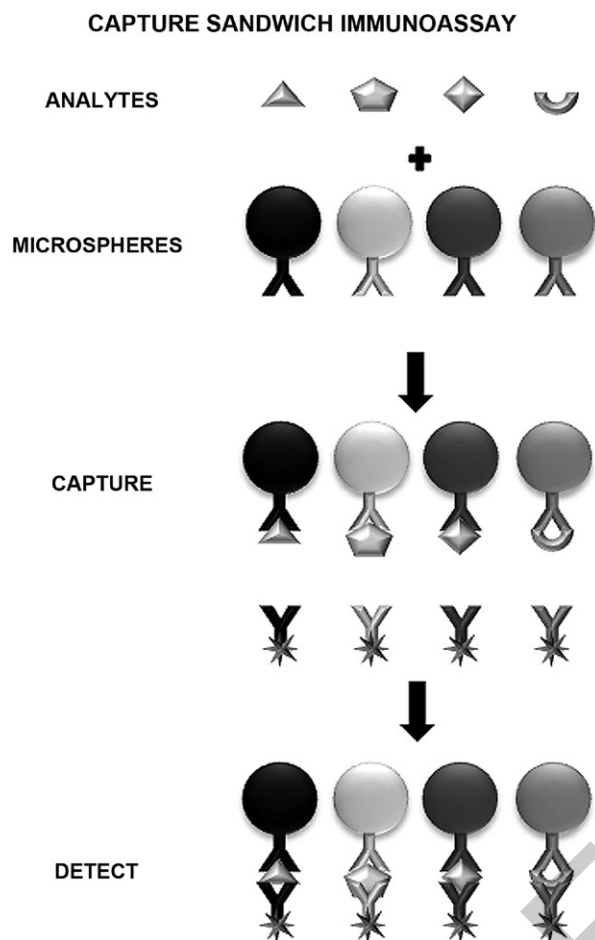


**FIGURE 4** Immunometric (sandwich) assay. A sample containing a mixture of analytes is combined with antibody-coupled microsphere sets. Analytes are captured onto their respective microsphere sets and then detected by quantitation of bound fluorochrome-labeled detection antibodies.

Assay Buffer. 50  $\mu$ L of Working Microsphere Mixture is required for each reaction.

4. Aliquot 50  $\mu$ L of the Working Microsphere Mixture into the appropriate wells of a round-bottom well plate (CoStar 3789).
5. Add 50  $\mu$ L of Assay Buffer to each background well.
6. Add 50  $\mu$ L of standard or sample to the appropriate wells.
7. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
8. Cover the plate and incubate for 30 min at room temperature on a plate shaker set to approximately 800 rpm.
9. Place the plate into the magnetic separator and allow separation to occur for 30–60 s.
10. Use a multi-channel pipette to carefully aspirate the supernatant from each well. Take care not to disturb the microspheres.
11. Leave the plate in the magnetic separator for the following wash steps:
  - a. Add 100  $\mu$ L Assay Buffer to each well.
  - b. Use a multi-channel pipette to carefully aspirate the supernatant from each well or use the

manual inversion wash method. Take care not to disturb the microspheres.

- c. Repeat steps a and b above.
12. Remove the plate from the magnetic separator and resuspend the microspheres in 50  $\mu$ L of Assay Buffer by gently pipetting up and down several times using a multi-channel pipettor.
13. Dilute the biotinylated detection antibody to 4  $\mu$ g/mL in Assay Buffer. 50  $\mu$ L of diluted detection antibody is required for each reaction.
14. Add 50  $\mu$ L of the diluted detection antibody to each well.
15. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
16. Cover the plate and incubate for 30 min at room temperature on a plate shaker set to approximately 800 rpm.
17. Place the plate into the magnetic separator and allow separation to occur for 30–60 s.
18. Use a multi-channel pipette to carefully aspirate the supernatant from each well. Take care not to disturb the microspheres.
19. Leave the plate in the magnetic separator for the following wash steps:
  - a. Add 100  $\mu$ L Assay Buffer to each well.
  - b. Use a multi-channel pipette to carefully aspirate the supernatant from each well or use the manual inversion wash method. Take care not to disturb the microspheres.
  - c. Repeat steps a and b above.
20. Remove the plate from the magnetic separator and resuspend the microspheres in 50  $\mu$ L of Assay Buffer by gently pipetting up and down several times with a multi-channel pipettor.
21. Dilute SAPE reporter to 4  $\mu$ g/mL in Assay Buffer. 50  $\mu$ L of diluted SAPE is required for each reaction.
22. Add 50  $\mu$ L of the diluted SAPE to each well.
23. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
24. Cover the plate and incubate for 30 min at room temperature on a plate shaker set to approximately 800 rpm.
25. Place the plate into the magnetic separator and allow separation to occur for 30–60 s.
26. Use a multi-channel pipette to carefully aspirate the supernatant from each well. Take care not to disturb the microspheres.
27. Leave the plate in the magnetic separator for the following wash steps:
  - a. Add 100  $\mu$ L Assay Buffer to each well.
  - b. Use a multi-channel pipette to carefully aspirate the supernatant from each well or use the manual inversion wash method. Take care not to disturb the microspheres.
  - c. Repeat steps a and b above.
28. Remove the plate from the magnetic separator and resuspend the microspheres in 100  $\mu$ L of Assay Buffer by gently pipetting up and down several times with a multi-channel pipettor.
29. Analyze 50–75  $\mu$ L on the Luminex analyzer according to the system manual.

### Technical notes

- A. Microspheres should be protected from prolonged exposure to light throughout this procedure.
- B. See Table 1 for information on buffers.
- C. See Table 2 for information on magnetic separators.

### Sample Protocol for No-wash Immunometric (Sandwich) Immunoassay

1. Select the appropriate antibody-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for approximately 20 s.
3. Prepare a Working Microsphere Mixture by diluting the coupled microsphere stocks to a final concentration of 200 microspheres of each set/ $\mu\text{L}$  in Assay Buffer. 25  $\mu\text{L}$  of Working Microsphere Mixture is required for each reaction.
4. Aliquot 25  $\mu\text{L}$  of the Working Microsphere Mixture to the appropriate wells of a round-bottom microtiter plate (CoStar 3789).
5. Add 25  $\mu\text{L}$  of Assay Buffer to each background well.
6. Add 25  $\mu\text{L}$  of standard or sample to the appropriate wells.
7. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
8. Cover the plate and incubate for 30 min at room temperature with mixing on a plate shaker (400 rpm for non-magnetic microspheres or 800 rpm for MagPlex microspheres).
9. Dilute the biotinylated detection antibody to the appropriate concentration in Assay Buffer. 25  $\mu\text{L}$  of diluted detection antibody is required for each reaction.
10. Add 25  $\mu\text{L}$  of the diluted detection antibody to each well.
11. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
12. Cover the plate and incubate for 30 min at room temperature with mixing on a plate shaker (400 rpm for non-magnetic microspheres or 800 rpm for MagPlex microspheres).
13. Dilute SAPE to the appropriate concentration (typically  $\geq 4 \mu\text{g/mL}$ ) in Assay Buffer. 25  $\mu\text{L}$  of diluted SAPE is required for each reaction.
14. Add 25  $\mu\text{L}$  of the diluted SAPE to each well.
15. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
16. Cover the plate and incubate for 15–30 min at room temperature with mixing on a plate shaker (400 rpm for non-magnetic microspheres or 800 rpm for MagPlex microspheres).
17. Analyze 50–75  $\mu\text{L}$  on the Luminex analyzer according to the system manual.

### Technical notes

- A. Microspheres should be protected from prolonged exposure to light throughout this procedure.
- B. See Table 1 for information on buffers.

- C. Concentrations of the detection antibodies and SAPE should be optimized. The optimal concentrations tend to be higher than in a washed assay.
- D. If high backgrounds are observed, a final post-labeling wash step may be performed just prior to analysis. Refer to washed assay protocols for instructions for washing by vacuum filtration or magnetic separation.

### INDIRECT

The indirect (serological) immunoassay format has also been adapted for use with the xMAP Technology. Detection of specific antibodies in a test sample is accomplished by binding to the surface of antigen-coupled microsphere sets (Fig. 5). Antigen coupling can be accomplished using the various chemistries as described previously. Optimal amount of antigen for coupling will depend on the size of the antigen and should be determined by titration, but generally, 0.04–5  $\mu\text{g}$  per 1 million microspheres will perform well in serological assays. A labeled anti-species detection antibody detects the antigen-specific antibody captured on the microsphere surface. Typically, the anti-species

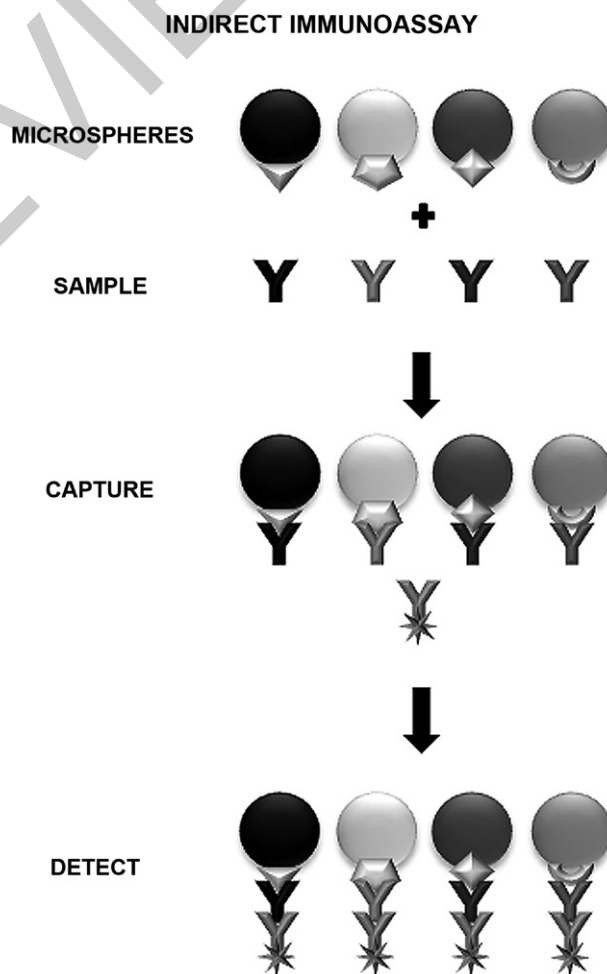


**FIGURE 5** Indirect immunoassay. A sample containing antigen-specific antibodies is combined with antigen-coupled microsphere sets. Antibodies are captured onto their respective microsphere sets and then detected by quantitation of bound fluorochrome-labeled anti-species detection antibodies.

detection antibody is directly conjugated to the PE reporter fluorophore or can be biotinylated and labeled by the addition of SAPE. Serological immunoassays are usually performed in a washed assay format to remove unbound antibody and other complex matrix proteins that can lead to interference and increase backgrounds (Luminex Corporation, 2006d, 2011c). Washing steps can be facilitated with the use of MagPlex microspheres and magnetic separation. A sample protocol for an indirect immunoassay with MagPlex Microspheres is detailed below.

### Sample Protocol for Indirect (Antibody Capture) Immunoassay

1. Select the appropriate antigen-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for approximately 20 s.
3. Prepare a Working Microsphere Mixture by diluting the coupled microsphere stocks to a final concentration of 100 microspheres of each set/ $\mu$ L in Assay Buffer. 50  $\mu$ L of Working Microsphere Mixture is required for each reaction.
4. Aliquot 50  $\mu$ L of the Working Microsphere Mixture into the appropriate wells of a round-bottom microtiter plate (CoStar 3789).
5. Add 50  $\mu$ L of Assay Buffer to each background well.
6. Add 50  $\mu$ L of standard or sample to the appropriate wells.
7. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
8. Cover the plate and incubate for 30–60 min at room temperature on a plate shaker (800 rpm for MagPlex Microspheres).
9. Place the plate into the magnetic separator and allow separation to occur for 30–60 s.
10. Use a multi-channel pipette to carefully aspirate the supernatant from each well. Take care not to disturb the microspheres.
11. Leave the plate in the magnetic separator for the following wash steps:
  - a. Add 100  $\mu$ L Assay Buffer to each well.
  - b. Use a multi-channel pipette to carefully aspirate the supernatant from each well or use the manual inversion wash method. Take care not to disturb the microspheres.
  - c. Repeat steps a and b above.
12. Remove the plate from the magnetic separator and resuspend the microspheres in 50  $\mu$ L of Assay Buffer by gently pipetting up and down several times with a multi-channel pipettor.
13. Dilute PE-labeled anti-species IgG detection antibody to 4  $\mu$ g/mL in Assay Buffer. 50  $\mu$ L of diluted detection antibody is required for each reaction.
14. Add 50  $\mu$ L of the diluted detection antibody into the appropriate wells of the filter plate.
15. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
16. Cover the filter plate and incubate for 30 min at room temperature on a plate shaker (800 rpm for MagPlex Microspheres).
17. Place the plate into the magnetic separator and allow separation to occur for 30–60 s.
18. Use a multi-channel pipette to carefully aspirate the supernatant from each well. Take care not to disturb the microspheres.
19. Leave the plate in the magnetic separator for the following wash steps:
  - a. Add 100  $\mu$ L Assay Buffer to each well.
  - b. Use a multi-channel pipette to carefully aspirate the supernatant from each well or use the manual inversion wash method. Take care not to disturb the microspheres.
  - c. Repeat steps a and b above.
20. Remove the plate from the magnetic separator and resuspend the microspheres in 100  $\mu$ L of Assay Buffer by gently pipetting up and down several times with a multi-channel pipettor.
21. Analyze 50–75  $\mu$ L on the Luminex analyzer according to the system manual.

### Technical notes

- A. Microspheres should be protected from prolonged exposure to light throughout this procedure.
- B. See Table 1 for information on buffers.
- C. See Table 2 for information on magnetic separators.

### COMPETITIVE

The use of a competitive immunoassay format may be preferred for detection of small analytes that contain only one or a few epitopes or where there are only one or few antibodies available. In this assay format, MFI signal decreases with increasing analyte concentration due to the presence of a competing analyte. Competitive immunoassays on the xMAP platform can be performed by two methods shown in Fig. 6. In one format (Format 1), antibody specific to the analyte is coupled to the microsphere surface and the analyte present in the sample competes with a labeled analyte for binding to the antibody coupled to the microsphere surface. Alternatively, the analyte can be coupled to the microsphere surface and compete with the analyte in the sample binding to a labeled detection antibody (Format 2). For both formats, the labeled detection reagent should be at a concentration that yields 70–80% of the maximum signal ( $[IC_{70}]$  or  $[IC_{80}]$ ) to ensure that binding is not saturated and the measurement occurs in the steepest portion of the standard curve. Determination of the  $[IC_{70}]$  or  $[IC_{80}]$  is accomplished *via* titration of the labeled reagent with the coupled microspheres, in the absence of the test sample. Example protocols for both formats (unwashed) are provided below and vary primarily in the order in which reagents are added to the reaction (Luminex Corporation, 2006b, 2007d).

### Sample Protocol for Competitive Immunoassay for Antibody-Coupled Microspheres

1. Select the appropriate antibody-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for approximately 20 s.

## COMPETITIVE IMMUNOASSAY

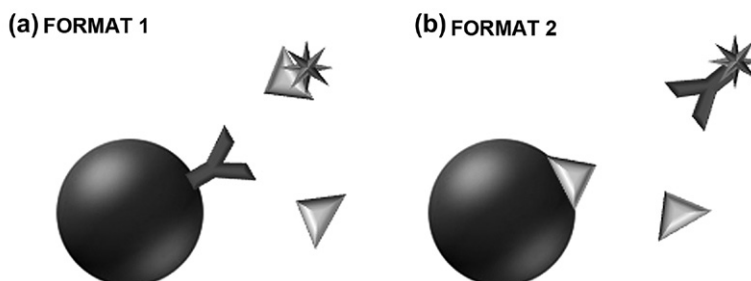


**FIGURE 6** Competitive immunoassay. (a) In Format 1, the analyte in the sample competes with a labeled analyte for binding to the specific antibody-coupled microspheres. (b) In Format 2, the analyte in the sample competes with the analyte-coupled microspheres for binding to the labeled detection antibody.

3. Prepare a Working Microsphere Mixture by diluting the coupled microsphere stocks to a final concentration of 200 microspheres of each set/ $\mu\text{L}$  in Assay Buffer. 25  $\mu\text{L}$  of Working Microsphere Mixture is required for each reaction.
4. Dilute the biotinylated competitor to the  $[\text{IC}_{70}]$  or  $[\text{IC}_{80}]$  in Assay Buffer. 25  $\mu\text{L}$  of diluted competitor is required for each reaction.
5. Add 25  $\mu\text{L}$  of Assay Buffer to each background well in a round-bottom microtiter plate (CoStar 3789).
6. Add 25  $\mu\text{L}$  of standard or sample to the appropriate wells.
7. Add 25  $\mu\text{L}$  of the diluted, biotinylated competitor to each well.
8. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
9. Add 25  $\mu\text{L}$  of the Working Microsphere Mixture to each well.
10. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
11. Cover the plate and incubate for 60 min at room temperature on a plate shaker (400 rpm for non-magnetic microspheres or 800 rpm for MagPlex microspheres).
12. Dilute the SAPE reporter to the appropriate concentration (typically  $\geq 4 \mu\text{g/mL}$ ) in Assay Buffer. 25  $\mu\text{L}$  of diluted SAPE is required for each reaction.
13. Add 25  $\mu\text{L}$  of the diluted SAPE to each well.
14. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
15. Cover the plate and incubate for 30 min at room temperature on a plate shaker (400 rpm for non-magnetic microspheres or 800 rpm for MagPlex microspheres).
16. Analyze 50–75  $\mu\text{L}$  on the Luminex analyzer according to the system manual.

### Technical notes

- A. Microspheres should be protected from prolonged exposure to light throughout this procedure.
- B. See Table 1 for information on buffers.

- C. The  $[\text{IC}_{70}]$  and  $[\text{IC}_{80}]$  are the concentrations of biotinylated competitor that yield 70% and 80% of the maximum obtainable signal, respectively. The  $[\text{IC}_{70}]$  or  $[\text{IC}_{80}]$  should be determined by titration in Assay Buffer.
- D. Concentrations of SAPE should be optimized. The optimal concentration tends to be higher than in a washed assay.
- E. If high backgrounds are observed, a final post-labeling wash step may be performed just prior to analysis. Refer to washed assay protocols for instructions for washing by vacuum filtration or magnetic separation.

### Sample Protocol for Competitive Immunoassay for Antigen-Coupled Microspheres

1. Select the appropriate antigen-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for approximately 20 s.
3. Prepare a Working Microsphere Mixture by diluting the coupled microsphere stocks to a final concentration of 200 microspheres of each set/ $\mu\text{L}$  in Assay Buffer. 25  $\mu\text{L}$  of Working Microsphere Mixture is required for each reaction.
4. Dilute the biotinylated detection antibody to the  $[\text{IC}_{70}]$  or  $[\text{IC}_{80}]$  in Assay Buffer. 25  $\mu\text{L}$  of biotinylated detection antibody is required for each reaction.
5. Add 25  $\mu\text{L}$  of Assay Buffer to each background well in a round-bottom microtiter plate (CoStar 3789).
6. Add 25  $\mu\text{L}$  of standard or sample to the appropriate wells.
7. Add 25  $\mu\text{L}$  of the Working Microsphere Mixture to each well.
8. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
9. Add 25  $\mu\text{L}$  of the diluted biotinylated detection antibody to each well.
10. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.

11. Cover the plate and incubate for 60 min at room temperature on a plate shaker (400 rpm for non-magnetic microspheres or 800 rpm for MagPlex microspheres).
12. Dilute the SAPE reporter to the appropriate concentration (typically  $\geq 4 \mu\text{g/mL}$ ) in Assay Buffer. 25  $\mu\text{L}$  of diluted SAPE is required for each reaction.
13. Add 25  $\mu\text{L}$  of the diluted SAPE to each well.
14. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
15. Cover the plate and incubate for 30 min at room temperature on a plate shaker (400 rpm for non-magnetic microspheres or 800 rpm for MagPlex microspheres).
16. Analyze 50–75  $\mu\text{L}$  on the Luminex analyzer according to the system manual.
5. Add 10  $\mu\text{L}$  of Assay Buffer to each background well of a round-bottom microtiter plate (CoStar 3789).
6. Add 10  $\mu\text{L}$  of standard or sample to the appropriate wells.
7. Add 5  $\mu\text{L}$  of the diluted competitor to each well.
8. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
9. Aliquot 5  $\mu\text{L}$  of the Working Microsphere Mixture to each well.
10. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
11. Cover the filter plate and incubate for 60 min at room temperature on a plate shaker (400 rpm for non-magnetic microspheres or 800 rpm for MagPlex Microspheres).
12. Dilute the biotinylated detection antibody to the appropriate concentration in Assay Buffer. 10  $\mu\text{L}$  of diluted detection antibody is required for each reaction.
13. Add 10  $\mu\text{L}$  of the diluted detection antibody to each well.
14. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
15. Cover the plate and incubate for 60 min at room temperature on a plate shaker (400 rpm for non-magnetic microspheres or 800 rpm for MagPlex Microspheres).
16. Dilute the SAPE reporter to the appropriate concentration (typically  $\geq 10\text{--}12 \mu\text{g/mL}$ ) in Assay Buffer. 10  $\mu\text{L}$  of diluted SAPE is required for each reaction.
17. Add 10  $\mu\text{L}$  of the diluted SAPE to each well.
18. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
19. Cover the plate and incubate for 30 min at room temperature on a plate shaker (400 rpm for non-magnetic microspheres or 800 rpm for MagPlex Microspheres).
20. Bring final volume of each reaction to 100  $\mu\text{L}$  with Assay Buffer.
21. Analyze 50–75  $\mu\text{L}$  on the Luminex analyzer according to the system manual.

### Technical notes

- A. Microspheres should be protected from prolonged exposure to light throughout this procedure.
- B. See Table 1 for information on buffers.
- C. The  $[\text{IC}_{70}]$  and  $[\text{IC}_{80}]$  are the concentrations of detection antibody that yield 70% and 80% of the maximum obtainable signal, respectively. The  $[\text{IC}_{70}]$  or  $[\text{IC}_{80}]$  should be determined by titration in Assay Buffer.
- D. Concentrations of the detection antibodies and SAPE should be optimized. The optimal concentrations tend to be higher than in a washed assay.
- E. If high backgrounds are observed, a final post-labeling wash step may be performed just prior to analysis. Refer to washed assay protocols for instructions for washing by vacuum filtration or magnetic separation.

## COMBINED IMMUNOMETRIC AND COMPETITIVE

Competitive immunoassays can also be multiplexed with the immunometric (sandwich) immunoassay (Luminex Corporation, 2006a). A sample protocol for this combined multiplexing strategy (unwashed) is provided below.

### Sample Protocol for Combined Immunometric/Competitive Immunoassay

1. Select the appropriate antibody- and/or antigen-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for approximately 20 s.
3. Prepare a Working Microsphere Mixture by diluting the coupled microsphere stocks to a final concentration of 1000 microspheres of each set/ $\mu\text{L}$  in Assay Buffer. 5  $\mu\text{L}$  of Working Microsphere Mixture is required for each reaction.
4. Dilute the biotinylated competitor to the  $[\text{IC}_{70}]$  or  $[\text{IC}_{80}]$  in Assay Buffer. 5  $\mu\text{L}$  of diluted competitor is required for each reaction.

### Technical notes

- A. Microspheres should be protected from prolonged exposure to light throughout this procedure.
- B. See Table 1 for information on buffers.
- C. The  $[\text{IC}_{70}]$  and  $[\text{IC}_{80}]$  are the concentrations of detection antibody that yield 70% and 80% of the maximum obtainable signal, respectively. The  $[\text{IC}_{70}]$  or  $[\text{IC}_{80}]$  should be determined by titration in Assay Buffer.
- D. Concentrations of biotinylated competitors, detection antibodies, and SAPE should be optimized. The optimal concentrations tend to be higher than in a washed assay.
- E. If high backgrounds are observed, a final post-labeling wash step may be performed just prior to analysis. Refer to washed assay protocols for instructions for washing by vacuum filtration or magnetic separation.

**TABLE 3** xMAP Immunodiagnostic Applications and Platforms

| Partner                      | Application Areas         | Products                      |                                |                                 | Website                                                            | References                                                                                                                                                       |
|------------------------------|---------------------------|-------------------------------|--------------------------------|---------------------------------|--------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                              |                           | Platforms                     | Assays                         | Other                           |                                                                    |                                                                                                                                                                  |
| Alere                        | Autoimmune disease        | AtheNA Multi-Lyte® Instrument | AtheNA Multi-Lyte®             |                                 | <a href="http://www.alere.com">www.alere.com</a>                   | (Dhiman <i>et al.</i> , 2010; Binnicker <i>et al.</i> , 2010; Martins <i>et al.</i> , 2008; Avannis-Aghajani <i>et al.</i> , 2007; Biagini <i>et al.</i> , 2007) |
|                              | Infectious disease        | AIMS®                         |                                |                                 |                                                                    |                                                                                                                                                                  |
| BMD                          | Autoimmune disease        | FIDIS™ system                 | FIDIS™/ Multiplex              | MLx Booster software            | <a href="http://www.bmd-net.com">www.bmd-net.com</a>               | (Vercammen <i>et al.</i> , 2007; Tozzoli <i>et al.</i> , 2006; Buliard <i>et al.</i> , 2005)                                                                     |
|                              | Infectious disease        | FIDIS™ 200 system             |                                | Fpx software                    |                                                                    |                                                                                                                                                                  |
|                              | Cytokines                 | CARIS™<br>CARIS™ <sub>μ</sub> |                                | Custom assays                   |                                                                    |                                                                                                                                                                  |
| Bio-Rad Laboratories         | Autoimmune disease        | BioPlex® 2200                 | BioPlex®                       | eFlex™ software                 | <a href="http://www.bio-rad.com">www.bio-rad.com</a>               | (Binnicker <i>et al.</i> , 2010; Loeffelholz <i>et al.</i> , 2011; Binnicker <i>et al.</i> , 2011; Hanly <i>et al.</i> , 2010)                                   |
|                              | Infectious disease        |                               |                                |                                 |                                                                    |                                                                                                                                                                  |
| Gen-Probe                    | HLA testing               | Gen-Probe Fluoroanalyzer      | LIFECODES antibody detection   |                                 | <a href="http://www.gen-probe.com">www.gen-probe.com</a>           | (Middleburg <i>et al.</i> , 2011; Jung <i>et al.</i> , 2009)                                                                                                     |
| InGen Biosciences            | Infectious disease        |                               | BJI InoPlex™                   | Distribution                    | <a href="http://www.ingen.fr">www.ingen.fr</a>                     |                                                                                                                                                                  |
| Inova Diagnostics            | Autoimmune disease        | QUANTA Plex™                  | QUANTA Plex™                   |                                 | <a href="http://www.innovadx.com">www.innovadx.com</a>             | (Ghillani <i>et al.</i> , 2007)                                                                                                                                  |
| ImmuneTech                   | Allergy                   |                               | My Allergy Test (send in test) | My Allergy Test testing service | <a href="http://www.immunetech.com">www.immunetech.com</a>         |                                                                                                                                                                  |
| Indoor Biotechnologies, Inc. | Allergy                   |                               | MARIA™                         | Testing service                 | <a href="http://www.inbio.com">www.inbio.com</a>                   | (King <i>et al.</i> , 2007; Earle <i>et al.</i> , 2007)                                                                                                          |
| Innogenetics                 | Neurodegenerative disease |                               | INNO-BIA                       |                                 | <a href="http://www.innogenetics.com">www.innogenetics.com</a>     | (Lewczuk <i>et al.</i> , 2008; Fagan <i>et al.</i> , 2011)                                                                                                       |
| Mikrogen                     | Infectious disease        |                               | recomBead®                     | Distribution                    | <a href="http://www.mikrogen.de">www.mikrogen.de</a>               |                                                                                                                                                                  |
| Multimetrix                  | Infectious disease        |                               | Multimetrix                    | Testing service                 | <a href="http://www.multimetrix.com">www.multimetrix.com</a>       |                                                                                                                                                                  |
|                              | Cytokines                 |                               |                                | Custom assays                   |                                                                    |                                                                                                                                                                  |
|                              |                           |                               |                                | Assay development reagents      |                                                                    |                                                                                                                                                                  |
| One Lambda, Inc.             | HLA testing               | LABScan™ 100                  | LABScreen®                     | HLA Visual™ software            | <a href="http://www.onelambda.com">www.onelambda.com</a>           | (Jung <i>et al.</i> , 2009; Irving <i>et al.</i> , 2011; Quillen <i>et al.</i> , 2011)                                                                           |
|                              |                           |                               |                                | HLA Fusion™ software            |                                                                    |                                                                                                                                                                  |
| Tellgen                      | Tumor markers             |                               | Tellgenplex™                   |                                 | <a href="http://www.tellgen.com">www.tellgen.com</a>               |                                                                                                                                                                  |
| ZEUS Scientific              | Autoimmune disease        | AtheNA Multi-Lyte® Instrument | AtheNA Multi-Lyte®             | Distributed by Alere            | <a href="http://www.zeusscientific.com">www.zeusscientific.com</a> | (Dhiman <i>et al.</i> , 2010; Binnicker <i>et al.</i> , 2010; Martins <i>et al.</i> , 2008; Avannis-Aghajani <i>et al.</i> , 2007; Biagini <i>et al.</i> , 2007) |
|                              | Infectious disease        | AIMS®                         |                                |                                 |                                                                    |                                                                                                                                                                  |

## COMMERCIAL IMMUNOASSAY APPLICATIONS AND PLATFORMS

The extensive adoption of xMAP Technology has led to the development of a wide range of applications and platforms for research and approved for IVD use in the immunodiagnostic laboratory. These applications include multiplex detection of proteins and antibodies for human leukocyte antigen (HLA) and tissue typing, autoimmune diseases, infectious disease serology, neurodegenerative diseases, and allergy testing (Luminex Corporation, 2011a).

Table 3 describes the various immunodiagnostics platforms, applications, and services available from Luminex partners, and references selected recent publications describing the use and performance of these products in immunodiagnostic applications.

## CONCLUSIONS

The Luminex xMAP system provides the technology and tools for multiplexing up to 500 analytes in a single reaction, optimizing throughput and bioassay performance.

xMAP Technology is used extensively in immunodiagnos-  
tics, preclinical/clinical trials, and research environments,  
and has been found to be a rapid, cost-effective, high-  
throughput platform for a variety of bioassays. The avail-  
able instrumentation, assay methodologies, and software  
provide easy implementation into most laboratory set-  
tings, and numerous protocols and publications are avail-  
able to facilitate assay development and use of the  
technology. The published literature demonstrates the  
flexibility and versatility of this unique analysis platform.  
Although here we have focused on immunoassay and  
immunodiagnosics applications and products of xMAP  
Technology, this technology is also widely used for nucleic  
acid applications, including single nucleotide polymor-  
phism genotyping, molecular genetics, gene expression  
profiling, molecular detection for infectious diseases, and  
microRNA profiling.

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