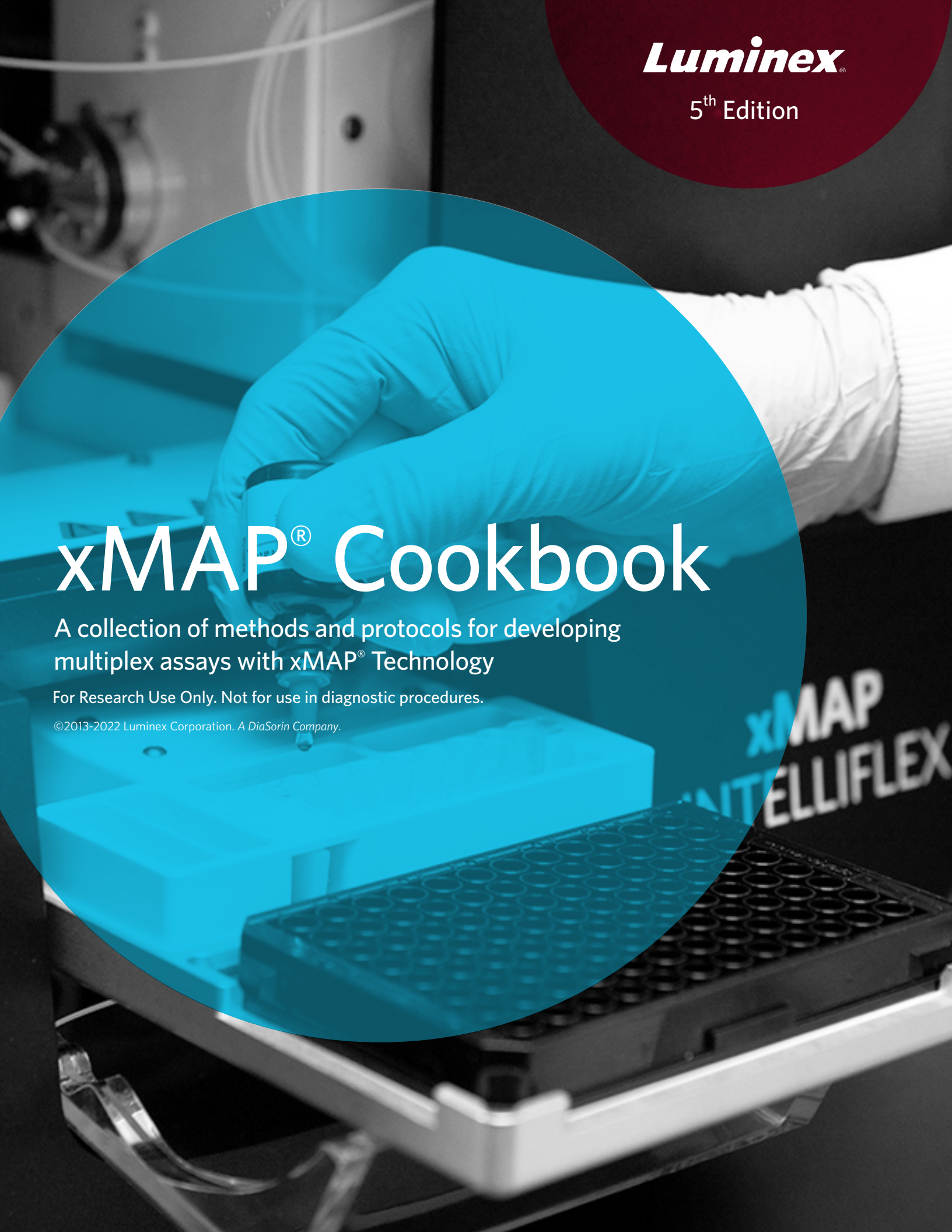
Luminex
5th Edition
xMAP® Cookbook
A collection of methods and protocols for developing multiplex assays with xMAP® Technology
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Chapter 1

Introduction

Chapter 1

Introduction

Biological assays have evolved from relatively large volume reactions to smaller volume, faster, highly automated tests. Whether in a test tube rack, a microwell plate, or a micro-volume chip, these are all considered 'arrays' of assays, where different samples are physically separated from one another.

Since biological assays are typically coupled to a colorimetric readout, the notion of 'multiplexing,' or reading multiple test results in a single sample volume, has been complicated primarily by spectral overlap, where color from one assay detection channel interferes with color in other detection channels. This limitation means multi-color assays are only useful for a few analytes per sample.

While microarrays (two-dimensional solid arrays) allow small-volume assaying of physically separated features, limitations such as slow solid-phase kinetics, instability of immobilized protein or nucleic acid capture molecules, and poor reproducibility may limit their broader application in clinical and research laboratories.

Solution-phase multiplex assays remain highly desirable to laboratories due to the following benefits:

- Reduced sample volume and other redundant consumables
- More data with less labor
- Faster results due to solution-phase kinetics

What is multiplexing?

Multiplexing describes assaying multiple analytes simultaneously within a single sample volume in a single cycle or run. While solid-phase microarrays technically meet this definition, multiplexing typically describes solution-phase assays such as xMAP® Technology or quantitative PCR.

Chapter 2

xMAP® Technology

Chapter 2

xMAP® Technology

In the late 1990s, scientists at Luminex invented xMAP® Technology, which was a major advancement for multiplexed biological assays. xMAP Technology draws from the strengths of solid-phase separation technology without the typical limitations of solid-phase reaction kinetics. By combining advanced fluidics, optics, and digital signal processing with proprietary microsphere ("bead") technology, xMAP Technology enables a high degree of multiplexing within a single sample volume. Featuring a flexible, open-architecture design, xMAP Technology can be configured to perform a wide variety of assays quickly, cost-effectively, and accurately.

How xMAP got its name

x = Biomarker or disease panel to be tested
MAP = Multi-analyte profiling
xMAP = Multiplex biological testing of up to 500 analytes in a single sample volume

How does xMAP Technology work?

xMAP Technology uses colored beads to perform biological assays similar to ELISA or nucleic acid hybridization assays. By color-coding microscopic beads into many spectrally distinct sets, each bead set can be coated with a nucleic acid or protein capture molecule specific to a particular biological target, allowing the simultaneous capture of multiple analytes from a single sample. Because of the microscopic size and low density of these beads, assay reactions exhibit nearly solution-phase reaction kinetics. However, once an assay is complete, the solid-phase characteristics allow each bead to be analyzed discretely. By incorporating magnetic properties into xMAP microspheres, assay washing is simplified while maintaining desirable solution-phase properties.

Figure 1.

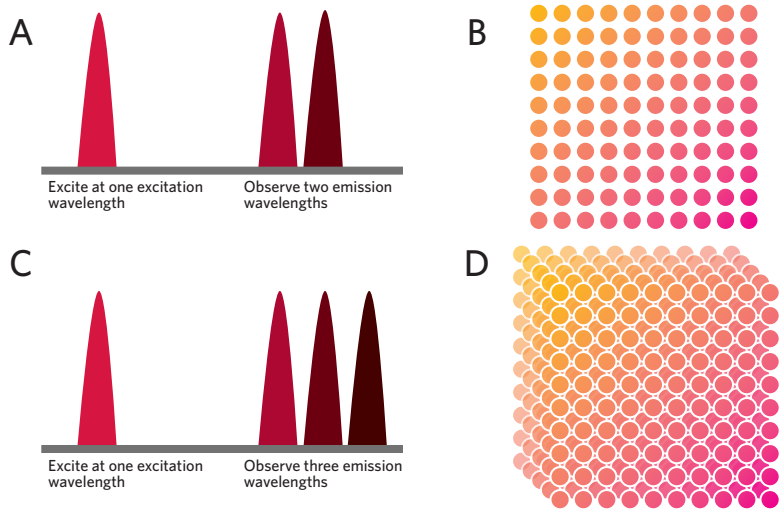


Figure 1 – xMAP® microspheres include two dyes where (**A**) one excitation wavelength allows the observation of two separate fluorescence emission wavelengths, yielding (**B**) 100 unique microsphere sets (10x10 dye matrix), or three dyes where (**C**) one excitation wavelength allows the observation of three separate fluorescence wavelengths, yielding (**D**) 500 unique microsphere sets (10x10x5 dye matrix).

Figure 2.

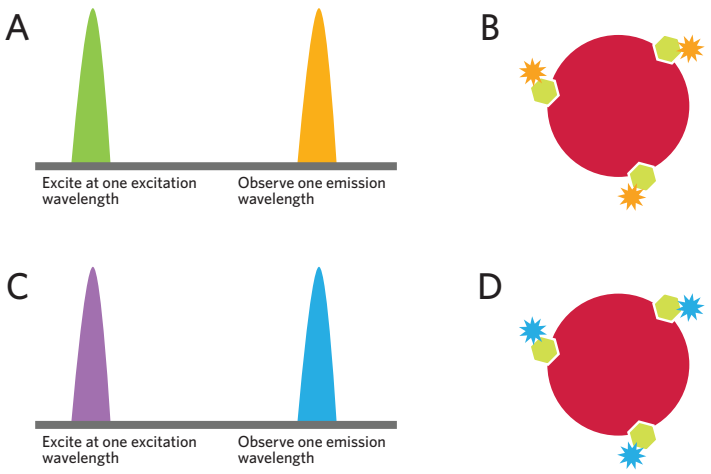


Figure 2 – In addition to the detection of internal bead dyes (shown in **Figure 1**), (**A**) a second excitation wavelength allows the observation of a separate fluorescent reporter molecule, (**B**) enabling the detection of the analyte captured on the surface of the microsphere. On the xMAP INTELLIFLEX® DR-SE System, (**C**) a third excitation wavelength permits the observation of an additional fluorescent reporter molecule, (**D**) enabling the detection of a second parameter or analyte captured on the surface of the microsphere.

Multiple light sources inside the Luminex analyzer excite the internal bead dyes that identify each microsphere particle, as well as any fluorescent reporter molecules captured during the assay. The instrument records dozens of readings for each bead set and produces a distinct result for each analyte in the sample. Using this process, xMAP Technology allows multiplexing of up to 500 unique bioassays within a single sample both rapidly and precisely.

This revolution in multiplex biological assays has been licensed by Luminex to a number of kit developers in the clinical diagnostic, pharmaceutical, and life sciences research markets. Commercially available kits exist for molecular diagnostics, immunodiagnostics, kinase profiling, cytokine/chemokine detection, genotyping, gene expression profiling, and other applications.

In addition to commercial kits, Luminex supports custom assay development. The xMAP Cookbook provides a summary of methods and protocols for developing multiplex biological assays with xMAP Technology.

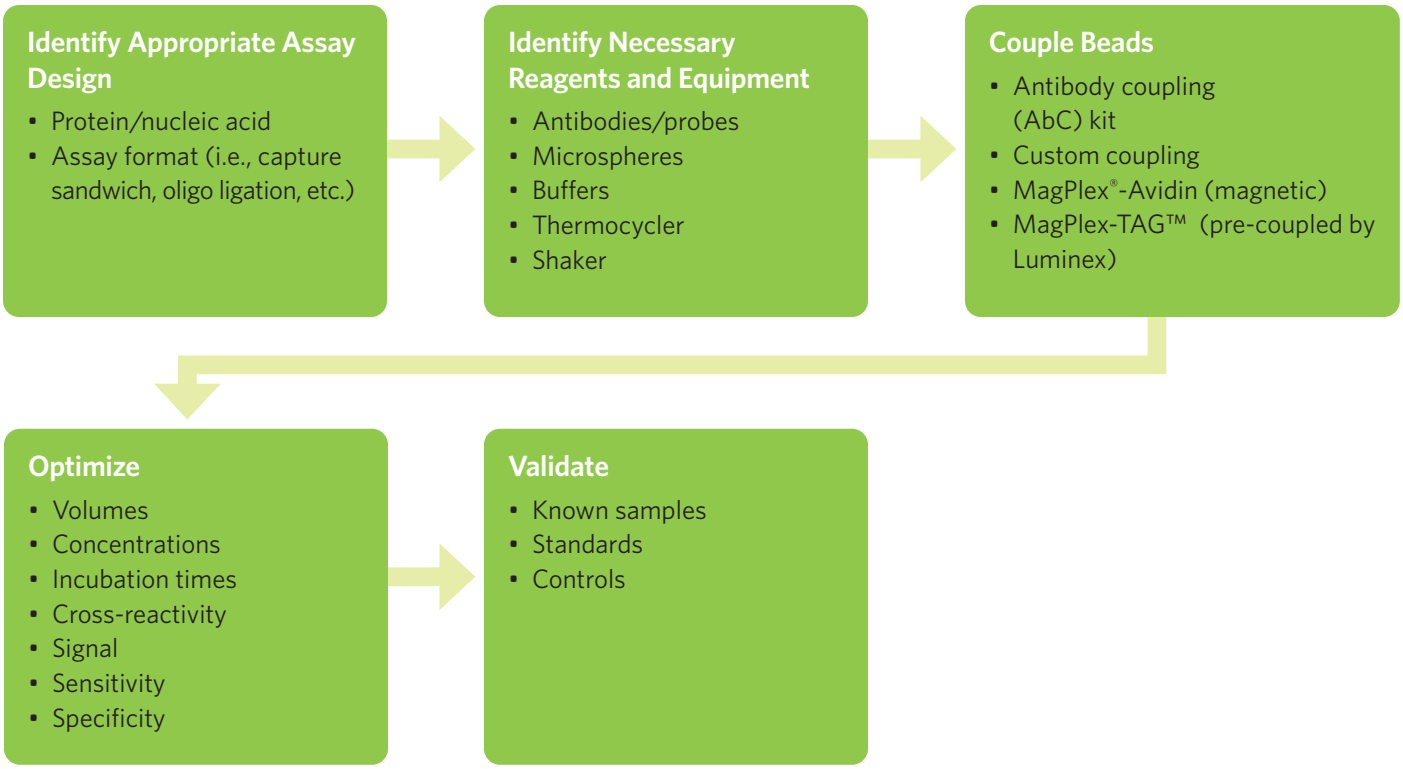
Chapter 3

Development of an xMAP® Assay

Chapter 3

Development of an xMAP® Assay

The development process for xMAP® multiplex assays is relatively simple, but does require a few unique considerations compared to singleplex assays. The following describes the general workflow of xMAP assay development.



This chapter covers the initial steps and considerations for developing an xMAP multiplex assay, including general assay design reagents and equipment needed. Additional assay development steps such as bead coupling, optimization, and validation are discussed separately in **Chapters 4 (Proteomic Applications)** and **5 (Genomic Applications)**.

Chapter 3.1

Assay Design

xMAP® Technology is adaptable to a number of biological assays, including immunoassays, nucleic acid assays, and enzyme activity assays. Common immunoassay formats include the capture sandwich, competitive, and indirect antibody assays. Nucleic acid assays are hybridization-based, where a probe sequence captures a labeled complementary target from your sample reaction. Enzyme activity assays typically involve labeling or cleaving a peptide substrate to introduce or release a fluorescent molecule.

Immunoassay	Nucleic Acid	Enzyme Activity
Capture sandwich	TAG incorporation	Kinase/phosphatase selectivity
Competitive	PCR-based	
Indirect assay	Primer extension	
	Probe ligation	

Immunoassays

An immunoassay is a biochemical test that measures the presence or concentration of a macromolecule in a solution through the use of an antibody or immunoglobulin. The macromolecule detected by the immunoassay is often referred to as an "analyte" and is, in many cases, a protein. This type of assay is also referred to as protein or proteomic assay.

Figure 3.

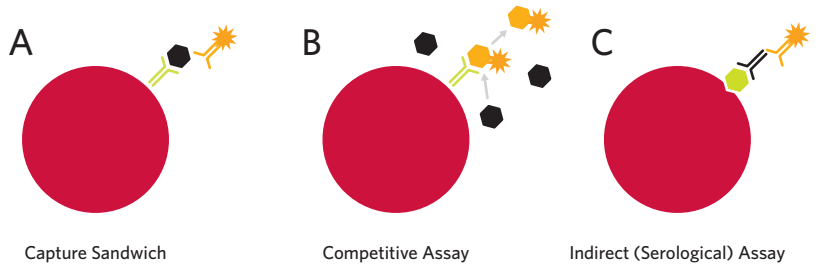


Figure 3 - Common assay formats for immunoassays include (**A**) the capture sandwich assay, which requires capture and detection antibodies to your analyte target (polyclonal capture antibodies should be purified and mono-specific); (**B**) the competitive (antibody) assay, which requires a single antibody and a labeled positive target; and (**C**) the indirect (or serology) assay, which requires both a target protein and an anti-antibody.

Immunoassays are used for the detection of biological substances and have become established as one of the most popular analytical techniques applied in clinical and veterinary medicine, drug discovery, and rapidly emerging areas such as biothreat and food safety research. Due to their ease of use, accuracy, specificity, and speed, immunoassays are commonly used to measure a large number of hormones, blood products, enzymes, drugs, disease markers, and other biological molecules. Many immunoassays can be performed directly on untreated samples, such as plasma, serum, urine, saliva, and cerebrospinal fluid. Single-analyte ELISA has been an industry standard for immunoassays for decades, and has led to the development of more novel techniques, including highly multiplexed immunoassays that can measure hundreds of analytes simultaneously. Multiplex assays are particularly useful for generating profiles of clinical samples, which can facilitate accurate disease diagnoses or the prediction of drug responses.

Nucleic acid assays

Multiplex nucleic acid assays require different optimization steps from immunoassays, although some similarities exist. The sensitivity of nucleic acid assays may be affected by the assay chemistry selected, the amount of capture oligonucleotide, and the amount of beads used. In order to distinguish similar nucleic acid sequences, standards and controls must be run to confirm that there is minimal cross hybridization or non-specific hybridization between sequences. Depending on whether the purpose of your assay is to measure gene expression, determine the genotype, or detect specific sequences, there are different requirements for the type of starting nucleic acid used in the assay and the chemistry required to generate reporter molecules. No matter which chemistry is used to generate the reporter molecules, the capture and detection of the reporter molecules is performed as illustrated in **Figure 4**.

Figure 4.

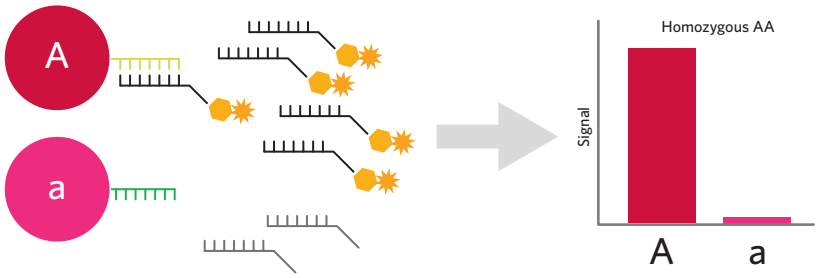
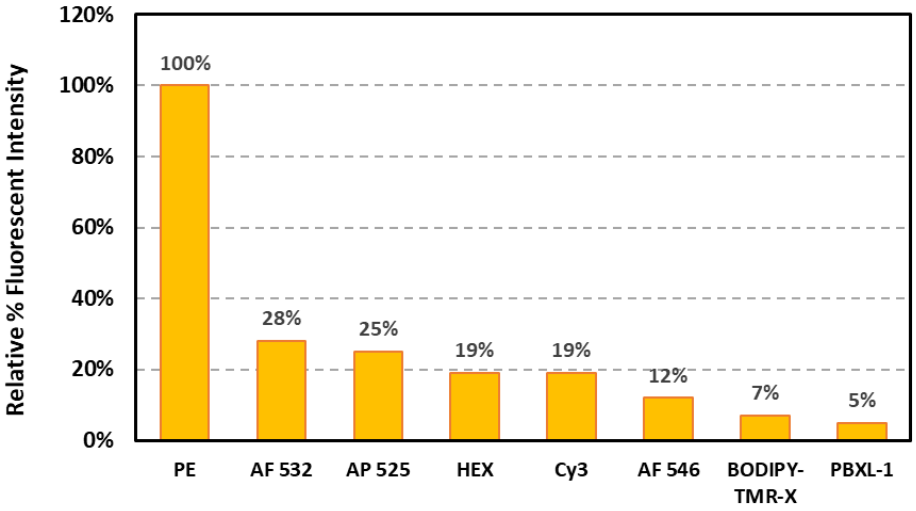


Figure 4 - Schematic of nucleic acid assay analysis on Luminex beads. Each bead has a unique capture sequence specific for a given marker sequence. If reporter molecules are generated and captured (bead A), a fluorescence signal is detected. If no reporter molecules are generated and captured (bead a), minimal or background signal is detected.

Reporter fluorophores

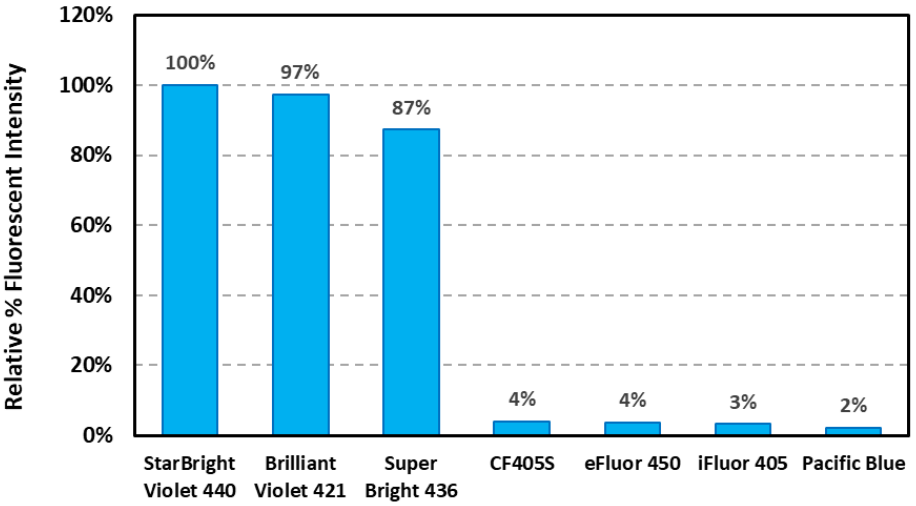
A variety of commercially available fluorescent reporter dyes may be suitable for xMAP assays using one or both reporter channels. For Reporter 1 (RP1), excitation by the green laser occurs at 532 nm, with emission measured at 565–585 nm. For Reporter 2 (RP2), excitation by the violet laser occurs at 405 nm, with emission measured at 421–441 nm. The relative reporter intensities for RP1 and RP2 are shown as a percentage of the brightest dye for all the reporter fluorophores we have tested as of this publication.

Figure 5. Relative Reporter 1 Intensities



R-phycoerythrin (PE) is the brightest reporter dye for the RP1 channel (100%), followed by Alexa Fluor® 532 (AF 532) at 28%, AquaPhluor® 525 (AP 525) at 25%, hexachlorofluorescein (HEX) and cyanine 3 (Cy3) at 19%, and Alexa Fluor® 546 (AF 546) at 12%. BODIPY-TMR-X and PBXL-1 each contributed less than 10% of the signal intensity observed with PE.

Figure 6. Relative Reporter 2 Intensities Biotinylated Microspheres

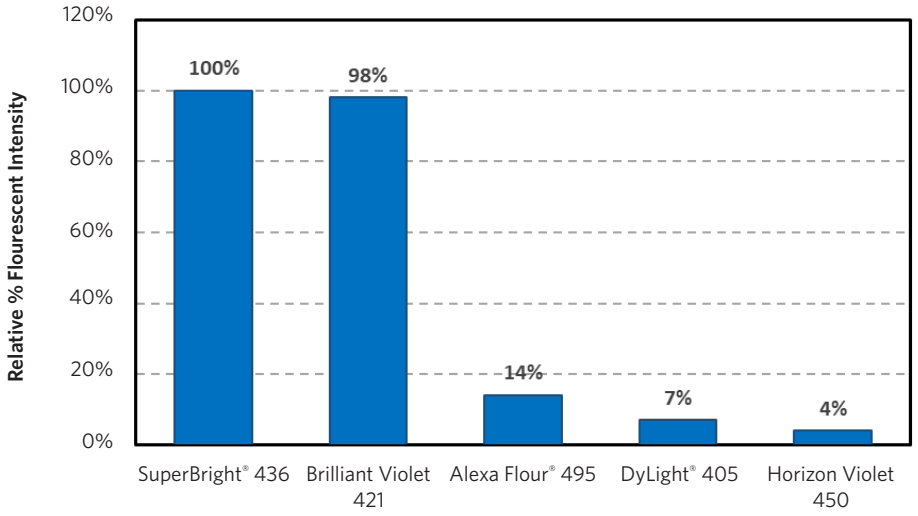


At the time of this publication, several preliminary experiments were conducted to compare reporter fluorophores in the RP2 channel for different assay formats.

While the relative intensities varied somewhat between assays, StarBright™ Violet 440 (Bio-Rad), Brilliant Violet 421 (BD Biosciences), and SuperBright® 436 (eBioscience – Thermo Fisher) were the brightest dyes in the RP2 channel. Reporter intensity is likely influenced by the assay format and detection reagents used. We recommend you test a few options and optimize concentrations by titration to select the best RP2 reporter dye for your assay.

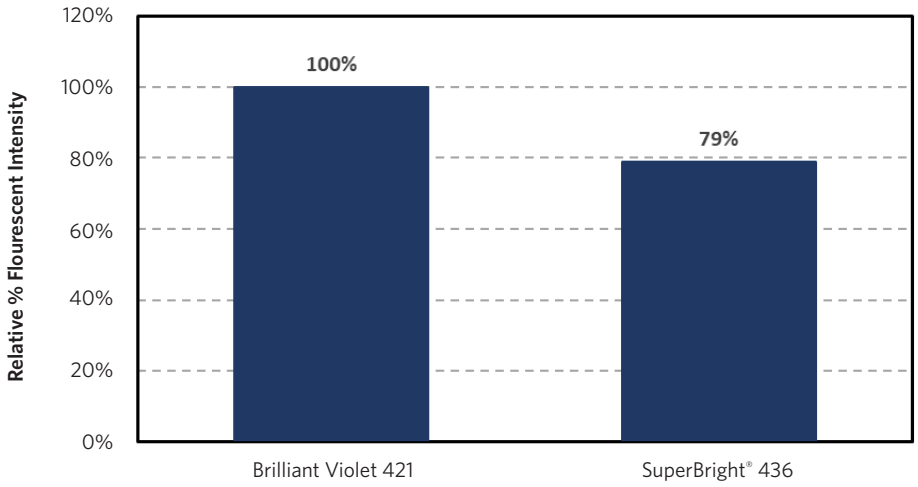
For the comparison of streptavidin conjugates for labeling biotinylated MagPlex® microspheres, StarBright Violet 440 was the brightest of the available dyes, followed by Brilliant Violet 421 (97%) and SuperBright 436 (87%). The remaining dyes (CF405S, eFluor 450, iFluor 405, and Pacific Blue) yielded less than 10% of the signal intensity of StarBright Violet 440. Each of the dyes were tested over a range of concentrations from 0.06–4 µg/mL, except for StarBright Violet 440, which comes as a 1X solution and was tested at final dilutions of 1:100 to 1:6,400.

Figure 7. Relative Reporter 2 Intensities Serological Immunoassay



In the indirect (serological) immunoassay, streptavidin conjugates were used with a biotinylated anti-human IgG detection antibody. In this experiment, SuperBright® 436 generated the highest signal, followed by Brilliant Violet 421 (98%) and Alexa Fluor® 405 (14%). The remaining dyes (DyLight® 405 and Horizon Violet 450) yielded less than 10% of the signal intensity of SuperBright 436.

Figure 8. Relative Reporter 2 Intensities Capture Sandwich Immunoassay



For the capture sandwich immunoassay, a seven-point standard curve was used with a biotinylated detection antibody for human insulin-like growth factor-binding protein-1 (IGFBP-1) to compare streptavidin conjugates of Brilliant Violet 421 and SuperBright® 436 at four working concentrations. SuperBright 436 gave the highest signal, with Brilliant Violet 421 at 79% of the SuperBright signal on average (range=73–84%).

Chapter 3.2

Reagents and Equipment

While Luminex has numerous partnerships with other companies for a variety of kits, reagents, and instrumentation, a number of products can be purchased directly from Luminex for the development and analysis of protein and genomic assays. These products include reagents, instruments, and supplies that can be found on the Luminex website (luminexcorp.com/reagents-and-accessories).

At the heart of the Luminex platform's flexibility and multiplexing capabilities are Luminex beads. Luminex's most popular variety of xMAP® beads are MagPlex® Microspheres, which are 6.5-micron superparamagnetic beads. The beads are dyed with different ratios of two or three dyes, allowing the generation of 500 different colored beads or bead regions for the development of multi-analyte assays up to 500-plex. The surface of these beads are embedded with iron-containing magnetite particles. This feature allows the use of magnets to rapidly remove the beads from reaction suspensions, speeding up processing protocols, minimizing bead loss, and resulting in more reproducible data generation.

Note: Bead colors are referred to as "regions" because beads are plotted in different regions of the bead map in the instrument software based on their dye ratios.

MagPlex Microspheres can be purchased from Luminex. The beads should be stored at 4°C and kept in the dark. They can be used directly from their vials as described in the coupling protocols. MagPlex Microspheres are available at concentrations of 2.5 million and 12.5 million beads/mL and can be ordered in 1 mL and 4 mL vial sizes. Special orders of larger size vials are also available upon request.

MagPlex-TAG™ Microspheres are MagPlex beads where each bead region is covalently coupled with a unique 24-base oligonucleotide, or 'anti-TAG' sequence. These beads enable you to quickly and easily design custom genomic assays by incorporating complementary 'TAG' sequences in primers or probes used in assay reactions. This allows reporter molecules generated in the reactions to be hybridized to specific anti-TAG sequences on specific beads regions. For a complete list of the TAG and anti-TAG sequences for each of the 150 available microspheres, visit luminexcorp.com/magplex-microspheres.

Note: The assays and protocols described in this cookbook are optimized for use with MagPlex® or MagPlex-TAG™ Microspheres unless otherwise noted. If using non-magnetic xMAP beads, contact Technical Support or your Luminex Field Application Scientist (FAS) for information on assay modifications needed for non-magnetic beads.

MagPlex and MagPlex-TAG Microspheres are compatible with all Luminex instruments, including MAGPIX® (up to 50-plex), Luminex® 200™ with xPONENT® Software (up to 80-plex), FLEXMAP 3D®, and xMAP INTELLIFLEX® (up to 500-plex for MagPlex, up to 150-plex for MagPlex-TAG). Basic reagents for Luminex equipment include Calibration and Verification kits, and Sheath or Drive Fluid. These products are also offered in a variety of custom volumes. For additional information on all xMAP reagents, contact your Luminex representative.

Whether you need to couple your own capture molecules to xMAP beads or use any of our pre-coupled bead types, developing a custom assay will require additional reagents and equipment that must be provided by the user. To assist with the development of different types of assays, the xMAP Cookbook is divided into sections for proteomic- and genomic-based assays. Each of the protocols in these two sections includes lists and recommended sources of reagents, equipment needed, and information on troubleshooting and validation. A list of common buffers and equipment required for different xMAP assays can be found in **Appendices A** and **B**, and a protocol for automated bead washing is found in **Appendix C**.

Chapter 4

Proteomic Applications

Chapter 4.1

Development of xMAP® Proteomic Assays

This section provides protocols for developing a number of different proteomic assays. These assays can be used for studying immunological responses, detecting concentrations of proteins and other molecules in different samples, analyzing protein-protein interactions, and other applications.

Before beginning the process of developing a new assay, be sure to search the hundreds of commercial assay kits already available through more than 50 of our Licensed Technology Partners. With dozens of new kits becoming available every month, many common biological targets can be found in pre-optimized panels developed by our partners. The xMAP® Kit Finder—an online catalog of research immunoassays—is a helpful tool for searching all of the commercial kits available from our partners and can be found at luminexcorp.com/kitfinder.

If there are no commercial kits available for your targets of interest and a new assay must be developed, the first step is to select an appropriate assay format such as the capture sandwich, competitive, or indirect (serological) assay. The assay format will largely depend on what antibodies are available and the size of the target analyte. Consult the literature to determine if any similar assays have already been developed and described. If an assay is already available on another platform, you can try the same format on your Luminex instrument.

The **capture sandwich** format is commonly used for xMAP protein assays (and for ELISA), where the analyte is "sandwiched" between a capture and detection antibody. The capture sandwich format is compatible with a wide variety of target analytes.

The **competitive format** is used when the target analyte is of low molecular weight and has only one or two antibody binding epitopes, or if only one antibody is available. Competitive xMAP assays can be developed using either antibody-coupled or antigen-coupled beads, and can be multiplexed with the capture sandwich format in the same assay.

The **indirect format** is used for serological assays to measure the amount of antigen-specific antibody present in biological samples. Once a format has been chosen, the beads can be coupled to the proteins used in the assay.

In the following sections, a standard carbodiimide coupling protocol is provided which can be used for coupling immunoglobulins, linkers, and other proteins as needed. Additional protocols for the use of these coupled beads for various applications are provided. Each protocol includes information that outlines the concept of the protocol, lists the required reagents, supplies, and equipment needed, and notes any troubleshooting, validation, scaling, or optimization tips.

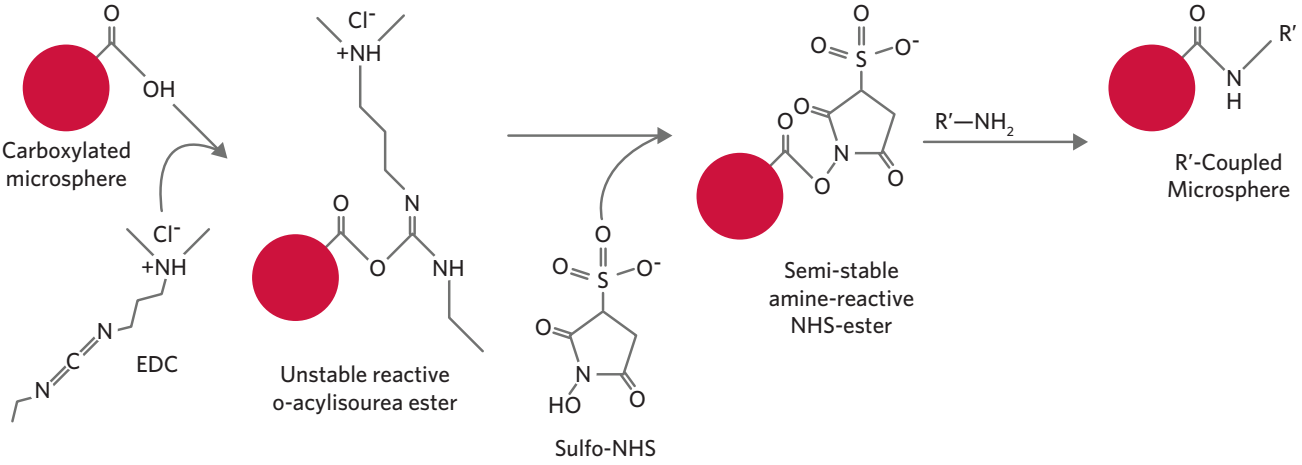
Chapter 4.2

Protein Coupling

The development of different proteomic applications with xMAP® microspheres requires the covalent coupling of capture molecules such as antibodies, proteins, or small molecule linkers to the beads. Carbodiimide coupling chemistry takes advantage of approximately 100 million carboxyl groups on each xMAP microsphere. The chemistry of the coupling process involves formation of a covalent bond between primary amines on an antibody, protein, or linker with activated carboxyl groups on the surface of xMAP microspheres.

Carbodiimide chemistry is a simple two-step process during which microsphere carboxyl groups are first activated with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride reagent in the presence of Sulfo-NHS (N-hydroxysulfosuccinimide) to form a Sulfo-NHS-ester intermediate. The reactive intermediate is then replaced by reacting with the primary amine of the coupling molecule (antibody, protein, linker, or peptide) to form a covalent amide bond.

Figure 9. Protein Coupling Chemistry



A number of factors can affect the efficiency of the coupling reaction. Some common additives to proteins and buffers that can interfere with coupling include amine-containing compounds such as trisaminomethane (Tris) or bovine serum albumin (BSA). In addition, glycerol, urea, imidazole, azide, and some detergents may also interfere with the coupling chemistry. If any of these compounds are present in the protein storage buffer, they should be removed using a suitable buffer exchange method.

For antibodies, common antibody purification methods that can be used for buffer exchange include Protein A, Protein G, ion exchange, size exclusion, and analyte-specific affinity chromatography. Affinity purification is the method of choice as it reduces nonspecific immunoglobulins and other interfering molecules. In some cases where interfering substances cannot be removed or are in low concentration (such as detergents, azide, or urea), a sufficient dilution can be performed to allow efficient coupling to proceed. For other protein or peptide preparations, ion exchange, size

exclusion, and/or dialysis methods are available to facilitate buffer exchange. When the molecule to be coupled is in a suitable buffer, the carbodiimide coupling reaction is most efficient at low pH (i.e., pH 5–6). However, for proteins sensitive to lower pH conditions, coupling reactions may be carried out at higher pH to ensure stability and functional conformation of the protein. For capture sandwich assays, monoclonal antibodies should be used for capturing the analyte to the microsphere surface to achieve maximum sensitivity and specificity. If a polyclonal antibody is used as a capture molecule, it should be monospecific and affinity-purified. The optimal amount of capture reagent may vary depending on the reagent used and should be titrated. For antibodies, about 5 µg of antibody per 1 million microspheres performs well. For other proteins, the optimum amount to couple (per million beads) will vary depending on the molecular weight and amino acid composition of the protein being coupled. Coupling a peptide may require a chemical linker. Protocols for coupling specific linkers and peptides are included in **Chapter 4.4 (Other Coupling Moieties)**.

To determine where reactive groups are on your protein, a number of bioinformatic tools are available to analyze a protein's sequence, and in some cases its 3D structure. Sites for searching for 3D structures include the NCBI Structure Group (ncbi.nlm.nih.gov/Structure/index.shtml), the RSCD Protein Data Bank (rcsb.org/pdb/home/home.do), and the SWISS-MODEL Repository (expasy.org/proteomics/protein_structure).

Chapter 4.2.1

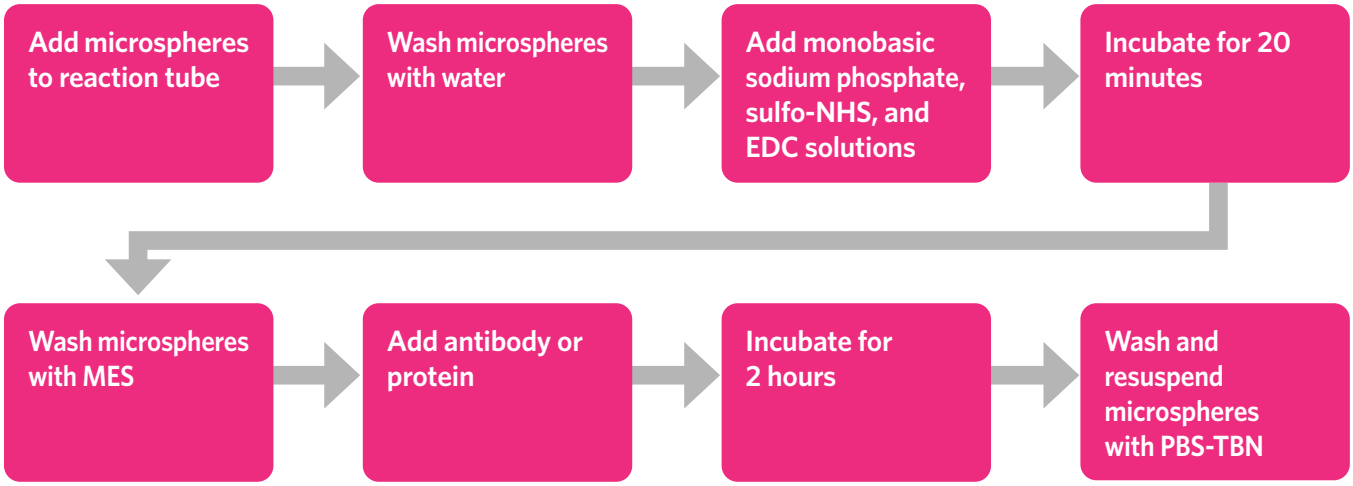
Carbodiimide Coupling Protocol for Antibodies and Proteins

General guidelines for coupling protein to xMAP® microspheres

Luminex has made coupling of antibodies or other proteins easy with the xMAP® Antibody Coupling (AbC) Kit. The kit contains all the reagents necessary to covalently couple antibodies (or other proteins) to MagPlex® Microspheres in approximately three hours. Alternatively, MagPlex®-Avidin Microspheres are magnetic microspheres pre-coupled with avidin to allow simple (non-covalent) binding of biotinylated molecules (such as peptides), without having to chemically couple capture molecules.

Whether using the xMAP AbC Kit or coupling with your own reagents, a summary of the standard coupling protocol can be found below. Once coupled, the stability of coupled microspheres is dependent on a number of factors, such as the stability of the coupled protein, buffer composition, and other storage conditions. When stored properly, coupled microspheres can be stable for more than one year.

Summary of protocol



Materials needed

Reagents and Consumables	Vendor
MagPlex® Microspheres	Luminex
Antibody/protein to be coupled	Any suitable source
1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC)	Thermo Scientific Pierce™ 77149 ProteoChem™ c1100-100mg
Sulfo-NHS	Thermo Scientific Pierce™ 24510 (500 mg) or 24520 (8x2 mg, No-Weigh Format)
Activation buffer* (0.1 M NaH_2PO_4, pH 6.2)	MilliporeSigma S3139
Coupling buffer† (50 mM MES, pH 5.0)	MilliporeSigma M2933
Phosphate buffered saline (PBS), pH 7.4‡	MilliporeSigma P3813
PBS-TBN buffer§	MilliporeSigma P3563 MilliporeSigma A7888 MilliporeSigma S8032
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Protein LoBind® 022431081
Disposable pipette tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

*Activation can be performed in 50 mM MES, pH 6.0–6.2, with similar results.
†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.
‡Alternative coupling buffer for proteins that do not couple well at pH 5–6.
§Microsphere blocking/storage buffer. May also be used as assay buffer.
¶May also be used as microsphere wash buffer.

The Antibody Coupling Kit (Luminex PN: 40-50016) includes buffers that perform well for most coupling applications.

For complete equipment and materials list, see **Appendix B**.

Protocol 4.2.1: Carbodiimide coupling

1. Resuspend the stock uncoupled microsphere suspension according to the instructions described in the product information sheet provided with your microspheres.
2. Transfer $5.0x10^6$ of the stock microspheres to a recommended microcentrifuge tube.
3. Place the tube into a magnetic separator and allow separation to occur for 30 to 60 seconds.
4. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the microspheres.
5. Remove the tube from the magnetic separator and resuspend the microspheres in 100 µL dH_2O by vortex and sonication for ~20 seconds.
6. Place the tube into a magnetic separator and allow separation to occur for 30 to 60 seconds.
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

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

Note: For scaling of coupling reactions, see **Scale Up Information Table** at the end of this section.

Note: This protocol is also used for coupling SeroMAP™ carboxylated microspheres.

microspheres in 80 µL 0.1 M sodium phosphate (monobasic), pH 6.2 by vortex and sonication for ~20 seconds.

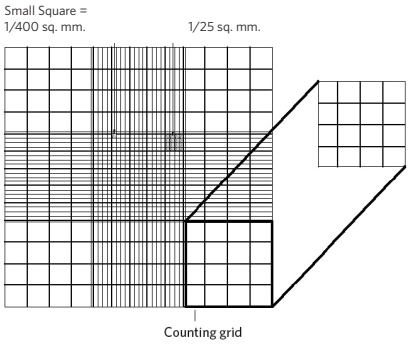
9. Add 10 µL of 50 mg/mL Sulfo-NHS (diluted in activation buffer) to the microspheres and mix gently by vortex.
10. Add 10 µL of 50 mg/mL EDC (diluted in activation buffer) to the microspheres and mix gently by vortex.
11. Incubate for 20 minutes at room temperature with gentle mixing by vortex at 10 minute intervals.
12. Place the tube into a magnetic separator and allow separation to occur for 30 to 60 seconds.
13. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the microspheres.
14. Remove the tube from the magnetic separator and resuspend the microspheres in 250 µL of 50 mM MES, pH 5.0 by vortex and sonication for approximately 20 seconds.
15. Repeat steps 12 and 13 for a total of two washes with 50 mM MES, pH 5.0.
16. Remove the tube from the magnetic separator and resuspend the activated and washed microspheres in 100 µL of 50 mM MES, pH 5.0 by vortex and sonication for approximately 20 seconds.
17. Add 25 µg protein to the resuspended microspheres (i.e., 5 µg/1 million microspheres). *(Note: 5 µg protein per 1 million beads typically performs well. We recommend titrating up and/or down as needed to achieve optimal assay performance.)*
18. Bring total volume to 500 µL with 50 mM MES, pH 5.0.
19. Mix coupling reaction by vortex.
20. Incubate for 2 hours with mixing (by rotation) at room temperature.
21. Place the tube into a magnetic separator and allow separation to occur for 30 to 60 seconds.
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 µL of PBS-TBN by vortex and sonication for ~20 seconds.
24. Optional blocking step – incubate for 30 minutes with mixing (by rotation) at room temperature. (*Note: 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 to 60 seconds.
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 PBS-TBN by vortex and sonication for ~20 seconds.
28. Repeat steps 25 and 26 for a total of two washes with 1 mL PBS-TBN.
29. Remove the tube from the magnetic separator and resuspend the coupled and washed microspheres in 250–1,000 µL of PBS-TBN.
30. Count the number of microspheres recovered after the coupling reaction using a cell counter or hemacytometer. (See figure and note to the right).
31. Store coupled microspheres refrigerated at 2–8°C in the dark.

Follow this coupling procedure with **Coupling Confirmation, Section 4.2.2**.

Simplify coupling your antibodies to MagPlex® Microspheres

xMAP® Antibody Coupling (AbC) Kit – The xMAP AbC Kit contains all of the necessary reagents and consumables needed to couple antibodies to MagPlex® Microspheres, along with an easy-to-use protocol (Catalog # 40-50016).

Luminex Magnetic Tube Separator – If you are only coupling a few bead sets at a time, try the Luminex Magnetic Tube Separator—it is a convenient tool for washing beads one 1.5 mL vial at a time (Catalog # CN-0288-01).



Note: To calculate the total number of beads when counting 10 µL with a hemacytometer, use the following formula: Total beads = count (1 corner of 4x4 section) x 10 x dilution factor x resuspension volume in µL. To get beads/mL, use the following calculation: Beads/mL = count (1 corner of 4x4 grid) x 10 x dilution factor x resuspension volume in µL)/ resuspension volume in mL.

Figure 10. Scale-Up Information for Bead Coupling

Microspheres (million)	EDC (mg)	Sulfo-NHS (mg)	Activation Volume (µL)	Coupling Volume (mL)	S-NHS (50 mg/mL) (µL)	EDC (50 mg/mL) (µL)	# of 96-Well Plates Using 2,500 Beads/Well
1	0.5	0.5	100	0.5	10	10	4
2.5	0.5	0.5	100	0.5	10	10	10
5	0.5	0.5	100	0.5	10	10	20
10	2.5	2.5	100	1	50	50	40
12.5	2.5	2.5	100	1	50	50	50
50	2.5	2.5	500	2	50	50	200
100	5	5	500	2	100	100	400
200	5	5	500	2	100	100	800
600	20	20	800	2	400	400	2,400

Note: Coupling reactions of 0.5 to 1 mL 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. See **Appendix B** for recommended tubes.

Chapter 4.2.2

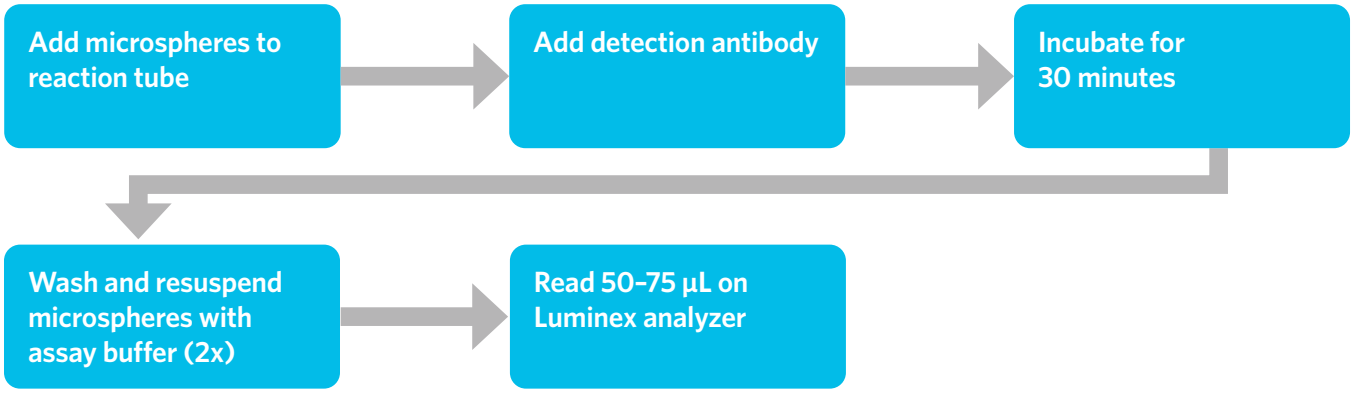
Coupling Confirmation

Once antibodies or proteins are coupled to xMAP® microspheres, it is strongly recommended to assess coupling efficiency before proceeding to assay development. The coupled microspheres can be incubated with suitable reporter-labeled antibodies specific for the proteins coupled. For example, if a mouse monoclonal antibody was coupled to the beads, a suitable goat, rabbit, or other anti-mouse reporter-labeled antibody can be used for analysis on a Luminex instrument. Alternatively, a biotinylated target antigen specific for the coupled antibody may be used and subsequently labeled with a streptavidin-conjugated reporter dye. Examples of coupling confirmation can be found in de Jager et al, 2003.[1]

If an antigen or other protein is coupled to the beads, a suitable reporter-labeled or biotinylated conjugated antibody to the protein can be used. In the case of coupling linkers, the efficiency is determined after the subsequent coupling of the peptide using reporter-labeled or biotin-conjugated antibodies that recognize the peptide. Details are provided in the protocols on coupling peptides to different linkers in **Chapter 4.4, Other Coupling Moieties**.

Keep in mind that proteins are typically coupled in random orientation as they may have many lysine groups available for coupling. In assays where retention of the conformation or activity of a protein needs to be maintained, functional testing of the coupled protein is also critical during assay development.

Summary of protocol



1. de Jager W, te Velthuis H, Prakken BJ, et al. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol. 2003;10(1);133-9.

Materials needed

Reagents and Consumables	Vendor
MagPlex® Microspheres (antibody-coupled)	Supplied by user
Assay/wash buffer (PBS, 1% BSA)	MilliporeSigma P3688
PBS-BN buffer*	MilliporeSigma P3688 MilliporeSigma S8032
PBS–TBN buffer*,†	MilliporeSigma P3563 MilliporeSigma A7888 MilliporeSigma S8032
96-well plate	See **Appendix B**
Reporter or biotin-labeled anti-species detection antibody	Any suitable source
Streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Thermo Fisher (Invitrogen S866) ProZyme®, or equivalent
Reporter 2 dyes for xMAP INTELLIFLEX® DR-SE: SuperBright® 436 Brilliant Violet 421 StarBright™ Violet 440	Thermo Fisher eBioscience™ Streptavidin SuperBright® 436 (62-4317-82), BD Biosciences BD Horizon™ BV421 Streptavidin (563259), Bio-Rad Streptavidin:StarBright™ Violet 440 (STAR210SBV440)
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Protein LoBind® 022431081
Disposable pipette tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

*Also used as assay buffer.
†Also used as wash buffer.

For complete equipment and materials list, see **Appendix B**.

Note: For dual reporter assays using the xMAP INTELLIFLEX® DR-SE System, one detection antibody must be directly conjugated to a reporter dye, but the other detection antibody may be biotinylated for use with a streptavidin-reporter dye conjugate. Alternatively, both detection antibodies may be directly conjugated to the relevant Reporter 1 or Reporter 2 dye.

A protocol for verifying coupling efficiency using phycoerythrin (PE) as the reporter is provided below. A dose response increase in MFI should be observed as the concentration of labeled detection antibody increases. In general, an antibody coupling should yield at least 10,000 MFI with PE (using standard PMT settings on the Luminex® 200™ and FLEXMAP 3D® instruments, or on a MAGPIX® instrument) at saturation for optimal use in immunoassays. The maximum MFI for other proteins will vary depending on the detection antibodies used. However, a saturation curve can still be obtained by a detection antibody titration to determine coupling efficiency. The example below uses a directly PE-labeled anti-species detection antibody for coupling confirmation.

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

Protocol 4.2.2: Antibody coupling confirmation

1. Select the appropriate antibody-coupled microsphere set or sets.
2. Resuspend the microspheres by vortex and sonication for ~20 seconds.
3. Prepare a working microsphere mixture by diluting the coupled microsphere stocks to a final concentration of 50 beads/µL in assay buffer.
4. Prepare a solution of PE-labeled anti-species IgG detection antibody at 4 µg/mL in assay buffer. 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.

Note: 50 µL per well of the microsphere solution is required for each reaction (16 wells = 800 µL)

Note: An Excel®-based bead calculator to facilitate determining the method and volumes needed in making the working microsphere mixture can be obtained **here**.

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

5. Aliquot 50 µL of the microsphere solution prepared in Step 3 into each well in two columns of the 96-well plate (16 wells total).
6. Add 50 µL of assay buffer, as a blank sample, into the wells A1 and A2 containing the microsphere solution.
7. Add 50 µL of each of the diluted detection antibody solutions prepared in Step 4 into the appropriate wells (as shown in the plate layout below).

Example of plate layout using columns 1 & 2

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

8. Mix the reactions gently by pipetting up and down several times with a pipettor.
9. Cover the plate and incubate for 30 minutes at room temperature on a plate shaker at <800 rpm.
10. Clip the plate in place on the Luminex magnetic plate separator. Allow magnetic separation to occur for 30–60 seconds. With the plate still on the separator, rapidly and forcefully invert the plate/separator over a biohazard receptacle or use a multi-channel pipettor to remove the liquid from the wells. *Note: For information on the MagPlex® Manual Wash Method, please visit the Magnetic Separators page at luminexcorp.com/magnetic-separators.*
11. Wash each well with 100 µL of assay buffer by gently pipetting up and down several times with a pipettor, and remove the liquid by using the procedure described in the previous step.
12. Repeat steps 10 and 11 for a total of 2 washes.
13. Resuspend the microspheres in 100 µL of assay buffer by gently pipetting up and down several times with a pipettor.
14. Analyze 50–75 µL on the Luminex analyzer according to the system manual. An example of typical results is shown in **Figure 11**.

Note: Be sure the plate is held tightly in place during magnetic separation/inversion and that the magnetic separator is in direct contact with the bottom of the plate. This method performs best with flat-bottom plates.

Figure 11.

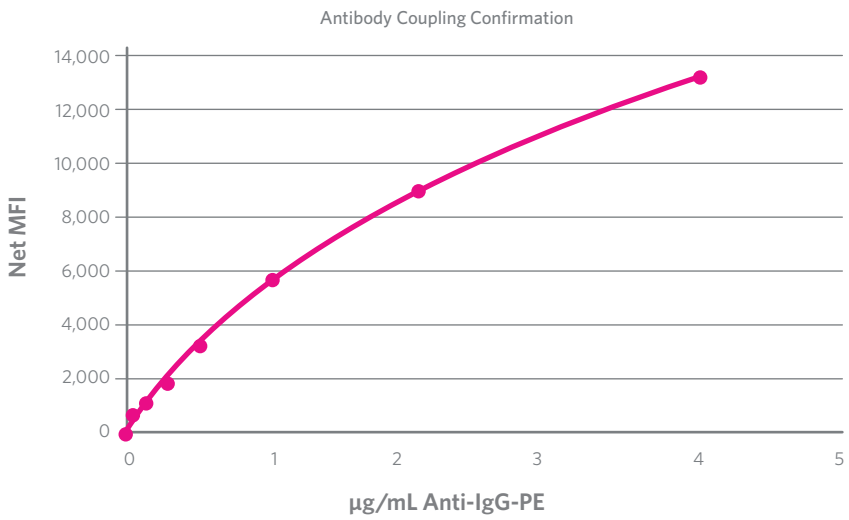


Figure 11 – Plot of typical results for anti-species IgG-phycoerythrin (PE) conjugate titration of antibody-coupled microspheres, as measured by a Luminex analyzer.

Antibody coupling references

General antibody coupling

- de Jager W, te Velthuis H, Prakken BJ, et al. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol. 2003;10:133-9.
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Antibody coupling confirmation

- Clotilde LM, Bernard C, Salvador A, et al. A 7-plex microbead-based immunoassay for serotyping Shiga toxin-producing *Escherichia coli.* J Microbiol Methods. 2013;92(2):226-30.

Antibody coupling kit

- Charlermroj R, Himananto O, Seepiban C, et al. Multiplex detection of plant pathogens using a microsphere immunoassay technology. PLoS One. 2013;8(4):U504-14.
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Chapter 4.3

Common xMAP® Immunoassay Formats

xMAP® microspheres offer a flexible means of developing a range of immunoassays. Antibodies, antigens, and proteins are easily coupled to xMAP microspheres, allowing the capture and quantitation of analytes in a range of sample types.

Figure 12.

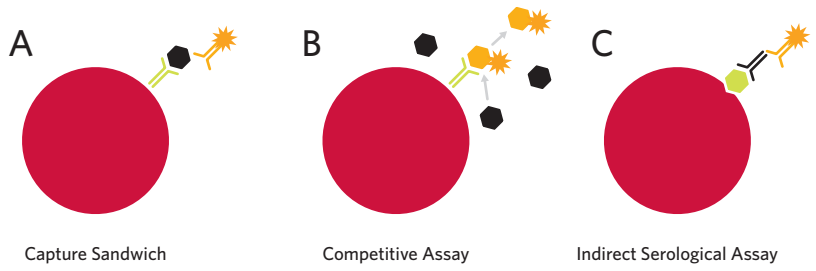


Figure 12 - Common assay formats include the (**A**) capture sandwich assay, requiring two antibodies to your analyte target, (**B**) competitive assay, requiring a single antibody and a labeled analyte (in this example), and (**C**) indirect (serological) antibody assay (or serology assay), requiring both a target protein and an anti-IgG antibody.

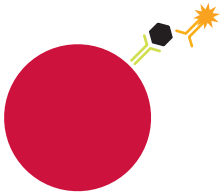
Depending on the availability of antibodies and the type of molecule to be measured, common assay formats include the capture sandwich, competitive, and indirect (serological) assays. The following sections cover each of these assays in detail, including assay principles and overviews, technical notes, materials needed, step-by-step protocols, and references.

Chapter 4.3.1

Capture Sandwich Immunoassay

The following immunoassay protocol presumes that the user is familiar with general assay development and optimization (including microsphere coupling). This example uses PE/SAPE as the reporter dye.

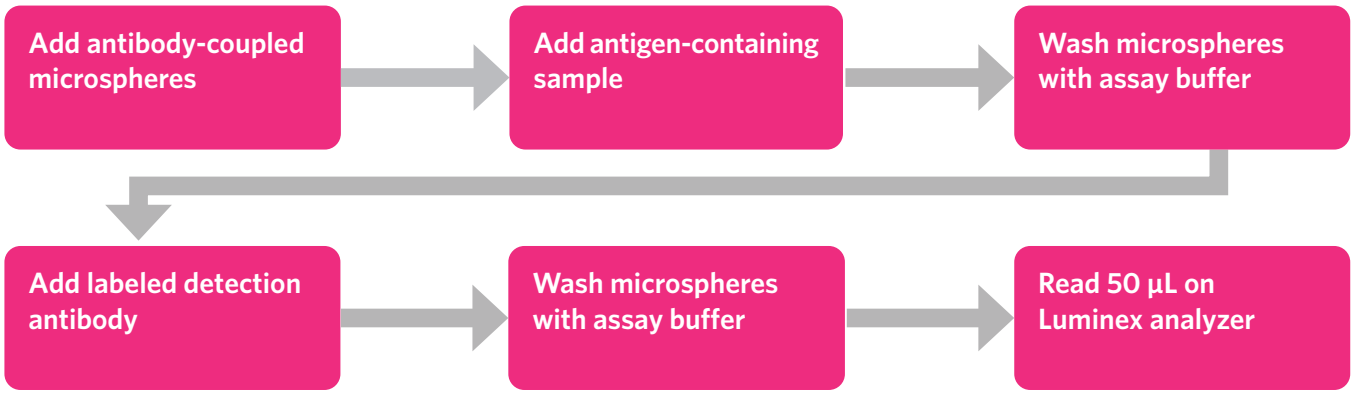
Figure 13. Capture Sandwich



Assay principle and overview

The capture sandwich immunoassay detects an antigen (analyte) through the use of a capture antibody attached to the surface of a microsphere and a detection antibody that incorporates a fluorescent label, forming a "sandwich." This assay is commonly used to measure hormones, blood products, enzymes, drugs, disease markers, and other biological molecules. The general steps for performing a sandwich immunoassay with xMAP® Technology are as follows:

Summary of protocol



Technical notes

- For capture sandwich immunoassays, 2–4 µg/mL detection antibody is usually sufficient; however, up to 5-fold more detection antibody may be required for a no-wash assay format. To optimize detection antibody concentration for washed assays, we recommend starting with 4 µg/mL and titrating down to 1 µg/mL by 2-fold dilutions.
- The optimal detection antibody concentration will depend on specific reagents and the level of multiplexing. Concentrations often need to be increased when increasing the number of multiplexed assays and when converting to a no-wash assay format.
- For SAPE, the reporter concentration should be approximately 1.5X the concentration of the detection antibody. When using SAPE at concentrations >8 µg/mL in a no-wash format, a dilution or wash step may be required to minimize background fluorescence prior to analysis on your Luminex instrument. The optimal reporter concentration should be determined by titration.

Materials needed

Reagents and Consumables	Vendor
MagPlex® Microspheres (antibody-coupled)	Supplied by user
Assay/wash buffer (PBS, 1% BSA)	MilliporeSigma P3688
PBS-BN buffer*	MilliporeSigma P3688 MilliporeSigma S8032
PBS–TBN buffer*,†	MilliporeSigma P3563 MilliporeSigma A7888 MilliporeSigma S8032
96-well plate	See **Appendix B**
Reporter or biotin-labeled detection antibody	Any suitable source
Streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Thermo Fisher (Invitrogen S866) ProZyme®, Thermo Fisher, or equivalent
Reporter 2 dyes for xMAP INTELLIFLEX® DR-SE: SuperBright® 436 Brilliant Violet 421 StarBright™ Violet 440	Thermo Fisher eBioscience™ Streptavidin SuperBright® 436 (62-4317-82), BD Biosciences BD Horizon™ BV421 Streptavidin (563259), Bio-Rad Streptavidin:StarBright™ Violet 440 (STAR210SBV440)
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendor Protein LoBind® 022431081
Disposable pipette tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

*Also used as assay buffer.
†Also used as wash buffer.

For complete equipment and materials list, see **Appendix B**.

Note: For dual reporter assays using the xMAP INTELLIFLEX® DR-SE System, one detection antibody must be directly conjugated to a reporter dye, but the other detection antibody may be biotinylated for use with a streptavidin-reporter dye conjugate. Alternatively, both detection antibodies may be directly conjugated to the relevant Reporter 1 or Reporter 2 dye.

Protocol 4.3.1: Capture sandwich immunoassay

1. Select the appropriate antibody-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for ~20 seconds.
3. Prepare a working microsphere mixture by diluting the coupled microsphere stocks to a final concentration of 50 microspheres of each set/µL in assay buffer.
4. Aliquot 50 µL of the working microsphere mixture into the appropriate wells of a 96-well plate.
5. Add 50 µL of assay buffer to each background well.
6. Add 50 µL 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 minutes at room temperature on a plate shaker set to ~800 rpm.
9. Place the plate into the magnetic separator and allow separation to occur for 30–60 seconds.
10. Use a multi-channel pipette to carefully aspirate the supernatant from each well. Take care not to disturb the microspheres.
11. Remove the plate from the magnetic separator for the following wash steps:
    a. Add 100 µL assay buffer to each well.
    b. Place the plate into the magnetic separator and allow separation to occur for 30–60 seconds.
    c. Use a multi-channel pipette to carefully aspirate the supernatant from each well, or use the Manual Inversion Wash Method (see **Protocol 4.2.2**). Take care not to disturb the microspheres.
    d. Repeat steps a–c above.
12. Remove the plate from the magnetic separator and resuspend the microspheres in 50 µL of assay buffer by gently pipetting up and down several times using a multi-channel pipettor.
13. Dilute the biotinylated detection antibody to 4 µg/mL in assay buffer.
14. Add 50 µL 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 minutes at room temperature on a plate shaker set to ~800 rpm.
17. Place the plate into the magnetic separator and allow separation to occur for 30–60 seconds.
18. Use a multi-channel pipette to carefully aspirate the supernatant from each well. Take care not to disturb the microspheres.
19. Remove the plate from the magnetic separator for the following wash steps:
    a. Add 100 µL assay buffer to each well.Place the plate into the magnetic separator and allow separation to occur for 30–60 seconds.
    b. Place the plate into the magnetic separator and allow separation to occur for 30–60 seconds.
    c. Use a multi-channel pipette to carefully aspirate the supernatant from each well or use the manual inversion wash method (see **Protocol 4.2.2**). Take care not to disturb the microspheres.
    d. Repeat steps a–c above.

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

Note: 50 µL of working microsphere mixture is required for each reaction.

Note: An Excel®-based bead calculator to help determine the method and volumes needed for making the working microsphere mixture can be obtained by contacting **Luminex Technical Support**.

Note: 50 µL of diluted detection antibody is required for each reaction.

20. Remove the plate from the magnetic separator and resuspend the microspheres in 50 µL of assay buffer by gently pipetting up and down several times with a multi-channel pipettor.
21. For the the reporter stock solution, dilute SAPE reporter to 4 µg/mL in assay buffer.
22. Add 50 µL of the diluted reporter solution 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 minutes at room temperature on a plate shaker set to ~800 rpm.
25. Place the plate into the magnetic separator and allow separation to occur for 30–60 seconds.
26. Use a multi-channel pipette to carefully aspirate the supernatant from each well. Take care not to disturb the microspheres.

- Remove the plate from the magnetic separator for the following wash steps:
    a. Add 100 µL assay buffer to each well.
    b. Place the plate into the magnetic separator and allow separation to occur for 30–60 seconds.
    c. Use a multi-channel pipette to carefully aspirate the supernatant from each well, or use the Manual Inversion Wash Method (see **Protocol 4.2.2**). Take care not to disturb the microspheres.
    d. Repeat steps a–c above.

27. Remove the plate from the magnetic separator and resuspend the microspheres in 100 µL of assay buffer by gently pipetting up and down several times with a multi-channel pipettor.
28. Analyze 50–75 µL on the Luminex analyzer according to the system manual.

Note: 50 µL of diluted reporter solution is required for each reaction.

Capture sandwich immunoassay references

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Chapter 4.3.2

Competitive Immunoassay

The following immunoassay protocol presumes that the user is familiar with general assay development and optimization (including microsphere coupling). The examples below use PE/SAPE as the reporter dye.

Figure 14. Competitive Assay

Format 1
Antibody-coupled
Microspheres

Format 2
Antigen-coupled
Microspheres

Assay principle and overview

The competitive immunoassay enables the detection of an antigen (analyte) through the use of a single capture antibody attached to the surface of a microsphere and a competitive, labeled antigen reversibly bound to the antibody (**Format 1**). Analyte in the sample is detected by competing away the bound, labeled antigen and causing a decrease in signal. The assay format may also be reversed with the antigen attached to the microsphere and the antibody labeled (**Format 2**). In this case, the analyte in the sample competes away the labeled antibody in solution rather than on the surface of the microsphere. The competitive assay is useful for smaller protein analytes (<3–4 kDa) with only a single (or very few) epitopes, or when only a single antibody is available. The general steps to performing this type of assay with xMAP® Technology are as follows:

For competitive immunoassays, the reporter signal is inversely proportional to the sample analyte concentration—i.e., the higher the sample target concentration, the lower the reporter signal. A major advantage of a competitive immunoassay is the ability to use crude or impure samples and still selectively bind any target that may be present.

Technical notes

- For competitive immunoassay Format 1, we recommend testing a range of labeled, competing analyte (0.2 to 5 µg) with increasing concentrations of reporter (e.g., SAPE). For Format 2, test a range of detection antibody concentrations starting with an excess (e.g., 4 µg/mL) and titrating down by 2-fold serial dilution. The competitor or detection antibody concentration that yields 70–80% of the maximum signal should provide the largest linear dynamic range for the assay. The reporter concentration should be approximately 1.5X the concentration of the competitor or detection antibody. When using SAPE at concentrations >8 µg/mL in a no-wash format, a dilution or wash step may be required to minimize background fluorescence prior to analysis on your Luminex instrument.

Materials needed

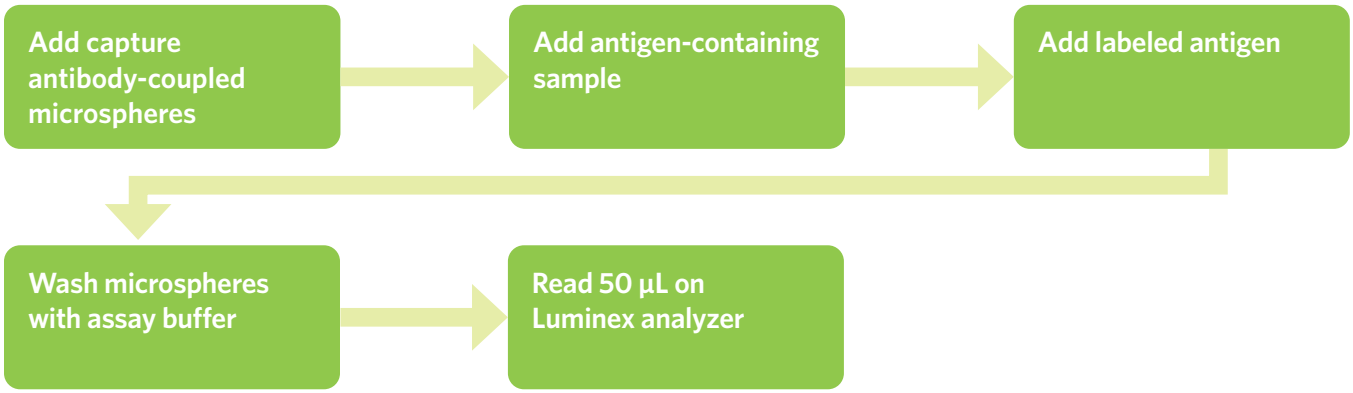
Reagents and Consumables	Vendor
MagPlex® Microspheres (antibody- or antigen-coupled)	Supplied by user
Assay/wash buffer (PBS, 1% BSA)	MilliporeSigma P3688
PBS-BN buffer*	MilliporeSigma P3688 MilliporeSigma S8032
PBS–TBN buffer*,†	MilliporeSigma P3563 MilliporeSigma A7888 MilliporeSigma S8032
96-well plate	See **Appendix B**
Reporter or Biotin-labeled detection antibody or analyte	Any suitable source
Streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Thermo Fisher (Invitrogen S866), ProZyme®, or equivalent
Reporter 2 dyes for xMAP INTELLIFLEX® DR-SE: SuperBright® 436 Brilliant Violet 421 StarBright™ Violet 440	Thermo Fisher eBioscience™ Streptavidin SuperBright® 436 (62-4317-82), BD Biosciences BD Horizon™ BV421 Streptavidin (563259), Bio-Rad Streptavidin:StarBright™ Violet 440 (STAR210SBV440)
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Protein LoBind® 022431081
Disposable pipette tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

*Also used as assay buffer.
†Also used as wash buffer.

For complete equipment and materials list, see **Appendix B**.

Note: For dual reporter assays using the xMAP INTELLIFLEX® DR-SE System, one detection antibody must be directly conjugated to a reporter dye, but the other detection antibody may be biotinylated for use with a streptavidin-reporter dye conjugate. Alternatively, both detection antibodies may be directly conjugated to the relevant Reporter 1 or Reporter 2 dye.

Summary of protocol (Format 1)



Protocol 4.3.2.1: Competitive immunoassay (Format 1)

1. Select the appropriate antibody-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for ~20 seconds.
3. Prepare a working microsphere mixture by diluting the coupled microsphere stocks to a final concentration of 100 microspheres of each set/µL in assay buffer. Note: 25 µL of working microsphere mixture is required for each reaction.
4. Dilute the biotinylated competitor to the [IC_{70}] or [IC_{80}] in assay buffer.
5. Add 25 µL of PBS-1%BSA to each background well.
6. Add 25 µL of standard or sample to the appropriate wells.
7. Add 25 µL 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 µL of the working microsphere mixture to the appropriate wells
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 minutes at room temperature on a plate shaker at 800 rpm.
12. For SAPE reporter, dilute the reporter stock solution reporter to 4 µg/mL in assay buffer. *Note: 25 µL of diluted reporter solution is required for each reaction.*
13. Add 25 µL of the diluted reporter solution 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 minutes at room temperature on a plate shaker at 800 rpm.
16. OPTIONAL—Include the following steps if high backgrounds occur:
    a. Use a magnetic plate separator to carefully remove the supernatant from each well by using either manual inversion (see **Protocol 4.2.2**), manual pipetting, or magnetic plate washer. Take care not to disturb the microspheres.
    b. Add 100 µL of wash buffer to each reaction well. Take care not to disturb the microspheres.
17. Repeat step 16 once more, for a total of 2 washes (if needed).
18. Bring final volume of each reaction to 100 µL with assay buffer.
19. Analyze 50–75 µL on the Luminex analyzer according to the system manual.

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

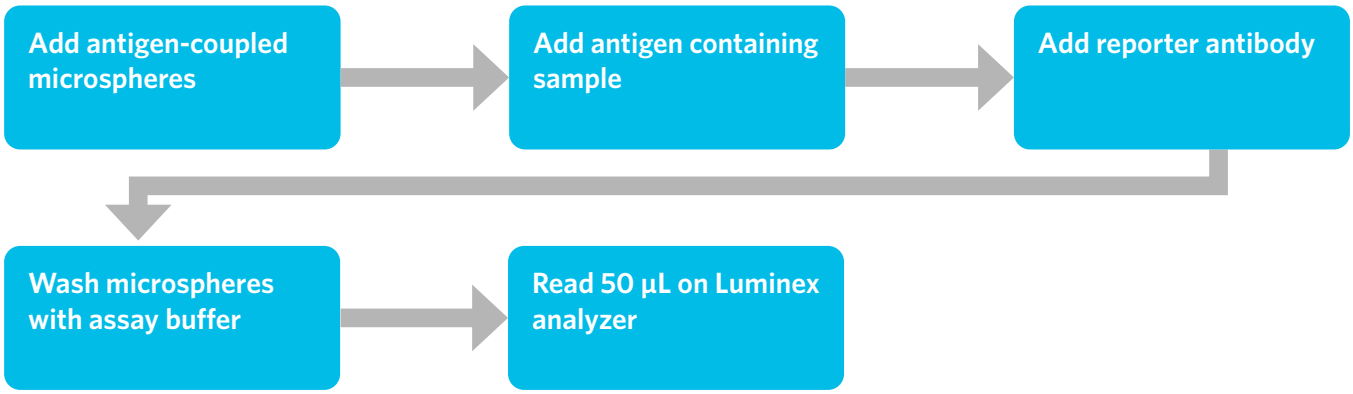
Note: 25 µL of diluted, biotinylated competitor is required for each reaction.

Note: An Excel®-based bead calculator to help you determine the method and volumes needed for making the working microsphere mixture can be obtained here.

Technical Note

The [IC_{70}] and [IC_{80}] are the concentrations of biotinylated competitor that yield 70% and 80% of the maximum obtainable signal, respectively. The [IC_{70}] or [IC_{80}] should be determined by titration in assay buffer.

Summary of protocol (Format 2)



Protocol 4.3.2.2: Competitive immunoassay (Format 2)

1. Select the appropriate antigen-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for ~20 seconds.
3. Prepare a working microsphere mixture by diluting the coupled microsphere stocks to a final concentration of 100 microspheres of each set/µL in assay buffer. 25 µL of working microsphere mixture is required for each reaction.
4. Dilute the biotinylated detection antibody to the [IC_{70}] or [IC_{80}] in assay buffer. 25 µL of biotinylated detection antibody is required for each reaction.
5. Add 25 µL of assay buffer to each background well.
6. Add 25 µL of standard or sample to the appropriate wells.
7. Add 25 µL 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 µL 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 minutes at room temperature on a plate shaker at 800 rpm.
12. Dilute the reporter dye to the appropriate concentration (typically ≥4 µg/mL for SAPE) in assay buffer. 25 µL of diluted reporter solution is required for each reaction.
13. Add 25 µL of the diluted reporter solution 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 minutes at room temperature on a plate shaker.
16. OPTIONAL: Include the following steps if high backgrounds occur:
    a. Use a magnetic plate separator to carefully remove the supernatant from each well by using either manual inversion, manual pipetting, or magnetic plate washer. Take care not to disturb the microspheres.
    b. Add 100 µL of wash buffer to each reaction well. Take care not to disturb the microspheres.
17. Repeat step 16 once more, for a total of 2 washes (if needed).
18. Bring final volume of each reaction to 100 µL with assay buffer.
19. Analyze 50–75 µL on the Luminex analyzer according to the system manual.

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

Note: An Excel-based bead calculator to help you determine the method and volumes needed for making the working microsphere mixture can be obtained **here**.

Technical Notes

The [IC_{70}] and [IC_{80}] are the concentrations of detection antibody that yield 70% and 80% of the maximum obtainable signal, respectively. The [IC_{70}] or [IC_{80}] should be determined by titration in assay buffer.

Concentrations of the detection antibodies and reporter dyes should be optimized. The optimal concentrations tend to be higher than in a washed assay.

Competitive assay references

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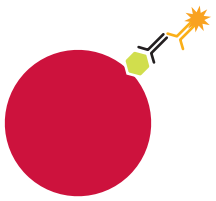
Chapter 4.3.3

Indirect (Serological) Immunoassay

The following immunoassay protocol presumes that the user is familiar with general assay development and optimization (including microsphere coupling). The example below uses PE/SAPE as the reporter dye.

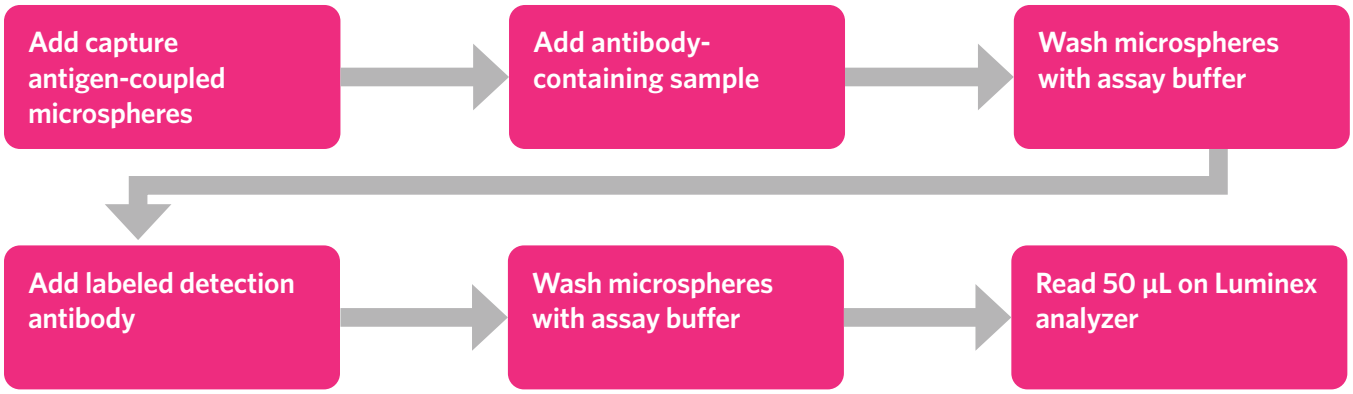
Figure 15. Indirect Serological Assay



Assay principle and overview

The indirect immunoassay enables the detection of an antibody through the use of a capture antigen and an anti-species detection antibody. This assay is useful for serology assays where serum antibodies are measured to determine infection status, vaccine responses, allergy or autoimmune activity, etc. The general steps to performing a serological immunoassay with xMAP® Technology are as follows:

Summary of protocol



Technical notes

- For indirect (serological) immunoassays, 2–4 µg/mL detection antibody is usually sufficient, however, up to 5-fold more detection antibody may be required for a no-wash assay format. To optimize detection antibody concentration for washed assays, we recommend starting with 4 µg/mL and titrating down to 1 µg/mL by 2-fold dilutions. The optimal detection antibody concentration will depend on specific reagents and the level of multiplexing. Concentrations often need to be increased when increasing the number of multiplexed assays and when converting to a no-wash assay format.
- For SAPE, the reporter concentration should be approximately 1.5X the concentration of the detection antibody. When using SAPE at concentrations >8 µg/mL in a no wash format, a dilution or wash step may be required to minimize background fluorescence prior to analysis on your Luminex instrument. The optimal reporter concentration should be determined by titration.

Materials needed

Reagents and Consumables	Vendor
MagPlex® Microspheres (antigen-coupled)	Supplied by user
Assay/wash buffer (PBS, 1% BSA)	MilliporeSigma P3688
PBS-BN buffer*	MilliporeSigma P3688 MilliporeSigma S8032
PBS–TBN buffer*,†	MilliporeSigma P3563 MilliporeSigma A7888 MilliporeSigma S8032
96-well plate	See **Appendix B**
Reporter or biotin-labeled detection antibody	Any suitable source
Streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Thermo Fisher (Invitrogen S866), ProZyme®, or equivalent
Reporter 2 dyes for xMAP INTELLIFLEX® DR-SE: SuperBright® 436 Brilliant Violet 421 StarBright™ Violet 440	Thermo Fisher eBioscience™ Streptavidin SuperBright® 436 (62-4317-82), BD Biosciences BD Horizon™ BV421 Streptavidin (563259), Bio-Rad Streptavidin:StarBright™ Violet 440 (STAR210SBV440)
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Protein LoBind® 022431081
Disposable pipette tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

**Also used as assay buffer.*
†Also used as wash buffer.

For complete equipment and materials list, see **Appendix B**.

Note: For dual reporter assays using the xMAP INTELLIFLEX® DR-SE System, one detection antibody must be directly conjugated to a reporter dye, but the other detection antibody may be biotinylated for use with a streptavidin-reporter dye conjugate. Alternatively, both detection antibodies may be directly conjugated to the relevant Reporter 1 or Reporter 2 dye.

Protocol 4.3.3.1: Indirect (serological) immunoassay

1. Dilute samples and controls using diluent (e.g., dilute 1 to 500).
2. Select the appropriate antigen-coupled microsphere sets. Prepare a working microsphere mixture by diluting the coupled microsphere stocks to a final concentration of 50 microspheres of each set/µL in assay buffer.
3. Resuspend the microspheres by vortex and sonication for ~20 seconds.
4. Aliquot 50 µL of the working microsphere mixture into the appropriate wells.
5. Add 50 µL of diluted controls and diluted samples to the appropriate wells.
6. If available, add 50 µL of standard to the appropriate wells.
7. Cover the plate to protect it from light and incubate for 60 minutes at room temperature on a plate shaker set to ~800 rpm.
8. Place the plate into the magnetic separator and allow separation to occur for 30–60 seconds.
    a. Use a magnetic plate separator to carefully remove the supernatant from each well by either manual inversion (see **Protocol 4.2.2**), manual pipetting, or magnetic plate washer. Take care not to disturb the microspheres.
    b. Add 100 µL of wash buffer to each reaction well.
9. Repeat step 8 once more for a total of 2 washes.
10. Remove the plate from the magnetic separator and add 100 µL of biotinylated detection antibody to each well of the plate.
11. Cover the plate to protect it from light and incubate for 30 minutes at room temperature on a plate shaker set to ~800 rpm.
12. Place the plate into the magnetic separator and allow separation to occur for 30–60 seconds.
    a. Use a magnetic plate separator to carefully remove the supernatant from each well by either manual inversion (see **Protocol 4.2.2**), manual pipetting, or magnetic plate washer. Take care not to disturb the microspheres.
    b. Add 100 µL of wash buffer to each reaction well.
13. Repeat step 12 once more for a total of 2 washes.
14. Remove the plate from the magnetic separator and add 100 µL of reporter conjugate (e.g., SAPE) to each well of the plate.
15. Cover the plate to protect it from light and incubate for 30 minutes at room temperature on a plate shaker set to ~800 rpm.
16. Place the plate into the magnetic separator and allow separation to occur for 60 seconds.
    a. Use a magnetic plate separator to carefully remove the supernatant from each well by either manual inversion (see **Protocol 4.2.2**), manual pipetting, or magnetic plate washer. Take care not to disturb the microspheres.
    b. Add 100 µL of wash buffer to each reaction well.
17. Repeat step 16 once more, for a total of 2 washes.
18. Remove the plate from the magnetic separator and add 100 µL of wash buffer to each well of the plate.
19. Resuspend the microspheres by pipetting up and down several times with a multichannel pipettor or placing the plate onto a plate shaker for approximately 15 seconds.
20. Analyze 50–75 µL on the Luminex analyzer according to the system manual.

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

Note: 50 µL of working microsphere mixture is required for each reaction.

Note: An Excel®-based bead calculator to help determine the method and volumes needed for making the working microsphere mixture can be obtained **here**.

Note: Many standards are supplied prediluted at working concentration and do not require further dilution.

Note: Directly reporter-conjugated anti-species antibody may be used for detection in place of a biotinylated detection antibody and streptavidin-reporter. Reporter-conjugated anti-species detection antibodies are commercially available from Jackson ImmunoResearch Laboratories, Rockland™ Immunochemicals, or equivalent.

Dual Reporter Antibody Isotyping on the Luminex xMAP INTELLIFLEX® DR-SE System Technical Notes

- Antibody isotyping assays are used to measure the classes and/or subclasses of antibodies in a serum sample and can be used to monitor the immune response in general or to a specific infection, vaccination, or drug therapy.
- The dual reporter functionality of the xMAP INTELLIFLEX® DR-SE System can be used for antibody isotyping with various pairs of reporter dyes.
- For assays using two reporters, one detection reagent must be directly labeled with the reporter dye, but the other detection reagent may be biotinylated to use with a streptavidin-reporter dye conjugate. Alternatively, both detection reagents may be directly labeled with the appropriate RP1 and RP2 dyes.
- The protocol below is a modified version of **Protocol 4.3.3.1** for using two reporters for the simultaneous measurement of antigen-specific IgG and IgM.

Materials needed

Reagents and Consumables	Vendor
MagPlex® Microspheres (antibody- or antigen-coupled)	Supplied by user
PBS–TBN Buffer* (PBS + 0.1% BSA + 0.02% Tween-20 + 0.05% sodium azide)	MilliporeSigma P3813 MilliporeSigma A7888 MilliporeSigma P9416 MilliporeSigma S8032
Reporter or biotin-labeled detection antibody	Any suitable source
Streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Thermo Fisher (Invitrogen S866), Prozyme, or equivalent.
Reporter 2 dyes for xMAP INTELLIFLEX® DR-SE: SuperBright® 436 Brilliant Violet 421 StarBright™ Violet 440	Thermo Fisher eBioscience® Streptavidin SuperBright® 436 (62-4317-82), BD Biosciences BD Horizon® BV421 Streptavidin (563259), Bio-Rad Streptavidin:StarBright™ Violet 440 (STAR210SBV440)
96-well plate	See Appendix B
96-well plate magnet	See Appendix B
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500, Eppendorf Protein LoBind®, 022431081, or equivalent
Disposable pipette tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

**Used as both assay buffer and wash buffer.*

Protocol 4.3.3.2: Indirect (serological) dual reporter isotyping immunoassay on the xMAP INTELLIFLEX® DR-SE System

1. Dilute the serum samples to appropriate concentrations (e.g., 1:400) using PBS-TBN buffer.
2. Add 50 µL of the diluted samples to the appropriate wells.
3. Select the appropriate antigen-coupled microsphere sets. Prepare a working microsphere mixture by diluting the coupled microsphere stocks to a final concentration of 50 microspheres of each set/µL in PBS-TBN buffer (~2,000 beads/set).
4. Resuspend the microspheres by vortex for ~30 seconds.
5. Aliquot 50 µL of the working microsphere mixture to the appropriate wells.
6. Cover the plate to protect it from light and incubate for 60 minutes at room temperature on a plate shaker set to ~800 rpm.
7. Place the plate onto the magnetic separator and allow separation to occur for 120 seconds.
8. With the plate still on the magnetic separator, carefully remove the supernatant from each well either by manual inversion, manual pipetting, or magnetic plate washer. Take care not to disturb the microspheres.
9. Remove the plate from the magnetic separator and add 100 µL of wash buffer to each reaction well. Mix gently using a pipette.
10. Repeat steps 7–8 once more for a total of two washes.
11. Remove the plate from the magnetic separator and add 100 µL of appropriate detection antibody/antibodies to each well.
12. Cover the plate to protect it from light and incubate for 60 minutes at room temperature on a plate shaker set to ~800 rpm.
13. Place the plate onto the magnetic separator and allow separation to occur for 120 seconds.
14. With the plate still on the magnetic separator, carefully remove the supernatant from each well either by manual inversion, manual pipetting, or magnetic plate washer. Take care not to disturb the microspheres.
15. Remove the plate from the magnetic separator and add 100 µL of wash buffer to each reaction well.
16. Repeat steps 13 and 14 once more for a total of two washes and proceed to either step 17 or 18.
17. **If using directly labeled detection antibody**, remove the plate from the magnetic separator and add 100 µL of wash buffer to each reaction well. Resuspend the microspheres by pipetting up and down several times with a multichannel pipette or by placing the plate onto a plate shaker for approximately 15 seconds, then proceed to step 19.
18. **If using biotinylated detection antibody for one of the reporter channels**, perform the following steps:
    a. Remove the plate from the magnetic separator and add 100 µL of reporter conjugate to each well of the plate.
    b. Cover the plate to protect it from light and incubate for 20 minutes at room temperature on a plate shaker set to ~800 rpm.
    c. Place the plate onto the magnetic separator and allow separation to occur for 120 seconds.
    d. With the plate still on the magnetic separator, carefully remove the supernatant from each well either by manual inversion, manual pipetting, or magnetic plate washer. Take care not to disturb the microspheres.

e. Remove the plate from the magnetic separator and add 100 µL of wash buffer to each reaction well.
f. Repeat steps c–e once more for a total of two washes.
g. Resuspend the microspheres by pipetting up and down several times with a multichannel pipette or placing the plate onto a plate shaker for ~15 seconds.

19. Analyze 75 µL on your xMAP INTELLIFLEX® DR-SE System according to the system manual.

Figure 16. Dual reporter indirect (serological) isotyping immunoassay example.

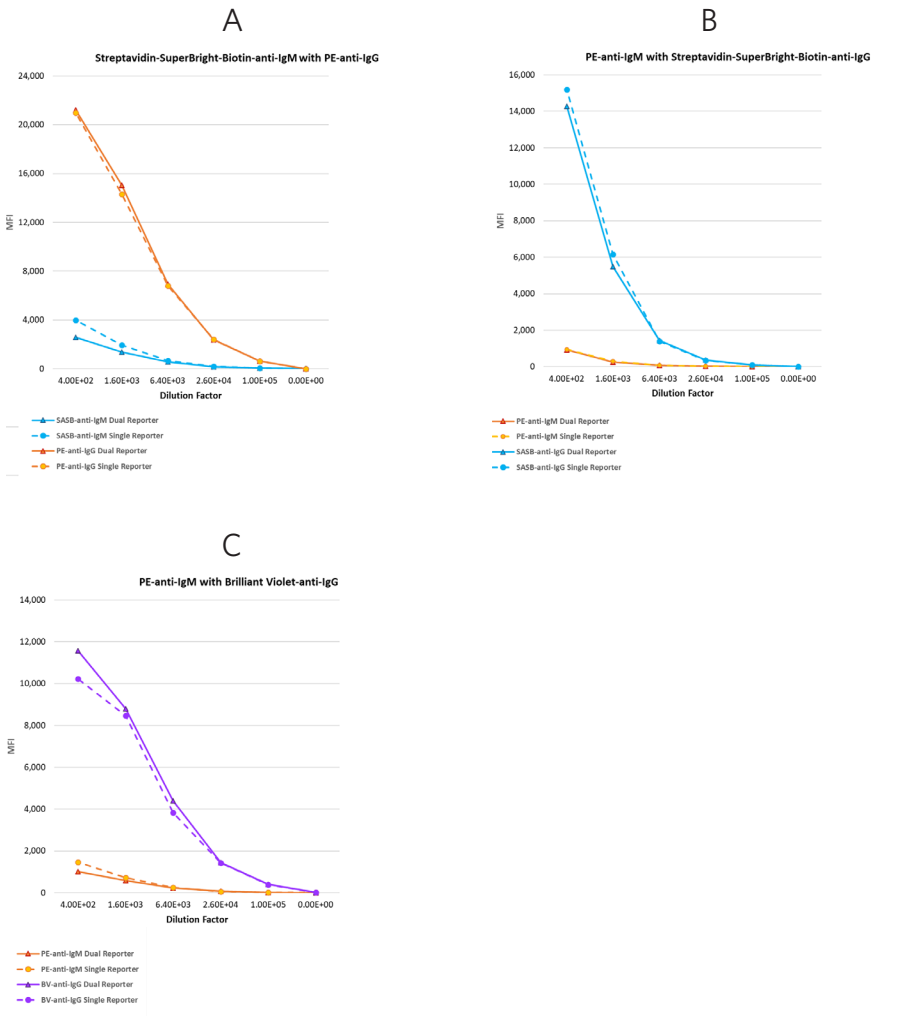


Figure 16 - Dual reporter indirect (serological) isotyping immunoassay example. (**A**) SASB-Biotin-anti-IgM detection antibody paired with a directly labeled R-PE-anti-IgG. (**B**) Directly labeled R-PE-anti-IgM detection antibody paired with SASB-Biotin-anti-IgG. (**C**) Directly labeled R-PE-anti-IgM detection antibody paired with a directly labeled BV-anti-IgG detection antibody. R-phycoerythrin (PE) was used for the RP1 channel and either Streptavidin SuperBright® 436 Conjugate (SASB) or Brilliant Violet 421 (BV) was used for the RP2 channel.

Indirect immunoassay references

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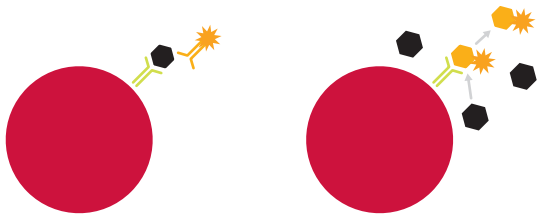
Chapter 4.3.4

Combined Capture Sandwich and Competitive Immunoassay

The following immunoassay protocol presumes that the user is familiar with general assay development and optimization (including microsphere coupling). The example below uses PE/SAPE as the reporter dye.

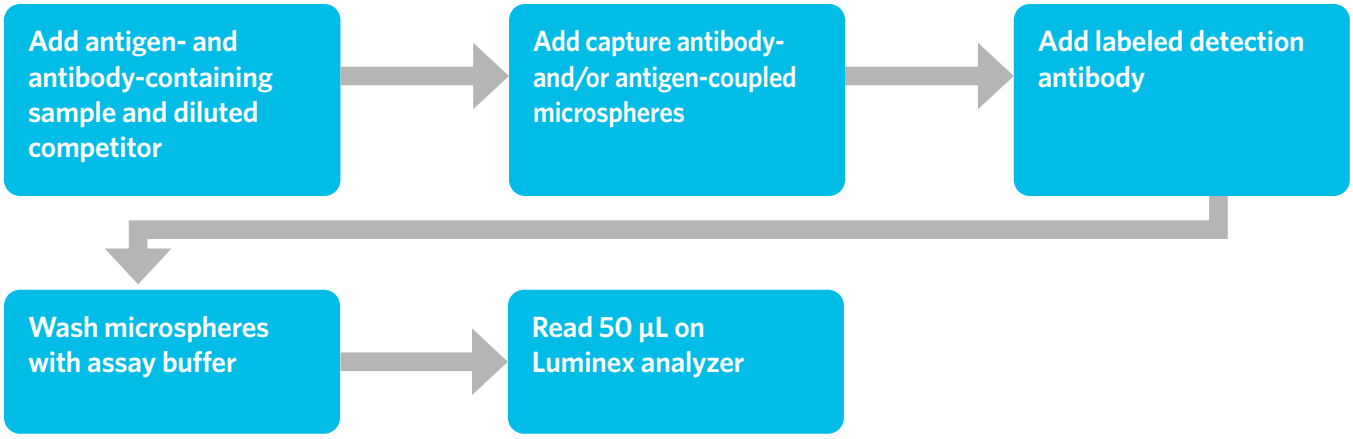
Figure 17. Combined Capture Sandwich and Competitive Antibody Assay



Assay principle and overview

Competitive immunoassays can be multiplexed with capture sandwich immunoassays, adding versatility to your multiplex assays. The general steps to performing a combined capture sandwich and competitive immunoassay with xMAP® Technology are as follows:

Summary of protocol



Materials needed

Reagents and Consumables	Vendor
MagPlex® Microspheres (antibody- or antigen-coupled)	Supplied by user
Assay/wash buffer (PBS, 1% BSA)	MilliporeSigma P3688
PBS-BN buffer*	MilliporeSigma P3688 MilliporeSigma S8032
PBS–TBN buffer*,†	MilliporeSigma P3563 MilliporeSigma A7888 MilliporeSigma S8032
96-well plate	See **Appendix B**
Reporter or Biotin-labeled detection antibody	Any suitable source
Streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Thermo Fisher (Invitrogen S866), ProZyme®, or equivalent
Reporter 2 dyes for xMAP INTELLIFLEX® DR-SE: SuperBright® 436 Brilliant Violet 421 StarBright™ Violet 440	Thermo Fisher eBioscience™ Streptavidin SuperBright® 436 (62-4317-82), BD Biosciences BD Horizon™ BV421 Streptavidin (563259), Bio-Rad Streptavidin:StarBright™ Violet 440 (STAR210SBV440)
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Protein LoBind® 022431081
Disposable pipette tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

*Also used as assay buffer.
†Also used as wash buffer.

For complete equipment and materials list, see **Appendix B**.

Note: For dual reporter assays using the xMAP INTELLIFLEX® DR-SE System, one detection antibody must be directly conjugated to a reporter dye, but the other detection antibody may be biotinylated for use with a streptavidin-reporter dye conjugate. Alternatively, both detection antibodies may be directly conjugated to the relevant Reporter 1 or Reporter 2 dye.

Protocol 4.3.4: Combined capture sandwich and competitive immunoassay

1. Select the appropriate antibody- and/or antigen-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for ~20 seconds.
3. Prepare a working microsphere mixture by diluting the coupled microsphere stocks to a final concentration of 500 microspheres of each set/µL in assay buffer. 5 µL of working microsphere mixture is required for each reaction.
4. Dilute the biotinylated competitor to the [IC_{70}] or [IC_{80}] in assay buffer. 5 µL of diluted competitor is required for each reaction.
5. Add 10 µL of assay buffer to each background.
6. Add 10 µL of standard or sample to the appropriate wells.
7. Add 5 µL 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 µL 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.

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

Note: An Excel®-based bead calculator to help determine the method and volumes needed for making the working microsphere mixture can be obtained by contacting **here**.

11. Cover the plate and incubate for 60 minutes at room temperature on a plate shaker at 800 rpm.
12. Dilute the biotinylated detection antibody to the appropriate concentration in assay buffer. 10 µL of diluted detection antibody is required for each reaction.
13. Add 10 µL 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 minutes at room temperature on a plate shaker at 800 rpm.
16. Dilute the reporter dye to the appropriate concentration (typically ≥10–12 µg/mL for SAPE) in assay buffer. 10 µL of diluted reporter solution is required for each reaction.
17. Add 10 µL of the diluted reporter solution 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 minutes at room temperature on a plate shaker at 800 rpm.
20. Bring final volume of each reaction to 100 µL with assay buffer.
21. OPTIONAL: Include the following steps if high backgrounds occur:
    a. Use a magnetic plate separator to carefully remove the supernatant from each well by either manual inversion (see **Protocol 4.2.2**), manual pipetting, or magnetic plate washer. Take care not to disturb the microspheres.
    b. Add 100 µL of wash buffer to each reaction well. Take care not to disturb the microspheres.
22. Repeat step 21 once more for a total of 2 washes (if needed).
23. Bring final volume of each reaction to 100 µL with assay buffer.
24. Analyze 50–75 µL on the Luminex analyzer according to the system manual.

Technical Notes

The [IC_{70}] and [IC_{80}] are the concentrations of detection antibody that yield 70% and 80% of the maximum obtainable signal, respectively. The [IC_{70}] or [IC_{80}] should be determined by titration in assay buffer.

Concentrations of biotinylated competitors, detection antibodies, and SAPE should be optimized. The optimal concentrations tend to be higher than in a washed assay.

If high backgrounds are observed, a final post-labeling wash step may be performed just prior to analysis.

Chapter 4.4

Other Coupling Moieties

Peptides, phospholipids, and other small molecules can be directly coupled to the microsphere surface,[1] but this may be accomplished more easily through the modification of the small molecule or microsphere to provide adequate spacing from the microsphere surface. This can be done using a linker or carrier protein attached to the small molecule, which can then be coupled to the microsphere surface using the standard one-step carbodiimide chemistry. If the small molecule is available in a biotinylated form, it can be bound to MagPlex®-Avidin Microspheres, where the avidin provides spacing from the microsphere surface.[2,3]

1. Komatsu N, Shichijo S, Nakagawa M, Itoh K. New multiplexed flow cytometric assay to measure anti-peptide antibody: a novel tool for monitoring immune responses to peptides used for immunization. Scand J Clin Lab Invest. 2004;64(6):535–45.

2. Iannone MA, Consler TG, Pearce KH, et al. Multiplexed molecular interactions of nuclear receptors using fluorescent microspheres. Cytometry. 2001;44(4):326–337.

3. Drummond JE, Shaw EE, Antonello JM, et al. Design and optimization of a multiplex anti-influenza peptide immunoassay. J Immunol Methods. 2008;334(1-2):11–20.

Common protein and chemical spacers used to couple peptides to xMAP® microspheres:

BSA—bovine serum albumin
KLH—keyhole limpet hemocyanin
TG—thyroglobulin
ADH—adipic acid dihydrazide
MPBH—(4-(4-N-maleimidophenyl) butyric acid hydrazide)
Maleimide
Biotin
Ni-NTA—(nickel-nitrilotriacetic acid)

Luminex recommends, in the following order

1. Couple molecules >10 kDa directly without using a linker.
2. Couple peptides via a carrier protein: Conjugating your small molecule to a carrier protein such as bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), or thyroglobulin (TG) may be done using commercially available cross-linking reagents, followed by coupling the peptide-carrier protein conjugate to the beads using our standard protein coupling protocol.
3. Biotinylate the peptide and bind it to MagPlex®-Avidin Microspheres. Your capture peptide may be available in biotinylated form or may be easily biotinylated using commercially available reagents. In this case, the detection reagent must be directly conjugated to the reporter dye.
4. Modify the microsphere surface with adipic acid dihydrazide (ADH) or 4-(4-N-maleimidophenyl) butyric acid hydrazide (MPBH), and couple the peptide via carboxyl or sulfhydryl groups using EDC.

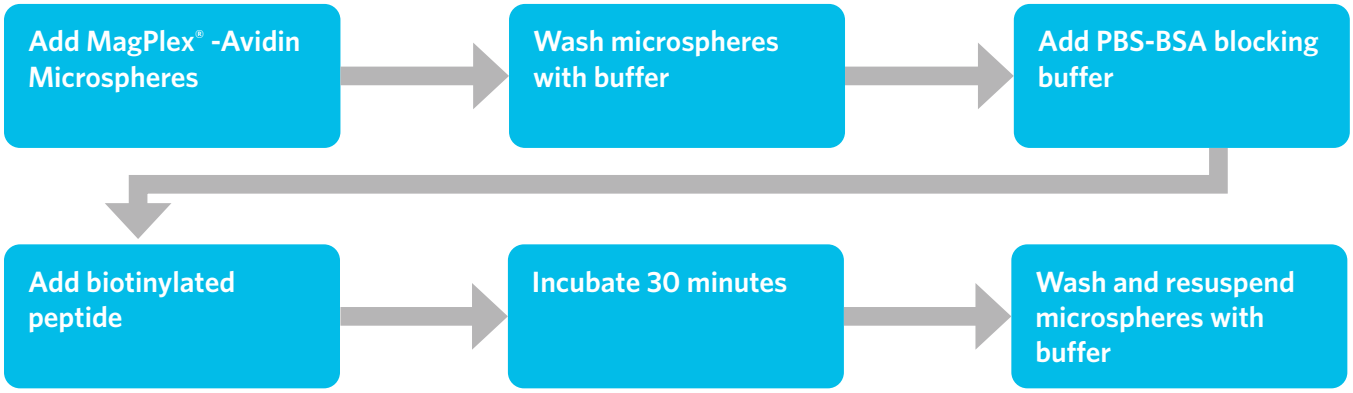
Other coupling moiety references

- Lauer SA, Nolan JP. Development and characterization of Ni-NTA-bearing microspheres. Cytometry. 2002;48(3):136-45.
- Pochechueva T, Chinarev A, Spengler M, et al. Multiplex suspension array for human anti-carbohydrate antibody profiling. Analyst. 2011;136(3):560-69.
- Schlottmann SA, Jain N, Chirmule N, Esser MT. A novel chemistry for conjugating pneumococcal polysaccharides to Luminex microspheres. J Immunol Methods. 2006;309(1-2)75–85.
- Wang H, Li H, Zhang W, et al. Multiplex profiling of glycoproteins using a novel bead-based lectin array. Proteomics. 2014;14(1):78–86.

Binding Biotinylated Peptides to MagPlex®-Avidin Microspheres

A sample protocol for binding biotinylated molecules to MagPlex®-Avidin Microspheres is described below. However, with this approach, a biotin–streptavidin system cannot be used for reporter labeling. An alternative reporter labeling method, such as a direct conjugation of the reporter dye to the detection reagent, would be necessary.

Summary of protocol



Materials needed

Reagents and Consumables	Vendor
MagPlex®-Avidin Microspheres	Luminex
Assay/wash buffer (PBS, 1% BSA)	MilliporeSigma P3688
Biotin-conjugated molecule	Any suitable brand
PBS-BN buffer[1]	MilliporeSigma P3688 MilliporeSigma S8032
PBS–TBN buffer*,†	MilliporeSigma P3563 MilliporeSigma A7888 MilliporeSigma S8032
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Protein LoBind® 022431081
Disposable pipette tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

For complete equipment and materials list, see **Appendix B**.

*Also used as assay buffer.

†Also used as wash buffer.

Protocol 4.4.1: Coupling biotinylated peptides to MagPlex®-Avidin Microspheres

1. Resuspend the stock MagPlex®-Avidin Microsphere suspension according to the instructions described in the product information sheet provided with your microspheres.
2. Transfer 1.0×10^6 of the stock microspheres to a recommended microcentrifuge tube.
3. Place the tube into a magnetic separator and allow separation to occur for 30 to 60 seconds.
4. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the microspheres.
5. Remove the tube from the magnetic separator and resuspend the pelleted microspheres in 250 µL of PBS–BSA by vortex and sonication for ~20 seconds.
6. Dilute the biotin-conjugated molecule in PBS–BSA. The optimal concentration should be determined by titration. (Note: 5 µg protein per 1 million beads typically performs well. We recommend titrating up and/or down as needed to achieve optimal assay performance.)
7. Add 250 µL of the biotin-conjugated molecule solution to the microsphere suspension and mix immediately by vortex.
8. Incubate for 30 minutes with mixing (by rotation) at room temperature.
9. Place the tube into a magnetic separator and allow separation to occur for 30 to 60 seconds.
10. With the tube still positioned in the magnetic separator, remove the supernatant. Take care not to disturb the microspheres.
11. Resuspend the pelleted microspheres in 500 µL of blocking/storage buffer (PBS-BN or PBS–TBN) by vortex.
12. Repeat steps 9 and 10 for a total of two washes with blocking/storage buffer.
13. Resuspend the microspheres in 250–1,000 µL blocking/storage buffer by vortex and sonication for ~20 seconds.
14. Store the bound MagPlex-Avidin Microspheres refrigerated at 2–8°C in the dark.

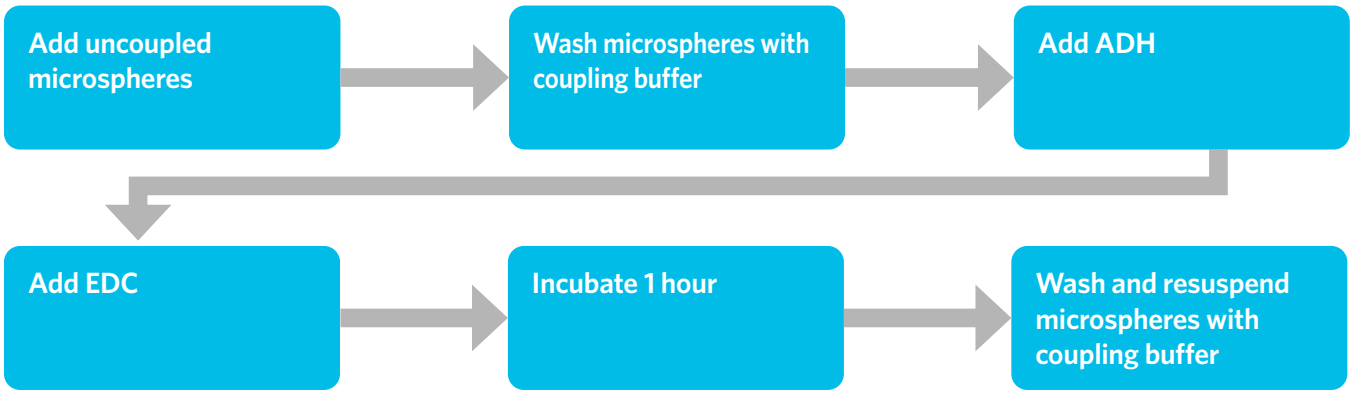
Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

Chapter 4.4.2

Modification of Microspheres with ADH

Adipic acid dihydrazide (ADH) is a suitable chemical spacer for extending the peptide away from the surface of xMAP® microspheres for optimum reactivity with sample and reagents. ADH provides a 10-atom spacer with an active amine group for coupling to peptide carboxyl groups. A sample protocol for modifying xMAP microspheres with ADH is described below.

Summary of protocol



Materials needed

Reagents and Consumables	Vendor
MagPlex® Microspheres	Luminex
Adipic acid dihydrazide (ADH)	MilliporeSigma A0638
Coupling buffer (0.1 M MES, pH 6.0)	MilliporeSigma M2933
1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC)	Thermo Scientific Pierce™ 77149 ProteoChem® c1100-100mg
Wash buffer (0.1 M MES, pH 4.5)	MilliporeSigma M2933
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Protein LoBind® 022431081
Disposable pipette tips	Any suitable brand

For complete equipment and materials list, see **Appendix B**.

Protocol 4.4.2: Modification of microspheres with adipic acid dihydrazide (ADH)

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 25×10^6 microspheres to a centrifuge tube and pellet by centrifugation at ≥4,000 x g for 2 minutes (or by using a magnetic separator) and remove supernatant.
3. Resuspend the pelleted microspheres in 1 mL of 0.1 M MES, pH 6.0, by vortex and sonication for ~20 seconds.
4. Transfer the resuspended microspheres to a recommended microcentrifuge tube and pellet the microspheres by microcentrifugation at ≥8,000 x g for 1–2 minutes (or by using a magnetic separator) and remove supernatant.
5. 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 µL 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 hour with mixing (by rotation) at room temperature.
8. Pellet the microspheres by microcentrifugation at ≥8,000 x g for 1–2 minutes (or by using a magnetic separator) and remove supernatant.
9. Resuspend the pelleted microspheres in 1 mL of 0.1 M MES, pH 4.5, by vortex.
10. Pellet the microspheres by microcentrifugation at 8,000 x g for 1–2 minutes (or by using a magnetic separator) and remove supernatant. Repeat twice, for a total of 3 washes with 1 mL of 0.1 M MES, pH 4.5.
11. 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.

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

Note: This protocol was developed for modification of 25 million microspheres.

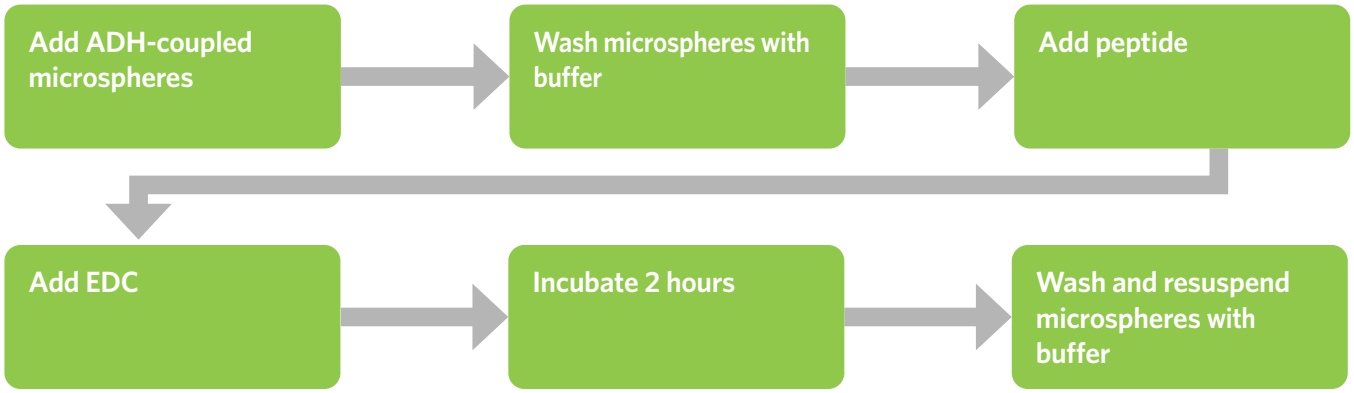
Note: To scale down the protocol for $1.25\text{x}10^6$ microspheres, use 1 mL of 3.5 mg/mL ADH (Step 5) and 20–25 mg/mL EDC (Step 6).

Chapter 4.4.3

Coupling Peptides to ADH-Modified Microspheres

Adipic acid dihydrazide (ADH) is a suitable chemical spacer for extending the peptide away from the surface of xMAP® microspheres for optimum reactivity with the sample and reagents. A sample protocol for coupling peptides to ADH-modified microspheres is described below.

Summary of protocol



Materials needed

Reagents and Consumables	Vendor
MagPlex® Microspheres (ADH-coupled)	Supplied by user
Peptide to couple	Any suitable source
Wash buffer (0.1 M MES, pH 6.0)	MilliporeSigma M2933
1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC)	Thermo Scientific Pierce™ 77149 ProteoChem™ c1100-100mg
Wash buffer (PBS-TBN buffer)	MilliporeSigma P3563 MilliporeSigma A7888 MilliporeSigma S8032
PBS, pH 7.4	MilliporeSigma P3813
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Protein LoBind® 022431081
Disposable pipette tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

For complete equipment and materials list, see **Appendix B.**

Protocol 4.4.3: Coupling peptides to ADH-modified microspheres

1. Resuspend stock ADH-modified microsphere suspension by vortex and sonication (15–30 seconds).
2. Remove an aliquot of $25x10^6$ ADH microspheres to a centrifuge tube and pellet by centrifugation at ≥8,000 x g, 1–2 minutes (or by using a magnetic separator) and remove supernatant.
3. Wash once with 1 mL 0.1 M MES, pH 6.0 and pellet by centrifugation at ≥8,000 x g, 1–2 minutes (or by using a magnetic separator) and remove supernatant, then resuspend ADH microspheres in 100 µL 0.1 M MES, pH 6.0, vortex.
4. Add 250 µg peptide to ADH microspheres and adjust volume to 500 µL with 0.1 M MES, pH 6.0.
5. Add 50 µL 200 mg/mL EDC (prepared immediately before use in 0.1 M MES, pH 6.0), vortex.
6. Incubate 2 hours at room temperature with rotation (protect from light).
7. Pellet by centrifugation at ≥8,000 x g, 1–2 minutes (or by using a magnetic separator) and remove supernatant, then resuspend coupled microspheres in 1 mL PBS, pH 7.4 and vortex.
8. Pellet by centrifugation at ≥8,000 x g, 1–2 minutes (or by using a magnetic separator) and remove supernatant. Repeat twice, for a total of 3 washes with 1 mL PBS-TBN.
9. Resuspend coupled microspheres in 1 mL PBS-TBN.
10. Count microsphere suspension by hemacytometer.*
    **Calculation: Total microspheres = count (1 corner of the 4x4 grid)x($1x10^4$) x(dilution factor)x(resuspension volume in mL).*

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

Note: This protocol was developed for coupling to 25 million ADH-modified microspheres.

Note: Performing wash steps 7 and 8 with PBS-Tween containing a low concentration of the unactivated peptide/protein used for coupling may improve assay performance. After these wash steps, PBS-TBN can be used as a storage buffer.

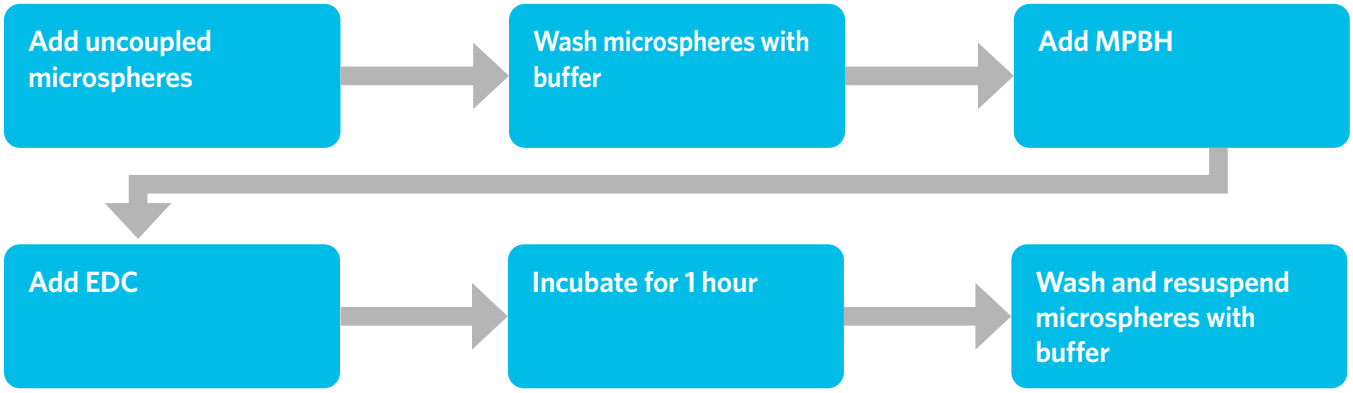
Note: For coupling polysaccharides (modified) to ADH-modified microspheres (see **Chapter 4.4.6**), remove an aliquot of $1.25x10^6$ ADH microspheres (Step 2) and in Step 4, add 12.5 µg of modified polysaccharide. Use 50 µL of 20–25 mg/mL EDC (Step 5).

Chapter 4.4.4

Modification of Microspheres with MPBH

4-(4-N-maleimidophenyl)butyric acid hydrazide (MPBH) is a suitable chemical spacer for extending the peptide away from the surface of xMAP® microspheres for optimum reactivity with the sample and reagents. MPBH provides an 8-atom spacer with a reactive maleimide group for coupling to cysteine sulfhydryls. A sample protocol for modifying xMAP microspheres with MPBH is described below.

Summary of protocol



Materials needed

Reagents and Consumables	Vendor
MagPlex® Microspheres	Luminex
4-(4-N-maleimidophenyl)butyric acid hydrazide (MPBH)	Thermo Scientific Pierce™ 22305
0.1 M MES, pH 6.0	MilliporeSigma M2933
DMSO	Any suitable source
1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC)	Thermo Scientific Pierce™ 77149 ProteoChem™ c1100-100mg
0.1 M MES, pH 4.5	MilliporeSigma M2933
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Protein LoBind® 022431081
Disposable pipette tips	Any suitable brand

For complete equipment and materials list, see **Appendix B**.

Protocol 4.4.4: Modification of microspheres with MPBH (maleimide)

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 25×10^6 microspheres to a centrifuge tube and pellet by centrifugation at ≥4,000 x g for 2 minutes (or by using a magnetic separator) and remove supernatant.
3. Resuspend the pelleted microspheres in 1 mL of 0.1 M MES, pH 6.0, by vortex and sonication for ~20 seconds.
4. Transfer the resuspended microspheres to a recommended microcentrifuge tube and pellet the microspheres by microcentrifugation at ≥8,000 x g for 1–2 minutes (or by using a magnetic separator) and remove supernatant.
5. Dissolve MPBH at 80 mM (28.3 mg/mL) with DMSO.
6. Dilute dissolved MPBH to 16 mM (5.7 mg/mL) with 0.1 M MES, pH 6.0.
7. Resuspend the microspheres in 250 µL of diluted MPBH by vortex.
8. Add 100 µL of 20 mg/mL EDC (prepared immediately before use in 0.1 M MES, pH 6.0) and mix by vortex.
9. Incubate for 1 hour with mixing (by rotation) at room temperature.
10. Add 1 mL of 0.1 M MES, pH 4.5, and mix by vortex.
11. Pellet the microspheres by microcentrifugation at ≥8,000 x g for 1–2 minutes (or by using a magnetic separator) and remove supernatant.
12. Resuspend the pelleted microspheres in 1 mL of 0.1 M MES, pH 4.5 by vortex and pellet by microcentrifugation at ≥8,000 x g for 1–2 minutes (or by using a magnetic separator) and remove supernatant.
13. Repeat step 12 for a total of 2 washes with 1 mL of 0.1 M MES, pH 4.5.
14. 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.

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

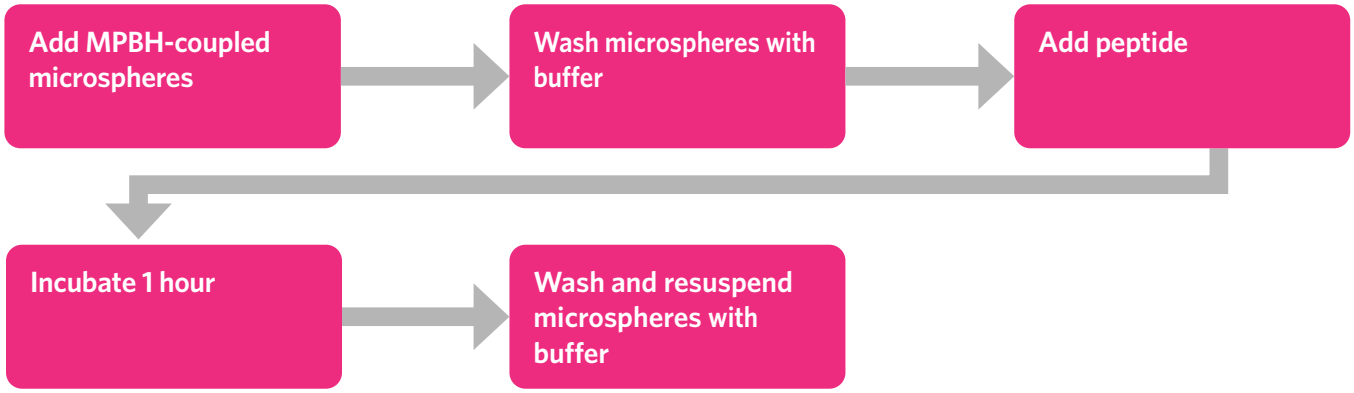
Note: This protocol was developed for modification of 25 million microspheres.

Chapter 4.4.5

Coupling Peptides to Maleimide-Modified Microspheres

A maleimide-containing linker, such as MPBH, is suitable for extending the free terminal cysteine-containing peptide away from the surface of xMAP® microspheres for optimum reactivity with the sample and reagents. A sample protocol for coupling peptides to MPBH-modified xMAP microspheres is described below.

Summary of protocol



Materials needed

Reagents and Consumables	Vendor
MagPlex® Microspheres (MBPH-modified)	Supplied by user
Peptide to couple	Any suitable source
100 mM Tris, pH 7.4	See **Appendix A**
Assay/wash buffer (PBS, 1% BSA)	MilliporeSigma P3688
Storage buffer (PBS-TBN)	MilliporeSigma P3563 MilliporeSigma A7888 MilliporeSigma S8032
0.1 M sodium phosphate, 50mM NaCl pH 7.0	See **Appendix A**
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Protein LoBind® 022431081
Disposable pipette tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

For complete equipment and materials list, see **Appendix B**.

Protocol 4.4.5: Coupling peptides to maleimide-modified microspheres

1. Resuspend stock maleimide-modified microsphere suspension by vortex and sonication (15–30 seconds).
2. Remove an aliquot of $1x10^5$ maleimide-modified microspheres and pellet by centrifugation at ≥8,000 x g, 1–2 minutes (or by using a magnetic separator) and remove supernatant.
3. Resuspend maleimide microspheres at $1x10^5$/mL in 0.1 M sodium phosphate, 50 mM NaCl, pH 7.0 by vortex.
4. Aliquot $1x10^4$ microspheres to each coupling reaction (100 µL).
5. Add peptide (100 µL, in 100 mM Tris, pH 7.4) to each $1x10^4$ microsphere reaction (see below).
6. Incubate for 1 hour at room temperature with shaking (protect from light).
7. Pellet by centrifugation at ≥8,000 x g, 1–2 minutes (or by using a magnetic separator) and remove supernatant.
8. Wash twice with assay buffer, (200–500 µL).
9. Resuspend coupled microspheres in PBS-TBN.

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

Note: Performing wash steps with PBS-Tween containing a low concentration of the unactivated peptide/protein used for coupling may improve assay performance.

Peptide Coupling Titration

1	16.5 nmol
2	1.65 nmol
3	0.165 nmol
4	0.0165 nmol
5	0.00165 nmol
6	0.000165 nmol
7	0.0000165 nmol

Coupling Polysaccharides to Carboxylated or ADH-Modified Microspheres

Overview of Pneumococcal Polysaccharides

Nomenclature		ATCC#	Source*	PnPs Vaccine†	Polysaccharide Modification‡	Microsphere Functional Group
Danish¶	USA#					
1	1	15-X 10-X	Merck Pfizer	PCV10 PCV13 PCV23	DMTMM	ADH
					DMTMM	COOH
					None	COOH
2	2	16-X 500-X	Merck Pfizer	PCV23	PLL	COOH
					DMTMM	ADH
3	3	17-X 31-X	Merck Pfizer	PCV13 PCV23	DMTMM	COOH
					PLL	COOH
4	4	18-X 34-X	Merck Pfizer	PCV7 PCV10 PCV13 PCV23	None	ADH
					DMTMM	COOH
					DMTMM	ADH
5	5	57-X 37-X	Merck Pfizer	PCV13 PCV23 PCV10	PLL	COOH
					DMTMM	COOH
					DMTMM	ADH
6A	6	14-X	Pfizer	PCV13	DMTMM	ADH
					PLL	COOH
6B	26	58-X 105-X	Merck Pfizer	PCV7 PCV10 PCV13 PCV23	DMTMM	COOH
					DMTMM	ADH
					None	ADH
7F	51	108-X	Pfizer	PCV10 PCV13 PCV23	PLL	COOH
					None	ADH
					DMTMM	COOH
8	8	20-X 503-X	Merck Pfizer	PCV23	DMTMM	COOH
					DMTMM	ADH
9N	9	21-X 506-X	Merck Pfizer	PCV23	PLL	COOH
					None	ADH
9V	68	305-X	Merck Pfizer	PCV7 PCV10 PCV13 PCV23	DMTMM	COOH
					PLL	COOH
					DMTMM	ADH

Nomenclature		ATCC#	Source*	PnPs Vaccine†	Polysaccharide Modification‡	Microsphere Functional Group§
Danish¶	USA#					
10A	34	60-X 509-X	Merck Pfizer	PCV23	None PLL	ADH COOH
11A	43	61-X 512-X	Merck Pfizer	PCV23	PLL DMTMM	COOH ADH
12F	12	22-X 515-X	Merck Pfizer	PCV23	PLL None	COOH ADH
14	14	23-X 73-X	Merck Pfizer	PCV7 PCV10 PCV13 PCV23	PLL DMTMM DMTMM	COOH ADH COOH
15B	54	62-X 518-X	Merck Pfizer	PCV23	None DMTMM	ADH COOH
17F	17	63-X 521-X	Merck Pfizer	PCV23	None PLL	ADH COOH
18C	56	285-X	Pfizer	PCV7 PCV10 PCV13 PCV23	None DMTMM PLL	ADH ADH COOH
19A	57	64-X 301-X	Merck Pfizer	PCV13 PCV23	DMTMM None DMTMM	ADH ADH COOH
19F	19	84-X	Pfizer	PCV7 PCV10 PCV13 PCV23	DMTMM PLL DMTMM	COOH COOH ADH
22F	22	527-X	Pfizer	PCV23	PLL	COOH
23F	23	25-X 102-X	Merck Pfizer	PCV7 PCV10 PCV13 PCV23	DMTMM None DMTMM	ADH ADH COOH
33F	70	67-X 533-X	Merck Pfizer	PCV23	PLL DMTMM	COOH ADH

*Source of pneumococcal antigens. Data based on patent WO2011 117844A2. All strains are available from SSI Diagnostica (https://www.ssidiagnostica.com/antigens/).

†Strains based on either:

- Prevnar® (PVC7) 7-valent polysaccharides conjugate vaccine (Pfizer) containing PnPS 4, 6B, 9V, 14, 18C, 19F, and 23F.
- Synflorix™ (PCV10) 10-valent polysaccharides conjugate vaccine (GlaxoSmithKline) containing PnPS 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F.
- Prevnar® (PVC13) 13-valent polysaccharides conjugate vaccine (Pfizer) containing PnPS 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F.
- Pneumovax® (PCV23) 23-valent polysaccharide conjugate vaccine (Merck) containing PnPS 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F.

‡PnPS modification: None, no conjugation of PnPS, DMTMM; DMTMM conjugated PnPS; PLL: poly-L-lysine conjugated PnPS (see **Protocol 4.6.6.1**).

¶The Danish designation uses an alphanumeric system to indicate any known cross-reactivity. The number refers to the sero-capsular antigen group and the letter represents related serotypes that cannot be distinguished.

#The United States designations chronologically indicate the antigens in order of recognition.

Protocol 4.4.6.1: Modification of Pneumococcal Polysaccharides with Poly-L-lysine (PLL)

Materials Needed

Reagents and Consumables	Vendor
NaOH powder (Sodium hydroxide)	Merck/MilliporeSigma 106498
PnPs target to couple (see **Table 1**)	Any suitable source
Phenolphthalein (PPT, pH indicator)	Merck/MilliporeSigma 105945
Cyanuric chloride	Merck/MilliporeSigma C95501
Poly-L-lysine hydrobromide (PLL)	Merck/MilliporeSigma P2636
Phosphate buffered saline, pH 7.4 (PBS)	Merck/MilliporeSigma P3813
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 Eppendorf Protein LoBind® 022431081
5 mL tubes	Any suitable brand
Sephadex G-25M PD 10 Desalting Column	Merck/MilliporeSigma GE17-0851-01
0.22 µm filters	Merck/MilliporeSigma SLGS033SB
Rotator	Any suitable brand
Disposable pipette tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable brand

Stock solution: PnPs (5 mg/mL)

1. Prepare a PnPs stock solution of 5 mg/mL by adding ddH_2O (e.g., 10 mg PnPs in 2 mL of ddH_2O).
2. Mix carefully until it is completely dissolved.
3. Place the PnPs solution tube on a rotator and rotate overnight at 4°C (8–12 hours).
4. Aliquot and freeze at -80°C or use fresh.

Stock solution: 0.01% NaOH/0.001% PPT

1. Prepare a stock solution by dissolving 100 mg NaOH and 10 mg PPT in 1,000 mL ddH_2O.
2. Store at 2–8°C no longer than 7 days.

Stock solution: PLL (0.25 mg/mL)

1. Prepare a fresh PLL stock solution by dissolving 2.5 mg PLL in 10 mL ddH_2O, use fresh.

Stock solution: PBS

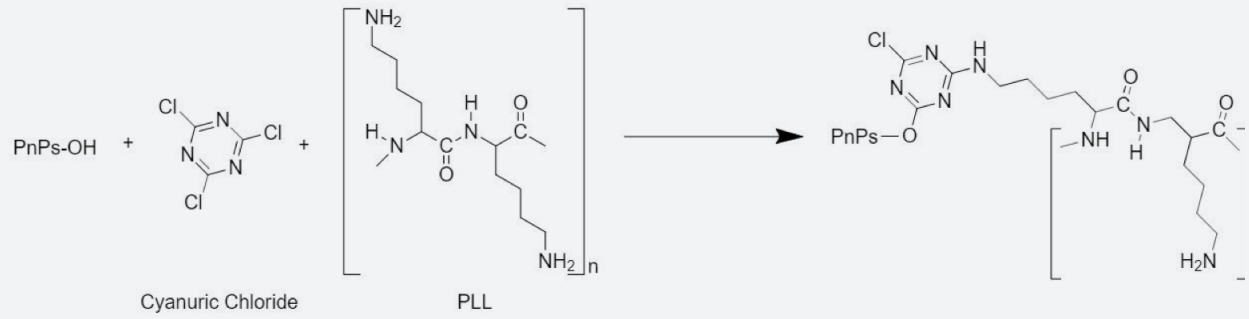
1. Filter PBS through 0.22 µm filter.

Stock solution: NaOH/PLL/PnPs

1. Add 1.25 mL 0.01% NaOH/0.001% PPT solution into a 5 mL tube.
2. Add 0.5–0.7 mL (2.5–3.5 mg per reaction) PnPs (amount should be determined for individual polysaccharide).
3. Vortex for 10 seconds.
4. Add 0.7–1.05 mL ddH_2O to the 5 mL tube.
5. Vortex for 10 seconds.
6. Add 12.5 mg cyanuric chloride in a 50 mL tube.
7. Add the NaOH/PPT/PnPs solution to the cyanuric chloride powder.
8. Vortex for 10 seconds.
9. When the solution turns from pink to colorless, add 50 µL of freshly prepared PLL solution.

10. Incubate static at 4°C overnight.
11. On the next day, prepare a Sephadex G25 column for each PnPs by adding 25 mL of freshly filtered PBS. Run the entire volume, but make sure the column doesn't run dry.
12. Add the NaOH/PPT/PnPs/PLL solution to the column. Once it stops dripping, discard the eluate.
13. Place a clean 5 mL tube under the column.
14. Add 3.5 mL fresh filtrated PBS and collect the eluate.
15. Mix the eluate and aliquot in appropriate volume.
16. Store the PLL conjugated PnPs at ≥ -70°C, or use direct for coupling to COOH xMAP® microspheres (see **Protocol 4.2.1: Carbodiimide coupling**).
    Note: 5 µg of modified polysaccharide per million beads typically performs well in coupling reactions. We recommend titration up and/or down as needed to achieve optimal assay performance.

Figure 18. Poly-L-Lysine (PLL) Chemistry



Further Reading:

1. Schlottmann SA, Jain N, Chirmule N, and Esser MT. A novel chemistry for conjugating pneumococcal polysaccharides to Luminex microspheres. J Immunol Methods. 2006;309(1-2), 75-85.
2. Tan CY, Immermann FW, Sebastian S, et al. Evaluation of a Validated Luminex-Based Multiplex Immunoassay for Measuring Immunoglobulin G Antibodies in Serum to Pneumococcal Capsular Polysaccharides. mSphere. 2018;3(4), e00127-18.
3. Pavliakova D, Giardina PC, Moghazeh S, et al. Development and Validation of 13-plex Luminex-Based Assay for Measuring Human Serum Antibodies to Streptococcus pneumoniae Capsular Polysaccharides. mSphere. 2018;3(4), e00128-18.

Protocol 4.4.6.2: Modification of Pneumococcal Polysaccharides with DMTMM

Materials Needed

Reagents and Consumables	Vendor
4-(4,6-dimethoxy[1,3,5]triazin-2-yl)-4-methylmorpholinium chloride (DMTMM)	Merck/MilliporeSigma 74104
PnPs target for coupling (see **Table 1**)	Any suitable source
Phosphate buffered saline, pH 7.4 (PBS)	Merck/MilliporeSigma P3813
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 Eppendorf Protein LoBind® 022431081
5 mL tubes	Any suitable brand
Sephadex G-25M PD 10 Desalting Column	Merck/MilliporeSigma GE17-0851-01
0.22 µm filters	Merck/MilliporeSigma SLGS033SB
Rotator	Any suitable brand
Disposable pipette tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable brand

Stock solution: PnPs (10 mg/mL)

1. Prepare a PnPs stock solution of 10 mg/mL by adding ddH_2O (e.g., 10 mg PnPS in 1 mL of ddH_2O).
2. Mix carefully until it is dissolved completely.
3. Place the PnPs solution tube on a rotator, and rotate overnight at 4°C (8–12 hours).
4. Aliquot and freeze at -80°C, or use fresh.

Stock solution: DMTMM (200 mg/mL)

1. Prepare a DMTMM stock solution of 200 mg/mL by adding ddH_2O (e.g., 1 g DMTMM in 5 mL ddH_2O).
2. Mix carefully until it is completely dissolved.
3. Prepare aliquots of 1 mL and store at -80°C, or use fresh.

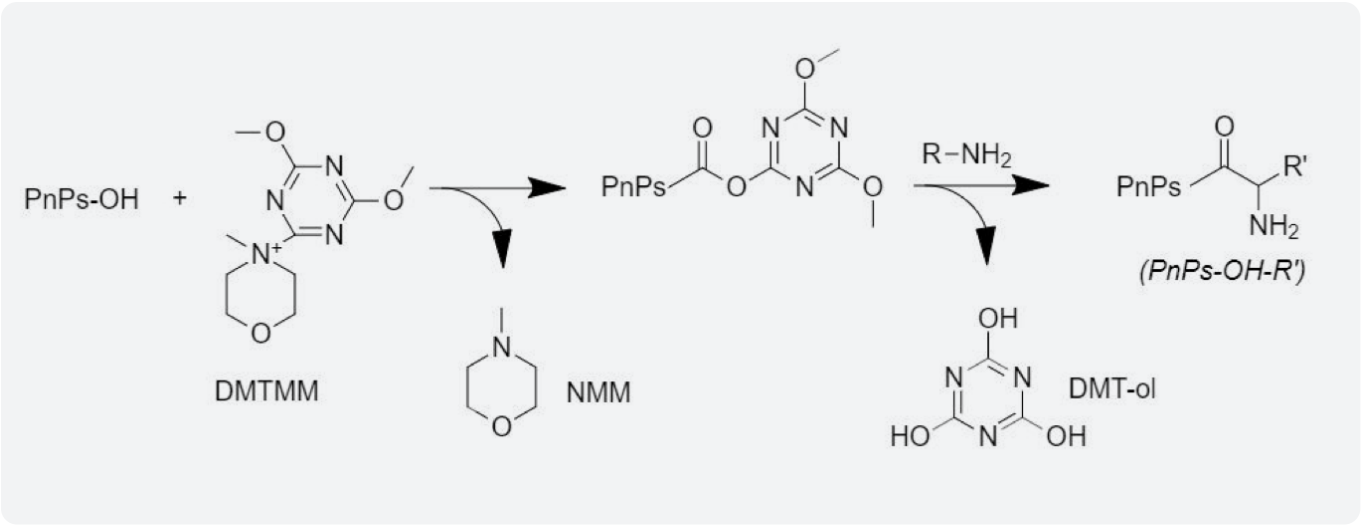
Stock solution: PBS

1. Filter 100 mL PBS through 0.22 µm filter.

DMTMM modification of PnPS

1. Prepare 2.5 mL of 1 mg/mL PnPS in a 5 mL tube.
2. Add 200 µL of 200 mg/mL DMTMM stock solution.
3. Place tube on a rotator and rotate for 1 hour at room temperature.
4. Rinse a Sephadex G25 column by adding 25 mL of fresh filtered PBS. Run the entire volume, but make sure the column doesn't run dry.
5. Add the PnPS-DMTMM mixture to the column. Once it stops dripping, discard the eluate.
6. Elute the PnPS/DMTMM complex with 3.5 mL filtrated PBS.
7. Collect the eluate in a 5 mL tube.
8. Aliquot the eluate in MICRONIC tubes in increments of 500 µL and store at -20°C, or use fresh for coupling to either COOH- (see **Protocol 4.2.1: Carbodiimide coupling**) or ADH-modified microspheres (see **Protocol 4.4.3: Coupling peptides to ADH-modified microspheres**) .
   Note: 5 µg of modified polysaccharide per million beads typically performs well in coupling reactions. We recommend titration up and/or down as needed to achieve optimal assay performance.

Figure 19. DMTMM Chemistry



Further Reading:

1. Schlottmann SA, Jain N, Chirmule N, et al. A novel chemistry for conjugating pneumococcal polysaccharides to Luminex microspheres. J Immuno Methods. 2006;309(1-2), 75-85.

Protocol 4.4.6.3: Indirect Serological Immunoassay for Detection of Pneumococcal IgG Antibodies

Materials Needed

Reagents and Consumables	Vendor
MagPlex® Microspheres (conjugated with polysaccharide)	Supplied by user
Antibody Depleted Human Serum (ADHS) (See Note)	Any suitable source
Pneumococcal Cell Wall Polysaccharide (CWPS Multi)	SSI Diagnostica 68866
PBS, pH 7.4	Merck/MilliporeSigma P3813
TWEEN®-20	Merck/MilliporeSigma P9416
Wash buffer (PBS, 0.05% TWEEN®-20, pH 7.4)	Merck/MilliporeSigma P3563
Human anti-pneumococcal capsule reference serum (007sp or equivalent)	WHO (www.nibsc.org) or other suitable source
Disposable pipette tips	Any suitable brand
Centrifuge or magnetic separator	Any suitable brand
2.0 mL microcentrifuge tubes	Any suitable brand
Plate sealer	Any suitable brand

** ADHS stock solution can be replaced by Surmodics™ Assay Diluent (SM01) supplemented with 2% fetal calf serum.*

Stock solution: Antibody depleted human serum (ADHS)

1. Aliquot 2 mL ADHS per vial.
2. Store up to 3 years at <-70°C.

Stock solution: Pneumococcal cell wall polysaccharide (CWPS multi, 15 mg/mL)

1. Make a stock solution of 15 mg/mL by dissolving a vial of 10 mg CWPS multi in 667 µL PBS.
2. Aliquot 20 µL into stock vials.
3. Store up to 1 year at <-70°C (*Note: 5–10 µg/mL CWPS multi in combination with 5–10 ug/mL PnPs 22F may be used*).

Dilution buffer (10% ADHS, 30 µg/mL CWPS multi in PBS)

1. Prepare 10 mL for a full 96-well plate. Add 1 mL ADHS to 10 µL Multi CWPS stock solution to 9 mL PBS (*Note: 0.05% TWEEN®-20 may also be included in the Dilution Buffer*).

   Note: If using Surmodics Assay Diluent (SM01), add 10 µL of Multi CWPS stock solution to 10 mL of SM01.
2. Mix carefully until it is completely dissolved to prevent the formation of bubbles.
3. Prepare fresh and keep at 2–8°C.

Preparation of standards

1. Prepare a stock pre-dilution of the reference serum (007sp), or equivalent biological standard, in dilution buffer and prepare aliquots at 1:80 to 1:100.
2. Store up to 2 years at < -70°C.

PnPs-coupled microsphere mixture (2,500 beads per target per well in 50 µL PBS)

1. Suspend stock PnPs-modified microspheres by vortex and sonication (15–30 seconds).
2. Calculate the volume needed to make a stock solution containing 2,500 microspheres per set in a total volume of 50 µL PBS per well
3. Example for 96 wells: 96 wells x 50 µL = 4,800 µL. This solution should contain 240,000 beads per set (96 wells x 2,500 microspheres); (*Note: for sufficient excess volume, prepare 5,000 µL total volume*).
4. If there is an excess volume after combining the bead set, pellet microspheres by centrifugation at ≥8,000 x g, 1–2 minutes (or by using a magnetic separator).
5. Remove the supernatant and adjust to the correct volume with PBS.

Indirect serological immunoassay for detection of anti-pneumococcal IgG antibodies

1. Make sure that all reagents, including biological samples, are at room temperature for at least 30 minutes.
2. Vortex all biological samples (including the stock dilution 007sp, or equivalent) before use.
3. Prepare a linear standard curve using 007sp sample or equivalent in dilution buffer.
4. Dilute the samples and controls (e.g. dilute 1:50–1:1000) in dilution buffer.
5. Add 100 µL of standards, samples, and controls to the appropriate wells of a 96-well plate and seal the plate with a plate sealer.
6. Incubate for 1–5 hours at room temperature on a plate shaker at 800 rpm (alternatively, incubation can be done for 30 minutes at 37°C, or overnight at 4°C on a plate shaker).
7. During incubation, prepare the PnPs-coupled microsphere mixture (see **"PnPs-coupled microsphere mixture"** section above).
8. After incubation, if condensation is present on the plate sealer, centrifuge the plate for 1 minute at 2,000 x g.
9. Remove 50 µL of the incubated standards, samples, and controls and transfer to a new 96-well plate.
10. Aliquot 50 µL of PnPS-coupled microsphere mixture to each well.
11. Cover the plate to protect it from light and incubate for 60 minutes at room temperature on a plate shaker set to ≤800 rpm.
12. Place the plate into the magnetic separator and allow separation to occur for 30–60 seconds.
13. Carefully remove the supernatant from each well by magnetic plate separator using either manual inversion (See **Protocol F** above, Step 10), manual pipetting or magnetic plate washer. Take care not to disturb the microspheres.
14. Add 100 µL of wash buffer to each reaction well.
15. Repeat steps 12–14 for a total 2–4 washes. Remove supernatant from each well.
16. Prepare an appropriate dilution of the goat anti-human IgG-PE conjugate in PBS (e.g., dilute 1:50–1:1,000).
17. Remove the plate from the magnetic separator and add 25–100 µL anti-human IgG-PE to each well of the plate.
18. Cover the plate to protect it from light and incubate for 30–60 minutes at room temperature on a plate shaker set ≤800 rpm.
19. Place the plate into the magnetic separator and allow separation to occur for 30–60 seconds.
20. Carefully remove the supernatant from each well by magnetic plate separator using either manual inversion (See **Protocol F** above, Step 10), manual pipetting or magnetic plate washer. Take care not to disturb the microspheres.
21. Add 100 µL of wash buffer to each reaction well.
22. Repeat steps 19–21 for a total 2–4 washes. Remove supernatant from each well.
23. Remove the plate from the magnetic separator and add 100 µL wash buffer to each well of the plate.
24. Resuspend the microspheres by pipetting up and down several times with a multichannel pipette or placing the plate onto a plate shaker at 800 rpm for ~15–60 seconds.
25. Analyze 50–100 µL on your Luminex analyzer according the system manual.

Further Reading:

1. Biagini RE, Schlottmann SA, Sammons DL, et al. Method for simultaneous measurement of antibodies to 23 pneumococcal capsular polysaccharides. Clin Diagn Lab Immunol. 2003;10(5)744–50.
2. Schlottmann S, Jain N, Esser M. A novel chemistry for conjugating pneumococcal polysaccharides to Luminex microspheres. J Immunol Methods. 2006 Feb 20;309 (1-2);75-85.
3. Elberse KR, Tcherniaeva I, Berbers GA, et al. Optimization and application of amultiplex bead-based assay to quantify serotype-specific IgG against Streptococcus pneumoniae polysaccharide: response to the booster vaccine after immunization with the pneumococcal 7 valent conjugate vaccine. Clin Vaccine Immunol. 2010 17(4):674-682.
4. Tan CY, Immerman FW, Sebastian S, et al. Evaluation of a Validated Luminex-Based Multiplex Immunoassay for Measuring Immunoglobulin G Antibodies in Serum to Pneumococcal Capsular Polysaccharides. mSphere. 2018 Aug 8;3(4) 00127-18.
5. Pavliakova D, Giardina PC, Moghazeh S, et al. Development and Validation of 13-plex Luminex-Based Assay for Measuring Human Serum Antibodies to Streptococcus pneumoniae Capsular Polysaccharides. mSphere. 2018 Aug 8;3(4)00128-18.
6. den Hartog G, Schepp RM, Kuijer M, et al. SARS-CoV-2-Specific Antibody Detection for Seroepidemiology: A Multiplex Analysis Approach Accounting for Accurate Seroprevalence. J Infect Dis. 2020;222(9):1452-1461.

Chapter 4.5

Optimization of Immunoassays

Multiplex immunoassays offer a number of advantages over singleplex assays, but these robust assays require some optimization. For multiplex immunoassays, the effective biological range of each analyte must be considered to ensure that reporter fluorescence will fall into the dynamic range of your assay. Singleplex assays address this by serial dilution of the sample, but a multiplex assay must take a different approach. Some analytes may exist in such a low range of concentrations that a more sensitive assay is needed, while another analyte in the same multiplex assay may be abundant and therefore require a lower-sensitivity assay. Assay sensitivity can be affected by the affinity of the capture antibody, the abundance of the capture antibody, and the amount of capture beads used for that analyte.

A higher affinity antibody for capture and/or detection may improve the lower limit of detection (LLOD) for an assay, whereas a lower affinity antibody may improve the upper limit of detection (ULOD) by extending the linear dynamic range. Decreasing the amount of antibody coupled to the beads can also improve sensitivity by improving linearity at low analyte concentrations. Conversely, increasing the amount of capture antibody can increase the signal for an assay but may provide less linearity at low analyte concentrations. For multiplexing assays with widely disparate concentration ranges, increasing the number of beads per set or using a weaker reporter for the high concentration analytes can bring these assays into the detectable dynamic range while allowing a minimal sample dilution to ensure optimal sensitivity for the low-concentration analytes.

Another consideration for microsphere-based immunoassays is the biological matrix and non-specific binding. Serum samples typically have extraneous proteins that may non-specifically bind to polystyrene and other materials. xMAP® microspheres are polystyrene beads that appear relatively smooth under a microscope, but on a molecular level, have irregular, porous surfaces. Microsphere pores range in size from 100 to 2,000 angstroms, allowing them to trap proteins, which typically range from 50 to 100 angstroms in diameter. As a result, microsphere assays sometimes employ blocking agents optimized for each biological matrix to reduce non-specific binding of non-target molecules.

Users must ensure that the optimum amount of capture molecule is bound to the microsphere and that capture and detection reagent pairs allow maximum binding and detection capacity. For capture sandwich immunoassays, it's important to confirm that the pair of capture and detection antibodies used binds to different epitopes. 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 can then be tested in multiplex with the individual candidate detection antibodies and analytes. This enables the rapid identification of the best-performing capture and detection antibody pair for a particular analyte.

TIP

High-quality reagents are particularly important in multiplex assays, where contamination by a single component may affect results of many assays. When possible, consider additional purification or filtration steps for antibodies, peptides, and buffers.

Figure 20. Antibody Pair Optimization

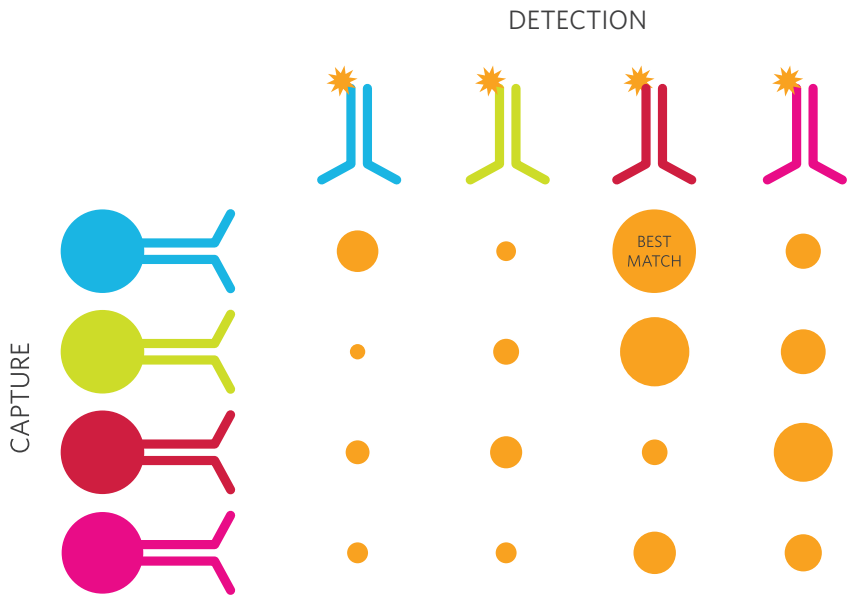


Figure 20 - Determining the best capture antibody and detection antibody by pairwise analysis. Using a small test quantity of antibody and microspheres, each candidate capture antibody (CAPTURE) is coupled to a separate microsphere and tested with target antigen and multiple candidate detection antibodies (DETECTION). Because of xMAP® multiplexing capabilities, the above 16 reactions can be performed in 4 wells. The best-matched pair can easily be determined, allowing the greatest assay sensitivity.

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 a streptavidin-dye conjugate as the reporter, but detection antibodies may also be directly conjugated to reporter dye, which eliminates the need for a separate reporter labeling step in the assay protocol.

General immunoassay tips

Coupling tips

- Coupling should be allowed to proceed for 2 hours with end-over-end mixing on a rotator.
- For scaling up to 50–600 million microspheres per coupling reaction, couple in 2 mL using a 15 mL polypropylene centrifuge tube or a 4 mL microcentrifuge tube. Place 15 mL centrifuge tubes at a 33–45 degree angle in a tube rack and mix on a plate rotator for the 2-hour coupling incubation.
- After washing, allow microspheres to block overnight in Blocking/Storage Buffer at 4°C in the dark (if possible).
- Too much antibody in coupling reactions may lead to passive adsorption and can manifest as a very high signal initially, with continued decline over time as the antibody becomes detached. Additionally, cross-reactivity can occur if beads are stored as a multiplexed mixture.

Assay tips

- Run at least 2 background samples.
- Run all samples at least in duplicate if sample volume allows.
- Minimize the presence of detergents in samples. Some antibodies may be sensitive to detergents, even at low concentrations (e.g., 0.1% SDS).
- Dilute concentrated biological samples at least 1:5 (final) to overcome matrix (serum) effects that can interfere with analysis of the microspheres. If samples cannot be diluted at least 1:5, try using a small initial reaction volume and diluting the final reaction prior to analysis.

- For capture sandwich and indirect (serological) immunoassays, 2–4 µg/mL detection antibody is usually sufficient.
- Up to 5-fold more detection antibody may be required for a no-wash assay format.
- The optimal detection antibody concentration will depend on specific reagents and the level of multiplexing. Concentrations often need to be increased when increasing the number of multiplex assays and when converting to a no-wash assay format.
- For a competitive immunoassay using antibody-coupled beads (Format 1, see **Chapter 4.3.2**), we recommend testing a range of competing analyte (0.2 to 5 µg) with increasing concentrations of detection antibody. For antigen-coupled beads (Format 2, see **Chapter 4.3.2**), test a range of detection antibody concentrations starting with an excess (e.g., 4 µg/mL) and titrating down by 2-fold serial dilution. The competitor or detection antibody concentration that yields 70–80% of the maximum signal should provide the largest linear dynamic range for the assay.
- For SAPE, the reporter concentration should be approximately 1.5X the concentration of the detection antibody.
- Use PE/SAPE as your reporter 1 (RP1) molecule if possible (it yields the highest signal of all the RP1 dyes we have compared).
- Use Brilliant Violet 421, SuperBright® 436, or StarBright™ Violet 440 as your Reporter 2 (RP2) molecule if possible (in our initial studies, they yielded very robust signals).
- Use either PBS-1%BSA or PBS-TBN as the assay buffer.
- When using SAPE at a >8 µg/mL final concentration in a no-wash assay, a dilution or post-labeling wash step may be required to minimize background fluorescence prior to analysis on the Luminex instrument.

Factors affecting multiplex assays

The assay dynamic range, cross-reactivity, and biological matrix are factors that need to be uniquely and specifically addressed in multiplex assays in order to ensure optimal results. Understanding the biological range of each analyte, the binding specificity of assay reagents, and the unique makeup of your sample (plasma, culture media, urine, etc.) allows you to develop the most effective multiplex assay.

Assay conditions, including the buffer system, blocking agents, sample volume, sample dilution, total reaction volume, number of microspheres per reaction (2,000–5,000 per region per well), concentration of capture reagent for coupling, detection antibody and reporter concentration, assay format (washed vs. unwashed), and incubation times, should be optimized to provide the best results according to the specific assay requirements. The final assay performance should be 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 (final) 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 a multiplex assay. These factors and others are described in more detail below.

Coupling optimization

To improve the sensitivity of an assay, try using a higher affinity antibody for capture and/or detection. Sensitivity may also be improved by decreasing the amount of antigen or antibody coupled to the microspheres, and/or decreasing the detection antibody concentration. The median fluorescent signal will tend to be lower and saturate at a lower analyte concentration, but with improved linearity at lower analyte concentrations.

To increase the median fluorescent signal of an assay, try increasing the amount of antigen or antibody coupled to the microspheres and using the detection antibody at 70–80% saturating concentration. The median fluorescent signal will tend to be higher and saturate at higher analyte concentration, but with less linearity at lower analyte concentrations.

Using a lower affinity antibody for capture and/or detection may also increase the upper limit of detection by extending the linear dynamic range of the assay.

An example of these effects is shown in **Figure 21**.

Figure 21.

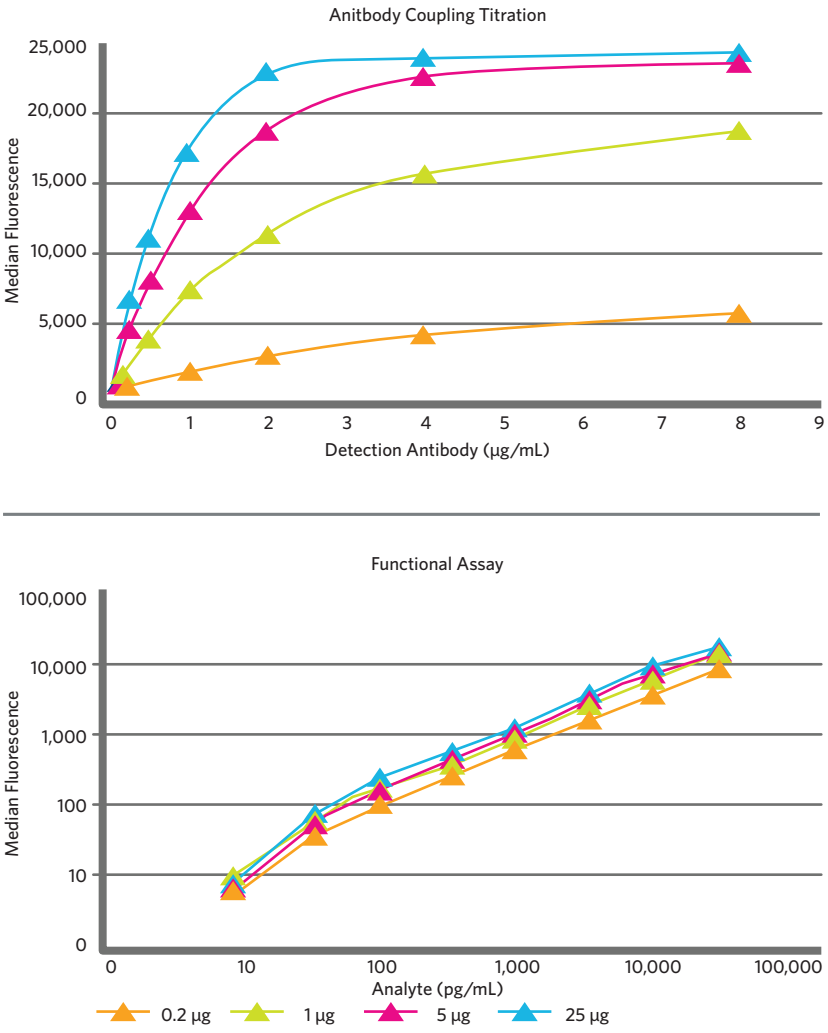


Figure 21 - (**A**) Antibody coupling titration. Capture antibody was coupled to individual microsphere sets at 0.2, 1, 5, and 25 µg/1 million microspheres. Coupling efficiency was assessed using the Coupling Confirmation assay protocol as described in **Protocol 4.2.2**. (**B**) Functional assay. Assay performance was assessed by a standard curve using the Capture Sandwich Immunoassay protocol as described in **Protocol 4.3.1**.

To achieve both maximum sensitivity and a broad dynamic range for an assay, try coupling different amounts of the antigen or antibody (or antibodies of different affinities) to different bead sets and/or using a combination of detection antibodies to generate a multiplexed standard curve. See **Figure 22**.

Figure 22.

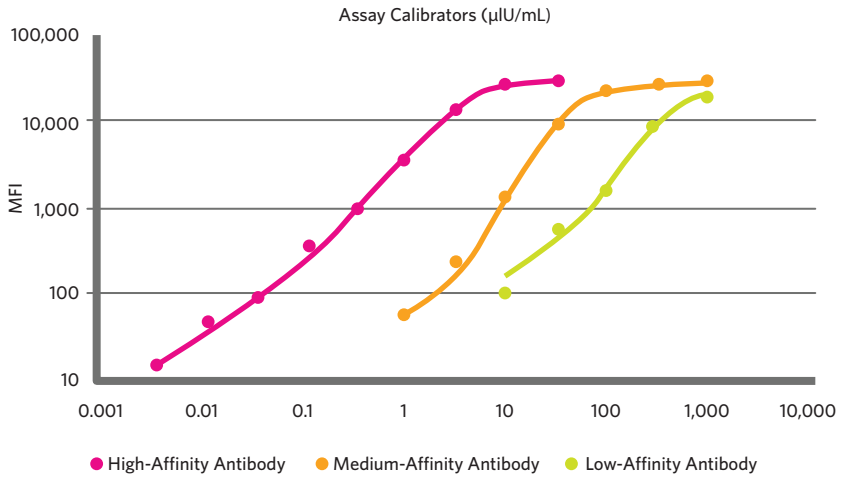


Figure 22 - Capture antibodies of different affinities were coupled to individual bead sets and then combined to generate a multiplexed standard curve.

For multiplexing assays with widely disparate concentration ranges, increasing the number of beads per set or using a weaker reporter for the high concentration analytes can bring these assays into the detectable dynamic range while ensuring a minimal sample dilution to provide optimal sensitivity for the low concentration analytes.

Amount of detection antibody

Multiplexed microspheres should be 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., 2-fold serial dilution from 4 to 1 µg/mL), but generally, 2–4 µg/mL is adequate. Detection antibody concentrations may need to be increased in multiplex as compared to the concentration used in singleplex 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 5-fold to compensate for excess unbound analyte in the supernatant. Typically, reporter fluorophore (e.g., SAPE) concentration should be 1.5–2X the concentration of detection antibody. Final concentrations above 8 µg/mL of SAPE may interfere with the background subtraction performed by the analyzer and thus may require a post-labeling wash step. The optimal reporter concentration should be determined by titration.

Cross-reactivity

Multiplex assays must be tested for various types of cross-reactivity to determine if the reagents are of high enough specificity to yield true positive signals. For example, the following types of cross-reactivity can occur:

- Antigen-capture antibody cross-reactivity
- Detection-capture antibody cross-reactivity
- Antigen-detection antibody cross-reactivity
- Capture antibody-conjugate cross-reactivity
- Antigen-conjugate cross-reactivity

If cross-reactivity between antibodies for different targets is observed, it may be necessary to replace these with other reagents. Selecting other antibodies to be pre-screened during coupling optimization steps may be required. As illustrated in **Figure 23**, multiplex assays should be tested for specificity and cross-reactivity testing:

- Individual analytes with corresponding reporter antibodies (to determine if analytes/antibodies cross-react with non-target beads
- Multiplexed analytes with individual reporter antibodies
- Individual analytes with multiplexed reporter antibodies (to determine if cross-reactivity is due to analytes and/or reporter antibodies)
- Multiplexed analytes and multiplex detection antibodies (to determine sensitivity and to confirm there is no cross-reactivity/interference in the fully multiplexed assay)

Sample cross-reactivity protocol

Figure 23. 3-Plex Cytokine Assay

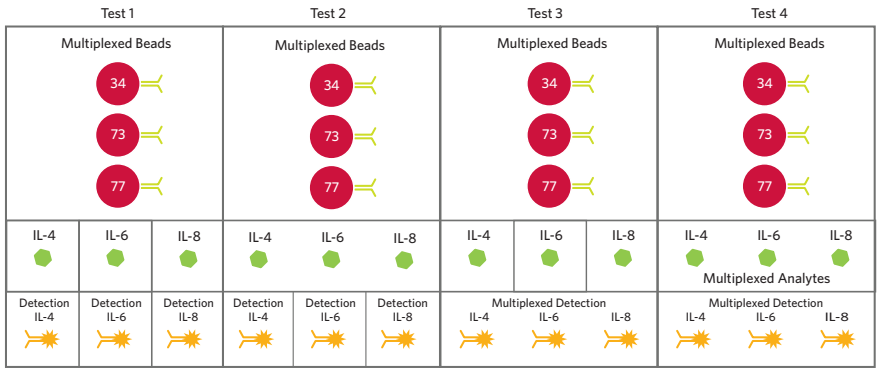


Figure 23 - Immunoassay cross-reactivity protocol. (**Test 1**) Test multiplex capture beads with 3 separate titrations of IL-4, IL-6, and IL-8 using appropriate singleplex reporter antibody for each titration. (**Test 2**) Test multiplex capture beads with multiplexed titrations of IL-4, IL-6, and IL-8 using individual reporter antibodies. (**Test 3**) Test multiplex capture beads with 3 separate titrations of IL-4, IL-6, and IL-8 using multiplexed reporter antibodies. (**Test 4**) Test multiplex capture beads with multiplex titration of IL-4, IL-6, and IL-8 and multiplex reporter antibodies (i.e., the full multiplex reaction).

Test 1 is for individual analytes and corresponding reporter antibodies to determine if there is cross-reactivity with non-target beads. Tests 2 and 3 determine if cross-reactivity is due to binding of non-target reporter antibodies or non-target analytes, respectively. Test 4 is for the fully multiplexed analytes and multiplexed reporter antibodies to determine sensitivity and to assess any interference in the fully multiplexed assay. Each test should be run as a standard curve with a blank and 7 concentrations of analyte as shown in the dilution table on the next page.

Three-fold serial dilution of individual and multiplexed cytokines

Tube	IL-4 (pg/mL)	IL-6 (pg/mL)	IL-8 (pg/mL)	IL-4, IL-6, IL-8 (pg/mL)
1	10,000	10,000	10,000	10,000
2	3,333.3	3,333.3	3,333.3	3,333.3
3	1,111.1	1,111.1	1,111.1	1,111.1
4	370.4	370.4	370.4	370.4
5	123.5	123.5	123.5	123.5
6	41.2	41.2	41.2	41.2
7	13.7	13.7	13.7	13.7

Replace any reagents that show interference, cross-reactivity, or poor performance and determine the optimal sample and reaction volumes, microspheres per reaction (within the range of 2,000–5,000 microspheres per region), incubation times, detection antibody and reporter concentrations, coupling amounts for capture reagents, and assay format (washed vs. homogeneous), then evaluate the performance of the optimized assay with test samples.

The assay results below are specific with <1% cross-reactivity among the cytokine targets. Cross-reactivity was calculated using the net median fluorescent intensity (net MFI) of individual and multiplexed detection antibodies, and capture antibody-coupled beads in the presence of single antigen at the third highest concentration in the standard curve.

Cross-reactivity results: percentage of total signal (net MFI)

Target	IL-4	IL-6	IL-8	IL-4, IL-6, IL-8
IL-4	90	-0.3	-0.2	90
IL-6	0	93	-0.4	93
IL-8	0.1	-0.4	98	98

Sensitivity, limit of detection, precision, and linearity

Several resources are available for guidance in determining the analytical performance characteristics for an assay. The information provided below serves as an example for determining some of those characteristics. Be sure to consult the references appropriate for your laboratory and assay to ensure all required parameters have been incorporated into your study design.

The working assay range will need to be determined during assay development. Limit of blank (LoB), limit of detection (LoD), and limit of quantitation (LoQ) measurements are used to determine the smallest concentration that can be measured by an analytical procedure.

- LoB: The LoB is the highest measurement result that is likely to be observed (with a stated probability [alpha]) for a blank sample.
- LoD: The LoD is the smallest quantity of an analyte that can be reproducibly and statistically distinguished from the background (including variation in background), or a zero calibrator in a given assay system.

- LoQ: The LoQ is the lowest amount of analyte in a sample that can be quantitatively determined with stated acceptable precision and trueness, under stated experimental conditions.
- The lower limit of quantitation (LLoQ) is the lowest analyte concentration that can be quantified with acceptable precision and accuracy.
- The upper limit of quantitation (ULoQ) is the highest analyte concentration that can be quantified with acceptable precision and accuracy.

Targets (3-Plex Assay)	LLoQ (pg/mL)	ULoQ (pg/mL)	LoD (pg/mL)	Intra-assay (%CV)	Inter-assay (%CV)
IL-4	3.1	7,394	0.8	5	4
IL-6	3.4	1,958	0.8	6	11
IL-8	24.1	3,066	7	6	4

The assay results (above) are sensitive, accurate, and precise. The LLoQ and ULoQ values define the working assay range—accuracy (80–120% recovery) and precision (<10% intra-assay CV). The LoD is defined here as the lowest measurable concentration obtained from the MFI of 3 replicate wells for 8 standard points. The inter-assay %CV is measured from the CV of observed concentrations of 8 standard points for 3 independent assays.

Precision

Precision is the closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions. Precision is determined by assessing the repeatability and reproducibility of the assay.

Intra-assay and inter-assay repeatability, as well as run-to-run and site-to-site reproducibility, should be determined in the appropriate sample matrix and diluent (i.e., simulated, spiked samples). NOTE: Intra/inter-assay CV may vary between site, user, and assay. The following recommendations can be used as a guideline:

- **Intra-assay CV <10% is usually acceptable within the working range of the assay.** A minimum of 3 separate runs should be performed to determine the intra-assay precision. The intra-assay precision should be calculated from a minimum of 2 replicates at each of 2 spiked concentrations.
- **Inter-assay CV <20% is usually acceptable within the working range of the assay.** A minimum of 5 separate runs should be performed to determine inter-assay precision.

Linearity

A linear relationship should also be evaluated across the range of the assay. The linearity of dilution provides confidence that the analytes present are within the assay range and that assay values are directly proportional to the concentration of the analyte in the expected sample type(s) or matrix.

Linearity of Sample Dilutions (R^2)

Matrix	IL-4	IL-6	IL-8
Plasma	0.9999	0.9999	0.9962
Serum	0.9958	0.9995	0.9986
Cell Culture	0.9998	0.9996	0.9955

The R^2 values were determined by linear regression analysis of analytes measured in a 3-fold serial dilution of standard 'spiked' samples within assay range in 3 matrices.

Parallelism is related to dilutional linearity. That is, if the sample is diluted and the standards are diluted in the same manner, the results from the standard curves should follow the same pattern as the dilutions, or be 'parallel.' Note that sample matrices may affect linearity and parallelism and should be considered in the study design.

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Binding kinetics and assay sensitivity

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 analytical sensitivity. Though seemingly paradoxical, improved sensitivity can sometimes be 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 (**Figure 21A**).

Figure 24.

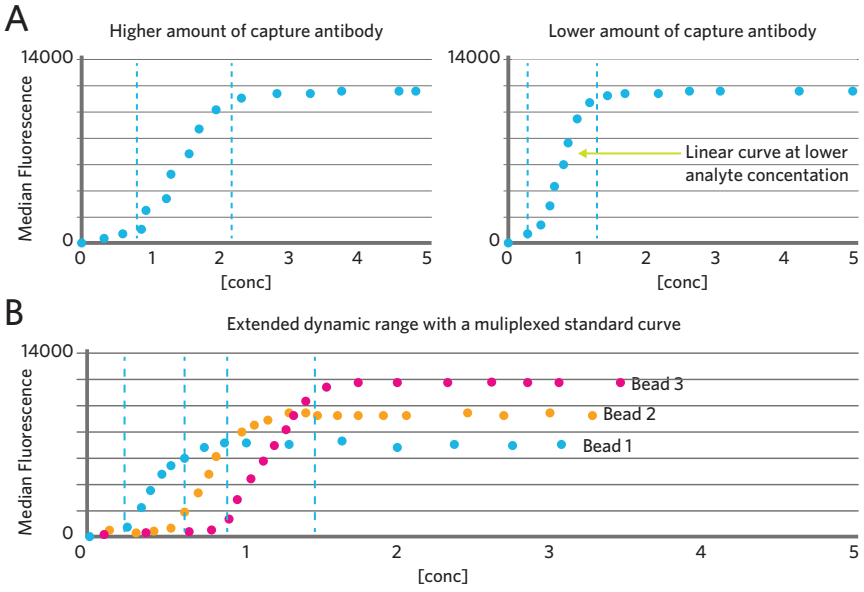

Figure 24 – Dynamic range and assay sensitivity may be affected by (**A**) reducing the amount of capture antibody on the bead to make lower concentration range more linear, and (**B**) by using different amounts of capture antibody (or antibodies with different affinities) on multiple bead sets to form a multiplexed standard curve.

Antibody affinity and sensitivity

Antibodies with higher affinity can also improve analytical 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. 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 (**Figure 21B**). The same effect can be accomplished by coupling different concentrations of the same capture reagent to different microsphere color sets.

Matrix effects

Highly concentrated serum or plasma can lead to 'matrix effects' presenting as poor bead recovery, instrumentation clogging, low signals, and variable results. Matrix effects can play a major role in assay performance and the type of sample tested may therefore have effects on assay performance. Labs developing immunoassays should include replicates of samples, as well as negative and positive (low, medium, and high) controls with known concentrations of the analytes of interest to aid in the interpretation of results. Dilutions of controls should be included that reflect the diluents used to reconstitute the standards and the sample matrix tested in order to account for possible matrix effects. This will allow the assessment of linearity, precision, and recovery and will aid in the choice of best standard curve regression and optimal calibration. An interference study may help in determining the effects of potentially interfering substances.[1] Luminex recommends that plasma and serum be diluted at least 1:5. If samples

1. EP7-A2: Interference Testing in Clinical Chemistry; Approved Guideline–Second Edition (Clinical Laboratory Standards Institute, www.clsi.org).

cannot be diluted at least 1:5, try using a small initial reaction volume and diluting the final reaction prior to analysis on your Luminex instrument. In addition, if non-specificity remains after diluting the serum samples, try adding additional blockers to the assay buffers. If BSA is not helping or could interfere with the assay, you may opt for albumin from another species (porcine), milk casein, ChemiBLOCKER™, StabilGuard®, Prionex®, etc., or switch to a washed assay format if using a no-wash.

Washed versus no-wash assays

Conversion of a washed assay to a no-wash assay format can reduce hands-on time as well as decrease total assay time. To convert to a no-wash 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.

Instrument settings and reporter fluorescence

When developing a multiplex assay, optimization of assay performance and meeting requirements for sensitivity, dynamic range, ease of use, and time to results should be kept in mind. There are many factors to consider to improve sensitivity or increase signal, including adjusting the PMT setting on Luminex® 200™ and FLEXMAP 3D® instruments, selecting different vendors or types of reporter, incorporating dendrimers or other signal amplification techniques, or additional reporter labeling steps.

Other reagent optimization

Finally, the fully multiplexed assay is performed to determine sensitivity and interference when all analytes and reagents are present in the reaction. Multiplex assay development can be an iterative process, requiring further optimization as complex interactions between assay components are observed. Assay conditions, such as the buffer system, blocking agents, sample volume and dilution, total reaction volume, number of microspheres per reaction (2,000–5,000 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.

Using dried blood spot samples in xMAP assays

Dried blood spot testing (DBS) is a form of sampling where a biological sample, such as blood, is blotted and dried on filter paper. The method was first described by Ivar Christian Band in 1913 when he measured glucose from dried blood spots eluted from cellulose paper. The concept that capillary blood, obtained from pricking the heel or finger and blotting it onto filter paper, could be used to screen for metabolic diseases in neonates was introduced by Robert Guthrie in 1963.

Since then, blood spot cards (also known as Guthrie cards) have been routinely collected from infants across the world to screen for congenital and infectious diseases. Dried samples can be easily shipped to an analytical laboratory for serological testing, cytokine and hormone measurements, and other tests. Despite their advantages, limitations in sensitivity and specificity from screening such small volumes of blood restricted the use of dried blood spots for many years. Now, this issue can easily be overcome using xMAP Technology.

Materials Needed

Reagents and Consumables	Vendor
Dried blood spot cards	Any suitable source
Protease inhibitor cocktail	Roche 04693116001, or equivalent
96-well flat bottom plate	Any suitable brand
Blood spot card puncher (manual or automated)	Any suitable source
PBS, 0.05% Tween® 20, pH 7.4	MilliporeSigma P3563

Sample processing

1. Spot the sample onto the card according to the manufacturer's instructions.
2. Dry the sample at room temperature for 2–24 hours or, if needed, samples can be inactivated by incubating at 56°C for 1 hour.
3. Seal the dried sample card in a plastic bag and store at -20°C until further use.

Extraction buffer

PBS, 0.05% Tween® 20, pH 7.4, supplemented with a protease inhibitor cocktail

Extraction of DBS samples

1. Visually inspect the card to verify the spots were correctly filled.
2. Harvest 1–2 filter disks (3–8 mm in diameter) by manually punching or using an automated dried blood spot puncher. (Note: clean the punching device between samples with water followed by 70% ethanol or isopropanol to prevent cross-contamination between samples.)
3. Place the punched spots into the appropriate wells of a flat bottom 96-well plate (any suitable brand).
4. Add 100–200 µL extraction buffer and incubate at room temperature 1–4 hours with continuous shaking at 200 rpm.
5. Centrifuge the plate for 3 minutes at 3,000 rpm.
6. Retrieve the supernatant eluate by pipette for direct use in your xMAP assay, or dilute further using an assay buffer (such as PBS with 0.05% Tween-20 and 1–3% BSA) as needed.

Extracted DBS samples can be used in a variety of xMAP assays, including capture sandwich immunoassays (**Chapter 4.3.1**), indirect (serological) immunoassays (**Chapter 4.3.3**), and mutation analysis via nucleic acid assays (**Chapter 5**).

Note: Cellulose-based collection media release small fibers of cellulose from the filter paper during sample extraction. These fibers are small enough to be introduced into the optics of the system during sample acquisition and can lead to clogs in the cuvette (flow analyzers) or imaging chamber (MAGPIX®). After each run using DBS samples, clean your xMAP analyzer using either the appropriate Stringent Clean routine or the post-cellulase treatment Flushing Routine, as described below. Also be sure to clean and sonicate the sample probe, and perform the maintenance procedures as recommended for your xMAP analyzer.

Stringent Clean routine

1. Fill the appropriate reservoir with freshly prepared 0.1N NaOH and run the Sanitize command 2 times.
2. Fill the appropriate reservoir with freshly prepared 10–20% bleach and run the Sanitize command 2 times.
3. Run the Backflush command.
4. Run the Drain command.
5. Repeat steps 3–4 two additional times.
6. Fill the appropriate reservoir with 70% ethanol or isopropanol and run the Alcohol Flush command 2 times.
7. Fill the appropriate reservoir with deionized H2O and run the Rinse command 3 times.

Then, once per week (or more often if needed), we recommend running the Cellulase Cleaning procedure. Cellulase achieves enzymatic hydrolysis of cellulose fibers into soluble saccharides in aqueous solutions, enabling it to be flushed out of your xMAP System.

Cellulase solution, 100 mL

Reagent	Source	Amount
Citric acid monohydrate	MilliporeSigma C1909	1.1 g
ProClin™ 300	MilliporeSigma 48912-U	53.2 µL
Cellulase from Aspergilllus spp.	MilliporeSigma C2605	6.4 mL
Cellulase from Trichoderma reesei ATCC26921	MilliporeSigma C8546	63.8 mg
5N Sodium hydroxide – to adjust pH to 4.5	MilliporeSigma S5881	~1.5 mL
dH20	Any suitable source	QS to 100 mL

Cellulase cleaning protocol

1. Load a full 96-well plate with 125 µL Cellulase Solution per well.
2. Turn on the heater on your xMAP instrument and heat to 50°C.
3. Acquire the full plate with an acquisition volume of 50 µL and Time Out set to 30 seconds.
4. Remove the plate and turn off the heater.
5. Allow the instrument to cool to ambient temperature.
6. Run the Cellulose Flushing Routine as described below.

Cellulase flushing routine

1. Fill the appropriate reservoir with 0.1N NaOH and run the Sanitize command 2 times.
2. Fill the appropriate reservoir with 10% bleach and run the Sanitize command 2 times.
3. Run the Backflush command.
4. Run the Drain command.
5. Repeat steps 3–4 two additional times.
6. Fill the appropriate reservoir with 0.1N NaOH and run the Sanitize command 2 times.
7. Run the Backflush command.
8. Run the Drain command.
9. Repeat steps 3–4 two additional times.
10. Fill the appropriate reservoir with 70% ethanol or isopropanol and run the Alcohol Flush command 2 times.
11. Fill the appropriate reservoir with deionized H2O and run the Rinse command 4 times.

Further reading

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Chapter 4.6

Immunoassay Validation

After confirming successful coupling and sufficient signal from a multiplex assay, microspheres should be further tested with standard or control materials. These are often recombinant proteins as known positive and negative samples. Protein samples should be prepared in the appropriate sample matrix to match the composition of the test samples as closely as possible.

Validation of xMAP® immunoassays is described in detail in multiple publications.[1-7] The parameters you select and optimize will be determined by your particular research needs. The National Institutes of Health Chemical Genomics Center is a good resource for general immunoassay validation (ncbi.nlm.nih.gov/books/NBK92434). The Clinical and Laboratory Standards Institute (clsi.org) is also a good source for consensus standards and guidelines for laboratories (clsi.org/standards). There are also publications that give "fit-for-purpose" guidelines for validating assays.[8,9] The information described here is intended to serve as an example to immunoassay validation. Be sure to consult the references appropriate for your laboratory and assay to ensure all required parameters have been considered in your validation protocol.

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Spike-and-recovery immunoassay sample validation protocol

Spike-and-recovery and linearity-of-dilution experiments are methods for validating and assessing the accuracy analytical techniques (such as ELISA) for particular sample types. Spike-and-recovery is used to determine whether analyte detection is affected by differences between the diluent used to prepare the standard curve and the biological sample matrix. Linearity of dilution refers to the predictability of spiked or natural sample recovery for known dilution factors in the desired assay range. Performing spike-and-recovery and linearity testing on biological samples helps show the accuracy of the reported values from these samples.

A. Performing a spike-and-recovery experiment

To perform a spike-and-recovery experiment, a known amount of analyte is added to the sample matrix and the standard diluent, and the two sets of responses are compared based on values calculated from a standard curve. If the recovery observed for the spiked sample ("spike") is identical to the recovery obtained in standard diluent, the sample matrix is considered valid for the assay procedure. If the recovery differs, then components in the sample matrix are affecting the recovery and adjustments must be made to minimize the discrepancy.

B. Performing a linearity-of-dilution experiment

There are two ways to perform a linearity-of-dilution experiment. The usual method involves using either a low-level sample containing a known amount of spiked analyte or a high-level sample without spike and then testing several different dilutions of that sample in the chosen sample diluent. An alternative method involves preparing several different dilutions of a low level sample first and then spiking the same known amount of analyte into each one before testing. Assay recovery is assessed by comparing observed vs. expected values based on non-spiked and/or neat (undiluted) samples. If a sample does not exhibit linear dilution, this indicates that a sample component is interfering with detection of the analyte at a given dilution. It is important also to find the linear dilution range for an unvalidated sample type, as this will allow the comparison of sample values from samples run at different dilutions.

The plate could be set up as follows:

	1	2	3	4	5	6
A	Std 1		Neat Spiked sample		Control spike	
B	Std 2		1:2 Spiked sample		1:2 Control spike	
C	Std 3		1:4 Spiked sample		1:4 Control spike	
D	Std 4		1:8 Spiked sample		1:8 Control spike	
E	Std 5		Neat sample (unspiked)			
F	Std 6		1:2 Sample (unspiked)			
G	Std 7		1:4 Sample (unspiked)			
H	Blank		1:8 Sample (unspiked)			

- **Spiked sample**: A sample to which known concentrations of specific analytes have been added in such a manner as to minimize the change in the matrix of the original sample.
- **Neat sample**: A sample that is not diluted with any diluent.
- **Control spike sample**: A control spike sample is a known matrix spiked with compound(s) representative of the target analyte(s). This is used to show that the sample preparation procedure does not contribute to loss of analytes.

Calculations:

1. Spike/Recovery

$$\% \text{ Recovery} = \frac{\text{Observed - Neat}}{\text{Expected}} \times 100$$

Observed = Spiked sample value
Neat = Sample (unspiked) value
Expected= Amount spiked into sample

Note: Recovery should be in the range of 80–120%. The control spike should have a recovery value within 80–120%. If not, this indictaes a problem in preparation of control spike.

2. Linearity

Use the spiked sample value as the expected value if testing linearity of the spiked sample. Use the neat sample value as the expected value if testing linearity of the unspiked sample.

$$\% \text{ Recovery of 1:2} = \frac{\text{Observed value (pg/mL) of 1:2 dilution}}{\text{Expected value (pg/mL) divided by 2}} \times 100$$

$$\% \text{ Recovery of 1:4} = \frac{\text{Observed value (pg/mL) of 1:4 dilution}}{\text{Expected value (pg/mL) divided by 4}} \times 100$$

$$\% \text{ Recovery of 1:8} = \frac{\text{Observed value (pg/mL) of 1:8 dilution}}{\text{Expected value (pg/mL) divided by 8}} \times 100$$

Note: Recovery of spiked and neat samples should be in the range of 80–120%. Diluting the control spike is a good control for serial dilutions. Recovery for the control spike should be in the range of 80–120%. If not, this indicates a problem in preparation of the control spike dilutions.

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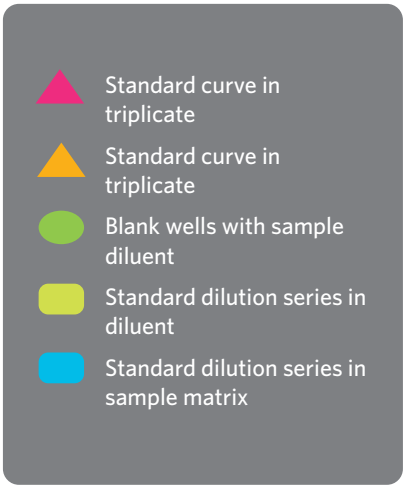
Design of assay validation

Typical Performance Parameters

Limit of Detection	≤1 pg/mL
Precision Intra-assay CV Inter-assay CV	 <10% ≤15%
Accuracy (% recovery)	80–120%
Cross-reactivity	<1%
Working Assay Range	Varies from target-to-target
Matrices	Plasma, serum, culture supernatant, lysates, other biological fluids

Sample Plate Layout

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



Above is a general plate layout for assay validation. Columns 1–3 and 10–12 are standards, ideally a 7- or 8-point standard curve in standard diluent. The data from these 6 standard curves will be used to determine standard curve recovery, intra-assay CV, LLOQ, and ULOQ. The wells marked with a B are blank wells, i.e., assays run only with sample diluent. The average of these 12 should be used to determine background levels. In columns 4–6, samples 1–6 are standard dilution series run in standard diluent to determine spike control recovery and linearity of dilution. In columns 7–9, samples 1–6 are standard diluted in sample matrix to determine sample spike recovery and inter-assay CV, although a minimum of 3 plates needs to be run for inter-assay CV. The sample matrix should be depleted of target proteins, i.e., use depleted serum or plasma.

Immunoassay validation references

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- Kofoed K, Schneider U, Scheel T, et al. Development and validation of a multiplex add-on assay for sepsis biomarkers using xMAP® technology. Clin Chem. 2006;52(7):1284-93.
- Lal G, Balmer P, Stanford E, et al. Development and validation of a nonaplex assay for the simultaneous quantitation of antibodies to nine *Streptococcus pneumoniae* serotypes. J Immunol Methods. 2005;296(1-2)135-147.
- Lee JW, Devanaryan V, Barrett YC, et al. Fit-for-purpose method development and validation for successful biomarker measurement. Pharm Res. 2006;23(2):312-28.
- Lee JW and Hall M. Method validation of protein biomarkers in support of drug development or clinical diagnosis/prognosis. J Chromatogr B Analyt Technol Biomed Life Sci. 2009;877:1259-71.
- Cox K, Devanarayan V, Kriauciunas JM, et al. Immunoassay Methods. Assay Guidance Manual (Internet). 2012; Updated 2019. PMID: 22553884.
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- Ravindran R, Khan I, Krishnan V, et al. Validation of multiplex microbead immunoassay for simultaneous serodetection of multiple infectious agents in laboratory mouse. J Immunol Methods. 2010;363(1):51-9.
- van Gageldonk P, van Schaijk F, van der K, et al. Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to *Bordetella pertussis,* diphtheria and tetanus. J Immunol Methods. 2008;335 (1-2):79-89.
- Wagner B, Freer H, Rollins A, et al. Development of a multiplex assay for the detection of antibodies to *Borrelia burgdorferi* in horses and its validation using Bayesian and conventional statistical methods. Vet Immunol Immunopathol. 2011;144(3-4):374-81.

Chapter 4.7

Proteomics FAQs

For immunoassays, should you couple a polyclonal or monoclonal antibody to the microspheres?

Monoclonal antibodies are recommended because of their specificity. Polyclonal antibodies can be used after an affinity purification step. If options are limited, use what is available and check for possible cross-reactivity with other analytes in the sample. The success of the assay with the desired sensitivity and specificity will depend on the quality of the reagents.

Can I measure protein-protein interactions on Luminex microspheres?

Yes, however, protein-protein interaction applications can be difficult for proteins that have low binding affinity. See additional references for protein-protein interactions:

- Bida AT, Gil D, and Schrum AG. Multiplex IP-FCM (immunoprecipitation-flow cytometry): Principles and guidelines for assessing physiologic protein-protein interactions in multiprotein complexes. Methods. 2012 Feb;56(2):154-60.
- Smith SE, Bida AT, Davis TR, et al. IP-FCM measures physiologic protein-protein interactions modulated by signal transduction and small-molecule drug inhibition. PLoS One. 2012;7(9):e45722.

Does Luminex recommend sources for antibody pairs?

Luminex has used several sources, including Bio-Techne (R&D Systems), BD Pharmingen, Jackson ImmunoResearch Laboratories, Inc, Rockland Immunochemincals, OEM, and Fitzgerald Industries but we recommend that you consult your preferred vendor. OriGene has validated antibody pairs for Luminex assays (see origene.com/products/antibodies/primary-antibodies/luminex-antibody-pairs). Quality and purity are of utmost importance. Manufacturers of ELISA kits often sell matched pairs that are easily transferable to microspheres. Examples include, but are not limited to DuoSets® from Bio-Techne and eBioscience. Additionally, several publications list the source, catalog number, and clone number for their antibodies used. Many of the assays built in these publications are common, so using these as a starting point can help you save time (see below for antibody pair references).

Websites such as the Antibody Directory (antibodydirectory.com), Antibodies Online (antibodies-online.com), and Antibody Resource (antibodyresource.com) are good resources for searching antibody suppliers. The Antibody Search Engine (citeab.com) offers the ability to browse antibodies by Luminex application. Other websites such as linscottsdirectory.com and biocompare.com may also be good sources for antibody selection. When choosing raw materials (antibodies and recombinant proteins), select vendors that have rigorous quality control

procedures and provide as much information as possible about the antibodies or proteins. Request that the vendor provide purity information from SDS- and/or non-denaturing-PAGE. Also, request profiles of the antibody from capillary isoelectric focusing to compare lots from the same vendor. Luminex recommends that you devise your own incoming materials quality control procedure to compare lots from antibody suppliers. See other references for sources for antibody pairs:

- Bjerre M, Hansen TK, Flyvbjerg A, Tonnesen E. Simultaneous detection of porcine cytokines by multiplex analysis: Development of magnetic Bioplex assay. Vet Immunol Immunopathol. 2009;130:53-8.
- Carson RT, Vignali DAA. Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. J Immunol Methods. 1999;227:41-52.
- de Jager W, te Velthuis H, Prakken BJ, et al. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol. 2003;10:133-9.
- de Jager W, Prakken BJ, Bijlsma JWJ, et al. Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. J Immunol Methods. 2005;300:124-35.
- de Jager W, Bourcier K, Rijkers GT, et al. Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. BMC Immunol. 2009;10:U1-U11.
- Dernfalk J, Waller KP, Johannisson A. Commercially available antibodies to human tumour necrosis factor-alpha tested for cross-reactivity with ovine and bovine tumour necrosis factor-alpha using flow cytometric assays. Acta Vet Scand. 2004;45:99-107.
- Dernfalk J, Waller KP, Johannisson A. The xMAP® technique can be used for detection of the inflammatory cytokines IL-1a, IL-6 and TNF-alpha bovine samples. Vet Immunol Immunopathol. 2007; 118:40-9.
- Faucher S, Crawley AM, Decker W, et. al. Development of a quantitative microsphere capture assay for soluble IL-7 receptor alpha in human plasma. PLoS One. 2009;4:U66-U71.
- Lawson S, Lunney J, Zuckermann F, et. al. Development of an 8-plex Luminex assay to detect swine cytokines for vaccine development: Assessment of immunity after porcine reproductive and respiratory syndrome virus (PRRSV) vaccination. Vaccine. 2010;28:5356-64.
- Ray CA, Bowsher RR, Smith WC, et. al. Development, validation, and implementation of a multiplex immunoassay for the simultaneous determination of five cytokines in human serum. J Pharm Biomed Anal. 2005;36:1037-44.
- Rizzi G, Zhang YJ, Latek R, et al. Characterization and development of a Luminex-based assay for the detection of human IL-23. Bioanalysis. 2010;2:1561-72.
- Skogstrand K, Thorsen P, Norgaard-Pedersen B, Schendel, et. al. Simultaneous measurement of 25 inflammatory markers and neurotrophinsin neonatal dried blood spots by immunoassay with xMAP technology. Clin Chem. 2005;51:1854-66.
- Wood B, O'Halloran K, VandeWoude S. Development and validation of a multiplex microsphere-based assay for detection of domestic cat (Felis catus) cytokines. Clin Vaccine Immunol. 2011,doi:10.1128/CVI.00289-10.

Does Luminex recommend specific assay controls?

Many customers have successfully incorporated commercially available assay controls like the AssayCheX® Process Control Panel from Radix Biosolutions in their xMAP® assays. Luminex MagPlex® Monitoring Microspheres are designed to assist with assay development and make it possible for users to monitor RP1 and RP2 reporter values and reporter channel performance well-by-well.

Should peptides be synthesized with a linker?

Putting the linker on the beads makes peptide synthesis easier and cheaper. Adding a linker with a terminal amine on the peptide for coupling to carboxylated microspheres is a suitable alternative.

Does an assay involving peptides require alternate assay buffers?

Buffers used in peptide-based assays will depend on the assay format. If assaying for peptide-specific antibodies, then a normal immunoassay buffer should suffice (e.g., PBS-BSA). When using peptides to measure enzymatic reactions, a buffer optimal for that assay would be required.

Do you have any recommendations for labeling proteins?

Biotin labeling can be performed using the EZ-Link™ Sulfo-sNHS-LC-Biotin labeling kit from Thermo Fisher (piercenet.com/product/ez-link-sulfo-nhs-lc-biotin-biotinylation-kits). Reagents may be ordered directly labeled with reporter fluorophores. We have had success labeling proteins with phycoerythrin using PhycoLink® kits from Agilent (ProZyme®, agilent.com) and the Moss PE-IgG Conjugation Kit (mosssubstrates.com).

Can polysaccharides be coupled to microspheres?

For information regarding the coupling of polysaccharides to microspheres, please refer to the following publications:

- Pickering JW, Martins TB, Greer RW, et al. A multiplexed fluorescent microsphere immunoassay for antibodies to pneumococcal capsular polysaccharides. Am J Clin Pathol. 2002;117:589-596.
- Biagini RE, Schlottmann SA, Sammons DL, et al. Method for simultaneous measurement of antibodies to 23 pneumococcal capsular polysaccharides. Clin Diagn Lab Immunol. 2003;10:744-750.
- Schlottmann S, Jain N, Chirmule N, Esser M. A novel chemistry for conjugating pneumococcal polysaccharides to Luminex microspheres. J Immunol Methods. 2006;309:75-85.

Are there alternatives to BSA for blocking?

Any non-specific protein can be used for blocking, including many of the commercially available blocking buffers. Bovine serum albumin (BSA) is a common blocking agent. If BSA is insufficient or interfers with the assay, you

may opt for albumin from another species (e.g. porcine, goat, etc.), milk casein, ChemiBLOCKER™, StabilGuard®, Prionex®, fish, or porcine collagen, etc., or switch to a washed assay format if using a no-wash. **Do not use unpurified blockers such as non-fat milk.**

Can xMAP® be used for enzyme activity assays?

Yes, substrates can be coupled to the beads and labeled. Enzyme activity is typically detected using an antibody either to the substrate or to a final product on the bead, monitored by an increase or decrease in MFI. For an example, see:

- Albrecht A, Rahmoune H, Leedjaerv K, et al. Development of a novel assay for proprotein converting enzyme activity on a multiplex bead-based array system. Proteomics. 2013;13(20):2976-2979.

Can streptavidin-conjugated reporter dyes be used in assays when the samples are in tissue culture media?

Tissue culture generally contains a large amount of biotin. The streptavidin-reporter will bind to the biotin in the media and not onto the detection antibody. Here are some suggestions for a no-wash format with tissue culture samples:

- Use a detection antibody that is directly labeled with reporter dye.
- Pre-combine the detection antibody and the streptavidin-reporter (30–60 minutes) before addition to the sample. By doing this, the detection antibody and reporter can bind first before the free biotin can interfere.

How many PE molecules equal 1 MFI?

Based on experiments using PE standards, the approximate molecules of PE per MFI is:

- For Luminex® 200™, 23 PE/MFI at standard (low) PMT; 5 PE/MFI at high PMT.
- For FLEXMAP 3D®, 15 PE/MFI at standard (low) PMT; 3 PE/MFI at high PMT.
- For MAGPIX®, 23 PE/MFI.
- For xMAP INTELLIFLEX®, 0.2 PE/MFI in High Sensitivity mode. All other modes as described for their counterparts above.

If you have multiple instruments or collaborator(s) with different instruments, be sure to optimize on all instrument types as the raw MFI results may differ between instruments.

How many reporter molecules equal 1 MFI in the second reporter channel?

Preliminary experiments using Streptavidin-SuperBright® 436 (Thermo Fisher) with biotinylated beads have indicated there are approximately 40 molecules/MFI.

What third-party data processing tools does Luminex recommend?

- Bio-Rad - BioPlex® Manager™ bio-rad.com/en-us/category/bio-plex-software?ID=45938d9d-c2ec-4ae4-9ed3-e7358a98d30b
- Microsoft Excel®
- MilliporeSigma Belysa™ Analysis Software sigmaaldrich.com/life-science/immunoassay-platform-solutions/curve-fitting-software-download.html
- Systat Software Sigma Plot®

How do samples containing biological fluids perform on Luminex instruments?

As long as the reagents function in the biological matrix, the assay should work on xMAP Technology with the proper optimization. A 1:5 dilution is a recommended starting point for samples containing serum or plasma. Urine, CSF, and synovial fluid may be run without dilution following centrifugation or filtration. Assays can be performed in undiluted biological fluids and then diluted before running on the Luminex platform. Often, routine assay processing includes a sufficient dilution with the addition of the microspheres, detection antibody, and fluorescent reporter. Assays may not be as sensitive in biological fluids as they are in buffer because the fluids are less purified and may contain interferents. When running biological fluids, remember to sanitize with 10–20% bleach and wash and soak with distilled water at the end of the day to prevent clogging. Also use the stringent cleaning routine (with 0.1–0.2N NaOH) daily to weekly as needed to minimize clogs.

What signal is considered a 'positive' signal?

A general recommendation is that a positive signal should be at least 2 to 3 standard deviations (SD) above background (or the negative control). Similarly, a signal-to-noise ratio (SNR) of 2 to 3 may be considered a positive result. Appropriate cutoff values for positive MFI should be determined for each assay by running known positive and negative samples. Typically, a confidence interval (i.e., 95% confidence interval) is used to determine the reliability of the cutoff value.

Which buffers/solvents are compatible with the Luminex system?

During the coupling procedure, avoid buffers that contain free amines that might interfere with the coupling to the COOH sites on the beads. As the salt concentration of the assay buffer increases, the classification of the beads in the flow analyzers may be effected causing the beads to spread out of the region (i.e., SSC buffer). Also avoid organic solvents as they will cause the internal classification dyes of the beads to leach out, causing misclassification. See **Appendix A** for a list of incompatible buffers.

Can formaldehyde be used to stop reactions on Luminex microspheres?

Stop solutions are generally not needed, particularly for washed assays. Assays can be held for several hours prior to analysis if a final wash is performed to remove unbound reporter and the reactions (resuspended in assay buffer) are held refrigerated and protected from light. Formaldehyde or paraformaldehyde can usually be used to stop reactions in microsphere assays. The final concentration should be less than 1%. We recommend testing it with a small sample of beads coupled to reagents before incorporating it fully into the procedure.

How much should you increase the amount of reporter when converting from singleplex to multiplex?

Use the cumulative concentration of the singleplex assays, generally 1.5X the detection antibody concentration (based on experience with SAPE). There is no prescribed ratio of biotin to streptavidin-reporter concentration because the extent of biotinylation of detection antibodies is usually not known. Titration is recommended. More streptavidin-reporter must be added for no-wash than for washed assays to compensate for unbound biotin in the reaction.

What does it mean when there is a high RP1 peak in the zero bin of the histogram?

This may occur in no-wash assays where the RP1 signal on the bead is less than the background signal. The background subtract algorithm of the software takes a background reading before and after each bead. If the amount of fluorescence present in solution is greater than the fluorescence on the beads themselves, zero values will be reported. This can be corrected by reducing the amount of reporter dye or washing samples before running them.

Can the Luminex platform analyze whole cell assays?

The xMAP platform is not designed to analyze whole cells, especially eukaryotic cells, which are much larger than xMAP microspheres. However, xMAP Technology has been used for capture of bacterial cells. If you run cell-based assays on MAGPIX, for example, there is a possibility of a biofilm layer forming in the imaging cell. Routine cleaning with 0.1 N NaOH is a necessity in order to minimize bacterial growth. These papers describe the capture whole bacterial cells:

- Stroot JM, Leach KM, Stroot PG, Lim DV. Capture antibody targeted fluorescence in situ hybridization (CAT-FISH): Dual labeling allows for increased specificity in complex samples. J Microbiol Methods. 2012;88(2):275-84.
- Clotilde L, Bernard C, Hartman G, et al. Microbead-based immunoassay for simultaneous detection of Shiga toxins and isolation of *Escherichia coli* O157 in foods. J Food Prot. 2011;74(3):373-9.
- Clotilde LM, Bernard C, Salvador A, et al. A 7-plex microbead-based immunoassay for serotyping Shiga toxin-producing *Escherichia coli.* J Microbiol Methods. 2013;92(2):226-30.

Cell lysates can be analyzed as long as the viscosity of the sample is sufficiently reduced before aspirating into the analyzer. This must be determined empirically

by the user. The user needs to be aware of potential shifting of the beads out of their regions due to composition differences between the sample core and sheath fluid in the flow analyzers.

How many events should be collected per analyte?

50–100 events is sufficient to obtain accurate results. For a detailed study on the effect of counting bead events and assay results, see:

- Jacobson J, Oliver K, Weiss C, Kettman J. Analysis of individual data from bead-based assays ('bead arrays'). Cytometry A. 2006;69(5):384-90.

Carson and Vignali (1999) concluded, "The data derived from as few as 100 beads per cytokine assay was sufficient to obtain accurate results. While all subsequent data presented here represent the collection of approximately 100 events per cytokine per sample, comparable results from fewer events may be possible."

- Carson RT, Vignali DA. Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. J Immunol Methods. 1999;227(1-2):41-52.

Several studies (not published) have concluded that a minimum of 35 events is required to obtain a statistically valid median result. Customers may want to carry out a similar study to determine the optimal number of events to collect. In determining how many microsphere events to collect in your sample, keep in mind that the output of the assay is median fluorescence, which is determined from the sampled microspheres.

Is there an alternative to washing microspheres with dH_2O prior to activation?

While Luminex recommends washing microspheres with dH_2O prior to activation with sulfo-NHS and EDC, customers can choose to wash microspheres with MES, pH 6.0.

Can I multiplex assays when the analytes are found in widely different biological concentrations (e.g., µg/mL vs. pg/mL)?

Low concentration analytes require the sample to be diluted as little as possible (1:5 or less) in the initial incubation step. This can cause high concentration analytes to be outside of the dynamic range of the system. To multiplex assays for analytes with widely disparate concentration ranges, try increasing the number of beads per set for the high-conentration analytes to use the beads to "dilute" the analyte. Alternatively, using a weaker reporter (Alexa Fluor® 532) for the high concentration analytes can bring these assays into the detectable dynamic range. The FLEXMAP 3D® analyzer has an extended dynamic range that may make it possible to multiplex assays with different dynamic ranges without any modifications at all. The xMAP INTELLIFLEX® DR-SE analyzer has a second reporter channel that may be used with appropriate dyes to perform these types of assays in multiplex as well.

Chapter 5

Genomic Applications

Chapter 5.1

Development of Nucleic Acid Assays

This section provides protocols for nucleic acid assay development, including oligo coupling, different SNP genotyping chemistries, miRNA assays, and hybridization. Protocols or kits for multiplex gene expression and other applications can be found in the published literature or may be provided by certain Liscensed Technlogy Partners.

If you need to develop your own multiplex assays, xMAP® Technology features an open-architecture design that allows it to be used for the development of a number of genomic applications, including gene expression analysis, miRNA analysis, SNP analysis, specific sequence detection, and more. Multiplex genomic assays may be developed by coupling user-defined, sequence-specific capture oligos to MagPlex® beads, or, to shorten assay development times, you can purchase MagPlex-TAG™ beads, which are already coupled to unique capture sequences that can be used for most genomic applications. For any Luminex-based multiplex genomic assay, analysis is based on the analyte signal intensity detected on the different analyte specific bead regions in a multiplex reaction mix.

Figure 25.

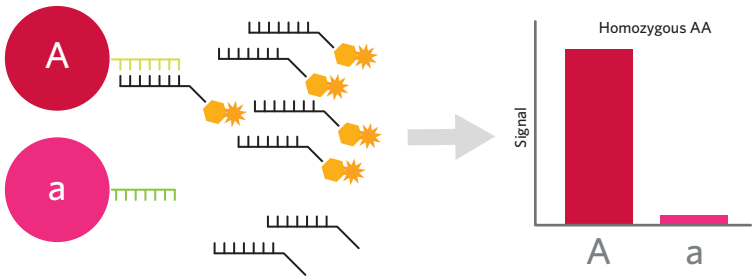


Figure 25 - Schematic of nucleic acid assay analysis on Luminex beads. Each bead has a unique capture sequence specific for a marker sequence. If reporter molecules are generated and captured (**bead A**), a fluorescence signal is detected. If no reporter molecules are generated and captured (**bead a**), minimal or background signal is detected.

Many of the protocols in this cookbook focus on several genomic chemistries where target molecules are captured with MagPlex-TAG Microspheres. However, with certain modifications, some of these applications can be used with beads coupled with capture sequences designed by the user.

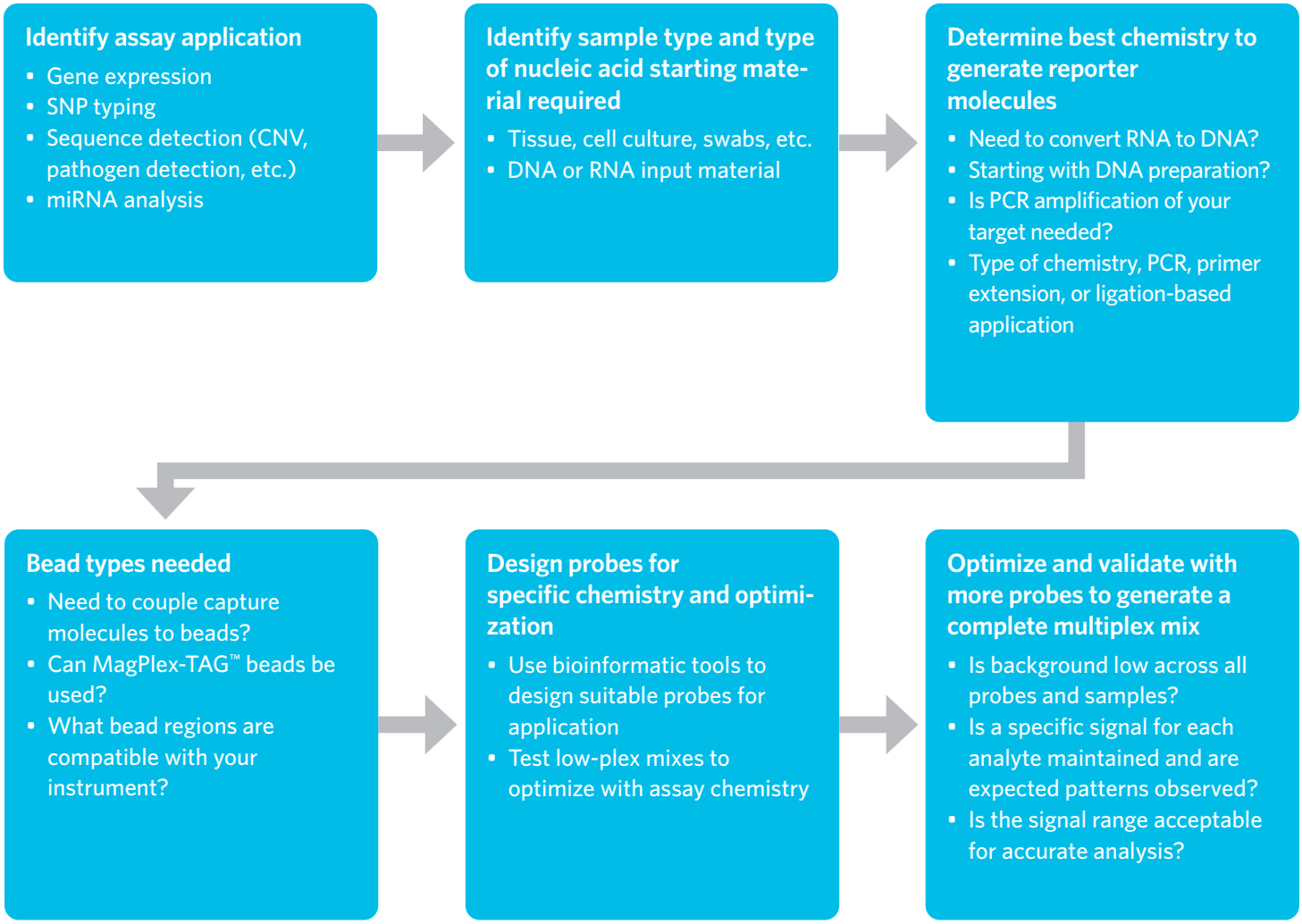
Whether using MagPlex-TAG Microspheres or coupling user-defined capture sequences, developing an effective xMAP-based nucleic acid assay depends on a number of factors. One of the first things to consider is the purpose of the assay. Depending on the application, different types of starting nucleic acid types and specific chemistries may be required to generate labeled target molecules for data collection with a Luminex instrument.

Several different reporter dyes are available for use with xMAP nucleic acid assays. The protocols described in this chapter primarily use SAPE as the reporter dye for detection of biotinylated targets, because in our studies, PE was the brightest RP1 reporter of the dyes we tested. Primers and probes can also be synthesized with an Alexa Fluor™ 532 reporter dye as an alternative to biotin.

If using the second reporter channel on xMAP INTELLIFLEX® DR-SE, streptavidin conjugates are available for several RP2-compatible dyes, including SuperBright®

436 (Thermo Fisher), Brilliant Violet 421 (BD Biosciences), and StarBright™ 440 (Bio-Rad). Alternatively, primers and probes can be synthesized with an Alexa Fluor™ 405 reporter dye. See **Chapter 3.1, "Reporter Fluorophores."**

In general, the steps involved with designing a xMAP-based nucleic acid assay are described in the following workflow:



Chapter 5.2

Nucleic Acid Coupling

General guidelines for nucleic acid coupling to xMAP® microspheres

Coupling oligonucleotides (oligos) to xMAP® microspheres is a straightforward process, but may require some optimization. While Luminex supports custom oligo coupling, for some applications, Luminex has developed MagPlex-TAG™ Microspheres. These are beads that are already coupled with unique 24-base DNA sequences ('anti-TAGs'), which capture complementary 'TAG' sequences generated on target molecules with several different genomic chemistries. These beads circumvent the need for assay developers to go through the process of coupling oligos to beads, or validating the in-house coupled beads when developing a multiplex assay.

Figure 26.

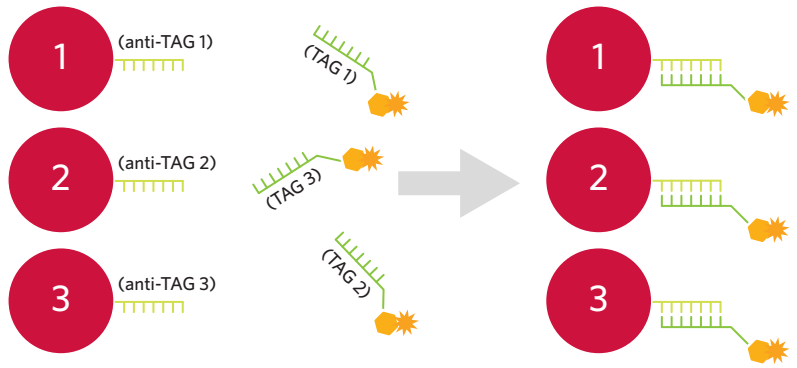
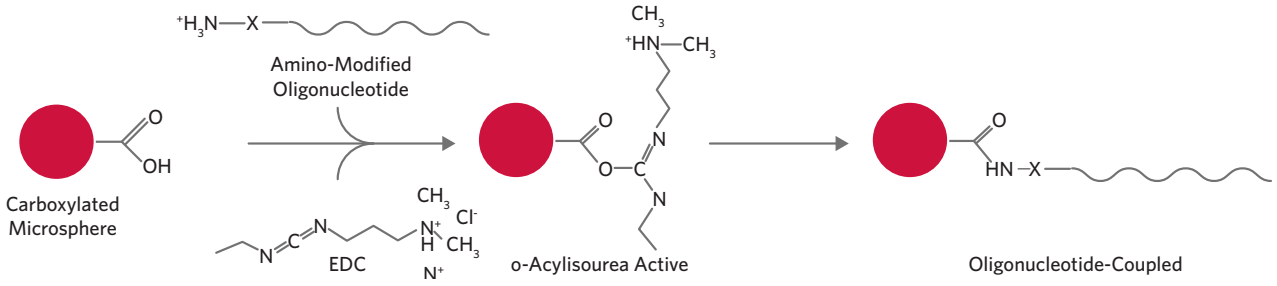


Figure 26 – MagPlex-TAG™ Microspheres are pre-coupled with anti-TAG sequences, allowing you to incorporate TAG tails into assay reactions to facilitate bead capture without the need to chemically couple oligos.

If you want to couple custom capture oligonucleotide sequences to xMAP beads, below is a standard carbodiimide coupling protocol. Oligo probes must have a primary amine for coupling to bead carboxyl groups. For best performance, we recommend that oligonucleotides are synthesized with a 5′ end amine-spacer (e.g., amino-modifier C12). Having this spacer ensures that the coupled oligo is positioned off of the bead surface, facilitating the interaction with the target and reporter molecules it needs to capture in an assay. Refer to **Chapter 5.4, Optimization of Nucleic Acid Assays** for additional information and recommendations.

Figure 27. Nucleic Acid Coupling Chemistry



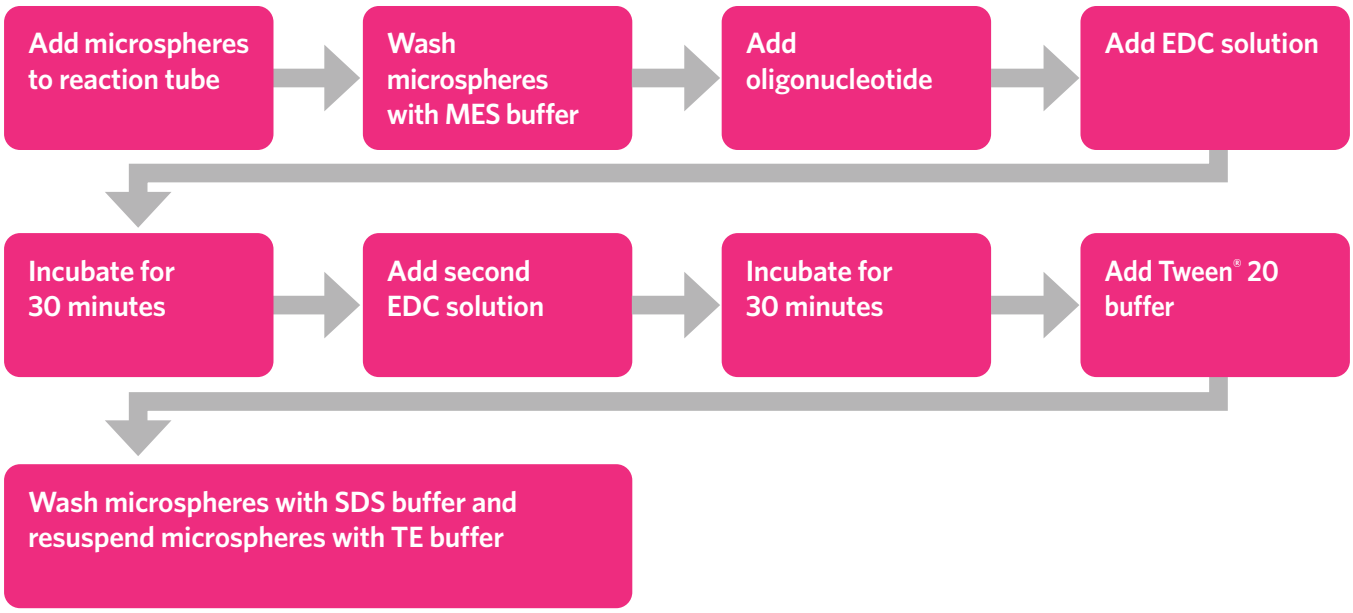
Oligonucleotide coupling is a one-step process where microsphere carboxyl groups are activated by EDC and form covalent bonds with primary amines on amine-modified oligonucleotides. Because of the reactivity with primary amines, modified oligo preparations should not be stored in buffers with amine-containing compounds such as Tris, BSA, azide, glycerol, urea, and some detergents. It is best to resuspend oligos in molecular grade H_2O.

Chapter 5.2.1

Standard Nucleic Acid Coupling to xMAP® Microspheres

Below is a standard coupling protocol for 5 million MagPlex® Microspheres per reaction. Coupled microsphere stability depends on the stability of the coupled nucleic acid, but when properly stored, coupled microspheres are usually stable for more than 1 year. The protocol for coupling amine-modified oligos to beads only takes a few hours and can be scaled up or down as needed. The optimal ratio of oligo to beads will depend on several factors, including length of probe, size of target, and secondary structure; however, 0.2 to 0.5 nmol oligo performs best in a 5 million microsphere coupling reaction. Additional recommendations for oligo to bead ratios can be found in **Chapter 5.4 (Optimization of Nucleic Acid Assays)**.

Summary of protocol



Materials needed

Reagents and Consumables	Vendor
MagPlex® Microspheres	Luminex
5′ amine C-12 spacer oligonucleotides (special order)	Integrated DNA Technologies or other vendor
0.1 M MES buffer pH 4.5	MilliporeSigma M2933
0.02% Tween® 20	MilliporeSigma P9416
1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC)	Thermo Scientific Pierce™ 77149
TE buffer pH 8.0	MilliporeSigma T9285
0.1% SDS	MilliporeSigma 71736
RNase/DNase-free microcentrifuge tubes 1.5 mL	USA Scientific 1415-2500 or Eppendorf Protein LoBind® 022431081
Disposable pipette tips; multi- and single-channel (2–1,000 µL)	Any suitable brand
Barrier pipette tips	Any suitable brand
Distilled deionized H2O (ddH2O)—molecular grade	Any suitable source

For complete equipment and materials list, see **Appendix B**.

Protocol 5.2.1: Nucleic acid coupling

1. Bring a fresh aliquot of -20°C, desiccated Pierce EDC powder to room temperature.
2. Resuspend the amine-substituted oligonucleotide (“probe” or “capture” oligo) to 1 mM (1 nanomole/µL) in dH_2O.
3. Resuspend the stock uncoupled microspheres by vortexing and sonication according to the instructions described in the product information sheet provided with your microspheres.
4. Transfer $5.0x10^6$ of the stock microspheres to a USA Scientific microcentrifuge tube.
5. Pellet the stock microspheres with a tube magnet or by microcentrifugation at ≥8,000 x g for 1–2 minutes.
6. Remove the supernatant and resuspend the pelleted microspheres in 50 µL of 0.1 M MES, pH 4.5 by vortexing and sonication for approximately 20 seconds.
7. Prepare a 1:10 dilution of the 1 mM capture oligo in dH_2O (0.1 nanomole/µL).
8. Add 2 µL (0.2 nanomole) of the 1:10 diluted capture oligo to the resuspended microspheres and mix by vortex.
9. Prepare a fresh solution of 10 mg/mL EDC in dH_2O.
10. One by one for each coupling reaction, add 2.5 µL of fresh 10 mg/mL EDC to the microspheres (25 µg or ≅ $[0.5\ µg/µL]_{final}$) and mix by vortex.
11. Incubate for 30 minutes at room temperature in the dark.
12. Prepare a second fresh solution of 10 mg/mL EDC in dH_2O.
13. One by one for each coupling reaction, add 2.5 µL of fresh 10 mg/mL EDC to the microspheres and mix by vortex.

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

Note: See Recommendations for Scaling Oligonucleotide-Microsphere Coupling in next section.

Note: We recommend using a fresh aliquot of EDC powder for each EDC addition.

Note: EDC is very labile with a rate constant of hydrolysis of seconds. Thus, two additions of fresh EDC are required for maximal coupling efficiency.

14. Incubate for 30 minutes at room temperature in the dark.
15. Add 1.0 mL of 0.02% Tween® 20 to the coupled microspheres.
16. Pellet the coupled microspheres with a tube magnet or by microcentrifugation at ≥8,000 x g for 1–2 minutes.
17. Remove the supernatant and resuspend the coupled microspheres in 1.0 mL of 0.1% SDS by vortex.
18. Pellet the coupled microspheres with a tube magnet or by microcentrifugation at ≥8,000 x g for 1–2 minutes.
19. Remove the supernatant and resuspend the coupled microspheres in 100 µL of TE, pH 8.0 by vortex and sonication for ~20 seconds.
20. Count the coupled microspheres by hemacytometer or other particle/cell counter.
21. Store coupled microspheres refrigerated at 2–8°C in the dark.

Note: If using a hemacytometer, proceed as follows:
a. Dilute the resuspended, coupled microspheres 1:100 in dH_2O.
b. Mix thoroughly by vortexing.
c. Transfer 10 µL to the hemacytometer.
d. Count the microspheres within the 4 large corners of the hemacytometer grid.
e. Microspheres/µL = (Sum of microspheres in 4 large corners) x 2.5 x 100 (dilution factor).
f. Maximum is 50,000 microspheres/µL.

Chapter 5.2.2

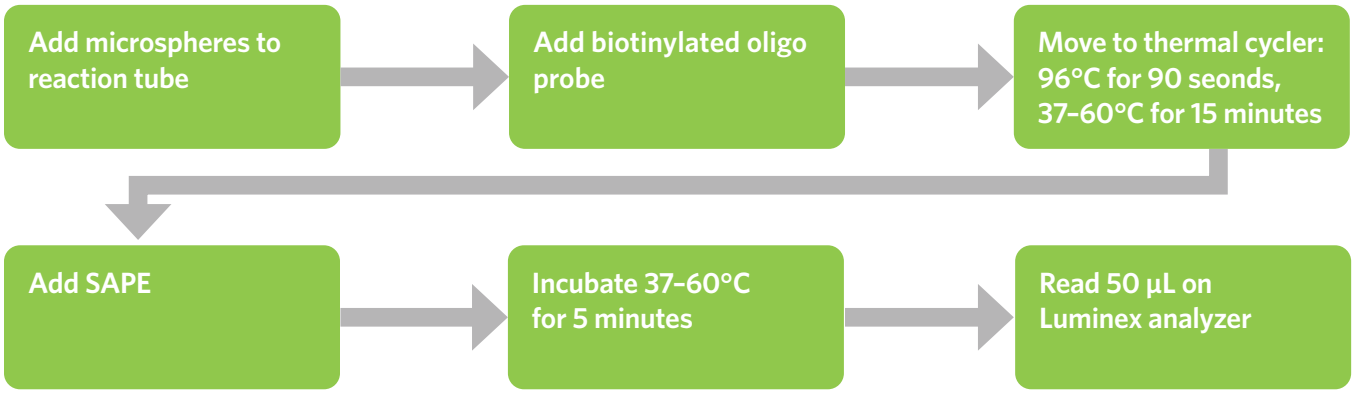
Oligonucleotide Coupling Confirmation

Once capture oligonucleotides have been coupled to xMAP® microspheres, it is strongly recommended to assess the coupling efficiency before proceeding to assay development. To do this, the coupled microspheres can be hybridized to biotinylated target oligonucleotides, labeled with streptavidin-reporter, and analyzed on your Luminex instrument.

Determining the optimum amount of oligo that provides the highest signal may require several coupling reactions at different oligo amounts. Beads from each coupling reaction can then be hybridized with a complementary biotin-labeled oligonucleotide (5 to 200 fmols) to analyze coupling efficiency and signal intensity. We recommend purchasing sequence-specific 5′ biotinylated complementary oligos for each capture sequence as the most reliable method, since it allows precise amounts of target with a single biotin group to be used in the hybridization reaction.

The following protocol may be used for confirmation of oligonucleotide coupling reactions. In this example, SAPE is used as the reporter.

Summary of protocol



Materials needed

Reagents and Consumables	Vendor
MagPlex® Microspheres (oligonucleotide-coupled)	Supplied by user
1.5X TMAC hybridization solution	See **Appendix A**
1X TMAC hybridization solution	See **Appendix A**
TE buffer pH 8.0	MilliporeSigma T9285
96-well bead hybridization plate	Corning Thermowell™ 6509
Biotin-labeled complementary oligonucleotide targets	Any suitable source
Streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Thermo Fisher (Invitrogen S866), ProZyme®, or equivalent
RNase/DNase-free microcentrifuge tubes 1.5 mL	USA Scientific 1415-2500 or Eppendorf Protein LoBind® 022431081
Microseal® 'A' film	Bio-Rad MSA5001
Brayer roller, soft rubber, or silicon	USA Scientific 9127-2940
Disposable pipette tips; multi- and single-channel (2–1,000 µL)	Any suitable brand
Distilled deionized H_2O (ddH_2O)—molecular grade	Any suitable source

For complete equipment and materials list, see **Appendix B**.

Note: Luminex xTAG Buffers are specific for MagPlex-TAG™ (low G-C) hybridization reactions. TMAC buffers are best suited for custom coupling (typically used for direct hybridization assays).

If shipping/receiving TMAC is problematic, 2X xTAG buffer may be a suitable substitute for direct hybridization assays run on MAGPIX® only. See **Appendix A**.

An example protocol for verifying oligonucleotide coupling is provided below. A dose response increase in MFI should be observed as the concentration of labeled target oligonucleotide increases. In general, oligonucleotide coupling should yield at least 10,000 MFI for SAPE (standard PMT or MAGPIX®) at saturation for optimal use in hybridization assays.

Protocol 5.2.2: Oligonucleotide coupling confirmation

1. Select the appropriate individual MagPlex® coupled bead sets and resuspend by vortexing and sonication for 20 seconds each.
2. Dilute/concentrate the coupled bead sets to prepare a mixture containing 75 microspheres of each set/µL in 1.5X TMAC hybridization solution. Vortex and sonicate for ~20 seconds. (Note: 33 µL are required for each reaction to give 2,500 beads of each set/reaction.)
3. Add 33 µL of the microsphere mixture to each well of a bead hybridization plate as needed for each reaction.
4. Add 17 µL of dH_2O to each background well.
5. Add 5 to 20 µL of complementary biotin-oligonucleotide (5 to 200 femtomoles) to appropriate sample wells.
6. Adjust the total volume to 50 µL by adding the appropriate volume of dH_2O or TE to each sample well.
7. Cover the plate with Microseal® 'A' film to prevent evaporation. Process in a thermal cycler according to the following program.
   96°C for 90 seconds
   37–60°C for 15 minutes
8. Dilute SAPE to 10 µg/mL in 1X TMAC hybridization solution. (Note: 25 µL is required for each reaction.)
9. Add 25 µL of 10 µg/mL SAPE to each well and mix by gently pipetting up and down several times. (Note: Final concentration of SAPE should be 2–4 µg/mL.)
10. Incubate at hybridization temperature for 5 minutes.
11. Analyze 50 µL at hybridization temperature on the Luminex analyzer according to the system manual.

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

Note: Coupling confirmation should be performed at the anticipated assay hybridization temperature.

Figure 28. Typical Results of an Oligonucleotide Coupling Reaction

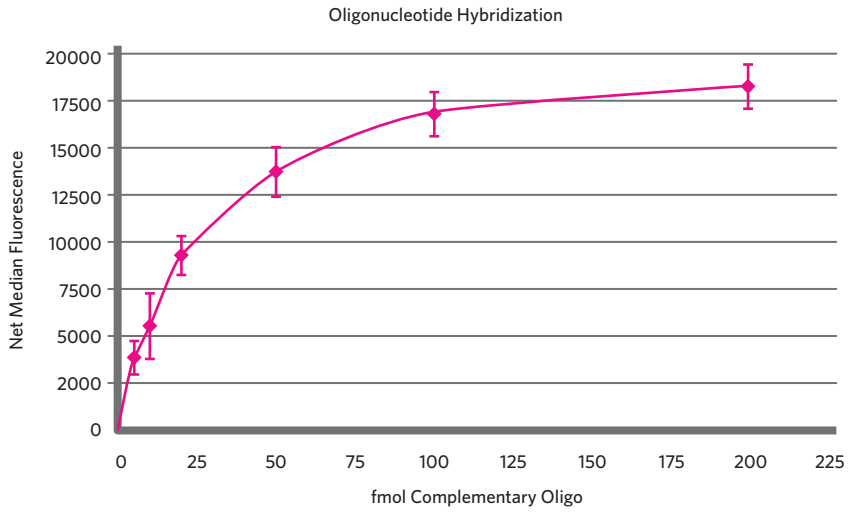


Figure 28 – Plot of experimental results for oligo coupling, as measured by a Luminex analyzer. Results shown are typical for successful coupling reactions using SAPE reporter. MFI values increase as more complementary oligo is added; the MFI values exceed 10,000 as the hybridization reaction reaches saturation. Optimal amount for typical coupling is usually 0.2 to 1 nanomole per 5 million microspheres.

Oligonucleotide coupling references

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Chapter 5.3

Common Nucleic Acid Assay Formats

xMAP® Technology supports a number of genomic assay formats, including gene expression analysis, microRNA analysis, single nucleotide polymorphism (SNP) analysis, specific sequence detection, and other applications. Assays may be developed by coupling sequence specific capture oligos to MagPlex® beads, or TAG sequences may be incorporated into assay reactions to capture to MagPlex-TAG™ beads without the need to couple oligos.

Figure 29.

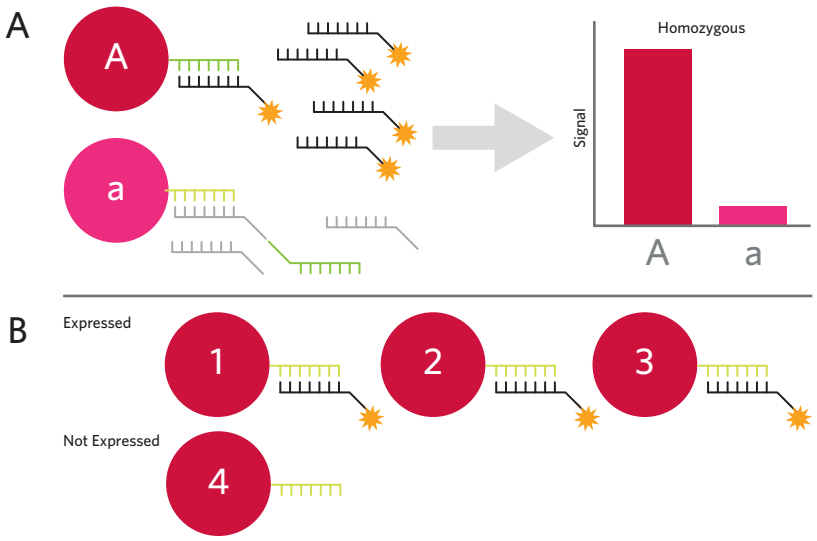

Figure 29 - Common assay formats for nucleic acid assays: **A**) genotyping (qualitative) and **B**) gene expression (quantitative).

Common nucleic assay formats include oligo ligation assay (OLA) SNP typing, allele-specific primer extension (ASPE) SNP typing, target-specific PCR sequence detection, direct DNA hybridization sequence detection, and microRNA analysis. The following sections cover each of these assays in detail, including assay overview, materials needed, buffer and reagent recipes, step-by-step protocols, recommendations for optimization and troubleshooting, and references.

Typically, a no-wash protocol is used for nucleic acid hybridization assays, however each of the following sections describes both unwashed and washed protocols. The ability to use a no-wash format will depend on the number of targets, the level of multiplexing, and the reporter concentration needed to be able to eliminate wash steps.

Chapter 5.3.1

Oligo Ligation Assay (OLA) SNP Typing

The oligonucleotide ligation assay (OLA) is a flexible, inexpensive, and simple approach for detecting SNPs and other sequence variations. The flexibility of this assay allows it to be used for genotyping a number of different genes and organisms.[1-5]

In OLA chemistry, one or more forward probes containing TAG sequences bind adjacently to a common biotinylated downstream probe (**Figure 30**). The close proximity of a bound OLA-TAG probe 3′ end with the OLA reporter probe 5′ end allows enzymatic ligation to join them into a complete biotinylated reporter molecule. If the OLA-TAG probe 3′ base is not complementary to the SNP variant base, ligation does not occur and no significant signal is generated for analysis.

Note that OLA probes do not have primer binding sites for PCR amplification, as seen with other ligation chemistries. With this chemistry, signal amplification is achieved by prior amplification of genomic target regions and multiple cycles of the ligation reaction.

Figure 30. OLA Probe Design

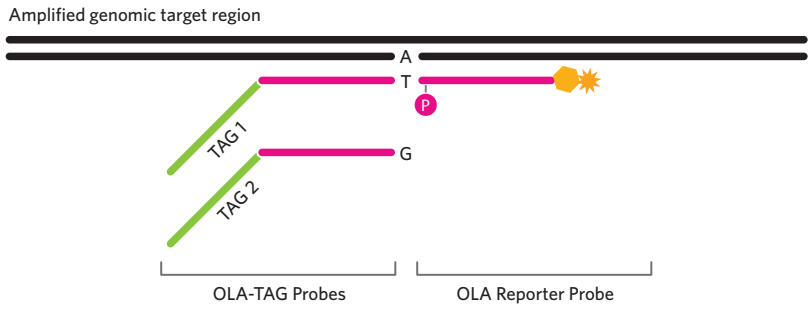

Figure 30 - The oligo ligation assay (OLA) involves two target-specific probes that align adjacent to one another, allowing enzymatic ligation at the 5′ phosphate if an exact match occurs at the target SNP site. Incorporation of TAG sequences enables capture of each possible allele to a unique MagPlex-TAG™ Microspheres.

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Materials needed

Reagents and Consumables	Vendor
MagPlex-TAG™ Microspheres	Luminex
Primers for PCR amplification of gDNA target regions	Integrated DNA Technologies or other vendor
OLA-TAG primers with 5′ TAG sequence and biotinylated OLA reporter primers	Integrated DNA Technologies or other vendor
QIAGEN HotStarTaq® 2X master mix	Qiagen 203443 or equivalent
Taq DNA Ligase and 10X ligase buffer	New England BioLabs M0208S or equivalent
2X xTAG® hybridization buffer	See Buffer and Reagent Recipes section
1X xTAG® hybridization buffer	See Buffer and Reagent Recipes section
Streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Thermo Fisher (Invitrogen S866), ProZyme®, or equivalent
0.2 mL PCR tubes	Any suitable brand
96-well PCR plate	Bio-Rad MSP9601
Microseal® 'A' film	Bio-Rad MSA5001
Magnetic separation plate (special order)	V&P Scientific VP771LD-4CS or equivalent
Disposable pipette tips; multi- and single-channel (2–1,000 µL)	Any suitable brand
RNase/DNase-free microcentrifuge tubes, 1.5 mL	USA Scientific or equivalent
Barrier pipette tips	Any suitable brand
Vortex mixer	Any suitable brand
Microcentrifuge	Any suitable brand
Bath sonicator (40-55 kHz)	Any suitable brand
Centrifuge with microplate swinging bucket rotor	Eppendorf 5804 or equivalent
Brayer roller, soft rubber or silicon	USA Scientific 9127-2940
Thermocycler with 96-well head and heated lid	Any suitable brand
Luminex instrument with xPONENT® or higher software	Luminex

For complete equipment and materials list, see **Appendix B**.

Note: Molecular grade ddH_2O should be used for all nucleic acid protocols.

OLA buffer and reagent recipes

Step	Notes
MagPlex-TAG™ Microspheres from Luminex	Required microsphere regions should be purchased from Luminex. They should be stored at 4°C in the dark. For multiplex assays, combine different bead regions as directed in the protocol. To assist with making a multiplex microsphere stock mix from individual bead stocks, an Excel®-based bead calculator is available for determining the method and volumes needed for making the bead mix.

Enzymes and enzyme reaction buffers	All enzymes and their reaction buffers can be used as directed in the protocol.
PCR amplification primers for gDNA targets	Proper design of these primers for amplification of target regions is required for optimum assay performance. Recommendations for primer designs include: 1. PCR primers should be designed to amplify gDNA target regions containing the SNP(s) of interest. 2. PCR primers should not be labeled. 3. A small amplicon size is not required, as the amplicon is not directly hybridized to the bead surface. However, amplicon size may be restricted by the efficiency of the polymerase used and proximity of the SNPs being studied. If amplifying multiple genomic regions, consider designing amplicons to be similar in size. These primers can be purchased from multiple vendors. Upon receipt, the primers should be dissolved or diluted with molecular grade distilled-deionized H_2O (ddH_2O) to a concentration of 1 mM (1 nanomole/µL). They can be stored as individual stocks at -20°C. Pooled mixes can be made to deliver 0.2 µM of each primer/PCR reaction or as your gDNA protocol requires.
OLA-TAG and biotinylated OLA reporter primers	OLA primers can be ordered from several oligo manufacturers, such as IDT. Upon receipt, they should be resuspended in molecular grade ddH_2O to 1 mM (1 nanomole/µL). Make a 100 nM OLA-TAG probe mix by combining and diluting each 1 mM stock 1:10,000 into one tube with molecular grade ddH_2O. Make a 5 µM OLA reporter probe mix by combining and diluting each 1 mM stock 1:200 into one tube with molecular grade ddH_2O. Individual stocks and probe mixes should be stored at -20°C.
OLA primer design factors	1. OLA probes should be synthesized for all sequence variants and all OLA-TAG and OLA reporter pairs for each target should be from the same DNA strand. 2. OLA probes should be matched for melting temperature at 51–56°C. 3. OLA-TAG probes should extend out to and include the SNP as the 3′ nucleotide. 4. Use oligo design software to select an appropriate TAG sequence or contact Luminex Technical Support for assistance in selecting TAG sequences. 5. The OLA-TAG probe should be synthesized with the TAG sequence incorporated at the 5′ end. 6. The OLA reporter probe should have a melting temperature of 51–56°C. 7. The OLA reporter probe's 5′ base should be the nucleotide immediately downstream from the SNP variant nucleotide. 8. The OLA reporter probe must be modified with phosphate at the 5′ end and with biotin at the 3′ end. 9. If two SNPs are close enough so that OLA-TAG and reporter probes overlap, target the second SNP on the opposite strand.
2X xTAG® hybridization buffer	The buffer's composition is 0.4 M NaCl, 0.2 M Tris, 0.16% Triton™ X-100, pH 8.0. It should be filter sterilized and stored at 4°C.
1X xTAG® hybridization buffer	The buffer's composition is 0.2 M NaCl, 0.1 M Tris, 0.08% Triton™ X-100, pH 8.0. It should be filter sterilized and stored at 4°C.
Streptavidin-R-phycoerythrin (SAPE)	SAPE at can be purchased from a number of suppliers, such as Moss Incorporated (SAPE-001G75), Thermo Fisher (Invitrogen S866), ProZyme® (various), or equivalent. A working aliquot should be made fresh by diluting with 1X Tm hybridization buffer to the required volume and concentrations needed as indicated in the washed or no-wash protocols.

Protocol 5.3.1: OLA SNP typing

Genomic Target Sequence Amplification

Multiplexed PCR amplification of genomic target regions containing SNPs of interest should be performed under optimized conditions. The parameters listed below are an example and may not be optimum for your samples or any specific genomic amplification kit you may be using.

Each final reaction contains:

Reagent	Amount
gDNA template	50 ng
QIAGEN PCR reaction buffer	1X
$MgCl_2$	1.5 mM
dNTPs	200 µM each
Primers	0.2 µM each
QIAGEN HotStarTaq® or other Taq polymerase	2.5 units

Amplification of target regions can be done with the following PCR cycling parameters:

Temperature	Time	Cycle
95°C	15 minutes (for enzyme activation)	
94°C	30 seconds	
55°C	30 seconds	35 cycles
72°C	30 seconds	
72°C	7 minutes	
4°C	Hold	

Multiplex OLA reaction

Prior to making the 2X OLA master mix, make stocks of 100 nM OLA-TAG primer mix and 5 µM OLA reporter mix as directed in the OLA Buffer and Reagent Recipes section.

1. Make a 2X OLA master mix as follows:

Reagent	Amount
10X Taq Ligase buffer	2.0 µL
Taq DNA Ligase (40,000 U/mL)	0.25 µL
OLA-TAG primer mix (100 nM each)	1.0 µL
OLA reporter mix (5 µM each)	1.0 µL
ddH_2O (molecular grade)	5.75 µL
Total volume =	10 µL

2. Assemble OLA reactions in 20 µL total volume for each sample as follows:

Reagent	Amount
2X OLA master mix	10 µL
Amplified genomic targets (0.5 to 5 µL)	Y µL
ddH_2O (molecular grade) as needed (to 20 µL)	X µL
Total volume =	20 µL

3. Mix OLA reactions by pipetting up and down several times.
4. Cover plate with a plate sealer and perform multiple rounds of ligation in a thermal cycler with the following parameters:

Temperature	Time	Cycle
96°C	2 minutes	
94°C	15 seconds	30 cycles
37°C	1 minute	
4°C	Hold	

5. Proceed to hybridization with MagPlex-TAG™ Microspheres using a washed or no-wash protocol. The examples below use SAPE as the reporter dye. Optimal reporter concentration should be determined by titration.

5.3.1.1 Hybridization to MagPlex-TAG™ Microspheres: No-wash protocol

1. Select the appropriate MagPlex-TAG™ microsphere sets and resuspend according to the instructions described in the product information sheet provided with your microspheres.
2. Combine 2,500 microspheres of each set per reaction.
3. Dilute/concentrate the MagPlex-TAG microsphere mixture to 111 microspheres of each set per µL in 1X xTAG hybridization buffer and mix by vortex and sonication for ~20 seconds.
4. Aliquot 22.5 µL of the MagPlex-TAG microsphere mixture to each well. (Note: This will provide 2,500 beads of each set/reaction.)
5. Add 2.5 µL of dH_2O to each background well.
6. Add 2.5 µL of each sample to the appropriate wells.
7. Cover the plate with Microseal 'A' film to prevent evaporation and hybridize in a thermal cycler with the following parameters:
   96°C for 90 seconds
   37°C for 30 minutes
8. Prepare SAPE mix by diluting SAPE to 10 µg/mL in 1X xTAG hybridization buffer.
9. Add 100 µL SAPE mix to each well. Mix gently.
10. Incubate at 37°C for 15 minutes.
11. Analyze 100 µL at 37°C on your Luminex analyzer according to the system manual.

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

Note: An Excel®-based bead calculator to facilitate determining the method and volumes needed in making the working microsphere mixture can be obtained **here**.

5.3.1.2 Hybridization to MagPlex-TAG™ Microspheres: Washed protocol

1. Select the appropriate MagPlex-TAG™ microsphere sets and resuspend according to the instructions described in the product information sheet provided with your microspheres.
2. Combine 2,500 microspheres of each set per reaction. (Note: If needed, an Excel-based bead calculator is available for determining the method and volumes needed for making the bead mix.)
3. Dilute/concentrate the MagPlex-TAG microsphere mixture to 100 microspheres of each set per µL in 2X xTAG hybridization buffer and mix by vortex and sonication for ~20 seconds.
4. Aliquot 25 µL of the MagPlex-TAG microsphere mixture to each well. (Note: This will provide 2,500 beads of each set/reaction.)
5. Add 1–5 µL of each OLA reaction to appropriate wells and add 25 µL of dH_2O to each background well.
6. Adjust the total volume to 50 µL by adding the appropriate volume of dH_2O to each sample well that received less than 25 µL of OLA reaction.
7. Cover the plate with Microseal® 'A' film to prevent evaporation and hybridize in a thermal cycler with the following parameters:
   96°C for 90 seconds
   37°C for 30 minutes
8. Pellet the MagPlex-TAG Microspheres by placing the plate on a magnetic separator for 30 to 60 seconds.
9. After the beads have pelleted, remove the supernatant being careful not to disturb them.
10. Resuspend the pelleted MagPlex-TAG Microspheres in 75 µL of 1X xTAG hybridization buffer on a magnetic separator for 30 to 60 seconds.
11. After the beads have pelleted, remove the supernatant being careful not to disturb them.
12. Repeat steps 8 to 11 for a total of 2 washes.
13. Resuspend microspheres in 75 µL of 1X xTAG hybridization buffer containing 2–8 µg/mL SAPE.
14. Incubate at 37°C for 15 minutes.
15. Analyze 50 µL at 37°C on your Luminex analyzer according to the system manual.

Note: An Excel®-based bead calculator to facilitate determining the method and volumes needed in making the working microsphere mixture can be obtained **here**.

Recommendations for optimization and troubleshooting

Low Reporter Intensity

1. Verify the production of the genomic target PCR products (OLA templates) by electrophoresis.
2. Verify the hybridization assay by direct hybridization to 5 (low dose) and 50 (high dose) femtomoles of labeled oligonucleotide targets (i.e., biotinylated TAGs).
3. Titrate the gDNA input for target region generation to determine the optimal amount for OLA reaction.
4. Titrate the gDNA amplified template input into OLA reaction to determine the optimal amount of template.
5. Titrate the allele-specific and reporter probe inputs to determine optimal concentrations for OLA.
6. Increase the number of cycles in the OLA reaction.
7. Decrease and/or increase the OLA annealing temperature.

Note: An allele-specific to reporter probe ratio of 1:50 improves the probability that an allele-specific probe will anneal adjacent to a reporter probe.

8. Check the primer and template sequences for potential secondary structures.
9. Redesign the PCR primers.
10. Redesign the OLA probes for the opposite DNA strand.
11. Lengthen the OLA probes.

Poor Discrimination

1. Increase the OLA annealing temperature.
2. Redesign the OLA probes for the opposite DNA strand.
3. Shorten the "leaky" OLA probe.

Poor Reporter Distribution Between Alleles

1. Redesign the OLA probes for the opposite DNA strand.
2. Lengthen the OLA probes to increase signal on the "low" allele.
3. Shorten the OLA probes to decrease signal on the "high" allele.

High Background

1. Determine the optimal reporter concentration by titration. Sometimes lowering the reporter concentration may help reduce background MFI.
2. If using SAPE that does not already contain BSA (e.g., Moss SAPE-001G75), dilute the SAPE in 1X xTAG buffer containing BSA. Final BSA concentration in the hybridization reaction should be 0.1%.
3. If the high background is due to contamination of the PCR reaction, replace the PCR reagents.
4. If high background is observed for the hybridization negative control, replace the hybridization reagents.
5. If high background is observed for the OLA negative control, replace the OLA reagents.

OLA references

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Chapter 5.3.2

Allele-Specific Primer Extension (ASPE) SNP Typing

Allele-specific primer extension (ASPE) is a flexible, inexpensive, and simple chemistry for detecting SNPs and other sequence variations. The flexibility of the assay has made it useful for genotyping typing a number of different genes and organisms.[1-5]

Figure 31. ASPE Mechanism

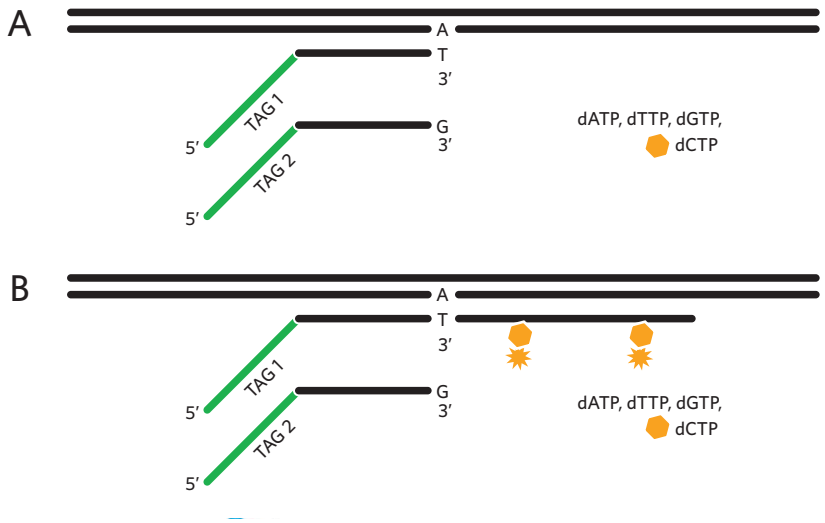

Figure 31 - Allele-specific primer extension (ASPE) involves target-specific primers with 3′ bases matching the possible SNP alleles of interest. **A**) A primer anneals to the target region with its 3′ end aligned at the SNP site. **B**) If the primer finds a perfect match at the 3′ base (as with the TAG1 probe) then DNA polymerase is able to extend the primer, thereby incorporating labeled dNTPs downstream. Primers with a 3′ mismatch at the SNP site (as with the TAG2 probe) are not extended and no labeled dNTPs are incorporated. Inclusion of TAG sequences enables capture of each possible allele to a unique MagPlex-TAG™ Microsphere.

ASPE chemistry takes advantage of a primer's ability to act as a primer for DNA polymerase when the 3′ base is complementary to the target SNP's base (**Figure 31A**). When the 3′ base is complementary, the polymerase can use it as a start site to synthesize new DNA containing biotin-labeled nucleotides, but a primer cannot promote this extension if its 3′ base is mismatched (**Figure 31B**). With several rounds of primer extension, significant quantities of labeled targets are produced to generate the signals required for analysis of multiple genotypes in one reaction.

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To use ASPE chemistry with xTAG® Technology, each ASPE primer that identifies a SNP variation needs a unique TAG sequence on its 5′ end (**Figure 31**). In addition, for each SNP, gDNA targets containing SNPs of interest must be amplified before testing with ASPE probe mixes. This is achieved using standard multiplex PCR amplification methods (**Figure 32**). Target genomic amplicons can be of various sizes and can contain multiple SNP targets.

Figure 32. Amplifications of gDNA Target Regions of Interest

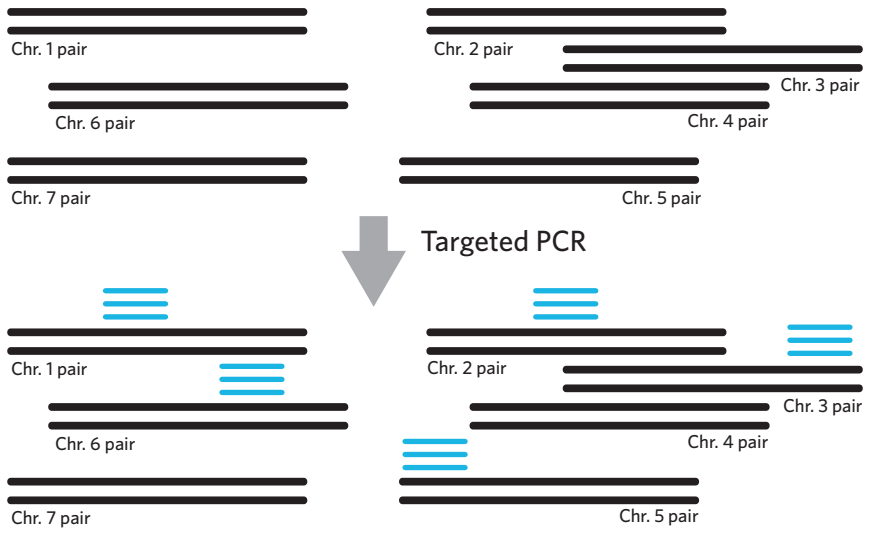

Figure 32 - Genomic DNA is amplified in the region of each SNP using standard multiplex PCR amplification prior to performing the ASPE assay. Amplified targets may be of various lengths and may even contain multiple SNPs.

Materials needed

Reagents and Consumables	Vendor
MagPlex-TAG™ Microspheres	Luminex
Primers for PCR amplification of gDNA target regions	Integrated DNA Technologies or other vendor
ASPE primers with 5′ TAG sequences	Integrated DNA Technologies or other vendor
QIAGEN HotStarTaq® 2X master mix	QIAGEN 203443 or equivalent
ExoSAP-IT® or separate Exo I and SAP	Thermo Fisher Applied Biosystems 78200 or equivalent
Platinum™ Tsp DNA polymerase, 10X PCR Buffer, 50 mM $MgCl_2$	Thermo Fisher (Invitrogen 11448-024) or equivalent
Biotin-14-dCTP	Thermo Fisher (Invitrogen 19518-018) or equivalent
dNTPs stocks	Thermo Fisher (Invitrogen 10297-018) or equivalent
2X xTAG® hybridization buffer	See Buffer and Reagent Recipes section
1X xTAG® hybridization buffer	See Buffer and Reagent Recipes section
Streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, ThermoFisher (Invitrogen S866), ProZyme® or equivalent
0.2 mL PCR tubes	Any suitable brand
96-well PCR plate	Bio-Rad MSP9601

Note: Molecular grade ddH_2O should be used for all nucleic acid protocols.

Technical Note
Platinum™ Tfi Exo(-) DNA Polymerase (Life Technologies, 60684-050) and TaKaRa Taq™ Hot Start DNA Polymerase (Takara Bio, R007A) also perform well in xTAG® ASPE applications. The same **Exo(-)** polymerase can be used for both PCR and ASPE for most applications.

96-well bead hybridization plate (optional)	Corning Thermowell™ 6509
Microseal® 'A' film	Bio-Rad MSA5001
Magnetic separation plate (special order)	V&P Scientific VP771LD-4CS or equivalent
Disposable pipette tips; multi- and single-channel (2–1,000 µL)	Any suitable brand
25 mL reservoirs (divided well)	Any suitable brand
RNase/DNase-free microcentrifuge tubes 1.5 mL	USA Scientific or equivalent
Barrier pipette tips	Any suitable brand
Vortex mixer	Any suitable brand
Microcentrifuge	Any suitable brand
Bath sonicator (40-55 kHz)	Any suitable brand
Centrifuge with microplate swinging bucket rotor	Eppendorf 5804 or equivalent
Brayer roller, soft rubber, or silicon	USA Scientific 9127-2940
Thermocycler with 96-well head and heated lid	Any suitable brand
Luminex instrument with xPONENT® or higher software	Luminex

ASPE buffer and reagent recipes

Step	Notes
MagPlex-TAG™ Microspheres from Luminex	Required microsphere regions should be purchased from Luminex. They should be stored at 4°C in the dark. For multiplex assays, combine different bead regions as directed in the protocol.
Enzymes and enzyme reaction buffers	All enzymes and their reaction buffers can be used as directed in the protocol.
PCR amplification primers for gDNA targets	Proper design of these primers for amplification of target regions is required for optimum assay performance. Recommendations for primer designs include: 1. PCR primers should be designed to amplify gDNA target regions containing the SNP(s) of interest. 2. PCR primers should not be labeled. 3. A small amplicon size is not required, as the amplicon is not directly hybridized to the bead surface. However, amplicon size may be restricted by the efficiency of the polymerase used and proximity of the SNPs being studied. If amplifying multiple genomic regions, consider designing amplicons to be similar in size. These primers can be purchased from multiple vendors. Upon receipt, the primers should be dissolved or diluted with molecular grade ddH_2O to a concentration of 1 mM (1 nanomole/µL). They can be stored as individual stocks at -20°C. Pooled mixes can be made to deliver 0.2 µM of each primer/PCR reaction or as your gDNA protocol requires.
ASPE primers with 5′ TAG sequence	ASPE primers can be ordered from several oligo manufacturers, such as IDT. Upon receipt, they should be resuspended in molecular grade ddH_2O to 1 mM (1 nanomole/µL). Individual stocks and concentrated mixes should be stored at -20°C.

When designing ASPE primers, the following factors should be considered	1. ASPE primers should be synthesized for all sequence variants and should be from the same DNA strand for each target sequence. 2. ASPE primers should be matched for melting temperatures at 51–56°C. 3. ASPE primers should extend out to and include the SNP variant base as the 3′ nucleotide. 4. Use oligo design software to select an appropriate TAG sequence or contact Luminex Technical Support for assistance in selecting TAG sequences. 5. The ASPE primer should be designed to include the TAG sequence at its 5′ end. 6. If two SNPs are close enough such that the TAG-ASPE primers will overlap, target the second SNP on the opposite strand.
dNTPs	These can be purchased from several vendors such as Thermo Fisher (Invitrogen 10297-018). A 33 mM stock mix can be made by mixing equal amounts of 100 mM dATP, dTTP, and dGTP for the 3 dNTP stock mix for ASPE. Individual stocks and concentrated mixes should be stored at -20°C.
Biotin-14-dCTP	Biotin-labeled dCTP can be purchased from several sources, including Thermo Fisher (Invitrogen 19518-018). It is supplied at 0.4 mM in 100 mM Tris-HCl (pH 7.5), 0.1 mM EDTA. Store at -20°C and use as indicated in the protocol.
2X xTAG® hybridization buffer	The buffer's composition is 0.4 M NaCl, 0.2 M Tris, 0.16% Triton™ X-100, pH 8.0. It should be filter sterilized and stored at 4°C.
1X xTAG® hybridization buffer	The buffer's composition is 0.2 M NaCl, 0.1 M Tris, 0.08% Triton™ X-100, pH 8.0. It should be filter sterilized and stored at 4°C.
Streptavidin-R-phycoerythrin (SAPE)	SAPE can be purchased from a number of suppliers, such as Moss Incorporated (SAPE-001G75), Thermo Fisher (Invitrogen S866), ProZyme® (various), or equivalent. A working aliquot should be made fresh by diluting with 1X xTAG® hybridization buffer to the required volume and concentrations needed as indicated in the washed or no-wash protocols.

Protocol 5.3.2: ASPE SNP typing

Genomic Target Sequence Amplification

Multiplexed PCR amplification of genomic target regions containing SNPs of interest should be performed under optimized conditions. The parameters listed below are an example and may not be optimum for your samples or any specific genomic amplification kit you may be using.

Each final reaction contains:

Reagent	Amount
gDNA template	50 ng
QIAGEN PCR reaction buffer	1X
$MgCl_2$	1.5 mM
dNTPs	200 µM each
Primers	0.2 µM each
QIAGEN HotStarTaq® or other Taq polymerase	2.5 units

Amplification of target regions can be done with the following PCR cycling parameters:

Temperature	Time	Cycle
95°C	15 minutes (for enzyme activation)	
94°C	30 seconds	
55°C	30 seconds	35 cycles
72°C	30 seconds	
72°C	7 minutes	
4°C	Hold	

EXO/SAP Treatment for the Removal of Unused Primers

Treat 7.5 µL of each PCR reaction with ExoSAP-IT according to the following procedure:

Reagent	Amount
PCR Reaction	7.5 µL
ExoSAP-IT	3.0 µL
Total volume =	10.5 µL

Mix and incubate in a thermal cycler with the following protocol:

Temperature	Time
37°C	30 minutes
80°C	15 minutes
4°C	Hold (or store at -20°C)

Detailed Multiplex ASPE Reaction Protocol

Prior to making the 2X ASPE master mix, dilute the 1 mM ASPE-TAG primer mix 1:2,000 (500 nM each) and the 33 mM 3 dNTP stock 1:330 (100 µM each).

1. Make a 2X ASPE master mix as follows:

Reagent	Amount
10X PCR reaction buffer	2.0 µL
50 mM $MgCl_2$	0.5 µL
TAG-ASPE primer mix (500 nM each)	1.0 µL
Tsp DNA polymerase (5 U/µL)	0.15 µL
3 dNTP mix (-dCTP) (100 µM each)	1.0 µL
400 µM biotin-dCTP	0.25 µL
ddH_2O (molecular grade)	5.1 µL
Total volume =	10 µL

2. Assemble the ASPE reactions in 20 µL total volume for each sample as follows:

Reagent	Amount
2X ASPE master mix	10 µL
Target EXO treated PCR Use up to	Y µL
ddH_2O (as needed to 20 µL)	X µL
Total volume =	20 µL

3. Mix each ASPE reaction by pipetting up and down several times.
4. Perform multiple rounds of primer extension in a thermal cycler with the following parameters.

Temperature	Time	Cycle
96°C	2 minutes	
94°C	30 seconds	
55°C	1 minute	30 cycles
72–74°C	2 minutes	
4°C	Hold	

Note: The temperature of the 1 minute annealing step can be adjusted to what is needed for different probe mixes.

5. Proceed to hybridization with MagPlex-TAG™ Microspheres using a washed or no-wash protocol. The examples below use SAPE as the reporter. Optimal reporter concentrations should be determined by titration.

5.3.2.1 Hybridization to MagPlex-TAG Microspheres: No wash protocol

1. Select the appropriate MagPlex-TAG microsphere sets and resuspend according to the instructions described in the product information sheet provided with your microspheres (beads).
2. Combine 2,500 microspheres of each set per reaction.
3. Dilute/concentrate the MagPlex-TAG microsphere mixture to 111 microspheres of each set per µL in 1X xTAG hybridization buffer, and mix by vortex and sonication for ~20 seconds.
4. Aliquot 22.5 µL of the MagPlex-TAG microsphere mixture to each well.
5. Add 2.5 µL of dH_2O to each background well.
6. Add 2.5 µL of each sample to the appropriate wells.
7. Cover the plate with MicroSeal 'A' film to prevent evaporation and hybridize in a thermal cycler with the following parameters:
   96°C for 90 seconds
   37°C for 30 minutes
8. Prepare reporter mix by diluting SAPE to 10 µg/mL in 1X xTAG hybridization buffer.
9. Add 100 µL reporter mix to each well. Mix gently.
10. Incubate at 37°C for 15 minutes.
11. Analyze 100 µL at 37°C on your Luminex analyzer according to the system manual.

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

5.3.2.2 Hybridization to MagPlex-TAG™ Microspheres: Washed protocol

Note: An Excel®-based bead calculator to facilitate determining the method and volumes needed in making the working microsphere mixture can be obtained **here**.

12. Select the appropriate MagPlex-TAG™ microsphere sets and resuspend according to the instructions described in the product information sheet provided with your microspheres (beads).
13. Combine 2,500 microspheres of each set per reaction. (Note: If needed, an Excel®-based bead calculator is available for determining the method and volumes needed for making the bead mix. Download it here.)
14. Dilute/concentrate the MagPlex-TAG microsphere mixture to 100 microspheres of each set per µL in 2X xTAG hybridization buffer and mix by vortex and sonication for ~20 seconds.
15. Aliquot 25 µL of the MagPlex-TAG microsphere mixture to each well.
16. Add 1–5 µL of each ASPE reaction to appropriate wells and add 25 µL of dH_2O to each background well.
17. Adjust the total volume to 50 µL by adding the appropriate volume of dH_2O to each sample well that received less than 20 µL of extension reaction.
18. Cover the plate with Microseal® 'A' film to prevent evaporation and hybridize in a thermal cycler with the following parameters:
    96°C for 90 seconds
    37°C for 30 minutes
19. Pellet the MagPlex-TAG Microspheres by placing the plate on a magnetic separator for 30 to 60 seconds.
20. After the beads have pelleted, remove the supernatant being careful not to disturb the bead pellets.
21. Resuspend the pelleted MagPlex-TAG Microspheres in 75 µL of 1X xTAG hybridization buffer on a magnetic separator for 30 to 60 seconds.
22. After the beads have pelleted, remove the supernatant being careful not to disturb the bead pellets.
23. Repeat steps 8 to 11, for a total of 2 washes.
24. Resuspend microspheres in 75 µL of 1X Tm hybridization buffer containing 2–8 µg/mL SAPE.
25. Incubate at 37°C for 15 minutes.
26. Analyze 50 µL at 37°C on your Luminex analyzer according to the system manual.

Recommendations for optimization and troubleshooting xTAG® with ASPE assays

Low Reporter Intensity

1. Verify the production of the PCR products (ASPE templates) by electrophoresis.
2. Verify the hybridization assay by direct hybridization to 5 (low dose) and 50 (high dose) femtomoles of labeled oligonucleotide targets (i.e., biotinylated TAGs).
3. Titrate the target input to determine the optimal amount for hybridization.
4. Titrate the template input to determine the optimal amount for ASPE.
5. Titrate the biotinylated dCTP input to determine the optimal concentration for ASPE.
6. Increase the number of cycles in the ASPE reaction.
7. Decrease and/or increase the ASPE annealing temperature.
8. Check the primer and template sequences for potential secondary structures.

9. Redesign the PCR primers.
10. Redesign the ASPE primers for the opposite DNA strand.
11. Lengthen the ASPE primers.

Poor Discrimination

1. Increase the ASPE annealing temperature.
2. Redesign the ASPE primers for the opposite DNA strand.
3. Shorten the "leaky" ASPE primer.

Poor Reporter Distribution between Alleles

1. Redesign the ASPE primers for the opposite DNA strand.
2. Lengthen the ASPE primer to increase signal on the "low" allele.
3. Shorten the ASPE primer to decrease signal on the "high" allele.

High Background

1. Determine the optimal reporter concentration by titration. Sometimes lowering the reporter concentration may help reduce background MFI.
2. If using SAPE that does not already contain BSA (e.g., Moss SAPE-001G75), dilute the SAPE in 1X xTAG buffer containing BSA. Final BSA concentration in the hybridization reaction should be 0.1%.
3. If high background is observed for the PCR-negative control, verify performance of the Exo/SAP step.
4. If the high background is due to contamination of the PCR reaction, replace the PCR reagents.
5. If high background is observed for the hybridization negative control, replace the hybridization reagents.
6. If high background is observed for the ASPE negative control, replace the ASPE reagents.

ASPE references

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Chapter 5.3.3

Target-Specific PCR Sequence Detection with MagPlex-TAG™ Microspheres

There are several different PCR approaches for generating reporter molecules to detect sequences sequences in different types of samples. These approaches have been used for a number of applications, including the detection of various pathogens.[1] With standard PCR reaction chemistries, double stranded PCR amplicons are generated along the whole length of the target sequence and primers. These double stranded amplicons can generate low signals in xTAG® applications since the biotin-labeled TAG strand will preferentially bind its complementary anti-TAG strand rather than the complementary anti-TAG sequence on the beads. To address this, elimination or reduction of the amount of unlabeled complementary strand could be accomplished with more complex protocols, typically involving enzyme treatments or asymmetric PCR chemistries.

A simpler and more straightforward approach is to prevent synthesis of the complementary anti-TAG portion of the amplicon during the PCR reaction. This can be achieved with the use of a TAG-containing primer, where the TAG sequence is separated from the sequence-specific portion of the primer with an internal spacer (**Figure 33A**). When this TAG primer is combined with a sequence-specific 5′ biotinylated reverse primer, an amplicon containing a single-stranded TAG overhang and a sequence specific double-stranded biotinylated region is generated (**Figure 33B**). The TAG portions of these amplicons do not have a competing anti-TAG complementary strand to inhibit binding to the anti-TAG sequences on the MagPlex-TAG beads (**Figure 33C**).

Figure 33. Outline of Target-Specific PCR Primer Design and Chemistry

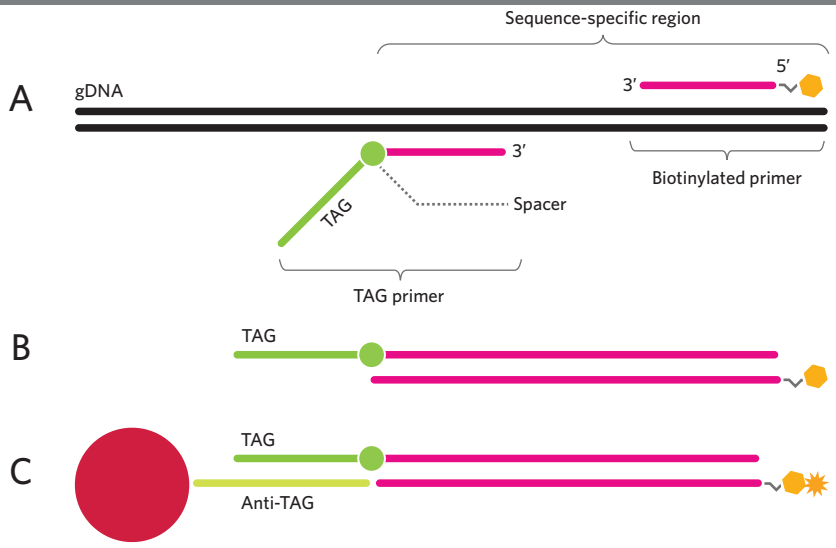

Figure 33 - Target-specific PCR sequence detection involves (**A**) a TAG-containing primer separated from the sequence-specific portion by an internal spacer. When combined with a biotinylated sequence-specific reverse primer, (**B**) a double-stranded amplicon is created with a single-stranded TAG overhang. (**C**) The TAG overhang allows hybridization to MagPlex-TAG™ microspheres without a competing complementary anti-TAG strand generated in the PCR reaction.

1. Babady NE, Mead P, Stiles J, et al. Comparison of the Luminex® xTAG® RVP Fast Assay and the Idaho Technology FilmArray RP assay for detection of respiratory viruses in pediatric patients at a cancer hospital. J Clin Microbiol. 2012;50(7):2282-2288.

Materials needed

Reagents and Consumables	Vendor
MagPlex-TAG™ Microspheres	Luminex
Spacer-modified TAG PCR primers	Integrated DNA Technologies or other vendor
5′ biotinylated reverse PCR primers	Integrated DNA Technologies or other vendor
QIAGEN HotStarTaq® 2X master mix	QIAGEN 203443 or equivalent
2X xTAG® hybridization buffer	See Target-Specific PCR Buffer and Reagent Recipes section
1X xTAG® hybridization buffer	See Target-Specific PCR Buffer and Reagent Recipes section
Streptavidin-R-phycoerythrin (SAPE)	Moss, Inc. SAPE-001G75, Thermo Fisher (Invitrogen S866), ProZyme®, or equivalent
Reporter 2 dyes for xMAP INTELLIFLEX® DR-SE: SuperBright® 436 Brilliant Violet 421 StarBright™ Violet 440	Thermo Fisher eBioscience Streptavidin SuperBright® 436 (62-4317-82), BD Biosciences BD Horizon™ BV421 Streptavidin (563259), Bio-Rad Streptavidin:StarBright™ Violet 440 (STAR210SBV440)
0.2 mL PCR tubes	Any suitable brand
96-well PCR plate	Bio-Rad MSP9601
96-well bead hybridization plate (optional)	Corning Thermowell™ 6509
Microseal® 'A' film	Bio-Rad MSA5001
Magnetic separation plate (special order)	V&P Scientific VP771LD-4CS or equivalent
Disposable pipette tips; multi- and single-channel (2–1,000 µL)	Any suitable brand
25 mL reservoirs (divided well)	Any suitable brand
RNase/DNase-free microcentrifuge tubes 1.5 mL	USA Scientific or equivalent
Barrier pipette tips	Any suitable brand
Vortex mixer	Any suitable brand
Microcentrifuge	Any suitable brand
Bath sonicator (40-55 kHz)	Any suitable brand
Centrifuge with microplate swinging bucket rotor	Eppendorf 5804 or equivalent
Brayer roller, soft rubber, or silicon	USA Scientific 9127-2940
Thermocycler with 96-well head and heated lid	Any suitable brand
Luminex instrument with xPONENT® or higher software	Luminex

For complete equipment and materials list, see **Appendix B**.

Note: Molecular grade ddH_2O should be used for all nucleic acid protocols.

Note: For dual reporter assays using the xMAP INTELLIFLEX® DR-SE System, one sequence-specific reverse primer must be directly conjugated to a reporter dye, but the other sequence-specific reverse primer may be biotinylated for use with a streptavidin-reporter dye conjugate. Alternatively, both sequence-specific reverse primers may be directly conjugated to the relevant Reporter 1 or Reporter 2 dye. We recommend Alexa Fluor™ 532 for RP1 and Alexa Fluor™ 405 for RP2.

Target-specific PCR buffer and reagent recipes

Step	Notes
MagPlex-TAG™ Microspheres from Luminex	Required microsphere regions should be purchased from Luminex. They should be stored at 4°C in the dark. For multiplex assays, combine different bead regions as directed in the protocol.
Enzymes and enzyme reaction buffers	All enzymes and their reaction buffers can be used as directed in the protocol.
PCR primer design	PCR primers should be designed to amplify a region containing any sequence of interest and the pairs should be matched for melting temperatures at 51–56°C. Primers should amplify a region in the 100–150 bp range for best performance. The TAG containing primer should have a 12 to 18 atom spacer separating the TAG sequence on its 5′ end from its sequence specific 3′ end. The reverse primer should be biotinylated on its 5′ end. When designing these primers, use oligo design software to select an appropriate TAG sequence to include on the TAG primers, or contact Luminex Technical Support for assistance in selecting TAG sequences. These primers can be purchased from multiple vendors, such as IDT. Upon receipt, the primers should be dissolved or diluted with sterile molecular grade ddH_2O to a concentration of 1 mM (1 nanomole/µL). They can be stored as individual stocks at -20°C. Pooled mixes can be made to deliver 0.2 µM of each primer/ PCR reaction, or as your optimized PCR protocol requires.
1X xTAG® hybridization buffer	The buffer's composition is 0.2 M NaCl, 0.1 M Tris, 0.08% Triton™ X-100, pH 8.0. It should be filter sterilized and stored at 4°C.
Streptavidin-R-Phycoerythrin (SAPE)	SAPE can be purchased from a number of suppliers, such as Moss Incorporated (SAPE-001G75), Thermo Fisher (Invitrogen S866), ProZyme® (various), or equivalent. A working aliquot should be made fresh by diluting with 1X xTAG® Hybridization buffer to the required volume and concentrations needed as indicated in the washed or no-wash protocols.
Reporter 2 dyes for xMAP INTELLIFLEX® DR-SE: SuperBright® 436 Brilliant Violet 421 StarBright™ Violet 440	Reporter 2 dyes can be purchased from various suppliers, including Thermo Fisher (eBioscience™ Streptavidin SuperBright® 436, Catalog # 62-4317-82), BD Biosciences (BD Horizon™ BV421 Streptavidin, Catalog # 563259), Bio-Rad (Streptavidin:StarBright™ Violet 440, Catalog # STAR210SBV440), or an equivalent. A working aliquot should be made fresh by diluting with 1X xTAG® Hybridization buffer to the required volume and concentrations needed as indicated in the washed or no-wash protocols.

Protocol 5.3.3: Target-specific PCR sequence detection

Target Sequence Amplification

Multiplexed PCR amplification of target regions should be performed under optimized conditions. The parameters listed below are for an example only and may not be optimum for your samples or any specific genomic amplification protocol you may be using.

1. Assemble PCR reactions. Each final reaction contains:

Reagent	Amount
DNA template	50 ng
QIAGEN PCR reaction buffer	1X
$MgCl_2$	1.5 mM
dNTP	200 µM each
xTAG® and biotinylated primer	0.2 µM each
QIAGEN HotStartTaq® or other Taq polymerase	2.5 units

2. Perform PCR with the following program:

Temperature	Time	Cycle
95°C	15 minutes	
94°C	30 seconds	
55°C	30 seconds	35 cycles
72°C	30 seconds	
72°C	7 minutes	
4°C	Hold	

Note: The temperature of the 55°C annealing step can be adjusted as needed.

3. Proceed to hybridization with MagPlex-TAG™ Microspheres using the no-wash protocol. This example protocol uses SAPE as the reporter. Optimal reporter concentration should be determined by titration.

5.3.3.1 Hybridization to MagPlex-TAG Microspheres: No-wash protocol

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

1. Select the appropriate MagPlex-TAG microsphere sets and resuspend according to the instructions described in the product information sheet provided with your microspheres.
2. Combine 2,500 microspheres of each set per reaction.
3. Dilute/concentrate the MagPlex-TAG microsphere mixture to 125 microspheres of each set per µL in 1X xTAG hybridization buffer and mix by vortex and sonication for ~20 seconds.
4. Aliquot 20 µL of the MagPlex-TAG microsphere mixture to each well including those for bead background. This will provide 2,500 beads of each set per reaction.
5. Add 1 to 5 µL of each PCR reaction to appropriate wells and add 5 µL of dH_2O to each background well.
6. Adjust the total volume to 25 µL by adding the appropriate volume of dH_2O to each sample well that received less than 5 µL of PCR reaction.
7. Prepare reporter mix by diluting SAPE to 8–10 µg/mL in 1X xTAG hybridization buffer.
8. Add 70–75 µL SAPE to each well. Mix gently.
9. Cover the plate to prevent evaporation and hybridize in a thermal cycler or a temperature controlled bench top plate shaker with the following parameters;
   37–45°C for 25 to 45 minutes (up to 45°C may be used to improve specificity of TAG/anti-TAG annealing).
10. Analyze 70 µL at the hybridization temperature on your Luminex analyzer according to the system manual.

Note: If needed, an Excel®-based bead calculator is available for determining the method and volumes needed for making the bead mix. **Contact Luminex Technical Support** for a copy.

Recommendations for optimization and troubleshooting

Low Reporter Intensity

1. Verify production of amplified products by electrophoresis.
2. Verify labeling of amplified target.
3. Try increasing and decreasing the target input to determine the optimal amount.
4. Check primer and target sequences for potential secondary structures.
5. Check primer and target sequences for specific sequence complementarity.
6. Redesign PCR primers to target a different region if needed.
7. Try increasing amount of SAPE.

Poor Discrimination

1. Decrease the target input.
2. Increase the hybridization temperature to 45°C.
3. Verify PCR primer sequence specificity and binding characteristics.
4. Redesign PCR primers to target more unique regions.

High Background

1. Determine the optimal reporter concentration by titration. Sometimes lowering the reporter concentration may help reduce background MFI.
2. If using SAPE that does not already contain BSA (i.e., Moss SAPE-001G75), dilute the SAPE in 1X xTAG buffer containing BSA. The final concentration in the hybridization reaction should be 0.1% BSA.
3. If high background is isolated to one or a few microsphere sets, test individual PCR TAG amplicons with the bead mix to determine if the high background is related to specific target cross hybridization with the microspheres.
4. Redesign targets with high background.
5. If high background occurs on all microsphere sets, try decreasing the target input to determine optimal amount.
6. Try decreasing amount of reporter (e.g., SAPE).

Target-specific PCR references

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Dual Reporter Hybridization to MagPlex-TAG™ Microspheres on the xMAP INTELLIFLEX® DR-SE

The xMAP INTELLIFLEX® DR-SE System can be used for detecting two reporter signals in a hybridization assay with various pairs of reporter dyes. The protocol below is a modified version of the Hybridization to MagPlex-TAG™ Microspheres: No-Wash Protocol (described in **Protocols 5.3.1–5.3.3**) that explains how to use two reporters to detect two signals on the xMAP INTELLIFLEX DR-SE. Note: For assays using two reporters, one detection reagent must be directly labeled with the reporter dye, while the other may be biotinylated for use with a streptavidin-reporter dye conjugate. Alternatively, both detection reagents may be directly labeled with the appropriate RP1 and RP2 dyes.

Materials needed:

Reagents and Consumables	Vendor
MagPlex-TAG™ microspheres	Luminex
1X xTAG® hybridization buffer (0.1 M Tris, pH 8.0, 0.2 M NaCl, 0.08% Triton™ X-100)	MilliporeSigma T3038 MilliporeSigma S5150 MilliporeSigma T8787
1X TMAC hybridization buffer (3M TMAC, 0.1% Sarkosyl solution, 50 mM Tris-HCL, 4 mM EDTA, pH 8.0)	Sigma T3411 Sigma L7414 Sigma T3038 Thermo Fisher 15575020
Streptavidin SuperBright® 436 conjugate (for biotinylated targets)	Thermo Fisher 62-4317-82
Bovine serum albumin solution	MilliporeSigma B8667-5ML
96-well PCR plate	Thermo Fisher AB0600
Thermocycler with 96-well head and heated lid	Any suitable brand
Microseal® 'A' film	Bio-Rad MSA5001
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500, Eppendorf Protein LoBind®, 022431081 or equivalent
Disposable pipette tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable brand

Protocol 5.3.4: No-wash hybridization to MagPlex-TAG microspheres using two reporters on the xMAP INTELLIFLEX DR-SE System

1. Select the appropriate MagPlex-TAG microsphere sets and mix by vortex and sonication for ~20 seconds.
2. Resuspend in 1X xTAG® hybridization buffer at a concentration of 2,500 microspheres of each set per 25 µL (100 microspheres/µL). Mix by vortex.
3. Aliquot 25 µL of the MagPlex-TAG™ microsphere mixture to each well (2,500 beads of each set/reaction).
4. Add 25 µL of 1X xTAG hybridization buffer to each background well.

5. Dilute the targets in 1X xTAG hybridization buffer to obtain the appropriate amount in 25 µL.
6. Add 25 µL of each sample to the appropriate wells. Mix gently by pipette.
7. Cover the plate with Microseal 'A' film to prevent evaporation and hybridize in a thermal cycler with the following parameters:
   a. 96°C for 90 seconds
   b. 37°C for 15–30 minutes
   c. Hold at 37°C"
8. Prepare reporter mix (if needed) by diluting the Streptavidin SuperBright® 436 (SASB) reporter to 1.3 µg/mL in 1X xTAG hybridization buffer containing 0.2% BSA.
   Note: Final concentrations are 0.625 µg/mL SASB and 0.1% BSA.
9. Add 50 µL of reporter mix or hybridization buffer to the appropriate wells of the plate in the thermal cycler (total volume 100 µL/well). Mix gently by pipette.
10. Incubate at 37°C for 10–15 minutes in the thermal cycler with heated lid.
11. Analyze 75 µL at 37°C on your xMAP INTELLIFLEX DR-SE analyzer according to the system manual.

Figure 34.

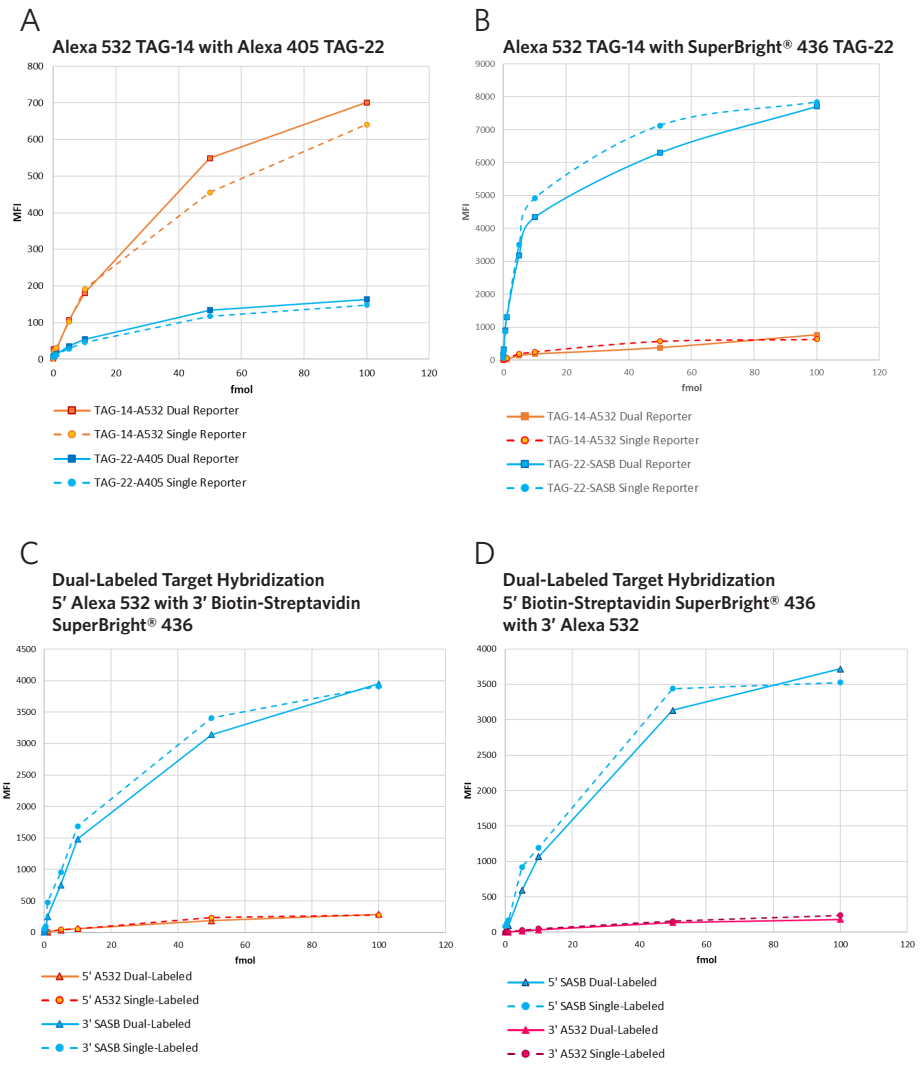

Figure 34 - Dual Reporter MagPlex-TAG™ hybridization example. (**A**) An A532-labeled TAG-14 oligo target was evaluated with an A405-labeled TAG-22 oligo in Single and Dual Reporter modes. (**B**) An A532-labeled TAG-14 oligo target was evaluated with a biotinylated TAG-22 oligo + SASB in Single and Dual Reporter modes. (**C**) Single- and dual-labeled targets with 5′ A532 and 3′ biotin + SASB were analyzed in Dual Reporter mode. (**D**) Single- and dual-labeled targets with 5′ biotin + SASB and 3′ A532 were analyzed in Dual Reporter mode. Alexa Fluor™ 532 (A532) was used for the RP1 channel and either Alexa Fluor™ 405 (AF405) or Streptavidin SuperBright® 436 (SASB) were used for the RP2 channel.

Direct DNA Hybridization Sequence Detection

For some applications, an xMAP-based genomic assay may require coupling the beads with specific capture sequences that are complementary to organism-specific sequences in the labeled reporter molecules generated by an assay chemistry. These different approaches can be used for gene expression analysis, genotyping, specific sequence detection, or other applications.[1-3] In these situations, specificity may require coupling capture probes of different lengths and/or similar base compositions to different beads in the multiplex mix.

For these types of applications, the hybridization of labeled target sequences to the beads requires stringent hybridization conditions to ensure a high degree of specificity with robust signal strength and low background. To meet these needs, the use of TMAC-containing buffers has proven to be a good alternative to other buffer systems.[3-5]

This protocol outlines a TMAC-based hybridization procedure that can be used for these types of direct hybridization assays, as well as other applications. This example protocol uses SAPE as the reporter.

Figure 35. Direct DNA Hybridization Sequence Detection

Figure 35 - Direct DNA Hybridization Sequence Detection relies on sequence-specific hybridization to capture target sequences. Labeled reporter molecules are incubated with microspheres coupled with a complementary capture sequence. Stringent hybridization conditions are required to discriminate positive reactions from non-specific binding.

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Materials needed

Reagents and Consumables	Vendor
MagPlex® Microspheres (oligo-coupled)	Supplied by user
1.5X TMAC hybridization solution	See **Direct Hybridization Buffer and Reagent Recipes** section
1X TMAC hybridization solution	See **Direct Hybridization Buffer and Reagent Recipes** section
TE, pH 8.0	See **Direct Hybridization Buffer and Reagent Recipes** section
Streptavidin-R-phycoerythrin (SAPE)	Moss, Inc. SAPE-001G75, Thermo Fisher (Invitrogen S866), ProZyme®, or equivalent
96-well PCR plate	Bio-Rad MSP9601
96-well bead hybridization plate (optional)	Corning Thermowell™ 6509
Microseal® 'A' film	Bio-Rad MSA5001
Silicon mat (optional)	Phenix Research products SMX-CM
Magnetic separation plate	Any suitable magnet
Disposable pipette tips; multi- and single-channel (2–1,000 µL)	Any suitable brand
25 mL reservoirs (divided well)	Any suitable brand
RNase/DNase-free microcentrifuge tubes 1.5 mL	USA Scientific or equivalent
Barrier pipette tips	Any suitable brand
Vortex mixer	Any suitable brand
Microcentrifuge	Any suitable brand
Bath sonicator (40-55 kHz)	Any suitable brand
Centrifuge with microplate swinging bucket rotor	Eppendorf 5804 or equivalent
Brayer roller, soft rubber, or silicon	USA Scientific 9127-2940
Thermocycler with 96-well head and heated lid	Any suitable brand
Luminex instrument	Luminex

For complete equipment and materials list, see **Appendix B**.

Note: At the time of release, preliminary testing performed on the xMAP INTELLIFLEX® DR-SE with Alexa Fluor™ 532- and Alexa Fluor™ 405-labeled targets in TMAC hybridization solutions performed well. However, experiments with streptavidin-conjugated SuperBright® 436 have shown high background when used with TMAC hybridization solutions. We have not yet developed a procedure for using this RP2 reporter dye with TMAC and have not tested other streptavidin-RP2 dye conjugates with TMAC.

Direct hybridization buffer and reagent recipes

Step	Notes
MagPlex® Microspheres coupled with desired capture sequences	Required microspheres region should be purchased from Luminex and coupled with desired capture probes as outlined in the Standard Nucleic Acid Coupling to xMAP® microspheres protocol (**Chapter 5.2.1**). The coupled beads should be resuspended as recommended in the coupling protocol and stored at 4°C in the dark. For multiplex assays, combine different coupled bead regions as indicted in the following direct hybridization protocols.
1.5X TMAC hybridization solution	The composition of this solution is 4.5 M TMAC (MilliporeSigma T3411), 0.15% Sarkosyl (MilliporeSigma L7414), 75 mM Tris, and 6 mM EDTA. The solution should be stored at room temperature.
1X TMAC hybridization solution	The composition of this solution is 3M TMAC (MilliporeSigma T3411), 0.1% Sarkosyl (MilliporeSigma L7414), 50 mM Tris, and 4 mM EDTA. The solution should be stored at room temperature.
TE, pH 8.0	This is a 1X Tris-EDTA Buffer, pH 8.0. It can be purchased directly from any suitable vendor or made from more concentrated stocks. It should be filter sterilized (if diluted from concentrate) and stored at room temperature.
Streptavidin-R-phycoerythrin (SAPE)	SAPE can be purchased from a number of suppliers, such as Moss Incorporated (SAPE-001G75), Thermo Fisher (Invitrogen S866), or ProZyme® (various). A working aliquot should be made fresh by diluting with 1X TMAC Hybridization buffer to the required volume and concentrations needed as indicated in the washed or no-wash protocols.

Protocol 5.3.5.1: Direct DNA hybridization: No-wash protocol

1. Select the appropriate oligonucleotide-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for ~20 seconds.
3. Prepare a working microsphere mixture by diluting coupled microsphere stocks to 76 microspheres of each set/µL in 1.5X TMAC hybridization solution. Since 33 µL of the working microsphere mixture is required for each reaction, this will provide about 2,500 beads of each region/reaction.
4. Mix the working microsphere mixture by vortex and sonication for ~20 seconds.
5. To each sample or background well, add 33 µL of the working microsphere mixture.
6. To each background well, add 17 µL TE, pH 8.0.
7. To each sample well, add volumes of labeled target reaction and TE, pH 8.0, to a total volume of 17 µL. (Note: For most assay chemistries, 1-5 µL of a robust PCR or labeled target reaction will be sufficient for detection.)
8. Mix reaction wells gently by pipetting up and down several times.
9. Cover the plate to prevent evaporation and hybridize in a thermal cycler with the following parameters:
   95°C for 5 minutes (denaturation step)
   45 to 60°C for 15 to 20 minutes (hybridization step)
10. During the hybridization incubation, prepare fresh reporter mix by diluting SAPE to 6–24 µg/mL in 1X TMAC hybridization solution (which will provide a final SAPE concentration of 2–8 µg/mL).
11. Add 25 µL of reporter mix to each well and mix gently by pipetting up and down several times.

Note: An Excel®-based bead calculator is available for determining the method and volumes needed for making the working microsphere mixture. **Download it here**.

Note: Use the optimum hybridization temperature for the target sequences in the mix.

Note: 25 µL of reporter mix is required for each reaction.

12. Incubate the reaction plate at the hybridization temperature for 5 minutes.
13. Analyze 50 µL at the hybridization temperature on your Luminex analyzer according to the system manual.

Protocol 5.3.5.2: Direct DNA hybridization washed protocol

1. Select the appropriate oligonucleotide-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for ~20 seconds.
3. Prepare a working microsphere mixture by diluting coupled microsphere stocks to 76 microspheres of each set/µL in 1.5X TMAC hybridization solution. Since 33 µL of the working microsphere mixture is required for each reaction, this will provide about 2,500 beads of each region/reaction.
4. Mix the working microsphere mixture by vortex and sonication for ~20 seconds.
5. To each sample or background well, add 33 µL of the working microsphere mixture.
6. To each background well, add 17 µL TE, pH 8.0.
7. To each sample well, add volumes of labeled target reaction and TE, pH 8.0, to a total volume of 17 µL. (Note: For most assay chemistries, 1–5 µL of a robust PCR or labeled target reaction will be sufficient for detection.)
8. Mix reaction wells gently by pipetting up and down several times.
9. Cover the plate to prevent evaporation and hybridize in a thermal cycler with the following parameters:
   95°C for 5 minutes (denaturation step)
   45 to 60°C for 15 to 20 minutes (hybridization step)
10. During the hybridization incubation, prepare fresh reporter mix by diluting SAPE to 2–8 µg/mL in 1X TMAC hybridization solution.
11. Place plate on plate magnet for 30–60 seconds to pellet the microspheres.
12. After beads have collected on side of wells, carefully remove the supernatant.
13. Remove plate from the plate magnet and return the sample plate to the hybridization temperature.
14. Add 75 µL of reporter mix to each well and mix gently by pipetting up and down several times.
15. Incubate the reaction plate at the hybridization temperature for 5 minutes.
16. Analyze 50 µL at the hybridization temperature on your Luminex analyzer according to the system manual.

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

Note: An Excel®-based bead calculator is available for determining the method and volumes needed for making the working microsphere mixture. **Download it here**.

Note: Use the optimum hybridization temperature for the target sequences in the mix.

Note: 75 µL of the reporter mix is required for each reaction.

Note: An 8-channel pipettor can be used to extract the supernatant in 8 wells simultaneously.

Recommendations for optimization and troubleshooting

Low Reporter Intensity

1. Verify coupling and hybridization assay components by direct hybridization to labeled reverse complementary oligonucleotides (0 to 200 femtomoles).
2. Verify production of amplified target.
3. Verify labeling of amplified target.
4. Try increasing and decreasing the target input to determine the optimal amount.
5. Decrease the hybridization temperature.
6. Check probe and target sequences for a potential secondary structure.
7. Increase probe length.
8. Decrease size of target.
9. Redesign probes and target for the opposite DNA strand.

Note: Some of these problems and solutions will be unique to the chemistry used to generate labeled targets and not all are addressed in the following recommendations.

Poor Discrimination

1. If using SAPE that does not already contain BSA (i.e., Moss SAPE-001G75), dilute SAPE in 1X TMAC buffer containing BSA. The final concentration in the hybridization reaction should be 0.1% BSA.
2. Decrease the target input.
3. Increase the hybridization temperature.
4. Decrease probe length.
5. Redesign probes and target for the opposite strand of DNA.

High Background

1. Determine the optimal reporter concentration by titration. Sometimes lowering the reporter concentration may help reduce background MFI.
2. If using SAPE that does not already contain BSA (i.e., Moss SAPE-001G75), dilute SAPE in 1X TMAC buffer containing BSA. The final concentration in the hybridization reaction should be 0.1% BSA.
3. If high background is isolated to one or a few microsphere sets, recouple the probes to different microsphere sets to determine if the high background is related to the probes or the microspheres.
4. Resynthesize probes with high background.
5. If high background occurs on all microsphere sets from the same coupling reaction, use coupled microspheres with low background to test hybridization buffers for contamination.
6. Replace all coupling buffers and recouple.

Low Bead Count

1. Microsphere mix was diluted incorrectly. Make sure the microsphere mix is vortexed thoroughly and prepared correctly. *Note: If needed, an Excel®-based bead calculator is available for determining the method and volumes needed for making the bead mix. Download it here.*
2. Beads were lost during washes. When using a manual wash, make sure the assay plate is properly seated in the magnetic separator. Make sure you selected a suitable magnetic separator based on the type of plate and reaction volumes used in the assay. Guidelines for plate selection can be found at luminexcorp.com/blog/selecting-the-right-plate-magnet-for-luminex-assays/ and in **Appendix B**. Be careful not to hold the pipette tip immediately above the beads/bead pellet; be sure to aim away from the pelleted beads. Carefully remove the supernatant slowly. When using automatic plate washers, make sure the washer settings are programmed according to the User's Manual for your plate washer and the appropriate separator is used.

3. Incorrect probe height adjustment. Adjust probe height according to the instruments User's Manual.
4. Incorrect protocol set-up on the Luminex instrument. Make sure correct bead regions are selected based on your particular bead mix.
5. Beads shifting out of region in the bead map on the Luminex instrument. Ensure hybridization/wash buffer was made properly and washes (if any) are performed thoroughly. Make sure the bead solution is stored in the dark at 4°C to prevent photobleaching.

Direct DNA hybridization references

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Chapter 5.3.6

MicroRNA Analysis

A number of PCR-based and direct hybridization assays are available for the analysis of miRNA expression levels. Most of the PCR-based approaches can only be run as singleplex assays in individual reactions or on costly chips, increasing processing times, requiring more sample, and limiting the number of samples that can be processed rapidly.[1-3] Hybridization assays can be multiplexed to different degrees with the use of special costly probes, cassettes, and analysis instruments.[4-5] Many of these chemistries are suitable for analysis of expression levels, but often lack the ability to distinguish between closely related miRNA targets that differ by a single base. In addition to the lack of single base resolution, these assays can also be costly per sample, with low sample throughput capabilities.

To overcome these obstacles, the Luminex-based nuclease protection approach takes advantage of a unique combination of three essential assay characteristics:

1. **Use of MagPlex-TAG™ Microspheres.** Users can create their own mixes as needed. These magnetic beads are available from Luminex with unique TAG sequences already coupled to them. These sequences are universal array sequences that do not cross-hybridize with each other or with native sequences.

2. **Biotin-labeled chimeric probes.** These are composed of RNA sequences that are 100% complementary to their mature miRNA targets, and a DNA sequence which is 100% complementary to specific anti-TAG sequences on the MagPlex-TAG Microspheres. The probes can be easily designed by the user, making the assay more cost effective and flexible.

3. **Nuclease protection chemistry.** This chemistry, when combined with the assay's step-down hybridization protocol, results in single-base resolution of nucleotide differences, even in miRNA species that cannot be distinguished with other chemistries.

This combination of characteristics also contributes to the miRNA assay having a short one-day protocol without sacrificing single nucleotide specificity, even without PCR amplification.[6] This is achieved by the ability of the biotinylated chimeric probes to specifically bind their miRNA targets in a short period of time with the protocol's step-down hybridization approach. The chimeric probe/miRNA complexes are then rapidly captured on MagPlex-TAG beads, followed by a short nuclease reaction that degrades mismatched and unbound probes. Following a short SAPE labeling step and some washes, the samples are ready for analysis. The following protocol is based on the former Luminex FLEXMIR® v2 product and uses SAPE as the reporter. The optimal reporter concentration should be determined by titration.

Figure 36. miRNA Assay Workflow

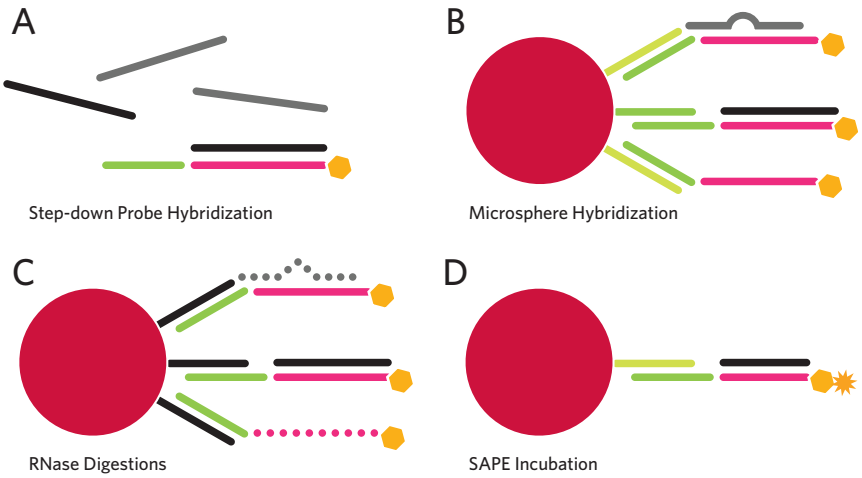

Figure 36 - (**A**) Step-down probe hybridization - DNA/RNA chimeric probes hybridize to target miRNAs during incremental reductions in annealing temperature; 2 hours. (**B**) Microsphere hybridization - miRNA-chimeric probe complexes are hybridized to microspheres; 30 minutes. (**C**) RNase digestion - Excess probes, single-stranded RNAs, and mismatched probes are digested. Only perfectly-matched probes are protected; 30 minutes. (**D**) SAPE incubation - A brief incubation with SAPE incorporates reporter molecules; 30 minutes. Detection - Targets of interest are quantified on an xMAP® instrument; <5 hours total to results.

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Materials needed

Reagents and Consumables	Vendor
MagPlex-TAG™ Microspheres	Luminex
Chimeric probes	Integrated DNA Technologies or other vendor
Stock RNase One™	Promega M4265
Wash and hybridization buffer	See miRNA Buffer and Reagent Recipes section
Streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Thermo Fisher (Invitrogen S866), ProZyme®, or equivalent
96-well PCR plate	Bio-Rad MSP9601
96-well bead hybridization plate (optional)	Corning Thermowell™ 6509
Microseal® 'A' film	Bio-Rad MSA5001
Silicon mat	Phenix Research products SMX-CM

For complete equipment and materials list, see **Appendix B**.

Note: Molecular grade ddH_2O should be used for all nucleic acid protocols.

Magnetic separation plate (special order)	V&P Scientific VP771LD-4CS or equivalent
Disposable pipette tips; multi- and single-channel (2–1,000 µL)	Any suitable brand
25 mL reservoirs (divided well)	Any suitable brand
RNase/DNase-free microcentrifuge tubes 1.5 mL	USA Scientific or equivalent
Barrier pipette tips	Any suitable brand
Vortex mixer	Any suitable brand
Microcentrifuge	Any suitable brand
Bath sonicator (40–55 kHz)	Any suitable brand
Centrifuge with microplate swinging bucket rotor	Eppendorf 5804 or equivalent
Brayer roller, soft rubber, or silicon	USA Scientific 9127-2940
Thermocycler with 96-well head and heated lid	Any suitable brand
Luminex instrument	Luminex

Note: At the time of release, preliminary testing was performed on xMAP INTELLIFLEX® DR-SE with directly labeled chimeric probes using Alexa Fluor™ 405 for RP2. For other RP2 dye options for miRNA analysis, contact your Luminex Field Applications Scientist or **Luminex Technical Support**.

miRNA buffer and reagent recipes

Enzymes and enzyme buffers	All enzymes and their buffers can be purchased from the recommended manufacturers. Use of each enzyme and its buffer in different master mixes are indicated in the protocol.
Chimeric probe design strategy	Proper design of the chimeric probes is critical for proper assay performance. (See Note). 1. Identify the miRNAs desired for the assay and obtain their sequences. 2. Select MagPlex-TAG™ bead regions desired for the plex required for cover all miRNAs of interest and internal controls. 3. 3. Design the chimeric capture probes so that each chimeric probe will capture the miRNA desired, as well as the anti-TAG sequence on the beads. Note that the capture end for the probe should be RNA and the end for the xTAG® sequence should be DNA. This chimeric oligo should be biotinylated at the RNA 5′ end (see table below). 4. Order the biotinylated chimeric DNA/RNA probes from an oligo vendor. See the Luminex chimeric probe design Excel® Workbook for additional information for probe design characteristics.

Note: To ensure proper probe design, an easy to use Excel®-based probe design tool is available. The tool ensures a more accurate design of your chimeric probe sequences in a format suitable for ordering from different oligo manufacturers. The tool can be downloaded from **the Luminex website**.

Note: The probes MUST be designed to be a perfect match to the miRNA under investigation, since the high specificity of the method allows single base resolution of mismatches between closely related miRNAs.

Probe Portion	Sequence
miRNA sequence for mmu-miR-34b-5p	5′-AGGCAGUGUAAUUAGCUGAUUGU-3′
Reverse complement of miRNA	5′-ACAAUCAGCUAAUUACACUGCCU-3′ (RNA)
Anti-TAG sequence on MagPlex-TAG MTAG-A015	5′-GTTGTAAATTGTAGTAAAGAAGTA-3′
Reverse complement of anti-TAG Sequence 15	5′-TACTTCTTTACTACAATTTACAAC-3′ (DNA)
Oligo to order	5′-Biotin-ACAAUCAGCUAAUUACACUGCCUTACTTCTTTACTACAATTTACAAC-3′

Chimeric probe mix	Individual chimeric probe preparations from the manufacturer can be dissolved to 100 µM with TE buffer, pH 8.0. If you need to make probe mixes higher than 100-plex, the individual probe should be dissolved to higher concentrations (200 µM if possible). The individual dissolved probes can be aliquoted and stored frozen at this point (as individual concentrates). To create an equimolar mix of chimeric probes, a concentrated mixed probe stock at 1 µM for each probe is first made and then further diluted to generate a working mix where 1.25 µL of probe mix for each reaction delivers 10 nM for each probe. Example: To use a 5-plex miRNA profile to analyze 100 samples, the total number of samples will require 100x1.25 µL = 125 µL of a 10nM probe mix with all 5 probes. Each of the individual chimeric probes are at 100 µM and need to be diluted 1:100 to generate the 1 µM concentrated probe mix. This 1 µM mix can be made by adding 1.25 µL of each probe into an empty tube with 118.75 µL of TE buffer (5x1.25 µL = 6.25 µL + 118.75 µL TE buffer creates 125 µL of 1 µM probe mix). The working stock is made fresh by making a 1:100 dilution of this 1 µM probe mix with hybridization buffer to bring the probe mix to the 10 nM concentration. This working dilution is the Chimeric Probe Mix that is used at 1.25 µL/reaction as described in the protocol.
Hybridization and wash buffer	The same buffer is used for hybridization to Luminex beads and washes. It is a pH 7.7 buffer consisting of 10 mM Tris, 200 mM sodium acetate, 5 mM EDTA, and 0.05% Tween® 20. This buffer can be made from 3 M sodium acetate stock solution, a 0.5 M EDTA solution (pH 8.0), a 1 M Tris pH 7.5, and a 10% Tween® 20 solution – with a final adjustment to the proper pH as needed. Filter sterilize and store at 4°C.
MagPlex-TAG™ magnetic bead mixes	Pre-defined MagPlex-TAG™ bead mixes come in a concentration of $2.5x10^6$ beads per region per mL. Since each region should contribute 1,000 beads per reaction, 0.4 µL of the bead stock would be needed for each reaction and can be diluted into a maximum volume of 4.0 µL (see protocol). Creation of a master mixture that is added as 4.0 µL/reaction to deliver 1,000 beads/reaction for each region can be made as follows, assuming the bead stock purchased has all the bead regions required for the assay. Multiply the number of samples by 0.4 µL. For example, if 100 samples are to be tested, this would be 40.0 µL of bead stock. Since 0.4 µL of this stock is needed per reaction, it can be diluted to the 4.0 µL volume required for each reaction as follows. For 100 samples, add 360 µL of TE, pH 8.0 buffer to the 40 µL of bead mix. Place the tube on a magnet or spin to pellet the beads. Remove all of the supernatant. Resuspend the beads in 400 µL of hybridization buffer. This will achieve a mix where 4 µL will deliver 1,000 beads per region to each well for 100 wells/reactions. To compensate for slight fluid loss during pipetting, a 20% overage can be calculated for these volumes. Note: For assays above 10-plex, the use of multiple bead stocks of individual bead regions will require additional concentration steps to make a master mix that can deliver all the required bead regions at 1,000 beads per region in 4 µL. If needed, an Excel®-based bead calculator is available for determining the method and volumes needed for making the bead mix, download it here.

Sample requirements for the assay:	Only purified total RNA should be used. Purified miRNA or "small RNA" is not recommended. Purification methods used to isolate miRNAs may introduce unwanted bias by selectively purifying some miRNA species over others, resulting in losses that may be universal or specific. Isolation of total RNA has no bias toward particular miRNA species. Traditional methods such as phenol/chloroform extraction may also be use for total RNA extraction. Some older bind-and-elute methods are not suitable as they do not recover miRNAs. Please make sure that a total RNA extraction method that maximizes the recovery of miRNAs is used. Adjust the sample's total RNA concentration to deliver from 250 ng to 500 ng per sample in a volume of 2.5 µL or less. Example: If the sample RNA concentration is 1 mg/mL (1,000 ng/µL), then combine 0.5 µL RNA with 2.0 µL of hybridization buffer to achieve 500 ng in the 2.5 µL recommended sample volume.

Protocol 5.3.6.1: miRNA Analysis

1. In a nuclease-free tube, for each sample and a no-RNA negative control, make a sample master mix as follows with 20% overage. The total RNA samples should be delivering 250 ng to 500 ng of RNA in 2.5 µL.

Reagent	1 rxn	N rxns + 20%
Hybridization buffer	16.25 µL	(16.25 µL x N) + 20%
Sample (total RNA or H_2O for neg. control)	2.5 µL	(2.5 µL x N) + 20%
Chimeric probe mix (10 nM each probe)	1.25 µL	(1.25 µL x N) + 20%
Total volume =	20.0 µL	(20.0 µL x N) + 20%

Note: Microspheres should be protected from prolonged exposure to light throughout this procedure.

2. Pipette 20.0 µL of the sample master mix to appropriate wells of a 96-well PCR plate.
3. Seal plate with Microseal® 'A' film using a brayer to secure the seal. Vortex for 5 seconds followed by quick spin to ensure all reagents are at the bottom of the wells.
4. Cover plate with silicon mat and place in thermal cycler programmed with the step-down profile using the following parameters:
   - 90°C for 3 minutes
   - 80°C for 6 minutes
   - Program to drop 1°C every 6 minutes until 60°C is achieved (i.e., steps will be 80° for 6 minutes, then 79°C for 6 minutes, then 78°C for 6 minutes, etc. to 60°C)
   - 37°C and HOLD until user intervention (see steps 5 and 6 below)
   - 37°C for 30 minutes after user intervention
   - 30°C and HOLD for user intervention (see steps 7 to 9 below)
   - 30°C for 30 minutes
   - END
5. At the 37°C HOLD step, pause the thermal cycler and add 4 µL of the bead mix to each well. Mix well by pipetting up and down or remove the re-sealed plate, vortex for 10 to 15 seconds and quick spin for 1–2 seconds. Note: See miRNA Buffer and Reagent Recipes on how to make the bead mix. The bead mix should deliver at least 1,000 beads/region for each reaction.
6. Resume step-down program (37°C for 30 minutes).

7. Nuclease enzyme digestion: 5 min prior to the completion of the 37°C bead hybridization step, prepare a 1:500 dilution of the stock nuclease enzyme using the hybridization buffer as the diluent. At the 30°C HOLD, pause the thermal cycler, remove Microseal® 'A' film and add 2.5 µL of diluted nuclease enzyme to each reaction while the plate remains in the cycler. It is important that the enzyme is pipetted into the bottom of the tube, not onto the walls. After addition, seal with new Microseal® 'A' film.
8. Remove the plate briefly from the thermal cycler and mix well by gentle vortexing, followed by a brief spin (1 to 2 seconds) to bring the all contents down into the bottom of the wells.
9. Return plate to the thermal cycler for the final step of 30°C for 30 minutes.
10. Five minutes prior to the end of the 30°C step, prepare a 1:500 dilution master mix of SAPE in hybridization buffer. Calculate the volume by using 75 µL per well with an overage of 20%. Example: If 10 samples are tested, make 10x75 µL of reporter solution plus 20% overage= 900 µL.
11. Before adding SAPE, remove supernatant and wash as follows: Place the plate on a magnetic separator. Let the magnetic beads migrate for 2 minutes.
12. Remove Microseal® 'A' film. With a multi-channel pipette, remove the fluid gently from the wells without disturbing the bead pellet.
13. Add 200 µL of wash buffer to each well and resuspend the beads by pipetting up and down 3 or 4 times.
14. Return the plate to the magnetic separator and again allow the microspheres (beads) to migrate and form a pellet for 2 minutes.
15. Remove the supernatant gently and carefully, as described in Step 12.
16. Add 75 µL of the diluted SAPE solution and mix by pipetting up and down several times. Seal the plate with Microseal® 'A' film.
17. Shake the sealed plate on a plate shaker for 30 minutes at room temperature. Shake at a speed that insures a mixing vortex is formed in each well.
18. Remove plate from plate shaker and place it on the magnetic separator, allowing the microspheres to migrate for 2 minutes.
19. Remove all of the solution from the wells by pipetting without disturbing the pellet.
20. Add 200 µL of wash buffer to each well and resuspend the beads by pipetting up and down 3 to 4 times
21. Return the plate to the magnetic separator and allow beads to migrate for 2 minutes and remove all the supernatant without disturbing the bead pellets.
22. Repeat steps 20 and 21 for another wash.
23. Remove plate form magnet and add 100 µL wash buffer and resuspend beads by pipetting up and down 3 to 4 times. Note: Avoid making foam or bubbles.
24. Read the plate in a suitable Luminex instrument which has been adjusted for the type of plate used. If you prefer, you may transfer the 100 µL of bead suspension to a standard bead hybridization plate (Corning Thermowall™ 6509) for analysis.

Recommendations for optimization and troubleshooting

High Background

1. Wash steps were not performed thoroughly. Ensure that as much supernatant is removed as possible during each wash step while taking care to avoid disturbing the microsphere pellet.
2. Possible reagent contamination. Replace all buffers first. Use only nuclease-free barrier pipette tips for all reagent additions and mixing.

Unexpected Results from Control Samples

3. Reagents were not stored at the recommended temperatures. Make sure all reagents are stored at the recommended temperatures. For reagents that are frozen, avoid multiple freeze-thaw cycles. Place reagent master mixes that contain enzymes on ice during preparation. If indicated, pre-warm other reagents to room temperature immediately before use.
4. Vortexing enzyme stocks. You should never vortex enzyme stocks. Instead, flick tube to mix. Gently vortex and quick-spin the enzyme solution only after you have made the recommended dilution.
5. High signal on Negative Control sample due to contamination. Make sure all consumables such as tubes and pipette tips are nuclease-free, as well as general reagents such as PBS, 10 mM Tris pH 7.0, and nuclease-free dH_2O. If problem persists, replace all buffer reagents.
6. Positive Control sample signal is too low. Verify that the purified control RNA concentration is correct and the RNA is not degraded.

Low MFI Signal

1. RNA concentration too low or RNA degraded. Verify that the RNA concentration is correct and the RNA is not degraded.
2. Thermal cycler not functioning properly or error in program. Ensure all actual incubation temperatures are within ±2°C of the recommended incubation temperature. Make sure the step-down protocol and other steps in the PCR program are entered correctly.
3. Incorrect probe hybridization temperature and/or annealing temperature. Ensure probe hybridization and temperature and annealing temperatures are optimum for the particular probe mix.
4. Nuclease enzyme too active. Make sure the enzyme is properly diluted and not at too high a concentration. Decrease concentration if needed.
5. Either streptavidin-reporter not added or incorrect reporter dilution used. Make sure reporter is stored at 4°C in the dark, do not freeze, ensure reporter dilutions are prepared as described in the protocol and the working stock is protected from light.
6. Luminex instrument was not set for detection using high PMT. Ensure Luminex analyzer is set to high reporter gain setting (high PMT) for detection in the RP1 channel.
7. Severe agitation. Avoid foam formation when pipetting reagents. You should perform all reagent additions and mixing gently and to the bottom of the well.
8. Sample evaporation. Make sure all wells are sealed properly, especially during incubations.
9. Reagent additions not performed correctly. Make sure all reagents are added at the bottom of each well. Accurate pipetting is critical for achieving tight %CVs between replicates.

Low Microsphere Count

1. Microsphere mix was diluted incorrectly. Make sure you thoroughly vortex the microsphere mix vial and prepare the dilution as described in the protocol.
2. Microspheres were lost during washes. Use the recommended magnetic plate separator (V&P Scientific VP771LD-4CS) or suitable substitute (see **Appendix B**). When performing a manual wash, make sure the plate sits properly on the magnetic separator. Be careful not to hold the pipette tip directly above or near where the microspheres are pelleted. Remove the supernatant carefully and slowly. When using automatic plate washers, make sure the washer settings are programmed according to the plate washer instruction manual. Make sure the washing protocol has been optimized for the magnetic separator and plate type used.
3. Incorrect probe height adjustment on instrument. Adjust the probe height according to the instructions in the Adjust the Probe Height section.
4. Incorrect protocol set up on the Luminex instrument. Make sure you enter assay parameters and bead regions correctly when you create your protocol.
5. Microspheres shifting out of region in the bead map on the Luminex instrument. Make sure wash buffer was prepared correctly. Make sure the microsphere solution is stored in the dark at 4°C to prevent photobleaching.

Low Specificity

1. Wrong concentration of probe mix in the reactions. Make sure the probe mix dilution is prepared correctly.
2. Pipetting errors. Verify that pipettes are calibrated and volumes measured are accurate.

Low Sensitivity

1. RNA concentration not correct or RNA degraded. Verify that the RNA concentration is not too high or too low and that the RNA is not degraded.
2. Carryover contamination. Make sure you carefully perform the manual washes to avoid sample transfer mistakes or carryover contamination. While removing plate sealers, make sure well contents do not splash over adjacent wells.
3. Chimeric probe hybridization to RNA needs to be optimized. Probe concentration or hybridization temperatures need to be adjusted. A chimeric probe titration series and a different probe hybridization step-down temperature range may need to be tested.
4. Chimeric probe sequences not accurate. Make sure the chimeric probe sequences are the correct reverse complement to the target RNA sequence. Use the Excel®-based miRNA chimeric probe design tool for proper probe design. This tool can be downloaded here.
5. Bead hybridization temperature too high or low. A temperature gradient may be needed to determine the optimum bead hybridization temperature.

Chapter 5.4

Optimization of Nucleic Acid Assays

Probe design strategy for direct hybridization

1. All probes should be exactly the same length per target sequence (using TMAC hybridization buffer).
2. For detection of point mutations, use probes between 18 and 24 nucleotides in length. 20 nucleotides is a good starting point.
3. If point mutations (or SNPs) are expected in a sequence, they should be positioned at the center of the probe sequence (i.e., position 10 or 11 for a 20-nucleotide probe). Multiple polymorphisms should be equally spaced throughout the probe sequence. Point mutations may be positioned off-center if necessary to avoid secondary structures in the probe sequence. Usually, adequate specificity can be achieved if a point mutation is at nucleotide position 8-14 in a 19- or 20-nucleotide probe.
4. Probes should be synthesized for all variants (all mutant and wild type sequences) and should be from the same DNA strand (per target sequence).
5. For unrelated sequences, probes may be lengthened. Better sensitivity may be achieved with longer probes (50 or 70 nucleotides).
6. Probes must have a primary amino group for coupling to the carboxyl group on the microsphere. We suggest synthesizing the oligonucleotide with a 5′ amine-spacer (See step 7).
7. Probes must have a spacer between the reacting amine and the hybridizing sequence. We recommend synthesizing capture probes with 5′ Amino Modifier C12.

Tips, important points, and critical factors for direct hybridization

1. Amine-substituted oligonucleotide probes should be resuspended and diluted in dH_20. Tris, azide, or other amine-containing buffers must not be present during the coupling procedure. If oligonucleotides were previously solubilized in an amine-containing buffer, desalting by column or precipitation and resuspension into dH_2O is required.
2. We recommend using EDC from Pierce or Thermo Scientific for best results. EDC is labile in the presence of water. The active species is hydrolyzed in aqueous solutions at a rate constant of just a few seconds, so care should be taken to minimize exposure to air and moisture. EDC should be stored desiccated at -20°C in dry, single-use aliquots with secure closures. A fresh aliquot of EDC powder should be used for each addition. Allow the dry aliquot to warm to room temperature before opening. Prepare a fresh 10 mg/mL EDC solution immediately before each of the two additions and discard after use.

3. Uncoupled microspheres tend to be somewhat sticky and will adhere to the walls of most microcentrifuge tubes, resulting in poor post-coupling microsphere recovery. We have found that copolymer microcentrifuge tubes from USA Scientific (#1415-2500) perform best for coupling and yield the highest microsphere recoveries post-coupling. Eppendorf Protein LoBind® (#022431081) also perform well for microsphere coupling.
4. 100 mM MES, pH 4.5 should be filter-sterilized and either prepared fresh or stored at 4°C between uses. Do not store at room temperature. The pH must be in the 4.5–4.7 range for optimal coupling efficiency.
5. The optimal amount of a particular oligonucleotide capture probe for coupling to carboxylated microspheres is determined by coupling various amounts in the range of 0.04–1 nmol per $5x10^6$ microspheres. Usually, 0.2 to 1 nmol per $5x10^6$ microspheres in a 50 µL reaction is optimal. The coupling procedure can be scaled up or down. Above $5x10^6$ microspheres, use the minimum volume required to resuspend the microspheres. Below $5x10^6$ microspheres, maintain the microsphere concentration and scale down the volume accordingly.
6. We use 5-M TMAC (Tetramethylammonium chloride) solution from MilliporeSigma (T-3411) for preparation of 1.5X and 1X TMAC hybridization solutions. We find that this TMAC formulation usually does not have a strong "ammonia" odor. Alternatively, 6M TMAC solution from AppliChem (DarmStadt Germany 5456) has also performed well. TMAC of lower purity and with a strong odor sometimes leads to higher backgrounds in direct hybridization assays. TMAC hybridization solutions should be stored at room temperature to prevent precipitation of sarkosyl. TMAC hybridization solutions can be warmed to hybridization temperature to re-solubilize precipitated sarkosyl.
7. Denaturation and hybridization can be performed in a thermal cycler. Use a heated lid and a spacer (if necessary) to prevent evaporation. Maintain the hybridization temperature throughout the labeling and analysis steps.
8. The hybridization kinetics and thermodynamic affinities of matched and mismatched sequences can be driven in a concentration-dependent manner. At concentrations beyond the saturation level, the hybridization efficiency can decrease, presumably due to competition of the complementary strand and renaturation of the PCR product. Therefore, it is important to determine the range of target concentrations that yield efficient hybridization without sacrificing discrimination.
9. Whether it is necessary to perform a wash step before the labeling step is dependent on the amount of biotinylated PCR primers and unhybridized biotinylated PCR products that are present and available to compete with the hybridized biotinylated PCR product for binding to the SAPE reporter.

Oligonucleotide coupling optimization

It is recommended that you check the efficiency of each coupling with a range of biotinylated complementary oligonucleotide concentrations. For example, if you coupled xMAP® microsphere #1 to 4 different amounts of oligo #1 (ranging from 0.04 nmol to 5.0 nmol), each of these couplings should be hybridized with several amounts of biotinylated complementary oligonucleotide target as shown below:

5 fmols Labeled Complementary Target	25 fmols Labeled Complementary Target	50 fmols Labeled Complementary Target	100 fmols Labeled Complementary Target
Bead 1- no oligo	Bead 1- no oligo	Bead 1- no oligo	Bead 1- no oligo
Bead 1- 0.04nmol oligo #1	Bead 1- 0.04nmol oligo #1	Bead 1- 0.04nmol oligo #1	Bead 1- 0.04nmol oligo #1
Bead 1- 0.20 nmol oligo #1	Bead 1- 0.20 nmol oligo #1	Bead 1- 0.20 nmol oligo #1	Bead 1- 0.20 nmol oligo #1
Bead 1- 1.00 nmol oligo #1	Bead 1- 1.00 nmol oligo #1	Bead 1- 1.00 nmol oligo #1	Bead 1- 1.00 nmol oligo #1
Bead 1- 5.00 nmol oligo #1	Bead 1- 5.00 nmol oligo #1	Bead 1- 5.00 nmol oligo #1	Bead 1- 5.00 nmol oligo #1
H_2O background (no beads)	H_2O background (no beads)	H_2O background (no beads)	H_2O background (no beads)

Figure 37. Oligo Coupling Optimization

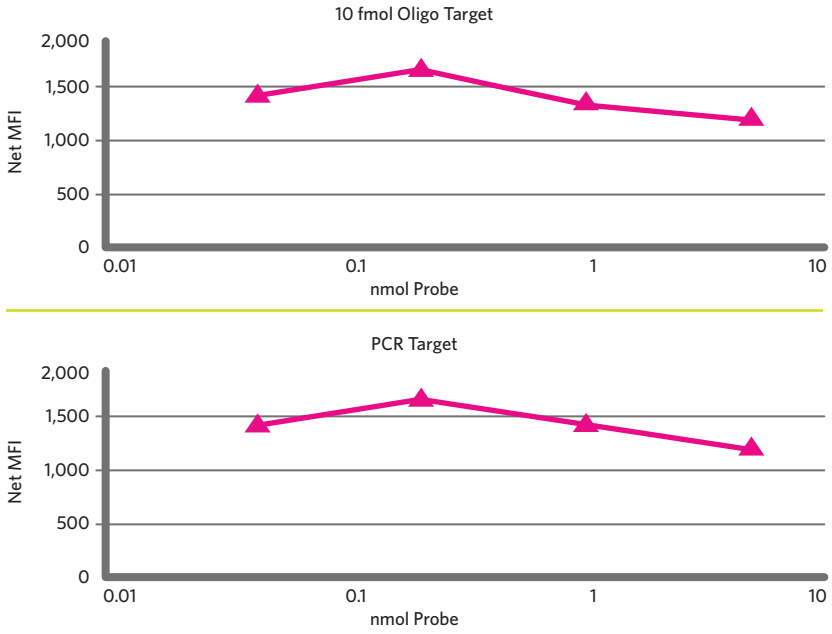

Figure 37 – Plot of experimental results for oligo coupling, as measured by a Luminex analyzer. Results show that 0.2 nmol for a 5 million microsphere coupling yielded the highest MFI values. The optimal amount for a typical coupling is usually 0.2 to 1 nanomoles per 5 million microspheres.

To determine the optimum ratio for scaled coupling reactions, use the following table as a guide for adjusting the amount of reagents used in the coupling reactions:

Recommendations for Scaling Oligonucleotide-Microsphere Coupling

Number of Microspheres	Reaction Volume (µL)	Probe Input* (nmol)	EDC Concentration (mg/mL)	Tween® 20 Wash Volume (mL)	SDS Wash Volume (mL)	Final Resuspension Volume†
$1x10^6$	10	0.04–0.1	0.5–1	0.5	0.5	20 µL
$2.5x10^6$	25	0.1–0.2	0.5–1	0.5	0.5	50 µL
$5x10^6$	50	0.2–1	0.5–1	1.0	1.0	100 µL
$10x10^6$	50	0.5–1	0.5–1	1.0	1.0	200 µL
$50x10^6$	50–100	1–4	0.5–1	1.0	1.0	1.0 mL
$100x10^6$	100–200	1–4	0.5–1	1.0	1.0	2.0 mL
$200x10^6$	200–300	2	0.5–1	1.0	1.0	4.0 mL
$400x10^6$	400	4	0.5–1	1.0	1.0	8.0 mL

*We recommend titrating the probe input to optimize coupling for the particular application when needed.
†Resuspension volume of TE, pH 8.0 for 50,000 microspheres/µL assuming 100% recovery. Adjust as needed.

Optimizing alternative sample types for genotyping assays

Genotyping methods across different sample types typically differ only in the genomic isolation step. Some samples may have contaminants or inhibitors that can affect downstream quantification or PCR reactions, and these components need to be removed to improve results. Once the DNA has been isolated and quantified, PCR reactions are essentially independent of sample type. Genomic DNA isolation involves cell lysis followed by purification of the DNA. Cell disruption can be accomplished in several ways, generally using a lysis buffer that includes a detergent to break apart the plasma membrane and a protease to break down proteins in the sample to allow further purification. Purification of the DNA may involve alcohol precipitation, filtration, or other available kit protocols.

Sample Protocol for Extraction of Human Normal Skin and Hypertrophic Scar Tissue for Genotyping Assays[1]

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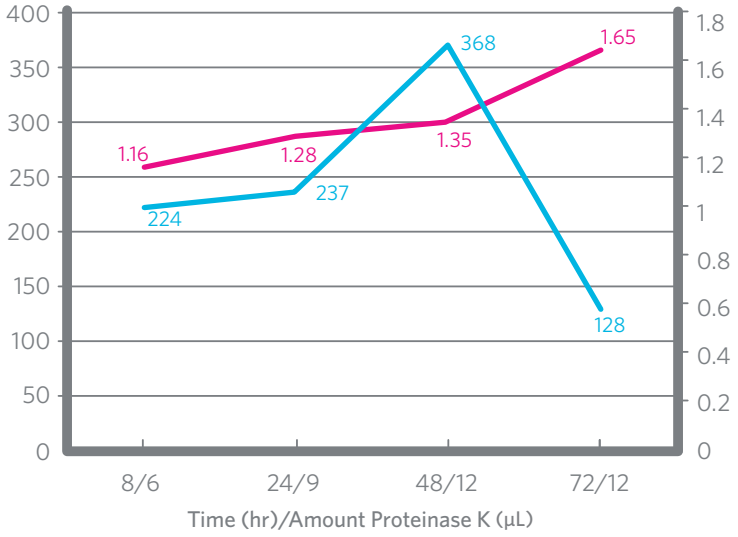
Skin tissue contains contractile proteins, collagen, and other components that may require more robust protease treatment to facilitate disruption and purification.[2] DNA extraction of tissue samples can be performed using using the QIAamp® DNA Mini commercial kit (QIAGEN, USA), but with modifications to the Proteinase K amount and incubation time to optimize the yield and purity of the extracted DNA. As shown in **Figure 38** below, Proteinase K amounts were tested at several input volumes and incubation times. An input volume of 12 µL with a 72-hour incubation provided the highest purity with a final A260/A280 nm ratio of 1.65 and a concentration of 128 mg/mL. This was found to be suitable for downstream genotyping applications.

Figure 38.



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Chapter 5.5

Nucleic Acid Assay Validation

After confirmation of successful coupling and sufficient signal from a multiplex assay, coupled microspheres should be further validated as a genomic assay. Each assay may have specific validation requirements, but typical parameters include accuracy, limit of detection (LoD), limit of background (LoB), input range, precision, reproducibility, and potential interfering substances. The examples for genomic/genotyping assays described below are for informational purposes only. Several resources are available for guidance in determining the performance characteristics for an assay, such as those listed at the end of this section. Be sure to consult the references appropriate for your laboratory and assay to ensure all required parameters have been incorporated into your validation study design. The Clinical and Laboratory Standards Institute (clsi.org) is a good source for consensus standards and guidelines for molecular methods.

Accuracy

Generally, assay results should be compared to a 'gold standard' method. For genotyping assays, this standard is sequencing.

Limit of Detection (LoD)

The LoD is defined as the lowest amount of genomic DNA in a sample for which the assay can detect genotypes with a given accuracy and precision. For example, one may choose to use a probability of 95% with a false-negative level of 5%. Smaller error levels may also be considered.

Limit of Background (LoB)

The LoB is defined as the highest measurement result (MFI) that is likely to be observed for a sample that is negative for a mutation.

Input Range

Input range is defined as the range of input DNA for which the assay can accurately detect genotypes with a stated probability, for example, at a 95% positivity rate with a 95% confidence interval (CI).

Precision and Reproducibility

Evaluate pre- and post-extraction reproducibility, but also look at within-run and between-run reproducibility. Some examples are listed below.

- Precision
  - One site, 21 runs, 3 reagent lots and 3 instruments
  - Multiplexed assays vs. 'X' specimens with single analyte, 4 specimens with 2 analytes (1 high, 1 low) and 1 negative control specimen in sample matrix

- Reproducibility
  - 3 sites, 5 runs, 'X' single-positive specimens at 3 dilutions, 4 specimens with dual-positive specimens and one negative specimen
  - Dilutions at LoD, 0.1 x LoD NS 10, or 100 X LoD
  - Each sample tested in 6 replicates per run
  - Extraction method—'X' samples tested with 3 lots each of 3 different extractions methods
- LoD/LoB
  - 25–80 replicates per dilution; non-Gaussian—nonparametric ranking methods are used.
  - LoD for each target, >95%positive calls (beta or type II error risk <5%)
  - LoB for each target, <5% positive calls (alpha or type I error risk <5%)

Interfering Substances

Examine the effects of potential interfering substances that might be expected to be found in samples. For example, in whole blood samples, potential interfering substances include hemoglobin, bilirubin, and various triglycerides.

Genomics assay validation references

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- MM12-A: Diagnostic Nucleic Acid Microarrays, Approved Guideline. Clinical Laboratory Standards Institute (Internet). Cited 2021 June. Available from: www.clsi.org.
- MM17-A: Verification and Validation of Multiplex Nucleic Acid Assays, Approved Guideline. Clinical Laboratory Standards Institute (Internet). Cited 2021 June. Available from: www.clsi.org.

Chapter 5.6

Genomics FAQs

What primer design software should I use?

Luminex has not tested every primer design software available on the market. Customers have used PrimerPlex from Premier Biosoft, as well as others.

References using PrimerPlex software for xMAP® genomics assays:

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How do I choose which genomic format to use?

The table below provides a general guidelines on which method to choose.

Nucleic Acid Format Comparison

Feature	Direct Hybridization	xTAG®/ASPE	xTAG®/OLA	Target Specific PCR Sequence Detection
Usage	SNPs, multiple polymorphisms, unrelated sequences	SNPs, multiple polymorphisms, unrelated sequences	SNPs, multiple polymorphisms, unrelated sequences	SNPs, multiple polymorphisms, unrelated sequences
Plex	Best for low-mid plex (1–50), more possible	Up to 500-plex	Up to 500-plex	Best for low-mid plex (1–50)
Oligos Required	PCR primers, Capture probes	PCR primers, Target-specific primers	PCR Prim primers ers, Target-specific primers	Target-specific PCR primers
Coupling Required	Yes	No	No	No
Workflow	PCR, <300 bp, Biotinylate target strand, hybridization & detection	PCR, all sizes, Exo-SAP, enzymatic genotyping, hybridization & detection	PCR, all sizes, enzymatic genotyping, hybridization & detection	Target-specific PCR <300 bp, hybridization & detection
Hybridization Conditions	TMAC Buffer, 45–55°C (optimize) for 15–30 minutes, wash/no-wash	Tm buffer, 37°C for 30 minutes, wash/no-wash	Tm buffer, 37°C for 30 minutes, wash/no-wash	Tm buffer, 37–45°C for 25–45 minutes, wash/no-wash
Total Time	~3.5 hours	~6–7 hours	~4 hours	~4.5 hours

Can I use TMAC hybridization buffers for xTAG® assays with MagPlex-TAG™ assays?

Yes. Although the xTAG® sequences were designed and optimized for use with Tm hybridization buffers, many investigators use them with the TMAC buffers.[1-4] TMAC stabilizes AT base pairs, minimizing the effect of base composition on hybridization, but we have also found that it enhances the signal of nucleic acid assays when using phycoerythrin reporter dye.

1. Peck D, Crawford ED, Ross KN, et al. A method for high-throughput gene expression signature analysis. Genome Biol. 2006;7(7):R61. doi: 10.1186/gb-2006-7-7-r61.
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3. McNamara DT, Kasehagen LJ, Grimberg BT, et al. Diagnosing infection levels of four human malaria parasite species by a polymerase chain reaction/ligase detection reaction fluorescent microsphere-based assay. Am J Trop Med Hyg. 2006 Mar;74(3):413-21.
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How can I reduce the background signal in hybridization assays?

Try adding BSA (e.g., BSA 30% solution, MilliporeSigma, A7284) to the SAPE reporter mixture so that the final concentration in the hybridization reaction is ≤0.1% BSA. Moss SAPE-001G75 comes with BSA included at 75 mg/mL. Results from hybridization of MagPlex® microspheres in TMAC buffer and MagPlex-TAG™ microspheres in Tm buffer with and without 0.1% BSA are shown below.

MagPlex®, TMAC Hybridization Buffer

Treatment	Sample	Target 1	Target 2	Target 3	Target 4
No BSA	Background	**752**	**324**	**554**	**479**
	Sample 1	786	285	497	376
	Sample 2	754	486	559	445
	Sample 3	1,713	3,444	1,374	1,373
	Sample 4	3,979	15,149	1,771	3,830
	Sample 5	5,721	19,678	4,282	7,055
	Sample 6	5,673	20,545	4,265	7,173
	Sample 7	6,390	21,012	5,059	7,491
0.1% BSA	Background	**59**	**85**	**85**	**47**
	Sample 1	57	94	94	90
	Sample 2	102	302	302	238
	Sample 3	1,008	3,158	3,158	1,017
	Sample 4	3,238	15,395	15,395	3,269
	Sample 5	5,092	19,602	19,602	6,573
	Sample 6	5,488	20,841	20,841	6,933
	Sample 7	5,770	21,072	21,072	6,962

MagPlex-TAG™, Tm Hybridization Buffer

Treatment	Sample	Target 1	Target 2	Target 3	Target 4
No BSA	Background	**513**	**195**	**294**	**396**
	Sample 1	6,091	7,002	3,320	4,750
	Sample 2	15,313	16,257	10,934	13,907
0.1% BSA	Background	**36**	**47**	**28**	**42**
	Sample 1	4,504	5,510	2,203	4,517
	Sample 2	11,900	12,399	8,323	10,232

Also be sure to use the Internal Sample Wash option when setting up MAGPIX® Protocols in xPONENT® Software. Internal Wash will rinse away unbound reporter in the supernatant from the imaging chamber before reading the microspheres.

What are the advantages or disadvantages of using washed vs. no-wash protocols for nucleic acid hybridization assays?

Whether wash steps after the hybridization and/or labeling steps are needed depends on the amount of excess biotinylated primers and unhybridized products that are present to compete for binding to streptavidin-reporter. Generally, the no-wash protocol works well as the recommended product input and reporter concentrations are optimized for highest signal. A final post-labeling wash step is also generally not required because of the background subtraction algorithm used by the Luminex analyzers, and MAGPIX is capable of performing an internal sample wash step (set up in the Protocol in xPONENT Software). We recommend starting with the no-wash protocols, adjusting target input and reporter concentrations as needed, and switching to a washed protocol when necessary for optimal assay performance.

What hybridization temperature should I use for xTAG assays?

The xTAG sequences have been designed and optimized for use with the Tm hybridization buffers at 37°C. However, sometimes better performance can be achieved at 45°C when using the xTAG system with either the Tm or TMAC hybridization buffer systems.

Are there other methods for genomic assays that are not described in the xMAP Cookbook?

Yes, and a few examples are listed below. xMAP Technology is very flexible and scientists have been creative in designing a multitude of methods. Here are some examples of other genomics application references:

- Aste-Amezaga M, Zhang N, Lineberger J, et al. Characterization of Notch1 antibodies that inhibit signaling of both normal and mutated Notch1 receptors. PLoS One. 2010;5(2):U69-U81.
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- Haining W, Angelosanto J, Brosnahan K, et al. High-throughput gene expression profiling of memory differentiation in primary human T cells. BMC Immunol. 2008;9(U1-U11).
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- Stegmaier K, Wong J, Ross K, et al. Signature-based small molecule screening identifies cytosine arabinoside as an EWS/FLI modulator in Ewing sarcoma. PLoS Med. 2007;4(4):702-14.
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Appendix A

Common Buffers Used in xMAP® Protocols

xMAP® buffers

Buffer	Composition	Use(s)	Source	Notes
Activation buffer*	0.1 M NaH_2PO_4, pH 6.2	Microsphere ("bead") activation buffer for protein coupling	MilliporeSigma S3139	Adjust to pH 6.2 with 5N NaOH Filter sterilize Store at 4°C
Coupling buffer†	50 mM MES, pH 5.0	Microsphere-protein coupling buffer	MilliporeSigma M2933	Adjust to pH 5.0 with 5N NaOH Filter sterilize Store at 4°C
Phosphate buffered saline (PBS), pH 7.4‡	138 mM NaCl, 2.7 mM KCl, pH 7.4	Alternate microsphere-protein coupling buffer	MilliporeSigma P3813	Filter sterilize Store at 4°C
PBS-Tween buffer	PBS, 0.05% Tween-20, pH 7.4	Microsphere wash buffer	MilliporeSigma P3563	Filter sterilize Store at 4°C
PBS-BN buffer§	PBS, 1% BSA, 0.05% sodium azide	Microsphere blocking/ storage buffer Assay buffer	MilliporeSigma P3688 MilliporeSigma S8032	Filter sterilize Store at 4°C
PBS–TBN buffer§,¶	PBS, 0.1% BSA, 0.02% Tween-20, 0.05% sodium azide	Microsphere blocking/ storage buffer Microsphere wash buffer Assay buffer	MilliporeSigma P3563 MilliporeSigma A7888 MilliporeSigma S8032	Filter sterilize Store at 4°C
Assay/wash buffer	PBS, 1% BSA, pH 7.4	Assay buffer	MilliporeSigma P3688	Filter sterilize Store at 4°C
Maleimide coupling buffer	0.1 M Sodium Phosphate, 50mM NaCl pH 7.0	Coupling peptides to maleimide-modified microspheres.	MilliporeSigma S3139	Adjust to pH 7.0 with 5N NaOH Filter sterilize Store at 4°C
Peptide resuspension buffer	100 mM Tris, pH 7.4	Resuspension of peptides for coupling to maleimide-modified microspheres.	MilliporeSigma T3038	Prepare from 1M Trizma® hydrochloride solution, pH 8
0.1 M MES buffer pH 4.5	0.1 M MES	Oligonucleotide-microsphere coupling buffer	MilliporeSigma M2933	Adjust pH w/ 5N NaOH Filter sterilize Store at 4°C
0.02% Tween-20 wash	0.02% Tween-20	Oligo coupling wash buffer	MilliporeSigma P9416	Filter sterilize Store at room temperature
0.1% SDS wash	0.1% SDS	Oligo coupling wash buffer	MilliporeSigma 71736	Filter sterilize Store at room temperature
EDC	1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC)	Microsphere coupling activation	Thermo Scientific Pierce™ 77149 ProteoChem™ c1100-100mg	Store desiccated at -20°C
TE buffer pH 8.0	TE	General purpose nucleic acid buffer	MilliporeSigma T9285	Filter sterilize Store at room temperature
2X xTAG® hybridization buffer	0.2 M Tris pH 8.0, 0.4 M NaCl, and 0.16% Triton™ X-100	xTAG® DNA hybridization reactions		Filter sterilize Store at 4°C

1X xTAG® hybridization buffer	0.1 M Tris pH 8.0, 0.2 M NaCl, and 0.08% Triton™ X-100	xTAG® labeling and wash buffer		Filter sterilize Store at 4°C
1.5X TMAC hybridization solution	4.5 M TMAC, 0.15% Sarkosyl solution, 75 mM Tris-HCL, 6 mM EDTA (pH 8.0)	Direct DNA hybridization microsphere diluent	Sigma T3411 Sigma L7414 Sigma T3038 Thermo Fisher (Invitrogen 15575-020)	Filter sterilize Store at room temperature
1X TMAC hybridization solution	3 M TMAC, 0.1% Sarkosyl solution, 50 mM Tris-HCL, 4 mM EDTA (pH 8.0)	Direct DNA hybridization labeling and wash buffer		Add 1 part molecular grade ddH_2O to 2 parts 1.5X TMAC Hybridization solution Filter sterilize Store at room temperature

*Activation can be performed in 50 mM MES, pH 6.0–6.2, with similar results.
†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.
‡Alternative coupling buffer for proteins that do not couple well at pH 5–6.
§Also used as assay buffer.
¶Also used as wash buffer.

Incompatible buffers

The following solvents should not be used with Luminex microspheres as they will affect the classification dyes in the microspheres:

Aromatic Hydrocarbons

- Benzene
- Toluene
- Xylene
- Ethylbenzene
- Chlorinated aliphatic

Hydrocarbons

- Methylene chloride
- Chloroform
- Carbon tetrachloride

Others

- Pyridine
- Dioxane
- Dimethylformamide
- Methyl ethyl ketone
- Diisopropyl ketone
- Cyclohexanone
- Tetrahydrofuran
- N-butyl phthalate
- Methyl phthalate
- Ethyl phthalate
- Tetrahydrofurfuryl alcohol
- Ethyl acetate
- Butyl acetate
- 1-nitro-propane
- Carbon disulfide
- tributyl phosphate
- cyclohexane
- methylcyclohexane
- ethylcyclohexane
- acetone
- DMSO

Note: Luminex R&D has tested 10% DMSO at 37°C (98.6°F) for 1 month with no obvious change in microsphere properties. Testing of 20% DMSO was shown to have no effect over a period of several hours, but no long-term studies have been done at this concentration.

High Salt Buffers

High salt concentrations will affect the classification of the microspheres on the Luminex® 200™, FLEXMAP 3D®, and xMAP INTELLIFLEX®. As the salt concentration of the buffer increases, the microspheres will tend to spread out on the bead map. High salt buffers (6X SSC, >0.2M NaCl) should be diluted or exchanged prior to analysis, as they can interfere with microsphere classification.

Appendix B

Equipment Needed for xMAP® Protocols

Immunoassay Equipment	Vendor
Luminex xMAP® analyzer*	Luminex
Magnet for 1.5 mL microcentrifuge tube washing†	Dynabeads™ MPC®-S Magnetic Particle Concentrator (Thermo Fisher 120-20D or equivalent)
Magnet for 96 well plate washing‡	See list below
Balance	Any suitable brand capable of measurements down to 0.1 mg
Microcentrifuge	Any suitable brand
Hemacytometer or cell counter	Cellometer® Auto 2000, TC10, TC20 Cell Counter, Countess® Automated Cell Counter
Vortex mixer	Any suitable brand
Sonicator bath	Ultrasonic Cleaner (Cole-Parmer® EW-08848-15 or equivalent)
Rotator	Any suitable brand capable of 15-30 rpm
Microtiter plate shaker	Any suitable brand capable of 800 rpm
96-well plates	Any suitable brand

*MAGPIX® has the ability to perform a final wash step prior to reading the plate.

†If a magnet is not available, use a microcentrifuge (8,000 x g for 1–2 minutes).

‡If a magnet not available, use a centrifuge compatible with 96 well plates (8,000 x g for 1–2 minutes).

Magnetic separators for MagPlex® Microspheres with compatible tubes and plates

Nucleic Acid Assay Equipment	Vendor
96-well thermocycler with heated lid	Any suitable brand

Product	Use	Source	Compatible Tube
Luminex magnetic tube separator	Coupling	Luminex Corporation, CN-0288-01	1.5 mL, co-polymer microcentrifuge tubes (USA Scientific 1415-2500)
Dynabeads™ MPC®-S magnetic particle concentrator	Coupling	Thermo Fisher (Applied Biosystems A13346)	1.5 mL, co-polymer microcentrifuge tubes (USA Scientific 1415-2500)

Product	Use	Source	Compatible Plates
Luminex magnetic plate separator	Assays	Luminex Corporation CN-0269-01	96-well, round bottom polystyrene solid plates (Corning 3789, 3792, or equivalent) 96-well, flat bottom polystyrene plates (Corning 8915BC, 3600, 3650, 3651, Greiner Bio-One 655096, 655900, or equivalent)
LifeSep™ 96F magnetic separation unit	Assays	Dexter Magnetic Technologies, Inc. 2501008	96-well, round-bottom polystyrene solid plates (Corning 3789 or 3792)
Ambion® 96-well magnetic ring stand	Assays	Thermo Fisher (Invitrogen AM10050)	96-well, round-bottom polystyrene solid plates (Corning 3789 or 3792)
96-well plate magnet	Assays	PerkinElmer (Customer Care) 5083175	96-well, round-bottom polystyrene solid plates (Corning 3789 or 3792) 96-well, Thermowell P polycarbonate PCR plates (Corning 6509) 96-well, F-bottom, (chimney well), µclear, med. Binding, black (Greiner Bio-One® bio-one 655096) Aluminum Foil Seals (Beckman Coulter® 538619) or equivalent
96-well side pull bar magnet 96-well PCR separation plate	Assays	Permagen Labware	96-well, Thermowell P polycarbonate PCR plates (6509)

Compatible plates and consumables

Description	Use	Analyzer(s)	Source	Catalog Number	Notes
1.5 mL copolymer microcentrifuge tubes	Coupling		USA Scientific	1415-2500	
1.5 mL Protein LoBind microcentrifuge tubes	Coupling		Eppendorf	22431081	
Extended fine tip transfer pipette	Coupling		Thermo Scientific Samco™	233	Good for removing supernatant from coupling wash steps
96-well, flat bottom, polystyrene solid plates	Protein/unheated assays	Luminex® 200™, FLEXMAP 3D®, MAGPIX®	Corning (Costar®)	3912, 3915	
96-well, round bottom, polystyrene solid plates	Protein/unheated assays	Luminex® 200™, FLEXMAP 3D®, MAGPIX®	Corning (Costar®)	3789, 3792	
96-well, Thermowell polycarbonate PCR plates, model P	Nucleic acid/heated	Luminex® 200™, FLEXMAP 3D®, MAGPIX®	Corning (Costar®)	6509	
96-well, multiscreen-BV 1.2 mm filter plates	Protein/unheated	Luminex® 200™, FLEXMAP 3D®, MAGPIX®	EMD Millipore	MABVN1250	Can be used for washes with vacuum filtration for non-magnetic beads
96-well, uClear, flat bottom, chimney well plates	Protein/unheated	Luminex® 200™, FLEXMAP 3D®, MAGPIX®	Greiner Bio-One	655096	
384-well, uClear, flat bottom, chimney well plates	Protein/unheated	FLEXMAP 3D®	Greiner Bio-One	781906	
384-well, Thermowell GOLD polypropylene microplates	Nucleic acid/heated	FLEXMAP 3D®	Corning (Costar®)	3757	
384-well, Armadillo PCR Plates	Nucleic acid/heated	FLEXMAP 3D®	Thermo Scientific	AB-2384	
384-well, hard-shell, thin wall, skirted PCR plates	Nucleic acid/heated	FLEXMAP 3D®	Bio-Rad	HSP-3805	
384-well, twin.tec PCR plate	Nucleic acid/heated	FLEXMAP 3D®	Eppendorf	951020702	
96-well microplate aluminum sealing tape	Nucleic acid/heated	FLEXMAP 3D®, MAGPIX®	Corning (Costar®)	6570	
Microseal 'A' film	Nucleic acid/heated	Luminex® 200™, FLEXMAP 3D®, MAGPIX®	Bio-Rad	MSA-5001	

Appendix C

Automated Bead Washing Option

BioTek® ELx405 and ELx406 microplate washer—keypad programming instructions

Note: For use with BioTek®-provided magnet P/N 7103016 (Dexter LifeSep™ 96F technology) and round-bottom 96-well microplate.

Luminex® Mag Bead Assay Wash 96-Well Protocol
2-Cycle Wash with No Final Dispense

1) Create SOAK program.

DEFINE → CREATE → MORE → SOAK → name (Ex. SOAK60) *note: to get letters, scroll through with OPTIONS button* → ENTER.
SOAK DURATION: **60 SEC** → ENTER.
SHAKE BEFORE SOAK?: **YES** → ENTER.
SHAKE DURATION: **1 SEC** → ENTER.
SHAKE INTENSITY: **1** → ENTER.
OK TO SAVE PROGRAM? **YES** → ENTER.

Note: Program delays for 60 seconds with a 96-well plate on the magnet prior to aspiration, followed by a 2-cycle wash with 60 second delays after each dispense, and ends with a final aspiration.

2) Create ASPIRATION program.

DEFINE → CREATE → MORE → ASPIR → name (Ex. ASPIR) *note: to get letters, scroll through with OPTIONS button* → ENTER.

PLATE TYPE: **96** → ENTER.
ASPIRATE HEIGHT: **58** → ENTER.
HORIZONTAL ASPR POS: **-50** → ENTER.
HORIZ Y ASPR POS: **0** → ENTER.
ASPIRATION RATE: **1** → ENTER.
ASPIRATE DELAY: **0** → ENTER.
CROSSWISE ASPIR: **NO** → ENTER.
OK TO SAVE PROGRAM? **YES** → ENTER.

Note: These instructions were created 1/6/2011 by J. Greene, BioTek® Instrument settings were determined by H. Baker, Luminex Corporation.

3) Create WASH program.

DEFINE → CREATE → WASH → name (Ex. WASH2X) *note: to get letters scroll through with OPTIONS button* → ENTER.

SELECT REAGENT BOTTLE: **A** → ENTER.
PLATE TYPE: **96** → ENTER.

METHOD → NUMBER OF CYCLES: **2** → ENTER → SOAK/SHAKE: **YES** → ENTER → SOAK DURATION: **60 SEC** → ENTER → SHAKE BEFORE SOAK: **NO** → ENTER → PRIME AFTER SOAK: **NO** → ENTER.

DISP →
DISPENSE VOLUME: **100 UL/WELL** → ENTER.
DISPENSE FLOW RATE: **9** → ENTER.
DISPENSE HEIGHT: **128** → ENTER.
HORIZONTAL DISP POS: **0** → ENTER.
HORIZ Y DISP POS: **0** → ENTER.
DISABLE ASPIRATE? **NO** → ENTER.

BOTTOM WASH FIRST?: **NO** → ENTER.
PRIME BEFORE START?: **NO** → ENTER.

ASPIR →
ASPIRATE HEIGHT: **58** → ENTER.
HORIZONTAL ASPR POS: **-50** → ENTER.
HORIZ Y ASPR POS: 0 → ENTER.
ASPIRATION RATE: **7** → ENTER.
ASPIRATE DELAY: **0** → ENTER.
CROSSWISE ASPIR: **NO** → ENTER.
FINAL ASPIRATION: **YES** → ENTER.
FINAL ASPIR DELAY: **0** → ENTER.

MAIN MENU → OK TO SAVE PROGRAM?: **YES** → ENTER.

4) Create LINK program.

DEFINE → CREATE → MORE → LINK → name (Ex. LUMINEX) n*ote: to get letters, scroll through with OPTIONS button* → ENTER → MORE → SOAK *(scroll through using OPTIONS button to find SOAK program you made in step # 1)* → ENTER → MORE → ASPIR *(scroll through using OPTIONS button to find ASPIR program you made in step # 2)* → ENTER → WASH *(scroll through using OPTIONS button to find WASH program you made in step # 3)* → ENTER.

MAIN MENU → OK TO SAVE PROGRAM?: **YES** → ENTER.

5) Run LINK program.

- *After creating LINK program, this is what you will actually run when washing plates.*
- RUN → MORE → LINK *(scroll through with OPTIONS button to find LINK program you made in step # 4)* → ENTER.

6) Run basic maintenance.

- Before each use:
  RUN → PRIME (using your wash buffer) → scroll through using OPTIONS to **PRIME 200** → ENTER.
- End of the day:
  Switch to bottle containing rinse liquid (ex. deionized water).
  MAINT → *scroll through using OPTIONS to* **OVERNIGHT LOOP** → ENTER.

Note: Automation options from other other vendors, including Gilson® (gilson.com), Hamilton® (hamiltoncompany.com), PerkinElmer® (perkinelmer.com), Tecan® (tecan.com), Thermo Scientific™ (thermoscientific.com) etc., may also be suitable for xMAP® Technology.

Appendix D

Buffer Exchange and Purification

Some common additives to proteins can interfere with the coupling reaction, including amine-containing compounds such as Tris, bovine serum albumin (BSA), and azide, some detergents, glycerol, urea, and imidazole. 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 azide, detergents, or urea, a sufficient dilution of the protein to be coupled can be performed to improve coupling efficiency.

Removing BSA from protein solutions prior to coupling and biotinylation

BSA is a carrier protein that is added to protein solutions to provide stability to proteins at low concentrations and for long-term storage. BSA will interfere in bead coupling and biotinylation, and should be removed prior to conjugation and labeling. One of the quickest and easiest methods to purify antibody solutions is by affinity chromatography using Protein A, Protein G, or Protein A/G resins. In this method, antibodies bind to the resin and BSA passes through to waste. The antibodies are then eluted, neutralized, and desalted, resulting in a BSA-free antibody stock solution.

Another commonly used protein purification method includes desalting products such as Zeba™ Spin Desalting Columns and PD-10 Desalting Columns. Zeba™ columns consist of high performance size-exclusion chromatography resins that help in processing very dilute protein samples.[1] PD-10 columns contain Sephadex® G-25, which allows rapid separation of high molecular weight substances from low molecular weight substances.[2] The Pierce™ Antibody Clean-up Kit[3] also provides an effective way to remove BSA, gelatin, and other stabilizer proteins from antibody stock solutions.

Removing glycerol

Glycerol is added to protein stock solutions to provide stability and preserve function during long-term storage. However, glycerol can also interfere in coupling and biotinylation. Depending on the ratio of protein to glycerol and the volume of the stock solutions, low amounts of glycerol can be diluted out. Coupling and biotinylating proteins of low concentration in solutions containing high glycerol should be avoided, if possible.

A common method of removing glycerol from protein stock solutions is buffer exchange by dialysis. Rapid and effective dialysis can be achieved using products like the Pierce™ 96-well Microdialysis Plate[4] and Slide-A-Lyzer™ Dialysis Cassettes.[5]

Removing sodium azide and Tris

Sodium azide is added to protein stock solutions as an antimicrobial agent. Tris is added to neutralize protein solutions after acidic elution during purification. Both will interfere with coupling and biotinylation. A common and efficient way of removing these is by desalting columns, such as Zeba™ Spin Desalting Columns and PD-10 Desalting Columns. This method uses size exclusion chromatography, where small molecules are retained and the large ones pass through the column.

Note: It is important to measure the concentration of the protein prior to and after buffer exchange to determine the recovery and concentration. This can be done by spectrophotometry at A280 using the Pierce™ BCA Protein Assay Kit,[6] or other methods.

- Instructions—Zeba™ Micro Spin Desalting Columns, 7K MWCO. Thermo Scientific (Internet). Cited 2021 Jun. Available from: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011522_Zeba_Spin_Desalt_Colomn_7K_MWCO_UG.pdf.
- Instructions 52-1308-00 BB—PD-10 Desalting Columns. GE Healthcare (Internet). Cited 2016 January. Available from: http://wolfson.huji.ac.il/purification/PDF/dialysis/AMERSHAM_PD10Desalting.pdf.
- Pierce™ Antibody Clean-up Kit. ThermoFisher Scientific (Internet). Cited 2021 Jun. Available from: https://www.thermofisher.com/order/catalog/product/44600.
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- Pierce™ BCA Protein Assay Kit. ThermoFisher Scientific (Internet). Cited 2021 Jun. Available from: https://www.thermofisher.com/order/catalog/product/23225.

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