Luminex® xMAP® Technology

1st Edition

xMAP® Cookbook

A collection of methods and protocols for developing multiplex assays with xMAP Technology.

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Effective Date: 01 July 2013

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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 may all be 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. Color from one assay detection channel interferes with color in other detection channels. This limitation has made such multi-color assays useful only to a few analytes per sample.

While microarrays (2-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 its broader application in the clinical or research laboratory.

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

- reduced sample volume and other redundant consumables
- more data from the same amount of labor
- faster results due to solution-phase kinetics

What is multiplexing?
Multiplexing describes assaying multiple analytes simultaneously within a single sample volume and within 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

In the late 1990's scientists at Luminex® invented xMAP Technology, a major advance in multiplexed biological assays. xMAP Technology draws from the strengths of solid-phase separation technology but 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.

xMAP Technology Name Origin

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 carry 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 virtually solution-phase 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.

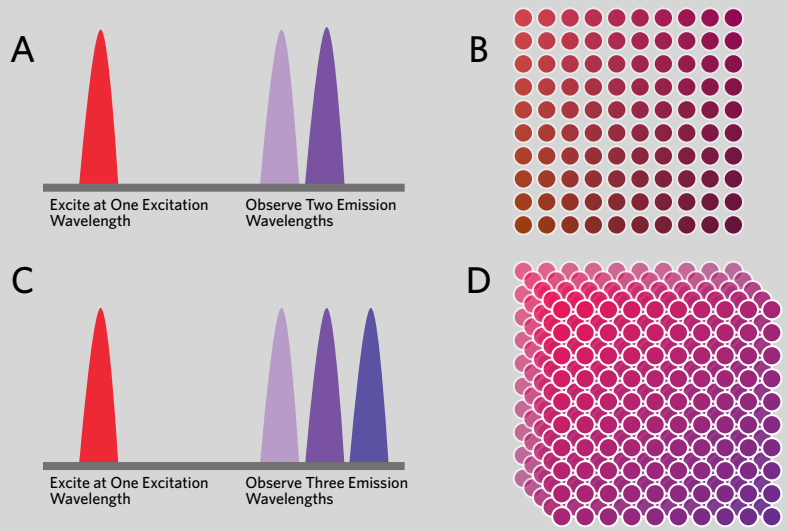

Figure 1 – xMAP Microspheres include two-dye products where (A) one excitation wavelength allows observation of two separate fluorescence emission wavelengths, yielding (B) 100 unique microsphere sets (10x10 dye matrix); and three-dye products where (C) one excitation wavelength allows observation of three separate fluorescence wavelengths, yielding (D) 500 unique microsphere sets. (10x10x5 dye matrix)

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Figure 2.

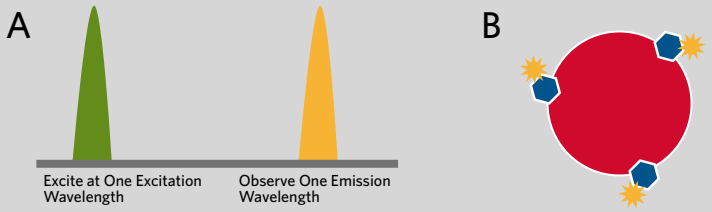


Figure 2 – In addition to detection of internal bead dyes (shown in Figure 1), (A) a second excitation wavelength allows observation of a separate fluorescent reporter molecule, {B) that allows detection of a biological assay on the surface of the microsphere.

Multiple light sources inside the Luminex analyzer excite (1) the internal bead dyes that identify each microsphere particle and (2) 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.

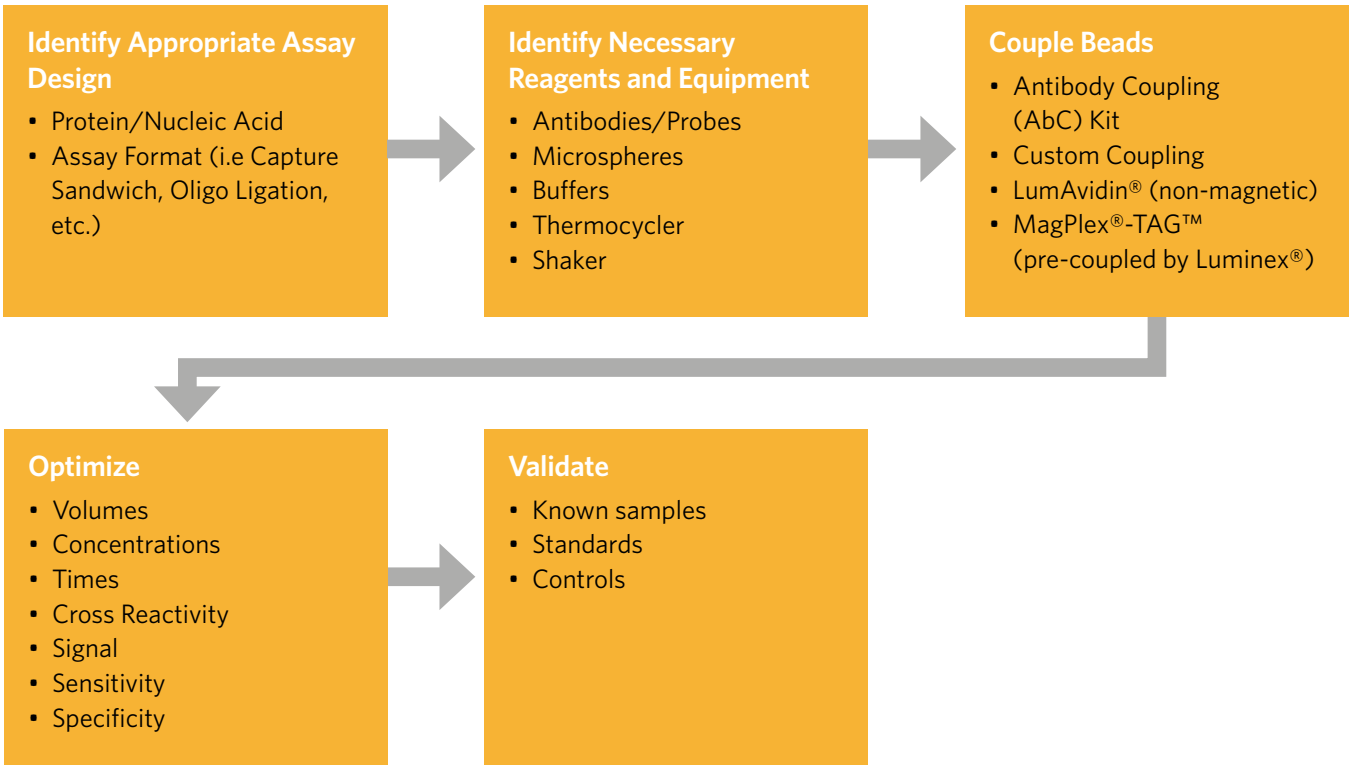
This revolution in multiplex biological assays has been licensed by Luminex to a number of kit developers in the clinical diagnostics, pharmaceutical and life science research markets. Commercially available kits include molecular diagnostics, immunodiagnostics, kinase profiling, cytokine/chemokine,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

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

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 monoplex assays. The following describes the general workflow of xMAP assay development.



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 are 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

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. Due to the 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 decades and has led to more novel techniques such as highly multiplexed immunoassays for measuring potentially hundreds of analytes simultaneously. Such multiplex assays can be used to generate profiles of clinical samples that can facilitate accurate disease diagnoses or prediction of drug responses.

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. Monoplex 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 for that analyte, while another analyte in the same multiplex assay may be abundant and therefore require a lower-sensitivity assay. Sensitivity of each antibody assay may 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.

Figure 3.

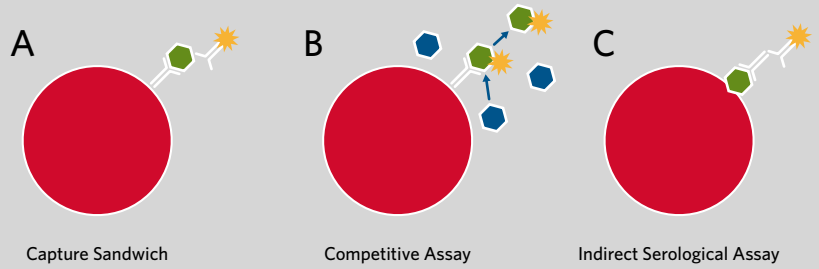


Figure 3 - Common assay formats for immunoassays include (A) capture sandwich assay, requiring capture and detection antibodies to your analyte target (polyclonal capture antibodies should be purified and mono-specific); (B) competitive (antibody) assay, requiring a single antibody and a labeled positive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

A second consideration for polystyrene 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 ("beads") 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 2000 angstroms, allowing them to trap proteins, which typically range from 50 to 100 angstroms in diameter. Microsphere assays can employ blocking agents optimized for each biological matrix to reduce non-specific binding of non-target molecules.

Nucleic acid assays

Multiplex nucleic acid assays require mostly different optimization steps from immunoassays, although some similarities exist. Similar to immunoassays, sensitivity of nucleic acid assays may be affected by 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 and non-specific hybridization between sequences. Depending on whether the purpose of your assay is gene expression, genotyping or simply sequence detection, 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 diagrammed in Figure 4.

Figure 4.

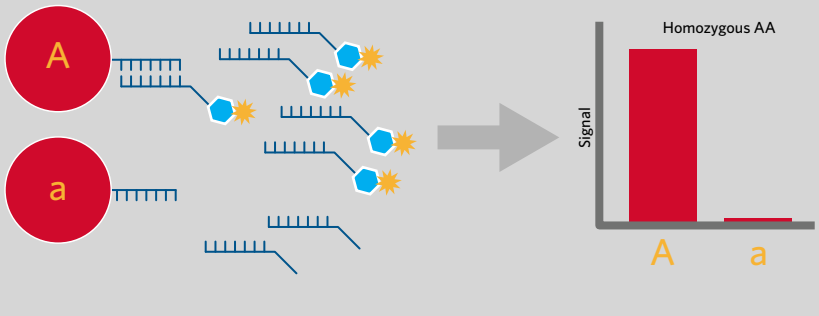


Figure 4 - Schematic of nucleic acid assay analysis on Luminex® beads. Each bead has a unique capture sequence specific for a marker sequence. If reporter molecules are generated and captured (bead A) a fluorescence signal is detected. If no reporter molecules are generated and captured (bead a) minimal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

Chapter 3.2

Reagents and Equipment

The newest generation of xMAP® beads are MagPlex® Microspheres*("beads") which are superparamagnetic microspheres, 6.5 microns in diameter. These beads are impregnated with different ratios of two or three dyes allowing the generation of 500 different bead regions for the development of assays up to 500 plex. The surface of these beads are also impregnated with iron containing magnetite particles. This feature allows the use of magnets to rapidly remove the beads from reaction suspensions to speed up processing during different protocols and minimize bead loss 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 should be purchased from Luminex®. They should be stored at 4°C and be kept in the dark. They can be used directly from their vials as described in the coupling protocol. MagPlex Microspheres are available at concentrations of 2.5 million and 12.5 million beads/mL and can be ordered in 1mL and 4mL vial sizes.

MagPlex®-TAG™ Microspheres are MagPlex beads covalently coupled with unique 24 base oligonucleotide 'anti-TAG' sequences. These beads enable the user to quickly and easily design custom bead arrays, simply by adding a complementary 'TAG' sequence to primers or probes used in assay reactions and then using the TAG/anti-TAG hybridization to capture each assay product to a unique MagPlex bead. For a complete list of the TAG and anti-TAG sequences for each of the 150 available microspheres, visit www.luminexcorp.com.

MagPlex and MagPlex®-TAG™ Microspheres are compatible with all Luminex instruments, including MAGPIX® (up to 50-plex), Luminex® 100/200™ (up to 80-plex) and FLEXMAP 3D® (up to 500-plex for MagPlex; up to 150-plex for MagPlex-TAG). Basic Luminex equipment reagents include Calibration and Verification kits and Sheath or Drive Fluid. They are also offered in a variety of custom volumes. For additional information on all xMAP reagents contact your sales manager or visit http://www.luminexcorp.com/Products/ReagentsMicrospheres/

Depending on the type of multiplex assay you are developing, certain additional reagents and equipment must be provided by you. For a list of materials needed for typical assays, see each specific protocol. For a list of common buffers and equipment used in xMAP assays, please see Appendices A and B.

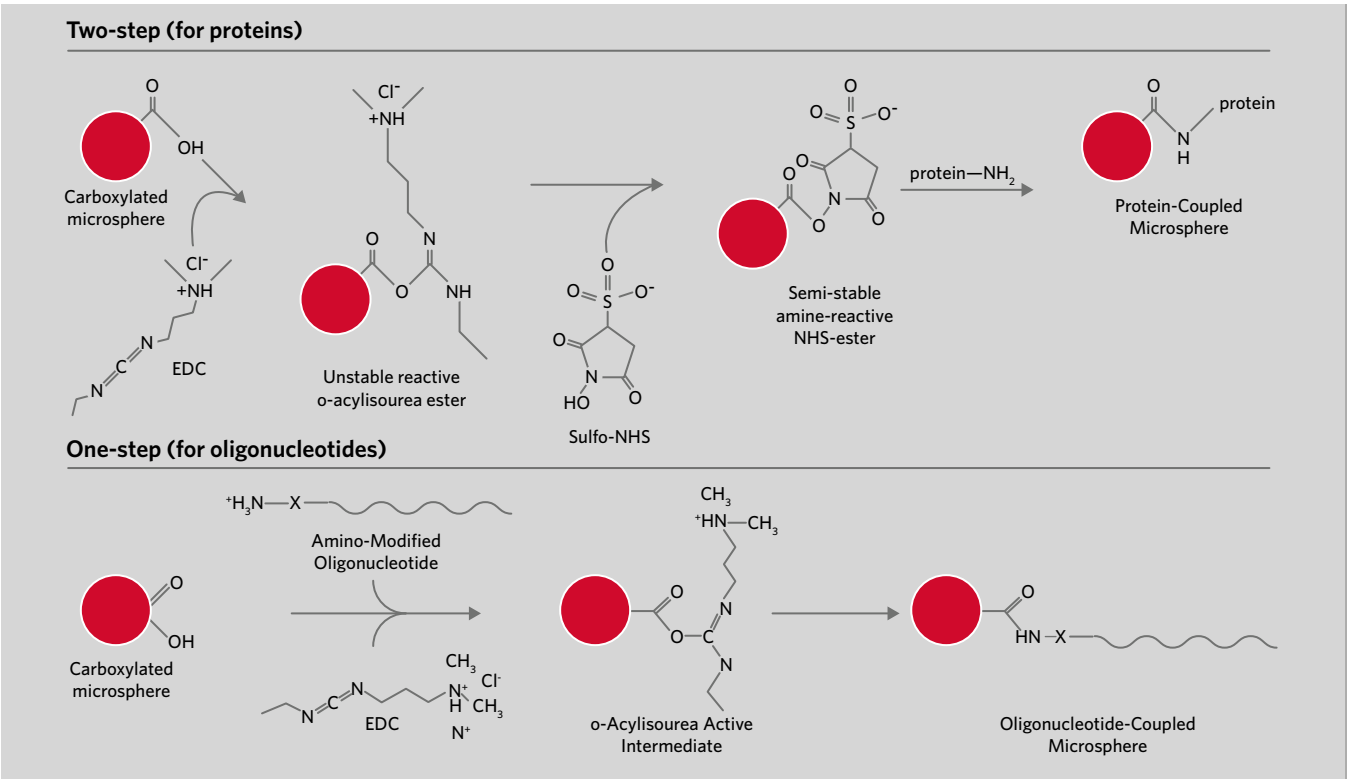
*Assays and protocols are described for MagPlex or MagPlex-TAG Microspheres unless otherwise noted.

Chapter 3.3

Microsphere Coupling

xMAP® Microspheres ("beads") require chemical coupling of a capture agent (e.g., antibody or oligonucleotide) or enzyme substrate (e.g., peptide or protein) in order to perform an assay. There are approximately 100 million carboxyl groups on each xMAP microsphere. The chemistry of the coupling process involves formation of a carbodiimide bond between primary amines on an antibody, protein, peptide or oligonucleotide and carboxyl groups on the surface of xMAP Microspheres:

Coupling Chemistry



Antibody coupling for proteins is a simple two-step carbodiimide procedure during which microsphere carboxyl groups are first activated with EDC (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 reaction with the primary amine of the target molecule (antibody, or peptide) to form a covalent amide bond. Oligonucleotide coupling is a one-step process where microsphere carboxyl groups activated by EDC form a covalent amide bond with primary amines on amine-modified oligonucleotides.

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Luminex® provides several products to support coupling and assay development with xMAP Microspheres:

- **xMAP Antibody Coupling (AbC) Kit** – contains all of the reagents necessary to covalently couple antibodies to Luminex MagPlex Microspheres (beads) in approximately three hours. Does not include microspheres or labeling reagent. [Catalog Number 40-50016]
- **MagPlex®-TAG™ Microspheres** – magnetic microspheres pre-coupled with unique 24-base DNA sequences ('anti-TAGs') to allow incorporation of complementary 'TAG' sequences into custom oligonucleotide targets for hybridization capture. [Catalog Number MTAG-XXXX]
- **LumAvidin® Microspheres** – xMAP Microspheres pre-coupled with avidin to allow simple non-covalent binding of biotinylated targets such as peptides. Non-magnetic [Catalog Number L100-LXXX]

A number of factors affect the coupling chemistry. Some common additives to proteins and buffers can interfere with the coupling reaction, including amine-containing compounds such as Tris, BSA, or azide. In addition, glycerol, urea, imidazole and some detergents may also interfere with coupling chemistry. Any of these compounds should always be removed from the protein, peptide or oligonucleotide as purified materials are most commonly used.

Common antibody purification methods 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, such as detergents or urea, a sufficient dilution of the agent to be coupled can be performed to improve coupling efficiency. Oligonucleotide purification methods are typically desalting or HPLC purifications.

The carbodiimide coupling reaction is most efficient at low pH level (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. Oligonucleotide coupling performs best at pH 4.5.

Monoclonal antibodies should be used for capturing the analyte to the microsphere surface to achieve best sensitivity and specificity. If a polyclonal antibody is used for capture, it should be monospecific and affinity-purified. The optimal amount of capture reagent may vary depending on the reagent used and should be titrated. For antibodies, 5 µg of antibody per 1 million microspheres performs well. For antigens, the amount will vary depending on the size and composition of the antigen being coupled.

Chapter 3.3.1

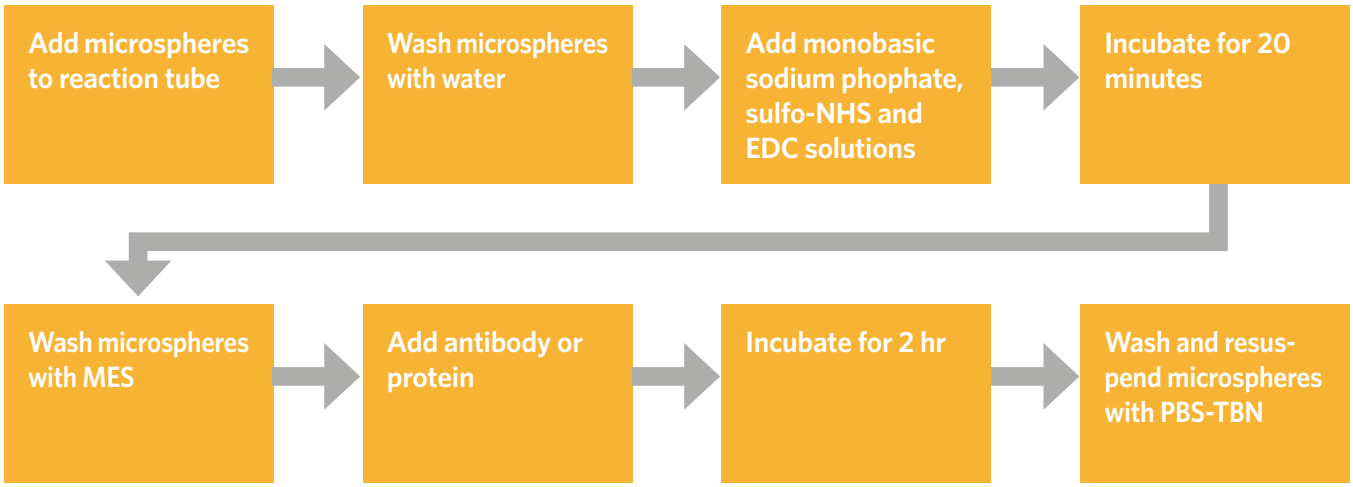
Antibody and Protein Coupling

General guidelines for antibody coupling to xMAP® Microspheres

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

For users wishing to couple xMAP Microspheres with antibodies or other proteins without the xMAP Antibody Coupling (AbC) Kit, below is a standard coupling protocol for 5 million MagPlex Microspheres. Coupled microsphere stability depends on the stability of the coupled protein but when properly stored, coupled microspheres are usually stable for more than 1 year.

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)	Pierce 77149
Sulfo-NHS	Pierce 24510 (500 mg) or 24520 (8 x 2 mg, No-Weigh Format)
Activation Buffer 1 (0.1 M NaH_2PO_4, pH 6.2)	Sigma S3139
Coupling Buffer 2 (50 mM MES, pH 5.0)	Sigma M2933
Assay/Wash Buffer (PBS, 1% BSA)	Sigma P3688
Phosphate buffered saline (PBS), pH 7.4[3]	Sigma P3813 Sigma P3563
PBS-BN buffer[4]	Sigma P3688
PBS–TBN buffer[4,5]	Sigma P3813 Sigma A7888 Sigma P9416
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Lo Bind 022431081
Disposable pipettes tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

For complete equipment and materials list see Appendix B

1. Activation can be performed in 50 mM MES, pH 6.0–6.2, with similar results.
2. 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.
3. Alternative coupling buffer for proteins that do not couple well at pH 5–6.
4. Also used as assay buffer.
5. Also used as wash buffer.

Protocol 3.3.1 (antibody coupling)

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

1. Resuspend the stock uncoupled microsphere suspension according to the instructions described in the Product Information Sheet provided with your microspheres.
2. Transfer 5.0 x 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 microspheres in 100 µL dH_2O by vortex and sonication for approximately 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 microspheres in 80 µL 100 mM Monobasic Sodium Phosphate, pH 6.2 by vortex and sonication for approximately 20 seconds.
9. Add 10 µL of 50 mg/mL Sulfo-NHS (diluted in dH_20) to the microspheres and mix gently by vortex.
10. Add 10 µL of 50 mg/mL EDC (diluted in dH_20) 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. See Technical Note 2.
15. Repeat steps 13 and 14 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 125, 25, 5 or 1 µg protein to the resuspended microspheres. *(Note: We recommend titration in the 1 to 125 µg range to determine the optimal amount of protein per specific coupling reaction.)*
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 approximately 20 seconds.
24. Optional – 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 approximately 20 seconds.
28. Repeat steps 25. and 26. This is 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 250-1000 µL of PBS-TBN.
30. Count the number of microspheres recovered after the coupling reaction using a cell counter or hemacytometer.
31. Store coupled microspheres refrigerated at 2-8°C in the dark.

Follow this coupling procedure with Coupling Confirmation (section 3.3.2).

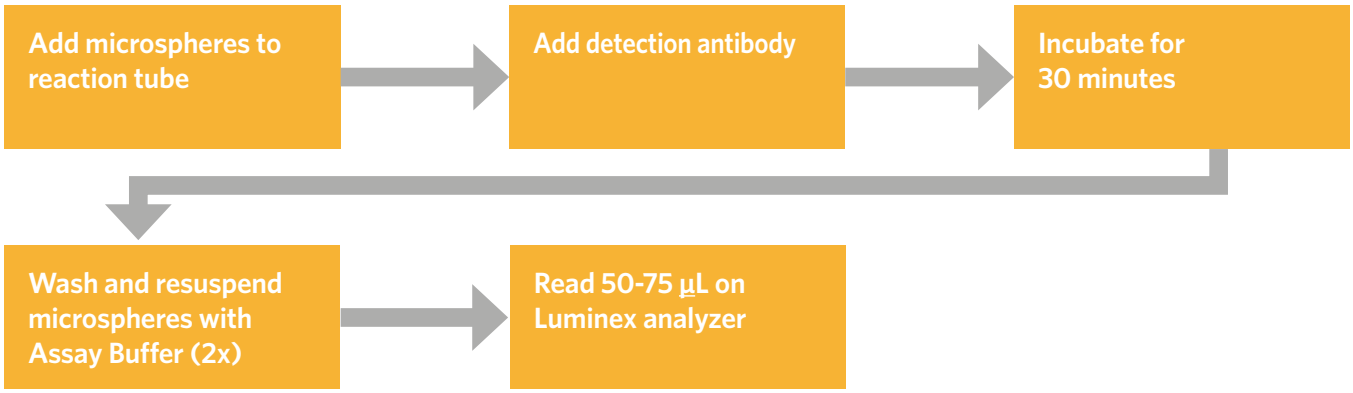
Note: When counting on a hemacytometer use the following calculation: Total microspheres = count (1 corner of 4 x 4 section) x (1 x 10^4) x (dilution factor) x (resuspension volume in mL)

Chapter 3.3.2

Antibody Coupling Confirmation

Once antibodies have been coupled to xMAP® Microspheres ("beads"), it is strongly recommended to assess coupling efficiency before proceeding to assay development. The coupled microspheres can be reacted with phycoerythrin (PE)-labeled anti-species antibody and analyzed on a Luminex® instrument. Alternatively, target antigen may be biotinylated and subsequently labeled with streptavidin-R-phycoerythrin (SAPE). Proteins are typically coupled in random orientation as they have many lysine groups available for coupling. Functional testing is also critical during assay development. Examples of coupling confirmation can be found in *de Jager et al. 2003. (see reference on page 19)*.

Summary of Protocol



Materials Needed:

For complete equipment and materials list see Appendix B

Reagents and Consumables	Vendor
MagPlex® Microspheres (antibody-coupled)	
Assay/Wash Buffer (PBS, 1% BSA)	Sigma P3688
PBS-BN buffer[1]	Sigma P3813 Sigma A7888 Sigma P9416
PBS–TBN buffer[1,2]	Sigma P3813
96 well plate	See Appendix B
PE or Biotin labeled anti-species detection Antibody	Any suitable source
streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Life Technologies S-866 or equivalent
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Lo Bind 022431081
Disposable pipettes tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

1. Also used as assay buffer.
2. Also used as wash buffer.

A protocol for verifying antibody coupling is provided below. A dose response increase in MFI should be observed as concentration of labeled detection antibody increases. In general, an antibody coupling should yield at least 10,000 MFI (at standard PMT setting or on MAGPIX® instrument) at saturation for optimal use in immunoassays.

Protocol 3.3.2 (antibody coupling confirmation)

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

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

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

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 ↄ columns of the 96-well plate (16 wells total).
6. Add 50 µL of Assay Buffer, as a blank sample, into the wells in 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).

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

(Example of plate layout using columns 1 & 2)

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

10. Clip the plate in place on the Luminex Magnetic Plate Separator and rapidly and forcefully invert over a biohazard receptacle to evacuate the liquid from the wells.
    NOTE: For information on the MagPlex Manual Wash Method, please visit: http://www.luminexcorp.com/Products/ReagentsMicrospheres/MagneticSeparators/.
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 step 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 below.

Figure 5.

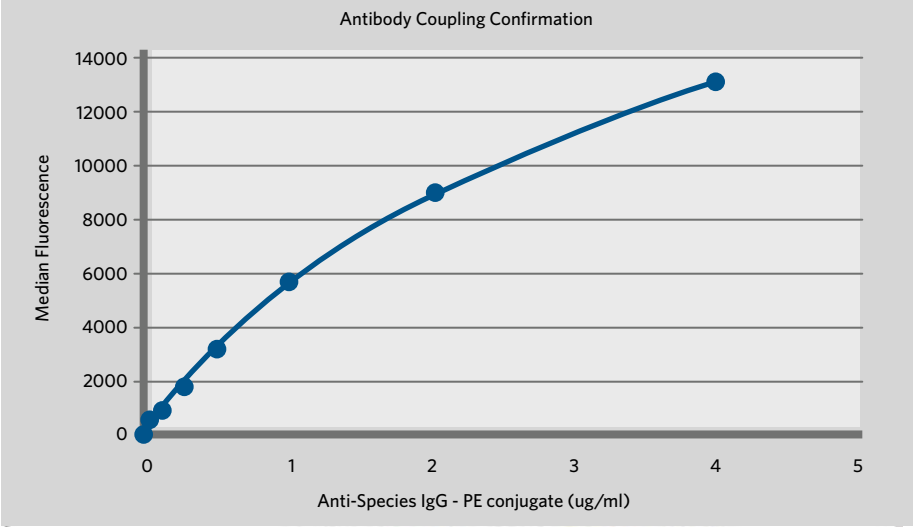


Figure 5 – Plot of typical results for anti-species IgG-PE conjugate titration of antibody-coupled microspheres, as measured by a Luminex analyzer.

Antibody coupling references

- de Jager, W., te Velthuis, H., Prakken, B. J., Kuis, W., and Rijkers, G. T. "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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- Lawson, S., Lunney, J., Zuckermann, F., Osorio, F., Nelson, E., Welbon, C., Clement, T., Fang, Y., Wong, S., Kulas, K., and Christopher-Hennings, J. "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.

Chapter 3.3.3

Nucleic Acid Coupling

General guidelines for nucleic acid coupling to xMAP® Microspheres

Coupling of oligonucleotides (oligos) to xMAP Microspheres ("beads") is a straightforward process but does require a number of optimization steps to ensure best results in a multiplex assay. Luminex® supports custom oligo coupling, but has also innovated a pre-coupled, pre-optimized, universal microsphere set that circumvents the need for assay developers to couple oligos to beads. MagPlex®-TAG™ Microspheres are magnetic microspheres pre-coupled with unique 24-base DNA sequences ('anti-TAGs') to allow incorporation of complementary 'TAG' sequences into custom oligonucleotide targets for hybridization capture.

Figure 6.

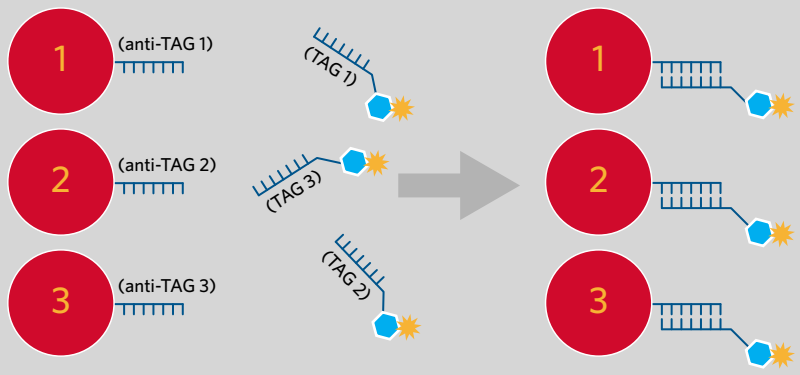
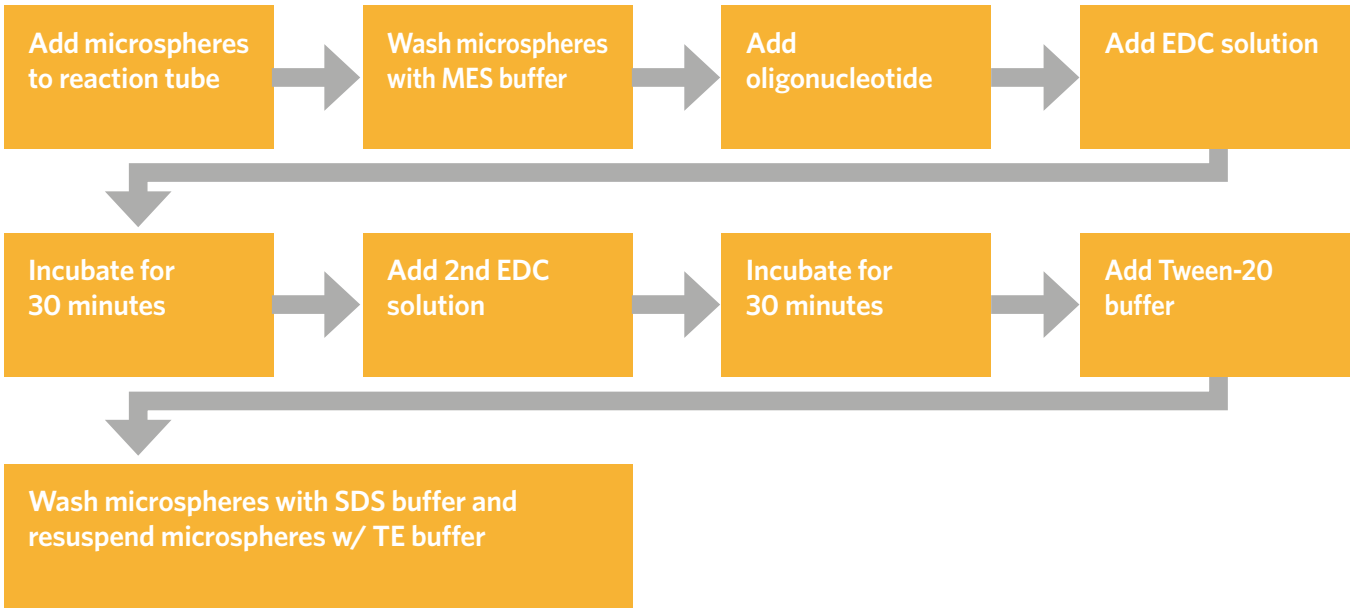


Figure 7– MagPlex-TAG Microspheres are pre-coupled with anti-TAG sequences, allowing the user to incorporate TAG tails into assay reactions to facilitate bead capture without the need to chemically couple oligos.

For users wishing to couple custom oligonucleotide sequences to xMAP Microspheres themselves, below is a standard protocol. It is recommended to use oligonucleotides synthesized with a 12-carbon amine containing group on the 5′ end. Having this spacer insures that the coupled oligo is raised off of the bead surface to facilitate interaction with the target and reporter molecules it needs to capture in an assay.

Below is a standard coupling protocol for 5 million MagPlex® Microspheres. 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 when the optimum ratio of beads to oligo preparation has been determined.

Summary of Protocol



Materials Needed:

Reagents and Consumables	Vendor
MagPlex® Microspheres	Luminex
5′ amine C-12 spacer Oligonucleotides (Special order)	IDT or other vendor
0.1 M MES Buffer pH 4.5	Sigma M2933
0.02% Tween-20	Sigma P9416
1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC)	Pierce 77149
TE Buffer pH 8.0	Sigma T9285
0.1% SDS	Sigma L4522
RNase/DNase-Free Microcentrifuge Tubes 1.5 mL	USA Scientific 1415-2500 or Eppendorf Lo Bind 022431081
Disposable pipette tips; multi- and single-channel (2-1000 mL)	Any suitable brand
Barrier pipettes tips	Any suitable brand
Distilled deionized H2O (ddH2O) - molecular grade	Any suitable source

For complete equipment and materials list see Appendix B

Protocol 3.3.3 (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 5.0 x 10^6 of the stock microspheres to a USA Scientific microcentrifuge tube.
5. Pellet the stock microspheres with a tube magnet or by microcentrifugation at ≥ 8000 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 ≅ $[0.5\ \mu g/\mu L]_{final}$) 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.
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 ≥ 8000 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 ≥ 8000 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 approximately 20 seconds.
20. Enumerate the coupled microspheres by hemacytometer or other particle/cell counter.
21. Store coupled microspheres refrigerated at 2-8°C in the dark.

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: 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. Note: maximum is 50,000 microspheres/µL.

Chapter 3.3.4

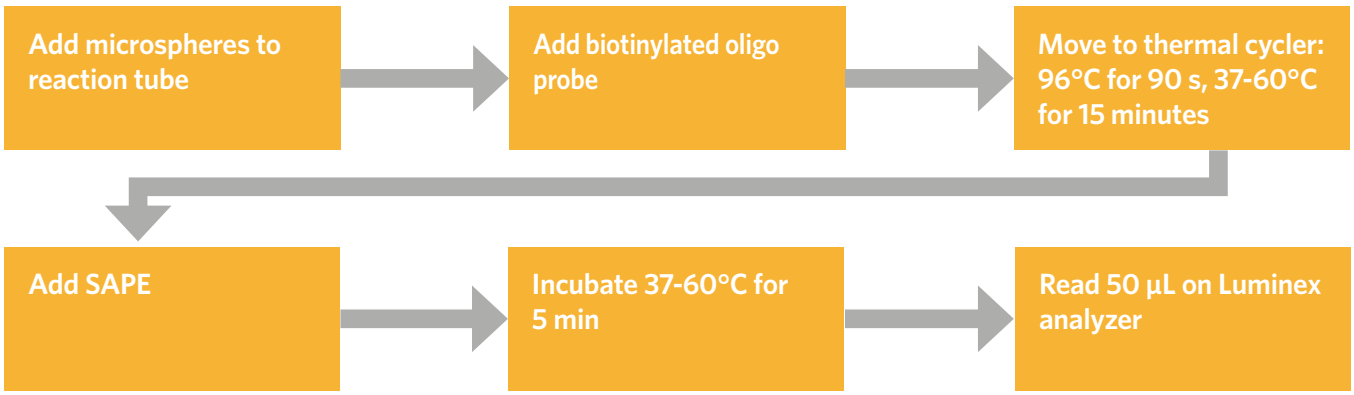
Oligonucleotide Coupling Confirmation

Once oligonucleotides have been coupled to xMAP® Microspheres ("beads"), it is strongly recommended to assess coupling efficiency before proceeding to assay development. The coupled microspheres can be reacted with biotinylated target oligonucleotide, labeled with streptavidin-R-phycoerythrin (SAPE) and analyzed on a Luminex® instrument (followed by SAPE labeling).

To determine the optimum amount of oligo that provides the highest signal several coupling reactions at different oligo concentrations should be carried out. 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 optimization. We recommend purchasing sequence-specific 5′ biotinylated complementary oligos for each capture sequence as the most reliable method, since precise amounts of probe with a single biotin group can be used in the hybridization reaction.

The following protocol may be used for confirmation of oligonucleotide coupling reactions.

Summary of Protocol



BR_574.01_0613

Materials Needed:

Reagents and Consumables	Vendor
MagPlex® Microspheres (oligonucleotide-coupled)	
1.5X TMAC Hybridization Solution	See Appendix A
1X TMAC Hybridization Solution	See Appendix A
TE Buffer pH 8.0	Sigma T9285
96 well bead hybridization plate	Corning Costar 6509
Biotin labeled complementary oligonucleotide targets	Any suitable source
streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Life Technologies S-866 or equivalent
RNase/DNase-Free Microcentrifuge Tubes 1.5 mL	USA Scientific 1415-2500 or Eppendorf Lo Bind 022431081
MicroSeal A	BioRad MSA5001
Brayer roller, soft rubber or silicon	USA Scientific 9127-2940
Disposable pipette tips; multi- and single-channel (2-1000 mL)	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 Tm 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).

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

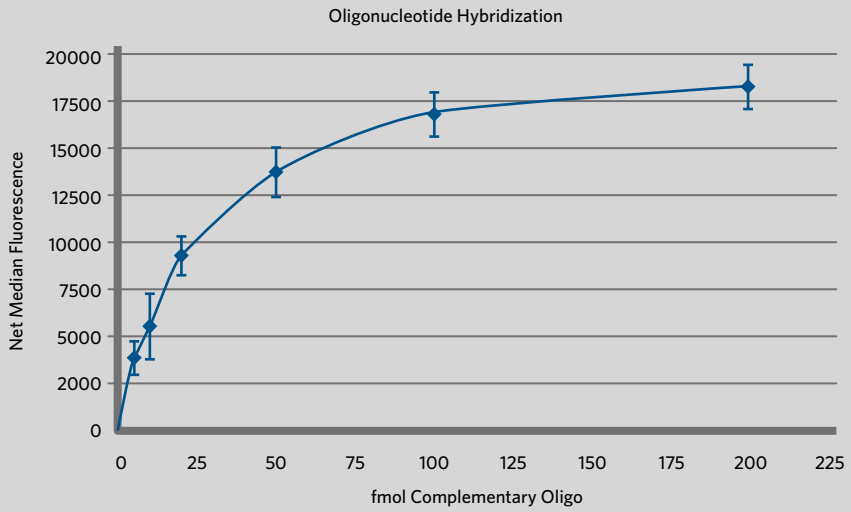
Protocol 3.3.4 (oligonucleotide coupling confirmation)

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.

1. Select the appropriate individual MagPlex coupled bead sets and resuspend by vortexing and sonication for 20 seconds each.
2. Dilute/concentrate an aliquot of each to 75 microspheres/µL in 1.5X TMAC Hybridization Solution. Vortex and sonicate for approximately 20 seconds. (Note: 33 µL are required for each reaction to give 2,500 beads/reaction.)
3. Add 33 µL of the 75 microspheres/µL aliquot 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 with 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 are 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.

Figure 7. Typical results of an oligonucleotide coupling reaction



Recommendations for Optimization and Troubleshooting

Probe Design Strategy

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 prevent secondary structure in 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 sequence 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 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 or 5′ Uni-Link Amino Modifier.

Tips, Important Points & Critical Factors

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 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 coupling episode. 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 close the dry aliquot tightly and return to desiccant between preparations. The dry aliquot should be discarded after the second addition.
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.
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 5 x 10^6 microspheres. Usually, 0.2 to 1 nmol per 5 x 10^6 microspheres in a 50 µL reaction is optimal. The coupling procedure can be scaled up or down. Above 5 x 10^6 microspheres, use the minimum volume required to resuspend the microspheres. Below 5 x 10^6 microspheres, maintain the microsphere concentration and scale down the volume accordingly.
6. We use 5 M TMAC (Tetramethylammonium chloride) solution from Sigma (T-3411) for preparation of 1.5X and 1X TMAC hybridization solutions. We find that this TMAC formulation does not have a strong "ammonia" odor. TMAC hybridization solutions should be stored at room temperature to prevent precipitation of the 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 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 remove the hybridization supernatant before the labeling step is depend 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 #1	Bead 1- no oligo #1	Bead 1- no oligo #1	Bead 1- no oligo #1
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 8.

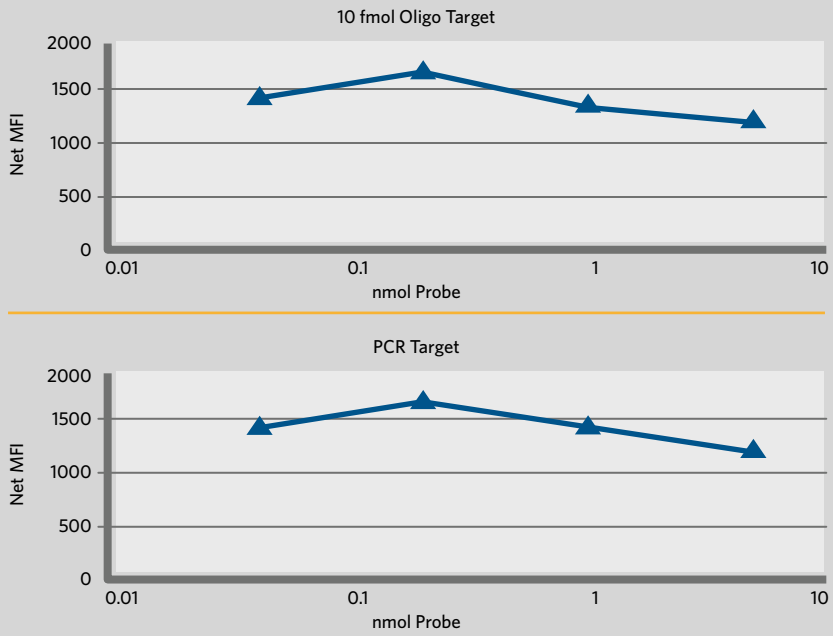


Figure 8 – Plot of experimental results for oligo coupling, as measured by a Luminex analyzer. Results show that 0.2 nmol for a 5 million microsphere coupling yielded highest MFI values. Optimal amount for typical coupling is usually 0.2 to 1 nanomole per 5 million microspheres.

To determine the optimum ratio for scaled up or for smaller 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	Probe Input[a]	EDC Concentration[b]	Tween-20 Wash Volume	SDS Wash Volume	Final Volume[c]
1 x 10^6	10 µL	0.04-0.1 nmol	0.5-2.5 mg/mL	0.5 mL	0.5 mL	20 µL
2.5 x 10^6	25 µL	0.1-0.2 nmol	0.5-1 mg/mL	0.5 mL	0.5 mL	50 µL
5 x 10^6	50 µL	0.2-1 nmol	0.5-1 mg/mL	1.0 mL	1.0 mL	100 µL
10 x 10^6	50 µL	0.5-1 nmol	0.5-1 mg/mL	1.0 mL	1.0 mL	200 µL
50 x 10^6	50-100 µL	1-4 nmol	0.5-1 mg/mL	1.0 mL	1.0 mL	1000 µL
100 x 10^6	100 µL	1-4 nmol	0.5-1 mg/mL	1.0 mL	1.0 mL	2000 µL

a We recommend titrating the probe input to optimize coupling for the particular application.
b EDC input was not adjusted for reactions containing less than 5x10^6 microspheres.
c Resuspension volume of TE, pH 8.0 for 50,000 microspheres/µL assuming 100% recovery.

Oligonucleotide coupling references

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Chapter 3.4

Optimization of Multiplex

Once the best antibody pairs or nucleic acid reagent ratios are determined for each analyte, assays should be combined into a multiplex and checked for cross-reactivity at each step. The multiplexed coupled microsphere ("bead") set should be tested with each individual analyte and detection antibody or oligonucleotide target to evaluate performance and determine specificity. Factors such as specific cross-reactivity among individual assays, non-specific binding of other reagent components, or interference of sensitivity due to excess non-bound reagents may affect your multiplex assay.

Optimization of Immunoassays

The first steps in optimizing an assay are to ensure that the optimum amount of capture molecule is bound to the microsphere ("bead") and that capture reagent pairs allow maximum binding and detection capacity. For capture sandwich immunoassays, it is important to confirm that the pair of antibodies used bind 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 then tested in multiplex with the individual candidate detection antibodies and analytes. This allows 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 oligonucleotides, antibodies, peptides and buffers.

Figure 9.

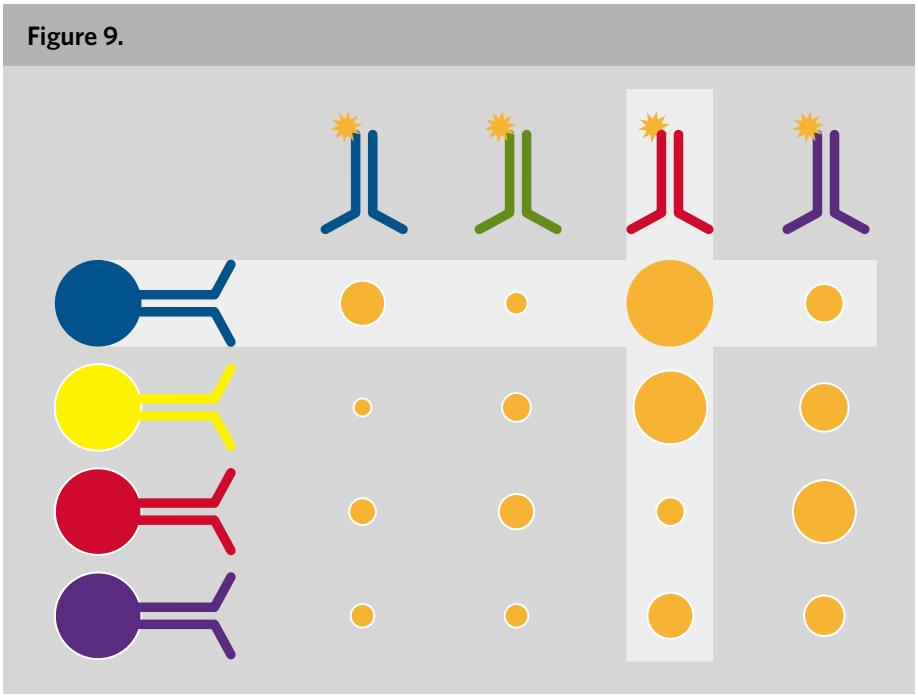


Figure 9 - Determining the best capture antibody and detection antibody by pairwise analysis. Using a small test quantity of antibody and microspheres, each candidate capture antibody is coupled to a separate microsphere and tested with target antigen and multiple candidate detection antibodies. Because 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 streptavidin-R-phycoerythrin (SAPE) as the reporter but detection antibodies may also be directly conjugated to PE, which eliminates the need for a separate reporter labeling step in the assay protocol.

General Immunoassay Tips

- Run at least 2 background samples.
- Run all samples at least in duplicate if possible whenever sample 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 1:5 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 five-fold more detection antibody may be required for a no-wash assay format.
- The optimal detection antibody concentration will depend on specific reagents and 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 Competitive Immunoassay format, we recommend testing a range of competing analyte (0.2 to 5 µg) with increasing concentrations of antibody. The 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 one and one-half times the concentration of the detection antibody.
- Use SAPE as your reporter molecule (gives highest signal of all the dyes we have compared).
- Use either PBS-1%BSA or PBS-TBN as the Assay Buffer.
- When using SAPE at > 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.
- Coupling should be allowed to proceed for 2 hours with end-over-end mixing on a rotator.
- For scaling up to 50-200 million microspheres per coupling reaction, couple in 2 mL using a 15 mL polypropylene centrifuge tube or a 4 mL microcentrifuge tube. Place tube 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 over night in Blocking/Storage Buffer at 4°C in the dark if possible.
- Too high of an antibody concentration may lead to passive adsorption and can manifest itself 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 multiplex.

Factors affecting multiplex assays

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, such as buffer system, blocking agents, sample volume and dilution, total reaction volume, number of microspheres per reaction (2000–5000 per region per well), concentration of capture reagent for coupling, detection antibody and reporter concentration, assay format (washed vs. unwashed), and incubation times should be optimized to provide best results according to the specific assay requirements. The final assays 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 to prevent interference or microsphere agglutination from matrix effects. Any reagents that show interference, cross-reactivity, or poor performance should be replaced.

Optimization of assay performance and meeting requirements for sensitivity, dynamic range, ease of use, and time to result should be kept in mind when developing the multiplexed assay. These factors and others are described in more detail below.

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., two-fold serial dilution from 4 to 1 µg/mL), but generally, 2–4 µg/mL is adequate. Detection antibody concentration may need to be increased in multiplex as compared to the concentration used in monoplex due to interactions between various detection antibodies.

In general, as the level of multiplexing increases, the amount needed for each detection antibody may also increase. In unwashed assay formats, detection antibody concentrations may need to be increased by up to as much as five-fold to compensate for excess unbound analyte in the supernatant. Typically, reporter fluorophore (SAPE) concentration should be one and one-half to two times the concentration of detection antibody.
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.

Cross-reactivity

If cross-reactivity between antibodies for different targets is observed, it may be necessary to replace with other reagents. Selecting other antibodies pre-screened during coupling optimization steps may be required. Multiplexed assays should be tested for specificity and cross-reactivity with (see illustration below):

1. individual analytes and corresponding reporter antibodies (to determine if analytes cross-react with non-target beads)
2. individual analytes and multiplexed detection antibodies (to determine if reporter antibodies cross-react with non-target analytes)
3. multiplexed analytes and multiplex detection antibodies (to determine sensitivity and to confirm there is no cross-reactivity or interference in the complete assay).

Sample cross-reactivity protocol

Figure 10 - 3-plex cytokine assay

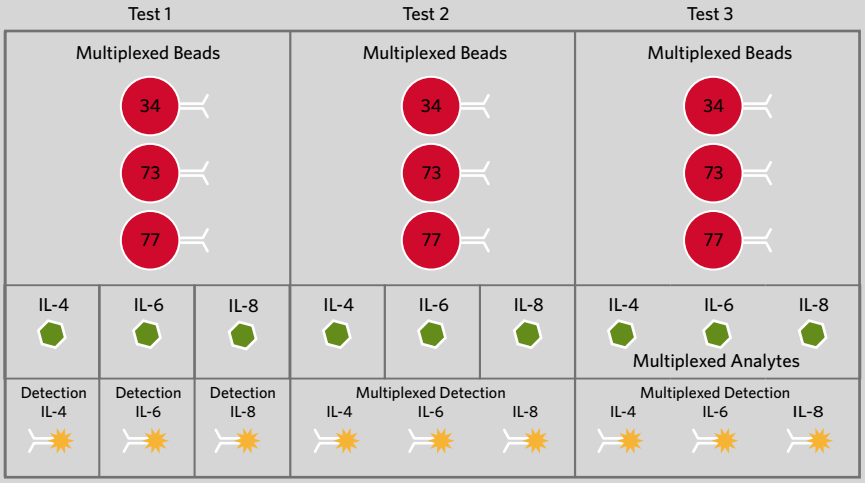


Figure 10 - Immunoassay cross-reactivity protocol. (Test 1) Test multiplex capture beads with 3 separate titrations of IL-4, IL-6 and IL-8 using appropriate monoplex reporter antibody for each titration. (Test 2) Test multiplex capture beads with 3 separate titrations of IL-4, IL-6 and IL-8 using multiplexed reporter antibodies. (Test 3) Test multiplex capture beads with multiplex titration of IL-4, IL-6 and IL-8 with multiplex reporter antibodies (i.e., the full multiplex reaction)

Test 1 is for individual analytes and corresponding reporter antibodies to determine if analytes cross-react with non-target beads. Test 2 is for individual analytes and multiplexed detection antibodies to determine if reporter antibodies cross-react with non-target analytes. Test 3 is for multiplexed analytes and multiplex detection antibodies to determine sensitivity and to confirm there is no cross-reactivity in the complete assay. Each test should be run as a standard curve with a blank and 7 concentrations of analyte as shown in the dilution table below:

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	3333.3	3333.3	3333.3	3333.3
3	1111.1	1111.1	1111.1	1111.1
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 2000 - 5000 microspheres per region), incubation times, detection antibody and reporter concentrations, coupling amount for capture reagents, and assay format (washed vs. homogeneous), and 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 median fluorescence intensity (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.

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

The working assay range will also need to be determined during assay development. Limit of Blank (LoB), Limit of Detection (LoD), and Limit of Quantitation (LoQ) are used to determine the smallest concentration of a measurand that can be reliably measured by an analytical procedure.

- LoD is the lowest analyte concentration likely to be reliably distinguished from the LoB and at which detection is feasible. LoD is determined by utilizing both the measured LoB and test replicates of a sample known to contain a low concentration of analyte.
- LoB is the highest apparent analyte concentration expected to be found when replicates of a blank sample containing no analyte are tested.
- LoQ is the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met. The LoQ may be equivalent to the LoD or it could be at a much higher concentration.

Targets	LLoQ	ULoQ	LOD	Intra-assay %CV	inter-assay %CV
3-Plex Assay					
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 at 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

Intra-assay and inter-assay reproducibility should be determined in sample diluent. NOTE: Intra/Inter assay CV may vary between site (user) and assay. The following can be used as guidelines:

Within assay working range:

Intra assay CV < 10% is usually acceptable.
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
A minimum of 5 separate runs should be performed to determine inter-assay precision.
NOTE: LLoQ may vary between site (user) and target (analyte).

For additional examples, calculations, and information, refer to the following documents:

- EP17-A: Protocols for Determination of Limits of Detection and Limits of Quantitation
- MM06-A2: Quantitative Molecular Methods for Infectious Diseases
- Ligand–Binding Assays (Development, Validation, and Implementation in the Drug Development Arena) Edited by Masood N. Khan and John W. A. Findlay (Wiley)

Linearity

A linear relationship should also be evaluated across the assay. The linearity of dilution provides confidence that the analytes present are within the assay range can be diluted and accurately for relative quantitation.
Linearity of Sample Dilutions (R2)

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

The R2 was determined by linear regression analysis of analytes measured in a 3-fold serial dilution of standard 'spiked' samples within assay range in 3 matrices.

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 sensitivity. Though seemingly paradoxical, improved sensitivity can be sometimes accomplished by decreasing the amount of capture reagent coupled to the microspheres. While this may result in saturation at lower analyte concentrations and lower the maximum achievable signal, it may improve linearity at low concentrations, thus improving the limit of detection (Fig. 11A).

Figure 11.

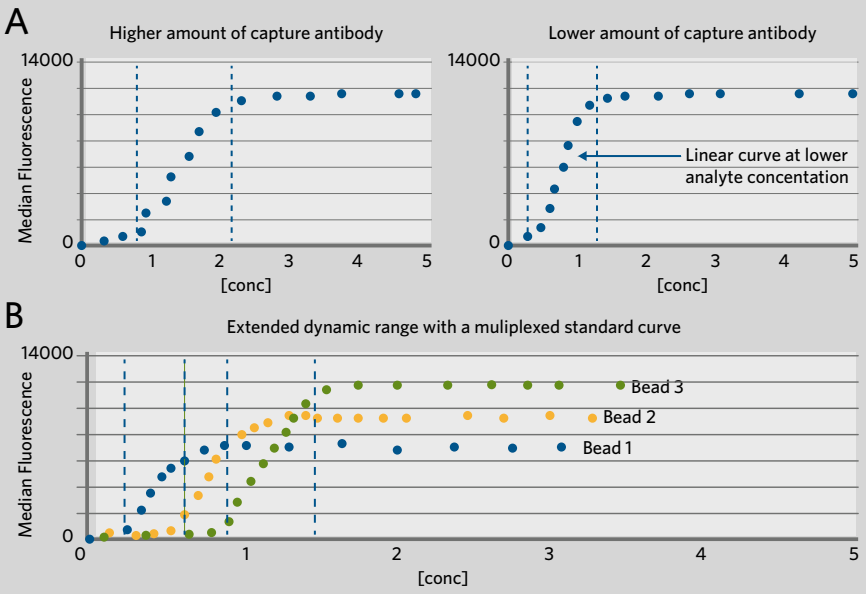


Figure 11 – Dynamic range and assay sensitivity may be affected by (A) reducing the amount of capture antibody on the bead to make lower concentration range more linear, and (B) by using different amounts of capture antibody (or antibodies with different affinities) on multiple bead sets to form a multiplexed standard curve.

Antibody affinity and sensitivity

Antibodies with higher affinity can also improve 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 (Fig. 11B). 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 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 and recovery and aid in the choice of best standard curve regression and optimal calibration. Luminex recommends that plasma and serum be diluted at least 1:5. If samples cannot be diluted at least 1:5, try using a small initial reaction volume and diluting the final reaction prior to analysis on the 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 might interfere with the assay, you may opt for other species albumin (porcine), milk casein, ChemiBLOCKER, etc. or switch to a washed assay format if using a no-wash.

Washed versus unwashed assays

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

Instrument settings and reporter fluorescence

Optimization of assay performance and meeting requirements for sensitivity, dynamic range, ease of use, and time to result should be kept in mind when developing the multiplexed assay. To improve sensitivity or increase signal many factors may be considered, including adjustment of the PMT setting on Luminex® 100/200™ and FLEXMAP 3D® instruments; selection of different vendors or types of SAPE reporter; and incorporation of dendrimers, rolling circle amplification, 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. Multiplexed assay development can be an iterative process, requiring further optimization as complex interactions between assay components are observed. Assay conditions, such as buffer system, blocking agents, sample volume and dilution, total reaction volume, number of microspheres per reaction (2000–5000 per region per well), concentration of capture reagent for coupling, detection antibody and reporter concentration, assay format (washed vs. unwashed), and incubation times, are optimized to provide best results according to the specific assay requirements, and the performance is evaluated and validated with known samples. Concentrated biological samples and samples of a highly complex nature, such as serum, plasma, or tissue lysates, should be diluted at least 1:5 to prevent interference or microsphere agglutination from matrix effects. Any reagents that show interference, cross-reactivity, or poor performance should be replaced.

Chapter 3.5

Assay Validation

After confirmation of successful coupling and sufficient signal from a multiplex assay, microspheres ("beads") 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.

Chapter 3.5.1

Immunoassay Validation

Spike and recovery immunoassay sample validation protocol

Preparing sample/control spike serial dilutions (testing sample linearity)

To test samples for linearity, make serial dilutions of the sample spike and control spike. If the neat sample has a value greater than 60% of the high standard, test the sample for natural linearity using the same dilution series described below. Vortex briefly between each dilution.

1:2 dilution

Add 0.5 mL of sample spike, control spike, or neat sample to 0.5 mL standard curve diluent.

1:4 dilution

Add 0.5 mL of 1:2 dilution to 0.5 mL standard curve diluent.

1:8 dilution

Add 0.5 mL of 1:4 dilution to 0.5 mL standard curve diluent.

These dilutions will be read off the standard curve to determine if dilutions of unvalidated samples are parallel to the standard curve and if the values of the sample dilutions are accurate.

Calculations

1. Spike/Recovery
   - % Recovery = Observed - Neat x 100
   - Observed = Spiked sample value
   - Neat = Unspiked sample value
   - Expected = Amount spiked into sample
   - Note: The neat sample may read 0 pg/mL.
   - Recovery should be in the range of 80-120%.
   - Control spike should have a recovery value within 80-120%. If not, this indicates there was a problem in preparing the 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.
     - % Recovery (1:2) = Observed value (pg/mL) of 1:2 dilution x 100
     - Expected value (pg/mL) divided by 2
     - % Recovery (1:4) = Observed value (pg/mL) of 1:4 dilution x 100
     - Expected value (pg/mL) divided by 4
     - % Recovery (1:8) = Observed value (pg/mL) of 1:8 dilution x 100
     - Expected value (pg/mL) divided by 8
   - 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 there was a problem in preparing the control spike.

Note: Recovery of spiked/ neat samples should be in the range of 80-120%.

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

R&D Systems. 2006. Spike and Recovery Immunoassay Sample Validation Protocol. http://www.woongbee.com/0NewHome/RnD/ELISA/RnD_%20SPIKEandREC2006.pdf.

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 six 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 ten 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 three plates needs to be run for inter-assay CV. Sample matrix should be depleted of target proteins, e.g. use depleted serum or plasma.

Chapter 4

Proteomic Applications

BR_574.01_0613

Chapter 4.1

Common xMAP® Immunoassays

The following immunoassay protocols presume that the user is familiar with general assay development and optimization (including microsphere ("bead") coupling). This introduction is intended to review the immunoassay development process only at a high level.

First, the assay format should be selected, such as capture sandwich, competitive or indirect assay. Next is the acquisition of necessary reagents, including antibodies, analytes, standards/controls, and buffers. The capture reagent (e.g., antibody or protein, etc.) should then be purified of extraneous primary amines prior to chemical coupling to the microspheres, which are then washed and resuspended in buffer. Coupled beads should be enumerated with a hemocytometer or other device to estimate the resulting bead count in the stock solution.

Figure 12.

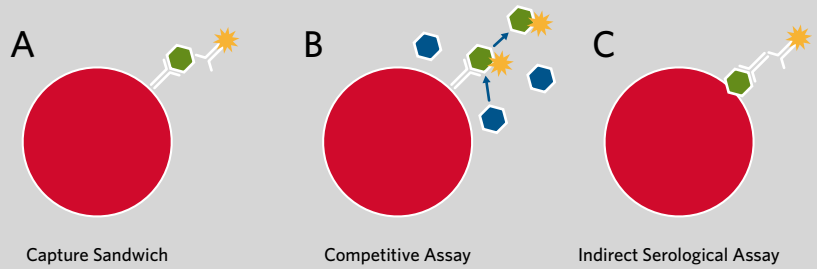


Figure 12 - Common assay formats for immunoassays include (A) capture sandwich assay, requiring two monoclonal antibodies to your analyte target; (B) competitive (antibody) assay, requiring a single antibody and a labeled positive target; and (C) indirect antibody assay (or, serology assay), requiring both a target protein and an anti-IgG antibody.

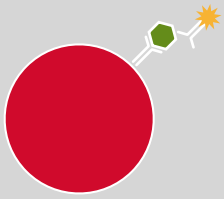
Microsphere coupling reactions are then confirmed in the assay buffer system using an appropriate detection reagent. The background fluorescence should be assessed in the target matrix to evaluate nonspecific binding and cross-reactivity, and positive control reagents similar to the sample should be used to assess positive binding reactions, sensitivity, and specificity. Monoplex microspheres can then be combined into a multiplex assay, testing for new non-specific binding, cross-reactivity or loss of signal in multiplex conditions.

Chapter 4.1.1

Capture Sandwich Immunoassay

The following immunoassay protocol presumes that the user is familiar with general assay development and optimization (including microsphere ("bead") coupling).

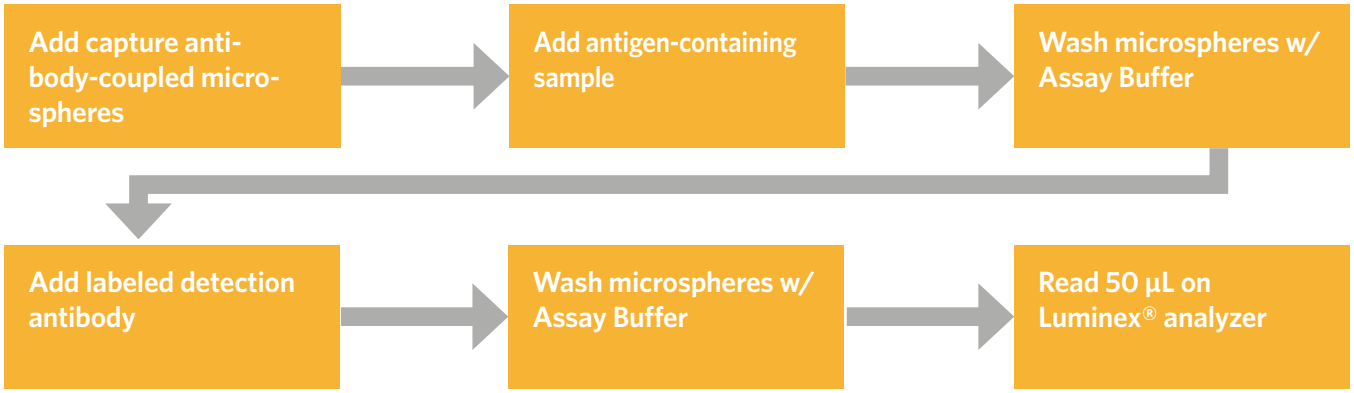
Capture Sandwich



Assay Principle and Overview

A capture sandwich immunoassay is used to detect an antigen (target) with 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 a large number of hormones, blood products, enzymes, drugs, disease markers and other biological molecules. The general steps to 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 five-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 two-fold dilutions.
- The optimal detection antibody concentration will depend on specific reagents and 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.
- The reporter concentration should be approximately one and one-half times the concentration of the detection antibody. When using streptavidin-R-phycoerythrin (SAPE) at concentrations > 8 µg/mL final concentration in a no wash format, a dilution or wash step may be required to minimize background fluorescence prior to analysis on the Luminex® instrument.

Materials Needed:

For complete equipment and materials list see Appendix B

Reagents and Consumables	Vendor
MagPlex® Microspheres (antibody coupled)	
Assay/Wash Buffer (PBS, 1% BSA)	Sigma P3688
PBS-BN buffer[1]	Sigma P3688
PBS–TBN buffer[1,2]	Sigma P3813 Sigma A7888 Sigma P9416
96 well plate	See Appendix B
PE or Biotin labeled Detection Antibody	Any suitable source
streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Life Technologies S-866 or equivalent
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Lo Bind 022431081
Disposable pipettes tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

1. Also used as assay buffer.
2. Also used as wash buffer.

Protocol 4.1.1 - Capture Sandwich Immunoassay

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

1. Select the appropriate antibody-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for approximately 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. NOTE: 50 µL of Working Microsphere Mixture is required for each reaction.

4. Aliquot 50 µL of the Working Microsphere Mixture into the appropriate wells of a round-bottom 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 approximately 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. Leave the plate in the magnetic separator for the following wash steps:
    a. Add 100 µL Assay Buffer to each well.
    b. Use a multi-channel pipette to carefully aspirate the supernatant from each well or use the manual inversion wash method. Take care not to disturb the microspheres.
    c. Repeat steps a and b above.
12. Remove the plate from the magnetic separator and resuspend the microspheres in 50 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 approximately 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. Leave the plate in the magnetic separator for the following wash steps:
    a. Add 100 µL Assay Buffer to each well.
    b. Use a multi-channel pipette to carefully aspirate the supernatant from each well or use the manual inversion wash method. Take care not to disturb the microspheres.
    c. Repeat steps a and b above.
20. Remove the plate from the magnetic separator and resuspend the microspheres in 50 µL of Assay Buffer by gently pipetting up and down several times with a multi-channel pipettor.
21. Dilute SAPE reporter to 4 µg/mL in Assay Buffer.
22. Add 50 µL of the diluted SAPE to each well.
23. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
24. Cover the plate and incubate for 30 minutes at room temperature on a plate shaker set to approximately 800 rpm.
25. Place the plate into the magnetic separator and allow separation to occur for 30-60 seconds.
26. Use a multi-channel pipette to carefully aspirate the supernatant from each well. Take care not to disturb the microspheres.

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

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

27. Leave the plate in the magnetic separator for the following wash steps:
    a. Add 100 µL Assay Buffer to each well.
    b. Use a multi-channel pipette to carefully aspirate the supernatant from each well or use the manual inversion wash method. Take care not to disturb the microspheres.
    c. Repeat steps a and b above.
28. Remove the plate from the magnetic separator and resuspend the microspheres in 100 µL of Assay Buffer by gently pipetting up and down several times with a multi-channel pipettor.
29. Analyze 50–75 µL on the Luminex analyzer according to the system manual.

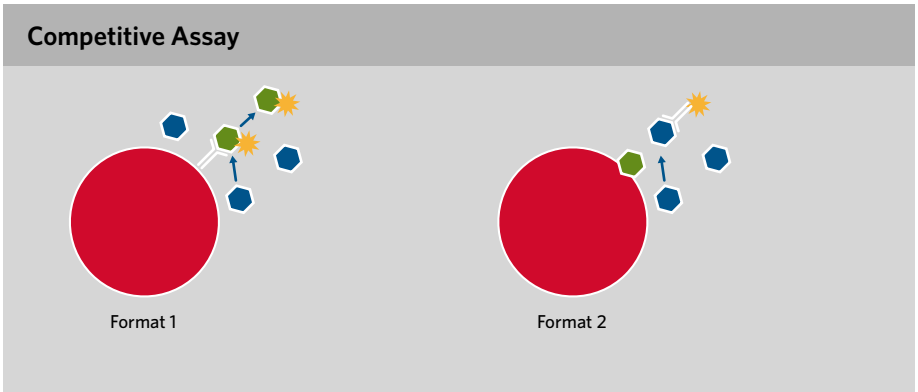
Capture Sandwich Immunoassay References:

- Bjerre M, Hansen TK, Flyvbjerg A, Tønnesen E. "Simultaneous detection of porcine cytokines by multiplex analysis: development of magnetic bioplex assay." *Vet Immunol Immunopathol.* 2009;130:53-58.

Chapter 4.1.2

Competitive Immunoassay

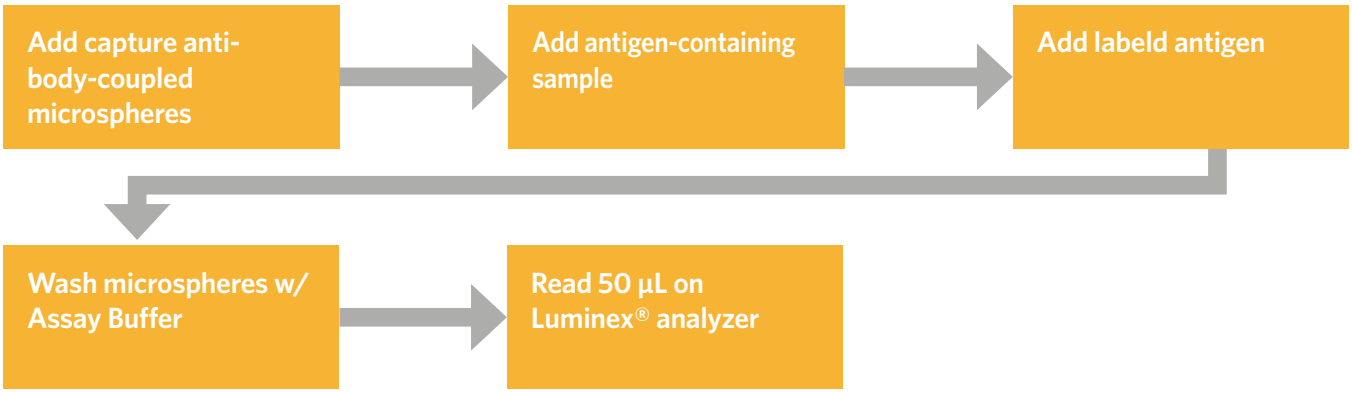
The following immunoassay protocol presumes that the user is familiar with general assay development and optimization (including microsphere ("bead") coupling).



Assay Principle and Overview

A competitive immunoassay enables detection of an antigen (target) with 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. This assay is useful for smaller protein analytes (<3-4 kD) 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 on xMAP® Technology are as follows:

Summary of Protocol (Format 1)



For competitive immunoassays, the higher the sample target concentration, the weaker the eventual signal. The 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, we recommend testing a range of competing analyte (0.2 to 5 µg) with increasing concentrations of antibody. The 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 one and one-half times the concentration of the detection antibody. When using streptavidin-R-phycoerythrin (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 the Luminex® instrument.

Materials Needed:

Reagents and Consumables	Vendor
MagPlex® Microspheres (antibody- or antigen-coupled)	
Assay/Wash Buffer (PBS, 1% BSA)	Sigma P3688
PBS-BN buffer[1]	Sigma P3688
PBS–TBN buffer[1,2]	Sigma P3813 Sigma A7888 Sigma P9416
96 well plate	See Appendix B
PE or Biotin labeled Detection Antibody or Analyte	Any suitable source
streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Life Technologies S-866 or equivalent
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Lo Bind 022431081
Disposable pipettes tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

1. Also used as assay buffer.
2. Also used as wash buffer.

For complete equipment and materials list see Appendix B

Protocol 4.1.2.1 – Competitive Immunoassay (Format 1)

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

1. Select the appropriate antibody-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for approximately 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 PBS-1% BSA. 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 PBS-1% BSA.

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

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. Dilute the SAPE reporter to 4 µg/mL in PBS-1% BSA. Note: 25 µL of diluted SAPE is required for each reaction.
13. Add 25 µL of the diluted SAPE to each well.
14. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
15. Cover the plate and incubate for 30 minutes at room temperature on a plate shaker at 800 rpm.
16. OPTIONAL Include the following steps if high backgrounds occur:
    *Carefully remove the supernatant from each well by magnetic plate separator using either manual inversion, manual pipetting or magnetic plate washer. Take care not to disturb the microspheres. Add 100 µL of Wash Buffer (PBS-TBN) to each reaction well. Take care not to disturb the microspheres.
17. Repeat step 16 once more for a total of two washes.
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.

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 PBS-1% BSA (or PBS-BN).*

Summary of Protocol (Format 2)

Protocol 4.1.2.2 – Competitive Immunoassay (Format 2)

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

1. Select the appropriate antigen-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for approximately 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 multichannel 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 multichannel pipettor.
11. Cover the plate and incubate for 60 minutes at room temperature on a plate shaker (800 rpm for MagPlex Microspheres).
12. Dilute the SAPE reporter to the appropriate concentration (typically ≥4 µg/mL) in Assay Buffer. 25 µL of diluted SAPE is required for each reaction.
13. Add 25 µL of the diluted SAPE to each well.
14. Mix the reactions gently by pipetting up and down several times with a multichannel pipettor.
15. Cover the plate and incubate for 30 minutes at room temperature on a plate shaker (400 rpm for non-magnetic microspheres or 800 rpm for MagPlex Microspheres).
16. OPTIONAL – Include the following steps if high backgrounds occur: *Carefully remove the supernatant from each well using either manual inversion, manual pipetting or magnetic plate washer. Take care not to disturb the microspheres. Add 100 µL of Wash Buffer (PBS-TBN) to each reaction well. method. Take care not to disturb the microspheres.
17. Repeat step 16 once more for a total of two washes
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.

Technical Notes

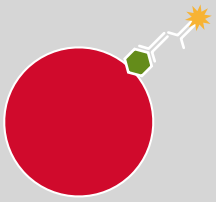
- *The [IC_{70}] and [IC_{80}] are the concentrations of detection antibody that yield 70% and 80% of the maximum obtainable signal, respectively. The [IC_{70}] or [IC_{80}] should be determined by titration in Assay Buffer.*
- *Concentrations of the detection antibodies and SAPE should be optimized. The optimal concentrations tend to be higher than in a washed assay.*

Chapter 4.1.3

Indirect (Serological) Immunoassay

The following immunoassay protocol presumes that the user is familiar with general assay development and optimization (including microsphere ("bead") coupling).

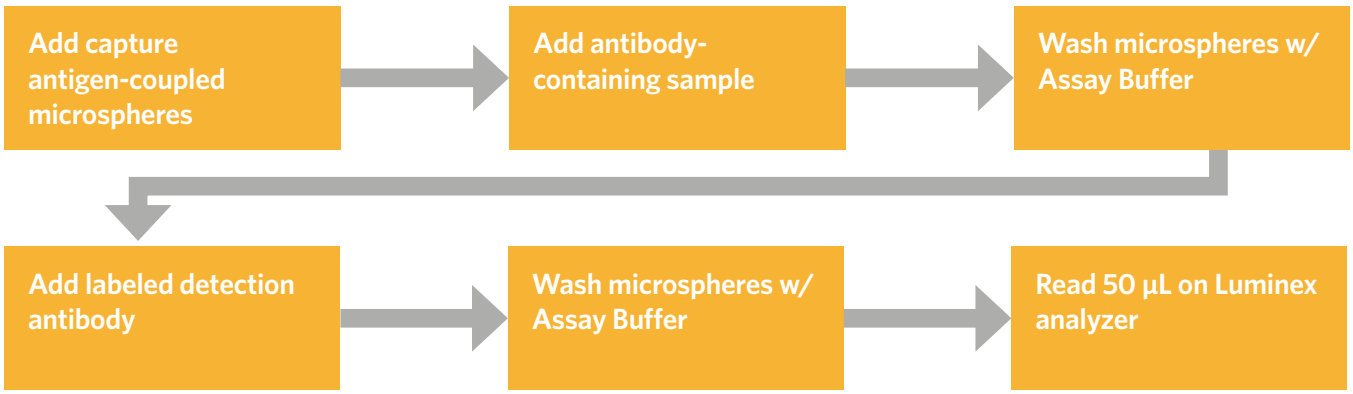
Indirect Serological Assay



Assay Principle and Overview

An indirect immunoassay enables the detection of an antibody (target) with the use of a capture protein antigen and a detection antibody. This assay is useful for serology assays where serum antibodies are measured to determine infection, allergy or autoimmune activity. The general steps to performing a serology 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 five-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 two-fold dilutions. The optimal detection antibody concentration will depend on specific reagents and 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.
- The reporter concentration should be approximately one and one-half times the concentration of the detection antibody. When using streptavidin-R-phycoerythrin (SAPE) at concentrations > 8 µg/mL final concentration, in a no wash format, a dilution or wash step may be required to minimize background fluorescence prior to analysis on the Luminex instrument.

Materials Needed:

Reagents and Consumables	Vendor
MagPlex® Microspheres (antigen-coupled)	
Assay/Wash Buffer (PBS, 1% BSA)	Sigma P3688
PBS-BN buffer[1]	Sigma P3688
PBS-TBN buffer[1,2]	Sigma P3813 Sigma A7888 Sigma P9416
96 well plate	See Appendix B
PE or Biotin labeled Detection Antibody	Any suitable source
streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Life Technologies S-866 or equivalent
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Lo Bind 022431081
Disposable pipettes tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

1. Also used as assay buffer.
2. Also used as wash buffer.

For complete equipment and materials list see Appendix B

Protocol 4.1.3 – Indirect (Serological) Immunoassay

1. Dilute samples and controls using diluent (for example, dilute 1 to 500).
2. Select the appropriate antigen-coupled microsphere mixture. Prepare a Working Microsphere Mixture by diluting the coupled microsphere stocks to a final concentration of 50 microspheres of each set/µL in PBS-1% BSA.
3. Resuspend the microspheres by vortex and sonication for approximately 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 approximately 800 rpm.
8. Place the plate into the magnetic separator and allow separation to occur for 30-60 seconds.
   *Carefully remove the supernatant from each well by magnetic plate separator using either manual inversion, manual pippetting or magnetic plate washer. Take care not to disturb the microspheres. Add 100 µL of Wash Buffer (PBS-TBN) to each reaction well.
9. Repeat step 8 once more for a total of two washes
10. Remove the plate from the magnetic separator and add 100 µL of 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 approximately 800 rpm.
12. Place the plate into the magnetic separator and allow separation to occur for 30-60 seconds.
    *Carefully remove the supernatant from each well by magnetic plate separator using either manual inversion, manual pipetting or magnetic plate washer. Take care not to disturb the microspheres. Add 100 µL of Wash Buffer (PBS-TBN) to each reaction well.
13. Repeat step 14 once more for a total of two washes.
14. Remove the plate from the magnetic separator and add 100 µL of reporter conjugate (e.g SA-PE) 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 approximately 800 rpm.
16. Place the plate into the magnetic separator and allow separation to occur for 60 seconds.
    *Carefully remove the supernatant from each well by magnetic plate separator using either manual inversion, manual pipetting or magnetic plate washer. Take care not to disturb the microspheres. Add 100 µL of Wash Buffer (PBS-TBN) to each reaction well.
17. Repeat step 16 once more for a total of two 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 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: Many standards are supplied prediluted at working concentration and do not require further dilution.

Indirect Immunoassay References:

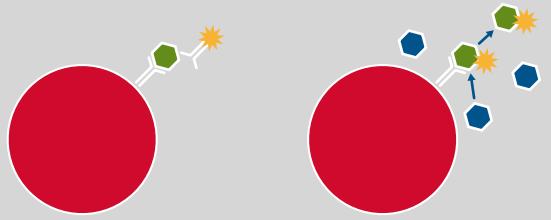
- Biagini, R.E., Schlottmann, S.A., Sammons, D.L., Smith, J.P., Snawder, J.C., Striley, C.A., MacKenzie, B.A. and Weissman, D.N. "Method for simultaneous measurement of antibodies to 23 pneumococcal capsular polysaccharides." Clin. Diagn. Lab. Immunol. 10, 744–750 (2003).
- Pickering, J.W., Martins, T.B., Greer, R.W., Schroder, M.C., Astill, M.E., Litwin, C.M., Hildreth, S.W. and Hill, H.R. "A multiplexed fluorescent microsphere immunoassay for antibodies to pneumococcal capsular polysaccharides." *Am. J. Clin. Pathol.* 117, 589–596 (2002).
- Shichijo, S., Keicho, N., Long, H.T., Quy, T., Phi, N.C., Ha, L.D., Ban, V.V., Itoyama, S., Hu, C.J., Komatsu, N., Kirikae, T., Kirikae, F., Shirasawa, S., Kaji, M., Fukuda, T., Sata, M., Kuratsuji, T., Itoh, K. and Sasazuki, T. "Assessment of synthetic peptides of severe acute respiratory syndrome coronavirus recognized by long-lasting immunity." *Tissue Antigens* 64, 600–607 (2004).

Chapter 4.1.4

Combined Capture Sandwich and Competitive Immunoassay

The following immunoassay protocol presumes that the user is familiar with general assay development and optimization (including microsphere ("bead") coupling).

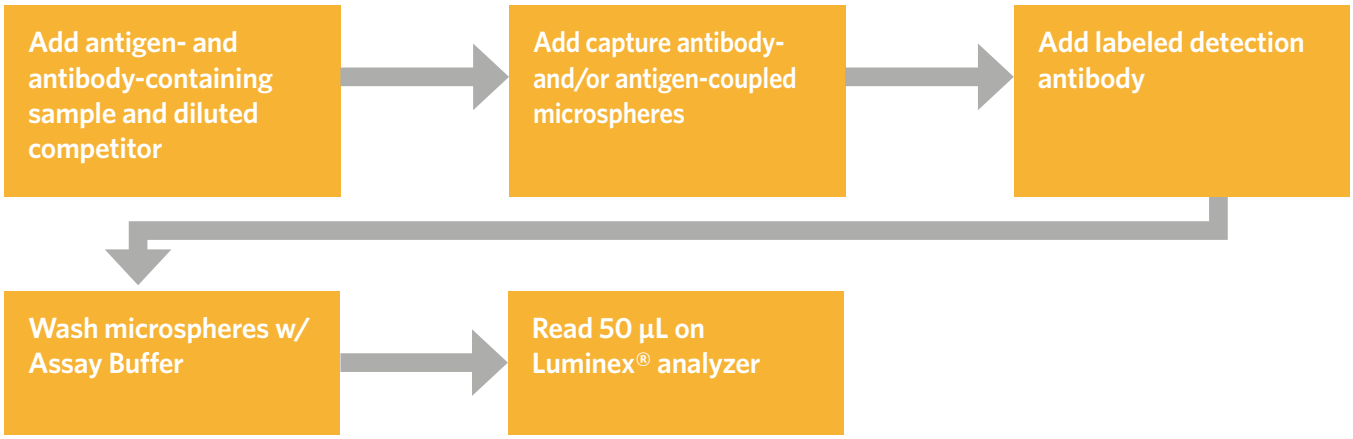
Combined Capture Sandwich and Competitive Antibody Assay



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)	
Assay/Wash Buffer (PBS, 1% BSA)	Sigma P3688
PBS-BN buffer[1]	Sigma P3688
PBS-TBN buffer[1,2]	Sigma P3813 Sigma A7888 Sigma P9416
96 well plate	See Appendix B
PE or Biotin labeled Detection Antibody	Any suitable source
streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Life Technologies S-866 or equivalent
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Lo Bind 022431081
Disposable pipettes tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

1. Also used as assay buffer.
2. Also used as wash buffer.

For complete equipment and materials list see Appendix B

Protocol 4.1.4 – 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 approximately 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.
11. Cover the filter plate and incubate for 60 minutes at room temperature on a plate shaker (800 rpm for MagPlex Microspheres).
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 (800 rpm for MagPlex Microspheres).

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

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.*

16. Dilute the SAPE reporter to the appropriate concentration (typically ≥10–12 µg/mL) in Assay Buffer. 10 µL of diluted SAPE is required for each reaction.
17. Add 10 µL of the diluted SAPE to each well.
18. Mix the reactions gently by pipetting up and down several times with a multi-channel pipettor.
19. Cover the plate and incubate for 30 minutes at room temperature on a plate shaker (800 rpm for MagPlex Microspheres).
20. Bring final volume of each reaction to 100 uL with assay buffer.
21. OPTIONAL – Include the following steps if high backgrounds occur: – Carefully remove the supernatant from each well using either manual inversion, manual pipetting or magnetic plate washer. Take care not to disturb the microspheres. Add 100 µL of Wash Buffer (PBS-TBN) to each reaction well. Take care not to disturb the microspheres.
22 Repeat step 21 once more for a total of two washes
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.

Chapter 4.2

Other Coupling Moieties

Peptides, phospholipids, and other small molecules can be directly coupled to the microsphere ("bead") surface (Komatsu et al., 2004; Shichijo et al., 2004) but may be more efficiently accomplished through modification of the small molecule or the microsphere to provide adequate spacing from the microsphere surface. This can be accomplished through the use of a linker or carrier protein attached to the small molecule, which can then be coupled to the microsphere surface using the standard one-step carbodiimide chemistry. If the small molecule is available in a biotinylated form, it can be bound to LumAvidin® Microspheres where the avidin provides spacing from the microsphere surface (Iannone et al., 2001; Drummond et al., 2008; Gu et al., 2008).

Common 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-N-Maleimidophenyl) butyric acid hydrazide hydrochloride
Maleimide
Biotin

Luminex® recommends, in the following order:

1. Coupling the peptide to a carrier protein – Conjugating your small molecule to a carrier protein such as bovine serum albumn (BSA), keyhole limpet hemocyanin (KLH), or thyroglobulin (TG) may be done using commercially available crosslinking reagents followed by coupling the modified capture moiety to the beads using our standard protein coupling protocol.
2. Biotinylate the peptide and bind it to LumAvidin beads (non-magnetic) – Your capture peptide may be available in biotinylated form, or may be easily modified using commercially available reagents. In this case the detection reagent must be directly conjugated with PE.
3. Modify the microsphere surface with adipic acid dihydrazide (ADH) or 4-(4-N-Maleimidophenyl)butyric acid hydrazide hydrochloride (MPBH) and couple the peptide via carboxyl or sulfhydryl groups using EDC.

Other Coupling Moiety References:

- Iannone, M.A., Consler, T.G., Pearce, K.H., Stimmel, J.B., Parks, D.J. and Gray, J.G. "Multiplexed molecular interactions of nuclear receptors using fluorescent microspheres." *Cytometry* 44, 326–337 (2001).
- Drummond, J.E., Shaw, E.E., Antonello, J.M., Green, T., Page, G.J., Motley, C.O., Wilson, K.A., Finnefrock, A.C., Liang, X. and Casimiro, D.R. "Design and optimization of a multiplex anti-influenza peptide immunoassay." *J. Immunol. Methods* 334, 11–20 (2008).
- Komatsu, N., Shichijo, S., Nakagawa, M. and 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.* 64, 535–545 (2004).
- Schlottmann, S.A., Jain, N., Chirmule, N. and Esser, M.T. "A novel chemistry for conjugating pneumococcal polysaccharides to Luminex microspheres." *J. Immunol. Methods* 309, 75–85 (2006).

Chapter 4.2.1

Coupling Biotinylated Peptides with LumAvidin® Microspheres

A sample protocol for binding of biotinylated molecules to LumAvidin Microspheres ("beads") is described below. However, with this approach a biotin–streptavidin system cannot be used for reporter labeling and an alternative reporter labeling method, such as a direct conjugation of PE to the detection reagent, would be necessary.

Summary of Protocol

Materials Needed:

Reagents and Consumables	Vendor
LumAvidin® Microspheres	Luminex®
Assay/Wash Buffer (PBS, 1% BSA)	Sigma P3688
Biotin conjugated molecule	Any suitable brand
PBS-BN buffer[1]	Sigma P3688
PBS–TBN buffer[1,2]	Sigma P3813 Sigma A7888 Sigma P9416
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 Eppendorf Lo Bind 022431081
Disposable pipettes tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

1. Also used as assay buffer.
2. Also used as wash buffer.

For complete equipment and materials list see Appendix B

Protocol 4.2.1 Coupling biotinylated peptides with LumAvidin Microspheres

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

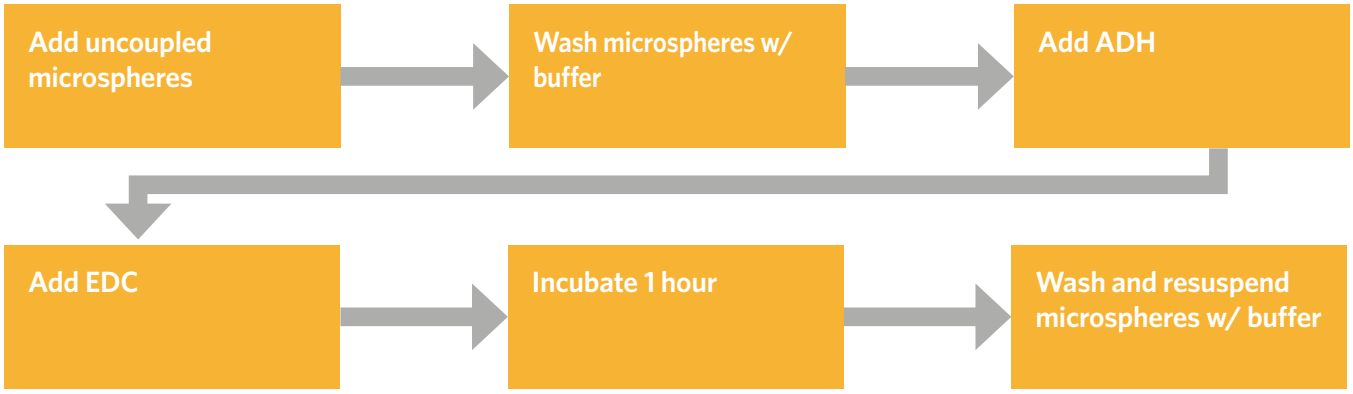
1. Resuspend the stock LumAvidin microsphere suspension according to the instructions described in the Product Information Sheet provided with your microspheres.
2. Transfer 1.0×10^5 of the stock microspheres to a recommended microcentrifuge tube.
3. Pellet the stock microspheres by microcentrifugation at ≥8000 g for 1–2 miniutes (or by using a magnetic separator) and remove supernatant.
4. Remove the supernatant and resuspend the pelleted microspheres in 250 µL of PBS–BSA by vortex and sonication for approximately 20 seconds.
5. Dilute the biotin-conjugated molecule in PBS–BSA. The optimal concentration should be determined by titration in the 4–4000 nM range.
6. Add 250 µL of the biotin-conjugated molecule solution to the microsphere suspension and mix immediately by vortex.
7. Incubate for 30 minutes with mixing (by rotation) at room temperature.
8. Pellet the bound microspheres by microcentrifugation at ≥8000 g for 1–2 minutes (or by using a magnetic separator) and remove supernatant.
9. Resuspend the pelleted microspheres in 500 µL of Blocking/Storage Buffer (PBS-BN or PBS–TBN) by vortex.
10. Repeat steps 8 and 9 for a total of two washes with Blocking/Storage Buffer.
11. Remove the supernatant and resuspend the microspheres in 250–1000 µL Blocking/Storage Buffer by vortex and sonication for approximately 20 seconds.
12. Store the bound LumAvidin microspheres refrigerated at 2–8 °C in the dark.

Chapter 4.2.2

Coupling Peptides with ADH

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

Summary of Protocol



Materials Needed:

Reagents and Consumables	Vendor
MagPlex® Microspheres	Luminex
Adipic acid dihydrazide (ADH)	Sigma A0368
Coupling Buffer (0.1 M MES, pH 6.0)	Sigma M2933
1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC)	Pierce 77149
PBS pH7.4	See Appendix A
Storage Buffer PBS–TBN buffer	Sigma P3813 Sigma A7888 Sigma P9416
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Lo Bind 022431081
Disposable pipettes tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

For complete equipment and materials list see Appendix B

Protocol 4.2.2 Modification of microspheres with adipic acid dihydrazide (ADH)

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

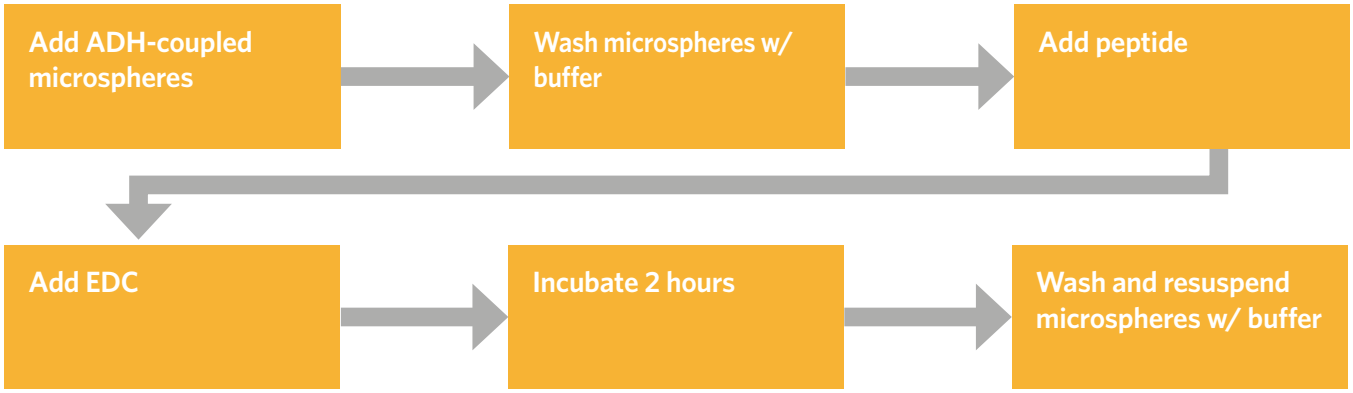
1. Resuspend the stock microsphere suspension according to the instructions described in the Product Information Sheet provided with your microspheres.
2. Remove an aliquot of 25×10^6 of microspheres and pellet by centrifugation at ≥4000 g for 2 min (or by using a magnetic separator) and remove supernatant.
3 Resuspend the pelleted microspheres in 1 mL of 0.1 M MES, pH 6.0, by vortex and sonication for approximately 20 seconds.
4. Transfer the resuspended microspheres to a recommended microcentrifuge tube and pellet the microspheres by microcentrifugation at ≥8000 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 ≥8000 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 8000 g for 1–2 minutes (or by using a magnetic separator) and remove supernatant. Repeat for a total of 3 washes with 1 mL of 0.1 M MES, pH 4.5.
12. Resuspend the ADH-modified microspheres in 1 mL of 0.1 M MES, pH 4.5, and store refrigerated at 2–8 °C in the dark.

Chapter 4.2.3

Peptide Coupling to ADH-Modified Microspheres

Adipic acid dihydrazide (ADH) is a suitable chemical spacer for extending the peptide away from the surface of xMAP® Microspheres ("beads") for optimum reactivity with 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)	
Protein to couple	Any suitable source
Wash Buffer (0.1 M MES, pH 6.0)	Sigma M2933
1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC)	Pierce 77149
Wash Buffer PBS-TBN Buffer	Sigma P3813 Sigma A7888 Sigma P9416
PBS pH7.4	See Appendix A
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Lo Bind 022431081
Disposable pipettes tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

For complete equipment and materials list see Appendix B

Protocol 4.2.3 Coupling peptides to ADH-Modified Microspheres

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

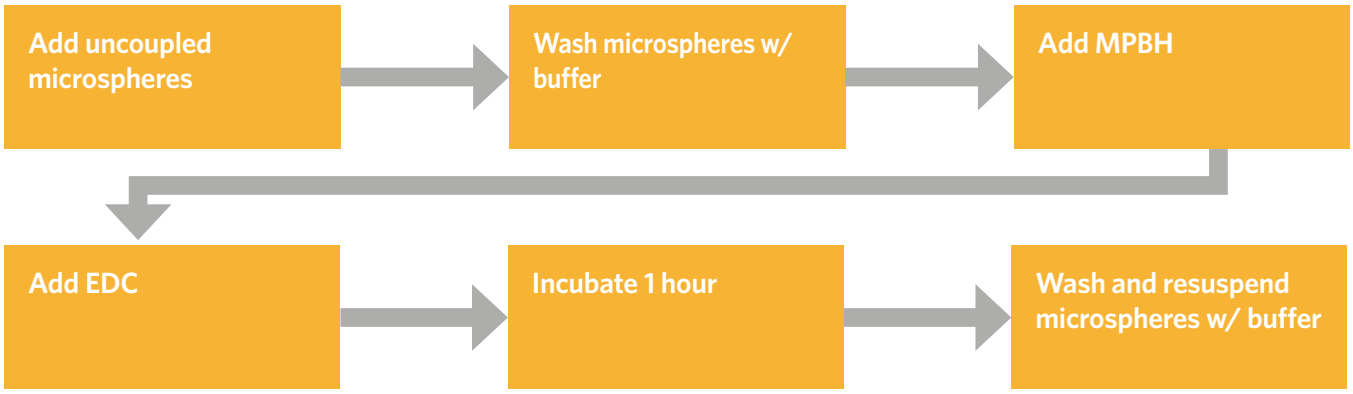
1. Resuspend stock ADH-modified microsphere suspension by vortex and sonication (15-30 seconds)
2. Remove an aliquot of 25 x 10^6 ADH microspheres and pellet by centrifugation at ≥ 8000 x g, 1-2 minutes (or by using a magnetic separator) and remove supernatant.
3. Wash once with 1 mL 0.1 M MES, pH 6.0 and pellet by centrifugation at ≥ 8000 x g, 1-2 minutes (or by using a magnetic separator) and remove supernatant, and resuspend ADH microspheres in 100 µL 0.1 M MES, pH 6.0, vortex.
4. Add 250 µg protein 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 ≥ 8000 x g, 1-2 minutes or by using a magnetic separator and remove supernatant, Resuspend coupled microspheres in 1 mL PBS, pH and vortex.
8. Pellet by centrifugation at ≥ 8000 x g, 1-2 minutes, or by using a magnetic separator and remove supernatant, wash twice 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 4x4 section) x (1 x 10^4) x (dilution factor) x (resuspension volume in mL)*

Chapter 4.2.4

Coupling Peptides with MPBH

4-(4-N-Maleimidophenyl)butyric acid hydrazide hydrochloride (MPBH) is a suitable chemical spacer for extending the peptide away from the surface of xMAP® Microspheres ("beads") for optimum reactivity with sample and reagents. MPBH provides an 8-atom spacer with a reactive maleimide group for coupling to cysteine sulfhydryls. A sample protocol for modifiying 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 hydrochloride (MPBH)	Pierce 22305
0.1 M MES, pH 6.0	Sigma M2933
DMSO	Any suitable source
1-Ethyl-3-[3-dimethylaminopropyl]carboiimide hydrochloride (EDC)	Pierce 77149
0.1 M MES, pH 4.5	Sigma M2933
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Lo Bind 022431081
Disposable pipettes tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

For complete equipment and materials list see Appendix B

Protocol 4.2.4 Modification of microspheres with MPBH (maleimide)

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

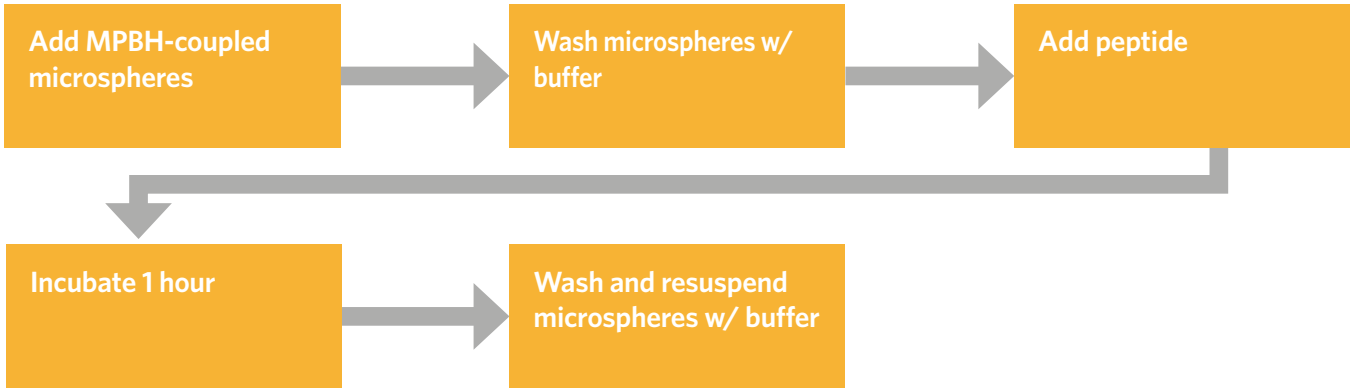
1. Resuspend the stock microsphere suspension according to the instructions described in the Product Information Sheet provided with your microspheres.
2. Remove an aliquot of 25×10^6 of microspheres and pellet by centrifugation at ≥4000 g for 2 min (or by using a magnetic separator) and remove supernatant.
3. Resuspend the pelleted microspheres in 1 mL of 0.1 M MES, pH 6.0, by vortex and sonication for approximately 20 seconds.
4. Transfer the resuspended microspheres to a recommended microcentrifuge tube and pellet the microspheres by microcentrifugation at ≥8000 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 ≥8000 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 ≥8000 g for 1–2 minutes (or by using a magnetic separator) and remove supernatant.
13. Repeat step 14 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.

Chapter 4.2.5

Coupling Peptides with Maleimide

Maleimide (MPBH) is a suitable chemical spacer for extending the free terminal cysteine-containing peptide away from the surface of xMAP® Microspheres ("beads") for optimum reactivity with 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)	
Peptide to couple	Any suitable source
100 mM Tris, pH 7.4	See Appendix A
Assay/Wash Buffer (PBS, 1% BSA)	See Appendix A
Storage Buffer (PBS-TBN)	Sigma P3813 Sigma A7888 Sigma P9416
0.1M Sodium Phosphate, 50mM NaCl pH 7.0	See Appendix A
1.5 mL microcentrifuge tubes	USA Scientific 1415-2500 or Eppendorf Lo Bind 022431081
Disposable pipettes tips	Any suitable brand
Distilled deionized water (ddH_2O)	Any suitable source

For complete equipment and materials list see Appendix B

Protocol 4.2.5 Coupling peptides to maleimide-modified microspheres

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

1. Resuspend stock maleimide-modified microsphere suspension by vortex and sonication (15-30 seconds)
2. Remove an aliquot of 1 x 10^5 maleimide microspheres and pellet by centrifugation at ≥ 8000 x g, 1-2 minutes (or by using a magnetic separator) and remove supernatant.
3. Resuspend maleimide microspheres at 1 x 10^5/mL in 0.1 M Sodium Phosphate, 50 mM NaCl, pH 7.0 by vortex
4. Aliquot 1 x 10^4 microspheres to each coupling reaction (100 µL)
5. Add peptide (100 µL, in 100 mM Tris, pH 7.4) to each 1 x 10^4 microsphere reaction
6. Incubate 1 hour at room temperature with shaking (protect from light)
7. Pellet by centrifugation at ≥ 8000 x g, 1-2 minutes (or by using a magnetic separator) and remove supernatant.
8. Wash twice with PBS-1% BSA, (200-500 µL).
9. Resuspend coupled microspheres in PBS-TBN.

Reaction	Peptide 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

Chapter 4.3

Proteomics FAQ's

For immunoassays, should you couple a polyclonal or monoclonal antibody to the microspheres ("beads")?

- 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.
- As always, the success of the assay achieving the desired sensitivity and specificity will depend on the quality of the reagents.

Do protein-protein interactions work on Luminex® Microspheres?

- Yes. An example of protein-protein interactions is a transcription complex where proteins interact to influence gene expression.
- The difficulty with protein-protein interactions is the low affinity that proteins have for each other.
- Multiplex IP-FCM (immunoprecipitation-flow cytometry): Principles and guidelines for assessing physiologic protein-protein interactions in multiprotein complexes. Bida AT, Gil D, Schrum AG. Methods. 2012 Feb;56(2):154-60. doi: 10.1016/j.ymeth.2011.09.005. Epub 2011 Sep 16. PMID: 21945581
- IP-FCM measures physiologic protein-protein interactions modulated by signal transduction and small-molecule drug inhibition. Smith SE, Bida AT, Davis TR, Sicotte H, Patterson SE, Gil D, Schrum AG. PLoS One. 2012;7(9):e45722. doi: 10.1371/journal.pone.0045722. Epub 2012 Sep 21. PMID: 23029201

Does Luminex recommend sources for antibody pairs?

- Luminex has used several sources including R&D Systems, Pharmingen, Rockland, OEM, and Fitzgerald but recommends that customers consult their preferred vendor. 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 R&D Systems (Wood et al. 2011) and eBioscience (Rizzi et al. 2010). Many publications list the source, catalog number and clone number for their antibodies used (Bjerre et al. 2009; Carslon & Vignali 1999; de Jager et al. 2003; de Jager et al. 2005; de Jager et al. 2009; Dernfalk, et al. 2004; Dernfalk et al. 2007; Lawson et al. 2010; Ray et al. 2005; Skogstrand et al. 2005). Many of the assays built in these publications are common and Luminex suggests you use these as a starting point to save time. The Antibody Resource website (http://www.antibodyresource.com) is a good starting point to search for antibody suppliers.
- 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 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, T. K., Flyvbjerg, A., and Tonnesen, E. "Simultaneous detection of porcine cytokines by multiplex analysis: Development of magnetic bioplex assay." *Vet Immunol Immunopathol* 2009; 130:53-8.
  - Carson,R. T. and Vignali, D. A. A. "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, B. J., Kuis, W., and Rijkers, G. T. "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, B. J., Bijlsma, J. W. J., Kuis, W., and Rijkers, G. T. "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, G. T., Prakken, B. J., and Seyfert-Margolis, V. "Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays." *BMC Immunology* 2009;10:U1-U11.
  - Dernfalk, J., Waller, K. P., and Johannisson, A. "Commercially available antibodies to human tumour necrosis factor-? tested for cross-reactivity with ovine and bovine tumour necrosis factor-? using flow cytometric assays." *Acta Vet Scand* 2004; 45:99-107.
  - Dernfalk, J., Waller, K. P., and Johannisson, A. "The xMAP technique can be used for detection of the inflammatory cytokines IL-1?, IL-6 and TNF-? bovine samples." *Vet Immunol Immunopathol* 2007; 118:40-9.
  - Faucher, S., Crawley, A. M., Decker, W., Sherring, A., Bogdanovic, D., Ding, T., Bergeron, M., Angel, J. B., and Sandstrom, P. "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., Osorio, F., Nelson, E., Welbon, C., Clement, T., Fang, Y., Wong, S., Kulas, K., and Christopher-Hennings, J. "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, C. A., Bowsher, R. R., Smith, W. C., Devanarayan, V., Willey, M. B., Brandt, J. T., and Dean, R. A. "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, Y. J., Latek, R., Weiner, R., and Rhyne, P. W. "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, D. E., Sorensen, L. C., and Hougaard, D. M. "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. and 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

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?

- It depends upon the assay format.
- If assaying for peptide specific antibodies then a normal immunoassay buffer should suffice (like 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?

- Reagents may be ordered with reporter fluorophores directly labeled.
- We have had success labeling proteins with phycoerythrin using "PhycoLink" kits from Prozyme (http://www.prozyme.com).

Coupling Polysaccharides

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

    - Pickering, J.W., T.B. Martins, R.W. Greer, M.C. Schroder, M.E. Astill, C.M. Litwin, S.W. Hildreth, and H.R. Hill. 2002. "A multiplexed fluorescent microsphere immunoassay for antibodies to Pneumococcal capsular polysaccharides." *American Journal of Clinical Pathology* 117: 589-596.

    - Biagini RE, Schlottmann SA, Sammons DL, Smith JP, Snawder JC, Striley CA, MacKenzie BA, Weissman DN. "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., (2006) "A Novel Chemistry for Conjugating Pneumococcal Polysaccharides to Luminex Microspheres." *Journal Of Immunological Methods* 309:75-85.

Alternatives to BSA for blocking.

- Any non-specific protein can be used for blocking including many of the commercially available blocking buffers.
- Do not use unpurified blockers such as non-fat milk.

Can streptavidin-R-phycoerythrin (SAPE) be used in assays when the samples are in tissue culture media?

- Tissue culture generally contains a large amount of biotin.
- The SAPE 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 directly coupled detection antibody.
  - Pre-combine the detection antibody and the SAPE (30-60 minutes) before addition to the sample. By doing this the detection antibody and SAPE can bind first before the free biotin can interfere.

How many PE molecules per 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.

What third-party data processing tools does Luminex recommend?

- Millipore - MILLIPLEX Analyst
  http://www.millipore.com/bmia/flx4/multiplex-assay-analysis-software
- Bio-Rad - BioPlex Manager
  http://www.bio-rad.com/evportal/en/US/LSR/Category/45938d9d-c2ec-4ae4-9ed3-e7358a98d30b/Bio-Plex-Software
- MiraiBio - MasterPlex QT
  http://www.miraibio.com/masterplex-qt-qt-luminex-quantitative-data-analysis-software/
- Excel
- Sigma Plot

How do samples containing biological fluids perform on the Luminex Instruments?

- As long as the reagents function in the biological matrix, the assay should work on the Luminex platform 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.

What signal is considered a 'positive' signal?

- Base this on the positive control. A general recommendation is that a positive signal should be at least 2 standard deviations above background (or the negative control).

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 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).
- Avoid organic solvents as they will cause the internal classification dyes of the beads to leach out causing misclassification.

Can formaldehyde be used to stop reactions on the Luminex Microspheres?

- Formaldehyde or Para-formaldehyde 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 PE when converting from monoplex to multiplex?

- Use cumulative concentration of simplex assay.
- There is no prescribed ratio of biotin to SA-RPE concentration because the extent of biotinylation of detection antibodies is usually not known.
- Titration is recommended.
- More PE must be added for no-wash than wash.

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 fluorescence or washing samples before running them.

Can the Luminex platform analyze whole cell assays?

- The Luminex platform is not designed to analyze whole cells.
- Cell lysates can be analyzed as long as the viscosity of the sample is sufficiently reduced before aspirating into the analyzer.
- This will have to be an empirical determination on the part of the user.
- The user needs to be aware of potential shifting of the beads due to composition differences between sample core and sheath fluid.

How many events should be collected per analyte?

- 50-100 events is sufficient to obtain accurate results.
- Refer to Carson and Vignali (Carson, R.T. and D.A.A. Vignali, 1999, "Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay." *Journal of Immunological Methods*). From the comparison, they 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."
- Several studies (not published) have concluded that a minimum of 35 events is required to obtain a statistically valid median result.
- Customer 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 determined from the sample microspheres.

Chapter 5

Genomic Applications

BR_574.01_0613

Chapter 5.1

Introduction: Development of Nucleic Acid Assays

The following nucleic acid assay protocols presume that the user is familiar with general assay development and optimization (including microsphere ("bead") coupling). This introduction is intended to review the genomic assay development process only at a high level.

xMAP Technology is capable of a number of genomic assay formats such as 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 onto MagPlex®-TAG™ beads without the need to couple oligos.

Figure 13.

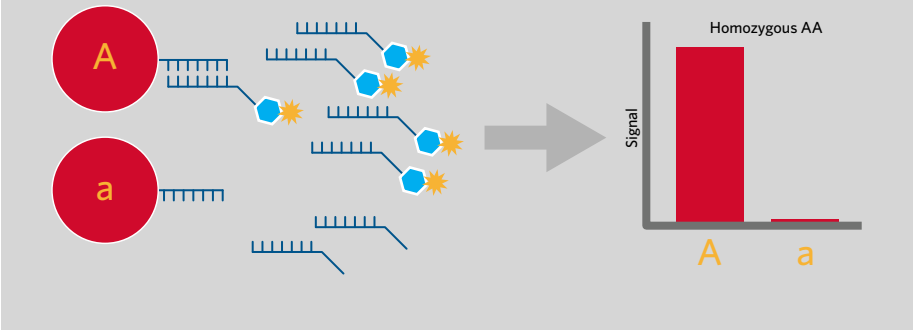
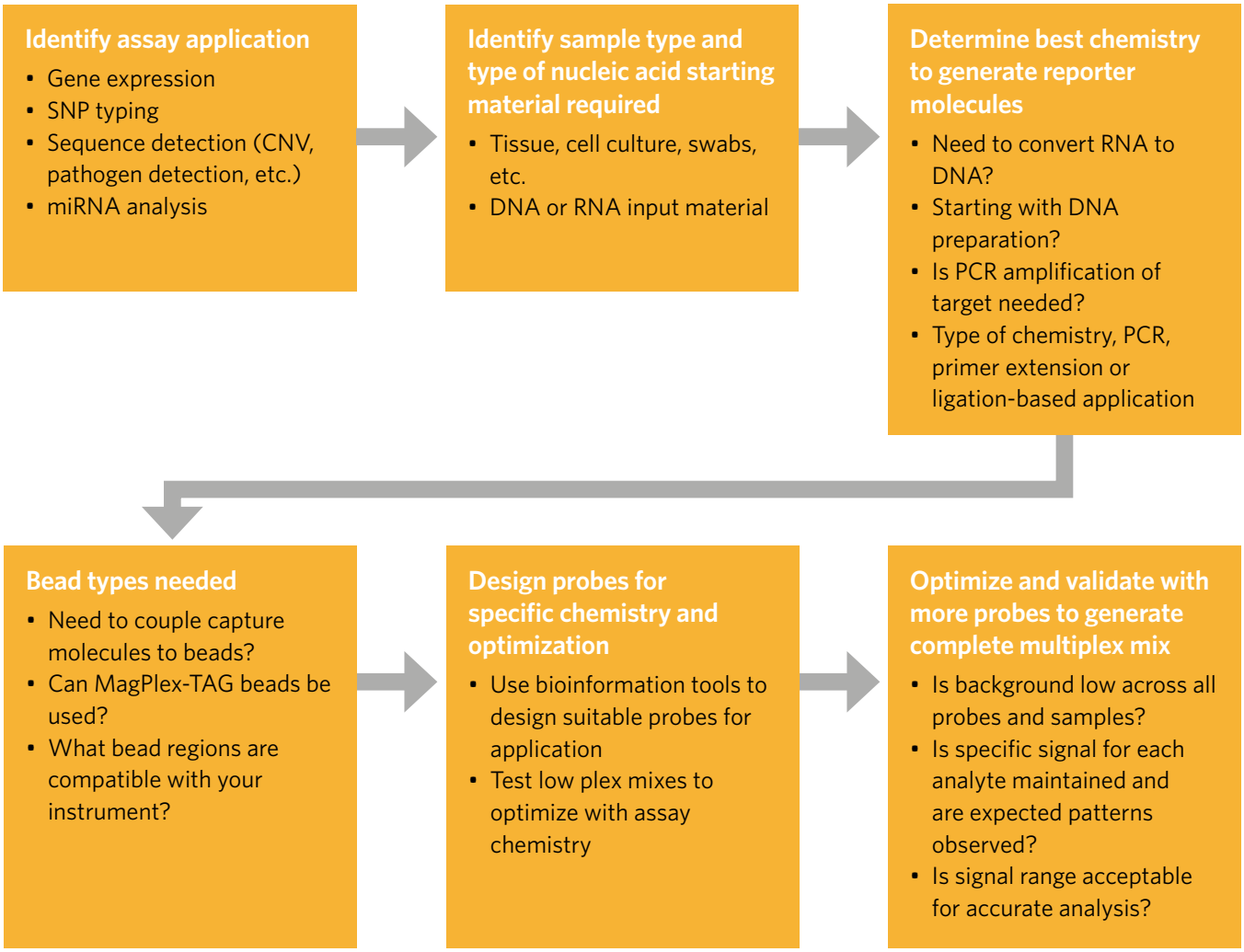


Figure 13 - Schematic of nucleic acid assay analysis on Luminex® beads. Each bead has a unique capture sequence specific for a marker sequence. If reporter molecules are generated and captured (bead A) a fluorescence signal is detected. f no reporter molecules are generated and captured (bead a) minimal or background signal is detected.

While there are a large number of scientific publications describing a variety of Luminex genomic assays (www.luminexcorp.com/bibliography), the protocols outlined in this guide describe a number of applications developed and tested in the Luminex laboratory. These protocols focus on several genomic chemistries captured to MagPlex-TAG Microspheres, but some may be applied, with suitable modifications, to beads coupled with other sequences by the user.

Development of an effective xMAP-based nucleic acid assay is dependent on a number of factors. One of the first things to consider is the purpose of the assay. Depending on the application chosen, different types of starting nucleic acid and other chemistry will be required to generate reporter molecules for data collection with the Luminex platform.

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



Chapter 5.1.1

Oligo Ligation Assay (OLA) SNP Typing

The oligonucleotide ligation assay (OLA) is flexible, inexpensive and simple approach for detecting SNPs and other sequence variations. The flexibility of the assay has allowed its use for genotyping a number of different genes and organisms (Bruse, Moreau et al. 2008, Schwartz, Pike-Buchanan et al. 2009, Henry-Halldin, Nadesakumaran et al. 2012).

In OLA chemistry, one or more forward probes containing TAG sequences bind adjacent to a common biotinylated downstream probe (Figure 14). The close proximity of a bound OLA-TAG probe 3′ end with the OLA reporter probe 5′ end enables 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, little or no ligation occurs and no significant signal is generated for analysis.

Note that OLA probes do not have primer binding sites for PCR amplification as 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 14. OLA Probe Design

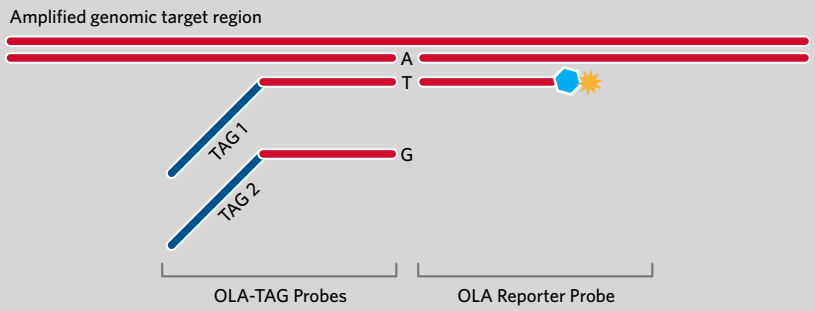


Figure 14 - Oligo Ligation Assay (OLA) involves two target-specific probes that align adjacent to one another allowing enzymatic ligation if an exact match occurs at the target SNP site. Incorporation of TAG sequences enables capture of each possible allele to a unique MagPlex-TAG microsphere ("beads").

Materials Needed:

Reagents and Consumables	Vendor
MagPlex®-TAG™ Microspheres	Luminex®
Primers for PCR amplification of gDNA target regions.	IDT or other vendor
OLA-TAG primers with 5′ TAG sequence and biotinylated OLA reporter primers	IDT 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
dNTPs stocks	Life Technologies™ 10297-018 or equivalent
2X Tm Hybridization Buffer	See Buffer and Reagent Recipes section
1X Tm Hybridization Buffer	See Buffer and Reagent Recipes section
streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Life Technologies S-866 or equivalent
96-well PCR Plate	BioRad MSP9601
MicroSeal A	BioRad MSA5001
Magnetic separation plate (special order)	V&P Scientific VP771LD-4CS or equivalent
Disposable pipette tips; multi- and single-channel (2-1000 mL)	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, frequency waves)	Any suitable brand
Centrifuge with Microplate Swinging Bucket Rotor	Eppendorf 5704 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 3.1 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 Microspheres 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 multiplex a 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 contact your Luminex representative or visit www.luminexcorp.com.
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. Amplicon size is not restricted. 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. 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. 5. The OLA-TAG probe is 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.
dNTPs	These can be purchased from several vendors such as Life Technologies™ (10297-018). A 25 mM stock mix can be made by mixing equal amounts of 100 mM dATP, dTTP, dCTP, and dGTP for the 4dNTP stock mix for gDNA amplification. Individual stocks and concentrated mixes should be stored at -20°C.
2X Tm 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 Tm 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), Life Technologies™ (S866) 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 wash or no wash protocols.

Protocol 5.1.1 – 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 for example purposes only 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
dNTP	200 µM each
primer	0.2 µM each
Qiagen HotStar 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	
37°C	1 minute	30 cycles
4°C	Hold	

5. Proceed to hybridization with MagPlex-TAG Microspheres using a wash or no wash protocol.

Hybridization to MagPlex-TAG Microspheres- 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 (beads).
2. Combine 2500 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. Contact your Luminex representative or visit www.luminexcorp.com.)
3. Dilute/concentrate the MagPlex-TAG microsphere mixture to 100 of each microsphere set per µL in 2X Tm Hybridization Buffer and mix by vortex and sonication for approximately 20 seconds.
4. Aliquot 25 µL of the MagPlex-TAG microsphere mixture to each well.
5. Add 5 to 25 µL of each OLA reaction to appropriate wells (Note: 1-5 µL is usually sufficient) 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 the bead pellets.
10. Resuspend the pelleted MagPlex-TAG Microspheres in 75 µL of 1X Tm 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 the bead pellets.

12. Repeat steps 8 to 11 for a total of two washes.
13. Resuspend microspheres in 75 µL of 1X Tm Hybridization Buffer containing 2-8 µg/mL SAPE.
14. Incubate at 37°C for 15 minutes.
15. Analyze 50 µL at 37°C on the Luminex analyzer according to the system manual.

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 2500 microspheres of each set per reaction.
3. Dilute/concentrate the MagPlex-TAG microsphere mixture to 111 of each microsphere set per µL in 1X Tm Hybridization Buffer and mix by vortex and sonication for approximately 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 SAPE Mix by diluting SAPE to 10 µg/mL in 1X Tm 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 a 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 your Luminex representative or visit www.luminexcorp.com.

Recommendations for Optimization and Troubleshooting

Low Reporter Intensity

1. Verify the production of the genomic target PCR products (OLA templates) on agarose gels.
2. Verify the hybridization assay by direct hybridization to 5 and 50 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.
8 Check the primer and template sequences for potential secondary structure.
9. Redesign the PCR primers.
10. Redesign the OLA probes for the opposite DNA strand.
11. Lengthen the OLA probes.

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.

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. If not using SAPE already containing BSA (i.e., Moss SAPE-001G75), dilute SAPE in 1X Tm buffer containing BSA. Final BSA concentration in the reaction should be 0.1% BSA in the reaction.
2. If the high background is due to contamination of the PCR reaction, replace the PCR reagents.
3. If high background is observed for the hybridization negative control, replace the hybridization reagents.
4. If high background is observed for the OLA negative control, replace the OLA reagents.

References

- Bruse, S., et al. (2008). "Improvements to bead-based oligonucleotide ligation SNP genotyping assays." *Biotechniques* 45(5): 559 - 571.
- Henry-Halldin, C. N., et al. (2012). "Multiplex Assay for Species Identification and Monitoring of Insecticide Resistance in Anopheles punctulatus Group Populations of Papua New Guinea." *American Journal Of Tropical Medicine And Hygiene* 86(1): 140-151.
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Chapter 5.1.2

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 allowed its use for genotyping typing a number of different genes and organisms (Francis DM 2012,Koo, Ong et al. 2007, Li, Luo et al. 2011, Li, Jortani et al. 2011, Marcil, Sinnett et al. 2012).

Figure 15. ASPE mechanism

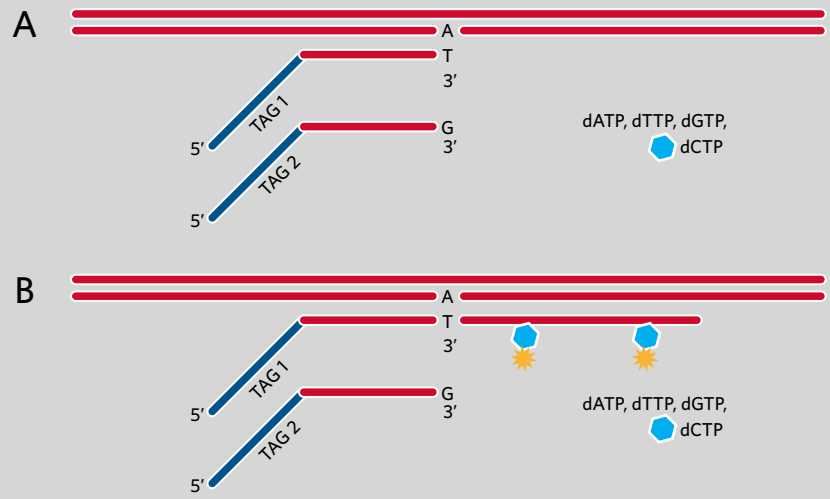

Figure 15 - Allele-Specific Primer Extension (ASPE) involves target-specific primer with 3' bases matching the possible SNP alleles of interest. A) A primer anneals to the target region with its 3' end aligned at the SNP site. B) If the primer finds a perfect match at the 3' base (as with TAG1 probe) then DNA polymerase is able to extend the primer, thereby incorporating labeled dNTP's downstream. Primers with a 3' mismatch at the SNP site (as with TAG2 probe) are not extended and no labeled dNTP's are incorporated. Inclusion of TAG sequences enables capture of each possible allele to a unique MagPlex-TAG Microsphere ("bead").

The chemistry takes advantage of a primer's ability to act as a suitable primer for a DNA polymerase when the probe 3′ base is complementary to the target SNP's base (Figure 15A). When a probe 3′ base is complementary, the polymerase can use it to synthesize new DNA containing biotin labeled nucleotides, but a primer cannot promote this extension if its 3′ base is mismatched (Figure 15B). With several rounds of primer extension, significant quantities of labeled molecules are produced to generate the signals required for analysis of multiple genotypes in one reaction.

To take advantage of this chemistry with the xMAP platform, each ASPE primer that identifies a SNP variation needs a unique TAG sequence on its 5′ end (Figure 15). 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 16). Target genomic amplicons can be of various sizes containing multiple SNP targets.

This combination of gDNA target amplification, primer function for variant detection, and multiplexing with xMAP beads, often allows this chemistry to be more reliable than other applications for typing SNP variations in the same reaction.

Figure 16. Amplifications of gDNA target regions of interest

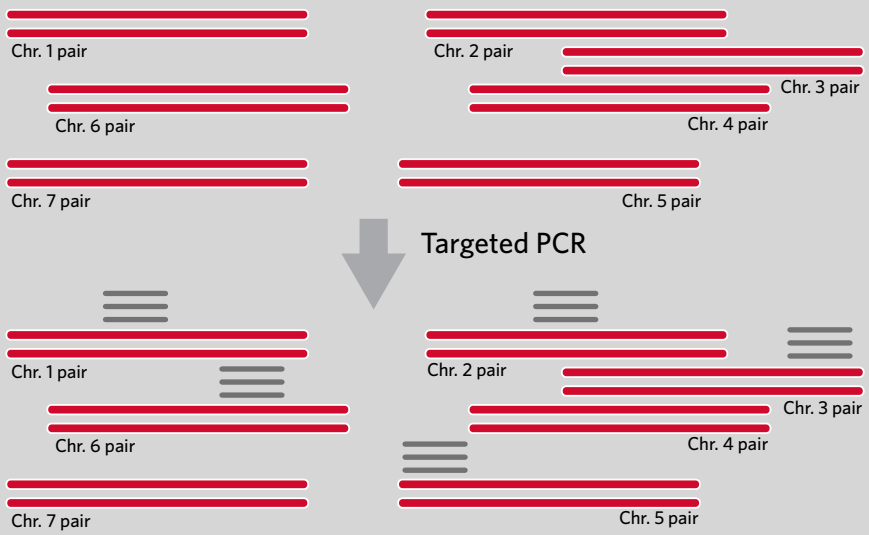

Figure 16 - Genomic DNA is amplified in the region of each SNP prior to performing ASPE assay using standard multiplex PCR amplification. Amplified targets may be of various lengths and may even contain multiple SNPs.

Materials Needed:

For complete equipment and materials list see Appendix B

Reagents and Consumables	Vendor
MagPlex®-TAG™ Microspheres	Luminex
Primers for PCR amplification of gDNA target regions.	IDT or other vendor
ASPE primers with 5′ TAG sequences	IDT or other vendor
Qiagen HotStarTaq 2X Master Mix	Qiagen, 203443 or equivalent
ExoSAP-IT or separate Exo I and SAP	GE Healthcare US78200, or equivalent
Platinum Tsp DNA polymerase, 10X PCR Buffer, 50 mM $MgCl_2$	Invitrogen, 11448-024 or equivalent
Biotin-14-dCTP	Invitrogen, 19518-018 or equivalent
dNTPs stocks	Life Technologies™ 10297-018 or equivalent
2X Tm Hybridization Buffer	See Buffer and Reagent Recipes section
1X Tm Hybridization Buffer	See Buffer and Reagent Recipes section
streptavidin-R-phycoerythrin (SAPE)	Moss SAPE-001G75, Life Technologies S-866 or equivalent
96-well PCR Plate	BioRad MSP9601
96-well Bead Hybridization Plate (optional)	Corning Costar 6509
MicroSeal A	BioRad MSA5001
Magnetic separation plate (special order)	V&P Scientific VP771LD-4CS or equivalent
Disposable pipette tips; multi- and single-channel (2-1000 mL)	Any suitable brand
25mL reservoirs (divided well)	Any suitable brand
RNase/DNase-Free Microcentrifuge Tubes 1.5 ml	USA Scientific or Equivalent
Barrier Pipette Tips	Any suitable brand

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

Vortex Mixer	Any suitable brand
Microcentrifuge	Any suitable brand
Bath Sonicator (40 - 55 kHz, frequency waves)	Any suitable brand
Centrifuge with Microplate Swinging Bucket Rotor	Eppendorf 5704 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 3.1 or higher software	Luminex

ASPE Buffer and Reagent Recipes

Step	Notes
MagPlex-TAG Microspheres from Luminex	Required Microspheres 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. Amplicon size is not restricted. These primers can be purchased from multiple vendors. Upon receipt, the primers should be dissolved or dilute 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 the 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 temperature 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. 5. The ASPE primer is designed to include the TAG sequence in 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 Life Technologies™ (10297-018). A 25 mM stock mix can be made by mixing equal amounts of 100 mM dATP, dTTP, dCTP, and dGTP for the 4dNTP stock mix for gDNA amplification. Individual stocks and concentrated mixes should be stored at -20°C.
Biotin-14-dCTP	Biotin labeled dCTP can be purchased from several sources including Life Technologies™. (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 Tm 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 Tm 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 1 mg/ml can be purchased from a number of suppliers such as Moss Incorporated (SAPE-001G75), Life Technologies™ (S866) 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 wash or no wash protocols.

Protocol 5.1.2 - for 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 for example purposes only 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
dNTP	200 µM each
primer	0.2 µM each
Qiagen HotStar 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 1mM ASPE-TAG primer mix 1:2000 (500 nM each) and the 100 mM 3 dNTP stock 1:1000 (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. Cover plate with a plate sealer and perform multiple rounds of primer extension in a thermal cycler with the following parameters: (Note: The temperature of the 1 minute hybridization step can be adjusted to what is needed for different probe mixes.)

Temperature	Time	Cycle
96°C	2 minutes	
94°C	30 seconds	
55°C	1 minute	30 cycles
37°C	2 minutes	
4°C	Hold	

5. Proceed to hybridization with MagPlex-TAG Microspheres using a wash or no wash protocol.

Hybridization to MagPlex-TAG Microspheres- 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 (beads).
2. Combine 2500 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. Contact your Luminex representative or visit www.luminexcorp.com.)
3. Dilute/concentrate the MagPlex-TAG microsphere mixture to 100 of each microsphere set per µL in 2X Tm Hybridization Buffer and mix by vortex and sonication for approximately 20 seconds.
4. Aliquot 25 µL of the MagPlex-TAG microsphere mixture to each well.
5. Add 5 to 25 µL of each ASPE reaction to appropriate wells (Note: 1-5 µL is usually sufficient) 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 20 µL of extension 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 the bead pellets.
10. Resuspend the pelleted MagPlex-TAG Microspheres in 75 µL of 1X Tm 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 the bead pellets.
12. Repeat steps 8 and 11. This is a total of two washes.
13. Resuspend microspheres in 75 µL of 1X Tm Hybridization Buffer containing 2-8 µg/mL SAPE.
14. Incubate at 37°C for 15 minutes.
15. Analyze 50 µL at 37°C on the Luminex analyzer according to the system manual.

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 2500 microspheres of each set per reaction.
3. Dilute/concentrate the MagPlex-TAG microsphere mixture to 111 of each microsphere set per µL in 1X Tm Hybridization Buffer and mix by vortex and sonication for approximately 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 Tm 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 the 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 your Luminex representative or visit www.luminexcorp.com.

Recommendations for Optimization and Troubleshooting xTAG with ASPE Assays

Low Reporter Intensity

1. Verify the production of the PCR products (ASPE templates) on agarose gels.
2. Verify the hybridization assay by direct hybridization to 5 and 50 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 structure.
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. 1. If not using SAPE already containing BSA (i.e., Moss SAPE-001G75), dilute SAPE in 1X Tm buffer containing BSA. Final BSA concentration in the reaction should be 0.1% BSA in the reaction.
2. If high background is observed for the PCR negative control, verify performance of the Exo/SAP step.
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 ASPE negative control, replace the ASPE reagents.

References

- Francis DM, 2012. "DNA Sequence Variation (SNP) Genotyping Using Allele Specific Primer Extension (ASPE) with the Luminex platform." *The Ohio State University web site* at http://www.extension.org/pages/32476/dna-sequence-variation-snp-genotyping-using-allele-specific-primer-extension-aspe-with-the-luminex-pl
- Koo, S., T. Ong, K. Chong, C. Lee, F. Chew and E. Lee (2007). "Multiplexed genotyping of ABC transporter polymorphisms with the Bioplex suspension array." *Biological Procedures Online*: 27-42.
- Li, G., X. Luo, J. He, Z. Zhu, G. Yu, H. Qin, T. Zeng, Z. Liu, S. Wu, J. Xu and L. Ren-Heidenreich (2011). "A novel liquid chip platform for simultaneous detection of 70 alleles of DNA somatic mutations on EGFR, KRAS, BRAF and PIK3CA from formalin-fixed and paraffin-embedded slides containing tumor tissue." *Clinical Chemistry And Laboratory Medicine* 49(2): 191-195.
- Li, Y., S. Jortani, B. Ramey-Hartung, E. Hudson, B. Lemieux and H. Kong (2011). "Genotyping three SNPs affecting warfarin drug response by isothermal real-time HDA assays." *Clinica Chimica Acta* 412: 79-85.
- Marcil, V., D. Sinnett, E. Seidman, F. Boudreau, F. P. Gendron, J. F. Beaulieu, D. Menard, M. Lambert, A. Bitton, R. Sanchez, D. Amre and E. Levy (2012). "Association between genetic variants in the HNF4A gene and childhood-onset Crohn's disease." *Genes And Immunity* 13(7): 556 - 565.

Chapter 5.1.3

Target-Specific PCR Sequence Detection with MagPlex®-TAG™ Microspheres ("beads")

There are a number of different PCR approaches to generate reporter molecules for detecting sequences in different types of samples. These approaches have been used for a number of applications including the detection of various pathogens (Babady, Mead et al. 2012). 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 since the biotin labeled TAG strand will preferentially bind its complementary anti-TAG strand rather than the complementary anti-TAG sequence on the beads. Elimination or reduction of the amount of unlabeled complementary strand can be achieved with more complex protocols involving enzyme treatments or asymmetric PCR chemistries.

A simpler and more straightforward approach is to prevent synthesis of the anti-TAG complementary 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 17A). 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 17B). 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 17C).

Figure 17. Outline of Target-Specific PCR probe design and chemistry

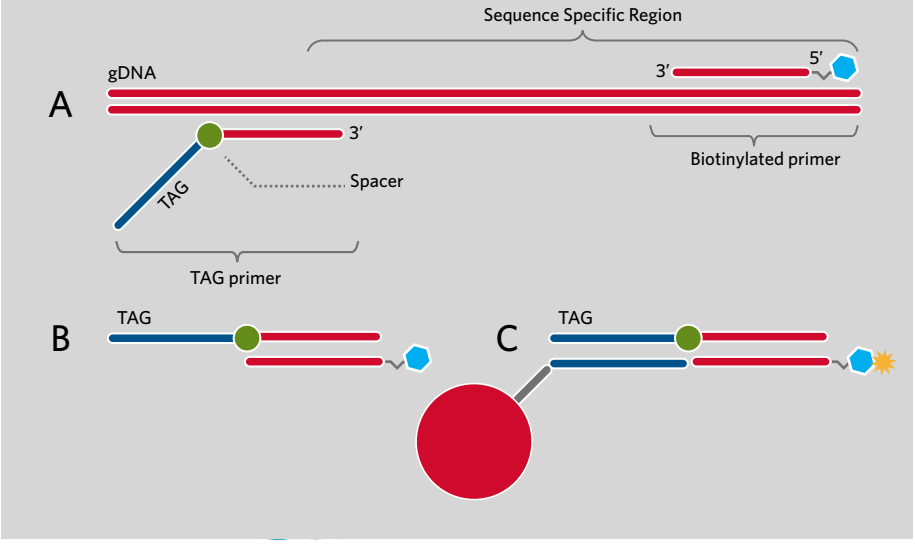

Figure 17 - Target-Specific PCR sequence detection involves (A) a TAG-containing primer separated from the sequence-specific portion by an internal spacer. When combined with a biotinylated sequence-specific reverse primer, (B) a double-stranded amplicon is created with a TAG overhang. (C) The TAG overhang allows hybridization capture to MagPlex-TAG microsphere without a competing anti-TAG complementary strand generated in the PCR reaction.

Materials Needed:

Reagents and Consumables	Vendor
MagPlex-TAG Microspheres	Luminex®
Spacer modified TAG PCR primers	IDT or other vendor
5′ biotinylated reverse PCR primers	IDT or other vendor
Qiagen HotStarTaq 2X Master Mix	Qiagen 203443 or equivalent
dNTPs stocks	Life Technologies™ 10297-018 or equivalent
2X Tm Hybridization Buffer	See Target-Specific PCR Buffer and Reagent Recipes section
1X Tm Hybridization Buffer	See Target-Specific PCR Buffer and Reagent Recipes section
streptavidin-R-phycoerythrin (SAPE)	Moss, Inc. SAPE-001G75, Life Technologies S-866 or equivalent
96-well PCR Plate	BioRad MSP9601
96-well Bead Hybridization Plate (optional)	Corning Costar 6509
MicroSeal A	BioRad MSA5001
Magnetic separation plate (special order)	V&P Scientific VP771LD-4CS or equivalent
Disposable pipette tips; multi- and single-channel (2-1000 mL)	Any suitable brand
25mL 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, frequency waves)	Any suitable brand
Centrifuge with Microplate Swinging Bucket Rotor	Eppendorf 5704 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 3.1 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.

Target-Specific PCR Buffer and Reagent Recipes

Step	Notes
MagPlex-TAG Microspheres from Luminex	Required Microspheres 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. These primers can be purchased from multiple vendors , such as IDT. Upon receipt, the primers should be dissolved or dilute 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 Tm 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), Life Technologies™ (S866) or equivalent. A working aliquot should be made fresh by diluting with 2X Tm Hybridization buffer to the required volume and concentrations needed as indicated in the wash or no wash protocols.

Protocol 5.1.3 – 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 example purposes only and may not be optimum for your samples or any specific genomic amplification protocol you may be using.

1. Assembly of 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 HotStar or other Taq polymerase	2.5 Units

2. Cover plate with a plate sealer and place in a thermal cycler. Perform PCR with the following program: (Note: The temperature of the 55°C hybridization step can be adjusted as needed).

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	

3. Proceed to hybridization with MagPlex-TAG Microspheres using the no wash protocol.

Hybridization to MagPlex-TAG Microspheres- no wash protocol

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

Note: If needed, an Excel based bead calculator is available for determining the method and volumes needed for making the bead mix. Contact your Luminex representative or visit www.luminexcorp.com.

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 2500 microspheres of each set per reaction.
3. Dilute/concentrate the MagPlex-TAG microsphere mixture to 125 of each microsphere set per µL in 1x Tm Hybridization Buffer and mix by vortex and sonication for approximately 20 seconds.
4. Aliquot 20 µL of the MagPlex-TAG microsphere mixture to each well including those for bead background.
5. Add 1 to 5 µL of each PCR reaction to appropriate wells and add 5 µL of dH_2O to each bead only 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 Tm 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 hybridization temperature on the Luminex analyzer according to the system manual.

Recommendations for Optimization and Troubleshooting

Low Reporter Intensity

1. Verify production of amplified products on agarose gels.
2. Verify labeling of amplified target.
3. Try increasing and decreasing the target input to determine optimal amount.
4. Check primer and target sequences for potential secondary structure.
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. If not using SAPE already containing BSA (i.e., Moss SAPE-001G75), dilute SAPE in 1X Tm buffer containing BSA. Final BSA concentration in the reaction should be 0.1% BSA in the reaction.
2. 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.
3. Redesign targets with high background.
4. If high background occurs on all microsphere sets try decreasing the target input to determine optimal amount.
5. Try decreasing amount of SAPE.

References

- Babady, N. E., et al. (2012). "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." *Journal Of Clinical Microbiology* 50(7): 2282 - 2288.

Chapter 5.1.4

Direct DNA Hybridization Sequence Detection

For some applications a Luminex® based genomic assay may require coupling Luminex beads with specific capture sequences that are complementary to organism specific sequences in the labeled reporter molecules generated by an assay's chemistry. These different approaches can be used for gene expression analysis, genotyping, specific sequence detection or other applications (Yang, Tran et al. 2001, Itoh, Mizuki et al. 2005, Oehrmalm, Eriksson et al. 2012). In these situations specificity requires 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 (Dunbar and Jacobson 2007, Oehrmalm, Jobs et al. 2010, Oehrmalm, Eriksson et al. 2012).

This protocol outlines a TMAC based hybridization procedure that can be used for these types of direct hybridization assays as well as other applications.

Materials Needed:

Reagents and Consumables	Vendor
MagPlex® Microspheres (oligo-coupled)	
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, Life Technologies S-866 or equivalent
96-well PCR Plate	BioRad MSP9601
96-well Bead Hybridization Plate (optional)	Corning Costar 6509
MicroSeal A	BioRad MSA5001
Silicon Mat	Phenix Research products SMX-CM
Magnetic separation plate	Any suitable magnet
Disposable pipette tips; multi- and single-channel (2-1000 mL)	Any suitable brand
25mL 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, frequency waves)	Any suitable brand
Centrifuge with Microplate Swinging Bucket Rotor	Eppendorf 5704 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 3.1 or higher software	Luminex

For complete equipment and materials list see Appendix (B)

Direct Hybridization Buffer and Reagent Recipes

Step	Notes
MagPlex Microspheres coupled with desired capture sequences	Required Microspheres regions should be purchased from Luminex and coupled with desired capture probes as outlined in the Nucleic Acid Coupling protocol (Chapter 3.3.4). 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 (Sigma T3411), 0.1% Sarkosyl (Sigma L7414), 50 mM Tris and 4 mM EDTA. The solution should be stored at room temperature.
1X TMAC Hybridization solution	The composition of this solution is 3 M TMAC (Sigma T3411), 0.15% Sarkosyl (Sigma L7414), 75mM Tris and 6mM EDTA. The solution should be stored at room temperature. 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 and stored at room temperature.
Streptavidin-R-phycoerythrin (SAPE)	SAPE at 1 mg/ml can be purchased from a number of suppliers such as Moss Incorporated (SAPE-001G75) or Life Technologies™ (S866). 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 wash or no wash protocols.

Protocol 5.1.4.1 – Direct DNA Hybridization - no wash protocol

1. Select the appropriate oligonucleotide-coupled microsphere sets.
2. Resuspend the microspheres (beads) by vortex and sonication for approximately 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 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 approximately 20 seconds.
5. To each sample or background well, add 33 µL of Working Microsphere Mixture.
6. To each background well, add 17 µL TE, pH 8.
7. To each sample well add volumes of labeled target reaction and TE, pH 8.0 to a total volume of 17 µL.
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 to 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: If needed, an Excel based bead calculator is available for determining the method and volumes needed for making the Working Microsphere Mixture.

Note: for most assay chemistries 2-5 µL of a robust PCR or labeled target reaction will be sufficient for detection.

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 hybridization temperature for 5 minutes.
13. Analyze 50 µL at hybridization temperature on the Luminex analyzer according to the system manual.

Protocol 5.1.4.2 - Direct DNA Hybridization Washed Protocol

1. Select the appropriate oligonucleotide-coupled microsphere sets.
2. Resuspend the microspheres by vortex and sonication for approximately 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 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 approximately 20 seconds.
5. To each sample or background well, add 33 µL of Working Microsphere Mixture.
6. To each background well, add 17 µL TE, pH 8.
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 2-5 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 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 hybridization temperature for 5 minutes.
16. 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: If needed, an Excel based bead calculator is available for determining the method and volumes needed for making the Working Microsphere Mixture.

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

Note: 75 µL of 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 optimal amount.
5. Decrease the hybridization temperature.
6. Check probe and target sequences for 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 not using SAPE already containing BSA (i.e., Moss SAPE-001G75), dilute SAPE in 1X Tm buffer containing BSA. Final BSA concentration in the reaction should be 0.1% BSA in the reaction.
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. If not using SAPE already containing BSA (i.e., Moss SAPE-001G75), dilute SAPE in 1X Tm buffer containing BSA. Final BSA concentration in the reaction should be 0.1% BSA in the reaction.
2. 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.
3. Resynthesize probes with high background.
4. If high background occurs on all microsphere sets from the same coupling, use uncoupled microspheres and coupled microspheres with low background to test hybridization buffers for contamination.
5. 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.
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 http://www.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 plate washer's User's Manual 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 buffer wash made properly and washes (if any) are performed thoroughly. Make sure the bead solution is stored in the dark at 4°C to prevent photo bleaching.

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Chapter 5.1.5

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 single plex 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 (Jang, Simon et al. 2011; Taylor, Satoor et al. 2012; Marco Ragusa 2013). Hybridization assays can be multiplexed to different degrees with the use of special costly probes, cassettes and analysis instruments (Cascione, Gasparini et al. 2013; Naduparambil Korah Jacob 2013). 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™ microsphere ("beads") mixes. 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-hybridizing with each other or with any known sequence in the biome.

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 (beads). The probes can be easily designed by the user making the assay more cost effective and flexible to meet the user's needs.

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 with miRNA species that cannot be distinguished with other chemistries.

This combination of characteristics also contributes to the assay having a short 1 day protocol without sacrificing single nucleotide specificity even without PCR amplification (Sorensen 2011).

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.

Figure 18. miRNA assay work flow

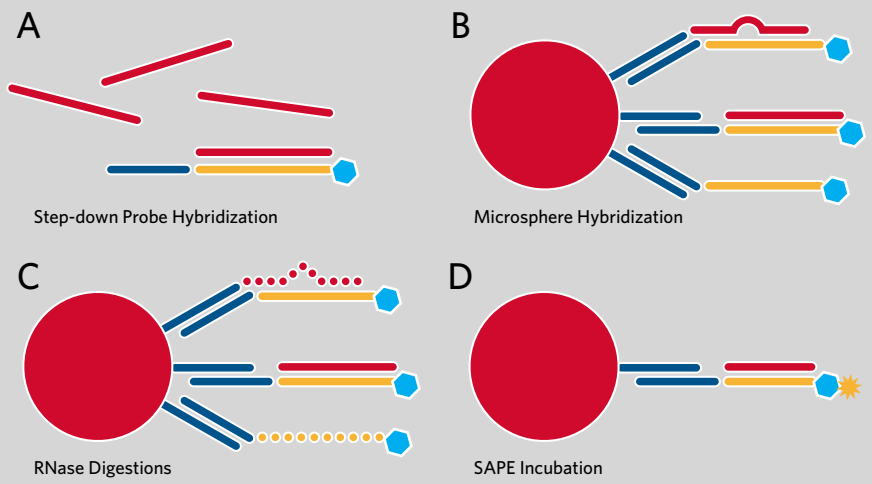

Figure 18 - (A) Step-down Probe Hybridization - DNA/RNA chimeric probes hybridize to target miRNAs during incremental reductions in annealing temperature. 2 hours. (B) Microsphere Hybridization - miRNA-chimeric probe complexes are hybridized to microspheres. 30 minutes. (C) RNase Digestion - Excess probes, single-stranded RNAs and mismatched probes are digested. Only perfectly-matched probes are protected. 30 minutes. (D) SAPE Incubation - A brief incubation with steptavidin-conjugated R-Phycoerythrin (SAPE) incorporates reporter molecules. 30 minutes.

Detection - Targets of interest are quantified on an xMAP instrument

< 5 hours total to results.

Materials Needed:

Reagents and Consumables	Vendor
MagPlex®-TAG™ Microspheres	Luminex
Chimeric probes	IDT 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, Life Technologies S-866 or equivalent
96-well PCR Plate	BioRad MSP9601
96-well Bead Hybridization Plate (optional)	Corning Costar 6509
MicroSeal A	BioRad MSA5001
Silicon Mat	Phenix Research products SMX-CM
Magnetic separation plate (special order)	V&P Scientific VP771LD-4CS or equivalent
Disposable pipette tips; multi- and single-channel (2-1000 mL)	Any suitable brand
25mL 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, frequency waves)	Any suitable brand
Centrifuge with Microplate Swinging Bucket Rotor	Eppendorf 5704 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 3.1 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.

miRNA Buffer and Reagent Recipes

Title	Title
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. An excel workbook for designing chimeric probes is available (Contact your Luminex representative or visit www.luminexcorp.com.) To design probes with or without the workbook, use the following procedure. 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. 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.

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′	

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.

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 then further diluted to generate a working mix where 1.25 µl of probe mix for each reaction delivers 10nM for each probe. Example: A 5 plex miRNA profile is to be analyzed on 100 samples. The total number of samples will require 100 * 1.25 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 ul of each probe into an empty tube with 118.75 µl of TE buffer (5 * 1.25 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 3M sodium acetate stock solution, a 0.5 M EDTA solution pH 8.0, a 1M Tris pH 7.5, and a 10% Tween 20 solution – with a final adjustment to the proper pH as needed. Filters sterilize and store at 4°C.
MagPlex-TAG Magnetic bead mixes	Pre-defined MagPlex-TAG bead mixes come in a concentration of 2.5 x10^6 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/rxn 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 ul 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 high plex bead mixes are required the purchase of bead stocks containing multiple bead regions is recommended to simplify or eliminate the concentration steps needed to make the bead mix that is added to the reaction. Whatever the bead source, the final bead dilution for use in the assay should be in hybridization buffer. Also, if needed, an Excel based bead calculator is available for determining the method and volumes needed for making the bead mix.
Samples 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 5.2 – 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.

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

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%

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 with 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 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. 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. Remove the reaction supernatant prior to the addition of SAPE 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. Again, remove MicroSeal A film and remove the supernatant gently and carefully.
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 to 22 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. Caution: 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 Costar 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

1. 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.
2. 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.
3. 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.

4. 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 or degradation. 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, 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 SAPE not added or incorrect SAPE dilution used. Make sure SAPE is stored at 4°C in the dark, do not freeze SAPE solutions, and ensure SAPE dilutions are prepared as described in the protocol and SAPE 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).
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 according to the instruction manual.
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 photo-bleaching.

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 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 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 compliment to the target RNA sequence. Use the Excel based FlexmiR 200 Chimeric probe design tool for proper probe design. Contact your Luminex representative or visit www.luminexcorp.com.
5. Bead hybridization temperature too high or low. A temperature gradient may be needed to determine the optimum bead hybridization temperature.

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Appendix A

Common buffers used in xMAP® protocols

xMAP Buffers

Buffer	Composition	Use(s)	Source	Notes
Activation Buffer [1]	0.1 M NaH_2PO_4, pH 6.2	Microsphere ("bead") activation buffer for protein coupling	Sigma S3139	Adjust to pH 6.2 with 5 N NaOH Filter sterilize Store at 4 °C
Coupling Buffer[2]	50 mM MES, pH 5.0	Microsphere-protein coupling buffer	Sigma M2933	Adjust to pH 5.0 with 5 N NaOH Filter sterilize Store at 4 °C
Phosphate buffered saline (PBS), pH 7.4 [3]	138 mM NaCl, 2.7 mM KCl, pH 7.4	Alternate microsphere-protein coupling buffer	Sigma P3813	Filter sterilize Store at 4 °C
PBS-Tween buffer	PBS, 0.05% Tween-20, pH 7.4	Microsphere wash buffer	Sigma P3563	Filter sterilize Store at 4 °C
PBS-BN buffer[4]	PBS, 1% BSA, 0.05% sodium azide	Microsphere blocking/ storage buffer Assay buffer	Sigma P3688 Sigma S8032	Filter sterilize Store at 4 °C
PBS-TBN buffer[4,5]	PBS, 0.1% BSA, 0.02% Tween-20, 0.05% sodium azide	Microsphere blocking/ storage buffer Microsphere wash buffer Assay buffer	Sigma P3813 Sigma A7888 Sigma P9416 Sigma S8032	Filter sterilize Store at 4 °C
Assay/Wash Buffer	PBS, 1% BSA, pH 7.4	Assay buffer	Sigma P3688	Filter sterilize Store at 4 °C
0.1 M MES Buffer pH 4.5	0.1 M MES	Oligonucleotide-microsphere coupling buffer	Sigma 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	Sigma P9416	Filter sterilize Store at room temperature
0.1% SDS Wash	0.1% SDS	Oligo coupling wash buffer	Sigma L4522	Filter sterilize Store at room temperature
EDC	1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC)	Microsphere coupling activation	Pierce 77149	Store desiccated at -20 °C
TE Buffer pH 8.0	TE	General purpose nucleic acid buffer	Sigma T9285	Filter sterilize Store at room temperature
2X Tm 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 Tm 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 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

1. Activation can be performed in 50 mM MES, pH 6.0–6.2, with similar results.
2. 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.
3. Alternative coupling buffer for proteins that do not couple well at pH 5–6.
4. Also used as assay buffer.
5. Also used as wash buffer.

Appendix B

Equipment needed for xMAP® protocols

Immunoassay Equipment	Vendor
Luminex® xMAP analyzer with xPONENT software[1]	Luminex
Magnet for 1.5 mL microcentrifuge tube washing[2]	(Dynal MPC®-S Magnetic Particle Concentrator, Invitrogen 120-20D) or equivalent.
Magnet for 96 well plate washing[3]	(See list below)
Balance	Any suitable brand capable of weighing down to 0.1 mg
Microcentrifuge	Any suitable brand
Hemacytometer or Cell Counter	Cellometer Auto 1000, TC10 Cell Counter, Countess® Automated Cell Counter
Vortex mixer	Any suitable brand
Sonicator bath	Ultrasonic Cleaner, Cole-Palmer, A-08849-00 or equivalent
Rotator	Any suitable brand capable of 15-30 rpm
Microtiter plate shaker	Any suitable brand capable of 800 rpm
96-well plate	

Nucleic Acid Assay Equipment	Vendor
Thermocycler with 96-well Head and Heated Lid	Any suitable brand

1. Note : MAGPIX® has the ability to perform a final wash step prior to reading the plate.
2. Note: If a magnet is not available, use a microcentrifuge (8000 x g for 1-2 minutes).
3. Note : If a magnet not available, use a centrifuge compatible with 96 well plates (8000 x g for 1-2 minutes)
4. Note: LumAvidin beads are not magnetic and require MultiScreen Filter Plates (Millipore, MABV N12) and vacuum pump system manifold for vacuum pump system, such as the MultiScreen™ Resist Vacuum Manifold from Millipore (MAVM0960R).

Magnetic Separators for MagPlex Microspheres with compatible tubes and plates*

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)
Dynal MPC®-S magnetic particle concentrator	Coupling	Life Technologies™ 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 (Costar 3789 or 3792)
LifeSep™ 96F magnetic separation unit	Assays	Dexter Magnetic Technologies, Inc. 2501008	96-well, round-bottom polystyrene solid plates (Costar 3789 or 3792)
Ambion® 96-well magnetic ring stand	Assays	Life Technologies™ AM10050	96-well, round-bottom polystyrene solid plates (Costar 3789 or 3792)
96-well plate magnet	Assays	PerkinElmer (Customer Care) 5083175	96-well, round-bottom polystyrene solid plates (Costar 3789 or 3792) 96-well, Thermowell P polycarbonate PCR plates (Costar 6509) 96-well, F-bottom, (chimney well), µclear, med. Binding, black (Greiner bio-one 655096) Aluminum Foil Lids (Beckman 538619) or equivalent

*Note: LumAvidin® beads are not magnetic and require MultiScreen Filter Plates (Millipore, Cat. No. MABV N12) and vacuum pump system manifold for vacuum pump system, such as the MultiScreen™ Resist Vacuum Manifold from Millipore (MAVM0960R)

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		Samco Scientific	233	Good for removing supernatant from coupling wash steps
96-well, flat bottom, polystyrene solid plates	Protein/Unheated assays	Luminex 100/200, FLEXMAP 3D, MAGPIX	Corning (Costar)	3912, 3915	
96-well, round bottom, polystyrene solid plates	Protein/Unheated assays	Luminex 100/200, FLEXMAP 3D, MAGPIX	Corning (Costar)	3789, 3792	
96-well, Thermowell polycarbonate PCR plates, Model P	Nucleic Acid/Heated	Luminex 100/200, FLEXMAP 3D, MAGPIX	Corning (Costar)	6509	
96-well, Multiscreen-BV 1.2 mm filter plates	Protein/Unheated	Luminex 100/200, FLEXMAP 3D, MAGPIX	EMD Millipore	MABVN1250	Can be used for washes with vacuum filtration for LumAvidin/ nonmagnetic beads
96-well, uClear, flat bottom, chimney well plates	Protein/Unheated	Luminex 100/200, 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 100/200, FLEXMAP 3D, MAGPIX	Bio-Rad	MSA-5001	