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Improving Reliability of Filter-Based Luminex® xMAP® Multiplex Assays Through Filter Plate and Membrane Material Selection

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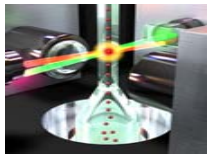
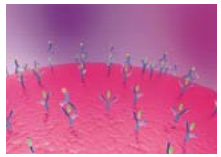
Abstract

Luminex xMAP Multiplex technology, utilizing microspheres as a solid support, continues to grow in its application for immunological research and diagnostics. This technology is frequently used, for instance, in serological assays to assess the presence of autoimmune disease. Optimization of factors such as bead recovery and non-specific reactivity can each have a significant impact on the reliability of these assays by ensuring statistically relevant analysis and preventing false positive results, respectively.

We demonstrate the impact of filter plate and membrane materials on bead recovery, assay signal, and data reliability. In addition, we successfully show reduced false positive frequency in a known, clean, yet atypical serum sample subjected to concurrent detection for specific antigens. Initial findings support the role of surface interactions in the reduction of non-specific reactivity. Thus, filter plate and membrane materials have a significant impact on the prevalence of false-positives when used with the described technology for serological assays.

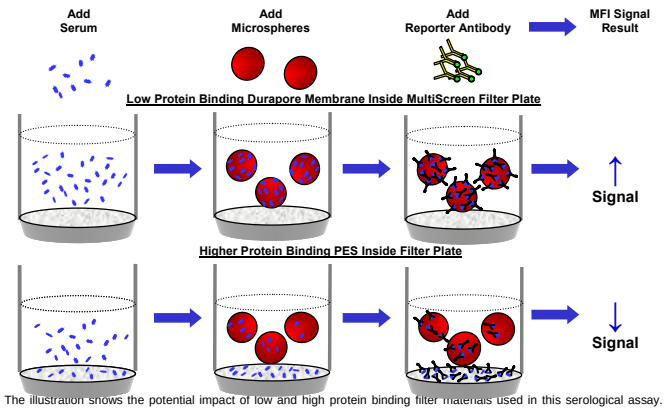




Introduction

Optimal reaction conditions are critical when performing multiplexed bead-based serological assays to minimize any misdiagnosis or repeat testing. Each well of a multiwell filtration plate is an independent vessel for the reaction to take place. Therefore, key factors to consider when trying to reduce the non-specific background signal, and 'false positive' results are filter plate properties (filter material, filter protein binding, pore size, etc.), reporter antibody concentration, serum concentration, and microsphere recovery.

Luminex has developed a serological assay to allow observations and determine reactivity. Using the Luminex 100™ IS analyzer, the median fluorescent intensity (MFI) signal strength of two microspheres types (high and low reactivity) are generated, and used as the measure for all the described assays. For this assay, MFI values greater than 2,000 are considered to be a positive response, and below 600, a negative response. When a known negative serum sample results in MFI readings greater than 600, it is considered a 'false positive,' which is likely caused by non-specific protein binding of serum proteins to the microsphere's surface.

MultiScreen® filter plates with hydrophilic 1.2 µm BV and 0.45 µm HV Durapore® Polyvinylidene fluoride (PVDF) membrane are low protein binding filter plates (data not shown). We hypothesize that higher protein binding filter plate types, such as Polyethersulfone (PES), would result in lower MFI signals due to sequestration of the serum and reporter antibody to the filter membrane (see figures below). The Durapore membrane MultiScreen filter plates do not show this same binding phenomenon. Through the various experiments outlined here, we show that incorrect MFI response can result from a filter plate's interference with the serological test being performed.



The illustration shows the potential impact of low and high protein binding filter materials used in this serological assay. High binding filter materials demonstrate non-specific binding of serum proteins and subsequently reporter antibody to the membrane, thus reducing effective antibody concentration available to the microspheres and lowering MFI signal.

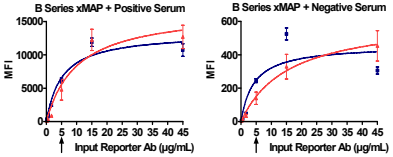
Methods

Pre-wet	Reaction		Filter	Wash 2x	Reaction		Wash 2x	Resuspend	Analyze
50 µL BSA/PBS Vacuum @ 8-12"Hg	20 µL Serum + 10 µL Microspheres	Mix 1 hour	Vacuum @ 8-12"Hg	100 µL BSA/PBS Vacuum @ 8-12"Hg	50 µL BSA/PBS + 50 µL Reporter Ab (5 ug/mL)	Mix 1 hour	100 µL BSA/PBS Vacuum @ 8-12"Hg	100 µL BSA/PBS	Read on Luminex 100 IS Analyzer

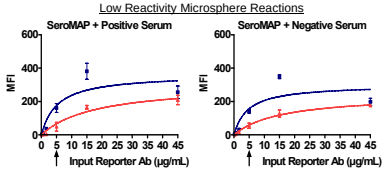
Multiwell filter plates are pre-wet with BSA to block non-specific binding. Microspheres (high reactivity B series xMAP® type and low reactivity SeroMAP™ type) are combined and added to filter wells with known positive (autoimmune disease) and negative (normal) serum samples. The filter plates are incubated on plate shaker and washed. Buffer and fluorescent reporter antibody are added to the reaction, incubated on plate shaker, and washed. The microspheres are resuspended and read on the Luminex 100 IS analyzer to determine MFI.

Reporter Antibody Optimization

High Reactivity Microsphere Reactions



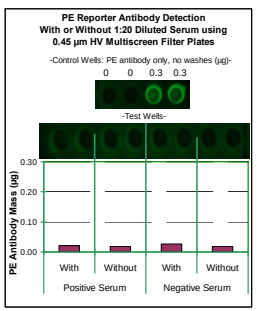
Low Reactivity Microsphere Reactions



$K_{d\ App}$ Values (µg/mL)	0.45 µm HV MultiScreen Filter Plate	1.2 µm PES Brand P Filter Plate
B Series xMAP type + Positive Serum	5.2	10.1
B Series xMAP type + Negative Serum	4.4	15.8
SeroMAP type + Positive Serum	5.3	18.0
SeroMAP type + Negative Serum	4.7	15.6

The optimal apparent dissociation constant ($K_{d\ App}$) for two filter plates are shown for all microsphere and serum types. The typical concentration of input PE (phycoerythrin conjugated goat anti-human IgG) reporter antibody used in the assay is identified by the arrow in the graphs (5 µg/mL). Overall, MFI signal increases as the PE reporter antibody increases for all bead and serum type reactions. More reporter antibody is needed when using Brand P-PES filter plates.

Effects of Serum Concentration on Non-Specific Binding



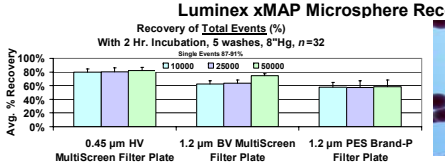
Panel A

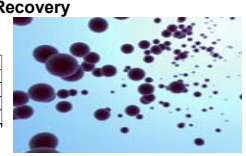
Input PE Reporter Ab Concentration per well (µg/mL)	PE Reporter Antibody Mass Detection with 2 Serum Concentrations (µg)							
	0.45 µm HV MultiScreen Filter Plate				1.2 µm PES Brand P Filter Plate			
	Positive Serum		Negative Serum		Positive Serum		Negative Serum	
	1:20	1:100	1:20	1:100	1:20	1:100	1:20	1:100
0.2	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
0.6	0.03	0.02	0.02	0.03	0.02	0.02	0.02	0.02
1.7	0.03	0.03	0.03	0.03	0.02	0.03	0.02	0.03
5.0	0.03	0.03	0.03	0.03	0.03	0.03	0.04	0.04
15.0	0.03	0.03	0.04	0.03	0.09	0.05	0.11	0.07
45.0	0.04	0.03	0.04	0.03	0.33	0.05	0.27	0.04

Panel B

A digital imaging system (Kodak® IS4000MM system, 2x10 sec. exposure, 465 nm/600 nm) was used to detect fluorescent PE reporter antibody present on the filter membrane. A fluorescence plate reader (Spectramax® Gemini plate reader, 490 nm/580 nm) was used to quantitate reporter antibody non-specifically bound to the membrane. As predicted (Pitt, 1987), no PE reporter antibody was observed or detected in the 0.45 µm HV MultiScreen filter plate in the presence or absence of serum (Panel A). The experiment was extended by using two serum concentrations with the 0.45 µm HV MultiScreen and 1.2 µm PES Brand P filter plates (Panel B). PE Antibody was only detected on Brand P-PES filter plate (shown in red).

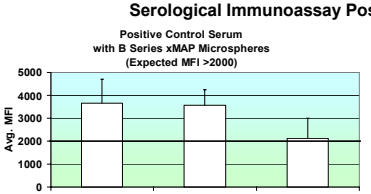
Luminex xMAP Microsphere Recovery





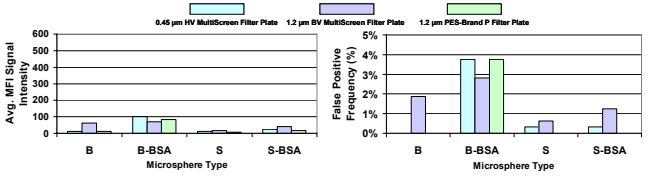
Filter plates were challenged with three microsphere levels (10K, 25K, & 50K) per well. The greatest microsphere recovery was achieved with the smaller pore sized filter plate. Total microsphere recovery is typically not an issue because in most assays only a fraction of the total microspheres are counted. In addition, when using the 0.45 µm MultiScreen filter plate, sample acquisition time is expected to be reduced.

Serological Immunoassay Positive Control Results



Positive control serum samples' average MFI responses were generated during three test days. Higher MFI signals are confirmed with high reactivity B series xMAP Microspheres with positive serum using two pore sizes of the MultiScreen filter plates and the Brand P-PES filter plate.

Serological Immunoassay Negative Serum Test Results



A total of 320 diluted serum samples (1:100) were evaluated using uncoupled and BSA-coupled B series xMAP microspheres (B, B-BSA) and SeroMAP microspheres (S, S-BSA). The average MFI signal intensity (Panel A) and frequency of 'false positives' are shown (Panel B). In general, higher signals are generated from the MultiScreen filter plates. The 0.45 µm MultiScreen HV filter plates demonstrate a low frequency of 'false positives.'

Conclusions

- Use of low binding 0.45 µm HV and 1.2 µm BV MultiScreen filter plates with Durapore PVDF membrane:
  - ✓Allows for higher free concentration of antibody needed in the assay due to decreased non-specific binding to filter material and plate.
  - ✓Equivalent results observed over multiple serum concentrations.
- Minimizing serum concentration reduces non-specific protein binding to microspheres and filter material.
- Total microsphere recovery over a broad range of concentrations increases when the smaller pore size 0.45 µm HV MultiScreen filter plates are used.
- 'False positive' results are not impacted when using low binding filter plates.
- Serological immunoassay results confirm that low reactive SeroMAP type microspheres perform as expected.

Reference: Pitt, AM. The nonspecific protein binding of polymeric microporous membranes. *J. Parenter. Sci. Technol.* 1987 May-Jun;41(3):110-3.