

Abstract

Next generation xMAP® technology, based on the use of magnetic MagPlex microspheres, has been available for a number of years. The use of MagPlex® eases sample processing through the use of magnets to immobilize the microspheres during wash processes. Recently, a new paradigm of xMAP Reader has been provided by Luminex® which does not utilize flow cytometry principles for detection. The MAGPIX® Reader System has been developed by Luminex exclusively for MagPlex microspheres. The washing of MagPlex beads used with the MAGPIX reader technology has been accomplished using a manual hand-held magnet or vacuum apparatus. The wash steps can be automated using an appropriately configured microplate washer equipped for biomagnetic separation. The spatial relationship between the localization of the magnetic beads by the magnet and the aspiration tubes is paramount for bead recovery. Here we describe the washer parameters associated with low residual volumes, good bead recovery and optimal assay performance. Human multiplex assay kits from Millipore were used to optimize the ELx50™. Washer settings and resultant assay data will be provided.

Introduction

Diagnostic biomarkers are a key element in cancer research. Research has focused primarily on intracellular biomarkers such as HER2 or BRAC1 that identify specific tumors and provide genetic or phenotypic information that can clarify the process of oncogenesis. Recently more attention is being placed on identifying soluble extracellular circulating biomarkers, which can provide information on the body's response to cancer, as well as the relationship between a tumor cell and its environment. Because cancer is a series of different disease states, the study of individual biomarkers is usually inadequate to study the complex relationship between a tumor and its environment. Only with a large panel of different biomarkers can one discern the autocrine and paracrine interactions of the tumor and its host. While some biomarkers are tumor specific, such as PSA, others such as IL-8, are found in tumors of many different origins.

Using a panel of known tumor biomarkers to characterize tumor cell lines of known lineage under different conditions a better understanding of the biology specific to different tumor types can be determined.

The assay is based on Luminex xMAP® technology. Distinct internally color-coded magnetic microspheres each coated with a specific antibody capture and quantitate different analytes. By using multiplexed bead sets several analytes can be quantitated simultaneously. Using traditional Luminex technology, microspheres were allowed to pass rapidly and individually through a laser which excites the internal dyes identifying the bead and as such the analyte, while a second laser excites the reporter molecule and quantifies the analyte. The MAGPIX reader used in this study employs CCD fluorescent imaging to identify and quantitate the analyte. Because of the new reader technology, important optimization of the automated parameters to provide equivalent results was performed.

Assay Process

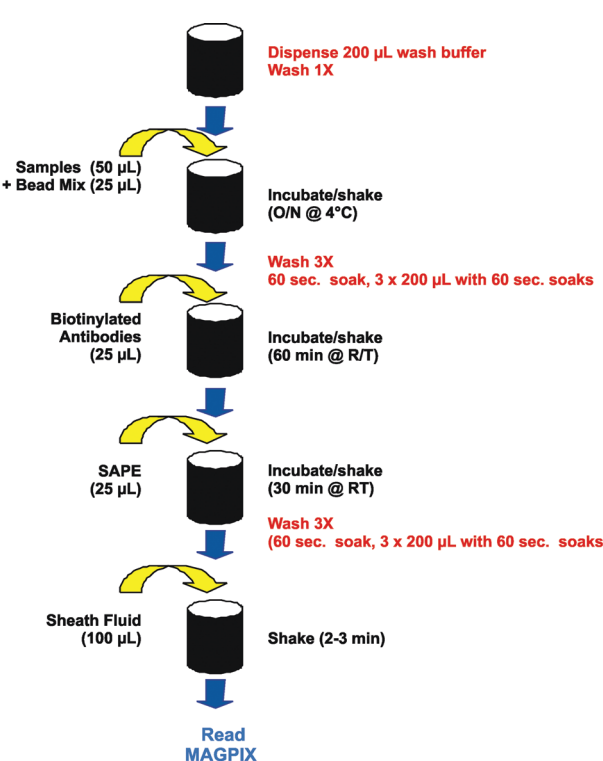


Figure 1 – Millipore MAGPIX Cytokine Assay Process.

The assay plate was first washed one time using the supplied assay wash buffer to remove any residue. Eight working multiplex standards were generated by serial dilution (1:3) of the reconstituted human cytokine standard. These standards contained 22 different analytes. After reconstitution, 50 µL each of standards and samples were pipetted into bead containing wells of the assay microplate. In parallel, the bead master mix was prepared by combining 150 µL of each individual bead suspension. 25 µL aliquots of the master mix were added to each well and the reactions were allowed to incubate overnight at 4°C with agitation on a plate shaker. After incubation the plate was washed 3 times as described in the washing instructions (See Table 2). After washing, 25 µL of detection or secondary antibody reagent was added and allowed to incubate for 60 minutes at RT with agitation. The beads were again washed three times followed by the addition of 25 µL of SAPE reagent. After a 30-minute incubation with agitation to allow for reporter tag binding to occur, the plate was again washed as described in the washing instructions. The samples and standards were then resuspended in 100 µL of sheath fluid. Samples were then read on either a Luminex MAGPIX or a Luminex® 100™ (LX100) reader with xPONENT® software using the parameters outlined in the assay kit instructions (Figure 1).

Calibration Curves

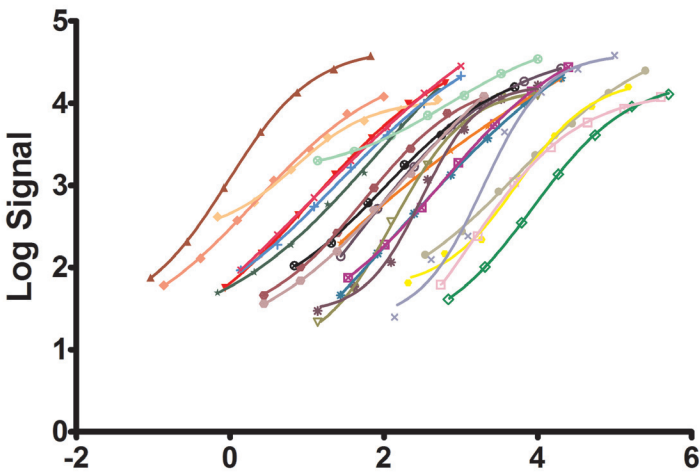


Figure 2 – Standard Curves for the Millipore MILLIPLEX 22-Plex Human Circulating Cancer Biomarker Panel using ELx50 Microplate Strip Washer.

Using known concentrations of analyte, a series of standard curves were generated for each cytokine by plotting the median fluorescent intensity (MFI) signal against concentration. These standard curves can then be interpolated to determine the concentrations of unknown samples. As with ELISA reactions, in order to obtain useable results efficient washing to remove nonspecific antibody binding is critical. As shown in Figure 2, using the ELx50 to wash magnetic bead based multiplex assays in a 96-well microplate format results in very reliable data. These standard curves can be used to calculate unknown sample concentrations with a high degree of confidence.

Assay Quality Control

Analyte	QC 1		QC2	
	Expected Range	Result	Expected Range	Result
AFP	3319-6893	5035	16333-33921	28457
Beta-HCG	0.73-1.52	1.07	4.36-9.06	7.97
CA125	7.0-14.6	11.77	38-79	62.47
CA15-3	1.17-2.44	1.99	6.4-13.2	12.14
CA19-9	6.8-14.2	14.2	35-73	60.91
CEA	231-480	473	1204-2502	2308
CYFRA 21-1	2206-4581	2665	11498-23881	18518
FGF2	123-255	143	596-1238	1045
HE4	7878-16361	10793	38636-80244	73437
HGF	224-465	254	1240-2574	1684
IL-6	4.56-9.48	9.29	26-54	46.9
IL-8	12-25	19.78	65-136	119
Leptin	1296-2691	1758	6399-13291	12430
MIF	330-685	647	1314-2729	2697
OPN	5592-11614	10907	28302-58782	56651
SCF	62-129	98.3	305-633	564.9
sFas	247-513	409.4	1340-2783	2497
TGF-alpha	24-50	35.5	131-273	205.2
TNF-alpha	12-26	22.9	67-140	117.8
Total PSA	125-261	255.5	650-1350	1196
TRAIL	26-54	45.4	136-283	219.9
VEGF	120-249	120.3	675-1402	899.8

Table 1 – Quality Control Analysis of Human Circulating Cancer Biomarker 22 Plex Panel.

Automated Washer Settings

Link File	Millipore MAGPIX		
	SOAK60	MAGX3	FINAL
Program Name	Soak	Wash	Aspiration
File Type			
Method			
Wash Buffer			
Plate Type			
Number of Cycles			
Soak/Shake			
Soak Duration	60 sec	60 sec	
Shake before soak	No	No	
Prime	No	No	
Prime Volume			
Prime Flow Rate			
Dispense			
Dispense Volume		200	
Dispense Flow Rate		5	
Dispense Height		130	
Horizontal			
Dispense Position		00	
Horizontal Y			
Position		00	
Bottom Wash First		No	
Bottom Dispense Volume			
Bottom Flow Rate			
Bottom Dispense Height			
Bottom Dispense Position			
Prime	No	No	
Prime Volume			
Prime Flow Rate			
Aspiration			
Aspiration Height		45	38
Aspiration Position			
Aspiration Rate	-20	-20	
Aspiration Delay	6	6	
Crosswise	00	00	
Aspirate	No	No	
Crosswise			
Aspirate On			
Crosswise Height			
Crosswise			
Horizontal			
Position	No	Yes	
Final Aspiration	0000	0000	
Delay	msec	msec	

Table 2 – ELx50 Automated Microplate Strip Washer settings. Beads were immobilized using a strong rare-earth magnet integrated into the ELx50 washer. An initial 60 second soak allows for bead capture, while a stepped aspiration scheme reduces bead loss from aspiration.

Cell Line Characterization

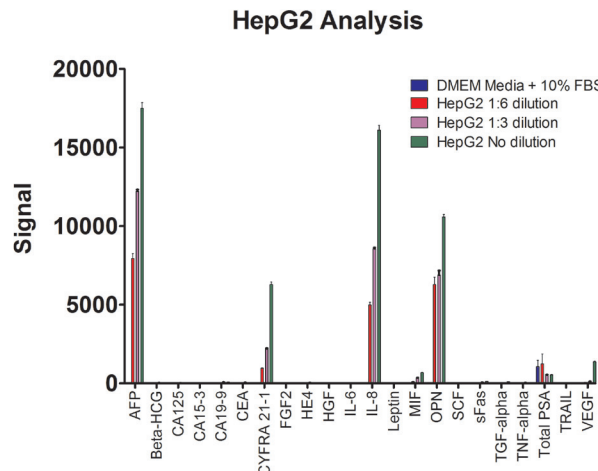


Figure 3– HepG2 Cell Line Biomarker Characterization. Tissue culture cell conditioned media supernatant was obtained from HepG2 cultures. The conditioned media was diluted 1:3 and 1:6 with fresh media (DMEM + 10 % FBS) and aliquots (25 µL) were assayed in parallel with undiluted conditioned media. Data are the mean of 8 determinations.

MAGPIX vs. LX100

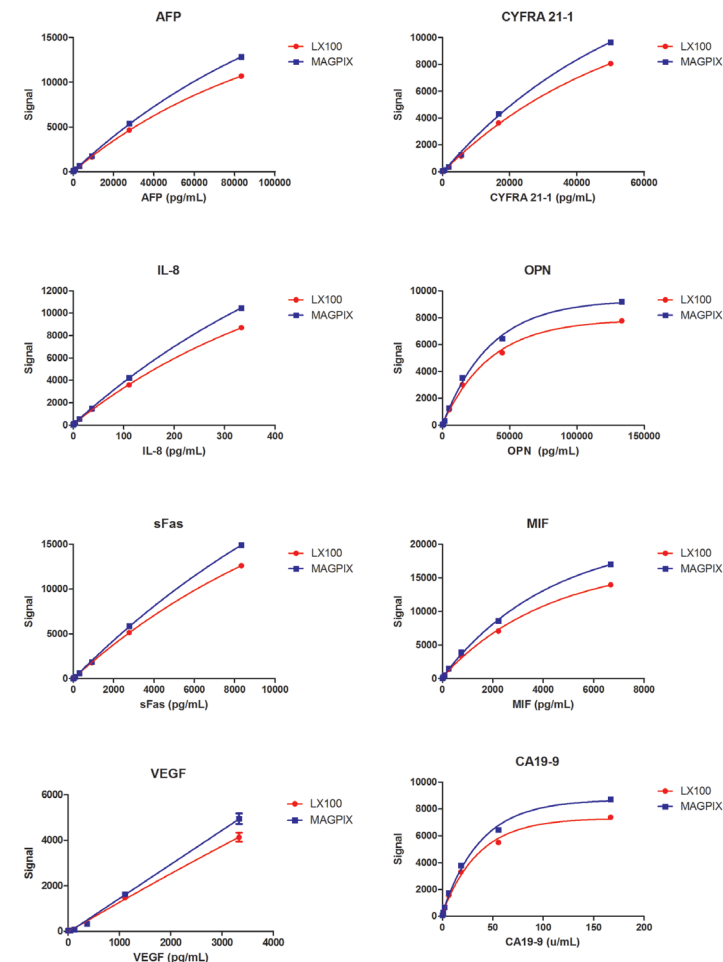


Figure 4– Comparison of Select Calibration Curves. Calibration curves generated using either an LX100 or a MAGPIX reader from equivalent samples.

When selected standard curves generated using either the established LX100 reader or the new MAGPIX reader are compared very similar results are obtained. While the MAGPIX reader reported slightly higher signal values than the LX100, the overall shape of the curves were very similar (Figure 4). More importantly, when calculated concentration results of samples are compared between the two readers equivalent results are obtained (Figure 5).

MAGPIX vs. LX100 (Continued)

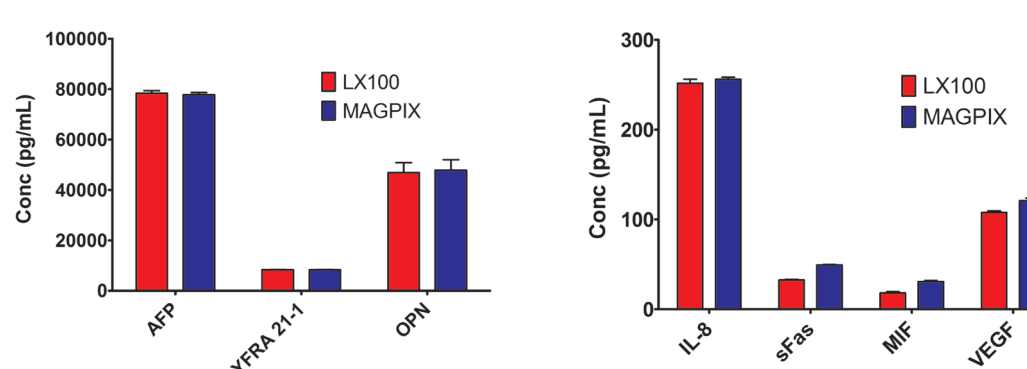


Figure 5 – Comparison of LX100 and MAGPIX Calculated Biomarker Concentrations. Multiplex reactions of HepG2 cell supernatant were assayed for the indicated cancer biomarkers along with standard curves in parallel using LX100 and MAGPIX readers. Determined concentrations from both readers are compared.

	Read Time	Events
MAGPIX	32.5 sec	2544
LX100	16.8 sec	3047

Table 3 – Comparison of read time and number of events. Using MAGPIX and LX100 readers, the average per well read time and number of measured events from equivalent experiments were determined for a 96-well plate.

Instrumentation



Figure 6 – MAGPIX Fluorescent Imaging System



Figure 7 – ELx50 Automated Microplate Strip Washer

Conclusions

- A multiplex assay was developed for simultaneous measurement of twenty-two human circulating cancer markers in cell culture samples using the MAGPIX reader.
- Automated wash procedures using the ELx50 Microplate Washer were developed.
- Hep G2 Cell Line Biomarker secretions were characterized.
- The sample and standard values measured with this assay were correlated with laser based LX100 reader.
- The availability of the assay provides a useful tool for further investigation of biological functions of these biological markers.