Automated Washing of Multiplex Bead Based Assays for the Luminex MAGPIX® Reader System

Comparison of Luminex 100® and MAGPIX® Luminex Readers

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ELx50

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The MAGPIX Reader System has been developed exclusively for use with magnetic MagPlex® microspheres. Here we compare the results obtained with the Luminex MAGPIX reader to that of the Luminex 100 (LX100) using the BioTek ELx50™ to automate the wash steps of an EMD Millipore Cancer Biomarker multiplex assay.

Introduction

Luminex xMAP® technology provides the means to measure multiple analytes simultaneously from the same sample. Originally designed around polystyrene MicroPlex® beads which required vacuum aspiration for washing, the latest generation of MagPlex® beads use embedded ferrite particles to allow for the use of magnets to immobilize the microspheres during the wash steps.   Both beads can be read using a flow cytometry based reader that interprets the bead type as well as quantitate the analyte. Recently, a new paradigm of xMAP reader has been developed by Luminex which does not utilize flow cytometry principles for detection. The MAGPIX® reader system has been developed by Luminex exclusively for use with MagPlex® microspheres. With both reader technologies, distinct internally color-coded magnetic microspheres coated with a specific antibody, capture and quantitate different analytes. Using traditional Luminex flow cytometry technology, microspheres were channeled to pass rapidly and individually through a laser beam which excites the internal dyes identifying the bead and analyte, while a second laser excites the reporter molecule, quantifying the analyte. The MAGPIX® reader used in this study employs CCD fluorescent imaging to identify and quantitate the analyte. Both technologies require the same assay process prior to multiplex analyte determination by the reader. While we have established that the wash steps for MagPlex beads can be automated using an appropriately configured microplate washer using the LX100 reader, little data exists using the MAGPIX reader in conjunction with automated wash systems. Here we compare the results obtained with the MAGPIX reader to that of the LX100 using the BioTek ELx50 Automated Microplate Strip Washer to automate the wash steps of a EMD Millipore Cancer Biomarker 22-plex multiplex assay.
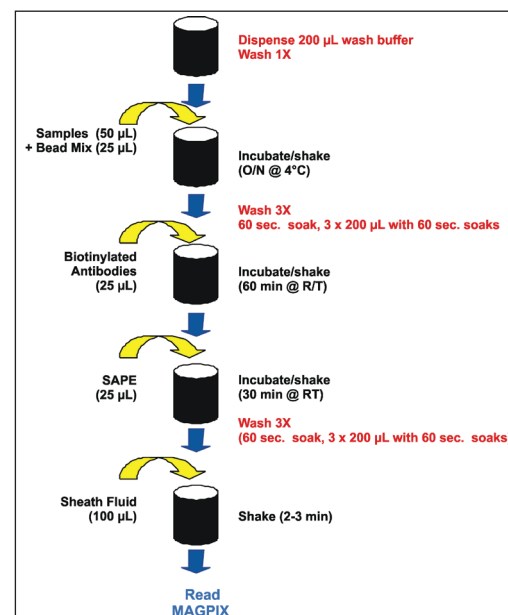

Figure 1. EMD Millipore cancer biomarker assay process. Red text indicates automated processes carried out by the ELx50 Automated Microplate Strip Washer.

Materials and Methods

The human tissue culture cell line HepG2 was obtained from ATCC. A MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel was obtained from EMD Millipore. HepG2 cells were maintainedin DMEM +10% FBS, which were obtained from Invitrogen. Aliquots (1.5 mL) of conditioned media were obtained from cultures in log phase growth and centrifuged at 14,000 RPM in an Eppendorf 5415C microfuge to remove any particulates.

The MILLIPLEX assay was performed according to the kit instructions (Figure 1). Briefly, the assay plate was first washed once 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 Circulating Cancer Biomarker Panel. This 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. The following day 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 LX100 reader with XPONENT software using the parameters outlined in the assay kit instructions.

The ELx50 Microplate Strip Washer (BioTek Instruments) used in these experiments was configured with a 96-well flat magnet (P/N 7103016). Wash programs were configured via the keypad using the "Link" utility to join three separate routines. An initial 60 second soak allows for bead capture, while a stepped aspiration scheme reduces bead loss from aspiration (See Table 2). Beads were immobilized using a strong rare-earth magnet integrated into the ELx50 washer.

Results

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.
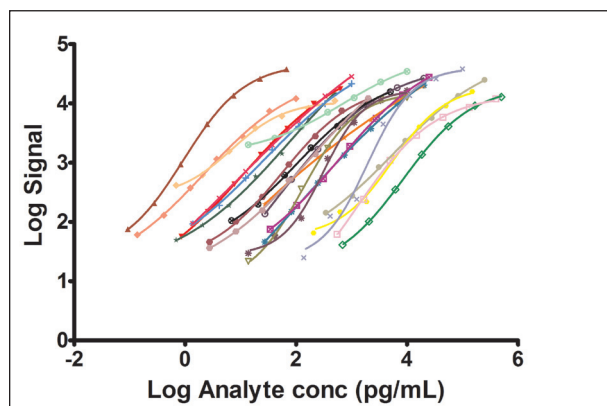

Figure 2. Standard curves for the Millipore 22-Plex Human Circulating Cancer Biomarkers using ELx50 Automated Microplate Strip Washer.

The assay kit provides quality control (QC) samples at two different concentration levels for all twenty-two analytes examined. By interpolation of the calibration curves generated for each analyte, concentrations for the QC samples can be calculated and compared to the expected range provided with the assay kit. As demonstrated in Table 1, the calculated results for all of the tested analytes falls within the expected range values.

Analyte	QC1 Expected Range	QC1 Result	QC2 Expected Range	QC2 Result
AFP	3319-6893	5035	16333-33921	28457
β-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
CYFRA21-1	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-α	24-50	35.5	131-273	205.2
TNF-α	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 Biomarker 22-plex assay.

Link File	Millipore MAGPIX		
Program Name	SOAK 60	MAGX3	FINAL
File Type	Soak	Wash	Aspiration
Method			
Wash Buffer		A	
Plate Type		96	
Number of Cycles		3	
Soak/Shake		Yes	
Soak Duration	60	60 sec	
Shake before soak		No	
Prime		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	
Prime Volume			
Prime Flow Rate			
Aspiration			
Aspiration Height		45	38
Horizontal Aspiration position		-20	-20
Aspiration Rate		6	6
Aspiration Delay		00	00
Crosswise Aspirate		No	No
Crosswise Aspirate on			
Crosswise Height			
Crosswise horizontal position			
Final aspiration		No	Yes
Final aspiration delay		0000 msec	0000 msec

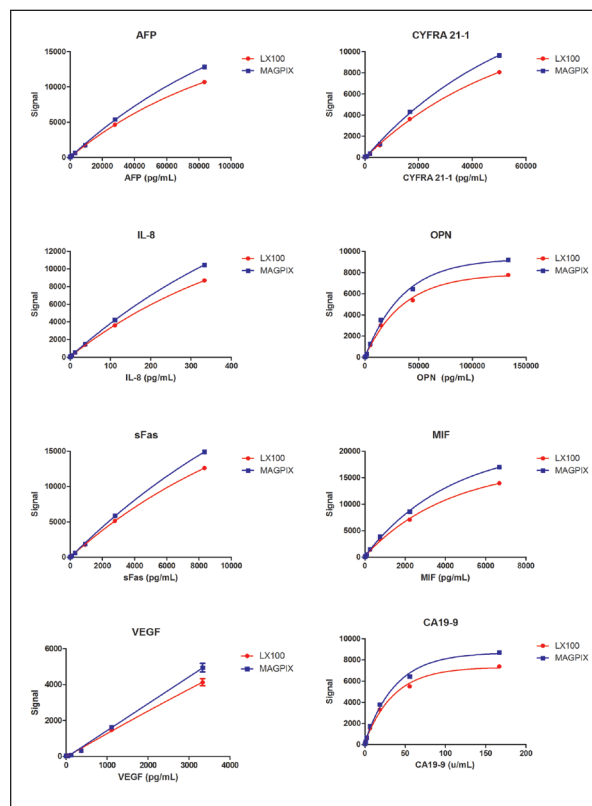*Table 2.* ELx50 Automated Microplate Strip Washer settings.

Figure 3. 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 shapes of the curves were very similar (Figure 3). More importantly, when calculated concentrations of samples are compared between the two readers, equivalent results are obtained (Figure 5 and 6).

Specific cancer marker secretion is observed when dilutions of conditioned media supernatant from HepG2 cells were assayed using the multiplex assay. HepG2 cells demonstrate measurable amounts of AFP, CYFRA21-1, IL-8, OPN and VEGF that correlates with the concentration of the input material. The signal observed with Total PSA is the result of a high 0 standard signal and does not correspond to expected input concentrations based on the starting dilution (Figure 4). 25 µL of 6X agonist (EC_{80}) was added to the 96-well plate, and incubated for 10 minutes at 37°C/5% CO_2. The remainder of the procedure is as described for the agonist assay.
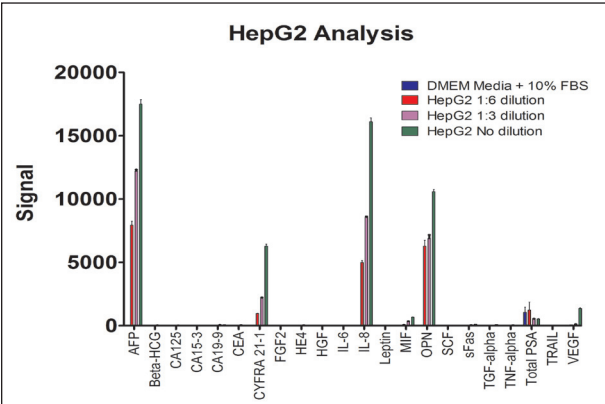
Figure 4. HepG2 cell line biomarker characterization. Tissue culture cell conditioned media supernatant was obtained from HepG2 cultures. The conditioned media was diluted 1:3 and 1:6 with fresh media (DMEM + 10 % FBS) and aliquots (25 µL) were assayed in parallel with undiluted conditioned media. Data are the mean of 8 determinations.

Comparison of the calculated concentrations made by the LX100 and MAGPIX readers with the same sample demonstrates the equivalence of the two reader platforms. Because of differences between the analyte concentrations in the multiplex assays, two different bar charts were used to compare the results with the LX100 and MAGPIX readers.
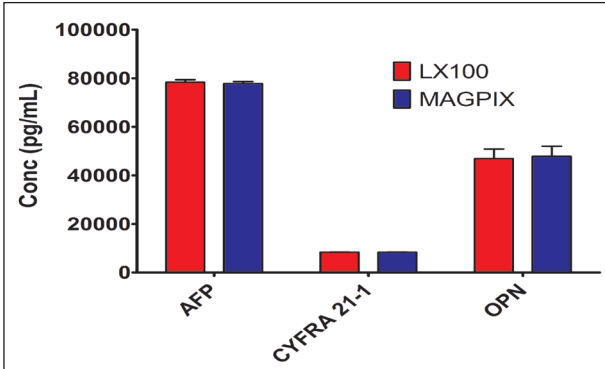

Figure 5. Comparison of LX100 and MAGPIX calculated biomarker concentrations. Multiplex reactions of HepG2 cell supernatant were assayed for the indicated cancer biomarkers along with standard curves in parallel using LX100 and MAGPIX readers. Determined concentrations from both readers are compared.

The analytes, AFP, CYFRA21-1, and OPN, which previously have been shown to provide a significant fluorescent signal when HepG2 cell supernatants were assayed, return concentrations in the high pg/mL range. At these ranges, the LX100 and the MAGPIX readers produced equivalent calculated concentrations when their respective calibration curves are interpolated (Figure 5). When analytes that have expected low pg/mL values, such as IL-8, sFas, MIF, and VEGF, are compared similar results are observed as well (Figure 6).
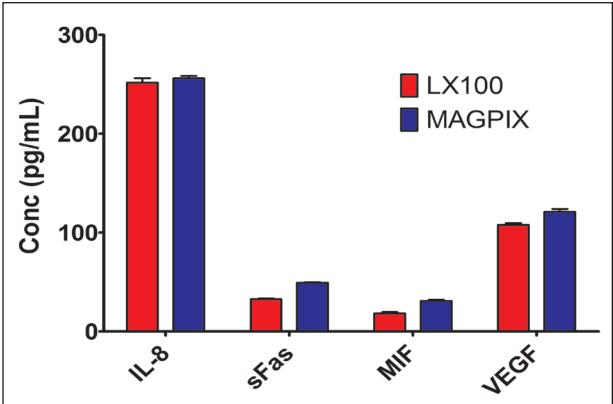

Figure 6. Comparison of LX100 and MAGPIX calculated biomarker concentrations. Multiplex reactions of HepG2 cell supernatant were assayed for the indicated cancer biomarkers along with standard curves in parallel using LX100 and MAGPIX readers. Determined concentrations from both readers are compared.

	Read Time	Events
MagPIX	32.5 sec	2544
LX100	16.8 sec	3047

Table 3. Comparison of read time and number of events.

When the average read time of the two different readers is compared, the flow cytometer based LX100 has a distinct advantage (Table 3). Because flow cytometry interprets one bead at a time, the quantity/concentration of beads available has the greatest influence on read time for the LX100. Whereas the MAGPIX interprets the beads simultaneously, using a fixed read time.
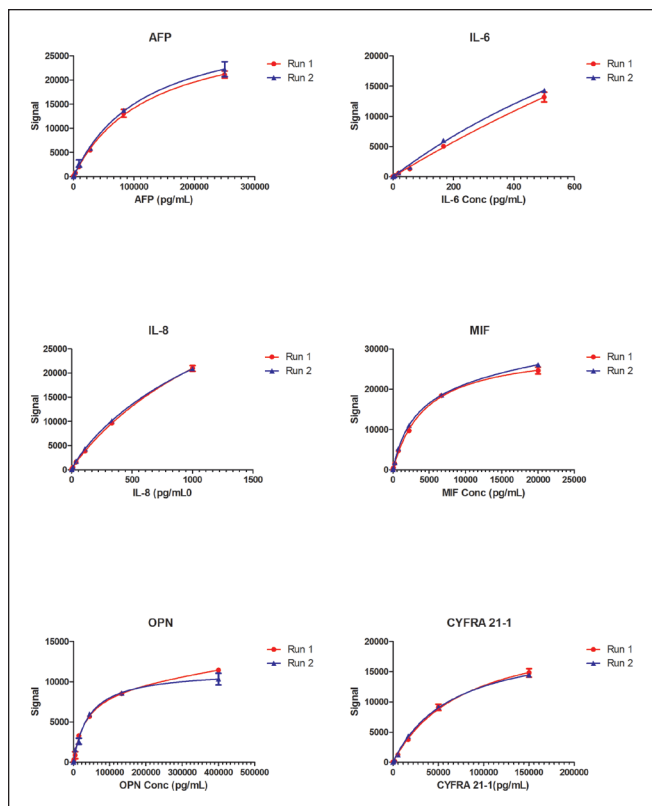
Figure 7. Inter-assay repeatability. Results from two separate assay runs for representative analytes are depicted. Data points represent the mean of duplicate determinations.

The multiplex cancer biomarker assay was found to be very repeatable in multiple assay runs carried out on separate days. Comparison of the signal of representative calibration curves from separate assay runs shows almost identical standard curves (Figure 7).

Discussion

These data indicate the BioTek ELx50 Automated Microplate Strip Washer is capable of automating the wash steps for both the LX100 and the MAGPIX Luminex readers. The LX100, based on flow cytometry, uses lasers to excite the bead and the analyte tracer and PMTs for detection, while the MAGPIX uses LEDs for excitation and CCD technology for detection. Because of the marked difference in read technology between the two readers, validation of similar wash protocols was necessary.

Effective washing needs to be balanced against bead retention. While the beads are immobilized by the rare earth magnet, they are not physically attached to the microplate and have the potential to be aspirated during the wash cycles. We have found that the spatial relationship between the localization of the magnetic beads by the magnet and the aspiration tubes is paramount for bead recovery. By offsetting the aspiration tube to one edge of the well, exposure of the majority of the beads to the fluid vortex of the aspiration tube is minimized and aspiration efficiency, as measured by low residual volume is maximized. The use of a stepped aspiration height, where the final aspiration uses a lower aspiration height serves to reduce bead loss, while allowing an effective wash.

Regardless of the reader employed this methodology can be used to characterize the cancer biomarker secretion of cells in tissue culture. HepG2 cells demonstrate a specific pattern of cancer biomarkers not present in the media alone. The differences in signal, which translate to differences in concentration, with differing sample dilutions indicate that the multiplex assay can be used quantitatively. Thus, the assay can be used to characterize other cells lines or tumor cells under various conditions. Cells are known to modulate their phenotype in response to external stimuli such as low serum drug and growth factor exposure, and oxidative conditions. Changes in their cancer biomarker secretions would also be expected.

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