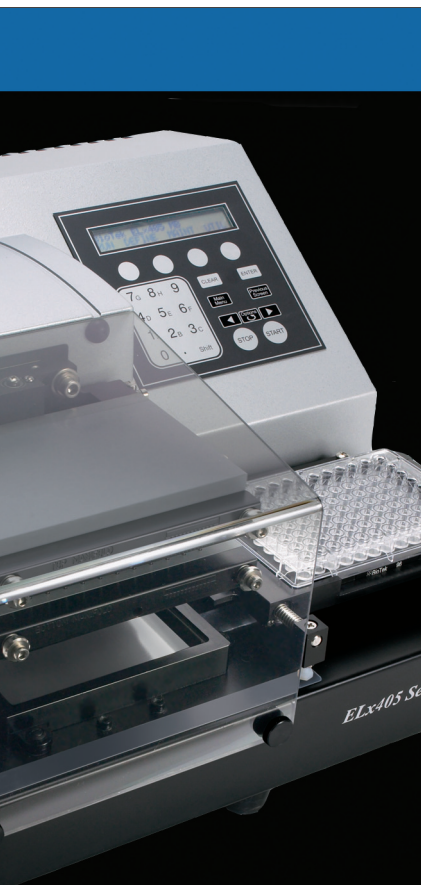


Multiplexing Magnetic Bead-based Human Cytokine Assays Automated Magnetic Bead Washing Using the ELx405™

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Cytokines and growth factors are cell signaling proteins that mediate a number of physiological responses. In addition they are also associated with a number of diseases including tumor growth and metastasis, infections and inflammation. Because these molecules rarely act unilaterally it is important to assess a number of them in parallel. The advent of multiplex assays using Luminex xMAP® bead technology has simplified the task of performing multiple assays on samples. Here we describe the use of the BioTek ELx405 Magnetic Bead Washer to automate the wash steps of the Bio-Rad Human Cytokine 8-plex assay kit.

Introduction

Cytokines and growth factors are small (8-30 kDa) water soluble proteins and glycoproteins that are involved in cell signaling. Each of the many different cellular cytokines and growth factors has a matching cell-surface receptor which serves to transmit the signal through subsequent intracellular signaling cascades to alter cell function and elicit a cellular response. Cytokines and growth factors have been associated with inflammation, autoimmune diseases and Cancer [1].

Almost all cytokines are pleiotropic effectors with a myriad of biological activities. In addition, multiple cytokines often have overlapping activities and a single cell frequently interacts with multiple cytokines with seemingly identical responses. That every cell type may have different responses to the same growth factor or cytokine can be explained, in part, by different spectrums of genes expressed in these cells and the availability and levels of various transcription factors that drive gene expression [1].

Specific receptors for cytokines have been identified and classified on the basis of function. These include: (1) the immunoglobulin (Ig) superfamily, typified by IL-1 receptor are found throughout the body; (2) the haemopoietic growth factor family, such as the IL-2 receptor; (3) the interferon type, which includes INF- α and INF- γ receptors; (4) the tumor necrosis factor (TNF) family of receptors, which share a cystein-rich extracellular binding domain; and (5) lastly there is the seven transmembrane helix family of receptors that include the G-protein coupled receptors (GPCRs). In addition to GPCRs, the chemokine receptors CXCR4 and CCR5, which are also interact with HIV binding proteins are included in this family [1].

Cytokines have been assayed by several different means, the most notable of which is ELISA. With ELISA, antibodies capture and quantitate specific cytokines individually from samples. These assays are typically performed in microplates (96-, 384-, and 1536-well). The ELISPOT (Enzyme-linked ImmunoSPOT) assay, which was developed as a modification of ELISA, measures the secreted cytokine products from live cytokine secreting cells. Cells are deposited onto a membrane coated with an antibody specific for the cytokine of interest. After a sufficient incubation period the protein of interest is detected in the environment immediately surrounding the secreting cell with a second antibody specific for a different epitope. The signal is generated by the HRP enzyme/substrate results in a colored footprint of the secreting cells and is quantitating by visual scoring. Cytokine bioassays, both direct and those using neutralizing antibodies, measure biological activity by some sort of proliferation assay (e.g. XTT, MTT) of primary cells or cell lines dependent and/or responsive to the cytokine of interest. Immunofluorescence staining and flow cytometry use fluorescently labeled antibodies to detect intracellular cytokines and surface receptors at the single cell level. These assays, as well as others suffer from either the length of time required to run the assay or the fact that each analyte needs to be quantitated separately. The bead based technology employed by the Bio-Rad multiplex kits allows multiple analytes to be assayed concurrently in as little as 1.5 hours. Here we describe the use of the ELx405™ Magnetic Bead Automated Microplate Washer to automate the wash steps in the Bio-Rad Bio-Plex Pro human Cytokine 8-Plex Assay.

Key Words:

Magnetic Beads

Washing

Luminex

xMAP®

Flow Cytometry

Cytokine

Basis of the Assay

The Bio-Rad Bio-Plex Pro™ assay uses beads comprised of polystyrene microspheres of about 6.5 microns in diameter that have been impregnated with ferrite particles and a mixture of two fluorescent dyes. The relative volumes of the dyes are carefully adjusted to provide 100 distinct colors that can be used to differentiate beads and provide a means to simultaneously measure up to 100 analytes in a microplate well. Capture of analyte molecules is provided by conjugating analyte specific primary antibodies to the surface of the microsphere and using fluorescently-labeled reporter tags that bind to the captured molecule. In the case of metabolic hormone assays, the capture molecule would be a primary antibody against the cytokine analyte and the reporter tag would be a secondary antibody to a different epitope on the protein labeled with biotin. Fluorescent readout would then be provided using streptavidin labeled with phycoerythrin, which binds directly to the biotin moiety of the secondary antibody. The amount of bound phycoerythrin is proportional to the amount of analyte.

Materials and Methods

A Bio-Rad Bio-Plex Pro™ Human Cytokine Magnetic Bead Panel, catalog number M50-000007A, was used to evaluate the performance of the ELx405™ Magnetic Bead Washer. A series of calibration curves were generated and assayed according to the assay kit instructions. Briefly, the bead mixture was first washed two times using the ELx405 Magnetic Bead Washer with the supplied assay wash buffer to remove any residual storage solution from the beads. Eight working multiplex standards were generated by serial dilution (1:4) of the reconstituted human cytokine standard. These standards contained 8 different analytes. After reconstitution, 50 µL each of standards and samples were pipetted into bead containing wells of the assay microplate. The reaction was allowed to incubate for 30 minutes at room temperature (RT) with agitation on a plate shaker. After incubation the plate was washed using an ELx405 Magnetic Bead Automated Microplate Washer (BioTek Instruments) as described in the washing instructions below. After washing, 25 µL of detection or secondary antibody reagent was added and allowed to incubate for 30 minutes at RT with agitation. The beads were again washed three times followed by the addition of 50 µL of SAPE reagent. After 10-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 125 µL of assay buffer. Samples were then read on a Luminex 100 reader with XPONENT 3.1 software using the parameters outlined in the assay kit instructions (Figure 1).

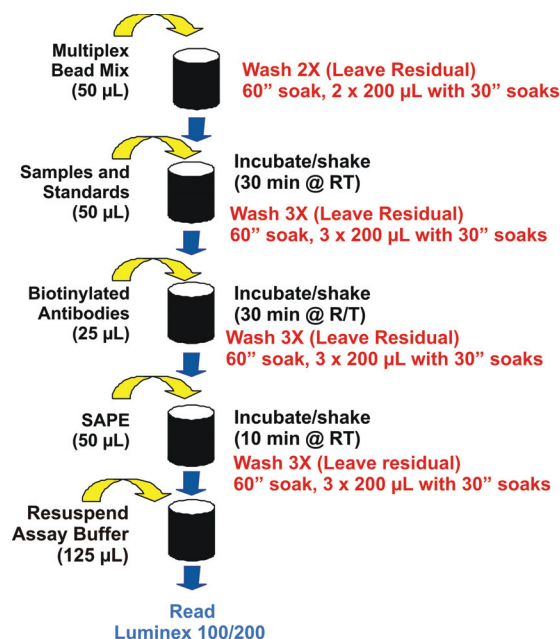


Figure 1. Cytokine assay workflow.

Washing Instructions

Automated plate washing was performed using the ELx405 Magnetic Bead Microplate Washer (BioTek Instruments). The onboard keypad programming "Link" function of the washer was used to link three wash procedures together, that when used together define a "stepped" aspiration (Table 1). An initial soak routine (SOAK60) was linked to one of two different wash procedures (MAGX2 or MAGX3) depending on the assay procedure required followed by a final aspiration program (FINAL). The soak routine allows for a 60 second bead capture by the magnet, while the wash procedures dispense and aspirate 200 µL for two or three cycles.

The wash routines have also utilized short soak routines (30 seconds) between cycles to allow the recapture of any beads resuspended during the fluid dispense. Both of these wash procedures do not call for a final aspiration. Final aspiration is accomplished with a third routine that uses aspiration heights that are lower than those used with the wash procedures, leave significantly less residual fluid. The specific parameters for each procedure are listed in Table 1 and have been optimized for both bead retention and washing efficiency.

Link File	Bio-Rad			
Program Name	SOAK60	MAGX3	MAGX2	FINAL
File Type	Soak	Wash	Wash	Aspiration
Method				
Wash Buffer		A	A	
Plate Type		96	96	
Number of Cycles		3	2	
Soak/Shake		Yes	Yes	
Soak Duration	60	30 sec	30 sec	
Shake before soak		No	No	
Prime		No	No	
Prime Volume				
Prime Flow Rate				
Dispense				
Dispense Volume		200	200	
Dispense Flow Rate		5	5	
Dispense height		130	130	
Horizontal dispense position		00	00	
Horizontal Y position		00	00	
Bottom wash first		No	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	45	40
Horizontal Aspiration position		-50	-50	-50
Aspiration Rate		6	6	6
Aspiration Delay		00	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 1. ELx405™ Magnetic Bead Wash Protocol for Bio-Rad Bio-Plex Pro™ 8-plex Cytokine Assay. Parameters have been optimized for the Greiner assay plate supplied by the kit.

Results

Initial experiments focused on bead recovery. Recovery was assessed using the read step acquisition times. The amount of time necessary to acquire 50 beads of each bead type is inversely proportional to the number of beads present in the sample. On two separate experiments the Luminex Reader required an average of 7.22 or 6.19 seconds respectively to interpret 50 beads each of eight different bead analytes of the Bio-Plex Pro™ human Cytokine assay when the samples were washed using the ELx405™ Magnetic Bead Washer (Table 2). In both cases an average of approximately 500 events were counted from each well, providing an acquisition rate between 70-80 beads per second (Table 2).

	Exp 1	Exp 2
Acquisition Time (sec)	7.22	6.19
Total Events (beads)	498.2	500.88
Acquisition Rate (beads/sec)	69	81

Table 2. Comparison of Acquisition time and rate from separate assays.

Bead recovery was also assessed by observing the bead count of different bead types in the 8-plex assay as part of the data assay analysis. The Luminex reader was configured to either count at least 50 beads of each type. As demonstrated in Figure 2, the different bead types from two separate assay experiments each with 27 different wells of the 8-plex assay averaged 55 beads or greater. The two separate runs exhibited very similar bead counts, indicating that washer performance is consistent.

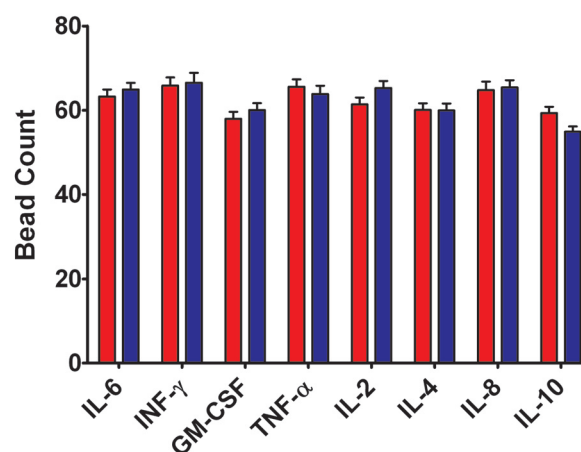


Figure 2. Comparison of Bead Count by Individual Bead Type for Two Assay Runs. Each bar graph represents the average of six wells.

When total bead counts from individual wells are examined an average of 495 ± 39 beads were counted (Figure 3). Individual wells from various regions on the plate had virtually the same total number of beads, which indicates that the washes performed were uniform across the entire 96-well plate.

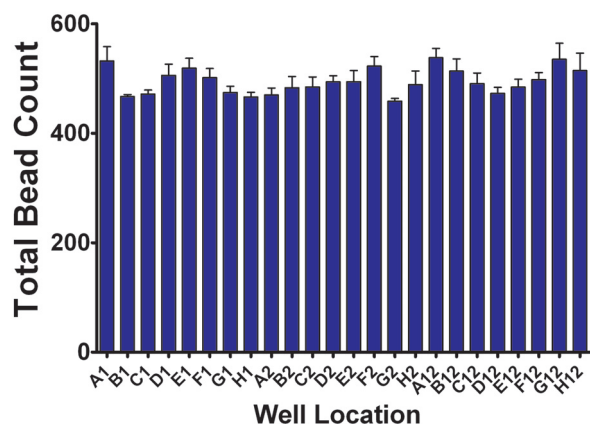


Figure 3. Total Bead Count at Specific Well Locations. Data represents the average of four separate determinations.

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. However, unlike ELISA reactions where the solid surface is the microplate itself, with bead based assays retention of the bead is equally important. As shown in Figure 4, using the ELx405™ Microplate Bead Washer 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.

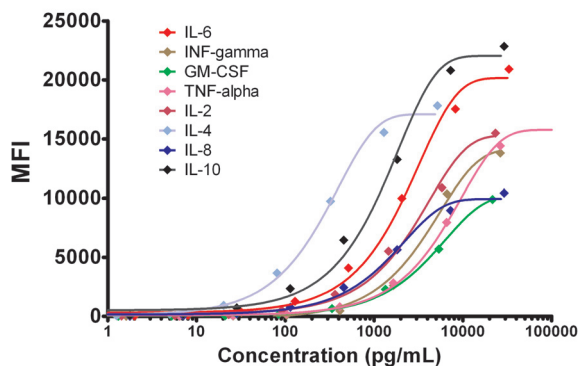


Figure 4. Standard curves for the Bio-Plex™ 8-plex human Cytokine Panel from Bio-Rad.

Consistency of the assay results was tested by running the assay on subsequent days. As shown in Figure 5, day to day consistency for the assay is quite good. The median fluorescence intensity (MFI) of calibrations curves for IL-4 experiments run on two different days is virtually identical. Similar results for the other analytes of the 8-plex assay were also observed (data not shown).

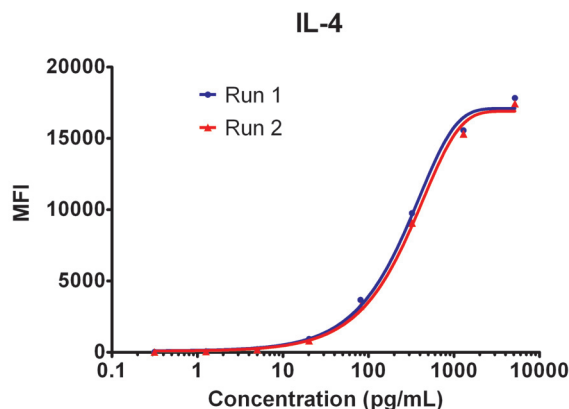


Figure 5. Comparison of IL-4 standard curve from multiple runs.

Assay precision is a good indicator of washer efficiency. As seen in Figure 6, the CVs of replicate samples are generally quite low. The distribution is such that over 40% of the samples had replicate CVs less than 2.5% and over 75% had CVs less than 5%. These data indicate that the ELx405 is washing the beads thoroughly, as well as consistently from well-to-well.

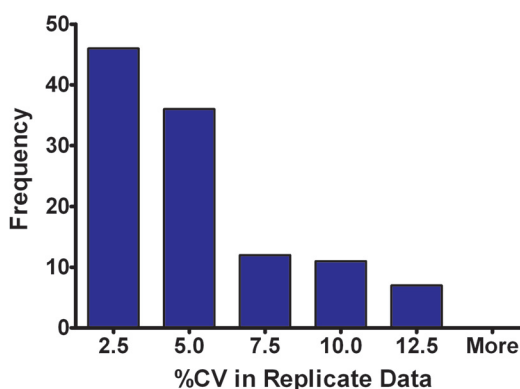


Figure 6. Histogram depicting the frequency of data points in the standard curves with CVs depicted on the abscissa.

Discussion

There are several parameters that need to be optimized in order to achieve the optimum results with the automated magnetic bead washers. Correct positioning of the aspiration tubes is critical for good bead recovery. While almost all 96-well plates conform to the SBS defined footprint, each plate manufacture employs a slightly different well geometry that requires optimization. The ELx405™ Microplate Bead Washer from BioTek has the ability to adjust the aspiration and dispense tubes in three different axis of movement, which greatly simplifies this task.

Proper washer maintenance is important for good washer performance. Aspiration and dispense-tubes need to be free of any obstructions. Obstructions are usually caused by a failure to rinse the fluidics with de-ionized water at the end of the session. Dissolved salts and/or proteins will crystallize and coat the inner and outer surfaces of the tubes resulting in full or partial occlusions. This not only affects the well(s) directly associated with the plug, but also the other wells on the plate. Occluded wells will result in increased pressure or fluid flows in the non-occluded tubes.

A critical component of washing magnetic beads is bead recovery. Because the beads are not physically attached to the microplate, the use of inappropriate washer parameter settings can result in significant bead loss. In these experiments we have utilized stepped aspiration heights to maximize bead recovery, while minimizing the residual fluid remaining in the well at the completion of the wash. Close proximity of the aspiration tubes to the beads will result in significant bead loss as a result of scour. Using a higher Z-offset for all but the final aspiration reduces the cumulative bead loss by minimizing the scour effect on all but the final aspiration.

Beads will cluster in regions of the microplate well that are closest to the capture magnet. In these experiments, a flat 96-well format magnet (BioTek P/N 7103016) was used to immobilize the beads in a thin film across the bottom of the entire well. Aspiration from one edge of the well in conjunction with the appropriate z-height seemed to retain more beads than center aspiration.

References

1. Mantovani, A. B. Savino, M. Locati, L. Zammataro, P. Allavena, and R. Bonecchi. (2010) The Chemokine System in Cancer Biology and Therapy. Cytokine and Growth Factor Reviews, 21: 27-39.

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