Bio-Plex Pro™/Bio-Plex Pro II Wash Stations: Validation for Bead-Based Immunosandwich Suspension Array Assays

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Introduction

Bead-based suspension array assays such as the Bio-Plex® assays have become the method of choice for the multiplex detection of cytokines, chemokines, and other potential soluble, protein-based and disease-related biomarkers. However, a large part of the actual assay process still remains dependent on cumbersome manual vacuum filtration-based washing steps. With the introduction of magnetic beads, increased automation and the elimination of manual washing are now possible.

Bio-Rad's Bio-Plex Pro assays are the first multiplex immunosandwich assays that use magnetic beads for easier and more reliable separations. Recently, Bio-Rad has introduced the Bio-Plex Pro Wash Station product line consisting of two distinct instruments: the Bio-Plex Pro wash station and Bio-Plex Pro II wash station (Figure 1A, 1B; Table 1). The Bio-Plex Pro wash station incorporates a magnetic plate carrier, which allows for quick magnetic separations, aspirations, and dispensations of wash buffer to achieve hands-free washing in 96-well plates. The Bio-Plex Pro II wash station includes both the magnetic carrier and an interchangeable vacuum manifold thus providing the added flexibility of utilizing either vacuum filtration or magnetic separation during washing. Both options include preset wash programs that have been optimized for Bio-Plex immunosandwich assays. The implementation of Bio-Plex Pro wash stations in the assay workflow can reduce the manual intervention involved in Bio-Plex assays and consequently help decrease the variability between operators, and increase the consistency between independent experiments.

The purpose of this study is to validate both the Bio-Plex Pro and Bio-Plex Pro II wash stations with a variety of bead-based assays and to present information associated with overall instrument and assay performance.

Fig. 1A. Bio-Plex Pro wash station.

Fig. 1B. Bio-Plex Pro II wash station.

Table 1. Bio-Plex Pro/Bio-Plex Pro II Wash Station Technical Specifications.

Microplate types	Bio-Plex Pro flat bottom plate (Bio-Rad, 171-025001) MultiScreen$_{HTS}$-BV (Millipore, MSBCS1210) Other microplates (need plate definition for that plate)
Magnetic field	4,500 ± 200 gauss
Vacuum range*	–50 to –150 mbar
Number of dispensing and aspirating channels	8 each
Dispense volume	50 – 400 µl in 50 µl increments
Dispense precision	8-way manifold, <4%CV over the plate
Soak time	1 to 600 sec
Residual volume	Single aspiration, 8-way manifold, <4 µl/well (%CV <30, 96 wells)
Onboard software	5 washing programs, 5 maintenance programs
PC software	For wash protocol programming
Power	100 to 240 VAC, 50/60 Hz
Dimensions (W x D x H)	28 x 37 x 18 cm
Weight	6.6 kg

* Applies to Bio-Plex Pro II only.

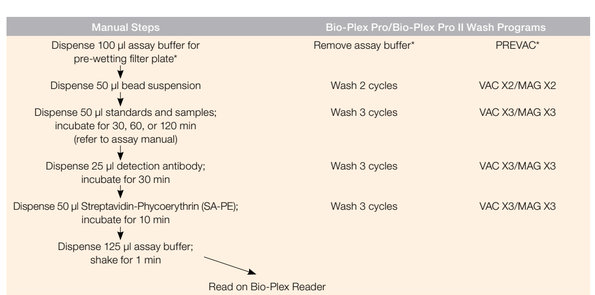
Materials and Methods

Three separate magnetic bead-based assays were validated on the Bio-Plex Pro and Bio-Plex Pro II wash stations. Assays utilizing either Bio-Rad 8.2 µm diameter magnetic beads that are included in all Bio-Plex Pro and Precision Pro™ assays or Luminex 6.4 µm diameter MagPlex C beads were evaluated. In addition, a nonmagnetic Bio-Plex human cytokine 27-plex assay was validated with the vacuum manifold option of the Bio-Plex Pro II washer. The workflow of the assay is summarized in Figures 2 and 3.

Each assay panel was run in 4 parallel workflows which differed only in the execution of the wash steps within the assay protocol. A control workflow utilized vacuum filtration with a Millipore MultiScreen$_{HTS}$-BV vacuum manifold (Millipore Corporation). The three remaining workflows employed separate test Bio-Plex Pro or Bio-Plex Pro II wash stations. Overall, each assay was run in each of these four workflows. Replicate parallel assays were then run on two additional days to provide information on inter-assay variability.

Vacuum filtration workflows utilized MultiScreen$_{HTS}$-BV 96-well filter plates. Magnetic separation workflows utilized Bio-Rad Bio-Plex Pro flat bottom 96-well plates (Bio-Rad Laboratories, Inc.).

A general assay protocol for either magnetic or nonmagnetic separation is shown in Figure 2.



* For low pressure vacuum manifold with filter plate.

Fig. 2. Bio-Plex assay procotol and wash programs for bead-based immunosandwich assay.

Instrument Performance

Instrument performance was evaluated on the basis of residual volume left in each well of the 96-well plate at the conclusion of each wash program and the recovery of magnetic beads following the final wash procedure. Inter-instrument variability was also examined for each assay. The Bio-Plex Pro wash station firmware settings and preinstalled wash programs were designed to maximize bead recovery and minimize residual volume.

Determination of Residual Volume

Residual volume was measured with 2 mM fluorescein dye. Bio-Plex wash buffer (50 µl) containing 0.75 mg/ml fluorescein dye was added to a Bio-Plex Pro flat bottom plate. This volume of dye was then aspirated with the final cycle setting of bead assay wash programs. Bio-Plex wash buffer (100 µl) was then added to the residual volume left in each well, and the plate was read on a Benchmark microplate reader (Bio-Rad) at a dual wavelength setting of 490/630 nm. The OD was converted to residual volume based on a four-point absorbance/dye volume standard curve prepared with the same fluorescein dye and Bio-Plex wash buffer.

Determination of Post-Washing Bead Recovery

Bead recovery was tested for all three types of beads: Bio-Rad 8.2 µm magnetic beads, Luminex 6.4 µm MagPlex C magnetic beads, and 5.6 µm polystyrene beads. 50 µl of antibody-coupled beads went through 4 series of washes (a total of 11 washes — 2 washes for capture antibody-beads, 3 washes for antigen, 3 washes for detection antibody, 3 washes for SA-PE). After these serial washes, beads were re-suspended in 150 µl of assay buffer and bead concentration was measured on a Beckman Z2 COULTER COUNTER (Beckman Coulter Inc.), and recovery was calculated against the control wells which did not undergo washing steps.

Assay Performance

Assay performance was evaluated on the basis of intra-assay and inter-assay precision, and recovery in both serum samples and spiked serum controls.

Bio-Plex Assays

All Bio-Plex assays were performed according to the manufacturer's guidelines. In particular, each assay was washed four serial times with the same protocols as a standard manual Bio-Plex assay, but with the actual wash steps automated by the Bio-Plex Pro/Bio-Plex Pro II wash stations (automatic magnetic bead washes for magnetic bead-based assays or automatic low vacuum filtration washes for polystyrene bead-based assays). Assays that were washed manually with a MultiScreen$_{HTS}$-BV vacuum manifold were treated as controls. Four parallel assays (one on MultiScreen$_{HTS}$-BV vacuum manifold and three on Bio-Plex Pro wash stations) were run side by side. Assays from each panel were also repeated.

On each assay plate, a four-fold dilution of low concentration standards from 3,200 – 0.2 pg/ml or high concentration standards from 32,000 – 1.95 pg/ml (S1 – S8) were run in triplicates in columns 1, 2, and 3, human serum diluted 1:4 in sample diluent, background (human-serum based), 0 pg/ml of bovine serum standard diluent and spiked sample at 200 pg/ml were run in column 4, 8, and 12 respectively (Figure 3).

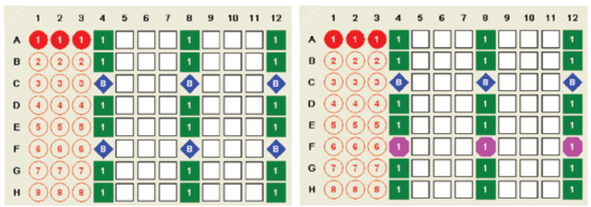


Fig.3. Assay plate layouts. A, cytokine assay plate layout; **B**, human diabetes assay plate layout.

The intra-assay, inter-assay, inter-instrument-assay coefficient of variation (CV), and recoveries (pg/ml) were calculated from 18 replicates of endogenous targets in human serum or standards spiked into serum. All calculations and analysis were done with the Bio-Plex Manager™ 5.0 software. Standard serum spike recoveries were also compared between automatic bead washers and manual MultiScreen$_{HTS}$-BV vacuum manifold.

The intra-assay %CV was determined based on median fluorescence intensity (MFI) from the variance of serum samples and serum spiked standards within 18 replicates. The inter-assay %CV was determined based on observed concentration from the variance of serum samples and spikes of the three-day experiments washed with the same washer. Inter-instrument-assay %CV was calculated based on observed concentration from the variance of serum samples and serum spike standards of same day experiments.

Results

Automatic wash programs used for magnetic bead wash and vacuum wash were developed and optimized based on percent bead recovery, well residual volume, and assay performance. To ensure that the residual volume and bead recovery meet the technical specifications, residual wash buffer left behind from the Bio-Plex Pro wash stations was measured using dye absorbance. By comparing the absorbance at 490/630 nm of Bio-Plex wash buffer that had been dispensed into wells of a 96-well microplate to a previously prepared standard curve (volume vs. absorbance), the residual volume of each well could be determined. As demonstrated in Figure 4 and Table 2, residual volumes in a 96-well Bio-Plex Pro flat-bottomed microplate were found to be less than 4 µl. The impact of residual wash buffer volume of <4 µl on Bio-Plex assay sensitivity is negligible, and the high percentage of bead recovery (better than that manually washed ~60%) after a series of four automatic washes improves the speed and efficiency of sample acquisition on a Bio-Plex 200/Luminex 200 detector.

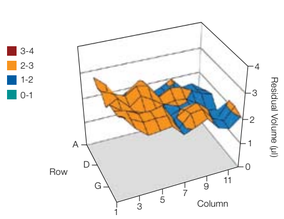


Fig. 4. Three-dimensional residual volume plot comparing relative areas of a 96-well Bio-Plex Pro flat bottom plate.

Table 2. Mean residual volume of buffer after each wash program.

Washer #	Type	Mean Residual Volume (µl)
801001924	Bio-Plex Pro	3.5
802002062	Bio-Plex Pro	3.7
802002063	Bio-Plex Pro II	2.7

The bead concentration after four wash series was measured with a Beckman Z2 COULTER COUNTER. Recovery was calculated against the control wells, which contained the same amount of beads and did not go through a series of washes. Data collected from six replicates on each plate/washer showed both Bio-Rad 8.2 µm magnetic beads and Luminex 6.4 µm MagPlex C magnetic beads have better recovery than the manually washed polystyrene beads (Table 3). Retention of polystyrene beads after washing on the Bio-Plex Pro II vacuum wash station is comparable to that of beads manually washed with manual vacuum manifold.

Table 3. Bead recovery after series washes. Data presented represent the average recovery determined from three washers. The %CV for 18 determinations obtained from three different plates is represented.

Type of Bead	Diameter (µm)	% Bead Recovery	%CV
Polystyrene	5.6	63	9
Bio-Rad Magnetic	8.2	79	16
MagPlex C Magnetic	6.4	92	12

Intra-assay, inter-assay (between days) and inter-washer precision were determined with human cytokines and human diabetes assays. In particular, intra-assay precision was measured in 18 replicates of human serum. Intra-assay %CV was <11, inter-assay %CV was <30, and inter-washer %CV was <15 for all measured human cytokine targets (Table 4A, B and C).

Additional precision and recovery tests were performed with human serum spiked with 200 pg/ml of exogenous human diabetes markers (Table 5). In these experiments high level of endogenous leptin, PAI-1, and resistin precluded accurate measurement of the spiked proteins.

Table 4. Comparison of precision in quantifying picogram levels of human cytokines and growth factors. Human sera were diluted 1:4 with serum sample diluent, and analyzed with Bio-Plex assays on magnetic beads or polystyrene beads. The assays were washed on Bio-Plex Pro/Bio-Plex Pro II magnetic bead wash stations or on Bio-Plex Pro II vacuum wash stations, respectively. The control assays for each panel were washed manually with MultiScreen$_{HTS}$-BV vacuum manifold. Data from detectable analytes in the tested samples are presented. Some targets (4 out of 10) were not detected in the samples used.

Table 4A. Bio-Plex Precision Pro human cytokine 10-plex assays on Bio-Rad 8.2 µm magnetic beads, washed on Bio-Plex Pro/Pro II magnetic bead wash stations.

		Precision*								
		Manual Wash		Washer 1		Washer 2		Washer 3		
Analyte	Observed Concentration (pg/ml)**	Intra-Assay	Inter-Assay	Intra-Assay	Inter-Assay	Intra-Assay	Inter-Assay	Intra-Assay	Inter-Assay	Inter-Washer Precision***
IL-6	497.1	12.8	3.7	5.3	3.9	4.9	7.1	7.2	0.6	14.7
IFN-γ	28.7	13.0	7.1	5.1	9.8	5.5	14.1	8.0	16.4	11.8
IL-4	14.8	12.0	1.3	6.0	8.3	5.9	10.4	7.8	7.6	8.8
TNF-α	14.4	13.8	1.6	6.9	9.5	6.9	10.6	8.4	9.8	10.0
IL12-p70	12.8	7.0	9.3	6.9	12.8	6.5	12.3	8.1	10.2	13.4
IL-1β	7.2	13.8	6.5	5.6	16.5	6.1	15.8	7.4	11.8	3.9

* Calculated as %CV. ** Observed concentrations are represented by the mean of the 18 replicates for 12 microplates using three wash stations and manual washes. *** Calculated as %CV of assays for three wash stations.

Table 4B. Bio-Plex human cytokine 27-plex assay on 6.4 µm MagPlex C beads, washed on Bio-Plex Pro/ Bio-Plex Pro II magnetic bead wash stations.

		Intra-Assay Precision*				
Analyte	Serum Cytokine (pg/ml)**	Manual Wash	Washer 1	Washer 2	Washer 3	Inter-Washer Precision***
IL-1ra	1959.4	6.3	8.0	7.9	7.8	4.8
IL-8	1449.0	7.3	5.8	4.8	5.8	6.9
PDGF-bb	1444.7	4.5	5.0	5.7	4.8	8.5
IFN-γ	924.2	7.4	9.8	5.8	9.2	12.0
IP-10	483.8	4.4	6.3	4.7	5.2	2.8
GM-CSF	469.6	6.0	9.0	7.4	6.7	6.0
TNF-α	249.4	6.6	10.5	8.1	7.9	6.9
IL-9	116.2	6.3	8.4	6.2	8.7	4.0
IL-2	73.4	5.4	9.5	6.3	7.3	6.1
FGF basic	66.6	6.8	7.3	7.4	8.6	6.2
IL-6	56.1	6.1	8.6	7.7	8.0	5.7
MIP-1β	52.1	6.1	5.4	4.7	4.6	1.6
IL-12 (p70)	48.2	7.8	6.8	5.5	9.2	3.5
G-CSF	47.3	7.9	10.6	8.0	10.3	6.5
IL-17	24.7	4.8	6.5	5.5	7.8	11.6
Eotaxin	23.2	5.0	8.1	7.1	6.3	8.0
IL-1β	19.7	4.8	8.8	7.8	7.8	4.7
IL-13	18.0	9.8	8.5	10.4	10.1	11.1
VEGF	17.1	5.3	8.8	10.8	8.5	9.2
IL-4	12.4	8.3	9.3	9.6	10.1	2.1
IL-7	11.6	7.0	8.0	5.6	8.4	4.3
MIP-1α	8.6	4.5	6.1	5.4	7.4	12.3
IL-10	6.0	6.1	9.3	6.6	8.2	10.2
IL-5	3.9	10.8	10.9	11.9	16.9	3.2

* Calculated as %CV. ** Observed concentrations are represented by the mean of the 18 replicates for 8 microplates using three wash stations and manual washes. *** Calculated as %CV of assays for three wash stations.

Table 4C. Bio-Plex human cytokine 27-plex assay on 5.6 µm Polystyrene beads, washed with Bio-Plex Pro II low-vacuum wash stations.

		Precision*						
		Manual Wash		Washer 1		Washer 3		
Analyte	Serum Cytokine (pg/ml)**	Intra-Assay	Inter-Assay	Intra-Assay	Inter-Assay	Intra-Assay	Inter-Assay	Inter-Washer Precision***
IL-1ra	8441.3	5.0	14.1	8.3	19.1	7.5	9.0	12.1
IFN-γ	2112.3	7.0	7.6	9.0	5.4	9.8	5.6	7.5
IL-2	1165.7	7.0	14.1	8.7	17.1	8.5	5.8	10.0
IL-8	1162.8	3.2	0.9	3.1	6.9	2.8	21.0	12.9
GM-CSF	929.8	5.7	10.9	7.6	15.6	7.0	7.9	7.4
TNF-α	910.1	5.5	21.6	7.8	16.7	8.2	19.3	2.9
PDGF-bb	845.1	5.2	10.1	6.6	7.8	5.4	18.0	6.8
Eotaxin	712.7	6.8	20.6	8.4	7.1	10.7	14.5	6.3
IL-13	508.2	4.8	14.7	6.2	9.2	4.8	15.9	5.8
IL-12 (p70)	465.9	4.9	23.8	6.7	19.9	5.5	25.2	5.0
G-CSF	370.7	6.7	19.8	9.1	12.2	9.1	19.0	6.3
IP-10	347.7	5.7	23.7	6.5	17.0	6.4	23.6	4.7
IL-6	290.8	7.2	29.2	7.8	5.3	7.4	26.2	10.8
IL-10	283.1	4.9	19.1	6.1	10.5	5.0	19.4	5.5
IL-7	269.1	5.5	25.9	8.1	22.3	7.0	29.4	6.9
IL-15	213.4	6.9	11.6	7.5	4.8	6.6	7.5	7.2
IL-1β	115.0	5.5	19.9	9.0	12.7	6.1	17.0	9.7
MIP-1β	81.8	5.3	25.1	4.5	13.9	5.8	29.9	13.5
IL-4	6.4	7.3	31.1	10.1	29.8	7.7	27.2	2.5

* Calculated as %CV. ** Observed concentrations are represented by the mean of the 18 replicates for 9 microplates using three wash stations and manual washes. *** Calculated as %CV of assays for three wash stations.

Table 5. Comparison of accuracy and precision of quantitation of human diabetes and obesity marker standards in human serum sample. Bio-Plex human diabetes 12-plex assays were processed using pooled human serum samples, and serum samples which were spiked with known amounts of standards (200 pg/ml), and automatically washed with Bio-Plex Pro magnetic bead wash stations. Assays washed manually were treated as controls. Numerical values in assay range indicate %CV among replicates (N = 18). Absence of recovery value indicates some target concentrations outside the LOQ of the assay. Accuracy was defined as % recovery relative to spiked value. Precision calculated as %CV.

		Manual Wash				Washer 1				Washer 2				Washer 3				
			Accuracy	Precision			Accuracy	Precision			Accuracy	Precision			Accuracy	Precision		
Analyte	Expected Concentration (pg/ml)	Spike Standard (pg/ml)	% Recovery	Intra-Assay Precision	Inter-Assay Precision	Spike Standard (pg/ml)	% Recovery	Intra-Assay Precision	Inter-Assay Precision	Spike Standard (pg/ml)	% Recovery	Intra-Assay Precision	Inter-Assay Precision	Spike Standard (pg/ml)	% Recovery	Intra-Assay Precision	Inter-Assay Precision	Inter-Washer Precision
Visfatin	2,121	2,589	107	2	21	2,630	109	1	21	2,599	107	2	19	2,772	114	2	16	19
PAI-1*	443	171	–	2	–	–	–	1	–	227	–	1	–	280	–	1	–	–
Ghrelin	328	256	94	5	13	253	93	6	20	262	95	4	2	284	105	6	16	13
Leptin*	253	897	–	4	–	–	–	6	–	–	–	7	–	–	–	5	–	–
Resistin*	241	185	129	1	–	–	–	1	–	–	–	1	–	–	–	1	–	–
GLP-1**	212	69	–	7	–	–	–	4	–	–	–	14	–	–	–	12	–	–
C-peptide	189	110	85	8	6	125	95	8	13	105	81	8	11	123	95	20	3	9
Insulin	179	156	94	5	20	142	85	10	16	130	78	6	21	149	88	6	6	14
Glucagon	176	130	101	5	35	135	105	13	34	136	107	7	39	134	104	9	36	36
GIP	127	153	106	3	24	157	109	6	27	150	104	2	21	154	106	4	20	23
IL-6	12	14	107	6	19	12	98	10	16	13	99	7	11	12	95	7	9	12
TNF-α	10	12	108	7	15	11	96	14	20	11	97	7	13	11	94	14	10	15

* Spike human serum contain a high concentration of these analytes. ** Located on the low end of the curve with low recovery.

Conclusions

The Bio-Plex Pro wash stations from Bio-Rad are automated microplate washers, designed to carry out both magnetic bead separation wash and vacuum filtration wash for all aspects of a variety of xMAP assays. The Bio-Plex Pro II can also be configured with a vacuum manifold plate carrier to perform wash steps for nonmagnetic bead assay. This is an alternative washing system to the standard xMap assays that utilize manual vacuum manifold systems, and may simplify the implementation of automation in customer applications.

Validation data presented confirm that the Bio-Plex Pro wash stations with preloaded wash programs are compatible with both 8.2 µm Bio-Rad magnetic beads and 6.4 µm Luminex magnetic beads and are capable of supporting all standard xMap assays that utilize magnetic microspheres. The Bio-Plex Pro wash stations enhance the bead-based assay workflow with the implementation of magnetic separations during wash steps. Such automation can both reduce manual intervention and improve precision.

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