Development and Validation of Two Human Cytokine Multiplex Assay Panels on Magnetic Microspheres

Hui Zhou[1], Li Ma[1], Joe Fedynyshyn, Jessica Dines, and Qian-Shu Wang,
Bio-Rad Laboratories, Inc., Hercules, CA
[1] These two authors contributed equally to this study.

Introduction

Cytokines, chemokines, and growth factors play important roles in a wide range of physiological processes, including immune response, inflammation, hematopoiesis, and carcinogenesis. Changes in the circulating levels of these proteins have been linked to a spectrum of disease states, making them valuable diagnostic and therapeutic biomarkers.

The Bio-Plex Pro™ human cytokine assay panels contain assays for 50 well characterized human cytokines, chemokines, and growth factors and offer excellent performance in diverse matrices such as serum, plasma, and tissue culture supernatant. The Bio-Plex Pro assays are based on magnetic beads (MagPlex beads from Luminex Corporation), which allow the option of implementing automated wash steps (using the Bio-Plex Pro Wash Station), thus simplifying assay processing by eliminating the need for vacuum manifold-based wash steps. The assays have been conveniently configured into all-in-one kits that contain all the necessary reagents and diluents for preparing and running the assay. The antibodies and buffer compositions are the same as for the original nonmagnetic, polystyrene bead-based assays that have been available from Bio-Rad in the past.

This article describes the development and validation of the new Bio-Plex Pro human cytokine, chemokine, and growth factor panels — the human group I panel and the human group II panel. Comparisons of the standard curves between the new magnetic bead-based assays and the original nonmagnetic bead-based assays are shown. The curves are comparable for most targets. Measurements of the working assay range — the lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) — sensitivity (limit of detection or LOD), and examples of assay parallelism and linearity are also provided for the new magnetic assays. For some targets, the new magnetic assays show improved assay range.

Magnetic Bead-Based Bio-Plex Assays

The magnetic bead-based Bio-Plex assay platform uses a series of color-coded magnetic beads, each coupled to a unique antibody specific for a biochemical marker. Each magnetic bead is dyed with two fluorophores (classification dyes) that absorb maximally at 635 nm and emit at two distinct wavelengths. The capture antibody-coupled beads serve as solid phase for the capture of analytes, followed by binding of a second biotinylated antibody in a sandwich-like assay. Quantitation is performed using the reporter dye, streptavidin-phycoerythrin, a fluorophore that absorbs maximally at 532 nm and emits at a third distinct wavelength. The Bio-Plex array system consists of a laminar flow cell designed such that the beads flow in single file through a region illuminated by two lasers. The particles emit light at three wavelengths, two from the classification dyes and one from the reporter dye, as schematically represented in Figure 1.

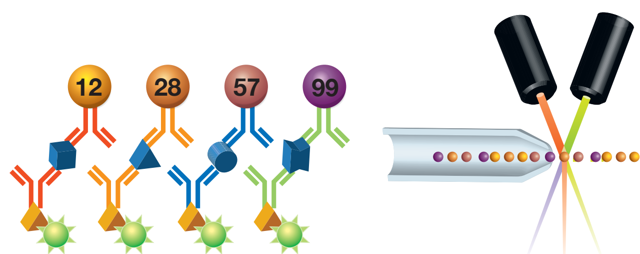


Fig. 1: Schematic representation of Bio-Plex immunoassay. A, Color-coded magnetic beads coupled to specific antibodies bind analytes. Bound analytes are detected using a biotinylated antibody and quantitated using streptavidin coupled to a fluorophore (PE). **B**, Fluorescence of the beads and of PE are measured simultaneously.

Methods

Evaluation of Assay Sensitivity (Limit of Detection)

The Limit of Detection (LOD) was calculated as the concentration of analyte on the standard curve for which the corresponding MFI value is two standard deviations above the background measured in the blank. The mean LOD of five independent assays was calculated using standard diluent or culture media as a matrix.

Evaluation of Assay Precision

Intra-assay precision was calculated as the coefficient of variation (%CV) among MFI values of six replicate wells of standard curve points on a single assay plate. Inter-assay precision was calculated as the %CV of the observed concentration of spike controls from five independent assays.

Evaluation of Assay Accuracy

Assay accuracy (recovery) was calculated as the percentage of the observed value of a spiked standard of known concentration relative to its expected value. Spike concentrations were measured at six different points within the assay range.

Determination of Assay Working Ranges

Working ranges for Bio-Plex assays are determined based on standard and spike recovery and assay precision. The assay working range is the range of concentrations in which the assay is both precise (intra-assay %CV < 20% and inter-assay %CV < 30%) and accurate (70-130% recovery). Assay working range is described as the Lower Limit of Quantitation (LLOQ) and Upper Limit of Quantitation (ULOQ).

Evaluation of Assay Linearity of Dilution

Linearity of dilution ensures that analytes present in concentrations above the LLOQ can be diluted and measured accurately within the assay working ranges. Human serum/plasma diluent was prepared from 4-fold dilution of pooled human serum/plasma with sample diluent. Standard antigens were reconstituted at the highest concentration point of the standard curve (S1), followed by 3-fold serial dilutions using the prepared human serum/plasma diluent for a series of six dilution points. The highest concentration of spiked multiplexed human cytokines (Dilution 1) is 1/3 of S1. The linear relationship of observed concentration and expected concentration of each dilution point within assay working range is plotted for each target. The R^2 value of each linear plot reflects the linearity of sample dilution for that assay.

Evaluation of Assay Parallelism

Assay parallelism is a measure of the impact of matrix effect on the binding characteristics of an assay. This was investigated by comparing slopes of spiked standard concentration-response curve in human serum or plasma with those of standard concentration-response curve in standard diluent. The concentration-response curves were prepared with six points of 3-fold serial dilutions of standards in either standard diluent or in human serum or plasma. The difference of the curve slopes (slope of the tangent at midpoint) between the two types of curves demonstrates the assay parallelism between standard diluent and natural matrices such as human serum and plasma.

Determination of Assay Specificity (% Cross-Reactivity)

The group I and group II assay panels were tested on different plates with the recommended concentration of 27-plex capture beads or 23-plex beads (21-plex plus ICAM-1 and VCAM-1), and with the concentration of 27-plex or 23-plex antigen standards at the second dilution point. Detection antibodies were added individually. Nonspecific, cross-reacting signal was defined as the percentage of signal detected relative to the specific signal for that analyte.

Data Analysis

Bio-Plex Pro human cytokine assays were analyzed using Bio-Plex Manager™ software version 5.0.

Results

Assay Sensitivity, Working Ranges, and Precision

Assay sensitivity for each target is reflected by the LOD. Assay working ranges (LLOQ – ULOQ) are defined as the concentration ranges in which the assays are both precise and accurate. Assay precision is measured by intra- and inter-assay %CV. Assay accuracy is determined by spike recovery.

The assay working ranges, LOD, intra- and inter-assay %CV data for all targets of Bio-Plex Pro human cytokine assays in group I (27-plex) and group II (21-plex plus ICAM-1 and VCAM-1) in serum-based matrix are summarized in Table 1A and Table 1B. Similar results were obtained in RPMI cell culture media matrix (not shown).

© 2009 Bio-Rad Laboratories, Inc.

Table 1A. Representative assay working ranges, assay sensitivity, and precision of Bio-Plex Pro human cytokine 27-plex group I panel. The LLOQ, ULOQ, LOD, and intra-/inter-assay %CV are mean data determined from five assays in serum-based matrix. These assays were performed using the vacuum-based wash method. %CV is expected to be comparable or lower with the magnet-based wash method.

	Assay Working Ranges, pg/ml	Assay Sensitivity, pg/ml	Assay Precision	
Targets	**LLOQ ULOQ**	**LOD**	**Intra-Assay %CV**	**Inter-Assay%CV**
IL-1β	3.2 – 3,261	0.6	6	8
IL-1Rα	81.1 – 70,487	5.5	9	8
IL-2	2.1 – 17,772	1.6	7	9
IL-4	2.2 – 3,467	0.7	9	8
IL-5	3.1 – 7,380	0.6	8	10
IL-6	2.3 – 18,880	2.6	7	11
IL-7	3.1 – 6,001	1.1	6	8
IL-8	1.9 – 26,403	1.0	9	4
IL-9	2.1 – 7,989	2.5	8	9
IL-10	2.2 – 8,840	0.3	5	6
IL-12 (p70)	3.3 – 13,099	3.5	6	6
IL-13	3.7 – 3,137	0.7	8	7
IL-15	2.1 – 2,799	2.4	5	6
IL-17	4.9 – 12,235	3.3	8	6
Eotaxin	40.9 – 5,824	2.5	8	11
FGF basic	27.2 – 7,581	1.9	8	8
G-CSF	2.4 – 11,565	1.7	10	5
GM-CSF	63.3 – 6,039	2.2	12	6
IFN-γ	92.6 – 52,719	6.4	15	9
IP-10	18.8 – 26,867	6.1	11	9
MCP-1	2.1 – 1,820	1.1	9	7
MIP-1α	1.4 – 836	1.6	7	8
MIP-1β	2.0 – 1,726	2.4	8	8
PDGF-BB	7.0 – 51,933	2.9	9	8
RANTES	2.2 – 8,617	1.8	9	6
TNF-α	5.8 – 95,484	6.0	8	6
VEGF	5.5 – 56,237	3.1	9	7

Table 1B. Representative assay working ranges, assay sensitivity, and precision of Bio-Plex Pro human cytokine, group II 21-plex panel and of ICAM-1 and VCAM-1. The LLOQ, ULOQ, LOD, and intra-/inter-assay %CV are mean data determined from five assays in serum-based matrix.

	Assay Working Ranges, pg/ml	Assay Sensitivity, pg/ml	Assay Precision	
Targets	**LLOQ ULOQ**	**LOD**	**Intra-Assay %CV**	**Inter-Assay%CV**
CTACK	13 – 16,874	3.4	5	6
GRO-α	19 – 2,711	6.3	5	8
HGF	9.5 – 25,357	4.9	5	6
IFN-α2	307 – 3,438	4.3	7	3
IL-1α	1.4 – 22,569	0.5	4	4
IL-2Rα	299 – 7,338	2.1	6	4
IL-3	12 – 22,803	4.8	7	4
IL-12 (p40)	41 – 28,662	23.3	5	8
IL-16	190 – 12,133	0.4	6	4
IL-18	1.8 – 28,677	0.2	4	5
LIF	12 – 22,184	5.5	4	3
MCP-3	79 – 11,234	1.0	7	8
M-CSF	2.0 – 32,200	0.9	4	5
MIF	231 – 24,373	1.5	5	8
MIG	2.7 – 8,584	1.2	6	6
β-NGF	1.0 – 4,247	0.2	4	7
SCF	1.5 – 25,078	1.0	5	4
SCGF-β	66 – 47,862	45.4	6	8
SDF-1α	10 – 10,391	8.7	6	6
TNF-β	1.5 – 24,505	0.3	4	4
TRAIL	4.4 – 44,769	2.1	4	8
ICAM-1	13 – 26,368	2.4	4.3	3.8
VCAM-1	38 – 21,430	0.6	6.7	5.5

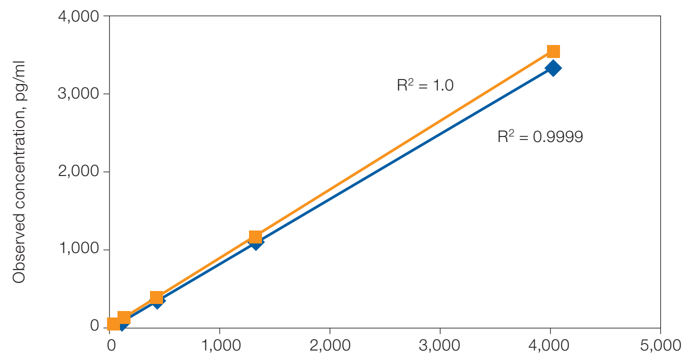
© 2009 Bio-Rad Laboratories, Inc.

Assay Linearity of Dilution

The linear relationship of observed concentration and expected concentration of dilution points within the assay working range was plotted for each target. The R^2 value of each plot reflects the linearity of sample dilution for that assay. The R^2 values for 27 targets in group I and 21 targets in group II are all above 0.95. Figures 2A and 2B are representative assay linearity of dilution plots for IL-10 of group I and IL-1α of group II.

Assay Parallelism

Assay parallelism was analyzed by comparing slopes of spiked standard concentration-response curves in human serum or plasma with those of standard concentration response curves in standard diluent. The curves are prepared with six points of 3-fold serial dilutions. The difference (% values) of the curve slopes between the concentration response curves in standard diluent and in human serum are <15% for all target assays in group I and group II 21-plex panels. The difference (% values) of the curve slopes between the concentration response curves in standard diluent and in human plasma are ≤25% for all assays, except for RANTES (56%) due to its high endogenous level in human plasma sample. Figures 3A and 3B demonstrate the parallelism of spike concentration response curves of IL-10 in group I and IL-1α in group II.



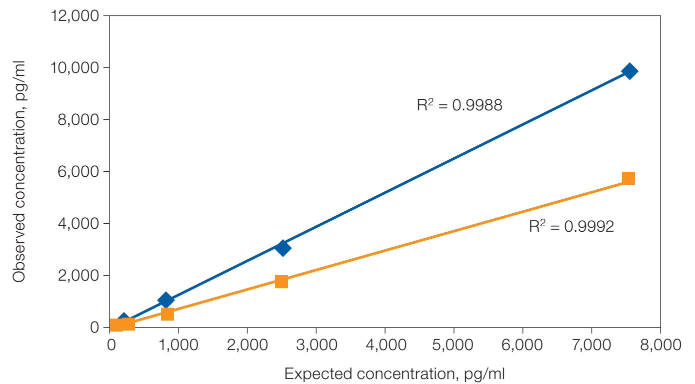
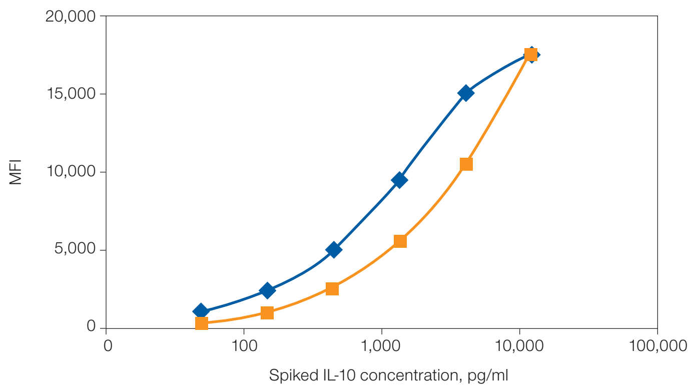
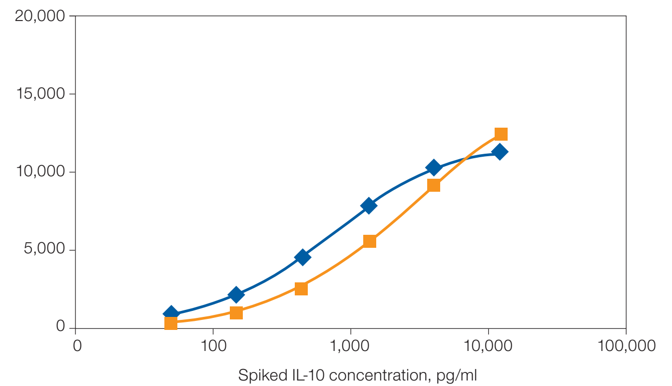


Fig. 2. Representative assay linearity of dilution plots in human serum and plasma using 6-point spike concentrations. A, IL-10 as an example of 27-plex group I panel; **B,** IL-1α as an example of group II 21-plex panel. (■), human serum matrix; (■), human plasma matrix.

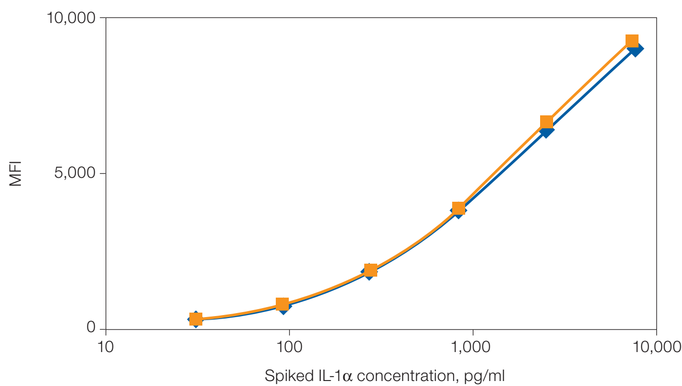
A. IL-10 — Human Serum Matrix



B. IL-10 — Human Plasma Matrix



C. IL-1α — Human Serum Matrix



D. IL-1α — Human Plasma Matrix

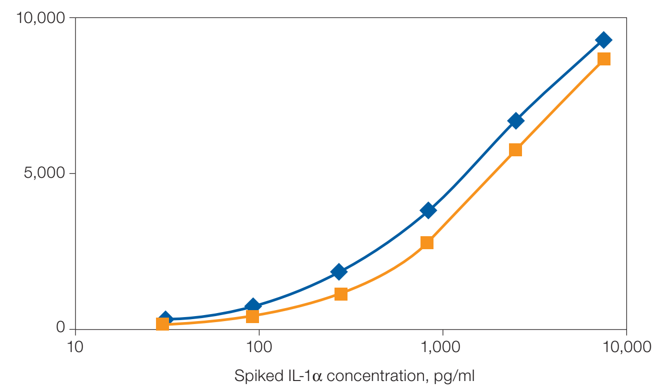
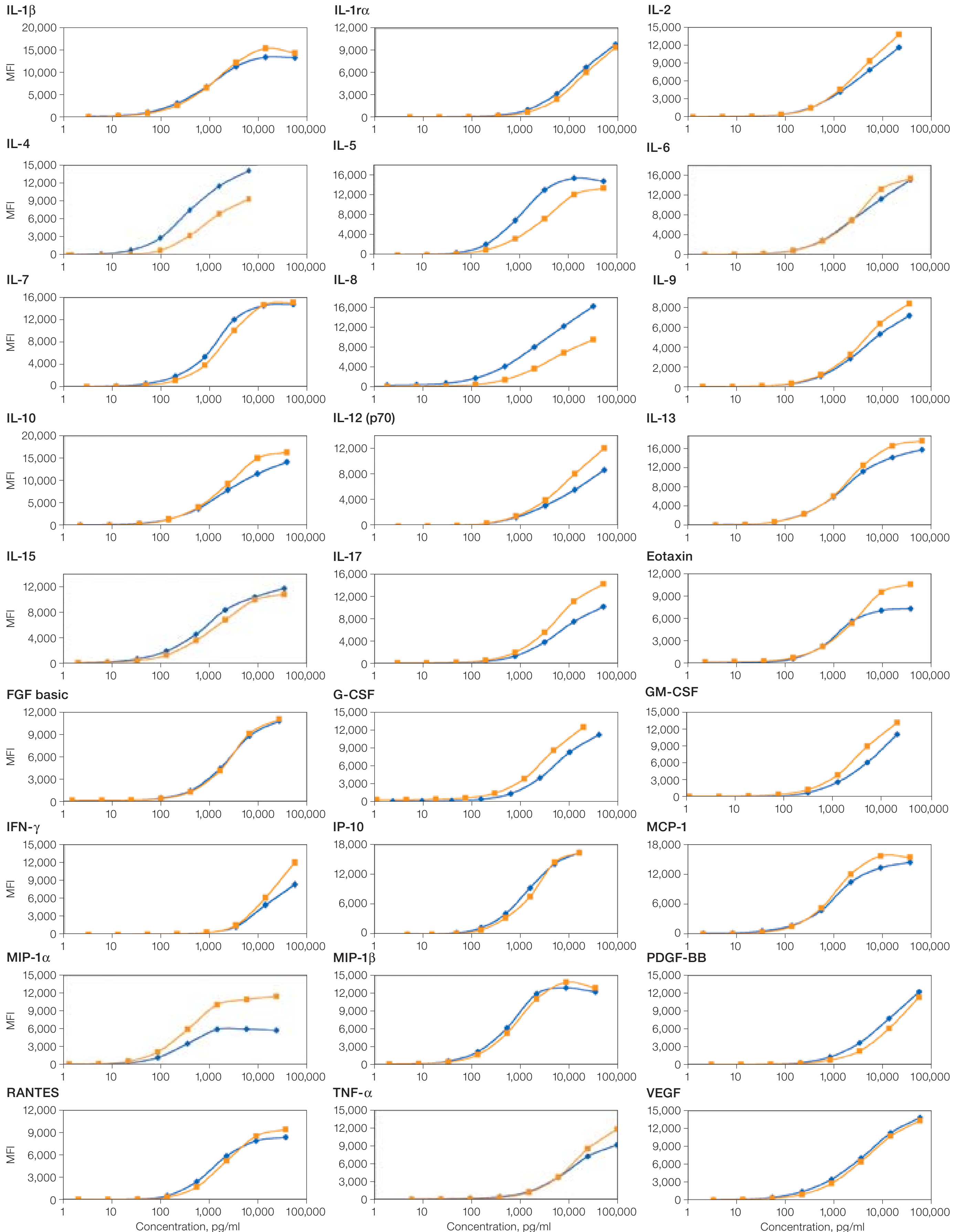


Fig. 3. Representative assay parallelism assessed by comparisons between concentration response curves in standard diluent (■) and in human serum or plasma samples (■). Parallelism analysis of IL-10 from the group I panel (A and B) and IL-1α from the group II panel (C and D), using 6-point concentration-response curves with 4PL curve fitting (4PL curve fitting is used when comparing slopes between two different curves). The curve slope difference between standard diluent and human serum is 3.2% for IL-10 (A) and 1.5% for IL-α (C). The curve slope difference between standard diluent and human plasma is 2.7% for IL-10 (B) and 12% for IL-1α (D).

Assay Standard Curves Comparison: Magnetic vs. Nonmagnetic Microsphere Bio-Plex Assays

Standard curves for the magnetic bead-based assays (Bio-Plex Pro human cytokine assay, 27-plex group I and 21-plex group II) were compared to the polystyrene bead-based assays. The 27 standard curve graphs of group I and 21 standard curve graphs of group II are displayed in Figures 4A and 4B. Standard curve comparisons showed that magnetic bead-based assays are mostly comparable or superior to the polystyrene bead-based assays.

© 2009 Bio-Rad Laboratories, Inc.



© 2009 Bio-Rad Laboratories, Inc.

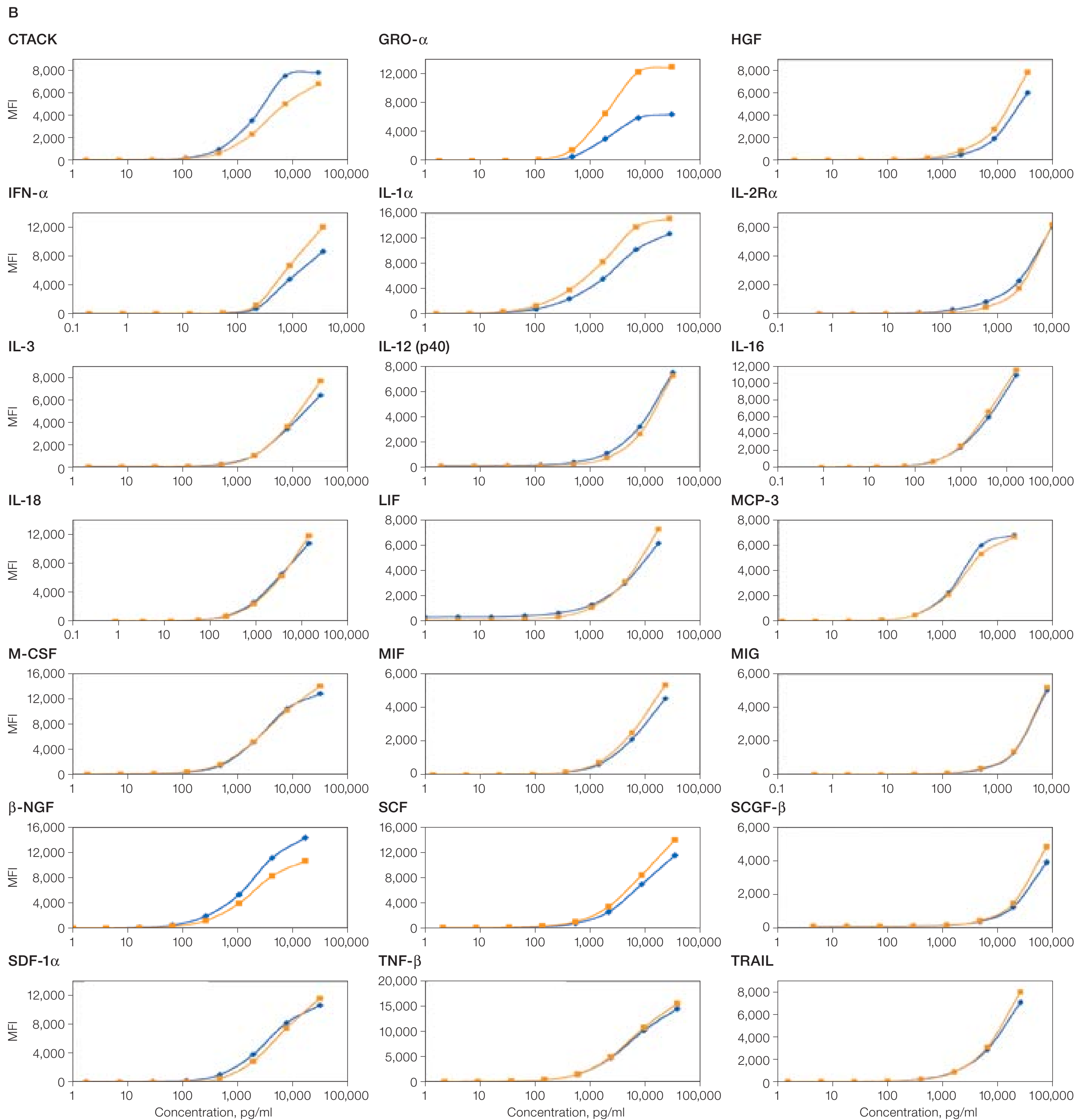


Fig. 4. Representative comparison of standard curves obtained for magnetic and polystyrene bead-based assays. A, Bio-Plex human cytokine 27-plex group I panel; **B**, human cytokine 21-plex group II panel. (■), Bio-Plex Pro assay (magnetic beads); (■), original Bio-Plex assay (polystyrene beads).

© 2009 Bio-Rad Laboratories, Inc.

Conclusions

This report describes the development and validation of two magnetic bead-based cytokine assay panels, Bio-Plex Pro human cytokine group I (27-plex) and group II (21-plex plus ICAM-1 and VCAM-1). These panels allow simultaneous measurement of multiple cytokine, chemokine, and growth factors in a single sample in serum, plasma, and cell culture medium matrices, thus significantly reducing the time and cost on screening of biological samples for these biomarkers. In addition, the implementation of magnetic bead-based assays allows for the automation of assay wash steps (using the Bio-Plex Pro wash station), which helps reduce inter-assay variations by eliminating the manual vacuum manifold washes. The assays have been shown to achieve a working range that is sufficiently broad for a wide variety of applications. The performance of these assays is mostly comparable or superior to the original Bio-Plex polystyrene bead-based assays.

The Bio-Plex suspension array system includes fluorescently labeled microspheres and instrumentation licensed to Bio-Rad Laboratories, Inc. by the Luminex Corporation.

MagPlex is a trademark of Luminex Corporation.

Information in the tech note was current as of the date of writing (2008) and not necessarily the date this version (rev A, 2009) was published.

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *www.bio-rad.com* ***USA*** *800 4BIORAD* ***Australia*** *61 02 9914 2800* ***Austria*** *01 877 89 01* ***Belgium*** *09 385 55 11* ***Brazil*** *55 21 3237 9400* ***Canada*** *905 364 3435* ***China*** *86 21 6426 0808* ***Czech Republic*** *420 241 430 532* ***Denmark*** *44 52 10 00* ***Finland*** *09 804 22 00* ***France*** *01 47 95 69 65* ***Germany*** *089 318 84 0* ***Greece*** *30 210 777 4396* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 1 455 8800* ***India*** *91 124 4029300* ***Israel*** *03 963 6050* ***Italy*** *39 02 216091* ***Japan*** *03 6361 7000* ***Korea*** *82 2 3473 4460* ***Mexico*** *52 555 488 7670* ***The Netherlands*** *0318 540666* ***New Zealand*** *0508 805 500* ***Norway*** *23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700* ***Russia*** *7 495 721 14 04* ***Singapore*** *65 6415 3188* ***South Africa*** *27 861 246 723* ***Spain*** *34 91 590 5200* ***Sweden*** *08 555 12700* ***Switzerland*** *061 717 95 55* ***Taiwan*** *886 2 2578 7189* ***United Kingdom*** *020 8328 2000*