Bio-Plex® suspension array system

tech note 5800

Development and Validation of Two Magnetic Bead-Based Mouse Cytokine Multiplex Assay Panels

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Introduction

Cytokines, chemokines, and growth factors play an important role in a wide range of physiological processes, including immune response, inflammation, and hematopoiesis. They have great utility as biomarkers of a spectrum of disease states, including cancer. This article describes the validation of two reconfigured Bio-Plex mouse cytokine assay panels, now available on magnetic beads: the Bio-Plex Pro™ mouse 23-plex group I panel and the Bio-Plex Pro mouse 9-plex group II panel. A pilot study using these assays to measure cytokine levels in a mouse model for breast cancer is shown to demonstrate the utility of these panels in experiments in which there is a desire to measure many markers but sample volume is limiting.

The Bio-Plex Pro mouse cytokine assay panels contain assays for 32 well characterized mouse cytokine, chemokine, and growth factors and offer excellent performance in diverse matrices such as serum, plasma, and tissue culture supernatants. 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 buffers 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.

In this article, comparisons of the standard curves between the new magnetic bead-based assays and the original nonmagnetic bead-based assays are shown. The curves are very similar for most targets. Measurements of the working assay range — the lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) — sensitivity, and examples of parallelism and assay linearity are also provided. 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 phases 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 suspension 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 3 wavelengths, two from the classification dyes and one from the reporter dye, as schematically represented in Figure 1.

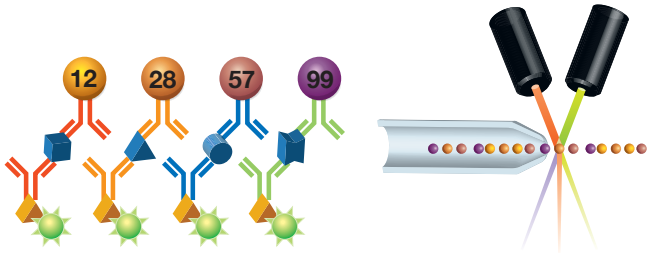


Fig. 1. Schematic representation of Bio-Plex immunoassay. Color-coded magnetic beads coupled to specific antibodies bind analytes. Bound analytes are detected using a biotinylated antibody and quantified using streptavidin coupled to a fluorophore (PE). Fluorescence of the beads and of PE are measured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

Application in a Pilot Study

In a pilot study, we used the Bio-Plex suspension array system to measure the levels of 32 biomarkers in plasma collected from transgenic (C3TAG) mice with breast tumors caused by overexpression of the SV40 large tumor antigen (T-antigen). Targeted expression of the T-antigen has been used previously to produce a breast cancer model in female mice by stimulating viral oncogenesis (Maroulakou et al. 1994).

The C3TAG transgenic model, in which all stages of cancer development are observed, is ideal for examining the association between tumor growth and plasma cytokine levels. Female C3TAG mice develop ductal epithelial atypia at 8 weeks, progress to intra-epithelial neoplasia at 12 weeks (resembling human ductal carcinoma in situ), and invasive carcinoma and grossly palpable tumors at 16 weeks (Maroulakou et al. 1994). These mice serve as an excellent model to examine different stages of breast cancer and to investigate candidate cancer biomarkers in humans.

In this study, we selected two different age groups of female transgenic mice: 1.5 months old, which are free of detectable tumors, and 6 months old, which have an extremely high tumor burden and metastases. We compared the cytokine levels in the plasma of 1.5 month-old and 6 month-old C3TAG transgenic mice to wild type control FVB/N mice that do not develop tumors. Results suggested an association between the presence of tumors and the expression of specific cytokines.

Methods

The Bio-Plex Pro mouse group I and II panels were analyzed in 23-plex and 9-plex, respectively. Standard curves were generated according to the Bio-Plex Pro cytokine instruction manual.

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 mean fluorescence intensity (MFI) value is two standard deviations above the background measured in the blank. The mean 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 three 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 were 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). Working assay 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. Mouse serum/plasma diluent was prepared from 4-fold dilution of pooled mouse 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 mouse serum/plasma diluent for a series of six dilution points. The highest concentration of spiked multiplexed mouse 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 matrix effect on the binding characteristics of an assay. This was investigated by comparing slopes of spiked standard concentration-response curves in mouse serum or plasma with those of standard concentration-response curves in standard diluent. The spiked standard concentration-response curve was prepared with six points of 3-fold serial dilutions of standards in mouse 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 mouse serum and plasma.

Determination of Assay Specificity (% Cross-Reactivity)

The group I and group II assay panels were tested on different plates in standard diluent with the recommended concentration of 23-plex or 9-plex capture beads and with the concentration of 23-plex or 9-plex standards at the second standard 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.

Mouse Model of Breast Cancer

The C3TAG transgenic mice overexpressing the SV40 virus large tumor antigen were used as a mouse model of breast cancer. The mice used in this study (Table 1) were bred in Dr Kalpna Gupta's laboratory from breeder pairs obtained from NCI.

Table 1. Age, weight, and tumor size of the mice used in this study.

Mouse	Age, months	Total Weight, g	Total Tumor Weight, g
FVB/N1	1.5	21.8	—
FVB/N2	1.5	20.7	—
FVB/N3	1.5	22.4	—
FVB/N4	1.5	19.9	—
FVB/N5	6	29.1	—
FVB/N6	6	33.1	—
FVB/N7	6	30.2	—
FVB/N8	6	30.9	—
C3TAG1	1.5	20.1	—
C3TAG2	1.5	18.9	—
C3TAG3	1.5	19.2	—
C3TAG4	1.5	18.8	—
C3TAG5	6	29.7	2.981
C3TAG6	6	28.7	1.893
C3TAG7	6	29.1	2.914
C3TAG8	6	32.4	4.282

Determination of Biomarker Profiles in Mouse Plasma Samples

Profiles of 32 cytokines from transgenic mice with tumors induced by T-antigen expression and from control mice were determined using the Bio-Plex Pro mouse 23-plex group I panel and the Bio-Plex Pro mouse 9-plex group II panel on a Bio-Plex suspension array system. Immunoassays were performed as described in the Bio-Plex Pro cytokine assay instruction manual. Assay standards were reconstituted in the standard diluent provided and an 8-point standard curve was generated using a 1:4 dilution series. The expected concentrations were assigned to the points using the normalized values provided with the kit. Samples were diluted 1:4 in the sample diluent provided. Both standards and samples were plated in duplicate. The vacuum-based wash method was used (the Bio-Plex Pro wash station was not yet available). All standards and samples were measured in duplicate and the MFI was used to calculate analyte concentrations using Bio-Plex Manager™ software.

Data Analysis

Bio-Plex Pro mouse cytokine assays data 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 mouse cytokine assays in 23-plex group I panel and 9-plex group II panel in serum-based matrix are summarized in Table 2. Similar results were obtained in RPMI cell culture media matrix (not shown). These experiments were performed using the traditional vacuum wash method. Precision is expected to be comparable or better when using magnetic separation as with the Bio-Plex Pro wash station (Bulletins 5629 and 5703).

Table 2. Representative assay working range*, sensitivity, and precision.** Values are mean data determined from five assay runs.

Target	Assay Range, pg/ml	Sensitivity, pg/ml	Intra-Assay, %CV	Inter-Assay, %CV
Mouse Group I: 23-plex				
IL-1α	1.84–21,093	0.2	3	29
IL-1β	10.36–60,631	9.4	4	7
IL-2	3.72–51,857	0.6	3	19
IL-3	1.55–21,632	0.2	2	15
IL-4	6.98–9,372	2.1	3	20
IL-5	3.57–13,315	0.3	4	14
IL-6	0.74–12,053	0.2	3	16
IL-9	6.89–28,208	12.5	4	9
IL-10	2.95–12,066	1.0	4	5
IL-12 (p40)	1.53–25,024	0.4	2	7
IL-12 (p70)	1.62–26,507	2.3	3	7
IL-13	47.2–57,011	38.7	4	5
IL-17	2.65–43,337	0.8	3	10
Eotaxin	257.9–4,636	147.4	4	5
G-CSF	5.1–84,244	0.6	3	12
GM-CSF	21.2–3,401	5.6	3	24
IFN-γ	1.84–30,164	1.2	4	6
KC	3.2–18,202	0.3	3	30
MCP-1	22.4–41,873	3.7	5	7
MIP-1α	256.2–15,565	36.3	3	11
MIP-1β	3.33–24,798	0.8	3	8
RANTES	2.78–8,759	0.6	4	4
TNF-α	5.8–59,626	1.4	3	6
Mouse Group II: 9-plex				
IL-15	5.7–37,501	6.6	6	3
IL-18	81.4–29,761	31.8	8	18
Basic FGF	4.8–35,500	2.2	8	8
LIF	3.5–57,366	0.6	4	12
M-CSF	1.5–24,221	0.4	4	14
MIG	183–46,393	3.4	5	21
MIP-2	32–8,574.2	0.4	5	13
PDGF-BB	10.4–36,939	4.3	5	10
VEGF	27.2–32,771	1.6	4	15

* Assay range is LLOQ and ULOQ calculated from five independent assays.

** Data were generated using vacuum manifold. %CV is expected to be lower with magnetic bead washer.

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 dilutions for that assay. Linearity was demonstrated in both serum and plasma matrices with $R^2 > 0.95$ for all the targets except MIP-1α (0.84 – 0.87). Figure 2 shows representative assay linearity of dilution plots for IFN-γ in serum and plasma.

Assay Parallelism

Parallelism was evaluated by comparing slopes of spiked standard concentration-response curves in mouse serum or plasma with the standard concentration-response curves in diluent. Figure 3 shows parallelism of the IL-6 assay between 8-point standard curve in standard diluent and a 6-point spiked standard concentration-response curve in serum and plasma, demonstrating robustness in these matrices. The slopes were parallel (<30% difference) for all the targets in serum and plasma tested, with the exception of MIP-1α (slope difference ~ 60%).

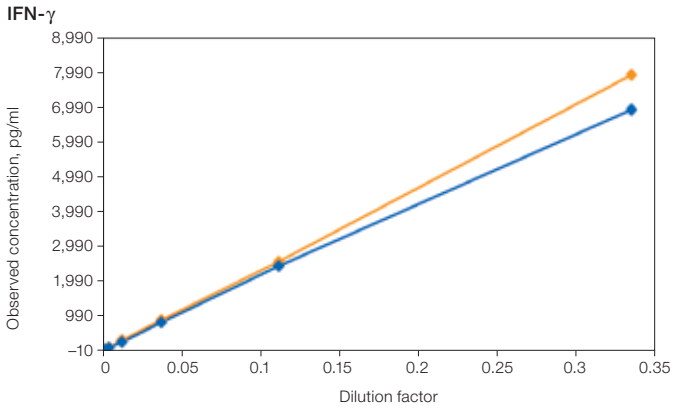


Fig. 2. Example of linearity of dilution in mouse serum and plasma using 6-point spike concentrations. (■), serum; (■), plasma.

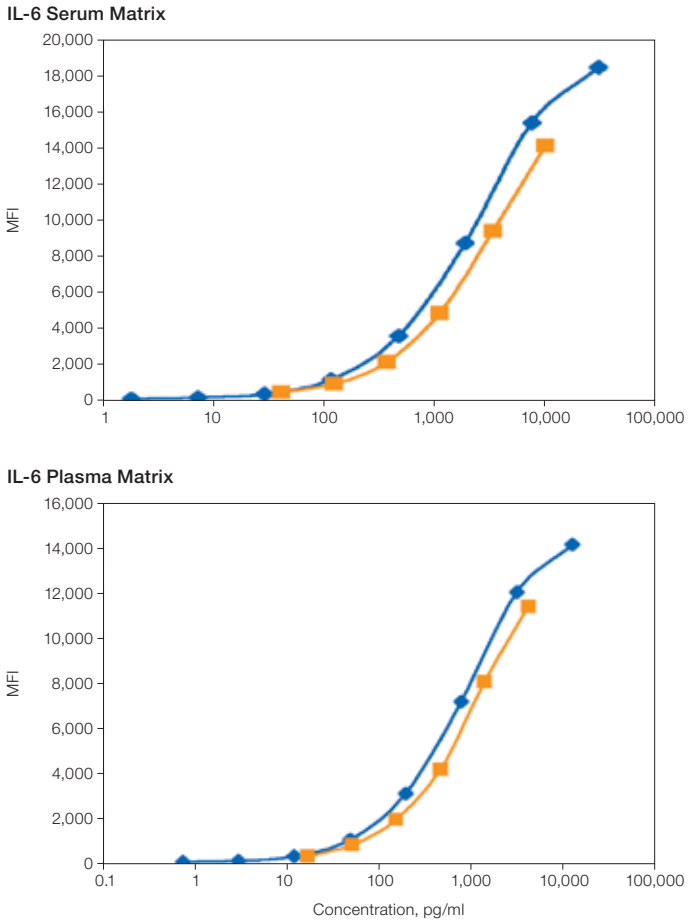


Fig. 3. Examples of parallelism in serum and plasma matrices. Comparison of an 8-point standard curve (■) and 6-point spiked standard concentration-response curve (■) with 4-PL curve fitting, in a serum matrix (top panel) and a plasma matrix (bottom panel).

Biomarker Levels in Samples From Transgenic Mice With Tumors

The Bio-Plex Pro mouse cytokine assay panels were used to measure the levels of the 32 cytokines, chemokines, and growth factors in plasma from transgenic mice. The 23-plex panel and the 9-plex panel were used to quantitate cytokines in the samples in two separate experiments and assays were performed as described in the instruction manual.

Figures 4A and 4B show plasma levels of the 32 cytokines, chemokines, and growth factors that were measured in transgenic (C3TAG) mice expressing the large T-antigen and control mice (FVB/N), at the age of 1.5 months (Figure 4A) and of 6 months (Figure 4B). At 1.5 months, which is earlier than the onset of detectable tumors in the transgenic mice, there is no significant difference in cytokine levels between transgenic and control mice. However, at 6 months, when tumors were detected in the transgenic mice, the levels of at least five cytokines were increased in the plasma of transgenic mice (n=4) as compared to the control FVB/N mice (n=4) that do not have the transgene: VEGF (30-fold), G-CSF (8-fold), IL-12 (p40) (4-fold), RANTES (3-fold), and KC (2-fold).

In Figure 4C, comparisons of cytokine levels in the plasma of 1.5 month old and 6 month old transgenic mice are shown. The tumor burden and metastases were highly advanced in the 6 month old mice (not shown). The plasma levels of seven analytes were elevated in the tumor-bearing 6 month old mice relative to the 1.5 month old tumor-free mice: VEGF (16-fold), G-CSF (7-fold), IL-2 (6-fold), IL-6 (6-fold), IL-12 (p40) (6-fold), RANTES (2-fold), and KC (3-fold). While the difference in levels is not visible for all analytes in the figure due to the scale of the Y axis, the measurements were all within the assay range indicated in Table 2.

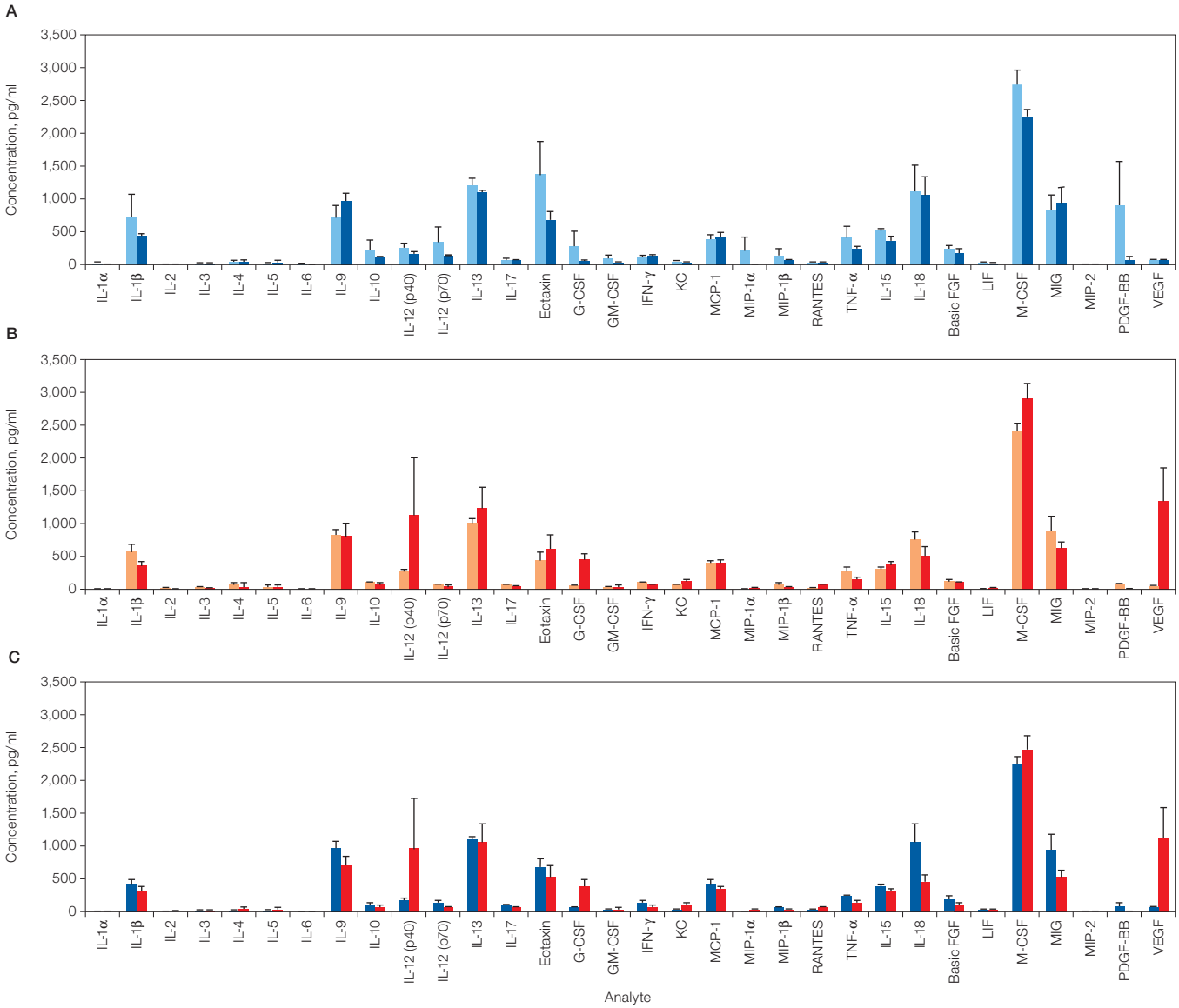
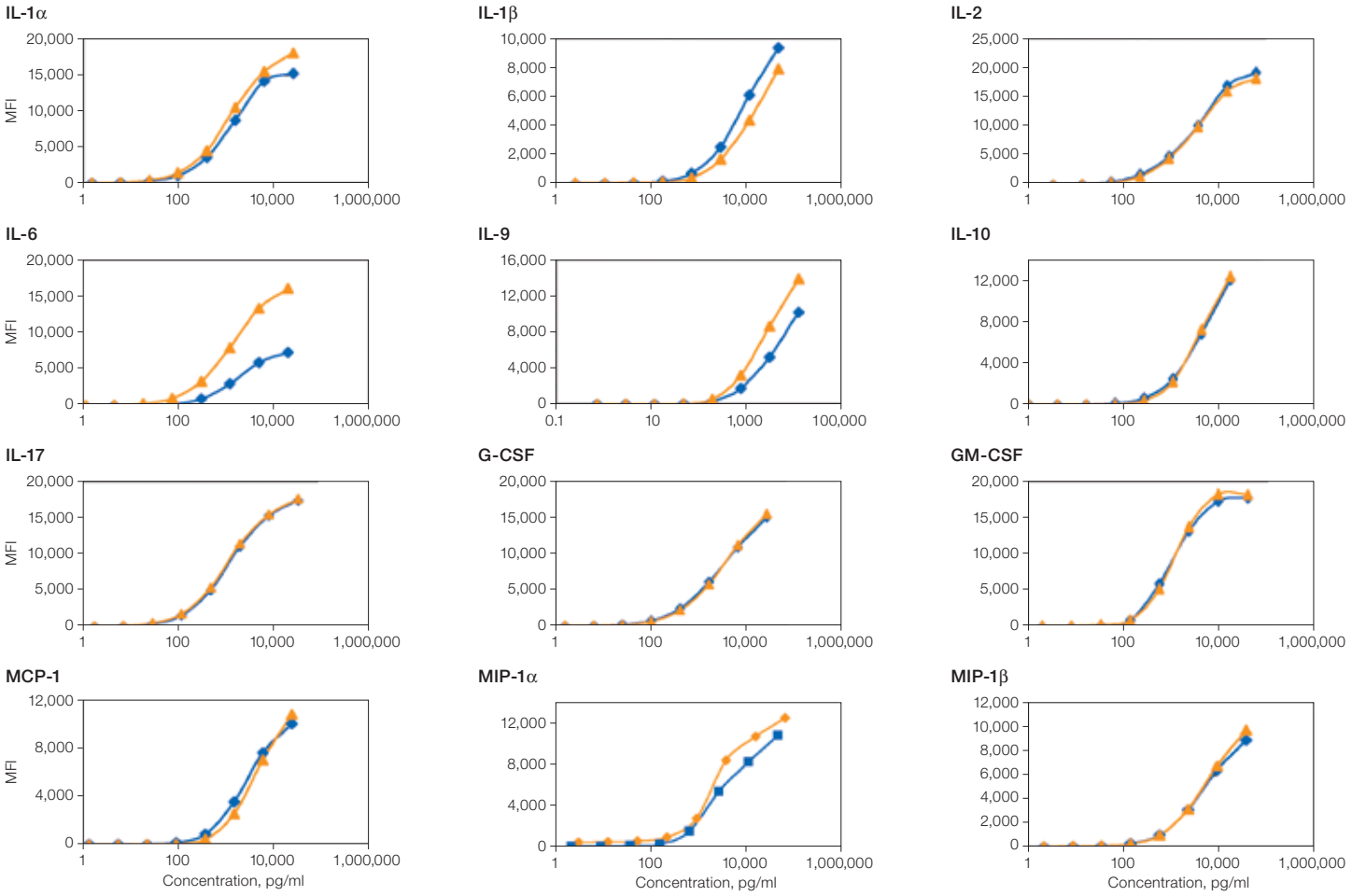


Fig. 4. Comparison of plasma cytokine levels in FVB/N (control) and C3TAG (transgenic) mice. A, 1.5 month old FVB/N ( ) vs. C3TAG mice ( ); **B**, 6.0 month old FVB/N ( ) vs. tumor-bearing C3TAG mice ( ); **C**, 1.5 month ( ) old vs. 6.0 month old ( ) C3TAG mice.

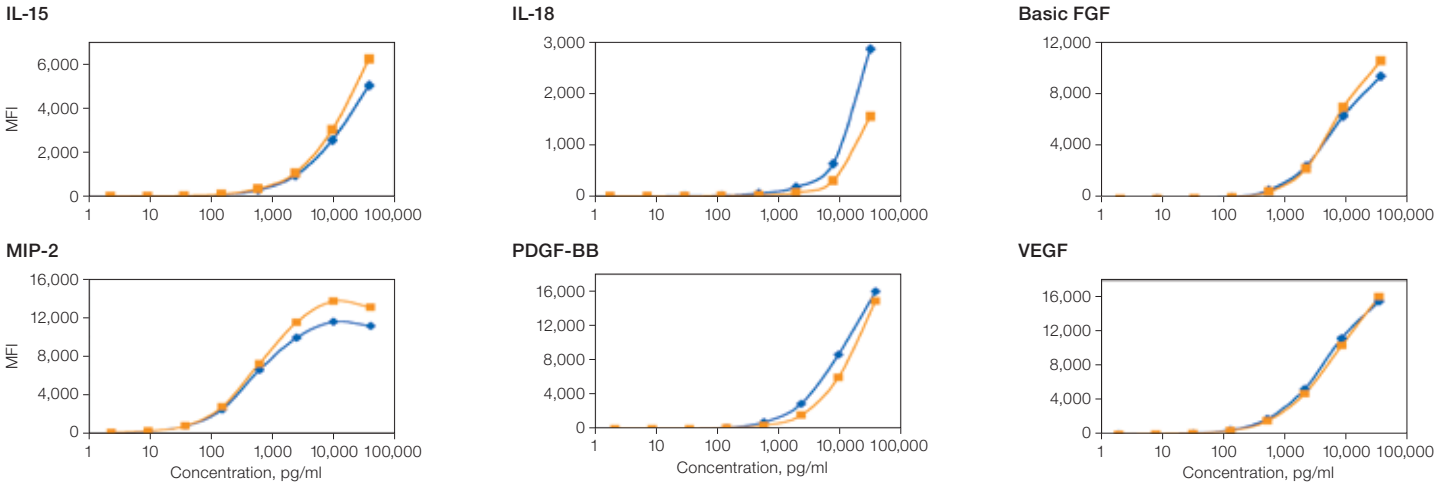
Comparison of Bio-Plex Pro Assay Standard Curves to the Nonmagnetic Assay Standard Curves

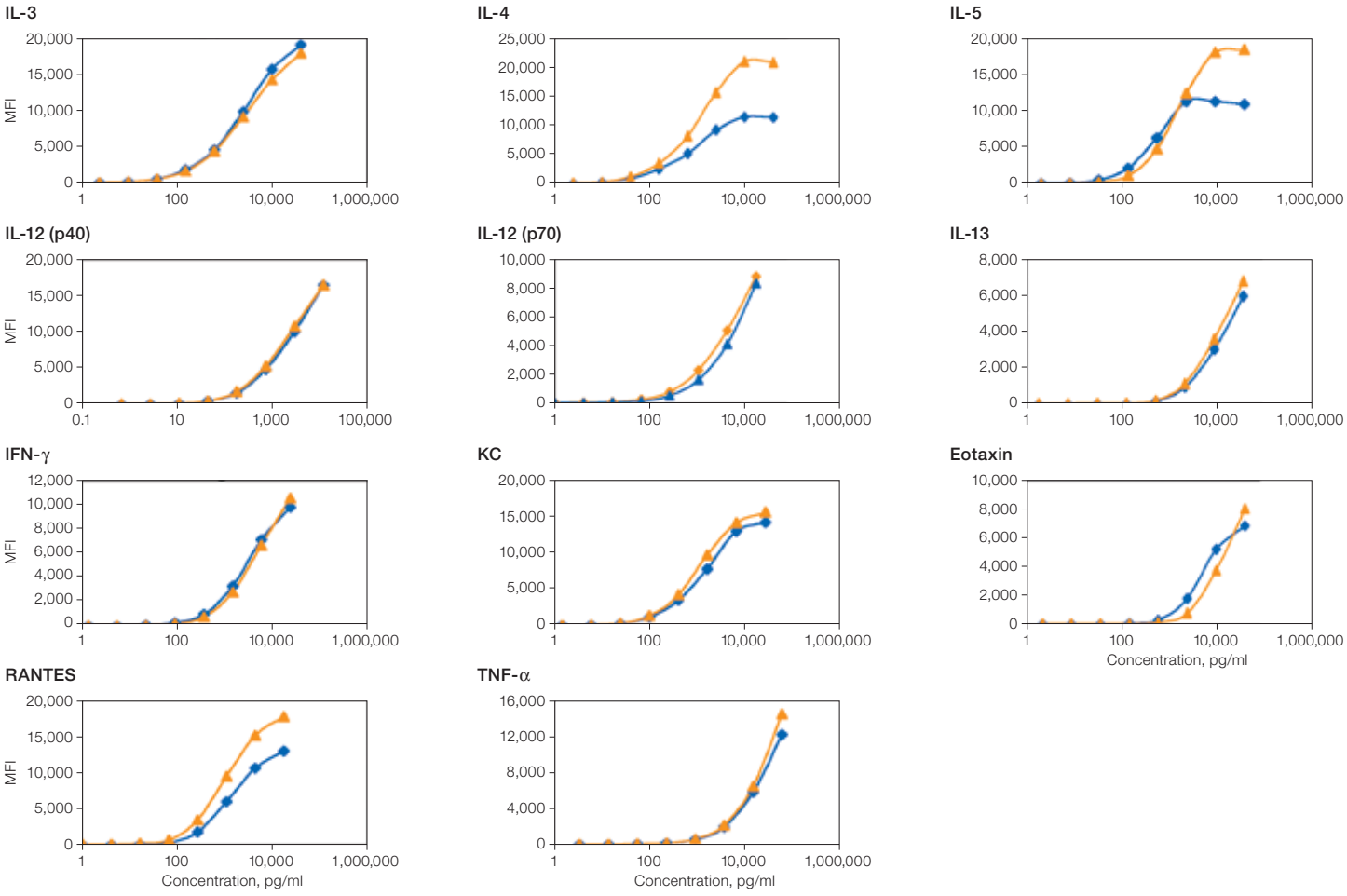
Standard curves for the Bio-Plex Pro magnetic assays were mostly comparable to standard curves obtained with the corresponding nonmagnetic bead-based assays (Figure 5). Some of the magnetic assays show improved assay range over the nonmagnetic assays.

Group I Targets



Group II Targets





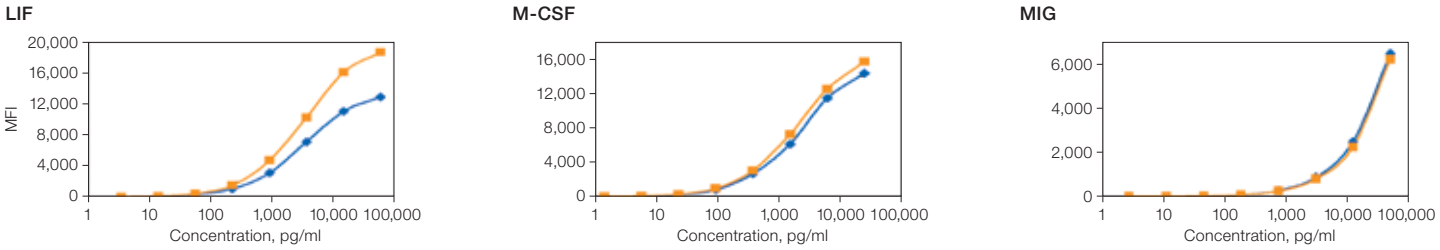


Fig. 5. Comparison of magnetic Bio-Plex Pro and nonmagnetic Bio-Plex assay standard curves for 32 mouse cytokines. The upper panel includes targets from group I. (▲) Bio-Plex Pro assay (magnetic beads), (◆) original Bio-Plex assay (polystyrene beads). The lower panel includes targets from group II. (■) Bio-Plex Pro assay (magnetic beads), (◆) original Bio-Plex assay (polystyrene beads).

Conclusion

This report describes the development and validation of two robust magnetic bead-based mouse cytokine, chemokine, and growth factor assay panels. The Bio-Plex Pro mouse cytokine 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 of screening on 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.

Preliminary analysis of the samples from transgenic mice with breast tumors showed elevation of multiple cytokines, including VEGF, IL-12 (p40), G-CSF, KC, and RANTES. The relevance of these cytokines in breast cancer is supported by the literature (Abbate et al. 1992, Chavey et al. 2007, Dehqanzada et al. 2007, Derin et al. 2007, Jin et al. 1997, Maroulakou et al. 1994, Potter et al. 2008, Premkumar et al. 2007, Youngs et al. 1997).

There is a strong association between advanced tumor growth and metastases and expression of KC, RANTES, VEGF, G-CSF, and KC (Vazquez-Martin et al. 2008). Increased circulating levels of KC have recently been linked to estrogen receptor inaction, endocrine resistance of HER-2 overexpressing breast carcinoma, increased cell invasion, and angiogenesis. These cytokines can thus serve as excellent biomarkers of cancer progression and may even be suggestive of response to therapy.

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