Bio-Plex® suspension array system

tech note 5654

Profiling of Human Angiogenesis Biomarkers in Sera of Cancer Patients Using the Bio-Plex® Suspension Array System

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Introduction

Angiogenesis is defined as the formation of new blood vessels from preexisting blood vessels. This process is considered natural during wound healing, pregnancy, and the female reproductive cycle. Angiogenesis becomes pathological during early tumor formation. Angiogenic transformation is a multistep process consisting of extracellular matrix remodeling, endothelial cell migration and proliferation, capillary differentiation, and abnormal production and interaction of various angiogenic factors. These processes subsequently lead to tumor vasculature and metastasis. A variety of angiogenic factors (soluble as well as membrane bound) involved in regulating these steps have been identified. The levels of these factors could be elevated or reduced in cancer patients. These angiogenic factors are potential candidate drug targets relevant for the development of cancer therapies. Development of cancer therapies often involves the measurement of levels of these angiogenic factors.

In this study, we evaluated the ability of the Bio-Plex suspension array system to detect the levels of nine biomarkers — angiopoietin-2 (Ang-2), VEGF, PDGF-BB, leptin, PECAM-1, IL-8, follistatin, HGF, and G-CSF in sera collected from four different types of cancer patients. These nine biomarkers were chosen for their potential clinical relevance to cancer development (Bach et al. 2007, Bauhofer et al. 2007, Grusch et al. 2006, Jackson et al. 2003, Schips et al. 2007, Shih et al. 2006, Somasundar et al. 2004, Vairaktaris et al. 2007, Wang et al. 2006, Yoshiji et al. 2005).

Methods

A magnetic bead-based technology was used to determine profiles of the nine angiogenic biomarkers. This technology utilizes an 8 µm magnetic bead coupled with two fluorophores. These classification dyes exhibit emission spectra at distinct wavelengths and absorption maxima at 635 nm. The reporter dye is a third fluorophore, phycoerythrin (PE), which shows emission at a third distinct wavelength and maximum absorption at 532 nm. PE was chosen for its high molar extinction coefficient, quantum yield, resistance to photobleaching, minimal self-quenching, and excellent stability. The detector unit consists of a flow cell that enables magnetic beads to travel in a single file (laminar flow) through a region illuminated by a pair of lasers. The beads emit light at three wavelengths, two from the classification dyes and one from the reporter dye.

Profiles of the nine angiogenic biomarkers collected from sera of four types of cancer patients, colorectal cancer (n = 10), breast, lung, and prostate cancer (n = 15), were evaluated using a magnetic bead and flow-based assay on a Bio-Plex suspension array system. This platform integrates a series of color-coded beads, each of which is coupled to a unique antibody specific for a biochemical marker. These capture antibody-coupled beads serve as solid phases for the capture of desired serum or plasma proteins, using a standard sandwich-based detection format, as schematically represented in Figure 1. The levels of biomarkers in cancer patients were compared to those in control sera, collected from healthy individuals (n = 117).

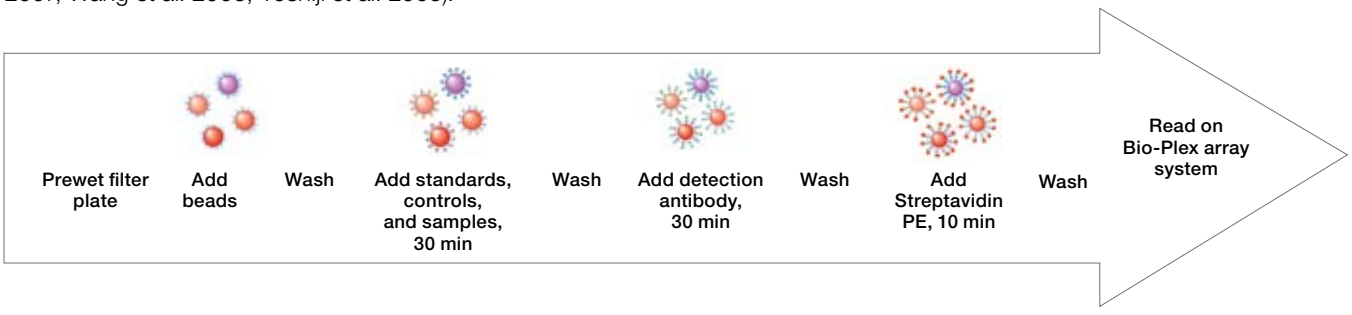

Fig. 1. A schematic representation of a multiplex bead-based sandwich immunoassay for the experimental workflow.

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Results

Assay Specificity

To study assay specificity, the nine recombinant antigens were independently evaluated with multiplexed capture and detection antibodies. This format determines the overall target specificity that is conferred by the capture antibody. Results are summarized in Table 1. Data showed <1% cross-reactivity for 8 of the 9 targets. The antibody pair for Ang-2 exhibited <3.4% cross-reactivity with IL-8, VEGF, PDGF-BB, and HGF antigens. Percentages of cross-reactivity were calculated using median fluorescence intensities (MFIs) of the multiplexed capture beads and detection antibodies in the presence of a single antigen.

Assay Sensitivity and Limit of Detection

In a study designed to evaluate assay sensitivity and limits of detection, the standard curve for each target was tested in duplicate from eight independent assays for serum matrix and from five independent assays for RPMI matrix. The limit of detection (LOD) is defined as the concentration obtained at background MFI for the standard curve. Figure 2 depicts LOD values for both serum and RPMI matrices. Table 2 shows the range of concentrations in which the assay is both precise (intra-assay ≤15% CV and inter-assay ≤25% CV) and accurate (70–130% standard and spike recovery). These parameters define the upper and lower limits of quantitation for each target. The standard curves for each target are shown in Figure 3.

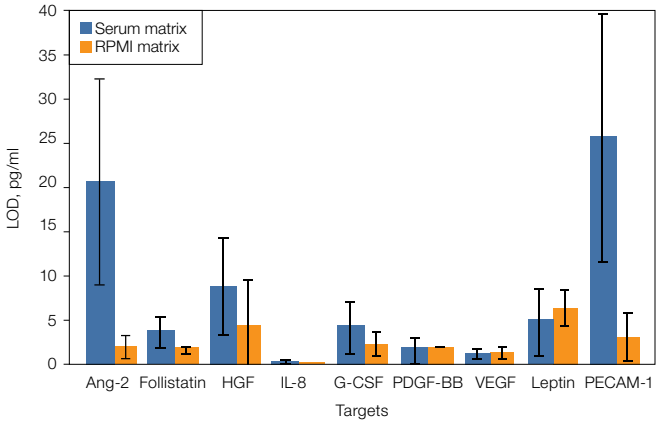

Fig. 2. Assay sensitivity of targets on serum and RPMI matrices.

Precision of Measurements

Values were determined from the variance of MFI of replicate wells for eight standard points. The intra-assay precision was measured from five replicate wells in one assay and the inter-assay precision was measured from duplicate wells in five independent assays. All targets recorded ≤15% CV.

Table 1. Percentage of cross-reactivity for different angiogenic factors.

Target	Ang-2	Follistatin	HGF	IL-8	G-CSF	PDGF-BB	VEGF	Leptin	PECAM-1
Ang-2	110.9	0.1	0.2	0.8	0.1	0.0	0.0	0.1	0.2
Follistatin	1.5	112.5	0.2	0.7	0.1	0.0	0.0	0.2	0.2
HGF	3.4	0.4	104.5	0.9	0.1	0.1	0.1	0.3	0.2
IL-8	2.4	0.2	0.2	98.8	0.0	0.0	0.0	0.5	0.3
G-CSF	0.0	0.1	0.1	0.7	107.5	0.0	0.0	0.0	0.1
PDGF-BB	3.3	0.3	1.1	0.9	0.2	106.1	0.3	1.4	0.9
VEGF	1.2	0.2	0.3	0.8	0.1	0.0	106.5	0.2	0.2
Leptin	0.3	0.0	0.0	0.7	0.0	0.0	0.0	116.4	0.0
PECAM-1	0.2	0.1	0.0	0.7	0.0	0.0	0.0	0.0	117.0

Table 2. Assay range, as determined from the mean of five independent assays.

	Assay Range, pg/ml	
Targets	LLOQ*	ULOQ**
Ang-2	59	59,784
Follistatin	25	25,584
HGF	20	20,201
IL-8	1	7,475
G-CSF	11	25,665
PDGF-BB	3	24,423
VEGF	1	16,906
Leptin	8	36,256
PECAM-1	45	32,691

* Lower limit of quantitation.
** Upper limit of quantitation.

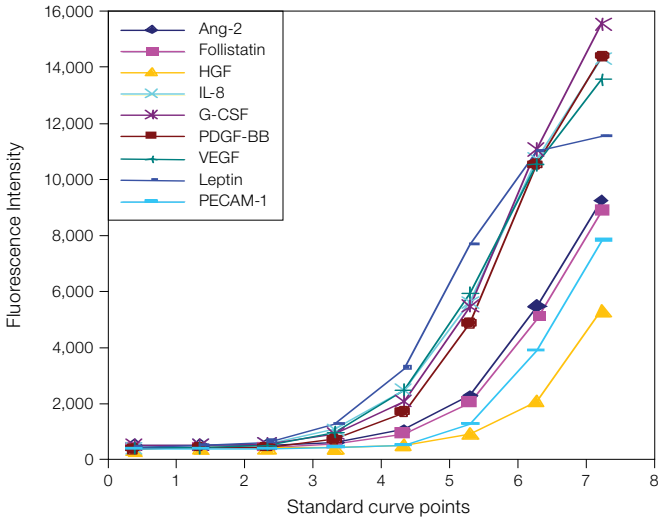

Fig. 3. Plot of 8-point standard curves for each target.

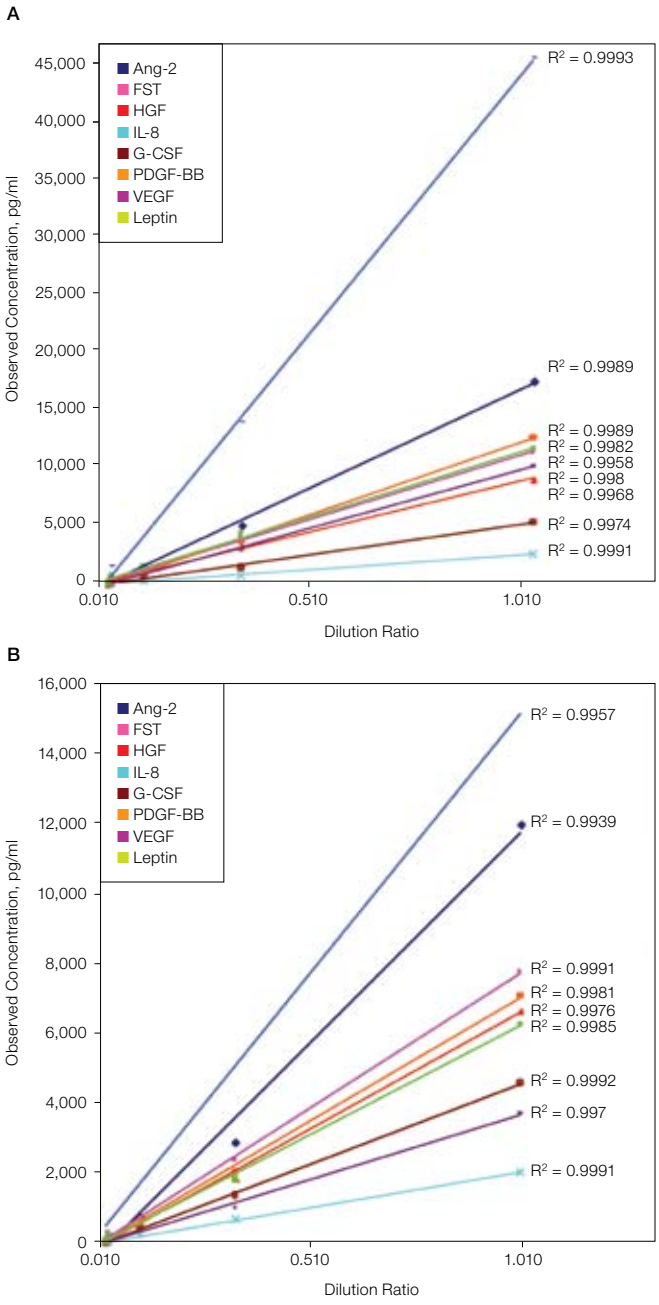

Fig. 4. Linearity of dilution in different targets. A, concentration of samples measured from serum matrix; **B**, concentration of samples measured from a plasma matrix.

Dilutional Linearity and Parallelism

Dilutional linearity ensures analyte present in concentrations above the ULOQ can be diluted linearly and measured accurately within the assay range. Linearity was demonstrated in both serum and plasma samples with $R^2 > 0.99$ for all nine targets. Figure 4 shows one representative serum and plasma sample tested by means of spiked sampling.

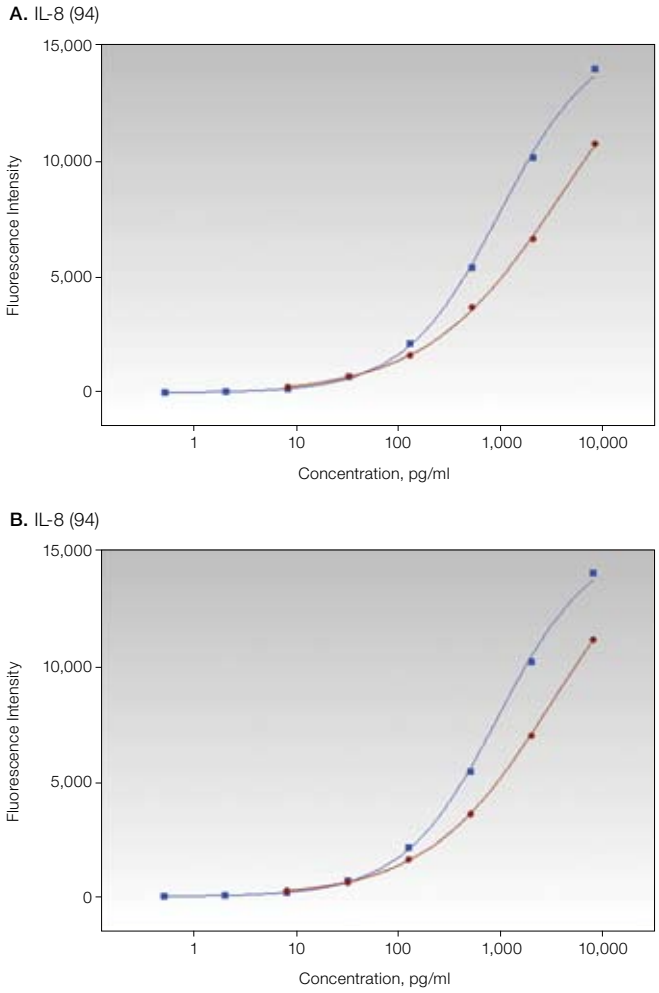

Fig. 5. Examples of parallelism in serum and plasma matrices using an 8-point standard (■) and 6-point spiked serum or plasma sample (●) dilutions with 4-PL curve fitting.

Parallelism ensures that the standards and samples have the same binding characteristics. This is demonstrated by comparing a spiked sample dilution-response curve with the standard concentration-response curve. Figure 5 shows parallelism between an 8-point standard and a 6-point spiked dilution using a representative serum or plasma sample with 4-PL curve fitting. With the exception of PECAM-1, the slope difference was <20% for the remaining 8 targets in the serum and plasma samples tested.

Physiological Levels of Angiogenesis Biomarkers

The physiological levels of these markers were determined in 117 serum samples collected from apparently healthy donors. The mean and median values were compared to serum levels of samples collected from four cancer types. Seven of the nine targets showed marked elevation across all four cancer types. In contrast, the levels of leptin and PECAM-1 were reduced (Figure 6).

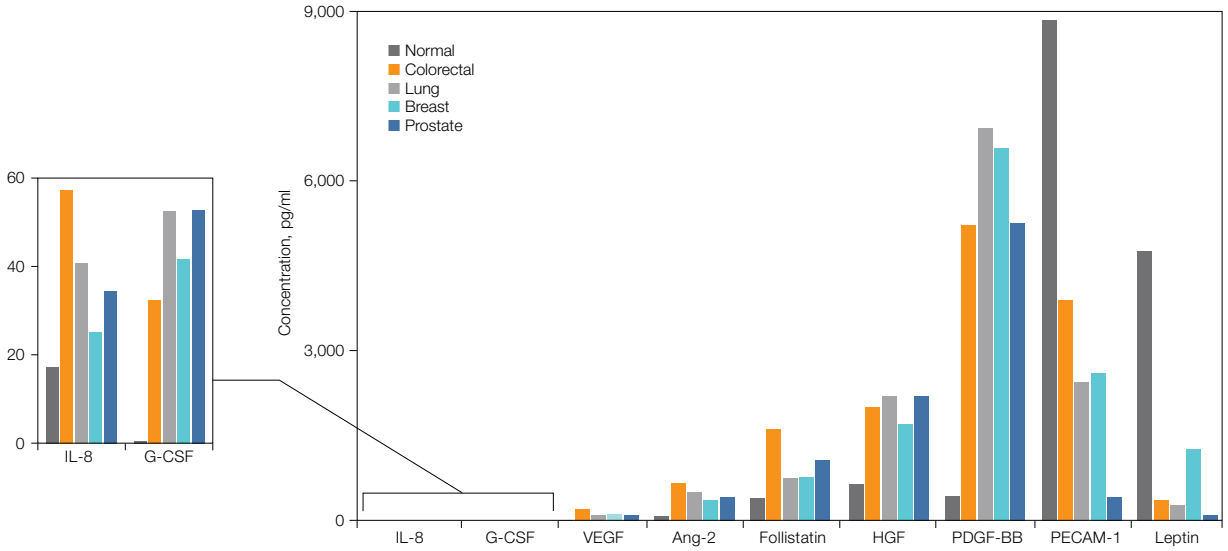

Fig. 6. Changes in biomarker levels in sera of different cancer groups. Concentration levels represent median values.

Conclusion

Initial screenings of cancer biomarkers are both costly and labor-intensive. In many cases, studies generated from different test formats often yield conflicting, and therefore, inconclusive results. The Bio-Plex system is capable of measuring the levels of multiple targets simultaneously in less than 2 hours, using as little as 12.5 µl of serum, plasma, and other matrices. This feature significantly reduces the time and cost spent on preliminary screening of serum samples for biomarker profiling. Overall, the assays are specific, sensitive, and reproducible. The system also provides a broad range for measurements, which can be utilized depending on specific sensitivity requirements.

Preliminary analysis of different cancer samples using the Bio-Plex suspension array system showed marked elevation of 7 of the 9 biomarkers studied, suggesting the importance of these markers as key regulators of angiogenesis. The reduction of the levels of leptin and PECAM-1 also provide interesting insight into the modulation of these markers in the associated cancers.

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