Bio-Plex® suspension array system

tech note 6156

Composite Profiling of Angiogenic Factors Using Bio-Plex Pro™ Human Cancer Biomarker Panel 1

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Introduction

Angiogenesis is a fundamental process required for multiple physiological and pathological events. It is also a hallmark of over 50 different disease states, including cancer, rheumatoid arthritis, cardiovascular diseases, diabetes, and psoriasis (Staton et al. 2004, 2009). Methods developed to study these diseases are important tools for testing potential therapeutics. These methods include both in vivo and in vitro assays. In vivo assays are considered the most informative because of the complex nature of vascular responses to test reagents. However, these assays are often costly and laborious. In contrast, in vitro assays can be carried out expeditiously, are less expensive, and are easier to interpret. Often, these in vitro assays provide maximum benefits when developed as multivariate index assays where the data of multiple assays yield a composite profile of clinically relevant protein biomarkers.

Using Luminex xMAP technology (Dale et al. 2008 and bulletin 5404), we have developed a multiplex Bio-Plex Pro human cancer biomarker panel that employs a magnetic bead-based workflow to measure angiogenesis biomarkers in diverse matrices including serum, plasma, cell culture supernatant, and many other sample types. In combination with the Bio-Plex® suspension array and the Bio-Plex Pro wash station, the multiplexing feature makes it possible to quantify multiple angiogenesis targets in a single well of a 96-well microplate in just three hours, using as little as 12.5 µl of serum or plasma.

The panel of 16 markers comprises sEGFR, FGF-basic, follistatin, G-CSF, HGF, sHER2/neu, sIL-6Rα, leptin, osteopontin, PECAM-1, PDGF-AB/BB, prolactin, SCF, sTIE-2, sVEGFR-1, and sVEGFR-2 (Table 1). These markers were selected because of their direct relevance to tumor-associated angiogenesis (Bridges et al. 2011). However, the panel is relevant to other disease-related processes such as metastasis, cell proliferation, cell adhesion, apoptosis, and inflammation.

Table 1. Bio-Plex Pro human cancer biomarker panel 1 assay menu.

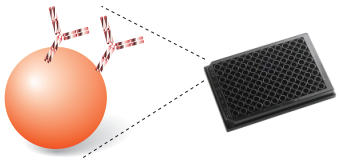
	16-Plex	Bead Regions
sEGFR	•	15
FGF-basic	•	44
Follistatin	•	26
G-CSF	•	57
HGF	•	62
sHER2/neu	•	12
sIL-6Rα	•	19
Leptin	•	78
Osteopontin	•	77
PECAM-1	•	46
PDGF-AB/BB	•	47
Prolactin	•	52
SCF	•	65
sTIE-2	•	64
sVEGFR-1	•	76
sVEGFR-2	•	45

The assays were validated on human serum, plasma, and cell culture matrices. Validation criteria include working assay range (LLOQ/ULOQ), sensitivity (LOD), intra- and inter-assay precision, specificity and cross-reactivity, linearity of dilution, and parallelism to evaluate robustness in the key sample matrices mentioned above.

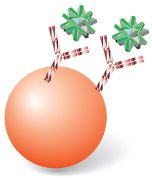
Method

The principle of these 96-well plate-formatted bead-based assays is similar to a capture sandwich immunoassay (Figure 1). The capture antibody–coupled beads were first incubated with antigen standards or samples followed by incubation with biotinylated detection antibodies. After washing away the unbound biotinylated antibodies, the beads were incubated with a reporter streptavidin-phycoerythrin (SA-PE) conjugate. Following removal of excess SA-PE, the beads were passed through the Bio-Plex array reader (Bio-Plex 200 system), which measures the fluorescence of the bead and of the bound SA-PE. Assay incubations were performed at room temperature according to the settings shown in Table 2. All washes were performed using a Bio-Plex Pro wash station. Data acquisition was performed using Bio-Plex Manager™ 6.0 at a low PMT setting.

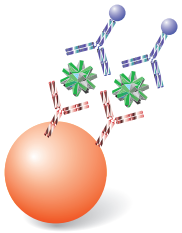
Step One:
Dispense capture beads
Wash plate 2 times



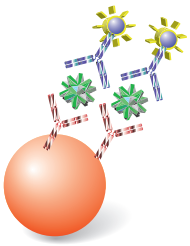
Step Two:
Add samples
Incubate
Wash plate 3 times



Step Three:
Add biotinylated detection antibody
Incubate
Wash plate 3 times



Step Four:
Add streptavidin-PE reporter dye
Incubate
Wash plate 3 times



Step Five:
Resuspend beads
Perform fluorescent sorting
Analyze data

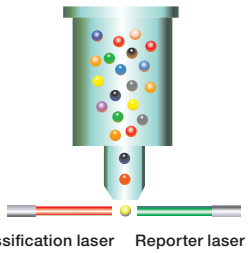


Fig. 1. Schematic representation of a sandwich-based Bio-Plex assay workflow.

Table 2. Bio-Plex Pro human cancer biomarker panel 1 assay settings.

Step	Parameters
2 (Samples + Capture Beads)	60 min incubation
3 (Detection Antibody)	30 min incubation
4 (SA-PE)	10 min incubation
5 (Data Acquisition)	PMT setting: Low RP1
	Shaker speed: 850 ± 50 rpm

Assay Performance Characteristics

The angiogenesis assays were evaluated according to the following parameters: assay sensitivity, precision, accuracy, assay working range, cross-reactivity, matrix effects, and validation with biological samples. Assay sensitivity, defined as limit of detection (LOD), was evaluated by adding two standard deviations to the mean median fluorescence intensity (MFI) of ten zero standard replicates. With the exception of osteopontin and PECAM-1, all the targets reported concentrations below 10 pg/ml in both serum-based standard diluent and RPMI cell culture media (Table 3). Osteopontin recorded 56.4 pg/ml in standard diluent. PECAM-1 recorded 24.4 pg/ml and 39.6 pg/ml in standard diluent and RPMI, respectively.

Assay reproducibility evaluates both intra-assay %CV and inter-assay %CV. Intra-assay %CV assesses variation among the replicates within the assay. Inter-assay %CV measures the variability across three independent assays. Intra-assay %CV was calculated from the median fluorescent intensity (MFI) of all three replicates at each standard antigen dilution point from a representative assay in serum-based standard diluent and RPMI matrices. All the targets demonstrated <10 %CV in both serum and RPMI matrices. Inter-assay %CV for multiplex assays was determined from three independent plates using the observed concentration of a 6-level spike control and the observed concentration of within assay range standard points. All the targets demonstrated <15 %CV in both serum and RPMI matrices (Table 4).

Table 3. Limit of detection (pg/ml) in serum and RPMI tissue culture media.

	Analyte	
	Standard diluent	RPMI
sEGFR	3.5	8.2
FGF-basic	5.1	2.2
Follistatin	1.5	2.0
G-CSF	1.1	1.0
HGF	1.8	1.0
sHER2/neu	0.5	0.5
sIL-6Rα	0.5	0.2
Leptin	1.1	3.3
Osteopontin	56.4	10.5
PECAM-1	24.4	39.6
PDGF-AB/BB	0.7	4.6
Prolactin	7.0	1.7
SCF	0.1	0.2
sTIE-2	6.6	4.0
sVEGFR-1	0.8	7.1
sVEGFR-2	7.4	4.3

The LOD values from each matrix represent means of 4 independent assays.

Table 4. Precision profile — intra- and inter-assay %CV.

	Intra-Assay %CV			Inter-Assay %CV			
	Multiplex	Singleplex	Multiplex	Mean Spike Control %CV		Mean Standard Curve %CV	
Analyte	Standard Diluent	Standard Diluent	RPMI	Standard Diluent	RPMI	Standard Diluent	RPMI
sEGFR	3.6	3.8	4.5	3.8	21.8	4.9	4.2
FGF-basic	2.9	3.2	3.7	4.7	3.8	6.8	3.2
Follistatin	3.1	3.2	2.8	3.6	3.5	3.3	2.8
G-CSF	3.7	5.5	5.1	7.7	7.8	3.5	3.1
HGF	3.2	3.2	3.0	6.1	7.6	4.6	1.4
sHER2/neu	3.2	6.1	3.3	3.9	1.9	2.9	1.8
sIL-6Rα	3.1	2.5	2.9	5.3	2.1	5.1	1.4
Leptin	2.8	3.8	4.9	9.6	13.9	5.8	2.9
Osteopontin	4.0	3.9	3.5	7.8	8.2	3.2	3.7
PECAM-1	4.2	4.0	3.3	5.9	6.6	4.2	1.9
PDGF-AB/BB	3.2	2.4	2.7	7.9	5.0	4.9	3.1
Prolactin	3.0	3.2	3.7	6.8	8.0	9.3	4.3
SCF	2.9	3.2	3.5	3.6	9.2	4.0	1.7
sTIE-2	3.0	4.6	3.4	5.8	10.7	4.8	5.0
sVEGFR-1	2.9	2.8	4.2	3.3	12.8	6.4	10.8
sVEGFR-2	3.1	2.1	2.8	3.5	11.7	2.9	2.6

Notes: The mean intra-assay %CV values were derived from 16 multiplex and singleplex assays in a serum matrix and RPMI cell culture medium, and from 16 multiplex assays in RPMI cell culture medium. For inter-assay %CV (derived in multiplex format), the mean spike control %CV was derived from 6 serially diluted concentration levels. The mean standard curve %CV was derived from working assay range standard points.

Assay accuracy (also defined as recovery) was calculated as the percentage of the observed concentration value of a target antigen relative to the expected value. This parameter was evaluated using standard points and spiked controls in both multiplex and singleplex configurations, and in both serum and RPMI. Overall, the standard recovery was comparable in both matrices, with most targets recovering 80–120% within assay working ranges (Table 5). The recovery in both single- and multiplex settings was also comparable (data not shown).

Assay working range is defined as the range between the lowest level of quantification (LLOQ) and the upper level of quantification (ULOQ) in which an assay is both precise and accurate. The ranges of these assays were determined for both serum and RPMI cell culture medium. Table 6 lists the assay ranges in multiplex and singleplex assays in a serum matrix and RPMI cell culture medium supplemented with 10% FBS. The reproducibility of these ranges is dictated by the overall precision in preparing the assay reagents.

Table 5. Standard curve and spike recovery.

	sEGFR	FGF-basic	Follistatin	G-CSF	HGF	sHER2/neu	sIL-6Rα	Leptin	Osteopontin	PECAM-1	PDGF-AB/BB	Prolactin	SCF	sTIE-2	sVEGFR-1	sVEGFR-2
Serum	Standard Curve Recovery %															
S1	90	67	101	105	100	97	99	90	65	99	86	101	95	98	100	92
S2	107	146	98	98	100	104	102	115	146	101	113	99	106	103	101	110
S3	98	95	102	101	100	99	97	93	95	99	96	101	97	98	100	97
S4	101	98	99	100	101	98	103	103	98	101	99	99	100	100	100	99
S5	100	103	99	100	99	102	100	99	103	101	105	101	102	101	101	102
S6	100	100	101	100	99	102	99	101	101	97	98	98	99	100	98	102
S7	101	94	99	101	103	96	101	100	92	109	98	104	100	98	102	95
S8	99	118	101	99	98	103	99	100	120	80	101	97	100	102	99	103
	Spike Recovery %															
Spike 1	103	100	100	101	100	101	101	102	101	101	101	100	101	100	100	101
Spike 2	97	100	98	98	99	98	97	97	97	98	98	99	98	98	99	99
Spike 3	104	100	103	105	102	103	106	103	103	103	104	101	103	102	103	102
Spike 4	97	100	97	95	98	98	96	99	99	97	97	99	98	98	97	98
Spike 5	103	100	102	105	101	101	102	100	101	102	102	100	101	101	102	101
Spike 6	99	100	99	98	100	100	99	100	100	99	100	100	100	100	100	100

Note: RPMI data are not shown.

Table 6. Assay working ranges.

	Serum Matrix				RPMI	
	Multiplex		Singleplex		Multiplex	
Analyte	LLOQ	ULOQ	LLOQ	ULOQ	LLOQ	ULOQ
sEGFR	11.9	195,000.0	11.9	195,000.0	11.9	192,950.5
FGF-basic	10.2	4,500.0	17.6	13,279.0	13.2	17,745.3
Follistatin	4.6	42,750.0	2.6	42,650.0	5.2	35,674.3
G-CSF	1.1	18,000.0	1.1	18,000.0	3.3	18,754.0
HGF	4.5	42,124.0	2.6	42,124.0	2.6	37,301.3
sHER2/neu	1.4	23,500.0	1.4	23,500.0	1.4	25,034.8
sIL-6Rα	1.4	12,985.0	2.6	12,985.0	0.8	74,738.8
Leptin	7.3	119,429.0	7.3	119,429.0	7.3	95,693.8
Osteopontin	72.5	216,000.0	52.7	229,036.0	26.4	32,503.3
PECAM-1	51.5	135,000.0	131.8	135,000.0	33.0	141,887.8
PDGF-AB/BB	2.1	35,000.0	7.3	31,296.0	8.5	58,892.3
Prolactin	12.2	200,000.0	12.2	200,000.0	73.2	157,490.0
SCF	2.0	32,000.0	2.0	32,000.0	2.0	70,046.0
sTIE-2	12.2	200,000.0	2.2	200,000.0	24.4	156,428.8
sVEGFR-1	4.3	35,324.0	2.2	35,324.0	51.7	67,794.5
sVEGFR-2	12.1	197,681.0	12.1	197,681.0	12.1	156,607.5

Assay specificity was examined by performing single-antigen and single-detection cross-reactivity studies. The single-antigen study evaluates the specificity of a capture antibody. This was conducted by testing an individual antigen in the presence of multiplexed capture beads and detection antibodies. The single-detection study evaluates the specificity of the detection antibody. This was conducted by testing the individual detection antibody in the presence of multiplexed antigens and capture beads (Table 7). Data analysis was weighted on the second highest standard concentration point. The results showed that the degree of cross-reactivity across the entire panel was below 2%.

Linearity of dilution was assessed by spiking a known quantity of recombinant antigens into human serum and plasma samples with 1:3 serial dilutions. For targets with high endogenous analytes, serum or plasma samples (with no spikes) were serially diluted one-fourth with sample diluent. This was followed by a subsequent dilution in standard diluent to preserve a 25% serum content. The observed sample concentrations were plotted as a function of their expected concentrations within the assay working range to derive correlation coefficient (R^2) values. The results in both single- and multiplex assays in serum and plasma are presented in Table 8.

Table 7. Single-detection antibody cross-reactivity profile.

	Capture Antibody															
Detection	sEGFR	sHER2/neu	sIL-6Rα	Osteopontin	sTIE-2	sVEGFR-1	sVEGFR-2	Prolactin	PECAM-1	Follistatin	Leptin	FGF-basic	HGF	G-CSF	PDGF-AB/BB	SCF
sEGFR		0.2	0.2	0.1	0.3	0.3	0.0	0.3	0.4	0.9	0.4	0.3	0.1	0.2	0.1	0.3
sHER2/neu	0.0		0.0	0.1	0.0	0.2	0.1	0.0	0.0	0.0	0.0	0.1	0.3	0.0	0.0	0.0
sIL-6Rα	1.7	0.0		0.0	0.0	1.0	0.1	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Osteopontin	0.8	0.0	0.6		0.4	0.9	0.3	0.9	0.0	0.5	0.3	0.0	0.3	0.1	0.0	0.3
sTIE-2	0.2	0.0	0.2	0.0		0.0	0.2	0.4	0.0	0.4	0.2	0.0	0.2	0.1	0.0	0.3
sVEGFR-1	0.0	0.0	0.0	0.1	0.0		0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0
sVEGFR-2	0.0	0.1	0.1	0.1	0.1	0.1		0.3	0.3	0.1	0.0	0.1	0.3	0.0	0.1	0.0
Prolactin	0.0	0.0	0.0	0.0	0.0	0.0	0.4		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PECAM-1	0.2	0.0	0.4	0.1	0.0	0.4	0.2	0.5		0.1	0.0	0.0	0.0	0.0	0.0	0.0
Follistatin	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.1		0.0	0.0	0.0	0.0	0.0	0.0
Leptin	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0
FGF-basic	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0
HGF	0.1	0.1	0.0	0.0	0.1	0.0	0.3	0.0	0.0	0.1	0.1	0.1		0.1	0.0	0.0
G-CSF	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0
PDGF-AB/BB	0.1	0.1	0.1	0.0	0.1	0.1	0.0	0.1	0.0	0.0	0.1	0.0	0.1	0.1		0.1
SCF	0.0	0.0	0.0	0.1	0.2	0.0	0.0	0.0	0.0	0.5	0.1	0.0	0.1	0.0	0.0	

Table 8. Linearity of dilution and parallelism.

	Linearity, R^2				Assay Parallelism			
					Percent Difference in Slope Value			
	Serum		Plasma		Multiplex		Singleplex	
Analyte	Multiplex	Singleplex	Multiplex	Singleplex	Serum	Plasma	Serum	Plasma
sEGFR	0.96	0.99	0.99	0.97	12.3	1.7	12.1	6.2
FGF-basic	0.99	0.99	0.99	0.99	24.8	14.3	13.9	25.2
Follistatin	0.99	0.99	0.99	0.99	3.0	0.0	11.1	3.0
G-CSF	0.99	0.99	0.99	0.99	2.7	3.6	14.2	8.5
HGF	0.99	1.00	0.99	0.99	1.0	0.0	6.2	1.1
sHER2/neu	0.99	0.99	0.99	0.97	1.0	14.9	2.1	14.6
sIL-6Rα	0.99	0.94	0.99	0.94	1.1	0.0	4.4	8.9
Leptin	0.80	0.87	0.99	0.87	11.1	5.1	1.0	9.4
Osteopontin	0.99	0.98	0.97	0.88	20.3	14.3	12.1	11.3
PECAM-1	1.00	0.99	0.98	0.99	18.2	16.9	24.2	25.8
PDGF-AB/BB	0.97	0.99	0.99	0.99	8.7	8.7	2.0	21.6
Prolactin	0.99	0.99	0.99	0.99	5.1	15.2	0.0	17.0
SCF	0.99	0.99	0.99	0.99	5.2	3.1	7.4	7.
sTIE-2	0.99	0.99	0.99	0.97	7.1	5.4	9.2	7.1
sVEGFR-1	0.99	0.99	0.99	0.99	2.1	6.3	5.2	3.1
sVEGFR-2	0.91	0.96	0.99	0.96	24.2	18.3	25.0	37.6

Assay parallelism was investigated by comparing the slope of a spike concentration-response curve in a human serum or plasma matrix with that of the standard curve using 4-PL curve fitting (Table 8). The percentage difference for most targets was less than 25%. The data are presented for information as the results may vary based on sample quality.

Assay Validation with Biological Samples

The multiplex assays were further validated with measurements from serum samples collected from individuals with colon, breast, and lung cancer plus matching controls. Figure 2 is a heat map representation of the relative levels of each marker. Statistical differences in measured values were generated using Bio-Plex Data Pro™ software with the Mann-Whitney method. P values below 0.05 were considered statistically significant.

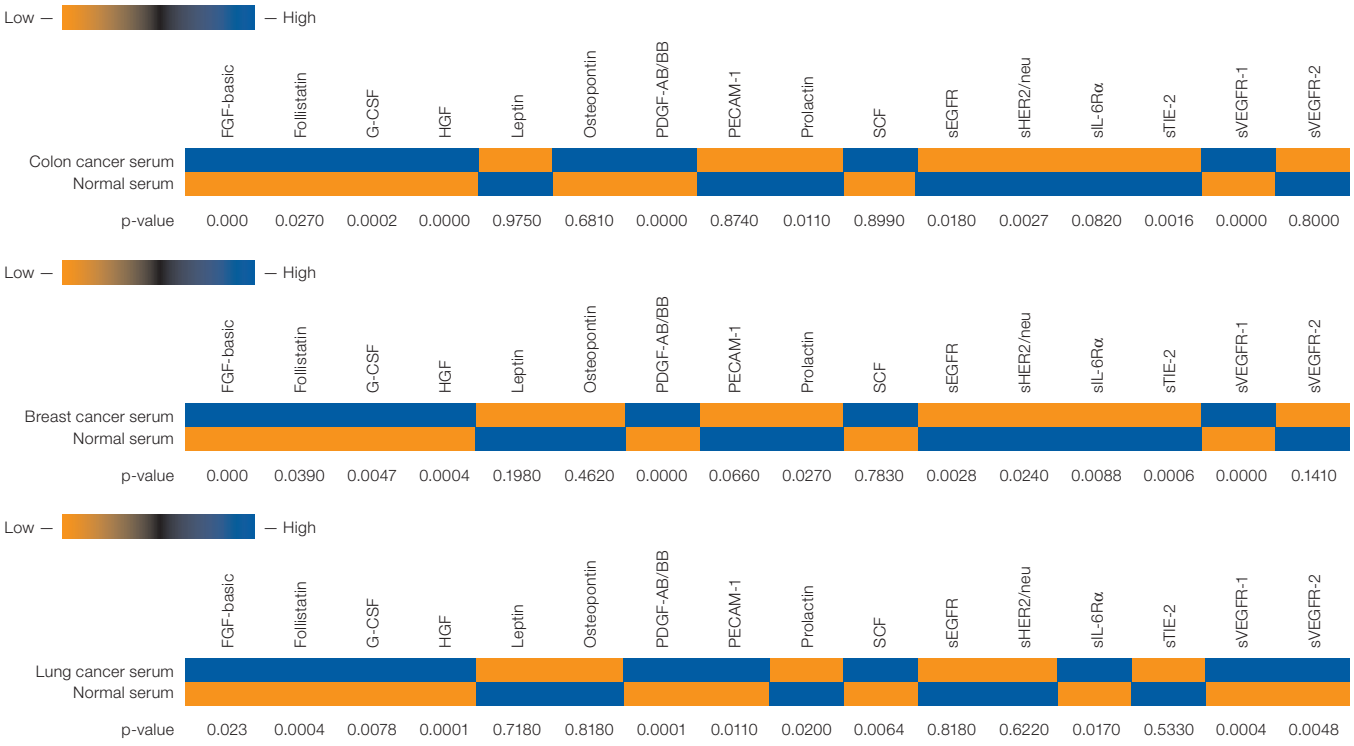


Fig. 2. Angiogenic profile in colon, breast, and lung cancer samples.

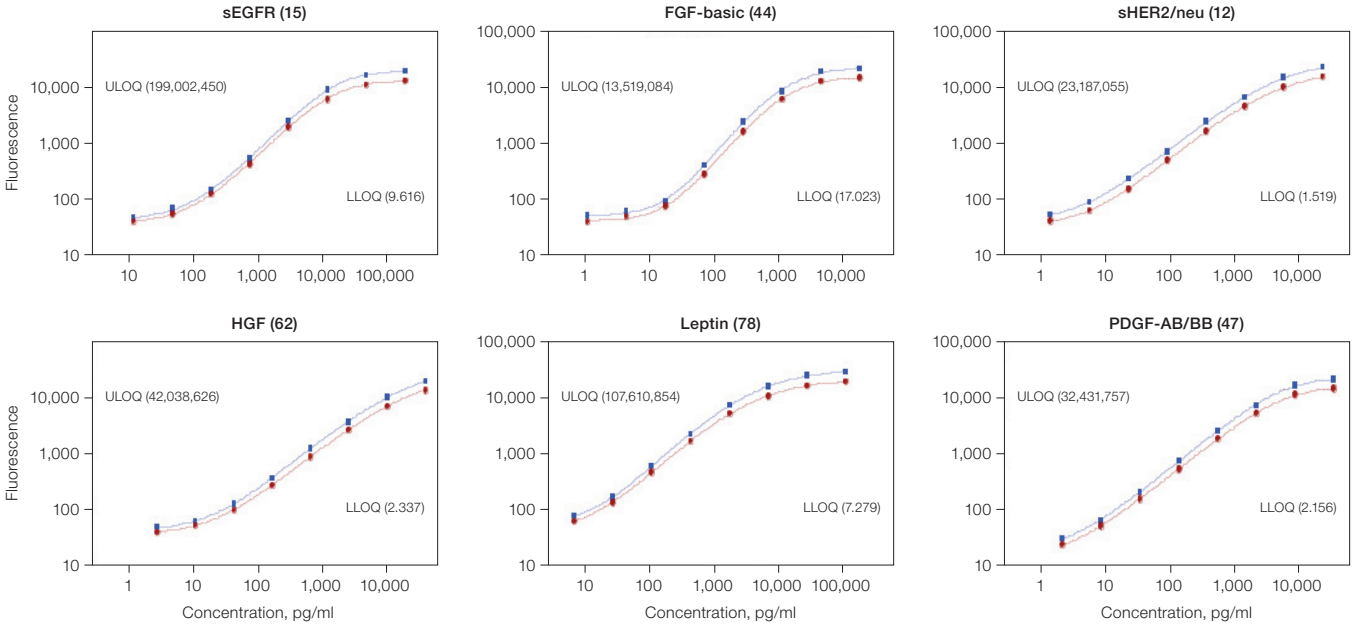


Fig. 3. Alignment between the Bio-Plex 200 (●) and Luminex FlexMAP 3D systems (■).

Agreement with other Luminex Platforms

The angiogenesis panel was also validated on a Bio-Plex 3D system to provide users the option of using alternative Luminex platforms. Overall, the standard curves generated with these two instruments are comparable (Figure 3), with all samples recording a <20% difference in observed concentration (data not shown).

Conclusions

In recent years, academic and pharmaceutical research communities have adopted the Luminex platform as mainstream technology in the life science industry. The newly configured Bio-Plex Pro human cancer biomarker panel 1 is a magnetic bead-based assay developed to gain a global view of angiogenesis and other important cancer-related processes in one simple assay. It offers improved quantification, rapid interpretation, ease of execution, and reproducibility.

The validation studies described here demonstrate the robustness of the panel of 16 assays. Assay precision, accuracy, specificity, sensitivity, and linearity were evaluated according to standard industry guidelines on validation of immunoassays. By measuring multiple markers simultaneously, the human cancer panel reduces time, cost, and sample volume compared to more traditional systems such as ELISA.

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