Development of Multiplexed Immunoassay Panels for Human Growth Factors and Growth Factor Receptors: bFGF, sFlt-1, PlGF, VEGF, KDR and c-Kit

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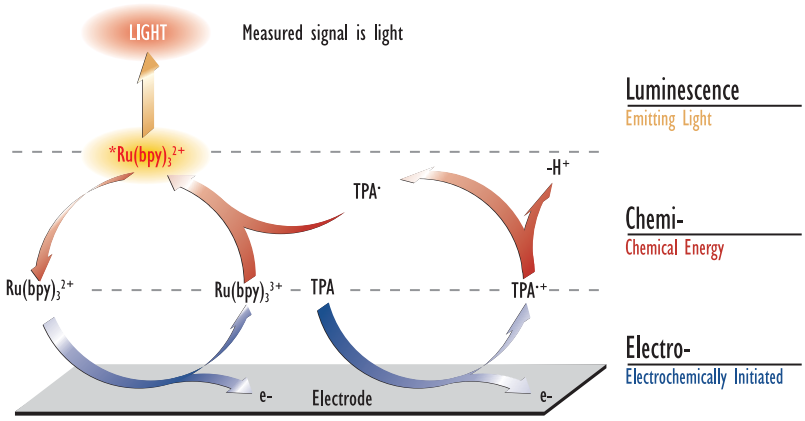
Growth factors and growth factor receptors are targets for cancer therapy and potential biomarkers to monitor disease progression. Electrochemiluminescence-based multiplexed immunoassay panels were developed for simultaneous measurement of multiple analytes per well in a 96-well format. Panel 1 detects the low-abundance analytes – Basic Fibroblast Growth Factor (bFGF), Placental Growth Factor (PlGF), Vascular Endothelial Growth Factor (VEGF), and soluble VEGF Receptor 1 (sFlt-1/VEGFR1), and was optimized for a 25 µL sample (serum or EDTA plasma). Panel 2 detects the more abundant analytes – soluble VEGF Receptor 2 (KDR/VEGFR2) and soluble Stem Cell Factor Receptor (c-Kit), and was optimized for 50 µL of a 50-fold diluted sample. The assay format is simple: diluent and sample are added to blocked and washed plates, and after a two-hour incubation with agitation, plates are washed and detection antibody reagent is added. After a second two-hour incubation, plates are washed and read on a MSD® SECTOR™ Imager 6000 instrument (throughput of one plate per minute).

The lower and upper limits of the assay ranges in the following table represent the analytical sensitivity and the highest calibrator level, respectively. The linear range extends substantially beyond the highest calibrator level. Intra-plate CVs were approximately 4–8%. The assays are sensitive enough to measure these biomarkers in normal samples, and the dynamic range extends well beyond the elevated levels expected in disease states. Each analyte in the multiplexed panels is measured accurately even in the presence of a high abundance of other analytes, as demonstrated in an experiment where an elevated concentration of each analyte was spiked individually into a normal serum sample. Spike recovery and dilution linearity were in the range of 80% to 120%. In conclusion, multiplexed assays for simultaneous measurement of growth factors and growth factor receptors were successfully developed and validated.

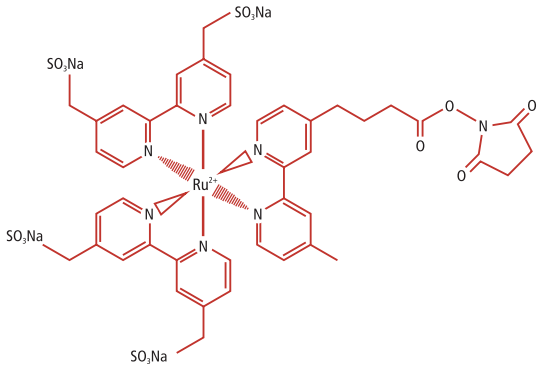
Analyte	Assay Range	Analyte Concentration in Normal Pooled Serum & Plasma Samples (not paired)	
		Serum	EDTA Plasma
bFGF	1 - 9,000 pg/mL	< 1 pg/mL	7 pg/mL
sFlt-1	8 - 9,000 pg/mL	63 pg/mL	201 pg/mL
PlGF	1 - 9,000 pg/mL	13 pg/mL	16 pg/mL
VEGF	9 - 9,000 pg/mL	31 pg/mL	148 pg/mL
KDR	0.8 - 750 ng/mL	31 ng/mL	28 ng/mL
c-Kit	7 - 7,500 ng/mL	165 ng/mL	140 ng/mL

Electrochemiluminescence (ECL)



- Selective
- Convenient chemistry
- Robust, stable
- Few interferences

Ruthenium (II) tris-bipyridine-(4-methylsulfonate) NHS ester (MSD SULFO-TAG™ label)



- Size, MW: ~1200 daltons
- Stability: Years
- Solubility: Aqueous, DMSO
- Functionality: Hydrophilic
- Specificity: High

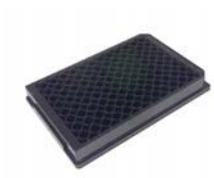
Meso Scale Discovery MULTI-ARRAY® Technology

SECTOR Imager 6000

SECTOR PR 400 Reader

Instrument Features

- Highly sensitive
- SECTOR Imager designed for high-throughput screening (HTS)
- SECTOR Imager or SECTOR PR ideal for assay development
- Custom optics
- High-speed motion control systems
- Electrochemiluminescent (ECL) detection



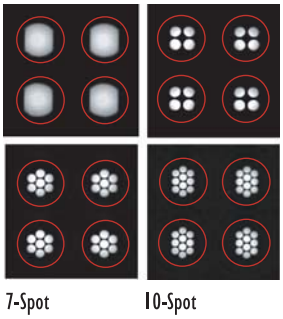
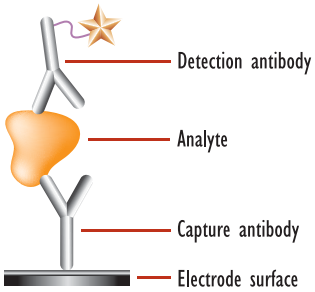


Plate Features

- Disposable plates
- Carbon electrodes with high binding capacity
- Suitable electrochemistry for ECL
- Biocompatible: direct immobilization of IgG, membrane fragments, intact cells, etc.
- Functional assays: simple binding reactions, GPCRs, enzyme cascades, post-translational modification, etc.

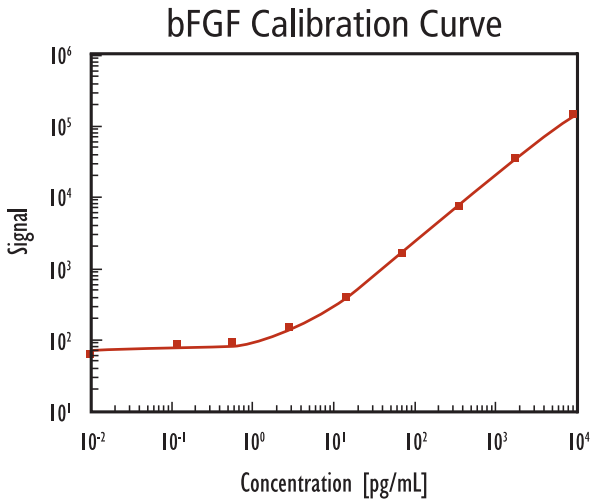
Assay Format

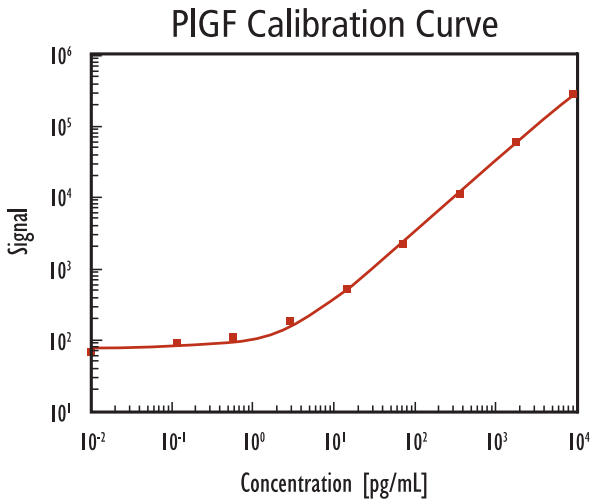


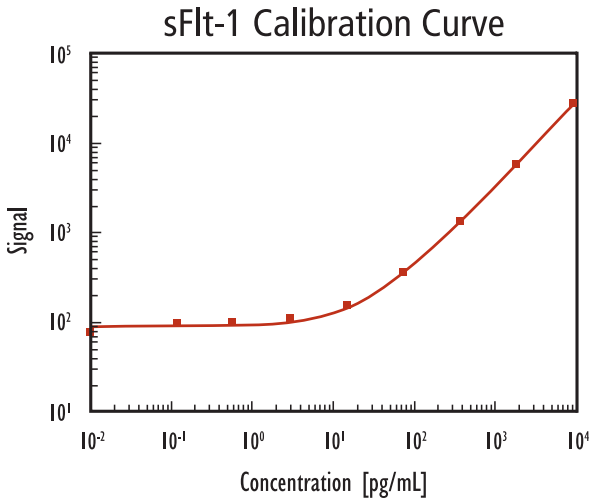
- Block plates for 1 hr at room temperature or overnight at 4°C and wash (optional)
- Add 25 µL of assay diluent and 25 µL of sample to each well*
- Incubate for 2 hrs with shaking; wash
- Add 25 µL of detection antibody reagent
- Incubate for 2 hrs with shaking; wash
- Add MSD Read Buffer T; read

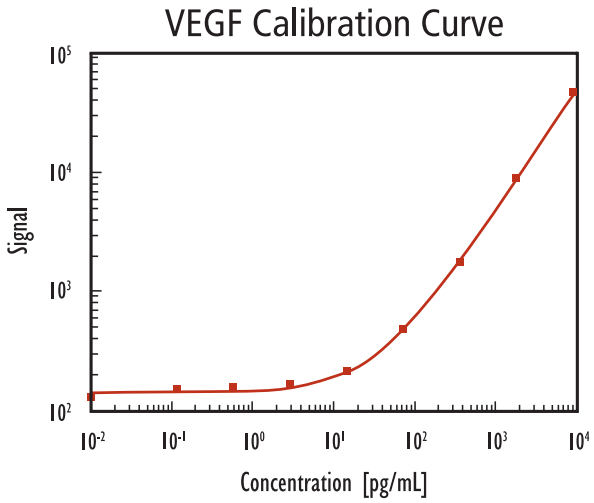
*For the Growth Factor 2-plex assay, 50 µL of a 50X diluted sample is added to each well

Calibration Curves: Growth Factor 4-Plex



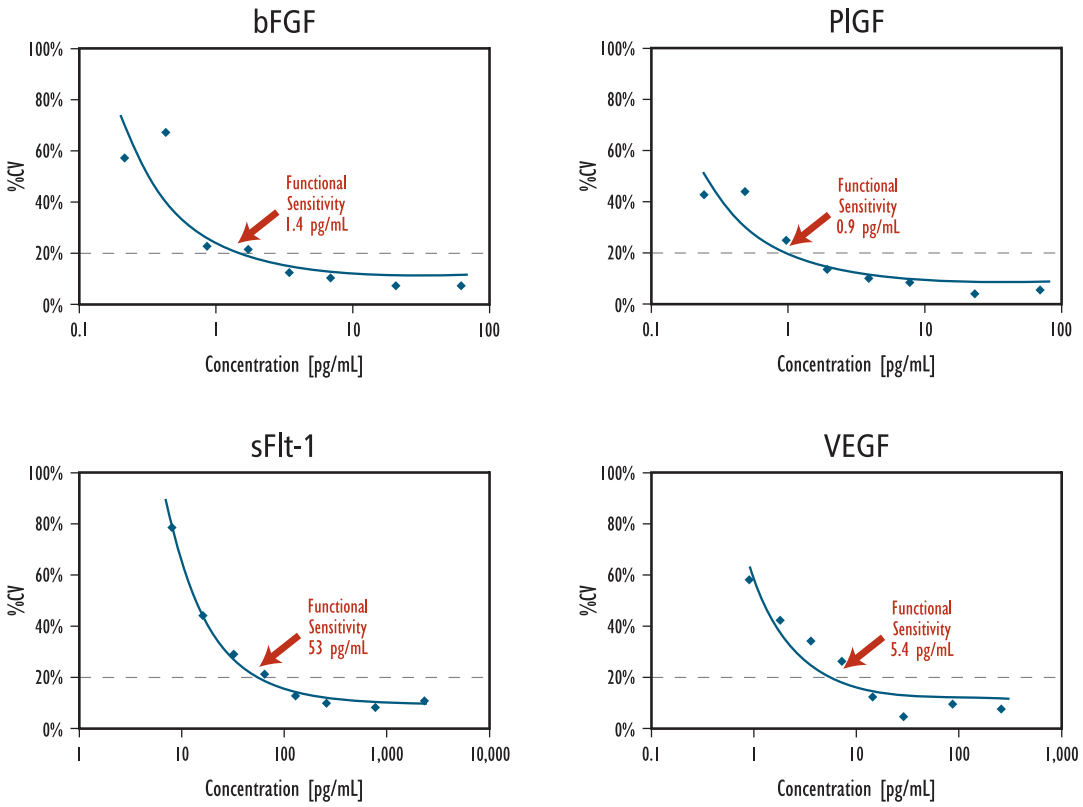






Curve represents a 4-parameter fit: $$y = b_1 + \frac{b_2 - b_1}{1 + (x/b_3)^{b_4}}$$

Functional Sensitivity: Growth Factor 4-Plex

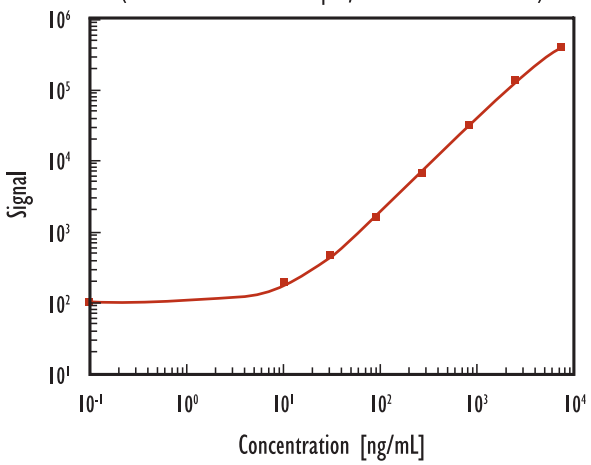


Functional Sensitivity: Lowest analyte concentration that can be measured with a within-plate CV of not more than 20%.

Calibration Curves & Functional Sensitivity: Growth Factor 2-Plex

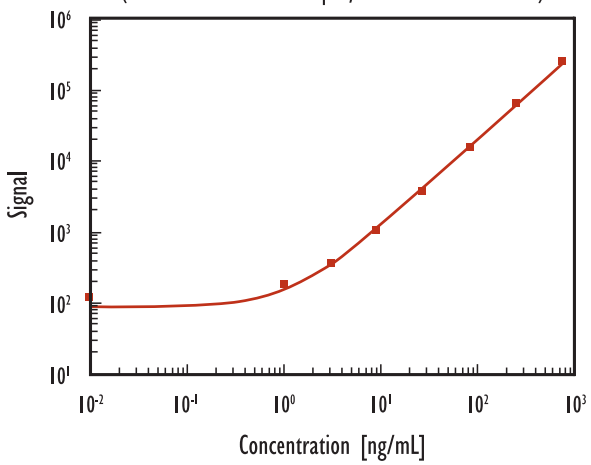
c-Kit Calibration Curve

(concentration in sample, before 50X dilution)

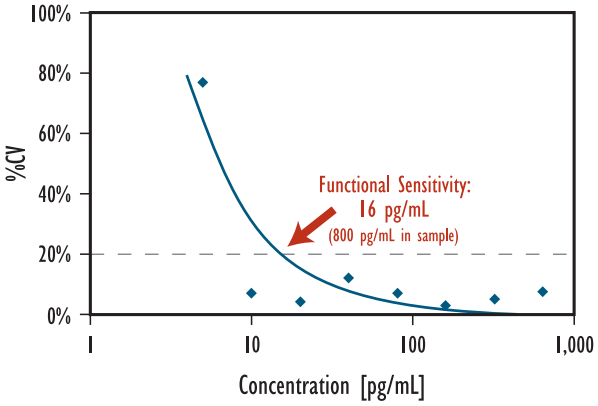


KDR Calibration Curve

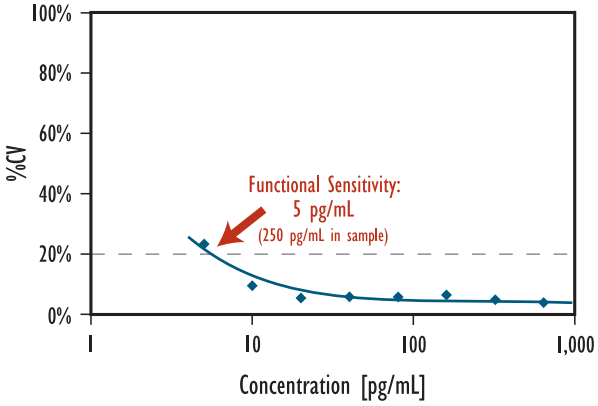
(concentration in sample, before 50X dilution)



c-Kit



KDR



Reproducibility (Between-Plate)

	bFGF		sFlt-1		PlGF		VEGF	
	Average (pg/mL)	Between-Plate CV	Average (pg/mL)	Between-Plate CV	Average (pg/mL)	Between-Plate CV	Average (pg/mL)	Between-Plate CV
QC1	23	3%	209	6%	22	2%	2,893	3%
QC2	n/d	69%	89	6%	18	3%	30	9%
QC3	n/d	80%	55	7%	12	5%	25	7%
QC4	n/d	103%	63	10%	13	3%	31	12%
QC5	946	1%	4,778	2%	353	1%	2,987	1%
Detection Limit	1.1		8		0.9		9	

	c-Kit		KDR	
	Average (ng/mL)	Between-Plate CV	Average (ng/mL)	Between-Plate CV
QC-A	162	3%	32	2%
QC-B	220	3%	33	1%
QC-C	142	1%	49	1%
QC-D	140	3%	28	1%
QC-E	165	3%	31	1%
Detection Limit	6		0.6	

For analyte concentrations sufficiently above the detection limits, between-plate variability was low.

To determine between-plate reproducibility, five QC samples were run on six plates. QC1–QC4 and QC-A–QC-D were run in quadruplicates per plate; QC-5 and QC-E in 32 replicates per plate. Each plate contained a calibration curve, and the concentrations of the QC samples was calculated for each plate. Between-plate variability was estimated as the CV of the average concentrations of the five QCs for the six plates.

Multiplexing: Cross-Reactivity

	Zero Calibrator	1,000 pg/mL bFGF	1,000 pg/mL sFlt-1	1,000 pg/mL PlGF	1,000 pg/mL VEGF
bFGF Spot	28	25,880	65	49	19
sFlt-1 Spot	26	222	20,216	36	17
PlGF Spot	18	43	68	23,894	43
VEGF Spot	33	40	125	35	9,030

	% Cross-Reactivity			
	1,000 pg/mL bFGF	10,000 pg/mL sFlt-1	1,000 pg/mL PlGF	1,000 pg/mL VEGF
bFGF Spot	X	0.3%	0.1%	-0.1%
sFlt-1 Spot	0.8%	X	0%	-0.1%
PlGF Spot	0.1%	0.2%	X	0.3%
VEGF Spot	0%	0.5%	0%	X

	Zero Calibrator	5,000 pg/mL KDR	50,000 pg/mL c-Kit
bFGF Spot	73	25,073	135 (0.04%)
sFlt-1 Spot	61	109 (0.2%)	162,524

No significant cross-reactivity is observed (<1%)

Percent cross-reactivity is defined here as the increase in background on a given spot divided by the signal on the analyte-specific spot, (e.g. cross-reactivity on the bFGF spot due to sFlt-1 analyte is (65-28)/20216 = 0.3%).

Demonstration of Assay Specificity by Analyte Depletion

Stripped with	% Analyte Detected vs. Control			
	bFGF	sFlt-1	PlGF	VEGF
bFGF Ab	27%	102%	100%	99%
sFlt-1 Ab	87%	13%	99%	97%
PlGF Ab	113%	106%	18%	94%
VEGF Ab	100%	86%	97%	19%

Stripped with	bFGF	sFlt-1
bFGF Ab	27%	102%
sFlt-1 Ab	87%	13%
PlGF Ab	113%	106%
VEGF Ab	100%	86%

The antibodies used for the assays specifically recognize their respective analytes.

After the depletion of the bFGF and sFlt-1 analytes, their respective signals were at or below the analytical sensitivities of these assays.

A serum pool was affinity-stripped with antibodies for either bFGF, sFlt-1, PlGF, or VEGF. Different antibodies than those used as capture or detection were used. The stripped serum was then assayed for all four analytes, and the concentration of the affinity depleted analyte was compared to a control that had been exposed to a BSA surface instead of a specific antibody. An analogous stripping experiment was performed for KDR and c-Kit.

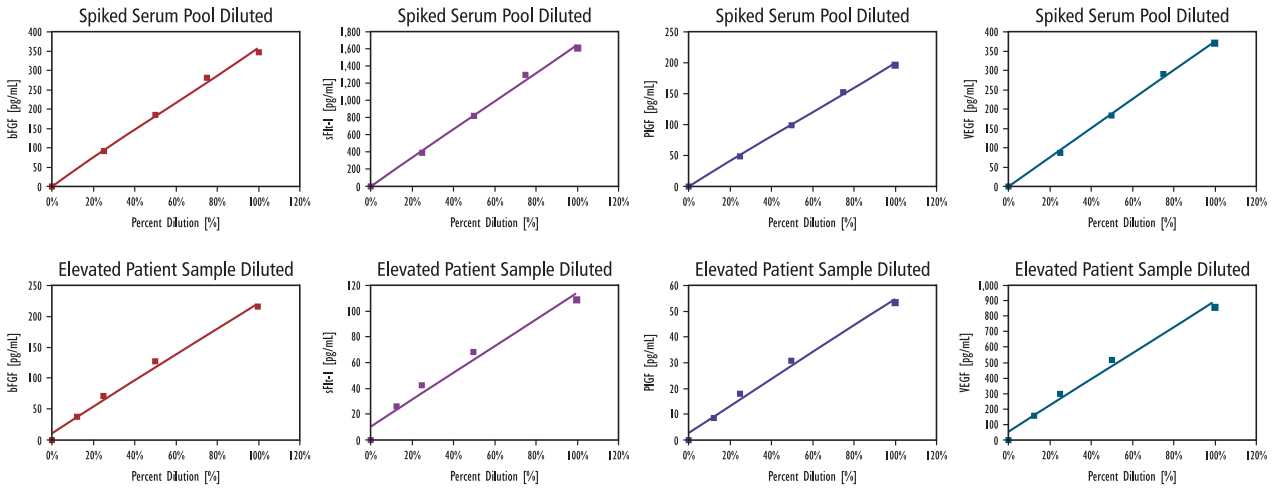
Spike Recovery: bFGF, sFlt-1, PlGF and VEGF

	bFGF (pg/mL)			sFlt-1 (pg/mL)			PlGF (pg/mL)			VEGF (pg/mL)		
	Measured	Expected	Recovery	Measured	Expected	Recovery	Measured	Expected	Recovery	Measured	Expected	Recovery
Plasma +100	81.7	106	77%	1,115	1,181	94%	112	115	98%	221	233	95%
Plasma +33	34.4	39	87%	518	514	101%	46	48	96%	162	166	97%
Plasma +10	17.2	17	101%	321	292	110%	27	26	103%	150	144	104%
Plasma	6.7			201			16			148		
Serum +100	99.7	101	99%	1,172	1,121	105%	125	113	111%	138	122	114%
Serum +33	39.1	34	115%	504	455	111%	53	46	114%	67	55	122%
Serum +10	15.5	12	131%	265	232	114%	28	24	119%	39	33	118%
Serum	0.8			135			14			24		
Detection Limit	2.0			37			1.8			8		

Recovery was acceptable.

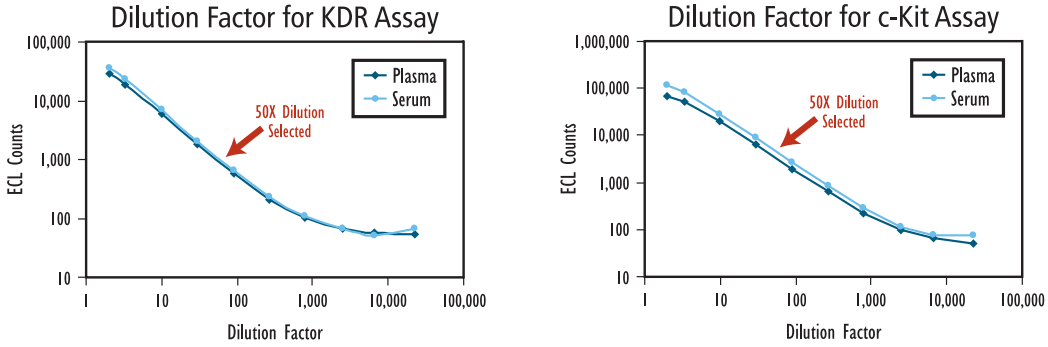
Pooled serum and EDTA plasma samples were spiked with three levels of combined calibrator: 100, 33, or 10 pg/ml bFGF, PlGF, & VEGF, and 1,000, 333, and 100 pg/ml sFlt-1, respectively. Recovery was defined as measured concentration divided by expected concentration.

Dilution Linearity: Growth Factor 4-Plex



- To demonstrate dilution linearity, a spiked pool and an elevated patient sample were diluted.
- All analytes in the 4-Plex diluted linearly.

Dilution Factor for KDR and c-Kit Duplex



- A serum and a plasma pool were diluted over a wide dilution range.
- Samples diluted linearly from a 10X to a 1,000X dilution.
- A dilution factor of 50X was selected.

KDR and c-Kit Panel: Spike Recovery

Recovery was demonstrated by spiking c-Kit or KDR calibrator into serum and plasma samples. The samples were diluted 50X and analyzed. Percent recovery was between 80 and 120% for all samples.

		c-Kit (ng/mL)			KDR (ng/mL)		
		Measured	Expected	Recovery	Measured	Expected	Recovery
PLASMA 1	Neat	91			13		
	Low Spike	231	247	94%	193	206	93%
	Medium Spike	700	716	98%	755	788	96%
	High Spike	2,697	2,591	104%	3,308	3,113	106%
PLASMA 2	Neat	83			14		
	Low Spike	202	240	84%	170	208	82%
	Medium Spike	583	708	82%	617	789	78%
	High Spike	2,672	2,583	103%	3,264	3,114	105%
PLASMA 3	Neat	78			12		
	Low Spike	232	234	99%	216	205	105%
	Medium Spike	722	703	103%	787	787	100%
	High Spike	2,915	2,578	113%	3,714	3,112	119%
PLASMA 4	Neat	144			20		
	Low Spike	284	300	95%	206	214	96%
	Medium Spike	766	769	100%	775	795	97%
	High Spike	2,904	2,644	110%	3,567	3,120	114%
PLASMA 5	Neat	116			12		
	Low Spike	328	272	120%	191	206	93%
	Medium Spike	838	741	113%	761	787	97%
	High Spike	2,923	2,616	112%	3,362	3,112	108%

Conclusions

- Two multiplex panels for 6 growth factor markers were developed in a 96-well format: GF 4-plex containing bFGF, PlGF, sFlt-1 & VEGF; and GF 2-plex containing KDR and c-Kit.
- Required sample volume is 25 µL for the low-abundance analyte GF 4-plex and 1 µL (i.e. 50 µL at 50X dilution) for the high-abundance analyte GF 2-plex.
- Detection limits for low abundance analytes are in the 1–10 pg/mL range. Typical CVs are <10%.
- Dynamic ranges for each of the 6 analytes span 3–4 orders of magnitude.
- Cross-reactivity is low (<1%).
- Simple two-step assay format.