Development and Validation of a Multiplexed Assay for Human Angiogenesis Biomarkers

Daisy Roy, Bruk Leta, Robert Umek, Pankaj Oberoi, and Jacob N. Wohlstadter

1 Abstract

Purpose: Angiogenesis markers consist mainly of proangiogenic proteins, most notably VEGF, VEGF-C, VEGF-D, Tie-2, sFlt-1, PlGF, and bFGF. These are potential prognostic markers for disease activity and indicators of response to chemotherapy. This poster describes the validation of a multiplex assay for these key biomarkers that can be used to detect signs of angiogenesis.

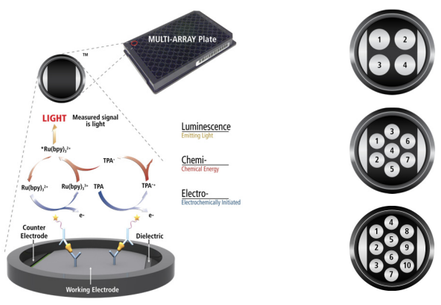
Methods: MESO SCALE DISCOVERY® (MSD) developed a 7-plex panel of assays according to fit-for-purpose assay development and validation principles. The assays were tested for sensitivity, precision, specificity, accuracy, dilution linearity, and robustness. Samples of normal human serum were measured to establish a normal range, then serum samples from 2 disease states were assayed to quantify biomarker modulation. No more than 100 µl of serum or plasma was required to quantify all 7 biomarkers in duplicate.

Results: The quantitative range for the assay was established, and all subsequent assay validation tests were conducted within this functional range of the assay. The panel was tested across 12 plates at low, medium, and high analyte levels and exhibited intra- and inter-plate variability of 10% or less. Matched serum and plasma samples were spiked with analyte at 4 levels across the dynamic range of the assay; recovery for 6 of 7 analytes was between 80% and 120% at all concentrations (we noted lower levels of recovery for Tie-2 extremely high analyte levels). Data for the remaining assay validation parameters are presented in this poster. Additionally, normal and diseased samples were tested to functionally assess the utility of this multiplex panel. For 6 of 7 analytes, all samples were quantifiable at a 2-fold dilution (bFGF was below the quantitative range for multiple samples). When comparing normal samples and the 2 different disease conditions, multiple analytes showed significant differences in analyte expression levels.

Conclusions: The 7-plex panel illustrated here will be a useful tool for researchers studying angiogenesis. Multiplexed assays mean simpler protocols and faster results with reduced sample volume requirements. The assays were tested in both serum and plasma matrices according to specifications detailed in fit-for-purpose validation principles.

2 Methods

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.



Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

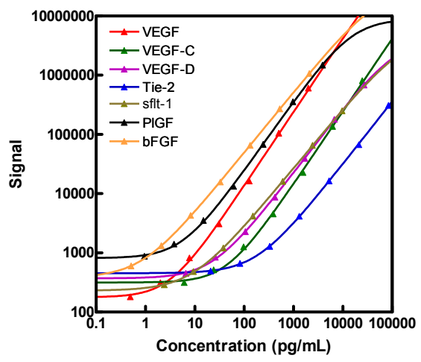
Protocol

1. Block MSD MULTI-SPOT plate with 150 µl blocking solution for 1 hour.
2. Wash plate and add 50 µl calibrator or sample.* Incubate at room temperature with shaking for 2 hours.
3. Wash plate and add 25 µl detection antibody solution. Incubate at room temperature with shaking for 2 hours.
4. Wash plate and add 150 µl Read Buffer T.
5. Analyze the plate with MSD SECTOR® Imager.

* MSD recommends a 2-fold dilution of serum and plasma samples

3 Standard Curves, Sensitivity, and Precision

These standard curves illustrate the sensitivity and dynamic range of the 7 assays in the MSD Angiogenesis Panel 1 (human). The graphs display representative data from a single run. Reproducibility (precision) across runs is shown in the table. Reproducibility was assessed with matrix based controls run across 6 plates over 3 days of testing.



Assay	Control	Runs	Avg Conc (pg/mL)	Avg Intra-plate %CV	Inter-plate %CV
VEGF	High	6	515	3.4	5.2
	Mid	6	105	7.6	6.3
	Low	6	14	5.9	9.0
VEGF-C	High	6	7138	2.2	4.6
	Mid	6	476	4.0	8.1
	Low	6	319	7.0	7.7
VEGF-D	High	6	10154	3.0	5.3
	Mid	6	2085	4.3	4.4
	Low	6	73	1.1	8.0
Tie-2	High	6	17574	2.5	6.0
	Mid	6	5425	2.9	8.3
	Low	6	1966	5.8	6.4
sFlt-1	High	6	4259	5.6	7.0
	Mid	6	653	6.5	10.9
	Low	6	252	5.0	10.2
PlGF	High	6	1708	5.5	6.3
	Mid	6	298	1.6	6.9
	Low	6	21	3.5	10.8
bFGF	High	6	816	2.3	2.6
	Mid	6	74	2.5	6.1
	Low	6	10	2.5	5.9

The lower limit of detection (LLOD) is a calculated concentration based on a signal that is 2.5 standard deviations over the blank (N=24). The average and range for the LLOD is calculated over at least 6 runs. The lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) were established using kits from a single lot. Six plates were run over multiple days to establish the LLOQ and ULOQ with acceptable precision (≤25%) and accuracy (between 75% and 125% of expected concentrations). Serum contains endogenous analyte levels that are too high to establish an LLOQ. A concentration range of calibrator spiked into diluent was used to assess the LLOQ and ULOQ.

	VEGF	VEGF-C	VEGF-D	Tie-2	sFlt-1	PlGF	bFGF
LLOD Range (pg/mL)	0.32-0.96	4.9-17	1.3-6.8	7.7-30	1.4-5.5	0.25-25	0.043-0.31
Avg. LLOD (pg/mL)	0.60	8.0	3.4	19.0	3.0	2.9	0.10
LLOQ (pg/mL)	2.2	74	77	83	27	11	0.8
ULOQ (pg/mL)	1475	16 510	17 269	55 967	6123	2460	1679

4 Specificity

To determine capture antibody specificity, individual calibrators were tested with blended detection antibodies. The non-specific interactions were below 1% for all seven analytes. Non-specific interaction is defined as (background-subtracted non-specific signal/background subtracted specific signal)∗100.

Interference was tested by adding related proteins at supra-physiological levels. 50 ng/mL of Ang-1, Ang-2, Ang-4, Angptl-1, NRP-1, NRP-2, or VEGF-B was added to the indicated concentrations of VEGF, VEGF-C, VEGF-D, and Tie-2 calibrators. Percent recovery was calculated with respect to the no-interference condition. The only significant cross reactivity was observed between Ang-2 and Tie-2. Ang-1 and Ang-2 are both ligands of the Tie-2 receptor. The Tie-2 signal was elevated by 20% in the presence of 50 ng/ml Ang-2, endogenous levels of Ang-2 are significantly lower than 50 ng/ml in both normal and diseased states (data not shown).

	VEGF	VEGF-C	VEGF-D	TIE-2	sFlt-1	PlGF	FGF
Calibrator Conc. Tested (pg/mL)	625	5000	10 000	25 000	5000	3750	625

	Single Calibrator with Blended Detectors						
Spot	VEGF	VEGF-C	VEGF-D	Tie-2	sFlt-1	PlGF	bFGF
VEGF	100%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%
VEGF-C	<0.1%	100%	<0.1%	<0.1%	0.41%	<0.1%	<0.1%
VEGF-D	<0.1%	<0.1%	100%	<0.1%	<0.1%	<0.1%	<0.1%
Tie-2	<0.1%	<0.1%	<0.1%	100%	0.48%	0.27%	<0.1%
sFlt-1	<0.1%	<0.1%	<0.1%	0.15%	100%	<0.1%	<0.1%
PlGF	<0.1%	<0.1%	<0.1%	0.66%	<0.1%	100%	<0.1%
bFGF	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	100%

5 Samples

Serum, EDTA plasma, and heparin plasma samples were tested at 2-fold dilutions. Shown are the median and range of concentrations for each sample set. Concentrations have been corrected for sample dilution. The LLOQ value for bFGF is 1.0 pg/mL for the sample set tested.

Sample Type	Statistic	VEGF	VEGF-C	VEGF-D	Tie-2	sFlt-1	PlGF	bFGF
Serum	Median (pg/mL)	234	268	2141	3659	932	267	2.4
	Range (pg/mL)	77-598	143-377	1391-3613	837-4215	519-1241	183-402	<LLOQ -6.3
	Number of Samples	13	13	13	13	13	13	13
	Number of Samples in quantitative range	13	13	13	13	13	13	4
EDTA Plasma	Median (pg/mL)	22	52	1734	3587	1057	202	<LLOQ
	Range (pg/mL)	2-407	28-156	1304-3514	2463-4615	519-1465	157-386	<LLOQ
	Number of Samples	13	13	13	13	13	13	13
	Number of Samples in quantitative range	13	13	13	13	13	13	None
Heparin Plasma	Median (pg/mL)	111	119	1252	4167	1560	259	
	Range (pg/mL)	22-448	4-443	900-1811	795-5290	654-2316	175-411	
	Number of Samples	13	13	13	13	13	13	
	Number of Samples in quantitative range	13	13	13	13	13	13	

6 Matrix Tolerance

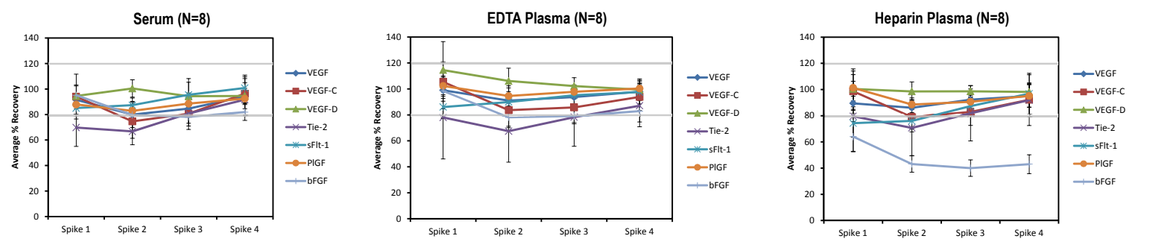
Spike Recovery

Eight matched normal human serum, EDTA plasma, and heparin plasma samples were spiked with calibrators at multiple levels throughout the range of the assay. Spikes were made into neat samples then diluted 2-fold. The spike levels shown in the table (at right) are dilution-corrected values.

The graphs below show the average % recovery for each analyte at each spike level.

% Recovery = measured/expected∗100

	Spike Concentration (pg/mL)			
	Spike 1	Spike 2	Spike 3	Spike 4
VEGF	2000	250	62.5	15.6
VEGF-C	16000	2000	500	125
VEGF-D	32000	4000	1000	250
Tie-1	80000	10000	2500	625
sFlt-1	16000	2000	500	125
PlGF	12000	1500	375	93.8
bFGF	2000	250	62.5	15.6



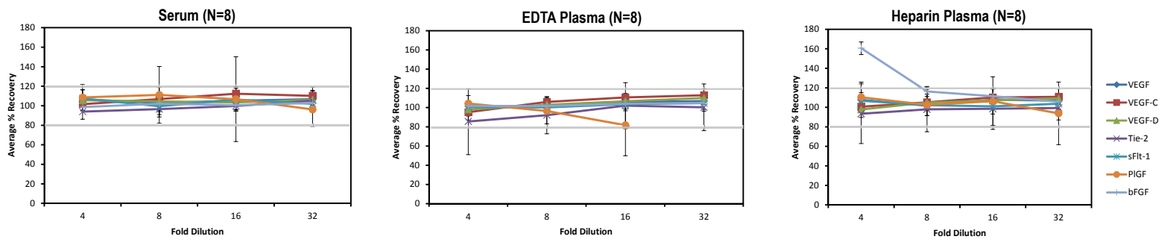
Spike recovery was outside of ±20% for multiple concentrations of Tie-2 in all three sample types. Interference from Ang-2 may be contributing to the low recovery. Most of the other analytes recovered between 80% and 120% at each spike level.

Heparin induces bFGF oligomerization through a known heparin binding domain, resulting in a dramatic under-recovery of bFGF.[3] Heparin samples are not recommended for measuring bFGF. Most of the other analytes recovered between 80% and 120% at each spike level.

Dilution Linearity

To assess dilution linearity, eight matched serum, EDTA plasma, and heparin plasma samples were spiked with VEGF, VEGF-C, sFlt-1, and bFGF calibrators. Endogenous analyte levels for VEGF-D, Tie-2, and PlGF did not require a spike of additional calibrator. The samples were diluted 2-fold, 4-fold, 8-fold, 16-fold, and 32-fold. The 2-fold dilution was set as 100% recovery. The average % recovery for all analytes for all samples fell between 80% and 120%, with the exception of bFGF in heparin plasma which is known to be problematic.

% Recovery = (measured x dilution factor)/expected x 100

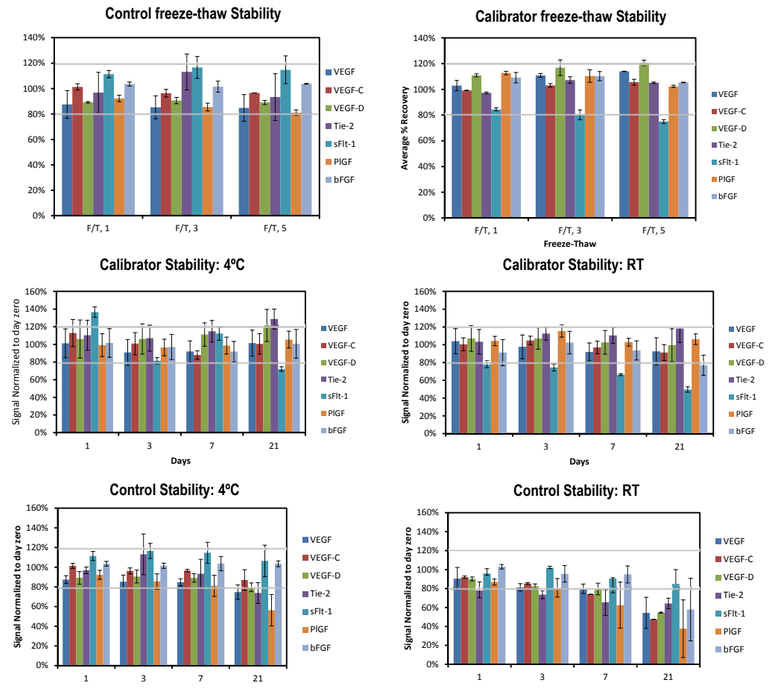


7 Assay Robustness

Controls were prepared by spiking analytes into pooled EDTA plasma. Calibrators and controls were tested for stability through 5 freeze–thaw cycles. Percent recovery was calculated in reference to initial use. All calibrators/controls were stable through 5 freeze–thaw cycles with the exception of sFlt-1 calibrator. MSD doesn't recommend re-freezing calibrators. Controls should be stored frozen at ≤−70ºC for maximum stability and thawed immediately before use. Any unused thawed controls should be discarded.

The calibrator blend was subjected to accelerated stability testing at 2–8ºC and room temperature (RT) for 21 days. Percent signal was calculated in reference to calibrators stored at ≤−70ºC (day zero). Calibrators are stable at 2–8ºC up to 7 days. Most of the calibrators, with the exception of sFlt-1, are stable at RT for 7 days. For maximum stability, MSD recommends that calibrators be stored frozen at −70ºC.

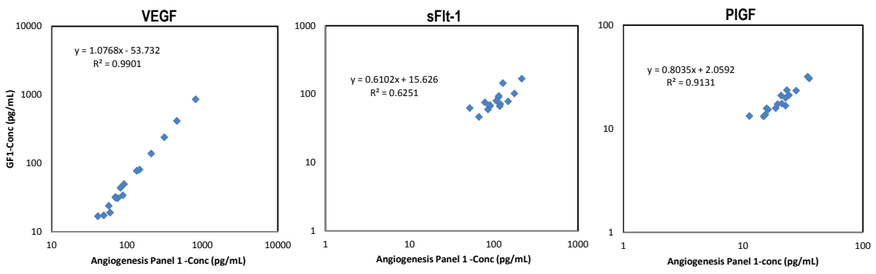
Matrix based controls were subjected to accelerated stability testing at 2–8ºC and room temperature (RT) for 21 days. Percent signal was calculated in reference to controls stored at ≤−70ºC (day zero). Controls are stable at 2–8ºC up to 7 days. Most of the controls showed significant decreases in signal when stored at RT for 7 days. For maximum stability, MSD recommends that controls be stored frozen at −70ºC.



SULFO-TAG labeled detection antibodies were tested for stability at room temperature (in both light and dark conditions) and at 2–8ºC in the light (data not shown) . Detection antibodies are normally stored at 2–8ºC in the dark. Detection antibodies are sensitive to light exposure, but are stable in the dark for 21 days at RT. For maximum stability, MSD recommends that detection antibodies be kept in the dark at 2–8ºC.

8 Assay Performance

MSD Human Growth Factor Panel 1 is a multiplex panel containing bFGF, VEGF, sFlt-1, and PlGF. This panel was directly compared to the Angiogenesis Panel 1. Normal EDTA samples were run across both panels. bFGF results are not shown because the majority of samples fell below the LLOQ.



Assay	NIBSC code	Units/ampoule	Concentration/ ampoule	Conversion determined from 3 day experiments run on Angiogenesis Panel-1 (Human) Kit
VEGF	02/286	13000 units	13 µg/mL	485 units of NIBSC = 1 µg/mL of MSD VEGF standard
PlGF	09/272	5000 units	10 µg/mL	480 units of NIBSC = 1 µg/mL of MSD PlGF standard
bFGF	90/712	1600 IU	4 µg/mL	680 IU of NIBSC = 1 µg/mL of MSD bFGF standard

National Institute for Biological Standards and Control (NIBSC) reference standards for VEGF, PlGF, and bFGF were calibrated against MSD blended calibrators supplied with the Angiogenesis Panel 1. Conversion factors are listed in the table to the left.

9 Conclusions

MSD Angiogenesis Panel 1 (human) is a robust panel of assays that quantitatively measure disease-relevant pro-angiogenic biomarkers. During assay development and validation, the accuracy, precision, sensitivity, and stability of the multiplex assay panel were established. All the assays have a broad dynamic range allowing accurate quantification of samples without the need for multiple dilutions. The panel uses a simple and rapid protocol with minimal sample volume requirements resulting in increased sample conservation and decreased assay time. The panel quantifies the levels of all 7 analytes in serum and plasma samples from normal and diseased populations and offers a powerful tool for measuring responses to anti-angiogenesis treatment.

References

Woolard J, Bevan HS, Harper SJ, Bates DO. Molecular diversity of VEGF-A as a regulator of its biological activity. Microcirculation. 2009;16(7):572-92.

Lee JW, Devanarayan V, Barrett YC, Weiner R, Allinson J, Fountain S, Keller S, Weinryb I, Green M, Duan L, Rogers JA, Millham R, O'Brien PJ, Sailstad J, Khan M, Ray C, Wagner JA. Fit-for-purpose method development and validation for successful biomarker measurement. Pharm Res. 2006; 23(2):312-28.

Spivak-Kroizman T, Lemmon MA, Dikic I, Ladbury JE, Pinchasi D, Huang J, Jaye M, Crumley G, Schlessinger J, Lax I. Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. Cell. 1994; 79(6):1015-24

Meso Scale Discovery®
A division of Meso Scale Diagnostics, LLC.
www.mesoscale.com®