MSD® MULTI-SPOT Assay System

Angiogenesis Panel 1 (human) Kit

1-Plate Kit	K15190D-1
5-Plate Kit	K15190D-2
25-Plate Kit	K15190D-4

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MSD Biomarker Assays

Angiogenesis Panel 1 (human) Kit

VEGF, VEGF-C, VEGF-D, Tie-2, sFlt-1, PlGF, bFGF

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

MESO SCALE DISCOVERY®
A division of Meso Scale Diagnostics, LLC.
9238 Gaither Road
Gaithersburg, MD 20877 USA
www.mesoscale.com

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Ordering Information

MSD Customer Service

Phone: 1-301-947-2085
Fax: 1-301-990-2776
Email: CustomerService@mesoscale.com

MSD Scientific Support

Phone: 1-301-947-2025
Fax: 1-240-632-2219 attn: Scientific Support
Email: ScientificSupport@mesoscale.com

Introduction

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.[1] This product insert describes a multiplex panel of these key biomarkers that can be used to detect signs of angiogenesis.

Several different strategies have been designed to target vascular endothelial growth factor receptor (VEGFR) signal transduction. Several VEGF-neutralizing antibodies have been successful in combination with chemotherapy to limit angiogenesis and tumor progression. Other strategies to target the VEGF/VEGFR pathway include soluble VEGFRs (Traps), receptor tyrosine-kinase inhibitors that target VEGFR2/Flk-1, and neutralizing aptamers.[2] Clinicians are interested in promoting angiogenesis in conditions such as stroke and ischemia. Therapeutic angiogenesis through gene transfer of various pro-angiogenic molecules, including VEGFs, is currently under evaluation in clinical trials.

Despite the success of anti-angiogenic therapy using VEGF inhibitors in delivering clinical benefit for many cancer patients, significant challenges remain for those patients who do not respond to the anti-VEGF treatment. Therefore, it would be desirable to have reliable markers to identify patients who are most likely to respond to anti-VEGF treatment. Furthermore, a better understanding of signaling pathways in tumor angiogenesis, in addition to VEGF-receptors signaling, may help achieve an even more effective anticancer therapy.[2]

Vascular endothelial growth factor-A (VEGF/VEGF-A) is widely expressed in the extracellular matrix and can stimulate both physiological and pathological angiogenesis.[1]

Vascular endothelial growth factor-C (VEGF-C) is expressed most prominently in the heart, placenta, muscle, ovary, and small intestine. VEGF-C transduces different signals through the receptors VEGFR2/Flk-1 and VEGFR3/Flt-4 in vasculoangiogenesis.[3] This growth factor is active in angiogenesis and endothelial cell growth, stimulating cell proliferation and migration; it also affects the permeability of blood vessels. VEGF-C expression is associated with hematological malignancies. In leukemia, VEGF-C, like VEGF, is a survival factor for cancer cells. Together with the expression of their receptors, VEGF and VEGF-C result in the generation of autocrine loops that may support cancer cell survival and proliferation.[1]

Vascular endothelial growth factor-D (VEGF-D), also known as c-Fos-induced growth factor, is structurally and functionally related to VEGF-C.[4] Like VEGF-C, VEGF-D is a ligand for VEGFR2/Flk-1 and VEGFR3/Flt-4. It is initially synthesized as a disulfide-linked prepropeptide containing N- and C-terminal extensions not found in other VEGF polypeptides, flanking a central receptor-binding VEGF homology domain.[5] VEGF-D is a growth factor active in angiogenesis, lymphangiogenesis, and endothelial cell growth, stimulating their proliferation and migration; it also affects the permeability of blood vessels. It helps in the formation of the venous and lymphatic vascular systems during embryogenesis and in the maintenance of differentiated lymphatic endothelium in adults.[6]

Tyrosine kinase-2 (Tie-2), also called TEK, is a receptor-like tyrosine kinase expressed almost exclusively in the endothelium of actively growing blood vessels. Tie-2 is also produced by early hematopoietic cells and is found in the plasma and serum of healthy human individuals.[7]

Soluble fms-like tyrosine kinase 1 (sFlt-1), also known as vascular endothelial growth factor receptor 1 (VEGFR1), is highly expressed in vascular endothelial cells and functions mainly in angiogenesis. sFlt-1 is required for the recruitment of haematopoietic precursors and migration of monocytes and macrophages.[8] sFlt-1 has been shown to mediate monocyte migration, recruit endothelial cell progenitors, improve hematopoietic stem cell survival, and release growth factors from liver endothelial cells.[9] sFlt-1 signaling has been implicated in tumor metastasis.

Placental Growth Factor (PlGF), a member of the VEGF family, shares receptors with VEGF and stimulates angiogenesis. PlGF was first identified in human placenta and is upregulated under certain pathological conditions including wound healing and tumor formation. PlGF is a growth factor active in angiogenesis and endothelial cell growth, stimulating their proliferation and migration. It binds to the receptor sFlt-1.[10]

Basic Fibroblast Growth Factor (bFGF) stimulates the growth and development of new blood vessels (angiogenesis) that contribute to the pathogenesis of several diseases (e.g. cancer, atherosclerosis). The mammalian FGFs mediate numerous developmental programs during embryogenesis and play critical roles in adult tissue repair and maintenance.[11-14] bFGF has a heparin binding domain and oligimerizes upon binding to heparin making it less available to detection. Heparin plasma samples should not be tested for FGF.

Principle of the Assay

MSD vascular assays provide a rapid and convenient method for measuring the levels of protein targets within a single, small-volume sample. The assays in the Human Angiogenesis Panel 1 Kit are sandwich immunoassays (Figure 1). MSD provides a plate pre-coated with capture antibodies. The user adds the sample and a solution containing detection antibodies conjugated with electrochemiluminescent labels (MSD SULFO-TAG™) over the course of one or more incubation periods. Analytes in the sample bind to capture antibodies immobilized on the working electrode surface; recruitment of the detection antibodies by the bound analytes completes the sandwich. The user adds an MSD buffer that provides the appropriate chemical environment for electrochemiluminescence and loads the plate into a SECTOR® Imager where a voltage applied to the plate electrodes causes the captured labels to emit light. The instrument measures the intensity of emitted light to provide a quantitative measure of analytes in the sample.

1. VEGF
2. VEGF-C
3. VEGF-D
4. Tie-2
5. sFlt-1
6. PlGF
7. bFGF

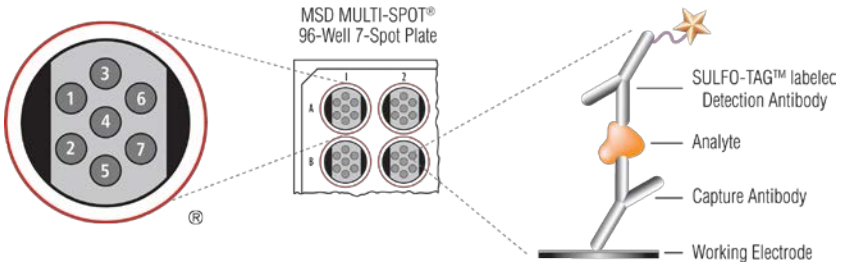


Figure 1. Spot diagram showing placement of analyte capture antibodies. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. A unique bar code label on each plate allows complete traceability back to MSD manufacturing records.

Reagents Supplied

Product Description	Storage	Quantity per Kit K15190D-1	K15190D-2	K15190D-4
MULTI-SPOT 96-Well 7-Spot Angiogenesis Panel 1 (human) Plate N75190A-1	2–8°C	1 plate	5 plates	25 plates
SULFO-TAG Anti-hu VEGF Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-hu VEGF-C Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-hu VEGF-D Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-hu Tie-2 Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-hu sFlt-1 Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-hu PlGF Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
SULFO-TAG Anti-hu bFGF Antibody[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
Angiogenesis Panel 1(human) Calibrator Blend (20X)	≤-70°C	1 vial (20 µL)	5 vials (20 µL ea)	25 vials (20 µL ea)
Diluent 7 R54BB-3 (50 mL)	≤-10°C	1 bottle (50 mL)	2 bottles (50 mL ea)	10 bottles (50 mL ea)
Diluent 11 R50AA-4 (50 mL), R50AA-2 (200 mL)	≤-10°C	1 bottle (50 mL)	2 bottles (50 mL ea)	10 bottles (50 mL ea)
Blocker A Kit (Blocker A [dry] in 250 mL bottle and 50 mL bottle of 5X Phosphate Buffer) R93AA-2 (250 mL)	RT	1 kit (250 mL)	1 kit (250 mL)	5 kits (250 mL ea)
Read Buffer T (4X) R92TC-3 (50 mL)	RT	1 bottle (50 mL)	1 bottle (50 mL)	5 bottles (50 mL ea)

Required Material and Equipment (not supplied)

- ❑ Appropriately sized tubes for reagent preparation
- ❑ Microcentrifuge tubes for preparing serial dilutions
- ❑ Phosphate buffered saline plus 0.05% Tween-20 (PBS-T) for plate washing
- ❑ Liquid handling equipment for desired throughput, capable of dispensing 10 to 150 µL/well into a 96-well microtiter plate
- ❑ Plate washing equipment: automated plate washer or multichannel pipette
- ❑ Adhesive plate seals
- ❑ Microtiter plate shaker
- ❑ Deionized water

[1] SULFO-TAG–conjugated detection antibodies should be stored in the dark.

Optional Material

Angiogenesis Control Pak 1 (available for separate purchase from MSD, catalog # C4190-1)

Safety

Use safe laboratory practices and wear gloves, safety glasses, and lab coats when handling kit components. Handle and dispose of all hazardous samples properly in accordance with local, state, and federal guidelines.

Reagent Preparation

Bring all reagents to room temperature. *This is especially important for Diluent 7, as some components are not soluble below room temperature.* Thaw the stock calibrator on ice.

> Important: Upon first thaw, separate Diluent 7 and Diluent 11 into aliquots appropriate for the size of your needs before refreezing.

Prepare Blocker A Solution

Follow the Blocker A instructions included in the kit.

Prepare Standards

MSD supplies blended calibrator for the Angiogenesis Panel 1 (human) Kit at 20-fold higher concentration than the recommended highest standard. We recommend a 7-point standard curve with 4-fold serial dilution steps and a zero calibrator blank. Signals from the blank should be excluded when generating the curve. Thaw the stock calibrator and keep on ice, then add to diluent at room temperature to make the standard curve solutions. Calibrators should be prepared at room temperature no more than 20 minutes before use. For the actual concentration of each calibrator in the blend, refer to the certificate of analysis (C of A) supplied with the kit. You may also find a copy of the lot-specific C of A at www.mesoscale.com by entering K15190D in the search box.

To prepare 7 standard solutions plus a zero calibrator blank for up to 4 replicates:

1) Prepare the highest standard by adding 10 µL of stock calibrator to 190 µL of Diluent 7. Mix well.
2) Prepare the next standard by transferring 60 µL of the highest standard to 180 µL of Diluent 7. Mix well. Repeat 4-fold serial dilutions 5 additional times to generate 7 standards.
3) Use Diluent 7 as the blank.

Dilute Samples

For human serum and plasma samples, MSD recommends a 2-fold dilution in Diluent 7; however, you may adjust dilution factors for the sample set under investigation. Heparin plasma samples should not be tested for FGF.

To dilute sample 2-fold, add 100 µL of sample to 100 µL of Diluent 7.

Prepare Detection Antibody Solution

MSD provides each detection antibody as a 50X stock solution. The working detection antibody solution is 1X.

For 1 plate, combine:

- ❑ 60 µL of 50X SULFO-TAG Anti-hu VEGF Antibody
- ❑ 60 µL of 50X SULFO-TAG Anti-hu VEGF-C Antibody
- ❑ 60 µL of 50X SULFO-TAG Anti-hu VEGF-D Antibody
- ❑ 60 µL of 50X SULFO-TAG Anti-hu Tie-2 Antibody
- ❑ 60 µL of 50X SULFO-TAG Anti-hu sFlt-1 Antibody
- ❑ 60 µL of 50X SULFO-TAG Anti-hu PlGF Antibody
- ❑ 60 µL of 50X SULFO-TAG Anti-hu bFGF Antibody
- ❑ 2580 µL of Diluent 11

Note: You may omit detection antibody for any analyte not being measured; add 60 µL of Diluent 11 for each omitted antibody.

Prepare Read Buffer

MSD provides Read Buffer T as a 4X stock solution. The working solution is 2X.

For 1 plate, combine:

- ❑ 10 mL of Read Buffer T (4X)
- ❑ 10 mL of deionized water

You may prepare diluted read buffer in advance and store it at room temperature in a tightly sealed container.

Prepare MSD Plate

MSD plates are pre-coated with capture antibodies (Figure 1) and exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies. Plates can be used as delivered; no additional preparation (e.g., pre-wetting) is required.

Protocol

Notes

1. **Add Blocker A Solution:** Add 150 µL of Blocker A solution to each well. Seal the plate with an adhesive plate seal and incubate for 1 hour with vigorous shaking (300–1000 rpm) at room temperature.

Shaking the plate typically accelerates capture at the working electrode.

2. **Wash and Add Sample:** Wash the plate 3 times with 300 µL/well of PBS-T. Add 50 µL of sample (standards, controls, or unknowns) per well. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.

   You may prepare detection antibody solution during incubation.

3. **Wash and Add Detection Antibody Solution:** Wash the plate 3 times with 300 µL/well of PBS-T. Add 25 µL of 1X detection antibody solution to each well. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.

   You may prepare diluted read buffer during incubation.

You may keep excess diluted read buffer in a tightly sealed container at room temperature for later use.

4. **Wash and Read:** Wash the plate 3 times with 300 µL/well of PBS-T. Add 150 µL of 2X Read Buffer T to each well. Analyze the plate on the SECTOR Imager. No incubation in read buffer is required before reading the plate.

Bubbles introduced when adding read buffer will interfere with imaging of the plate and produce unreliable data. Use reverse pipetting technique to avoid creating bubbles.

Due to the varying nature of each research application, you should assess assay stability before allowing plates to sit with read buffer for extended periods.

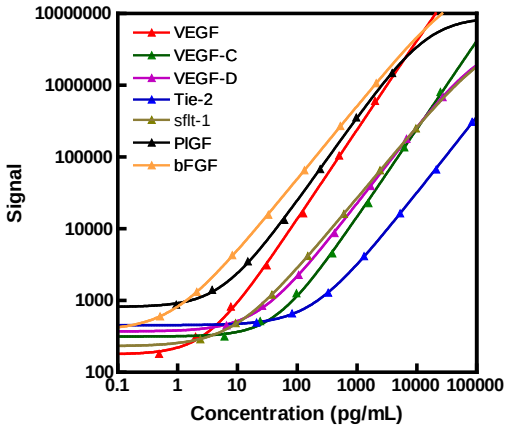
Curve Fitting

MSD DISCOVERY WORKBENCH® software uses least-squares fitting algorithms to generate a standard curve that will be used to calculate the concentration of analyte in the samples. The assays have a wide dynamic range (3–4 logs) that allows accurate quantification without the need for dilution in many cases. The software uses a 4-parameter logistic model (or sigmoidal dose-response) and includes a $1/Y^2$ weighting function. The weighting function is important because it provides a better fit of data over a wide dynamic range, particularly at the low end of the standard curve.

Typical Data

The following standard curve graph illustrates the dynamic range of the assay. Actual signals will vary. Best quantification of unknown samples will be achieved by generating a standard curve for each plate using a minimum of 2 replicates of standards. For each kit lot, refer to the C of A for the actual concentration of the calibrator.

VEGF		
Conc. (pg/mL)	Average Signal	%CV
0	113	7.0
0.51	169	4.7
2.1	311	5.7
8.2	902	4.0
33	3345	2.5
132	14 184	2.0
527	70 869	4.5
2107	378 404	4.1



VEGF-C		
Conc. (pg/mL)	Average Signal	%CV
0	366	11.0
5.8	421	6.5
23	611	3.6
92	1300	8.7
369	4314	7.8
1474	19 420	6.3
5897	116 073	7.2
23 586	669 091	6.8

VEGF-D		
Conc. (pg/mL)	Average Signal	%CV
0	270	11.6
6.0	374	9.6
24	788	4.9
96	2220	5.3
385	8808	1.2
1542	42 300	1.3
6168	207 607	2.0
24 670	710 421	4.0

Tie-2		
Conc. (pg/mL)	Average Signal	%CV
0	328	4.3
20	355	11.2
78	473	4.0
312	838	6.0
1249	2408	4.8
4997	8963	1.5
19 988	37 896	2.1
79 953	163 252	3.0

sFlt-1		
Conc. (pg/mL)	Average Signal	%CV
0	168	12.5
2.1	225	3.5
8.5	311	10.6
34	641	4.2
137	2064	5.6
547	7866	4.8
2187	34 065	4.7
8747	129 052	5.6

PlGF		
Conc. (pg/mL)	Average Signal	%CV
0	671	5.7
0.86	813	9.6
3.4	1290	1.6
14	2875	3.1
55	8949	7.2
220	40 455	4.2
879	208 508	8.4
3514	859 344	4.2

bFGF		
Conc. (pg/mL)	Average Signal	%CV
0	259	22.7
0.51	486	9.6
2.0	1298	4.3
8.2	3916	1.6
33	14 618	3.3
131	58 108	2.2
525	227 349	1.8
2099	786 861	1.1

Sensitivity

The lower limit of detection (LLOD) is a calculated concentration based on a signal 2.5 standard deviations above the background (zero calibrator blank). The LLOD shown below was calculated based on 34 runs on 2 lots of plates.

	VEGF	VEGF-C	VEGF-D	Tie-2	sFlt-1	PlGF	bFGF
Average LLOD (pg/mL)	0.60	8.0	3.4	19	3.0	2.9	0.10
LLOD Range (pg/mL)	0.32–0.96	4.9–17	1.3–6.8	7.7–30	1.4–5.5	0.25–2.5	0.043–0.31

Precision

Control samples of high, medium, and low levels of each analyte were measured using a minimum of 2 replicates on 6 runs over 3 days.

Controls were made by spiking calibrator into human EDTA plasma.

Average intra-run %CV is the average %CV of the control replicates within an individual run.

Inter-run %CV is the variability of controls across 6 runs.

	Control	Runs	Average Conc. (pg/mL)	Average Intra-run %CV	Inter-run %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	10 154	3.0	5.3
	Mid	6	2085	4.3	4.4
	Low	6	73	1.1	8.0
Tie-2	High	6	17 574	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

Dilution Linearity

To assess linearity, normal human serum, EDTA plasma, and heparin plasma samples were spiked with the VEGF, VEGF-C, sFlt-1, and bFGF calibrators and further diluted 4-fold, 8-fold, 16-fold, and 32-fold before testing. Percent recovery at each dilution was calculated by dividing the measured concentration by the expected concentration, i.e., the concentration of the previous dilution. The average percent recovery shown below was calculated from samples with values above the LLOD.

% Recovery=measured/expected∗100

		VEGF		VEGF-C		VEGF-D		Tie-2	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=8)	4	107	101–110	102	91–109	106	98–123	94	89–105
	8	103	98–119	107	91–125	105	101–109	97	84–101
	16	105	97–112	112	100–128	103	89–116	100	95–104
	32	107	100–122	110	100–129	106	96–117	105	96–118
EDTA Plasma (N=8)	4	102	96–108	95	84–110	98	91–109	86	82–90
	8	101	96–107	106	99–112	103	94–109	92	88–97
	16	106	102–114	111	105–115	107	100–116	102	83–145
	32	107	101–115	113	103–126	110	104–116	100	64–124
Heparin Plasma (N=8)	4	108	102–115	101	92–122	98	94–103	93	85–100
	8	102	96–108	105	84–122	105	100–109	98	91–109
	16	108	104–111	110	97–128	107	103–113	99	88–116
	32	107	103–111	111	100–120	109	105–115	99	87–118

		sFlt-1		PlGF		bFGF	
Sample Type	Fold Dilution	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
Serum (N=8)	4	108	100–127	108	100–127	99	94–103
	8	100	92–116	100	92–116	102	97–110
	16	106	97–130	106	97–130	101	95–106
	32	102	94–118	102	94–118	103	96–115
EDTA Plasma (N=8)	4	101	93–107	101	93–107	102	97–106
	8	100	94–109	100	94–109	102	94–108
	16	103	98–116	103	98–116	104	97–115
	32	105	99–116	105	99–116	105	97–122
Heparin Plasma (N=8)	4	107	96–122	107	96–122	*	*
	8	102	97–112	102	97–112	*	*
	16	101	85–111	101	85–111	*	*
	32	104	98–115	104	98–115	*	*

*Heparin plasma samples are not tested for bFGF.

Spike Recovery

Normal human serum, EDTA plasma, and heparin plasma samples were spiked with the calibrators at multiple levels throughout the range of the assay. Heparin plasma samples showed poor recovery when tested with bFGF assay. Spikes were made into neat samples, and then diluted 2-fold. The average percent recovery shown below was calculated from samples with values above the LLOD. % Recovery=measured/expected∗100

	VEGF			VEGF-C			VEGF-D		
Sample Type	Spike Conc. (pg/mL)	Average % Recovery	% Recovery Range	Spike Conc. (pg/mL)	Average % Recovery	% Recovery Range	Spike Conc. (pg/mL)	Average % Recovery	% Recovery Range
Serum (N=8)	13	94	82–101	147	96	79–109	154	95	62–108
	53	85	75–92	590	81	59–95	617	94	64–105
	211	80	76–85	2359	75	56–93	2467	100	89–107
	1686	92	85–95	18 869	94	66–112	19 736	95	77–102
EDTA Plasma (N=8)	13	98	92–106	147	94	91–102	154	100	90–110
	53	94	85–103	590	86	81–96	617	102	93–110
	211	99	82–104	2359	84	82–100	2467	106	90–102
	1686	99	90–104	18 869	105	99–119	19 736	115	90–158
Heparin Plasma (N=8)	13	95	81–102	147	92	84–101	154	98	88–105
	53	92	80–103	590	83	79–95	617	99	91–103
	211	86	76–99	2359	79	74–89	2467	99	91–110
	1686	89	84–97	18 869	99	92–122	19 736	100	89–127

	Tie-2			sFlt-1			PlGF		
Sample Type	Spike Conc. (pg/mL)	Average % Recovery	% Recovery Range	Spike Conc. (pg/mL)	Average % Recovery	% Recovery Range	Spike Conc. (pg/mL)	Average % Recovery	% Recovery Range
Serum (N=8)	500	92	89–94	55	101	82–111	22	93	73–108
	1999	81	76–86	219	95	83–105	88	89	73–97
	7995	67	61–71	875	87	79–94	351	83	77–96
	63 962	70	59–81	6998	85	78–96	2811	88	75–99
EDTA Plasma (N=8)	500	87	80–102	55	98	77–106	22	100	89–109
	1999	78	66–99	219	95	87–111	88	98	90–105
	7995	67	49–90	875	90	76–104	351	95	81–105
	63 962	78	44–110	6998	86	73–97	2811	102	93–113
Heparin Plasma (N=8)	500	92	76–101	55	97	67–121	22	95	80–106
	1999	82	71–91	219	87	70–96	88	91	87–99
	7995	71	59–85	875	76	66–84	351	89	82–93
	63 962	80	65–107	6998	74	64–96	2811	101	88–116

	bFGF		
Sample Type	Spike Conc. (pg/mL)	Average % Recovery	% Recovery Range
Serum (N=8)	13	82	74–92
	52	78	73–90
	210	80	69–93
	1679	95	84–102
EDTA Plasma (N=8)	13	83	74–97
	52	79	72–90
	210	78	66–89
	1679	98	77–117
Heparin Plasma (N=8)	13	43	33–54
	52	40	33–50
	210	43	36–52
	1679	64	48–81

Specificity

Each assay in the panel recognizes both native and recombinant proteins. The kit is tested for cross-reactivity to other related analytes: Ang-1, Ang-2, Ang-4, Flk-1, MMP-2, MMP-9, Gro-alpha, NRP-1, NRP-2, VEGF-B, Flt-4, and ANGPTL1 at concentrations of 0, 10, 100, and 1000 ng/mL. All analytes in the MSD Angiogenesis Panel 1 (human) have less than 1% signal cross-reactivity with other tested analytes as well as with analytes within the panel. The only exceptions are with Tie-2 and VEGF-C.

The VEGF-C assay was tested with samples and recombinant protein using an individual detection antibody. All assays have <0.5% cross-reactivity with native samples; however, approximately 3.0% cross-reactivity was observed with VEGF-D recombinant protein. Although VEGF binds to the Flt-1 receptor, cross-reactivity testing showed that sFlt-1 signals were not affected with 5 ng/mL of VEGF, and VEGF signals were not affected with 20 ng/mL of sFlt-1.

Ang-1 and Ang-2 ligands usually bind to the Tie-2 receptor. When Ang-1 and Ang-2 were tested at 50 ng/mL on the Tie-2 assay, the observed Tie-2 signal was elevated by 20%. The same effect was not observed with Ang-4 or ANGPTL-1.

Interference

Since murine monoclonal antibodies are used in the assays, different lots of HAMA+ and RF+ samples were run with the Angiogenesis Panel 1 (human) using individual and blended detection antibodies to test their interference with the analytes.

No cross-reactivity or interference from HAMA or RF samples was detected.

Stability

Kit components were tested for freeze-thaw stability. Results (not shown) demonstrated good stability for 5 freeze-thaws for all components with the exception of sFlt-1 calibrator which is stable for 3 freeze-thaws.

Tested Samples

Serum, EDTA plasma, and heparin plasma samples were tested at 2-fold dilutions with the Angiogenesis Panel-1 (human). Median and range of concentrations for each sample set are displayed below. Concentrations are corrected for sample dilution. The lower limit of quantification (LLOQ) value for bFGF is 1.0 pg/mL for the sample set tested. Heparin plasma samples are not recommended to be used with bFGF assay.

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
	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
	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	-
	Samples in Quantitative Range	13	13	13	13	13	13	-

**The LLOQ is determined as the lowest concentration where the %CV of the calculated concentration is less than 20% and the percent recovery of the standard is between 80% and 120%.

Reference Standard

National Institute for Biological Standards and Control (NIBSC) reference standards for VEGF, PIGF, and bFGF recombinants were calibrated against MSD blended calibrator for all of the 3 assays. Shown below are the conversions determined for all 3 assays based on experiments run over 3 days.

	WHO Standard[3]			Conversion determined from 3-day experiments run on Angiogenesis Panel-1 (human) Kit
Assay	NIBSC code	Units/ampoule	Concentration/ ampoule	
VEGF	02/286	13 000 units	13 µg/mL	485 units of NIBSC = 1 µg/mL of MSD VEGF standard
PIGF	09/272	5000 units	10 µg/mL	480 units of NIBSC = 1 µg/mL of MSD PIGF standard
bFGF	90/712	1600 IU	4 µg/mL	680 IU of NIBSC = 1 µg/mL of MSD bFGF standard

[3]NIBSC standards are reconstituted and aliquoted as per the world health organization (WHO) specification sheet.

Assay Components

Calibrators

The assay calibrator blend uses the following recombinant human proteins:

VEGF (residues 27-191) expressed in baculovirus based *Sf* 21 insect cells

VEGF-C (residues 103-227) expressed in murine myeloma cells

VEGF-D (residues 93-201) expressed in baculovirus based *Sf* 21 insect cells

Tie-2 (residues 23-745) expressed in murine myeloma cells

sFlt-1 (residues 27-687) expressed in baculovirus based *Sf* 21 insect cells

PlGF (residues 21-149) expressed in E.coli

bFGF (residues 132-288) expressed in E.coli

Antibodies

	Source Species	
Analyte	MSD Capture Antibody	MSD Detection Antibody
VEGF	Mouse Monoclonal	Mouse Monoclonal
VEGF-C	Mouse Monoclonal	Goat Polyclonal
VEGF-D	Mouse Monoclonal	Goat Polyclonal
Tie-2	Mouse Monoclonal	Goat Polyclonal
sFlt-1	Mouse Monoclonal	Goat Polyclonal
PlGF	Mouse Monoclonal	Goat Polyclonal
bFGF	Mouse Monoclonal	Mouse Monoclonal

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Summary Protocol

MSD 96-well MULTI-SPOT Angiogenesis Panel 1 (human) Kit

MSD provides this summary protocol for your convenience. Please read the entire detailed protocol prior to performing the Angiogenesis Panel 1 (human) assays.

Sample and Reagent Preparation

Bring all reagents to room temperature and thaw the calibrator on ice.

Prepare Blocker A solution.

Prepare 7 standard solutions using the supplied calibrator:

- Dilute the stock calibrator 20-fold in Diluent 7.
- Perform a series of 4-fold dilution steps and prepare a zero calibrator blank.

Dilute samples 2-fold in Diluent 7 before adding to the plate.

Prepare combined detection antibody solution by diluting each stock detection antibody 50-fold in Diluent 11.

Prepare 2X Read Buffer T by diluting 4X Read Buffer T 2-fold with deionized water.

Step 1: Add Blocker A Solution

Add 150 µL/well of Blocker A Solution.

Incubate at room temperature with vigorous shaking (300–1000 rpm) for 1 hour.

Step 2: Wash and Add Sample

Wash plate 3 times with 300 µL/well of PBS-T.

Add 50 µL/well of sample (standards, controls, or unknowns).

Incubate at room temperature with vigorous shaking (300–1000 rpm) for 2 hours.

Step 3: Wash and Add Detection Antibody Solution

Wash plate 3 times with 300 µL/well of PBS-T.

Add 25 µL/well of 1X detection antibody solution.

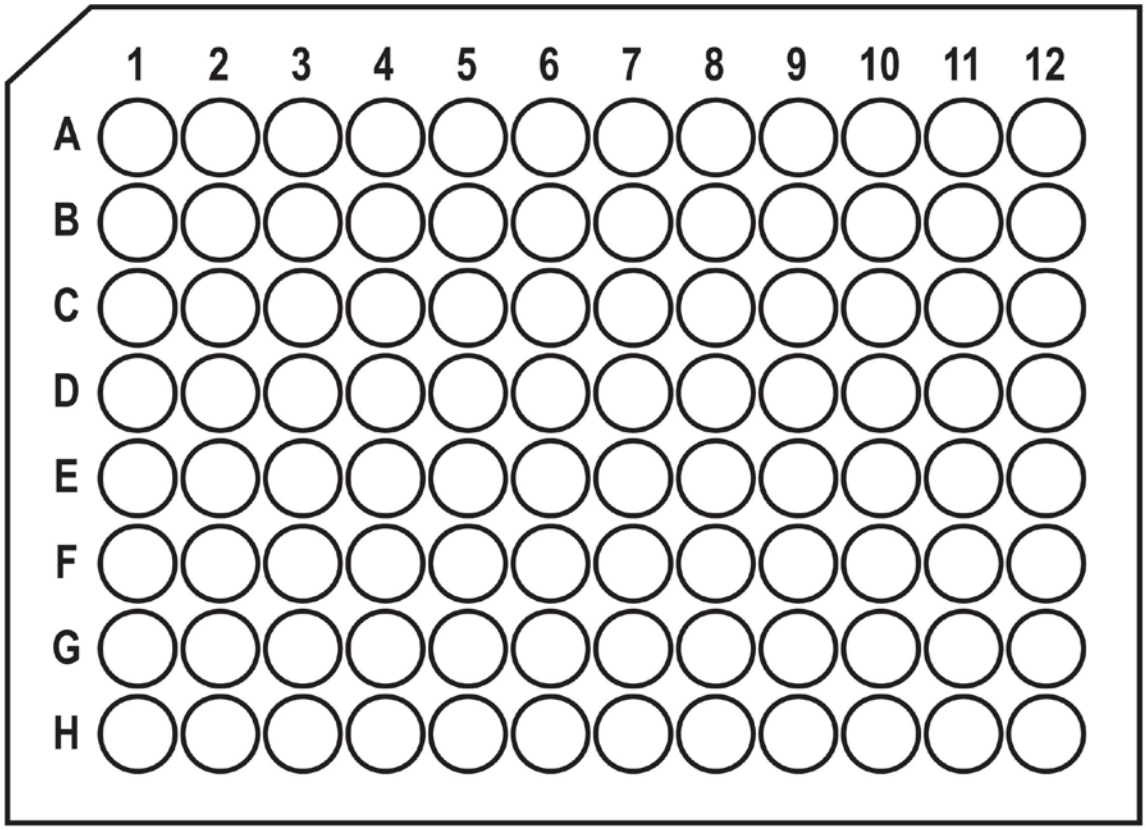
Incubate at room temperature with vigorous shaking (300–1000 rpm) for 2 hours.

Step 4: Wash and Read Plate

Wash plate 3 times with 300 µL/well of PBS-T.

Add 150 µL/well of 2X Read Buffer T.

Analyze plate on SECTOR Imager.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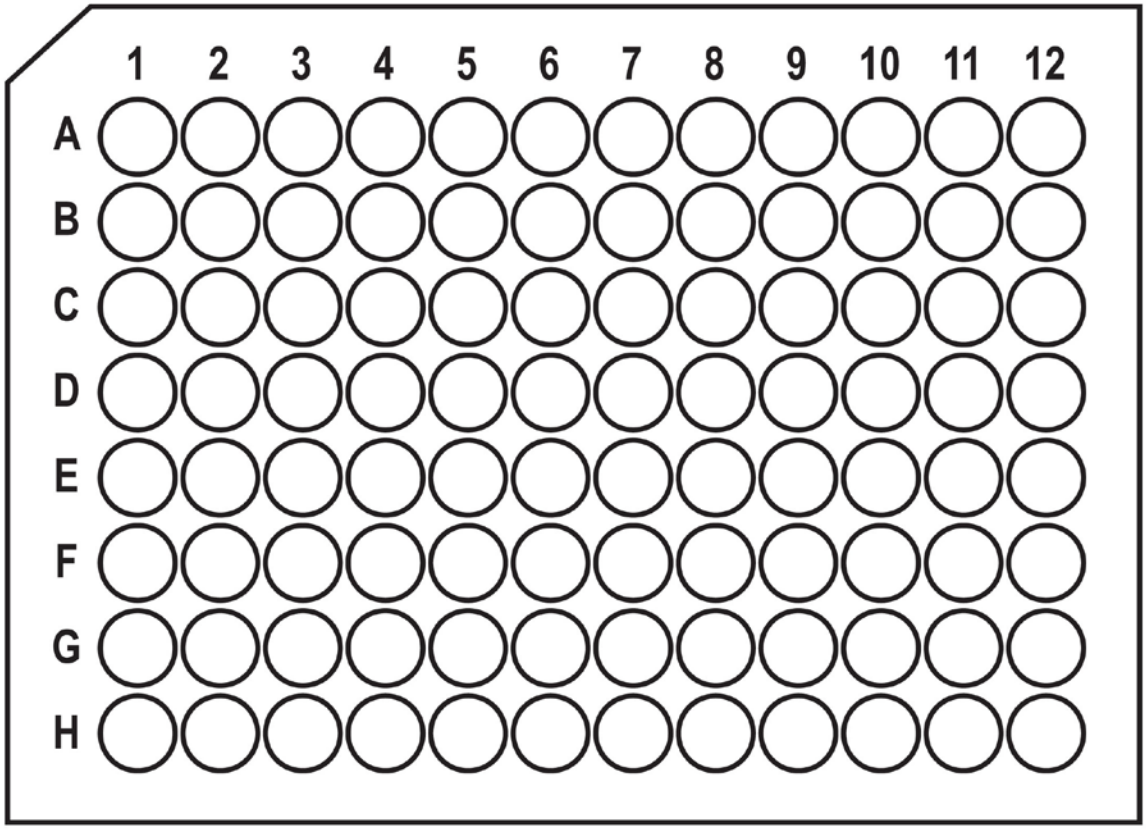
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