Sword ELISA Booster

For Human VEGF

Catalog No. SB-HVEGF02-05

For use with the

Human VEGF DuoSet ELISA from R&D Systems (Cat# DY293B)

This package insert must be read before using this product.
For research use only.
Not for use in diagnostic procedures.

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MANUFACTURED AND DISTRIBUTED BY:

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INTRODUCTION

Vascular endothelial growth factor (VEGF) was identified as a cell specific mitogen, and a mediator of both angiogenesis and vasculogenesis.[1] It contributes to the development of tumors, and elevated levels of VEGF production are able to be detected in tumor cells at the extreme periphery of the tumor.[2] In response to tumor-induced hypoxia there have been high levels of VEGF found in various types of tumors, but this is not the case in normal tissue.[3] Elevated levels of VEGF result in bronchial asthma, diabetic retinopathy, rheumatoid arthritis, and tumor growth and, the biological properties of VEGF make it an important therapeutic target. This Sword ELISA Booster has been optimized for use with the R&D Systems Human VEGF DuoSet ELISA (Cat#DY293B).

MATERIALS

Booster Components

Part	Volume	Supplied
Sword ELISA Booster - Component A (10X)	**9 mL**	**1 - bottle**
Sword ELISA Booster - Component B (10X)	**9 mL**	**1 - bottle**
Sword Diluent - Component C (10X)	**9 mL**	**1 - bottle**
Sword Development Solution - Component D (5X)	**18 mL**	**1 - bottle**
Sword ELISA Blocker for VEGF - Product no. SBL-503-05	**110 mL**	**1 - bottle**
Sword ELISA Sample Diluent for VEGF Product no. SDI-801-05	**110 mL**	**1 - bottle**
Sword ELISA HRP Conjugate - Product no. SCO-005	**30 µL**	**1 - tube**

Each Booster provides sufficient reagents for approximately 500 wells using a final reaction volume of 300 µL per assay. **Do not mix reagent components from different Reagent Booster lots.**

STORAGE AND HANDLING

Upon receipt, the booster should be stored at 2-8°C, protected from light. Stored properly, the booster components should remain stable until the expiration date designated with the booster. Allow reagents to warm to room temperature before opening component containers.

ADDITIONAL MATERIAL REQUIRED

R&D Systems Human VEGF DuoSet ELISA (Cat# DY293B)

96 well microplates, high binding, clear or black (Recommended: Nunc™ Maxisorp™ or similar)

Plate seals

Tris Buffered Saline (TBS)

Wash Buffer: TBS + 0.05% Tween® 20

Reagent Diluent: 2% BSA, Fraction V in TBS

Deionized water

Proper pipettes and pipette tips

Multimode plate reader compatible with Sword ELISA Booster chemistry (see Appendix 1)

EXPERIMENTAL PROTOCOL

The following protocol is designed for use with the R&D Systems Human VEGF DuoSet ELISA (Cat# DY293B). Qualified detectors are listed in Appendix 1.

Preparation of Working Solutions for Sword ELISA Booster

1.1 **Preparation of Sword ELISA Booster substrate solution:**

The following example is to prepare 16 mL Sword ELISA Booster substrate solution, enough for one 96 well plate. If you are not using a full plate, scale accordingly.

- Add the following to 11.2 mL deionized water:
- 1.6 mL Sword Booster - Component A (10X)
- 1.6 mL Sword Booster - Component B (10X)
- 1.6 mL Sword Diluent - Component C (10X)

Sufficient 10X Sword Diluent, Sword Booster Component A, and Sword Booster Component B have been provided to prepare sufficient Sword Booster to run 500 tests. This mixture is stable at 2-8º C for 3 days, but it is best if used within 1 - 3 hours. Protect this solution from air and light. The Sword substrate solution should be yellow in color.

Note: Salts in the Sword Diluent Component C (10X) may precipitate upon prolonged storage at 2-8°C. These salts readily re-dissolve by gentle inversion when the 10X solution is brought to Room Temperature. Check vial before using this reagent.

1.2 **Preparation of 1X Sword Development Solution:**

The following example is to prepare 16 mL Sword Development Solution, enough for one 96 well plate. If you are not using a full plate, scale accordingly.

- Add 3.2 mL 5X Sword Development Solution (Component D) to 12.8 mL deionized water.

Caution: *Both the 5X and 1X Development Solutions are caustic and should not come in contact with the skin*.

Assay Procedure

2.1 Reconstitute the Human VEGF Capture Antibody with 0.5 mL of TBS to prepare a 120 µg/mL concentrate of mouse anti-human VEGF antibody. Dilute the concentrated Human VEGF Capture Antibody to a working concentration of 4.0 µg/mL in TBS, without carrier protein. Immediately coat a 96-well microplate with 100 µL/well of the diluted Human VEGF Capture Antibody. Seal the plate and incubate overnight at 2-8°C.

2.2 Aspirate each well and wash with 400 µL per well Wash Buffer (TBS + 0.05% Tween® 20), repeating the process 2 times for a total of 3 washes. Complete removal of liquid at each step is essential to good performance. For each wash, allow Wash Buffer to sit in plate for 15 - 30 seconds prior to aspiration. Use of an automated plate washer is recommended. After the last wash, remove any

excess wash buffer by aspirating or decanting. Invert plate and blot it against clean paper towels.

2.3 Block plates by adding 200 µL Sword ELISA Blocker for VEGF to each well. Cover with a plate seal and incubate at room temperature for a minimum of 1 hour.

2.4 Reconstitute Human VEGF Standard with 0.5 mL of Sword ELISA Sample Diluent for VEGF to prepare a 100 ng/mL concentrate of recombinant human VEGF. Prepare dilutions of standard in Sword ELISA Sample Diluent for VEGF. A seven-point standard curve, with a high standard of 1000 pg/mL and a low standard of 0.24 pg/ml, is recommended. Prepare sufficient volumes to add 25 µl standard or sample per well.

2.5 Repeat the aspiration/wash as in Step 2.2. The plates are now ready for sample addition.

2.6 Add 75 uL of Sword ELISA Sample Diluent for VEGF per well.

2.7 Add 25 µL of standard or sample per well. Cover with a plate seal and incubate 2 hours at room temperature on a rotator/shaker.

2.8 Repeat the aspiration/wash as in Step 2.2.

2.9 Reconstitute the Human VEGF Detection Antibody with 1.0 mL Reagent Diluent (2% BSA in TBS) to prepare a 6.0 µg/mL concentrate of biotinylated goat anti-human VEGF antibody. Dilute to a working concentration of 100 ng/mL in Reagent Diluent (2% BSA in TBS).

2.10 Add 100 µL of the diluted Human VEGF Detection Antibody to each well. Cover with a plate seal and incubate 2 hours at room temperature on a rotator/shaker.

2.11 Repeat the aspiration/wash as in Step 2.2.

2.12 Dilute Sword ELISA HRP Conjugate 1:5000 in Reagent Diluent (2% BSA in TBS) (i.e. To prepare sufficient volume for one plate, add 2.4 µl Sword ELISA HRP Conjugate to 12 mL Reagent Diluent. Mix well). Add 100 µL of the diluted Sword ELISA HRP Conjugate to each well. Cover with a plate seal and incubate for 20 minutes at room temperature on a rotator/shaker. Avoid placing the plate in direct light.

2.13 Repeat the aspiration/wash as in Step 2.2, for a total of 6 washes.

2.14 Add 150 µL of the Sword ELISA Booster substrate solution (prepared in step 1.1) to each well. Incubate for 15 minutes at room temperature in the dark with no shaking.

2.15 Add 150 µL of Sword Development solution (prepared in step 1.2). The assay mixture should turn pink upon the addition of Sword Development solution. Incubate for 30 minutes in the dark. Due to the high volume in the wells, it is critical that caution is taken to avoid spilling contents. ***Do not put a lid on the plate. Do not shake.***

2.16 Determine the relative fluorescence units (RFU) of each well, using a microplate reader set to fluorescence with excitation and emission settings depending on the detector as listed in Appendix 1. Use the auto calculate function to determine the optimal gain setting. Signal will be stable for up to 90 minutes after development.

EVALUATION OF RESULTS

A standard curve is generated by plotting the mean signal values from the standard samples against the concentration of the standard samples.

3.1 For the most accurate results, the standard sample data should be fit to a four-parameter logistic curve (4PLC) using the appropriate computer software for this iterative fitting process.

The 4PLC equation: $$Y = D + \frac{(A-D)}{\left[1+\left(\frac{X}{C}\right)^{B}\right]}$$

communicated by D. Rodbard[4] has been used by Sword Diagnostics for this fitting process.

PERFORMANCE

Sword Booster Performance Human VEGF		DuoSet® Insert	Sword Boost	Quantikine® Insert	Sword Boost
Assay Range:	0.40-1000 pg/mL	31.2-2000 pg/mL	78.0x	15.6-1000 pg/mL	39.0x
LLOQ:	0.49 pg/mL	31.2 pg/mL	63.7x	15.6 pg/mL	31.8x
Sample Size:	100 µL	100 µL		100 µL	
Spike Recovery: (Plasma)	0.24 pg/mL				
Spike Recovery: (Serum)	0.24 pg/mL				

Sensitivity

Figure 1. Sword ELISA Booster for Human VEGF

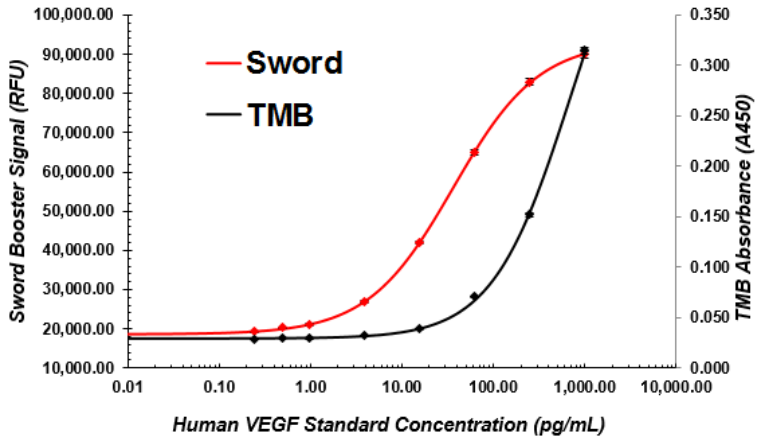

Figure 1. Human VEGF standard curve (red line) generated as described in “Experimental Protocol” section of this document in comparison to TMB (black line). The curve was fit to a four-parameter logistic curve (4PLC). Standard error bars are shown with each data point.

The **Limit of Detection** (LOD) of Human VEGF with this procedure is **0.40 pg/mL**. The limit of detection, as defined by Sword Diagnostics, is the lowest concentration of standard with signal greater than the sum of the mean zero standard and the standard deviation of the zero standard values. The **Lower Limit of Quantitation** (LLOQ) is the lowest concentration of standard at or above the LOD, with back-calculated accuracy of 80 - 120% and %CV of 25% or less. The LLOQ with this procedure is **0.49 pg/mL.** The recommended assay range for this procedure is 0.24 – 1000 pg/mL.

The reported assay range for TMB in the R&D Systems Human VEGF DuoSet ELISA is 31.20 - 2000 pg/mL.[5] The reported assay sensitivity for TMB in the R&D Systems Human VEGF Quantikine ELISA Kit is 9 pg/mL and the reported assay range is 15.6 – 1000 pg/mL.[6]

Precision

Table 1: Precision of VEGF Quantification in Human Serum with Sword ELISA Booster

	VEGF Quantified (pg/ml)				
Donor	**Run 1**	**Run 2**	**Run 3**	**Average**	**CV%**
Very Low	0.05	0.42	0.53	0.33	75.0%
Low	1.14	1.38	1.92	1.48	27.1%
Medium	3.46	3.86	3.88	3.73	6.4%
High	9.15	10.30	10.75	10.07	8.2%

Table 1. VEGF levels were quantified in human serum from healthy donors using the R&D Systems Human VEGF DuoSet ELISA (DY293B) with Sword ELISA Booster for Human VEGF. Donor samples were tested in duplicate in three separate runs.

Table 2: Precision of VEGF Quantification in Human Plasma EDTA with Sword ELISA Booster

Plasma EDTA

	VEGF Quantified (pg/ml)				
Donor	**Run 1**	**Run 2**	**Run 3**	**Average**	**CV%**
Very Low	0.71	0.31	0.85	0.63	44.9%
Low	0.69	0.96	1.31	0.98	31.3%
Medium	2.90	3.57	3.69	3.39	12.6%
High	7.30	7.95	10.02	8.42	16.8%

Table 2. VEGF levels were quantified in human plasma EDTA from healthy donors using the R&D Systems Human VEGF DuoSet ELISA (DY293B) with Sword ELISA Booster for Human VEGF. Donor samples were tested in duplicate in three separate runs.

Recovery

Table 3: Spike Recovery with Sword ELISA Booster for Human VEGF in Serum

VEGF Spike (pg/ml)	Quantified VEGF (pg/ml)	Accuracy %	CV%
0.00	4.12	-	9.2%
0.24	4.38	100%	2.6%
0.49	4.57	99%	1.8%
0.98	4.80	94%	4.0%
3.91	7.05	88%	3.2%
15.63	16.69	85%	1.4%
62.50	52.73	79%	1.7%
250.00	223.70	88%	9.6%
1000.00	1193.54	119%	21.2%

Table 3. Human VEGF Reference Standard was spiked into pooled human serum from healthy donors. Human VEGF levels were quantified using the R&D Systems Human VEGF DuoSet ELISA (DY293B) with Sword ELISA Booster for Human VEGF.

Table 4: Spike Recovery with Sword ELISA Booster for Human VEGF in Plasma EDTA

VEGF Spike (pg/ml)	Quantified VEGF (pg/ml)	Accuracy %	CV%
0.00	2.51	-	8.0%
0.24	2.61	95%	5.4%
0.49	2.94	98%	8.3%
0.98	3.13	90%	0.5%
3.91	5.83	91%	3.1%
15.63	17.13	94%	3.1%
62.50	59.72	92%	1.7%
250.00	235.51	93%	4.6%
1000.00	1089.75	109%	25.6%

Table 4. Human VEGF Reference Standard was spiked into pooled human plasma EDTA from healthy donors. Human VEGF levels were quantified using the R&D Systems Human VEGF DuoSet ELISA (DY293B) with Sword ELISA Booster for Human VEGF.

Linearity

Table 5: Linearity with Sword ELISA Booster for Human VEGF in Diluted Serum and Plasma EDTA

	VEGF Quantified (pg/mL)	
Dilution	Serum	Plasma
1:1	1176.23	1100.30
1:2	525.25	465.98
1:4	218.71	217.63
1:8	119.67	116.98
1:16	64.10	60.36

Table 5. Human VEGF Reference Standard was spiked into pooled human serum and plasma EDTA from healthy donors. Spiked samples were diluted in Sword ELISA Sample Diluent for VEGF. Human VEGF levels were quantified using the R&D Systems Human VEGF DuoSet ELISA (DY293B) with Sword ELISA Booster for Human VEGF.

Quantification

Table 6: Quantification of VEGF in Healthy Human Serum

	VEGF Quantified (pg/mL)	
Donor	**Range**	**Average**
1	8.99 - 14.33	11.08
2	4.39 - 5.84	5.07
3	1.41 - 2.66	1.88
4	4.70 - 6.64	5.55
5	5.74 - 8.57	6.95
6	0.05 - 0.53	0.33
7	1.14 - 1.92	1.48
8	3.46 - 3.88	3.73
9	0.73 - 1.92	1.47
10	5.75 - 8.59	7.21
11	9.15 - 10.75	10.07
12	ND - 0.83	0.29

Table 6. Human VEGF was quantified in human serum from twelve healthy donors using the R&D Systems Human VEGF DuoSet ELISA (DY293B) with Sword ELISA Booster for Human VEGF. Measured human VEGF levels varied from not detectable (ND) to 14.33 pg/ml. Mean and median human VEGF levels were 4.59 pg/ml and 4.40 pg/ml, respectively.

Table 7: Quantification of VEGF in Healthy Human Plasma

	VEGF Quantified (pg/mL)	
Donor	**Range**	**Average**
1	1.92 - 3.28	2.47
2	2.90 - 3.69	3.39
3	0.69 - 1.31	0.98
4	1.62 - 2.51	2.10
5	0.70 - 1.45	1.04
6	1.59 - 2.69	2.14
7	0.19 - 1.00	0.56
8	4.52 - 7.09	5.39
9	7.30 - 10.02	8.42
10	0.83 - 1.67	1.27
11	0.31 - 0.85	0.63
12	4.37 - 5.93	5.06

Table 7. Human VEGF was quantified in human plasma EDTA from twelve healthy donors using the R&D Systems Human VEGF DuoSet ELISA (DY293B) with Sword ELISA Booster for Human VEGF. Measured human VEGF levels varied from 0.19 pg/ml to 10.02 pg/ml. Mean and median human VEGF levels were 2.79 pg/ml and 2.12 pg/ml, respectively.

REFERENCES

[1]Muller YA, et al.: The crystal structure of vascular endothelial growth factor (VEGF) refined to 1.93 A resolution: multiple copy flexibility and receptor binding. Structure 1997, 5 (10):1325–1338

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[3]Soker, S, et al.: Characterization of Novel Vascular Endothelial Growth Factor (VEGF) Receptors on Tumor Cells that Bind VEGF166 via its Exon 7-encoded Domain. The Journal of Biological Chemistry 1996, 271:5761-5767.

[4]Rodbard D: Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. Clinical chemistry 1974, 20:1255-70.

[5]www.rndsystems.com. R&D Systems DuoSet ELISA for Human VEGF, 2016.

[6]www.rndsystems.com. R&D Systems Human VEGF Quantikine ELISA Kit, 2016.

TECHNICAL SUPPORT

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APPENDIX 1. COMPATIBLE DETECTORS

Vendor	Model	Sword Compatible	Filter Mode	Monochromater	Red PMT
BioTek	Cytation 5	Yes	Best	Ok	Best
BioTek	Cytation 3	Yes	Good	Ok	Best
BioTek	Synergy H1, H4, 2	Yes	Good	Ok	Best
BioTek	NEO, NEO2	Yes	Best	Good	Best
Tecan	Infinite M1000 Pro	Yes	n/a	Best	n/a
Tecan	Infinite F500 Pro	Yes	Best	n/a	n/a
Tecan	Infinite 200 Pro	Yes	n/a	Good	n/a
Tecan	Spark 10m / 20M	Yes	n/a	Best	n/a
Molecular Devices	SpectroMax Paradigm	Yes	Good	Ok	
Molecular Devices	SpectraMax M Series	No			
Perkin Elmer	Envision	No			
Perkin Elmer	Victor	No			

APPENDIX 2. INSTRUMENT SETTINGS

BioTek Cytation 5 Parameter	Setting
Detection Method	Fluorescence Intensity
Read Type	Endpoint/Kinetic
Optics Type	Filter
Excitation Wavelength	530 nm
Excitation Bandwidth	25 nm
Emission Wave Length	730 nm
Emission Bandwidth	40 nm
Optics Position	Top 570 nm
Gain	Extended
Read Height	Calibrate for high well (usually A1)

Note: A red-shifted PMT is recommended for best results, but not critical.

BioTek Synergy H4 Parameter	Setting
Detection Method	Fluorescence Intensity
Read Type	Endpoint/Kinetic
Optics Type	Filter
Excitation Wavelength	530 nm
Excitation Bandwidth	25 nm
Emission Wave Length	680 nm*
Emission Bandwidth	30 nm
Optics Position	570
Gain[a]	Extended
Read Height	Calibrate for high well (usually A1)

*Note: *The 680 nm emission filter is recommended for in the instruments without a red-shifted PMT. If the H4 being used has a red-shifted PMT, we recommend the 730 nm/40 nm BW emission filter.*

BioTek NEO2 Parameter	Setting
Detection Method	Fluorescence Intensity
Read Type	Endpoint/Kinetic
Optics Type	Filter
Excitation Wavelength	530 nm
Excitation Bandwidth	25 nm
Emission Wave Length	730 nm
Emission Bandwidth	40 nm
Optics Position	Top 570 nm
Gain	Autoscale to high well (usually A1)
Read Height	Calibrate for high well (usually A1)

Note: The NEO2 is equipped with the red-shifted PMT, and it should be used for our application.

Tecan Infinite M1000 Pro Parameter	Setting
Measurements	Fluorescence Intensity
Mode	Top
Excitation Wavelength	530 nm
Excitation Bandwidth	20 nm
Emission Wave Length	700 nm
Emission Bandwidth	20 nm
Flashes	25 (100 Hz)
Integration	20 µsec
Lag Time	0 µsec
Gain[a]	Auto based on high well (usually A1)
Z-Position	Auto based on high well (usually A1)