BioLegend®

LEGEND MAX™

ELISA Kit with Pre-coated Plate

Human VEGF

(free-form)

Cat. No. 446507 1 Plate

ELISA Kit for Accurate Quantitation of Human Free VEGF from Cell Culture Supernatant, Serum, and Plasma

BioLegend, Inc.
biolegend.com

It is highly recommended that this manual be read in its entirety before using this product. Do not use this kit beyond the expiration date.

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Introduction:

VEGF (also known as VEGF-A) is a signaling protein with strong vascular permeability activity that stimulates the formation of new blood vessels during the processes of vasculogenesis and angiogenesis. It is a member of the vascular endothelial growth factor family, which in mammals is comprised of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF (placental growth factor). There are multiple isoforms of VEGF that result from alternative splicing of mRNA. VEGF binds and activates two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1), through which VEGF exerts its mitogenic effects. VEGF is highly expressed in solid tumors of breast, lung, renal, colorectal, and hepatic origin. Additionally, it significantly contributes to ascites tumor formation. Beyond cancer, VEGF has also been associated with multiple pathogenic conditions including rheumatoid arthritis, age-related macular degeneration, Crow-Fukase syndrome, sclerosis and Alzheimer's disease.

The BioLegend LEGEND MAX™ Human VEGF Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) kit includes a 96-well strip plate that is pre-coated with a mouse monoclonal anti-human VEGF capture antibody. The detection antibody is a biotinylated goat polyclonal anti-human VEGF antibody. This kit is specifically designed for the accurate quantitation of free-form human VEGF from cell culture supernatant, serum, plasma, and other biological fluids. It is analytically validated with ready-to-use reagents.

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Materials Provided:

Description	Quantity (1 plate)	Volume (per bottle)	Part #
Anti-human VEGF Precoated 96-well Strip Microplate	1 plate		750000191
Human VEGF Detection Antibody	1 bottle	12 mL	750000192
Human VEGF Standard	1 vial	lyophilized	750000193
Avidin-HRP	1 bottle	12 mL	77897
Assay Buffer A	1 bottle	25 mL	78232
Assay Diluent D	1 bottle	25 mL	76384
Wash Buffer (20X)	1 bottle	50 mL	78233
Substrate Solution F	1 bottle	12 mL	79132
Stop Solution	1 bottle	12 mL	79133
Plate Sealers	4 sheets		78101

Materials to be Provided by the End-User:

- Microplate reader able to measure absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 1 µL to 1,000 µL
- Deionized water
- Wash bottle or automated microplate washer
- Log-Log graph paper or software for data analysis
- Polypropylene tubes to prepare standard dilutions
- Timer
- Plate Shaker
- Polypropylene vials

Storage Information:

Store unopened kit components between 2°C and 8°C. Do not use this kit beyond its expiration date.

<table>
<tr><th colspan="2">Opened or Reconstituted Components</th></tr>
<tr><td>Microplate Strips</td><td>If not all microplate strips are used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal. Store between 2°C and 8°C for up to one month.</td></tr>
<tr><td>Standard</td><td>The remaining reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Detection Antibody</td><td rowspan="7">Store opened reagents between 2°C and 8°C and use within one month.</td></tr>
<tr><td>Avidin-HRP</td></tr>
<tr><td>Assay Buffer A</td></tr>
<tr><td>Assay Diluent D</td></tr>
<tr><td>Wash Buffer (20X)</td></tr>
<tr><td>Substrate Solution F</td></tr>
<tr><td>Stop Solution</td></tr>
</table>

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online at BioLegend's website for details (www.biolegend.com/msds).
2. Substrate Solution F is harmful if inhaled or ingested. Avoid skin, eye and clothing contact.
3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum, plasma and other biological fluids in accordance with NCCLS regulations.
4. Stop Solution contains strong acid and is corrosive. *Wear eye, hand, and face protection.*
5. Before disposing of the plate, rinse it with an excess amount of tap water.

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Specimen Collection and Handling:

Specimens should be clear and non-hemolyzed. If possible, unknown samples should be run at a number of dilutions to determine the optimal dilution factor that will ensure accurate quantitation.

Cell Culture Supernatant: If necessary, centrifuge all samples to remove debris prior to analysis. It is recommended that samples be stored at < -70°C. Avoid repeated freeze-thaw cycles.

Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 20 minutes at 1,000 x *g*. Remove serum layer and assay immediately or store serum samples at < -70°C. Avoid repeated freeze-thaw cycles.

Plasma: Collect blood samples in citrate, heparin or EDTA containing tubes. Centrifuge for 20 minutes at 1,000 x *g* within 30 minutes of collection. Assay immediately or store plasma samples at < -70°C. Avoid repeated freeze-thaw cycles. Heparin plasma is not recommended as a sample type due to poor spike recovery and linearity.

Reagent and Sample Preparation:

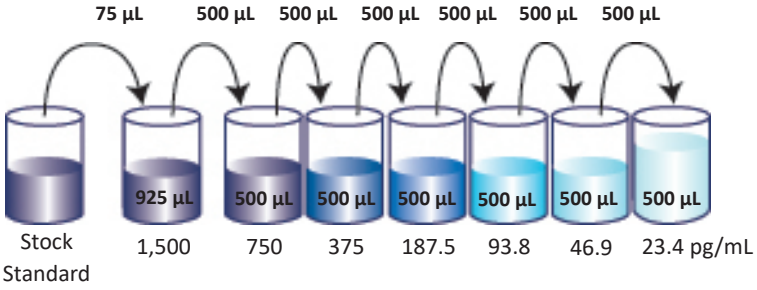
Note: All reagents should be diluted immediately prior to use.

1. Dilute the 20X Wash Buffer to 1X with deionized water. For example, make 1 liter of 1X Wash Buffer by adding 50 mL of 20X Wash Buffer to 950 mL of deionized water. If crystals have formed in the 20X Wash Buffer, bring to room temperature and vortex until dissolved.
2. Reconstitute the lyophilized Human VEGF Standard by following the instructions described in LEGEND MAX™ Kit Lot-Specific Certificate of Analysis/LEGEND MAX™ Kit Protocol enclosed in the kit. Allow the reconstituted standard to sit at room temperature for 15 to 20 minutes, then briefly vortex to mix completely.
3. In general, samples are analyzed without dilutions. If dilutions are required, use Assay Buffer A as the sample diluent.

Assay Procedure:

Note: Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this kit.

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.
2. If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.
3. Prepare 1,000 µL of the 1,500 pg/mL top standard by adding 75 µL of the standard stock in 925 µL of Assay Buffer A. Perform six two-fold serial dilutions of the 1,500 pg/mL top standard in separate tubes using Assay Buffer A as the diluent. Thus, the Human VEGF standard concentrations in the tubes are 1,500 pg/mL, 750 pg/mL, 375 pg/mL, 187.5 pg/mL, 93.8 pg/mL, 46.9 pg/mL, and 23.4 pg/mL, respectively. Assay Buffer A serves as the zero standard (0 pg/mL).

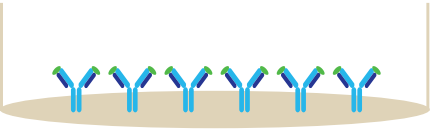


4. Wash the plate 4 times with 300 µL of 1X Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on clean, absorbent paper. All subsequent washes should be performed similarly.
5. Add 50 µL of Assay Diluent D to each well that will contain the standard dilutions or samples.
6. Add 50 µL of standard dilutions or samples to the appropriate wells.
7. Seal the plate with a Plate Sealer included in the kit and incubate the plate for 2 hours at room temperature with shaking.
8. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.
9. Add 100 µL of Human VEGF Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour with shaking.

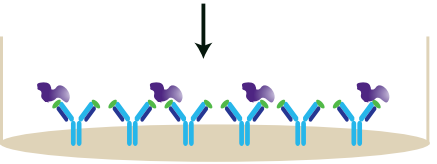
10. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.
11. Add 100 µL of Avidin-HRP solution to each well, seal and incubate the plate at room temperature for 30 minutes with shaking.
12. Discard the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 4. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.
13. Add 100 µL of Substrate Solution F to each well and incubate at room temperature for 10 to 15 minutes in the dark. Wells containing human VEGF should turn blue in color with an intensity proportional to its concentration. It is not necessary to seal the plate during this step.
14. Stop the reaction by adding 100 µL of Stop Solution to each well. The solution color should change from blue to yellow.
15. Read absorbance at 450 nm immediately. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

Assay Procedure Summary

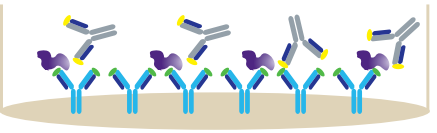
1. Wash 4 times
   Add 50 µL Assay Diluent D to standard wells and sample wells



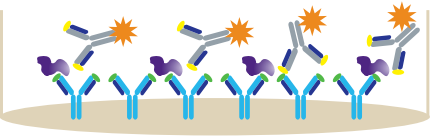
2. Add 50 µL diluted standards or samples,
   Incubate 2 hr, RT, shaking



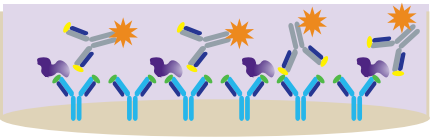
3. Wash 4 times
   Add 100 µL Detection Antibody solution
   Incubate 1 hr, RT, shaking



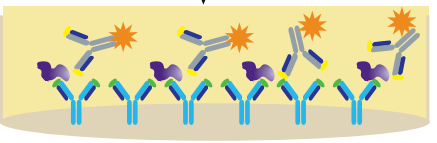
4. Wash 4 times
   Add 100 µL Avidin-HRP solution
   Incubate 30 min, RT, shaking



5. Wash 5 times
   Add 100 µL Substrate Solution F
   Incubate 10 to 15 min, RT, in the dark



6. Add 100 µL Stop Solution



7. Read absorbance at 450 nm and 570 nm

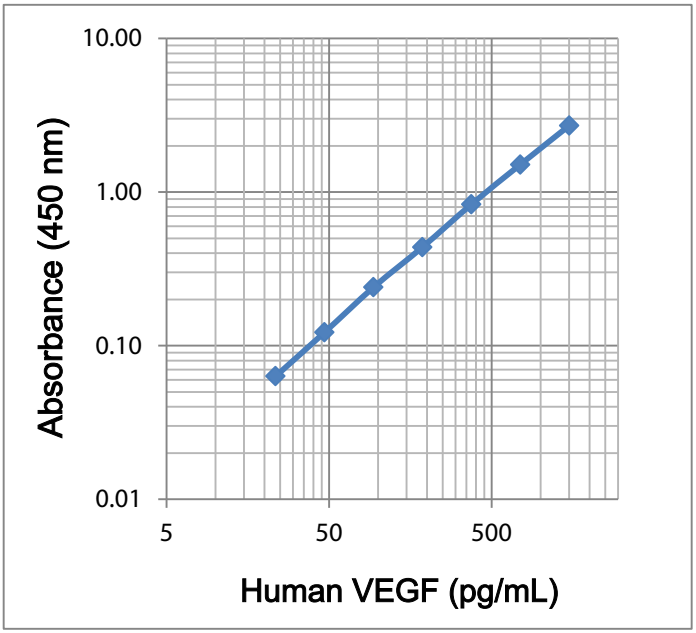
Calculation of Results:

The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If an appropriate software is not available, use log-log graph paper to determine sample concentrations. Determine the mean absorbance for each set of duplicate or triplicate standards, controls, and samples. Plot the standard curve on log-log graph paper with protein concentration on the X-axis and absorbance on the Y-axis. Draw a best fit line through the standard points. To determine the unknown protein concentrations, find the mean absorbance value of the unknown concentration on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the protein concentration.

If samples were diluted, multiply the concentration by the appropriate dilution factor. If a test sample's absorbance value falls outside the linear portion of the standard curve, the test sample needs to be re-analyzed at a higher (or lower) dilution as appropriate.

Typical Data:

This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.



Performance Characteristics:

Specificity: No cross-reactivity was observed when this kit was used to analyze the following recombinant proteins, each at 50 ng/mL.

Human	IFN-γ, IGF-I, IGF-II, PDGF-AA, PDGF-AB, PDGF-CC, PDGFRB Fc, PlGF-1, PlGF-2, PlGF-3, TGF-α, TGF-β1, TGF-β2, TGF-β3, VEGF-B167, VEGF-C, VEGF-D, EG-VEGF, VEGFR1, VEGFR2-Fc chimera
Mouse	VEGF-120, VEGF-164, VEGF-C, PDGF-BB
Rat	VEGF-164

Recombinant human VEGF-165 and VEGF-121 show 100% and 7.2% reactivity with this assay respectively.

Recombinant human VEGF/PlGF heterodimer shows 0.94% cross-reactivity at 50 ng/mL.

Recombinant human VEGFR-1 interferes at all levels when various concentrations of recombinant human VEGFR-1 were added to a sample of known VEGF concentration (750 pg/mL).

Concentration of Human VEGFR-1	VEGF: VEGFR-1 Molar Ratio	% Interference
150 pg/mL	1:0.1	8.4
300 pg/mL	1:0.2	19.0
1,500 pg/mL	1:1	69.6
3,000 pg/mL	1:2	90.4
7,500 pg/mL	1:5	100.0

Recombinant human VEGFR-2 starts to interfere at concentrations greater than 44,000 pg/mL.

Sensitivity: The average minimum detectable concentration is 4.1 ± 2.2 pg/mL.

Recovery: Recombinant human VEGF (720, 180, and 45 pg/mL) was spiked into human samples, then analyzed with the LEGEND MAX™ Human VEGF ELISA Kit. Heparin plasma is not recommended as a sample type.

Sample Type	N	% Recovery
Serum	10	100.0
Citrate Plasma	5	74.6
EDTA Plasma	5	78.3
Heparin Plasma	5	15.9

Linearity: Human serum and plasma samples spiked with recombinant human VEGF were diluted to produce samples within the dynamic range, and then assayed with the kit to determine the dilutional linearity. Heparin plasma is not recommended as a sample type.

Sample Type	N	% Linearity
Serum	10	110.8
Citrate Plasma	5	120.5
EDTA Plasma	5	113.5
Heparin Plasma	5	178.4

Intra-Assay Precision: Two serum samples were spiked with different recombinant human VEGF concentrations and tested with 16 replicates in one assay.

Concentration	Sample 1	Sample 2
Number of Replicates	16	16
Mean Concentration (pg/mL)	819.0	106.6
Standard Deviation	47.4	5.9
% CV	5.8	5.1

Inter-Assay Precision: Two human samples were spiked with different concentrations of recombinant human VEGF and assayed in four independent assays.

Concentration	Sample 1	Sample 2
Number of Assays	4	4
Mean Concentration (pg/mL)	667.8	89.2
Standard Deviation	94.5	13.3
% CV	14.1	14.9

Human Biological Samples:
Normal Ranges: 75 human serum and plasma (Citrate, EDTA and Heparin) samples from human donors were tested for endogenous human VEGF. Heparin plasma is not recommended as a sample type.

Sample Type	N	% Detectable	Min (pg/mL)	Max (pg/mL)	Median (pg/mL)
Serum	27	100	20	645.9	103.1
Citrate	16	62.5	ND	20.6	11.9
EDTA	16	87.5	ND	36.5	19.9
Heparin	16	75.0	ND	12.0	6.0

Cell Culture Supernatants:
Peripheral blood mononuclear cells (PBMC) (1 x 10^6 cells/mL) from different donors and HT29 cells (1 x 10^6 cells/mL) were cultured for 0 to 3 days. The cell supernatants were collected and assayed for human VEGF.

Cell Type	Concentration (pg/mL)
PBMC (Donor #1, Day 1, unstimulated)	5.4
PBMC (Donor #2, Day 2, unstimulated)	132.0
PBMC (Donor #3, Day 2, unstimulated)	69.5
HT29 (Day 0, unstimulated)	ND
HT29 (Day 3, unstimulated)	775.5

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Troubleshooting Guide:

Problem	Probable Cause	Solution
High Background	Background wells were contaminated	Avoid cross-well contamination by using the provided plate sealers. Use multichannel pipettes and change tips between pipetting samples and reagents.
	Insufficient washes	Increase number of washes. Increase soaking time between washes prior to addition of substrate solution.
	TMB Substrate Solution was contaminated	TMB Substrate Solution should be clear and colorless prior to addition to wells. Use a clean container prior to pipetting substrate solution into wells.
No or poor signal	Detection Antibody, Avidin-HRP or Substrate solution were NOT added	Rerun the assay and follow the protocol.
	Wrong reagent or reagents were added in wrong sequential order	
	Insufficient plate agitation	The plate should be agitated during all incubation steps using a plate shaker at a speed where solutions in wells are within constant motion without splashing.
	The wash buffer contains Sodium Azide (NaN3)	Avoid Sodium Azide contamination in the wash buffer as it inhibits HRP activity.
	Incubations were done at an inappropriate temperature, timing or without agitation	Rerun the assay and follow the protocol.
Low or poor standard curve signal	The standard was incorrectly reconstituted or diluted	Adjust the calculations and follow the protocol.
	Standard was inappropriately stored	Store the reconstituted standard stock solution in polypropylene vials at -70°C. Avoid repeated freeze-thaw cycles.
	Reagents added to wells with incorrect concentrations	Check for pipetting errors and the correct reagent volume.

Problem	Probable Cause	Solution
Signal is high, standard curves have saturated signal	Standard reconstituted with less volume than required	Reconstitute new lyophilized standard with the correct volume of solution recommended in the protocol.
	Standards/samples, detection antibody, Avidin-HRP or substrate solution were incubated for too long	Rerun the assay and follow the protocol.
Sample readings are out of range	Samples contain no or below detectable levels of the analyte	If samples are below detectable levels, it may be possible to use a larger sample volume. Contact technical support for appropriate protocol modifications.
	Samples contain analyte concentrations greater than highest standard point	Samples may require dilution and analysis.
High variation in samples and/or standards	Multichannel pipette errors	Confirm that pipette calibrations are accurate.
	Plate washing was not adequate or uniform	Ensure pipette tips are tightly secured. Ensure uniformity in all wash steps.
	Non-homogenous samples	Thoroughly mix samples before assaying.
	Samples may have high particulate matter	Remove particulate matter by centrifugation.
	Cross-well contamination	Do not reuse plate sealers. Always change tips for reagent additions. Ensure that pipette tips do not touch the reagents on the plate.

ELISA Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

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BioLegend®

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