Human VEGF ELISA Kit

Cat# NB-06-0717

INTRODUCTION

Human VEGF, also known as Vascular Endothelial Growth Factor, is a 232 amino acid growth factor protein encoded from the *VEGFA* gene located at locus 6p12 on chromosome 6. After initial synthesis, the 26 residue signal sequence is cleaved from the N-terminal end, allowing for the 206 residue VEGF protein to fold properly into its mature form. VEGF is known to be a growth factor active in angiogenesis, vasculogenesis and endothelial cell growth. Through binding to the FLT1/VEGFR1 and KDR/VEGFR2 receptors, heparin sulfate and heparin, homodimeric VEGF is able to induce endothelial cell proliferation, promote cell migration, as well as inhibit apoptosis and induce permeabilization of blood vessels. Moreover, NRP1/Neuropilin-1 binds isoforms VEGF165 and VEGF145, where isoform VEGF165B binds to KDR but does not activate downstream signaling pathways and does not activate angiogenesis, while inhibiting tumor growth. VEGF121 is acidic and freely secreted. VEGF165 is more basic, has heparin-binding properties and, although a significant proportion remains cell-associated, most is freely secreted. VEGF189 is very basic; it is cell-associated after secretion and is bound avidly by heparin and the extracellular matrix, although it may be released as a soluble form by heparin, heparinase or plasmin. Defects in VEGFA are a cause of susceptibility to microvascular complications of diabetes type 1 (MVCD1). These are pathological conditions that develop in numerous tissues and organs as a consequence of diabetes mellitus. They include diabetic retinopathy, diabetic nephropathy leading to end-stage renal disease, and diabetic neuropathy. Diabetic retinopathy remains the major cause of new-onset blindness among diabetic adults. It is characterized by vascular permeability and increased tissue ischemia and angiogenesis.

Source: Entrez Gene: VEGFA vascular endothelial growth factor A [Homo sapiens]; Swiss-Prot: P15692

ASSAY PRINCIPLES

The Human VEGF ELISA Kit contains the components necessary for quantitative determination of natural or recombinant hVEGF concentrations within any experimental sample including cell lysates, serum and plasma. This particular immunoassay utilizes the quantitative technique of a “Sandwich” Enzyme-Linked Immunosorbent Assay (ELISA) where the target protein (antigen) is bound in a “sandwich” format by the primary capture antibodies coated to each well-bottom and the secondary detection antibodies added subsequently by the investigator. The capture antibodies coated to the bottom of each well are specific for a particular epitope on the Human VEGF cytokine while the user-added detection antibodies bind to epitopes on the captured target protein. Amid each step of the procedure, a series of wash steps must be performed to ensure the elimination of non-specific binding between proteins to other proteins or to the solid phase. After incubation and “sandwiching” of the target antigen, a peroxidase enzyme is conjugated to the constant heavy chain of the secondary antibody (either covalently or via Avidin/Streptavidin-Biotin interactions), allowing for a colorimetric reaction to ensue upon substrate addition. When the substrate TMB (3, 3’, 5, 5’-Tetramethylbenzidine) is added, the reaction catalyzed by peroxidase yields a blue color that is representative of the antigen concentration. Upon sufficient color development, the reaction can be terminated through addition of Stop Solution (2 N Sulfuric Acid) where the color of the solution will turn yellow. The absorbance of each well can then be read by a spectrophotometer, allowing for generation of a standard curve and subsequent determination of protein concentration.

ASSAY RESTRICTIONS

- This ELISA kit is intended for research purposes only, NOT diagnostic or clinical procedures of any kind. Materials included in this kit should NOT be used past the expiration date on the kit label.
- Reagents or substrates included in this kit should NOT be mixed or substituted with reagents or substrates from any other kits.
- Variations in pipetting technique, washing technique, operator laboratory technique, kit age, incubation time or temperature may cause differences in binding affinity of the materials provided.
- The assay is designed to eliminate interference and background by other cellular macromolecules or factors present within any biological samples. However, the possibility of background noise cannot be fully excluded until all factors have been tested using the assay kit.

MATERIALS INCLUDED

Reagent	Quantity Per Plate	Container	Reconstitution	Dilution
96-Well Microplate or Strips Coated w/ Capture Antibody	12 x 8 Strips	-	-	-
Biotin-Conjugated Detection Antibody	Lyophilized	Yellow	25 µl H_2O	Use Detection Antibody Diluent
Ready-to-Use Avidin-HRP Conjugate Solution	11 ml	Clear	-	-
Cytokine Protein Standard	Lyophilized (100 ng)	Red	100 µl H_2O	Use Protein Standard Diluent
Ready-to-Use Substrate	11 ml	Brown	-	-
Stop Solution	11 ml	Clear	-	-
Adhesive Plate Sealers	4 Sheets	-	-	-
Wash Buffer (10X)	50 ml	Clear	-	Dilute to 1X Using Pure H_2O
Protein Standard Diluent	11 ml	Clear	-	-
Detection Antibody Diluent	11 ml	Clear	-	-

ADDITIONAL MATERIALS REQUIRED

The following materials and/or equipment are NOT provided in this kit but are necessary to successfully conduct the experiment:

- Microplate reader able to measure absorbance at 450 nm (with correction wavelength set to 540 nm or 570 nm) Micropipettes with capability of measuring volumes ranging from 1 µl to 1 ml
- Deionized or sterile water
- Squirt bottle, manifold dispenser, multichannel pipette reservoir or automated microplate washer Graph paper or computer software capable of generating or displaying logarithmic functions Absorbent paper or vacuum aspirator

- Test tubes or microfuge tubes capable of storing ≥1 ml Bench-top centrifuge (optional)
- Bench-top
- vortex (optional)
- Orbital shaker (optional)

HEALTH AND SAFETY PRECAUTIONS

- Reagents provided in this kit may be harmful if ingested, inhaled or absorbed through the skin. Please carefully review the MSDS for each reagent before conducting the experiment.
- Stop Solution contains 2 N Sulfuric Acid (H_2SO_4) and is an extremely corrosive agent.
- Please wear proper eye, hand and face protection when handling this material. When the experiment is finished, be sure to rinse the plate with copious amounts of running water to dilute the Stop Solution prior to disposing the plate.

STORAGE INFORMATION

Note: If used frequently, reagents may be stored at 2-8°C. If used infrequently, reagents should be stored at -20°C.

Condition	Component	Storage Information	Storage Time
Sealed, Unopened Assay Kit	-	2-8°C	1 month
Reconstituted, Opened Assay Kit	96-Well Microplate (Capture Antibody Coated)	2-8°C	1 month
	Detection Antibody		
	Ready-to-Use Avidin-HRP Conjugate Solution		
	Cytokine Protein Standard		
	Ready-to-Use Substrate		
	Stop Solution		
	Wash Buffer (10X)		
	Protein Standard Diluent		
	Detection Antibody Diluent		
	Plate Sealers		

REAGENT RECONSTITUTION AND PREPARATION

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

IMMUNOASSAY PROTOCOL

Note: If possible, all incubation steps should be performed on an orbital shaker to equilibrate solutions when added to the microplate wells. Also, all provided solutions should be at ambient temperature prior to use.

Note: Avoid adding solutions into wells at an angle, always keep pipette tip perpendicular to plate bottom.

Reconstitution of Provided Materials

Please see tables above regarding reagent reconstitution and storage information.

Addition of Known Standard and Unknown Sample to Immunoassay

Prior to applying an unknown sample to the Sandwich ELISA, the immunoassay must be performed using a serial dilution of a known standard sample in order to determine the standard curve. This is necessary to allow for the interpretation of results generated by the unknown samples.

Dilute the known standard sample from 1 ng/ml to 0 ng/ml in a series of microfuge tubes. Mix each tube thoroughly by inverting several times or by vortexing lightly to ensure proper equilibration. Add 100 µl of each serial dilution step into the wells of a specified row or column of the 96-well microtiter plate in duplicate or triplicate and incubate at room temperature for 2 hours. Seal the microplate air-tight using one of the microplate adhesive seals provided in this kit or Parafilm if readily available. **Note:** If a standard curve has already been generated, substitute the standard with the unknown sample of interest.

Application of Detection Antibody to Capture Antibody-Bound Samples

1. Aspirate the protein standard solution out of the microplate wells. If your lab does not have a vacuum-based aspirator, you may dump the solutions from the microplate into a waste container and blot 3-4 times on a stack of paper towels until most or all of the liquid is removed from the wells. Dilute the 10X wash buffer to 1X using pure H_2O. Add 300-400 µl of Wash Buffer to each well being used and gently shake for 5-7 minutes on an orbital shaker. Perform this wash step 4 times consecutively.

2. After the 4th wash step, dilute the detection antibody solution 1:400 in detection antibody diluent to a concentration of 0.25 µg/ml. Mix the test tube either by inverting several times or vortexing to ensure proper equilibration. Ensure that there is enough detection antibody solution for all wells being used. Add 100 µl of the diluted detection antibody solution into each well, seal the plate and incubate at room temperature for 2 hours.

Conjugation of Avidin-Horseradish Peroxidase Enzyme with Detection Antibody

1. Remove the detection antibody solution out of the microplate wells by either vacuum-based aspirator or paper towel blotting. Perform 4 consecutive wash steps with gentle shaking between each wash.

2. After the 4th wash step, add 100 µl of Ready-to-Use Avidin-HRP Conjugate Solution into each well and incubate at room temperature for 30 minutes.

Application of Liquid Substrate for Colorimetric Reaction

1. Remove the Avidin-HRP conjugate solution out of the microplate wells by either vacuum-based aspirator or paper towel blotting. Prepare the TMB substrate solution by bringing it to room temperature without exposure to fluorescent or UV light as these may degrade the TMB. Perform 4 consecutive wash steps with gentle shaking between each wash.

2. After the 4th wash step, add 100 µl of TMB substrate solution into each well and incubate at room temperature for color development. The microplate should be kept out of direct light by either covering with an opaque object or putting it into a dark room. Closely monitor the color development as some wells may turn blue very quickly depending on analyte and/or detection antibody-HRP concentrations. Once the blue color has ceased to develop further, immediately add 100 µl of Stop Solution to each well being used. The color in the wells should immediately change from blue to yellow.

3. The microplate is now ready to be read by a microplate reader. Within 30 minutes of adding the Stop Solution, determine the optical density (absorbance) of each well by reading the plate with the microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. **Caution:** Readings made directly at 450 nm without correction may be higher and less accurate.

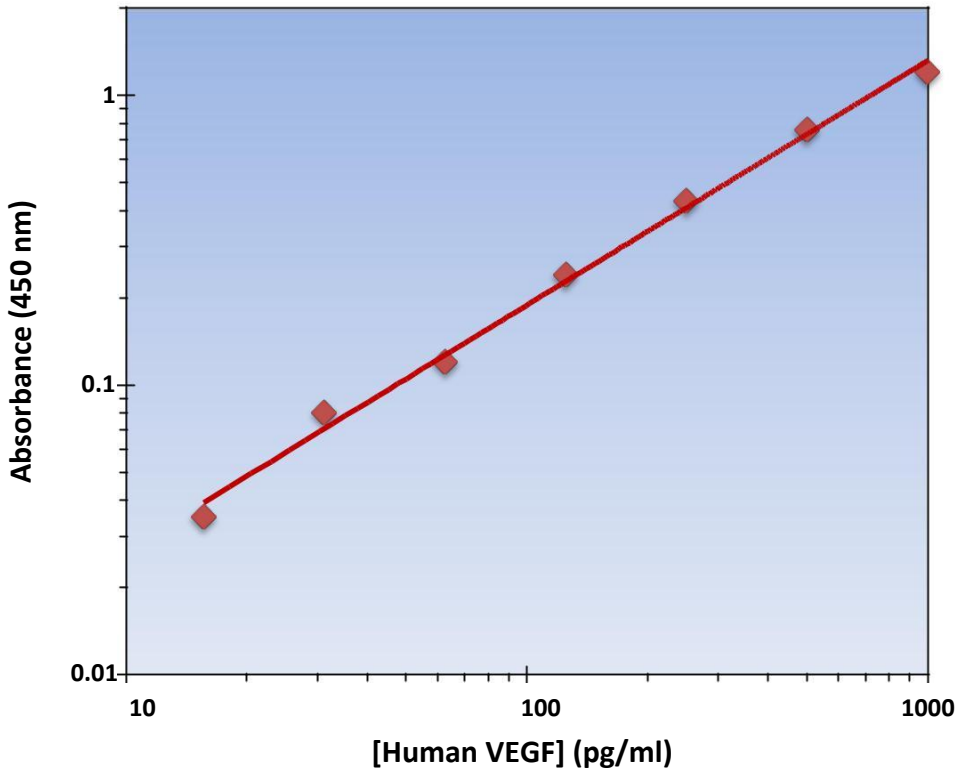
Generation of Standard Curve and Interpretation of Data

1. Average the duplicate or triplicate readings for each standard, control and sample and subtract the average zero standard optical density.

2. Generate a standard curve by using Microsoft Excel or other computer software capable of establishing a 4-Parameter Logistic (4-PL) curve fit. If using Excel or an alternative graphing tool, plot the average optical density values in absorbance units (y-axis) against the known standard concentrations in pg/ml (x-axis). **Note:** Only use the values in which a noticeable gradient can be established. Afterwards, generate a best fit curve or “trend-line” through the plotted points via regression analysis. **Note:** Shown on the next page is an example of typical data produced by analysis of the standard sample.

The data and subsequent graph was obtained after performing a cytokine ELISA for Human VEGF. Each known sample concentration was assayed in triplicate.

Human VEGF Standard Curve	
Concentration (pg/ml)	**Average OD 450nm**
1000	1.2
500	0.76
250	0.43
125	0.24
62.5	0.12
31.25	0.08
15.625	0.035



CROSS REACTIVITY AND SPECIFICITY

The Human VEGF ELISA is capable of recognizing both recombinant and naturally produced Human VEGF proteins. The antigens listed below were tested at 50 ng/ml and exhibited 100% cross reactivity.

Human: $VEGF_{121}$
Murine: VEGF
Rat: VEGF

The antigens listed below were tested at 50 ng/ml and did not exhibit significant cross reactivity or interference.

Human: EGF, EG-VEGF, FGF-16, GM-CSF, PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, RANTES, SCF, VEGF-B, VEGF-C
Murine: EGF, GM-CSF, PDGF-AA, PDGF-BB, SCF
Rat: EGF, SCF

TECHNICAL SUPPORT

For troubleshooting, information or assistance, please go online to www.neo-biotech.com or contact us at:

183, avenue Georges Clemenceau
92000 Nanterre – France

Tel: +33 9 77 40 09 09

Fax: +33 9 77 40 10 11

Email: info@neo-biotech.com

ELISA PLATE TEMPLATE