



human VEGF

Enzyme Immunometric Assay Kit

Catalog No. 900-080

96 Determination Kit

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Description

Assay Designs' human Vascular Endothelial Cell Growth Factor (VEGF) Enzyme Immunometric Assay (EIA) kit is a complete kit for the quantitative determination of human VEGF in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a monoclonal antibody to human VEGF immobilized on a microtiter plate to bind the VEGF in the standards or sample. A recombinant human VEGF Standard is provided in the kit. After a short incubation, the excess sample or standard is washed out and a polyclonal antibody to human VEGF labeled with the enzyme Horseradish peroxidase is added. This labeled antibody binds to the human VEGF captured on the plate. After a short incubation, the excess labeled antibody is washed out and substrate is added. The substrate reacts with the labeled antibody bound to the human VEGF captured on the plate. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450 nm. The measured optical density is directly proportional to the concentration of human VEGF in either standards or samples. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard¹ or Tijssen².

Introduction

Vascular Endothelial Growth Factor (VEGF, VEGF-165) is the predominant member of a family of endothelial cell-specific mitogens that are related by structure and function. The 165 amino acid variant is the most common soluble secreted form found in human tissues. Biologically active as a homodimer or a heterodimer with VEGF-B, this paracrine growth factor stimulates angiogenesis by specifically activating vascular endothelial cells through a MAP kinase cascade³⁻⁵. VEGF is not only essential for normal vascular embryonic and reproductive angiogenesis but also is central to the growth and dissemination in a number of cancerous states⁶⁻⁸. Because of these pleiotropic effects, VEGF is valued for its predictive and therapeutic target applications for variety of pathological states including breast cancer, non-Hodgkins lymphoma, melanoma and rheumatoid arthritis⁹⁻¹².

Precautions

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1. Stop Solution is a 1 normal (1N) sulfuric acid solution. This solution is caustic; care should be taken in use.
2. The activity of the Horseradish peroxidase conjugate is affected by nucleophiles, such as azide, cyanide and hydroxylamine.
3. We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
4. The human VEGF Standard provided, Catalog No. 80-0688, should be handled with care, because of the known and unknown effects of VEGF.
5. The human VEGF Standard and Labeled Antibody should be stored at -20°C. Do not repeatedly freeze-thaw.

Materials Supplied

1. **human VEGF Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0690**
A strip microtiter plate coated with mouse antibody specific to human VEGF.
2. **human VEGF Labeled Antibody, 1 vial, Catalog No. 80-0689**
Rabbit antibody to human VEGF conjugated to Horseradish peroxidase.
3. **Assay Buffer, 30 mL, Catalog No. 80-0170**
Phosphate buffered saline containing proteins and detergents.
4. **Labeled Antibody Diluent, 10 mL, Catalog No. 80-0182**
Phosphate buffered saline containing proteins and detergents.
5. **Wash Buffer Concentrate, 50 mL, Catalog No. 80-0171**
Phosphate buffered saline containing detergents.
6. **human VEGF Standard, 2 vial, Catalog No. 80-0688**
A vial containing 1,000 pg of recombinant human VEGF.
7. **TMB Substrate, 15 mL, Catalog No. 80-1342**
A solution of 3,3',5,5' tetramethyl benzidine (TMB) and hydrogen peroxide. Ready to use.
8. **Stop Solution, 12 mL, Catalog No. 80-0176**
A 1N solution of sulfuric acid in water. Keep tightly capped. Caution: **Caustic**.
9. **human VEGF Assay Layout Sheet, 1 each, Catalog No. 30-0143**
10. **Plate Sealer, 2 each, Catalog No. 30-0012**

Storage

All components of this kit, **except the Labeled Antibody and Standard**, are stable at 4°C until the kit's expiration date. The Labeled Antibody and Standard **must** be stored at -20°C.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 100 μ L and 1,000 μ L.
3. Disposable test tubes for dilution of samples and standards.
4. Repeater pipet for dispensing 100 μ L.
5. Disposable beakers for diluting buffer concentrates.
6. Graduated cylinders.
7. Adsorbent paper for blotting.
8. Microplate reader capable of reading at 450 nm., preferably with correction between 570 nm and 590 nm.
9. Graph paper for plotting the standard curve.

Sample Handling

Assay Designs' EIA is compatible with human VEGF samples in tissue culture media and serum. Samples diluted sufficiently into Assay Buffer can be read directly from the standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions.

Culture fluids or serum are suitable for use in the assay. Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens. Samples in the majority of tissue culture media, including those containing fetal bovine serum, can be read in the assay if diluted into Assay Buffer. Users should only use standard curves generated in Assay Buffer to calculate concentrations of human VEGF.

Samples must be stored frozen to avoid loss of bioactive human VEGF. If samples are to be run within 24 hours, they may be stored at 4°C, otherwise samples must be stored frozen at -70°C. Up to three freeze/thaw cycles of serum has been shown to have no effect on human VEGF levels. Nonetheless, excessive freeze/thaw cycles should be avoided. Prior to assay, frozen sera should be brought to room temperature slowly and gently mixed by hand. Do not thaw samples in a 37°C incubator. Do not vortex or sharply agitate samples.

Procedural Notes

1. Do not mix reagents from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses plates with removable strips. Unused strips must be kept desiccated at 4°C in the sealed foil bag. The strips should be used in the frame provided.
8. **Prior to addition of standard, antibody, and substrate, ensure that there is no residual wash buffer in these wells. Any remaining wash buffer may cause variation in assay results.**

Reagent Preparation

1. Wash Buffer

Prepare Wash Buffer by diluting 25 mL of the supplied concentrate with 975 mL of deionized water. This can be stored at 4°C until the kit expiration date, or for 3 months, whichever is earlier.

2. human VEGF Standards

Add 500 µL of deionized water to the human VEGF Standard. Let it sit at room temperature for 5 minutes. Mix it gently. This solution contains 2,000 pg/mL human VEGF.

Label seven 12 x 75 mm glass tubes #1 through 7. Pipet 220 µL of Assay Buffer into tubes #1 through #7. Add 220 µL of the 2,000 pg/mL standard to tube #1. Vortex. Add 220 µL of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #7.

The concentration of human VEGF in tubes #1 through #7 will be 1,000, 500, 250, 125, 62.5, 31.3 and 15.6 pg/mL respectively. See human VEGF Assay Layout Sheet for dilution details. STORE STANDARD AT -20°C, avoid repeated freeze/thaws.

3. Preparation of Labeled Antibody Conjugate

Prepare labeled antibody solution **immediately before use**. Do not store prepared labeled antibody solution. For each strip used, mix 30 µL of labeled antibody concentrate with 870 µL of labeled antibody diluent.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the foil pouch and seal the ziploc. Store unused wells at 4°C.
2. Pipet 100 µL of Assay Buffer into the S0 (0 pg/mL Standard) wells.
3. Pipet 100 µL of Standards #1 through #7 into the appropriate wells.
4. Pipet 100 µL of the Samples into the appropriate wells.
5. Tap the plate gently to mix the contents.
6. Seal the plate and incubate at 4°C for 18-24 hours .
7. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 6 more times for a total of **7 washes**. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
8. Pipet 100 µL of the Labeled Antibody into each well, except the Blank.
9. Seal the plate and incubate at 4°C for 30 minutes. Prepare Substrate (See page 5, Section 4).
10. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 8 more times for a total of **9 washes**. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
11. Add 100 µL of the Substrate Solution to each well.
12. Incubate at room temperature for 30 minutes in the dark.
13. Add 100 µL of Stop Solution to each well.
14. Blank the plate reader against the Blank wells, read the optical density at 450nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the blank wells from all readings.

Calculation of Results

Several options are available for the calculation of the concentration of human VEGF in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of human VEGF can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

$$\text{Average Net OD} = \text{Average OD} - \text{Average Blank OD}$$

2. Using linear graph paper, plot the Average Net OD for each standard versus human VEGF concentration in each standard. Approximate a straight line through the points. The concentration of human VEGF in the unknowns can be determined by interpolation.

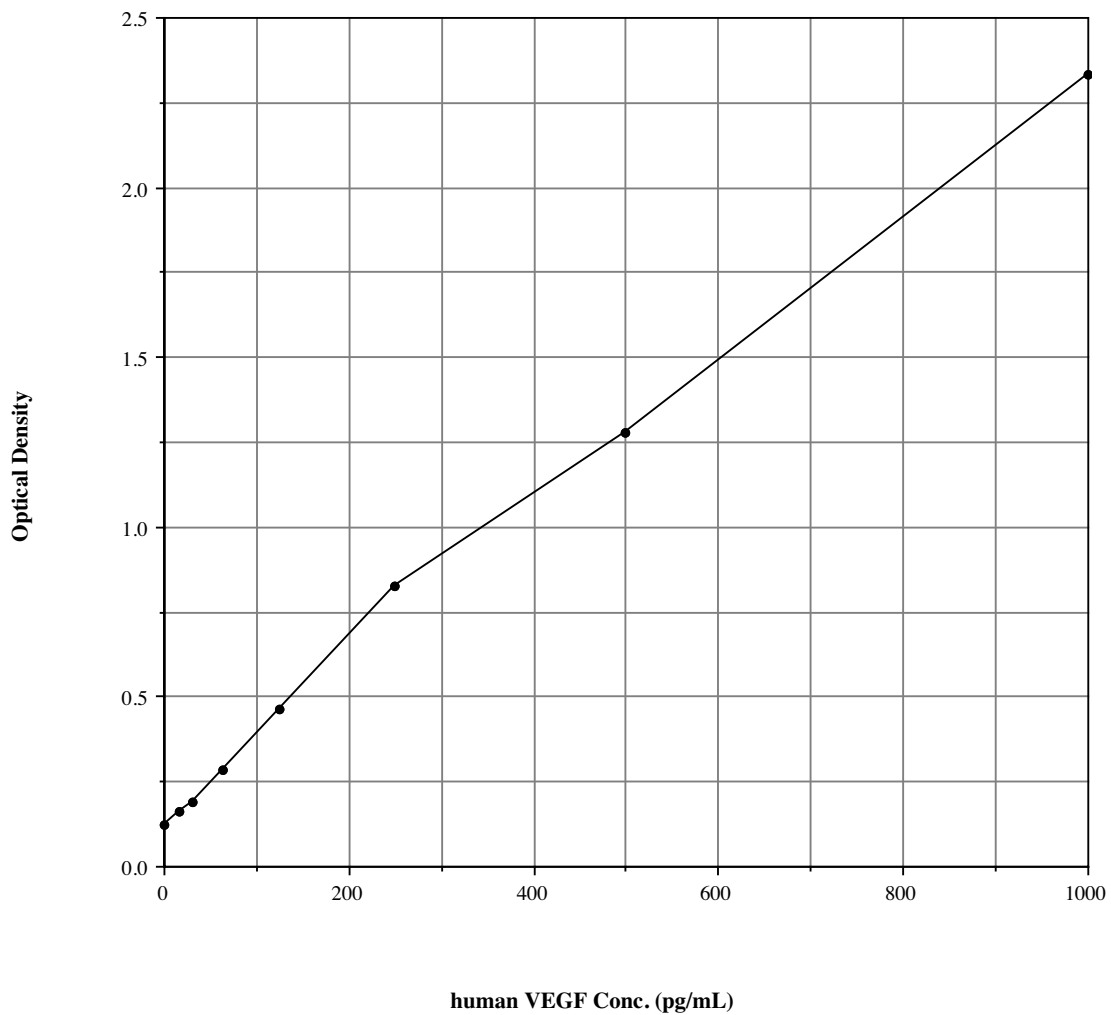
Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

<u>Sample</u>	<u>Average OD</u>	<u>Net OD</u>	human VEGF (pg/mL)
Blank	0.067		
0 standard	0.187	0.120	0
S1	2.402	2.335	1,000
S2	1.344	1.277	500
S3	0.890	0.823	250
S4	0.532	0.465	125
S5	0.351	0.284	62.5
S6	0.255	0.188	31.3
S7	0.231	0.164	15.6

Typical Standard Curve

The typical standard curve shown below **must not** be used to calculate human VEGF concentrations; each user must run a standard curve for each assay.



Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols¹³.

Sensitivity

Sensitivity was calculated by determining the average optical density bound for fourteen (14) wells run at 0 pg/mL human VEGF, and comparing to the average optical density for fourteen (14) wells run with Standard #7. The detection limit was determined as the concentration of human VEGF measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

Average Optical Density for the S0 = 0.110 ± 0.009 (8.16%)

Average Optical Density for Standard #7 = 0.130 ± 0.009 (6.95%)

Delta Optical Density (15.6 pg/mL -0 pg/mL) = 0.020

2 SD's of the 0 pg/mL Standard = 2 x 0.009 = 0.018

Sensitivity = $\frac{0.018}{0.020} \times 15.6 \text{ pg/mL} = \mathbf{14.04 \text{ pg/mL}}$

Linearity

A sample containing 741 pg/mL human VEGF was diluted 5 times 1:2 into Assay Buffer and measured in the assay. The data was plotted graphically as actual human VEGF concentration versus measured human VEGF concentration.

The line obtained had a slope of 0.9854 and a correlation coefficient of 0.9997.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of human VEGF and running these samples multiple times (n=24) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of human VEGF in multiple assays (n=8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of human VEGF determined in these assays as calculated by a curve fitting program.

	<u>human VEGF</u> (pg/mL)	<u>Intra-assay</u> <u>%CV</u>	<u>Inter-assay</u> <u>%CV</u>
Low	20.084	5.8	
Medium	73.55	2.9	
High	348.54	2.6	
Low	20.69		5.3
Medium	73.50		2.8
High	350.67		2.1

Cross Reactivities

The cross reactivities for a number of related compounds was determined by dissolving the cross reactant in Assay Buffer. These samples were then measured in the human VEGF assay, and the measured human VEGF concentration calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

<u>Compound</u>	<u>Cross Reactivity</u>
human VEGF 165	100%
human VEGF 121	6.0%
human PDGF	≤0.1%
human IL-10	≤0.1%
human GRO	≤0.1%
human Endothelin-1	≤0.1%
human Big Endothelin-1	≤0.1%
human G-CSF	≤0.1%
human SCF	≤0.1%
human IL-8	≤0.1%
human HGF	≤0.1%
human Endothelin-3	≤0.1%

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

Human VEGF concentrations were measured in tissue culture media (10% FSB added RPMI-1640) and human serum. Human VEGF was spiked into the undiluted samples of these matrices which were then diluted with the kit Assay Buffer and assayed in the kit. The following results were obtained:

<u>Sample</u>	<u>% Recovery*</u>	<u>Recommended Dilution*</u>
Tissue Culture Media	104.6	1:2 - 1:32
human Serum	100.7	1:2 - 1:64

* See Sample Handling instructions on page 4 for details.

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LIMITED WARRANTY

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Material Safety Data Sheet (MSDS) available on our website or by fax.

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Catalog No. 25-0356

March 23, 2007

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