



Human VEGF165 ELISA Kit

Enzyme Immunoassay for the quantification of Human VEGF165 in serum,
plasma, cell culture supernatants

Catalog number: ARG81305

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INTRODUCTION

VEGF is a member of the PDGF/VEGF growth factor family and encodes a protein that is often found as a disulfide linked homodimer. This protein is a glycosylated mitogen that specifically acts on endothelial cells and has various effects, including mediating increased vascular permeability, inducing angiogenesis, vasculogenesis and endothelial cell growth, promoting cell migration, and inhibiting apoptosis. Elevated levels of this protein is linked to POEMS syndrome, also known as Crow-Fukase syndrome. Mutations in this gene have been associated with proliferative and nonproliferative diabetic retinopathy. Alternatively spliced transcript variants, encoding either freely secreted or cell-associated isoforms, have been characterized. There is also evidence for the use of non-AUG (CUG) translation initiation sites upstream of, and in-frame with the first AUG, leading to additional isoforms. [provided by RefSeq, Jul 2008]

VEGF is a growth factor activated in angiogenesis, vasculogenesis and endothelial cell growth. Induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and induces permeabilization of blood vessels. Binds to the FLT1/VEGFR1 and KDR/VEGFR2 receptors, heparan sulfate and heparin. NRP1/Neuropilin-1 binds isoforms VEGF-165 and VEGF-145. Isoform VEGF165B binds to KDR but does not activate downstream signaling pathways, does not activate angiogenesis and inhibits tumor growth. [UniProt]

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for VEGF-A has been pre-coated onto a microtiter plate.

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Standards or samples are pipetted into the wells and any VEGF165 present is bound by the immobilized antibody. After washing away any unbound substances, a biotin-conjugated antibody specific for VEGF165 is added to each well and incubate. Following a washing to remove unbound substances, streptavidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After washing away any unbound antibody-enzyme reagent, a substrate solution (TMB) is added to the wells and color develops in proportion to the amount of VEGF165 bound in the initial step. The color development is stopped by the addition of acid and the intensity of the color is measured at a wavelength of 450nm \pm 2nm. The concentration of VEGF165 in the sample is then determined by comparing the O.D of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8 °C. Use the kit before expiration date.

Component	Quantity	Storage information
Antibody-coated microplate	8 X 12 strips	4°C. Unused strips should be sealed tightly in the air-tight pouch.
Standard (Lyophilized)	2 vials (1000 pg/ml)	4°C
Standard/Sample diluent	1 X 25 ml	4°C
Antibody conjugate concentrate	1 vial (400 μ l)	4°C
Antibody diluent buffer	7 ml	4°C
HRP-Streptavidin concentrate	2 vials (5 μ l)	4°C (Protect from light)
HRP-Streptavidin diluent buffer	23 ml	4°C
200X Wash buffer	10 ml	4°C

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TMB substrate	11 ml	4°C (Protect from light)
STOP solution	11 ml	4°C
Plate sealer	2 strips	Room temperature

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm (optional: read at 610-650 nm as the reference wave length)
- Pipettes and pipette tips
- Deionized or distilled water
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 4°C at all times.
- Briefly spin down the antibody conjugate concentrate and HRP-Streptavidin concentrate before use.
- If crystals are observed in the 200X Wash buffer or Standard/Sample diluent concentrate, warm to RT until the crystals are completely dissolved.
- Ensure complete reconstitution and dilution of reagents prior to use.
- It is highly recommended that the standards and samples be assayed in duplicates.
- Change pipette tips between the addition of different reagent or samples.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernatants - Remove particulates by centrifugation (1000 X g, 10 min) and aliquot & store samples at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Serum- Use a serum separator tube (SST) and allow samples to clot for few minutes before centrifugation for 10 minutes at 1000 x g. Collect serum and assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma on ice using EDTA, citrate or heparin as an anticoagulant. Centrifuge (1000 x g) for 30 minutes at $2-8^{\circ}\text{C}$ within 30 minutes of collection. Collect the supernatants and assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Note:

- It is recommended that thaw the samples at room temperature and make sure that sample is completely thawed and homogeneous before use. Do not thaw by heating at 37°C or 56°C .
- Avoid using haemolysed or lipemic samples.
- If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

REAGENT PREPARATION

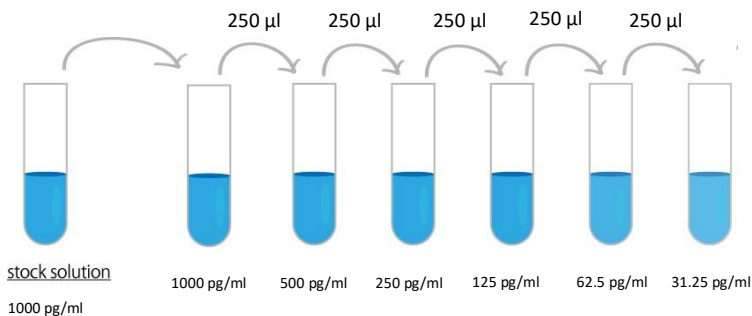
- **1X Wash buffer:** Dilute 200X Wash buffer into distilled water to yield 1X Wash buffer (e.g. 10 ml 200X Wash buffer + 1990 ml distilled water). Store the 1X wash buffer at 2-8°C for up to 1 week.
- **1X Antibody conjugate:** Prepare immediately before use, dilute 27.5X antibody conjugate concentrate into 1X antibody diluent buffer with Antibody diluent buffer to yield 1X detection antibody solution (e.g. for 16 wells, add 40µl Antibody conjugate concentrate into 1060µl Antibody diluent buffer). Use 1X Antibody conjugate immediately and do not store.
- **HRP-Streptavidin working solution:** Prepare immediately before use, it is recommended to centrifuge the vial for a few seconds to spin down all the volume at the bottom. Add 0.5 ml HRP-Streptavidin diluent buffer in the vial contains 5µl of HRP-Streptavidin concentrate solution, mix completely to get the 1st diluted HRP-Streptavidin Solution (67.67X). Then further dilute the 1st diluted solution with HRP-Streptavidin diluent buffer to yield 1X HRP-Streptavidin working Solution. (e.g. for 16 wells, add 30µl of 1st diluted HRP-Streptavidin Solution (67.67X) into 2ml HRP-Streptavidin diluent buffer) to yield 1X HRP-Streptavidin working Solution. Use 1X HRP-Streptavidin working Solution immediately and do not store.
- **1X Standard/Sample Diluent Buffer:** Dilute 10X Standard/Sample Diluent Buffer into distilled water to yield 1X Standard/Sample Diluent Buffer (e.g. 25 ml Standard/Sample Diluent Buffer + 225 ml distilled water). Store the 1X Standard/Sample Diluent Buffer at 2-8°C for up to 1 week.

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- **Sample:** Human serum or plasma samples have to be diluted 1:2 in 1X Standard/Sample Diluent Buffer. If the initial assay found samples contain VEGF165 higher than the highest standard, the samples can be diluted with Standard/Sample diluent and then re-assay the samples. For the calculation of the concentrations this dilution factor has to be taken into account.

(It is recommended to do pre-test to determine the suitable dilution factor).

- **Standards:** immediately before use, reconstitute the standard with **1.18 ml** 1x Standard/Sample diluent to yield a stock concentration of 1000 pg/ml. Mix the reconstituted standard gently by inversion only, and make sure the standard is dissolved completely before making serial dilutions. The 1x Standard/Sample diluent serves as zero standard (0 pg/ml), and the rest of the standard serial dilution can be diluted with 250µl 1x Standard/Sample diluent as according to the suggested concentration below: 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.25 pg/ml, .



ASSAY PROCEDURE

All materials should be equilibrated to room temperature (18 - 25°C) before use, each vial should be mixed thoroughly without foaming prior to use. Standards, samples and controls should be assayed in duplicates.

1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Add 100 µl of standards, samples and zero controls (Standard/Sample diluent) into wells. Cover wells and incubate for 2 hours at room temperature.
3. Remove the cover and aspirate each well and wash, repeating the process two times for a total three washes. Wash by filling each well with 1× Wash Buffer (300 µl) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.
4. Add 50 µl 1X Antibody conjugate into each well. Cover wells and incubate for 1 hour at room temperature.
5. Aspirate each well and wash as step 3.
6. Add 100 µl of 1X HRP-Streptavidin working solution to each well. Cover wells and incubate for 30 minutes at room temperature.
7. Aspirate each well and wash as step 3.
8. Add 100 µl of TMB substrate reagent to each well. Incubate for 10-15 minutes at room temperature in dark.
9. Add 100 µl of Stop Solution to each well. The color of the solution should

change from blue to yellow. Gently tap the plate to ensure thorough mixing

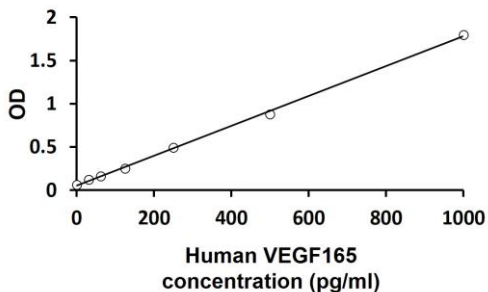
10. Read the OD with a microplate reader at 450 nm immediately. (optional: read at 610-650 nm as the reference wave length)

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and patient samples.
2. Using linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.
4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.
5. If the samples have been diluted, the concentration read from the standard curve must be further converted by the appropriate dilution factor according to the sample preparation procedure as described above.

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Sensitivity

The minimum detectable dose (MDD) of Human VEGF165 ranged from 31.25-1000 pg/ml. The mean MDD was 15.9 pg/ml.

Specificity

This assay recognizes natural and recombinant Human VEGF165. No significant cross-reactivity or interference with the factors below was observed:

VEGF-121, VEGF-145, VEGF-B, VEGF-C, VEGF-D, VEGF-R1, Ang-2, GM-CSF, IFN gamma, IL-29.

This VEGF-165 ELISA kit shows cross reactivity with VEGF-189 isoform

Intra-assay and Inter-assay precision

Intra-assay: 3.8%.

Inter-assay: 7.6%

Recovery range:

72 - 117%