Human VEGF-A ELISA Kit

Instructions for use

Catalogue numbers: 1x96 tests: 650.080.096
2x96 tests: 650.080.192

For research use only

Fast Track Your Research……

Version 10 23/01/2013

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Human VEGF-A ELISA Kit

1. Intended use

The Human VEGF-A ELISA is an enzyme-linked immunosorbent assay for quantitative detection of human VEGF-A in cell culture supernatants, rat serum, plasma or other body fluids.

This kit has been configured for research use only.

2. Introduction

2.1. Summary

Normal tissue function depends on a regular supply of oxygen through the blood vessels. Understanding the formation of blood vessels has become the focus of a major research effort throughout the last decade. Vasculogenesis in the embryo is the process by which new blood vessels are generated *de novo* from primitive precursor cells. Angiogenesis is the process of new blood vessel formation from pre-existing vasculatures. It plays an essential role in development, normal tissue growth, wound healing, the female reproductive cycle (placental development, ovulation, corpus luteum) and also plays a major role in various diseases (1). Special interest is focused on tumor growth, since tumors cannot grow more than a few millimeters in size without developing a new blood supply. This process is described as tumor angiogenesis which is also essential for the spread and growth of tumor cell metastasis. One of the key molecules for angiogenesis and for the survival of the endothelium is vascular endothelial growth factor (VEGF-A) (2). It is a specific endothelial cell mitogen and a strong vascular permeability factor (VPF) (3). VEGF-A is a heparin-binding glycoprotein, secreted as a homodimer of 45 kDa by many different cell types. VEGF-A also causes vasodilation through the nitiric oxide synthase pathway in endothelial cells and can activate migration in monocytes. Many different splice variants of VEGF-A have been described, but $VEGF_{165}$ is the most predominant protein and anchors with its heparin binding domain to extracellular matrix and to heparin sulfate. During the past few years, several other members of the VEGF family have been cloned, including VEGF-B, -C- and -D. In terms of vascular angiogenesis, which mainly is regulated by VEGF-A, lymphangiogenesis is mainly regulated by VEGF-C and -D (4).
VEGF-A transcription is highly activated by hypoxia and by oncogenes (5) like H-ras and several transmembrane tyrosine kinases, such as epidermal growth factor receptor and ErbB2 (6). Together these pathways account for a marked upregulation of VEGF-A in tumors compared to normal tissues and are often of prognostic importance and relevance (7,8). VEGF-A can be detected in both plasma and serum samples of patients, with much higher levels in serum (9). Extremely high levels can be detected in the cystic brain fluid of brain tumor patients (10,11) or in ascites fluid of patients. Platelets release VEGF-A upon aggregation and may be another major source of VEGF-A delivery to tumors (12). Several other studies have shown that association of high serum levels of VEGF-A with poor prognosis in cancer patients may be correlated with an elevated platelet count (13). Tumors can release cytokines and growth factors that stimulate the production of megakaryocytes in the marrow and elevate the platelet count. This can result in another, indirect increase of VEGF-A delivery to tumors (14). Furthermore, VEGF-A is implicated in several other pathological conditions associated with enhanced angiogenesis or enhanced vascular permeability. Examples where VEGF-A plays an important role are psoriasis and rheumatoid arthritis (15), as well as the ovarian hyperstimulation syndrome (16). Diabetic retinopathy is associated with high intraocular levels of VEGF-A, and inhibition of VEGF-A function may result in infertility by blockage of corpus luteum function (17). Direct demonstration of the importance of VEGF-A in tumor growth has been achieved using dominant negative VEGF receptors to block in vivo proliferation (18), as well as blocking antibodies to VEGF or to one of the VEGF receptors (19). Interference with VEGF-A function has therefore become of major interest for drug development to block angiogenesis and metastasis. More than 110 pharmaceutical companies world-wide are involved in the development of such antagonists. Their approaches include antagonists of VEGF-A or its receptors, selective tyrosine kinase inhibitors, targeting of drugs and toxins to VEGF receptors and gene therapy regulated by the same hypoxia pathway that controls VEGF-A production, Targeting the VEGF signalling may be of major therapeutic importance for many diseases (20) and serves as a basis for the design of future (anti)-angiogenic treatments.

2.2. Principle of the method

The VEGF-A kit is a solid phase sandwich Enzyme Linked-Immuno- Sorbent Assay (ELISA). A monoclonal antibody specific for VEGF-A has been coated onto the wells of the microtiter strips provided. Samples, including standards of known VEGF-A concentrations and unknowns are pipetted into these wells.

During the first incubation, the VEGF-A antigen is added to wells. After washing, a biotinylated monoclonal antibody specific for VEGF-A is incubated. Then the enzyme (streptavidin-peroxydase) is added. After incubation and washing to remove all unbound enzyme, a substrate solution which actes on the bound enzyme is added to induce a coloured reaction product. The intensity of this coloured product is directly proportional to the concentration of VEGF-A present in the samples.

3. Reagents provided and reconstitution

REAGENTS (store at 2-8°C)	Quantity 1x96 well kit 650.080.096	Quantity 2x96 well kit 650.080.192	RECONSTITUTION
96-wells precoated microtiter plate	1	2	Ready-to-use
Plate covers	2	4	
Biotin-Conjugate anti- human VEGF-A polyclonal antibody	1 vial	2 vials	Dilute 100 times in Assay Buffer (120µl)
Streptavidin-HRP	1 vial	2 vials	Dilute 100 times in Assay Buffer (150µl)
h. VEGF-A Standard: 2 ng/ml	2 vials	4 vials	See label on the vial
Assay Buffer Concentrate	1 vial	2 vials	(5 ml) 20X concentrate. Dilute in distilled water
Wash Buffer Concentrate	1 vial	2 vials	(50 ml) 20X concentrate. Dilute in distilled water
Substrate Solution	1 vial	2 vials	(15 ml) Ready-to-use
Sample Diluent	1 vial	2 vials	(12 ml) Ready-to-use
Stop Solution (1M Phosphoric acid)	1 vial	2 vials	(12 ml) Ready-to-use
Blue Dye	1 vial	2 vials	(0.4 ml) Make a 1/250 dilution in the appropriate diluent
Green Dye	1 vial	2 vials	(0.4 ml) Make a 1/100 dilution in the appropriate diluent
Red dye	1 vial	2 vials	(0.4 ml) Make a 1/250 dilution in the appropriate diluent

4. Materials required but not provided

- Microtiter plate reader fitted with appropriate filters (450nm required with optional 630nm reference filter)
- Microplate washer or wash bottle
- 10, 50, 100, 200 and 1,000µl adjustable single channel micropipettes with disposable tips
- 50-300µl multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Miscellaneous laboratory plastic and/or glass, if possible sterile

5. Specimen collection, processing & storage

Cell culture supernatants, rat serum, plasma or other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

Cell culture supernatants: Remove particulates and aggregates by spinning at approximately 1000 x g for 10 min.

Storage: If not analyzed shortly after collection, samples should be aliquoted (250-500µl) to avoid repeated freeze-thaw cycles and stored frozen at –70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

Recommendation: Do not thaw by heating at 37°C or 56°C. Thaw at room temperature and make sure that sample is completely thawed and homogeneous before use. When possible avoid use of badly haemolysed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

6. Safety & precautions for use

- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures , e.g.CDC/NIH Health manual : " Biosafety in Microbiological and Biomedical Laboratories" 1984
- Laboratory gloves should be worn at all times
- Avoid any skin contact with H_2SO_4 and TMB. In case of contact, wash thoroughly with water
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used
- Do not pipette by mouth
- When not in use, kit components should be stored refrigerated or frozen as indicated on vials or bottles labels
- All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration
- Cover or cap all reagents when not in use
- Do not mix or interchange reagents between different lots
- Do not use reagents beyond the expiration date of the kit
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of Stop reagent and substrate solution, avoid pipettes with metal parts
- Use a clean plastic container to prepare the washing solution
- Thoroughly mix the reagents and samples before use by agitation or swirling
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells
- The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning TMB is toxic avoid direct contact with hands. Dispose off properly
- If a dark blue colour develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded. Read absorbance’s within 1 hour after completion of the assay
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells
- Follow incubation times described in the assay procedure
- Dispense the TMB solution within 15 min of the washing of the microtitre plate

7. Assay Preparation

Bring all reagents to room temperature before use

7.1. Assay Design

Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard and zero should be tested **in duplicate**. Remove sufficient Microwell Strips for testing from the aluminium pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8^{o}C storage.

Example plate layout (example shown for a 6 point standard curve)

	Standards		Sample Wells									
	1	2	3	4	5	6	7	8	9	10	11	12
A	1000	1000										
B	500	500										
C	250	250										
D	125	125										
E	62.5	62.5										
F	31.25	31.25										
G	Blank	Blank										
H												

All remaining empty wells can be used to test samples in duplicate

7.2. Preparation of Wash Buffer

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** into a clean 1,000 ml graduated cylinder. Bring final volume to 1,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that the Wash Buffer is stable for 30 days.

7.3. Preparation of Assay Buffer

Mix the contents of the bottle well. Add contents of **Assay Buffer** Concentrate (5.0 ml) to 95 ml distilled or deionized water and mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer is stable for 30 days.

7.4. Preparation of Standard

Standard vials must be reconstituted with the volume of distilled water shown on the vial immediately prior to use. This reconstitution gives a stock solution of 2000pg/ml of VEGF-A. **Mix the reconstituted standard gently by inversion only**. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 1000 to 31.25pg/ml. A fresh standard curve should be produced for each new assay.

- Add 100µl of Sample Diluent to all standard and blank wells
- Immediately after reconstitution add 100µl of the reconstituted standard to wells A1 and A2, which provides the highest concentration standard at 1000pg/ml. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells
- Transfer 100µl from wells A1 and A2 to B1 and B2. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells
- Continue this 1:1 dilution using 100µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 1000 to 31.25pg/ml
- Discard 100µl from the final wells of the standard curve (F1 and F2)

Alternatively these dilutions can be performed in separate clean tubes and immediately transferred directly into the relevant wells.

7.5. Preparation of Biotin Conjugate

Make a 1:100 dilution with Assay Buffer in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (µl)	Assay Buffer (ml)
1 - 6	60	5.94
1 - 12	120	11.88

7.6. Preparation of Streptavidin-HRP

Make a 1:100 dilution of the concentrated **Streptavidin-HRP** solution in **Assay Buffer** as needed according to the following table:

Number of Strips	Streptavidin-HRP (µl)	Assay Buffer (ml)
1 - 6	60	5.94
1 - 12	120	11.88

7.7. Addition of Color Dyes

In order to help our customers to avoid any mistakes in pipetting, color dyes help to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure.

This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.
Alternatively, the dye solutions from the stocks provided (***Blue-Dye, Green-Dye, Red dye***) can be added to the reagents according to the following guidelines:

A. Diluent:

Before sample dilution add the ***Blue-Dye*** at a dilution of 1:250 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of ***Blue-Dye***, proceed according to the instruction booklet.

5 ml Assay Buffer	20 µl ***Blue-Dye***
12 ml Assay Buffer	48 µl ***Blue-Dye***

B. Biotin-Conjugate:

Before dilution of the concentrated Biotin-Conjugate, add the ***Green-Dye*** at a dilution of 1:100 (see table below) to the Assay Buffer (1x) used for the final conjugate dilution. Proceed after addition of ***Green-Dye*** according to the instruction booklet, preparation of Biotin-Conjugate.

3 ml Assay Buffer	30 µl ***Green-Dye***
6 ml Assay Buffer	60 µl ***Green-Dye***

C. Streptavidin-HRP:

Before dilution of the concentrated Streptavidin-HRP, add the ***Red-Dye*** at a dilution of 1:250 (see table below) to the Assay Buffer (1x) used for the final Streptavidin-HRP dilution. Proceed after addition of ***Red-Dye*** according to the instruction booklet, preparation of Streptavidin-HRP.

6 ml Assay Buffer	24 µl ***Red-Dye***
12 ml Assay Buffer	48 µl ***Red-Dye***

8. Method

We strongly recommend that every vial is mixed thoroughly without foaming prior to use except the standard vial which must be mixed gently by inversion only.

Prepare all reagents as shown in section 7.

Assay Step		Details
1.	Wash	Remove the pre-coated plate from the sealed pouch, removed any un-needed strips and wash the plate as follows: a) Dispense 0.3 ml of **1x washing solution** into each well b) Aspirate the contents of each well c) Repeat step a and b
2.	Addition	**Prepare Standard curve** as shown in section 7.4
3.	Addition	Add 100µl of Sample Diluent in duplicate to the blank wells
4.	Addition	Add 50µl of Sample Diluent to the sample wells
5.	Addition	Add 50µl of each sample in duplicate to the designated wells
6.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **2 hours** on a rotator set at 200rpm if available
7.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of **1x washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c another two times
8.	Addition	Add 50µl of diluted **biotinylated Conjugate** to all wells including blanks
9.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hour** on a rotator set at 200rpm if available
10.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of **1x washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c another two times
11.	Addition	Add 100µl of **Streptavidin-HRP** solution into all wells
12.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hour**
13.	Wash	Repeat wash step 7.
14.	Addition	Add 100µl of ready-to-use **TMB Substrate Solution** into all wells
15.	Incubation	Incubate for **20-30 minutes*** at room temperature on a rotar set at 200rpm if available. Avoid direct exposure to light by wrapping the plate in aluminium foil
16.	Addition	Add 100µl of **Stop Reagent** into all wells
Read the absorbance value of each well (immediately after step 16.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 630 nm as the reference wave length (610 nm to 650 nm is acceptable).		

***Incubation time of the substrate solution is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range*

9. Data Analysis

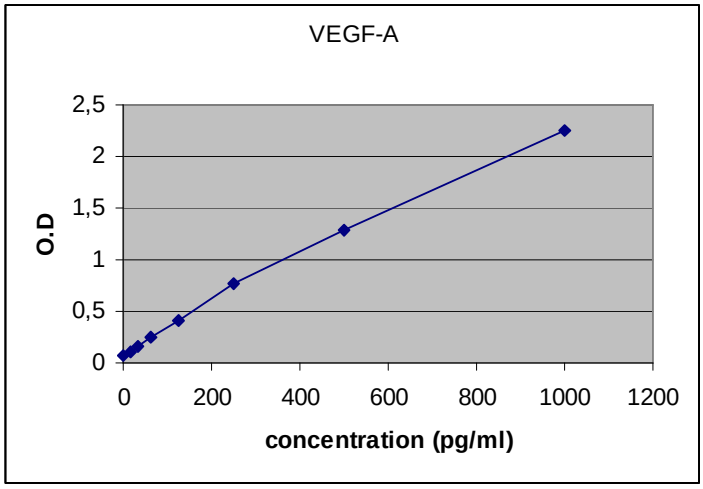
Calculate the average absorbance values for each set of duplicate standards and samples**.** Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding human VEGF-A standard concentration on the horizontal axis.

The amount of human VEGF-A in each sample is determined by extrapolating OD values against VEGF-A standard concentrations using the standard curve**.**

Example human VEGF-A
Standard Curve

Standard	human VEGF-A **Conc**	**OD (450nm) mean**	**CV (%)**
1	1000	2.254	2.4
2	500	1.286	3.2
3	250	0.771	0.6
4	125	0.419	2.5
5	62.5	0.258	0.1
6	31.3	0.163	0.3
Zero	0	0.069	-



Note; curve shown above should not be used to determine results. Every laboratory must produce a standard curve for each set of microwell strips assayed.

For samples which have been diluted according to the protocol (1:2), the calculated concentration should be multiplied by the dilution factor.

10. Assay limitations

Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard diluent or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.

The influence of various drugs on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results.

Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Washing Buffer, fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

As with most biological assays conditions may vary from assay to assay therefore **a fresh standard curve must be prepared and run for every assay.**

11. Performance Characteristics

11.1. Sensitivity

The limit of detection of human VEGF-A defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (mean plus two standard deviations) was determined to be 7.9 pg/ml (mean of 6 independent assays).

11.2. Specificity

The interference of circulating factors of the immune systems was evaluated by spiking these proteins at physiologically relevant concentrations into a human VEGF-A positive serum. There was no detectable cross reactivity.
Interference was detected for VEGF-R1 at concentrations > 200 pg/ml, and not for VEGF-R2.

11.3. Precision

Intra-assay

Reproducibility within the assay was evaluated in three independent experiments. **The overall intra-assay coefficient of variation has been calculated to be 6.2%.**

Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in three independent experiments by three technicians. **The overall inter-assay coefficient of variation has been calculated to be 4.3%.**

11.4. Dilution Parallelism

Serum, plasma and cell culture supernatant samples with different levels of human VEGF-A were analysed at serial 2 fold dilutions with 4 replicates each.

Sample matrix	Recovery of Exp. Val.	
	Range (%)	Mean (%)
Serum	74-104	90
Plasma (EDTA)	93-147	110
Plasma (citrate)	83-99	90
Plasma (heparin)	98-119	108
Cell culture supernatant	72-103	91

11.5. Spike Recovery

The spike recovery was evaluated by spiking 3 levels of human VEGF-A into serum, plasma and cells culture supernatant.Recoveries were determined with 4 replicates each. The amount of endogenous human VEGF-A in unspiked samples was substracted from the spike values.

Sample matrix	Spike high (%)	Spike medium (%)	Spike low (%)
Serum	88	85	81
Plasma (EDTA)	77	77	79
Plasma (citrate)	92	90	94
Plasma (heparin)	106	88	64
Cell culture supernatant	98	92	88

11.6. Stability

Storage Stability

Aliquots of spiked serum were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the human VEGF-A level determined after 24 h. There was a significant loss of human VEGF-A immunoreactivity during storage at room temperature and 37°C.

Freeze-thaw Stability

Aliquots of spiked serum were stored frozen at –20°C and thawed up to 5 times, and human VEGF-A levels determined. There was a significant loss of human VEGF-A by repeated freezing and thawing.

11.7. Expected serum values

A panel of 40 sera as well as EDTA, citrate and heparin plasma samples from apparently healthy blood donors (males and females) was tested for VEGF-A. The normal levels measured may vary with the sample collective used. (nd= non detectable, measured below the sensitivity).

Sample Matrix	Number of samples evaluated	Range (pg/ml)	% detectable	Mean of detectable (pg/ml)
Serum	40	nd-42.6	2.5	--
Plasma (EDTA)	40	nd-128.9	7.5	45.7
Plasma (citrate)	40	nd- 66.2	7.5	47.3
Plasma (Heparin)	40	nd- 311.4	7.5	144.3

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13. Assay Summary

Total procedure length : 4h30 mn

Add 100µl of sample or diluted standard

⇓

Incubate 2 hours at room temperature

⇓

Wash three times

⇓

Add 50µl of biotinylated detection antibody

⇓

Incubate 1 hour at room temperature

⇓

Wash three times

⇓

Add 100µl of Streptavidin-HRP

⇓

Incubate 1 hour at room temperature

⇓

Wash three times

⇓

Add 100 µl of ready-to-use TMB
Protect from light. Let the color develop for 20-30 mn.

⇓

Add 100 H_2SO_4

⇓

Read Absorbance at 450 nm

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