Human VEGF-A ELISA

650 080 096 **1 x 96 tests**
650 080 096 **2 x 96 tests**

INTENDED USE

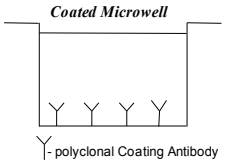
The VEGF-A ELISA is an enzyme-linked immunosorbent assay for quantitative detection of active human Vascular Endothelial Growth Factor A in cell culture supernatants, human serum, plasma, or other body fluids. **The VEGF-A ELISA is for research use only. Not for use in diagnostic or therapeutic procedures.**

SUMMARY

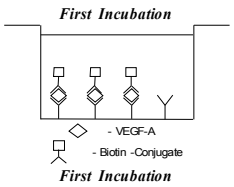
Normal tissue function depends on a regular supply of oxygen through the blood vessels. Understanding the formation of blood vessels has become the focus of a major research effort throughout the last decade. Vasculogenesis in the embryo is the process by which new blood vessels are generated *de novo* 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.

PRINCIPLES OF THE TEST

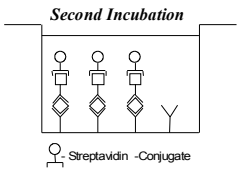
An anti VEGF-A polyclonal coating antibody is adsorbed onto microwells.



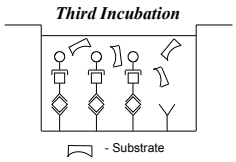
VEGF-A present in the sample or standard binds to the antibodies adsorbed to the microwells; a biotin-conjugated polyclonal VEGF-A antibody is added and binds to VEGF-A captured by the first antibody.

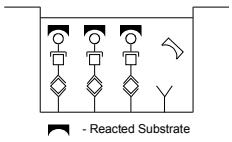


Following incubation unbound biotin conjugated VEGF-A is removed during a wash step. Streptavidin-HRP is added and binds to the biotin conjugated VEGF-A. Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.



A coloured product is formed in proportion to the amount of VEGF-A present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from seven VEGF-A standard dilutions and VEGF-A sample concentration determined.





REAGENTS PROVIDED

REAGENTS (store at 2-8°C)	QTY 1 plate	QTY 2 plates	RECONSTITUTION
96-wells precoated microtiter plate	1	2	Ready-to-use
Plate covers	4	8	
VEGF-A Standard: 4 ng /ml	2 vials	4 vials	Reconstitute with the volume of distilled water indicated on the vial
Biotin-Conjugate anti VEGF-A polyclonal (goat) antibody	1 vial	2 vials	(0.1ml) Dilute 100 times in Assay Buffer
Streptavidin-HRP	1 vial	2 vials	(150 µl) Dilute 200 times in Assay Buffer
Sample Diluent	1 bottle	2 bottles	(12 ml) Ready-to-use
Assay Buffer Concentrate	1 vial	2 vials	(5 ml) 20X concentrate. Dilute in distilled water
Wash Buffer Concentrate	1 bottle	2 bottles	(50 ml) 20X concentrate. Dilute in distilled water
Substrate Solution	1 vial	2 vials	(15 ml) Ready-to-use
Stop Solution (1 M 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

MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes
- 10 µl to 1,000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform linear regression analysis

SAFETY

- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Reagents containing thimerosal as preservative may be toxic if ingested.
- Avoid contact of substrate solutions with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagents.
- Exposure to acids will inactivate the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solutions must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as if they could contain infectious agents.The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0 % sodium hypochlorite. Allow 30 minutes for effective

decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

STORAGE INSTRUCTIONS

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on box front labels.
The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

SPECIMEN COLLECTION, PROCESSING AND STORAGE

Cell culture supernatants, human serum, EDTA, heparin and citrate plasma, and 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.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be frozen at -20°C, unless they will be assayed the day of collection. **Excessive freeze-thaw cycles should be avoided.** Prior to assay, frozen sera should be brought to room temperature slowly and mixed gently. Do not thaw samples in a 37°C water bath. Do not vortex or sharply agitate samples.

PREPARATION OF REAGENTS

Prepare Wash Buffer (reagent A) and Assay Buffer (reagent B) before starting with the test procedure.

1. 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. Wash Buffer may be prepared as needed according to the following table.

Number of Strips	Wash Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

2. 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. Assay Buffer may be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

3. Preparation of VEGF-A-Standards

Reconstitute **VEGF-A Standard** by addition of distilled water. Reconstitution volume is stated on the label of the standard vial. Mix gently to ensure complete solubilization. Discard reconstituted Standard after use.

4. Preparation of Biotin Conjugate

Dilute the Biotin-Conjugate 1:100 just prior to use with **Assay Buffer** (reagent B.) in a clean plastic tube.

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution. The dilution (1:100) of the Biotin-Conjugate may be prepared as needed according to the following table:

Number of Strips	Biotin-Conjugate (µl)	Assay Buffer (ml)
1 - 6	30	2.97
1 - 12	60	5.94

5. Preparation of Streptavidin-HRP

Make a 1:200 dilution of the concentrated **Streptavidin-HRP** solution as needed according to the following table:

Number of Strips	Streptavidin-Conjugate (µl)	Assay Buffer (ml)
1 - 6	30	5.97
1 - 12	60	11.94

6. Addition of Colour-giving Dyes

In order to help our customers to avoid any mistakes in pipetting, DIACLONE now offers a new tool that helps 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 Sample Diluent	20 µl ***Blue-Dye***
12 ml Sample Diluent	48 µl ***Blue-Dye***

B. Biotin-Conjugate:

Before dilution of the concentrated 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***

TEST PROTOCOL

a. Mix all reagents thoroughly without foaming before use.

b Determine the number of Microwell Strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank, and optional control sample should be assayed in duplicate. Remove extra **Microwell Strips coated with anti VEGF-A polyclonal antibody** from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

c Wash the microwell strips twice with approximately 300 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells.

After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

d Add 100 µl of **Sample Diluent** in duplicate to all standard wells. Prepare standard dilutions by pipetting 100 µl of **VEGF-A Standard**, in duplicate, into well A1 and A2 (see Figure 1 and 2). Mix the contents of wells A1 and A2 by repeated aspiration and ejection, and transfer 100 µl to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure five times, creating two rows of VEGF-A standard dilutions ranging from 2000 to 31.25 pg/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of VEGF-A standard dilutions:

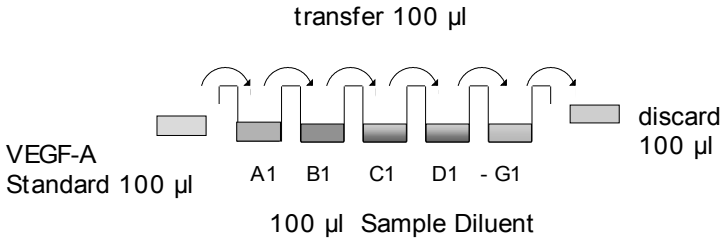


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

Standard Concentrations pg/mL		Sample wells										
	1	2	3	4	5	6	7	8	9	10	11	12
A	2000	2000										
B	1000	1000										
C	500	500										
D	250	250										
E	125	125										
F	62.5	62.5										
G	31.25	31.25										
H	Blank	Blank										

e. Add 100 µl of **Sample Diluent,** in duplicate, to the blank wells.

f. Add 50 µl of **Sample Diluent** to the sample wells.

g. Add 50 µl of each **Sample**, in duplicate, to the designated wells and mix the contents.

h. Prepare **Biotin-Conjugate**.

i. Add 50 µl of diluted **Biotin-Conjugate** to all wells, including the blank wells.

j. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 3 hours, if available on a rotator set at 100 rpm.

k. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.

l. Prepare **Streptavidin-HRP**

m. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.

n. Cover with a **Plate Cover** and incubate at room temperature (18º to 25ºC) for 1 hour, if available on a rotator set at 100 rpm**.**

o. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.

p. Pipette 100 µl of **TMB Substrate Solution** to all wells, including the blank wells.

q. Incubate the microwell strips at room temperature (18° to 25°C) for about 20 minutes, if available on a rotator set at 100 rpm. Avoid direct exposure to intense light.
The colour development on the plate should be monitored and the substrate reaction stopped (see point s. of this protocol) before positive wells are no longer properly recordable.
It is recommended to add the stop solution when the highest standard has developed a dark blue colour.
Alternatively the colour development can be monitored by the ELISA reader at 260 nm. The substrate reaction should be stopped ad soon as an OD of 0.6 – 0.65 is reached.

r. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

s. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both, the samples and the VEGF-A standards.

Note: In case of incubation without shaking the obained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.

- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the VEGF-A concentration on the abscissa. Draw a best fit curve through the points of the graph.

- To determine the concentration of circulating VEGF-A for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding VEGF-A concentration.

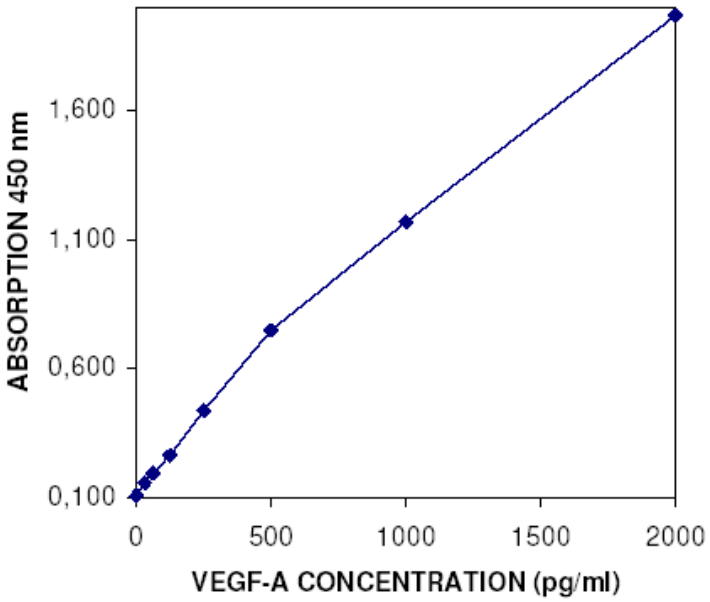
- **For samples which have been diluted according to the instructions given in this manual 1:2, the concentration read from the standard curve must be multiplied by the dilution factor (x 2).**

Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low VEGF-A levels. Such samples require further dilution of 1:4 - 1:8 with Sample Diluent in order to precisely quantitate the actual VEGF-A level.

- It is suggested that each testing facility establishes a control sample of known VEGF-A concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.

– A representative standard curve is shown in Figure 3. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 3. Representative standard curve for VEGF-A ELISA. Symbols represent the mean of three parallel titrations.
Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.



Typical data using the VEGF-A ELISA

Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	VEGF-A Concentration (pg/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	2000	1.933	1.968	2.0
	2000	2.003		
2	1000	1.164	1.168	1.3
	1000	1.172		
3	500	0.762	0.747	2.9
	500	0.732		
4	250	0.410	0.435	5.4
	250	0.459		
5	125	0.265	0.263	8.5
	125	0.261		
6	63	0.202	0.193	6.9
	63	0.184		
7	32	0.160	0.156	5.6
	32	0.152		
Blank	0	0.103	0.106	
	0	0.108		

LIMITATIONS OF THE PROCEDURE

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- 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 Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- Excessive freeze – thaw cycles may result in loss of bio-active VEGF-A. Avoid repeated freeze – thaw cycles, preferentially use freshly collected samples.

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PERFORMANCE CHARACTERISTICS

Sensitivity

The limit of detection of VEGF-A defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (mean plus three standard deviations) was determined to be 13.5 pg/ml (mean of 6 independent assays).

Reproducibility

a. Intra-assay

Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 5 serum samples containing different concentrations of VEGF-A. Two standard curves were run on each plate. Data below show the mean VEGF-A concentration and the coefficient of variation for each sample. The overall intra-assay coefficient of variation has been calculated to be 6.8 %.

Positive Sample	Experiment	VEGF-A Concentration (pg/ml)	Coefficient of Variation (%)
1	1	2003.4	4.6
	2	2131.6	6.5
	3	2172.5	8.0
2	1	1398.1	9.6
	2	1486.2	11.3
	3	1289.7	10.1
3	1	2596.7	4.6
	2	2088.3	5.5
	3	2161.7	6.7
4	1	2647.7	3.2
	2	2859.3	8.3
	3	2578.6	4.4
5	1	2019.6	7.5
	2	1569.8	4.5
	3	1856.7	7.5

b. Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in three independent experiments by three technicians. Each assay was carried out with 6 replicates of 5 serum samples containing different concentrations of VEGF-A. Two standard curves were run on each plate. Data below show the mean VEGF-A concentration and the coefficient of variation calculated on 18 determinations of each sample. The overall inter-assay coefficient of variation has been calculated to be 8.3 %.

Sample	VEGF Concentration (pg/ml)	Coefficient of Variation (%)
1	2102.5	4.2
2	1391.4	7.1
3	2282.2	12.0
4	2695.2	5.4
5	1815.4	12.5

Spike Recovery

The spike recovery was evaluated by spiking recombinant human VEGF-A into different human serum samples. Recoveries were determined in three independent experiments with 8 serum samples. The amount of endogenous VEGF-A in unspiked serum was substracted from the two spike values. Recoveries ranged from 81.5 to 91.9 % with an overall mean recovery of 86.3 %.
Recoveries were shown to depend on the serum used.

Dilution Parallelism

Three serum samples with different levels of VEGF-A were assayed at three serial two-fold dilutions (1:2-1:16) with 4 replicates each. In the table below the per cent recovery of expected values is listed. Recoveries ranged from 96.6 % to 117.6 % with an overall mean recovery of 111.9 %. Recoveries were shown to depend on the serum used.

		VEGF-A Concentration (pg/ml)		
Sample	Dilution	Expected Value	Observed Value	% Recovery of Exp. Value
1	1:2	--	7468,9	--
	1:4	3734,4	4391,9	117,6%
	1:8	2195,9	2471,1	112,5%
	1:16	1235,5	1371,3	111%
2	1:2	--	4666,0	--
	1:4	2333,0	2728,5	117%
	1:8	1364,2	1569,3	115%
	1:16	784,6	923,0	117,6%
3	1:2	--	8245,8	--
	1:4	4122,9	4608,7	111,8%
	1:8	2304,4	2226,3	96,6%
	1:16	1113,2	1201,2	107,9%
4	1:2	--	774,5	--
	1:4	387,3	437,8	113%
	1:8	218,9	244,7	111,8%
	1:16	122,3	135,4	110,7%

Sample stability

a. Freeze-Thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored at -20°C and thawed up to 5 times, and VEGF-A levels determined. There was a significant loss of active VEGF-A by repeated freezing and thawing, which therefore should be avoided.

b. Storage stability

Aliquots of a serum sample (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the VEGF-A level determined after 24 h. There was a significant loss of active VEGF-A immunoreactivity during storage at RT and 37°C, which therefore should be avoided.

Comparison of serum and plasma

Serum as well as plasma are suitable for the test. It is highly recommended to assure the uniformity of blood preparations.

Specificity

The interference of circulating factors of the immune systems was evaluated by spiking these proteins at physiologically relevant concentrations into a VEGF-A positive serum. There was no interference with any of the spiked proteins.

Expected serum values

A panel of 8 sera from apparently healthy blood donors (males and females) was tested for VEGF-A. The detected VEGF-A levels ranged between 49-877 pg/ml with a mean level of 323 pg/ml. The normal levels measured may vary with the sample collective used.

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REAGENT PREPARATION SUMMARY

A. Wash Buffer Add **Wash Buffer Concentrate** 20 x (50 ml) to 950 ml distilled water

B. Assay Buffer

Number of Strips	Assay Buffer Concentr. (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

C. Standard Reconstitute **Standard** by addition of distilled water. Reconstitution volume is stated on label of the standard vial.

D. Biotin-Conjugate Make a 1:100 dilution according to the table.

Number of Strips	Biotin-Conjugate (µl)	Assay Buffer (ml)
1 - 6	30	2.97
1 - 12	60	5.94

E. Streptavidin-HRP

Number of Strips	Streptavidin-HRP (µl)	Assay Buffer (ml)
1 - 6	30	5.97
1 - 12	60	11.94

TEST PROTOCOL SUMMARY

- Wash microwell strips twice with **Wash Buffer**
- Add 100 µl **Sample Diluent**, in duplicate, to standard wells
- Pipette 100 µl **VEGF-A Standard** into the first wells and create standard dilutions ranging from 2000 to 31.25 pg/ml by transferring 100 µl from well to well. Discard 100 µl from the last wells
- Add 100 µl **Sample Diluent**, in duplicate, to the blank wells
- Add 50 µl **Sample Diluent** to sample wells.
- Add 50 µl **Sample**, in duplicate, to designated wells
- Prepare **Biotin-Conjugate**
- Add 50 µl of diluted **Biotin-Conjugate** to all wells
- Cover microwell strips and incubate 3 hours at room temperature
- Prepare **Streptavidin-HRP**
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl diluted **Streptavidin-HRP** to all wells
- Cover microwell strips and incubate 1 hour at room temperature (18º to 25ºC)
- Prepare **TMB Substrate Solution** few minutes prior to use
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl of mixed **TMB Substrate Solution** to all wells including blank wells
- Incubate the microwell strips for about 20 minutes at room temperature (18°to 25°C)
- Add 100 µl **Stop Solution** to all wells including blank wells
- Blank microwell reader and measure colour intensity at 450 nm

Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low VEGF-A levels. Such samples require further dilution of 1:4 - 1:8 with Sample Diluent in order to precisely quantitate the actual VEGF-A level.