ELISA PRODUCT INFORMATION & MANUAL

Human VEGF ELISA Kit (Colorimetric)

NBP1-91272

Enzyme-linked Immunosorbent Assay for quantitative detection. For research use only.
Not for diagnostic or therapeutic procedures.

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1 Intended Use

The human VEGF ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human VEGF. **The human VEGF ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

2 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. 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). It is a specific endothelial cell mitogen and a strong vascular permeability factor (VPF). VEGF is a heparin-binding glycoprotein, secreted as a homodimer of 45 kDa by many different cell types. VEGF also causes vasodilation through the nitiric oxide synthase pathway in endothelial cells and can activate migration in monocytes. Many different splice variants of VEGF 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, lymphangiogenesis is mainly regulated by VEGF-C and -D.
VEGF transcription is highly activated by hypoxia and by oncogenes like H-ras and several transmembrane tyrosine kinases, such as epidermal growth factor receptor and ErbB2. Together these pathways account for a marked upregulation of VEGF in tumors compared to normal tissues and are often of prognostic importance and relevance. VEGF can be detected

in both plasma and serum samples of patients, with much higher levels in serum. Extremely high levels can be detected in the cystic brain fluid of brain tumor patients or in ascites fluid of patients. Platelets release VEGF-A upon aggregation and may be another major source of VEGF delivery to tumors. Several other studies have shown that association of high serum levels of VEGF with poor prognosis in cancer patients may be correlated with an elevated platelet count. 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 delivery to tumors. Furthermore, VEGF is implicated in several other pathological conditions associated with enhanced angiogenesis or enhanced vascular permeability. Examples where VEGF plays an important role are psoriasis and rheumatoid arthritis, as well as the ovarian hyperstimulation syndrome. Diabetic retinopathy is associated with high intraocular levels of VEGF, and inhibition of VEGF function may result in infertility by blockage of corpus luteum function. Direct demonstration of the importance of VEGF in tumor growth has been achieved using dominant negative VEGF receptors to block in vivo proliferation, as well as blocking antibodies to VEGF or to one of the VEGF receptors. Interference with VEGF 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 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 production, Targeting the VEGF signalling may be of major therapeutic importance for many diseases and serves as a basis for the design of future (anti)-angiogenic treatments. =

3 Principles of the Test

An anti-human VEGF coating antibody is adsorbed onto microwells.

Figure 1

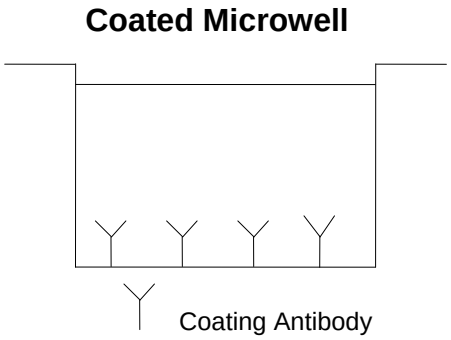


Figure 2

Human VEGF present in the sample or standard binds to antibodies adsorbed to the microwells.

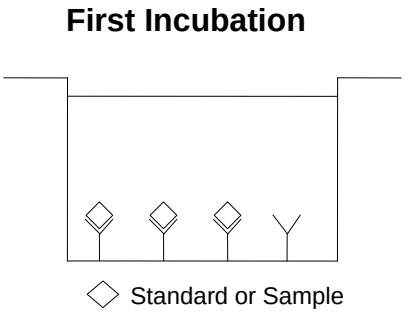


Figure 3

Following incubation unbound biological components are removed during a wash step. A biotin-conjugated anti-human VEGF antibody is added and binds to human VEGF captured by the first antibody.

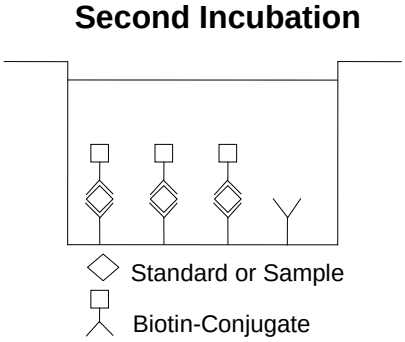
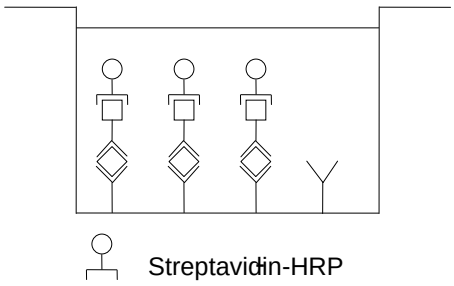


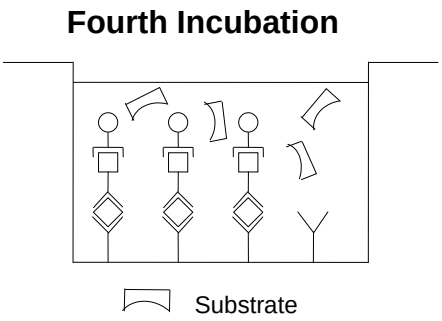
Figure 4

Following incubation unbound biotin-conjugated anti-human VEGF antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human VEGF antibody.



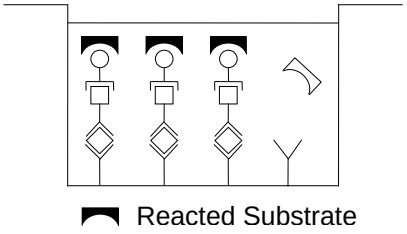
Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

Figure 5



A coloured product is formed in proportion to the amount of human VEGF present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human VEGF standard dilutions and human VEGF-A sample concentration determined.

Figure 6



4 Reagents Provided

4.1 Reagents for human VEGF ELISA NBP1-91272 (96 tests)

1 aluminium pouch with a **Microwell Plate coated** with polyclonal antibody to human VEGF

1 vial (120 µl) **Biotin-Conjugate** anti-human VEGF polyclonal antibody

1 vial (150 µl) **Streptavidin-HRP**

2 vials human VEGF **Standard** lyophilized, 2 ng/ml upon reconstitution

1 vial (12 ml) **Sample Diluent**

1 vial (5 ml) **Assay Buffer Concentrate** 20x
(PBS with 1% Tween 20, 10% BSA)

1 bottle (50 ml) **Wash Buffer Concentrate** 20x
(PBS with 1% Tween 20)

1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)

1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)

6 **Adhesive Films**

4.2 Reagents for human VEGF ELISA NBP1-91272
(10x96 tests)

10 aluminium pouches with a **Microwell Plate coated** with polyclonal antibody to human VEGF

10 vials (120 µl) **Biotin-Conjugate** anti-human VEGF polyclonal antibody

10 vials (150 µl) **Streptavidin-HRP**

10 vials human VEGF **Standard** lyophilized, 2 ng/ml upon reconstitution

7 vials (12 ml) **Sample Diluent**

3 vials (5 ml) **Assay Buffer Concentrate** 20x
(PBS with 1% Tween 20, 10% BSA)

1 bottle (500 ml) **Wash Buffer Concentrate** 20x
(PBS with 1% Tween 20)

10 vials (15 ml) **Substrate Solution** (tetramethyl-benzidine)

1 vial (100 ml) **Stop Solution** (1M Phosphoric acid)

30 **Adhesive Films**

5 Storage Instructions – ELISA Kit

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 labels.

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, this reagent is not contaminated by the first handling.

6 Specimen Collection and Storage Instructions

Cell culture supernatant, serum* and plasma (EDTA, citrate, heparin) were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells 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 aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human VEGF. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 13.5). Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

* Pay attention to a possibly elevated serum level of human VEGF due to VEGF release by platelets during platelet activation (sampling process).

7 Materials Required But Not Provided

- 5 ml and 10 ml graduated pipettes
- 5 µl to 1000 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)
- Microplate shaker
- 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 regression analysis

8 Precautions for Use

- All reagents should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- 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.
- Avoid contact of substrate solution 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 reagent.

- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as 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.

9 Preparation of Reagents

Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure.
If crystals have formed in the **Buffer Concentrates**, warm them gently until they have completely dissolved.

9.1 Wash Buffer (1x)

Pour 50 ml of the **Wash Buffer Concentrate** (20x) into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water.
Mix gently to avoid foaming.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.

Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950
10x (1 – 12)	500	10000

9.2 Assay Buffer (1x)

Pour the entire contents (5 ml) of the **Assay Buffer Concentrate (20x)** into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming.

Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.

Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

9.3 Biotin-Conjugate

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **Biotin-Conjugate** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

9.4 Streptavidin-HRP

Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **Streptavidin-HRP** solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

9.5 Human VEGF Standard

Reconstitute **human VEGF standard** by addition of distilled water. Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 2 ng/ml).
Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.

After usage remaining standard cannot be stored and has to be discarded.

Standard dilutions can be prepared directly on the microwell plate (see 10.c) or alternatively in tubes (see 9.5.1).

9.5.1 External Standard Dilution

Label 7 tubes, one for each standard point.

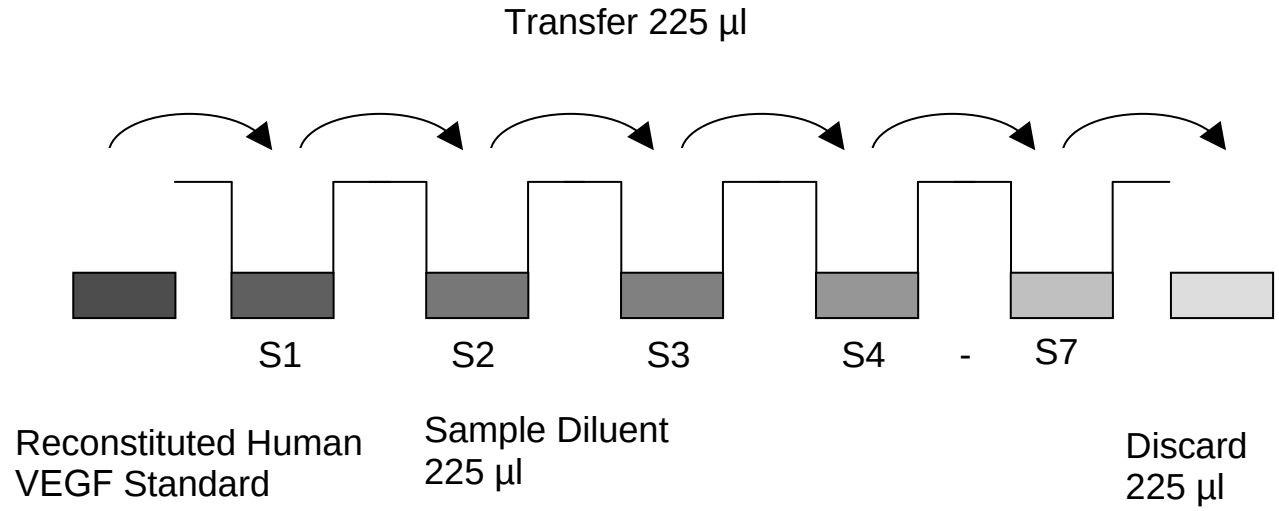
S1, S2, S3, S4, S5, S6, S7

Then prepare 1:2 serial dilutions for the standard curve as follows:
Pipette 225 µl of Sample Diluent into each tube.

Pipette 225 µl of reconstituted standard (concentration of standard = 2 ng/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 1 ng/ml).
Pipette 225 µl of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer.
Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 7).

Sample Diluent serves as blank.

Figure 7


Transfer 225 µl
S1
S2
S3
S4
-
S7
Reconstituted Human VEGF Standard
Sample Diluent 225 µl
Discard 225 µl

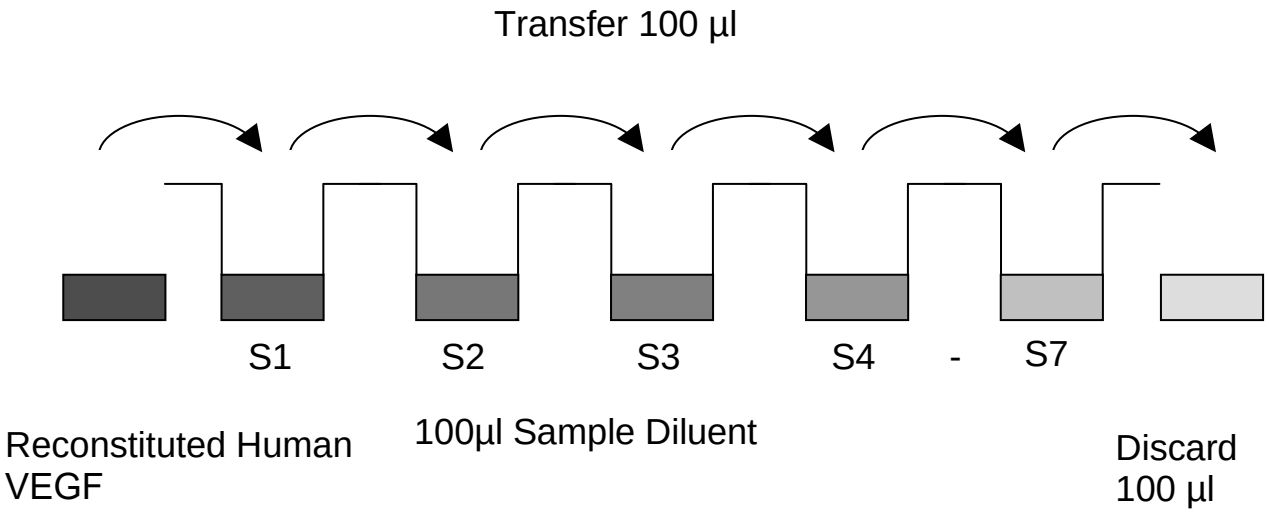
10 Test Protocol

a. 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 from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

b. Wash the microwell strips twice with approximately 400 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells.
After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. **Do not allow wells to dry**.

c. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes - see 9.5.1.):
Add 100 µl of Sample Diluent in duplicate to all **standard wells**.
Pipette 100 µl of prepared **standard** (see Preparation of Standard 9.5, concentration = 2000 pg/ml) in duplicate into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 1000 pg/ml), and transfer 100 µl to wells B1 and B2, respectively (see Figure 8). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human VEGF standard dilutions ranging from 1000 to 15.6 pg/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 8


Transfer 100 µl
S1
S2
S3
S4
-
S7
Reconstituted Human VEGF
100µl Sample Diluent
Discard 100 µl

In case of an **external standard dilution** (see 9.5.1), pipette 100 µl of these standard dilutions (S1 - S7) in the standard wells according to Table 1.

Table 1
Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	**2**	**3**	**4**
A	Standard 1 (1000.0 pg/ml)	Standard 1 (1000.0 pg/ml)	Sample 1	Sample 1
B	Standard 2 (500.0 pg/ml)	Standard 2 (500.0 pg/ml)	Sample 2	Sample 2
C	Standard 3 (250.0 pg/ml)	Standard 3 (250.0 pg/ml)	Sample 3	Sample 3
D	Standard 4 (125.0 pg/ml)	Standard 4 (125.0 pg/ml)	Sample 4	Sample 4
E	Standard 5 (62.5 pg/ml)	Standard 5 (62.5 pg/ml)	Sample 5	Sample 5
F	Standard 6 (31.3 pg/ml)	Standard 6 (31.3 pg/ml)	Sample 6	Sample 6
G	Standard 7 (15.6 pg/ml)	Standard 7 (15.6 pg/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

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

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

f. Add 50 µl of each **sample** in duplicate to the **sample wells**.

g. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 2 hours on a microplate shaker set at 400 rpm. **(Shaking is absolutely necessary for an optimal test performance.)**

h. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate 9.3).

i. Remove adhesive film and empty wells. **Wash** microwell strips 6 times according to point b. of the test protocol. Proceed immediately to the next step.

j. Add 100 µl of **Biotin-Conjugate** to all wells.

k. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour on a microplate shaker set at 400 rpm. **(Shaking is absolutely necessary for an optimal test performance.)**

l. Prepare **Streptavidin-HRP** (refer to Preparation of Streptavidin-HRP 9.4).

m. Remove adhesive film and empty wells. **Wash** microwell strips 6 times according to point b. of the test protocol. Proceed immediately to the next step.

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

o. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour on a microplate shaker set at 400 rpm. **(Shaking is absolutely necessary for an optimal test performance.)**.

p. Remove adhesive film and empty wells. **Wash** microwell strips 6 times according to point b. of the test protocol. Proceed immediately to the next step.

q. Pipette 100 µl of **TMB Substrate Solution** to all wells.

r. Incubate the microwell strips at room temperature (18° to 25°C) for **about 30 min**. Avoid direct exposure to intense light.

   The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay.

   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 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

s. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well. 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.

t. 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 standards.

11 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 value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human VEGF concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating human VEGF 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 human VEGF concentration.
- **If instructions in this protocol have been followed, samples have been diluted 1:2 (50 µl sample + 50 µl Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x 2).**
- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human VEGF levels. Such samples require further external predilution according to expected human VEGF values with Sample Diluent in order to precisely quantitate the actual human VEGF level.**
- It is suggested that each testing facility establishes a control sample of known human VEGF concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 9. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 9

Representative standard curve for human VEGF ELISA. Human VEGF-A was diluted in serial 2-fold steps in Sample Diluent. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

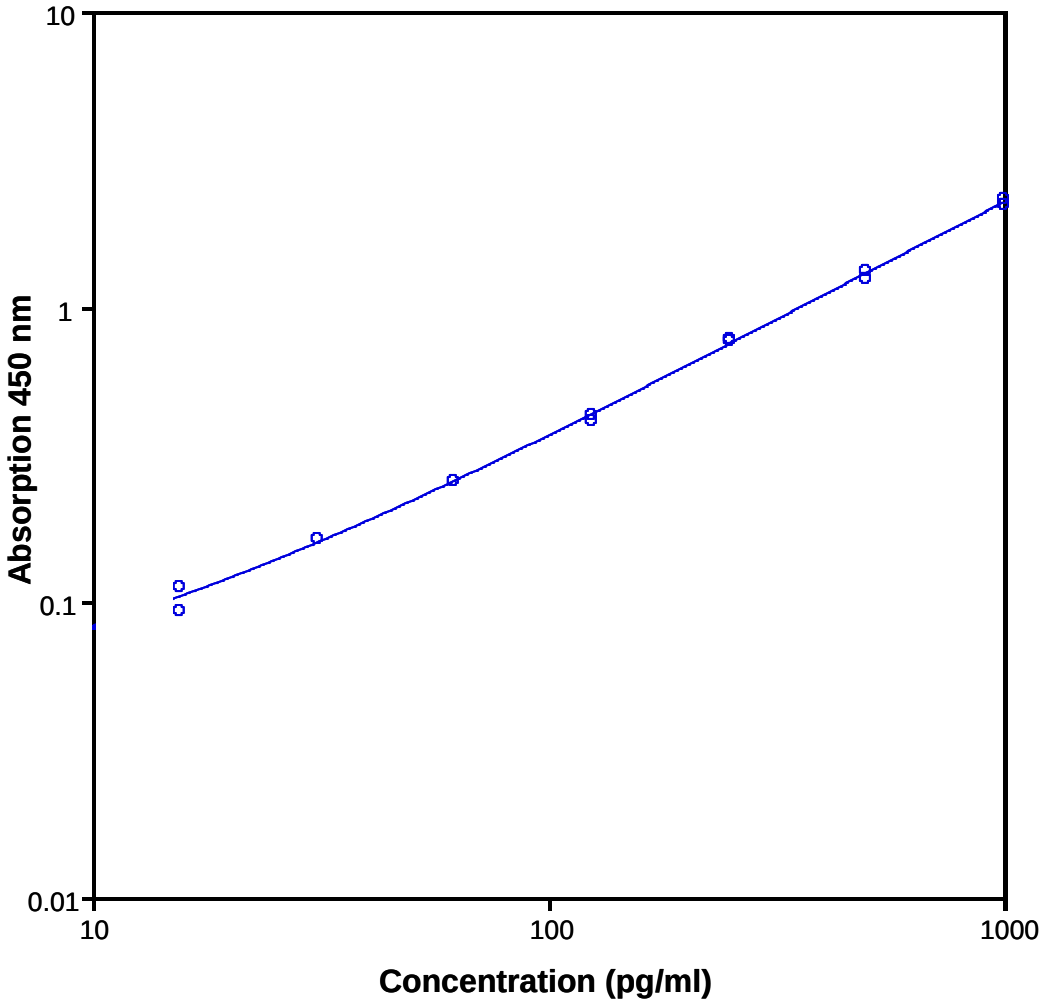


Table 2

Typical data using the human VEGF ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	Human VEGF Concentration (pg/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	1000.0	2.201	2.254	2.4
		2.308		
2	500.0	1.244	1.286	3.2
		1.327		
3	250.0	0.766	0.771	0.6
		0.775		
4	125.0	0.409	0.419	2.5
		0.429		
5	62.5	0.258	0.258	0.1
		0.258		
6	31.3	0.162	0.163	0.3
		0.163		
7	15.6	0.112	0.102	9.4
		0.093		
Blank	0	0.066	0.069	4.9
		0.073		

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

12 Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen 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. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

13 Performance Characteristics

13.1 Sensitivity

The limit of detection of human VEGF defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 7.9 pg/ml (mean of 6 independent assays).

13.2 Reproducibility

13.2.1 Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human VEGF. 2 standard curves were run on each plate. Data below show the mean human VEGF concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 6.2%.

Table 3

The mean human VEGF concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human VEGF Concentration (pg/ml)	Coefficient of Variation (%)
1	1	1596	3.7
	2	1496	2.9
	3	1507	1.8
2	1	881	2.6
	2	853	7.7
	3	888	5.3
3	1	439	3.5
	2	452	6.2
	3	469	10.3
4	1	897	7.1
	2	849	6.5
	3	844	5.7
5	1	119	11.7
	2	118	13.3
	3	130	7.8
6	1	85	8.3
	2	78	8.7
	3	83	2.6
7	1	256	5.2
	2	295	9.9
	3	304	3.1
8	1	371	3.9
	2	350	4.2
	3	352	6.2

13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human VEGF. 2 standard curves were run on each plate. Data below show the mean human VEGF concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 4.3%.

Table 4
The mean human VEGF concentration and the coefficient of variation of each sample

Sample	Mean Human VEGF Concentration (pg/ml)	Coefficient of Variation (%)
1	1533	3.6
2	874	2.1
3	453	3.4
4	864	3.4
5	122	5.2
6	82	4.6
7	285	8.9
8	358	3.1

13.3 Spike Recovery

The spike recovery was evaluated by spiking 3 levels of human VEGF into serum, plasma and cell culture supernatant. Recoveries were determined with 4 replicates each. The amount of endogenous human VEGF in unspiked samples was subtracted from the spike values. For recovery data see Table 5.

Table 5

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

13.4 Dilution Parallelism

Serum, plasma and cell culture supernatant samples with different levels of human VEGF were analysed at serial 2 fold dilutions with 4 replicates each.
For recovery data see Table 6.

Table 6

Sample matrix	Recovery of Exp. Val.	
	Range (%)	Mean (%)
Serum	76 - 104	90
Plasma (EDTA)	93 - 147	110
Plasma (citrate)	83 - 99	90
Plasma (heparin)	98 - 119	108
Cell culture supernatant	72 - 103	91

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C and thawed 5 times, and the human VEGF levels determined. A significant decrease of human VEGF immunoreactivity was detected. Therefore samples should be stored in aliquots at -20°C and thawed only once.

13.5.2 Storage Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the human VEGF level determined after 24 h. There was no significant loss of human VEGF immunoreactivity detected during storage at -20°C and 2-8°C.
A significant loss of human VEGF immunoreactivity was detected during storage at RT and 37°C after 24 h.

13.6 Specificity

The assay detects both natural and recombinant human VEGF.
Crossreactivity and interference of circulating factors of the immune systeme were evaluated by spiking these proteins at physiologically relevant concentrations into a human VEGF positive serum.
There was no crossreactivity detected, notably not with human VEGF-B, VEGF-C, VEGF-D and PfGF.
Interference was detected for VEGF-R1 at concentrations > 200 pg/ml, and not for VEGF-R2.

13.7 Expected Values

Panels of 40 serum as well as EDTA, citrate and heparin plasma samples from randomly selected apparently healthy donors were tested for human VEGF.
The levels measured may vary with the sample collection used.
For detected human VEGF levels see Table 7.

Table 7

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

* n.d. = non-detectable, samples measured below the lowest standard point are considered to be non-detectable.

14 Reagent Preparation Summary

14.1 Wash Buffer (1x)

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

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

14.2 Assay Buffer (1x)

Add **Assay Buffer Concentrate 20x** (5 ml) to 95 ml distilled water.

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

14.3 Biotin-Conjugate

Make a 1:100 dilution of **Biotin-Conjugate** in Assay Buffer (1x):

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

14.4 Streptavidin-HRP

Make a 1:100 dilution of **Streptavidin-HRP** in Assay Buffer (1x):

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

14.5 Human VEGF Standard

Reconstitute lyophilized **human VEGF standard** with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

15 Test Protocol Summary

1. Determine the number of microwell strips required.
2. Wash microwell strips twice with Wash Buffer.
3. Standard dilution on the microwell plate: Add 100 µl Sample Diluent, in duplicate, to all standard wells. Pipette 100 µl prepared standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells.
   Alternatively external standard dilution in tubes (see 9.5.1): Pipette 100 µl of these standard dilutions in the microwell strips.
4. Add 100 µl Sample Diluent, in duplicate, to the blank wells.
5. Add 50 µl Sample Diluent to sample wells.
6. Add 50 µl sample in duplicate, to designated sample wells.
7. Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C). **(Shaking is absolutely necessary for an optimal test performance.)**
8. Prepare Biotin-Conjugate.
9. Empty and wash microwell strips 6 times with Wash Buffer.
10. Add 100 µl Biotin-Conjugate to all wells.
11. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C). **(Shaking is absolutely necessary for an optimal test performance.)**
12. Prepare Streptavidin-HRP.
13. Empty and wash microwell strips 6 times with Wash Buffer.
14. Add 100 µl diluted Streptavidin-HRP to all wells.
15. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C). **(Shaking is absolutely necessary for an optimal test performance.)**
16. Empty and wash microwell strips 6 times with Wash Buffer.
17. Add 100 µl of TMB Substrate Solution to all wells.
18. Incubate the microwell strips for about 30 minutes at room temperature (18° to 25°C).
19. Add 100 µl Stop Solution to all wells.
20. Blank microwell reader and measure colour intensity at 450 nm.

Note: If instructions in this protocol have been followed, samples have been diluted 1:2 (50 µl sample + 50 µl Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x 2).