



ELISA PRODUCT INFORMATION & MANUAL

Human VEGFR2/KDR/Flk-1 ELISA Kit (Colorimetric)

NBP1-83729

Enzyme-linked Immunosorbent Assay for quantitative
detection. For research use only.

Not for diagnostic or therapeutic procedures.

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Novus kits are guaranteed for 6 months from date of receipt

TABLE OF CONTENTS

1	Intended Use	3
2	Summary	3
3	Principles of the Test	5
4	Reagents Provided	7
5	Storage Instructions – ELISA Kit	9
6	Specimen Collection and Storage Instructions	9
7	Materials Required But Not Provided	10
8	Precautions for Use	11
9	Preparation of Reagents	13
10	Test Protocol	17
11	Calculation of Results	22
12	Limitations	25
13	Performance Characteristics	26
14	Reagent Preparation Summary	31
15	Test Protocol Summary	32

1 Intended Use

The human VEGFR2/KDR/Flk-1 ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human VEGFR2/KDR/Flk-1. **The human VEGFR2/KDR/Flk-1 ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

2 Summary

Angiogenesis is a fundamental physiological process, both during the development of the organism and in adult life, requiring the well coordinated action of a variety of growth factors and adhesion molecules in endothelial cells. So far, VEGF-A (also called VEGF) and its receptors represent the best characterized signalling pathway in developmental and tumour angiogenesis. VEGF-A binds to two receptor-tyrosine kinases: VEGFR-1/Flt-1 and VEGFR-2/KDR. VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF-A.

The VEGFR's possess an approximately 750 amino acid residue extracellular domain, which is organized into seven immunoglobulin (Ig)-like folds. This extracellular domain (also called ectodomain) is followed by a single transmembrane region, a juxtamembrane domain, a split tyrosine-kinase domain interrupted by a 70-amino acid kinase insert and a C-terminal tail. Alternative splicing or proteolytic processing of VEGFR's give rise to secreted variants of VEGFR-2, also called soluble VEGFR-2 (sVEGFR-2). Although the VEGFR's are primarily expressed in the vascular system, sensitive methods like sandwich ELISA's have allowed the detection of VEGFR expression in non-endothelial cells like hematopoietic stem cells.

The important role of VEGFR-2 signalling during development and in neo-vascularization in physiological or pathological conditions in vivo has allowed the design of clinically beneficial therapies. A soluble form of VEGFR-2 protein can be detected in human and murine plasma. Studies confirmed that the detected soluble fragment was a truncated form of VEGFR-2, shed from mouse and human endothelial cells. Since the activation of VEGFR-2 plays an important role in tumour angiogenesis, there is broad clinical interest in monitoring plasma soluble VEGFR-2 levels in cancer patients with a focus on its potential as a surrogate biomarker for disease progression as well as monitoring marker of the efficiency of anti-angiogenesis drugs. Using mouse

models with human tumours a reverse relationship could be shown between the levels of VEGFR-2 and tumour size.

Besides its putative role as a surrogate marker for tumour angiogenesis, naturally occurring VEGFR-2 is a molecular regulator for VEGF and VEGFR signalling. Further investigations will reveal if VEGFR-2 can arrest solid tumour angiogenesis and modulate metastasis.

The sandwich ELISA to detect, measure and quantify soluble and solubilized VEGFR-2 levels will help to explain recent clinical results for anti-angiogenic therapy and will allow further understanding of VEGFR-2 as biomarker for monitoring cancer progression and its possible role in modulation of vessel growth.

3 Principles of the Test

An anti-human VEGFR2/KDR/Flk-1 coating antibody is adsorbed onto microwells.

Human VEGFR2/KDR/Flk-1 present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human VEGFR2/KDR/Flk-1 antibody is added and binds to human VEGFR2/KDR/Flk-1 captured by the first antibody.

Following incubation unbound biotin-conjugated anti-human VEGFR2/KDR/Flk-1 antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human VEGFR2/KDR/Flk-1 antibody.

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

Figure 1

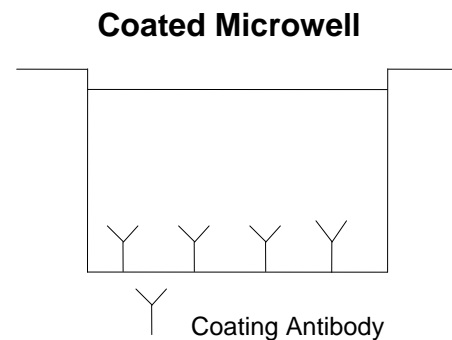


Figure 2

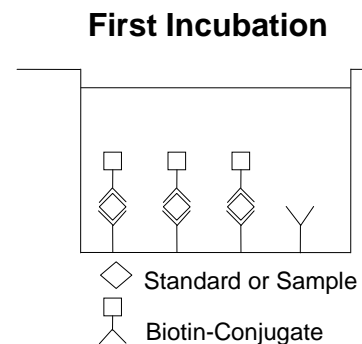


Figure 3

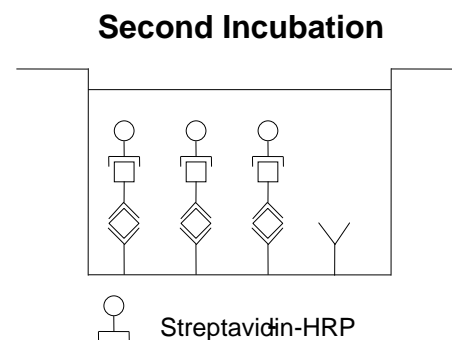
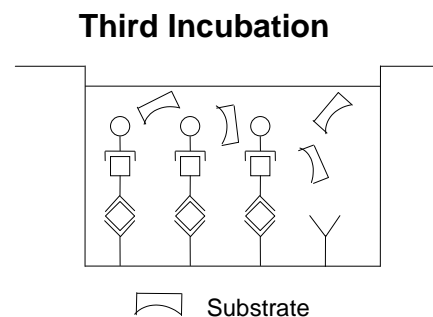
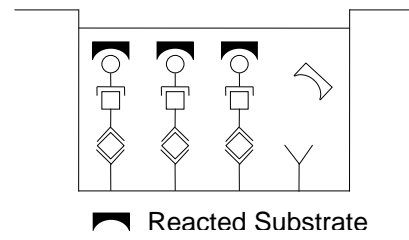


Figure 4



A coloured product is formed in proportion to the amount of human VEGFR2/KDR/FIk-1 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 VEGFR2/KDR/FIk-1 standard dilutions and human VEGFR2/KDR/FIk-1 sample concentration determined.

Figure 5



4 Reagents Provided

4.1 Reagents for human VEGFR2/KDR/FIk-1 ELISA NBP1-83729 (96 tests)

- 1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to human VEGFR2/KDR/FIk-1
- 1 vial (70 µl) **Biotin-Conjugate** anti-human VEGFR2/KDR/FIk-1 monoclonal antibody
- 1 vial (150 µl) **Streptavidin-HRP**
- 2 vials human VEGFR2/KDR/FIk-1 **Standard** lyophilized, 10 ng/ml upon reconstitution
- 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)
- 4 **Adhesive Films**

4.2 Reagents for human VEGFR2/KDR/FIk-1 ELISA NBP1-83729 (10x96 tests)

- 10 aluminium pouches with a **Microwell Plate coated** with monoclonal antibody to human VEGFR2/KDR/FIk-1
- 10 vials (70 µl) **Biotin-Conjugate** anti-human VEGFR2/KDR/FIk-1 monoclonal antibody
- 10 vials (150 µl) **Streptavidin-HRP**
- 10 vials human VEGFR2/KDR/FIk-1 **Standard** lyophilized, 10 ng/ml upon reconstitution
- 7 vials (5 ml) **Assay Buffer Concentrate 20x**
(PBS with 1% Tween 20, 10% BSA)
- 6 bottles (50 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)
- 20 **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 VEGFR2/KDR/Flk-1. 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.

7 Materials Required But Not Provided

- 5 ml and 10 ml graduated pipettes
- 5 μ l to 1000 μ l adjustable single channel micropipettes with disposable tips
- 50 μ l to 300 μ l 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 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 entire contents (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

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	0.03	2.97
1 - 12	0.06	5.94

9.4 Streptavidin-HRP

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

Make a 1:200 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	0.03	5.97
1 - 12	0.06	11.94

9.5 Human VEGFR2/KDR/FIk-1 Standard

Reconstitute **human VEGFR2/KDR/FIk-1 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 = 10 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.d) 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 μ l of Assay Buffer (1x) into each tube.

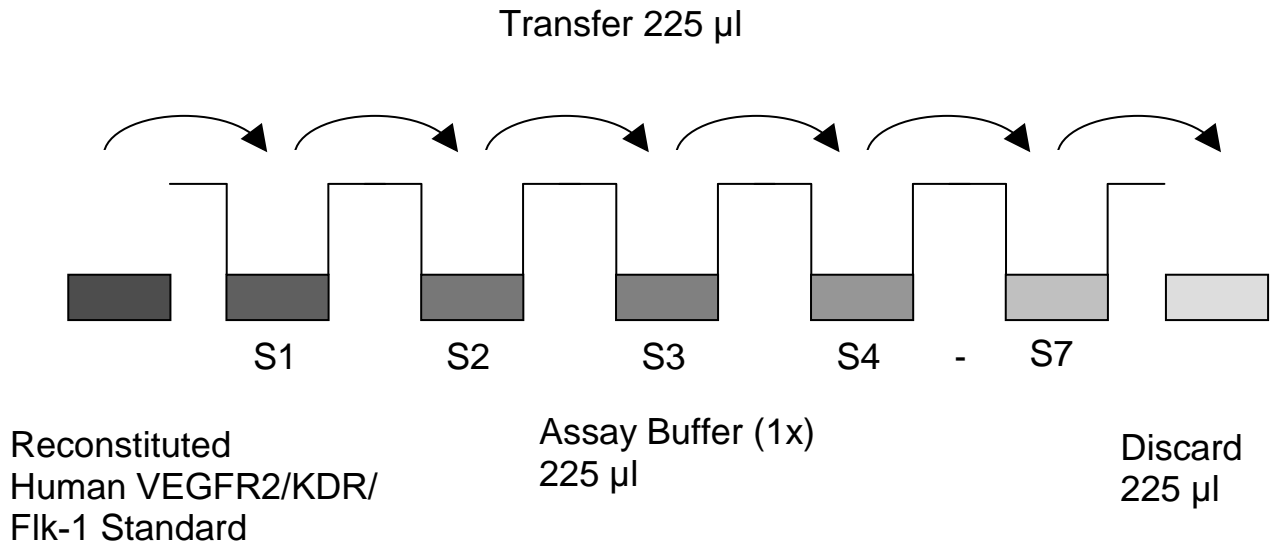
Pipette 225 μ l of reconstituted standard (concentration of standard = 10 ng/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 5 ng/ml).

Pipette 225 μ l 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 6).

Assay Buffer (1x) serves as blank.

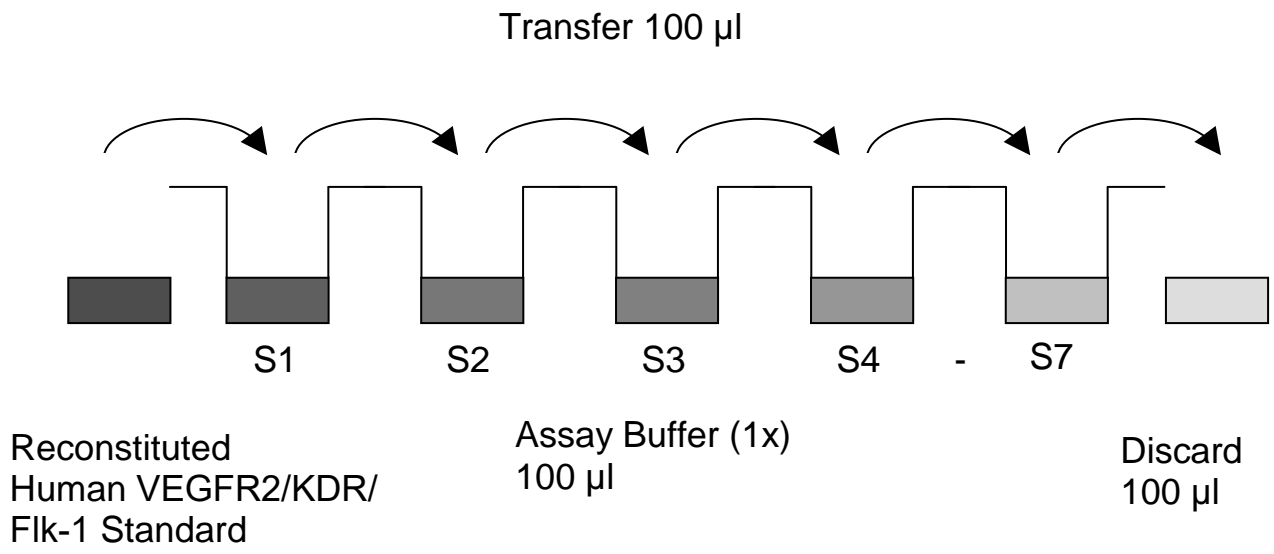
Figure 6



10 Test Protocol

- a. Predilute your samples before starting with the test procedure. Dilute serum, plasma and cell culture samples 1:25 with Assay Buffer (1x) according to the following scheme: 20 μ l sample + 480 μ l Assay Buffer (1x).
- 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 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 400 μ l **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
- d. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes - see 9.5.1)
Add 100 μ l of Assay Buffer (1x) in duplicate to all **standard wells**. Pipette 100 μ l of prepared **standard** (see Preparation of Standard 9.5, concentration = 10000 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 = 5000 pg/ml), and transfer 100 μ l to wells B1 and B2 (see Figure 7). Take care not to scratch the inner surface of the microwells.
Continue this procedure 5 times, creating two rows of human VEGFR2/KDR/Flk-1 standard dilutions ranging from 5000 to 78 pg/ml. Discard 100 μ l of the contents from the last microwells (G1, G2) used.

Figure 7



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 (5000 pg/ml)	Standard 1 (5000 pg/ml)	Sample 1	Sample 1
B	Standard 2 (2500 pg/ml)	Standard 2 (2500 pg/ml)	Sample 2	Sample 2
C	Standard 3 (1250 pg/ml)	Standard 3 (1250 pg/ml)	Sample 3	Sample 3
D	Standard 4 (625 pg/ml)	Standard 4 (625 pg/ml)	Sample 4	Sample 4
E	Standard 5 (313 pg/ml)	Standard 5 (313 pg/ml)	Sample 5	Sample 5
F	Standard 6 (156 pg/ml)	Standard 6 (156 pg/ml)	Sample 6	Sample 6
G	Standard 7 (78 pg/ml)	Standard 7 (78 pg/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

- e. Add 100 µl of **Assay Buffer (1x)** in duplicate to the **blank wells**.
- f. Add 50 µl of **Assay Buffer (1x)** to the **sample wells**.
- g. Add 50 µl of each prediluted **sample** in duplicate to the **sample wells**.
- h. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate 0).
- i. Add 50 µl of **Biotin-Conjugate** to all wells.
- j. 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.)**
- k. Prepare **Streptavidin-HRP** (refer to Preparation of Streptavidin-HRP 9.4).
- l. Remove adhesive film and empty wells. **Wash** microwell strips 6 times according to point c. of the test protocol. Proceed immediately to the next step.
- m. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.
- n. 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.)**
- o. Remove adhesive film and empty wells. **Wash** microwell strips 6 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.
- q. 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.

- r. 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.
- 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 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 VEGFR2/KDR/Flk-1 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 VEGFR2/KDR/Flk-1 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 VEGFR2/KDR/Flk-1 concentration.
- **If instructions in this protocol have been followed, samples have been diluted 1:50 (1:25 external predilution, 1:2 dilution on the plate: 50 µl sample + 50 µl Assay Buffer (1x)) and the concentration read from the standard curve must be multiplied by the dilution factor (x 50).**
- **Calculation of 1:25 prediluted samples with a concentration exceeding standard 1 may result in incorrect, low human VEGFR2/KDR/Flk-1 levels (Hook Effect). Such samples require further external predilution according to expected human VEGFR2/KDR/Flk-1 values with Assay Buffer (1x) in order to precisely quantitate the actual human VEGFR2/KDR/Flk-1 level.**
- It is suggested that each testing facility establishes a control sample of known human VEGFR2/KDR/Flk-1 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 8. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 8

Representative standard curve for human VEGFR2/KDR/Flk-1 ELISA. Human VEGFR2/KDR/Flk-1 was diluted in serial 2-fold steps in Assay Buffer (1x). Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

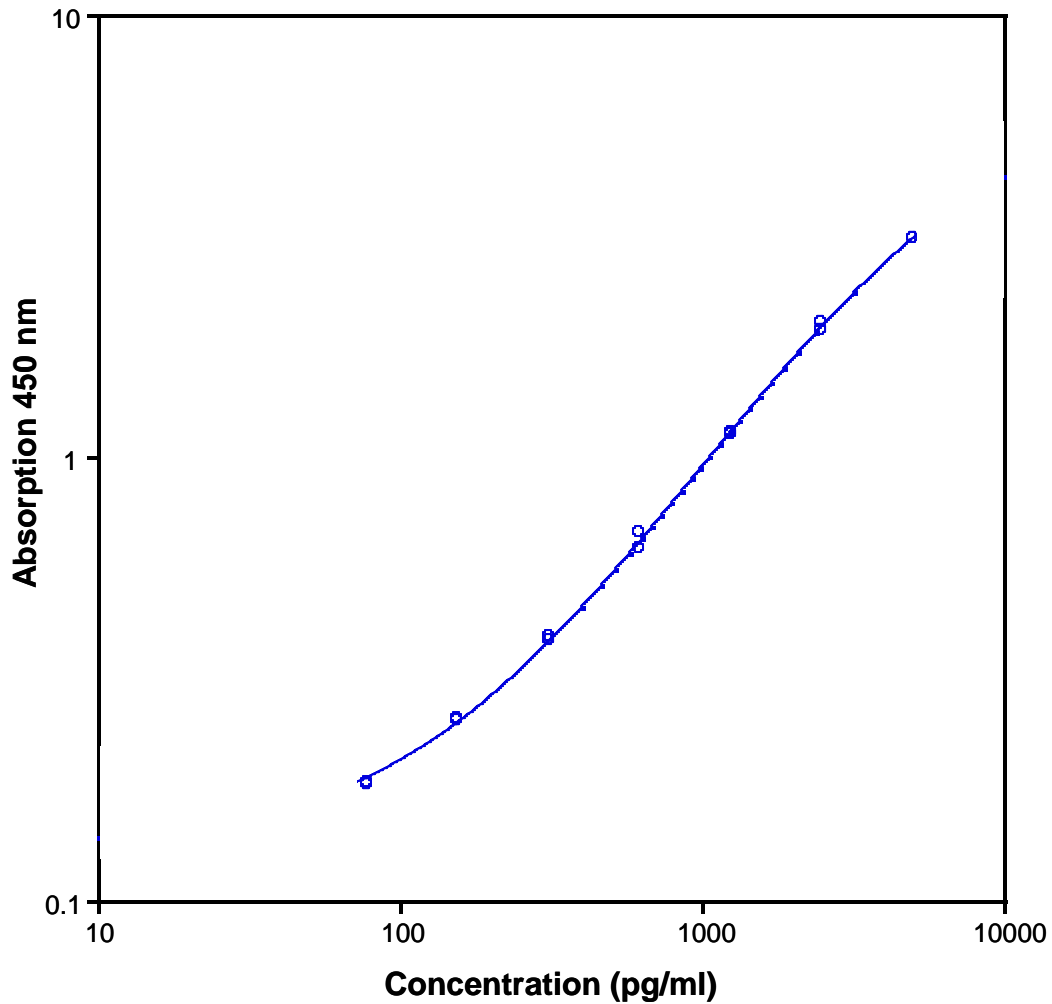


Table 2

Typical data using the human VEGFR2/KDR/Flk-1

ELISA Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Human VEGFR2/KDR/ Flk-1 Concentration (pg/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	5000	3.122 3.118	3.120	0.1
2	2500	2.020 1.936	1.978	2.1
3	1250	1.142 1.127	1.135	0.6
4	625	0.675 0.623	0.649	4.0
5	313	0.386 0.393	0.389	1.0
6	156	0.257 0.255	0.256	0.5
7	78	0.185 0.182	0.183	0.6
Blank	0	0.093 0.092	0.093	0.2

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.
- The use of radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

13 Performance Characteristics

13.1 Sensitivity

The limit of detection of human VEGFR2/KDR/Flk-1 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 7.0 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 VEGFR2/KDR/Flk-1. 2 standard curves were run on each plate. Data below show the mean human VEGFR2/KDR/Flk-1 concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 5.3%.

Table 3

The mean human VEGFR2/KDR/Flk-1 concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human VEGFR2/KDR/Flk-1 Concentration (pg/ml)	Coefficient of Variation (%)
1	1	152357	6.0
	2	119914	1.8
	3	117181	2.2
2	1	92056	5.3
	2	81321	3.3
	3	77605	4.0
3	1	47453	5.8
	2	40261	4.0
	3	36194	6.1
4	1	19234	5.3
	2	16679	3.7
	3	14978	4.8
5	1	15509	8.5
	2	13796	3.9
	3	13131	5.5
6	1	12818	5.4
	2	11928	6.1
	3	10974	6.0
7	1	6225	10.1
	2	6068	3.9
	3	5294	6.7
8	1	6805	7.6
	2	7132	5.4
	3	6795	6.8

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 VEGFR2/KDR/Flk-1. 2 standard curves were run on each plate. Data below show the mean human VEGFR2/KDR/Flk-1 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 9.8%.

Table 4

The mean human VEGFR2/KDR/Flk-1 concentration and the coefficient of variation of each sample

Sample	Mean Human VEGFR2/KDR/Flk-1 Concentration (pg/ml)	Coefficient of Variation (%)
1	129817	15.1
2	83661	9.0
3	41303	13.8
4	16964	12.6
5	14145	8.7
6	11906	7.7
7	5862	8.5
8	6911	2.8

13.3 Spike Recovery

The spike recovery was evaluated by spiking 3 levels of human VEGFR2/KDR/Flk-1 into serum, plasma and cell culture supernatant samples. Recoveries were determined with 4 replicates each. The amount of endogenous human VEGFR2/KDR/Flk-1 in unspiked serum was subtracted from the spike values. For recovery data see Table 5.

Table 5

Sample matrix	Spike high (%)	Spike medium (%)	Spike low (%)
Serum	90	90	89
Plasma (EDTA)	86	91	107
Plasma (citrate)	92	97	108
Plasma (heparin)	96	93	106
Cell culture supernatant	86	93	98

13.4 Dilution Parallelism

Serum, plasma and cell culture supernatant samples with different levels of human VEGFR2/KDR/FIk-1 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	83 - 97	89
Plasma (EDTA)	84 - 99	88
Plasma (citrate)	80 - 97	89
Plasma (heparin)	83 - 97	88
Cell culture supernatant	84 - 107	97

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C and thawed 3 times, and the human VEGFR2/KDR/FIk-1 levels determined. There was no significant loss of human VEGFR2/KDR/FIk-1 immunoreactivity detected by freezing and thawing.

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 VEGFR2/KDR/Flk-1 level determined after 24 h. There was no significant loss of human VEGFR2/KDR/Flk-1 immunoreactivity detected during storage under above conditions.

13.6 Specificity

The assay detects both natural and recombinant human VEGFR2/KDR/Flk-1. Cross reactivity and interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human VEGFR2/KDR/Flk-1 positive sample. There was no cross reactivity detected, notably not with VEGF-R1 at physiological concentrations and not with VEGF-R3/FLT-4.

There was no interference detected, notably not for VEGF-A and VEGF-C.

13.7 Expected Values

Panels of 40 serum as well as EDTA, citrate and heparin plasma samples from randomly selected apparently healthy donors (males and females) were tested for human VEGFR2/KDR/Flk-1.

The levels measured may vary with the sample collection used.

For detected human VEGFR2/KDR/Flk-1 levels see Table 7.

Table 7

Sample Matrix	Number of Samples Evaluated	Range (pg/ml)	Mean (pg/ml)	Standard Deviation (pg/ml)
Serum	40	7104 - 30861	17553	5082
Plasma (EDTA)	40	7824 - 22688	14017	3426
Plasma (Citrate)	40	9255 - 30288	18471	5560
Plasma (Heparin)	40	7101 - 21177	13207	3324

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	0.03	2.97
1 - 12	0.06	5.94

14.4 Streptavidin-HRP

Make a 1:200 dilution of **Streptavidin-HRP** in Assay Buffer (1x):

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

14.5 Human VEGFR2/KDR/FIk-1 Standard

Reconstitute lyophilized **human VEGFR2/KDR/FIk-1 standard** with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

15 Test Protocol Summary

1. Predilute sample with Assay Buffer (1x) 1:25.
2. Determine the number of microwell strips required.
3. Wash microwell strips twice with Wash Buffer.
4. Standard dilution on the microwell plate: Add 100 µl Assay Buffer (1x). 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.
5. Add 100 µl Assay Buffer (1x) in duplicate. to the blank wells.
6. Add 50 µl Assay Buffer (1x) to sample wells.
7. Add 50 µl prediluted sample in duplicate to designated sample wells.
8. Prepare Biotin-Conjugate.
9. Add 50 Biotin-Conjugate to all wells.
10. Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C). **(Shaking is absolutely necessary for an optimal test performance.)**
11. Prepare Streptavidin-HRP.
12. Empty and wash microwell strips 6 times with Wash Buffer.
13. Add 100 µl diluted Streptavidin-HRP to all wells.
14. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C). **(Shaking is absolutely necessary for an optimal test performance.)**
15. Empty and wash microwell strips 6 times with Wash Buffer.
16. Add 100 µl of TMB Substrate Solution to all wells.
17. Incubate the microwell strips for about 30 minutes at room temperature (18° to 25°C).
18. Add 100 µl Stop Solution to all wells.
19. Blank microwell reader and measure colour intensity at 450 nm.

Note: If instructions in this protocol have been followed, samples have been diluted 1:50 (50 µl 1:25 prediluted sample + 50 µl Assay Buffer (1x)) and the concentration read from the standard curve must be multiplied by the dilution factor (x 50).