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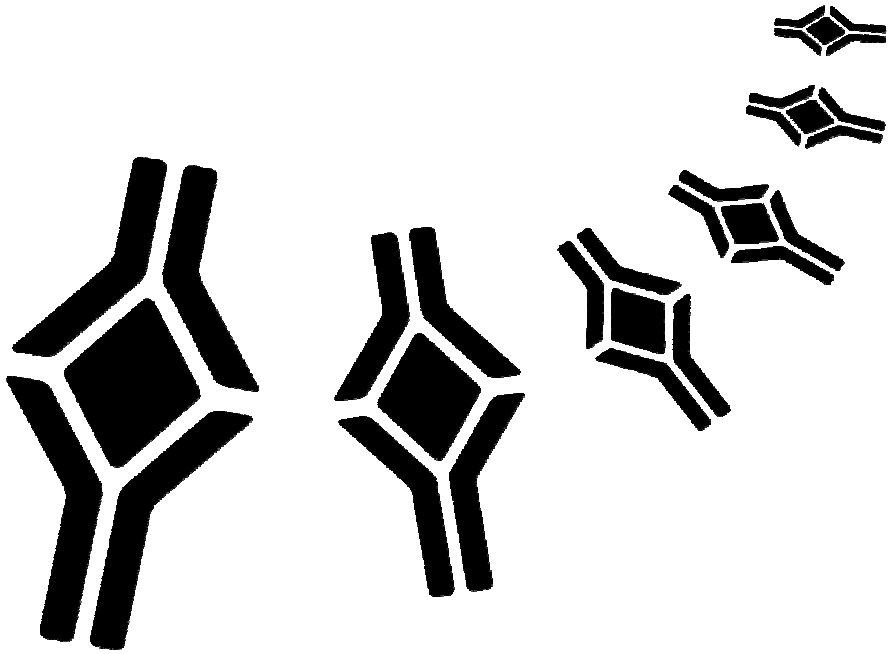


HUMAN VEGF-R3/FLT-4 ELISA

Product Data Sheet

Cat. No.: RBMS2064R

For Research Use Only

CONTENTS

This kit is manufactured by:
BioVendor – Laboratorní medicína, a.s.

Use only the current version of Product Data Sheet enclosed with the kit!

1 INTENDED USE

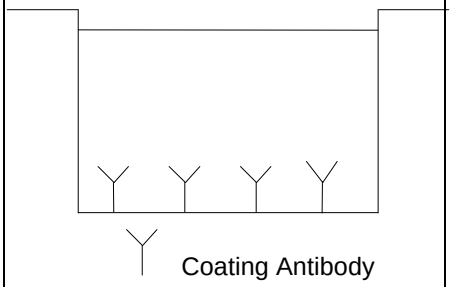
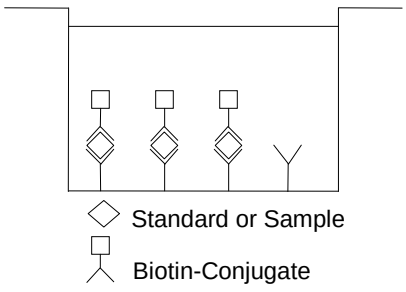
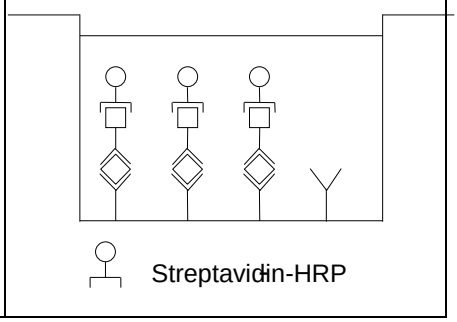
The human VEGF-R3/FLT-4 ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human VEGF-R3/FLT-4. **The human VEGF-R3/FLT-4 ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

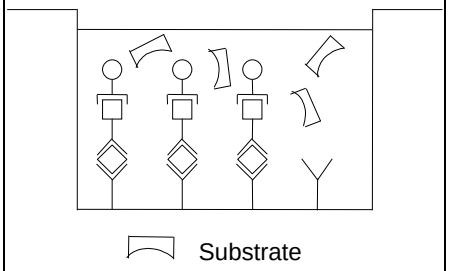
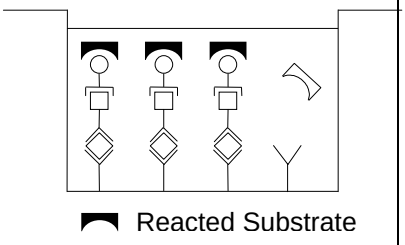
2 SUMMARY

The main function of the lymphatic system is to return plasma back to the circulating blood. Plasma fluid, macromolecules and cells, such as leucocytes and activated antigen-presenting cells, enter the blind-ended lymphatic vessels. Lymph is then transported toward collecting lymphatic vessels and is returned to the blood circulation.
First growth factors and molecular markers specific for lymphatic vessels were discovered only about ten years ago. This is rather astonishing, given the importance of the lymphatic system in maintaining fluid homeostasis and its involvement in the pathogenesis of many diseases, including cancer. Scientific studies over the past years have revealed a signal-transduction system for lymphatic endothelial cell growth, migration and survival. This system is formed by VEGF-C and VEGF-D and their shared receptor VEGFR-3/FLT-4. Homozygous deletion of VEGFR-3 in mice leads to defects in blood-vessel remodelling and embryonic death at mid-gestation, indicating an early vascular function for the receptor. Furthermore mouse studies suggest that upregulation and activation of VEGFR-3 have functional roles in sprouting angiogenesis and that VEGFR-3 is an important effector of the vascular phenotype resulting from Notch inhibition.
Heterozygous missense point mutations leading to receptor kinase inactivation have been found in VEGFR-3 of patients with Milroy disease, a rare autosomal dominant lymph oedema. Metastatic tumour spread through the blood or lymphatic vessels in human cancer, often associated with regional lymph-node metastasis, represents the most important prognostic factor for carcinoma patients. Several studies have shown positive correlations between VEGF-C, VEGF-D and VEGFR-3 expression and vascular invasion, lymphatic vessel and lymph node involvement. VEGF-C expression in tumour cells may be induced by growth factors or proinflammatory cytokines. High levels of VEGF-C or VEGF-D also enhance lymphatic metastasis in various experimental models. In some tumours, processed VEGF-C or VEGF-D may be generated, which mainly target VEGFR-3, but also VEGFR-2, which is often upregulated in tumour blood vessels.
In lymphoma patients, high expression of VEGFR-3 can been found in lymph endothelial cells isolated from the tumour tissue. In corneal inflammation mouse models, dendritic cells expressing both VEGFR-3 and VEGF-C could be detected, suggesting that immune cells may respond to lymphangiogenic signals and induce lymphangiogenesis.
Lymphangiogenesis research has revealed important therapeutic options for human diseases such as lymph oedema and other oedemas that will enter clinical development in the near future.

The importance of lymph-node metastasis in the spread of cancer to distant organs needs better understanding before applying the newly acquired knowledge to patients. In this context, the roles of VEGF-C and VEGFR-3 upregulation in tumour angiogenesis need to be explored for additional therapeutical application.
The sandwich ELISA for detecting and reliable measuring of solubilized VEGFR-3 from tumour tissue, lymphatic vessels and different kind of endothelial cells will certainly help to understand the mechanisms of lymphatic metastasis including the identification of tumour determinants that are important for the spread of tumour cells.

3 PRINCIPLES OF THE TEST

An anti-human VEGF-R3/FLT-4 coating antibody is adsorbed onto microwells.	Figure 1 
Human VEGF-R3/FLT-4 present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human VEGF-R3/FLT-4 antibody is added and binds to human VEGF-R3/FLT-4 captured by the first antibody.	Figure 2 
Following incubation unbound biotin-conjugated anti-human VEGF-R3/FLT-4 antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human VEGF-R3/FLT-4 antibody.	Figure 3 

Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.	Figure 4 **Third Incubation** 
A coloured product is formed in proportion to the amount of human VEGF-R3/FLT-4 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-R3/FLT-4 standard dilutions and human VEGF-R3/FLT-4 sample concentration determined.	Figure 5 

4 REAGENTS PROVIDED

1 aluminium pouch with a **Antibody Coated Microtiter Strips** with monoclonal antibody to human VEGF-R3/FLT-4
1 vial (60 µl) **Biotin-Conjugate** anti-human VEGF-R3/FLT-4 monoclonal antibody
1 vial (150 µl) **Streptavidin-HRP**
2 vials human VEGF-R3/FLT-4 **Standard** lyophilized, 80 ng/ml upon reconstitution
1 vial (50 ml) **Sample Diluent**
1 vial (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20 and 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)
1 vial (0.4 ml) **Blue-Dye**
1 vial (0.4 ml) **Green-Dye**
1 vial (0.4 ml) **Red-Dye**
4 **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 (citrate) 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-R3/FLT-4. 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 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 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. 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 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 1:100 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

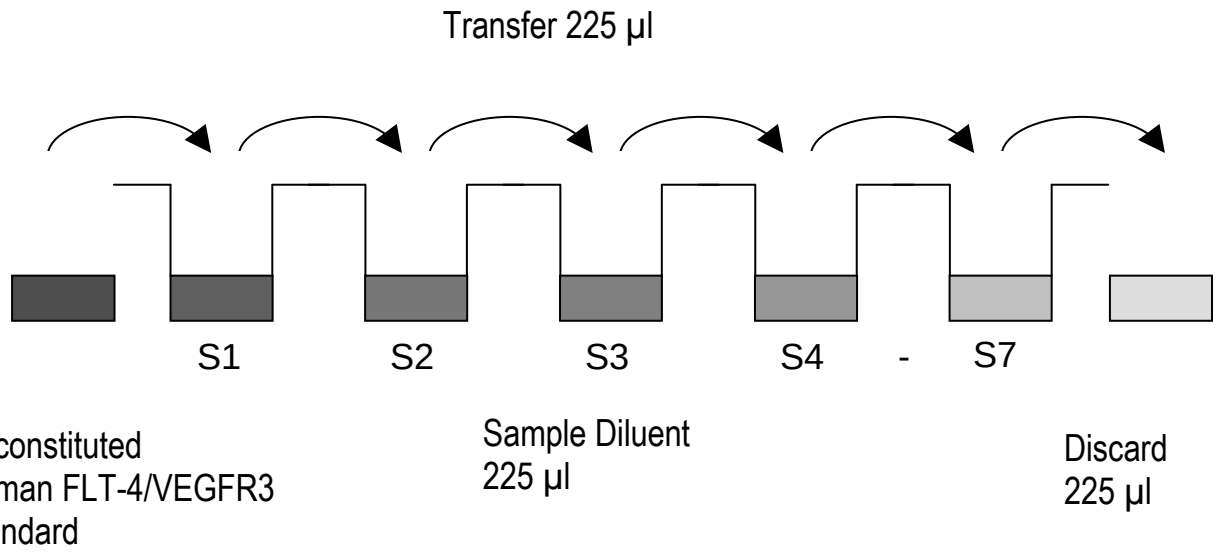
9.5 Human VEGF-R3/FLT-4 Standard

Reconstitute **human VEGF-R3/FLT-4 standard** by addition of Sample Diluent. Reconstitution volume is stated in the Certificate of Analysis. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 80 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 µl of Sample Diluent into each tube
Pipette 225 µl of reconstituted standard (concentration of standard = 80 ng/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 40 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 6).
Sample Diluent serves as blank.

Figure 6



9.6 Addition of Colour-giving Reagents: Blue-Dye, Green-Dye, Red-Dye

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:

1. Diluent: Before standard and 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***
50 ml Sample Diluent	200 µl ***Blue-Dye***

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

3 ml Assay Buffer (1x)	30 µl ***Green-Dye***
6 ml Assay Buffer (1x)	60 µl ***Green-Dye***
12 ml Assay Buffer (1x)	120 µl ***Green-Dye***

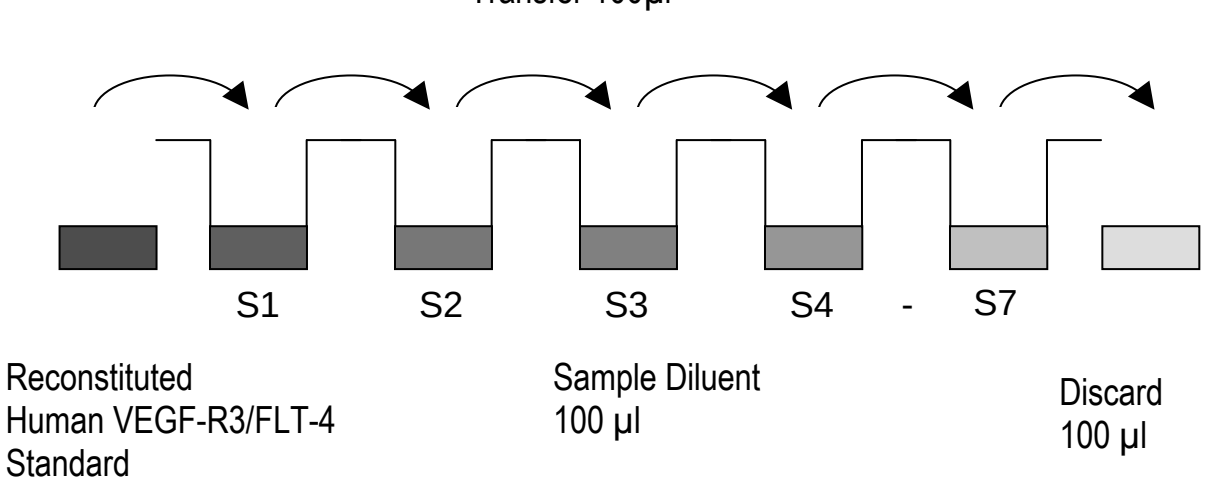
3. Streptavidin-HRP: Before dilution of the concentrated Streptavidin-HRP, add the ***Red-Dye*** at a dilution of 1:250 (see table below) to the Assay Buffer (1x) used for the final Streptavidin-HRP dilution. Proceed after addition of ***Red-Dye*** according to the instruction booklet: Preparation of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

6 ml Assay Buffer (1x)	24 µl ***Red-Dye***
12 ml Assay Buffer (1x)	48 µl ***Red-Dye***

10 TEST PROTOCOL

a. Predilute your samples before starting with the test procedure. Dilute serum, plasma and cell culture samples 1:10 with Sample Diluent according to the following scheme: 20 µl sample + 180 µl Sample Diluent
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 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**.
d. **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 0, concentration = 80 ng/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 = 40 ng/ml), and transfer 100 µl 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 VEGF-R3/FLT-4 standard dilutions ranging from 40.0 to 0.6 ng/ml. Discard 100 µl 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(40.0 ng/ml)	Standard 1 (40.0 ng/ml)	Sample 1	Sample 1
B	Standard 2 (20.0 ng/ml)	Standard 2 (20.0 ng/ml)	Sample 2	Sample 2
C	Standard 3 (10.0 ng/ml)	Standard 3 (10.0 ng/ml)	Sample 3	Sample 3
D	Standard 4 (5.0 ng/ml)	Standard 4 (5.0 ng/ml)	Sample 4	Sample 4
E	Standard 5 (2.5 ng/ml)	Standard 5 (2.5 ng/ml)	Sample 5	Sample 5
F	Standard 6 (1.3 ng/ml)	Standard 6 (1.3 ng/ml)	Sample 6	Sample 6
G	Standard 7 (0.6 ng/ml)	Standard 7 (0.6 ng/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

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 prediluted **sample** in duplicate to the **sample wells**.
h. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate 9.3).
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 100 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 100 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 VEGF-R3/FLT-4 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-R3/FLT-4 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-R3/FLT-4 concentration.
- **If instructions in this protocol have been followed samples have been diluted 1:20 (1:10 external predilution, 1:2 dilution on the plate: 50 µl sample + 50 µl Sample Diluent), the concentration read from the standard curve must be multiplied by the dilution factor (x 20).**
- **Calculation of 1:10 prediluted samples with a concentration exceeding standard 1 may result in incorrect, low human VEGF-R3/FLT-4 levels. Such samples require further external predilution according to expected human VEGF-R3/FLT-4 values with Sample Diluent in order to precisely quantitate the actual human VEGF-R3/FLT-4 level.**
- It is suggested that each testing facility establishes a control sample of known human VEGF-R3/FLT-4 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 VEGF-R3/FLT-4 ELISA. Human VEGF-R3/FLT-4 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.

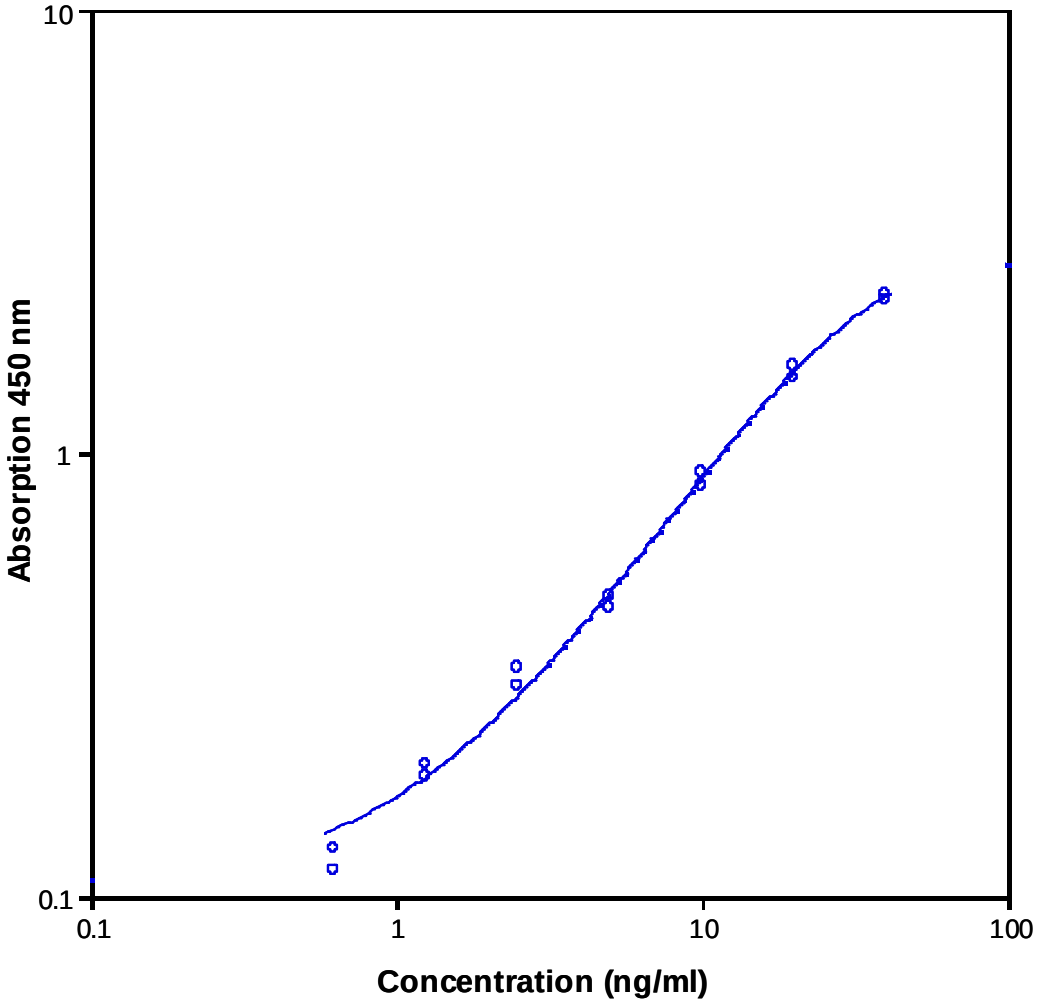


Table 2
Typical data using the human VEGF-R3/FLT-4 ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	Human VEGF-R3/FLT-4 Concentration (ng/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V.(%)
1	40.0	2.288 2.211	2.250	1.7
2	20.0	1.571 1.477	1.524	3.1
3	10.0	0.905 0.845	0.875	3.4
4	5.0	0.446 0.477	0.461	3.3
5	2.5	0.328 0.298	0.313	4.8
6	1.3	0.198 0.187	0.192	2.8
7	0.6	0.129 0.114	0.122	6.0
Blank	0	0.052 0.051	0.051	1.5

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 VEGF-R3/FLT-4 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 0.03 ng/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 7 serum samples containing different concentrations of human VEGF-R3/FLT-4. 2 standard curves were run on each plate. Data below show the mean human VEGF-R3/FLT-4 concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 5.7%.

Table 3
The mean human VEGF-R3/FLT-4 concentration and the coefficient of variation for each sample.

Sample	Experiment	Mean Human VEGF-R3/FLT-4 Concentration (ng/ml)	Coefficient of Variation (%)
1	1	106.3	5.0
	2	120.8	6.3
	3	110.5	2.5
2	1	56.8	5.6
	2	63.0	10.9
	3	59.5	4.0
3	1	55.6	5.4
	2	54.5	4.2
	3	52.8	2.4
4	1	38.8	3.1
	2	36.9	3.5
	3	39.3	5.3
5	1	38.9	2.6
	2	38.1	6.6
	3	35.3	2.8
6	1	27.7	9.2
	2	28.1	10.1
	3	25.5	6.7
7	1	8.8	3.7
	2	8.6	14.1
	3	7.7	5.9

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 7 serum samples containing different concentrations of human VEGF-R3/FLT-4. 2 standard curves were run on each plate. Data below show the mean human VEGF-R3/FLT-4 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 5.0%.

Table 4
The mean human VEGF-R3/FLT-4 concentration and the coefficient of variation of each sample

Sample	Mean Human VEGF-R3/FLT-4 Concentration (ng/ml)	Coefficient of Variation (%)
1	113	6.6
2	60	5.2
3	54	2.6
4	38	3.2
5	37	5.1
6	27	5.1
7	8	7.2

13.3 Spiking Recovery

The spiking recovery was evaluated by spiking 4 levels of human VEGF-R3/FLT-4 into serum, plasma and cell culture supernatant samples. Recoveries were determined with 4 replicates each. The amount of endogenous human VEGF-R3/FLT-4 in unspiked serum was subtracted from the spike values.
For recovery data see Table 5.

Table 5

Sample matrix *	Spike high		Spike medium	
	Range (%)	Mean (%)	Range (%)	Mean (%)
Serum	83 – 100	92	86 - 108	97
Plasma (citrate)	53 – 82	70	48 – 80	70
Cell culture supernatant		86		81

* Due to high endogen human VEGF-R3/FLT-4 levels data for low spikes are not indicated.

13.4 Dilution Linearity

Serum, plasma and cell culture supernatant samples with different levels of human VEGF-R3/FLT-4 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	72 - 105	86
Plasma (citrate)	80 - 119	100
Cell culture supernatant	70 - 106	93

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 VEGF-R3/FLT-4 levels determined. There was no significant loss of human VEGF-R3/FLT-4 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 VEGF-R3/FLT-4 level determined after 24 h. There was no significant loss of human VEGF-R3/FLT-4 immunoreactivity detected during storage under above conditions.

13.6 Specificity

The assay detects both natural and recombinant human VEGF-R3/FLT-4.
Cross reactivity and interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human VEGF-R3/FLT-4 positive sample.
There was no cross reactivity detected, notably not with VEGF-R1 and VEGF-R2/KDR.
Interference was detected for VEGF-C at concentrations > 7 ng/ml, and not for VEGF-D.

13.7 Expected Values

Panels of 40 serum as well as plasma (citrate) samples from randomly selected apparently healthy donors (males and females) were tested for human VEGF-R3/FLT-4.
The levels measured may vary with the sample collection used.
For detected human VEGF-R3/FLT-4 levels see Table 7.

Table 7

Sample Matrix	Number of Samples Evaluated	Range (ng/ml)	Mean (ng/ml)	Standard Deviation (ng/ml)
Serum	40	33 - 167	97	39
Plasma (Citrate)	40	31 - 200	112	34

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 1:100 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-R3/FLT-4 Standard

Reconstitute lyophilized **human VEGF-R3/FLT-4 standard** with Sample Diluent. (Reconstitution volume is stated in the Certificate of Analysis.).

15 TEST PROTOCOL SUMMARY

1. Predilute sample with Sample Diluent 1:10.
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 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.
5. Add 100 µl Sample Diluent, in duplicate, to the blank wells.
6. Add 50 µl Sample Diluent to sample wells.
7. Add 50 µl prediluted sample in duplicate, to designated sample wells.
8. Prepare Biotin-Conjugate.
9. Add 50 µl 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 1:20 (50 µl 1:10 prediluted sample + 50 µl Sample Diluent), the concentration read from the standard curve must be multiplied by the dilution factor (x 20).

NOTES

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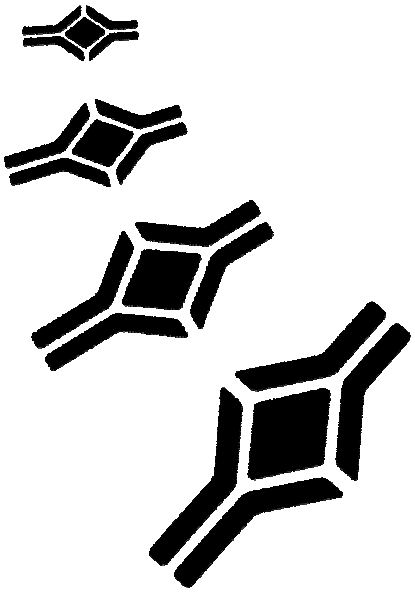

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