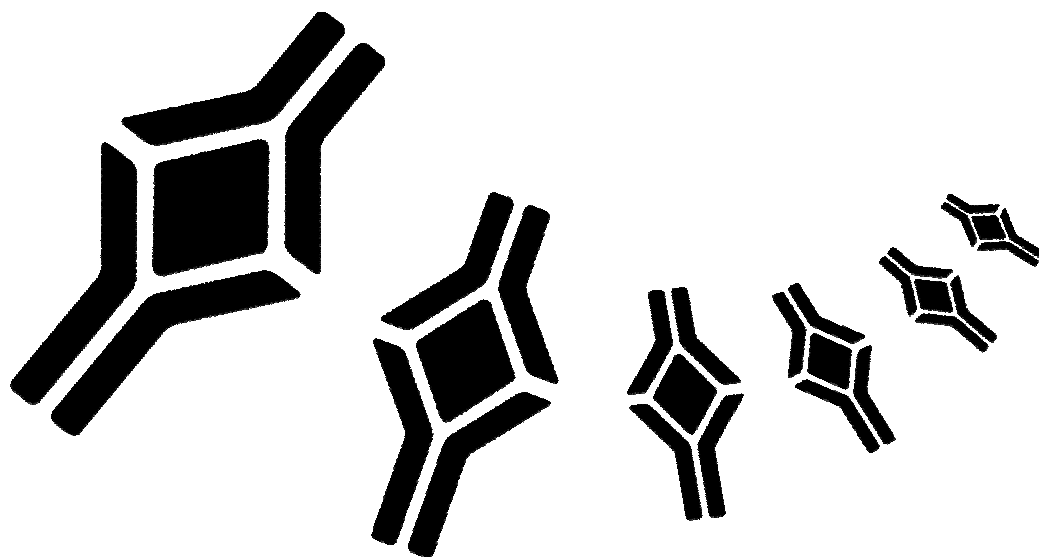


**BioVendor**

Research  
and Diagnostic Products



## **RAT VEGF-C ELISA**

Product Data Sheet

Cat. No.: RBMS626R

For Research Use Only

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**»» 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

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

## 2 SUMMARY

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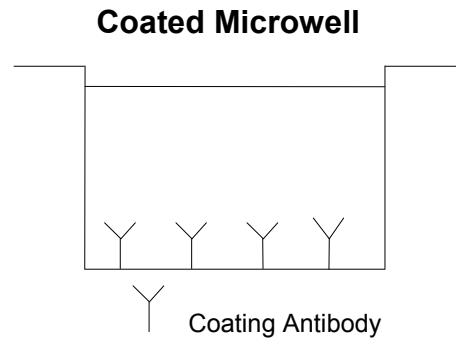
Vascular endothelial growth factor-C (VEGF-C) is a member of the VEGF family of cytokines. It has been shown to exhibit angiogenic and lymphangiogenic actions. The VEGF family of growth factors and receptors is involved in the development and growth of the vascular endothelial system. Two of its family members, VEGF-C and VEGF-D, regulate the lymphatic endothelial cells via their receptor VEGFR3, thus acting as mitogens for these cells.

VEGF-C expression is associated with hematological malignancies. Like VEGF it acts as survival factor on leukemia. Together with the expression of their receptors, VEGF and VEGF-C result in the generation of autocrine loops that may support cancer cell survival and proliferation. Further VEGF-C expression has been shown in gastrointestinal tract malignancies where it correlates with lymphatic invasion, lymphnode metastasis and reduced survival.

### 3 PRINCIPLES OF THE TEST

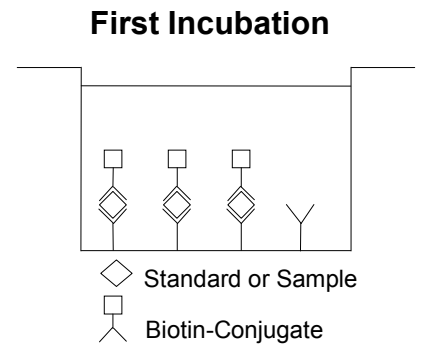
An anti-rat VEGF-C coating antibody is adsorbed onto microwells.

Figure 1



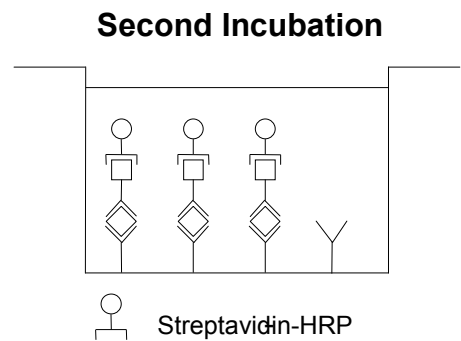
Rat VEGF-C present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-rat VEGF-C antibody is added and binds to rat VEGF-C captured by the first antibody.

Figure 2



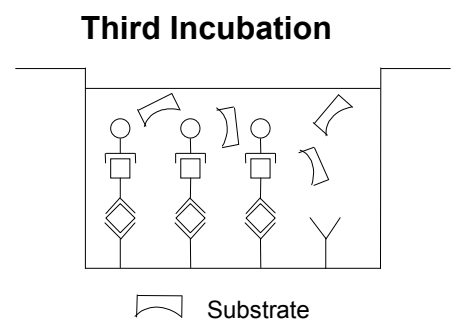
Following incubation unbound biotin-conjugated anti-rat VEGF-C antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-rat VEGF-C antibody.

Figure 3



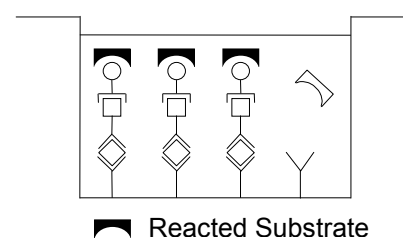
Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

Figure 4



A coloured product is formed in proportion to the amount of rat VEGF-C 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 rat VEGF-C standard dilutions and rat VEGF-C sample concentration determined.

Figure 5



#### 4 REAGENTS PROVIDED

- 1 aluminium pouch with a **Antibody Coated Microtiter Strips** with polyclonal antibody to rat VEGF-C
- 1 vial (100 µl) **Biotin-Conjugate** anti-rat VEGF-C polyclonal antibody
- 1 vial (150 µl) **Streptavidin-HRP**
- 2 vials rat VEGF-C **Standard** lyophilized, 6000 pg/ml upon reconstitution
- 1 vial (12 ml) **Sample Diluent**
- 1 bottle (20 ml) **Conjugate Diluent**
- 1 bottle (50 ml) **Wash Buffer Concentrate 20x** (PBS with 1% Tween 20)
- 1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)
- 1 vial (12 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

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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

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Cell culture supernatant and serum were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum from the clot as soon as possible after clotting.

Pay attention to a possible “**Hook Effect**” due to high sample concentrations (see chapter 11).

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 rat VEGF-C. 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. Do not thaw samples in a 37°C water bath. Do not vortex or sharply agitate samples.

## 7 MATERIALS REQUIRED BUT NOT PROVIDED

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- 5 ml and 10 ml graduated pipettes
- 5  $\mu$ l to 1000  $\mu$ l adjustable single channel micropipettes with disposable tips
- 50  $\mu$ l to 300  $\mu$ 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

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- All chemicals 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

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**Buffer concentrates** should be brought to room temperature and should be diluted before starting the test procedure.

### 9.1 Wash Buffer

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

Pour entire contents (50 ml) of the Wash Buffer Concentrate into a clean 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 is stable for 30 days.

Wash Buffer may also be prepared as needed according to the following table:

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

### 9.2 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 Conjugate Diluent in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (ml)	Conjugate Diluent (ml)
1 - 6	0.030	2.970
1 - 12	0.060	5.940

### 9.3 Streptavidin-HRP

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

Make a 1:400 dilution of the concentrated **Streptavidin-HRP** solution with Conjugate Diluent in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Conjugate Diluent (ml)
1 - 6	0.015	5.985
1 - 12	0.030	11.970

### 9.4 Rat VEGF-C Standard

Reconstitute **rat VEGF-C standard** by addition of distilled water. Refer to the Certificate of Analysis for current volume of Distilled water needed for reconstitution of standard. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 6000 pg/ml).

The standard has to be used immediately after reconstitution and cannot be stored.

**Standard dilutions** can be prepared directly on the microwell plate (see 10.c) or alternatively in tubes (see 9.4.1).

### 9.4.1 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:

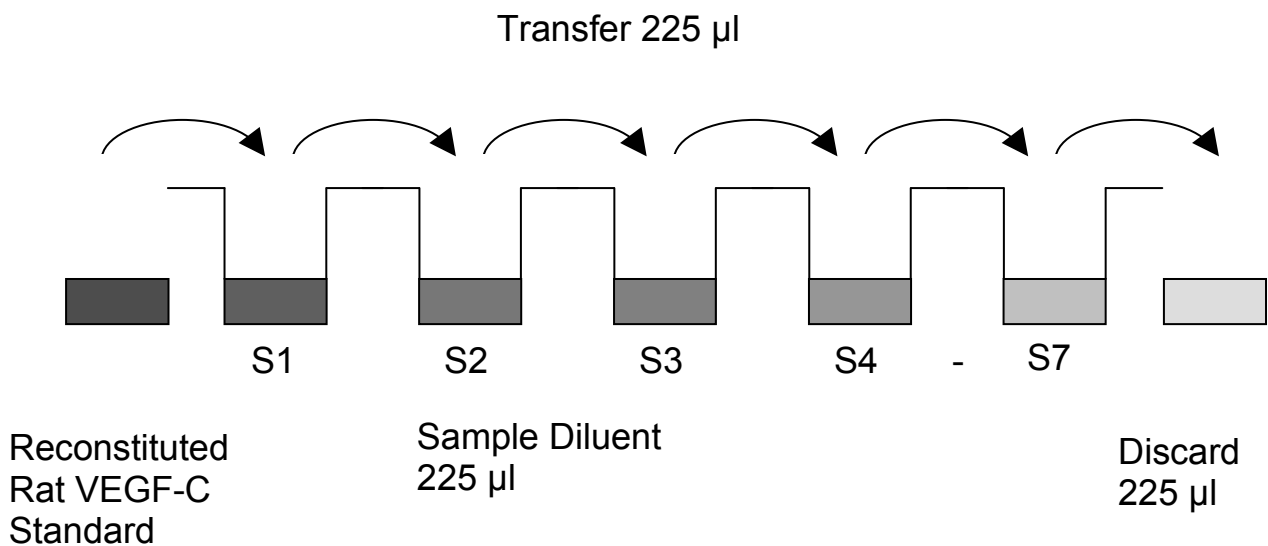
Pipette 225  $\mu$ l of Sample Diluent into each tube.

Pipette 225  $\mu$ l of reconstituted standard (concentration = 6000 pg/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 3000 pg/ml). Pipette 225  $\mu$ 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).

Sample Diluent serves as blank.

Figure 6



## 9.5 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 <b>Blue-Dye</b>
12 ml Sample Diluent	48 µl <b>Blue-Dye</b>
50 ml Sample Diluent	200 µl <b>Blue-Dye</b>

### 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 Conjugate Diluent used for the final conjugate dilution. Proceed after addition of **Green-Dye** according to the instruction booklet: Preparation of Biotin-Conjugate.

3 ml Conjugate Diluent	30 µl <b>Green-Dye</b>
6 ml Conjugate Diluent	60 µl <b>Green-Dye</b>

### 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 Conjugate Diluent used for the final Streptavidin-HRP dilution. Proceed after addition of **Red-Dye** according to the instruction booklet: Preparation of Streptavidin-HRP.

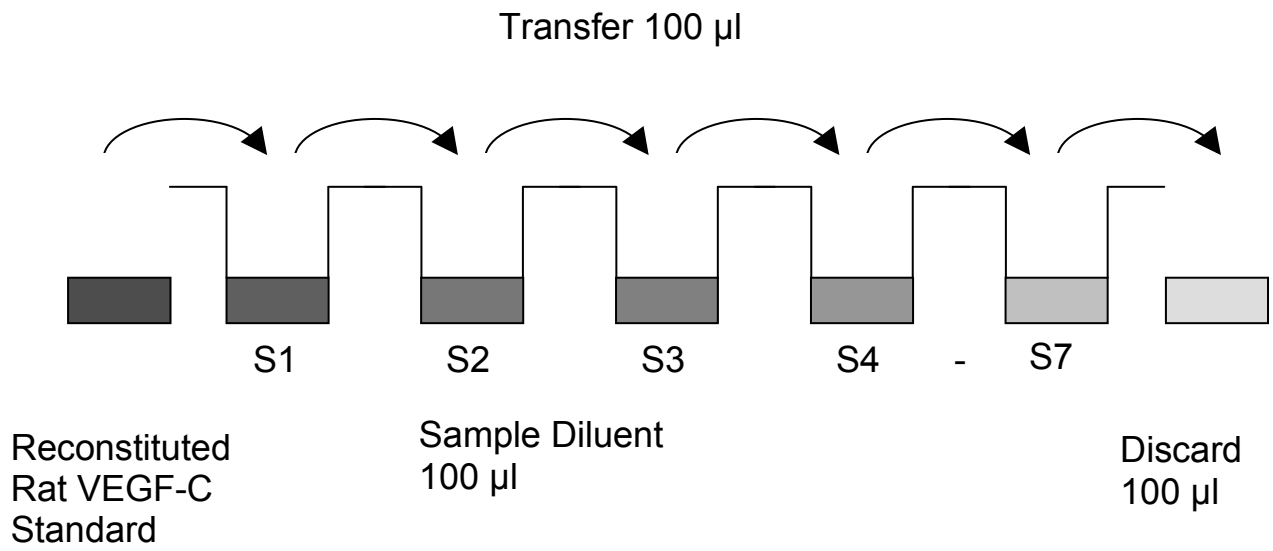
6 ml Conjugate Diluent	24 µl <b>Red-Dye</b>
12 ml Conjugate Diluent	48 µl <b>Red-Dye</b>

## 10 TEST PROTOCOL

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- 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 9.4.1): Add 100 µl of Sample Diluent in duplicate to all **standard wells**. Pipette 100 µl of prepared **standard** (see Preparation of Standard 9.4, concentration = 6000 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 = 3000 pg/ml), and transfer 100 µl to wells B1 and B2, respectively (see Figure 7). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of rat VEGF-C standard dilutions ranging from 3000 to 47 pg/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 7



In case of an **external standard dilution** (see 9.4.1), 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:

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
<b>A</b>	Standard 1 (3000 pg/ml)	Standard 1 (3000 pg/ml)	Sample 1	Sample 1
<b>B</b>	Standard 2 (1500 pg/ml)	Standard 2 (1500 pg/ml)	Sample 2	Sample 2
<b>C</b>	Standard 3 (750 pg/ml)	Standard 3 (750 pg/ml)	Sample 3	Sample 3
<b>D</b>	Standard 4 (375 pg/ml)	Standard 4 (375 pg/ml)	Sample 4	Sample 4
<b>E</b>	Standard 5 (188 pg/ml)	Standard 5 (188 pg/ml)	Sample 5	Sample 5
<b>F</b>	Standard 6 (94 pg/ml)	Standard 6 (94 pg/ml)	Sample 6	Sample 6
<b>G</b>	Standard 7 (47 pg/ml)	Standard 7 (47 pg/ml)	Sample 7	Sample 7
<b>H</b>	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. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate 9.2).
- h. Add 50 µl of **Biotin-Conjugate** to all wells.
- i. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours, if available on a microplate shaker set at 100 rpm.
- j. Prepare **Streptavidin-HRP** (refer to Preparation of Streptavidin-HRP 9.3).
- k. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point b. of the test protocol. Proceed immediately to the next step.
- l. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.
- m. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour, if available on a microplate shaker set at 100 rpm.
- n. Remove adhesive film and empty wells. **Wash** microwell strips 3 times according to point b. of the test protocol. Proceed immediately to the next step.
- o. Pipette 100 µl of **TMB Substrate Solution** to all wells.

- p. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 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 0.6 – 0.65.

- q. 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.
- r. 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.

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

## 11 CALCULATION OF RESULTS

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- 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 rat VEGF-C 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 rat VEGF-C 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 rat VEGF-C concentration.
- **If instructions in this protocol have been followed samples have been diluted 1:2 (50 µl sample + 50 µl Sample Diluent), 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 rat VEGF-C levels (Hook Effect). Such samples require further external predilution according to expected rat VEGF-C values with Sample Diluent in order to precisely quantitate the actual rat VEGF-C level.**
- It is suggested that each testing facility establishes a control sample of known rat VEGF-C 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 rat VEGF-C ELISA. Rat VEGF-C 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.

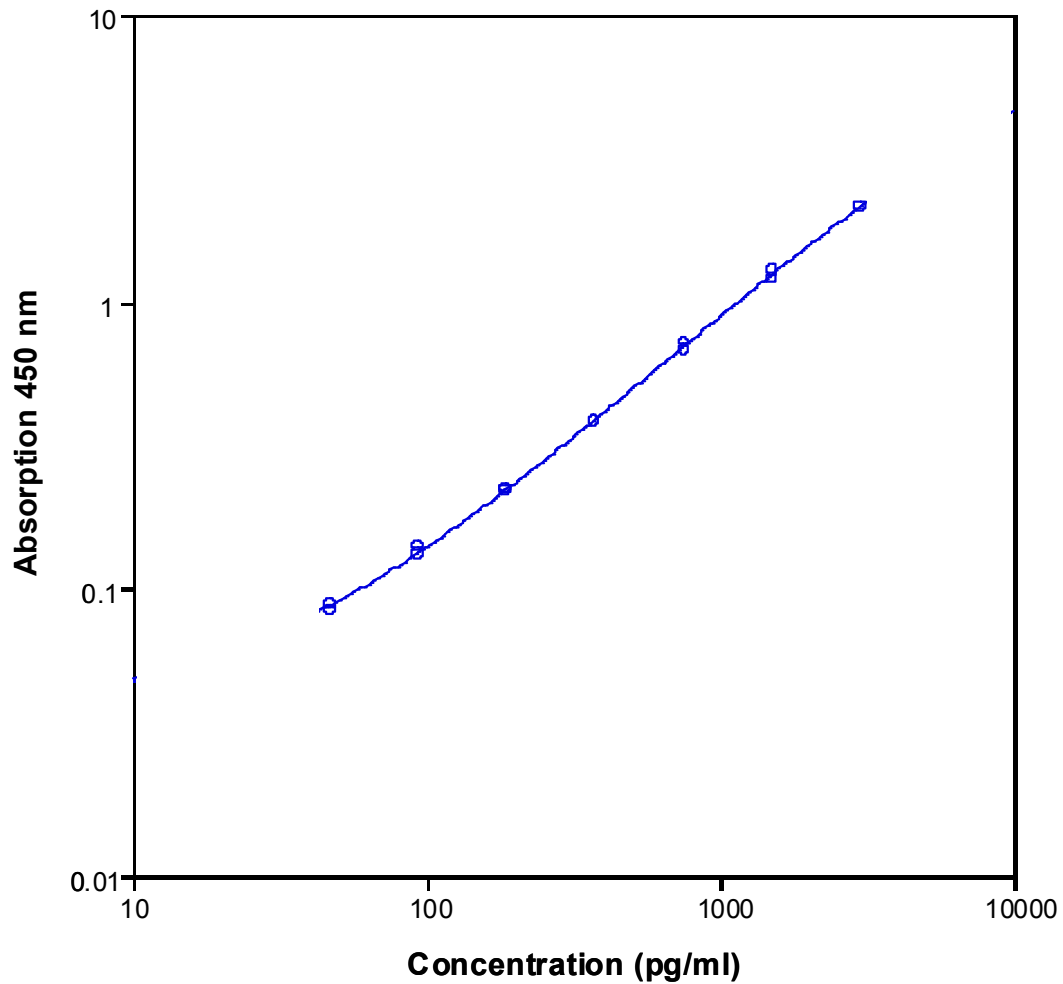


Table 2

## Typical data using the rat VEGF-C ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Rat VEGF-C Concentration (pg/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	3000	2.147	2.151	0.2
	3000	2.155		
2	1500	1.283	1.248	2.8
	1500	1.213		
3	750	0.677	0.701	3.4
	750	0.724		
4	375	0.388	0.389	0.1
	375	0.389		
5	188	0.220	0.223	1.1
	188	0.225		
6	94	0.131	0.136	3.3
	94	0.140		
7	47	0.084	0.086	2.3
	47	0.088		
Blank	0	0.041	0.044	
	0	0.047		

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

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- 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

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### 13.1 Sensitivity

The limit of detection of rat VEGF-C 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 4 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 rat VEGF-C. 2 standard curves were run on each plate. The calculated overall intra-assay coefficient of variation was 8.2%.

#### 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 rat VEGF-C. 2 standard curves were run on each plate. The calculated overall inter-assay coefficient of variation was 6.5%.

### 13.3 Spiking Recovery

The spiking recovery was evaluated by spiking 4 levels of rat VEGF-C into normal pooled rat serum samples. Recoveries were determined in 3 independent experiments with 4 replicates each.

The amount of endogenous rat VEGF-C in unspiked serum was subtracted from the spike values.

The overall mean recovery was 79%.

## **13.4 Dilution Linearity**

4 serum samples with different levels of rat VEGF-C were analysed at serial 2 fold dilutions with 4 replicates each.  
The overall mean recovery was 106%.

## **13.5 Sample Stability**

### **13.5.1 Freeze-Thaw Stability**

Aliquots of spiked serum samples were stored at -20°C and thawed 5 times, and the rat VEGF-C levels determined. There was no significant loss of rat VEGF-C immunoreactivity detected by freezing and thawing.

### **13.5.2 Storage Stability**

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

## **13.6 Specificity**

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a rat VEGF-C positive serum.  
There was no crossreactivity detected.

## 14 REFERENCES

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- 7) Olofsson B, Jeltsch M, Eriksson U, Alitalo K. Current biology of VEGF-B and VEGF-C. *Curr Opin Biotechnol.* 1999 Dec; 10 (6): 528-35.
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## 15 REAGENT PREPARATION SUMMARY

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### 15.1 Wash Buffer

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

### 15.2 Biotin-Conjugate

Make a 1:100 dilution of **Biotin-Conjugate** in Conjugate Diluent:

Number of Strips	Biotin-Conjugate (ml)	Conjugate Diluent (ml)
1 - 6	0.030	2.970
1 - 12	0.060	5.940

### 15.3 Streptavidin-HRP

Make a 1:400 dilution of **Streptavidin-HRP** in Conjugate Diluent:

Number of Strips	Streptavidin-HRP (ml)	Conjugate Diluent (ml)
1 - 6	0.015	5.985
1 - 12	0.030	11.970

### 15.4 Rat VEGF-C Standard

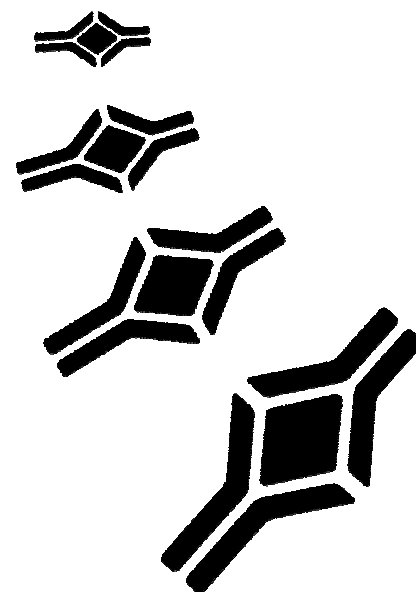
Reconstitute lyophilized **rat VEGF-C standard** with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

## 16 TEST PROTOCOL SUMMARY

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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 9.4.1): 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. Prepare Biotin-Conjugate.
8. Add 50 µl Biotin-Conjugate to all wells.
9. Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C).
10. Prepare Streptavidin-HRP.
11. Empty and wash microwell strips 3 times with Wash Buffer.
12. Add 100 µl diluted Streptavidin-HRP to all wells.
13. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C).
14. Empty and wash microwell strips 3 times with Wash Buffer.
15. Add 100 µl of TMB Substrate Solution to all wells.
16. Incubate the microwell strips for about 10 minutes at room temperature (18°to 25°C).
17. Add 100 µl Stop Solution to all wells.
18. 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), the concentration read from the standard curve must be multiplied by the dilution factor (x 2).**



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