

PRODUCT INFORMATION AND MANUAL

human VEGF-C ELISA

BMS297

Enzyme-linked immunosorbent assay for quantitative detection of active human Vascular Endothelial Growth Factor C.

For research use only.

Not for diagnostic or therapeutic procedures.

96 Tests

***Human VEGF-C ELISA
BMS297***



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1. INTENDED USE

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

2. SUMMARY

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.

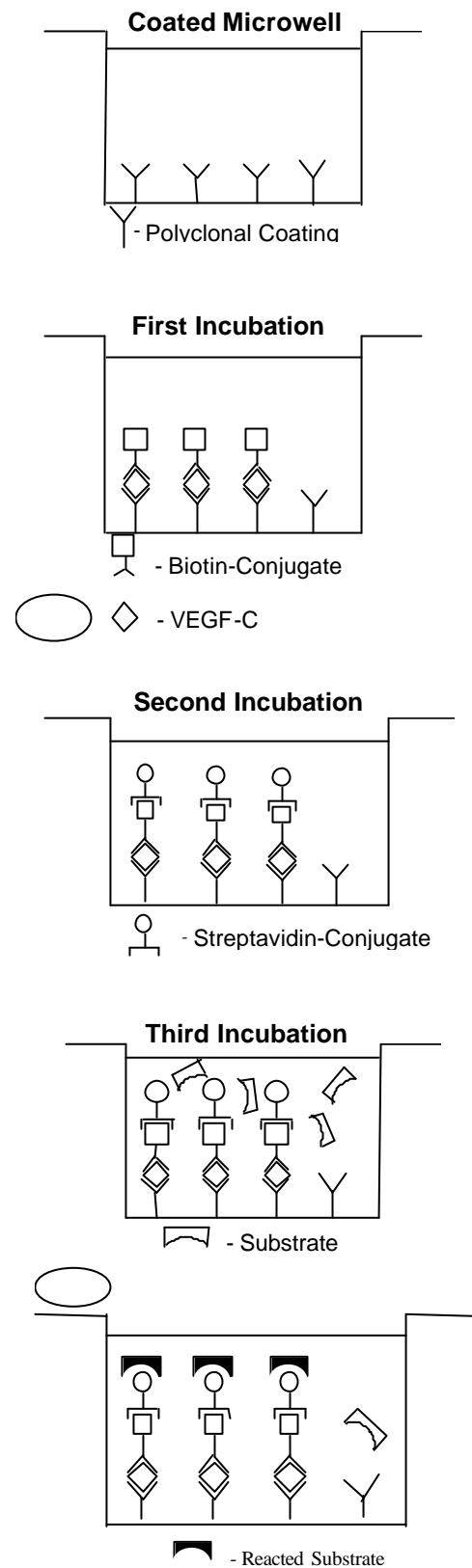
3. PRINCIPLES OF THE TEST

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

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

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

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



4. REAGENTS PROVIDED

- 1 aluminium pouche with a **Microwell Plate coated with anti-VEGF-C polyclonal antibody**
- 1 vial (100 µl) **Biotin-Conjugate** anti-VEGF-C polyclonal (goat) antibody*
- 2 vials **VEGF-C Standard**, 6 ng/ml upon reconstitution
- 1 vial (150µl) **Streptavidin-HRP***
- 1 bottle (12 ml) **Sample Diluent** (Protein matrix)
- 1 bottle (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)*
- 1 bottle (20 ml) **Conjugate Diluent**
- 1 vial (7 ml) **Substrate Solution I** (tetramethyl-benzidine)
- 1 vial (7 ml) **Substrate Solution II** (0.02 % buffered hydrogen peroxide)
- 1 vial (12 ml) **Stop Solution** (1M Phosphoric acid)
- 1 vial (0.4 ml each) **Blue-Dye, Green-Dye, Red-Dye**
- 4 adhesive **Plate Covers**

Reagent Labels

* reagents contain preservative

5. STORAGE INSTRUCTIONS

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels.

The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

6. SPECIMEN COLLECTION

Cell culture supernatants, human serum, EDTA, heparin and citrate plasma, and other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

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

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

7. MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes
- 10 μ l to 1,000 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 linear regression analysis.

8. PRECAUTIONS FOR USE

- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Reagents containing thimerosal as preservative may be toxic if ingested.
- Avoid contact of substrate solutions with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.

- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagents.
- Exposure to acids will inactivate the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solutions must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0 % sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

9. PREPARATION OF REAGENTS

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

A. Wash Buffer

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

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** into a clean 1,000 ml graduated cylinder. Bring final volume to 1,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that the Wash Buffer is stable for 30 days. Wash Buffer may be prepared as needed according to the following table:

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

B. Preparation of VEGF-C Standard

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

C. Preparation of Biotin-Conjugate

Dilute the Biotin-Conjugate 1:100 just prior to use with **Conjugate Diluent** in a clean plastic tube.

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

Number of Strips	Biotin-Conjugate (ml)	Conjugate Diluent (ml)
1 - 6	0.03	3.0
1 - 12	0.06	6.0

D. Preparation of Streptavidin-HRP

Make a 1:400 dilution of the concentrated **Streptavidin-HRP** solution as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Conjugate Diluent (ml)
1 - 6	0.015	6.0
1 - 12	0.03	12.0

E. TMB Substrate Solution

Using clean pipettes and containers known to be metal free, dispense an equal volume of **Substrate Solution I** into **Substrate Solution II** and swirl gently to mix. The TMB Substrate Solution may develop a yellow tinge over time. This does not seem to affect product performance. A blue colour present in the TMB Substrate Solution, however, indicates that it has been contaminated and must be discarded. The TMB Substrate Solution must be used within a few minutes after mixing. Warm to room temperature before use. Avoid direct exposure of TMB reagents to intense light and oxidizing agents during storage or incubation.

Substrate preparation by assay size:

BMS297 VEGF-C

Number Of Strips	Substrate Solution I (ml)	Substrate Solution II (ml)
1 - 6	3.0	3.0
1 - 12	6.0	6.0

F. Addition of colour-giving reagents: **Blue-Dye, Green-Dye, Red-Dye**

In order to help our customers to avoid any mistakes in pipetting the Bender MedSystems ELISAs, Bender MedSystems now offers a new tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure.

This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.

Alternatively, the dye solutions from the stocks provided (**Blue-Dye, Green-Dye, Red-Dye**) can be added to the reagents according to the following guidelines:

- 1. Diluent:** Before sample dilution add the **Blue-Dye** at a dilution of 1:250 (see table below) to the appropriate diluent 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

- 2. Biotin-Conjugate:** Before dilution of the concentrated 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 μ l Green-Dye
6 ml Conjugate Diluent	60 μ l 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 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 μ l Red-Dye
12 ml Conjugate Diluent	48 μ l Red-Dye

10. TEST PROTOCOL

- a. Mix all reagents thoroughly without foaming before use.
- b. Determine the number of Microwell Strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank, and optional control sample should be assayed in duplicate. Remove extra **Microwell Strips coated with anti-VEGF-C polyclonal antibody** from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
- c. Wash the microwell strips twice with approximately 300 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells.

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

- d. Add 100 µl of **Sample Diluent** in duplicate to all standard wells. Prepare standard dilutions by pipetting 100 µl of **VEGF-C Standard**, in duplicate, into well A1 and A2 (see Figure 1 and 2). Mix the contents of wells A1 and A2 by repeated aspiration and ejection, and transfer 100 µl to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure five times, creating two rows of VEGF-C standard dilutions ranging from 3000 to 46.9 pg/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of VEGF-C standard dilutions:

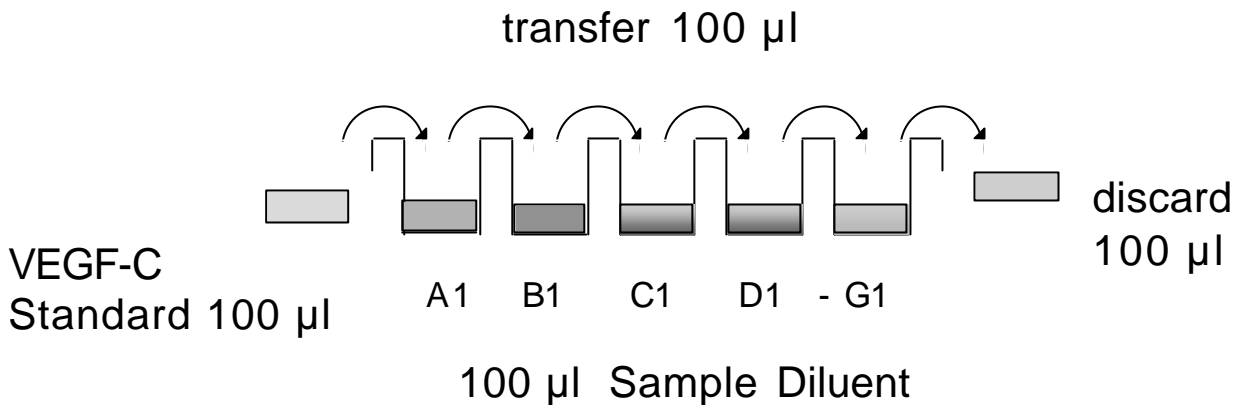


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (3000 pg/ml)	Standard 1 (3000 pg/ml)	Sample 1	Sample 1
B	Standard 2 (1500 pg/ml)	Standard 2 (1500 pg/ml)	Sample 2	Sample 2
C	Standard 3 (750 pg/ml)	Standard 3 (750 pg/ml)	Sample 3	Sample 3
D	Standard 4 (325 pg/ml)	Standard 4 (325 pg/ml)	Sample 4	Sample 4
E	Standard 5 (187.5 pg/ml)	Standard 5 (187.5 pg/ml)	Sample 5	Sample 5
F	Standard 6 (93.75 pg/ml)	Standard 6 (93.75 pg/ml)	Sample 6	Sample 6
G	Standard 7 (46.9 pg/ml)	Standard 7 (46.9 pg/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

- e. Add 100 μ l of **Sample Diluent** in duplicate to the blank wells.
- f. Add 50 μ l of **Sample Diluent** to the sample wells.
- g. Add 50 μ l of each **Sample**, in duplicate, to the designated wells.
- h. Prepare **Biotin-Conjugate** (refer to preparation of reagents 9.C.).
- i. Add 50 μ l of diluted **Biotin-Conjugate** to all wells, including the blank wells.
- j. Cover with a **Plate Cover** and incubate 2 hours at room temperature, if available on a microplate shaker set at 100 rpm.
- k. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.
- l. Prepare **Streptavidin-HRP** (refer to preparation of reagents D).
- m. Add 100 μ l of diluted **Streptavidin-HRP** to all wells, including the blank wells.
- n. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 1 hour, if available on a microplate shaker set at 100 rpm.
- o. Prepare **TMB Substrate Solution** a few minutes prior to use (refer to preparation of reagents 9.E.).
- p. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.
- q. Pipette 100 μ l of mixed **TMB Substrate Solution** to all wells, including the blank wells.

- r. Incubate the microwell strips at room temperature (18° to 25°C) for about 20 minutes, if available on a microplate shaker set at 100 rpm. Avoid direct exposure to intense light. The point at which the substrate reaction is stopped is often determined by the ELISA reader being used. Many ELISA readers record absorbance only up to 2.0 O.D. **Therefore the colour development within individual microwells must be watched by the person running the assay and the substrate reaction stopped before positive wells are no longer properly recordable.**
- s. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.
- t. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both, the samples and the VEGF-C 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.

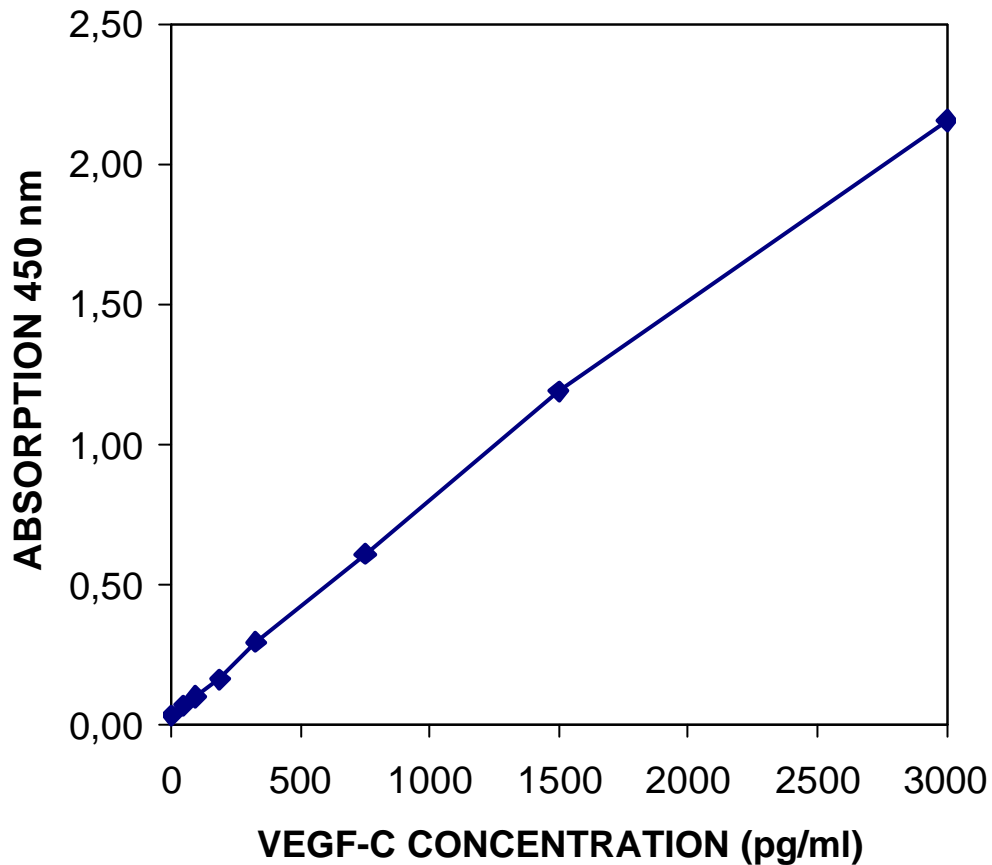
11. CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the VEGF-C concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating 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 VEGF-C concentration.
- **For samples which have been diluted according to the instructions given in this manual 1:2, the concentration has to be multiplied by the dilution factor (x2).**

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

- It is suggested that each testing facility establishes a control sample of known VEGF-C concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.
- A representative standard curve is shown in Figure 3. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

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



Typical data using the VEGF-C ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	VEGF-C Concentration (pg/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	3000	2.153	2.155	0.1
	3000	2.157		
2	1500	1.141	1.187	5.5
	1500	1.233		
3	750	0.593	0.606	2.9
	750	0.618		
4	325	0.287	0.294	3.1
	325	0.300		
5	187.5	0.159	0.161	1.3
	187.5	0.162		
6	93.75	0.098	0.098	0.7
	93.75	0.097		
7	46.9	0.067	0.068	2.1
	46.9	0.069		
Blank	0	0.034	0.034	
	0	0.033		

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. Completely empty wells before dispensing fresh Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- Excessive freeze – thaw cycles may result in loss of bio-active VEGF-C. Avoid repeated freeze – thaw cycles, preferentially use freshly collected samples.

13. PERFORMANCE CHARACTERISTICS

A. Sensitivity

The limit of detection of VEGF-C defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (mean plus two standard deviations) was determined to be 12.8 pg/ml (mean of 6 independent assays).

B. Reproducibility

a. Intra-assay

Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of VEGF-C. Two standard curves were run on each plate. Data below show the mean VEGF-C concentration and the coefficient of variation for each sample. The overall intra-assay coefficient of variation has been calculated to be 8.2 %.

b. Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in three independent experiments by three technicians. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of VEGF-C. Two standard curves were run on each plate. The overall inter-assay coefficient of variation has been calculated to be 6.5 %.

C. Spike Recovery

The spike recovery was evaluated by spiking recombinant human VEGF-C into different human serum samples. Recoveries were determined in three independent experiments with 8 serum samples. The amount of endogenous VEGF-C in unspiked serum was subtracted from the two spike values. Recoveries ranged from 78.7 to 107.6 % with an overall mean recovery of 85.6 %.

Recoveries were shown to depend on the serum used.

D. Dilution Parallelism

Three serum samples with different levels of VEGF-C were assayed at four serial two-fold dilutions (1:2-1:16) with 4 replicates each. Recoveries ranged from 71.8 % to 118 % with an overall mean recovery of 104.1 %. Recoveries were shown to depend on the serum used.

E. Sample Stability

a. Freeze-Thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored at -20°C and thawed up to 5 times, and VEGF-C levels determined. There was no significant loss of active VEGF-C by repeated freezing and thawing.

b. Storage Stability

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

F. Comparison of Serum and Plasma

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

G. Specificity

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

H. Expected Values

A panel of 8 sera from apparently healthy blood donors (males and females) was tested for VEGF-C. The detected VEGF-C levels ranged between 54 - 647 pg/ml with a mean level of 170 pg/ml. The normal levels measured may vary with the sample collective used.

14. BIBLIOGRAPHY

- 1) Clauss M. Molecular biology of the VEGF and the VEGF receptor family.
Semin Thromb Hemost. 2000; 26 (5): 561-9.
- 2) Duff SE, Li C, Jeziorska M, Kumar S, Saunders MP, Sherlock D, O'Dwyer ST, Jayson GC. Vascular endothelial growth factors C and D and lymphangiogenesis in gastrointestinal tract malignancy.
Br J Cancer. 2003 Aug 4; 89 (3): 426-30.
- 3) Ferrara N. Vascular endothelial growth factor and the regulation of angiogenesis.
Recent Prog Horm Res. 2000; 55: 15-35; discussion 35-6.
- 4) Jussila L, Alitalo K. Vascular growth factors and lymphangiogenesis.
Physiol Rev. 2002 Jul; 82 (3): 673-700.
- 5) Karkkainen MJ, Petrova TV. Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis.
Oncogene. 2000 Nov. 20; 19 (49): 5598-605.
- 6) Larrivee B, Karsan A. Signaling pathways induced by vascular endothelial growth factor (review).
Int J Mol Med. 2000 May; 5 (5): 447-56.
- 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.
- 8) Orpana A, Salven P. Angiogenic and lymphangiogenic molecules in hematological malignancies.
Leuk Lymphoma. 2002 Feb; 43 (2): 219-24.
- 9) Pepper MS. Lymphangiogenesis and tumor metastasis: myth or reality?
Clin Cancer Res. 2001 Mar; 7 (3): 462-8.

15. ORDERING INFORMATION

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Cat.No. BMS297 VEGF-C ELISA

16. REAGENT PREPARATION SUMMARY

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

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

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

Number of Strips	Biotin-Conjugate (ml)	Conjugate Diluent (ml)
1 - 6	0.03	3.0
1 - 12	0.06	6.0

D. Streptavidin-HRP

Number of Strips	Streptavidin-HRP (ml)	Conjugate Diluent (ml)
1 - 6	0.015	6.0
1 - 12	0.03	12.0

**E. TMB Substrate
Solution**

Number of Strips	Substrate Solution I (ml)	Substrate Solution II (ml)
1 - 6	3.0	3.0
1 - 12	6.0	6.0

17. TEST PROTOCOL SUMMARY

- Wash microwell strips twice with **Wash Buffer**
- Add 100 µl **Sample Diluent**, in duplicate, to standard wells
- Pipette 100 µl **VEGF-C Standard** into the first wells and create standard dilutions ranging from 3000 to 46.9 pg/ml by transferring 100 µl from well to well. Discard 100 µl from the last wells
- Add 100 µl **Sample Diluent**, in duplicate, to the blank wells
- Add 50 µl **Sample Diluent** to sample wells.
- Add 50 µl **Sample**, in duplicate, to designated wells
- Prepare **Biotin-Conjugate**
- Add 50 µl of diluted **Biotin-Conjugate** to all wells
- Cover microwell strips and incubate 2 hours at room temperature on a microplate shaker set at 100 rpm
- Prepare **Streptavidin-HRP**
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl diluted **Streptavidin-HRP** to all wells
- Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C) on a microplate shaker set at 100 rpm
- Prepare **TMB Substrate Solution** few minutes prior to use
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl of mixed **TMB Substrate Solution** to all wells including blank wells
- Incubate the microwell strips for about 20 minutes at room temperature (18°to 25°C)
- Add 100 µl **Stop Solution** to all wells including blank wells
- Blank microwell reader and measure colour intensity at 450 nm

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