Human VEGF-R1 ELISA

DIACLONE

650 030 096 **1 x 96 tests**
650 030 192 **2 x 96 tests**

INTENDED USE

The sVEGF-R1 ELISA is an enzyme-linked immunosorbent assay for quantitative detection of soluble human vascular endothelial Growth Factor Receptor-1 in cell culture supernatants, human serum, plasma or other body fluids. **The sVEGF-R1 ELISA is for research use only. Not for use in diagnostic or therapeutic procedures.**

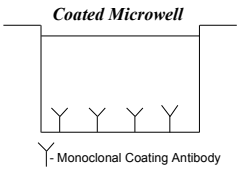
SUMMARY

Soluble VEGF-R1 (sFLT-1) is a naturally occurring endogenous form of the VEGF-R1 and was originally found in the supernatant of human vascular endothelial cells. It is generated by differential splicing of the flt-1 gene. In vitro sVEGF-R1 is used to inhibit VEGF-A mediated signals in endothelial cells and in vivo it can be used to block physiological angiogenesis in several organs, e.g. in the ovary or in bones. Tumor cells transfected with the flt-1 gene are growth restricted in vivo because of the limitation in developing tumor blood vessels via VEGF-A signalling. Very recent studies have shown that this molecule is present endogenously at ng/ml concentrations in biologicals fluids of normal human subjects or in the conditioned media of FLT-1 positive cell types. The measurement of sFLT-1 in a variety of clinical conditions may open up new insights in health and disease.

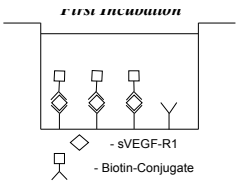
Characterization of angiogenic activity, such as embryonic development, placental vascularization, cancer and wound healing is measured by comparing the ratio of angiogenic stimulators (e.g. FGF-2, FGF-1, VEGF-A, Ang-1) to angiogenic inhibitors (e.g. sFLT-1, angiostatin, endostatin, thrombospondin). Several independently published data of both normal and pathogenic subjects have confirmed endogenous levels of VEGF-A and bFGF in pg/ml ranges. These factors have been thought to work unopposed to cause blood vessel formation. The finding that sVEGF-R1, a strong VEGF-A antagonist, is present in normal subjects suggests a finely tuned balance of signal transduction, the workings of which can now be explored. Together with other similar assay systems, positive and negative angiogenic regulators can now be explored in many different physiological and pathological settings using human cell culture supernatants and biologicals fluids.

PRINCIPLES OF THE TEST

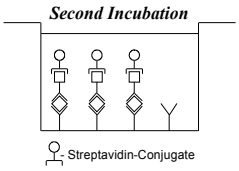
An anti-sVEGF-R1 coating antibody is adsorbed onto microwells.

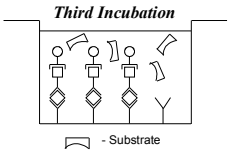


sVEGF-R1 present in the sample or standard binds to antibodies adsorbed to the microwells; a biotin conjugated anti-sVEGF-R1 antibody is added and binds to sVEGF-R1 captured by the first antibody.

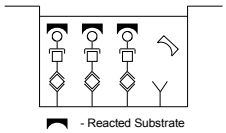


Following incubation unbound biotin conjugated anti-sVEGF-R1 is removed during a wash step. Streptavidin-HRP is added and binds to the biotin conjugated anti-sVEGF-R1. Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.





A coloured product is formed in proportion to the amount of sVEGF-R1 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 sVEGF-R1 standard dilutions and sVEGF-R1 sample concentration determined.



REAGENTS PROVIDED

REAGENTS (store at 2-8°C)	QTY 1 plates	QTY 2 plates	RECONSTITUTION
96-wells precoated microtiter plate	1	2	Ready-to-use
Plate covers	4	8	
VEGF-R1 Standard: 20 ng/ml	2 vials	4 vials	Reconstitute with distilled water. Volume is stated on the label of the standard vial
Biotin conjugate anti VEGF-R1 polyclonal antibody	1 vial	2 vials	(100 µl) Make a 1/100 dilution in Conjugate Diluent
Streptavidin-HRP*	1 vial	2 vials	(150 µl) Make a 1/ 200 dilution in Conjugate Diluent
Assay Buffer	1 bottle	2 bottles	(5ml) 20X concentrate. Dilute in distilled water
Conjugate Diluent	1 bottle	2 bottles	(20 ml) Ready to use
Substrate Solution	1 bottle	2 bottles	(15 ml) tetrametthyl-benzidine
Wash Buffer Concentrate	1 bottle	2 bottles	(50 ml) 20X concentrate. Dilute in distilled water
Stop Solution (1 M Phsophoric acid)	1 vial	2 vials	(15 ml) Ready-to-use
Blue Dye	1 vial	2 vials	(0.4 ml). Make a 1/250 dilution in the appropriate diluent
Green Dye	1 vial	2 vials	(0.4 ml). Make a 1/100 dilution in the appropriate diluent
Red Dye	1 vial	2 vials	(0.4 ml). Make a 1/250 dilution in the appropriate diluent

* Reagents contain preservative

MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes
- 10 µl to 1,000 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)
- 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

SAFETY

- 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.

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 box front 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.

SPECIMEN COLLECTION, PROCESSING AND STORAGE

Cell culture supernatants, murine serum, plasma or 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.

Pay attention to a possible "**Hook effect**" due to high sample concentrations.

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

Clinical samples should be kept at 2° to 8°C and separated rapidly before storing at -20°C to avoid loss of bioactive sVEGF-R1. If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles.

PREPARATION OF REAGENTS

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 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

2. Assay Buffer

Mix the contents of the bottle well. Add contents of **Assay buffer Concentrate** (5.0ml) to 95 ml distilled or deionized water and mix gently to avoid foaming. Store at 2° to 8°C. Please note that the assay buffer is stable for 30 days. Assay bottle may be prepared as needed according to the following table :

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

3. Biotin Conjugate

Make a 1:100 dilution 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	0.03	2.97
1 - 12	0.06	5.94

4. Preparation of VEGF-R1 Standard

Reconstitute sVEGF-R1 Standard by addition of distilled water. Reconstitution volume is stated on the label of the standard vial. Make sure the contents entirely dissolve by gentle swirling. The standard solution obtained is 20 ng/ml.

5. Preparation of Streptavidin-HRP

Make a 1:200 dilution of the concentrated Streptavidin-HRP solution as needed according to the following table:

Strips	Streptavidin-HRP (ml)	Conjugate Diluent (ml)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

6. Addition of Colour-giving Dyes

In order to help our customers to avoid any mistakes in pipetting, DIACLONE 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:

A. Diluent: Before 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 Diluent	20 µl ***Blue-Dye***
12 ml Diluent	48 µl ***Blue-Dye***
50 ml Diluent	200 µl ***Blue-Dye***

B. 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 µl ***Green-Dye***
6 ml Conjugate Diluent	60 µl ***Green-Dye***
12 ml Conjugate Diluent	120 µl ***Green-Dye***

C. 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 ***Red-Dye***
12 ml Conjugate Diluent	48 µl ***Red-Dye***

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 Antibody to human sVEGF-R1 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 reconstituted (refer to preparation of reagents, 9.D.) sVEGF-R1 Standard, in duplicate, into wells A1 and A2. 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 sVEGF-R1 standard dilutions ranging from 10 ng to 0.16 ng/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of sVEGF-R1 standard dilutions:

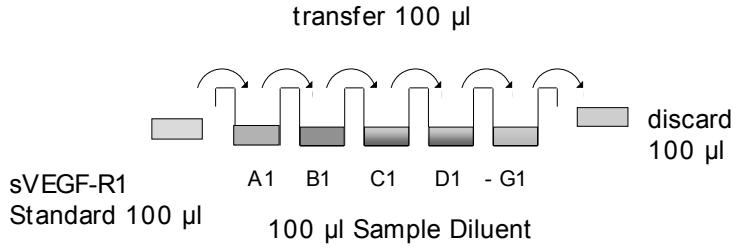


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	Standard Concentrations ng/mL		Sample wells									
	1	2	3	4	5	6	7	8	9	10	11	12
A	10	10										
B	5	5										
C	2.5	2.5										
D	1.25	1.25										
E	0.63	0.63										
F	0.32	0.32										
G	0.16	0.16										
H	Blank	Blank										

e. Add 100 µl of **Assay Buffer** in duplicate to the blank wells.

f. Add 50 µl of **Assay Buffer**, in duplicate, to the sample wells.

g. Add 50 µl of each **Sample**, in duplicate, to the designated wells.

h. Prepare **Biotin-Conjugate** (refer to preparation of reagents).

i. Add 50 µl of diluted **Biotin-Conjugate** to all wells, including the blank wells.

j. Cover with a Plate Cover and incubate at room temperature (18° to 25°C) for 2 hours on a microplate shaker set at 100 rpm.

k. Remove Plate Cover and empty wells. Wash microwell strips 4 times according to point c. of the test protocol. Proceed immediately to the next step.

l. Prepare **Streptavidin-HRP** (refer to preparation of reagents).

m. Add 100 µl 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 on a microplate shaker at 100 rpm.

o. Remove Plate Cover and empty wells. Wash microwell strips 4 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, including the blank wells.

q. Incubate the microwell strips at room temperature (18° to 25°C) for about 20 minutes. On a microplate shaker at 100 rpm. Avoid direct exposure to intense light. The point at which the substrate reaction need

to be 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. The substrate reaction must be stopped before positive wells are no longer properly recordable.

r. 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.

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 sVEGF-R1 standards.

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

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 sVEGF-R1 concentration on the abscissa. Draw a best fit curve through the points of the graph.

- To determine the concentration of circulating sVEGF-R1 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 sVEGF-R1 concentration.

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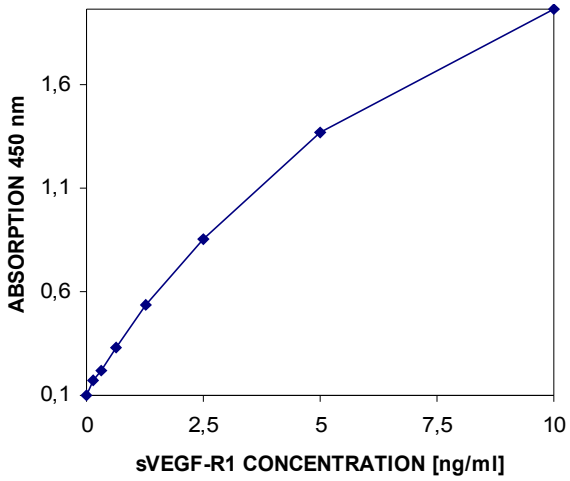
For samples which have been diluted according to the instructions given in this manual 1:2, the concentration read from the standard curve must be multiplied by the dilution factor (x2).

Note: Calculation of samples with an O.D. exceeding the range of the standard curve may result in incorrect, low sVEGF-R1 levels. Such samples should be re-analyzed at higher dilution rate in order to precisely quantitate the actual sVEGF-R1 level.

It is suggested that each testing facility establishes a control sample of known sVEGF-R1 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 sVEGF-R1 ELISA. sVEGF-R1 was diluted in serial two-fold steps in Sample Diluent, 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 sVEGF-R1 ELISA

Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	VEGF-R1 Concentration (ng/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	10	2.027	2.046	1.3
	10	2.064		
2	5	1.208	1.243	4.0
	5	1.278		
3	2.5	0.684	0.677	1.6
	2.5	0.669		
4	1.25	0.384	0.384	0.2
	1.25	0.383		
5	0.625	0.237	0.230	4.3
	0.625	0.223		
6	0.312	0.148	0.152	3.7
	0.312	0.156		
7	0.156	0.106	0.107	1.3
	0.156	0.108		
Blank	0	0.080	0.077	
	0	0.074		

LIMITATIONS OF THE PROCEDURE

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either 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.

PERFORMANCE CHARACTERISTICS

Sensitivity

The limit of detection of sVEGF-R1 defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (mean plus three standard deviations) was determined to be 0.1 ng/ml (mean of 6 independent assays).

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 sVEGF-R1. Two standard curves were run on each plate. Data below show the mean sVEGF-R1 concentration and the coefficient of variation for each sample. The overall intra-assay coefficient of variation has been calculated to be 5.1 %.

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 sVEGF-R1. Two standard curves were run on each plate. Data below show the mean sVEGF-R1 concentration and the coefficient of variation calculated on 18 determinations of each sample. The overall inter-assay coefficient of variation has been calculated to be 10.

Spike Recovery

The spike recovery was evaluated by spiking four levels of sVEGF-R1 into 4 pooled normal human sera. The amount of endogenous sVEGF-R1 in unspiked serum was substracted from the spike values. Recoveries ranged from 71 % to 83 % with an overall mean recovery of 77 %.

Dilution Parallelism

Four spiked serum samples with different levels of sVEGF-R1 were assayed at four serial two-fold dilutions with 4 replicates each. In the table below the per cent recovery of expected values is listed. Recoveries ranged from 90% to 116% with an overall mean recovery of 105%.

		sVEGF-R1 Concentration (ng/ml)		
Sample	Dilution	Expected Value	Observed Value	% Recovery of Exp. Value
1	1:2	--	13.2	--
	1:4	6.6	6.7	101.8%
	1:8	3.3	3.7	113.1%
	1:16	1.7	1.8	110.7%
2	1:2	--	6.1	--
	1:4	3.0	3.6	120.3%
	1:8	1.5	1.8	118.8%
	1:16	0.8	0.8	109.6%
3	1:2	--	11.5	
	1:4	5.7	6.8	118.3%
	1:4	2.9	3.8	134.3%
	1:8	1.4	2.0	141.2%
4	1:2	--	8.7	--
	1:4	4.4	4.1	94.1%
	1:8	2.2	1.9	89.4%
	1:16	1.1	1.0	92.2%

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 sVEGF-R1 levels determined. There was no significant loss of sVEGF-R1 freezing and thawing up to 5 times.

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 sVEGF-R1 level determined after 24 h. There was no loss of sVEGF-R1 immunoreactivity during storage at –20°C and 4°C. Storage at RT and 37°C gave rise to 20-50 % loss of sVEGF-R1 immunoreactivity.

Comparison of serum and plasma

From eight individuals, serum as well as EDTA and citrate, and heparin plasma obtained at the same time point were evaluated. sVEGF-R1 concentrations were not significantly different and therefore all these body fluids are suitable for the assay. It is nevertheless highly recommended to assure the uniformity of blood preparations.

Specificity

The interference of circulating factors of the immune systems was evaluated by spiking these proteins at physiologically relevant concentrations into a serum sample. There was no detectable cross reactivity.

Expected serum values

There are no detectable sVEGF-R1 levels found in healthy donors.

BIBLIOGRAPHY

1. Banks K, Forbes M, Searles J, Pappin D, Canas B, Rahman D, Kaufmann S, Walters C, Jacksin A, Eves P, Linton G, Keen J, Walker J, Selby P. (1998). Evidence for the existence for a novel pregnancy-associated soluble variant of the vascular endothelial growth factor receptor, FLT-1. Molec. Hum. Reprod. 4, 377-386.

2. Barleon B, Totzke F, Herzog C, Blank S, Kremmer E, Siemeister G, Marmè D, Martiny-Baron G. (1997). Mapping of the sites for ligand binding and receptor dimerization at the extracellular domain of the vascular endothelial growth factor receptor FLT-1. J. Biol. Chem. 272, 10382-10388.

3. Clark DE, Smith SK, He Y, Day KA, Licence DR, Corps AN, Lammoglia R, Charnock-Jones DS. (1998). A vascular endothelial growth factor antagonist is produced by the human placenta and released into the maternal circulation. Biol. Reprod. 59(6):1540-1548.

4. Goldman C, Kendall RL, Cabrera G, Soroveanu L, Heike Y, Gillespie G, Siegal G, Mao X, Bett A, Huckley W, Thomas K, Curiel D. (1998). Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. Proc. Natl. Acad. Sci. 95, 8795-8800.

5. He Y, Smith SK, Day KA, Clark DE, Licence DR, Charnock-Jones DS. (1999). Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. Mol. Endocrinol 13, 537-545.

6. Hornig C, Behn T, Bartsch W, Yayon A, Weich HA. (1999). Detection and quantification of complexed and free soluble human vascular endothelial growth factor receptor-1 (sVEGF-R1) by ELISA. J. Immun. Meth. 226 (1-2) 169-177.

7. Hornig C, Weich HA. (1999). Soluble VEGFR receptors. Angiogenesis 3(1) 33-39.

8. Kendell RL, Thomas, KA. (1993). Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc. Natl. Acad. Sci. 90, 10705-10709.

9. Kendell RL, Wang G and Thomas KA. (1996). Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. Biochem. Biophys. Res. Commun. 226, 324-328.

10. Röckl W, Hecht D, Sztajer H, Waltenberger J, Yayon A, Weich HA. (1998). Differential binding characteristics and cellular inhibition by soluble forms of KDR and FLT-1. J. Exp. Cell Res. 241, 161-170.
Eur J Neurosci 1994 May 1;6(5):766-78.

REAGENT PREPARATION SUMMARY

A. Wash Buffer	Add **Wash Buffer Concentrate** 20 x (50 ml) to 950 ml distilled water		
B. Assay Buffer	Number	Assay Buffer concentrate	Distilled water
	1-6	2.5	47.5
	1-12	5.0	95.0
C. Biotin-Conjugate	Make dilution according to the table.		
	Number	Biotin-Conjugate	Conjugate Diluent (ml)
	1 - 6	0.03	2.97
	1 - 12	0.06	5.94
D. Standard	h vial of lyophilized **sVEGF-R1 Standard** as needed.		
E. Streptavidin-HRP	Number	Streptavidin-HRP (ml)	Conjugate Diluent(ml)
	1 - 6	0.03	5.97
	1 - 12	0.06	11.94

TEST PROTOCOL SUMMARY

- Wash microwell strips twice with Wash Buffer
- Add 100 µl **Assay Buffer**, in duplicate, to all standard wells
- Pipette 100 µl reconstituted sVEGF-R1 Standard into the first wells and create standard dilutions ranging from 10 to 0.16 ng/ml by transferring 100 µl from well to well. Discard 100 µl from the last wells
- Add 100 µl **Assay Buffer**, in duplicate, to the blank wells
- Add 50 µl **Assay Buffer** to the 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 (18° to 25°C) on microplate shaker
- Prepare **Streptavidin-HRP**
- Empty and wash microwell strips 4 times with **Wash Buffer**
- Add 100 µl of diluted **Streptavidin-HRP** to all wells
- Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C) on microplate shaker
- Empty and wash microwell strips 4 times with Wash Buffer
- Add 100 µl of **TMB Substrate Solution** to all wells including blank wells
- Incubate the microwell strips for about 10 minutes at room temperature (18°to 25°C) on microplate shaker
- 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 sVEGF-R1 levels. Such samples require further dilution with Sample Diluent in order to precisely quantitate the actual sVEGF-R1 level.