

## Total Soluble Vascular Endothelial Growth Factor Receptor-1 (sVEGFR-1<sub>total</sub>) ELISA Kit

Anti-Angiogenesis Test Kit for the quantitative determination of recombinant and naturally occurring, human total soluble vascular endothelial growth factor receptor-1 (sVEGFR-1<sub>total</sub>) in cell culture supernatants and complex biological fluids.

<b>Catalog Number:</b>	CKV000
<b>Quantity:</b>	1 x 96 determinations. The kit includes enough reagents for one 96-well ELISA plate. We recommend running the standard and the samples in duplicate.
<b>Sensitivity:</b>	<0.16 ng/ml
<b>Range of detection:</b>	0.3 ng/ml to 10 ng/ml
<b>Intra-assay variation:</b>	8%
<b>Inter-assay variation:</b>	7 - 8%
<b>Storage:</b>	STORE AT 4°C

### Introduction:

A Soluble VEGFR-1 (sFLT-1) is a naturally occurring endogenous form of the VEGFR-1 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* sVEGFR-1 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 biological fluids of normal human subjects or in the conditioned media of *flt-1* positive cell types. The measurement of sVEGFR-1 in a variety of clinical conditions may open up new insights in health and disease.

We are pleased to announce that through a collaboration with several laboratories we were able to develop the first simple, accurate immunoassay for the detection of sVEGFR-1 in research samples and complex biological fluids such as cell culture supernatants, amniotic fluids and cystic brain fluids. The assay allows the detection of the total amount of sVEGFR-1 (free and complexed with ligands). The product has been developed using state-of-the-art methods for sandwich-ELISA developments and using specific, high affinity binding proteins for sVEGFR-1. This new assay has been validated under various conditions and is quantitatively calibrated to a verified recombinant molecular standard.



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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 to ng/ml ranges as measured by commercially available sandwich ELISA kits. These factors have been thought to work unopposed to cause blood vessel formation. The finding that sVEGFR-1, 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 biological fluids.

## Human Total Soluble VEGFR-1 ELISA

Enzyme linked immunosorbent assay (ELISA) for the detection of naturally occurring, total soluble vascular endothelial growth factor receptor-1 (sVEGFR-1<sub>total</sub>) in cell culture supernatants or complex biological fluids. The assay has been successfully used for the detection of sVEGFR-1<sub>total</sub> in human primary cell culture supernatants, human tumour cell lysates, amniotic fluids, cystic brain fluids, wound fluids and follicle fluids. Recombinant forms as sVEGFR-1 D1-3, D1-4, D1-5 and D1-6 can also be measured. This improved version of the ELISA offers for the first time the possibility to measure sVEGFR-1 in serum and plasma samples. The assay recognizes both ligand-complexed and free forms of the soluble receptor. Cross-reactivity with sVEGFR-2 and -3 and sPDGFR- $\beta$  have not been observed. A schematic representation of the assay system is given in Fig. 1.

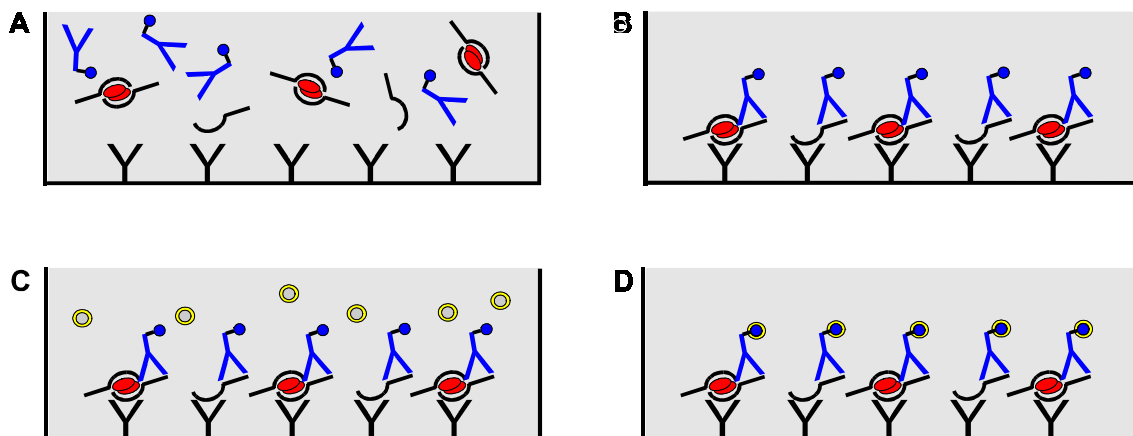


Figure 1: Schematic representation of sVEGFR-1 sandwich ELISA



Briefly, precoated mouse monoclonal antibodies are used to capture sVEGFR-1 from the samples. The antibodies recognize free and complexed forms of sVEGFR-1 (A). A biotinylated, polyclonal rabbit antiserum, which is incubated together with the samples, is used to detect bound sVEGFR-1 (B). After incubation with peroxidase conjugated streptavidin (C), the color developing reagent is added. Horseradish peroxidase catalyzes the oxidation of TMB into a blue oxidation product of TMB. By addition of 1M H<sub>2</sub>SO<sub>4</sub> the reaction is stopped and the color changes to yellow.

## Kit Materials

Microtiter plate:	One precoated and saturated 96-well microtiter ELISA plate, removable 16-well racks
Plate sealer/Manual:	Resealable bag containing two adhesive strips and manual
Wash buffer:	30ml of 20x concentrate (1x Wash buffer: 50mM Tris-Cl, 0.2% TWEEN 20, pH 8.0)
Assay buffer:	10ml of 10x concentrate
Sample diluent:	12ml of 1x diluent
sVEGFR-1 standard:	Two vials containing 6ng lyophilized hu. sVEGFR-1
Biotinylated detector:	One vial containing 60µl pre-diluted rabbit IgG.
Streptavidin enzyme:	One vial containing 120µl pre-diluted poly-HRP conjugated streptavidin
Color reagent:	12ml one-component TMB (tetramethyl-benzidine) solution
Stop solution:	12ml 1M Sulphuric acid (H <sub>2</sub> SO <sub>4</sub> )



## Materials required but not provided:

- Multichannel or repeating pipettes
- Pipettes capable of accurately measuring 1-1000µl
- Orbital shaker
- Clean 10-15ml serological tubes and Eppendorf tubes for preparation of working dilutions
- 96-well microtiter plate reader with 450nm and 650nm filter
- Distilled water
- Computerized data plotting or graph paper for manual plotting of data

WARNING: SOME LIQUID MATERIALS CONTAIN SODIUM AZIDE OR THIMEROSAL AS PRESERVATIVE. AVOID SKIN CONTACT. H<sub>2</sub>SO<sub>4</sub> CAUSES SKIN IRRITATIONS. TMB IS HIGHLY TOXIC. AVOID BREATHING IT. PROTECT EYES, FACE, HANDS AND CLOTHES WHILE WORKING WITH ALL ELISA COMPONENTS.

## Manual plate washing

**Washing and complete removal of all liquid at the end of each incubation step is very important to obtain low background values. The following washing procedure is recommended:**

1. Remove existing fluid from each well by flicking the plate over a sink.
2. Blot the plate on clean paper towels.
3. Forcefully pipet 250µl diluted wash buffer into each well.
4. Repeat steps 1-3 twice.
5. Always remove wash buffer immediately. Do not incubate plate in wash buffer.

## Preparation of reagents and dilutions

Reagents supplied as small volumes must be spinned down before opening the tubes to avoid loss of reagents. Prepare all reagents right before usage and keep cool until application.

1. *Wash- and assay buffer:* Dilute 10x assay buffer concentrate and 20x wash buffer concentrate with distilled water.
2. *Microtiter plate:* Unpack ELISA plate. Reconstitute the plate by pipetting 100µl 1x wash buffer into each well, wait 5 min, flick and blot the plate. Use plate sealer to avoid drying of plate.



3. *Standard*: Reconstitute sVEGFR-1 by addition of 300µl assay buffer to make up 40ng/ml. Mix well. Serial dilutions are prepared within the wells. The reconstituted standard should not be stored for longer than 24h at 4°C.
4. *Biotinylated detector*: Dilute 1/100 in assay buffer (final volume 6ml).
5. *Streptavidin enzyme*: Dilute 1/100 in assay buffer (final volume 12ml).

HALF OF THE SOLUTION IS NEEDED FOR WORKING WITH A HALF PLATE.  
USE A NEW LYOPHOLIZED STANDARD PROTEIN EVERY TIME.  
WE RECOMMEND RUNNING STANDARD AND SAMPLES IN DUPLICATE.

## Assay procedure

1. Start with the reconstituted plate. Remove the plate sealer and add 100µl sample diluent in duplicate to the standard wells (A1/A2 to H1/H2).
2. Add sample diluent to the sample wells. Samples should be measured diluted 1/2 **or** 1/4. For 1/2 dilution add 50µl diluent to all sample wells, for 1/4 dilution add 75µl diluent to all sample wells (Some samples may be diluted higher, e.g. 1/8).
3. Add 100µl of the reconstituted standard to wells H1/H2. Prepare 1/2 dilutions within the wells by mixing and pipetting 100µl to wells G1/G2, F1/F2, ..., B1/B2. Discard 100µl from wells B1/B2 (see Figure below). Wells A1/A2 are background controls. Do not add standard protein to wells A1/A2. Avoid touching the bottom of the wells with the pipette-tips.



4. Add samples to the sample wells. Use 50µl of the samples for 1/2 dilution and 25µl of the samples for 1/4 dilution.
5. Add 50µl biotin-conjugate (diluted 1/100 in assay buffer) to each well including the background controls (A1/A2). Seal the plate and incubate for 2h at room temperature.
6. Wash plate four times.
7. Add 100µl streptavidin-enzyme (diluted 1/100) in assay buffer) to each well. Seal plate and incubate for 1h at room temperature.
8. Wash plate four times.
9. Add 100µl TMB-substrate solution to each well. Allow the blue color to develop for 10-30 minutes. Do not shake plate during this incubation step. Stop the reaction by adding 50µl stop-solution to each well. The blue color is turned to yellow as a result of the pH- shift.
10. Measure plate in a 96-well microplate reader using 450nm as measuring and 650nm as reference wavelength.

## ASSAY PROCEDURE SUMMARY

Prepare samples and standard, reconstitute plate. Apply standard, samples and biotinylated dector.



Incubate 2h



Wash three times. Add streptavidine-enzyme to each well.



Incubate 1h



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Wash plate three times and add TMB-substrate to each well.



Wait 10-30min for color development. Stop reaction and read the plate at 450nm and 650nm as reference.

## Calculation of results

Plot the standard curve on semi-logarithmic paper. Known concentrations of sVEGFR-1 are plotted on the log-scale (X-axis), the corresponding OD is plotted on the linear scale. The standard curve should have a sigmoidal shape (Fig.2). The concentrations of sVEGFR-1 in unknown samples may be determined by plotting the sample OD on the Y-axis and drawing a horizontal line that intersects with the standard curve. A vertical line dropped from the point of intersection with the standard curve to the X-axis intersects the X-axis at the point of the concentration of the unknown sample. Multiply this value with the dilution factor of the sample to get the original concentration of the undiluted sample.

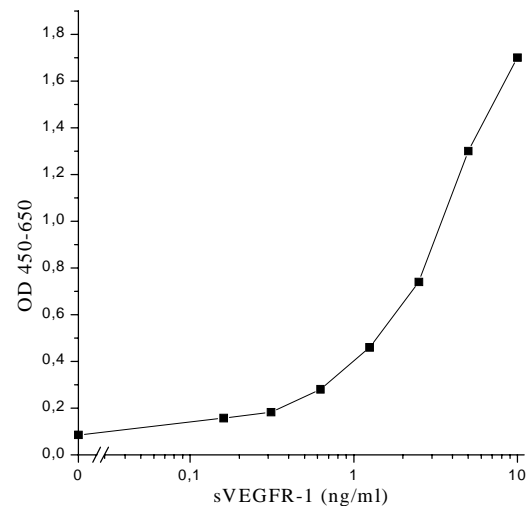


Fig 2: Standard curve

## Storage and application of samples

1. Clear samples by centrifugation prior to storage.
2. For short term storage (<4 weeks) keep samples at  $-20^{\circ}\text{C}$ . For long term storage (>4 weeks) keep samples at  $-70^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.
3. Samples containing low amounts of sVEGFR-1 can be concentrated by ultrafiltration (>50 kDa).
4. We recommend desalting of samples with a high salt content prior to measurement.
5. Samples can be depleted of sVEGFR-1 by incubation with Heparin sepharose.

## Approximate sample values (Table 1)

Sample	Range (ng/ml)	Average (ng/ml)
Amniotic fluid	0-40	15
Serum / Plasma	0.1-0.5	0.3
Follicle fluid	0-5	1.5
Wound fluid	0-7	2.5
Endothelial cell (EC) CM	0-12	10
Activated EC CM	5-50	20
Monocyte CM	0-3	2

## Soluble VEGFR-1 references



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