Human VEGF-R1 ELISA Kit

Instructions for use

Catalogue numbers: 1x96 tests: 650.030.096
2x96 tests: 650.030.192

For research use only

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Human VEGF-R1 ELISA KIT

1. Intended use

The VEGF-R1 ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human transforming growth factor Receptor-1 levels in cell culture supernatants, human serum, plasma, or other body fluids.
This kit has been configured for research use only.

2. Introduction

2.1. Summary

Soluble VEGF-R1 (FLT-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 VEGF-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 FLT-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. FLT-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 VEGF-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.

2.2. Principle of the method

An anti-human VEGF-R1 coating antibody is adsorbed onto microwells. Human VEGF-R1 present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human VEGF-R1 antibody is added and binds to human VEGF-R1 captured by the first antibody.

Following incubation unbound biotin-conjugated anti-human VEGF-R1 antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human VEGF-R1 antibody. 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 human VEGF-R1 present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm.

3. Reagents provided and reconstitution

REAGENTS (store at 2-8°C)	Quantity 1x96 well kit 650.030.096	Quantity 2x96 well kit 650.030.192	RECONSTITUTION
96-wells precoated microtiter plate	1	2	Ready-to-use
Plate covers	2	4	
Biotin-Conjugate anti-human VEGF-R1	1 vial	2 vials	(100µl). Make a 1/100 dilution in Conjugate Diluent
Streptavidin-HRP	1 vial	2 vials	(150µl). Make a 1/100 dilution in Conjugate Diluent
VEGF-R1 Standard: 20ng/ml	2 vial	4 vials	Reconstitute with distilled water. Volume is stated on the label of the standard vial
Assay Buffer Concentrate	1 vial	2 vials	(5 ml) 20X concentrate. Dilute in distilled water
Conjugate Diluent	1 vial	2 vials	(20 ml) Ready to ude
Wash Buffer Concentrate	1 vial	2 vials	(50 ml) 20X concentrate. Dilute in distilled water
Substrate Solution	1 vial	2 vials	(15 ml) Ready-to-use
Stop Solution (0.5M Phosphoric acid)	1 vial	2 vials	(15 ml) Ready-to-use
Blue Dye	1 vial	2 vials	(0.4 ml). Make a 1/2000 dilution in the appropriate diluent
Green Dye	1 vial	2 vials	(0.4 ml). Make a 1/800 dilution in the appropriate diluent
Red Dye	1 vial	2 vials	(0.4 ml). Make a 1/2000 dilution in the appropriate diluent

4. Materials required but not provided

- Microtiter plate reader fitted with appropriate filters (450nm required with optional 630nm reference filter)
- Microplate washer or wash bottle
- 10, 50, 100, 200 and 1,000µl adjustable single channel micropipettes with disposable tips
- 50-300µl multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Miscellaneous laboratory plastic and/or glass, if possible sterile

5. Specimen collection, processing & storage

Cell culture supernatants, 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.

Cell culture supernatants: Remove particulates and aggregates by spinning at approximately 1000 x g for 10 min.

Storage: If not analyzed shortly after collection, samples should be aliquoted (250-500µl) to avoid repeated freeze-thaw cycles and stored frozen at –70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

Recommendation: Do not thaw by heating at 37°C or 56°C. Thaw at room temperature and make sure that sample is completely thawed and homogeneous before use. When possible avoid use of badly haemolysed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

6. Safety & precautions for use

- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures, e.g.CDC/NIH Health manual : " Biosafety in Microbiological and Biomedical Laboratories" 1984
- Laboratory gloves should be worn at all times
- Avoid any skin contact with H_2SO_4 and TMB. In case of contact, wash thoroughly with water
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used
- Do not pipette by mouth
- When not in use, kit components should be stored refrigerated or frozen as indicated on vials or bottles labels
- All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration
- Cover or cap all reagents when not in use
- Do not mix or interchange reagents between different lots
- Do not use reagents beyond the expiration date of the kit
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of H_2SO_4 and substrate solution, avoid pipettes with metal parts
- Use a clean plastic container to prepare the washing solution
- Thoroughly mix the reagents and samples before use by agitation or swirling
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells
- The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning TMB is toxic avoid direct contact with hands. Dispose off properly
- If a dark blue colour develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded. Read absorbance's within 1 hour after completion of the assay
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells
- Follow incubation times described in the assay procedure
- Dispense the TMB solution within 15 min of the washing of the microtitre plate

7. Assay Preparation

Bring all reagents to room temperature before use

7.1. Assay Design

Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard and zero should be tested **in duplicate**. Remove sufficient Microwell Strips for testing from the aluminium pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8^{o}C storage.

Example plate layout (example shown for a 6 point standard curve)

	Standards		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	Blank	Blank										
H												

All remaining empty wells can be used to test samples in duplicate

7.2. Preparation of 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.

7.3. Preparation of Assay Buffer

Mix the contents of the bottle well. Add contents of **Assay Buffer** Concentrate (5.0 ml) 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.

7.4. Preparation of Standard

Standard vials must be reconstituted with the volume of distilled water shown on the vial immediately prior to use. This reconstitution gives a stock solution of 20000pg/ml of VEGF-R1. **Mix the reconstituted standard gently by inversion only**. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 10000 to 320 pg/ml. A fresh standard curve should be produced for each new assay.

- Add 100µl of Assay Buffer to all standard and blank wells
- Immediately after reconstitution add 100µl of the reconstituted standard to wells A1 and A2, which provides the highest concentration standard at 20000pg/ml. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells
- Transfer 100µl from wells A1 and A2 to B1 and B2. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells
- Continue this 1:1 dilution using 100µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 10000 to 320 pg/ml
- Discard 100µl from the final wells of the standard curve (F1 and F2)

Alternatively these dilutions can be performed in separate clean tubes and immediately transferred directly into the relevant wells.

7.5. Preparation of Biotin Conjugate

Make a 1:100 dilution with Assay Buffer in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (µl)	Assay Buffer (ml)
1 - 6	30	2.97
1 - 12	60	5.94

7.6. Preparation of Streptavidin-HRP

Make a 1:100 dilution of the concentrated **Streptavidin-HRP** solution in **Assay Buffer** as needed according to the following table:

Number of Strips	Streptavidin-HRP (µl)	Assay Buffer (ml)
1 - 6	60	5.94
1 - 12	120	11.88

7.7. Addition of Color Dyes

In order to help our customers to avoid any mistakes in pipetting, color dyes help 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 1:2000 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of ***Blue-Dye***, proceed according to the instruction booklet.

12 ml Diluent	6 µl ***Blue-Dye***
50 ml Diluent	25 µl ***Blue-Dye***

B. Biotin-Conjugate:

Before dilution of the concentrated Biotin-Conjugate, add the ***Green-Dye*** at a dilution of 1:800 (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.

6 ml Conjugate Diluent	8 µl ***Green-Dye***
12 ml Conjugate Diluent	15 µl ***Green-Dye***

C. Streptavidin-HRP:

Before dilution of the concentrated Streptavidin-HRP, add the ***Red-Dye*** at a dilution of 1:2000 (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.

12 ml Assay Buffer	6 µl ***Red-Dye***

8. Method

We strongly recommend that every vial is mixed thoroughly without foaming prior to use except the standard vial which must be mixed gently by inversion only.

Prepare all reagents as shown in section 7.

Assay Step		Details
1.	Wash	Remove the pre-coated plate from the sealed pouch, removed any un-needed strips and wash the plate as follows: a) Dispense 0.3 ml of **1x washing solution** into each well b) Aspirate the contents of each well c) Repeat step a and b
1.	Addition	**Prepare Standard curve** as shown in section 7.4
2.	Addition	Add 100µl of **Assay Buffer (1X)** in duplicate to the blank wells Add 50µl of **Assay Buffer (1X)** in duplicate in sample wells Add 50 µl of each **sample** in duplicate to the designated wells
3.	Addition	Add 50µl of diluted **biotinylated anti-VEGF-R1** to all wells
4.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **2 hours** on a rotator set at 100 rpm
5.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of **1x washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c another two times
6.	Addition	Add 100µl of **Streptavidin-HRP** solution into all wells
7.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hour** on a rotator set at 100 rpm
8.	Wash	Repeat wash step 5.
9.	Addition	Add 100µl of ready-to-use **TMB Substrate Solution** into all wells
10.	Incubation	Incubate in the dark for **30 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil
11.	Addition	Add 100µl of **H_2SO_4:Stop Reagent** into all wells
Read the absorbance value of each well (immediately after step 11.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 630 nm as the reference wave length (610 nm to 650 nm is acceptable).		

***Incubation time of the substrate solution is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range*

9. Data Analysis

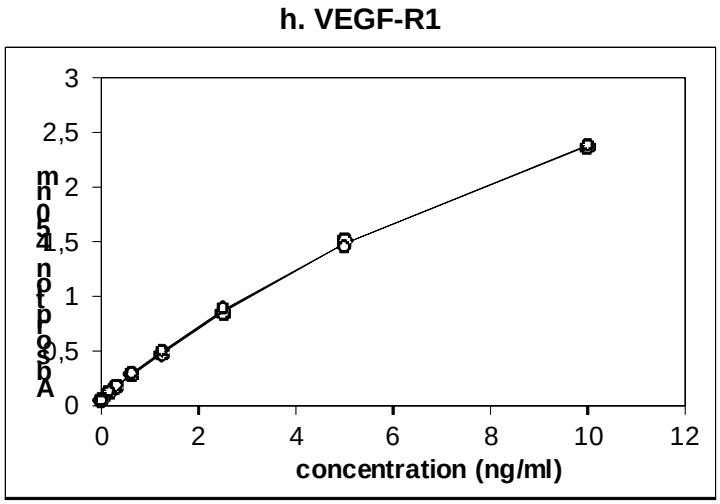
Calculate the average absorbance values for each set of duplicate standards and samples**.** Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding human VEGF-R1 standard concentration on the horizontal axis.

The amount of human VEGF-R1 in each sample is determined by extrapolating OD values against VEGF-R1 standard concentrations using the standard curve**.**

Example human VEGF-R1 Standard Curve

Standard	human VEGF-R1 Conc (ng/ml)	**OD (450nm) mean**	**CV (%)**
1	10	2.381	0.4
2	5	1.488	2.0
3	2.5	0.876	2.8
4	1.25	0.496	3.1
5	0.63	0.297	3.0
6	0.31	0.186	2.9
Zero	0	0.069	-



Note; curve shown above should not be used to determine results. Every laboratory must produce a standard curve for each set of microwell strips assayed.

For samples which have been diluted according to the protocol (1:2), the calculated concentration should be multiplied by the dilution factor.

10. Assay limitations

Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard diluent or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.

The influence of various drugs on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results.

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 Washing Buffer, fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

As with most biological assays conditions may vary from assay to assay therefore **a fresh standard curve must be prepared and run for every assay.**

11. Performance Characteristics

11.1. Sensitivity

The limit of detection of human VEGF-R1 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 0.03 ng/ml (mean of 6 independent assays).

11.2. 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.

11.3. Precision

Intra-assay

Reproducibility within the assay was evaluated in three independent experiments. **The overall intra-assay coefficient of variation has been calculated to be 5.5%.**

Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in three independent experiments by three technicians. **The overall inter-assay coefficient of variation has been calculated to be 5.1%.**

11.4. Dilution Parallelism

Serum, plasma and cell culture supernatant samples with different levels of human **sVEGF-R1** were analysed at serial 2 fold dilutions with 4 replicates each.

Linearity of dilution was measured in various samples. For Recovery data see table below:

Sample Matrix	Recovery of Exp. Val.	
	Range (%)	Mean (%)
Serum	112-148	128
Plasma (EDTA)	114-142	125
Plasma (citrate)	77-121	102
Cell culture supernatant	105-127	116

11.5. Spike Recovery

The spike recovery was evaluated by spiking 3 levels of human sVEGF-R1 into 4 serum, plasma, and cell culture supernatant. Recoveries were determined with 4 replicates each. The amount of endogenous sVEGF-R1 in unspiked samples was substracted from the spike values.

For Recovery data see table below

Sample Matrix	Spike high (%)	Spike medium (%)	Spike low (%)
Serum	46	50	67
Plasmas (EDTA)	73	72	91
Plasmas (citrate)	148	115	155
Cell culture Supernatant	47	56	79

11.6. Stability

Storage Stability

Aliquots of spiked serum were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the human VEGF-R1 level determined after 24 h. There was no loss of human VEGF-R1 immunoreactivity during storage at -20°C and 2-8°C. Storage at RT and 37°C show 20-50 % loss of VEGF-R1 immunoreactivity.

Freeze-thaw Stability

Aliquots of spiked serum were stored frozen at –20°C and thawed up to 5 times, and human VEGF-R1 levels determined. There was no significant loss of human VEGF-R1 by repeated freezing and thawing up to 5 times.

11.7. Expected serum values

There no detectable VEGF-R1 levels found in healthy donors.

12. Bibliography

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13. Assay Summary

Total procedure length : 3h30mn

Add 100µl of sample or diluted standard or control

⇓

Add 50µl of diluted biotinylated Detection antibody to all wells

⇓

Incubate 2 hours at room temperature

⇓

Wash three times

⇓

Add 100µl of streptavidin-HRP to all wells

⇓

Incubate 1 hour at room temperature

⇓

Wash three times

⇓

Add 100 µl of ready-to-use TMB
Protect from light. Let the color develop for 30 min.

⇓

Add 100 µl H_2SO_4

⇓

Read Absorbance at 450 nm

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