# Human VEGF-R1 ELISA Kit

Instructions for use

Catalogue numbers: 1x96 tests: 650.030.096

**For research use only**

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# Human VEGF-R1 ELISA KIT

## 1. Intended use

The Diaclone VEGF-R1 ELISA kit is a solid phase sandwich ELISA for the *in-vitro* qualitative and quantitative determination of soluble VEGF-R1 in supernatants, buffered solutions or serum and plasma samples. This assay will recognise both natural and recombinant human VEGF-R1.

**This kit has been configured for research use only. Not suitable for use in therapeutic procedures.**

## 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.

### 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@2-8°C) | **Quantity 1x96 well kit** Cat no. 650.030.096 | **Reconstitution** |
|---|---|---|
| 96 well microtiter strip plate | 1 | Ready to use (Pre-coated) |
| Plastic plate covers | 2 | n/a |
| VEGF-R1 Standard: 20 ng/ml | 2 | Reconstitute as directed on the vial (see Assay preparation, section 8) |
| Biotin-Conjugate anti-human VEGF-R1 | 1 (70µl) | Dilute in Conjugate Diluent (see Assay preparation, section 8) |
| Streptavidin-HRP | 1 (150µl) | Dilute in Conjugate Diluent (see Assay preparation, section 8) |
| Conjugate Diluent | 1 (20ml) | Ready to use |
| Assay Buffer | 1 (5ml) | 20x Concentrate dilute in distilled water (see Assay preparation, section 8) |
| Wash Buffer | 1 (50ml) | 20x Concentrate dilute in distilled water (see Assay preparation, section 8) |
| Substrate Solution | 1 (15ml) | Ready to use |
| Stop Solution | 1 (15ml) | Ready to use |

## 4. Materials required but not provided

- Microtiter plate reader fitted with appropriate filters (450 nm required with optional 620 nm 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. Storage Instructions

Store kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2-8°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.

**Wash buffer 1X:** Once prepared, store at 2-8°C for up to 30 days.

**Assay Buffer 1X:** Once prepared, store at 2-8°C for up to 30 days.

**Reconstituted Standard :** Once prepared use immediately and do not store.

**Diluted Biotin Conjugate:** Once prepared use immediately and do not store.

**Diluted Streptavidin-HRP:** Once prepared use immediately and do not store.

## 6. Specimen collection, processing & storage

Cell culture supernatants, human 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.

**Serum:** Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. Following clotting, centrifuge at approximately 1000 x g for 10 min and remove serum.

**Plasma:** EDTA, citrate and heparin plasma can be assayed. Spin samples at 1000 x g for 30 min to remove particulates. Harvest plasma.

**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.

## 7. 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 Stop solution and Substrate Solution. 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 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 Stop Solution 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 Substrate Solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the Substrate Solution with metal to prevent colour development. Warning Substrate Solution 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 Substrate 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 Substrate Solution within 15 min of the washing of the microtiter plate.

# 8. Assay Preparation

**Bring all reagents to room temperature before use**

## 8.1. 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, zero should be tested **in duplicate**. Remove sufficient microwell strips for testing from the pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8ºC storage.

**Example plate layout** (example shown for a 6 points 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 | 0.31 | 0.31 | | | | | | | | | | |
| G | Blank | Blank | | | | | | | | | | |
| H | | | | | | | | | | | | |

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

## 8.2. 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.

## 8.3. 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.

## 8.4. Preparation of Standard

Standard vials must be reconstituted with the volume of standard diluent shown on the vial immediately prior to use. This reconstitution gives a stock solution of 20 ng/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 10 ng/ml to 0.32 ng/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 10 ng/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 10 to 0.32 ng/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 into the relevant wells.

## 8.5. Preparation of Biotin conjugate anti-VEGF-R1

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.

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 (µl) | Conjugate Diluent (ml) |
|---|---|---|
| 1 - 6 | 30 | 2.97 |
| 1 - 12 | 60 | 5.94 |

## 8.6. Preparation of Streptavidin-HRP

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

Make a 1:300 dilution of the concentrated **Streptavidin-HRP** solution with Conjugate Diluent as needed according to the following table:

| Number of Strips | Streptavidin-HRP (µl) | Conjugate Diluent (ml) |
|---|---|---|
| 1 - 6 | 20 | 5.98 |
| 1 - 12 | 40 | 11.96 |

# 9. Method

**We strongly recommend that every vial is mixed thoroughly without foaming prior to use.**

**Prepare all reagents as shown in section 8.**
**Note: final preparation of Biotin Conjugate (section 8.5) and Streptavidin-HRP (section 8.6) should occur immediately before use.**

| 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:<br>a) Dispense 0.3 ml of **1x washing solution** into each well<br>b) Aspirate the contents of each well<br>c) Repeat step a and b |
| 2. | Addition | Prepare **Standard curve** as shown in section 8.4<br>Add 100µl of **Assay Buffer** in duplicate to the blank wells |
| 3. | Addition | Add 50µl of **Assay Buffer** in duplicate in sample wells<br>Add 50µl of each **Cample** in duplicate to the designated wells |
| 4. | Addition | Add 50µl of diluted **Biotin conjugate** to all wells |
| 5. | Incubation | Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **2 hours,** if available on a microplate shaker |
| 6. | Wash | Remove the cover and wash the plate as follows:<br>a) Aspirate the liquid from each well<br>b) Dispense 0.3 ml of **1x washing solution** into each well<br>c) Aspirate the contents of each well<br>d) Repeat step b and c another two times |
| 7. | Addition | Add 100µl of diluted **Streptavidin-HRP** solution into all wells |
| 8. | Incubation | Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hour,** if available on a microplate shaker |
| 9. | Wash | Repeat wash step 6. |
| 10. | Addition | Add 100µl of **Substrate Solution** into all wells |
| 11. | Incubation | Incubate in the dark for **30 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil |
| 12. | Addition | Add 100µl of **Stop Solution** into all wells |
| **Read the absorbance** value of each well (immediately after step 12.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 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.*

## 10. Data Analysis

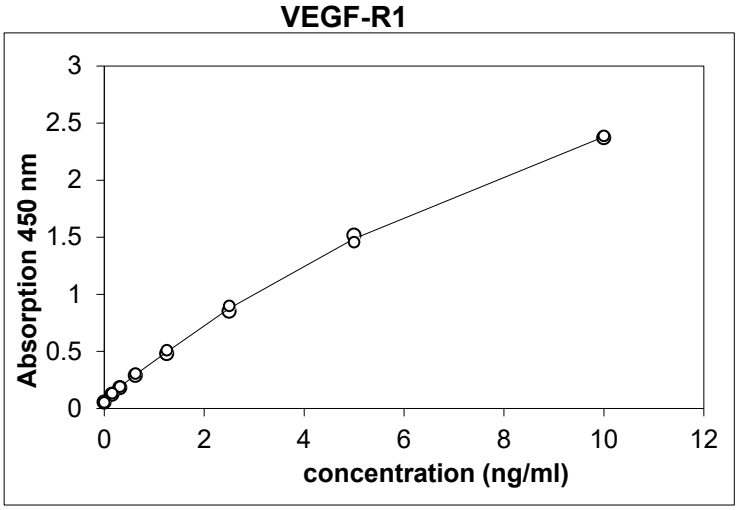
Calculate the average absorbance values for each set of duplicate standards and samples. Ideally duplicates should be within 20% of the mean.

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

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

**Example 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 | 1.6 |




**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.

## 11. 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 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.

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.**

# 12. Performance Characteristics

## 12.1. 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).

## 12.2. Specificity

The assay detects both natural and recombinant human VEGF-R1.
The cross-reactivity and interference of circulating factors of the immune systems was evaluated by spiking these proteins at physiologically relevant concentrations into a human VEGF-R1 positive sample.
Interference was detected for VEGF-A at concentrations > 0.15 ng/ml and for PLGF-1 at concentrations > 2.5 ng/ml.
There was no cross reactivity or interference detected for KDR, PDGFAA, PDGF-BB, VEGF-B, VEGF-C, VEGF-D, HGF, EGF.

## 12.3. Precision

**Intra-assay**
Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 6 serum samples containing different concentrations of human VEGF-R1. **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 two independent experiments. Each assay was carried out with 6 replicates of serum samples containing different concentrations of human VEGF-R1. **The calculated overall coefficient of variation was** 5.1%.

## 12.4. Dilution Parallelism

Serum, plasma and cell culture supernatant samples with different levels of human **VEGF-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 |

## 12.5. 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 human VEGF-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 |
| Plasma (EDTA) | 73 | 72 | 91 |
| Plasma (citrate) | 148 | 115 | 155 |
| Cell culture Supernatant | 47 | 56 | 79 |

## 12.6. 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 significant loss of human VEGF-R1 immunoreactivity detected during storage at -20°C and 2-8°C. A significant loss of human VEGF-R1 immunoreactivity was detected during storage at RT and 37°C.

**Freeze-thaw Stability**
Aliquots of serum samples (spiked or unspiked) were stored frozen at –20°C and thawed 5 times, and human VEGF-R1 levels determined. There was no significant loss of human VEGF-R1 immunoreactivity detected by freezing and thawing.

## 12.7. Expected serum values

Panels of 40 serums as well as EDTA and citrate plasma samples from randomly slected apparently healthy donors were tested for human VEGF-R1.
There is no detectable VEGF-R1 levels found.

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## 14. Assay Summary

**Total procedure length: 3h30min**

**Add standard and samples**
↓
**Add 50µl of Biotin conjugate**
↓
**Incubate 2 hours at room temperature**
↓
**Wash three times**
↓
**Add 100µl of Streptavidin-HRP**
↓
**Incubate 1 hour at room temperature**
↓
**Wash three times**
↓
**Add 100µl of Substrate Solution**
**Protect from light. Let the color develop for 30 min.**
↓
**Add 100µl Stop Solution**
↓
**Read Absorbance at 450 nm**

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