

## Human VEGF-D ELISA Kit

**Catalog No.:** BEK1231

**Size:** 96T

**Range:** 10 pg/ml-300 pg/ml

**Storage and Expiration:** Store at 2-8°C for 6 months.

**Application:** For quantitative detection of VEGF-D in human serum, plasma, urine, cell culture supernatant or tissue samples.

### Introduction

C-fos induced growth factor (FIGF) (or vascular endothelial growth factor D, VEGF-D) is a member of the platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) family. It is active in angiogenesis, lymphangiogenesis, and endothelial cell growth. VEGF-D has been established to be over-expressed in both tumor tissues and patients' serum samples in several types of human cancer. In addition, VEGF-D expression has been implicated with increased incidence of regional lymph node metastasis. In experimental mice study, genetically modified tumor cell that was forced to produce VEGF-D protein have been established to boost up regional lymph nodes metastases.

### Principle of the Assay

This kit was based on standard sandwich enzyme-linked immune-sorbent assay technology. The purified anti-VEGF-D antibody was pre-coated onto 96-well plates. And the HRP conjugated anti-VEGF-D antibody was used as detection antibodies. The standards, test samples and HRP conjugated detection antibody were

added to the wells subsequently, mixed and incubated, then, unbound conjugates were washed away with wash buffer. TMB substrates (A & B) were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the VEGF-D amount of sample captured in plate. Read the O.D. absorbance at 450nm in a microplate reader, and then the concentration of VEGF-D can be calculated.

### Kit components

1. One 96-well plate pre-coated with anti-human VEGF-D antibody
2. Standard: 0.5ml (360pg /mL)
3. Standard diluent buffer: 1.5 ml
4. Wash buffer (30×): 20 ml. Dilution: 1:30
5. Sample diluent buffer: 6 ml
6. HRP conjugated anti-human VEGF-D antibody (RTU): 6ml
7. Stop solution: 6 ml
8. TMB substrate A: 6ml
9. TMB substrate B: 6ml
10. Plate sealer: 2
11. Hermetic bag: 1

### Material Required But Not Provided

1. 37°C incubator
2. Microplate reader (wavelength: 450nm)
3. Precise pipette and disposable pipette tips
4. Automated plate washer
5. ELISA shaker
6. 1.5ml of Eppendorf tubes
7. Absorbent filter papers
8. Plastic or glass container with volume of above 1L

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## Protocol

### ● Preparation of sample and reagents

#### 1. Sample

Isolate the test samples soon after collecting, then, analyze immediately (within 2 hours). Or aliquot and store at -20°C for long term. Avoid multiple freeze-thaw cycles.

- ✧ **Serum:** Coagulate at room temperature for 10-20 min, then, centrifuge at the speed of 2000-3000 r.p.m. for 20 min to collect supernatant. If precipitation appeared, centrifuge again.
- ✧ **Plasma:** Collect plasma using EDTA or citrate plasma as an anticoagulant, and mix for 10-20 min, centrifuge at the speed of 2000-3000 r.p.m. for 20 min of collection. If precipitation appeared, centrifuge again.
- ✧ **Urine:** Collect urine using a sterile container, centrifuge at the speed of 2000-3000 r.p.m. for 20 min to collect supernatant. If precipitation appeared, centrifuge again. For collection of hydrothorax and cerebrospinal fluid, take reference to this operation.
- ✧ **Cell culture supernatant:** For secretory components: use a sterile container to collect. Centrifuge at the speed of 2000-3000 r.p.m. for 20 min to collect supernatant. For intracellular components: Dilute cell suspension with PBS (pH7.2-7.4) to make the cell concentration reached 1 million / ml. Damage cells and release of intracellular components through repeated freeze-thaw cycles. Centrifuge at the speed of 2000-3000 r.p.m. For 20 min to collect supernatant. If precipitation appeared, centrifuge again.
- ✧ **Tissue samples:** Cut samples and weight, add certain volume of PBS (pH7.4), rapidly frozen with liquid nitrogen. After melting, store samples at -2-8 °C. Add certain volume of PBS (pH7.4), homogenize thoroughly, centrifuge at the speed of 2000-3000 r.p.m. for 20 min to collect supernatant.

**Note:** 1. Coagulate blood samples completely, then, centrifuge, and avoid hemolysis and particle.  
2. NaN<sub>3</sub> can not be used as test sample preservative, since it is the inhibitor for HRP.  
3. After collecting samples, analyze immediately or aliquot and store frozen at -20°C. Avoid repeated freeze-thaw cycles.

#### 2. Wash buffer

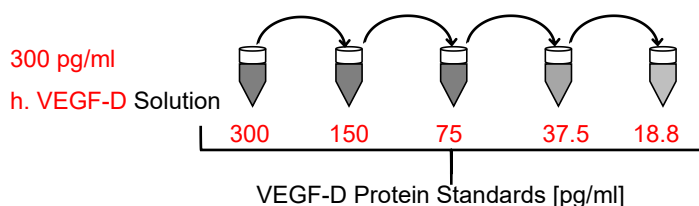
Dilute concentrated Wash buffer (Kit Component 4) 30-fold (1:30) with distilled water (i.e. add 20 ml of concentrated wash buffer into 580 ml of distilled water).

#### 3. Standard

Dilution of the Human VEGF-D standard (Kit Component 2): standard solution should be prepared no more than 2 hours prior to the experiment. (Note: Do not dilute the standard directly in the plate)

- a. 300 pg/ml of standard solution: Add 200 µl of the 360pg/ml standard (Kit Component 2) into 40 µl Standard diluent buffer (Kit Component 3) and mix thoroughly.
- b. 150 pg/ml → 18.8 pg/ml of standard solutions: Label 4 Eppendorf tubes with 150pg/ml, 75pg/ml, 37.5pg/ml, 18.8 pg/ml, respectively. Aliquot 100 µl of the Standard diluent buffer (Kit Component 3) into each tube. Add 100 µl of the above 360 pg/ml standard solution into 1st tube and mix thoroughly. Transfer 100 µl from 1st tube to 2nd tube and mix thoroughly. Transfer 100 µl from 2nd tube to 3rd tube and mix thoroughly, and so on.

100 µl    100 µl    100 µl    100 µl  
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**Note:** The standard solutions are best used within 2 hours. Avoid repeated freeze-thaw cycles.

#### ● Assay procedure

1. Equilibrate kit components for 15-30 min at room temperature.
2. Set standard, test sample and control (zero) wells on the pre-coated plate respectively, and then, record their positions. Add 50 $\mu$ l of diluted standards (300 pg/ml, 150 pg/ml, 70 pg/ml, 37.5 pg/ml, 18.8 pg/ml) into the standard wells. Add 50 $\mu$ l of Standard diluent buffer (Kit Component 3) into the control (zero) well. Do not add sample and HRP conjugated antibody into the control (zero) well.
3. For test sample wells, add 40 $\mu$ l of Sample diluent buffer (Kit component 5) first, then, add 10 $\mu$ l of sample. Add the solution at the bottom of each well without touching the side wall. Shake the plate mildly to mix thoroughly.
4. Cover the plate with Plate sealer (Kit Component 10) and incubate at 37°C for 30 min.
5. Remove the sealer, and wash plate using one of the following methods:  
**Manual Washing:** Discard the solution in the plate without touching the side walls. Clap the plate on absorbent filter papers. Fill each well completely with Wash Buffer (1 $\times$ ) and vortex mildly on ELISA shaker for 2 min, then aspirate contents from the plate, and clap the plate on absorbent filter papers. Repeat this procedure four more times for a **total of FIVE washes**.  
**Automated Washing:** Aspirate all wells, then wash plates **FIVE times** using Wash Buffer (1 $\times$ ). After the final wash, invert plate, and clap the plate on absorbent filter papers until no moisture remained. It is recommended that the washer be set for a soaking time of 10 seconds or shaking.
6. Add 50 $\mu$ l of HRP conjugated anti-VEGF-D antibody (Kit Component 6) into each well (except control well).
7. Cover the plate with Plate sealer (Kit Component 10) and incubate at 37°C for 30 min.
8. Remove the sealer, and wash the plate. (See Step 5)
9. Add 50 $\mu$ l of TMB chromogenic reagent A (Kit Component 8) into each well, and then, add 50 $\mu$ l of TMB chromogenic reagent B (Kit Component 9), vortex gently the plate on ELISA shaker for 30 seconds (Or shake gently by hand for 30 seconds), and incubate in dark at 37°C for 15 min. The shades of blue can be seen in the wells.
10. Add 50 $\mu$ l of Stop solution (Kit Component 7) into each well and mix thoroughly. The color changes into yellow immediately.
11. Read the O.D. absorbance at 450nm in a microplate reader within 15 min after adding the stop solution.

For calculation, (the relative O.D.<sub>450</sub>) = (the O.D.<sub>450</sub> of each well) – (the O.D.<sub>450</sub> of Zero well). The standard curve can be plotted as the relative O.D.<sub>450</sub> of each standard solution (Y) vs. the respective concentration of the standard solution (X). The Human VEGF-D concentration of the samples can be interpolated from the standard curve.

**Note:** If the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

#### Precautions

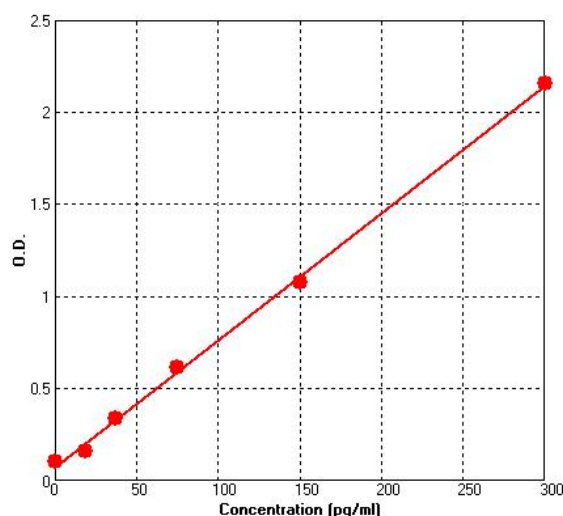
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1. Before the experiment, centrifuge each kit component for several minutes to bring down all reagents to the bottom of tubes.
2. It is recommend to measure each standard and sample in duplicate.
3. Do NOT let the plate completely dry at any time! Since the dry condition can inactivate the biological material on the plate.
4. Do not reuse pipette tips and tubes to avoid cross contamination.
5. Do not use the expired components and the components from different batches.
6. Store the TMB substrate A & B (Kit Component 8 & 9) in dark.
7. Prolong the incubation time if the hypochromasia obtained. Heat the water in the water bath during diluting if the crystalloid appeared in Wash buffer (Kit Component 4).
8. Do not remove microplate from the storage bag until needed, and the unused strips should be stored at 2-8℃ in their pouch or the provided Hermetic bag (Kit Component 11).

#### Typical Data & Standard Curve

Results of a typical standard run of a Human VEGF-D ELISA Kit are shown below. This standard curve was generated at our lab for demonstration purpose only. Each user should obtain their own standard curve as per experiment. (N/A=not applicable)

X	Pg/ml	0	18.8	37.5	75	150	300
Y	OD450	0.105	0.158	0.338	0.617	1.079	2.154



#### Reference

1. Achen, M. G., Jeltsch, M., Kukk, E., Makinen, T., Vitali, A., Wilks, A. F., Alitalo, K., Stacker, S. A. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc. Nat. Acad. Sci. 95: 548-553, 1998.
2. Stacker, S. A., Caesar, C., Baldwin, M. E., Thornton, G. E., Williams, R. A., Prevo, R., Jackson, D. G., Nishikawa, S., Kubo, H., Achen, M. G. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nature Med. 7: 186-191, 2001.

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