

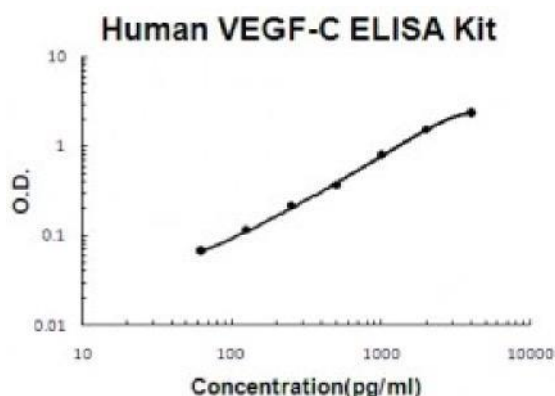
**IK4258 Human VEGF-C ELISA Kit****Size:** 96wells/kit, with removable strips.**Sandwich High Sensitivity ELISA kit for Quantitative Detection of Human VEGF-C. 96wells/kit, with removable strips.****Typical Data Obtained from Human VEGF-C**

(TMB reaction incubate at 37°C for 15-20min)

Concentration(pg/ml)	0	62.5	125	250	500	1000	2000	4000
O.D.	0.016	0.067	0.114	0.213	0.363	0.794	1.524	2.332

Typical Human VEGF-C PELISA Kit Standard Curve

This standard curve was generated at IS for demonstration purpose only. A standard curve must be run with each assay.

**Range:** 62.5pg/ml-4000pg/ml**Sensitivity:** <3pg/ml**Specificity:** Natural and recombinant Human VEGF-C**Cross-reactivity:** There is no detectable cross-reactivity with other relevant proteins.

Storage

Store at 4°C for 6 months, at -20°C for 12 months. Avoid multiple freeze-thaw cycles(Shipped with wet ice.)

Intra/Inter Assay Precision

Intra-Assay Precision (Precision within an assay) Three samples of known concentration were tested on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays) Three samples of known concentration were tested in separate assays to assess inter-assay precision.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	16	16	16	24	24	24
Mean(pg/ml)	198	1915	3096	236	2534	3497
Standard deviation	6.93	80.43	145.5	10.62	134.3	202.8
CV(%)	3.5	4.2	4.7	4.5	5.3	5.8

Assay Principle

IS' Human VEGF-C ELISA Kit was based on standard sandwich enzyme-linked immune-sorbent assay technology. A monoclonal antibody from mouse specific for VEGF-C has been precoated onto 96-well plates. Standards(Expression system for standard: NSO; Immunogen sequence: T103-R227) and test samples are added to the wells, a biotinylated detection polyclonal antibody from goat specific for VEGF -C is added subsequently and then followed by washing with PBS or TBS buffer. Avidin-Biotin-Peroxidase Complex was added and unbound conjugates were washed away with PBS or TBS buffer. HRP substrate TMB was 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 Human VEGF-C amount of sample captured in plate.

Kit Components

Materials included in the kit

Description	Quantity
96-well plate precoated with anti-Human VEGFC antibody	1
lyophilized recombinant Human VEGFC standard	10ng/tubex2
biotinylated anti-Human VEGFC antibody	130ul(dilution 1:100)
Avidin-Biotin-Peroxidase Complex(ABC)	130ul(dilution 1:100)
sample diluent buffer	30ml
antibody diluent buffer	12ml
ABC diluent buffer	12ml
TMB color developing agent	10ml

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TMB stop solution	10ml
Adhesive cover	4

PBS washing buffer	Powder for 1000ml
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Washing buffer Preparation: Dissolve to 1000ml distilled water and adjust pH to 7.2~7.6. Finally, adjust the total volume to 1L.

Materials Required But Not Provided

1. Microplate reader in standard size.
2. Automated plate washer.
3. Adjustable pipettes and pipette tips. Multichannel pipettes are recommended in the condition of large amount of samples in the detection.
4. Clean tubes and Eppendorf tubes.

Notice Before Application

Please read the following instructions before starting the experiment.

1. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, pilot experiment using standards and a small number of samples is recommended.
2. The TMB Color Developing agent is colorless and transparent before using, contact us freely if it is not the case.
3. Before using the Kit, spin tubes and bring down all components to the bottom of tubes.
4. Duplicate well assay is recommended for both standard and sample testing.
5. Don't let 96-well plate dry, for dry plate will inactivate active components on plate.
6. Don't reuse tips and tubes to avoid cross contamination.
7. Avoid using the reagents from different batches together.
8. In order to avoid marginal effect of plate incubation due to temperature difference (reaction may be stronger in the marginal wells), it is suggested that the diluted ABC and TMB solution will be pre-warmed in 37°C for 30 min before using.
9. Take precautionary measures to prevent operator contamination (such as saliva and other body fluids) of kit reagents while running this assay.

Preparation

1. Sample Preparation and Storage

Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

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- **Cell lysates:** After sufficient splitting, there should be no obvious cell sediment. Centrifuge cell lysates at approximately 10000 X g for 5 min. Collect the cell lysate supernates to go ahead.
- **Serum:** Allow the serum to clot in a serum separator tube (about 4 hours) at room temperature. Centrifuge at approximately 1000 X g for 15 min. Analyze the serum immediately or aliquot and store samples at -20°C.
- **Cell culture supernates:** Remove particulates by centrifugation, assay immediately or aliquot and store samples at -20°C.
- **Plasma:** Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 min at 1500 x g within 30 min of collection. Assay immediately or aliquot and store samples at -20°C.

2. Sample Dilution Guideline

the linear regime in the standard curve. Dilute the sample using the provided diluent buffer. The following is a guideline for sample dilution. Several trials may be necessary in practice. **The sample must be well mixed with the diluents buffer.**

- **High target protein concentration (40000pg/ml-400000pg/ml).** The working dilution is 1:100. i.e. Add 1µl sample into 99 µl sample diluent buffer.
- **Medium target protein concentration (4000pg/ml-40000pg/ml).** The working dilution is 1:10. i.e. Add 10µl sample into 90 µl sample diluent buffer.
- **Low target protein concentration (62.5pg/ml-4000pg/ml).** The working dilution is 1:2. i.e. Add 50µl sample to 50 µl sample diluent buffer.
- **Very low target protein concentration (0pg/ml-62.5pg/ml).** No dilution necessary, or the working dilution is 1:2.

3. Reagent Preparation and Storage

- A. Reconstitution of the Human VEGF-C standard: VEGF-C standard solution should be prepared no more than 2 hours prior to the experiment. Two tubes of VEGF-C standard (10ng/tube) are included in each kit. Use one tube for each experiment.
- a. 10000pg/ml of Human VEGF-C standard solution: Add 1ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
 - b. 4000pg/ml of Human VEGF-C standard solution: Add 0.4ml of the above VEGFC standard solution into 0.6 ml sample diluent buffer and mix thoroughly.
 - c. 2000pg/ml→62.5pg/ml of Human VEGF-C standard solutions: Label 6 Eppendorf tubes with 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml respectively. Aliquot 0.3ml of the sample diluent buffer into each tube. Add 0.3ml of the above 4000pg/ml VEGF-C standard solution into 1st tube and mix. Transfer 0.3ml from 1st tube to 2nd tube and mix. Transfer 0.3ml from 2nd tube to 3rd tube and mix, and so on.

Note: The standard solutions are best used within 2 hours. The 10000pg/ml standard solution should be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.

- B. Preparation of biotinylated anti-Human VEGF-C antibody working solution: The solution should be prepared no more than 2 hours prior to the experiment.
- a. The total volume should be: 0.1ml/well x (the number of wells). (Allowing

The user needs to estimate the concentration of the target protein in the sample and select a proper dilution factor so that the diluted target protein concentration falls near the middle of

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- 0.2 ml more than total volume)
- b. Biotinylated anti-Human VEGF-C antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly. (i.e. Add 1 μ l Biotinylated anti-Human VEGF-C antibody to 99 μ l antibody diluent buffer.)
- C. Preparation of Avidin-Biotin-Peroxidase Complex (ABC) working solution: The solution should be prepared no more than 1 hour prior to the experiment.
- a. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
 - b. Avidin- Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly. (i.e. Add 1 μ l ABC to 99 μ l ABC diluent buffer.)

Assay Procedure

The ABC working solution TMB color developing agent and TMB stop solution must be kept warm at 37°C for 30 min before use. When diluting samples and reagents, they must be mixed completely and evenly. Standard VEGF-C detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of VEGF-C amount in samples.

1. Aliquot 0.1ml per well of the 4000pg/ml, 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml Human VEGF-C standard solutions into the precoated 96-well plate. Add 0.1ml of the sample diluent buffer into the control well (Zero well). Add 0.1ml of each properly diluted sample of Human cell culture supernates, cell lysates, serum or plasma (heparin, EDTA) to each empty well. **See "Sample Dilution Guideline" above for details.** It is recommended that each Human VEGF-C standard solution and each sample be measured in duplicate.
2. Seal the plate with a new adhesive cover provided and incubate at 37°C for 90 min.
3. Remove the cover, discard plate content, and blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.
4. Add 0.1ml of biotinylated anti-Human VEGF-C antibody working solution into each well, seal the plate with a new adhesive cover provided and incubate at 37°C for 60 min.
5. Wash plate 3 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. (**Plate Washing Method:** Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1~2 minutes. Repeat this process two additional times for a total of THREE washes. Note: For automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling wells with PBS or TBS buffer. Blot the plate onto paper towels or other absorbent material.)
6. Add 0.1ml of prepared ABC working solution into each well, seal the plate with a new adhesive cover provided and incubate at 37°C for 30 min.
7. Wash plate 5 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. (See Step 5 for plate washing method.)
8. Add 90 μ l of prepared TMB color developing agent into each well, seal the plate with a new adhesive cover and incubate at 37°C in dark for 15-20min (**Note:** For reference only, the optimal incubation time should be determined by end user. And the shades of blue can be seen in the wells with the four most concentrated Human VEGF-C standard solutions; the other wells show no obvious color).
9. Add 0.1ml of prepared TMB stop solution into each well. The color changes into yellow immediately.
10. Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution.

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

Summary

1. Add samples and standards and incubate the plate at 37°C for 90 min. Do not wash.
2. Add biotinylated antibodies and incubate the plate at 37°C for 60 min. Wash plate 3 times with 0.01M TBS.
3. Add ABC working solution and incubate the plate at 37°C for 30 min. Wash plate 5 times with 0.01M TBS.
4. Add TMB color developing agent and incubate the plate at 37°C in dark for 15-20min.
5. Add TMB stop solution and read.

Background

Vascular endothelial growth factor C is a VEGF. The human gene encoding it is VEGFC. The protein encoded by this gene is a member of the platelet-derived growth factor/vascular endothelial growth factor(PDGF/VEGF) family, is active in angiogenesis, lymphangiogenesis and endothelial cell growth and survival, and can also affect the permeability of blood vessels. This secreted protein undergoes a complex proteolytic maturation, generating multiple processed forms which bind and activate VEGFR-3 receptors. Only the fully processed form can bind and activate VEGFR-2 receptors. This protein is structurally and functionally similar to vascular endothelial growth factor D(VEGF-D). The C terminus of VEGFC has cysteine-rich repeat units characteristic of the Balbiani ring 3 protein(BR3P) of the midge *Chironomus tentans*.^{1,2} The standard product used in this kit is recombinant human VEGF-C, consisting of 135 amino acids with the molecular mass of 23Kda after glycosylation.