VEGF (Human) ELISA Kit

Catalog Number KA0650

96 assays

Version: 03

Intended for research use only

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Introduction

Intended Use

VEGF (Human) ELISA Kit is an enzyme-linked immunosorbent assay for the quantitative detection of human VEGF levels in cell culture supernatants, human serum, plasma (EDTA, heparin and citrate), CSF and urine.

Background

VEGF, also known as vascular permeability factor (VPF), is characterized by its highly specific mitogenic activity for endothelial cells and by its angiogenic effect observed in vitro and in vivo (1). The VEGF family consists of four isoforms; VEGF-A (also called VEGF), VEGF-B, VEGF-C, and VEGF-D (2, 3). VEGF-A is considered the most important of these with respect to tumor angiogenesis. Especially the splice variant isoforms VEGF121 and VEGF165 were found to be secreted by a wide spectrum of cell types, including smooth muscle cells (4), fibroblasts and epithelial cells (5), keratinocytes (6), macrophages (7), cardiac myocytes (8) and various tumor cells (1). Both environmental and epigenetic factors can cause up-regulation of VEGF in tumor cells (5, 6). It has been shown that inhibition of VEGF with a specific monoclonal antibody can suppress tumor growth in vivo.

Principle of the Assay

This VEGF (Human) ELISA Ki is an in vitro enzyme-linked immunosorbent assay (ELISA) for quantitative measurement of human VEGF in cell culture supernatants, serum, plasma, CSF and urine. This assay employs an antibody specific for human VEGF coated onto a 96-well plate. Standards, samples and biotinylated anti-human VEGF are pipetted into the wells and VEGF present in a sample is captured by the antibody immobilized to the wells and by the biotinylated VEGF-specific detection antibody. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted into the wells. The wells are again washed. Following this second wash step, TMB substrate solution is added to the wells, resulting in blue color development proportional to the amount of VEGF bound. The stop solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

General Information

Materials Supplied

List of component

Test components	Amount/Volume
96 Well Plate with 12 Strips Break-apart microtiter test strips each with 8 VEGF antibody coated single wells. Ready for use.	1 frame
$VEGF_{165}$ Standard 4200 pg/ml Lyophilized & stabilized recombinant human $VEGF_{165}$ Reconstitute with the sample diluents volume shown on the label.	2 vials
Biotinylated VEGF antibody. Stabilized. Ready for use.	10 ml
HRP-Conjugated Avidin. Stabilized. Ready for use.	12 ml
20x Wash buffer concentrate (sufficient for 1000ml) Dilute 1:20	50 ml
Sample Diluent Ready for use.	100 ml
Stop solution 0.9 N H_2SO_4 Ready for use.	8 ml
TMB-Substrate Ready for use.	8 ml

Storage Instruction

Reagent	Storage	Stability
Antibody coated 96 well plate with 12 strips. Break-apart microtiter test strips each with 8 antibody coated single wells.	Store at 2-8℃ in closed aluminum bag with desiccant. Strips which are not used must be stored in the re-sealable aluminum bag in humidity free and airtight conditions.	3 months after opening.
$VEGF_{165}$ Standard Lyophilized	Store at 2-8℃.	Until date of kit expiry in lyophilized format. Unstable. Use immediately after dissolving. Keep on ice if not used within 1 hr after dissolving.
Biotinylated antibody Ready for use.	Store at 2-8℃. *Avoid contamination.* (Use clean sterile tips)	3 months after opening.
HRP-Conjugated Avidin Ready for use.	Store at 2-8℃. *Avoid contamination.* *(Use clean sterile tips)*	3 months after opening.
Sample Diluent	Store at 2-8℃. *Avoid contamination.* *(Use clean sterile tips or pipettes)*	3 months after opening.
20x Concentrated Wash Buffer	Store at 2-8℃. To avoid crystal formation, wash buffer concentrate, may also be stored at Room Temperature.	Until expiry date.
Diluted Wash Buffer	1x working dilution. *Bottles used for the working dilution should be cleaned regularly, discard cloudy solutions.*	1 week at room temperature or one month at 2-8 ℃.
TMB-Substrate Solution	Store at 2-8℃, protected from light! *Avoid contamination.* *(Use clean sterile tips)*	Until expiry date.
Stop Solution	Store at 2-8℃. May also be stored at Room Temperature.	Until expiry date.

Materials Required but Not Supplied

- ✓ Microplate reader capable of measuring absorbance at 450 nm.
- ✓ Precision pipettes (2 µl to 1 ml volumes).
- ✓ Multi-channel pipette (25 µl to 350 µl).
- ✓ Adjustable 1-25 ml pipettes for reagent preparation.
- ✓ 100 ml and 1 liter graduated cylinders.
- ✓ Absorbent paper.
- ✓ Distilled or de-ionized water.
- ✓ Log-log graph paper or computer and software for ELISA data analysis.
- ✓ Tubes to prepare standard or sample dilutions.
- ✓ Timer.

Precautions for Use

- ✓ When not in use, kit components should be kept refrigerated. All reagents should be warmed to room temperature before use.
- ✓ Microtiter plates should be allowed to come to room temperature before opening the foil bags.
- ✓ Once the desired number of strips has been removed, immediately return unused strips to the bag, reseal the bag and store at 2-8℃ to maintain plate integrity. Protect from humidity.
- ✓ Samples should be collected in pyrogen/endotoxin-free tubes.
- ✓ Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
- ✓ When possible, avoid the use of badly hemolyzed or lipemic sera. If large amounts of particulate matter are present, centrifuge or filter prior to analysis.
- ✓ It is recommended that all standards, controls and samples are run in duplicate.
- ✓ Samples that are >700pg/mL should be diluted with Sample Diluent.
- ✓ When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
- ✓ Cover or cap all reagents when not in use.
- ✓ Do not use reagents past their expiration date.
- ✓ Read absorbencies within 20 minutes of assay completion.
- ✓ In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
- ✓ All residual wash buffer 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.
- ✓ Because TMB substrate solution is light sensitive, avoid prolonged exposure to light. Also avoid contact between TMB substrate solution and metal, or color may develop.

Assay Protocol

Reagent Preparation

Note: All reagents and samples must be allowed to equilibrate to room temperature (18-25℃) before use.

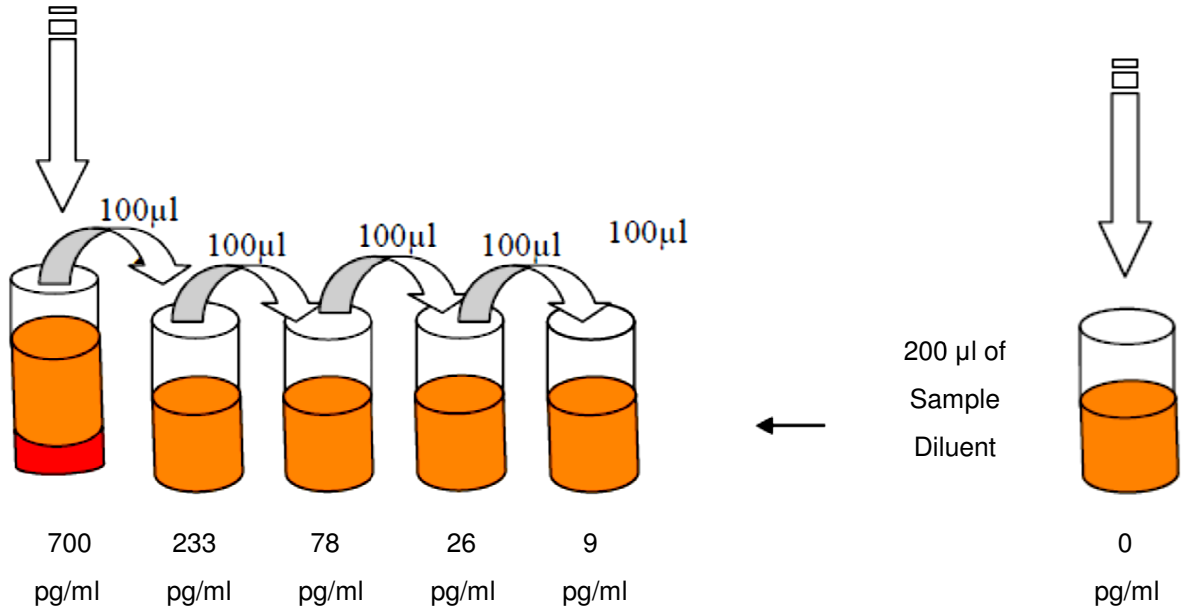
- Antibody coated plate: Before opening the foil pouch, determine the number of strips required to test the desired number of samples, plus 16 wells needed for running standards and blanks in duplicate. Remove non-used strips from the plate-frame and return them to the foil pouch containing the desiccant for up to 3 months at 2-8℃.

- Dilution of test standard: Dissolve the lyophilised VEGF standard with "Sample Diluent" volume shown on the label. VEGF standard is unstable after dissolving. Use immediately or keep on ice if not used within 1 hr after dissolving.
  - ✓ Take 100 µl of $VEGF_{165}$ from kit standard tube containing 4200 pg/ml of VEGF and pipette into Standard tube 1. Add 500 µl of sample diluent to obtain VEGF concentration of 700 pg/ml in the first dilution tube (total volume 600 µl).
  - ✓ Add 200 µl of Sample Diluent to the other 4 dilution tubes. Take 100 µl from the first tube (700 pg/ml) and start 3-fold serial dilutions in dilution tubes as described in the figure by mixing several times with the pipette in each tube (Total of 5 dilution tubes).
  - ✓ 200 µl of sample Diluent serves as zero standard (0 ng/ml) in tube 6.

100 µl of Standard (4200 pg/ml)

+

500 µl of Sample Diluent



- Wash Buffer: If the 20x concentrated Wash Buffer contains visible crystals, warm it at 37℃ and mix gently until dissolved. Dilute 1:20 with de-ionized or distilled water (e.g. 25 ml of Wash Buffer Concentrate and 475 ml distilled water to yield 500 ml of 1x Wash Buffer). Check the pH of the diluted wash buffer and adjust to 7.4 if necessary.

- Vortex mix green colored Biotinylated antibody solution gently before use.

- Vortex mix blue colored peroxidase (HRP) labeled avidin gently before use.

Caution: TMB substrate (Tetramethylbenzidine) and Stop solution (H2SO4) are toxic or corrosive and should be handled with care. Use gloves during handling.

- Amounts of the reagents needed to perform the test

	Reagents				
No of strips used (8 wells each)	Biotinylated antibody 50 ul/well	Avidin-HRP 50 ul/well	TMB Substrate 50 ul/well	Stop Solution 25 ul/well	Wash Buffer 300 ul/well
1 (8 wells)	500 ul	500 ul	500 ul	300 ul	30 ml
2 (16 wells)	1 ml	1 ml	1 ml	600 ul	55 ml
4 (32 wells)	2 ml	2 ml	2 ml	1.2 ml	110 ml
6 (48 wells)	3 ml	3 ml	3 ml	1.8 ml	165 ml
8 (64 wells)	4 ml	4 ml	4 ml	2.4 ml	220 ml
12 (96 wells)	6 ml	6 ml	6 ml	4 ml	350 ml

Sample Preparation

- Sample preparation and dilution: Dilution of samples is not required for initial screening. Samples that exceed the measuring range should be diluted serially in sample diluent and measured again. Samples with absorbance values >1.900 can be serially diluted 1:2, 1:4, 1:8, or further if necessary. The dilution factor must be taken in account when calculating the results.
  Dilute and store all samples in tubes or plates made of material with low binding surface, such as polypropylene.

- Sample collection and storage: Serum, EDTA, heparin or citrate anti-coagulated plasmas, cerebrospinal fluid, cell culture supernatants and urine are suitable sample types for use in the assay (caution: separate plasma/serum and blood cells within 4 hours after collection, non-separated samples must be kept in temperatures from 2-8℃). Do not use grossly haemolyzed or lipemic specimens. If samples are to be run within 24 hours, they may be stored at 2-8℃; otherwise samples should be stored frozen (at least between -18 to -32℃, but preferably < -70℃). Up to 3 freeze-thaw cycles have no effect on the VEGF levels of samples. Nonetheless, excessive freeze-thaw cycles should be avoided. Prior to the assay,

frozen samples should be thawed as quickly as possible in tap water (18-25℃). Do not use 37℃ or 56℃ water bath for this purpose.

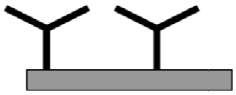
Assay Procedure

1. Bring all reagents and samples to room temperature (18-25℃) before use. It is recommended that all standards and samples are run at least in duplicate. Leave some wells as a reagent blank (2 to 4 wells).
   FIRST STEP: STANDARD, SAMPLES AND BLANK+ BIOTINYLATED ANTIBODY
2. Pipette 50 µl of sample and 50 µl of each diluted standard starting from 1200 pg/mL into appropriate wells. Pipette 50 µl of sample diluent to the wells which will be used as a blank. Incubate 1 hr at room temperature without shaking.
   SECOND STEP: BIOTINYLATED ANTIBODY
3. Wash 5 times with 1x Wash Solution (300 µl each).
   To wash manually: Empty plate contents. Use a multi-channel pipette to fill each well with 300 µl of diluted wash buffer, then empty plate contents again. Repeat procedure 4 additional times for a total of FIVE washes. Gently blot plate onto paper towels or other absorbent material. Never let reaction wells dry. Continue to the next step without delay or interruption.
   For automated washing: Aspirate all wells and wash 5 times with 300 µl diluted wash buffer. Blot plate onto paper towels or other absorbent material. Never let reaction wells dry. Continue to the next step without delay or interruption.
4. Promptly add 50 µl of green colored Biotinylated VEGF detection antibody to all wells. Tap the plate gently by hand to homogenize your mixture. Avoid touching the reaction wells with the pipette tip. Incubate at room temperature for 1 hr without shaking.
   THIRD STEP: HRP-CONJUGATED AVIDIN
5. Wash 5 times as described in Step 3.
   Add 50 µl of prepared HRP-conjugated Avidin solution (ready to use) to each well. Incubate for 30 minutes at room temperature.
   FOURTH STEP: TMB SUBSTRATE
6. Wash 5 times as described in Step 3.
7. Using a multichannel pipet, promptly add 50 µl of TMB ready to use Substrate Solution to each well. Incubate for 20 minutes at room temperature in the dark.
8. Add 25 µl of Stop Solution to each well. Read at 450 nm within 15 minutes.
   Correcting for optical imperfections in the microplates by subtracting $A_{630\ nm}$ is recommended, but not an essential procedure.
   FIFTH STEP: READING AND CALCULATION
9. Calculate the mean of reagent blank absorbance values and subtract it from all test well values (standard and test samples). Mean reagent blank absorbance value at 450 nm should be less than 0.200.
10. Calculate your results against standard curve, as outlined below.

- **Summary**

1. Prepare all reagents, samples and standards.
   Dilution of samples is not required at initial screening.
2. Add 50 µl standard (starting from 700 pg/ml), test samples and sample diluent as a blank into the appropriate wells of the strips.
   Incubate 1 hour at room temperature. Wash 5x.
3. Add 50 µl ready for use biotin antibody promptly to each well.
   Incubate 1 hour at room temperature. Wash 5x.
4. Add 50 µl ready for use HRP-Streptavidin solution.
   Incubate 30 minutes at room temperature. Wash 5x.
5. Add 50 µl TMB Substrate Reagent to each well.
   Incubate 20 minutes at room temperature.
6. Add 25 µl Stop Solution to each well.
   Read at 450 nm against *630 nm immediately.
   Subtract blank values from values for standards and test samples.

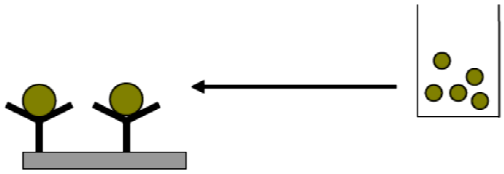
*Correcting for optical imperfections in the microplates by subtracting A630 nm is recommended, but not an essential procedure.



VEGF Antibody coated test well

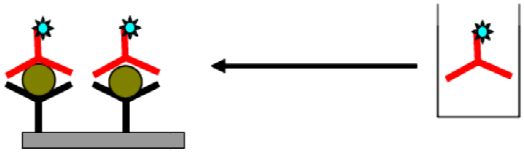
STEP 1

Add 50 µL of standards and test samples to test well and incubate 1 hr at Room Temperature



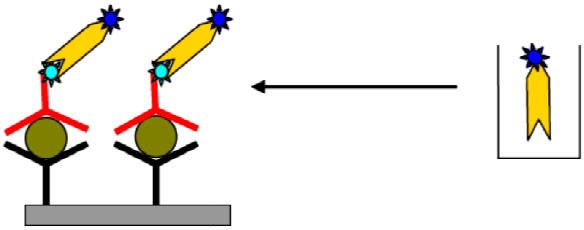
STEP 2

Add 50 µL of Biotinylated VEGF antibody to test well and incubate 1 hr at Room Temperature



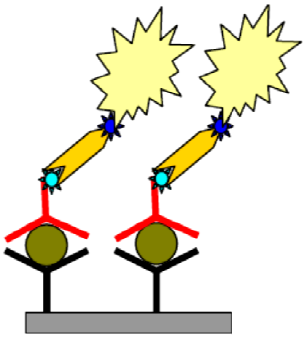
STEP 3

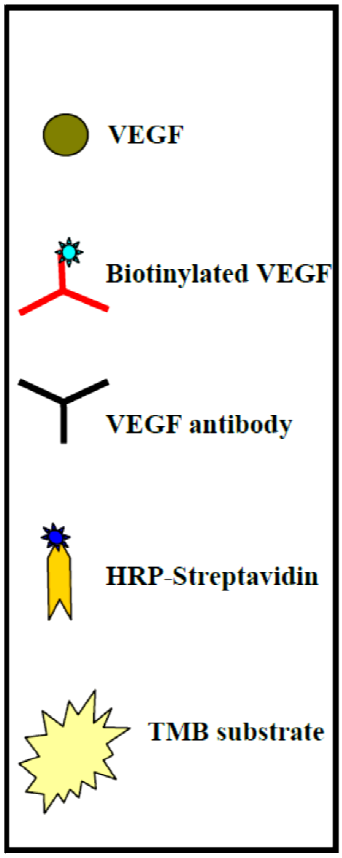
Add 50 µL of HRP-Streptavidin to test well and incubate 30 minutes at Room Temperature



STEP 4

Add 50 µL of TMB substrate to test well incubate 20 minutes at Room Temperature





Data Analysis

Calculation of Results

The standard curve must be determined individually for each experiment. Correct the absorbance values of all standards by subtracting from them the mean O.D. value of the reagent blank (Bl = only sample diluent). Calculate the mean absorbance value for each standard from the duplicates.

The standard curve is used to determine the amount of VEGF in an unknown sample. The standard curve is generated by plotting the average O.D. (450 nm) obtained for each of the standard concentrations on the vertical (Y) axis versus the corresponding VEGF concentration (pg/mL) on the horizontal (X) axis.

Construct the standard curve using graph paper or statistical software.

If samples generate values higher than the highest standard, dilute the samples with sample diluent and repeat the assay. Note that the concentration read from the standard curve must be multiplied by the dilution factor.

- **Typical Data**

The following standard curve is obtained for the various VEGF standards over the range of 0 to 1200 pg/ml. The graph also shows the calibration data of WHO reference reagent compared to Orgenium standard.

Please note: This graph is an example only. A standard curve should be generated each time the assay is performed. Do not use this table in your calculations.

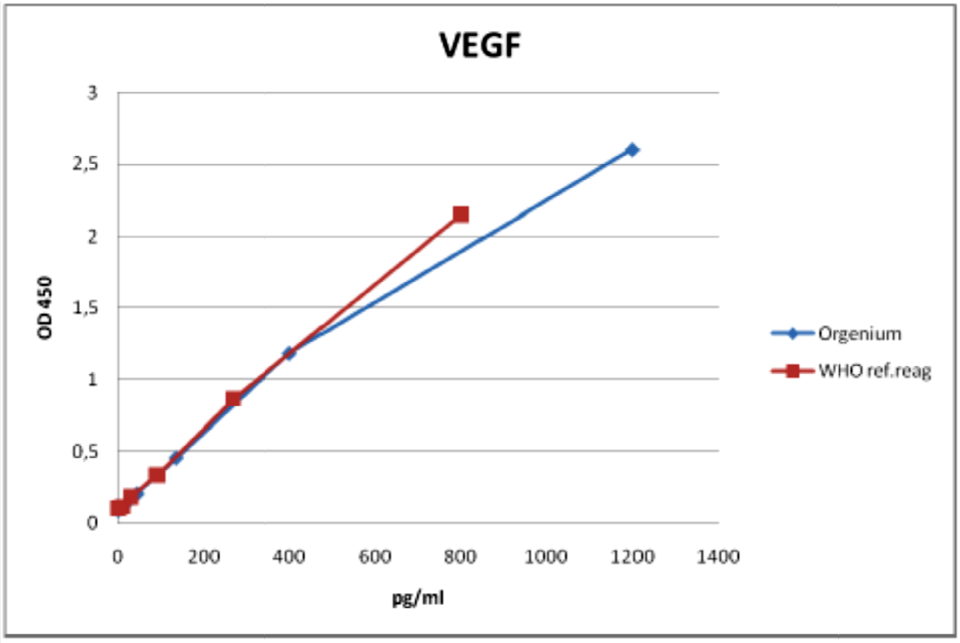


Figure 1: Typical Standard Curve for VEGF (Human) ELISA Kit.

Performance Characteristics

	VEGF
Assay range	9-700 pg/ml
Standard curve points	700, 233, 78, 26, 9 and 0 pg/ml
Intra-Assay-Precision	≦6%
Inter-Assay-Precision	≦10%
Inter-Lot-Precision	≦12%
Cross-Reactivity	No cross-reactivity was observed with the following recombinant human proteins: IL-1, IL-1ß, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, TNF α, TARC.
Specificity	Recognizes both natural and recombinant human $VEGF_{165}$. Isoform VEGF121 cross-reacts 100% in the assay.
Sensitivity	<5 pg/ml.

Resources

Troubleshooting

Problem	Cause	Solution
Poor standard Curve	1. Inaccurate pipetting or pipetting error 2. Improper standard dilution	Check pipettes and calibrate regularly. Vortex the stock before use and dilute carefully in an eppendorf tube.
Low signal	1.Shorter incubation than recommended 2. Inadequate reagent volumes or improper dilution or pipetting error	Ensure sufficient incubation time; Check pipettes and ensure correct performance.
Large CV	Inaccurate pipetting and drying of wells during test procedure.	Check pipettes Fill the wells promptly with wash buffer and reagents.
High background	1. Plate is insufficiently washed 2. Contaminated wash Buffer 3. Wash buffer volume is less than advised	Use multichannel pipet for washings. If using a plate washer, check that all ports are unobstructed and clean. Make a fresh wash buffer Use 300µl per well
Low sensitivity	1.Improper storage of the ELISA kit 2. Stop solution 3. Contamination of reagents	Store test kit components as advised in this user manual. Keep substrate solution protected from light. Stop solution should be added to each well before measure. Use clean sterile tips. Discard contaminated reagents.

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Plate Layout

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