

Rat VEGF ELISA Kit

Catalog Number: EK0523



For the quantitative determination of rat vascular endothelial growth factor (VEGF) concentrations in cell culture supernates, serum, and plasma.

This instruction must be read in its entirety before using this product.

For research use only. Not for use in diagnostic procedures.

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PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VEGF present is bound by the immobilized antibody. Following incubation unbound samples are removed during a wash step, and then a detection antibody specific for VEGF is added to the wells and binds to the combination of capture antibody- VEGF in sample. Following a wash to remove any unbound combination, and enzyme conjugate is added to the wells. Following incubation and wash steps a substrate is added. A coloured product is formed in proportion to the amount of VEGF present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from seven VEGF standard dilutions and VEGF sample concentration determined.

DETECTION RANGE

15.6 pg/ml - 1000 pg/ml

SENSITIVITY

The minimum detectable dose was 7pg/mL.

SPECIFICITY

This assay recognizes both natural and recombinant rat VEGF. The factors listed below were prepared at 50ng/ml in Standard /sample Diluent and assayed for cross-reactivity and no significant cross-reactivity or interference was observed.

Table 1: Factors assayed for cross-reactivity

Recombinant human	Recombinant mouse	Recombinant Rat
VEGF ₁₆₅	VEGF ₁₁₅	GM-CSF
VEGF-B ₁₆₇	VEGF-B ₁₆₇	IFN- γ
VEGF-C	VEGF-B ₁₈₆	TNF- α
VEGF-D	VEGF-D	IL-2
VEGF R3	VEGF R3	IL-4
		IL-6
		IL-10

PRECISION

The coefficient of variation of both intra-assay and inter-assay were less than 10%.

MATERIALS PROVIDED

1. Aluminium pouches with a Microwell Plate coated with antibody to rat VEGF (8x12)
2. 2 vials rat VEGF Standard lyophilized, 1000 pg/vial upon reconstitution
3. 2 vials concentrated Biotin-Conjugate anti-rat VEGF antibody
4. 2 vials Streptavidin-HRP solution
5. 1 bottle Standard /sample Diluent
6. 1 bottle Biotin-Conjugate antibody Diluent
7. 1 bottle Streptavidin-HRP Diluent
8. 1 bottle Wash Buffer Concentrate 20x (PBS with 1% Tween-20)
9. 1 vial Substrate Solution
10. 1 vial Stop Solution
11. 4 pieces Adhesive Films
12. package insert

STORAGE

Table 2: Storage of the kit

Unopened Kit	Store at 2 – 8°C. Do not use past kit expiration date.	
Opened/ Reconstituted Reagents	Standard /sample Diluent	May be stored for up to 1 month at 2 – 8°C.**
	Concentrated Biotin-Conjugate	
	Streptavidin-HRP solution	
	Biotin-Conjugate antibody Diluent	
	Streptavidin-HRP Diluent	
	Wash Buffer Concentrate 20x	
	Substrate Solution	
	Stop Solution	
	Standard	Aliquot and store for up to 1 month at -20°C. Avoid repeated freeze-thaw cycles. Diluted standard shall not be reused.
Microplate Wells	Return unused wells to the foil pouch containing pack, desiccant reseal entire edge of zip-seal. May be stored for up to 1 month at 2 –8°C.**	

****Provided this is within the expiration date of the kit.**

THE REQUIRED ITEMS (not provided, but can help to buy):

1. Microplate reader (450nm).
2. Micro-pipette and tips: 0.5-10, 2-20, 20-200, 200-1000 μ L.
3. 37 °C incubator, double-distilled water or deionized water, coordinate paper, graduated cylinder.

PRECAUTIONS FOR USE

1. Store kit regents between 2°C and 8°C. After use all reagents should be immediately returned to cold storage(2°C to 8°C).
2. Please perform simple centrifugation to collect the liquid before use.
3. To avoid cross contamination, please use disposable pipette tips.
4. The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material. Avoid contact of skin or mucous membranes with kit reagents or specimens. In the case of contact with skin or eyes wash immediately with water.

5. Use clean, dedicated reagent trays for dispensing the washing liquid, conjugate and substrate reagent. Mix all reagents and samples well before use.
6. After washing microtiter plate should be fully pat dried. Do not use absorbent paper directly into the enzyme reaction wells.
7. Do not mix or substitute reagents with those from other lots or other sources. Do not use kit reagents beyond expiration date on label.
8. Each sample, standard, blank and optional control samples should be assayed in duplicate or triplicate.
9. Adequate mixing is very important for good result. Use a mini-vortexer at the lowest frequency or Shake by hand at 10min interval when there is no vortexer.
10. Avoid microtiter plates drying during the operation.
11. Dilute samples at the appropriate multiple, and make the sample values fall within the standard curve. If samples generate values higher than the highest standard, dilute the samples and repeat the assay.
12. Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time and temperature, and kit age can cause variation in binding.

13. This method can effectively eliminate the interference of the soluble receptors, binding proteins and other factors in biological samples.

SAMPLE COLLECTION AND STORAGE

1. **Cell Culture Supernates** - Remove particulates by centrifugation.
2. **Serum** - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 x g. Remove serum, avoid hemolysis and high blood lipid samples.
3. **Plasma** - Recommended EDTA as an anticoagulant in plasma. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection.
4. Assay immediately or aliquot and store samples at -20°C. Avoid repeated freeze-thaw cycles.
5. Dilute samples at the appropriate multiple (recommended to do pre-test to determine the dilution factor).

Note: The normal rat serum or plasma samples are suggested to make a 1:2 dilution.

REAGENT PREPARATION

1. Bring all reagents to room temperature before use.
2. **Wash Buffer** - Dilute 10mL of Wash Buffer Concentrate into deionized or distilled water to prepare 200mL of Wash Buffer. If crystals have formed in the concentrate Wash Buffer, warm to room temperature and mix gently until the crystals have completely dissolved.
3. **Standard** - Reconstitute the Standard with 1.0mL of Standard /sample Diluent. This reconstitution produces a stock solution of 1000 pg /mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Pipette 500 μ L of Standard/sample Diluent into the 500 pg/mL tube and the remaining tubes. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 1000 pg/mL standard serves as the high standard. The Standard/ sample Diluent serves as the zero standard (0 pg/mL).

If you do not run out of re-melting standard, store it at -20°C. Diluted standard shall not be reused.

4. Working solution of Biotin-Conjugate anti-rat VEGF antibody: Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with the Biotin-Conjugate antibody Diluent in a clean plastic tube.

The working solution should be used within one day after dilution.

5. Working solution of Streptavidin-HRP: Make a 1:100 dilution of the concentrated Streptavidin-HRP solution with the Streptavidin-HRP Diluent in a clean plastic tube.

The working solution should be used within one day after dilution.

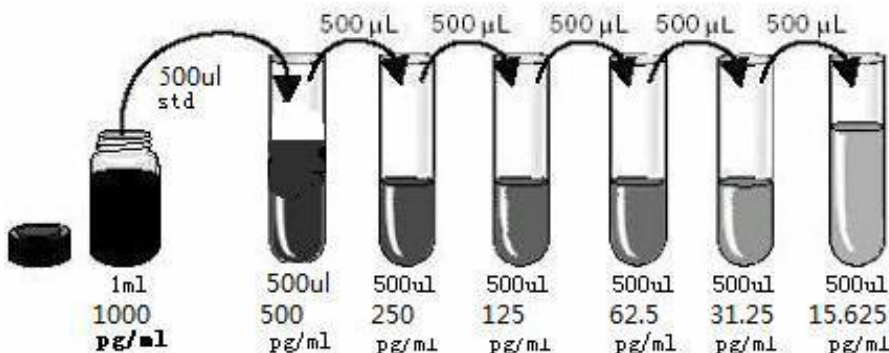


Figure 1: Preparation of VEGF standard dilutions

GENERAL ELISA PROTOCOL

1. Prepare all reagents and working standards as directed in the previous sections.
2. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2-8°C sealed tightly.
4. Add 100µL of Standard, control, or sample, per well. Cover with the adhesive strip provided. Incubate for 1.5 hours at 37°C. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (350µL) using a squirt bottle, manifold dispenser or auto-washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

5. Add 100 μ L of the working solution of Biotin-Conjugate to each well. Cover with a new adhesive strip and incubate 1 hours at 37 $^{\circ}$ C.
6. Repeat the aspiration/wash as in step 4.
7. Add 100 μ L of the working solution of Streptavidin-HRP to each well. Cover with a new adhesive strip and incubate for 30 minutes at 37 $^{\circ}$ C. Avoid placing the plate in direct light.
8. Repeat the aspiration/wash as in step 4.
9. Add 100 μ L of Substrate Solution to each well. Incubate for 10-20 minutes at 37 $^{\circ}$ C. Avoid placing the plate in direct light.
10. Add 100 μ L of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well immediately, using a microplate reader set to 450 nm.(optionally 650nm as the reference wave length;610-650nm is acceptable)

ASSAY PROCEDURE SUMMARY

Prepare all reagents and standards as directed.↵



Add 100µL standard or samples to each well, incubate 90 minutes, 37°C.↵

↵ Aspirate and wash 4 times.↵

Add 100µL working solution of Biotin-Conjugate anti-rat VEGF antibody to each well, incubate 60 minutes, 37°C.↵

↵ Aspirate and wash 4 times.↵

Add 100µL working solution of Streptavidin-HRP to each well, incubate 30 minutes, 37°C.↵

↵ Aspirate and wash 4 times.↵

Add 100µL Substrate solution to each well, incubate 10-20 minutes, 37°C. Protect from light.↵



Add 100µL Stop solution to each well. Read at 450nm within 30 minutes.↵

Figure 2: Assay procedure summary

TECHNICAL HINTS

1. When mixing or reconstituting protein solutions, always avoid foaming.
2. To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
4. Substrate Solution should remain colorless until added to the plate. Stop Solution should be added to the plate in the same order as the Substrate Solution. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
5. A standard curve should be generated for each set of samples assayed. According to the content of tested factors in the sample, appropriate diluted or concentrated samples, it is best to do pre-experiment.

CALCULATION OF RESULTS

1. Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.
2. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph.
3. The data may be linearized by plotting the log of the VEGF concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
4. This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Table 3: Typical data using the VEGF ELISA (Measuring wavelength: 450nm, Reference wavelength: 630nm)

Standardized(pg/ml)	OD.	OD.	Average	Corrected
0	0.047	0.041	0.044	—
15.625	0.124	0.129	0.127	0.128
31.25	0.202	0.209	0.206	0.209
62.5	0.357	0.349	0.353	0.361
125	0.632	0.651	0.642	0.632
250	1.087	1.091	1.089	1.087
500	1.766	1.757	1.762	1.767
1000	2.680	2.587	2.634	2.632

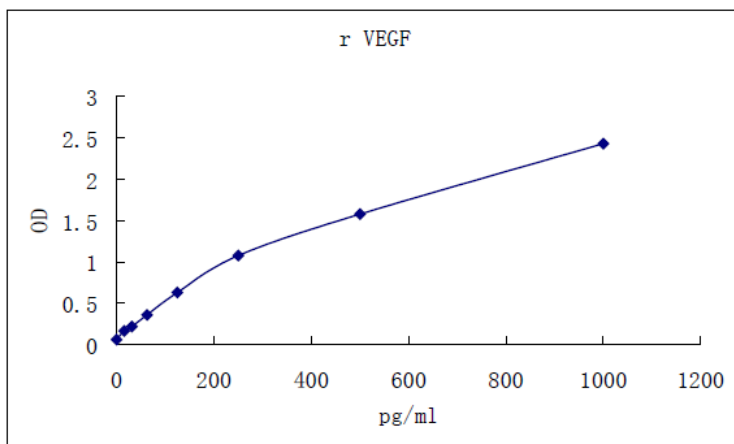


Figure 3: Representative standard curve for VEGF ELISA. VEGF was diluted in serial two-fold steps in Sample Diluent.

Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

BACKGROUND

Vascular endothelial growth factor (VEGF or VEGF-A), also known as vascular permeability factor (VPF), is a potent mediator of both angiogenesis and vasculogenesis in the fetus and in adults (1-3). It is a member of the PDGF family that is characterized by the presence of eight conserved cysteine residues in a cystine knot structure and formation of anti-parallel disulfide-linked dimers (4). Alternately spliced isoforms of 120, 164, and 188 amino acids (aa) have been found in rats and mice, while 121, 145, 165, 183, 189, and 206 aa isoforms have been identified in humans (2, 4). In humans, VEGF165 appears to be the most abundant and potent isoform, followed by VEGF121 and VEGF189 (3, 4). The same pattern may exist in rats and mice. Isoforms other than VEGF120 and VEGF121 contain basic heparin-binding regions and are not freely diffusible (4). Rat VEGF164 shares 97% aa sequence identity with corresponding regions of mouse, 88% with human and bovine, 89% with porcine and canine, and 90% with feline and equine

VEGF. VEGF is expressed in multiple cells and tissues including skeletal and cardiac muscle (5, 6), hepatocytes (7), osteoblasts (8), neutrophils (9), macrophages (10), keratinocytes (11), brown adipose tissue (12), CD34+ stem cells (13), endothelial cells (14), fibroblasts, and vascular smooth muscle cells (15). VEGF expression is induced by hypoxia and cytokines such as IL-1, IL-6, IL-8, Oncostatin M, and TNF- α (3, 4, 9). The isoforms are differentially expressed during development and in the adult (3). VEGF dimers bind to two related receptor tyrosine kinases, VEGF R1 (also called Flt-1) and VEGF R2 (Flk-1/KDR) and induce their homodimerization and autophosphorylation (3, 4, 7, 17, 18). These receptors have seven extracellular immunoglobulin-like domains and an intracellular split tyrosine kinase domain. They are expressed on vascular endothelial cells and a range of non-endothelial cells. Although VEGF affinity is highest for binding to VEGF R1, VEGF R2 appears to be the primary mediator of VEGF angiogenic activity (3, 4). VEGF₁₆₅ also binds the semaphorin receptor, neuropilin-1, which promotes complex formation with VEGF R2 (19).

VEGF is best known for its role in vasculogenesis. During embryogenesis, VEGF regulates the proliferation, migration, and

survival of endothelial cells (3, 4), thus regulating blood vessel density and size but playing no role in determining vascular patterns. VEGF promotes bone formation through osteoblast and chondroblast recruitment and is also a monocyte chemoattractant (20-22). In postnatal life, VEGF maintains endothelial cell integrity and is a potent mitogen for micro- and macro-vascular endothelial cells. In adults, VEGF functions mainly in wound healing and the female reproductive cycle (3). In diseased tissues, VEGF promotes vascular permeability. It is thus thought to contribute to tumor metastasis by promoting both extravasation and tumor angiogenesis (23, 24). Various strategies have been employed therapeutically to antagonize VEGF-mediated tumor angiogenesis (25). Circulating VEGF levels correlate with disease activity in autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus (26).

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