ab119566 – VEGFA Human ELISA Kit

Instructions for Use

For the quantitative measurement of Human VEGFA concentrations in Cell culture supernatant, serum and plasma (EDTA, citrate, heparin).

This product is for research use only and is not intended for diagnostic use.

Version 5 Last Updated 11 January 2019

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1. BACKGROUND

Abcam's VEGFA Human *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for accurate quantitative measurement of Human VEGFA concentrations in Cell culture supernatant, serum and plasma (EDTA, citrate, heparin).

VEGFA specific antibodies have been precoated onto 96-well plates. Standards and test samples are added to the wells and then incubated at room temperature. After washing, a Biotin-conjugated anti-Human VEGFA detection antibody is added then incubated at room temperature. Following washing Streptavidin-HRP conjugate is added to each well, incubated at room temperature then again washed. TMB is added and then catalyzed by HRP to produce a blue color product that changes into yellow after addition of an acidic stop solution. The density of yellow coloration is directly proportional to the amount of VEGFA captured on the plate.

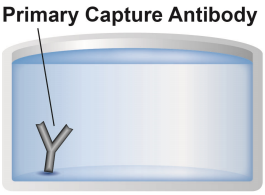
Normal tissue function depends on a regular supply of oxygen through the blood vessels. Understanding the formation of blood vessels has become the focus of a major research effort throughout the last decade. Vasculogenesis in the embryo is the process by which new blood vessels are generated de novo from primitive precursor cells. Angiogenesis is the process of new blood vessel formation from pre-existing vasculatures. It plays an essential role in development, normal tissue growth, wound healing, the female reproductive cycle (placental development, ovulation, corpus luteum) and also plays a major role in various diseases. Special interest is focused on tumor growth, since tumors cannot grow more than a few millimeters in size without developing a new blood supply. This process is described as tumor angiogenesis which is also essential for the spread and growth of tumor cell metastasis. One of the key molecules for angiogenesis and for the survival of the endothelium is vascular endothelial growth factor (VEGFA). It is a specific endothelial cell mitogen and a strong vascular permeability factor (VPF). VEGFA is a heparin-binding glycoprotein,

secreted as a homodimer of 45 kDa by many different cell types. VEGFA also causes vasodilation through the nitric oxide synthase pathway in endothelial cells and can activate migration in monocytes. Many different splice variants of VEGFA have been described, but VEGF165 is the most predominant protein and anchors with its heparin binding domain to extracellular matrix and to heparin sulfate. During the past few years, several other members of the VEGF family have been cloned, including VEGFB, VEGFC and VEGFD. In terms of vascular angiogenesis, which mainly is regulated by VEGFA, lymphangiogenesis is mainly regulated by VEGFC and VEGFD.

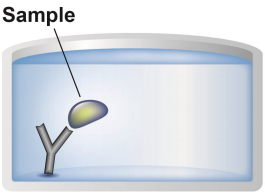
VEGFA transcription is highly activated by hypoxia and by oncogenes like H-ras and several transmembrane tyrosine kinases, such as epidermal growth factor receptor and ErbB2. Together these pathways account for a marked up regulation of VEGFA in tumors compared to normal tissues and are often of prognostic importance and relevance. VEGFA can be detected in both plasma and serum samples of patients, with much higher levels in serum. Extremely high levels can be detected in the cystic brain fluid of brain tumor patients or in ascites fluid of patients. Platelets release VEGFA upon aggregation and may be another major source of VEGFA delivery to tumors. Several other studies have shown that association of high serum levels of VEGFA with poor prognosis in cancer patients may be correlated with an elevated platelet count. Tumors can release cytokines and growth factors that stimulate the production of megakaryocytes in the marrow and elevate the platelet count. This can result in another, indirect increase of VEGFA delivery to tumors. Furthermore, VEGFA is implicated in several other pathological conditions associated with enhanced angiogenesis or enhanced vascular permeability. Examples where VEGFA plays an important role are psoriasis and rheumatoid arthritis, as well as the ovarian hyperstimulation syndrome. Diabetic retinopathy is associated with high intraocular levels of VEGFA, and inhibition of VEGFA function may result in infertility by blockage of corpus luteum function. Direct demonstration of the importance of VEGFA in tumor growth has been achieved using dominant negative

VEGF receptors to block in vivo proliferation, as well as blocking antibodies to VEGF or to one of the VEGF receptors.

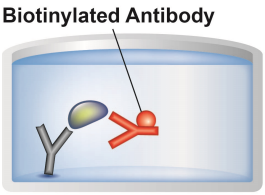
2. ASSAY SUMMARY



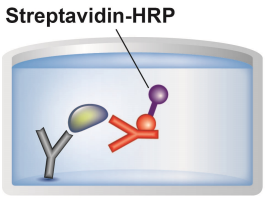
Prepare all reagents, samples and standards as instructed.



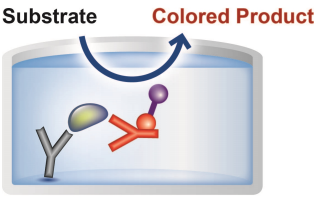
Add standards or samples to each well used. Incubate the plate.



Wash wells and add Biotin-Conjugated anti-Human VEGFA antibody to appropriate wells. Incubate the plate.



Wash wells and add prepared Streptavidin-HRP Conjugate to appropriate wells. Incubate at room temperature.



Wash wells and add TMB Substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at 2-8ºC immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 9. Reagent Preparation.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Human VEGFA coated microplate (12 x 8 wells)	96 wells	2-8 ºC
Biotin-Conjugate anti-Human VEGFA polyclonal antibody	120 µL	2-8 ºC
VEGFA Standard Lyophilized	2 Vials	2-8 ºC
20X Wash Buffer Concentrate	50 mL	2-8 ºC
20X Assay Buffer Concentrate	5 mL	2-8 ºC
TMB Substrate Solution	15 mL	2-8 ºC
Stop Solution (1 M Phosphoric Acid)	15 mL	2-8 ºC
Streptavidin-HRP	150 µL	2-8 ºC
Sample Diluent	12 mL	2-8 ºC
Adhesive Films	6 units	2-8 ºC

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- 5 mL and 10 mL graduated pipettes
- 5 µL to 1000 µL adjustable single channel micropipettes with disposable tips
- 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microplate strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted

8. TECHNICAL HINTS

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers
- Avoid foaming or bubbles when mixing or reconstituting components
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps
- Complete removal of all solutions and buffers during wash steps.
- As exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of radio immunotherapy has significantly increased the number of patients with Human anti-Human IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analyzed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.
- **This kit is sold based on number of tests.** A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18-25°C) prior to use. If crystals have formed in the Buffer Concentrates, warm them gently until they have completely dissolved.

9.1 1X Wash Buffer

Prepare 1X Wash Buffer by diluting the 20X Wash Buffer Concentrate with distilled or deionized water. To make 500 mL 1X Wash Buffer, combine 25 mL 20X Wash Buffer Concentrate with 475 mL distilled or deionized water. Mix thoroughly and gently to avoid foaming.

Note: The 1X Wash Buffer should be stored at 2-8 ºC and is stable for 30 days.

9.2 1X Assay Buffer

Prepare 1X Assay Buffer by diluting the 20X Assay Buffer Concentrate with distilled or deionized water. To make 50 mL 1X Assay Buffer, combine 2.5 mL 20X Assay Buffer Concentrate with 47.5 mL distilled or deionized water. Mix thoroughly and gently to avoid foaming.

Note: The 1X Assay Buffer should be stored at 2-8 ºC and is stable for 30 days.

9.3 1X Biotin-Conjugate Antibody

To prepare the 1X Biotin Conjugated Antibody, dilute the Polyclonal anti-Human VEGFA Antibody 100-fold with 1X Assay Buffer. Use the following table as a guide to prepare as much 1X Biotin Conjugated Antibody as needed by adding the required volume (µL) of the Polyclonal anti-Human VEGFA Antibody to the required volume (mL) of distilled water. Mix gently and thoroughly.

Number of strips	Volume of Biotin-Conjugate Concentrate (µL)	Volume of 1X Assay Buffer (mL)
1 - 6	60	5.94
7 - 12	120	11.88

Note: The 1X Biotin-Conjugated Antibody should be used within 30 minutes after dilution.

9.4 1X Streptavidin-HRP Conjugate

To prepare the Streptavidin-HRP Conjugate, dilute the anti-Streptavidin-HRP Conjugate 100-fold with 1X Assay Buffer. Use the following table as a guide to prepare as much 1X Streptavidin-HRP Conjugate as needed by adding the required volume (µL) of the Streptavidin-HRP Conjugate to the required volume (mL) of distilled water. Mix gently and thoroughly.

Number of strips	Volume of Streptavidin-HRP solution Concentrate (µL)	Volume of 1X Assay Buffer (mL)
1 - 6	60	5.94
7 - 12	120	11.88

Note: The 1X Streptavidin-HRP should be used within 30 minutes after dilution.

- All other solutions are supplied ready to use

10. STANDARD PREPARATIONS

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use.

10.1 Prepare a 2,000 pg/mL **Stock Standard** by reconstituting one vial of the Human VEGFA standard with the volume of distilled water stated on the label. Hold at room temperature for 10-30 minutes. The 2,000 pg/mL **Stock Standard** cannot be stored for later use.

10.2 Label eight tubes with numbers 1 - 8.

10.3 Add 225 µL Sample diluent into all tubes.

10.4 Prepare a 1,000 pg/mL **Standard 1** by adding 225 µL of the 2,000 pg/mL Stock Standard to 225 µL sample diluent to tube 1. Mix thoroughly and gently.

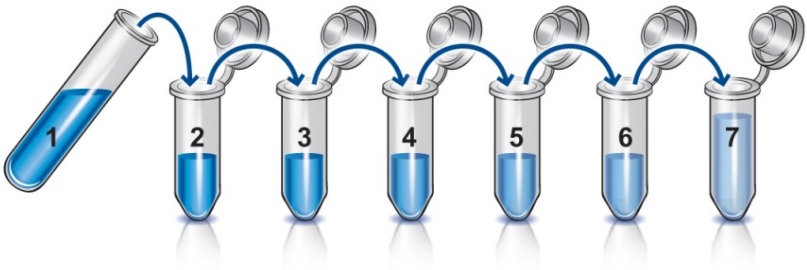
10.5 Prepare **Standard 2** by transferring 225 µL from Standard 1 to tube 2. Mix thoroughly and gently.

10.6 Prepare **Standard 3** by transferring 225 µL from Standard 2 to tube 3. Mix thoroughly and gently.

10.7 Using the table below as a guide, repeat for tubes number 4 through to 7.

10.8 **Standard 8** contains no protein and is the Blank control

Standard	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	Stock	225	225	2,000	1,000
2	Standard 1	225	225	1,000	500
3	Standard 2	225	225	500	250
4	Standard 3	225	225	250	125
5	Standard 4	225	225	125	62.5
6	Standard 5	225	225	62.5	31.3
7	Standard 6	225	225	31.3	15.6
8	None	-	225	-	0



11. SAMPLE COLLECTION AND STORAGE

- Cell culture supernatant and serum were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum from the clot or cells as soon as possible after clotting and separation.

- Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

- Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive Human VEGFA. If samples are to be run within 24 hours, they may be stored at 2° to 8°C.

- Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently and properly diluted with 1X Sample Diluent.

- Aliquots of serum samples (spiked or unspiked) were stored at -20°C and thawed 5 times, and the Human VEGFA levels determined. A significant decrease of Human VEGFA immunoreactivity was detected. Therefore samples should be stored in aliquots at -20°C and thawed only once.

- Aliquots of serum samples (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the Human VEGFA level determined after 24 h. There was no significant loss of Human VEGFA immunoreactivity detected during storage at -20°C and 2-8°C. A significant loss of Human VEGFA immunoreactivity was detected during storage at RT and 37°C after 24 h.

12. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use.
- Unused well strips should be returned to the plate packet and stored at 2-8°C
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)
- Well effects have not been observed with this assay.

13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

13.1. Prepare all reagents, working standards, and samples as directed in the previous sections. Determine the number of microplate strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards.

13.2. Wash the microplate twice with approximately 400 µL 1X Wash Buffer per well with thorough aspiration of microplate contents between washes. Allow the 1X Wash Buffer to remain in the wells for about 10 - 15 seconds before aspiration. Take care not to scratch the surface of the microplate.

13.3. After the last wash step, empty wells and tap microplate on absorbent pad or paper towel to remove excess 1X Wash Buffer. Use the microplate strips immediately after washing. Alternatively the microplate strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

13.4. Add 100 µL of each standard to the appropriate standard wells (including the no standard blank control).

13.5. Add 50 µL of 1X Sample Diluent to all the sample wells.

13.6. Add 50 µL of each sample in duplicate to the sample wells.

13.7. Cover with adhesive film and incubate at room temperature (18° to 25°C) for 2 hours (microplate can be incubated on a shaker set at 400 rpm).

13.8. Remove adhesive film and empty wells. Wash microplate strips 6 times according to step 13.2. Proceed immediately to step 13.9.

13.9. Add 100 µL of Biotin-Conjugated Antibody to all wells.

13.10. Cover with adhesive film and incubate at room temperature (18° to 25°C) for 1 hour (microplate can be incubated on a shaker set at 400 rpm).

13.11. Remove adhesive film and empty wells. Wash microplate strips 6 times according to step 13.2. Proceed immediately to step 13.12.

13.12. Add 100 µL of Streptavidin-HRP to all wells.

13.13. Cover with adhesive film and incubate at room temperature (18° to 25°C) for 1 hour (microplate can be incubated on a shaker set at 400 rpm).

13.14. Remove adhesive film and empty wells. Wash microplate strips 6 times according to step 13.2. Proceed immediately to step 13.15.

13.15. Add 100 µL of TMB Substrate Solution to all wells.

13.16. Incubate the microplate strips at room temperature (18 to 25°C) for 30 minutes. Avoid direct exposure to intense light.

Note: The color development on the plate should be monitored and the substrate reaction stopped (see step 13.17) before the signal in the positive wells becomes saturated. Determination of the ideal time period for color development should to be done individually for each assay. It is recommended to add the stop solution when the highest standard has developed a dark blue color. Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 - 0.95.

13.17. Stop the enzyme reaction by adding 100 µL of Stop Solution into each well.

Note: It is important that the Stop Solution is mixed quickly and uniformly throughout the microplate to completely inactivate the enzyme. Results must be read immediately

after the Stop Solution is added or within one hour if the microplate strips are stored at 2 - 8°C in the dark.

13.18. Read absorbance of each microplate on a spectrophotometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

14. CALCULATIONS

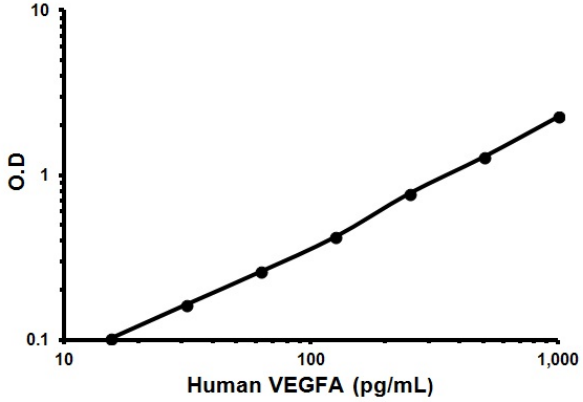
Average the duplicate reading for each standard, sample and control blank. Subtract the control blank from all mean readings. Plot the mean standard readings against their concentrations and draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A five parameter algorithm (5PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 5-parameter logistic). Extrapolate protein concentrations for unknown and control samples from the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

If samples have been diluted 1:2, as stated in step 13.6, the concentration obtained from the standard curve must be multiplied by the dilution factor (x 2) to obtain an accurate value, in addition to any sample dilution factor undertaken by the user.

Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low Human VEGFA levels. Such samples require further external predilution according to expected Human VEGFA values with Sample Diluent in order to precisely quantitate the actual Human VEGFA level.

15. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



Standard Curve Measurements			
Conc. (pg/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.066	0.073	0.069
15.6	0.112	0.093	0.102
31.3	0.162	0.163	0.163
62.5	0.258	0.258	0.258
125	0.409	0.429	0.419
250	0.766	0.775	0.771
500	1.244	1.327	1.286
1,000	2.201	2.308	2.254

Figure 1. Example of Human and the Human VEGFA standard protein standard curve.

16. TYPICAL SAMPLE VALUES

SENSITIVITY –

The limit of detection for VEGFA defined as the analyte concentration resulting in an absorption significantly higher than the absorption of the dilution medium (mean plus two standard deviations) was determined to be 7.9 pg/mL (mean of 6 independent assays).

RECOVERY –

The spike recovery was evaluated by spiking 3 levels of Human VEGFA into serum, plasma and cell culture supernatant. Recoveries were determined with 4 replicates each. The amount of endogenous Human VEGFA in unspiked samples was subtracted from the spike values.For recovery data see the table below.

Sample Matrix	Spike High (%)	Spike Medium (%)	Spike Low (%)
Serum	88	85	81
Plasma (EDTA)	77	77	79
Plasma (Citrate)	92	90	94
Plasma (heparin)	106	88	64
Cell culture supernatant	98	92	88

DILUTION PARALLELISM –

Serum, plasma and cell culture supernatant samples with different levels of Human VEGFA were analyzed at serial 2 fold dilutions with 4 replicates each.

Sample Matrix	Recovery of Exp. Value	
	Range (%)	Mean (%)
Serum	76 – 104	90
Plasma (EDTA)	93 – 147	110
Plasma (Citrate)	83 – 99	90
Plasma (heparin)	98 – 119	108
Cell culture supernatant	72 – 103	91

PRECISION –

Intra- and Inter-assay reproducibility was determined by measuring samples containing different concentrations of Human VEGFA.

	Intra-Assay	Inter-Assay
n=	8	8
%CV	6.2	4.3

EXPECTED VALUES –

Panels of 40 serum as well as EDTA, citrate and heparin plasma samples from randomly selected apparently healthy donors were tested for Human VEGFA. The levels measured may vary with the sample collection used. The detected Human VEGFA levels are shown in the table below.

Sample Matrix	Number of Samples Evaluated	Range (pg/mL)	% Detectable	Mean of Detectable (pg/mL)
Serum	40	nd *- 42.6	2.5	-
Plasma (EDTA)	40	nd *- 128.9	7.5	45.7
Plasma (Citrate)	40	nd *- 66.2	7.5	47.3
Plasma (Heparin)	40	nd *- 311.4	7.5	144.3

* n.d. = non-detectable,

17. ASSAY SPECIFICITY

The assay detects both natural and recombinant Human VEGF-A. Cross reactivity and interference of circulating factors of the immune system were evaluated by spiking these proteins at physiologically relevant concentrations into a Human VEGF-A positive serum. There was no cross reactivity detected, notably not with Human VEGFB, VEGFC, VEGFD and PfGF. Interference was detected for VEGF-R1 at concentrations > 200 pg/ml, and not for VEGF-R2.

18. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high.	Dilute the specimens and repeat the assay
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the all components as directed.

19. NOTES

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