arigoPLEX® Human Angiogenic Marker Multiplex ELISA kit (VEGF, FGF basic, HGF, IL6)

arigoPLEX® Human Angiogenic Marker Multiplex ELISA kit (VEGF, FGF basic, HGF, IL6) is an Enzyme Immunoassay kit for the quantification of Human Angiogenic Marker (VEGF, FGF basic, HGF, IL6) in Human serum, plasma and cell culture supernatants.

Catalog number: ARG83510

Package: 96 wells

Lot. 374160

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

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INTRODUCTION

VEGF: Growth factor active in angiogenesis, vasculogenesis and endothelial cell growth. Induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and induces permeabilization of blood vessels. Binds to the FLT1/VEGFR1 and KDR/VEGFR2 receptors, heparan sulfate and heparin. NRP1/Neuropilin-1 binds isoforms VEGF-165 and VEGF-145. Isoform VEGF165B binds to KDR but does not activate downstream signaling pathways, does not activate angiogenesis and inhibits tumor growth. Binding to NRP1 receptor initiates a signaling pathway needed for motor neuron axon guidance and cell body migration, including for the caudal migration of facial motor neurons from rhombomere 4 to rhombomere 6 during embryonic development (By similarity). [UniProt]

FGF basic: Acts as a ligand for FGFR1, FGFR2, FGFR3 and FGFR4 (PubMed:8663044). Also acts as an integrin ligand which is required for FGF2 signaling (PubMed:28302677). Binds to integrin ITGAV:ITGB3 (PubMed:28302677). Plays an important role in the regulation of cell survival, cell division, cell differentiation and cell migration (PubMed:8663044, PubMed:28302677). Functions as a potent mitogen in vitro (PubMed:3732516, PubMed:3964259). Can induce angiogenesis (PubMed:23469107, PubMed:28302677). [UniProt]

HGF: Potent mitogen for mature parenchymal hepatocyte cells, seems to be a hepatotrophic factor, and acts as a growth factor for a broad spectrum of tissues and cell types. Activating ligand for the receptor tyrosine kinase MET by binding to it and promoting its dimerization. [UniProt]

IL6: Cytokine with a wide variety of biological functions. It is a potent inducer of the acute phase response. Plays an essential role in the final differentiation of B-cells into Ig-secreting cells Involved in lymphocyte and monocyte differentiation. Acts on B-cells, T-cells, hepatocytes, hematopoietic progenitor cells and cells of the CNS. Required for the generation of T(H)17 cells. Also acts as a myokine. It is discharged into the bloodstream after muscle contraction and acts to increase the breakdown of fats and to improve insulin resistance. It induces myeloma and plasmacytoma growth and induces nerve cells differentiation. [UniProt]

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. The antibodies specific to VEGF, FGF basic, HGF and IL6 have been pre-coated onto a microtiter plate. Standards Mixture and samples are pipetted into the wells and any target cytokines present is bound by the immobilized specific antibodies. After incubation, added Mixed Antibody-Conjugate to each well and incubate. After washing away any unbound substances, the HRP Conjugate is added into the wells and incubated. The wells are thoroughly washed to remove all unbound HRP Conjugate. A TMB substrate is added to the wells and color develops in proportion to the amount of target cytokines in the initial step. The color development is stopped by the addition of STOP solution and the intensity of the color is measured at a wavelength of O.D. 450 nm. The concentration of rat proinflammatory cytokines in the samples is then determined by comparing the O.D. 450 nm of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Upon received, store 10X Antibody Conjugate Mixture at ≤ -20°C.

Store other components at 2-8°C at all times. Use the kit before expiration date.

Component	Quantity	Storage information
Antibody Coated Microplate [1]	8 X 12 strips	4°C
Standards Mixture [2]	3 vial (lyophilized)	4°C
10X Antibody Conjugate Mixture	1.2 mL	≤ -20°C
40X HRP-Streptavidin concentrate	300 µL	4°C
Standard / Sample Diluent Buffer	30 mL	4°C
Antibody Diluent Buffer	35 mL	4°C
10X Wash Buffer	50 mL	4°C
TMB substrate	12 mL (ready to use)	4°C (protect from light)
STOP solution	6 mL (ready to use)	4°C
Plate sealer	3 adhesive strips	RT

Note:

1. The Antibody Coated microplate contains twelve 8-well ELISA strips. Each of the eight wells has been coated with a different antibody specific to one of the 4 cytokines as shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF
B	FGF basic	FGF basic	FGF basic	FGF basic	FGF basic	FGF basic	FGF basic	FGF basic	FGF basic	FGF basic	FGF basic	FGF basic
C	HGF	HGF	HGF	HGF	HGF	HGF	HGF	HGF	HGF	HGF	HGF	HGF
D	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6
E	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF	VEGF
F	FGF basic	FGF basic	FGF basic	FGF basic	FGF basic	FGF basic	FGF basic	FGF basic	FGF basic	FGF basic	FGF basic	FGF basic
G	HGF	HGF	HGF	HGF	HGF	HGF	HGF	HGF	HGF	HGF	HGF	HGF
H	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6	IL-6

2. Standards Mixture each vial contains a buffered protein base and four cytokines at different amount: VEGF: 31.25- 2000 pg/ml, FGF basic: 15.6 - 1000 pg/ml, HGF: 125- 8000 pg/ml, IL-6: 15.6- 1000 pg/ml.

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of reading at 450 nm
- Deionized or distilled water
- Pipettes and pipette tips
- Multichannel micropipette reservoir
- Microtiter plate washer (optional)

TECHNICAL NOTES AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Upon received, store 10X Antibody Conjugate Mixture at ≤ -20°C. Store other component at 2-8°C at all times.
- Prior to beginning the assay procedure, bring all reagents and required number of strips to room temperature (20-25°C).
- Unused wells must be stored at 2-8 °C in the sealed foil pouch and used in the frame provided.
- All reagents must be mixed without foaming and briefly spin down the all vials before use.
- If crystals are observed in the 10X Wash Buffer, warm to 37°C until the crystals are completely dissolved.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
- Ensure complete reconstitution and dilution of reagents prior to use.

- Take care not to contaminate the mixed TMB Substrate. Do not expose the mixed TMB solution to glass, foil or metal. If the solution is blue before use, DO NOT USE IT.
- All reagents must be mixed without foaming before use.
- Change pipette tips between the addition of different reagent or samples.
- Taping the well strips together with lab tape can be done as an extra precaution to avoid plate strips from coming loose during the procedure.

SAMPLE COLLECTION & STORAGE INFORMATION

The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design.

Cell Culture Supernatants - Remove particulates by centrifugation for 10 min at 1500 x g at 4°C and aliquot & store samples at-20°C up to 1 month or-80°C up to 6 months. Avoid repeated freeze-thaw cycles.

Serum- Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Collect serum and assay immediately or aliquot & store samples at-20°C up to 1 month or-80°C up to 6 months. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g. within 30 minutes of collection. Collect the supernatants and assay immediately or aliquot and store samples at -20°C up to 1 month or -80°C up to 6 months. Avoid repeated freeze-thaw cycles.

Note:

a) Do not use haemolytic, icteric or lipaemic specimens.
b) Samples containing sodium azide should not be used in the assay.
c) Avoid disturbing the white buffy layer when collection serum / plasma sample.

d) To obtain the data of each cytokine, at least 0.2 mL of the sample is needed to complete one run of the assay. It is recommended to have a larger volume available in case that the experiments have to be repeated.

REAGENT PREPARATION

- 1X Wash Buffer: Dilute 10X Wash Buffer into distilled water to yield 1X Wash Buffer. (E.g., add 50 mL of 10X Wash Buffer into 450 mL of distilled water to a final volume of 500 mL) The 1X Wash Buffer is stable for up to 4 weeks at 2-8°C. Mix well before use.
- 1X Antibody Conjugate Mixture: It is recommended to prepare this reagent immediately prior to use and use it within 20 min after preparation. Dilute 10X Antibody Conjugate Mixture concentrate into Antibody Diluent Buffer to yield 1X detection antibody solution. (e.g. 100 µl of 1000X Antibody Conjugate Mixture concentrate + 900 µl of Antibody Diluent Buffer)
- 1X HRP-Streptavidin Solution: It is recommended to prepare this reagent immediately prior to use and use it within 20 min after preparation. Dilute 40X HRP-Streptavidin concentrate solution into Antibody Diluent Buffer to yield 1X HRP-Streptavidin Solution buffer. (e.g. 30 µl of 40X HRP-Streptavidin concentrate solution + 1170 µl of Antibody Diluent Buffer)
- Sample: Before assay, serum and plasma are recommended to dilute with equal volume of Standard / Sample Diluent Buffer. (eg. Premix 250 µL sample with 250 µL Standard / Sample Diluent Buffer.)

- Standards Mixture:
  A. Add **1 mL** of **Standard / Sample Diluent Buffer** to reconstitute the lyophilized Standard Mixture to obtain the high concentration stock of 4 cytokines at different concentrations (see table below). Brief vortex and allow the stock standard to sit for at least 15 minutes with gentle agitation to make sure the standard is dissolved completely before making serial dilutions. The reconstituted Standard Mixture high concentration stock could be stored at-80°C for up to 30 days.
  B. Use the above high concentration Standards Mixture and a 32-fold diluted low concentration Standards Mixture to test together with up to 22 test samples. If more accurate results are required, a two-fold serial dilution with Dilution Buffer can generate a more accurate standard curve. However, the number of test samples will be reduced.

The concentrations of the 4 cytokines in different dilutions of the Standards Mixture are listed as below:

Cytokines (pg / mL)	High Conc. Standard Stock	1:2	1:4	1:8	1:16	1:32 (Low Conc. Standard Mixture)	1:64
VEGF	2000	1000	500	250	125	62.5	31.2
FGF basic	1000	500	250	125	62.5	31.25	15.6
HGF	8000	4000	2000	1000	500	250	125
IL-6	2000	1000	500	250	125	62.5	31.2

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT, 20-25°C) before use.

1. Add **100 µL** of the **Standards Mixture** or **diluted samples** to the Antibody Coated microplate.

   Note: To obtain the approximate concentrations of 4 cytokines on 22 test samples, the low concentration standard mixture (**S1, 1:32 from high concentration mixture**), the high concentration Standards Mixture (**S2, stock**) and test samples (T1 to T22) can be added as the scheme as below:

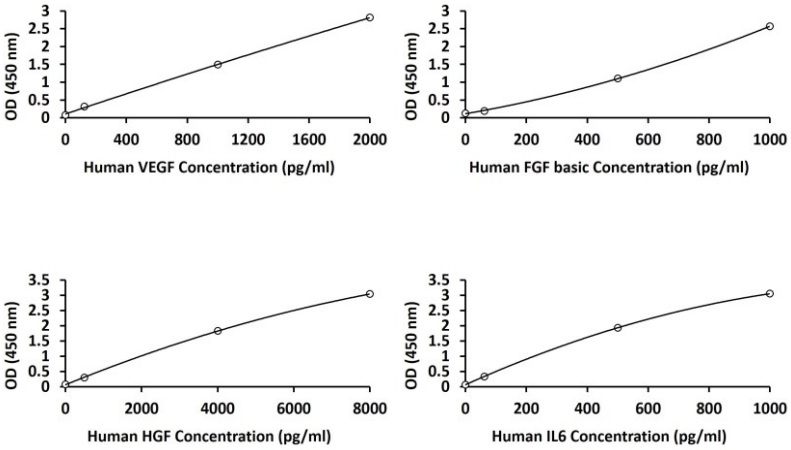
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
B	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
C	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
D	S1	T1	T3	T5	T7	T9	T11	T13	T15	T17	T19	T21
E	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22
F	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22
G	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22
H	S2	T2	T4	T6	T8	T10	T12	T14	T16	T18	T20	T22

2. Cover the plate and incubate for **2 hours** at **room temperature**.
3. Aspirate each well and wash, repeating the process 4 times for a **total 5 time washes**. Wash by filling each well with **1× Wash Buffer (300 µL)** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.
4. Add **100 µL** of **1X Antibody Conjugate Mixture** to each wells.
5. Cover the plate and Incubate for **2 hour** at **room temperature**.
6. Aspirate each well and **wash as step 3.**

7. Add **100 µL** of **1X HRP-Streptavidin Solution** to each well. Cover the plate and incubate for **20 minutes** at **room temperature**.
8. Aspirate each well and **wash as step 3**.
9. Add **100 µL** of **TMB Substrate** to each well. Cover and incubate for **15-25 minutes** at **room temperature** in the dark.
10. Immediately Add **50 µL** of **Stop Solution** to each well. The color of the solution should change from blue to yellow.
11. Read the OD with a microplate reader at **450 nm** immediately. It is recommended reading the absorbance **within 30 minutes** after adding the stop solution.

EXAMPLE OF TYPICAL STANDARD VALUES

The following table shows the OD readings of a run of this multiplex ELISA with serial diluted standards. It is for demonstration purpose only and cannot be used to replace the standard curve for testing. For each investigation, standards have to be assayed along with test samples and only the curve generated from the same test can be used.



CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards and samples.
2. The 4 curves for 4 targets can be generated from OD readings of high concentration standard and low concentration standard mixture. The standard curves might not be perfectly straight. To obtain more accurate results, more dilution points can be used when generating standard curves.
3. 4 Parameter Logistics is the preferred method for the result calculation. Other data reduction functions may give slightly different results.
4. arigo provides GainData®, an in-house development ELISA data calculator, for ELISA data result analysis. Please refer our GainData® website for details. (https://www.arigobio.com/elisa-analysis)
5. Serum/plasma sample has multiplied the dilution factor (Dilution factor =2). If the samples have been further diluted, the concentration read from the standard curve must be further converted by the appropriate dilution factor according to the sample preparation procedure.