CELL APPLICATIONS, INC.

Human PLGF ELISA Kit

Cat. No. CL0490
96-wells

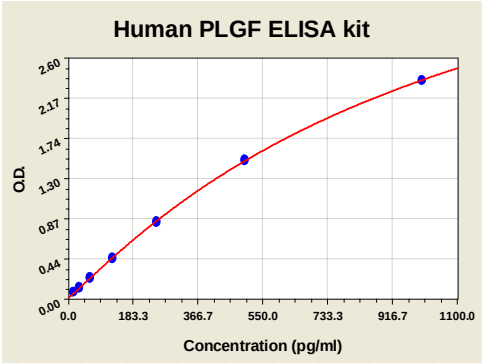
Target Protein Species:	Human
Range	15.6 pg/ml – 1000pg/ml
Specificity	no detectable cross-reactivity with any other cytokine.

KIT COMPONENTS

Component	Amount
96-well plate precoated with anti-human PLGF antibody	1 Plate
Protein Standard: Lyophilized recombinant human PLGF	2 tubes, 10 ng/tube
Sample Diluent Buffer	30 ml
Biotinylated Antibody (Anti-human PLGF)	130 µl (100x)
Antibody Diluent Buffer	12ml
Avidin-Biotin-Peroxidase Complex (ABC) Solution	130 µl (100x)
ABC Diluent Buffer	12 ml
Tetramethylbenzidine (TMB) Color Developing Agent	10 ml
TMB Stop Solution	10 ml

Storage

Store at 4°C. Cell Applications, Inc. recommends using the kit within 6 months of order.



X	pg/ml	0.0	15.6	31.3	62.5	125	250	500	1000
Y	O.D.450	0.013	0.077	0.130	0.241	0.451	0.842	1.508	2.368

Figure 1: PLGF **Standard Curve.** Using the human PLGF ELISA Kit, O.D. data was graphed against PLGF protein concentration. The TMB reaction was incubated at 37° C for 10 min.

BACKGROUND

Placenta growth factor (PlGF) is a close homolog of vascular endothelial growth factor (VEGF), shares receptors with VEGF, and stimulates angiogenesis. It is a pleiotropic cytokine that stimulates endothelial cell (EC) growth, migration, and survival; chemoattracts angiocompetent macrophages and bone marrow progenitors; and determines the metastatic niche. Unlike VEGF, PlGF together with VEGF-B belong to a subgroup of ligands within the VEGF family, which only binds to VEGFR1 and its coreceptors neuropilin-1 and -2. Consistent with the receptor binding patterns, PlGF and VEGF-B do not seem to display significant physiological functions.[1] For example, plgf or vegf-b-null mice develop normally and lack obvious vascular and non-vascular defects during the adulthood. However, recent studies show that PlGF might significantly contribute to pathological angiogenesis such as tumor neovascularization, vascular regeneration under tissue ischemia, and wound healing. Besides indirect effects, PlGF signals directly via VEGFR-1, thus, acting independently of VEGF in ECs, macrophages, bone marrow progenitors, and tumor cells, which primarily express VEGFR-1.[2] In addition to its positive roles in regulation of pathological angiogenesis, PlGF has also been reported as a negative regulator of tumor angiogenesis and tumor growth. The mechanism of negative regulation of angiogenesis involves the formation of VEGF-PlGF heterodimers that do not display significantly angiogenic activity relative to VEGF homodimers. Four isoforms of human PlGF and six isoforms of human VEGF-A generated by alternative splicing from the same genes could potentially form 24 different heterodimers with different affinities to heparan sulfate proteoglycans, and thus create complex gradients around their producing cells.[3] It was shown that PlGF levels in plasma and tumors correlate with tumor stage, vascularity, recurrence, metastasis, and survival in various tumors. Notably, PlGF is upregulated in cancer patients treated with VEGF(R) inhibitors therapy as well as in human tumors after radio-immunotherapy, suggesting a key role of PlGF in the angiogenic rescue.[4] Genetic studies show that PlGF is redundant for vascular development and maintenance, but contributes to the angiogenic switch in disease. This raised the question whether PlGF inhibitors might reduce pathological angiogenesis but, unlike VEGF(R) inhibitors are, without affecting healthy blood vessels, and thus provide an attractive drug with a better safety profile.

ELISA OVERVIEW

Cell Applications ELISA Kits are based on standard sandwich enzyme-linked immunosorbent assay technology. Freshly prepared standards, samples, and solutions are recommended for best results.

1. Prepare test samples.
2. Prepare a protein standard of the target protein.
3. Add test samples and standards to the pre-coated 96-well plate. Do not wash.
4. Add biotinylated detection antibodies. Wash.
5. Add Avidin-Biotin-Peroxidase Complex (ABC) Solution. Wash.
6. Add Tetramethylbenzidine (TMB) Color Developing Agent, containing HRP substrate.
7. Add TMB Stop Solution
8. Subject the plate to analysis.

NOTES:

- Before using the kit, quick spin tubes to bring down all solution to the bottom of tube.
- Duplicate assay wells are recommended for both standard and sample testing.
- Do not let the 96-well plate dry, this will lead to inactivation of plate components.
- When diluting samples and reagents, ensure that they are mixed completely and evenly.
- Pre-warm diluted ABC and TMB solutions at 37°C for 30 min before use to avoid variable temperature effects.
- For washes, use TBS or PBS. Do not touch well walls.
- A protein standard is included in the kit. A protein standard detection curve should be generated with each experiment, no more than 2 hours prior to the experiment.
- The user will determine sample dilution fold by estimation of target protein amount in samples.

Reference

1. Iyer, S. et al: J. Biol. Chem. 276:12153-61, 2001
2. Loges, S. et al: Clin. Cancer Res. 15:3648-53, 2009
3. Hedlund, E.M. et al: Proc. Natl. Acad. Sci. USA 106:17505-10, 2009
4. Fischer, C. et al: Cell 131:463-75, 2007

ELISA PROTOCOL

Preparation of Test Samples

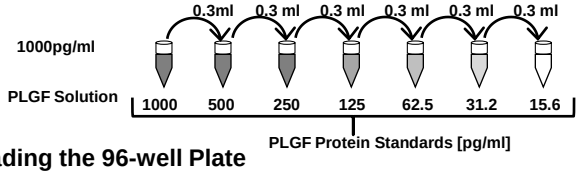
1. Process Test Samples in the following manner:
   - **Cell culture supernate, tissue lysate or body fluids**: Remove particulates by centrifugation. analyze immediately or aliquot and store at -20° C
   - **Serum**: Allow the serum to clot in a serum separator tube (about 30 min) at room temperature. Centrifuge at approximately 1000 x g for 15 min. Analyze the serum immediately or aliquot and store frozen at -20° C.
     Plasma: Collect plasma using heparin, EDTA, citrate as an anticoagulant. Centrifuge for 15 min at 1000 x g within 30 min of collection. Analyze immediately or aliquot and store frozen at -20° C..
2. Estimate the concentration of the target protein in the sample and select a proper dilution factor such that the diluted target protein concentration falls within the 15.6-1000 pg/ml standard curve range. Depending on the sample, several trial dilutions may be necessary. Dilute the sample using the provided diluent buffer, mixing well. Suggested working dilutions of samples are as follows:

Target Protein Concentration Range	Sample Working Dilution	Sample Vol.	Diluent Buffer Vol.
10-100 ng/ml	1:100	1 µl	99 µl
1-10 ng/ml	1:10	10 µl	90 µl
15.6-1000 pg/ml	1:2	50 µl	50 µl
≤15.6 pg/ml	n/a	100µl	n/a

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

Preparation of Standard Solutions (15.6-1000 pg/ml)

4. Reconstitute the Lypophilized Recombinant Protein to make a 10.000 pg/ml human PLGF solution. Add 1 ml Sample Diluent Buffer to a tube of lypophilized protein, keep the tube at room temperature for 10 min. Mix thoroughly.
5. Add 0.1 ml of the mixed 10,000 pg/ml PLGF solution to the eppendorf tube containing 0.9 ml diluent buffer and mix to make a 1000 pg/ml PLGF solution.
6. Label 6 eppendorf tubes with the human PLGF protein concentrations to be prepared by serial dilution :500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml, 15.6pg/ml
7. Aliquot 0.3 ml of the Sample Diluent Buffer to the labeled tubes.
8. Serially dilute the protein standards into their respectively labeled tubes. Transfer 0.3 ml from the 1000pg/ml PLGF Solution to the 500pg/ml eppendorf tube and mix thoroughly. Transfer 0.3 ml of the 500 pg/ml solution to the 250pg/ml tube and mix thoroughly, Transfer 0.3 ml of the 250pg/ml solution to the 125pg/ml tube and mix, and so on to make the 62.5, 31.3 and 15.6 pg/ml solutions.
9. Store at 4° C until use.



Loading the 96-well Plate

10. Aliquot 0.1 ml of the sample diluent buffer into a control well to serve as the Blank. This will yield the O.D.450(Blank) reading.
11. Aliquot 0.1 ml of the standard solutions of the **Preparation of Standard Solutions** (15.6-1000pg/ml) into empty wells of the precoated 96-well plate. Duplicate measurements of standards are recommended.
12. Aliquot 0.1 ml of each properly diluted test sample to empty wells prepared in **Step 2**. Duplicate measurements of each test sample are recommended.
13. Cover the 96-well plate and incubate at 37° C for 90 min.
14. During the **Step 13** incubation period, prepare a stock of Biotinylated 1:100 Antibody Working Solution. Count the number of reactions and multiply by 0.1 ml/well for the Working Solution total volume (preparation of 1-2 reactions in excess of the number of wells is recommended). Dilute the Biotinylated Antibody to 1:100 in Antibody Diluent Buffer and mix thoroughly. Use the working solution within 2 hours.
15. Upon completion of the 90 min incubation of **Step 13**, remove the cover of the 96 well plate and discard plate well contents. Blot the plate onto paper towels or other absorbent material. DO NOT let the wells completely dry at any time.
16. Add 0.1 ml of the Biotinylated 1:100 Antibody Working Solution (prepared in **Step 14**) to each well and incubate the plate at 37° C for 60 min.
17. During the incubation period of **Step 16**, prepare a stock of ABC Working Solution. Count the number of reactions and multiply by 0.1 ml/well for the Working Solution total volume (preparation of 1-2 reactions in excess of the number of wells is recommended). Dilute the ABC Stock Solution to 1:100 in ABC Diluent Buffer and mix thoroughly. Pre-warm the ABC working solution at 37° C for 30 min before use. Use the working solution within 1 hour.
18. Upon completion of the 60 min incubation of **Step 16**, wash the plate 3 times with 0.3 ml TBS or PBS. For each wash, leave washing buffer in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
19. Add 0.1 ml of prepared ABC Working Solution (prepared in **Step 17**) to each well and incubate the plate at 37° C for 30 min.
20. Add 90 µl of the pre-warmed TMB Color Developing Agent into each well and incubate at 37° C for 10-15 min (shades of blue can be seen in the wells with the four most concentrated Protein Standard Solutions; the other control wells should show no obvious color).
21. During the incubation period of **Step 19**, pre-warm TMB Color Developing Agent at 37° C for 30 min before use.
22. Upon completion of the 30 min incubation of **Step 19**, wash the plate 5 times with 0.3 ml TBS or PBS. For each wash, leave the washing buffer in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
23. Add 0.1 ml of the TMB Stop Solution to each well. The acidic stop solution will change the mixture color to yellow. The yellow intensity is proportional to the amount of target protein captured by the plate.
24. Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution. These readings are the O.D.450(Reading).

Calculating Protein Concentration

- For all wells, determine O.D.450(Relative):
  O.D.450(Relative) = O.D.450(Reading) – O.D.450(Blank)
- Plot the standard curve: plot O.D.450(Relative) of each standard solution (Y) vs. the respective concentration of the standard solution (X). See **Figure 1** for a typical standard curve.
- The human PLGF concentration of the samples can be interpolated from the standard curve. Multiply the interpolated concentration by the dilution factor to obtain the target protein concentration in the sample.