CELL APPLICATIONS, INC.

Human PAI-1 ELISA Kit

Cat. No. CL0859
96-wells

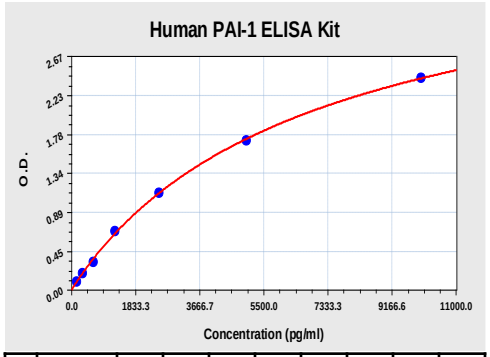
Target Protein Species:	Human
Range	156 pg/ml – 10ng/ml
Specificity	No detectable cross-reactivity with other cytokines

KIT COMPONENTS

Component	Amount
96-well plate precoated with anti-human PAI-1 antibody	1 Plate
Protein Standard: Lyophilized recombinant human PAI-1	2 tubes, 10 ng/tube
Sample Diluent Buffer	30 ml
Biotinylated Antibody (Anti-Human PAI-1)	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	156	313	625	1250	2500	5000	10000
Y	O.D.450	0.006	0.105	0.203	0.329	0.678	1.121	1.722	2.432

Figure 1: PAI-1 Standard Curve. Using the human PAI-1 ELISA Kit, O.D. data was graphed against PAI-1 protein concentration. The TMB reaction was incubated at 37° C for 25 min.

BACKGROUND

Plasminogen activator inhibitor 1 (PAI-1, Serpin E) belongs to the serine proteinase inhibitors (serpin) superfamily. PAI-1 is produced by endothelial cells, smooth muscle cells, adipocytes, spleen cells, platelets, and liver cells as well as some tumor cell lines including hepatoma, melanoma, and fibrosarcoma cells. PAI-1 is the primary physiological inhibitor of uPA and tPA. It not only regulates the proteolytic activity of uPA, but also determines the level of uPA bound to uPAR by promoting the rapid endocytosis of the trimolecular uPA–PAI-1–uPAR complex. Consequently, PAI-1 plays an important role in cardiovascular diseases (mainly through inhibition of t-PA), and in cell migration and tumor development.[1] At first, PAI-1 modulates cell migration by regulating ECM proteolysis. In addition, by blocking the interaction between vitronectin (VN), uPAR, and integrins, PAI-1 may induce cell detachment from the extracellular matrix and thereby promote cellular migration and tumor invasion. Furthermore, PAI-1, through its ability to titer active plasmin, promotes syndecan-1 dependent migration on unprocessed laminin-332 by preventing cleavage of the syndecan binding site LG4/5. PAI-1 inhibition of plasmin activation facilitates migration on unprocessed laminin-332 by reducing the shedding of syndecan-1 from the cell surface. Moreover, PAI-1 binding to the LRP1 in a non-uPA/uPAR dependent manner, triggers Jak/Stat1 signaling events that culminate in enhanced cell migration.[2] High levels of circulating PAI-1 are associated with a number of thrombotic diseases. Furthermore, inhibition of PAI-1 activity prevents thrombus formation in animal models. The antithrombotic effects of PAI-1 inhibition are achieved by enhancing endogenous fibrinolytic activity without directly affecting blood coagulation and platelet function. Phenotypic analysis of PAI-1 deficiency in both human and mouse suggests that inhibition of PAI-1 will not lead to severe bleeding or other major adverse effects. Thus, PAI-1 inhibitors represent a new class of antithrombotic drugs with a possible wider therapeutic index than conventional antiplatelet and anticoagulant agents.[3]

References

1. Bajou, K. et al: J. Cell Biol. 152:777-84, 2001
2. Gils, A. & Declerck, P.J. : Thromb. Haemost. 91: 425–37, 2004
3. Wilkins-Port, C.E. et al: Cell Communication Insights 3:1–10, 2010

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.

ELISA PROTOCOL

Preparation of Test Samples

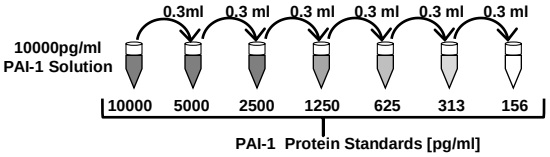
1. Process Test Samples in the following manner:
   - **Cell culture supernate, tissue lysate or body fluids**: Remove particulates by centrifugation, analyze immediately or aliquot and store at -20°C.
   - **Plasma**: Collect plasma using heparin/EDTA as an anticoagulant. Centrifuge for 15 min at 1000 x g at 2-8° C 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 156-10000 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.
100-1000 ng/ml	1:100	1 µl	99 µl
10-100 ng/ml	1:10	10 µl	90 µl
156-10000 pg/ml	1:2	50 µl	50 µl
≤156pg/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 (156-10000 pg/ml)

4. Reconstitute the Lypophilized Recombinant Protein to make a 10,000 pg/ml PAI-1 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. Label 6 eppendorf tubes with the PAI-1 protein concentrations to be prepared by serial dilution: 5000pg/ml, 2500pg/ml, 1250pg/ml, 625pg/ml, 313pg/ml, 156pg/ml.
6. Aliquot 0.3 ml of the Sample Diluent Buffer to the labeled tubes.
7. Serially dilute the protein standards into their respectively labeled tubes. Transfer 0.3 ml from the 10000pg/ml PAI-1 Solution to the 5000pg/ml eppendorf tube and mix thoroughly. Transfer 0.3 ml of the 5000 pg/ml solution to the 2500pg/ml tube and mix thoroughly, Transfer 0.3 ml of the 2500pg/ml solution to the 1250pg/ml tube and mix, and so on to make the 625, 313 and 156 pg/ml solutions.
8. Store at 4° C until use.



Loading the 96-well Plate

9. 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.
10. Aliquot 0.1 ml of the standard solutions of the **Preparation of Standard Solutions** (156-10000pg/ml) into empty wells of the precoated 96-well plate. Duplicate measurements of standards are recommended.
11. 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.
12. Cover the 96-well plate and incubate at 37° C for 90 min.
13. During the **Step 12** 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.
14. Upon completion of the 90 min incubation of **Step 12**, 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.
15. Add 0.1 ml of the Biotinylated 1:100 Antibody Working Solution (prepared in **Step 13**) to each well and incubate the plate at 37° C for 60 min.
16. During the incubation period of **Step 15**, 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.
17. Upon completion of the 60 min incubation of **Step 15**, 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.
18. Add 0.1 ml of prepared ABC Working Solution (prepared in **Step 16**) to each well and incubate the plate at 37° C for 30 min.
19. During the incubation period of **Step 18**, pre-warm TMB Color Developing Agent at 37° C for 30 min before use.
20. Upon completion of the 30 min incubation of **Step 18**, 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.
21. Add 90 µl of the pre-warmed TMB Color Developing Agent into each well and incubate at 37° C for 25-30 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).
22. 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.
23. 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 PAI-1 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.