

INSTRUCTIONS FOR USE

PAI-1 ELISA

Item No. 06489914

The logo for WILEX, featuring the word "WILEX" in a bold, black, sans-serif font. A small red triangle is positioned above the letter "X".

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Manufactured by:
WILEX Inc.
Cambridge, MA 02140 USA

Intended Use

The PAI-1 ELISA is an enzyme-linked immunoassay used to quantitate PAI-1 (plasminogen activator inhibitor, type 1) found in human plasma.

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Method Principle	Solid Phase Sandwich ELISA
Analytical Range	0 ng/mL to 1.5 ng/mL
Specimen Types	Human plasma
Sample Test Volume	100 microliters
Sensitivity	0.10 ng/mL
Purchase of this kit licenses its use under the following patents: United States patent 5,422,245 and European patent 0229116	

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Background

PAI-1 is a 50-kDa glycoprotein member of the serine protease inhibitor (SERPIN) superfamily and is the principal physiological inhibitor of both forms of plasminogen activators (PAs), uPA (urokinase-type plasminogen activator), and tPA (tissue-type plasminogen activator). It is secreted in an active form, which spontaneously converts to a latent form [1], but it can be stabilized in the active form by binding to the plasma protein vitronectin [2,3]. Both tumor cells and capillary endothelial cells express higher levels of PAI-1 than other cell types [4]. It is hypothesized that high levels of PAI-1 serve to protect the tumor stroma from degradation by high amounts of secreted uPA [5,6]. High PAI-1 also may contribute to tumor-induced angiogenesis [7, 8].

Initial studies focused on PAI-1 levels in tumor lysates and cytosols. Since PAI-1 is an inhibitor, it was surprising that increased levels were detected in tumor tissue. For example, a study in 2002 determined PAI-1 to be a strong prognostic indicator in tumors of primary breast cancer patients [9]. Tissue findings were then extended to plasma studies and similar results were seen. Specifically, elevated plasma levels of PAI-1 have been documented in research studies of colon cancer patients [10,11], and reports suggest that PAI-1 may be a potential marker for monitoring disease progression. Another study showed increased levels of PAI-1 above the normal cutoff in 24% of breast cancer plasmas, 30% of colon cancer plasmas, 40% of lung cancer plasmas, and 12% of prostate cancer plasmas [12]. More recently, it was shown that metastatic breast cancer patients who had elevated pretreatment plasma PAI-1 had a shorter overall survival than metastatic breast cancer patients with normal levels of PAI-1 [13].

In order to understand the potential clinical utility of measuring PAI-1 in plasma, additional studies are necessary. Plasma samples can be analyzed for PAI-1 levels using the PAI-1 ELISA. This assay detects all forms of human PAI-1.

The availability of a quantitative, reproducible, standardized assay measuring PAI-1 in plasma allows researchers to examine the potential clinical value of measuring this circulating marker at any time during cancer. Although previous research on PAI-1 levels was conducted using tissue cytosols, plasma sample testing is more convenient and less invasive than tissue cytosol analysis and could provide investigators with the ability to measure this analyte in various cancer types.

Principle of the Assay

4 The PAI-1 ELISA measures all forms of human PAI-1. It is a sandwich-type immunoassay that utilizes a monoclonal antibody to human PAI-1 as the capture reagent. The Capture Antibody has been immobilized on the interior surface of microtiter plate wells. To perform the test, an appropriate volume of specimen is incubated in the coated well to allow binding of the antigen by the Capture Antibody. The immobilized antigen is then reacted with the PAI-1 detector rabbit antiserum. The amount of Detector Antibody bound to antigen is measured by binding it with a goat-anti-rabbit IgG-horseradish peroxidase (HRP) Conjugate. Color development by incubation with OPD (o-phenylenediamine) Substrate enables quantitation of captured PAI-1. The colored

reaction product is quantitated by spectrophotometry and reflects the amount of PAI-1 protein in the sample.

For instructions, see the Detailed Protocol and Evaluation of Results sections of this booklet.

Summary of Procedure

Steps	Incubations
1. Add samples and Standards to wells	3 hours, RT*
2. Wash	
3. Add Detector Antibody to wells	1.5 hours, RT*
4. Wash	
5. Add Conjugate to wells	30 minutes, RT*
6. Wash	
7. Add Substrate to wells	45 minutes, RT* (dark)
8. Add Stop Solution to wells	
9. Read plate at 490 nm	

*Room temperature = (20–27°C)

Materials Provided

Samples and Standards should be assayed in duplicate. A standard curve must be included each time samples are analyzed. The following ELISA components are supplied:

Microtiter plate—One (1) precoated microtiter plate supplied ready to use, with 96 wells (12 strips of eight wells) in a zip-lock bag with a desiccant pack. Wells are coated with an anti-PAI-1 monoclonal antibody.

PAI-1 Standards—Six (6) separate vials containing PAI-1. Standards levels have been calibrated in nanograms of PAI-1 per mL using highly purified PAI-1.

Standard#	Concentration	Volume/Vial
6	1.50 ng/mL	1 mL
5	1.00 ng/mL	1 mL
4	0.60 ng/mL	1 mL
3	0.30 ng/mL	1 mL
2	0.10 ng/mL	1 mL
1	0.00 ng/mL	1 mL

Sample Diluent—One (1) bottle containing 100 mL of buffer containing BSA and 0.1% sodium azide.

Detector Antibody—One (1) bottle supplied ready to use, containing 12 mL of rabbit anti-PAI-1 antiserum in 0.01 M PBS (pH 7.4), a protein stabilizer, and 0.1% sodium azide.

Conjugate Diluent—One (1) bottle containing 12 mL of 0.01 M PBS (pH 7.4), BSA, and 0.01% chloroacetamide.

Conjugate Concentrate—One (1) vial containing 0.4 mL of 50X goat-anti-rabbit IgG-horseradish peroxidase in buffer. Must be diluted to 1X with Conjugate Diluent to make Working Conjugate. See **Table 1**, p. 16.

Substrate Diluent—Two (2) bottles of 9 mL each containing 0.1 M citrate buffer (pH 5.0) and 0.01% H₂O₂ (hydrogen peroxide).

Substrate Tablets—One (1) vial containing eight (8) OPD tablets. These must be dissolved in Substrate Diluent (1 tablet/2 mL) to make Working Substrate. See **Table 1**, p. 16.

Stop Solution—One (1) bottle supplied ready to use, containing 12 mL of 2.5 N H₂SO₄ (sulfuric acid).

Platwash Concentrate (20X)—One (1) 100-mL bottle. Dilute one (1) part concentrate in 19 parts high-quality deionized water prior to use.

Materials Required but Not Provided

- Pipettors: 2–20 μL , 20–200 μL , and 200–1000 μL precision pipettors with disposable tips
- Precision repeating pipettor
- Wash bottle or multichannel dispenser for plate washing
- Microcentrifuge, ultracentrifuge, and tubes for sample preparation
- Vortex mixer
- Plate reader or spectrophotometer capable of measuring absorbance in 96-well plates at a wavelength of 490 nm
- 500- or 1000-mL graduated cylinder
- Reagent reservoirs
- Deionized water of high quality
- Plastic wrap or adhesive plate sealers
- Liquid household bleach for inactivating clinical specimens and decontamination of plate washer
- Disposable paper towels or lint-free laboratory wipes

PAI-1 ELISA Controls—Controls have been developed to provide customers with control material for quality monitoring of day-to-day assay performance. PAI-1 ELISA Controls consisting of PAI-1 in buffer are sold separately. Refer to PAI-1 ELISA Controls, Item No. 06489922, when ordering. Controls are supplied ready to use. Volumes are 1.0 mL each. Store undiluted PAI-1 ELISA Controls at 2–8°C. Protect from light.

Precautions and Recommendations

- Store components at 2–8°C. Do not expose reagents to excessive light. Do not freeze any of the kit components.
- Do not use kit components beyond the indicated kit expiration date.
- Use only the microtiter wells provided with the kit.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents used in this kit contain either sodium azide or chloroacetamide as preservatives. Care should be taken to avoid direct contact with these reagents.
- Do not mouth pipet or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.

- Human samples may be contaminated with infectious agents. Do not ingest, expose to open wounds, or breathe aerosols. Wear protective gloves and dispose of biological samples properly.
- Do not handle the Substrate Tablets with fingers or permit contact with skin, metal, or oxidizing agents. Dispose of OPD-containing solutions in compliance with local regulations.
- Wear disposable gloves and eye protection when handling Stop Solution (2.5 N sulfuric acid).

Sample Preparation

PLASMA

Remove all flocculent material from specimens by centrifugation in a tabletop microcentrifuge. Dilute plasma samples in Sample Diluent to 1:50 before assay. Add 100 μ L of diluted sample in duplicate to the microtiter plate.

Detailed Protocol

RECOMMENDED PROCEDURES

1. Addition of reagents must be in the order specified.
2. All six (6) Standards and the test specimens should be run in duplicate. For greater accuracy, test each sample at more than one concentration. Change tips during this process. Avoid carry-over of one Standard into another.

3. Before addition of the Detector Antibody, Conjugate, or Substrate, equilibrate all reagents to room temperature (20–27°C) for at least 10 minutes prior to use.
4. CAUTION: When inverting the microtiter plate to decant or blot, press the side tabs of the frame inward to prevent the strips from falling out. Fill the open portion of the frame with uncoated or used strips when mechanical washers are used.
5. Preparation of Platewash:
 - a. If the Platewash Concentrate is cold, allow it to reach room temperature (20–27°C) before use (approximately 45 minutes). Make sure all crystals are dissolved. If necessary, warm at 37°C and mix or shake.
 - b. Dilute one (1) volume of Platewash Concentrate with 19 volumes of distilled or deionized water. Mix well. This solution is Platewash. The total volume required will depend on the washing method/instrument used. Approximately 1 L of this solution is required to prime an automated washer and run one microtiter plate; about 700 mL is required for each microtiter plate when manual washing is performed.
 - c. Platewash must be freshly prepared each day. Do not store Platewash.
6. Microtiter plate washing may be automated, semi-automated, or manual but must be carried out with care to ensure optimal performance of the assay. Plate washing equipment must be properly adjusted, cleaned, and maintained. Whichever method is used, the solution used to wash plates must be Platewash:

- a. Automatic microtiter plate washer—Set the fill volume to 300 μL /well. Prime the instrument with Platewash. Use two 3-cycle washes. After the initial 3-cycle wash, rotate the plate 180° and repeat.
 - b. Manual microtiter plate washer—Wash six times, using 300 μL per well per wash. Fill the entire plate, then aspirate in the same order.
 - c. Hand-Held Syringe—Wash six times, using 300 μL per well per wash. Blot the plate upside-down between washes.
7. After the final wash, invert the microtiter plate and tap it on an absorbent surface to remove excess liquid. Wells should not be completely dry. Residual liquid protects the reagents from desiccation. This is particularly important for maintaining HRP enzyme activity.

Assay Procedure

1. Remove the microtiter plate from the bag. From the number of samples to be tested, calculate the number of strips required. (Remember that each sample dilution or Standard requires two wells, and two wells are needed for Substrate blank determination. The standard curve requires 12 wells.) Store unused strips in the zip-lock bag with desiccant at 2–8°C (see Reagent Stability and Storage).

2. Dilute samples in Sample Diluent. (See Sample Preparation. Do NOT dilute Standards.)
3. Briefly, without foaming, mix the Standard or sample dilution and add 100 μL each to duplicate wells. Set up four wells with the 0 ng/mL Standard, two to measure the background absorbance and two for use as the Substrate blank wells.
4. Cover the microtiter plate with a piece of plastic wrap or plate sealer and **incubate for 3 hours at room temperature (20–27°C)**.
5. Remove the plastic wrap or plate sealer and wash the microtiter plate with Platewash as in Step 6 in Detailed Protocol.
6. Add 100 μL of Detector Antibody to all wells **except the Substrate blank wells. Incubate at room temperature (20–27°C) for 1.5 hours.**
7. During the incubation with Detector Antibody, prepare Working Conjugate 15–30 minutes prior to use by diluting 50X Conjugate Concentrate 1:50 in Conjugate Diluent, and dispense into a clean reagent reservoir. See **Table 1**, p. 16.
8. Wash the microtiter plate with Platewash.
9. Add 100 μL of Working Conjugate prepared in Step 7 to all wells **except the Substrate blank wells. Incubate for 30 minutes at room temperature (20–27°C)**.

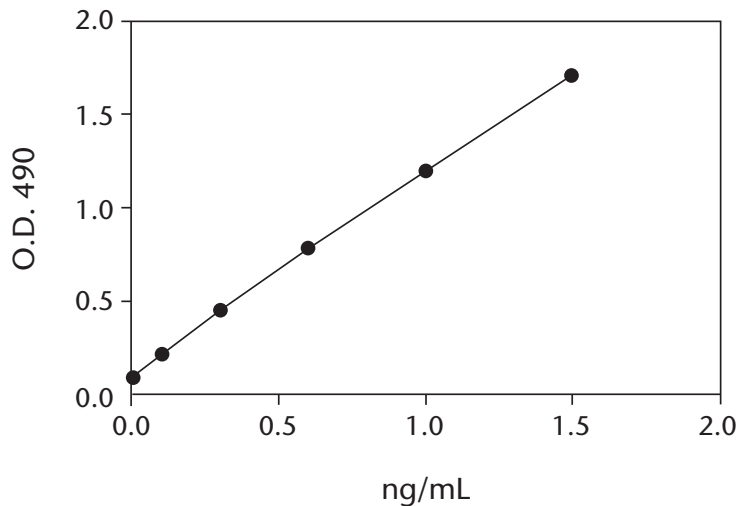
10. During the incubation with Conjugate, prepare Substrate by dissolving Substrate Tablets in Substrate Diluent. See **Table 1**, p. 16, for the quantities to use for the number of strips being run. During resuspension, use dark opaque or foil-covered tubes to prevent light leakage. Vortex vigorously to assure complete solubilization. Once prepared, Substrate should be used within 30 minutes. Avoid exposure to light.
11. Wash the microtiter plate with Platewash.
12. Including the Substrate blank wells, add 100 μ L of Substrate to all wells. **Incubate the microtiter plate in the dark at room temperature (20–27°C) for 45 minutes.**
13. Add 100 μ L of Stop Solution to each well to stop the reaction.
14. Read the absorbance at 490 nm within 30 minutes. If plate is not read immediately after Stop Solution is added, keep plate in the dark at room temperature (20–27°C) until the absorbance is read.

TABLE 1. PAI-1 ELISA—PREPARATION OF ASSAY REAGENTS

# Strips Used	Conj. Concentrate	Conj. Diluent	Substrate Tablets	Substrate Diluent
1	20 μ L	0.98 mL	2	4 mL
2	40 μ L	1.96 mL	2	4 mL
3	60 μ L	2.94 mL	2	4 mL
4	80 μ L	3.92 mL	2	4 mL
5	100 μ L	4.90 mL	4	8 mL
6	120 μ L	5.88 mL	4	8 mL
7	140 μ L	6.86 mL	4	8 mL
8	160 μ L	7.84 mL	4	8 mL
9	180 μ L	8.82 mL	6	12 mL
10	200 μ L	9.80 mL	6	12 mL
11	220 μ L	10.78 mL	6	12 mL
12	240 μ L	11.76 mL	6	12 mL

Figure 1

Sample Standard Curve



Evaluation of Results

CONCENTRATION OF STANDARDS

PAI-1 Standards have been calibrated in mass units (nanograms/mL) using a preparation of immunoaffinity purified PAI-1.

CONCENTRATION OF UNKNOWNNS

1. Average the absorbance values for each Standard and specimen dilutions to obtain the mean absorbance.
2. Determine the concentration of PAI-1 for each specimen dilution by interpolation from the standard curve. Software packages are available (such as SoftMax Pro™ from Molecular Devices, Sunnyvale, CA and KC4™, Bio-Tek Instruments, Inc., Winooski, VT) that can simplify this process. Use a quadratic (second order polynomial) curve fitting algorithm.

NOTE: Do not assign “blank” wells using software. This will subtract the average blank readings from all other wells. It is useful for quality control and troubleshooting purposes to be able to inspect the absorbance values reported for all wells without adjustments applied to the raw data.

3. Results for plasma samples and Controls can be expressed in ng/mL in the original sample by correcting the value obtained from the standard curve by the dilution factor used in the assay.

Sample Values

The levels listed should be used as a guideline only. The determination of normal ranges should be carried out by each laboratory using appropriate samples.

SAMPLE GROUP: HEALTHY MALES AND FEMALES (N=100)

Mean recovery of PAI-1:	Recovery range:
25.9 ng/mL plasma	5.6–150 ng/mL plasma

Assay Characteristics

SENSITIVITY

The PAI-1 ELISA will detect 0.10 ng/mL of PAI-1 in the sample tested. The signal of the 0.10 ng/mL Standard is greater than two times the zero (background) signal.

SPECIFICITY

The PAI-1 ELISA is capable of quantitating PAI-1 in the active and latent forms as well as in complex with tPA and uPA. The assay has been tested for cross-reactivity by challenging with PAI-2, tPA, and Ovalbumin, which show no cross-reactivity at high-challenge doses. Also, the assay has been analyzed for parallel response of tumor samples over a range of concentrations and shows excellent agreement for replicate sample dilutions.

RECOVERY

Two individual 1-mL samples of EDTA plasma were spiked with PAI-1, one at a final dose of 50 ng/mL plasma, and the second at 25 ng/mL. Replicate preparations of the above spiked samples were made in Sample Diluent. All samples were assayed at a starting dilution of 1:50. After correction for endogenous PAI-1 levels measured in the unspiked samples, the recovery was determined to be 99% of the Sample Diluent Control at the 50-ng dose and 101% at the 25-ng dose.

Troubleshooting

A. ASSAY DOES NOT DEVELOP COLOR OR ODs ARE LOW

- Plate allowed to dry out after Conjugate step.
- Step(s) omitted or in wrong sequence.
- Room temperature during incubations fell below 20°C.
- Substrate was not warmed to room temperature (20–27°C) prior to addition to wells.

B. HIGH BACKGROUND SIGNAL

- Insufficient washing between steps.
- The Substrate blank well should read ≤ 0.08 absorbance units. Zero Standard should read ≤ 0.13 absorbance units. Higher readings indicate deterioration of Substrate or exposure of Substrate to light before or during the incubation step.

- Be certain the plate is read at the correct wavelength.

C. POOR DUPLICATES

- Insufficient washing, especially when accompanied by high background. Take special care when washing plates by hand, or have automatic washer serviced.
- Sporadic high signals may indicate contamination of Substrate by Conjugate. Be sure to use a fresh piece of plastic wrap or adhesive plate sealer for this step. Residual droplets of Conjugate on re-used plastic wrap or adhesive plate sealer may lead to false positive signals.
- Generating bubbles in wells on addition of reagents. Use care in pipetting.
- Splashing of reagents between wells will lead to erroneous results. Avoid jarring the plate.

Reagent Stability and Storage

All of the reagents included in the PAI-1 ELISA have been tested for stability. Reagents should not be used beyond the stated expiration date. Kit reagents should be stored at 2–8°C with the exception of Stop Solution and Platewash Concentrate, which may be stored at room temperature (20–27°C). Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

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