

### INTENDED USE

This dog PAI-1 activity assay is intended for the quantitative determination of active plasminogen activator inhibitor type 1 (PAI-1) in dog plasma. **For research use only.**

### BACKGROUND

Plasminogen activator inhibitor-1 (PAI-1) is a central regulator of the blood fibrinolytic system [1]. Clinical studies have indicated that increased PAI-1 levels increase the risk for thrombosis, whereas decreased levels may cause recurrent bleeding [2].

### ASSAY PRINCIPLE

Functionally active PAI-1 present in plasma reacts with urokinase coated and dried on a microtiter plate. Latent or complexed PAI-1 will not bind to the plate and will not be detected. After appropriate washing steps, primary antibody binds to the captured protein. Excess antibody is washed away and bound polyclonal antibody is reacted with horseradish peroxidase conjugated secondary antibody. TMB substrate is used for color development at 450nm. A standard calibration curve is prepared along with the samples to be measured using dilutions of dog PAI-1. The amount of color development is directly proportional to the concentration of active PAI-1 in the sample.

### REAGENTS PROVIDED

- **96-well urokinase coated microtiter strip plate** (removable wells 8x12) containing urokinase, blocked and dried.
- **10X Wash buffer:** 1 bottle of 50ml
- **Dog PAI-1 standard:** 1 vial lyophilized standard
- **Anti-dog PAI-1 primary antibody:** 1 vial lyophilized monoclonal antibody
- **Anti-mouse horseradish peroxidase secondary antibody:** 1 vial concentrated HRP labeled antibody
- **TMB substrate solution:** 1 bottle of 10ml solution

### STORAGE AND STABILITY

Store all kit components at 4°C upon arrival. Return any unused microplate strips to the plate pouch with desiccant. Reconstituted standard and primary may be stored at -80°C for later use. Do not freeze-thaw the standard and primary antibody more than once. Store all other unused kit components at 4°C. This kit should not be used beyond the expiration date.

### OTHER REAGENTS AND SUPPLIES REQUIRED

- Microtiter plate shaker capable of 300 rpm uniform horizontally circular movement
- Manifold dispenser/aspirator or automated microplate washer
- Microplate reader capable of measuring absorbance at 450 nm
- Pipettes and Pipette tips
- Deionized or distilled water
- Polypropylene tubes for dilution of standard
- Paper towels or laboratory wipes
- 1N H<sub>2</sub>SO<sub>4</sub> or 1N HCl
- Bovine Serum Albumin Fraction V (BSA)
- Tris(hydroxymethyl)aminomethane (Tris)
- Sodium Chloride (NaCl)

### PRECAUTIONS

- FOR LABORATORY RESEARCH USE ONLY. NOT FOR DIAGNOSTIC USE.
- Do not mix any reagents or components of this kit with any reagents or components of any other kit. This kit is designed to work properly as provided.
- Always pour peroxidase substrate out of the bottle into a clean test tube. Do not pipette out of the bottle as contamination could result.
- Keep plate covered except when adding reagents, washing, or reading.
- DO NOT pipette reagents by mouth and avoid contact of reagents and specimens with skin.
- DO NOT smoke, drink, or eat in areas where specimens or reagents are being handled.

## PREPARATION OF REAGENTS

- TBS buffer:** 0.1M Tris, 0.15M NaCl, pH 7.4
- Blocking buffer (BB):** 3% BSA (w/v) in TBS
- 1X Wash buffer:** Dilute 50ml of 10X wash buffer concentrate with 450ml of deionized water

## SAMPLE COLLECTION

Collect 9 volumes of blood in 1 volume of 0.1M trisodium citrate, acidified citrate, or EDTA. Immediately after collection of blood, samples must be centrifuged at 3000Xg for 15 minutes. It is important to ensure a platelet free preparation since platelets can release PAI-1 [1,4,6]. The plasma must be transferred to a clean plastic tube and stored on ice prior to analysis. Collected samples should be stable for up to 24 hours or stored at -20°C for up to one month and thawed three times without loss of PAI-1 activity.

## ASSAY PROCEDURE

Perform assay at room temperature. Vigorously shake plate (300rpm) at each step of the assay.

### Preparation of Standard

Reconstitute standard by adding 1ml of blocking buffer directly to the vial and agitate gently to completely dissolve contents. This will result in a 1000ng/ml standard solution.

Dilution table for preparation of dog PAI-1 standard:

PAI-1 concentration (ng/ml)	Dilutions
100	900µl (BB) + 100µl (from vial)
50	500µl (BB) + 500µl (100ng/ml)
20	600µl (BB) + 400µl (50ng/ml)
10	500µl (BB) + 500µl (20ng/ml)
5	500µl (BB) + 500µl (10ng/ml)
2	600µl (BB) + 400µl (5ng/ml)
1	500µl (BB) + 500µl (2ng/ml)
0.5	500µl (BB) + 500µl (1ng/ml)
0.2	600µl (BB) + 400µl (0.5ng/ml)
0	500µl (BB) Zero point to determine background

**NOTE: DILUTIONS FOR THE STANDARD CURVE AND ZERO STANDARD MUST BE MADE AND APPLIED TO THE PLATE IMMEDIATELY.**

## Standard and Unknown Addition

Remove microtiter plate from bag and add 100µl PAI-1 standards (in duplicate) and unknowns to wells. Carefully record position of standards and unknowns. Shake plate at 300rpm for 30 minutes. Wash wells three times with 300µl wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

NOTE: The assay measures active PAI-1 in the 0.2-100 ng/ml range. If the unknown is thought to have high PAI-1 levels, dilutions may be made in blocking buffer. No dilution is recommended for normal dog plasma.

### Primary Antibody Addition

Reconstitute primary antibody by adding 11ml of blocking buffer directly to the vial and agitate gently to completely dissolve contents. Add 100µl to all wells. Shake plate at 300rpm for 30 minutes. Wash wells three times with 300µl wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

### Secondary Antibody Addition

Briefly centrifuge vial before opening. Dilute 2µl of conjugated secondary antibody in 10ml of blocking buffer and add 100µl to all wells. Shake plate at 300rpm for 30 minutes. Wash wells three times with 300µl wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

### Substrate Incubation

Add 100µl TMB substrate to all wells and shake plate for 2-10 minutes. Substrate will change from colorless to different strengths of blue. Quench reaction by adding 50µl of 1N H<sub>2</sub>SO<sub>4</sub> or HCl stop solution to all wells when samples are visually in the same range as the standards. Add stop solution to wells in the same order as substrate upon which color will change from blue to yellow. Mix thoroughly by gently shaking the plate.

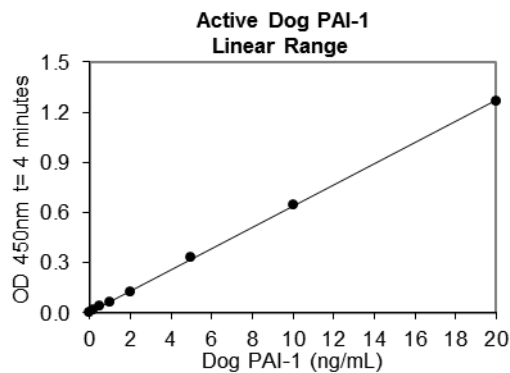
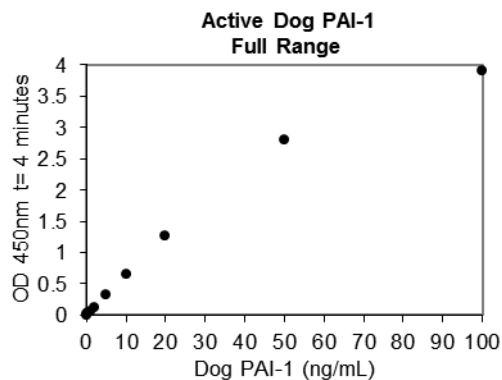
### Measurement

Set the absorbance at 450nm in a microtiter plate spectrophotometer. Measure the absorbance in all wells at 450nm. Subtract zero point from all standards and unknowns to determine corrected absorbance (A<sub>450</sub>).

## Calculation of Results

Plot  $A_{450}$  against the amount of PAI-1 in the standards. Fit a straight line through the linear points of the standard curve using a linear fit procedure if unknowns appear on the linear portion of the standard curve. Alternatively, create a standard curve by analyzing the data using a software program capable of generating a four parameter logistic (4PL) curve fit. The amount of PAI-1 in the unknowns can be determined from this curve. If samples have been diluted, the calculated concentration must be multiplied by the dilution factor.

A typical standard curve (EXAMPLE ONLY):



## EXPECTED VALUES

Abnormalities in PAI-1 levels have been reported in the following conditions:

- Vascular thrombosis: Increased PAI-1 levels may contribute to venous thrombosis [1].
- Myocardial Infarction: Increased PAI-1 levels may contribute to myocardial infarction [1].
- Endotoxemia: Endotoxin induces a large increase in PAI-1 levels (80-fold) [4].
- Hyperglycemia, hyperinsulinemia, and insulin resistance: Elevated PAI-1 levels in obese and diabetic mice contribute to these metabolic disorders [5,6].

## PERFORMANCE CHARACTERISTICS

**Sensitivity:** The minimum detectable dose (MDD) was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates (range  $OD_{450}$ : 0.046-0.062) and calculating the corresponding concentration. The MDD was 0.104 ng/ml.

**Intra-assay Precision:** These studies are currently in progress. Please contact us for more information.

**Inter-assay Precision:** These studies are currently in progress. Please contact us for more information.

**Recovery:** These studies are currently in progress. Please contact us for more information.

**Linearity:** These studies are currently in progress. Please contact us for more information.

**Specificity:** This assay recognizes natural and recombinant active dog PAI-1. Pooled normal plasma from human and pig was assayed and significant cross-reactivity was observed.

**Sample Values:** Samples were evaluated for the presence of the antigen at varying dilutions.

Sample Type	Dilution	Mean (ng/mL)
Mixed Breed EDTA Plasma	Undiluted	41.43
Beagle EDTA Plasma	Undiluted	7.58

## DISCLAIMER

This information is believed to be correct but does not claim to be all-inclusive and shall be used only as a guide. The supplier of this kit shall not be held liable for any damage resulting from handling of or contact with the above product.

## REFERENCES

1. Eitzman DT, *et al.*: Blood. 2000, 95(2): 577-580.
2. Kawasaki T, *et al.*: Blood. 2000, 96(1): 153-160.
3. Declerck PJ, *et al.*: Blood. 1988, 71(1): 220-225.
4. Declerck PJ, *et al.*: Thromb Haemostas. 1995, 74(5): 1305-9.
5. Schafer K, *et al.*: FASEB. 2001, 15: 1840-2.
6. Samad F, *et al.*: PNAS. 1996, 96(12): 6902-7.

### Example of ELISA Plate Layout

96 Well Plate: 20 Standard wells, 76 Sample wells

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.2 ng/ml	0.5 ng/ml	1 ng/ml	2 ng/ml	5 ng/ml	10 ng/ml	20 ng/ml	50 ng/ml	100 ng/ml		
B	0	0.2 ng/ml	0.5 ng/ml	1 ng/ml	2 ng/ml	5 ng/ml	10 ng/ml	20 ng/ml	50 ng/ml	100 ng/ml		
C												
D												
E												
F												
G												
H												

SAMPLE INSERT  
Refer to kit box for  
lot specific instructions