Enzyme Immunoassay for
Porcine Tissue Plasminogen Activator (tPA) Activity

For Research Use Only

INTRODUCTION

This porcine tPA activity assay is intended for the quantitative determination of active tissue plasminogen activator in porcine plasma and other biological fluids.

Tissue plasminogen activator is a serine protease that catalyzes the activation of plasminogen to plasmin[1]. Clinical studies have indicated that high tPA levels may increase the risk for thrombosis[2], whereas decreased levels may cause neuronal plasticity and degeneration[3].

PRINCIPLES OF PROCEDURE

Functionally active tPA will form a covalent complex with the biotinylated porcine PAI-1 which is bound to the avidin on the plate. After appropriate washing steps, polyclonal anti-porcine tPA primary antibody binds to the captured tPA. Excess antibody is washed away and bound polyclonal antibody is then reacted with the secondary antibody conjugated to horseradish peroxidase. Following an additional washing step, TMB is used for color development at 450nm. The amount of color development is directly proportional to the concentration of active tPA in the sample.

MATERIALS PROVIDED

Component	Contents	Quantity	Storage	Cat. No.
Coated Plate	Avidin coated 96-well plate	1 plate	4°C	UP40a
Standard	Porcine tPA activity standard, lyophilized	1 vial	4°C	UP40b
Assay Buffer	Buffer used to raise the pH of samples	10 mL	4°C	UP40c
Wash Buffer	10x solution for washing plate	50 mL	4°C	UP40d
Biotinylated PAI-1	Biotinylated porcine PAI-1, lyophilized	1 vial	4°C	UP40e
Primary Antibody	Rabbit anti-porcine tPA antibody, lyophilized	1 vial	4°C	UP40f
Secondary Antibody	Anti-rabbit HRP conjugated antibody	1 vial	4°C	UP40g
Substrate	TMB substrate	10 mL	4°C	UP40h

MATERIALS NEEDED BUT NOT PROVIDED

1. Pipettes covering 0-10 μl and 200-1000 μl and tips
2. 12-channel pipette covering 30-300 μl
3. 1N H_2SO_4
4. DI water
5. Microtiter plate spectrophotometer with a 450 nm filter
6. Microtiter plate shaker with uniform horizontally circular movement up to 300 rpm
7. TBS Buffer (see Reagent Preparation)
8. Blocking Buffer (see Reagent Preparation)

STORAGE CONDITIONS

1. Store this kit and its components at 4°C until use.
2. Reconstituted standards and primary may be stored at -70°C for later use. **DO NOT** freeze/thaw the Standards and Primary Antibody more than once.

PROCEDURAL NOTES

1. Use aseptic technique when opening and dispensing reagents.
2. This kit is designed to work properly as provided and instructed. Additions, deletions, or substitutions to the procedure or reagents are not recommended, as they may be detrimental to the assay.
3. Exercise universal precautions during the performance or handling of this kit or any component contained therein.

SAMPLE COLLECTION, STORAGE AND PREPARATION

Samples of porcine plasma in citrate or EDTA may be assayed with this kit. Plasma in heparin is not recommended. It is important to ensure a platelet free preparation as platelets can release PAI-1, which in turn could potentially form a complex with active tPA. Serum and cell culture media at neutral pH may also be used.

For best results, collect 9 volumes of blood in 1 volume of 0.1M acidified citrate. The low pH of the resulting plasma insures that PAI-1 is inhibited from quenching tPA activity[5,6]. Immediately after collection of blood, samples must be centrifuges at 2500Xg for 15 minutes. The plasma must be transferred to a clean plastic tube and stored on ice prior to analysis. The tPA activity samples are stable for up to 5 hours on ice, up to one month frozen at -20°C, or up to 5 months frozen at -70°C.

This assay measures active tPA in the 0.02-10 ng/mL range. If the sample is thought to have high tPA levels, dilutions may be made in a similar biological fluid devoid of tPA or in Blocking Buffer.

REAGENT PREPARATION

1. **TBS Buffer:** 0.10 M TRIS, 0.15 M NaCl, pH 7.4
2. **Blocking Buffer:** 3% BSA in TBS Buffer.
3. **10x Wash Buffer:** Dilute the 50 mL of concentrate to 1x with 450 mL of DI water prior to use.
4. **Biotinylated PAI-1:** Reconstitute with Blocking Buffer as directed on the vial and vortex gently to mix. Prepare immediately prior to use.
5. **Standard:** Reconstitute with Blocking Buffer as directed on the vial and vortex gently to mix. Prepare according to include Standard Dilution Table immediately prior to use.
6. **Primary Antibody:** Reconstitute with Blocking Buffer as directed on the vial and vortex gently to mix. Prepare immediately prior to use.
7. **Secondary Antibody:** Dilute with Blocking Buffer as directed on the vial and vortex gently to mix. Prepare immediately prior to use.

STANDARD PREPARATION

Reconstitute the Standard according to the attached dilution table. **Do not prepare the Standards until you are ready to apply them to the plate.**

ASSAY PROCEDURE

1. Add 100 μL of diluted Biotinylated PAI-1 to each well. Shake the plate at 300 rpm for 30 minutes.
2. Wash wells according to the following wash procedure:
   a. Remove contents of the plate by inversion into an appropriate disposal device.
   b. Tap out remaining contents of the plate onto a lint free paper towel.
   c. Add 300 µL of Wash Buffer to each well.
   d. Let stand for 2-3 minutes.
   e. Remove contents of the plate by inversion into an appropriate disposal device.
   f. Repeat procedure 2 more times and proceed to step "g".
   g. Tap out the remaining contents of the plate onto a lint free paper towel and proceed to step 3.
3. Add 100 µL of standards or samples to each well. See **Scheme I** for a suggested plate layout. Shake the plate at 300 rpm for 30 minutes.

   NOTE: Samples that are at neutral pH can be added straight to the plate. If the pH of the samples is below pH 6.0, first add 40 μl of Assay Buffer to all wells, then add 60 μl of standards and samples to each well.
4. Wash wells according to step 2.
5. Add 100 µL of diluted Primary Antibody to each well. Shake the plate at 300 rpm for 30 minutes.
6. Wash wells according to step 2.
7. Add 100 µL of diluted Secondary Antibody to each well. Shake the plate at 300 rpm for 30 minutes.
8. Wash wells according to step 2.
9. Add 100 µL of TMB Substrate to each well and incubate for 2-5 minutes with shaking.
10. Stop the reaction with 50 µL per well of 1 N H_2SO_4 and read plate at 450 nm.

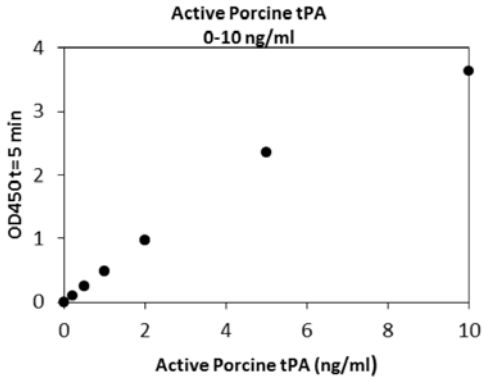
Scheme I:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S_0	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	U_1
B	S_0	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	U_1
C	U_2	U_3	U_4	U_5	U_6	U_7	U_8	U_9	U_{10}	U_{11}	U_{12}	U_{13}
D	U_2	U_3	U_4	U_5	U_6	U_7	U_8	U_9	U_{10}	U_{11}	U_{12}	U_{13}
E	U_{14}	U_{15}	U_{16}	U_{17}	U_{18}	U_{19}	U_{20}	U_{21}	U_{22}	U_{23}	U_{24}	U_{25}
F	U_{14}	U_{15}	U_{16}	U_{17}	U_{18}	U_{19}	U_{20}	U_{21}	U_{22}	U_{23}	U_{24}	U_{25}
G	U_{26}	U_{27}	U_{28}	U_{29}	U_{30}	U_{31}	U_{32}	U_{33}	U_{34}	U_{35}	U_{36}	U_{37}
H	U_{26}	U_{27}	U_{28}	U_{29}	U_{30}	U_{31}	U_{32}	U_{33}	U_{34}	U_{35}	U_{36}	U_{37}

CALCULATIONS

1. Average the O.D. values for each pair of duplicate wells.
2. Plot the A_{450} against the concentration of uPA in the standards.
3. Fit a straight line through the points using a linear fit procedure.
4. Calculate the uPA concentrations in the unknowns using the standard curve. See Figure 1 on the following page for an example of a typical standard curve.

Figure 1: Typical Standard Curve
(Do Not Use For Calculations)



EXPECTED VALUES

The basal level of tPA activity in experimental pigs was found to be 2 IU/ml (n=6)[8]. 1 IU = 1.64 ng of tPA based on the WHO International Standard for Human tPA. The control plasma concentration of tPA antigen in a porcine model of cardiopulmonary bypass was 1.69 ng/ml (range=1.06-2.73, n=10)[9]. The control plasma concentration of tPA antigen in a porcine model of sepsis varied by collection site[10]:

Site	Mean	SEM (ng/mL)
Aortic Artery	11.1	1.7
Pulm. Artery	12.5	1.7
Hepatic Vein	8.9	1.3
Portal Vein	15.9	2.1
Renal Vein	13.1	2.1

Abnormalities in tPA levels have been reported in the following conditions:

- Neuronal plasticity and degeneration: Decreased levels of tPA have been implicated in the process of neuronal plasticity and degeneration[1,3].
- Arthritis: Decreased tPA levels may exacerbate arthritis[4].
- Deep venous thrombosis: Increased tPA levels may contribute to deep venous thrombosis[2].
- Coronary heart disease: Increased tPA levels may contribute to severe coronary heart disease[2].
- Pregnancy: Increased tPA levels are observed during pregnancy[7].

REFERENCES

1. Sallés, Fernando J., *et al.*: (2002) J. Neuroscience **22(6)**:2125-2134
2. Wiman, Björn, *et al.*: (1984) J. Biol. Chem. **259(6)**:3644-47
3. Hastings, Gregg A., *et al.*: (1997) J. Biol. Chem. **272(52)**:33062-33067
4. Yang, Y.H., *et al.*: (2001) J. Immunol **167(2)**:1047-52
5. Ranby, M., *et al.*: (1989) Thromb Haemost **62(3)**:917-22
6. Chmielewska, J., *et al.*: (1986) Clin. Chem. **32(3)**:482-5
7. Kruithof, E.K., *et al.*: (1987) Blood **69(2)**:460-466
8. Aspelin, T., *et al.*: (2005) J. Thromb. Haemost. **3(9)**:1947-1954
9. Adrian, K., *et al.*: (2006) Artif. Organs **30(12)**:922-928
10. Nyberg, A., *et al.*: (2003) Acta. Anaesthesiol. Scand. **47**:1125-1131

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