

Rat tPA Activity Assay

Strip well format. Reagents for up to 96 tests.

For Research Use Only.

INTENDED USE

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

BACKGROUND

Tissue plasminogen activator (tPA) is a serine protease that converts plasminogen to the active serine protease plasmin in the blood fibrinolytic system [1,2]. It also plays an important role in the removal of incipient thrombi [3]. tPA is widely used for the thrombolytic treatment of acute myocardial infarction [3].

ASSAY PRINCIPLE

Functionally active rat tPA will form a covalent complex with the biotinylated human PAI-1 which is bound to the avidin on the plate. Only free active tPA will react with the PAI-1 bound to the plate. After appropriate washing steps, polyclonal anti-murine 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 substrate is used for color development at 450nm. The amount of color development is directly proportional to the concentration of active tPA in the sample.

REAGENTS PROVIDED

- ◆ **Avidin Coated Plate:**
1-96 well immulon strip plate (8x12 removable strips) coated with avidin, blocked, and dried
- ◆ **10X Wash Buffer:**
1 bottle of 50mL wash; bring up to 1X using DI water
- ◆ **10X TBS Buffer pH 7.4:**
1 vial of 5ml TBS buffer
- ◆ **Biotinylated PAI-1:**
1 vial of lyophilized biotinylated PAI-1
- ◆ **Rat tPA activity standard:**
1 vial of lyophilized standard
- ◆ **Anti-murine tPA primary antibody:**
1 vial of lyophilized monoclonal antibody
- ◆ **Anti-mouse secondary antibody:**
1 vial of concentrated horseradish peroxidase conjugated antibody
- ◆ **TMB substrate solution:**
1 bottle of 10 ml solution

STORAGE AND STABILITY

All kit components must be stored at 4°C. Store unopened plate and any unused microtiter strips in the pouch with desiccant. Reconstituted reagents may be stored at -70°C for later use. **DO NOT** freeze/thaw the reagents more than once. All other unused kit components must be stored at 4°C. Kit should be used no later than the expiration date.

REAGENTS AND EQUIPMENT REQUIRED

- 1-channel pipettes covering 1-10µl, 20-200µl, 200-1000µl and 500-5000µl
- 12-channel pipette for 30-300µl
- Paper towels or kimwipes

- 1.5ml microcentrifuge tubes
- 1N H₂SO₄
- DI water
- Magnetic stirrer and stir-bars
- Plastic containers with lids
- TBS buffer
- Blocking buffer
- Microtiter plate spectrophotometer operable at 450nm
- Microtiter plate shaker with uniform horizontally circular movement up to 300rpm

WARNINGS

Warning – Avoid skin and eye contact when using TMB One substrate solution since it may be irritating to eyes, skin, and respiratory system. Wear safety goggles and gloves.

PRECAUTIONS

- 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.
- DO NOT** pipette reagents by mouth.
- Always pour substrate out of the bottle into a clean test tube. **DO NOT** pipette out of the bottle as you could contaminate the substrate.
- Keep plate covered except when adding reagents, washing, or reading.
- DO NOT** smoke, drink, or eat in areas where specimens or reagents are being handled.

PREPARATION OF REAGENTS

- TBS buffer:** 0.1M Tris-HCl 0.15M NaCl, pH 7.4
- Blocking buffer (BSA):** 3% BSA in TBS buffer

SPECIMEN COLLECTION

Samples of rat 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 tPA. Serum and cell culture media at neutral pH may also be used.

ASSAY PROCEDURE

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

Biotinylated Human PAI-1 Addition:

Remove microtiter plate from bag. Add 10ml of 3% BSA blocking buffer directly to the biotinylated human PAI-1 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.

Preparation of Standard:

Prepare the tPA standard according to the dilution table. Reconstitute standard as directed on vial to give a 1,000ng/ml standard solution.

tPA concentration (ng/ml)	Dilutions
50	950µl (BSA) + 50µl (standard from vial)
25	500µl (BSA) + 500µl (50ng/ml)
10	600µl (BSA) + 400µl (25ng/ml)
5	500µl (BSA) + 500µl (10ng/ml)
2	600µl (BSA) + 400µl (5 ng/ml)
1	500µl (BSA) + 500µl (2 ng/ml)
0.5	500µl (BSA) + 500µl (1ng/ml)
0.25	500µl (BSA) + 500µl (0.5ng/ml)
0.1	600µl (BSA) + 400µl (0.25ng/ml)
0	500µl (BSA) 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:

If using acidified citrate samples with a pH lower than 6.0, add 30 μ l of 10X TBS buffer in each well and construct the standard curve in the same format. If using samples at a neutral pH this step should be omitted.

Add 100 μ l standards and unknowns to wells, in duplicates. Carefully record position of standards and unknowns. Shake plate at 300rpm for 30 minutes. Wash wells three times with 300 μ l wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

NOTE: This assay measures active tPA in the 0.1-50ng/mL range. If the unknown is thought to have high tPA levels, dilutions may be made in a similar biological fluid devoid of tPA, or in 3% BSA blocking buffer.

Primary Antibody Addition:

Add 10ml of 3% BSA blocking buffer directly to the primary antibody vial and agitate gently to completely dissolve contents. Add 100 μ l to all wells. Shake plate at 300rpm for 30 minutes. Wash wells three times with 300 μ l wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

Secondary Antibody Addition:

Dilute 1 μ l of conjugated secondary antibody into 10ml of 3% BSA blocking buffer and add 100 μ l to all wells. Shake plate at 300rpm for 30 minutes. Wash wells three times with 300 μ l wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

Substrate Incubation:

Add 100 μ l TMB substrate to all wells and shake plate for 2-10 minutes. Quench the reaction by the addition of 50 μ l of 1N H₂SO₄ and read final absorbance values at 450nm.

NOTE: Time for substrate development is dependent on needs of researcher.

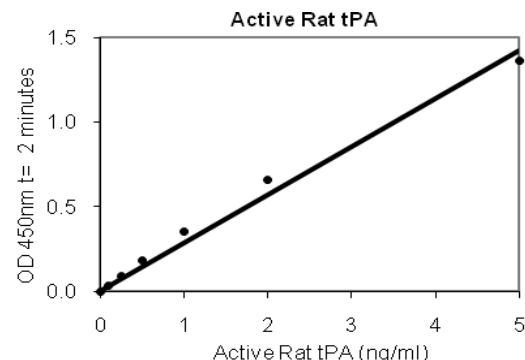
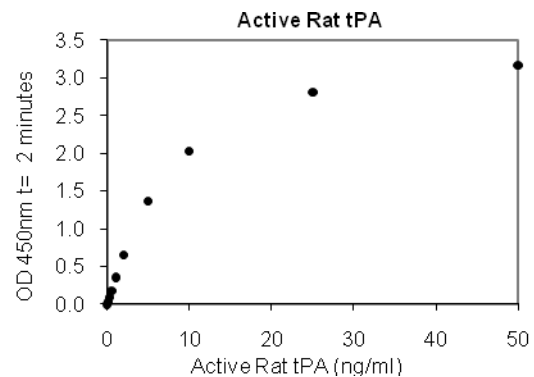
Measurement:

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 (A₄₅₀).

Assay Calibration:

Plot A₄₅₀ against the amount of tPA in the standards. Fit a straight line through the points using a linear fit procedure. The tPA activity in the unknowns can be determined by from this curve.

A typical standard curve:
(EXAMPLE ONLY, DO NOT USE)

**EXPECTED VALUES**

NOTE: No specific data has been reported for rat tPA concentrations. Please refer to references for mouse tPA.

The concentration level of tPA antigen in murine plasma has been reported to be 2.5 \pm 1.0 ng/ml [4].

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

- ◆ Venous Thrombosis: Locally applied tPA reduces thrombus formation after vascular injury [9].
- ◆ Ischemic Diseases: tPA may affect the course of ischemic diseases [5].
- ◆ Pathological Infarction: tPA may prevent or limit pathological infarction and improve neurological functions [6]. Usage of tPA at the onset of ischemic stroke improves clinical outcome [7].
- ◆ Blood-Brain Barrier: tPA is necessary and sufficient to directly increase the vascular permeability in the early stages of BBB opening [8].

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 or from contact with the above product.

REFERENCE

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4. Declerck PJ, *et al.*: Immunoassay of murine t-PA, u-PA, and PAI-1 using monoclonal antibodies raised in gene-inactivated mice. Thromb Haemostas., Nov**74**(5): 1305-9, 1995.
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6. Sakurama T, *et al.*: Tissue-type Plasminogen Activator Improves Neurological Functions in a Rat Model of Thromboembolic Stroke. Stroke, **25**, 451-456, 1994.
7. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. Tissue Plasminogen Activator for Acute Ischemic Stroke. N Engl J Med., Dec14; **333**(24), 1581-1588, 1995.
8. Yepes M, *et al.*: Tissue-type Plasminogen Activator Induces Opening of the Blood-Brain Barrier via the LDL Receptor-Related protein. J Clin Invest, Nov;**112**(10), 1483-5, 2003.
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Sample Plate Layout: 96 Well Plate Standards: 20 wells Samples: 76 wells

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.1ng/ml	0.25 ng/ml	0.5 ng/ml	1 ng/ml	2 ng/ml	5 ng/ml	10 ng/ml	25 ng/ml	50 ng/ml		
B	0	0.1ng/ml	0.25 ng/ml	0.5 ng/ml	1 ng/ml	2 ng/ml	5 ng/ml	10 ng/ml	25 ng/ml	50 ng/ml		
C												
D												
E												
F												
G												
H												