

Human tPA activity assay

Strip well format. Reagents for up to 96 tests.

For Research Use Only.

INTENDED USE

Human tPA activity assay is intended for the quantitative determination of active tissue plasminogen activator in human plasma.

BACKGROUND

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].

ASSAY PRINCIPLE

Functionally active tPA present in plasma reacts with the biotinylated human PAI-1 capture. Latent or complexed tPA will not bind to the plate and will not be detected. Unbound tPA samples are washed away and an anti-tPA primary antibody is added. Excess primary antibody is washed away and bound antibody, which is proportional to the original active tPA present in the samples, is then reacted with the horseradish peroxidase secondary antibody. Following an additional washing step, TMB is then used for color development at 450nm. The amount of color development is directly proportional to the concentration of active tPA in the sample.

DEFINITION OF tPA UNIT

The tPA activity standard provided contains human single chain tPA and is calibrated against the International Standard for tPA, lot 98/714 distributed by NIBSC, South Mimms, Potters Bar, Hertfordshire, UK.

REAGENTS PROVIDED

- ◆ **8 coated 12-well microtiter strips:** containing Avidin dried and blocked on the strip well surface.
- ◆ **Biotinylated Human PAI-1 Capture:**
1 vial lyophilized biotinylated capture
- ◆ **10X Wash Buffer:**
1 bottle of 50ml wash; bring to 1X using DI water
- ◆ **General assay diluent:**
1 bottle of 10ml diluent
- ◆ **Human tPA activity standard:**
1 vial lyophilized standard
- ◆ **Anti-human tPA primary antibody:**
1 vial lyophilized polyclonal anti-human tPA antibody
- ◆ **Anti-rabbit horseradish peroxidase secondary antibody:**
1 vial concentrated HRP labeled antibody
- ◆ **TMB substrate solution:**
1 bottle of 10ml 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 standards and primary may be stored at -70°C for later use. **DO NOT** freeze/thaw the standards and primary antibody 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 0-10 μ l and 200-1000 μ l
- 12-channel pipette covering 30-300 μ l
- Paper towels or kimwipes
- 50ml tubes
- 1N H₂SO₄
- DI water
- Magnetic stirrer and stir-bars
- Plastic containers with lids
- TBS buffer
- 3% Blocking buffer
- Microtiter plate spectrophotometer operable at 450nm
- Microtiter plate shaker with uniform horizontally circular movement up to 300rpm.

WARNINGS

Warning - The tPA standards are of human origin. Each donor unit has been tested and found negative for the presence of HBsAg, anti-HIV 1+2, anti-HBc, and anti-HCV.

Since no tests are currently available to assure that no infectious agents are present, the standard must be treated as is recommended at the Biosafety Level 2 as potentially infectious human serum or blood specimen in the Centres for Disease Control/National Institutes of Health manual, "Biosafety in Microbiological and Biomedical Laboratories", 1984.

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:** 0.10M TRIS, 0.15M NaCl, pH 7.4
- **Blocking buffer:** 3% BSA in TBS buffer

SPECIMEN COLLECTION

Collect 9 volumes of blood in 1 volume of 0.1M trisodium citrate or acidified citrate, preferably using Stabilyte™ evacuated vials (Biopool, cat# 102080) [6]. This insures that the fast-acting inhibitor for single-chain tPA which is usually present in large excess, is inhibited from quenching tPA activity [5]. 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. If sample was collected in acidified citrate, check the pH and if it's less than 6.0, then the pH needs to be brought up to neutral with the General Assay Diluent provided in the kit. The tPA activity samples collected in the Stabilyte™ media is stable for up to 5 hours or frozen at -20°C for up to one month or up to 5 months at -70°C.

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 3% BSA blocking buffer directly to the biotinylated human PAI-1 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.

Preparation of Standard:

Prepare the tPA standard in blocking buffer according to the dilution table.

Reconstitute standard as directed on vial to give a 6.9 IU/ml standard solution.

tPA concentration (IU/ml)	µl of "6.9 IU/ml" tPA standard	µl of "Blocking" Buffer
1	73	427
0.5	36	464
0.4	29	471
0.25	18	482
0.1	7	493
0.05	4	496
0	0	500

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

Standard and Unknown Addition:

NOTE: If the unknown is thought to have high tPA levels, dilutions may be made in 3% BSA blocking buffer.

If the pH of your samples is less than 6.0, then add 40µl general assay diluent to all wells. Add 60µl tPA standards and 60µl unknowns to wells, in duplicates. If the samples are at neutral pH, then only add 100µl of standards and 100µl of unknowns to wells, in duplicates. Carefully record the 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.

Primary Antibody Addition:

Reconstitute primary antibody by adding 10ml blocking buffer to vial. 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:

Dilute 2µl conjugated secondary antibody in 10ml BSA 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 of substrate solution to all wells and shake plate for 5-15 minutes. Quench the reaction with the addition of 50µl of 1N H₂SO₄ and read final absorbance values at 450nm.

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

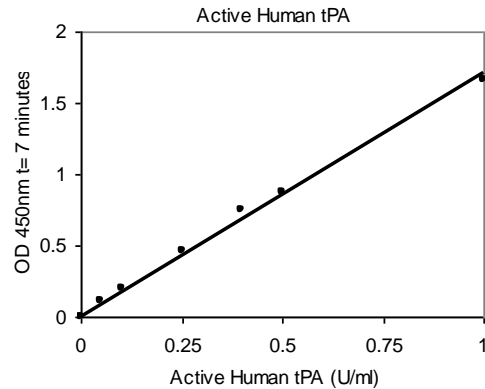
Measurement:

Set the absorbance at 450nm in a microtiter plate spectrophotometer. Measure the absorbance in all wells at 450nm, 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 from this curve.

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



EXPECTED VALUES

The basal level of tPA in healthy humans was found to be between 0.2-2 IU/ml [8].

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].

CONVERSION FACTOR

1 tPA IU = 1.45 ng

PERFORMANCE CHARACTERISTICS

Sensitivity = 0.006 IU/ml

(calculated by determining the OD of 20 reps of So and 20 reps of the low standard)

Linearity

The slope = 0.9776

Correlation coefficient = 0.9971

Intra Assay Precision

High 3.8%, Medium 4.0%, Low 9.8%
(calculated by running 20 reps of each concentration in an assay)

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

1. Sallés Fernando J, *et al.*: Localization and Regulation of the Tissue Plasminogen Activator – Plasmin System in the Hippocampus. The Journal of Neuroscience., March;**22(6)**: 2125-2134, 2002.
2. Wiman Björn, *et al.*: Inactivation of Tissue Plasminogen Activator in Plasma. The Journal of Biological Chemistry, March;**259(6)**: 3644-47, 1984.
3. Hastings Gregg A., *et al.*: Neuroserpin, a Brain-associated Inhibitor of Tissue Plasminogen Activator Is Localized Primarily in Neurons. The Journal of Biological Chemistry, Dec;**272(52)**: 33062-33067, 1997.
4. Yang YH, *et al.*: Tissue-type Plasminogen Activator Deficiency Exacerbates Arthritis. J Immunol, July15;**167(2)**: 1047-52, 2001.
5. Chmielewska J, *et al.*: Determination of Tissue Plasminogen Activator and its “fast” Inhibitor in Plasma. Clin Chem, Mar**32(3)**: 482-5, 1986.
6. Ranby M, *et al.*: Blood Collection in Strong Acidic Citrate Anticoagulant used in a Study of Dietary Influence on Basal tPA Activity. Thromb Haemost, Nov24;**62(3)**: 917-22, 1989.
7. Kruithof EK, *et al.*: Fibrinolysis in Pregnancy: a study of plasminogen activator inhibitors. Blood, Feb;**69(2)**: 460-466, 1987.
8. Eliasson, *et al.*: Influence of Gender, Age and Sampling Time on Plasma Fibrinolytic Variables and Fibrinogen. Fibrinolysis 7: 316-323, 1993.