For Research Use Only.

**INTENDED USE**
The Murine tPA activity assay is intended for the quantitative determination of active mouse tPA such as that found in tissue extracts and cell culture media. The endogenous level of active tPA in mouse plasma has been reported to be very low [1] and is at or below the sensitivity threshold of this kit. This assay is suitable for measuring mouse tPA in plasma where the subject mouse has been treated with exogenous mouse tPA.

**BACKGROUND**
Tissue plasminogen activator (tPA) is a serine protease that converts plasminogen to plasmin in the blood fibrinolytic system [1,2,3,9]. It also plays an important role in the nervous system, including the processes of neuronal migration, neurite outgrowth, and neuronal plasticity [1,2,4,7,10]. tPA has been suggested to have a role in several neuropathological conditions such as cerebral ischemia, seizures, and demyelinating diseases [1,3,5].

**ASSAY PRINCIPLE**
Functionally active mouse tPA will bind to the biotinylated human PAI-1 coated on the microtiter plate. Only free active enzyme will react with the PAI-1 on the plate. After appropriate washing steps, polyclonal anti-murine tPA primary antibody binds to the captured enzyme. Excess antibody is washed away and bound polyclonal antibody is then reacted with the secondary antibody conjugated to horseradish peroxidase. 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 tPA.

**REAGENTS PROVIDED**

- ♦ Avidin coated plate: 1-96 well immulon strip plate coated with Avidin, blocked, and dried
- ♦ 10X Wash Buffer: 1 bottle of 50ml wash; bring to 1X using DI water
- ♦ Biotinylated PAI-1: 1 vial lyophilized biotinylated PAI-1
- ♦ 10X TBS Buffer pH 7.4: 1 vial of 5ml TBS buffer
- ♦ Murine tPA activity standard: 1 vial lyophilized
- ♦ Anti-murine tPA primary antibody: 1 vial lyophilized polyclonal anti-mouse antibody
- ♦ Anti-rabbit horseradish peroxidase conjugate secondary antibody: 1 vial concentrated HRP labeled antibody
- ♦ TMB substrate solution: 10 ml

**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 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.

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. Immediately after collection of blood, samples must be centrifuged at 3000Xg for 15 minutes. 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. Also sample must be at a neutral pH to be used in the assay. If sample was collected in acidified citrate the pH needs to be brought up to neutral with the 10X TBS provided in the kit. The plasma must be transferred to a clean plastic tube and must be stored on ice prior to analysis. The tPA activity samples are stable for up to 24 hours or stored at -20°C for up to one month and thawed three times without loss of tPA activity.

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 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:**
Reconstitute standard as directed on vial and agitate gently to completely dissolve contents. Prepare the PAI-1 standard according to the dilution table insert found in the kit.
NOTE: DILUTIONS FOR THE STANDARD CURVE MUST BE MADE AND APPLIED TO THE PLATE IMMEDIATELY.

Reconstitute standard as directed on vial to give a 1,000ng/ml standard solution.

Dilution table for preparation of mouse tPA standards:

<table>
<thead>
<tr>
<th>tPA concentration (ng/ml)</th>
<th>Dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>990µl (BSA) + 10µl (standard from vial)</td>
</tr>
<tr>
<td>5</td>
<td>500µl (BSA) + 500µl (10ng/ml)</td>
</tr>
<tr>
<td>2</td>
<td>600µl (BSA) + 400µl (5ng/ml)</td>
</tr>
<tr>
<td>1</td>
<td>500µl (BSA) + 500µl (2ng/ml)</td>
</tr>
<tr>
<td>0.5</td>
<td>500µl (BSA) + 500µl (1ng/ml)</td>
</tr>
<tr>
<td>0.25</td>
<td>500µl (BSA) + 500µl (0.5ng/ml)</td>
</tr>
<tr>
<td>0.1</td>
<td>600µl (BSA) + 400µl (0.25ng/ml)</td>
</tr>
<tr>
<td>0.05</td>
<td>500µl (BSA) + 500µl (0.1ng/ml)</td>
</tr>
</tbody>
</table>

Standard and Unknown Addition:
If using acidified citrate samples with a pH lower than 6.0, add 30µl of 10X TBS buffer in each well. If using samples at a neutral pH this step should be omitted. Add 100µl 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 300µl wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

NOTE: Samples giving tPA levels above 10ng/ml should be diluted in plasma devoid of active tPA, or 3% BSA blocking buffer.

Primary Antibody Addition:
Add 10ml 3% BSA blocking buffer directly to the primary antibody 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:
Dilute 2.5µl into 10ml of 3% 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 TMB substrate to all wells and shake plate for 2-10 minutes. Quench the reaction by the addition of 50µl of 1M H₂SO₄ and read final absorbance values at 450nm.

NOTE: Time for substrate development is dependent on the needs of the 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 by from this curve.
A typical standard curve.
(EXAMPLE ONLY, DO NOT USE)

The concentration level of endogenous tPA antigen in murine plasma has been reported to be 2.5 +/- 1.0 ng/ml [15].

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

♦ Venous Thrombosis: Endogenous tPA plays a key role in restoring cerebral blood flow and limiting infarct size after thrombosis [6].
♦ Spinal Cord Contusion: Suppression of tPA production may help decrease secondary injury after spinal cord injury [1].
♦ Ischemic Diseases: tPA may attenuate neuronal injury after mild focal cerebral ischemia [5]. tPA may be involved in the regulation of blood vessel tone, which may affect the course of ischemic diseases [3].
♦ Bone Formation: A decreased in tPA may result in an increase of bone formation [14].
♦ Diabetic Retinopathy: Increased tPA levels have been associated with proliferative diabetic retinopathy [8].
♦ Adipose Tissue Development: A decrease in tPA may increase the development of adipose tissue in diet-induced obesity [11].
♦ Stress-induced Anxiety: tPA is critical for the development of anxiety-like behavior after stress [12].

PERFORMANCE CHARACTERISTICS

The assay measures total tPA in the 0.05-10 ng/ml range.

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

7. Manuel Yepes, et al.: Regulation of Seizure Spreading By Neuroserpin and...


