

ACTICHROME® TFPI Activity Assay

Product No. 848

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INTENDED USE

The ACTICHROME® Tissue Factor Pathway Inhibitor (TFPI) Activity Assay is a chromogenic assay intended for the measurement of active TFPI (EPI, LACI)¹ in plasma where TFPI exhibits an inhibitory effect on the Tissue Factor/FVIIa complex. The kit is for research use only.

BACKGROUND

TFPI circulates in human plasma as complexes with LDL, HDL and VLDL² and can be found in several forms: a 36,000 D molecule, a 43,000 D molecule and as truncated moieties. This heterogeneity of size appears, in part, to be the result of the formation of mixed disulfide complexes between TFPI and apolipoprotein AII.³ In humans, approximately 10% of total TFPI is carried by platelets which release TFPI once they are activated by thrombin.⁴ Thus, at the site of a wound, where platelets aggregate, elevated levels of TFPI are present. Based on the initial isolation of the inhibitor⁵ it was found that TFPI inhibits Tissue Factor (TF) procoagulant activity; i.e., the TF/FVIIa complex, and directly inhibits factor Xa by binding at or near its serine active site.⁶ The inhibitory mechanism of TFPI is a two step process. In the first step TFPI binds to factor Xa via its Kunitz domain 2, followed by a second step in which the TFPI/FXa complex binds to the TF/FVIIa complex via its Kunitz domain 1, forming an inactive quaternary TFPI/FXa/TF/FVIIa complex.

Quantitation of TFPI, with its dual inhibitory role against factor Xa and factor VIIa/TF, offers insight into the mechanism of disseminated intravascular coagulation (DIC) triggered by Tissue Factor. It has been observed that infusion of TFPI mitigates DIC and prevents reocclusion following thrombolysis induced by vascular injury. Clinical conditions in which thrombolysis appears to be initiated by TF and in which TFPI treatment may be potentially useful include sepsis, inflammatory disease and transplant rejection. TFPI has potential as an antithrombotic agent. Heparin is known to release TFPI from the vascular endothelium, therefore measurement of TFPI levels upon initial heparinization may provide an indication of patient heparin response.

PRINCIPLE OF THE METHOD

The ACTICHROME TFPI Activity Assay measures the ability of TFPI to inhibit the catalytic activity of the TF/FVIIa complex to activate factor X to factor Xa. After incubation of test samples with TF/FVIIa and FX, the residual activity of the TF/FVIIa complex is measured using SPECTROZYME® FXa, a highly specific chromogenic substrate cleaved only by FXa generated in the assay, releasing a p-nitroaniline (pNA) chromophore. The absorbance of the pNA in the reaction solution at 405 nm is measured and compared to those values obtained from a standard curve constructed using known TFPI activity levels. This assay may be performed in either end-point or kinetic mode.

REAGENTS

This kit contains reagents sufficient to perform 100 test points.

1 vial of Assay Buffer, 5 mL (5X concentrate)
1 vial of SPECTROZYME® FXa, 5 µmoles (lyophilized)
2 vials of TFPI Depleted Plasma, 0.5 mL (lyophilized)

1 vial of TFPI Reference Plasma, 0.5 mL, ca. 1 unit/mL (lyophilized)
1 vial of Human Factor X, 25 µg (lyophilized)
1 vial of Lipidated Tissue Factor, 50 ng (lyophilized)
1 vial of Human Factor VIIa Reagent (lyophilized)
1 vial of TFPI Standard, 0.2 unit/mL (lyophilized)

ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED

EDTA (trisodium, dihydrate)
1 x 96 microwell test plate (round bottom)
0.22 µm filtered deionized or distilled H₂O
200 - 1000 µL single pipette
10 - 100 µL single pipette
50 - 100 µL eight channel multi-pipette
Microwell plate reader at 405 nm
Glacial acetic acid

WARNING

The TFPI Depleted Plasma and TFPI Reference Plasma provided are of human origin. Each donor unit used in the manufacture of these reagents has been tested by an FDA licensed method and found to be non-reactive for Hepatitis B surface Antigen (HBsAg), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus (HIV). As no known method can offer complete assurance that products derived from human blood will not transmit HBsAg, HCV, HIV or other blood-borne pathogens, this plasma should be handled as recommended for any potentially infectious human serum or blood specimen.

REAGENT RECONSTITUTION AND STABILITY

Unreconstituted reagents are stable until the expiration date indicated on the label when stored at +2° - +8°C.

- Assay Buffer:** Add the contents of the vial to 20 mL of cold (+2 - +8°C) filtered deionized H₂O and mix thoroughly.
- EDTA:** Dissolve 48 mg of EDTA (trisodium, dihydrate) in 2.0 mL of filtered distilled water. Mix thoroughly, adjust the pH to 9.9 with NaOH and q.s. to 2.5 mL with filtered distilled H₂O.
- SPECTROZYME FXa:** Add 2.1 mL of filtered deionized H₂O to the vial and mix thoroughly. Reconstituted material may be stored at -20°C or colder for up to one year.
- TFPI Depleted Plasma:** Add 0.5 mL of Assay Buffer to each vial of TFPI Depleted Plasma, mix thoroughly and place on melting ice. Combine the contents of these vials and add to 19 mL of Assay Buffer. Aliquot this 5% TFPI Depleted Plasma into labeled plastic cryotubes, placing aliquots for immediate use on melting ice. Store unused aliquots immediately at -20°C for up to one month.
- TFPI Reference Plasma:** Add 0.5 mL of filtered deionized H₂O to the vial of TFPI Reference Plasma, mix thoroughly and place the vial on melting ice for 3 minutes. Aliquot into labeled plastic cryotubes. Place aliquots for immediate use on melting ice. Store unused aliquots immediately at -20°C for up to one month.
- Human Factor X:** Add 2.5 mL of filtered distilled water to the vial of Human Factor X and mix thoroughly. Aliquot into labeled plastic cryotubes. Place aliquots for immediate use on melting ice. Store unused aliquots immediately at -20°C for up to one month.
- Lipidated Tissue Factor:** Add 100 µL of Assay Buffer to the vial of Lipidated Tissue Factor and mix thoroughly. Aliquot into labeled plastic cryotubes. Place aliquots for immediate use on melting ice. Store unused aliquots immediately at -20°C for up to one month.
- Human Factor VIIa Reagent:** Add 2.25 mL of filtered distilled H₂O to the vial of Human Factor VIIa reagent and mix thoroughly. Aliquot into labeled plastic cryotubes. Place aliquots for immediate use on melting ice. Store unused aliquots immediately at -20° for up to one month.

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9. **TFPI Standard:** Add 1.0 mL of filtered deionized H₂O to the TFPI Standard vial to generate a 0.2 unit/mL standard. Prepare TFPI Standard Concentrations of 0.2, 0.1, 0.08, 0.06, 0.04 and 0.02 unit/mL by diluting the 0.2 unit/mL TFPI Standard as shown in **Table 1**. Label cryotubes and aliquot accordingly. Store unused standards immediately at -20°C for up to one month.

Table 1 - TFPI Standard Dilutions

TFPI Standard Concentration unit/mL	Volume of 0.2 unit/mL Standard	Volume of TFPI Depleted Plasma
0.20	As needed	0 µL
0.10	100 µL	100 µL
0.08	80 µL	120 µL
0.06	60 µL	140 µL
0.04	40 µL	160 µL
0.02	20 µL	180 µL
0	0 µL	As needed

10. **Tissue Factor/FVIIa Reagent:** Add 26.6 µL of lipidated tissue factor per mL of factor VIIa. Prepare the TF/FVIIa reagent fresh each time the assay is run. Discard any unused factor TF/FVIIa reagent.

SPECIMEN COLLECTION AND PREPARATION

Blood samples may be drawn using syringes or evacuated siliconized tubes. Mix nine parts of blood with one part of 3.2 or 3.8% (109 or 129 mM) of the dihydrate form of trisodium citrate. After mixing, centrifuge the samples at a minimum of 3,000 x g for 10 minutes. The collected plasma may then be stored at +2° - +8°C but should be tested within four hours. Alternatively, plasma may be rapidly frozen and stored at -70°C for up to six months. Frozen plasmas must be thawed rapidly at 37°C before use, and tested immediately or stored up to two hours at +2° - +8°C.⁷

Dilute the TFPI Reference Plasma and each test sample 1:20 (i.e., add 15 µL reference plasma/test sample to 285 µL TFPI Depleted Plasma) prior to performing the assay.

PROCEDURE

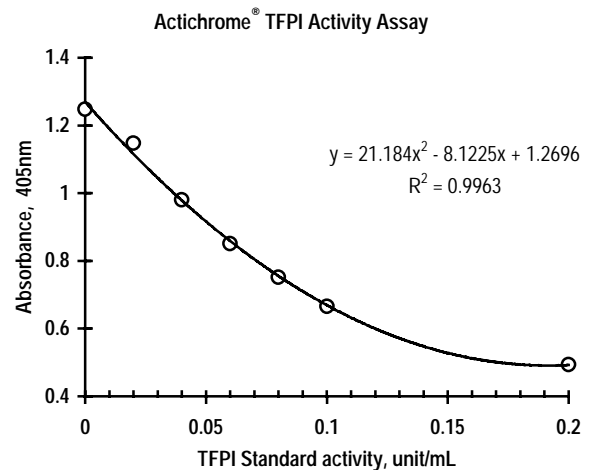
Micro-titer End-point Method

1. Add 20 µL of TFPI Standard, diluted Reference Plasma or diluted test sample to each well.
2. Add 20 µL of TF/FVIIa complex to the wells of a micro-test plate.
3. Cover the micro-test plate and incubate at 37°C for 30 minutes.
4. Add 20 µL of Human Factor X to each well.
5. Cover and incubate at 37°C for 15 minutes.
5. Add 20 µL of EDTA to each well.
6. Add 20 µL of SPECTROZYME Fa substrate to each well.
7. The reaction will begin immediately upon addition of the SPECTROZYME FXa and the solution will turn yellow as the reaction continues. Read the absorbances of the solution at a wavelength of 405 nm. Color development may be monitored by reading the plate every 5 minutes. Stop the reaction at 25 minutes by adding 50 µL of glacial acetic acid to each well.

REPRESENTATIVE STANDARD CURVE

The standard curve is constructed by plotting the mean absorbance value calculated for each TFPI standard at 405 nm versus the corresponding concentration. Interpolate the TFPI concentrations for the diluted samples directly from the standard curve. Multiply the results of the tested samples and the Reference Plasma by the dilution factor of 20 to obtain the actual TFPI concentration of the sample. If a higher or lower dilution has been used, multiply the final result by the appropriate dilution factor. A standard

curve should be generated each time the assay is performed. The following curve is for demonstration purposes only.



LIMITATIONS OF PROCEDURE

The kit reagents contain agents that neutralize heparin up to and including 5 unit/mL. Testing on plasmas containing higher heparin levels may produce inaccurate results.

EXPECTED VALUES

Citrated plasma samples from 300 normal human volunteers were determined to contain a mean immunologic assay TFPI value of 55 ng/mL plasma with a two standard deviation range of 40-70 ng/mL. On this basis the ACTICHROME TFPI Activity Assay Standard has been assigned a value and contains 0.2 unit TFPI activity. The vial of Reference Plasma included in the kit contains approximately 55 ng/mL, or 1 unit of TFPI activity.

INTERPRETATION OF RESULTS

The significance of measuring plasma TFPI activity has not been fully explored. The main role of TFPI involves the inhibition of small amounts of Tissue Factor, which is an essential component for maintaining normal hemostasis.⁸ The role of TFPI in accelerating heparin's actions may also be important in regulating anticoagulant therapy. Other observations such as the increased synthesis of TFPI by malignant cells remain to be fully explored.

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