DEPARTMENT OF THE AIR FORCE
59th Medical Wing (AETC)
Clinical Research Squadron (MSRL)
Lackland AFB, Texas 78236-5319

Medical Service

THE DETERMINATION OF TISSUE FACTOR PATHWAY INHIBITOR (TFPI) BY MICROTITER

1. **Purpose/Principle/Introduction:** The TFPI ACTICHROME microtiter method 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 end-point mode.

2. **Specimen:**

2.1 Type: Citrated whole blood or citrated plasma. Whole blood must be collected to give a 9:1 ratio of blood to anticoagulant.

2.2 Amount: 1.0 ml of citrated platelet-poor plasma (PPP).

2.3 Timing: The collection tube may be the last tube obtained to avoid contamination by tissue thromboplastin by a traumatic venipuncture. However, recent studies have shown that this practice does not need to be adhered to except under protocols that specifically request it (9). Administration of oral anticoagulants or heparin could influence any coagulation assay. Information on the time of the dosing of any anticoagulant medication should accompany the specimen.

2.4 Preservations: The sample should be run within 4 hours of collection. Keep the specimen at 2-4°C until ready to separate. If storage is required, separate the supernatant platelet-poor plasma (PPP) and place in a plastic 12 x 75-mm test tube and freeze at -70°C, ± 10°C until ready for testing. Thaw the specimen rapidly at 37°C ± 2°C just before analysis.

2.5 Anticoagulant: 3.2% sodium citrate.

2.6 Special Handling: Centrifuge the citrate whole blood for 15 minutes at approximately 2500 g in a R3C3 Sorvall centrifuge or equivalent to obtain PPP.

2.7 Unacceptable Specimen: Samples where the blood to anticoagulant ratio is not 9:1 may yield incorrect values. Clotted or hemolyzed samples may yield incorrect values. There is a possibility of heparin interference if the concentration in the subject sample is above 5 units/mL. Testing on plasmas containing higher heparin levels may produce inaccurate levels.

3. Reagents:

3.1 Assay Buffer: 1 vial of 5.0 mL, 5X concentrate.

3.2. TFPI Depleted Plasma: 2 vials of 0.5 mL, lyophilized.

3.3. TFPI Reference Plasma: 1 vial of 0.5 mL, ca. 1 unit/mL, lyophilized. (See label for amount)

3.4. Human Factor X: 1 vial of 25 μg, lyophilized.

3.5. Lipidated Tissue Factor: 1 vial of 50 ng, lyophilized.

3.6. Human Factor VIIa Reagent: 1 vial of 2.25 mL, lyophilized.

3.7. SPECTROZYME fXa: 1 vial, 5μM, lyophilized.

3.8. TFPI Standard: 1 vial of TFPI, 0.2 unit/mL, lyophilized.

Additional materials required but not provided:

3.9. EDTA: Dissolve 48 mg of EDTA (trisodium, dihydrate) in 2.0 mL of filtered distilled water. Mix thoroughly, adjusting the pH to 9.9 with NaOH and q.s. to 2.5 mL with filtered distilled water.

3.10. Glacial acetic acid (20.0%).

3.11. Ice

4. **Calibration:** This assay uses a 6 point standard curve based on a reference value assigned by the manufacturer. A point-to-point curve was used to calculate the results. The manufacturer originally wanted a 2nd order polynomial to be used. Our reader does not have this capability but I plugged in the data on the MLA 900C analyzer and this gave a least squares regression coefficient of 0.998. Also the CV's of the duplicate standards were consistently below 10.0%.

5. **Quality Control:** Assay controls consist of reference plasma provided by the manufacturer and a spiked heparinized plasma patient sample. (150 μL 1000 unit/mL heparin to 1.0 mL of normal plasma).

5.1. Assay both controls with each run.

5.2 All controls should be within the ± 2 SD range stated in the quality control and stored with the protocol file.

6. **Procedure:**

6.1. Reconstitute all reagents according to manufacturer's instructions with the some noted exceptions:

6.1.1 Add the vial of Assay Buffer to 20 mL of deionized water and mix thoroughly.

6.1.2. Dissolve 48 mg of EDTA (trisodium dihydrate) in 2.0 mL of distilled water. Mix thoroughly, adjust the pH to 9.9 with NaOH and q.s. to 2.5 mL with distilled water.

6.1.3. SPECTROZYME fXa: Add 2.5 mL of deionized water to the vial of reagent and mix thoroughly. Reconstituted material may be stored at -20°C or colder for up to one year. NOTE: This is an exception from the manufacturer's instructions. (2.1 mL of deionized water).

6.1.4. 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 5.0% of 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.

6.1.5. TFPI Reference Plasma. Add 0.5 mL of deionized water 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.

6.1.6. TFPI Standard: Add 1.0 mL of deionized 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 units/mL TFPI Standard as show below:

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

6.1.7. Human Factor X. Add 2.5 mL of deionized water to the vial of Human Factor X and mix thoroughly. Aliquot into labeled plastic cryotubes. Place aliquots on melting ice. Store unused aliquots immediately at -20°C. Stable for up to one month.

6.1.8. Lipidated Tissue Factor. Add 100 uL 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. Stable for up to one month.

6.1.9. Human Factor VIIa Reagent. Add 2.25 mL of deionized water 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°C. Stable for up to one month.

6.1.10. Factor VIIa/Tissue Factor Reagent. Add 26.6 uL of lipidated tissue factor/mL of factor VIIa. Prepare the factor VIIa/TF reagent fresh each time the assay is run. Discard any unused factor VIIa/TF reagent.

6.2. Dilute the TFPI Reference Plasma and each test sample 1:21 (i.e., add 15 uL reference plasma/test sample to 300 uL TFPI Depleted Plasma) prior to performing the assay.

6.3. Add 20 uL of TFPI Standard, diluted Reference Plasma or diluted test sample to each microtiter plate test well.

6.4. Add 20 uL of factor VIIa/TF complex to the wells of a micro-test plate.

6.5. Cover the microtiter test plate and incubate at 37°C for 30 minutes.

6.6. Add 20 uL of Human Factor X to each well. Cover and incubate at 37°C for 15 minutes.

6.7. Add 20 uL EDTA to each well.

6.8. Immediately add 20 uL of SPECTROZYME fXa substrate to each well.

6.9. The reaction will begin immediately upon addition of the SPECTROZYME fXa and the solution will turn yellow as the reaction continues. Stop the reaction at 25 minutes by adding 50 uL of 20% glacial acetic acid to each well. Read the absorbances of the solution at a wavelength of 405 nm.

7. Calculations:

7.1. **Calibration Curve**: Plot the absorbance obtained with each of the TFPI Standards at 405 nm against the TFPI Standard (unit/mL). Use a point to point curve using the LP400 Microplate reader. Interpolate the TFPI activity directly from the standard curve. Multiply the results of the test samples and the Reference Plasma by the dilution factor of 21 to obtain the correct TFPI value. If a high or lower dilution has been used, multiply the final results by the appropriate dilution factor.

7.2. A new Standard Curve must be created each time the assay is performed. See Attachment One for an example of the calibration curve.

8. Reporting Results:

8.1. Results are reported out in units /mL (Example: 3.1 units/mL).

8.2. Our laboratory determined the reference ranges of a commercial normal donor set of 20 normal donors (Precision Biologic, NDS 315) to be 0.72-1.99 units/mL on humans. The swine range was 0.10-0.65 units/mL on 28 baseline samples.

9. Procedure Notes: Any QC results that are out of range (see range in protocol notes) must be rerun and be within acceptable range before proceeding with the assay. The TFPI Reference Plasma included in the kit should be tested each time the assay is performed.

10. Limitations of Procedure: There is a possibility of heparin interference if the concentration in the subject sample is above 5 units/mL. Testing on plasmas containing higher heparin levels may produce inaccurate results.

11. References:

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