

american diagnostica inc.

500 West Avenue, P.O. Box 110215 • Stamford, CT. 06911-0215
Tel. (203) 602-7777 Fax. (203) 602-2221
www.americandiagnostica.com

IMUBIND[®] Total TFPI ELISA Kit

Product No. 849

INTRODUCTION

The IMUBIND[®] Total Tissue Factor Pathway Inhibitor (TFPI) ELISA kit is an enzyme-linked sandwich immunoassay for the quantitation of TFPI (EPI, LACI)¹ in plasma as well as in cell culture supernatants. This ELISA detects both intact and truncated forms of TFPI as well as complexes with Tissue Factor (TF) and factor VIIa (TF/VIIa/TFPI). Binary complexes with factor Xa (TFPI/Xa) and quaternary complexes with TF, factor VIIa and factor Xa (TF/VIIa/TFPI/Xa) are also recognized by this ELISA, but with slightly lower sensitivity. The lower limit of detection for this assay is 0.360 ng TFPI/mL sample. The assay is for research use only.

BACKGROUND

Tissue Factor Pathway Inhibitor (TFPI) circulates in plasma as a complex with LDL>HDL>>VLDV.² In plasma, TFPI is found in several forms: one at 36,000 kD, one at 43,000 kD, and the various truncated forms. This size heterogeneity appears in part to be the result of the formation of mixed disulfide complexes between TFPI and apolipoprotein AII.³ Approximately 10% of 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. TFPI has dual inhibitory function (against factor Xa and factor VIIa/TF). This is consistent with the presence of multiple Kunitz-type domains: (Kunitz 1 for factor VIIa/TF and Kunitz 2 for factor Xa interaction). Based on the initial isolation of the inhibitor⁵, it was found that TFPI inhibits factor VIIa/TF and directly inhibits factor Xa by binding at or near its serine active site.⁶ The mechanism of action for factor Xa dependent inhibition of factor VIIa/TF by TFPI involves the formation of a quaternary factor Xa/TFPI/factor VIIa/TF complex. This results from the initial binding of factor Xa to TFPI, with subsequent binding of the factor Xa-TFPI complex to factor VIIa/TF. Alternatively, TFPI might bind to the factor Xa-VIIa/TF complex.

BIOLOGICAL CONTENT

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 abrogates DIC and prevents reocclusion following thrombolysis induced by injury. Clinical conditions in which thrombosis 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.

PRINCIPLE

The IMUBIND Total TFPI ELISA is a "sandwich" ELISA employing a rabbit anti-human TFPI polyclonal antibody as the capture antibody. Specificity of the capture antibody for native, complexed and truncated TFPI was confirmed by Western blot analysis, visualizing a single band at 34 kD, corresponding to the mobility of intact native TFPI and visualizing a single band at 21 kD, corresponding to the mobility of a truncated form of TFPI. Diluted plasma samples or cell culture supernatants incubate in micro-test wells precoated with this capture antibody. TFPI is detected using a biotinylated monoclonal antibody specific for the Kunitz domain 1 of TFPI. The subsequent binding of the streptavidin conjugated horseradish peroxidase (HRP) completes the formation of the antibody enzyme detection complex. The addition of TMB substrate and its subsequent reaction with HRP provides a blue color. Sensitivity is increased by addition of a 0.5M sulfuric acid stop solution, yielding a yellow color. TFPI levels are determined by measuring sample solution absorbance at 450 nm and comparison against those of a standard curve developed using native TFPI.

REAGENTS

6 x 16 well precoated micro-test strips with holder and lid
2 vials TFPI Standard, 5 ng/mL (lyophilized)
1 vial TFPI Depleted Plasma (lyophilized)
1 vial TFPI Reference Plasma (lyophilized)
2 vials Detection Antibody, biotinylated anti-human TFPI F(ab')₂ (lyophilized)
1 vial Enzyme Conjugate, Streptavidin-horseradish peroxidase (60 µL)
1 vial Enzyme Conjugate Diluent (lyophilized)
1 vial Substrate, TMB (11 mL)
1 packet of Wash Buffer, PBS with 0.1% Triton X-100, pH 7.4

ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED

Distilled H₂O
50-200 µL eight channel multi-pipette
10-200 µL single pipette
Micro-test plate reader at 450 nm
0.5M H₂SO₄
Bovine Serum Albumin (BSA, e.g. Sigma A-7030)

WARNING

The TFPI depleted plasma is of human origin. Each donor unit has been tested by an FDA approved method and found to be non-reactive for HBsAg, HIV-1 and HCV. As no known method can offer complete assurance that products derived from human blood will not transmit disease, this plasma should be handled as recommended for any potentially infectious human serum or blood specimen.

Reagents supplied in this kit contain sodium azide (NaN₃), which may form explosive metallic azides upon reaction with copper and lead plumbing. Flush with large volumes of water during disposal to prevent azide build-up.

REAGENT PREPARATION

Procedural Notes:

1. Reconstitute standards and references immediately before adding to the microtest wells. Do **not** prepare standards in advance.
2. Reconstituted standards and reference material should be used within 1 hour. Aliquot remaining standards, TFPI depleted plasma and TFPI reference plasma and store at -20°C.

A. Standards

1. Add 0.5 mL of cold (2-8°C) distilled H₂O to the TFPI Depleted Plasma vial. Allow the vial to stand on ice for 2-3 minutes. Vortex the vial to achieve adequate mixing.
2. Prepare a solution of 5% TFPI depleted plasma by diluting the TFPI depleted plasma 1:20 with Sample Buffer. Gently vortex and let stand for 5 minutes.
3. Hold the 5 ng/mL TFPI standard vial upright and tap the vial to settle its contents (lyophilized under vacuum). Release the vacuum by slowly removing the vial stopper.
4. Add 1 mL of the 5% TFPI depleted plasma to the 5 ng/mL standard vial.
5. Generate standards of 2.5, 1.25, 0.625 and 0.312 ng/mL concentration by serially diluting the 5 ng/mL TFPI plasma standard. Label tubes accordingly and pipette 0.5 mL of 5% TFPI depleted plasma into each tube. Pipette 0.5 mL of the 5 ng/mL TFPI plasma standard into the 2.5 ng/mL labeled tube and mix. Transfer 0.5 mL from the 2.5 ng/mL tube into the 1.25 ng/mL labeled tube and mix. Continue this process for the 0.625 ng/mL and 0.312 ng/mL labeled tubes.
6. Use the 5% TFPI depleted plasma as the "0" standard.

B. TFPI Reference Plasma

Add 0.5 mL of cold filtered deionized H₂O to the vial and gently mix for 2 minutes.

C. Detection Antibody

Add 5.5 mL filtered deionized H₂O per vial and agitate gently for 3 minutes.

D. Enzyme Conjugate Diluent

Add 20 mL filtered deionized H₂O to the vial and mix well.

E. Wash Buffer

1. Dissolve contents of PBS packet in 900 mL of filtered deionized H₂O.
2. Mix well.
3. Dilute to a final volume of 1 Liter with filtered deionized H₂O.

F. Sample Buffer

Prepare an appropriate amount of Sample Buffer by adding BSA to Wash Buffer to a final concentration of 1% w/v (1 gm BSA/100 mL Wash Buffer).

REAGENT STABILITY

Store unused micro-test strip-wells and unreconstituted reagents at +2°-+8°C until expiration dates indicated on label.

Store reconstituted reagents at -20°C for up to one month. Remember to aliquot and freeze reconstituted standards and plasmas **immediately**.

SAMPLE PREPARATION

A. Plasma (Note: Heparinized plasma can be used in this assay)

1. Collect blood into 3.8% trisodium citrate anticoagulant solution in the proportion of 9 volumes of blood to 1 volume of anticoagulant solution.
2. Centrifuge the blood sample at 6,000 rpm for 10 minutes and store frozen.
3. Frozen plasma should be thawed at 37°C for 15 minutes.
4. Dilute TFPI Reference Plasma and plasma samples 1:40 in Sample Buffer.

B. Tissue Culture Supernatant

Dilute samples 1:5 (recommended initial dilution) in Sample Buffer.

Note: some cell systems may require a higher dilution factor (up to 1:500).

ASSAY PROCEDURE

DAY ONE

1. Remove the necessary number of precoated micro-test strips from the foil pouch and place them in the plate holder. Reseal the foil pouch with the desiccant inside and store at +2° - +8°C.
2. Add 100 µL of TFPI Standard, TFPI Reference Plasma or diluted sample to micro-test wells, cover with lid and incubate overnight at +4°C. Perform measurements in duplicate.

DAY TWO

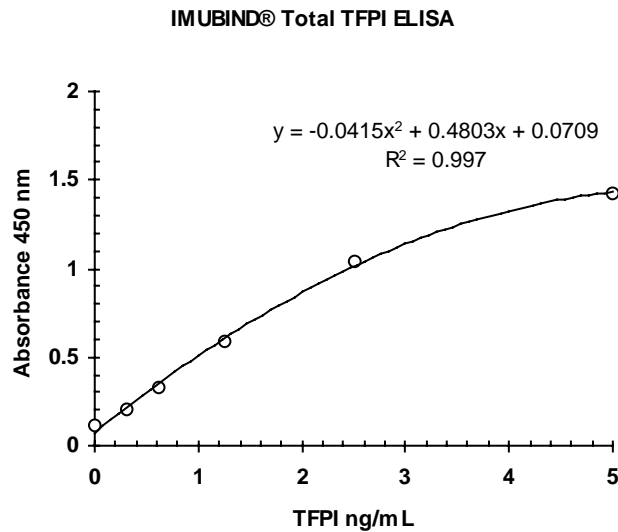
3. Wash wells 4 times with Wash Buffer.
4. Add 100 µL of Detection Antibody to each well, cover with lid and incubate for 1 hour at room temperature.
5. Wash wells 4 times with Wash Buffer.
6. For running all 96 wells at one time, add 12 µL of Enzyme Conjugate to 12 mL of Enzyme Conjugate Diluent (add 2 µL of conjugate to 2 mL of diluent for each 16 well strip when running less than 96 wells). Add 100 µL of diluted enzyme conjugate to each well, cover with lid and incubate for 1 hour at room temperature.
7. Wash wells 4 times with Wash Buffer.
8. Add 100 µL of Substrate solution to each well, cover with lid and incubate for 20 minutes at room temperature. A blue color will develop.
9. Stop the enzymatic reaction by adding 50 µL of 0.5M H₂SO₄. Tap the sides of the strip-wells to ensure even distribution of the H₂SO₄. The solution color will turn yellow. Read the absorbances on a micro-test plate reader at a wavelength of 450 nm within 30 minutes. Deduct the background average of the blanks from the standards and sample readings.

INTERPRETATION OF RESULTS

A normal range for human plasma has yet to be established. Citrated plasma samples from healthy volunteers (n=40) were found to have a mean value of 89.5 ng TFPI/mL plasma with a range of 75-120 ng TFPI/mL plasma when using this kit.

REPRESENTATIVE STANDARD CURVE

The standard curve is constructed by plotting the mean absorbance value for each TFPI standard versus the corresponding concentration of TFPI in ng/mL. Interpolate the TFPI concentrations for diluted samples directly from the standard curve. A standard curve should be generated each time the assay is performed. The following standard curve is for demonstration purposes only.



CALCULATION OF RESULTS

Average the TFPI concentrations obtained for each test sample, as interpolated from the standard curve. Multiply this concentration by the dilution factor of the sample to calculate the TFPI concentration of the original sample. For example, if the test sample was diluted 1:40 as recommended, multiply the concentration of the diluted sample read from the standard curve by 40 to obtain actual TFPI concentration. The calculation would be,

$$\text{Concentration of Diluted Test Sample} \times 40 = \text{Concentration of Test Sample}$$

PERFORMANCE CHARACTERISTICS

Specificity: This assay recognizes native and recombinant human TFPI complexed with HDL, LDL, and VLDL, and truncated forms of human TFPI. Preparations of various coagulation factors, in both 5% TFPI depleted plasma and Sample Buffer, at 10-fold concentrations to TFPI standard concentration were assayed. No significant cross-reactivity or interference was observed.

Specificity: This assay recognizes native and recombinant human TFPI complexed with HDL, LDL, and VLDL, and truncated forms of human TFPI. Preparations of various coagulation factors, in both 5% TFPI depleted plasma and Sample Buffer, at 10-fold concentrations to TFPI standard concentration were assayed. No significant cross-reactivity or interference was observed.

Sensitivity: The lower limit of detection was determined by adding 2 standard deviations to the mean OD value for the "zero" standard (n=15) and calculating the corresponding concentration from the standard curve. For plasma samples, the lower limit of detection was found to be 180 pg/mL (0.180 ng/mL).

Precision: Intra-assay and Inter-assay variations for plasma samples were:

TFPI Level (ng/mL)	Intra-Assay C.V. (n=10)	Inter-Assay C.V. (n=10)
1.25	6.2	6.7
2	7.1	7.3
5	6.5	5.5

REFERENCES

- Rapaport, S., I. The extrinsic pathway inhibitor: a regulator of tissue factor-dependent blood coagulation. *Thrombosis and Haemostasis* 1991; **66**: 6-15.
- Hubbard, A., R., and Jennings, C., A. Inhibition of the tissue factor-factor VII complex: Involvement of factor Xa and lipoproteins. *Thrombosis Research* 1987; **46**: 527-537.
- Novotny, W., F., Girard, T., and Miletich, J., P. Purification and characterization of the lipoprotein-associated coagulation inhibitor from human plasma. *Journal of Biological Chemistry* 1989; **264**: 18832-18837.
- Novotny, W., F., Girard, T., J., and Miletich, J., P. Platelets secrete a coagulation inhibitor functionally and antigenically similar to the lipoprotein-associated coagulation inhibitor. *Blood* 1988; **72**: 2020-2025.
- Broze, G., J., and Miletich, J., P. Isolation of the tissue factor inhibitor produced by HepG2 Hepatoma cells. *Proceedings of the National Academy of Science USA* 1987; **84**: 1886-1890.
- Broze, G., J., Warren, L., A., and Novotny, W., F. The lipoprotein associated coagulation inhibitor that inhibits factor VII tissue factor complex also inhibits factor Xa. Insight into the possible mechanism of action. *Blood* 1988; **71**: 335-343.