



AssayMax Human Tissue Factor Pathway Inhibitor (TFPI) ELISA Kit

Catalog No. ET1005-1

Introduction

Tissue factor pathway inhibitor (TFPI) is an endogenous protease inhibitor that regulates the initiation of the extrinsic coagulation pathway by producing factor Xa-mediated feedback inhibition of the tissue factor/factor VIIa (TF/FVIIa) catalytic complex (1). TFPI has a negatively charged amino-terminus, three tandem Kunitz proteinase inhibitory domains, and a positively charged carboxy-terminus. The first Kunitz domain is the binding site for the TF/FVIIa complex and the second domain for factor Xa. The resultant quaternary complex of TFPI/FXa/TF/FVIIa lacks TF/FVIIa catalytic activity (2). The third Kunitz-type domain and the carboxy-terminus of TFPI mediate its binding to heparin and cell surfaces including the endothelium (3). TFPI is synthesized mainly by endothelial cells and present in three pools *in vivo*: 10% in platelets, in endothelium associated with endothelial glyco-saminoglycans, and in plasma circulating as free or lipoprotein associated forms (4). The plasma TFPI contains mostly 34 and 40 kDa forms and the concentration is approximately 50 to 100 ng/ml (5, 6). Measurement of TFPI could be important in thrombogenesis, atherosclerosis and heparinization studies. Higher plasma levels of TFPI were found in older individuals, pregnant women and patients with advanced cancer (7, 8, 9).

Principal of the Assay

The AssayMax Human TFPI ELISA kit is designed for detection of human TFPI in plasma, serum, and cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay technique that measures TFPI in less than 4 hours. A polyclonal antibody specific for TFPI has been pre-coated onto a 96-well microplate with removable strips. TFPI in standards and samples is sandwiched by the immobilized polyclonal antibody and biotinylated polyclonal antibody specific for TFPI, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- **Prepare all reagents (working diluent buffer, wash buffer, standards, biotinylated-antibody, and SP conjugate) as instructed, prior to running the assay.**
- **Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this protocol. However, the user should determine the optimal dilution factor.**
- **Spin down the SP conjugate vial and the biotinylated-antibody vial before opening and using contents.**
- This kit is for research use only.

- The kit should not be used beyond the expiration date.
- The Stop Solution is an acid solution.

Reagents

- **TFPI Microplate:** 96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against TFPI.
- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes, which can be cut to fit the format of the individual assay.
- **TFPI Standard:** Human TFPI in a buffered protein base (160 ng, lyophilized).
- **Biotinylated TFPI Antibody (100x):** A 100-fold concentrated Biotinylated antibody against human TFPI (80 µl).
- **MIX Diluent Concentrate (10x):** A 10-fold buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x):** A 20-fold concentrated buffered surfactant (30 ml, 2 bottles).
- **Streptavidin-Peroxidase Conjugate (100x):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate:** A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution:** A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Store components of the kit at 2-8⁰C or -20⁰C upon arrival up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody at -20⁰C
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8⁰C
- Opened unused microplate wells may be returned to the foil pouch with the desiccant packs. Reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.
- Diluent (1x) may be stored for up to 1 month at 2-8⁰C.
- Store Standard at 2-8⁰C before reconstituting with Diluent and at -20⁰C after reconstituting with Diluent.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm.
- Pipettes (1-20 µl, 20-200 µl, 200-1000µl and multiple channel)
- Deionized or distilled reagent grade water.

Sample Collection, Preparation and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 2000 x g for 10 minutes and assay. Dilute samples 1:20 with MIX Diluent. The undiluted samples can be stored at -20⁰C or below for up to 3 months. Avoid repeated freeze-thaw cycles. (EDTA and Heparin can also be used as a anticoagulant)
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2000 x g for 10 minutes. Remove serum and assay. Dilute samples 1:20 into MIX Diluent. The undiluted samples can be stored at -20⁰C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

- **Cell Culture Supernatants:** Centrifuge cell culture media at 2000 x g for 10 minutes to remove debris. Collect supernatants and assay. The samples can be stored at -20°C or below. Avoid repeated freeze-thaw cycles.

Reagent Preparation

- Dilute all reagents freshly and bring all reagents to room temperature before use.
- **MIX Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the MIX Diluent 1:10 with reagent grade water. Store for up to 1 month at 2-8°C.
- **Standard Curve:** Reconstitute the 160 ng of human TFPI Standard with 4 ml of MIX Diluent to generate a 40 ng/ml of standard solution. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the TFPI standard solution (40 ng/ml) 1:2 with equal volume of MIX Diluent to produce 20, 10, 5, 2.5, 1.25, 0.625 and 0.313 ng/ml. MIX Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C.

Standard Point	Dilution	[TFPI] (ng/ml)
P1	1 part Standard (40 ng/ml) + 1 part MIX Diluent	20.000
P2	1 part P1 + 1 part MIX Diluent	10.000
P3	1 part P2 + 1 part MIX Diluent	5.000
P4	1 part P3 + 1 part MIX Diluent	2.500
P5	1 part P4 + 1 part MIX Diluent	1.250
P6	1 part P5 + 1 part MIX Diluent	0.625
P7	1 part P6 + 1 part MIX Diluent	0.313
P8	MIX Diluent	0.000

- **Biotinylated TFPI Antibody (100x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:100 with MIX Diluent. Any remaining solution should be frozen at -20°C.
- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with MIX Diluent. Any remaining solution should be frozen at -20°C.

Assay Procedure

- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30°C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of standard or sample per well. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300 µl of Wash Buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.

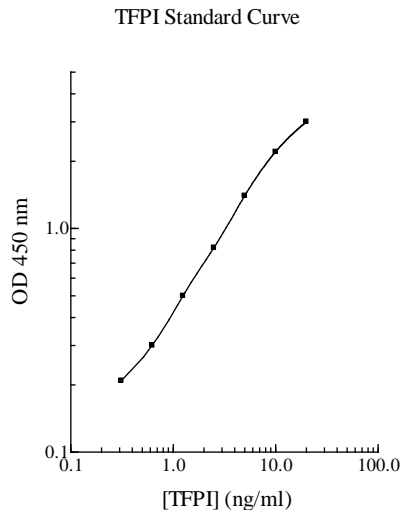
- Add 50 μ l of Biotinylated Human TFPI Antibody to each well and incubate for 1 hour.
- Wash the microplate as described above.
- Add 50 μ l of Streptavidin-Peroxidase Conjugate to each well and incubate for 30 min at room temperature. Turn on the microplate reader and set up the program in advance.
- Wash a microplate as described above.
- Add 50 μ l of Chromogen Substrate to each well and incubate for about 10 minutes at room temperature or till the optimal blue color density develops. Gently tap the plate to ensure thorough mixing and break the bubbles if there is any in the well with pipette tip.
- Add 50 μ l of Stop Solution per well. The color will change from blue to yellow.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample and subtract the mean value of zero standard readings.
- To generate a Standard Curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.



Precision, Sensitivity and Specificity

- The minimum detectable level of TFPI is typically \sim 0.3 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 4.6 % and 7.3 % respectively.
- This kit measures total TFPI concentration.

Linearity

Sample Dilution	Average Percentage of Expected Value	
	Plasma	Serum
1:10	98%	96%
1:20	100%	98%
1:40	103%	105%

Recovery

Standard Added Value	0.5 - 5 ng/ml
Recovery %	94 – 104%
Average Recovery %	97%

Cross-Reactivity

Species	% Cross Reactivity
Canine	None
Bovine	None
Monkey	< 30 (suggest 1:5 dilution for plasma/serum)
Mouse	None
Rat	None
Swine	< 5

References

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