



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 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

- 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 (30 ng, lyophilized).
- **Biotinylated TFPI Antibody (50x):** A 50-fold concentrated Biotinylated polyclonal antibody against human TFPI (160 µl).
- **EIA 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 kit at 2-8⁰C or -20⁰C upon arrival up to the expiration date.
- Opened EIA Diluent may be stored for up to 1 month at 2-8⁰C. Store reconstituted reagents at -20⁰C or below.
- Opened unused strip wells may return to the foil pouch with the desiccant pack, reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.

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 EIA 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 -20⁰C or below. Avoid repeated freeze-thaw cycles.

Reagent Preparation

- Dilute all reagents freshly and bring all reagents to room temperature before use. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.
- **EIA Diluent Concentrate (10x):** Dilute the EIA Diluent 1:10 with reagent grade water. Store for up to 1 month at 2-8⁰C.
- **Standard Curve:** Reconstitute the 30 ng of human TFPI Standard with 1.5 ml of EIA Diluent to generate a 20 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 (20 ng/ml) twofold with equal volume of EIA Diluent to produce 10, 5, 2.5, 1.25 and 0.625 ng/ml. EIA 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 (20 ng/ml)	20.00
P2	1 part P1 + 1 part EIA Diluent	10.00
P3	1 part P1 + 1 part EIA Diluent	5.00
P4	1 part P1 + 1 part EIA Diluent	2.50
P5	1 part P1 + 1 part EIA Diluent	1.25
P6	1 part P1 + 1 part EIA Diluent	0.63
P7	EIA Diluent	0.00

- **Biotinylated TFPI Antibody (50x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:50 with EIA Diluent. Any remaining solution should be frozen at -20°C.
- **Wash Buffer Concentrate (20x):** 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 EIA 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 µl of Biotinylated Human TFPI Antibody to each well and incubate for 1 hour.
- Wash a microplate as described above.
- Add 50 µl 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 µl of Chromogen Substrate to each well and incubate for about 15 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 µl 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

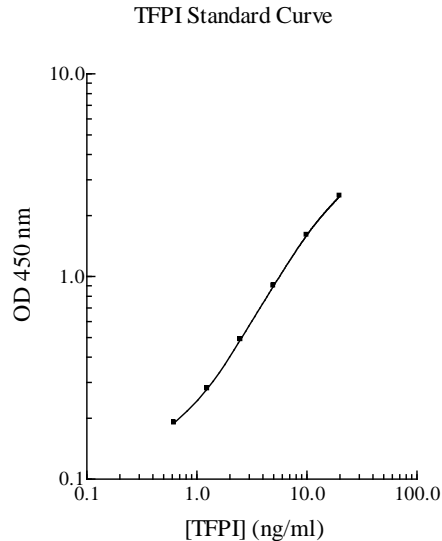
unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings

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 < 0.5 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 4.3 % and 7.7% respectively.
- This kit measure total TFPI concentration.

Linearity

Sample Dilution	Average Percentage of Expected Value
	Plasma
1:10	99%
1:20	100%
1:40	106%

Recovery

Standard Added Value	1-10 ng/ml
Recovery %	92 – 106%
Average Recovery %	98%

Cross-Reactivity

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

Note: Reference Plasma TFPI concentration is averaged at 85 ng/ml.

References

- (1) Broze, G.J. Jr. (1995) *Annu. Rev. Med.* 46:103
- (2) Rapaport, S.I. *et al.* (1990) *Adv. Exp. Med. Biol.* 281:97
- (3) Wesselschmidt, R.I. *et al.* (1993) *Blood Coag. Fibrinol.* 4:661
- (4) Novotny, W.F. *et al.* (1989) *J. Biol. Chem.* 264:18831
- (5) Broze, G.J. *et al.* (1994) *Blood Coag. Fibrinol.* 5:551
- (6) Sandset, P.M. *et al.* (1991) *Haemostasis* 22:219
- (7) Sandset, P.M. *et al.* (1989) *Haemostasis* 19:189
- (8) Novotny, W.F. *et al.* (1989) *J. Biol. Chem.* 264:18832
- (9) Lindahl, A.K. *et al.* (1989) *Acta. Chir. Scand.* 155:389

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