INTENDED USE

The IMUBIND® Tissue Factor ELISA Kit is an enzyme-linked immunoassay for the quantitative determination of human tissue factor (TF, Thromboplastin, factor III) in plasma, tumor tissue extracts and cell culture supernatants (e.g. LPS stimulated monocytes). The lower detection limit is approximately 10 pg/mL. This assay recognizes TF-apo, TF and TF-VII complexes and is designed such that there is no interference from other coagulation factors or inhibitors of procoagulant activity. This assay is for research use only. It is not intended for diagnostic or therapeutic procedures.

BACKGROUND

Tissue Factor is an integral membrane-bound glycoprotein (47 kD) on SDS-PAGE that requires the presence of specific phospholipids to function. It serves as both the receptor and essential cofactor(s) for VII and VIIa in initiating cell surface procoagulant activity (PCA).1,2 Once Tissue Factor was understood to initiate the extrinsic pathway of coagulation (directly activating VII that in turn activates factor X). Now, it is also known to activate factor X through the intrinsic pathway by activating factor IX which in turn activates factor X.3 The bimolecular complex of TF and factor(s) VII or VIIa activates factors IX and X by limited proteolysis, leading ultimately to thrombin generation and fibrin formation. As a potent initiator of coagulation, TF is believed to have a critical function in hemostasis and thrombogenesis.

Tissue Factor, while predominantly found in lung, brain, placenta and some neoplastic tissues (e.g. breast carcinoma, melanoma), a recent investigation has shown increased Tissue Factor levels in patients diagnosed with malignant solid tumor diseases.10

BIOLOGICAL CONTENT

Generally, Tissue Factor is absent on cells within the vascular endothelium or, if present, its conformation is not suitable to react with factor VII. However, when tissue injury disrupts the vascular endothelium, the physical barrier separating intravascular factor VII from TF is broken and factor VII/TF complexes can be formed. Furthermore, when monocytes, macrophages and endothelial cells are stimulated by endotoxins, cytokines and lectins the TF is "upregulated" in these cells with an increase in PCA. Some studies have found elevated levels of TF in plasma samples of patients with endotoxin induced DIC.4,9 However, it should be stressed that TF or the apo form of TF may not be fully active. The normal expression of TF reported in isolated trophoblastic microvilli, located at the interface of placentas and maternal blood, shows this discordance.9,6 In addition, recombinant TF shows little to no PCA, unless it is properly lipidated.
PRINCIPLE

The IMUBIND Tissue Factor ELISA Kit employs a murine anti-human tissue factor monoclonal antibody for antigen capture. This antibody recognizes and neutralizes human brain thromboplastin. Specificity of the ELISA was confirmed by the presence of a single 47 kD band on Western blot analysis. This was found not only for SDS gels of purified tissue factor apoprotein (recombinant TF) but also Triton X-100 extract from human brain tissue.7,8

Tissue or plasma samples incubate in micro-test wells precoated with capture antibody. Once captured the TF is detected using a biotinylated antibody fragment that specifically recognizes bound TF. 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 creates a blue colored solution. Sensitivity is increased by the addition of a 0.5M sulfuric acid stop solution, yielding a yellow color. TF levels are determined by measuring solution absorbances at 450 nm and comparing the values with those of a standard curve.

REAGENTS

6 x 16 well precoated micro-test strips with holder and lid
6 vials TF Standards, 0-1000 pg/mL (lyophilized)
2 vials Detection Antibody, biotinylated anti-human TF 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

0.2 µm filtered deionized or distilled H2O
TBS, Tris Buffered Saline, pH 7.4 (50 mM Tris, 100 mM NaCl)
50-200 µL eight channel multi-pipette
10-200 µL single pipette
Microwell plate reader at 450 nm
Microwell plate washer
0.5M H2SO4
Bovine Serum Albumin (BSA, e.g. Sigma A-7030)

REAGENT PREPARATION

A. Standards
1. Add 1.0 mL distilled H2O to 50, 100, 200, 500 and 1000 pg/mL standard vials.
2. Add 2.0 mL distilled H2O to 0.0 pg/mL standard vial.
3. Agitate gently for 3 minutes.

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

C. Enzyme Conjugate Diluent
Add 20 mL filtered deionized H2O to the vial and mix well.

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

E. 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 strips and unreconstituted reagents at 2°-8°C until expiration dates indicated on label. Store reconstituted reagents at 2°-8°C for up to one month. Standards must be aliquoted and frozen.

SAMPLE PREPARATION

A. Cell Lysates

Cells may be disrupted by repeated freeze-thaw cycles or sonication and the tissue factor extracted with a buffer of Tris Buffered Saline (50 mM Tris, 100 mM NaCl), pH 7.4 containing 0.1% Triton X-100. An extraction for 18 hours at 2°-8°C is recommended.

Centrifuge the lysed cells to remove the cell debris. Cell lysates should be stored at -70°C until assayed.
SAMPLE PREPARATION (continued)

C. Plasma
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 3,000 rpm for 10 minutes and store frozen.
3. Frozen plasma should be thawed at 37°C for 15 minutes.
4. Dilute plasma samples 1:4 in Sample Buffer.

D. Extracts of Tissue Samples
1. Suspend the powder from pulverized frozen tissue samples (100-300 mg wet weight) in 1.8 mL of TBS, pH 8.5.
2. Add 0.2 mL 10% Triton X-100 in TBS, pH 8.5, to the tissue suspension to yield a 1% Triton X-100 final preparation.
3. Stir for 12 hours at 4°C.
4. Centrifuge the suspension at 100,000 x g for 60 minutes at 4°C to separate cell debris.
5. Decant the supernatant/tissue extract and measure the total protein content of the extract using a BCA protein assay. If necessary, adjust the total protein content to 2-3 mg/mL with TBS, pH 8.5. Aliquot the extract into 100 µL portions.
6. Dilute the tissue extract 1:20 in Sample Buffer for immediate use or freeze the extract at -70°C or in liquid nitrogen for long term storage.

ASSAY PROCEDURE (continued)

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.

REPRESENTATIVE STANDARD CURVE

The standard curve is constructed by plotting the mean absorbance value for each TF standard versus the corresponding concentration of TF in pg/mL. Interpolate unknown values directly from the standard curve. For diluted samples multiply the value from the standard curve by the dilution factor to calculate the corrected sample value. A standard curve should be generated each time the assay is performed.

**IMUBIND® Tissue Factor ELISA**

\[ y = -7E-07x^2 + 0.0023x + 0.130 \]

\[ R^2 = 0.9996 \]
EXPECTED VALUES

IMUBIND Tissue Factor ELISA is an excellent screening tool for determining antigenic TF in biological fluids. A normal range for human plasma remains to be established. Since disrupted membrane proteins are removed by the reticuloendothelial system, low levels of circulating TF are expected in both normal plasma and in most diseased samples. In addition, the assay measures TF complexed to factor VII or VIIa.

This ELISA measures TF in plasma and other biological fluids, however, plasma samples may give a slightly higher background signal. In sepsis and some diseased states TF levels may vary owing to certain stimulants (e.g., endotoxins, etc.). This may be reflected in variable levels following the course of the disease.

References


