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IMUBIND® Tissue Factor ELISA

REF 845

INTENDED USE

The IMUBIND® Tissue Factor ELISA 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). 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.

EXPLANATION OF THE TEST

Tissue Factor (TF) is a 45 kD transmembrane cell surface glycoprotein known for its role in initiating coagulation for the past 90 years.[1] TF is comprised of three domains: an extracellular domain (aa 1-219), followed by a hydrophilic spanning domain (aa 220-242) and a cytoplasmic tail (aa 243-263).[2] It functions as a receptor and cofactor for the latent serine proteases factor VII and VIIa.[3,4] Contact between TF and blood is sufficient to initiate the extrinsic pathway of coagulation. TF is located on the cells in the adventitia and variably on cells in cell culture.

In vitro studies reveal that once TF complexes with factor VII, factor VII is efficiently activated by factor Xa. As with all vitamin K-dependent zymogens, activation requires the presence of calcium ions and phospholipids. Factor VII activation differs from the activation of other vitamin K-dependent zymogens by its binding to TF, which causes a major allosteric change, and its upregulation by TF. Formation of this TF/FVIIa complex renders the factor VII bond at Arg152 - Ile153 susceptible to cleavage by trace amounts of factor Xa and factor IXa. Activation by factor Xa is profoundly enhanced by lipidated TF but not by soluble TF (aa 1-219). TF lipidation by acidic phospholipids is more efficient than vesicles made with phosphatidylcholine (PC). This suggests that factor Xa mediated activation of factor VII requires binding of the Gla-domain to the phospholipid head groups.[5,6]

Factor VIIa possesses little proteolytic activity by itself; only when bound to TF does factor VIIa possess sufficient proteolytic activity to activate factor IX and factor X. Although factor VIIa possesses some activity towards peptidyl substrates in the presence of apo TF protein and calcium ions, this is not the case for physiological substrates such as factor IX and factor X. These factors require negatively charged phospholipids for full expression of factor VIIa proteolytic activity.[7] Gamma carboxylation of the factor VII Gla-domain is crucial for manifestation of full biologic activity. Factor VII mutants lacking the Gla-domain have a markedly diminished capacity to bind to tissue factor. The interaction of factor VIIa with TF involves multiple binding sites: the N-terminal Gla-domain, the EGF domain ((aa 51-88) and amino acid residues located in the factor VII protease domain.

While predominantly found in lung, brain, trophoblastic microvilli, placenta and some neoplastic tissues (e.g. benign breast carcinomas and melanomas), recent investigations have revealed increased tissue factor levels in patients diagnosed with malignant solid tumors.[8,9] When monocytes and macrophages are stimulated by endotoxins, cytokines and lectins, TF is upregulated in these cells with an increase in procoagulant activity (PCA).

The cellular distribution of TF in the extravascular compartments (e.g. epidermis, placenta and organ capsules and in the central nervous system) suggests that TF represents a hemostatic barrier. Studies have reported patients diagnosed with Disseminated Intravascular Coagulation (DIC) as having an increase in TF plasma levels.[10]

Tissue Factor is released into the blood stream following disruption of the endothelium. The initiation of the coagulation pathways requires the participation of a series of molecules; TF and its ability to complex with, factor VII[11], factor X or factor IX, charged phospholipids and calcium (the catalytic activity of factor VIIa in comparison to VII is insignificant.[12]). The TF/FVIIa complex efficiently activates both factor X and factor IX, thus initiating both the intrinsic and extrinsic coagulation pathways.[13] The extrinsic pathway is quickly dampened by tissue factor pathway inhibitor (TFPI). TFPI is the only effective inhibitor of TF/FVIIa allowing intrinsic pathway via IX to activate factor X.[14]

PRINCIPLE OF THE METHOD

The IMUBIND Tissue Factor ELISA employs a murine anti-human tissue factor monoclonal antibody for antigen capture. This antibody recognizes human brain thromboplastin. Specificity of the antibody 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 microwells precoated with capture antibody. Once captured the TF is detected using a biotinylated antibody 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

96 antibody coated microwells (6 x 16 well strips) in a frame and cover sheet
6 vials of TF Standards, 0-1000 pg/mL (lyophilized)
2 vials of Detection Antibody, biotinylated anti-human TF (lyophilized)
1 vial of Enzyme Conjugate, Streptavidin-horseradish peroxidase (60 µL)
1 vial of Enzyme Conjugate Diluent (lyophilized)
1 vial of Substrate, TMB (11 mL)
1 packet of Wash Buffer, PBS with 0.1% Triton X-100, pH 7.4

WARNINGS AND PRECAUTIONS

FOR RESEARCH USE ONLY. Do not use kit components beyond the printed expiry date. Do not mix reagents from different kit lots. Avoid microbial contamination of kit components. Do not mouth pipette or ingest any of the reagents.. Do not smoke, eat or drink in areas where specimens or kit reagents are handled.

TF standards	Danger (US) Warning (EU)		CONT Polyethylene glycol octylphenol ether H317, H319, H334, H412, P261, P264, P272, P273, P280, P305 + P351 + P338, P337 + P313
Detection Antibody	Warning		H319, P264, P280, P305 + P351 + P338, P337 + P313
Enzyme Conjugate	Warning		CONT 2-methyl-4-isothiazol-3 one H317, P261, P272, P280, P302 + P352, P333 + P313
Enzyme Conjugate Diluent	Danger		CONT Polyethylene glycol octylphenol ether H317, H318, H334, H412, P261, P273, P280, P302+P352, P305 + P351 + P338
Wash buffer, PBS + Triton	Danger		CONT Polyethylene glycol octylphenol ether H318, H412, P273, P280, P305 + P351 + P338

Hazard Statements:
H317 May cause an allergic skin reaction.
H318 Causes serious eye damage.
H319 Causes serious eye irritation.
H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled.
H412 Harmful to aquatic life with long lasting effects.

Precautionary Statements:
P261 Avoid breathing mist or vapor.
P264 Wash thoroughly after handling.
P272 Contaminated work clothing must not be allowed out of the workplace.
P273 Avoid release to the environment.
P280 Wear eye protection/face protection. Wear protective gloves.
P302 + P352 IF ON SKIN: Wash with plenty of water.
P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P333 + P313 If skin irritation or rash occurs: Get medical advice/attention.
P337 + P313 If eye irritation persists: Get medical advice/attention.

REAGENT PREPARATION AND STORAGE

A. Standards

1. Add 1.0 mL distilled H_2O to 50, 100, 200, 500 and 1000 pg/mL standard vials.
2. Add 2.0 mL distilled H_2O to 0.0 pg/mL standard vial.
3. Agitate gently for 3 minutes.

B. Detection Antibody

Add 5.5 mL filtered deionized H_2O per vial and agitate gently for 3 minutes.

C. Enzyme Conjugate Diluent

Add 20 mL filtered deionized H_2O to the vial and mix well.

D. Wash Buffer

1. Dissolve contents of the Wash Buffer packet in 900 mL of filtered deionized H_2O.
2. Mix well.
3. Dilute to a final volume of 1 Liter with filtered deionized H_2O.

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 microwells 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.

SPECIMEN COLLECTION AND 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.

B. Tissue Culture Supernatants

Dilute samples 1:5 (recommended initial dilution) in Sample Buffer. Note: some cell systems may require a higher dilution factor (up to 1:500).

C. Plasma

Only citrate collected platelet poor plasma may be used for this assay. Do Not Use EDTA collected plasma. See "Collection, Transport and Processing of Blood Specimens for Testing Plasma-based Coagulation Assays and Molecular Hemostasis Assays; Approved Guidelines-Fifth Edition", CLSI Document H21-A5, Vol. 28, No. 5, January 2008. Plasma collection should be performed as follows:

1. Collect 9 parts of blood into 1 part of 3.2% (0.109 M) trisodium citrate anticoagulant solution.
2. Centrifuge the blood sample at 3,000 x g for 15 minutes.
3. Plasma should be stored at room temperature and assayed within 2 hours. Alternatively, plasma may be stored at –80°C for up to 6 months
4. Frozen plasma should be thawed rapidly at 37°C. Thawed plasmas should be stored at room temperature and assayed within 2 hours.
5. 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.

PROCEDURE

Materials Provided – See Reagents

Materials Required But Not Provided

0.2 µm filtered deionized or distilled H_2O
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 H_2SO_4
Bovine Serum Albumin (BSA, e.g. Sigma A-7030)

Assay Procedure

Day One

1. Remove the necessary number of antibody coated microwells from the foil pouch. Return unused microwells to the pouch and reseal it with the desiccant inside.
2. Add 100 µL of Tissue Factor Standard or diluted sample to micro-test wells, cover with lid and incubate overnight at 2°-8°C. All standards and samples should be assayed in duplicate.

Day Two

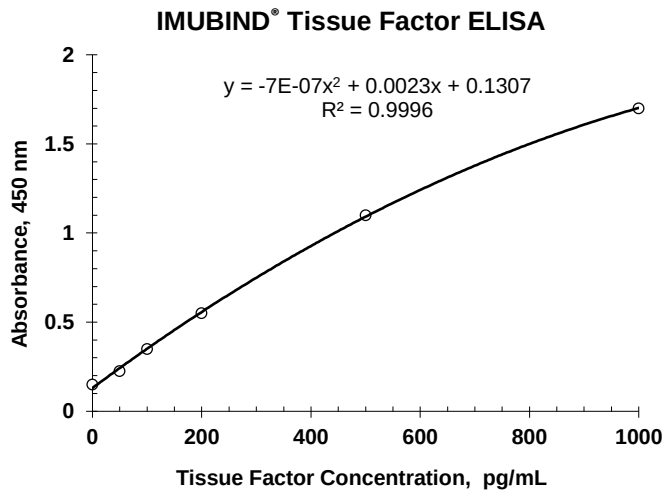
3. Wash wells 4 times with Wash Buffer.
4. Add 100 µL of Detection Antibody to each well, cover and incubate for 1 hour at room temperature.
5. Wash wells 4 times with Wash Buffer.
6. For running all 96 wells at one time, add 12 µL of Enzyme Conjugate to 12 mL of Enzyme Conjugate Diluent (add 2 µL of 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 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 H_2SO_4. Tap the sides of the wells to ensure even distribution of the H_2SO_4. The solution color will turn yellow. Read the absorbances on a microwell plate reader at a wavelength of 450 nm within 30 minutes.

RESULTS

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. The following curve is for demonstration purposes only.



EXPECTED VALUES

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 normal plasma. Plasma samples may present a slightly higher background signal than other sample media.

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DEFINITION OF SYMBOLS

	Consult instructions for use	LOT	Lot number	⚠	Refer to SDS
CONT	contains		Expiration Date		
	Manufactured by	REF	Catalog number		
2°C 8°C	Store at 2°C to 8°C	Σ n	Contains sufficient for <n> tests		