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ZYMUTEST Tissue Factor

 # RK042A

(Two-site, enhanced, ELISA immunoassay for measuring Tissue Factor)

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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INTENDED USE:

The ZYMUTEST Tissue Factor kit is a two-site, enhanced, immuno-assay for measuring Tissue Factor (TF), in plasma and purified milieu, or in any biological fluid where TF can be present. This kit does not recognize alternatively spliced Tissue Factor (asTF).
This kit is for research use only and should not be used for patient diagnosis or treatment.

ASSAY PRINCIPLE:

In a first step, the TF-Sample Diluent and the sample or calibrator are introduced into a microwell coated with a highly purified mouse monoclonal antibody specific for human TF. When present, TF is captured onto the solid phase. Then, following a washing step, a sheep polyclonal antibody specific for human TF coupled to biotin (BIOT Ab) is introduced into the microwells and binds to another free epitope of immobilized TF. Subsequently, following a new washing step, a Horseradish Peroxidase-Streptavidin conjugate (HRP-S) is introduced. Due to its high affinity for biotin, streptavidin binds to the biotinylated antibody. Following a last washing step, the highly sensitive peroxidase substrate (TMB-HS), in presence of hydrogen peroxide (H_2O_2), is introduced and a blue colour develops. When the reaction is stopped with Sulfuric Acid, a yellow colour is obtained. The amount of colour developed is directly proportional to the concentration of human TF in the tested sample.

TEST SAMPLE:

- Trisodium Citrate human plasma.
- Cell culture supernatants.
- Any biological fluid where TF must be measured.

REAGENTS:

1. **COAT: Micro ELISA plate,** containing 12 strips of 8 wells, coated with a mouse monoclonal antibody specific for human TF, then stabilised; the plate is packed in an aluminium pouch hermetically sealed in presence of a desiccant.
2. **SD-TF:** 2 vials containing 40 ml each, of the specific **TF-Sample Diluent,** green colored, containing 0.1% of Triton X-100, ready to use.
3. **Cal:** 3 vials of lyophilised **Tissue Factor Calibrator,** containing **"C" pg/ml** of TF (about 500 pg/ml) following reconstitution, in a purified milieu.
4. **CI:** 1 vial containing **1 ml** of lyophilised **Tissue Factor Control I** (high level).
5. **CII**: 1 vial containing **1 ml** of lyophilised **Tissue Factor Control II** (low level).

Note: The TF concentrations and acceptancy ranges for calibrator and controls can vary from lot to lot, and are indicated on the flyer provided in the kit.

6. **BIOT Ab (4X):** 1 vial of **Anti-(h)-TF biotinylated sheep polyclonal antibody**, **4 fold concentrated**, lyophilised.
7. **HRP-S:** 3 vials of **HRP-Streptavidin conjugate**, lyophilised.
8. **CD-TF:** 1 vial of 25 ml of specific TF **Conjugate Diluent**, ready to use.
9. **WS:** 1 vial of 50 ml of 20 fold concentrated **Wash Solution**.
10. **TMB-HS:** 1 vial of highly sensitive peroxidase substrate: **3,3',5,5' – Tetramethylbenzidine** containing hydrogen peroxide. Ready to use.
11. **SA:** 1 vial of 6 ml of **0.45M Sulfuric acid** (Stop solution). Ready to use.

***Note*:** Use only components from a same kit lot. Do not mix components from different lots, when running the assay.

REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED:

- 8-channel or repeating pipette allowing dispensing 50-300 µl.
- 1-channel pipettes at variable volumes from 20 to 200 µl and 200 to 1000 µl.
- Micro ELISA plate washing equipment and shaker.
- Micro ELISA plate reader with a wavelength set up at 450 nm.
- Distilled water.

TRACEABILITY TO THE REFERENCE MATERIAL:

The concentration of Tissue Factor, for each new lot of calibrators and controls, is established against the internal reference which concentration has been precisely determined. The calibrator and controls are prepared with recombinant full length human Tissue Factor (1-263).

REAGENTS PREPARATION, STORAGE AND STABILITY:

In their original packaging box, before use, when stored at 2-8°C, the unopened reagents are stable until the expiration date printed on the box.

1. **Micro ELISA plate:** open the aluminium pouch and take off the required amounts of 8 well strips for the test series. When out of the pouch, the strips must be used within 30 minutes. Unused strips can be stored at **2-8°C** for **4 weeks** in their original aluminium pouch, in presence of the desiccant, hermetically closed and protected from any moisture, and stored in the provided microplate storage bag (minigrip).
2. **TF Sample Diluent:** It is ready to use. When opened, it can be used for **4 weeks**, stored at **2-8 °C**, and provided that any bacterial contamination is avoided during use. This reagent contains 0.05% Kathon CG and 0.1% Triton X-100.
3. **Tissue Factor Calibrator:** restore each vial with exactly **2 ml of TF Sample Diluent** in order to obtain a solution containing **"C" pg/ml of recombinant human TF** (see the flyer provided in the kit)**.**
4. **Tissue Factor Control I** (high): restore with exactly **1 ml** of distilled water to obtain a solution containing about 350 pg/ml of recombinant human TF (see the flyer provided in the kit).
5. **Tissue Factor Control II** (low): restore with exactly **1 ml** of distilled water to obtain a solution containing about 75 pg/ml of recombinant human TF (see the flyer provided in the kit).

Note: when restored, Tissue Factor calibrator and controls are stable for **8 hours** at room temperature (18-25°C), **72 hours at 2-8°C** or **2 months** frozen **at –20°C** or below.

6. **Biotinylated Antibody (4X)**: each vial must be restored with **6 ml of TF Sample Diluent**. Let the pellet completely dissolve before use, and shake the vial (vortex) in order to homogenize the contents, **then withdraw the quantity of biotinylated antibody necessary for the test and dilute it 4 fold in SD-TF**. For example, for 4 strips an adapted dilution is 1.8 ml of biotinylated antibody and 5.4 ml of SD-TF. In its original vial, the restored biotinylated antibody is stable for at least **24 hours** at room temperature (18-25°C), **4 weeks at 2-8°C or 6 months frozen at -20°C or below**.
7. **HRP-Streptavidin conjugate**: each vial must be restored with **7.5 ml of TF Conjugate Diluent**. Let the pellet completely dissolve before use, and shake the vial gently in order to homogenize the contents. The restored conjugate is stable for at least **24 hours** at room temperature (18-25°C), **2 weeks at 2-8°C or 2 months frozen at -20°C or below**.
8. **TF Conjugate Diluent**: It is ready to use. When opened, it can be used for **4 weeks**, stored at **2-8 °C**, and provided that any bacterial contamination is avoided during use. This reagent contains 0.05% Kathon CG.
9. **Wash Solution**: Incubate the vial for 15-30 minutes in a water bath **at 37°C** until complete dissolution of solids, when present. Shake the vial and dilute the amount required **1:20 in distilled water** (the 50 ml contained in the vial allow preparing 1 liter of Wash Solution). The Wash Solution must be stored at **2-8°C** in its original vial and used within **4 weeks** following opening. The diluted Wash Solution must be used within **7 days**, when protected from any contamination and stored at **2-8°C**. This reagent contains 0.05% Kathon CG.
10. **TMB-HS substrate:** It is ready to use. When opened, it can be used for **4 weeks**, stored at **2-8°C**, and provided that any bacterial contamination is avoided during use.
11. **Stop solution:** It is ready to use.

Cautions: Sulfuric acid, although diluted to 0.45M is caustic. As for any similar chemical, handle Sulfuric acid with great care. Avoid any skin and eye contact. Wear protection glasses and gloves when handling.

***Note*:** Bring the kit at room temperature, at least 30 min. before use. Store the unused reagents at 2-8°C. The stability studies at 30°C show that the reagents can be shipped at room temperature without damage.

SPECIMEN COLLECTION, HANDLING AND STORAGE:

Blood (9 vol.) must be collected on 0.109M citrate anticoagulant (1 vol.); plasma supernatant is decanted following a 20 min. centrifugation at 2,500 g; citrated plasma should be tested within **8 hours** or stored frozen at –20°C or colder for up to 6 months, and thawed for 15 min. at 37°C just before use. Thawed specimen must be tested within **4 hours**.

Note: Refer to GEHT or NCCLS/CLSI recommendations for further instructions on specimen collection, handling and storage. Discard any plasma presenting an unusual aspect (haemolysed, lipaemic aspect…).

PROCEDURE:

Tested plasmas or samples or controls:

The plasma samples and the Controls I and II must be tested **undiluted**. For other types of sample, the dilution factor must be adjusted to have a final TF concentration between 25 and 500 or "**C**" pg/ml in the tested samples. Dilutions of tested samples must be done in TF Sample diluent (TF-SD).

Calibration:

Using the **TF** calibrator at **"C"** pg/ml provided in the kit, prepare the following calibrators:

TF concentration (pg/ml)	C	C/2	C/4	C/10	C/20	0
Vol. of TF calibrator	1.0 ml	0.5 ml	0.25 ml	0.10 ml	0.05 ml	0 ml
Vol. of Sample Diluent	0 ml	0.5 ml	0.75 ml	0.90 ml	0.95 ml	1 ml

Mix gently for a complete homogenization.
The standard dilutions are stable for at least **4 hours** at room temperature (18-25°C).

Assay procedure:

Remove the required number of strips from the aluminium pouch, for the series of measures to be performed. Then put the strips in the frame provided. In the different wells of the micro ELISA plate introduce the reagents and perform the various assay steps as indicated on the following table:

Reagent	Volume	Procedure
TF-Sample Diluent (SD-TF)	**100 µl**	Introduce the TF-Sample Diluent in the micro ELISA plate wells
TF calibrator, or tested sample or controls or SD-TF (blank)	**100 µl**	Introduce the standard solutions or the tested samples in the corresponding micro ELISA plate well
Incubate for 2 hours at 37°C (or overnight at room temperature (18-25°C)) (a)		
Wash Solution (20 fold diluted in distilled water before use)	**300 µl**	Proceed to 5 successive washings **(b)**
Biotinylated Antibody (restored with 6ml of SD-TF and **diluted 4 fold in SD-TF)**	**200µl**	Introduce the Biotinylated Antibody diluted 1:4 in SD-TF in the micro ELISA plate wells
Incubate for 2 hours at 37°C (a)		
Wash Solution (20 fold diluted in distilled water before use)	**300 µl**	Proceed to 5 successive washings **(b)**
HRP-Streptavidin (restored with 7.5ml of Conjugate Diluent	**200µl**	Introduce the HRP-Streptavidin in the micro ELISA plate wells
Incubate for exactly 30 minutes at room temperature (18-25°C) (a)		
Wash Solution (20 fold diluted in distilled water before use)	**300 µl**	Proceed to 5 successive washings **(b)**
TMB-HS Substrate	**200 µl**	Immediately after the washing, introduce the substrate into the wells. **(b)** *Note:* The substrate distribution, row by row, must be accurate and at exact time intervals **(c)**.
Incubate for **exactly 15 minutes** at room temperature **(18-25 °C) (a)**		
0.45 M Sulfuric Acid (5)	**50 µl**	Following exactly the same time intervals than for the addition of substrate, stop the colour development by introducing the 0.45M sulfuric acid **(c).**
Wait for **10 minutes** in order to allow the colour to stabilize and measure absorbance at **450 nm. (A450)**. Subtract the blank value **(d)**.		

Note: Distribute calibrators, controls and tested specimen as rapidly as possible, in order to obtain homogeneous immunological kinetics for TF binding. A too long delay (>10 min) between the distribution of the first and the last wells may have incidence on immunological kinetics and produce inaccurate results (underestimated value for the last wells).

a. Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. An incubation temperature of 18-25°C must be respected. Results can be affected by a too high (>25°C) or too low (<18°C) temperature, and measured A450 could then be too high or too low. It has to be considered when analyzing the results. A450 values generated in the assay are susceptible to be significantly increased if shaking is used throughout the incubation steps.
b. Never let the plates empty between the addition of the reagents or following the washing step. The next reagent must be added **within 3 minutes**, in order to prevent the plate from drying, which could damage the immobilised components. If necessary, keep the plate filled with Wash Solution and empty it just before the introduction of the next reagent. The washing instrument must be adjusted in order to wash the plates gently, and to avoid a too drastic emptying, which could lower plate reactivity.
c. For addition of the substrate, the time interval between each row must be accurate and exactly determined. It must be the same when stopping the reaction with sulphuric acid.
d. For bichromatic readings, a reference wavelength at 690 nm or at 620 nm can be used

EXPRESSION OF RESULTS:

- On a linear graph paper, plot the **TF concentration (pg/ml)** on abscissa and the corresponding absorbance (**A450**) on ordinates. Draw the calibration curve that best fits your data.
- Users must construct their own calibration curve, obtained using their calibrators (See model on the flyer).
- For Controls and plasma samples tested undiluted, deduce directly the TF concentrations from the calibration curve obtained. For obtaining the TF concentration, the value read on the calibration curve must be multiplied by the dilution factor (i.e., x2 if the samples are tested at the 1:2 dilution…). The calibration is validated when quality controls are measured within their acceptance range, indicated for each lot on the flyer provided in the kit.
- Alternatively, an ELISA software (i.e., Dynex, Biolise, etc.) can be used for the calculation of concentrations. Choose the most suitable interpolation method. (The target values as well as acceptance ranges of controls must be verified in the exact laboratory working conditions and adjusted if required).

The results obtained should be for research purposes only and not used for patient diagnosis or treatment.

PERFORMANCES AND CHARACTERISTICS:

- **Detection threshold**: ≤ 10pg/ml.
- **Intra-assay reproducibility** (N= 12): CV = 9% for CI, CV = 8% for CII.
- **Inter-assay repeatability** (N= 10): CV = 6% for CI, CV = 7% for CII.
- **Recovery of full length TF in plasma**: about 80% for undiluted plasmas.
- **Recovery of truncated TF (1-219) in plasma**: about 80% for undiluted plasmas.
- **Specificity**: the reactivity is inhibited in plasmas following the addition of a monoclonal antibody specific for human TF.
- **Interferences**: the kit has been optimized to minimize the interference of the potential presence of heterophilic antibodies in some particular plasmas, which could otherwise result in an overestimation of TF concentration.
- **Reactivity**: the kit recognizes full length TF (1-263) and the extracellular domain of TF (1-219). It does not recognize asTF.

BIOCHEMISTRY:

Tissue Factor, (also known as coagulation factor III, or thromboplastin) is the physiologic trigger of blood coagulation. TF binds Factor VIIa to form FVIIa-TF complexes that cleave factors X and IX, initiating the whole coagulation cascade. TF is a 47KDa transmembrane protein (SDS-PAGE) constitutively expressed in sub-endothelial cells such as adventitial fibroblasts or smooth muscle cells. TF has three domains: an extracellular domain (aa 1-219), a transmembrane domains (aa 220-242), and a cytoplasmic tail (aa 243-263).

REFERENCES:

1. Parhami-seren et al. "Immunologic quantitation of tissue factors". J. Thromb. Haemost. 4:1747-1755, 2006.
2. Mackman, "The many faces of Tissue Factor". J. Thromb. Haemost. 7(Suppl. 1):136-139, 2009.
3. Edgington TS et al, "The structural biology of expression and function of tissue factor", Thromb Haemost 1991, 66(1): 67-79.