For Research Use Only

September/2023

SMAT – TFPI Assay Kit

Assay of Tissue Factor Pathway Inhibitor Activity in Plasma

The kit is for research purpose only, not for in vitro diagnostics

INTENDED USE

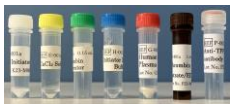
The smart analysis of thrombin production (SMAT) – tissue factor pathway inhibitor (TFPI) is a novel coagulation assay for determination of TFPI anticoagulant activity in plasma. The kit can be used to assess the profile of plasma TFPI activity in patients with thrombotic and bleeding disorders.

ASSAY PRINCIPLE

The tissue factor (TF)-FVIIa complex produces FXa in an initiation phase of coagulation that forms the initial prothrombinase complex with FVa. The initial prothrombinase activates prothrombin to a key coagulation enzyme thrombin (FIIa). TFPI controls the initial prothrombinase generation (PTaseG) through inhibition of activation of FX and FV by the TF-FVIIa complex and FXa, respectively. In assay, TFPI activity in plasma is defined as an ability to inhibit the initial PTaseG. The levels of the initial PTaseG in plasma are determined using discontinuous FIIa generation-based assay. The initial PTaseG is induced by adding TFPI assay initiator reagent including TF into recalcified plasma, followed by incubation at 37°C for 2.5 min. After incubation, the levels of the initial PTaseG are determined by kinetically monitoring the generated FIIa activity with a fluorogenic substrate. The levels are expressed as a relative ratio (%) to that of control plasma. TFPI activity is indicated as arbitrary unit that is determined by calculating the ratio between the initial PTaseG (%) in TFPI-depleted plasma with anti-TFPI blocking antibody as the numerator and the initial PTaseG (%) in plasma without adding anti-TFPI antibody as the denominator.

MATERIALS

Materials Supplied in the Kit



The kit for 20 determinations contains 7 reagents (2 with lyophilization and 5 in solution) in microtubes with different color caps for identification:

Kit Components

Cap colors	Tubes	Reagents with Lyophilization
White	1	TFPI Assay Initiator Including TF
Red	1	Human Control Plasma
Cap colors	**Volume**	**Reagents in Solution**
Yellow	1 × 1 mL	$CaCl_2$
Green	1 × 0.15 mL	Thrombin Calibrator (4,000 pM)
Blue	1 × 1 mL	Reconstitution Buffer
Brown	2 × 1.4 mL	Fluorogenic FIIa substrate/EDTA
Transparent	1 × 0.045 mL	Anti-TFPI Polyclonal Antibody

Materials Required – Not Supplied in the Kit

- Fluorescent plate reader (~360 nm excitation/~460 nm emission) with temperature control at 37°C and suitable software capable to monitor changes of fluorescence intensity over time
- Single - and repeater pipettes
- 96-well microtiter plate (flat bottom)

Preparation of Blood and Plasma Samples

Blood samples are prepared by carefully mixing 9 parts venous blood with 1 part 3.2% sodium citrate solution. Platelet-poor plasma samples are prepared by centrifugation of blood at 25°C for 10 min at 2,500 × g. Immediately after centrifugation, plasma is frozen and stored below -70°C.

PRECAUTIONS FOR USING OR HANDLING

- For research use only
- Control plasma included in the kit is tested and found negative for Hb_SAg, HIV 1/2 antibodies and HCV antibodies, but control plasma and plasma samples have to be handled as potentially infectious materials with appropriate care and in compliance with the respective biosafety regulations.
- All materials contaminated with blood or plasma must be disposed as biohazardous waste.

STORAGE

The kit should be stored at 4-8°C before use. The expiry date printed on the box label is only applicable to storage of the kit. The reconstituted initiator reagent can be stored at 4-8°C and used within 7 days.

ASSAY PROCEDURE

Preparation of Reagents for TFPI Activity Assay

- Preparation of the TFPI assay initiator reagent solution: the initiator reagent solution (white cap) is prepared by adding **0.64 mL** of reconstitution buffer (blue cap) and then mixing (vortex). The reagent solution can remain at ~25°C.
- Reconstitution of control plasma: control plasma (red cap) is reconstituted with **0.15 mL** of distilled water for 5 min at ~25°C. Plasma can remain at ~25°C for a maximum for 4 h.
- The reagents in solution supplied in the kit: the reagents stored at 4-8°C should be warmed to ~25°C before use. The $CaCl_2$ solution (yellow cap) and FIIa substrate/EDTA (brown cap) are kept at 37°C prior to the assay.

Preparation of TFPI-depleted plasma

TFPI-depleted plasma is prepared by incubation of **38 µL** of control plasma or plasma sample with **2 µL** of anti-TFPI polyclonal antibody (PoAb) blocking TFPI activity for 15 min at ~25°C.

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PERFORMANCE OF THE INITIAL PTASEG ASSAY FOR DETERMINING TFPI ACTIVITY IN PLASMA

1. Assay of the Initial PTaseG in Plasma Controlled by TFPI

(1) Add **35 µL** of control plasma or plasma sample to each 96-well microtiter plate wells.
(2) Insert plate into plate reader to incubate plate at 37°C for 2 min.
(3) Remove plate from plate reader.
(4) For each plasma, add **10 µL** the TFPI assay initiator reagent solution (white cap) to a plate well.
(5) Insert plate into plate reader to incubate plate at 37°C for 2 min.
(6) Remove plate from plate reader.
(7) Add **10 µL** $CaCl_2$ solution (yellow cap) to each plate well.
(8) Insert plate into plate reader to incubate plate at 37°C for 2.5 min.
(9) Remove plate from plate reader.
(10) Add **50 µL** FIIa substrate/EDTA solution (brown cap) to each plate well.
(11) Insert plate into 37°C plate reader. Measure fluorescence intensity generated by FIIa cleavage of the fluorogenic substrate at 37°C for ~1 min in ~10 sec intervals.
(12) Monitor relative fluorescence units (RFU) over time and plot data with plate reader operation software.
(13) Based on plotted RFU data over time, a mean value of slope (ΔRFU/second) is calculated using a linear regression analysis. Slope value is equal to the levels of PTaseG. The result is expressed as a relative ratio to control plasma: the initial PTaseG (%) = (the slope value of plasma sample ÷ the slope value of control plasma) × 100.

Attention!

Plasma sample should be tested simultaneously with control plasma.

2. Assay of the Initial PTaseG in TFPI-Depleted Plasma

The levels of the initial PTaseG in TFPI-depleted plasma are determined as above following addition of **35 µL** of control plasma or plasma sample including anti-TFPI PoAb to each 96-well microtiter plate wells. The levels are expressed as a relative ratio (%) to that of control plasma with anti-TFPI PoAb.

3. Assay with the FIIa Calibrator

Prior to testing plasma, assay of the FIIa calibrator activity (green cap) is recommended to verify that the fluorescence measurement with plate reader works correctly.

(1) Add **55 µL** of the FIIa calibrator and reconstitution buffer (blue cap) to each plate wells.
(2) Insert plate into plate reader to incubate plate at 37°C for 6.5 min.
(3) Remove plate from plate reader.
(4) Add **50 µL** FIIa substrate/EDTA solution (brown cap) to each plate well.
(5) Insert plate into 37°C plate reader. Measure fluorescence intensity generated by FIIa cleavage of the fluorogenic substrate at 37°C for ~1 min in ~10 sec intervals.
(6) Monitor RFU over time and plot data with plate reader operation software.

EVALUATION OF TFPI ACTIVITY IN PLASMA

TFPI activity is denoted as arbitrary unit. Arbitrary unit is determined by calculating the ratio between the initial PTaseG (%) in TFPI-depleted plasma as the numerator and the initial PTaseG (%) in plasma without adding anti-TFPI antibody as the denominator.

VALIDATION OF THE TFPI ACTIVITY ASSAY

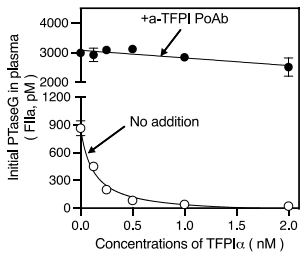

Fig.1 Dose-response of TFPIα effect on the initial PTaseG in plasma in the either presence or absence of inhibitory anti-TFPI PoAb. Data point denotes mean ± SD (n=2) of the generated FIIa.

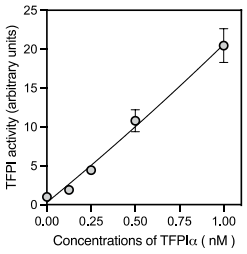

Fig.2 Determination of TFPIα anticoagulant activity in inhibition of the initial PTaseG. Data point denotes mean ± SD (n=2) of TFPI activity.

REFERENCE

(1) Selective factor VIII activation by the tissue factor-factor VIIa-factor Xa complex. Blood 130: 1661-1670, 2017

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