



SMAT – FVIII/FIX kit

Assay for Determination of the Intrinsic Tenase Complex - Driven Initial Prothrombinase Generation

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

INTENDED USE

The smart analysis of thrombin production (SMAT) – FVIII/FIX is the assay system with high sensitivity for determination of the prothrombinase complex generation (PG) in plasma in an initiation phase of coagulation induced by the intrinsic tenase complex (FVIIIa/FIXa) pathway. The kit can be used to monitor the anticoagulation drugs such as direct oral anticoagulants and the hemostatic agents for hemophilia patients. The kit is also intended to evaluate abnormality in blood coagulation in patients with thrombotic and hemorrhagic disorders and to investigate association of cancer and infectious diseases such as COVID-19 with thrombosis.

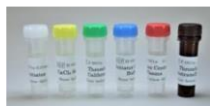
ASSAY PRINCIPLE

Prothrombinase complex activates prothrombin to an active enzyme FIIa. In the SMAT, the levels of the initial PG in plasma are determined by the FIIa generation-based assay with the FVIII/FIX initiator reagent. In assay, to specifically access the intrinsic tenase complex - driven PG, the initiator reagent including TF, phospholipids, and FXIa is incubated with citrated plasma in the presence of calcium ion. After incubation, the PG is terminated with ethylenediaminetetraacetic acid (EDTA) solution and then fluorescence generated by FIIa cleavage of fluorogenic substrate is kinetically measured. The PG is expressed as a relative ratio (%) to that of human control plasma. Of note, the assay is completed within 10 min.

MATERIALS

Materials Supplied in the Kit

The kit for 20 determinations contains 6 reagents (2 with lyophilization and 4 in solution) in microtubes with different color caps for identification:



Kit Components

Cap colors	Tubes	Reagents with Lyophilization
White	1	FVIII/FIX Initiator
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	1 × 1.4 mL	Fluorogenic FIIa substrate/EDTA

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 3 days.

ASSAY PROCEDURE

Preparation of Reagents for the PG Assay

- Preparation of the FVIII/FIX initiator reagent solution: The initiator reagent solution (white cap) is prepared by adding **0.32 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 **0.12 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 CaCl₂ solution (yellow cap) and FIIa substrate /EDTA (brown cap) are kept at 37°C prior to the assay.

Plate Reader Setting

- Temperature during assay procedures and fluorescence measurement: 37°C
- Fluorometer wavelength: ~360 nm/~460 nm (excitation/emission)



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PERFORMANCE OF THE PG ASSAY

The PG assay is carried out according to the following workflow. To create a workflow program using the plate reader operation software is strongly recommended for more accurate and consistent results.

1. Workflow for the PG Assay with Control Plasma and Plasma Sample

- (1) Add **35 μL** of control plasma (red cap) and 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, use a repeater pipette to add **10 μL** the FVIII/FIX 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 utilizing repeater pipette.
- (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 utilizing repeater pipette.
- (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.

Attention!

Plasma sample should be tested simultaneously with control plasma.

2. Workflow for Assay with the FIIa Calibrator

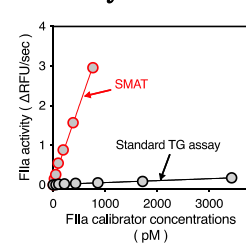
Prior to testing plasma, assay of the FIIa calibrator activity (green cap) is recommended to verify that the fluorescence measurement with plate reader and workflow program works correctly. The assay is performed in the same way as in the PG with plasma, but the steps (4) and (7) for testing plasma are skipped.

- (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 2 min.
- (3) Remove plate from plate reader.
- (4) Insert plate into plate reader to incubate plate at 37°C for 2 min.
- (5) Remove plate from plate reader.

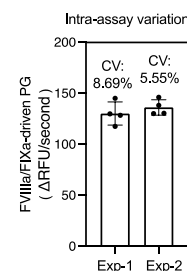
- (6) Insert plate into plate reader to incubate plate at 37°C for 2.5 min.
- (7) Remove plate from plate reader.
- (8) Add **50 μL** FIIa substrate/EDTA solution (brown cap) to each plate well utilizing repeater pipette.
- (9) Insert plate into 37°C plate reader. Measure fluorescence intensity generated by FIIa cleavage of the fluorogenic substrate at 37°C for ~1 min in ~10 sec intervals.
- (10) Monitor RFU over time and plot data with plate reader operation software.

EVALUATION OF THE PG IN PLASMA SAMPLE

Based on plotted RFU data over time, a mean value of slope ($\Delta\text{RFU}/\text{second}$) is calculated by a linear regression analysis of data with plate reader operation software or Excel (after data are exported). Slope value is equal to the levels of prothrombinase complex generated in plasma. The intrinsic tenase complex - driven TG is evaluated by dividing the slope value of plasma sample by that of control plasma. The result is expressed as a relative ratio (%) to control plasma: PG (%) = (the slope value of plasma sample \div the slope value of control plasma) \times 100.

ASSAY SENSITIVITY AND PRECISION**1. Assay Sensitivity**

Compared to the standard FIIa generation (TG)-based assay, the SMAT has higher sensitivity for detection of FIIa.

2. Precision

Testing intra-assay variation in the TG assay of control plasma indicates high reproducibility.

REFERENCES

- (1) Selective factor VIII activation by the tissue factor-factor VIIa-factor Xa complex. Blood 130: 1661-1670, 2017
- (2) <https://youtu.be/zUFNLGRioOs>

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