# TGMeasurement*


Innovation

Automated System

Complete Solution

Standardisation

## An introduction to the concise measurement of thrombin forming potential in plasma

*TG: Thrombin Generation

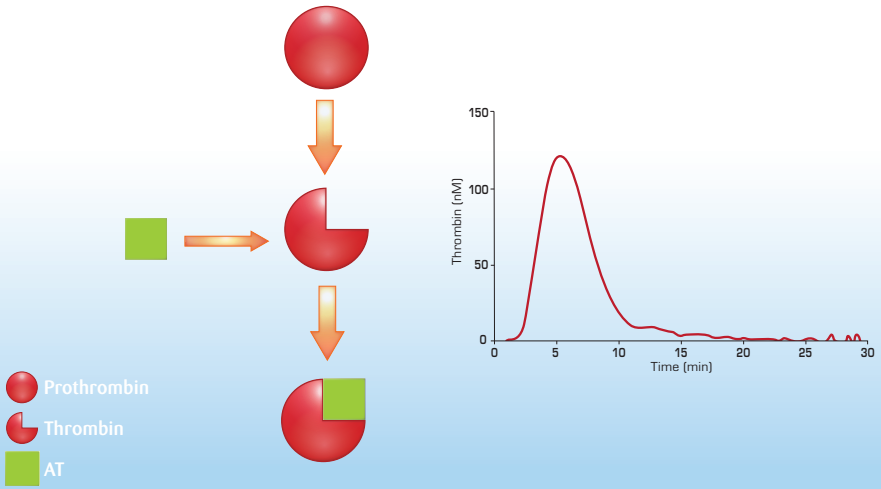
Stago

# Why Thrombin and its generation are important?

Thrombin is the central player in Haemostasis and Thrombosis[1], acting on various cells (in addition to platelets), and playing a role in inflammation. In addition, it retards fibrinolysis by activating thrombin activatable fibrinolysis inhibitor (TAFI).

In the body, thrombin is formed immediately after vessel damage in a complicated series of intricate reactions in which plasma proteins, blood platelets and the vessel wall participate. Tissue Factor (TF) initiates a series of interactions between the clotting factors of plasma, leading to the formation of *prothrombinase*; the latter rapidly converts prothrombin into thrombin. Newly formed thrombin is slowly inactivated by the several *antithrombins* of plasma[2].

## Central role of Thrombin




The situation is not unlike quickly emptying a pail of water in a washbasin with a small, open outlet. At first the level of water rises, slowly decaying to zero following the cessation of the inflow.

# Regulation of Thrombin Generation

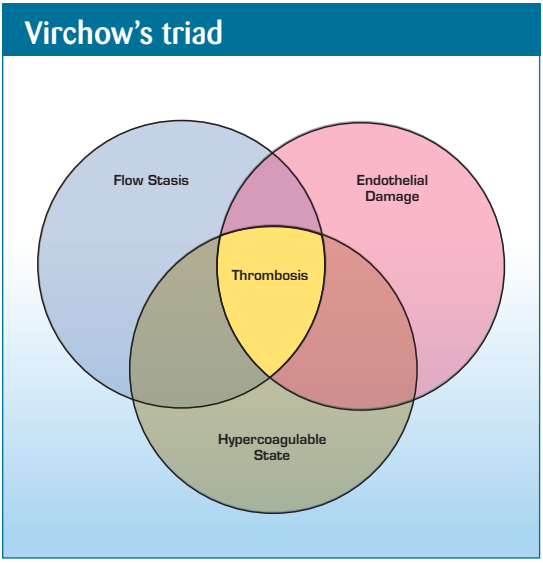
TF is the principle physiologic activator of the prothrombinase complex upon response to tissue injury. Prothrombinase activation is governed by the interaction of procoagulant clotting factors (FV, FVII, FVIII, FIX, FX, and FXI) with the inhibitory proteins tissue factor pathway inhibitor (TFPI), protein S (PS), and activated protein C (APC). APC formation is triggered by a complex of thrombin and vessel wall-bound thrombomodulin (TM). APC downregulates the coagulation pathway with the help of the cofactor PS.

Virchow's triad is a model describing the interplay of blood flow, vessel wall surface, and blood coagulability in the development of thrombosis.

Available evidence shows the thrombin forming potential of blood is the primary contributor to bulk coagulability, and can be measured by TG.

TG allows for an entirely new approach to hemostasis disorders, which have been thought of as "nobody's disease but everybody's complication (A. Kakkar)."

"Hypercoagulability" can be evidenced and quantified whatever its cause(s): an excess of one or more procoagulant factors and/or a defect in the inhibitory process.

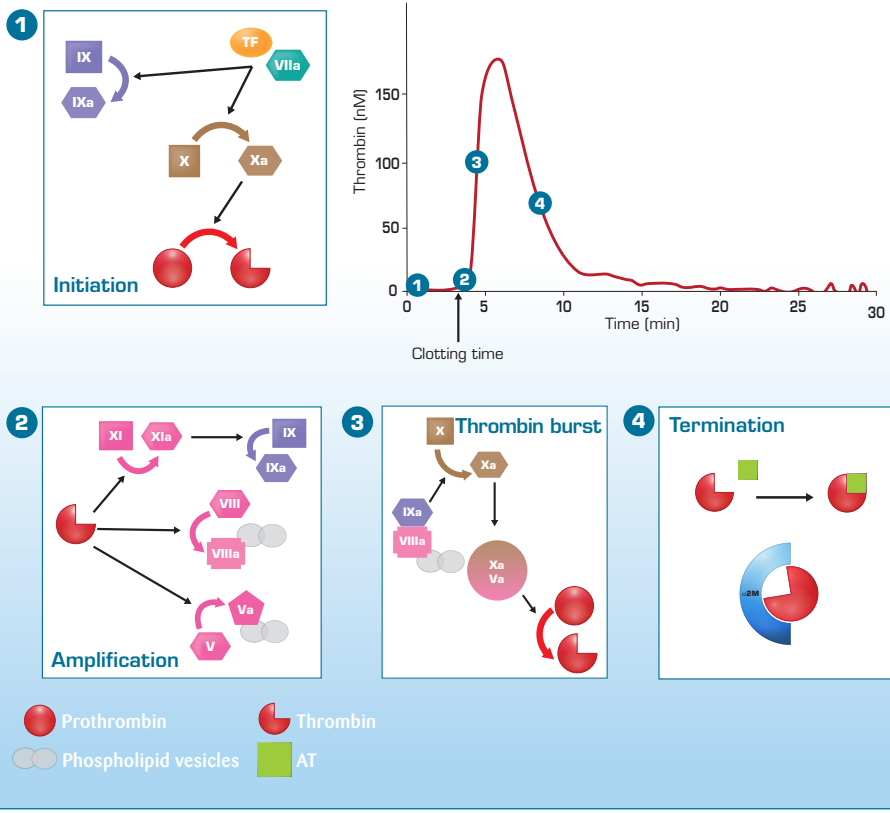



"The more thrombin the less bleeding but the more thrombosis, (and conversely) the less thrombin the more bleeding but the less thrombosis (H.C Hemker)".

# A typical normal thrombogram with its underlying phenomena

TG *in vitro* can be conceptualized as a functional test on a piece of "isolated organ" i.e. a sample of plasma to which essential elements of the vessel wall such as TF and TM are added. The thrombin-in-time curve is the *thrombogram*, which serves to quantify the complete thrombin potential available in the system.

## Four phases on the TG profile



TG in PPP is initiated with a low picomolar concentration of TF in the presence of artificial procoagulant vesicles of suitable phospholipids and calcium ions designed to mimic the surface of activated platelets.

The initiation phase (1) corresponds to the generation of the first traces of thrombin *via* Xa, and is under the control of TFPI. At the same time, the complex of FVIIa and TF activates small amounts of FIX which go on to form the intrinsic tenase. The intrinsic tenase is formed by the complex of FIXa and its activated cofactor FVIIIa, which then go on to activate FX.

Amplification (2) comprises the positive feedback loops of thrombin:

- formation of additional IXa as a result of the generation of XIa by thrombin;
- activation of factors VIII and V, acting as cofactors in the complexes intrinsic tenase and prothrombinase, respectively.

Physiologically, thrombin activates platelets, allowing for a procoagulant contribution of this surface to thrombin activation. In TG, the aforementioned process only occurs in PRP.

The thrombin burst (3) occurs when a fully active prothrombinase is assembled with further FXa generated by the intrinsic tenase.

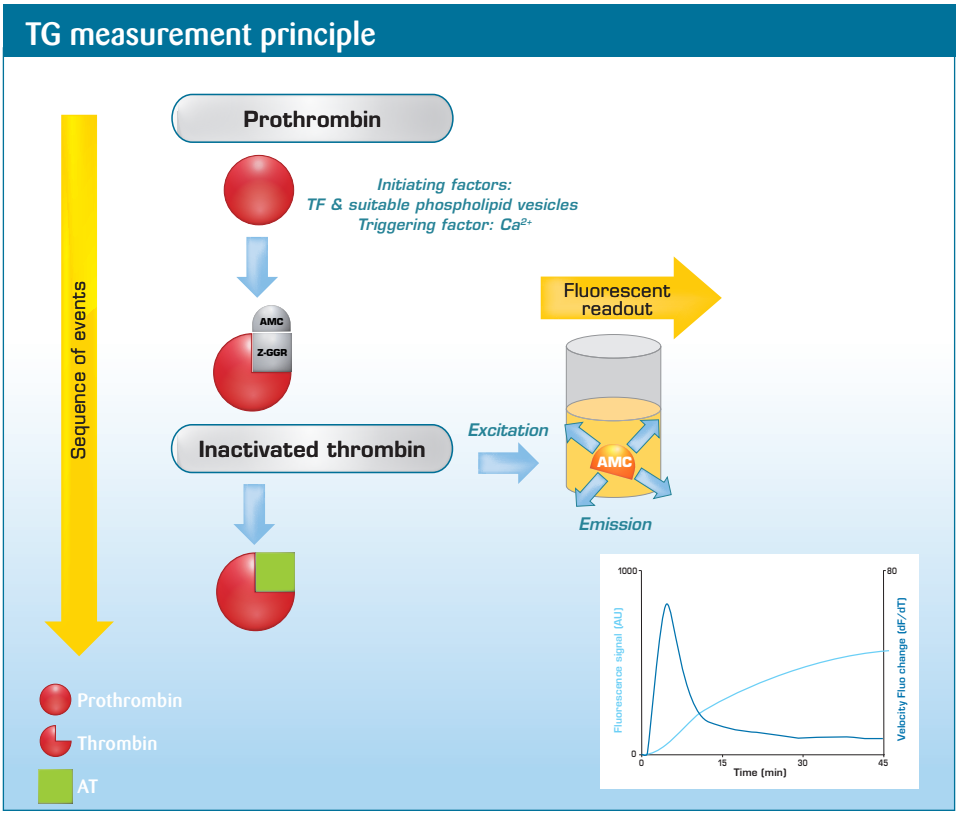
Importantly, clotting occurs at the very beginning of the thrombin burst. The clinical picture is determined by the bulk of thrombin generation, which occurs after clotting and is missed by traditional clotting assays.

The termination phase (4) corresponds to the neutralisation of thrombin by antithrombin, when thrombin is no longer generated. The bulk of thrombin inactivation is also missed when performing traditional clotting assays.

# TG Measurement

The TG measurement principle was invented by HC Hemker, with a small substrate of thrombin (Z-GGR-AMC) added to the sample plasma. The substrate is converted slowly to avoid complete consumption during the assay. With the low affinity of the substrate for thrombin, physiologically occurring clotting mechanisms are minimally disturbed[3].

## TG measurement principle

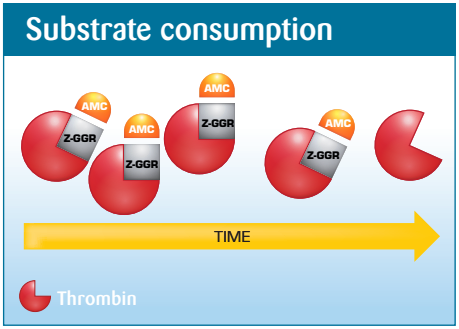


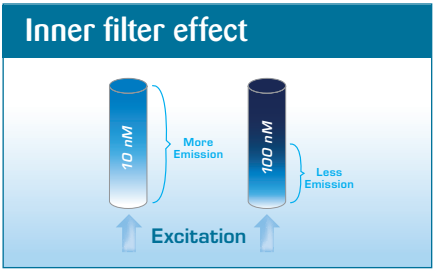

The substrate is split by thrombin and is said to be fluorogenic since the product - AMC – can be detected by its fluorescence (light blue line).

The velocity (first derivative) of the increase in fluorescence is the dark blue line.

# How to determine the TG from fluorescence changes? (I)

While fluorescence velocity increase resembles the TG curve, corrections must be made to produce the final TG curve. The first reason is due to the reaction velocity not only being dependent on the amount of thrombin at each timepoint but also on the substrate concentration, which diminishes during the reaction.

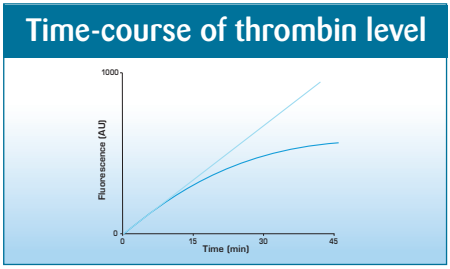



The second reason is that fluorescence is not proportional to the concentration of the cleavage product. AMC molecules are already emitting light, shielding other molecules from producing the maximal light emission, as illustrated in the following experiment.

Two solutions of AMC were prepared and exposed to a light beam from the bottom (arrows) at the excitation wavelength indicated. Light output (shown in light blue) decreases from bottom to top, with the difference in the output increasing as AMC concentration increases. The phenomenon is known as the “inner filter effect.”

As a consequence both of substrate consumption and inner filter effect, the calibration curve, i.e. the fluorescence trace resulting from a fixed amount of thrombin (100 nM), is not straight (light blue) but curved (dark blue line).




Using the combination of the corrected calibration curve and the fluorescence tracing, the time course of the thrombin concentration in the sample is calculated[5].

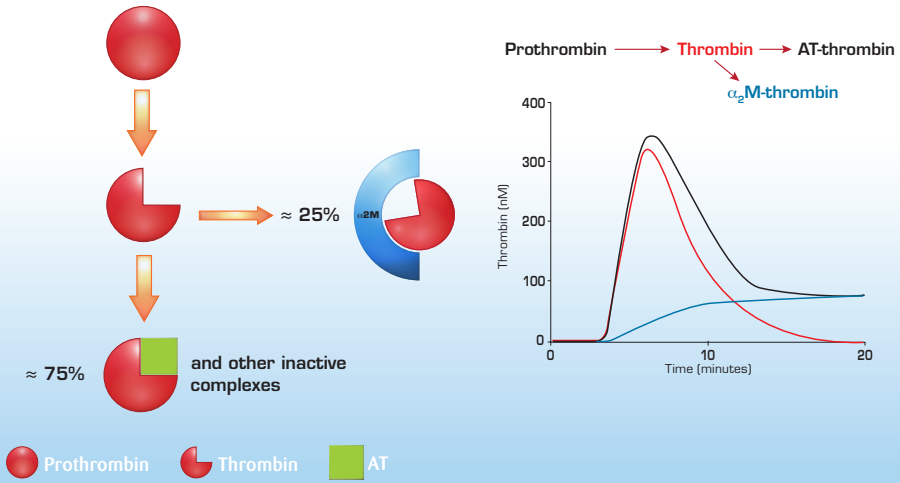
# How to determine TG from fluorescence changes? (II)

In order to determine the activity curve of free thrombin, another factor needs to be taken into consideration.

Newly formed thrombin is inactivated by several different antithrombins, the most important of which is antithrombin, which inactivates approximately 65% of all thrombin. Antitrypsin and various other inhibitors inactivate another 10%.

Thrombin complexed to $\alpha_2$M ($\alpha_2$M-thrombin) has lost physiological activity with respect to macromolecular substrates but can still cleave small fluorogenic substrates.

## $\alpha_2$-macroglobulin correction

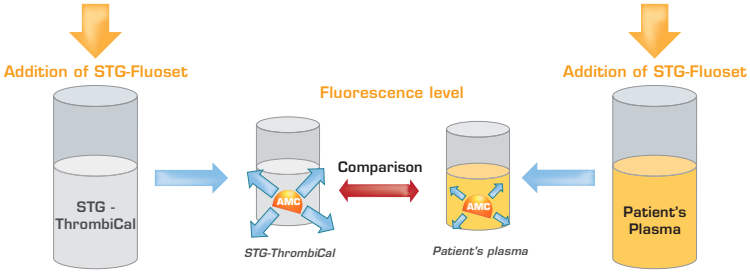



During TG, $\alpha_2$M-thrombin (blue curve) builds up and its activity adds up to that of free uninhibited thrombin (red curve). The activity probed by the substrate is thus the sum of the two (black curve). A simple algorithm enables splitting of the blue curve into its consituent two components, allowing for an isolation of the activity of free thrombin (red curve).

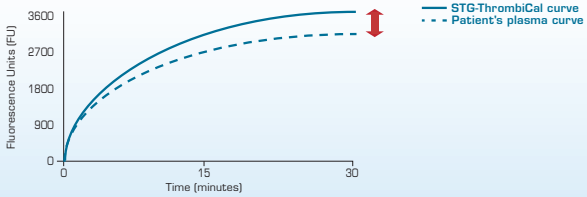
# How is the intensity of the plasma colour evaluated?

Fluorescence recorded is affected by the colour of the plasma (optical characteristics), differing between individual samples.

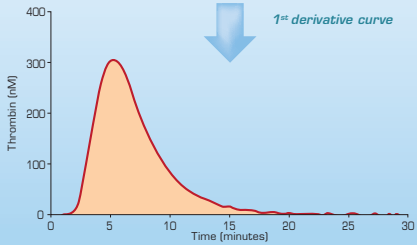
## Role of STG-Fluoset




Fluorescence level in neutral liquid




Adjustment of the calibration curve according to a correction factor related to the plasma sample colour

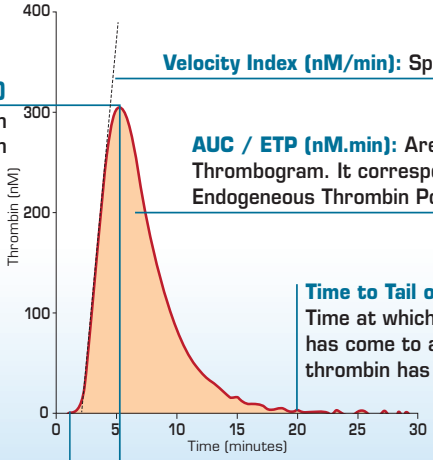



Thrombogram in plasma sample after calibration curve adjustment

In the plasma sample, a known amount of AMC fluorescent substrate provided from the STG-Fluoset allows for an accurate determination of the thrombin levels after the calibration curve is adjusted for the plasma colour. In this way, the thrombin activity and plasma colour are simultaneously evaluated for every individual sample run. This automated plasma colour correction is specific to the ST Genesia.

# Thrombogram parameters

## Six parameters of the Thrombogram




**Velocity Index (nM/min):** Speed to reach the peak

**Peak Height (nM)**
Maximal thrombin concentration

**AUC / ETP (nM.min):** Area under the Thrombogram. It corresponds to the patient Endogeneous Thrombin Potential (ETP)

**Time to Tail or Start Tail (min):**
Time at which thrombin generation has come to an end. All the generated thrombin has been inhibited

**Lag Time (min):**
Timepoint when fibrin clot is formed (=clotting time)

**Time To Peak (min)**

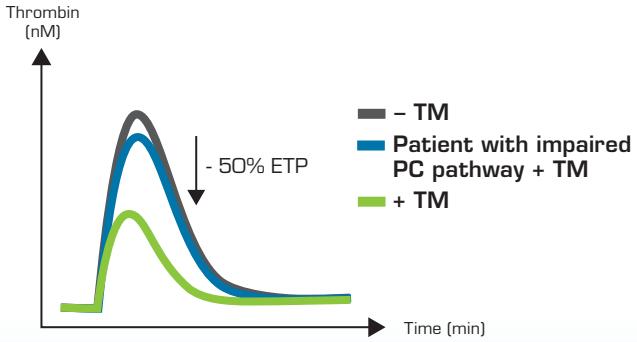
# Reagents

| Name | Cat Nr. | Kit contents | Packaging | Stability on board | Composition |
|---|---|---|---|---|---|
| STG-Bleedscreen* | 01131 | STG-BleedScreen | 3x1mL | 8H | Human TF at low picomolar level, balanced for sensitivity to factor deficiencies and while minimizing contact activation.<br>Procoagulant phospholipids . |
| | | STG-RefPlasma BLS | | 4H | |
| | | STG-QualiTest Low BLS | | | |
| | | STG-QualiTest Norm BLS | | | |
| STG-DrugScreen* | 01132 | STG-DrugScreen | 3x1mL | 8H | Human TF at high picomolar level to assess the anticoagulant effect at prophylactic and therapeutic doses.<br>Procoagulant phospholipids. |
| | | STG-RefPlasma DS | | 4H | |
| | | STG-QualiTest Low DS | | | |
| | | STG-QualiTest Norm DS | | | |
| STG-Thromboscreen | 01133 | STG-Thromboscreen - TM | 3x1mL | 8H | Human TF at medium picomolar level balanced for sensitivity to deficiencies in natural anticoagulants. No interfering contact activation.<br>Yields an average of 50% inhibition in normal plasma.<br>Procoagulant phospholipids. |
| | | STG-Thromboscreen +TM | | | |
| | | STG-RefPlasma TS | | 4H | |
| | | STG-QualiTest Low TS | | | |
| | | STG-QualiTest Norm TS | | | |
| | | STG-QualiTest High TS | | | |
| STG-Cal&Fluo | 01141 | STG-ThrombiCal | 3x2mL | 8H | STG-Thrombical:<br>Fixed amount of thrombin<br>STG-Fluoset:<br>Fixed amount of AMC<br>STG-Fluostart:<br>$Ca^{2+}$ and Z-GGR-AMC |
| | | STG-FluoStart | 3x1.5mL | 8H | |
| | | STG-FluoSet | | | |
| STG-ThrombiClean | 01140 | STG- ThrombiClean | 6x2mL | 4H | Decontaminating solution |

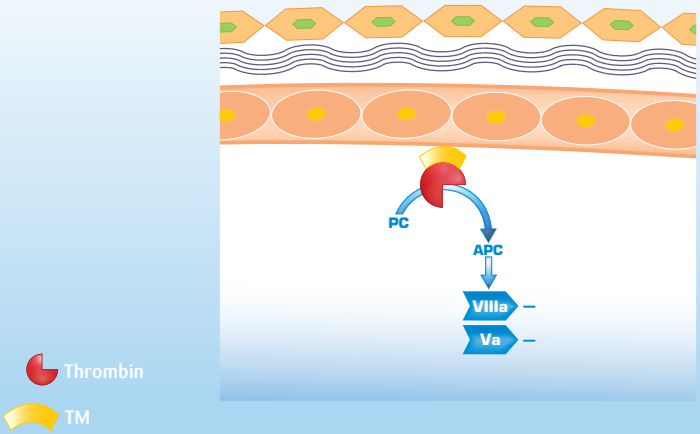
Availability depending upon countries.
*Reagent under development.

# TG in the presence of Thrombomodulin

## Normal Donor



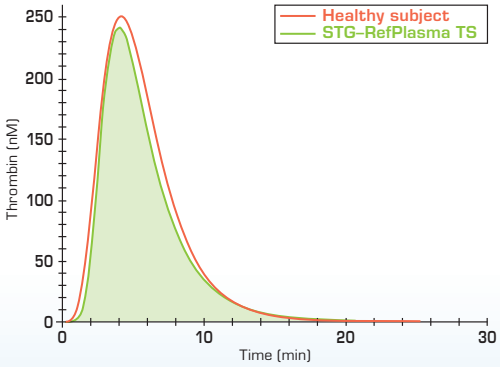

## PC inhibitory pathway



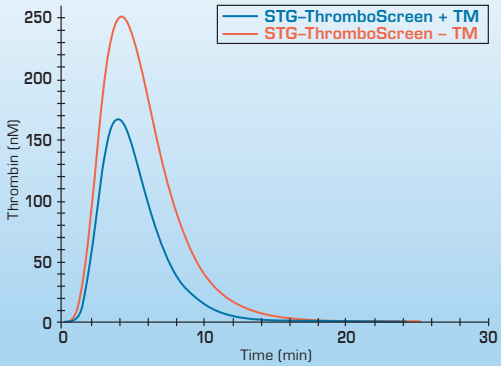

In case of a defect in the TM - APC - protein S system (inherited defects, oral contraceptives, cancer, etc.), ETP in the presence of TM is less reduced compared to normal plasma. In other words ETP in the presence of TM is higher for those patients than for normal subjects.

# TG in the presence of Thrombomodulin: normal plasma from a healthy subject

## Healthy subject: STG–ThromboScreen –TM




## Healthy subject: STG–ThromboScreen +TM

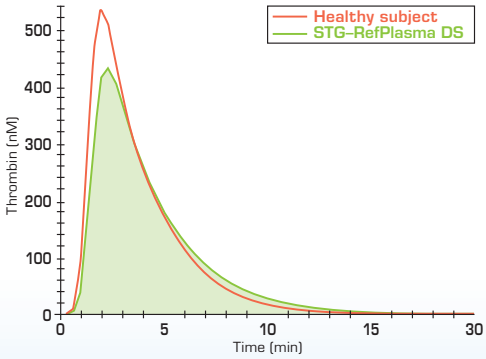



Internal data

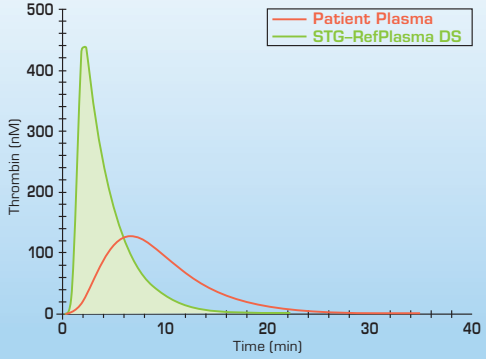
In a healthy subject and in the presence of TM, the TG profile is affected. Decreased Peak Height, ETP (in this case, ETP: 528 nM.min versus 946 nM.min without TM), and Lag Time values are slightly longer (in this case, 1.5 min versus 1.2 min without TM).

# Example of Thrombogram using STG-DrugScreen* and ST Genesia

## Healthy subject




## Direct Xa inhibitor treated patient



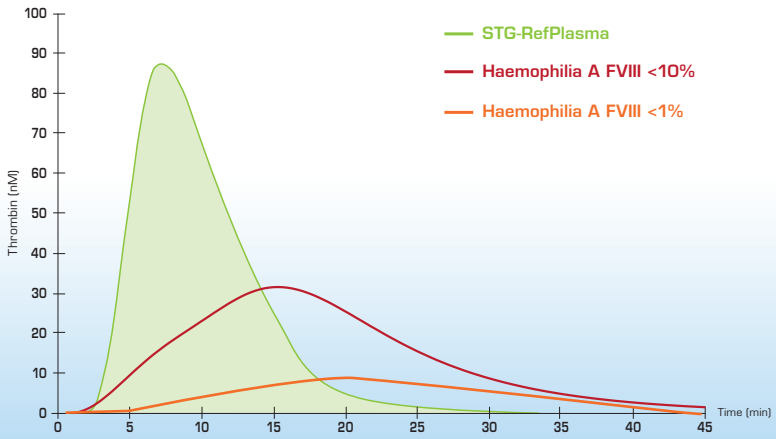

Internal data

TG profiles are diminished in all forms of anticoagulant treatment. As shown in the picture, for a patient treated with a direct oral anticoagulant (DOAC), ETP and peak height are diminished and the lag-time prolonged. Such decreases are also seen with heparin treatment and VKA-based oral anticoagulation.

*reagent under development.

# Example of Thrombogram with STG-BleedScreen* and ST Genesia

## TG profile is function of FVIII level




Internal data

Deficiencies of any physiologically relevant clotting factor, notably of the antihaemophilic factors A and B, causes a decrease in the thrombin generating potential. Here are shown two examples for two haemophiliacs with different severities of factor VIII deficiency.

An ETP lower than 20% of normal appears to indicate a substantial risk of bleeding[3].

*reagent under development.

# Standardisation

Automation of TG measurement allows standardisation through:

- Precise temperature control at 37°C
- Dedicated combinations of trigger reagents and QC
- Up to three QC levels to cover the entire working range
  ✓ STG-QualiTest Low, Norm and High
- Assayed reference plasma for result normalisation and improved overall precision [6, 7]
  ✓ STG-RefPlasma

| | CAT system [6] PPP-reagent LOW™ | | ST Genesia* STG–BleedScreen** | |
|---|---|---|---|---|
| | Normalised CV External Ref Plasma from Stago | Robust mean (N=34) | Normalised CV STG–Ref Plasma | Robust mean (N=10) |
| Lag Time (min) | **8%** | 3.6 | **3.1%** | 2.44 |
| Time to Peak (min) | **7%** | 9.0 | **3.7%** | 5.86 |
| ETP (nM.min) | **10%** | 1167 | **3.2%** | 774 |
| Peak Height (nM) | **17%** | 108 | **6.3%** | 94.1 |
| Velocity Index (nM/min) | **27%** | 20 | **11.4%** | 38.02 |

* unpublished internal data

Two different normal plasmas have been tested with two different reagents at low picomolar TF levels on either the CAT or ST Genesia systems. These data were obtained in different experimental conditions. They are not intended to compare performances of the 2 systems.

The STG-RefPlasma is useful for improvement of lab-to-lab inter-assay reproducibility and standardisation[7]. It should not be confused with the ST Genesia specific calibrator STG-ThrombiCal enabling the conversion of the fluorescent signal into thrombin activity.

** reagent under development

# Conclusion and a few words on preanalytical aspects

"Thrombin, you cannot live without it and you will probably die from it (K.G. Mann)."

ST Genesia is an integrated automated solution for measurement of plasma thrombin generating potential. It is a sensitive method to detect the entire function of the plasmatic contribution to the haemostatic - thrombotic system.

It is greatly influenced by preanalytical conditions. Care should be taken that blood is drawn from a clean venipuncture with minimal stasis. Centrifugation should not be delayed and should always be performed consistent with published recommendations[8].

Automated TG measurement is at the present time performed with platelet-poor plasma. Since low concentrations of TF (picomolar range) are used to initiate thrombin generation, it is important to minimise contact activation and TF contamination[8, 9].

"It took a while before we realised that the difficult part was not getting a signal out of clotting plasma but getting thrombin concentrations out of the signal. Finally after some 15 years we have a method that could be used beyond the limited circle of specialised laboratories (H.C. Hemker)."

## Abbreviations

**APC:** Activated Protein C
**$\alpha_2$M:** $\alpha_2$-macroglobulin
**AMC**: Fluorophore
**AT:** Antithrombin
**AUC**: Area Under the Curve
**$Ca^{2+}$:** Calcium ions
**ETP:** Endogenous Thrombin Potential
**PPP:** Poor-platelet plasma
**PRP:** Platelet-rich plasma
**PC:** Protein C
**TF:** Tissue Factor
**TFPI:** Tissue Factor Pathway Inhibitor
**TG:** Thrombin Generation
**TM:** Thrombomodulin
**Z-GGR-AMC:** Fluorogenic substrate

# References


(1) Mann KG. Thrombin Can't live without it; Probably die from it. Chest 2003; 124:1S–3S

(2) Tripodi A. Thrombin Generation and its Applications in the clinical laboratory. Clinical Chemistry 2016; 62(5): 699-707

(3) Al Dieri R., Peyvandi F., Santagostino E, *et al.* The Thrombogram in Rare Inherited Coagulation Disorders: Its Relation to Clinical Bleeding. Thromb Haemost 2002; 88(4): 576-82.

(4) Hemker H. C., Wielders S, Kessels H., *et al.* Continuous Registration of Thrombin Generation in Plasma, its use for the Determination of the Thrombin Potential. Thromb Haemost 1993; 70(4): 617-24.

(5) Hemker H. C., Giesen P., Al Dieri R. *et al.* Calibrated Automated Thrombin Generation Measurement in Clotting Plasma. Pathophysiol Haemost Thromb 2003; 33(1): 4-15.

(6) Dargaud Y, Wolberg AS, Luddington R *et al.* Evaluation of a standardized protocol for thrombin generation measurement using the calibrated automated thrombogram: An international multicenter study. Thromb Res 2012; 130: 929-934

(7) Perrin J, Depasse F, Lecompte T *et al.* Large external quality assessment survey on thrombin generation with CAT: further evidence of the usefulness of normalization with an external reference plasma. Thromb Res 2015; 136: 125-130

(8) Loeffen R, Kleinegris MCF, Loubele STBG *et al.* Preanalytic variables of thrombin generation: towards a standard procedure and validation of the method. J Thromb Haemost 2012; 10: 2544-54

(9) Baglin T, Besser M, Cattaneo M *et al.* Towards a recommendation for the standardization of the measurement of platelet-dependent thrombin generation. J Thromb Haemost 2011; 9(9): 1859–1861

# Notes


Document developed in collaboration with Pr H.C. Hemker, Synapse, Maastricht and Pr T. Lecompte, University of Geneva and University Hospital of Geneva.





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