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## ORIGINAL ARTICLE



# Assessment of the analytical performances and sample stability on ST Genesis system using the STG-DrugScreen application

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## Abstract

**Background:** Thrombin generation testing has been used to provide information on the coagulation phenotype of patients. The most used technique is the calibrated automated thrombogram (CAT) but it suffers from a lack of standardization, preventing its implementation in routine. The ST Genesis is a new analyzer designed to assess thrombin generation based on the same principle as the CAT. Unlike the CAT system, the ST Genesis is a benchtop, fully automated analyzer, able to perform the analyses individually and not by batch, with strict control of variables such as temperature and volumes, ensuring, theoretically, maximal reproducibility.

**Objectives:** This study aimed at assessing the performance of the STG-DrugScreen application on the ST Genesis analyzer. We also aimed at exploring stability of plasma samples after freezing and defining a reference normal range.

**Results:** Results demonstrated the excellent interexperiment precision of the ST Genesis and confirmed that the use of a reference plasma helps reducing the

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inter-experiments variability. Stability revealed that plasma samples are stable for at least 11 months at  $-70^{\circ}\text{C}$  or lower, except for those containing low molecular weight heparins which have to be tested within 6 months. Freezing had no effect on the majority of thrombin generation parameters except on time to peak.

**Conclusions:** Our results suggest an easy implementation of thrombin generation with the use of ST Genesis in the routine laboratory. This will facilitate the design of multicentric studies and enable the establishment of reliable and evidence-based thresholds, which may improve the management of patients treated with anticoagulants.

#### KEYWORDS

anticoagulants, blood coagulation tests, clinical laboratory techniques, normal range, reproducibility

## 1 | INTRODUCTION

Thrombin generation has been used since the early 1950s.<sup>1</sup> At the start, whole blood or plasma was supplemented with triggers such as tissue factor or activator of the intrinsic pathway of the coagulation combined with calcium chloride to initiate coagulation. Thrombin generation was then evaluated by sampling the clotting mixture at regular intervals into a test tube containing fibrinogen. The clotting times were then recorded and the quantity of thrombin generated was derived from a calibration curve constructed with a known amount of thrombin.<sup>2</sup> Changes have been made and the test was improved by replacing fibrinogen by a chromogenic substrate specific for thrombin. This required the use of defibrinated plasma and a computer was used to calculate the parameters stemming from the thrombogram.<sup>3</sup> Finally, the chromogenic substrate was replaced by a fluorogenic substrate, which permits the measurement of thrombin in samples that were not defibrinated because fluorescence is not hampered by the turbidity generated during the clotting process. These improvements have laid the groundwork for what is now known as calibrated automated thrombogram (CAT).<sup>4</sup> Currently, different solutions are available on the market to measure thrombin generation.<sup>5</sup> These are the Innovance ETP (Siemens Healthcare), the only one still based on the cleavage of a chromogenic substrate as readout, the Technothrombin TGA (Technoclone) and the Thrombinoscope CAT (Diagnostica Stago). These last two both use a fluorogenic substrate<sup>5</sup>; however, some limitations have been highlighted, such as the lack of established standardization of the methods and reagents, and missing quality controls. In addition, batch-to-batch variations have been reported which further hampers study-to-study comparisons.<sup>6-8</sup>

As a consequence, efforts have been made since the past decade to reduce the interlaboratory variation, such as the use of a reference plasma to normalize the results.<sup>9</sup> Without the use of this reference plasma, coefficient of variation (CVs) for the most global parameter of CAT (i.e., the endogenous thrombin potential [ETP]), were often higher than 15%. This also raised the question of different local

### Essentials

- Thrombin generation testing is widely used to provide information on the coagulation phenotype of patients.
- Some limitations have been highlighted such as the lack of established standardization.
- This is the first study reporting precision results of the ST Genesis.
- Normal range of the STG-DrugScreen application has been assessed in healthy subjects.
- The ST Genesis may facilitate the implementation of thrombin generation in the routine laboratory.

practices which may explain, in part, this high CV.<sup>10</sup> Even if this strategy appeared to efficiently reduce the interlaboratory variation and showed the potential benefit of results normalization, improvements are still needed to ensure a proper standardization of the method and its implementation in the routine daily care of patient. Namely, it has been reported that standardization of the tested conditions (e.g., control of the temperature throughout the process, preheating of the sample and the reagents, collection tube or centrifugation) may significantly affect the CAT parameters.<sup>8-11</sup> Thus, for its implementation in routine daily care, the CAT system had to move to a next generation of analyzers able to perform the analyses individually and not by batch, with strict control of all variables to ensure maximal reproducibility.

The newly developed ST Genesis is a fully automated system intended to measure thrombin generation. It provides capacity to continuously load patients' samples for unitary testing. This new system has been designed to offer enhanced reproducibility compared with CAT and also to provide traceability and standardization, two criteria necessary to fulfill the requirements of an in vitro diagnostics format and to enter the clinical laboratory.

Numerous studies showed that thrombin generation measurement is sensitive enough to detect the presence of anticoagulants in

patients' plasma and to evaluate the intensity of the treatment<sup>12-19</sup> therefore, Diagnostica Stago aimed at developing a kit for the assessment of thrombin generation in anticoagulated patients, the STG-DrugScreen. The first aim of this study was to determine the precision of the device with this kit and the utility of the normalization of the results. Second, this study aimed at assessing the stability of the plasma samples over time after freezing as well as defining the normal range of the STG-DrugScreen application in a normal healthy population.

## 2 | MATERIALS AND METHODS

The study intends to evaluate the precision performance of the device under final user setting and investigate the stability of thrombin generation results on samples stored at  $-70^{\circ}\text{C}$  or lower. The study was in accordance with the Declaration of Helsinki and has been approved by the Ethical Committee of the CHU UCL Namur, Yvoir, Namur, Belgium. The study took place from June 2016 to September 2017 at the CHU UCL Namur, Yvoir, Belgium.

### 2.1 | Plasma collection of patients' and healthy donors' samples

Blood was taken by venipuncture in the antecubital vein and collected into 0.109 M sodium citrate (9:1 v/v) tubes (Vacurette, Greiner, Austria) without corn trypsin inhibitor using a 21-gauge needle (Terumo). The first tube was always discarded and the first centrifugation was performed within 30 minutes. The platelet-poor plasma (PPP) was obtained from the supernatant fraction of blood tubes after a double centrifugation for 15 minutes at  $1500 \times g$  at room temperature. The centrifuge brake was set to the minimum position at the end of the process. The residual platelet count was assessed every week to ensure the centrifugation procedure provides plasma with a platelet count  $<10,000$  platelets/ $\mu\text{L}$ . Immediately after centrifugation, PPP was aliquoted by  $600 \mu\text{L}$  ( $n = 12$  for the stability study,  $n = 4$  for the normal range definition study) and frozen at  $\leq -70^{\circ}\text{C}$  without any delay (except for one aliquot tested fresh in the stability study). Frozen PPP samples were thawed, heated to  $37^{\circ}\text{C}$  for 2-3 minutes and mixed gently just before the experiment. All tests were performed within 4 hours after thawing.

### 2.2 | Normal and targeted population and samples

Six healthy individuals and 23 samples from patients treated with an anticoagulant have been included in the stability study (apixaban  $n = 4$ ; dabigatran  $n = 3$ ; low-molecular-weight heparins [LMWH]  $n = 5$ ; rivaroxaban  $n = 5$ ; vitamin K antagonists [VKA]  $n = 6$ ). For patients on apixaban, dabigatran, and rivaroxaban, plasma had to be taken at peak (i.e., between 30 minutes and 4 hours after drug intake) to obtain the highest effect on thrombin generation. For patients under LMWH, the blood had to be taken between 1 and 6 hours after the administration while for patients under VKA, the targeted international normalized ratio (INR) was between 2 and 3.

For the definition of the normal range, 42 healthy individuals (mean age =  $20 \pm 3$  years; min-max range = 18-32 years), not taking any antithrombotic therapy and not having any hemostasis disorders were included in the study.

### 2.3 | Assessment of thrombin generation

Thrombin generation has been assessed on an ST Genesia analyzer using the STG-DrugScreen application. More information about the analyzer, its methodology and the reagents are provided in the supplementary material.

### 2.4 | Assessment of anticoagulant activity with specific tests

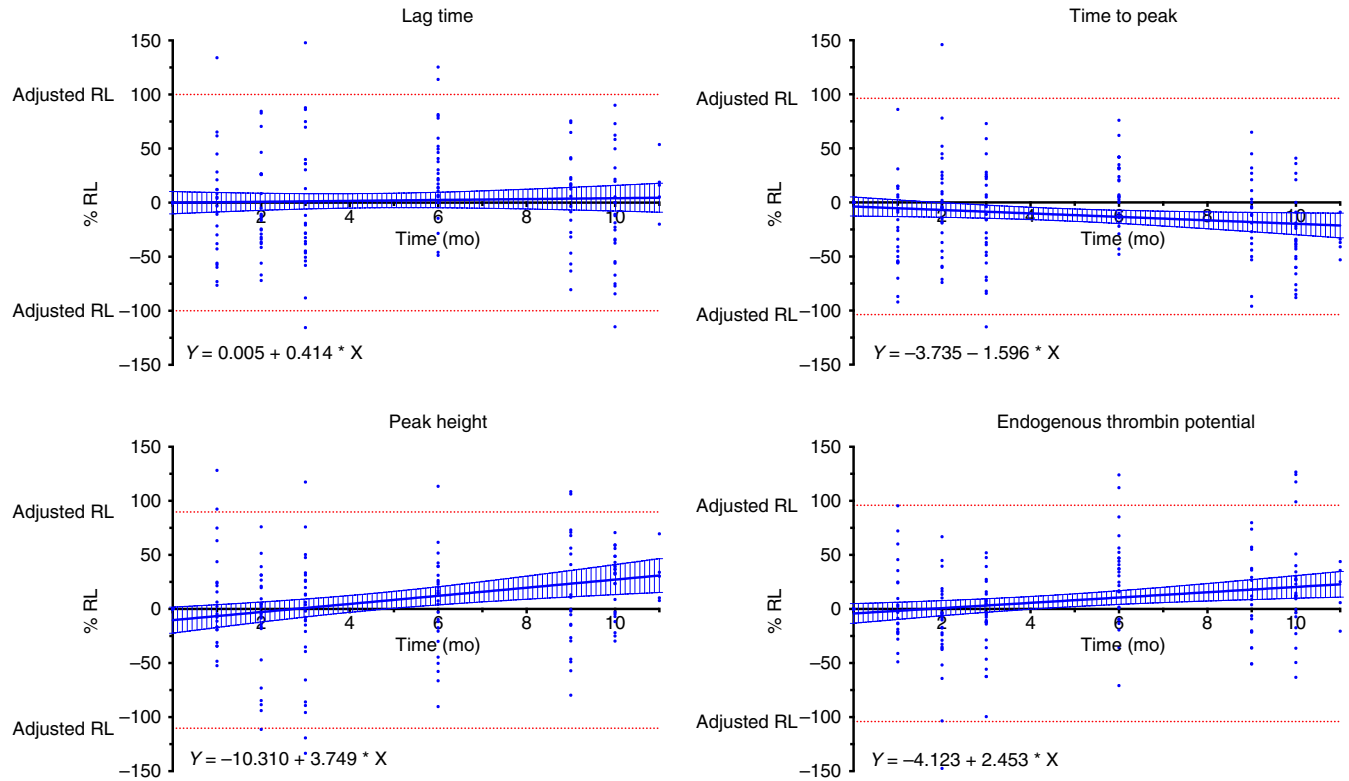
For patients on apixaban, rivaroxaban, and LMWH, the estimated concentrations or the anti-Xa activity have been measured using the STA-Liquid Anti-Xa (Diagnostica Stago) and the corresponding calibrators and controls according to the recommendations of the manufacturer on an STA-R Max analyzer (Diagnostica Stago). For dabigatran samples, the estimated dabigatran plasma concentrations have been measured using the STA-ECA II (Diagnostica Stago) with the corresponding calibrators and controls. For VKA patients, the INRs have been measured with the STA-Neoplastine R (Diagnostica Stago) according to the recommendations of the manufacturer. For healthy individuals, the prothrombin time (PT) and the activated partial thromboplastin time (aPTT) have been assessed using the STA-Neoplastine R (Diagnostica Stago) and the STA-C.K. Prest (Diagnostica Stago) according to the recommendations of the manufacturer.

### 2.5 | Determination of the precision of the ST Genesia in its STG-DrugScreen application

Each day of testing ( $n = 97$ ), a new calibration is set and the quality controls (two levels) and the reference plasma are assessed. During the study period, two batches of reagent kits were used (batch 201797, from 21 June 2016 to 3 November 2016, and batch 202028, from 4 November 2016 to 29 September 2017). The precision has been assessed by collecting the daily values of the quality controls (two levels [i.e., the STG-QualiTest Norm DS and the STG-QualiTest Low DS]) and the reference plasma (i.e., the STG-RefPlasma DS) over this period. Both the absolute and normalized results of the lag time, time-to-peak, ETP, and peak height were extracted, and the mean, standard deviation and CV computed from those results.

### 2.6 | Determination of the stability after freezing of the plasma samples for thrombin generation measurement

Fresh plasma from the six healthy individuals, six patients on VKA, five on LMWH, four on apixaban, three on dabigatran, and five on rivaroxaban were tested at different time points to assess the stability

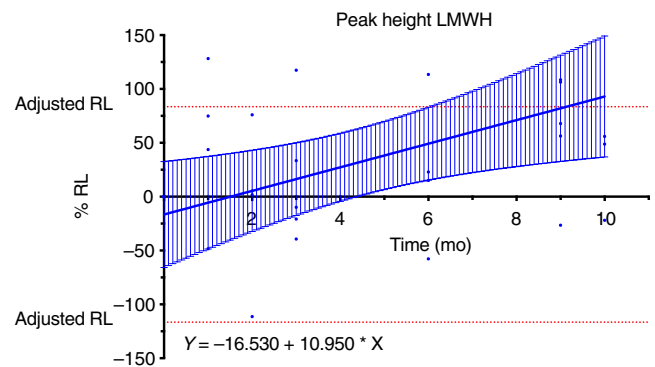


**FIGURE 1** Stability of the different thrombin generation parameters. The 100% rejection limit (RL) has been defined as the maximal allowable error (MAE). For the endogenous thrombin potential (ETP), the MAE was 20% and for the lag time, time to peak, and the peak height; the MAE was 18% based on the data from the precision study. The Y-intercept was used to determine the adjusted RL (upper adjusted RL = 100 + Y-intercept - lower adjusted RL = -100 + Y-intercept). The 95% confidence interval of the linear regression (blue hashed zone) do not cross the adjusted RL, which means that the samples are stable over the analyzed period (i.e., 11 months for healthy subject and 10 months for anticoagulated patients)

of the different thrombin generation parameters over time. Testing was performed on follows.

- On a fresh sample (the day of plasma collection after centrifugation)
- At day 1, month 1, month 3, month 6, month 9, month 10, and month 11 (for healthy individuals only) after freezing at  $-70^{\circ}\text{C}$  or lower. The maximal deviation tolerated was 7 days except for day 1 for which no deviation was tolerated.

The stability of thrombin generation results once the plasma has been frozen at  $-70^{\circ}\text{C}$  or lower was assessed on the lag time, time-to-peak, ETP, and peak height using the method of the rejection limit (RL). This method aims to establish the length of time during which the measurement of the thrombin generation is acceptable, when carefully following laboratory's established sample handling and storage conditions. Briefly, the percent bias (or the %RL) was plotted vs time for each time point. The regression line of this plot represents the average percent bias (proportional change) over time. For each parameter, the 100% RL is defined based on the maximal allowable error (MAE). To take into account method precision, reagents aging, and sample aging, the MAE was set at 20% for the ETP and at 18% for the lag time, time-to-peak, and the peak height. The RL is also adjusted according to the value of



**FIGURE 2** Stability results for samples from patients treated with low molecular weight heparin (LMWH). The upper 95% confidence interval limit of the linear regression crosses the adjusted rejection limit (RL) after 6 months determining the maximal stability of the samples

the intercept of the linear regression with the y axis. The maximal sample stability is defined as the intercept of the 95% confidence interval (CI) of the linear regression with the adjusted rejection limit. If there is no interception, the maximal duration limit is defined as the last point of measurement (see the Results section and Figures 1 and 2 for graphical representation).

The impact of freezing was assessed comparing the results of each sample at day 0 (i.e., fresh sample) to the results obtained at day 1. To provide evidence of the normal distribution of the results, a Shapiro-Wilk test using an  $\alpha$ -risk of 5% (two-sided CI) has been performed. If the population had not a normal distribution, then the paired results were compared using a Wilcoxon matched rank-signed test. If the distribution was normal, then the means were compared with a *t* test for paired samples. In both cases, a *P* value below 5% leads to the conclusion of significant impact of freezing on results.

### 3 | RESULTS

#### 3.1 | Thrombin generation values of the reference plasma and controls and precision of the ST-Genesia

The within-batch variability was always below the between-batches relative differences, except for the normalized ETP of the STG-QualiTest Low DS (Table 1). The within-batch variability was always below 6%. All results are summarized in Table 1.

#### 3.2 | Impact on freezing on thrombin generation parameters

On the total cohort ( $n = 29$  samples), Shapiro-Wilk normality test showed normal distribution for the lag time and the ETP parameters, whereas the normality failed for the peak height and the time to peak. This means that, according to the statistical analysis plan, a *t* test for paired samples was used for the lag time and the ETP, whereas a Wilcoxon matched rank-signed test was used for the peak height and the time-to-peak. Because of a small sample size, the Wilcoxon matched rank-signed test was used when the results were stratified by treatment (Table 2).

Wilcoxon matched rank-signed test revealed no significant difference between the two conditions on lag time, peak height, and ETP, whereas the time-to-peak was impaired (mean of difference =  $-0.15$  minutes). When results are analyzed by treatment, there is no significant difference between fresh and frozen plasma (Table 2).

#### 3.3 | Stability of the plasma samples once conserved at $-70^{\circ}\text{C}$ or below

For the lag time, the adjusted R<sub>L</sub>s are  $-100\%$  to  $100\%$ , the slope of the linear regression was equal to  $0.414$  (95% CI,  $-1.358$  to  $2.185$ ). The 95% CI of the linear regression did not cross the adjusted R<sub>L</sub>s (Figure 1) and the maximal storage duration was defined as the latest point assessed in this study (i.e., 11 months for healthy volunteers and 10 months for anticoagulated patients). Stratification by treatment did not provide different results (Table 3). For the time to peak, the adjusted R<sub>L</sub>s were  $-104\%$  to  $96\%$ , the slope of linear regression was equal to  $-1.596$  (95% CI,  $-3.113$  to  $-0.078$ ; significant nonzero). The 95% CI of the linear regression did not

cross the adjusted R<sub>L</sub>s (Figure 1) and the maximal storage duration for this parameter was also defined at the latest point assessed in this study (i.e., 11 months). Again, stratification by treatment did not provide different results (Table 3). For the peak height, the adjusted R<sub>L</sub>s were  $-110\%$  to  $90\%$ , the slope of linear regression was equal to  $3.749$  (95% CI,  $-1.675$  to  $5.822$ ). The 95% CI of the linear regression did not cross the adjusted R<sub>L</sub>s (Figure 1) and the maximal storage duration for this parameter was also defined at the latest point assessed in this study (i.e., 11 months). Stratification by treatment revealed that the peak height is only stable for 6 months in samples from patients with LMWH (Table 3 and Figure 2). For the ETP, the adjusted R<sub>L</sub>s were  $-104\%$  to  $96\%$ , the slope of linear regression was equal to  $2.453$  (95% CI,  $0.886$ - $4.019$ ). The 95%CI of the linear regression did not cross the adjusted R<sub>L</sub>s (Figure 1) and the maximal storage duration for this parameter was defined at the latest point assessed in this study (i.e., 11 months). Stratification by treatment did not provide different results (Table 3).

#### 3.4 | Thrombin generation in the normal and targeted population

The reference values obtained in the healthy population revealed thrombin generation values close to the value of the reference plasma (Table 4). Screening coagulation testing, namely the PT and aPTT, were normal in all healthy individuals (PT: mean =  $13.7 \pm 0.7$  seconds; min-max range =  $11.9$ - $15.10$  seconds; and aPTT: mean =  $31.5 \pm 2.5$  seconds; min-max range =  $27.1$ - $36.7$  seconds). For apixaban, measured drug plasma levels through anti-Xa method were between  $158$  and  $370$  ng/mL, the normalized thrombin generation parameters were all affected by the treatment (Table 5). No clear correlation could be drawn from this small population ( $n = 4$ ) between thrombin generation parameters and the plasma level. For dabigatran, peak plasma levels were between  $51$  and  $187$  ng/mL, the normalized lag time and time to peak were affected in all subjects, whereas the ETP and the peak height were not different from the healthy subjects at the lowest concentration observed (i.e.,  $51$  ng/mL) (Table 5). For LMWH, the peak anti-Xa effect were between  $0.35$  IU/mL and  $1.37$  IU/mL. All thrombin generation parameters were affected. In the patient with the highest exposure (i.e.,  $1.37$  IU/mL), the peak height and the ETP were highly affected (8% and 14% the value of the reference plasma, respectively) (Table 5). For rivaroxaban, the peak plasma concentrations were between  $197$  and  $300$  ng/mL. The most affected thrombin generation parameters were the peak height and the time to peak (Table 5). In patient 1, the rivaroxaban concentration was  $229$  ng/mL, the peak height dropped to 37% with significant prolongation of the time to peak but the ETP was not affected (i.e., 101% the value of the reference plasma). In patients treated with VKA, the INR varied between  $1.86$  and  $3.31$ . The peak and the ETP were the most influenced thrombin generation parameters while lag time and time to peak were less influenced (Table 5).

**TABLE 1** Coefficient of variation of the different quality controls over 2 batches of STG®-DrugScreen kit

	STG-QuallTest Norm DS			STG-QuallTest Low DS			STG-RefPlasma DS			Mean coef- ficient of variation
	Mean value	SD	CV	Mean value	SD	CV	Mean value	SD	CV	
Lag time (min - absolute/ratio - normalized)										
Absolute										
All (n = 97)	0.87 min	0.06 min	6.53%	1.05 min	0.12 min	11.03%	0.95 min	0.05 min	5.11%	7.56%
Batch 201797 (n = 37)	0.93 min	0.04 min	4.03%	1.19 min	0.05 min	3.97%	0.98 min	0.05 min	5.25%	4.42%
Batch 202028 (n = 60)	0.84 min	0.04 min	4.36%	0.96 min	0.03 min	3.14%	0.93 min	0.03 min	3.38%	3.63%
Difference, %	11			24			5			
Normalized										
All (n = 97)	0.92	0.04	4.71%	1.10	0.09	8.47%				6.59%
Batch 201797 (n = 37)	0.95	0.04	3.83%	1.21	0.05	4.22%				4.03%
Batch 202028 (n = 60)	0.91	0.04	4.42%	1.04	0.04	3.69%				4.06%
Difference, %	4			16						
Time-to-peak (min - absolute/ratio - normalized)										
Absolute										
All (n = 97)	1.91 min	0.08 min	4.07%	2.14 min	0.17 min	7.85%	2.13 min	0.07 min	3.26%	5.06%
Batch 201797 (n = 37)	1.97 min	0.07 min	3.60%	2.33 min	0.10 min	4.25%	2.17 min	0.08 min	3.61%	3.82%
Batch 202028 (n = 60)	1.86 min	0.05 min	2.60%	2.02 min	0.05 min	2.28%	2.11 min	0.05 min	2.50%	2.46%
Difference, %	6			15			3			
Normalized										
All (n = 97)	0.90	0.02	2.70%	1.00	0.06	6.19%				4.45%
Batch 201797 (n = 37)	0.91	0.02	2.09%	1.08	0.03	2.55%				2.69%
Batch 202028 (n = 60)	0.89	0.02	2.58%	0.96	0.03	2.56%				2.57%
Difference, %	2			13						
Peak height (nmol/L - absolute/% - normalized)										
Absolute										
All (n = 97)	480.1 nmol/L	22.6 nmol/L	4.71%	215.9 nmol/L	13.3 nmol/L	6.17%	472.5 nmol/L	18.6 nmol/L	3.95%	4.94%
Batch 201797 (n = 37)	501.8 nmol/L	16.5 nmol/L	3.30%	206.2 nmol/L	10.1 nmol/L	4.89%	485.8 nmol/L	17.0 nmol/L	3.50%	3.90%
Batch 202028 (n = 60)	466.7 nmol/L	13.7 nmol/L	2.93%	221.9 nmol/L	11.5 nmol/L	5.16%	464.3 nmol/L	14.5 nmol/L	3.12%	3.74%
Difference, %	8			7			5			
Normalized										
All (n = 97)	101.6%	2.9%	2.81%	45.7%	3.2%	6.94%				4.88%
Batch 201797 (n = 37)	103.2%	2.2%	2.08%	42.4%	1.4%	3.30%				2.69%

(Continues)

TABLE 1 (Continues)

	STG-Qualitest Norm DS			STG-Qualitest Low DS			STG-RefPlasma DS			Mean coefficient of variation
	Mean value	SD	CV	Mean value	SD	CV	Mean value	SD	CV	
Batch 202028 (n = 60)	100.5%	2.8%	2.75%	47.8%	2.0%	4.15%				3.45%
Difference, %	3			11						
Endogenous thrombin potential (ETP) (nmol/L/min - absolute/% - normalized)										
Absolute										
All (n = 97)	1531 nmol/L/min	96 nmol/L/min	6.26%	523 nmol/L/min	22 nmol/min	4.27%	1722 nmol/L/min	72 nmol/L/min	4.19%	4.91%
Batch 201797 (n = 37)	1607 nmol/L/min	65 nmol/L/min	4.07%	524 nmol/L/min	19 nmol/min	3.53%	1736 nmol/L/min	64 nmol/L/min	3.67%	3.76%
Batch 202028 (n = 60)	1483 nmol/L/min	80 nmol/L/min	5.40%	522 nmol/L/min	25 nmol/min	4.69%	1714 nmol/L/min	76 nmol/L/min	4.45%	4.85%
Difference, %	8			0			1			
Normalized										
All (n = 97)	88.9%	4.9%	5.56%	30.4%	1.3%	4.18%				4.87%
Batch 201797 (n = 37)	92.6%	3.7%	3.95%	30.2%	1.1%	3.47%				3.71%
Batch 202028 (n = 60)	86.6%	4.2%	4.85%	30.5%	1.4%	4.55%				4.70%
Difference, %	7			1						

Note: Results are presented as CV expressed in percentage. For the 2 levels of controls, results are presented as absolute or normalized (against STG -RefPlasma DS). The use of a reference plasma titrated by a manufacturer reduces the interbatch variation. Difference in % represents the relative difference between the two batches of reagents and controls for each TGT parameter. CV, coefficient of variation; SD, standard deviation; TGT, thrombin generation test.



**TABLE 2** Impact of freezing on thrombin generation parameters

Fresh (D0) vs frozen (D1)	Passed normality testing?	Mean difference (D1-D0)	Wilcoxon matched-pairs signed rank test or paired t test (P value)	Significant?
All results (n = 29)				
Lag time <sup>a</sup>	Yes <sup>a</sup>	-0.08 min	0.7179 <sup>a</sup>	No
Peak height	No	3.29 nmol/L	0.096	No
Time-to-peak	No	-0.15 min	0.020	Yes
ETP <sup>a</sup>	Yes <sup>a</sup>	1 nmol/L/min	0.9702 <sup>a</sup>	No
Apixaban (n = 4)				
Lag time	N too small	-0.09 min	0.375	No
Peak height		19.38 nmol/L	0.250	No
Time-to-peak		-0.24 min	0.250	No
ETP		-4 nmol/L/min	0.875	No
Dabigatran (n = 3)				
Lag time	N too small	-0.25 min	0.250	No
Peak height		7.53 nmol/L	0.500	No
Time-to-peak		-0.23 min	0.500	No
ETP		27 nmol/L/min	0.250	No
Healthy (n = 6)				
Lag time	N too small	-0.02 min	>0.999	No
Peak height		-20.17 nmol/L	0.688	No
Time-to-peak		-0.03 min	>0.999	No
ETP		-116 nmol/L/min	0.438	No
LMWH (n = 5)				
Lag time	N too small	-0.00 min	>0.999	No
Peak height		2.78 nmol/L	0.625	No
Time-to-peak		-0.09 min	0.474	No
ETP		28 nmol/L/min	0.313	No
Rivaroxaban (n = 5)				
Lag time	N too small	-0.21 min	0.063	No
Peak height		14.77 nmol/L	0.125	No
Time-to-peak		-0.53 min	0.063	No
ETP		79 nmol/L/min	0.188	No
Vitamin K antagonists (n = 6)				
Lag time	N too small	-0.02 min	0.750	No
Peak height		4.79 nmol/L	0.313	No
Time-to-peak		-0.04 min	0.313	No
ETP		22 nmol/L/min	0.219	No

Note: Wilcoxon matched pairs signed-rank test have been performed to compare the impact on freezing (i.e., at D0 on fresh and at D + 1 once the samples have been frozen). When considering all results (n = 29), freezing has an effect on time to peak. Results are also stratified by treatment but the number of samples is too small to drive any conclusion.

Abbreviations: CI, confidence interval; ETP, endogenous thrombin potential; LMWH, low-molecular-weight heparin; RL, rejection limit; SE, standard error; VKA, vitamin K antagonist.

<sup>a</sup>For these analyses, a paired t test was used as the data passed the normality testing using the Shapiro-Wilk test.

## 4 | DISCUSSION

This is the first study evaluating the performances of the ST Genesis, a benchtop analyzer for thrombin generation in the routine setting. This instrument aims at introducing thrombin generation testing in

the routine laboratory. Indeed, to date, the CAT system is probably one of the most used thrombin generation technique in research laboratories but the use of a microtiter plate, the manual placing of the sample and the reagents into the wells, added to the lack of standardized procedure and reagents have prevented its introduction into

**TABLE 3** Stability of plasma samples once frozen at -80°C

Parameter	Linear regression						Time at which RL is reached <sup>a</sup>		
	Slope	SE	95% CI	Slope significantly nonzero?	Intercept	SE		95% CI	Adjusted RL (%)
Lag time									
All	0.414	0.898	-1.358 to 2.185	No	0.005	5.219	10.290 to 10.300	-99.995 to 100.005	11 mo
Apixaban	-3.210	3.243	-9.903 to 3.483	No	20.180	19.250	-19.540 to 59.910	-79.820 to 120.18	10 mo
Dabigatran	-1.412	1.853	-5.340 to 2.516	No	6.077	11.150	-17.560 to 29.720	-93.923 to 106.08	10 mo
Healthy	2.165	1.316	-0.504 to 4.834	No	-17.280	8.207	-33.920 to -0.632	-117.280 to 82.72	11 mo
LMWH	-0.383	2.028	-4.520 to 3.753	No	-9.564	11.810	-33.830 to 14.320	-109.564 to 90.436	10 mo
Rivaroxaban	1.453	2.485	-3.603 to 6.509	No	-0.721	13.190	-27.560 to 26.120	-100.721 to 99.279	10 mo
VKA	2.216	2.069	-1.994 to 6.426	No	9.868	11.590	-13.710 to 33.450	-90.132 to 109.87	10 mo
Time to peak									
All	-1.596	0.769	-3.113 to -0.078	Yes	-3.735	4.471	-12.560 to 5.086	-103.735 to 96.265	11 mo
Apixaban	-0.031	2.089	-4.334 to 4.271	No	2.476	12.190	-22.640 to 27.590	-97.524 to 102.746	10 mo
Dabigatran	-3.204	3.012	-9.589 to 3.181	No	-21.720	18.13	-60.140 to 16.710	-121.720 to 78.280	10 mo
Healthy	-1.465	1.197	-3.892 to 0.9618	No	-17.370	7.463	-32.510 to 2.238	-117.37 to 82.630	11 mo
LMWH	-3.527	1.870	-7.342 to 0.2870	No	-3.726	10.890	-25.930 to 18.480	-103.726 to 96.274	10 mo
Rivaroxaban	-0.9155	1.585	-4.140 to 2.309	No	0.691	8.412	-16.420 to 17.810	-99.309 to 100.691	10 mo
VKA	-0.055	1.737	-3.592 to 3.483	No	7.920	9.870	-12.180 to 28.020	-92.080 to 107.920	10 mo
ETP									
All	2.453	0.794	0.886 to 4.019	Yes	-4.123	4.615	-13.230 to 4.980	-104.123 to 95.877	11 mo
Apixaban	2.873	1.989	-1.191 to 6.937	No	-4.348	11.690	-28.470 to 19.770	-104.348 to 95.652	10 mo
Dabigatran	-1.850	2.721	-7.618 to 3.918	No	17.650	16.380	-17.060 to 52.360	-82.350 to 117.65	10 mo
Healthy	3.015	1.097	0.7903 to 5.240	Yes	-6.215	6.842	-20.090 to 7.661	-106.215 to 93.785	11 mo
LMWH	5.053	2.586	-0.221 to 10.330	No	-12.630	15.050	-43.330 to 18.060	-112.630 to 87.37	10 mo
Rivaroxaban	3.280	2.321	-1.441 to 8.002	No	-9.106	12.320	-34.170 to 15.950	-109.106 to 90.894	10 mo
VKA	1.697	7.429	-2.515 to 2.882	No	1.697	7.429	-13.420 to 16.810	-98.303 to 101.7	10 mo
Peak height									
All	3.749	1.051	-1.675 to 5.822	Yes	-10.310	6.108	-22.360 to 1.745	-110.310 to 89.69	11 mo
Apixaban	-0.064	2.204	-4.612 to 4.484	No	-20.860	13.080	-47.860 to 6.136	-120.860 to 79.14	10 mo
Dabigatran	0.952	2.244	-3.805 to 5.708	No	11.470	13.500	-17.150 to 40.100	-88.530 to 111.47	10 mo
Healthy	3.252	0.682	1.870 to 4.634	Yes	7.126	4.250	-1.494 to 15.750	-92.874 to 107.13	11 mo
LMWH	10.950	4.141	2.506 to 19.400	Yes	-16.530	24.100	-65.690 to 32.630	-116.530 to 83.47	6 mo

(Continues)

**TABLE 3** (Continues)

Linear regression									
Parameter	Slope	SE	95% CI	Slope significantly nonzero?	Intercept	SE	95% CI	Adjusted RL (%)	Time at which RL is reached <sup>a</sup>
Rivaroxaban	1.657	2.245	-2.911 to 6.224	No	-22.670	11.920	-46.910 to 1.573	-122.670 to 77.33	10 mo
VKA	2.904	1.591	-0.334 to 6.141	No	-10.460	8.912	-28.590 to 7.673	-110.460 to 89.54	10 mo

Abbreviations: ETP, endogenous thrombin potential; LMWH, low-molecular-weight heparin; VKA, vitamin K antagonists.

<sup>a</sup>When the regression slope is not statistically significant ( $P > 0.05$ ) from zero, this indicates parameter is not changing as a function of time and the maximum storage time is equal to  $T_{MAX}$  (last point of the linear regression).

When the regression slope is statistically significant ( $P \leq 0.05$ ) from zero, and the one-sided 95% confidence limit of the regression line intersects the adjusted rejection limit before reaching  $T_{MAX}$ , the maximum storage time is determined as this intersection.

**TABLE 4** Normal range definition of the STG-DrugScreen application on the ST Genesis system using plasma sample from 42 healthy individuals

Healthy (n = 42)	Lag time absolute	Peak height absolute	Time-to-peak absolute	ETP absolute	Lag time normalized (ratio)	Peak height normalized	Time-to-peak normalized (ratio)	ETP normalized
Mean	0.93 min	456.20 nmol/L	2.03 min	1418 nmol/L	1.16	97%	0.98	83%
SD	0.09 min	56.35 nmol/L	0.16 min	270 nmol/L	0.10	12%	0.08	15%
CV (%)	9.8	12.4	7.9	19.1	9.6	12.1	7.9	18
Minimum	0.73 min	355.90 nmol/L	1.80 min	1027 nmol/L	0.81	75%	0.87	56%
Maximum	1.22 min	571.40 nmol/L	2.69 min	1993 nmol/L	1.33	120%	1.29	118%
10%	0.82 min	380.20 nmol/L	1.87 min	1122 nmol/L	0.90	81%	0.90	68%
90%	1.00 min	538.90 nmol/L	2.17 min	1851 nmol/L	1.11	115%	1.29	109%

Note: All samples were tested on the same day, explaining the few differences in term of variability between absolute and normalized results.

Abbreviations: CV, coefficient of variation; ETP, endogenous thrombin potential; SD, standard deviation.

**TABLE 5** Thrombin generation results at T0 of all patients and healthy donors included in the stability study

Patient no.	Lag time absolute	Peak height absolute	Time-to-peak absolute	ETP absolute	Lag time normalized (ratio)	Peak height normalized	Time-to-peak normalized (ratio)	ETP normalized	Anticoagulant ng/mL or IU/mL or INR
Healthy 1	0.96 min	561.90 nmol/L	2.12 min	1903 nmol/L/min	0.98	113%	0.96	105%	
Healthy 2	1.11 min	382.90 nmol/L	2.37 min	1263 nmol/L/min	1.12	77%	1.08	69%	
Healthy 3	1.00 min	395.00 nmol/L	2.36 min	1509 nmol/L/min	1.01	80%	1.07	83%	
Healthy 4	0.95 min	488.20 nmol/L	2.01 min	1844 nmol/L/min	0.96	98%	0.91	101%	
Healthy 5	1.14 min	410.00 nmol/L	2.64 min	1589 nmol/L/min	1.15	83%	1.20	87%	
Healthy 6	1.06 min	427.10 nmol/L	2.38 min	1716 nmol/L/min	1.07	86%	1.08	94%	
Apixaban 1	2.48 min	153.90 nmol/L	4.69 min	1295 nmol/L/min	2.62	31%	2.23	77%	307 ng/mL
Apixaban 2	1.90 min	112.40 nmol/L	6.79 min	1214 nmol/L/min	2.01	23%	3.23	72%	269 ng/mL
Apixaban 3	2.77 min	247.90 nmol/L	4.82 min	1437 nmol/L/min	2.83	46%	2.19	74%	158 ng/mL
Apixaban 4	2.14 min	169.20 nmol/L	4.29 min	995 nmol/L/min	2.27	33%	2.08	56%	195 ng/mL
Dabigatran 1	2.71 min	325.30 nmol/L	3.84 min	1150 nmol/L/min	2.64	69%	1.74	68%	89 ng/mL
Dabigatran 2	2.22 min	401.50 nmol/L	3.35 min	1361 nmol/L/min	2.22	87%	1.50	83%	51 ng/mL
Dabigatran 3	2.64 min	312.80 nmol/L	3.66 min	1015 nmol/L/min	2.86	68%	1.78	60%	187 ng/mL
LMWH 1	1.87 min	43.83 nmol/L	3.81 min	260 nmol/L/min	1.93	8%	1.81	14%	1.37 IU/mL
LMWH 2	1.41 min	331.70 nmol/L	2.81 min	1179 nmol/L/min	1.45	64%	1.34	65%	0.35 IU/mL
LMWH 3	1.33 min	334.00 nmol/L	2.54 min	1092 nmol/L/min	1.39	69%	1.25	64%	0.35 IU/mL
LMWH 4	1.38 min	259.70 nmol/L	2.79 min	879 nmol/L/min	1.41	57%	1.27	49%	0.52 IU/mL
LMWH 5	1.35 min	122.70 nmol/L	3.49 min	661 nmol/L/min	1.51	27%	1.64	40%	1.26 IU/mL
Rivaroxaban 1	2.47 min	177.90 nmol/L	7.01 min	1695 nmol/L/min	2.49	37%	3.23	101%	229 ng/mL
Rivaroxaban 2	3.33 min	93.67 nmol/L	9.67 min	965 nmol/L/min	3.65	20%	4.61	58%	197 ng/mL
Rivaroxaban 3	2.88 min	191.30 nmol/L	6.29 min	1454 nmol/L/min	3.12	38%	3.01	82%	218 ng/mL
Rivaroxaban 4	3.28 min	146.10 nmol/L	7.35 min	1187 nmol/L/min	3.43	30%	3.62	70%	296 ng/mL
Rivaroxaban 5	1.95 min	106.40 nmol/L	8.03 min	1064 nmol/L/min	2.12	23%	3.90	63%	300 ng/mL
VKA 1	2.06 min	104.80 nmol/L	3.43 min	451 nmol/L/min	2.01	22%	1.55	27%	2.57
VKA 2	2.14 min	109.80 nmol/L	3.35 min	391 nmol/L/min	2.24	23%	1.65	23%	3.31
VKA 3	2.87 min	137.90 nmol/L	4.56 min	525 nmol/L/min	3.01	29%	2.16	29%	1.86
VKA 4	2.39 min	118.70 nmol/L	3.74 min	451 nmol/L/min	2.44	26%	1.71	25%	2.91
VKA 5	2.10 min	91.17 nmol/L	3.26 min	260 nmol/L/min	2.14	20%	1.49	15%	2.68
VKA 6	1.82 min	130.90 nmol/L	3.36 min	459 nmol/L/min	2.04	29%	1.60	27%	1.79

Note: Results of the intensity of anticoagulation (expressed as ng/mL for DOACs, IU/mL for LMWH, and INR for VKA) are provided. Abbreviations: DOACs, direct oral anticoagulants (i.e., apixaban, dabigatran, rivaroxaban); INR, international normalized ratio; LMWH, low-molecular-weight heparin; VKA, vitamin K antagonist.

the routine practice.<sup>2</sup> By providing a complete kit of reagent and controls, the STG-DrugScreen application of the ST Genesis simplifies the evaluation of the thrombin generation in patients treated with anticoagulants in the routine laboratory. This is made possible by the standardized and validated methodology of the ST Genesis. The ST Genesis is derived from the CAT principle, but is not strictly equivalent. Yet, as already shown by others,<sup>20</sup> there is a direct correlation between the two systems under comparable triggering conditions. This study is not intended to provide the clinical performance of the STG-DrugScreen application to predict any clinical events, but to provide the analytical performance of the system in the routine setting and to evaluate the stability of the samples once conserved at  $-70^{\circ}\text{C}$  or below.

The precision of the ST Genesis has been evaluated using the data from the daily calibration and the value of the two levels of control and reference plasma (i.e., the STG-QualiTest Low DS and STG-QualiTest Norm DS and STG-RefPlasma DS, respectively) over a 15-month period. Two batches of reagents were provided by the manufacturer during the course of this study. Main results revealed that the coefficient of variation of the different parameters were always  $<10\%$  and for the majority of these results the CV was even  $<5\%$  (Table 1). As previously reported,<sup>10</sup> this study also demonstrates that the use of a reference plasma reduced the interexperiment variability. Regarding the stability of the samples, our results demonstrate that freezing has no impact on most thrombin generation parameters except the time to peak, especially in samples containing apixaban, dabigatran, or rivaroxaban. Our data suggest that the plasma, once frozen, is stable during at least 11 months, except for LMWH samples, for which the maximal duration storage is 6 months.

As mentioned, this study has limitations. First, this is a monocentric study and, thus, the interlaboratory performance of the ST Genesis has not been assessed. Second, results of external quality assurance program have not been included in this validation because the current thrombin generation EQA program is not designed for thrombin generation assessment of anticoagulant treatment<sup>21</sup> and thus the high tissue factor activity of the STG-DrugScreen trigger may not be comparable to that of the other reagents and methodologies. Finally, the study was not powered to drive any conclusions regarding the effect of anticoagulants on thrombin generation because only peak plasma samples were collected for this part of the study. Only trends can be drawn on the overall effect of the different anticoagulant agents; further work is necessary to define the minimal concentration of anticoagulants that would yield results outside the reference range.

#### 4.1 | Reference plasma

Many previous publications recommended the use of a reference plasma to reduce the interlaboratory variability.<sup>6,7,9,10</sup> The choice of providing a reference plasma within the STG-DrugScreen kit agrees with the literature because it has been reported that the use of local plasma (i.e., a reference plasma performed at the laboratory

facilities) were generally unable to improve the interlaboratory variability or the CV is even worsen after normalization.<sup>6,8,10</sup> The choice of a commercially available and certificated plasma is therefore more appropriate than using local plasma. On the other hand, it has been reported a certain heterogeneity between the different reference plasma which may impede the benefit of the use of such reference plasma.<sup>6</sup> Therefore, the best approach would be to use a reference plasma specifically dedicated for the application. This strategy improves the between-batch variation. In this study, we report relative differences between batches from 0% to 24% when results are not normalized (Table 1; see results of the controls). Once results are normalized most of the parameters show better inter-batch CVs (except for the peak height with the STG-QualiTest Low DS), demonstrating the efficiency of this approach. The choice of a freeze-dried plasma can be challenged because it has been reported that these plasmas have higher thrombin generation capacity than frozen plasma from multiple preanalytical reasons.<sup>6,22</sup> In any case, the conditions in which these reference plasmas are produced are probably far from the best recommendations of blood sample collection for thrombin generation testing because these reference plasmas have to be produced on a large scale. Thus, one solution may be to assess the response of the commercial reference plasma to a smaller pool of plasma from healthy donors collected in ideal conditions and then to apply a correction factor to the commercial plasma to compensate for these differences. In the package insert of the STG-DrugScreen kit, an assay value of the reference plasma is provided by the manufacturer to correct the reference plasma activity. The results of this study demonstrate that this approach is efficient since our healthy subjects, collected in ideal conditions for this application, were close to 100% or a ratio of 1 depending on the parameter (Table 5). In addition, the difference between the two batches of the STG-RefPlasma DS was  $\leq 5\%$  for all thrombin generation parameters demonstrating that the use of a correction factor specific to each batch of reagent and reference plasma is useful to reduce the batch-to-batch variation (Table 1).

#### 4.2 | Quality controls

It has been reported that internal quality control is essential to produce consistent results for thrombin generation<sup>23</sup> however, in the era of thrombin generation, the confusion between internal quality control and a reference is often made. Namely, most studies have proposed to normalize the results according to a reference plasma,<sup>6,7,9,10,23</sup> which is not the same as true control plasma. To comply with local legislation, regulations, guidelines, or standards issued by relevant bodies, at least two levels of controls are recommended. Thus, this is of utmost importance for thrombin generation to propose such different level of controls to allow the implementation of thrombin generation into routine testing. This will ensure the technique is under control for both normal and abnormal sample analysis.

Over our series of 97 measurements of the reference plasma and the quality controls, we demonstrate that the CVs of the normalized

results were always below 10% and for the majority of the parameters were even below 5%. These interassay precision results are in line with the intra- and interassay reproducibility data provided by the manufacturer in the insert-sheet of the STG-DrugScreen kit (Table S2). All of these data demonstrate that the STG-DrugScreen performed on the ST Genesis is a precise thrombin generation application, which will allow thrombin generation to be performed routinely to assess patients treated with anticoagulants. All the requirements are now met to implement this technique in the routine laboratory.

### 4.3 | Impact of freezing and stability of the samples

A previous study evaluating the effect of preanalytical parameters on the measurement of circulating microparticles with thrombin generation revealed that plasmas are stable for 1 year at  $-80^{\circ}\text{C}$ .<sup>24</sup> Apart from this information, few studies been done to assess the stability of plasma samples for thrombin generation testing. In this previous study of Lacroix et al., the authors assessed the stability of thrombin generation using the CAT system for the measurement of the procoagulant activity of circulating microparticles with the MP-reagent from Thrombinoscope bv, a reagent that does not contain tissue factor to trigger the coagulation. There is a different setting than the one assessed in this study because the STG-DrugScreen reagent contains tissue factor at concentration higher than 5 pM. This higher tissue factor concentration provides robustness to the analytical procedure. Indeed, in another study of Loeffen et al., thrombin generation triggered with tissue factor at a concentration of 5 pM has not demonstrated to be affected by the two centrifugation protocols tested nor by the temperature at which the plasma is stored until analysis ( $4^{\circ}\text{C}$ , room temperature or  $37^{\circ}\text{C}$ ).<sup>8</sup>

Freezing has no effect on the majority of the thrombin generation parameters using the STG-DrugScreen application, except for time to peak (Table 2). Once frozen, plasma samples are stable during at least 11 months, except for LMWH samples for which an impact is seen after 6 months (Table 3, Figure 2). A previous report also demonstrates that LMWH samples are not stable beyond 6 months.<sup>25</sup> This may be explained by the release of PF-4 from residual platelets. Namely, according to the CLSI document H21-A5, heparin neutralization may occur in sample not collected on CTAD tubes from the release of PF4 from platelets.<sup>26</sup> It is possible that residual platelets released PF4 during storage and thus neutralize heparin. This is consistent with the increase of the peak observed. For the other subgroups, all parameters were stable which is in correlation with the findings of Woodhams et al.,<sup>27</sup> who demonstrated that all plasma coagulation proteins were stable for at least 18 months once stored at  $-74^{\circ}\text{C}$ . If all the coagulation proteins are stable at  $-74^{\circ}\text{C}$ , it can be expected that a global test such as thrombin generation, which relies on these coagulation proteins, can also be stable for a such long period. Our results confirm this assumption for a period of 11 months for healthy subjects and 10 months for anticoagulated patients.

### 4.4 | Normal range definition

In this study, 42 samples from healthy donors were tested to provide grounds for the definition of a normal for the STG-DrugScreen application on the ST-Genesis. All these samples were normal according to PT and aPTT screening. The small correction between absolute and normal value is certainly because all 42 samples were tested on the same day with the first batch of reagents (i.e., batch 201797). For all the parameters, the interindividual variation was always below 20% and was below 10% for the lag time and the time-to-peak. When comparing with the results obtained in patients treated with an anticoagulant, the 10th-90th percentile range (Table 4) could serve as a basis for the classification of normal and abnormal plasma. It could be proposed that samples below the 10th percentile (for peak height and ETP) and above the 90th percentile (for lag time and time to peak) may represent samples in which anticoagulant therapy is suspected. However, further studies in larger cohorts of patients taking anticoagulants are needed before adopting this assumption.

### 4.5 | Usefulness of thrombin generation to assess anticoagulated patients

Tailoring drug dosages to fit the needs of individual patients could be helpful to make old and new drugs more effective and safer.<sup>2</sup> Preliminary observations showed that thrombin generation testing is affected by all anticoagulant drugs and therefore it could be the candidate assay.<sup>18,19,28-31</sup> The test has been found to be very sensitive to all kind of anticoagulants<sup>13-16,18,32,33</sup> and may best represent the interindividual response than just exploring plasma drug concentrations.<sup>28</sup> In addition to considering the interindividual response to an antithrombotic drug, thrombin generation testing is also able to explore in more detail the effect of anticoagulants on the coagulation process. Namely, depending the type of drug, our results confirmed that the fingerprint of the thrombin generation differs, revealing their different pharmacodynamics (Table 5).<sup>17,18,32,34</sup> This is of particular importance because bleeding or thrombosis have been reported within the “on-therapy” range, demonstrating that the drug level alone may not be sufficient to identify those who are more at risk.<sup>35</sup> However, further investigation on patients who bleed or who have recurrent thrombosis while on a fixed dose of anticoagulants is needed to show the benefit of thrombin generation and provide cutoffs for bleeding and thrombotic complications. Thrombin generation has also been reported as an informative tool to document on antidote administration in polytrauma models with direct implication for patient care.<sup>36</sup> This is particularly important because it may help in adjusting the dose of prothrombin complex concentrate to administer.<sup>37</sup> The ST Genesis will provide a step forward to this aim in allowing thrombin generation to be done routinely with reduced interexperiments and interlaboratory variations. In this way, it will be easier to recruit patients, design multicenter studies, and provide data for the establishment of reliable and evidence-based thresholds.

## 5 | CONCLUSIONS

This is the first study presenting performance data of the ST Genesis, a new benchtop analyzer for thrombin generation testing. The study aimed at evaluating the performances of the STG-DrugScreen application, a kit of reagents designed for the quantitative determination of thrombin generation in plasma from patients treated with an anticoagulant. Results revealed an excellent interexperiment precision thanks to the standardized methodology, the excellent reproducibility of the analyzer and the use of the reference plasma included in the kit. Stability study revealed that plasma samples are stable for at least 11 months after freezing and storage at  $-70^{\circ}\text{C}$  or lower, except for those containing LMWH, which have to be tested within 6 months. Freezing has no impact of the majority of thrombin generation parameters except on time-to-peak. Thus, our results support the hypothesis that the ST Genesis may facilitate the implementation of thrombin generation in the routine laboratory; this way, it may offer a serious input in the individual screening of patients requiring anticoagulants. However, multicentric studies are required to assess the interlaboratory reproducibility of the ST Genesis and confirm its usefulness for standardizing thrombin generation testing. In addition, the STG-DrugScreen application of the ST Genesis should be part of the propositions of external quality assurance programs.

### CONFLICTS OF INTERESTS

The study was financed by Diagnostica Stago group. Among the authors, J. Douxfils is chief executive officer and founder of QUALblood s.a. and reports personal fees from Diagnostica Stago, Roche, Roche Diagnostics, and Daiichi-Sankyo, outside the submitted work. F. Mullier reports institutional fees from Diagnostica Stago, Werfen, Nodia, Sysmex, and Bayer. He also reports speaker fees from Boehringer Ingelheim, Bayer Healthcare, and Bristol-Myers Squibb-Pfizer, all outside the submitted work. The other authors have no conflicts of interest to disclose.

### AUTHOR CONTRIBUTIONS

Among the authors, J. Douxfils and F. Mullier designed the study. C. Devroye, M. Guldenpfennig, and J. Baudar performed the analyses. J-M. Dogné, C. Devroye, M. Guldenpfennig, and J. Baudar collected data. J. Dogné, L. Morimont, and C. Bouvy analyzed and interpreted data. J. Douxfils, L. Morimont, and C. Bouvy performed statistical analysis. J. Dogné, L. Morimont, C. Bouvy, M. de Saint-Hubert, B. Devalet, A-S. Dincq, J-M. Dogné, A-S. Larock, S. Lessire, and F. Mullier wrote the manuscript.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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