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## Regular Article

## Effect of Pre-Analytical Conditions on the Thrombodynamics Assay

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## ABSTRACT

**Introduction:** Standardization of pre-analytical conditions is the obligatory step for all potential diagnostic tests. Spatial clot growth (Thrombodynamics) is a new global hemostasis assay that considers spatial organization of coagulation. The principal parameter is rate of fibrin clot growth from the tissue-factor coated surface. In this work we studied the pre-analytical variables of Thrombodynamics assay that include conditions of blood collection, sample preparation and storage.

**Materials and Methods:** Blood of apparently healthy volunteers was used. Eight types of citrate blood collection tubes were tested, centrifugation conditions for plasma preparation were evaluated and impact of plasma freezing/thawing was tested.

**Results:** Among the blood collection tubes tested, BD Vacutainer glass tubes showed a significantly higher clot growth rate compared to plastic tubes. There was no difference between 3.2% and 3.8% of sodium citrate. For plasma preparation, a single 15 min centrifugation at 1 600 g shows significantly increased clot growth rate compared to plasma obtained by two sequential centrifugations (15 min 1 600 g, 5 min 10 000 g). There was no significant difference between 1 600 g and 2 100 g if the second centrifugation was performed. For the second centrifugation there was no difference between 20 min at 1 600 g and 5 min at 10 000 g. Frozen-thawed plasma showed increased clot growth rate compared to fresh plasma.

**Conclusion:** The data represent the necessary steps for the standardization of Thrombodynamics assay and for the formulation of the operating guide.

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## Introduction

During last years a new global hemostasis assay (Thrombodynamics) has been developed that is based on monitoring spatial clot formation initiated by immobilized tissue factor [1,2]. This assay considers not only biochemical reactions of the coagulation cascade but also the spatial organization of clot formation as the activator is not mixed with the plasma sample [3].

Coagulation in a thin layer of non-stirred plasma is initiated by immobilized TF and the fibrin clot growth into the bulk of plasma is monitored with a videomicroscopy system [1,4–6].

Spatial clot growth parameters were previously shown to be sensitive to hemophilia A, B and C [4–6] and coagulation state in sepsis [1]. It was also sensitive to the changes in the coagulation system due to platelet microparticles [7], recombinant activated factor VII [2,8] and to tissue factor pathway inhibitor inhibition [2].

To be used as a diagnostic method for coagulation disorders this method requires a validated standardization of the operating procedure. In this study we analyzed the effects of pre-analytical conditions (blood collection, plasma preparation and storage) on the results of spatial clot growth as a necessary step of this standardization.

## Materials and methods

## Blood donors

55 apparently healthy informed volunteers in total (29 males and 26 females; age range 22–65 years) who were recruited from the laboratory staff participated in this study.

**Abbreviations:** TF, tissue factor; APTT, activated partial thromboplastin time; PFP, platelet free plasma; PPP, platelet poor plasma.

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### Plasma preparation

Unless otherwise stated, blood was collected into plastic tubes with sodium citrate at a 9:1 volume ratio using conventional straight Vacuette 21G needle, 0.8 × 38 mm (Greiner Bio-One, Kremsmunster, Austria). The first tube collected after the venipuncture was discarded. The blood was processed according to the standard protocol at our laboratory: the first centrifugation at 1 600 g during 15 min to obtain platelet-poor plasma (PPP) was followed by the second centrifugation 5 min at 10 000 g to obtain platelet-free plasma (PFP).

### Blood collection tubes comparison

Commercially available citrated blood collection tubes from four manufacturers were used: Monovette plastic 4.5 ml 3.2% citrate (Sarstedt, Nümbrecht, Germany), Vacutainer glass 4.5 ml 3.2% citrate and plastic 2.7 ml 3.2% citrate (Becton Dickinson, Plymouth, UK), Vacuette plastic 4.5 ml 3.2%, 3.8% citrate and CTAD (Greiner Bio-One, Kremsmunster, Austria) and Venosafe plastic 4.5 ml 3.2% and 3.8% citrate (Terumo Europe N.V., Leuven, Belgium). The information about the tubes is summarized in Table 1.

Blood was collected from healthy volunteers in a random order into 4–6 different tubes. The first tube collected after the venipuncture was discarded. Sample preparation was performed as described in Plasma preparation section. Paired comparison was performed only for the tubes collected from the same volunteer.

### Comparison of centrifugation protocols

All centrifugations were performed at room temperature. To compare the parameters of the first centrifugation two Monovette tubes (Sarstedt, Nümbrecht, Germany) were taken from each donor ( $n = 8$ ). Immediately after blood collection the tubes were centrifuged 15 min at 1600 g or 2100 g. The plasma supernatant from the tubes was then centrifuged 5 min at 10 000 g. Twice-centrifuged plasma was used for the experiment.

To compare the parameters of the second centrifugation blood was collected into 5 ml Vacuette tubes (Greiner Bio-One, Kremsmunster, Austria) and centrifuged for 15 min at 1600 g. The supernatant was divided into two parts and centrifuged for 5 min at 10 000 g or for 15 min at 1600 g. The resulting PFP was treated as described above.

### Plasma freezing

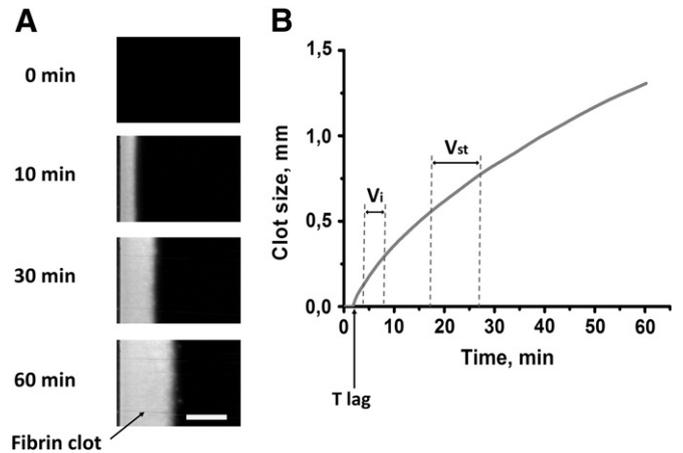
Blood was collected into 5 ml Monovette tubes (Sarstedt, Nümbrecht, Germany) and fresh PFP was obtained using two centrifugations: 15 min 2100 g followed by 5 min 10 000 g at room temperature.

300  $\mu$ l of PFP were frozen at  $-80^{\circ}\text{C}$  and then thawed in water bath at  $37^{\circ}\text{C}$ . The plasma samples were incubated at room temperature for 1 hour. Afterwards the samples were handled as fresh ones.

**Table 1**

List of the blood collection tubes used.

Tube name	Anticoagulant	Designation	Wall material
BD Vacutainer	105 mM (3.2%) of sodium citrate	Vacutainer glass	Siliconized glass
	109 mM (3.2%) of sodium citrate	Vacutainer plastic	Plastic
Sarstedt Monovette	106 mM (3.2%) of sodium citrate	Monovette	Plastic
Greiner Bio-One Vacuette	3.2% of sodium citrate	Vacuette 3.2%	Plastic
	3.8% of sodium citrate	Vacuette 3.8%	Plastic
	CTAD, 110 mM sodium citrate (3.2%) with theophylline, adenosine and dipyrindamole,	Vacuette CTAD	Plastic
Terumo Europe Venosafe	109 mM (3.2%) of sodium citrate	Venosafe 3.2%	Plastic
	129 mM (3.8%) of sodium citrate	Venosafe 3.8%	Plastic



**Fig. 1.** Design of the Thrombodynamics assay. A. Typical images of growing fibrin clot. Coagulation is activated by immobilized TF (on the left) and fibrin clot grows into the bulk of plasma. Scale bar is 1 mm. B. Clot size vs. time plot calculated from the images. Lag time (T lag) is calculated as time between the moment of activation and actual start of clot growth. Rates of clot growth are calculated as a slope of the curve within the interval 2–6 min of clot growth (initial rate,  $V_i$ ) and 15–25 min after beginning of clot formation (stationary rate,  $V_{st}$ ).

### Thrombodynamics assay

The general idea of the test was previously described in [1–3,6]. The scheme of the assay is shown on Fig. 1. Briefly, coagulation is activated in a thin layer of plasma when it is brought in contact with TF immobilized on a plastic surface. Clot formation starts on the activator and propagates into the bulk of plasma where there is no TF present.

Light scattering by fibrin allows observation of spatial clot formation in a real time by using a time lapse imaging (Fig. 1A).

The main parameters of clot growth in space are lag time, initial and stationary rates of clot growth. Lag time (Tlag) is defined as time between clotting initiation and actual appearance of the fibrin clot (Fig. 1B). This parameter is mostly dependent on tissue factor density on the activating surface [9,10] and on the factors of the TF pathway. Initial rate of clot growth ( $V_i$ ) is measured as a slope of the curve on a clot size vs. time graph during 2–6 minutes of clot growth when coagulation occurs in the region where diffusion of the active factors from the activator play the major role. The most peculiar parameter is stationary rate of clot growth ( $V_{st}$ ) measured as a slope of the curve on a clot size vs. time graph within the interval 15–25 min after clot growth beginning. Coagulation process occurs far from the activating surface without direct contact with TF and it is determined only by plasma protein properties. Factors of intrinsic pathway were shown to be responsible for self-sustained coagulation propagation in this system [3,5,6].

Thrombodynamics Analyser and Thrombodynamics kit (Hemacore LLC, Moscow, Russia) that includes the necessary reagents for the sample preparation and coagulation activation were used for all experiments [1]. For clotting activation TF immobilized to the plastic surface was used [9].

Activated partial thromboplastin time measurement

Activated partial thromboplastin time (aPTT) was measured using Helena C2 coagulometer (Helena Biosciences Europe, UK) and aPTT measurement kit “Coagulo-test” (Renam, Moscow, Russia) or using ACL 9000 coagulometer using HemoSL APPT-SP kit (Instrumentation laboratory, Bedford, MA, USA). aPTT values were normalized to the mean value of the reference range for each instrument. Normalized results for the samples measured using these two techniques did not change significantly (data not shown). Paired comparison was performed only using samples measured with the same technique.

Statistical analysis

Spatial clot growth parameters were statistically analyzed using Origin 8.0 software (OriginLab Corporation, Northampton, MA, USA). Paired Student t-test was used for statistical analysis. A p-value < 0.05 was considered statistically significant. Spearman correlation coefficient was used to evaluate the correlation.

Results

Comparison of blood collection tubes

Sodium citrate venous blood collection tubes of four major manufacturers (BD, Greiner Bio-One, Sarstedt and Terumo Europe) were compared. The information about the tubes used is presented in Table 1.

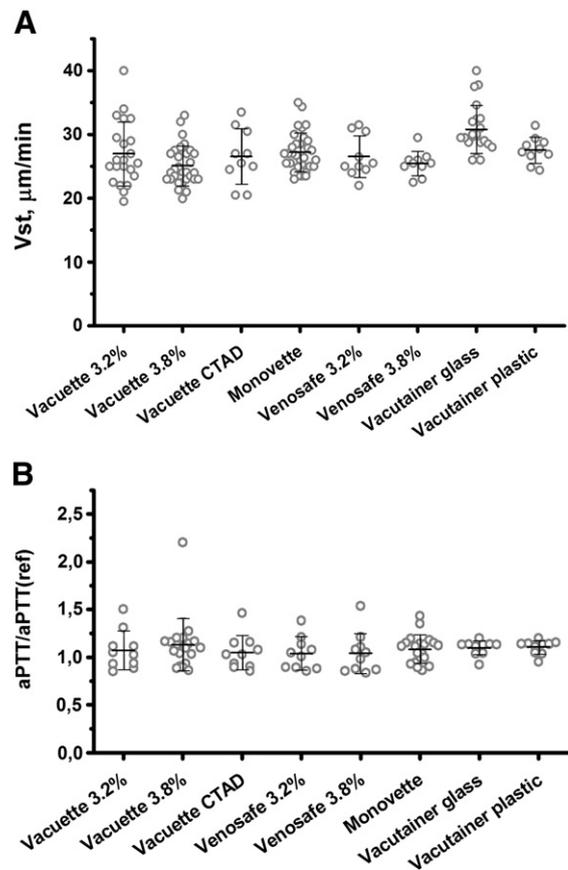
The Thrombodynamics parameters for all the tubes are presented in Table 2 and Fig. 2A.

BD Vacutainer glass tubes showed significantly increased rate of clot growth compared to all the plastic tubes. There was no significant difference between the tubes of the same manufacturer with 3.2% and 3.8% sodium citrate. There was no significant difference for the plastic tubes with 3.2% of sodium citrate from different manufacturers. This holds true for aPTT values (Table 2, Fig. 2B). Statistically significant difference (p < 0.05) between glass and plastic BD Vacutainer tubes shows that the main difference arises from the wall material of the tube. There was no significant correlation between clot growth parameters between plastic and glass BD Vacutainer tubes that shows randomness of error introduced by the tubes choice.

Difference in aPTT values between glass and plastic tubes, though statistically significant, was less than 1%. that cannot be considered

**Table 2**  
Thrombodynamics parameters and normalized aPTT values for the blood collection tubes tested.

Blood collection tube	N	Tlag, min ± SD	Vi, µm/min ± SD	Vst, µm/min ± SD	aPTT normalized ± SD
Vacurette 3.2%	22	0.8 ± 0.3	49.6 ± 6.4	27.0 ± 5.0	1.07 ± 0.20 n = 10
Vacurette 3.8%	32	0.8 ± 0.3	46.4 ± 4.1	25.1 ± 3.1	1.13 ± 0.28 n = 20
Vacurette CTAD	10	1.0 ± 0.5	47.9 ± 4.9	26.6 ± 4.3	1.05 ± 0.18 n = 10
Venosafe 3.2%	10	1.0 ± 0.3	53.8 ± 5.2	26.6 ± 3.3	1.04 ± 0.17 n = 10
Venosafe 3.8%	10	1.0 ± 0.3	50.2 ± 4.5	25.5 ± 1.9	1.04 ± 0.22 n = 10
Monovette	32	0.9 ± 0.3	53.4 ± 4.4	27.2 ± 3.1	1.09 ± 0.15 n = 20
Vacutainer glass	22	0.9 ± 0.3	54.7 ± 4.2	30.8 ± 3.8	1.10 ± 0.08 n = 10
Vacutainer plastic	10	1.0 ± 0.2	54.5 ± 3.9	27.6 ± 2.1	1.11 ± 0.07 n = 10



**Fig. 2.** Comparison of the blood collection tubes. Individual and mean values ± SD are shown. A. Stationary rate of clot growth, B. Normalized aPTT values.

diagnostically relevant and is in the agreement with the results from previous studies [11,12] (Table 2, Fig. 2.B).

Sample preparation

The use of plasma in coagulation assay requires standardization of the centrifugation protocol. Additional centrifugation of plasma is known to decrease variability of the results in coagulation tests [13].

We compared for 31 healthy individuals the results with plasma obtained by single blood centrifugation for 15 min at 1600 g (PPP) and plasma that was additionally centrifuged 5 min at 10 000 g (PPF). A statistically significant difference in Thrombodynamics parameters was observed for these different conditions of plasma preparation (Table 3).

Values of initial and stationary rates were significantly higher (p < 0.05) when one centrifugation was used compared to two centrifugations. Data distribution for Vst is shown in Fig. 3A. A significant correlation was found for Vst between PPP and PFP (r = 0.50, p < 0.05) that shows a systematic shift of values obtained with a single centrifugation (Fig. 3B).

Two centrifugation conditions for PFP preparation were tested: 5 min at 10 000 g and 15 min at 1 600 g (n = 7). The values of stationary clot growth rates were 25.3 ± 4.2 µm/min and 25.6 ± 4.1 µm/min, respectively. No significant difference in Thrombodynamics parameters was observed. If the second centrifugation was performed there was no difference between 1600 g and 2100 g for the first 15 min centrifugation (n = 8), Vst = 25.6 ± 2.6 µm/min for 1 600 g and 26.0 ± 2.7 µm/min for 2 100 g.

These data indicate that a range of centrifugation protocols can be used depending on laboratory equipment available. But different ranges of normal values should be used for PPP and PFP.

**Table 3**  
Thrombodynamics parameters for platelet poor (PPP) and platelet free (PFP) plasmas.

Plasma type	N	Tlag, min ±SD	Vi, $\mu\text{m}/\text{min}$ ± SD	Vst, $\mu\text{m}/\text{min}$ ± SD
PPP	31	0.8 ± 0.2	50.2 ± 3.3	27.8 ± 1.9
PFP	31	0.9 ± 0.2	46.6 ± 3.6	24.6 ± 1.7
P value*	31	0.03	<0.001	<0.001

\* P value is for paired sample t-test.

### Sample storage

Sample freezing is a common way to preserve the plasma samples for a delayed analysis [14]. We compared the Thrombodynamics parameters for fresh and frozen samples of PFP from  $n = 35$  healthy individuals (Table 4, Fig. 3C,D). PFP was frozen at  $-80^\circ\text{C}$  and then thawed in water bath at  $37^\circ\text{C}$  as described in Materials and Methods section. Then plasma was incubated at room temperature for 1 hour. After incubation, the samples were handled as fresh ones.

While Vi and Vst were significantly higher ( $p < 0.05$ ) for frozen samples compared to fresh ones, the difference was about 2% and thus was not diagnostically relevant (Table 4, Fig. 3C). Tlag did not change significantly. Also there was a significant correlation (Fig. 3D) between Vst for fresh and frozen-thawed plasma ( $r = 0.72$ ,  $p < 0.05$ ). We then concluded that frozen samples can be used for the assay at least when using the described protocol.

### Discussion

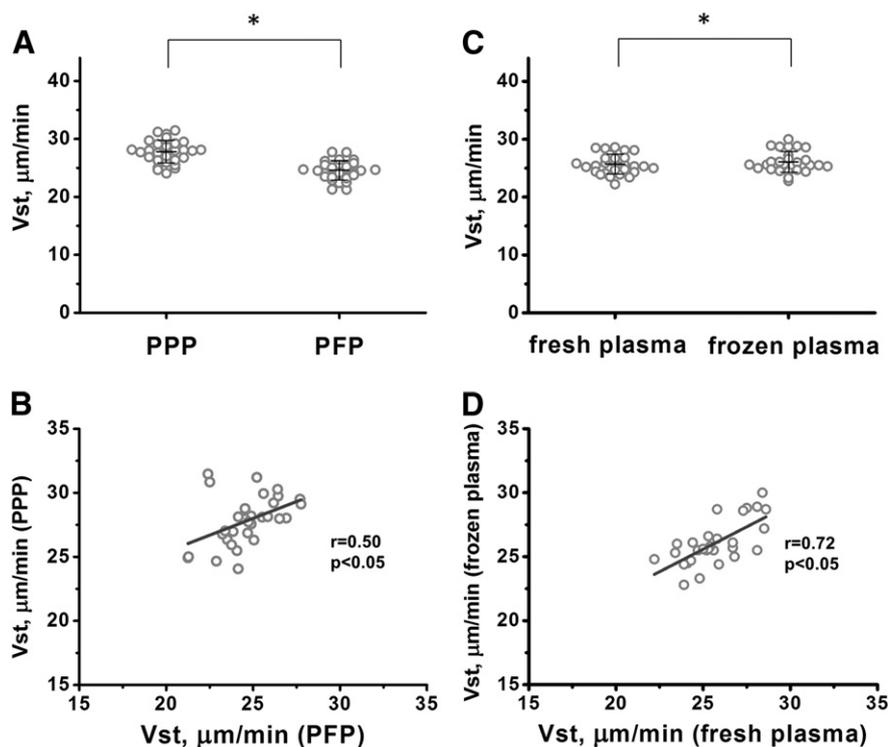
In this work we completed the first stage for the standardization of pre-analytical conditions to decrease variability of the results obtained in Thrombodynamics assay starting from blood collection.

General recommendations for blood collection and sample preparation for coagulation assays were already developed [14] and should be followed. However, new assays that claim higher sensitivity could require additional precautions, which have to be tested [15,16]. We found the steps in the sample preparation for Thrombodynamics assay to be extremely important for robust and reproducible results.

In this regard, we hypothesize that blood collection tubes can be the reason for result deviation. One of the tubes tested (BD Vacutainer glass) showed a significantly different result compared to the other tubes; this difference was not detectable by aPTT. A potential reason is the use of silicized glass as a wall material that can lead to additional contact activation [13]. We did not test the effect of the tubes on the diagnostic sensitivity, but using BD Vacutainer glass tubes for Thrombodynamics would at the very least require additional standardization, and it is important to recognize that the range of normal values determined for other tubes would not be applicable.

Previous studies showed that preparation of PPP has a great influence on the results of coagulation tests [13,17] and the second centrifugation resulting in PFP production is sometimes recommended. We compared the results with one or two centrifugation steps and identified significant differences between these two protocols. Despite this difference, a strong correlation between these two protocols was observed showing that both can be used. However, the direct comparison of the results is impossible and the separate range of normal values should be obtained for each protocol. We recommend using additional centrifugation of plasma to remove the remaining blood cells that can potentially increase the dispersion. The parameters of the second centrifugation can be adjusted according to the available equipment without significantly affecting the results.

In coagulation laboratories, sample freezing is usually performed for a further analysis. Results obtained with the frozen-thawed samples have a good correlation with fresh ones and the use of frozen samples



**Fig. 3.** Impact of the centrifugation conditions and freezing. A. Stationary rate of clot growth for PPP and PFP for  $n = 31$  healthy individuals. Mean values  $\pm$  SD are shown with whiskers. Mean values are significantly different ( $p < 0.05$ ). B. Correlation between stationary rate values for PFP and PPP plasma. Correlation coefficient  $r = 0.50$  is significant at  $p < 0.05$  level. Linear approximation is shown with the straight line. C. Stationary rate of clot growth for fresh and frozen-thawed plasma for  $n = 35$  healthy individuals. Mean values are significantly different ( $p < 0.05$ ). D. Correlation between stationary rate values for fresh and frozen-thawed plasma. Linear approximation is shown with the straight line. Correlation coefficient  $r = 0.72$  is significant at  $p < 0.05$  level.

**Table 4**

Thrombodynamics parameters for fresh and frozen-thawed PFP samples.

Plasma type	N	Tlag, min ± SD	Vi, μm/min ± SD	Vst, μm/min ± SD
fresh	35	0.8 ± 0.2	51.5 ± 3.8	25.6 ± 1.7
frozen-thawed	35	0.9 ± 0.1	52.8 ± 3.2	26.0 ± 1.7
<i>P</i> value*	35	0.31	0.01	0.04

\* *P* value is for paired sample t-test.

is therefore possible for Thrombodynamics assay at least for normal samples. However it would still require determination of the separate range of normal values.

In summary, the data presented show that, while sensitive to protocol specificities, Thrombodynamics assay exhibits stable results for a range of pre-analytical conditions. It should yet be demonstrated that the same holds true for patients with coagulation disorders, and to provide evidence that the sensitivity and specificity of the assay correlates with the clinical phenotype.

### Conflict of interest disclosure

N.M.D., N.P.S., M.A.P., and F.I.A. are employees and/or founders of HemaCore LLC, which holds several patents and patent applications on the diagnostic use of coagulation assays in spatially distributed systems, which are currently developed under the trade name of Thrombodynamics®. T.A.V., R.A.O., and S.S.S. are supported by the grants from HemaCore LLC.

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