An international study on the feasibility of developing a standardized combined plasma clot turbidity and lysis assay: methodological and clinical considerations

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Background and aim

Abnormal fibrin clot structure, in particular increased fibrin fibre density and stiffness and resistance to fibrinolysis, are reported to be linked to cardiovascular disease (CVD) and a higher incidence of venous and arterial thromboembolic events [1, 2]. Clot structure is, to a large extent, kinetically controlled and determined by the early stages of fibrin assembly, polymerization and lateral aggregation [3]. Knowledge of the transformation of soluble polymers to an insoluble elastic gel is fundamentally important in understanding blood clotting, fibrinolysis, wound healing, and thrombosis [4]. In addition several clinical diagnostic tools currently rely on fibrin polymerization as molecular markers of thrombin generation and/or intravascular fibrin deposition by monitoring changes in D-dimer, fibrinopeptide A or soluble fibrin precursors [5]. In the research setting, turbidity analysis is used as a quantitative high throughput technique to monitor clot formation and structure in large datasets [6]. In a similar, but separate assay, clot lysis time (CLT) can be calculated to determine the plasma fibrinolytic potential of an individual with application in both hypercoagulable and hyperfibrinolytic conditions [6-13]. Combining the turbidity and clot lysis assays has the added benefit of directly relating lysis times to clot formation and structure, ultimately resulting in a more comprehensive characterization of plasma clot properties (Figure 1).

The absence of an internationally accepted (combined) standardized methodology has resulted in numerous different approaches being described in the literature, which varied considerably in terms of activator, the concentration of reagents, dilution of plasma and recorded measurements. Not surprisingly, when we

performed a review of these published assays [6, 14-34], we found large inter-laboratory variation for the respective healthy control groups including; lag times that ranged from 27 to 399s; rate of lateral aggregation (slope) that ranged from 0.02 to 0.43 change in absorbance units per unit of time (min); and maximum absorbance that ranged from 0.32 to 1.22 absorbance units. This exceptionally large variation precludes inter-laboratory comparison and prevents the establishment of normal and disease ranges, which are available for other CVD risk factors. This necessitates the need to establish an internationally accepted standardized procedure that would 1) facilitate interpretation of results, 2) allow direct comparison of data from different laboratories and 3) permit the determination of parameters of clot formation and lysis for healthy normal and pro-thrombotic individuals. Our aim was therefore to perform a pilot international study to investigate the development of a combined turbidity and clot lysis assay. A method was established based on protocols currently used by participating laboratories (Supplement 1). The proposed protocol was tested by nine laboratories in an experimental study aimed at determining 1) the practicality of combining the turbidity and clot lysis assays and 2) comparing inter-laboratory variation using centrally provided lyophilized plasma from the NIBSC, as well as in-house normal plasma (generated locally from normal healthy individuals or a commercial preparation). In addition, we analysed turbidity data from one laboratory on 50 healthy controls, 50 patients following acute myocardial infarction and 50 individuals with stable coronary artery disease (CAD) to assess the sensitivity of this assay as a tool for detecting differences between healthy individuals and those with CVD. The data demonstrate significant inter-laboratory variation for the proposed method, with similar variation obtained for the centrally provided NIBSC plasma as for the in-house pooled healthy plasma. This suggests that the variability inherent to

the protocol is larger than the contribution of different plasma pools. The assay was, however, able to detect significant differences between the healthy and CAD patients, highlighting its usefulness in the clinical setting. Although the current method cannot be proposed as an internationally accepted standardized protocol, we clearly demonstrate that the turbidity and clot lysis assays can successfully be combined. Crucially, we identified critical steps that should be addressed in all versions of the respective protocols and future standardization endeavors.

Findings and Recommendations

Each laboratory followed the proposed protocol to analyze the reconstituted lyophilized pool plasma as well as their respective in-house plasmas on three consecutive days. One run consisted of a 96-well plate of which all wells were filled with the same plasma.

Six of the nine test laboratories successfully performed the analysis providing evidence that it is possible to combine traditional turbidity and clot lysis assays. Three of the laboratories were unable to generate accurate turbidity and lysis curves. This was attributed to the requirement of a spectrophotometer capable of performing 4-6 readings per minute of a 96-well plate at a temperature of 37 °C without causing significant sample evaporation. The addition of liquid paraffin to the clotting sample (as is done in some clot lysis assays) could limit said evaporation. In addition, the commercial preparation of plasma used by one of the laboratories did not generate reproducible turbidity curves, suggesting that plasma preparation and/or storage may impact on assay performance and reproducibility.

Table 1 provides the mean ± standard deviation and between-day coefficient of variation (CV) of the NIBSC and in-house pool plasmas for the six participating laboratories. There is significant inter-laboratory variation (CV's of up to 50%) despite the use of the same protocol and plasma (in the case of the NIBSC). Also, unexpectedly, the inter-laboratory CV's for the different in-house plasma's were similar to that of the centrally provided NIBSC plasma. There was, however a good correlation between data generated from the in-house pool and NIBSC plasma within each respective laboratory (Table 1). These data indicate that subtle methodological, equipment or reagent differences between laboratories contribute significantly to the outcome of the protocol. In light of this, it may be more feasible to report patient data relative to that of a reference plasma.

One critical factor that contributes to variation is timing, both in terms of mixing and pipetting reagents as well as measurement of data points (as detailed in Supplement 1). Assay conditions were selected with the specific focus of obtaining turbidimetric curves from which all the different variables calculated can be clearly visualized and quantified. To achieve this, a thrombin concentration was required that was sufficiently low to visualize the lag time but high enough to generate a clear plateau for the calculation of maximum absorbance. In addition to the absolute thrombin concentration, the thrombin – tPA ratio is a crucial factor to ensure adequate clot formation before onset of lysis (again ensuring the formation of a clear plateau). The selection of the tPA concentration is furthermore based on the achievement of a clot lysis time (60-100min) that allows the presence and functionality of the different components of the fibrinolytic system and precludes fibrinogenolysis (Table 1 of Supplement 2 provides details on the tPA concentrations used by the participating

laboratories). The assay is sensitive to the incubation time of plasma with tPA, as it is rapidly inactivated by inhibitors, and therefore the protocol specifies that tPA be added to the plasma just prior (<60 seconds) to the addition of the activation mix (thrombin and calcium chloride). If these stringent protocol guidelines are not adhered to, or the assay performed by an inexperienced operator, it can result in significant changes in CLT.

An additional source of variation is the calculation of the different parameters from the data. Detailed instructions for calculating the different parameters are included in Supplement 1, however, it can be difficult to accurately pinpoint them due to variability in the shape of the turbidity curves and there will be individual to individual variability in this process. Furthermore, curve fitting programs, such as Origin and Prism, are often used but can have limitations as they were on occasion not able to successfully fit a curve to the data points, while values could be obtained using a more “manual” approach in Excel, such as determining the midpoint of the ascending curve by adding half of the increase in absorbance to the baseline absorbance. To test the impact of software choice and operator contribution on variability the raw data of the participating laboratories was centrally calculated using Excel or Origin (using the Boltzmann sigmoidal curve fitting option for CLT) which resulted in an average decrease of 5% in the inter-laboratory CVs. This exemplifies the need for a program to standardize data calculation, one such online tool for analysis of turbidity and lysis, termed the Shiny App, was recently developed by Longstaff [35] which could significantly minimize the variability associated with interpretation of data of this nature.

One laboratory tested healthy controls and CAD patients (Supplement 2, Table 2) using the provided turbidity and clot lysis assay and demonstrated significantly faster rates of lateral aggregation (slope), increased clot density (maximum absorbance) and longer CLTs (acute MI only) in the CAD patients compared to the healthy controls. This illustrates that despite the inter-assay variation this combined method of clot formation and lysis has considerable usefulness in comparing data sets of clinical samples to normal individuals.

In conclusion, we have shown that development of a combined turbidity and clot lysis assay is feasible and has potential for clinical use to examine fibrin clot properties in CVD. The study highlights the huge analytical challenges that exist with regard to assays examining clot lysis. It is likely that normalization of findings will be necessary to allow direct comparison between laboratories. Nevertheless, the identification of the critical steps that introduce variation in this assay allow the realization of our ultimate goal; to generate a standardized assay which will aid in the establishment of healthy and pro-thrombotic ranges and facilitate inter-laboratory comparisons of large data sets.

Addendum

M. Pieters made a substantial contribution to concept and design, analysis and interpretation of data; critical writing of the intellectual content; and final approval of the version to be published. H. Philippou, A. Undas, N.J. Mutch and D.C. Rijken made a substantial contribution to concept and design, analysis of data; critical revision of the intellectual content; and final approval of the version to be published. Z. De Lange made a substantial contribution to analysis and interpretation of data;

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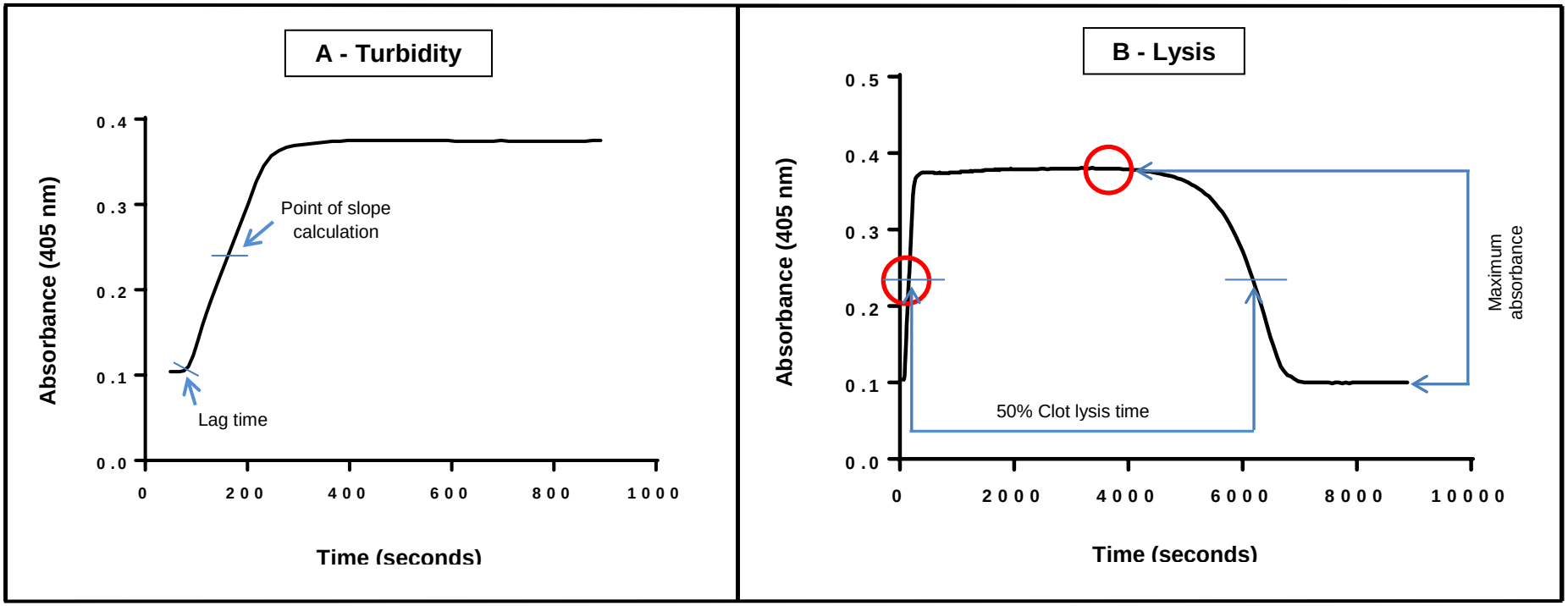

Figure 1 Variables calculated from turbidimetric curves. The clotting and lysis parts of the curve are broken down for ease of interpretation (Fig 1A and 1B - note the difference in the scale of the x-axis).

Data reported in A and B were obtained from healthy pool plasma samples and experiments containing tPA.

Lag time – time taken from the start to when the absorbance increases 0.015 from baseline. Slope - calculated from the slope of the line at the midpoint (----) between initial baseline and maximum absorbance at plateau. Maximum absorbance - calculated as the increase in absorbance from baseline to maximum absorbance at plateau. The plateau is often not a perfect horizontal line and the area of the plateau where the line is closest to horizontal should be selected for the calculation. 50% Clot lysis time (CLT) - calculated as the time from the midpoint, from clear to

maximum turbidity, to the midpoint in the transition from maximum turbidity to the final baseline turbidity. Please refer to Supplement 1 for detailed description of calculations.

Table 1 Variation in turbidity and lysis of NIBSC and in-house pool plasma within the 6 participating laboratories. Data is shown as mean ± std and the coefficient of variation (CV)

Variable	Lab 1 mean ± std CV (%)	Lab 2 mean ±std CV (%)	Lab 3 mean ± std CV (%)	Lab 4 mean ± std CV (%)	Lab 5 mean ± std CV (%)	Lab 6 mean ± std CV (%)	Labs combined mean ± std CV (%)	Pearson correlation between NIBSC and pool plasma (r)
NIBSC plasma								
Lag time (s)	79.7 ± 11.2	87.5 ± 6.9	32.0 ± 8.5	96.6 ± 12.9	195.0 ± 24.3	33.3 ± 7.3	92.7 ± 49.6	0.95
	14.0	7.9	26.4	13.4	12.5	22.1	53.5	
Slope (abs/min)	0.090 ± 0.028	0.072 ± 0.018	0.10 ± 0.01	0.048 ± 0.012	0.0660 ± 0.0004	0.17 ± 0.05	0.078 ± 0.024	0.82
	23.2	21.4	11.0	19.1	0.7	29.5	30.3	
Max absorbance	0.12 ± 0.03	0.15 ± 0.01	0.12 ± 0.01	0.130 ± 0.008	0.06 ± 0.01	0.21 ± 0.03	0.13 ± 0.04	0.88
	23.6	4.0	4.1	5.9	11.2	15.5	29.3	
CLT (min)	72.7 ± 9.9	98.3 ± 5.9	157.0 ± 6.3	103.0 ± 3.5	115.0 ± 10.7	88.4 ± 10.5	104.0 ± 26.8	0.96
	13.6	6.0	4.0	3.4	9.3	11.8	25.8	
In-house pool plasmas								
Lag time (s)	72.3 ± 9.7	76.9 ± 6.9	18.3 ± 6.0	82.1 ± 9.5	120.0 ± 12.3	27.0 ± 1.5	72.4 ± 34.4	
	13.4	9.0	33.0	11.5	10.2	5.4	47.5	
Slope (abs/min)	0.130 ± 0.042	0.036 ± 0.006	0.13 ± 0.03	0.090 ± 0.006	0.060 ± 0.006	0.180 ± 0.054	0.096 ± 0.048	
	29.5	10.1	22.9	9.2	1.0	30.6	50.9	
Max absorbance	0.27 ± 0.07	0.25 ± 0.01	0.22 ± 0.02	0.30 ± 0.01	0.17 ± 0.01	0.34 ± 0.03	0.25 ± 0.06	
	24.9	3.0	9.1	4.3	8.5	10.1	23.0	
CLT (min)	52.0 ± 10.3	103.0 ± 2.6	168.0 ± 10.0	101.0 ± 5.7	99.9 ± 9.7	94.2 ± 2.3	105.0 ± 37.4	
	19.8	2.6	5.9	5.6	9.8	8.8	35.8	

CLT – clot lysis time; CV – coefficient of variation; std – standard deviation; s – seconds; abs – absorbance; min – minutes; Max - maximum

All data reported, were obtained from experiments containing tPA

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Supplement 1

Protocol for the determination of the feasibility of developing a standardized combined plasma clot turbidity and lysis assay

Objective

The purpose of this project is to provide a standardized assay that can be used for the determination of plasma clot turbidity and lysis. Currently many laboratories perform turbidity and lysis assays, but each laboratory uses its own methodology which is in essence a modification of the same assay. Standardization of these assays will allow better comparison of results between laboratories and will aid in the establishment of healthy normal ranges that can be used to compare with disease states, in particular cardiovascular disease. In order to allow the use of this assay in studies with large sample sizes, it was decided to standardize the method for use in 96-well plates.

Development of proposed protocol

The proposed standardized protocol was developed based on in-house turbidity and clot lysis protocols obtained from different laboratories world-wide. Although both turbidity and clot lysis assays are turbidimetric by design, turbidity assays are designed to visualize the clot formation process while clot lysis protocols focus on the lysis of formed clots. This is achieved by varying assay conditions to allow the visualization of the area of interest on the turbidimetric curve. From these curves specific variables (as detailed below) are calculated to provide information on clot formation, structure and lysis. The clotting agent of choice for turbidity assays is thrombin as it is the direct activator of fibrinogen and the purpose of the turbidity assay is to characterize the activation of fibrinogen and the consequent formation of the fibrin network. Turbidity assays are typically performed without the addition of a lytic agent. The combination of the two techniques will allow better characterization of the fibrin clot as information on clot lysis can now be added and directly related to the clot properties obtained from turbidity. Although thrombin is used as clotting agent, endogenous thrombin generation might be important, particularly for clot lysis because of the activation of thrombin

activatable fibrinolysis inhibitor (TAFI). To eliminate a varying amount of phospholipids in different plasma samples an excess of external phospholipids is added.

The assay conditions of the available turbidity and clot lysis protocols were combined and refined until the optimal conditions that allowed clear visualization of all parts of the turbidimetric curve, were achieved / selected. These are now presented as the protocol to be used in the standardization project, which includes the following experiments.

Experimental layout

As these assays are often performed in laboratories also performing other coagulation tests, the reagents required for the proposed protocol would typically be available in-house. Consequently we do not stipulate the use of a specific brand name but rather specify the specific activity of the reagents to be used, to allow the use of readily available reagents but with standardized functionality. For determination of the tissue plasminogen activator (tPA) concentration to be used, it was decided to standardize the clot lysis times (CLT), rather than use a defined tPA concentration. Differences in the specific activity of commercially available tPA preparations, as well as batch differences within the same preparation would make a concentration approach unfeasible. A CLT of 100 ± 5 minutes (min) was selected as the reference point as CLTs of 60-100 min are sensitive to many activators and inhibitors in the coagulation and fibrinolytic pathways and 100 min demonstrated the highest repeatability. The assay procedure below will be performed in three phases:

Phase 1

Perform the plasma clot turbidity and fibrinolysis assays using lyophilized NIBSC plasma and different tPA concentrations until a CLT of 100 ± 5 min is obtained. Calculation of CLT is described in Figure 1. For this phase, the full 96 well plate need not be used. It is recommended that different tPA concentrations are tested in 12 wells each and therefore five (5) different tPA concentrations can be tested in one plate (the outer wells of the plates are not to be used to avoid possible temperature differences between these and the inner rows). It is suggested to start the experiments with tPA concentrations in the range of 20-40 ng/mL.

Phase 2

Once the correct tPA concentration has been determined (i.e. a concentration that will achieve a CLT of 100 ± 5 min using the NIBSC lyophilized plasma), perform 3 repeats of the assay using the lyophilized NIBSC plasma. One run = one 96-well plate. The assay will be performed both with and without the addition of tPA, as described below. Fill rows B, D, and F of the plate with plasma-buffer mix without tPA and rows C, E, G with plasma-buffer mix containing tPA.

Phase 3

Use the same tPA concentration and perform 3 repeats of the assay using local pooled plasma, commercial or prepared in house from normal healthy volunteers. One run = one 96-well plate. The assay will be performed both with and without the addition of tPA. Fill rows B, D, and F of the plate with plasma-buffer mix without tPA and rows C, E, G with plasma-buffer mix containing tPA.

Collection of local pool plasma

If using an in house preparation of normal pool, the following guidelines are suggested. Please disregard if using a commercial preparation of normal pool plasma.

Collect 3.2 % citrated plasma from at least n=8 fasting healthy individuals before 11:00. Exclusion criteria are pregnancy, use of oral contraceptives, use of acute or chronic medication, elevated temperature or known blood disorders. Blood samples should be kept at room temperature after collection for no longer than 30 min and then centrifuged at 2000g for 20 min at 15 °C to prepare platelet poor plasma. Plasma of all individuals should be pooled, mixed and aliquoted before freezing at -65°C or below.

Equipment

96-well plate reader with a 405 nm filter and capacity to read at 37 °C with a plate shaker on board.

Materials & Reagents provided

1. Lyophilized NIBSC plasma (18 vials of 1 mL)

2. 1 x 3 mL vial Phospholipids, (Rossix, Cat no: PL604T, 0.5 mmol/L)

Storage of reagents provided

1. Lyophilized plasma will be shipped at room temperature to the address provided and should be stored at 2-8 °C.

2. Phospholipids will be shipped on ice and should be stored at 2-8 °C.

Additional Materials & Reagents required

1. Human α-thrombin
2. $CaCl_2$
3. 20 mL frozen 3.2 % citrated pool plasma obtained from healthy individuals or a commercial preparation
4. tPA
5. 10 x 96-well untreated polystyrene plates
7. Buffer = 50 mM Tris; 140 mM NaCl; 1mg/mL BSA pH 7.4

Reconstitution of reagents

1. Lyophilized NIBSC plasma: Allow plasma to reach room temperature and then reconstitute with 1000 µL MilliQ water. Mix by gently swirling vial and allow standing at room temperature for 15 min before use.

2. Frozen pool plasma locally collected: Thaw plasma at 37 °C for at least 5 min.

3. Phospholipids: Keep undiluted stock at 2-8ºC. On day of experiment, dilute stock 1:9 to 0.05 mM with assay buffer.

4. Human α-thrombin: Prepare stock of 12.5 NIH U/mL with assay buffer, aliquot and store frozen. Thaw once only and keep on ice until addition to activation mix, to prevent inactivation. Use within 30 minutes.

5. $CaCl_2$: Prepare stock of 250 mM with MilliQ water

6. tPA: Reconstitute according to package insert and dilute with assay buffer to obtain a final concentration in the clot of 20-40 ng/mL. (Preliminary experiments have indicated this to be the tPA concentration range likely to give lysis times of 100 ± 5 min). An additional dilution step may be required depending on the final tPA concentration selected. Aliquot and store frozen. Thaw once only. Lysis time is defined as the time from the midpoint, from clear to maximum turbidity, which is representative of clot formation, to the midpoint in the transition from maximum turbidity to the final baseline turbidity, which represents the lysis of the clot (as depicted in Figure 1.).

Procedure

1. Start the micro plate reader and set the temperature at 37°C.
2. Fill the outer wells with water.
3. Prepare the plasma-buffer mix with and without tPA as well as the Activation Mix (see below). Prepare the Activation Mix before plasma is transferred to 96-well plate and add immediately afterwards to minimize incubation time of tPA with plasma.

Plasma-buffer Mix without tPA

TO BE PIPETTED INTO ROWS B, D AND F OF THE 96-WELL PLATE

Stock solution	Volume for 3800 µL Plasma-buffer mix (µL)	Concentration in Plasma-buffer Mixture	Final concentration in clot
Phospholipids	950	12.5 µM	10 µM
Plasma	1425	-	30%
Buffer	1425	-	-

Plasma-buffer Mix with tPA

TO BE PIPETTED INTO ROWS C, E, AND G OF THE 96-WELL PLATE

Stock solution	Volume for 3800 µL Plasma-buffer mix (µL)	Concentration in Plasma-buffer Mixture	Final concentration in clot
Phospholipids	950	12.5 µM	10 µM
Plasma	1425	-	30%
Buffer	95	-	-
tPA*	1330		

***Add the tPA to the plasma-buffer mix just before (<60 seconds) it is transferred to the 96-well plate – use a multichannel pipette for the transfer to decrease pipetting time.**

Activation Mix – keep on ice for no longer than 30 min

Stock solution	Volume for 4 000 µL Activation mix (µL)	Concentration in Activation Mixture	Final concentration in clot
Thrombin	800	2.5 NIH U/mL	0.5 NIH U/mL
$CaCl_2$	1 200	75 mM	15 mM
Buffer	2 000		

4. Add 80 µL of the two Plasma-Buffer Mixes to the wells of the micro plate (untreated) – do not use the outer wells for samples. Add the Plasma-Buffer Mix without tPA to the wells of rows B, D and F and the Plasma-Buffer Mix with tPA to the wells of rows C, E and G of the micro plate. *When using this assay in future for individual patient samples, perform assay in duplicate (i.e. 4 wells per individual). Mix 180 µL plasma and 120 µL phospholipids and pipette 50 µL thereof into each of the 4 wells. Then mix buffer and tPA, or for samples without tPA, use buffer only (enough for all the*

samples on the plate), and pipette 30µL into the wells using a multichannel, to prevent long incubation times of tPA with plasma.

5. Check if the plate reader has reached 37°C. (**note!!!** The next steps have to be performed quickly because the reaction starts immediately when the activation mixture is added to plasma).
6. Add 20 µL activation mix to each well with a 6-well multi-channel (start at column 2 and work towards column 11). Note time required for pipetting of activation mix to plate until measurements are started to include in time point calculations as detailed below. The time required for pipetting of the activation mix, should be less than the lag time to ensure that the lag time of the first sample is visible on the turbidity curves.
7. Place the plate into the plate reader and start with a 2 sec shake step at 900 rpm before taking the first reading. Start measuring the absorbance at 405 nm every 10 seconds up to 30 min (180 readings) and then every minute until the clots have broken down or up to 180 minutes (n=150 readings).

Example of plate layout and numbering

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		1	7	13	19	25	31	37	43	49	55	
C		2	8	14	20	26	32	38	44	50	56	
D		3	9	15	21	27	33	39	45	51	57	
E		4	10	16	22	28	34	40	46	52	58	
F		5	11	17	23	29	35	41	47	53	59	
G		6	12	18	24	30	36	42	48	54	60	
H												

Fill with water

Fill with plasma-buffer mix without tPA

Fill with plasma-buffer mix with tPA

Calculate lag time, slope, maximum absorbance and 50% clot lysis time from graphs using a software program such as Origin, Prism or Excel as indicated in Figure 1 and 2 below. A Boltzmann sigmoidal curve fitting should be used.

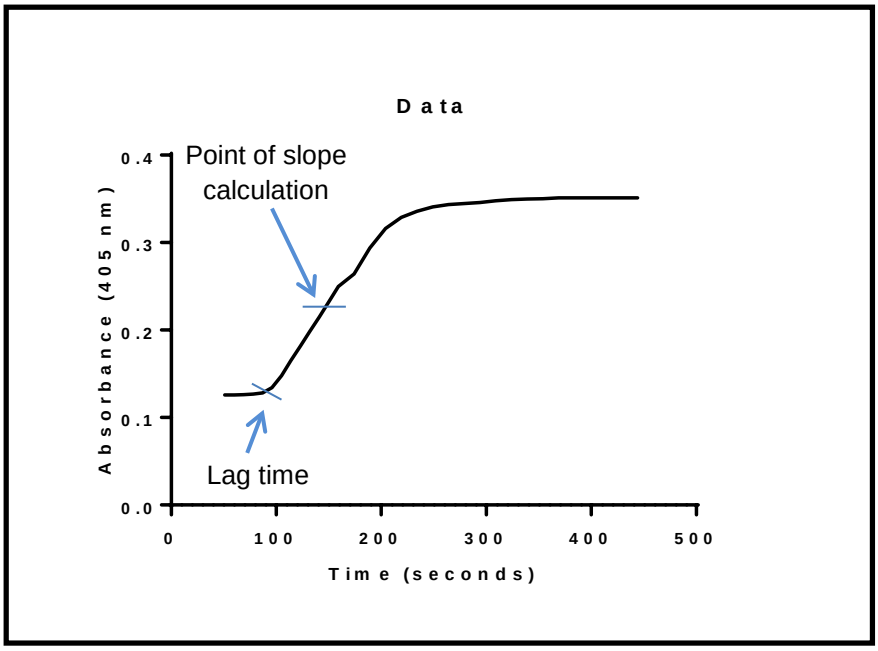

Figure 1. Positions on curve to be used for determination of slope and lag time

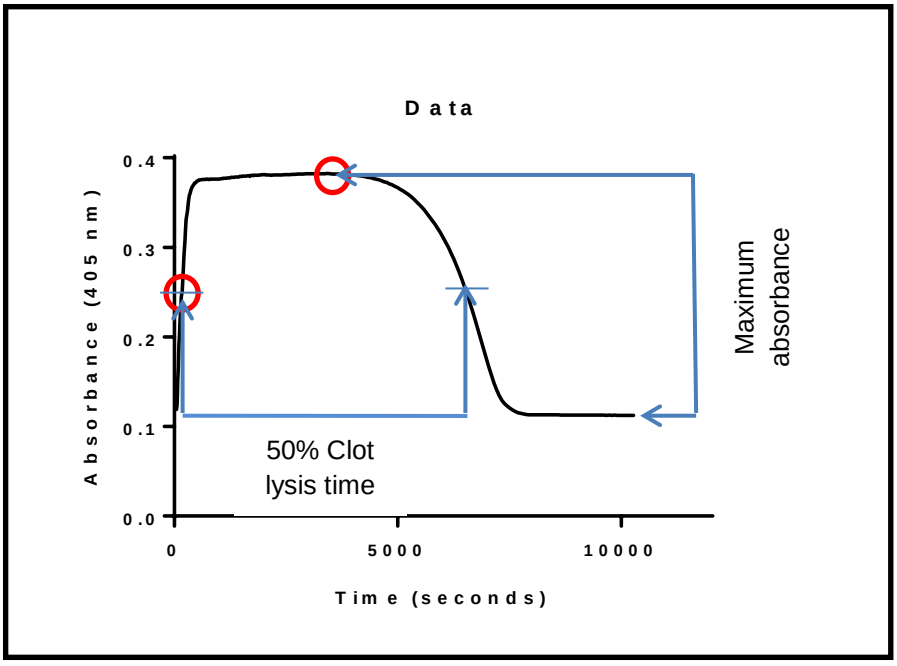

Figure 2. Positions on curve to be used for determination of maximum absorbance and 50% clot lysis time.

From the data the following measurements are obtained.

1. Lag time – time in seconds taken from the start to when the absorbance increases 0.015 from baseline.
2. Slope - calculated from the slope (Δ absorbance units/min) of the line at the midpoint (----) between initial baseline and maximum absorbance at plateau by making use of the slope function in Excel using the absorbance readings (y) and time points (x) of the 5 measurements below and above the midpoint . The midpoint can be identified through adding ½ maximum absorbance (see point 3 below) to the baseline absorbance or by curve fitting as described below.
3. Maximum absorbance - calculated as the increase in absorbance from baseline to maximum absorbance at plateau. The plateau is often not a perfect horizontal line and the area of the plateau where the line is closest to horizontal should be selected for the calculation.
4. 50% Clot lysis time – calculated as the time in minutes from the midpoint, from clear to maximum turbidity, to the midpoint in the transition from maximum turbidity to the final baseline turbidity. These midpoints are identified by applying two separate sigmoidal curve fittings to the ascending and descending parts of the turbidity curves separately. Software programmes such as Prism or Origin can calculate the midpoints from the fitted curves. The time of the midpoint of the ascending curve is then subtracted from the time of the midpoint of the descending curve to provide the 50% CLT.

Note: remember to include the time required for pipetting of activation mix to plasma in all calculations as the reaction starts immediately.

Supplement 1

Protocol for the determination of the feasibility of developing a standardized combined plasma clot turbidity and lysis assay

Objective

The purpose of this project is to provide a standardized assay that can be used for the determination of plasma clot turbidity and lysis. Currently many laboratories perform turbidity and lysis assays, but each laboratory uses its own methodology which is in essence a modification of the same assay. Standardization of these assays will allow better comparison of results between laboratories and will aid in the establishment of healthy normal ranges that can be used to compare with disease states, in particular cardiovascular disease. In order to allow the use of this assay in studies with large sample sizes, it was decided to standardize the method for use in 96-well plates.

Development of proposed protocol

The proposed standardized protocol was developed based on in-house turbidity and clot lysis protocols obtained from different laboratories world-wide. Although both turbidity and clot lysis assays are turbidimetric by design, turbidity assays are designed to visualize the clot formation process while clot lysis protocols focus on the lysis of formed clots. This is achieved by varying assay conditions to allow the visualization of the area of interest on the turbidimetric curve. From these curves specific variables (as detailed below) are calculated to provide information on clot formation, structure and lysis. The clotting agent of choice for turbidity assays is thrombin as it is the direct activator of fibrinogen and the purpose of the turbidity assay is to characterize the activation of fibrinogen and the consequent formation of the fibrin network. Turbidity assays are typically performed without the addition of a lytic agent. The combination of the two techniques will allow better characterization of the fibrin clot as information on clot lysis can now be added and directly related to the clot properties obtained from turbidity. Although thrombin is used as clotting agent, endogenous thrombin generation might be important, particularly for clot lysis because of the activation of thrombin activatable fibrinolysis inhibitor (TAFI). To eliminate a varying amount of phospholipids in different plasma samples an excess of external phospholipids is added.

The assay conditions of the available turbidity and clot lysis protocols were combined and refined until the optimal conditions that allowed clear visualization of all parts of the turbidimetric curve, were achieved / selected. These are now presented as the protocol to be used in the standardization project, which includes the following experiments.

Experimental layout

As these assays are often performed in laboratories also performing other coagulation tests, the reagents required for the proposed protocol would typically be available in-house. Consequently we do not stipulate the use of a specific brand name but rather specify the specific activity of the reagents to be used, to allow the use of readily available reagents but with standardized functionality. For determination of the tissue plasminogen activator (tPA) concentration to be used, it was decided to standardize the clot lysis times (CLT), rather than use a defined tPA concentration. Differences in the specific activity of commercially available tPA preparations, as well as batch differences within the same preparation would make a concentration approach unfeasible. A CLT of 100 ± 5 minutes (min) was selected as the reference point as CLTs of 60-100 min are sensitive to many activators and inhibitors in the coagulation and fibrinolytic pathways and 100 min demonstrated the highest repeatability. The assay procedure below will be performed in three phases:

Phase 1

Perform the plasma clot turbidity and fibrinolysis assays using lyophilized NIBSC plasma and different tPA concentrations until a CLT of 100 ± 5 min is obtained. Calculation of CLT is described in Figure 1. For this phase, the full 96 well plate need not be used. It is recommended that different tPA concentrations are tested in 12 wells each and therefore five (5) different tPA concentrations can be tested in one plate (the outer wells of the plates are not to be used to avoid possible temperature differences between these and the inner rows). It is suggested to start the experiments with tPA concentrations in the range of 20-40 ng/mL.

Phase 2

Once the correct tPA concentration has been determined (i.e. a concentration that will achieve a CLT of 100 ± 5 min using the NIBSC lyophilized plasma), perform 3 repeats of

the assay using the lyophilized NIBSC plasma. One run = one 96-well plate. The assay will be performed both with and without the addition of tPA, as described below. Fill rows B, D, and F of the plate with plasma-buffer mix without tPA and rows C, E, G with plasma-buffer mix containing tPA.

Phase 3

Use the same tPA concentration and perform 3 repeats of the assay using local pooled plasma, commercial or prepared in house from normal healthy volunteers. One run = one 96-well plate. The assay will be performed both with and without the addition of tPA. Fill rows B, D, and F of the plate with plasma-buffer mix without tPA and rows C, E, G with plasma-buffer mix containing tPA.

Collection of local pool plasma

If using an in house preparation of normal pool, the following guidelines are suggested. Please disregard if using a commercial preparation of normal pool plasma.

Collect 3.2 % citrated plasma from at least n=8 fasting healthy individuals before 11:00. Exclusion criteria are pregnancy, use of oral contraceptives, use of acute or chronic medication, elevated temperature or known blood disorders. Blood samples should be kept at room temperature after collection for no longer than 30 min and then centrifuged at 2000g for 20 min at 15 °C to prepare platelet poor plasma. Plasma of all individuals should be pooled, mixed and aliquoted before freezing at -65°C or below.

Equipment

96-well plate reader with a 405 nm filter and capacity to read at 37 °C with a plate shaker on board.

Materials & Reagents provided

1. Lyophilized NIBSC plasma (18 vials of 1 mL)

2. 1 x 3 mL vial Phospholipids, (Rossix, Cat no: PL604T, 0.5 mmol/L)

Storage of reagents provided

1. Lyophilized plasma will be shipped at room temperature to the address provided and should be stored at 2-8 °C.

2. Phospholipids will be shipped on ice and should be stored at 2-8 °C.

Additional Materials & Reagents required

6. Human α-thrombin
7. $CaCl_2$
8. 20 mL frozen 3.2 % citrated pool plasma obtained from healthy individuals or a commercial preparation
9. tPA
10. 10 x 96-well untreated polystyrene plates
7. Buffer = 50 mM Tris; 140 mM NaCl; 1mg/mL BSA pH 7.4

Reconstitution of reagents

1. Lyophilized NIBSC plasma: Allow plasma to reach room temperature and then reconstitute with 1000 µL MilliQ water. Mix by gently swirling vial and allow standing at room temperature for 15 min before use.

2. Frozen pool plasma locally collected: Thaw plasma at 37 °C for at least 5 min.

3. Phospholipids: Keep undiluted stock at 2-8ºC. On day of experiment, dilute stock 1:9 to 0.05 mM with assay buffer.

4. Human α-thrombin: Prepare stock of 12.5 NIH U/mL with assay buffer, aliquot and store frozen. Thaw once only and keep on ice until addition to activation mix, to prevent inactivation. Use within 30 minutes.

5. $CaCl_2$: Prepare stock of 250 mM with MilliQ water

6. tPA: Reconstitute according to package insert and dilute with assay buffer to obtain a final concentration in the clot of 20-40 ng/mL. (Preliminary experiments have indicated

this to be the tPA concentration range likely to give lysis times of 100 ± 5 min). An additional dilution step may be required depending on the final tPA concentration selected. Aliquot and store frozen. Thaw once only. Lysis time is defined as the time from the midpoint, from clear to maximum turbidity, which is representative of clot formation, to the midpoint in the transition from maximum turbidity to the final baseline turbidity, which represents the lysis of the clot (as depicted in Figure 1.).

Procedure

8. Start the micro plate reader and set the temperature at 37°C.
9. Fill the outer wells with water.
10. Prepare the plasma-buffer mix with and without tPA as well as the Activation Mix (see below). Prepare the Activation Mix before plasma is transferred to 96-well plate and add immediately afterwards to minimize incubation time of tPA with plasma.

Plasma-buffer Mix without tPA

TO BE PIPETTED INTO ROWS B, D AND F OF THE 96-WELL PLATE

Stock solution	Volume for 3800 µL Plasma-buffer mix (µL)	Concentration in Plasma-buffer Mixture	Final concentration in clot
Phospholipids	950	12.5 µM	10 µM
Plasma	1425	-	30%
Buffer	1425	-	-

Plasma-buffer Mix with tPA

TO BE PIPETTED INTO ROWS C, E, AND G OF THE 96-WELL PLATE

Stock solution	Volume for 3800 µL Plasma-buffer mix	Concentration in Plasma-buffer	Final concentration in clot

	(µL)	Mixture	
Phospholipids	950	12.5 µM	10 µM
Plasma	1425	-	30%
Buffer	95	-	-
tPA*	1330		

***Add the tPA to the plasma-buffer mix just before (<60 seconds) it is transferred to the 96-well plate – use a multichannel pipette for the transfer to decrease pipetting time.**

Activation Mix – keep on ice for no longer than 30 min

Stock solution	Volume for 4 000 µL Activation mix (µL)	Concentration in Activation Mixture	Final concentration in clot
Thrombin	800	2.5 NIH U/mL	0.5 NIH U/mL
$CaCl_2$	1 200	75 mM	15 mM
Buffer	2 000		

11. Add 80 µL of the two Plasma-Buffer Mixes to the wells of the micro plate (untreated) – do not use the outer wells for samples. Add the Plasma-Buffer Mix without tPA to the wells of rows B, D and F and the Plasma-Buffer Mix with tPA to the wells of rows C, E and G of the micro plate. *When using this assay in future for individual patient samples, perform assay in duplicate (i.e. 4 wells per individual). Mix 180 µL plasma and 120 µL phospholipids and pipette 50 µL thereof into each of the 4 wells. Then mix buffer and tPA, or for samples without tPA, use buffer only (enough for all the*

samples on the plate), and pipette 30µL into the wells using a multichannel, to prevent long incubation times of tPA with plasma.

12. Check if the plate reader has reached 37°C. (**note!!!** The next steps have to be performed quickly because the reaction starts immediately when the activation mixture is added to plasma).
13. Add 20 µL activation mix to each well with a 6-well multi-channel (start at column 2 and work towards column 11). Note time required for pipetting of activation mix to plate until measurements are started to include in time point calculations as detailed below. The time required for pipetting of the activation mix, should be less than the lag time to ensure that the lag time of the first sample is visible on the turbidity curves.
14. Place the plate into the plate reader and start with a 2 sec shake step at 900 rpm before taking the first reading. Start measuring the absorbance at 405 nm every 10 seconds up to 30 min (180 readings) and then every minute until the clots have broken down or up to 180 minutes (n=150 readings).

Example of plate layout and numbering

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H												

Fill with water

Fill with plasma-buffer mix without tPA

Fill with plasma-buffer mix with tPA

Calculate lag time, slope, maximum absorbance and 50% clot lysis time from graphs using a software program such as Origin, Prism or Excel as indicated in Figure 1 and 2 below. A Boltzmann sigmoidal curve fitting should be used.

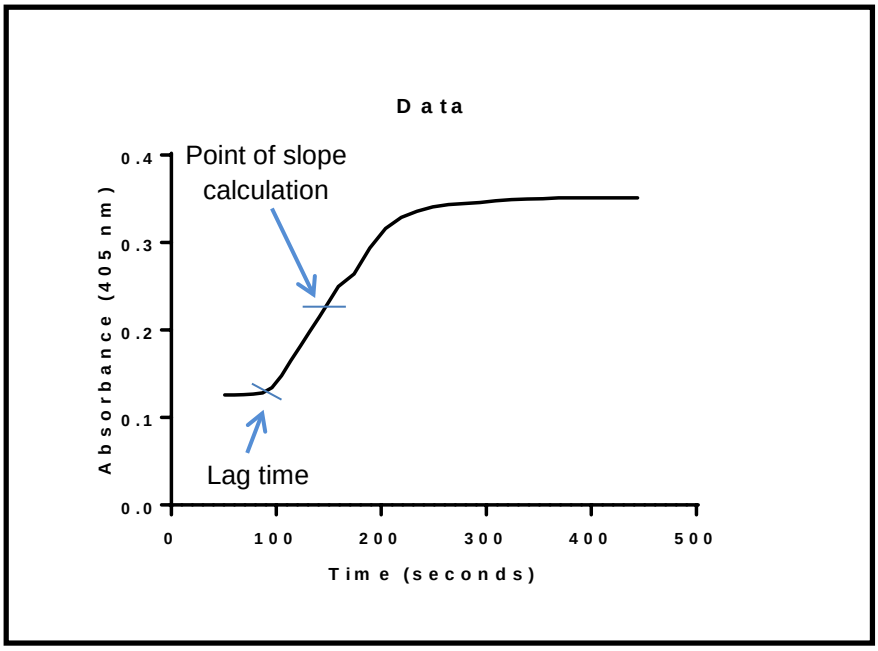

Figure 1. Positions on curve to be used for determination of slope and lag time

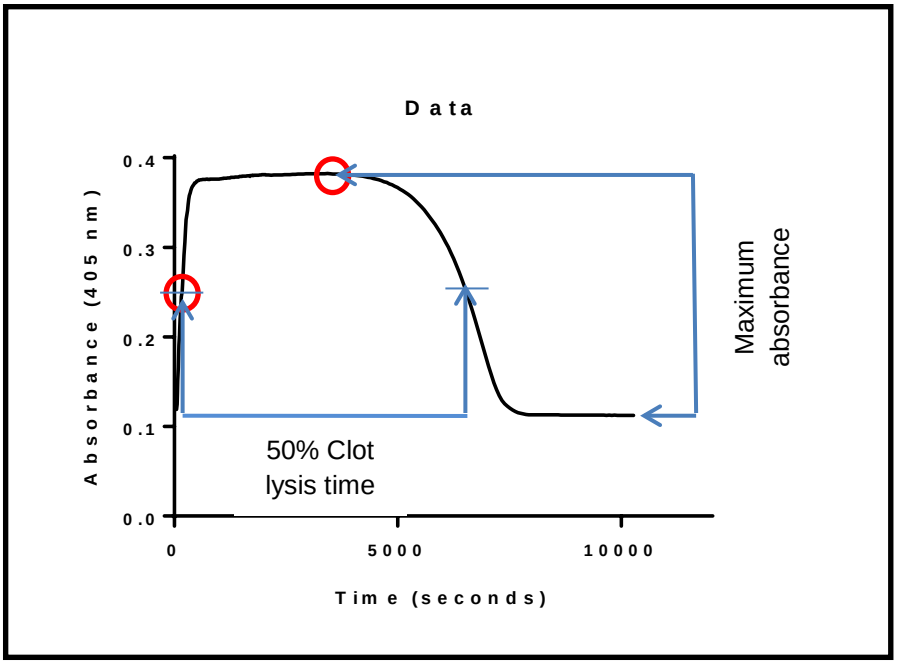

Figure 2. Positions on curve to be used for determination of maximum absorbance and 50% clot lysis time.

From the data the following measurements are obtained.

4. Lag time – time in seconds taken from the start to when the absorbance increases 0.015 from baseline.
5. Slope - calculated from the slope (Δ absorbance units/min) of the line at the midpoint (----) between initial baseline and maximum absorbance at plateau by making use of the slope function in Excel using the absorbance readings (y) and time points (x) of the 5 measurements below and above the midpoint . The midpoint can be identified through adding ½ maximum absorbance (see point 3 below) to the baseline absorbance or by curve fitting as described below.
6. Maximum absorbance - calculated as the increase in absorbance from baseline to maximum absorbance at plateau. The plateau is often not a perfect horizontal line and the area of the plateau where the line is closest to horizontal should be selected for the calculation.
4. 50% Clot lysis time – calculated as the time in minutes from the midpoint, from clear to maximum turbidity, to the midpoint in the transition from maximum turbidity to the final baseline turbidity. These midpoints are identified by applying two separate sigmoidal curve fittings to the ascending and descending parts of the turbidity curves separately. Software programmes such as Prism or Origin can calculate the midpoints from the fitted curves. The time of the midpoint of the ascending curve is then subtracted from the time of the midpoint of the descending curve to provide the 50% CLT.

Note: remember to include the time required for pipetting of activation mix to plasma in all calculations as the reaction starts immediately.