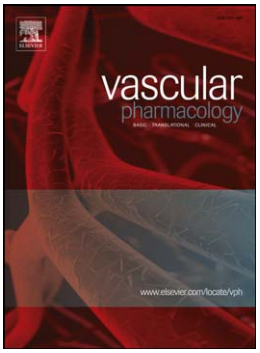
Development and validation of a new assay for assessing clot integrity

Ranjit, Praveen; Lau, Yee; Lip, G.y.h.; Blann, A.d.

DOI:
10.1016/j.vph.2015.02.013



Development and validation of a new assay for assessing clot integrity

P. Ranjit, Y. Lau, G.Y.H. Lip, A.D. Blann

PII: S1537-1891(15)00058-0
DOI: doi: 10.1016/j.vph.2015.02.013
Reference: VPH 6162

To appear in: *Vascular Pharmacology*

Received date: 8 October 2014
Revised date: 29 January 2015
Accepted date: 20 February 2015

Please cite this article as: Ranjit, P., Lau, Y., Lip, G.Y.H., Blann, A.D., Development and validation of a new assay for assessing clot integrity, *Vascular Pharmacology* (2015), doi: 10.1016/j.vph.2015.02.013

VPH-D-14-00185R1

Development and validation of a new assay for assessing clot integrity

P Ranjit*, Y Lau*, GYH Lip, AD Blann

University of Birmingham Centre for Cardiovascular Sciences

Department of Medicine, City Hospital, Dudley Road, Birmingham B18 7QH, UK

CHEMICAL COMPOUNDS

Thrombin

Tissue plasminogen activator

Kaolin

Correspondent: Dr AD Blann PhD FRCPath

Tel/fax 00 44 121 507 5076

a.blann@bham.ac.uk

Key words thrombelastograph, thrombosis, fibrinolysis, haemostasis

Disclosures The authors have no financial disclosures.
There was no specific funding source.

*Drs Ranjit and Lau contributed equally to the project

Abstract

Introduction: Research and routine laboratory assessment of clot integrity can be time consuming, expensive, and cannot be batched as it is generally performed in real time. To address these issues, we developed and validated a micro-titre based assay to quantify thrombogenesis and fibrinolysis, the purpose being to assess patients at risk of cardiovascular events by virtue of hypercoagulability. In further validation, thrombogenesis results were compared to similar indices from the thrombelastograph (TEG).

Methods: Our assay determines three indices of thrombogenesis (lag time to the start of thrombus formation (LT), rate of clot formation (RCF), and maximum clot density (MCD)) and two of fibrinolysis (rate of clot dissolution (RCD) and time for 50% of the clot to lyse (T50). Plasma was tested fresh and again after being frozen at -70^{o}C. Some samples were tested immediately, others after being left at room temperature for up to 24 hours.

Results: The intra-assay coefficients of variation (CVs) of the three thrombogenesis measures (LT, RCF, MCD) and two fibrinolysis measures (RCD, T50) varied between 2.7-12.0% in fresh plasma and between 1.3%-10.8% in frozen plasma respectively. Similarly, the inter-assay coefficients of variation of the thrombogenesis and fibrinolysis measures were 4.9-10.8% in fresh plasma and 2.2-6.5% in frozen plasma respectively. TEG assays intra- and inter assay CVs were around 25%. There were no significant differences in all plate assay indices up to 6 hours at room temperature. Certain plate assay thrombogenesis data were comparable to TEG indices after analysis by Pearson's correlation. The reagent processing cost per sample is £15 for TEG and £2 for the plate assays.

Conclusion: Our micro-titre based assay assessing plasma thrombogenesis and fibrinolysis has good intra- and inter-assay CVs, can assess plasma up to 6 hours after venepuncture, is more efficient (in terms of throughput) and is more economical than that of the TEG.

Introduction

Correct haemostasis functioning (the balance between thrombosis and fibrinolysis) is an essential physiological process. Increased thrombosis and/or impaired fibrinolysis leads to life-threatening conditions such as ischaemic stroke, myocardial infarction and pulmonary embolism. Conversely, failure of thrombosis (perhaps to due over-anticoagulation) and/or excessive fibrinolysis leads to life threatening haemorrhage, such as of the gastro-intestinal tract and in causing haemorrhagic stroke (1,2). The need for information regarding the potential occurrence of these events, their treatment with anti-coagulants, in stable thrombotic disease such as coronary artery disease and atrial fibrillation, in critical conditions such as disseminated intravascular coagulopathy and trauma, and in investigating cardiovascular pharmacology, such as the effects of anti-coagulants, call for reliable laboratory tests of haemostasis (3,4).

The thrombelastograph (TEG) is an established laboratory tool for the investigation and management of haemostasis, simultaneous delivering numerous indices on various aspects of clot formation and fibrinolysis, a selection of which are shown in table 1 (5,6). Despite its flexibility in being able to assess haemostasis in trauma, haemorrhage and the effects of anticoagulation and fibrinolysis (7-9), it has several disadvantages. These include the requirement of whole blood to be assessed in real time, a maximum of two samples to be assessed per analyser at the same time, relatively high coefficients of variation, poorly standardized methodologies, quality control/assurance issues, and limitations on the stability of whole blood samples (10-12). These issues also mean it is a poor choice of assay in clinical research and drug development. Although used most commonly with whole blood, some of these problems may be addressed by using plasma, and the use of frozen plasma allows non-urgent assessments to be batched, possibly to be processed out of hours.

However, slow and limited throughput of samples, and other problems leave room for alternative technologies.

The insoluble nature of the fibrin clot, and so the interruption of beam of light whilst it forms, it an established feature of the laboratory assessment of thrombogenesis (13-16). Based on these principles, we set out to develop and validate a micro-method for a high-throughput assay that can assess thrombogenesis and fibrinolysis in citrated plasma, and that may be useful in clinical practice and research. We determined that the most convenient and efficient platform for this would be a standard 96-well microtitre plate, and we compared our method with parallel data obtained from an established haemostasis device, i.e. the TEG.

Subjects, Materials and Methods

Subjects

Following local research ethics committee approval and written informed consent, whole blood was obtained from 19 healthy (i.e. not taking prescription medications) volunteers (mean age 35, standard deviation 8, range 26 – 52 years old, 12 males) into citrated vacutainers. A portion of whole blood was processed on the TEG. Plasma was obtained following centrifugation of the latter for 20 minutes at 3000 rpm: an aliquot of the fresh plasma was processed promptly while some plasma was frozen immediately and stored at -70^{o}C. Prior to assay, frozen plasma was thawed in a 37^{o}C water bath.

General principles of the assays

When plasma is exposed to thrombin, the polymerisation of fibrin can be monitored by measuring the amount of light passing through the solution. As fibrinogen is converted to protofibril monomers and fibrin, this solution will become more turbid and thus cause more

scattering and absorption of light (16). With subsequent addition of exogenous tissue plasminogen activator (tPA), the solution becomes less turbid due to dissolution of fibrin threads by plasmin, so that light passage is restored. We adopted and amended this process, developing a technique for concurrently assessing both thrombogenesis and fibrinolysis, as these share common features.

The plate assay for thrombogenesis

The method for the thrombogenesis assay calls for 25 µL of plasma to be added to the well of a standard ELISA-quality 96-well microtitre plate (R&D Systems Europe Ltd, Abingdon. UK), followed by 75 µL of a TRIS-NaCl buffer (1.51 g Tris-HCl, 1.75 g NaCl, 200 mL distilled water). Coagulation is initiated by the addition of 50 µL of a thrombin/calcium solution. For a batch of 20 patient samples in triplicate (total of sixty samples), this reagent will consists of 20 µL of a solution of 1000 IU thrombin [Sigma catalogue number T9549] reconstituted in 4 ml phosphate buffered saline/0.1% bovine serum albumin (BSA), 500 µL of 500 mM calcium chloride in the Tris/NaCl buffer, and 3 ml of the Tris/NaCl buffer (all reagents Sigma-Aldrich, Gillingham, Kent). Bulk addition of buffers to multiple wells of the micro-titre plate is facilitated by an 8-channel micro-pipette. The thrombin/calcium solution can be bulk reconstituted and stored frozen at -70^{o}C in aliquots.

The plate is immediately loaded into a Tecan Sunrise (Tecan Group Ltd, Männedorf, Switzerland) plate reader at 37^{o}C programmed to measure the optical density (OD) at 340 nm every six seconds (with an intermediate two-second shaking period) for 30 minutes. The raw OD data at each time point can be printed out as an excel file and as a graphic. A typical graphical print-out is presented in Figure 1, and shows change in OD over time as the fibrin clot forms. From these, three key indices can be obtained: (a) the lag time (LT), which is the time in seconds from the initial measurement (addition of the calcium/thrombin) to the start

of the exponential part of the thrombogenesis curve, (b) the rate of clot formation (RCF), calculated as the change in OD unit/second, being [The OD at the end of the exponential phase minus the OD at the start of the exponential phase]/[The time in seconds between these two points], and (c) the maximum increase in OD taken at 30 minutes, which give us the maximum clot density (MCD).

The plate assay for fibrinolysis

A strength of our method is that many of the features of the thrombogenesis assay are part of the fibrinolysis assay. In the fibrinolysis assay, 75 µL of plasma is added to the well of a microtitre plate. To this is added 75 µL of a Tris/NaCl/calcium buffer supplemented with thrombin and tPA, to be made up fresh per assay run. For a batch of 20 patient samples in triplicate (therefore sixty wells), this buffer will consist of 9 ml Tris/NaCl buffer, 400 µL calcium chloride solution, 20 µL thrombin (as above) and 800 µL tPA (Technoclone TC41072: stock solution of 100 µg tPA reconstituted in 400µL of 1 molar potassium bicarbonate/1% BSA (both Sigma). The tPA solution can be bulk reconstituted and stored frozen at -70^{o}c in aliquots.

The plate is immediately loaded into a Tecan Sunrise plate reader as for the thrombogenesis assay, and data collected for 30 minutes. Again, the raw data of the OD at each time point can be printed out as an excel file and as a graphic. A typical graphical print-out is presented in Figure 2, and shows change in OD over time as the fibrin clot is initially formed and then lysed. The data is post-processed to plot into line charts, and from these the rate of clot dissolution (RCD), being the slope of the right hand portion of the graph, and the time for 50% clot lysis (T50) can be determined (as demonstrated in Figure 2).

As the parameters and settings for the plate reader are identical for each assay, they can be performed in parallel on the same plate. However, should it be necessary, each assay can be

run independently. The duration of time required for both plate assays is dependent on the thrombogenesis and fibrinolysis potential of the samples, and thus processing time may be variable. However, for most samples, results may be obtained within 20 minutes, at which point the assay can be terminated. Our data shows results at 30 minutes, as this is one of the TEG endpoints.

The TEG

Manufacturer's instructions were followed. Briefly, 340 µL of citrated whole blood or plasma is added to a reaction cuvette, to which is added 20 µL of 0.1 M calcium chloride solution and kaolin (Haemonetics, Watford, UK). The reaction proceeds immediately and is monitored in real time by the analyser with results fed directly to a microcomputer. A modified TEG graphical printout is presented as Figure 3, and shows the formation of clot, the increasing physical strength of the developing clot on the vertical axis over time, and finally clot autolysis. Together with the graphical printout, numerous TEG indices are generated, although we have focussed on the R, K, Angle, MA and LY30 indices as these are in common with our plate assay indices (table 2). Note the similarity of the TEG printout (Figure 3) with that of the thrombogenesis assay (Figure 1).

Comparing TEG vs Plate assay

By comparing the results from TEG and thrombogenesis assay, several equivalent data can be observed. Firstly, the Reaction time (R-time) in TEG and the Lag time (LT) in thrombogenesis assay both describes the time taken for clot formation to commence, either through assessment of tensile strength in TEG or through the changes in turbidity in thrombogenesis assay. Second, the α-angle in TEG is markedly similar to rate of clot formation (RCF) in thrombogenesis assay, as both describe the rate of clot growth as clot fibres polymerise to form stronger and denser fibres. Finally, maximum amplitude (MA) in

TEG and maximum clot density (MCD) in thrombogenesis assay represent the final formation of the mature clot. As the TEG reports autolysis, whereas in our plate assay, lysis is by exogenously added tPA, we did not feel the two assays to be comparable. Indeed, it can be noted that autothrombolysis is both slower and less marked (figure 3) compared to fibrinolysis by added tPA in the plate assay (figure 2).

Validation

The intra- and inter-assay coefficients (CV) of the plate assay indices were determined by testing 5 samples of fresh and frozen plasma 5 times each. The same plasma (and matched whole blood) was used to generate CVs on the TEG. To determine the effect of time-delay on clotting, whole anticoagulated blood was obtained from 5 individuals, and plasma collected after set periods of time had elapsed (T=0, T +3 hours, T+6 hours, T+12 hours and T+24 hours) at room temperature.

Statistics

Data are presented as mean and standard deviation (when data normally distributed) or median and inter-quartile range (non-normally distributed). Data were correlated according to Pearson's or Spearman's method, dependent on distribution. Data at different time points was analysed by repeated measures analysis of variance, and overall by linear trend. All analyses were performed on Minitab release 16 and $p<0.05$ was taken to assume significance.

Results

Reproducibility

The intra-assay and inter-assay CVs for the microplate assays are shown in Table 2, those of the TEG assay indices in Table 3. Note that there is no data for the LY30 in the TEG assay data. This is because there was no change in the MA at 30 minutes – i.e. no clot lysis had occurred. We speculate therefore that the freezing and thawing of plasma and the addition of the kaolin/calcium chloride essentially destroys autolytic potential. This may possibly be of the plasma's own tPA, because exogenous tPA was able to lyse the clot in the plate assay.

The median (interquartile range, IQR) CV of the 20 indices from the plate assays (table 2) is 5.1 (2.7-8.7)%. As a bio-assay, which generally have large CVs (see TEG data), we regard these data as very good. For the plasma CVs in the TEG assay in table 3 the data is 21.1 (8.6-27.2)% ($p<0.001$ to the plate assay CVs). The data for CVs for the whole blood is 17.4 (9.8-43.6)% ($p=0.615$ to CVs of the TEG plasma assays), demonstrating significant variability within and between analyses, regardless of whole blood, fresh plasma or frozen plasma. As the CVs for the thrombogenesis aspect of plate assay are markedly smaller (by a factor of at least 4) than that of the TEG, we conclude that plate assay is superior to TEG in assessing various aspects of clot formation and stability. The fresh plasma and frozen plasma TEG indices are of equal reproducibility (median CVs 21.5% and 21.3% respectively).

Time between venepuncture and processing

The effect of time on the haemostasis indices is shown in Table 4 for the plate assays and Table 5 for the TEG. The plate assay (Table 4) demonstrated that processing samples collected over time results in a progressive shortening of the lag time (LT), a slower rate of clot formation (RCF) but increased maximum clot (MCD). Resultant clot formed over time was also demonstrated to be more resistant to lysis by tPA, shown by increased T50% and reduced rate of clot dissolution (RCD). This relationship existed for both fresh and frozen plasma in plate assay (all indices, $p< 0.001$). Although the results of the plasma samples were

statistically not diferent 12 hours after preparation, there is a clear change from the six hour to the 12 hour samples. We therefore recommend that samples are processed not longer than six hours after preparation.

Parallel TEG data are shown in Table 5. Broadly speaking, most samples were stable to 24 hours after preparation. The only index that did show a statistical change was the α-angle, assessing the rate of thrombogenesis, which showed that samples clotted more rapidly at 3 and 24 hours after preparation (both p=0.019). As there is no clear physiological mechanism for these variations, we suggest this may simply reflect assay variability (as demonstrated by the high CVs). There is also a trend towards reduction of R-time, but this change is not statistically significant.

The lag time, rate of clot formation and maximum clot density measurements in the thrombogenesis plate assay are in essence similar measures of coagulation in respects to indices of the TEG, namely K-time, angle and maximum amplitude. Pearson's correlation coefficients of paired indices are shown in table 6. These indicate a significant correlation between MA and MCD (p=0.008), none between the R-time and the LT, or between the α-angle and the RCF.

Economic Evaluation

Excluding the cost of purchase or rental of microplate reader or TEG analyser, there is a significant difference in cost of processing samples. Taking catalogue costs for reagents, the cost of processing a sample on the TEG is £14.33 versus £1.65 by plate assays when samples are processed in triplicates. By processing huge numbers of samples, the cost per sample in plate assays will be expected to fall, due to the initial start-up cost of bulk purchase of laboratory chemicals. For 5000 samples, cost of each sample processed will remain at £15 for TEG but fall to under £0.35 for plate assays. However, the plate assay demands much more

operator input in preparing reagents, developing the assay and in calculating the results. In this respect the semi-automatic TEG, with minimal operator time, is markedly more attractive

Discussion

The ability to reliably, rapidly and accurately assess haemostasis is an important part of clinical and laboratory medicine and vascular pharmacology, and within this process, the value of determining thrombotic and fibrinolytic potential is becoming recognised (1-4). One of the most useful tools in this respect is the TEG, although it has several disadvantages (5-12), some of which have led to the development of alternative methods (13-16).

The TEG technology is well-established, and has been in use for decades in clinical setting as a "point-of-care" assessment of coagulation potential in critically ill patients and those who have experienced poly-trauma (17,18). Nevertheless, in some circumstances, such as haemophilia and in point of care testing, other methods may be better (19,20). Indeed, as shown in this paper, the large CVs in the TEG data have a significant impact on the reproducibility of the results. The large CVs, the fairly long processing time, the limited number of channels per analyser and the cost of the assay are a significant drawback of this method. Hence, in comparing the TEG with our thrombogenesis and fibrinolysis plate assays, the latter have lower CVs, faster running speed and cheaper processing cost, so may potentially be a viable alternative to TEG. The plate assays also allow for batch analysis after prior freezing and still provides for reliable results, but a drawback is the need for manual post-processing to obtain the results.

The two systems do not draw exact comparison. Correlation analysis shows that despite the similarities in certain components of both the TEG and plate assays, such as the relationship

between maximum amplitude (reflecting the tensile strength of developed clot) and maximum clot density (reflecting the thickness of fibrin clot monomers), both methods are not identical, but may be complementary. The TEG measures the haemostasis of whole blood or plasma (to which is added kaolin) through coagulation kinetics, clot tensile strength and subsequent clot retraction and autolysis. These contrast with the thrombogenesis and fibrinolysis plate assay, which provides data affecting coagulation, such as direct measurement of the rate of fibrin clot formation and polymerisation, together with the fibrin clot monomer thickness, and a further test of fibrin clot lysis assisted by added tPA. It remains to be seen whether or not the plate assays offer a more useful assessment of haemostasis than the TEG in a clinical setting. A limitation of our data is that it is based on samples from healthy subjects much younger than those whose haemostasis is likely to be assessed in a clinical setting.

Conclusion

We present a microplate assay for measuring thrombogenesis and fibrinolysis which has significantly better intra-assay and inter-assay CVs than TEG technology. Furthermore, results are stable for up to 6 hours from venepuncture, and the plate assay is cheaper and faster to operate. Table 7 summarises the advantages and disadvantages of the two methods. We believe our new method has potential in both clinical and research (drug development) settings where the quantifiable effects of anticoagulants are important considerations.

Acknowledgements

We thank Haemonetics Ltd for help with the TEG.

References

1. Versteeg HH, Heemskerk JWM, Levi M, Reitsma PH. New fundamentals in hemostasis. Physiol Rev. 2013;93:327-58.
2. Funk DM. Coagulation assays and anticoagulant monitoring. Hematology Am Soc Hematol Educ Program. 2012;2012:460-5.
3. Mann KG. Thrombin generation in hemorrhage control and vascular occlusion. Circulation. 2011;124:225-35.
4. Favaloro EJ, Lippi G. The new oral anticoagulants and the future of haemostasis laboratory testing. Biochem Med 2012;22:329-41.
5. Luddington RJ. Thrombelastography/thromboelastometry. Clin Lab Haematol. 2005;27:81-90.
6. Salooja N, Perry DJ. Thrombelastography. Blood Coagul Fibrinolysis. 2001;12:327-37.
7. Traverso CI, Arcelus JI, Caprini JA. Role of thromboelastography in evaluating other anticoagulant agents: warfarin and heparin-dihydroergotamine. Semin Thromb Hemost. 1995;21 Suppl 4:59-62.
8. Summaria L. Thromboelastographic study of fibrinolytic agents. Semin Thromb Hemost. 1995;21 Suppl 4:63-71.
9. Johansson PI, Stissing T, Bochsen L, Ostrowski SR. Thrombelastography and tromboelastometry in assessing coagulopathy in trauma. Scand J Trauma Resusc Emerg Med. 2009 Sep 23;17:45. doi: 10.1186/1757-7241-17-45
10. Chen A, Teruya J. Global hemostasis testing thromboelastography: old technology, new applications. Clin Lab Med. 2009;29:391-407.
11. Chitlur M, Lusher JM. Standardization of thromboelastography: values and challenges. Semin Thromb Hemost. 2010;36:707-11.

12. Kitchen DP, Kitchen S, Jennings I, Woods TA, Walker ID. Quality assurance and quality control of thrombelastography and rotational thromboelastometry: the UK NEQAS for blood coagulation experience. Semin Thromb Hemost. 2010;36:757-63.
13. Undas A, Ariëns RAS. Fibrin clot structure and function: a role in the pathophysiology of arterial and venous thromboembolic diseases. Arterioscler Thromb Vasc Biol. 2011; 31: e88-99.
14. Ryan EA, Mockros LF, Weisel JW, Lorand L. Structural origins of fibrin clot rheology. Biophys J. 1999; 77: 2813-26.
15. Weisel JW, Nagaswami C. Computer modeling of fibrin polymerization kinetics correlated with electron microscope and turbidity observations: clot structure and assembly are kinetically controlled. Biophys J. 1992;63:111-28.
16. Carr ME Jr, Hermans J. Size and density of fibrin fibers from turbidity. Macromolecules 1978:11:46-50.
17. Karon BS. Why is everyone so excited about thromboelastrography (TEG)? Clin Chim Acta. 2014;436:143-8.
18. Da Luz LT, Nascimento B, Shankarakutty AK, Rizoli S, Adhikari NKJ. Effect of thromboelastography (TEG®) and rotational thromboelastometry (ROTEM®) on diagnosis of coagulopathy, transfusion guidance and mortality in trauma: descriptive systematic review. Crit Care. 2014;18:518.
19. Bowyer AE, Van Veen JJ, Goodeve AC, Kitchen S, Makris M. Specific and global coagulation assays in the diagnosis of discrepant mild hemophilia A. Haematologica. 2013;98:1980-7.
20. Perry DJ, Fitzmaurice DA, Kitchen S, Mackie IJ, Mallett S. Point-of-care testing in haemostasis. Br J Haematol. 2010;150:501-14.

Table 1: Major TEG indices

Index	Function in ex-vivo haemostasis
R-time	The time from when the sample is put on the TEG until the first sign of clot formation (amplitude of 2 mm) is reached.
K- time	The time from the R or beginning of clot formation to a fixed level of clot firmness (amplitude of 20 mm) is reached.
Angle (α)	The rate of clot growth.
MA (Maximum Amplitude)	Maximum strength or stiffness (maximum shear modulus) of the developed clot. MA measures the strength or elasticity of the clot in mm.
LY30	Measures percent lysis at 30 minutes after MA is reached.

Table 2: Intra- and inter- assay CV's of plate assay indices using fresh plasma or frozen plasma

Index (unit)	Fresh plasma Intra-assay	Fresh plasma Inter-assay	Frozen plasma Intra-assay	Frozen plasma Inter-assay
Thrombogenesis				
Lag time (sec)	2.7	10.3	2.4	6.1
Rate of clot formation (OD unit/sec)	9.0	7.7	5.1	6.5
Maximum clot density (OD unit)	2.6	4.9	1.3	2.2
Fibrinolysis				
Rate of clot dissolution (OD unit/sec)	12.0	10.8	3.5	5.3
T50% (sec)	3.6	9.9	2.8	5.1
Median (IQR)	3.6 (2.6-10.5)	9.9 (6.2-10.5)	2.8 (1.8-4.3)	5.3 (3.6-6.3)

Data are %. CV = coefficient of variation, OD = optical density
IQR = inter-quartile range

Table 3: TEG intra- and inter-assay CVs

Index (unit)	Whole blood		Fresh plasma		Frozen plasma	
	Intra - Inter		Intra - Inter		Intra - Inter	
R (min)	21.8	16.4	19.3	24.5	24.6	29.5
K (min)	18.4	24.0	23.0	23.7	31.0	26.5
Angle (degrees)	10.4	14.3	9.4	10.0	12.4	11.4
MA (mm)	5.5	7.8	12.2	8.6	17.2	17.0
LY30 (%)	102.5	637.9	73.8	94.3	*	*
Median (IQR)	18.4 (8.0-62.2)	16.4 (11.1-330.1)	19.3 (10.8-48.4)	23.7 (9.3-59.4)	20.9 (13.6-29.4)	21.7 (12.8-28.7)

Data are %. *No reliable data obtained
MA = maximum amplitude, IQR = interquartile range
CV = coefficient of variation

Table 4: Effect of time on haemostasis indices of the plate assay

4(a) Fresh plasma

	Thrombogenesis assay			Fibrinolysis assay	
Time point (hours)	**LT (secs)**	**RCF (OD/sec)**	**MCD (OD)**	**RCD (OD/sec)**	**T50 (secs)**
T = 0	540 (92)	11.4 (3.1)	0.59 (0.1)	2.7 (0.9)	132 (19)
T + 3	535 (93)	10.3 (2.4)	0.59 (0.1)	2.6 (0.8)	143 (24)
T + 6	535 (104)	9.5 (2.0)	0.61 (0.1)	2.6 (0.9)	143 (21)
T + 12	449 (83)	7.5 (1.0)	0.62 (0.12)	2.4 (0.8)	173 (17)
T +24	378 (26)*	6.3 (1.2)*	0.67 (0.12)	1.5 (0.6)	207 (22)*
P for linear trend	0.0021	0.0002	0.703	0.0332	0.0048

4 (b) Frozen plasma

	Thrombogenesis assay			Fibrinolysis assay	
Time point (hours)	**LT (secs)**	**RCF (OD/sec)**	**MCD (OD)**	**RCD (OD/sec)**	**T50 (secs)**
T = 0	548 (90)	10.3 (1.6)	0.59 (0.1)	2.7 (0.9)	136 (19)
T + 3	551 (99)	9.3 (0.95)	0.60 (0.1)	2.7 (0.9)	144 (23)
T + 6	533 (102)	8.9 (0.65)	0.62 (0.1)	2.6 (0.8)	146 (17)
T + 12	461 (78)	7.9 (1.3)	0.63 (0.1)*	2.4 (0.8)	177 (17)*
T +24	388 (45)*	5.9 (1.5)*	0.69 (0.1)*	1.5 (0.5)*	215 (23)*
P for linear trend	0.0024	<0.0001	0.216	0.0251	<0.0001

Date are mean (standard deviation). LT = lag time, MCD = maximum clot density, RCD = rate of clot dissolution, RCF = rate of clot formation, T50 = time for 50% of the clot to be lysed, OD = optical density. *p<0.001 compared to baseline.

Table 5: Effect of time on haemostasis indices of TEG

5 (a) Fresh sample

	Thromboelastography (TEG)				
Time point (hours)	**R (minutes)**	**K (minutes)**	**Angle (degree)**	**MA (mm)**	**LY 30 (%)**
T = 0	12.9 (4.6)	3.4 (1.5)	45.3 (9.9)	59.7 (7.3)	2.32 (1.9)
T + 3	12.0 (3.3)	2.7 (0.8)	55.8 (9.0)*	59.7 (7.2)	2.32 (2.5)
T + 6	10.4 (2.4)	3.2 (1.0)	51.3 (8.8)	54.2 (11.5)	3.00 (1.1)
T + 12	10.0 (2.00)	3.2 (1.2)	48.6 (17.7)	56.8 (10.2)	2.98 (1.7)
T +24	9.5 (2.2)*	2.7 (1.1)	55.4 (10.9)*	59.1 (6.1)	1.52 (0.5)
P for linear trend	0.0525	0.624	0.442	0.742	0.816

5 (b) Frozen sample

	Thromboelastography (TEG) †			
Time point (hours)	**R (minutes)**	**K (minutes)**	**Angle (degree)**	**MA (mm)**
T = 0	8.3 (1.7)	1.6 (0.8)	67.1 (8.8)	30.3 (6.5)
T + 3	7.6 (1.6)	1.9 (0.5)	65.9 (7.4)	29.7 (6.6)
T + 6	7.0 (1.3)	2.3 (0.9)	63.3 (8.3)	27.5 (8.0)
T + 12	7.7 (0.8)	1.9 (0.7)	63.7 (8.3)	28.9 (5.9)
T +24	6.8 (1.5)	2.3 (1.4)	70.2 (4.0)	27.2 (4.8)
P for linear trend	0.170	0.285	0.712	0.451

Data are mean (standard deviation). R = Reaction time, K = K time, LY 60 = percent lysis at 60 minutes after MA is reached, MA = Maximum amplitude. *p<0.05 compared to baseline. † No lysis for all frozen samples.

Table 6: Correlation of Major Indices in TEG and Plate Assay

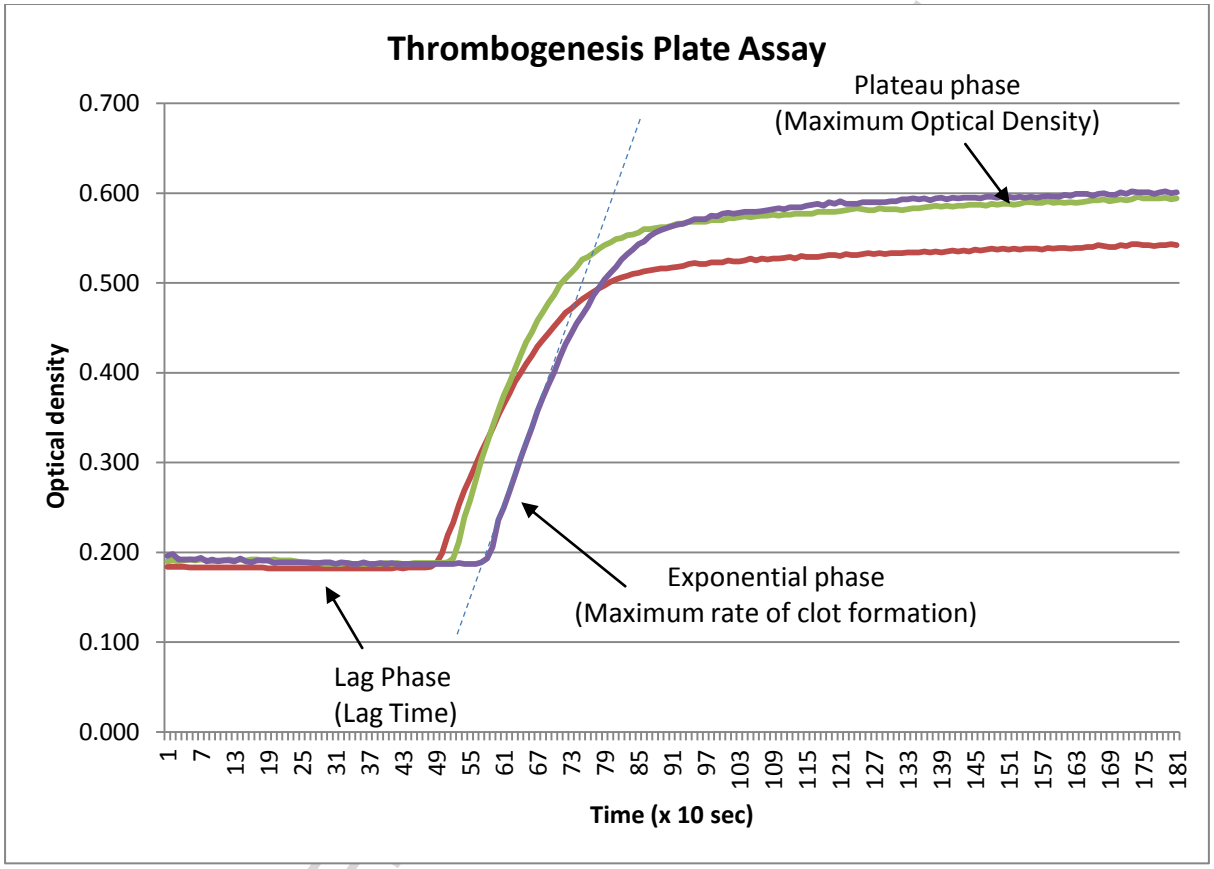
TEG index – Plate index	Correlation coefficient	P value
Reaction time with Lag time	-0.12	0.630
Angle with Rate of clot formation	0.31*	0.190
Maximum amplitude with Maximum clot density	0.59	0.008

Data from analyses of 19 samples of normal plasma. *Spearman correlation coefficient

Table 7: Advantages and disadvantage of the two methods

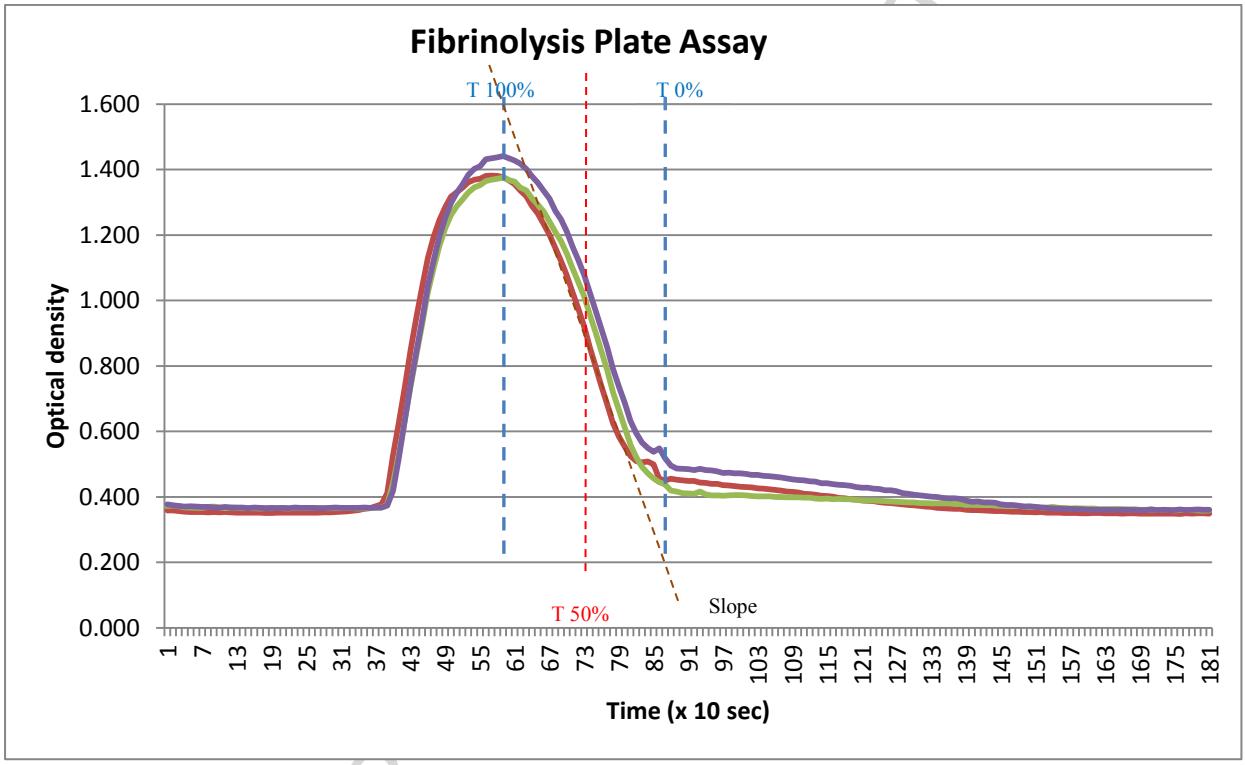
	Plate assay	TEG
Reagent cost	Low	High
Reproducibility	Good (can run in duplicate, triplicate, or more)	Poor (but can run in duplicate)
Operator time	High	Low
Operates on whole blood	No	Yes
Fully operational on frozen plasma	Yes	No
Unit processing	Rapid: Up to 16 patient tests per plate	Slow: Only two channels

Figure 1: The thrombogenesis assay



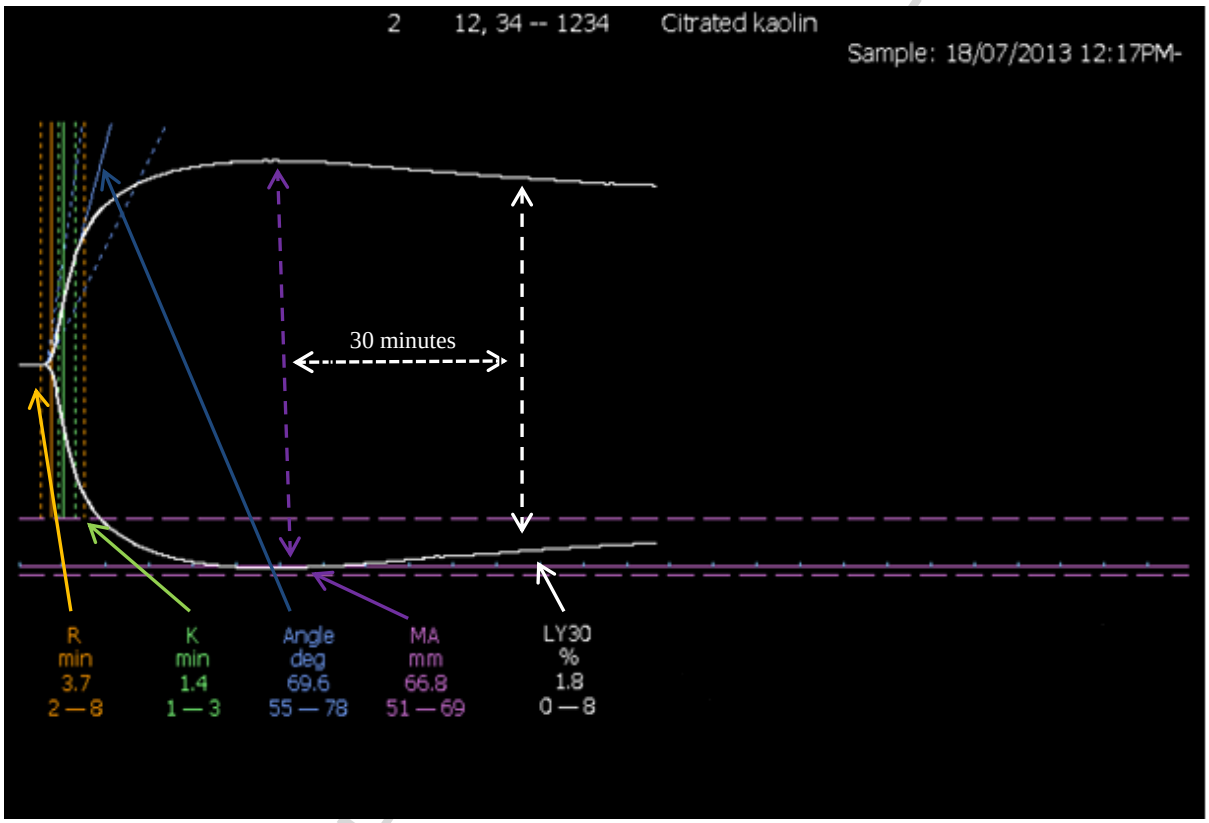
The plot shows changes in optical density as the fibrin clot forms. Triplicate plots are shown.

Figure 2: The fibrinolysis assay



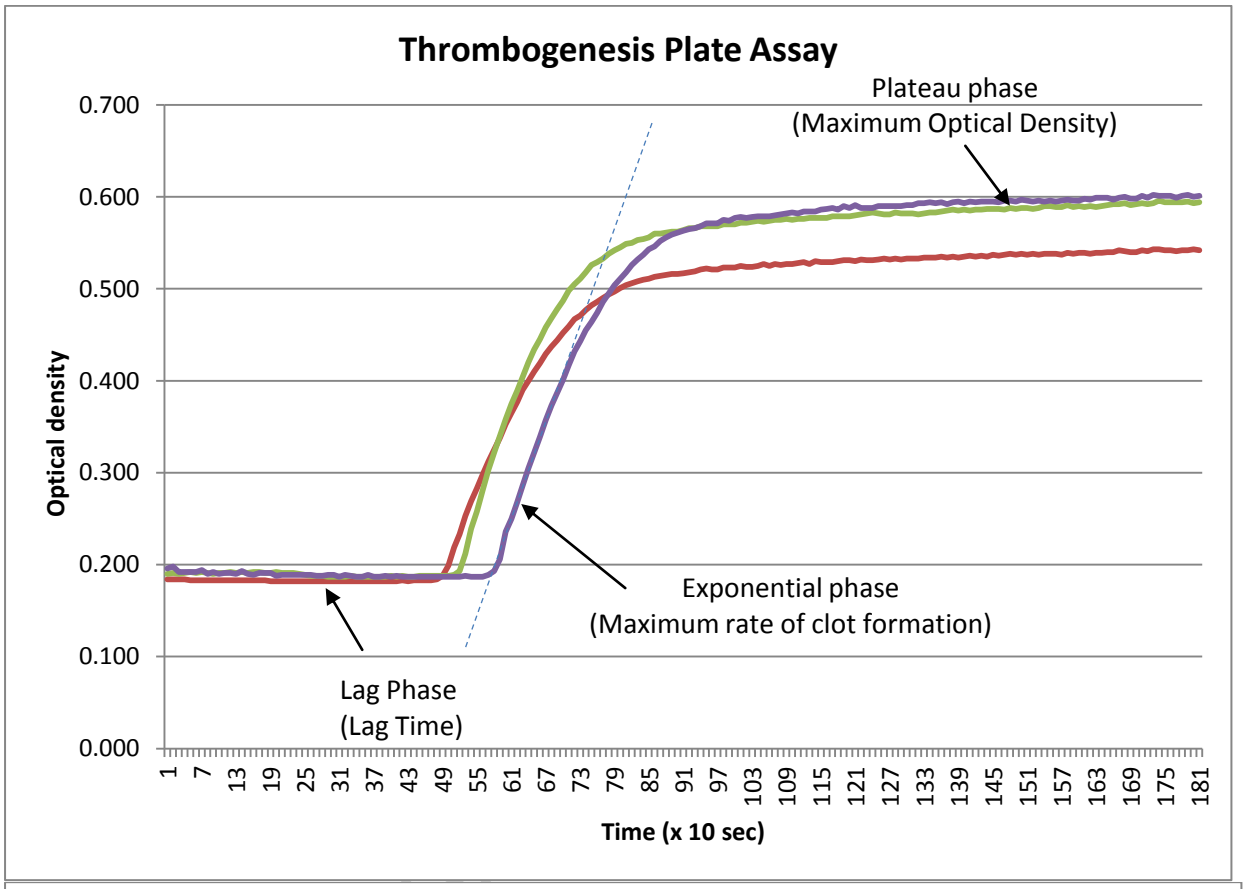
The plot shows changes in optical density as the fibrin clot forms. Triplicate plots are shown. T100% is the time to maximum absorbance, T0% is the return of the optical denity to near-baseline. T50% is (T100% - T0%)/2. The slope is the sharpest fall in optical density over time under the effect of exogenous tPA, effectively the reverse of the rate of clot formation in Figure 1.

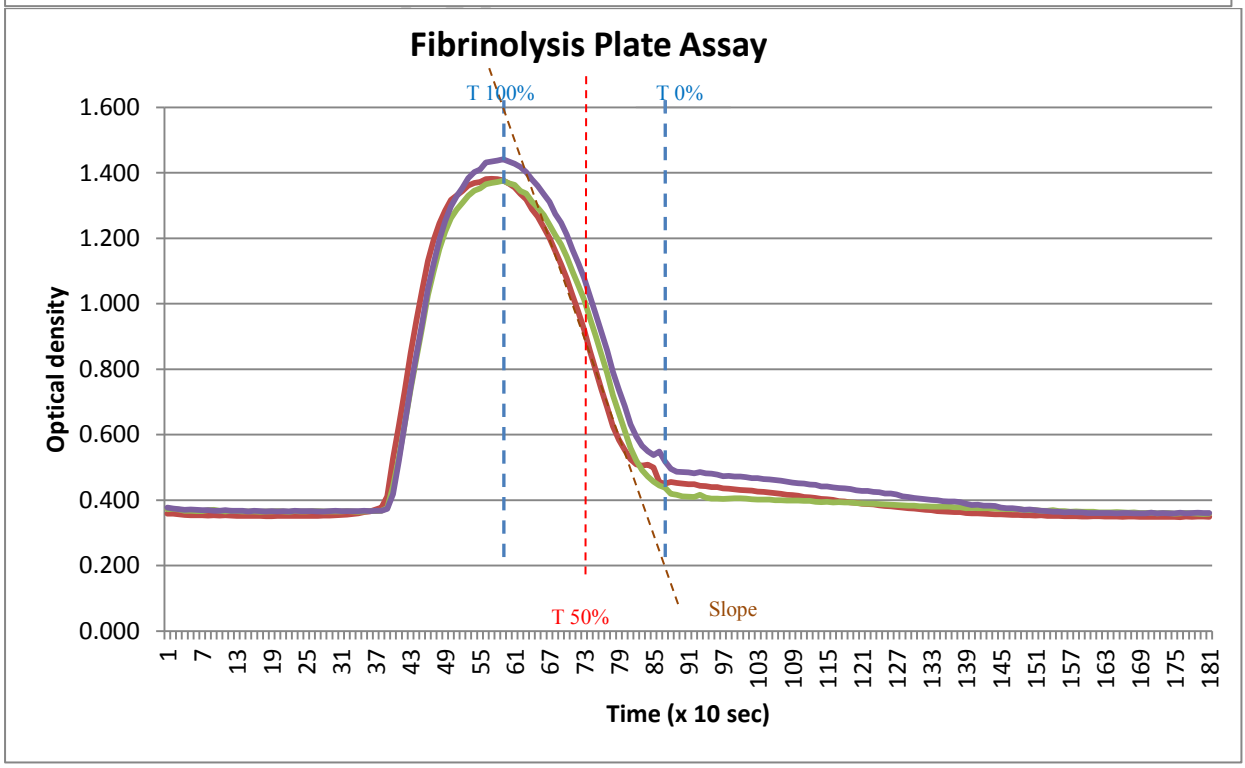
Figure 3: A modified TEG printout



See table 1 for an explanation of the indices.

VPH-D-14-00185R1





Grahical abstract