Development of a paper-based lateral flow prothrombin assay

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Disorders of haemostasis result in both excessive bleeding and clotting and are a major global cause of morbidity and mortality, particularly in the developing world. A small number of simple tests can be used to screen and monitor for such dysfunctions, one of which is the prothrombin time (PT) test and associated International Normalisation Ratio (INR). PT/INR is routine in hospital laboratories in developed countries, and can also be performed using point-of-care instruments. However, neither of these approaches is appropriate in low-resource settings. Significant interest has grown in paper-based devices to form the basis of simple and low-cost assays that may have the potential for application in such environments. This study describes the development of a simple, low-cost, paper-based lateral flow prothrombin assay. The assay employed wax printing on chromatography paper to define test channels, with deposition of thromboplastin reagent and calcium chloride onto the resulting strips. These were placed in a test housing and measurement of the flow rates of deposited plasma samples were performed in triplicate. The flow dynamics of the assay was optimised according to the type of paper substrate used, the nature and quantity of the thromboplastin reagent, the amount of calcium chloride required, and the volume of sample employed. An optimised assay configuration demonstrated a dynamic range of 6 mm between normal and factor-deficient plasmas. The assay showed good correlation with laboratory-based PT assay (Yumizen G200) in artificial plasmas in the 9.8 to 36 s range (r^2=0.8112). The assay also demonstrated good dynamic range and correlation in patient plasma samples in comparison with hospital PT, with a range of 9.8 to 45 s (r^2=0.7209).

Introduction

Haemostatic disorders lead to excessive bleeding conditions such as post-partum haemorrhage (PPH), prolonged menstrual periods, gastrointestinal bleeding, and frequent epistaxis; or clotting problems such as deep vein thrombosis (DVT), thromboembolic stroke, arterial thrombosis, and myocardial infarction[1]. They are among the leading causes of morbidity and mortality worldwide. The problem is more serious in the developing world where suitable facilities for diagnosis and treatment are lacking.

The prothrombin time (PT) test, along with its standardised format, the International Normalised Ratio (INR) is an essential test used for the clinical assessment of bleeding and clotting disorders. It involves the use of thromboplastin (tissue factor, phospholipids, and calcium) to trigger coagulation in patient plasmas to evaluate factor activity in the extrinsic and common pathways of the coagulation cascade[2,3]. In a PT test, thromboplastin is added to a plasma sample and the time taken for the onset of clot formation is measured. Among other things, PT is indicated for assessing unexplained bleeding, diagnosing disseminated intravascular coagulation (DIC), detecting extrinsic and common coagulation factor (II, VII, IX, X) deficiency[4], and antiphospholipid antibodies, monitoring oral anticoagulant therapies such as vitamin-K antagonists (VKAs such as warfarin)[5,6], and assessing liver disease and function[6]. It is also used in pre-, peri-, and post-operative coagulation monitoring[7].

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

Conventional PT assays are conducted in hospital clinical laboratories using benchtop analysers, which is excellent for inpatient care, but unsuitable for community-based support[8]. The need for improved monitoring at primary care and at home has been addressed through the development of point-of-care devices. Current devices use a number of techniques for PT measurement. CoaguCheck® XS (Roche Diagnostics Cooperation) uses electrochemical detection of a thrombin substrate, CoagMax (Microvisk Ltd) uses elasto-mechanical detection, and Hemochron Signature (Werfen) uses blood flow cessation for detection. Depending on the detection technique, these devices use disposable cartridges or test strips in combination with an instrument. Most of these devices have good correlations with routine lab methods[9]. However, their complexity and associated cost limits their accessibility, both in the developed world, but particularly in developing countries[7]. A number of devices continue to be developed on principles such as impedance[10], quartz crystal microbalance[11], fluorescence[12], and centrifugal force [13]. However, these do not address the requirements of low cost and simplicity demanded for low-resource applications.

Recent attempts to develop simplified approaches to PT testing include a polymer microfluidic device which propels sample with a pump based on effervescence generated from carbon dioxide[7]. Other polymer microfluidic approaches to the development of coagulation assays have employed micropillar-embossed chips for activated partial thromboplastin (aPTT) assays [12]and fibrinogen[14], employing either fluorescence transduction, or visual readout. However, such polymeric platforms are not environmentally sustainable, with approaches based on paper rapidly increasing in interest due to their low cost, simplicity, and sustainability[15]. A nitrocellulose paper-based assay has been developed for measuring coagulation, and although it did not employ thromboplastin reagent, was correlated with PT/INR[16]. A paper-based device

using pencil graphite electrodes has also been employed to measure impedance changes during clotting[17]

This study represents the first demonstration of a truly passive, cellulose paper-based prothrombin assay employing thromboplastin coagulation activation with visual readout. The device employed wax-printed chromatography paper modified with thromboplastin and calcium chloride and was able to measure PT in the range of 9.8 to 45 s in clinical samples.

Materials and methods

Materials

Whatman No. 1 chromatography paper (460 mm x 570 mm, 1001–197) was from Sigma-Aldrich, UK, while Whatman™ SG81 grade chromatography paper (11370744), Whatman™ grade 3MM Chr cellulose chromatography paper (10085370), and Calcium chloride fused granule (10043-52-4) were from Fisher Scientific, UK. Human citrated plasma standards: Routine Control N (5186, PT approx. 9 s), Routine Control A (5187, PT approx. 17 s), Routine Control SA (5183, PT approx. 34 s), Immunodepleted factor VII-deficient plasma (5792), pooled normal human plasma (5185) and Thromboplastin LI (5248) were from Helena Biosciences, UK. Prothrombin Time assay reagent Yumizen G PT Reco 10 (1300036376) and calibration reagents Yumizen G CTRL I & II (1300036412) were from Horiba, UK. Dade® Innovin® (B4212-40) and Thromborel® S were from Sysmex, UK, while Hemosil RecombiPlasTin 2G (0020003050) was purchased from Instrumentation Laboratory, UK. Sixty human citrated plasma samples were supplied by North Bristol NHS Trust with NRES approval (20/SC/0393). Silica gel desiccant sachets (10 g) were from CelloExpress, and aluminium foil Mylar bags (X000Y9UA0L, 12 × 8 cm) were from Fresherpack, UK.

Device fabrication

Assay strips with a test channel of 3 x 36 mm and sample application zone of 5 mm diameter were designed using Microsoft PowerPoint (Fig. 1A), and printed onto A4 sheets of chromatography paper using a ColorQube 8570 wax printer (Xerox Corporation, Malaysia)[18]. First, the boundary of the test channel was deposited, followed by curing at 100°C which allowed the wax to penetrate the paper, forming a hydrophobic barrier. An additional wax layer was then printed onto the first 11 mm of the underside of the strips. Assay strips were cut from the sheet, suspended on a wire rack, and modified with assay reagents, as appropriate.

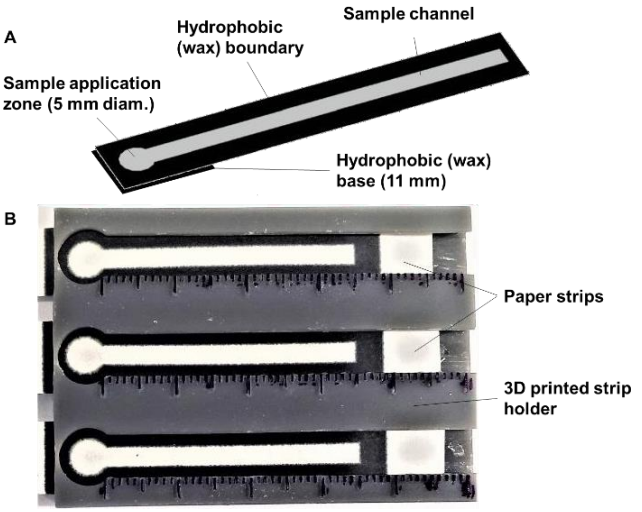

Figure 1. Assay strip design. A) Test channels (3 x 36 mm) and sample application zone (5 mm diam.) were formed by using wax printing followed by curing at 100°C for 2 min to create a hydrophobic boundary, followed by printing an 11 mm wax layer on the base of the strip. B) Test strips were housed in a 3D-printed holder and all measurements were conducted in triplicate.

Flow rate measurements

All assay flow rate measurements were performed in triplicate using strips placed inside a 3D-printed strip holder fabricated using a Formlabs Form2 printer with grey resin. The edges of the strips were supported on elevated rails on which the strips were freely suspended. (Fig, 1B). The strip holder was placed on a dry heating block at 37°C. Samples were pre-incubated at 37°C for 5 min before deposition at the sample application zone. Sample flow was captured via video recording and the distance travelled was measured using the printed scaling on the strip housing.

Measurement of prothrombin time

The PT and INR values of plasma standards and samples were measured using the Yumizen G200 coagulation analyser (Horiba, UK), according to manufacturer's instructions.

Storage of test strips

For stability studies, prepared test trips were placed between sheets of protective paper and vacuum sealed in aluminium foil bags with a sachet of silica gel desiccant (10 g).

Results and discussion

Optimisation of the assay substrate and device design

Paper has become an extremely versatile material for the development of low-cost bioassays and has seen a resurgence

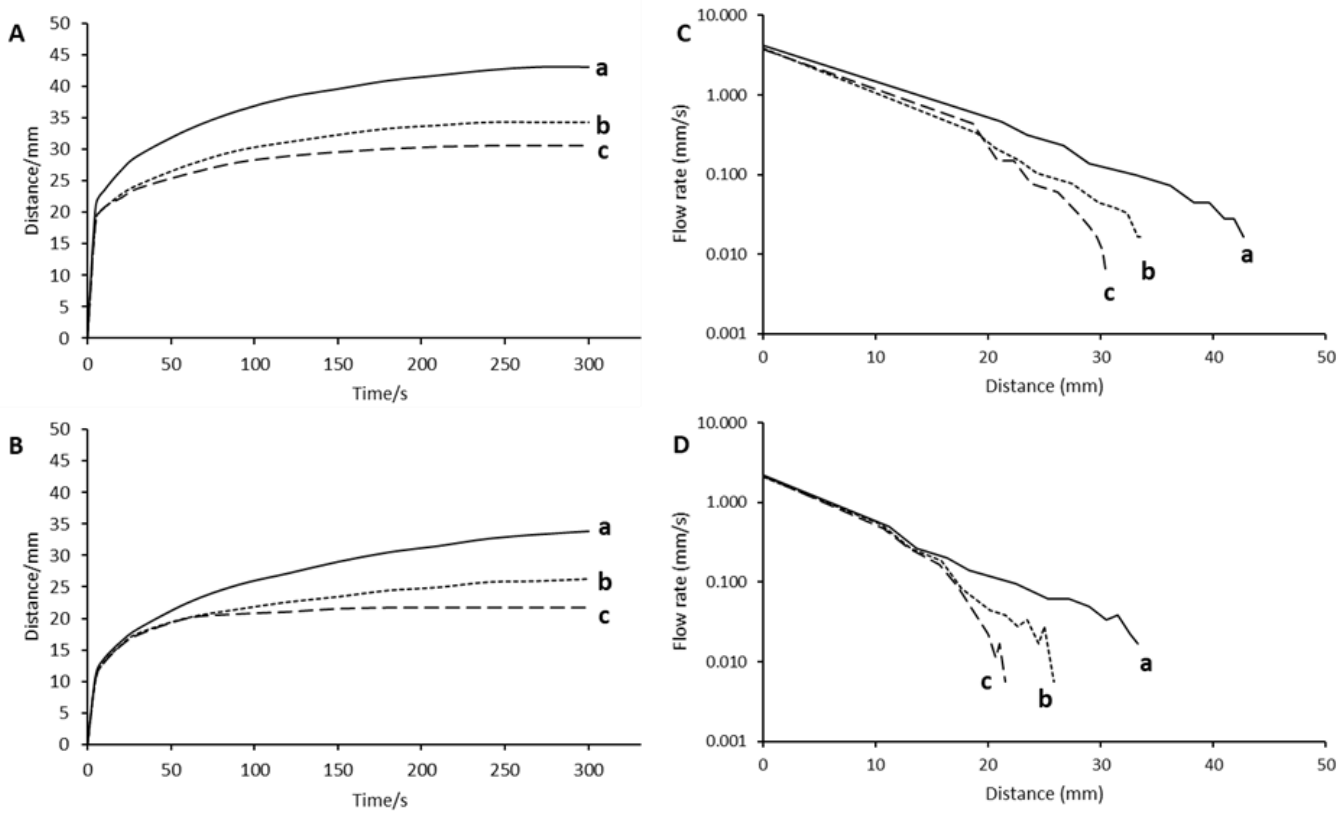

Figure 2. Fluid dynamic properties of sample viscosity controls (22 µL of a. Owren's buffer, b. serum, and c. normal plasma) on paper assay strips coated with 30 µL (A and C) Owren's buffer or (B and D) PT Reco 10 thromboplastin reagent (n=3). A and B show time vs. distance travelled by the fluid front, while C and D show the sample flow rate vs its distance along the strip.

in interest due to issues of environmental sustainability with respect to plastic alternatives[19]. However, paper is a simple term for a very complex material and diverse range of materials with many and varying properties [20]. While many devices have been developed based on nitrocellulose due to its excellent behaviour, it is relatively costly, does not biodegrade and slowly decomposes into toxic and explosive by-products. Cellulosic paper is of specific interest due to its low cost and wide availability. It also varies in many characteristics such as thickness, pore size, and water flow rate. All these factors can influence the suitability of the substrate for test strip fabrication and as well as for good assay performance. Several types of paper have become widely used in paper-based fluidic assay devices, particularly analytical chromatography papers, as these are manufactured to clearly defined and high-quality production standards to ensure a reliable source of substrate for analytical applications. Chief among these has been Whatman No. 1, with reproducible surface properties, a thickness of 0.18 mm and a linear water flow rate of 130 mm/30 min. It has been found to be easy to use in fabricating test strips and has also been found to be effective in the development of a number of lateral flow assays.[19,10,21] However, it might be that other substrate properties may provide better performance, and so should be evaluated.

Two additional substrates were investigated in addition to Whatman No. 1 for their potential application to this assay. These were Cytiva Whatman SG81 ion exchange chromatography paper[22] with greater thickness of 0.27 mm and slower linear flow rate of 110 mm/30 min and Whatman 3MM with a much greater thickness of 0.34 mm[23] and a linear water flow rate of 130 mm/30 min and is mainly used for general chromatography and electrophoresis. While the SG81 produced high quality prints of the wax layer, the wax would not penetrate the paper to provide a hydrophobic seal. The 3MM paper was very difficult to print on due to its increased thickness, and the wax was not capable of forming a complete seal through the paper, thus not providing a hydrophobic barrier. As a result, Whatman No. 1 chromatography paper was found to be the most suitable of the three tested and was used for further assay development.

Several strip design variables were explored, including the presence of a wax underside. It was found that this improved device reproducibility and prevented 'blebbing' of the sample beneath the paper at the application zone. It also increased hydrophobicity, which was useful at the sample zone to increase interaction with sample and reagent, but adversely reduced flow rate if used all along the strip. Coating the first 11 mm of the underside was found to provide an optimum balance of these effects.

Effect of sample and reagent properties on assay kinetics

The kinetics of liquid samples on lateral flow test devices such as the paper-based assay employed in this study will be influenced by a number of factors relating to both the sample and the test device. In the context of designing an assay based

on measurement of coagulation, establishing an understanding of these factors is critical to determine the specific contribution from the clotting process. With respect to aqueous biological samples, this relates predominantly to their viscosity, while with respect to the test strip, it relates to the wettability and hydrophobicity of the paper itself, as well as additional properties of the solubility of any reagents that may be deposited on the strip, in this instance thromboplastin reagents. To isolate and identify the contribution of the activation of coagulation on assay kinetics, a series of controls were performed to determine the various contributions of sample and substrate properties on strip flow rates. This was conducted using initial sample and reagent volumes of 22 and 30 µL, respectively (Fig. 2).

Owren's assay buffer acts as a low viscosity control, similar to that of water. While serum and plasma have similar viscosity ranges of approx. 1.1 to 1.8 mPa s,[24,25] serum has been depleted of coagulation factors and fibrinogen, which will have the effect of lowering viscosity and preventing the formation of a fibrin mesh, while plasma retains its clotting factors and has the ability to generate fibrin, which has the potential to modify sample viscosity. However, clotting can only occur in the presence of an activating reagent, such as a thromboplastin. Whether samples were on strips modified with buffer or thromboplastin, their flow velocities were in the order buffer>serum>plasma, as might be expected from their viscosities. All samples on control strips flowed faster and further than on strips modified with thromboplastin, illustrating the delay in sample flow brought about by the wetting and rehydration of the reagent. However, plasma sample flow on the reagent-modified strips had ceased at approx. 22 mm, while serum continued to travel beyond 26 mm, demonstrating that a specific contribution from the clotting process could be identified. However, this initial difference of approx. 1.5 mm was very small and required further optimization.

Optimisation of thromboplastin reagent and sample volume

The thromboplastin reagent used in prothrombin assays has typically been designed for use in solution, and so its concentration has been adjusted to achieve optimal performance in a particular volume ratio of reagent to sample. In the case of the PT Reco 10 reagent used here initially, this was 2:1. For its use in a lateral flow assay in which it has been dried onto the strip, this restricts the optimisation of the reagent based on its concentration. While some optimisation with respect to the volume deposited on the strip is possible, there are limits as to how much solution can be absorbed into the paper on a single deposition. These same volume limits also apply to the sample, along with additional factors. For instance, a minimum volume of sample is required to traverse the strip. However, above this minimum, the volume will also influence the ratio of interaction with the deposited reagent, as well as the flow rates and path of the sample through the strip. An increased sample volume will increase velocity, but with increased fluid flow across the surface of the strip, rather than within the porous matrix.

Based on the initial volumes employed in the strip configuration in Fig. 2, plasma sample and reagent volumes were cross-optimised in the range of 30 to 60 µL for the reagent and 12 to 22 µL for the sample, by determining the distance travelled by the sample after 5 min. As a part of this, plasma standards with a range of PT values were employed to assess the dynamic range of the assay. The dynamic range was determined by the difference in distance covered by normal plasma (N) with a PT of 9 s, compared to that of severe abnormal (SA) plasma with a PT of 34 s (Table 1). Increasing the volume, and therefore quantity of thromboplastin reagent on the strips did not appear to enhance the rate of coagulation, indicating that adequate reagent was available for sample activation at 30 µL. Sample volume had a more significant impact on the distance travelled which, not surprisingly, increased with sample volume for all plasma types. However, the optimal impact of sample volume on assay dynamic range was found to be at 15 µL, which may relate to having sufficient volume to fill the strip in a reasonable time frame, in combination with an optimum interaction of sample with reagent on the strip. At higher volumes, sample may well escape the influence of the reagent before it has become dissolved and dispersed fully throughout the sample. As a result, a reagent and sample volume combination of 30 µL and 15 µL, respectively resulted in a maximum dynamic range of 4.7 mm, and these values were employed for further development.

Optimisation of calcium chloride concentration

A particular issue with respect to coagulation assay measurements and associated assay development is that blood plasma samples destined for coagulation measurement are typically collected in a 9:1 v/v ratio with 3.2% (109 mM) trisodium citrate to chelate Ca^{2+} ions and so prevent clotting prior to testing. As part of the test procedure, $CaCl_2$ (25 mM, 100 µL) is added back into the sample to counter the effect of citrate and allow coagulation to proceed. Coagulation reagents are either formulated with $CaCl_2$ present or require its further addition.

Table 1. Distance travelled by Normal (N), Abnormal (A) and Severe Abnormal (SA) control plasmas after five minutes for strips modified with PT Reco 10 (n=3). Reagent volume was optimised with 22 µL sample, while sample volume was optimised with 30 µL reagent.

	Distance travelled by plasma after five min /mm							
	Reagent volume with 22 µL sample				Sample volume with 30 µL reagent			
Plasma (PT/s)	**60 µL**	**50 µL**	**40 µL**	**30 µL**	**22 µL**	**18 µL**	**15 µL**	**12 µL**
Normal (9)	23.3	24.3	20.7	22.2	23.7	18.8	14.0	9.2
Abnormal (17)	24.3	22.5	22.2	21.3	22.5	20.8	17.2	10.3
Severe Abnormal (34)	24.0	25.0	21.7	24.0	25.0	21.7	18.7	11.3
Dynamic range	0.7	0.7	1.0	1.8	1.3	2.8	4.7	2.2

In the case of PT Reco 10, Ca^{2+} ions are present in the reconstituting solvent at a concentration appropriate for the mixing of reagent and sample in a 2:1 v/v ratio. However, in the context of lateral flow assay development, the interaction of the reagent and sample does not take place in bulk, but rather, they are progressively introduced to one another as the latter traverses the strip. As a consequence, the concentration/time/location profile of calcium may be complex and may not reach optimal concentrations at all times and places within the sample to eliminate the anti-coagulant effect of sodium citrate. A more optimal concentration of $CaCl_2$ may be required to ensure the most effective on-strip clotting.

Previous work on measurement of calcium in blood based on coagulation and sample travel distance on lateral flow assays demonstrated that addition of calcium (15 µL) to sample at a range of concentrations (50–500 mM) elicits different coagulation rates and consequently leads to different sample travel distances[26]. Based on this, the quantity of deposited $CaCl_2$ was further investigated here. Assay test strips were first supplemented with a range of volumes of 400 mM $CaCl_2$ followed by 30 µL PT Reco 10 and assay dynamic range (difference between Normal Control and Severe Abnormal Control) was assessed. While the assay range at 2.5 µL was found to be effective at 5.5 mm, a slightly improved range of 6.17 mm was achieved with two depositions of 2.5 µL and may relate to the non-linear distributions of both plasma and Ca^{2+} that result during the assay, and this approach was adopted for further optimisation.

Investigation of the effect of thromboplastin reagent

Thromboplastins are essentially composed of tissue factor and phospholipids, which mimic vascular damage and platelet membrane, respectively[27]. Depending on their source, processing, and preparation, these can vary in their composition and potency. While details of their precise composition may be proprietary, principal differences are whether the material is from animal, human or recombinant and synthetic sources. Thromboplastin reagents are typically assigned an international sensitivity index (ISI) value by the WHO, depending on their clotting time behaviour with respect to an international standard. In the context of lateral flow assays, the composition may also have an influence on sample flow rates. While, thus far, investigation using the PT Reco 10 reagent had shown the potential for a lateral flow prothrombin assay, the combination of variations in activity, as well as potential impacts on lateral flow behaviour necessitated the investigation of the functional performance of a number of available thromboplastins. In addition to PT Reco 10, two other thromboplastins were investigated which were also based on human recombinant tissue factor and synthetic phospholipids. These were RecombiPlastin 2G and Innovin. In addition, Thromboplastin L (rabbit brain) and Thromborel S (human placenta) were also evaluated.

Employing the optimised $CaCl_2$ (2 x 2.5 µL 400 mM), reagent (30 µL), and sample (15 µL) volumes, five thromboplastin reagents were investigated for their assay performance. In this case, Normal Control, Abnormal Control and Factor VII-deficient (FVIID) plasmas were employed, the latter in which no coagulation should take place. The Prothrombin Time and INR values were also determined using the Yumizen G 200 coagulometer, although, it should be noted that the instrument is only calibrated for use with the PT Reco 10 reagent (Table 2).

With respect to PT and INR, PT Reco 10 gave the lowest values for both Normal Control and Abnormal Control plasmas, with Innovin second fastest. RecombiPlastin 2G, Thromborel S and Thromboplastin L were all generally more prolonged, although they varied minimally in their specific sensitivities to Normal and Abnormal Control plasmas. All thromboplastins, with the exception of Thromborel S did not return a clotting time for FVII-deficient plasma, which was as expected. While Thromborel S did return a value, this may have been an artefact of the operation of the Yumizen G200 with a reagent for which it was not designed.

Table 2. PT and INR values for Normal, Abnormal and FVII-deficient (FVIID) plasmas measured using different thromboplastin reagents with Yumizen G 200, and their corresponding travel distances and dynamic ranges on assay test strips modified with 30 µL of reagent.

Plasma	PT/s and INR									
	Thromboplastin L		PT Reco 10		RecomPlastin 2G		Innovin		Thromborel S	
	PT	INR	PT	INR	PT	INR	PT	INR	PT	INR
Normal	13.0	1.34	8.9	0.93	11.1	1.15	9.3	0.97	11.9	1.23
Abnormal	23.6	2.39	17.1	1.75	23.9	2.42	21.3	2.16	28.7	2.89
FVIID	>200	>200	>200	>200	>200	>200	>200	>200	56.7	5.6
	Assay test strips, distance travelled/mm									
Normal	10.83		9.50		16.33		10.83		12.00	
Abnormal	11.50		11.33		18.00		11.17		16.67	
FVIID	12.67		14.33		19.67		12.33		18.00	
Dynamic range	1.8		4.8		3.3		1.5		6.0	

With respect to responses on the assay strips (Table 2 and Fig. 3), these were again evaluated in terms of the overall dynamic range achievable and could be contextualised in terms of the difference between how far the sample travelled in the absence of coagulation due to the impact of reagent physical properties versus how effectively the reagent could arrest flow in a normal coagulating sample. With regard to the former, Thromboplastin L and Innovin had a strong impact on the flow of the FVIID plasmas, which would significantly limit the dynamic range achievable with these reagents. The impact of PT Reco 10 was more intermediate, while Thromborel S and RecombiPlastin 2G allowed the furthest travel of the non-clotting control, with RecombiPlastin 2G exhibiting the largest distances. However, RecombiPlastin 2G did little to bring about the early arrest of flow in the Normal Control plasma. While PT Reco 10 had the greatest impact on the Normal Control, Thromborel S had the greatest overall assay dynamic range, due to a combination of reagent potency and impact on flow kinetics. With regards to the source of thromboplastin, although the recombinant/synthetic forms generally appeared to perform better for PT/INR measurement, there was no consistent pattern with respect to their behaviour on the assay strips.

Given the improved dynamic range of the Thromborel S over PT Reco 10, further assay development was undertaken with the former. The assay was re-optimised using Thromborel S, which continued to achieve a dynamic range of approx. 6 mm (Table 3). Relative standard deviations (RSDs) of <10% (n=4) were achieved. It is also well-established that clotting time variability increases with clotting time, due to the stochastic nature of the clotting process[28], as was illustrated here in the increase in RSD with plasma clotting time.

Based on the mean distances and 95 % CI and PT values determined for the plasma standards, the relationship between these two parameters was derived (Table 4). These were found to fall into appropriate ranges, with Normal Control plasma resulting in PT between 8.4 and 14 s and distance ranging from 10 to 14 mm, while for Abnormal, this was 15 to 30 s and 14.5 to 17.5 mm, and for Severe Abnormal was >30 s and ≥16 mm.

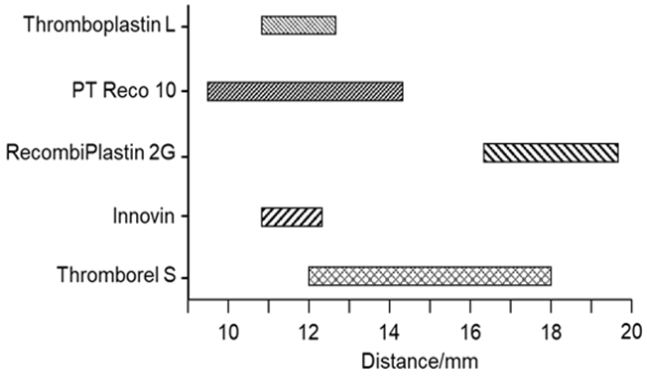

Figure 3. Distance ranges travelled by Normal Control versus Factor VII-deficient plasmas on assay strips modified with different thromboplastin reagents.

Table 3. Distance travelled by 15 µL Normal, Abnormal and Severe Abnormal Control plasma samples after 5 min in strips optimised with 30 µL Thromborel £, 15 µL 400 mM $CaCl_2$ (n=4).

Plasma	Mean/ mm	RSD (%)	95% CI
Normal	12.3	5.27	0.63
Abnormal	16.3	6.41	1.02
Severe abnormal	18.0	9.35	1.65

Table 4. Distance ranges and PT values for plasmas of different clotting capacities.

Plasma	Distance range/mm	PT/s
Low	<10.0	<8.5
Normal	10.0 - 14.0	8.5 - 14
Abnormal	14.5 - 17.5	15 - 30
Severe Abnormal	≥16.0	>30

Assay validation in clinical plasma samples

The assay was validated using both artificial and clinical plasma samples. Initially, fifteen lyophilised commercial plasma samples with varying PT values (9.8 – 36 s) were reconstituted and tested (n=3) simultaneously with the paper-based assay and the Yumizen G200 (Fig. 4A). There was good correlation between the paper-based assay and the reference Yumizen G200 method (r^2=0.8112). More importantly, all of the normal plasma samples had mean distances that fell within the normal distance range (9.5 – 14 mm) and those of all abnormal plasma samples fell above the normal distance range (>14 mm). However, there was greater variation in the distance values from the method for the plasmas with moderately prolonged (17-20 s) PT values.

For further validation in clinical samples, patient plasmas were initially selected from across a broad range of PT values from 9.8 to 95.4 s and assessed using the lateral flow assay. However, above PT values of approx. 45 s, there was a loss of linearity from the paper-based assay (r^2=0.0013), demonstrating that the paper-based assay could not quantify clotting prolongation above this value (Fig. 4B). However, since values above 37 s would be considered critical [29], quantification beyond this range would be less important or unnecessary, particularly in a low resource context. Following this, samples were taken for analysis in the range of 9.8 to 45 s (n = 47 samples). This resulted in good agreement between the hospital method and the paper-based assay (r^2 = 0.7209).

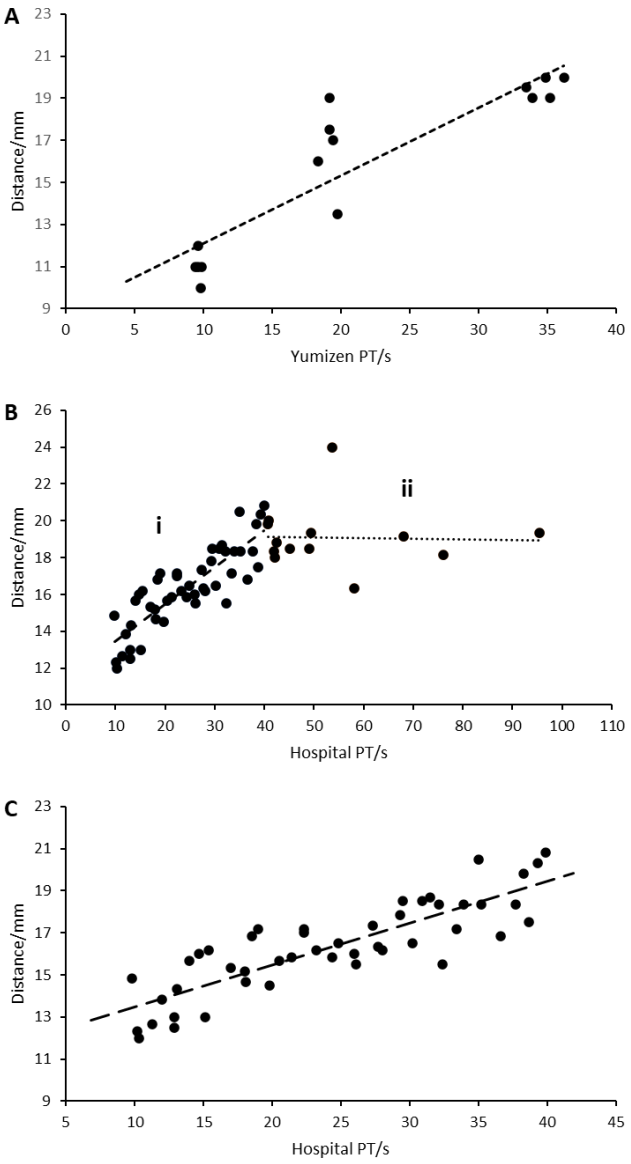

Figure 4. Validation of paper-based lateral flow prothrombin assay (n = 3 repeats). (A) Laboratory validation using artificial plasma samples with PT values 9.8 – 36 s, comparing Yumizen G200 PT with distance travelled on the paper device (mm) (r^2=0.8112). B) Correlation between hospital PT and the paper-based lateral flow assay in plasma samples (n = 60 samples) with PT values in the range 9.8 – 95.4 s, showing a correlative range (i) between 9.8 and 45 s (r^2=0.7209) and non-correlative range (ii) >45 s (r^2=0.0013). C) Correlation between hospital PT assay and paper-based lateral flow assay for plasmas with PT values ≤40 s (r^2=0.7209).

While improvements to assay dynamic range and reproducibility would enhance the performance of the assay further, this result shows remarkable comparative performance between an automated hospital instrument and a simple, disposable paper strip, and so demonstrates significant potential for low resource applications.

Assay ranges and cut offs

Based on the clinical correlations established in Fig. 4, ranges of distance travelled were assigned to relate to critical clinical ranges (Fig. 5A). For instance, those in the range 11 to 13 mm could be assigned as Normal, while those from 13 to 15 mm as Normal or slightly prolonged and coincide with Normal PT and INR 1-2. For 15 to 18, these could be designated moderate to significant prolongation, coinciding with an Abnormal PT and INRs from 2 to 3, while >18 mm would be significantly prolonged and coincide with INR ≥3 and Severe Abnormal and a critical cut off of 37 s [29].

Storage and operational temperature and stability

The temperature of both device storage and operation have the potential to impact multiple assay variables including enzymatic activity, stability, and viscosity, among others. For devices which might be used in low resource regions, the vast majority of which lie within the torrid zone, understanding the potential challenges likely to be encountered will support further assay development to improve stability in a manner that does not require a cold chain, or other support infrastructures.

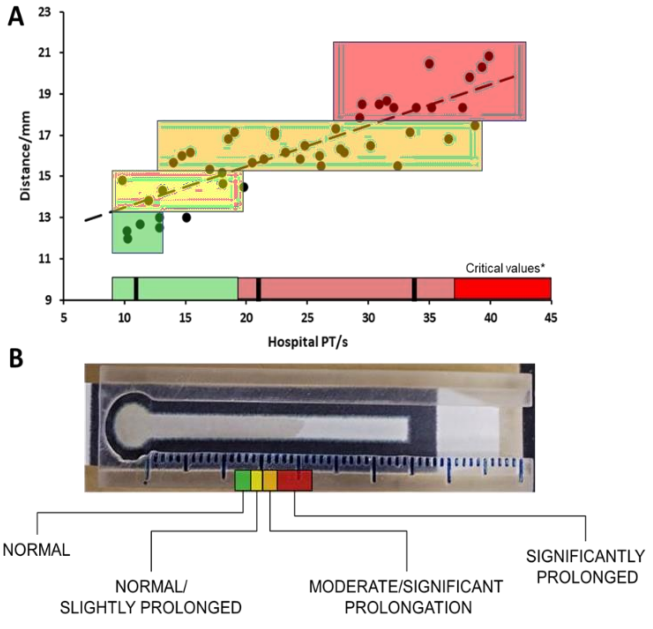

Figure 5. Correlation of hospital PT with paper-based assay distances with overlayed test ranges of normal (green), normal/slightly prolonged (yellow), moderate/significant prolongation (orange) and significantly prolonged (red), in conjunction with current clinical PT ranges of 9-19 s (normal, green), 19-37 s (prolonged, pink) and >37 s (severely prolonged, red), along with mean INRs of 1, 2 and 3 (black bars). B) Overlay of prothrombin assay ranges onto the paper-based assay strip.

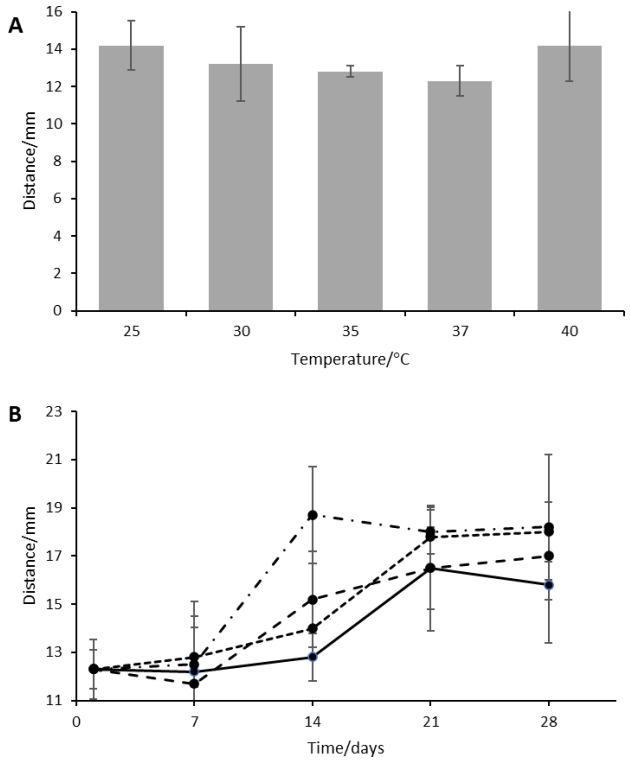

Figure 6. Effects of operational and storage temperature on assay device behaviour. Distance travelled by Normal control plasma with operation of the optimised assay at: A) different temperatures, and B) at 37°C following storage at -20°C (solid line), 6°C (short dash), ambient (long dash) and 37°C (dot-dash) for up to 28 days (n=3).

The impact of the change in operational temperature of the assay can be seen in Fig. 6A. Although not statistically significant based on the size of the data set, there was a clear pattern of behaviour with respect to temperature that would be expected for an enzymatic process optimised for operation at 37°C. In general, as well as the prothrombin distance increasing away from this temperature, there appeared to be moderate increases in variability. Not unexpectedly, a similar trend has been observed in the development of a paper-based lateral flow fibrinogen assay[30]. Variability due to operational temperature could be addressed through the use of specific temperature calibration ranges.

The storage stability of the device was also investigated by placing the paper test strips in vacuum-sealed aluminium pouches with desiccant, with storage at different temperatures (-20 to 37°C) and testing them over time with normal plasma (Fig. 6B). After one week, although the effects were small, strips stored under elevated and ambient temperatures fell outside the normal control assay range. After 14 days, all devices had deviated from the normal range, although there was a clear temperature-dependence on this, with lower storage temperatures deviating least from normal. However, after 21 and 28 days, strips stored at all temperatures were essentially no longer responding to activation of coagulation, demonstrating that, irrespective of storage temperature, additional development is required to prevent loss of reagent activity over time, and will relate to the stability of both tissue factor and phospholipids. While beyond the scope of this work, there are many approaches to improving dried enzyme assay reagents stability, which could be employed to address this issues [31].

Conclusion

A paper-based lateral flow device for human plasma prothrombin assay was developed. It was demonstrated that activation of plasma samples with immobilised thromboplastin reagent can modify the lateral flow properties of the sample on the strip, and that this was found to be proportional to the clotting capacity of the plasma sample. When performed in triplicate, assays correlated well with laboratory standards using commercial PT assay devices (Yumizen G200), and it also demonstrated good correlation with hospital-measured PT for patient plasmas with PT values ≤40 s ($r^2 = 0.7209$). While the device was less precise in comparison with routine PT assay methods, its simplicity and cost offer potential for use in low resource environments. However, further improvements to assay precision, dynamic range, and stability are essential to achieving this objective.

Author Contributions

Jerro Saidykhan: Methodology and investigation
Louise Pointon: Provision of clinical samples and data
Stefano Cinti: Supervision and paper expertise
Jennifer E. May: Supervision and haematology expertise
Anthony J. Killard: Conceptualisation, project administration, supervision and bioanalytical expertise

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We wish to recognise the support offered by the UK Commonwealth Scholarship Commission for funding the project

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