Engineering and testing novel fibrinogen paper diagnostics for blood analysis

Marek Bialkower

Bachelor of Biomedical Sciences/Engineering (Honours)

A thesis submitted for the degree of Doctor of Philosophy at

Monash University in 2021

Bioresource Processing Institute of Australia

New page

Copyright notice

I certify that I have made all reasonable efforts to secure copyright permissions for third-party content included in this thesis and have not knowingly added copyright content to my work without the owner's permission.

I certify that I have made all reasonable efforts to secure copyright permissions for third-party content included in this thesis and have not knowingly added copyright content to my work without the owner's permission.

© Marek Bialkower (*2021*).

New page

Abstract

This thesis develops the science and engineering behind novel diagnostics for measuring blood fibrinogen concentration on paper. It aims to quantify their sensitivity, determine their mechanisms and understand their limitations. These diagnostics are designed to be rapid, simple to use and cheap to easily determine wherever critically bleeding patients have low blood fibrinogen concentrations. Hence, it can allow them to receive life-saving fibrinogen replacement therapy (FRT) and save millions of lives a year.

Fibrinogen is the effector clotting protein in blood. It is normally present in the plasma at concentrations between 2-4 g/L. When bleeding is induced, fibrinogen is polymerised into fibrin at the site of blood vessel breakage. It then forms the backbone of clots to stop bleeding. Patients with hypofibrinogenemia are at a significantly greater risk of mortality due to critical bleeding. The early depletion of fibrinogen can impair the formation of clots.

When hypofibrinogenemia is detected in critically bleeding patients, FRT is used. European clinical guidelines have stated that FRT should be implemented when plasma fibrinogen concentrations fall below 2 g/L. However, FRT needs to be handled carefully to prevent unnecessary fibrinogen supplementation. Over-supplementation can lead to thrombotic episodes, induce iatrogenic effects and waste expensive resources.

The main clinical assays currently used to detect hypofibrinogenemia contain difficulties. The Clauss assays are the most common standard laboratory technique for quantifying fibrinogen concentration. They do so by taking a plasma sample, adding thrombin and measuring the time taken for fibrin formation to occur. Longer times correlate to lower fibrinogen concentrations. Likewise, viscoelastic haemostatic assays have risen in popularity in the last 20 years. This is because they can show the effect of numerous clotting factors in whole blood per assay. Viscoelastic haemostatic assays measure fibrinogen through the change in viscoelasticity when fibrin is formed. However, both assays require well controlled, sensitive and expensive instruments which cannot be transported to the patient. As the patient must be transported to the equipment, the assays consume critical time before FRT can be administered.

Therefore, a rapid, cheap, portable, hand-held plasma fibrinogen concentration diagnostic is needed to greatly reduce the time to treatment needed for FRT.

This thesis describes numerous fibrinogen concentration tests on paper. The first is a vertical wicking test where plasma-wetted paper strips are inserted into an aqueous dye bath and the fibrinogen concentration dictates the length the dye wicks up the strip. The second is a horizontal wicking test where plasma-wetted paper strips are oriented horizontally, an aqueous dye is pipetted onto the strips and the fibrinogen concentration of the plasma dictates the wicking length of the blue dye. The third is another horizontal wicking test where a droplet of whole blood is formed on a solid surface, a paper strip is placed on top and the fibrinogen concentration dictates the distance the blood wicks down.

New page (thesis including published works only)

Thesis including published works declaration

This declaration is to be included in a 'thesis including published works'. You must provide details of all publications included in your thesis in the section below. If you have not completed a thesis including published works, please remove this section and include a standard thesis declaration.

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 4 original papers published in peer reviewed journals and 1 submitted publications. The core theme of the thesis is Paper Fibrinogen Diagnostics. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Bioresource Processing Institute of Australia under the supervision of Gil Garnier.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of all chapters my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status *(published, in press, accepted or returned for revision, submitted)*	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
1	*Fibrinogen Diagnostics in Major Hemorrhage*	*Accepted*	*80% - Lead Author*	Gil Garnier *20% Advising*	*No*
2	*Rapid Paper Diagnostic for Plasma Fibrinogen Concentration.*	Accepted	80% - Lead Author	Heather Mcliesh *5% Various Roles* Clare Manderson *5% Various Roles* Rico Tabor *5% Various Roles* Gil Garnier *5% Various Roles*	No No No No
3	*Rapid, hand-held paper diagnostic for measuring Fibrinogen Concentration in blood.*	Accepted	80% - Lead Author	Heather Mcliesh *5% Various Roles* Clare Manderson *5% Various Roles* Rico Tabor *5% Various Roles* Gil Garnier *5% Various Roles*	No No No No
4	*Paper Diagnostic for Direct Measurement of Fibrinogen Concentration in Whole Blood*	Accepted	80% - Lead Author	Heather Mcliesh *2% Various Roles* Clare Manderson *10% Various Roles* Rico Tabor *3% Various Roles*	No No No No

				Gil Garnier *5% Various Roles*	

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student name: Marek Bialkower

Student signature: **Date:** 26/03/2021

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor name: Gil Garnier

Main Supervisor signature: **Date:** 26/03/2021

New page

Acknowledgements

Special thanks are given to various wonderful people and groups. For if it was not for them, the many milestones accomplished in this thesis wouldn't have been achieved.

First of all, the industry sponsor for this project: Haemokinesis.

Second of all, the Bioresource Processing Institute of Australia (BioPRIA) research group.

This includes as follows:

- Diana Alves, Rodrigo Curvello and Scot Sharman for performing various experiments.
- Michael Hertaeg, Mahdi Naseri-Nosar, Laila Hossain, Heather McLiesh and Clare Manderson for supplying materials and methodology ideas needed to complete experiments.

Finally, the supervisors for this project: Rico Tabor and Gil Garnier. For not only their continual mentoring and feedback, but also their willingness to provide emotional support needed to complete the PhD candidature and thesis.

Engineering and Testing Novel Fibrinogen Paper Diagnostics for Blood Analysis

Marek Bialkower

Summary

This thesis develops the science and engineering behind novel diagnostics for measuring blood fibrinogen concentration on paper. It aims to quantify their sensitivity, determine their mechanisms and understand their limitations. These diagnostics are designed to be rapid, simple to use and cheap so that medical personnel can easily determine wherever critically bleeding patients have low blood fibrinogen concentrations or not. Hence, it can allow these patients to receive life-saving fibrinogen replacement therapy (FRT) at the site of injury and save millions of lives a year.

Fibrinogen is the effector clotting protein in blood. It is normally present in the plasma at concentrations between 2-4 g/L (1, 2). When bleeding is induced, fibrinogen is polymerised into fibrin at the site of blood vessel breakage (3, 4). It then forms the backbone of clots to stop bleeding (5-9). Patients with hypofibrinogenemia are at a significantly greater risk of mortality due to critical bleeding (10, 11). This is because the early depletion of fibrinogen can impair the formation of clots (12, 13).

When hypofibrinogenemia is detected in critically bleeding patients, FRT is used. European clinical guidelines have stated that FRT should be implemented when plasma fibrinogen concentrations fall below 2 g/L (14). Whilst the topic remains controversial, there is ongoing evidence to show the early detection of hypofibrinogenemia and FRT can improve outcomes in haemorrhagic patients (15-17).

However, at the same time, FRT needs to be handled carefully to prevent unnecessary fibrinogen supplementation. Over-supplementation can lead to thrombotic episodes, induce iatrogenic effects and waste expensive resources (18, 19).

The main clinical assays currently used to detect hypofibrinogenemia contain difficulties. The Clauss assays are the most common standard laboratory technique for quantifying fibrinogen concentration. They do so by taking a plasma sample, adding thrombin and measuring the time taken for fibrin formation to occur. Longer times correlate to lower fibrinogen concentrations (20). Likewise, viscoelastic haemostatic assays (VHAs) have risen in popularity in the last 20 years. This is due to their ability to quantify numerous clotting factors in whole blood per assay. VHAs measure fibrinogen concentration through the change in viscoelasticity when fibrin is formed (21). However, both assays require well controlled, sensitive and expensive instruments which cannot be transported to the patient. As the patient must be transported to the equipment, the assays consume critical time before FRT can be administered (22).

Therefore, a rapid, cheap, portable and hand-held plasma fibrinogen concentration diagnostic is needed to greatly reduce the time to treatment needed for FRT.

The use of paper in diagnostics has become pronounced in the last two decades. Paper is a cheap and lightweight material that can facilitate the rapid movement of biological samples (due to its network of capillaries) and biochemical reactions. Much of the research in paper diagnostics has been completed in detecting antigens, measuring enzymatic activity and blood typing (23-27). However, no research currently exists in the use of paper for measuring fibrinogen concentrations in the blood.

This thesis describes numerous fibrinogen concentration tests on paper. The first is a vertical wicking test where plasma-wetted paper strips are inserted into an aqueous dye bath and the fibrinogen concentration dictates the length the dye wicks up the strip. The second is a horizontal wicking test where plasma-wetted paper strips are oriented horizontally, an aqueous dye is pipetted onto the strips and the

fibrinogen concentration of the plasma dictates the wicking length of the dye down the strip. The third diagnostic is another horizontal wicking test where a droplet of whole blood is deposited on a solid surface, a paper strip is placed on top of it and the fibrinogen concentration dictates the distance the blood wicks down.

The first two tests can distinguish low (<2 g/L) from normal fibrinogen concentrations in minutes. They work by reacting the plasma wetted paper strips with thrombin to form fibrin. The conversion of plasma fibrinogen to fibrin significantly increases the hydrophobicity of the paper strip. Therefore, it makes it much harder for the aqueous dye to move through. Hence, higher fibrinogen concentrations cause the aqueous dye to wick to shorter distances.

The third diagnostic invented can work with whole blood. It can easily distinguish fibrinogen concentrations below and above 1.6 g/L in minutes. It works by pre-mixing the blood droplet with a thrombin solution to clot it. The higher the fibrinogen concentration, the more fibrin that is formed and hence the lower the permeability of the droplet. Hence, when the paper strip is placed on top of the droplet, the ability of the blood to wick down the strip is impeded. Because the test utilises pre-mixing, it means that the ratio of blood:thrombin solution in the mixed droplet can be changed. A lower ratio effectively means the blood is pre-diluted. When this occurs, the effect of interfering substances in the blood can be minimised. This includes haematocrit and thrombin inhibitors.

Additionally, this thesis also describes a test that can measure prothrombin time (PT). PT relates to the time required for a clot to form after blood clotting has been induced. It measures the activity of enzymes involved in the tissue factor (or extrinsic) pathway of clotting (28). The test is an adaptation of the third test. However, instead of using thrombin as the reagent, it uses tissue factor instead (to initiate the extrinsic pathway). As the test can clearly tell when a clot has formed, it can distinguish wherever the blood droplet has above or below biomedically relevant PTs. Therefore, the test can perform a variety of services. This includes, informing whether a patient may need a liver transplant or if a patient on anticoagulant medication needs to adjust their dose.

These novel paper diagnostics are inexpensive, easy to use, robust and sensitive. They can minimise the time needed before FRT is administered amongst other applications. Hence, they can optimise survival and save well over 20 million lives per year.

Acknowledgments

Special thanks are given to various wonderful people and groups. For if it was not for them, the many milestones accomplished in this thesis wouldn't have been achieved.

First of all, the industry sponsor for this project: Haemokinesis.

Second of all, the Bioresource Processing Institute of Australia (BioPRIA) research group. This includes as follows:

- Diana Alves, Rodrigo Curvello and Scot Sharman for performing various experiments.
- Michael Hertaeg, Mahdi Naseri-Nosar, Laila Hossain, Heather McLiesh and Clare Manderson for supplying materials and methodology ideas needed to complete experiments.

Finally, the supervisors for this project: Rico Tabor and Gil Garnier. For not only their continual mentoring and feedback, but also their willingness to provide emotional support needed to complete the PhD candidature and thesis.

Contents

Introduction

Fibrinogen is central to the process of blood clotting. It is a large protein that forms the building blocks needed to create blood clots. In its inactive state, fibrinogen is present in the blood as a monomer. However, when the vascular endothelium is disrupted and bleeding is induced, fibrinogen self-polymerises into a sticky protein network. It then adheres across the site of the broken blood vessel and forms the backbone of the clot to stop further bleeding (29). Fibrinogen in healthy non-pregnant adults is present in the plasma at concentrations of 2–4 g/L (1, 2). However, in many coagulopathies, fibrinogen can drop significantly below these levels. This can exacerbate major haemorrhage and even cause death (15-17).

Fibrinogen plays a critical role in maintaining haemostasis during major haemorrhage (16, 30). It has only been known since 1995 that fibrinogen is usually one of the first coagulation factors to fall to critically low levels in the blood during many coagulopathies (31). Haemorrhagic patients that present with hypofibrinogenemia are at a significantly greater risk of massive transfusion requirements and mortality (10, 11). European guidelines state that fibrinogen replacement therapy (FRT) should be implemented when plasma fibrinogen levels fall below 1.5–2 g/L (14). Whilst the topic remains controversial, there is ongoing evidence to show the early detection of hypofibrinogenemia and FRT can improve outcomes in haemorrhagic patients (15-17).

The main clinical assays currently used to detect hypofibrinogenemia contain difficulties. The Clauss assays are the most common standard laboratory technique for quantifying fibrinogen concentration. They do so by taking a plasma sample, adding thrombin and measuring the time taken for fibrin formation to occur. Longer times correlate to lower fibrinogen concentrations (20). Likewise, viscoelastic haemostatic assays (VHAs) have risen in popularity in the last 20 years. This is due to their ability to quantify numerous clotting factors in whole blood per assay. VHAs measure fibrinogen concentration through the change in viscoelasticity when fibrin is formed (21). However, both assays require well controlled, sensitive and expensive instruments which cannot be transported to the patient. As the patient must be transported to the equipment, the assays consume critical time before FRT can be administered (22).

Currently, there is no rapid, cheap, portable, and easy to use hand-held blood fibrinogen concentration diagnostic that can be used for rapid, point of care (PoC) hypofibrinogenemia testing.

The use of paper in diagnostics has become pronounced in the last two decades. Paper is a cheap and lightweight material that can facilitate the rapid movement of biological samples (due to its network of capillaries) and biochemical reactions. Paper diagnostics have been successfully developed for detecting antigens, measuring enzymatic activity and blood typing (23-27). Therefore, paper has the potential to be a suitable medium for detecting hypofibrinogenemia in PoC setting.

This thesis develops a series of novel paper-based fibrinogen diagnostics - to facilitate rapid PoC testing for hypofibrinogenemia.

The first chapter reviews the available methods of diagnosing early hypofibrinogenemia. In the first part, the biochemistry of fibrinogen is summarised to provide a fundamental understanding of the science behind fibrinogen concentration assays. In the second part, these assays are discussed. In the third part, more advanced concepts and recent point of care diagnostics are presented.

The second chapter explores a test that utilises a vertical wicking setup. It initially investigates the mechanism of the test. It then aims to quantify the effect of varying several controlling parameters on the

sensitivity of the test. The parameters that are analysed include: the paper conditions, the concentrations of reagents used as well as the reaction and elution times. This chapter also measures the effect of non-specific blood proteins on the test.

The third chapter investigates a hand-held test that works with blood plasma. It explores the effect that different human plasma samples have on the test - to conclude whether the test varies between healthy individuals. It compares the performance of the test to a hospital-grade Clauss assay. Finally, it explores the effect of common blood abnormalities and weather conditions. This includes the effects of acidosis, alcohol, heparin, warfarin, haemolysis and dietary lipids as well as surrounding temperatures and humidities.

The fourth chapter develops a hand-held test that functions with whole blood. It initially investigates the conditions needed for the test to work properly. It then observes the effect of blood dilution. This is to determine whether dilution can improve the sensitivity of the test and minimise the effect of inhibitors found in blood. It also provides insight on the mechanism behind the test. Additionally, this chapter demonstrates a hand-held prototype of the diagnostic applicable for PoC testing.

Finally, the fifth chapter converts the whole blood diagnostic into one that measures prothrombin time (PT). It first proves the concept - using blood samples with different PTs. Afterwards, it analyses if the diagnostic can be used for liver disease detection and anticoagulant monitoring. Lastly, because the test may be used in developing nations with minimal temperature regulation, it also observes the effect of temperature.

These novel paper diagnostics are inexpensive, easy to use, robust and sensitive. They can minimise the time needed before FRT is administered amongst other clotting-based health issues. Hence, they can optimise survival and save well over 20 million lives per year.

Chapter 1: Literature Review

Publication Details

Publication Status: Published

Journal: Critical Reviews in Analytical Chemistry *(Q1 – Analytical Chemistry)*

Reference: Bialkower M, Garnier G. *Fibrinogen Diagnostics in Major Hemorrhage.* DOI: https://doi.org/10.1080/10408347.2020.1793098

Abstract

Fibrinogen is one of the first factors to fall to critically low levels in the blood in many coagulopathic events. Patients with hypofibrinogenemia are at a significantly greater risk of major haemorrhage and death. The rapid replacement of fibrinogen early on in hypofibrinogenemia may significantly improve outcomes for patients. Fibrinogen is present at concentrations between 2 and 4 g/L in the plasma of healthy people. However, hypofibrinogenemia is diagnosed when the fibrinogen level drops below 1.5–2 g/L. This review analyses different types of fibrinogen assays that can be used for diagnosing hypofibrinogenemia. The scientific mechanisms and limitations behind these tests are then presented. Additionally, the current state of clinical major haemorrhage protocols (MHPs) is presented and the structure, function and physiological role of fibrinogen is summarised.

1. Introduction

Fibrinogen is central in the process of blood clotting. It is a large protein that forms the building blocks needed to create blood clots. In its inactive state, Fibrinogen is present in the blood as a monomer. However, when the vascular endothelium is disrupted and bleeding is induced, fibrinogen self-polymerises into a sticky protein network. It then adheres across the site of the broken blood vessel and forms the backbone of the clot to stop further bleeding (29). Fibrinogen in healthy non-pregnant adults is present in the plasma at concentrations of 2–4 g/L (1, 2). However, in many coagulopathies, fibrinogen can drop significantly below these levels. This can lead to major haemorrhage and even death. Whilst the topic remains controversial, there is ongoing evidence to show the early detection of hypofibrinogenemia and replenishment of fibrinogen levels can improve outcomes in haemorrhagic patients (15-17).

Coagulopathy is a major clinical issue. It is characterised by tissue injury and low tissue perfusion. This results in coagulation factor (CF) depletion, platelet dysfunction, hypocoagulation and hyperfibrinolysis (32, 33). Furthermore, it has been shown that acidosis, hypocalcaemia and hypothermia can all exacerbate the effects of coagulopathy even further (34). Not only can coagulopathy lead to the onset of major haemorrhage, but it can also compound and exacerbate existing haemorrhage – thus worsening the outcome of patients. Hypofibrinogenemia is present in coagulopathies such as those in trauma, post-partum haemorrhage, gastrointestinal bleeding, cardiac surgery, general surgery and liver transplant patients (35-39).

Fibrinogen plays a critical role in maintaining haemostasis during major haemorrhage (16, 30). It has only been known since 1995 that fibrinogen is usually one of the first CFs to fall to critically low levels in the blood during many coagulopathies (31). Haemorrhagic patients that present with hypofibrinogenemia are at a significantly greater risk of massive transfusion requirements and mortality (10, 11). Raza *et al.* and Davenport *et al.* reported that low fibrinogen concentrations can impair the formation of clots (12, 13). European guidelines state that fibrinogen replacement therapy should be implemented when plasma fibrinogen levels fall below 1.5–2 g/L (14).

This review aims to analyse the available methods of diagnosing early hypofibrinogenemia. In the first part, the biochemistry of fibrinogen is summarised to provide a fundamental understanding of the science behind fibrinogen concentration assays. In the second part, these assays are discussed. In the third part, more advanced concepts and recent point of care diagnostics are presented. We hope this review will give an overview of the current state of early hypofibrinogenemia diagnostics.

2. Biochemistry of Fibrinogen

2.1. Fibrinogen Structure

Fibrinogen is a dimeric 340 kDa glycoprotein that is synthesised in the liver and circulates in the blood at concentrations of 2–4 g/L (1, 2). Each fibrinogen molecule consists of multiple polypeptide chains that are linked together through disulfide bridges and coiled-coil segments. This causes them to span 47.5 nm in length and 6.5 nm in diameter in a rod-like shape (40).

Fibrinogen is composed of two sets of Aα, Bβ and ϒ polypeptide chains arranged dimerically. Each chain is 610, 461 and 411 amino acids in length, respectively (41). Half-molecules of fibrinogen are formed through 24 disulfide bonds between parallel Aα, Bβ and ϒ chains. Full molecules are formed through 5 disulfide bonds between the N-termini of antiparallel Aα, Bβ and ϒ chains in each half molecule (41-43). This is shown in **Figure 1**.

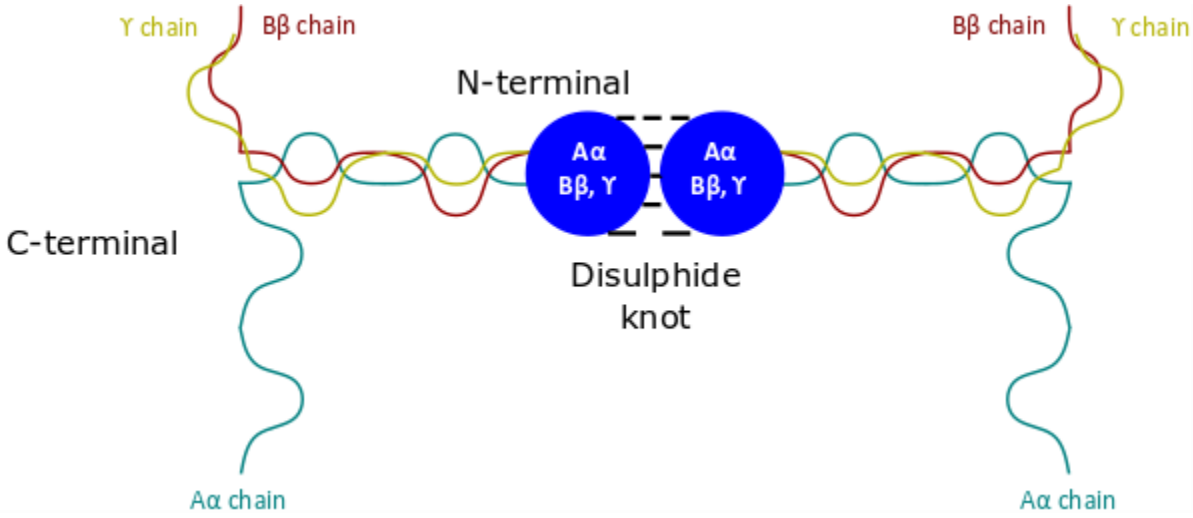


Figure 1: Fibrinogen is composed of 2 half molecules of Aα, Bβ and ϒ chains. 24 disulfide bonds and coiled coil segments hold each half molecule together. The full molecule is formed when 2 half molecules are held together by 5 disulfide bonds between the N-termini of antiparallel Aα, Bβ and ϒ chains. *Reproduced from:* Neutralization of AntiCitrullinated Protein Antibodies in Rheumatoid Arthritis - A Way to Go? (44). Adapted: 03/10/2017.

One molecule of fibrinogen consists of 5 domains: a central E domain and two distal D domains and an outer αC-domain. The E domain consists of a 5 nm diameter node that incorporates the N-Termini of all Aα, Bβ and ϒ chains. The D domains consist of 6.5 nm diameter nodes that incorporates part of the Aα chain as well as parallel C-termini of Bβ and ϒ chains (40, 41). The αC-domains consist exclusively of the C-termini of the Aα chain (45). The E domain and D domains are separated by 16 nm long, 1.5 nm thick coiled-coil triple helix segments in each half-molecule (40, 46, 47). This is illustrated in **Figure 2**.

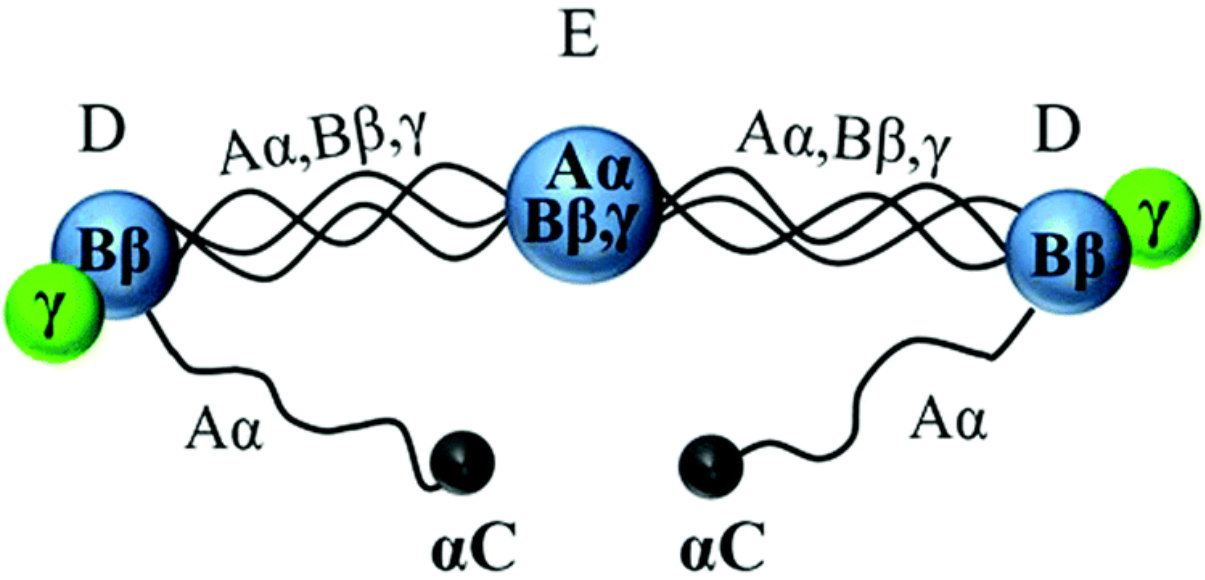


Figure 2: The fibrinogen molecule consists of 5 domains: a central E domain and two distal D domains and an outer αC-domain. Coiled coil segments separate the E and D domains. *Copied with permission from: Fibrinogen: a journey into biotechnology (48)*. Adapted: 24/06/2019.

The isoelectric point of fibrinogen is 5.8 (47). This means that in healthy plasma (which has a pH of approximately 7.4), fibrinogen has a net negative surface charge. However, because the αC-domain is heavily composed of positively charged amino acids, this domain is able to maintain a positive surface charge in healthy plasma (49).

Additionally, there is an alternative version of the ϒ chain known as the ϒ' chain incorporated into some molecules of fibrinogen. Whilst the C-termini of the ϒ-chain ends with a 4 amino acid long sequence from residue position 408, the ϒ' chain instead replaces it with a 20 amino acid long sequence (50). The unique amino acid sequence features sulfated tyrosine residues as well as several negatively charged residues. This allows the ϒ'-chain to bind to the proteolytic enzymes: thrombin and Factor XIII (FXIII) (51).

Because the liver produces 2 different forms of the ϒ-chain, fibrinogen is usually present in the body as 2 variations of the molecule: Fibrinogen 1 and Fibrinogen 2. Fibrinogen 1 is homodimeric and contains a ϒ-chain/ϒ-chain combination. On the other hand, Fibrinogen 2 is heterodimeric and contains a ϒ-chain/ϒ'-chain combination (52-54). Because ϒ'-chains are expressed at much lower levels than ϒ-chains, Fibrinogen 2 generally makes up only 8–15% of all fibrinogen molecules in the body (55).

The expression of Aα and Bβ-chains is regulated through housekeeping mechanisms to maintain constant fibrinogen concentrations in the body (56). However, the relative expression of ϒ-chains and ϒ'-chains in the body is controlled by induced response mechanisms (57). Anti-inflammatory and anti-thrombotic responses are able to up-regulate the expression of the ϒ'-chain (58). Thus, this increases the ratio of Fibrinogen 2 to Fibrinogen 1 circulating in the body.

Overall fibrinogen is a large, bilateral protein with different surface charges at different loci. This means that: a) it has many epitopes for antibodies to bind to and b) it precipitates earlier than other blood proteins when heated/salted out. Hence, these have formed the assaying principle behind antigenic and precipitation-based assays, respectively (see **Section 4.2** for more information).

2.2. Fibrinogen Function

Whilst fibrinogen has several functions in the body, its main one is to prevent blood loss upon vessel damage. Fibrinogen is the effector protein for a couple of haemostatic processes. These include the formation of hard fibrin clots and the aggregation of platelets into the clot network (4). The process is summarised below in **Table 1**.

Table 1: Summary of the function of fibrinogen

Function	Process
Clot Formation	1. Blood Vessel Injury – Contact activation (or Intrinsic) and Tissue Factor (or Extrinsic) Pathway activated. 2. Blood enzymes thrombin and actor XIIIa activated. 3. Thrombin converts fibrinogen into fibrin monomers. 4. Fibrin monomers self-associate – to form soft fibrin clot. 5. Factor XIIIa creates covalent bonds between fibrin monomers – to form hard fibrin clot.
Platelet Aggregation	1. Platelets bind to exposed collagen and Von Willebrand Factor (VWF) from vessel rupture. 2. Platelets secrete over 300 substances that activates nearby platelets. 3. Activated platelets change shape and dimerise their GPIIb/IIIa receptors. 4. Fibrinogen binds to GPIIb/IIIa receptors – aggregating platelets. 5. Hard fibrin clot binds to aggregated platelets incorporating them into a clot.

2.2.1. Clot Formation

Hard fibrin clots are formed when the blood enzyme - thrombin - converts fibrinogen into fibrin fibrils (4). In normal physiological conditions, thrombin circulates the blood in its inactive precursor form (pro-thrombin). However, when blood vessel damage occurs, 2 different coagulation mechanisms are activated: the tissue factor (or extrinsic) and contact activation (or intrinsic) pathway. The extrinsic pathway is activated when blood is exposed to the tissue that surrounds the site of blood vessel damage. The cells of these tissues express a protease receptor called tissue factor (TF) (28). This receptor can activate one cascade of plasma proteins to induce coagulation. The plasma proteins can either be activated on the surface of blood cells (such as platelets) or in the bloodstream (as unbound molecules). On the other hand, the intrinsic pathway is activated when the vessel rupture itself exposes the collagen embedded in the endothelial layer. The collagen then activates another set of coagulation-inducing plasma proteins (59). Regardless of which pathway is activated, the same outcome is achieved: a rapid and efficacious conversion pro-thrombin to thrombin.

Once thrombin is formed, it cleaves 2 key peptides off the E domain of fibrinogen molecules: Fibrinopeptide A (FPA) and Fibrinopeptide B (FPB) (4). This forms fibrin monomers. FPA is located on the N-terminus of the Aα-chain of fibrinogen (60). Once FPA is cleaved, it reveals a polymerization site on each monomer (called E_A). E_A is then able to interact with a neighboring fibrin monomer. It does so via the neighboring molecule's binding pocket located in the D domain of the ϒ chain (called Da) (61). FPB on the other hand is located on the N-terminus of the Bβ-chain of fibrinogen. Once cleaved, it reveals a different polymerization site on each monomer (called E_B) which binds to the D domain of Bβ chains in neighboring monomers (called Db) (62, 63). Additionally, the cleavage of FPB breaks the covalent tethering between the αC-domain and the E-domain. This allows the αC-domains from neighboring monomers to interact

with each other (64, 65). All 3 thrombin-induced interactions allow fibrin monomers to be arranged into motifs of double stranded fibrils capable of branching. This creates a clot structure which is soft (3).

Additionally, thrombin can activate another blood enzyme: Factor XIII (FXIII) by cleaving it and converting it to FXIIIa (66). FXIIIa introduces covalent bonds in-between neighboring fibrin monomers of the soft clot. It achieves this by cross-linking specific amino acid residues between adjacent ϒ-ϒ and ϒ-Aα chains throughout the E_A-Da interaction site (5, 7). This stabilises the soft clot into a hard clot (8, 9). To further maintain the stability of the hard clot, FXIIIa can also catalyse the formation of crosslinks between fibrin and antifibrinolytic proteins (67-69). Once the hard clot is properly formed, it behaves as a scaffold capable of binding to red blood cells (RBCs), endothelial cells and platelets (4). This allows the vessel damage site to be blocked thus preventing further blood loss. A graphical summary on the polymerization of fibrinogen is provided in **Figure 3**.

Unlike the ϒ-chain, the ϒ'-chain contains a high affinity thrombin and FXIII binding site in its C-terminus (70-73). Therefore, Fibrinogen 2 can sequester both enzymes significantly better than Fibrinogen 1. Not only can Fibrinogen 2 serve as the carrier for these enzymes around the blood, but it can also regulate their activity. Siebenlist *et al.* demonstrated that FXIIIa crosslinks Fibrinogen 1 3.5 times faster than Fibrinogen 2 (74). Accordingly, Omarova *et al.* showed that Fibrinogen 2 reduces thrombin generation when compared to the Fibrinogen 1 (75). Due to the perturbing effects that Fibrinogen 2 has on thrombin and FXIII, it causes hard clots to have unique morphologies. Clots with higher compositions of Fibrinogen 2 generally contain smaller volumes, thinner fibres and more branching (53, 76).

The overall conversion of fibrinogen into fibrin clots makes blood significantly more viscoelastic, hydrophobic and opaque. These physical changes have formed the basis of many assaying principles to diagnose fibrinogen concentration (see **Section 4.1** and **Section 5** for more information). Assays that rely on these principles add external sources of tissue factor, thrombin and FXIIIa to cause the clotting of patient plasma samples. Furthermore, these assays can account for increases and decreases in Fibrinogen 2 (and other fibrinogen molecules with lower clotting activity) so that the concentration of functional fibrinogen in a patient can be determined.

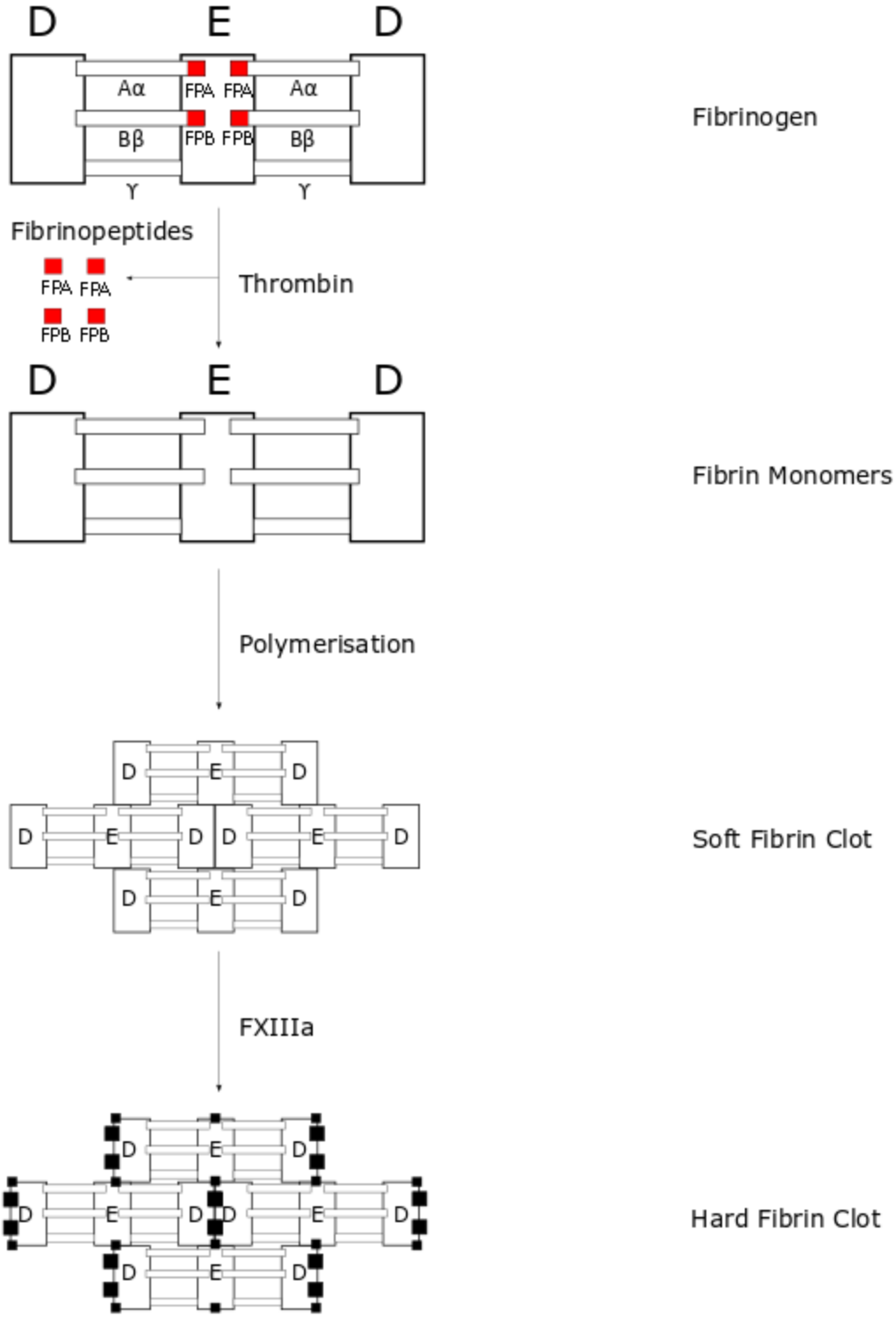


Figure 3: Thrombin cleaves Fibrinopeptides A and B in the E domain of fibrinogen to form sticky fibrin monomers. The fibrin monomers then associate through secondary bonds with other neighboring monomers forming E:D domain interactions to form a soft clot. Finally, Factor XIIIa (FXIIIa) introduces covalent bonds within the E:D domain interactions converting the soft clot into a hard clot (29). *Reproduced from: PDQ Biochemistry, Volume 1.* Adapted 11/10/2017.

2.2.2. *Platelet Aggregation*

Platelet aggregation occurs without requiring the conversion of fibrinogen to fibrin. Platelets normally circulate in an inactivated state. However, when vessel rupture occurs, platelets bind to the exposed collagen and Von Willebrand Factor (VWF) (from the endothelial layer) resulting in their activation (77). They then proceed to secrete over 300 substances that facilitates the surface-activation of activate other platelets nearby the site of damage (78).

The main event that occurs upon platelet activation is the calcium-dependent association of the cell surface glycoprotein (GP) receptors: GPIIb and GPIIIa with each other. When one of these GPs associates with the other, the activated complex: GPIIb/IIIa is formed (79, 80). GPIIb/IIIa is capable of binding to the ϒ chain of fibrinogen (81). Due to the bilateral nature of fibrinogen, one fibrinogen molecule is capable of binding to 2 individual GPIIb/IIIa receptors. Therefore, this allows activated platelets to aggregate in the presence of fibrinogen (82, 83).

The activation of platelets also causes them to change morphology from a flat disc, to an amorphous structure with extensions. This provides an increased membrane surface area so that the platelets can aggregate more efficiently and form a firmer network (84).

The resulting interactions between activated platelets and collagen/VWF/fibrinogen allow a strong platelet plug to be formed at the vessel rupture site (85, 86). Not only is the plug capable of mending the damage to the site, but it is also able to act as a platform for the hard clot to bind onto. This ensures that blood loss is averted at the site of blood vessel damage. An overall schematic of the formation of a platelet plug is illustrated in **Figure 4**.

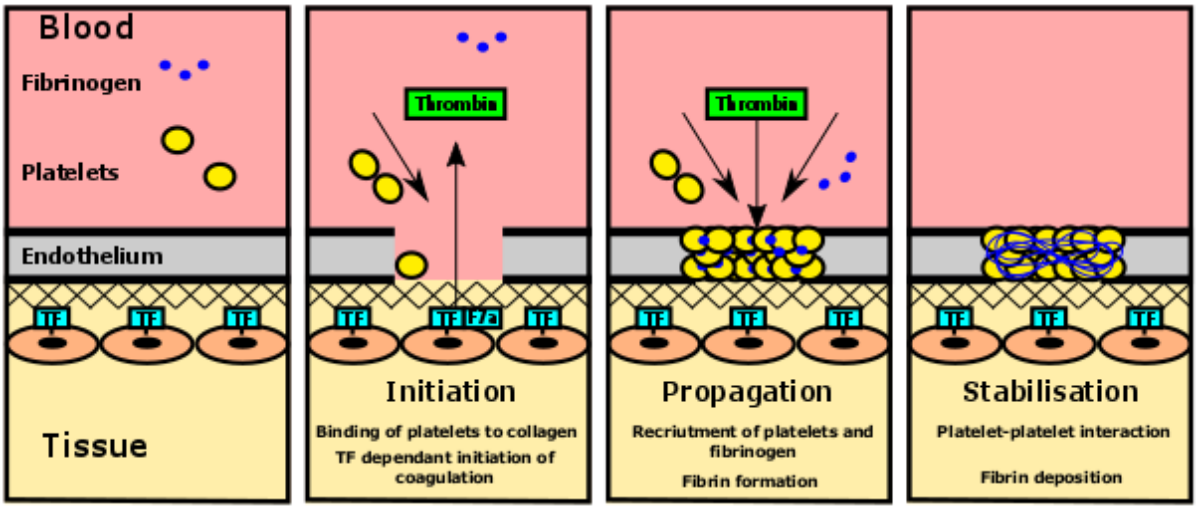


Figure 4: When vessel rupture occurs, exposed tissue factor and collagen initiates the extrinsic and intrinsic pathways of coagulation resulting in the formation of a hard clot. Additionally, platelets can bind to the exposed collagen and Von Willebrand Factor to become activated. They then secrete over 300 substances that activate and recruit near-by platelets. This causes the platelets to undergo fibrinogen-induced aggregation of which a platelet plug forms. The platelet plug serves as a scaffold for the hard clot to bind onto thus blocking off the site of vessel damage. *Reproduced from: Role of the Extrinsic Pathway of Blood Coagulation in Hemostasis and Thrombosis* (87). Adapted: 11/10/2017.

Brinkhous *et al.* identified that platelet aggregation occurs if sufficient fibrinogen is present in plasma (88). Therefore, the use of reagent platelets may be a diagnostic tool for measuring hypofibrinogenemia.

However, at the same time, platelets found in the blood samples of patients are problematic during fibrinogen assays. This is because these platelets can interact with the fibrinogen present and modify its physical properties. Depending on the platelet activity of a particular blood sample, this in turn may alter the measured fibrinogen concentration. Therefore, fibrinogen assays must nullify the effect of these platelets to obtain accurate results. This is done by 1) centrifuging and collecting the platelet-free plasma fraction in whole blood before assaying (see **Section 4.1**) or 2) using platelet inhibitors in whole blood during assaying (see **Section 5**).

Additionally, activated platelets can also facilitate coagulation protein activation on their surface. Therefore, the processes of clot formation and platelet aggregation are not mutually exclusive (82, 83).

3. Diagnostics Used in Clinic

Clinically, plasma fibrinogen concentrations are determined through standard laboratory techniques (SLTs) and viscoelastic haemostatic assays (VHAs). **Section 6** provides a clinical overview. The unique structure and function of fibrinogen (see **Section 2**) gives rise to many assays that quantifies concentrations through different mechanisms. **Table 2** summarises the main assays in clinical use, their concept and their limitations.

Table 2: Summary of commonly used clinical assays for measuring fibrinogen concentration.

Assay	Tests with (+ Activating reagent)	Concept	Measurement that implies low fibrinogen	Limitations
Clauss Assay	Plasma (+ thrombin)	Thrombin clots plasma. Fibrinogen concentration alters thrombin kinetics.	↑ clotting time	-Other blood substances can also affect thrombin kinetics -Heavy plasma dilution needed -Time consuming -Many steps required
Prothrombin Time Assay	Plasma (+ TF)	Tissue Factor (TF) initiates clotting. Clotting alters plasma opacity.	↓ opacity of clot	-Time consuming -Overestimates fibrinogen concentration
Precipitation Type Assays	Plasma (+ heat or salts)	Fibrinogen precipitates earlier than other blood proteins (due to structure). Precipitation alters plasma opacity.	↓ opacity of precipitate	-Time consuming -Affected by other blood parameters. -Precipitates other blood proteins.
Immunological Type Assays	Plasma (+ antibodies)	Antibodies bind to epitopes on fibrinogen. Binding is measured through precipitation or colorimetric assays.	↓ precipitation ↓ colorimetric labelling	-Many steps required -Extremely time consuming. -Affected by fibrin degradation products.
Viscoelastic Haemostatic Assays	Whole Blood (+ TF and platelet inhibitors)	TF initiates clotting. Clotting alters blood viscoelasticity.	↓ viscoelasticity of clot	-Platelet inhibitors imperfect -Extremely Expensive

4. Standard Laboratory Testing

There are more than 60 Standard Laboratory Tests (SLTs) procedures available that can determine patient plasma fibrinogen concentration (89).

Fibrinogen Concentration SLTs can be divided into 2 main assay types: functional assays (FAs) and antigenic assays (AAs). FAs measure the concentration of plasma fibrinogen that is clottable via the conversion fibrinogen into fibrin. The most clinically common of these includes the Clauss assays and prothrombin time (PT) derived fibrinogen assays. Whereas, antigenic assays (AAs) measure the total plasma fibrinogen without converting fibrinogen into fibrin. AAs consist of tests such as immunological and precipitation based assays (20). Due to the many fibrinogen assays that exist, this section will only review the ones in common clinical use. This analysis includes the concept and shortcomings behind each one.

4.1. Functional Assays

Functional assays (FA) have remained popular since the 1950 due to fibrin's unique properties (90-93). When compared to uncoagulated plasma, Fibrin is significantly more hydrophobic, viscoelastic and opaque (94-96). All FAs attempt to exploit and measure the change in at least one of these properties to determine the plasma fibrinogen concentration.

Ratnoff and Menzie *et al.* pioneered FAs by measuring the total quantity of plasma protein that is clottable. These assays involve adding thrombin into plasma samples, centrifuging off the fibrin clot, washing them and dissolving them in urea for spectrophotometric analysis (90). Whilst clottable protein assays can produce accurate results, the steps required are very laborious, time consuming and prone to human error. Because of this, Clauss assays have arisen and been preferred in emergency situations for diagnosing early hypofibrinogenemia.

Clauss *et al.* noticed an inverse correlation between plasma fibrinogen concentration and fibrin clotting time when anticoagulated plasma samples become incubated with excessive levels of exogenous thrombin. This property was exploited to determine the patient plasma fibrinogen concentration (92). In this test, the patient plasma is collected, diluted in isotonic buffer, heated to 37 °C before thrombin is added. The time taken for clot formation is then recorded, where longer clotting times correlates with lower fibrinogen concentrations (20). A visual summary of a Clauss assay is shown in **Figure 1**. In a study comparing the Clauss assay to other SLT procedures, Miesbach *et al.* concluded that the Clauss assay was the diagnostic tool of choice for measuring low fibrinogen levels. This was due to its significantly greater accuracy in identifying patients with low plasma fibrinogen concentrations (97). However, despite this conclusion, utilizing Clauss assays for early hypofibrinogenemia still poses many significant problems.

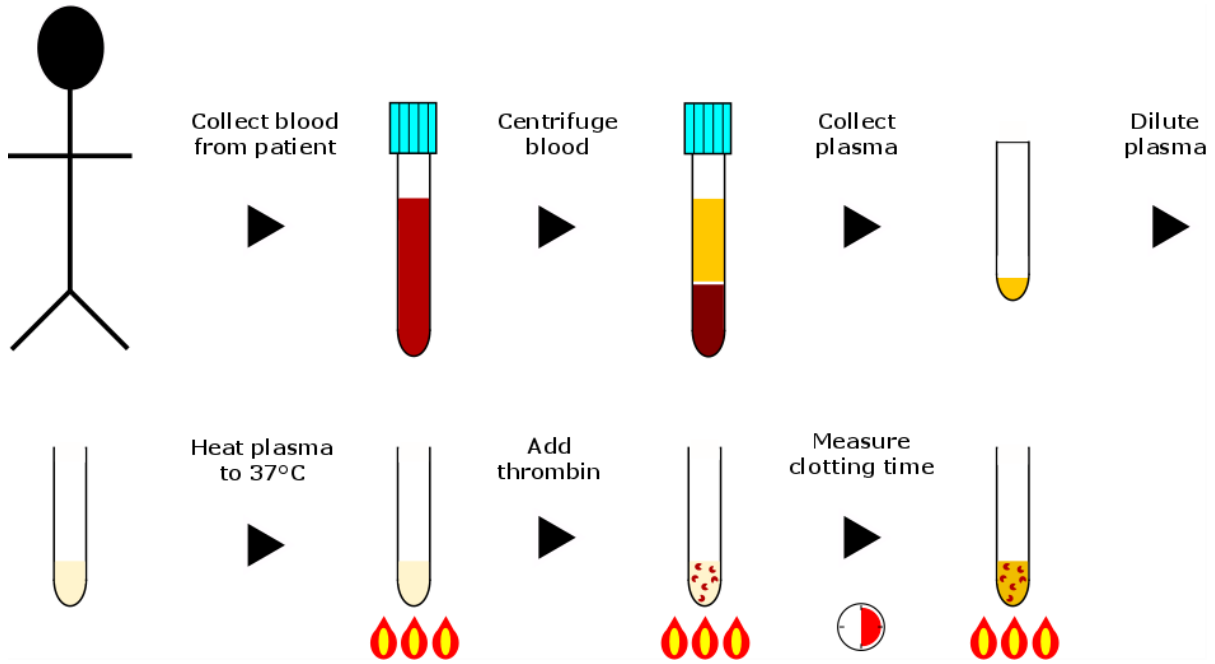


Figure 1: Standard Technique of Clauss assays. The Clauss assays cannot diagnose early hypofibrinogenemia fast enough due to the requirement of multiple and time-consuming steps which require sophisticated laboratory based analytical instrumentation. This includes an unavoidable 10–15 min pre-centrifugation step to separate blood cells from plasma. Adapted: 08/07/2019.

First, Clauss assays can only accurately estimate fibrinogen concentration when it has an inversely linear relationship with the clotting time. Mackie *et al.* reported that this occurs within a concentration range of ten to thirty times below normal physiological concentrations of plasma fibrinogen (98). Therefore, all samples must be heavily diluted in isotonic buffers to be prepared properly. An example of a calibration curve displaying the dilutions used for measurement is shown in **Figure 2**. Second, many endogenous substances already in the plasma can affect the measured clotting time. The World Health Organization (WHO) reported in 1999 that endogenous thrombin (i.e., thrombin that is already present in the plasma) can cause fibrin formation even before the start of the assay. Hence, this can increase the measured clotting time (99). On the other hand, natural thrombin inhibitors can have the opposite effect. Seifried *et al.* and Lowe *et al.* commented that molecules such as heparin, Fibrinogen 2 and fibrin degradation products (FDPs) can decrease the measured clotting time (100, 101). If plasma samples contain sizable amounts of these substances, inaccurate readings can occur.

Prothrombin time (PT) derived fibrinogen assays are another FA for measuring fibrinogen concentration. The primary purpose of a PT assay is to evaluate the tissue factor (or extrinsic) pathway of a plasma sample. This is achieved by incubating the plasma sample at 37 °C, before adding tissue factor and measuring the time for clot formation to occur (102). Hence, an elevation in the duration indicates that coagulopathy is present in the extrinsic pathway. However, whilst running PT assays, Chantarangkul *et al.* coincidentally showed that the concentration of fibrinogen can also be correlated to the total change in turbidimetry (light absorbance) and nephelometry (light scattering) of the formed clot (103). An illustration showing how these properties are measured is given in **Figure 3**. PT assays are highly favored by clinics for measuring fibrinogen concentration because the result comes at no extra cost to determining the extrinsic pathway functionality (20, 97).

Despite the economic advantages of PT assays, their ability to detect hypofibrinogenemia is very poor. Many studies comparing PT assays to other FAs have consistently found that PT assays produce significantly higher fibrinogen concentration measurements (97, 104-108). Furthermore, Mackie *et al*. found that this discrepancy becomes especially pronounced when fibrinogen concentrations drop below 1 g/L. He believed that the discrepancy is due to the fact that PT assays falsely measure FDPs and non-functional fibrinogen (98). Because PT assays can misdiagnose hypofibrinogenemic plasma samples as having normal fibrinogen concentrations, they can delay hypofibrinogenemic patients from receiving fibrinogen replacement therapy.

Recently however, PT assays have been recommended to measure total fibrinogen concentration instead. Miesbach *et al.* concluded that PT-derived fibrinogen assays estimate the fibrinogen concentration to be similar to fibrinogen antigenic assays (97). Whilst, Xiang *et al.* had found suitability in using PT assays in diagnosing dysfibrinogenemia (see **Section 4.2** for more information) (109).

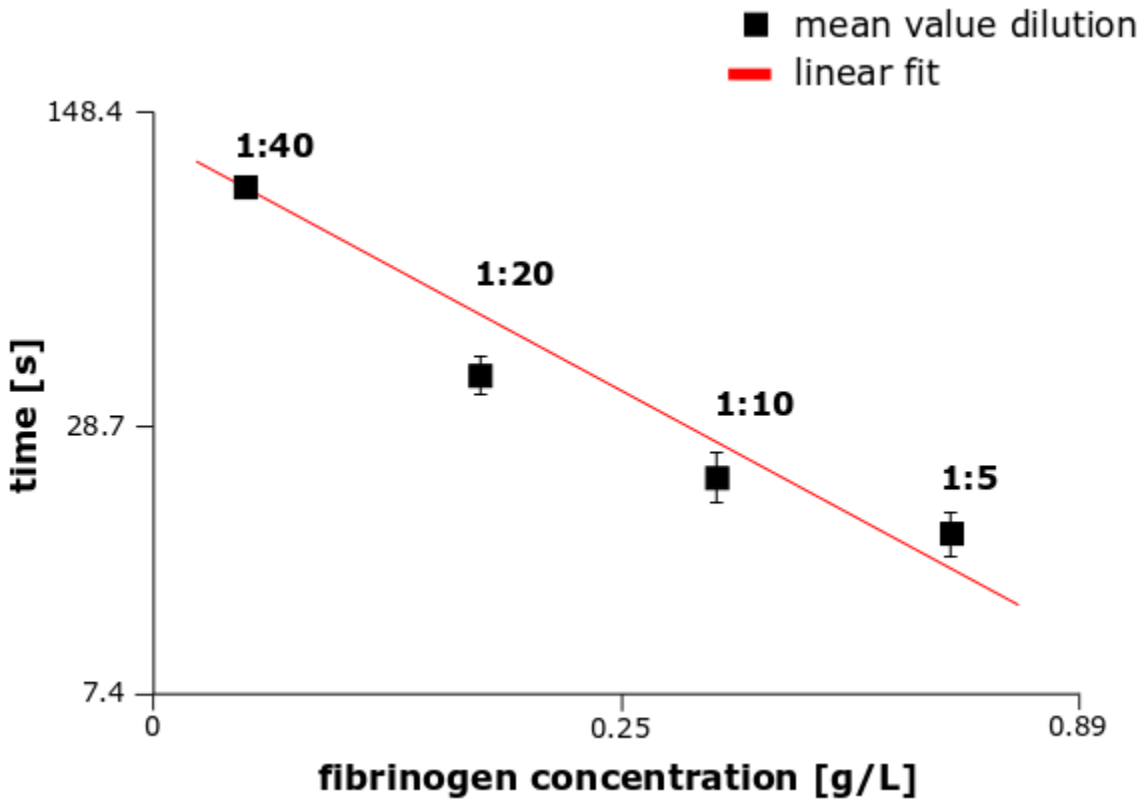


Figure 2: Turbidimetric and nephelometric assays. In these assays, fibrinogen is selectively precipitated in a plasma sample by: a) heating, b) salting out, c) adding antibodies or d) inducing coagulation. Then, a beam of incident light is passed through the sample. As precipitation occurs, the amount of light that transmits through decreases and the angle at which light scatters through increases. These types of assays are highly sensitive to changes in pH, viscosity and FDPs. Hence, they are poor at diagnosing hypofibrinogenemia. *Copied with permission from: Adsorption of cationic copolymer nanospheres onto cotton fibers investigated by a facile nephelometry (110).* Adapted: 05/01/2018.

4.2. Antigenic Assays

Because antigenic assays (AA) do not measure the functionality of a patient's fibrinogen, these are generally used as secondary assays to functional assays (FA). Whilst AAs in major haemorrhage protocols (MHPs) can be used to diagnose patient fibrinogen concentrations on their own, these are mainly implemented complementary to FAs to determine the ratio of functional fibrinogen to total fibrinogen in patients. Hence, this can clarify if they suffer from hypofibrinogenemia or dysfibrinogenemia (111, 112). AAs broadly fall into 2 categories: precipitation and immunological type assays.

Precipitation assays are formed on the principle that fibrinogen has the lowest solubility of all plasma proteins. This is due to its large, symmetrical structure (see **Section 2.1** for more information). Fredericq *et al.* noticed that fibrinogen precipitates at a temperature (i.e., 56 °C) much lower than other proteins (i.e., 75 °C) (113). Since then, heat precipitation assays have been created around this phenomenon (114-122). These assays heat plasma samples at 56 °C for several minutes to suspend out the fibrinogen. Traditionally, the fibrinogen concentration was then determined by centrifuging the plasma and quantifying the precipitate through gravimetric or volumetric analysis (114-119). However, because of the labour involved in isolating the precipitate for analysis, modern assays estimate the fibrinogen concentration by using turbidimetry and nephelometry to compare the plasma before and after heating instead (120-122). An illustration of how turbidimetry and nephelometry are measured is provided in **Figure 3**. Due to the requirement of heating equipment, chemical precipitation assays have also been developed to simplify the assay. Parfentjev *et al.* created an assay that correlates the plasma fibrinogen concentration to the resulting turbidity when plasma samples become precipitated with 12% ammonium sulfate (123). Since then, newer chemical precipitation assays have been developed which use sodium sulfite and glycerol as the precipitant instead (124, 125).

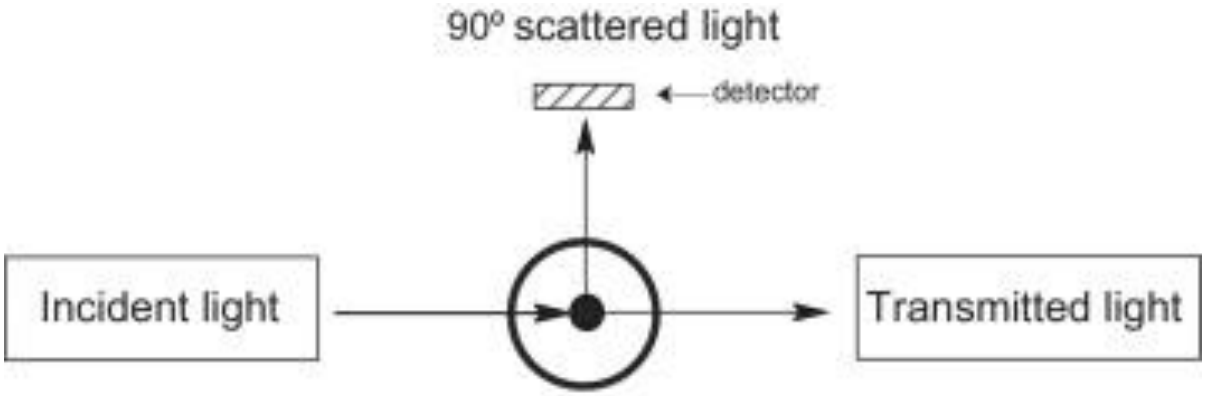


Figure 3: Turbidimetric and nephelometric assays. In these assays, fibrinogen is selectively precipitated in a plasma sample by: a) heating, b) salting out, c) adding antibodies or d) inducing coagulation. Then, a beam of incident light is passed through the sample. As precipitation occurs, the amount of light that transmits through decreases and the angle at which light scatters through increases. These types of assays are highly sensitive to changes in pH, viscosity and FDPs. Hence, they are poor at diagnosing hypofibrinogenemia. *Copied with permission from: Adsorption of cationic copolymer nanospheres onto cotton fibers investigated by a facile nephelometry (126).* Adapted: 17/01/2018.

Whilst precipitation assays can be rapid (provided they do not require sample preparation or manual analysis), these perform poorly at differentiating between low and normal plasma fibrinogen concentrations (127-131). Furthermore, Dintenfass *et al.* and Desvignes *et al.* noticed that factors such as cryoglobulins, high plasma viscosity, fibrin degradation products (FDPs) and pH variations can all greatly perturb the accuracy of heat precipitation assays (121, 132). Finally, Grannis *et al.* concluded that

chemical precipitation assays incompletely precipitate fibrinogen and partially precipitate other plasma proteins (133). For these reasons, precipitation assays are avoided in diagnosing early hypofibrinogenemia.

Immunological assays utilise anti-fibrinogen antibodies to measure plasma fibrinogen concentrations. Ouchterlony *et al.* in 1949 first demonstrated that antibodies and antigens can diffuse through agar gels and form visible white immunoprecipitates when they reach each other (134). Eventually, Mancini *et al.* modified this procedure to quantify the concentration of antigen present in a solution. By pre-embeding antibodies in agar gels, cutting holes in it, adding antigen and observing the formation of precipitin rings around the hole, she concluded that the size of the rings are proportional to the antigen concentration added to each hole (135). This This procedure would eventually become known as a radial immunodiffusion (RID) assay. Finally, Brittin *et al.* illustrated that RID assays are suitable for quantifying plasma fibrinogen concentration when he concluded that they have accurate correlations when compared to Clauss assays (136). One major problem with using RID assays to determine fibrinogen concentrations, is that they require 48 h (121, 135). Because of this, alternative immunological assays have been developed to diagnose plasma fibrinogen concentrations more rapidly. These include immunonephelometry assays, immunoelectrophoresis assays, enzyme linked immunosorbent assays (ELISAs) (137-139).

Despite the improvements to newer immunological assays, some still require significant amounts of time. Mackie *et al.* reported that ELISAs and immunoelectrophoretic assays take “many hours to perform” (20). This is far too long to diagnose early hypofibrinogenemia. Other immunoassays such as immunonephelometry are also extremely sensitive to FDPs. Hoffman *et al.* and Jelić-Ivanoić *et al.* both found that the presence of FDPs in plasma cause the fibrinogen concentration to be over-estimated in immunonephelometry (140, 141). This can be detrimental toward differentiating between low and normal levels of fibrinogen. Therefore, immunological assays provide significant issues when diagnosing early hypofibrinogenemia.

4.3. Standard Laboratory Tests (SLT) Overview

All standard laboratory tests (SLTs) require large, expensive and complicated equipment for plasma fibrinogen concentration analysis. This includes a pre-centrifugation step to retrieve plasma from blood samples. Furthermore, important variations can arise in the measured parameters each time an SLT is performed on a source of plasma. This occurs even when the same combination of reagents and equipment is used. Therefore, most SLTs require serial dilutions of known fibrinogen concentrations to be simultaneously measured with patient plasma for calibration. An example of this is shown in **Figure 2** which illustrates a calibration curve prepared for a Clauss assay. Depending on the nature of the assay, significantly more time, resources and labour can be required just to prepare these extra samples. Finally, there can be important differences in the equipment and reagents that each SLT and clinic uses. This can result in variation with the measured fibrinogen concentration between clinics. For these reasons, these tests are inadequate for emergency and point of care applications.

5. Viscoelastic Testing

Because of the criticisms surrounding fixed-ratio transfusions (**Section 6**) and SLTs, recent major haemorrhage protocols (MHPs) make use of viscoelastic haemostatic assays (VHAs). The formation of clots in whole blood samples upon coagulation causes an increase in viscoelasticity. Therefore, VHAs can quantify plasma fibrinogen concentration by measuring changes in blood viscoelasticity after clotting. Hartert *et al.* first described that viscoelastic (VE) changes in blood occur during fibrin polymerization. Furthermore, these could be graphically recorded as changes in clot strength (21). However, Kang *et al.* first documented the use of VHAs in clinics when he monitored the blood coagulation system of 66 patients during liver transplantation. By doing this, he saved 33% in blood product transfusions (142). A VE diagnostic consists of a cup and a suspended pin connected to a detector system. An anticoagulated patient whole blood sample is incubated in the cup at 37 °C and coagulation is induced by the addition of tissue factor (ie. extrinsic) or contact activation (ie. intrinsic) pathway activators. The cup and the pin are oscillated relative to each other. As hard fibrin clot formation occurs between the cup and pin, it is picked up by the detector system and graphed (143, 144). This is illustrated in **Figure 4**. There are 2 types of VE diagnostics used in clinics: thromboelastography (TEG®) and thromboelastometry (ROTEM®). TEG® features a rotating cup in which the fibrin formed attaches to it and transmits movement to the pin (145). ROTEM® features a rotating pin in which the fibrin formed binds to the cup and impedes pin movement (146).

Both TEG® and ROTEM® can be used to test many different parameters of the blood. However, this section will focus on the clinical tests both diagnostics use to measure plasma fibrinogen concentrations as well as their limitations.

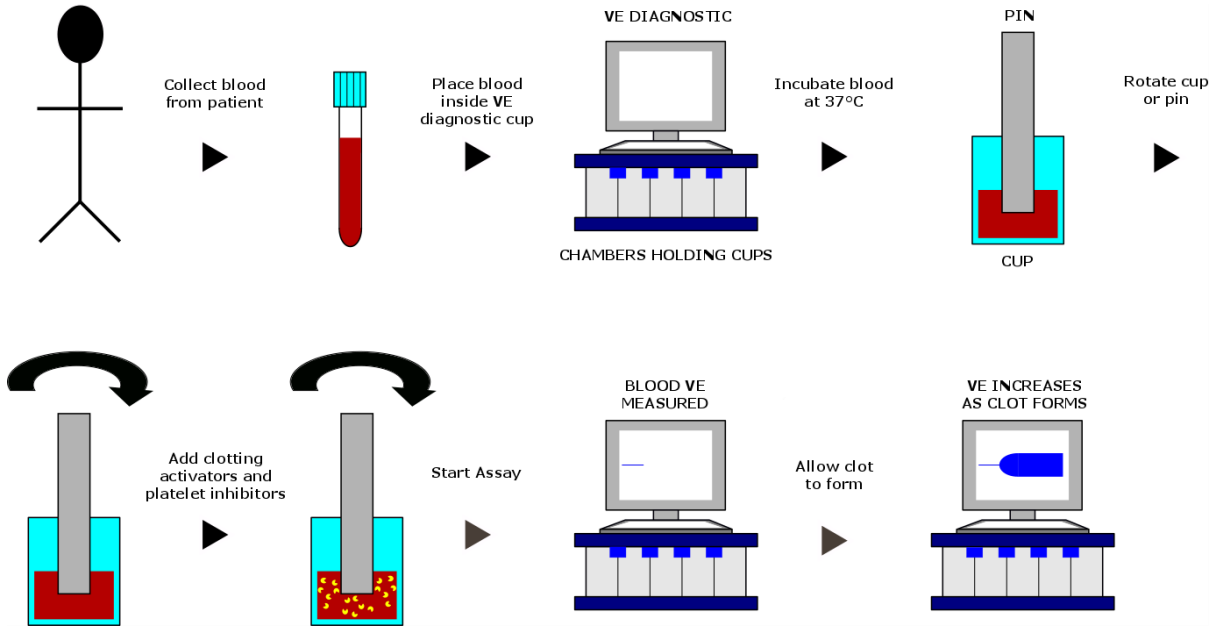


Figure 4: Standard technique for VHA. VHAs can measure the viscoelasticity of blood during clotting to determine plasma fibrinogen concentration. However, it is complicated by time delays, very expensive infrastructure and the imperfect efficacy of platelet inhibitors. Adapted: 10/07/2019.

The clinical tests used by TEG® and ROTEM® to measure plasma fibrinogen concentration are called functional fibrinogen (FF) and FIBTEM™, respectively (147, 148). Khurana *et al.* reported that there are

two factors in blood that contribute to the strength of blood clots in viscoelastic testing: platelets and fibrin. Therefore, to exclusively measure the fibrin contribution toward clot strength on TEG®, these tests pre-treat whole blood with the platelet inhibitors: abciximab and cytochalasin D (149). Whilst each inhibitor inhibits platelets through different mechanisms, they attempt to nullify any contribution of platelets toward the clot strength. Today, FF routinely uses abciximab whilst FIBTEM™ consistently relies on cytochalasin D instead (150). Once coagulation is induced, FF and FIBTEM™ can measure the maximum amplitude (MA) or maximum clot firmness (MCF) of the blood clot, respectively. Both parameters indicate the maximum hard fibrin clot strength (151). Hence, maximum clot firmness (MCF) directly correlates with the fibrinogen concentration. An example displaying the graphs produced from FF and FIBTEM is shown in **Figure 5**.

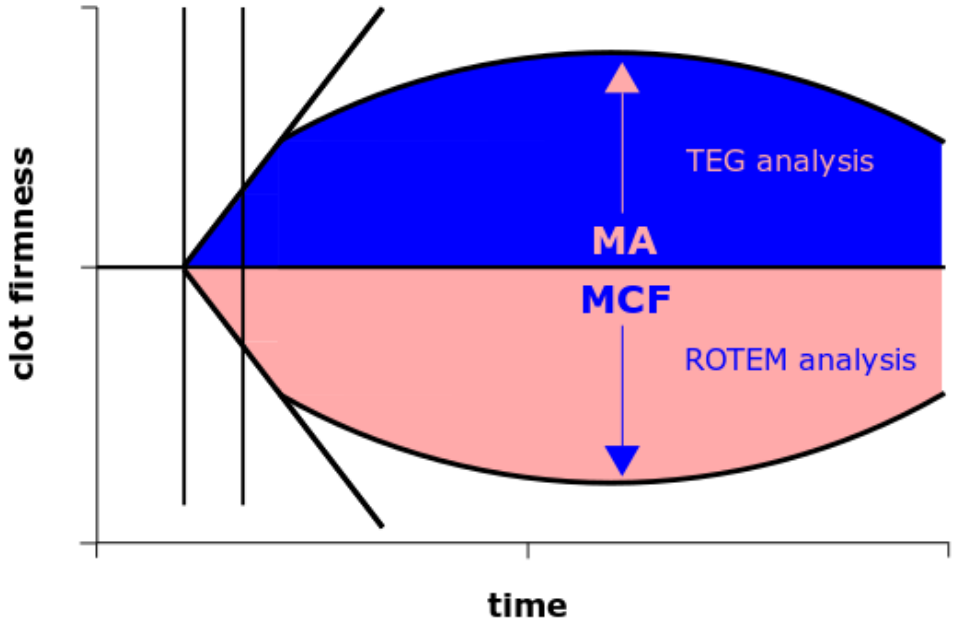


Figure 5: Graphical output of TEG® (above) and ROTEM® (below). Both diagnostics measure clot strength (firmness) over time. Since clot strength depends on the contribution of both platelets and hard fibrin, both diagnostics require the use of platelet inhibitors to measure fibrinogen concentration. The tests that measure fibrinogen concentration are called FF and FIBTEM™, respectively. The parameters MA and MCF are measurements of the maximum clot strength. They directly correlate to plasma fibrinogen concentration in both tests. *Reproduced from: TEG® and ROTEM® in trauma: similar test but different results? (152).* Adapted: 12/02/2018.

VHAs have been shown to diagnose hypofibrinogenemia in a timely manner. Huissoid *et al.* and Schöchl *et al.* both reported that FIBTEM and FF can achieve a result 5-15 min after taking blood from the patient (153, 154). Therefore, these tests have been the driving force for many clinics to implement goal directed MHPs over fixed-ratio or SLT-based MHPs. However, despite this fact, VHAs still have shortcomings.

First, the efficacy of the platelet inhibitors has come under criticism. In 2004, a study was performed to quantify the effect of abciximab and cytochalasin D on TEG®. The authors found that when both platelet inhibitors were used individually, they produced higher MAs than when used in combination (155). Since, both FIBTEM™ and FF only use one type of inhibitor, these methods can overestimate fibrinogen

concentrations. Årgen *et al.* found that FF over diagnosed patient plasma fibrinogen concentrations by 1 g/L when compared to Clauss assays (156). The imperfect efficacy of platelet inhibitors renders both tests problematic for correctly diagnosing hypofibrinogenemia.

Second, the price of VE diagnostics are very high. Whiting *et al.* calculated that a 4 channel TEG® or ROTEM® device of 3 year life expectancy with software, connections, printer and trolley costs £20,000 (AU$35,168) or £32,584 (AU$57,285) (157). Furthermore, because fresh cups, pins and other reagents must be used for each test, operating expenses are also particularly high. Winerals *et al.* commented that a set of tests on a 4 channel ROTEM® device costs approximately AU$44 (158). Finally, VE diagnostics require highly trained specialists to calibrate, troubleshoot, operate, interpret results and conduct maintenance. Whiting *et al.* estimated that costs involved in training and maintenance exceeded £2,000 (AU$3,517) per year (157). Therefore, not only do these costs act as major deterrents for some clinics to transition from fixed-ratio to VHA-guided MHP, they also prohibit clinics in developing countries from implementing them (159, 160).

Overall, the implementation of VHAs in clinical MHP still remains a controversial topic. VHA-guided MHPs have not yet demonstrated a survival advantage against SLT-guided MHPs or fixed-ratio MHPs (158, 161-163).

6. Clinical Overview of Major Haemorrhage Protocols (MHPs)

Early hypofibrinogenemia diagnostics are used mostly as a part of clinical major haemorrhage protocols (MHPs). These diagnostics determine whether a haemorrhagic patient requires fibrinogen replacement therapy (FRT) or not. FRT is commonly implemented by clinics when their fibrinogen levels fall below 1.5 g/L to 2 g/L (14). The method of FRT varies between clinics around the world and can be achieved using fresh frozen plasma, cryoprecipitate or fibrinogen concentrate. Depending on the MHP selected, some clinics may not use hypofibrinogenemia diagnostics and instead transfuse the patient with either fresh frozen plasma or cryoprecipitate immediately to "empirically replenish all blood factors at once."

Major Haemorrhage Protocols (MHPs) are utilised worldwide once a patient with active critical bleeding is identified. They are used to mitigate the correlated coagulopathy (164). Holcomb *et al.* demonstrated that the successful implementation of MHPs improved outcomes (165). However, much controversy lies between different clinics around the world regarding what an "ideal" MHP might be (166, 167).

Since 2010, many clinics' MHPs have used fixed volumetric ratios of fresh frozen plasma (FFP), cryoprecipitate, platelet concentrate (PC) and packed red blood cell (PRBC) transfusions (168). However, fixed ratio MHPs that use FFPs and cryoprecipitate pose several problems. First, the time needed to thaw and transfuse both products is long. Stanworth *et al.* reported a median FFP time delivery time of 68 min and a median cryoprecipitate delivery time of more than 2 h across UK trauma centres (169). Second, criticism has arisen as these generic fixed ratio MHPs cannot be tailored to correct individual patients' coagulopathies (170, 171). Schöchl *et al.* argued that the FFP and cryoprecipitate used in fixed ratio MHPs is not standardised. Hence, it can leave different patients being unknowingly over or under-transfused with CFs at random (170). Third, the use of FFP or cryoprecipitate has been reported to have side effects in patients (170, 172-175). The recent RETIC Trial studied the effects of FFP on reversing trauma induced coagulopathy. However, it had to be terminated early due to adverse effects in the group treated with plasma (176). Finally, fixed ratio transfusions in part dilutes fibrinogen to concentrations well below the threshold levels for clotting (177-179). This has caused prolonged clotting times and significantly increased massive transfusion requirements. The recent PROPPR study found that the mortality rate of fixed ratio transfusions varied between 22-26% after 30 days (**Figure 6**) (180). As there is a strong association between fixed ratio MHPs and the proportion of deaths that have occurred, alternative MHPs have been used instead.

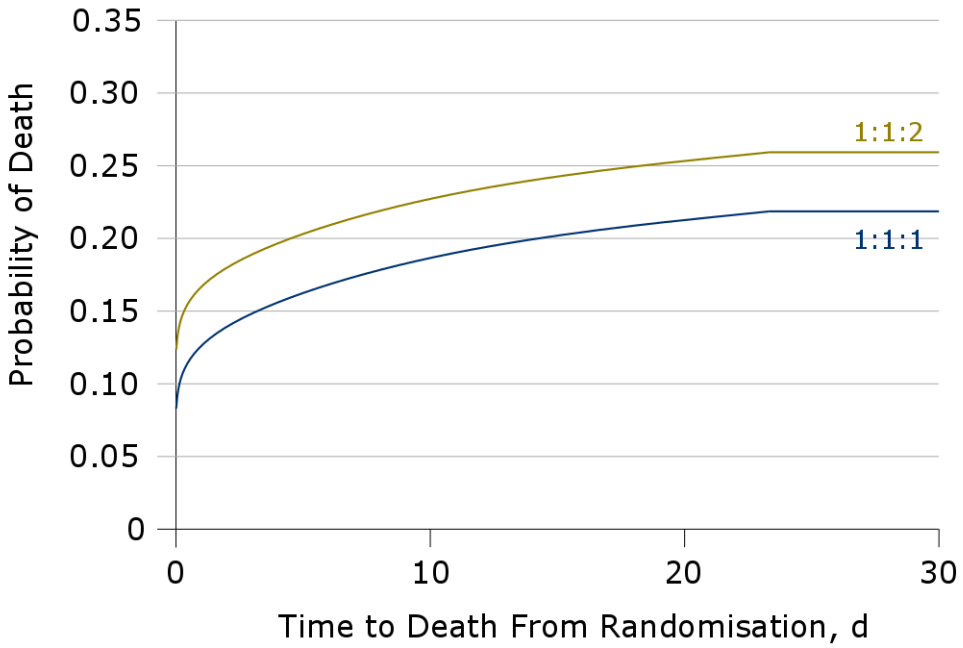


Figure 6 from the Pragmatic, Randomised Optimal Platelet and Plasma Ratios (PROPPR) study which compared the difference in mortality for 2 different standard fixed ratio transfusions. One group of trauma patients was given a fixed FFP:PC:PRBC ratio of 1:1:1 units and the other group was given a fixed ratio of 1:1:2 units. The 30 day mortality was between 22-26% in both groups respectively. *Reproduced from: Transfusion of Plasma, Platelets, and Red Blood Cells in a 1:1:1 vs a 1:1:2 Ratio and Mortality in Patients With Severe Trauma: The PROPPR Randomized Clinical Trial* (180). Adapted: 25/09/2017.

Goal-directed MHPs provide more flexibility than fixed ratio MHPs. They diagnose the specific coagulopathies of haemorrhagic patients and allow directed therapy to correct the specific conditions. Assays that diagnose coagulopathy have traditionally relied on standard laboratory techniques (SLTs) (181). These SLTs are generally time consuming and resource intense (182). Given the life-threatening nature of major haemorrhage, there has been minimal evidence that SLTs provide any survival advantage in haemorrhagic patients (183-186). However, with the recent advancement of Viscoelastic Haemostatic Assay (VHA) diagnostics and the increased availability of individual lyophilised CFs, countries around the world have started using goal directed MHPs instead (187, 188).

Once hypofibrinogenemia has been diagnosed, this condition can be quickly resolved through the use of fibrinogen concentrate (FC). Whilst FC requires reconstitution prior to admission, Rahe-Meyer *et al.* reported that an administration time of 5 min was possible for FC (174). Thus, the combination of VHAs and FC may improve survival in patients suffering from early hypofibrinogenemia when compared to fixed ratio MHPs (15, 16).

However, the routine use of VHA diagnostics in emergency departments and “in the field” is still problematic due the high cost of equipment. Furthermore, “in the field” use of VHA diagnostics still requires generated electricity and the proper training of key paramedical personnel (160, 189). Therefore, the ability of VHAs to diagnose early hypofibrinogenemia still has its limitations.

The different types of SLTs used to diagnose hypofibrinogenemia are discussed in **Section 4**. Likewise, the specific VHAs use to diagnose hypofibrinogenemia are discussed in **Section 5**.

7. Point of Care Diagnostics

Both SLTs and VHAs alike are disadvantaged by their inability to be used outdoors directly at the site of traumatic injury. Hagemo *et al*. reported that ROTEM devices could be set up in field hospitals and air ambulances (190). However, these devices still require electrical and tabletop infrastructure to be operational.

Trauma induced major haemorrhage alone is the leading cause of mortality for individuals aged 18–39, contributing over 2 million deaths annually worldwide (174, 191-196). Furthermore, the early depletion of fibrinogen in trauma is also prevalent. McQuilten *et al.* identified that 21.2% of major trauma patients had fibrinogen levels below 2 g/L (197).

The development of portable point of care (POC) diagnostics for measuring early hypofibrinogenemia has only been recent. The best-known example is the dry hematology method (DRIHEMATO®) (198). This test method was reported to show nearly identical readings to a Clauss assay. However, it still requires, complicated infrastructure and electricity to operate.

Recently, we have developed two paper-based diagnostics that can detect hypofibrinogenemia. One which analyses plasma and another which works with whole blood. The plasma diagnostic is a two-step procedure: first, plasma is added onto thrombin-treated paper strips where fibrinogen is converted to fibrin, then the strips are placed into an aqueous dye bath where elution occurs. The test operates by measuring the change in hydrophobicity – which increases with fibrinogen concentration (199). We have also engineered this diagnostic into a POC hand-held diagnostic (200). The whole blood diagnostic is also a two-step procedure: first, a pre-mixed, droplet of whole blood and thrombin is formed on a solid substrate, then a paper strip is placed on top of the droplet where blood wicking occurs. This test operates by measuring the change in permeability of the droplet – which decreases with increasing fibrinogen concentration (201).

The field of POC diagnostics for early hypofibrinogenemia can potentially revolutionise clinical MHPs for trauma - by allowing diagnosis to take place “in the field”, at the site of patient retrieval. Therefore, any further development in this field may improve the outcome of trauma patients.

8. Overview

Fibrinogen is essential for the process of blood clotting. It is present at plasma concentrations of 2–4 g/L in healthy non-pregnant adults. In coagulopathic patients, it can fall to critically low levels (<1.5–2 g/L) and cause haemorrhage and death. Fibrinogen is one of the first blood proteins to fall below critical levels in many coagulopathies. Therefore, the early detection of hypofibrinogenemia and replenishment may significantly improve survival outcomes.

Early hypofibrinogenemia has been traditionally diagnosed through standard laboratory tests (SLTs). SLTs are restricted by the slow and resource intensive steps that need to be carried out. This includes the need to transport blood samples to a laboratory, pre-separate the plasma from blood cells by centrifugation and the subsequent assaying steps to measure the fibrinogen concentration of these plasma samples. Therefore, they have never been commonly implemented in clinical major haemorrhage protocols (MHPs). However, viscoelastic haemostatic assays (VHAs) – which have emerged in the last 20 years – can work directly with whole blood. This has seen some clinics use FIBTEM™ and functional fibrinogen to diagnose early hypofibrinogenemia instead. Overall, neither mode of diagnosis has ever demonstrated a conclusive survival advantage over fixed-ratio transfusions.

Fibrinogen has a unique structure and function. It is a large and bilateral protein with different surface charges at different loci. Hence, it can precipitate before other plasma proteins. Furthermore, fibrinogen can specifically interact with thrombin and factor XIIIa to form fibrin clots – and make blood significantly more viscoelastic, hydrophobic and opaque. The unique structure of fibrinogen and its functional interactions form the basis of a wide range of methods for measuring its concentrations. This includes the methods behind recently developed point of care (POC) diagnostics.

POC testing offers an opportunity for the early recognition of hypofibrinogenemia in critical bleeding settings. POC testing removes requirements of sample transportation and medical laboratory infrastructure needed for SLTs and VHAs. Therefore, they can be used outdoors in critical situations such as trauma. This enables medical personnel to diagnose early hypofibrinogenemia at the site of injury so that fibrinogen replacement therapy (FRT) can be given immediately.

9. Nomenclature

AA – Antigenic Assay
CF – Coagulation Factor
E_A-Da/E_B-Db – Binding interfaces between 2 neighbouring fibrin monomers.
FA - Functional Assay
FC - Fibrinogen Concentrate
FDP – Fibrin Degradation Product
FF - TEG® test that measures fibrinogen concentration
FFP – Fresh Frozen Plasma
Fibrinogen 1 – Fibrinogen molecule containing ϒ/ϒ chain combination
Fibrinogen 2 - Fibrinogen molecule containing ϒ/ϒ’ chain combination
FIBTEM™ - ROTEM® test that measures fibrinogen concentration
FPA/FPB – Fibrinopeptide A/Fibrinopeptide B
FXIII/FXIIIa – Factor 13/Factor 13a
GPIIb/IIIa – Glycoprotein receptors on platelets that interact with fibrinogen, collagen and Von Willebrand Factor.
Hypofibrinogenemia – Low Fibrinogen Concentration
MA – Maximum clot strength measurement on TEG®
MCF – Maximum clot strength measurement on ROTEM®
MHP – Major Haemorrhage Protocol
Nephlometry - Light scattering
PC – Platelet Concentrate
PRBC – Packed Red Blood Cells
PROPPR study - Pragmatic, Randomised Optimal Platelet and Plasma Ratios study
PT – Prothrombin Time
RETIC Trial - Reversal of trauma-induced coagulopathy Trial
ROTEM® - Thromboelastometry
SLT - Standard Laboratory Technique
TEG® - Thromboelastography
TF – Tissue Factor
Thrombin – Enzyme that cleaves fibrinogen
Turbidimetry - Light absorbance
VE - Viscoelastic
VHA - Viscoelastic Haemostatic Assay
ϒ’ chain – Longer variant of the Fibrinogen ϒ chain

Chapter 2: Vertical Wicking Lateral Flow Assay

Publication Details

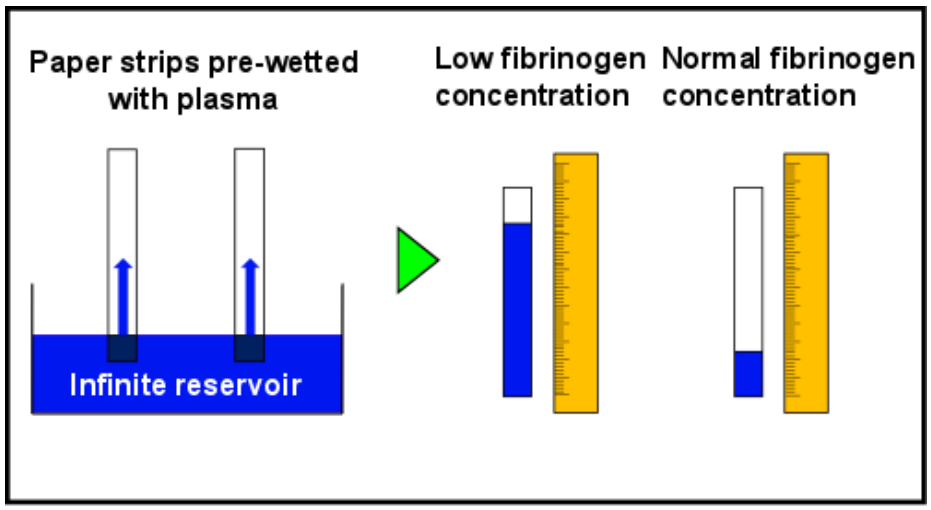
Publication Status: Published

Journal: Analyst *(Q1 – Analytical Chemistry)*

Reference: Bialkower M, McLiesh H, Manderson C, Tabor R, Garnier G. *Rapid Paper Diagnostic for Plasma Fibrinogen Concentration.* DOI: https://doi.org/10.1039/C9AN00616H

Abstract

Fibrinogen is a blood protein that is essential for the formation of a stable clot. It is converted into the polymer fibrin by the blood enzyme thrombin and crosslinked by the blood enzyme factor XIIIa. Fibrinogen is one of the first proteins to be depleted in heavily bleeding patients. Patients with early hypofibrinogenemia need urgent fibrinogen replenishment to prevent the onset of haemorrhage and death. However, currently there is no rapid, sensitive, cheap and easy-to-use fibrinogen assay that can detect fibrinogen concentrations. In this study, we have developed a new paper-based diagnostic to quantify the fibrinogen concentration in blood at room temperature. This diagnostic is a 2-step process: first, plasma is added onto thrombin-treated paper strips where fibrinogen is converted to fibrin; then the strips are placed into an aqueous dye bath where elution occurs. The test operates by measuring the change in hydrophobicity, which increases with fibrinogen concentration under otherwise constant conditions. The diagnostic can precisely measure fibrinogen concentration within the range of 0–2 g/L, which is ideal for the clinical diagnosis of hypofibrinogenemia. Furthermore, testing needs only 12 µL of plasma, 60 mU of thrombin and 7.5 mins of testing. This diagnostic has the potential to revolutionise point of care testing and save many lives.



1. Introduction

This study aims to develop a rapid and easy to use paper-based diagnostic to measure fibrinogen concentration in human plasma. Martinez *et al.* in 2007 first demonstrated that patterned paper was an inexpensive medium that could wick fluids through internal capillary actions and facilitate colorimetric biochemical reactions (23). This has influenced the emergence of many paper-based diagnostics for detecting and quantifying analytes in blood, urine and saliva (24) and for blood typing (25-27). However, there has been no development of paper-based fibrinogen concentration diagnostics to date.

Fibrinogen is essential in the process of haemostasis. It is a plasma protein that usually circulates between 2–4 g/L in the human body (1, 2). When vessel breakage occurs, the coagulation cascade is activated that results in the activation of two key blood enzymes: thrombin and factor XIIIa (FXIIIa) (59, 66). Thrombin converts fibrinogen into fibrin monomers that self-polymerise (3, 4). FXIIIa then covalently links the neighbouring fibrin monomers to each other, forming a hard clot network (5-9). This hard clot network can adhere to the site of breakage where it is capable of entrapping red blood cells and binding to the site of damage. Hence, this allows complete blockage of the damaged site and hence prevents further blood loss until the site has healed (1).

Low fibrinogen concentrations are critical in the onset of major haemorrhage. In 1995, Hiippala *et al.* reported that fibrinogen is usually the first plasma protein that becomes depleted in haemorrhagic patients (31). Patients that have early hypofibrinogenemia are at a significantly greater risk of mortality (10, 11). The implementation of fibrinogen concentrate (FC) into clinical major haemorrhage protocols is gaining strong interest. In observational studies, the ability to diagnose early hypofibrinogenemia and treat it rapidly with reconstituted FC strongly increases survival in haemorrhagic patients (15-17). However, there is no early hypofibrinogenemia assay currently available in clinics that is sufficiently rapid, sensitive, cheap or easy to use, particularly in cases of critically bleeding patients.

Traditionally, plasma fibrinogen concentrations have been determined through the use of standard laboratory techniques (SLTs) (181). However, SLTs are all problematic as they involve complex instrumentation for plasma analysis. Additionally, they require large volumes of plasma (ie. >200 µL) to test with (202). Finally, most SLTs need serial dilutions of known fibrinogen concentrations to be simultaneously measured with patient plasma for calibration (20, 92, 101). Depending on the assay, this can require significant time and labour to perform in emergency situations.

Given the life-threatening nature of major haemorrhage and the significant time SLTs require, there has been minimal evidence that SLTs provide any survival advantage in hypofibrinogenemic patients (183-186). Hence, clinics have avoided implementing a fibrinogen concentration diagnostic step in their major haemorrhage protocols. Instead, they have replenished fibrinogen (and all other coagulation proteins) by transfusing donated fresh frozen plasma (FFP) and cryoprecipitate into patients (168, 180, 203-205). However, as these products are unstandardised and have not undergone viral inactivation, they have been criticised as therapeutics due to their iatrogenic effects (including TRALI, TACO and organ failure) (170, 172-175).

The recent advancement of Viscoelastic Haemostatic Assays (VHAs) has allowed clinics to measure hypofibrinogenemia more easily than SLTs (188). However, VHAs still poses clinical problems. First, assaying times of 5-15 mins are required before a result is achieved (153, 154). Second, the efficacy of the required platelet inhibitors are imperfect and may result in overestimation of plasma fibrinogen concentrations (155, 156). Finally, the devices used to perform VHAs (ie. TEG® or ROTEM®) require very significant outlay in terms of initial infrastructure requirements and costs (157, 158).

Likewise, the dry hematology method (DRIHEMATO®) is another emerging method (198). It has shown nearly identical fibrinogen measurements to a Clauss assay – the most widely used SLT for diagnosing hypofibrinogenemia in clinics. Furthermore, it can acquire results in a minute. However, it still requires the use of electricity to operate and complicated infrastructure to acquire fibrinogen readings.

Paper-based diagnostics present a great opportunity to address the many shortcomings associated with current early hypofibrinogenemia assays.

In this study, we demonstrate a novel concept for measuring fibrinogen concentrations on paper for diagnostic application. Previously, Li *et al.* (206). and Guan *et al.* (207). had developed diagnostics that could determine clotting time and blood type respectively through lateral flow wicking. This concept works similarly. By adding fibrinogen and thrombin solutions to paper strips, allowing thrombosis to occur, placing the strips into an aqueous dye bath and allowing elution to proceed, we show that the distance the dyed fluid elutes up each strip correlates precisely to the fibrinogen concentration of the solution. Therefore, this study aims to quantify the effect that varying the controlling parameters has on the sensitivity of the test. The parameters that are analysed in this study include: the type of paper, the strip width, the volume of fibrinogen solution added, the individual concentrations of thrombin and FXIIIa used as well as the reaction and elution times. Additionally, this study also measures the effect of non-specific blood proteins on the test to emulate plasma-like conditions.

2. Materials and Methodology

2.1. Materials

Low density (tissue) and high density (filter) papers were selected to create the paper strips. The filter paper used was Whatman 41, whereas the tissue papers were produced in the Kimberly Clark Experimental Forming Unit (EFU), Neenah (WI), USA. Tissues consist of eucalyptus fibres formed with a 3 layer headbox. 10 Kg/T PAE wet strength agent was added to each layer. Tissues were moulded and thoroughly air-dried. Paper properties are listed in Supplementary Table 3. A 1×1 mm^2 square steel rod was cut 35 mm long to hold the paper strips over the elution bath tub. Two elution tanks (13.1 cm tall) were used as support to position the steel rod over the bath tub. The bath tub consisted of a petri dish (15.5 cm in diameter and 2.7 cm tall). MilliQ water was used as the bulk fluid of the elution bath. Brilliant Blue FCF (from Queen Blue Food Colouring) was used as the aqueous dye to colour the elution bath. Thrombin vials and fibrinogen powder were sourced from Sigma Aldrich. FXIIIa was purchased from Zedira GmbH, Darmstadt, Germany. Bovine Serum Albumin (BSA) concentrate was bought from CSL Behring.

For hydrophobicity testing, Menzel Gläser 22 × 22 mm coverslips were used as the substrate. Analytical grade sodium methoxide solution and cellulose acetate was sourced from Sigma Aldrich. Acetone was purchased from Ajax Finechem. Methanol was sourced from Merck. All reagents and solvents were used as supplied without further purification.

2.2. Methodology

2.2.1. Paper Cutting

Low density (tissue) and high density (filter) papers were selected to create the paper strips. The filter paper used was Whatman 41, whereas the tissue papers were produced in the Kimberly Clark Experimental Forming Unit (EFU), Neenah (WI), USA. Tissues consisted of eucalyptus fibres formed with a 3 layer headbox. 10 kg per T PAE wet strength agent was added to each layer. Tissues were moulded and thoroughly airdried. Paper properties are listed in **Table S3**. A 1 × 1 mm^2 square steel bar was cut 35 mm long to hold the paper strips over the elution bath. Two elution tanks (13.1 cm tall) were used as mounts to position the steel bar over the elution bath. The elution bath consisted of a Petri dish (15.5 cm in diameter and 2.7 cm tall) filled with an aqueous dye. MilliQ water was used as the bulk fluid of the elution bath. Brilliant Blue FCF (from Queen Blue Food Colouring) was used as the aqueous dye to colour the elution bath. Ponceau 6R (from Queen Red Food Colouring) was used as the aqueous dye to stain cellulose and fibrin fibres for microscopic imaging. Thrombin vials and fibrinogen powder were sourced from Sigma Aldrich. FXIIIa was purchased from Zedira GmbH, Darmstadt, Germany. Plasma (anticoagulated with EDTA) and serum was sourced from the Australian Red Cross. Bovine Serum Albumin (BSA) concentrate was bought from CSL Behring. Glycerol was sourced from Astral Scientific. For hydrophobicity testing, Menzel Gläser 22 × 22 mm coverslips were used as the substrate. Analytical grade sodium methoxide solution and cellulose acetate was sourced from Sigma Aldrich. Acetone was purchased from Ajax Finechem. Methanol was sourced from Merck. All reagents and solvents were used as supplied without further purification.

2.2.2. Fibrinogen Solution Preparation

A 4 g/L fibrinogen solution was prepared by dissolving fibrinogen powder into PBS at 37 °C. First, 90 mg of fibrinogen was dissolved in 10 mL of PBS. As the powder contained an inconsistent mixture of fibrinogen and other salts, the fibrinogen concentration had to be determined on a UV-Vis spectrometer (Agilent Cary 60). The fibrinogen concentration was determined using Beer's law at 280 nm with a 1 cm cuvette. An extinction coefficient of 1.51 was used to convert absorbance units to g/L fibrinogen. The fibrinogen

solution was adjusted to exactly 4 g/L by measuring its concentration on the UV-Vis spectrometer and diluting it with the appropriate amount of PBS. From there serial dilutions of 2 g/L, 1 g/L, 0.5 g/L and 0.25 g/L fibrinogen solution were also prepared. All of these solutions were made into small aliquots, snap frozen in liquid nitrogen and stored at −80 °C until used.

2.2.3. Test Setup

The elution bath was placed in between the 2 mounts. The elution bath was prepared by pouring 250 mL solution of 0.1% Brilliant Blue FCF in MilliQ water into the Petri dish. A schematic of this is provided in **Figure 7**. The thrombin solution was prepared by reconstituting a vial of thrombin with 300 µL of MilliQ water. This made a vial of 30 NIH units per mL thrombin (these units are simplified to U/mL in this chapter). However, different thrombin concentrations could be made by reconstituting each vial with different volumes of MilliQ water.

FXIIIa was also used to see if could improve the sensitivity of the test. It was hypothesised to do so because it could form covalent cross-links between the fibrin monomers and strengthen the fibrin. First, a 60 U/mL solution of FXIIIa was prepared by dissolving 150 µL of MilliQ water into a vial of FXIIIa. Then, serial dilutions of 30 U/mL, 15 U/ml, 8 U/mL FXIIIa were prepared. Afterwards, a 60 U/mL vial of thrombin was prepared. Finally, equal volumes of thrombin and FXIIIa solutions were mixed into separate Eppendorf tubes. This created 30 U/mL thrombin solutions with different FXIIIa concentrations.

2.2.4. Test Procedure

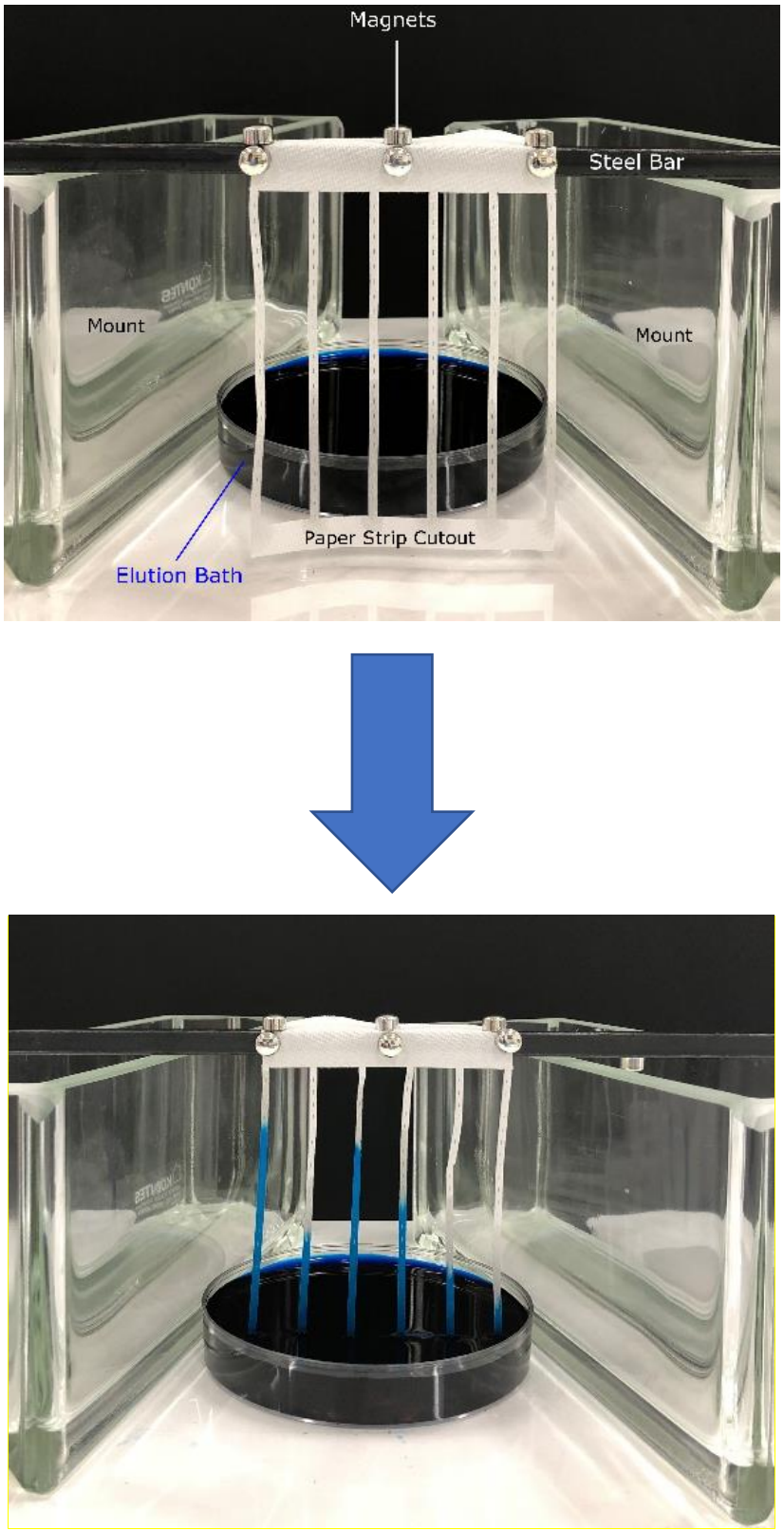


Figure 7: Set up of paper strips. Thrombin solution is applied to the strips followed by fibrinogen solution (a). The strips are placed in the elution bath and allowed to elute. Different fibrinogen concentrations causes the dye to elute at different rates (b).

A paper strip cut-out was attached perpendicularly to the steel bar with magnets. The steel bar's bottom and top edges were positioned 12.5 and 13.5 cm from the bottom of the cut-out respectively. The slits 13.5 cm from the bottom of the cut-out (as shown in **Supplementary Figure 1**) helped align the steel bar. The steel bar was placed on the mounts (but not directly over the elution bath) with the cut-out vertically orientated. This prevented the cut-out from making contact with the table. Thrombin solution was pipetted near the bottom of each strip (see **Supplementary Figure 4** for exact positionings). The fibrinogen solution was then pipetted 5 mm above each wetting of thrombin solution. As the fibrinogen solution wicked in both directions, it meant that only half of it could mix with the enzymes. However, it ensured that: (1) The fibrinogen and thrombin mixed properly with each other, (2) The fibrin barrier formed at the bottom of each strip. The cut-out was left for 30, 60 or 90 s to react the fibrinogen and thrombin. Afterwards, the paper strip cut-out was moved into the elution bath. The 0 mm marking of the paper strips sat level with the dye surface of the elution bath. The fibrin barrier formed at the bottom of each strip caused elution to be perturbed immediately. The paper strips were eluted for 1, 3, 5, 7 or 10 mins – times less than and equal to those found in VHAs. Finally, the cut-out was removed from the elution bath and dried on paper towel. The elution height of each strip was measured by finding the mid-point of each elution front and rounding to the nearest millimetre. Images of the cut-outs were taken by photocopier (Epson V370 Photo Scanner). A summary of the procedure is given in **Figure 8**.

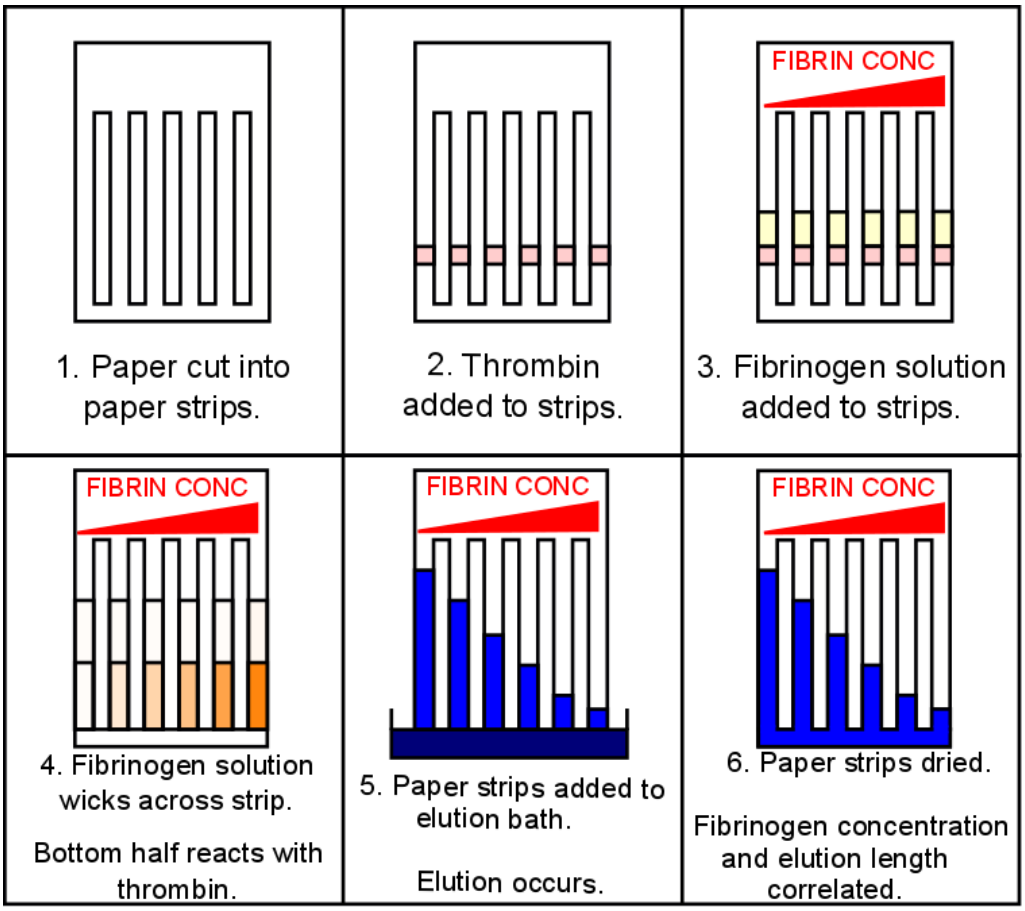


Figure 8: Summary of Test Procedure. Paper strips are wetted with the thrombin solution. Then the fibrinogen solution is added where it wicks across the strips and half of it reacts with the thrombin. Finally, the cut-out is placed into the elution bath where elution occurs. The elution height up each strip can be correlated to the fibrinogen concentration. Dimensions of the paper strip cut-out used can be found in **Supplementary Figure 1**.

2.2.5. Coverslip Preparation

Coverslips were prepared to test for hydrophobicity.

Cellulose acetate solution was made by dissolving 0.25% w/v cellulose acetate in acetone. Coverslips were plasma cleaned (Harrick Plasma Cleaner PDC-002-HP) for 3 mins on one side – to create a smooth, dust-free surface for accurate measurements. Cellulose acetate was spin-coated (Laurell WS-650-23 Spin Coater) on the plasma cleaned sides for 20 s at 2000 rpm. Sodium methoxide solution in methanol was prepared at 0.5% w/v. The cellulose acetate coated slides were immersed into the sodium methoxide bath for 10 h. This converted the cellulose acetate into a cellulose film. Afterwards, the coverslips were washed with methanol followed by MilliQ water and were left to dry.

Thrombin solution was made to 10 NIH Units/mL in MilliQ water and a layer was spin-coated on top of the cellulose film for 20 s at 2000 rpm. Likewise, fibrinogen solution was made to 2 g/L in PBS and a layer was spin-coated on top of the thrombin layer for 20 s at 2000 rpm. This converted the fibrinogen into fibrin.

2.2.6. Hydrophobicity Testing

Hydrophobicity testing was completed to decipher the physical mechanism behind how the test works.

Contact angle (Dataphysics OCA35) was used to measure the hydrophobicity of the spin-coated coverslips. Growing water droplets were created by dripping MilliQ water at a rate of 2 µL/s on top of each coverslip surface. The dispensing needle was in contact with the growing droplet throughout each recording. The advancing contact angle of the droplet was used to quantify hydrophobicity, with the general observation that larger angles corresponded to a more hydrophobic surface.

2.2.7. Viscosity Testing

Viscosity testing was completed to decipher the physical mechanism behind how the test works.

Glycerol:water mixtures of composition 0:100%, 85:15% and 100:0% were made. Their viscosities were measured in Cannon-Fenske viscometers of Size 200 (~0.1 cSt/s), Size 450 (~2.5 cSt/s) and converted to centipoise. The glycerol:water mixtures were then added to the paper strips and eluted as per the test procedure. Full details can be found in **Table S1**.

3. Results

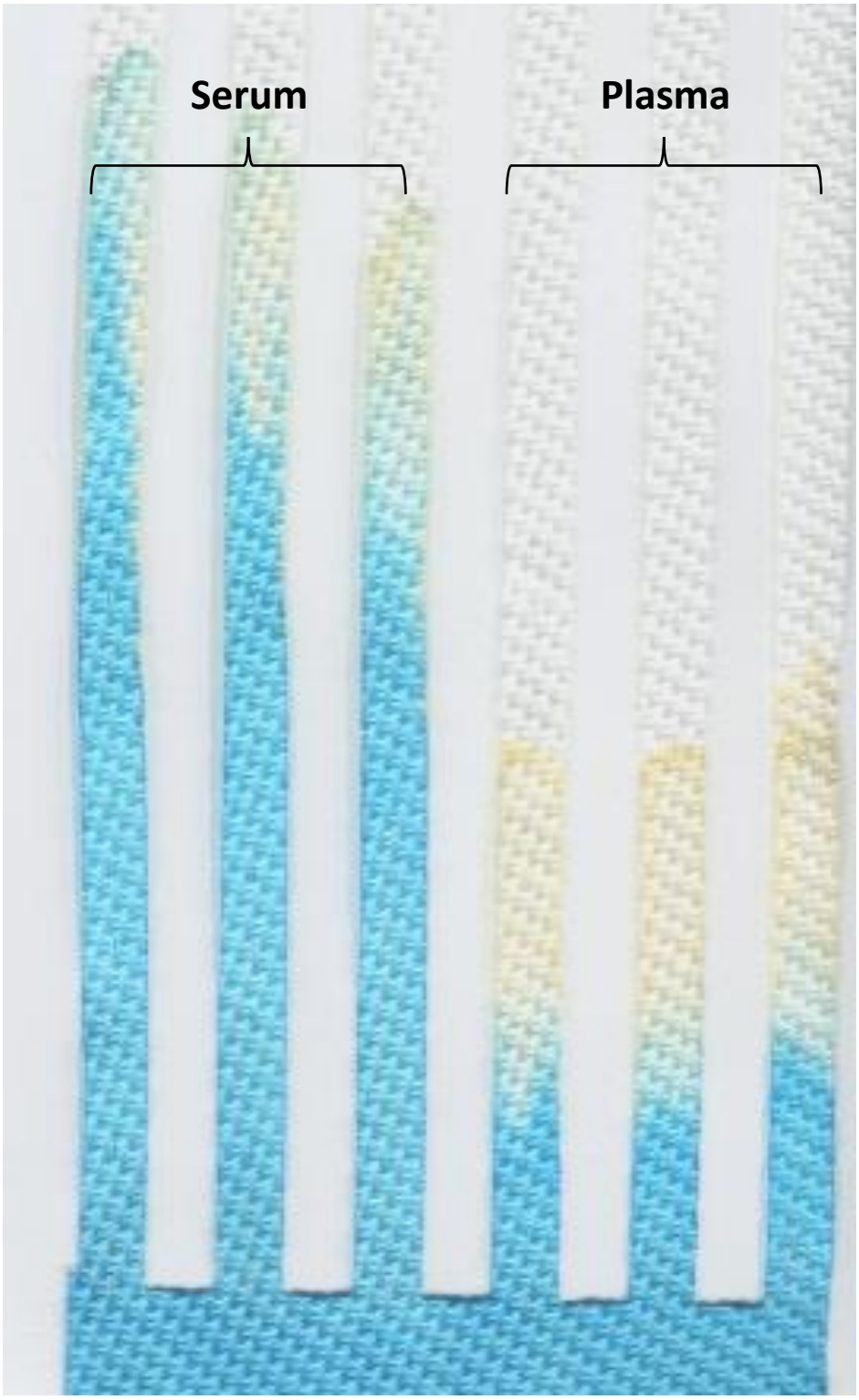
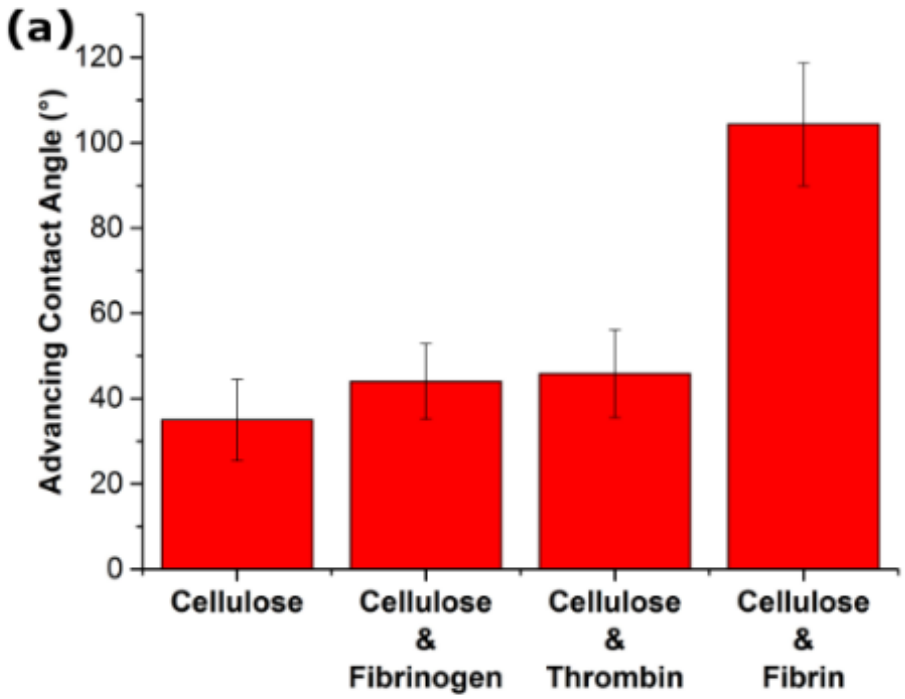


Figure 9: Elution of Serum vs Plasma. Both blood products were collected from the Australian Red Cross. Test Conditions can be found in **Table S9**. Each test was performed in triplicate.

Figure 9 demonstrated that the elution of plasma samples (which contains fibrinogen) eluted much less than the serum samples (which does not contain fibrinogen). Therefore, this phenomenon was explored in further detail. Thrombin and Factor XIIIa (FXIIIa) were deposited onto paper strips. Then fibrinogen solution was added where it reacted with the enzyme to form fibrin. Finally, the strips were placed vertically in a blue dye bath, eluted for a set period of time and dried afterwards. There was a clear and reproducible correlation between the height that the blue dye eluted up each strip and the concentration of the fibrinogen solution. Higher fibrinogen concentrations resulted in lower elution heights. This discovery formed the concept of a sensitive, rapid and reproducible paper diagnostic for quantifying fibrinogen concentrations in blood. The variables estimated to be important were tested individually and their impact on the diagnostic's sensitivity were quantified. The variables investigated included: paper structure, strip width, fibrinogen solution volume, thrombin concentration, FXIIIa concentration, reaction time and elution time, and the results of this testing were discussed in detail below. This analysis elucidates the controlling mechanisms and optimises the conditions for developing a robust blood-based diagnostic.

3.1. Mechanism

The effect of fibrinogen, thrombin and fibrin coating on cellulose film hydrophobicity was quantified (**Figure 10a**). The film becomes significantly more hydrophobic when it is coated with fibrin. This is the concept behind the paper diagnostic. The coating of un-polymerised fibrinogen on the cellulose film did not contribute significantly to the hydrophobicity. The effect of viscosity is also quantified using glycerol-water mixtures as model solutions (**Figure 10b**). Viscosities above 100 cP do perturb elution. However, its exact contribution towards perturbation after fibrin polymerisation is unknown. Additionally, the potential effect of paper pore blockage (**Supplementary Figure 9** and **10**) was investigated by microscopy and surface topography. Paper pore and surface structure was slightly and not affected by fibrin coating, respectively. Fibrin coating effect on the diagnostic is rated as minimal. Therefore, the diagnostic is driven predominantly by the increase in hydrophobicity that results from the polymerisation of fibrinogen.



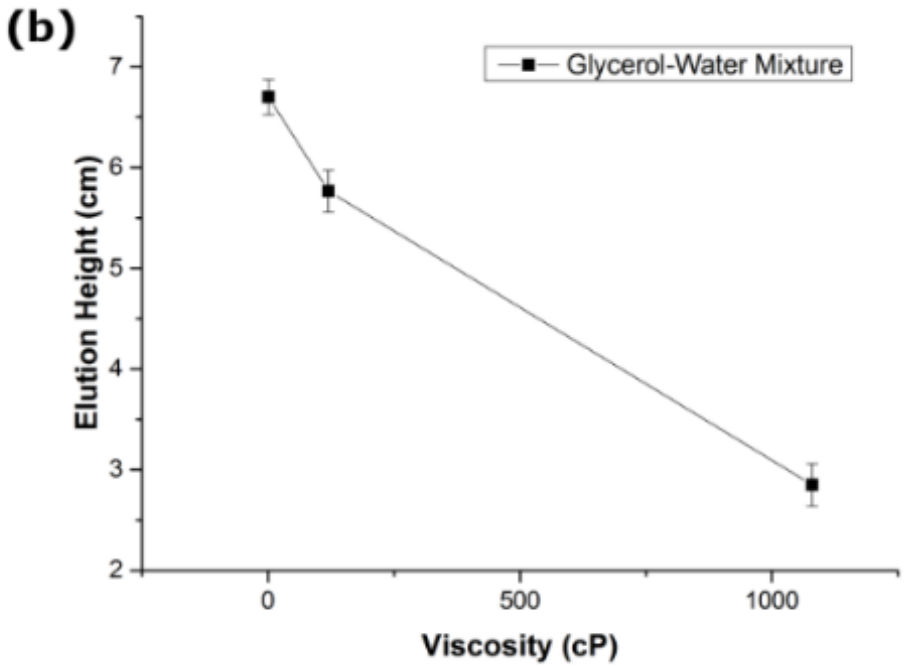


Figure 10: Effect of hydrophobicity on the different cellulose surfaces (a). The contact angle of water droplets deposited on the spin-coated coverslips is measured. Effect of glycerol viscosity of elution height (b). Effect of solution viscosity on elution height. Glycerol-water mixtures are made to different viscosities and tested. Test conditions can be found in **Table S1**. Each test was performed in quadruplet and the average and standard deviations are reported as the bar and error bar respectively.

3.2. Effect of Paper Structure Elution Rate

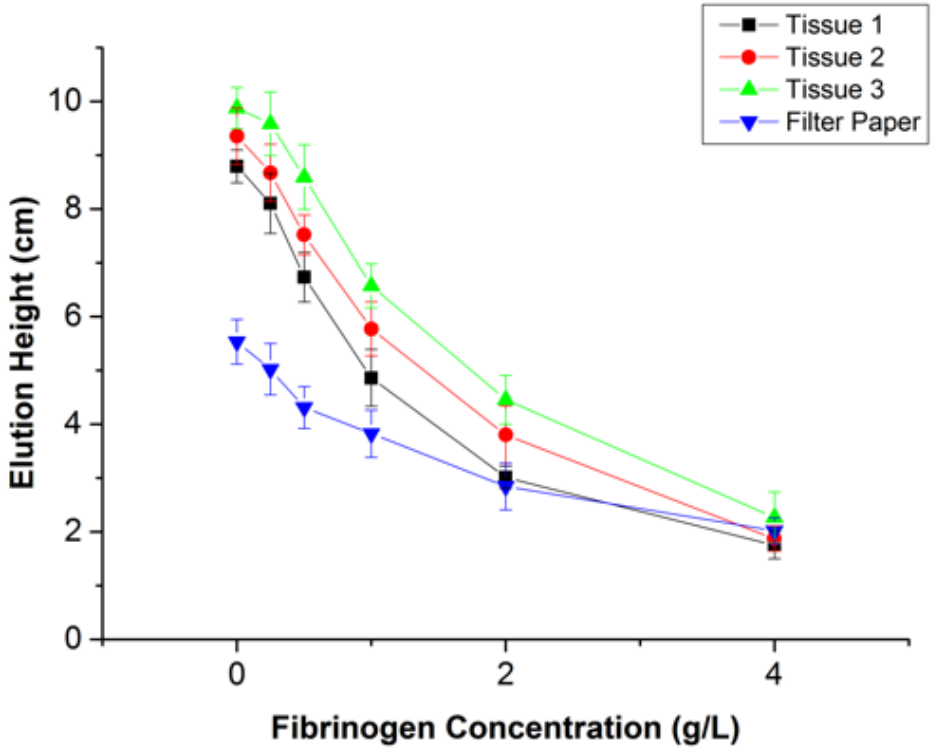


Figure 11: Effect of paper structure and fibrinogen concentration on elution height. Test conditions can be found in **Table S2** and **S4**. Each test was performed in quadruplet and the average and standard deviations are reported.

The effect of fibrinogen concentration on elution height for four papers differing in structure was investigated (**Figure 11**). **Table S3** reports the structural properties of these papers. There are three observations of interest. First, elution height strongly decreases with fibrinogen concentration, with the steepest decrease in the desired 0–2 g/L concentration range, and these results are very reproducible across the four papers. Second, the sensitivity is a strong function of paper density, with the tissue papers showing much higher sensitivity than the filter papers (see **Supplementary Figure 2**). Third, the sensitivity increases with basis weight for the tissue papers. This can be correlated to **Table S3** which shows that higher basis weighted papers are thicker.

3.3. Effect of Fibrinogen Solution Volume and Thrombin Concentration

The effect of thrombin concentration on elution height for different fibrinogen concentrations was investigated (**Figure 12b**). Higher thrombin concentrations significantly increase sensitivity (see **Supplementary Figure 4**). With no thrombin, the fibrinogen concentration–elution height relationship is virtually horizontal. However, at higher thrombin concentrations, sensitivity drastically increases and the elution height decreases with fibrinogen concentration. Insignificant changes are observed with thrombin concentrations of 30 U/mL (60 mU total enzyme) or higher.

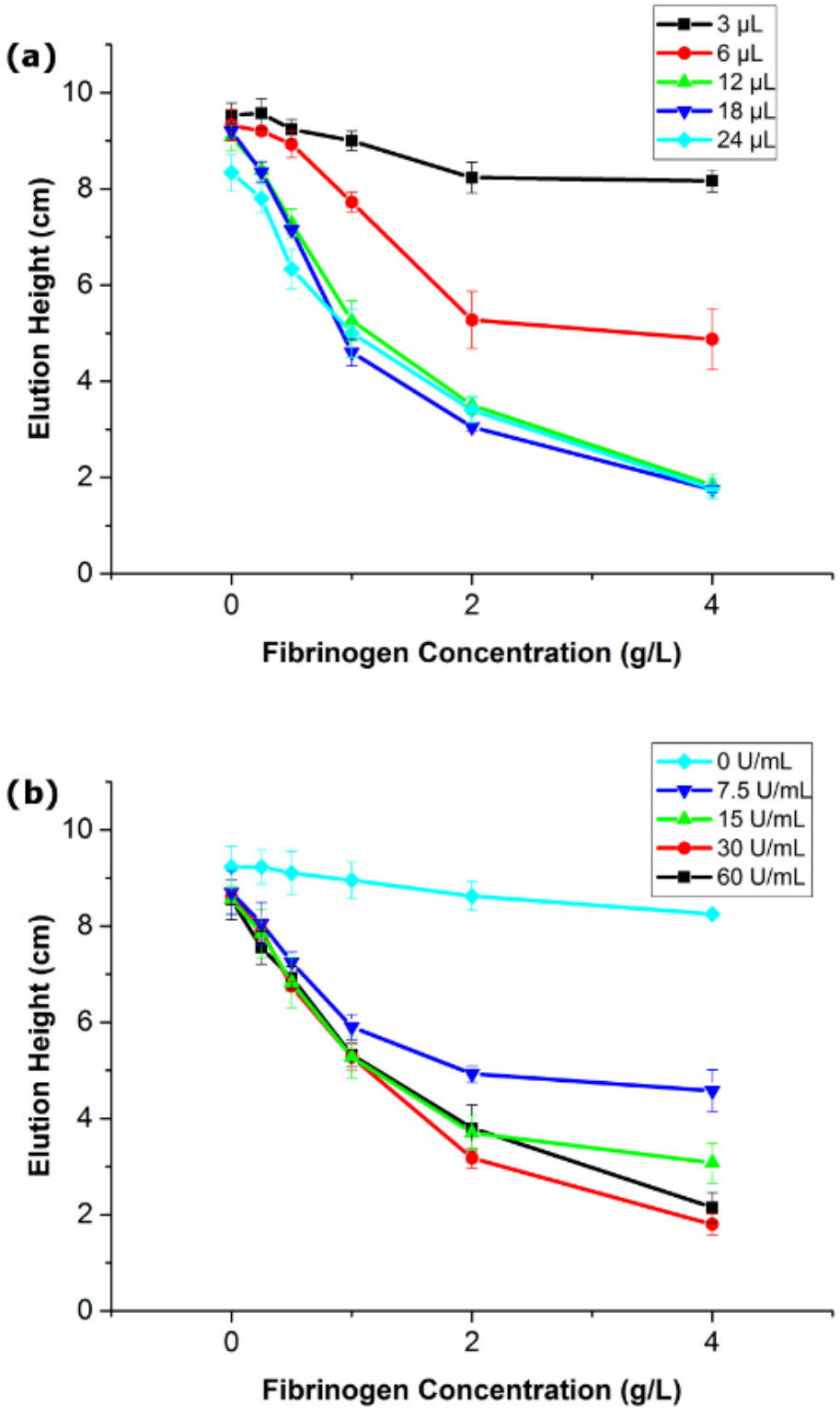


Figure 12: Effect of fibrinogen solution volume and fibrinogen concentration on elution height **(a)**. Effect of thrombin concentration and fibrinogen concentration on elution height **(b)**. Test conditions can be found in **Table S2**, **S5** and **S6**. Each test was performed in quadruplet and the average and standard deviations are reported.

The effect of fibrinogen solution volumes on elution height for different fibrinogen concentrations was also analysed (**Figure 12a**). A very similar effect is observed. At 3 µL volume, the fibrinogen concentration–elution height relationship is flat and insensitive, as denoted by the low slope. At higher fibrinogen volumes, sensitivity drastically increases and the elution height decreases with fibrinogen concentration. Insignificant changes are observed with volumes of 12 µL and higher.

3.4. Effect of Elution and Reaction Time

The effect of reaction time on elution height for different fibrinogen concentrations was investigated (**Figure 13a**). The reaction time is the time allowed for the fibrinogen solution to react with the thrombin before it is placed in the elution bath. Longer reaction times increase sensitivity. However, once the reaction time goes above 30 s, insignificant changes occur.

The effect of elution time on elution height for different fibrinogen concentrations was also investigated (**Figure 13b**). Longer elution times cause a remarkable increase in sensitivity (see **Supplementary Figure 5**). By 1 min, the fibrinogen concentration– elution height relationship is flat and sensitivity is only present between 0–1 g/L fibrinogen. By 7 mins, sensitivity is present between 0–2 g/L fibrinogen. Finally, by 10 mins, clear sensitivity can be seen across 0–4 g/L fibrinogen.

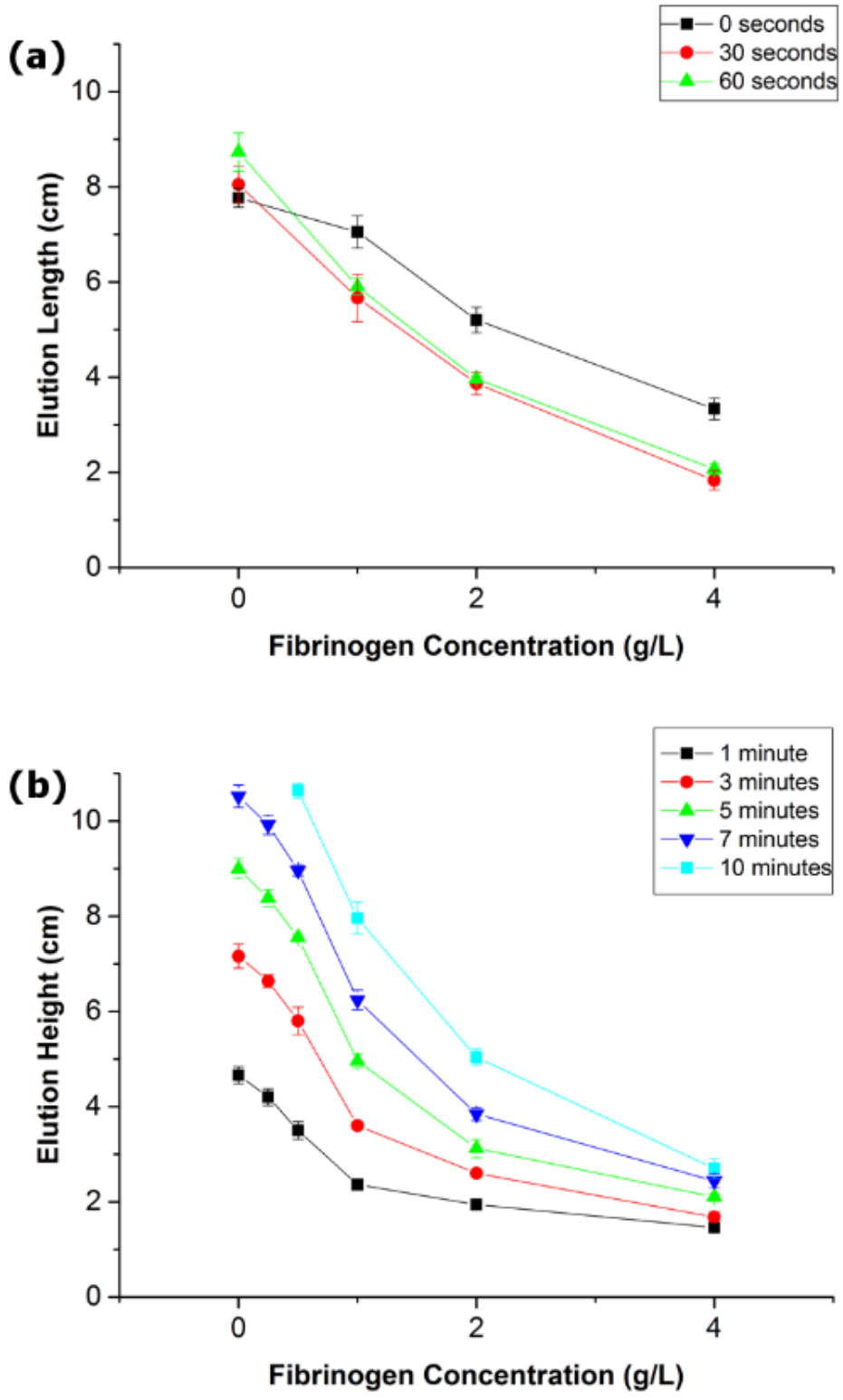


Figure 13: Effect of reaction time and fibrinogen concentration on elution height **(a)**. Effect of elution time and fibrinogen concentration on elution height **(b)**. Test conditions can be found in **Table S2** and **S6**. Each test was performed in quadruplet and the average and standard deviations are reported.

3.5. Effect of Other Plasma Proteins

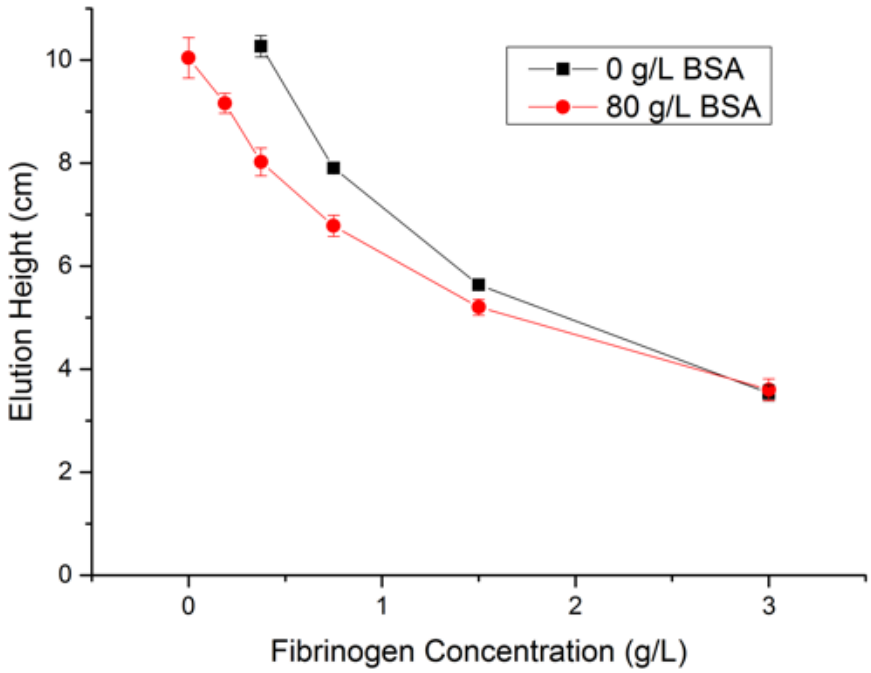


Figure 14: Effect of BSA concentration and fibrinogen concentration on elution height. Test conditions can be found in **Table S2** and **S7**. Each test was performed in quadruplet and the average and standard deviations are reported.

The effect of BSA concentration on elution height for different fibrinogen concentrations was investigated (**Figure 14**). Albumin is the most abundant protein in plasma constituting up to 70% of total plasma protein (208). Therefore, fibrinogen solutions in 80 g/L BSA were prepared to emulate plasma-like conditions in human blood. The effect of BSA is moderate. At low fibrinogen concentrations, the solution with BSA does elute less than the solution without. However, at high fibrinogen concentrations, this effect is nullified. The paper thickness also plays a critical role in the visibility of the elution front with the plasma-like samples (see **Supplementary Figure 6**). Paper 2 produced a fading blue gradient at the elution front. This made it difficult to interpret. However, Paper 3 produced a much clearer elution front.

3.6. Optimal Conditions

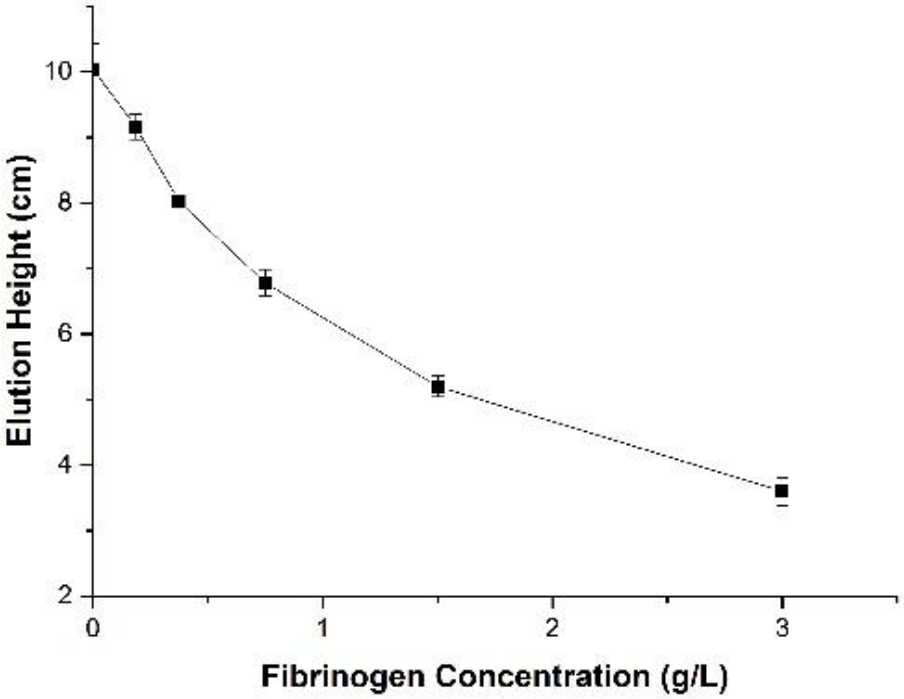


Figure 15: Optimal Test Conditions. Fibrinogen in 80 g/L BSA was used as the model fluid to emulate plasma-like conditions. Test conditions can be found in **S8**. Each test was performed in quintuplet and the average and standard deviations are reported.

Optimal conditions were decided based on the parameters (from **Figures 11, 12, 13** and **14**) that could best distinguish fibrinogen concentrations between 0-2 g/L in plasma-like conditions. Parameters are shown in **Table S8**.

4. Discussion

4.1. Concept

In this study, we have developed a new paper-based diagnostic to quantify the fibrinogen concentration in plasma. The diagnostic concept relies on fibrin forming a hydrophobic barrier to fluid flow in a porous and wettable medium, such as paper. While not linear, the sensitivity of the test in the concentration range of 0–2 g/L is highest, which perfectly fits clinical needs for diagnosing early hypofibrinogenemia. The total duration needed for the test is less than 8 mins (**Figure 15**).

Unlike other assays that require laborious preparations or specialist training (157, 182), using the paper diagnostic is only a two-step process. First blood plasma is added onto thrombin-treated paper strips where the fibrinogen present is converted to fibrin. This is an enzyme triggered polymerization process operating at room temperature. Then the paper strips are eluted with a standard aqueous dye. We discovered that as fibrinogen is converted into fibrin upon interaction with thrombin, the elution barrier it induces on paper drastically increases. Therefore, the maximum elution height of the dye drastically decreases.

Whilst this test itself is not hand-held, it is convertible into one (as shown in **Chapter 3**).

The test follows Washburn kinetics of imbibition through a porous medium (**Supplementary Figure 11**). Despite the elution barrier effect that fibrin exerts on the test, it does not cause it to deviate away from Washburn kinetics.

Hydrophobicity, viscosity and physical pore blockage were all tested to see how the elution barrier works.

The hydrophobicity increases remarkably when fibrinogen is converted into fibrin. When 2 g/L fibrinogen and thrombin reacts, it increases the water–surface contact angle by over 60° (**Figure 10a**). Paper elution is governed by the capillary pressure:

$$P_C = \frac{2\gamma}{r}\cos(\theta) \quad \textbf{(1)}$$

Where γ is the air-water surface tension, r is the capillary radius, θ is the water-capillary surface contact angle and P_C is the capillary pressure (209, 210). The higher P_C is, the greater the extent of elution. As the polymerisation of fibrinogen into fibrin caused such a large increase in θ, it induces a major decrease in P_C. Therefore, the test is predominantly driven by hydrophobicity. Although the roughness and fibre structure of paper would certainly result in additional complexities when compared to the hydrophobicity measurements undertaken on idealised smooth cellulose films, the basic controlling concept remains the same.

The effect of viscosity was investigated using a series of glycerol-water solutions (**Figure 10b**). As glycerol is a hydrophilic substance, it cannot act as a hydrophobic barrier to fluid flow. Therefore, it can only restrict elution purely on viscosity. Elution is impeded at viscosities over 100 cP and can be significant once viscosities rise over 1000 cP. However, the extent to which this applies to fibrin polymerisation is unknown. This is because the viscosity of fibrin is affected by the shear rate exerted on it (211). However, viscosity effects are expected to play a secondary role in the diagnostic. This is because the viscosity of the dye solution is unaffected by the formation of fibrin.

The effects of physical pore blockage due to fibrin formation was also deciphered (**Supplementary Figures 9** and **10**). Images from **Supplementary Figure 9** show that the paper pore size can exceed 100 µm and

that the fibrin formed cannot fill the pores. Profilometry data from **Supplementary Figure 10** shows that the surface roughness does not decrease when fibrin is formed on paper. Therefore, physical pore blockage does not occur.

In essence, the test predominantly consists of measuring the change in hydrophobicity, which increases with fibrinogen concentration under otherwise constant conditions. Various methods to measure paper hydrophobicity already exist. These include the contact angle test and water droplet adsorption time (212, 213). However, this elution-based test was chosen due to its superior efficacy and easy-to-read result to the naked eye.

4.2. Variables Affecting Sensitivity

Paper structure, fibrinogen volume, thrombin concentration, reaction time and elution time are the main variables affecting the sensitivity of the test. Strip width and FXIIIa, however, show no impact. Additionally, BSA concentration has a small but non-detrimental effect.

The sensitivity of the test correlates strongly with the elution rate of the paper (**Figure 11**). The sensitivity is significantly better for the tissue papers than for the filter paper, indicating that low density papers are preferred. The elution height difference for the tissues between 0–2 g/L fibrinogen are all around 6 cm. This is easy to visualise and hence these papers enable determination of the fibrinogen concentration using only the naked eye. The sensitivity is also much higher than the diagnostic reported by Li *et al.* which can only distinguish clotted from non-clotted blood samples in the range of 4 mm (206). Whereas, filter paper only shows 2.5 cm difference between this range and hence requires more subjective interpretation of results. Low elution rate papers thereby provide lower sensitivity than high elution rate papers. **Table S3** highlights the effect of paper structure properties and how they influence the elution rate. A clear difference is demonstrated in basis weight and density between the tissue and filter papers. As the tissue papers have a lower density than the filter paper, they are able to elute a lot faster. In 2012, Su *et al.* investigated the paper structure needed to separate agglutinated from non-agglutinated blood cells and reported a similar finding.

Thin and porous papers were reported to provide greater sensitivity than thick and dense papers (214). Paper of low density and high porosity is needed for such fibrinogen diagnostics.

The effect of fibrinogen solution volume provides the basis for the amount of blood plasma required (**Figure 12a**). Likewise, the effect of thrombin concentration determines the amount of thrombin to be used per test (**Figure 12b**). The trend is the same for both relationships. Increased fibrinogen solution volume and thrombin concentration both increase the sensitivity of the test. The sensitivity of the test becomes optimised once the volume and concentration reaches certain threshold values: 12 µL and 30 U/mL, respectively. This means that the reagents needed to perform such a test are significantly lower in quantity than any other currently available fibrinogen assay. For instance, Clauss assays require 100 µL thrombin solutions (of up to 200 U/mL) and 200 µL of diluted plasma in order to provide reliable results (215). Likewise, FIBTEM tests require 300 µL of blood and 40 µL of extra reagents (216). As our test needs only 60 mU of thrombin and a small drop of plasma (~12 µL), it can save significant reagent usage from the clinic and the heavily bleeding patient.

The effect of reaction and elution time combined define the testing time (**Figure 13**). The reaction time shows optimal sensitivity at only 30 s. In 1953, Biggs *et al.* stated that normal plasma could be clotted at 37 °C in 10 s (217). The fact that our study was performed at room temperature (instead of 37 °C) indicates that temperature has little impact on the rapidity of the test. Likewise, the sensitivity improves with larger elution times. By 7 mins, concentrations between 0–2 g/L are very easy to distinguish. Therefore, this was selected as the optimal time needed for the test. It is quicker than FIBTEM which can require up to 11 mins to get a reading (153, 154). This paper test can give clear results in half the time of a FIBTEM.

We hypothesised that including FXIIIa would improve the sensitivity of the test. This is because FXIIIa forms covalent cross-links between fibrin monomers that strengthens the fibrin. Therefore, we thought that this would increase the hydrophobicity of the elution barrier and impede further elution. However, FXIIIa has no clear impact on the sensitivity of our test (**Supplementary Figure 7**). The effect of BSA concentration provides the basis for nonspecific effects on the test (**Figure 14**). The reference range for total protein concentration for human plasma is 60–80 g/L (208).

Therefore, 80 g/L solutions of BSA were used to emulate human plasma-like conditions in the high concentration range. BSA does perturb the sensitivity of the test. However, this is only evident at low fibrinogen concentrations (i.e. below 1.5 g/L). At higher concentrations, the effects of BSA are negligible. Given that this experiment tested between an 80 g/L difference of BSA, whereas total plasma protein concentration between normal individuals varies by 20 g/L, such small effect would be nullified even further. This indicates that nonspecific effects in normal plasma would be insignificant on the sensitivity of the test.

4.3. Perspectives

Non-specific effects of other plasma proteins, especially albumins and globulins, are a concern in this test. Both of these proteins have been known to adsorb to cellulose (218) and contribute to plasma viscosity (219).

Therefore, they may retard elution. Whilst we found that physiologically relevant levels of these proteins had measurable but not detrimental effects on the elution height when compared to fibrin, abnormally high levels of these proteins were not considered. Therefore, individuals with hyperviscous blood may be susceptible to giving falsely high fibrinogen concentration readings.

Non-homogeneous elution can cause the elution front to move further and hence cause a falsely low reading for fibrinogen concentration (see **Supplementary Figure 3**). The main phenomenon behind inhomogeneous elution is the creation of local areas of air pockets across the width of the strip. As these air pockets are lower in pressure than the surrounding clotted plasma, the aqueous dye prefers to move through them. These air pockets are most likely formed due to variability within the paper pore structure. **Supplementary Figure 8** shows that 5 mm strips produce larger variations in elution height than 3 mm strips. This is probably due to the greater chance of encountering an air pocket in a wider strip than in a narrower strip. Therefore, our test requires a high-quality paper with no variability in pore structure or density.

A rapid and simple method to pre-separate blood cells from plasma must be considered before applying plasma onto our test. We found that whilst serum and plasma showed clear elution differences to each other (**Figure 9**), whole blood did not perform as well (result not included). This is in contrary to Li *et al.*'s (220) diagnostic which could distinguish whole blood samples with prolonged clotting parameters from

samples with normal clotting parameters. In 2017, Kim *et al.* recently developed a high purity and high throughput pipette tip that could separate plasma and blood from each other (220). In this study, we only looked at the effects at room temperature and relative humidities between 30–50%. However, given that our test is intended to be a hand-held, point of care (POC) diagnostic for emergency fibrinogen transfusions, it is likely to be used in both indoor and outdoor environments. In Australia, the climate can vary from hot and dry conditions in Central Australia to mild and damp conditions in Victoria and Tasmania. Therefore, such a diagnostic may have to be stored and operated under controlled temperature and humidity conditions, indicating the need for additional testing.

5. Conclusion

In this study, we have developed a rapid, simple, sensitive and cheap paper-based diagnostic that can quantify fibrinogen concentrations. The test is a two-step process. First, dissolved fibrinogen is added onto thrombin-treated paper strips. The thrombin converts the fibrinogen into fibrin, and then the strips are eluted with a standard elution dye. The aqueous dye will elute to a certain height, depending on the quantity of fibrin present on the strip. This is through a hydrophobicity-controlled mechanism. This diagnostic is well adapted for clinical major haemorrhage protocols as it can detect within the range of 0–2g/L, necessary for diagnosing early hypofibrinogenemia.

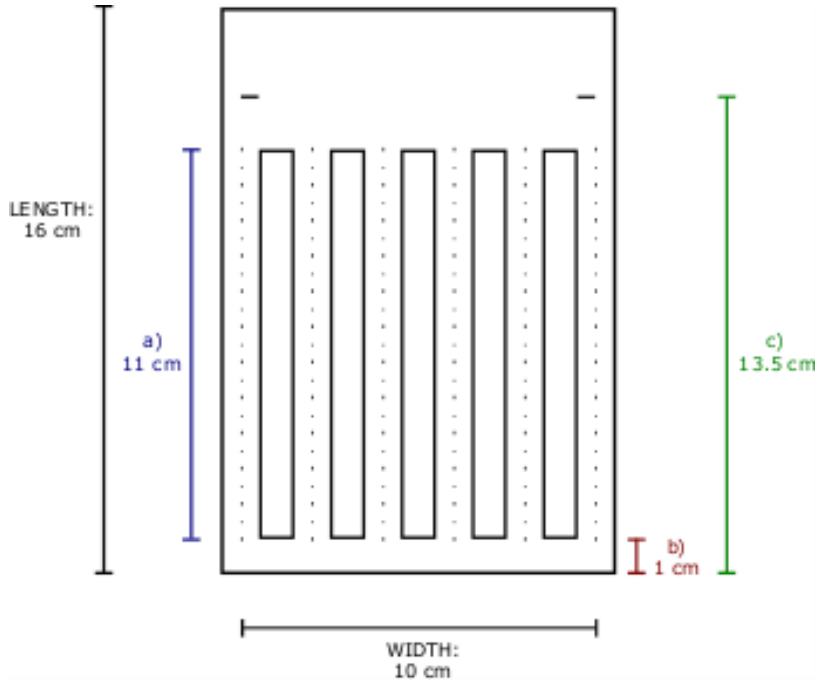
We found that the paper type, fibrinogen solution volume, thrombin concentration, reaction time and elution time all affected the sensitivity of the test. Whilst the test requires low density paper for rapid elution, it can achieve sensitivity with low reagent quantities (12 µL fibrinogen solution and 60 mU thrombin) and short assaying times (30 s reaction time and 7 mins elution time). This is significantly quicker and cheaper than any current laboratory tests, including FIBTEM. We also tested the non-specific effects of other blood proteins in plasma. We found that at physiologically relevant concentrations, its impact on the test was small. However, abnormally high levels were not tested and could be a limitation. Other limitations of the test include non-homogenous elution patterns (which causes the dye to elute further than it should), the requirement to pre-separate blood cells from plasma and the potential effects of temperature and humidity. Hence, further optimization is still required to develop the test into a robust POC commercial diagnostic.

The future of this test is exciting. Such a diagnostic could be used to detect early hypofibrinogenemia and save many lives. It also has the potential to revolutionise major haemorrhage protocols by introducing rapid, simple and cheap point of care diagnostics that are suitable for targeted treatment.

Supplementary Data

2.1. Experimental Method:

Paper Strip Cut-Out:



Supplementary Figure 1: Paper strip cut-out prepared on CorelDRAW X6. a) The total height of the strip. In the middle of each strip are markings 5 mm apart. These are used to measure the elution height up each strip. b) The bottom section of the cut-out. This was included to hold the strips in place during elution. c) The height up the cut-out that the rod was attached to. This is indicated by the horizontal slits on either side of the cut-out.

2.2. Test Conditions:

Table S1: Conditions for hydrophobicity and viscosity testing

Experiment	Relevant Figure	Conditions	
Effect of hydrophobicity on different surfaces	Figure 10a	**Cellulose**	0.25 % w/v cellulose 0 U/mL Thrombin 0 g/L Fibrinogen
		Cellulose & Fibrinogen	0.25 % w/v cellulose 0 U/mL Thrombin 2 g/L Fibrinogen
		Cellulose & Thrombin	0.25 % w/v cellulose 10 U/mL Thrombin 0 g/L Fibrinogen
		Cellulose & Fibrin	0.25 % w/v cellulose 10 U/mL Thrombin 2 g/L Fibrinogen
Effect of Glycerol Viscosity on Elution Height	Figure 10b	**Paper Type**	Tissue 1
		Strip Width	3 mm
		Position of Glycerol from Bottom of Strip (cm)	1 cm
		Glycerol-Water Mixture Volume	6 µL
		Glycerol Wicking Time	60 sec
		Elution Time	3 min

Table S2: Conditions for variable-sensitivity testing

Experiment	Relevant Figure	Conditions	
Effect of Paper Structure and Fibrinogen Concentration on Elution Height	Figure 11	**Strip Width**	5 mm
		Thrombin Solution Volume	3 µL
		Thrombin Concentration	30 U/mL
		FXIIIa Concentration	0 U/mL
		Fibrinogen Solution Volume	20 µL
		Reaction Time	60 sec
		Elution Time	5 min
Effect of Strip Width and Fibrinogen Concentration on Elution Height	Supplementary Figure 8	**Paper Type**	Tissue 2
		Thrombin Solution Volume (3 mm/5 mm)	2/3 µL
		Thrombin Concentration	30 U/mL
		FXIIIa Concentration	0 U/mL
		Fibrinogen Solution Volume (3 mm/5 mm)	12/20 µL
		Reaction Time	60 sec
		Elution Time	5 min
Effect of Fibrinogen Solution Volume and Fibrinogen Concentration on Elution Height	Figure 12a	**Paper Type**	Tissue 2
		Strip Width	3 mm
		Thrombin Solution Volume	2 µL
		Thrombin Concentration	30 U/mL
		FXIIIa Concentration	0 U/mL
		Reaction Time	60 sec

		Elution Time	5 min
Effect of Thrombin Concentration and Fibrinogen Concentration on Elution Height	Figure 12b	**Paper Type**	Tissue 2
		Strip Width	3 mm
		Thrombin Solution Volume	2 µL
		FXIIIa Concentration	0 U/mL
		Fibrinogen Solution Volume	12 µL
		Reaction Time	60 sec
		Elution Time	5 min
Effect of FXIIIa Concentration and Fibrinogen Concentration on Elution Height	Supplementary Figure 7	**Paper Type**	Tissue 2
		Strip Width	3 mm
		Thrombin Solution Volume	2 µL
		Thrombin Concentration	30 U/mL
		Fibrinogen Solution Volume	24 µL
		Reaction Time	60 sec
		Elution Time	5 min
Effect of Reaction Time and Fibrinogen Concentration on Elution Height	Figure 13a	**Paper Type**	Tissue 2
		Strip Width	3 mm
		Thrombin Solution Volume	2 µL
		Thrombin Concentration	60 U/mL
		FXIIIa Concentration	0 U/mL
		Fibrinogen Solution Volume	12 µL
		Elution Time	5 min
Effect of Elution Time and Fibrinogen Concentration on Elution Height	Figure 13b	**Paper Type**	Tissue 2
		Strip Width	3 mm
		Thrombin Solution Volume	2 µL
		Thrombin Concentration	60 U/mL
		FXIIIa Concentration	0 U/mL
		Fibrinogen Solution Volume	12 µL
		Reaction Time	30 sec
Effect of BSA Concentration and Fibrinogen Concentration on Elution Height.	Figure 14	**Paper Type**	Tissue 3
		Strip Width	3 mm
		Thrombin Solution Volume	2 µL
		Thrombin Concentration	60 U/mL
		FXIIIa Concentration	0 U/mL
		Fibrinogen Solution Volume	12 µL
		Reaction Time	30 sec
		Elution Time	7 min

Table S3: Structural Properties of Paper Substrates

	Grammage (GSM)	**Thickness (mm)**	**Density (kg/m^3)**
Tissue 1	25	0.14	180
Tissue 2	30	0.16	190
Tissue 3	41	0.24	180
Filter	91	0.23	400

Table S4: Positionings of fibrinogen and thrombin solution up paper strips for each test condition in **Figure 11.** Each condition was reacted for 60 s and eluted for 5 mins.

	Position of Thrombin Solution from Bottom of Strip (cm)	Position of Fibrinogen Solution from Bottom of Strip (cm)
Tissue 1	2.5	3
Tissue 2	2	2.5
Tissue 3	1.5	2
Filter Paper	1.5	2

Table S5: Positionings of fibrinogen and thrombin solution up paper strips for each test condition in **Figure 12a.** Each condition was reacted for 60 s and eluted for 5 mins.

	Position of Thrombin Solution from Bottom of Strip (cm)	Position of Fibrinogen Solution from Bottom of Strip (cm)
3 µL Fibrinogen Solution	0.5	1
6 µL Fibrinogen Solution	1	1.5
12 µL Fibrinogen Solution	2	2.5
18 µL Fibrinogen Solution	3	3.5
24 µL Fibrinogen Solution	4	4.5

Table S6: Positionings of fibrinogen and thrombin solution up paper strips for each test condition in **Figure 12b, 13, S7** and **S8.** Each condition except **Figure 13** was reacted for 60 s and eluted for 5 mins.

	Position of Thrombin Solution from Bottom of Strip (cm)	Position of Fibrinogen Solution from Bottom of Strip (cm)
All Conditions	2	2.5

Table S7: Positionings of fibrinogen and thrombin solution up paper strips for each test condition in **Figure 14.** Each condition was reacted for 30 s and eluted for 7 mins.

	Position of Thrombin Solution from Bottom of Strip (cm)	Position of Fibrinogen Solution from Bottom of Strip (cm)
0 g/L BSA	1.5	2
80 g/L BSA	1	1.5

Table S8: Optimal Test Conditions in **Figure 15.**

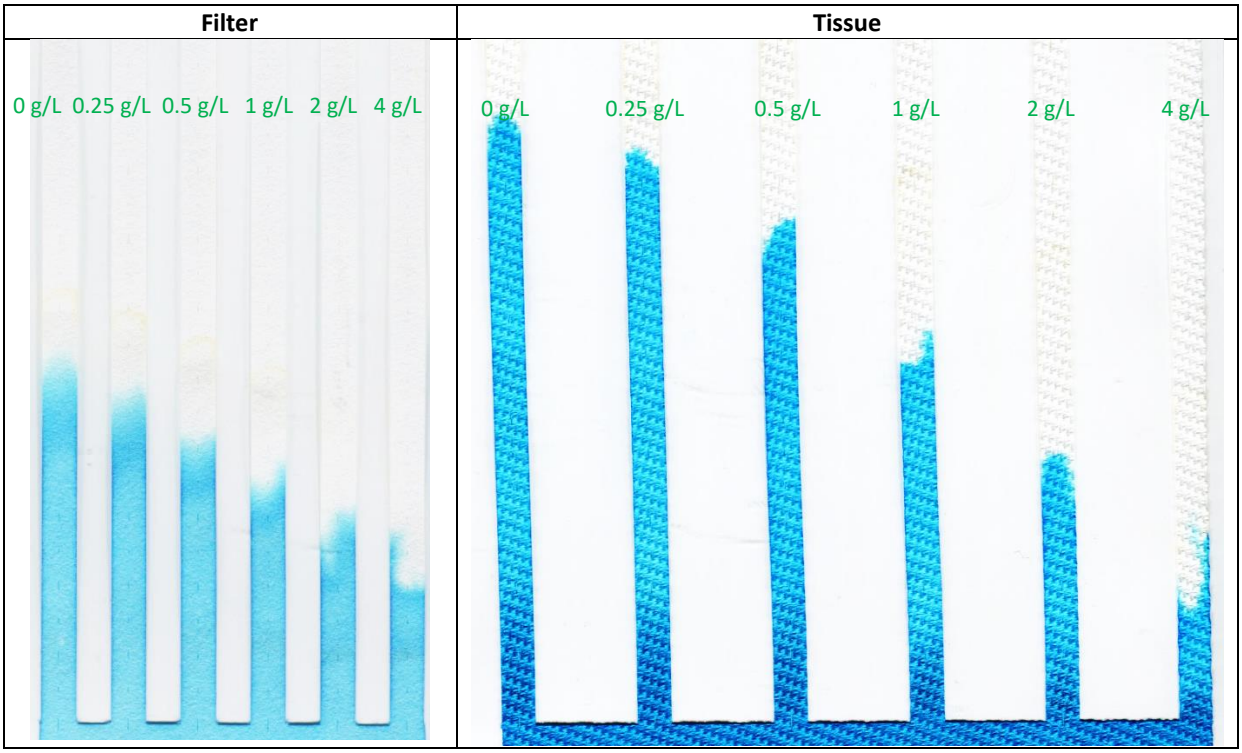
Optimal Test Conditions	Figure 15	**Paper Type**	Tissue 3
		Strip Width	3 mm
		Thrombin Solution Volume	2 µL
		Thrombin Concentration	60 U/mL
		FXIIIa Concentration	0 U/mL
		Fibrinogen Solution Volume	12 µL
		Reaction Time	30 sec
		Elution Time	7 min
		Position of Thrombin Solution from Bottom of Strip (cm)	1
		Position of Fibrinogen Solution from Bottom of Strip (cm)	1.5

Table S9: Conditions for Serum vs Plasma testing in **Figure 9.**

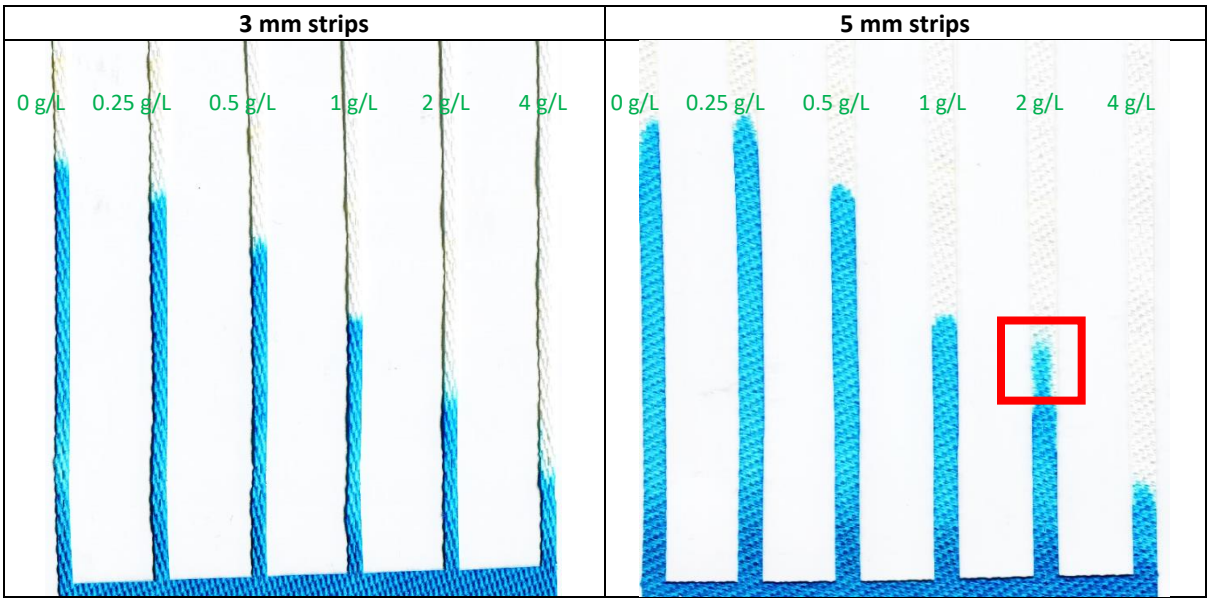
Elution of Serum vs Plasma	Figure 9	**Paper Type**	Tissue 2
		Strip Width	5 mm
		Thrombin Solution Volume	3 µL
		Thrombin Concentration	30 U/mL
		FXIIIa Concentration	0 U/mL
		Serum/Plasma Volume	20 µL
		Reaction Time	60 sec
		Elution Time	5 min
		Position of Thrombin Solution from Bottom of Strip (cm)	1.5
		Position of Fibrinogen Solution from Bottom of Strip (cm)	2

RESULTS:

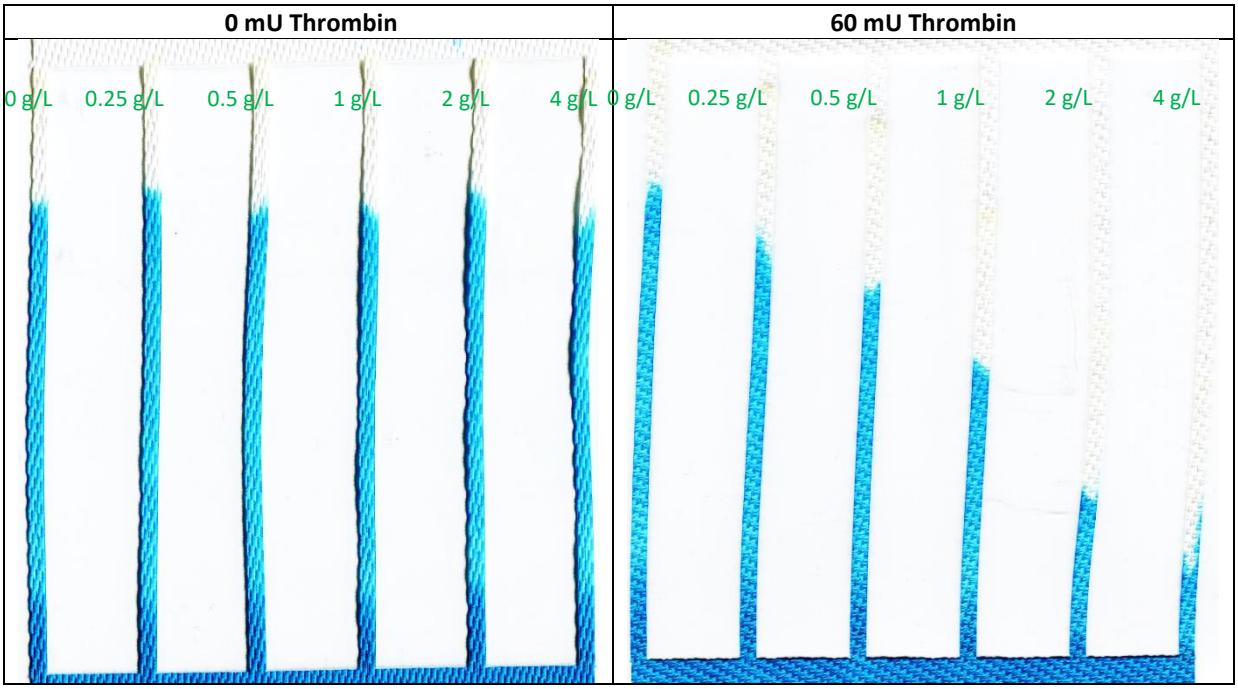
2.3. Raw Data:



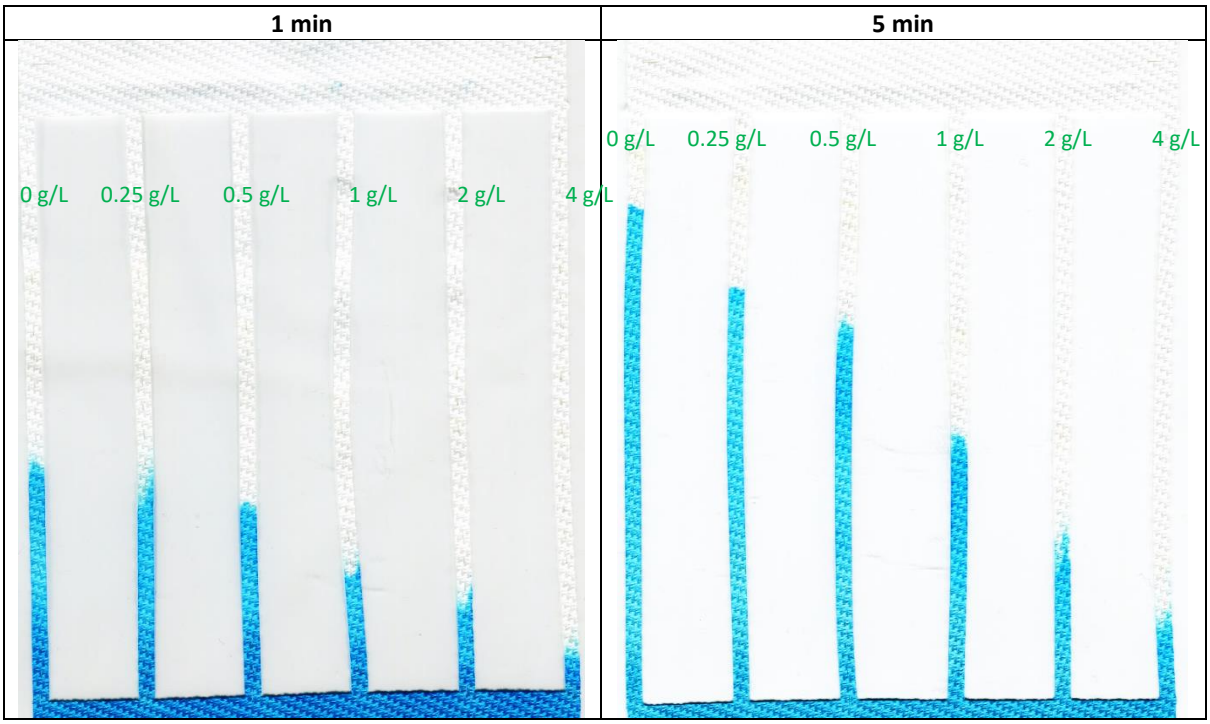
Supplementary Figure 2: Elution of Filter (High Density) Paper vs Tissue (Low Density) Paper. Tissue Paper elutes a lot faster than Filter Paper. Therefore, it is far more sensitive at differentiating between different fibrinogen concentrations. Green numbers indicate fibrinogen solution added to each strip.



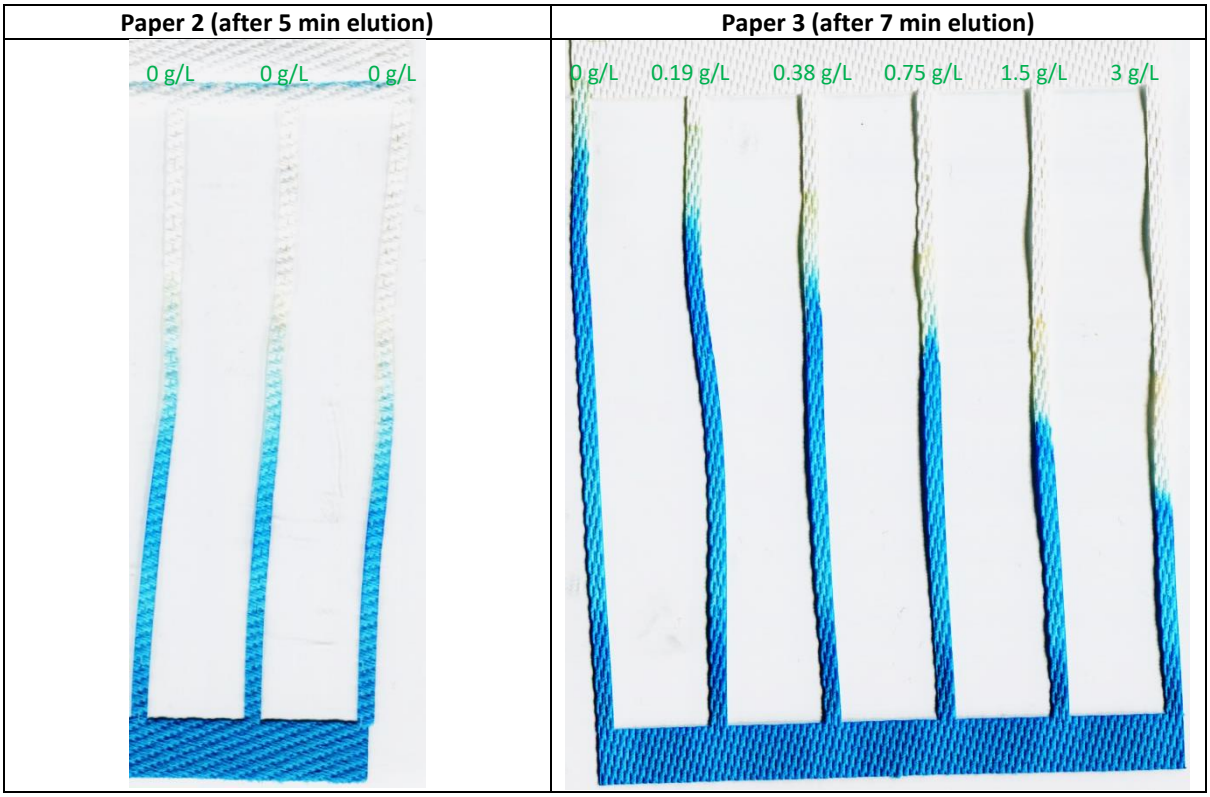
Supplementary Figure 3: Elution of 3 mm strips vs 5 mm strips. 3 mm strips produces greater accuracy than 5 mm strips. This is due to the lower frequency of non-homogeneous elution. Non-homogenous elution (indicated by the red square) is attributed to the inconsistencies in paper pore structure and causes the aqueous dye to elute further than expected. Green numbers indicate fibrinogen solution added to each strip.



Supplementary Figure 4: Elution of 0 mU Thrombin vs 60 mU Thrombin. Thrombin is essential for this test. 0 mU thrombin shows negligible elution height differences between different fibrinogen concentrations. 60 mU thrombin however strongly differentiates between different fibrinogen concentrations. Green numbers indicate fibrinogen solution added to each strip.

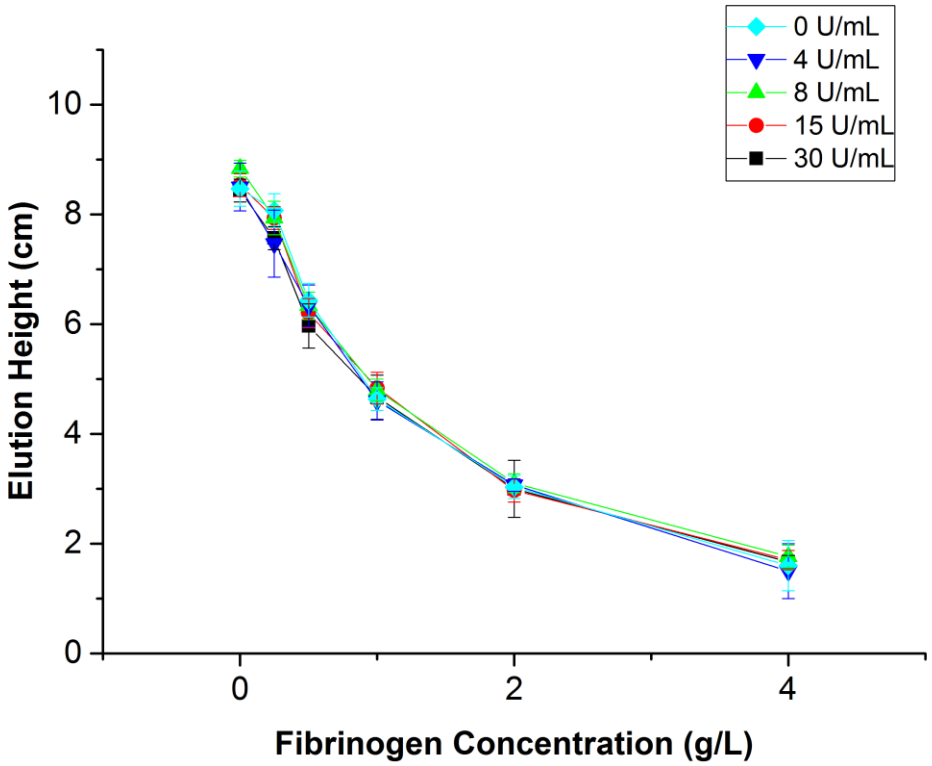


Supplementary Figure 5: 1 min elution time vs 5 min elution time. Elution Time plays a large impact on sensitivity. At 1 min separation between different fibrinogen concentrations was poor. However, by 5 mins, separation was clear between different fibrinogen concentrations. Green numbers indicate fibrinogen solution added to each strip.



Supplementary Figure 6: Paper 2 (0.16 mm thickness) vs Paper 3 (0.24 mm thickness). Paper Thickness impacts on the visibility of the elution front for Fibrinogen Solutions in 80 g/L BSA. Paper 2 produces an elution front gradient, making results difficult to read. Paper 3 however produces a much sharper elution front making results easier to read.

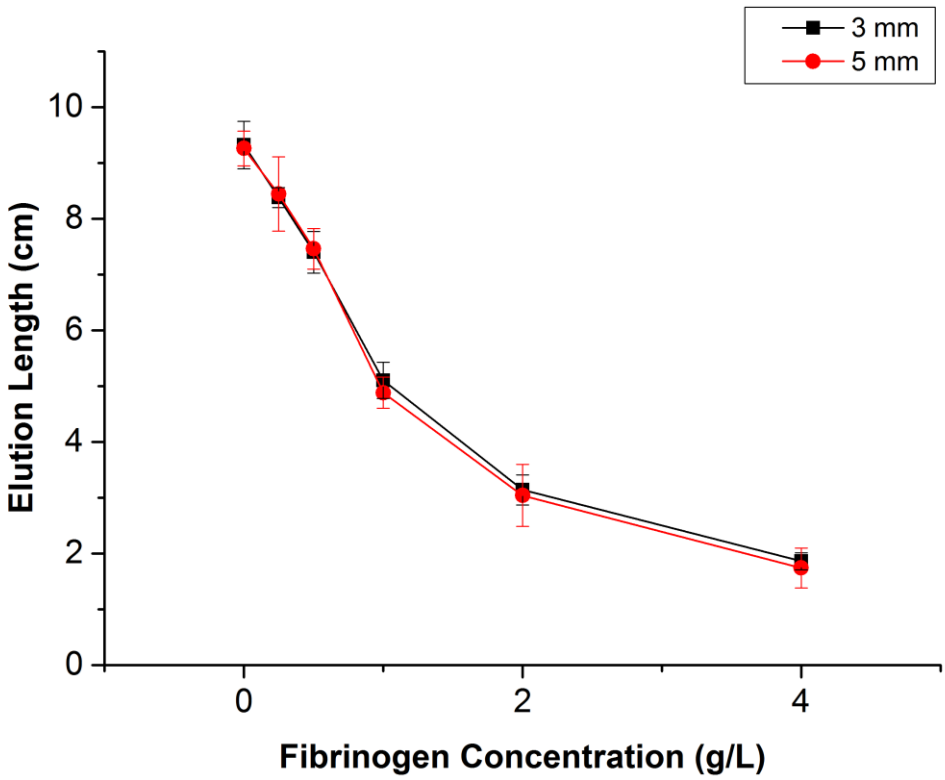
2.4. Effect of FXIIIa Concentration:



Supplementary Figure 7: Effect of FXIIIa Concentration and Fibrinogen Concentration on Elution Height. Test conditions can be found in **Table S2** and **S6**. Each test was performed in quadruplet and the average and standard deviations are reported.

The effect of Factor XIIIa concentration on elution height for different fibrinogen concentrations is investigated (**Supplementary Figure 8**). Factor XIII has no effect on the sensitivity of the test.

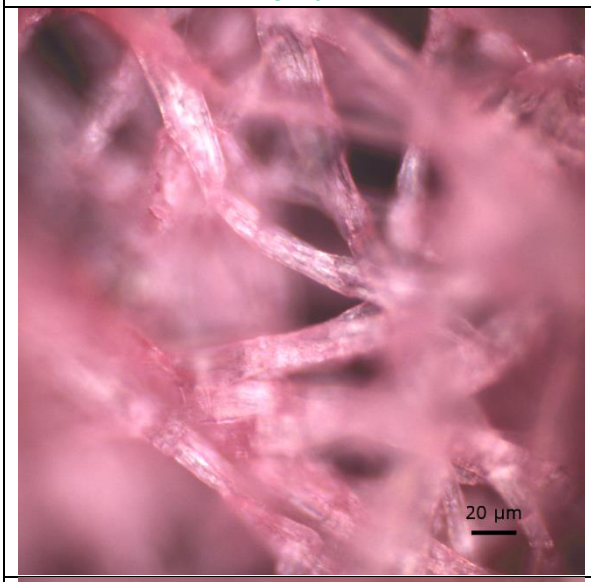
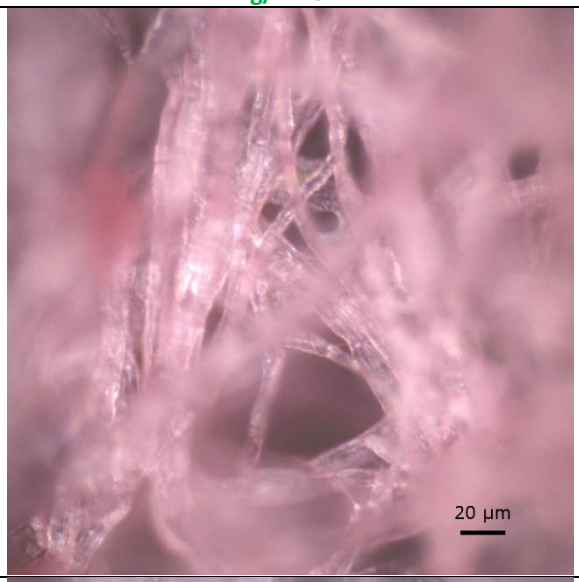
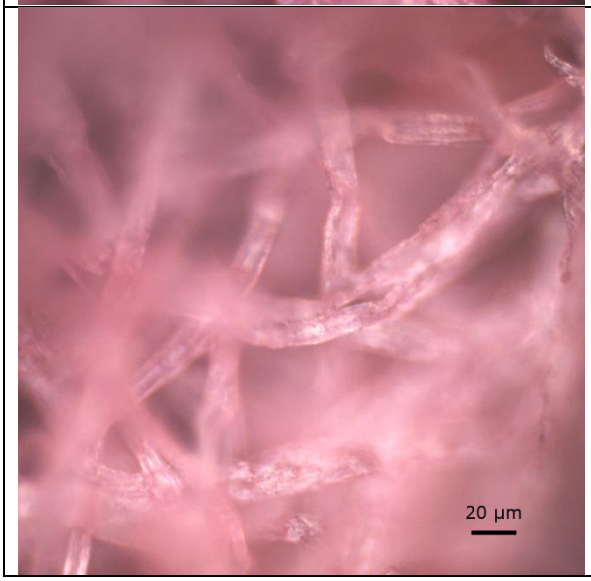
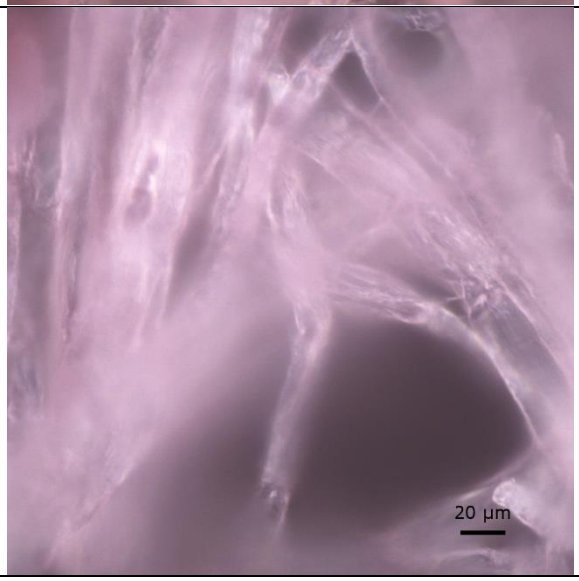
2.5. Effect of Strip Width:

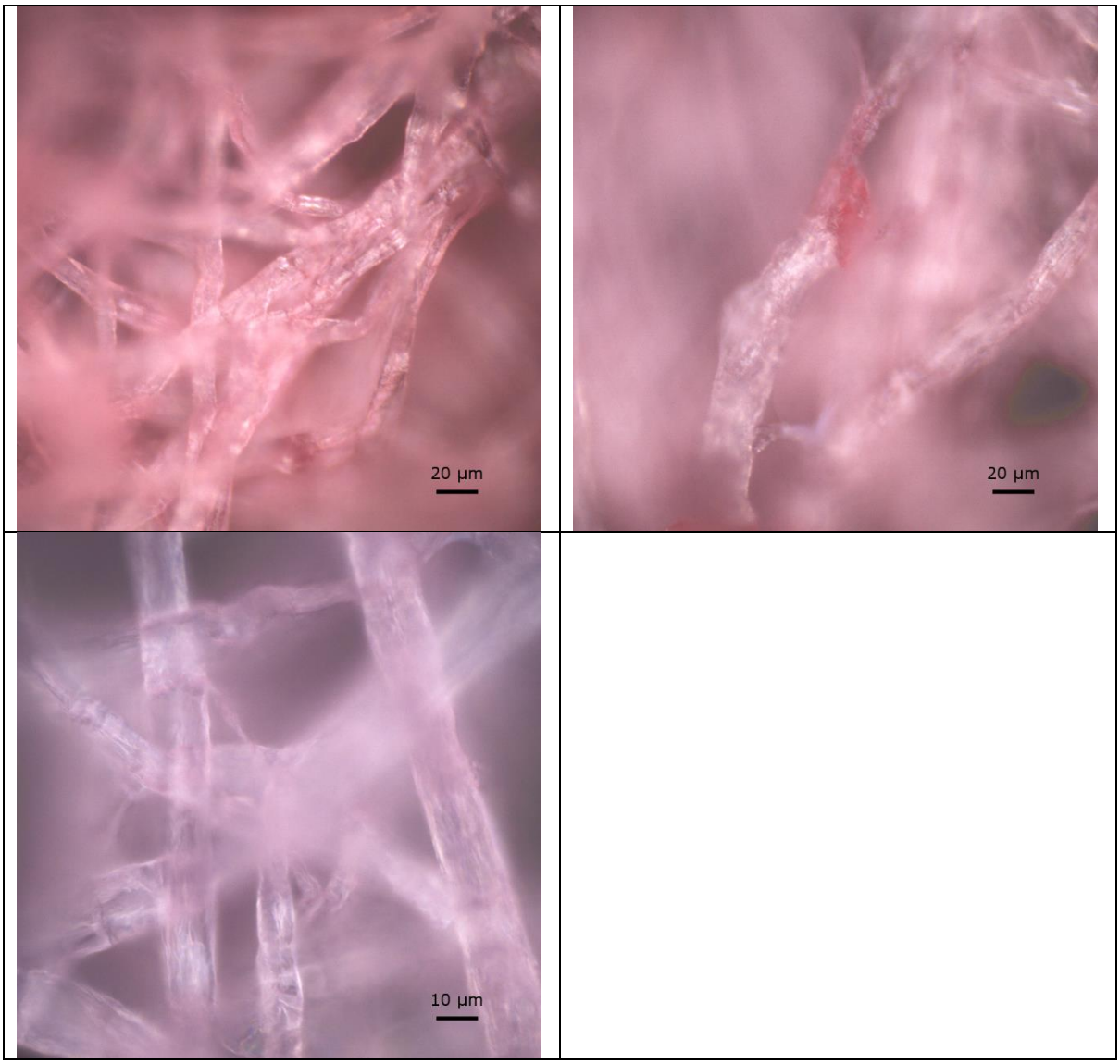


Supplementary Figure 8: Effect of Strip Width and Fibrinogen Concentration on Elution Height. Test conditions can be found in **Table S2** and **S6**. Each test was performed in quadruplet and the average and standard deviations are reported.

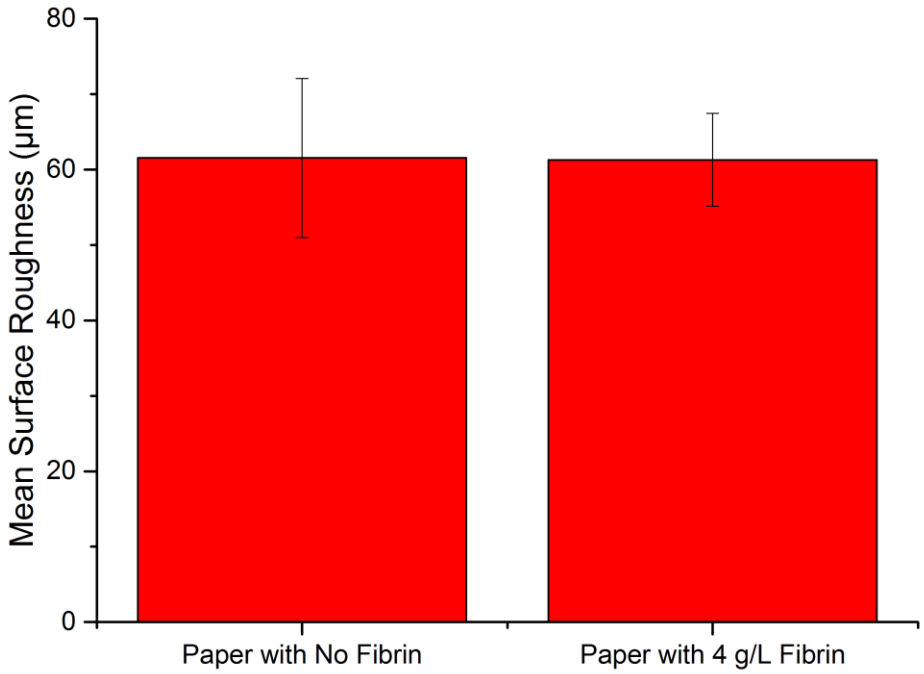
The effect of fibrinogen concentration on elution height for different strip widths is investigated (**Supplementary Figure 8**). 2 mm strips were too fragile for proper diagnostic manipulation. 3 mm and 5 mm wide strips show no significant difference in elution sensitivity. However, the thinner strips show better reproducibility between different fibrinogen concentrations due to an elution front that is clearer and easier to read (see **Supplementary Figure 3**). This is attributed to wider strips introducing more inconsistencies in paper pore structure. In turn, this causes the aqueous dye to elute further than expected. Therefore, 3 mm strips were selected for their more reliable performance.

2.6. Paper Pore Blockage Analysis:

No Fibrin	4 g/L Fibrin
	
	



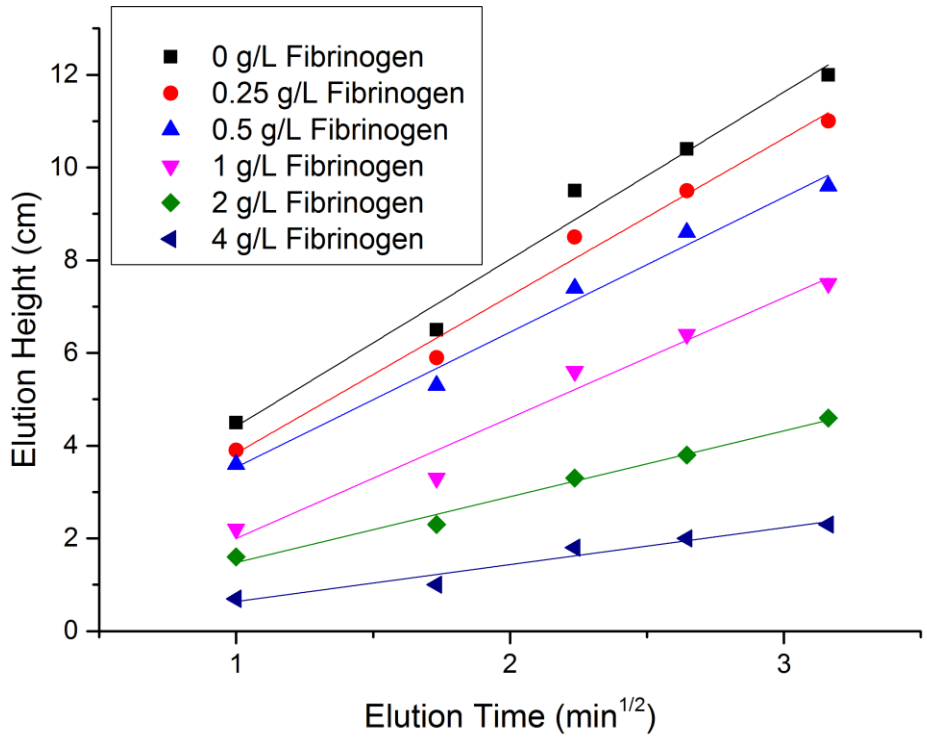
Supplementary Figure 9: Images of Tissue Paper 3 coated with and without fibrin as captured under a laser microscope. Paper squares 1 X 1 cm^2 were cut on Epilog Laser Cutter. Thrombin vials were reconstituted with 0.3% Ponceau 4R (from Queens Red Food Dye) diluted in water. 2 µL of Thrombin was quickly mixed with 24 µL of Fibrinogen solutions (0 g/L and 4 g/L in PBS) in a PCR tube before being pipetted onto the paper. They were then imaged under a LEXT 3D Measuring Laser Microscope OLS5000 at 50X and 100X.



Supplementary Figure 10: Mean surface roughness of Tissue Paper 3 coated with and without fibrin as measured under a profilometer. Thrombin vials were reconstituted with water. 2 µL of Thrombin was quickly mixed with 24 µL of Fibrinogen solutions (0 g/L and 4 g/L in PBS) in a PCR tube before being pipetted onto the paper. The mean surface roughness was measured using a LEXT 3D Measuring Laser Microscope in triplicate.

Supplementary Figure 9 and **10** verifies wherever the physical blockage of the tissue paper pores occurred due to fibrin formation. Light microscope images are shown in **Supplementary Figure 9**. 2 observations are noticed: 1) The paper pores are very large and can exceed 100 µm. 2) There is no observed decrease in pore size due to the formation of fibrin. Mean surface roughness measurements are also shown in **Supplementary Figure 10.** If fibrin did fill the voids of the paper pores, then the paper surface would smoothen and the measured mean surface roughness would decrease. However, that was not the case as the paper surface roughness was similar regardless of the formation of fibrin. the paper surface roughness was similar regardless of the formation of fibrin. This means that the physical blockage of the pores is not a driving mechanism of the diagnostic.

2.7. Washburn Flow Kinetic Analysis:



Supplementary Figure 11: Washburn kinetics analysis derived from the data of **Figure 13b**. Each test was performed in quadruplet and the linear line of best fit though each set of data is given.

Table S10: Coefficient of Determination calculations corresponding to the linear lines of best fits in **Supplementary Figure 11**.

Fibrinogen Concentration (g/L)	Coefficient of Determination (R^2)
0	0.98
0.25	0.99
0.5	0.99
1	0.97
2	0.99
4	0.95

Supplementary Figure 11 and **Table S10** verifies wherever the polymerisation of fibrinogen in the test causes elution to deviate away from Washburn Kinetics. The Lucas-Washburn Equation is summarised below:

$$L = \sqrt{\frac{\gamma r t \cos\theta}{2\eta}} \quad (2)$$

Where L is the elution height γ is the apparent γ is the air-blue dye surface tension, r is the capillary radius, θ is the water-capillary surface contact angle t is the elution time and η is the blue dye viscosity. Curves that follow Washburn Kinetics will show a linear trend when L is plotted against $t^{\frac{1}{2}}$.

In **Supplementary Figure 11,** all fibrinogen concentrations give linear trend-lines. In **Table S10**, all trend-lines have an R^2 value above 0.95. This means that the formation of fibrin in the test does not cause elution to deviate away from Washburn kinetics.

Chapter 3: Horizontal Wicking Lateral Flow Assay

Publication Details

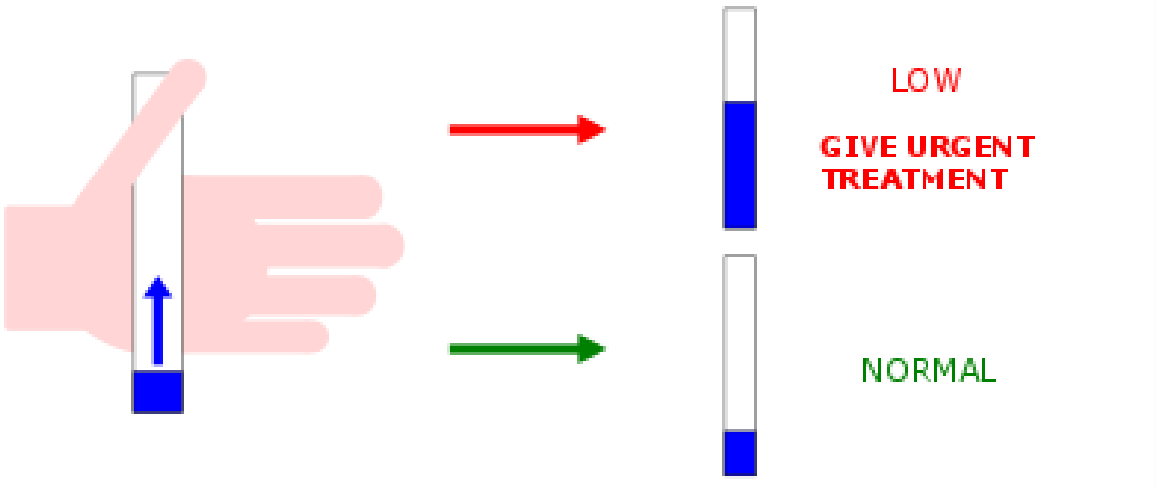
Publication Status: Published

Journal: Analytica Chimica Acta *(Q1 – Analytical Chemistry)*

Reference: Bialkower M, McLiesh H, Manderson C, Tabor R, Garnier G. *Rapid, hand-held paper diagnostic for measuring Fibrinogen Concentration in blood.* DOI: https://doi.org/10.1016/j.aca.2019.12.046

Abstract

Critical bleeding causes over 2 million deaths a year. Early hypofibrinogenemia is a strong predictor of mortality in critically bleeding patients. The early replenishment of fibrinogen may significantly improve outcomes. However, over replenishment can also be dangerous. Furthermore, there is no rapid, cheap, hand-held diagnostic that can aid critically bleeding patients in fibrinogen replacement therapy. In this study, we have developed a hand-held paper diagnostic that measures plasma fibrinogen concentrations. The diagnostic has the potential to be used as a point of care device both inside and outside of hospital settings. It can vastly reduce the time to treatment for fibrinogen replacement therapy. The diagnostic is a two-step process. First, thrombin and plasma are added onto horizontally-orientated paper strips where the fibrinogen is converted into fibrin, drastically increasing the plasma's hydrophobicity. Second, an aqueous blue dye is pipetted onto the strips and allowed to wick through the fibrin. The distance the blue dye wicks through the strip correlates precisely to the fibrinogen concentration. The diagnostic can provide results within a minute. It can distinguish low fibrinogen concentrations (ie. <2 g/L) from normal fibrinogen concentrations. It shows remarkable reproducibility between healthy individuals. It is unaffected by common blood conditions such as acidosis, blood alcohol, severe hypertriglyceridemia, severe haemolysis and warfarin administration. Finally, it is unaffected by humidity and can withstand cold temperatures.



1. Introduction

This study aims to develop a rapid hand-held paper diagnostic for measuring fibrinogen concentrations in plasma. The diagnostic can revolutionise the treatment of heavily bleeding patients. It can vastly reduce the time to treatment before fibrinogen replacement therapy. Furthermore, it can be used as a point of care device in a multitude of locations - not only in hospital emergency rooms, but also in ambulances or outdoors at the site of injury.

Fibrinogen is the effector clotting protein in blood. It is normally present in the plasma at concentrations between 2 and 4 g/L (1, 2). When bleeding is induced, fibrinogen is polymerised into fibrin at the site of blood vessel breakage (3, 4). Patients with hypofibrinogenemia are at a significantly greater risk of mortality due to critical bleeding (10, 11). The early depletion of fibrinogen can impair the formation of clots (12, 13).

Critical bleeding can occur anywhere in the world. Outside clinics, trauma-induced major haemorrhage is the leading cause of death for people aged 18-39 - contributing two million deaths worldwide a year (174, 191-196). Inside clinics, critical bleeding can arise as a result of childbirth (75% of all cases), cardiac surgery, liver transplant and postoperative complications (35-39).

The incidence of hypofibrinogenemia in critical bleeding is common. In 2017, McQuilten *et al.* identified that 21.2% of major trauma patients had fibrinogen levels below 2 g/L (197). Fibrinogen is one of the first proteins to be depleted in critically bleeding patients (31). Hence, the early replenishment of fibrinogen levels may significantly improve outcomes (15-17).

When hypofibrinogenemia is detected in critically bleeding patients, fibrinogen replacement therapy (FRT) is used. European clinical guidelines state that FRT should be implemented when plasma fibrinogen concentrations fall below 2 g/L (14). However, FRT needs to be handled carefully to prevent unnecessary fibrinogen supplementation. Over-supplementation may lead to thrombotic episodes, induce iatrogenic effects and waste expensive resources (18, 19).

Traditionally, fibrinogen replacement therapy (FRT) was provided indirectly through fixed volumetric transfusions (168). This consisted of fixed volumetric ratios of donor red blood cells, platelets, cryoprecipitate and fresh frozen plasma (FFP) being transfused into critically bleeding patients. Since these components had all the blood products needed for clotting, the diagnostic step to determine the causes of bleeding was often overlooked. However, fixed volumetric transfusions have been scrutinised because: 1) The time required to thaw and transfuse both cryoprecipitate and FFP is over an hour (169). 2) The contents of the cryoprecipitate and FFP are unstandardised (170, 171). 3) The transfusion of cryoprecipitate or FFP can cause dangerous side effects (170, 172-175). 4) Even though the FFP and cryoprecipitate contains fibrinogen, the transfused red blood cells and platelets dilute the patient's fibrinogen to be below the threshold levels for clotting (177-179).

Recently, the availability of lyophilised clotting factors has allowed major haemorrhage protocols to be carried out more safely. Therefore, FRT has been successfully implemented by using fibrinogen concentrate (FC). Rahe-Meyer *et al.* reported that FC only needs a supplementation time of 5 min (174). However, a means of diagnosing hypofibrinogenemia must be executed to prevent the inappropriate supplementation of FC.

The main clinical assays currently used to detect hypofibrinogenemia are unreliable. The Clauss assays are the most common standard laboratory technique for quantifying fibrinogen concentration. They do so by taking a plasma sample, adding thrombin and measuring the time taken for fibrin formation to occur. Longer times correlate to lower fibrinogen concentrations (20). Likewise, viscoelastic haemostatic assays have risen in popularity in the last twenty years. This is due to their ability to quantify numerous clotting factors in whole blood per assay. Viscoelastic haemostatic assays measure fibrinogen through the change in viscoelasticity when fibrin is formed (21). However, both assays require well controlled, sensitive and expensive instruments with long assaying times (22).

Therefore, a rapid, cheap, portable and hand-held plasma fibrinogen concentration diagnostic is needed to greatly reduce the time to treatment needed for fibrinogen replacement therapy.

In our previous study, we have developed a paper test based on the principles of vertical wicking (199). First, plasma is added onto paper strips pre-wetted with thrombin. Second, the test is placed vertically into an infinite reservoir of blue dye. The fibrinogen concentration can be measured by how far the blue dye wicks up the strip over a set amount of time. This is because when the fibrinogen in plasma reacts with the thrombin to form fibrin, it increases the hydrophobicity of the strip. This in turn decreases the blue dye wicking rate. The higher the fibrinogen concentration, the stronger the strip hydrophobicity and hence the lower the blue dye wicking height. Therefore, the tests of patients with hypofibrinogenemia will produce further wicking than healthy people.

However, the vertical wicking test has limitations that confines it to research laboratories. First, the test-setup is not easy-to-use. The infinite reservoir must rest on a stable horizontal surface and the paper strips need to be placed stationary in it for accurate reading. This is not feasible in outdoor scenarios. Second, the test is limited by gravity. As the strip must be held vertically, the rate at which the blue dye moves up the strip is slower than what it would be in a flatter orientation. Therefore, we aim to modify this test so that it is better suited as a rapid, hand-held point of care device. We intend to achieve this by not-only substituting the infinite reservoir with pipetting droplets of blue dye on the strip, but also by orientating the strips horizontally instead of vertically.

In this paper, we develop a rapid and sensitive paper-based test that can be hand-held horizontally for point of care use. We investigate the effect that different human plasma samples have on the test - to conclude wherever the test varies between healthy individuals. We compare its performance to a hospital-grade Clauss assay. Finally, we investigate the effect of common blood abnormalities and weather conditions. This includes the effects of acidosis, alcohol, heparin, warfarin, haemolysis and dietary lipids as well as surrounding temperatures and humidities. It is our objective to show the proof of concept of a point of care paper fibrinogen diagnostic.

2. Materials and Methodology

2.1. Materials

Tissue paper of 30 GSM and 0.16 mm was used to create the paper strip cut-outs. They were produced in the Kimberly Clark Experimental Forming Unit (EFU), Neenah (WI), USA. Tissues consist of eucalyptus fibres formed with a 3 layer headbox. 10 kg/T Kymene, a PAE wet strength agent, was added to each layer. Tissues were moulded and thoroughly air-dried. Two 1 X 1 mm^2 square steel rods were cut into 35 mm lengths to hold the paper strips horizontally. J Burrows Magnets were used to attach the paper strip cut-outs to the steel bar. A 7.5 X 12.6 X 26.7 cm^3 Falcon tube holder was used to hold the metal bars in place and keep paper strip cut-out suspended over the tabletop. A schematic of the setup is shown **Supplementary Figure 12**.

Brilliant Blue FCF (from Queen Blue Food Colouring) was used to make the blue dye (for wicking). Ponceau 4R (from Queen Red Food Colouring) was used to make the red dye (for colouring the thrombin solution).

3 lots of citrated plasma from healthy samples (with fibrinogen concentrations of 2.8 g/L, 3.2 g/L and 3.3 g/L) were sourced from R2 diagnostics. Each plasma sample was pooled from the platelet-poor plasma of at least fifteen different donors. Thrombin (T8885), unfractionated heparin (H5515), warfarin (PHR1435), triglycerides (17810), hydrochloric acid solution (150696), sodium chloride (S7653) and the haemoglobin assay kit (MAK115) was sourced from Sigma Aldrich. Absolute ethanol (AJA214) was sourced from Ajax Finechem. Ethylenediaminetetraacetic acid (EDTA) anticoagulated whole blood was sourced from the Australian Red Cross. Soy wax (NatureWax® C3 Container Blend) was sourced from Candle Supply.

MilliQ water was used to dilute the dyes to the required concentrations and reconstitute vials of citrated plasma and thrombin.

"Tubes PCR Strip Tubes 0.2 ml Thin Wall & Flat Caps" from Axygen were used as the tubes for mixing. They are referred to as PCR tubes throughout the paper.

An iPhone 8 with the app ProMovie was used to record wicking. A Yellowstone 6000 Tripod was used to hold the iPhone in place during recording. A Manfrotto Universal Smartphone Clamp was used to attach the iPhone to the tripod.

2.2. Methodology

2.2.1. Paper Strip Cut-Out Preparation

Paper strip cut-outs were prepared as such on CorelDRAW X6 with the dimensions shown in **Supplementary Figure 13**. Paper was cut on a Laser Cutter (60 Watt Epilog Helix) using the cut-outs prepared on CorelDRAW X6. The print settings used were 100% speed, 5% Power and 500 Hz Frequency. A Petri dish filled with soy wax was melted at 150°C on a Corning hot plate (#6795-420D). Once molten, 10 mL of molten wax was applied across the bottom of each strip below the dye pad. This ensured that the blue dye only travelled in one direction.

2.2.2. Plasma Sample Preparation

Vials of citrated plasma were fully reconstituted with 1 mL of distilled water each. This plasma is referred to as normal or healthy plasma throughout the paper. Then, 50 mL aliquots of normal plasma were pipetted into Eppendorf Tubes and snap-frozen in liquid nitrogen. They were later stored at -86°C until used.

Defibrinogenated plasma was prepared through heat precipitation. Normal plasma was transferred to Eppendorf Tubes and heated in a water bath (Thermo Scientific Precision GP20) at 56-58°C for 30 min - to selectively precipitate the fibrinogen. Afterwards, the Eppendorf Tubes were centrifuged at 12500 rpm using a 11124-H rotor for 10 min in a Sigma 2-16P centrifuge. The supernatant - which contained no fibrinogen - was collected, aliquoted 50 mL each into Eppendorf Tubes, snap frozen in liquid nitrogen and stored at -86°C until used.

When both normal and defibrinogenated plasma samples were thawed, they had to be re-mixed using a pipette - to ensure their contents were uniformly distributed. Plasma samples of different fibrinogen concentrations were prepared by mixing normal plasma and defibrinogenated plasma to the appropriate ratios.

2.2.3. Thrombin Solution Preparation

50 mL of Queen Red Food Colouring was diluted in 450 mL MilliQ Water. One vial of thrombin was reconstituted with 300 mL of the solution to make vials of 30 NIH U/mL Thrombin. The vials were then aliquoted 50 mL each into Eppendorf Tubes, snap-frozen in liquid nitrogen and stored at 86°C until used. (NB: Any food colouring can be used. We used red colouring as it contrasted with the blue dye used in the test and allowed its microfluidics to be observed more easily).

When these thrombin solution samples were unfrozen and thawed, they had to be re-mixed using a pipette - to ensure their contents were uniformly distributed.

From **Chapter 2**, Factor XIIIa did not influence the test (despite crosslinking fibrin monomers). Therefore, it was not observed in this chapter.

2.2.4. Main Test

Experiments were performed in a temperature and humidity-controlled room at 23°C and 50% relative humidity. 0.5 mL of Thrombin Solution was pipetted into the bottom of a PCR tube. Using a pipette, 3 mL of plasma sample was quickly mixed with the thrombin solution until the red colouring was homogenous. It was then applied across the 0 mm marking of the paper strip. The mixed plasma had to be evenly applied across the width of the strip to prevent gaps forming in the fibrin network. This was achieved by quickly pipetting the plasma dropwise onto the left-hand side, right-hand side and centre of the strip e thus forming a zone of hydrophobicity.

After 30 s, a 30 mL droplet of 5% Queen Blue Food Colouring was applied from the bottom of the dye pad. It was important to ensure that the shear force of the blue dye did not destroy the fibrin network that was formed. Therefore, the blue dye wicking through the network was controlled in two ways. 1) The blue dye was added slowly and continuously over a period of 10 s. 2) The blue dye was added from the bottom of the dye pad (to provide enough room for the droplet to sink in). The wicking length was measured 30 s after the blue dye began to be added to the strip. **Figure 16** illustrates this procedure.

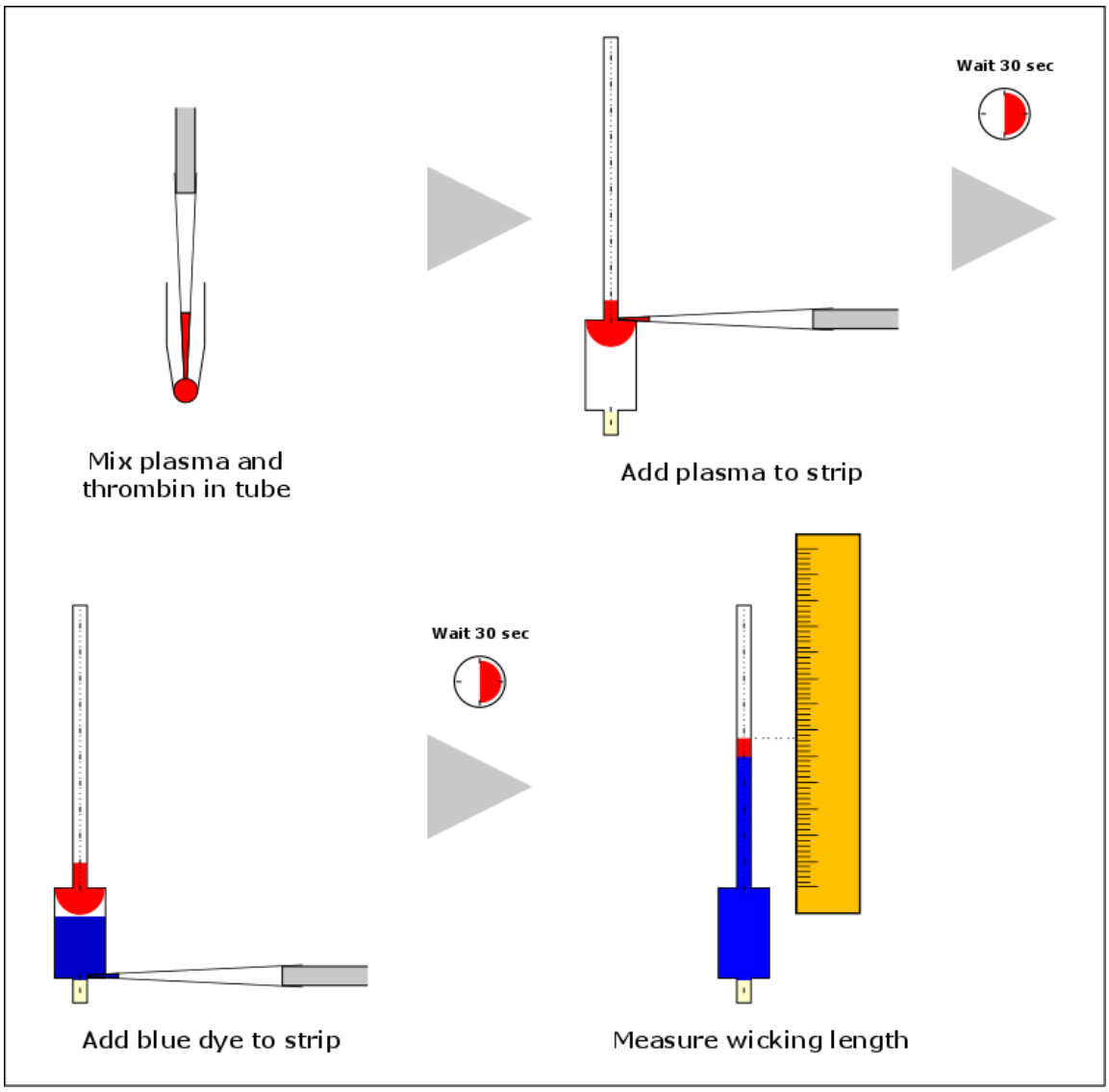


Figure 16: Schematic Illustration of the main test procedure

2.2.5. Wicking Length Measurement

The midpoint for the wicking front was used to measure the wicking length. The iPhone was held directly over the paper strips and ProMovie (downloaded from the App Store) was used to film the main test. ProMovie was used because it could manually focus on the paper strip markings for analysis (see **Supplementary Figure 14**). To measure wicking length, snapshots of each strip in the recorded videos were taken 30 s after blue dye was first applied. They were then analysed on ImageJ to determine their wicking length - as shown in **Supplementary Figure 15**.

2.2.6. Testing of Different Substances

Heparin, warfarin, alcohol and lipids were all tested as they are all common substances found in plasma that may have impacted on the test. Solutions of 14 U/mL heparin, 60 mg/L warfarin, 4 g/dL ethanol and 25,000 mg/dL triglycerides were all prepared by dissolving or diluting in MilliQ water.

Each substance was diluted 1:20 times in either normal or defibrinogenated plasma. This created plasma samples with physiologically significant concentrations of each substance. These samples were then tested.

2.2.7. Testing of Acidosis

A pH Meter (Thermo Fisher) was used to record the pH of 2 mL of normal plasma. This was performed in a 15 mL Falcon tube - because it could allow the plasma to reach a high enough level for pH measurement. The pH was measured to be 7.6. 500 mL of normal plasma was initially collected and kept aside.

1 M hydrochloric acid was added carefully to drop the pH of the remaining plasma to 7.3 and another 500 mL was collected. This process was repeated twice more with the remaining plasma further dropped to 7.1 and 6.9 and collected each at 500 mL. The final concentration of hydrochloric acid in the pH 6.9 sample was calculated. Then, the equivalent molar concentration of sodium chloride was added to 250 mL of normal plasma with 1 M sodium chloride. This created a control sample that determined whether any observed effects were driven by decreased pH or increased salt concentration.

Half the volume of each sample was heated at 56-58°C for 30 min to also prepare defibrinogenated samples at different pHs. These samples were then tested.

2.2.8. Testing of Haemolysis

1 mL of whole blood was pipetted into an Eppendorf tube. It was then centrifuged at 12500 rpm using a 11124-H rotor for 10 min in a Sigma 2-16P centrifuge. The plasma was carefully discarded using a pipette - leaving behind the red blood cells (RBCs). The RBCs were lysed by snap-freezing them in liquid nitrogen and re-thawing them at room temperature three times. This created haemoglobin concentrate. To confirm that the cells had successfully lysed, a 1 mL sample of haemoglobin concentrate was viewed under a Nikon H600L microscope and verified by the lack of cells present.

The concentration of the haemoglobin concentrate was quantified by using the haemoglobin assay kit and following the technical bulletin instructions. The assay was performed on a Tecan Infinite M Nano microplate reader. The haemoglobin concentrate was measured to be 36 g/dL. 1.8 g/dL haemoglobin plasma samples were created by diluting the haemoglobin concentrate 1:20 times in normal and defibrinogenated plasma. Furthermore, a 0.9 g/dL haemoglobin plasma sample was created by diluting the haemoglobin concentrate 1:40 times in defibrinogenated plasma. These samples were then tested.

2.2.9. Testing of Humidity

A dessication chamber (Dry Keeper Sanplatec Corp Ce3B) was used and filled with three bottles of silica gel contained in petri dishes to drop the relative humidity from 50% to 30%. The test as per normal was carried out inside the chamber. The door was opened when adding reagents onto the strip but was closed immediately to prevent the humidity from rising.

2.2.10. Testing of Temperature

An incubator (Carun Diurnal Incubator Model 6042) was used to change the temperature to 7°C, 23°C, 36°C and 44°C. The test setup and all reagents were placed inside the incubator and allowed to equilibrate with the set temperature for 10 min. Afterwards, the test as per normal was carried out inside the chamber. The door was opened when adding reagents onto the strip but was closed immediately to prevent the temperature from returning to room temperature.

2.2.11. Clauss Assay

A Sta-R Analyser - with commercial reagents from Diagnostica Stago (STA® Fibrinogen 5) - was used. This was then compared to the paper test.

3. Results

Paper strips were prepared as shown in **Supplementary Figure 13**. The dye pad design is deciphered in **Supplementary Figure 18**. **Figure 17** illustrates the concept behind the test.

Plasma and thrombin (dyed red) were quickly mixed with a pipette in a PCR tube until the mixture became homogenously red. The mixture was then quickly pipetted across the 0 mm marking and allowed to react for 30 s. This formed a zone of hydrophobicity.

Accurate plasma volumes were confirmed by measuring the equilibrium stain size on paper (**Supplementary Figure 21**) (218).

A droplet of blue dye was then deposited on the dye pad. The wicking length on paper was measured 30 s after the blue dye was first applied. This was chosen as the most suitable wicking time for this study (**Supplementary Figure 19** and **20**). It was important that the blue dye passed the fibrin network at a controlled velocity to prevent it from causing damage. This was achieved by: 1) adding it slowly over a period of 10 s, and 2) adding it from the bottom of the strip (to ensure that the droplet had sunk into the paper before it reached the fibrin network).

The further the wicking front migrates to, the lower the plasma fibrinogen concentration. The mechanism is predominantly hydrophobicity-driven. The conversion of fibrinogen into fibrin increases the hydrophobicity of the paper. Therefore, when the blue dye reaches this hydrophobic zone, its contact angle with the paper increases significantly and wicks at a much slower rate. This decreases the wicking length after 30 s. The amount of fibrin present on paper dictates the extent of hydrophobicity (in this hydrophobic zone), which controls the wicking lengths reached by the dye droplet. There is a clear and reproducible correlation between the length that the droplet wicks along each paper strip and the plasma fibrinogen concentration. The discovery forms the concept of a hand-held diagnostic for measuring plasma fibrinogen concentrations in blood.

The test was diagnosed using three different healthy plasma samples. Each sample of plasma was pooled from at least fifteen different donors and citrated. The test's sensitivity was compared to an automated Clauss assay (Stago STA® Fibrinogen 5). Finally, the effects of haemolysis, acidosis, alcohol, heparin, warfarin and dietary lipids as well as surrounding temperatures and humidities were also compared.

	0 g/L Fibrinogen	3.2 g/L Fibrinogen
Paper strip		
Plasma + Thrombin added to strip **(Zone of hydrophobicity formed)**		
Blue Dye added after 30 s		
Wicking length measured after 30 s		

Figure 17: Summary of wicking test for plasma samples without fibrinogen (0 g/L) and with fibrinogen (3.2 g/L).

3.1. Comparison Between Plasma Samples

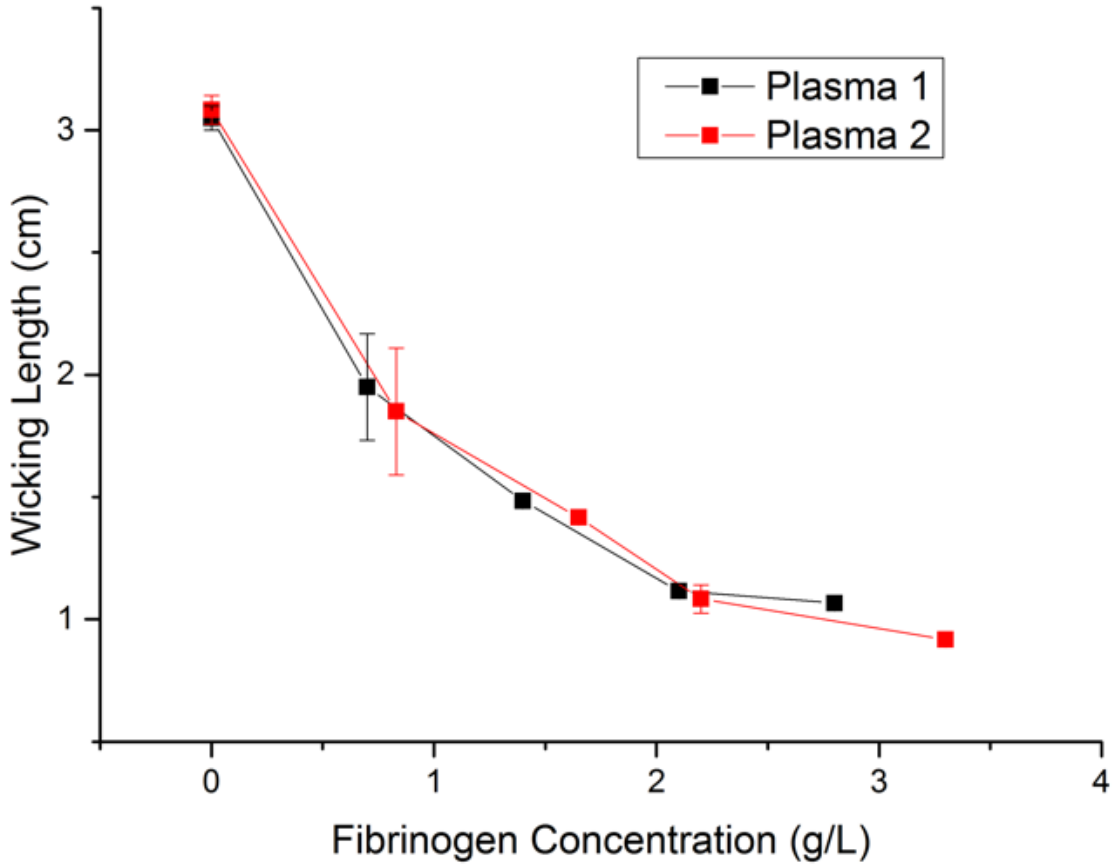


Figure 18: Testing of plasma samples with different fibrinogen concentrations. Plasma from samples 1 and 2 contained 2.8 g/L and 3.3 g/L fibrinogen respectively before they were diluted with defibrinogenated plasma to different fibrinogen concentrations. Each data-point was tested three times and the mean and standard deviations are reported.

Different plasma samples were experimented with to verify whether the test varies between healthy individuals. Results are shown in **Figure 18** and **19**.

Plasmas from two different samples were artificially made to different fibrinogen concentrations by diluting with defibrinogenated plasma. The wicking length on paper was then measured.

This experiment was performed in triplicates and the average and standard deviation are reported in **Figure 18**. Each trendline was created from the normal and defibrinogenated plasmas of a single sample. For example, the Plasma 1 trendline featured the dilution of plasma sample 1 with defibrinogenated plasma created from plasma sample 1. There are two observations of interest. First, a non-linear relationship is observed for both curves. Fibrinogen concentrations below 2 g/L displayed optimal sensitivity, as denoted by the strong gradients of the curves. Second, no variability is observed between the curves. The sample the plasma is sourced from does not impact wicking length. The use of defibrinogenated plasma as an appropriate dilutant is shown in **Supplementary Figures 17** and **23**.

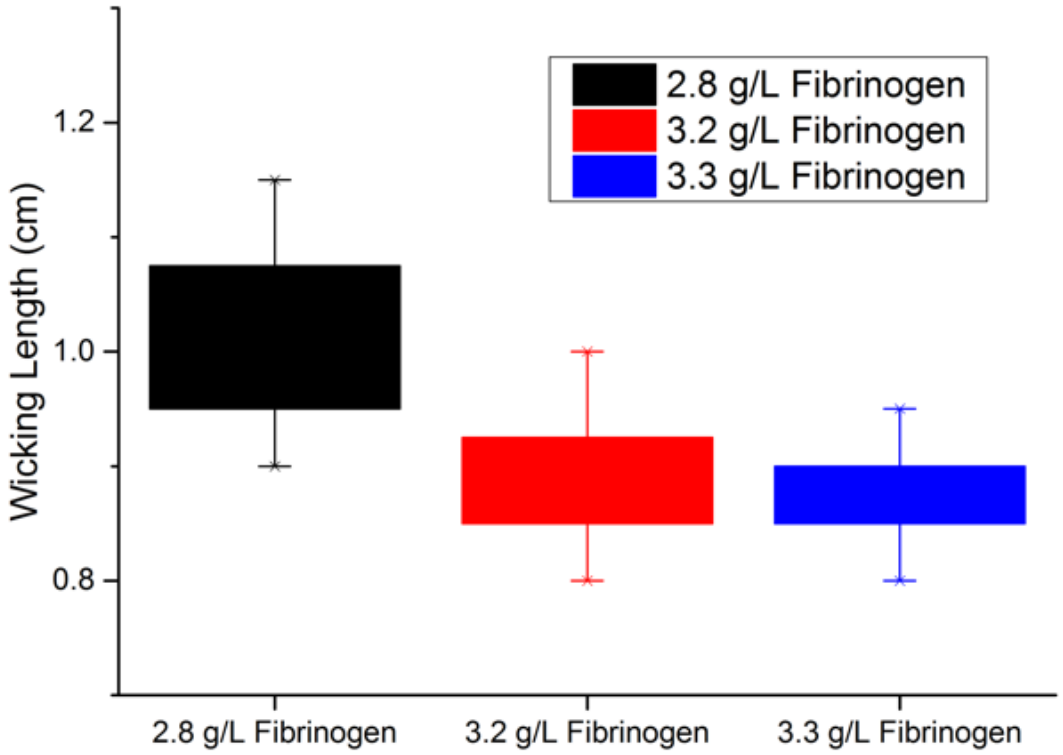


Figure 19: Testing of normal plasma from three samples. Citrated plasma from samples 1, 2 and 3 contained 2.8 g/L, 3.2 g/L and 3.3 g/L fibrinogen respectively. Each plasma was tested up to twenty times. The normal range (ie. mean ± standard deviation) is denoted by the solid block, the total range (ie. range of entire set of data) is denoted by the line bars.

Plasma from three different samples was tested without dilution. Each sample was tested up to twenty times and the normal range (solid block) and total range (line bars) are reported in **Figure 19**.

Two observations are present. First, there is a clear relationship between plasma fibrinogen concentration and wicking length. Plasma samples of higher fibrinogen concentrations wick less. Second, even non-optimal fibrinogen concentrations (ie. above 2 g/L) still produced a clear difference in wicking. This is demonstrated by the non-overlapping normal ranges between the 2.8 g/L fibrinogen sample and the other two samples. Therefore, plasma samples in the optimal fibrinogen concentration range (ie. below 2 g/L) are unlikely to overlap at all.

3.2. Comparison to Clauss Assay

The sensitivity of the paper test was compared to a hospital grade automated Clauss assay (Stago STA® Fibrinogen 5). Plasma from sample 1 (fibrinogen concentration: 2.8 g/L) was diluted to 1.4 g/L and 2.1 g/L fibrinogen with defibrinogenated plasma. Each sample was tested in triplicate on both the paper test and the Stago STA® Fibrinogen. An analysis of variance was then performed to quantify the ability of each assay to distinguish between the three concentrations.

The t-value was used as the metric for the Analysis of Variance. It quantifies how well both devices could distinguish a 1.4 g/L (low) fibrinogen sample from a 2.1 g/L (borderline-normal) fibrinogen sample as well as a 2.1 g/L sample from a 2.8 g/L (normal) sample. The higher the t-value, the greater the sensitivity between two fibrinogen concentrations. Results are shown in **Figure 20**. The Clauss assay displays greater

sensitivity in samples across all fibrinogen concentrations. An example showing how the t-value is calculated is provided in **Table S11**.

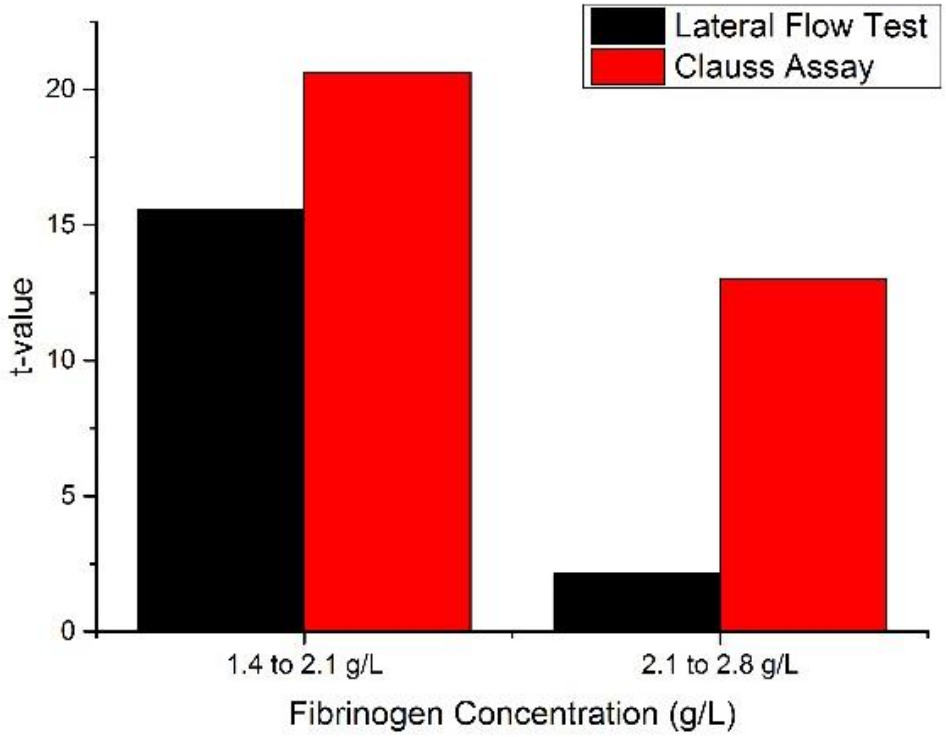


Figure 20: Analysis of variance between the horizontal wicking assay and the automated Clauss assay (Stago STA® Fibrinogen 5). Defibrinogenated plasma from sample 1 was made to different fibrinogen concentrations and tested on both diagnostics. Samples (1.4, 2.1 and 2.8 g/L) were tested on each diagnostic three times. The means and standard deviations were taken from each diagnostic and used to calculate the t-values across a range of fibrinogen concentrations.

3.3. Patient Condition: Effect of Chemical Substances in Plasma

Patients requiring the measurement of blood fibrinogen concentration might be on anticoagulants, have drunken alcohol, or suffer from high cholesterol. These conditions should not affect the test. Extreme plasma concentrations of heparin, warfarin, alcohol and dietary fats were therefore all tested individually to observe whether they impact on wicking distance. These substances were added to defibrinogenated plasma to determine whether they influenced the blue dye migration rate. These additives were also added to normal plasma to investigate if they interfere with the fibrin formation process and subsequent wicking impedance. Each substance was tested five times in both defibrinogenated and normal plasma and the mean and standard deviations are reported in **Figure 21**.

Two observations are noted: 1) No substance alone affects the blue dye migration rate (as shown in the top graph). It was hypothesised that triglycerides would at least impair the wicking of defibrinogenated plasma due to its hydrophobic properties. However, this is not the case. 2) Heparin is the only substance to diminish the level of fibrin formation and cause further migration of blue dye than expected (as shown in the bottom graph). This is due to its ability to inhibit already activated thrombin. Heparin can activate the plasma protein anti-thrombin, which in turn can inhibit thrombin. This can drop the thrombin activity

and subsequentially the amount of fibrin formed. Although both warfarin and alcohol can act as anti-coagulants, they cannot do so by inhibiting active thrombin. For example, warfarin exerts its effects by inhibiting the vitamin K dependent synthesis of coagulation factors in the liver (including thrombin, factor VII, IX and X).

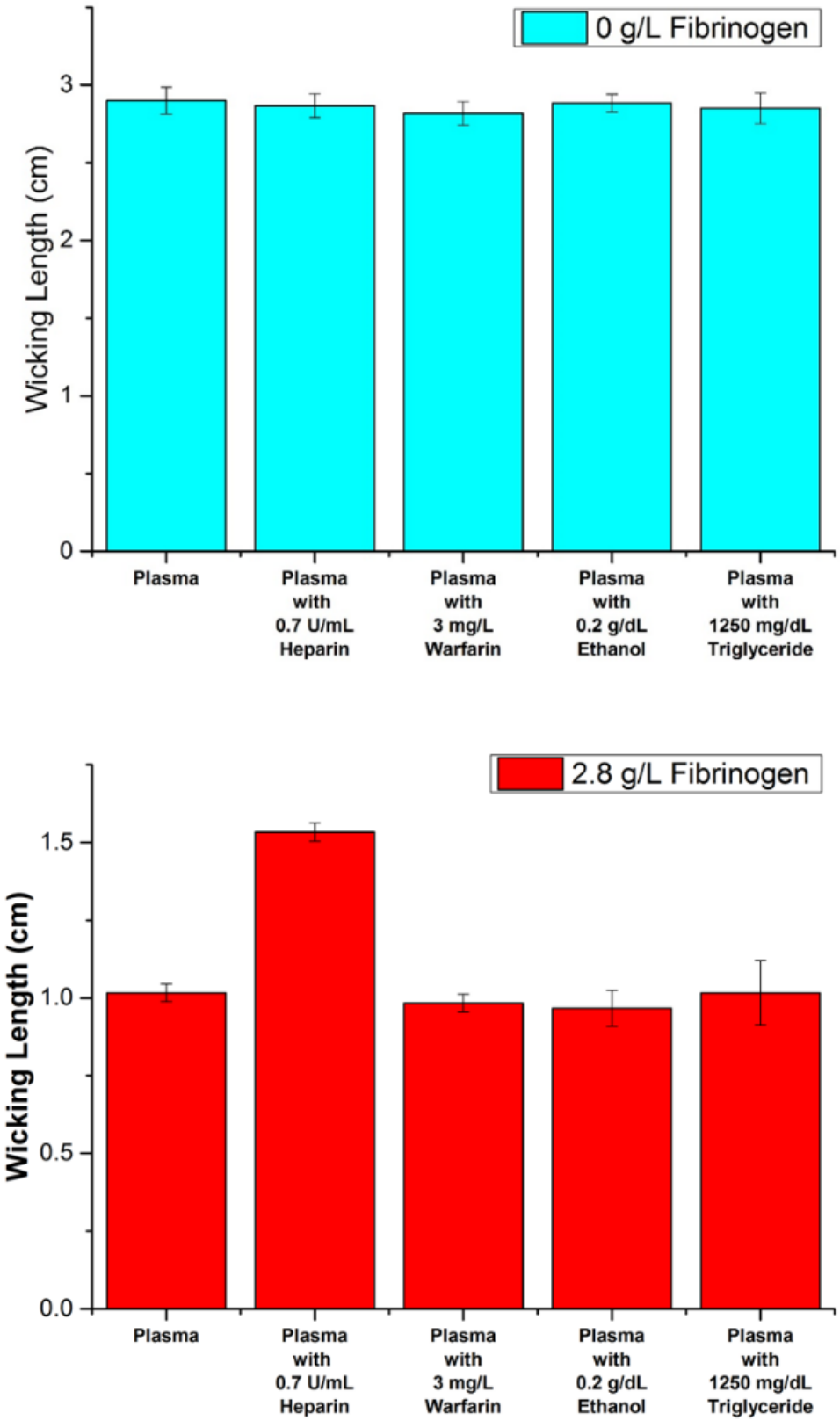


Figure 21: Testing of different substances on defibrinogenated plasma (top) and normal plasma (bottom). Each substance was tested five times in both plasmas. The means and standard deviations are reported accordingly.

3.4. Effect of Acidosis

Acidosis is commonly present in heavily bleeding patients. It results from the build-up of lactic acid due to insufficient blood flow to the tissues. Acidosis is fatal as it impairs coagulation when the blood pH drops below 7. The pH of normal plasma was decreased by mixing it with small volumes of concentrated hydrochloric acid and subsequentially measuring the pH with a pH meter. The initial pH of the normal plasma was 7.6. The effect of acidosis was observed by dropping the pH of the plasma to three key levels: 7.3 (moderate acidosis), 7.1 (critical acidosis), 6.9 (fatal acidosis). Each pH was tested five times and the mean and standard deviations are reported in **Figure 22**.

The effect of acidosis is insignificant. At moderate acidosis levels, no effect is observed. However, at extreme levels a small decrease in wicking length is noted. To validate whether this small decrease was due to the drop in pH or increase in chloride ion concentration, sodium chloride was also added to pH 7.6 plasma. No difference is shown between the pH 7.6 or pH 7.6 + NaCl plasmas, verifying that the effect is solely pH driven.

The effect of acidosis and increase in osmolarity was also observed in defibrinogenated plasma (result not shown). No effect is observed by decreasing the pH or increasing the sodium chloride concentration.

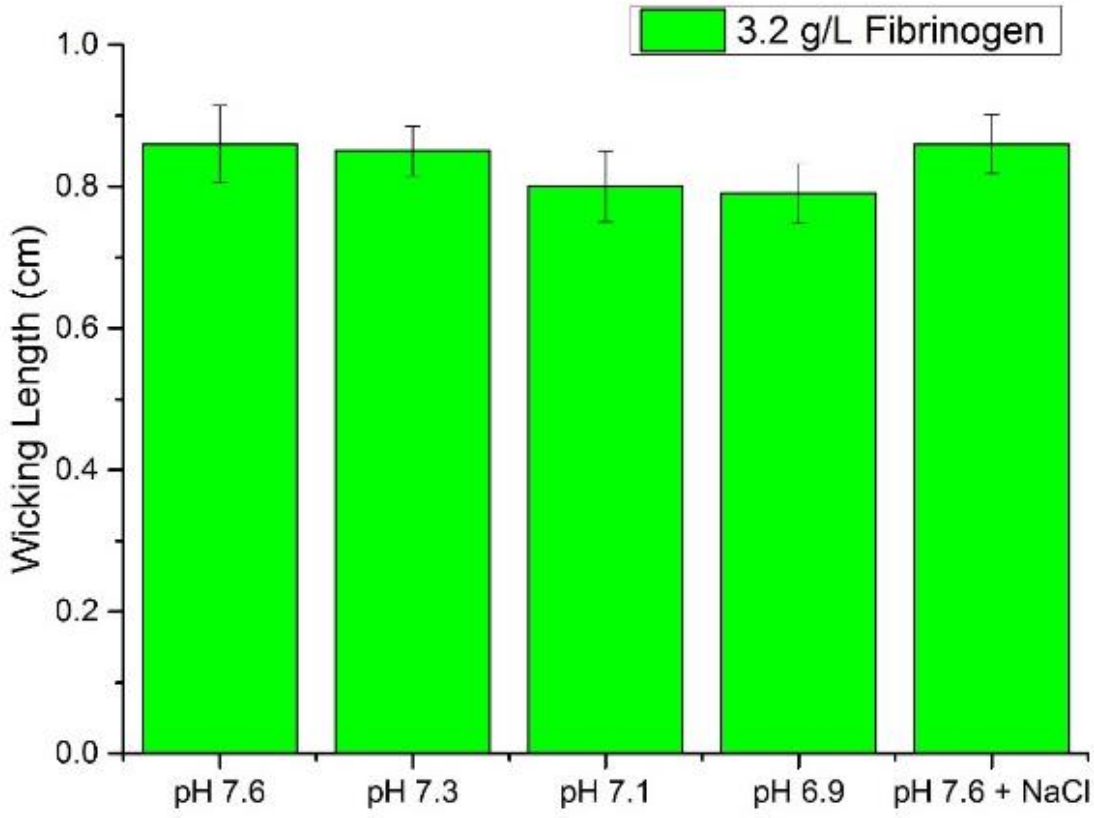


Figure 22: The effect of acidosis on normal plasma was tested five times. The means and standard deviations are reported accordingly.

3.5. Effect of Haemolysis

The destruction of red blood cells commonly occurs in bleeding disorders such as disseminated intravascular coagulation. The resulting effect is the release of haemoglobin into the plasma. Plasma haemoglobin concentrations above 0.5 g/dL are classified as severely haemolysed.

The release of haemoglobin can increase the viscosity of the plasma. Therefore, the effect on the blue dye migration rate was observed. Haemoglobin concentrate was created by lysing red blood cells. Its haemoglobin concentration was then quantified. Normal and defibrinogenated plasmas of different haemoglobin concentrations were made by mixing different quantities of haemoglobin concentrate into the plasma samples. Each concentration was tested four times and the mean and standard deviations are reported in **Figure 23**.

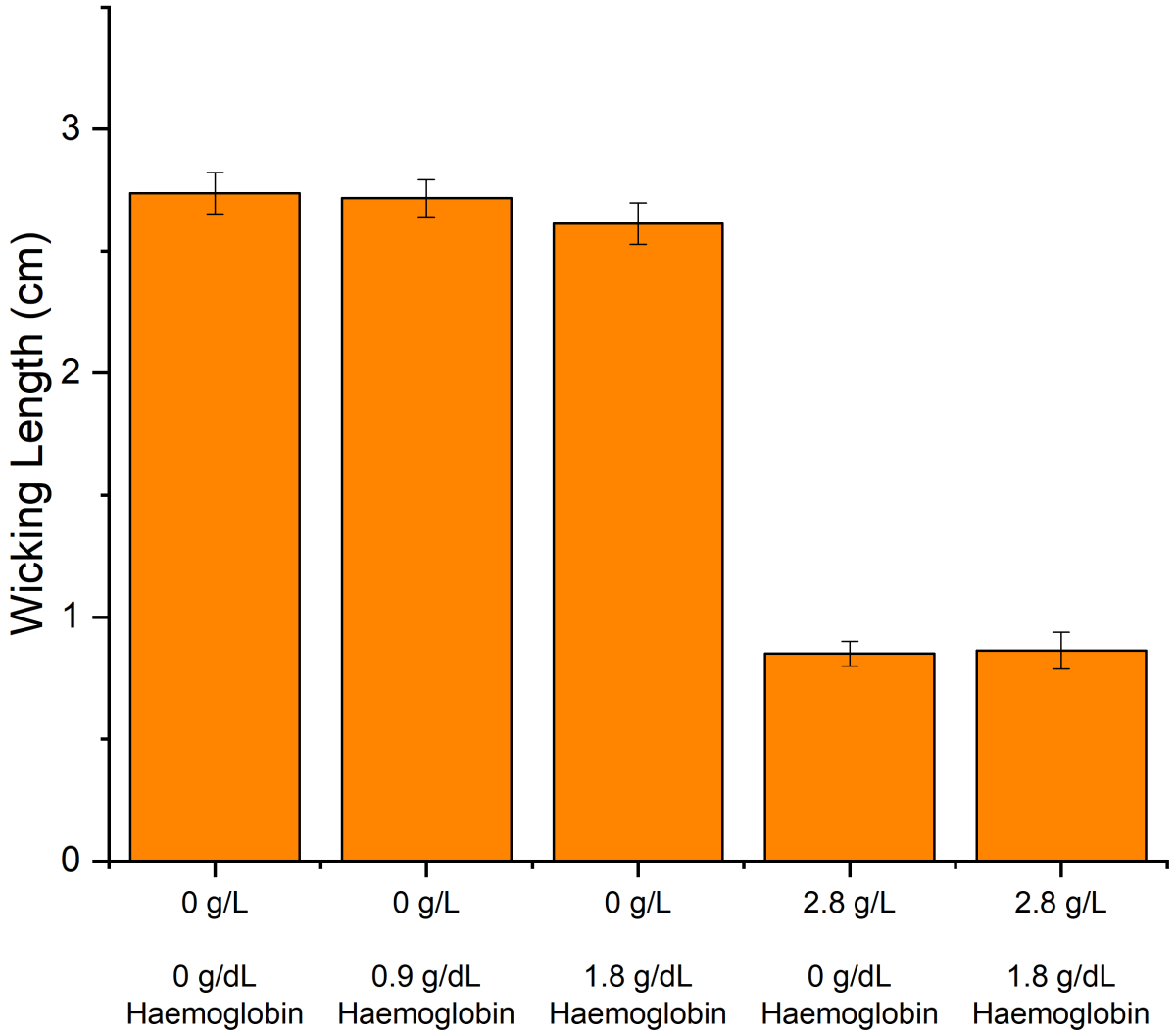


Figure 23: The effect of haemolysis on defibrinogenated (0 g/L) plasma and normal (2.8 g/L) plasma was tested four times. The means and standard deviations are reported accordingly.

3.6. Effect of Temperature and Humidity

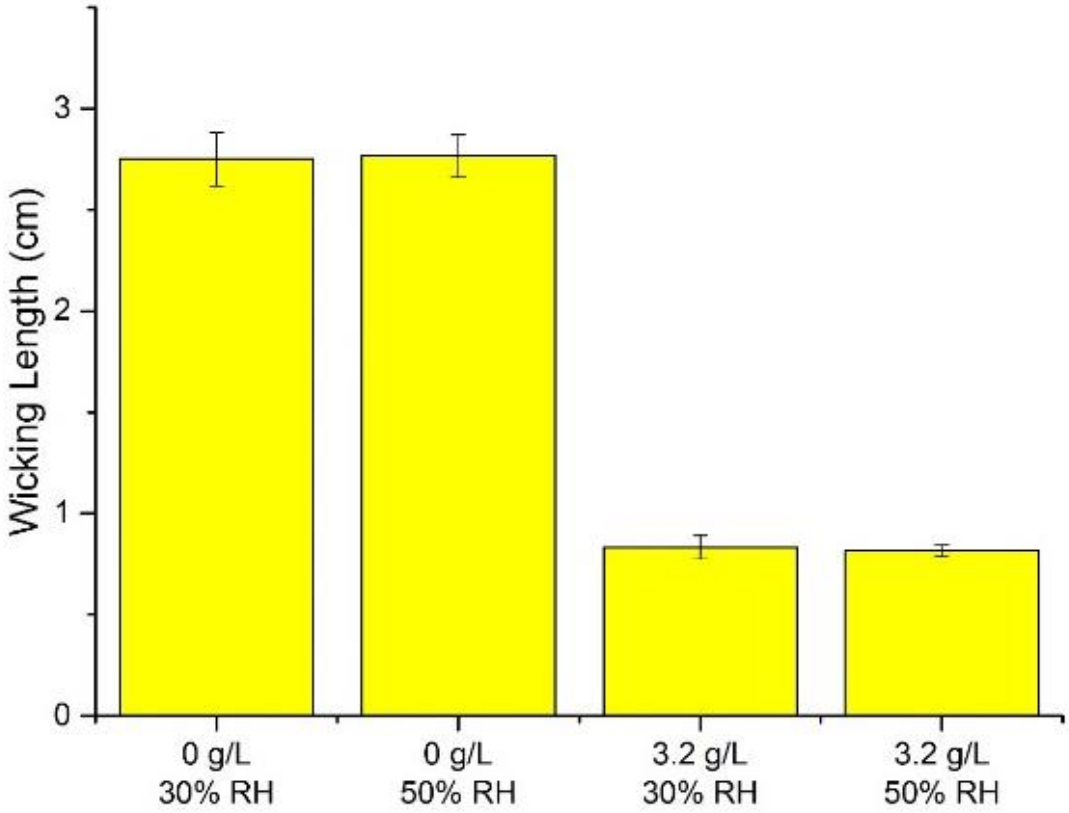


Figure 24: The effect of humidity on defibrinogenated (0 g/L) plasma and normal (3.2 g/L) plasma was tested four times. The temperature was kept constant at 23°C. The means and standard deviations are reported accordingly.

Because critical bleeding can occur anywhere in the world, the test was experimented at different humidities (**Figure 24**) and temperatures (**Figure 25**). This is because humidity and temperature affect the mechanical and structural properties of paper.

Experiments at 50% relative humidity were performed in a temperature and humidity-controlled room. A desiccator cabinet filled with silica gel was used to drop the relative humidity to 30%. Defibrinogenated plasma was used to test the direct effect humidity had on the blue dye migration rate due to drying. Normal plasma was used to test the effect humidity had on fibrin formation. Each condition was tested in quadruplets in both defibrinogenated and normal plasma and the mean and standard deviations are reported in **Figure 24**.

No difference in wicking length for either the defibrinogenated or the normal plasma is observed (**Figure 24**).

Experiments at different temperatures were performed using a temperature incubator. Four temperatures were tested: cold (7°C), temperate (23°C), hot (36°C), and extremely hot (44°C). Defibrinogenated plasma was used to test the direct effect temperature had on the blue dye migration rate due to viscosity changes. Normal plasma was used to test the effect temperature had on fibrin formation. Each condition was tested in quadruplets in both defibrinogenated and normal plasma and the mean and standard deviations are reported in **Figure 25**.

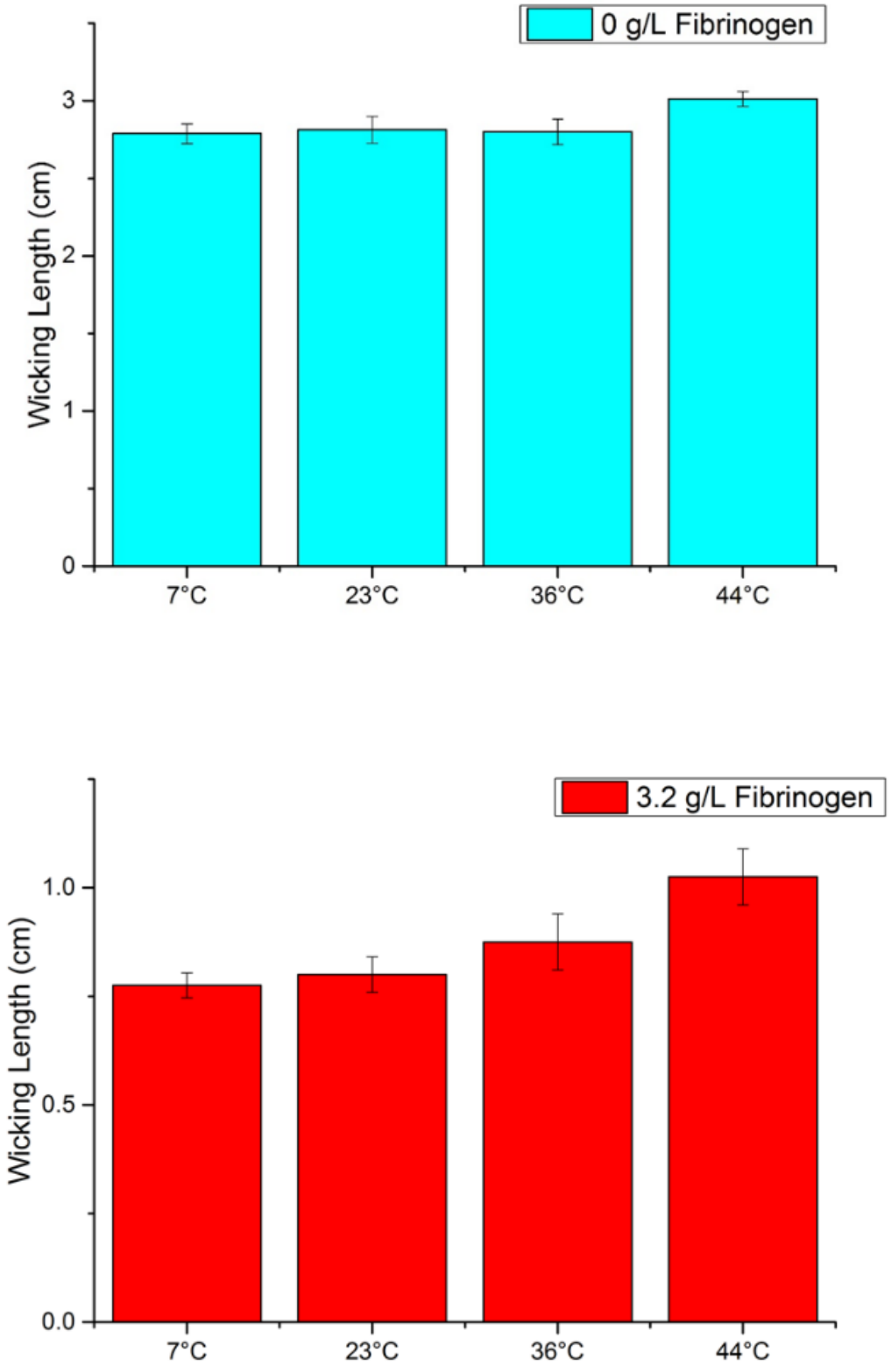


Figure 25: The effect of temperature on defibrinogenated plasma (top) and normal plasma (bottom) was tested four times. The means and standard deviations are reported accordingly. The relative humidities for each temperature condition are summarised in the Appendix.

Two observations are noted. 1) The blue dye wicking rate is unaffected by cold to hot temperatures, but slightly increases at extremely hot temperatures. 2) The fibrin formation is unaffected by cold to moderate temperatures, but is affected by hotter temperatures.

4. Discussion

4.1. Impact

In this study, we have developed the first ever paper based hand-held test for diagnosing hypofibrinogenemia. Clear results can be obtained in only a minute. Therefore, it can strongly reduce the time to treatment before fibrinogen replacement therapy. As discussed in our first study (199), the test predominantly works on the basis of hydrophobicity. When plasma and thrombin react with each other on paper, they convert fibrinogen into fibrin. This creates a zone of hydrophobicity. When the droplet of blue dye is applied onto the strip, the zone of hydrophobicity retards its capabilities to wick through the paper strip (199). The amount of fibrinogen present dictates the magnitude of the hydrophobicity. Therefore, the plasma fibrinogen concentration can control the rate at which the blue dye can wick down the strip. This is modelled through the Lucas-Washburn equation which states:

$$L = \sqrt{\frac{\gamma r t \cos\theta}{2\eta}} \quad \textbf{(2)}$$

where L = length, γ = surface tension, r = pore radius of paper, η = blue dye viscosity, t = wicking time and θ = contact angle between blue dye and paper. The zone of hydrophobicity increases the contact angle between the wicking front of the blue dye and the paper surface. Therefore, it decreases the length that the blue dye wicking front can travel through the strip. This means that the plasma fibrinogen concentration can dictate the length of the wicking front as demonstrated in **Figure 17**.

This study has achieved two objectives over the previous study (199). The first is by successfully replacing the infinite reservoir in the vertical wicking test for a step where a droplet of blue dye can be applied by hand. The second is by creating a horizontal setup that can distinguish physiologically relevant plasma fibrinogen concentrations from each other and accurately measure them. Furthermore, a blue dye wicking time of only 30 s is required to provide clear results. This is significantly better than the 7 min wicking time previously needed for the vertical wicking test. This means that the new test can be engineered into a portable hand-held device and has the potential to save more than two million lives worldwide every year (174, 191-196).

Although the plasmas tested were artificially diluted to different fibrinogen concentrations, the paper diagnostic shows optimal sensitivity below 2 g/L fibrinogen, the range of clinical interest (**Figure 18**). The results are very reproducible. These trends are consistent with those observed with the vertical wicking test (199). This new methodology can rapidly identify whether a bleeding patient has a blood fibrinogen concentration below 2 g/L or not. It can also distinguish cases of mild hypofibrinogenemia (1.5–2 g/L) from severe hypofibrinogenemia (<0.5 g/L).

This new test is unaffected by the source of plasma. The trendlines generated in **Figure 18** were created from the normal and defibrinogenated plasmas of two different citrated plasma samples. They show clear similarity to each other. Furthermore, undiluted plasma from all three healthy plasma samples were tested twenty times each (**Figure 19**). The plasmas containing 3.2 g/L and 3.3 g/L fibrinogen do not show noticeable separation. On the other-hand, the plasma containing 2.8 g/L fibrinogen wicks further than the other two similarly concentrated plasmas. This means that the wicking length is controlled only by the plasma fibrinogen concentration in healthy individuals.

4.2. Performance

The paper test was compared to a hospital-grade automated Clauss assay (Stago STA® Fibrinogen 5). Even though the paper test has a lower sensitivity than the hospital grade Clauss assay (as shown in **Figure 20**), it still possesses five important advantages over other hospital grade assays that make it particularly suitable for emergency situations.

First, the test can be transported easily to a patient. This makes it suitable as a point of care device. Whereas, other hospital grade assays require bulky and heavy table-top systems dedicated to a laboratory environment. Second, the paper test clearly works within the physiological range of plasma fibrinogen. In contrast, other hospital grade assays including Clauss assays require up to 1:30 times dilutions to achieve clear results (98). As the paper test does not require pre-dilutions, it saves a considerable amount of time, complexity and reduces human errors. Third, the paper test can be performed immediately at room temperature. However, other hospital grade assays require that the blood or plasma sample be pre-warmed at 37 °C for 4–6 min before the assay starts (221). Given the urgent situation of critical bleeding, these pre-incubation times are disturbing. Fourth, hospital grade assays require complicated endpoint determination techniques to identify when clot formation has occurred (222). The endpoint refers to the point of time when fibrin formation has completed. For example, the Stago STA® Fibrinogen 5 detects when fibrin formation has been completed by monitoring the motion of metal ball bearings. A metal ball bearing is added to the plasma sample once the assay has started and is allowed to move around with the aid of magnets. The formation of fibrin impedes the movement of the ball bearing. The moment the ball bearing stops moving is when fibrin formation is completed. However, this requires complicated apparatus such as ball bearing dispensers, applied magnetic fields and detectors to achieve this. However, as the paper test measures the fibrinogen concentration after 30 s of wicking, it takes out the requirement for endpoint determination altogether. Finally, the machine requires a computer screen to display fibrinogen concentration readings. On the contrary, the paper test is interpreted directly unassisted to the naked eye.

Given that clinics utilise the same fibrinogen replacement therapy procedures once they find a patient has below 2 g/L plasma fibrinogen, further sensitivity below those values is not as important. Therefore, this test is better suited for diagnosing hypofibrinogenemia than typical hospital grade assays.

4.3. Adaptability

Since the paper test could successfully measure plasma fibrinogen concentrations in healthy individuals, we also quantified the effect that important physiological and pharmaceutical conditions can have on the test. We looked at those most likely to cause deviations away from the results expected with healthy and defibrinogenated plasmas. This included anticoagulant drugs, alcohol, dietary lipids, acidosis and haemolysis (**Figure 21**, **22** and **23**). Furthermore, because the test is suitable as an outdoor point of care device, we also tested the effects of different temperatures and humidities (**Figure 24** and **25**). Only conditions known to alter thrombin activity affect the test. Heparin exerts the most potent effect. However, extreme heat does negatively affect the test also.

Anticoagulant drugs such as heparin and warfarin are used during and after cardiac surgery to prevent acute coronary syndrome. Warfarin is preferred over heparin due to its ability to be administered orally and its longer half-life. However, warfarin takes longer to exert its effect (223). Therefore, heparin and

warfarin are both initially used for the first five days to prevent anticoagulation (224). Once anticoagulation has stabilised, then heparin therapy is discontinued and patients can continue taking warfarin.

Heparin is clinically used as either unfractionated heparin or low molecular weight heparin. Because unfractionated heparin has a much greater inhibitory effect on thrombin, it was used in this study (225). Furthermore, the use of unfractionated heparin also indicates how other pathophysiological thrombin inhibitors - such as fibrin/fibrinogen degradation products - or other thrombin inhibiting anticoagulants - such as direct oral anticoagulants - might behave (226). Clinical target therapeutic concentrations of heparin in plasma are as high as 0.7 U/mL (227). Therefore, we added heparin in healthy plasma to that concentration and quantified its effect. The effect of heparin is significant (**Figure 21** - bottom graph). When compared to a healthy plasma sample of 2.8 g/L fibrinogen, the heparin causes an extra 5 mm of further wicking. From **Figure 18**, this correlates with a reading of roughly 1.5 g/L fibrinogen. The inhibitory mechanism of heparin on thrombin is well documented. Heparin binds to the enzyme anti-thrombin and activates it. This in turn inactivates thrombin (228). Given that the amount of thrombin used in the paper test was not in huge excess, the inhibitory effect is not surprising. The inhibitory effects on thrombin (of heparin or other inhibitors) can be fixed through several means. The simplest solution is by using higher thrombin concentrations for sufficient competition.

Likewise, we added 3 mg/L warfarin to healthy plasma as it is the upper threshold of clinical target therapeutic concentrations. However, no effect is observed (**Figure 21**). This is expected as warfarin does not form inhibitory complexes with thrombin like heparin does. Instead, its mode of action is to prevent the vitamin K dependent liver synthesis of coagulation factor precursors into their active forms, including thrombin (229). Given that the thrombin adsorbed on the paper strip is already active, warfarin cannot exert any effect on it.

High blood alcohol concentrations are problematic in situations such as drink-driving traffic accidents. This represents a significant cause of trauma. In 2017, Mitra *et al.* reported that 16% of trauma patients involved in traffic accidents on Australian roads had blood alcohol concentrations above 0.05 g/dL (230). Ethanol is known to impair the initial clot formation time but has no reported effect on thrombin (231). We tested plasma mixed with four times the Australian legal driving limit of 0.05 g/dL blood alcohol concentration. However, we observed no effect compared to healthy plasma (**Figure 21**). This means that the test is unaffected by alcohol.

In our first manuscript we concluded that the mechanism behind the test is largely hydrophobicity driven. Therefore, we wanted to assess wherever blood lipids exerted their own effect on blue dye wicking. This is because blood lipids have hydrophobic components that may impede wicking if concentrations get too high (232). Triglycerides make up 50% of all plasma lipids in the healthy population. Normal baseline triglyceride concentrations do not exceed 150 mg/dL. However, in cases of very severe hypertriglyceridemia, they can exceed 1000 mg/dL (233). When we add 1250 mg/dL of triglycerides to defibrinogenated plasma, we do not observe any changes in results (**Figure 21**). This indicates that plasma lipids do not impact on wicking.

Acidosis is another complication in heavily bleeding patients. It is caused by the build-up of lactic acid from anaerobic metabolism due to decreased blood flow and oxygen delivery to tissues (234). Because the

healthy plasma samples used were citrated, their pH levels were recorded to be 7.6. By adding hydrochloric acid to healthy plasma, we dropped its pH from 7.6, to 7.3 (mild acidosis), 7.1 (critical acidosis) and 6.9 (fatal acidosis). When the pH is dropped to 7.3, no difference in wicking is observed when compared to a pH of 7.6 (**Figure 22**). This means that the test can adapt to blood pH levels between those two values. However, when the pH is dropped to fatal levels, a small decrease in wicking length - averaging roughly 0.5 mm - occurs. To verify wherever this effect is due to the drop in pH or the increase in chloride ions, sodium chloride was also added to healthy plasma. The added sodium chloride does not impede further wicking, thus confirming that the slight decrease in wicking is pH driven. Although the effect is insignificant, it is likely because thrombin works optimally at a pH of 6.5 (235). Hence, low blood pH can enhance thrombin activity and further impair wicking.

Microangiopathic haemolytic anaemia is a common symptom in bleeding disorders such as disseminated intravascular coagulation. Vessel rupture and fibrin deposition is thought to cause the formation of abnormal flow channels and hence the mechanical destruction of red blood cells (RBCs). This results in RBCs releasing their cytosolic contents into the plasma (including haemoglobin) (236). As haemoglobin is a large and globular protein, it was hypothesised that increasing the haemoglobin concentration in plasma would also increase its viscosity. Hence, it may have perturbed blue dye wicking. Severe haemolysis is diagnosed when plasma haemoglobin concentrations rise above 0.5 g/dL (237). When defibrinogenated plasma samples are made to 0.9 g/dL haemoglobin, no impediment in wicking is observed (**Figure 23**). It is only when the plasma haemoglobin concentration is made to 1.8 g/dL - a concentration extremely rarely reached (237) - that an insignificant wicking perturbation is observed. Furthermore, the addition of haemoglobin does not affect the wicking of normal plasma - proving that it has no impact on fibrin formation. Therefore, the test is unaffected by clinically relevant levels of haemolysis.

Traumatic injury can occur in different geographical environments around the world. These environments can all have different weather conditions. Therefore, we measured the effect of temperature and humidity on the test. We originally hypothesised that dropping the humidity would hasten drying and hence retard wicking. However, **Figure 24** shows that no effect is observed when the humidity is dropped from 50% to 30%. Likewise, we also hypothesised changing temperature would exert two different effects. One was that increasing temperature would decrease blue dye viscosity and cause further wicking. The other was that either increasing or decreasing the temperature to extreme values would decrease thrombin activity and cause further wicking. **Figure 25** (top graph) shows that the blue dye wicking rate on its own is insensitive to temperature change. Furthermore, **Figure 25** (bottom graph) demonstrates that fibrin formation is unaffected at low to moderate temperatures. However, at high temperatures, fibrin formation is perturbed and further wicking occurs. This can lead to fibrinogen readings being falsely low. A solution is to use a correction factor based on the ambient temperature the test is performed at. Alternatively, a means to protect the test from extreme heat can also be used. Otherwise, the test is suitable in a wide range of different weather conditions and locations around the world.

4.4. Limitations

The main limitation of the paper test is the distribution of plasma and thrombin evenly through the paper strip. When the fibrinogen in plasma reacts with thrombin on paper, it forms a fibrin network that perturbs the wicking of the blue dye. However, when the fibrinogen and thrombin are not perfectly distributed on paper, it creates areas of unreacted plasma. Because these areas are more hydrophilic than the surrounding fibrin network, the blue dye travels through them preferentially and wicks further than

expected. This is demonstrated in **Supplementary Figure 16**. Initially, we intended to add the thrombin onto paper first and then add the plasma on top of the thrombin afterwards. However, the plasma would frequently push the thrombin out of its way instead of mixing with it. This created results that were difficult to reproduce. Therefore, a means to keep the thrombin in place (such as enzyme immobilisation on paper) is needed for the test in the future. However, our solution to this issue for this study was to pre-mix the plasma with thrombin before applying to paper. The pre-mixing caused extra limitations in our study.

First, the concentrations of thrombin we used had to be low (30 U/mL) to provide enough time to mix with the plasma and apply to the paper before it could clot. We tried using concentrations of 600 U/mL (similar to that of a Clauss assay) (20). However, it causes the plasma to clot immediately in the pipette tip before it could be applied to paper. Additionally, the low thrombin concentrations used in the test may be the reason why the test did not work with therapeutic unfractionated heparin concentrations. By finding a way to remove the pre-mixing step, we may be able to react the plasma uniformly with higher thrombin concentrations on paper.

Second, the mixed plasma has to be applied evenly across the width of the strip. If the mixed plasma is added to one section of the strip, it creates a gap surrounding the fibrin network for the blue dye to easily go through. Hence, the blue dye wicks further than expected as shown in **Supplementary Figure 16**. In our methodology, we fixed this issue by pipetting approximately one third of the mixed plasma on the left-hand side, one-third on the right-hand side and the other third in the centre of the strip. Because pipetting technique varies from individual to individual, a means of distributing the plasma evenly across the strip is needed for the test.

The resulting fibrin network formed on the strip is fragile and susceptible to damage from excessive shear stresses. Therefore, the velocity of the blue dye has to be controlled as it wicks through the network. In this study, we achieved this through three mechanisms. 1) By expanding the width of the dye pad (see **Supplementary Figures 19** and **20**). 2) By adding the blue dye from the bottom of the dye pad (to provide settling distance between the dye and the fibrin network). 3) By adding the blue dye slowly over a period of 10 s. Performing the test with this technique does not create outliers.

Our study was also limited by the choice of plasma reagent. We worked with platelet-poor citrated plasma of healthy donors. This was because no extra substances were added into the plasma samples that could affect wicking rates or thrombin activity. Hence, we can claim that the results observed in **Figure 19** are due only to fibrinogen concentration. However, as these samples were derived from healthy donors with minimal modifications, the fibrinogen concentrations were all around 3 g/L. Therefore, they had to be artificially diluted in defibrinogenated plasma to reach the desired fibrinogen concentrations for testing. The use of defibrinogenated plasma as an appropriate dilution medium is validated in **Supplementary Figures 17** and **23**. These results support the hypothesis that the trend observed in **Figure 18** would be similar if we used unmodified plasma samples of different concentrations instead.

We did not have access to samples from patients already containing the substances tested in **Figure 21**. Therefore, we added those substances into normal and defibrinogenated plasmas instead. Whilst most of these substances exert a direct effect in the plasma, warfarin does not. Instead, warfarin inhibits the synthesis of coagulation factors in the liver. This normally takes up to five days to have an effect. Whilst

warfarin is shown not to impact the test, further work is needed to determine whether the plasma of a patient on warfarin for five days produces the same effect. In this study, we used thrombin concentrations similar to a thrombin time assay (ie, 30 U/mL). As it is known that any depletion of endogenous thrombin (due to the effects of warfarin) does not impact a thrombin time assay, we assume this diagnostic will not be impacted by it either.

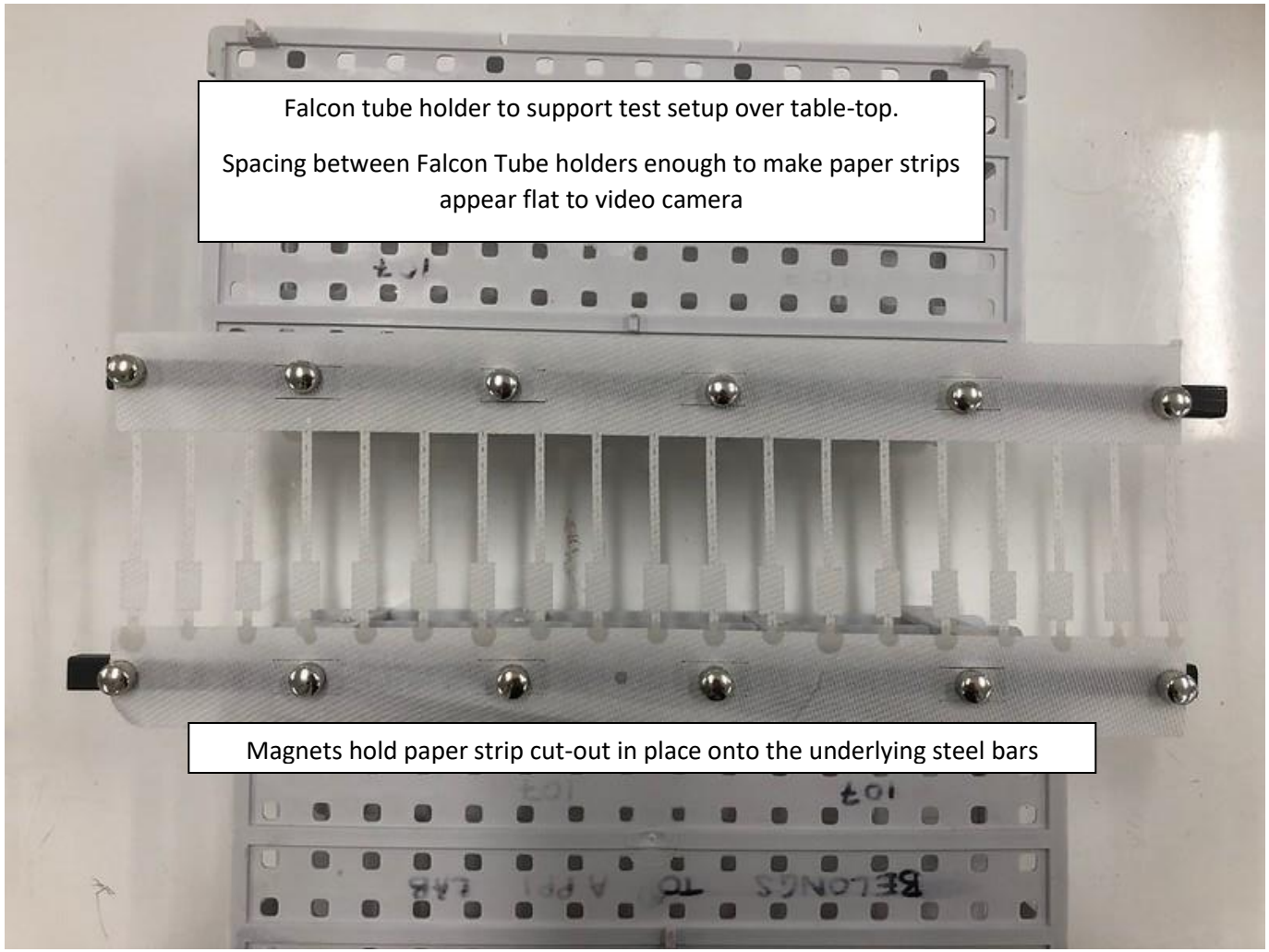
Finally, the effects of dysfibrinogenemia were not considered in this study. We assumed that if a patient suffered from dysfibrinogenemia and their fibrinogen could not react with the thrombin in our test, then it could not react with the thrombin in their plasma either. Hence, they would need fibrinogen replacement therapy anyway. Whilst there are some types of dysfibrinogenemia that cause thrombosis, Korte *et al.* found that there was no definitive evidence to suggest that fibrinogen replacement therapy increases the risk of thrombosis in these patients (238). Therefore, we prioritised the development of a diagnostic that could measure the concentration of clottable fibrinogen as opposed to total fibrinogen.

5. Conclusions

In this study, we have developed a rapid, hand-held paper-based diagnostic for measuring fibrinogen concentrations in human plasma. The test can provide a result within a minute. This time is suitable for facilitating fibrinogen replacement therapy. The test has optimal sensitivity for fibrinogen concentrations below 2 g/L - the critical diagnostic concentration - and shows reproducibility between healthy individuals. Whilst the diagnostics does not have the same sensitivity as a hospital-grade automated Clauss assay, it is portable, much faster, requires no plasma pre-treatment, pre-incubation or clot formation detection mechanism and has a simple visual readout. The test can also withstand common blood conditions such as acidosis, blood alcohol, severe hypertriglyceridemia, severe haemolysis and warfarin administration. Finally, the test is unaffected by humidity and can withstand cold temperatures. This makes it suitable as a point of care diagnostic to detect hypofibrinogenemia early - both inside and outside of the hospital setting. Therefore, it can greatly reduce the time to treatment needed for fibrinogen replacement therapy and save more than two million lives a year.

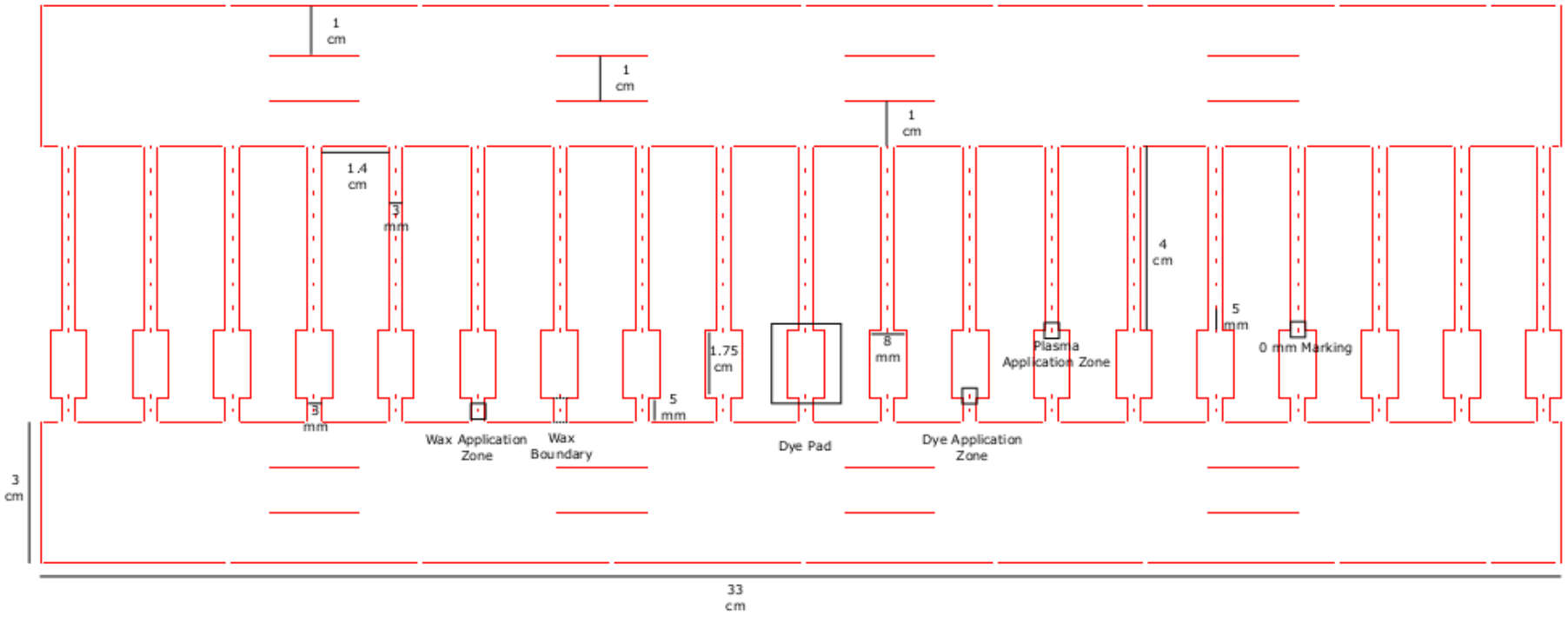
Supplementary Data

3.1. Test Setup:



Supplementary Figure 12: Test setup used to record experiments with.

3.2. Paper Strip Cut-Out:

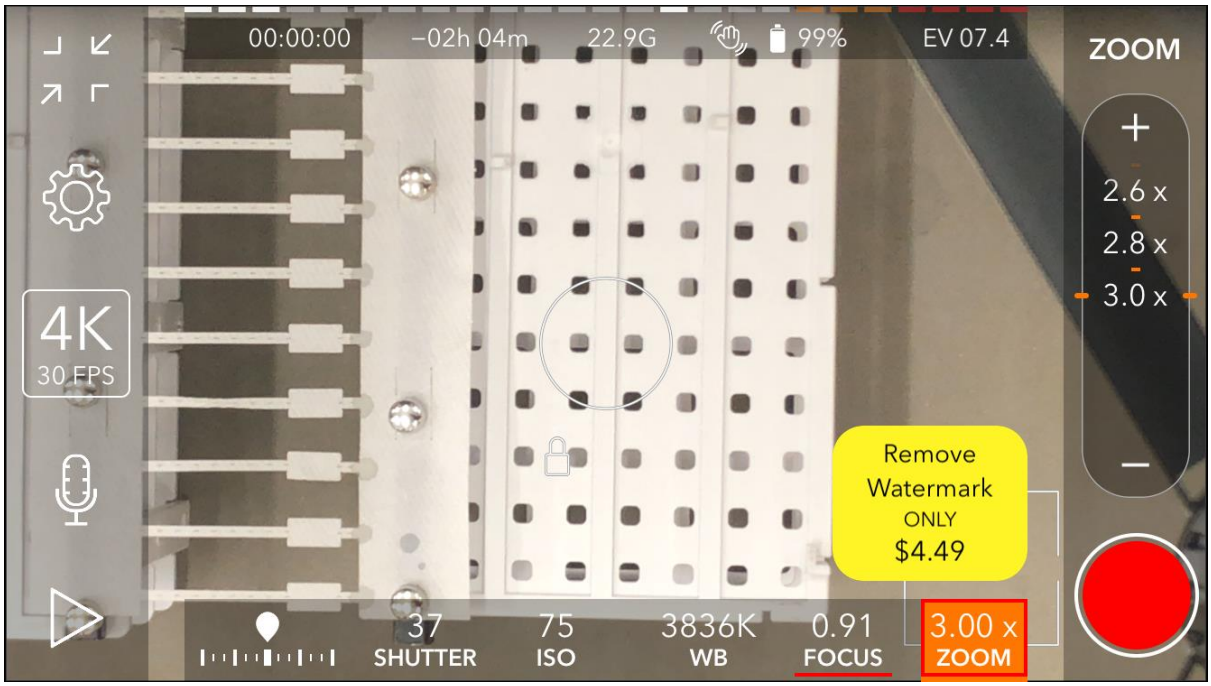


Supplementary Figure 13: Cut-out of Paper Strips showing dimensions, markings and locations chemicals are pipetted onto the paper. The paper strip cut-out stencil was drawn on CorelDRAW X6 and a 60 Watt Epilog Helix was used to laser cut the paper.

3.3. Manual Camera Setup:

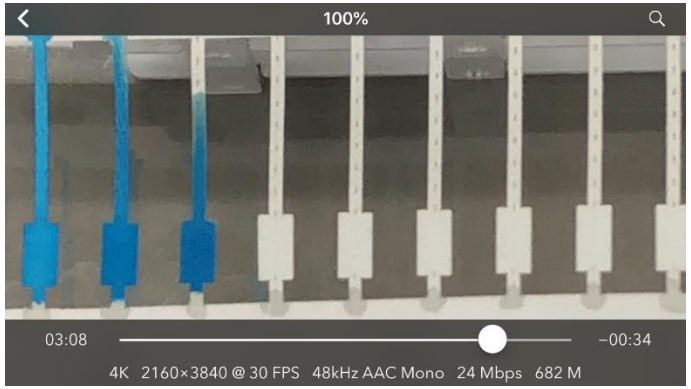
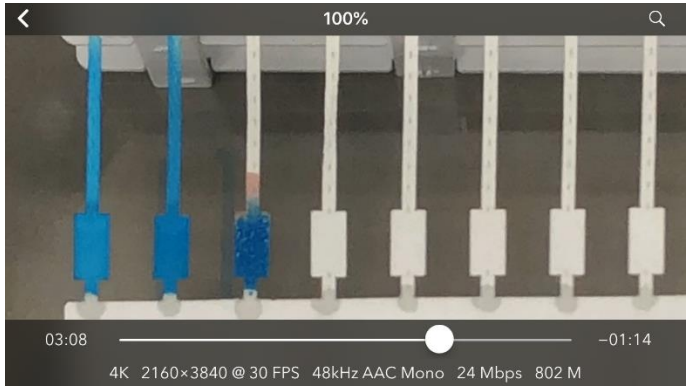
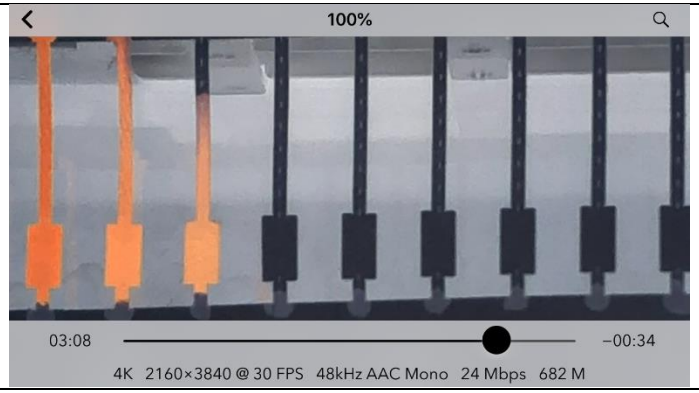
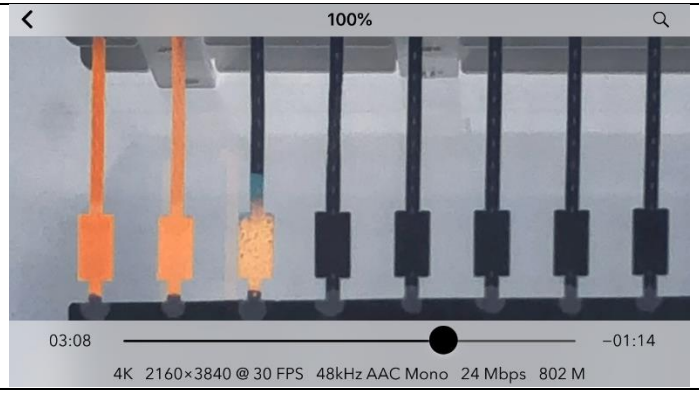
In order to capture videos which manually focussed on the strip's markings, ProMovie was used on an iPhone 6. The zoom was adjusted to 3.00 X to get maximum zoomage and the focus was changed until the markings on the paper strips could be clearly seen.

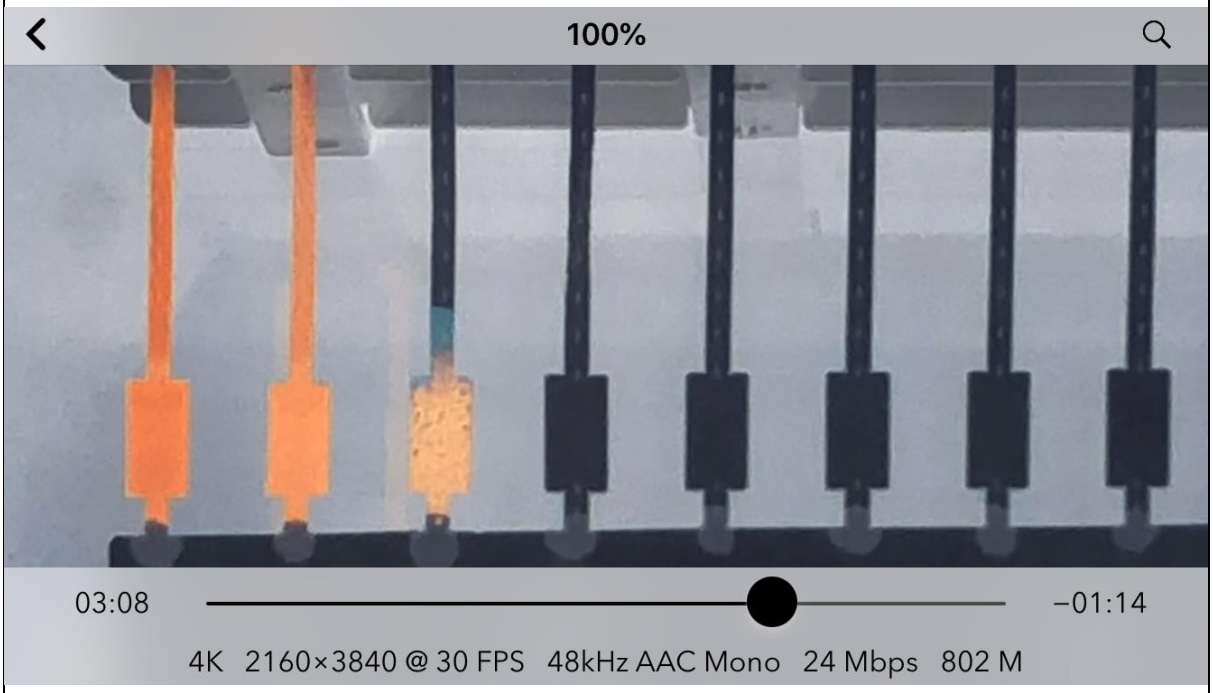
The videos recorded could then be screenshotted and used for analysis on ImageJ.

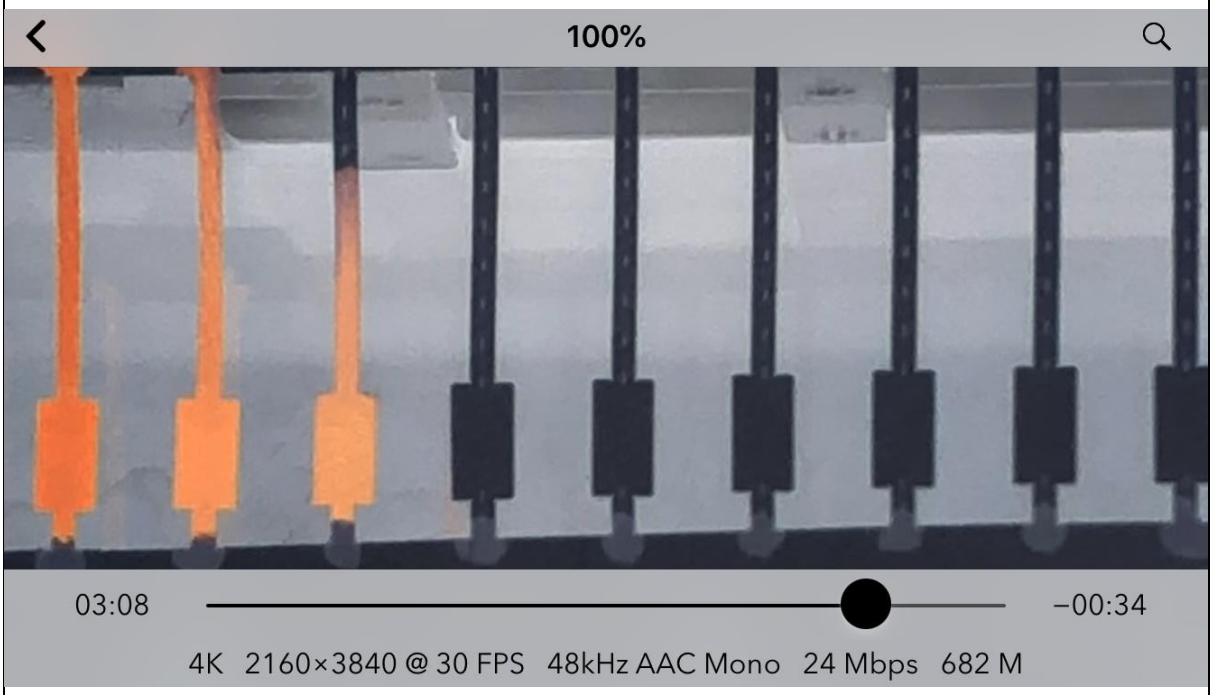


Supplementary Figure 14: Settings on ProMovie used to manually focus and zoom on the paper strip's markings.

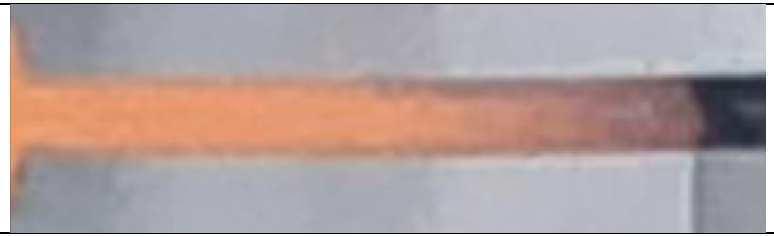
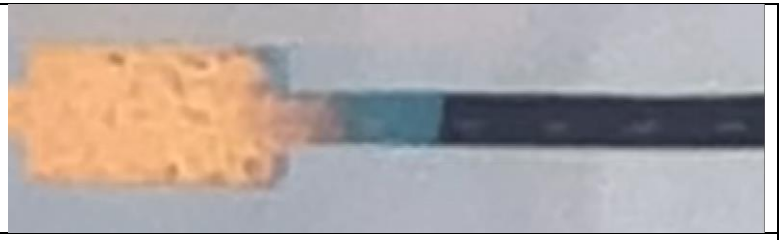
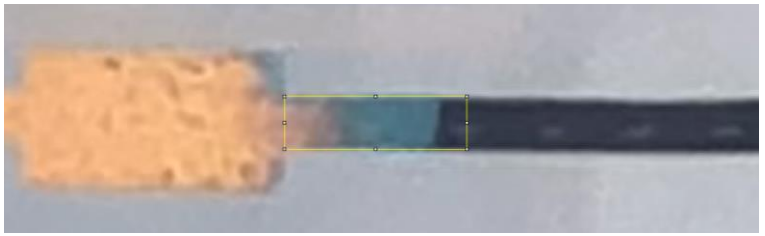
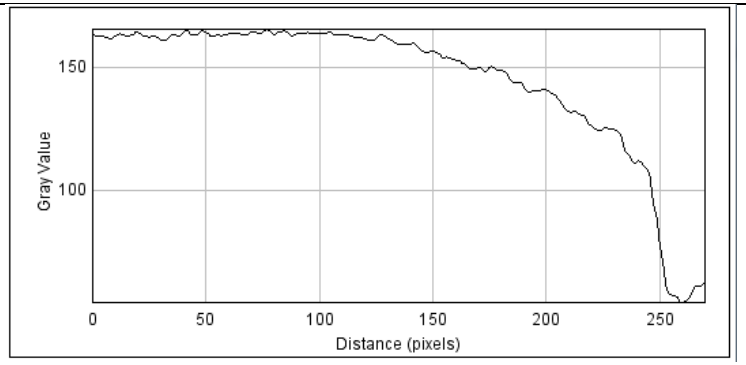
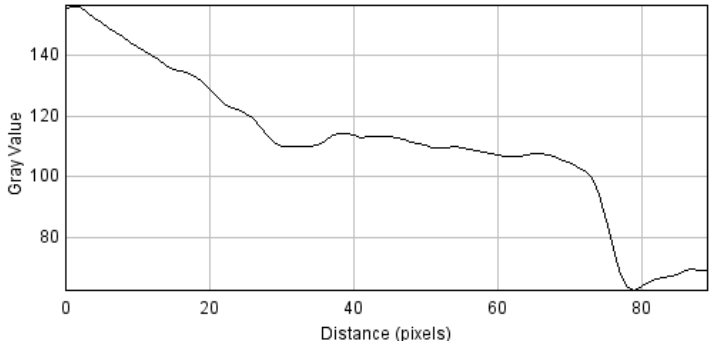
3.4. Measuring Process:

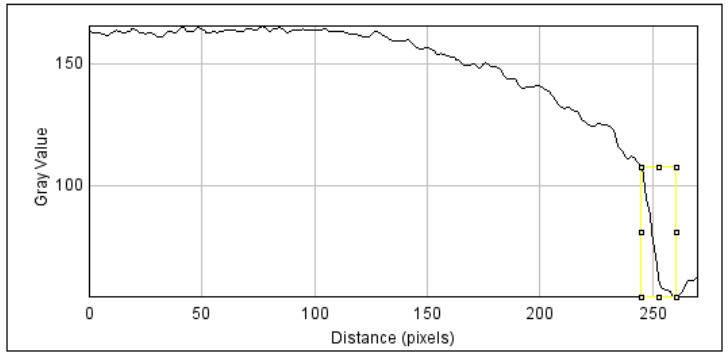
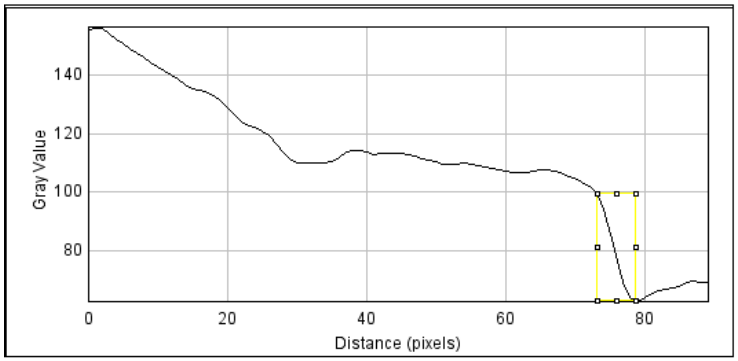
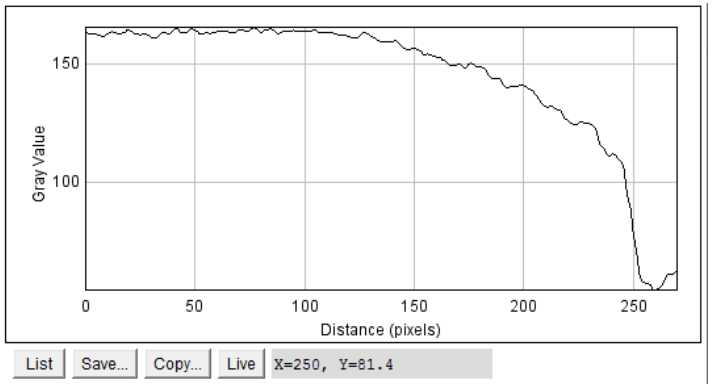
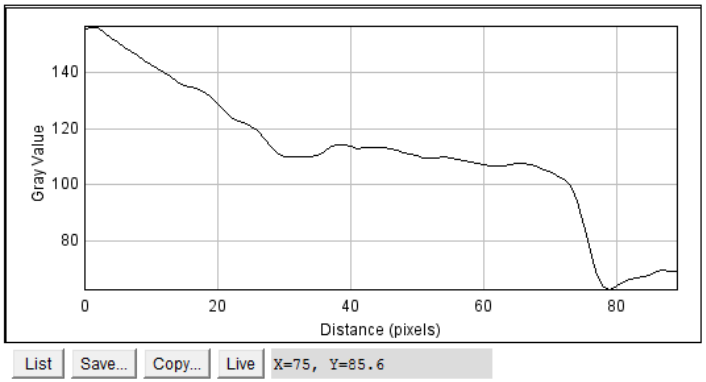
	0 g/L Fibrinogen	3.2 g/L Fibrinogen
1. Take Screenshot 30 seconds after blue dye is applied to strip **2. Download image on computer and open on Image J**		
3. Invert		





4. **Flip 90° Right**

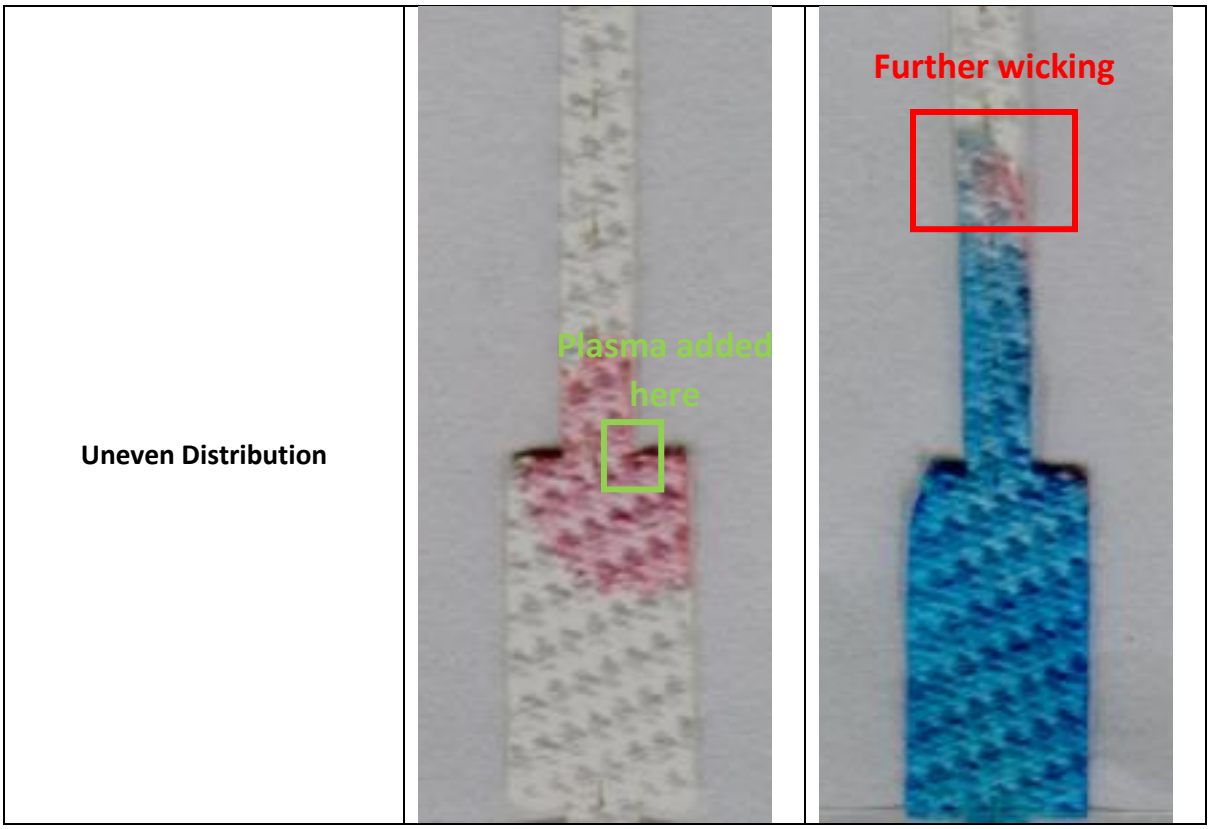
5. Zoom into strip to be analysed		
6. Draw box from 0 cm marking to marking rounded up from wicking front. **7. Take note of 0.5 cm marking selected and number of corresponding horizontal pixels**	 270 pixels 3.0 cm	 90 pixels 1.0 cm
8. Plot Profile of box		

9. Identify area of steepest decrease. This correlates with the wicking front	 LHS: x - 245 pixels; y – 109.0 Gray Value RHS x – 261 pixels; y – 55.5 Gray Value Median gray value: y – 82.25	 LHS: x - 73 pixels; y – 99.8 Gray Value RHS x – 79 pixels; y – 62.4 Gray Value Median gray value: y - 81.1
10. Find closest corresponding x-point of median gray value. This is the midpoint of the wicking front.	 X midpoint = 250 pixels	 X midpoint = 75 pixels

11. Convert pixels into cm	$Length = \frac{250\ Pixels}{270\ Pixels} \times 3\ cm$ $Length = 2.8\ cm$	$Length = \frac{75\ Pixels}{90\ Pixels} \times 1\ cm$ $Length = 0.83\ cm$

Supplementary Figure 15: Step by step process of calculating wicking length on Image J.

3.5. Distribution of Plasma on Strip:



Supplementary Figure 16: Difference between even and uneven distribution of plasma across strip. The evenly distributed plasma was added as suggested in method section. The unevenly distributed plasma was added only on the right-hand side of the strip.

3.6. Calculating t-value:

To calculate and compare the sensitivity of the paper test and the automated Clauss assay between 2 concentrations, the t-value was used. The equation for the t-value is given below:

$$t - value = \frac{c_2 - c_1}{\sqrt{\frac{{\sigma_2}^2}{N_2} + \frac{{\sigma_1}^2}{N_1}}}$$

Where:

$$c_1 = mean\ reading\ of\ sample\ 1$$

$$c_2 = mean\ reading\ of\ sample\ 2$$

$$\sigma_1 = standard\ deviation\ of\ sample\ 1$$

$$\sigma_1 = standard\ deviation\ of\ sample\ 2$$

$$N_1 = number\ of\ repeats\ done\ for\ sample\ 1$$

$$N_2 = number\ of\ repeats\ done\ for\ sample\ 2$$

The higher the t-value, the greater the sensitivity between 2 concentrations.

Calculations are provided below to find the t-values between a sample with a fibrinogen concentration of 1.4 g/L (Sample 1) and 2.1 g/L (Sample 2).

Table S11: Calculations of t-value for paper test and Clauss assay between a sample with 1.4 g/L fibrinogen and a sample with 2.1 g/L fibrinogen.

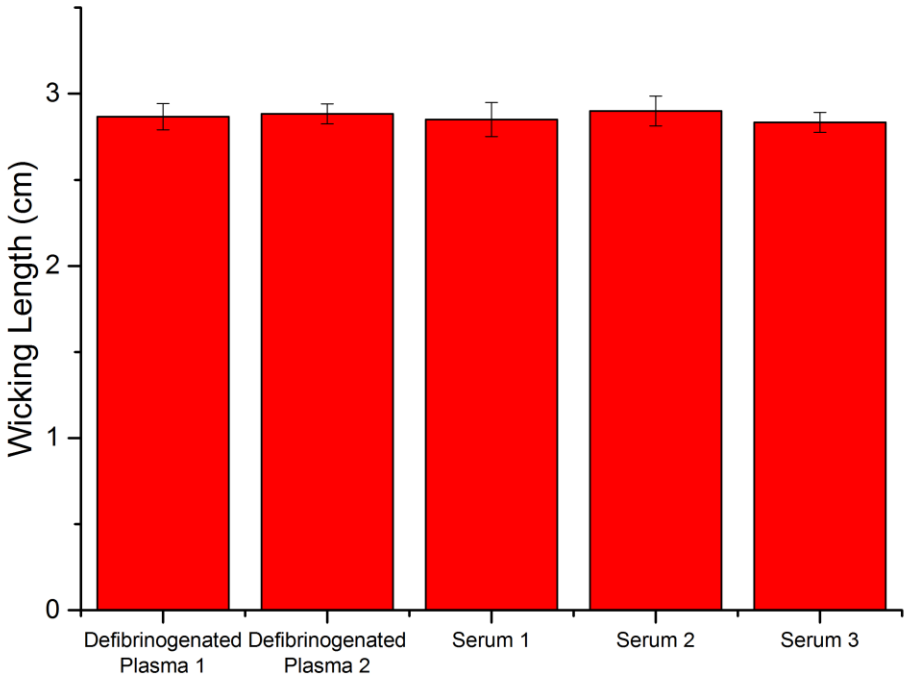
	Paper Test		**Clauss Assay**	
Raw Data	**Sample 1 - Wicking Distance (cm)**	**Sample 2 - Wicking Distance (cm)**	**Sample 1 – Clot Formation Time (s)**	**Sample 2 - Clot Formation Time (s)**
	1.50	1.07	26.5	17.4
	1.59	1.18	28.1	17.0
	1.49	1.11	27.1	16.9
Statistics	**Sample 1**	**Sample 2**	**Sample 1**	**Sample 2**
	C	1.53 cm	1.12 cm	C	27.1 s	17.1 s
	σ	0.0551 cm	0.0557 cm	σ	0.808 s	0.265 s
	N	3	3	N	3	3
t-value	15.6		20.6	

As 20.6 > 15,6, the Clauss assay has a greater sensitivity between the two samples than fibrinogen does.

3.7. Wicking of Defibrinogenated Plasmas vs Serum:

The defibrinogenated plasmas produced to create the dilutions necessary for **Figure 18** were compared to 3 donated serums from the Australian Red Cross. The serums were used to determine wherever the defibrinogenated plasma samples mimicked the properties of afibrinogenemic plasma samples well enough.

Serum is plasma with the clotting factors taken out. Blood samples from donors were collected, allowed to clot and centrifuged to collect the serum. As all the fibrinogen had been clotted off, the serum contained **no** fibrinogen. However, because it had clotted, it also contained fibrin/fibrinogen degradation products which inhibited thrombin. Therefore, it was not suitable to dilute the normal plasma samples with.



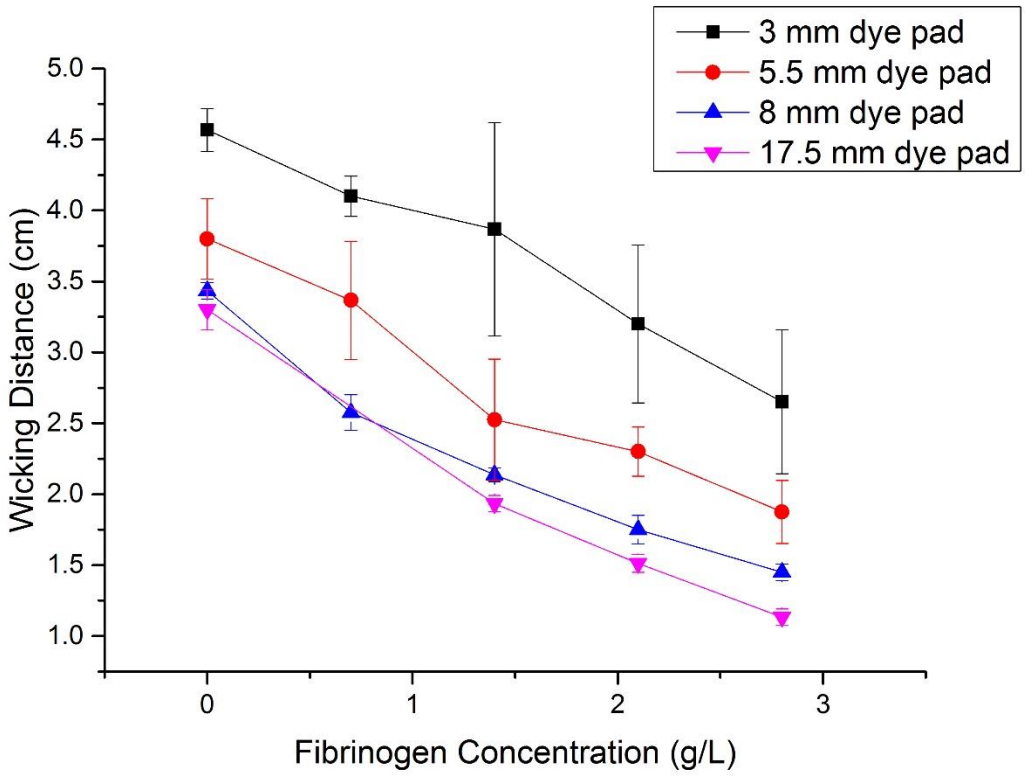
Supplementary Figure 17: Testing of defibrinogenated plasmas and donated serum. Each sample was tested 3 times. The means and standard deviations are reported accordingly.

Defibrinogenated plasma samples wicks to the exact same lengths as donated serum samples, indicating that the contents of both samples are similar.

As the 2 defibrinogenated plasmas wicked similarly to the 3 serum samples, they were suitable to dilute the normal plasma samples in.

Furthermore, as the 3 serum samples themselves wicked similarly, afibrinogenemic plasma exerts no direct effect on wicking.

3.8. Design of Dye Pad:



Supplementary Figure 18: Testing of different dye pad widths. Approximately 4 µL per mm dye pad width of blue dye was added to each strip and allowed to wick for 30 s. Each substance was tested 4 times in both plasmas. The means and standard deviations are reported accordingly.

A dye pad was introduced to control the flow of the blue dye through the fibrin network. To ensure that the Lucas-Washburn kinetics was achieved across all dye pad widths, a pseudo-infinite reservoir was required. This was achieved by adding a large enough volume of blue dye to attain this condition. Lucas-Washburn kinetics was considered achieved when the 0 g/L fibrinogen data points achieved optimal wicking after 30 s. In other words, if a larger blue dye volume was added to the strip, the 0 g/L fibrinogen data point would not wick any further after 30 s. A table describing the volumes needed to obtain the pseudo Lucas-Washburn kinetics for each dye pad width in **Table S12** is given below; as a general rule, 4 µL per mm dye pad appeared to be enough:

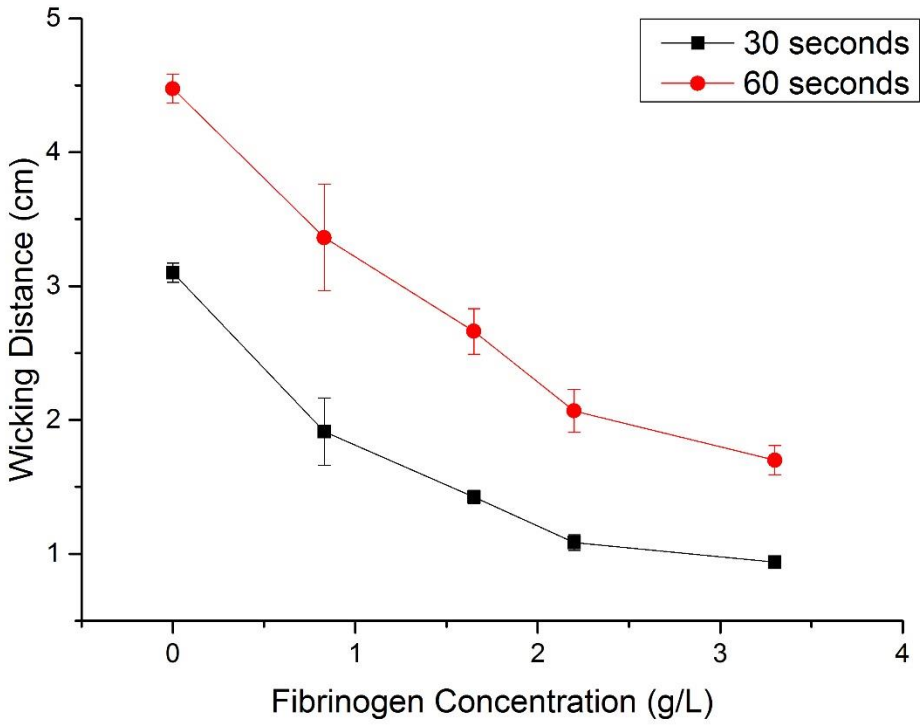
Table S12: Blue dye volumes needed to achieve Lucas-Washburn Kinetics for each dye pad width used.

Dye Pad Width (mm)	Blue Dye Volume (µL)
3 mm	12
5.5 mm	18
8 mm	30
17.5 mm	70

Wider dye pads allow for better fibrinogen concentration separation. We hypothesise that this is due to 2 reasons. 1) The fibrin network stretches out too linearly when the plasma and thrombin mixture is added to the strip. This provides large, elongated gaps that the blue dye can travel through easily. 2) The plasma and thrombin mixture wicks too close to the point where the blue dye is applied. This causes damage to the network due to the excessive shear stresses applied on the resulting fibrin network by the blue dye.

The wider the dye pad is, the more laterally the fibrin network can spread over it. By achieving this, not only can the fibrin network become more compressed (to minimise gaps), but it also can provide some distance between the point at which the blue dye is applied and the fibrin network. Once the dye pad is wide enough to allow the fibrin network to form a semi-circle on it, the separation between different fibrinogen concentrations becomes maximised.

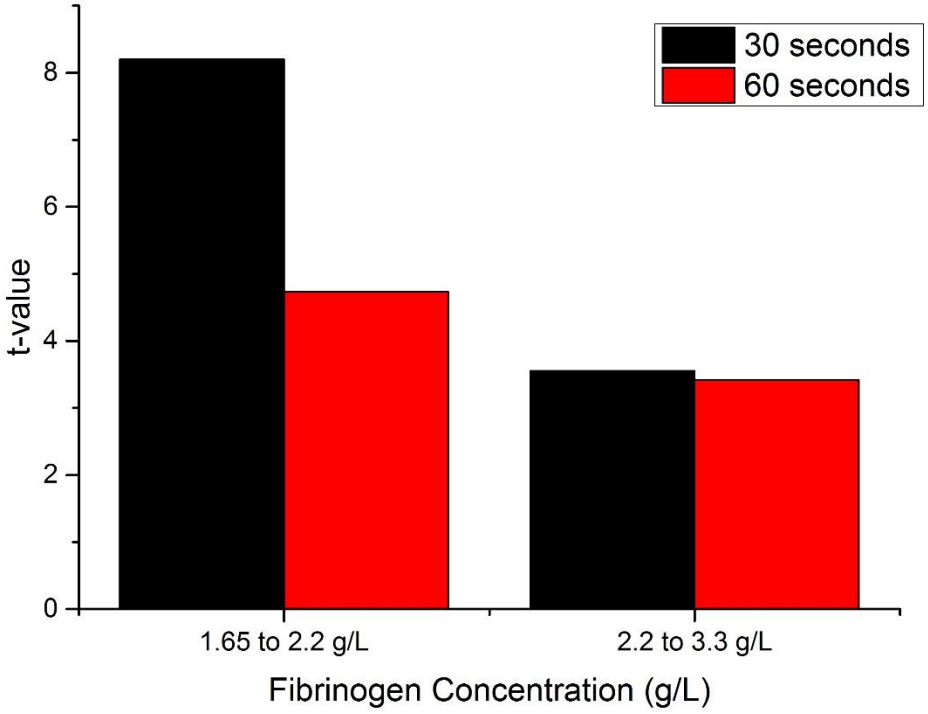
3.9. Effect of Wicking Time:



Supplementary Figure 19: Testing of different wicking times. Plasma from sample 2 (containing 3.3 g/L fibrinogen) was diluted with defibrinogenated plasma to different fibrinogen concentrations. Each data-point was tested 3 times and the mean and standard deviations are reported.

To determine a suitable wicking time, wicking distances at 30 s were compared to wicking distances at 60 s. Times below 30 s did not provide clear separation for concentrations above 0.83 g/L.

Wicking at 60 s causes larger separation to occur between different fibrinogen concentrations. However, it does so at the expense of higher standard deviations. When an analysis of variance is performed, the 30 s wicking interval produces equal or higher t-values, indicating better separability. Therefore, a wicking time of 30 s was chosen for this study.



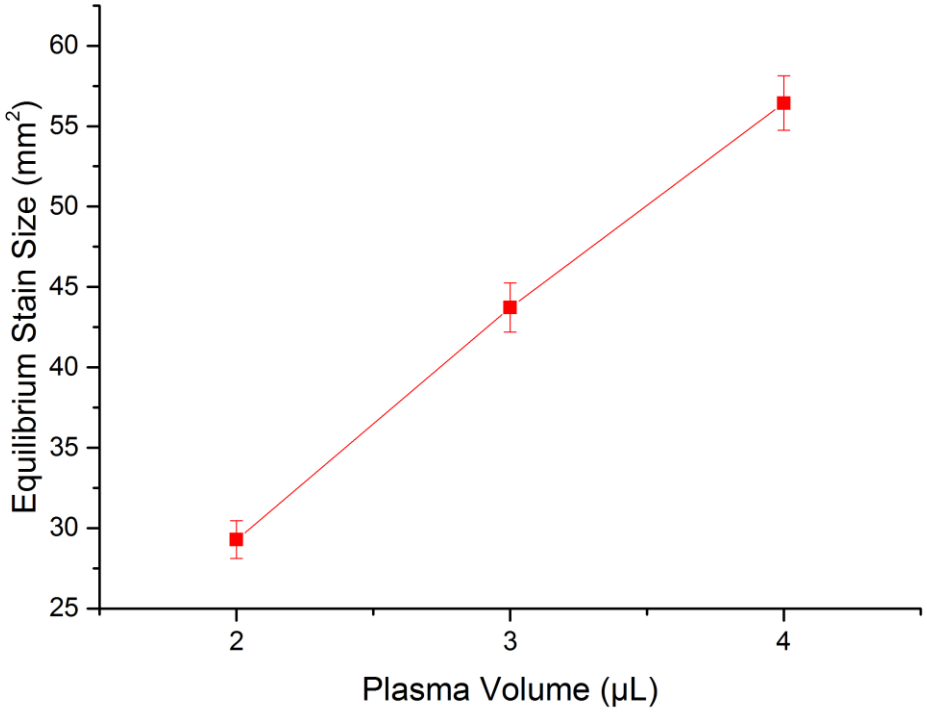
Supplementary Figure 20: Analysis of Variance derived from the results of **Supplementary Figure 19.**

3.10. Confirmation of Accurate Volumes:

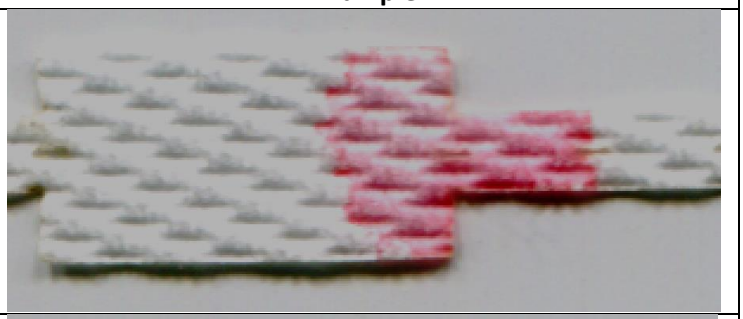
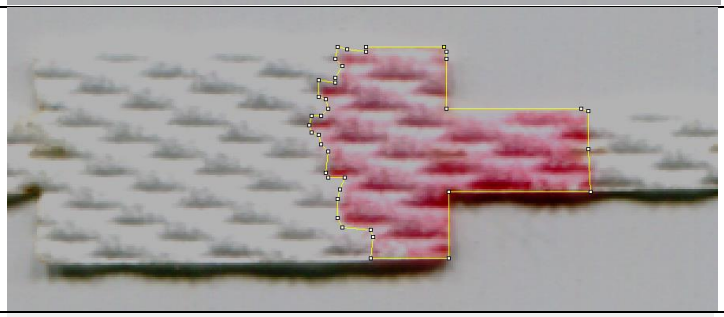
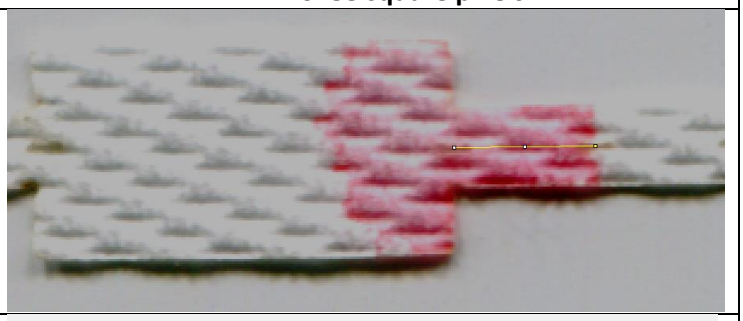
Since the test in its current state uses a low plasma volume (3 µL), we confirmed whether the quantity used was accurate by measuring the equilibrium stain size it formed on when added onto paper. Hertaeg *et al.* found a linear relationship between the 2 parameters (218). The equilibrium stain size here was defined as the final area occupied by the zone of hydrophobicity on the paper strips before blue dye was added.

A visual summary over how the equilibrium stain size was calculated can be found in **Supplementary Figure 22**.

The adding of 3 µL of plasma onto paper is very accurate and can easily be distinguished from volumes ±1 µL.



Supplementary Figure 21: Plasma Volume versus Equilibrium Stain Size on Paper. Each data-point was tested 4 times and the mean and standard deviations are reported.

Step	Example
1. Zoom into strip on ImageJ	
2. Draw polygon around zone of hydrophobicity	
3. Measure Pixel Area	 | | Area | Mean | Min | Max | | 1 | 6288 | 141.182 | 34 | 179 | **AREA = 6288 square pixels**
4. Draw Line between midpoint of 5 mm marking	
5. Measure Length	| | Area | Mean | Min | Max | | 1 | 6288 | 141.182 | 34 | 179 | | 2 | 59 | 114.651 | 79.494 | 163.439 | **LENGTH = 59 pixels (for every 5 mm)**

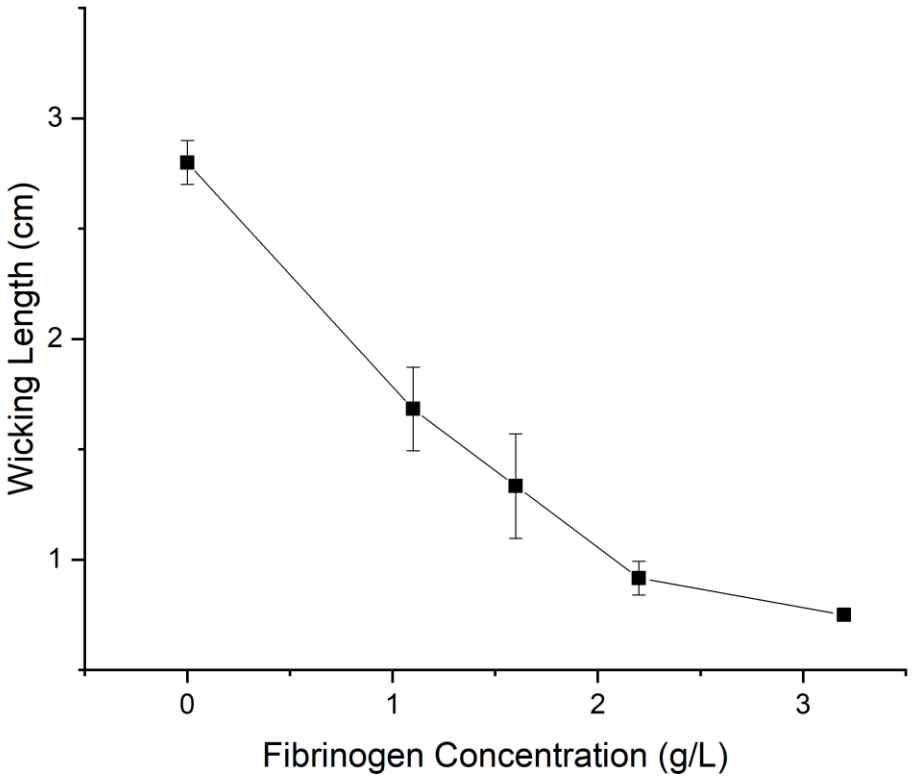
6. Convert square pixels to mm²	$Area = 6288\ square\ pixels\ \times \frac{5^2\ mm^2}{59^2\ square\ pixels}$ $Area = 6288\ square\ pixels\ \times \frac{5^2\ mm^2}{59^2\ square\ pixels}$ $Area = 45\ mm^2$

Supplementary Figure 22: Step by step process of calculating Equilibrium Stain Size on Image J.

3.11. Wicking of Refibrinogenated Plasma:

Since the samples tested in **Figure 18** were diluted with variable amounts of defibrinogenated plasma, it needed to be verified that the trend observed was due to different fibrinogen concentrations and not due to any antithrombotic effects that might have arisen during the heating of defibrinogenated plasma at 56°C.

Fibrinogen solutions in PBS were made according to the methodology provided by Bialkower *et al.* (199). Fibrinogen solutions consisting of 64, 44, 32 and 22 g/L were created and subsequently diluted 1:20 in defibrinogenated plasma from a single source.



Supplementary Figure 23: Testing of defibrinogenated plasma from Sample 3 reconstituted with fibrinogen to different concentrations. Each data-point was tested 3 times and the mean and standard deviations are reported.

The trend observed in **Supplementary Figure 23** is no different to the trend observed in **Figure 18.** Fibrinogen concentrations were distinguishable over a physiologically relevant range. This indicates that no anti-thrombotic effects occurred due to the heating of defibrinogenated plasma. Therefore, it was a suitable to dilute plasma with.

Additionally, given that there has been no published information which states that there is an increase in anti-clotting activity after plasma has been heated to 56°C (239, 240), we conclude that the trend observed in **Figure 18** is indeed driven by fibrinogen concentration.

Chapter 4: Whole Blood Assay

Publication Details

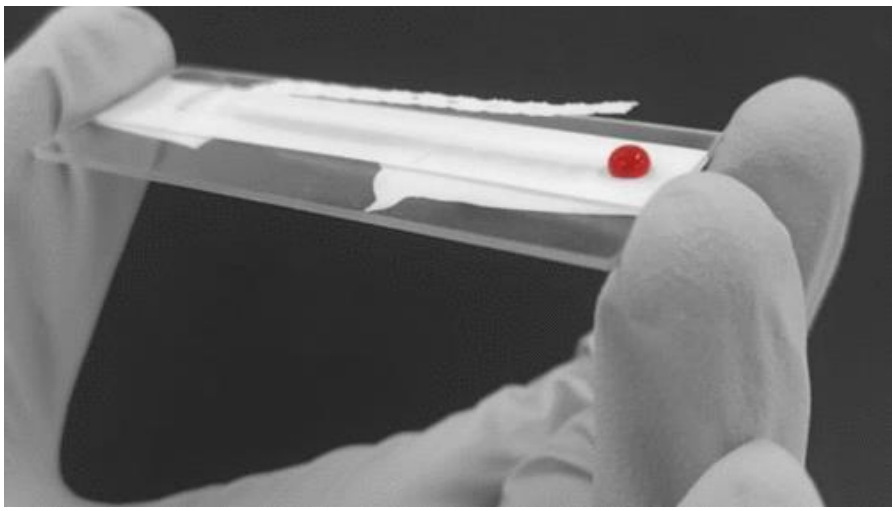
Publication Status: Published

Journal: ACS Sensors *(Q1 – Bioengineering)*

Reference: Bialkower M, Manderson CA, McLiesh H, Tabor RF, Garnier G. *Paper Diagnostic for Direct Measurement of Fibrinogen Concentration in Whole Blood.* DOI: https://pubs.acs.org/doi/10.1021/acssensors.0c01937

Abstract

The ability to diagnose and treat critically bleeding patients can save more than 2 million lives a year. Diagnosing hypofibrinogenemia is essential in these patients. Recently, with the development of new hand-held diagnostics, fibrinogen concentration can be measured rapidly at the point of care. However, these diagnostics can only work with plasma and hence need blood cells to be separated before use. In this study, we demonstrate a hand-held fibrinogen diagnostic that works with whole blood. The test works by (1) forming a premixed droplet of a whole blood sample and thrombin solution on a solid surface, (2) allowing it to clot, and (3) dropping a paper strip on top. The further that blood moves down the strip, the lower the fibrinogen concentration. The diagnostic can easily measure plasma fibrinogen concentrations below 1.6 g/L for blood samples with haematocrits between 40 and 50%. Furthermore, diluting blood samples not only increases the test's sensitivity but also eliminates the effect of haematocrit and thrombin inhibitors. The test can be completed in 3−4 min, making it suitable for diagnosing early hypofibrinogenemia and allowing for fibrinogen replacement therapy in critically bleeding patients.



1. Introduction

This study aims to develop a rapid, hand-held paper diagnostic for measuring fibrinogen concentration directly in whole blood. The diagnostic can take out the requirement of separating blood cells from plasma that other hand-held fibrinogen diagnostics are limited by. Hence, it can eliminate the preparation time, labour and transportation difficulties that traditional techniques like centrifugation have. The implications for this diagnostic are significant. It can diagnose hypofibrinogenemia in critically bleeding patients anywhere in the world. This can drastically reduce the time to treatment needed for fibrinogen replacement therapy (FRT).

Critical bleeding can occur anywhere in the world. Outside clinics, trauma-induced major haemorrhage causes 2 million deaths worldwide a year (174, 191-196). Hypofibrinogenemia (that is, insufficient fibrinogen to enable effective clotting) in critical bleeding is common. McQuilten *et al*. identified that 21.2% of major trauma patients had fibrinogen levels below 2 g/L. (197). Fibrinogen is one of the first proteins to be depleted in critically bleeding patients (31). Therefore, the early diagnosis of hypofibrinogenemia and FRT may stop bleeding in these patients and save their lives.

There are more than 60 tests that can measure fibrinogen concentration (89). However, most of these tests can only work with plasma and not whole blood. This means that a way of separating blood cells from plasma is needed before the diagnostic can be used. Typically, plasma is separated from blood cells by centrifugation. Whole blood is collected in a tube and spun at 2000−4000g for 10 to 20 min. Afterward, the plasma is collected and used. The centrifuge itself is a specialised instrument that requires a stable environment and electricity to use. This is often impossible at the site of injury.

There are some tests that do use whole blood for diagnosing hypofibrinogenemia, the most notable of which are viscoelastic haemostatic assays (VHAs) and DRIHEMATO (21, 198). VHAs measure fibrinogen concentration through the difference in viscoelasticity (VE) of a blood sample before and after it has clotted (21). Hypofibrinogenemia is diagnosed when the change in VE magnitude is low. The VE requires well controlled, sensitive, and expensive electrical instruments to measure. Hence, VHAs cannot be transported. DRIHEMATO measures fibrinogen concentration through the time taken for a blood sample to clot (198). Thrombin (an enzyme that converts fibrinogen into fibrin − the backbone polymer found within clots) is added to a blood sample to induce clotting. Longer clotting times are indicative of hypofibrinogenemia. However, DRIHEMATO is also limited by prohibitively expensive items and electrical equipment.

In our previous study, we developed a cheap, hand-held paper diagnostic for measuring the fibrinogen concentration in blood (200). First, thrombin and plasma are added onto horizontally orientated paper strips where the fibrinogen is converted into fibrin. This drastically increases the plasma's hydrophobicity. Second, an aqueous blue dye is pipetted onto the strips and allowed to wick through the fibrin. The distance that the blue dye wicks through the strip correlates precisely to the fibrinogen concentration. The diagnostic can provide results within a minute, and it can distinguish low fibrinogen concentrations (i.e., <2 g/L) from normal fibrinogen concentrations. The diagnostic is not affected by blood conditions such as acidosis, blood alcohol, hypertriglyceridemia, haemolysis, and warfarin administration. However, like most other fibrinogen tests, the diagnostic is only able to work with plasma and not whole blood.

Therefore, a rapid, cheap, portable, and hand-held fibrinogen concentration diagnostic that works directly with whole blood remains an outstanding and critical requirement.

In this manuscript, we develop a rapid and sensitive paper test that can work directly with whole blood. The test works by forming a premixed droplet of whole blood and thrombin on a solid surface, allowing it to clot, and finally placing a paper strip on top to visualise results. The further that blood moves down the

strip, the lower the fibrinogen concentration is. We initially investigate (1) the contact angle that the droplet requires for the test to work, (2) the time the reaction needs before completion, (3) the optimal time the blood needs to wick for, and (4) the haematocrit required for the test to work properly. We then observe the effect of dilution to determine whether it can improve the sensitivity of the test and minimise the effect of haematocrit and thrombin inhibitors. We also provide insight on the mechanism behind the test.

Finally, we demonstrate a hand-held prototype of the diagnostic applicable for point-of-care testing. It is our objective to demonstrate the proof of concept of this new diagnostic so it may later be used clinically to diagnose and treat early hypofibrinogenemia.

2. Materials and Methodology

2.1. Materials

Tissue paper of 30 g/m^2 and 0.16 mm was used to create the paper strip cut-outs. They were produced in the Kimberly Clark Experimental Forming Unit (EFU), Neenah (WI), USA. Tissues consist of eucalyptus fibres formed with a three-layer headbox. 10 kg/T Kymene, a polyaminoamide-epichlorohydrin (PAE) wet strength agent, was added to each layer. Tissues were moulded and thoroughly air-dried.

Two batches of citrated platelet-poor plasmas were sourced from Affinity Biologicals. One of these was a fibrinogen-free plasma (FGDP), and the other was a normal plasma (FRNCP0105). Both lots derive from a pool of 20 donors, buffered with 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer to stabilise the pH at around 7.4. The fibrinogen-free plasma batch had trace amounts of thrombin added to it to remove all the available fibrinogen before the plasma is frozen at −80 °C. No residual thrombin or fibrinogen remained after the reaction. The normal plasma batch (which had a fibrinogen concentration of 3.2 g/L) was frozen immediately at −80 °C. Ethylenediaminetetraacetic acid (EDTA) anticoagulated donor blood tubes were sourced from the Australian Red Cross.

Ponceau 4R (from Queen red food colouring) was used to make the red dye (for colouring the plasma in the plasma vs whole blood experiment). Brilliant Blue FCF (from Queen blue food colouring) was used to colourise wicking fronts with poor visibility (in the testing of dilution). Thrombin (T8885), unfractionated heparin (H5515), and phosphate-buffered saline (PBS) tablets (79382) were sourced from Sigma Aldrich.

MilliQ water was used to dissolve PBS tablets, reconstitute vials of thrombin, and perform Carstair's staining.

"Tubes PCR Strip Tubes 0.2 mL Thin Wall & Flat Caps" from Axygen were used as the tubes for mixing. They are referred to as PCR tubes throughout the manuscript.

An iPhone 8 with the app ProMovie was used to record wicking. A Yellowstone 6000 Tripod was used to hold the iPhone in place during recording. A Manfrotto Universal Smartphone Clamp was used to attach the iPhone to the tripod.

Kinetic 12 mm × 10 m white standard thread seal tape (PTFE tape) was used to provide a hydrophobic surface for beaded droplets to form on top. Westlab microscope glass slides (25 × 75 × 1.0–1.2 mm) were used to provide a hydrophilic surface for unbeaded droplets to form on top (and for the PTFE tape to be wrapped around). Menzel Gläser 22 × 22 mm coverslips were used to attach the PCR tube to the glass slide for assembling the prototype diagnostic. Selley's Power Grip glue was used to glue together the prototype diagnostic.

Several reagents were used to perform Carstair's stain. These can be found in the **Supplementary Section 4.1.**

2.2. Methodology

2.2.1. Paper Strip Cut-Out Preparation

Paper strip cut-outs were designed using CorelDRAW X6 with the dimensions shown in **Supplementary Figure 24**. Paper was cut on a laser cutter (60 Watt Epilog Helix) using the cut-outs prepared on CorelDRAW X6. The print settings used were 100% speed, 5% power, and 500 Hz frequency. It was important to ensure that the strips were cut parallel to the machine direction of the paper for uniform and maximum wicking. To ensure that the test produced consistent results, the paper strips had to be cut thin enough to prevent gaps from being introduced into its structure. Otherwise, the blood could wick

further than it should. In our previous study, we found that 3 mm paper strips produced precise results after wicking (199). Therefore, this width was chosen for the paper strips in this study as well.

2.2.2. Phosphate Buffer Saline (PBS) Solution Preparation

Five tablets of PBS were added to a 1 L Schott bottle. 1 L of MilliQ water was added into the Schott bottle. The PBS tablets were dissolved in solution by gently rocking the Schott bottle with a WiseShake SHR rocker at 40 rpm overnight.

2.2.3. Whole Blood Sample Preparation

Plasma samples of different fibrinogen concentrations were prepared from normal and fibrinogen-free plasmas. Both plasma samples were thawed, continuously filled, and emptied with a pipette. This ensured that their contents were uniformly distributed. The two plasmas were then remixed with each other in different ratios to obtain plasma samples with different fibrinogen concentrations used for experimentation. These samples were then aliquoted 150 µL each into Eppendorf tubes, snap-frozen in liquid nitrogen, and stored at −86 °C until use. When these samples were used, they were again mixed up and down with a pipette to distribute their contents uniformly.

Red blood cells (RBCs) were sourced from EDTA anticoagulated donor blood. A donor blood tube was centrifuged for 15 min at 3000 rpm on an Eppendorf 5702 centrifuge. This completely fractionated the blood components. The plasma supernatant was discarded with manual pipetting.

The RBCs were then washed with PBS solution. 3 mL of PBS solution was added to the RBCs, resuspended, and recentrifuged for 15 min at 3000 rpm. The PBS supernatant was then discarded with manual pipetting. The washing step was repeated until the PBS supernatant appeared clear. The final clear PBS supernatant was discarded, and the washed RBCs were retained and used on the day of experimentation.

Whole blood samples of different fibrinogen concentration were prepared by the mixing of washed RBCs and plasma samples. They were most often mixed half-half to create whole blood samples with haematocrits of 50% (unless stated otherwise). Whole blood samples with different haematocrits were prepared by changing the mixing ratio of washed RBCs and plasma. The whole blood samples were used on the day of experimentation. Because blood cells in whole blood samples sediment over time due to gravity, they were uniformly suspended each time before use.

2.2.4. Thrombin Solution Preparation

One vial of thrombin was reconstituted in 300 µL of MilliQ water and separated into vials containing each 30 NIH U/mL thrombin. The vials were then aliquoted 50 µL each into Eppendorf tubes, snap-frozen in liquid nitrogen, and stored at −86 °C until use.

When these thrombin solution samples were unfrozen, they were remixed using a pipette. This ensured that their contents were uniformly distributed.

From **Chapter 2**, Factor XIIIa did not influence the test (despite crosslinking fibrin monomers). Therefore, it was not observed in this chapter.

2.2.5. Hydrophilic and Hydrophobic Surface Preparation

Beaded and unbeaded droplets of mixed blood were formed on top of hydrophobic and hydrophilic surfaces, respectively.

Hydrophilic surfaces consisted of glass slides and used as they were.

Hydrophobic surfaces were prepared by wrapping one piece of PTFE tape around a glass slide with the tape lying flat.

2.2.6. Main Test

Experiments were performed in standard laboratory conditions (i.e., 23 °C, 50% relative humidity).

A hydrophobic surface glass slide was generally used unless stated otherwise.

5 µL of thrombin solution was added into a PCR tube. 25 µL of whole blood (or plasma) was mixed into the thrombin solution. This was done by pipetting up and down at least 10 times to ensure that the blood and thrombin were mixed uniformly (without creating bubbles). The mixture was then deposited on top of the glass slide before it could clot (i.e., convert fibrinogen into fibrin). The mixing and deposition time afforded before clotting are summarised in **Supplementary Figure 37.**

Mixtures with lower fibrinogen concentration took longer to clot and hence could be deposited on top of the spot more easily.

It was important to ensure that the mixture was not spread across the glass slide and added onto one spot. This ensured that a 25 µL droplet was formed on top of each surface.

The droplet reacted on the slide for a set period to ensure that it had clotted. This generally occurred after 3 min unless stated otherwise.

Once the reaction time was complete, a paper strip was carefully placed on top of the clotted droplet. This was done by ensuring that (1) the dominant hand levitated the paper strip millimetres above the clotted droplet with tweezers, (2) the nondominant hand pinned the far end of the paper strip to the glass slide (to hold it in place), and (3) the tweezers were instantly opened (releasing the paper strip to fall on top of the clotted droplet at a low velocity). An illustration of the landing position of the paper strip on the clotted droplet is provided in **Supplementary Figure 31.**

The wicking length was measured 30 s after the paper strip landed on top of the clotted droplet (unless stated otherwise). **Figure 26** illustrates this entire procedure.

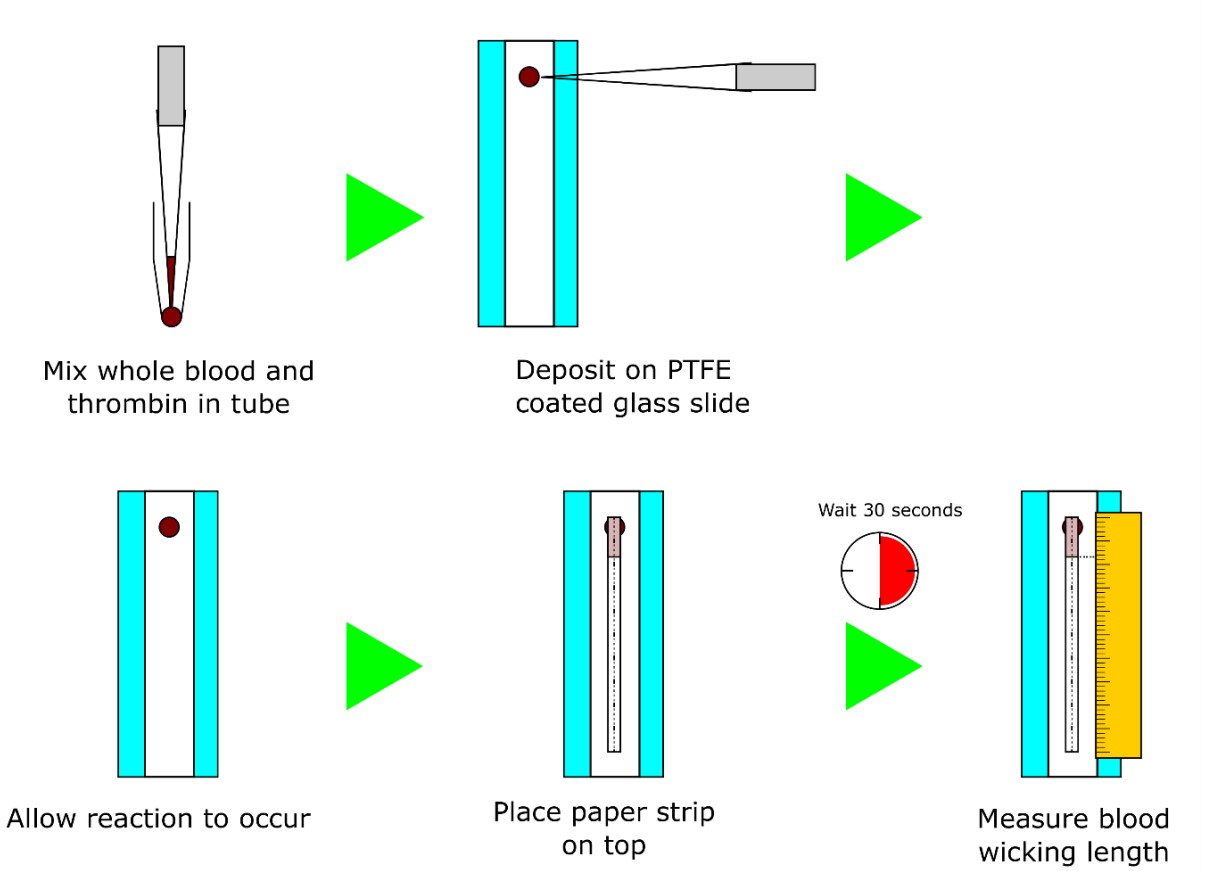


Figure 26: Schematic illustration of the main test procedure.

2.2.7. Wicking Length Measurement

The midpoint for the wicking front was used to measure the wicking length. The iPhone was held directly over the paper strip, and ProMovie (downloaded from the App Store) was used to film the main test. To achieve accurate recordings, the markings on the paper strips were manually focused on before filming (see **Supplementary Figure 25**). To measure wicking length, snapshots of each strip in the recorded videos were generally taken 30 s after the paper strip made contact with the clotted droplet (unless specified otherwise).

Images were then analysed using ImageJ to determine their wicking length (see **Supplementary Figure 26**).

2.2.8. Testing of Dilution

Whole blood samples were diluted to determine wherever the sensitivity of the test could be improved. Whole blood samples of different fibrinogen concentrations were diluted in PBS buffer and tested.

The dilutions tested were 1:0, 1:1, 1:3, 1:7, and 1:15. The left-hand number refers to the whole blood fraction, and the right-hand number refers to the PBS fraction. For example, 1:3 means one part whole blood to three parts PBS (or 25% whole blood).

Since diluted blood samples (i.e., 1:3, 1:7, and 1:15) produced wicking fronts with poor visibility, a minute amount of Queen blue food dye was added to each paper strip to aid in the image analysis. As blood

wicked down the strip, it mixed with the blue dye and enhanced the visibility of the wicking front. An example of the preparation is shown in **Supplementary Figure 40**.

Diluted whole blood samples then followed the procedure of the main test and subsequent image analysis.

Alternatively, the dilution of whole blood samples could be achieved by changing the mixing ratio between the whole blood and thrombin solution instead.

2.2.9. Testing of Thrombin Inhibitors

Since the diagnostic uses thrombin as an active reagent, endogenous thrombin inhibitors (such as heparin) were tested to determine whether they impacted the test.

Whole blood samples of different fibrinogen concentrations were mixed with heparin (a well-known thrombin inhibitor) and tested.

A solution of 14 U/mL heparin was prepared by dissolving unfractionated heparin in MilliQ water. It was then mixed into whole blood samples at a ratio of 1:19 (creating whole blood samples with 0.7 U/mL heparin).

Whole blood samples without heparin were used as controls.

Additionally, whole blood samples (with and without heparin) were diluted in PBS at a ratio of 1:7. This was to determine wherever dilution could reduce the effect of thrombin inhibitors.

All these blood samples followed the procedure of the main test and subsequent image analysis.

2.2.10. Staining and Imaging of Fibrin

To decipher the mechanism of the test, it was necessary to determine wherever fibrin moved down the paper strip or remained in the clotted droplet during wicking.

Plasma samples (both normal and fibrinogen-free) were tested as normal except they wicked until drying. The dried paper strips then underwent Carstair's staining procedure (shown in **Supplementary Section 4.1**). The paper strips were contained within a mini colander (while performing the procedure) to hold them in place.

After the staining procedure, the DPX mounted paper strips were imaged under a Nikon Eclipse Ni-E upright microscope. Images were taken across the length of the paper strip to determine where fibrin was located. Fibrin was indicated by the presence of red bodies.

2.2.11. Testing Plasma vs Whole Blood

Plasma samples were tested against whole blood samples to determine whether the sensitivity of the test was governed by fibrin−RBC interactions or by fibrin on its own.

Plasma samples were mixed with Queen red food dye at a ratio of 1:19 (to colourise the plasma). These samples followed the procedure of the main test and subsequent image analysis.

Whole blood samples were diluted in PBS to a ratio of 1:3.

This was done to neutralise the non-fibrinogen effects of the whole blood sample. Non-fibrinogen effects refer to the effect that the RBCs and plasma proteins (other than fibrinogen) have on blood wicking by themselves. RBCs and plasma proteins both interact with the cellulose fibres and increase the viscosity of a blood sample. Thus, they are both able to drastically decrease the wickability of whole blood irrespective of the fibrinogen concentration.

The diluted whole blood samples followed the procedure of the main test and subsequent image analysis.

2.2.12. Diagnostic Assembly

A hydrophobic surfaced glass slide was used.

A black marker was used to mark two points 5.8 cm apart on the PTFE tape (as shown in **Figure 27 – top left**).

A paper strip was placed over the two markings. The bottom edge was aligned to one marking and attached with sticky tape. A bent paper clip was wedged between the other end of the paper strip and the glass slide. This allowed the paper strip to levitate over the glass slide. The shape and position of the paper clip could be altered to change the levitation height of the paper strip. A single PCR tube was glued side-on to a coverslip. After drying, the coverslip was glued underneath the glass slide and left to dry. This allowed the PCR tube to be attached to the diagnostic. An image of an assembled diagnostic is shown in **Figure 27 – top right**.

2.2.13. Diagnostic Testing

An assembled diagnostic was hand-held horizontally using fingertips as shown in **Figure 27 – bottom**.

1:7 diluted whole blood and thrombin solution was mixed in the PCR tube (as described in the procedure of the main test). The thrombin solution was pipetted into the very back of the PCR tube so that the blood could be easily mixed with it.

The mixture was deposited directly on top of the free marking. The diagnostic was steadily held for a 3 min reaction time (to avoid the droplet from spreading).

After the reaction, the paper clip was quickly pulled out of the diagnostic. This allowed the paper strip to fall on top of the clotted droplet. The wicking length was determined 30 s after the paper strip landed on top of the clotted droplet.

The wicking length was measured as the approximate midpoint of the wicking front observed by the naked eye. This was rounded off to the nearest half millimetre.

3. Results

Terminology defining various blood terms is provided in **Table S13**.

Whole blood samples were mixed with thrombin solution in PCR tubes until they were uniformly distributed. Each mixture was then deposited as a droplet onto a hydrophilic- or hydrophobic-surfaced glass slide. After reacting for 3 min, a paper strip was carefully placed on top of the clotted droplet and blood wicked down it for 30 s. This is shown in **Figure 28**.

The further the blood wicks, the lower the fibrinogen concentration. This is strongly hypothesised to be controlled by the permeability of the clotted droplet. Clotted droplets with lower fibrinogen concentrations most likely have higher permeabilities. When the droplet becomes clotted, two phases are formed: a clotted phase (i.e., the fibrin scaffold) and an unclotted phase (i.e., the remaining blood fluid). The unclotted phase can freely wick through the paper; however, the clotted phase cannot. The clotted phase impedes the movement of the unclotted phase down the strip. This impediment is strongly enhanced by the presence of RBCs. The RBCs likely get entrapped into the clotted phase and strongly reduce its permeability through volume occupancy and surface drag. Therefore, whole blood samples with lower fibrinogen concentrations form clotted phases with fewer entrapped RBCs and most likely higher permeabilities.

Several factors were investigated to understand the requirements to adapt the test into a hand-held prototype diagnostic. These included the effects of droplet contact angle, blood wicking time, relative humidity, droplet reaction time, haematocrit, whole blood sample dilution, and thrombin inhibitors.

The effect of droplet contact angle is investigated as shown in **Supplementary Figure 32**. Droplets were dispensed on hydrophilic- and hydrophobic-surfaced glass slides to produce unbeaded and beaded droplets, respectively. However, the contact angle of the droplet does not significantly affect the test.

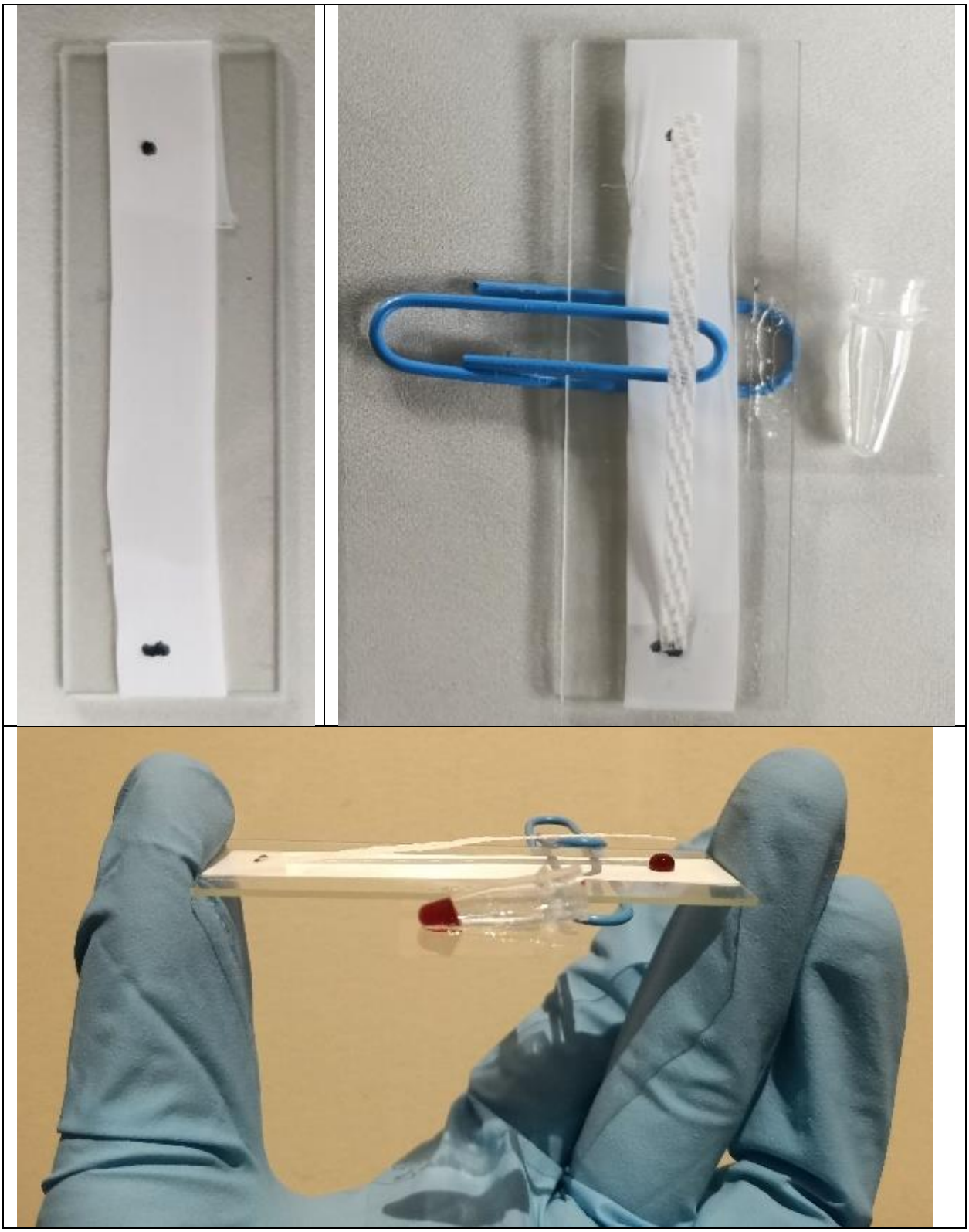


Figure 27: Assembling of diagnostic. **Top Left:** Hydrophobic surfaced glass slide showing markings 5.8 cm apart. **Top Right:** Assembled diagnostic with the PCR tube attached to side of diagnostic and paper strip attached and levitated above diagnostic. **Bottom:** 25 µL droplet steadily deposited on the diagnostic directly below paper strip.

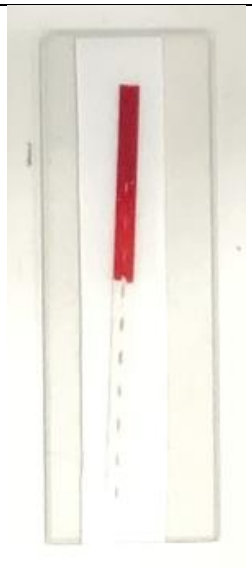
	0 g/L Fibrinogen in Plasma	3.2 g/L Fibrinogen in Plasma
Droplet added to PTFE coated glass slide and allowed to react		
Paper strip placed on top of clotted droplet. **Wicking Length measured after 30 s**		

Figure 28: Summary of wicking test for whole blood samples without fibrinogen (0 g/L) and with fibrinogen (3.2 g/L).

3.2. Effect of Wicking Time

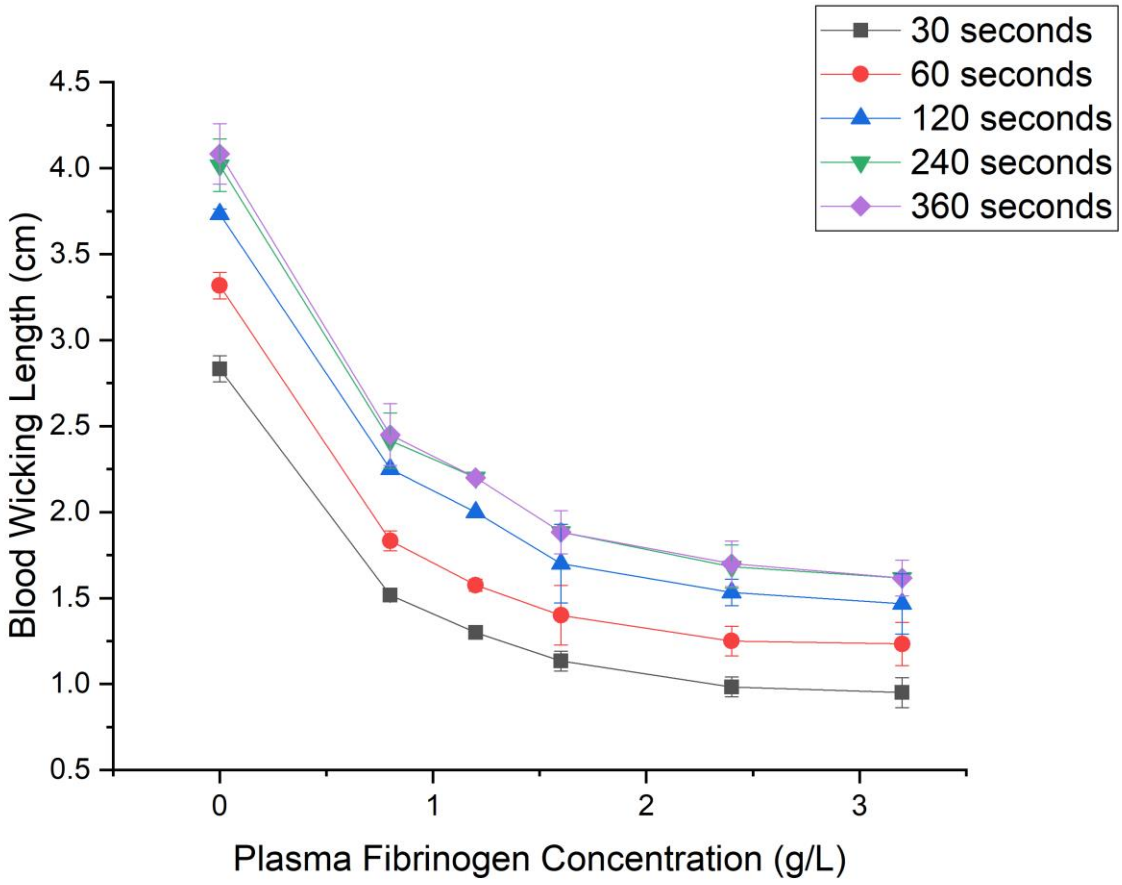


Figure 29: Testing of whole blood samples under different blood wicking times. Whole blood reacted with thrombin for 3 min and the blood wicking length was measured using ImageJ. The test was carried out at 23°C and 50% relative humidity. Each data point was tested 3 times and the mean and standard deviation are reported.

Figure 29 investigates the effect of blood wicking time to determine the optimum time needed for wicking. Whole blood droplets of different fibrinogen concentrations were wicked through paper until they dried. The wicking length was measured at different time points after the paper strip was placed on top of the clotted droplet. The longer the wicking time, the greater the separation that occurs between whole blood samples of different fibrinogen concentrations. However, this also comes at the cost of greater standard deviation. Hence, the test is better suited for wicking for shorter times (i.e., 30 s) than for longer times (i.e., 1 min or more).

Furthermore, **Supplementary Figure 27** evaluates the effect of wicking time at different humidities. Whole blood droplets at lower relative humidities dry faster than those at higher relative humidities. Hence, they stop wicking at both shorter wicking times and lengths. To minimise the effects of humidity, the test is more suitable to wicking for short times (i.e., 30 s) than for long times (i.e., 2 min or more).

Therefore, a wicking time of 30 s was used in this study.

Additionally, raw images deciphering **Figure 29** after drying are displayed in **Supplementary Figure 28**. A clear relationship between fibrinogen concentration and wicking length is displayed.

Finally, the effect of reaction time is evaluated as shown in **Supplementary Figure 29**. The reaction is completed in 2–3 min. Therefore, a reaction time of 3 min was used for this study.

3.3. Effect of Dilution

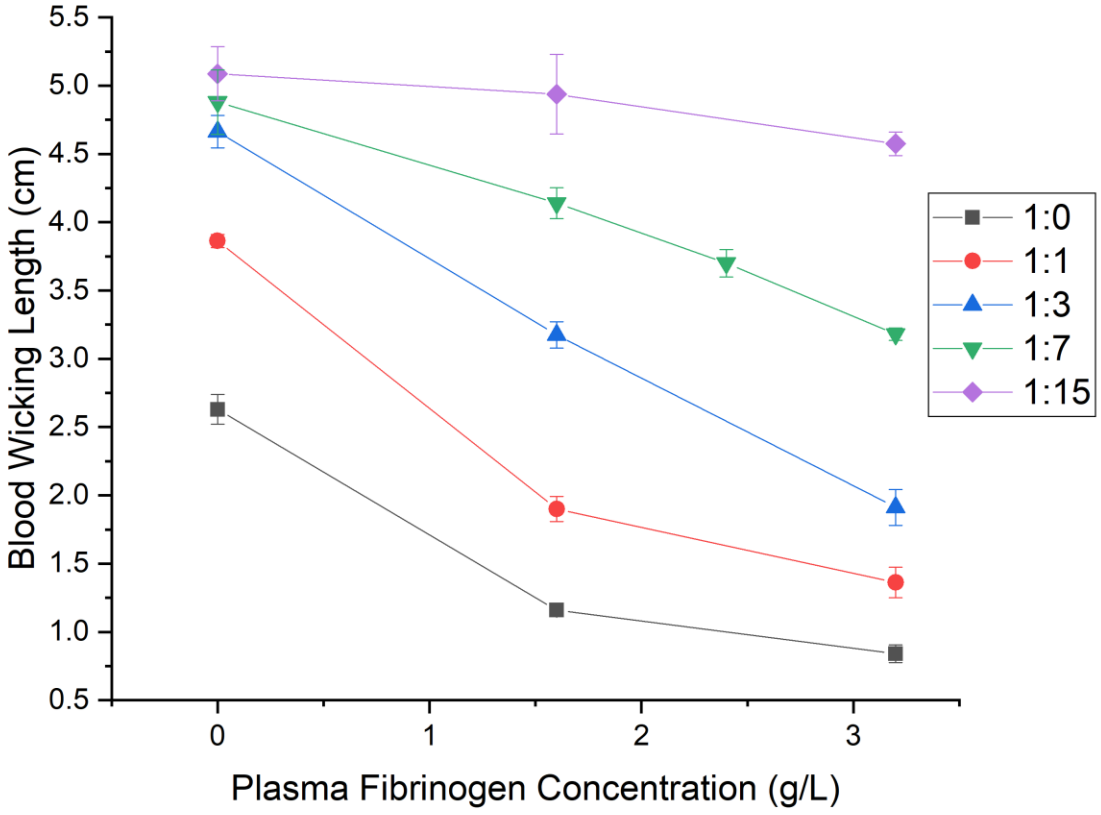


Figure 30: Testing of blood samples diluted to different ratios in Phosphate Buffer Solution (PBS). Whole blood reacted with thrombin for 3 min and the blood wicking length was measured after 30 s of wicking using ImageJ. Each data point was tested 5 times and the mean and standard deviation are reported.

Figure 30 investigates the effect of whole blood dilution to determine whether it could improve test results. As shown in **Supplementary Figure 37**, droplets with lower fibrinogen concentrations take longer to clot. Hence, more time is available for the end-user to mix and dispense the droplet carefully onto the glass slide.

Whole blood samples of different fibrinogen concentrations were diluted in phosphate-buffered saline (PBS) and tested. The x-axis refers to the plasma fibrinogen concentrations of the whole blood samples before dilution in PBS.

Diluting whole blood vastly improves the sensitivity of the test. When whole blood is diluted between 1:3 to 1:7 with PBS, the relationship between plasma fibrinogen concentration and length becomes more linear. Hence, fibrinogen readings can be more easily obtained. The linearization is explained in more

detail in **Supplementary Figures 35** and **36**. However, once the whole blood is diluted 1:15 in PBS, the post-dilution fibrinogen concentration drops too low and the sensitivity becomes diminished.

Additionally, the non-fibrinogen effects at higher dilutions become significantly weaker. Non-fibrinogen effects refer to the effect that the RBCs and plasma proteins have on blood wicking without fibrinogen. RBCs and plasma proteins both interact with the cellulose fibres of paper and also increase the viscosity of a blood sample. Thus, they are both able to drastically decrease the wickability of whole blood on paper. As demonstrated with the 0 g/L plasma fibrinogen samples, the non-fibrinogen effect drastically decreases when whole blood is serially diluted in PBS. As whole blood samples become more diluted, less RBCs and plasma proteins are present to impede the wicking of the blood sample's unclotted phase through the paper strip. When dilution reaches between 1:3 and 1:7, the wicking perturbation becomes negligible (reaching similar wicking lengths of 4.7 ± 0.1 and 4.9 ± 0.2 cm, respectively).

Therefore, a dilution ratio background of 1:3 to 1:7 is optimal for such a test.

3.4. Mechanism

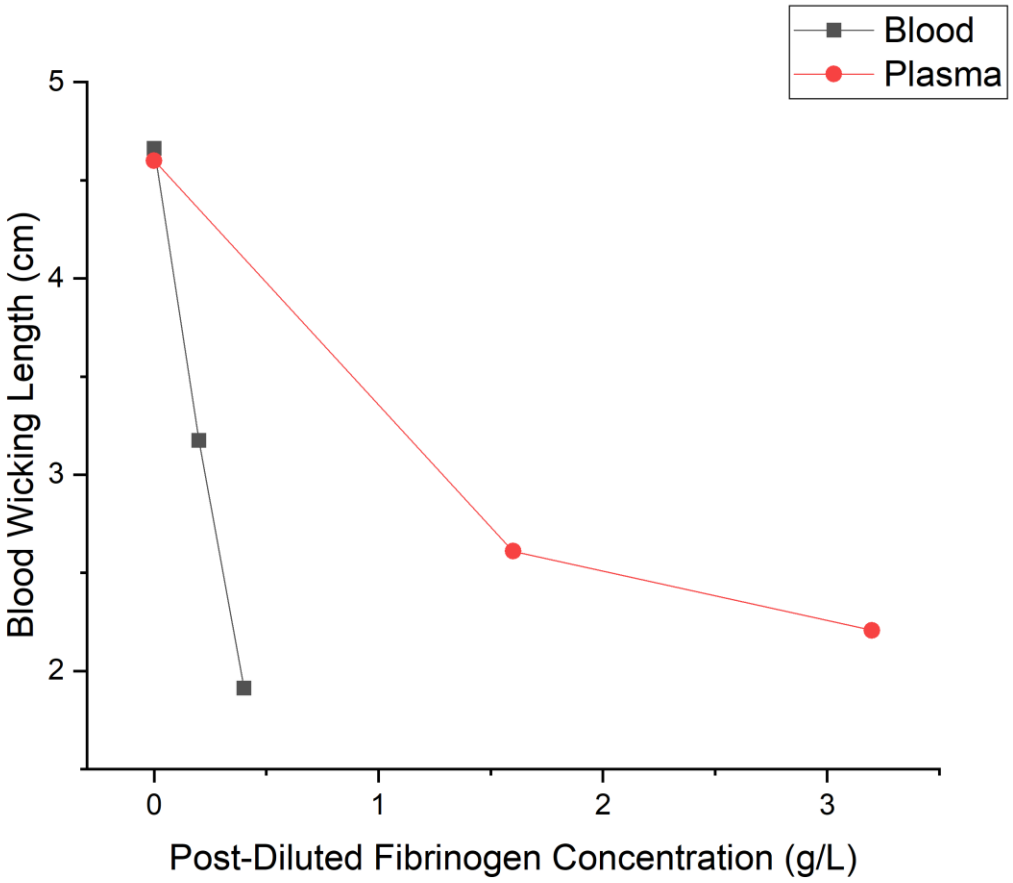


Figure 31: Comparison of plasma wicking to that of whole blood (diluted in PBS) with similar background effects. Plasma and diluted whole blood reacted with thrombin for 3 min and the blood wicking length was measured after 30 s of wicking using ImageJ. Each data point was tested 5 times and the mean is reported.

Supplementary Figure 30 and **Figure 31** provide insight into how the test works.

Supplementary Figure 30 illustrates whether the fibrin moves down the paper strip or remains in the clotted droplet during wicking. The fibrin stays within the clotted droplet, while other blood components move down the strip.

Therefore, the mechanism is as follows. Once the droplet has reacted with thrombin, two phases are formed: a clotted phase and an unclotted phase. When the strip is placed on the clotted droplet, only the unclotted phase can wick down the strip.

Figure 31 quantifies wicking of plasma and diluted whole blood samples of different fibrinogen concentrations. This is to determine whether the test's sensitivity is driven by fibrin−RBC interactions or by fibrin on its own. Undiluted plasma samples were compared to blood samples diluted 1:3 in PBS because they both show similar non-fibrinogen effects (i.e., wicking lengths in samples without fibrinogen). The x-axis refers to fibrinogen concentrations of both samples after dilution. The effects of RBCs are significant. Diluted whole blood samples show an 8-fold increase in sensitivity compared to plasma samples. Hence, the test is predominantly driven by fibrin−RBC interactions.

It is well known that fibrin scaffold can entrap RBCs (241). This in turn occupies pore volume and induces surface drag. Hence, we strongly hypothesise that in the test, the RBCs become entrapped within the clotted phase and reduce its permeability. As the unclotted phase attempts to wick down the paper strip, the entrapped RBCs impede it from doing so. Therefore, it limits the unclotted phase volume that can move down the paper strip. This causes the unclotted phase to wick to shorter lengths.

As the fibrinogen concentration (in the unclotted droplet) dictates the mass of the fibrin scaffold formed (in the clotted droplet), it also dictates the number of RBCs that become entrained (within the clotted droplet). Therefore, higher fibrinogen concentration leads to more RBC entrapment and subsequent blood wicking impedance.

3.5. Effect of Haematocrit

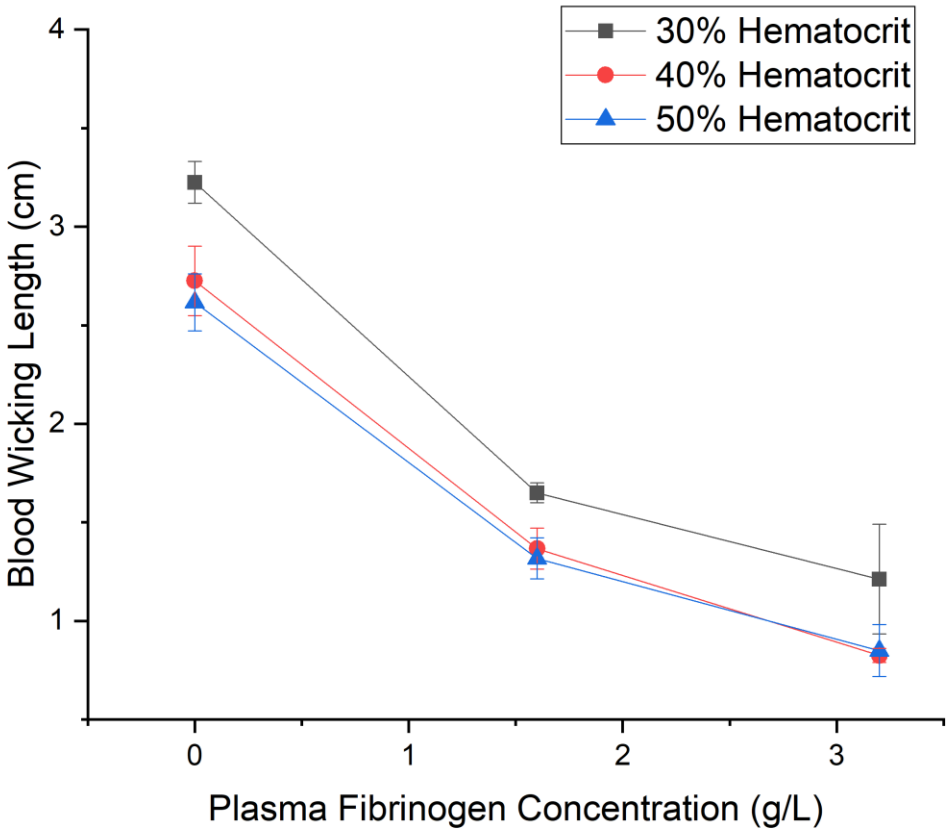


Figure 32: Testing of whole blood samples with different haematocrits. Whole blood reacted with thrombin for 3 min, and the blood wicking length was measured after 30 s of wicking using ImageJ. Each data point was tested four times, and the mean and standard deviation are reported.

Figure 32 investigates the effect of haematocrit interference on test results. The haematocrit refers to the volumetric percentage of red blood cells in a sample of whole blood. Normal adult populations typically have haematocrits ranging between 40 and 50%. Plasma samples of different fibrinogen concentrations were mixed with varying amounts of red blood cells to produce whole blood samples with different haematocrits. These were then tested. Whole blood samples varying between 40 and 50% haematocrits do not produce any significant differences. This suggests that the test can be used in the normal population without issue. However, at lower haematocrits, the entire curve shifts upward. As red blood cells have a significantly higher viscosity than plasma, the haematocrit of a blood sample dictates its viscosity. Therefore, blood samples with low haematocrits wick further due to their lower viscosity. A summary comparing volumetric RBC concentration, viscosity, and blood wicking length can be found in **Supplementary Figure 34**.

Figure 33 quantifies the effect of haematocrit in whole blood samples after dilution in PBS to determine whether dilution reduces or eliminates the viscosity-dependent effects seen in **Figure 32**. Plasma samples

of different fibrinogen concentrations were mixed with varying amounts of red blood cells to produce whole blood samples with different haematocrits. They were then diluted 1:7 with PBS and tested. Dilution significantly reduces the effect that haematocrit has on blood wicking length. Samples with a lower haematocrit do not display an upward shift in the curve (like in **Figure 32**). Hence, dilution can drop the viscosity of blood samples to levels that have a negligible effect on wicking. This is further verified in **Supplementary Figure 34**. As blood samples below 10% haematocrit have negligible differences in viscosity, they also have negligible effects on blood wicking length. Because a 1:7 dilution effectively produces a blood sample with a haematocrit of approximately 6%, it will also produce negligible differences in wicking regardless of what the original haematocrit of the blood sample is.

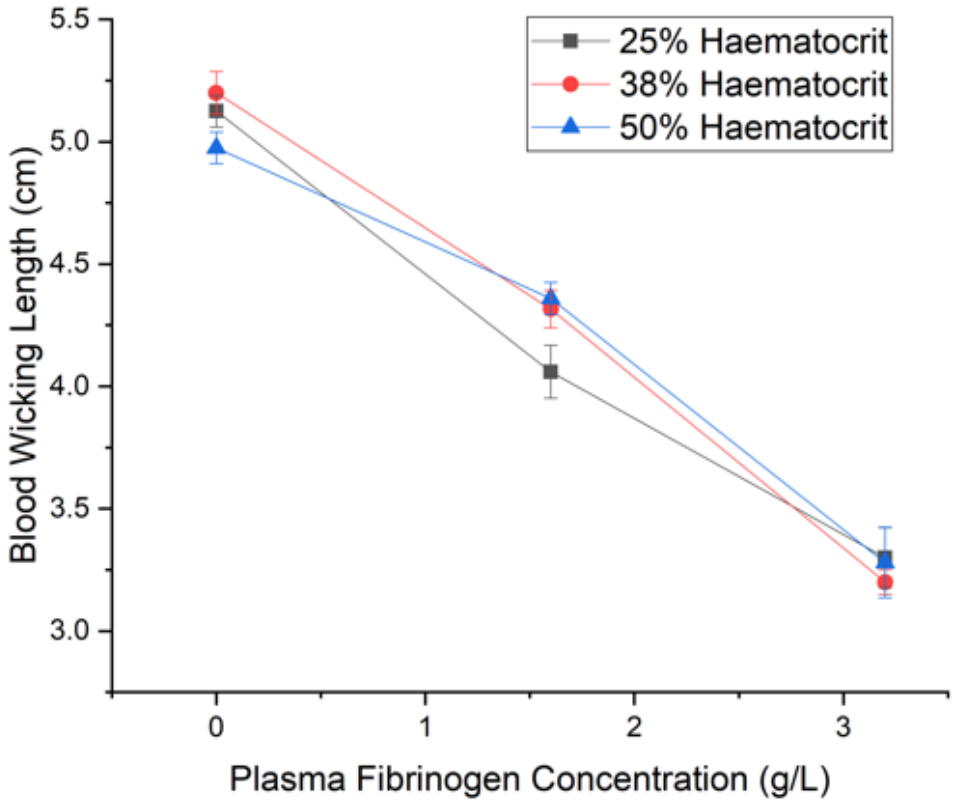


Figure 33. Testing of whole blood samples with different haematocrits after a 1:7 dilution with PBS. Diluted whole blood reacted with thrombin for 3 min, and the blood wicking length was measured after 30 s of wicking using ImageJ. Each data point was tested four times, and the mean and standard deviation are reported.

3.6. Effect of Thrombin Inhibitors

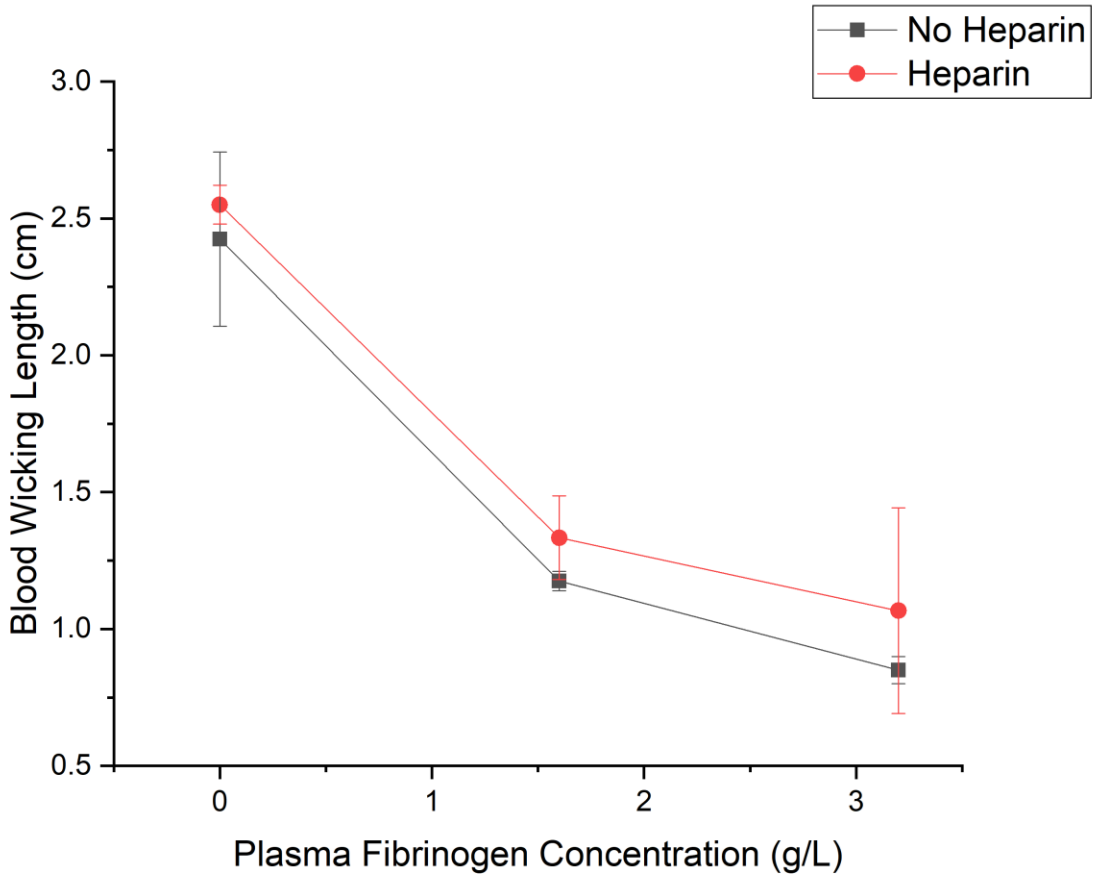


Figure 34: Testing of undiluted whole blood samples with and without 0.7 U/mL Heparin. Whole blood reacted with thrombin for 3 min and the blood wicking length was measured after 30 s of wicking using ImageJ. Each data point was tested 5 times and the mean and standard deviation are reported.

Figure 34 quantifies the effect of heparin in undiluted whole blood samples to determine if the test is affected by thrombin inhibitors. Undiluted whole blood samples of different fibrinogen concentrations were made to contain 0.7 U/mL heparin (levels similar to therapeutic levels found in patients recovering from cardiac surgery) and tested. These samples were compared to undiluted whole blood samples without heparin. Heparin causes the standard deviation to increase significantly. Some of the time, the test works as normal, but other times, blood leakage occurs from the clotted droplet and causes the fluid to wick much further than it should have. Yeromonahos *et al.* discovered that heparin causes the porosity of fibrin in clots to increase (242). Therefore, we hypothesise that the presence of heparin forms large and heterogeneous pores through which the blood can wick more easily. Therefore, the current test cannot be accurate with undiluted whole blood samples that contain thrombin inhibitors.

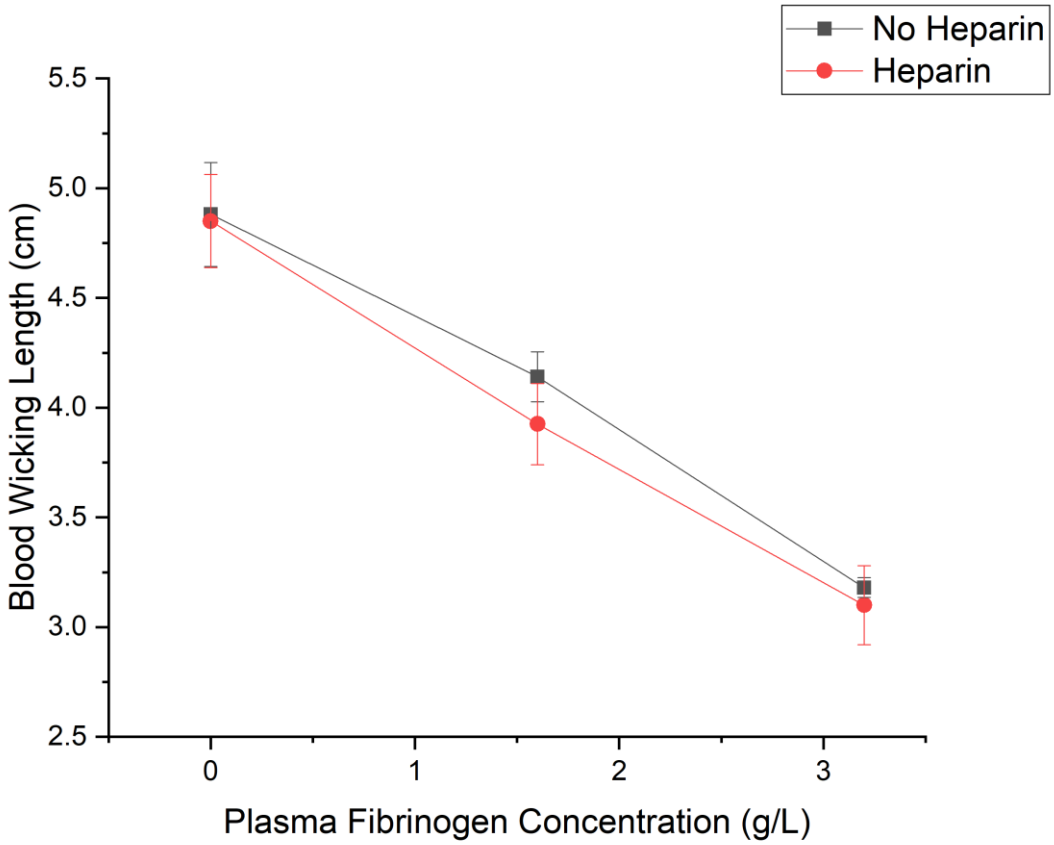


Figure 35: Testing of whole blood samples with and without 0.7 U/mL heparin before a 1:7 dilution with PBS. Whole blood reacted with thrombin for 3 min and the blood wicking length was measured after 30 s of wicking using ImageJ. Each data point was tested 5 times and the mean and standard deviation are reported.

Figure 35 quantifies the effect of heparin in whole blood samples diluted in PBS to determine whether dilution can reduce or eliminate the effects of thrombin inhibitors. Whole blood samples of different fibrinogen concentrations were prepared with 0.7 U/mL heparin, diluted 1:7 with PBS, and tested. They were compared to whole blood samples without heparin diluted equivalently. The effect of dilution is significant. Blood leakage no longer occurs, and the whole blood samples without heparin give the same result as the samples with heparin. Therefore, dilution can eliminate the effects that thrombin inhibitors have on the test.

3.7. Evaluation of Diagnostic

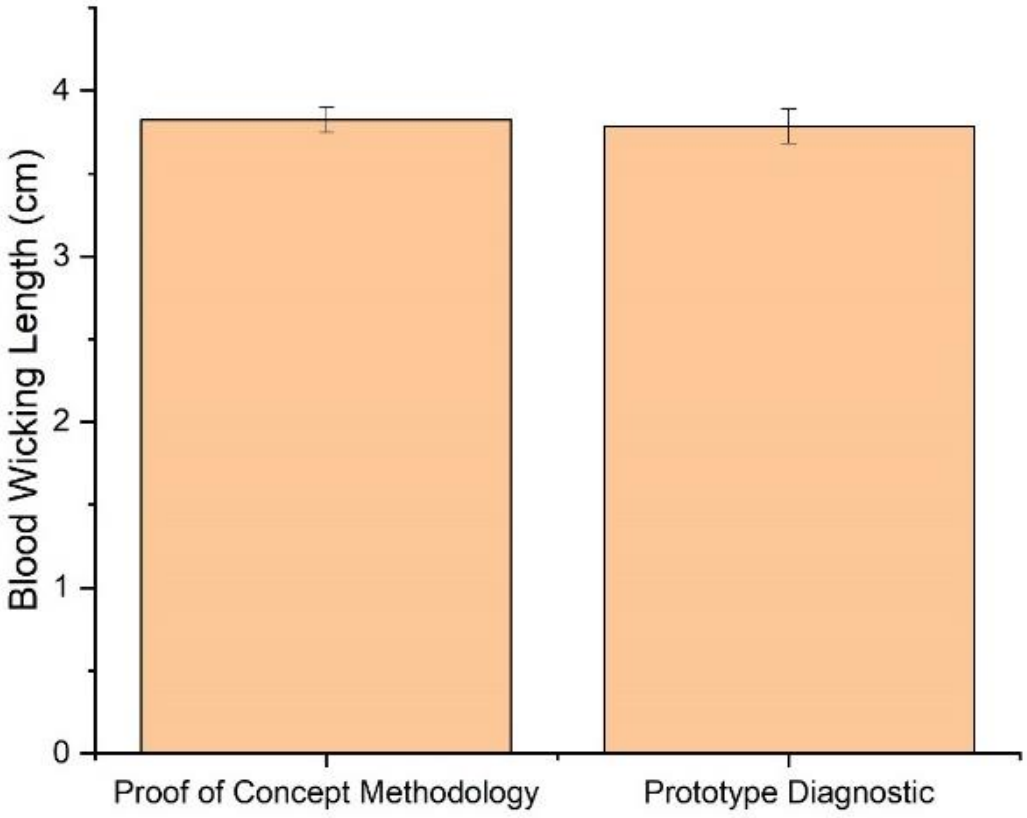


Figure 36. Comparison of a whole blood sample (diluted 1:7) using the proof-of-concept methodology (as described in Sections 2.2.6 and 2.2.7) and the prototype diagnostic (as described in Section 2.2.13). The paper strip in the diagnostic was released at a height of 1 cm above the glass slide. Each data point was tested four times, and the mean and standard deviation are reported.

Figure 36 compares the wicking and measurement of the prototype diagnostic methodology (i.e., Section 2.2.13) to the proof-of-concept methodology (i.e., Sections 2.2.6 and 2.2.7). This is to demonstrate that the test can be converted to a hand-held diagnostic that can be measured with the naked eye. An EDTA whole blood sample was taken and diluted 1:7 with PBS and tested. The dilution provided ample mixing and depositing time before clotting could occur (see **Supplementary Figure 37**). The paper strip was levitated 1 cm above the glass slide. This was to provide enough space to deposit the droplet and prevent accidental contact while the droplet was reacting. The effect of levitating the diagnostic's paper strip to different heights can be found in **Supplementary Figure 39**. To prevent bias, (1) the fibrinogen concentration of the EDTA sample was not known. (2) The prototype diagnostic was measured first, and the proof-of-concept methodology was measured afterward. Both actions prevented retrospective subjectivity from impacting the naked eye readings of the prototype diagnostic.

The assembled diagnostic wicks similarly to the proof-of-concept methodology. Both show blood wicking lengths of 3.8 cm and standard deviations of 0.1 cm. Hence, the prototype diagnostic can be used at the point of care without the need for other external equipment other than pipettes and reagents.

4. Discussion

We have developed the first hand-held fibrinogen diagnostic for whole blood. The test works by premixing whole blood with thrombin and depositing it on a solid surface as a droplet for 2−3 min. Afterward, a paper strip is carefully dropped on top of the droplet, and the fluid is allowed to wick for 30−60 s. The wicking length measured after this time correlates precisely to the fibrinogen concentration. As the test is completed in 3−4 min, it can allow fibrinogen replacement therapy to be rapidly implemented into hypofibrinogenemic patients, with the potential to save millions of lives each year.

Unlike our previous hand-held fibrinogen diagnostic, this one demonstrates sufficient sensitivity with whole blood. Therefore, it does not require the separation of blood cells and plasma before use. With undiluted whole blood, the test shows clear sensitivity to fibrinogen concentrations below 1.6 g/L and can work with a haematocrit in the range of 40−50%. This is suitable for the normal population. With diluted whole blood, the sensitivity of the test can not only be optimised but can also reduce or eliminate the effect of haematocrit and problematic thrombin inhibitors (such as heparin). Therefore, this paper achieved two aims. First, a rapid, hand-held fibrinogen diagnostic suitable for diagnosing hypofibrinogenemia in whole blood was demonstrated. Second, a means of reducing the effect of haematocrit and thrombin inhibitors was achieved.

4.2. Primary Factors

A full table describing the blood terms used in this section is provided in **Table S13**.

The primary factor modulating the blood wicking length is the permeability of the clotted droplet.

Although the test utilises a similar lateral flow readout to our previous diagnostic, the mechanism is completely different. When the blood droplet becomes clotted, two phases are formed. One is the clotted phase, which consists of the fibrin scaffold and red blood cells entrapped within it. The other is the unclotted phase, which contains the majority of the water, salt, macromolecules, and red blood cells that do not bind to the fibrin. Once the paper strip is placed on top of the clotted droplet, only the unclotted phase wicks down the strip. The clotted phase remains behind. This is shown in **Supplementary Figure 30**, which microscopically depicts the distribution of fibrin through a paper strip when the test is performed on clotted droplets of plasma.

The formation of the clotted phase perturbs the movement of the unclotted phase down the strip. If the clotted phase is treated as a soft, porous matrix for the unclotted phase to travel through, Darcy's law can be used to describe it as such:

$$q = -\frac{k}{\mu}\frac{\Delta P}{L} \quad \textbf{(3)}$$

where q is the flux of the unclotted phase, μ is the viscosity of the unclotted phase, k is the permeability of the clotted phase, ΔP is the capillary pressure on the unclotted phase and L relates to the diameter of the clotted droplet. The higher the q is, the further the unclotted phase wicks down the paper strip.

Clotted phases with entrapped RBCs perturb the movement of the unclotted phase significantly more than clotted phases without RBCs. This is shown in **Figure 31**, where whole blood samples produce the same levels of wicking as plasma samples with only an eighth of the total fibrinogen concentration. The whole blood samples were diluted in PBS to produce similar background effects as plasma samples.

Therefore, the fibrin-RBC interactions within the clotted phase are responsible for the sensitivity of the test.

The incorporation of red blood cells into the clotted phase causes a major decrease in its permeability. Varin *et al.* concluded that the permeability of whole blood clots was 6.7 times higher than that from its corresponding plasma clot (243). This factor correlates very well with the result shown in **Figure 31**. Therefore, we hypothesise that this steep decrease in clotted phase permeability is responsible for the subsequent lowering of the unclotted phase flux and shorter blood wicking lengths.

Van Gelder *et al.* reported that RBCs decrease the permeability of the fibrin scaffold by becoming entrapped within it. This in turn increases the volume occupancy and surface drag within the network (244). Therefore, we suggest that the reason that the test is sensitive to fibrinogen concentration is because fibrinogen is responsible for the mass of the fibrin scaffold formed and the resulting quantity of entrapped RBCs. The varying quantity of fibrin-entrapped RBCs at different fibrinogen concentrations modifies the clotted phase's permeability and, ultimately, the unclotted phase's wicking length.

4.3. Secondary Factors

Other factors modulating the blood wicking length include the stiffness of the clotted droplet and the viscosity of the unclotted phase.

The stiffness of the clotted droplet dictates the contact area between the droplet and the paper strip. As the fibrinogen concentration in the droplet decreases, so does the eventual stiffness of the clotted droplet. When the fibrinogen concentration becomes very low (i.e., <0.4 g/L), the clotted droplet compresses underneath the weight of the paper strip. This compression increases the contact area between the clotted droplet and the paper strip and causes further wicking.

The relationship between the droplet's fibrinogen concentration and the interfacial contact area is shown in **Supplementary Figures 35** and **36**.

As shown in Figure 32, there is a haematocrit-dependent effect on wicking in fibrinogen-free whole blood samples. Samples with 30% haematocrit wick further than samples with 40 or 50% haematocrit. It is well known that the haematocrit dictates the viscosity of a blood sample (245). This in turn controls the blood wicking length. **Supplementary Figure 34** demonstrates the relationship between haematocrit, blood sample viscosity, and blood wicking length. There is an exponential relationship between haematocrit and viscosity. When the haematocrit rises above 10%, the effect on blood sample viscosity becomes significant and affects the test.

The contact angle at which the droplet interacts with the solid surface does not affect the test's sensitivity. This is despite the vast difference in droplet contact angle between hydrophilic and hydrophobic surfaces (**Supplementary Figures 31** and **32**). After 30s of wicking, the hydrophilic surface causes blood to move slightly further down the strip than the hydrophobic surface (**Supplementary Figures 33 – top graph**). We hypothesise this to be due to the unbeaded droplet being able to wet the paper strip more rapidly than the beaded droplet. However, by 60 s, that initial wetting effect becomes nullified and both surfaces display similar blood wicking distances (**Supplementary Figures 33 – bottom graph**).

This means that the test can work with a variety of solid surface materials and droplet contact angles.

4.4. Perspective

We looked at several factors that impact the performance of the test including reaction time, wicking time, haematocrit, predilution, and thrombin inhibitors. The test is ideally suited to longer reaction times (>2 min), shorter wicking times (<1 min), and haematocrits of the reference range (40−50%). Furthermore, large dilution factors (4 to 8 times) optimise the efficacy and minimise the effect of both haematocrit and thrombin inhibitors.

Both the reaction time (**Supplementary Figure 29**) and blood wicking time (**Figure 29**) determine the total time the test needs until completion. Overall, the test takes 3−4 min. This is significantly shorter than other assays, which typically take 10−20 min to obtain a fibrinogen concentration reading (153, 154).

Since droplets with lower fibrinogen concentrations make the test easier for the end-user to perform (see **Supplementary Figure 37**), we observed the effect of prediluting whole blood samples in PBS (**Figure 30**). Not only does diluting whole blood samples increase the efficacy of the test, but it also linearises the curve. **Supplementary Figures 35** and **36** demonstrate the mechanism behind this linearization. The optimal sensitivity occurs when there is a 4-to 8-fold dilution of whole blood. However, dilutions above this cause the sensitivity to diminish. This is most likely due to the continual reduction in the mass of fibrin formed after clotting and hence its subsequent effects on blood wicking.

In this study, we prediluted whole blood samples to ensure that the conditions in **Figure 30** were properly controlled. However, the effect of dilution can be mimicked by changing the ratio of thrombin solution and whole blood that get mixed together − something more practical in real-life emergency situations.

RBCs have a major impact on the test. Therefore, their concentrations in whole blood samples were tested to quantify the impact that they had on the test (**Figures 32** and **33**).

The blood wicking length remains relatively unchanged for haematocrits between 40 and 50% in undiluted whole blood (Figure 32). However, when the haematocrit falls below this range, the entire curve shifts upward. This is problematic in instances like trauma where the whole blood haematocrit can drop to 25−35% due to intravenous fluid admission and subsequent haemodilution (246, 247).

On the contrary, the same upward curve shift is not observed when whole blood samples become diluted in PBS (**Figure 33**). The curve remains similar for haematocrits between 25 and 50%, making it suitable for testing haemodiluted blood. **Supplementary Figure 34** deciphers the relationship between haematocrit and blood sample viscosity. When the haematocrit of a blood sample drops below 10%, any change in viscosity becomes negligible. Hence, the blood wicking length does not change. This means that diluting whole blood samples below this range causes the effects of haematocrit to become insignificant on the test.

In our previous hand-held diagnostic, we tested a wide variety of blood conditions. We found that it is not impacted by common conditions such as acidosis, blood alcohol, hypertriglyceridemia, haemolysis, and warfarin administration (200). However, it is impacted by the presence of thrombin inhibitors. Because of this, we observed the effect of heparin in this test (**Figures 34** and **35**). Heparin is a well-known thrombin inhibitor found in the blood. It is used therapeutically directly after cardiac surgery at concentrations up to 0.7 U/mL. Therefore, we used that concentration in this study. Heparin causes major increases in variability for undiluted blood (**Figure 34**). This is most likely due to an increase in the porosity of the clot (242). However, heparin shows no effect after the blood becomes diluted (**Figure 35**). This is because the

effect of dilution reduces the amount of heparin that can interact with thrombin and fibrinogen in the test.

Aside from heparin, there are other thrombin inhibitors in the blood that we did not test in this study. These include direct oral anticoagulants and fibrinogen/fibrin degradation products (248, 249). Other fibrinogen assays that use thrombin as an active reagent often dilute plasma 10- to 30-fold in buffer solution to neutralise any thrombin inhibitors (98). Given that the dilution factor used in this test is similar, we hypothesise that it can nullify the effect of other thrombin inhibitors (aside from heparin) as well.

We also tested the effect of temperature in our previous diagnostic. We found that the test is unaffected by temperatures ranging from 7 to 36 °C (200). However, at extreme temperatures (i.e., 44 °C), the test wicks further than normal and hence needs a means of protection. This test and the previous diagnostic both use the same lateral flow mechanism to measure the blood fibrinogen concentration. Therefore, we expect this test to show the same effects with temperature.

Finally, we developed a prototype hand-held diagnostic and compared it to the proof-of-concept methodology (**Figure 36**). The proof-of-concept methodology was performed on tabletop using both hands and an iPhone to record and analyse the blood wicking length. However, the hand-held diagnostic was held in one hand for the entirety of the test with the other hand doing the assaying work. Furthermore, the blood wicking length was determined by the naked eye. Despite the differences in methodology, the hand-held diagnostic shows near identical readings to the proof-of-concept methodology. Not only can the test be facilitated entirely within the fingertips, but the wicking (after 30 s) is slow enough to be accurately measured by eye. This means that the test can be easily transported to the point of care and give readings that do not need external equipment to validate.

4.5. Limitations

The test must pre-clot the droplet before a paper strip can be placed on top of it. Unlike other paper-based diagnostics that simply react with biological fluids on bioactive paper, it is not possible to do the same with this test. This means that the whole blood sample must be uniformly premixed and clotted with thrombin solution to achieve the sensitivity the test requires.

Additionally, tests where thrombin and blood have not been uniformly mixed led to instances of blood leakage from the clotted droplet. We hypothesise this to be due to the formation of an inhomogeneous fibrin clot with gaps in it that the unclotted phase can easily travel through. Therefore, we ensured that the blood sample and thrombin were suctioned up and down at least 10 times before depositing.

We did not readily have access to fresh (i.e., <1 day old) blood from donors to work with. The closest to this were ethylenediaminetetraacetic acid (EDTA) anticoagulated whole blood samples from the Australian Red Cross that were delivered to us at least 1 week post-collection. Since we were not sure how much the samples had degraded by then, we instead chose to make whole blood samples artificially. This involved taking red blood cells from these samples, washing them with PBS (to get rid of debris) and recombining them with Quality Control (QC) plasma. QC plasma is a good representation of actual plasma as there are only three subtle differences. First, the QC plasma is pooled from 20 donors to normalise levels of all proteins in the blood. Second, the QC plasma is stabilised in small amounts of HEPES buffer to keep the pH at 7.4. Third, the QC plasma is citrated to prevent coagulation. As citrate works to prevent coagulation by chelating calcium ions in blood, the test can also work with other anticoagulants that do the same (such as EDTA or oxalates).

In this study, we briefly observed the effect of platelet activity (**Supplementary Figure 41**). When coagulation is induced, thrombin activates platelets by cleaving protease-activated receptors on their membrane (250). Three events occur afterward. The first is the rapid polymerization of actin filaments within the platelets' cytoskeleton (251). The second is the binding of platelets (through their IIb/IIIa receptors) to fibrin strands within the fibrin network. The third is the contraction (through the polymerised actin filaments within the platelets) of the fibrin network (252). This causes the entrapped RBCs to compress and further decrease the clot's permeability to blood flow (253).

We found that platelets do not exert an effect when donated blood is collected in citrated tubes. However, the extent to which the citrate inhibits the platelets is unknown. Therefore, further work with uncoagulated whole blood is needed to verify the effect of platelets.

However, irrespective of the effect that activated platelets may cause on the test, they can be neutralised with platelet inhibitors. VHAs routinely use cytochalasin D and abciximab to inhibit platelets (150). Cytochalasin D works by inhibiting the polymerization of actin after thrombin stimulation (254). Abciximab binds to the IIb/IIIa receptors and prevents platelets from binding to the fibrin strands (255). Lang *et al.* showed that the use of both inhibitors can eliminate all platelet activity (256).

Therefore, we strongly hypothesise that the use of these inhibitors is sufficient to make the test insensitive to platelet activity.

4.6. Future Work

Whilst we successfully developed a prototype of the diagnostic (**Figure 36**), further steps are still required.

The first is by improving the ergonomics of the test. Currently, the diagnostic is stabilised by holding it still in one hand for 3 minutes as the droplet clots. However, this holding time can be decreased by increasing the concentration of thrombin solution used (and hence allowing for a faster reaction). Furthermore, scaffolding to assist in the both the deposition/positioning of the droplet and positioning/landing of the paper strip can also help.

The second is by implementing a means to stop the blood from moving further down the strip after the 30 second wicking time. A simple way could be by cutting the paper strip at certain distances. For example, **Figure 35** illustrates that a blood sample with a fibrinogen concentration of 1.5 g/L will wick 4.3 cm down the strip after 30 seconds. Therefore, by cutting the strip at that position then, the end user can clearly tell wherever the patient has above or below 1.5 g/L fibrinogen.

The third is by performing large scale clinical trials. This includes testing both healthy and coagulopathic whole blood samples and comparing them to an established clinical assay (such as a Clauss Assay).

The fourth is by scaling up economically with proper quality controls in place. This includes ensuring: 1) the paper structure is manufactured consistently enough to allow for reproducible results, 2) the thrombin produced has a high enough activity so the droplet can clot completely in a timely manner.

5. Conclusion

We have developed a hand-held diagnostic suitable for determining the fibrinogen concentration in whole blood. The diagnostic subtracts the requirement of needing to separate blood cells from plasma. Results can be achieved in only 3−4 min to allow for rapid fibrinogen replacement therapy. The test works by premixing a blood sample with thrombin solution, depositing it as a droplet on a solid surface, allowing the droplet to clot, and applying a paper strip on top. The distance that the blood wicks down the strip precisely correlates to the fibrinogen concentration. The overall permeability of the clotted droplet is most likely responsible for this phenomenon. Lower fibrinogen concentrations cause the clotted droplet to have higher permeabilities and allows for more wicking. The test can determine wherever undiluted whole blood samples of normal haematocrit (40−50%) have plasma fibrinogen concentrations below 1.6 g/L. However, the test is dramatically improved by diluting whole blood samples. This is because dilution not only optimises the sensitivity of the test but also eliminates the effect of any thrombin inhibitors and abnormal haematocrits that may be present. The test can rapidly diagnose hypofibrinogenemia in critically bleeding patients at the site of injury and save their lives.

Supplementary Data

4.1. Carstair's Stain:

REAGENTS

i) Bouin's Fixative

(1) 250 mL of Formalin (37%)

(2) 750 mL of saturated aqueous picric acid

(3) 50 mL of glacial Acetic acid

ii) Weigert's Iron Haematoxylin

(1) Solution A

(a) 5 g of Haematoxylin powder

(b) 500 mL of absolute Ethanol

(2) Solution B

(a) Add 6 grams of ferric chloride to 520mL of distilled water, and then add 5mL of concentrated hydrochloric acid. Stir continuously until the ferric chloride is completely dissolved.

(3) Mix solution A and B in equal parts, does not keep beyond a day

iii) Picric Acid – Orange G

(1) 200 ml isopropanol

(2) 2.8 g saturated picric acid

(3) 20 ml saturated aqueous picric acid

(4) 0.2 g Orange G

iv) Ponceau-fuschin

(1) 1 ml 100% acetic acid

(2) 0.5 g acid fuschin

(3) 0.5 g Ponceau 2R

v) Aniline blue staining solution

(1) 25 grams of Aniline Blue

(2) 25 mL of glacial Acetic acid

(3) 1,000 mL of distilled water

vi) 1% Phosphotungstic acid

(1) 10 grams of phosphotungstic acid

(2) 1,000 mL of distilled water

PROCEDURE

1. Bring paper strips to water.

2. Place paper strips in Bouin's fixative either overnight at room temperature or 1 h at 60°C.

3. Wash the paper strips in running water.

4. Stain paper strips in Weigert's haematoxylin for 15 min.

5. Differentiate briefly in acid alcohol.

6. Wash in running tap water for 10 min.

7. Stain paper strips in Picric acid-orange G solution for 45 min.

8. Wash in running tap water.

9. Stain paper strips in Ponceau-fuschin solution for 5 min.

10. Wash in running tap water.

11. Apply 1% tungstophosphoric acid for 15 min (not required to wash sections after this step).

12. Stain with Aniline blue staining solution for 20 min.

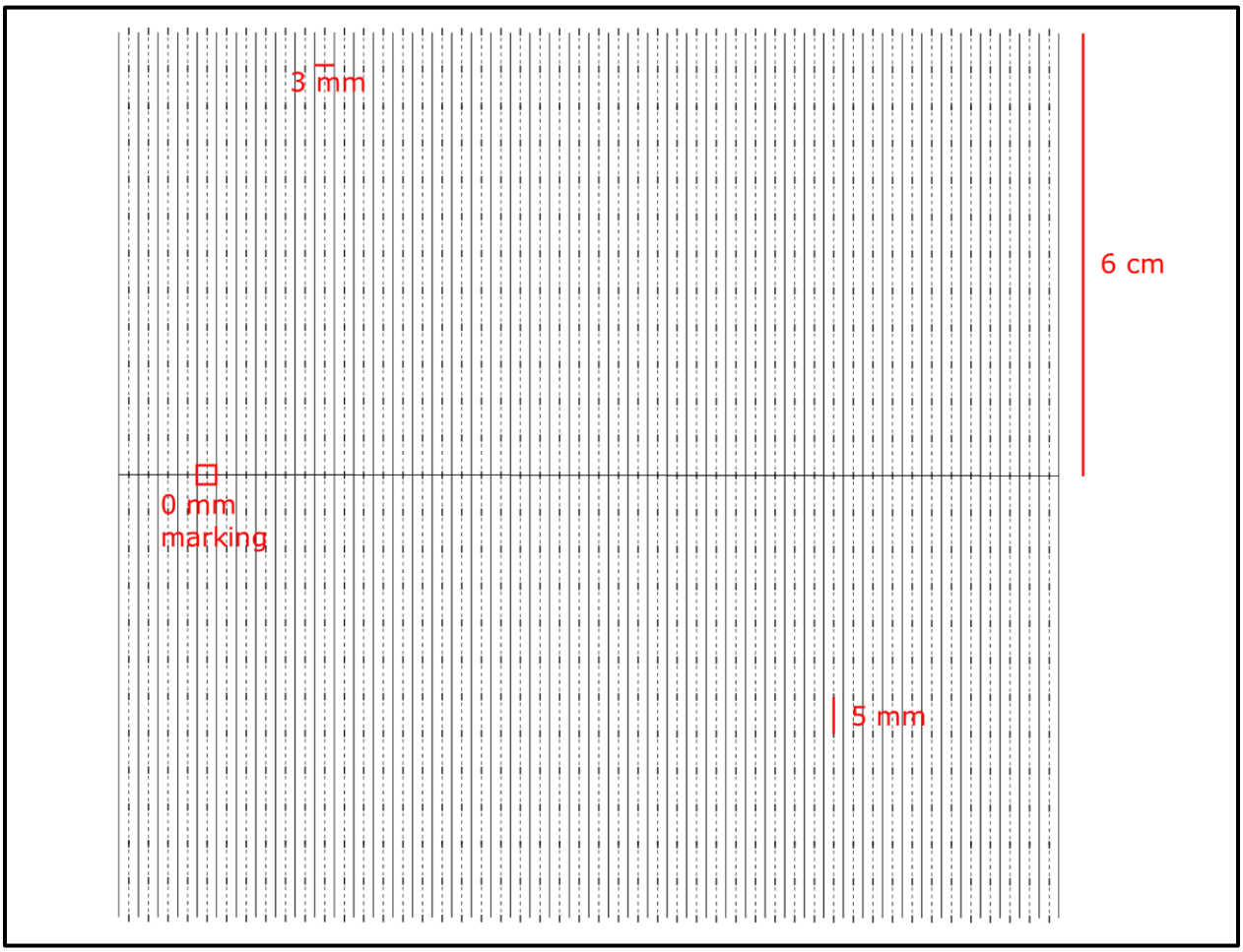
13. Wash in running tap water.

14. Dehydrate through 3 changes of ethanol.

15. Clear in 3 changes of xylene.

16. Mount with DPX.

4.2. Paper Strip Cut-Out:



Supplementary Figure 24: Cut-out of Paper Strips showing dimensions, markings and locations. The paper strip cut-out stencil was drawn on CorelDRAW X6 and a 60 Watt Epilog Helix was used to laser cut the paper.

After laser cutting the paper with the stencil used in **Supplementary Figure 24**, paper strips were ripped out individually with tweezers and experimented with.

The 0 mm marking refers to the location where the paper strip was dropped on top of the clotted droplet. The 0 mm marking aligned with the edge of the clotted droplet covering it in whole.

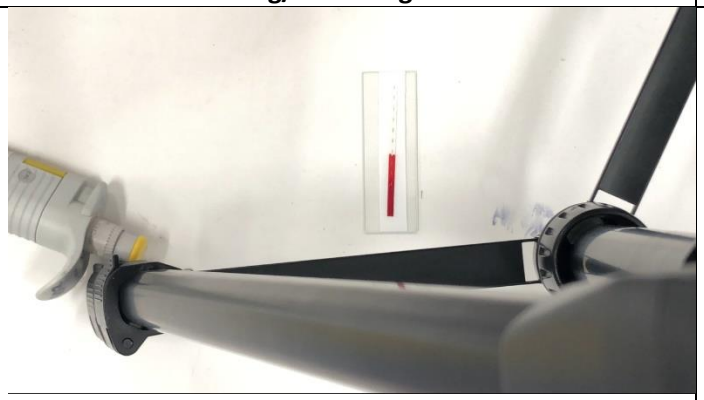
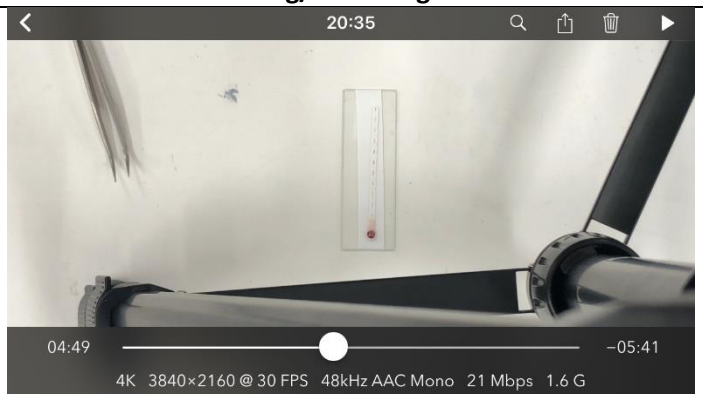
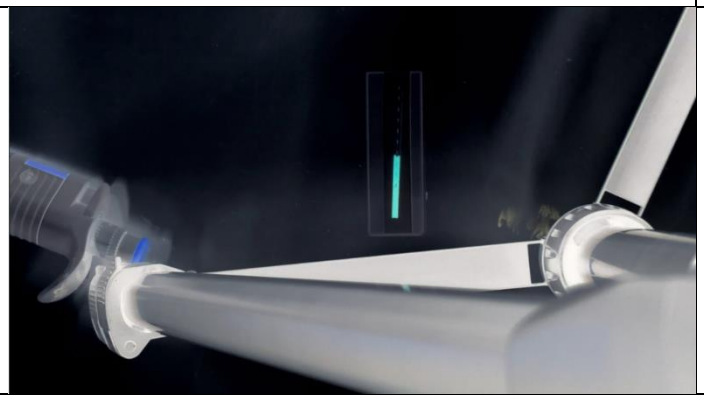
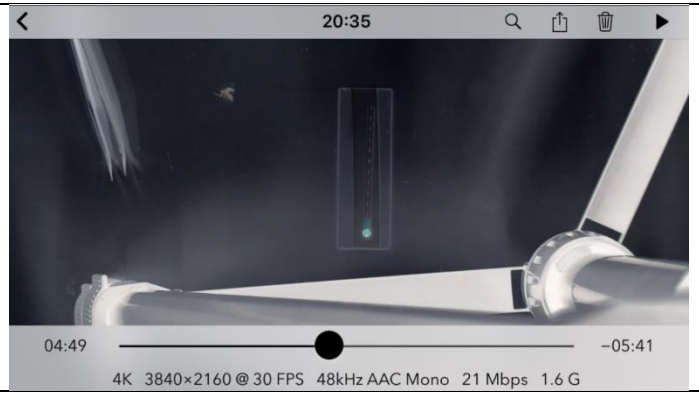
4.3. Manual Camera Setup:

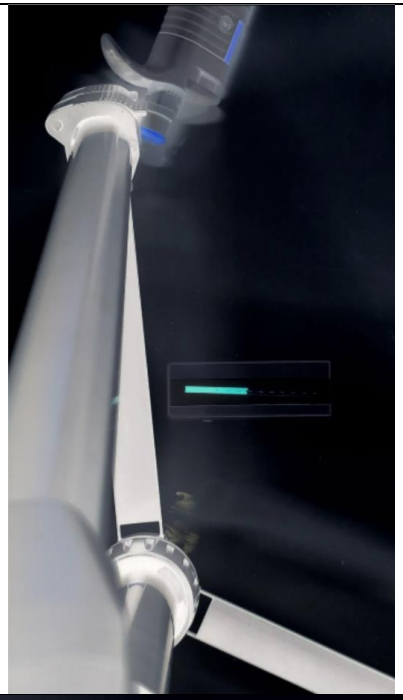
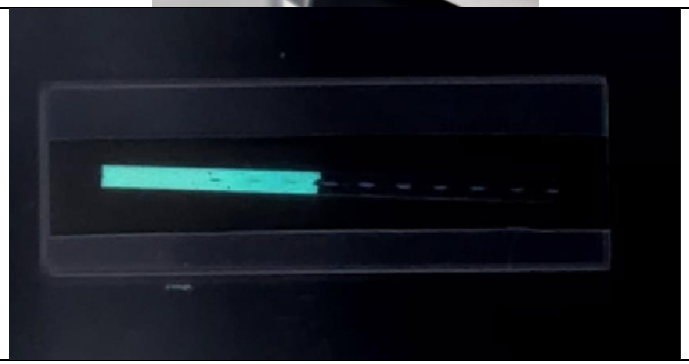
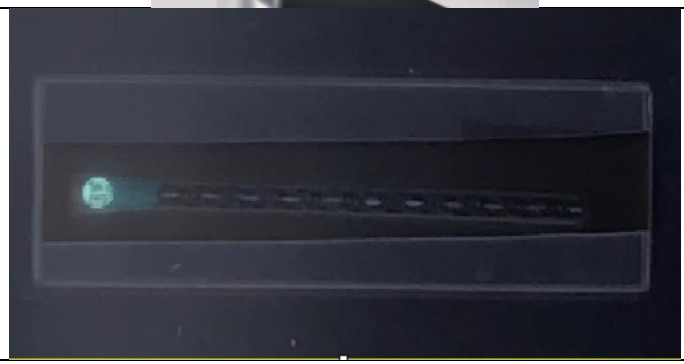
In order to capture videos which manually focused on the strip's markings, ProMovie was used on an iPhone 6. The zoom was adjusted to 3.00 X to get maximum zoomage and the focus was changed until the markings on the paper strips could be clearly seen (as shown in **Supplementary Figure 25**).

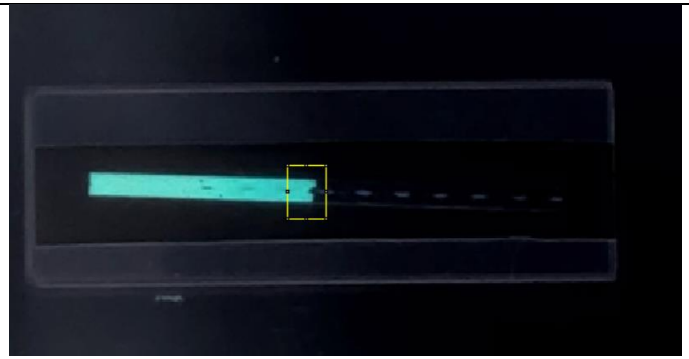
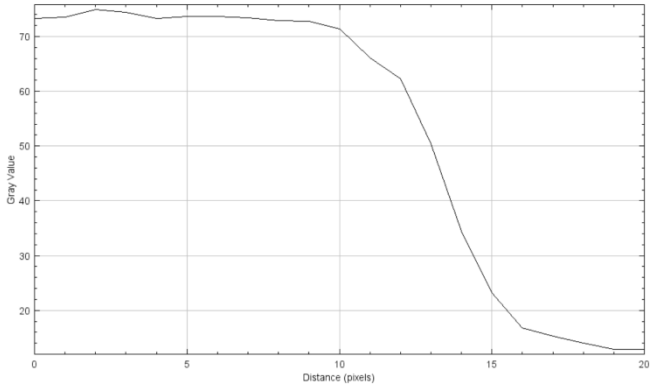
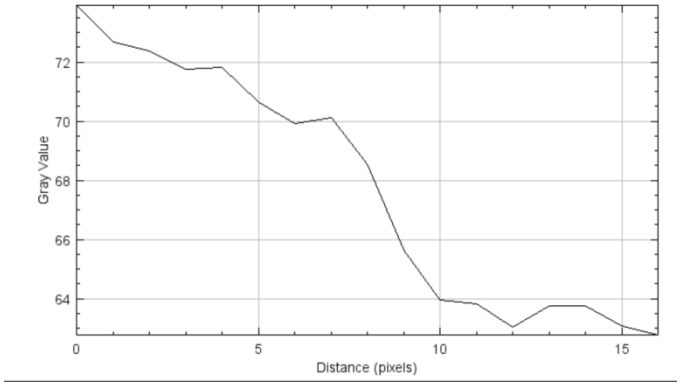
The videos recorded could then be screenshotted and used for analysis on ImageJ.

Supplementary Figure 25: Settings on ProMovie used to manually focus and zoom on the paper strip's markings.

4.4. Measuring Process:

	0 g/L Fibrinogen	3.2 g/L Fibrinogen
1. Take Screenshot 30 seconds after blue dye is applied to strip 2. Download image on computer and open on Image J		
3. Invert		

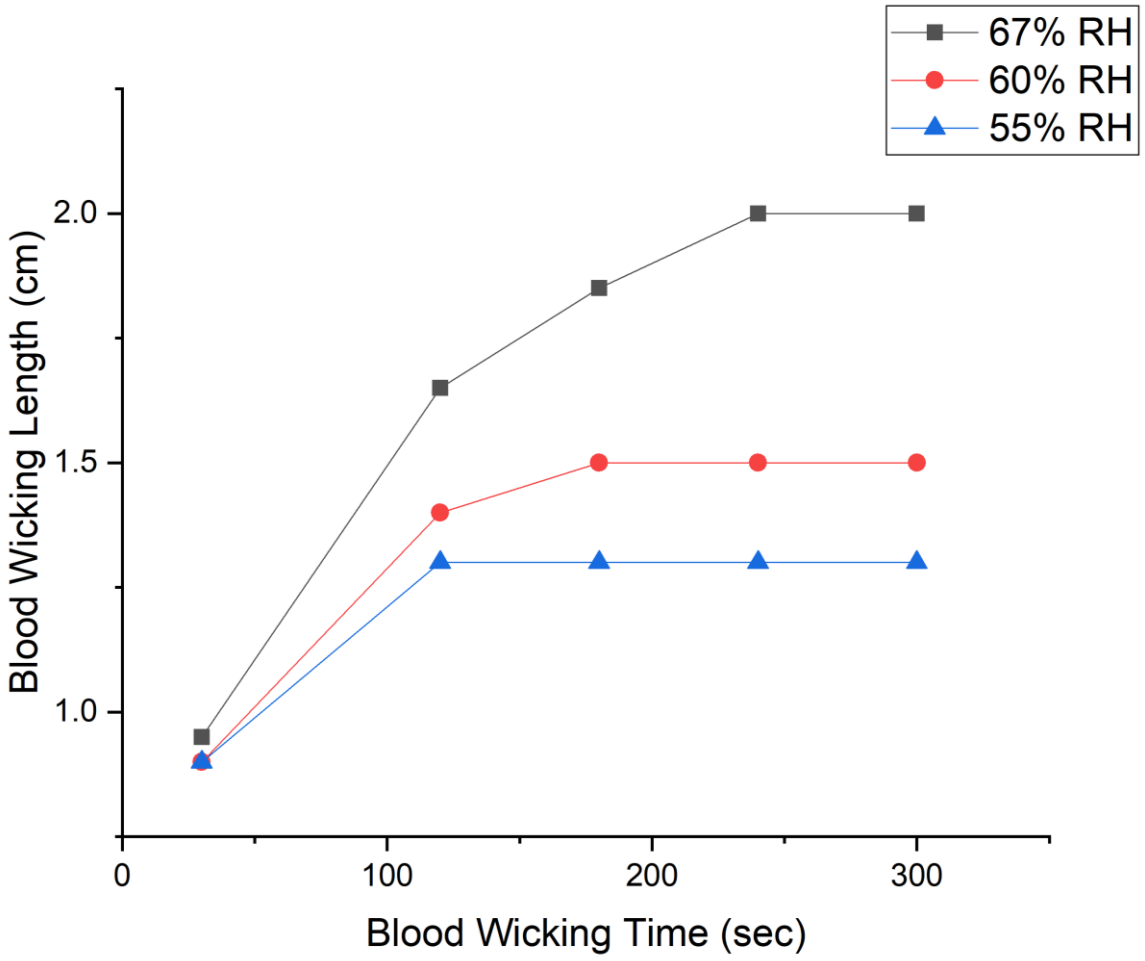
4. Flip to this orientation		
5. Zoom into strip		
6. Draw box between the 0.5 cm markings the wicking front		

midpoint is located between. **7. Take note of the actual 0.5 cm increments used down the strip**	 Wicking front midpoint is located between 2.5 – 3 cm.	 Wicking front midpoint is located between 0.5 – 1 cm.
8. Plot Profile of box **9. Take note of the number of horizontal pixels the 0.5 cm increment covers.**	 **0.5 cm = 20 pixels**	 **0.5 cm = 16 pixels**
10. Identify area of steepest decrease. This correlates with the wicking front	LHS: x - 12 pixels; y – 62 Gray Value RHS x – 16 pixels; y – 17 Gray Value Median gray value: y – 44.5	LHS: x - 7 pixels; y – 70. Gray Value RHS x – 10 pixels; y – 64 Gray Value Median gray value: y - 67

11. Find closest corresponding x-point of median gray value. This is the midpoint of the wicking front.	X midpoint = 13 pixels	X midpoint = 9 pixels
12. Convert pixels into cm	$Length = 2.5\ cm + 0.5\ cm \times \frac{13\ pixels}{20\ pixels}$ $Length = 2.83\ cm$	$Length = 0.5\ cm + 0.5\ cm \times \frac{9\ pixels}{16\ pixels}$ $Length = 0.78\ cm$

Supplementary Figure 26: Step by step process of calculating wicking length on Image J.

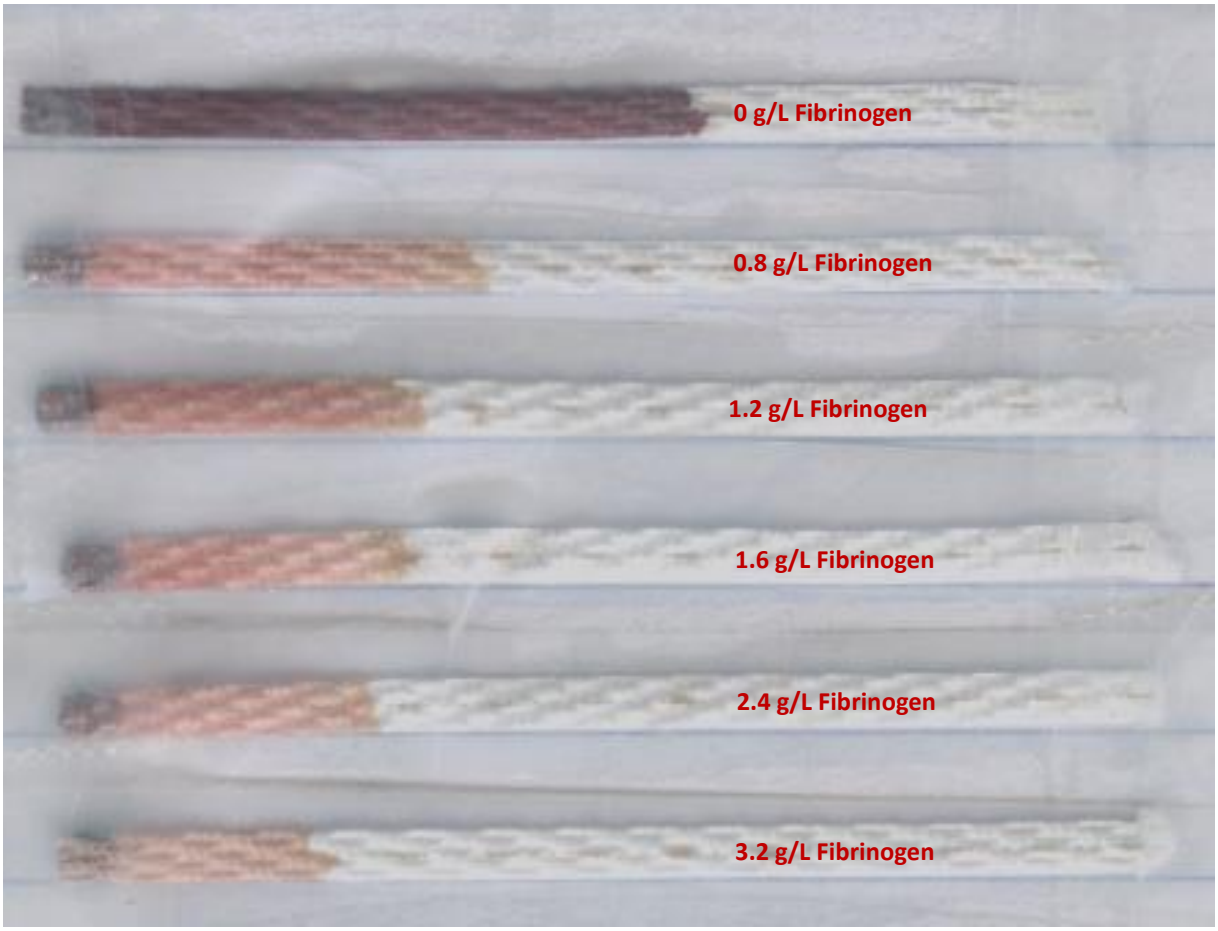
4.5. Effect of Humidity:



Supplementary Figure 27: Testing of 3.2 g/L whole blood sample at different relative humidities. Whole blood reacted with thrombin for 3 min and blood wicking length was measured using ImageJ. Each data point was tested 1 time.

Supplementary Figure 27 demonstrates a single whole blood sample wicking at different relative humidities to observe the effects of drying on wicking blood. At smaller wicking times, the blood wicking lengths are quite similar. However, at longer wicking times the effect of drying becomes more pronounced. Whole blood droplets at low relative humidities dry out faster than at high relative humidities. Hence, they stop wicking at both shorter wicking times and lengths. Therefore, to minimise the effects of humidity, the test is more suitable to wicking for short times (ie. 30 s) than long times.

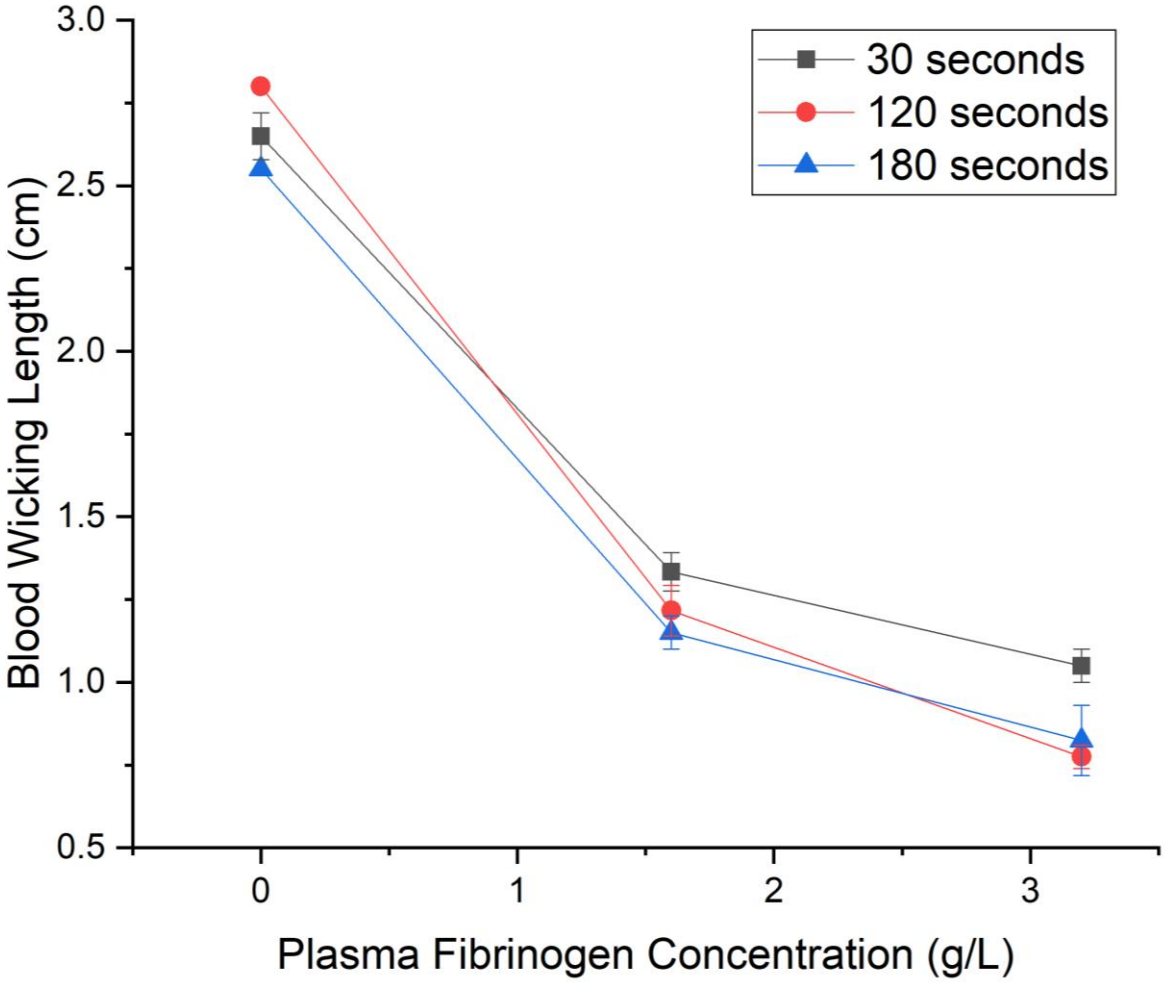
4.6. Raw Results After Drying:



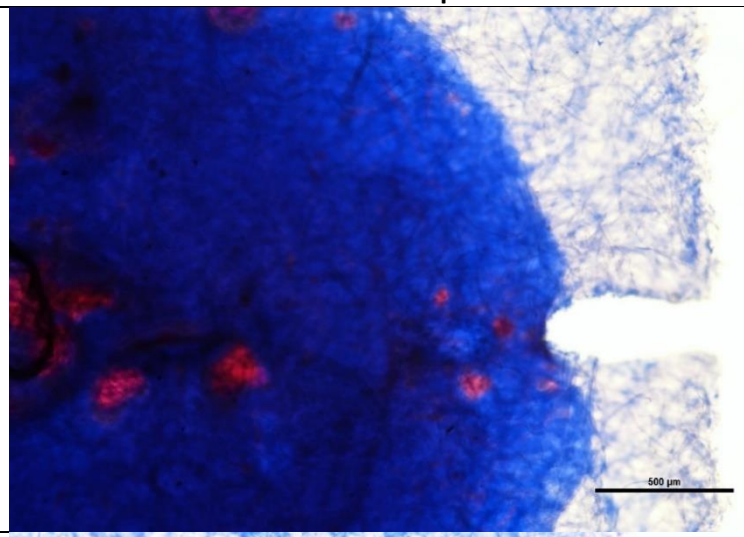
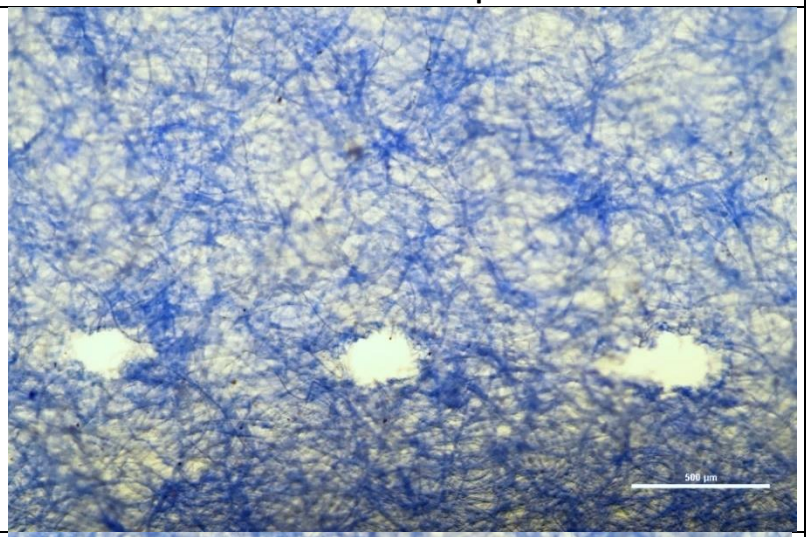
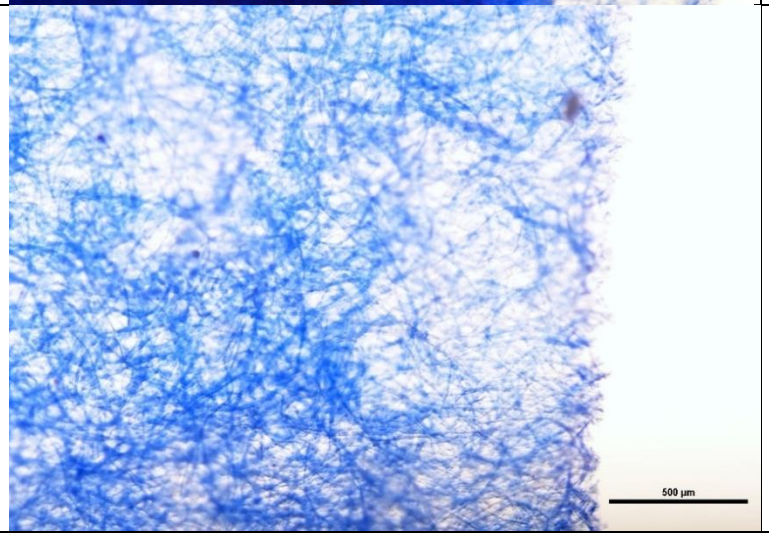
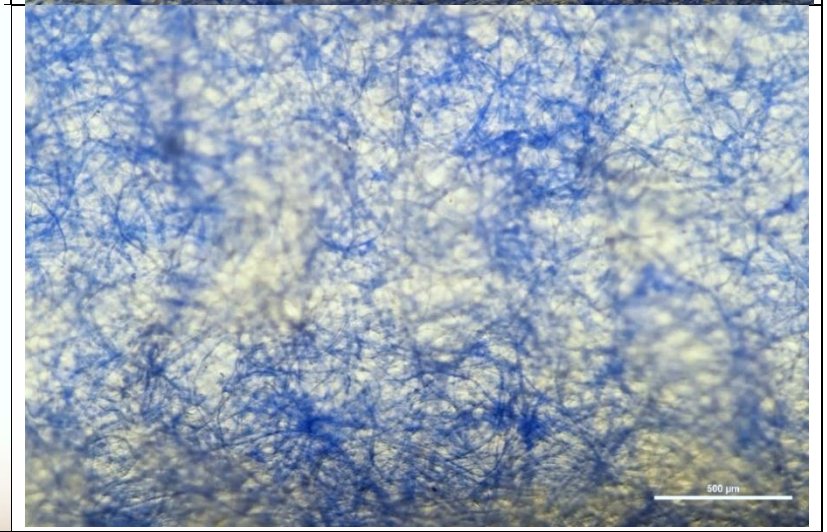
Supplementary Figure 28: Raw results for the wicking of clotted droplets of different fibrinogen concentrations. Whole blood samples of different fibrinogen concentrations were tested. Paper strips were collected after drying and scanned. Lower fibrinogen concentrations cause further blood wicking to occur.

4.7. Effect of Reaction Time:

Supplementary Figure 29 investigates the effect of reaction time to observe how long it takes for the reaction to complete. Whole blood droplets reacted with thrombin for different periods of time before a paper strip was placed on top of them. The longer the reaction time the greater the wicking length is perturbed. At 30 s, a plasma fibrinogen concentration vs blood wicking length curve is already developing. However, it is not developed until at least 120 s. At 180 s, no more significant change occurs in the curve. This indicates that the reaction is completed in between 120 s and 180 s.



Supplementary Figure 29: Testing of whole blood samples under different reaction times. Blood wicking length was measured after 30 s of wicking using ImageJ. Each data point was tested 3 times and the mean and standard deviation are reported.

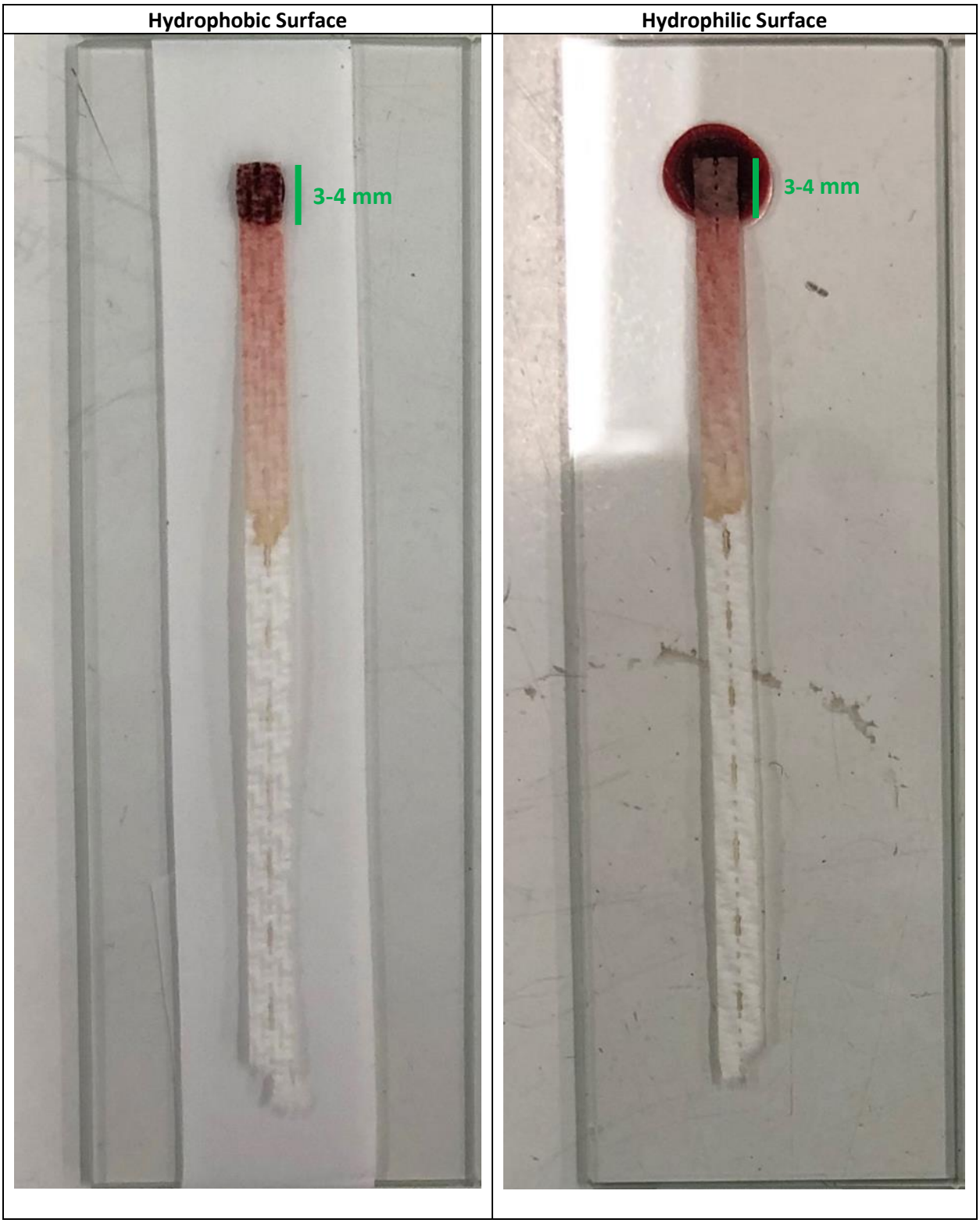
	Start of Strip	Middle of Strip
Positive Control **(Plasma with Fibrinogen)**		
Negative Control **(Plasma without Fibrinogen)**		

Supplementary Figure 30: Histological staining and imaging of plasma samples on paper with (positive control) and without (negative control) fibrinogen. Plasma droplets were deposited on a PTFE coated glass slide, mixed with thrombin and reacted for 3 min, before a paper strip was placed on top of the droplet. After drying, the paper strips underwent a Carstairs stain to visualise the location of fibrin throughout the paper. Imaging was performed on a Nikon Eclipse Ni-E Upright Microscope at (5X zoom). Red = Fibrin; Blue = Cellulose.

4.8. Fibrin Staining:

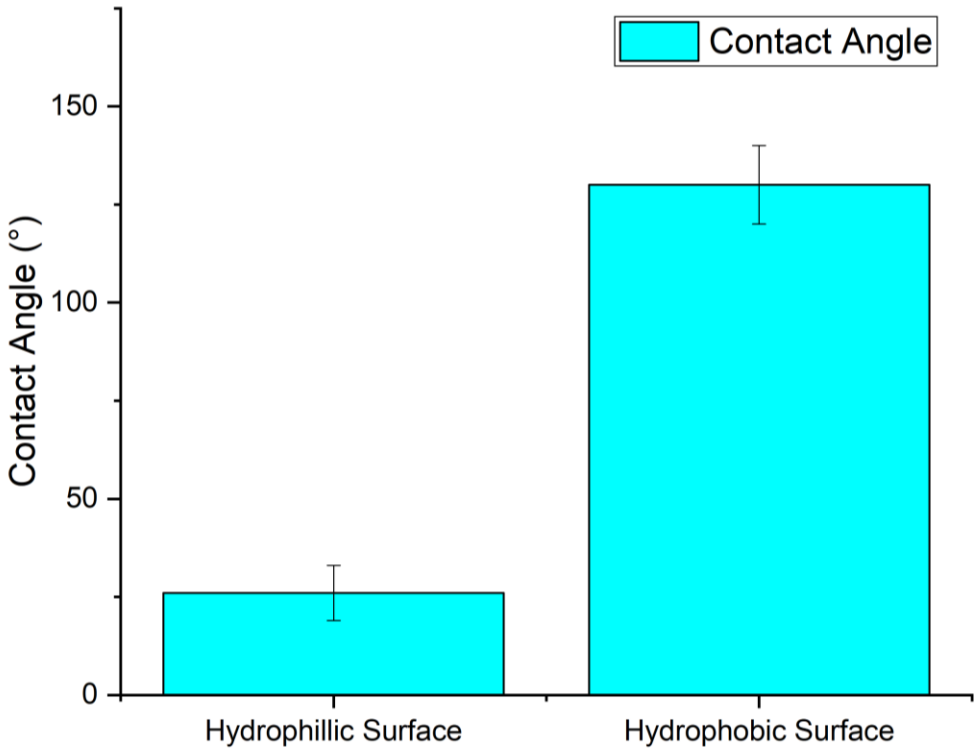
Supplementary Figure 30 illustrates the location of fibrin along the strip after drying to determine whether the fibrin moves with the wicking fluid as one phase or remains in the droplet during wicking. Both a positive control (plasma with fibrinogen) and a negative control (plasma without fibrinogen) were imaged. A Carstair's stain was performed to visualise the location of fibrin along the paper strip (See **Supplementary Section 4.1**). Fibrin deposits are coloured red, cellulose fibres are coloured blue. The start of the strip refers to the position where the paper strip was placed on top of the clotted droplet. The middle of the strip refers to a location approximately 3 cm down the strip away from the clotted droplet - where blood has clearly wicked to. There is a strong presence of fibrin deposits at the start of the strip for the positive control, but not the negative control. However, there is no presence of fibrin in the middle of either strip. This means that the fibrin remains in the clotted droplet and does not wick down the strip. Only the remaining serum fluid from the clotted droplet wicks down the strip. Therefore, once the droplet has reacted with thrombin, 2 phases are formed: a clotted phase and an unclotted phase. When the strip is placed on the clotted droplet, only the unclotted phase can wick down the strip.

4.9. Effect of Droplet Contact Angle:



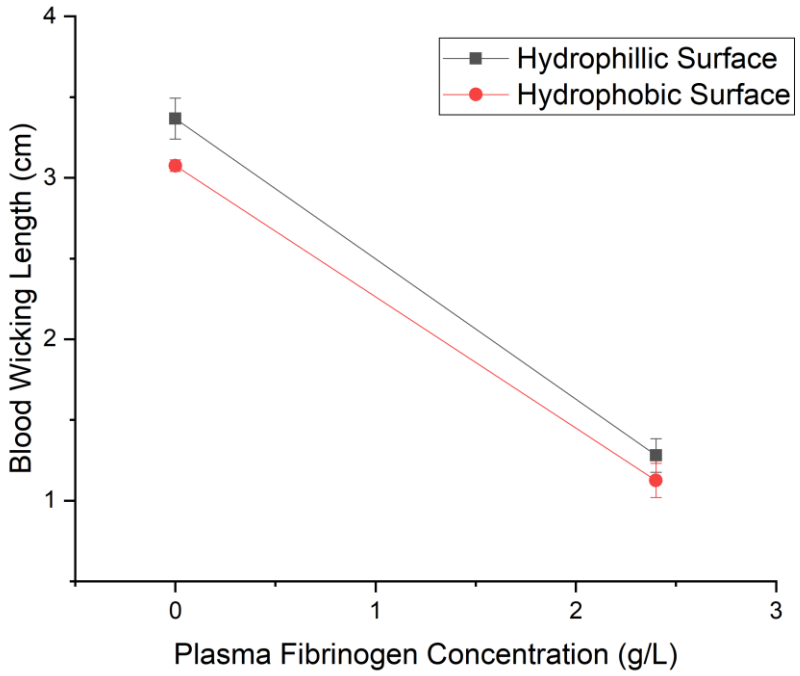
Supplementary Figure 31: Comparison of the test performed on hydrophobic and hydrophilic-surfaced glass slides after it has completely dried and wicked no more.

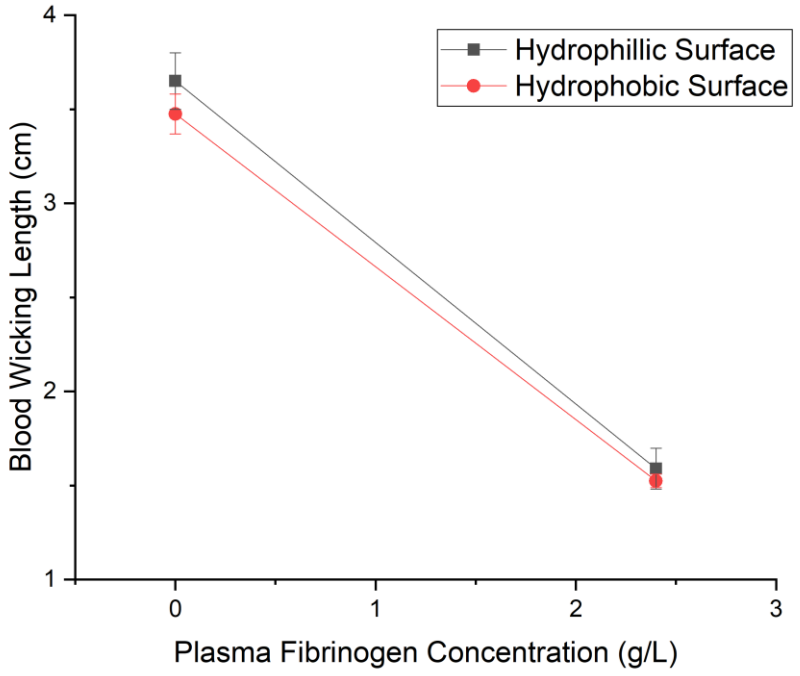
Supplementary Figure 31 compares the wicking of blood on hydrophobic surfaces and hydrophilic surfaces. Since hydrophilic surfaces produce droplets with lower contact angles, it also causes the droplet to spread out much further. To ensure the comparison was controlled, the contact area between the paper strip and the clotted droplet was kept consistent. This was achieved by allowing an interfacial contact length between 3-4 mm to form.



Supplementary Figure 32: Measurement of water droplet contact angle on hydrophobic and hydrophilic-surfaced glass slides. Each data point was tested 3 times and the mean and standard deviation are reported.

Supplementary Figure 32 determines the water droplet contact angle of both the hydrophobic and hydrophilic-surfaced glass slides. The contact angle was measured using a Dataphysics OCA35 device. A 2 µL droplet of water was deposited onto the surface and the contact angle was measured afterwards. The difference in contact angle is significant. The hydrophilic surface produced a contact angle averaging 25° whilst the hydrophobic surface produced a contact angle averaging 130°. Respectively, these angles are indicative of the angles of the unbeaded and beaded droplets formed in the actual test.

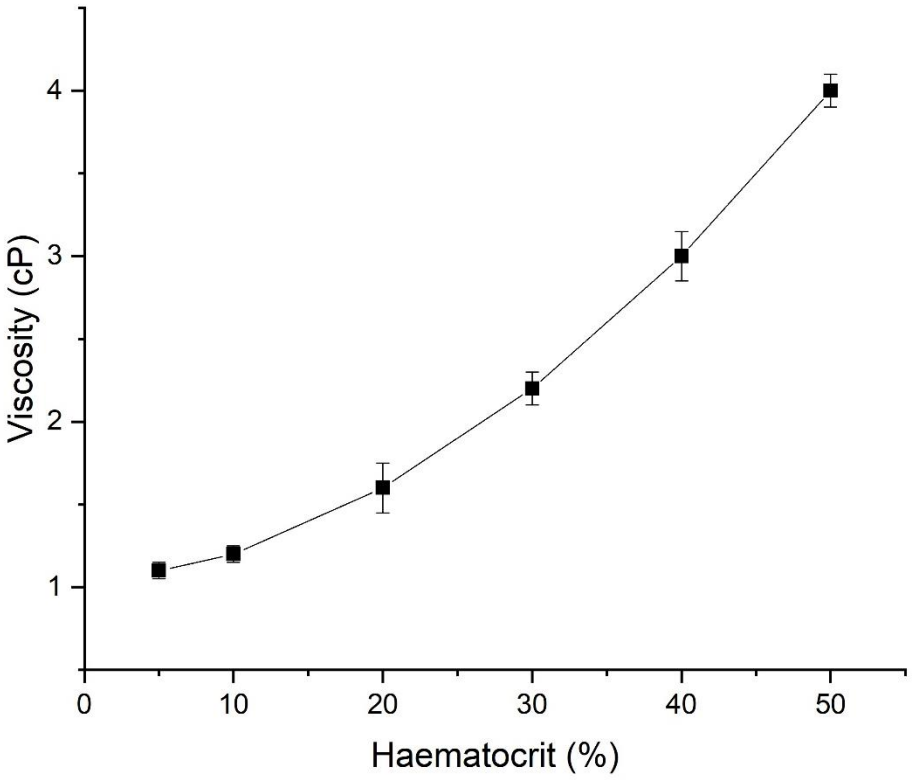


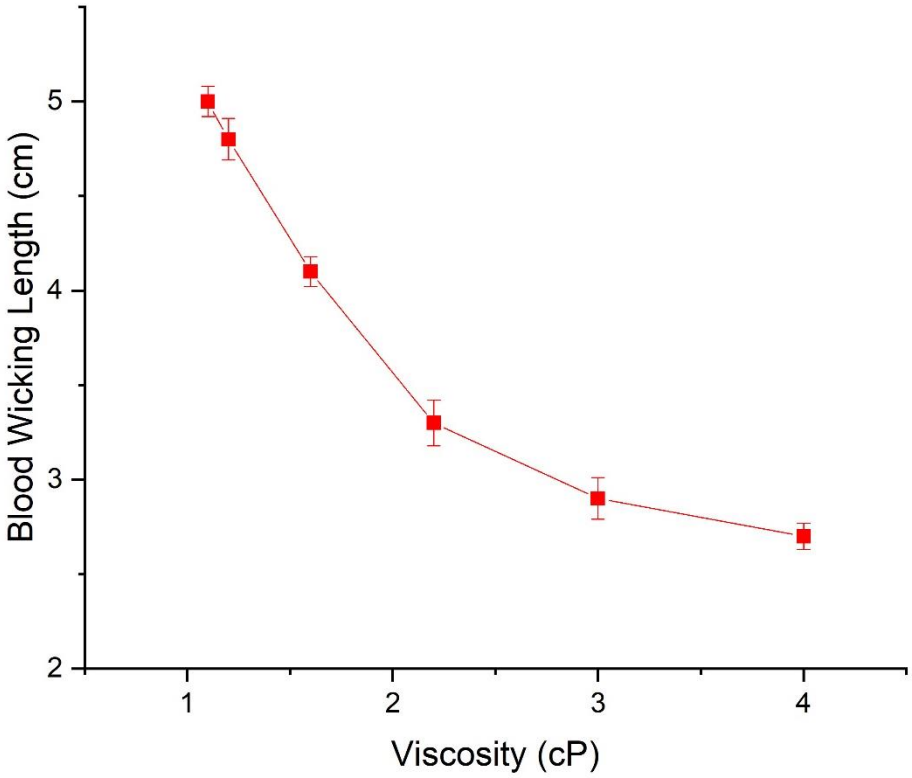


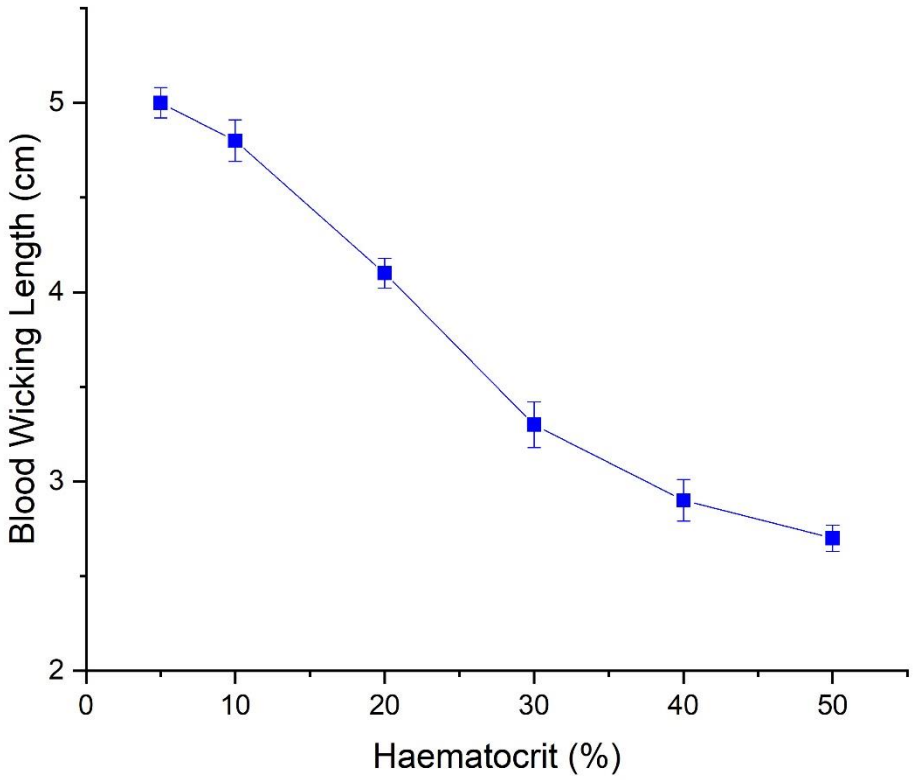
Supplementary Figure 33: Testing of hydrophobic and hydrophilic-surfaced glass slides. Whole blood reacted with thrombin for 3 min and the blood wicking length was measured after 30 s (top) and 60 s (bottom) of wicking using ImageJ. Each data point was tested 3 times and the mean and standard deviation are reported.

Supplementary Figure 33 investigates the effects of droplet contact angle and measures the blood wicking length after different wicking times. Unbeaded droplets and beaded droplets are tested on hydrophobic and hydrophilic surfaces, respectively. After 30 s (top graph) there are insignificant differences in wicking. Unbeaded droplets wick slightly further than beaded droplets. This is hypothesised to be due to the unbeaded droplets ability to wet the paper more efficiently. However, no difference in sensitivity is observed. After 60 s, the initial wetting effect becomes neutralised. Both unbeaded and beaded droplets wick to the same lengths. Despite the significantly different contact angle unbeaded and beaded droplets display in **Supplementary Figure 32**, the overall effect it has on the test is subtle. Hence, the test can work with different solid surface materials and droplet contact angles.

4.10. Relationship between Haematocrit, Viscosity and Blood Wicking Length:







Supplementary Figure 34: The relationship between haematocrit, viscosity and blood wicking length deciphered from artificially prepared blood samples. Top Graph: haematocrit vs viscosity. Middle graph: viscosity vs blood wicking length. Bottom graph: haematocrit vs blood wicking length. Each data point was tested 3 times and the mean and standard deviation are reported.

Supplementary Figure 34 determines how haematocrit, viscosity and blood wicking length all relate to each other. Blood samples were prepared by washing red blood cells (as described in the methodology section) and diluted in PBS to different haematocrits. The viscosity of each blood sample was measured using a Cannon-Fenske viscometer of size 200 (~0.1 cSt/s). Since the density of whole blood (ie. 1.06 g/mL) is very similar to that of PBS solution (ie. 1.00 g/mL), the density used to convert cSt to cP was assumed to be 1.00 g/mL for all blood samples. The blood wicking length was measured using the main test procedure (described in the methodology section).

The top graph shows an exponentially increasing relationship between haematocrit and viscosity. Haematocrits above 10% show significant changes in viscosity. However, haematocrits below this value do not change significantly.

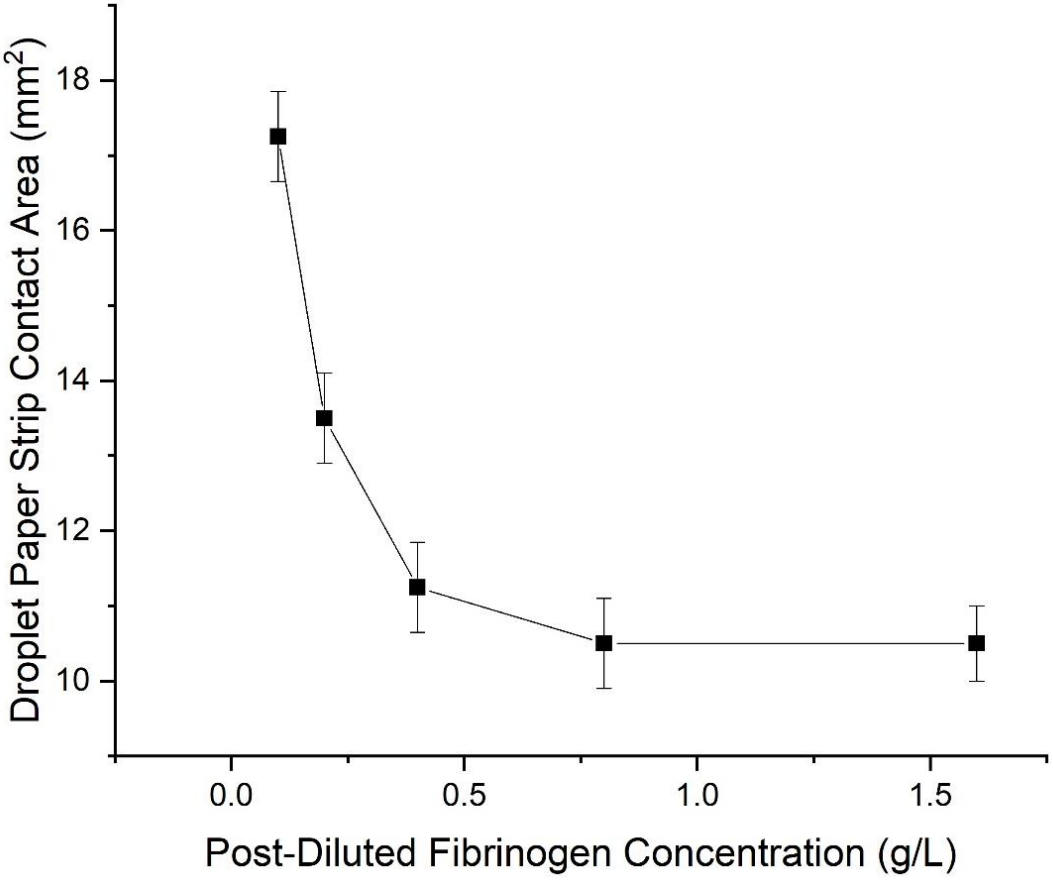
The middle graph shows an exponentially decaying relationship between viscosity and blood wicking length. The effect of viscosity changes significantly up until 3 cP. After that, the effect of viscosity becomes insignificant.

The bottom graph shows a sigmoidal relationship between haematocrit and blood wicking length. This shape arises due to: 1) the insignificant changes in viscosity below 10% haematocrit (as shown in the top graph); 2) the insignificant effect of viscosity on blood wicking length above 40% haematocrit (as shown in the middle graph).

4.11. Relationship Between Droplet Fibrinogen Concentration and Contact Area:

Fibrinogen Concentration	Fibrinogen Concentration
0.4 g/L Fibrinogen	 ≈3 mm
0.2 g/L Fibrinogen	 ≈4.5 mm
0.1 g/L Fibrinogen	 ≈6 mm

Supplementary Figure 35: Comparison of the contact length between the paper strip and clotted droplets of different fibrinogen concentrations. Images were taken after they had completely dried and wicked no more.



Supplementary Figure 36: The relationship between the clotted droplet fibrinogen concentration and the contact area with the paper strip. Each data point was tested 4 times and the mean and standard deviation are reported.

Supplementary Figures 35 and **36** explores the relationship between the clotted droplet fibrinogen concentration and the contact area with the paper strip. This in turn explains how the droplet stiffness impacts on blood wicking length and why **Graph 4** linearises at higher dilutions.

As the clotted droplet's fibrinogen concentration decreases, so does its stiffness (as less fibrin is present). At fibrinogen concentrations below 0.4 g/L, the stiffness drops so low that it compresses underneath the weight of the paper. As the fibrinogen concentration drops, the compression increases exponentially. Consequentially, the contact area between the paper strip and the clotted droplet also increases. This causes more blood to wick out of the droplet than it would have otherwise if the clotted droplet resisted compression.

This can be explained with Darcy's Law:

$$q = -\frac{k}{\mu}\frac{\Delta P}{L}$$

And the volumetric flux equation:

$$q = \frac{L_2 A_2}{t A_1}$$

Where L_2 is the blood wicking length, A_2 is the paper cross sectional area, t is the wicking time and A_1 is the contact area between the clotted droplet and the paper strip.

From **Supplementary Figure 36,** the clotted droplet resists compression when its fibrinogen concentration ranges from 0.4-1.6 g/L. This is relevant for the curves created for the 1:0 and 1:1 dilutions.

The only factor modulating the blood wicking length between these concentrations is the permeability of the droplet (k). Hence, the permeability of the droplet is proportional to the blood wicking length:

$$k \propto L_2$$

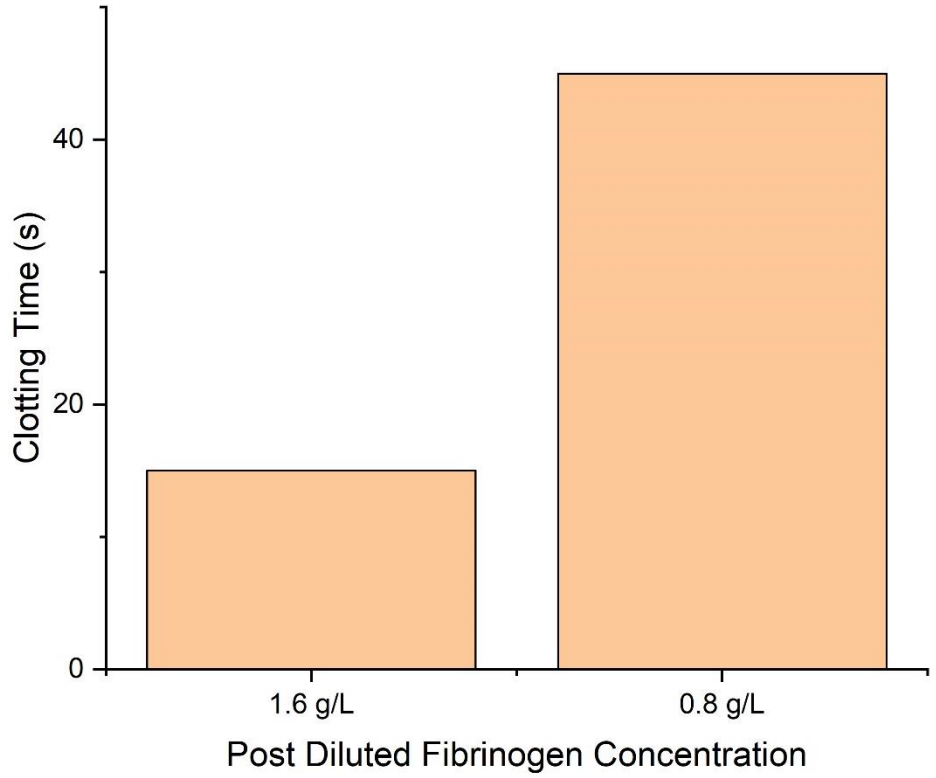
Given that higher fibrinogen concentrations (c) create droplets with lower permeabilities, the 2 parameters are inversely proportional to each other. Hence the blood wicking length will also be inversely proportional to the fibrinogen concentration:

$$c^n \propto \frac{1}{k}$$

Hence the fibrinogen concentration vs blood wicking length relationship seen for these concentrations is non-linear.

At larger dilutions (ie. 1:3 and 1:7), the curve linearises. These dilutions correspond to clotted droplet fibrinogen concentrations below 0.4 g/L. As the fibrinogen concentration drops below this range, the blood wicking length becomes modulated by both the clot permeability and contact area. Each time the clotted droplet's fibrinogen concentration halves, the contact area increases by roughly 25%. Hence, it wicks 25% further than it would on permeability alone. This increase is enough to convert the non-linear relationship seen with the 1:1 dilution curve to a linear relationship seen with the 1:3 dilution curve.

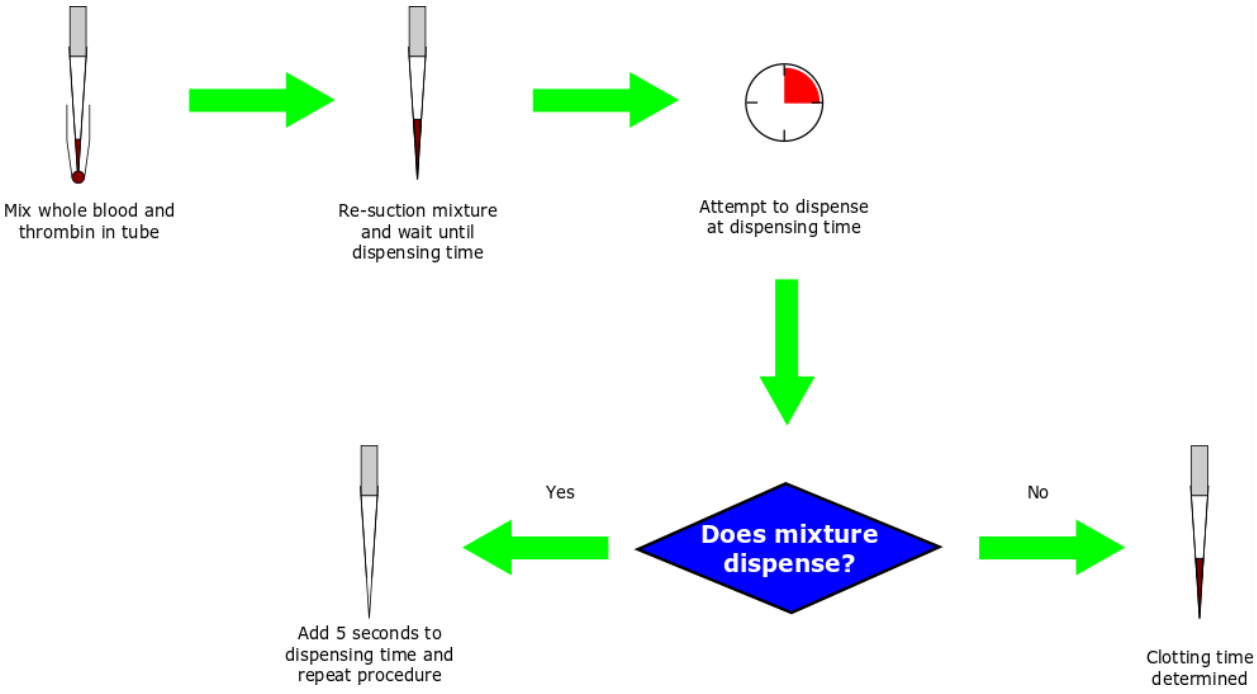
4.12. Relationship between Fibrinogen Concentration and Clotting Time:



Supplementary Figure 37: The relationship between the droplet fibrinogen concentration and the time before it clots. The procedure for determining the clotting time is given in **Supplementary Figure 38**. The clotting time for each data point was verified 3 times.

Supplementary Figure 37 demonstrates the time taken to clot a droplet after it has been first mixed with the thrombin solution. The longer the clotting time the better, as more time is afforded for the end-user to be able to successfully mix and deposit the droplet onto the glass slide. A whole blood sample was prepared from normal plasma (as described in **Chapter 4 Section 2.2.3** of the methodology). This sample was then serially diluted with PBS (as described in **Chapter 4 Section 2.2.9**) to produce other samples of lower fibrinogen concentrations.

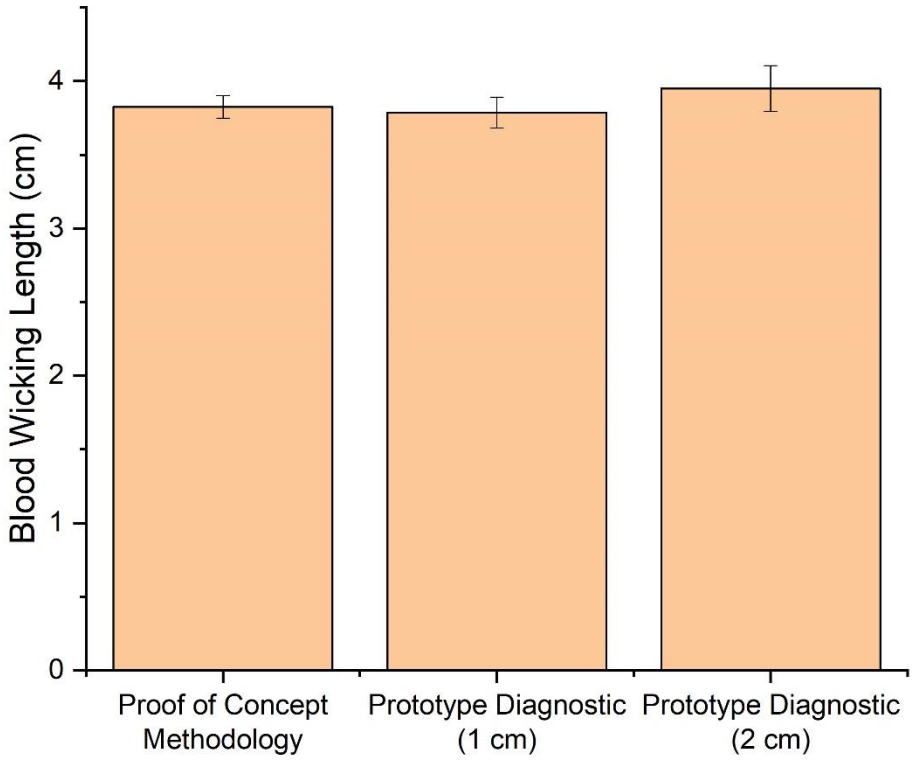
To measure clotting time, 5 µL of thrombin was added to a PCR tube. It was then mixed repetitively with a pipette containing 25 µL of blood. The timing started the moment the blood and thrombin solution first mixed. Once the blood and thrombin solution mixture appeared uniform, it was re-suctioned into the pipette so that the clotting time could be determined. The clotting time was determined as the time the mixture could no longer escape from the pipette tip. The formation of the clot prevented the mixture from being dispensable from the tip. The clotting time was calculated by iteration using the procedure given in **Supplementary Figure 38**:



Supplementary Figure 38: Summary of methodology to determine the clotting time for droplets of different fibrinogen concentrations. The clotting time was determined as the time the mixture could no longer be dispensed from the pipette. Dispensing times began 10 s after the starting time and determined iteratively. 5 s was added to each iteration until clotting was evident.

The clotting time increases with decreasing fibrinogen concentration. This is a well-known phenomenon seen in Clauss assays. An undiluted whole blood sample of normal fibrinogen concentration clots after 15 s. However, diluting by half, causes the clotting time to increase to 45 s. Hence, by diluting, the time afforded to the end user before clotting increases. This makes the test easier to perform.

4.13. Effect of Paper Strip Dropping Height in Prototype Diagnostic:



Supplementary Figure 39: Comparison of a whole blood sample (diluted 1:7) using the proof-of-concept methodology (as described in **Section 3.6** and **3.7**) and the prototype diagnostic (as described in **Section 3.13**). The paper strip in the diagnostic was released at variable heights (i.e. 1 cm and 2 cm) above the glass slide. Each data point was tested 4 times and the mean and standard deviation are reported.

Supplementary Figure 39 compares the wicking and measurement of the prototype diagnostic at variable dropping heights to that of the proof-of-concept methodology. It determines the optimal dropping height of the diagnostic.

Regardless of the dropping height, the prototype diagnostic wicks very similarly to the proof-of-concept methodology (where the paper strip is manually released millimetres above the clotted droplet).

Although minor, the 2 cm dropping height causes the diagnostic to wick slightly further than the 1 cm dropping height. We hypothesise this to be due to the greater impact between the paper strip dropped at that height and the clotted droplet. Hence, **Figure 10** displays the results of the prototype diagnostic with a 1 cm dropping height.

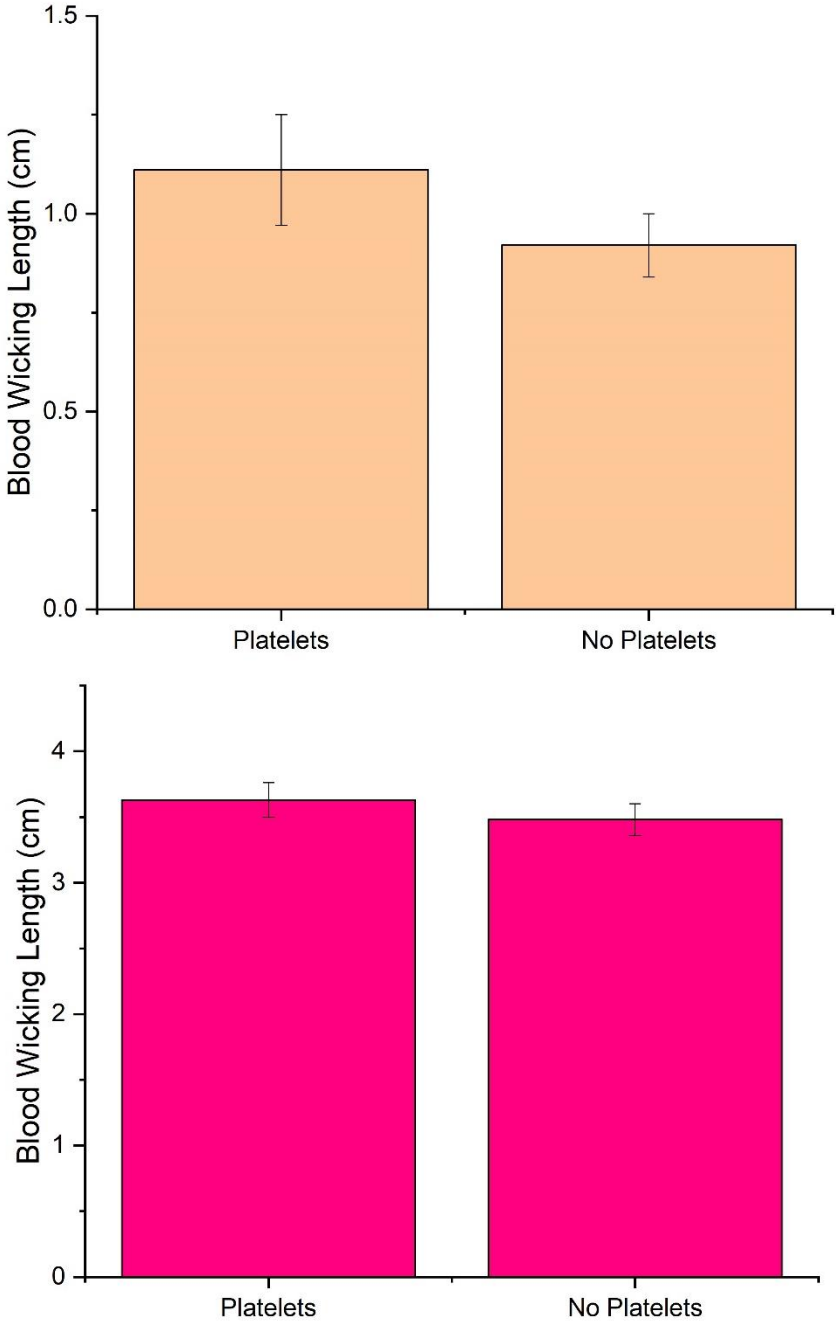
4.14. Wicking Front Enhancement:

Supplementary Figure 40: Wicking front enhancement for diluted blood samples (ie. 1:3, 1:7 and 1:15). 0.1 µL of Queens Blue Food Dye (undiluted) was pipetted 1.5 cm from the top of the paper strip.

Supplementary Figure 40 shows how wicking fronts from blood samples with poor visibility were enhanced. Diluted blood samples produced wicking fronts that were difficult to see and analyse. Hence, a minute amount of blue dye was added to the strip - downstream from where the paper strip landed on top of the blood sample.

After the paper strip was placed on the blood sample, the wicking front mixed with the blue dye so that it could be easily seen.

4.15. Effect of Platelets:



Supplementary Figure 41: Testing of whole blood with and without platelets. Whole blood reacted with thrombin for 3 min, and the blood wicking length was measured after 30 s of wicking using ImageJ. Top Graph: whole blood tested without dilution. Bottom Graph: whole blood tested after a 1:7 dilution in PBS.

Supplementary Figure 41 illustrates the effect of platelets on the whole blood test. Whole blood was collected from a donor - not on aspirin – in citrated tubes and used within 4 hours.

Samples with platelets consisted of the collected blood used without further modification. Samples with no platelets involved removing the platelets from the collected blood through manual pipetting.

First, 2 blood samples were centrifuged at 3000 rpm for 15 min. In one sample, the volume of plasma and volume of blood cells was measured to calculate the haematocrit of the collected blood. In the other sample, the plasma layer was separated into another tube, the platelets were removed (by pipetting them out) and the remaining RBCs and plasma were recombined (to the correct haematocrit) to create blood with no platelets.

2 scenarios were tested: 1) whole blood used undiluted (top graph); 2) whole blood used after a 1:7 dilution ratio in PBS (bottom graph).

Both graphs show that platelets do not have a significant impact on the test. However, the extent to which the citrate inhibits the platelets is unknown. Therefore, further work with uncoagulated whole blood is needed in order to reach a conclusion.

4.16. Terminology:

Table S13: Definition of common terms as used in the manuscript to provide clarity.

Term	Description
Blood	A sample/fluid composed of blood cells and/or components of plasma of any composition. It is used in this manuscript as an umbrella term (e.g. blood wicking length refers to the distance that fluid from a blood sample has wicked down the paper strip).
Whole Blood	Blood sample which contains a mixture of blood cells and plasma at physiologically relevant compositions (i.e. compositions that are typically found in the blood stream). In this manuscript, a whole blood sample refers to a pre-made mixture of 50% red blood cells and 50% plasma (unless the composition is stated otherwise).
Droplet	A mixture of thrombin solution and whole blood or plasma that is immediately deposited on the PTFE-coated glass slide.
Unbeaded Droplet	A droplet with an acute contact angle (< 90 degrees).
Beaded Droplet	A droplet with an obtuse contact angle (> 90 degrees).
Clotted Droplet	A droplet after the fibrinogen in it (if present) has polymerised into fibrin and formed the fibrin scaffold.
Non-fibrinogen Effects	The effect that RBCs and plasma proteins (other than fibrinogen) have on blood wicking. As RBCs and plasma proteins both interact with cellulose fibres and increase the viscosity of a blood sample, they are able to slowdown the movement of blood through the paper strips (irrespective of what the fibrinogen concentration of the blood sample is).
When the **Clotted Droplet** is formed, 2 phases are created:	
Clotted Phase	The viscoelastic phase which consists of the fibrin scaffold and entrapped RBCs.
Unclotted Phase	The liquid phase which consists of RBCs and unbound plasma molecules that did not get entrapped by the fibrin scaffold.

Chapter 5: A Simple Prothrombin Time Paper Diagnostic

Publication Details

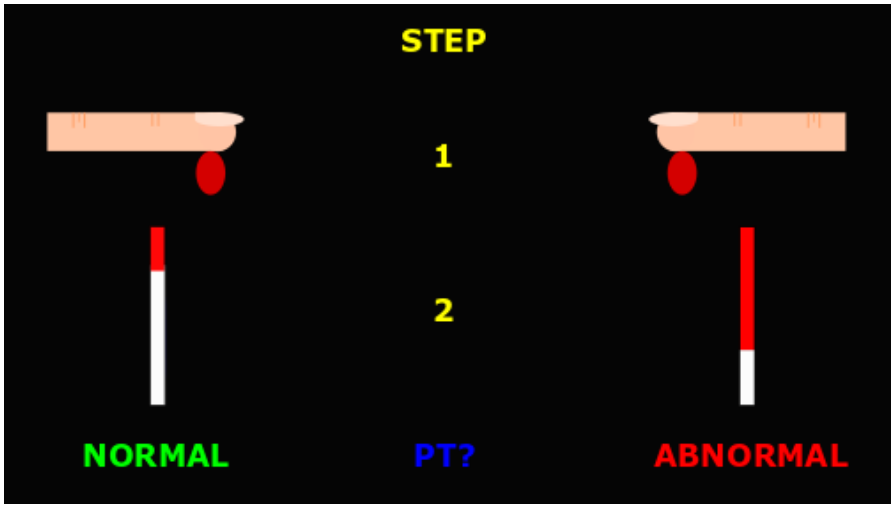
Publication Status: Submitted

Journal: ACS Sensors

Reference: N/A

Abstract

Prothrombin time (PT) assaying is essential to detect liver disease and monitor anticoagulant therapy in patients susceptible of thrombosis or stroke. Globally, over 200 million PT tests are performed per year – putting major strain on biomedical laboratories. The concept of point of care (PoC) diagnostics for monitoring PT has grown in popularity. This is due to the ability for patients to self-test with only a fingerprick of blood. However, there is currently no test available that is rapid, cheap and requires no external equipment. In this manuscript, we develop a novel hand-held and easy to use paper-based PT diagnostic that works with whole blood. The test functions by pre-mixing a small blood sample with tissue factor (TF) solution in a PCR tube, depositing it on a glass slide as a droplet, reacting for a certain period and then dropping a paper strip on top of the droplet. The concept is simple: blood samples that have clotted after interacting with the TF will wick significantly less than those that have not clotted yet. The test can be completed in a minute. Hence, it can be used to rapidly measure whether samples are above or below the key internal normalised ratios (INRs) - the current measuring standard. The paper sensor can easily distinguish between INRs of 1, 1.5, 2 and 3 - the values of biomedical importance. Therefore, this new test can allow for liver disease diagnosis or anticoagulant drug monitoring in resource limited places. Thus, it can help save 20 million lives per year.



1. Introduction

Prothrombin time (PT) quantifies the time taken for blood to clot once bleeding is induced. It measures the activity of coagulation factor (CF) enzymes involved in the tissue factor (or extrinsic) pathway of clotting. The extrinsic pathway is activated when blood is exposed to the tissue that surrounds the site of blood vessel damage. The cells of these tissues express a protease receptor called tissue factor (TF) (28). After exposure, TF activates the cascade of extrinsic pathway enzymes. Eventually, this converts fibrinogen into fibrin and allows bleeding to stop (257).

PT is currently measured by collecting a patient blood sample, centrifuging it, extracting the plasma, heating it to 37°C and adding a TF reagent to it. This process requires a full biomedical laboratory to perform and usually requires 10-20 min. The PT is measured as the time required until a clot has formed. Healthy samples generally take 12 s to clot upon addition of the reagent (258). However, since different laboratories use different reagents and equipment to measure PT, the measurement of healthy samples vary as well. Because of this, reference plasmas (of known PT) are used to achieve standardisation. Thus, PT can be converted into an internal normalised ratio (INR) as such:

$$INR = \left(\frac{PT_{Patient}}{PT_{Healthy}}\right)^{ISI} \quad \textbf{(4)}$$

Where $PT_{Patient}$ is the prothrombin time of the patient being tested, $PT_{Healthy}$ is the prothrombin time of a healthy reference plasma and ISI is the activity of the TF reagent. Due to its definition, the INR of a healthy blood sample, will always range narrowly from 0.9-1.1 (259).

PT tests are heavily implemented in monitoring thrombotic and liver diseases. Acute liver failure is diagnosed when the INR of a patient rises above 2. Since the extrinsic pathway CF enzymes are synthesised in the liver, a deficiency of them in the blood is a clear indicator of liver disease. However, when patients also show signs of encephalopathy, their INR only needs to measure above 1.5 to be diagnosed with acute liver failure (260). In both these situations, patients may require liver transplantation (261). Likewise, patients who are at risk of thrombosis or stroke require their INR to become elevated above 2. Anticoagulant therapy is used to achieve this. The most common anticoagulant used to prolong INR is warfarin. Warfarin works by preventing the synthesis of extrinsic pathway CF enzymes (229). Hence, it impairs the ability of the patient to form clots. However, excessive anticoagulant therapy is also dangerous as it exposes the patient to a risk of bleeding excessively. Therefore, to minimise the risk of under or over-dosage, patients often require their INR to be kept between 2 and 3 (262).

Globally, these diseases cause an estimated 20 million mortalities per year (263, 264). Because of this, more than 200 million PT tests must be performed annually (265). This puts major resource strains on biomedical laboratories that use current PT assays. Hence, the large demand for PT testing has made day-to-day monitoring of patients infeasible in health centres.

More recently however, the development of point of care (PoC) devices has allowed patients to test their own blood daily. PoC devices - such as the Coaguchek - require the patient to take a finger prick of their own blood, then apply it on a test card and finally insert it into an analysis instrument. This analysis equipment then relies on an electrochemical reaction to determine the INR (266). However, these devices are prohibitively expensive for personal use. The analysis system cost more than AU$700. Furthermore, the test strips cost AU$6-8 each. This makes self-testing unaffordable in both developing nations and

developed nations where healthcare is not publicly subsidised. The USA alone has 15 million warfarin users that require daily anticoagulant monitoring (267).

The most affordable PoC PT diagnostic to date has been developed by Li *et al.* (206). The test induces clotting of a blood sample, before immediately adding it to a paper-based lateral flow strip. The concept is simple; the further the blood travels up the strip, the longer it takes to clot. Hence, further wicking distances correlate to longer PT/INRs. The test is easy to use, quick and results can clearly be seen to the naked eye. However, it relies on wicking and clotting to occur simultaneously. This severely restricts the maximum sensitivity that can be achieved. Because of this issue, the test is strongly impacted by haematocrit (i.e. the red blood cell concentration). The haematocrit of a blood sample dictates its viscosity. Hegener *et al.* performed clinical trials on this device with patients over a wide range of haematocrits (≈15%) (268). They found that blood samples of lower haematocrit wick further and falsely elevate the measured INR. Overall, the test cannot easily distinguish INRs between 1-3 over that range. Therefore, a separate haematocrit measuring device is required to calibrate and interpret the result - which makes it difficult to execute as a PoC diagnostic (269).

Currently, there is no paper-based PT diagnostic that can distinguish INRs between 1-2 (for liver disease) or 2-3 (for anticoagulant monitoring) without requiring extra equipment. There have been no convenient and affordable PoC PT diagnostics developed yet.

In our previous study, we developed a hand-held fibrinogen concentration diagnostic that operates with whole blood (201). In the test, whole blood samples get mixed with thrombin solution in PCR tubes until they are uniformly distributed. Each mixture is then deposited as a droplet onto a glass slide. After reacting for 3 min, a paper strip is placed on top of the clotted droplet and blood wicks down it for 30 s. The further the blood wicks, the lower the fibrinogen concentration.

Given that this test utilises pre-mixing, it allows fibrinogen to be completely converted into fibrin, before the paper strip is placed on top – thus optimising its sensitivity. Hence, the test can clearly detect if a sample has clotted or not – regardless of haematocrit.

In this study, we demonstrate a rapid, reliable and cheap paper-based PT diagnostic that functions directly with whole blood. The test performs similarly to the previously developed whole blood fibrinogen concentration diagnostic. However, instead of using a thrombin solution as the reagent, it relies on a tissue factor (TF) solution instead. The test looks at whether a blood sample has clotted or not after reacting with TF for a selected time. The paper strip - once placed on top of the sample - determines whether it has clotted. This is indicated by a clear throttling of wicking distance. Hence, it can be used to clearly determine if a blood sample is above or below key INRs - which makes it suitable for monitoring liver disease and anticoagulant coagulation.

In this study, we first prove the concept - by observing the reaction time vs wicking distance relationship using blood samples with different INRs. We then investigate the effect of haematocrit on the test and analyse ways to mitigate its effect. Afterwards, we analyse if the diagnostic can be used for liver disease detection and anticoagulant monitoring. Finally, because the test may be used in developing nations with minimal temperature regulation, we also observe the effect of temperature.

2. Materials and Methodology

2.1. Materials

Tissue paper of 30 g/m^2 and 160 µm thickness was used to create the paper strip cut-outs. They were produced in the Kimberly Clark Experimental Forming Unit (EFU), Neenah (WI), USA. Tissues consist of eucalyptus fibres formed with a three-layer headbox. 10 kg/ T Kymene, a polyaminoamide-epichlorohydrin (PAE) wet strength agent, was added to each layer. Tissues were moulded and thoroughly air-dried.

Three batches of lyophilised citrated platelet-poor plasmas and tissue factor (TF) reagent were sourced from Affinity Biologicals (Ancaster, ON, Canada). The plasmas used represented healthy (Plasmacon N), mildly abnormal (Plasmacon L1) and highly abnormal (Plasmacon L2) prothrombin time (PT) parameters. Their internal normalisation ratios (INRs - see **Equation 4**) were 1, 1.7 and 5.2, respectively. Each lot derived from a pool of 20 donors, buffered with 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer to stabilise the pH at around 7.4. The TF reagent used was Phosphoplastin RL. It had an international sensitivity index (ISI – see **Equation 4**) of 1.13. Ethylenediaminetetraacetic acid (EDTA) anticoagulated donor blood tubes were sourced from the Australian Red Cross.

Phosphate-buffered saline (PBS) tablets (79382) were sourced from Sigma Aldrich. MilliQ water was used to dissolve the PBS tablets and reconstitute vials of plasma.

"PCR Strip Tubes 0.2 mL Thin Wall & Flat Caps" from Axygen (Union City, CA, USA) were used as the tubes for mixing. They are referred to as PCR tubes throughout the manuscript.

Westlab microscope glass slides (25 × 75 × 1.0−1.2 mm) were used to provide a surface for blood droplets to form on top of. Kinetic 12 mm × 10 m white standard thread seal tape (PTFE tape, Bunnings, Australia) was used to wrap around the glass slides and allow the deposited droplets to become beaded.

2.2. Methodology

2.2.1. Paper Strip Cut-Out Preparation

Paper strip cut-outs were designed using CorelDRAW X6 with the dimensions shown in **Supplementary Figure 42**. Paper was cut on a laser cutter (60 Watt Epilog Helix) using the cut-outs prepared on CorelDRAW X6. The print settings used were 100% speed, 5% power, and 500 Hz frequency. It was important to ensure that the strips were cut parallel to the machine direction of the paper for uniform and maximum wicking. To ensure that the test produced consistent results, the paper strips had to be cut thin enough to prevent gaps from being introduced into its structure. Otherwise, the blood could wick further than it should. In our previous study, we found that 3 mm paper strips produces precise results after wicking (199). Therefore, this width was chosen for the paper strips in this study as well.

2.2.2. Phosphate-Buffered Saline (PBS) Solution Preparation

Five tablets of PBS were added to a 1 L Schott bottle. 1 L of MilliQ water was added into the Schott bottle. The PBS tablets were dissolved in solution by gently rocking the Schott bottle with a WiseShake SHR rocker at 40 rpm overnight.

2.2.3. Whole Blood Solution Preparation

Vials of Plasmacon N, L1 and L2 were reconstituted with 1 mL of distilled water. Since none of these plasmas had INRs of 2 or 3, they had to be created by mixing the plasmas to the correct ratios. This is summarised in **Supplementary Section 5.2**.

Red blood cells (RBCs) were sourced from EDTA anticoagulated donor blood. A donor blood tube was centrifuged for 15 min at 3000 rpm on an Eppendorf 5702 centrifuge. This completely fractionated the blood components. The plasma supernatant was discarded with manual pipetting. The RBCs were then washed with PBS solution. 3 mL of PBS solution was added to the RBCs before resuspension and recentrifugation for 15 min at 3000 rpm. The PBS supernatant was then discarded with manual pipetting. The washing step was repeated until the PBS supernatant appeared clear. The final clear PBS supernatant was discarded, and the washed RBCs were retained and used on the day of experimentation.

Whole blood samples of different fibrinogen concentration were prepared by mixing washed RBCs and plasma samples together. Whole blood samples with different haematocrits were prepared by changing the mixing ratio of washed RBCs and plasma. The whole blood samples were used on the day of experimentation. Because blood cells sediment in whole blood samples over time due to gravity, they were uniformly suspended each time before use.

2.2.4. Diagnostic Assembly

Diagnostics were prepared by wrapping one piece of PTFE tape around a glass slide with the tape lying flat.

A black marker was used to mark two points 5.8 cm apart on the PTFE tape. A paper strip was placed over the two markings. The bottom edge was aligned to one marking and attached with sticky tape. A bent paper clip was wedged between the other end of the paper strip and the glass slide. This allowed the paper strip to levitate over the glass slide.

2.2.5. Main Test

Experiments were performed in standard laboratory conditions (i.e., 23 °C, 50% relative humidity) unless stated otherwise.

25 µL of whole blood was typically mixed in 50 µL of Phosphoplastin RL inside a PCR tube. This created a 2:1 ratio of TF solution to blood. The mixing was done by pipetting up and down at least 10 times to ensure that the blood and thrombin were dispersed uniformly in each other (without creating bubbles). The resulting 25 µL mixture was deposited (as a beaded droplet) directly on top of an assembled diagnostic - at the site of the free marking.

To test for prothrombin time (PT), the blood mixture was left to react on the diagnostic over variable times. After the desired reaction time was completed, the paper clip was pulled from the diagnostic. This allowed the paper strip to fall on top of the mixture and initiate wicking.

The blood wicking length was determined 30 s afterwards by the naked eye. 30 s was chosen as the wicking time as it was long enough to produce clear wicking differences between clotted and unclotted samples. However, at the same time, it was not long enough to be affected by the drying rates of humidity.

The reaction time was defined as the time the blood first mixed with the TF solution to the time the paper strip fell on top of the droplet.

To ensure timing was accurate, a timer was used. After starting the timer, the times at which mixing, pulling and wicking were all noted. Hence, this minimised the need to continuously stop-start the timer and induce human error.

A visual demonstration of the entire test is presented in **Figure 40**.

2.2.6. Changing Mixing Ratio

The mixing ratio of TF solution to whole blood was done by changing the volume of Phosphoplastin PL used. For example, a 1:1 ratio was achieved by using 25 µL of Phosphoplastin PL. 25 µL of whole blood was still used. This ensured that the mixture deposited onto the diagnostic maintained at 25 µL.

2.2.7. Effect of Temperature

Experiments were performed in a Memmert UF 55 incubator. All reagents and apparatus were left in the incubator at 32°C for an hour beforehand. This ensured that they were equilibrated at the correct temperatures.

Afterwards the main test was carried out inside the chamber. As the chamber door was left open over the duration of testing, ≈0.5-1°C was lost from the chamber every time it was performed. Therefore, the chamber door was re-closed for 3 min between tests to allow the chamber and reagents to return to 32°C.

3. Results

Whole blood samples were mixed with the tissue factor (TF) solution in a PCR tube until uniformly distributed. The sample was then deposited as a droplet onto a glass slide with a paper strip levitating above it (via a paper clip). After certain reaction time intervals, the paper clip was released and the strip fell on top of the droplet. This is shown in **Figure 40**.

Prothrombin time (PT) is indicated by the formation of clots at specific time intervals. As clot formation throttles the wicking of blood, it is detected by a decrease in the lateral flow of blood down the strip. Whole blood samples with larger Internal Normalisation Ratios (INRs) will take longer to clot than samples with smaller INRs. Hence, they will require a larger reaction time interval to experience lateral flow perturbation.

The test relies on a similar clotting/wicking mechanism as that previously developed for the whole blood fibrinogen diagnostic (201). After the blood droplet has clotted on the glass slide, 2 phases are created: the clotted phase and the unclotted phase. The clotted phase consists of the fibrin and particles attached to it - which includes the red blood cells. The unclotted phase consists of the rest of the blood sample.

When the paper strip is placed on top of the clotted droplet, only the unclotted phase can wick and travel through the strip. However, the clotted phase is a soft, porous solid with its own permeability. Hence, it can throttle the migration of the unclotted phase down the strip. Darcy's Law can be used to explain it as such:

$$q = -\frac{k}{\mu}\frac{\Delta P}{L} \quad \textbf{(2)}$$

where q is the flux of the unclotted phase, μ is the viscosity of the unclotted phase, k is the permeability of the clotted phase, ΔP relates to the paper capillary suction force and L relates to the diameter of the clotted droplet. As the clot becomes formed, k decreases which subsequently decreases q. This in turn causes a wicking perturbation clearly visible to the naked eye. Thus, the end user can clearly distinguish between a clotted and unclotted sample.

Several factors were studied to understand the performance and limitations of the test. These include the effect of haematocrit, its sensitivity for INRs between 1, 1.5, 2 and 3 (the values of biomedical importance for liver disease and anticoagulation monitoring) as well as the effect of temperature.

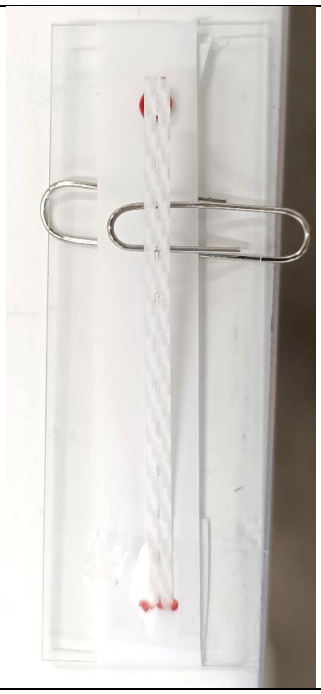
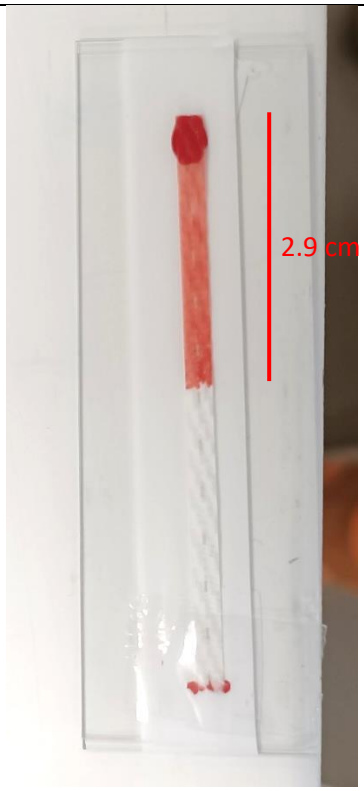
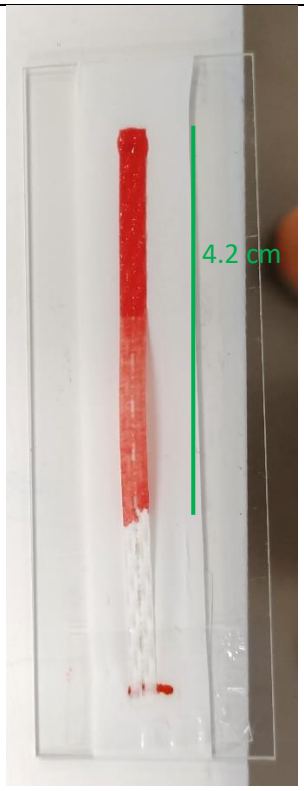
<table>
<tr><td>Deposit Blood Mixture on Diagnostic</td><td colspan="2">  </td></tr>
<tr><td>Select Reaction Time</td><td colspan="2">40 s</td></tr>
<tr><td>Pull Paper Clip</td><td colspan="2"></td></tr>
<tr><td rowspan="3">Wick for 30 s and Measure Length</td><td>INR: 1</td><td>INR: 1.7</td></tr>
<tr><td> 
</td><td> 
</td></tr>
<tr><td>CLOTTED</td><td>NOT CLOTTED</td></tr>
</table>

Figure 40: Illustration of the Prothrombin Time (PT) test using healthy (INR: 1) and abnormal (INR: 1.7) samples.

3.1. Concept

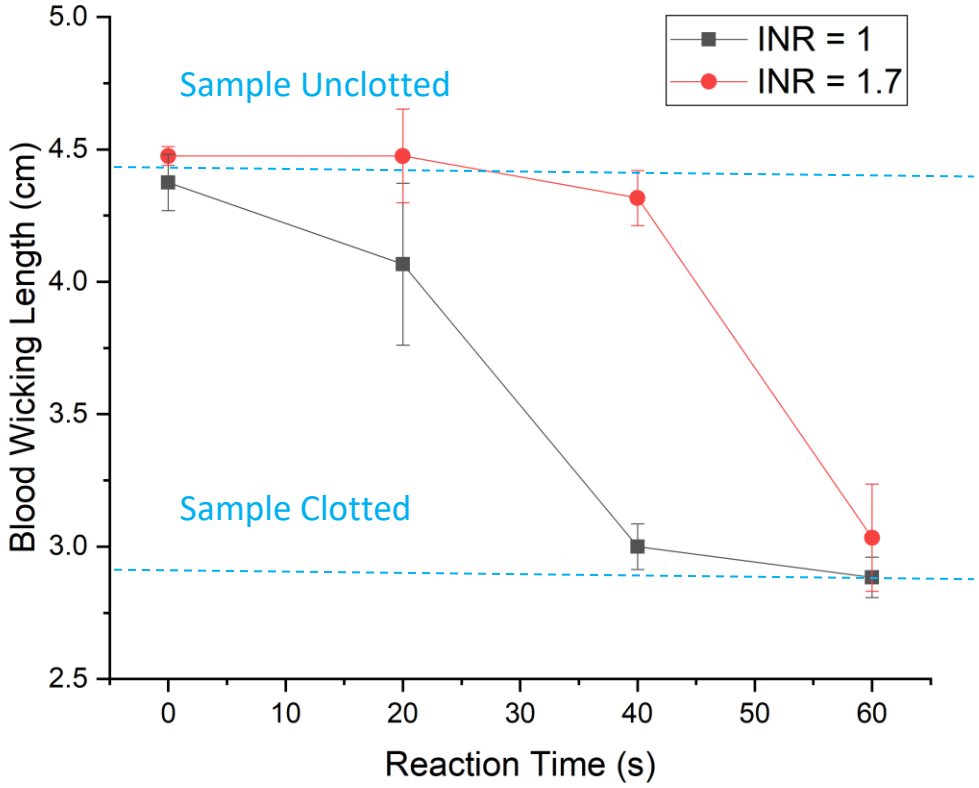


Figure 41: Blood wicking length as a function of Reaction time for 2 whole blood samples of different INRs. The ratio of TF solution to whole blood used was 2:1. Both whole blood samples had haematocrits of 50%. Each test was performed in triplicate and the average and standard deviations are reported.

Figure 41 shows the concept for a paper-based prothrombin time (PT) test. It deciphers the differences in wicking of two whole blood samples - after they have been mixed with tissue factor (TF). Both whole blood samples had different Internal Normalised Ratios (INRs). One sample clotted normally (i.e. INR: 1) and the other took slightly longer to clot (i.e. INR: 1.7).

The reaction time was measured from the instance the whole blood sample first mixed with TF, to the instance a paper strip was placed on top of it (as a deposited droplet). The blood wicking length was measured 30 s after the paper strip was dropped on top of the blood sample.

The samples clearly wick differently. After 20 s, the normal blood sample begins to clot. This causes the wicking of blood down the strip to be throttled. After 40 s, the normal sample clots completely whilst the abnormal sample remains unclotted. The difference in wicking length between the 2 samples is greater than 1 cm. Therefore, this provides the premise for a sensitive paper-based PT test.

After 60 s, the abnormal sample clots. Hence, wicking is throttled to lengths like that of the normal sample.

3.2. Effect of Haematocrit

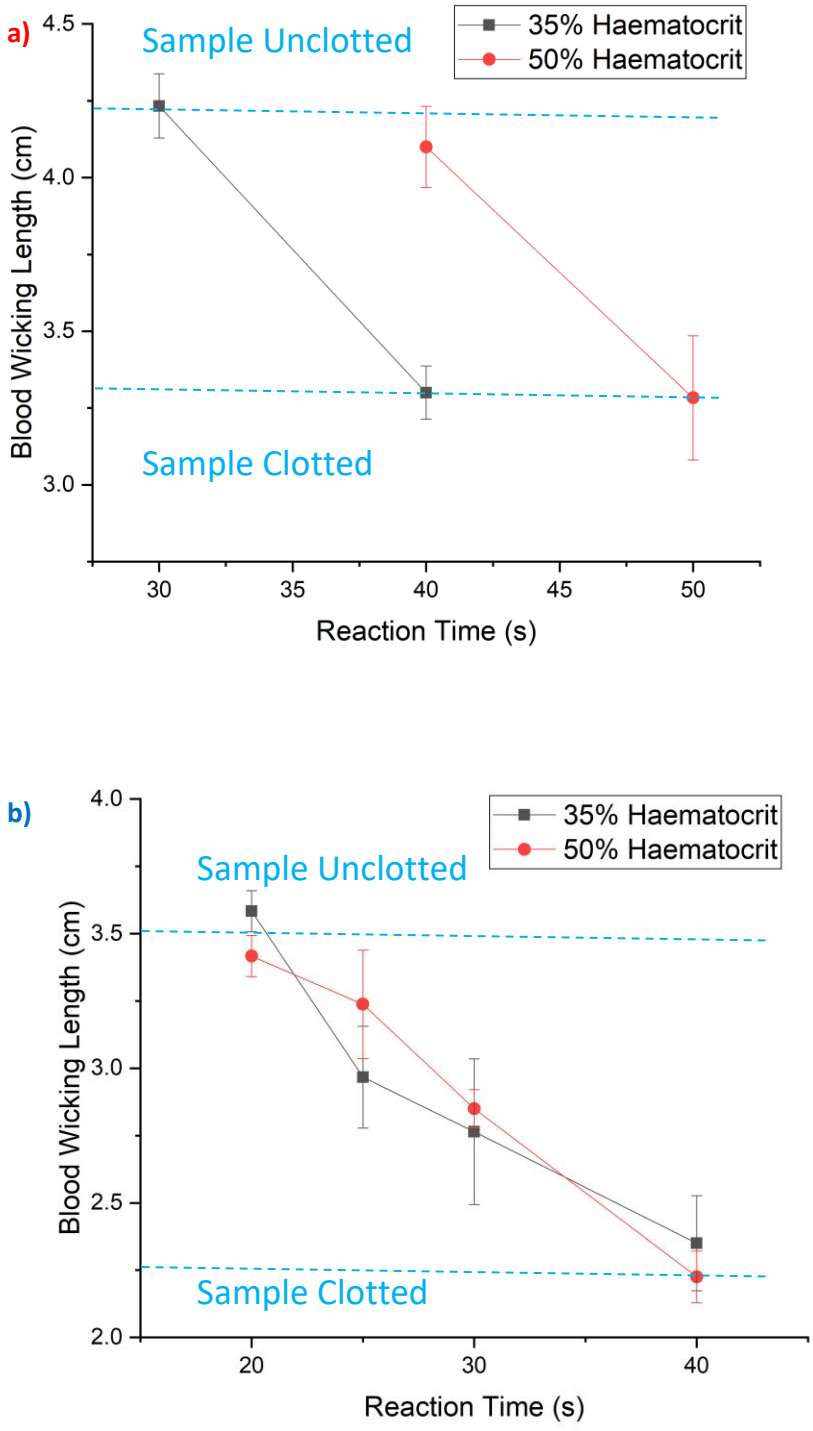


Figure 42: Effect of haematocrit concentration on blood wicking length. Blood wicking length as a function of Reaction time for 2 whole blood samples with different haematocrit. The ratio of TF solution to whole blood used was 2:1 (**a**) and 1:1 (**b**). Both whole blood samples had INRs of 1.7. Each test was performed in triplicate and the average and standard deviations are reported.

Figure 42 deciphers the effect of haematocrit. The haematocrit refers to the volumetric concentration of red blood cells in whole blood. It is expressed as % v/v. As different individuals possess different haematocrits (normally in the range between 35-50%), it was desired to test its effects.

Two whole blood samples with the same INR but different haematocrits were tested. Whole blood samples with lower haematocrits have lower viscosities. Hence, they are expected to wick further on paper. This in turn could falsely detect samples as being unclotted. However, in both figures, this is not the case. A clot could be clearly detected regardless of the haematocrit of the blood. In our previous study (201), we found that pre-mixing large amounts of reagent with small amounts of whole blood neutralised the whole blood's viscosity completely. Samples between 25-50% haematocrit did not wick differently when mixed with reagents to ratios smaller than 1:1 (201). As this test dilutes whole blood to similar ratios, it is also unaffected by the viscous effects of haematocrit.

However, haematocrit does affect the test in another way. **Figure 42(a)** shows that samples with 35% haematocrit clot 10 s earlier than samples with 50% haematocrit. As samples with 35% haematocrit contain more plasma than samples with 35% haematocrit, they also contain more coagulation factor (CF) enzymes as well. Therefore, they clot faster. This is further explained in **Supplementary Section 5.3**.

However, **Figure 42(b)** shows that this phenomenon is reduced when the whole blood sample is mixed with less TF solution. A mathematical explanation for this is provided in **Supplementary Section 5.4**. Basically, the volume of plasma dictates the amount of CFs present in the droplet, whilst the volume of TF solution dictates the extent to which they get diluted. Since the volume of plasma present in the droplet varies with haematocrit, the extent to which the CFs get diluted varies more when pre-mixed with greater volumes of TF solution. Hence, less TF solution causes less dilutional effects to occur. This in turn causes less variation with haematocrit.

3.3. Sensitivity Testing

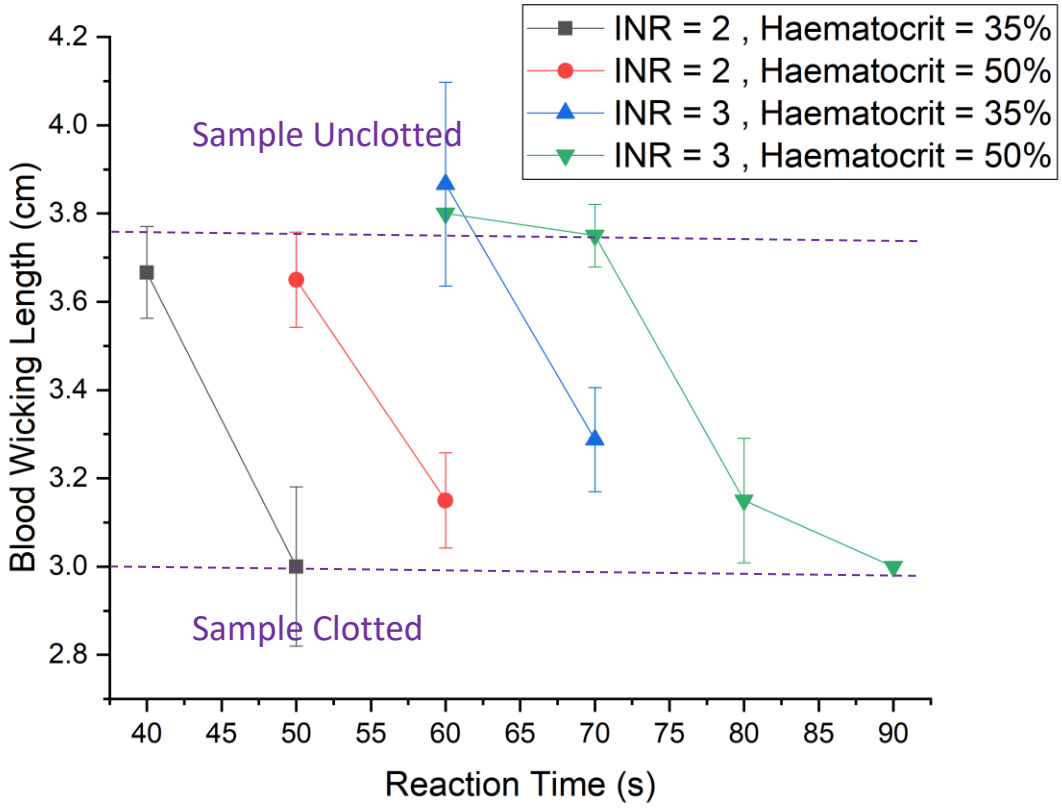


Figure 43: Testing of sensitivity for anticoagulant therapy. Blood wicking length as a function of reaction time for blood samples of different haematocrit and INRs. The ratio of TF solution to whole blood used was 2:1. Each data-point was performed five times and the average and standard deviations are reported.

In this section, we tested the diagnostic's sensitivity for measuring different pathologies. This included anticoagulation monitoring and liver disease. Anticoagulation monitoring requires the diagnostic to accurately determine whether the patient's INR is maintained in-between 2-3. Liver disease monitoring requires the diagnostic to distinguish between a normal INR (ie. 1) from a diseased INR (ie. >1.5). Hence, we tested physiologically relevant blood samples with INRs of 1, 1.7, 2 and 3. Furthermore, the diagnostic's sensitivity may be impacted by the haematocrit. Therefore, we tested physiologically relevant blood samples with normal reference haematocrit ranges (ie. 35-50%) – since they are most likely to be encountered by medical personnel during testing.

The diagnostic can accurately monitor anticoagulant dosage (**Figure 43**). Patients who are at risk of life-threatening thrombosis require anticoagulants - such as warfarin - to keep their INR above 2. However, at the same time, they need to limit their intake of these anticoagulants so that they do not risk haemorrhage. Because of this, their INR also needs to be kept below 3. Hence, it is desired to keep the INR range in-between 2 to 3.

Two factors were considered when monitoring anticoagulant dosage:

1. The test's ability to distinguish between INRs of 2 and 3.
2. The test's ability to work within the normal haematocrit range - ie. 35-50% (see **Figure 42**).

First, the INR was compared (by keeping haematocrit constant). A 20 second clotting time lag is shown between INRs of 2 to 3. This clearly tells patients whether they are overdosed or underdosed.

Second, haematocrit was compared (this time by keeping INR constant). Haematocrit can have an impact on the test (**Figure 42**). Larger haematocrits correlate to longer clotting times. This is because the extra RBCs decreases the volume of plasma, and hence the amount of coagulation factors used in the test. Despite this, only a 10 second clotting time lag is present between haematocrits of 35-50%.

Finally, to stress test the diagnostic, a sample with an INR of 2 and 50% haematocrit was compared to a sample with an INR of 3 and 35% haematocrit. Despite the significantly different haematocrit, a 10 second time lag is still present. Hence, the test is suitable for monitoring anticoagulation.

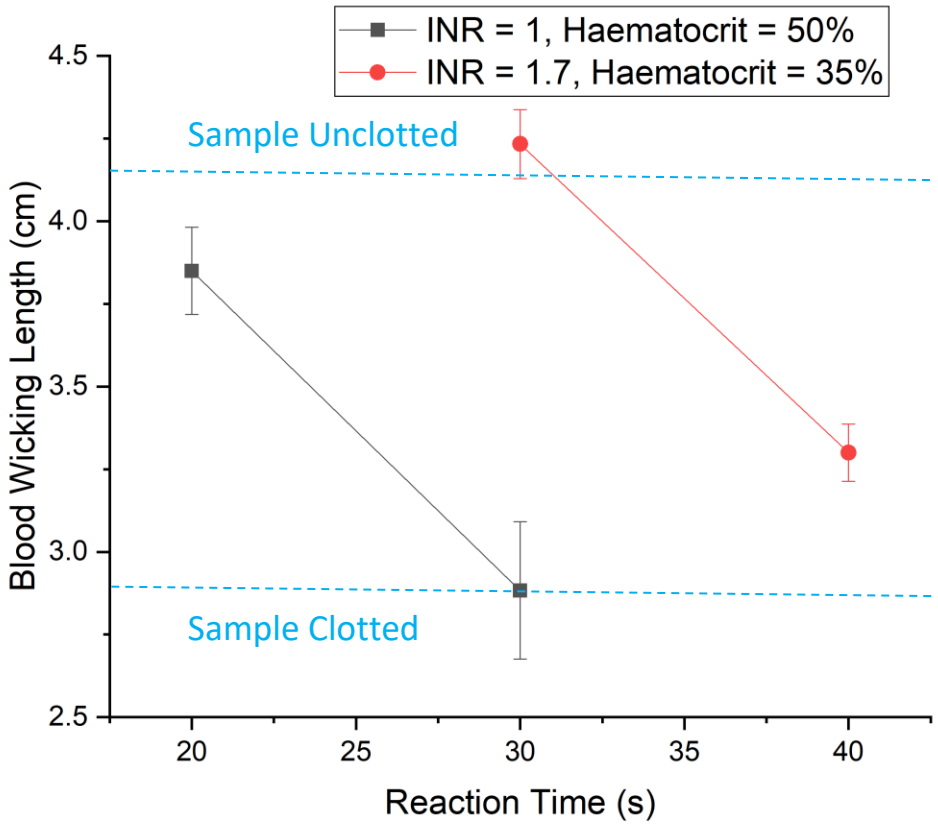


Figure 44: Testing of sensitivity for liver disease monitoring. Blood wicking length as a function of reaction time for blood samples of different haematocrit and INRs. The ratio of TF solution to whole blood used was 2:1. Each data-point was performed in triplicate and the average and standard deviations are reported.

Figure 44 illustrates the test's ability to detect liver disease. The coagulation factor enzymes involved in the extrinsic pathway are synthesised in the liver. Hence, depleted levels in the blood marks liver disease. Liver disease is commonly diagnosed when a patient's INR rises above 1.5 if they have encephalopathy, and 2 if they do not.

Larger haematocrits cause the test to clot slower (see **Figure 42**). Hence, a normal blood sample (of INR: 1) with a 50% haematocrit was compared to an abnormal blood sample (of INR: 1.7) with a 35% haematocrit to stress test.

Despite the difference in haematocrit, the test still displays sensitivity between the two samples - with a clotting time lag of 10 s.

Therefore, the diagnostic can clearly indicate when blood samples have liver disease.

3.4. Effect of Temperature

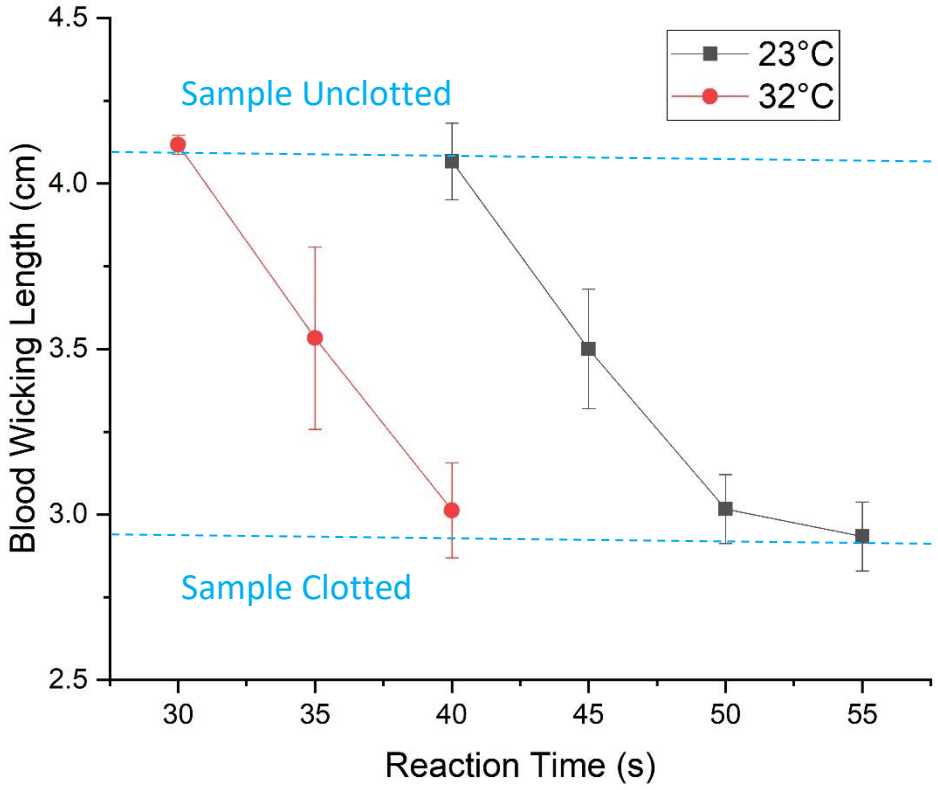


Figure 45 Effect of temperature. Blood wicking length as a function of reaction time. The ratio of TF solution to whole blood used was 2:1. The whole blood sample had a haematocrit and INR of 50% and 1.7, respectively. Each data-point was performed in triplicate and the average and standard deviations are reported.

Figure 45 depicts the effect of temperature on a single blood sample. Much of the developing world lives in warmer climates without access to air conditioning. Hence, the test was performed at a standard Equatorial temperature (ie. 32°C) and compared to standard lab conditions (ie. 23°C).

Warmer temperatures affect the test. As the external temperature increases, wicking becomes throttled. This was thought to be due to either an increase in the reaction rate of the coagulation enzymes or the rate of evaporation. In our previous studies (201), we found that evaporation becomes pronounced if the test wicks on paper for more than 2 min. However, given this test only wicks for 30 s, evaporation is unlikely to have any significant effect (201). Hence, the throttling of wicking is most likely due to the increased kinetics of the coagulation enzymes. As blood coagulation factor enzymes work optimally at 37°C (270), it is expected for clotting to occur faster at 32°C than 23°C.

The test clots 10 s faster at 32°C than it does at 23°C. Therefore, the test needs to be protected from heat. Alternatively, a means of calibrating the test at different working temperatures can also be used.

4. Discussion

We have developed a novel low cost, paper-based and easy to use hand-held prothrombin time (PT) diagnostic that gives results in around a minute. This is the first portable point of care (PoC) PT device that does not require external equipment. This diagnostic can be used to detect liver disease and monitor anticoagulant dosage. First, a whole blood sample is collected. Second, it is pre-mixed with Tissue Factor (TF) - a blood protease - in a PCR tube to initiate clotting. Third, the mixture is deposited as a droplet on a glass slide. Finally, a paper strip is placed on top of the droplet. The wicking of the blood through the paper strip is clearly perturbed when it has clotted. Hence, it can be used to precisely measure whether patients are above or below key prothrombin times (**Figure 41**).

PT is typically measured as an internal normalised ratio (INR) value. The INR is a multiple of the time taken for a patient to clot blood compared to that of a healthy person. For example, a patient with an INR of 2 will take twice as long to clot blood compared to a healthy person. The conversion of PT to INR is summarised in **Equation 4**.

PT is most used to detect liver disease and monitor anticoagulant dosage. Since the coagulation factor enzymes involved in the extrinsic pathway are synthesised in the liver, their depletion commonly marks liver disease. Acute liver failure is detected when the INR of a patient is at least 2. It is reduced to 1.5 if the patient suffers from encephalopathy (271).

Likewise, anticoagulant dosage monitoring is essential for patients at risk of stroke, heart attack or deep vein thrombosis. Globally, 423 million people are diagnosed with these cardiovascular diseases per year (263). Hence, oral anticoagulants are used to prevent these diseases. The most used anticoagulant is warfarin. 15 million Americans alone use warfarin (267). Warfarin works by inhibiting the synthesis of coagulation factor (CF) enzymes produced in the liver. This drops the concentration of these enzymes found in the blood. Hence, when coagulation is induced, patients clot more slowly – thus decreasing the risk of thrombosis. However, warfarin must also be carefully administered to prevent over-dosage and subsequent haemorrhage. In warfarin patients, INRs below 2 are generally considered at risk of thrombosis and thus require increased warfarin dosage. Likewise, INRs above 3 are generally considered at risk of haemorrhage and thus require decreased warfarin dosage (262). Furthermore, in extremely high risk patients – such as those with prosthetic valves – the INR needs to be adjusted between 3 to 4 (272). Whilst we did not test blood with INRs as high as 4, we strongly hypothesise that the test can distinguish between samples in this range also.

The test can clearly detect when patients have acute liver disease and may require transplantation (**Figure 44**). Furthermore, the test can accurately determine whether warfarin patients are at risk of overdosing or underdosing by directly using a whole blood sample (**Figure 43**). This subtracts the usual pre-centrifugation requirement limited to biomedical laboratory settings. Furthermore, due to the test's utilisation of cheap and lightweight materials, it eliminates the prohibitive cost barriers normally associated with the few currently available PoC PT devices such as the Coagucheck.

Therefore, the test can be used in many different testing environments that have not been previously possible. Those include: 1) resource limited settings which do not have accessible electricity, 2) developed nations - such as the USA - where public healthcare is not well subsidised. Hence, it can greatly reduce the burden of the 200 million PT tests that must be performed annually around the world (265).

4.1. Comparison

As our test pre-mixes whole blood samples with TF solution, a clot can clearly be detected the moment the paper strip lands on top of the droplet (**Figure 40**). Li *et al.* also developed a hand-held lateral flow paper diagnostic that monitors coagulation (206). The test takes a whole blood sample, pre-induces clotting, adds the sample to a paper strip and causes wicking to occur. As the sample clots, its viscosity increases and thus its movement down the strip becomes impeded. Hence, the prothrombin time (PT) can be indicated by the distance it travels down the strip. Whilst the test is simple to use, it requires clotting to occur whilst the test is the process of wicking. This creates several limitations.

First, the test of Li *et al.* wicks for a duration of 4 min. From our previous results, this amount of time causes humidity to impact results via drying (201). Hence, Li et al.'s test may experience enhanced rates of blood evaporation in dry conditions and vice versa in humid settings. On the contrary, our test only utilises wicking for 30 s – a time not affected by humidity as it is too short for the paper structure to be influenced.

Second, Li. *et al*'s test is more susceptible to the haematocrit of blood. Hegener *et al.* performed clinical trials of the diagnostic and found that it cannot easily distinguish patients with INRs between 1-3. This is due to the haematocrit of different individuals affecting their blood viscosity (269). On the contrary, our test can clearly distinguish between INRs of 1 to 1.7 over a similarly tested haematocrit range (ie. 15%) (**Figure 44**) - making it suitable for detecting the lower limit of liver disease. Furthermore, it is unaffected by the viscosity of the blood (**Figure 42**). Hence, on its own, our test provides a significantly greater accuracy.

4.2. In-Home Testing Potential

An ideal paper-based whole blood prothrombin time (PT) test is unaffected by temperature and haematocrit. Furthermore, it requires the patient to perform a minimum number of steps (for simplicity) in point of care (PoC) self-testing.

Figure 45 shows that the test is impacted by warmer temperatures (above 30°C). Much of the developing world without electricity lies on the Equator where temperatures commonly exceed 30°C. Therefore, in these environments, the test may need a way to insulate it from heat.

Alternatively, the test can be calibrated at different temperatures. Since warfarin dosage requires keeping the INR within a certain range (rather than to an exact value), calibration is not difficult. Once the temperature is known, the reaction times to produce INRs of 2 and 3 (or any other value) can be easily worked out – without the need of using mathematical equations.

In this study, we were limited to using quality control plasma anticoagulated in citrate. Therefore, we required the use of reagents to initiate clotting. However, a test which does not require premixing with reagents is ideal. This is because it would minimise the number of steps that the test requires.

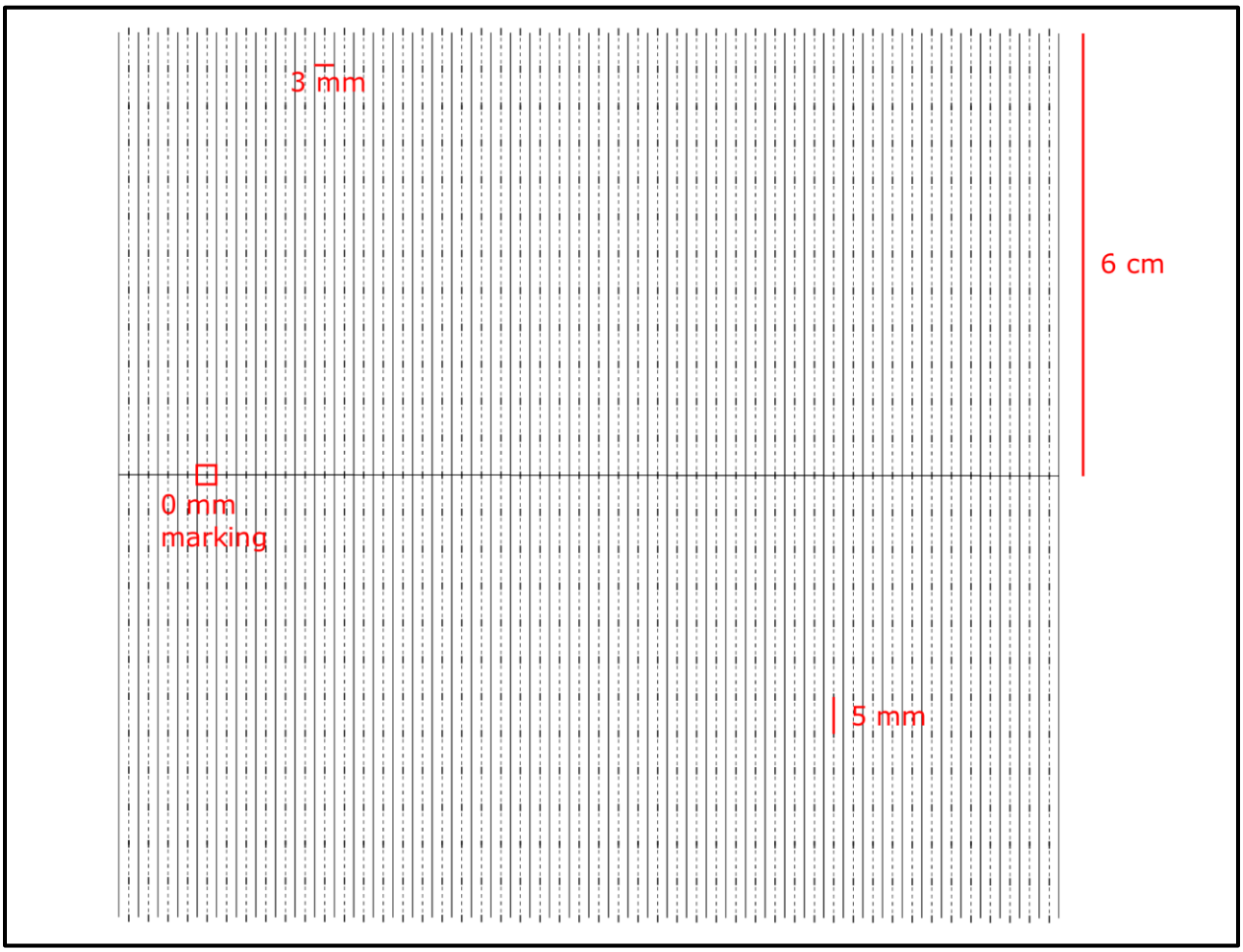
When blood is collected from an individual, the breakage of vessels initiates clotting. This is due to the exposure of endogenous tissue factor (TF) from tissues surrounding the ruptured blood vessels. This means that unless the collected blood is treated with anticoagulants, it does not require any more reagents to form a clot. Therefore, the test can be optimised by collecting whole blood via fingerprick and allowing it to clot on its own – without extra reagents.

The next step to make the diagnostic practical for in-home use should involve the testing of whole blood collected by fingerprick. Any extra steps (such as pre-mixing) may induce user errors and needs to be minimised.

5. Conclusion

We have developed the world's first hand-held and equipment-free paper-based diagnostic suitable for measuring prothrombin time (PT) from whole blood samples. The test can provide accurate results in about a minute. The test works by premixing a blood sample and tissue factor (TF) solution in a PCR tube, depositing it on a glass slide, reacting for a certain period and dropping a paper strip on top of the mixture. A clot is clearly detected when wicking is perturbed through the strip. Hence, it can be used to detect if blood samples are above or below key PTs or internal normalised ratios (INRs). Despite the test being slightly affected by haematocrit (ie. red blood cell concentration), it can still easily distinguish between INR values of 1, 1.5, 2 and 3 in whole blood. These are the values of biomedical importance. Hence, it can be used to detect critical liver disease and monitor anticoagulant drug dosage. Furthermore, the test only utilises 30 s of wicking – a time unaffected by the effects of evaporation. This means that the test can work in a wide range of humidities. However, the test currently clots faster in warm temperatures (ie. 32°C) than it does at room temperature (ie. 23°C). Hence, a means of either insulating the test or calibrating the results of the test is beneficial in warmer geographical settings with no temperature regulation. The test can be used as a point of care (PoC) device in many different testing environments. This includes limited resource settings that do not have the finances or infrastructure to utilise current prothrombin time diagnostics. Therefore each year, it can ease the burden of performing over 200 million PT tests and help save over 20 million lives.

5.1. Paper Strip Cut-Out:



Supplementary Figure 42: Cut-out of Paper Strips showing dimensions, markings and locations. The paper strip cut-out stencil was drawn on CorelDRAW X6 and a 60 Watt Epilog Helix was used to laser cut the paper.

After laser cutting the paper with the stencil used in **Supplementary Figure 42**, paper strips were ripped out individually with tweezers and experimented with.

The 0 mm marking refers to the location where the paper strip was dropped on top of the clotted droplet. The 0 mm marking aligned with the edge of the clotted droplet covering it in whole.

5.2. Creating Plasmas with INRs of 2 and 3:

There were no plasmas with internal normalization ratios (INRs) of 2 and 3 (the key INRs for anticoagulant monitoring) directly available for this study. Therefore, they had to be made by mixing other plasmas to the correct proportions.

The 3 plasmas readily available had of INRs 1 (healthy), 1.7 (slightly abnormal) and 5.2 (highly abnormal). Their prothrombin times (PT) were 12.3 s, 19.6 s and 64.4 s respectively.

The INR is defined as such:

$$INR = \left(\frac{PT_{Sample}}{PT_{Healthy}}\right)^{ISI}$$

Where ISI is the activity of the TF reagent (In this study, ISI = 1.13).

To create INRs of 2 and 3, the prothrombin times needed to be calculated:

$$2 = \left(\frac{PT_{INR=2}}{12.3}\right)^{1.13} \text{and } 3 = \left(\frac{PT_{INR=3}}{12.3}\right)^{1.13}$$

Hence,

$$PT_{INR=2} = 22.7\ sec \text{ and } PT_{INR=3} = 32.5\ sec$$

Prothrombin time is assumed to be inversely related to the concentration of clotting factors (c) in the blood:

$$PT \propto \frac{1}{c}$$

To calculate the proportions of slightly (x) and highly abnormal plasma (y) needed to create plasmas with INRs of 2 and 3, the right concentration of clotting factors had to be achieved:

$$c_{INR=2} = c_{slighly\ abnormal}x_{INR=2} + c_{highly\ abnormal}y_{INR=2}$$

$$c_{INR=3} = c_{slightly\ abnormal}x_{INR=3} + c_{highly\ abnormal}y_{INR=3}$$

As $y = 1 - x$:

$$\frac{1}{PT_{INR=2}} = \frac{1}{PT_{Slightly\ Abnormal}}x_{INR=2} + \frac{1}{PT_{Highly\ Abnormal}}(1 - x_{INR=2})$$

$$\frac{1}{PT_{INR=3}} = \frac{1}{PT_{Slightly\ Abnormal}}x_{INR=3} + \frac{1}{PT_{Highly\ Abnormal}}(1 - x_{INR=3})$$

Hence, by substituting known values, proportions were calculated as such:

Desired INR of Plasma	Proportion of Slightly Abnormal Plasma Required (x)	Proportion of Highly Abnormal Plasma Required (y)
2	0.81	0.19
3	0.43	0.57

5.3. Effect of Haematocrit on Diagnostic:

The clotting time (PT) is dictated by the concentration of coagulation factors (c) in the deposited blood droplet:

$$PT \propto \frac{1}{c_{in\ droplet}}$$

The higher the concentration of clotting factors, the faster the measured clotting time.

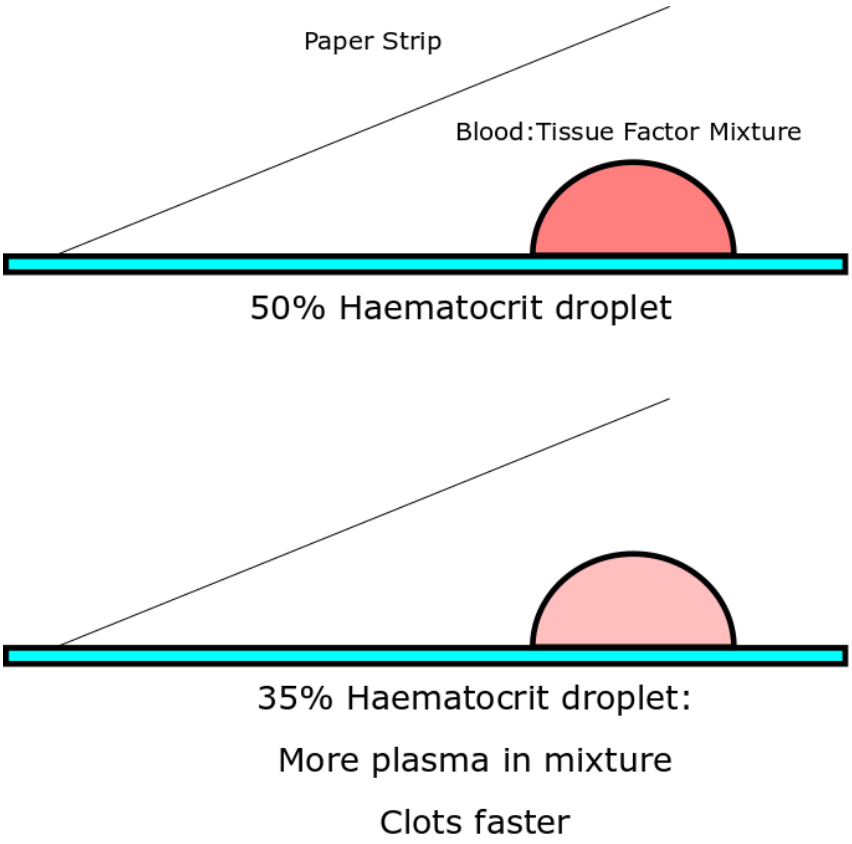
Blood samples with lower haematocrit subsequentially contain more plasma. For example, a blood sample with a 35% haematocrit contains 65% plasma. Likewise, a blood sample with a 50% haematocrit contains 50% plasma.

As a 35% haematocrit blood sample contains 30% more plasma than a 50% haematocrit sample, it also contains 30% more coagulation factors.

After mixing and reacting with tissue factor (TF) solution:

$$1.3 \times PT_{35\%\ Haematocrit} = PT_{50\%\ Haematocrit}$$

Hence, a 50% haematocrit sample is expected to clot 30% slower than a 35% haematocrit sample. **Supplementary Figure 43** illustrates this.



Supplementary Figure 43: Simplified illustration of how lower haematocrits cause faster clotting times. More plasma gets mixed in tissue factor solution thus causing a higher concentration of clotting factors to be present when the mixture is deposited on the diagnostic. This causes the mixture to clot faster.

5.4. Relationship Between the Clotting Time and Droplet Composition:

For simplicity, **Supplementary Section 5.3** assumes that the concentration of coagulation factors in the deposited blood droplet dictates clotting time. However, it does not consider that red blood cells (RBCs) occupy their own volume in the droplet. As the coagulation factors cannot travel into the RBCs, the clotting time (PT) is restricted to the concentration of coagulation factors (c) present in the aqueous phase of the droplet:

$$PT \propto \frac{1}{c_{in\ aqueous\ phase\ of\ droplet}}$$

The concentration is defined as:

$$c_{in\ aqueous\ phase\ of\ droplet} = \frac{m_{coagulation\ factors\ in\ droplet}}{V_{aqueous\ phase\ of\ droplet}}$$

$$c_{in\ aqueous\ phase\ of\ droplet} = \frac{m_{coagulation\ factors\ in\ plasma} + m_{coagulation\ factors\ in\ TF\ reagent}}{V_{Plasma\ in\ droplet} + V_{TF\ Reagent\ in\ droplet}}$$

As the TF reagent contains no known coagulation factors:

$$c_{in\ aqueous\ phase\ of\ droplet} = \frac{m_{coagulation\ factors\ in\ plasma}}{V_{Plasma\ in\ droplet} + V_{TF\ Reagent\ in\ droplet}}$$

Since $m_{coagulation\ factors\ in\ plasma} = V_{Plasma\ in\ droplet} \times c_{Plasma}$

$$c_{in\ aqueous\ phase\ of\ droplet} = \frac{V_{Plasma\ in\ droplet} \times c_{Plasma}}{V_{Plasma\ in\ droplet} + V_{TF\ Reagent\ in\ droplet}}$$

Relating this back to the clotting time:

$$PT \propto \frac{V_{Plasma\ in\ droplet} + V_{TF\ Reagent\ in\ droplet}}{V_{Plasma\ in\ droplet} \times c_{Plasma}}$$

$$PT \propto \frac{1}{c_{Plasma}} + \frac{V_{TF\ Reagent\ in\ droplet}}{V_{Plasma\ in\ droplet} \times c_{Plasma}}$$

The plasma volume ($V_{Plasma\ in\ droplet}$) is related to the whole blood volume ($V_{Blood\ in\ droplet}$) and the haematocrit (h) as such:

$$V_{Plasma\ in\ droplet} = (1 - h)V_{Blood\ in\ droplet}$$

Hence:

$$PT \propto \frac{1}{c_{Plasma}} + \frac{V_{TF\ Reagent\ in\ droplet}}{(1-h)V_{Blood\ in\ droplet} \times c_{Plasma}}$$

The mixing ratio of TF solution to whole blood (r) in the test can be simplified as such:

$$r = \frac{V_{TF\ Reagent\ in\ droplet}}{V_{Blood\ in\ droplet}}$$

$$PT \propto \frac{1}{c_{Plasma}} + \frac{r}{(1-h)c_{Plasma}} \textbf{ (6)}$$

From this equation, larger mixing ratios causes the effect of haematocrit to be enhanced.

Conclusion

In this thesis, we have developed the fundamental understanding and a series of rapid, hand-held, cheap and easy to use paper-based diagnostics that can measure blood fibrinogen concentration in emergency bleeding. Although these tests require extra ergonomic design for clinicians, clinical trials and scaling up of production before they can be of commercial use, they have huge implications.

Major haemorrhage causes over 2 million deaths per year. The clinical assays currently used to measure blood fibrinogen concentrations are unreliable for major haemorrhage. This is because they require large, table bound and expensive electrical instruments - in the setting and expertise of biomedical laboratories. They cannot be taken to the site of haemorrhage to diagnose hypofibrinogenemia. Thus, haemorrhagic patients must be transported to emergency centres with these diagnostics – causing significant time delays until fibrinogen replacement therapy (FRT) is administered.

The use of paper has been recently implemented in diagnostics. This is due to its low cost and weight – combined with its high performance and very reproducible results. This makes it ideal for point of care (PoC) testing. Hence, this thesis explored several concepts for measuring fibrinogen concentrations with paper and subsequentially developing them into diagnostics.

The first diagnostic developed is a vertical wicking test where plasma-wetted paper strips are inserted into an aqueous dye bath and the fibrinogen concentration dictates the length the dye wicks up the strip. The second diagnostic is a horizontal wicking test where plasma-wetted paper strips are oriented horizontally, an aqueous dye is pipetted onto the strips and the fibrinogen concentration of the plasma dictates the wicking length of the dye down the strip. The third diagnostic is another horizontal wicking test where a droplet of whole blood is deposited on a solid surface, a paper strip is placed on top of it and the fibrinogen concentration dictates the distance the blood wicks down.

The first two diagnostics can distinguish low (<2 g/L) from normal fibrinogen concentrations in minutes. They work by reacting the plasma wetted paper strips with thrombin to form fibrin. The conversion of plasma fibrinogen to fibrin significantly increases the hydrophobicity of the paper strip. Therefore, it makes it much harder for the aqueous dye to move through. Hence, higher fibrinogen concentrations cause the aqueous dye to wick to shorter distances.

The third diagnostic invented can work with whole blood. It can easily distinguish fibrinogen concentrations below and above 1.6 g/L in minutes. It works by pre-mixing the blood droplet with a thrombin solution to clot it. The higher the fibrinogen concentration, the more fibrin that is formed and hence the lower the permeability of the droplet. Hence, when the paper strip is placed on top of the droplet, the ability of the blood to wick down the strip is impeded. Because the test utilises pre-mixing, it means that the ratio of blood:thrombin solution in the mixed droplet can be changed. A lower ratio effectively means the blood is pre-diluted. When this occurs, the effect of interfering substances in the blood can be minimised. This includes haematocrit and thrombin inhibitors.

Additionally, the third diagnostic was modified and adapted to measure prothrombin time (PT). PT relates to the time required for a clot to form after blood clotting has been induced. It measures the activity of enzymes involved in the extrinsic pathway of clotting. However, instead of using thrombin as the reagent, it uses tissue factor (TF) instead (to initiate the extrinsic pathway). As the test can clearly tell when a clot has formed, it can distinguish whether the blood droplet is above or below biomedically relevant PTs.

Therefore, the test can perform a variety of services. This includes, informing whether a patient may need a liver transplant or if a patient on anticoagulant medication needs to adjust their dose.

The implications of these diagnostics are huge. They can be used within hospitals or transported to sites of major haemorrhage to directly diagnose hypofibrinogenemia. Hence, patients no longer need to be transported to testing centres for diagnosis. This new generation of PoC tests brings the emergency room to the site of trauma - where it is most needed. This can significantly reduce the time needed until FRT is administered – thus optimising the chances of survival.

Furthermore, as these diagnostics utilise clotting, they can also be used to diagnose other clotting based disorders - such as PT-related diseases. Hence, they can be re-purposed as PoC clotting diagnostics for many different testing environments. This includes resource-limited settings that do not have the finances or infrastructure to utilise current clotting diagnostics. Therefore, each year, it can ease the burden of performing hundreds of millions of clotting tests and help save tens of millions of lives.

References

1. Takada Y. Fibrinogen. In: Schwab M, editor. Encyclopedia of Cancer. Berlin, Heidelberg: Springer Berlin Heidelberg; 2011. p. 1395-7.
2. Zhang Q, Zhou S, Zhou J. Tigecycline treatment causes a decrease in fibrinogen levels. Antimicrobial agents and chemotherapy. 2015;59(3):1650-5.
3. Roach HD, Laufman H. Relationship Between Pulmonary Embolism and Pulmonary Infarction: An Experimental Study. Annals of Surgery. 1955;142(1):82-91.
4. MOSESSON MW. Fibrinogen and fibrin structure and functions. Journal of Thrombosis and Haemostasis. 2005;3(8):1894-904.
5. Chen R, Doolittle RF. γ-γ Cross-linking sites in human and bovine fibrin. Biochemistry. 1971;10(24):4486-91.
6. Doolittle RF, Chen R, Lau F. Hybrid fibrin: proof of the intermolecular nature of γ-γ crosslinking units. Biochemical and Biophysical Research Communications. 1971;44(1):94-100.
7. Siebenlist KR, Mosesson MW. Evidence for Intramolecular Cross-Linked Aα·γ Chain Heterodimers in Plasma Fibrinogen. Biochemistry. 1996;35(18):5817-21.
8. Maurer MC, Trumbo TA, Isetti G, Turner BT. Probing interactions between the coagulants thrombin, Factor XIII, and fibrin(ogen). Archives of Biochemistry and Biophysics. 2006;445(1):36-45.
9. Turner BT, Sabo TM, Wilding D, Maurer MC. Mapping of Factor XIII Solvent Accessibility as a Function of Activation State Using Chemical Modification Methods. Biochemistry. 2004;43(30):9755-65.
10. Schöchl H, Cotton B, Inaba K, Nienaber U, Fischer H, Voelckel W, et al. FIBTEM provides early prediction of massive transfusion in trauma. Critical Care. 2011;15(6):R265.
11. Inaba K, Karamanos E, Lustenberger T, Schöchl H, Shulman I, Nelson J, et al. Impact of fibrinogen levels on outcomes after acute injury in patients requiring a massive transfusion. Journal of the American College of Surgeons. 2013;216(2):290-7.
12. Davenport R. Coagulopathy following major trauma hemorrhage: lytic, lethal and a lack of fibrinogen. Critical Care. 2014;18(3):151.
13. Raza I, Davenport R, Rourke C, Platton S, Manson J, Spoors C, et al. The incidence and magnitude of fibrinolytic activation in trauma patients. Journal of Thrombosis and Haemostasis. 2013;11(2):307-14.
14. Rossaint R, Bouillon B, Cerny V, Coats TJ, Duranteau J, Fernández-Mondéjar E, et al. Management of bleeding following major trauma: an updated European guideline. Critical care. 2010;14(2):R52.
15. Pavord S, Maybury H. How I treat postpartum hemorrhage. Blood. 2015;125(18):2759-70.
16. Rourke C, Curry N, Khan S, Taylor R, Raza I, Davenport R, et al. Fibrinogen levels during trauma hemorrhage, response to replacement therapy, and association with patient outcomes. Journal of Thrombosis and Haemostasis. 2012;10(7):1342-51.
17. Soleimani M, Masoumi N, Nooraei N, Lashay A, Safarinejad M. The effect of fibrinogen concentrate on perioperative bleeding in transurethral resection of the prostate: a double-blind placebo-controlled and randomized study. Journal of Thrombosis and Haemostasis. 2017;15(2):255-62.
18. Wong H, Curry N. Do we need cryoprecipitate in the era of fibrinogen concentrate and other specific factor replacement options? ISBT Science Series. 2018;13(1):23-8.
19. MANCO-JOHNSON MJ, DIMICHELE D, CASTAMAN G, FREMANN S, KNAUB S, KALINA U, et al. Pharmacokinetics and safety of fibrinogen concentrate. Journal of Thrombosis and Haemostasis. 2009;7(12):2064-9.
20. Mackie IJ, Kitchen S, Machin SJ, Lowe GDO, on behalf of the H, Thrombosis Task Force of the British Committee for Standards in H. Guidelines on fibrinogen assays. British Journal of Haematology. 2003;121(3):396-404.

21. Hartert H. Blutgerinnungsstudien mit der Thrombelastographie, einem neuen Untersuchungsverfahren. Klinische Wochenschrift. 1948;26(37):577-83.
22. Chitolie A, Mackie I, Grant D, Hamilton J, Machin S. Inaccuracy of the'derived'fibrinogen measurement. Blood coagulation & fibrinolysis: an international journal in haemostasis and thrombosis. 1994;5(6):955-7.
23. Martinez AW, Phillips ST, Butte MJ, Whitesides GM. Patterned Paper as a Platform for Inexpensive, Low Volume, Portable Bioassays. Angewandte Chemie (International ed in English). 2007;46(8):1318-20.
24. Sher M, Zhuang R, Demirci U, Asghar W. Paper-based analytical devices for clinical diagnosis: recent advances in the fabrication techniques and sensing mechanisms. Expert review of molecular diagnostics. 2017;17(4):351-66.
25. Khan MS, Thouas G, Shen W, Whyte G, Garnier G. Paper diagnostic for instantaneous blood typing. Analytical chemistry. 2010;82(10):4158-64.
26. Then W, Li M, McLiesh H, Shen W, Garnier G. The detection of blood group phenotypes using paper diagnostics. Vox sanguinis. 2015;108(2):186-96.
27. Al-Tamimi M, Shen W, Zeineddine R, Tran H, Garnier G. Validation of Paper-Based Assay for Rapid Blood Typing. Analytical Chemistry. 2012;84(3):1661-8.
28. Mackman N. The Role of Tissue Factor and Factor VIIa in Hemostasis. Anesthesia and analgesia. 2009;108(5):1447-52.
29. Baker RR, Murray RK. PDQ Biochemistry: BC Decker; 2001.
30. Fries D, Martini W. Role of fibrinogen in trauma-induced coagulopathy. British journal of anaesthesia. 2010;105(2):116-21.
31. Hiippala ST, Myllyla GJ, Vahtera EM. Hemostatic factors and replacement of major blood loss with plasma-poor red cell concentrates. Anesthesia & Analgesia. 1995;81(2):360-5.
32. Davenport RA, Brohi K. Cause of trauma-induced coagulopathy. Current Opinion in Anesthesiology. 2016;29(2):212-9.
33. Maegele M, Nardi G, Schöchl H. Hemotherapy algorithm for the management of trauma-induced coagulopathy: the German and European perspective. Current Opinion in Anesthesiology. 2017;30(2):257-64.
34. Cap A, Hunt B. The pathogenesis of traumatic coagulopathy. Anaesthesia. 2015;70(s1):96.
35. Kozek-Langenecker SA, Afshari A, Albaladejo P, Santullano CAA, De Robertis E, Filipescu DC, et al. Management of severe perioperative bleeding: guidelines from the European Society of Anaesthesiology. European Journal of Anaesthesiology (EJA). 2013;30(6):270-382.
36. Sniecinski RM, Levy JH. Bleeding and management of coagulopathy. The Journal of thoracic and cardiovascular surgery. 2011;142(3):662-7.
37. Cleland S, Corredor C, Ye JJ, Srinivas C, McCluskey SA. Massive haemorrhage in liver transplantation: Consequences, prediction and management. World journal of transplantation. 2016;6(2):291.
38. Butwick AJ, Goodnough LT. Transfusion and coagulation management in major obstetric hemorrhage. Current opinion in anaesthesiology. 2015;28(3):275.
39. Collins P, Abdul‐Kadir R, Thachil J. Management of coagulopathy associated with postpartum hemorrhage: guidance from the SSC of the ISTH. Journal of Thrombosis and Haemostasis. 2016;14(1):205-10.
40. Cacciafesta P, Humphris AD, Jandt KD, Miles MJ. Human plasma fibrinogen adsorption on ultraflat titanium oxide surfaces studied with atomic force microscopy. Langmuir. 2000;16(21):8167-75.
41. Henschen A, Lottspeich F, Kehl M, Southan C. Covalent structure of fibrinogen. Annals of the New York Academy of Sciences. 1983;408(1):28-43.

42. Hoeprich Jr PD, Doolittle RF. Dimeric half-molecules of human fibrinogen are joined through disulfide bonds in an antiparallel orientation. Biochemistry. 1983;22(9):2049-55.
43. Huang S, Cao Z, Davie E. The role of amino-terminal disulfide bonds in the structure and assembly of human fibrinogen. Biochemical and biophysical research communications. 1993;190(2):488-95.
44. Cerqueira CF, Klareskog L, Jakobsson PJ. Neutralization of AntiCitrullinated Protein Antibodies in Rheumatoid Arthritis–A Way to Go? Basic & clinical pharmacology & toxicology. 2014;114(1):13-7.
45. Weisel JW, Medved L. The structure and function of the αC domains of fibrinogen. Annals of the New York Academy of Sciences. 2001;936(1):312-27.
46. Hall CE, Slayter HS. The fibrinogen molecule: its size, shape, and mode of polymerization. The Journal of Cell Biology. 1959;5(1):11-27.
47. Cieśla M, Adamczyk Z, Barbasz J, Wasilewska M. Mechanisms of fibrinogen adsorption at solid substrates at lower pH. Langmuir. 2013;29(23):7005-16.
48. Bratek-Skicki A, Żeliszewska P, Ruso JM. Fibrinogen: a journey into biotechnology. Soft matter. 2016;12(42):8639-53.
49. Jung S-Y, Lim S-M, Albertorio F, Kim G, Gurau MC, Yang RD, et al. The Vroman effect: a molecular level description of fibrinogen displacement. Journal of the American Chemical Society. 2003;125(42):12782-6.
50. Wolfenstein-Todel C, Mosesson MW. Carboxy-terminal amino acid sequence of a human fibrinogen. gamma.-chain variant (. gamma.'). Biochemistry. 1981;20(21):6146-9.
51. Meh DA, Siebenlist KR, Brennan SO, Holyst T, Mosesson MW. The amino acid sequence in fibrin responsible for high affinity thrombin binding. Thrombosis and haemostasis. 2001;85(3):470-4.
52. Ismail AE. Purification of fibrinogen from human plasma. 2012.
53. Siebenlist KR, Mosesson MW, Hernandez I, Bush LA, Di Cera E, Shainoff JR, et al. Studies on the basis for the properties of fibrin produced from fibrinogen-containing γ′ chains. Blood. 2005;106(8):2730-6.
54. Siebenlist KR, Meh DA, Mosesson MW. Plasma Factor XIII Binds Specifically to Fibrinogen Molecules Containing γ ‘Chains. Biochemistry. 1996;35(32):10448-53.
55. Muthard RW, Welsh JD, Brass LF, Diamond SL. Fibrin, γ′-Fibrinogen, and Transclot Pressure Gradient Control Hemostatic Clot Growth During Human Blood Flow Over a Collagen/Tissue Factor WoundSignificance. Arteriosclerosis, thrombosis, and vascular biology. 2015;35(3):645-54.
56. Fort A, Borel C, Migliavacca E, Antonarakis SE, Fish RJ, Neerman-Arbez M. Regulation of fibrinogen production by microRNAs. Blood. 2010;116(14):2608-15.
57. Mannila MN, Lovely R, Kazmierczak S, Eriksson P, Samnegård A, Farrell D, et al. Elevated plasma fibrinogen γ′ concentration is associated with myocardial infarction: effects of variation in fibrinogen genes and environmental factors. Journal of Thrombosis and Haemostasis. 2007;5(4):766-73.
58. Mosesson M, Hernandez I, Raife T, Medved L, Yakovlev S, SIMPSON‐HAIDARIS P, et al. Plasma fibrinogen γ′ chain content in the thrombotic microangiopathy syndrome. Journal of Thrombosis and Haemostasis. 2007;5(1):62-9.
59. Gailani D, RennÉ T. The intrinsic pathway of coagulation: a target for treating thromboembolic disease? Journal of Thrombosis and Haemostasis. 2007;5(6):1106-12.
60. Scheraga HA, Laskowski M. The Fibrinogen-Fibrin Conversion11Part of this review was written while one of us (H.A.S.) was at the Carlsberg Laboratorium, Copenhagen, Denmark, as a Fulbright Research Scholar and John Simon Guggenheim Memorial Foundation Fellow. In: Anfinsen CB, Anson ML, Bailey K, Edsall JT, editors. Advances in Protein Chemistry. 12: Academic Press; 1957. p. 1-131.

61. Olexa SA, Budzynski AZ. Evidence for four different polymerization sites involved in human fibrin formation. Proceedings of the National Academy of Sciences of the United States of America. 1980;77(3):1374-8.
62. Spraggon G, Everse SJ, Doolittle RF. Crystal structures of fragment D from human fibrinogen and its crosslinked counterpart from fibrin. Nature. 1997;389(6650):455-62.
63. Pechik I, Yakovlev S, Mosesson MW, Gilliland GL, Medved L. STRUCTURAL BASIS FOR SEQUENTIAL CLEAVAGE OF FIBRINOPEPTIDES UPON FIBRIN ASSEMBLY. Biochemistry. 2006;45(11):3588-97.
64. Veklich YI, Gorkun OV, Medved LV, Nieuwenhuizen W, Weisel JW. Carboxyl-terminal portions of the alpha chains of fibrinogen and fibrin. Localization by electron microscopy and the effects of isolated alpha C fragments on polymerization. J Biol Chem. 1993;268(18):13577-85.
65. Mosesson MW, Hainfeld J, Wall J, Haschemeyer RH. Identification and mass analysis of human fibrinogen molecules and their domains by scanning transmission electron microscopy. Journal of Molecular Biology. 1981;153(3):695-718.
66. Schwartz ML. The subunit structures of human plasma and platelet factor XIII (fibrin stabilizing factor). J Biol Chem. 1971;246:5851-4.
67. Siebenlist K, W Mosesson M, Meh D, Diorio J, M Albrecht R, D Olson J. Coexisting dysfibrinogenemia (γR275C) and factor V Leiden deficiency associated with thromboembolic disease (fibrinogen Cedar Rapids)2000. 293-304 p.
68. Siebenlist K, W Mosesson M. Progressive cross-linking of fibrin γ chains increases resistance to fibrinolysis1994. 28414-9 p.
69. Fraser SR, Booth NA, Mutch NJ. The antifibrinolytic function of factor XIII is exclusively expressed through α₂-antiplasmin cross-linking. Blood. 2011;117(23):6371.
70. Schroeder V, Vuissoz J-M, Caflisch A, Kohler HP. Factor XIII activation peptide is released into plasma upon cleavage by thrombin and shows a different structure compared to its bound form. Thrombosis and Haemostasis. 2007;97(6):890-8.
71. Siebenlist KR, Meh DA, Mosesson MW. Plasma Factor XIII Binds Specifically to Fibrinogen Molecules Containing γ‘ Chains. Biochemistry. 1996;35(32):10448-53.
72. Meh D, Siebenlist K, Brennan S, Holyst T, W Mosesson M. The amino acid sequence in fibrin responsible for high affinity thrombin binding2001. 470-4 p.
73. Lovely R, Moaddel M, Farrell DH. Fibrinogen γ‘ chain binds thrombin exosite II2003. 124-31 p.
74. Siebenlist KR, Meh DA, Mosesson MW. Protransglutaminase (Factor XIII) Mediated Crosslinking of Fibrinogen and Fibrin*. Thrombosis and Haemostasis. 2001;86(5):1221-8.
75. Omarova F, Uitte De Willige S, Ariëns RAS, Rosing J, Bertina RM, Castoldi E. Inhibition of thrombin-mediated factor V activation contributes to the anticoagulant activity of fibrinogen γ′. Journal of Thrombosis and Haemostasis. 2013;11(9):1669-78.
76. Magwenzi SG, Ajjan RA, Standeven KF, Parapia LA, Naseem KM. Factor XIII supports platelet activation and enhances thrombus formation by matrix proteins under flow conditions. Journal of Thrombosis and Haemostasis. 2011;9(4):820-33.
77. Nuyttens BP, Thijs T, Deckmyn H, Broos K. Platelet adhesion to collagen. Thrombosis Research.127:S26-S9.
78. Golebiewska EM, Poole AW. Platelet secretion: From haemostasis to wound healing and beyond. Blood Reviews. 2015;29(3):153-62.
79. Rivas GA, González-Rodríguez J. Calcium binding to human platelet integrin GPIIb/IIIa and to its constituent glycoproteins. Effects of lipids and temperature. Biochemical Journal. 1991;276(Pt 1):35-40.
80. Sims PJ, Ginsberg MH, Plow EF, Shattil SJ. Effect of platelet activation on the conformation of the plasma membrane glycoprotein IIb-IIIa complex. Journal of Biological Chemistry. 1991;266(12):7345-52.

81. Kloczewiak M, Timmons S, Lukas TJ, Hawiger J. Platelet receptor recognition site on human fibrinogen. Synthesis and structure-function relationship of peptides corresponding to the carboxy-terminal segment of the .gamma. chain. Biochemistry. 1984;23(8):1767-74.
82. Mustard JF, Packham MA, Kinlough-Rathbone RL, Perry DW, Regoeczi E. Fibrinogen and ADP-induced platelet aggregation. Blood. 1978;52(2):453.
83. Marguerie GA, Edgington TS, Plow EF. Interaction of fibrinogen with its platelet receptor as part of a multistep reaction in ADP-induced platelet aggregation. Journal of Biological Chemistry. 1980;255(1):154-61.
84. Park K, Mao FW, Park H. Morphological characterization of surface-induced platelet activation. Biomaterials. 1990;11(1):24-31.
85. Rodvien R, Mielke CH. Role of Platelets in Hemostasis and Thrombosis. Western Journal of Medicine. 1976;125(3):181-6.
86. Lüscher EF. Platelets in Haemostasis and Thrombosis*. British Journal of Haematology. 1967;13(1):1-9.
87. Mackman N, Tilley RE, Key NS. Role of the Extrinsic Pathway of Blood Coagulation in Hemostasis and Thrombosis. Arteriosclerosis, Thrombosis, and Vascular Biology. 2007;27(8):1687.
88. Brinkhous K, Read M, Rodman N, Mason R. Need of fibrinogen for thrombin-induced aggregation of porcine platelets. The Journal of laboratory and clinical medicine. 1969;73(6):1000-10.
89. Weinstock N, Ntefidou M, The Isth/Ssc Fibrinogen S, The Gth Fibrinogen Working P. SSC International Collaborative Study to establish the first high fibrinogen plasma reference material for use with different fibrinogen assay techniques. Journal of Thrombosis and Haemostasis. 2006;4(8):1825-7.
90. Ratnoff O, Menzic C. A new method for the determination of fibrinogen in small samples of plasma. Journal of Laboratory and Clinical Medicine. 1951;37:516-20.
91. Jacobsson K. Studies on the determination of fibrinogen in human blood plasma: Carl Bloms Boktryckeri; 1955.
92. Clauss A. Gerinnungsphysiologische schnellmethode zur bestimmung des fibrinogens. Acta haematologica. 1957;17(4):237-46.
93. Vermylen C, De Vreker R, Verstraete M. A rapid enzymatic method for assay of fibrinogen fibrin polymerization time (FPT test). Clinica chimica acta. 1963;8(3):418-24.
94. Laurens N, Koolwijk P, De Maat MPM. Fibrin structure and wound healing. Journal of Thrombosis and Haemostasis. 2006;4(5):932-9.
95. van Oss CJ. Surface properties of fibrinogen and fibrin. Journal of Protein Chemistry. 1990;9(4):487-91.
96. Roberts WW, Lorand L, Mockros L. Viscoelastic properties of fibrin clots. Biorheology. 1973;10(1):29-42.
97. Miesbach W, Schenk J, Alesci S, Lindhoff-Last E. Comparison of the fibrinogen Clauss assay and the fibrinogen PT derived method in patients with dysfibrinogenemia. Thrombosis Research. 2010;126(6):e428-e33.
98. Mackie J, Lawrie A, Kitchen S, Gaffney PJ, Howarth D, D O Lowe G, et al. A Performance Evaluation of Commercial Fibrinogen Reference Preparations and Assays for Clauss and PT-derived Fibrinogen2002. 997-1005 p.
99. Ehret W, Organization WH. Use of Anticoagulants in Diagnostic Laboratory Investigations: World Health Organization; 1999.
100. Seifried E, Oethinger M, Tanswell P, Hoegee-de Nobel H, Nieuwenhuizen W. Studies on the functionality of fibrinogen during rt-PA therapy: results of three different methods of fibrinogen determination. Blood coagulation & fibrinolysis. 1992;3(1):81-8.
101. Lowe GD, Rumley A, Mackie IJ. Plasma fibrinogen. Annals of clinical biochemistry. 2004;41(6):430-40.

102. Brinkhous K. Plasma prothrombin; vitamin K. Medicine. 1940;19(3):329-416.
103. Chantarangkul V, Tripodi A, Mannucci P. Evaluation of a fully automated centrifugal analyzer for performance of hemostasis tests. Clinical chemistry. 1987;33(10):1888-90.
104. Chitolie A, Mackie I, Grant D, L Hamilton J, Machin S. Inaccuracy of the 'derive' fibrinogen measurement1995. 955-7 p.
105. Palareti G, Maccaferri M, Manotti C, Tripodi A, Chantarangkul V, Rodeghiero F, et al. Fibrinogen assays: a collaborative study of six different methods. CISMEL Comitato Italiano per la Standardizzazione dei Metodi in Ematologia e Laboratorio. Clinical chemistry. 1991;37(5):714-9.
106. Chantarangkul V, Tripodi A, Mannucci P. Results of a collaborative study for fibrinogen measurement. Evidence that the use of a common calibrator improves interlaboratory agreement. Blood coagulation & fibrinolysis. 1994;5(5):761-6.
107. Sobas F, Hanss M, Ffrench P, Trzeciak M, Dechavanne M, Negrier C. Human plasma fibrinogen measurement derived from activated partial thromboplastin time clot formation. Blood coagulation & fibrinolysis. 2002;13(1):61-8.
108. De Cristofaro R, Landolfi R. Measurement of plasma fibrinogen concentration by the prothrombin-time-derived method: applicability and limitations. Blood coagulation & fibrinolysis. 1998;9(3):251-9.
109. Xiang L, Luo M, Yan J, Liao L, Zhou W, Deng X, et al. Combined use of Clauss and prothrombin time-derived methods for determining fibrinogen concentrations: Screening for congenital dysfibrinogenemia. Journal of clinical laboratory analysis. 2018;32(4):e22322.
110. Oberfrank S, Drechsel H, Sinn S, Northoff H, Gehring F. Utilisation of Quartz Crystal Microbalance Sensors with Dissipation (QCM-D) for a Clauss Fibrinogen Assay in Comparison with Common Coagulation Reference Methods. Sensors. 2016;16(3):282.
111. Rodgers GM, Garr SB. Comparison of functional and antigenic fibrinogen values from a normal population. Thrombosis research. 1992;68(3):207-10.
112. Krammer B, Anders O, Nagel H-R, Burstein C, Steiner M. Screening of dysfibrinogenaemia using the fibrinogen function versus antigen concentration ratio. Thrombosis Research. 1994;76(6):577-9.
113. Fredericq L. Recherches sur la coagulation du sang. Première partie. Bulletin de l'Académie Royale des Sciences, des Lettres et des Beaux-Arts de Belgique Sciences. 1877;44(7):56.
114. Schulz F. Eine einfache Bewertungsmethode von Leberparenchymschäden (volumetrische Fibrinbogenbestimmung). Acta Hepatol. 1955;3:306-13.
115. Whipple GH, Hurwitz SH. FIBRINOGEN OF THE BLOOD AS INFLUENCED BY THE LIVER NECROSIS OF CHLOROFORM POISONING. The Journal of Experimental Medicine. 1911;13(1):136-61.
116. Gram H. A new method for the determination of the fibrin percentage in blood and plasma. Journal of Biological Chemistry. 1921;49(2):279-95.
117. Goodwin JF. An evaluation of technics for the separation and estimation of plasma fibrinogen. Clinical chemistry. 1965;11(1):63-73.
118. Low E, Hill H, Searcy R. Simple method for detection of abnormal plasma fibrinogen levels. Technical bulletin of the Registry of Medical Technologists. 1967;37(3):72-4.
119. Foster JBT, Denatale A, Dotti LB. Determination of Plasma Fibrinogen by Means of Centrifugation After Heating. American Journal of Clinical Pathology. 1959;31(1):42-5.
120. Thorp JM, Horsfall GB, Stone MC. A new red-sensitive micronephelometer. Medical and biological engineering. 1967;5(1):51-6.
121. Desvignes P, Bonnet P. Direct determination of plasma fibrinogen levels by heat precipitation. A comparison of the technique against thrombin clottable fibrinogen with spectrophotometry and radial immuno-diffusion. Clinica Chimica Acta. 1981;110(1):9-17.
122. Rice EW, Muesse D. Clinical nephelometry: 1. New, accurate, heat-precipitation nephelometric method for rapid determination of plasma fibrinogen. Clinical chemistry. 1972;18(1):73-5.

123. Parfentjev IA, Johnson ML, Cliffton EE. The determination of plasma fibrinogen by turbidity with ammonium sulfate. Archives of Biochemistry and Biophysics. 1953;46(2):470-80.
124. Exner T, Burridge J, Power P, Rickard KA. An evaluation of currently available methods for plasma fibrinogen. American journal of clinical pathology. 1979;71(5):521-7.
125. Kazal LA, Amsel S, Miller OP, Tocantins LM. The Preparation and Some Properties of Fibrinogen Precipitated from Human Plasma by Glycine. Proceedings of the Society for Experimental Biology and Medicine. 1963;113(4):989-94.
126. Liu D, Hao L, Fang K. Adsorption of cationic copolymer nanospheres onto cotton fibers investigated by a facile nephelometry. Colloids and Surfaces A: Physicochemical and Engineering Aspects. 2014;452:82-8.
127. Smith BP. Large Animal Internal Medicine-E-Book: Elsevier Health Sciences; 2014.
128. Thrall MA, Weiser G, Allison R, Campbell T. Veterinary hematology and clinical chemistry: John Wiley & Sons; 2012.
129. Walton RM. Equine clinical pathology: John Wiley & Sons; 2013.
130. Bassert JM, Thomas J. McCurnin's Clinical Textbook for Veterinary Technicians-E-Book: Elsevier Health Sciences; 2014.
131. Reed SM, Bayly WM, Sellon DC. Equine Internal Medicine-E-Book: Elsevier Health Sciences; 2009.
132. Dintenfass L, Kammer S. Re-evaluation of heat precipitation method for plasma fibrinogen estimation: effect of abnormal proteins and plasma viscosity. Journal of Clinical Pathology. 1976;29(2):130-4.
133. Grannis GF. Plasma fibrinogen: determination, normal values, physiopathologic shifts, and fluctuations. Clinical chemistry. 1970;16(6):486-94.
134. Ouchterlony O. Antigen—antibody reactions in gels. APMIS. 1949;26(4):507-15.
135. Mancini G, Carbonara AO, Heremans JF. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry. 1965;2(3):235-IN6.
136. Brittin G, Rafinia H, Raval D, Werner M, Brown D. Evaluation of single radial immunodiffusion for quantitation of plasma fibrinogen. American journal of clinical pathology. 1972;57(1):89-94.
137. Hoegee-de Nobel E, Voskuilen M, Briet E, Brommer E, Nieuwenhuizen W. A monoclonal antibody-based quantitative enzyme immunoassay for the determination of plasma fibrinogen concentrations. Thrombosis and haemostasis. 1988;60(3):415-8.
138. Kolar OJ, Dyken ML. Fibrinogen Concentration and Immunoelectrophoresis in the Internal Jugular Vein and the Corresponding Arterial Blood in Ischemic Brain Afflictions. Stroke. 1973;4(5):807.
139. Cremer P, Nagel D, Labrot B, Mann H, Muche R, Elster H, et al. Lipoprotein Lp (a) as predictor of myocardial infarction in comparison to fibrinogen, LDL cholesterol and other risk factors: results from the prospective Göttingen Risk Incidence and Prevalence Study (GRIPS). European journal of clinical investigation. 1994;24(7):444-53.
140. Hoffmann J, Verhappen M. Automated nephelometry of fibrinogen: analytical performance and observations during thrombolytic therapy. Clinical chemistry. 1988;34(10):2135-40.
141. Jelić-Ivanović Z, Pevcević N. Fibrinogen determination by five methods in patients receiving streptokinase therapy. Clinical chemistry. 1990;36(4):698-9.
142. Kang YG, Martin DJ, Marquez J, Lewis JH, Bontempo FA, Shaw BW, et al. Intraoperative Changes in Blood Coagulation and Thrombelastographic Monitoring in Liver Transplantation. Anesthesia and analgesia. 1985;64(9):888-96.
143. Luddington RJ. Thrombelastography/thromboelastometry. Clinical & Laboratory Haematology. 2005;27(2):81-90.

144. Da Luz LT, Nascimento B, Shankarakutty AK, Rizoli S, Adhikari NK. Effect of thromboelastography (TEG®) and rotational thromboelastometry (ROTEM®) on diagnosis of coagulopathy, transfusion guidance and mortality in trauma: descriptive systematic review. Critical Care. 2014;18(5):518.
145. Shore-Lesserson L, Manspeizer HE, DePerio M, Francis S, Vela-Cantos F, Ergin MA. Thromboelastography-guided transfusion algorithm reduces transfusions in complex cardiac surgery. Anesthesia & Analgesia. 1999;88(2):312-9.
146. Armstrong S, Fernando R, Ashpole K, Simons R, Columb M. Assessment of coagulation in the obstetric population using ROTEM® thromboelastometry. International Journal of Obstetric Anesthesia. 2011;20(4):293-8.
147. Innerhofer P, Streif W, Kühbacher G, Fries D. Monitoring of Perioperative Dilutional Coagulopathy Using the ROTEM® Analyzer: Basic Principles and Clinical Examples. Transfusion Medicine and Hemotherapy. 2004;31(4):244-9.
148. Gottumukkala VN, Sharma SK, Philip J. Assessing platelet and fibrinogen contribution to clot strength using modified thromboelastography in pregnant women. Anesthesia & Analgesia. 1999;87(6):1453.
149. Khurana S, Mattson JC, Westley S, O'neill WW, Timmis GC, Safian RD. Monitoring platelet glycoprotein IIb/IIIa—fibrin interaction with tissue factor—activated thromboelastography. Journal of Laboratory and Clinical Medicine. 1997;130(4):401-11.
150. Hänecke P, Klouche M. Thrombelastography Today: Practicability and Analytical Power. Transfusion Medicine and Hemotherapy. 2007;34(6):421-8.
151. Solomon C, Sørensen B, Hochleitner G, Kashuk J, Ranucci M, Schöchl H. Comparison of Whole Blood Fibrin-Based Clot Tests in Thrombelastography and Thromboelastometry. Anesthesia & Analgesia. 2012;114(4):721-30.
152. Sankarankutty A, Nascimento B, Teodoro da Luz L, Rizoli S. TEG® and ROTEM® in trauma: similar test but different results? World Journal of Emergency Surgery. 2012;7(1):S3.
153. Huissoud C, Carrabin N, Benchaib M, Fontaine O, Levrat A, Massignon D, et al. Coagulation assessment by rotation thrombelastometry in normal pregnancy. Thrombosis and haemostasis. 2009;102(04):755-61.
154. Schöchl H, Forster L, Woidke R, Solomon C, Voelckel W. Use of rotation thromboelastometry (ROTEM®) to achieve successful treatment of polytrauma with fibrinogen concentrate and prothrombin complex concentrate. Anaesthesia. 2010;65(2):199-203.
155. Lang T, Toller W, Gütl M, Mahla E, Metzler H, Rehak P, et al. Different effects of abciximab and cytochalasin D on clot strength in thrombelastography. Journal of Thrombosis and Haemostasis. 2004;2(1):147-53.
156. Ågren A, Wikman AT, Östlund A, Edgren G. TEG® functional fibrinogen analysis may overestimate fibrinogen levels. Anesthesia & Analgesia. 2014;118(5):933-5.
157. Whiting P, Al M, Westwood M, Ramos IC, Ryder S, Armstrong N, et al. Viscoelastic point-of-care testing to assist with the diagnosis, management and monitoring of haemostasis: a systematic review and cost-effectiveness analysis. 2015.
158. Winearls J, Reade M, Miles H, Bulmer A, Campbell D, Görlinger K, et al. Targeted coagulation management in severe trauma: the controversies and the evidence. Anesthesia & Analgesia. 2016;123(4):910-24.
159. Schöchl H, Nienaber U, Maegele M, Hochleitner G, Primavesi F, Steitz B, et al. Transfusion in trauma: thromboelastometry-guided coagulation factor concentrate-based therapy versus standard fresh frozen plasma-based therapy. Critical Care. 2011;15(2):R83.
160. Schlimp CJ, Voelckel W, Inaba K, Maegele M, Schöchl H. Impact of fibrinogen concentrate alone or with prothrombin complex concentrate (+/− fresh frozen plasma) on plasma fibrinogen level and

fibrin-based clot strength (FIBTEM) in major trauma: a retrospective study. Scandinavian journal of trauma, resuscitation and emergency medicine. 2013;21(1):74.
161. David J-S, Durand M, Levrat A, Lefevre M, Rugeri L, Geay-Baillat M-O, et al. Correlation between laboratory coagulation testing and thromboelastometry is modified during management of trauma patients. Journal of Trauma and Acute Care Surgery. 2016;81(2):319-27.
162. Wikkelsø A, Wetterslev J, Møller AM, Afshari A. Thromboelastography (TEG) or thromboelastometry (ROTEM) to monitor haemostatic treatment versus usual care in adults or children with bleeding. The Cochrane Library. 2016.
163. Haas T, Fries D, Tanaka K, Asmis L, Curry N, Schöchl H. Usefulness of standard plasma coagulation tests in the management of perioperative coagulopathic bleeding: is there any evidence? British journal of anaesthesia. 2015;114(2):217-24.
164. Patil V, Shetmahajan M. Massive transfusion and massive transfusion protocol. Indian Journal of Anaesthesia. 2014;58(5):590-5.
165. Holcomb JB, Gumbert S. Potential value of protocols in substantially bleeding trauma patients. Current Opinion in Anesthesiology. 2013;26(2):215-20.
166. Dutton R. Management of traumatic haemorrhage–the US perspective. Anaesthesia. 2015;70(s1):108.
167. Schöchl H, Voelckel W, Schlimp C. Management of traumatic haemorrhage–the European perspective. Anaesthesia. 2015;70(s1):102.
168. Repine TB, Perkins JG, Kauvar DS, Blackborne L. The use of fresh whole blood in massive transfusion. Journal of Trauma and Acute Care Surgery. 2006;60(6):S59-S69.
169. Stanworth S, Davenport R, Curry N, Seeney F, Eaglestone S, Edwards A, et al. Mortality from trauma haemorrhage and opportunities for improvement in transfusion practice. British Journal of Surgery. 2016;103(4):357-65.
170. Schöchl H, Maegele M, Voelckel W. Fixed ratio versus goal-directed therapy in trauma. Current Opinion in Anesthesiology. 2016;29(2):234-44.
171. Schöchl H, Maegele M, Solomon C, Görlinger K, Voelckel W. Early and individualized goal-directed therapy for trauma-induced coagulopathy. Scandinavian journal of trauma, resuscitation and emergency medicine. 2012;20(1):15.
172. Pandey S, Vyas GN. Adverse effects of plasma transfusion. Transfusion. 2012;52(s1).
173. Heddle NM, Klama L, Singer J, Richards C, Fedak P, Walker I, et al. The role of the plasma from platelet concentrates in transfusion reactions. New England Journal of Medicine. 1994;331(10):625-8.
174. RAHE‐MEYER N, Sørensen B. Fibrinogen concentrate for management of bleeding. Journal of Thrombosis and Haemostasis. 2011;9(1):1-5.
175. Sørensen B, Bevan D. A critical evaluation of cryoprecipitate for replacement of fibrinogen. British journal of haematology. 2010;149(6):834-43.
176. Innerhofer P, Fries D, Mittermayr M, Innerhofer N, von Langen D, Hell T, et al. Reversal of trauma-induced coagulopathy using first-line coagulation factor concentrates or fresh frozen plasma (RETIC): a single-centre, parallel-group, open-label, randomised trial. The Lancet Haematology. 2017.
177. Ponschab M, Schöchl H, Gabriel C, Süssner S, Cadamuro J, Haschke-Becher E, et al. Haemostatic profile of reconstituted blood in a proposed 1:1:1 ratio of packed red blood cells, platelet concentrate and four different plasma preparations. Anaesthesia. 2015;70(5):528-36.
178. Tisherman SA. Is fibrinogen the answer to coagulopathy after massive transfusions? Crit Care. 2010;14(3):154-.
179. Kozek-Langenecker S. Clinical efficacy of fresh frozen plasma compared with coagulation factor concentrates for treating coagulopathy in patients with massive bleeding. Medicina intensiva. 2016;40(6):371-3.

180. Holcomb JB, Tilley BC, Baraniuk S, Fox EE, Wade CE, Podbielski JM, et al. Transfusion of Plasma, Platelets, and Red Blood Cells in a 1:1:1 vs a 1:1:2 Ratio and Mortality in Patients With Severe Trauma: The PROPPR Randomized Clinical Trial. JAMA. 2015;313(5):471-82.
181. Lancé MD. A general review of major global coagulation assays: thrombelastography, thrombin generation test and clot waveform analysis. Thrombosis journal. 2015;13(1):1.
182. Haas T, Fries D, Tanaka K, Asmis L, Curry N, Schöchl H. Usefulness of standard plasma coagulation tests in the management of perioperative coagulopathic bleeding: is there any evidence? British journal of anaesthesia. 2014;114(2):217-24.
183. Fries D, Innerhofer P, Perger P, Gütl M, Heil S, Hofmann N, et al. Coagulation management in trauma-related massive bleeding.-Recommendations of the Task Force for Coagulation (AGPG) of the Austrian Society of Anesthesiology, Resuscitation and Intensive Care Medicine (OGARI). Anasthesiologie, Intensivmedizin, Notfallmedizin, Schmerztherapie: AINS. 2010;45(9):552-61.
184. Nuttall GB, C; Connis, R; Gessner, J; Harrison, C; Miller, R; Niickinovich, D; Nussmeier, N; Rosenberg, A; Spence, R. Practice Guidelines for Perioperative Blood Transfusion and Adjuvant Therapies An Updated Report by the American Society of Anesthesiologists Task Force on Perioperative Blood Transfusion and Adjuvant Therapies. Anesthesiology. 2006;105(1):198-208.
185. Spahn DR, Bouillon B, Cerny V, Coats TJ, Duranteau J, Fernández-Mondéjar E, et al. Management of bleeding and coagulopathy following major trauma: an updated European guideline. Critical Care. 2013;17(2):R76.
186. Gaarder C, Naess P, Christensen EF, Hakala P, Handolin L, Heier H, et al. Scandinavian Guidelines—"The massively bleeding patient". Scandinavian Journal of Surgery. 2008;97(1):15-36.
187. Levy JH, Goodnough LT. How I use fibrinogen replacement therapy in acquired bleeding. Blood. 2015;125(9):1387-93.
188. Rizoli S, Min A, Perez Sanchez A, Shek P, Grodecki R, Veigas P, et al. In trauma, conventional ROTEM and TEG results are not interchangeable but are similar in clinical applicability. Military medicine. 2016;181(5S):117-26.
189. Gonzalez E, Moore EE, Moore HB, Chapman MP, Chin TL, Ghasabyan A, et al. Goal-directed hemostatic resuscitation of trauma-induced coagulopathy: a pragmatic randomized clinical trial comparing a viscoelastic assay to conventional coagulation assays. Annals of surgery. 2016;263(6):1051.
190. Hagemo JS. Prehospital detection of traumatic coagulopathy. Transfusion. 2013;53(S1):48S-51S.
191. Rossaint R, Bouillon B, Cerny V, Coats TJ, Duranteau J, Fernández-Mondéjar E, et al. The European guideline on management of major bleeding and coagulopathy following trauma. Critical care. 2016;20(1):100.
192. Cothren CC, Moore EE, Hedegaard HB, Meng K. Epidemiology of urban trauma deaths: a comprehensive reassessment 10 years later. World journal of surgery. 2007;31(7):1507-11.
193. Norton R, Kobusingye O. Injuries. New England Journal of Medicine. 2013;368(18):1723-30.
194. Castellucci LA, Shaw J, Le Gal G, Hawken S, McIntyre L, Carrier M. Delayed Major Bleeding in Critically Ill Trauma Patients. Am Soc Hematology; 2015.
195. Jacob M, Kumar P. The challenge in management of hemorrhagic shock in trauma. Medical Journal, Armed Forces India. 2014;70(2):163-9.
196. Floccard B, Rugeri L, Faure A, Saint Denis M, Boyle EM, Peguet O, et al. Early coagulopathy in trauma patients: an on-scene and hospital admission study. Injury. 2012;43(1):26-32.
197. McQuilten ZK, Wood EM, Bailey M, Cameron PA, Cooper DJ. Fibrinogen is an independent predictor of mortality in major trauma patients: A five-year statewide cohort study. Injury. 2017;48(5):1074-81.
198. Ogawa S, Tanaka KA, Nakajima Y, Nakayama Y, Takeshita J, Arai M, et al. Fibrinogen measurements in plasma and whole blood: a performance evaluation study of the dry-hematology system. Anesthesia & Analgesia. 2015;120(1):18-25.

199. Bialkower M, McLiesh H, Manderson C, Tabor R, Garnier G. Rapid Paper Diagnostic for Plasma Fibrinogen Concentration. Analyst. 2019;144:4848 - 57.
200. Bialkower M, McLiesh H, Manderson CA, Tabor RF, Garnier G. Rapid, hand-held paper diagnostic for measuring Fibrinogen Concentration in blood. Analytica Chimica Acta. 2020;1102:72-83.
201. Bialkower M, Manderson CA, McLiesh H, Tabor RF, Garnier G. Paper Diagnostic for Direct Measurement of Fibrinogen Concentration in Whole Blood. ACS Sensors. 2020.
202. Millar H, Simpson J, Stalker A. An evaluation of the heat precipitation method for plasma fibrinogen estimation. Journal of Clinical Pathology. 1971;24(9):827-30.
203. Holcomb JB, Del Junco DJ, Fox EE, Wade CE, Cohen MJ, Schreiber MA, et al. The prospective, observational, multicenter, major trauma transfusion (PROMMTT) study: comparative effectiveness of a time-varying treatment with competing risks. JAMA surgery. 2013;148(2):127-36.
204. Stinger HK, Spinella PC, Perkins JG, Grathwohl KW, Salinas J, Martini WZ, et al. The ratio of fibrinogen to red cells transfused affects survival in casualties receiving massive transfusions at an army combat support hospital. Journal of Trauma and Acute Care Surgery. 2008;64(2):S79-S85.
205. Baraniuk S, Tilley BC, Del Junco DJ, Fox EE, Van Belle G, Wade CE, et al. Pragmatic randomized optimal platelet and plasma ratios (PROPPR) trial: design, rationale and implementation. Injury. 2014;45(9):1287-95.
206. Li H, Han D, Pauletti G, Steckl A. Blood coagulation screening using a paper-based microfluidic lateral flow device. Lab on a Chip. 2014;14(20):4035-41.
207. Guan L, Tian J, Cao R, Li M, Cai Z, Shen W. Barcode-Like Paper Sensor for Smartphone Diagnostics: An Application of Blood Typing. Analytical Chemistry. 2014;86(22):11362-7.
208. Merrell K, Southwick K, Graves SW, Esplin MS, Lewis NE, Thulin CD. Analysis of Low-Abundance, Low-Molecular-Weight Serum Proteins Using Mass Spectrometry. Journal of Biomolecular Techniques : JBT. 2004;15(4):238-48.
209. Washburn EW. The Dynamics of Capillary Flow. Physical Review. 1921;17(3):273-83.
210. Laplace P. Treatise on capillary action. Mechanique Celleste, Supplement to Book. 1806;10:1806.
211. Ranucci M, Laddomada T, Ranucci M, Baryshnikova E. Blood viscosity during coagulation at different shear rates. Physiol Rep. 2014;2(7):e12065.
212. Modaressi H, Garnier G. Mechanism of Wetting and Absorption of Water Droplets on Sized Paper: Effects of Chemical and Physical Heterogeneity. Langmuir. 2002;18(3):642-9.
213. Yuan Y, Lee TR. Contact angle and wetting properties. Surface science techniques: Springer; 2013. p. 3-34.
214. Su J, Al-Tamimi M, Garnier G. Engineering paper as a substrate for blood typing bio-diagnostics. Cellulose. 2012;19(5):1749-58.
215. J. MI, Steven K, J. MS, O. LGD. Guidelines on fibrinogen assays. British Journal of Haematology. 2003;121(3):396-404.
216. Ortmann E, Rubino A, Altemimi B, Collier T, Besser MW, Klein AA. Validation of viscoelastic coagulation tests during cardiopulmonary bypass. Journal of Thrombosis and Haemostasis. 2015;13(7):1207-16.
217. Biggs R, Douglas AS, Macfarlane RG. The formation of thromboplastin in human blood. The Journal of Physiology. 1953;119(1):89-101.
218. Hertaeg MJ, Tabor RF, Garnier G. Effect of Protein Adsorption on the Radial Wicking of Blood Droplets in Paper. Journal of colloid and interface science. 2018.
219. Késmárky G, Kenyeres P, Rábai M, Tóth K. Plasma viscosity: a forgotten variable. Clinical hemorheology and microcirculation. 2008;39(1–4):243-6.
220. Li H, Han D, Pauletti GM, Steckl AJ. Blood coagulation screening using a paper-based microfluidic lateral flow device. Lab on a Chip. 2014;14(20):4035-41.

221. Stang LJ, Mitchell LG. Fibrinogen. In: Monagle P, editor. Haemostasis: Methods and Protocols. Totowa, NJ: Humana Press; 2013. p. 181-92.
222. Mark T. Cunningham, John D. Olson, Wayne L. Chandler, Elizabeth M. Van Cott, Charles S. Eby, Jun Teruya, et al. External Quality Assurance of Fibrinogen Assays Using Normal Plasma: Results of the 2008 College of American Pathologists Proficiency Testing Program in Coagulation. Archives of Pathology & Laboratory Medicine. 2012;136(7):789-95.
223. Patel S, Patel N. Warfarin. StatPearls. 2019.
224. Rosen R. Heparin and warfarin: use of anticoagulants in the prevention and treatment of venous thrombosis and pulmonary embolism. The Journal of family practice. 1979;8(5):923-7.
225. Buyue Y, Misenheimer TM, Sheehan JP. Low molecular weight heparin inhibits plasma thrombin generation via direct targeting of factor IXa: contribution of the serpin-independent mechanism. J Thromb Haemost. 2012;10(10):2086-98.
226. Mischke R, Wolling H. Influence of fibrinogen degradation products on thrombin time, activated partial thromboplastin time and prothrombin time of canine plasma. Pathophysiology of Haemostasis and Thrombosis. 2000;30(3):123-30.
227. Smythe MA, Priziola J, Dobesh PP, Wirth D, Cuker A, Wittkowsky AK. Guidance for the practical management of the heparin anticoagulants in the treatment of venous thromboembolism. J Thromb Thrombolysis. 2016;41(1):165-86.
228. Jin L, Abrahams JP, Skinner R, Petitou M, Pike RN, Carrell RW. The anticoagulant activation of antithrombin by heparin. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(26):14683-8.
229. Gray E, Hogwood J, Mulloy B. The Anticoagulant and Antithrombotic Mechanisms of Heparin. In: Lever R, Mulloy B, Page CP, editors. Heparin - A Century of Progress. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012. p. 43-61.
230. Mitra B, Charters KE, Spencer JC, Fitzgerald MC, Cameron PA. Alcohol intoxication in non-motorised road trauma. Emergency Medicine Australasia. 2017;29(1):96-100.
231. Howard BM, Kornblith LZ, Redick BJ, Vilardi RF, Balhotra KS, Crane JM, et al. The effects of alcohol on coagulation in trauma patients: interpreting thrombelastography with caution. Journal of trauma and acute care surgery. 2014;77(6):865-72.
232. de Meyer F, Smit B. Effect of cholesterol on the structure of a phospholipid bilayer. Proceedings of the National Academy of Sciences. 2009;106(10):3654.
233. Diakoumakou O, Hatzigeorgiou G, Gontoras N, Boutsikou M, Kolovou V, Mavrogeni S, et al. Severe/extreme hypertriglyceridemia and LDL apheretic treatment: review of the literature, original findings. Cholesterol. 2014;2014.
234. Lim S. Metabolic acidosis. Acta Med Indones. 2007;39(3):145-50.
235. Aldrich S. Thrombin from Human Plasma: Product Information. 2017.
236. Kottke‐Marchant K. Diagnostic approach to microangiopathic hemolytic disorders. International journal of laboratory hematology. 2017;39:69-75.
237. Mansour MM, Azzazy HM, Kazmierczak SC. Correction factors for estimating potassium concentrations in samples with in vitro hemolysis: a detriment to patient safety. Archives of pathology & laboratory medicine. 2009;133(6):960-6.
238. Korte W, Poon MC, Iorio A, Makris M. Thrombosis in Inherited Fibrinogen Disorders. Transfusion Medicine and Hemotherapy. 2017;44(2):70-6.
239. Seegers WH, Miller KD, Andrews EB, Murphy RC. Fundamental interactions and effect of storage, ether, adsorbants and blood clotting on plasma antithrombin activity. American Journal of Physiology-Legacy Content. 1952;169(3):700-11.
240. Biggs R, Denson K, Akman N, Borrett R, Hadden M. Antithrombin III, antifactor Xa and heparin. British Journal of Haematology. 1970;19(3):283-305.

241. Lominadze D, Dean WL. Involvement of fibrinogen specific binding in erythrocyte aggregation. FEBS Lett. 2002;517(1-3):41-4.
242. Yeromonahos C, Marlu R, Polack B, Caton F. Antithrombin-Independent Effects of Heparins on Fibrin Clot Nanostructure. Arteriosclerosis, Thrombosis, and Vascular Biology. 2012;32(5):1320-4.
243. Varin R, Mirshahi S, Mirshahi P, Klein C, Jamshedov J, Chidiac J, et al. Whole blood clots are more resistant to lysis than plasma clots - greater efficacy of rivaroxaban. Thrombosis Research. 2013;131(3):e100-e9.
244. van Gelder JM, Nair CH, Dhall DP. The significance of red cell surface area to the permeability of fibrin network. Biorheology. 1994;31(3):259-75.
245. Li H, Han D, Hegener MA, Pauletti GM, Steckl AJ. Flow reproducibility of whole blood and other bodily fluids in simplified no reaction lateral flow assay devices. Biomicrofluidics. 2017;11(2):024116.
246. Nijboer J, Horst I, Hendriks H, Duis H-J, Nijsten M. Myth or Reality: Hematocrit and Hemoglobin Differ in Trauma. The Journal of trauma. 2007;62:1310-2.
247. Perel A. Iatrogenic hemodilution: a possible cause for avoidable blood transfusions? Crit Care. 2017;21(1):291-.
248. Mischke R, Wolling H. Influence of Fibrinogen Degradation Products on Thrombin Time, Activated Partial Thromboplastin Time and Prothrombin Time of Canine Plasma. Haemostasis. 2000;30:123-30.
249. Mitrophanov AY, Rosendaal FR, Reifman J. Mechanistic Modeling of the Effects of Acidosis on Thrombin Generation. Anesthesia and analgesia. 2015;121(2):278-88.
250. Brass LF. Thrombin and Platelet Activation. CHEST. 2003;124(3):18S-25S.
251. Bearer EL, Prakash JM, Li Z. Actin dynamics in platelets. International Review of Cytology. 217: Academic Press; 2002. p. 137-82.
252. Weisel JW, Litvinov RI. Red blood cells: the forgotten player in hemostasis and thrombosis. Journal of Thrombosis and Haemostasis. 2019;17(2):271-82.
253. Litvinov RI, Weisel JW. What Is the Biological and Clinical Relevance of Fibrin? Semin Thromb Hemost. 2016;42(04):333-43.
254. Casella JF, Flanagan MD, Lin S. Cytochalasin D inhibits actin polymerization and induces depolymerization of actin filaments formed during platelet shape change. Nature. 1981;293(5830):302-5.
255. Schneider DJ. Anti-platelet therapy: glycoprotein IIb-IIIa antagonists. British Journal of Clinical Pharmacology. 2011;72(4):672-82.
256. Lang T, Toller W, Gütl M, Mahla E, Metzler H, Rehak P, et al. Different effects of abciximab and cytochalasin D on clot strength in thrombelastography. Journal of Thrombosis and Haemostasis. 2004;2(1):147-53.
257. Morrissey JH. Tissue factor: an enzyme cofactor and a true receptor. Thrombosis and haemostasis. 2001;86(07):66-74.
258. Kline SS, Atkins MO. Medical liability risk avoidance: a case for adopting the International Normalized Ratio (INR) system. The American journal of managed care. 1997;3:59-63.
259. Mavros MN, Coburn NG, Davis LE, Zuk V, Hallet J. Risk of venous thromboembolism in patients with elevated INR undergoing hepatectomy: an analysis of the American college of surgeons national surgical quality improvement program registry. HPB. 2020.
260. Kortsalioudaki C, Taylor RM, Cheeseman P, Bansal S, Mieli‐Vergani G, Dhawan A. Safety and efficacy of N‐acetylcysteine in children with non‐acetaminophen‐induced acute liver failure. Liver transplantation. 2008;14(1):25-30.
261. Bilir BM, Guinette D, Karrer F, Kumpe DA, Krysl J, Stephens J, et al. Hepatocyte transplantation in acute liver failure. Liver Transplantation. 2000;6(1):32-40.

262. Schein JR, White CM, Nelson WW, Kluger J, Mearns ES, Coleman CI. Vitamin K antagonist use: evidence of the difficulty of achieving and maintaining target INR range and subsequent consequences. Thrombosis Journal. 2016;14(1):14.
263. Roth GA, Johnson C, Abajobir A, Abd-Allah F, Abera SF, Abyu G, et al. Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. J Am Coll Cardiol. 2017;70(1):1-25.
264. Asrani SK, Devarbhavi H, Eaton J, Kamath PS. Burden of liver diseases in the world. Journal of hepatology. 2019;70(1):151-71.
265. Tripathi MM, Egawa S, Wirth AG, Tshikudi DM, Van Cott EM, Nadkarni SK. Clinical evaluation of whole blood prothrombin time (PT) and international normalized ratio (INR) using a Laser Speckle Rheology sensor. Scientific Reports. 2017;7(1):9169.
266. Greenway A, Ignjatovic V, Summerhayes R, Newall F, Burgess J, DeRosa L, et al. Point-of-care monitoring of oral anticoagulation therapy in children. Thrombosis and haemostasis. 2009;102(07):159-65.
267. Nam YH, Han X, Brensinger CM, Bilker WB, Leonard CE, Hennessy S. Sulfonylureas and metformin were not associated with an increased rate of serious bleeding in warfarin users: a self-controlled case series study. Clinical Pharmacology & Therapeutics. 2020.
268. Hegener MA, Li H, Han D, Steckl AJ, Pauletti GM. Point-of-care coagulation monitoring: first clinical experience using a paper-based lateral flow diagnostic device. Biomedical Microdevices. 2017;19(3):64.
269. Li H, Han D, Pauletti GM, Hegener MA, Steckl AJ. Correcting the effect of hematocrit in whole blood coagulation analysis on paper-based lateral flow device. Analytical Methods. 2018;10(24):2869-74.
270. Shimokawa M, Kitaguchi K, Kawaguchi M, Sakamoto T, Kakimoto M, Furuya H. The Influence of Induced Hypothermia for Hemostatic Function on Temperature-Adjusted Measurements in Rabbits. Anesthesia & Analgesia. 2003;96(4):1209-13.
271. Wei G, Bergquist A, Broomé U, Lindgren S, Wallerstedt S, Almer S, et al. Acute liver failure in Sweden: etiology and outcome. Journal of internal medicine. 2007;262(3):393-401.
272. Saksena D, Mishra YK, Muralidharan S, Kanhere V, Srivastava P, Srivastava CP, et al. Follow-up and management of valvular heart disease patients with prosthetic valve: a clinical practice guideline for Indian scenario. Indian Journal of Thoracic and Cardiovascular Surgery. 2019;35(1):3-44.