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# Leveraging turbidity and thromboelastography for complementary clot characterization

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| **Corresponding Author:** | Nathan Alves<br>Indiana University School of Medicine<br>Indianapolis, IN UNITED STATES |
| **Corresponding Author's Institution:** | Indiana University School of Medicine |
| **Corresponding Author E-Mail:** | nalves@iu.edu |
| **Order of Authors:** | Ziqian Zeng |
| | Tanmaye Nallan Chakravarthula |
| | Nathan J Alves |
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**TITLE:**

# Leveraging Turbidity and Thromboelastography for Complementary Clot Characterization

**AUTHORS AND AFFILIATIONS:**

Ziqian Zeng[1,2], Tanmaye Nallan Chakravarthula[1,2], Nathan J Alves[1,2]

[1] Emergency Medicine Department, Indiana University School of Medicine, Indianapolis, IN
[2] Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN

Corresponding author:
Nathan J. Alves
nalves@iu.edu

Email addresses of Co-authors:
Ziqian Zeng (zeng155@purdue.edu)
Tanmaye Nallan Chakravarthula (tnallanc@purdue.edu)

**KEYWORDS:**

Thromboelastography, Turbidity, Thrombosis, Fibrin, Clot, Clot strength, Clot turbidity

**SUMMARY:**

Fibrin is responsible for clot formation during hemostasis and thrombosis. Turbidity assays and thromboelastograhy (TEG) can be utilized as synergistic tools that provide complementary assessment of a clot. These two techniques together can give more insight into how clotting conditions affect fibrin clot formation.

**ABSTRACT:**

Thrombosis is a leading cause of death worldwide. Fibrin(ogen) is the protein primarily responsible for clot formation and thrombosis. Therefore, characterizing fibrin clot formation is beneficial to the study of thrombosis. Turbidity and thromboelastography (TEG) are both widely utilized in vitro assays for monitoring clot formation. Turbidity dynamically measures the light transmittance through a fibrin clot structure via a spectrometer and is often used in research laboratories. TEG is a specialized viscoelastic technique that directly measures blood clot strength and is primarily utilized in clinical settings to assess patients' hemostasis. With the help of these two tools, this study describes a method for characterizing an in vitro fibrin clot using a simplified fibrinogen/thrombin clot model. Data trends across both techniques were compared under various clotting conditions. Human and bovine fibrin clots were formed side-by-side in this study as bovine clotting factors are often used as substitutes to human clotting factors in clinical and research settings. Results demonstrate that TEG and turbidity track clot formation via two distinct methods and when utilized together provide complementary clot strength and fiber structural information across diverse clotting conditions.

**INTRODUCTION:**

Thrombosis is the pathological formation of a blood clot in the body that blocks blood circulation

leading to high morbidity and mortality worldwide. There are 1 to 2 cases of venous thromboembolism and 2 to 3 cases of thrombosis-induced vascular diseases per 1000 people annually[1,2]. Presented here is a method leveraging thromboelastography and turbidity to monitor clot formation under various clotting conditions. Fibrin(ogen) is the primary protein that is responsible for clot formation in the body. In the final steps of the coagulation cascade, fibrinopeptides are cleaved from fibrinogen by thrombin initiating the polymerization of insoluble fibrin monomers as the clot develops[3,4]. To understand clot formation in pathological thrombosis, it is necessary to characterize fibrin formation under diverse clotting circumstances. Multiple clot monitoring assays have been utilized to study fibrin clot formation in vitro. Prothrombin time (PT/INR) and activated partial thromboplastin time (aPTT) are two common clinical assays that measure the integrity of a specific coagulation pathway. However, they use time as the only variable that gives no indication of physical clot properties[5]. Electron microscopy allows visualization of the micro-structure of a completely formed fibrin clot but provides no information about the clot forming process itself[6]. Among all assays, turbidity assays and TEG offer the ability to track clot characteristics dynamically over time. These techniques enable the measure of comprehensive clotting profiles and therefore, provide some benefit over other fibrin clot characterization tools.

Specifically, turbidity assays (or clot turbidimetry) is widely used for research and clinical applications due to its simplistic implementation and the wide accessibility of spectrometers in research laboratories. This assay allows a dynamic measurement of light transmittance through a forming clot by taking individual repetitive readings at a defined wavelength (most commonly at a wavelength in the range of 350 – 700 nm)[7]. Temperature in the reading chamber can also be adjusted. As fibrin gel forms, the amount of light that travels through the protein network is reduced causing an increase in absorbance over time. Similarly, absorbance decreases when the clot network degrades. Turbidity assays can easily be multiplexed using a multi-well plate format to allow for high throughput sample screening in both 96- and 384-well plates. Several clot characteristics can be derived from a turbidity tracing curve (absorbance over time measurement) that include: maximum turbidity, time to maximum turbidity, time to clot onset, and clot formation rate (Vmax). A fibrin fiber mass/length ratio can also be derived from raw turbidity data to estimate fibrin fiber thickness[8–10].

TEG is primarily utilized in the clinical setting to assess patients' hemostasis and clot lysis. It is also commonly used in surgical applications to determine when anti-fibrinolytic drugs or hemostatic blood products should be administered[11,12]. Clot formation occurs inside a TEG cup with all the clotting components being added to the cup prior to the initiation of the assay. The cup, with evolving clot, physically rotates against a pin that is inserted into its center and an electromechanical torsion sensor measures the increasing viscoelastic strength of the clot. This assay is typically carried out at the physiological temperature of 37 °C; however, the temperature can be manually adjusted on the instrument. Maximum amplitude (MA), reaction rate (R), kinetics time (K), α-angle (Angle), and time to maximum amplitude (TMA) are extracted by the TEG software from the dynamic TEG tracing. These values are typically compared with clinical normal ranges to assess a patient's coagulation state. While TEG is not precisely a viscometer, as it measures clot strength in millimeter units, it does provide important viscoelastic clot data and

functions as a valuable clinical decision making tool for physicians to decide to administer specific blood products and adjust therapeutic dosing[13]. When both TEG and turbidity assays are utilized together, it provides complementary clot characterization information as clot strength and kinetics are easy extracted from TEG and fibrin fiber thickness can be accessed by optical turbidity measurements.

As fibrin is a critical component of a blood clot, fibrin clot characterization under diverse clot formation conditions can provide valuable insight into how a specific variable contributes to the clot formation process and ultimate clot properties. Understanding this can provide guidance for thrombosis diagnosis and the development of therapeutics. To obtain a more representative fibrin clot characterization, plasma can be substituted to monitor clot formation as it resembles in vivo clotting conditions more closely than a simplified fibrinogen/thrombin model system. However, due to the intricate nature of the coagulation cascade, clot formation using plasma adds to the complexity, making it more difficult to isolate the impact of individual factors. Utilizing a simplified fibrinogen/thrombin model prevents the need to initiate the entire clotting cascade allowing for isolation of the final fibrin formation step. By including two major fibrin forming components (fibrinogen and thrombin), this setup creates a highly controlled clot formation condition. It is also important to note that while the simplified clot model is used here, this protocol can also be utilized to characterize more complex clots by including additional clotting factors. In this study, fibrin clot characterization using turbidity and TEG are carried out by varying fibrinogen and thrombin concentrations, ionic strength, pH, and total protein concentration in the clotting solution to mimic different in vivo clotting circumstances[14]. Details regarding these variations to the protocol have been included in Section 5.

**PROTOCOL:**

## 1. Preparation of phosphate buffer saline (PBS)

NOTE: PBS was used throughout this study as the described assays did not require the addition of calcium. It is important to note that when adding calcium, often utilized to re-calcify citrated blood products, PBS should be avoided as calcium is known to precipitate in phosphate buffers.

1.1. Make a 0.01 M, pH 7.4 PBS buffer by mixing 137 mM sodium chloride, 1.8 mM potassium phosphate monobasic, 10 mM sodium phosphate dibasic and 2.7 mM potassium chloride in DI water.

1.2. Verify buffer pH using a pH probe and adjust the pH using sodium hydroxide or hydrochloric acid as needed.

1.3. Use this PBS for preparation of fibrinogen and clotting assays (unless otherwise specified).

NOTE: Buffer is suggested to reconstitute fibrinogen powder since rehydration in DI water can result in fibrinogen precipitation even at 37 °C.

## 2. **Preparation and storage of proteins**

NOTE: Throughout the protocol the protein stock concentrations are prepared at different concentrations for turbidity and TEG to allow for the consistent ratio of salt, DI water, PBS and other residual factors in the final clotting solutions.

### 2.1. Preparation and storage of fibrinogen

NOTE: Contaminants in commercially available fibrinogen include a significant amount of factor XIII, residual amounts of other clotting factors, storage buffer and salts. In the presence of calcium, factor XIII is known to crosslink the fibrin clot network. This effect contributes to a tightened clot structure and enhanced clot strength affecting fibrin characterization. Commercially available activity kits can be used to determine active factor XIIIa levels. To minimize variability caused by factor XIIIa, experiments should be designed with the exclusion of calcium or additional steps to remove factor XIIIa should be incorporated into this protocol. In addition, protein storage salt and buffer type should be assessed and if necessary dialysis can be carried out to transfer to a preferred assay working buffer.

2.1.1. Weigh and aliquot lyophilized fibrinogen powder (bovine or human) in 2 mL tubes at 20 mg of protein per tube and store aliquots at -20 °C for up to 6 months.

2.1.2. Allow fibrinogen aliquot to acclimate to RT (room temperature) for 10 minutes on the day of the experiment. Reconstitute fibrinogen by adding 600 µL of PBS to the aliquot 20 min prior to use.

2.1.3. Take 10 µL of fibrinogen and dilute it with 190 µL of PBS in a UV transparent 96-well plate or a cuvette. Determine fibrinogen concentration by taking absorbance at 280 nm via a commercial spectrometer and its software.

2.1.4. Calculate fibrinogen concentration (mg/mL) using Beer's law. Prepare 12 mg/mL (for turbidity assays) and 3.2 mg/mL (for TEG) fibrinogen stock solutions by further dilution with PBS (unless otherwise specified).

NOTE: Fibrinogen concentration determination (mg/mL) by Beer's law: $C = D * \left(\frac{A}{\varepsilon L}\right) * (\frac{Mw}{1000})$
Molar extinction coefficient: **ε** = 513,400 L $mol^{-1}$ $cm^{-1}$ at 280 nm; Pathlength (L); Dilution factor (D); Molecular Weight (MW) = 340,000 Da. **ε** is derived by multiplying $E^{1\%}$ = 15.1 (280 nm) (extinction coefficient, given by the supplier) with MW.

### 2.2. Preparation and storage of thrombin

2.1.5. Reconstitute lyophilized thrombin (bovine or human, 1000 U stock) in 200 µL of deionized (DI) water to make 200 µL of 5000 U/mL thrombin stock solution.

2.1.6. Make 5 µL (20 U/tube) aliquots of the solution and keep aliquots frozen at −20 °C.

2.1.7. Thaw thrombin aliquots at RT for 15 min on the day of experiment and make thrombin working solution by diluting it to 20 U/mL for turbidity assays and 18 U/mL for TEG with DI water (unless otherwise specified).

NOTE: Precautions should be taken to maintain enzyme activity which can be accomplished by maintaining enzymes on ice during thawing and use; however, no reduction in thrombin activity was observed when utilized directly after thawing at RT.

## 3. Turbidity

3.1. Use any commercially available spectrometer that has an absorbance range of 350 - 700 nm and a corresponding software to monitor clot turbidity over time (see **Table of Materials**).

3.2. Turn on the spectrometer and open the corresponding analysis software.

3.3. Select **plate 1** and open plate settings tab. Click **ABS** mode and **Kinetic** to monitor a dynamic absorbance reading over time.

3.4. Select 550 nm (or any value in the range of 350 – 700 nm) in the wavelength tab and adjust the total run time to be 60 min with an interval of 30 s in the timing tab. Select wells of interest for reading by highlighting the wells. Adjust other settings if needed.

NOTE: Selecting a wavelength at the lower end of the range (around 350 nm) brings a better sensitivity but absorbance might also exceed the detection limit of the spectrometer. The most commonly utilized wavelength for turbidity measurement is 405 nm in literature; however, this protocol uses 550 nm to ensure the dynamic turbidity values are within the detection limit for all experiments. The selected reading interval should be as short as possible to achieve the highest level of assay sensitivity. This will depend upon the spectrometer and number of wells being read during a given assay.

3.5. Take a UV transparent 96-well plate. Pipette 140 µL PBS in a well that is selected for reading. Add and mix 10 µL of thrombin (20 U/mL) in the well.

NOTE: Do not use high binding assay plates to minimize nonspecific protein binding to the well surface. This could affect protein dispersion in the solution and result in high assay variability.

3.6. Initiate clotting immediately by adding 50 µL of fibrinogen (12 mg/mL) in the well to obtain a 200 µL clotting solution with a final concentration of 3 mg/mL fibrinogen and 1 U/mL thrombin. Mix the contents in the well by pipetting up and down five times taking care to avoid the creation of bubbles in the solution as they will impact absorbance by scattering light.

NOTE: Use a multichannel pipette when running multiple clot samples on the same plate at the same time. Record time differences across wells and the time period prior to the first read by the instrument to offset clotting times.

3.7. Place the 96-well plate in the holder and click **Start** in the software to start turbidity reading at RT.

NOTE: If carrying out the assay at an elevated then the spectrometer, plate, and reagents must all be maintained at the desired temperature prior to clot initiation.

3.8. Once completed, retrieve the turbidity data and obtain a turbidity tracing curve by plotting absorbance change over time in a plotting software.

3.9. Derive $Turb^{Max}$ (maximum turbidity indicative of fibrin fiber thickness and fibrin network density) by taking the max absorbance value of the curve over time.

NOTE: Fibrin fiber mass/length ratio can be calculated from turbidity values using the equation provided in the following manuscript[8].

3.10. Calculate 90% maximum turbidity by multiplying $Turb^{Max}$ by 90%. Derive $Turb^{Time}$ by computing time from clot initiation to 90% maximum turbidity.

NOTE: The time to 90% maximum turbidity is a more reliable metric than time to absolute maximum turbidity as it better represents clot time by eliminating the highly variable final clot formation period. Additional clotting parameters such as clot onset time (time from start of test to when absorbance starts to increase), and clot formation rate (Vmax, the largest slope of the linear region in the turbidity tracing curve) can also be extracted from the turbidity tracings.

## 4. Thromboelastography (TEG)

4.1. Turn on the thromboelastograph analyzer and wait for the temperature to stabilize at 37 °C.

4.2. Open TEG - software. Once logged in, create an experiment name under the ID section.

4.3. Conduct an e-test for all channels by following the on-screen software prompts. Place the lever back to the load position once all checks are complete.

NOTE: A TEG e-test is required and should be conducted every time when using the instrument. TEG coagulation control assays (using TEG level 1 and level 2 controls) are required at the regular manufacturer suggested intervals when utilized for clinical samples.

4.4. Click the **TEG** tab, input sample information for channels that will be used. Place a clear uncoated TEG cup in its corresponding channel. Slide the carrier up to the top and press the cup

bottom 5-times to affix the pin to the torsion rod. Lower the carrier and press the cup downward into the carrier base until it "clicks".

4.5. Pipette 20 µL of thrombin solution (18 U/mL) into the TEG cup. Initiate clotting immediately by adding 340 µL of fibrinogen (3.2 mg/mL) into the TEG cup to obtain a 360 µL clotting solution with a final concentration of 3 mg/mL fibrinogen and 1 U/mL thrombin in the cup. Mix the contents by pipetting up and down five times.

NOTE: Potential clotting factors or other components of interest should be added during this step being careful to always maintain a final volume of 360 µL in the TEG cup.

4.6. Slide the cup loaded carrier up, move the lever to the read position and click **Start** in the software to initiate the TEG reading.

4.7. Once TEG is completed (after about an hour), retrieve TEG parameters and obtain a TEG tracing curve by plotting amplitude over time in a plotting software.

4.8. Collect MA as $TEG^{Max}$ (maximum amplitude is indicative of clot strength) and TMA as $TEG^{Time}$ (time to maximum amplitude) from the software.

NOTE: MA is calculated by the software as the maximum amplitude at the time at which the amplitude has less than a 5% deviation over a 3-minute period of time. TMA is determined as the time from the maximum thrombus generation rate (near the split point) to the MA. Other parameters might also be useful to assess when performing the clot analysis. Some examples of these parameters include: R-time (the time from start of test to when amplitude reaches 2 mm), K (the time from the end of R to when amplitude reaches 20 mm), alpha (slope of line between R and K), and CLT (clot lysis time).

## 5. Fibrin characterization under different clotting conditions

NOTE: Perform fibrin characterization experiments by modulating a specific variable in the clotting solutions such as: fibrinogen and thrombin concentrations, ionic strength, pH, and total protein concentrations. Experimental preparations with these example variables are described in this section; however, other clotting factors and conditions of interest can be substituted as well. Carefully select a suitable buffer system taking into consideration each unique assay requirements. For turbidity and TEG assays, include a buffer only control to ensure an accurate background subtraction while analyzing the effect of these variables.

5.1. Varying fibrinogen concentration (1, 2, 3, 4, 5 mg/mL)

5.1.1. Adjust step 2.1.4 to prepare the fibrinogen stock at different concentrations (4, 8, 12, 16, 20 mg/mL for turbidity assays and 1.1, 2.1, 3.2, 4.2, 5.3 mg/mL for TEG).

5.1.2. Adjust step 3.6 to "add 50 µL fibrinogen (4, 8, 12, 16, 20 mg/mL) into multiple wells of

the 96 well-plate" for turbidity assays.

5.1.3. Adjust step 4.5 to "add 340 µL fibrinogen (1.1, 2.1, 3.2, 4.2, 5.3 mg/mL) into clear TEG cups" for TEG.

5.2. Varying thrombin concentration (0.1, 0.3, 0.6, 0.8, 1, 2.5, 5, 10 U/mL)

5.2.1. Adjust step 2.2.3 to prepare thrombin stock at different concentrations (2, 6, 12, 16, 20, 50, 100, 200 U/mL for turbidity assays and 1.8, 5.4, 10.8, 14.4, 18, 45, 90, 180 U/mL for TEG).

5.2.2. Adjust step 3.5 to "add 10 µL thrombin (2, 6, 12, 16, 20, 50, 100, 200 U/mL) into multiple wells of the 96 well-plate" for turbidity assays.

5.2.3. Adjust step 4.5 to "pipette 20 µL thrombin (1.8, 5.4, 10.8, 14.4, 18, 45, 90, 180 U/mL) into clear TEG cups" for TEG.

5.3. Varying ionic strength (0.05, 0.13, 0.14, 0.15, 0.16, 0.17 and 0.3 M)

5.3.1. Dissolve sodium chloride (21, 101, 111, 121, 131, 141, and 271 mM) along with 1.8 mM potassium phosphate monobasic, 10 mM sodium phosphate dibasic and 2.7 mM potassium chloride in DI water to make 0.01 M PBS solutions with varying ionic strengths.

5.3.2. Adjust step 1.3 to use PBS made at different ionic strengths to prepare fibrinogen and clotting solutions for both turbidity and TEG assays.

5.4. Varying pH (5.8, 6.6, 7.3, 7.4, 7.5, and 8.0)

5.4.1. Dissolve sodium phosphate dibasic (0.7, 3.2, 7.7, 8.1, 8.4, 9.5 mM) and potassium phosphate monobasic (8.2, 6.0, 2.0, 1.7, 1.4, 0.5 mM) along with 2.7 mM potassium chloride and sodium chloride (153, 147, 138, 137, 136, 134 mM) to make 0.01 M PBS solutions with varying pH and a final ionic strength at 0.165 M.

5.4.2. Verify buffer pH value via pH probe and adjust pH if needed.

5.4.3. Adjust step 1.3 to use PBS made at different pH to prepare fibrinogen and clotting solution for both turbidity and TEG assays.

5.5. Varying albumin concentration (0, 20, 40, 50, 60, 80, 100 mg/mL)

5.5.1. Dissolve 2 g of lyophilized albumin in 500 µL of PBS at RT for 20 min on the day of experiment.

5.5.2. Determine albumin concentration using the same procedure mentioned in step 2.1.3 and 2.1.4 with a molar extinction coefficient of 43,800 L $mol^{-1}$ $cm^{-1}$ (at 280 nm) for albumin.

5.5.3. Prepare albumin stock at different concentrations.

5.5.4. Adjust step 3.5 to "Pipette 40 µL PBS in a well that is selected for reading and add 10 µL thrombin (20 U/mL)". Adjust step 3.6 to "Initiate clotting immediately by adding 50 µL fibrinogen (12 mg/mL) with 100 µL albumin (0, 40, 80, 100, 120, 160, 200 mg/mL) into multiple wells to a final concentration of 3 mg/mL fibrinogen, 1 U/mL thrombin and 0, 20, 40, 50, 60, 80, 100 mg/mL albumin in wells ."

5.5.5. Adjust step 4.5 to "Initiate clotting immediately by adding a mixture of 200 µL albumin (36, 72, 90, 108, 144, 180 mg/mL) and 140 µL 7.7 mg/mL fibrinogen into TEG cups to obtain a 360 µL clotting solution with a final concentration of 3 mg/mL fibrinogen, 1 U/mL thrombin and 0, 20, 40, 50, 60, 80, 100 mg/mL albumin in TEG cups."

**REPRESENTATIVE RESULTS:**

The experiments shown in **Figure 1** are representative turbidity tracing curves of human and bovine fibrin clots at different fibrinogen levels. Representative TEG tracing curves for fibrin clot formation at different fibrinogen levels are shown in **Figure 2**. Both tracing curves demonstrate that after a lag period following clot initiation, clot turbidity or clot amplitude increases over time and levels off at the end of clot formation. An endpoint value of maximum clot formation and the time to maximum clot formation from each assay are used to assess the features of a completely formed clot and the overall clotting process. Maximum turbidity ($Turb^{Max}$) and time to maximum turbidity ($Turb^{Time}$) are the two parameters derived from turbidity while maximum amplitude ($TEG^{Max}$) and time to maximum amplitude ($TEG^{Time}$) are derived from TEG.

In addition, $Turb^{Max}$ is an optical measure of clot structure which is indicative of fibrin fiber thickness and fibrin network density. $TEG^{Max}$ is a mechanical measure that reflects absolute clot strength. They represent different aspects of a clot that can change independent of each other based on our previous findings[14]. Taken together, the two values provide complementary insight about the clot microstructures, such as how dense the fibers are packed in the fibrin network. To explicitly see how modulating a clotting variable affects the results, data were further organized and presented using trend plots. Representative examples of both turbidity and TEG data trends are shown in **Figure 3**. At a higher level of fibrinogen in the clotting solution, all four values ($Turb^{Max}$, $TEG^{Max}$, $Turb^{Time}$ and $TEG^{Time}$) increase. From these results, it can be interpreted that a higher fibrinogen substrate level results in a denser fibrous network limiting light transmission through the clot (larger $Turb^{Max}$). This tightened network also enhances clot strength (larger $TEG^{Max}$). The elongation of both $Turb^{Time}$ and $TEG^{Time}$ indicates that fibrin polymerization is hampered at increased fibrinogen levels. The trends of $Turb^{Max}$ and $TEG^{Max}$ for variables tested by our group and their interpretations are shown in **Table 1**.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Representative examples of clot turbidity tracing curve (0 to 30 min).** Turbidity tracings of bovine (A) and human (B) fibrin formation over time at different fibrinogen concentrations (1, 2, 3, 4, 5 mg/mL) with 1 U/mL species matched thrombin.

**Figure 2: Representative examples of TEG tracing curve (0 to 30 min).** TEG amplitude tracings of bovine (**A**) and human (**B**) fibrin formation over time at different fibrinogen concentrations (1, 2, 3, 4, 5 mg/mL) with 1 U/mL species matched thrombin.

**Figure 3: Representative examples of turbidity and TEG data trends.** Data trends of $Turb^{Max}$ and $Turb^{Time}$ (**A**) and trends of $TEG^{Max}$ and $TEG^{Time}$ (**B**) for different bovine or human fibrinogen concentrations (1, 2, 3, 4, 5 mg/ml) with 1U/mL species matched thrombin. All data points and error bars are averages and standard deviations of triplicates (Turbidity) and duplicates (TEG). This figure has been reused from [Zeng, 2020][14].

**Table 1. Results and interpretations of $Turb^{Max}$ and $TEG^{Max}$**

**DISCUSSION:**

This protocol demonstrates the utilization of two distinct clot characterization tools testing a simplified fibrinogen/thrombin clot model using commercially available components. Both TEG and turbidity assays are easy to conduct. They not only provide end point clot examinations such as max clot formation ($Turb^{Max}$ and $TEG^{Max}$) and clot formation times ($Turb^{Time}$ and $TEG^{Time}$) but also assess the dynamic clot forming process. This makes TEG and turbidity valuable tools for clot characterization to add to alternative methods such as: SEM, PT, aPTT, or clot rheology, which can have complicated experimental procedures or focus only on testing of a single clot aspect. Turbidity and TEG results together also offer a more complete profile of how a clotting variable impacts clot characteristics.

In addition to the clotting variables detailed in this protocol, there are many other variables that can be studied leveraging these techniques. This protocol can be modified by adjusting: temperature, calcium levels, coagulation factor levels, the addition of clot activators or inhibitors, or the addition of pharmaceutical agents, to name a few. These variables can potentially be studied using both the simplified fibrinogen/thrombin model system or using plasma. Studying coagulation factors requires careful consideration and setup being sure to provide for proper upstream clot activation initiating the coagulation pathway. Turbidity and TEG are also effective tools used to monitor clot digestion. The protocol can be modified to characterize fibrin clot formation and lysis in the presence of anticoagulants or thrombolytic agents. It is important to note that the therapeutic agent must be added prior to clot initiation when using TEG as the clotting system is functionally closed when the instrument is in operation with the pin seated in the TEG assay cup. With that said, TEG is unable to be used to test the therapeutic profile of a thrombolytic agent in an existing clot.

In this protocol, both assays were conducted under their commonly utilized experimental conditions. TEG is commonly performed at 37 °C and turbidity assays are carried out at room temperature (RT). The emphasis of this characterization method is to determine and interpret the trend results that a specific variable has on clotting. Importantly, controlling temperature utilizing TEG is straightforward as reagents are pipetted directly into a temperature controlled TEG cup. Temperature control in a turbidity assay is less straightforward as clotting reagents are

mixed and initiated in a well-plate prior to being placed within a heated spectrometer. The rate at which the plate and clotting solution warm within the chamber is slow relative to the clot formation rate and maintaining all reagents and the plate at elevated temperature prior to placing into the heated chamber can be difficult and often results in poor assay reproducibility. Maintaining a steady temperature other than room temperature for turbidity assays is further complicated when multiplexing across many wells. Another critical step to ensure a reliable and reproducible turbidity result is mixing the well to initiate clotting. As direct thrombin-fibrinogen cleavage is rapid, mixing the well before clot initiation can circumvent non-uniform fibrin formation.

This protocol can also be easily modified to characterize a clot formed by clinical samples such as platelet-rich plasma or platelet-poor plasma. Citrated blood draws are recommended as they have been validated previously by standard TEG protocols. Platelet-rich and platelet-poor plasma can be separated from citrated whole blood via centrifugation at 200 and over 2,000 x *g* for 15 min at RT, respectively. In turbidity or TEG assays, clots can be initiated with the addition of excess calcium chloride (11 mM). However, since phosphate can bind to calcium resulting in precipitation, PBS should be avoided when calcium is used as the clot initiator. When studying platelet rich plasma clot formation, it is important to consider platelet settling as a significant confounder when running clot formation assays under conditions in which clot formation is slow. Issues regarding platelet settling are less impactful when clot formation is rapid. This is particularly important for turbidity assays where the assay accuracy and reproducibility largely rely on the homogeneity of the reagents in the well. If experimental design permits, kaolin (often utilized as a clot initiator for TEG) or other negatively charged particle activators can be used to speed up plasma clot initiation by providing additional surface area for faster contact pathway activation of the coagulation cascade. Importantly, activator suspensions should be mixed thoroughly with clotting solutions to avoid settling. When utilizing a surface area or charged particle-based clot activator ensure that the necessary background controls are taken into account as the reagents themselves may contribute to absorptivity in turbidity assays. Additionally, settling may be an issue with larger particle-based additives such as kaolin.

Whole blood samples should be processed carefully when considering the use of turbidity assays since red blood cells contribute significantly to absorbance at standard turbidity wavelengths. For this reason, the measurement of whole blood via turbidity often exceeds the detection limit of most spectrometers and is typically not feasible. Alternative methods to characterize whole blood may be necessary or dilution prior to running turbidity assays on blood samples that contain red blood cells is also possible. TEG and turbidity, when used together, provide for comprehensive clot formation and digestion characterization by combining distinct measurements for clot strength and fibrin fiber/network morphology for both highly controlled minimal protein clotting assays and clinical plasma samples.

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**DISCLOSURES:**

The authors have nothing to disclose.

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Figure 1



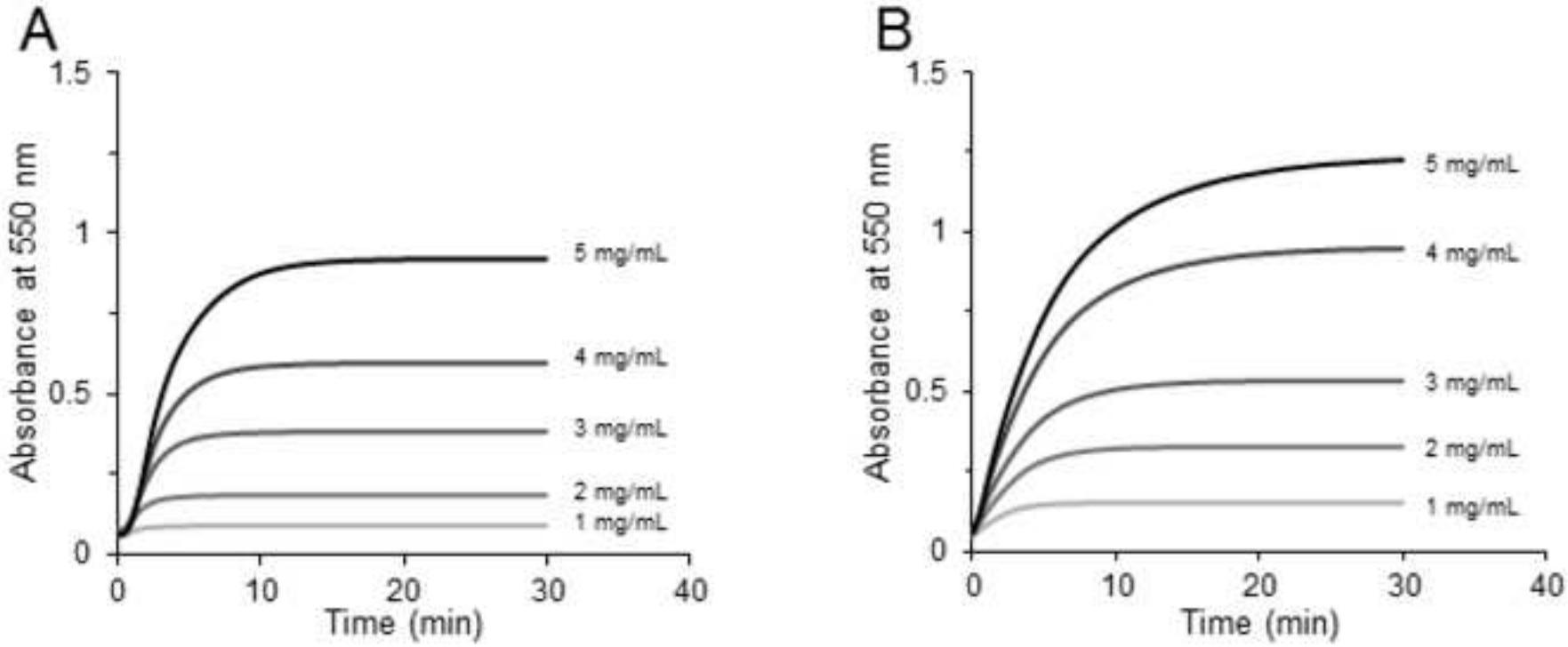

Figure 2



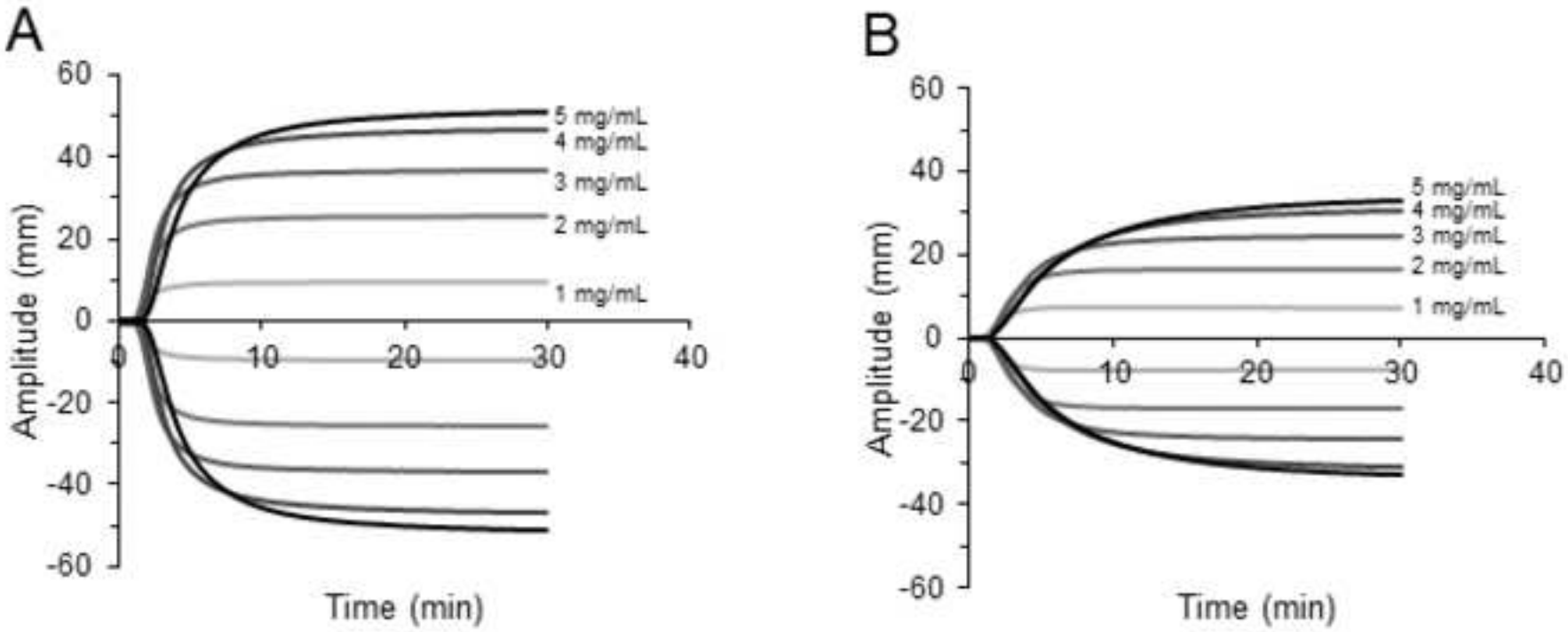

Figure 3



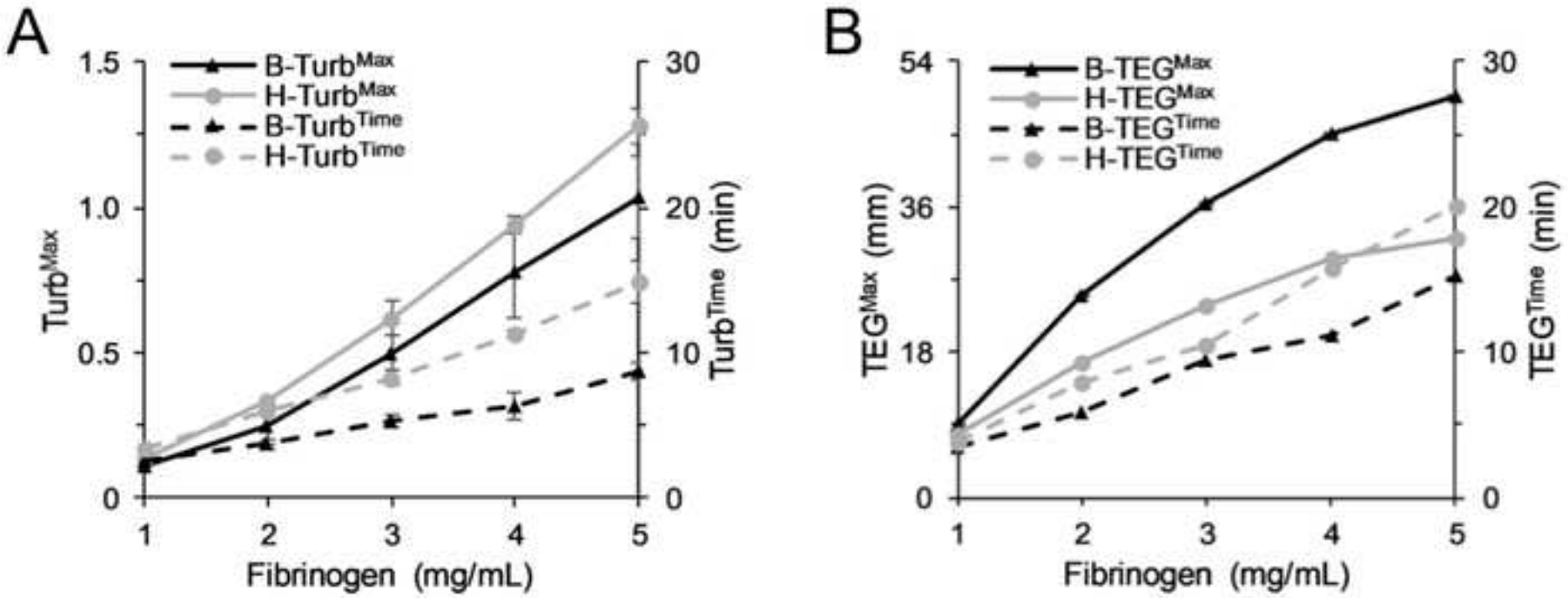

**Table 1. Results and interpretations of $Turb^{Max}$ and $TEG^{Max}$**

| Variables | Trend Results |
|---|---|
| Increased fibrinogen level | Increased $Turb^{Max}$ and $TEG^{Max}$ |
| Increased thrombin level, pH and ionic strength | Decreased $Turb^{Max}$, increased $TEG^{Max}$ |
| Increased albumin level | Increased $Turb^{Max}$, Decreased $TEG^{Max}$ |

| Interpretations |
| --- |
| Formation of tighter fibrin fibers and denser fibrous network |
| Formation of thinner and tighter fibrin fibers |
| Formation of thicker and looser fibrin fibers |

| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 96-Well Clear Flat Bottom UV-Transparent Microplate | Corning | 3635 | Non-treated acrylic copolymer, non-sterile |
| Albumin from human serum | Millipore Sigma | A1653 | ≥96%, lyophilized powder |
| Arium Mini Plus Ultrapure Water System | Sartorius | NA | DI water source |
| Bovine serum albumin | Millipore Sigma | A2153 | ≥96%, lyophilized powder |
| Disposable Cups and Pins for TEG 5000 (Clear) | Haemonetics | REF 6211 | |
| Fibrinogen, Bovine Plasma | Millipore Sigma | 341573 | contains more than 95% clottable protein |
| Fibrinogen, Plasmingogen-Depleted, Human Plasma | Millipore Sigma | 341578 | Contains ≥ 95% clottable proteins. |
| Phosphate buffered saline | Millipore Sigma | P3813 | Powder, pH 7.4, for preparing 1 L solutions |
| Potassium chloride | Millipore Sigma | 60130 | ≥99.5% purity |
| Potassium phosphate monobasic | Millipore Sigma | P9791 | ≥98% purity |
| SevenEasy pH Meter | Mettler Toledo | S20 | |
| Sodium chloride | Millipore Sigma | 71378 | ≥99.5% purity |
| Sodium phosphate dibasic | Millipore Sigma | 71636 | ≥99.5% purity |
| SpectraMax M5 multi-detection microplate reader system | Molecular Devices | M5 | |
| TEG 5000 Thrombelastograph Hemostasis analyzer system | Haemonetics | 07-022 | |
| Thrombin, Bovine | Millipore Sigma | 605157 | |
| Thrombin, Human Plasma, High Activity | Millipore Sigma | 605195 | |

INDIANA UNIVERSITY
SCHOOL OF MEDICINE
Department of Emergency Medicine

April 22, 2020

Dear Dr. Alisha DSouza,

Please find enclosed the following revised submission of a method for consideration at JOVE: "Leveraging turbidity and thromboelastography for complementary clot characterization". This article is being exclusively considered for publication at JOVE and is based on the recently published article that was recently accepted at Thrombosis Research titled "Fibrin clot formation under diverse clotting conditions: Comparing turbidimetry and thromboelastography".

The revised manuscript addresses all reviewer concerns and details how to run turbidity and thromboelastography utilizing a simplified fibrinogen/thrombin model providing for clot analysis across many different clot formation conditions. Additionally, the discussion regarding alternatives, pitfalls, protocol modifications, and data analysis associated with these techniques has been expanded. We look forward to hearing from you regarding this submission. The authors have no conflicts of interest to disclose. If there are any questions or concerns feel free to email (nalves@iu.edu) or call me at 203-565-2049. Thank you for your consideration.

Best Regards,

Nathan J. Alves, PhD
Assistant Professor
Department of Emergency Medicine
Indiana University School of Medicine
635 Barnhill Dr. Rm. 371
Indianapolis, IN 46202
nalves@iu.edu

**Editorial Comments:**

**• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.**

The manuscript has been proofread. Spelling and grammatical errors have been corrected.

**• Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.**

Authors have checked all procedures and made efforts to include all necessary details in the video script.

**• Protocol Highlight:**
**1) Please focus the title on the highlighted portions.**
**2) Notes cannot be filmed and should be excluded from highlighting.**

The manuscript title reflects the highlighted portion. Authors have removed highlighting from notes.

**• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.**

Authors have modified/ added content in the discussion of the manuscript and it should now cover all the details mentioned above:

"However, since phosphate can bind to calcium resulting in precipitation, PBS should be avoided when calcium is used as the clot initiator. When studying platelet rich plasma clot formation, it is important to consider platelet settling as a significant confounder when running clot formation assays under conditions in which clot formation is slow. Issues regarding platelet settling are less impactful when clot formation is rapid. This is particularly important for turbidity assays where the assay accuracy and reproducibility largely relies on the homogeneity of the reagents in the well. If experimental design permits, kaolin (often utilized as a clot initiator for TEG) or other negatively charged particle activators can be used to speed up plasma clot initiation by providing additional surface area for faster contact pathway activation of the coagulation cascade. Importantly, activator suspensions should be mixed thoroughly with clotting solutions to avoid settling. When utilizing a surface area or charged particle-based clot activator ensure that the necessary background controls are taken into account as the reagents themselves may contribute to absorptivity in turbidity assays. Additionally, settling may be an issue with larger particle-based additives such as kaolin." (discussion section)

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**1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.**

Authors have removed or replaced all commercial sounding languages.

**• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."**

Authors reuse one figure (figure 3) in this manuscript and it is properly cited. A reuse permission has been obtained and the file is saved in a separate word document.

**Reviewer #1:**
**Manuscript Summary:**
**The procedure of the tests is really clearly described in the present manuscript However in Zeng et al Thrombosis Research 2020 article all the variables listed as possible changes in the present manuscript: albumin concentration, ionic strength and pH, are very well described there but not is this one. This manuscript seems to be the model and the possibilities of changing the variables are listed, but without details.**

The authors would like to draw the attention of the reviewer to Section 5 in the protocol. The aim of this manuscript was to introduce experimental procedures and analysis used in basic thromboelastography and turbidity assays for clot characterization. To maintain the focus on methodology, procedures omitting the complexities that accompany changing of the listed variables was first described in the initial four sections of the protocol. A separate section (section 5) was added to elaborate experimental procedures of how to modify the protocol and change variables, such as fibrinogen, thrombin and albumin concentrations, pH, ionic strength.

A sentence has been added to the end of the introduction to guide the reader that details regarding the specific clotting variations that are listed will be discussed following the

description of the techniques. “Details regarding these variations to the protocol have been included in Section 5.”

**Minor Concerns:**
**Considering the discussion of the possibility to use these methods for clinical samples, the authors should to be more careful and explained that turbidimetric assay will be very hard to standardize if Platelet reach plasma is used do to the fact that platelets will decant in the well base during the 60 min of Absorbance reading. In the same way Kaolin is a heavy powder that is useful to is not useful in photooptical coagulometers because of the turbidity and the need to mix all over the time because it decants in well base as well. Perhaps some colloidal silica suspensions or celite suspension as negatively charged intrinsic coagulation pathway activators could be more adequate for these experiments. So, it is recommended to consider these limitations in the discussion. PRP or whole blood, as well as Kaolin or other negatively charged materials are able to be processed by TEG instrument without problems.**

To address reviewer’s concerns the following sentences are modified and added in the discussion session of the manuscript:

“When studying platelet rich plasma clot formation, it is important to consider platelet settling as a significant confounder when running clot formation assays under conditions in which clot formation is slow. Issues regarding platelet settling are less impactful when clot formation is rapid. This is particularly important for turbidity assays where the assay accuracy and reproducibility largely relies on the homogeneity of the reagents in the well. If experimental design permits, kaolin (often utilized as a clot initiator for TEG) or other negatively charged particle activators can be used to speed up plasma clot initiation by providing additional surface area for faster contact pathway activation of the coagulation cascade. Importantly, activator suspensions should be mixed thoroughly with clotting solutions to avoid settling. When utilizing a surface area or charged particle-based clot activator ensure that the necessary background controls are taken into account as the reagents themselves may contribute to absorptivity in turbidity assays. Additionally, settling may be an issue with larger particle-based additives such as kaolin.”

**Reviewer #2:**
**Manuscript Summary:**
**The authors describe the use of two techniques to investigate clot structure, namely turbidity and thromboelastography. The methodology described by the authors is sufficiently detailed, leading to the formation of fibrin clots from which the specific polymerisation and clot formation parameters can be determined. However, the reasoning for how the parameters obtained from each method (maximum absorbance or maximum amplitude, and time to maximum absorbance/amplitude) are complementary when utilised together are not made clear.**

Additional sentences have been added throughout the manuscript to detail how TEG can be used to assess clot strength and turbidity can be utilized a surrogate to fibrin fiber thickness and fibrin fiber density. When taken together the two techniques provide synergistic clot characterization.

”Results demonstrate that TEG and turbidity track clot formation via two distinct methods and when utilized together provide complementary clot strength and fiber structural information across diverse clotting conditions.”

“When both TEG and Turbidity assays are utilized together it provides complementary clot characterization information as clot strength and kinetics are easy extracted from TEG and fibrin fiber thickness can be accessed by optical turbidity measurements.”

“In addition, $Turb^{Max}$ is an optical measure of clot structure which is indicative of fibrin fiber thickness and fibrin network density. $TEG^{Max}$ is a mechanical measure that reflects absolute clot strength. They represent different aspects of a clot that can change independent of each other based on our previous findings[14]. Taken together, the two values provide complementary insight about the clot microstructures, such as how dense the fibers are packed in the fibrin network.”

**General comments:**
**1) As mentioned above, the way in which the parameters calculated from turbidity and TEG are complementary are not obvious. The authors might consider updating the tittle of the manuscript. Alternatively, a designated section describing what fibrin clot characteristic the chosen parameters are used to measure (e.g., maximum absorbance in turbidity is indicative of fibre thickness, and maximum amplitude in TEG reflects absolute clot strength), and how these parameters complementary, can be included in the manuscript.**

See above added statements to strengthen the complementary claim of the two unique clot characterization techniques.

**2) The use of 'turbidity' and/or 'turbidimetry' is not continuous throughout the manuscript. For instance, 'turbidimetry' is used in the abstract and 'turbidity' in the introduction. The authors do mention that mass/length ratio can be calculated from turbidity data, which justifies the term 'turbidimetry'. However, the actual method described in the manuscript is for turbidity analysis, and with that I would suggest the authors use 'turbidity' throughout the manuscript.**

To maintain uniformity, the word “turbidimetry” has been replaced with “turbidity” throughout the manuscript.

**Specific comments:**
**3) In the abstract and first paragraph of the introduction the authors refer to fibrin as the primary protein responsible for clot formation. This should be updated to fibrinogen or fibrin(ogen) for a more accurate description.**

In both the abstract and introduction, the word “fibrin” has been replaced with “fibrin(ogen).”

**4) Following from the previous comment, on lines 50-51 the authors state 'In the final step of the coagulation cascade, fibrin monomers are cleaved from fibrinogen by thrombin…'.**

**This sentence in incorrect and needs careful revision! Fibrinogen, which is the final product of the coagulation cascade, has its fibrinopeptides cleave by thrombin to form fibrin monomers that are able to polymerise.**

This sentence has been modified to "In the final steps of the coagulation cascade, fibrinopeptides are cleaved from fibrinogen by thrombin initiating the polymerization of insoluble fibrin monomers as the clot develops."

**5) Section 1: Why did the authors decide the use PBS? This buffer choice could become problematic with the addition of calcium, as it would cause it to precipitate. In the sections where the authors mention the potential addition of calcium or use of citrated plasma this issue needs to be highlighted! (e.g., section 5 and discussion).**

Since the experiments in this manuscript do not involve the addition of calcium, PBS was used as it can operate at physiological pH and can provide a large pH range to study the effect of pH on clot formation. However, we agree that PBS can cause calcium precipitation and therefore, included the following sentences to highlight this issue with PBS.

"Note: PBS was used throughout this study as the described assays did not require the addition of calcium. It is important to note that when adding calcium, often utilized to re-calcify citrated blood products, PBS should be avoided as calcium is known to precipitate in phosphate buffers."(In section 1.)

"A suitable buffer system should be carefully selected taking into consideration each unique assay requirements." (In the note of section 5)

"However, since phosphate can bind to calcium, PBS should be avoided when calcium is used as the clot initiator." (discussion section)

**6) Section 1.3: a degree symbol is missing in between 37 C.**

This error has been corrected.

**7) Section 2.1.4: was the extinction coefficient used in the concentration calculation provided by the supplier? This is very dissimilar to the extinction coefficient for human plasma purified fibrinogen from Sigma.**

Yes, the extinction coefficient was provided by the supplier. However, it was converted to molar extinction coefficient to determine concentration using Beer's law. For better understanding, "Extinction coefficient" has been replaced with "Molar extinction coefficient" throughout the manuscript. The following statement has also been added in section 2.1.4 note to clarify the difference between molar extinction coefficient and extinction coefficient:

"ε is derived by multiplying $E^{1\%}$ = 15.1 (280 nm) (extinction coefficient, given by the supplier) with MW."

**8) Section 2.2: Why is thrombin thawed at RT? This is likely drastically decreasing its**

**activity! I would suggest it be thawed as quickly as possible and be kept on ice. Keeping this enzyme on ice in order to keep high levels of activity should be highlighted in the protocol**

We agree that measures to retain thrombin activity should be taken. A statement to address this issue has been added in the note of section 2.2.3

"Note: Precautions should be taken to maintain enzyme activity which can be accomplished by maintaining enzymes on ice during thawing and use; however, no reduction in thrombin activity was observed when utilized directly after thawing at RT."

**9) Section 3.4: If compatible with the instruments I would suggest an interval and short as possible, which allows for more accurate overall data to be obtained.**

To address this concern, the following sentence has been added to the note of section 3.4:
"The selected reading interval should be as short as possible to achieve the highest level of assay sensitivity. This will depend upon the spectrometer and number of wells being read during a given assay."

**10) Section 3.6: Was a multichannel pipette used for this step to ensure the measurements can start as soon as fibrinogen is mixed in? In other words, is the time taken between mixing the 1st to the last well taken into account when obtaining the parameters once the run is complete?**

Yes, a multichannel pipette was used. We also took into account the time difference across wells (if there was any) as well as the time period prior to the first read by the instrument in the analysis. To address this concern, a sentence has been added in the note of section 3.6:

"Note: Use a multichannel pipette when running multiple clot samples on the same plate at the same time. Record time differences across wells and the time period prior to the first read by the instrument to offset clotting times."

**11) Section 4.5: is this mixed at all prior to the start of the run? This would be particularly important when other agents added (such as tPA, calcium, etc.)**

Yes, the contents were mixed prior to the start of the run. A sentence has been added at the end of section 4.5 to address this:
"Mix the contents by pipetting up and down five times."

**12) For both methods a buffer only control (i.e., no fibrinogen) should be included. This is particularly important when other proteins, such as albumin, are added to the clotting mixture.**

To address this concern, a sentence has been added to the note of section 5:
"For turbidity and TEG assays, a buffer only control should also be included to ensure an accurate background subtraction while analyzing the effect of these variables."

**13) Section 5.5.2: A clear distinction between extinction coefficient and molar extinction coefficient would be helpful.**

“Extinction coefficient” has been replaced with “molar extinction coefficient” throughout the manuscript.

**14) Figure 1 legend: The legend should read '…examples of clot turbidity tracing curve', as per the remaining legend.**

Turbidimetry has been replaced with turbidity in the Figure 1 legend.

**15) Table 1: In trend results of increased albumin level an 'x' is missing from TEGMax.**

The word has been corrected in the table file.

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Apr 21, 2020

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