Microkinetic coagulation assays for human and zebrafish plasma

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Coagulation assays, prothrombin time (PT), and partial thromboplastin time (PTT) are tests to measure the clotting ability of plasma and used in evaluating patients suffering from bleeding disorders. These assays require 100 μl of human plasma. In zebrafish, dilute plasma with exogenously added human fibrinogen was used. Our objective is to create a microkinetic coagulation assay for human and zebrafish plasmas using 1 μl plasma under conditions similar to PT and PTTs. Here, we developed an assay using the Take3 plate with wells holding up to 6 μl, which can be loaded in a microplate reader for measuring the absorbance of fibrin formation. In this assay, we used 1 μl of citrated zebrafish or human plasma followed by the addition of either thromboplastin or Dade ACTIN or factor X activator from Russell viper venom as an activating agent and CaCl$_2$. We found 4 or 3 μl of the final volume of reaction was optimal. Our results showed both zebrafish and human plasmas yielded kinetic PT, kinetic PTT, and kinetic Russel's viper venom time curves similar to previously established curves using dilute plasma. This kinetic coagulation was inhibited by heparin and was reduced significantly in coagulation factor deficient plasmas. These results validated our microkinetic coagulation assays. Moreover, we derived clotting times from these kinetic curves, which were identical to human PT, PTT, and Russel's viper venom time. In conclusion, we established a microkinetic assay that could measure blood coagulation activity in models like zebrafish and human blood samples obtained from a finger prick in adults or heel prick in infants. *Blood Coagul Fibrinolysis* 32:50–56 Copyright © 2020 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

In humans, after injury, the ruptured blood vessel exposes the subendothelial matrix and tissue factor (TF) that initiates blood coagulation [1]. The TF binds to the preexisting activated coagulation factor VII (FVII). This complex cleaves coagulation factor X to generate FXa, which further converts small amounts of prothrombin to thrombin. The minuscule amount of thrombin generated converts fibrinogen to fibrin, which is not sufficient to plug the wound. This process is known as the initiation pathway of blood coagulation, also called the extrinsic pathway. The thrombin generated by the above pathway then cleaves coagulation factor XI (FXI) to FXIa, which subsequently converts coagulation factor IX (FIX) to FIXa. FIXa, along with activated coagulation factor VIII, cleaves larger amounts of factor X to FXa, which converts explosive amounts of prothrombin to thrombin. This thrombin converts large quantities of fibrinogen to fibrin that seals the injured site and prevents bleeding. As the above pathway enhances the production of clots, it is called the propagation pathway. However, *in vitro* in the presence of a negative charge, coagulation factor XII (FXII) is converted to FXIIa, which then cleaves FXI to FXIa and continues the cascade to generate fibrin [2]. This in-vitro FXII-mediated pathway is called the intrinsic pathway. The FXa generated by both the extrinsic and intrinsic pathways convert prothrombin to thrombin in the presence of activated coagulation factor V. The cascade of events downstream of FXa, generating fibrin, is known as the common pathway. A similar mechanism of clot initiation and propagation has been observed in zebrafish, although FXII is missing, and there may be FXII-like molecule in the zebrafish [3].

The extrinsic, intrinsic, and common pathways are measured *in vitro* by prothrombin time (PT), partial thromboplastin time (PTT), and Russel's viper venom time (RVVT) which are activated by thromboplastin, Dade ACTIN and Russel's viper venom-Xa, respectively [4]. The assays generally require 100 μl of human plasma in clinical laboratories. As we can only obtain 1 μl of plasma in zebrafish, a method that measures PT, PTT, and RVVT using 1 μl of zebrafish plasma in 100 μl of total reaction volume (100 times diluted zebrafish plasma) spiked with human fibrinogen was established [5]. These assays are called kinetic PT (kPT), kinetic PTT (kPTT),

and kinetic RVVT (kRVVT) assays. In classical PT, PTT, and RVVT measurements, a final 33% plasma concentration is used without exogenously added fibrinogen. Here, using Take3 plates, we developed modified kPT, kPTT, and kRVVT assays with a final 25% zebrafish plasma concentration and eliminated the addition of exogenous human fibrinogen, making it comparable with the conditions used in human PT, PTT, and RVVT assays. We have also applied this assay to human coagulation using 33% plasma, which is identical to the classical coagulation assays. In conclusion, the assays developed here could be used not only to measure blood coagulation activity in mice and zebrafish but also in pediatric clinics where the volume of blood collected from the infants is a limiting factor.

Materials and methods
Materials
Human citrated plasma, human factor X deficient plasma, rabbit thromboplastin, purified factor X activator from Russell viper venom (RVV-Xa), and MS222 were purchased from Millipore Sigma (St. Louis, Missouri, USA) and reconstituted for use as per manufacturer's instructions. Human FVII deficient plasma and FXII deficient plasma were purchased from Haematologic Technologies, LLC (Essex Junction, Vermont, USA). Dade ACTIN was purchased from Trinity Biotech (Jamestown, New York, USA). All analytical reagents were from Fisher Scientific (Waltham, Massachusetts, USA).

Zebrafish thromboplastin was prepared from the zebrafish muscle, as described previously [6]. Briefly, 10 zebrafish, each weighing approximately 0.5 g were used; their scales, fins, internal organs, and head were removed, and the rest of the zebrafish muscle was suspended in 0.1 mol/l NaCl, 50 mmol/l Tris-HCl, pH 7.5 on ice and homogenized for 30 s. The homogenate was stirred at 37 °C for 30 min and centrifuged at $11\,000 \times g$ for 15 min. EDTA was added to the supernatant to achieve a final concentration of 20 mmol/l and centrifuged at $48\,000 \times g$ for 60 min. The pellet was washed twice by high-speed centrifugation at $48\,000 \times g$ for 60 min and suspended in 1 ml buffer containing 0.1 mol/l NaCl and 50 mmol/l Tris-HCl at a pH of 7.5. The thromboplastin was stored at −80 °C for use in modified kPT/PT assay. The volume of thromboplastin added was adjusted according to the maximal activation of coagulation.

Zebrafish husbandry
Wild-type zebrafish were obtained from Ekwill farms through a local vendor (Fish n Chirp Pets, Denton, Texas, USA). Fish were maintained at 28 °C in a recirculating water system supplemented with the Instant Ocean under a 14-h light/10-h dark cycle. They were fed live brine shrimp and fish flakes thrice a day. All zebrafish procedures were approved by the University of North Texas Institutional Animal Care and Use Committee, and animal experiments were performed with humane care in compliance with institutional guidelines.

Zebrafish plasma preparation
Adult zebrafish were placed horizontally on a dry paper towel, and the head was covered with a wet Kim Wipe. The body was wiped dry with another Kim Wipe, and a small incision was made using a pair of dissecting scissors in to the region of the dorsal aorta and posterior cardinal vein, located in between the posterior dorsal and ventral fins. An amount of 2 μl of blood welling up at the site of incision were quickly collected using a micropipette and added to 0.5 μl of 3.8% sodium citrate in a microcentrifuge tube. The tube was finger tapped to ensure complete mixing. It was then centrifuged at $1000 \times g$ at 4 °C for 5 min. The top 1 μl of clear citrated plasma was pipetted out without disturbing the red cell layer and used for kinetic coagulation assays [7]. After blood collection, the fish was immediately euthanized by MS222 overdose [8].
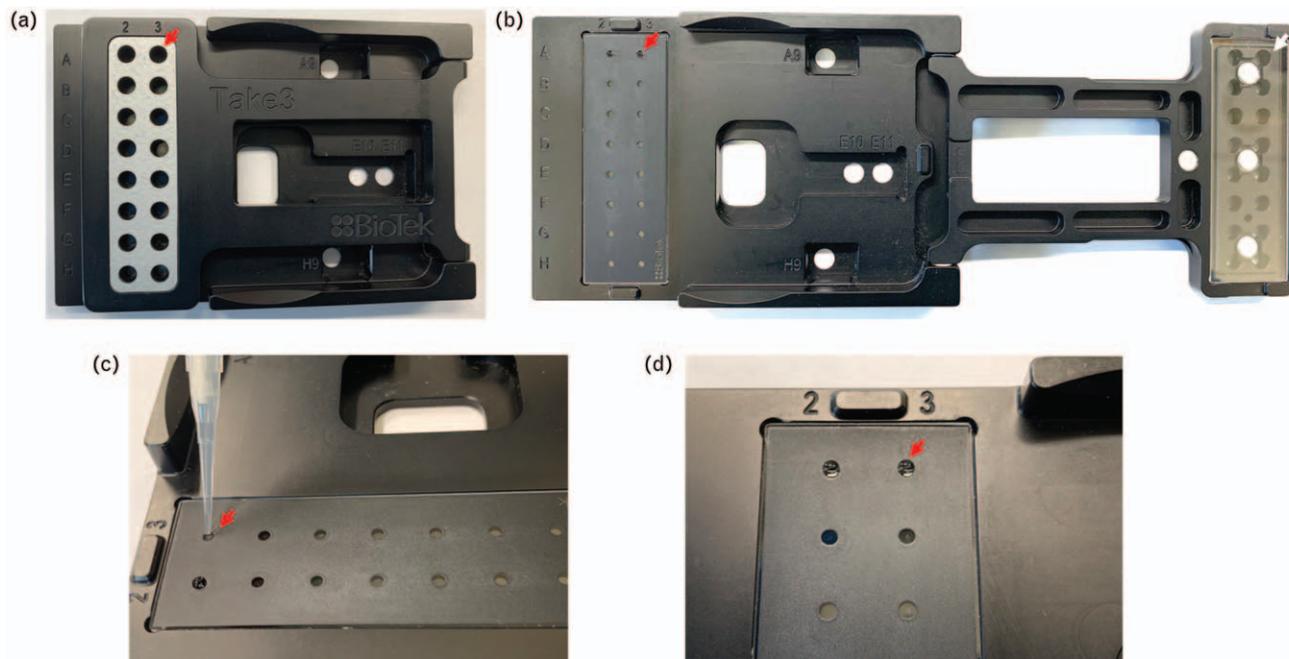
Kinetic blood coagulation assays
Blood coagulation assays were performed in the Take3 Micro-Volume Plate (Biotek, Winooski, Vermont, USA) capable of holding a maximum of 6 μl in each well. All reagents used for human and zebrafish plasma assays were incubated at 37 and 25 °C, respectively, before use. 1 μl of human plasma or 1 μl zebrafish plasma mixed with 1 μl 1× PBS was added to a well followed by the addition of 1 μl of either thromboplastin (rabbit or zebrafish) or Dade ACTIN or RVV-Xa. The reaction was recalcified by adding 1 μl of 100 mmol/l CaCl$_2$. Next, the plate was immediately placed in the Epoch 2 Microplate Spectrophotometer (Biotek). The plate was shaken for 2 s and absorbance of each sample was recorded at 405 nm every 5 s for 7 min for human plasma (at 37 °C) and every 5 s for 2 min for zebrafish plasma (at 25 °C). Fibrin generated using thromboplastin (rabbit or zebrafish), Dade ACTIN, and RVV-Xa were called kPT, kPTT, and kRVVT, respectively. The kPT, kPTT, and kRVVT assays were also carried out in the presence of heparin. One-tenth of a microliter of heparin (50 and 1 mg/ml for human and zebrafish plasma samples, respectively) was added in every assay before the addition of CaCl$_2$. Negative controls were run by replacing thromboplastin (rabbit or zebrafish) or Dade ACTIN or RVV-Xa with 1× PBS. The kinetic curves were obtained by plotting the absorbance at 405 nm as a function of time. The time taken for half-maximal fibrin formation (TTHFF) and maximal absorbance were calculated and compared between normal and experimental plasma groups.

Statistical analysis
Statistical calculations were performed using GraphPad Prism version 8.4.3, GraphPad Software, San Diego, California, USA. Normal and experimental groups were

Loading of Take3 plate. Take3 plate used for performing the kinetic assays and its loading with plasma and coagulation reagents are shown. (a) Take3 plate in the closed state showing the path for light transmission (looks like holes, shown by the red arrow), (b) Take3 plate when the lid is opened towards the right, exposing the microwells (shown by the red arrow on the left) and the thin glass plate that covers the microwells in the closed state (shown by the white arrow on the right), (c) adding plasma and reagents to the microwell (shown by the red arrow) and (d) Take3 plate after sample loading (shown by the red arrow). The lid is then closed and will be like (a) where the sample can be viewed from the top.

analyzed using unpaired t test or ordinary one-way analysis of variance followed by Dunnett's multiple comparisons test. P value less than 0.05 was considered statistically significant. The mean $\pm$ SD was plotted.

Results and discussion
Previously kinetic coagulation assays were developed using dilute plasma with exogenously added human fibrinogen as the amount of plasma collected in zebrafish is limiting [5]. However, these conditions were not similar to those used in human clinical PT, PTT, and RVVT, where the final concentrations of plasma are 33%, and the fibrinogen present intrinsically is sufficient to form the fibrin clot for detection. To establish similar conditions without adding fibrinogen, we miniaturized the existing kinetic blood coagulation assays. This miniaturization was achieved by using the Take3 plate that has wells, each holding 6 µl total volume (Fig. 1). The Take3 plate can be placed in any Biotek microplate reader to monitor the absorbance at 405 nm. However, other plate readers could be used if they are programmed to align the plate for detecting the wells for reading the absorbance values. We performed the kPT, kPTT, and kRVVT assays using zebrafish plasma in these plates. The kinetic curves were obtained (Fig. 2a–c) and were used to calculate the TTHFF and maximal absorbance for each assay. The

results showed the average TTHFFs for kPT, kPTT, and kRVVT of zebrafish plasma were 29 (Fig. 2d), 26 (Fig. 2e), and 35 (Fig. 2f) s, respectively. To test whether zebrafish plasma is inhibited by heparin, we performed the kPT and kPTT assays in the presence of 1 mg/ml heparin. The kPT and kPTT assays of zebrafish plasma showed inhibition with heparin as expected with average TTHFF of 22 (Fig. 2d) and 33 (Fig. 2e) s, respectively. We also found that the average maximal absorbance was 0.05 for kPT (Fig. 2g), kPTT (Fig. 2h), and kRVVT (Fig. 2i) with zebrafish plasma. Similarly, the average maximal absorbances for kPT and kPTT assay with 1 mg/ml heparin were 0.014 (Fig. 2g) and 0.026 (Fig. 2h), respectively. The negative control curve obtained using wild-type zebrafish plasma did not show any coagulation activity and is shown in Fig. 3.

To test whether this microassay works with human plasma, we also performed the kPT, kPTT, and kRVVT assays using normal and coagulation factor deficient human plasmas in the Take3 plate. The kinetic curves obtained (Fig. 4a–c) were used to calculate the TTHFF and maximal absorbance for each assay. The average TTHFF for kPT, kPTT, and kRVVT of normal human plasma was found to be 23 (Fig. 4d), 51 (Fig. 4e), and 34 (Fig. 4f) s, respectively. To test whether kPT and kPTT

Fig. 2
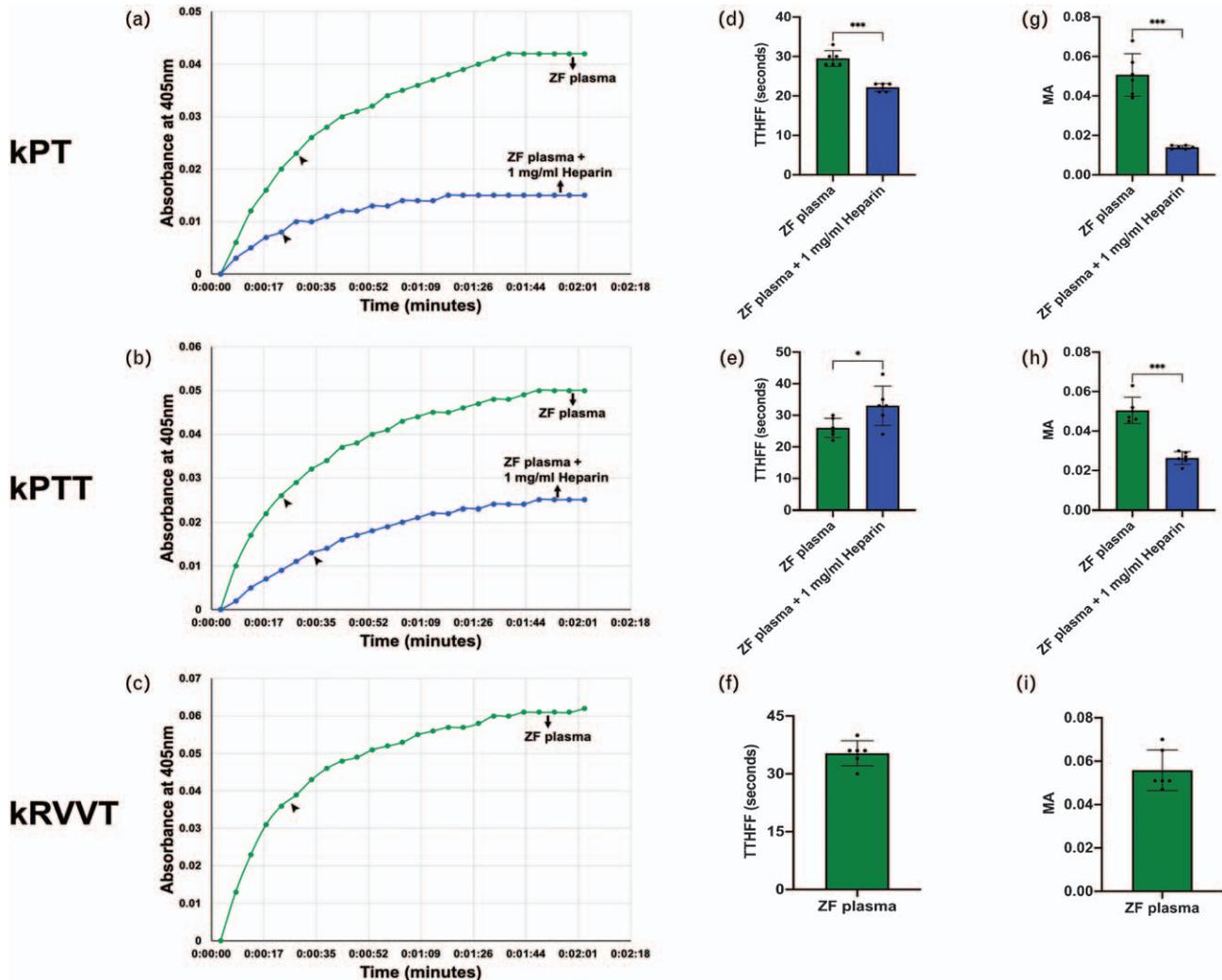
Kinetic assays performed using zebrafish plasma. Left panels represent kinetic curves of fibrin formation, shown in green for normal zebrafish plasma generated using (a) zebrafish thromboplastin (kinetic prothrombin time), (b) Dade-ACTIN (kinetic partial thromboplastin time) and (c) factor X activator from Russell viper venom (kinetic Russel's viper venom time). The absorbance at 405 nm is plotted as a function of time, showing increases in fibrin formation. Heparin inhibition, shown in blue for kinetic prothrombin time and kinetic partial thromboplastin time (zebrafish plasma + 1 mg/ml heparin), are represented in (a) and (b). Arrowheads point to the time to half-maximal fibrin formation for each curve. The middle panels show a significant difference between the time to half-maximal fibrin formation of normal plasma and plasma inhibited with heparin in (d) kinetic prothrombin time, and (e) kinetic partial thromboplastin time. (f) Time to half-maximal fibrin formation for the kinetic Russel's viper venom time. Right panels show a significant reduction in the maximal absorbance between normal plasma and plasma inhibited with heparin in (g) kinetic prothrombin time, and (h) kinetic partial thromboplastin time. (i) Maximal absorbance, for the kinetic Russel's viper venom time. Normal and heparin treated plasma groups were analyzed using unpaired *t* test and plotted as mean ± SD ($n = 6$). *$P < 0.05$ and ***$P < 0.001$.

are inhibited by heparin, we performed these assays in the presence of 50 mg/ml heparin. The normal human plasma showed inhibition with heparin as expected, with average TTHFF for kPT and kPTT of 15 (Fig. 4d) and 18 (Fig. 4e) s, respectively. To test whether coagulation factor deficient plasmas have impaired fibrin formation, we used FVII, FXII, and factor X deficient plasmas and performed the kPT, kPTT, and kRVVT assays, respectively. We found a prolongation for the initiation of clot formation with average TTHFF of 360 (Fig. 4d), 86

(Fig. 4e), and 55 (Fig. 4f) s, respectively. We also found that the average maximal absorbance was 0.035 (Fig. 4g), 0.056 (Fig. 4h), and 0.031 (Fig. 4i) for kPT, kPTT, and kRVVT with human plasma. Similarly, the average maximal absorbances for kPT and kPTT assays with 50 mg/ml heparin were 0.013 (Fig. 4g) and 0.011 (Fig. 4h), respectively. Moreover, the average maximal absorbances for FVII, FXII, and factor X deficient plasmas were found to be 0.041 (Fig. 4g), 0.013 (Fig. 4h), and 0.018 (Fig. 4i), respectively. The negative control curve

Fig. 3
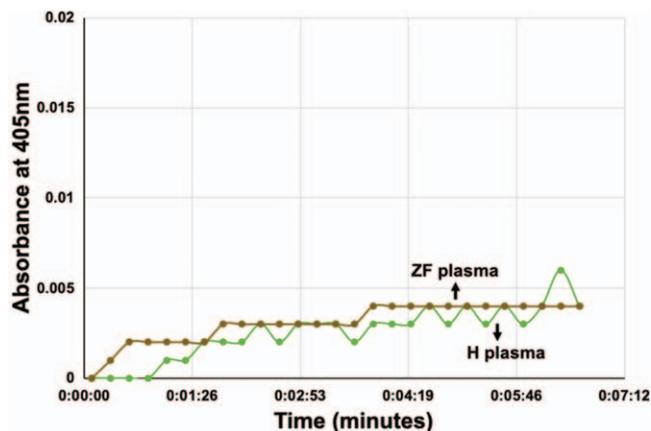
Negative control for kinetic assays. The kinetic curve obtained using zebrafish plasma or human plasma without thromboplastin or Dade ACTIN or factor X activator from Russell viper venom.

obtained using normal human plasma did not show any coagulation activity and is shown in Fig. 3.

As the human PT, PTT, and RVVT are in the range of 10–12, 25–35, and 29–44 s, respectively, we calculated the absorbance values in a representative curve corresponding to 10, 30, and 40 s, for PT, PTT, and RVVT, respectively. The results showed 0.012, 0.029, and 0.025 for PT, PTT, and RVVT, respectively. Subsequently, we used these absorbance values and calculated the corresponding times in seconds for the normal human plasma curves. We found the average times to attain the above absorbance values were 13.6, 31.6, and 43 s for human kPT, kPTT, and kRVVT, respectively (Fig. 5). Similarly, for zebrafish, we used the above absorbance values and obtained 12.8, 33.1, and 40 s for kPT, kPTT, and kRVVT, respectively (Fig. 5).

In this article, we have developed a microkinetic coagulation assay for human and zebrafish samples using 1 μl of

Fig. 4
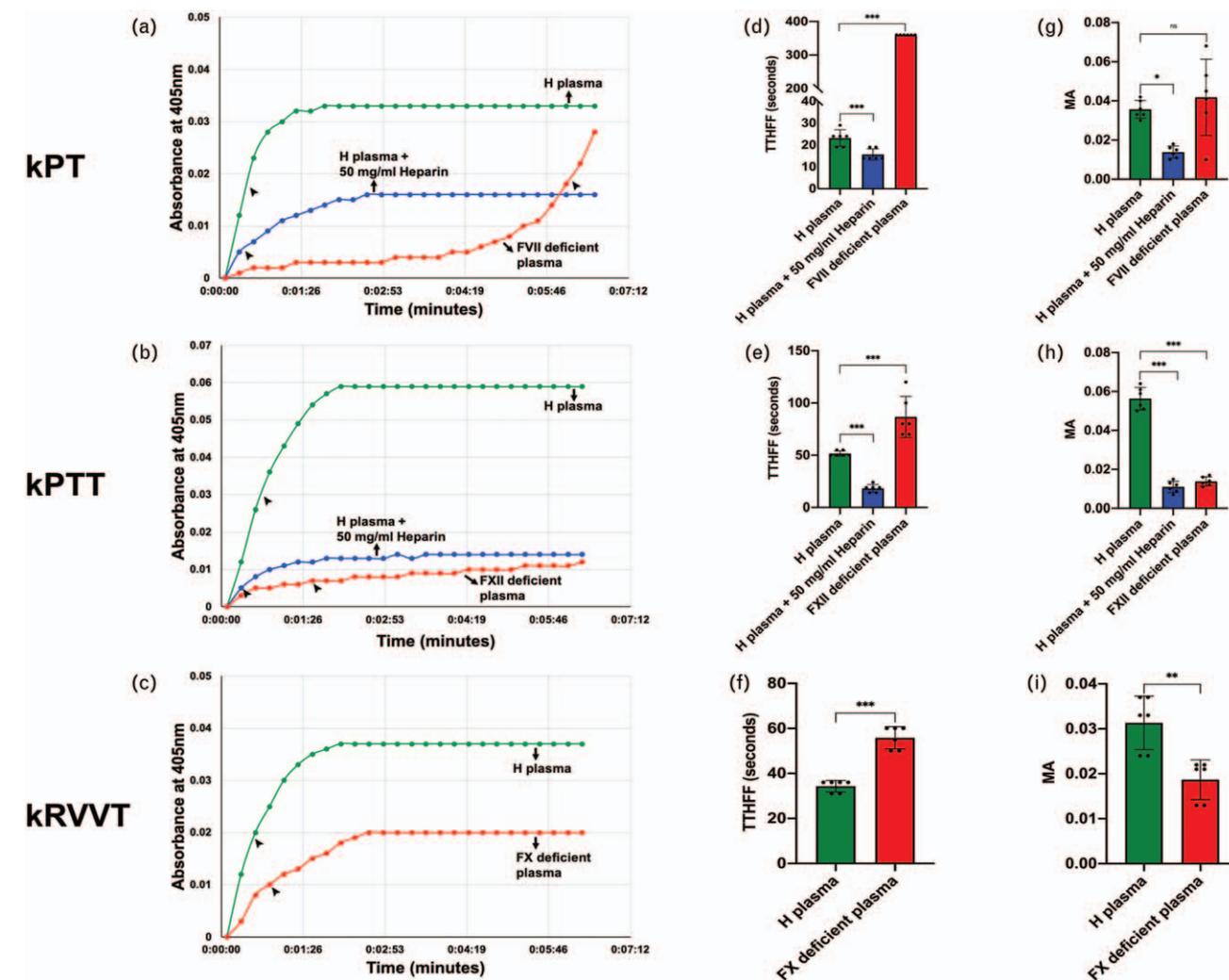
Kinetic assays performed using human plasma. Left panels represent kinetic curves of fibrin formation, shown in green for normal human plasma generated using (a) rabbit brain thromboplastin (kinetic prothrombin time), (b) Dade-ACTIN (kinetic partial thromboplastin time), and (c) factor X
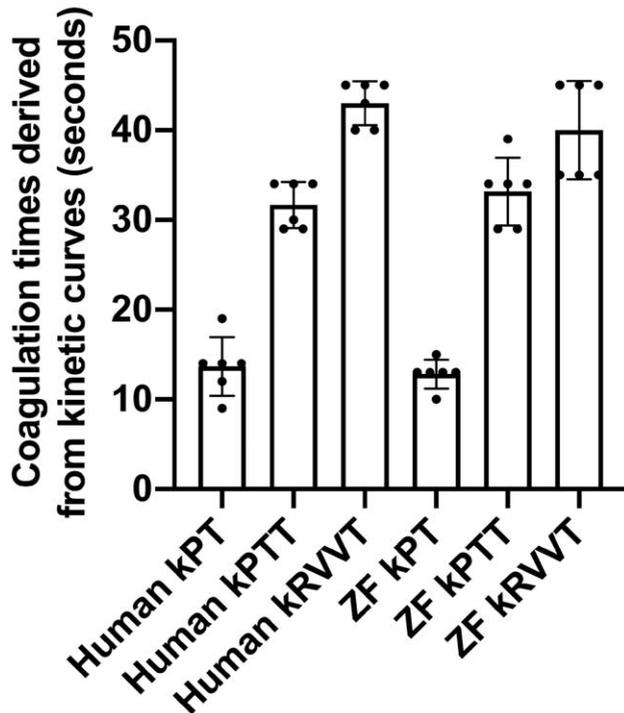
Coagulation times derived from the kinetic curves. The time in seconds, at a particular absorbance value, derived from the corresponding kinetic curves (kinetic prothrombin time, kinetic partial thromboplastin time, kinetic Russel's viper venom time) for both human and zebrafish plasmas, were plotted. The mean values represent prothrombin time, partial thromboplastin time, and Russel's viper venom time, respectively.

plasma. In this assay, the final concentration of plasma was 25–33%, which is similar to the percentages of plasma used in classical clinical PT, PTT, and RVVT. This assay with a reduced amount of plasma was enabled by the availability of Take3 microvolume plate. Therefore, this assay could be used in infants, where a small volume of blood is collected. We could also use this in the blood collected after finger prick, as long as it is collected rapidly into a citrated tube in less than 10 s.

In zebrafish coagulation assays, 33% plasma was not successful because the fish plasma clots faster than human plasma. Therefore, we had to adjust the final

plasma concentration to 25%. Although this concentration is less than 33%, the coagulation curves are strikingly similar to the curves obtained using 33% human plasma. The confirmation of the assay curves by the inhibition of coagulation activation in heparin treated samples as well as in deficiency plasma samples lends support for this assay.

We have analyzed the curves by both TTHFF and maximal absorbance to check whether both these analyses would yield results compatible with activation and inhibition of coagulation. Here, we found that both these approaches yielded similar data supporting the activation and inhibition of coagulation. Significantly, we observed prolongation of TTHFF of kPTT of zebrafish plasma treated with heparin, in contrast to the kPT results. This is probably due to the differences between the rate of reaction of kPT and kPTT in the presence of heparin. Similarly, we also found the maximal absorbance of kPT using FVII deficient plasma departed from maximal absorbances of kPTT of FXII deficient plasma and kRVVT of factor X deficient plasma. Again, this discrepancy could be explained due to the differences in the rate of the enzymatic reactions. Despite these differences, the kinetic curves distinctly show a shift to the right side of the normal human plasma curves indicating the inhibition by heparin as well as the coagulation factor deficient plasmas. Significantly, the times obtained at a given absorbance unit in both human and zebrafish plasmas are similar to the classical PT, PTT, and RVVT. These results once again emphasize the validity of this method.

In this assay, pipetting reagents into the Take3 plate and loading the plate into the microplate reader must be quick to obtain the first initiation points of absorbance in coagulation activation. To accomplish this goal, we have limited our assays to only two wells at a time. However, the use of an eight-channel pipette could improve the efficiency of this method.

In conclusion, in this article, we established a microkinetic coagulation assay method using 1 μl of plasma from both human and zebrafish. We could also derive coagulation times in seconds from the kinetic curves, which were similar to classical PT, PTT, and RVVT. Furthermore, as microvolumes of blood are needed, blood collected from either finger prick or from the infant's heel

activator from Russell viper venom (kinetic Russel's viper venom time). The absorbance at 405 nm is plotted as a function of time, showing increases in fibrin formation. Heparin inhibition, shown in blue for kinetic prothrombin time and kinetic partial thromboplastin time (human plasma + 50 mg/ml heparin) are represented in (a) and (b). Impaired fibrin formation, shown in red for (a) kinetic prothrombin time, (b) kinetic partial thromboplastin time and (c) kinetic Russel's viper venom time performed using factor VII deficient plasma, factor XII deficient plasma, and factor X deficient plasma, respectively. Arrowheads point to the time to half-maximal fibrin formation for each curve. The middle panels show a significant difference between the time to half-maximal fibrin formations of normal human plasma and plasma inhibited with heparin or coagulation factor deficient plasmas in (d) kinetic prothrombin time, (e) kinetic partial thromboplastin time and (f) kinetic Russel's viper venom time. Right panels show significant reduction in the maximal absorbance between normal human plasma and plasma inhibited with heparin or coagulation factor deficient plasmas in (g) kinetic prothrombin time, (h) kinetic partial thromboplastin time, and (i) kinetic Russel's viper venom time, except the maximal absorbance of kinetic prothrombin time of factor VII deficient plasma which did not show a significant reduction compared with normal human plasma. Normal, heparin treated, and coagulation factor deficient plasma groups were analyzed using ordinary one-way analysis of variance followed by Dunnett's multiple comparisons test and plotted as mean ± SD ($n = 6$). ns, nonsignificant. **$P < 0.01$ and ***$P < 0.001$.

prick should be sufficient to perform these assays. Moreover, single mutant zebrafish blood would be adequate for conducting these assays.

Acknowledgements

Conflicts of interest

The authors declare no competing financial interests.

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