PART 5 Initial Evaluation of Hemostasis

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TOPICS COVERED

- ✓ Bleeding Time Test
- ✓ Prothrombin Time (PT)
- ✓ Activated Partial Thromboplastin Time (APTT)
- ✓ Mixing Tests for Further Investigation of Abnormal PT and APTT
- ✓ Thrombin Clotting Time
- ✓ Thrombin Time in the Presence of Protamine Sulphate to Detect Presence of Heparin
- ✓ Reptilase Time
- ✓ Fibrinogen (Modified Clauss Assay)
- ✓ Removal of Heparin from Plasma

Bleeding Time Test: The bleeding time test was historically developed to pre-operatively assess patients' capability to maintain a normal bleeding pattern while undergoing major surgical procedures, or patients suspected to have a bleeding disorder due to platelet dysfunction. Unfortunately, the test has been confirmed to be unreproducible and insensitive, and therefore, if it must be used, it should be in combination with a comprehensive family and clinical history, with accompanying coagulation screening tests, including platelet count and morphology. The PFA 100/200 has largely replaced the bleeding time test in the assessment of platelet function despite its observed inadequacies in thrombocytopenic patients (Rodgers and Levin, 2023; Undas, 2023).

The bleeding time is the time taken for a standardized skin cut of fixed depth and length to stop bleeding. Prolongation of the bleeding time occurs in patients with thrombocytopenia, von Willebrand disease (VWD) type 3 and type 2B, Glanzmann's thrombasthenia, Bernard-Soulier syndrome, storage pool disease, other platelet disorders, sepsis (Williams et al, 2024), autoimmune diseases, vitamin deficiency, severe anemia, hematological malignancies (e.g. myeloproliferative disorders that results in factor V deficiency), and reaction to drugs (Vinholt et al, 2019). Fibrinogen is required to stop bleeding, and a role for FV has been suggested. The bleeding time can therefore be prolonged in patients deficient in fibrinogen or FV. Prolongation of bleeding time also occurs in some patients with renal disease, dysproteinemias, and vascular disorders (Russeau et al, 2023; Bourguignon et al, 2022).

Materials and equipment:

- ✓ Sphygmomanometer
- ✓ Cleansing swabs
- ✓ Template bleeding time device
- ✓ 1 mm thick filter paper
- ✓ Stopwatch

Method:

- ✓ The sphygmomanometer cuff is placed around the upper arm positioned at the level of the heart and inflated to 40 mm of mercury. This pressure is maintained throughout the test.
- ✓ The dorsal surface of the forearm is cleaned, and the bleeding time device placed firmly against the skin without pressing. The trigger is depressing and the stopwatch started.
- ✓ Superficial veins, scars, and bruises should be avoided.

✓ At 30-second intervals, blot the flow of blood with filter paper. Bring the filter paper close to the incisions without touching the edge of the wound.
✓ Record the time from puncture to cessation of bleeding.

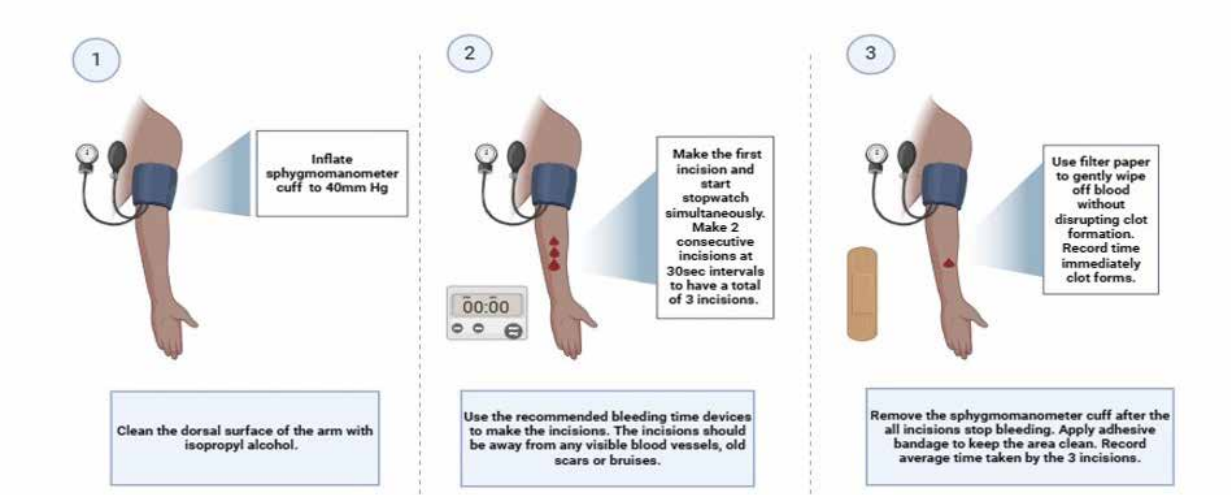

Figure 2. Flow diagram demonstrating bleeding time test. (Adopted from Sally Kim's template for "Measurement of blood pressure: The Auscultatory Method" in biorender.com.)

Interpretation: The normal range in adults is 2 to 7minutes (up to 8 minutes), but may vary according to the method used.

Notes: A normal range should be established locally, regardless of the device used. The incision should be made in a direction parallel to the length of the arm. Cuts made perpendicular bleed longer. An abnormal result should be repeated. It is not necessary to record endpoints if bleeding continues beyond 20 minutes. The effect of drugs interfering with platelet function should be considered. For example, drugs containing Aspirin can cause prolongation. So, where possible, these should not be taken for seven days prior to testing. There is a possibility of scarring and sometimes hematoma formation at the site of bleeding time incisions. This should be brought to the attention of patients prior to performing the incision. Bleeding time in pregnant females can be misleading due to physiologically elevated levels of FVIII and VWF. The sphygmomanometer cuff in use should be regularly calibrated as per the quality standard guidelines in use. The Duke method of bleeding time has a higher rate of inaccuracy with increased risk of hematoma development (Russeau et al, 2023). Since bleeding time is performed at the bedside, all relative standard procedures regarding POCT quality requirements should be applied to promote patient safety (ISO 15189:2022). In case a patient is suspected to have VWD and your laboratory has the capability, the following tests/assays should be performed in addition to obtaining a comprehensive bleeding history: VWF antigen, VWF activity, FVIII assay, total blood count (including platelet counts and morphology), and basic coagulation screening tests (PT, APTT, TT, fibrinogen). These tests/assays would give a better understanding of what the clinical diagnosis might be in the absence of bleeding time or a special coagulation laboratory capable of making a definitive VWD diagnosis.

Prothrombin Time (PT): PT evaluates the integrity of the extrinsic system. It is very useful in detecting coagulation factor deficiencies that could be qualitative or quantitative of the extrinsic and common pathways. It is also useful for monitoring vitamin K antagonist (VKA) anticoagulants such as warfarin, and detecting liver disease, vitamin K deficiency, FX deficiency due to amyloidosis, disseminated intravascular coagulation (DIC), presence of direct oral anticoagulants (DOACs) in a dose-dependent manner, or antibodies against factors of the extrinsic pathway. These conditions can prolong the PT test results (Dorgalaleh et al, 2021). PT is sensitive to changes in factors V, VII, and X, and less so to FII (prothrombin). It is unsuitable for detecting minor changes in fibrinogen level, as results may be abnormal if the fibrinogen level is very

low or if an inhibitor is present. The sensitivity of the test is influenced by the reagents and technique used, and it is important to establish a reference range locally. The pathway measured by PT is shown in Figure 3. The PT reagent, often termed thromboplastin, contains tissue factor and phospholipids. Many suitable reagents are commercially available.

Reagents:

- ✓ Thromboplastin (this may contain calcium chloride)
- ✓ 25mM calcium chloride (required only if thromboplastin reagent does not contain calcium)

Method: The manual tilt-tube technique is described in Part 4 of this manual. The reagent manufacturer's recommendations should be followed. When using a new thromboplastin reagent that has a different lot number from the previous one, a fresh calibration curve must be plotted.

Interpretation: Clotting times are normally influenced by using different coagulometers, depending on how and when the end point is detected. This further emphasizes the importance of establishing normal ranges for the method currently in use in the laboratory and based on the local population. In the presence of mild deficiencies of factor II, V, VII, or X, the degree of prolongation may be minimal. In the case of FII deficiency, the PT may be within the normal range. Some PT reagents can be affected by the presence of lupus anticoagulants/anti-phospholipid antibodies, and some rare types of antibodies may prolong the PT without any prolongation of APTT. Reagents with lower phospholipid concentrations are more likely to be affected, including some reagents that are constructed by lipidating recombinant tissue factor. The presence of activated FVII, either following therapy with recombinant FVIIa or when native FVII has been activated, can shorten the PT. The effect is dependent on the tissue factor reagent used. Reagents containing bovine tissue factor are particularly susceptible to this effect (Kitchen et al, 1992). Whole blood for PT determination may be stable for at least 24 hours, depending on the reagent used (Baglin and Luddington, 1997). PTs determined with reagents containing human tissue factor may be different from those obtained with reagents containing tissue factor from other species, such as rabbit. In such cases, the result obtained with human tissue factor reagents may be more indicative of bleeding risk. For a full discussion of issues related to determination of PT, please read the current CLSI guidelines on one-stage PT and APTT (2023).

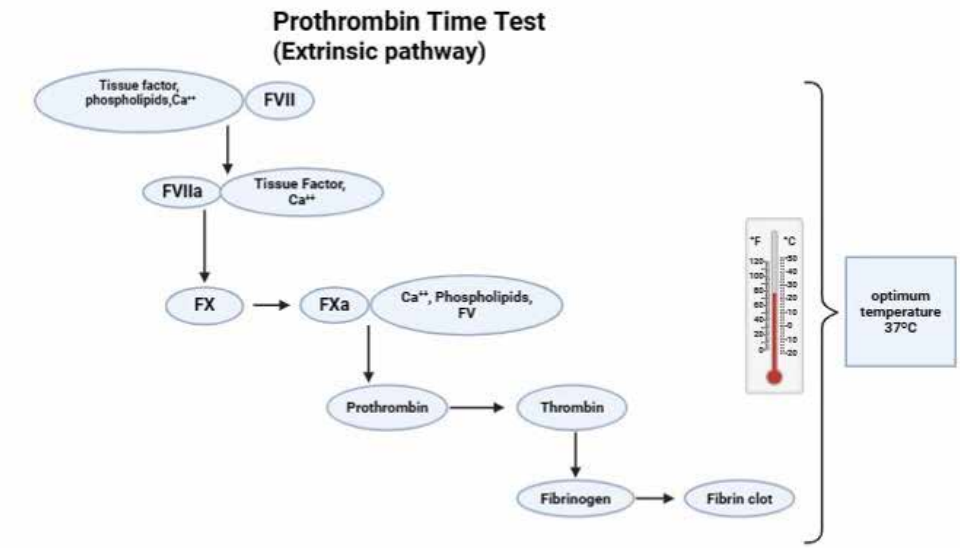

Figure 3. Pathway measured by the PT test

Activated Partial Thromboplastin Time (APTT): This is a clot formation-based assay that helps to identify clotting factor deficiencies or inhibitors of the intrinsic and common pathways. Taken in conjunction with a normal prothrombin time, it is the most useful screening test for detecting deficiencies of factors VIII, IX, XI, and XII. The APTT will also be prolonged in any deficiency involving the common pathways (deficiencies of factors V, X, and II, and fibrinogen) and in the presence of inhibitors. The presence of some therapeutic inhibitors of coagulation such as heparin will also prolong APTT. It is important to exclude the possibility that such treatments have been employed in the initial investigation of prolonged APTTs. The APTT is prolonged in the presence of PKK or HMWK deficiency, unless the test is performed using a reagent that contains ellagic acid as the activator (Turi, 1986). In that case, the APTT will be normal, even in the complete absence of these factors. It is advisable to note that each laboratory must ascertain their APTT normal ranges based on the local population, the type of APTT reagent, and coagulometer in use. The APTT reagent contains diluted phospholipids and contact activators such as silica, ellagic acid, and kaolin. This is added to the platelet poor citrated plasma at 37°C. This mixture is incubated at 37°C for a specified time to permit activation of the contact factors to occur, then the addition of calcium chloride leads to the formation of the fibrin clot. The length of time taken to form a clot is recorded in seconds. The pathway measured by the APTT is shown in Figure 4.

Reagents:

- ✓ APTT reagent
- ✓ 25mM calcium chloride

Method: The manual tilt-tube technique is described in Part 4 of this manual. The reagent manufacturer's recommendations should be followed.

Interpretation: A normal range should always be established locally. A long APTT with a normal PT indicates a possible deficiency of factors VIII, IX, XI, XII, HMWK, PKK, or the presence of an inhibitor. In cases of a long APTT, an equal mixture of normal and test plasma should be tested (i.e. a mixture of 1 part test plasma to 1 part normal plasma, called a 50:50 mix, below). If the APTT corrects by more than 50% of the difference between the clotting times of the normal and test plasma, a factor deficiency is indicated (details are described in specific topic below). Poor correction suggests an inhibitor, possibly to one of the clotting factors in the system or of the non-specific type, such as lupus anticoagulant.

Table 7. An example of interpretation of prolonged APTT

Sample	Result
APTT control	35 seconds
Test	60 seconds
If 50:50 mix	42 seconds (this is a good correction, so there is probably a factor deficiency)
If 50:50 mix	52 seconds (this is a poor correction, so an inhibitor is probably present)

Investigation of isolated prolonged APTT: For patients with a normal PT and prolonged APTT, the normal sequence of investigation to follow is:

- ✓ Determine thrombin time. If it is normal, proceed to follow steps. If thrombin time is prolonged, repeat in the presence of protamine sulphate. If the thrombin time is corrected to normal, this suggests that heparin is present, and further tests below are not required. If the patient is not known to be receiving heparin of any kind, a repeat sample should be requested.
- ✓ Determine APTT on mixtures of normal and patient plasma using a 1:1 (50%) mixture of normal:patient. Failure of the 50% mixture to correct the APTT to normal may indicate the presence of an inhibitor (details are described below).
- ✓ Determine APTT with a second reagent that contains high concentration phospholipids, such as Actin FS (Dade Behring). If the initial APTT is clearly prolonged (by at least three seconds

over the upper limit of normal in use) and the Actin FS is normal, then lupus anticoagulant is the likely cause. This can be confirmed later by specific tests such as dilute Russell's viper venom time, although this is not normally necessary in the absence of any requirement to investigate possible lupus anticoagulant as a risk factor for thrombosis. Very rarely, deficiency of PKK is the other possible cause of normal APTT with Actin FS and marked prolongation of APTT with a reagent that uses silica or kaolin as activator. Like most cases of lupus anticoagulant, this is not associated with any bleeding risk. Therefore, again, confirmation may not be required. When initial APTT is clearly prolonged (three or more seconds) and Actin FS APTT is normal, there is no need to perform factor assays.

- ✓ If both APTTs are prolonged, perform FVIII:C, FIX, and FXI assays. A FXII assay can be performed if required, since deficiency is relatively common and detection of this can then explain the prolongation of APTT. This is not necessary to exclude the presence of a bleeding disorder, since deficiency of FXII is not associated with increased bleeding risk.
- ✓ Reagents such as Actin FS, which employ ellagic acid as the contact activator, are associated with normal results in the presence of even severe deficiency of PKK.

Notes: Many suitable reagents are commercially available. These include materials with different sensitivities. As for PT, clotting times can be influenced by the use of a coagulometer. Historically, research has proven that there is a lot of variability in APTT testing as observed in variation of results of different reagents, variations of results from different coagulometers, and variation of results testing similar samples in different laboratories. This evidence prompts the necessity to adhere to establishment of local normal ranges for specific coagulometers and APTT reagents. Within test plasmas, high levels of one clotting factor can compensate for lower levels of other factors. For example, a markedly raised FVIII during acute phase reaction can lead to a normal APTT in the presence of reductions of FIX or FXI, which could be clinically important. If a patient has the appropriate personal or family history suggestive of a bleeding disorder, further investigation, including specific factor assays, may be justified in the presence of a normal APTT, particularly if the result lies in the upper part of the reference range. The concentration of phospholipid varies markedly between reagents. This is one reason why reagents vary markedly in their sensitivity to the presence of lupus anticoagulants. If a lupus-sensitive reagent is used for the initial APTT, it is useful to perform a second APTT using a reagent such as Actin FS (Dade Behring, Marburg, Germany), which has a very high phospholipid concentration (Kitchen et al, 1999). If the prolongation with the first reagent is caused by lupus anticoagulant, then the second APTT is almost always normal, since very few lupus anticoagulants prolong APTT when Actin FS is used.

A normal APTT with Actin FS, combined with an initial prolonged APTT, normally excludes the presence of FVIII, FIX, or FXI deficiency, and in this case, there is no need for factor assays. Rarely, a normal APTT with any reagent can occur when FIX or FXI are mildly reduced (30 to 50 U/dl) and FVIII is markedly elevated. APTT with Actin FS is frequently normal when FXII is reduced in the range of 20 to 50 U/dl and APTT with kaolin or silica-based activation is mildly elevated. This defect has no clinical relevance. A few powerful lupus anticoagulants prolong APTT with Actin FS. Specific antibodies to FVIII, FIX, or FXI prolong APTT, irrespective of reagent. For a full discussion of issues related to determination of APTT, see CLSI (2023).

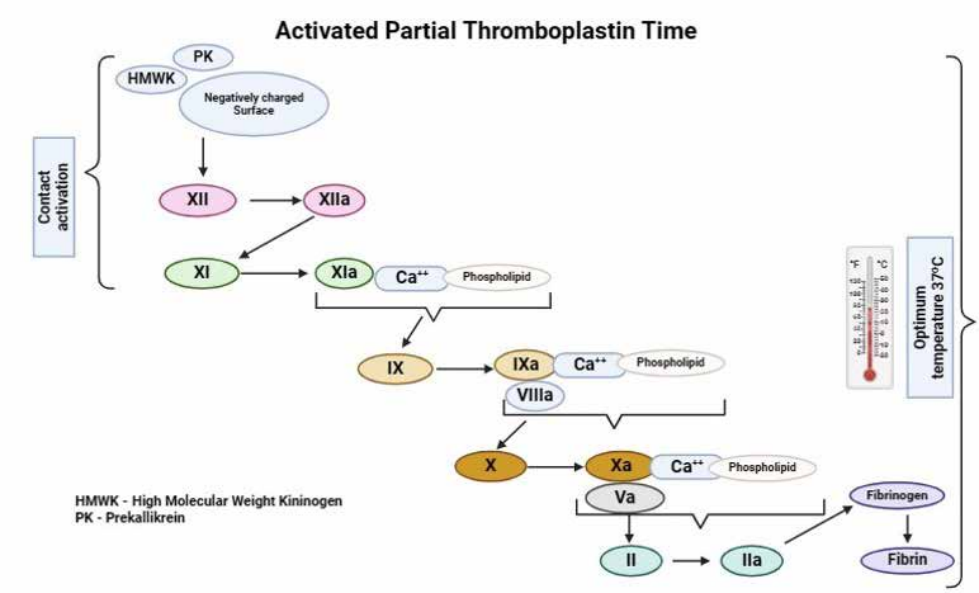

Figure 4. The pathway measured by APTT

Mixing Tests for Further Investigation of Abnormal PT and APTT: Mixing tests are largely performed when the baseline PT or APTT is prolonged, and the cause of the prolongation needs to be identified so that further tests can be performed to arrive at a diagnosis. There has been a lot of variability identified in the way mixing tests are conducted and interpreted. Knowledge about factor deficiencies and inhibitors behavior in mixing studies is paramount in addition to factors that can influence the performance and interpretation of mixing tests (Figure 5) (Favaloro, 2020; Adcock et al, 2023). Patient plasma and PNP are mixed in equal portions (i.e. 50:50) and the previously prolonged test is performed on this mix. The appropriate controls (PNP mixed with plasma containing inhibitor and PNP mixed with plasma containing a factor deficiency) should be used. There are various interpretation methods and each laboratory should establish their cut-off ranges. Each laboratory should additionally establish the normal reference intervals for PT and APTT based on the local population to guide accurate interpretation of results (Adcock et al, 2023).

Procedure:

- ✓ Mix equal portions of PNP and patient plasma (50:50) and set up for the previously prolonged test (APTT will be applied in this case).

If the APTT result obtained from the normal control and patient plasma mix is corrected compared to the original prolonged APTT result, further mixing tests can be done to identify the factor that is deficient in the patient. A second batch of mixing tests can be conducted using one tube containing a mix of the patient's plasma and FVIII deficient plasma in equal portions, and a second tube containing an equal mix of FIX deficient plasma with the patient's plasma. An APTT test is performed on both mixes. The APTT result that shows a correction indicates a FVIII or FIX deficiency (Table 8).

These two mixes are only performed if the patient's clinical history is suggestive of an intrinsic pathway factor deficiency in laboratories that do not have the capacity to do factor assays.

Interpretation of mixing tests: If the APTT corrects by more than 50% of the difference between the clotting times of the normal and test plasma, a factor deficiency is indicated, as discussed previously. Poor correction suggests an inhibitor, possibly to one of the clotting factors in the system or of the non-specific

type, such as lupus anticoagulant. We can also use the calculation percentage (%) of correction to interpretation of mixing test, as below.

$$(\%)\textbf{Correction} = \frac{(\textbf{patient APTT} - \textbf{mix APTT})}{(\textbf{patient APTT} - \textbf{NPP APTT})} \times \textbf{100}$$

Correction = %value is above/equal to the locally established cut-off point

No correction = %value is below the locally established cut-off value

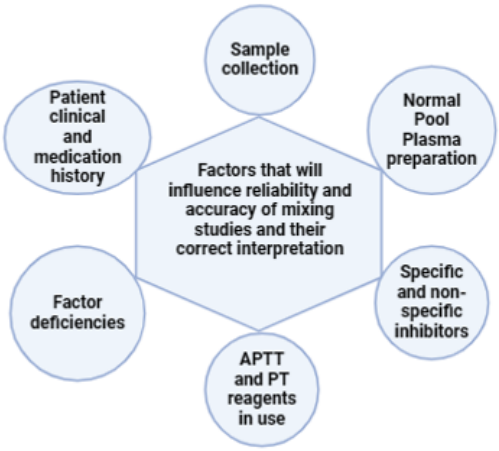

Figure 5. Diagram developed in biorender.com using information obtained from Adcock et al. (2023).

As highlighted in Adcock et al (2023), there are several factors that influence mixing test accuracy. One important factor is knowing the patient clinical and medication history. Other important factors include whether sample collection was conducted per accepted standards, whether the patient has a previous history of a factor deficiency, the type of reagents used and their sensitivity (especially to mild factor deficiencies and inhibitors), establishment of normal reference intervals for screening tests using normal samples from the local population, the preparation of the normal pool plasma and concentration levels of coagulation factors (at least 80%), and whether specific or non-specific inhibitors are endogenous or exogenous in nature. A better understanding of these factors will enhance the laboratories' capability to interpret mixing test results correctly.

FVIII/FIX-deficient plasma for mixing study: Plasma from patients with isolated severe deficiency (<1 IU/dl) of FVIII or FIX are very useful for mixing studies. Plasma selected for this purpose should have a normal PT, confirming that the other clotting factors synthesized in the liver are likely to be at normal levels. Such plasmas can be lyophilized for long-term storage or stored as plasma at -35°C (or lower) for at least three months. By using 50:50 mixtures of additive and patient's plasma, an abnormality can be characterized. In situations where there is an isolated prolongation of the APTT, FVIII-deficient plasma is preferable to aged plasma. Similarly, FIX-deficient plasma is preferable to adsorbed plasma.

It is ethical to obtain the patient's informed consent before obtaining a sample for use in mixing tests. A negative inhibitor status should be established before collecting samples for mixing tests.

Table 8. Pattern of mixing test results in the presence of individual factor deficiencies

Defect in test plasma	APTT	FVIII- deficient	FIX-deficient	Normal plasma
FVIII	abn	no corr	corr	corr
FIX	abn	corr	no corr	corr
FXI/FXII	abn	corr	corr	corr
Inhibitor	abn	no corr	no corr	no corr

Defect in test plasma	PT	APTT	Normal plasma
FII	abn	abn	corr
FV	abn	abn	corr
FVII	abn	norm	corr
FX	abn	abn	corr

abn = abnormal; no corr = no correction; corr = correction

Notes: The coagulation sample should contain a platelet count of <10 x 10^9/l to provide minimal phospholipid content and allow detection of weak lupus anticoagulants (Toulon et al, 2016). The mixing studies should only be performed on the same plasma that had provided a prolonged result. If for some reason a new sample was drawn from the patient, the abnormal baseline test should be repeated before the mixing test is done. Validation and verification of new reagents with new lot numbers should always be performed to ensure the reagent sensitivity to inhibitors and factor deficiencies is still in the acceptable range (Toulon et al, 2016). All coagulation samples should be collected as described in Part 3 of this manual. Non-specific inhibitors affecting APTT (e.g. lupus anticoagulant) typically show no correction, although plasmas containing weaker or low titer inhibitors may be partially corrected by normal plasma.

Specific inhibitors against FVIII: Specific inhibitors against FVIII may be associated with lack of immediate correction of APTT following addition of normal plasma. In other cases, there is an immediate correction by normal plasma, followed by lengthening of APTT in the mixture over time. A mix of test and normal plasma should be tested after one hour at 37°C, together with APTT determinations on normal and test plasmas that have been incubated separately at the same time. Specific inhibitors against other clotting factors are particularly rare but can occur. It is not possible to generalize their behavior in mixing experiments, except that FIX inhibitors are typically fast acting. Figure 6 shows how APTT changes when 20% and 50% PNP is added to samples from subjects with acquired hemophilia A with anti-FVIII antibodies. The upper limit of the normal range for this APTT method was 37 seconds. This example illustrates that in the presence of anti-FVIII antibodies, there can be full correction in a 50:50 mix of patient and PNP in some cases. If these mixtures are incubated at 37°C, there is a progressive increase in the APTT, as anti-FVIII antibodies inhibits FVIII. The Nijmegen Bethesda assay is the "gold standard" test in quantification of inhibitors present in a plasma sample following a positive screen. The chromogenic Bethesda assay is the test of choice to assess inhibitors in patients on emicizumab as the APTT-based inhibitor screen results have been known to be shortened in patients on emicizumab (Lowe et al, 2020). Other clotting factor inhibitors such as FXa inhibitors can be detected using an anti-FXa activity assay.

Table 9. Mixing studies in acquired hemophilia A

Bethesda titer (U/ml)	APTT (seconds)	APTT + 20% normal plasma (seconds)	APTT + 50% normal plasma (seconds)
1.0	210	137	77
1.1	83	52	38
2.0	82	43	34
6.6	107	51	37
8.4	150	55	39
21	145	62	48
23	123	127	55
120	69	50	38

Thrombin Clotting Time: Thrombin clotting time (also known as thrombin time) is useful in identifying hereditary fibrinogen abnormalities as well as acquired quantitative or qualitative abnormalities. Thrombin time has been known to be very sensitive to presence of heparin or drugs such as direct thrombin inhibitors (DTIs) (Bonar et al, 2017). Thrombin time reflects the reaction between thrombin and fibrinogen. It is prolonged when the fibrinogen level is very low (less than 1.0 g/l); in the presence of heparin, heparin-like substances, DTIs, or other inhibitors (e.g. fibrin/fibrinogen degradation products [FDPs]); and when fibrinogen is qualitatively abnormal (dysfibrinogenemia), including both congenital and acquired defects secondary to liver disease (Mackie et al, 2024).

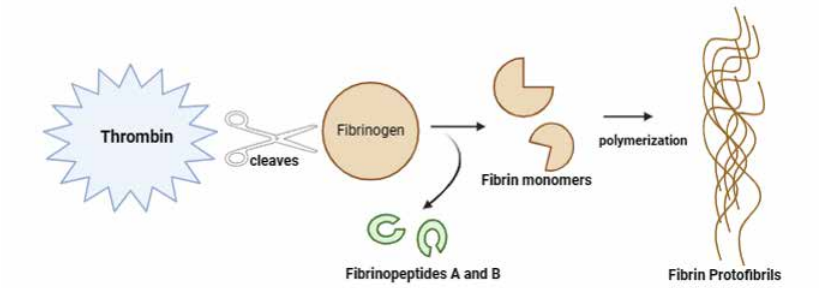

Figure 6. Flow diagram depicting the conversion of fibrinogen to fibrin in the presence of thrombin. Developed using Biorender.

Reagents:

- ✓ Thrombin solution, which induces clotting of normal plasma in about 15 seconds.
- ✓ Stronger solutions give shorter clotting times and may be normal in the presence of mild defects.

There are various thrombin reagents that are commercially available. Some have two levels of screening dilutions of thrombin reagent with the buffer, and a third concentration of thrombin reagent for samples with a high heparin content suggestive of patient on therapeutic heparin. As usual, it is recommended to follow the manufacturer's instructions and local normal reference ranges should be established by the laboratory.

Method: The manual tilt-tube method is described in Part 4 of this manual. The reagent manufacturer's *recommendations should be followed.*

Notes: The thrombin concentration used should be that which gives a clotting time of around 15 seconds with PNP (control). If concentrated thrombin is used, it should be diluted to around 10 to 15 U/ml in saline,

and further diluted as required, until the appropriate control time is obtained. For all commercially acquired thrombin reagents, the manufacturer's instructions on reconstitution, use, and storage should be followed. Reconstituted thrombin can be stored at -35°C or lower and diluted prior to use. Diluted thrombin kept at room temperature will deteriorate. A PNP control should be included with each group of tests. Plasma samples for thrombin time should be tested within 4 hours after sample collection and within 2 hours if heparin presence is suspected. High levels of heparin in a patient sample being tested for thrombin time will lead to lack of clot formation. This is also possible with some DTIs, such as dabigatran. Use the reptilase time test to confirm the presence of DTIs or heparin contamination/presence in the patient sample. There has been variation in results of thrombin time methods based on the type of reagents and the coagulation instrument used, therefore it is recommended that each patient result should be reported together with a reference range specific to that reagent and instrument.

Thrombin Time in the Presence of Protamine Sulphate to Detect Presence of Heparin: The presence of UFH can cause prolongation of thrombin time. The larger forms of heparin, which prolong thrombin time, can be neutralized by the addition of protamine sulphate. Protamine sulphate is available from many hospital pharmacies, where it is used as a therapeutic agent for reversal of heparin effect. The concentration of drug in therapeutic preparations is normally much higher than is useful for laboratory testing purposes. Therefore, if necessary, the drug should be diluted in saline to a concentration of 40 mg% as a working solution. A working solution of thrombin with protamine sulphate is prepared by mixing nine parts of thrombin reagent with one part of 40 mg% protamine sulphate. This is then used in place of the thrombin solution. Normal control should be analyzed. If the thrombin time is prolonged but corrects to within two seconds of control result, the presence of heparin is confirmed.

Reagents (Hogwood et al 2017):

- ✓ Barbitone buffer pH 7.4 (Fritsma, 2019)
- ✓ Protamine sulphate stock – use 5 ml of 10 mg/ml protamine sulphate to make a 1/20 dilution using barbitone buffer. Serial dilutions of various concentrations of protamine sulphate can be prepared from this solution. They remain stable when stored at 4°C.
- ✓ Patient PPP
- ✓ Thrombin

Method:

- ✓ Make serial dilution of protamine sulphate in barbitone buffer.
- ✓ Prepare diluted thrombin using barbitone buffer. When 100 µl of the diluted thrombin is added to 200 µl of patient plasma in buffer at 37°C should enable clot formation at 10seconds.

Interpretation: If the clot forms within 10 seconds, there is no UFH present in the patient plasma. If the time is prolonged, there is UFH in the patient plasma. The patient plasma is mixed with diluted protamine sulphate, and the test is redone. The goal is to measure thrombin time on different dilutions of patients' plasma and protamine sulphate that allow clot formation within a normal range.

Reptilase Time: Reptilase is a snake venom obtained from *Bothrops atrox*. It is a thrombin-like enzyme referred to as Batroxobin that acts by cleaving fibrinogen to form fibrinopeptide A, leading to the formation of a fibrin monomer and clot formation by polymerization. It is not inhibited by antithrombin, so it is not affected by the presence of heparin or DTIs. Therefore, it can be used to assess the rate of fibrinogen conversion to fibrin in the presence of heparin and DTIs (Mackie et al, 2024). It is useful to check whether a prolonged thrombin time is caused by the presence of heparin or DTIs in the sample. If thrombin time is prolonged and reptilase is normal, the most likely cause is the presence of heparin or a DTI. In the presence of dysfibrinigonemia, the reptilase time may be more sensitive (i.e. more prolonged) than thrombin time. Reptilase added to patient's plasma (PPP) provides Batroxobin that cleaves fibrinogen releasing fibrinopeptide A to form a fibrin monomer with clot formation following polymerization (Karapetian, 2013).

Reagents: Reptilase (Sigma Aldrich, Code V5375) dissolved at a concentration of 25 mg in 15 ml Owren's buffer. This crude venom is hazardous, and care must be taken to avoid inhaling the powder. The operator should wear gloves and a mask while handling the crude venom. The stock solution should be stored deep-frozen at -70°C in 0.5 ml aliquots. It is stable for at least two years under these conditions. To prepare ready-to-use reagent, thaw and dilute stock reagent 1/10 in Owren's buffer, aliquot, and refreeze at -70°C for further use. This ready-to-use reagent is stable under these conditions for at least three months. Ready-to-use frozen aliquots should be thawed in a 37°C water bath for at least three minutes. This is then stable for use for at least 12 hours at ambient temperatures of 20 to 25°C. There are various commercially available preparations of Batroxobin.

Method:

- ✓ Perform all tests in duplicate.
- ✓ Place sufficient 75 x 10 mm glass clotting tubes in a water bath at 37°C (two per patient, plus two for the control).
- ✓ Pipette 0.3 ml plasma (control or patient) into warm clotting tubes.
- ✓ Warm for one to two minutes.
- ✓ Add 0.1 ml reptilase dilution and start stopwatch.
- ✓ Tilt three times to mix, then three times every five seconds until clot formation.
- ✓ Record clotting time.
- ✓ The control time should be 15 to 18 seconds. (If shorter, adjust by further diluting the reptilase reagent with Owren's buffer.)
- ✓ If no clotting occurs, report as >90 seconds.

Normal range: Patient's time should be within three seconds of the control time. Control time should be reported with patient time.

Interpretation: Interpretation of prolonged thrombin time and reptilase time is shown in Table 10.

Table 10. Interpretation of prolonged thrombin time

Thrombin time	Reptilase time	Cause	Remarks
Prolonged	Equally prolonged	Hypo- or afibrinogenemia	Measure fibrinogen
Prolonged	Strongly prolonged	Dysfibrinogenemia	Congenital or acquired
Prolonged	Normal	Heparin	
Prolonged	Slightly prolonged	Heparin with some hypo- or dysfibrinogenemia	Rare case of dysfibrinogenemia may give this pattern
Prolonged	Equally Prolonged	Disseminated intravascular coagulation (DIC)	Measure D-dimers
Prolonged	Normal	Direct Thrombin Inhibitors	

Note: Reptilase reagents are available at a ready-to-use concentration from several commercial manufacturers. The advantage of this is that there is no need to handle the crude venom, thereby avoiding its health and safety issues. If using one of these, follow the manufacturer's instructions for use. Where reptilase is an expensive reagent, the protamine neutralization/thrombin time method can be used to confirm the presence of heparin in the test sample.

Fibrinogen (Modified Clauss Assay): Dilutions of standard normal plasma with known fibrinogen content are prepared in glyoxaline buffer. The clotting time is measured after the addition of thrombin, and a graph is constructed. The clotting time is proportional to the concentration of fibrinogen, and the 1/10

dilution is taken to represent the value in the standard preparation. The test plasma is diluted 1/10, and the result read from the standard line.

Reagents:

- ✓ Standard or reference plasma with known fibrinogen concentration
- ✓ Thrombin high concentration of 100 – 200 U/ml (concentration may vary according to source).
- ✓ Imidazole buffer (glyoxaline) or Owren's buffer pH 7.35

Method: The manual tilt-tube technique is described in Part 4 of this manual. The reagent manufacturer's recommendation should be followed.

This test is not affected by heparin at the levels used for the treatment of venous thromboembolism. The use of a higher concentration of thrombin is supposed to overwhelm the effect of high concentrations of UFH on thrombin, such as those applied in cardiopulmonary bypass enabling clot formation. However, caution should be applied when interpreting results from such patients since prolonged clotting times can be observed, leading to an underestimation of fibrinogen, unless the reagent contains heparin neutralizers to counter-this. The effect of DTIs will be highly dependent on the concentration of the DTI in the patient plasma and the type of reagents used. DTIs in the therapeutic range do not affect the high concentration thrombin used in Clauss fibrinogen assay (Mackie et al, 2024).

Typical calibration data:

(Note: a calibration curve must be established with the reagents in local use.)

Standard plasma: 2.1 g/l fibrinogen

Table 11. Example of a fibrinogen calibration

Dilution of standard	Concentration of fibrinogen (g/l)	Clotting time (seconds)
1/5	4.2	8.5
1/10	2.1	14
1/15	1.4	19.5
1/20	1.05	24.5

Examples:

Test plasma 1: diluted 1 in 10, clotting time 15 seconds.

fibrinogen = 1.9 g/l (from calibration graph)

Test plasma 2: diluted 1 in 5, clotting time 16 seconds.

fibrinogen = 1.8 g/l from calibration graph x 5/10 (since 1/5 dilution rather than 1/10)

= 0.9 g/l

Notes on Clauss fibrinogen assay: Fibrinogen level can be underestimated in presence of high concentrations of fibrin/fibrinogen degradation products, therefore careful interpretation of results is advised when this is suspected. Collection of coagulation blood samples from heparin contaminated venous or arterial devices should be avoided to exclude possibilities of obscure heparin interference in the results obtained. Samples from patients on high doses of UFH should be avoided when quantifying fibrinogen levels to prevent obtaining false low results.

PT-Derived Fibrinogen Test: A number of coagulation analyzers can estimate the level of fibrinogen during determination of prothrombin time. This is possible because the change in light scatter or transmission due to clot formation is proportional to the initial fibrinogen concentration. These methods are commonly referred to as PT-derived fibrinogen. There are limitations to most PT-derived methods. In particular, the results obtained are often much higher than those obtained by the Clauss assay when there are either very low levels (<1.5 g/l) or elevated levels (above 5 g/l) of fibrinogen. Results are usually above normal in the presence of dysfibrinogenemia (Mackie et al, 2024; Miesbach et al, 2010). There are Clauss fibrinogen methods that are suitable for assaying undiluted test plasma, but results may not be interchangeable with the results of the widely used Clauss assays employing diluted test plasma (Jennings et al. 2009).

Factors that affect the use of PT-derived fibrinogen: For patients on anticoagulant therapy, it is advisable not to use PT-derived fibrinogen estimation for quantifying their fibrinogen levels. Certain oral anticoagulants affect thrombin generation, eventually reducing thrombin production and causing formation of thick fibrils that are sensed as turbidity in the sample by the optical sensors and relayed as increased fibrinogen (Chitolie et al, 1994). There have been cases of patients with hypodysfibrinogenemia being identified as having normal PT-derived fibrinogen while these levels were actually low when measured by the Clauss method (Chitolie et al, 1994). Reference plasma turbidity leads to higher estimates of fibrinogen levels. In addition, patient plasma that is lipemic and turbid can lead to erroneous high estimation of fibrinogen. These inaccurate fibrinogen levels can be observed in chronically, severely ill patients. Based on the current international guidelines on fibrinogen quantification, the Clauss fibrinogen method is recommended and, due to the inaccuracies associated with PT-derived fibrinogen, should not be used for screening fibrinogen deficiencies, should not be used on patients known to be on anticoagulant therapy, and the results should be interpreted with careful consideration.

Removal of Heparin from Plasma: Heparinase 1 (the active component of Hepzyme®) is specific for heparin, which it cleaves at multiple sites per molecule, producing oligosaccharides that have lost their antithrombotic activity. Hepzyme® is a purified bacterial heparinase 1 produced in *Flavobacterium heparinum*. It can remove up to 2 IU heparin per ml in plasma. Hepzyme® can be used to neutralize the effect of heparin in a sample, so that the underlying coagulation status can be assessed. It is particularly used in instances of heparin contamination (Forte and Abshire, 2000).

Reagents:

- ✓ Hepzyme®, a vial containing dried preparation of heparinase 1 with stabilizers
- ✓ Manufacturer: Dade Behring
- ✓ Storage: 4°C
- ✓ Stability: as per manufacturer's expiry date. Each vial is used for one test patient only

Method:

- ✓ Add 1.0 ml of platelet-poor citrated plasma to a vial of Hepzyme®.
- ✓ Re-stopper and invert gently 5 to 10 times.
- ✓ Leave at room temperature for 15 minutes.
- ✓ Transfer to a 2-ml plastic sample cup, and allow a few moments for any bubbles to disappear.
- ✓ Place on CA1500 and perform required test.

The thrombin time should be included to check that all the heparin has been successfully removed. Tests should be performed as soon as possible (i.e., within testing guidelines for that procedure).

Interpretation: This enzyme does not remove any clotting factors (unlike some of the alternative techniques for removing heparin), so substantial shortening of clotting times in APTT, thrombin time, or PT after treatment with hepzyme indicates that heparin was present. Both UFH and low-molecular forms are degraded by this enzyme.

References

Adcock DM, Gosselin RC. The danger of relying on the APTT and PT in patients on DOAC therapy, a potential patient safety issue. *Int J Lab Hematol* 2017; 39 Suppl 1: 37-40.

Adcock DM, Moore GW, Montalvão SL, Kershaw G, Gosselin RC. Activated partial thromboplastin time and prothrombin time mixing studies: Current state of the art. *Semin Thromb Hemost* 2023; 49(6): 571-579.

Baglin T, Luddington R. Reliability of delayed INR determination: Implications for decentralized anticoagulant care with off-site blood sampling. *Br J Haematol* 1997; 96(3): 431-434.

Bonar RA, Lippi G, Favaloro EJ. Overview of Hemostasis and Thrombosis and Contribution of Laboratory Testing to Diagnosis and Management of Hemostasis and Thrombosis Disorders. In: Favaloro EJ, Lippi G, eds. *Hemostasis and Thrombosis: Methods and Protocols.* New York, NY: Springer New York; 2017: 3-27.

Bourguignon A, Tasneem S, Hayward CP. Screening and diagnosis of inherited platelet disorders. *Crit Rev Clin Lab Sci* 2022; 59(6): 405-444.

Chitolie A, Mackie IJ, Grant D, Hamilton JL, Machin SM. Inaccuracy of the 'derived' fibrinogen measurement. *Blood Coagul Fibrinolysis* 1994; 5(6): 955-957.

Clinical and Laboratory Standards Institute. One-stage prothrombin time (PT) test and activated partial thromboplastin time (APTT) test, 3rd edition. CLSI standard H47. 2023. https://clsi.org/standards/products/hematology/documents/h47.

Dorgalaleh A, Favaloro EJ, Bahraini M, Rad F. Standardization of prothrombin time/international normalized ratio (PT/INR). *Int J Lab Hematol* 2021; 43(1): 21-28.

Favaloro EJ. Coagulation mixing studies: Utility, algorithmic strategies and limitations for lupus anticoagulant testing or follow up of abnormal coagulation tests. *Am J Hematol* 2020; 95(1): 117-128.

Favaloro EJ. Optimizing the verification of mean normal prothrombin time (MNPT) and international sensitivity index (ISI) for accurate conversion of prothrombin time (PT) to international normalized ratio (INR). *Methods Mol Biol* 2017; 1646: 59-74.

Forte K, Abshire T. The use of hepzyme in removing heparin from blood samples drawn from central venous access devices. *J Pediatr Oncol Nurs* 2000; 17(3): 179-181.

Fritsma GA. Antithrombotic Therapies and Their Laboratory Assessment. In: *Rodak's Hematology: Clinical Principles and Applications.* 2019: 746-764.

Hogwood J, Mulloy B, Gray E. Precipitation and neutralization of heparin from different sources by protamine sulfate. *Pharmaceuticals (Basel)* 2017; 10(3): 59.

International Organization for Standardization (ISO). ISO 15189:2022(en) Medical laboratories — Requirements for quality and competence. https://www.iso.org/obp/ui/#iso:std:iso:15189:ed-4:v1:en. Accessed March 23, 2025.

Jennings I, Kitchen DP, Woods T, Kitchen S, Walker ID. Differences between multifibrin U and conventional Clauss fibrinogen assays: Data from UK National External Quality Assessment Scheme surveys. *Blood Coagul Fibrinolysis* 2009; 20(5): 388-390.

Karapetian H. Reptilase time (RT). *Methods Mol Biol* 2013; 992: 273-277.

Kitchen S, Adcock DM, Dauer R, Kristoffersen AH, Lippi G, Mackie I, Marlar RA, Nair S. International Council for Standardisation in Haematology (ICSH) recommendations for collection of blood samples for coagulation testing. *Int J Lab Hematol* 2021; 43(4): 571-580.

Kitchen S, Cartwright I, Woods TA, Jennings I, Preston FE. Lipid composition of seven APTT reagents in relation to heparin sensitivity. *Br J Haematol* 1999; 106(3): 801-808.

Kitchen S, Malia RG, Preston FE. A comparison of methods for the measurement of activated factor VII. *Thromb Haemost* 1992; 68(3): 301-305.

Lowe A, Kitchen S, Jennings I, Kitchen DP, Woods TAL, Walker ID. Effects of emicizumab on APTT, FVIII assays and FVIII inhibitor assays using different reagents: Results of a UK NEQAS proficiency testing exercise. *Haemophilia* 2020; 26(6): 1087-1091.

Mackie I, Casini A, Pieters M, Pruthi R, Reilly-Stitt C, Suzuki A. International Council for Standardisation in Haematology recommendations on fibrinogen assays, thrombin clotting time and related tests in the investigation of bleeding disorders. *Int J Lab Hematol* 2024; 46(1): 20-32.

Mackie IJ, Kitchen S, Machin SJ, Lowe GD. Guidelines on fibrinogen assays. *Br J Haematol* 2003; 121(3): 396-404.

Maier CL, Sniecinski RM. Anticoagulation monitoring for perioperative physicians. *Anesthesiology* 2021; 135(4): 738-748.

Mielke CH. Measurement of the bleeding time. *Thromb Haemost* 1984; 52(2): 210-211.

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. *Thromb Res* 2010; 126(6): e428-433.

Miller CH. Laboratory testing for factor VIII and IX inhibitors in haemophilia: A review. *Haemophilia* 2018; 24(2): 186-197.

Pipe SW. Functional roles of the factor VIII b domain. *Haemophilia* 2009; 15(6): 1187-1196.

Practical-Haemostasis.com. Protamine sulphate neutralization test. https://practical-haemostasis.com/. Accessed March 23, 2025.

Rodgers RPC, Levin J. A critical reappraisal of the bleeding time. *Semin Thromb Hemost* 2024; 50(3): 499-516.

Russeau AP, Vall H, Manna B. Bleeding Time. [Updated 2023 Aug 8]. In: *StatPearls [Internet]*. Treasure Island, FL: StatPearls Publishing: https://www.ncbi.nlm.nih.gov/sites/books/NBK537233/. Accessed March 23, 2025.

Toulon P, Eloit Y, Smahi M, Sigaud C, Jambou D, Fischer F, Appert-Flory A. In vitro sensitivity of different activated partial thromboplastin time reagents to mild clotting factor deficiencies. *Int J Lab Hematol* 2016; 38(4): 389-396.

Undas A. The bleeding time test in 2024: A glorious past and current challenges. *Semin Thromb Hemost* 2024; 50(3): 517-519.

Vandiver JW, Vondracek TG. Antifactor Xa levels versus activated partial thromboplastin time for monitoring unfractionated heparin. *Pharmacotherapy* 2012; 32(6): 546-558.

Vinholt PJ. The role of platelets in bleeding in patients with thrombocytopenia and hematological disease. *Clin Chem Lab Med* 2019; 57(12): 1808-1817.

Williams B, Lin Z, Pittet JF, Chao W. Sepsis-induced coagulopathy: a comprehensive narrative review of pathophysiology, clinical presentation, diagnosis, and management strategies. *Anesth Analg* 2024; 138(4): 696-711.