# SEED Coagulation

## Sysmex Educational Enhancement and Development
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# The Thrombin Time Test and Reptilase Test – what is their role in coagulation testing?

### Baseline screening tests of coagulation

The bulk of prothrombin time (PT) and activated partial thromboplastin time (APTT) test requests worldwide are for the monitoring of anticoagulant therapy. There are however a number of instances where such baseline tests are performed with no indication that a specific haemostatic disorder is present. The main indication for such requests is to evaluate whether a patient who is scheduled to undergo elective surgery or invasive procedure is at risk of bleeding. Screening tests are also the starting point of investigation of patients that present with a clinical history of a suspected bleeding diathesis. In such instances, the thrombin time (TT) and reptilase test (RT) are a valuable adjunct to interpretation of results and for guiding more definitive testing where indicated.

### The principle of the thrombin time

The thrombin time, although not strictly speaking a screening test, is a commonly performed test that is widely available. It is a very simple clot-based test that involves only the addition of exogenous thrombin (the reagent) to platelet poor plasma which triggers the conversion of fibrinogen to fibrin. The thrombin time therefore measures the rate of fibrin clot formation after the addition of a standard amount of thrombin to citrated plasma.

The test exclusively measures the conversion of fibrinogen to fibrin. Any prolongation of the thrombin time is therefore directly related to this reaction; either in the form of a fibrinogen abnormality or the presence of any substance that may inhibit this reaction, most notably heparin (Fig. 1).

### The conversion of fibrinogen to fibrin

The addition of thrombin initiates the cleavage of fibrinopeptide A and fibrinopeptide B from the N-terminal ends of the alpha and beta chains of the fibrinogen molecule. This action of thrombin converts fibrinogen into fibrin monomers. These fibrin monomers each of which is comprised of a so-called E-domain and two D-domains align to form fibrin dimers and then polymers. These fibrin polymers in turn are stabilised through the process of cross linkage mediated via activated factor XIII to form an insoluble fibrin clot. This cross linkage takes the form of a covalent bond which irreversibly binds the D-domains of two adjacent fibrin molecules to each other (Fig. 2).

The resultant fibrin is insoluble and forms a clot. The time taken for clot formation is recorded as the thrombin time.

In health, the process of clot formation is finely balanced with the simultaneous initiation of clot breakdown, so-called fibrinolysis to ensure that the blood vessel lumen is not occluded. The process of fibrinolysis is mediated via the enzyme plasmin which has the ability to cleave both fibrin and fibrinogen into a heterogeneous mixture of so-called fibrin(ogen) degradation products (FDPs). The covalent bond formed between two D-domains is however resistant to plasmin degradation. FDPs containing 2 such D-domains are referred to as D-dimers and are exclusively produced from cross-linked fibrin.

Any interference with the polymerisation process will affect the thrombin time.

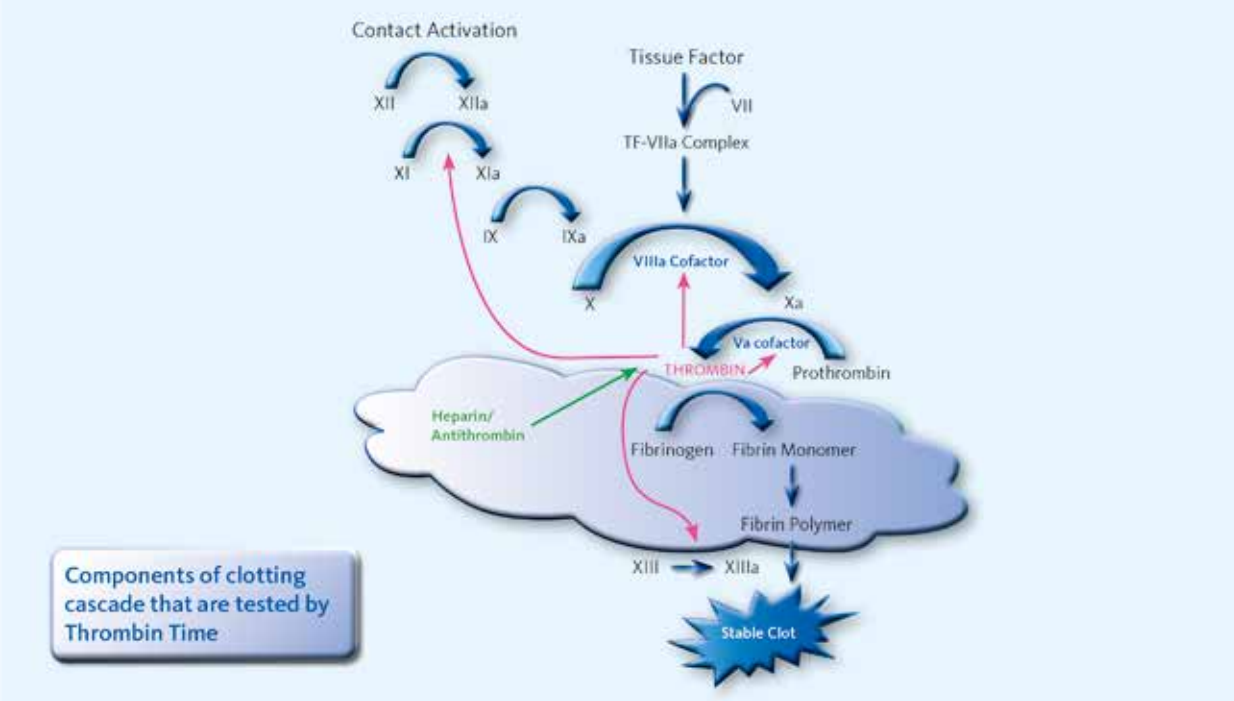


***Fig. 1*** *Schematic representation of the clotting pathways highlighting the components that are tested by the thrombin time. Thrombin is excluded as the test is activated by the addition of the test reagent which contains exogenous thrombin. Pink arrows indicate the activation effects of thrombin additional to its conversion of fibrinogen to fibrin. The green arrow indicates the inhibitory effect on thrombin by heparin/antithrombin complex.*

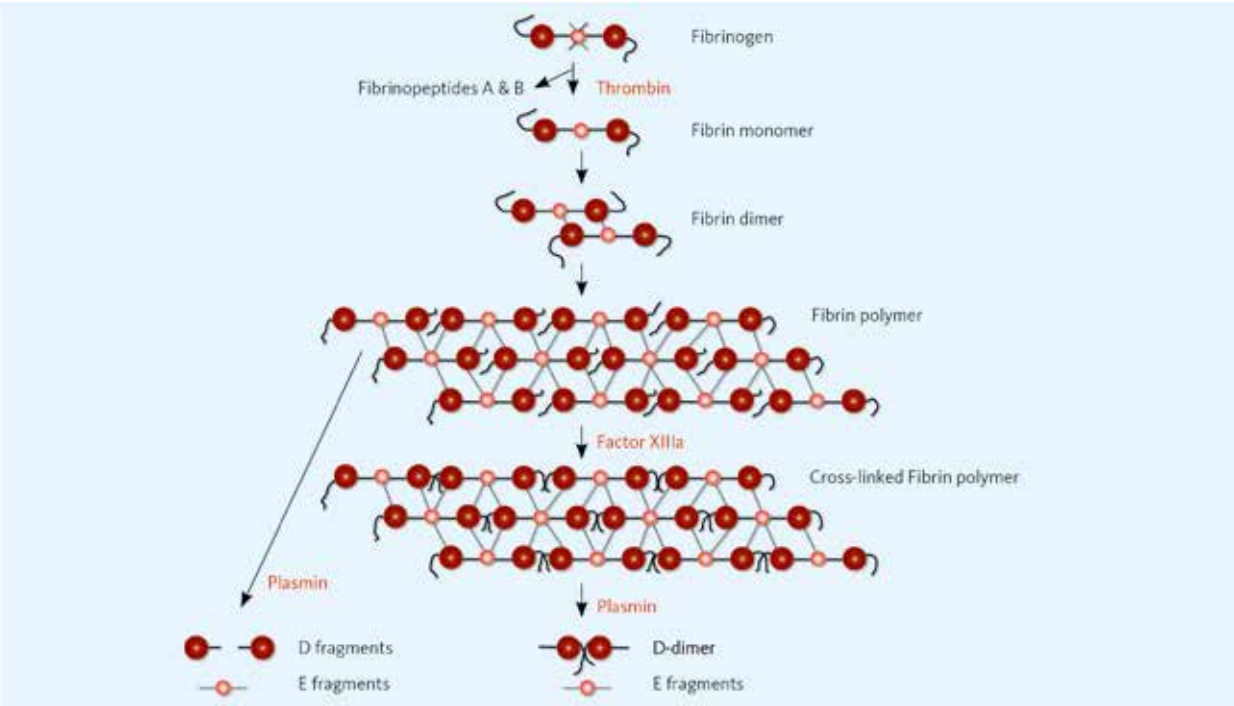


***Fig. 2*** *Schematic representation of fibrin polymerisation.*

## The thrombin time test method

The test sample is platelet poor plasma obtained from whole blood which has been collected with citrate as the anticoagulant (the same as for all routine clot-based tests of haemostasis). The test reagent containing thrombin, which can be of human or bovine origin, is added to platelet poor plasma at 37°C. The time taken (in seconds) for the clot to form, is recorded. The test can be performed manually in a water bath or set up on an automated coagulation analyser.

## Interpretation of thrombin time

Normal values for thrombin time are highly dependent on the concentration of thrombin used as well as the clot detection principle of the analyser. The concentration of thrombin is not uniform in commercially available thrombin time reagents, the package inserts of which confirm that the expected values are accordingly different. To illustrate this, published thrombin time reference range values sometimes do not even overlap; for example 13–15 seconds, in contrast to 15–22 seconds. Consequently, it is critically important that thrombin time results are interpreted strictly only against local laboratory established reference ranges.

A prolonged thrombin time is considered abnormal.

If a shortened thrombin time is obtained, the possibility of an activated sample must be excluded before this result is accepted as biologically representative of the patient's clinical status.

## Causes of prolonged thrombin times

These can be divided into 3 broad categories.

1. A problem with fibrinogen
2. Anything interfering with fibrin polymerisation
3. Anything inhibiting the reaction by interfering with thrombin

### a. Fibrinogen abnormalities

***i. Quantitative abnormalities***

As the thrombin time measures the conversion of fibrinogen to fibrin, an adequate amount of fibrinogen must be present in the patient plasma. Patients with absent fibrinogen or a reduced concentration of fibrinogen will manifest a prolonged or unclottable thrombin time, depending on the severity. Generally speaking, fibrinogen levels need to be below 1 g/L before the thrombin time will become abnormal. Fibrinogen deficiencies can be either congenital or acquired. The inherited deficiencies are termed afibrinogenaemia, where fibrinogen is absent or severely reduced (generally <0.2 g/L), or hypofibrinogenaemia if reduced. Acquired deficiencies of fibrinogen are observed in disseminated intravascular coagulopathy, following thrombolytic therapy for life threatening thrombosis, liver disease and in certain malignancies.

***ii. Quantitative abnormalities***

A functional abnormality of fibrinogen may likewise manifest with a prolonged thrombin time. This can occur as a result of impaired thrombin activation of fibrinogen, i.e. reduced conversion of fibrinogen to fibrin, or due to impaired fibrin polymerisation, leading to clot instability (see below). These conditions, called dysfibrinogenaemias, are rare and can be either genetically inherited or acquired. Acquired dysfibrinogenaemias occur most commonly in the context of liver disease.

The thrombin time in neonates is commonly prolonged because of the presence of foetal fibrinogen. Although the concentration of fibrinogen is normal, there is an increase in carbohydrate content which delays polymerisation similar to what is observed in the acquired dysfibrinogenaemia of liver disease. In this context it is imperative to ensure that the reference range used is age specific. Infants less than 6 months old can be expected to have a thrombin time that is approximately 2–3 seconds longer than the normal adult reference range.

***iii. Other causes***

Very rarely, patients with amyloidosis may present with a prolonged thrombin time due to the inhibition of conversion of fibrinogen to fibrin by the presence of abnormal circulating proteins. This in essence would manifest similarly to a quantitative deficiency of fibrinogen.

### b. Interference with fibrin polymerisation

***i. Dysfibrinogenaemias and FDPs***

As the end point of detection of the thrombin time is clot formation, anything that will interfere with the stability of the clot may cause a prolongation of the clotting time. As previously described, the fibrin polymer is progressively built up from individual evenly sized and structurally identical fibrin monomers. The presence of any structural variants, as in dysfibrinogenaemia, or FDPs such as D-dimers, which contain only part of the structure, may result in an unstable clot if these are incorporated into the growing polymer. A very high concentration of D-dimers may result in a prolonged thrombin time.

The aetiology of the impairment of fibrinogen, which is produced in the liver, in acquired dysfibrinogenaemias associated with liver disease, is a structural defect caused by an increased carbohydrate content which interferes with fibrin polymerisation.

***ii. Other causes***

Paradoxically, a very high fibrinogen (> 14 g/L), but not elevated levels associated with the acute phase response, can be associated with a prolonged thrombin time which is thought to occur because the excess fibrinogen interferes with the assembly of fibrin.

The presence of abnormal circulating proteins, such as paraproteins observed in myeloma may interfere with fibrin polymerisation and cause a prolonged thrombin time.

Hypoalbuminaemia (albumin <30 g/L) may also interfere with fibrin polymerisation and cause a prolongation of the thrombin time.

### c. Thrombin inhibition

***i. Heparin***

As the thrombin time is initiated by the addition of exogenous thrombin, it is insensitive to deficiencies of all upstream clotting factors that are involved in the generation of endogenous thrombin. The assay is however sensitive to any substances present in the patient plasma that may inhibit the exogenous thrombin added to the plasma as a reagent. Even a very small amount of heparin, whether given therapeutically or introduced as a contaminant during blood collection from a heparinized central intravenous (IV) line, will result in a thrombin time that invariably will fail to clot.

The test is so exquisitely sensitive to heparin that one of the commonest uses of the thrombin time is to confirm the presence of heparin when this is suspected to be present and is interfering with the interpretation of other coagulation tests. As such it is most commonly used as a quality control check to establish the presence or absence of unfractionated heparin in a sample prior to undertaking more complex tests of coagulation rather than for the investigation of clinically suspected abnormalities of fibrinogen. The reptilase test, which has a similar test principle to thrombin time, is used to confirm that the cause of prolongation of a thrombin time is indeed heparin (see below).

Low molecular weight heparins will generally not affect the thrombin time unless the patient has been significantly overdosed.

***ii. Other anticoagulant drugs***

The direct thrombin inhibitors hirudin, argatroban and the new oral agent dabigatran all cause a prolongation of the thrombin time. The thrombin time shows a linear dose response to these drugs although most commercially available thrombin time assays will be too sensitive to provide any quantitative information. A normal thrombin time would generally exclude the presence of a direct thrombin inhibitor whereas a markedly prolonged or unclottable thrombin time cannot differentiate between sub-therapeutic, therapeutic or supra-therapeutic levels.

Rivaroxaban, being a direct factor Xa inhibitor has no effect on the thrombin time. Warfarin also has no effect on the thrombin time as it exerts its anticoagulant action by impeding the synthesis of functional prothrombin, and other vitamin K dependent clotting factors, as these are all upstream from the test reaction.

***iii. Rare antibodies***

Patients previously exposed to bovine thrombin, may develop inhibitors that prolong bovine-based thrombin times, and if there is cross-reactivity to human thrombin, may also prolong the thrombin times where the thrombin reagent is

of human origin. Patients may be exposed to bovine thrombin in the form of 'tissue glue'. Tissue glue is a unique adhesive material that is being used with increasing frequency in a variety of surgical situations to rapidly secure haemostasis, independent of the patient's own coagulation status, in anatomically vulnerable sites. In practice, it is a two-component system in which a solution of concentrated fibrinogen and factor XIII are combined with a solution of thrombin and calcium in order to form a clot, simulating the final stage of the clotting cascade. Once the thrombin, calcium, fibrinogen and factor XIII are all combined, a fibrin clot forms in seconds.

Heparin-like anticoagulants have been reported, albeit rarely, in patients with malignant neoplasms and will manifest with a prolonged thrombin time.

### Causes of shortened thrombin times

In very rare instances, a shortened thrombin time may be observed. The commonest cause of this would be the use of colloidal fluids, such as dextran or hydroxyethyl starch, as circulatory volume expanders during surgery. In such cases the clotting times are shortened although clot strength has been shown to be weaker.

### The reptilase time

The reptilase test, also known as the atroxin time, is very similar in principle to the thrombin time in that it assesses the conversion of fibrinogen to fibrin. The difference is that in the reptilase test, thombin is replaced with the thrombin-like enzyme reptilase which is purified from the venom of the snake Bothrops atrox. This enzyme, like thrombin converts fibrinogen to fibrin. The difference however is that it cleaves only fibrinopeptide A, and not fibrinopeptide B, from fibrinogen. The main difference that is observed is that this assay is insensitive to the presence of heparin and other drugs that act as thrombin inhibitors. A sample in which the thrombin time is prolonged, a normal reptilase time would (in most cases) be consistent with the presence of unfractionated heparin or one of the newer direct thrombin inhibitor drugs. A prolonged thrombin time is therefore commonly associated with a normal reptilase time.

All other causes of a prolonged thrombin time would also affect the reptilase time. There is only one scenario that would give rise to the combination of a normal thrombin time with a prolonged reptilase time, namely elevated fibrinogen levels. This is commonly observed as plasma samples submitted for coagulation testing are frequently received from patients that have had an acute thrombosis which would elicit an acute phase reaction, and hence a rise in fibrinogen. Even mildly elevated levels of fibrinogen (4–7 g/L) commonly cause a prolongation of the reptilase time, in contrast to the thrombin time where this is only manifest with extreme hyperfibrinogenaemia (> 14 g/L). The reason for this difference is not clear but it is well documented that the reptilase times revert back to normal once the fibrinogen levels normalise.

As for thrombin time, reference ranges for reptilase time are dependent on the assay system and hence local reference ranges must be used for interpretation. Such a reference should be established from plasma obtained from normal individuals with confirmed normal fibrinogen levels. When using such reference values, about 30% of patients with an elevated fibrinogen level will have a prolonged reptilase time, depending on the degree of fibrinogen elevation.

### What are the indications for thrombin time and reptilase time testing?

A thrombin time test is generally a test that the laboratory will perform if the baseline PT and APTT are abnormal. As the commonest cause of prolongation of both PT and APTT is heparin, the thrombin time is performed as a quick test to exclude this possibility. If heparin (or heparin like substances and other thrombin inhibitors) is the cause of the prolonged thrombin time, the normal reptilase time will confirm this. The reptilase time is therefore generally only ever indicated if the thrombin time is abnormal.

Thrombin time testing is also commonly performed as part of a panel of tests to determine the possible cause of the underlying hypercoagulability in patients with thrombosis. In this case, the aim is two-fold.

Firstly, to exclude heparin as above, as its presence would interfere with other assays and make them inconclusive. In such cases, the plasma may have to be treated with a special enzyme called hepzyme to eliminate this interference before commencing with additional tests. More commonly however, further testing will be delayed until such time that the patient is no longer heparinised and a fresh blood sample submitted to the laboratory.

Secondly, the thrombin time is used to assess for dysfibrinogenaemia, which may manifest as thrombosis, although it is an infrequent finding accounting for less than 1% of cases.

Whilst the thrombin time is generally prolonged in deficiencies of fibrinogen and the dysfibrinogenaemias, in such cases a Clauss fibrinogen test, which is sensitive to both quantitative and functional abnormalities, would generally be more informative. In dysfibrinogenaemia, the Clauss fibrinogen result (an activity assay) would be lower than an antigen based fibrinogen assay (e.g. ELISA), whereas both would be low in a deficiency of fibrinogen with normal structure and function. If a dysfibrinogenaemia is suspected, the fibrinogen activity-antigen ratio is used for confirmation in most cases. Liver function tests would support an acquired aetiology whereas family studies and more specialized investigations are required to confirm an inherited abnormality.

## What tests does Sysmex have on offer?

Sysmex has reagents and controls available for thrombin time and reptilase time for manual testing or for automated testing (Tab. 1). Test protocols are available for all Sysmex coagulation analysers.

Sysmex only provides a normal control for these tests. As a general rule, it is not considered necessary to perform an abnormal control for thrombin time and reptilase time tests as these are primarily used to see if there is heparin contamination in the sample in which case the thrombin usually fails to clot, and the reptilase time is normal. If a laboratory wishes to include an abnormal control this can be sourced from another company. The laboratory however will have to establish its own target range as values may vary significantly from one testing platform to the next.

## Take home message

- The thrombin time and reptilase time are basic coagulation tests that evaluate fibrin formation from fibrinogen in plasma.
- The thrombin time is most commonly used to exclude the presence of heparin before commencing more extensive coagulation tests which would otherwise be difficult to interpret.
- The advantage of the reptilase time is that it is insensitive to heparin and other inhibitors of thrombin.
- Causes of prolonged thrombin and reptilase times include fibrinogen abnormalities and anything that may interfere with fibrin polymerisation.
- The thrombin time and reptilase time are used for screening for dysfibrinogenaemia but it must be noted that both are susceptible to a high rate of false positivity as they are nonspecific tests.
- If dysfibrinogenaemia is suspected on the basis of a prolonged thrombin and/or reptilase time, this must be confirmed with a fibrinogen activity:antigen ratio determination.

| | Thrombin Time | Reptilase Time |
|---|---|---|
| Reagent | Test Thrombin (OWHM135) or | Batroxobin reagent (OUOV215) |
| | Thromboclotin (281007) | |
| Normal Control | Control Plasma N (ORKE415) or | Control Plasma N (ORKE415) or |
| | Citrol 1 (291070) | Citrol 1 (291070) |
| Calibrator | Not required | Not required |

***Tab. 1*** *Thrombin time and reptilase time reagents available from Sysmex*

## References

*[1]* ***Favaloro EJ and Lippi G.*** *(2013): The new oral anticoagulants and the future of haemostasis laboratory testing. Biochemia Medica 22:329–341.*

*[2]* ***Curvers J. et al.*** *(2012): Measuring direct thrombin inhibitors with routine and dedicated coagulation assays. Which assay is helpful? Am J Clin Pathol 138:551–558.*

*[3]* ***Cunningham MT et al.*** *(2002): Laboratory diagnosis of dysfibrinogenemia. Arch Pathol Lab Med 126(4):499–505.*

*[4]* ***Van Cott EM et al.*** *(2002): Elevated fibrinogen in an acute phase reaction prolongs the reptilase time but typically not the thrombin time. Am J Clin Pathol 118:263–268.*

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