Examination of blood coagulation

Basic examination of coagulation employs **group tests** that target certain parts of the coagulation system – intrinsic, extrinsic, or common pathways. The group tests can be complemented by **special tests** that examine function and amount of specific coagulation or fibrinolytic factors. There are also **global tests** that reflect function of the whole coagulation system and hemostasis.

According to the principle employed for the test evaluation, three analytical approaches are found:

- **Functional** – measure period of time needed for the formation of fibrin clot.
- **Biochemical** – utilize various synthetic substrates (e.g. chromogenic or fluorogenic), from which the factors with proteolytic activity cleave colored or fluorescent products, whose amount is then analyzed by means of spectrophotometry or spectrofluorometry, respectively.
- **Immunochemical** – by means of various immunochemical methods that use specific antibodies to examine certain components of coagulation system.

Further explanation will focus on selected functional coagulation tests and their evaluation.

Functional coagulation tests

Functional coagulation tests are initiated by activation of coagulation, whose way depends on which part of the coagulation cascade is tested. Regardless of the way of activation monitoring of the fibrin clot formation follows. The clot formation is associated with a change of sample properties (e.g. viscosity or turbidity), which is detected by various means:

- Visual
- Optical methods
- Electromechanical detection

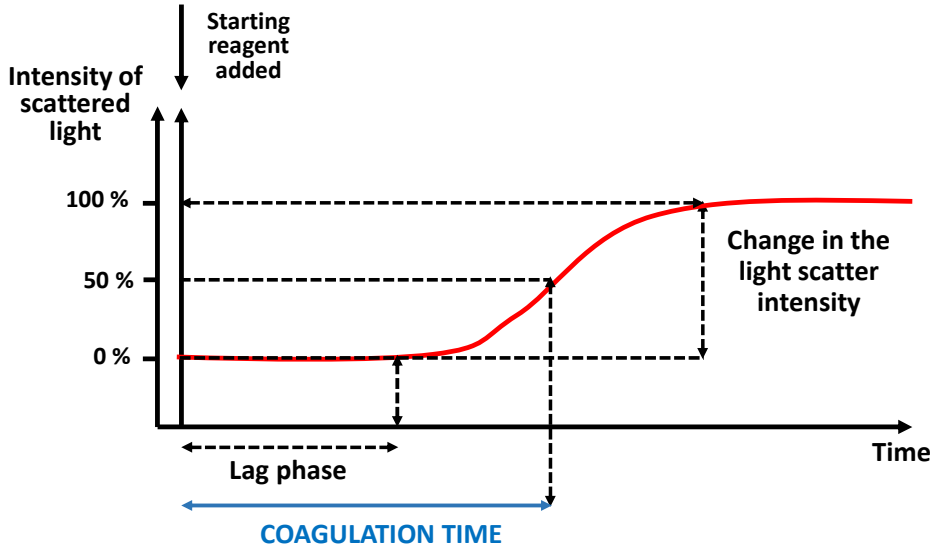
In the manually performed tests the formation of clot can be assessed visually, e.g. while moving the test tube back and forth in the water bath. Manual examination of coagulation with visual assessment of the clot formation is replaced with examination using coagulometers, which are based on different principles and employ electromechanical or optical methods for the clot detection.

Electromechanical detection can record change in the sample viscosity e.g. by measuring movement of a steel ball placed to the blood plasma sample in cuvette under the influence of electromagnetic field.

The change in optical properties is usually followed by means of turbidimetry (measurement of absorbance of monochromatic light passing the cuvette) or nephelometry (measurement of intensity of scattered light). In the nephelometric detection the change in the light scatter takes a characteristic course during the coagulation tests. At the beginning the changes are minimal (lag phase). Once a sufficient amount of thrombin is formed, enabling conversion of fibrinogen to fibrin, an increase in the intensity

of scattered light is observed. As soon as fibrinogen is exhausted, the intensity of light scatter stabilizes and the measurement can be finished. The obtained values of scattered light intensity are used for construction of coagulation curve, from which the coagulation time can be determined using various algorithms. One possibility is per cent detection method shown in Fig. 1. The value of light scatter immediately after the addition of starting reagents is set as 0 %, and following attainment of the equilibrium state as 100 %. The exact coagulation time is usually read at defined intensity of scattered light, often 50%.

Fig. 1 Determination of coagulation time with per cent detection method

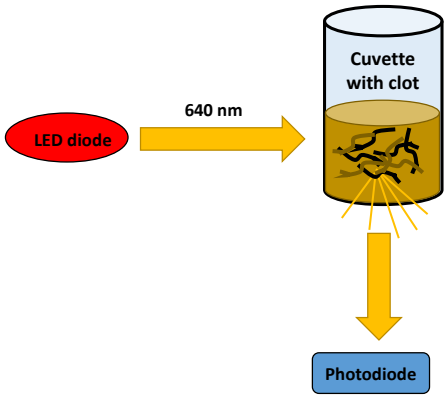


If turbidimetry or nephelometry is used, the results of coagulation tests are affected by plasma chylosity, to a lesser extent also in case of hemolytic or icteric plasma.

The results of the following tests can also be affected by other factors during the pre-analytical phase or the actual test performance (reagents used, technique). Therefore, it is recommended that each laboratory sets its own reference values. Preferably, the results are given as a ratio of the values get by examining patients' plasma to those obtained with normal plasma.

In the practical lesson we will get acquainted with semiautomatic coagulometer (type ECL 105m, Erba Lachema, s.r.o., Brno, ČR), which detects clot formation by nephelometry. The instrument contains a diode emitting light at 640 nm. This light reaches the cuvette where coagulation takes place. The intensity of light scattered by the clot is measured by a photodiode placed at the angle of 90 ° (Fig. 2).

Fig. 2 Nephelometric detection of clot formation



Selected coagulation tests

The basic tests that can performed with the semiautomatic coagulometer ECL 105m will be described. Blood samples for these tests are taken to the test tubes containing citrate, which binds calcium. This inhibits blood clotting. The recommended ratio is 9 parts of blood and 1 part of citrate (3.2%). After separation of blood cells by centrifugation a decalcified plasma is obtained.

1. Prothrombin time (PT , thromboplastin time, Quick test)

Principle

Prothrombin time is a basic coagulation test that evaluates function of the **extrinsic** (factor VII) **and common pathways** (factors X, V, II and fibrinogen) of the coagulation cascade. It evaluates the coagulation processes that include activation of prothrombin (factor II) to thrombin (factor IIa) up to conversion of fibrinogen to fibrin employing the extrinsic pathway. The key process of coagulation, cleavage of prothrombin to thrombin, results from the action of prothrombinase, consisting of a complex of activated factors Xa and Va, which are bound to negatively charged phospholipid surfaces (on thrombocytes, endothelium) in the presence of Ca^{2+} ions.

Reagent

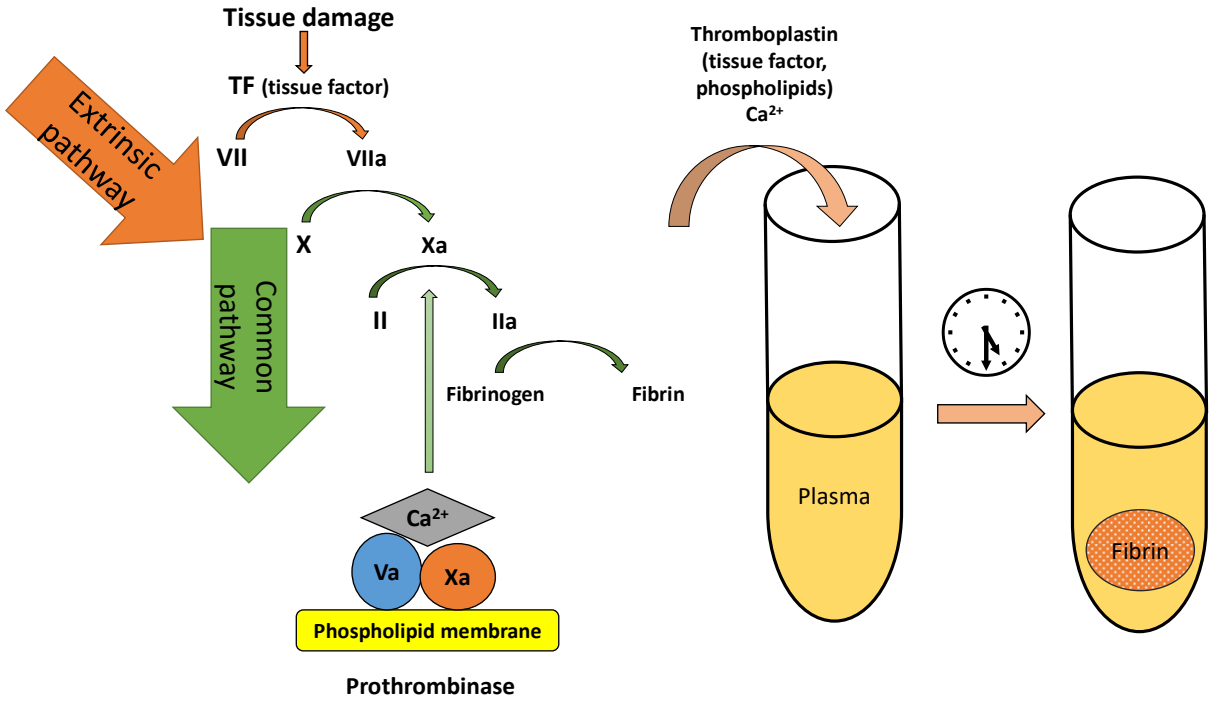
The main components of the starting reagent for PT are **thromboplastin and calcium ions**. By thromboplastin we mean the **tissue factor**, which binds to factor VII and initiates coagulation, together with **phospholipids** that replace the function of thrombocytes. Extracts from tissues rich in these two components, e.g. from rabbit brain or human placenta, can be used as thromboplastin. Another possibility is the human recombinant tissue factor supplemented with phospholipids. Calcium ions are necessary for recalcification of plasma. Some kit reagents contain also substances that neutralize heparin and eliminate its effect on the test result. The reagent also contains stabilizers and buffers.

Performance of the test

Activation of the extrinsic pathway requires presence of Ca^{2+} ions and the complete thromboplastin. In the one-step performance of the test the reagent is added to the decalcified plasma devoid of thrombocytes[1]. Time needed for the clot formation is evaluated by various ways described above (visually, optical methods, electromechanical detection). Coagulation time in the range 15 – 20 seconds is reported. Reaction is performed at 37 °C. The cascade of subsequent activation of factors of the extrinsic and common pathway during the PT test is shown in Fig. 3.

If an abnormally extended prothrombin time is found, a correction test is performed. The examined plasma is combined with an equal volume of normal plasma. Normal PT following the addition of normal plasma suggests lack of coagulation factors II, V, VII, X, or fibrinogen. If, however, the PT remains long, its extension is caused by the presence of coagulation factors inhibitors, which can be specified by further tests.

Fig. 3 Course and performance of prothrombin time (PT) test



[1] For obtaining a plasma devoid of platelets a longer centrifugation (15 min at 2,000–2,500 g) is recommended.

Test evaluation

Reporting of results is different for patients treated with coumarin anticoagulants and for patients not on this therapy. Information about the patient treatment should be included in the request for this test.

- **Using R**

  In case of patients not treated with coumarins the prothrombin time is evaluated by means of R, which is a ratio of coagulation time of patient's plasma in seconds to the coagulation time of normal plasma in seconds:

$$\mathrm{R} = \frac{\text{Coagulation time of patient's plasma in s}}{\text{Coagulation time of normal plasma in s}}$$

  The normal plasma can be obtained either as plasma pooled from minimally 20 healthy donors, or is obtainable commercially.

- **Calculation of INR**

  The results of patients treated with coumarin drugs are expressed by means of the internationally standardized ratio INR (international normalized ratio). It enables comparison of results from different laboratories that use different reagents (especially thromboplastin) and equipment.

  The base of INR calculation is the presented ratio of the measured coagulation time in seconds to the coagulation time of normal plasma in seconds (R). This ratio is exponentiated with index of sensitivity of thromboplastin (ISI). The value of ISI is included to each diagnostic kit. It is estimated by comparison of the thromboplastin used in the kit with a reference thromboplastin.

$$\mathrm{INR} = \left(\frac{\text{Coagulation time of patient's plasma in s}}{\text{Coagulation time of normal plasma in s}}\right)^{\mathrm{ISI}}$$

- **Reference range**

  R: 0.8 – 1.2

  INR: Reference range is not given – the attending physician considers INR in specific patients with respect to their condition and diagnosis. The value of treated patients should be in the range 2–3. A higher value of INR means the coagulation time is extended and coagulation takes a rather slow course.

Prothrombin test is used for monitoring **therapy with coumarin preparations (antagonists to vitamin K** – e.g. warfarin) and for guidance also **therapy with inhibitors of factor Xa** (e.g. rivaroxaban). Next, it can be used in disorders of the factors involved in the extrinsic coagulation pathway, in deficiency of vitamin K, and also in **liver diseases** for **assessment of proteosynthesis**, because majority of coagulation factors is synthesized by the liver. In these conditions an extension of PT is found.

2. Activated partial thromboplastin time (APTT)

Principle

Activated partial thromboplastin time is another basic coagulation test. Unlike the prothrombin time the APTT examines the group of factors that participate in the **intrinsic** (factors XII, XI, IX, VIII, high-molecular-weight-kininogen – HMWK, and prekallikrein – PK) **pathway** and subsequently also in the **common pathway** (factors X, V, II, and fibrinogen) of the conversion of prothrombin to thrombin.

Reagent

The reagent for estimation of APTT contains **contact activators** and **partial thromboplastin,** which is the thromboplastin without tissue factor. The role of activators is fulfilled by negatively charged surfaces in the form of silicates, kaolin, ellagic acid (a polyphenolic compound), or other substances. The activators are needed for activation of factors XII and XI. They enlarge the contact surface and hence accelerate the test. As the partial thromboplastin, which substitutes for the function of phospholipids from platelets, phospholipids either synthetic, or from animal or plant sources (e.g. cephalin from rabbit brains or soya phospholipids) are used. The reagents contain also stabilizers and buffers.

Performance of the test

Addition of the contact activators, together with the partial thromboplastin, to the examined decalcified, platelet-poor plasma initiates the activation of intrinsic coagulation pathway. Further progression requires **addition of Ca^{2+} ions** (calcium chloride), which trigger the coagulation. Time needed for the production of fibrin clot (25 – 40 second) is measured since the addition of Ca^{2+} ions. The clot formation can be detected in similar ways as in the PT (Fig. 4).

In case of finding an extended time a correction test is done as described above for PT. If the test normalizes following addition of normal plasma, it means a deficit of coagulation factors of the intrinsic pathway and the patient is threatened by an increased risk of bleeding. If normalization does not occur, the extended time is caused by presence of an inhibitor.

Evaluation

- **Using R**

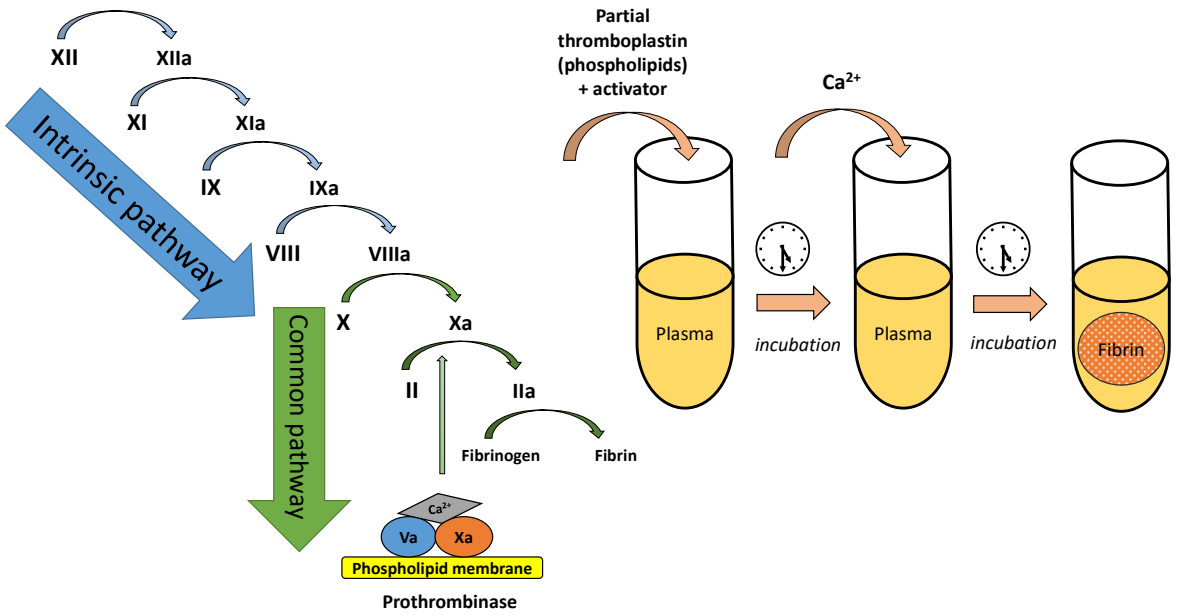
The results of APTT are expressed as the ratio of the coagulation time values for tested and normal plasma samples (R):

$$R = \left(\frac{\text{Coagulation time of patient's plasma in s}}{\text{Coagulation time of normal plasma in s}}\right)$$

- **Reference range:**

  R: 0.8 – 1.2

Fig. 4 Course and performance of activated partial thromboplastin time (APTT) test



The extension of APTT occurs in patients treated with **unfractionated heparin**, but not low-molecular-weight heparins, and accompanies also therapy with coumarins. Another cause of an APTT extension may be lack of factors of the intrinsic coagulation pathway, either inherited (hemophilia A, B, or C) or acquired (decreased liver synthesis, increased loss or enhanced consumption), or presence of an inhibitor. Usually, for an APTT extension a participating factor must decrease below 40 % of its normal levels.

3. Thrombin time (TT)

Principle

Thrombin time is a routine test that evaluates the **last phase of coagulation by measuring the rate of conversion of fibrinogen to fibrin** by thrombin. The active thrombin cleaves from fibrinogen chains the fibrinopeptides A and fibrinopeptides B; following their separation fibrinogen monomers are formed. Their spontaneous polymerization produces soluble fibrin, which is further stabilized by factor XIII in the presence of Ca^{2+}. This further stabilization of fibrin is not measured by TT test. Thrombin time is not affected by lack of coagulation factors that participate in the coagulation cascade before thrombin, nor is affected by endogenous thrombin.

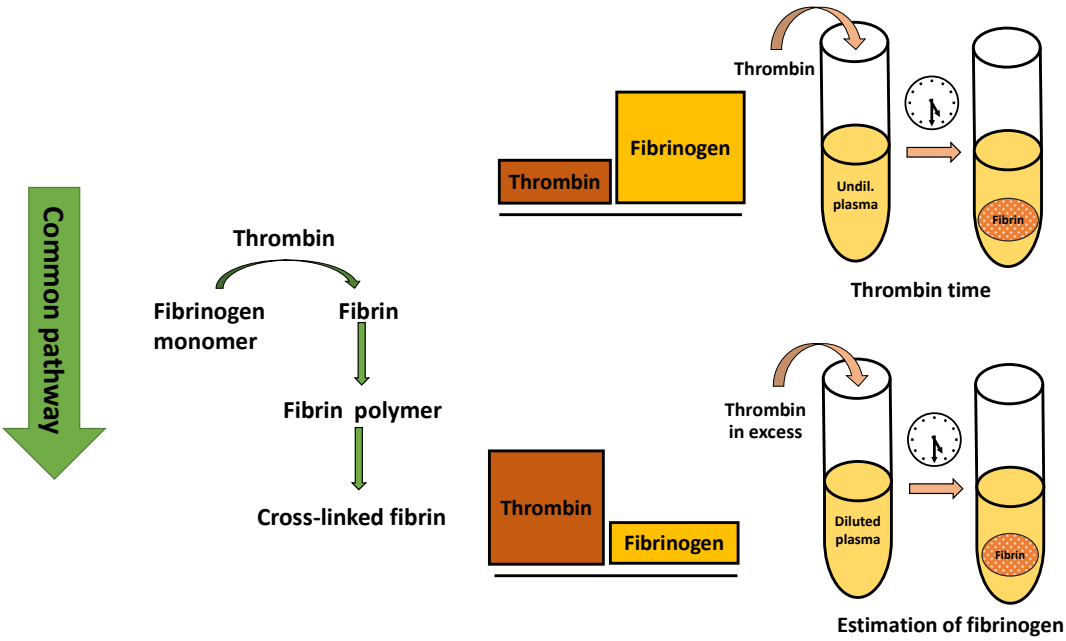
Reagent

The reagent for TT test contains bovine or human thrombin. It may also contain Ca^{2+} ions, but recalcification of plasma is not necessary.

Performance of the test

To **undiluted** citrate plasma thrombin is added in low concentration. Time until formation of coagulum is measured, its normal values range between 14 – 18 (up to 23) seconds (Fig. 5).

Fig. 5 Course and performance of thrombin time (TT) test and estimation of fibrinogen



Evaluation

The values depend on the used thrombin. The results are expressed as in the previous tests in the form of a ratio between the values of TT measured with the patient's and normal plasma.

- **Reference range**

  R: 0.8 – 1.2

Thrombin time serves for detection of fibrinogen abnormalities – acquired or inherited fibrinogen deficiency (hypofibrinogenemia) or qualitative changes of fibrinogen associated with impaired function (dysfibrinogenemia). Thrombin test responds to the presence of heparin, especially unfractionated, or direct inhibitors of thrombin (such as dabigatran) by extension of the coagulation time. An extension occurs also in the presence of other inhibitors with anti-thrombin effect, such as fibrin-degradation products, which further limit polymerization of fibrin monomers. The test can therefore be used for monitoring patients on thrombolytic therapy.

4. Estimation of fibrinogen

Principle

Fibrinogen is a coagulation factor, glycoprotein in nature, which has the highest concentration of all the coagulation factors in blood plasma. Cleavage of fibrinogen to fibrin monomers by thrombin closes the whole coagulation cascade. Polymers produced from fibrin monomers form the basis of the final hemostatic thrombus. Fibrinogen behaves as the positive acute phase reactant.

Reagent

Bovine or human thrombin with stabilizers is used. Ions of Ca^{2+} can be added.

Performance of the test

Fibrinogen, which serves as a substrate in the coagulation system, can be estimated by a functional test that evaluates its ability to get converted to fibrin – method according to Clauss. To a **diluted** plasma a high concentration of thrombin is added, unlike the thrombin test, in which thrombin is used in low concentration (Fig. 5). Condition at which the reaction mixture contains several times lower concentration of fibrinogen than is physiological, ensures that the coagulation time will not be affected by concentration of thrombin, rather, **time needed for clot formation will be inversely proportional to the amount of fibrinogen**.

Alternatively, fibrinogen can be estimated as antigen by immunochemical methods using specific antibodies. This approach will not differentiate functional molecules of fibrinogen from those with impaired function (dysfibrinogenemia). In case of dysfibrinogenemia, which can be associated with both decreased blood coagulation and increased susceptibility to thrombosis, the concentrations of fibrinogen estimated by an immunochemical method will usually be higher in comparison to a functional test. In contrast, in hypofibrinogenemia we obtain similarly low results from both analysis of fibrinogen as antigen and from a functional test.

Evaluation

Results are given in g/l and are read from a calibration curve constructed from several dilutions of commercial calibrator plasma.

- **Reference range:**

  1.8–4.2 g/l

Decreased values of fibrinogen may point to an acquired or inherited hypofibrinogenemia or dysfibrinogenemia. When evaluating fibrinogen concentration, the fact that fibrinogen is one of the acute phase proteins needs to be taken into account. As part of the acute phase response (e.g. inflammation, surgery) the fibrinogen synthesis in the liver increases and its concentration rises 3–5×.

It also increases in pregnancy. An elevated concentration of fibrinogen is considered as one of the risk factors for development of thrombosis.

Selected coagulation tests – basic information

Test	Reagents	Tested phase of coagulation	Note
Prothrombin time (PT, Quick)	Thromboplastin (tissue factor, phospholipids) Ca^{2+}	Extrinsic and common pathways	Monitoring therapy with coumarins (warfarin), for guidance a check of effect of inhibitors of factor Xa (e.g. rivaroxaban)
Activated partial thromboplastin time (APTT)	Partial thromboplastin (phospholipids) Ca^{2+} Contact activators (e.g. silicates, kaolin, ellagic acid)	Intrinsic and common pathways	Monitoring therapy with unfractionated heparin, for guidance a check of effect of direct thrombin inhibitor dabigatran
Thrombin time (TT)	Thrombin (low concentration)	Conversion of fibrinogen to fibrin	Detection of hypofibrinogenemia or dysfibrinogenemia
Fibrinogen	Method by Clauss: thrombin (high concentration)	Conversion of fibrinogen to fibrin	dtto acute phase reagent

Literature

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