Procedure for the Determination of Fibrinogen in Plasma; Approved Guideline—Second Edition

1 Introduction

This document specifies a technique to assay fibrinogen in plasma, based on the method of Clauss.[1]

2 Scope

H30-A2 contains guidelines for the collection, transportation, handling, and storage of blood specimens or plasma samples and general guidelines for performing the fibrinogen assay by the Clauss method.[1] It is primarily directed toward laboratory and/or clinical personnel responsible for obtaining and processing blood specimens, performing the fibrinogen assay and quality control procedures, and reporting fibrinogen assay results. It is also intended as a guide for manufacturers of the reagents and instruments. The guideline does not cover prothrombin-time (PT)-derived fibrinogen determination which can be performed using various automated coagulation instruments.[14]

3 Standard Precautions

Because it is often impossible to know what might be infectious, all human blood specimens are to be treated as infectious and handled according to "standard precautions." Standard precautions are new guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals*. Infection Control and Hospital Epidemiology. CDC. 1996;Vol 17;1:53-80.), [MMWR 1987;36(suppl 2S):2S-18S] and (MMWR 1988;37:377-382, 387-388). For specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure, refer to NCCLS document M29—*Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue*.

4 Definitions[a]

Control (plasma), *n* - A batch of citrated plasma used to monitor the stability of the laboratory test system, which includes reagents, instruments, reconstituting and diluting fluids, and pipettes; **NOTES**: a) "Normal control plasma" gives test results within the range of the reference interval; b) "Abnormal control plasmas" for factor assays should contain factor concentrations below the reference interval values due to abnormally low factor concentrations; c) If factors are clinically elevated, the "abnormal control plasma" should contain factor concentrations above the reference interval; d) Normal and abnormal control plasmas may be prepared in the laboratory or obtained commercially.

Reference curve, *n* - A line, typically a straight line, that defines the quantitative relationship between an independent variable and a dependent variable; **NOTE**: From this line the observed output of an analytic procedure (e.g., APTT test) can be converted to the units of measurement of the analyte of interest (e.g., coagulation factor activity).

[a] Some of these definitions are found in NCCLS document NRSCL8—*Terminology and Definitions for Use in NCCLS Documents*. For complete definitions and detailed source information, please refer to the most current edition of that document.

Reference plasma, *n* - Citrated normal pooled plasma of known coagulant factor activity prepared in-house or available from a manufacturer; **NOTE**: This plasma is used to construct the reference curve.

Sample (patient), *n* - A sample taken from the patient specimen and used to obtain information by means of a specific laboratory test.

Specimen (patient), *n* - The discrete portion of a body fluid (e.g., blood) or tissue taken for examination, study, or analysis of one or more quantities or characteristics to determine the character of the whole.

Test plasma, *n* - Plasma (from a patient or unknown source) that is analyzed by a specific laboratory test.

5 Principle

In normal blood coagulation, fibrinogen is converted to fibrin in a two-step process. The first step is the thrombin-mediated limited proteolysis of two small peptides from fibrinogen-creating fibrin monomers. In the second step, these monomers (when present in sufficient concentration) spontaneously polymerize to form fibrin strands or polymers. The formation of fibrin polymers is the end point of the fibrinogen assay which is used to calculate fibrinogen concentration. In normal plasma, fibrin polymers are rapidly cross-linked by thrombin-activated factor XIII (fibrin stabilizing factor) to form insoluble fibrin. However, this reaction is not measured in the fibrinogen assay.

The most commonly used method for the determination of fibrinogen concentration is the Clauss thrombin clotting rate assay described in this document. A standard amount of thrombin is added to diluted plasma, and the time required for clot formation is recorded. The clotting time of dilute, citrated plasma is inversely proportional to the fibrinogen concentration of the plasma when a relatively high concentration of thrombin is used. The clotting time obtained is then compared with the clotting times of a series of diluted reference plasma of known fibrinogen concentration to yield the fibrinogen concentration of the test plasma.

The standard method recommended by DIN is essentially the same as the method described herein. Some differences do occur based on differences between available and standard reagents.[12,13]

6 Equipment

6.1 Containers

Using a semiautomated or automated end point detection system, the test is performed using nonactivating surface containers.

6.2 Delivery Systems

Delivery systems supplied with an instrument system should be used. The user should demonstrate and document accurate calibration of all delivery systems.

6.3 Heating Block

A heating block or water bath should be available to preheat and maintain reagents at 37 ± 1 °C.

7 Specimen Handling

7.1 Patient Information

A test requisition (computer order or hard copy) should accompany all specimens and should include patient demographic information, a unique patient identification number, and indication for testing. Pertinent patient information, such as treatment with heparin or warfarin, should be indicated as clinical information when appropriate and possible.

7.2 Specimen Collection, Transportation, Processing, and Storage

Blood should be collected, transported, processed, and stored according to the most current edition of NCCLS document H21— *Collection, Transport, and Processing of Blood Specimens for Coagulation Testing and General Performance of Coagulation Assays*.

7.3 Post-test Specimen Management

Please refer to the most current edition of NCCLS document M29—*Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue* for recommendations on specimen disposal and medical waste management.

8 Reagents

8.1 Commercial Reagents

There are several commercial reagent systems that are widely used for this assay. If one of these systems is used, the manufacturer's recommendations and instructions for reagents, procedures, and performance of the test should be strictly followed.

8.2 Non-Commercial Reagents

If noncommercial reagents are to be used in the determination of fibrinogen, the reagent preparations listed below are recommended.

8.2.1 Barbital Buffer (Owren's buffer; 0.02 mol/L, pH 7.4 ± 0.05)

To a 2-L volumetric flask, approximately 1 L of Type I distilled water (Section 12.2), 430 mL of 0.1 mol/L HCl, 11.7 g sodium diethylbarbiturate, and 14.7 g sodium chloride are added, and the mixture is stirred until complete dissolution is achieved. A sufficient volume of water is added to bring the volume to the 2-L mark. The pH of the buffer at 25 °C is adjusted to pH 7.4 with 1N HCl. The buffer is stable for at least three months when stored at 4 °C. Equivalent buffers may be used.

8.2.2 Thrombin

Thrombin (bovine or human), of known National Institutes of Health (NIH) units and prepared in barbital buffer, should be kept in a lyophilized form or frozen in aliquots in nonactivating surface containers. A frozen thrombin stock solution of 1,000 NIH units/mL is stable for at least one year at –70 °C. The stability of frozen thrombin at temperatures above –70 °C has not been well documented; however, 98% of clinical laboratories use commercially available lyophilized thrombin preparations with well-documented stability and clearly marked expiration dates. The expiration date criteria specified by the manufacturer should be strictly followed. (If thrombin is stored in a frozen state, then it is extremely important to aliquot the working thrombin solution in volumes appropriate for individual assay runs,

because repeated freeze-thaw cycles will denature the enzyme.) Before use, aliquots of thrombin should be reconstituted or thawed, the proper dilution should be made in barbital buffer, and it should be kept at 2 to 8 °C until needed for the test. Thrombin in working dilutions is typically stable for no more than one hour at 2 to 8 °C.

8.2.3 Normal Plasma Control

Normal plasma controls can be derived from a pool of commercial citrated normal plasmas, or prepared in the laboratory and stored in aliquots. If lyophilized, this material should be kept at 2 to 8 °C until the expiration date and, if frozen, stored in aliquots at ≤ -20 °C for up to six months. An aliquot of pooled normal plasma should be thawed or reconstituted and tested at the usual dilution used for normal plasma controls with each aliquot of reconstituted working thrombin solution. The measured fibrinogen concentration in thawed or reconstituted plasma is stable for six hours at a temperature of 2 to 8° C.

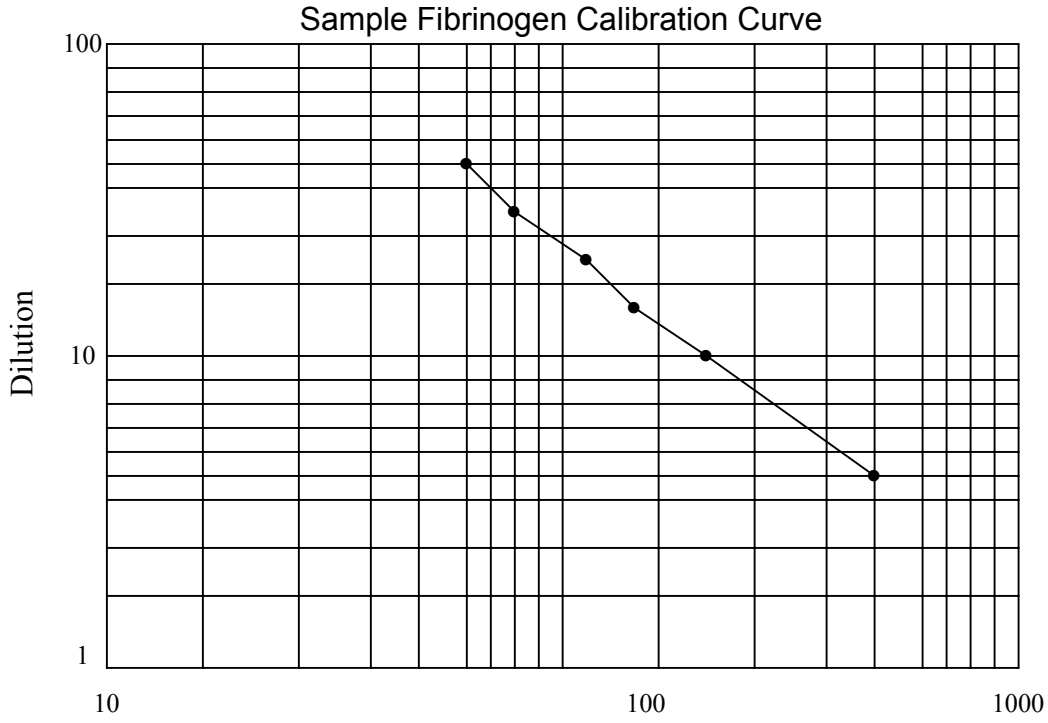
8.2.4 Abnormal Control

At least one abnormal control with a decreased fibrinogen level [80 to 100 mg/dL (0.8 to 1.0 g/L)] should also be assayed with every batch of specimens tested. In laboratories where many fibrinogen determinations are performed, normal and abnormal controls should be tested at a minimum of every 20 patient samples. Laboratories should follow the kit manufacturers' recommendations. An aliquot should be thawed or reconstituted and tested at the usual dilution used for normal plasma controls with each aliquot of reconstituted working thrombin solution. Commercial abnormal controls are available. Abnormal controls can be prepared by diluting pooled normal plasma with barbital buffer. The prepared abnormal control pool should be assayed, aliquoted, and stored at ≤ -20 °C.

9 Reference Curve

The reference curve should be prepared from reference plasma calibrated against a standard plasma with a known fibrinogen concentration. Reference plasmas may differ from those reported in the DIN procedures.[12,13] Reference plasmas are available from theWorld Health Organization and coagulation reagent manufacturers.[15] The manufacturer's recommendations and instructions should be followed. New reference curves should be prepared with each change of reagent lots, any change of instrument, or with any deviation from quality control or proficiency testing limits. Calibration should be performed on each new lot of pooled reference plasma.

At lease five points are recommended in the construction of the reference curve within the acceptable measuring range. The reference curve is prepared by plotting the logs of the clotting times of reference plasma dilutions against the logs of the fibrinogen concentrations. An example of a reference curve is shown in Figure 1. At least five points should be used in the construction of the reference curve. The fibrinogen concentration in diluted test plasma is read from the reference curve.



Dilution	Concentration (mg/dL)	Mean Clotting Time (sec)
1:5	476	5.4
1:10	238	9.8
1:15	159	14.9
1:20	119	21.7
1:30	79	31.3
1:40	60	47.1

Figure 1. Curve for Quantitative Fibrinogen Procedure

10 Procedure

(1) Collect blood into citrate anticoagulant according to the method described in the current edition of NCCLS document H21—*Collection, Transport, and Processing of Blood Specimens for Coagulation Testing and General Performance of Coagulation Assays.*

(2) Treat control and patient plasma in an identical manner. Dilute the plasma 1:10 with barbital buffer (see Section 8.2.1). Place the diluted plasma in the reaction container and warm to 37 ± 1 °C for five minutes.

(3) Prepare thrombin working solution by diluting stock thrombin [of known NIH units] with barbital buffer (see Section 8.2.2) to the desired concentration. The thrombin working solution must not be frozen before use.

(4) Add 0.1 mL of thrombin working solution to 0.2 mL of diluted test plasma, and simultaneously start a timer.

(5) Read the values of the fibrinogen concentration in mg/dL from the reference curve, and multiply by the appropriate plasma dilution to obtain the final mg/dL value.

(6) If the clotting time falls within the linear portion of the reference curve, read the result directly from the curve. If the thrombin clotting time is outside the linear portion of the curve, repeat the test using different test plasma/buffer dilutions until a clotting time is obtained that falls within the linear portion of the curve. When thrombin clotting times below the linear portion of the curve are obtained, a greater dilution (e.g., 1:20 to 1:40) is used. When thrombin clotting times are above the linear portion of the curve, a lower dilution (i.e., 1:5 or 1:3) is used. The lowest dilution that may be used with any degree of acceptable accuracy is 1:3; undiluted plasma cannot be used, because inaccurate results may be obtained due to the presence of interfering substances.

When the plasma fibrinogen concentration is below the detectable range with a 1:3 plasma dilution, the result should be reported as less than the value determined by the greatest dilution on the linear portion of the reference curve (corrected for the dilution of the patient sample).

(7) Although NCCLS documents generally use units that are fully acceptable within the Système International d'Unités (SI), these do not always coincide with the units recommended by the International Union of Pure and Applied Chemistry (IUPAC) and by the International Federation of Clinical Chemistry (IFCC) for reporting results of clinical laboratory measurements.[16] SI units are used worldwide, but there is not yet a consensus as to their use in the United States; NCCLS documents include the IUPAC/IFCC recommended units of volume (L) and substance (molecular) concentration (mol/L) in parentheses, where appropriate. Because of the uncertainty in the value of the relative molecular mass of fibrinogen, IFCC-IUPAC recommends that results be reported in grams per liter.

11 Test Result Management

Users should follow institutional policy for entering patient test results into the existing laboratory information system (LIS) to minimize/eliminate clerical errors and to ensure prompt and accurate result reporting.

Low critical values should be established by the laboratory and a procedure put in place for communicating these critical values to the appropriate clinical staff caring for the patient. Notification of the critical value (date/time/individual notifying/notified individual) should be documented.

12 Considerations in Performing the Fibrinogen Assay

12.1 Manufacturers' Instructions

Manufacturers' instructions for reagents and equipment should be strictly followed.

12.2 Water

Type I reagent grade water should be used (see the current edition of NCCLS document C3—*Preparation and Testing of Reagent Water in the Clinical Laboratory*).

12.3 Cleaning

All collection and storage tubes, pipets, and delivery systems should be clean.

12.4 Temperature

The test should be performed at 37 ± 1 °C.

12.5 End Point Determination

The end point can be measured by a variety of optical or electromechanical methods.

12.6 Quality Control

The laboratory should follow generally accepted quality control practices and quality control requirements of the appropriate regulatory agencies. Specifically, laboratory personnel with appropriate experience in performing fibrinogen assays should inspect the quality control results to evaluate for trends or shifts, as well as out-of-limit results. In addition, the plots of the reference plasmas and the individual patient/test plasmas should be reviewed for correctness. There should be periodic review of quality control data to look for long-term changes in the analytic system.

12.6.1 Controls Outside Stated Limits

If the test values for the control plasmas are not within stated limits, all reagents, control plasmas, and equipment should be checked. The actions undertaken to identify and correct the problem should be documented before any patient plasma is analyzed in the system.

12.7 Reference Interval

Each laboratory should develop its own reference interval for the fibrinogen assay.[16] The details of the procedures for determination of reference intervals for coagulation proteins can be found in the current edition of NCCLS document H21—*Collection, Transport, and Processing of Blood Specimens for Coagulation Testing and General Performance of Coagulation Assays*.

13 Sources of Preanalytical Error

13.1 Inappropriate Specimen Collection

Problems of inappropriate specimen collection include the following:

- incorrect collection tube (i.e., lack of anticoagulant or incorrect anticoagulant, e.g., heparin, EDTA);
- overfill or underfill of collection tubes;
- failure to adjust the citrate volume for persons with very high (>0.55) hematocrit (packed cell volume) (see the current edition of NCCLS document H21—*Collection, Transport, and Processing of Blood Specimens for Coagulation Testing and General Performance of Coagulation Assays*);
- clotted, hemolyzed, icteric, or lipemic specimens;
- inadequate or too vigorous agitation of a specimen; or
- contaminated collection or storage tubes.

14 Sources of Analytical Error

14.1 Inappropriate Thrombin Preparation

Problems with thrombin solution may include:

- contaminated buffer or reagent;
- reconstitution with an incorrect volume of buffer;
- use of thrombin working solution after freezing;
- defects in the commercial thrombin reagent;
- storage in glass; or
- prolonged (> 1 hour) storage or storage at ≥ 8 °C of thrombin at working solution dilution.

14.2 Incorrect Conditions

Incorrect conditions that may affect test results include using the wrong incubation time, temperature, buffer pH, volumes, or instrumentation procedures.

14.3 Paraproteins

High levels of some paraproteins may interfere with the polymerization of fibrin monomers, leading to underestimation of fibrinogen.

14.4 Bovine Thrombin Antibodies

The clinical use of topical bovine thrombin may lead to the development of antibodies to thrombin. These antibodies may lead to artifactual reduction in the rate of thrombin formation and underestimation of fibrinogen.

14.5 Fibrin/Fibrinogen Degradation Products (FDP)

Proteolytic products of fibrinogen and fibrin in high concentration may interfere with fibrin polymerization. At fibrinogen concentrations below 150 mg/dL (1.5 g/L), FDP greater than 75 µg/mL (75 mg/L) decrease the rate of fibrin polymerization and underestimate fibrinogen concentration.[17]

14.6 Heparin

Heparin, a potent activator of antithrombin, which irreversibly inhibits clotting-active serine proteases including thrombin, may interfere with the thrombin clotting time, thus causing erroneously low estimates of fibrinogen concentration. It may require higher concentrations of heparin (greater than 5.0 U/mL) to accomplish this inhibition.[17] Although these concentrations are infrequently seen in the clinical use of heparin, these concentrations may be found in blood that is collected incorrectly in heparinized tubes or through heparinized lines (high local concentration), and in patients undergoing cardiopulmonary bypass surgery, or during hemodialysis.

14.7 Dysfibrinogenemia

In certain patients with acquired or inherited biochemical abnormalities of fibrinogen which may inhibit the action of thrombin on fibrinogen and/or fibrin polymerization (i.e., dysfibrinogenemia), fibrinogen levels may be underestimated.