Assays of von Willebrand Factor Antigen and Ristocetin Cofactor Activity; Approved Guideline

This guideline describes the following: appropriate test specimens; reagents and materials; methods of platelet agglutination and ELISA; preparation of reference curves; determination of reference intervals; quality control procedures; result interpretation; and sources of error for assays of von Willebrand factor antigen and ristocetin cofactor activity. A brief description of von Willebrand disease and its various subtypes is included, as well as a list of references to more comprehensive reviews of this commonly inherited and rarely acquired bleeding disorder.

A guideline for global application developed through the NCCLS consensus process.
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Assays of von Willebrand Factor Antigen and Ristocetin Cofactor Activity; Approved Guideline

Abstract

NCCLS document H51-A—Assays of von Willebrand Factor Antigen and Ristocetin Cofactor Activity; Approved Guideline is part of a series of guidelines that address methods in hemostasis testing. The assay of ristocetin cofactor is the most common single test used in the diagnosis of von Willebrand disease and its classification into different subtypes. It is a functional assay of von Willebrand factor activity that measures the ability of the antibiotic ristocetin to induce platelet agglutination in the presence of von Willebrand factor. Thus, the rate and extent of platelet agglutination is a function of the concentration and functional integrity of von Willebrand factor.

Determination of von Willebrand factor antigen is another common single test used in the diagnosis of von Willebrand disease and its classification into numerous subtypes. The method described allows the quantitation of von Willebrand factor antigen (protein) by an enzyme-linked immunosorbent assay (ELISA).

This guideline describes appropriate test specimens, reagents and materials, methods of platelet agglutination and ELISA, preparation of reference curves, determination of reference intervals, quality control procedures, result interpretation, and sources of error for assays of von Willebrand factor antigen and ristocetin cofactor activity. A brief description of von Willebrand disease and its various subtypes is included, as well as a list of references to more comprehensive reviews of this commonly inherited and rarely acquired bleeding disorder.


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Number 20

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Swedish Medical Center – Providence Campus (WA)
Tampa General Hospital (FL)
Temple University Hospital (PA)
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**Foreword**

von Willebrand Disease (vWD) is the most commonly inherited bleeding disorder. It is characterized by mucocutaneous bleeding, such as increased bruising, menorrhagia, and epistaxis. Surveys from several countries indicate that 1% or more of the population may be affected. However, less than half of affected individuals have abnormal bleeding manifestations. vWD is caused by a deficiency and/or a qualitative abnormality of the protein, von Willebrand factor (vWF). Plasma vWF is a very high molecular weight, multimeric glycoprotein composed of identical subunits. The number of subunits per molecule varies. vWF, especially the higher molecular weight forms, mediates platelet adhesion to subendothelial connective tissue following vascular injury. To a lesser extent, it supports platelet aggregation. In addition, vWF is the carrier protein in plasma for Factor VIII and stabilizes its coagulant activity. With a deficiency or abnormality of vWF, Factor VIII activity is often reduced due to accelerated degradation.

vWF is synthesized in endothelial cells and megakaryocytes under the control of a gene located on chromosome 7. vWF is liberated from endothelial cells bidirectionally into plasma and subendothelium continuously (constitutive liberation) and in response to endothelial cell activation (release) from a specific storage organelle (Weibel-Palade body) in the form of large multimers (1,000 to 20,000 KD).

vWD is inherited autosomally and is a heterogeneous disorder caused by a large number of different mutations resulting in different phenotypes classified broadly into three major subtypes. Most patients (>80%) have Type 1 vWD in which there is a decrease in plasma of qualitatively normal vWF. Typically, persons with Type 1 vWD have mild bleeding symptoms; some are asymptomatic and may never be diagnosed. Type 2 vWD is characterized by qualitative abnormalities of vWF. Most of these are associated with deficiency of the higher molecular weight multimers. Patients with Type 2 vWD may have mild to severe bleeding manifestations. Type 3 vWD is a severe hemorrhagic diathesis in which plasma vWF is severely reduced or absent from plasma and platelets.

The laboratory diagnosis and subtyping of vWD may be difficult. It is based on the following tests: bleeding time or other screening tests; Factor VIII; vWF antigen (vWFAg); ristocetin cofactor activity (R:CoF); ristocetin-induced platelet agglutination (RIPA); the multimeric analysis of vWF; and DDAVP-response test. This document describes a procedure for performing assays of vWFAg and R:CoF. vWFAg is quantitated by immunoassay. R:CoF, a measure of vWF function, is commonly measured by determining the extent to which test plasma is able to support agglutination of fixed, normal platelets.

vWFAg and R:CoF assays may be affected by several preanalytical (mostly patient-related) and analytical variables. This guideline is intended to minimize the effects of some of these variables and to reduce variability in test results.

This document is written for laboratory professionals responsible for the performance of tests for von Willebrand disease (vWD). It is also intended for the manufacturers of the reagents and instruments used in these tests.

**Key Words**

Factor VIII, Laurell electroimmunoassay, ristocetin cofactor activity (R:CoF), von Willebrand disease (vWD), von Willebrand factor (vWF), von Willebrand factor antigen (vWFAg), von Willebrand factor multimers
Path of Workflow

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, GP26-A2 defines a clinical laboratory path of workflow which consists of three sequential processes: preanalytical, analytical, and postanalytical. All clinical laboratories follow these processes to deliver the laboratory’s services, namely quality laboratory information. The arrow depicts the sequence, from left to right, that any clinical laboratory follows. In addition, the necessary steps or subprocesses are listed below them.

Adapted from NCCLS document HS1—A Quality System Model for Health Care.

H51-A Addresses the Indicated Steps Within the Clinical Laboratory Path of Workflow

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<tr>
<td>Patient Assessment</td>
<td>Testing Review</td>
<td>Results Report</td>
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<td>Laboratory Interpretation</td>
<td>Post-test Specimen Management</td>
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Adapted from NCCLS document HS1—A Quality System Model for Health Care.
Assays of von Willebrand Factor Antigen and Ristocetin Cofactor Activity; Approved Guideline

1 Introduction

von Willebrand Factor (vWF) is a multimeric, high molecular weight protein present in plasma and platelets that mediates platelet adhesion to subendothelium and platelet aggregation in response to vascular injury (primary hemostasis).\(^1\) vWF also serves as a carrier protein and stabilizer for Factor VIII (FVIII).\(^1,2\) von Willebrand factor antigen (vWF\(\text{Ag}\)) is the protein that expresses vWF activity (commonly called ristocetin cofactor activity [R:CoF]).\(^2\) vWF\(\text{Ag}\) can be measured by several immunologic techniques, including electroimmunoassay (EIA), enzyme-linked immunosorbent assay (ELISA), and latex immunoassay (LIA) using antibodies against vWF.\(^2\) The method described in this document is an ELISA method.

R:CoF is the property of vWF that supports platelet agglutination in the presence of ristocetin and is the most common in vitro test used for vWF function.\(^1\) vWF in plasma does not bind to its platelet receptor, glycoprotein Ib-IX (GP Ib-IX), unless it is structurally modified by binding to subendothelial connective tissue structures such as collagen.\(^1,2\) Ristocetin is thought to mimic this modifying action of subendothelium on vWF.\(^3\) The method described in this document is based on ristocetin-induced platelet agglutination of formaldehyde- or glutaraldehyde-fixed, normal platelets in the presence of test plasma.\(^2\)

2 Scope

This guideline provides selected methods for measuring vWF\(\text{Ag}\) and R:CoF. Specimen requirements, reagents and materials, preparation of reference curves, establishment of reference intervals, result reporting, quality control, and common sources of error are addressed. A brief description of von Willebrand Disease (vWD) and its various subtypes is included, as well as references to more comprehensive reviews of this commonly inherited and rarely acquired bleeding disorder.

The method described for measuring vWF\(\text{Ag}\) is an ELISA technique. The method described for measuring R:CoF utilizes a turbidimetric platelet aggregometer to measure changes in light transmission and the extent of agglutination of formalin-fixed platelets by ristocetin in the presence of test plasma. The R:CoF assay described here must be distinguished from the ristocetin-induced platelet agglutination assay (RIPA), which is performed in freshly prepared, citrated, platelet-rich, test plasma using different concentrations of ristocetin to distinguish between different types of vWD.

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 for recommendations for the management of blood-borne exposure, refer to the most current edition of NCCLS document M29—Protection of Laboratory Workers from Occupationally Acquired Infections.
4 Definitions*

In this publication, the following definitions of terms are used:

**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, etc. **NOTES:** a) “Normal control plasma” should yield test results within the range of the normal reference interval; b) “Abnormal control plasma” should yield test results below the normal reference interval; c) Normal and abnormal control plasmas may be prepared in the laboratory or obtained commercially.

**Diluent, n** - The material used to make a concentrated material weaker; **NOTES:** a) The diluent is usually a liquid or a gas; b) A liquid diluent may also be used to reconstitute a dried material to its original concentration.

**Enzyme linked immunosorbent assay, ELISA, n** - A heterogeneous (requires separation of bound and free) immunoassay in which an analyte is captured by its corresponding antigen or antibody, then detected by an enzyme-conjugated reactant.

**Factor VIII, n** - A plasma glycoprotein which, when activated by thrombin, functions as a cofactor in the conversion of Factor X to Factor Xa; **NOTE:** It is normally carried and stabilized by von Willebrand factor; its reduction in plasma is the cause of hemophilia A.

**Fibrinogen, n** - A plasma glycoprotein which is converted to fibrin by thrombin and supports platelet aggregation.

**Glycoprotein Ib-IX, GPIb-IX, n** - The major platelet membrane receptor for von Willebrand factor.

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

**Platelet agglutination, n** - The clumping of intact or fixed platelets by von Willebrand factor in the presence of ristocetin.

**Platelet aggregation, n** - Platelet clumping, largely mediated by fibrinogen binding to the platelet receptor, GPIIb/IIIa, following activation of intact platelets by soluble agonists such as adenosine diphosphate (ADP) and thrombin, or particular agents such as a suspension of collagen, or sheer stress.

**Platelet aggregometer, n** - A device that measures changes in light transmission through a suspension of platelets; **NOTE:** As platelets clump, more light passes through the suspension, and the increase in light transmission is recorded continually.

**Reference plasma, n** - A citrated, normal, pooled plasma with known von Willebrand factor antigen or ristocetin cofactor activity prepared in-house or commercially available; **NOTE:** This plasma is used to construct the reference curve.

**Ristocetin, n** - A substance originally developed as an antibiotic and then found to cause platelet clumping in the presence of normal von Willebrand factor; **NOTE:** The extent of platelet clumping in the presence of an optimal concentration of ristocetin is directly proportional to the concentration of functional von Willebrand factor.

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* 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.

**Ristocetin-induced platelet agglutination, RIPA, n** - Platelet agglutination in citrated platelet-rich plasma in the presence of different concentrations of ristocetin.

**Slope, n** - The relationship between the change in $y$ and the change in $x$ between any two points along a line; **NOTE:** Slope is represented by the symbol “m” in the linear equation $y = mx + b$ and may be either positive or negative.

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

**von Willebrand disease, vWD, n** – A hereditary or acquired defect in the quantity and/or function of von Willebrand factor.

**von Willebrand factor, vWF, n** - A high molecular weight, multimeric glycoprotein present in endothelial cells, megakaryocytes, platelets, and plasma; **NOTE:** This protein is present in reduced amounts and/or is abnormal in von Willebrand disease.

**von Willebrand factor antigen, vWFAg, n** - von Willebrand factor as measured by an immunologic assay.

5 **Determination of von Willebrand Factor Antigen**

5.1 **Principle**

The method described here for determining vWFAg is an enzyme-linked immunosorbent assay (ELISA) that is an immunologically based assay used to detect small amounts of protein. The test antigen is captured with an antibody coated on a solid-phase surface (plastic surface of a microtiter plate). Then, a specific antibody recognizing a specific antigenic epitope(s) (different from the capture antibody, if it is used) is added and allowed to react with the antigen. After removal of excess antibody by washing, a specific anti-immunoglobulin with an enzyme coupled to the nonfunctional portion of the antibody is allowed to react. Following a second washing to remove excess secondary antibody-enzyme reagent, a chromogenic substrate is added and allowed to react with the coupled enzyme. After a standard time interval, the reaction is stopped and the amount of product is determined. The amount of product is proportional to the amount of enzyme present (and the amount of secondary antibody). Thus, the amount of color is proportional to the amount of antigen present. A standard curve is used to determine the amount of protein present in the sample.

The ELISA can be established using several different variations based on the number of antibodies used and the order of addition of the antibodies and antigen. These methods are detailed in general references for ELISA methods.

5.2 **Equipment**

Calibrated pipettes and/or calibrated delivery systems that deliver ±3% of the stated volume should be used. The desired temperature of incubators, refrigerators, freezers, and any other equipment should be verified with calibrated thermometers. An ELISA plate washer or manual wash method should be standardized to allow a consistent amount of wash solution to be added to each microtiter plate well. The ELISA plate reader should be calibrated to allow absorbance (OD) readings at the appropriate wavelengths.
5.3 Specimen Collection, Transport, Processing, and Sample Storage

Blood should be collected, transported, processed, and the samples 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.

5.4 Reagents and Materials

5.4.1 Manufacturers’ Instructions

Manufacturers’ instructions for reagents and equipment should be followed.

5.4.2 Reagents and Supplies

Most reagents and supplies are provided in commercially available kits or can be purchased separately. Materials and supplies which may be required and are not commercially available are:

- Control plasma (as described above);
- Normal pooled plasma; and
- Reference plasma, to be prepared from at least 20 apparently healthy males and nonpregnant females of known blood type, within specified age limits, not taking any medications, including estrogen (e.g., oral contraceptives). Reference plasma should have 100 ± 20 units/dL (100 ± 20%) of vWFAg. It should be calibrated to a World Health Organization (WHO) standard or a secondary standard.

5.5 Procedure for the Determination of von Willebrand Factor Antigen

Details of the procedures vary depending on the commercial kit used. The manufacturer’s recommendations must be followed.

5.6 Reference Intervals

The normal reference intervals for vWFAg vary according to blood type, ranging from 50 to 150 units/dL plasma (blood type O) to 90 to 200 units/dL plasma (blood type non-O).

Each laboratory should determine its own blood-type-specific reference interval for vWFAg and R:CoF activity. The reference interval should be reported with each test result and in the same units. For more information on reference intervals, see the most current edition of NCCLS document C28—How to Define and Determine Reference Intervals in the Clinical Laboratory. Ideally, reference intervals should be determined for each blood type, but due to the rarity of certain blood types in the population, blood types other than O may be grouped as blood type non-O. Every result should have an added statement expressing the problems of the variability of values within patients due to the effects of blood type and the acute phase reaction. All results should be interpreted in the context of the clinical setting for each patient.

5.7 Quality Control

5.7.1 Precision Analysis

The results of vWFAg testing may show considerable variability. Coefficients of variation (CV) of 10% or less are acceptable for the normal control.
5.7.2 Reproducibility of Duplicates

Determinations are commonly performed in duplicate, and the mean of the two values is reported. Duplicate results should agree within 10% or less of the average value. With improvements in the precision of automated instruments, singlet testing may be acceptable.

5.7.3 Frequency of Control Testing

Normal and abnormal control plasmas should be assayed with each test run. Results should be recorded and a statistically valid mean and standard deviation established. See the most current edition of NCCLS document C24—Statistical Quality Control for Quantitative Measurements: Principles and Definitions.

5.7.4 Controls Outside of Stated Limits

If control values do not fall within established limits, the source of error must be identified, corrected, and documented before patient results are reported.

5.7.5 Control Materials

See the most current version of NCCLS document H21—Collection, Transport, and Processing of Blood Specimens for Coagulation Testing and General Performance of Coagulation Assays, for information about the collection, handling, and storage of coagulation control plasmas.

5.7.6 General Quality Control Procedures

The laboratory should follow generally accepted quality control practices and fulfill the quality control requirements of the appropriate regulatory agencies. There should be periodic review of quality control data to look for long-term changes in the analytic system. Please refer to the most current versions of NCCLS documents H21—Collection, Transport, and Processing of Blood Specimens for Coagulation Testing and General Performance of Coagulation Assays and C24—Statistical Quality Control for Quantitative Measurements: Principles and Definitions for more information.

5.8 Potential Sources of Error

Potential sources of error include:

- Incorrect incubation time and/or temperature;
- Substrate not prepared freshly or discolored;
- Pipetting error;
- Incomplete washing; and
- Contaminated buffer.

6 Determination of Ristocetin Cofactor Activity

6.1 Principle

R:CoF is measured by the rate of agglutination of fixed, normal platelets. Dilutions of reference plasma, calibrated against a reference preparation of R:CoF, are added to suspensions of fixed, normal
platelets. Platelet agglutination, induced by a standard concentration of ristocetin, is measured by changes in light transmission. The procedure is repeated with dilutions of test plasma, and the rates of agglutination are compared to the rates obtained with the reference plasma dilutions.

6.2 Equipment

The following equipment is necessary to perform the procedure:

- Instrument capable of measuring the rate of turbidimetric platelet agglutination (e.g., platelet aggregometer);
- Automatic pipets (delivery systems [see Section 5.2]);
- Platelet-counting equipment;
- Instrument-specific cuvettes and stirring bars or magnets;
- 10 x 75-mm glass tubes with caps;
- Plastic containers (12 x 75 mm); and
- Disposable plastic transfer pipets.

6.3 Specimen Collection, Transport, Processing, and Sample Storage

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

6.4 Reagents and Materials

6.4.1 Manufacturers’ Instructions

Manufacturers’ instructions for reagents and equipment should be strictly followed. Different instrument and reagent combinations may have different testing characteristics. Manufacturers’ kits may call for materials, reagents, and reagent concentrations that differ from those presented in the procedures below. It is the responsibility of the laboratory and manufacturers to establish the optimal conditions for each assay system.

6.4.2 Platelet Suspension

Fixed, lyophilized platelets are available commercially. Fixed platelets may also be prepared in the laboratory from fresh blood or fresh or outdated platelet concentrates by differential centrifugation and fixed with formaldehyde or glutaraldehyde. The “quality” of platelet preparations (commercially available or locally prepared) may vary considerably in regard to results of standard curve preparation and test result reproducibility.

Fixed platelets are resuspended in Tris-buffered saline (TBS [0.05 mol/L Tris HCl, 0.15 mol/L NaCl, pH 7.4]) and gently mixed (i.e., inverted) for one minute to suspend platelets evenly.
6.4.3 Reference Plasma

Reference plasma should be prepared from at least 20 apparently healthy males and nonpregnant females of known blood type, within specified age limits, not taking any medications, including estrogen (e.g., oral contraceptives). Reference plasma should have 100 ± 20 units/dL (100 ± 20%) of R:CoF. It should be calibrated against a standard.

Since blood type affects vWF levels, the distribution of blood types should be similar to the blood type distribution of the population tested.

6.4.4 Ristocetin

Ristocetin, obtained from Nocardia lurida, is prepared as a stock solution of 10 mg/mL in water, which is stable at 5 °C for at least three months or for at least a year when stored at –20 °C or below. Thaw at 37 °C before use. Aliquots may be frozen and thawed up to three times. Allow 30 minutes at room temperature for complete dissolution. Prepare working solution freshly each day diluted in TBS at a concentration of ristocetin to yield a maximum rate of platelet agglutination (usually 1.0 to 1.2 mg/mL). The optimum concentration of ristocetin must be determined with each lot and verified periodically.

There is variability among different commercial preparations and lots of ristocetin. The concentration of each new lot or preparation of ristocetin should be titrated against previous lots so that the values obtained for normal and abnormal control plasmas are within the reproducibility of the assay, using the same reference plasma and platelet preparation.

6.5 Reference Intervals

The normal reference intervals for R:CoF vary according to blood type, ranging from 50 to 150 units/dL plasma (blood type O) to 90 to 200 units/dL plasma (blood type non-O). For the establishment of the reference intervals of R:CoF, see Section 5.6.

6.6 Quality Control

6.6.1 Precision Analysis

The results of R:CoF testing may show considerable variability. Coefficients of variation (CV) of 15% or less are acceptable.

6.6.2 Reproducibility of Duplicates

Tests are commonly performed in duplicate, and the mean of the two values is reported. Duplicate results should agree within 15% or less of the average value. With improvements in the precision of automated instruments, singlet testing may be acceptable, if appropriate quality standards are met.

6.6.3 Frequency of Control Testing

Normal and abnormal control plasmas should be assayed with each test run. Results should be recorded and a statistically valid mean and standard deviation established. See the most current edition of NCCLS document C24—Statistical Quality Control for Quantitative Measurements: Principles and Definitions.

6.6.4 Controls Outside of Stated Limits

If control values do not fall within established limits, the source of error must be identified, corrected, and documented before patient results are reported.
6.6.5 Control Materials

See the most current version of NCCLS document H21—Collection, Transport, and Processing of Blood Specimens for Coagulation Testing and General Performance of Coagulation Assays for information about the collection, handling, and storage of coagulation control plasmas.

6.6.6 General Quality Control Procedures

The laboratory should follow generally accepted quality control practices and fulfill the quality control requirements of the appropriate regulatory agencies. There should be periodic review of quality control data to look for long-term changes in the analytic system. Please refer to the current version of NCCLS document H21—Collection, Transport, and Processing of Blood Specimens for Coagulation Testing and General Performance of Coagulation Assays and NCCLS document C24—Statistical Quality Control for Quantitative Measurements: Principles and Definitions for more information.

6.7 Procedure for the Performance of Ristocetin Cofactor Assay

6.7.1 Preparation of Reference Curve

Reference curves are prepared with standard dilutions of reference plasma. Two types of reference curves are employed—an extended curve and a working curve. The extended curve is used to establish the range of linearity for the laboratory's specific analytic system. An extended curve should be prepared when initially setting up the procedure or with each change of reagent lot, reference plasma, or instrument. A working curve should be prepared with each run of assays. The working curve generally has fewer dilutions than the extended curve, and the dilutions are limited to those that fall within the linear portion of the extended curve. The working curve is used as the analytic reference against which test samples are measured.

6.7.1.1 Preparation of the Extended Reference Curve

At least seven dilutions of the reference plasma prepared with buffered saline should be stored in ice water or maintained at 0 to 4 °C. Typically, the lowest dilution is 1:2 or 1:5, and each subsequent dilution is double that of the previous. To minimize systematic error due to pipetting, all plasma dilutions should be prepared independently and not serially.

The rate of agglutination (i.e., the maximum slope of the agglutination curve) for each dilution is plotted against the dilution of the reference plasma on log/log graph paper. Use a three- or four-cycle graph. Alternatively, a suitable computer graphics program can be used to plot the data.

The resulting plots should form an S-shaped curve with flattening at both extremes. (See Figure 1.) If this is not accomplished, further dilutions should be tested. The plots should be observed to determine the range of dilutions that form a straight line in the center of the “S.” Figure 1 shows an example of a log/log extended reference curve.
6.7.1.2 Preparation of the Working Reference Curve

Using a set of at least three dilutions of reference plasma within the linear range, the samples are processed as described in Section 6.7.1.1. (See Figure 2.) The same lot of reference plasma is used that was used to construct the extended reference curve. The X-axis is labeled in terms of units of activity. The lowest dilution is designated an activity value of either 100 unit/dL (100%) or the assayed value of the reference plasma. From this designation, activity values for the other dilutions are derived. The rates of agglutination are indicated on the Y-axis. For each test sample, the rates of agglutination are plotted against units of R:CoF activity, and the line of best fit is drawn.

Figure 1. Log/Log Extended Reference Curve

Figure 2. Working Reference Curve
6.7.2 Preparation of the Reference Plasma Dilutions

At least three dilutions of reference plasma should be prepared with TBS as follows:

- (50%) or 1:2 (50 µL of reference plasma and 50 µL of TBS);
- (25%) or 1:4 (50 µL of reference plasma and 150 µL of TBS); and
- (12.5%) or 1:8 (50 µL of reference plasma and 350 µL of TBS).

Using this technique, the total volume in each tube will be different. To minimize systematic error due to pipetting, all dilutions should be prepared independently and not serially.

6.7.3 Performance of Assay

Perform the assay as follows:

1. Dilute control and test plasma 1:2 and 1:4 in TBS.

2. Add 400 µL of platelet suspension (200 µL of platelet suspension and 200 µL of TBS) into a cuvette containing a stirring bar, and adjust the instrument to yield 0% light transmission.

3. Add 400 µL of TBS into a second cuvette containing a stirring bar, and adjust the instrument to yield 100% light transmission.

4. Add 50 µL of ristocetin working solution to the cuvette containing the platelet suspension.

5. Incubate for three minutes at 37 °C.

6. Add 50 µL of test plasma or test plasma dilution to the cuvette, and record changes in light transmission for five minutes.

7. Determine the maximum slope of agglutination curve.

8. Determine the concentration of R:CoF activity by reading the agglutination slope value off the reference curve.

6.8 Evaluation of Results

The measured values of test plasmas should be corrected for sample dilution, averaged, and multiplied by the observed value of the reference preparation to yield the reportable result.

The reference and test plasma plots should be inspected for outliers, parallelism, and linearity. Four points (undiluted, 1:2, 1:4, 1:8) are required to establish the reference curve. At least three points are needed to determine linearity.

For results to be accepted, the maximum rate of agglutination should fall within the range encompassed by those of the highest and the lowest dilutions of the reference plasma. This represents the readable range of the reference curve.
If the maximum rate of agglutination of the test plasma is less than the highest dilution of the 1:8 reference plasma, one of the following options may be taken:

- The result may be reported as less than 12.5 units/dL or 12.5% activity.

- A further dilution (1:16) of the reference plasma is made and assayed. If the result falls within the linearity of the reference curve, the readable range is effectively extended to a level of 6.25 units/dL or 6.25% activity. If the R:CoF level remains below the readable range, it should be reported as less than 6.25 units/dL or 6.25% activity.

When the maximum rate of agglutination is greater than that of the 1:1 reference plasma, further dilutions should be made until a value is obtained that falls within the readable range.

### 6.9 Potential Sources of Error

Potential sources of error include:

- Incorrect incubation time, temperature, and stirring bar rotation speed;
- Incorrect preparation and handling of the ristocetin working solution;
- Incorrect preparation and storage of fixed-platelet suspension;
- Pipetting error;
- Contaminated buffer;
- Lipemic plasma;
- Icteric plasma;
- Agglutination responses in platelet suspensions may vary considerably among platelet donors, and some platelet preparations may not be usable; and
- Stirring bars may vary slightly in size, and some may yield inadequate stirring, which may lead to erroneous, low test results.

### 7 Interpretation of Results

Decreased levels of vWF Ag and/or R:CoF in plasma are consistent with the diagnosis of inherited or acquired vWD. In inherited vWD Type 1, the levels of vWF Ag and R:CoF are generally decreased to the same extent. However, in mild Type 1 vWD, vWF Ag and R:CoF may fluctuate substantially and may at times both be normal, or either one may be abnormal. Thus, it has been recommended that the diagnosis of mild vWD not be excluded unless vWF Ag and R:CoF are well within the blood-type-specific reference interval on at least two different occasions. A number of conditions and factors which can increase the levels of vWF Ag and R:CoF (such as a physical or emotional stress; liver disease; uremia; acute inflammatory states; pregnancy; and certain medications, in particular synthetic and conjugated estrogen) may make it difficult or impossible to confirm a diagnosis of mild vWD. Because of these difficulties in laboratory diagnosis, the patient’s personal and family history of abnormal bleeding must be taken into account in decisions concerning the diagnosis of vWD and clinical management of patients.

In Types 2A and 2B vWD, R:CoF is frequently appreciably lower than vWF Ag. However, additional tests such as platelet agglutination in response to different concentrations of ristocetin (RIPA) and vWF multimer analysis are required for verification of this subtype.

In Type 2N vWD, the vWF Ag and R:CoF may be normal or slightly decreased. Diagnosis of Type 2N requires demonstration of a disproportionate decrease in Factor VIII as compared to vWF Ag and specific molecular abnormalities. Diagnosis of Type 2M, a very rare type, usually has all multimers present, but may show abnormal bands (i.e., larger than normal bands or other abnormal patterns on high resolution...
vWF multimer analysis). The diagnosis of type 2M is reserved for cases in which there is evidence for an abnormal vWF molecule (lacking or additional satellite banding and/or molecular biological evidence) and the criteria for Types 2A, 2B, and 2N are not fulfilled.

In Type 3 vWD, vWF Ag and R:CoF are usually absent or severely reduced.\(^8,9\)

In platelet-type vWD, which is due to abnormal binding of normal vWD to abnormal platelet glycoprotein Ib/IX receptors on platelets, VWF and R:CoF are reduced to a variable extent and sometimes disproportionately.\(^11\)

In acquired vWD, the reductions of vWF Ag and R:CoF are usually severe with R:CoF frequently reduced to a greater extent than vWF Ag.

Mild to marked increases in the levels of vWF Ag and R:CoF may be observed in a number of conditions, including liver disease; uremia; vasculitis; generalized severe atherosclerosis; acute and chronic inflammatory states; physical or emotional stress; and in premenopausal and postmenopausal women taking synthetic or conjugated estrogen.

**Table 1. Classification of vWD\(^8\)**

<table>
<thead>
<tr>
<th>Type</th>
<th>vWF Ag</th>
<th>R:CoF</th>
<th>VIII:C</th>
<th>RIPA</th>
<th>Multimers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>↓</td>
<td>↓</td>
<td>↓ or N</td>
<td>N</td>
<td>All multimers decreased</td>
</tr>
<tr>
<td>2A</td>
<td>↓</td>
<td>↓↓</td>
<td>↓</td>
<td>↓</td>
<td>Large and middle multimers decreased</td>
</tr>
<tr>
<td>2B</td>
<td>↓</td>
<td>↓</td>
<td>↓ or N</td>
<td>↑</td>
<td>Large multimers decreased</td>
</tr>
<tr>
<td>2M</td>
<td>↓</td>
<td>↓</td>
<td>↓ or N</td>
<td>N</td>
<td>Normal multimers; satellite banding abnormal</td>
</tr>
<tr>
<td>2N</td>
<td>N</td>
<td>N</td>
<td>↓</td>
<td>N</td>
<td>Normal multimers</td>
</tr>
<tr>
<td>3</td>
<td>↓↓ or undet.</td>
<td>↓↓ or undet.</td>
<td>↓↓</td>
<td>Absent</td>
<td>All multimers absent</td>
</tr>
</tbody>
</table>

↓, decreased; ↓↓, markedly decreased; N, normal; ↑, increased; undet., undetectable
References


Additional References


Summary of Consensus Comments and Subcommittee Responses

NCCLS consensus procedures include an appeals process that is described in detail in Section 9 of the Administrative Procedures. For further information contact the Executive Offices or visit our website at www.nccls.org.

H51-P: Assays of von Willebrand Factor Antigen and Ristocetin Cofactor Activity; Proposed Guideline

General

1. The “tagline” should include mention of vWF Antigen.

   • The “tagline” has been modified to read, “This guideline describes appropriate test specimens, reagents and materials, methods of platelet agglutination and ELISA, preparation of reference curves, determination of reference intervals, quality control procedures, result interpretation, and sources of error for assays of von Willebrand factor antigen and ristocetin cofactor activity. A brief description of von Willebrand disease and its various subtypes is included, as well as a list of references to more comprehensive reviews of this commonly inherited and rarely acquired bleeding disorder.”

Foreword

2. Modify the wording of the fourth paragraph, third sentence to read, “However, less than half of affected…”

   • This editorial correction has been made to the text.

Section 3 (Now Section 4)

3. The document refers to factor VIII while the definition list this as factor VIII:C. The definition should be changed to reflect the term used throughout the document.

   • The text and definition have been modified for consistency (i.e., “Factor VIII”).

Section 5.6

4. There are clear differences in the reference intervals when performed within blood groups. There is, however, the inherent danger of using the reference ranges in a “strict” sense. Using the example given, a non-O patient with a value of 80 U/dL, on repeated testing, would be labeled “abnormal;” however, this may be perfectly normal in a patient who does not have the clinical syndrome. In the case of vWF and vWAg measurements, the number alone is not very useful without the clinical “story” for assistance. The data may (and often are) generated by a reference laboratory, remote from the patient, for a family practitioner or general internist who may not be very familiar with the nuances of diagnosing vWD. Because of all of the difficulties pointed out in Section 7 with variability in patients, each report should carry a qualifying explanation of these issues. Thus, we recommend the following addition:

   “Every result should have an added statement expressing the problems of the variability of values within patients and the effects of the acute phase reaction.”

   • The text has been modified as suggested.
Section 6.4.2

5. The phrase “prepared by the laboratory” should be modified to read, “prepared in the laboratory.”

- This editorial correction has been made to the text.

6. This section suggests gently vortexing the platelets. Most manufacturers suggest gently “rocking” platelets, and warn against vortexing.

- The subcommittee agrees with the comment. The text has been modified to read, “...gently mixed (i.e., inverted) for one minute...”

Section 6.4.3

7. This section states that individuals comprising the reference plasma pool should be of known blood type, however, the desired blood-type mix is not stated.

- The following sentence has been added for clarity:

  “Since blood type effects vWF levels, the distribution of blood types should be similar to the blood type distribution of the population tested.”

Section 7

8. The diagnosis of type 2M is reserved for all cases in which there is evidence for an abnormal vWF molecule and the criteria for types 2A, 2B, and 2N are not fulfilled. The demonstration of satellite banding is an example, but not a requirement. As an example, a case in which there is increased affinity of the vWF molecule for its receptor on the platelet, but in which there is normal vWAg multimeric structure, formerly called vWD Malmo. This case would, in the new classification, be called type 2M. Thus, we recommend the following modification:

The diagnosis of type 2M is reserved for all cases in which there is evidence for an abnormal vWF molecule and the criteria for Types 2A, 2B, and 2N are not fulfilled.

- The following sentence has been added, following the third paragraph of this section, to address the commenter's concerns:

  “The diagnosis of type 2M is reserved for cases in which there is evidence for an abnormal vWF molecule (lacking or additional satellite banding and/or molecular biological evidence) and the criteria for Types 2A, 2B, and 2N are not fulfilled.”

9. Paragraph 3 of this section states that the diagnosis of type 2M requires the demonstration of abnormal satellite banding on high-resolution multimeric analysis. I wonder if this statement should be expanded to state that multimeric analysis may be normal (abnormal banding not seen) in type 2M as I believe this is the more typical finding?

- The text has been modified to read, “…type 2M requires demonstration of abnormal satellite banding in high-resolution vWF multimer analysis, however normal banding is the more typical finding.”

10. In Table I, in the classification of 2M, should R:CoF be listed with two “downward” arrows?
• The use of one or two “downward” arrows is essentially arbitrary as R:CoF levels may vary somewhat; therefore, the subcommittee believes a single “downward” arrow is appropriate.

11. In Table 1 under the RIPA column should the "N" associated with type I, 2M and 2N be replaced with the word "none."

• In these types of abnormal vWD, the RIPA functions usually within the normal reference range. They do not have undetectable RIPA activity. Therefore, the designation has been expressed as “N” for “normal.”
Summary of Delegate Comments and Subcommittee Responses

H51-A: Assays of von Willebrand Factor Antigen and Ristocetin Cofactor Activity; Approved Guideline

Sections 5.6 and 6.5

1. There is some concern about group O and nongroup O reference ranges. The scenario will occur in which the value of 80 µ/dL in a group O patient be interpreted as below the reference interval and a value of 45 µ/dL in a group O patient be interpreted as within the reference interval. The clinical situation in the patients may indicate the opposite, that is, that the first patient does not have a clinical bleeding disorder and that the second does. If it is to be recommended that blood group specific reference intervals are to be developed, then there needs to be some statement about the interpretation in the context of the clinical setting. Often, one would say that is the case with all laboratory tests, but this is an area in which clinicians are often unclear about the testing and should be treated that way.

- **Section 5.6 has been modified for clarity.** It reads, "Every result should have an added statement expressing the problems of the variability of values within patients due to the effects of blood type and the acute phase reaction. All results should be interpreted in the context of the clinical setting for each patient."

Section 7

2. The third sentence is contradictory. This sentence states that normal banding is the more typical finding in Type 2M, but that demonstration of abnormal satellite banding is required for diagnosis of Type 2M. Clarification is needed.

- **The text has been modified to read,** "Diagnosis of Type 2M, a very rare type, usually has all multimers present, but may show abnormal bands (i.e., larger than normal bands or other abnormal patterns on high resolution vWF multimer analysis)."
Related NCCLS Publications*

C3-A3 Preparation and Testing of Reagent Water in the Clinical Laboratory; Approved Guideline—Third Edition (1997). This document provides guidelines on water purified for clinical laboratory use; methods for monitoring water quality and testing for specific contaminants; and water system design considerations.

H3-A4 Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture; Approved Standard—Fourth Edition (1998). This document examines methods for the collection of blood specimens by venipuncture and an appropriate training program aimed at increasing analyte integrity and minimizing laboratory error; a 24-step protocol for specimen collection, recommendations for "order of draw," and considerations for performing venipuncture on children are also included.


H30-A2 Procedure for the Determination of Fibrinogen in Plasma; Approved Guideline—Second Edition (2001). A performance guideline for laboratory and/or clinical healthcare professionals responsible for the routine performance of fibrinogen assays. This guideline describes a technique that is practical, precise, and widely used in the clinical laboratory. Preanalytical and analytical factors and conditions that may alter results are discussed.

H47-A One-Stage Prothrombin Time (PT) Test and Activated Partial Thromboplastin Time (APTT) Test; Approved Guideline (1996). This document provides guidelines for performing the PT and APTT tests in the clinical laboratory, for reporting results, and for identifying sources of error.

H48-A Determination of Factor Coagulant Activities; Approved Guideline (1997). A consolidation of Factor VIII and Factor IX assays guidelines, this document addresses the performance, quality control, and reporting of assays for coagulation factor activity based upon conventional APTT and PT coagulation tests.

M29-A2 Protection of Laboratory Workers from Occupationally Acquired Infections; Approved Guideline—Second Edition (2001). This document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; 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.

NRSC8-A Terminology and Definitions For Use in NCCLS Documents; Approved Standard (1998). This document provides standard definitions for use in NCCLS standards and guidelines, and for submitting candidate reference methods and materials to the National Reference System for the Clinical Laboratory (NRSC).