Diagnosis of Hemophilia and Other Bleeding Disorders: A Laboratory Manual

Third edition

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PART 1 General Quality Planning in the Hemostasis Laboratory

Pierre Toulon

TOPICS COVERED

- ✓ Control of Potentially Hazardous Substances to Health
- ✓ Laboratory Safety
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- ✓ Safety Measures–Universal Precautions
- ✓ Laboratory General Equipment
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- ✓ Evaluation and Use of Coagulometers
- ✓ Reagents

Control of Potentially Hazardous Substances to Health: The EN ISO 15189 standard/norm specifies the quality and competence requirements specific to medical biology analysis laboratories. ISO 15189 is intended to be used in all disciplines practiced by medical laboratories. Its application is therefore fundamental for laboratories because their services must meet the needs of both patients and clinicians responsible for the care provided to their patients. Those services include processing requirements, patient preparation and identification, and sample collection, transportation, storage, pre-processing, and analysis, followed by validation of results, their interpretation, reporting, and advice, while ensuring staff safety and respect for ethics.

Laboratory Safety: Laboratories that handle chemicals and biological samples are potentially hazardous places. In recent years, there has been an increasing appreciation of the importance of safe working practices in industry, for both health and environmental reasons. This awareness has led to greater stress on issues such as safety documentation, staff training, and risk assessment. Employers have a responsibility to provide the necessary protective clothing and equipment, and are required to provide training in safe working practices. Implementing such safe working practices should greatly reduce the probability of serious injury to yourself, your colleagues, and members of the public.

Safety Officers: It is important to appoint a safety officer or officers for each department. These people will take on the responsibilities of introducing and maintaining safety procedures. Nevertheless, safety is the responsibility of all staff in the laboratory.

Safety Manual: There should be a comprehensive safety manual that covers all aspects of safe working practices for the whole department. All staff members must read the manual and sign a declaration to indicate that they have understood it. Copies should be kept with the safety officers and also made available in places that are easily accessible to all staff members, either in print, or more preferably, in electronic form, to ensure the latest version is available.

Safety Measures–Universal Precautions: The system of universal precautions requires that any danger of infection from any source will be avoided or minimized by good working practices. All blood samples, blood products (including plasma-based reagents and kits), and other human body materials should be regarded as posing a possible danger of infection. The fullest possible protective measures should always be taken when working with any material. No other classification of risk should be made. All body fluids and materials other than blood, whether collected or brought into the unit for testing or any other purpose, should be handled with the same care as that given to blood.

The laboratory: The laboratory should always be clean and tidy. Paperwork should be kept separate from laboratory testing areas. Try not to use the laboratory for storage of bulk items. Try to ensure that everyone participates in keeping the laboratory orderly.

Protective clothing: Everyone who enters the laboratory, including visitors, should wear a laboratory coat. They should immediately replace the coat if it becomes contaminated.

Disposable gloves: Even though many people do not like to wear gloves, it is recommended to wear latex or polyacrylamide disposable gloves, as every sample handled in the laboratory is potentially hazardous. Gloves should always be worn when handling toxic material. Gloves and coats will obviously not protect against a needle stick-type accident, but they will prevent, for example, any cuts or abrasions on your skin being in contact with HIV positive serum or plasma. It is mandatory to always replace gloves immediately if they are broken or punctured.

Eye washing: Wash your eyes immediately with lots of cold running water if contact with a possible infectious material may have occurred, as many infections can be easily acquired by contact with the mucous membranes of the eyes.

Sharps: Sharps, in the form of needles and broken glass, present a great danger. Use a sharp box capable of containing sharps without being punctured. There have been cases of workers becoming infected as a result of needle stick injuries.

Aerosols: Avoid all practices in the open laboratory that may cause splashing or the release of airborne droplets or dust. Operations that cause aerosols must always be carried out in a suitable fume cupboard, and safety glasses must be worn. All spills should be cleaned up immediately, using bleach or a neutralizing agent as necessary.

Toxic and flammable substances: Toxic or flammable materials must always be contained within a fume cupboard or suitable safe box.

Electrical equipment: Take special care with any equipment that uses liquids, such as electrophoresis tanks and water baths. Always leave installation, servicing, and repairs to qualified personnel.

Personal possessions and behavior: Never take personal items, such as pens, bags, and combs, into the laboratory. Avoid bringing your hands into contact with your face or mucosae (eyes, nose, and mouth) while in the laboratory, but if you must do this, always wash your hands first. Always wash hands thoroughly before leaving the laboratory. Never mouth a pipette. Food, cigarettes, and cosmetics must never be brought into the laboratory. This implies that eating, drinking, and smoking must be avoided in the laboratory.

Accidents: All accidents should be reported immediately and should be recorded in an accident book kept by the unit Safety Officer. This is particularly important in relation to needle stick injuries. In these situations, follow local hospital systems for recording and reporting, along with any locally recommended or mandated actions.

Control of Potentially Hazardous Substances to Health: Laboratories must comply with the local regulation, which often issues useful guide in identifying risks and hazards, such as the Control of Substances Hazardous to Health (COSHH) in UK laboratories.

Hazard and risk: The hazard presented by a substance is its potential to cause harm. The risk from that substance is the likelihood of its harming someone under the actual conditions of use.

Identification of hazards: The identification of hazards is an essential prerequisite of risk assessment. The time spent identifying the hazards will vary according to the substance.

Risk assessment: Consider the following facts:

- Hazards
- Conditions of use
- Amounts to be used
- Likely routes or sites of exposure (inhalation, ingestion, skin, or eyes)

The outcome of the risk assessment will determine:

- Storage conditions
- Handling procedures
- Disposal procedures
- Requirement of monitoring and health surveillance
- Emergency procedures

Risk assessment must be reviewed annually and updated if necessary. An example of how to record information for risk assessments, using the COSHH procedure used in UK laboratories, is shown in Table 1. The purpose of such forms is to identify the hazards and control measures associated with equipment used in a particular procedure. Only staff documented as competent should perform any procedure, and they should perform that procedure only after reviewing the health and safety documentation related to that particular test.

Table 1. Control of potentially hazardous substances to health (COSHH) for prothrombin time and APTT-based clotting factor (F) assays

COSHH Ref. No. Assays 1		**Lab. Ref. One-Stage clotting assays for FII, FV, FVII, FVIII, FIX, FX, FXI and FXII**
Title of Procedure/Experiment:		
Substance	**Approximate quantity**	**Hazard identified**
Glyoxaline (imidazole) buffer, contains (see**)	<5 ml	Harmful if ingested.
**Imidazole	3.4 g/l	Corrosive: causes burns. Harmful if inhaled, ingested, or absorbed through skin. Irritating to eyes.
**Sodium chloride	5.85 g/l	Irritating to eyes and lungs. Avoid skin contact.
Factor-deficient plasma	1 ml	Risk of infection
Thromboplastin	2 ml	Low risk
APTT Reagent	2 ml	Low risk
0.025M calcium chloride	5 ml	Low risk
Owren's buffer	<500 ml	Contains barbitone. Harmful if swallowed. May cause sensitization by contact to skin or inhalation.
Coagulation analyzer wash solution 1	<50 ml	Causes burns: harmful to eyes, skin, etc. Do not mix with other disinfectants. Corrosive. Contact with combustible materials may cause fire. Contact with acid liberates toxic gas. Reacts violently with ammonium salts; organic solvent - explosive risk.
Coagulation analyzer wash solution 2	<50 ml	Contains 0.16% hydrochloric acid and detergent. Irritant: may harm eyes and skin.
Standard/control/patient plasma	<1000 µl	Risk of infection.

Laboratory General Equipment: Any laboratory involved in the diagnosis and treatment monitoring of bleeding disorders employing some or all the techniques described in this manual will require a minimum of basic equipment.

General equipment: The basic equipment requirements are:

1) A 4°C refrigerator for reagent storage. Reagents should normally be maintained at 2–8°C unless otherwise stated by the manufacturer. A good-quality domestic-grade unit may be adequate.
2) A deep freezer able to maintain at least -20°C (preferably -35°C). A lower temperature, such as -70°C, is useful for more prolonged storage, as clotting factors are stable at this temperature for at least 6 months. Freezers with an auto defrost cycle are completely unsuitable.
3) Regulated water bath(s) capable of maintaining temperatures of 37 ± 0.5°C. Temperature is normally better maintained in a water bath than in dry hot blocks, which may or may not be suitable, depending on the unit.
4) A pH meter.
5) A light source.
6) Stopwatch (es).
7) Calibrated automatic pipettes capable of accurate and precise delivery of sample and reagent volume in the range of 0 µl–200 µl and up to 1000 µl. It is important to check the accuracy of these pipettes regularly.
8) A calibrated pipette for delivery of liquid volumes up to 5 ml.
9) A centrifuge capable of generating at least 1700g, and preferably 2200 to 2500g. For most coagulation analyses, centrifugation at room temperature (20–25°C) is acceptable, even though centrifugation at 4°C is recommended in some techniques.
10) A calibrated analytical weighing scale/balance capable of accurate measurement of grams to three decimal places.

Additional equipment is required for some procedures, including:

11) A coagulation analyzer (coagulometer).
12) A microplate reader for enzyme-linked immunosorbent assays (ELISAs).
13) A platelet aggregometer. Equipment specified on particular method sheets.

Air conditioning in each room is a great advantage in countries where temperatures are high.

There should be an adequate supply of consumables. Reusing laboratory test tubes and pipette tips after washing should be avoided, since residual material can adversely affect results, causing wastage of reagents and time. The same applies to collection tubes, which are designed for single use and must not be reused even after extensive washing.

Metrology: To aid quality management, pipette volume and balance calibrations should be checked on a regular basis, such as every 3–6 months. Apparatus significantly out of calibration should be immediately removed from use until recalibration has been done. All pipettes should carry a unique identifier.

Method for checking pipette calibration: Pipettes may be for a single volume, for two or three volumes, or have a continuous range of volumes.

- Pipettes with one or two fixed settings are checked at each setting.
- Pipettes with three fixed settings are checked at minimum and maximum setting.
- Pipettes with a continuous range of volume settings: check the maximum setting as well as a volume of around 25% of the maximum setting. That is:
  - 10 ml pipette – 10 ml and 2.5 ml
  - 5 ml pipette – 5 ml and 1.25 ml
  - 1 ml pipette – 1 ml (1000 µl) and 0.25 ml (250 µl)

- 0.2 ml pipette – 0.2 ml (200 µl) and 0.05 ml (50 µl)
- 0.1 ml pipette – 0.1 ml (100 µl) and 0.025 ml (25 µl)
- 50 µl pipette – 50 µl and 15 µl

Check calibration by weighing five replicate volumes of distilled water (at room temperature) on a balance. Each weight is recorded in grams (with three decimal places). For practical purposes, 1.000 ml distilled water weighs 1.000 g.

Results and any action taken should be recorded. Pipettes should preferably be accurate within significantly less than 10% (see examples below). When a pipette is shown to be inaccurate because the mean pipetted volume differs by more than 10% from stated volume, it must be taken out of use immediately and not used until re-calibrated following manufacturer's instructions.

Note: If a pipette is inaccurate beyond the following limits (mean weight), it must be taken out of use immediately.

10 ml pipette
10 ml: 9.000 – 11.000 g
2.5 ml: 2.250 – 2.750 g

5 ml pipette
5 ml: 4.500 – 5.500 g
1.25 ml: 1.125 – 1.375 g

1 ml pipette
1 ml: 0.900 – 1.100 g
0.25 ml: 0.225 – 0.275 g

0.2 ml pipette
0.2 ml: 0.180 – 0.220 g
0.05 ml: 0.045 – 0.055 g

0.1 ml pipette
0.1 ml: 0.090 – 0.110 g
025 ml: 0.225 – 0.0275 g

50 µl pipette
50 µl: 0.045 – 0.055 g
15 µl: 0.013 – 0.165 g

For additional information, see ISO 8655-2-2002 document, available in different languages.

Method for checking balances: To ensure their accuracy, calibrated weights are weighed at six-month intervals, and the values recorded.

1) Zero the balance.
2) Weigh three calibrated weights, one at a time. Record the weights to three decimal places (e.g. 1.003 g).
3) If any weights are outside the stated limits (by >2%), remove them from use until the problem is fixed.

Method for checking temperature of refrigerated enclosures: Internal temperature in the refrigerators must be kept at +4°C (usually in the range from +2°C to +7°C) using a temperature probe, and should ideally be recorded constantly, either using a local printed disk or electronically. The same applies to freezers, which must keep at temperatures of -20°C, -35°C, or even below -70°C.

Evaluation and Use of Coagulometers: Automation in coagulation laboratories is now in widespread use in most parts of the world. It has contributed to improvements in standardization and facilitating tests that demand specific training and special working conditions, so that laboratories may improve their efficiency and repertoire. Automation in hemostasis is relatively recent. Manual methods based on visual detection of the fibrin clot and using incubators at 37°C were once the only techniques for coagulation studies. Then, in the 1970s, new semi-automatic equipment appeared based on photometric or mechanical principles to detect fibrin clots. More recently, fully automated instruments have become common in modern laboratories. New equipment connected to laboratory information systems, usually including specific data processing systems, can perform clotting, chromogenic, and immunological tests.

Two main methodologies are available today, which are based on mechanical and optical detection systems. Mechanical systems only allow performing clotting assays, whereas optical systems allow performing chronometric, chromogenic, and immunological assays based on photo-optical, nephelometric, chromogenic, and immunological principles. In addition, fluorescence- and chemiluminescence-based analyzers are becoming available on the market, allowing specific assays with a wide range of measurements to be performed.

Mechanical principle: Electromagnetic methods are based on the detection of an increase in plasma viscosity when fibrin is formed. Two variations to this principle are applied to laboratory equipment today.

The first uses an electromagnetic field applied to test cuvettes that detect movement within a stainless steel sphere placed in the plasma sample. The steel sphere follows a pendulum movement, swinging from one side to the other in a plasma reagent solution with a constant movement. As the fibrin begins to form, viscosity increases, and the sphere's movement is delayed. When the sphere's oscillation movement reaches a predetermined level, the chronometer stops, indicating the time of plasma coagulation.

A second mechanical detection method also uses a stainless steel sphere, located this time in a single point slot. A magnetic sensor detects the sphere's position, and as it rotates, the sphere maintains its inclination while the liquid sample remains fluid. When fibrin is formed, the clot captures the sphere, moving it from its original position. As it moves outside the sensor's range, the circuit is interrupted and the chronometer stops.

Optical or spectrophotometric principles:

Photo-optical principle: Optical systems are based on the notion that clot formation induces change in the plasma's optical density. As the clot is formed, there are changes in the optical characteristics from the initial reading of the plasma/reagents. These changes are monitored and used to derive the time taken for a particular degree of change to occur.

Nephelometric principle: The nephelometric principle is employed by some systems. In coagulation assays, a monochromatic laser light source is transmitted, for example, by fiber optics. The light dispersion readings are made possible by a sensor that may be installed at 90 or 180 degrees from the light path, depending on the system, which then measures scattered light at an angle or records the change in light transmission. When the light reaches insoluble complexes such as fibrin fibers, it disperses in forward scattered angles (180 degrees) and lateral scattered angles (90 degrees). The chronometer stops when the amount of scattered light or transmitted light reaches a specific predetermined level. The difference between light scattered or transmitted before and after the clot formation is normally proportional to the amount of fibrin formed.

Chromogenic principle: This is based on the use of a color-specific generating substance known as chromophore, of which para-nitroaniline (pNA) is the most common. It has a maximum absorbance at 405 nm. The principle of chromogenic testing resides in adherence of pNA to synthetic substrates. pNA is attached to a series of amino acids that mimics the target sequence of the activated coagulation factor we want to determine. The coagulation protein cleaves the chromogenic substrate at a specific site between a defined amino acid sequence and releases the pNA. The intensity of the yellow color is proportional to the amount of pNA released. This is measured by photo detection at 405 nm wavelength. As more pNA is cleaved and freed, the absorbance capacity of the sample increases, which leads to greater change in the solution's optical density. The first coagulation equipment could only provide a single definition parameter, such as a mechanical or photo-optical one. The photo-optical tools were initially designed for reading at a single wavelength (for example, 500 nm or 600 nm) that could only be used for the detection of clot formation. More recently, some coagulometers can read at two or more wavelengths, often including 405 nm, thereby increasing the capacity for newer reactions (chromogenic substrate methods). In the 1990s,

a number of manufacturers successfully included multiple detection methods, which now give a single laboratory the possibility of using the same equipment for different methodologies.

Immunological principle: Latex microparticles coated with a specific antibody are generally used against the analyte (antigen) being measured. A beam of monochromatic light goes through a latex microparticle suspension. When the wavelength is greater than the suspension particle diameter, the particles absorb a small amount of light. Yet, when the specific antigen-coated latex microparticles come in contact with the antigen present in the solution, they adhere to the antibody, forming links between the particles, which produces agglutination. When the particles' diameter approaches the wavelength of the monochromatic light beam, a greater amount of light is absorbed. This increase in light absorbance is proportional to the agglutination, which, in turn, is proportional to the amount of the antigen present in the sample. This type of technology is available in more sophisticated coagulation analyzers introduced in the market in the 1990s. Usually time-consuming standard immunological assays can be performed in minutes when using any of these automated tools.

Table 2. Advantages and disadvantages of detection methods in defining parameters

Method	Advantages	Disadvantages
Mechanical	No interference due to physical characteristics such as lipemia or icterus May use small sample volumes	Impossible to observe graphics of clot formation May present problems of endpoint detection in some samples with low fibrinogen
Photo-optic	Possibility of graphics on clot formation Optical checks for hemolysis/lipemia/icterus on some optical systems May use small sample volumes	Interference due to lipemia, hemolysis, hyperbilirubinemia, or protein increase on some systems Some systems may present difficulties with clot detection when using some completely transparent reagents Very short coagulation periods may go undetected owing to delay prior to initiation of monitoring
Nephelometric	Can measure antigen-antibody reactions in proteins present in very small amounts	Limits number of available tests Cost of reagents
Chromogenic	Fully specific assays may be easier Additional parameters not suitable for measurement by clot detection may be possible Increases the repertoire of possible tests Possible improvements in precision compared to clot-based analyses	Limited by the instrument's wavelength Requires large test volumes for positive cost-benefit ratio Cost of instruments and reagents
Immunological	Can automate time-consuming, manual methods Increases the number of possible tests	Limited number of tests available Cost of instruments Cost of reagents

Advantages of automation in the coagulation laboratory:

1) Improves the capacity and flexibility of professional time spent (Rodak, 1995).
2) Improves the performance of the tests. In the past, manual coagulation tests were inaccurate, with variation coefficients greater than 20%; the semi-automatic equipment provided greater accuracy in coagulation testing. However, with manual dispatch of samples and reagents, testing had to be done in duplicate. With fully automated equipment, accuracy improved, attaining

variation coefficients of less than 5%, and even 1% for some tests. This has led authors to introduce the notion of single tests and the possibility of reducing reagent costs and cuvettes by half.

3) Reduces cost in samples and reagents, by allowing the use of lower volumes of plasma and reagents (at least half).
4) Facilitates data storage and recovery systems by means of computer programs.
5) Allows automatic replay of results when mistakes are made in the first run.
6) Offers the possibility of running different tests using a single sample.
7) Permits sampling from a closed tube (so-called "cap-piercing"), which improves safety and efficiency in coagulation tests. This reduces, largely, the possibility of exposing the operator to sprays or patient sample spills, or mistakes in labelling. Anecdotally, one manufacturer offered a patented screening system that automatically separates plasma from erythrocytes before tests without previous centrifugation.
8) Provides capacity to dilute samples, calibrators, and controls. The equipment can be programmed for additional dilutions if the initial results escape the method's linearity. It can also automatically carry out other tests without the operator's intervention if clinically indicated or because of initial run results.
9) Most analyzers include alarm systems that warn the operator of readings in excess of pre-established limits, which may identify equipment problems (e.g. small amount of reagent, temperature failure, too small sample volume, and quality control errors), as well as pre-analytical errors (under-filled tubes, hemolysis, icterus, lipemia, and presence of clots).

The different methodological types available have advantages and disadvantages that should be known and understood to guarantee precision and validity of test results. It is important to consider that laboratories are responsible for trustworthy results. A laboratory's main concern is to select the coagulation equipment that will generate appropriate results in spite of budget restraints. Such instruments demand regular technical maintenance, permanent knowledge, and system control, since a mistake or failure may decisively influence a number of results. Control systems that guarantee analytical confidence are therefore compulsory.

Many laboratories may be fortunate enough to be able to evaluate equipment before purchasing. If this is not possible, it is very important to obtain adequate information and advice from a reference laboratory in addition to the review of the literature.

When evaluating new equipment before purchase, first compare analyzers according to criteria such as:

- equipment and maintenance costs
- inactivity period and reliability
- repair response time
- ease of use
- availability of adequate maintenance within an appropriate timeframe
- validation process and throughput
- cost of disposable elements
- flexibility in using reagents from other manufacturers
- possibility of adding new tests protocols
- ability and cost of connection with the laboratory information system
- training courses and continuous training support

The sensitivity of different types of equipment to multiple parameters will differ depending on how the machines are calibrated and how endpoints are detected. Laboratories have different needs, and it is advisable to rank priorities. For example, see Table 3.

Table 3. Characteristics of specialized equipment (adapted from Rodak, 1995)

Characteristics	Description
Random access	With patients' sample, various different tests are possible in any order and at the same time.
Sample primary tube	Plasma sample is directly taken by aspiration in an opened collection tube placed in the analyzer.
Penetrating plug and closed sampling tube	The analyzer vacuums the plasma sample within the collection tube with the rubber plug in place.
Barcode	Allows identification of reagent, patient samples, or both by means of a barcode. This reduces manual data entry.
Bidirectional inter-phase	The analyzer queries a centralized computer to determine the requested number of tests. The operator does not need to manually program the information in the equipment.
Sample indicator	Warns the operator of problems with sample integrity.
Liquid level sensor	Warns the operator of insufficient sample or reagent volume for adequate testing, or if the equipment did not vacuum enough from sample to perform the requested test.
Integrated quality control programs	Instrument's computer program stores and organizes quality control data. It may include the complete application of Westgard rules to indicate off-limit results.
STAT capacities	Allows the operator to cancel the test verification sequence in order to place a new STAT sample in the verification isle.
Refrigeration capacity of integrated samples	Preserves the integrity of samples, reagent, or both during the verification process.
Storage capacity of integrated samples	Indicates the amount of patient sample that can be loaded in the analyzer at any given time.
Reflex testing capacity	Makes it possible to program the equipment to repeat or add tests under specific parameters set by the operator.
Patient data storage	Analyzer capacity to store test results that can be recalled at any given moment. May store clot formation curves.
Reagent volume monitoring	Warns the operator of insufficient reagent for programmed tests.
Processing	Number of tests that can be processed within a given period (generally classified as number of tests per hour).
Clot formation curve	Allow the operator to visualize the clot formation within the cuvette. Helps detect certain unruly conditions or morbid states, or the location and solution of deviant test result failures.
Pre-analytical checks	Detection of under-filled tubes, hemolysis, icterus, lipemia, clot.

Technology is on the rise and growing daily demands generate the need for instruments of this nature in the laboratory. They will constitute a great step forward in the lab field, given the possibility of undertaking tests in a reliable, accurate, and precise manner, and delivering results more quickly (shorter turn-around time) and under better control. The advantages of automation are numerous. Technology is continuously advancing to meet new developments in the field and to reduce turnaround times, allowing tests to be reliable, accurate, and precise, while maintaining quality.

Reagents: In addition to specific reagents dedicated to specific assays, which will be detailed in the corresponding chapters, some reagents are widely used in the hemostasis laboratory (e.g. calcium chloride solution, various buffers). They can be purchased from reagent manufacturers or locally prepared from bulk reagents or concentrated solutions.

25 mM calcium chloride solution: For example, if a molar solution is purchased, to obtain a 25mM solution, dilute 25 ml 1M solution to 1 liter in volumetric flask with distilled water.

Buffers:

- Owren's barbiturate buffer pH 7.35

Weigh 5.875 g sodium diethylbarbiturate (barbitone sodium) and 7.335 g sodium chloride.

Place in a volumetric flask and dissolve in approximately 780 ml distilled water.

Add 215 ml 0.1M hydrochloric acid.

Adjust volume to 1 liter with distilled water.

Check pH and adjust to pH 7.35, if necessary.

- Owren's buffered saline

200 ml Owren's barbiturate buffer (see above).

Add 800 ml normal saline (0.9% sodium chloride).

- Imidazole (glyoxaline) buffer

Weigh 2.72 g imidazole (glyoxaline) and 4.68 g sodium chloride.

Place in volumetric flask and dissolve in approximately 650 ml distilled water.

Add 148.8 ml 0.1M HCl and adjust pH to 7.3.

Adjust volume to 1 liter with distilled water, if necessary.

Reagents for coagulation screening tests: In the initial stages of investigation and diagnosis of bleeding disorders, selection and application of suitable screening test reagents, particularly for prothrombin time (PT) and activated partial thromboplastin time (APTT) tests, are of great importance. Many different reagents are available throughout the world. Where a wide choice is available, selection should take into account the variation in sensitivity. In screening for a bleeding disorder by PT and APTT, the following sources of information in relation to the likely performance of a particular reagent can be considered:

- Comparative data in relation to other reagents from external quality assessment (EQA) schemes, such as the International EQA scheme
- Published data
- Local testing of plasma from patients with known defects
- Manufacturers' data sheets

Local production of PT and APTT reagents may be financially attractive, but can cause standardization difficulties, and therefore must be avoided. It should also be noted that some manufacturers offer different reagents. In addition, the composition of reagents bearing the same name may be altered from time to time. This means that recommendations for the use of a particular source of material cannot be given.

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PART 2 Hemostasis Test Validation, Reference Intervals, and Performance

Silmara Montalvão

TOPICS COVERED

- ✓ Method for Preparation of PNP
- ✓ How to Validate Coagulation Equipment and Tests
- ✓ Samples for Equipment/Reagent Validation Processes
- ✓ Validation or Verification
- ✓ Precision and Accuracy
- ✓ Establishing a Reference Interval
- ✓ Validation Versus Verification of Reference Interval
- ✓ Statistical Analysis of Reference Interval
- ✓ Internal Quality Control and External Quality Assessment

Pooled normal plasma (PNP) is an essential component for the hemostasis laboratory, as it is used in different testing protocols, from evaluating a prolonged APTT to evaluating specific and non-specific inhibitors. It can also be used as reference material for calibration and normal control if ideal conditions are observed for this purpose. The following is a protocol for how PNP should be prepared.

Table 4. Requirements for the preparation of PNP

Donors	Minimum 20 normal healthy individuals not taking medications that interfere with clotting factors and coagulation reaction. It is acceptable to include women taking oral contraceptives. An approximately equal number of males and females is desirable. The age range should be 20 to 50 years.
Anticoagulant	0.109 M (3.2%) trisodium citrate dihydrate buffered with N-2-hydroxyethylpiperazine. N-2-ethanesulphonic acid (HEPES) at 5 g per 100 ml trisodium citrate.
Collection	Donors are bled between 9:00 a.m. and 11:00 a.m. using 60 ml plastic disposable syringes and 21-gauge butterfly needles.

Method for Preparation of PNP:

- ✓ Collect 54 ml blood and mix with 6 ml anticoagulant in plastic containers.
- ✓ Store sample on melting ice during preparation of pool.
- ✓ Centrifuge at 4°C for 15 minutes at 2500g.
- ✓ Pool plasma in plastic non-contact container.
- ✓ Aliquot in 1.5 ml plastic vials in 0.5 ml aliquots.
- ✓ Snap freeze on dry ice/solid CO_2 if available. Alternatively, place immediately on an open shelf at -70°C.
- ✓ Complete the above procedure within four hours.
- ✓ Stable at -70°C for > six months.

A PNP prepared in this way will have levels of factor II (FII), factor V (FV), factor VII (FVII), factor IX (FIX), factor X (FX), factor XI (FXI), factor XII (FXII), high-molecular-weight kininogen (HMWK), and prekallikrien (PKK) of around 1 U/ ml or 100 U/dl, although the levels of FVIII and von Willebrand factor (VWF) vary widely in different pools of PNP. A local PNP should be calibrated in International Units (IU), since international standards are now available for all the above-mentioned clotting factors, except for FXII. The pool can be used uncalibrated with an assumed potency of 100 U/dl or 1 U/ml for FXII. To calibrate in IU, it is

necessary either to obtain calibrated World Health Organisation (WHO) reference preparations (which are held at the National Institute for Biological Standards and Control, South Mimms, Potters Bar, Herts, U.K.) or to purchase suitable commercial reference plasma that has been calibrated in IU by the manufacturer. Consideration should be given to replacing such a plasma pool every 12 to 18 months, unless there is evidence from internal quality control (IQC) results that stability has been maintained.

Method for calibration of local PNP:

- ✓ Obtain a calibrated standard, such as WHO international standard (IS) (minimum two vials).
- ✓ On two different days, use one vial of IS and four aliquots of local PNP.
- ✓ On day one test IS, local, local, local, local, IS, and repeat this using fresh dilutions of each plasma.
- ✓ On day two test local, local, IS, IS, local, local, local, and repeat this using fresh dilutions of each plasma.

Calculate potency of each aliquot of local standard against average of results with the two IS.

- ✓ The mean result of 4 aliquots x 2 dilutions x 2 days (n = 16) is assigned to the local standard as its potency.

How to Validate Coagulation Equipment and Tests: Before a new method is used, it is essential that it be evaluated for its suitability for the intended purpose. This chapter provides a general recommendation on how to plan and carry out the processes required for the selection and evaluation of hemostasis analyzers/testing systems. These recommendations are not intended to replace regulations or standards, but rather to provide guidance on the steps necessary to meet good laboratory practice.

The extent of the evaluation of a test system will depend on several factors, including: (a) the intended use of the test system, (b) whether the test system has been deemed approved for clinical use by regional bodies or statutes, (c) the available resources of the laboratory. When choosing a test system, research should be carried out to determine which hemostasis analyzers are available. A list of requirements should be drawn up to identify the best system for your laboratory, detailing the physical characteristics of the laboratory as well as the required performance of the equipment. Validation planning is an important stage and is related to the results obtained since good planning can generate a technically adequate validation. To this end, it is important to estimate a realistic time frame for the evaluation, based on the resources available and the scope of the evaluation. It is necessary to define the details of the assessment process (in stages), which must be reviewed and approved by the person in charge of the sector. The validation plan should detail the evaluation parameter (e.g. imprecision), the test(s) to be performed and the desired result (e.g. statistical limits). As some test systems may require unusual samples or those covering a wide range of values, it may be advisable to start collecting and freezing samples weeks or even months in advance. The quantities of consumables (including reagents) required for the evaluation of the test system should be estimated with room for contingency plans in case additional work is required. The laboratory must document each stage of the evaluation process including preventive instrument maintenance, temperature evaluations, and data generated in the validation or verification stages. The laboratory manager must analyze the data and record the result of the analysis. It is advisable to have a dedicated and labelled digital record folder for the validation and evaluation of the system. Regardless, any form of test system evaluation should be readily available for any need to verify laboratory suitability.

Samples for Equipment/Reagent Validation Processes: The sample processing used in the validation process should be the same as for patient test samples. For the PT test, studies show that the stability of processed plasma is 24 hours when kept at room temperature, however, the faster it is processed, the better the quality assurance. Plasma samples for other tests must be tested within 4 hours of collection. If samples cannot be tested within acceptable stability limits, platelet-poor plasma (PPP) samples should be produced, aliquoted, and stored frozen at -70°C (Favaloro et al, 2008). Before analysis, frozen samples

must be thawed at 37°C (3 to 5 minutes for aliquots of up to 1 ml) and mixed immediately before testing (Kitchen et al, 2021).

Validation or Verification: In general, validation is a process that must be carried out on completely new test systems or a test developed in the laboratory. Verification, on the other hand, is a process that can be applied to already validated test systems that have recently been introduced to the diagnostics market. Verification can also be applied to evaluation after equipment has been relocated. If a test system has been cleared by the regional regulatory authority, only the verification process of the test system can be carried out locally. In this case, verification can be defined as providing objective evidence that a given test system locally meets the specifications set by the manufacturer. Deviation from the manufacturer's instructions for use for a test system will require follow up for system validation (Castellone, 2017).

Precision and Accuracy: After installing the equipment, it is important to assess the degree of intra-run imprecision of the tests to be evaluated. This practice is useful for identifying outliers and variability in the measurement system. The number of tests required for an evaluation will depend on whether the test system(s) require validation or verification (Gardiner et al, 2021a). For accuracy studies, the samples tested will depend on the type of patient samples expected to be tested in the lab. Accuracy is an important assessment, defined as the agreement between a measurement and the true value. Accuracy is usually carried out by comparing new instruments or reagent systems with an existing or predetermined method (Eusebi, 2013). The comparison between systems should be carried out using statistical analysis. Comparability between systems can be analyzed using linear regression (normal, weighted, Deming, or Passing-Bablok, as appropriate), Bland Altman trend graphs, and paired t-tests (or Mann Whitney U-tests if the data is not normally distributed) (Jensen and Kjelgaard-Hansen, 2006). The acceptability criteria will be specific to the test. For the APTT test, for example, two different test systems using different reagents could generate results with differences that could be clinically significant (Montalvão et al, 2020). Assessments of the sensitivity, specificity, and negative and positive predictive value of the test are also relevant and fundamental information for some tests. Calibrated assays for which there is an international standard (e.g. FVIII) and standardized methodology, should produce a regression line with a slope close to 0.90–1.10, with a strong correlation ($r > 0.95$) and no clinically significant bias (Gardiner et al, 2021b). The exact number of samples for the statistical evaluation of the verification/validation process will depend on the acceptability criteria for each test. Participating in an EQA program can be useful for establishing the accuracy of the system before putting it into a patient routine (Montalvão et al, 2022).

Establishing a Reference Interval: Establishing a normal reference interval is one of the most important tasks carried out in the laboratory, as most medical decisions are made based on laboratory results. Coagulation tests present a unique set of challenges. The reagents used in routine tests can have different sensitivities to coagulation based on the concentration and type of phospholipid and activators. A classic example of this is reagents used for the APTT test, which use particles (e.g. kaolin, cellite, silica) or chemicals (e.g. ellagic acid) that directly affect the sensitivity and specificity of the test. In this context, the different particles, as well as the phospholipid class and fatty acid composition, are not standardized. Therefore, reagents from different suppliers may have different compositions. All these properties of the reagents need to be considered when establishing a normal reference interval. Therefore, it is essential for the laboratory to carry out a local determination of the normal reference range so that the set of reagents, equipment, and procedures can be taken into account when assessing the patient. Health is not a well-defined condition and is often a relative term. The ideal group in some cases could be closely matched with the population under investigation with respect to age and sex. However, such careful selection is not essential for many coagulation tests. In practice, the selection of healthy normal subjects for establishment of a normal range will be influenced by practical considerations. Healthy hospital employees who do not receive any medication and healthy blood donors can be successfully used. There are important considerations in relation to normal ranges, which are given below. The condition of the normal subjects when blood is collected can influence the results obtained. This includes a review of the evidence for the effects of physical stress (e.g. up to 10-hour persistence of a 2.5-fold increase in FVIII/VWF), mental stress (e.g. increase in FVIII

and VWF after acute mental stress), hormone effects, circadian variations, and the effects of posture and diet. Some general recommendations were made, which were not restricted to investigation of female patients. These were as follows:

- ✓ Abstain from intense physical exercise for 24 hours prior to venipuncture.
- ✓ Use an environment where physical and mental stress are reduced.
- ✓ Abstain from fatty foods and smoking on the morning of venipuncture.
- ✓ Obtain samples early in the morning (7 a.m. to 9 a.m.), after the subject has been seated in a relaxed position for 20 to 30 minutes.

Validation Versus Verification of Reference Interval: The results of the reference interval are statistically evaluated, and the type of statistical evaluation is based on the number of individuals used. The process can include validation or full verification, where the reference interval is previously established. Validation requires a study of a minimum of 120 individuals, while verification of a reference range requires only 20 individuals to demonstrate that a test performs as previously established. The reference interval must be verified with any change of reagent, lot number, and instrument or collection system. The mean and standard deviation (SD) can be calculated (Gardiner et al, 2021a).

Statistical Analysis of Reference Interval: The SD is the dispersion of the data around the mean. The more dispersed the data, the greater the deviation. The confidence interval measures the level of uncertainty. If the 95% confidence level is chosen, the intervals will be estimated at the 2.5th and 97.5th percentiles of the distribution of results. This ensures that your confidence interval of values contains the true average of 95% of the population. Higher confidence levels will have wider reference intervals, while lower confidence intervals are narrower (Henny et al, 2016).

Different statistical methods can be used to evaluate data:

(1) Parametric method: is used when the population distribution is normal or Gaussian.

(2) Non-parametric method: does not require probability laws due to careful selection of subjects and a sufficient number of (≥120) individuals tested.

(3) Robust method: for use on a limited number of individuals without requiring the distribution to be Gaussian and measures position (location) and dispersion (spread) instead of the mean and standard deviation.

Position meters sort the data from smallest to largest in equal parts, while dispersion analyses the distance between the values of the distribution are from the center. To check for outliers, the data can be visually inspected and evaluated in a method proposed by Dixon (Henny et al, 2016). With a sample size of 20 (reference interval check), two outliers are allowed. If there are more than two outliers, an additional 20 samples should be tested. If there are another two outliers, other sources of error, such as reagents, analyzer problems, or biological variation, should be investigated (Henny et al, 2016). It may be necessary to carry out a full reference interval.

Internal Quality Control and External Quality Assessment: Quality assurance (QA) is an overall term that may be used to describe all measures taken to ensure the reliability of laboratory testing and reporting. This includes the choice of test, the collection of a valid sample from the patient, analysis of the specimen and the recording of results in a timely and accurate manner, through to interpretation of the results, where appropriate, and communication of these results to the referring clinicians. IQC and external quality assessment (EQA) (sometimes referred to as proficiency testing) are two distinct, yet complementary, components of a laboratory quality assurance program. IQC is used to establish whether a series of techniques and procedures are performing consistently over a period. It is therefore deployed to ensure day-to-day laboratory consistency. EQA is used to identify the degree of agreement between one laboratory's results and those obtained by other centers.

Internal quality control: IQC is used to establish whether a series of techniques and procedures perform consistently over time. The expression "quality control" is commonly used to describe the set of procedures used to check that the results of laboratory investigations are reliable enough to be released to assist clinical decision making, monitoring of therapy, and diagnosis of hemostatic abnormalities. Quality control procedures should be applied in a way that ensures immediate and constant control of results generation.

Within a laboratory setting, the quality of results obtained is influenced by many factors, including:

- Appropriate sample collection and handling
- Selection of suitable techniques and maintenance of an up-to-date manual of standard operational procedures
- Use of reliable reagents and reference materials
- Selection of suitable automation and adequate maintenance
- Adequate records
- Reporting system for results

In addition, the quality of results obtained in routine practice is highly dependent on the selection, training, and motivation of an appropriate complement of suitable personnel.

IQC is particularly useful to identify the degree of precision of a particular technique—precision being the degree of agreement among repeat measurements on one sample. To ensure that the results obtained in the laboratory are reliable, they must be precise in their analyses. IQC guarantees the daily consistency of an analytical process and thus helps determine whether patient results are reliable enough to be released. An IQC program should refer to institutions that support laboratory quality guidelines, such as International Organisation for Standardisation (ISO) or Clinical and Laboratory Standards Institute (CLSI). Laboratory tests must be able to identify physiological and pathological results regardless of when the test is carried out. In the ISO 15189:2022 recommendations, the laboratory shall have an IQC procedure for monitoring the ongoing validity of examination results, according to defined criteria, that verifies the attainment of the intended quality and ensures consistent validity pertinent to clinical decision making. IQC shall be performed at a frequency that is based on the stability and robustness of the examination method and the risk of harm to the patient from an erroneous result. In CLSI H47-A2 Vol 28 No 207.8, for all non-manual coagulation test systems, minimally, the laboratory must include at least two levels of control material for every 8 hours of operation and each time a reagent is changed.

Internal quality control materials: To assess the precision of a particular method, it is necessary to perform repeated analyses of aliquots of the same sample. It is important to include quality control (QC) samples with normal and abnormal values to ensure that a method is under control at different levels of a particular analyte, since relatively minor changes in an analytical process may be more apparent when testing an abnormal control. The control material should be similar in properties to test samples and be analyzed concurrently. Quality control materials of human origin are more likely to closely resemble human test samples. All vials or aliquots of the control material should be practically identical, so that any variation in test results is not a consequence of vial-to-vial variation. The QC material should also be stable for its intended period of use. With respect to hemostatic tests and assays, plasma samples must be deep frozen (preferably at -35°C or lower) or lyophilized in order ensure adequate stability for use as QC material. For reconstitution of lyophilized samples, it is important to use distilled water with pH 6.8–7.2 and to allow at least five minutes for reconstitution. If commercial QC material is used, this should be reconstituted according to manufacturer's instructions using an accurate pipetting system. If deep frozen QC material is used, this should be thawed rapidly at 37°C for five minutes. In the selection of QC material, the risk of transmission of blood-borne viruses should be considered. High-risk material should not be used. At least one QC material should be included with each group of screening tests or assays. For screening tests, it may be most appropriate to include a normal QC in this way and to test abnormal QC materials once per day or shift, or when doubt exists about whether a method is under control. A QC material with a reduced level should be included with tests used for the diagnosis and monitoring of congenital

deficiency states associated with bleeding. In all cases, the control material must be treated exactly like test samples, if possible

Acceptable limits of variation: For commercial IQC, samples manufacturers often provide a target range of acceptable values. In the case of screening tests and occasional assays, the results obtained will depend on the reagents and endpoint detection system used to perform the tests. The target range must take these effects into account. Where a target range is not available for a particular technique, this can be established locally. The IQC material is tested repeatedly (minimum 10 times) on different days when the method is known to be under control (as indicated, for example, by within target results on an alternative QC material). The mean and SD of these results are then calculated. The SD is the square root of the sum of d2 divided by n-1, where d is the difference of individual results from the mean and n is the number of determinations. The SD is a measure of the spread of results; the larger the SD, the greater the spread of results. Another important parameter is the coefficient of variation (CV), which is the SD expressed as a percentage of the mean (CV = SD divided by mean, multiplied by 100%). The CV of results on different days for PT and APTT of a QC sample should always be less than 8%, and preferably lower. For assays such as FVIII:C and FIX, CVs of less than 10% should be attainable for tests performed over several days. In most cases, results obtained for an IQC sample will show a normal (Gaussian) distribution. It is common practice to set the target range for IQC results as the mean +/- 2 SD, since this should include 95% of values. Individual results should be recorded on a chart that identifies the target range. Results outside this range indicate that the QC material has deteriorated or been handled incorrectly, or that the method is not properly controlled. Repeat testing of further QC material will then differentiate between these two possibilities, with further out-of-limit results confirming that the test system is out of control.

External quality assessment: In large EQA schemes, retrospective analysis of results obtained by participating laboratories permits the identification, not only of poor individual laboratory performance, but also of reagents and methods that produce unreliable or misleading results. The primary function of EQA is proficiency testing of individual laboratory testing. The World Federation of Hemophilia (WFH) International External Quality Assessment Scheme (IEQAS) includes analyses of particular relevance to the diagnosis and management of bleeding disorders (for further information, contact the WFH). Data from this scheme have been published in the following references:

- Jennings I, Kitchen S, Woods TAL, Preston FE. Development of a World Federation of Hemophilia External Quality Assessment Scheme: results of a pilot study. *Haemophilia* 1996; 2: 4–46.
- Jennings I, Kitchen S, Woods TAL, Preston FE. Laboratory performance of haemophilia centres in developing countries: 3 years' experience of the World Federation of Hemophilia External Quality Assessment Scheme. *Haemophilia* 1998; 4: 739-746.
- Jennings I, Kitchen DP, Woods TA, Kitchen S, Walker ID, Preston FE. Laboratory performance in the WFH EQA programme 2003-2008. *Haemophilia*. 2009; 15:571-7.
- Silmara Montalvão, Ian Jennings, Christopher Reilly-Stitt, Dianne Kitchen, Steve Kitchen. Quality of diagnosis and lab monitoring of people with hemophilia and other bleeding disorders across the continents: WFH IEQAS programme 2016-2023.

The WFH IEQAS was launched in 2004 to monitor and improve laboratory performance in hemophilia treatment centers (HTCs) worldwide. Laboratories can participate in this scheme to assess their quality assurance systems and the reliability of their test results. IEQAS improves and standardizes laboratory diagnosis by auditing the effectiveness of the internal quality assurance systems in place and providing a measure of the laboratory's competence. The United Kingdom's National External Quality Assessment Scheme (UK NEQAS) operates the scheme for Blood Coagulation, based in Sheffield, which has been inspected by the United Kingdom Accreditation Service Ltd (UKAS) and granted full accreditation to ISO 17043 for all listed tests. The mandate of the WFH IEQAS is to provide an EQA for tests of blood coagulation and to promote high standards of performance and practice. EQA, together with IQC procedures, are vital components of overall laboratory quality assurance. In addition, the WFH IEQAS provides an advisory service to participants through exchanges on lab diagnosis, including a participants' meeting during the

biennial WFH World Congress and onsite/virtual visits to provide training, as needed. The WFH IEQAS Committee is responsible for overseeing the IEQAS program. The committee is comprised of an independent chair appointed by the WFH, the Scheme program director, IEQAS program staff in the Sheffield Teaching Hospitals (host institution), and WFH staff and volunteers. The IEQAS Committee oversees all operational aspects of the program, reviews participation in the scheme, analyzes results, monitors global laboratory performance, and provides advisory support for centers registered in the scheme. The WFH IEQAS surveys are distributed three times per year, typically in March, July, and November. All surveys usually include PT, APTT, FVIII assay, and FIX assay. Two of the three surveys include VWF antigen assay and ristocetin cofactor/VWF activity assay. One out of the three surveys include two other factor assays so that FII, FV, FVII, FX, and FXI assays are all assessed at some stage, along with fibrinogen. For details, contact neqas@coageqa.org.uk.

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PART 3 Sample Integrity and Preanalytical Variables

Kieron Hickey

TOPICS COVERED

- ✓ Blood Collection
- ✓ Platelet Poor Plasma (PPP)
- ✓ Interfering Substances
- ✓ Storage
- ✓ Thawing

Prior to blood collection, there are a few factors that must be taken into account. Fasting is not required prior to blood collection for most bleeding and thrombotic investigations. However, one exception is the homocysteine test which does require fasting. Exercise (Venema et al, 2017) and stress (Austin et al, 2012) can cause temporary increases in FVIII and VWF. Exercise can also affect the D-dimer test (Huskens D et al, 2016). Inflammation can affect clotting factors and other hemostatic parameters (Hardy et al, 2024). Pregnancy affects a variety of parameters including FVIII (Castaman, 2013), VWF (Delbrück et al, 2019), and D-dimer (Blombäck et al, 2007). Numerous pharmaceutical products and anticoagulants can interfere with hemostasis tests, therefore information regarding patient treatments is essential for the laboratory (Gosselin et al, 2019).

Blood Collection: Several guidelines are available describing best practices for sample collection and processing for hemostatic testing (CLSI, 2024; CLSI, 2017). Blood collection should use an evacuated collection system or plastic syringe with a 19- to 21-gauge needle (adults) or 22- to 23-gauge needle (children) (Srivastava et al, 2021). Tube types are not all the same, therefore centers should use a single tube type and generate reference intervals based on these tubes (Bowen et al, 2016). Even within a collection tube type, constitution is important; plastic and glass tubes are not interchangeable (Fiebig et al, 2005). Blood collection tubes should contain 0.105 to 0.109 M (3.2%) trisodium citrate (CLSI, 2024). The sequence of draw is important to prevent EDTA (Lima-Oliveira et al, 2015) or heparin cross contamination (Keppel et al, 2019); as such best practice in sample draw should be followed (WHO, 2010; Simundic et al, 2018). Samples require immediate anticoagulation after venipuncture, with filling to minimum 80% target volume (Kitchen et al, 2021) to achieve 9:1 blood:anticoagulant ratio. Gentle inversion (3 to 5 times) of blood tubes post phlebotomy allows suitable mixing of samples. Unwanted hemostatic changes occur in underfilled tubes (Lippi et al, 2012). Samples with hematocrit levels >55% require an adjusted citrate solution to compensate for the high packed cell volume and to obtain the correct 9:1 ratio. Significant changes in PT, APTT, and anticoagulant monitoring (INR) can be seen if this ratio is not maintained (Marlar et al, 2006). The recommend formula for readjusting citrate levels is shown below (Kitchen et al, 2021). Samples should be suitably labelled immediately pre- or post-phlebotomy following local regulatory or institutional policies.

$C = (1.85 \times 10^{-3})(100 - HCT)(V)$

C = volume of citrate in milliliters (ml) that should be added to a volume of blood (V)

HCT = is the hematocrit of the patient

V = is the volume of blood added in ml

And 1.85×10^{-3} is constant

An example using HCT of 70% and 4.5 ml of collected blood prior to addition of anticoagulant, gives the following calculation that is, 0.25 ml of citrate is mixed with 4.5 ml of blood.

$(1.85 \times 10^{-3})(100 - HCT)(V) = C$

$(1.85 \times 0.001)(100 - 70)(4.5\ ml) = 0.25\ ml\ of\ citrate$

Platelet Poor Plasma (PPP): Most coagulation tests can be performed with PPP after centrifugation at >1700g for 10 minutes (CLSI, 2024; Kitchen et al, 2021). Refrigerated centrifuges should be avoided, as cold activation of platelet factor 4 can affect heparin monitoring, platelet function tests, FVIII tests, and VWF tests (Favaloro, 2004). Some tests, such as unfractionated heparin (UFH) and Lupus anticoagulant, require double spun platelet free plasma ($<10 \times 10^9$) if testing is carried out on previously frozen samples. In this instance, plasma is removed from the spun blood tube into a secondary suitable container and re-spun with subaliquots removed for freezing. Platelet function testing requires platelet rich plasma (PRP), prepared after centrifugation at 170g for 15 minutes or 250g for 10 minutes (Gomes et al, 2021).

Interfering Substances: Hemolyzed samples should not be tested, as significant changes, particularly in APTT, can be seen (Woolley et al, 2016; Lippi et al, 2013), except when hemolysis is intravascular (Arachchillage et al, 2014). Routine assays are usually unaffected by jaundice/icterus (Woolley et al, 2016) and lipemia can be overcome by ultracentrifugation (Lippi et al, 2013, Dimeski and Jones, 2011).

Storage: Sample testing is time sensitive. Sample processing and testing should be conducted within the assay stability window after venipuncture, and samples should be stored at room temperature in the intervening time. Guidelines recommend testing within 4 hours (CLSI, 2024) for all samples, unless local data confirm extended stability for a specific tube/assay combination (Kitchen et al, 2021; Linskens et al, 2018). Higher temperature storage can lead to loss of clotting factors, such as FVIII (Omidkhoda et al, 2011). Release of platelet factor 4 can cause heparin neutralization of unfractionated heparin in samples, therefore these tubes should be centrifuged within 1 hour and analyzed within 4 hours (Baker et al, 2020). If plasma is stored for later testing, storage conditions can affect some assays. Plasma can be acceptably stored at -24°C for 3 months. However, for longer-term storage (approximately 6 months), samples should be stored -70°C (Woodhams et al, 2001; Fenclova et al, 2023).

Thawing: Frozen samples for testing should be thawed in a water bath at 37°C for 3 to 5 minutes and inverted several times prior to testing to homogenize the sample (Jo et al, 2020). Re-freezing once-thawed plasma for further testing should be avoided.

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PART 4 Laboratory Investigation Using Only Manual Tests

Steve Kitchen

TOPICS COVERED

- ✓ When to Use Manual Tilt-Tube Technique
- ✓ How to Perform Manual Tilt-Tube Technique
- ✓ PT by Manual Tilt-Tube Technique
- ✓ APTT by Manual Tilt-Tube Technique
- ✓ Thrombin Time and Fibrinogen by Manual Tilt-Tube Technique
- ✓ PT-Derived Fibrinogen Test

There are advantages to using coagulometers for coagulation testing, including speed of analysis/throughput and consistency of analysis, which deliver precise and accurate results in a timely fashion. Although many different instruments for coagulation tests are available and in use throughout the world, the manual tilt-tube technique can still be employed successfully for determination of clotting time. This can be done for all samples if no suitable automated method is available, or for a subgroup of samples, either because automated analysis does not generate results on samples with specific features, causing incompatibility of sample with the instrument in use, or because the coagulometer method is temporarily unavailable. Tilt-tube methods are suitable as alternatives for clot-based methods and even comprehensive hemophilia center laboratories should have the manual tilt-tube method available for those very few samples where automated analysis fails, but where results are really needed for safe patient management. This may be the case in the presence of grossly elevated plasma lipid concentrations, or where the clot formation pattern in the sample differs markedly from normal samples, particularly when the fibrinogen to fibrin polymerization is markedly abnormal. Because of the many variables and possible sources of contamination associated with manual techniques, these may require duplicate tests. If the between-day CV of IQC results is >5%, then duplicate testing should be considered. Where tests are performed in duplicate, the two results should be within +/- 5% of the mean with practice.

When to Use Manual Tilt-Tube Technique: Then manual tilt-tube method can be successfully used for determination of PT, APTT, thrombin time, and fibrinogen, as well as clotting factor assays based on PT and APTT.

How to Perform Manual Tilt-Tube Technique: The method of performing the tilt-tube technique for coagulation testing has been recently harmonized in relation to PT testing as part of the calibration of reference thromboplastins for the international normalized ratio (INR) system used for monitoring vitamin K antagonist drugs (Van den Bessellaar et al, 2020). This harmonized method has improved agreement between tilt-tube PT results when testing is done by different operators and in different centers. This method can be used for tilt-tube testing for APTT, thrombin time, and fibrinogen analysis in addition to PT testing.

Materials required:

1) Water bath for keeping the test tubes at a constant temperature of 37°C. Dimensions close to 40 x 30 x 20 cm are convenient. The water in the bath should be circulated continuously by a pump if possible. The temperature should be 37°C (tolerance limits: 37 ± 0.5°C). The temperature should be controlled with a calibrated thermometer.
2) A light source, such as an angle poise lamp, mounted 20 cm above the water level can be used to illuminate the test tube during tilting which facilitates the endpoint clot detection by

the operator. LED as a light source is preferred over bulbs which generate heat since that can elevate the temperature of test tubes held close to the light source.

3) Test tubes should be non-siliconized, previously unused glass tubes. Disposable culture tubes (catalogue number 73500-1275, Kimble Chase Life Science and Research Products LLC, Vineland, New Jersey) with dimensions of 75 x 12 mm and wall thickness 0.8 mm were used in the work to harmonize PT tilt-tube testing (van den Besselaar et al, 2020) but 75 x 10 mm tubes can also be used. The test tubes should be made of borosilicate glass. Test tubes should be discarded after use and not washed for re-use. Different sources of glass test tubes can be used successfully, but they may influence the clotting times obtained, particularly in screening tests such as APTT. If the source (manufacturer or composition) of test tubes is changed, the possibility that results have been influenced should be considered. This could be assessed by comparing a small number of tests, such as APTT, with the two types of tube. If systematic differences are present, a new normal range should be established.

Technique:

- The temperature of the room in which the equipment is installed and where the technique is to be performed should be maintained at 20–22°C.
- Prior to commencing PT, APTT, TT, or fibrinogen manual tests in the water bath, the temperature should be checked and subsequently recorded.
- Empty test tubes should be kept in a vertical position in a rack in the water bath at 37°C for at least 4 minutes at a depth of 3.5 cm before addition of reagents and plasma.

PT by Manual Tilt-Tube Technique:

- For PT testing, add 200 µl of the thromboplastin/calcium reagent and incubate for 2 min.
- Then pipette 100 µl of not pre-warmed plasma, dispensing from a height of 1 cm above the level of thromboplastin, with the tip resting against the wall of the test tube, and immediately start the stop watch with the other hand.
- Shake the tube gently to mix the contents, with the tube immersed in the water.
- Put the tube in the rack in the water bath.
- Lay down the pipette.
- The test tube should manually be kept in the water, with the water covering the bottom 5 cm of the tube (Figure 1).
- Manual titling of the tube should commence 7 seconds after starting the stopwatch.
- The tube should be tilted at an angle of nearly 90° by taking the tube out of the water for 2 seconds and putting it back in the water for 1 second (Figure 1). Note that tilting the tube by 90° or more will usually lead to the reaction mixture spilling out from the tube.
- The tube should not be stationary during this cycle but continuously tilted with the operator's hand resting on the side of the water bath.
- This cycle is repeated until the clot forms.
- In the horizontal position, the tube is kept not more than 10 cm and not less than 2 cm above the water level (Figure 1).
- Before the mixture clots, the operator observes the mixture flowing from the bottom to three quarters of the length of the tube in the nearly horizontal position and back to the bottom.
- When clotting commences, the speed of flowing is reduced.
- When flow is stopped, the operator stops the timer and records the clotting time in seconds to one decimal place.

The operator performing the manual tilt-tube technique lifts the test tube regularly out of the water. Taking the test tube out of the water will result in a temperature drop of the reaction mixture. Immersing the tube back into the water will result in a temperature increase. The average temperature drop observed in

the manual tilt-tube technique using the method described above is limited to 0.4°C (van den Bessellar et al, 2020).

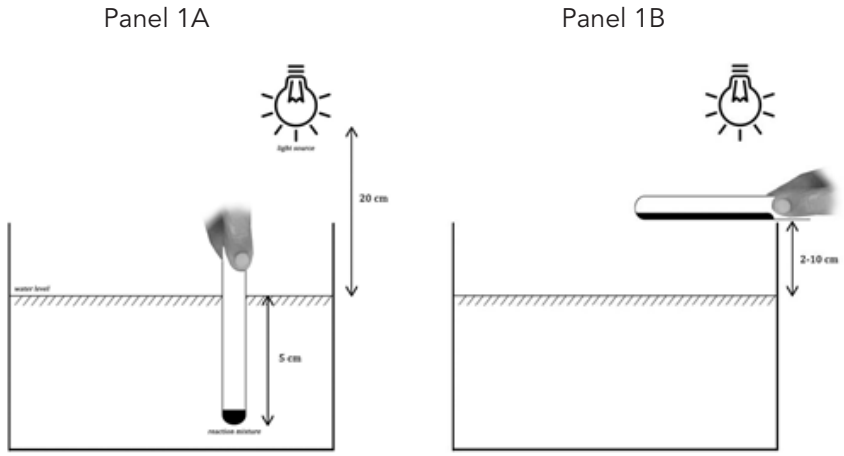

Figure 1. Schematic representation of the manual tilt-tube technique. Panel 1A: the test tube is in the vertical position in the water bath. Panel 1B: the test tube is in the horizontal position out of the water bath. The hand of the operator is resting on the edge of the water bath. Due to variation in the size of the operators' hands, the distance of the tube in the horizontal position to the water surface varies between 2 and 10 cm. The dimensions of the picture are not to scale. (Reproduced by kind permission of Elsevier Publishing, Amsterdam, Netherlands, from: Van den Besselaar et al. J Thromb Haemost. 2020; 18: 1986-1994.)

The above harmonized method was developed over two wet workshops assessing PT testing by up to seven operators from three centers, which identified a number of variables in technique (van den Besselaar et al, 2020). In particular, the workshops confirmed that dispensing the final component of the reaction mixture to initiate coagulation high in the upper part of the test tube was associated with longer clotting times then dispensing close to the surface of the reaction mixture lower down in the tube. Using the harmonized method described, the between-operator CV of Ts on the same test plasmas was 3% for a normal PT and 1.4% for a prolonged PT.

APTT by Manual Tilt-Tube Technique:

- Add 100 µl of APTT reagent to tube and incubate for 2 min.
- Then pipette 100 µl of not pre-warmed plasma, dispensing from a height of 1 cm above the level of APTT reagent, with the tip resting against the wall of the test tube and immediately start a stop watch with the other hand.
- Shake the tube gently to mix the contents, with the tube immersed in the water.
- Put the tube in the rack in the water bath.
- After the activation time recommended by the APTT reagent manufacturer (usually this is 3 mins, but can be 5 mins for others), add 100 µl calcium chloride which has been pre-warmed to 37°C in a separate tube in the water bath, dispensing from a height of 1 cm above the level of the reaction mixture, with the tip resting against the wall of the test tube, and immediately start a new stop watch with the other hand.
- Shake the tube gently to mix the contents, with the tube immersed in the water.
- Put the tube in the rack in the water bath.
- Lay down the pipette.
- The test tube should be kept manually in the water, with the water covering the bottom 5 cm of the tube (Figure 1).
- Manual titling of the tube should commence 15 seconds after starting the stopwatch.

- The tube should be tilted to an angle of nearly 90° by taking the tube out of the water for 2 seconds and putting it back in the water for 1 second (Figure 1). Note that tilting to 90° or more will usually lead to the reaction mixture spilling out from the tube.
- The tube should not be stationary during this cycle but continuously tilted with the operator's hand resting on the side of the water bath.
- This cycle is repeated until the clot forms.
- In the horizontal position, the tube is kept not more than 10 cm and not less than 2 cm above the water level (Figure 1).
- Before the mixture clots, the operator observes the mixture flowing from the bottom to three quarters of the length of the tube in the nearly horizontal position and back to the bottom.
- When clotting commences, the speed of flowing is reduced.
- When the flow is stopped, the operator stops the timer and records the clotting time in seconds to one decimal place.

Thrombin Time and Fibrinogen by Manual Tilt-Tube Technique: The manual tilt-tube methods should use the proportions of reagents and plasma or plasma dilutions recommend by the reagent manufacturer and follow the principles of the methods described for PT/APTT above.

Samples with lipidemia: Many coagulometers in current use that utilize photo-optical endpoints for analysis, are very tolerant of high lipid levels in the samples. However, samples may occasionally have such a high level of lipids that the analyzer cannot detect clot formation. Such samples can be analyzed manually and usually produce a solid clot that can be observed visually for PT, APTT, and thrombin time. It can be difficult to observe clot formation during Clauss fibrinogen analysis of such samples. If that is the case, another option is to subject the samples to centrifugation at 10,000g for 10 min at ambient temperature if available (ICSH guidance; Kitchen et al, 2021). After this ultracentrifugation, the lipid is sedimented and the sample can be analyzed on an automated coagulometer if available, or by the manual tilt-tube technique for PT, APTT, thrombin time, or fibrinogen.

Samples with fibrinogen abnormalities: Clot formation can be deranged in the presence of some fibrinogen abnormalities. For example, fibrinogen Longmont is associated with a weak and translucent clot during the Clauss fibrinogen assay. Photo-optical systems that monitor scattered light as the clot forms (as opposed to transmitted light monitoring) may be unable to detect the endpoint. Such samples can be analyzed using the tilt-tube method, but the operator must be aware that the clot formation may be difficult to discern visually. For some dysfibrinogenemia samples where automated analysis fails, the manual technique should be done with very careful scrutiny of the clotting process, since clots may be fragile and easily disrupted by additional mixing/tilting after the initial clot has formed.

Quality control: Quality control material, as described elsewhere in this manual for PT, APTT, thrombin time, and fibrinogen, is suitable for use with manual test methods. It is acceptable to test a single level of IQC, where the manual technique is reserved for occasional samples that fail automated analysis. Two levels should be available and tested according to the criteria described elsewhere in this manual, where manual testing is the primary analytical procedure in the laboratory.

The range of IQC results obtained by a small group of different operators experienced in the manual tilt-tube technique should typically be as follows:

Mean +/- 1 second for a QC with a mean PT in the range of 10 to 12 seconds,

Mean +/- 2.5 second for a QC with a mean APTT in the range 25 to 30 seconds,

Mean +/- 2.5 second for a QC with a mean thrombin time in the range 12 to 20 seconds,

Mean +/- 0.5 g/l for a QC with a mean fibrinogen of 2.5 to 3 g/l

Normal ranges: Manual test results are usually different from those generated by coagulometers. Typically, clotting times for PT and APTT are shorter for analyzers using photo-optics to monitor clotting compared with results that are obtained manually. The degree of difference is not consistent across different analyzers which may use transmitted or scattered light. Analyzers monitor change in light scatter/transmission over time after clotting is initiated and record clotting time as the time taken to exceed a particular threshold of change. This may be a threshold as low as 3% change over baseline or a high as 50% change. The lower the % change used in clotting curve analysis, the shorter the clotting time reported. This means that results of manual tests should not be reported alongside a reference range established for an automated technique, even when the same reagents are being used. There are two options for dealing with this issue. One is to establish a reference range for the manual technique using the process described elsewhere in this manual. This should be done if all testing in the lab is carried out using manual techniques. More often, manual testing is restricted to a small subgroup of samples where automated analysis has failed. In this case, most results being released by the lab will be issued with the relevant automated reference range. It is unhelpful to users of the service to see occasional PT or APTT results issued with a different reference range. In this case, the lab can take a pragmatic approach and use a conversion chart as described below, so that testing is done by manual technique, but the test result is converted to the result that would have been obtained if the sample had been analyzed using the automated method.

Converting Manual PT, APTT, and Thrombin Time Results into Automated Equivalent Numbers: A series of 20 to 30 samples covering a range of normal and abnormal results should be tested using both manual and automated methods. The data should be analyzed using regression analysis to establish the relationship between results obtained using the two methods. There should be a significant correlation between results, with a correlation coefficient >0.8. If so, the regression relationship can then be used to create a table which shows the manual result and the equivalent result that would have been obtained on the automated system. When a manual test is performed, the manual result is converted into the automated result, which is then reported alongside the usual automated method reference range. This means the users of the service will only see a single reference range for PT, APTT, or thrombin time. This is important, since such reference ranges are likely to be incorporated into clinical protocols used for patient management. Table 5 shows the manual and automated results obtained by testing the same 21 samples using both methods.

Table 5. Manual and automated APTTs on the same samples

Sample number	Manual APTT (sec)	Automated APTT (sec)	Sample number	Manual APTT (sec)	Automated APTT (sec)
1	31.7	27.0	12	31.7	30.1
2	51.1	46.9	13	31.9	28.9
3	27.2	26.5	14	36.2	33.2
4	42.2	39.4	15	33.1	28.5
5	34.5	30.4	16	40.0	36.4
6	44.2	43.2	17	37.1	30.7
7	33.0	30.5	18	29.2	25.6
8	31.9	30.0	19	35.2	28.1
9	22.2	19.6	20	37.1	34.7
10	34.0	27.8	21	36.5	31.4
11	32.5	31.3			

The linear regression relationship between the two data sets is calculated using a statistics package. In this example, the correlation coefficient (r) is 0.96 and the regression relationship is calculated as:

$$y = 0.9551x - 1.887$$

where

y is the automated APTT,
x is the manual APTT
0.9551 is the slope of the linear regression line
– 1.887 is the y intercept.

This equation is used to derive the automated APTT from the manually determined APTT of any test sample. It is convenient to prepare a table relating manual APTT to the automated equivalent result. Table 6 was derived using the above regression equation.

Table 6. Conversion chart: Manual APTT to Automated equivalent APTT

Manual APTT (sec)	Automated APTT (sec)	Manual APTT (sec)	Automated APTT (sec)	Manual APTT (sec)	Automated APTT (sec)
19	16.3	40	36.3	61	56.4
20	17.2	41	37.3	62	57.3
21	18.2	42	38.2	63	58.3
22	19.1	43	39.2	64	29.2
23	20.1	44	40.1	65	60.2
24	21.0	45	41.1	66	61.2
25	22.0	46	42.0	67	52.1
26	22.9	47	43.0	68	63.1
27	23.9	48	44.0	69	64.0
28	24.9	49	44.9	70	65.0
29	25.8	50	45.9	71	65.9
30	26.8	51	46.8	72	66.9
31	27.8	52	47.8	73	67.8
32	28.7	53	48.7	74	68.8
33	29.6	54	49.7	75	69.8
34	30.6	55	50.7	76	70.7
35	31.5	56	51.6	77	81.7
36	32.5	57	52.6	78	82.6
37	33.4	58	53.5	79	73.6
38	34.4	59	54.5	80	74.5
39	35.4	60	55.4		

Manual Clauss fibrinogen assay: The regents and methods used for the manual Clauss fibrinogen assay are the same as for the automated version (i.e. same buffer, thrombin, and test sample dilution). The clotting time of the manual tests is converted into fibrinogen concentration using a calibration curve. The calibration curve should be constructed using the same calibrator and calibrator dilutions as would be used for automated testing (see Clauss assay section elsewhere in this manual), however, the clotting times of each calibrator dilution used to construct the calibration curve is determined manually. The results are thereby converted into fibrinogen concentration and the result is reported in the same format as the automated version (g/l or mg/dl) using the same reference range as for the automated Clauss method in the same center.

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PART 5 Initial Evaluation of Hemostasis

Anastasia Khasiani

TOPICS COVERED

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

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

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

Materials and equipment:

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

Method:

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

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

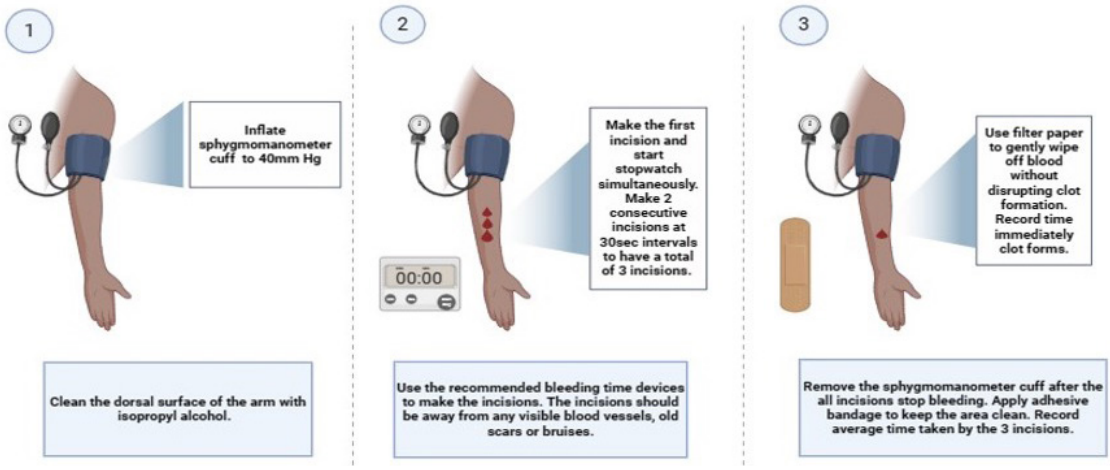

Figure 2. Flow diagram demonstrating bleeding time test. (Adopted from Sally Kim's template for "Measurement of blood pressure: The Auscultatory Method" in biorender.com.)

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

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

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

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

Reagents:

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

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

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

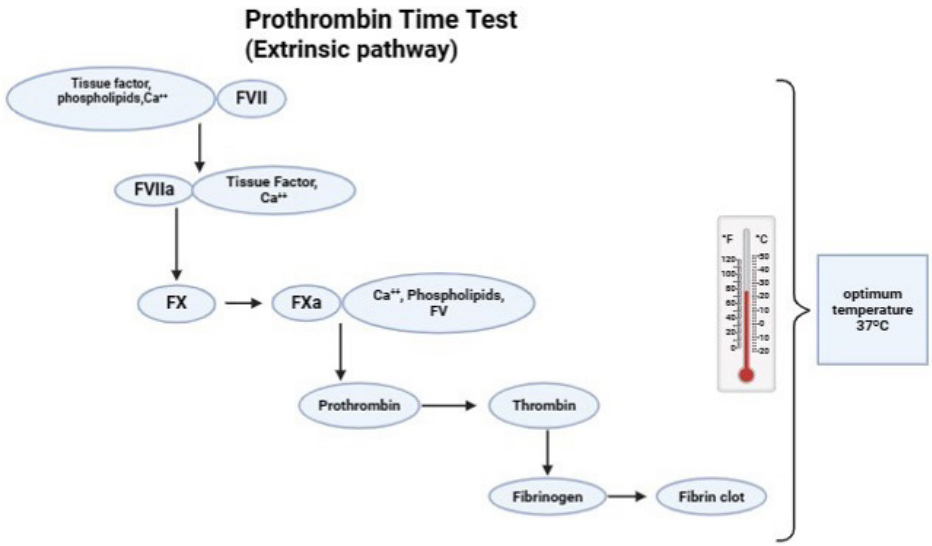

Figure 3. Pathway measured by the PT test

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

Reagents:

- ✓ APTT reagent
- ✓ 25mM calcium chloride

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

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

Table 7. An example of interpretation of prolonged APTT

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

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

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

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

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

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

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

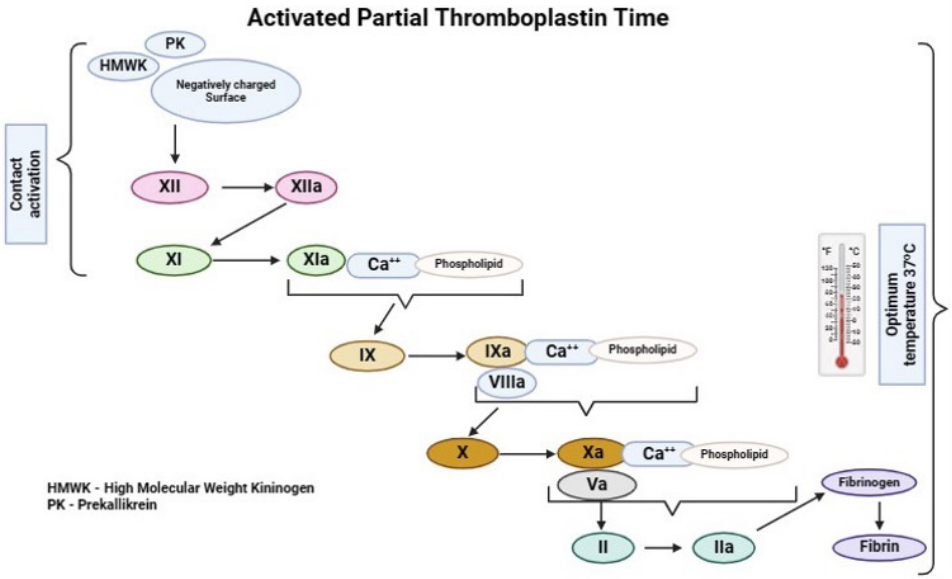

Figure 4. The pathway measured by APTT

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

Procedure:

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

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

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

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

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

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

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

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

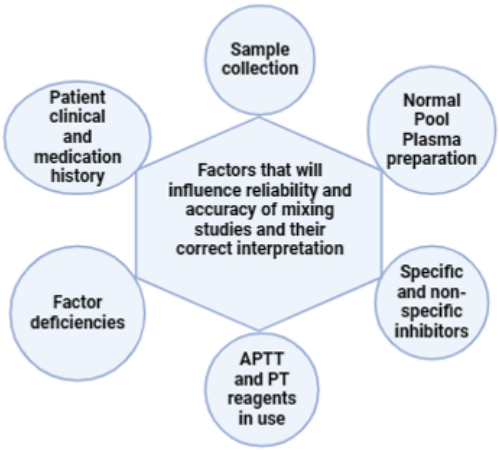

Figure 5. Diagram developed in biorender.com using information obtained from Adcock et al. (2023).

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

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

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

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

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

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

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

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

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

Table 9. Mixing studies in acquired hemophilia A

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

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

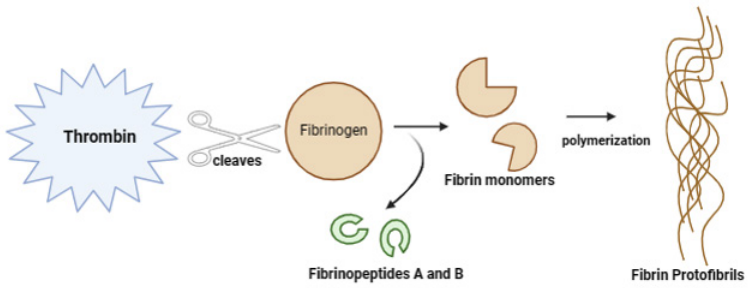

Figure 6. Flow diagram depicting the conversion of fibrinogen to fibrin in the presence of thrombin. Developed using Biorender.

Reagents:

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

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

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

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

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

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

Reagents (Hogwood et al 2017):

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

Method:

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

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

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

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

Method:

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

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

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

Table 10. Interpretation of prolonged thrombin time

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

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

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

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

Reagents:

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

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

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

Typical calibration data:

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

Standard plasma: 2.1 g/l fibrinogen

Table 11. Example of a fibrinogen calibration

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

Examples:

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

fibrinogen = 1.9 g/l (from calibration graph)

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

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

= 0.9 g/l

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

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

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

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

Reagents:

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

Method:

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

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

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

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PART 6 Hemophilia Lab Investigation

Annette Bowyer

TOPICS COVERED

- ✓ Assay of Factors VIII and IX: One-Stage and Chromogenic Methods
- ✓ Performing Factor Assays on Analyzers for Which the Software Uses a Single Dilution
- ✓ Bispecific Antibodies
- ✓ Post-FVIII and FIX Infusion Monitoring
- ✓ Gene Therapy
- ✓ Laboratory Aspects for Hemostatic Rebalancing Therapy Treatment

Assay of Factors VIII and IX: One-Stage and Chromogenic Methods: The laboratory diagnosis of hemophilia A or B is made by measurement of factor activity (Srivastava et al, 2020). The most commonly used methodology is the one-stage clotting assay based on the APTT. The one-stage assay for FVIII activity is described in this section. The assay is based on a comparison of the ability of dilutions of standard and test plasmas to correct the APTT of a plasma known to be totally deficient in FVIII but containing all other factors required for normal clotting. For factors IX, XI, and XII, the assay is essentially the same and is performed by substituting the relevant deficient plasma for FVIII-deficient plasma, and after selection of the appropriate reference plasma (Baker et al, 2020). A one-stage FVIII or FIX assay cannot be performed in the presence of bispecific antibodies such as emicizumab (Jenkins et al, 2020).

Reagents:

- ✓ Platelet-poor citrated test plasma
- ✓ Standard (reference/calibrator) plasma

The standard (reference) plasma used should be either a locally prepared plasma pool kept at -70°C or lower, or a commercial standard plasma. In either case, this reference plasma must be calibrated for clotting assay against the current international standard for FVIII or FIX in plasma. It is not acceptable to assume that a pooled normal plasma has 100 IU/dl.

- ✓ Internal quality control plasma (CLSI, 2016)
- ✓ FVIII-deficient plasma

This is available commercially or may be collected from a hemophilia donor under the following conditions:

- ✓ Level is less than 1 IU/dl
- ✓ No history of antibodies to FVIII
- ✓ Received no treatment for two weeks including extended-half life (EHL) or bispecific antibody therapy
- ✓ Normal liver function tests

Abnormal liver function could lead to a reduction in other clotting factors, which affect the specificity of the assay. This plasma can be stored in aliquots at -20°C or lower for approximately 1 month (Woodhams et al, 2001; Zhao et al, 2018). It is preferable to use FVIII/FIX-deficient plasma produced by immunodepletion of FVIII or FIX from normal plasma using a monoclonal antibody. This type of material is available commercially and has the advantage of enhanced viral safety compared with plasma sourced from patients with hemophilia who have been treated with plasma-derived products. However, not all immunodepleted

plasmas are found to be <1 IU/dl, and care should be taken to check this before use. Some experts hold the view that the presence of normal concentrations of VWF in FVIII/FIX-deficient plasma may be an advantage, and there is evidence to support this in relation to assays performed as part of inhibitor determinations (Verbruggen et al, 2001).

- ✓ APTT reagent that is sensitive to factor deficiencies (CLSI, 2016)
- ✓ Owren's buffered saline (OBS or glyoxaline buffer; see reagent session)
- ✓ 25 mM $CaCl_2$ (note that Werfen $CaCl_2$ supplied with SynthASil is 20 mM)

Method:

- ✓ Make 1/10 dilutions of standard, QC, and test plasma in buffered saline in plastic tubes. (If the test plasma is expected to have a very low level of FVIII, start at a 1/5 dilution.)
- ✓ Using 0.2 ml volumes, make doubling dilutions in OBS of standard, QC, and test plasma from 1/10 to 1/40 in plastic tubes. (Mix each dilution well before transferring to the next tube.) Plasma dilutions should be tested immediately after preparation. If room temperature exceeds 25°C, it may be necessary to keep dilutions on wet ice prior to testing.
- ✓ Pipette 0.1 ml of each standard dilution into a 75 x 10 mm glass tube.
- ✓ Add 0.1 ml of FVIII-deficient plasma and transfer to 37°C water bath.
- ✓ Add 0.1 ml of APTT reagent and incubate for 5 minutes.
- ✓ At 5 minutes, add 0.1 ml $CaCl_2$ and record the clotting time.
- ✓ A "blank" should also be set up using 0.1 ml of OBS in place of test plasma.

The clotting time of the blank should be longer than the time of 1% FVIII activity of standard from the calibration graph. If the time is shorter, this indicates that the substrate plasma is not totally deficient in FVIII and thus is not a suitable substrate plasma

Results: Plotting results requires double logarithmic or logarithmic/linear scale graph paper. The 1/10 dilution is arbitrarily assigned a value of 100%, the 1/20 dilution a value of 50%, and the 1/40 dilution a value of 25%. If used, the 1/5 dilution has a value of 200%. Straight lines, parallel to each other, should be obtained. Read off concentration of test sample as shown in Figure 7. In this example, the FVIII concentration in the test sample is 7% of that in the standard. If the standard has a FVIII concentration of 85 IU/dl, the test sample has a concentration of 85 IU/dl x 7% = 6 IU/dl. If the lines are not parallel, the assay should be repeated. Non-parallel lines may occur due to technical errors. If technical error has been eliminated, it may be due to the presence of an inhibitor, which may act specifically against FVIII or may be of the "lupus type", showing a converging pattern. Diverging lines are typical of an activated sample or presence of a DOAC (Baker et al, 2020).

Notes: If the test plasma FVIII (or FIX) concentration is close to zero (i.e. the clotting times of all dilutions are like the blank), non-parallel lines may occur. The presence of lupus anticoagulant can interfere with the phospholipids in APTT reagents and produce non-parallel factor assays (Ruinemans-Koerts et al, 2001). The normal range should be established locally but often has a lower limit of 50 to 65 IU/dl for both FVIII and FIX. The accurate monitoring of some EHL products by one-stage assay may affected by the APTT reagent used (Gray et al, 2020). See Part 6 on EHL. One-stage FVIII or FIX assays cannot be performed in the presence of bispecific antibodies such as emicizumab. These drugs artificially shorten the APTT. A short time to clot in a one-stage assay corresponds to a high factor activity (Jenkins et al, 2020; Bowyer et al, 2020; Bowyer et al, 2023). See Part 6 on bispecific antibodies. One-stage chromogenic FVIII discrepancy has been described in mild hemophilia A in some geographical areas and rarely for mild hemophilia B (Pouplard et al, 2009). If possible, a new diagnosis of mild hemophilia should have FVIII:C or FIX:C also measured by chromogenic assay Bowyer et al, 2018). One-stage FVIII assays can be used to measure recombinant porcine FVIII therapy Bowyer et al, 2022). There is a mutation in FIX, FIX Padua (p.R338L), reported to have eight-fold higher FIX activity than antigen (Simioni et al, 2009).

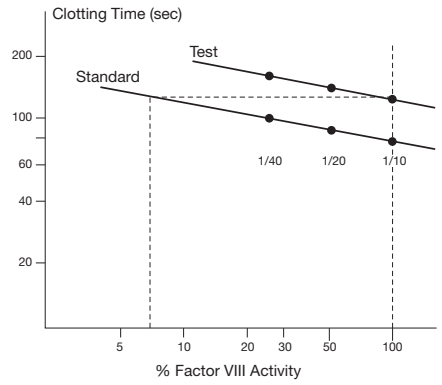

Figure 7. Graph of FVIII assay

Test samples should be assayed using three different sample dilutions as described above. This is a consistent recommendation in published national and international guidelines including those from WFH. It improves both assay accuracy and precision compared to use of a single test sample dilution. Factor assays are sometimes performed on analyzers with software designed for testing only a single test sample dilution. The following section describes a method that can be used to include three test sample dilutions on such analyzers.

Performing Factor Assays on Analyzers for Which the Software Uses a Single Dilution of Test Sample: The WFH recommends that one-stage APTT-based assays for FVIII and FIX should be done using three different dilutions of the test sample. This is described in the following document:

> WFH Guidelines for the Management of Hemophilia, 3rd edition. Srivastava A, Santagostino E, Dougall A, Kitchen S, Sutherland M, Pipe SW, Carcao M, Mahlangu J, Ragni MV, Windyga J, Llinás A, Goddard NJ, Mohan R, Poonnoose PM, Feldman BM, Lewis SZ, van den Berg HM, Pierce GF ; Chapter 3: Laboratory Diagnosis and Monitoring. Steve Kitchen, Francisco de Paula Careta, Silmara A de Lima Montalvao, Emna Gouider, Radoslaw Kaczmarek, Claude T. Tagny, Pierre Toulon, Glenn F. Pierce, Alok Srivastava. Haemophilia 2020; 26 (Suppl 1): 35-48.

The full document is available for free download on the WFH website via the following link:

Guidelines for the Management of Hemophilia - eLearning Platform (wfh.org)

The following text is reproduced from the WFH guideline:

Recommendation: For laboratory investigation due to clinical suspicion of hemophilia using one-stage FVIII/FIX assays, the WFH recommends analysis using 3 different dilutions of the reference and test plasma samples.

REMARK: The results of the test and standard plasma dilutions should be compared by parallel-line analysis. One way to assess this is to calculate the coefficient of variation (CV) of the 3 results using the equation CV = ([standard deviation/mean] x 100). If the CV of the 3 results is less than 15%, then the average of the 3 results should be reported. If the CV is greater than 15%, the results should be scrutinized. Presence of pathological inhibitors against specific clotting factors or lupus anticoagulants can interfere with some one-stage FVIII and FIX assays. Some therapeutic anticoagulants can also show this interference effect. In all of these settings, factor activity increases in the assay as the plasma is increasingly diluted. Factor activity is underestimated when the plasma is diluted less, and a more accurate activity result is obtained when the test plasma is diluted more.

The above principles should also be applied to one-stage assays of other clotting factors (i.e. FII, FV, FVII, FX, FXI, and FXII). Some analyzers have software that permits more than one dilution of test sample. For these analyzers, the test sample is presented for analysis and the analyzer constructs the different dilutions, runs the assay, and calculates the activity of the clotting factor being assayed. Other analyzers have software that permits only a single dilution of test sample. The procedure below can be used on such analyzers so that the recommendations of the WFH can be followed with improved assay accuracy and precision.

The examples given are for FVIII assays but can be used for one-stage assays of other factors.

- ✓ The test samples are presented to the analyzer undiluted (i.e. without any pre-dilution by the operator).
- ✓ The test sample is pre-diluted 1 in 2 by the operator using the same assay buffer as the analyzer uses, which is often Owren's buffer, but may be a different buffer.
- ✓ This can be done in any plastic tubes or vials that can be presented to the analyzer for analysis and which do not cause activation of the test sample.
- ✓ The test sample is pre-diluted 1 in 4 using the same assay buffer as the analyzer uses (see points 2 and 3 above).
- ✓ This means the analyzer is presented with three different materials derived from the same test sample.
- ✓ The analyzer is then requested to run a FVIII assay on each of these three materials (i.e. test sample undiluted, test sample pre-diluted 1 in 2, and test sample pre-diluted 1 in 4).
- ✓ There is one FVIII result obtained on the undiluted test sample.
- ✓ There is another result obtained on the test sample that was pre-diluted 1 in 2. This should be multiplied by 2 to correct for the pre-dilution.
- ✓ There is another result obtained on the test sample that was pre-diluted 1 in 4. This should be multiplied by 4 to correct for the pre-dilution
- ✓ The three numbers are then compared by the operator.
- ✓ Usually, the three numbers are very close to each other. If so, the operator can calculate and report the mean of the three numbers as the FVIII activity of the test sample.
- ✓ Sometimes, the three numbers are not close together. This can occur if the sample contains interfering substances or if coagulation has been activated in the sample (i.e. perhaps due to difficulties during sample collection).
- ✓ The operator must decide whether it is safe to use the mean of the three different answers.
- ✓ The WFH recommends using a simple mathematical assessment to decide.
- ✓ This is done by calculating the CV of the three different results.
- ✓ The mean of the three different results can be safely used if the CV is <15%.
- ✓ Using the mean in this way improves the precision of the test.
- ✓ If the CV is higher than 15%, the operator has more decisions to consider.
- ✓ When the factor level is 10 to 15 IU/dl, the CV of the three dilutions is higher than when the factor activity is at higher levels. In these samples, a CV of 20% can be accepted.
- ✓ When the factor level is below 5 IU/dl, a pre-dilution of 1 in 4 may reduce the factor activity below the lower limit of quantification, depending on the reagents. In these samples, it is acceptable to test only the undiluted test sample and the test sample pre-diluted 1 in 2. In this case, the lab should report the mean of the two results (after multiplying the analyzer result of the 1 in 2 dilution by 2), without calculation of CV.
- ✓ For samples with >15 IU/dl FVIII, if the CV is >15% and the result on the undiluted plasma is lower than the results on the third dilution, this indicates there may be an interfering substance in the test sample. In this case, the result obtained for the 1 in 4 dilution (after multiplication of analyzer result by 4 to correct for pre-dilution) will be the most accurate (see examples below).
- ✓ Examples of interfering substances that can cause this falsely low result in the undiluted test samples, are inhibitors such as lupus anticoagulant, UFH, DTIs, or direct FXa inhibitors.
- ✓ Use of a single dilution can lead to falsely low results and grossly inaccurate assays.

Some examples are given below.

Table 12. Example 1-FVIII assay with manual pre-dilution

	FVIII activity recorded by analyzer	FVIII activity after correcting for pre-dilution	Comments
Test samples without pre-dilution	25 IU/dl (%)	25 IU/dl (%)	No pre-dilution by operator so result does not change
Test samples diluted 1 in 2 before analysis	13.5 IU/dl (%)	27 IU/dl (%)	Analyzer result multiplied by 2
Test samples diluted 1 in 4 before analysis	6 IU/dl	24 IU/dl (%)	Analyzer result multiplied by 4

✓ Mean of three results is 25.3 IU/dl (%).
✓ Standard deviation of the three results is 1.53.
✓ CV of the three results is 6.0%.
✓ CV is <15%, so result reported is **25.3 IU/dl (%)**.

Table 13. Example 2-FVIII assay with manual pre-dilution

	FVIII activity recorded by analyzer	FVIII activity after correcting for pre-dilution	Comments
Test samples without pre-dilution	62 IU/dl (%)	62 IU/dl (%)	No pre-dilution by operator so result does not change
Test samples diluted 1 in 2 before analysis	33 IU/dl (%)	66 IU/dl (%)	Analyzer result multiplied by 2
Test samples diluted 1 in 4 before analysis	13 IU/dl	52 IU/dl (%)	Analyzer result multiplied by 4

✓ Mean of three results is 60.0 IU/dl (%).
✓ Standard deviation of the three results is 7.2.
✓ CV of the three results is 12.0%.
✓ CV is <15%.
✓ Result reported is **60.0 IU/dl (%)**.

Table 14. Example 3-FVIII assay with manual pre-dilution

	FVIII activity recorded by analyzer	FVIII activity after correcting for pre-dilution	Comments
Test samples without pre-dilution	7.0 IU/dl (%)	7.0 IU/dl (%)	No pre-dilution by operator so result does not change
Test samples diluted 1 in 2 before analysis	3.0 IU/dl (%)	6.0 IU/dl (%)	Analyzer result multiplied by 2
Test samples diluted 1 in 4 before analysis	2.1 IU/dl	8.4 IU/dl (%)	Analyzer result multiplied by 4

✓ Mean of three results is 7.1 IU/dl (%).
✓ Standard deviation of the 3 results is 7.1.
✓ CV of the 3 results is 16.9%.
✓ CV is <20%.
✓ Result reported is **7.1 U/dl (%)**.

Table 15. Examples with interfering substances present, FVIII assay results are in IU/dl (%)

Interference	Without pre-dilution	Pre-diluted 1 in 2		Pre-diluted 1 in 4		Results used to calculate CV	CV
		Analyzer results	Operator corrected result	Analyzer result	Operator corrected result		
Direct thrombin inhibitor	46.5	34.0	68.0	18.5	74.0	46.5, 68.0, 74.0	23.0%
Rivaroxaban	64.0	45.2	90.4	29.1	116.4	64.0, 90.4, 116.4	29.0%
Lupus anticoagulant	25.3	19.3	38.6	16.2	64.8	25.3, 38.6, 64.8	46.8%

- ✓ Note that similar patterns occur in other one-stage assays such as FIX.
- ✓ All three have CVs >15%.
- ✓ The effects of such interfering substances are usually lower in the 1 in 4 dilution sample than in the undiluted sample or in the 1 in 2 pre-dilution sample.
- ✓ The result for the 1 in 4 sample may still be an underestimation, but is the closest to an accurate result of the three tests performed. It may also be useful to add a 1 in 8 pre-dilution as a fourth test in such cases.
- ✓ The reportable results from the examples in the table could be >74 IU/dl (%), >116.4 IU/dl (%), or >64.8 IU/dl (%).

All three test samples in this example have FVIII activity that is not reduced below the normal range. Confirming that activity is not reduced, is sometimes sufficient for safe patient management.

Chromogenic FVIII:C and FIX assays: The laboratory diagnosis of hemophilia A or B is made by measurement of factor activity (Srivastava et al, 2020). The most commonly used methodology is the one-stage clotting assay based on the APTT (OSA). There are limitations to the one-stage assay, including interference if lupus anticoagulant, direct oral anticoagulants (DOACs) or extended half-life hemophilia therapies, including bispecific antibodies, are present (Gray et al, 2020; Jenkins et al, 2020; Bowyer et al, 2021; Moser et al, 2021; Ruinemans-Koerts et al, 2010). More importantly, mild hemophilia A is not excluded by the finding of a normal FVIII:C, and rarely FIX:C level, by OSA (Pouplard et al, 2009). Several groups have reported that a subgroup of mild hemophilia A patients have discrepancy between the activity of FVIII as determined using different types of assays (Pavlova et al, 2014). More than 20% of mild hemophilia A patients are associated with discrepancy in which chromogenic activity is two-fold or lower than OSA and the bleeding phenotype is commensurate with the chromogenic substrate assay (CSA) (Bowyer et al, 2018). A reverse form of assay discrepancy can also occur with two or more-fold lower FVIII:C by OSA than CSA. Reports of bleeding are much lower in these patients (Bowyer et al, 2018; Bowyer et al, 2011). Examples of results in such patients are shown in Table 16.

Table 16. Examples of patients with genetically confirmed mild hemophilia A and assay discrepancies

Case	One-stage assay (IU/dl)	Chromogenic assay (IU/dl)
A	101	13
B	88	28
C	15	69
D	55	40
E	58	33
F	72	36
G	84	45

Based on these results, it is advantageous for all hemophilia centers to have a chromogenic FVIII assay available. CSA should be performed on subjects with normal APTT and one-stage FVIIII activity in the presence of a personal or family history consistent with mild hemophilia. FVIII CSAs were first introduced in the early 1980s (Rosen et al, 1984) and a number of manufacturers have commercial kits for chromogenic assay of FVIII. Many of these are suitable for diagnosis of hemophilia A in the presence of normal one-stage FVIII activity. A small number of FIX CSA have been available since the mid-2010s and are mostly limited to research or specialist hemostasis laboratories (Kershaw et al, 2018). One-stage chromogenic FIX discrepancy has been described in mild hemophilia B but the discrepancy appears to compromise the classification of severity, with patients changing between moderate and mild hemophilia B (Pouplard et al, 2009; Truedsson et al, 2020)

Principle of analysis for FVIII CSA: Many automated coagulometers have the ability to perform CSA but since these assays were originally performed manually using microtiter plates, it is still possible to use a manual method. In some (but not all) chromogenic assays, all the FVIII in the sample is activated by thrombin. Activated FVIII then accelerates the conversion of FX to FXa in the presence of activated FIX, phospholipids, and calcium ions. The FXa activity is assessed by hydrolysis of a p-nitroanaline substrate specific to FXa. The initial rate of release of p-nitroanaline (yellow color) measured at 405 nm is proportional to the FXa activity and thus to the FVIII activity in the sample. The results of the patient plasma and quality control sample are compared to the standard (reference/calibrator) plasma to quantify the CSA using the same principles as with the OSA (Baker et al, 2020). The proteins used in FVIII CSA kits may be human or bovine sourced. For measurement of endogenous FVIII:C, or standard or extended half-life FVIII therapy, the source of proteins does not affect the CSA. The source of proteins is important when bi-specific antibodies are present in the plasma (see section Part 6 on bispecific antibodies).

Principle of analysis for FIX CSA: In some (but not all) chromogenic FIX assays, all the FIX in the sample is activated by FXIa. Activated FIX then accelerates the conversion of FX to FXa in the presence of activated FVIII, phospholipids, and calcium ions. The FXa activity is assessed by hydrolysis of a p-nitroanaline substrate specific to FXa. The initial rate of release of p-nitroanaline (yellow color) measured at 405 nm is proportional to the FXa activity and thus to the FIX activity in the sample as described above.

Notes: If the test plasma FVIII (or FIX) concentration is close to zero (i.e. the optical density of all dilutions are similar to the blank), non-parallel lines may occur. The normal range should be established locally but often has a lower limit of 50–65 IU/dl in both FVIII and FIX. The FIX CSA may not accurately measure recovery of some extended half-life FIX products (Gray et al, 2020; Bowyer et al, 2022). Chromogenic assays can be used to measure mimetic effect in plasma containing bispecific antibodies (Jenkins et al, 2020; Bowyer et al, 2020; Bowyer et al, 2023). Bovine FX-containing FVIII CSA can be used to measure recombinant FVIII therapy in plasma also containing bispecific antibodies. If possible, a new diagnosis of mild hemophilia A or B should have FVIII:C or FIX:C also measured by chromogenic assay (Bowyer et al, 2018). Some caution should be exercised when using CSA to measure recombinant Porcine FVIII as underestimation may occur (Bowyer et al, 2022). FIX CSA may underestimate recombinant standard half-life FIX therapies (Nederlof et al, 2020).

Measurement of EHL FVIII and FIX molecules: Modifications to recombinant FVIII or FIX have been made to extend the in vivo half-life of the therapy by altering the conformation of the molecule. Extension can be made by addition of polyethylene glycol (PEG) moieties, covalent linkage of the heavy and light chains of FVIII, fusion to albumin or covalently fused to the fc (fragment crystallizable) portion of human IgG1. The post infusion monitoring of recombinant EHL FVIII or FIX concentrates is necessary for clinical management of the patient with hemophilia. A lower-than-expected response or reduced half-life can indicate the requirement for additional therapy or possible development of anti-drug antibodies. Laboratory studies conducted during the pharmaceutical trials of each EHL, highlighted issues with the accurate laboratory measurement of some molecules. Over or underestimation was reported with some EHL FVIII or FIX but this was dependent on methodology or the APTT used in OSA. The method of molecule modification is not predictive of the factor assay response such that accurate monitoring of the three PEGylated FVIII

molecules may not be possible with the same APTT reagents. For currently licensed EHL FVIII molecules, chromogenic FVIII activity assays are all considered suitable for accurate monitoring but for OSA this may be reagent dependent. For EHL FIX molecules, there is no single assay methodology or reagent that will accurately measure all three currently licensed concentrates. An ultra long half-life concentrate, rFVIII-FC-VWF-XTEN, efanesoctocog alfa (Altuviiio/Altuvoct), was granted regulatory approval in the USA in 2023 and Europe in 2024. Monitoring is recommended by FVIII OSA using a particular APTT reagent, Siemens Actin FSL. The prevalent APTT reagents, Siemens Actin FS and Werfen Synthasil over and underestimated efanesoctocog alfa respectively. Chromogenic FVIII assays overestimate by 2-3 times the expected activity (Pipe, 2009). It is therefore necessary to carefully assess whether the assays available in each hemostasis laboratory are suitable for accurate monitoring of each EHL used as replacement therapy in their center. Tables 17 and 18 give examples of EHL rFVIII and rFIX concentrates, how the potency of the product was assigned and whether OSA or CSA are acceptable for use in monitoring the post infusion activity

Table 17. EHL FVIII molecules

Name	Company	Molecule	Potency label	OSA	CSA	References
Adynovi/ Adynovate Rurioctocog alfa pegol	Takeda	FVIII 2 x 10 kDa PEG	CSA	Varied results	Yes	Turecek et al, 2016 Bulla et al, 2017
Afstyla Ionoctocog alfa	CSL Behring	Single chain BDD FVIII	CSA	Results are approximately half of CSA	CSA	Bowyer et al, 2017 St Ledger et al, 2018
Elocta/Eloctate efmoroctocog	Sobi	FVIII FC fusion	CSA	yes	Yes	Sommer et al, 2014 Powell et al, 2012 Pouplard et al, 2020
Esperoct Turoctocog alpha pegol	Novo Nordisk	BD truncated rFVIII 40 kDa PEG	CSA	Varied results	Yes	Pickering et al, 2016 Hillarp et al, 2017
Jivi Damoctocog alfa pegol	Bayer	BDD rFVIII 60 kDa PEG	CSA	Not silica or kaolin APTT reagents	Yes	Gu et al, 2014 Church et al, 2018
Altuvoct/Altuviio Efanesoctocog alfa	Sanofi	rFVIII FC-VWF-XTEN	OSA	Actin FSL recommended	Overestimates 2-3 fold	Pipi et al, 2024

Table 18. EHL FIX molecules

Name	Company	Molecule	Potency label	OSA	CSA	References
Alprolix etrenonacog	Sobi	rFIX FC fusion	OSA	Not some silica or kaolin APTT reagents	Yes	Sommer et al, 2014 Bowyer et al, 2019 Persson et al, 2018
Idelvion albutrepenonacog	CSL Behring	rFIX albumin fusion	OSA	Varied results	no	Persson et al, 2018 St Ledger et al, 2016 Kitchen et al, 2017 Horn et al, 2019 Pouplard et al, 2019
Refixia/Rebinyn Nonacog beta pegol	Novo Nordisk	rFIX 40 kDa PEG	OSA	Only Cephascreen and Synthafax validated	Yes	Bowyer et al, 2016 Tiefenbacher et al, 2017 Young et al, 2016

Bispecifific Antibodies:

Measurement of bispecific antibodies: Bispecific antibodies are a class of non-replacement therapy for hemophilia A. They act to bridge human FIXa and human FX, in the absence of FVIII, to promote activation of FX. Bispecific antibodies differ from native FVIII in a number of intrinsic aspects, including a lack of regulatory mechanisms, which impact hemostasis assays (Lenting et al, 2017). Future, more potent, generations of bispecific antibodies may have a greater impact on hemostasis testing (Bowyer et al, 2023).

APTT and bispecific antibodies: Bispecific antibodies do not require activation to participate in the activation of FX. In the presence of bispecific antibodies, the APTT is dramatically shortened, often to below the bottom of the reference range (Bowyer et al, 2020; Bowyer et al, 2023). The APTT is not sensitive enough to changes in bispecific antibodies concentration to use in the monitoring of these therapies, however a prolongation to the APTT in a patient with a previously short APTT may indicate a loss of efficacy or compliance (Druzgal et al, 2020; Valsecchi et al, 2021).

OSA and bispecific antibodies: In the presence of bispecific antibodies, standard, plasma-calibrated, APTT-based assays, including FVIII, FIX, FXI, XII, protein C, protein S, and activated clot time (ACT), overestimate the amount of clotting factor or natural inhibitor so are unsuitable for use (Bowyer et al, 2023; EMA, 2018).

Modified FVIII OSA and bispecific antibodies: Commercial, product-specific calibrator (standard/reference) and quality control plasmas are available for the first generation of bispecific antibodies. The one-stage FVIII assay can be modified using these product-specific calibrators alongside an increased plasma dilution (dilutions of 1/40 or 1/80 instead of 1/10) to measure drug concentration of bispecific antibody in µg/ml. This modified assay will also measure any endogenous or replacement FVIII present in the plasma (Bowyer et al, 2020).

Chromogenic FVIII and bispecific antibodies: FVIII CSA that contain human FX and FIXa are sensitive to the presence of bispecific antibodies and measure some "mimetic or surrogate" FVIII-like activity (Bowyer et al, 2020; Bowyer et al, 2023). Human CSA can be used as a marker of the presence of bispecific antibodies in patients receiving prophylaxis. This is not interchangeable with the drug concentration level detailed above. The human CSA will also measure any endogenous or replacement FVIII present in the plasma (Bowyer et al, 2020). FVIII CSA that contain bovine FX and either human or bovine FIXa are insensitive to the presence of first-generation bispecific antibodies but may demonstrate some sensitivity to next generation bispecific antibodies (Bowyer et al, 2020; Bowyer et al, 2023). Bovine FX CSA should

be used to measure any endogenous FVIII or replacement FVIII therapy and to measure residual FVIII in the Bethesda inhibitor assay as detailed below.

Bethesda inhibitor assay and bispecific antibodies: The measurement of residual FVIII following incubation in the Bethesda inhibitor assays is usually by OSA (Verbruggen B, 1995) although chromogenic and fluorogenic measurement have been validated (Miller et al, 2021). In the presence of bispecific antibodies, OSA cannot be used so the CSA must be used. It is important that the CSA kit used contains bovine FX and either human or bovine FIXa to exclude the effect of the bispecific antibody, otherwise the inhibitor titer will be underestimated (Bowyer et al, 2021; Miller et al, 2021).

Post-FVIII and FIX Infusion Monitoring: The post-infusion monitoring of plasma-derived or recombinant standard half-life FVIII or FIX concentrates is necessary for clinical management of the patient with hemophilia. A lower-than-expected response or reduced half-life can indicate the requirement for additional therapy or possible inhibitor development. Measurement of replacement therapy should ideally be performed using the same assay method and reagents that were originally used to assign potency to the product. If this is not possible then an alternative, validated assay, should be used. In Europe, potency labelling for FVIII concentrates is by CSA (Barrowcliffe et al, 2002) and for FIX concentrates is by OSA (Kitchen et al, 2016). The United States Food and Drug Administration (FDA, 2020) recommends the use of OSA for FIX concentrates however, some FVIII concentrates have potency assigned by OSA and some by CSA. The WFH recommends the use of a FVIII or FIX assay that has been validated for use with the specific concentrate used for treatment and is calibrated with a plasma standard traceable to the current WHO international standard (Srivastava et al, 2020). Other guidelines recommend the use of OSA or CSA calibrated with plasma standards can be used to monitor plasma derived FVIII concentrates unless there is evidence to the contrary and use of a OSA for FIX concentrates (Gray et al, 2020). Tables 19 and 20 give examples of commonly available plasma derived and recombinant standard half-life FVIII and FIX concentrates, how the potency of the product was assigned and whether OSA or CSA are acceptable for use in monitoring the post infusion activity.

Table 19. Plasma-derived concentrate examples

Concentrate	Factor	Potency assignment	CSA	OSA	References
Factane	VIII	CSA	Yes	Yes	Adcock et al, 2018
Octanate	VIII	CSA	Yes	Yes	
FVIII 8Y	VIII	CSA	Yes	Yes	
Haemoctin	VIII	CSA	Yes	Yes	
Octaplex	VIII	CSA	Yes	Yes	
Recombinate	VIII	CSA	Yes	Yes	Jennings et al, 2007
Fanhdi	VIII	CSA	Yes	Yes	Peyvandi et al, 2016
Hemofil M	VIII	CSA	Yes	Yes	Peyvandi et al, 2016
Emoclot	VIII	CSA	Yes	Yes	Peyvandi et al, 2016
Replenine	IX	OSA		Yes	
Betafact	IX	OSA Pathromtin SL (silica)	Likely acceptable	Yes	Adcock et al, 2018
Mononine	IX	OSA Pathromtin SL (silica)	Yes	Yes	Bowyer et al, 2016 Wilmot et al, 2014
Octafix	IX	OSA Pathromtin (Kaolin)	No data	Yes	
Alphanine	IX	OSA		Yes	Aznar et al, 2009

Table 20. Recombinant standard half-life concentrates examples

Concentrate	Factor	Potency assignment	CSA	OSA	References
Advate	FVIII-full length	CSA	Yes	Yes	Kitchen et al, 2016 Kitchen et al, 2019
Refacto AF	FVIII-BDD	CSA	Yes	Yes, with Refacto lab standard	Kitchen et al, 2016 Morfini et al, 2003 Ingerslev et al, 2004
Novo8	FVIII-BDD	CSA	Yes	Yes, but some overestimation at trough levels	EMA, 2021 Viuff et al, 2011
Nuwiq	FVIII-BDD	CSA	Yes	Yes	Lissitchkov et al, 2016 EMA, 2022 Klukowska et al, 2016 Tiefenbacher et al, 2019
Xyntha	FVIII-BDD	OSA	No-overestimation	Yes	FDA, 2020
Kovaltry	FVIII	CSA	Yes	Yes	Mahlangu et al, 2018 Kitchen et al, 2016
Benefix	IX	OSA	Insufficient data	Yes	Bowyer et al, 2016 Kershaw et al, 2018 Sommer et al, 2014
Rixubis	IX	OSA Pathromtin SL validated	Yes	Yes	Kershaw et al, 2018 Gritsch et al, 2014

Gene Therapy: Hemophilia A and B are monogenic disorders and therefore ideal candidates for gene manipulation. A varied range of gene therapy strategies, including gene editing, have been researched in humans for both disorders since the early 2000s (De Wolf et al, 2023). In the mid 2020s, regulatory approval was granted in some countries for the limited use of gene therapy products for treatment of persons with hemophilia A or B. The measurement of FVIII or FIX transgene expression is essential to determine duration of response and whether additional therapies are required to achieve hemostasis. To minimize inter-laboratory variability, it is sensible to restrict routine monitoring to a limited number of specialized hemostasis laboratories in each country. Clinical trials of several gene therapies have reported assay variability with chromogenic FVIII and FIX activities 1.5 to 3.0-fold lower than one-stage activities. Differences have been reported between reagents in the same assay method. Due the constraints of adequate plasma volume it is difficult to conduct multicenter laboratory comparison studies in samples from patients who received gene therapy. However, there are limited data available regarding measurement of the candidate FVIII or FIX molecules using a range of methods or reagents. Wherever possible, to minimize variability, the reagents and methods used in the pharmaceutical clinical trials should be used by laboratories for monitoring post gene therapy expression (Table 21).

Table 21. Gene therapy for hemophilia A and B

	Pharmaceutical company	Trade name	Assay method	Reagents
Hemophilia A				
Valoctocogene roxaparvovec	Biomarin	Roctavian	CSA	Coatest SP4
Hemophilia B				
Etranacogene dezaparvovec	CSL Behring	Hemgenix	OSA	Synthasil
Fidanacogene elaparvovec	Pfizer	Beqvez	OSA	Synthasil

Gene therapy for hemophilia A: All approaches for hemophilia A have used AAV vectors and B-domain deleted (BDD) recombinant FVIII. Phase 1–3 trials of Roctavian (AAV5-FVIII-SQ, Valoctocogene roxaparvovec, Biomarin) measured transgene FVIII:C with Coatest SP4 CSA and OSA using Siemens Actin FSL APTT reagent on Siemens BCS XP analyzer (Rangarajan et al, 2017). CSA FVIII:C were approximately half of OSA FVIII:C (Mahlangu et al, 2023). Rosen et al reported comparable results between Coatest SP4 and Hyphen Biomed CSA when measuring AAV5-FVIII-SQ (4). A two-center comparison of FVIII:C measured by OSA or CSA in plasma from patients following therapy with Roctavian reported an approximate 1.65-fold difference between OSA and CSA. Similar FVIII:C results were obtained with Hyphen Biomed CSA and two other CSA (Platton et al, 2024). The authors concluded that OSA were not suitable for measuring FVIII following gene therapy with Roctavian and only CSA should be used.

Gene therapy for hemophilia B: The two currently approved hemophilia B products, Hemgenix (Etranacogene dezaparvovec, CSL Behring) and Beqvez (Fidanacogene elaparvovec, Pfizer), both use a naturally occurring, highly active FIX variant, FIX-Padua (R338L) (Simioni et al, 2009). Pharmaceutical program have only reported FIX activity by OSA in their study reports. Variability between reagents and methodologies have been reported when measuring FIX-Padua following gene expression and in plasma spiked with the FIX-Padua molecule. A global field study of plasma spiked with a recombinant R338L molecule (FLT180a, verbrinacogene setparvovec, by Freeline Therapeutics which is currently paused at the end of phase 1/2 testing) reported a 3-fold difference in FIX activity between 15 different OSA and CSA. A 1.8-fold variation was observed across 13 APTT reagents in the OSA whilst results from both CSA were approximately half that of expected activity measured by Synthasil OSA (Foley et al, 2023). Measurement of Beqvez transgene FIX-Padua activity highlighted assay differences between five APTT reagents in the OSA and between OSA and CSA (Robinson et al, 2021). In a global field study using plasma from participants in the phase 1/2a gene therapy trial, FIX:C was higher with the silica-activated APTT reagent, Synthasil, in the OSA than with the ellagic acid-activated APTT reagents, Actin FS and Actin FSL, or CSA (Pittman et al, 2024). There is minimal laboratory data available for the measurement of FIX activity following Hemgenix gene therapy. The Summary of Product Characteristics states that FIX activity is lower by CSA than OSA (EMA, 2024). Phase 1–3 clinical trials FIX used Synthasil APTT reagent in the OSA and an undisclosed CSA; OSA FIX activities were at least two-fold higher than with CSA (Pipe et al, 2023; Miesbach et al, 2018).

Laboratory Aspects for Hemostatic Rebalancing Therapy Treatment: Non-factor replacement therapies for hemophilia A or B aim to promote coagulation and rebalance hemostasis by targeting natural anti-coagulants or inhibitors of coagulation including antithrombin, tissue factor pathway inhibitor (TFPI), protein C, or protein S (Nogami and Shima, 2023). Some of these therapies have been approved for use in certain patient groups, others are currently in pharmaceutical trials.

Molecules that target antithrombin: Heparin-activated antithrombin (AT) exerts inhibitory actions on thrombin, FXa, FIXa, FXIa, and FXIIa (Rezaie et al, 2020). A small interfering RNA molecule, fitusiran, which targets AT synthesis in hepatocytes, has been developed to improve thrombin generation (Young et al, 2023). In clinical trials an 82–87% reduction in AT was associated with increased thrombin generation (Pasi et al, 2021). Target AT activity levels are 15–35 IU/dl. AT assays are well established as part of

thrombophilia testing, but it is rare to measure such low AT activities. A global comparative laboratory field study assessing the measurement of a range of AT activities (9–100 IU/dl) concluded that some AT assays should not be used to monitor AT during fitusiran therapy (Chhabra et al, 2024).

Molecules that target TFPI: Anti-TFPI antibodies target the Kunitz 2 domain of TFPI and prevent binding to activated FX thus enabling FXa generation to continue (Mast et al, 2022). The first anti-TFPI monoclonal antibody (concizumab, Novo Nordisk, Denmark) was approved for use in 2023 in Canadian hemophilia B patients with inhibitors. An alternative anti-TFPI antibody, marstacimab (Pfizer, USA) is under consideration for approval in USA and Europe for people with hemophilia A or B without inhibitors (Matino et al, 2023). TFPI assays are available in some research or specialist laboratories, but the clinical utility of measurement is unclear.

Molecules that target activated protein C (APC): APC, in conjunction with cofactor protein S, inactivates FVa and FVIIIa to prevent further thrombin generation. FV Leiden is a p.Arg506Gln (c.1691G>A) mutation at the primary APC cleavage site in activated FV. The presence of FV Leiden slows the inactivation of FVa by APC and is the most common cause of thrombophilia in humans (Van Cott et al, 2016). Alternative approaches to target APC are in clinical trials. A humanized monoclonal antibody that inhibits activated protein C has been reported to restore hemostasis in hemophilic mice (Jiang et al, 2023) and a serine protease inhibitor (serpin) which only targets APC, not the precursor protein C, has commenced human trials (Baglin et al, 2023). Assays for protein C and APC are routinely available in many tertiary hemostasis laboratories should measurement be required for drug monitoring.

Molecules that target protein S: Protein S is a cofactor for TFPI and APC which function to restrict thrombin generation. Targeting protein S using a small interfering RNA has been reported to improve hemostasis in hemophilia mice (Prince et al, 2020) and a protein S antibody has been used to enhance FIX replacement therapy in the thrombin generation of patients with hemophilia B (Wilson et al, 2024). Free protein S and total protein S, which also measures protein S complexed with the complement regulator, C4b-binding protein, are routinely available in many tertiary hemostasis laboratories should measurement be required for drug monitoring.

Thrombin generation assays: Thrombin generation (TG) assays are global assays which can assess overall hemostatic potential and highlight hyper- or hypocoagulability in plasma (Ninivaggi et al, 2021). There are several in-house and commercial chromogenic or fluorogenic TG assays available which commonly trigger TG using tissue factor, FXIa, or FIXa. Due to lack of standardization, there is poor correlation between them (Devreese et al, 2007). Despite these issues, assays of TG are often used in pharmaceutical trials to assess the effect of new molecules on hemostasis.

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PART 7 Detecting and Quantifying Congenital and Acquired Functional Inhibitors in Hemostasis

Silmara Montalvão

TOPICS COVERED

- ✓ Different Antibody Kinetics
- ✓ Samples for the FVIII/FIX Inhibitor Test: Collection, Dispatch, and Preparation
- ✓ Normal Pool Plasma for Inhibitor Testing
- ✓ Protocol for the FVIII and FIX Inhibitor Test
- ✓ von Willebrand Factor Inhibitors Assays
- ✓ Anti-Drug Antibody (ADA) in the Coagulation Laboratory - Emicizumab

Identifying the development of antibodies is vital if the hemophilia treatment program is to be able to provide adequate medical care (Peyvandi et al, 2016; Srivastava et al, 2020). In the context of hemophilia, inhibitors are polyclonal IgG antibodies to FVIII or FIX and mostly of the high-affinity IgG4 subclass (Montalvão et al, 2015). Inhibitors neutralize the factor concentrate administered to the patient, making it difficult to prevent and treat bleeding (Pratt et al, 2021). The appearance of inhibitors is the result of a multi-step process involving environmental and genetic determinants. In severe hemophilia A, FVIII inhibitors form in approximately 30% of patients, usually during the first 20–30 days of exposure. In severe hemophilia B, the cumulative incidence of inhibitor development is lower than in severe hemophilia A and reaches 4–5% 9–11 days after exposure (Ljung et al, 2019). The treatment of acute bleeding in patients with inhibitors depends on the titer of the inhibitor. Patients with a low inhibitor titer (<5 UB/ml) can be treated with standard replacement therapy, factor concentrate, although it requires higher doses to overcome the neutralizing effects of the inhibitor. For patients with high titer inhibitors (>5 UB/ml), the only effective therapies for treating bleeding are bypassing agents (Ljung et al, 2019). The three available bypassing agents used in hemophilia A and B are (1) activated prothrombin complex concentrate (aPCC), (2) two forms of recombinant activated FVII (rFVIIa), and (3) recombinant porcine FVIII. Newly developed hemostatic drugs, such as humanized bispecific antibodies (e.g. emicizumab), RNA interference (e.g. fitusiran), and anti-tissue factor inhibitor (anti-TFPI) agents, among others, are available in some countries to prevent bleeding. Immunological tolerance induction (ITI) is used to eradicate inhibitors and involves frequent intravenous injections of factor concentrate over a period of months. In hemophilia A, ITI is effective in around 65 to 70% of patients. Monitoring the inhibitor titer of these patients is essential for evaluating and managing the protocol. Laboratory investigation of the inhibitor should be carried out using the modified Bethesda method. Although this test has a high CV, it is the reference test used to titrate inhibitory antibodies. In some cases, screening for inhibitors using the APTT test can be carried out before titrating the antibodies. However, due to the limitations of the test, negative results should not be used to exclude the possible presence of an inhibitor. Further information can be found in chapter 6 of this manual. An alternative to screening for antibodies against FVIII or FIX, is the use of an immunological test. A variety of immunological assays have been studied, and even though these tests are more sensitive than functional tests, they do not discriminate between inhibitory and non-inhibitory antibodies and are therefore not yet useful in clinical practice for detecting or monitoring functional inhibitors. However, studies have shown that IgG4 subclass antibodies are correlated with functional inhibitors of FVIII and FIX (Awasthi et al, 2022; Montalvão et al, 2015; Moorehead et al, 2015).

Different Antibody Kinetics: Different kinetics of antibodies can affect the analysis of the data and consequently lead to misinterpretation. When FVIII or FIX inhibitors act in an inhibition test in a dose-dependent manner, such as completely inactivating FVIII or FIX, these inhibitors are called "Type I" inhibitors. Inhibitors that demonstrate a more complex kinetic behavior are usually called "Type II" inhibitors, incompletely

inactivating FVIII. Type I inhibitors usually develop in patients with congenital hemophilia A or B in response to FVIII or FIX concentrate, while type II inhibitors usually occur in patients with acquired hemophilia or mild hemophilia A. Type I FVIII inhibitors are time- and temperature-dependent because their target, FVIII, is complexed with its carrier protein, VWF. FIX inhibitors are not time and temperature dependent.

Samples for the FVIII/FIX Inhibitor Test: Collection, Dispatch, and Preparation:

Sample collection and dispatch: Samples for the FVIII and FIX inhibitor assays are collected in 3.2% trisodium citrate (0.105–0.109 M), the same type of sample used for most coagulation tests. The citrated whole blood should be centrifuged within 4 hours of blood collection, and the centrifugation should be at 1500g for 15 minutes. Plasma samples positive for FVIII and FIX inhibitors, not whole blood, can be stored at room temperature for 1 week, or frozen at -70°C and stored for up to 15 years. It is important to remember that, unlike traditional procedures for analyzing coagulation, in this type of sample, it is the antibody that must be preserved. This information is very useful for laboratories that need to send samples to another laboratory for testing, as transport does not depend on dry ice.

Heating the samples: Patient samples used for inhibitor detection may contain exogenous FVIII or FIX due to recent factor concentrate infusions, such as: (1) prophylaxis, (2) bleeding treatment, (3) ITI therapy, or (4) endogenous FVIII or FIX if the test is performed on mild or moderate hemophilia. The presence of exogenous FVIII or FIX can significantly affect the detection of inhibitors, with underestimation of the inhibitor titer and false-negative results (Batty et al, 2014; De Lima Montalvão et al, 2015). Heating the samples for 30 minutes at 56°C dissociates the antigen-antibody complex and denatures the factor. To standardize the test, it is recommended that all test samples are always pre-warmed, even if no exogenous FVIII or FIX is expected. The heating procedure should be followed by a centrifugation step, 2 minutes at 4000g, to remove the residues in the plasma caused by the heating. This step of heating the samples for the inhibitor test has not been evaluated for all the products available for patient treatment. Therefore, for each molecularly modified FVIII or FIX product, it must be demonstrated that residual FVIII or FIX deteriorates because of the heating procedure. Emicizumab is not destroyed by the preheating step, however, FVIII inhibitors can be measured in the presence of emicizumab using a bovine chromogenic FVIII method. It is worth remembering that some patients use factor concentrate and emicizumab concomitantly, so heat treatment is necessary.

Diluting the sample: To titrate the inhibitor, the test must be carried out with multiple dilutions of the test plasma. The way in which the dilution factor is selected is not limited to a specific number, as it depends on whether the previous inhibitor titer is known.

Normal Pool Plasma for Inhibitor Testing: To assess the activity of the inhibitor in the test sample, it is necessary to present an "external" source of FVIII or FIX for this inhibitor. This external source is based on the normal plasma pool, and so it is important to consider that any error at this stage can generate false positive and negative results. When preparing a pool for inhibitor testing, the FVIII or FIX activity should be measured and the deviation from this value should be monitored over storage time or when producing a new batch. A pool of normal plasma should be used to ensure that the level of FVIII or FIX is close to 1 IU/ml (100 per cent). A lower factor level in the normal plasma pool may result in an overestimation of the inhibitor titer, while a higher factor level may result in an underestimation of the inhibitor titer. A maximum deviation of 5% of 1 IU/ml of FVIII or FIX in the normal plasma pool is acceptable. The external source of FVIII or FIX can be made by preparing the normal plasma pool or from a commercial source and can be frozen or lyophilized. A minimum of 20 donors is suggested to obtain plasma with a level of FVIII or FIX close to 1 IU/ml. FVIII is a thermolabile clotting factor, which means that during a 2-hour incubation at 37°C, there will be a significant loss of FVIII activity due to a change in pH. To stabilize the pH during incubation, the normal plasma used must be buffered. This can be done using imidazole buffer or HEPES buffer.

Protocol for the FVIII and FIX Inhibitor Test: In 1975, Kasper et al described a method for determining FVIII and FIX inhibitors, and to date, this is still the most standardized test, known as the Bethesda test. In

1995, the Nijmegen method was described, a modification of the Bethesda assay, with some differences: (1) the introduction of buffered pool to improve the stability of FVIII during incubation, and (2) FVIII-deficient plasma to use in the control mixture. This modified Bethesda method was recommended by the International Society on Thrombosis and Haemostasis (ISTH) as the reference method for the FVIII inhibitor test (Verbruggen et al, 1995). The inhibitor test is an indirect method and is based on the principle of the inactivation of the coagulation factor from a known external source by the inhibitor in the test sample during an incubation period. One Bethesda unit is defined as the amount of inhibitor that will neutralize 50% of one unit of FVIII added in 2 hours at 37°C.

Reagents and equipment:

- Dilution buffer (part 1)
- Imidazole or HEPES buffer (below)
- Pool of normal plasma (part 2)
- FVIII-deficient plasma
- Cephalin (APTT reagent)
- Plastic tubes

Table 22. Imidazole or HEPES buffer

Buffer	
Imidazole	Mix 1 part of 4M imidazole buffer with 39 parts of the normal plasma pool. After mixing, the pH should be adjusted to between 7.3 and 7.5.
HEPES	Mix 1 part HEPES 1M buffer with 9 parts normal plasma pool. After mixing, the pH should be adjusted to between 7.3 and 7.5.

Method: Prepare dilutions of the test plasma in plastic tubes to a final volume of 0.2 ml using the dilution buffer. The dilutions required for each patient may change. A suggested starting point would be to start with an undiluted sample and then carry out dilutions of 1/2, 1/4, 1/8, etc.

Note: If the patient has previously had an inhibitor assay, the level can provide a rough guide as to which dilutions should be used. Pipette 0.2 ml of FVIII-deficient plasma into another plastic tube. This will serve as a control tube.

Note: In the original Bethesda assay, imidazole buffer was used to prepare a control mixture with the normal plasma pool. In the Nijmegen assay, the imidazole buffer is replaced with FVIII-deficient plasma. Some differences have been observed between the use of immunodepleted factor-deficient plasma, chemically depleted plasma, and congenitally deficient plasma. These differences may be due to the lack or presence of VWF in the plasma, the presence of antibodies, or the presence of FVIII fragments. As VWF is present in the normal plasma pool, it is not necessary for the diluent of the control mixture to also contain VWF, and to reduce costs, the factor-deficient plasma can be replaced with 4% bovine serum albumin (BSA). Four percent buffered BSA is a reliable and economical substitute for FVIII or FIX plasma and favors the standardization of the method.

✓ Add 0.2 ml of buffered normal plasma pool to the control tube and the test plasma dilutions. The FVIII level of all tubes will be approximately 0.5 IU/ml. This is considered to mean that the buffered normal plasma pool has 1 IU/ml of FVIII.
✓ Cap, mix, and incubate all the tubes at 37°C for 2 hours.
✓ After 2 hours, transfer all tubes to an ice bath, unless the FVIII assay is to be performed immediately.
✓ Perform the FVIII test on all incubation mixtures using the usual FVIII assay method, one-stage or chromogenic method (Part 6).
✓ Read the residual FVIII of each test mixture, using the control as 100% (0.5 IU/ml).

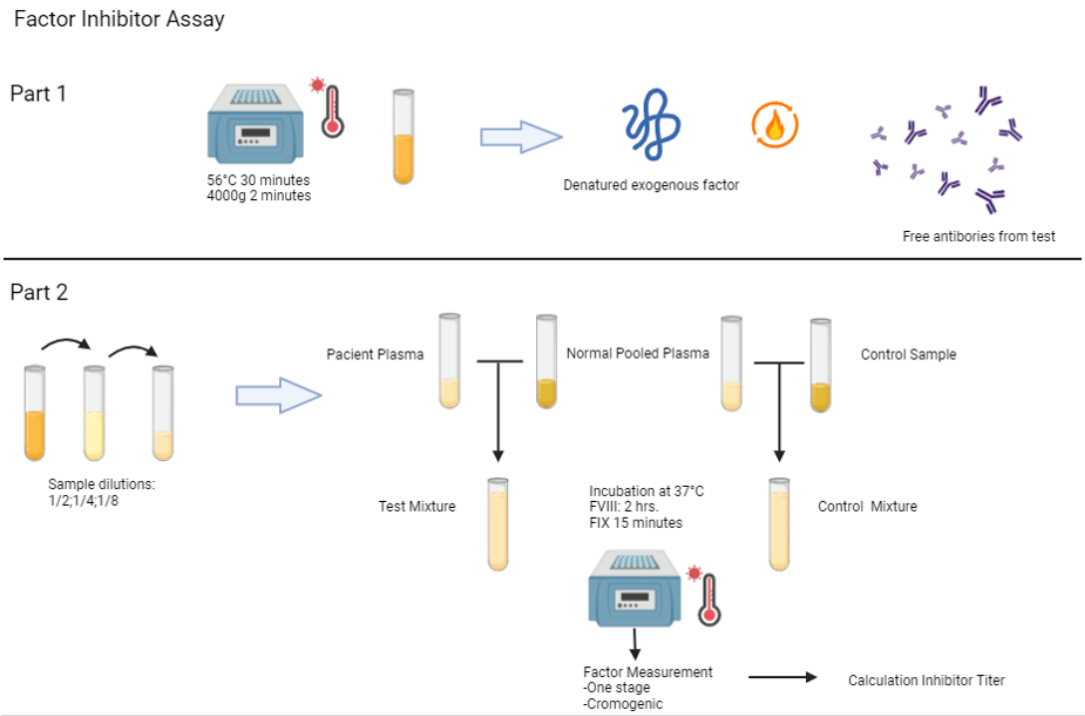

Figure 8. Inhibitor assay procedures

Results and interpretation: The dilution that provides a residual FVIII closest to 50%, but within the 25% to 75% range, is chosen for the inhibitor calculation. Any residual FVIII of <25% or >75% should not be used for inhibitor level calculations. A graph of % residual FVIII versus inhibitor units can be made on log-log paper from the inhibitor unit definition. Read the inhibitor level corresponding to the residual FVIII for each test mixture and correct the dilution. For example, if the value closest to 50% of the residual factor was found in the 1/4 dilution (i.e. in the mixture 1/4 + normal pool), the result, which will be close to 1 Bethesda Unit (BU), should be multiplied by 4.

- ✓ 1/4 dilution + normal pool
- ✓ FVIII residual = 50%
- ✓ Inhibitor unit (from graph) = 1 BU
- ✓ Multiply by the dilution factor (1/4) = 4 BU

Note: The inhibitor assay is based on determining the residual FVIII or FIX of the mixture of test and control plasma previously incubated. The Bethesda and Nijmegen assays were developed using a one-stage coagulation factor assay. However, testing for inhibitors using this assay has limitations. Clot formation in this test can be affected, for example, by the lupus anticoagulant (non-specific clotting inhibitors) and drugs such as emicizumab. An alternative to avoid these problems is the use of a chromogenic method. Another advantage of using a chromogenic method instead of a coagulation assay is the greater accuracy of the results. An undiluted patient sample with residual activity >75% can be reported as <0.4 BU/ml. For FVIII inhibitors, the Scientific and Standardisation Committee (SSC) of the ISTH recommends considering a result ≥0.6 BU/ml as positive. In addition to the calibration curve, the inhibitor titer can be calculated using the formula: (2-log %RA)/0.301. In the case of a type I inhibitor, a curve from a patient's test plasma shows parallelism with the calibration curve. Non-parallelism with the calibration curve indicates a different kinetic type II inhibitor standard. For inhibitors with type II kinetics, use the lowest dilution that comes close to 50% of the residual activity for the final calculation of the inhibitor titer.

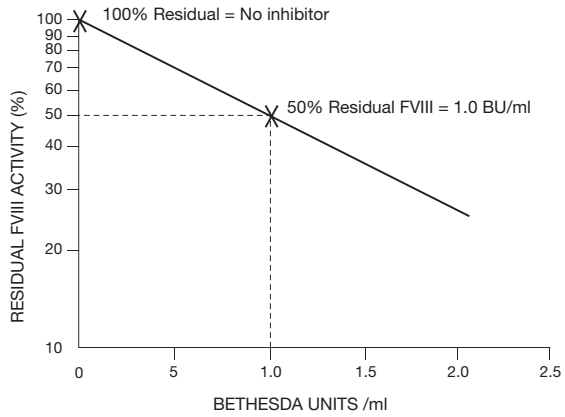

Figure 9. Residual factor activity calculation

Notes: The most frequently encountered functional inhibitors of hemostasis are lupus anticoagulants, which are not directed against specific coagulation factors and whose presence must be excluded before testing for specific factor inhibitors. Quantification of the inhibitor titer is carried out in the laboratory, preferably using the Bethesda assay modified by Nijmegen Bethesda, because this modification offers greater specificity and sensitivity compared to the original Bethesda assay. Positive FVIII inhibitor results below 2.0 BU can be confirmed with the chromogenic method, as it has less analytical interference and greater accuracy compared to the one-stage method. The chromogenic method is also the best choice if lupus anticoagulants are suspected in the test sample or if it contains therapeutic anticoagulants such as heparin or direct FXa or FIIa inhibitors. Non-neutralizing anti-FVIII antibodies that are not detected by the Nijmegen-Bethesda assay can be clinically relevant because they can increase FVIII clearance and can be measured by ELISA.

von Willebrand Factor Inhibitors Assays: VWD is considered the most common hereditary bleeding disorder known in humans, with a population prevalence of 1% and a symptomatic prevalence of 1 in 1000 (Bowman et al, 2010). Treatment options include the infusion of VWF concentrates, which usually also contain FVIII, administered to prevent or treat bleeding episodes. Alloantibodies against VWF have a prevalence of between 7 and 9.5% (James et al, 2013; Pagliari et al, 2023). In these cases, treatment with VWF concentrates is ineffective, and episodes of anaphylaxis have been reported with subsequent exposure to VWF (James et al, 2013). As we already know, VWF deficiency can be explained by different mechanisms resulting from the types of genetic defects identified. This variability of genetic defects contributes to a heterogeneity of inhibitors targeting different epitopes of the VWF molecule. For this reason, the laboratory detection of these antibodies is challenging (Connell et al, 2021; Miller, 2021; Sarji et al, 1974). In 1974, Sarji et al (1974) reported for the first time a case of alloantibody against VWF in a multi-transfused patient. The VWF inhibitor was measured using a method analogous to the Bethesda method for FVIII inhibitors (Sarji et al, 1974). Although there is no standardization for the identification of VWF inhibitors, the Bethesda method has been used by most laboratories (Favaloro et al, 2022), with the difference of using methods that assess VWF activity instead of the FVIII or FIX test. There are currently different types of methods available for detecting VWF activity, with different sensitivities and specificities, so it is important to consider that this variability of methods influences the sensitivity and specificity of detecting these inhibitors (Favaloro et al, 2022). Antibodies against VWF do not have the characteristics of being time- and temperature-dependent, so they can be evaluated immediately after the mixing test (Sarji et al, 1974). The classic ristocetin cofactor method, which evaluates the interaction of VWF with platelets fixed in the presence of ristocetin, as well as the collagen evaluation method and the gain-of-function method, are options that have already been evaluated and which have been shown to be methods with good sensitivity and stability, despite presenting different results as discussed above. Other available methods have not yet been evaluated for this type of investigation. Immunological methods have also been described and detect neutralizing and non-neutralizing antibodies. In relation to the development

of autoantibodies that characterize acquired VWD, patients with myeloproliferative neoplasms (MPNs) are a subgroup that present bleeding complications related to VWF activity. False results are observed in samples from patients with MPNs, depending on the technology applied. The laboratory investigation of VWF inhibitors characterized by alloantibodies and autoantibodies, should be carried out with caution in view of the different methodological possibilities. The performance of all the modern methods currently available is still unclear (Noye et al, 2024; Favaloro et al, 2022).

Anti-Drug Antibody (ADA) in the Coagulation Laboratory - Emicizumab: Emicizumab is a bispecific antibody that binds to human FIX/FIXa and FX/FXa and acts as a mimetic of FVIII function. However, it is not regulated by the mechanisms that regulate FVIII (Mahlangu et al, 2018, Mahlangu et al, 2022). The APTT screening test is considerably reduced by emicizumab (i.e. below the reference range, regardless of the reagents used). Emicizumab affects all APTT-based laboratory tests and assays. Emicizumab also interferes with chromogenic assays to measure FVIII using human FIXa and FX, but not those using FIXa and FX of bovine origin (Bowyer et al, 2021; Jenkins et al, 2020). Emicizumab can be measured and reported in µg/ml using a modified one-stage assay with higher sample dilution and calibrated with calibrators specific for emicizumab. Anti-drug antibodies (ADAs) can develop after a single dose or repeated administration of a therapeutic protein, and can affect the pharmacokinetics, pharmacodynamics, efficacy, and/or safety of this therapeutic protein. Studies evaluating the characteristics of ADA in patients being treated with emicizumab show that the APTT test can be prolonged in conjunction with hemorrhagic episodes in the presence of antibodies with a neutralizing action (Novembrino et al, 2023; Valsecchi et al, 2021). In this case, when the level of emicizumab was measured, it showed a significant reduction. The neutralizing activity of these antibodies was not clearly identified in functional methods, even for the modified Bethesda assay (Kaneda et al, 2021). The level of the Bethesda unit identified seems to be lower than expected when compared to the test that measures the level of emicizumab. The role of functional tests for ADA has not yet been established, but they may be complementary to measuring the plasma level of the drug in these cases. The WFH recommends measuring emicizumab levels using a modified one-step assay with higher sample dilution and calibrated with specific calibrators for emicizumab (Srivastava et al, 2020).

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PART 8 Von Willebrand Disease Lab Investigation, Tulasi Geevar

Nitty S. Mathews and Annette Bowyer

TOPICS COVERED

- ✓ Assays of Platelet-Binding Activity of VWF- *Nitty S. Mathews*
- ✓ Von Willebrand Antigen - *Tulasi Geevar*
- ✓ Von Willebrand Factor Collagen Binding Assay - *Tulasi Geevar*
- ✓ Factor VIII Binding Assay - *Tulasi Geevar*
- ✓ Von Willebrand Factor Multimers - *Annette Bowyer*
- ✓ Interpretation of von Willebrand Tests - *Tulasi Geevar*
- ✓ Preanalytical Variables in VWD Diagnosis - *Tulasi Geevar*
- ✓ Tests in the Repertoire for VWD Diagnosis - *Tulasi Geevar*
- ✓ Diagnosis of von Willebrand Disease in Resource-Poor Setting - *Tulasi Geevar*

Assays of Platelet-Binding Activity of VWF (VWF Activity Assays): With all guidelines using the VWF:RCo/VWF:Ag ratio to determine whether the patient has a type 1 (quantitative) or type 2 (qualitative) VWD, the role of VWF:glycoprotein Ib (GPIb)-binding activity is of utmost importance in the classification of VWD. A multidisciplinary guideline panel established by the American Society of Hematology (ASH), ISTH, National Hemophilia Foundation (NHF, now the National Bleeding Disorders Foundation [NBDF]), and WFH, suggests performing newer assays that measure the platelet-binding activity of VWF (e.g. VWF:GP1bM and VWF:GP1bR) over the conventional VWF ristocetin cofactor assay (VWF:RCo) [Recommendation 4] (James et al, 2021). The panel judged there to be moderate benefits of the newer assays, reflecting the lower CV and higher reproducibility compared with VWF:RCo. The VWF:GP1bR assay requires the presence of added ristocetin and therefore may be subject to the same issues as the VWF:RCo assay with respect to ristocetin-binding polymorphisms. However, it uses a recombinant GP1b fragment instead of platelets. In contrast, the VWF:GPIbM assay uses a recombinant GPIbα molecule that contains several gain-of-function mutations; consequently, this GP1b variant binds to the A1 domain of VWF even in the absence of ristocetin (Laffan et al, 2014). In a 2022 EQA study of the College of American Pathologists VWF proficiency testing program (Salazar et al, 2022), the overall CV for VWF:RCo ranged from 14.6% to 23.5% for results on normal samples and from 24.3% to 44.8% for results on samples with low activity. The CVs were less for VWF:GP1bM and VWF:Ab, ranging from 8.0% to 34.8%, including both normal and abnormal samples. A study comparing different VWF:GP1b binding activity assays reported the sensitivity and specificity for differentiating type 1 and type 2 VWD, based on activity/ antigen ratios using a cut-off of 0.7, were 92% and 72.4% for VWF:RCo, 84% and 89.7% for GP1bR, and 92 % and 85.1% for GP1bM (Vangenechten et al, 2018).

Table 23. Summary of commercially available automated VWF activity assays

VWF Activity- ISTH nomenclature	Principle	Assays	Detection method
VWF:RCo	Ristocetin-induced aggregation of lyophilized platelets	1.Siemens BC von Willebrand Reagent (Siemens, Marburg, Germany) 2. STA-VWF:Rco (Diagnostica Stago, France)	Agglutination of lyophilized platelets
VWF:GP1bR	Ristocetin-induced binding of VWF to a recombinant GPIb fragment	1. HemosIL VWF Ristocetin Cofactor Activity 2. HemosIL AcuStar VWF Ristocetin Cofactor Activity (Instrumentation Laboratory, Bedford, MA, USA)	Latex bead agglutination 2. Chemiluminescence
VWF:GP1bM	Binding of VWF to a gain-of-function mutant GPIb fragment without ristocetin	Siemens Innovance VWF Ac (Siemens, Marburg, Germany)	Latex bead agglutination
VWF:Ab	Binding of a monoclonal antibody to a VWF A1 domain epitope (platelet binding site)	HemosIL VWF Activity (Instrumentation Laboratory, Bedford, MA, USA)	Latex bead agglutination

General disclaimer: The reagent kits/protocols listed below have been validated on one or more coagulation analyzers by the manufacturer to optimize product performance and meet product specifications. User defined modifications may not be supported as they may affect the performance of the system and assay results. It is the responsibility of the user to validate modifications to these instructions or use of the reagents on analyzers other than those included in specific manufacturer's Application Sheets or Instructions for Use.

Automated VWF activity assays:

VWF ristocetin cofactor assay (VWF:RCo): Dilutions of a standard (the ristocetin cofactor value of which is known) are made and mixed with lyophilized platelets, so a known amount of cofactor is added to the platelets. Ristocetin-induced aggregation is then measured, and a standard curve is drawn. Test samples are treated similarly and the ristocetin cofactor value is calculated from the standard graph. Primary sample: Citrated plasma.

Reagents:

- ✓ Lyophilized fixed platelets (BC von Willebrand Reagent[5], Siemens Healthcare Diagnostics, Marburg, Germany)
- ✓ Normal saline
- ✓ Calibration plasma
- ✓ Patient plasma and control plasmas (normal control [e.g. control plasma N] and one low level abnormal control [e.g. control plasma P])
- ✓ Ristocetin reagent (e.g. Revohem, 25mg/ml; reconstitute with 0.625 ml of distilled water and mix well)
- ✓ Distilled water

Reagent preparation:

- ✓ Reconstitute the Von Willebrand reagent with 4 ml of distilled water first and dilute with 7 ml of saline. Check the platelet count, it should be ~2,000,000 to 2,050,000/cumm. Add 50.0 µl of ristocetin reagent for 950 µl of platelets.
- ✓ Reconstitute calibrator plasma with exactly 1 ml of distilled water. Allow the reconstituted material to stand at room temperature (18–25°C) for 30 minutes. Then, swirl the vial gently before use. The material is stable for 8 hours at 15–25°C. It can be frozen and stored at -80°C or below.
- ✓ Abnormal control plasma (control plasma P). Reconstitute control plasma P with exactly 1 ml of distilled or deionized water. Shake carefully to dissolve without foam formation and let stand at room temperature (18–25°C) for 15 minutes. Before use, again shake carefully.
- ✓ Normal control (control plasma N). Reconstitute control plasma N with exactly 1 ml of distilled or deionized water. Shake carefully to dissolve without foam formation and let stand at room temperature (18–25°C) for 30 minutes. Before use, again shake carefully.

Procedure:

- ✓ The required reagents (lyophilized platelets with ristocetin), normal saline, and calibrator are loaded on to the coagulation analyzer.
- ✓ The standard curve is run.
- ✓ After the calibration is complete, the calibration curve should be reviewed and validated. The validated curve is the calibration curve used for result determination.
- ✓ Run the test sample.

QC protocol:

Run normal control (control plasma N) and low-level abnormal control (control plasma P).

Note: Controls and calibrator plasma need to be purchased separately.

Possible interference:

- ✓ Platelets are not diluted properly.
- ✓ Change in the ristocetin concentration.

Result interpretation:

Results are reported in %.

Expected values (evaluated on the BCS system; Siemens, Marburg, Germany):

Blood group	% of normality
All (n = 185)	58–172
O	49–142
A+B+AB	66–183

VWF:GPIbR assay (HemosIL® AcuStar VWF Ristocetin Cofactor Activity[6] ; Instrumentation Laboratory, MA, USA): This assay is a two-step immunoassay that quantifies VWF:RCo activity in human citrated plasma using magnetic particles as solid phase and a chemiluminescent detection system. In the first step, the sample is mixed with the ristocetin-containing assay buffer and magnetic particles coated with a recombinant fragment of glycoprotein platelet receptor of VWF (rGPIbα) by means of a specific monoclonal antibody which orient the GPIbα fragment in the proper way to interact with the VWF of patient sample in the presence of ristocetin. VWF present in the sample binds to the magnetic particles proportionally to

its ristocetin cofactor activity. After magnetic separation and washing, an anti-WWF monoclonal antibody labelled with isoluminol is added and incubated in a second step. After a new magnetic separation and washing, two triggers are added and the resulting chemiluminescent reaction is measured as relative light units (RLUs) by the ACL AcuStar optical system. This is proportional to the VWF:RCo activity concentration in the sample.

Primary sample: Citrated plasma.

Reagents (kit composition):

- ✓ VWF:RCo cartridge for 25 determinations

Each cartridge contains 1 vial of magnetic particle suspension coated with a rGP1bα by means of a specific mouse monoclonal antibody, I vial of assay buffer containing ristocetin sulphate, 1 vial of a tracer containing an anti-VWF mouse monoclonal antibody labelled with isoluminol, and 1 vial of sample diluent. The reagents are in a citrate or HEPES buffer containing BSA, mouse IgG, stabilizers, and preservative.

- ✓ VWF:RCo calibrator 1: HEPES saline solution containing BSA, mouse IgG, stabilizers, and preservatives.
- ✓ VWF:RCo calibrator 2: Lyophilized human plasma containing buffer, stabilizers, and preservatives.

Preparation/procedure:

- ✓ Gently invert the cartridge 30 times avoiding the formation of foam.
- ✓ After complete resuspension of the microparticles, place the cartridge on a solid surface and gently remove the shipping tab from the cartridge.
- ✓ Press the two tabs on the sides of the piercing cap (gray color) and apply pressure to the top portion of the cartridge until it snaps locked.
- ✓ Once the cartridge is steady, load onto the instrument.
- ✓ Diluted calibrator vials are transferred to the respective plastic barcoded tubes and loaded onto the analyzer (ACL AcuStar).
- ✓ The standard curve is run.
- ✓ After the calibration is complete, the calibration curve should be reviewed and validated. The validated curve is the calibration curve used for result determination.
- ✓ Run the test sample.

Quality control:

- ✓ Two level controls recommended (to be purchased separately).
- ✓ Each lab should establish its own mean and standard deviation.

Traceability of calibrators and controls:

Reported values were determined over multiple runs on the ACL AcuStar system using specific lots of reagents and against an internal house standard, which has been value assigned against the current international reference material for VWF and FVIII.

Results:

VWF:RCo results are reported in % which is equivalent to IU/dl.

Table 24. Expected values to VWF:RCo

Blood ABO type	Number of samples tested	HemosIL AcuStar VWF:RCo (VWF:GP1bR)
All	287	45.6 – 176.3 %
O	163	43.8 – 161.5 %
A+B+AB	124	53.8 – 210.8 %

Note: It is recommended that each lab should establish its own VWF:RCo normal ranges depending on the population served and the technique, method and equipment used. Linearity: 0.5–200.0%. Detection limit: 0.17%.

WF:GPIbM assay (Innovance® VWF Ac; Siemens Healthcare Diagnostics, Marburg, Germany): The assay principle makes use of the binding of VWF to its receptor GPIb. Polystyrene particles are coated with an antibody against GPIb. Recombinant GPIb (two gain-of-function mutations included) is added and binds to the antibody as well as to the VWF of the sample. Due to the gain-of-function mutations, VWF binding to GPIb does not require ristocetin. This VWF binding induces particle agglutination which can be measured as an increase in extinction by turbidimetric measurements.

Primary sample: Citrated plasma.

Table 25. Reagents (kit composition) Innovance® VWF Ac

Innovance® VWF Ac	Ingredients	Concentration	Source
REAGENT I	Buffer, sucrose, polystyrene particles coated with anti-GPIb monoclonal antibodies, amphotericin B, gentamicin	2.2 g/l	Mouse
REAGENT II	Buffered saline solution, heterophilic blocking reagent, detergent, polyvinylpyrrolidone, sodium azide	<1 g/l	
REAGENT III	Buffered saline solution, recombinant GPIb, amphotericin B, gentamicin	≤80 mg/l	

Preparation/procedure:

- ✓ The reagents (RI, RII, and RIII), imidazole buffer, and calibrator are loaded onto the analyzer.
- ✓ The standard curve is run.
- ✓ After the calibration is complete, the calibration curve should be reviewed and validated. The validated curve is the calibration curve used for result determination.
- ✓ Run the test sample.

Calibration: A standard curve is generated by automatic determination of different dilutions of standard human plasma and Owren's veronal buffer. The standard curve must be re-generated if there is a change in the instrument or in the lot of Innovance® VWF Ac used, or if control results are out of the acceptable range. Assay calibration is performed with standard human plasma which is calibrated against the VWF:RCo value of the international standard for blood coagulation FVIII and VWF in plasma.

Samples initially above the calibration range are diluted by the instrument resulting in a measuring range up to 600% of normal.

Quality control:

- ✓ Controls (two levels) must be purchased separately
- ✓ Normal range: Control plasma N
- ✓ Pathological range: Control plasma P

Results:

- ✓ The results are reported as % normality
- ✓ 100 % = 1 IU/ml

Expected values: Fresh plasma specimens obtained from apparently healthy donors were tested using the Innovance® VWF Ac assay on the BCS®/BCS® XP System with results (2.5th to 97.5th percentile) as shown in Table 26.

Table 26. Expected values to Innovance® VWF Ac assay

Blood group	Number of samples tested	% of normality
All	263	47.8–173.2
O	129	46.3–145.6
A+B+AB	134	61.4–179.1

Note: It is recommended that each lab should establish its own VWF:RCo normal ranges depending on the population served and the technique, method, and equipment used.

Linearity: 4 to 150%

Detection limit: 2.2%

VWF:Ab assay (HemosIL® VWF Activity, Instrumentation Laboratory, Bedford, MA, USA): The VWF activity kit is a latex particle enhanced immunoturbidimetric assay to quantify VWF activity in plasma. The activity of VWF is determined by measuring the increase of turbidity produced by the agglutination of the latex reagent. A specific anti-VWF monoclonal antibody adsorbed onto the latex reagent, directed against the platelet binding site of VWF (GPIb receptor), reacts with the VWF of patient plasma. The degree of agglutination is directly proportional to the activity of VWF in the sample and is determined by measuring the decrease of transmitted light caused by the aggregates._

Primary sample: Citrated plasma.

Reagents (Kit composition):

- ✓ Latex Reagent: A lyophilized suspension of polystyrene latex particles coated with purified anti-VWF mouse monoclonal antibody directed against a functional epitope of VWF, containing bovine serum albumin, stabilizers and preservative.
- ✓ Buffer: Tris buffer containing bovine serum albumin, stabilizers and preservative.

Preparation:

- ✓ Buffer: Ready to use
- ✓ Latex reagent: Dissolve the contents of each vial by pouring the entire contents of one vial of Buffer into one vial of Latex Reagent. Replace the stopper and swirl gently for a minimum of 20 seconds to completely dissolve the lyophilized latex. Make sure of the complete reconstitution of the product. It must appear as a homogenous and slightly milky suspension. Keep the reagent at 15–25°C for 30 minutes and invert to mix before use. Do not shake. Note: Avoid foam formation when homogenizing reconstituted reagents. Bubbles on top of the liquids may interfere with the instrument's liquid sensors.

Quality control: Calibration plasma, normal, and abnormal controls (to be purchased separately) are recommended for a complete quality control program. Normal Control and Special Test Control Level 1 are designed for this program. Each laboratory should establish its own mean and standard deviation.

Results: VWF activity results are reported in % normality.

Table 27. Expected values to VWF:Ab assay HemosIL® VWF activity

Blood group	Number of samples tested	% VWF activity
O	132	40.3–125.9
A+B+AB	134	48.8–163.4

Note: Due to many variables, which may affect results, each laboratory should establish its own normal range for VWF activity.

- ✓ Linearity: 19–130%
- ✓ Detection limit: 3.2%

Von Willebrand antigen (VWF:Ag): The VWF:Ag assay is a quantitative assay that measures the total amount of VWF protein present in the sample, which includes both functional and dysfunctional forms. VWF:Ag levels can be quantified by immunological methods that include ELISA, automated latex immunoassay (LIA), and, more recently, chemiluminescent immunoassay (CLIA). VWF:Ag assays are generally very reliable and reproducible. The lower limit of detection varies between assays, with the LIA tending to have slightly higher and the CLIA assays have the lowest limit of detection. VWF:Ag is an essential test in the diagnosis of VWD, but is limited as it only assesses the presence of VWF and does not assess its function. Used alone, VWF:Ag can only identify type 3 VWD where there is undetectable levels of VWF:Ag, usually less than 3 IU/dl. As per British Society for Haematology (BSH) and United Kingdom Haemophilia Centre Doctors' Organisation (UKHCDO) guidelines, diagnosis of type 3 VWD requires a VWF:Ag assay capable of measuring levels to <1 IU/dl. Additional VWF functional or activity assays with calculation of ratios of VWF activity to antigen assays are required for diagnosis and differentiation of type 1 and type 2 VWD. The normal range of VWF:Ag value varies with each laboratory, but is generally accepted to be between 50 and 200 IU/dl. Levels below 50 IU/dl are considered to be low, although this needs to be correlated with bleeding history and other VWF activity assays for a diagnosis of VWD. It is well known that individuals with blood type O exhibit a 25% decrease in VWF levels when compared with non-O blood group individuals and hence are more likely to be diagnosed with type 1 VWD. However, the bleeding phenotype of individuals with VWD are similar irrespective of the blood group. Therefore, ABO-specific reference ranges are not required. The protocol for VWF:Ag ELISA assay by ELISA, LIA, and CLIA are listed below.

Von Willebrand antigen (VWF:Ag) by ELISA: ELISA assay for the quantitative determination of VWF antigen (VWF:Ag) in human citrated plasma. A microtiter well is coated with an antibody (capture antibody) specific to VWF. Dilutions of test and standard plasma are added and incubated, during which time VWF is bound by the capture antibody on the plate. After washing, a second enzyme labelled anti-VWF antibody (detection antibody) is added and binds to VWF bound to the plate. The amount of antibody bound, and therefore VWF present in the sample, is quantified by the addition of enzyme substrate followed by color development. Primary sample: 3.2% citrated blood.

Materials and Reagents:

- ✓ Microtiter plate
- ✓ Capture/coat antibody: Polyclonal rabbit anti-human VWF (Dako, Code No. 0082)
- ✓ Detection/tag antibody: Polyclonal rabbit anti-human VWF/HRP (Dako, code No. 0226)
- ✓ Bicarbonate coating buffer
- ✓ Dilution buffer
- ✓ Wash buffer
- ✓ Substrate buffer
- ✓ Substrate: O-phenylenediamine dihydrochloride (OPD) (Sigma P8287)
- ✓ 1.5 M H_2SO_4
- ✓ Calibrator: PNP or standard commercial plasma
- ✓ Citrated patient PPP

- ✓ Normal and abnormal control plasma
- ✓ 30% hydrogen peroxide
- ✓ ELISA plate washer and reader
- ✓ Pipettes and tips
- ✓ Water bath at 37°C
- ✓ Plate sealers
- ✓ Vortex mixer

Reagent preparation:

Bicarbonate coat buffer:

a. Sodium carbonate (Na_2CO_3): 0.16 g
b. Sodium bicarbonate ($NaHCO_3$): 0.294 g
c. Dissolve in distilled water and make up to 100 ml. Adjust pH to 9.6.
d. Add approximately 100 µl of red dye. Shelf life is 2–3 weeks.

Concentrated stock buffer:

a. Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$): 0.975 g
b. Disodium hydrogen orthophosphate ($Na_2HPO_4.12H_2O$): 6.7 g
c. Sodium chloride (NaCl): 70.55 g

Dissolve in distilled water, mix well by using a magnetic stirrer in flat bottom conical flask, and finally make up to 250 ml.

Wash buffer: Dilute concentrated stock buffer 1:10 (i.e. 100 ml of concentrated stock buffer to 900 ml of distilled water). Add 2 ml of Tween 20. Mix well and store at 4°C. Shelf life is 2 weeks.

Tag and dilution buffer: Dissolve 3 grams of PEG in 100 ml of high salt wash buffer. Add 100 ul of green dye. Shelf life is 2 weeks.

Substrate buffer: Dissolve 0.73 g of citric acid and 2.4 g of disodium hydrogen orthophosphate in distilled water and make up the volume to 100 ml. Adjust the pH to 5.0. Shelf life is 2 months.

1.5 M sulphuric acid: Add 16.5 ml of concentrated sulphuric acid to 180 ml of water. Always add acid to water.

Procedure:

Plate coating: Plates must be coated on the day before they are required. Plates can be coated for 16–96 hours before use. Dilute the coating antibody 1:1000 in COAT buffer, i.e. 12 µl into 12 ml COAT buffer. Mix gently and add 100 µl to each well. Seal with plastic cover and incubate at 40°C overnight.

Assay procedures: Prepare dilutions for the standard curve as shown:

- ✓ To make stock solution: Dilute 50 µl of the pooled normal plasma in 3.95 ml of dilution buffer (1.80). Prepare a range of standards from this stock as shown below:

a. Stock solution 125% (S1)
b. 0.8 ml stock + 0.2 ml buffer 100% (S2)
c. 0.6 ml stock + 0.4 ml buffer 75% (S3)
d. 0.4 ml stock + 0.6 ml buffer 50% (S4)
e. 0.2 ml stock + 0.8 ml buffer 25% (S5)
f. 0.1 ml stock + 0.9 ml buffer 12.5% (S6)

g. 0.05% stock + 0.95ml buffer 6.25% (S7)
h. 1.00 ml buffer blank

✓ Dilute patient and control samples in 2 dilutions, 1:100 and 1:200, in dilution buffer (i.e 10 µl in 990 µl and 500 µl of 1:100 diluted + 500 µl buffer).
✓ Wash three times with a wash buffer using the plate washer, leaving a three-minute soak between each wash. After the final aspiration, tap out excess liquid and check that there are no bubbles.
✓ Add 100 ul of the standards, tests, and blanks, in duplicate, to the wells of the coated plate using the following format:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	T1	T1	T5	T5	T9	T9	T13	T13	T17	T17
B	S2	S2	T1	T1	T5	T5	T9	T9	T13	T13	T17	T17
C	S3	S3	T2	T2	T6	T6	T10	T10	T14	T14	T18	T18
D	S4	S4	T2	T2	T6	T6	T10	T10	T14	T14	T18	T18
E	S5	S5	T3	T3	T7	T7	T11	T11	T15	T15	Q1	Q1
F	S6	S6	T3	T3	T7	T7	T11	T11	T15	T15	Q1	Q1
G	S7	S7	T4	T4	T8	T8	T12	T12	T16	T16	Q2	Q2
H	BK	BK	T4	T4	T8	T8	T12	T12	T16	T16	Q2	Q2

S1-S7-Dilutions of standard; T – Test plasma; BK - Blank

✓ Cover with plate sealer. Incubate for 1 hour in a water bath at 37°C.
✓ Wash three times with wash buffer using the plate washer, leaving a three-minute soak between each wash. After final aspiration, tap out excess liquid and check that there are no bubbles.
✓ Dilute VWF detection/tag antibody 1:4000 (i.e. 5 µl into 20 ml of Tag Buffer).
✓ Mix gently and add 100 µl of diluted tag antibody to each well. Cover with plate sealer. Incubate for 1 hour in a water bath at 37°C.
✓ Wash three times with the wash buffer using the plate washer, leaving a three-minute soak between each wash. After final aspiration, tap out excess liquid and check that there are no bubbles.
✓ During washing, prepare substrate solution as follows: Dissolve one 10 mg OPD tablet in 15 ml of substrate buffer. Bring prepared solution to room temperature. Just before use, add 7 µl of 30% hydrogen peroxide.
✓ Using a stopwatch, add 100 µl of substrate solution to each well. Incubate for 8–10 mins on bench.
✓ Stop the reaction by adding 100 µl of 1.5 M H_2SO_4 to each well at EXACTLY the same time interval as the substrate was added. Shake plate to mix.
✓ Select the appropriate program (492 nm) on Lab Systems plate reader and plot calibration curve using linear graph paper. Plates should be read within 30 mins of completion. If there is a delay, store plates in a dark cupboard for up to 4 hours.
✓ To calculate results: Read the 1:100 dilution directly from calibration, multiply the 1:200 by two, and average the results. Only include results that fall within the standard curve.

Quality control protocol: Run normal control and abnormal control (low level) along with every batch. If available, include a type 3 VWD sample as control.

Result interpretation: Results are reported as % or IU/dl.

Automated Von Willebrand antigen by LIA: The automated LIA is used for the quantitative determination of VWF antigen (VWF:Ag) in human citrated plasma. This is the most commonly used method to estimate VWF:Ag levels. The automated VWF:Ag kit is a latex particle enhanced immunoturbidometric assay to quantify VWF:Ag in plasma. When a plasma containing VWF:Ag is mixed with the latex reagent and the reaction buffer included in the kit, the coated latex particles agglutinate. The degree of agglutination is

directly proportional to the concentration of VWF:Ag in the sample and is determined by measuring the decrease of transmitted light caused by the aggregates. There are several commercially available LIA-based VWF:Ag assay kits. The protocol below is for HemosIL VWF:Ag kit from Instrumentation Laboratories, but other assays can also be used. The inclusion of this method is not an endorsement of a particular company's product. If you use a different commercial source, it is important to follow the manufacturer's instructions.

Reagents and materials:

- ✓ Latex reagent: 2 vials x 3 ml of a suspension of polystyrene latex particles coated with a rabbit polyclonal antibody directed against VWF containing bovine serum albumin, buffer, stabilizer, and preservative
- ✓ Reaction buffer: 2 vials x 4 ml of HEPES buffer containing bovine serum albumin, stabilizers, and preservative
- ✓ Factor diluent (imidazole buffer)
- ✓ Calibrator plasma (1l calibrator plasma)
- ✓ Patient citrated PPP
- ✓ Normal and abnormal (low level) control plasma
- ✓ Distilled water for reconstitution of reagents

Preparation of the reagents, and reagent storage and stability:

- ✓ Reconstitute calibrator plasma with exactly 1 ml of distilled water. Allow the reconstituted material to stand at room temperature (18–25°C) for 30 minutes. Then, swirl the vial gently before use. The preparation is stable for 8 hours at 15–25°C. It can be frozen and stored at -80°C or below.
- ✓ Abnormal control plasma (Dade P): Reconstitute control P with exactly 1ml of distilled or deionized water. Shake carefully to dissolve without foam formation and let stand at room temperature (18–25°C) for 15 minutes. Before use, again shake carefully.
- ✓ Normal control/pooled normal plasma: Keep PNP at 37°C water bath for 5 minutes, gently mix before use.
- ✓ Latex reagent: Add 2 ml of reaction buffer and mix well without air bubble. Once opened, it is stable for 3 months at 2–8°C in the original vial or 1 week at 15°C on the ACL Top Family. Do not freeze.
- ✓ Reaction buffer: Once opened, it is stable for 3 months at 2–8°C in the original vial or 1 week at 15°C on the ACL Top Family. Do not freeze.

Calibration details: Load the appropriate reagents (VWF:Ag latex, VWF:Ag buffer, calibrator, and factor diluent) onto the automated analyzer. Select the calibration program and run. Once the calibration is complete, review the results. If there are no errors/failures and the calibration is acceptable, validate the calibration curve. Calibration is performed when there is a change of reagent lot numbers or a change of major instrument components, per local regulatory requirements or at laboratory discretion.

Quality control protocol: Run at least two levels of control (normal control and abnormal control) along with each run. Each laboratory should establish their own mean and standard deviation and establish a quality control program.

Procedure: Select the appropriate program on the instrument and keep the required reagents (VWF:Ag latex, VWF:Ag buffer and factor diluent). Load the samples and run as per manufacturer's instructions.

Results: Results are reported in % or IU/dl.

Normal range:

- ✓ Blood group O: 42.0–140. 8 IU/dl
- ✓ Blood groups A, B, and AB: 66.1–176.3 IU/dl

Detection limit:

- ✓ ACL Family: 3.5 IU/dl
- ✓ ACL Top Family/ACL Top Family 5 series: 2.2 IU/dl

Linearity:

- ✓ ACL Family: 10–150 IU/dl
- ✓ ACL Top Family/ACL Top Family 5 series: 8.5–250 IU/dl
- ✓ If linear range is exceeded, samples should be diluted 1:4 with factor diluent (100 µl of sample + 300 µl of diluent). Further dilution of up to 1:16 can be done if required. Multiply the results by the respective dilution factor.

Automated Von Willebrand antigen by CLIA: The automated CLIA is used for the quantitative determination of VWF:Ag in human citrated plasma. The VWF:Ag CLIA assay is a two-step immunoassay to quantify VWF:Ag in human citrated plasma using magnetic beads as the solid phase, and chemiluminescent detection system. In the first step, the sample is mixed with anti-VWF polyclonal antibody coated magnetic particles and assay buffer. The VWF present in the sample binds to the anti-VWF coated magnetic particles. In the second step, after magnetic separation and washing, an anti-VWF polyclonal antibody labeled with isoluminol is added. The chemiluminescent reaction is measured as reactive light units which are directly proportional to the VWF:Ag concentration in the sample. Studies from EQA data have shown that the VWF:Ag CLIA method has the lowest CV and lowest limit of detection. Currently, the VWF:Ag by the CLIA method is only offered by HemosIL Acustar VWF:Ag and can be run only on the ACL Acustar instrument.

Reagents composition:

The VWF:Ag kit consists of:

a. VWF:Ag cartridge for 25 determinations: Each cartridge contains 1 vial of lyophilized magnetic particle suspension coated with a rabbit polyclonal anti-VWF antibody, 1 vial of assay buffer, 1 vial of tracer consisting of an anti-VWF rabbit polyclonal antibody labelled with isoluminol, and 1 vial of sample diluent. The reagents are in a phosphate buffer containing bovine serum albumin, rabbit polyclonal IgG, stabilizers, and preservatives.
b. VWF:Ag calibrator 1: Contains saline solution with preservatives.
c. VWF:Ag calibrator 2: 2 vials of lyophilized human plasma containing buffer, stabilizers, and preservative

Preparation and procedure:

VWF:Ag Cartridge: The first time the cartridge is used, gently invert the cartridge 30 times avoiding formation of foam, and check for complete resuspension of the microparticle vial. If the microparticles are not totally suspended, continue to invert the cartridge until it is completely resuspended. Follow the instructions provided to open the cartridge and load onto the ACL AcuStar System.

VWF:Ag Calibrator 1: Is liquid and must be mixed by gentle inversion several times before use to assure homogeneity of the calibrator.

VWF:Ag Calibrator 2: Dissolve the contents of the vial with 1 ml of clinical laboratory reagent (CLR)- type water or equivalent. Replace the stopper and swirl gently. Make sure of the complete reconstitution of the product. Keep the calibrator at 15–25°C for 30 minutes and gently invert it to mix before use. Do not shake. Once reconstituted pour the entire contents of the calibrator vial into the appropriately labelled empty barcoded plastic tube for use on the ACL AcuStar System.

Reagent storage and stability:

Unopened reagents and calibrators are stable until the expiration date shown on the cartridge and vial labels when stored at 2–8°C.

VWF:Ag Cartridge: Stability after opening at 2–8°C on board the ACL AcuStar is 8 weeks. Open cartridges should remain on-board the ACL Acustar.

VWF:Ag Calibrator 1 & 2: Stability after opening and/or reconstitution on board the ACL AcuStar is 4 hours. For optimal stability remove calibrators from the system and store them at 2–8°C in capped bar-coded plastic tubes.

Quality control protocol: Run at least two levels of control (normal control and abnormal control) along with each run. Each laboratory should establish their own mean and standard deviation and establish a quality control program.

Procedure: Select the appropriate program on the instrument and keep the required reagents. Load the samples and run as per manufacturer's instructions.

Results: Results are reported in % or IU/dl.

Normal range:

All blood groups: 52.2–177.9 IU/dl

Blood group O: 48.2–157.2 IU/dl

Blood groups A, B, and AB: 59.6–210.5 IU/dl

Detection limit:

ACL AcuStar: 0.13 IU/dl

Linearity:

ACL Family: 0.3–400 IU/dl

When the re-run capability of the instrument is activated, the instrument makes an automatic dilution and corrects the final result for the dilution factor (20x), thereby expanding the range 8000 IU/dl. The assay is not affected by prozone effect.

Von Willebrand Factor Collagen Binding Assay (VWF:CB): VWF is a plasma protein with multiple functions and activities. VWF acts as an adhesive bridge between the platelets and vessel wall. It binds to platelets, primarily through the GPIb receptor using its A1 domain, and to subendothelial collagen, mainly through A3 domain, bringing about platelet adhesion. The platelet binding ability of VWF can be assessed using ristocetin co factor assay (VWF:RCo), or other newer GPIb binding assays, and is the most commonly used VWF activity assay. However, VWF:RCo and VWF:CB assess different functions of VWF. VWF:CB relies on the ability of VWF to adhere to collagen and is dependent on the presence of high molecular weight (HMW) multimers and an intact collagen binding site. Collagen has low affinity for single VWF binding domains and requires large multimeric VWF for tight association with collagen. This property of VWF:CB is utilized to detect loss of HMW multimers, which can differentiate between type 2A/2B (loss of HMW multimer) versus type 2M (normal multimer distribution). The recent 2021 VWD guidelines suggest the use of either multimer analysis or VWF:CB to discriminate type 2M from type 2A/2B VWD. VWF:CB is most commonly performed using an ELISA-based method. Various commercial ELISA assays are available in the market. Care should be taken to choose the assay optimized to preferentially detect HMW VWF. More recently, it

can also be performed in the automated analyzer, Acustar, which uses a CLIA. The Acustar VWF:CB assay is a two-step immunoassay, wherein magnetic particles act as solid phase and are coated with type III collagen triple-helical peptide. The VWF present in the sample binds to the magnetic particles proportional to its collagen binding ability, which is measured by a chemiluminescent detection system.

Here we describe an in-house ELISA based method for VWF:CB: This assay tests the ability of patient plasma-derived VWF to bind to collagen which has been previously coated onto 96-well microtiter plates. The VWF adsorbed to the immobilized collagen is detected using an enzyme-labeled polyclonal antibody and subsequent substrate reaction, which is photometrically monitored with an ELISA reader. It is best to use a mixture of ~95% type I collagen (poor VWF binding) and ~5% type III collagen (good VWF binding) to obtain good selectivity for HMW VWF. Alternatively, a lower concentration of type III collagen (1–5 ug/ml) can also be used. Reduced VWF: CBA levels will be present in individuals with either quantitative defects or qualitative defects. Since this assay alone will not enable classification of VWD, it is important that this functional assay be run together with the VWF:Ag assay and VWF:RCo.

Primary Sample: Citrated blood.

Materials and reagents:

- ✓ Collagen with a mixture of ~95% type I collagen/~5% type III collagen (Horm Collagen, ICN collagen)
- ✓ Dilution buffer and wash buffer (PBS with Tween 20)
- ✓ Substrate buffer: 0.1 M sodium acetate/citric acid buffer
- ✓ 30% hydrogen peroxide H_2O_2
- ✓ HRP-conjugated polyclonal rabbit anti-human VWF antigen (Dako)
- ✓ 96-well EIA microtiter plates
- ✓ Patient citrated PPP, PNP, and abnormal control plasmas
- ✓ 2 M H_2SO_4
- ✓ Substrate: O-phenylenediamine dihydrochloride (OPD)

Reagent preparation:

PBS (pH 7.4) with Tween 20:

a. Sodium chloride (NaCl): 8.0 g
b. Potassium chloride (KCl): 0.20 g
c. Disodium hydrogen ortho phosphate (Na_2HPO_4-$12H_2O$): 2.90 g
d. Potassium dihydrogen phosphate (KH_2PO_4-H_2O): 0.20 g
e. Make up to 1.0 l and adjust to pH to 7.4. Add 500 µl of Tween 20.

Substrate Buffer:

0.1 M sodium acetate/citric acid buffer

Dissolve 13.6 g of sodium acetate to 100 ml distilled water, adjust the PH to 6.0 using 1 M citric acid (52.14 g per 250 ml of distilled water).

2 M H_2SO_4:

Add 10.65 ml of concentrated H_2SO_4 to 89.35 ml of distilled water.

Procedure:

- ✓ Coat ELISA plates with collagen. Gently mix the collagen by inversion prior to use. Dilute 200 µl stock collagen in 20 ml of PBS, mix well and then add 200 µl to each well. Seal and keep it in a wet box for 24–48 hours.
- ✓ Wash plate with PBS three times. (Three minutes interval for each wash.) Tap out excess buffer and check that there are no air bubbles.
- ✓ Make a 1:10 pre-dilution of each test sample (i.e. add 20 µl of sample to 180 µl of PBS in assay tubes). Also include controls in the run.
- ✓ The calibration curve runs from 400% to 0%. Label tubes from A to H. Take 180 µl of buffer in an assay tube and add 120 µl of PNP (this is tube A). Add 150 µl of buffer from tubes B to G. Serially dilute the tubes by taking 150 µl from tube A to B and up to tube G.
- ✓ The tube H serves as a blank. Add only 200 µl of buffer.
- ✓ Add 180 µl of buffer in all the wells after washing the collagen coated plates three times (as described in step 2).
- ✓ Add 20 µl of serially diluted standard from A to G.
- ✓ Add 20 µl pre-diluted sample and controls in triplicate.
- ✓ Seal and incubate at 22°C (room temp in a wet box) for 2 hours.
- ✓ Wash the plate three times with PBS and add pre-diluted peroxidase conjugated antibody (1:1000 dilution, i.e. 22 µl of VWF HRP-TAG Antibody to 22 ml of PBS). Add 200 µl to each well.
- ✓ Incubate for 2 hours at room temperature.
- ✓ Wash the plate three times with PBS.
- ✓ Add 200 µl of substrate. For this, dissolve one tablet of OPD 10 mg in 22.5 ml of distilled water and 2.5 ml of stock substrate buffer. Add 15.0 µl of 30% H_2O_2 just before use to the substrate.
- ✓ Wait for 15–20 minutes for color development and add 50 µl of 2M H_2SO_4 to stop the reaction. Read using an ELISA reader with filter of wavelength 492 nm.

Quality control protocol: Run normal control (PNP) and low-level abnormal control (Dade P) with each run. If available, known patient sample of VWD can also be included. Limit of detection: 0–400%.

Result interpretation: VWF:CB allows for more accurate diagnosis of VWD and reduces misclassification errors and potential missed diagnosis. Studies have shown that VWD diagnostic error rates are reduced by 50% by the addition of VWF:CB in a four-test panel (i.e. FVIII, VWF:RCo or GPIb-based assays, VWF:Ag, and VWF:CB) versus the most commonly used three-test panel (FVIII, VWF:RCo or GPIb-based assays, and VWF:Ag).

- ✓ Type 1 versus type 2 VWD: Type 1 VWD shows reduced levels of VWF:Ag with no discrepancy between activity and antigen assays. Hence, the VWF:CB to VWF:Ag ratio and VWF:RCo to VWF:Ag ratio are both normal. A ratio of 0.6 or 0.7 can be used to determine discrepancy between the various VWF activity and antigen assays. Studies have shown that VWF:CB can differentiate type 1 versus Type 2A/2B better than the VWF:RCo assay.
- ✓ Type 2A/2B versus 2M VWD: VWF:CB can be used as a substitute for multimer analysis to differentiate between Type 2A/2B versus 2M VWD as per the latest 2021 VWD guidelines. Reduced VWF:CB to VWF:Ag ratio suggests loss of HMW multimers which can be seen in type 2A and 2B VWD. These patients will also have reduced VWF:RCo to VWF:Ag ratio. Type 2M VWD will show a normal VWF:CB to VWF:Ag ratio but will have a reduced VWF:RCo to VWF:Ag ratio. A subset of type 2M with a defect in the collagen binding site can have normal VWF:CB to VWF:Ag ratio. Though the CLIA based VWF:CB can accurately discriminate type 1 versus type 2 VWD, it has less utility to discriminate type 2M VWD from type 2A VWD based on preliminary studies. Further studies are required to confirm these findings. An optimized ELISA based VWF:CB should be used for this purpose.

- ✓ Type 3 versus severe type 1 VWD: VWF:CB has a better lower limit of detection than VWF:RCo. VWF:CB can better detect absence of VWF in type 3 VWD and presence of very low levels of VWF in severe type 1 VWD.
- ✓ VWF:CB is an important tool in the diagnosis of VWD. However, an appropriate and optimized collagen binding assay should be used. The source and concentration of collagen can affect the results. When in-house assays are used, it is best to use a type I/III mixture (~95%/~5%), or else a type III collagen at low concentrations. Cost effectiveness: VWF:CB can be performed by in-house ELISA based assays which makes it a cost effective VWF activity assay. In combination with an in-house VWF:Ag ELISA assay, it provides a cheap and efficient method to identify subtypes of VWD (except for type 2M and type 2NVWD) in a resource constrained setting.

Factor VIII Binding Assay for Diagnosis of von Willebrand Disease Normandy: The Normandy variant (type 2N VWD) of VWD is a rare autosomal recessive disorder, first identified in 1989. The disease is characterized by a defect in VWF which results in a reduced capacity to bind FVIII, leading to increased clearance of unbound FVIII from the circulation. Clinical and laboratory manifestations of type 2N patients can resemble mild/moderate hemophilia A patients or hemophilia A carriers. It is likely that some of the type 2N patients are misdiagnosed unless a FVIII binding assay (VWF:FVIIIB) is carried out for confirmation. The distinction is important as replacement therapy with purified VWF will be far more effective than FVIII replacement in these patients. The inheritance patterns of the two diseases are also quite different—autosomal recessive in type 2N VWD and X-linked recessive of hemophilia A. Type 2N patients are either homozygotes (same type 2N mutations) or compound heterozygotes (two different type 2N mutations or a combination of 2N and other VWD mutations). Mutations are commonly seen in the D'D3 domain of VWF. FVIII levels are mildly to moderately reduced, usually in the range of 5 to 40 IU/dl. FVIII to VWF:Ag levels are reduced, usually <0.7. VWF parameters (VWF:Ag, VWF:RCo, VWF:CB, and multimer analysis) are usually normal, unless compound heterozygous for other VWD mutations. These latter patients tend to be more symptomatic. Heterozygotes for type 2N are generally asymptomatic and have normal or only mildly reduced levels of FVIII. Two in-house assays have been described for VWF:FVIIIB (Nesbitt et al, 1996; and Casonato et al, 1998). Both assays show good agreement and differ based on end point detection of residual FVIII. In both assays, the microtiter plate is coated with antibody against VWF. VWF-FVIII complex in the patient sample is immobilized onto the plate and endogenous FVIII is removed with calcium chloride. A recombinant preparation of pure FVIII (rFVIII) at a concentration of 100 IU/dl is added. The amount of rFVIII bound to the immobilized VWF is measured by a chromogenic assay (Nesbitt et al, 1996) or using conjugated anti-FVIII polyclonal antibody (Casonato et al, 1998). Only one commercial FVIII binding assay is available (Asserachrom:FVIIIB, Diagnostica Stago). Here we describe the in-house ELISA based method similar to that described by Casonato et al (1998).

Primary sample: Citrated blood.

Materials and reagents:

- ✓ Coat antibody: Polyclonal rabbit anti-human VWF (Dako)
- ✓ Primary antibody: Anti-human FVIII sheep, purified IgG, 10 mg/ml
- ✓ Tag antibody: Peroxidase-conjugated AffiniPure donkey anti-sheep IgG (Jackson Immunoresearch)

Alternatively, if available, HRP conjugated anti-FVIII antibody can be used directly in replacement for the primary and tag antibody.

- ✓ 0.4 M calcium chloride
- ✓ Bicarbonate coat buffer
- ✓ TBS buffer and dilution buffer
- ✓ Wash buffer
- ✓ Substrate buffer
- ✓ Substrate: O-phenylenediamine dihydrochloride (OPD)

✓ 2 M sulfuric acid (H_2SO_4)
✓ Hydrochloric acid
✓ FVIII concentrate (recombinate)
✓ Citrated platelet poor test plasma
✓ Pooled normal plasma, controls

Reagent preparation:

Bicarbonate coat buffer:

a. Sodium carbonate (Na_2CO_3): 0.16g
b. Sodium bicarbonate ($NaHCO_3$): 0.294g
c. Make up to 100 ml. Adjust pH to 9.6. Shelf life is 2–3 weeks.
d. Add approximately 200 µl of red dye.

Make up 150 mM TBS buffer as shown:

a. Sodium chloride, NaCl: 11.68 g
b. TRIZMA-base: 12.12 g
c. Dissolve in 1800 ml of water. Make up to 2000 ml. Adjust pH to 7.4 with hydrochloric acid.

Dilution buffer: Dissolve 1 g of bovine serum albumin (BSA) in 100 ml of the TBS buffer and Tween 20 to 0.2%. About 100 ml per plate is needed.

Wash buffer: Add Tween 20 to 0.2% to TBS. Add 2 ml Tween 20 to 1000 ml TBS.

0.4 M calcium chloride: Dissolve 0.588 g of $CaCl_2$ in 10 ml of dilution buffer.

Substrate buffer:

a. Citric acid. H_2O: 0.73 g
b. Disodium hydrogen orthophosphate ($Na_2HPO_4.12H_2O$): 2.4 g
c. Make up to 100 ml with distilled water. Adjust pH to 5.0. Shelf life is 2 months.

Substrate: Dissolve one 10 mg OPD tablet in 15 ml of substrate buffer.

2 M sulphuric acid: Add 10.65 ml of concentrated H_2SO_4 to 89.35 ml of distilled water.

Diluted FVIII concentrate (recombinate): Reconstitute the recombinate in 1 ml of deionized water and aliquot 500 ul in vials and store frozen at −80°C.

Procedure:

Plate coating:

✓ Dilute the coating antibody 1:1000
✓ Add 100ml/well. Incubate at 4°C overnight. Plates can be coated 16–96 hours before use.

Dilution of standards:

✓ To make stock solution, dilute 50 µl of PNP in 3.95 ml of dilution buffer (1:80).
✓ Prepare a range of standards from this stock.

a. Stock solution	125 % (S1)
b. 0.8 ml stock + 0.2 ml buffer	100 % (S2)
c. 0.6 ml stock + 0.4 ml buffer	75 % (S3)

d. 0.4 ml stock + 0.6 ml buffer — 50 % (S4)
e. 0.2 ml stock + 0.8 ml buffer — 25 % (S5)
f. 0.1 ml stock + 0.9 ml buffer — 12.5 % (S6)
g. 0.05 ml stock + 0.95 ml buffer — 0.25 % (S7)
h. 1.00 ml buffer — Blank

Dilution of patient samples and controls: Dilute patient samples and controls 1:100 in dilution buffer (10 µl of sample + 990 µl of buffer).

Assay procedure:

✓ Wash the plate three times with the wash buffer. Tap out excess buffer and check that there are no air bubbles.
✓ Add 100 µl of the standards and blanks in duplicates and tests in triplicates, to the wells of the plates, using the following format.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	T1	T1	T1	T9	T9	T9	T17	T17	T17	-
B	S2	S2	T2	T2	T2	T10	T10	T10	T18	T18	T18	-
C	S3	S3	T3	T3	T3	T11	T11	T11	T19	T19	T19	-
D	S4	S4	T4	T4	T4	T12	T12	T12	T20	T20	T20	-
E	S5	S5	T5	T5	T5	T13	T13	T13	T21	T21	T21	-
F	S6	S6	T6	T6	T6	T14	T14	T14	T22	T22	T22	-
G	S7	S7	T7	T7	T7	T15	T15	T15	T23	T23	T23	-
H	BK	BK	T8	T8	T8	T16	T16	T16	T24	T24	T24	-

S1-S7-Dilutions of standard; T – Test plasma; BK - Blank

✓ Seal the plate. Incubate for 1 hour in a water bath at exactly 37°C.
✓ Wash the plate three times with the wash buffer. Tap out excess buffer and check that there are no air bubbles.
✓ To remove endogenous FVIII, incubate with 100µl/well of 0.4M $CaCl_2$ for 1 hour at 37°C water bath. Shaking is not required.
✓ Wash the plate again with wash buffer three times. Tap out excess buffer.
✓ Thaw one of the frozen vials of FVIII concentrate (recombinate). Add 400 µl of FVIII concentrate to 9.6 ml of dilution buffer (concentration of ~100 IU/dl).
✓ Add 100 µl of diluted FVIII to each well. Incubate for 1 hour at 37°C.
✓ Wash the plate with wash buffer three times. Tap out excess buffer.
✓ Primary antibody is aliquoted in 25 µl quantities and stored at –80°C. Make 1:2000 dilution of the primary antibody in dilution buffer (5 µl in 10 ml). Replace the antibody aliquot immediately in the freezer for future use.
✓ Add 100 µl of diluted primary antibody to each well. Incubate for 1 hour at 37°C.
✓ Wash the plate with wash buffer three times. Tap out excess buffer.
✓ Tag antibody is aliquoted in 10 µl quantities and stored at –80°C. Make 1:5000 dilution of the tag antibody in dilution buffer (2 µl in 10 ml). Replace the antibody aliquot immediately in the freezer for future use.
✓ Add 100 µl of diluted tag antibody to each well. Incubate for 1 hour at 37°C.
✓ Wash the plate with wash buffer three times. Tap out excess buffer.
✓ Substrate solution is prepared only during the last washing step. Just before use, add 7 µl of 30% H_2O_2 to this solution.
✓ Add 100 µl of the above solution to each well. Start stopwatch. Incubate for 8–10 minutes.

- ✓ Stop reaction by adding 50 µl of 2M H_2SO_4 to each well at exactly the same time interval as the substrate was added.
- ✓ Select the appropriate program (492 nm) on the ELISA plate reader. Take the OD readings.

Quality protocol: Run normal control (PNP) and low-level abnormal control (Dade P). If known VWD Normandy controls are available, add one sample as control with each batch.

Result interpretation: Results are expressed as IU/dl. Limits of detection: less than 6.25% to more than 175%.

- ✓ Homozygotes or compound heterozygotes for type 2N: Absent or markedly reduced VWF:FVIIIB (<15%) and very low ratios of VWF:FVIIIB to VWF:Ag ratio (<0.3). FVIII:C levels are mild to moderately reduced, usually ranging from 5 to 40 IU/dl. FVIII:C to VWF:Ag ratios are also reduced, usually <0.7. This ratio can be normal, especially in compound heterozygotes with type 2N and quantitative/null VWF mutations. Hence, a VWF:FVIIIB assay or genetic testing may be required if clinical suspicion is high.
- ✓ Heterozygotes for type 2N: VWF:FVIIIB is moderately reduced or even normal but the VWF:FVIIIB to VWF:Ag ratio is reduced (<0.7). FVIII:C levels are usually normal or mildly reduced.
- ✓ Mild hemophilia A or female carriers of hemophilia: FVIII:C levels reduced or normal. Normal VWF:FVIIIB assay and normal VWF:FVIIIB to VWF:Ag ratio (>0.7).

Von Willebrand Factor Multimers: VWF is a large multimeric glycoprotein which circulates in the plasma as a series of identical polymer subunits called multimers. Multimers may be steadily released from endothelial cells or stored in Weibel-Palade bodies in platelets. Multimers comprise a variable number of subunits (500 kDa to more than 10,000 kDa in molecular weight) linked by disulphide bonds. Multimers are categorized according to size into low (1–5 multimers), intermediate (6–10 multimers), high (11–20 multimers), and ultra-large (>20 multimers) molecular weight multimers (LMWM, IMWM, HMWM, and ULMWM) (Stockschlaeder et al,2014; James et al, 2021). HMWM are important for binding to collagen and platelet receptors during primary hemostasis to facilitate platelet aggregation. Abnormalities in multimer assembly or number can cause lead to hemostatic complications. Multimer analysis is useful for classification of VWD which, in turn, may inform patient management. Type 1 VWD is a partial quantitative disorder demonstrating a quantitative reduction of normal multimers. Subtype 1C has reduced VWF survival/increased VWF clearance resulting in prolonged bleeding (Platton et al, 2024).

Type 2 VWD is caused by qualitative deficiencies of VWF, and type 3 VWD is a total absence of VWF. Type 2 VWD is subdivided into 4 subtypes:

- Type 2A in which there is defective multimer assembly, increased sensitivity to ADAMTS13 cleavage, or decreased synthesis leading to reduced or absent HMWM
- Type 2B is caused by a gain of function mutation that increases VWF binding to platelets. A loss of HMWM is reported in most, but not all, cases.
- Type 2M has decreased VWF-dependant platelet or collagen adhesion with a normal VWF multimer pattern.
- Type 2N is caused by reduced binding affinity to FVIII due to mutations in the FVIII binding site of VWF; a normal VWF multimer pattern is reported.

There are some exceptions; normal multimer patterns have been reported in some cases of type 2B and conversely, some loss of HMWM have been reported in a few variant type 2M cases. Ultra-large VWF multimers may be seen in type 1C VWD, in TTP, and occasionally in cases of type 2M VWD.

Traditional multimer visualization methods are complex, laborious and time-consuming involving sodium dodecyl sulphate (SDS) agarose gel electrophoresis followed by visualization using Western blotting, colorimetric immunodetection, autoradiography, chemiluminescence, or fluorescence. Evaluation of the multimers can be performed by visual inspection or by quantitation using densitometry (Platton et al, 2024).

At the time of writing, the most widely used technique based on participation in external quality assessment programs is a commercially available semi-automated assay which is substantially faster than manual SDS agarose gel methods. This method for the detection and analysis of the distribution of VWF multimers in human plasma by agarose gel electrophoresis and immunofixation with the semi-automated Sebia Hydrasys 2 instrument is described below (Bowyer et al, 2018).

Sebia Hydrasys VWF multimer analysis: H5VWM and H11VWM are agarose gel- based assays intended for the separation of plasma proteins according to their molecular weight. The electrophoretic separation of VWF multimers is carried out after sample treatment with an anionic detergent. In an excess of this anionic detergent, proteins are converted into anionic detergent-protein complexes. In these complexes, the native conformation of proteins is disrupted, and they all assume the same conformation and the same negative charge per mass unit. When such anionic detergent-proteins are electrophoresed on a medium with appropriate sieving properties, such as H5VWM or H11VWM gels, they separate according to their molecular weight. On H5VWM or H11VWM gels, VWF multimers are separated and immuno-precipitated with a specific anti-VWF antiserum. The different bands are then visualized into the gel with a peroxidase-labelled antibody and a specific substrate. The assay is carried out in two stages: 1) electrophoresis on an agarose gel to separate proteins contained in the plasma samples, and 2) immunofixation with an anti-VWF antiserum to visualize the different multimers.

Equipment required:

- 10ml, 100µl, 10µl pipettes and tips
- Eppendorf tubes
- Water bath
- Timer
- Vortex mixer
- Tissues
- Alcohol wipes
- Hydrasys analyser (Sebia, Lisses, France)

Table 28. Reagents to Sebia Hydrasys VWF Multimer analysis

Reagent	Catalog No.	Stability
Sebia Hydragel 5 or 11 VW multimers kit	4359	Till stated expiry date
Sebia Hydragel VW multimers Visualization kit	4747	Till stated expiry date
Hydrogen Peroxide 30%	Sigma-Aldrich 216763 100ml	Till stated expiry date
Destaining solution Dilute 5ml in 5L water	4540 (10 vials 100ml)	Working solution: 1 week at room temperature
Hydrasys wash solution Dilute 1 vial in 5L water (8mls in 500ml)	4541 (10 vials 80ml)	Working solution stable till stated expiry date
Normal QC		

The method for Hydragel 5 and Hydragel 11 varies only in volumes of reagent and size of masks/blotting paper.

Sample preparation:

✓ Heat small Grant water bath to 45°C.
✓ Thaw test plasma and QC at 37°C. Hydragel 5 will hold 4 patients and 1 QC. Hydragel 11 will hold 9 patients and 2 QC (but only QC 1 vial is required).

✓ Ensure VWF:Ag result is known before starting.
✓ Label Eppendorf tubes for patients and QC.
✓ Vortex test samples before use. Dilute the samples with sample diluent from multimer kit into the Eppendorf tubes according to the VWF:Ag of the sample. The limit of detection of VWF multimers is 0.05 IU/ml, limit of interpretation is 0.11 IU/ml).

VWF:Ag (IU/ml)	Dilution	Sample diluent (µl)	Plasma (µl)
<0.20	1/4	30	10
0.20–1.50	1/6	50	10
1.50–3.00	1/10	90	10
>3.00	1/20	95	5

✓ Vortex for 5 secs.
✓ Incubate for exactly 20 minutes at 45°C in the water bath.
✓ Remove the Eppendorf tubes from the water bath, **vortex**, and leave at room temperature for 10 minutes. During these 10 minutes, set up the migration.

Migration set up

1) Turn on Hydrasys 2 and VDU. Select phoresis programme: **u/n adm p/w sebia.**
2) Complete a worksheet. Record lot numbers of kits, wicks, and gel.
3) Select "5 VWF" (migration program #57) or 11 VWF (program #58) from the instrument menu (LHS screen).

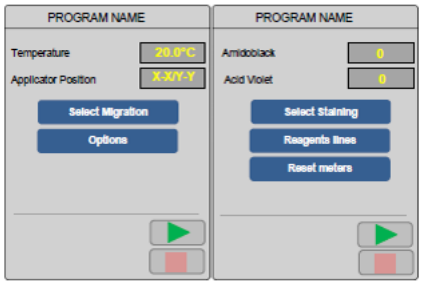

Figure 10. Hydrasys analyzer

4) Open the lid of the migration module, remove the applicator carrier, and raise the electrode carrier. ***Never close the lid when the carrier is raised***.
5) Using the plastic ends, remove the buffered wick strips from their packet (check for excess water). Attach via the holes to the carrier, with plastic backing touching the carrier (Figure 11).

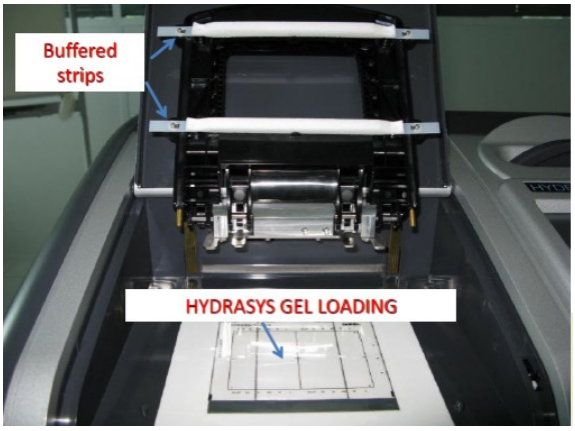

Figure 11. Hydrasys analyzer

6) Hydragel 5: Add 100 µl distilled water to the lower third of the frame printed on the migration module.
7) Hydragel 11: Add 200 µl distilled water to the lower third of the frame printed on the migration module.
8) Open the gel and wipe plastic gel support with tissue to remove excess water.
9) Place gel side up on the printed frame (Figure 12) with wells at the closest edge.
10) Roll a plastic mask onto the gel (different sizes for Hydragel 5 and 11), lined up with the markers on the gel (Figure 12). Avoid air bubbles, remove, and reapply immediately if present.

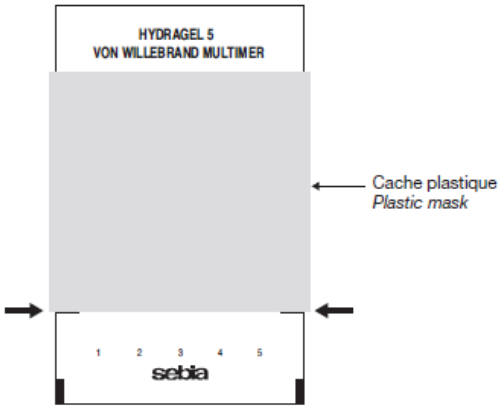

Figure 12. Hydrasys analyzer

11) Gently lower the electrode carrier onto the gel and close the migration module.
12) Press start (▶) on the screen, then confirm. This takes 5 minutes.
13) The beep signals the end. Open the migration module, raise the carrier.
14) Using a 10 µl pipette with thin tips, without touching the side or bottom of well, back pipette 5 µl sample to each well. Hydragel 5: add the normal QC control to well 5. Hydragel 11: add the normal QC control to wells 1 and 11.
****Avoid bubbles**.** This should be completed within 2 mins.
15) Gently lower the electrode carrier down and close the migration module.
16) Press start (▶) on screen. This takes approximately 100 minutes.
17) Remove TTF1 and TTF2 from fridge and leave at room temperature to liquefy.

Immunofixation 1 (60 mins)

18) At the beep, the following message is displayed ↓ANTISERUM VWF.
19) Open the module and raise, then remove electrode carrier. Discard the wicks and wipe electrodes with a soft wet tissue.
20) Hydragel 5: In a Z5, mix 2.5 ml antisera diluent with 60 µl anti-VWF antiserum.
21) Hydragel 11: In a Z5, mix 5 ml antisera diluent with 135 µl anti-VWF antiserum.
22) Remove the mask and discard. Place the yellow AS VWF mask onto the gel.
23) Hold pipette vertically. Gradually and carefully apply the antiserum mix in a single shot **avoid bubbles**.
24) Close the migration module and press start (60mins).
25) At the beep, open the lid and remove antiserum.
26) Hold pipette vertically, lightly press and withdraw antiserum then discard.
27) Remove mask and clean under water, a small brush is recommended. Allow to dry.

Gel blotting 1 (10 mins)

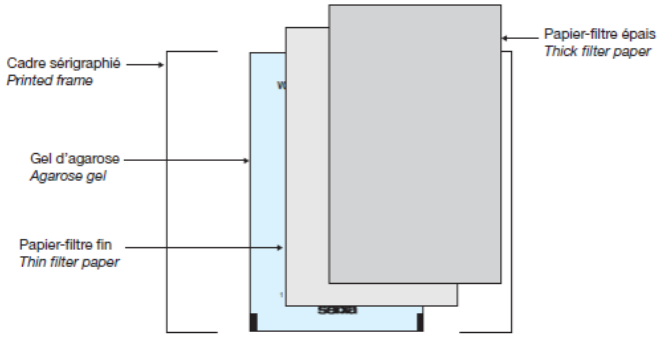

Figure 13. Hydrasys analyzer

28) Apply one thin and one thick filter paper (smooth side down) to the gel (Figure 13). Add the glass then the weight.
29) Close lid and press start.

Gel washing 1 (20 mins)

30) Remove the filter papers and add the orange washing/rehydration mask.
31) Hydragel 5: Add 4.5ml **red** wash solution to the mask. ****Avoid bubbles****.
32) Hydragel 11: Add 9.0 ml **red** wash solution to the mask. ****Avoid bubbles****.
33) Close lid and press start

Gel blotting 2 (10 mins)

34) Remove **red** wash solution then mask, and add thick and thin filter papers, glass, and weight.
35) Close lid and press start.
36) Wash mask.

Intermediate Gel Washing () then thin paper blot (10 mins + 5 secs)

37) Open lid. Remove filter papers and add orange washing/rehydration mask.
38) Hydragel 5: Add 4.5 ml **green** intermediate washing solution.
39) Hydragel 11: Add 4.5 ml **green** intermediate washing solution.
40) Close lid and press start.
41) Open lid and remove the green intermediate wash. Apply a **thin** filter paper only. Press start. After 5 secs remove thin paper.

Immunofixation 2 (30 mins)

42) Hydragel 5: Mix 4 ml antisera diluent with 2 µl anti-IgG-PER in a z5.
43) Hydragel 11: Mix 8 ml antisera diluent with 4 µl anti-IgG-PER in a z5.
44) Apply orange IgG-PER mask then add the anti-IgG-PER mix.
45) Close lid and press start.

Gel blotting 3 (10 mins)

46) Open lid, remove anti-IgG-PER, and discard. Remove and wash mask. Add thin and thick filter papers, glass, and weight.
47) Close lid and press start.

Gel washing 2 (20 mins)

48) Remove the filter papers and add the orange washing/rehydration mask.
49) Hydragel 5: Add 4.5 ml **red** wash solution to the mask. ****Avoid bubbles****.
50) Hydragel 11: Add 9.0 ml **red** wash solution to the mask. ****Avoid bubbles****.
51) Close lid and press start.

Gel blotting 4 (10 mins)

52) Remove rehydration solution then mask, and add thick and thin filter papers, glass, and weight.
53) Close lid and press start.
54) Wash mask.

Gel rehydration 1 (10 mins)

55) Remove the filter papers and add the orange washing/rehydration mask.
56) Hydragel 5: Add 4.5 ml **rehydration** solution to the mask. ****Avoid bubbles****.
57) Hydragel 11: Add 9.0 ml **rehydration** solution to the mask. ****Avoid bubbles****.
58) Close lid and press start.

Gel blotting 5 (10 mins)

59) Remove rehydration solution then mask, and add thick and thin filter papers, glass, and weight.
60) Close lid and press start.
61) Wash mask.

Gel rehydration 2 (10 mins)

62) Remove the filter papers and add the orange washing/rehydration mask.
63) Hydragel 5: Add 4.5 ml **rehydration** solution to the mask. ****Avoid bubbles****.
64) Hydragel 11: Add 9.0 ml **rehydration** solution to the mask. ****Avoid bubbles****.
65) Close lid and press start.
66) Vortex TTF 1 and TTF2.
67) Hydragel 5: Add 75 µl TTF1 to 3.0 ml TTF solvent in a z5. Invert to mix. Add 75 µl TTF2, invert to mix. Add 3 µl hydrogen peroxide (30%), invert to mix.
68) Hydragel 11: Add 150 µl TTF1 to 6.0ml TTF visualization solvent in a z5. Invert to mix. Add 150 µl TTF2, invert to mix. Add 6 µl hydrogen peroxide (30%), invert to mix.

Visualisation (10 mins)

69) Remove the rehydration solution the mask. Place orange TTF1/TTF2 mask onto gel.
70) Hydragel 5: Apply 2.5 ml TTF mix. ****Avoid bubbles****.
71) Hydragel 11: Apply 5 ml TTF mix. ****Avoid bubbles****.
72) Close lid and press start.

Gel blotting 6 (5 mins)

73) Remove TTF solution then mask, and add thick and thin filter papers, glass, and weight.
74) Close lid and press start.
75) Wash mask with water and alcohol wipe.

Gel rehydration 3 (5 mins)

76) Remove the filter papers and add the orange washing/rehydration mask.
77) Hydragel 5: Add 4.5 ml **rehydration** solution to the mask. ****Avoid bubbles****.

78) Hydragel 11: Add 9.0 ml **rehydration** solution to the mask. ****Avoid bubbles****.
79) Close lid and press start.

Gel blotting 7 (5 mins)

80) Remove rehydration solution then mask, and add thick and thin filter papers, glass, and weight.
81) Close lid and press start.
82) Wash mask.

Gel drying (10 mins)

83) Open lid and remove filter papers.
84) Close the lid and press start.

Wash and final processing (25 mins)

85) Open lid and remove gel.
86) The gel must be washed immediately in the staining compartment.
87) Open the gel holder and position gel as shown in Figure 14.

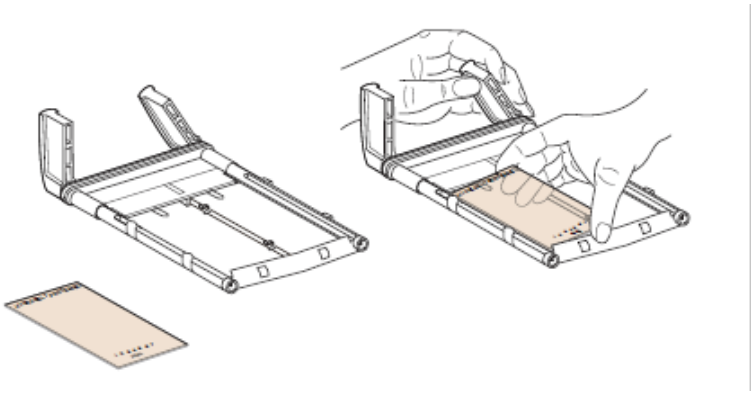

Figure 14. Hydrasys analyzer

88) Close holder and place into the gel processing /staining module.
89) Ensure at least 400 ml destaining solution is present and the waste container is empty.
90) Select VWF washing program from the instrument menu (RHS screen) and press start.

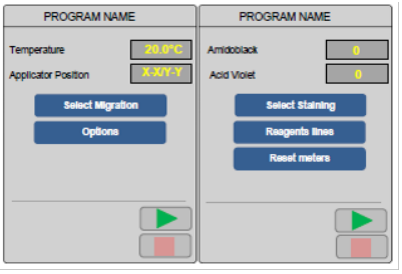

Figure 15. Hydrasys analyzer

Ensure that all masks are washed. The TTP mask, Hydrasys migration unit, and electrodes must be thoroughly cleaned with alcohol wipes.

Gel scanning

91) Remove the gel and holder from the gel processing/staining module and place into scanning module.
92) On the Phoresis programme create a worklist by table icon. Start at No.1 and add patient details including SID, name, DOB, sample date, hospital number, and requesting hospital (for RHH add consultant).
93) Hydrogel 5: Sample 5 is the normal control (QC).
94) Hydrogel 11: samples 1 and 11 are the normal control (QC).
95) Click on to open the gels and samples scanning window.
96) The following window will open:

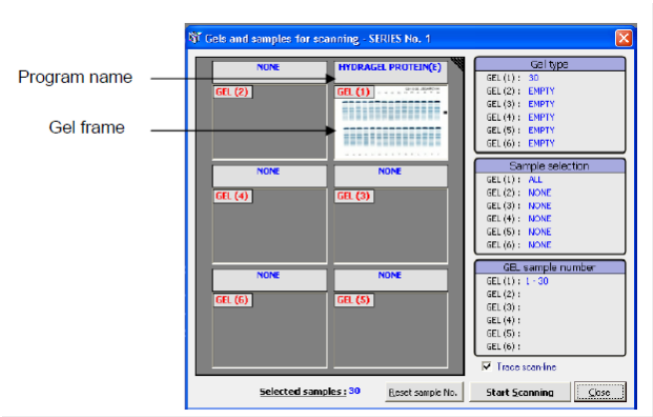

Figure 16. Hydrasys analyzer

97) Select the program of the first scanned gel in position 1. Press the top RHS image box once for Hydragel 5 and twice for Hydragel 11 (a third press resets back to 5).
98) Press start scanning.
99) Check that the scanner has correctly identified all the bands. On some occasions, it misses the first LMWM band. In this case choose "conservative gel localization mode to rescan the image", then rescan.
100) When the images appear, click on view scanning, then curve mosaic to access the densitometry.
101) The individual densitometry plots will appear in boxes:

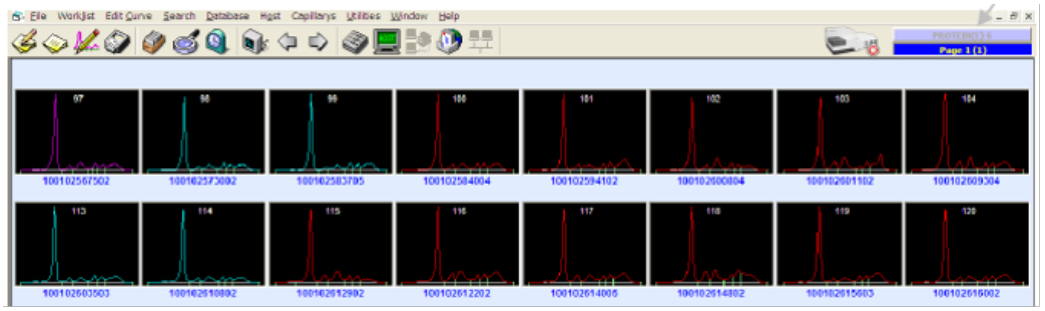

Figure 17. Hydrasys analyzer

Click on the first one to open. **Start with QC**–well 5 for Hydragel 5 or 1/11 for Hydragel 11. The QC results appear:

NB: If results cannot be evaluated on the day of testing, results can still be obtained retrospectively. Open the Phoresis program and select the date of testing where the black arrow is on Figure 18, then follow as below.

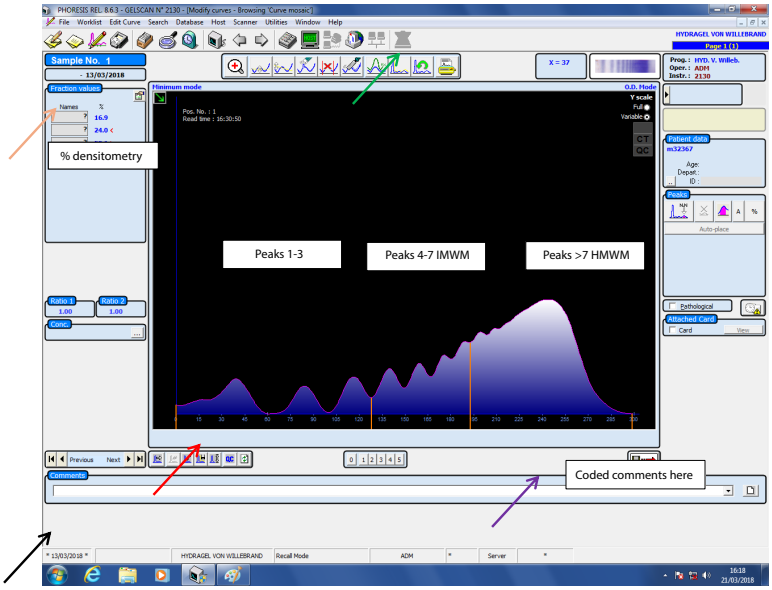

Figure 18. Hydrasys analyzer

Set Reference pattern by clicking 4th icon under the densitometry–**red** arrow. Set current curve as reference pattern. NB only choose one of the QC results for Hydragel 11.

102) Click on densitometry curve to set LMWM (peaks 1–3), IMWM (peaks 4–7), and HMWM (peaks >7).
103) The percentage of the peaks will appear and require labelling–**orange** arrow.
104) Check whether QC results are within expected ranges. If not, do not report patient results and speak to an assays senior. Save results (floppy disc icon) and move to patients.
105) Repeat 111+112 with each patient and for patient results click to overlay reference QC pattern–**green** arrow.
106) Using the adult reference ranges, decide whether densitometry is normal or abnormal, and choose the appropriate comments from the comments drop down box (**purple** arrow). Note only 1 comment can be added to each result. HMWM level is most clinically relevant.

Table 29. HMWM level and comment that can be added to results

Comment	Value
SRHMWM (slight reduction high molecular weight multimers)	HMWM 30%-bottom of normal range
RHMWM (reduction high molecular weight multimers)	HMWM 15-30%
GRHMW (Gross reduction high molecular weight multimers)	HMWM <15%
SIHMWM (slight increase high molecular weight multimers)	HMWM>top of normal range
SILMWM (slight increase low molecular weight multimers)	LMWM top of normal range to 26%
ILMWM (increase low molecular weight multimers)	LMWM >26%
QNORM/QNORM with reduced quantities	LMWM and HMWM are within normal ranges

107) Repeat testing may be required for samples with very high or low multimers. Speak to Senior BMS trained in this assay.
108) When all patients are complete, click edit curve tab, then print reports to PDF file via PDF creator.
109) This will export all results to a folder on the desktop (PDF shortcut). Find the current date and copy (or send) to the USB stick.
110) A JPEG copy of the gel is also saved in images. Copy (or send) to USB stick.
111) Ensure that the gel is labelled with testing date, stick to worksheet, and file in results folder.
112) Ensure that the normal QC results fall within acceptable limits,
113) Perform and record monthly cleaning as required,

Table 30. Adult reference range to LMWM, IMWM, and HMWM

	N	Mean (%)	Range in % (95% CI)
LMWM	51	17.3	11.8–23.6
IMWM	51	33.0	24.6–42.0
HMWM	51	49.9	35.0–58.5

Interpretation von Willebrand tests: VWD is the most common inherited bleeding disorder with an estimated prevalence of up to 1%. Only 10% of these individuals are symptomatic, and 10% of symptomatic individuals present to hospital. A conservative estimate of prevalence is 100 per million persons, with about 80% of them in the developing world. VWD is caused by a quantitative and/or qualitative deficiency of the plasma protein VWF. In contrast to most of the other coagulation factors, VWF functions in hemostasis as an adhesive protein that binds to several ligands that are critical components of the hemostatic process. It binds to GPIb receptor of platelets and subendothelial collagen, bringing about platelet adhesion. It also aggregates platelets by binding to the GPIIb/IIIa receptor. The other important function of VWF is acting as a carrier protein for FVIII and preventing proteolytic degradation of FVIII. In the absence of VWF, the half-life of FVIII is reduced from 8–12 hours to 2 hours. VWD can be classified into three main subtypes, according to quantitative (type 1 and 3 VWD) and qualitative (type 2 VWD) defects. Type 2 VWD is further subdivided into 4 subtypes type 2A, 2B, 2M, and 2N. The laboratory phenotypes of the different subtypes are summarized in Table 31.

1) Type 1 VWD: Partial quantitative deficiency of VWF.
2) Type 2 VWD: Qualitative defect. One or more functions of VWF is abnormal as assessed by VWF activity assay (described below).
    a. Type 2A VWD: Selective loss or deficiency of HMWM. The largest multimers are the functionally most active forms. Selective loss of these multimers leads to decrease in VWF dependent platelet adhesion and collagen binding ability.
    b. Type 2B VWD: Increased affinity of VWF to platelet GPIb leading to spontaneous binding of VWF to platelets which is cleared from the circulation, leading to loss of HMWM and thrombocytopenia. Peripheral smear may also show large platelets and platelets clumps. Platelet type VWD (pseudo VWD) can also show a similar phenotype.
    c. Type 2M VWD: Decrease in VWF dependent platelet adhesion without loss of HMWM. Normal multimer distribution.
    d. Type 2N VWD: Decreased in binding affinity of VWF for FVIII.
3) Type 3 VWD: Total absence/undetectable levels of VWF, usually VWF:Ag <3 IU/dl.

Preanalytical variables in VWD diagnosis: Pre-analytical issues can significantly influence the diagnosis of VWD. VWF and FVIII are acute phase reactants which can increase after inflammation, trauma, stress, pregnancy, and exercise, potentially masking a diagnosis of VWD. Collection of samples should be avoided at such times, or testing should be repeated at a different time point, before excluding a diagnosis of VWD. Improper sample collection, transportation, and processing (e.g. underfilled tubes, clotted sample or serum, delay in transport of samples, transport of refrigerated whole blood sample or in ice, and ineffective

freeze thaw or repeated freeze thaw cycles) can compromise VWD diagnosis. VWF samples should ideally be collected and processed at the same site to avoid such errors.

Tests in the repertoire for VW diagnosis:

Screening tests

Bleeding history and bleeding assessment tool (BAT): Bleeding history can be considered as the first screening test in the evaluation of a patient with bleeding symptoms. A careful history pertaining to the nature and frequency of bleeds, combined with family history, can give important clues to the diagnosis. Mucocutaneous bleeds (e.g. skin bleeds, gum bleeding, epistaxis, bleeding from minor wounds, GI bleed, and menorrhagia) are the typical pattern of bleeding seen in VWD. Hemarthrosis is rare and usually seen only in type 3 VWD when FVIII is significantly reduced. The new guidelines stress the importance of bleeding history and the use of BATs. There are many BATs available, such as Vicenza, MCMDM-1, Pictorial bleeding assessment chart (PBAC), and Paediatric bleeding questionnaire. In 2010, the ISTH proposed a new BAT which included 14 bleeding symptoms, each graded from 0 to 4. The reference range for the ISTH BAT, derived from 1040 healthy adults and 330 children, was <3 for children, <4 for adult males, and <6 for adult females. The main clinical value of BAT is to exclude a bleeding disorder. In a low prevalence setting, Tosetto et al found that a score of ≤3 had a negative predictive value of 99.2%, which essentially rules out a bleeding disorder. The ISTH recommends the use of a validated BAT to screen patients with a low probability of VWD (e.g. primary care setting). However, it is not reliable to use a BAT when the probability of VWD is intermediate (e.g. referred to a hematologist) or high (e.g. first degree relative with VWD).

Skin bleeding time (SBT): SBT is an in vivo test for primary hemostasis which was first described by French physician, Milian, in 1901 and later modified by Duke in 1910. The overall sensitivity of SBT for diagnosis of VWD is around 60% (ranging from 21% to 72%) but it has very good sensitivity for severe VWD (100% for type 3 VWD). Hence, it remains an useful test in resource limited setting to differentiate hemophilia A from severe VWD.

Platelet Function Analyzer 200: Platelet Function Analyzer (PFA100/200, Siemens, Dade Behring, Germany) is a device designed to measure primary hemostasis under high shear conditions. It records the time taken for a patient's whole blood to form a stable platelet plug at the device aperture, thus occluding it, and recorded as the "closure time (CT)". PFA100/200 showed an overall sensitivity of around 85–90%, with almost 100% sensitivity to type 3 and type 2 VWD. The sensitivity of type 1 VWD varied depending on the VWF levels. PFA 100/200 is an expensive instrument, not available in most low- and middle-income countries.

APTT: APTT is prolonged only in severe VWD cases when FVIII is also reduced, usually in type 3 VWD, type 2N VWD, and some subtypes of type 1 and 2, depending on VWF:Ag levels.

Specific VWF assays for diagnosis of VWD: The classification of VWD requires tests to quantify VWF protein and tests to assess the function of VWF. VWF binds to 1) platelets and subendothelial collagen to promote platelet adhesion, 2) activated platelets to promote platelet aggregation, and 3) FVIII to prevent its premature degradation. The functional assays for VWD assesses one or more of these functions of VWF. A discrepancy between functional assay and antigen assay is suggestive of a qualitative defect ascertained by calculating the ratio between VWF activity assay and antigen. A ratio <0.6 or <0.7 can be used depending on the preference of the laboratory.

Von Willebrand antigen (VWF:Ag): The assay measures the total amount of VWF protein present in the sample, both functional and non-functional. The most common methodology used is the automated LIA. ELISA-based methods and, more recently, CLIA in the HemosIL Acustar instrument can also be used.

Platelet- or GP1b-dependent VWF activity assays: These assays specifically measure the ability of VWF to bind to platelets, and hence they are used to differentiate between type 1 VWD and type 2A/2B/2M VWD.

1) *Ristocetin co-factor assay (VWF:RCo):* In the absence of any shear stress, ristocetin acts as a surrogate to induce alterations in VWF and cause platelet-VWF agglutination. The degree of agglutination is proportional to the amount of functional VWF present in the plasma, which can be measured either by using an aggregometer or the increase in turbidity by automated methods. The automated VWF:RCo assay has shown improved precision and better limit of detection when compared to aggregometry-based methods.

   The VWF:RCo assay has significant limitations. The assay has high inter- and intra-laboratory CV, with the potential for either falsely high or falsely low results. Also, the lower limit of detection is high, usually 8–20 IU/dl, which poses a problem in identifying type 2 variants when VWF:Ag levels are low. Certain polymorphisms commonly seen in the African population can lead to falsely low VWF:RCo levels even in the absence of VWD.

2) *VWF:GP1R:* This assay is similar to VWF:RCo, wherein platelets are replaced by microparticles (latex beads in LIA [HemosIL] and magnetic particles in CLIA [AcuStar]), coated with recombinant wild type GP1b fragments. The assay has reduced CV and lower limits of detection than the original VWF:RCo assay. Its clinical utility in VWD has been demonstrated in various studies.

3) *VWF:GP1M:* Unlike the other platelet-dependent VWF assays, this assay does not use ristocetin. Platelets are replaced by recombinant GP1b fragments with gain-of-function mutations to which VWF will "spontaneously" bind. VWF:GP1bM can be measured by LIA (Siemens Innovance VWF Ac assay) or even by few non-commercial ELISA based methods. The test has good reproducibility, low limit of detection, and provides comparable information to VWF:RCo and VWF:GP1bR assays for the diagnosis of type 2 VWD.

4) *VWF:Ab:* This assay is based on monoclonal antibodies directed against the platelet binding site (i.e. GP1b) of VWF. Though not a true functional assay, it can be considered as a surrogate for platelet binding activity. Several studies have demonstrated the utility of the automated VWF:Ab assay (HemosIL VWF Activity, IL, Bedford, Massachusetts) in the initial evaluation and subclassification of VWD.

New guidelines recommend the use of newer assays of platelet-dependent VWF activity, such as VWF:GP1bR and VWF:GP1bM over VWF:RCo (automated or non-automated).

Collagen binding assay (VWF:CB): The VWF:CB assesses the ability of VWF to bind to collagen and depends on the presence of HMWM and an intact collagen binding site. It can be used as a replacement for multimer analysis to differentiate type 2A versus type 2M. VWF:CB to antigen ratio is normal in type 2M and reduced in type 2A/2B. The VWF:CB assay has been shown to be more effective in distinguishing VWD type 1 from type 2 (except 2M) than the VWF:RCo assay. It can be used to distinguish severe type 1 from type 3 VWD due to its better limit of detection. VWF:CB can be measured by various commercial and non-commercial ELISA assays, and more recently by the CLIA method.

Ristocetin-induced platelet agglutination (RIPA): Agglutination response to ristocetin at normal (>1 mg/ml) and low dose (<1 mg/ml) ristocetin is one of the tests used in VWD diagnosis. While an absent response can be seen in type 3 and severe VWD subtypes, its major utility is in the identification of type 2B and platelet type VWD, where response is seen even at low doses of ristocetin. Differentiation of type 2B and platelet type VWD can be made out with cryoprecipitate challenge or mixing studies with normal plasma and/or control platelets. If available, genetic testing should be used.

FVIII binding assay: This test assesses the ability of VWF to bind to FVIII. Reduced VWF:FVIIIB assay and reduced VWF:FVIIIB to VWF:Ag ratio is suggestive of type 2 N VWD.

Multimer analysis: Assessment of VWF multimers is an established test in the evaluation of VWD, mainly to differentiate between type 2A versus type 2M. However, the test is very laborious, technically demanding, and is quickly disappearing from most laboratories.

Desmopressin trial: Type 1C Vicenza is a subtype of type 1 VWD associated with increased clearance of VWF. These patients show an exaggerated response to desmopressin, but with shortened life span. A higher than normal level of VWF propetide to VWF:Ag ratio is also seen in these patients.

Table 31. Laboratory phenotype of different subtypes of VWD

Type of VWD	FVIII	Von Willebrand antigen (VWF:Ag)	Platelet dependent VWF activity assays*	Collagen binding assay (VWF:CB)	Factor VIII binding assay (VWF:FVIIIB)	Platelet VWF assay/ Ag ratio$	VWF:CB/Ag ratio$	Multimer analysis	Ristocetin-induced platelet agglutination (RIPA)	Comments
Type 1 VWD	reduced or normal	reduced	reduced	reduced	Normal	Normal	Normal	Normal	Reduced or normal	VWF levels <30 IU/dl or VWF levels 30–50 IU/dl with abnormal bleeding is type 1 VWD as per new guidelines. Alternatively, VWF levels 30–50 IU/dl can be classified as low VWF with mild risk of bleeding.
Type 2A VWD	reduced or normal	reduced or normal	reduced	reduced	Normal	reduced	reduced	High to intermediate multimers lost	Usually reduced	Type 2A and 2B can be distinguished by RIPA.
Type 2B VWD	reduced or normal	reduced or normal	reduced	Reduced	Normal	Reduced	Reduced	Loss of HMW multimers	Response seen at low dose ristocetin	Type 2B vs platelet distinguished by RIPA mixing studies or genetic testing
Type 2M VWD	reduced or normal	reduced or normal	reduced	reduced	Normal	Reduced	Usually normal	Normal	Reduced or normal	Type 2M VWD with defect in collagen binding can have reduced VWF:CBA/ag ratio
Type 2N VWD	reduced (FVIII/ VWF:Ag ratio usually <0.7)	Usually normal#	Usually normal#	Usually normal#	Reduced VWF: FVIIIB/ Ag ratio is reduced (<0.7)	Normal	Normal	Normal	Normal	Phenotype similar to mild/moderate hemophilia A or carrier, confirmed with VWF;FVIIIB assay or genetic testing
Type 3 VWD	Reduced, usually 1–10 IU/dl	Markedly reduced <2 IU/dl	Markedly reduced	Markedly reduced	NA	NA	NA	Absent	Absent response which corrects on addition of cryoprecipitate	VWF activity to antigen ratios should not be calculated

*Platelet-dependent VWF assays include VWF:RCo, VWF:GP1M, VWF:GP1R, or VWF:Ab.

#VWF:Ag or activity assays can be reduced in type 2N VWD, when seen in a compound heterozygous state with associated VWF null/quantitative mutations

$ To determine a qualitative defect, the ratio of VWF activity assay to antigen assay is calculated. A ratio less than 0.6 or 0.7 can be used (depending on laboratory preference) to classify type 2 VWD.

Diagnosis of von Willebrand Disease in Resource-Poor Setting: VWD is the most common inherited bleeding disorder with an estimated prevalence of 1%. A conservative estimate of prevalence is 100 per million persons, with about 80% of them in the developing world. Only 2.6% of total reported VWD cases are from South Asia and Sub-Saharan Africa, though they contribute ~40% of total population worldwide. VWD is divided into three main subtypes. Type 3 VWD is due to severe quantitative deficiency of VWF due to undetectable levels of VWF. Type 1 VWD is due to a partial quantitative deficiency. Type 2 VWD is qualitative deficiency and is further subdivided into four types–2A, 2B, 2M, and 2N. There is wide disparity in the distribution of VWD subtypes in different parts of the world. In most of the high- and upper-middle-income countries (HIC and UMIC), type 1 is the most common subtype (60–80%) followed by type 2. Type 3 VWD is rare and usually constitutes less than 5% of cases. In contrast, the frequency of type 3 VWD is higher in many low- and low-middle-income countries (LIC and LMIC), as high as 64% in some parts which is nicely summarized in a review article by Favaloro et al. This striking difference in the distribution pattern can be attributed to the fact that most data from LMIC countries are from hospital records where only the most severe patients might present, and the milder ones go undetected. Another contributing factor is the increased consanguinity and marriages within small communities, which increases the incidence of type 3 VWD, an autosomal recessively inherited disease. There is considerable under-reporting of VWD, particularly in LIC and LMIC countries. Stonebraker et al reported the mean prevalence of VWD in HIC, UMIC, LMIC, and LIC was 60.3, 12.6, 2.5, and 1.1 per million, respectively, which was significantly different in relation to the income classification, often <1 per million in many LIC countries. However, the variability in prevalence of type 3 VWD is less marked, which suggests patients with type 3 VWD were diagnosed more frequently than other subtypes in these countries.

Roadblocks in the diagnosis of VWD in resource-poor setting

Low priority for health care and for bleeding disorders: Health care is assigned low priority, socially and politically, in most developing countries where only 1 to 2% of gross domestic product is allocated for healthcare. Of this limited budget, more pressing issues of public health significance, such as infectious diseases, infant and maternal mortality, and malnutrition, take precedence over relatively rarer conditions such as inherited bleeding disorders, including VWD.

Poor access to health care facilities and high costs of health care: In many developing countries, the health care infrastructure is poorly developed with fewer hospitals which are not easily accessible to all. This could be the reason for the higher incidence of severe VWD in developing countries where only the more severe bleeders present to the hospital. The high cost of investigations, inability to pay with travel distances involved, requirement to stop employment for investigations, and minimal medical insurance coverage, all contribute to underdiagnosis of mild VWD cases.

Non-availability of VWF reagents and lack of laboratory infrastructure: The new 2021 VWD guidelines suggest the use of newer platelet-dependent activity assays, such as VWF:GP1R and VWF:GP1M, for the diagnosis of VWD. However, these assay kits are not available or used in most LMIC and LIC countries. Further issues, such as delay in reagent supply, improper maintenance of transport conditions, low sample referrals, low shelf life of reagents, and fewer referral centers, also contribute to paucity of VWF testing facilities.

Overcoming the roadblocks in the diagnosis of VWD in resource-poor setting

Improve identification of VWD cases using BATs and use of cheap and easily accessible screening test: Screening tests for VWD include skin bleeding time (SBT) and closure time on Platelet Function Analyzer (PFA-200). In our center, we analyzed a large cohort of patients (n = 444) with reduced VWF <50 IU/dl among patients evaluated for a suspected bleeding disorder over a period of 7 years, from January 2012 to March 2019. The majority of patients were of the type 3 phenotype (48.3%) in accordance with other published studies from India. The patients were also subclassified according to severity based on the VWF:RCo levels as severe (<10%), moderate (10–30%), and mild (>30%), according to the classification

proposed by Federici et al (2014). The overall sensitivity of the SBT and PFA-200 was 72% and 95%, as shown in Figure 19. Importantly, SBT had 100% sensitivity in identifying type 3 VWD and a very high sensitivity of 92% in severe VWD, which included severe type 1 and type 2 cases. SBT was comparable to PFA-200 in both these categories. Overall, abnormal ISTH BAT score was seen in 75% of the VWD cases.

Since the advent of the PFA-200 in 1995, numerous publications support its use for diagnosis and monitoring of VWD, and it has replaced bleeding time as a screening test for VWD in most developed countries. All guidelines also discourage the use of SBT in the diagnostic workup of VWD. The sensitivity of SBT and PFA-100/200 are similar for platelet function disorders and for severe VWD. Although PFA-100/200 has good sensitivity to VWD, it is an expensive instrument which most laboratories in developing countries cannot use and therefore it cannot be a replacement for SBT in this setting. The majority of VWD cases in LIC and LMIC countries present with severe VWD where the diagnosis is often missed or individuals are misdiagnosed as hemophilia A. In resource restricted settings, where laboratory infrastructure, facilities, and reagent availability are limited, bleeding time is a cost-effective screening test to identify severe VWD in patients with significant bleeding history/family history. Potential cases should be preferably identified using a BAT, such as the ISTH BAT. It is important to note that SBT should only be used for patients suspected to have a bleeding disorder and not as a pre-operative screening test or to assess response to antiplatelets drugs. Also, milder cases of VWD can have a normal SBT and can be missed.

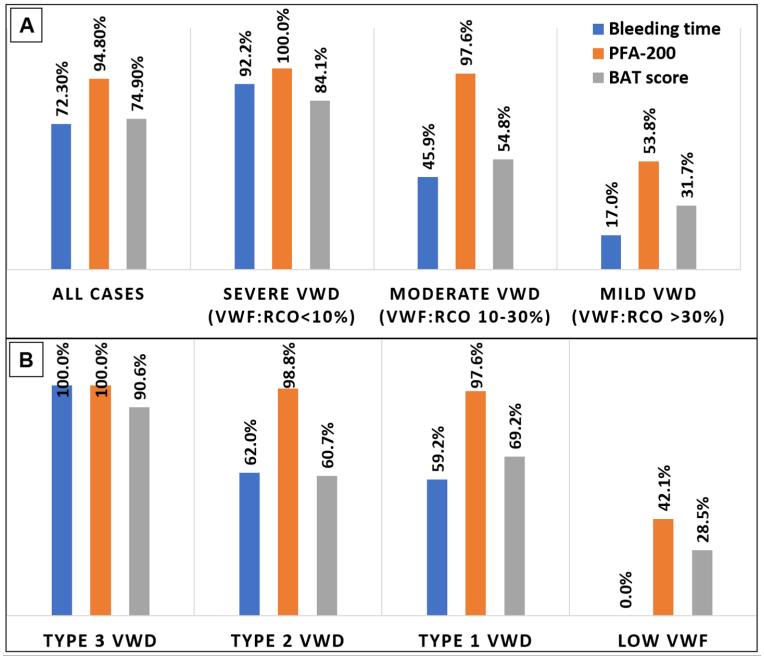

Figure 19. The overall sensitivity of the SBT and PFA-200

Cost effective approach to perform VWF assays: One of the major setbacks in performing tests of VWD is that most of the commercial kits are expensive and not accessible for most laboratories in LIC and LMIC countries. However, many of the tests for VWD, such as VWF antigen, collagen binding assay, and FVIII binding assays, can also be performed by manual in-house ELISA-based methods which decrease the cost considerably. Use of a minimalistic panel comprising SBT, APTT, FVIII, VWF:Ag, and VWF:CBA, can identify most subtypes of VWD (except type 2M VWD) and a provisional diagnosis of VWD can be made with reasonable confidence. The diagnosis of type 2M requires a platelet-dependent activity assay such as the ristocetin co factor assay and may be missed by using this panel alone.

The manual ristocetin co-factor assay by aggregometry is a very laborious and time-consuming test flawed with high CV and a very high limit of detection. Automation of VWF:RCo has markedly improved its CV and lower limit of detection. Another advantage of automation is the use of lower sample and reagent volumes

(ristocetin is an expensive reagent), which reduces the cost per test. However, the continued use of this test requires sufficient patient referrals and test requests. Few studies have been published on the use of non-commercial ELISA based methods for VWF:GP1bR and VWF:GP1bM which are also options that can be explored. However, this requires technical expertise, availability of reagents, and motivated personnel.

Since manual tests can be associated with errors, it is necessary to follow strict quality control measures such as running tests in duplicate or triplicate in ELISA runs, running normal (PNP) and abnormal samples in parallel with each run, and participation in EQAS program to ensure accuracy of results.

Recently, a point-of-care card-based device has been developed by Indian Council of Medical Research (ICMR)–National Institute of Immunohematology (NIIH), Mumbai, for the diagnosis of severe VWD and severe/moderate hemophilia with FVIII and VWF levels less than 5 IU/dl. This is a rapid and cost-effective test which can be used in outreach areas for a provisional diagnosis of severe/moderate hemophilia A and type 3 VWD. The device is currently awaiting validation studies before use in the market.

Conclusion: It is estimated that 80% of VWD cases are from developing countries. In many LIC and LMIC countries, more than 99% of the VWD cases are not identified and/or reported. Guidelines applicable for the rest of the world may not be appropriate in this setting due to lack of reagents, training centers, and laboratory infrastructure. The first step to improve reporting of cases is to increase awareness for the use of simple tools, from using BATs to identify patients with possible bleeding disorders, to screening suspected patients using easily available tests like SBT and APTT. Further testing can be done using ELISA-based VWF antigen and/or CBA testing, or referred to a tertiary center for more specialized tests wherever possible.

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PART 9 Laboratory Investigation of Other Coagulation Factors

Annette Bowyer

TOPICS COVERED

- ✓ Assays Based on PT (One-Stage Assay of FII, V, VII and X)
- ✓ Assays Based on APTT (One-Stage Assay of FXI, FXII, PKK, or HMWK)
- ✓ Factor XIII: Screening, Activity, and Antigen

Assays Based on PT (One-Stage Assay of FII, V, VII, and X): Deficiencies of clotting factors II, V, VII, or X are rare bleeding disorders (Mumford et al, 2014). The assays for factor II, V, VII, or X activity can be performed using a one-stage assay based on PT. The assay compares the ability of dilutions of a standard or reference plasma and test plasma to correct the PT of a plasma known to be totally deficient in the clotting factor being measured. In FV assay, for example (described below), the plasma is deficient in FV but contains normal amounts of all other clotting factors including II, VII, X, and fibrinogen. Clotting factors II, VII, and X may be assayed in a similar way, substituting FV-deficient plasma in the example given below with the corresponding deficient plasma, and using a reference (standard) plasma with a known concentration of the factor being assayed (Baker et al, 2020).

Reagents:

- ✓ FV-deficient plasma. This may be congenitally deficient or artificially deficient in FV (aged plasma).
- ✓ Owren's veronal buffer (OVB).
- ✓ Platelet-poor citrated test plasma.
- ✓ For the standard, a commercial reference (standard) plasma is preferred or if unavailable use a 20-donor platelet poor normal plasma pool (kept at -70°C or below).
- ✓ Internal quality control plasma-either commercial or locally sourced (CLSI, 2016).
- ✓ Thromboplastin reagent which should contain calcium chloride. It is best practice to use the same thromboplastin as used in the PT test but an alternative thromboplastin may be used for diagnosis of unusual patients.

Method: Decant sufficient thromboplastin reagent into a 75 x 12 mm glass tube. Allow to warm to 37°C for 5 minutes. If the thromboplastin reagent does not contain calcium, this needs to be added separately. Decant sufficient M/40 $CaCl_2$ into a 75 x 12 mm glass tube. Allow to warm to 37°C for 5 minutes. For both tests, QC, and standard plasmas, prepare dilutions in plastic tubes, as shown in Table 32.

Table 32. Preparation of test, QC, and standard plasma dilutions for one-stage assays of FII, FV, FVII, and FX

Dilution	Plasma (ml)	OVB (ml)
1/5	0.1	0.4
1/10	0.1	0.9
1/20	0.5 (1/10 dilution)	0.5
1/40	0.5 (1/20 dilution)	0.5

Note: Mix the 1/10 dilution well before using it to prepare the 1/20 dilution. Mix the 1/20 dilution well before using it to prepare the 1/40 dilution. Plasma dilutions should be tested immediately after preparation. If room temperature exceeds 25°C, it may be necessary to keep dilutions on melting ice prior to analysis.

Test each dilution of reference (standard/calibrator) plasma as follows:

✓ Pipette 0.1 ml of 1/10 dilution into a 75 x 10 mm glass tube.
✓ Add 0.1 ml FV-deficient plasma.
✓ Warm to 37°C for 2 minutes.
✓ Add 0.2 ml pre-warmed thromboplastin reagent.
✓ Start stopwatch and mix.

Note: If the thromboplastin reagent does not contain calcium, 0.1 ml of thromboplastin is added to the mixture of dilution and deficient plasma. After a 1- to 2-minute delay for warming to 37°C, the mixture is clotted with 0.1 ml pre-warmed (to 37°C) calcium.

Record clotting time:

- Repeat the 1/10 dilution then test 1/20 and 1/40 dilutions in duplicate.
- Repeat for dilutions of test plasma and QC. Test in duplicate.
- For test plasmas expected to be normal, test 1/10, 1/20, and 1/40 dilutions. For test plasmas expected to have reduced levels, test 1/5, 1/10, and 1/20 dilutions.
- A "blank" should also be tested as follows:
  - 0.1 ml OVB
  - 0.1 ml FV-deficient plasma
  - 0.2 ml thromboplastin/calcium reagent
- The clotting time of the "blank" reflects the quality of the deficient plasma and should be equivalent to less than 1 IU/dl (<0.01 IU/ml).

Results: Take an average of each duplicate result. The duplicate times must be within 10% of each other to be acceptable. On 3 cycle x 2 cycle logarithmic paper, plot clotting times of reference, test, and QC plasmas against percentage concentration of FV. The 1/10 dilution is arbitrarily assigned a value of 100%, thus the1/20 is equivalent to 50%, 1/40 to 25%, and 1/5 dilution to 200%. Alternatively, plot concentration on a logarithmic scale and clotting time on a linear scale. The relative amount of FV in the test plasma compared with the reference plasma is extrapolated from the graphs. An example of this is shown in the section on APTT-based assays. The clotting time equivalent to 100% test (the place where the test line passes through the 100% activity) is read from the standard line (therefore, the concentration of standard that could give that particular clotting time). This gives the concentration of the test in percentage of standard. This percentage is multiplied by the value of concentration of clotting factor in the standard plasma (in IU/dl) to give the concentration in the test (in IU/dl).

Notes: Low levels of FII, FV, FVII, or FX activity can be measured in patients with liver disease (Kujovich et al, 2015). The adult reference range for each of these clotting factors should be determined locally, but often has a lower limit of 50–70 IU/dl for FV, FVII, and FX. The lower reference limit for FII is higher (Appel et al, 2012; Wakeman et al, 2005). FVII activity can increase during pregnancy (Fu et al, 2022). Individuals with a reduced level of FV should also have a FVIII activity assay performed to exclude combined FV and FVIII deficiency (Zheng et al, 2013). The vitamin K-dependent factors II, VII, and X may naturally be reduced at birth, rising throughout childhood to reach adult levels. Pediatric reference ranges can be established locally or taken from the literature taking account of reagent variation (Toulon et al, 2016; Attard et al, 2013; Di Felice et al, 2022). In some cases of FVII deficiency (FVII Padua, FVII Nagoya, FVII Tondabayashi/Shinjo), there may be a discrepancy between the levels of FVII:C obtained, depending on the source of thromboplastin. The use of human thromboplastin is therefore advisable on the basis that the results are more likely to reflect the in vivo activity. In some rare cases, the result may be very low if rabbit thromboplastin is used, but higher or normal if the assay utilizes human or ox brain thromboplastin. This may be a reason why some cases of apparent severe FVII deficiency do not have bleeding symptoms (Balluet et al, 2020; Sevenet et al, 2017).

Assays Based on APTT (One-Stage Assay of FXI, FXII, PKK, or HMWK): FXI deficiency is a rare bleeding disorder (Mumford et al, 2014), whereas deficiencies of the contact factors FXII, PKK, and HMWK are not associated with bleeding (Maas et al, 2018), but do cause a significantly prolonged APTT. The one-stage APTT-based clotting assay for FXI is described in this section. The assay is based on a comparison of the ability of dilutions of standard and test plasmas to correct the APTT of a plasma known to be totally deficient in FXI but containing all other factors required for normal clotting. For factors FXII, PKK, and HMWK, the assay is essentially the same as that of the one-stage FXI but is performed by substituting the relevant deficient plasma for FXI-deficient plasma, and the selection of the appropriate reference plasma. The APTT reagent used for assay of PKK cannot use ellagic acid as an activator.

Reagents:

- ✓ Platelet-poor citrated test plasma.
- ✓ Standard (reference) plasma.
- ✓ The standard (reference/calibrator) plasma used should be either a locally prepared plasma pool kept at -40°C or lower, or a commercial standard plasma. In either case, this reference plasma must be calibrated for clotting FXI assay against the current international standard for FXI in plasma. It is not acceptable to assume that a pooled normal plasma has 100 IU/dl FXI activity.

Internal quality control plasma (Baker et al, 2020):

- ✓ FXI-deficient plasma.
- ✓ This is available commercially or may be collected from a severely deficient donor under the following conditions.
- ✓ FXI level is less than 1 IU/dl, no history of anti-FXI antibodies, received no treatment for 2 weeks including plasma normal liver function tests.
- ✓ Abnormal liver function could lead to reduction in other clotting factors, which affect the specificity of the assay. This plasma can be stored in aliquots at -20°C or lower for approximately 3 months (Zhao et al, 2018; Woodhams et al, 2001).
- ✓ It is preferable to use FXI-deficient plasma produced by immunodepletion of FXI from normal plasma using a monoclonal antibody. This type of material is available commercially and has the advantage of enhanced viral safety compared with plasma sourced from patients who have been treated with plasma-derived products.
- ✓ However, not all immunodepleted plasmas are found to be <1 IU/dl, and care should be taken to check this before use.
- ✓ APTT reagent that is sensitive to factor deficiencies (CLSI, 2016). Note that APTT reagents that are activated by ellagic acid are insensitive to PKK deficiency.
- ✓ Owren's buffered saline (OBS or glyoxaline buffer).
- ✓ 25mM $CaCl_2$ (note that Werfen $CaCl_2$ supplied with SynthASil is 20mM).

Method:

- ✓ For FXI, FXII, and HMWK: Make 1/10 dilutions of standard, QC, and test plasma in buffered saline in plastic tubes. (If the test plasma is expected to have a very low level of factor, start at a 1/5 dilution.) Using 0.2 ml volumes, make doubling dilutions in OBS of standard, QC, and test plasma from 1/10 to 1/40 in plastic tubes. (Mix each dilution well before transferring to next tube.) Plasma dilutions should be tested immediately after preparation. If room temperature exceeds 25°C, it may be necessary to keep dilutions on wet ice prior to testing.
- ✓ For PKK only, higher dilutions of 1/100, 1/200 and 1/400 are usually required. Test the standard plasma first. Pipette 0.1 ml of each dilution of standard into a 75 x 10 mm glass tube. Add 0.1 ml of FXI-deficient plasma, mix and transfer to 37°C water bath. Add 0.1 ml of APTT reagent, mix and incubate for 2-5 minutes depending on the recommended incubation time of the APTT reagent. At the end of the incubation time add 0.1 ml $CaCl_2$, mix tilt tube until a clot is visible.

Record the clotting time. Repeat steps 4-7 using QC then test plasma. A "blank" should also be set up using 0.1 ml of OBS in place of test plasma. The clotting time of the blank should be longer than the time of 1% FXI activity of standard read from the calibration graph. If the time is shorter, this indicates that the deficient plasma is not totally deficient in FXI and thus is not a suitable substrate plasma.

Results: Plotting of results is the same as for assays based on PT (described above), requiring double logarithmic or logarithmic/linear scale graph paper. For FXI, FXII, and HMWK, the 1/10 dilution is arbitrarily assigned a value of 100%, the 1/20 dilution a value of 50%, and the 1/40 dilution a value of 25%. If used, a 1/5 has a value of 200%. For PKK, the 1/100 dilution is arbitrarily assigned a value of 100%, the 1/200 dilution a value of 50%, and the 1/400 dilution a value of 25%. If used, a 1/50 has a value of 200%. Straight lines, parallel to each other, should be obtained. Read off concentration of QC and test sample as for assays based on PT (described above). In this example, the FXI concentration in the test sample is 7% of that in the standard. If the standard has a concentration of 85 IU/dl, the calculation is 85 IU/dl x 7% = the test sample has a concentration 6 IU/dl. If the lines are not parallel, the assay should be repeated. Check the clotting times of the test sample. If very long, then test a 1/5 (or 1/50 for PKK) dilution. Non-parallel lines may occur due to technical error. If technical error has been eliminated, it may be due to the presence of an inhibitor, which may act specifically against FXI or may be of the "lupus type", showing a converging pattern. Diverging lines are typical of an activated sample (Baker et al, 2020).

Notes: Deficiencies of FXII, PKK, or HMWK are not associated with an increased bleeding risk. Ellagic acid-activated APTT reagents are insensitive to PKK deficiency. If the test plasma FXI, FXII, PKK, or HMWK concentration is close to zero (i.e. the clotting times of all dilutions are similar to the blank), non-parallel lines may occur. The normal reference range should be established locally but often has a lower limit of 50–70 IU/dl for each of these factors. International Units have now been established for FXI and FXII in plasma, but there are no plans to establish international units for PKK or HMWK.

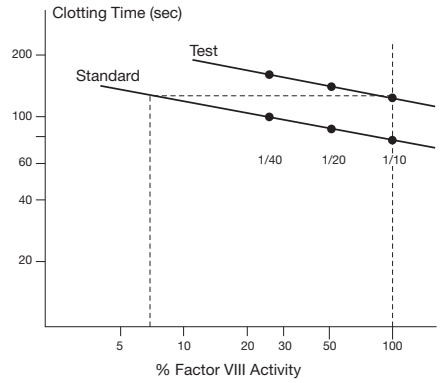

Figure 20. Graph of FXI assay

Factor XIII (FXIII): Screening, Activity, and Antigen

FXIII Screen (clot solubility tests): FXIII mediates cross linking of α and γ chains of fibrin. FXIII deficiency is classified as a rare bleeding disorder and may be caused by mutations in either FXIII-A catalytic subunits or FXIII-B carrier subunits (Karimi et al, 2018). Fibrin clot solubility qualitative tests are not currently recommended for use due to lack of standardization and poor sensitivity. It is recommended that FXIII deficiency is diagnosed using a functional FXIII activity assay rather than clot solubility (Mumford et al, 2014; Palla et al, 2015; Kohler et al, 2011) but in some areas of the world only clot solubility assays are available. Clot solubility tests containing 5M urea are more specific than those containing acetic acid, however, hypofibrinogenemia and dysfibrinogenemia can cause false positive results in the urea-based test (Dorgalaleh et al, 2016). Clot solubility tests containing 1% monochloroacetic acid (MCA) are more

sensitive and faster than urea-based tests (Dorgalaleh et al, 2016). It has been suggested that both urea- and acetic acid-based tests should be performed in parallel to optimize diagnosis (Dorgalaleh et al, 2016). A positive result will only be observed in severe FXIII deficiency, at FXIII activity of <5-10 IU/dl, depending on the method used.

Method: Thrombin and calcium are required to activate FXIII such that it will cross-link fibrin into a stable form. In this method, despite using citrated plasma, sufficient calcium ions are still available for FXIII activation. A normal ethylenediaminetetraacetic acid (EDTA) anticoagulated plasma is used for a control. In this plasma, EDTA results in a complete chelation of calcium ions, which means that the FXIII is not able to crosslink fibrin. Addition of 2% acetic acid or 5M urea results in the lysis of non-cross-linked clots, whereas citrated plasma with >10 U/dl of FXIII activity has an insoluble clot. The test is generally more sensitive if acetic acid (rather than urea) is employed, since the clot will dissolve at higher levels of FXIII in the presence of acetic acid (Jennings et al, 2003).

Materials/reagents:

- ✓ 75 x 10 mm glass tubes
- ✓ 0.9% saline
- ✓ 30 U/ml thrombin
- ✓ Normal EDTA plasma
- ✓ 2% acetic acid

Method: Add 0.2 ml test citrated plasma to 0.2 ml 0.9% saline in a glass tube. For positive control, repeat with 0.2 ml EDTA plasma. For a negative control, repeat with 0.2 ml normal citrated plasma. Add 0.1 ml of 30 U/ml thrombin, mix. Leave for 30 minutes at 37°C. Flick tubes to loosen clots from sides. Add 5 ml 2% acetic acid and stopper the tube. Leave at room temperature for 12 hours.

Results: EDTA plasma should have no visible clot. Normal citrated plasma should have an intact, visible clot. If clot is not visible, the subject has FXIII deficiency.

Normal range: Normal subjects have a visible clot after 12 hours in 2% acetic acid.

Notes: 5M urea can be used in place of 2% acetic acid. The incubation time for clot dissolution is then 18 hours. This method is less sensitive but more specific than acetic acid (described above). Clotting with calcium and lysis with urea produces abnormal results only when levels of FXIII are below 5 U/dl. By comparison, clotting with 30 U/ml thrombin followed by lysis with 2% acetic acid produces abnormal results at levels below 10 U/dl (Jennings et al, 2003). Occasionally, patients with FXIII levels above 5 U/dl may bleed (see Bolton-Maggs et al, 2004 for review). Patients with hypofibrinogenemia or afibrinogenemia must be excluded from testing by these tests.

FXIII activity assay: FXIII mediates cross linking of α and γ chains of fibrin. FXIII deficiency is classified as a rare bleeding disorder and may be caused by mutations in either FXIII-A catalytic subunits or FXIII-B carrier subunits (Karimi et al, 2018; Mumford et al, 2014). The currently available methods for clinical laboratory diagnoses of FXIII deficiency include clot-solubility assays, quantitative FXIIIa activity assays, FXIII antigen assays specific for the FXIII-A_2B_2 complex, FXIII-A_2, or FXIII-B_2, and genetic testing (Palla et al, 2015). It is recommended that FXIII deficiency is diagnosed using a functional FXIII activity assay rather than clot solubility (Mumford et al, 2014; Kohler et al, 2011; Dorgalaleh et al, 2016). Most FXIII activity assays are only sensitive to deficiencies of the FXIII-A subunit. Fibrinogen is coated on the surface of a microtiter plate. Non-specific binding is prevented by a special blocking agent. FXIII in the sample is activated by thrombin and calcium ions. In the incorporation step, FXIIIa in the test plasma incorporates the substrate, 5-biotinamidopentylamin (BAPA), into FXIII substrate fibrinogen coated on the plate in the presence of calcium. The amount of incorporated BAPA is proportional to the FXIII activity of the test sample. In the next step, a conjugate Strept-AP (streptavidine-alkaline phosphatase) is bound to the incorporated BAPA.

Alkaline phosphatase converts the synthetic substrate pNPP (p-nitro phenyl phosphate) into phosphate and p-nitrophenol, which can be measured at 405 nm. The reagents for the method described below are commercially available in kit form (Pefakit FXIII incorporation assay, Pentapharm Switzerland). Note that other activity assays are available from other manufacturers, including the Berichrom FXIII kit (Siemens, Marburg, Germany) ammonia release, and Technoflur FXIII activity fluorogenic assay (Technoclone, Vienna, Austria)

Reagents: All the required reagents are contained in the commercial kit.

Method:

Day 1

- ✓ Allow kit components to come up to room temperature for 30 minutes.
- ✓ Reconstitute coating reagent (R2) in distilled water, according to the volume recommended by the manufacturer.
- ✓ Add 100 µl coating reagent per well to empty wells of the microtiter plate strips.
- ✓ Freeze any excess coating reagent for subsequent use. It remains stable for 6 months at −20°C.
- ✓ Seal strips with provided plastic seal and incubate overnight (14 to 16 hours) at temperatures of 20–25°C.

Day 2

- ✓ Dilute 20x concentrated TBS R1 (tris buffered saline) 50 ml in 950 ml distilled water or lesser volume, if required.
- ✓ Dilute 3 ml of blocking reagent R3 with 27 ml diluted TBS R1. Freeze excess R3.
- ✓ Discard coating reagent from microtiter plate, invert the strip, and tap on tissue to remove residue.
- ✓ Add 300 µl diluted blocking reagent to each well.
- ✓ Incubate for 1–1.5 hours at 37°C in an incubator.
- ✓ Reconstitute calibrator R10 in 0.5 ml distilled water and the three controls, R11, R12, and R13, in 0.2 ml distilled water.
- ✓ Thaw any frozen test plasmas at 37°C for 5 minutes prior to analysis.
- ✓ Prepare a container with a few hundred ml of ice/water mixture as an ice bath.
- ✓ Make dilutions of all test and control plasmas, 10 µl plasma, and 1 ml diluted TBS R1 buffer (1:101 dilution). Vortex mix.
- ✓ Make calibrator dilutions as follows:

Cal 1: 30 µl R10 + 970 µl TBS R1

Cal 2: 20 µl R10 + 980 µl TBS R1

Cal 3: 75 µl R10 + 25 µl TBS R1

Cal 4: 25 µl R10 + 75 µl TBS R1

Cal 5: 10 µl R10 + 90 µl TBS R1

Note: Dilutions 1 and 2 are ready to use. Further dilute 10 µl of calibrator dilutions 3–5 in 1 ml TBS R1. Wash plate three times with 300 µl/well TBS R1. Invert and tap on tissue to remove excess liquid. Reconstitute activator reagent part A (R4) and part B (R5) in 5 ml distilled water each. Keep on melting ice/water ice bath for no more than 30 minutes. Add 25 µl each of calibrator, control, or test plasma into appropriate wells. Include a blank of TBS R1. Mix activator reagents part A and B (R4 and R5) to form the final incorporation reagent. Add 75 µl final incorporation reagent to each well, including blank well. Incubate for 30 minutes at 37°C in incubator. Add 200 µl/well incorporation stopping solution R6. Mix gently for 10 minutes on the plate shaker. Reconstitute detection reagent R7 by adding 12 ml distilled water. Freeze unused diluted

R7. Wash plate four times with 300 µl/well TBS. Tap to remove excess liquid. Add 100 µl/well detection reagent R7. Incubate for 15 minutes at 37°C in the incubator. Wash plate four times with 300 µl/well TBS. Tap to remove excess liquid. Make up substrate solution immediately before use:

- ✓ For 96 wells (full plate), add 9 tablets R8b to 22.5 ml diethanolamine buffer R8a
- ✓ For 64 wells (8 strips), add 6 tablets to 15 ml diethanolamine
- ✓ For 32 wells (4 strips), add 3 tablets to 7.5 ml diethanolamine
- ✓ For 24 wells (3 strips), add 2 tablets to 5 ml diethanolamine

Add 180 µl/well of substrate solution. Incubate for 11 minutes at 37°C in the incubator. Add 50 µl/well stopping solution (4M NaOH) R9. Read optical densities within 15 minutes at 405 nm in a microtiter plate reader.

Note: Several kit reagents can be stored deep-frozen for later use, as described above. However, the substrate, activator reagent parts A and B, calibrators, and controls should not be frozen. Partial reagent kits containing these latter materials can be purchased for use with any partially used reagents that have been frozen. This reduces the cost per test if test samples are analyzed in small batches.

Results calculation: Calibrator dilutions and control values are supplied with each kit. Using suitable data handling software or graph paper, construct a calibration curve by plotting the concentration against the optical density (OD) of the calibrator dilutions after subtracting the OD of the blank. Use a linear-linear scale. Subtract the blank OD from the ODs of the test sample/control dilutions, and convert the ODs to FXIII activity, using the calibration curve. The patient results can be accepted if the control sample values are within the acceptable range.

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PART 10 Platelet

Tulasi Geevar

TOPICS COVERED

- ✓ Platelet Count
- ✓ Platelet Function Testing
- ✓ Platelet Function Testing by Flow Cytometry

Platelet Count: Blood is mixed with a diluent that causes hemolysis of red cells. A hemocytometer is filled with the diluting fluid, and the platelets are counted under the microscope, preferably by using phase-contrast, if available.

Materials/equipment:

- ✓ Flat-bottom, thin counting chamber (phase-contrast hemocytometer with Neubauer ruling)
- ✓ Phase-contrast microscope equipped with long-working-distance phase condenser, if available; otherwise an ordinary light microscope
- ✓ 20 µl pipette
- ✓ 2 ml graduated pipette
- ✓ 12 x 75 mm tube
- ✓ Mechanical mixer

Reagent: Diluting fluid: 1% ammonium oxalate in distilled water. Store in the refrigerator and always filter just before using.

Specimen: If the blood sample is from a finger prick, the puncture must be clean and the blood free flowing. Wipe away the first drop of blood. If the blood sample is from venous blood, it must be collected into a dry plastic (or siliconized glass) syringe with a short needle not smaller than 21 gauge. The needle must be removed before the blood is delivered into a plastic container with EDTA. The blood and anticoagulant must be mixed gently, to avoid frothing, without any delay.

Method: Pipette 0.38 ml of diluting fluid into a test tube. Fill the 20 µl pipette to the mark and wipe off the outside of the pipette. Expel the contents of the pipette into the diluting fluid, and wash out the pipette by drawing up the blood and expelling it into the tube a few times. Mix for at least 10 minutes by hand or, preferably, by mechanical mixer. Fill the hemocytometer, as described below. Cover the chamber with a petri dish for 10 to 20 minutes to allow the platelets to settle. Leave a piece of wet cotton or filter paper in the dish to prevent evaporation. Using a microscope, count the platelets in the large 1 mm squares (= 0.1 µl). Count the platelets in as many squares as necessary to reach a count of at least 100. The platelets appear round or oval, and their internal granular structure and purple sheen allow them to be distinguished from debris. Ghosts of the red cells that have been lysed by the ammonium oxalate are seen in the background. If phase contrast is not available, an ordinary light microscope can be used, provided the condenser is racked down to provide a low intensity of light. Calculate the number of platelets per liter of blood according to the formula below.

The hemocytometer: The hemocytometer counting chamber, with Neubauer or improved Neubauer ruling, is constructed so that the distance between the underside of the cover glass and the surface of the chamber is 0.1 mm. The surface of the chamber contains two specially ruled areas with dimensions as

shown in Figure 21. The central 1 mm^2 has double or triple boundary lines. In the central areas, there are 25 squares in the improved Neubauer and 16 squares in the Neubauer ruling. Each square has an area of 0.04 mm^2 (0.2 x 0.2 mm). These squares are, in turn, divided into smaller squares, each 0.0025 mm^2 (0.05 x 0.05 mm). The outer quadrants of the ruled area are each 1 mm^2 and are divided into 16 squares.

Calculations:

The formula for calculating the cell count is:

Count (cells/l) = N x D/A x 10 x 10^6

Where N = total number of cells counted

D = dilution

A = total area counted (in mm^2)

10 = factor to calculate volume in µl from area (in mm^2) and depth of chamber (0.1 mm)

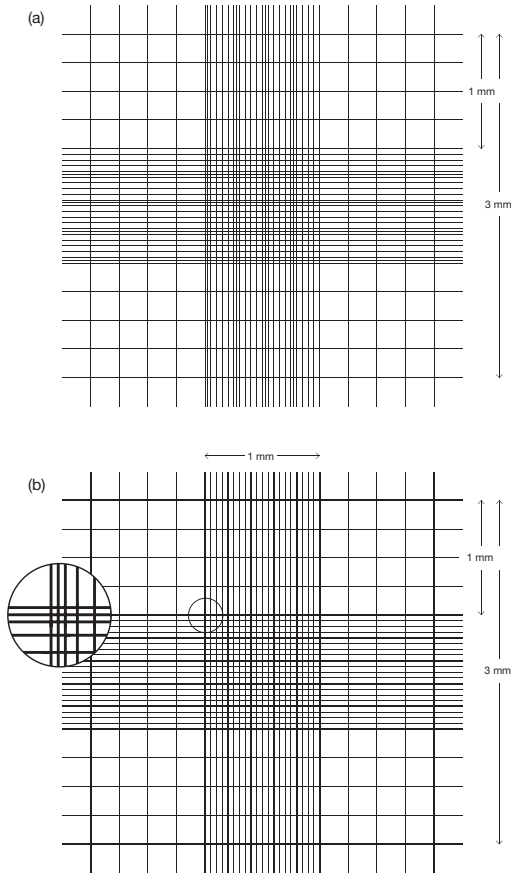
10^6 = factor to convert count/µl to count/l

Sources of error in cell counting: When capillary blood is used, a free-flowing drop must be obtained. When anti-coagulated blood is used, the specimen must be carefully mixed by inverting the tube of blood at least 20 times before a sample is taken. Do not shake the tube, because shaking introduces foam, which makes accurate pipetting impossible. Tilt the well-mixed tube to an angle of 45° or slightly more, and pipette from the lip of the tube, following the same procedures as for capillary blood. The blood-sampling pipettes must be clean and dry. The pipette must be filled quickly, and the blood must be drawn accurately by using a pipette suction device attached to the pipette, filling up to the desired line. If the line is overshot slightly, the excess blood may be expelled by touching the lip of the pipette on a piece of filter paper or soft tissue. If the line is overshot, a fresh pipette must be used. No air bubbles should be present in the blood column. The outside of the pipette must be wiped free of blood (being careful not to pull blood from the tip) before it is introduced into the diluting fluid. After the contents of the pipette have been discharged into the diluent, diluting fluid must then be drawn into the pipette with steady suction several times, to ensure that all the blood is discharged into the fluid. The tube containing the diluted blood must be shaken gently for at least two minutes by hand or, preferably, in a mechanical shaker. After the tube has been shaken, the chamber is immediately filled by means of a Pasteur pipette or capillary tube. The chamber is filled by capillary action, with the flow of fluid from the pipette or capillary regulated so that it fills quickly and smoothly. It must be filled completely, but fluid must not spill over into the moats. Allow the cells to settle in the counting area for 10 to 20 minutes, then proceed with the counting. The hemocytometer chamber and glass cover must be clean and dry before they are used. Important errors can be introduced by fingerprints or an oily film. A sufficient number of cells must be counted to reduce error due to chance distribution of cells. In practice, at least 100 cells should be counted. As a further check on correct distribution of cells in the chamber, the number of cells counted in each area (i.e. in the large squares) should not differ by more than 10%.

Controls: Two dilutions must be made, and the mean of the two counts taken; the two counts should agree within 10%.

Sources of error in platelet counting: Blood obtained by a venipuncture is preferable to capillary blood, because platelets adhere to the wound and successive dilutions from a finger prick are not always reproducible. The general errors of pipetting and hemocytometry are described above. In addition, special attention must be paid to ensuring that the counting chamber is scrupulously clean, since dirt and debris

may be counted as platelets. Wash the chamber with soapy water, then rinse with distilled water, allow to drain dry, and wipe with lint-free tissue. Be sure that the cover slip is clean before using it. The presence of platelet clumps precludes reliable counts. If the sample contains clumps, a fresh sample must be collected. The ammonium oxalate diluent should be kept refrigerated and must be discarded if there is evidence of bacterial contamination. The specimen must be counted within three hours of collection.



10.2 Platelet Function Testing

10.3 Clot Retraction

10.4 Measurement of Platelet Aggregation

Figure 21. Hemocytometer counting chamber (a) Neubauer and (b) Improved Neubauer

Platelet Function Testing: The primary role of platelets is to support hemostasis by formation of platelet plug at the sites of vascular injury. When the blood vessel is injured and the subendothelial microfibrils and collagen fibers are exposed, platelets bind to the vessel wall, a process called platelet adhesion. At sites of high shear, platelets bind to vessels indirectly with the help of high molecular weight VWF multimers. VWF binds to exposed collagen through its A3 domain, and the platelets bind to the collagen bound VWF through its GPIbα receptor. Platelets can also adhere directly to collagen in the sub endothelium with the glycoprotein VI (GPVI) and integrin α2β1 receptors. Once platelets adhere to the vessel wall, they undergo a series of changes: Through the reorganization of platelet cytoskeleton, they change from small disc shaped structures to spiculated spheres with the development of filopodia. Membrane flip flop translocates anionic procoagulant phospholipids, mainly phosphatidyl serine, to the outer surface of platelets, which forms a good platform for thrombin generation. Platelets release the contents of alpha (e.g. fibrinogen, FV, VWF, and growth factors) and dense granules (e.g. ADP, ATP, calcium, and serotonin). This causes further platelet activation. Formation of thromboxane A_2 (TxA_2) occurs from arachidonic acid via phospholipase A2, cyclooxygenase-1 (COX-1), and TXA_2 synthase. Platelet agonists (ADP, TxA_2, and thrombin) bind to specific membrane receptors and initiate platelet aggregation by activation of the integrin receptor, α2β3 (GPIIb-IIIa) that binds to fibrinogen and/or VWF to form the platelet plug. Abnormalities in any of these pathways causing platelet adhesion, activation, degranulation, and aggregation can cause

platelet dysfunction. Platelet disorders include both quantitative (thrombocytopenia) or qualitative defects and can be inherited or acquired. Bleeding symptoms are primarily mucocutaneous, like ecchymosis, gum bleeding, easy bruising, menorrhagia, post-partum hemorrhage, and gastrointestinal bleeding (malena, hemestemesis, or hematochezia). Symptoms can be mild or severe depending on the abnormality. A list of inherited platelet function disorders (IPFDs) with associated salient clinical and laboratory features are shown in Table 33. The true prevalence of platelet disorders is not known. It is thought that platelet function disorders are more common than previously appreciated due to underdiagnosis. In areas of the world where consanguinity is common, autosomal recessive disorders like Glanzmann thrombasthenia and Bernard Soulier syndrome have a higher prevalence. While diagnosis of Glanzmann thrombasthenia and Bernard Soulier syndrome are relatively easy due to typical aggregometry pattern, the diagnosis of most other IPFDs is cumbersome and requires complex assays. In the investigation of patients suspected of having an IPFD, it is important to collect a detailed clinical history, including personal and family history. The use of a validated BAT like the ISTH BAT is strongly encouraged. History should also include drug history, recent food intake which can possibly interfere with platelet function, and the presence of other features (e.g. eczema, recurrent infections, familial cases of myelodysplasia, AML) and potential syndromic features (e.g. hearing loss, heart defects, face or bone dysmorphism, albinism) which can help in diagnosis. Preliminary laboratory investigations should include full blood count including platelet count, examination of a blood film to look for platelet morphology and other blood cell features (see below), determination of the skin bleeding time (described in Section 11), screening tests for secondary hemostatic tests (PT and APTT), and fibrinogen assay to exclude other coagulation disorders. It is recommended to perform screening tests for VWD (i.e. VWF activity, VWF antigen) either concurrently or prior to more extensive work-up for platelet function disorders. VWD, type 2B VWD, and platelet type VWD can also present with macrothrombocytopenia and should also be considered in patients with low platelet counts. Another simple test that can give an indication that to the presence of a platelet function defect is the clot retraction method, described below.

Clot retraction: The retraction of the clot in clotted whole blood can give an indication of platelet number and function. When the clot retracts, serum is expressed, and the degree of clot retraction can be measured.

Method: Collect 1ml of blood into a glass test tube (75 mm x 10 mm) and place at 37°C. Examine the tube visually until a firm clot is present. Leave undisturbed at 37°C for another hour. Measure the distance from the base of the tube to the meniscus. Carefully remove the clot with a thin wooden stick (e.g. a cocktail stick), leaving the serum that has been expressed from the clot in the tube. Measure the distance from the base of the tube to the meniscus of the serum. Divide the serum distance by the total distance and multiply by 100 to give a percentage.

Interpretation: Normally, more than 40% serum is expressed. A decreased expression is present in some platelet defects, notably Glanzmann's thrombasthenia. It can also be abnormal in severe thromobocytopenia, Wiskott Aldrich syndrome, and Stormorken syndrome.

Notes: The tubes and wooden stick must be absolutely clean to prevent the clot from adhering to the tube. The clot must be removed carefully and gently to avoid squeezing and therefore more serum being expressed.

Platelet count, morphology, and examination of peripheral blood smear: Initial testing for a suspected platelet function disorder should include a complete blood count along with evaluation of peripheral blood smear. This helps to detect abnormalities of platelet number, size and morphology which can provide important clues for further laboratory testing. Platelet count can be measured by manual microscopic method, automated counting in hematology analyzers, and flow cytometry based immunological labelling. Estimation of the platelet count on the peripheral smear should be performed to confirm platelet count, especially in cases of macrothrombocytopenia or platelet anisocytosis. In a well prepared and stained smear, the average number of platelets are counted in 10 oil immersion fields (100x). This value should be multiplied by 15,000 to determine the platelet count/µL. It is important to check the smear to

evaluate platelet clumps, platelet size, morphology and granularity. Pseudo-thrombocytopenia due to EDTA-dependent platelet clumping and satellitism (platelets rosetting neutrophils) should be excluded. It is important to remember that platelet clumps can also be seen in platelet type or type 2B VWD and should not be mistaken for EDTA-induced clumping. Platelet type VWD can also show large platelets in the smear. An abnormal platelet size is often a feature of inherited platelet disorders (with or without platelet dysfunction) as shown below:

- Small platelets: Wiskott-Aldrich syndrome, X-linked thrombocytopenia, ADAP deficiency
- Large/giant platelets: Bernard Soulier syndrome (giant), MYH9-related disorders (giant), Grey platelet syndrome and α-granule deficiency syndromes, platelet type VWD, ITGA2B/ITGB3-related thrombocytopenia (GT variant), Filaminopathy, SLFN14-related thrombocytopenia, GATA1-defects, Velocardiofacial syndrome
- Normal platelets: All the remaining disorders

The mean platelet volume (MPV) is a readily available parameter on impedance-based platelet counting hematology analyzers and gives an estimate about the platelet size. Laboratories should establish their own reference ranges as MPV can vary between instruments. Small platelets can be easily missed by light microscopy and a reduced MPV might be a first clue for possible Wiskott Aldrich syndrome. Various factors can affect the measurement of MPV and interfere with impedance-based platelet counting, like abnormalities of red cells and platelets. The presence of microcytic and fragmented red cells can overestimate platelet counts while presence of large and/or giant platelets can be missed causing underestimation of platelet counts. In these scenarios, platelet counts and MPV will not be reliable. It is very useful to concomitantly look at platelet histogram which can identify platelet size abnormalities, and possible interferences/contamination. In macrothrombocytopenia, the optical or fluorescent platelet counts give truer platelet counts. Large pale platelets with bluish grey cytoplasm with absent or markedly reduced azurophilic granules can be seen in Gray platelet syndrome due to deficiency of α-granules. Paris Trousseau syndrome shows large to giant platelets, some of them showing clumped/fused α-granules forming one large, clumped granule. Platelet anisocytosis (i.e variation in platelet size) and anisogranularity (i.e variation of platelet granularity) with presence of pale staining empty looking platelets is a classical feature of Acquired platelet dysfunction with eosinophilia (APDE). APDE is a transient bleeding diathesis associated with eosinophilia, commonly presenting in children from South-East Asia, and reversed upon treatment of eosinophilia. Morphologic abnormalities can also be seen in other blood cells. Presence of Dohle-like inclusion bodies in neutrophils in MYH9-related disorders, giant peroxidase positive cytoplasmic granules in neutrophils and/or other leucocytes in Chediak Higashi syndrome, Howell Jolly bodies in Stormoken syndrome and dyserythropoiesis in GATA-1 mutations.

Measurement of platelet aggregation by light transmission aggregometry: Light transmission aggregometry (LTA) is the gold standard for platelet function testing. It was first independently described by O'Brien and Born in the 1960s. The principle is based on the changes in the optical density of platelet rich plasma (PRP) as platelets get activated and form aggregates. PRP is turbid due to the suspension of platelets compared to PPP. Once agonists are added to PRP, platelets form aggregates and settle down, thereby clearing the plasma and allowing more light to pass through. The change in transmitted light is measured over time by an aggregometer and represented graphically. By comparing the pattern of aggregation response to each agonist, the type of platelet function disorder can be suspected and diagnosed. A number of excellent reviews and guidelines for platelet function testing and diagnosis of IPFDs have been published.

Precautions prior to studying platelet aggregation (pre-analytical variables in platelet aggregation): Blood samples for LTA should be collected after a short period of rest. Subjects should refrain from smoking for at least 30 minutes before and from caffeine at least 2 hours before testing. A record of all drugs, including native/herbal medications taken during the last 10 days should be documented. For a list of drugs that can interfere with platelet function, see the British Society for Haematology's guidelines on platelet function testing referenced below. Drugs known to reversibly inhibit platelet function (e.g. NSAIDs) should be stopped at least 3 days before sampling. Drugs known to irreversibly inhibit platelet function (e.g. Aspirin,

thienopyridines) should be stopped at least 10 days before sampling, unless their effect is being specifically investigated. Patients should ideally be fasting, preferably after overnight fasting. If not fasting, avoid sampling after fatty meals. Many other "normal" dietary constituents, including alcohol, onions, garlic, pepper, and ginger, may also inhibit platelet aggregation. This should be kept in mind when evaluating results. Blood should be drawn with minimal, or no venostasis using a needle of at least 21 gauge into a 109 mM or 129 mM sodium citrate, buffered anticoagulant. The first 3-4 ml of blood drawn should be discarded or used for tests other than LTA (e.g. PT/APTT). When sample collection is difficult, under-filled tubes may only be used to exclude severe platelet function defects, such as Glanzmann thrombasthenia or Bernard-Soulier syndrome.

Preparation of platelet-rich and platelet-poor plasma: Blood samples should be allowed to 'rest' at room temperature for at least 15 minutes before centrifugation. Refrigeration of platelets can cause activation and hence samples should always be maintained at ambient temperature. PRP should be prepared by centrifuging blood samples at 200g for 10 minutes at ambient temperature (~21°C) without using a brake. For samples with very large platelets, like Bernard-Soulier syndrome, PRP should be prepared by blood sedimentation for 30 minutes. The PRP is carefully removed, avoiding contamination with red cells or the buffy coat (aggregation will be diminished if either are present), into a capped polypropylene tube kept upright at 20–25°C. PPP should be prepared by centrifuging whole blood, or the tubes of blood from which PRP was removed, at ambient temperature at 1500g for 15 minutes. Grossly hemolyzed samples should be rejected. If the sample is lipemic, indicate this in the final report. It is necessary to check the platelet count of the PRP. The results of LTA studies could be inaccurate when the platelet count in the PRP sample is lower than 150 x 10^9/l. Samples with low platelet counts should be interpreted with caution but may be tested to exclude severe platelet function disorders, like Glanzmann thrombasthenia, Bernard Soulier syndrome, type 2B VWD, and platelet VWD. The platelet count of PRP samples should NOT be adjusted to a standardized value with autologous PPP. Platelet count in PRP in samples from subjects with normal platelet count (200–600 x 10^9/l) do not affect the results of LTA studies. Adjusting the platelet count in PRP using autologous PPP within this range may inhibit platelet responsiveness. This is probably because the PPP may contain substances released from platelets during the additional trauma from the higher speed centrifugation used in PPP preparation. Uncertainty remains over what is the best practice to follow when the platelet count in PRP exceeds 600 x 10^9/l. For exceedingly high PRP counts (>1000 x 10^9/l), it may be advantageous to adjust the platelet count to a more suitable level with the patient's PPP.

Aggregating agents or agonists: There are two types of agonists, weak and strong agonists. Weak agonists (e.g. ADP and epinephrine) at critical concentrations show an initial primary wave of aggregation due to direct agonist induced effect, followed by a secondary wave of aggregation caused by the release of platelet granules and TXA_2 synthesis. Strong agonists (e.g. thrombin, collagen, TXA_2) on the other hand show only a single curve without distinction between primary and secondary aggregation as these agonists directly induce platelet aggregation, TXA_2 synthesis, and granule release. The different phases of platelet aggregation are shown in Figure 22, which is evident only with weak agonists.

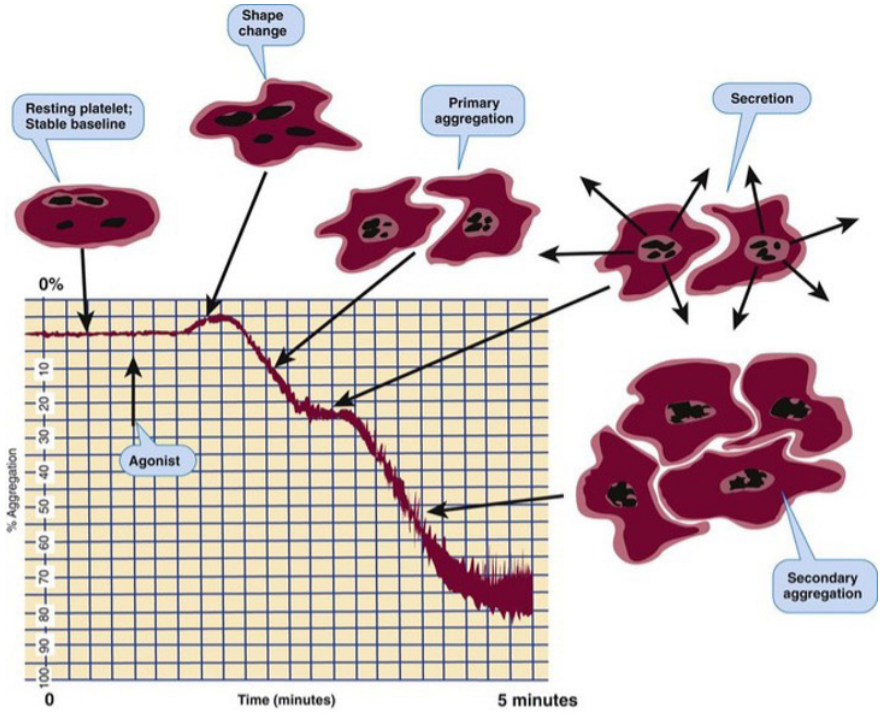


Figure 22. Image from Rodak's Hematology: Clinical principles and applications, 5th edition

Table 33. Summary of recommended agonists along with concentrations that can be used as an initial panel and extended panel (concentrations as per ISTH guidelines)

Agonist	Initial panel and concentration	Extended panel and concentration
ADP*	2 µM	5 µM, 10 µM
Epinephrine*	5 µM	10 µM
Collagen (Horm or type 1 fibrillary)	2 µg/ml	
Arachidonic acid	1mM	
Ristocetin (low dose)	0.5-0.7 mg/ml	
Ristocetin (high dose)	1.2-1.5 mg/ml	
Thrombin receptor activating peptide (TRAP)*, PAR1	-	10 µM
Thromboxane A2 mimetic U46619	-	1 µM

*Higher concentrations should be used if abnormal results are seen with the initial concentration.

Adenosine-5-diphosphate (ADP): A stock solution of 1mM/l of the disodium salt is prepared in OBS and stored in small amounts at -40°C. This is stable for at least three months. Once thawed, the solution should be used within three hours or discarded. For use, further dilutions are prepared in OBS. The pattern of response to ADP depends on its final concentration. At 2 µmol/l, clearly defined primary and secondary waves can be seen: the first represents the direct agonist-induced effect and the latter is due to release of endogenous ADP and generation of TXA_2, which itself aggregates platelets. Below 2 µmol/l, progressively fewer normal subjects show a secondary response, and the primary wave usually reverses as the ADP is enzymatically degraded. Above 3 µmol/l, the primary phase is usually so intense that the distinction between it and the secondary phase is masked. ADP induces a change in shape of the platelets from a disc to a spiky sphere. This initially causes a slight increase in the optical density of the platelet suspension, which can be seen only if primary aggregation is impaired.

Adrenaline (epinephrine): A stock solution of 1mM/l of the bitartrate salt is prepared in OBS. It should be stored and used as for ADP. With adrenaline, the concentrations used and the patterns of response are similar to those of ADP. However, in the absence of a secondary wave, the primary wave does not reverse, nor is it ever so intense that the secondary wave is masked.

Collagen: A very stable suspension of equine tendon collagen fibrils (1 mg/ml), available from Hormon-Chemie, Munich, Germany, is widely used. A number of other materials are equally suitable. It is stored at 4°C and must be well mixed immediately prior to dilution in the buffer packaged with it. It should be used at final concentration of 0.5–2.0 µg/ml in PRP, and diluted suspensions are stable for one week at 4°C. With collagen, no primary wave occurs. The response is usually defined by the duration of the lag phase prior to the onset of aggregation and by the intensity of the latter. A slight increase in the optical density caused by the shape change precedes aggregation. Collagen from a number of different sources is in use. Both the type of collagen and the species from which the preparation is prepared (e.g. equine or bovine) can have an important effect on the results obtained. Indeed, more than a hundred-fold range of concentrations are required, depending on the source material. It is therefore important to select a suitable source and to establish a local reference range for this material, which should be re-evaluated if the source is changed. For a review, see Jennings et al. (2008).

Ristocetin: At a final ristocetin concentration of 1 mg/ml in PRP, distinct primary and secondary waves are usually discernable, but above this the direct effect is so intense that the two phases merge. The primary wave is a measure of the amount of VWF present in the plasma, whereas the second wave is due to release of endogenous substances.

Arachidonic acid: Sodium arachidonate (99% purity) is dissolved in OBS to a concentration of 10mM/l. Small aliquots are placed in darkened glass vials that are flushed with nitrogen to prevent oxidation, then tightly capped and stored frozen below -20°C. Aggregation is generally monophasic and preceded by a short lag phase.

Reagents: Some examples of reagent concentrations and dilutions are shown below

Note: These concentrations are appropriate if one part is added to nine parts of PRP. Dilutions can be made in distilled water or saline, or as per manufacturer's instructions.

✓ ADP (Stock solution 1000 µM)

Make a 1 in 10 dilution = 100 µM (i.e. 0.1 ml 10,000 µM solution + 0.9 ml OBS). From this, make appropriate working strengths: 20 µM (i.e. 0.2 ml 100 µM + 0.8 ml OBS), PRP final concentration of 2–50 µM (i.e. 0.5 ml 100 µM + 0.5 ml OBS), PRP final concentration of 5 µM. In cases where hyperaggregability is being tested, working strengths of lower concentration may be needed (e.g. 10 µM, 5 µM).

- ✓ *Adrenaline (epinephrine):* Dilute as for ADP to make a working strength of 50 µM. This will give a final PRP concentration of 5 µM.
- ✓ *Collagen:* Mix well and dilute in OBS/other diluents: 1 in 500 (i.e. 0.1 ml stock + 4.9 ml OBS) = 20 µg/ml, PRP final concentration of 2 ug/ml
- ✓ *Ristocetin:* This is used at up to two different concentrations depending on the results obtained. Normal dose ristocetin at either 15 mg/ml or 12.5 mg/ml, and low ristocetin at either 7.5 mg/ml or 5 mg/ml. This will give a final PRP concentration of 1.5 mg/ml or 1.25 mg/ml, and low dose ristocetin of 0.75 mg/ml or 0.5 mg/ml respectively depending on the concentration used.

Method/procedure: LTA studies should be completed within a maximum of 4 hours after blood sampling. Because of platelet refractoriness to aggregation after centrifugation, PRP samples should be allowed to sit at ambient temperature for at least 15 minutes before testing. PRP should be stored in full, tightly stoppered tubes until tested. Before starting, bring the reagents/agonists to room temperature. Prepare fresh dilutions of reagents if required. Turn on the aggregometer and the computer/recorder and wait till

the temperature of the aggregometer reaches 37°C. Set the stirring speed to 1000 rpm unless otherwise specified by the manufacturer of aggregometer. Pipette 450 µl of PRP to glass cuvette with a magnetic stir bar. The number of cuvettes used depends on the number of agonists to be tested. Pre-warm the PRP cuvettes in the incubation wells surrounding the heater block for 2-5 minutes. Pipette 500 µl of PPP to another glass cuvette without stir bar and place in the corresponding blank reference well. PRP and autologous PPP should be used to set 0% and 100% light transmission in the aggregometer. Allow to run for 1 minute before adding the agonist. Pipette 50 µl of agonist to bottom of the PRP cuvette without producing bubbles. The volume of agonist added for LTA should be consistent, and never more than 10% of the total sample volume. A know control sample must be run parallel with the patient. Platelet aggregation should be monitored for a minimum of 3 minutes after adding an agonist, and up to 10 minutes for agonists that do not reach maximal aggregation by 3–5 minutes. Repeat the procedure for each agonist.

Notes: If an abnormal response is seen with initial panel of agonists, additional extended panel (Table 35) can be added on along with ATP release by lumiaggregometry (see below). Higher concentrations of agonists (e.g. ADP, epinephrine) should be used if abnormal response is seen at the initial concentration. If no aggregation response is seen with arachidonic acid, add on the agonist, TXA_2 receptor analogue (TRA), U46619. In TXA_2 synthesis defects (COX/TXA_2 synthase deficiency) or Aspirin effect, normal response is seen with TRA. In cases of TXA_2 receptor defect, abnormal responses are seen with both arachidonic acid and TRA. Isolated abnormal response with Epinephrine can be seen in a proportion of healthy subjects. However, this may be the only LTA feature of Quebec platelet syndrome. When studying platelet aggregation as part of a hyperaggregability assessment, ADP and adrenaline are used at lower concentrations to obtain a dose response curve. Concentrations used are: 2 µM, 1 µM, 0.5 µM, and 0.1 µM final concentration in PRP. A spontaneous aggregation is also performed before the rest of the agonists are tested. If the platelets hyperaggregate with 0.5 mg/ml ristocetin, this indicates possible type 2B VWD or platelet type VWD. Check for spontaneous aggregation by monitoring PRP under the same stirring conditions on the aggregometer without adding any agonist to stimulate aggregation. Perform mixing studies to differentiate between the two subtypes. This can be done by two methods. Basic procedure: Mix equal volumes of patient plasma with normal platelets (i.e. 225 µl of patient PPP + 225 µl of control PRP). This mixture is then assayed with ristocetin at 0.5 mg/ml. If agglutination is present, it suggests type 2B VWD. An absent agglutination may be due to platelet type VWD. It is important to note that an absent response may also be due to the additional dilution of PRP.

Alternate procedure: Wash the patient and control platelets three times with PBS-EDTA in 15 ml centrifuge tubes (low centrifugation speed to soft pellet the platelets). Carefully resuspend patient's platelets in normal plasma and normal platelets in patient's plasma. Adjust platelet count to 400 x 10^6 pelleted platelets. (Note that PBS-EDTA is prepared by adding 9 mM di-sodium EDTA to PBS and adjusting the pH to 7.0. PBS used contains 135 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, with a pH of 7.4.) Retest with ristocetin at 0.5 mg/ml. Reactions should fit one of the patterns below.

Table 34. Type 2B VWD and platelet type VWD interpretation

Tube 1	Washed patient platelets + Control PPP	No agglutination	Agglutination
Tube 2	Control washed platelets + patient PPP	Agglutination	No agglutination
	INTERPRETATION	Type 2 B VWD	Platelet type VWD

The 2021 VWD guidelines favor the use of targeted genetic testing for the diagnosis of type 2B and platelet type VWD. However, this may not be feasible for many laboratories due to cost constraints. RIPA mixing study is still retained as one of tests in the diagnostic algorithm according to ISTH.

Interpretation and reporting of results: The platelet aggregation tracing should be evaluated based on presence of shape change, length of lag phase (some agonists like collagen may have a longer lag phase), maximal aggregation (most convenient and commonly used method for reporting results), slope of aggregation (indicates rate of aggregation), visual examination of the aggregation tracings (i.e. disaggregation,

presence of secondary wave—particularly distinct for epinephrine and low doses of ADP). Disaggregation with ADP is particularly striking with inherited P2Y12 defects. PRP prepared in the same way from a healthy normal donor should be processed and run simultaneously with the patient as a check on the reagents. This is especially important if abnormal patient results are obtained, as some agonists are labile, particularly once diluted to working concentrations. Clinical laboratories must establish appropriate reference intervals for each agonist's concentration. Results from healthy normal subjects tested alongside the patient can be used to derive reference ranges. Great caution is required when interpreting platelet aggregation patterns. A number of technical factors may influence the results. Bear in mind that there are a number of important differences between aggregation determined by nephelometry and that occurring in the body. Nevertheless, useful diagnostic information can be obtained, and some examples of aggregation patterns are shown in Table 35.

Table 35. Platelet aggregation results in various disorders

Disorder	ADP	Collagen	Ristocetin 1.25 mg/ ml	Ristocetin 0.5 mg/ ml	Arachadonic Acid	Adrenaline
VWD type 1 and 2A	N	N	A/R**	A	N	N
VWD type 2B	N	N	N	H	N	N
Bernard-Soulier syndrome	N	N	A	A	N	N
Glanzmann's thrombasthenia	A	A	N	A	A	A
Storage pool disease	P/N	R/N	N	A	R/N	P/N
Cyclo-oxygenase defect*	R/N	R	N	A	R/A	R/N

N = Normal; A = Absent; R = Reduced; H = Heightened response; P = Primary wave only
*Or Aspirin effect
**Can be normal in mild VWD type 1

Further investigation of platelet function: If an abnormal aggregation pattern is observed in an individual, it is advisable to repeat the assessment on at least one further occasion to check for consistency of the abnormality. In the presence of abnormal aggregation, further investigation may be useful. These include the measurement of platelet nucleotide content and their release during platelet aggregation. Quantitation of membrane glycoproteins can be performed for the unequivocal diagnosis of Bernard-Soulier syndrome and Glanzmann's thrombasthenia (see below).

ATP release by lumiaggregometry: Lumiaggregometry is the most widely used method to assess platelet dense granule release function. It has the advantage that LTA and ATP release from dense granules can be measured simultaneously. It is based on the principle of luciferin-luciferase reaction. When platelets are activated, dense granules release their stored ATP and ADP. The released ATP reacts with luciferin in the presence of luciferase to give luminescence which can be quantified, relative to an ATP standard. Defective ATP secretion can be seen in both dense granule deficiency and primary secretion defects. Distinction between the two entities requires additional testing, like measurement of total platelet ATP-ADP content by bioluminescent assays, serotonin release assays with radiolabeled ^{14}C-5-HT, mepacrine assay by flow cytometry (see below) or assessment of dense granules by whole mount transmission electron microscopy.

Table 36. Technical factors that influence platelet function

Anticoagulant	1/10th volume of trisodium citrate.
Time	Start tests 30 minutes after preparation of PRP. Complete studies within four hours of blood collection.
Centrifugation	Should be sufficient to remove red cells and white cells, but not large platelets. Should be done at room temperature, not at 4°C. Large platelets can be separated by sedimentation.
Platelet count	Low counts <100 x 10^9/l cause slow, weak responses. High counts >1000 x 10^9/l may show reduced response.
pH	<pH 7.7 inhibits aggregation. >pH 8.0 enhances aggregation.
Mixing speed	<800 rpm shows reduced aggregation. >1200 rpm breaks up platelet clumps.
Hematocrit	>55% shows progressively less aggregation, especially second-phase inhibition due to increased citrate concentration.
Temperature	<35°C shows decreased aggregation with regular doses of all agonists, but increased response to low doses of ADP.
Lipemia	Increased chylomicrons cause reduced aggregation.
Dirty cuvette	May cause apparent spontaneous aggregation.
No stir bar	No response on addition of aggregating agent.
Air bubbles	Rapid, large oscillations of pen prior to aggregation. Also caused by low platelet count.

Differences between in vivo and in vitro conditions for platelet aggregation: With in vitro blood tests, blood is anticoagulated, RBC and WBC are removed, vascular components are not involved, coagulation is not involved, platelet population is selected, products of platelet activation and release are retained, reagents used are unphysiological in composition and dose, platelets are unstable out of the body, and drugs may show more or less marked effects than in vivo.

Platelet Function Testing by Flow Cytometry: Flow cytometry can provide useful information for the diagnosis and classification of platelet function disorders. It can be used for analysis of glycoprotein receptors, platelet function testing after agonist stimulation, measurement of platelet procoagulant function (e.g. annexin V binding) and assessment of alpha and dense granule content. The major advantages of flow cytometry are the requirement of small sample volumes, it can be performed on whole blood, and it is not limited by thrombocytopenia. This becomes particularly useful in pediatric and thrombocytopenic subjects.

Platelet surface glycoprotein analysis by flow cytometry: Glanzmann thrombasthenia, characterized by abnormal or absent αIIbβ3 can be detected with fluorescently labelled GPIIb (CD41) or CDIIIa (CD61) monoclonal antibodies, and Bernard Soulier syndrome, characterized by abnormal or absent GP1b/V/IX can be diagnosed using antibodies directed against GPIb (CD42b) or GPIX (CD42a) antibodies. Additionally, other less common IPFDs affecting the receptors α2β1 (CD49/CD29), GPIV (CD36) or GPVI can also be diagnosed using respective antibodies. Analysis of surface molecules can be done even up to 24 hours from collection. Flow cytometric platelet activation test (PACT)**.** PACT allows for simultaneous measurement of a broad range of different activation markers of platelet function thereby allowing for a more comprehensive analysis of various pathways involved in platelet function. Platelets can be identified using their characteristic forward side scatter (FSC) and side scatter (SSC) properties and preferably by the addition of a specific glycoprotein receptor (e.g. CD41). Some of the commonly used activation markers are: PAC-1 (monoclonal antibody that specifically binds to the activated conformation form of αIIbβ3), CD62P or P-selectin (one of the contents of α-granules, can be used as a marker of α-granule release), and CD63 (can be used as a marker of δ-granule release). Testing can be done using a combination of agonists to target different

pathways, such as ADP, thrombin receptor activating peptide (TRAP), and convulxin. Expression of activation markers on the platelets is measured (using MFI and/or %) at baseline (before adding agonist) and after agonist stimulation. A reduced expression after agonist stimulation suggests a defect/abnormality in the specific pathway. It is important to run a control sample simultaneously similar to LTA.

Ristocetin-induced VWF binding by flow cytometry: The function of the GP1b/V/IX receptor and VWF can also be assessed by flow cytometry by measuring VWF expression on platelets after the addition of normal and low doses of ristocetin. Monoclonal antibody against anti VWF can be used. Mixing studies with patient plasma and control plasma and vice versa at low doses of ristocetin can differentiate between type 2B and platelet type VWD.

Mepacrine uptake and release assay by flow cytometry: The mepacrine uptake and release assay can also be used to assess δ-granule defects. Mepacrine is a fluorescent acridine derivative that binds to platelet adenosine nucleotides with high affinity. After incubation of platelets with mepacrine at 37°C for 30 minutes, it is selectively taken up by dense granules which can be measured by increase in fluorescence by flow cytometry. The release function can also be assessed by measuring mepacrine fluorescence after agonist stimulation (e.g. convulxin or TRAP). Fluorescence is measured at baseline (only platelets), after addition of mepacrine (platelets + mepacrine) and after addition of agonist (platelets + mepacrine + agonist). Uptake and release ratios can be calculated. Decreased uptake and release are seen in dense granule deficiency, while a normal uptake with reduced release is seen in secretion defects.

Platelet procoagulant activity: Upon simultaneous stimulation of platelets with convulxin and thrombin, a subset of platelets expresses anionic procoagulant phospholipid, phosphatidyl serine on the surface of activated platelets. This can be assessed using fluorescently labelled annexin V or lactadherin which binds to phosphatidyl serine. The translocation of procoagulant phospholipids facilitates the assembly of coagulation factors for thrombin generation on the surface of platelets. Flow cytometry allows the detection of impaired (Scott Syndrome) or increased (Stormoken syndrome) procoagulant activity and its associated syndromes.

Table 37a. Summary of inherited platelet function disorders with clinical and laboratory features

Disorder	Genetic defect	Platelet count and morphology/ other PBS features	LTA	Additional laboratory features	Additional clinical (+/- syndromic features)
Defects of glycoprotein receptors					
Glanzmann thromb-asthenia	ITGA2B, ITGB3	Normal	Absent response to all agonists except agglutination to ristocetin	Flow cytometry identifies GPIIb/ GPIIIa deficiency	Autosomal recessive, severe bleeding
ITGA2B/ ITGB3-related thrombocytopenia	ITGA2B, ITGB3	Macrothrombocytopenia with anisocytosis	Absent/impaired response to all agonists except agglutination to ristocetin	Reduced GPIIb/ GPIIIa expression Defective PAC1 expression	Autosomal dominant, mild to moderate bleeding
Bernard Soulier syndrome	GP1BA, GP1BB, GP9	Macrothrombocytopaenia	Absent agglutination response to ristocetin	Flow cytometry identifies GP1b-IX-V deficiency	Autosomal recessive, moderate/severe bleeding
DiGeorge/ Velocardiofacial syndrome/ 22q11.2 deletion syndrome	Del22q11.2	Normal/mildly reduced platelet counts with large platelets.	Normal/variable aggregation (reduced response to Ristocetin in ~30% of cases)	Reduced GP1b/ IX/V expression by flow cytometry	Cleft palate, cardiac defects, abnormal facies, developmental disabilities, immunodeficiency, mild to significant bleeding
Platelet type VWD	Gain of function mutation in GP1BA	Macrothrombocytopenia, platelet clumps	Increased agglutination with low concentration of ristocetin	Reduced high molecular weight VWF multimers (reduced VWF activity)	Autosomal dominant, mild to moderate bleeding
Defects of collagen receptors	GP6 ND	Normal	Isolated reduced aggregation response to collagen	GPVI or α2β1 deficiency by flow cytometry	Autosomal recessive, mild bleeding

Table 37b. Summary of inherited platelet function disorders with clinical and laboratory features

Disorder	Genetic defect	Platelet count and morphology/ other PBS features	LTA	Additional laboratory features	Additional clinical (+/- syndromic features)
Abnormalities of G-protein-coupled receptors					
TXA_2 (TP) receptor defect	TBXA2R	Normal	Abnormal aggregation with AA and TXA_2 analog (U46619)		Autosomal recessive, mild bleeding diathesis
ADP receptor (P2Y12) defect	P2Y12	Normal	Markedly impaired aggregation response to ADP (only primary wave); reduced response with other agonists can also be seen	Decreased expression of activation markers on agonist stimulation by ADP	Autosomal recessive, mild bleeding after trauma/surgery
Defects of platelet granules (isolated/syndromic)					
Alpha granule defects					
Gray platelet syndrome	NBEAL2	Macrothrombocytopenia; large platelets with bluish grey cytoplasm and absent azurophilic granules	Variable, mild abnormalities; can be normal	Absent/ reduced α granules by EM Decreased/ absent P-selectin expression by flow cytometry/ELISA	Autosomal recessive, mild bleeding, progressive myelofibrosis with splenomegaly
Arthrogryposis, renal dysfunction, and cholestasis (ARC) syndrome	VPS33B, VIPAS39	Large grey platelets	Variable, mild abnormalities; can be normal	Absent/ reduced α granules by EM P-selectin is reduced in some and normal in others	Flexion contractures, hypotonia, cholestatic jaundice, renal tubular acidosis, failure to thrive, ichthyosis, infection, mild bleeding diathesis
Paris-Trousseau/ Jacobsen syndrome	Del11q23 including FL1	Large platelets, few with fused α-granules	variable	Giant fused α granules in EM, dysmegakaryopoi-esis	Mild bleeding diathesis, developmental delay, cardiac defects, craniofacial anomalies

Table 37c. Summary of inherited platelet function disorders with clinical and laboratory features

Disorder	Genetic defect	Platelet count and morphology/ other PBS features	LTA	Additional laboratory features	Additional clinical (+/- syndromic features)
Dense granule defects					
Isolated dense granule deficiency/δ-storage pool deficien cy		Normal	Variable. May be decreased to several agonists: ADP, collagen and epinephrine, or normal	Decreased ATP release by lumiaggregometry Reduced platelet ADP and increased ATP/ADP ratio Reduced/absent dense granules by whole mount TEM Reduced CD63 and reduced mepacrine uptake and release by flow cytometry	
Hermansky-Pudlak syndrome	HPS1, AP3B1 (HPS2), HPS3, HPS4, HPS5, HPS6, DNTBP1 (HPS7), BLOC1S3 (HPS8), and BLOC1S6 (HPS9)	Normal	Variable		Oculocutaneous albinism, immunodeficiency, mild bleeding diathesis, pulmonary fibrosis, granulomatous colitis, and neutropenia.
SLFN14-related thrombocytopenia	SLFN14	Macrothrombocytopenia	Variable		
Chediak-Higashi syndrome	LYST	Giant peroxidase positive cytoplasmic granules in leucocytes; lymphohistiocytosis	Variable		Variable oculocutaneous albinism, recurrent life threatening infections, mild bleeding diathesis
Combined α-granule/δ-granule disorders	ND	Pale grey platelets by light microscopy	Variable	Absent/reduced α and δ- granules on TEM, and other features as seen in alpha and dense granule disorders	Mild to moderate bleeding diathesis
Granule secretion defect or primary secretion defect	ND	Normal	Variable	Decreased ATP release by lumiaggregometry, normal mepacrine uptake with reduced mepacrine release by flow cytometry Reduced CD63 expression by flow cytometry; normal alpha and dense granules by EM	Mild to moderate bleeding diathesis.

Table 37d. Summary of inherited platelet function disorders with clinical and laboratory features

Disorder	Genetic defect	Platelet count and morphology/ other PBS features	LTA	Additional laboratory features	Additional clinical (+/- syndromic features)
Defects of transcription factors					
Familial platelet disorders with associated myeloid malignancy (FPD/ AML)	RUNX1	Thrombocytopenia	Abnormal aggregation to multiple agonists	δ-granule secretion defect	Predisposition to develop MDS and AML
FLI1-related δ granule defect	FLI1	Thrombocytopenia	Reduced aggregation to collagen and TRAP	δ-granule secretion defect	
GATA1- defect	GATA1	Macrothrombocytopenia	Reduced aggregation to collagen and ristocetin	Reduced α-granule content and release	
GFI1B-related defect	GFI1B	Macrothrombocytope-nia, dyserythropoiesis	Normal	Reduced α-granule release	Autosomal dominant
Signal transduction protein defects					
Cytosolic phospholipase A2 defect/ Cyclo-oxygenase deficiency/ TXA_2 synthase deficiency	PLA2G4A/ ND/TBXAS1	Normal	Absent response with AA and normal response with TXA_2		
CalDAG-GEFI defect	RASGRP2	Normal	Markedly reduced/absent response to ADP, Epinephrine, reduced/normal response to Collagen, TRAP and ristocetin	Normal GPIIb/ GPIIIa expression Defective PAC1 expression	Autosomal recessive, severe bleeding
Leukocyte adhesion deficiency-III	FERMT3	Leukocytosis	GT like pattern	Normal GPIIb/ GPIIIa expression Defective PAC1 expression	Autosomal recessive, severe bacterial infection, poor wound healing, severe bleeding

Table 37e. Summary of inherited platelet function disorders with clinical and laboratory features

Disorder	Genetic defect	Platelet count and morphology/ other PBS features	LTA	Additional laboratory features	Additional clinical (+/- syndromic features)
Defects of cytoskeletal proteins					
Wiskott Aldrich syndrome (WAS) /X-linked thrombocytopenia	WAS	Microthrombocytopenia		Decreased T-cell subsets, natural killer cell function, decreased α- and δ-granules by TEM	WAS: X-linked inheritance; Eczema, immune deficiency, malignancies and autoimmunity.
MYH9 related disease (formerly known as Sebastian, May-Hegglin, Fechtner, and Epstein syndromes)	MYH9	Macrothrombocytopenia, Dohle like inclusion in neutrophils	Normal		Autosomal dominant; variable association with sensorineural hearing loss, cataracts, and nephritis
ADAP defect	FYB	Microthrombocytopenia		Increased expression of P-selectin and PAC-1 but impaired expression upon activation	
Defects of membrane phospholipids					
Scott syndrome	TMEM16F	Normal	Normal	Reduced Annexin binding by flow cytometry	Autosomal recessive
Stormorken syndrome	STIM1, ORAI1	Anemia Howell Jolly bodies	Normal	Enhanced Annexin V binding and defective PAC-1 expression	Faical dysmorphism, ichtyhosis, myopathy
Enhanced platelet fibrinolytic activity					
Quebec platelet disorder	PLAU (duplication)	Variable thrombocytopenia	Abnormal response with Epinephrine	Excess platelet urokinase plasminogen activator causing proteolysis of α-granule proteins and fibrinolysis	Autosomal dominant, delayed onset bleeding after surgery/trauma, not response to antifibrinolytics but responsive to antifibrinolytics

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PART 11 Antiphospholipid Antibody Syndrome (APS) Lab Investigation

Silmara Montalvão

TOPICS COVERED

- ✓ Classification and Diagnostic Criteria for APS
- ✓ Anti-Phospholipid Antibodies
- ✓ Lupus Anticoagulant

A heterogeneous group of antibodies that can cause prolongation of the APTT test are antiphospholipid antibodies, which generally react with epitopes on proteins that are complexed with negatively charged phospholipids. Many of these antibodies require beta-2-glycoprotein 1, a protein that binds to phospholipids. Others can be directed against prothrombin. Proper identification of these antibodies will allow antiphospholipid antibody syndrome (APS) to be characterized (Ruiz-Irastorza et al, 2010; Schreiber et al, 2018). It is important to note that these antibodies can interfere with coagulation reactions in the laboratory, prolonging phospholipid-dependent tests such as the APTT and occasionally the PT, but they are not associated with bleeding, except in some rare cases where there is a significant acquired prothrombin deficiency. Paradoxically, these antibodies are clearly associated with venous and arterial thrombosis by mechanisms that are not well understood. In diagnostic centers for bleeding disorders, it is necessary to be able to detect these antibodies using specific tests for the investigation of patients with prolonged APTT (Barbosa et al, 2019). There are currently specific guidelines for the correct performance of the tests used for the laboratory diagnosis of APS are available and can be used to update lab information, see below.

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- Vandevelde A, Gris JC, Moore GW, Musiał J, Zuily S, Wahl D, Devreese KMJ. Toward harmonized interpretation of anticardiolipin and anti-β2-glycoprotein I antibody detection for diagnosis of antiphospholipid syndrome using defined level intervals and likelihood ratios: communication from the ISTH SSC Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibodies. J Thromb Haemost. 2024: S1538-7836(24)00236-8.

Classification and Diagnostic Criteria for APS: Since it became clear that antiphospholipid antibodies were significantly associated with vascular thrombosis and pregnancy morbidity, the need for consensus criteria for APS resulted in the Sydiney criteria, Table 38 (Miyakis et al, 2006). Patients are classified as having APS when a clinical event occurs together with at least one positive laboratory criterion. The laboratory criteria for defining APS are the presence of lupus anticoagulant, aCL IgG/IgM, or a2GPI IgG/IgM, persistently present for at least 12 weeks. Currently, a novel international initiative is being carried out to develop new criteria for classifying APS. The proposed laboratory criteria include only the antibodies from the current criteria (lupus anticoagulant, aCL IgG/IgM, and a2GPI IgG/IgM).

Table 38. Sydney 2006 criteria for classifying APS

Clinical Criteria	Laboratory Criteria
1. Vascular thrombosis	**1. Lupus anticoagulant**
Venous, arterial or microvascular;	≥2 positive results
Confirmed by objective validated criteria;	At least 12 weeks apart
No evidence of inflammation in vessel wall	
And/or	
2. Pregnancy morbidity	**2. Anticardiolipin antibody IgG and/or IgM**
≥1 unexplained fetal death _10th week of gestation or;	Serum and plasma
≥1 premature birth <34th week of gestation because of: Eclampsia or severe pre-eclampsia, placental insufficiency;	Medium or high titer (>40 GPL or MPL, or >99th percentile),
≥3 unexplained consecutive abortions <10th week of gestation.	Measured by ELISA Standardized
	≥2 positive results
	At least 12 weeks apart
And/or	
	3. Anti-β2 glycoprotein I antibody IgG and/or IgM
	Serum and plasma
	Medium or high titer (>40 GPL or MPL, or >99th percentile),
	Measured by ELISA Standardized
	≥2 positive results
	At least 12 weeks apart

Lupus anticoagulant

How to choose the test? Lupus anticoagulant can be detected by different phospholipid-dependent coagulation tests. The most recent update of the ISTH guidelines on lupus anticoagulant detection recommends using two tests in parallel, the diluted Russell viper venom time (dRVVT) and the APTT (Devreese et al, 2020). The dRVVT is more specific, while the APTT is more sensitive for lupus anticoagulant (depending greatly on the reagent used). The two assays are complementary because the antibodies do not always react in both assays. The dRVVT assay is based on the direct activation of FX by an enzyme present in the venom of Russell's vipers. The APTT assay is based on the activation of the contact (intrinsic) pathway of the coagulation cascade. The selection of appropriate reagents for lupus anticoagulant testing purposes is important, as there are several reagents available, especially for APTT (Favaloro et al, 2019). Two topics in the selection of the APTT reagent need to be addressed, the choice of activating agent and the composition and concentration of phospholipids. As an alternative to the APTT, the silica clotting time (SCT) test, can be used for lupus anticoagulant testing. The performance of lupus anticoagulant assays must be validated or verified before implementation in clinical practice. Part of the verification process should include testing samples with known lupus anticoagulant, and mean values well characterized (Gardiner et al, 2021a; Gardiner et al, 2021b).

How is the test carried out? Lupus anticoagulant assessment consists of a three-step procedure: screening, mixing, and confirmation (Devreese et al, 2020). PPP is necessary to avoid false-negative results due to the interaction of phospholipids and platelets. The screening stage includes tests with dRVVT and APTT reagents at low phospholipid concentrations. Coagulation factor deficiency or inhibitors other than lupus anticoagulant can cause a positive screening test, so mixing and a confirmation test step are necessary. The stepwise procedure can reduce costs, as it avoids carrying out the mixing and confirmation step unnecessarily if the screening step is negative. In the confirmation step, an excess of anionic phospholipid

is added to the test reagent, and the excess of phospholipid can reduce or neutralize the antibodies. In dRVVT tests, the screening and confirmation assays are carried out in parallel, and the result of the confirmation step is expressed as a normalized ratio according to the calculation: [(screening patient result)/(screening pool result)]/[(confirmation patient result)/(confirmation pool result)]. In the mixing step, the screening test is performed on a mixture of 1:1 patient plasma and normal plasma pool. The mixing test is expressed as the normalized ratio [(screen mix)/(screen pool of normal plasma)]. When the clotting time in the confirmation assay is prolonged, an additional mixing step with the confirmatory reagents (confirm mix) can be performed, and the ratio is more robust and less affected by interference from congenital or acquired factor deficiencies. There are integrated assays that carry out all three stages in a single procedure. In these assays, screening and confirmatory tests are performed parallel on the patient's plasma mixed with PNP and the results are mostly expressed as the difference between the two tests.

Cut-off values: To interpret the lupus anticoagulant results, it is necessary to determine cut-off values to define positivity in all stages. First, laboratories should determine the cut-off values using a population of healthy individuals with at least 120 people, determining the cut-off point as the 99th percentile after rejecting outliers (Devreese et al, 2020). However, the number of 120 normal individuals to calculate cut-off values can be difficult to obtain for many laboratories. An approach that requires fewer volunteers is the transfer of cut-off values recommended by the manufacturer. This assumes that the manufacture cut-off points are based on a large healthy reference population with adequate demographic data, a correct statistical method and a correct reagent-instrument combination (Castellone, 2017). When these conditions are satisfied, the manufacturer's cut-off values should be verified before transfer by testing 20 healthy volunteers representing the demographics of the local population. After rejecting outliers and replacing them with new results from healthy volunteers, the results (outlier-free population) should be compared with the suggested cut-off value.

Interferences and limitations: The C-reactive protein interferes in vitro with the APTT test through its affinity for phospholipids, leading to false-positive results. Although this effect has not been observed for the dRVVT assay, this can vary between reagents. In addition, the increased coagulant activity of FVIII is linked with a shorter APTT giving false-negative results. Elevated FVIII levels can be seen during pregnancy, surgery, inflammation, malignancy, and other conditions. Lupus anticoagulant testing during the thrombotic event or during anticoagulation treatment is not recommended (Devreese et al, 2020). The most recent ISTH guidelines do not advise pre-diluting samples for lupus anticoagulant testing in the presence of AVKs (Devreese et al, 2020). DOACs directly inhibit thrombin (e.g. dabigatran) or FXa (e.g. apixaban, betrixaban, edoxaban, and rivaroxaban), with various effects on coagulation tests, leading to the interpretation of false-negative and false-positive results. The APTT and PT should be performed before starting the lupus anticoagulant test in order to have more information about the sample, but this does not exclude the presence of DOACs or LMWH.

Anti-Phospholipid Antibodies:

How do I choose the test? The anti-cardiolipin and anti-beta-2-glycoprotein1 antibodies are identified by solid phase immunoassays. The SAF classification criteria indicate the measurement of these antibodies by standardized ELISA. However, alternative detection techniques for antibodies testing, such as chemiluminescence, fluorescence enzyme, and multiplex flow immunoassays have become available (Devreese et al, 2014). Compared to traditional manual ELISA methods, the newest techniques are easier to use and show better precision. Assays differ in terms of solid phase, detection principle, coating, source of antigens and antibodies, blocking agents to prevent non-specific binding, dilution protocol, calibration, and units (Devreese et al, 2014). It is recommended to carry out the patient's follow-up tests in the same laboratory, as the platforms cannot be used interchangeably.

How to carry out the test? Serum or PPP can be used for aCL and a2GPI testing (Devreese et al, 2018). The need to perform the test in duplicate depends on the performance characteristics of the assay. Duplicate testing is especially recommended for manual ELISAs or if the inter- and intra-run imprecision of the

assay is >10% (Devreese et al, 2014). In each run, internal quality control material needs to be analyzed at relevant titer levels. Calibration curves need to be determined in every single ELISA run or for each reagent lot in automated systems. Each calibration should be evaluated and rejected when it does not meet the manufacturer's requirements or when the correlation coefficient between test values and target values is less than 0.90 (Devreese et al, 2014). Unfortunately, there is no uniformity in reference material for test calibration. Efforts are being made to develop new monoclonal and polyclonal standards for aCL and a2GPI with the aim of creating WHO standards with IU/ml as the universal unit.

Cut-off values and antibody profile: The 40 GPL/MPL as the aCL cut-off point was based on studies showing better correlation of this point with APS (Levine et al, 1997). However, there may be a marked difference between 40 GPL/MPL and the 99th percentile for aCL (Vandevelde et al, 2024). And the ISTH-SSC does not recommend using 40 GPL/MPL as a cut-off point. It is recommended to calculate a laboratory-specific cut-off value for positivity based on a non-parametric 99th percentile of at least 120 reference subjects. Outlier rejection with the Dixon/Reed method is recommended to escape overestimation of cut-off values. Transferring the manufacturer's cut-off points after verification on 20 or more reference subjects is a valid alternative if the manufacturer's cut-off point is calculated on a sufficiently large reference population and an appropriate statistical methodology has been applied. Each aCL and a2GPI result above the cut-off point should be reported as positive, accompanied by the numerical value and the internal cut-off value (Vandevelde et al, 2024). Positivity in one of the tests (lupus anticoagulant, aCL IgG, aCL IgM, a2GPI IgG, or a2GPI IgM) is sufficient to diagnose APS. The combined interpretation of different aPL as antibody profiles has been suggested to identify high-risk patients, compared to individual assessment. In asymptomatic aPL carriers, double and triple positivity was a risk factor for the development of thrombotic events, but single positivity of aCL or a2GPI was not (Mustonen et al, 2014).

Interferences: The presence of rheumatoid factor can cause false-positive aCL IgM and a2GPI IgM results (Devreese et al, 2014; Forastiero et al, 2014). Unlike lupus anticoagulant assays, antibodies testing with solid phase immunoassays is not subject to analytical interference from acute phase reactor reagents or anticoagulation therapy. However, a transitory increase in aCL and a2GPI is observed in inflammatory conditions (Exner et al, 2020; Laureano and Crowthe, 2018).

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PART 12 Fibrinolysis Lab Iinvestigation

Silmara Montalvão

TOPICS COVERED

- ✓ Plasma Clot Lysis Time Induced by tPA
- ✓ Plasmin Generation Assay

Fibrinolysis is a series of enzymatic reactions that degrade insoluble fibrin and depends on the quantity and quality of various fibrinolytic enzymes, such as tissue-type plasminogen activator (tPA) and plasmin, their respective inhibitors, plasminogen activator inhibitor type 1 (PAI-1) and alpha-2-antiplasmin (α2AP), as well as the structure of the clot (Longstaff and Kolev, 2015). In a clinical setting, increasing the rate of fibrinolysis is used to reverse thrombotic occlusion (e.g. recombinant tPA for acute treatment of stroke, myocardial infarction, or pulmonary embolism), while lowering the fibrinolytic rate is used to reduce bleeding (e.g. tranexamic acid for acute treatment of stroke, myocardial infarction, or pulmonary embolism) (Draxler and Medcalf, 2015; Ilich et al, 2017; Kwaan et al, 2017). Recently, in hemophilia, assays measuring clot formation and fibrinolysis have been used to facilitate direct and functional comparisons between new and emerging hemostatic agents that present different mechanisms of action (Holle et al, 2024). However, global testing to identify an individual's fibrinolytic potential is not widely implemented. In contrast, basic fibrinolysis research is generally based on the quantification of the various fibrinolytic factors. Total concentrations are measured using antigen-based assays, and specific functional tests to determine their activity. Despite the variety of assays available, it remains a challenge to assign individual fibrinolytic factors that contribute to the overall fibrinolytic result due to the dynamic nature of the environment surrounding the clot (Longstaff, 2018). For decades, turbidity-monitored clot lysis assays have been used as a standard method to quantify the overall fibrinolytic potential of a sample, and variations of this global and simplistic assay have been developed to address the function(s) of fibrinolytic factors. In this context, two assays have been explored in the context of clinical practice and show promising results for use in assessing general fibrinolytic potential.

Plasma Clot Lysis Time Induced by tPA: Plasminogen activator-induced plasma clot lysis times are frequently reported to assess and quantify the general fibrinolytic property of a sample (Longstaff, 2018). This test is performed by simultaneously adding agonists to initiate coagulation (e.g. tissue factor and Ca2+) and fibrinolysis (e.g. tPA) to citrated plasma. Alternatively, clots can be generated first and then covered with tPA to mimic the clinical scenario in which tPA is infused to degrade existing ischemic thrombi (Longstaff et al, 2011). The simplicity of the reaction system and minimal sample processing make this assay an ideal method for investigating the susceptibility of plasma clots to fibrinolysis (Holle et al, 2024). The test is also sensitive to inhibitor molecules that target specific factors, such as PAI-1 and α2AP, which can be included to infer the respective roles of PAI-1 and α2AP in general fibrinolysis (Zheng et al, 2023).

Reagents and method:

- Phospholipids [4 μM final]
- $CaCl_2$ [10 mM final]
- Tissue factor [1:15,000 dilution of Innovin, 1 pM final tissue factor]
- HEPES-buffered saline (20 mM HEPES, pH7.4, 150 mM NaCl)
- tPA (0.5 μg/ml final)

1) Into a U-bottom 96-well plate, pipette 10 ul TF/PL/rtPA into the each well (reverse pipetting to the bottom of the well)
2) Add 40 ul of PPP into each well (reverse pipetting to the side of the well at the top)
3) Preheat the plate in incubator for 5 min
4) After 5 min preheating, use multichannel pipette to transfer 10 ul Ca, mix well and avoid creating bubbles.
5) Quickly put the plate into the plate reader and start reading
6) Monitor the reaction for 2 hours by measuring the turbidity at 405 nm every 12 seconds using a plate reader.

Plasmin Generation Assay: The plasmin generation assays developed by various groups share some common elements (Longstaff, 2018; Zheng et al, 2023). In general, procoagulant activity in the plasmin generation assay is initiated by the addition of exogenous tissue factor to the recalcified plasma, and fibrinolytic activity is triggered by the addition of exogenous tPA. Plasmin generation is detected through the cleavage of a fluorogenic substrate, and the parameters are defined from the accumulation of fluorescence or through a mathematical derivative of this fluorescence curve. Subtle differences between these assays include the concentrations of tissue factor and tPA used and whether diluted plasma is used or not. Some variations of these assays detect thrombin and plasmin simultaneously, while others carry out these measurements separately, but in parallel. Studies show that these assays are specific for plasmin and sensitive to α2AP, with the measured activity representing free plasmin. Plasmin generation is also sensitive to tranexamic acid. Considering the high concentrations of tPA required to trigger measurable plasmin generation, the test is not sensitive to plasma concentrations of PAI-1 (Miszta et al, 2021). However, the plasmin generation response to the addition of exogenous tPA is dose-dependent. A growing body of work suggests that plasmin generation assays, especially when used with thrombin generation and turbidity assays, produce a multidimensional impression of the integrated effects of procoagulant and fibrinolytic activities in health and disease (Miszta et al, 2021; Zheng et al, 2023).

Reagents and method:

- Tris-buffered saline (TBS), containing 66 mM of Tris and 130 mM NaCl
- 34 mM of $CaCl_2$
- 10 pM of tissue factor (lipidated recombinant human tissue factor, Innovin, Siemens Healthcare, Erlangen,Germany)
- 900 ng/ml of tPA (recombinant two-chain tissue plasminogen activator, Actylise, Boehringer Ingelheim, Biberach an der Riss, Germany)
- α-thrombin (BOC-VAL-PrO-ARG-MCA, Peptides International, 5 mg)
- Plasmin (BOC-GLU-LYS-LYS-MCA, Peptides International, 5 mg)

1) Prepare the reagent solution 34 mM of $CaCl_2$, 10 pM of tissue factor , and 900 ng/ml of tPA to the TBS solution. The final concentration of reactants in plasma were 5 pM TF and 450 ng/ml tPA.
2) Two substrates were prepared with a final concentration of 100 µM and used for the detection of the enzymes: α-thrombin and plasmin.
3) The first two wells of the plate (Grelner, 96-well, flat bottom, black clear) should be used as a blank, and the samples were run in duplicate in parallel rows for each substrate, avoiding potential interference and/or interaction in signal detection.
4) Add substrate solutions (20 µl) to the wells of the plate, followed by 90 µl of the samples and blank (TBS solution).
5) Using a multi-tip automated pipette, add 90 µl of the pre-warmed reaction solution (37°C for 3 min) to each well of the plate.
6) Finally, the plate should be read in the fluorometer wavelength of 340 nm excitation and 450 nm emission for 4 hours at 45-second intervals.
7) Data analysis could be performed using Microsoft® Excel® software. The curves for thrombin and plasmin should be generated by calculating the average at each time point for the duplicated

plasma wells, subtracting the reading values of the blank (for thrombin and plasmin separately). Parameters to be calculated in both assays, using Shiny app tools: onset (time to the inflection point before turbidity increase), maximum rate (slope of a line fitted to the maximum rate of turbidity increase using 5 to 10 points to determine the line), time to plateau/peak (time to the turbidity plateau [clot formation] or peak [fibrinolysis]), turbidity change (maximum clot turbidity less the starting turbidity), and area under the curve (AUC) (calculated as the sum of trapezoids formed by turbidity curves).

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PART 13 Coagulation Parameters in Pediatric Populations

Pierre Toulon

TOPICS COVERED

- ✓ Developmental Hemostasis
- ✓ Coagulation Parameters in Neonates and Children versus Adults

The pediatric hemostatic balance, which is different from that in adults, is an evolving process as the hemostatic system changes and matures from fetal to adult life, particularly during the early months of life. Understanding the concept of developmental hemostasis, which is now universally accepted, is critical to ensure optimal diagnosis and treatment of hemorrhagic and thrombotic diseases in children.

Developmental Hemostasis: Hemostasis is a complex mechanism involving both procoagulant and anticoagulant factors. It ultimately enables the blood to remain liquid when circulating in intact vessels. It also avoids both excessive bleeding by promoting clot formation after endothelial injury and excessive clotting by limiting clot formation to the site of injury. The hemostatic equilibrium mainly depends on many parameters including platelets as well as clotting factors and inhibitors, even though endothelial and blood cells play a significant role. Children are not just miniature adults, at least for hemostasis, as the pediatric hemostatic balance is different from that in adults. Moreover, it is an evolving process, as shown by Andrew M. et al (1987), more than 30 years ago both in pre-term and in full-term infants. These authors demonstrated that the hemostatic system changes and matures throughout the time from fetal life to adulthood, mainly during the early months of life, and promoted the concept of developmental hemostasis. Coagulation factors from maternal origin are unable to cross the placental barrier because of their size. The synthesis of clotting factors by the fetus starts early (e.g. during the fifth week of gestation for fibrinogen), and blood becomes clottable after eleven weeks of gestation. Fetal reference ranges for coagulation parameters were studied in different gestational age groups and the median plasma levels were between 10% and 30% of adult values, depending on the evaluated parameter, in fetuses aged 19 to 23 weeks, progressively increasing to levels between 10% and 50% between 30 and 38 weeks of gestation. The initial findings of Andrew M, et al (1987) were confirmed by several studies evaluating different pediatric populations in various technical conditions (i.e. reagents/analyzers combinations). The selection criteria of the subjects were relatively homogeneous among the studies, however, some had slightly different inclusion/exclusion criteria and age grouping. The main difference between studies was the number of evaluated subjects in each age group, which ranged from 10 to more than 500 individuals. The sampling process, which is a key point to take into account, as drawing blood from young infants or neonates could be more problematic than in adults, was comparable in the different studies, with blood collected by venipuncture into tubes containing 3.2% citrate (1 vol./9 vol.) through 18- to 24-gauge needles, depending on the age of the patients. Most of these studies focused mainly on activity assays for most parameters involved in the coagulation system, whereas one study evaluated antigen concentrations of various analytes. All these studies showed that, at birth, the plasma levels of most coagulation proteins were around half of those measured in adults, except for FVIII:C and VWF, which are elevated, the pre-term infants having lower levels than full-term infants. Adult values were reached between a few months of age and up to more than 16 years for specific parameters such as coagulation FVII or protein C, as shown in Table 39. Whereas the global trend is consistent across the studies, differences in absolute values are likely due to differences in the reagents and/or the instruments used to measure these parameters, particularly global coagulation tests such as PT or APTT. Accordingly,

it is recommended by the Subcommittee of the Scientific and Standardization Committee of the ISTH that each laboratory define its age-dependent reference ranges by using its own technical condition. To comply with the Clinical and Laboratory Standards Institute (CLSI) C28A3 guideline, reference ranges must be established by testing at least 30 different individuals, in each age group. Obtaining enough plasma to perform numerous tests from a high number of "apparently" healthy children, raises logistical issues that would be far beyond the capabilities of many laboratories. To circumvent that difficulty, it is common practice to refer to data from the literature, taking into account identical technical conditions**,** even though the pre-analytical process, and particularly blood collection, could be different from that used in a given institution. The technical conditions (i.e. combination of reagents and instruments) used in the main publications are reported in Table 40. Primary hemostasis was less studied. However, the platelet count is usually normal or elevated at birth, reaching adult values within 1 year after transient increases. Despite hyporeactive platelets, particularly in the neonatal period, the bleeding time and the platelet closure time (PFA-100®) were found to be shortened in newborns, suggesting an increased hemostatic potential. Normalization occurred before the end of the first month of life. Significantly higher levels of VWF were reported in newborns, which then decreased reaching adult values after 1 year of life, at a time when appears the significant increase in plasma levels in non-O blood groups versus O blood groups.

Conclusions

Understanding of the concept of developmental hemostasis, which is now universally accepted, is critical to ensure optimal prevention, diagnosis, and treatment of hemorrhagic and thrombotic diseases in children. Therefore, it is mandatory for the laboratory to use age-specific reference ranges for coagulation parameters. It seems impossible to ask every laboratory to establish its own references intervals for every coagulation parameter in its own technical conditions by testing at least 120 healthy individuals in each age-group, as it is recommended by the CLSI Guideline EP 28-A3C. Therefore, the best option for a laboratory would be to translate the findings of the literature to local reference ranges for neonates and children, by taking into account their specific technical environment. In that respect, data are already available for combinations of reagents and analyzers from current manufacturers. In the case of newcomers, specific, and preferably multicenter, studies would have to be carried out in order to establish the specific pediatric reference ranges using these new reagents/analyzers combinations.

Table 39. Coagulation parameters in neonates and children versus adults: Summary of test results and potential effect on hemostasis (adapted from Toulon et al, 2016)

Component	Parameter	Neonatal period (mean value)*	Normalization	Impact on hemostasis
Primary hemostasis	Platelets VWF Platelet closure time (PFA-100®)	Normal or increased Increased (153%)* Shortened	1 Y (after transient increases) 3 Mo 2–4 W	Enhanced primary hemostasis
Coagulation	FII, FVII, FIX, FX, FXI, FXII, PK, HMWK FV FVIII Fibrinogen PT aPTT	Decreased (40–66%)* Decreased (37–54%)* Normal or decreased (70%)* Normal or increased (100%)* Normal** Prolonged or normal Prolonged	1 Y (up to 16 Y for FVII) 1 Y 1 Y (up to 16 Y) 1 Mo 1 Y 1 Y 1 Y (up to 16 Y)	Decreased coagulation potential
Natural coagulation inhibitors	Antithrombin Protein C Protein S	Decreased (63%) Decreased (35%) Decreased (36%)*	3 Mo 16 Y 3 Mo	Decreased regulatory/ inhibitory potential
Fibrinolysis	Plasminogen alpha 2 antiplasmin tPA D-dimer	Decreased (36%)* Normal or decreased (85%)* Increased Increased	6 Mo 6 Mo 1 W 16 Y	Increased fibrinolytic activity

*In percentage (%) of adult values, from Andrew M, et al. (1987); **Fetal fibrinogen may be present; Mo: month; W: week; Y: year

Table 40. Technical conditions (instrument and reagents brands) used in the main studies reporting usual values of coagulation parameters in pediatric populations (*indicates instrument and/or reagents no longer commercially available)

Authors	Instruments	Reagents
Andrew et al (1987, 1988)	ACL (Werfen)*	Various*
Flanders et al (2005, 2006)	STA-R (Stago) BCS (Siemens)	Mainly Stago
Monagle et al (2006, 2011)	STA Compact (Stago)	Stago
Apple et al (2012)	BCS (Siemens) CA-1500 (Sysmex)	Siemens
Attard et al (2013)	Microplate reader	Stago
Toulon et al (2016)	ACL TOP 500/700 (Werfen)	Siemens

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PART 14 Molecular Genetic Analysis

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TOPICS COVERED

- ✓ Molecular Characteristics of Hemophilia-Related Genes and Proteins
- ✓ Spectrum of Causative Variants in Hemophilia
- ✓ Other Phenotypes Associated with the F8 Gene
- ✓ Other Phenotypes Associated with the F9 Gene
- ✓ Deep Vein Thrombosis (DVT) Protection
- ✓ Spectrum of Practical Approaches Applied in Genetic Testing
- ✓ Standardized Nomenclature and Pathogenic Potential of Variants

Genetic analysis of hemophilia is important in defining the underlying cause of the bleeding disorder in affected individuals and their family members. Understanding of the genetic variant associated with the phenotype can help to predict the severity of the disorder, including the risk of inhibitor development. It also aids in the identification of female carriers of hemophilia, who may be offered prenatal diagnosis. It is important that individuals who are proceeding to genetic investigation undergo appropriate genetic counselling prior to any testing. The recent WFH Guidelines for the Management of Hemophilia (3rd edition) (Srivastava et al, 2019) contain a chapter dedicated to the genetic assessment of individuals with hemophilia A and hemophilia B. This laboratory manual chapter describes the genetic basis of hemophilia A and B and highlights the heterogeneity of practical approaches that are currently available throughout the world that may be used to investigate the underlying genetic variants. It also describes the use of standardized nomenclature for the description of genetic variants, and their classification of pathogenicity, as well as highlighting the importance of a clear, concise interpretive report which outlines the genetic result and the implications for the individual and their family.

Molecular Characteristics of Hemophilia-Related Genes and Proteins: The molecular features of hemophilia genes, coagulation FVIII or F8, and coagulation FIX or F9, are shown in Table 41. Table 41 shows the genomic coordinates on GRCh38 (hg38), gene size and cytogenetic location of F8 and F9, their exon complexity (exon number), updated curated versions of RefSeq files (i.e., NG_..., NM_..., NP_...), and the relevant molecular size of the main gene transcripts and their derived polypeptide isoforms.

Table 41. Molecular features of F8 and F9 genes

HGNC official symbol	Gene name	Cytogenetic location	GRCh38 (hg38) (coordinates) NC_000023.11 (length [bp])	Genomic RefSeq (coordinate range)	Main transcript* RefSeq (length [nts]) (exons)	Main protein* RefSeq (length [aa])	OMIM #
F8	coagulation factor VIII	Xq28	complement (154,835,792-155,022,723) (186,931)	NG_011403.2 (5,001-191,932)	NM_000132.4 (9032) (26)	NP_000123.1 (2351)	300841
F9	coagulation factor IX	Xq27.1	(139,530,739-139,563,459) (32,720)	NG_007994.1 (5,001-37,723)	NM_000133.4 (2800) (8)	NP_000124.1 (461)	300746

HGNC: HUGO Gene Nomenclature Committee. Length [Units]: [bp], base pairs; [nts], nucleotides; [aa]; amino acids. *Only the longest (most significant) transcript variant and their derived main isoform are

indicated. OMIM: Online Mendelian Inheritance in Man (https://omim.org/); #, accession number. Data were collected from NCBI (National Center from Biotechnology Information) accessed Jan/18/2024 (https://www.ncbi.nlm.nih.gov/). RefSeq indicates Reference Sequence files from the NCBI browser platform.

Additionally, Table 42 shows the most relevant characteristics of the coagulation FVIII and FIX proteins. Table 42 compiles data from the main protein isoforms, the amino-acid (aa) cartography of recognizable protein domains (specified by UNIPROT database), and protein database (PDB) files associated with 3D-structure models (FVIIIa and FIXa atom 3-dimensional coordinates).

Table 42. Molecular features of coagulation factor VIII and IX proteins

Protein symbol	Protein Name main isoform	Isoform RefSeq (length [aa])	UNIPROT* #	Isoform domains: UNIPROT (aa coordinates)	3D-structure PDB id (aa coordinates)
FVIII	coagulation factor VIII isoform a preproprotein	NP_000123.1 (2351)	P00451	**Signal peptide**: (1-19) **A1**: F5/8 type A 1 (20-348) **A2**: F5/8 type A 2 (399-730) **B:** B-region (760-1667) **A3**: F5/8 type A 3 (1713-2040) **C1**: F5/8 type C 1 (2040-2188) **C2**: F5/8 type C 2 (2193-2345)	**Mature FVIIIa 2R7E.pdb**** A: Heavy chain A1-A2 (Legacy 1-725) B: Light chain A3-C1-C2 (Legacy 1689-2332)
FIX	coagulation factor IX isoform 1 preproprotein	NP_000124.1 (461)	P00740	**Signal peptide**: (1-28) – **Pro-peptide**: (29-46) **Gla**: γ-carboxyglutamate-rich (47-92) **EGF1:** Ca++-binding EGF-like 1 (93-129) **EGF2:** EGF-like 2 (130-171) **Act_peptide**: Activation peptide (192-226) **Tryp_SPc**: Trypsin-like serine protease (227-457)	**Homology model FIXa.pdb***** **L**: Light chain Gla-EGF1-EGF2 (47-171) **H**: Heavy chain Tryp_SPc (227-461)

*UNIPROT protein browser (URL: https://www.uniprot.org/). **Shen et al, 2008. ***Curators from the EAHAD Variant Databases Project (Rallapalli et al, 2013; McVey et al, 2020). Most data were collected from the EAHAD (European Association for Haemophilia and Allied Disorders) Coagulation Factor Variant Databases (URL: https://dbs.eahad.org/) accessed Jan/18/2024. Codons and amino acids (aa) are numbered following HGVS rules (i.e., codon +1 coding for the first residue (Met) of the primary polypeptide in FVIII and FIX). In Legacy numbering, codon/amino-acid +1 refers to that coding for the first amino-acid of the mature FVIII protein (excluding 19 aa of the signal-peptide) and FIX protein (excluding 46 aa of the signal-peptide and the pro-peptide). Although HGVS numbering is recommended, Legacy numbering has been extensively used in former publications.

Spectrum of Causative Variants in Hemophilia: Most pathogenic variants affecting the F8 gene cause hemophilia A, whilst most pathogenic F9 variants cause hemophilia B. The Online Mendelian Inheritance in Man (OMIM) database (https://www.omim.org) compiles a wide set of comprehensive information about human genes, indicates variants affecting their function, and describes and classifies their associated phenotypes. The OMIM database indicates that F8 variants are associated with two different phenotypes: hemophilia A (#306700) and thrombophilia 13 (X-linked, due to FVIII defect) (#301071) ((THPH13); while F9, with four phenotypes: hemophilia B (#306900), thrombophilia 8 (X-linked, due to FIX defect) (#300807) (THPH8), protection against deep venous thrombosis (DPV) (#300807), and warfarin sensitivity (#301052). Table 43 and Table 44, respectively, show the spectrum of F8 mutations causing hemophilia A according

to the coagulation FVIII activity levels (FVIII:C) and F9 mutations causing hemophilia B, associated with the FIX levels (FIX:C). Most relative prevalences of hemophilia A and hemophilia B causative variants, listed in Tables 43 and 44, were extracted from the European Association for Hemophilia and Allied Disorders (EAHAD) databases. The EAHAD F8 variant database excludes prevalent inversions associated with severe hemophilia A involving almost half of patients. In order to present an unbiased prevalence of severe hemophilia A causative mutations, their relative frequencies were merged taking into account the worldwide averages of the F8 intron 22 inversions (Inv22) reported by Antonarakis et al (1995), and those estimated for the F8 intron 1 inversion (Inv1) from an international hemophilia A patient series (Rossetti et al, 2004) (Table 43).

Table 43. Most typical hemophilia A causative F8 variants in hemizygous patients from international sources.

Variant type by effect	Severe non inversions # cases (%)	Severe Global # cases (%)	Moderate # cases (%)	Mild # cases (%)	References
Missense	1418 (30.2)	(16.2)	1340 (79.9)	3048 (95.8)	F8_var_db EAHAD*
In-frame-indel	70 (1.5)	(0.8)	19 (1.1)	32 (1.0)	F8_var_db EAHAD*
Frameshift-indel	1487 (31.7)	(17.0)	142 (8.5)	27 (0.9)	F8_var_db EAHAD*
Splicing defect	320 (6.8)	(3.7)	98 (5.8)	68 (2.1)	F8_var_db EAHAD*
Nonsense	968 (20.6)	(11.1)	59 (3.5)	4 (0.1)	F8_var_db EAHAD*
Large deletion (SV)	426 (9.1)	(4.9)	19 (1.1)	3 (0.1)	F8_var_db EAHAD*
Total non-inversions	4689 (100)	(53.7)	1677 (100)	3182 (100)	F8_var_db EAHAD*
Inv22 (SV) type 1 **Inv22 (SV) type 2** **Inv22 (SV) other types**		740 (35.4) 140 (6.7) 25 (1.1)			Antonarakis et al, 1995
Total Inv22		2093 (43.2)			
Inv1 (SV)		19 (3.1)			Rossetti et al, 2004
Total Inv1		622 (3.1)			

SV indicates structural variants including copy number variants (CNVs) as large F8 deletions and large F8 inversions (non-CNV) as the intron 22 inversion (Inv22) and intron 1 inversion (Inv1). Data from F8 inversions' uninformative patients were obtained from EAHAD (European Association for Haemophilia and Allied Disorders) databases registering individual patients. *F8 variant database (http://f8-db.eahad.org/).

The most characteristic and recurrent variant causing severe hemophilia A worldwide, is the F8 intron 22 inversion, a large perfect inversion of 600 kb mediated by recombination between inverted repeats of 10 kb (int22h or h) disrupting the F8 structure impeding the normal RNA splicing between exons 22 and 23 (Lakich et al, 1993; Naylor et al, 1993). There is a F8 intragenic copy of int22h within intron 22 (h1) and two extragenic copies (h2 and h3). Depending on which extragenic copy recombines with the intragenic one, the Inv22 shows a pattern type 1 (h1/h3) or a pattern type 2 (h1/h2). The Inv22 originates almost exclusively from male germ cells (Rossiter et al, 1995) and, consequently, the majority of mothers of patients with the Inv22 are carriers (Tizzano et al, 1995). The molecular mechanism of non-allelic homologous recombination between large inverted repeats in male meiosis supports the Inv22 recurrence as the most prevalent cause for severe hemophilia A worldwide (Table 43). Similarly, the F8 intron 1 inversion (Inv1) is a large perfect DNA inversion caused by recombination between 1 kb inverted repeats (int1h) disrupting the F8 structure at intron 1 (Bagnall et al, 2001) and involves an estimated average of 3% of severe hemophilia A patients worldwide (Table 43).

The remaining group of patients with severe, moderate, or mild hemophilia A (Table 43), uninformative for the F8 inversions, and all patients with hemophilia B (Table 44) show a typical spectrum of deleterious variants, including single nucleotide substitutions (SNV) predicting missense, nonsense, or splicing defects; small insertions/deletions (INDEL) predicting frameshifts or in-frame changes; or, less frequently, large copy number variations (CNVs), mostly large deletions.

Table 44. Most typical hemophilia B causative F9 variants in hemizygous patients from international sources

Variant type by effect	Severe # cases (%)	Moderate # cases (%)	Mild # cases (%)	References
Missense	999 (52.3)	1039 (85.1)	719 (95.0)	F9_var_db EAHAD*
In-frame-indel	27 (1.4)	9 (0.7)	1 (0.1)	F9_var_db EAHAD*
Frameshift-indel	185 (9.7)	42 (3.4)	2 (0.3)	F9_var_db EAHAD*
Splicing defect	135 (7.1)	66 (5.8)	30 (4.0)	F9_var_db EAHAD*
Nonsense	459 (24.0)	62 (5.1)	5 (0.7)	F9_var_db EAHAD*
Large deletion (SV)	107 (5.6)	3 (0.3)		F9_var_db EAHAD*
Total	1912 (100)	1221 (100)	757 (100)	F9_var_db EAHAD*

SV indicates structural variants as large deletions affecting partially or totally the F9 gene. Data from HB patients were obtained from EAHAD (European Association for Haemophilia and Allied Disorders) databases registering individual patients. *F9 variant database (https://f9-db.eahad.org/).

Information about F8 and F9 variants is compiled in publicly accessible databases, such as those developed by the CDC (Centers for Disease Control and Prevention) named CHAMP and CHBMP for hemophilia A and hemophilia B, respectively (https://www.cdc.gov/ncbddd/hemophilia/champs.html), and by EAHAD (European Association for Haemophilia and Allied Disorders) for F8 (http://f8-db.eahad.org/) and F9 (http://f9-db.eahad.org/) (Tables 43 and 44). Accessed on January 18, 2024, EAHAD databases contain information from 3052 unique F8 variants corresponding to 10144 individual cases, and 1244 unique F9 variants corresponding to 4713 individual cases. In Tables 43 and 44, genetic variants are classified by their predicted effect from the observed DNA nucleotide sequence evidence (i.e. missense, in-frame-indel, frameshift-indel, splicing defect, nonsense, large deletion). F8 and F9 variants, respectively, listed in Tables 43 and 44 represent those hemophilia causative variants with significant frequencies worldwide in contrast with those prevalent variants found in particular populations typically associated with non-severe phenotypes (e.g. F8 exon 13 duplication prevalent in the Italian population of mild hemophilia A (Acquila et al, 2004).

Other Phenotypes Associated with the F8 Gene

Thrombophilia 13 (X-linked, due to FVIII defect): Shen et al (2013) evaluated FVIII:C activity levels and F8 gene copy number in patients with venous thromboembolism (VTE) versus healthy controls. VTE patients showed significantly higher FVIII:C and greater number of copies of the F8 gene. Simioni et al (2021) reported two Italian families with thrombophilia 13 and identified a partial F8 tandem duplication, which is consistent with X-linked dominant inheritance pattern as hemizygous male patients are more severely affected than female carriers.

Other Phenotypes Associated with the F9 Gene

Thrombophilia 8 (due to FIX defect) is an X-linked recessive inherited phenotype associated with early onset VTE caused by a F9 missense defect, R338L or variant Padua, reported by Simioni et al (2009). FIX-Padua was reported to enhance the fibrinolytic resistance of plasma clots (Ammollo et al, 2014).

Deep Vein Thrombosis (DVT) Protection:

The common polymorphic variant FIX-Mälmo (minor allele frequency of 0.32), p.(Thr148Ala) due to a G>A single nucleotide substitution (SNV), associates with a DVT risk protection with odds ratios (OR) of 0.8 in male patients and 0.89 in female patients (Bezemer et al, 2008). However, the molecular mechanisms for DVT protection conferred by the FIX-Mälmo polymorphism remained unknown.

Warfarin is a widely prescribed anticoagulant for the prevention of thromboembolic events in 'at risk' patients. Warfarin sensitivity (X-linked, due to FIX variants) refers to a bleeding phenotype complication during anticoagulation therapy with vitamin K antagonists. Pezeshkpoor et al (2018) reported an association between F9 missense variants affecting the pro-peptide, such as p.(Ala37Thr) and p.(Ala37Val), and warfarin sensitivity characterized by a disproportionate reduction of FIX:C levels during anticoagulation therapy.

The causal relationship between a given genetic variant (e.g. F8 Inv22) and a particular phenotype (e.g. severe hemophilia A in a hemizygous patient) can be modified in rare cases by the involvement of a genetic mosaicism (GM), which is defined as a coexistence of at least two genetically different clones in an individual (e.g. Inv22-positive and -negative cells). A GM may involve partially or totally, some or all organs/tissues from an affected individual, resulting for example, in milder phenotypes if it affects somatic cells (e.g. hepatic endothelium-derived FVIII/FIX producing cells) and the gene variant heritability if it affects germ cells (e.g. a germinal mosaic male hemophilia patient may be the father of non-carrier daughters) (Abelleyro et al, 2018).

As historical paradigms of X-linked recessive disorders, hemophilia A (OMIM #306700) and hemophilia B (OMIM #306900) are typically expressed in hemizygous male patients (46,XY) and heterozygous females (46,XX) are usually asymptomatic. According to a consensual point of view among hematologists, a new classification of female hemophilia considers the factor coagulation activity levels, indicating severe disease when <1 IU/dl, moderate 1-5 IU/dl, and mild hemophilia 5-40 IU/dl; and when factor levels are >40 IU/dl, individuals are classified as symptomatic and non-symptomatic carriers (van Galen et al, 2021). The molecular basis of female hemophilia involves the impaired expression or silencing of F8 or F9 alleles mediated by the phenomenon of X-chromosome inactivation (XCI), which silences the gene expression in cis from one X in each cell to compensate doses with males. XCI takes place early in embryogenesis normally at random in each cell and this state is inherited clonally in the adult life of women. A homozygous female carrier and a compound heterozygote are expected to express hemophilia as well as heterozygous carriers with skewed XCI preferentially silencing the normal allele (Radic et al, 2015). Moreover, Garagiola et al (2021) proved a significant association between FVIII/FIX clotting activity levels and the pattern of XCI measured in peripheral blood leukocytes from heterozygous hemophilia A carriers with ≤50 IU/dl.

Spectrum of Practical Approaches Applied in Genetic Testing: Depending on the availability of resources and expertise, there are a variety of techniques that may be employed for the investigation of genetic variants associated with hemophilia A and hemophilia B. This chapter provides examples of these practical approaches and references, where available. A number of different techniques are available for the investigation of the F8 intron 22 inversion, including Southern blot, long range polymerase chain reaction (PCR), and inverse-shifting PCR (Lakich et al, 1993; Liu et al, 1998; Bagnall et al, 2006; Rossetti et al, 2008; Abelleyro et al, 2016; Ding et al, 2016; Hudecova et al, 2017; Pan et al, 2014; Kumar et al, 2015; Edison et al, 2016). The F8 intron 1 inversion can be detected by techniques such as double PCR or inverse shifting PCR (Bagnall et al, 2002; Rossetti et al, 2008). Analysis of SNVs in F8 and F9 can be performed by a range of techniques, including PCR and Sanger sequencing, or high throughput sequencing technologies, such as next generation sequencing (NGS) (Al-Allaf et al, 2019; Li et al, 2014; Lyu et al, 2016; Manderstedt et al, 2019; Edison et al, 2016). Where resources are limited, a screening approach prior to Sanger sequencing could be employed (Salviato et al, 2019), such as heteroduplex analysis using conformation sensitive gel electrophoresis (CSGE). For analysis of CNVs in F8 and F9, there are a number of techniques such as gap-PCR, multiplex ligation-dependent probe amplification (MLPA), quantitative real-time PCR, and NGS (Rossetti et al, 2004; Payne et al, 2012; Costa et al, 2004; Belvini et al, 2017; Kinkle et al, 2017; You et al,

2013; Wu et al, 2014; Fernandez-Lopez et al, 2007; Tizzano et al, 2005; Johnsen et al, 2017). In cases of hemophilia A and hemophilia B where an underlying genetic variant is not found in the essential regions of the F8 or F9 genes using the techniques described above, analysis of deep intronic regions for potential splicing defects may be available by targeted massive parallel sequencing (MPS) or whole genome sequencing (WGS) (Jourdy et al, 2018; Jourdy et al, 2020; Bach et al, 2015; Inaba et al, 2017; Castaman et al, (2011; Chang et al, 2019). Linkage analysis may also be considered for family studies where there is no identifiable F8 or F9 variant (Sun et al, 2015). The investigation of individuals with atypical phenotypes which may be due to complex genomic rearrangements may be by cytogenetic microarray analysis (Jourdy et al, 2016; Jourdy et al, 2017; Janczar et al, 2016; Lannoy et al, 2018). Analysis of X-chromosome inactivation may be performed by a number of techniques such as methylation specific restriction enzyme, PCR and fragment analysis, or other quantitative techniques (Nisen et al, 1989; Coleman et al, 1993; Johansson et al, 2023; Machado et al, 2014).

Standardized Nomenclature and Pathogenic Potential of Variants: Accuracy in the univocal description of genetic variants is essential for research and clinical care. To address this requirement, the Human Genome Variation Society (HGVS) Variant Nomenclature Committee (HVNC) under the auspice of the Human Genome Organization (HUGO) developed a set of recommendations. These include that genetic variants should be described at the most basic level, the DNA level, and descriptions at the RNA and/or protein level, in general predicted from the DNA evidence, may be given in addition (https://hgvs-nomenclature.org/stable/) (den Dunnen et al, 2016). HGVS nomenclature recommends a specific numbering for gene positions indicating codon +1 coding for the first residue (Met) of the primary polypeptide and nucleotide +1 for the A of the initiation codon AUG. In some former hemophilia publications, legacy numbering of codon/amino-acid +1 refers to that coding for the first amino-acid of the mature protein (i.e. in HGVS numbering, FVIII codon 20 and FIX codon 47). To adjust and normalize the nomenclature of variants according to HGVS, the Mutalyzer website offers efficient algorithms to check and verify their correct description from HGVS recommendations (https://mutalyzer.nl/) (Lefter et al, 2021). All detected variants should be classified according to their potential to cause the observed phenotype according to the guidelines produced by the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) (Richards et al, 2015). ACMG recommendations can be applied to conventional or next-generation sequencing-based genetic tests used in clinical laboratories and comprise a five-tier system of classification for variants relevant to Mendelian disorders: (1) pathogenic, (2) likely pathogenic, (3) uncertain significance, (4) likely benign, and (5) benign. To achieve this categorization, the ACMG/AMP recommends a thorough analysis of (a) population data, (b) computational data, (c) functional data, and (d) segregation data.

For example, the analysis of F8 and F9 variants involve:

(a) The study of the genotyped variant in the general population and its frequency in hemizygous, heterozygous individuals, etc. consulting gnomAD (https://gnomad.broadinstitute.org/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), and in the F8/HA and F9/HB gene specific databases, such as EAHAD and CHAMP (referenced above).

(b) The application of in silico bioinformatic tools to analyze missense changes by predicting eventual structural or functional changes using, for example, PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), MutationTaster (https://www.mutationtaster.org/) and Varsome (https://varsome.com/) among others; or to evaluate eventual splicing defects, such as NNSplice (https://www.fruitfly.org/seq_tools/splice.html), NetGene2 (https://services.healthtech.dtu.dk/services/NetGene2-2.42/) and ESEFinder for searching differences in exonic splicing enhancer sequences (esefinder.ahc.umn.edu); and many other computational tools to estimate the impact of variants on the promoter, 5′- or 3′-UTR, etc.

(c) Experimental data obtained from in vitro and in vivo functional studies of the mutated versus normal version to be tested, or a part of it, provide significant information to establish the impact of a genetic variant.

(d) Segregation data associated with an X-linked recessive mode of inheritance and co-segregation with hemophilia in multiple affected family members is indicative of pathogenicity. Taking into account this analysis, the ACMG/AMP indicate the criteria for classifying pathogenic variants scoring the evidence as very strong (PVS), strong (PS), moderate (PM) and supporting (PP); and the criteria for classifying benign or neutral impact of variants scoring the evidence as stand-alone (BA), strong (BS) and supporting (BP). Final classification in a category to estimate pathogenicity (1-5) results from the combination of P_ and B_ evidence scores (Richards et al, 2015).

Interpretive Reports: Interpretive reports should be clear and concise, and address the diagnosis of the person under investigation. Beyond its main focus stating its overall molecular conclusion in answering the genetic question, an interpretive report should include sufficient details to allow identification of the variant in other laboratories (i.e. indicate the practical approaches used, limitations of the techniques, genomic reference sequence used, and the classification of pathogenicity according to the ACMG guidelines, including the evidence applied for classification and references; ACGS reporting guidelines, 2020; Deans et al, 2022; Claustres et al, 2014; Gomez et al, 2019). Interpretive reports should include information explaining the actual extent of the molecular diagnosis in plain language with clear indication of, for example, specific risks for developing specific phenotypes in the family.

Quality assurance: In genetic testing, quality assurance covers all aspects of the diagnostic process, from nucleic acid extraction and analytical procedures, through to the classification and description of the variants detected and the production of an interpretive report. Internal quality control (IQC) of genetic tests should be routinely performed to ensure the validity of the results produced. Formal EQA schemes are available to ensure that diagnostic process and reporting procedures are in agreement with other laboratories (e.g. Genomics Quality Assessment [GenQA], and specifically for hemophilia genetic assessment by the U.K. National External Quality Assessment Service [UK NEQAS] for Blood Coagulation). Genetics laboratories should undergo periodic accreditation, if available, against internationally agreed standards, by an approved body. This ensures high quality provision of the genetic diagnostic service.

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PART 15 Troubleshooting Issues with Coagulation laboratory tests

Steve Kitchen

TOPICS COVERED

- ✓ Unexpected Results on an Individual Test Sample
- ✓ Out of Range IQC Results
- ✓ How to Investigate Out-of-Consensus Results in External Quality Assessment Surveys

Unexpected Results on an Individual Test Sample: Problems related to coagulation testing occur in all coagulation laboratories irrespective of which methods reagents and equipment are in use. Inaccurate results can occur as a consequence of issues with a particular sample due to issues with the sample itself. This can be a consequence of inadequate sample collection processing or storage prior to analysis. These issues are discussed in Part 3 of this manual. Recommendations on controlling preanalytical variables are also available, specifically related to hemophilia and allied disorders (Kitchen et al, 2020), and in relation to sample collection (Kitchen et al, 2021a) and processing (Kitchen et al, 2021b) in all aspects of coagulation laboratory testing, and are not discussed further here. Specific issues can occur during the analysis of a sample which relate to the reagent or sample handling during that specific test, despite successful analysis of adjacent samples just before or just after the sample with a questionable result. Many analyzers use a probe to automatically aspirate samples and such probes sometimes descend until detection of a liquid and then aspirate a suitable test sample volume to complete the testing. If that test sample has bubbles on the surface, this may lead to mis-sampling with inadequate volume, following be falsely abnormal results such as falsely prolonged screening tests results or falsely low activity in calibrated assays. Inaccurate pipetting of reagents during analysis can also lead to inaccurate results, for example, if a probe used for reagent movement in an analyzer has moved out of alignment. This would likely affect multiple sample results. It may be useful to run 10 replicates of the same sample to assess the precision of results, which is normally compromised if probe misalignment has occurred. Reagent areas on analyzers are usually maintained at a constant temperature, often chilled below ambient temperature but with reaction mixtures warmed to 37°C during analysis. Most coagulation test results are highly temperature dependent, therefore any drift outside a narrow acceptable range in the cooling of regents or in particular heating of reaction mixtures, can cause inaccurate results which are generally falsely abnormal. False normal results are much rarer than false abnormal ones, and are only regularly seen in relation to the false shortening of APTT as a consequence of pre-analytical issues such as in vitro hemolysis. Fully automated coagulometers often utilize wash/cleaning solutions that rinse through probes in between successive pipetting operations. This process normally avoids carryover of sample or reagent from one test into the following reaction mix, but such events have occurred. For example, partial sample carryover into a following sample reaction mixture has occurred in the past in relation to pathological components in the sample such as anti-phospholipid antibodies or paraproteins, or related to therapeutic drugs, including emicizumab, which can then cause false shortening of APTT in the following sample. Reagent components such as heparin neutralizer in reagents have carried over into following samples, which could lead to loss of heparin activity if present in the following sample. Reagent carryover effects have been largely eradicated when reagents and instruments from the same manufacturer are combined. This is more likely to be an issue if a reagent from one manufacturer is used on an analyzer from a different manufacturer, if that combination has not been validated for use. It is critical that laboratories follow diagnostic company guidance on permitted intervals between preventative maintenance visits to minimize risks of generating patient sample results that cannot be safely released for patient management decisions. It is important that laboratories receive sufficient patient/clinical information as is needed for experienced laboratory

staff to identify unexpected or unusual test results wherever possible. Any unexpected test result should be reanalyzed to exclude the possibility of analytical error. Where analytical error is excluded as an explanation for an unexpected result that does not seem to fit the patient's clinical picture, a repeat sample should be obtained for confirmation of the result.

Out-of-Range IQC Results: As described in Part 2 of this manual, it is convenient to keep a record of IQC results on each IQC material in the form of a chart. Many analyzers use the Levey-Jennings approach as shown in Figure 23. There are IQC systems available to assist laboratories in troubleshooting issues, such as the Westgard rules (www.westgard.com) which use 5 different control rules. These have few false alarms and give confidence in error detection. On the other hand, the patterns of IQC testing required for effective use of such systems are not well suited to regular use in most coagulation laboratories. The potential clinical consequences of laboratory error in management of patients with hemophilia and allied disorders, means a cautious approach to out-of-range IQC results is safer for patients. For this reason, any out-of-range IQC should be assessed and during investigation testing and reporting of patient results should be suspended. It is useful to identify the case of an out-of-limits IQC to help avoid future delays in processing samples. If a retest on the same vial of QC material generates a result which is clearly in range, then there may be an analyzer issue which can be assessed by performing 10 replicates on the same test sample. An analyzer issue is indicated if there is wide variability amongst the replicates. More often in routine coagulation testing, a repeat test on the same material is again out of range, and replacement of the material with a new vial or aliquot generates an in-range result, confirming that the IQC material itself was the source of the problem. In this case, patient results are safe to be released. If, on the other hand, testing a new vial/aliquot of IQC also generates a similar out-of-range result, there is an issue with the test system that would also impact patient results. In this case, the reagents used for the test should be replaced in sequence with a new IQC after each replacement of a reagent. Once a replacement reagent leads to an in- range IQC result, that reagent is identified as the source of the problem. This should be noted in IQC records so that patterns can be identified and a new assessment of reagent stability and use initiated. If replacement of all relevant reagents is still associated with out-of-range results, then that analyzer should be withdrawn from use pending review by the manufacturer and the laboratory should switch to a backup, ideally an alternative coagulometer giving the same results or, for tests with clotting endpoints, a manual technique (see Part 4 of this manual). Any patient results obtained since the previous in-range IQC result should be reviewed with repeat testing to establish where in the sequence of sample testing the problem may have commenced. If patient results from samples analyzed after the previous in-range IQC have been released, the laboratory should retest and recall any results that are not conformed, and should also review its IQC testing protocols since adequate IQC testing should avoid the necessity to recall any patient results whatsoever. Figure 23 shows the Levey-Jennings plot of APTT IQC results. The dotted red lines show the upper and lower limits of the acceptable range for this material. The first series of results are inside the range other than one on the lower limit. The second section shows a gradual and progressive increase in the clotting times. This trend occurs if one component of the test gradually changes over time. This is unlikely to occur in relation to commercial lyophilized IQC material stored according to manufacturer's instructions, but can occur if the IQC material has been locally prepared as described in Part 2. Alternative causes could be a gradual change in one of the reagents if not stored appropriately or by a gradual deterioration in some aspect of the endpoint detection system used (e.g. a deterioration in light source of a photo optical system). These types of problem are rare with modern automated coagulometers.

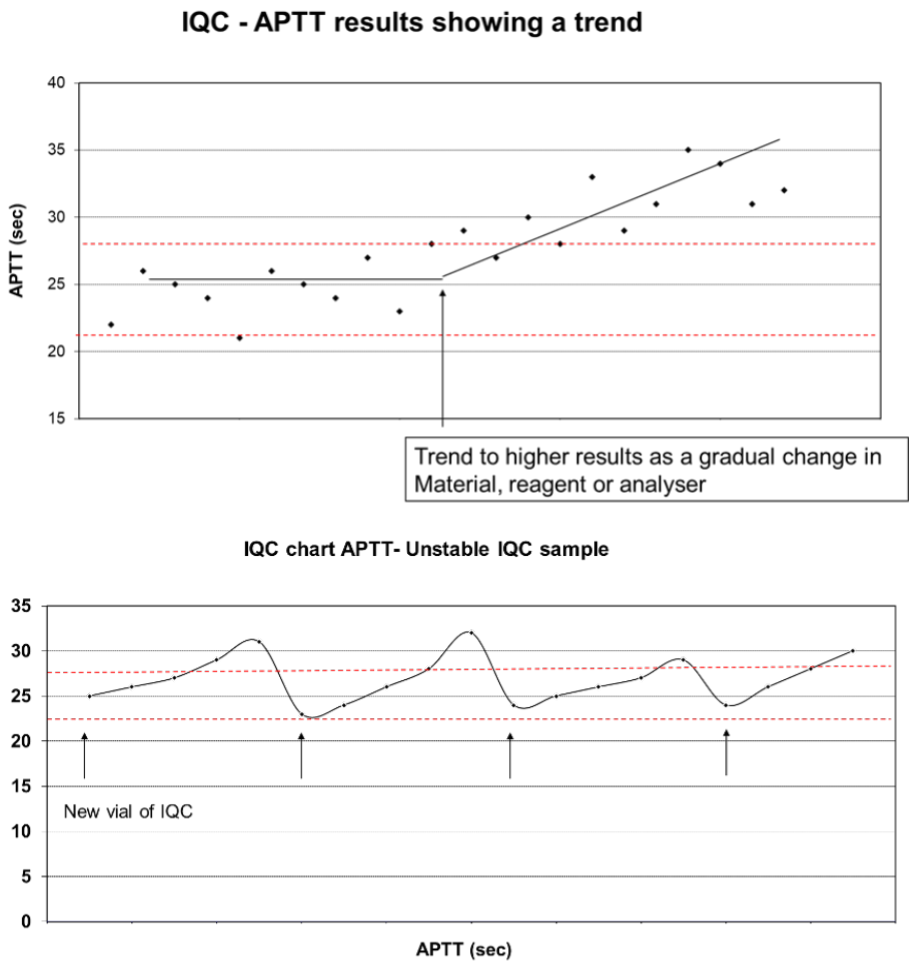

Figure 23. Levey-Jennings plot of APTT results on an unstable IQC material. The dotted red lines show the upper and lower limits of the acceptable range for this material. Each solid black arrow indicates when a new vial of IQC was loaded on to the analyzer. For each new vial there is a gradual increase in APTT over time. This example occurred because of a locally prepared frozen IQC material that was unstable after thawing. This can in principle also occur after reconstitution of lyophilized samples if not properly prepared or if the water used to reconstitute is contaminated.

How to Investigate Out-of-Consensus Results in External Quality Assessment Surveys: Participation in proficiency testing or EQA is an essential requirement for a laboratory to ensure it produces accurate results. Accreditation bodies who assess against ISO standards such as ISO 15189 (2022) require this for any tests where EQA is available. There is an IEQAS focused on hemophilia and allied disorders overseen by the WFH (see Part 2 of this manual). Results obtained in EQA exercises can be used to identify important issues related to the precision and accuracy of coagulation laboratory tests, provided the test material in the EQA program is commutable with patient samples (i.e. behaves in the same way in a particular method as patient samples would). Effective troubleshooting of EQA results that are not within the consensus of results in other centers, is important to ensure safe patient management. When considering outlying EQA results, there are a number of things that should be considered. A local result outside the target derived from the results in other centers, is less of a concern if the difference is not large enough to impact patient management. A clinically significant difference is much more of a problem than a statistical difference that would not be predicted to alter diagnosis or management of a patient.

A single result that is markedly different from the mean or median of results in other centers, to the extent that patient management would be affected had the sample been from a patient the laboratory, should be investigated further. This could include the following:

i) Checking that the sample was stored correctly on receipt, reconstituted correctly, and the test performed according to the written procedure for that method. If the problem was thought to be restricted to analysis of the EQA sample, then patient results would be unaffected.

ii) Checking that the internal QC at the time the EQA test was performed was satisfactory. If not, there was likely an issue with testing that could have affected patient results and patients results should be reviewed.

iii) Consideration of the particular details of the test sample. If abnormal, then the results obtained could be a consequence of the particular defect in the EQA sample.

When a laboratory has outlying results in consecutive surveys that occur over a number of surveys, investigation is needed and should take account of the pattern in the relationship between local results and the mean or median of results in other centers using similar methodology. In addition to the investigations after a single outlying result mentioned above, the following should be considered:

i) The clinical impact of the results should be evaluated. It is possible that, for an assay with very good precision, a laboratory can be persistently out of consensus, but still record results relatively close to the target, with no clinical consequences. It is also possible that if a locally determined reference range is employed, any bias in patient results is compensated by an appropriate reference range.

ii) Results which are consistently reading high or consistently reading low compared to the mean or median result in other centers, is often related to calibration. Typically, labs with such problems have a calibration curve established sometime in the past which is not appropriate for current testing, either because of a change in lot number of an assay component or because day to day variability in test results requires a fresh calibration alongside analysis of test samples. Recalibration typically resolves this issue in centers that are using a historical calibration curve. The possibility that an inappropriate potency has been assigned to the calibrator, although rare, should be considered. When investigating the possibility of a problem related to calibration, it can be useful to analyze a sample with an independently assigned value as a test sample to check how much the results of test samples are being over- or under-estimated. Such a material can also be used to perform a new calibration. The WFH IEQAS program has permission to supply a vial of the ISTH SSC plasma standard for this type of troubleshooting investigation. This has assigned values for a number of different coagulation parameters. The effect of a new calibration can be assessed by analyzing a small group of test samples before and after the new calibration. The SSC plasma standard is not available for routine use in calibration of local assay methods.

iii) Results above the mean or median in some surveys, and below the mean or median in others, suggest imprecision of the assay. This can occur as a consequence of poorly maintained instruments, inadequate reagent handling (i.e. reconstitution or storage), reagent instability, or issues related to staff training or competence.

Where possible, analysis of repeat samples after completion of investigation and after making any necessary improvements, is useful to confirming the success or otherwise of interventions. Retrospective review of past EQA survey results, prior to the problem of outlying results occurring, should be done alongside laboratory records of lot changes, calibrations, instrument service, and changes in methodology, to assess if the change in performance corresponds to any relevant internal changes. EQA data analysis and performance reports are by nature retrospective since analysis and reporting normally take place some time

after tests were performed in the laboratory. Therefore, any problem identified may have affected patient results over the same period of time. The laboratory should review with clinicians the past patient results for any tests where EQA indicates a clinically relevant inaccuracy could have been present. The review should consider whether any patient diagnosis or management could have been adversely affected. Retesting may be needed if the pattern of outlying results could have impacted patients adversely.

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