PART 8 Von Willebrand Disease Lab Investigation

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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).

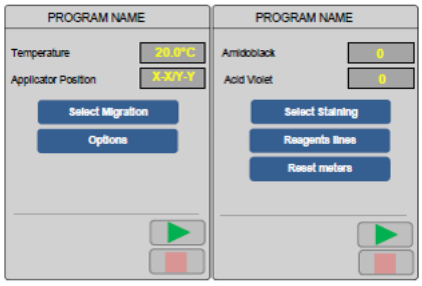

Figure 10. Hydrasys analyzer

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).

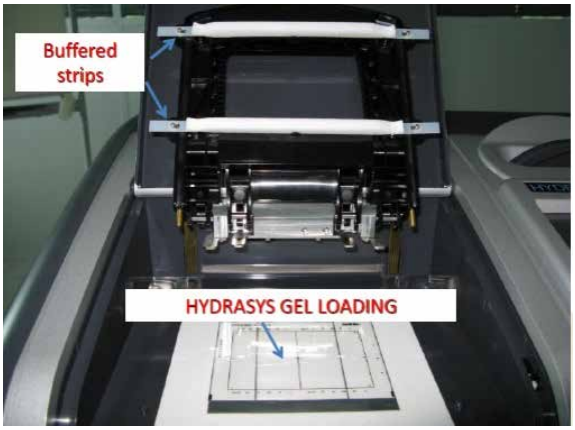

Figure 11. Hydrasys analyzer

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.

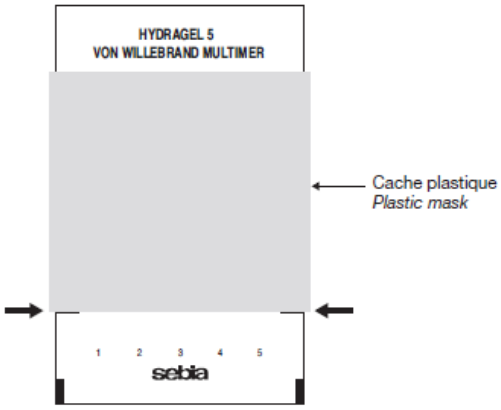

Figure 12. Hydrasys analyzer

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)

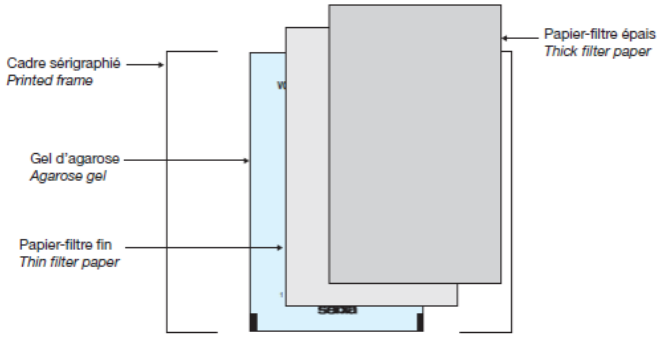

Figure 13. Hydrasys analyzer

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.

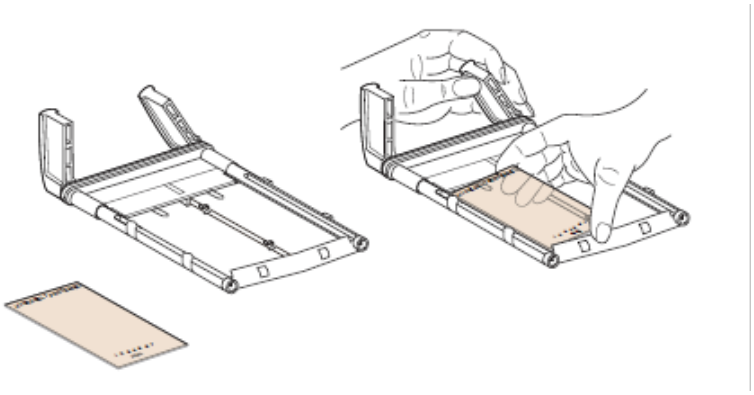

Figure 14. Hydrasys analyzer

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.

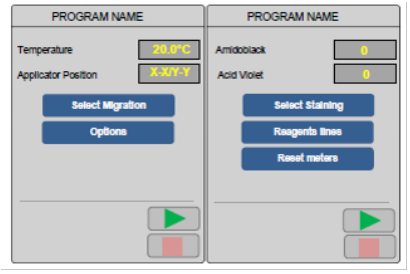

Figure 15. Hydrasys analyzer

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:

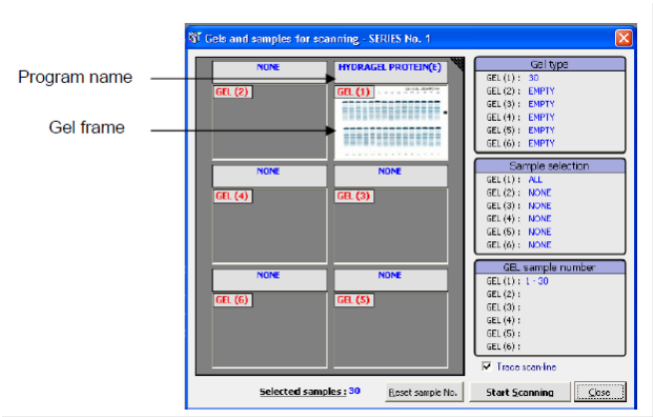

Figure 16. Hydrasys analyzer

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:

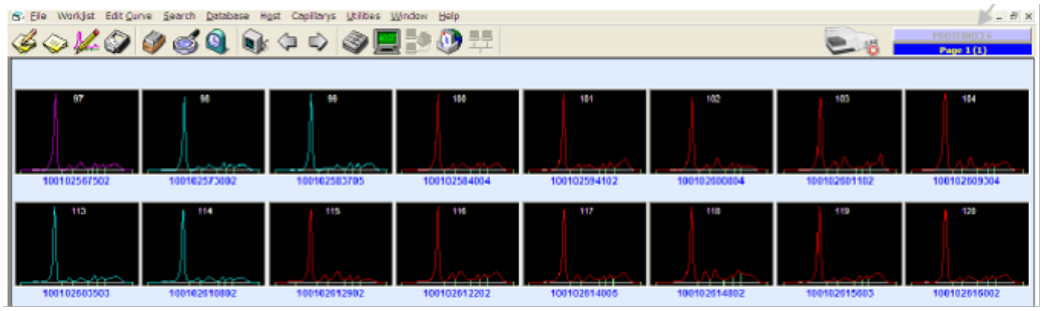

Figure 17. Hydrasys analyzer

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.

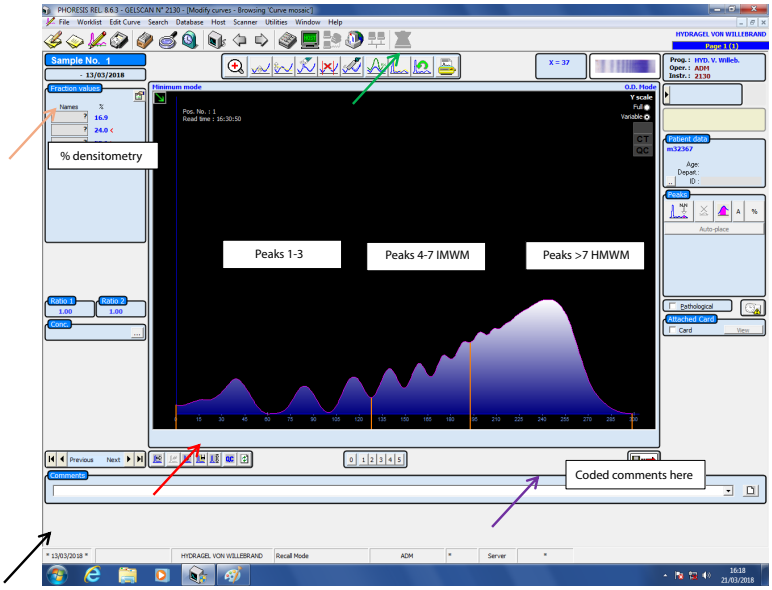

Figure 18. Hydrasys analyzer

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.

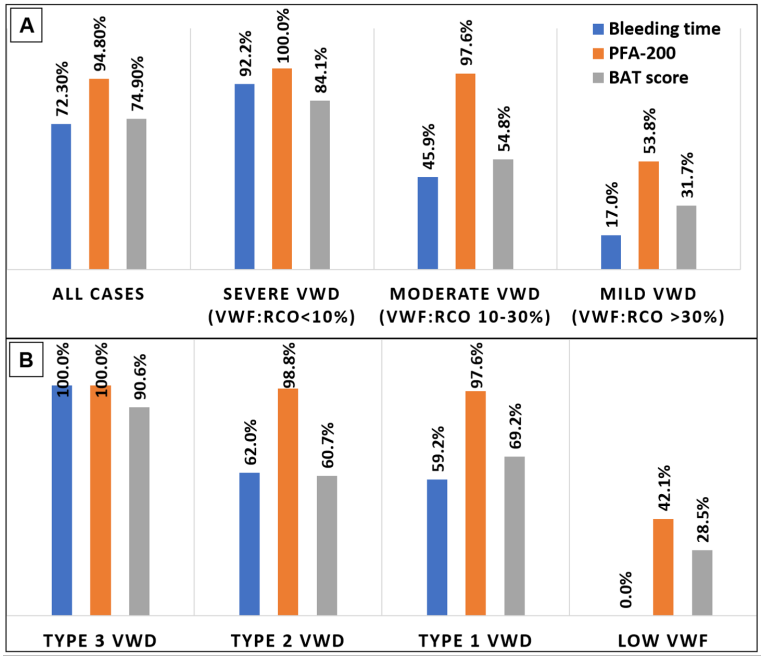

Figure 19. The overall sensitivity of the SBT and PFA-200

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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