VWF & Propeptide Assay

INTENDED USE

The VWF & Propeptide Assay is a quantitative solid phase enzyme linked immunosorbent assay (ELISA) for the measurement of von Willebrand Factor (VWF) and VWF propeptide in plasma.

For Research Use Only.

SUMMARY AND EXPLANATION

Von Willebrand Factor plays a pivotal role in successful recruitment and activation of platelets at the site of vascular injury. In addition, VWF serves as a carrier protein for the clotting factor, Factor VIII. Decreased levels of VWF is a hallmark of Type I von Willebrand Disease (VWD)[1].

The VWF propeptide is synthesized as part of a pro-VWF protein and is subsequently cleaved, stored and secreted at the same time as VWF. The level of VWF propeptide in the circulation can be used as a marker of VWF synthesis. In individuals with low VWF synthesis, the propeptide level is similarly decreased. In individuals with normal levels of VWF synthesis and reduced survival of their VWF, an increased propeptide: VWF ratio can be observed.[2] It is important to recognize this clearance phenotype clinically because the increased clearance of VWF reduces the efficacy of desmopressin treatment.[3,4]

PRINCIPLES OF THE PROCEDURE

Diluted calibrators, controls, and sample plasma are added to microwells to which monoclonal antibodies specific for VWF or propeptide have been immobilized. Incubation of the calibrators and samples with the microwells allows for binding of the VWF or propeptide to the immobilized antibodies. A wash step removes any unbound plasma proteins from the microwells. The microwells are then incubated with a biotinylated monoclonal antibody specific for the VWF or propeptide that has been captured in the wells. A wash step then removes any unbound antibodies. Subsequently the microwells are incubated with Streptavidin-labeled Horseradish Peroxidase. After a wash step which removes any unbound Streptavidin-labeled Horseradish Peroxidase from the microwell, a fluorescent substrate is added and incubated. Following this incubation, the reaction is stopped by addition of stopping solution. The fluorescence is measured in a fluorescent plate reader with excitation wavelengths between 315 – 340 nm and emission wavelengths between 370 – 470 nm. Values obtained from the calibrators are graphed to make a standard curve. Values for the positive controls and the patient samples are determined from this standard curve. Reportable results are given in IU/dL for VWF and U/dL for propeptide.

This kit contains two positive control samples. Expected values for both of the controls are lot-specific and can be found in the included Analysis Software. A significant difference between the obtained and the expected values would indicate an incorrectly performed assay.

REAGENTS

Maximum number of tests per kit: 41 in duplicate
All reagents should be stored as directed by the label.

MSVW / **MSPRP** 1. Microwells: Black, flat-bottomed microwell strips coated with antibodies specific for VWF (color-coded yellow) or propeptide (color-coded green). Contains murine and bovine source material. Ready for use. The microwell strips are enclosed in resealable foil pouches. Store at 2-8˚C.

5XCW 2. Concentrated Wash (5x): Phosphate buffered saline solution containing Tween 20 and 0.25% ProClin 300. Dilute with deionized or distilled water before use. Store at 2-8˚C.

VPC 3. Streptavidin-HRP (200x): Streptavidin labeled Horseradish Peroxidase in an HRP stabilizer. Must be diluted in Diluent before use. Store at 2-8˚C.

VPD 4. Diluent: Phosphate buffered saline solution containing bovine serum albumin and 0.05% ProClin 300. Ready for use. Store at 2-8˚C.

VCAL 5. Calibrator: Pooled Normal Plasma. For single use only. Assigned values can be found in the included Analysis Software. Dilute in Diluent before use. Store at ≤ -15°C.

VDA 6. VWF Detection Antibody (200x): Biotinylated murine monoclonal antibody with 0.05% ProClin 300. Dilute in Diluent before use. Store at 2-8˚C.

PDA	7.	Propeptide Detection Antibody (200x): Biotinylated murine monoclonal antibody with 0.05% ProClin 300. Dilute in Diluent before use. Store at 2-8˚C.
QA	8.	Substrate A Solution: Must be diluted 9 parts Substrate Solution A to 1 part Substrate Solution B before use. Store at 2-8˚C.
QB	9.	Substrate B Solution: Must be diluted 9 parts Substrate Solution A to 1 part Substrate Solution B before use. Store at 2-8˚C.
QSS	10.	Stopping Solution: Ready for use. Store at 2-8˚C.
PS	11.	Plate sealers.
AS	12.	VWF & Propeptide Analysis Software. Analysis Software on CD.

PRECAUTIONS

- Do not use reagents that are turbid or contaminated.
- Do not use reagents beyond their expiration date.
- Microwells and reagents contained in the kit are not to be used in conjunction with any other test system.
- Substitution of components other than those provided in this kit may lead to inconsistent or erroneous results.
- Discard any unused portions of Calibrators, Controls and used microwell strips after each run.
- Discard any unused, diluted Detector Antibodies, diluted Strep-HRP, and diluted Substrate Solution.
- When making dilutions, follow pipette manufacturer's instructions for appropriate dispensing and rinsing techniques.
- When making dilutions, do not pipette less than 5 µL of a reagent or sample. Using volumes less than 5 µL when diluting will increase dilutional imprecision.
- Accurately calibrated pipets should be used for reagent additions.
- The enzyme substrate reaction is temperature sensitive and should be performed in a controlled area at 22 to 25˚C.
- Reagents may contain more volume than stated on the container.

CAUTIONS

- All human plasma used in the Calibrator for this product has been tested and found negative for antibody to HIV, HCV and HBsAg by FDA approved methods. No test method, however, can offer complete assurance that HIV, Hepatitis C virus, Hepatitis B virus or other infectious agents are absent. Therefore, these materials should be handled as potentially infectious.
- Discard all components when completed according to local regulations.

SPECIMEN COLLECTION

Sample Collection and Preparation

NOTE: Only platelet poor plasma collected in 3.2% sodium citrate may be used for this assay. See Collection, Transport and Processing of Blood Specimens for Coagulation Testing and General Performance of Coagulation Assays. Approved Guideline H21-A4 NCCLS, Volume 23, Number 35, December 2003 for details.

Plasma collection should be performed as follows:

1. Collect blood in buffered sodium citrate (light blue top, 3.2%) plastic tubes (available in 4.5 mL, 2.7 mL, or 1.8 mL full draw tubes).

NOTE: Partial draw tubes should NOT be processed. Since the tubes are pre-calibrated to draw the specified amount of blood, the resulting sample, will not have the proper 9:1 ratio of blood to anticoagulant if a full sample is not collected.

2. After collection, store tube upright at room temperature until centrifugation.

NOTE: Blood samples should be centrifuged between fifteen minutes and two hours after blood collection for best results.

3. Remix the blood sample immediately prior to centrifugation by gently inverting the tube 8 to 10 times.

4. Centrifuge blood sample at room temperature in a horizontal rotor (swing-out rotor) for 15-20 minutes at 1500 to 1800 RCF (Relative Centrifugal Force).

WARNING: Excessive centrifuge speed (over 2000 RCF) may cause tube breakage and exposure to blood and possible injury.

5. Following centrifugation, pipet off the top 2/3 of plasma layer into a plastic tube.

6. Re-centrifuge the collected plasma at 1500 to 1800 RCF for 15-20 minutes to remove any remaining red cells or platelets.

7. Transfer the top 2/3 of plasma into a plastic tube, taking care not to disturb any cells at the bottom of the tube.

Sample Storage

1. Plasma should be stored at room temperature and assayed within 4 hours OR aliquoted and frozen at -15°C or colder until use.

2. Frozen plasma or Calibrator should be thawed rapidly at 37°C and then incubated at 37°C for 10 minutes prior to use in order to properly solubilize the antigens. Thawed plasma should be stored at room temperature and assayed within 4 hours.

PROCEDURE

Materials Provided:

Vials may contain more reagent than described on the labels. Be sure to measure the reagent with an appropriate device when making dilutions.

Box A:
1. 6 x 0.5 mL vials Calibrator

Box B:
1. 12 – 1 x 8 Black Microwell Strips for VWF detection (color coded-yellow) with holder
2. 12 – 1 x 8 Black Microwell Strips for propeptide detection (color coded-green) with holder
3. 3 x 50 mL Diluent
4. 1 x 125 mL Concentrated Wash
5. 1 x 40 µL VWF Detection Antibody
6. 1 x 40 µL Propeptide Detection Antibody
7. 1 x 75 µL Streptavidin-HRP
8. 1 x 14 mL Substrate A Solution
9. 1 x 2 mL Substrate B Solution
10. 1 x 14 mL Stopping Solution
11. 12 Plate Sealers
12. Recording Sheets
13. Reporting Card
14. Analysis Software on CD

Additional Materials Required:

1. Test tubes or microfuge tubes for patient sample and control dilutions and for reagent dilutions
2. Transfer pipets
3. Adjustable micropipets to deliver 1 – 10 µL, 10 – 100 µL and 100 - 1,000 µL and disposable tips
4. Timer
5. Fluorescent plate reader capable of measuring fluorescence at Excitation between 315 – 340 and Emission between 370–470 nm
6. Deionized or distilled water
7. Absorbent paper towels
8. Microplate washer or device
9. Centrifuge
10. 37°C waterbath or 37°C dry incubator
11. Computer with Microsoft®Excel® version 2003 or 2007

Test Procedure

1. Bring all assay reagents from Box B to room temperature (22 to 25°C).

2. Determine the number of patient samples to be tested. Using the Recording Sheet, assign each sample to a location consisting of two (duplicate) wells. Record the identity of each sample on the Recording Sheet.

3. Make Working Wash solution by diluting Concentrated Wash (5XCW). Add 1 volume of Concentrated Wash to 4 volumes of deionized or distilled water. Mix well. Working Wash solution can be stored at 2-8°C for 2 weeks.

Strips:	4 – 1x8	12 – 1 x8	24 – 1x8
Concentrated Wash	20 mL	60 mL	120 mL
DI Water	80 mL	240 mL	480 mL

4. Remove the appropriate number of VWF and propeptide strips as identified on the Recording Sheet. Promptly reseal unneeded strips in the protective pouch.

NOTE: *Only two frames are provided in the kit. Do not discard until all strips have been used.*

5. Quick thaw an aliquot of Calibrator stock from Box A as well as any frozen test plasma samples at 37°C.

6. Once thawed the Calibrator stock and samples should be incubated at 37°C for an additional 10 minutes to allow for proper VWF solubilization prior to dilution.

7. Use the following table to make Calibrators A-E by adding the specified plasma volume to the appropriate volume of Diluent (VPD). This dilution provides enough material to run the calibrators and controls in duplicate on both VWF and Propeptide plates.

Calibrator Level	**Plasma Volume**	**Diluent Volume**
Calibrator A	20 µL calibrator stock	1,080 µL VPD
Calibrator B	200 µL Calibrator A	80 µL VPD
Calibrator C	200 µL Calibrator A	260 µL VPD
Calibrator D	200 µL Calibrator A	1,000 µL VPD
Calibrator E	----	300 µL VPD

8. Use the following table to make the positive controls by adding the specified plasma volume to the appropriate volume of Diluent (VPD). This dilution provides enough material to test the sample in duplicate on both the VWF and propeptide strips.

Control Level	**Plasma Volume**	**Diluent Volume**
Positive Control High	5 µL calibrator stock	495 µL VPD
Positive Control Low	5 µL calibrator stock	995 µL VPD

9. For each patient sample to be tested, make a dilution in Diluent according to the following table. A 1:200 dilution is sufficient for most samples. To confirm a low or deficient sample a dilution of 1:20 is recommended.

Sample Dilution	**Patient Plasma Volume**	**Diluent Volume**
1:200	5 µL	995 µL VPD
1:20	13 µL	247 µL VPD

10. Add 50 µL of each Calibrator (A-E) (in duplicate) to the assigned microwells of the VWF and the propeptide strips.

11. Add 50 µL of the Positive Control, High (in duplicate) to the assigned microwells of the VWF and the propeptide strips.

12. Add 50 µL of the Positive Control, Low (in duplicate) to the assigned microwells of the VWF and the propeptide strips.

13. Add 50 µL of the diluted sample plasma solution prepared in step 9 (in duplicate) to the assigned microwells on the VWF and the propeptide strips.

NOTE: *If multiple patient samples are tested at the same time, only one set of calibrators and controls are required on each type of microwell strips.*

NOTE: *Label each strip to avoid errors.*

14. Seal the microwells with a plate sealer and incubate for 15 minutes in a 37˚C waterbath or incubator.

NOTE: *Since the incubation times are only 15 minutes, it is important to add all samples to the strips as quickly as possible starting with the same well each time.*

15. Dilute the VWF detection antibody (VDA) in Diluent (VPD) as described in the following table. The VDA will be added to all of the VWF microwells.

Strips:	2 – 1x8	4 - 1x8	12 - 1x8
VDA	6 µL	10 µL	30 µL
VPD	1.2 mL	2 mL	6 mL

16. Dilute the propeptide detection antibody (PDA) in Diluent (VPD) as described in the following table. The PDA will be added to all of the propeptide microwells.

Strips:	2 – 1x8	4 - 1x8	12 - 1x8
PDA	6 µL	10 µL	30 µL
VPD	1.2 mL	2 mL	6 mL

17. WASH STEP:

    a) Aspirate or decant contents of each well and blot on absorbent toweling.
    b) Add 300 µL Working Wash solution.
    c) Aspirate or decant.
    d) Repeat steps b + c for a total of 3 washes.
    e) Vigorously decant to remove all residual wash solution. Invert on absorbent toweling to prevent drying.

NOTE: It is important to completely remove all wash solution after the final wash.

18. To each well on the VWF plate (color coded yellow) add 50 µL of diluted VWF Detection Antibody (VDA).

19. To each well on the propeptide plate (color coded green) add 50 µL diluted Propeptide Detection Antibody (PDA).

20. Seal the microwells with a plate sealer and incubate for 15 minutes in a 37˚C waterbath or incubator.

21. Dilute the Streptavidin-HRP (VPC) in Diluent (VPD) as described in the following table. Use a polypropylene container. The diluted Streptavidin-HRP will be added to all the used wells on both types of microwell strips.

Strips:	4 - 1x8	8 - 1x8	24 - 1x8
VPC	10 µL	20 µL	60 µL
VPD	2 mL	4 mL	12 mL

22. WASH STEP:

    a) Aspirate or decant contents of each well and blot on absorbent toweling.
    b) Add 300 µL Working Wash solution.
    c) Aspirate or decant.
    d) Repeat steps b + c for a total of 3 washes.
    e) Vigorously decant to remove all residual wash solution. Invert on absorbent toweling to prevent drying.

23 Add 50 µL of diluted Streptavidin-HRP to all VWF and propeptide microwells.

24. Seal the microwells with a plate sealer and incubate for 15 minutes in a 37°C waterbath or incubator.

25. Add 9 parts Substrate A Solution (QA) to 1 part Substrate B Solution (QB). Use a polypropylene container. Mix well. This material is not light sensitive. This material will be added to all used wells on both types of microwell strips.

Strips:	4 - 1x8	8 - 1x8	24 - 1x8
Substrate A Solution (QA)	1.8 mL	3.6 mL	10.8 mL
Substrate B Solution (QB)	200 µL	400 µL	1.2 mL

26. WASH STEP:

   a) Aspirate or decant contents of each well and blot on absorbent toweling.
   b) Add 300 µL Working Wash solution.
   c) Aspirate or decant.
   d) Repeat steps b + c for a total of 3 washes.
   e) Vigorously decant to remove all residual wash solution. Invert on absorbent toweling to prevent drying.

NOTE: It is important to completely remove all wash solution after the final wash.

27. Add 50 µL of the mixed Substrate Solution from step 25 to each used well on both types of microwell strips.

28. Seal the microwells with a plate sealer and incubate for 15 minutes at ROOM TEMPERATURE (22 to 25°C). It is not necessary to protect the reaction from light.

29. Stop the reaction by adding 50 µL of Stopping Solution (QSS) to each well in the same sequence as the addition of substrate.

30. Read the plate in a fluorescent plate reader with Excitation wavelengths between 315 – 340 nm and Emission wavelengths between 370 – 470 nm at room temperature (22 to 25°C).

INTERPRETATION OF TEST RESULTS

Construct a calibration curve by plotting the mean fluorescence (n=2) value for each calibration standard versus its corresponding concentration of VWF or propeptide using the Analysis Software provided on the enclosed CD. A calibration curve for each type of plate should be generated each time the assay is performed.

CALCULATIONS

Determine the concentration of VWF and propeptide in the plasma sample using the Analysis Software provided on the enclosed CD. Instructions for using the software are on the first worksheet of the file. The Analysis Software assists with graphing and solving the equation to obtain IU/dL of VWF and U/dL for propeptide for the controls and the plasma samples.

If plasma samples give relative fluorescent values higher than that obtained for the highest level calibrator, then the sample must be repeated at a higher dilution to obtain a result that is within the calibration range. If the result for a plasma sample is less than 10 IU/dL of VWF or 10 U/dL of Propeptide, the sample can be tested at a lower dilution to obtain a reportable result that is within the calibration range. A minimal dilution of 1:20 is recommended for very low or deficient samples. More concentrated samples may yield incorrect results.

REFERENCES

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VWF & Propeptide Assay

- *For* Research *Use Only*
- STORE AT ≤ -15°C for Box A
- STORE AT 2 to 8°C for Box B

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