PRINCIPLE
Type 2 VWD is a qualitative form of VWD affecting VWF protein function. Type 2 Normandy (2N) is characterized by abnormally low FVIII:C levels caused by a reduced affinity of VWF for FVIII. As a consequence, less FVIII is bound and therefore less is protected from degradation/removal, leading to a lower concentration in plasma. Mutations responsible for type 2N VWD are found in the FVIII binding domain of VWF. Phenotypically, patients with type 2N VWD resemble patients with mild hemophilia — reduced FVIII:C, often with normal levels of VWF:RCo and VWF:Ag — but have an autosomal recessive pattern of inheritance.

The FVIII binding assay is an ELISA-based method to determine whether VWF binds FVIII normally. A monoclonal antibody is used to capture VWF, calcium chloride is used to remove endogenous FVIII from VWF, and a known amount of recombinant FVIII is added to the bound VWF and left to bind. The bound FVIII is then measured by a chromogenic FVIII assay. A method for FVIII binding based on a published technique (Nesbitt et al. 1996) is described below.

Data obtained using a recently developed commercial assay (Asserachrom VWF:FVIIIB, Diagnostica Stago) have been published in abstract form (Caron et al. 2009). This assay is similar to the one described below in the initial analytical steps. It utilizes microtitre wells coated with rabbit anti-VWF antibody, which bind VWF/FVIII from diluted patient plasma. After removal of endogenous (patient) FVIII, recombinant FVIII is added, which binds to the patient VWF depending on the nature of the patient VWF molecule. This assay differs from the one described below in relation to the method of detection of bound FVIII: in this commercial assay, a peroxidase conjugated mouse anti-human FVIII antibody is used. The assay was reported to have 100% sensitivity and specificity based on analysis of 37 previously diagnosed cases of type 2N VWD and 13 heterozygous mutation carriers (Caron et al. 2009), with an inter-assay coefficient of variation of <10% for measurements of % binding of FVIII.

REAGENTS
- MAS 533p monoclonal antibody to the GPIb binding site of VWF (Oxford Biotechnology OBT0085, Oxford U.K.)
Store at 4°C, per manufacturer’s instructions. Note that other antibodies from different sources can be successfully used.

- FVIII concentrate 2.5 U/ml (e.g. Advate, Baxter Pharmaceutical) 
  Store at -80°C.
  Concentrate is diluted in HEPES buffer made from:
  - 2.763 g/l HEPES acid
  - 2.188 g/l HEPES salt
  - 8.19 g/l NaCl
  - Add 1% BSA
  - Made up in 100 ml, pH to 7.35

- Coatest FVIII chromogenic kit (Instrumentation Laboratory, Lexington, MA, U.S.A.) One kit is sufficient for two plates. Make up kit and freeze remaining reagents at -80°C.

- 20% acetic acid

- Quality control samples
  Include a normal control and, if available, a known VWD Normandy control.

BUFFERS
These buffers can be colour-coded with food dye, which makes them easier to see in the microtitre plate. Make up fresh buffers for each assay.

- Citrate phosphate buffer 0.1M pH 5.0
  For 0.5 litre:
  - 3.65 g citric acid (BDH 10081)
  - 4.74 g anhydrous disodium hydrogen phosphate (BDH 102494C)

- 10xTris buffered saline (TBS): 50mM Tris, 100mM NaCl, pH 8.0
  For 0.5 litre:
  - 30.27 g Tris (Sigma)
  - 29.22 g NaCl (Sigma)
  Needs about 60 ml 1M HCL to pH.

- Wash buffer: TBS/0.1% BSA
  For 1 litre:
  - 100 ml 10 × TBS
  - 900 ml H₂O
  - 1 g bovine albumin (Sigma)

- Plasma diluent: TBS/3% BSA
  For 100 ml:
  - 10 ml 10 × TBS
  - 90 ml H₂O
  - 3 g bovine albumin (Sigma)
- Calcium chloride solution 0.35M
  For 50 ml:
  - 17.5 ml 1M calcium chloride solution
  - 32.5 ml H₂O

- FVIII diluent
  For 100 ml:
  - 100 ml wash buffer
  - 0.147 g calcium chloride
  - 0.002 ml Tween 20 (Sigma P5927): dip yellow tip into Tween and let 1 drop fall into the buffer.

**METHOD**

This assay takes three consecutive days. Ensure VWF:Ag results are available for each test patient prior to starting this assay.

**Day 1 (p.m.)**

1. Dilute MAS 533p monoclonal antibody (Oxford Biotechnology) in 0.1M citrate buffer pH 5.0 to a concentration of 2.5 µg/ml (exactly 50 µl antibody in 10 ml buffer).

2. Coat NUNC microtitre plate using 100 µl of the antibody/buffer mix and leave overnight at 4˚C covered in parafilm. There is only enough to coat 11 rows, so leave row 12 uncoated.

**Day 2 (p.m.)**

3. Wash four times with TBS (50mM Tris, 100mM NaCl, pH 8.0) containing 0.1% BSA, and blot excess liquid.

4. Add 100 µl of serial dilutions of plasma (~1 U/dl VWF:Ag- 0.125 U/dl VWF:Ag) in TBS/ 3% BSA and incubate at 4˚C overnight in a wet box. See “Dilution Protocol” below, and Figure 32.1 for plate layout.

**Dilution protocol**

- Four dilutions of each test plasma are produced by double dilution. Start with at least 0.6 ml of the first dilution.

- Each sample must be diluted to produce 1 U/dl (0.01 U/ml), so VWF:Ag must be known prior to starting this assay. For example, if VWF:Ag is 0.90 IU/ml, make a 1/90 starting dilution; if VWF:Ag is 0.06 IU/ml, make a 1/6 starting dilution, etc.

- For patient and control samples, dilute according to level of VWF:Ag to nearest 0.05 U/ml (i.e. VWF:Ag = 0.13 dilute 1/15, etc.).
Day 3 (start at about 9:30 a.m.)

5 Wash as before.

6 To remove endogenous FVIII, incubate twice with 100 µl 0.35M CaCl₂ for one hour each time at room temperature. Discard first CaCl₂ before adding second volume. Do not wash in between.

7 Wash as before.

8 Dilute 200 µl FVIII to 10 ml FVIII diluent to produce 0.05 U/ml FVIII. Add 100 µl to each well and incubate for two hours at 37°C.

9 Wash as before.

10 Use the Coatest SP4FVIII chromogenic kit as follows:
   i. Remove from cold room 30 minutes before use to warm up.
   ii. Make up reagents according to kit insert:
       •FIXa + FX in 3 ml distilled water, make up 2 vials of each
       •S-2765 in 12 ml distilled water
   iii. In a suitable disposable plastic vial, mix:
       • 5 parts FIXa + FX (4.12 ml) (fresh)
       • 1 part phospholipid (0.83 ml)
       • 3 parts 1/10 dilution of kit buffer (250 µl kit buffer and 2.25 ml water)
   iv. Add 75 µl of this mixture to each well, then incubate for five minutes at 37°C.

11 Without discarding the mixture, add 25 µl 0.025M CaCl₂ from kit to each well. Incubate for five minutes at 37°C.

12 Add 50 µl S-2765 chromogenic substrate to each well.

13 Leave at 37°C until colour develops (usually about 20 minutes) and control 1.0 U/dl has an optical density (OD) of 0.8–1.0. Read using the plate reader at 405 nm (filter 1).

14 Stop with 50 µl of 20% acetic acid (made from 2 ml glacial acetic acid and 8 ml water).

15 Re-read the absorbance of each sample using the plate reader at 405 nm (filter 1).

16 Plot the concentration of VWF antigen against absorbance at 405 nm of bound FVIII. Results should be reported in graph and report format (see “Data Interpretation”, on following page).
Figure 32.1. Layout of a 96-well microtitre plate

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B1</td>
<td>CTL</td>
<td>PT2</td>
<td>PT4</td>
<td>PT6</td>
<td>PT8</td>
<td>CTL</td>
<td>PT2</td>
<td>PT4</td>
<td>PT6</td>
<td>PT8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>B2</td>
<td>CTL</td>
<td>PT2</td>
<td>PT4</td>
<td>PT6</td>
<td>PT8</td>
<td>CTL</td>
<td>PT2</td>
<td>PT4</td>
<td>PT6</td>
<td>PT8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>B3</td>
<td>CTL</td>
<td>PT2</td>
<td>PT4</td>
<td>PT6</td>
<td>PT8</td>
<td>CTL</td>
<td>PT2</td>
<td>PT4</td>
<td>PT6</td>
<td>PT8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>B4</td>
<td>CTL</td>
<td>PT2</td>
<td>PT4</td>
<td>PT6</td>
<td>PT8</td>
<td>CTL</td>
<td>PT2</td>
<td>PT4</td>
<td>PT6</td>
<td>PT8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>B5</td>
<td>PT1</td>
<td>PT3</td>
<td>PT5</td>
<td>PT7</td>
<td>N</td>
<td>PT1</td>
<td>PT3</td>
<td>PT5</td>
<td>PT7</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>B6</td>
<td>PT1</td>
<td>PT3</td>
<td>PT5</td>
<td>PT7</td>
<td>N</td>
<td>PT1</td>
<td>PT3</td>
<td>PT5</td>
<td>PT7</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>B7</td>
<td>PT1</td>
<td>PT3</td>
<td>PT5</td>
<td>PT7</td>
<td>N</td>
<td>PT1</td>
<td>PT3</td>
<td>PT5</td>
<td>PT7</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>B8</td>
<td>PT1</td>
<td>PT3</td>
<td>PT5</td>
<td>PT7</td>
<td>N</td>
<td>PT1</td>
<td>PT3</td>
<td>PT5</td>
<td>PT7</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
<td></td>
</tr>
</tbody>
</table>

B = blank
CTL = control plasma
PT = test samples
N = known VWD Normandy control (if available)

Test eight patients on a single plate.

**DATA INTERPRETATION**

1. Calculate mean OD of each patient, control, and negative control.

2. In Excel, start a new worksheet and enter the following data:
   - Column A — in A1: VWF:Ag U/dl; in A2: 1.0; in A3: 0.5; in A4: 0.25; in A5: 0.125
   - Column B — in B1: Patient name; B2-5: mean ODs for the four patient dilutions
   - Column C — in C1: Normal control; C2-5: mean ODs for the four control dilutions
   - Column D — in D1: Normandy control; D2-5: mean ODs for the four RD dilutions
3 Choose ‘Chart wizard XY scatter’ with smoothed lines. Select all four columns and ODs, and ensure the series is in columns.

4 On the resulting graph, the x axis should be labelled VWF:Ag U/dl. Label the y axis FVIII binding 405 nm. Embed the graph as an object in sheet 1.

5 Add a linear trend line to the normal control and patient lines, then choose “display equation” on the chart. This will show a formula for each line similar to $y = 0....x + 0....y$. Use the $0...x$ part to calculate the ratio of patient gradient/control gradient.

REFERENCES
