Factor VIII (FVIII) Activity Assays

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Topics for Discussion

- Introduction to FVIII
- Clot-based FVIII Assays
- Chromogenic-based FVIII Assay
- Which Assay to Use (and for What Purpose)
Thrombin cleaves the heavy chain after arginine 740 to generate a polypeptide that is subsequently cleaved after arginine 372 exposing a functional Factor IXa interactive site resulting in cofactor activity.

Thrombin cleaves the light chain after arginine 1689 liberating FVIII from Von Willebrand Factor (VWF).

Activated FVIII is a heterotrimer of two heavy chain derived fragments and one light chain derived fragment (1689-2332)
- Phospholipid binding site lies between residues 2303-2332

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FVIII – VWF Partnership

- VWF regulates FVIII
  - Facilitates Thrombin cleavage of FVIII
  - Inhibits activation by Factor Xa (FXa)
  - Inhibits inactivation by activated Protein C
  - Prevents binding to phospholipid

- VWF stabilizes FVIII by competing with low density lipoprotein receptor-related proteins and thereby inhibiting clearance of FVIII
- VWF modifies conformational structure of FVIII by promoting association of the light and heavy chains
- VWF modulates immunogenicity of FVIII by interfering with both B- (A3 & C2 domains) and T-cell (C1/C2 domains) epitopes in FVIII

Federici AB. Haematologica 2003;88(Supp9):3-12.
FVIII Function

- FVIII in plasma is attached to VWF and circulates in an inactive form until proteolytically cleaved by either Thrombin or activated Factor X (FXa) to form activated FVIII (FVIIIa)
- FVIIIa binds to phosphatidyserine exposed on thrombin-activated platelet membrane surfaces
- FVIIIa, in its cofactor role, increases by nearly 100,000 fold the activation of FX by activated Factor IX (FIXa) when this macromolecular complex ("tenase" complex) forms on a phospholipid surface
  - FVIIIa facilitates a conformational change in the active site of FIXa which permits it to catalyze FX
- FVIIIa cofactor activity is dampened following "spontaneous" dissociation of the A2 subunit from the heterotrimer or by cleavage of the A2 subunit at Arg 562 by activated Protein C
  - Protein S abrogates the protection of this site by FIXa
Why Measure FVIII?

- Detect FVIII deficiency in Hemophilia A (HA) and Von Willebrand Disease (VWD)
  - Severity of bleeding in HA depends on levels of FVIII
    - Severe: less than 1% activity (<0.01 IU/ml) of normal
    - Moderate: activity levels between 1-5% (0.01-0.05 IU/ml)
    - Mild: greater than 5% to less than 40% activity levels
- Assign potency to FVIII concentrates used for treating patients with HA or VWD
- Monitor patients receiving FVIII replacement therapy
- Assess risk for thrombosis
  - Persistent FVIII activity levels of greater than 150% result in 5-6-fold higher risk for deep vein thrombosis (DVT) and recurrent DVT than FVIII activity levels less than 100%
- Documenting an acute phase response
Hemophilia A is a disorder of the Propagation Phase of coagulation.

Thrombin is initially generated via the TF/FVIIa Initiation Phase however, the large amounts of Thrombin necessary for adequate secondary hemostasis are not generated.
Clot-based
Factor VIII Assays
Coagulation in the Laboratory

Intrinsic Pathway

XII
XI
IX
VIII

Extrinsic Pathway

VII
Tissue Factor

APTT
Intrinsic + Common

PT
Extrinsic + Common

Fibrinogen
Fibrin Clot
Two-Stage Assay for FVIII

- Consists of two separate reactions that directly relate to amount of FVIII present in patient plasma

**Step 1**
Patient sample containing FVIII is added to human serum (contains activated Factor IX and Factor X) + bovine serum absorbed with barium salts (source of Factor V/Va) + calcium ions + phospholipids to generate prothrombinase activity

**Step 2**
Prothrombinase formed in Step 1 converts prothrombin to thrombin (substrate plasma is human normal pooled plasma) with the rate of fibrinogen conversion to fibrin measured as a clot-endpoint

- Test developed in the 1950’s in the United Kingdom
  - Infrequently performed
  - Precursor to the chromogenic method
One-Stage Assay for FVIII

- Most widely used FVIII assay
  - First described by Langdell, Wagner, & Brinkhous in 1953

- Based on the APTT
  - Requires both contact activation (APTT reagent activator) and recalcification (Ca^{+2} ions)
  - Design assumes that FVIII supplied by patient plasma is rate-limiting and that all other components (other clotting factors, phospholipid, and other cofactors) are present at saturating levels

- Assay is a "Mixing Study" that uses diluted patient plasma to "correct" the deficiency of FVIII (complete absence) in a FVIII deficient substrate plasma ("severe HA plasma")
**APTT Test**

- "Partial thromboplastin"
  - Tissue Factor not used to initiate (activate) coagulation
- Requires activation & re-calcification
- Reagent composition
  - Activator
    - Converts FXII to FXIIa
  - Phospholipid
    - Replaces *in vivo* platelet phospholipid surface on which coagulation reactions occur
  - Buffer (minimizes pH changes in plasma reaction mixture)
  - CaCl₂
    - Re-introduces calcium ions that were chelated by sodium citrate anticoagulant

**Time for clot formation**
~ 30 seconds

- 0.1 ml CaCl₂
- Incubate at 37 °C for ~5 minutes
- 0.1 ml Activator
- 0.1 ml Plasma
Classical Mixing Study

**IF baseline APTT is prolonged:**
- Mix patient platelet poor plasma (PPP) with normal pooled plasma (NPP) and repeat APTT
  - Most common mix is “1:1” Mix (50/50 Mix)
- Failure to “correct” (upper limit of reference interval) indicates presence of an inhibitor
- “Correction” indicates a factor deficiency

*Diagram showing mixing of plasma pools and resulting APTT changes.*

% NPP = Percent Normal Pooled Plasma
% PAT = Percent Patient Plasma
One-Stage Factor Assay - Step 1

Prepare 1:10 dilution of reference plasma or patient plasma in buffer.
One-Stage Factor Assay – Step 2

Prepare serial dilutions of plasma from 1:10 starting dilution:

- 1:10 Buffer Dil
- 1:20 Buffer Dil
- 1:40 Buffer Dil

ETC
One-Stage Factor Assay–Steps 3 & 4

Mix each serial dilution with factor deficient substrate plasma

Perform APTT at 37°C
Analytical Variables

- Diluent used for making dilutions
- Composition of FVIII deficient plasma
- Instrument clot detection method
- Reagent sensitivity to individual factors
- Nature of reference material used for preparing the reference (calibration) curve
  - Impacts FVIII assays irrespective of method (clot-based or chromogenic)
- Criteria for acceptability of reference curve
- Interpretation of test result
Reference plasma and patient plasma are diluted in order to optimize the assay

- Rationale is to prolong clotting times in order to create a larger discriminatory window for interpretation of results
  - First dilution (lowest) to which a plasma is diluted is referred to as the starting concentration
- If neat (undiluted) plasma is used, it would not be possible, for example, to separate a mild factor deficiency (36%) from normalcy (66%) since APTT readings may be very similar (ie, 32 or 33 seconds)

Dilutions are prepared using buffered (pH 7.4) diluents such as Imidazole Buffer or Owren-Koller Buffer

Dilutions can also be made with FVIII deficient plasma
Deficient (substrate) plasmas must be completely devoid of FVIII
- Serve as the “Mixing Study” component of the assay
  - Reference or patient plasmas “correct” the deficiency of this plasma and the degree of correction is directly proportional to the amount of FVIII in the reference/test plasmas

Most commercially available substrate plasmas (lyophilized or frozen) are prepared by selective immunadsorption of FVIII from human pooled normal plasma

Deficient plasmas are also obtained from patients congenitally deficient for FVIII
- Not all congenitally depleted plasmas may be totally deficient of FVIII
- Since FVIII deficient plasma is used undiluted and mixed with equal parts of relatively high dilutions of patient plasma, the presence of even minor traces of FVIII can impact the total concentration of FVIII in the test
How to determine FVIII deficiency of deficient plasma

- Recovery studies (see next slide)
- Measure FVIII antigen level
- “Buffer blank” test wherein the assay diluent is substituted for patient plasma and tested for FVIII activity
  - The only FVIII in the system is provided by the deficient plasma

CLSI (H48-A) indicates that activity levels for all remaining factors in FVIII deficient plasma should be greater than 0.5 IU/mL (50%) and contain more than 1.0 g/L (100 mg/dL) fibrinogen

- Question if greater than 80% activity for remaining factors would not be more appropriate when considering the degree of dilution
### Recovery Studies

Determine FVIII levels from mixtures prepared with an assayed reference plasma (known level of FVIII) and FVIII deficient plasma.

Plot recovered FVIII values against expected FVIII levels from various mixtures.

<table>
<thead>
<tr>
<th>Theoretical Yield</th>
<th>Normal Reference Plasma (ul)</th>
<th>FVIII Deficient Plasma (ul)</th>
<th>APTTT (seconds)</th>
<th>delta</th>
<th>Assay % FVIII</th>
<th>Recovered % FVIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;100%&quot;</td>
<td>1000</td>
<td>0</td>
<td>27.9</td>
<td></td>
<td>91.0</td>
<td>91</td>
</tr>
<tr>
<td>&quot;60%&quot;</td>
<td>600</td>
<td>400</td>
<td>30.4</td>
<td>2.5</td>
<td>54.6</td>
<td>58</td>
</tr>
<tr>
<td>&quot;50%&quot;</td>
<td>500</td>
<td>500</td>
<td>31.8</td>
<td>3.9</td>
<td>45.5</td>
<td>52</td>
</tr>
<tr>
<td>&quot;40%&quot;</td>
<td>400</td>
<td>600</td>
<td>33.4</td>
<td>5.5</td>
<td>36.4</td>
<td>42</td>
</tr>
<tr>
<td>&quot;30%&quot;</td>
<td>300</td>
<td>700</td>
<td>33.8</td>
<td>5.9</td>
<td>27.3</td>
<td>28</td>
</tr>
<tr>
<td>&quot;20%&quot;</td>
<td>200</td>
<td>800</td>
<td>36.4</td>
<td>8.5</td>
<td>18.2</td>
<td>20</td>
</tr>
<tr>
<td>&quot;0%&quot;</td>
<td>0</td>
<td>1000</td>
<td>59.8</td>
<td></td>
<td>0.0</td>
<td>0</td>
</tr>
</tbody>
</table>

**APTT Upper Limit of Reference Interval = 30.9 seconds**

\[ y = 1.0048x + 2.3616 \]

\[ R^2 = 0.992 \]
Analytical issues affecting the APTT will also impact factor assays

- Dilute systems such as factor assays challenge clot detection systems more so than their “undiluted” counterpart, the APTT
- Types of activators (kaolin, micronized or colloidal silica, celite, ellagic acid)
- Source, concentration, and types of phospholipid

Instruments use various methods for detecting clotting end-points

- Photo-optic, mechanical, nephelometric
- Consider imprecision of these various methods when contemplating the addition of factor assays to a laboratory’s test menu
APTT reagent should be sufficiently sensitive to FVIII such that APTT exceeds the upper limit of the reference interval when FVIII activity falls below 40%

### APTT Reagent FVIII Sensitivity

<table>
<thead>
<tr>
<th>Assay % FVIII</th>
<th>Recovered % FVIII</th>
<th>APTT (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>91.0</td>
<td>91</td>
<td>27.9</td>
</tr>
<tr>
<td>54.6</td>
<td>58</td>
<td>30.4</td>
</tr>
<tr>
<td>45.5</td>
<td>52</td>
<td>31.8</td>
</tr>
<tr>
<td>36.4</td>
<td>42</td>
<td>33.4</td>
</tr>
<tr>
<td>27.3</td>
<td>28</td>
<td>33.8</td>
</tr>
<tr>
<td>18.2</td>
<td>20</td>
<td>36.4</td>
</tr>
<tr>
<td>0.0</td>
<td>0</td>
<td>59.8</td>
</tr>
</tbody>
</table>

**APTT Upper Limit of Reference Interval = 30.9 seconds**

APTT reagent in this example showed a prolongation (31.8 seconds) of the APTT (upper limit of reference interval = 30.9 seconds) at 52% FVIII activity
Choice of reference plasma used in preparation of a reference curve affects accuracy

- Improper assignment of factor activity for reference plasma can result in gross over or under estimation of patient FVIII
- Reference material must be traceable to WHO (World Health Organization) international calibrator plasma

<table>
<thead>
<tr>
<th>WHO International Biological Reference Preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PREPARATION</strong></td>
</tr>
<tr>
<td>Blood Coagulation factor VIII and von Willebrand factor, plasma, human. Lyophilized. 0.68 IU/ampoule Factor VIII:C; 0.94 IU/ampoule factor VIII. Antigen; 0.91 IU/ampoule VWF:antigen; 0.78 IU/ampoule VWF:ristocetin cofactor; 0.94 IU/ampoule VWF:collagen binding</td>
</tr>
<tr>
<td>Blood Coagulation factor VIII concentrate, human. Lyophilized. 11.0 IU/ampoule</td>
</tr>
</tbody>
</table>

- Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis secondary FVIII plasma standard
  - Calibrated by multiple laboratories against WHO standard
Reference Curve Preparation

- Reference curve is prepared by selecting specific dilutions or by serial dilution
  - Starting concentrations vary by instrument/reagent systems but traditionally the optimal dilution is 1:10 (1 part plasma + 9 parts buffer)
    - Starting concentration is arbitrarily defined as “100%” but in actuality it is the manufacturer’s assigned value for the reference material (that is, 92% or 105%, etc)
  - Generally a curve consists of seven to eight points (1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280)
- “Low” reference curves can be prepared to measure FVIII levels found in severe FVIII deficiencies
- Unless otherwise validated, reference curves should be prepared for each run
  - Laboratories participating in UK NEQAS surveys (2001) showed less variation and increased accuracy for FVIII values if reference curves were freshly prepared versus using curves stored in the instrument
Reference Curve Construction

A reference curve is constructed using an XY scatterplot that plots percent factor activity/dilution on the “x-axis” (logarithmic) versus clotting times in seconds on the “y-axis” (arithmetic).

Automated coagulation analyzers contain statistical packages that generate both a regression line (reference curve) and a regression equation:
- Regression equation expresses the algebraic relationship between the two variables, percent factor activity and clotting times.
- A line that slopes down (from top left to bottom right) is “negative” and by that indicates an inverse relationship between variables (factor activities decrease with increasing clotting times).
Slope of regression line impacts precision and linearity of FVIII assay

- Slope is calculated by the spread in seconds between serial dilutions (change in y to the change in x)
- Average difference in clotting times between serial dilutions should be at least 3 seconds for APTT-based assays (mean delta seconds in example is 6.1)
  - The greater the change in y in relation to x, the steeper the slope and the better the assay precision

Reference curve slopes remain fairly consistent from run to run (provided there is no significant change in instrument/reagent system)

- Can establish a mean and 95% confidence interval for determining acceptability

<table>
<thead>
<tr>
<th>Dilution (x)</th>
<th>% Activity (x)</th>
<th>Seconds (y)</th>
<th>Delta Seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5</td>
<td>184</td>
<td>35.6</td>
<td></td>
</tr>
<tr>
<td>1:10</td>
<td>92</td>
<td>41.4</td>
<td>5.8</td>
</tr>
<tr>
<td>1:20</td>
<td>46</td>
<td>48.1</td>
<td>6.7</td>
</tr>
<tr>
<td>1:40</td>
<td>23</td>
<td>53.8</td>
<td>6.7</td>
</tr>
<tr>
<td>1:80</td>
<td>11.5</td>
<td>60.5</td>
<td>6.7</td>
</tr>
<tr>
<td>1:160</td>
<td>5.75</td>
<td>65.7</td>
<td>5.2</td>
</tr>
<tr>
<td>1:320</td>
<td>2.875</td>
<td>71.0</td>
<td>5.3</td>
</tr>
<tr>
<td>1:640</td>
<td>1.4375</td>
<td>76.2</td>
<td>5.2</td>
</tr>
<tr>
<td>1:1280</td>
<td>0.71875</td>
<td>83.1</td>
<td>6.9</td>
</tr>
<tr>
<td>1:2560</td>
<td>0.359375</td>
<td>85.2</td>
<td>2.1</td>
</tr>
</tbody>
</table>
Regression line represents a visual summary of the regression equation and three curve types can be determined

- Most commonly used curve is \([\text{Log}(x), \text{Lin}(y)]\) (Graph A)
- Graph B shows a linear relationship between the two variables due to log transformation, \([\text{Log}(x), \text{Log}(y)]\), of data on the y-axis
- Graph C \([\text{Lin}(x), \text{Lin}(y)]\) is non-linear
**Criteria for Curve Acceptability**

- **Regression correlation coefficient (R^2)**
  - Measure of the proportion of variability explained by, or due to, the regression in a sample of paired data
  - Expectation is that R^2 values for factor assay curves should be greater than 0.99 (for 4 or more points)

- **Slope (m) of the regression line (reference curve)**
  - y=mLn(x) + b provides the slope (m) and y-intercept (b) of the regression line and from that the unknown value of x (factor activity) for a known y (clotting time in seconds) can be mathematically determined

- **Linearity**
  - Correlation exists between degree of FVIII depletion & levels of other clotting proteins in the deficient plasma (affects slope)
  - Linear range corresponds to areas of the curve that lie between high and low end where curves tend to flatten
Interpretation of Test Result

**Example**

Patient value is determined by interpolating time, 50.1 seconds using a 1:10 dilution, from the reference curve to yield a FVIII activity of 38%. This indicates that the patient’s FVIII activity level is only 38% that of normal activity.

<table>
<thead>
<tr>
<th>Reference Dilution</th>
<th>% FVIII</th>
<th>Seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>92</td>
<td>41.4</td>
</tr>
<tr>
<td>1:20</td>
<td>46</td>
<td>48.1</td>
</tr>
<tr>
<td>1:40</td>
<td>23</td>
<td>53.8</td>
</tr>
<tr>
<td>1:80</td>
<td>11.5</td>
<td>60.5</td>
</tr>
<tr>
<td>1:160</td>
<td>5.7</td>
<td>65.7</td>
</tr>
<tr>
<td>1:320</td>
<td>2.9</td>
<td>71.0</td>
</tr>
<tr>
<td>1:640</td>
<td>1.4</td>
<td>76.2</td>
</tr>
</tbody>
</table>

Reference curve

\[ y = -8.1803 \ln(x) + 79.324 \]

\[ R^2 = 0.9964 \]
Non-Parallelism Due to LA Effect

**Fundamental principle of bioassay:** if two substances are comparable, they should behave as if one were a simple dilution of the other (basis for parallel line assay).

**Reference Curve**

<table>
<thead>
<tr>
<th>Reference Dilution</th>
<th>% FVIII X (log)</th>
<th>Seconds Y (ln)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>92</td>
<td>41.4</td>
</tr>
<tr>
<td>1:20</td>
<td>46</td>
<td>48.1</td>
</tr>
<tr>
<td>1:40</td>
<td>23</td>
<td>53.8</td>
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<td>5.7</td>
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<tr>
<td>1:320</td>
<td>2.9</td>
<td>71.9</td>
</tr>
<tr>
<td>1:640</td>
<td>1.4</td>
<td>76.2</td>
</tr>
</tbody>
</table>

**LA Sample showing inhibitor effect**

Factor activity 1:40 > 1:20 > 1:10 dilution

- $11\% \times 1 \ (1:10) = 11\%$
- $18\% \times 2 \ (1:20) = 36\%$
- $15.8 \times 4 \ (1:40) = 63\%$
- $12.4\% \times 8 \ (1:80) = 99\%$

**Normal Sample (parallel to reference curve)**

<table>
<thead>
<tr>
<th>Sample Dilution</th>
<th>% FVIII</th>
<th>Seconds</th>
<th>Interpolated Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>118</td>
<td>40</td>
<td>118</td>
</tr>
<tr>
<td>1:20</td>
<td>51.3</td>
<td>47</td>
<td>103</td>
</tr>
<tr>
<td>1:40</td>
<td>28.4</td>
<td>52</td>
<td>114</td>
</tr>
<tr>
<td>1:80</td>
<td>12.4</td>
<td>59</td>
<td>99</td>
</tr>
</tbody>
</table>
Chromogenic FVIII Assays
Chromogenic Substrate Technology

Technology was developed in the 1970’s

Chromogenic substrates are synthetic peptides designed to mimic the natural substrate for a particular enzyme
- Attached to the peptide is a chemical group (chromophore) which, when cleaved by an enzyme, will be released and give rise to a color that is read by a spectrophotometer
  - Color is directly proportional to the proteolytic activity of the cleaving enzyme and is directly or indirectly proportional to the activity of the initial protein to be tested

Enzyme specificity and substrate selectivity are critical
- Specificity describes how restrictive an enzyme is for its choice of substrate
- Selectivity indicates the degree to which a substrate is bound to and cleaved by different enzymes
  - Synthetic substrates are smaller than their natural counterparts
FVIII Chromogenic Principle

Amount of color released at 405 nm (para-nitroaniline [pNA]) is directly proportional to the amount of FVIII activity present in patient plasma.
Assay Characteristics

- Uses relatively high sample dilutions
  - Minimizes interferences due to heparin, warfarin, or other anticoagulants such as the Lupus Anticoagulant
- Does not use FVIII deficient plasma
- Majority of assays commercially available use non-human coagulation factors
- Second stage of assay measures hydrolysis of chromogenic substrate
  - Rates vary between methods depending on the substrate that is used
- Reagent contains a thrombin inhibitor in order to prevent interference by the enzyme
- Assays can be performed using microtiter plates or used on automated coagulation analyzers
Two methods can be used to assess color appearance

- Kinetic method
  - Read change in absorbance at 405 nm for 30-120 seconds
- Endpoint method
  - Reaction is stopped with an acid and absorbance read at 405 nm against a buffer blank

Calibrator issues are similar to those for one-stage clot-based FVIII assay (see slide 22)

Two curves required

- Normal range
  - 0.05 – 1.50 IU/ml (5 – 150%)
- Low range
  - 0.005 – 0.05 IU/ml (0.5% - 5%)
Which Assay to Use (and for What Purpose)
Challenges

- Assigning potency for FVIII replacement therapies has an impact on manufacturers of therapeutic concentrates, physicians managing HA patients, and patients
- Is the potency designation meaningful for determining dose?
- Does dosage provide a satisfactory clinical outcome? (and a financial one?)
- Does FVIII activity, post-infusion, show a correlation between dosage and efficacy?
- What *in vitro* assay can reliably predict *in vivo* hemostatic efficacy which is the key measure used for dosing considerations?
Replacement Therapies

- One-stage and chromogenic FVIII assays show discrepant results when assessing potency of some FVIII concentrates
  - More apparent with products of higher purity such as full-length or truncated recombinant FVIII (rFVIII) concentrates
  - Various components of both assay systems and the concentrates themselves have been extensively studied to determine the cause

- Over-estimation of potency may lead to lower dosing and potential to adversely impact treatment

- Under-estimation of potency may lead to higher dosing than required for efficacy and give rise to higher costs
Recommendations for Manufacturers

**Method** recommendation for assignment of FVIII potency for high purity and recombinant FVIII concentrates

- Chromogenic method recommended by Factor VIII and FIX Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis in 1993
- Chromogenic method adopted as the European Pharmacopoeia reference method in 1995 (replaced two-stage FVIII assay)

**Calibrator** recommendation

- Use a concentrate standard (not plasma standard) that is calibrated against the WHO concentrate standard

**Predilution** of concentrates to 1 IU/mL (working solution for testing) with severe HA plasma (or FVIII deficient plasma)

- Plasmas must have a normal VWF level

**Assay buffers** for FVIII testing (regardless of assay) should contain 1% BSA (bovine serum albumin)
Monitoring Therapy

Which assay should be used to monitor FVIII replacement?
- One-stage FVIII assay appears to underestimate FVIII levels in post-infusion samples \((in vivo\) recovery) by 20 to 50% when compared to a chromogenic method
  - Largest discrepancy is with truncated rFVIII (B domain- deleted recombinant FVIII \([\text{BDD-rFVIII}], \text{ReFacto}^\circ\) )
- Use assay similar to that used by manufacturer to assign potency?
  - Recombinate® (Baxter) and Kogenate® (Bayer) use one-stage clotting assay whereas ReFacto® (Wyeth) uses chromogenic assay

Calibrator
- Should the concentrate given to a patient also be used as calibrator for FVIII assay, that is “like-to-like”?
  - Rationale is that post-infusion samples are “concentrates” diluted in “hemophiliac” plasma
- Use ReFacto Laboratory Standard (RLS) for post-infusion samples from patients receiving BDD-rFVIII
Questions for the Laboratory

- Which assay is more “physiological”?  
  - One study asserted that the one-stage assay is subject to lipid artifacts (APTT reagent phospholipid derived from animal sources binds FVIII with higher affinity), however different calibrators were used in comparison studies

- Which assay is more accurate?  
  - One study showed that an ELISA assay for FVIII antigen was in better agreement with the chromogenic method than the one-stage assay, however FVIII antigen units were assigned using same reference standards used to assign activity units

- Which assay has been more extensively studied and compared?  
  - PubMed yields only 53 articles (majority related to FVIII replacement therapy) since 1984 when using the search terms "Factor VIII" and "chromogenic assay"
Recommendations

- Use a calibrator that is traceable to the WHO plasma international standard
- For ReFacto®, use the RLS calibrator (which is traceable to the WHO plasma standard)
- Prepare reference curve with each assay run
- Minimize analytical variables as noted

Laboratorians, globally, have “voted” with their pipettes and laboratories have “voted” with their pocketbooks to use the one-stage clot-based FVIII assay because it is fairly simple to perform, widely used, and relatively inexpensive
Summary

- It’s all about the Calibrator!
  - The SSC of the ISTH, along with the WHO, US FDA, and European Pharmacopoeia will continue to address standardization of FVIII assays via international reference standards for plasma FVIII/VWF, FVIII:C concentrates, potentially a separate standard for recombinant concentrates, and a FVIII inhibitor antibody standard

- Efforts will continue by these organizations to ascertain the best methods for assigning potency to FVIII concentrates and monitoring of FVIII in post-infusion samples

- Beware…….testing for FVIII via thrombin generation assays is “waiting in the wings”!!

Barrowcliffe TW. Factor VIII and Factor IX Sub-Committee Recommendations for the Assay of High Purity Factor VIII Concentrates. Thromb Haemost 1993;70:876-7


