PRINCIPLE
FVIII inhibitors resulting from treatment of people with hemophilia with FVIII therapy are time-dependent.

If FVIII is added to plasma containing an inhibitor and the mixture incubated, the FVIII will be progressively neutralized. If the amount of FVIII and the incubation period are standardized, the strength of the inhibitor may be defined in units according to how much of the added FVIII is neutralized.

The assay can be performed using human or porcine FVIII.

The presence of an inhibitor might be suspected from a reduced half-life and recovery of FVIII.

BETHESDA ASSAY
The source of FVIII is a pooled normal plasma for anti-human titres; porcine concentrate diluted in FVIII-deficient plasma for anti-porcine titres.

A Bethesda unit is defined as the amount of inhibitor that will neutralize 50% of one unit of added FVIII in two hours at 37°C.

 Patients not expected to have an inhibitor:
1. Add equal parts (0.2 ml) patient plasma to pooled normal plasma.
2. For a control, add 0.2 ml 0% FVIII:C to 0.2 ml pooled normal plasma.
3. Incubate at 37°C for two hours. Then perform FVIII:C assay.

 Patients expected to have an inhibitor:
1. Prepare dilutions using FVIII assay buffer of the patient’s plasma. It is preferable to put up too many dilutions than too few. They can always be kept on ice after the two-hour incubation for a second run of assays. If previous information in relation to the expected inhibitor titre is available, dilutions for testing should span either side of this.
2. A standardized amount of FVIII in the form of a normal plasma pool is added to each dilution of test plasma — this will normally contain around 100 U/dl. Thus, each incubation mix has a starting concentration of approximately 50 U/dl. The precise concentration is not important, because the same source is added to all incubation mixtures.
3. In the assay of FVIII carried out after the two-hour incubation, the control mix of normal plasma and FVIII-deficient plasma is used as the standard reference, and the FVIII concentration of other mixtures is calculated against this. This material is used as the 100% reference in the assay.

It is important to buffer the normal plasma, either by the addition of 0.1M imidazole pH 7.4 in the recently described Nijmegen modification, or by use of the buffered pooled normal plasma described in Section 7. This improves the sensitivity and specificity of the assay. For anti-porcine titres, porcine plasma is not readily available. Therefore, it is acceptable to use porcine concentrate diluted to 1 U/ml in FVIII-deficient plasma.

4. At the end of the incubation period, the residual FVIII level is measured and the inhibitor calculated from a graph of residual FVIII vs. inhibitor units. (See Figure 34.1, on page 112.)

**REAGENTS/EQUIPMENT**

- Glyoxaline buffer (see Section 9)
- Owren’s buffered saline (see Section 9)
- Normal plasma pool (see Section 7)
  
  *Note: This must be buffered to improve the stability of FVIII during the two-hour incubation during the assay.*
- Porcine concentrate
- FVIII-deficient plasma
- APTT reagent
- Ice bath
- Plastic tubes (75 × 12 mm)

**METHOD**

**Human anti-FVIII**

1. Prepare doubling dilutions of test plasma in plastic tubes in 0.2 ml volumes, using glyoxaline buffer as diluent. The dilutions required for each patient will vary. A suggested starting point would be from undiluted, 1/2, 1/4, etc.

*Note: If the patient has had an inhibitor assay performed previously, the level may provide a rough guide as to which dilutions should be used. It is also useful to note if they have received any treatment with FVIII recently, as this may have either increased or reduced the inhibitor level.*
2 Pipette 0.2 ml FVIII-deficient plasma into another plastic tube. This will serve as the standard.

3 Add 0.2 ml normal plasma pool to both the standard tube and the test plasma dilutions. The FVIII level of all tubes will be approximately 50 U/ml. This is considered to be 100% in the FVIII assay at the end of the incubation.

4 Cap, mix, and incubate all tubes at 37°C for two hours.

5 At two hours, transfer all tubes to an ice bath unless the FVIII assay is performed immediately.

6 Perform a FVIII assay on all incubation mixtures by the usual FVIII assay method, but using the tube set up as standard as 100%. Suitable dilutions to use for the FVIII assays would be 1/5, 1/10, 1/20.

7 Read off the residual FVIII of each test mixture, using the control as 100%.

RESULTS/INTERPRETATION

The dilution of test plasma that gives a residual FVIII nearest to 50% but within the range 30%-60% is chosen for calculation of the inhibitor. Alternatively, calculate the result from each dilution and take the average. Any residual FVIII of <25% or >75% should not be used for calculations of inhibitor level.

A plot may be made of % residual FVIII versus inhibitor units on log-log paper from the definition of the inhibitor unit (see Figure 34.1).

Read off inhibitor level corresponding to residual FVIII for each test mixture and correct for dilution. For example:

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

NOTES

- Quantitative inhibitor assays are most frequently performed on test plasmas from patients with severe hemophilia, therefore containing little or no measurable factor VIII:C. If the test plasma contains more than 5 U/dl FVIII, this must be taken into account during the calculation of inhibitor titre.
This can be done in three ways:

- The first is to add more factor to the control mixture than to the test mixture to compensate for the FVIII in test mixture. For example, if the test plasma contains 20 U/dl FVIII, the control mixture is made from 120 µl normal plasma and 80 µl 0% FVIII. (Both test and control mixtures then contain approximately 60 U/dl FVIII at the start of the incubation phase.) This approach can only be used if patient plasma is tested undiluted, and not after the dilution, which would alter the initial FVIII concentrations.

- Alternatively, the initial FVIII level in the test plasma can be taken into account during the calculation. In this case, the assay mixtures are constructed in the normal way.

- Another option is to heat the test plasma at 58°C for 90 minutes prior to analysis, which will destroy all the clotting factors, including FVIII. Since immunoglobulins are heat resistant, the inhibitor titre will be unaffected by this treatment.

- The FVIII-deficient plasma used in the construction of the control mixture is important. This should contain normal levels of VWF, since it has been shown that inhibitor titres are 30%–50% lower if the FVIII-deficient plasma does not contain VWF (Verbruggen et al. 2001).

**Example 1: Patient with 20 U/dl FVIII**

Control pooled normal plasma + 0% FVIII

a. Pooled normal plasma + test plasma undiluted – initial FVIII = 120%
b. Pooled normal plasma + test plasma diluted 1 in 2 – initial FVIII = 110%

After two-hour incubation, factor assays are performed.

a. Pooled normal plasma + test plasma undiluted – FVIII = 120% of control
   Residual FVIII: 120/120 = 100%; inhibitor result: negative
b. Pooled normal plasma + test plasma diluted 1 in 2 – FVIII = 110% of control
   Residual FVIII: 110/110 = 100%; inhibitor result: negative

**Example 2: Patient with 20 U/dl FVIII (and inhibitor present)**

Patient plasma tested undiluted

Pooled normal plasma + test plasma undiluted – initial FVIII = 120% of control

After two-hour incubation, FVIII assays are performed.

Pooled normal plasma + test plasma undiluted – FVIII = 90% of control

Residual FVIII = 90/120 = 75%
Porcine Anti-FVIII

The method for porcine FVIII inhibitors is essentially the same as for human inhibitors, except in the source of FVIII. This should be of porcine origin, but to date a reliable source of plasma porcine FVIII is not widely available. Therefore, porcine concentrate diluted in hemophilic plasma (human) to a concentration of 1 U/ml is used.

Note: If the residual FVIII is between 80% and 100% for a sample that was incubated undiluted with the porcine concentrate, it is interpreted as no inhibitor being present. Although plasma-derived porcine FVIII is not currently in use, a recombinant porcine FVIII concentrate is under development at the time of writing.

Figure 34.1. Relationship between residual FVIII and inhibitor titre

REFERENCES

