Chapter 24

Nijmegen-Bethesda Assay to Measure Factor VIII Inhibitors

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Abstract

Hemophilia A is an inherited bleeding disorder caused by a deficiency of factor VIII coagulant activity (FVIII:C). Patients are treated with infusions of either plasma-derived or recombinant factor VIII. However, some patients develop inhibitory antibodies (inhibitors) to infused factor VIII which render it ineffective. The original Bethesda method was developed to standardize measurement of inhibitors in a factor VIII neutralization assay. One Bethesda unit is defined as that amount of inhibitor that results in 50% residual FVIII:C activity of a defined test mixture. In the Nijmegen modification of the original Bethesda method, the pH and the protein concentration of the test mixture is further standardized. As a result, the FVIII:C in the test mixture is less prone to artifactual deterioration and the test has improved specificity. Even with a standardized procedure a number of factors can affect the performance of the test and it is important for laboratory staff to be aware of their impact on the result outcome.

Key words Factor VIII, Hemophilia, Coagulation, Inhibitor

1 Introduction

Hemophilia A is a sex-linked hereditary bleeding disorder characterized by a reduced level of factor VIII (FVIII) in blood. Bleeding episodes may occur spontaneously in the severe form of hemophilia or after trauma in the milder forms. Treatment for children and adults is available with various recombinant FVIII products and plasma-derived FVIII concentrate. However, treatment may cause inhibitory antibodies (inhibitors) to form against FVIII and the reported incidence ranges from 0 to 33% of patients (1–3). The inhibitors may be transient or persistent and in the latter case they become a lifelong problem. This complication most often develops in childhood and then it usually occurs after the first 10–20 days of treatment with FVIII. However inhibitors may develop later in life, for example in mild hemophilia patients who have had little or no previous exposure to FVIII treatment (4, 5), or in rare cases following a change in product formulation (6). Infused FVIII is relatively ineffective when given to hemophilia A patients with an inhibitor. This group of patients is very difficult to treat and they

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have a high rate of morbidity and mortality. Immune tolerance regimens have been developed to abolish factor VIII and IX inhibitors in up to 80% of selected patients, with best success rates if the program is started early and when the inhibitor titre is <10 Bethesda units/mL (BU/mL) (7). For those patients with persistent inhibitors >5 BU/mL, treatment with activated clotting factors (e.g., recombinant factor VIIa, activated prothrombin complex concentrates) has become the mainstay of therapy (7).

Acquired hemophilia is a serious bleeding disorder in which autoantibodies to FVIII develop in previously normal individuals and neutralize most of their available FVIII. The frequency of this condition is 1–2 per million per year. Treatment for bleeding is as for a patient with inherited hemophilia and an inhibitor. In addition, immunosuppressive treatment is used to suppress production of the antibody (7).

The detection and measurement of FVIII inhibitors are thus an important role of the haemostasis laboratory. It is necessary to diagnose inhibitors when they first arise and to measure the inhibitor concentration. Then it may be necessary to monitor the level and determine whether the inhibitor is persistent or transient (7, 8). For those patients receiving immune modulation therapy it is necessary to monitor inhibitor levels during and after treatment.

In this chapter we describe the original Bethesda method (9), with the Nijmegen modification proposed by Verbruggen et al. (10). The Scientific Standardization Committee of the International Society on Thrombosis and Haemostasis recommend the Bethesda assay with the Nijmegen modification for inhibitor measurement (10, 11). We will mainly focus on measurement of FVIII inhibitors. However, the procedure may be modified to quantitate inhibitors to factor IX or any other clotting factor measured using a functional assay, and this application will be briefly described (12–15).

In the original Bethesda method (9), test plasma is mixed with an equal volume of a normal plasma pool (NPP) and incubated for 2 h at 37°C. A control mixture is prepared by incubating an equal volume of the NPP with imidazole buffer. After 2 h the factor VIII coagulant activity (FVIII:C) of each mixture is measured using the one-stage assay. The FVIII:C of the test mixture is compared with that of the control and the percentage of residual FVIII:C is calculated. One Bethesda unit (BU) is defined as that amount of inhibitor that results in 50% residual FVIII:C activity. The BU/mL in the sample is determined from the theoretical inhibitor graph by interpolating the percentage residual activity against Bethesda units. Only percentage residual FVIII:C between 25 and 75% can be used to determine the inhibitor level. For an inhibitor >2 BU/mL, dilutions of patient plasma are also tested and the result corrected for the dilution factor.

The Nijmegen modification of the original Bethesda method (referred to here as the Nijmegen-Bethesda method) was developed to address two shortfalls of the assay and improve specificity (10). Firstly, the NPP used in the test and control mixtures is buffered to pH 7.4 using 0.1 M imidazole (final concentration). This is to maintain the test and control mixtures at pH 7.4 over the 2 h incubation period. Secondly, a source of immuno-depleted FVIII-deficient plasma is used in place of imidazole buffer to dilute NPP in the control mixture and to dilute patient samples. This maintains a similar protein concentration in control and test incubations. Together these modifications improve the stability of FVIII in the incubation mixtures and prevent artifactual deterioration of FVIII:C that may be falsely interpreted as being due to an inhibitor (10, 11). As in the original Bethesda method, in the Nijmegen modified method one Bethesda unit (BU) is defined as that amount of inhibitor that results in 50% residual FVIII:C activity (see Note 7) (10).

2 Materials

2.1 Nijmegen-Bethesda Assay

1. Buffered normal plasma pool (B-NPP).
   Commercial sources of buffered citrated plasma prepared from a pool of normal donors are available. It is essential to check the product information sheet to ensure that the lyophilized product is buffered and maintains a pH 7.4. Reconstitute according to the manufacturer's directions.

   To prepare the B-NPP in-house, collect blood from at least 20 normal donors and prepare plasma as described in Subheading 3.1. Screen individual plasma samples to ensure normal activated partial thromboplastin time, prothrombin time, and dilute Russell's viper venom time. Using polypropylene plastic containers throughout, pool the plasma, mix well, and measure the volume obtained. While stirring, add solid imidazole to achieve a final concentration of 0.1 M and then adjust the pH to 7.4 by slow addition of 1N HCl (10, 11). Dispense into aliquots of appropriate volume and freeze at −70°C until required. For safety reasons it is recommended that the B-NPP is checked for viral status and only used if negative for blood borne viruses, e.g., hepatitis B and C and HIV.

   An alternative method can be used when smaller volumes of B-NPP are processed. Prepare a 5.0 M imidazole solution and add 20 μL/mL of plasma while mixing well. Follow by immediately adjusting pH to 7.4 with 1N HCl (approximately 28 μL 1N HCl/mL plasma). This method is suitable to use with NPP that have been previously prepared and frozen for other purposes, and then required for use in an inhibitor assay.

2. Factor VIII-deficient plasma.
   Select a commercial source of FVIII-deficient plasma that is either immune-depleted or prepared from patients with severe hemophilia A. Reconstitute according to the manufacturer's directions. For many products it is possible to freeze any left-over plasma at −70°C for use in later inhibitor assays. When using an immuno-depleted FVIII-deficient plasma it is preferable to use one that contains relatively normal levels of von Willebrand factor (see Notes 16 and 17).

   Alternatively, the laboratory may prepare citrated plasma from a patient with severe hemophilia A, without an inhibitor, to use as the FVIII-deficient plasma. However the FVIII level must be <1 IU/dL which precludes donor patients who have received recent FVIII treatment. Also the viral status of the donor patient should be known to the user.
3. Quality control.
   In the absence of a commercially available inhibitor plasma, an in-house source from a patient with a FVIII inhibitor (inherited hemophilia or acquired) may be prepared and stored as described in Subheading 3.1.

   Another quality issue is the need for a program in the laboratory to ensure correct calibration of equipment. This includes regular maintenance of analyzers, pipette volume calibration, water bath temperature set at 37°C, and a timer available to ensure adherence to the 2 h incubation period.

2.2 Factor VIII 1-Stage Assay

Materials required are those that are used by the laboratory for the routine FVIII:C assay. They include FVIII-deficient plasma (see Note 16), APTT reagent, assay buffer, calibrator, and QC material (see Note 9). In the original Bethesda method the one-stage FVIII:C assay was recommended but a chromogenic assay can also be used and in some cases is preferred (see Note 19).

3 Methods

3.1 Preparation of Samples

1. The test requires a minimum of approximately 3 mL citrated whole blood to yield a 1.5 mL plasma sample. Collect blood into citrate anticoagulant concentration 0.105 or 0.129 M with a blood:citrate ratio of 9:1. In some circumstances, e.g., for children, a smaller sample can be used only if blood has been collected in the correct blood:citrate ratio and the extent of testing may be limited.
2. If the patient is also to be given an infusion of FVIII collect the sample before the infusion (see Note 14).
3. On receipt of sample in the laboratory, centrifuge specimen for at least 15 min at minimum of $2{,}000 \times g$, at 15–20°C. Remove plasma, taking care not to contaminate with platelet pellet, and

aliquot into approximately 0.5–1.0 mL volumes. Label adequately and store plasma at −70°C until required.

4. Samples may be used fresh or frozen. If frozen, thaw samples at 37°C for approximately 5–10 min, then mix by gentle inversion.
5. The calculations used in this assay are based on the assumption that there is no FVIII:C in the patient sample. In samples thought to contain low residual levels of FVIII this can be inactivated by heating approximately 1 mL of plasma in a small tube at 56°C for 10 min (16, 17). This inactivates FVIII present in the sample, but leaves the antibody and its inhibitory activity intact. Recent data shows complete inactivation of all other coagulation factors, even when the starting activity is 1.00 I.U./mL (or 100%) with a 90 min incubation at 58°C (18).
6. After heating, spin the sample for 1–2 min in a micro-centrifuge at full speed (approximately 20,000 × *g*) to remove any precipitated protein. Alternatively, spin for 15 min at 2,000 × *g* as above. Transfer supernatant to a fresh labelled tube, clearly stating "heat inactivated." Store at −70°C to await testing if required. This process does not apply to inhibitor detection for other factors as it does not adequately inactivate factors apart from FVIII. Other approaches are required for those factors (see Subheading 3.6).

3.2 Nijmegen-Bethesda Assay

1. For most automated analyzers a mix of equal volumes of 300 μL to yield a 600 μL final volume for all incubations will provide a sufficient final volume for analysis. This can be adjusted as required.
2. To prepare the control tube, mix an equal volume of VIII-deficient plasma (VIII-def) with B-NPP (see Note 1).
3. A full-strength test and a 1/2 dilution is recommended as a starting point for all patients previously negative for an inhibitor or those with an unknown history.
4. To test a patient sample at full strength, mix an equal volume of test plasma with B-NPP.
5. To test at 1/2, dilute test plasma 1/2 in VIII-def plasma. Then mix an equal volume of this with B-PN.
6. If a high titre of inhibitor is present, 1/5, 1/10, or higher dilutions may be required. These are also prepared by dilution of the test plasma in VIII-def plasma. Then mix with an equal volume of B-PN.
7. For very high levels of inhibitors prepare 1/10 dilution of patient plasma in FVIII-def plasma and then dilute this further to give 1/20, 1/50, and 1/100. Patient plasma can be diluted further as required.

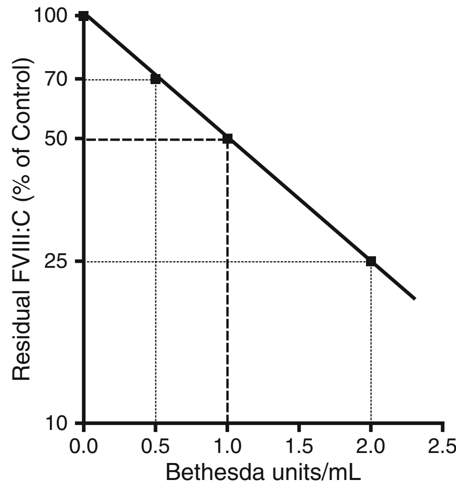

Fig. 1 Inhibitor graph to relate the percent Residual FVIII:C to the Bethesda Units/mL in test plasma

8. Prepare the mixtures in small tubes, cap and incubate at 37°C for 2 h.
9. After 2 h, measure the residual FVIII:C in the test and control mixtures using the routine method in use in your laboratory.
10. Calculate the ratio of residual factor VIII:

$$\text{Residual_FVIII_ratio} = \left(\frac{\text{FVIII : C_patient_mixture}}{\text{FVIII : C_control_mixture}} \right)$$

11. Convert the ratio to a percentage by multiplying by 100.
12. If the result falls between 25 and 75% residual FVIII:C, the BU/mL in the sample is determined from the theoretical inhibitor graph by interpolating the percentage residual activity against Bethesda units (Fig. 1).
13. Incubation mixtures with >75% residual FVIII:C have no detectable inhibitor, i.e., <0.5 BU/mL (see Note 2).
14. Incubation mixtures with high levels of inhibitor and <25% residual FVIII:C should have the test repeated using appropriate dilutions to lie in the 25–75% range. Multiply the answer by the dilution factor used in that mixture and average the results obtained for each valid dilution (see Notes 3–6).
15. If the inhibitor has type II kinetics it may not be possible to average a range of dilutions due to high variation of results. In this case take the lowest dilution of patient plasma to inactivate close to 50% of the FVIII in the incubation mixture, and calculate the BU/mL from this mixture (10, 19, 20).

16. One Bethesda unit/mL (BU/mL) is defined as the amount of inhibitor that causes a 50% reduction in the FVIII supplied in the control mixture (see Note 7) (9, 10).

3.3 Factor VIII Assay

Follow the usual protocol for FVIII:C assay in use in your laboratory. Generally this will be the one-stage FVIII:C assay although a chromogenic method can be used.

3.4 Internal Quality Control

1. Follow the usual quality control procedures for your laboratory for the factor VIII:C assay (see Note 9).
2. The buffered normal plasma, mixed with an equal volume of FVIII-def, should assay at approximately half of its pre-dilution value, within limits of ±10% (see Note 1).
3. If a source of sufficient patient plasma with an inhibitor is available, an internal inhibitor quality control material can be established (see Note 9). This is advisable to establish the imprecision of the assay and also to alert the laboratory of potential assay problems.

3.5 Measurement of Other Factor Inhibitors

The original Bethesda assay and the modified Nijmegen-Bethesda assay are not only applicable to measurement to FVIII inhibitors but can be used to measure inhibitors to other coagulation factors (12–15). Essentially, the test is the same as described above except that the sample is diluted in plasma deficient in the factor of interest. Also, the one-stage assay for the factor of interest is used to measure the percentage of residual factor.

3.6 Samples with Intrinsic Residual Activity of Factor of Interest

If the patient sample has only a mild or moderate reduction in factor level, it cannot be taken that the factor-deficient plasma mixed with an equal volume of B-NPP is the appropriate control for the mixture comprising patient plasma mixed with an equal volume of B-NPP. The factor activity in the test mixture must be corrected for the intrinsic residual activity (10).

1. In the case of FVIII inhibitors and low levels of FVIII:C in the patient sample, FVIII:C can be inactivated by heating the sample at 56°C for 10 min (16, 17) (see step 5 in Subheading 3.1).
2. In the case of specific inhibitors to other coagulation factors and where there are measurable levels of the factor of interest, full inactivation of all coagulation factors is achieved with a 90 min incubation at 58°C even when the starting activity is 1.00 I.U./mL (or 100%) (18).
3. A mathematical approach can be taken to the calculate percentage residual factor. Measure the factor level in the B-NPP and the patient sample before the incubation mixtures are set up. From this information calculate the theoretical factor level in the various mixtures of patient sample in the absence of an inhibitor.

This theoretical value is compared to the factor level after 2 h incubation to determine the percentage residual factor, instead of the value in the control mixture. For example, when testing a full-strength plasma containing 20 U/dL factor IX mixed in equal volume with B-NPP containing 100 U/dL factor IX, the final expected factor IX concentration is 60 U/dL. If, after 2 h incubation, the factor IX level in the mixture is 40 U/dL, this corresponds to 67% residual and approximately 0.55 BU/mL.

4 Notes

1. When testing samples from a number of patients in one batch assay a separate control mixture with every set of patient dilutions and use in the calculation for that patient. Each normal control, when mixed with an equal volume of FVIII-def, should contain approximately half of its starting FVIII:C value.
2. There is a lack of consensus between laboratories on the assay lower limit cutoff for the Nijmegen-Bethesda assay (8), with lower limits between 0.2 and 0.6 BU/mL reported in the literature. A widely used cutoff for a negative result is 0.5 BU/mL (11) which corresponds to 70% residual FVIII:C for full-strength plasma (0.4 BU/mL corresponds to 75% residual FVIII:C). The cutoff value will be influenced by the precision of the procedure (including preparation of incubation mixtures and FVIII:C assay) and should be assessed by each laboratory.
3. Where an inhibitor is detected, the final result is only valid if calculated from a percentage residual FVIII:C between 25 and 75%. If the residual FVIII:C is less than 25% the sample should be further diluted as required and retested. The inhibitor units read from the theoretical inhibitor graph should be multiplied by the dilution factor to obtain the final BU/mL (9, 10).
4. In the majority of patients with inherited hemophilia A and a FVIII inhibitor, the inhibitor shows type I kinetics, i.e., complete and irreversible binding of the antibody to FVIII. In these samples there is a linear correlation between the concentration of inhibitor and the logarithm of the percent residual FVIII:C (10, 21). Also, full-strength plasma or low dilutions of plasma will completely neutralize the added FVIII:C (21).
5. For patients with acquired FVIII Inhibitors and some hemophilia patients the sample dilutions may not show a linear correlation between concentration and percent residual FVIII:C (10, 19, 21). This is due to the type II kinetics, i.e., incomplete and reversible binding of the antibody to FVIII. In this situation, the least dilution of plasma giving close to 50% residual FVIII:C is taken to be the best estimate of the inhibitor level (10, 19). Alternatively an empirical estimation of inhibitor concentration can be used (see Note 6).

6. In another approach to calculation of results, Verbruggen et al. (10) have described an empirical method to calculate the inhibitor level for samples with >2 BU/mL. It is necessary to test a number of dilutions of the sample and prepare a log-log plot of the reciprocal of the plasma dilution (*x*-axis) against percent residual FVIII (*y*-axis). Where possible use at least four mixtures covering the range 25–75% residual FVIII:C. To determine the inhibitor level in the sample, draw a line of best fit and interpolate to the dilution factor that gives 50% residual FVIII:C—this is the BU/mL (10, 22).
7. The units used to report the Nijmegen-Bethesda assay are the same as those used for the original Bethesda method, i.e., Bethesda units/mL (BU/mL) (10). However some workers identify their use of the modified assay by reporting their results as Nijmegen-Bethesda units/mL (NBU/mL) (23, 24). Assay results and the units reported for both methods are calculated in the same way.
8. A FVIII inhibitor assay standard is under development. The aim of this standard material is to improve assay agreement between laboratories (8).
9. Internal quality control for the Nijmegen-Bethesda assay relies heavily on the quality control for the FVIII:C assay used in the measurement phase. A laboratory with access to a sufficient quantity of plasma from a patient with a FVIII inhibitor may be able to prepare in-house quality control material.
10. External quality assurance programs (QAP) for FVIII inhibitors are an ongoing challenge for organizing groups due to the need to source sufficient amounts of appropriate material. However such surveys are very instructive and help to identify assay variables which influence the result (25, 26). All laboratories performing the Nijmegen-Bethesda assay or original Bethesda assay are encouraged to enrol in a survey offering external quality control for this test, e.g., the ECAT Foundation External Quality Assessment Programme in Haemostasis and Thrombosis.
11. There is a high degree of variability between laboratories for results assayed by the Nijmegen-Bethesda method and also the original Bethesda method. In one report the Nijmegen-Bethesda method showed between-laboratory variation of 10–15% compared to variation of 13–33% for the original Bethesda method (25). We have reviewed external QAP reports received in our laboratory and observed much higher variation, with CVs from 20 to 80% for the original Bethesda method and with only a small improvement in CV shown for the Nijmegen modification.
12. The Nijmegen modification of the original Bethesda method improves the specificity of the procedure, such that a lesser

number of false-positive results in the cutoff range of 0.5 BU/mL are reported (11). However, both the original Bethesda method and the Nijmegen modified method might not detect some weak, but clinically significant inhibitors in patients with hemophilia (25, 27, 28).

13. Non-neutralizing antibodies will not be detected by the Bethesda-Nijmegen assay (17, 29). The incidence and clinical importance of such antibodies is uncertain (8, 17).
14. A FVIII inhibitor bound in an immune complex with FVIII will not be detected by the Bethesda-Nijmegen assay (30). Inhibitor detection after infusion of FVIII therapy in patients known or suspected to have an inhibitor may be unsuccessful.
15. The designation of Bethesda units in an inhibitor plasma does not imply that any specific number of factor VIII units infused into the patient would neutralize the circulating inhibitor (9).
16. The type and source of FVIII-deficient plasma used in the procedure can influence inhibitor assay results. Use of a FVIII-deficient plasma that contains relatively normal levels of von Willebrand factor is recommended (23). Also, use of the same FVIII-deficient plasma for both the sample dilution and FVIII:C assay is preferred (23). FVIII-deficient plasma prepared by chemical depletion should be avoided (11, 23).
17. Speculation has been raised that FVIII-deficient plasma prepared using immuno-depletion column technology may, on occasions, be contaminated with anti-FVIII monoclonal antibodies (23). These contaminating antibodies may cause a lower than usual FVIII:C level in the normal plasma control and decrease the inhibitor level determined (23). Inhibitor assessment of the FVIII-deficient plasma used in a procedure may be helpful for quality control or troubleshooting purposes.
18. To reduce the cost of the Nijmegen-Bethesda assay, a 4% bovine-serum albumin buffer can be used to dilute patient samples, instead of factor-deficient plasma (24). This modification maintains similar specificity to the method when factor-deficient plasma is used. However, it is unclear whether this modification causes a loss of the previous gain in performance from the presence of von Willebrand factor when FVIII-deficient plasma is used as the diluent (23).
19. Lupus anticoagulants (LA) may cause a false-positive result as they inhibit the phospholipid-dependent clotting pathway and cause prolongation of assay clotting times. LA can be excluded if the patient has a normal dilute Russell's viper venom clotting time. A coexisting LA and FVIII inhibitor may be suspected if, for example, the patient is positive for LA with a bleeding phenotype and has a FVIII:C that is substantially reduced compared to other clotting factor levels. A chromogenic FVIII

method can be used for the Bethesda assay as this method is not affected by LA and has been shown to be more specific (31). Also, a commercial ELISA developed to detect FVIII inhibitors can help to discriminate between these and a lupus anticoagulant or other factor inhibitors (22).

20. Heparin may cause a false-positive result or falsely elevates the inhibitor level due to inhibition of APTT-dependent tests (26, 32). Blood collected through a port or catheter line is at risk of heparin contamination. Performing a thrombin time (TT) using routine methods can be helpful. A TT within normal limits can exclude the presence of heparin, whereas a prolonged TT is suggestive of heparin, raised D-dimers, or an impairment of fibrinogen conversion to fibrin (e.g., low or dysfunctional fibrinogen, antibody interference at this step). A heparin assay using routine anti-Xa methods can both detect heparin and measure the concentration. If it is necessary to test a sample containing heparin, the sample can be pre-treated with heparinase (32).
21. Recombinant VIIa does not interfere with the determination of inhibitor level for factors VIII, IX, or XI in patients receiving treatment with this product. However, the measurement of inhibitors to factors II, V, VII, or X is unreliable in samples containing recombinant VIIa (33).
22. Recombinant porcine FVIII, for treatment of patients with inhibitors, is undergoing clinical trial (34). In future, laboratories may be required to check the cross-reactivity of inhibitors to porcine FVIII before a patient receives this form of therapy. This requires a source of porcine FVIII in the assay incubation mixture (16, 34, 35).

References

1. Ehrenforth S, Kreuz W, Scharrer I, Linde R, Funk M, Gungor T, Krackhardt B, Kornhuber B (1992) Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. Lancet 339:594–598
2. Scharrer I, Neutzling O (1993) Incidence of inhibitors in haemophiliacs. A review of the literature. Blood Coagul Fibrinolysis 4: 753–758
3. Wright J, Paisley S (2003) The epidemiology of inhibitors in haemophilia A: a systematic review. Haemophilia 9:418–435
4. Hay CRM (1998) Factor VIII inhibitors in mild and moderate-severity haemophilia A. Haemophilia 4:558–563
5. Oldenburg J, El-Maarri O, Schwaab R (2002) Inhibitor development in correlation to factor VIII genotypes. Haemophilia 8(Suppl 2):23–29
6. Peerlink K, Hermans C (2006) Epidemiology of inhibitor formation with recombinant factor VIII replacement therapy. Haemophilia 12:579–590
7. Hay CRM, Brown S, Collins PW, Keeling DM, Liesner R (2006) The diagnosis and management of factor VIII and IX inhibitors: a guideline from the United Kingdom Haemophilia Centre Doctors Organization. Brit J Haematol 133:591–605
8. Dimichele DM (2006) Inhibitor treatment in haemophilias A and B: inhibitor diagnosis. Haemophilia 12(Suppl 6):37–42
9. Kasper CK, Aledort LM, Counts RB, Edson JR, Frantantoni J, Green D et al (1975) A more uniform measurement of factor VIII inhibitors. Thrombos Diath Haemorrh 34:869–872
10. Verbruggen B, Novakova I, Wessels H, Boezeman J, van den Berg M, Mauser-

Bunschoten E (1995) The Nijmegen modification of the Bethesda assay for factor VIII:C inhibitors: improved specificity and reliability. Thromb Haemost 73:247–251

11. Giles AR, Verbruggen B, Rivard GE, Teitel J, Walker I, and the Association of Haemophilia Centre Directors of Canada (1998) A detailed comparison of the performance of the standard versus the Nijmegen modification of the Bethesda assay in detecting factor VIII:C inhibitors in the haemophilia A population in Canada. Thromb Haemost 79:872–875
12. Gadarowski JJ, Czapek EE, Ontiveros JD, Pedraza JL (1988) Modification of the Bethesda assay for factor VIII and IX inhibitors to improve efficiency. Acta Haematol 80:134–138
13. Hay CRM, Baglin TP, Collins PW, Hill FGH, Keeling DM (2000) The diagnosis and management of factor VIII and IX inhibitors: a guideline from the UK Haemophilia Centre Doctors' Organization (UKHCDO). Brit J Haematol 111:78–90
14. Salomon O, Zivelin A, Livnat T, Dardik R, Loewenthal R, Avishai O et al (2003) Prevalence, causes, and characterization of factor XI inhibitors in patients with inherited factor XI deficiency. Blood 101:4783–4788
15. Favaloro EJ, Posen J, Ramakrishna R, Soltani S, McRae S, Just S et al (2004) Factor V inhibitors: rare or not so uncommon? A multi-laboratory investigation. Blood Coagul Fibrinolysis 15:637–647
16. Gatti L, Mannucci PM (1984) Use of porcine factor VIII in the management of seventeen patients with factor VIII antibodies. Thromb Haemost 51:379–384
17. Ling M, Duncan EM, Rodgers SE, Street AM, Lloyd JV (2003) Low detection rate of antibodies to non-functional epitopes on factor VIII in patients with haemophilia A and negative for inhibitors by Bethesda assay. J Thromb Haemost 1:2548–2553
18. Verbruggen B, Novakova I, van Heerde W (2009) Detecting and quantifying functional inhibitors in haemostasis. In: Kitchen S, Olson JD, Preston FE (eds) Quality in laboratory haemostasis and thrombosis, 1st edn. Blackwell Publishing, Oxford, Chapter 20
19. Kasper CK (1995) Laboratory diagnosis of factor VIII inhibitors. In: Kessler C, Garvey MB, Green D, Kasper C, Lusher J (eds) Acquired haemophilia, 2nd edn. Excerpta Medica, Princeton, NJ, Chapter 2
20. Goldsmith J (1993) Diagnosis of factor VIII versus nonspecific inhibitors. Semin Haematol 30(Suppl 1):3–6
21. Ling M, Duncan EM, Rodgers SE, Somogyi AA, Crabb GA, Street AM, Lloyd JV (2001) Classification of the kinetics of factor VIII inhibitors in haemophilia A: plasma dilution studies are more discriminatory than time-course studies. Brit J Haematol 114:861–867
22. Sahud MA (2000) Laboratory diagnosis of inhibitors. Semin Thromb Hemostas 26:195–203
23. Verbruggen B, Giles A, Samis J, Verbeek K, Mensink E, Novakova I (2001) The type of factor deficient plasma used influences the performance of the Nijmegen modification of the Bethesda assay for factor VIII:C inhibitors. Thromb Haemost 86:1435–1439
24. Verbruggen B, van Heerde W, Novakova I, Lillicrap D, Giles A (2002) A 4% solution of bovine serum albumin may be used in the place of factor VIII:C deficient plasma in the control sample in the Nijmegen modification of the Bethesda factor VIII:C inhibitor assay. Thromb Haemost 88:362–364
25. Reber G, Aurousseau MH, Dreyfus M, Delahousse B, Caron C, Trzeciack MC et al (1999) Inter-laboratory variability of the measurement of low titer factor VIII:C inhibitor in haemophiliacs: improvement by the Nijmegen modification of the Bethesda assay and the use of common lyophilized plasmas. Haemophilia 5:292–293
26. Favaloro EJ, Bonar R, Duncan E, Earl G, Low J, Aboud M et al (2006) Identification of factor inhibitors by diagnostic haemostasis laboratories: a large multi-centre evaluation. Thromb Haemost 96:73–78
27. Ewing NP, Kasper CK (1982) In Vitro detection of mild inhibitors to factor VIII in hemophilia. Am J Clin Pathol 77:749–752
28. Keeling D, Beavis J, Sukhu K (2005) A simple inhibitor screen is more sensitive than a Bethesda assay in monitoring for the development of inhibitors in haemophilia A and B. Brit J Haematol 128:885
29. Dazzi F, Tison T, Vianello F, Radossi P, Zerbinati P, Carraro P et al (1996) High incidence of anti-FVIII antibodies against non-coagulant epitopes in haemophilia A patients: a possible role for the half-life of transfused FVIII. Brit J Haematol 93:688–693
30. White GC (1994) Factor VIII inhibitor assay: quantitative and qualitative assay limitations and development needs. Semin Haematol 31(Suppl 4):6–10
31. Blanco AN, Peirano AA, Grosso SH, Gennari LC, Bianco RP, Lazzari MA (2002) A chromogenic substrate method for detecting and titrating anti-factor VIII antibodies in the presence of lupus anticoagulant. Haematologica 87:271–278
32. Manco-Johnson MJ, Nuss R, Jacobson LJ (2000) Heparin neutralization is essential for

accurate measurement of factor VIII activity and inhibitor assays in blood samples drawn from implanted venous access devices. J Lab Clin Med 136:74–79

33. Keeney M, Allan DS, Lohmann RC, Chin Yee IH (2005) Effect of recombinant human factor 7 (Niastase) on laboratory testing of inhibitors of factors VIII and IX. Lab Haematol 11:118–123
34. Barrow RT, Lollar P (2006) Neutralization of antifactor VIII inhibitors by recombinant porcine factor VIII. J Thromb Haemost 4:2223–2229
35. Lloyd JV, Duncan EM, Street AM, Maxwell EL, Berry E, Rowell J et al (1997) Cross-reactivity to porcine factor VIII of factor VIII inhibitors in patients with haemophilia in Australia and New Zealand. Aust N Z J Med 27:658–664