PART 7 Detecting and Quantifying Congenital and Acquired Functional Inhibitors in Hemostasis

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TOPICS COVERED

- ✓ Different Antibody Kinetics
- ✓ Samples for the FVIII/FIX Inhibitor Test: Collection, Dispatch, and Preparation
- ✓ Normal Pool Plasma for Inhibitor Testing
- ✓ Protocol for the FVIII and FIX Inhibitor Test
- ✓ von Willebrand Factor Inhibitors Assays
- ✓ Anti-Drug Antibody (ADA) in the Coagulation Laboratory - Emicizumab

Identifying the development of antibodies is vital if the hemophilia treatment program is to be able to provide adequate medical care (Peyvandi et al, 2016; Srivastava et al, 2020). In the context of hemophilia, inhibitors are polyclonal IgG antibodies to FVIII or FIX and mostly of the high-affinity IgG4 subclass (Montalvão et al, 2015). Inhibitors neutralize the factor concentrate administered to the patient, making it difficult to prevent and treat bleeding (Pratt et al, 2021). The appearance of inhibitors is the result of a multi-step process involving environmental and genetic determinants. In severe hemophilia A, FVIII inhibitors form in approximately 30% of patients, usually during the first 20–30 days of exposure. In severe hemophilia B, the cumulative incidence of inhibitor development is lower than in severe hemophilia A and reaches 4–5% 9–11 days after exposure (Ljung et al, 2019). The treatment of acute bleeding in patients with inhibitors depends on the titer of the inhibitor. Patients with a low inhibitor titer (<5 UB/ml) can be treated with standard replacement therapy, factor concentrate, although it requires higher doses to overcome the neutralizing effects of the inhibitor. For patients with high titer inhibitors (>5 UB/ml), the only effective therapies for treating bleeding are bypassing agents (Ljung et al, 2019). The three available bypassing agents used in hemophilia A and B are (1) activated prothrombin complex concentrate (aPCC), (2) two forms of recombinant activated FVII (rFVIIa), and (3) recombinant porcine FVIII. Newly developed hemostatic drugs, such as humanized bispecific antibodies (e.g. emicizumab), RNA interference (e.g. fitusiran), and anti-tissue factor inhibitor (anti-TFPI) agents, among others, are available in some countries to prevent bleeding. Immunological tolerance induction (ITI) is used to eradicate inhibitors and involves frequent intravenous injections of factor concentrate over a period of months. In hemophilia A, ITI is effective in around 65 to 70% of patients. Monitoring the inhibitor titer of these patients is essential for evaluating and managing the protocol. Laboratory investigation of the inhibitor should be carried out using the modified Bethesda method. Although this test has a high CV, it is the reference test used to titrate inhibitory antibodies. In some cases, screening for inhibitors using the APTT test can be carried out before titrating the antibodies. However, due to the limitations of the test, negative results should not be used to exclude the possible presence of an inhibitor. Further information can be found in chapter 6 of this manual. An alternative to screening for antibodies against FVIII or FIX, is the use of an immunological test. A variety of immunological assays have been studied, and even though these tests are more sensitive than functional tests, they do not discriminate between inhibitory and non-inhibitory antibodies and are therefore not yet useful in clinical practice for detecting or monitoring functional inhibitors. However, studies have shown that IgG4 subclass antibodies are correlated with functional inhibitors of FVIII and FIX (Awasthi et al, 2022; Montalvão et al, 2015; Moorehead et al, 2015).

Different Antibody Kinetics: Different kinetics of antibodies can affect the analysis of the data and consequently lead to misinterpretation. When FVIII or FIX inhibitors act in an inhibition test in a dose-dependent manner, such as completely inactivating FVIII or FIX, these inhibitors are called "Type I" inhibitors. Inhibitors that demonstrate a more complex kinetic behavior are usually called "Type II" inhibitors, incompletely

inactivating FVIII. Type I inhibitors usually develop in patients with congenital hemophilia A or B in response to FVIII or FIX concentrate, while type II inhibitors usually occur in patients with acquired hemophilia or mild hemophilia A. Type I FVIII inhibitors are time- and temperature-dependent because their target, FVIII, is complexed with its carrier protein, VWF. FIX inhibitors are not time and temperature dependent.

Samples for the FVIII/FIX Inhibitor Test: Collection, Dispatch, and Preparation:

Sample collection and dispatch: Samples for the FVIII and FIX inhibitor assays are collected in 3.2% trisodium citrate (0.105–0.109 M), the same type of sample used for most coagulation tests. The citrated whole blood should be centrifuged within 4 hours of blood collection, and the centrifugation should be at 1500g for 15 minutes. Plasma samples positive for FVIII and FIX inhibitors, not whole blood, can be stored at room temperature for 1 week, or frozen at -70°C and stored for up to 15 years. It is important to remember that, unlike traditional procedures for analyzing coagulation, in this type of sample, it is the antibody that must be preserved. This information is very useful for laboratories that need to send samples to another laboratory for testing, as transport does not depend on dry ice.

Heating the samples: Patient samples used for inhibitor detection may contain exogenous FVIII or FIX due to recent factor concentrate infusions, such as: (1) prophylaxis, (2) bleeding treatment, (3) ITI therapy, or (4) endogenous FVIII or FIX if the test is performed on mild or moderate hemophilia. The presence of exogenous FVIII or FIX can significantly affect the detection of inhibitors, with underestimation of the inhibitor titer and false-negative results (Batty et al, 2014; De Lima Montalvão et al, 2015). Heating the samples for 30 minutes at 56°C dissociates the antigen-antibody complex and denatures the factor. To standardize the test, it is recommended that all test samples are always pre-warmed, even if no exogenous FVIII or FIX is expected. The heating procedure should be followed by a centrifugation step, 2 minutes at 4000g, to remove the residues in the plasma caused by the heating. This step of heating the samples for the inhibitor test has not been evaluated for all the products available for patient treatment. Therefore, for each molecularly modified FVIII or FIX product, it must be demonstrated that residual FVIII or FIX deteriorates because of the heating procedure. Emicizumab is not destroyed by the preheating step, however, FVIII inhibitors can be measured in the presence of emicizumab using a bovine chromogenic FVIII method. It is worth remembering that some patients use factor concentrate and emicizumab concomitantly, so heat treatment is necessary.

Diluting the sample: To titrate the inhibitor, the test must be carried out with multiple dilutions of the test plasma. The way in which the dilution factor is selected is not limited to a specific number, as it depends on whether the previous inhibitor titer is known.

Normal Pool Plasma for Inhibitor Testing: To assess the activity of the inhibitor in the test sample, it is necessary to present an "external" source of FVIII or FIX for this inhibitor. This external source is based on the normal plasma pool, and so it is important to consider that any error at this stage can generate false positive and negative results. When preparing a pool for inhibitor testing, the FVIII or FIX activity should be measured and the deviation from this value should be monitored over storage time or when producing a new batch. A pool of normal plasma should be used to ensure that the level of FVIII or FIX is close to 1 IU/ml (100 per cent). A lower factor level in the normal plasma pool may result in an overestimation of the inhibitor titer, while a higher factor level may result in an underestimation of the inhibitor titer. A maximum deviation of 5% of 1 IU/ml of FVIII or FIX in the normal plasma pool is acceptable. The external source of FVIII or FIX can be made by preparing the normal plasma pool or from a commercial source and can be frozen or lyophilized. A minimum of 20 donors is suggested to obtain plasma with a level of FVIII or FIX close to 1 IU/ml. FVIII is a thermolabile clotting factor, which means that during a 2-hour incubation at 37°C, there will be a significant loss of FVIII activity due to a change in pH. To stabilize the pH during incubation, the normal plasma used must be buffered. This can be done using imidazole buffer or HEPES buffer.

Protocol for the FVIII and FIX Inhibitor Test: In 1975, Kasper et al described a method for determining FVIII and FIX inhibitors, and to date, this is still the most standardized test, known as the Bethesda test. In

1995, the Nijmegen method was described, a modification of the Bethesda assay, with some differences: (1) the introduction of buffered pool to improve the stability of FVIII during incubation, and (2) FVIII-deficient plasma to use in the control mixture. This modified Bethesda method was recommended by the International Society on Thrombosis and Haemostasis (ISTH) as the reference method for the FVIII inhibitor test (Verbruggen et al, 1995). The inhibitor test is an indirect method and is based on the principle of the inactivation of the coagulation factor from a known external source by the inhibitor in the test sample during an incubation period. One Bethesda unit is defined as the amount of inhibitor that will neutralize 50% of one unit of FVIII added in 2 hours at 37°C.

Reagents and equipment:

- Dilution buffer (part 1)
- Imidazole or HEPES buffer (below)
- Pool of normal plasma (part 2)
- FVIII-deficient plasma
- Cephalin (APTT reagent)
- Plastic tubes

Table 22. Imidazole or HEPES buffer

Buffer	
Imidazole	Mix 1 part of 4M imidazole buffer with 39 parts of the normal plasma pool. After mixing, the pH should be adjusted to between 7.3 and 7.5.
HEPES	Mix 1 part HEPES 1M buffer with 9 parts normal plasma pool. After mixing, the pH should be adjusted to between 7.3 and 7.5.

Method: Prepare dilutions of the test plasma in plastic tubes to a final volume of 0.2 ml using the dilution buffer. The dilutions required for each patient may change. A suggested starting point would be to start with an undiluted sample and then carry out dilutions of 1/2, 1/4, 1/8, etc.

Note: If the patient has previously had an inhibitor assay, the level can provide a rough guide as to which dilutions should be used. Pipette 0.2 ml of FVIII-deficient plasma into another plastic tube. This will serve as a control tube.

Note: In the original Bethesda assay, imidazole buffer was used to prepare a control mixture with the normal plasma pool. In the Nijmegen assay, the imidazole buffer is replaced with FVIII-deficient plasma. Some differences have been observed between the use of immunodepleted factor-deficient plasma, chemically depleted plasma, and congenitally deficient plasma. These differences may be due to the lack or presence of VWF in the plasma, the presence of antibodies, or the presence of FVIII fragments. As VWF is present in the normal plasma pool, it is not necessary for the diluent of the control mixture to also contain VWF, and to reduce costs, the factor-deficient plasma can be replaced with 4% bovine serum albumin (BSA). Four percent buffered BSA is a reliable and economical substitute for FVIII or FIX plasma and favors the standardization of the method.

✓ Add 0.2 ml of buffered normal plasma pool to the control tube and the test plasma dilutions. The FVIII level of all tubes will be approximately 0.5 IU/ml. This is considered to mean that the buffered normal plasma pool has 1 IU/ml of FVIII.
✓ Cap, mix, and incubate all the tubes at 37°C for 2 hours.
✓ After 2 hours, transfer all tubes to an ice bath, unless the FVIII assay is to be performed immediately.
✓ Perform the FVIII test on all incubation mixtures using the usual FVIII assay method, one-stage or chromogenic method (Part 6).
✓ Read the residual FVIII of each test mixture, using the control as 100% (0.5 IU/ml).

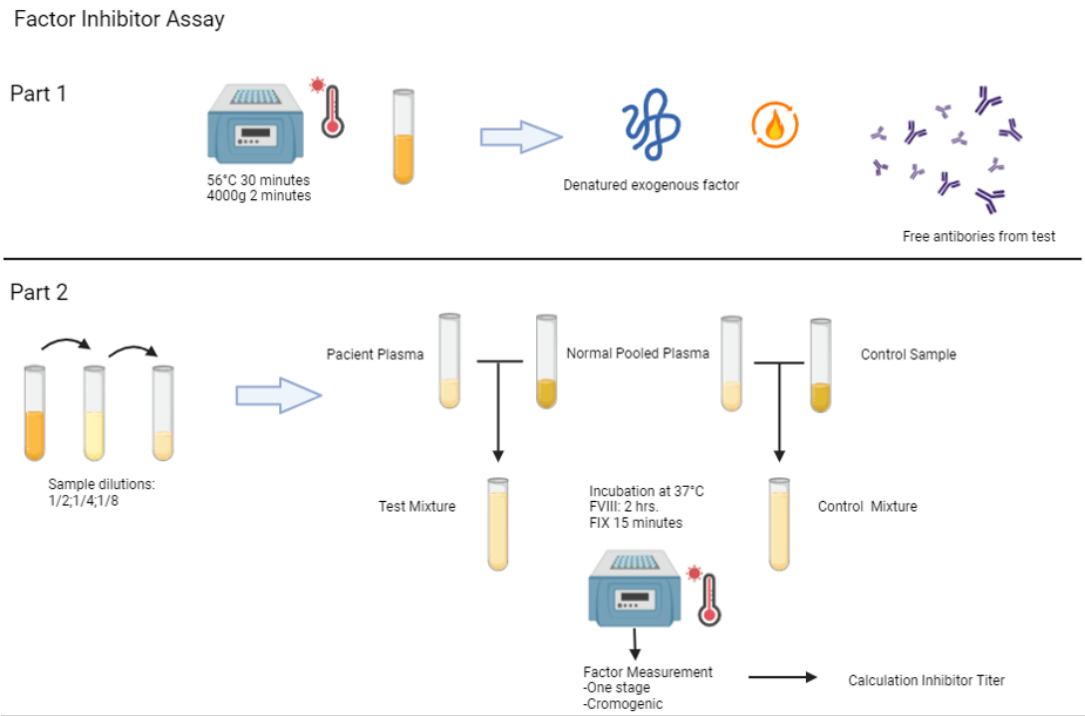

Figure 8. Inhibitor assay procedures

Results and interpretation: The dilution that provides a residual FVIII closest to 50%, but within the 25% to 75% range, is chosen for the inhibitor calculation. Any residual FVIII of <25% or >75% should not be used for inhibitor level calculations. A graph of % residual FVIII versus inhibitor units can be made on log-log paper from the inhibitor unit definition. Read the inhibitor level corresponding to the residual FVIII for each test mixture and correct the dilution. For example, if the value closest to 50% of the residual factor was found in the 1/4 dilution (i.e. in the mixture 1/4 + normal pool), the result, which will be close to 1 Bethesda Unit (BU), should be multiplied by 4.

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

Note: The inhibitor assay is based on determining the residual FVIII or FIX of the mixture of test and control plasma previously incubated. The Bethesda and Nijmegen assays were developed using a one-stage coagulation factor assay. However, testing for inhibitors using this assay has limitations. Clot formation in this test can be affected, for example, by the lupus anticoagulant (non-specific clotting inhibitors) and drugs such as emicizumab. An alternative to avoid these problems is the use of a chromogenic method. Another advantage of using a chromogenic method instead of a coagulation assay is the greater accuracy of the results. An undiluted patient sample with residual activity >75% can be reported as <0.4 BU/ml. For FVIII inhibitors, the Scientific and Standardisation Committee (SSC) of the ISTH recommends considering a result ≥0.6 BU/ml as positive. In addition to the calibration curve, the inhibitor titer can be calculated using the formula: (2-log %RA)/0.301. In the case of a type I inhibitor, a curve from a patient's test plasma shows parallelism with the calibration curve. Non-parallelism with the calibration curve indicates a different kinetic type II inhibitor standard. For inhibitors with type II kinetics, use the lowest dilution that comes close to 50% of the residual activity for the final calculation of the inhibitor titer.

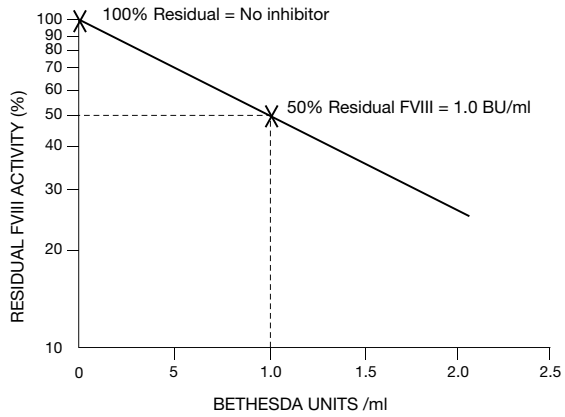

Figure 9. Residual factor activity calculation

Notes: The most frequently encountered functional inhibitors of hemostasis are lupus anticoagulants, which are not directed against specific coagulation factors and whose presence must be excluded before testing for specific factor inhibitors. Quantification of the inhibitor titer is carried out in the laboratory, preferably using the Bethesda assay modified by Nijmegen Bethesda, because this modification offers greater specificity and sensitivity compared to the original Bethesda assay. Positive FVIII inhibitor results below 2.0 BU can be confirmed with the chromogenic method, as it has less analytical interference and greater accuracy compared to the one-stage method. The chromogenic method is also the best choice if lupus anticoagulants are suspected in the test sample or if it contains therapeutic anticoagulants such as heparin or direct FXa or FIIa inhibitors. Non-neutralizing anti-FVIII antibodies that are not detected by the Nijmegen-Bethesda assay can be clinically relevant because they can increase FVIII clearance and can be measured by ELISA.

von Willebrand Factor Inhibitors Assays: VWD is considered the most common hereditary bleeding disorder known in humans, with a population prevalence of 1% and a symptomatic prevalence of 1 in 1000 (Bowman et al, 2010). Treatment options include the infusion of VWF concentrates, which usually also contain FVIII, administered to prevent or treat bleeding episodes. Alloantibodies against VWF have a prevalence of between 7 and 9.5% (James et al, 2013; Pagliari et al, 2023). In these cases, treatment with VWF concentrates is ineffective, and episodes of anaphylaxis have been reported with subsequent exposure to VWF (James et al, 2013). As we already know, VWF deficiency can be explained by different mechanisms resulting from the types of genetic defects identified. This variability of genetic defects contributes to a heterogeneity of inhibitors targeting different epitopes of the VWF molecule. For this reason, the laboratory detection of these antibodies is challenging (Connell et al, 2021; Miller, 2021; Sarji et al, 1974). In 1974, Sarji et al (1974) reported for the first time a case of alloantibody against VWF in a multi-transfused patient. The VWF inhibitor was measured using a method analogous to the Bethesda method for FVIII inhibitors (Sarji et al, 1974). Although there is no standardization for the identification of VWF inhibitors, the Bethesda method has been used by most laboratories (Favaloro et al, 2022), with the difference of using methods that assess VWF activity instead of the FVIII or FIX test. There are currently different types of methods available for detecting VWF activity, with different sensitivities and specificities, so it is important to consider that this variability of methods influences the sensitivity and specificity of detecting these inhibitors (Favaloro et al, 2022). Antibodies against VWF do not have the characteristics of being time- and temperature-dependent, so they can be evaluated immediately after the mixing test (Sarji et al, 1974). The classic ristocetin cofactor method, which evaluates the interaction of VWF with platelets fixed in the presence of ristocetin, as well as the collagen evaluation method and the gain-of-function method, are options that have already been evaluated and which have been shown to be methods with good sensitivity and stability, despite presenting different results as discussed above. Other available methods have not yet been evaluated for this type of investigation. Immunological methods have also been described and detect neutralizing and non-neutralizing antibodies. In relation to the development

of autoantibodies that characterize acquired VWD, patients with myeloproliferative neoplasms (MPNs) are a subgroup that present bleeding complications related to VWF activity. False results are observed in samples from patients with MPNs, depending on the technology applied. The laboratory investigation of VWF inhibitors characterized by alloantibodies and autoantibodies, should be carried out with caution in view of the different methodological possibilities. The performance of all the modern methods currently available is still unclear (Noye et al, 2024; Favaloro et al, 2022).

Anti-Drug Antibody (ADA) in the Coagulation Laboratory – Emicizumab: Emicizumab is a bispecific antibody that binds to human FIX/FIXa and FX/FXa and acts as a mimetic of FVIII function. However, it is not regulated by the mechanisms that regulate FVIII (Mahlangu et al, 2018, Mahlangu et al, 2022). The APTT screening test is considerably reduced by emicizumab (i.e. below the reference range, regardless of the reagents used). Emicizumab affects all APTT-based laboratory tests and assays. Emicizumab also interferes with chromogenic assays to measure FVIII using human FIXa and FX, but not those using FIXa and FX of bovine origin (Bowyer et al, 2021; Jenkins et al, 2020). Emicizumab can be measured and reported in µg/ml using a modified one-stage assay with higher sample dilution and calibrated with calibrators specific for emicizumab. Anti-drug antibodies (ADAs) can develop after a single dose or repeated administration of a therapeutic protein, and can affect the pharmacokinetics, pharmacodynamics, efficacy, and/or safety of this therapeutic protein. Studies evaluating the characteristics of ADA in patients being treated with emicizumab show that the APTT test can be prolonged in conjunction with hemorrhagic episodes in the presence of antibodies with a neutralizing action (Novembrino et al, 2023; Valsecchi et al, 2021). In this case, when the level of emicizumab was measured, it showed a significant reduction. The neutralizing activity of these antibodies was not clearly identified in functional methods, even for the modified Bethesda assay (Kaneda et al, 2021). The level of the Bethesda unit identified seems to be lower than expected when compared to the test that measures the level of emicizumab. The role of functional tests for ADA has not yet been established, but they may be complementary to measuring the plasma level of the drug in these cases. The WFH recommends measuring emicizumab levels using a modified one-step assay with higher sample dilution and calibrated with specific calibrators for emicizumab (Srivastava et al, 2020).

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