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REVIEW

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International Council for Standardization in Haematology recommendations for laboratory measurement of factor VIII and FIX type I inhibitors

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Abstract

This guidance document has been prepared on behalf of the International Council for Standardisation in Hematology. The aim of the document is to provide guidance and recommendations on the measurement of factor VIII (FVIII) and factor IX (FIX) inhibitors. After an introduction on the clinical background and relevance of factor VIII and factor IX inhibitor testing, the following aspects of laboratory testing are included: screening for inhibitors, assay principle, sample requirements, testing requirements and interpretation, quality assurance, interferences and recent developments. This guidance document focusses on recommendations for a standardised procedure for the laboratory measurement of FVIII and FIX type I inhibitors. The recommendations are based on published data in peer-reviewed literature and expert opinion.

KEYWORDS
factor IX, factor VIII, ICSH, inhibitors

1 | INTRODUCTION

The major complication of haemophilia therapy today is the development of anti-drug (anti-factor) antibodies termed inhibitors. These are polyclonal anti-factor VIII (FVIII) or factor IX (FIX) high-affinity IgG antibodies, which neutralize infused coagulation factor concentrate (CFC) replacement therapy, rendering prevention and treatment of bleeds difficult. The appearance of inhibitors is the outcome of a multi-step process that involves a cascade of interactions between environmental and genetic determinants.[1]

In severe haemophilia A, FVIII inhibitors form in approximately 30% of patients, usually during the first 20–30 days of CFC exposure,[2] but in severe haemophilia B, the cumulative incidence of FIX inhibitor development is lower than in severe haemophilia A and is as high as 4%–5% after a median of only 9–11 exposure days.[3]

The management of acute bleeding in patients with inhibitors depends on the inhibitor titre. A minority of patients with a low titre and low responding inhibitors can be treated with standard CFC replacement therapy, although it requires higher doses to overcome the neutralizing effects of the inhibitor. For patients with high titre inhibitors, the only effective therapies for treating bleeds are bypassing agents (BPA). The three available BPAs used in haemophilia A and B are activated prothrombin complex concentrate (aPCC) and two forms of recombinant activated factor VII (rFVIIa).[4] The role of recombinant porcine FVIII in management of congenital haemophilia A patient with inhibitors is evolving.[5] Recently developed novel haemostatic drugs, such as humanized bi-specific antibodies (e.g. emicizumab), short-interfering RNA (e.g. fitusiran) and anti-TFPI

agents among others are now or will be available in the future to prevent bleeding.[1] However, their role is confined to prevention, rather than treatment of bleeding episodes.

Immune tolerance induction (ITI) is used to eradicate inhibitors and involves frequent intravenous injections of CFC over many months.[6] In haemophilia A, ITI is effective in about 65–70% of patients.[7] Both a high daily dose and a lower dose every other day are equally effective in inducing tolerance; patients on the low-dose arm of an international study had more bleeding events in the first months of therapy.[7] For the remaining 30–35% of patients in whom ITI does not eradicate the inhibitor, alternative approaches for such patients include attempting ITI with a VWF-containing FVIII concentrate, with or without adjunctive immunosuppressants like rituximab.[8,9] Prevention of bleeding in haemophilia A with emicizumab has become the standard of care, whilst for those with haemophilia B, options for bleed prevention are quite limited.

Detection and monitoring of FVIII and FIX inhibitors are essential to patient management. Clinical presentation of such patients include increased frequency of bleeding in patients on prophylactic CFC therapy, refractory bleeding despite administration of CFC, or lower than expected post CFC infusion recovery of factor levels. Initial laboratory suspicion may also include finding of an unexplained prolonged activated partial thromboplastin time (APTT) that is inhibited in mixing studies with normal plasma. In these settings, it is important to pursue testing to detect factor inhibitors. For recent evidence-based recommendations on when to perform FVIII and IX inhibitor testing see the third edition of the WFH Guidelines for the Management of Hemophilia.[10]

1.1 | Type I and type II inhibitors

When FVIII or FIX inhibitors act in an inhibitor assay in a dose-dependent manner, these inhibitors are referred to as "Type I" inhibitors, while inhibitors which demonstrate a more complex kinetic behaviour are normally called "Type II" inhibitors, which incompletely inactivate FVIII. Type I inhibitors usually develop in congenital haemophilia A or B patients in response to FVIII or FIX CFC while Type II inhibitors typically occur in patients with acquired haemophilia or mild haemophilia A.[11,12] FVIII type I inhibitors are time- and temperature-dependent because the target of these inhibitors (FVIII) is in complex with its carrier-protein Von Willebrand Factor (VWF), while FIX inhibitors are not time- and temperature-dependent.[13,14]

For a recent review on the relevant characteristics of FVIII and FIX inhibitors see Miller.[15]

1.2 | Scope of the recommendations

This paper describes recommendations on a standardised procedure for the laboratory measurement of factor VIII (FVIII) and factor IX (FIX) type I inhibitors.

1.3 | Screening for inhibitors

1.3.1 | APTT mixing test

If there is a suspicion that FVIII or FIX inhibitors are present after CFC infusion of persons with haemophilia, the activated partial thromboplastin time (APTT) mixing test can be used to screen for the presence of such inhibitors.[16] The test is a relatively simple and inexpensive procedure when compared to a full inhibitor test. In general, the test plasma which presents an abnormally prolonged APTT result is mixed with an equal volume of normal pooled plasmas from healthy individuals (NPP) in order to verify the APTT normalisation. The reasoning for this kind of investigation is that when the patient has only clotting factor deficiency, the APTT on such a mixture is expected to correct to normal or nearly normal because of the contribution of the missing clotting FVIII or FIX from NPP. However, in the presence of an inhibitor to FVIII the APTT on an equal mixture may be prolonged on immediate mixing with NPP, or may normalize and prolong only after a 2 h incubation. For an inhibitor to FIX the APTT is prolonged in an immediate mixing test.[17–19] Note that APTT prolongation due to acquired haemophilia A (type II inhibitor kinetics) may also result in correction to normal or nearly normal. Repeating the APTT after a 2 h incubation of the mixture will result in a prolongation of the APTT.[20] A detailed description of the APTT mixing test is outside the scope of this paper. However, it should be realised that several studies have demonstrated a variation in the sensitivity of the APTT mixing test for the detection of inhibitors due to different kinds of reagents, the lot-to-lot variation of reagents and the cut-off levels used.[16,21] In addition, one-stage FVIII assay interferences described below, also affect the APTT (see Section 1.8). Performance of an APTT mixing test is not useful in the presence of emicizumab. It has been demonstrated that the interpretation of mixing studies in the presence of low titre anti-FVIII antibodies may be difficult.[22] Therefore APTT mixing studies alone should not be used to rule out the possible presence of low titre anti-FVIII or anti-FIX inhibitors.

1.3.2 | Immunological testing

An alternative to screening for the existence of FVIII or FIX antibodies is the use of an immunological assay. A variety of different immunological assays have been described, such as the enzyme-linked immunosorbent assay, fluorescence immunoassay, immunoprecipitation assay, surface plasmin resonance assay and luminex xMAP-based fluorescence-immunoassay.[23–33] Despite the fact that these tests are more sensitive than functional tests, they should only be used for screening purposes because they cannot discriminate between inhibitory and non-inhibitory antibodies and therefore not useful in clinical practice to detect or monitor functional inhibitors. Another application is to use an immunological test to confirm the presence of specific IgG-subclasses.[15] It has been demonstrated that IgG_4 subclass antibodies correlate with functional FVIII inhibitors.[24,34–38]

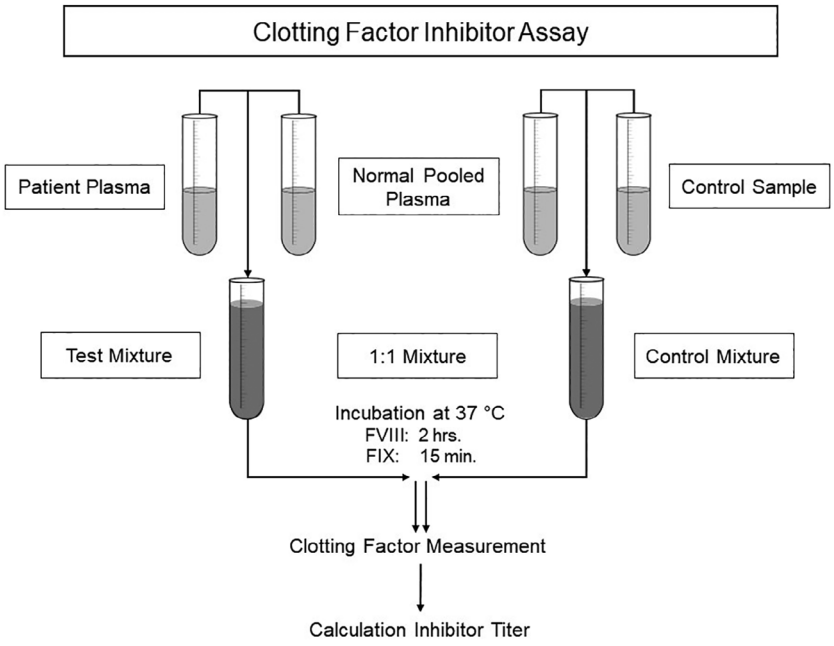

FIGURE 1 A schematic overview of the clotting factor inhibitor assay.

1.4 | Assay principle

1.4.1 | Introduction

The first functional laboratory method for the detection of FVIII inhibitors was published by Biggs et al and is known as the Oxford method.[13,39,40] A disadvantage of the Oxford method was the use of concentrate as the source for FVIII. Because of the variation of FVIII levels in plasma concentrates, test results show a wide variation. In 1975, Kasper et al. described a more standardised method using normal pooled plasma as the source for FVIII and Imidazole buffer as a control mixture, known as the Bethesda assay.[41] Also the use of a more uniform unit was introduced.[42] In 1995 the Nijmegen method, a modification of the Bethesda assay, was described, using buffered normal pooled plasma as the source of FVIII and FVIII-deficient plasma for the control mixture.[43] This method was recommended by the International Society on Thrombosis and Haemostasis as the reference method for FVIII inhibitor testing[44] and more recently by the World Federation of Haemophilia.[10] For a more detailed description of the differences between the Bethesda and Nijmegen assays, see Verbruggen et al.[45] More recently a simplification of the FVIII inhibitor test was suggested by Torita et al., by taking into account the remaining FVIII in the patient sample in the calculation of the inhibitor titre.[46] With this modification, known as the Osaka assay, the pre-heating procedure of the patient plasma can be avoided. In contrast, Miller et al., suggested including a pre-heating treatment of a patient sample as a standard procedure in their Center for Disease Control (CDC)-Modification of the Nijmegen-Bethesda assay.[47] Despite all efforts to improve the FVIII inhibitor assay further, there is still a considerable variation in test results among laboratories, which can be observed from results in external quality assessment programmes.[48–52] Also for FIX inhibitor testing a high variation in test results can be observed between laboratories.[53] Therefore, there is a need for detailed guidance regarding inhibitor testing in the plasma of congenital haemophilia A and B patients.

1.4.2 | Assay principle

A clotting factor inhibitor assay is based on the principle of inactivation of the clotting factor from a known source by the inhibitor from the patient sample during a fixed incubation period. The amount of inhibitor is quantified by comparing the residual clotting factor activity in the assay mixture with a control mixture. A schematic overview of the assay is given in Figure 1.

1.5 | Sample requirements

1.5.1 | General

Samples for FVIII and FIX inhibitor assays are collected in 3.2% (0.105–0.109 moL/L) trisodium citrate (citrated plasma). The standard procedure for blood collection and sample preparation can be used.[54] In summary, citrated whole blood is centrifuged within 4 h after blood collection. Standard centrifugation conditions for blood coagulation samples can be used (15 min at 1500×*g*). A recent study has demonstrated that both FVIII and FIX inhibitor positive sample can be stored at room temperature for 1 week, while stored at −70°C inhibitor positive samples can be stored for up to 15 years after their original analyses.[55] For FVIII:C and FIX:C it has been demonstrated that clotting activity should be measured within 2 and 4 h, respectively.[56] Storage of frozen plasma at −70°C for the measurement of FVIII:C and FIX:C is possible for 18 and 24 months, respectively.[57]

Miller et al have demonstrated that inhibitor positive samples that is, not whole blood, can be shipped to a central laboratory either frozen on dry-ice or on cold-packs.[47]

1.5.2 | Pre-analytical heat treatment

Patient samples used for inhibitor detection could contain exogenous FVIII or FIX because recent CFC infusions provided for prophylaxis or treatment of bleeding or during ITI therapy or endogenous FVIII or FIX if the test is performed in mild or moderate haemophilia. It has been demonstrated that the presence of exogenous FVIII or FIX could significantly affect inhibitor detection.[47,58–61] This results in an underestimation of the inhibitor titre and even also in false negative results. It has been demonstrated that pre-heat treatment of patient samples for 30 min at 56°C dissociates the antigen–antibody complex and denatures FVIII.[62] Alternatively, a more vigorous procedure was suggested, that is, heating for 90 min at 58°C.[58,63] However heating for 90 min at 58°C results in a significant decrease in the levels of anti-FVIII IgG_4.[64] Even heating for 30 min at 58°C may result in lower anti-FVIII IgG_4 levels.[64] Therefore, a preheating procedure of 30 min at 56°C is recommended.

For FIX it has been demonstrated that in addition to the heat treatment a cold pre-treatment by the addition of cold normal plasma for the mixture step (see Figure 1) results in a better sensitivity in detecting the inhibitor.[61]

To standardise the assay procedure, it is recommended that patient samples should always be preheated even if no residual FVIII or FIX is expected. Preheat treatment can be done on either fresh plasma or on or frozen and rapidly thawed plasma. The heating procedure should be followed by a centrifugation step (e.g. 2 min at 4000×*g*) to remove debris in the plasma caused by the heating procedure. The control sample (see below) should be treated using the same procedure.

It has been demonstrated that heat-inactivation destroys extended half-life FVIII and FIX concentrates.[65,66] However, not all products were studied. Therefore, in the case FVIII or FIX inhibitor testing is required, for each molecular modified FVIII or FIX product licensed for the treatment of the haemophilia A or B patient, it should be demonstrated that residual FVIII or FIX deteriorate as a result of the heating procedure. The novel product emicizumab is not destroyed by the preheat step.[67] However, FVIII inhibitors can be measured in the presence of emicizumab by using a bovine chromogenic FVIII method (see below).

Torita et al.[46] has described an alternative approach, known as the Osaka method, in which no pre-heat treatment is necessary and in calculating the inhibitor, the ratio between the actual and the theoretical remaining FVIII activity is taken into account. To the best of our knowledge, the validity of this approach has not been proven in any other study.

1.5.3 | Sample dilution

It has been shown that the dilution factor of the patient sample is an important contributor to the variation in inhibitor results between laboratories.[68] The residual FVIII or FIX activity in inhibitor positive patient samples should be between 25% and 75%.[41] (See also the section on the calculation of the inhibitor titre.) If the residual FVIII or FIX activity in an undiluted sample is larger than 75% this result should be used to calculate the inhibiter titre, taken into account the lower limit of quantification of the test (see also the section on assay positivity). If the residual activity is less than 25%, the patient samples must be retested after additional dilutions of the test sample. As a part of routine surveillance and pre-operative testing in previously inhibitor-free haemophilia A or B patients it is suggested that inhibitor measurement should be performed with undiluted test sample if surgery is required because testing is only meant to establish whether an inhibitor is present yes or no.[69] However, for unknown patient samples inhibitor testing should be performed with multiple dilutions of test plasma.[18,70] How dilution factors are selected depends on the expected inhibitor titre and is not limited to a specific number. For instance, a range of undiluted sample, 1 in 2 (=1 part patient sample + 1 part diluent), 1 in 5 (=1 + 4) and 1 in10 (=1 + 9) can be selected. However, even higher dilution factors may be necessary.[18,69,70] For patients known to have an inhibitor, *recent* results can guide dilution choice. To advance standardisation and the between-laboratory comparability of test results it is recommended to use standardised dilution factors.[68] In Table 1 Recommended dilutions factors are given.

TABLE 1 Dilution factors for known ranges of inhibitor titre.

Expected Inhibitor range (BU/mL)	Corresponding dilution factor
0–2.0	Neat (undiluted)
2.0–8.0	4 (=1 + 3)
5.0–20.0	10 (=1 + 9)
12.5–50.0	25 (=1 + 24)
25.0–100.0	50 (=1 + 49)

Because a single dilution could miss relatively small changes in titre by not capturing a residual level between 25% and 75%, in these cases additional dilutions are recommended.

The dilution matrix should be similar to the matrix of the control sample (see below).

The inhibitor titre calculated should be corrected for the dilution factor that is closest to the 50% residual FVIII:C to get the final titre. Note that any multiplication of the inhibitor titre also magnifies the error in testing. Therefore, the lowest possible sample dilution should be used for calculation.

1.5.4 | Consensus recommendations for sample requirements

1. Samples, both the clinical samples and the control plasma, should be preheated for 30 min at 56°C, followed by a centrifugation step for 2 min at 4000×*g*.

2. Unknown samples should be measured in a series of dilutions. Samples from patients with an inhibitor level in a known range should be measured with a fixed dilution factor.

TABLE 2 The effect of the factor level in the normal pooled plasma on the inhibitor titre.

Factor level normal pooled plasma (IU/mL)	Inhibitor titre (BU/mL)
1.10	0.88
1.00	1.00 (by definition)
0.95	1.07
0.90	1.17
0.80	1.42

TABLE 3 Buffers which can be used to buffer normal pooled plasma.

Buffer	
Imidazole	Mix 1 volume of 4 M Imidazole buffer with 39 volumes of normal pooled plasma. After mixing, the pH of the buffered normal pooled plasma should be verified and eventually adjusted between 7.3 and 7.5
Hepes	Mix 1 volume of 1 M Hepes buffer with 9 volumes of normal pooled plasma. After mixing, the pH of the buffered normal pooled plasma should be verified and eventually adjusted between 7.3 and 7.5

1.6 | Testing requirements and interpretation

1.6.1 | Normal pooled plasma

The basic principle of an inhibitor test is to mix the patient sample 1:1 with a normal pooled plasma.[41] A normal pooled plasma should be used to ensure that the level of FVIII or FIX is close to 1 IU/mL (=100%). A lower factor level in the normal pooled plasma may result in the overestimation of the inhibitor titre, while a higher factor level may give an underestimation of the inhibitor titre. A maximum deviation of 5% of 1 IU/mL FVIII or FIX in the normal pooled plasma is acceptable because in practice a factor level of exact 1 IU/mL cannot be guaranteed at any time. Table 2 shows the effect of the factor level in the normal pooled plasma on the inhibitor titre, which demonstrates the importance of a factor level close to 1 IU/mL.

The FVIII or FIX level of the normal pooled plasma should be known before it is used in the inhibitor test.

Normal pooled plasma can be prepared in-house from apparently healthy donors or be from a commercial source, and either be frozen or lyophilised. A minimum of 20 donors is suggested to obtain plasma with an FVIII or FIX level close to 1 IU/mL.[16]

1.6.2 | Buffering normal pooled plasma

In contrast to FIX it has been clearly demonstrated that FVIII is a thermolabile clotting factor.[56,71–74] During a 2-h incubation at 37°C there will be a significant loss of FVIII activity due to a change in the pH.[43,46] To stabilise the pH during incubation the normal plasma used in the mixture should be buffered. This can be done by using either imidazole buffer[43] or Hepes buffer.[46] Details are given in Table 3. Although it had been suggested that Hepes buffer gives better stability of FVIII activity,[46] recently it was demonstrated that Hepes buffer gives a higher change in the FVIII activity than imidazole buffer.[75]

1.6.3 | Control mixture

In the original Bethesda assay, Imidazole buffer was used to prepare a control mixture with normal pooled plasma.[41] In the Nijmegen assay imidazole buffer is replaced by FVIII deficient plasma.[43] This results in comparable protein concentrations in both the assay and control mixture. Differences have been observed between the use of immuno-depleted factor-deficient plasma, chemically depleted plasma and congenitally deficient plasma. This may be due to the lack of or presence of the von Willebrand Factor (VWF) in the plasma, the presence of preparatory antibodies or the presence of FVIII fragments.[44,76,77] Because VWF is present in the normal pooled plasma it is not necessary that the diluent of the control mixture also contains VWF.[47,77] To reduce costs deficient plasma can be replaced by 4% bovine serum albumin (BSA).[78,79] In a recent study the use of FVIII-depleted plasma and 4% BSA with and without buffering with imidazole or Hepes has been compared.[75] It was demonstrated that 4% BSA buffered with Imidazole gave more stable results than buffered FVIII-depleted plasma. Buffered 4% BSA is therefore a reliable and cost-effective substitute for FVIII-depleted plasma and supports method standardisation. Any other non-plasma substitute should be validated for change in FVIII and pH during the 2-h incubation at 37°C.

1.6.4 | Mixture

In a standard mixing test, the patient sample is mixed in 1:1 ratio with normal pooled plasma.[16,80] If an inhibitor is present, FVIII or FIX in the normal plasma is inhibited, resulting in a lower residual factor activity. The control mixture is mixed similarly with normal plasma.

1.6.5 | Incubation time and temperature

It has been demonstrated that an optimum FVIII inhibitor detection is reached with an incubation time of at least 2 h and an incubation temperature of 37°C.[63] An incubation lasting longer than 180 min results in a marked decrease in the FVIII activity in both the test sample and the control mixture and therefore produces unreliable inhibitor results. With an incubation time shorter than 120 min no optimum result in the inhibitor detection has yet been achieved. An incubation time of 2 h should therefore be used for FVIII inhibitor assays.

Because FIX inhibitors do not demonstrate a progressive character, like FVIII inhibitors, the incubation time can be reduced to 10–15 min.

1.6.6 | Residual factor VIII or FIX measurement

The inhibitor assay is based on the measurement of the residual FVIII or FIX in both the patient and the control mixture. Both the Bethesda and Nijmegen assays were developed by using a one-stage clotting factor assay. However, inhibitor testing using a one-stage clotting assay has certain limitations.[15] The clot formation in this test can be affected, for instance, by unfractionated heparin,[81] lupus anticoagulant,[82] non-specific coagulation inhibitors[83] as well as drugs like emicizumab.[84–86] See further the paragraph on interferences. An alternative for the measurement of residual FVIII or FIX is the use of a chromogenic method.

It has been demonstrated that chromogenic FVIII assays can be used to measure residual FVIII in Bethesda-type assays without the interference of non-FVIII-specific inhibitors.[29,87] Another advantage of the use of a chromogenic method instead of a one-stage clotting assay is the higher precision.[88] It has also been demonstrated for FIX that a false-positive FIX inhibitor can be excluded by the use of a chromogenic method because of an existing lupus anticoagulant.[89] Therefore, the use of chromogenic assays for the measurement of residual FVIII or FIX may reduce the number of false-positive inhibitor results.

Either the one-stage clotting assay or the chromogenic method should be performed as for regular plasma FVIII or FIX testing, including a plasma calibrator which is calibrated to the most recent Word Health Organisation International Standard for plasma FVIII or FIX.

1.6.7 | Calculation of the inhibitor titre

To assess the inhibitor titre in a patient sample the relative residual factor activity (%RA) has to be calculated. The %RA is the ratio of the factor activity in the test mixture to the control mixture. If no inhibitor is present in the patient sample the %RA is theoretically 100%, although the inherent imprecision in FVIII assays means that the %RA is usually in the 95–105% range in the absence of inhibitor. If an inhibitor is present the residual activity in the patient sample will be lower than in the control mixture, resulting in a %RA < 100%. By definition a %RA of 50% is considered as one Bethesda Unit (BU).[41] A theoretical calibration line can be prepared by plotting the residual factor activity (logarithmic) against the inhibitor titre (linear). This calibration line is fully defined by 0 and 1 BU/mL (Figure 2). In the case of a Type I inhibitor, a dilution curve of a patient plasma needs to show parallelism with the calibration curve. Non-parallelism with the calibration curve indicates a different kinetic inhibitor pattern (e.g. Type II inhibitors).[63]

To avoid errors in assessing the inhibitor titre manually from a graph, the titre can also be calculated by using the following formula: (2-log %RA)/0.301.[47]

An inhibitor titre should be calculated from a sample with a %RA between 25% and 75%. A %RA of 75% corresponds to an inhibitor titre of 0.4 BU/mL. In the standard inhibitor test this is the lowest level of quantification. An undiluted patient sample with a %RA > 75% should theoretically be reported as <0.4 BU/mL. A sample with a %RA < 25% should be tested in additional dilutions. In the case of multiple dilutions, the sample with the least dilution close to a %RA of 50% should be used. To calculate the final titre the result should be multiplied by the dilution factor used for the patient sample.[15,18]

1.6.8 | Assay positivity

For FVIII inhibitors the Scientific and Standardisation Committee of the International Society of Thrombosis and Haemostasis recommends considering a result ≥0.6 BU/mL as positive.[90] For the Nijmegen assay, however, a slightly lower threshold for positivity (≥0.5 BU/mL) was observed.[43,44] Miller et al demonstrated that their method modification of the Nijmegen assay may even be able to detect inhibitors down to 0.2 BU/mL.[91] For FIX inhibitors the same group has considered a threshold of ≥0.3 BU/mL.[47]

Because it has been shown that laboratories found false-negative results in samples up to an inhibitor level of approximately 1 BU/mL,

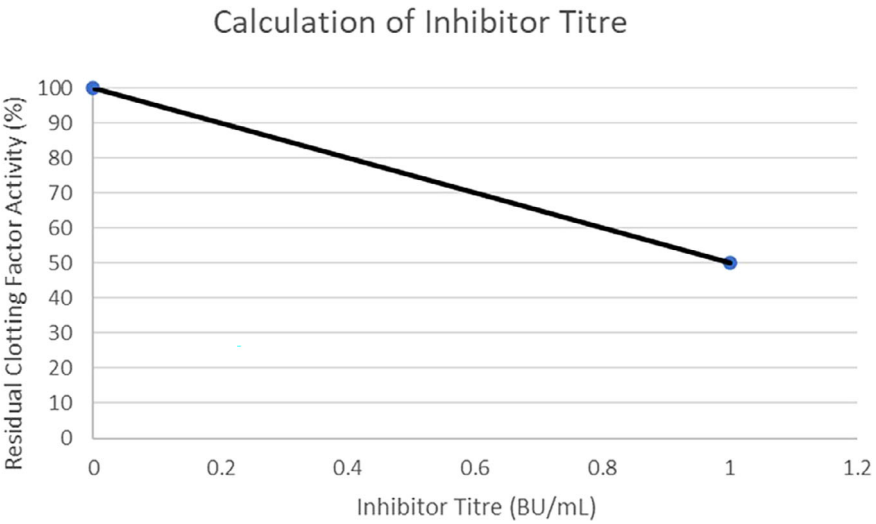

FIGURE 2 Graph demonstrating the calculation of the inhibitor titre.

it is important that laboratories establish the lowest level for positivity that is, the lower limit of quantification.[48,53]

On the other hand, also a relative high rate of false-positive inhibitor samples has been demonstrated in samples with a titre below 2 BU/mL when measured with a one-stage clotting assay. This has not been observed when using a chromogenic assay for the detection of residual clotting factor levels.[29,92] Therefore, inhibitor positive samples, measured with a one-stage clotting assay, with a titre below 2 BU/mL should be interpreted with caution and always be repeated.

1.6.9 | Consensus recommendations on testing requirements and interpretation

1. A normal pooled plasma with an FVIII or FIX level of 1 ± 0.05 IU/mL should be used.
2. Imidazole-buffered (final concentration: 0.1 M) normal pooled plasma with a pH between 7.3 and 7.5 should be used.
3. 4% Bovine serum albumin buffered with Imidazole and mixed with normal pooled plasma should be used as a control mixture.
4. Both patient sample and control mixture are mixed in a 1:1 ratio with normal pooled plasma.
5. Both the test sample and control mixture should be incubated for 120 min at 37°C for FVIII inhibitors. For FIX inhibitors an incubation of 15 min. is sufficient.
6. Either a one-stage clotting assay or a chromogenic assay can be used for the measurement of residual FVIII or FIX. However, the use of a chromogenic assay may reduce the number of false-positive inhibitor results. Chromogenic assay with bovine based reagents should be used for FVIII inhibitor measurements in patients treated with emicizumab.
7. An inhibitor titre should be calculated from a sample with a %RA between 25% and 75%.
8. In the case of multiple dilutions, the sample with the least dilution close to a %RA of 50% should be used.
9. The threshold for positivity is 0.5 BU/mL for FVIII inhibitors and 0.3 BU/mL for FIX inhibitors.

1.7 | Quality assurance

Quality assurance in laboratory medicine includes all activities used to assure the validity of the test result, detecting errors in the testing process and correcting errors before the test result is reported to the physician. This includes both internal quality control (IQC), which focuses on monitoring stable performance by detection of random and systematic errors using quality control samples with ranges of acceptable values, and external quality assessment (EQA), which focuses on monitoring accuracy by distributing samples to participants blinded to measurand values and evaluation by an external agency.[93–95]

For IQC it is recommended that both a negative control sample should be included, for example, FVIII or FIX-deficient plasma, and a positive control sample. As a positive sample, a patient sample with a known inhibitor titre can be used. A positive control sample of 1 BU/mL has been suggested.[47] Alternatively, commercial control samples can be used or FVIII or FIX-deficient plasma can be spiked with commercially purified inhibitors with known inhibitory characteristics. Small subsamples can be stored frozen and for each assay run a subsample thawed thoroughly in a water bath at 37°C. Control samples should be treated in the test like patient samples. So far only very limited data on the reproducibility and repeatability of inhibitor assays have been reported. A within-run reproducibility of between 5% and 6% and a repeatability of approximately 10% have been reported.[47]

Several studies have also demonstrated a considerable between-laboratory variation,[51,52,96,97] ranging from 20% and 80%. Variation in test methodology and reagents may contributes to this between-laboratory variation. It is known that some laboratories do not use either the Bethesda or Nijmegen assay, but a hybrid variant.[22,97,98] It has been demonstrated that the variation in dilution factors used by participants in EQA surveys significantly contribute to the between-laboratory variation.[68] This latter study has also demonstrated that standardisation of the inhibitor assay results in a significant decrease of the between-laboratory variation from 40% to 50% to less than 10%. This emphasises the urgent need for a more standardised performance of inhibitor testing.

Laboratories should participate in EQA surveys to ensure the accuracy of measurement.

1.8 | Interferences

It is well-known that certain circumstances may result in either false positive or false-negative inhibitor test results. It is of major importance for the management of a patient that a positive inhibitor test is specific to an FVIII or FIX inhibitor and not caused by a lupus anticoagulant, a non-specific inhibitor or anticoagulant treatment.[88,99] In this section these potential interferences are discussed briefly. For more details see Adcock et al[18] or Miller.[15]

1.8.1 | Anticoagulation therapy

Depending on the concentration and the sensitivity of the reagent, both heparin and direct oral anticoagulants (DOAC) may affect APTT test results. This may lead to decreased activity in an FVIII or FIX one-stage clotting assay and subsequently in a positive inhibitor test

result.[100–104] Interference of anticoagulants can be abolished by neutralising heparin by heparinase[105] and DOACs by pre-analytical DOAC absorbance,[106,107] although the effect has not been tested in a FVIII or FIX inhibitor test.

Especially in an emergency situation if the clinical background of a patient is not known, such a patient can be misdiagnosed as suffering from a factor inhibitor. To rule out a false positive inhibitor result, an anti-Xa assay to rule out the presence of an anti-Xa DOAC and thrombin time to rule out the presence of heparin and direct IIa inhibitors can be performed.

1.8.2 | Lupus anticoagulant

It has been reported that haemophilia patients with inhibitors coincidently can also have lupus anticoagulant.[29,82,99] In addition, strong lupus anticoagulant can result in a false positive inhibitor test.[96] It is therefore of major importance that a laboratory is able to distinguish between a lupus anticoagulant and FVIII or FIX inhibitor, because any misinterpretation may result in incorrect patient management. However, several studies have shown this can be difficult for laboratories.[51,108,109] One important difference between the characteristics of lupus anticoagulant and an FVIII inhibitor is a time- and temperature-dependent APTT prolongation in the mixing test for FVIII inhibitors. Lupus anticoagulant mostly acts as an immediate inhibitor. However, an FIX inhibitor also has an immediate inhibitory characteristic and therefore cannot be differentiated from a lupus anticoagulant. The most suitable solution is to use a chromogenic assay for the measurement of residual FVIII or FIX, where higher plasma dilution makes interference in the phospholipid dependant FXa generation unlikely. In the situation no chromogenic assay is available the potential presence of a lupus anticoagulant can be studied by a non-parallelism between a dilution curve of the patient sample and the calibration curve.[10] In summary, three different dilutions of the patient sample should be compared by parallel-line analysis with the calibration curve. For instance, this can be done by calculation of the coefficient of variation (CV) between the 3 test results of the patient sample. If the CV of the three results is less than 15% the presence of a lupus anticoagulant is unlikely. If the CV is greater than 15%, the presence of a lupus anticoagulant is likely (for details see Reference [10], page 38–39).

1.8.3 | Other specific factor inhibitors

It has been demonstrated that high-titre coagulation factor inhibitors other than an FVIII or FIX inhibitor may interfere in the inhibitor test, resulting in a false positive test result. In addition, a high FVIII inhibitor titre may also result in a positive FIX inhibitor test and vice versa.[18,110] Laboratories should be aware of this phenomenon. In a survey amongst 42 laboratories using a sample with a strong factor V inhibitor, about 5% reported the presence of a FVIII inhibitor.[111]

1.8.4 | Emicizumab

Emicizumab is a recombinant, humanised, bispecific monoclonal antibody which restores the missing function of activated factor VIII by bridging FIXa and FX.[112] It is used for prophylaxis of bleeding in haemophilia A patients, both with and without inhibitors.[113,114] It has been demonstrated that emicizumab interferes with both the one-stage clotting assay and a chromogenic assay using human coagulation factors resulting in an overestimation of FVIII activity and therefore potentially false negative FVIII inhibitor assay. Only a chromogenic assay using bovine FX is insensitive to emicizumab.[84–86,115,116] This implies that the FVIII inhibitors test is also affected by the presence of emicizumab when an assay sensitive to emicizumab is used.[85,86,115] It is also important to realise that emicizumab is not sensitive to heat treatment and is therefore not destroyed by the pre-heat treatment step in the inhibitor test.[15,115] This may result in false negative inhibitor titres.

Therefore, FVIII inhibitors can only be measured reliably in the presence of emicizumab if a chromogenic method with bovine coagulation factors is used.[67,117]

1.8.5 | Other causes

The use of EDTA plasma instead of citrated plasma may result in false positive inhibitor results.[18,111] Another study has shown that 8%–17% of representatives of laboratories that on a regular basis take part in FVIII inhibitor external quality assessment surveys of the ECAT Foundation, found in a workshop a false positive inhibitor result in an FVIII-deficient plasma.[108]

2 | SUMMARY OF A STANDARDISED FVIII AND FIX INHIBITOR ASSAY

In Table 4 a summary is given of a standardised FVIII and FIX assay.

2.1 | Recent developments

2.1.1 | Initiatives to standardisation

Recently several initiatives has been undertaken to develop assay kits for the measurement of FVIII inhibitors.[118,119] These initiatives may advance a more standardised approach for the measurement of FVIII inhibitors.

2.1.2 | Low-titre assay

Dardikh et al. have described a method for FVIII inhibitor testing which is 20 times more sensitive than the standard Nijmegen Assay.[120] This method is based on a concentration step of the test

TABLE 4 Procedure for a standardised FVIII and FIX inhibitor assay.

	Factor VIII inhibitor	Factor IX inhibitor
Sample	Citrated plasma	Citrated plasma
Pre-heat treatment	30 minutes at 56°C Centrifugation for 2 min at 4000×*g*	30 min at 56°C Centrifugation for 2 min at 4000×*g*
Sample dilution	Unknown sample: Dilution range Known inhibitor range: Fixed dilution factor (Table 1)	Unknown sample: Dilution range Known inhibitor range: Fixed dilution factor (Table 1)
Normal pooled plasma	FVIII level between 0.95 and 1.05 IU/mL	FIX level between 0.95 and 1.05 IU/mL
Buffering normal pooled plasma	Imidazole buffered (final concentration: 0.1 M); pH: 7.3–7.5	Imidazole buffered (final concentration: 0.1 M); pH: 7.3–7.5
Control mixture	4% Bovine albumin buffered with Imidazole or FVIII deficient plasma	4% bovine albumin buffered with Imidazole or FIX deficient plasma
Ratio for patient and control mixture	1:1	1:1
Incubation	120 min at 37°C	10–15 min at 37°C
Measurement of residual clotting factor activity	One-stage clotting assay or chromogenic assay To reduce the effect of interferences the use of a chromogenic method is recommended	One-stage clotting assay or chromogenic assay To reduce the effect of interferences the use of a chromogenic method is recommended
Residual factor activity	Between 25% and 75%	Between 25% and 75%
Calculation of inhibitor titre	(2-log %RA)/0.301	(2-log %RA)/0.301

plasma by selective protein filtration preceding the inhibitor test. This results in a lower limit of quantification of 0.03 BU/mL. The authors demonstrated in a small study that very low inhibitor titres might be of clinical relevance. In a clinical situation in which inhibitors are suspected and cannot be detected in a standard inhibitor assay, this test could be an interesting alternative. To our knowledge, this test has not been validated in any other study.

2.1.3 | South-MIMMS inhibitor assay

The South-MIMMS Inhibitor Assay (SMIA) was developed from the perspective of reducing the cost of the assay by replacing the FVIII-deficient plasma in the control mixture with normal pooled citrated plasma. This should also reduce a source of variation between different laboratories. Because the control mixture now consists only of normal pooled plasma the unknown inhibitor titre in the patient sample should now be expressed relative to 200% FVIII.[121] The authors claim a reduction in the lower limit of detection from 0.6 BU/mL in the Nijmegen Assay to 0.2 BU/mL in the SMIA. A recent survey has demonstrated that only a few laboratories are using this method.[98] To our knowledge, this test has not been validated in any clinical study.

AUTHOR CONTRIBUTIONS

Piet Meijer: Drafting manuscript. Flora Peyvandi, Guy Young, Rajiv Pruthi, Simara de Lima Montalvao and Steve Kitchen critically reviewed, edited and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest related to this publication.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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