

ORIGINAL ARTICLE

Impact of different factor VIII inhibitor kinetic profiles on the inhibitor titer quantification using the modified Nijmegen–Bethesda assay

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Funding information

Roche Diagnostics GmbH

Handling Editor: Dr Johnny Mahlangu

Abstract

Background: Coagulation factor VIII (FVIII) inhibitor titer quantification is vital for optimizing care in people with hemophilia A.

Objectives: This study analyzed the impact of the different kinetic profiles of four FVIII monoclonal antibodies on inhibitor titer quantification using the modified Nijmegen–Bethesda assay.

Methods: Concentration-related and time-related profiles of FVIII antibodies (4A4, BO2C11, 2-54, ESH-8) were evaluated in vitro. FVIII residual activity was measured using a one-stage clotting assay and chromogenic substrate assay. Profiles of the FVIII antibodies were compared with the theoretical kinetic model: the ideal log (residual activity)-linear (inhibitor concentration) relationship. Different theoretical kinetic model-dependent and -independent criteria to calculate FVIII inhibitor titer were compared.

Results: Factor VIII monoclonal antibodies had different concentration-related and time-related profiles, ideal for comparative analysis using the modified Nijmegen–Bethesda assay. The kinetic profile of 4A4 was similar to the theoretical kinetic model, while BO2C11 showed a steeper curve, and 2-54 and ESH-8 a flatter curve, than the model. In the modified Nijmegen–Bethesda assay, conversion of measured FVIII residual activities for different inhibitor dilutions into FVIII inhibitor titer is based on the theoretical kinetic model. Therefore, titer calculations for FVIII inhibitors that deviate from the model are prone to underestimation or overestimation. Calculating a theoretical dilution at 50% FVIII residual activity by sigmoidal regression reflecting different kinetic inhibition profiles can provide a more accurate titer result.

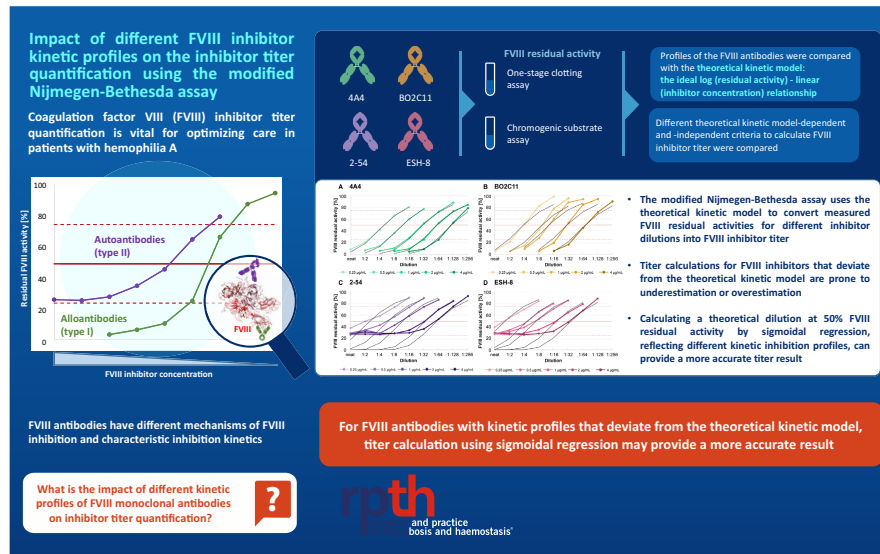
Conclusion: Kinetic profiles of FVIII antibodies can deviate from the theoretical kinetic model in the modified Nijmegen–Bethesda assay, leading to differences in FVIII inhibitor titer quantification.

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KEYWORDS

factor VIII, hemophilia A, human, kinetics, monoclonal antibodies



Essentials

- Factor VIII (FVIII) inhibitors are a complication of hemophilia A treatment and can cause acquired hemophilia.
- The modified Nijmegen–Bethesda assay is recommended to measure FVIII inhibitor titer.
- The modified Nijmegen–Bethesda assay does not reflect the variety of different FVIII inhibitors.
- A new method reflecting different kinetic profiles may provide a more accurate titer result.

1 | INTRODUCTION

Hemophilia A results from a deficiency in clotting factor VIII (FVIII), which can be inherited or acquired.^{1,2} Inherited hemophilia A is an X-linked disorder, with an estimated prevalence of 17.1 cases per 100,000 males worldwide.³ Many patients with hemophilia A develop neutralizing alloantibodies against FVIII when treated with FVIII replacement therapy.^{4,5} The presence of these FVIII antibodies impairs the efficacy of FVIII replacement therapy, leaving the patient vulnerable to bleeding symptoms and at increased risk of morbidity and mortality.⁵ Antibodies develop in 25%–35% of previously untreated patients with severe hemophilia A (FVIII activity, less than 1 IU/dl) and predominantly occur during the first 50 exposure days to FVIII replacement therapy.⁶ In contrast, acquired hemophilia A is caused by the formation of inhibitory autoantibodies against FVIII, a second type of FVIII inhibitor. Acquired hemophilia A is most commonly seen in elderly (age >60 years) people of either sex,¹ with a reported prevalence of up to six cases per million per year in Germany.⁷

FVIII human antibodies are primarily directed against epitopes in the A2 and C2 domains of the FVIII protein^{8,9} and different target epitopes lead to different mechanisms of FVIII inhibition.¹⁰ Moreover, FVIII antibodies can be differentiated depending on their characteristic inhibition kinetics.^{11,12} Type I antibodies have

second-order inactivation kinetics that can completely inactivate FVIII activity when present at sufficient concentrations; in contrast, Type II antibodies have more complex kinetics and cannot completely inhibit FVIII activity even at high concentrations.¹³ Type II FVIII antibodies can also form complexes with FVIII that retain some residual activity.¹²

The Nijmegen–Bethesda assay is recommended by the World Federation of Hemophilia (WFH) for the quantification of FVIII inhibitor titer.² The Nijmegen modification includes pH buffering of the normal plasma and comparable protein levels in all mixtures by using FVIII-deficient plasma or buffered albumin as a diluent, which improves specificity and sensitivity.^{14–17} In the modified Nijmegen–Bethesda assay an additional heat deactivation step prior to inhibitor testing is performed, recommended for samples with FVIII activity greater than 5 IU/dl.² The FVIII residual activity in the modified Nijmegen–Bethesda assay can be measured using a one-stage clotting assay (OSA) or chromogenic substrate assay (CSA). Despite these improvements, variable FVIII inhibitor titer results are reported in the literature.^{18,19} One reason may be a lack of standardization in the methodology used to calculate FVIII inhibitor titers across laboratories.^{20,21} In general, the titer calculation is based on a theoretical kinetic model displaying a linear relationship between the logarithm of the FVIII residual activity and the FVIII inhibitor

concentration (Figure S1). With this model, every measured residual activity can be directly converted into an inhibitor titer. The WFH recommends FVIII residual activities between 25% and 75% for titer quantification²; however, several different methodologies using FVIII residual activities to calculate the FVIII inhibitor titer have been reported (Table S1).

A reliable method for FVIII inhibitor titer quantification is vital for providing optimal care for people with hemophilia A. In people with congenital hemophilia A, measuring FVIII inhibitor titers is important in the first month after FVIII replacement therapy is initiated and should be followed up annually for detection of FVIII inhibitor development and to determine treatment efficacy.² In people with hemophilia A where FVIII antibodies have developed, monitoring can help distinguish between persistent and transient antibodies. Quantification of FVIII antibodies is important to categorize low (less than 5 Bethesda units [BU]/ml) and high (5 BU/ml or higher) titers,² assisting health care professionals in clinical decision making. Measurement of FVIII inhibitor titer is also required before surgery.² FVIII inhibitor titer may have prognostic relevance in people with acquired hemophilia A; therefore, it is important to confirm the presence of FVIII antibodies using the modified Nijmegen–Bethesda assay.²²

The aim of this study was to analyze the kinetic profiles of four different FVIII monoclonal antibodies and their impact on the inhibitor titer calculation in the modified Nijmegen–Bethesda assay, using different calculation criteria. In this article, a more standardized titer calculation will be introduced that uses sigmoidal regression to reflect different kinetic inhibition profiles.

2 | MATERIALS AND METHODS

This was a single-center study conducted on site at Roche Diagnostics GmbH, Penzberg, Germany (September 2020).

TABLE 1 Characteristics of selected FVIII monoclonal antibodies

Antibody	Isotype	Type	Domain (epitope)	Activity, BU/mg ^a	Mechanism of action	References
4A4	IgG2 κ	Type I	A2 (403–444)	27,000	Competitive inhibition of the tenase complex, which blocks FX activation	[24,32]
BO2C11	IgG4 κ	Type I	C2 (2170–2215; 2303–2332)	16,000	Inhibition of FVIIIa–VWF interaction and FVIIIa binding to phospholipids	[23]
2-54	IgG1 κ	Type II	A2 (604–740)	13,000	Inhibition of thrombin-catalyzed activation of FVIII	[24,25]
ESH-8	IgG2a	Type II	C2 (2248–2285)	8000	Inhibition of FVIII release from VWF	[33,34]

Abbreviations: BU, Bethesda unit; FVIII, factor VIII; FVIIIa, activated factor VIII; FX, factor X; IgG, immunoglobulin G; VWF, von Willebrand factor.

^aActivity values are calculated based on the titer calculation according to Criterion 5 for each concentration (0.25–4 μ g/ml). The represented values for each antibody are an average.

2.1 | FVIII monoclonal antibodies

Two Type I and two Type II monoclonal antibodies directed against the main epitopes (A2 and C2 domains) of FVIII were used in this study (Table 1): Mouse monoclonal antibodies 4A4 (GMA-8015) and 2-54 (GMA-8028) were purchased from Green Mountain Antibodies, a human monoclonal antibody BO2C11²³ was purchased from Creative Biolabs, and a mouse monoclonal antibody ESH-8 was purchased from ImmBioMed GmbH & Co. KG.

2.2 | Concentration-related and time-related study of different FVIII monoclonal antibodies

Type I and II FVIII inhibitors can be characterized in relation to either concentration or time.^{11,12} FVIII-immunodepleted plasma (Roche Diagnostics International Ltd) was spiked with the FVIII monoclonal antibodies (Table 1) at defined concentrations (concentration-related study: 2 μ g/ml [4.8–11.9 BU/ml]; time-related study: 0.5 μ g/ml [18.4–60.8 BU/ml]). Doubling dilutions of each inhibitor sample were prepared in imidazole-buffered bovine serum albumin (Precision BioLogic Inc.). Imidazole-buffered normal pooled plasma (Precision BioLogic Inc.) was added (1:1) to each diluted sample, resulting in a range of FVIII inhibitor concentrations (concentration-related study: 0.00195–1 μ g/ml; time-related study: 0.0625–0.25 μ g/ml). The samples were then incubated at 37°C until the reaction was stopped. For the concentration-related study, the FVIII residual activities were measured after 2 h of incubation using the OSA and CSA on cobas t 511 analyzers (Roche Diagnostics International Ltd.). For the time-related study, the reaction was stopped by shock freezing at –80°C after 0, 5, 10, 15, 30, 60, 90, 120, and 180 min. After thawing, FVIII residual activities were immediately measured using the OSA (Roche Diagnostics International Ltd) and CSA (Technoclone Herstellung von Diagnostika und Arzneimitteln GmbH).

2.3 | Inhibitor measurement using the modified Nijmegen–Bethesda assay

For modified Nijmegen–Bethesda assay measurements, human normal pooled plasma samples were spiked with the different FVIII monoclonal antibodies (Table 1) and incubated for 2 h at 37°C to allow for FVIII inhibitor complexes to be formed to model patient samples as closely as possible. The modified Nijmegen–Bethesda assay was performed using the CRYOcheck Factor VIII Inhibitor Kit (Precision BioLogic Inc.) according to manufacturer instructions, including a preanalytical heat inactivation to eliminate any remaining FVIII and the use of imidazole-buffered bovine serum albumin as diluent.

2.4 | Determination of FVIII residual activity

The FVIII residual activities were measured using the FVIII OSA or FVIII CSA. All FVIII activity measurements were performed on cobas t 511 analyzers. The OSA measurements were performed according to manufacturer instructions using the factor VIII ready-for-use cassette for cobas t analyzers. For CSA measurements, the TECHNOCHROM FVIII:C assay kit (Technoclone Herstellung von Diagnostika und Arzneimitteln GmbH) was used, and the reagents (A, B, substrate-buffer mixture) were prepared as described in the package insert. The samples were diluted 1:40 with NaCl (Roche Diagnostics GmbH), and reagents A and B were added in equivalent quantities. After incubation at 37°C for 2 min, the substrate-buffer mixture was added in a ratio of 1:5 to the diluted sample. The absorption increase was recorded at 408 nm for 150 s. For the calibration curve, six different dilutions of the Coagulation Reference (Technoclone) were prepared (1:423; 1:130; 1:80; 1:53.3; 1:40; 1:26.6) and measured in the same way as a patient sample.

2.5 | Inhibitor titer and slope calculation

The FVIII inhibitor titers were calculated according to the most commonly used methods (Table 2). In addition, titer calculation with Criterion 5 and slope calculations were performed using GraphPad Prism (GraphPad Software). For Criterion 5 (Table 2), all measured residual activities were plotted against the negative binary logarithm of the dilution (x). A sigmoidal regression with a top value of 100% FVIII residual activity and a constraint for the bottom value to be greater than 0% FVIII residual activity was used to determine the theoretical inhibitor dilution, giving 50% of FVIII residual activity. The reciprocal value of the determined inhibitor dilution corresponded to the inhibitor titer in BU/ml.

$$\text{Residual activity [\%]} = \text{bottom} + \frac{100 - \text{bottom}}{1 + 10^{(\text{LogEC}_{50} - x) \cdot \text{HillSlope}}}$$

$$x = -\log_2(\text{dilution})$$

The LogEC_{50} value represents the inhibitor dilution at which the inhibitory effect is halfway between the residual activity bottom value and residual activity of 100%. If the bottom value is calculated as equal to 0%, the LogEC_{50} value is equal to the x value that gives 50% residual activity. The variable HillSlope describes the steepness of the curve. In contrast, the theoretical kinetic model has a set HillSlope resulting in a fixed curve shape (Figure S1).

Using the calculated inhibitor titer (IT in the equation below) according to Criterion 5, the related residual activities for the theoretical kinetic model were calculated for each dilution.

$$\text{Residual activity [\%]} = 10^{2 - (\text{IT} \cdot (2 - \log(50)) \cdot \text{dilution})}$$

To explore the deviations from the theoretical kinetic model for each FVIII antibody and concentration (0.25–4 $\mu\text{g/ml}$), the slope values at 50% residual activity were calculated based on the first derivative of the sigmoidal regression.

3 | RESULTS

3.1 | Characterization of different FVIII monoclonal antibodies

In the concentration-related study, the 4A4 and BO2C11 antibodies showed a near-complete inactivation of FVIII (residual activity <9%) at high inhibitor concentrations (Figure 1). In contrast, FVIII residual activity >25% was seen even at high inhibitor concentrations for 2-54 and ESH-8 (Figure 1). Moreover, the slope for Type II antibodies (2-54, ESH-8) was flatter than for Type I antibodies (4A4, BO2C11; Figure 1). Comparing the OSA and CSA results, the inhibition of 2-54 was only detectable using the OSA to measure FVIII residual activity (Figure 1).

In the time-related study, only the Type I antibody BO2C11 (Figure 2B) showed a typical linear relationship between the logarithm of FVIII residual activity and incubation time at all three tested concentrations. The other antibodies (Figure 2A,C,D) showed a nonlinear relationship. The concentration- and time-related studies showed that the selected antibodies had different kinetic profiles, ideal for a comparative analysis in the modified Nijmegen–Bethesda assay. Inhibition by 2-54 is detectable only in the OSA; therefore, the FVIII residual activities were measured only with the OSA for the following analysis of the kinetic profiles in the modified Nijmegen–Bethesda assay.

3.2 | Comparative analysis of different inhibition kinetics in the modified Nijmegen–Bethesda assay

The kinetic profiles of the four FVIII antibodies were analyzed using the modified Nijmegen–Bethesda assay and compared with the theoretical kinetic model used for titer calculations (Figure 3). The kinetic profile of 4A4 was perfectly represented

TABLE 2 Overview and limitations of different criteria for inhibitor titer quantification

Criterion	FVIII inhibitor titer calculation	Limitations for FVIII antibodies with kinetic profiles that deviate from the theoretical kinetic model ^a
1 ³⁵	First dilution with residual activity >25%	B: selected dilution with residual activity close to 25% ↑ C, D: long plateau phase (>25% residual activity) ↓ selected dilution with residual activity close to 25% ↓
2 ²⁰	Mean value of all titers with residual activities between 25% and 75%	B: uneven distribution of values ↓↑ C, D: long plateau phase (>25% residual activity) ↓ uneven distribution of values ↓↑
3 ²⁰	Dilution closest to 50% residual activity	B, C, D: residual activities far from 50% ↓↑
4 ¹⁷	Semi-log(y)-plot: residual activities (25%–75%) vs. dilutions Titer calculation with dilution that gives 50% residual activity by interpolation	B: no calculation possible when only one value is between 25% and 75% residual activity
5	Semi-log(x)-plot: all residual activities vs. dilutions Titer calculation with dilution that gives 50% residual activity by sigmoidal regression $\text{Residual activity [\%]} = \text{bottom} + \frac{100 - \text{bottom}}{1 + 10^{\frac{\log(\text{EC}_{50-x}) - \text{HillSlope}}{\text{HillSlope}}}}$	–

Note: ↓↑ indicate a resulting underestimation or overestimation of the calculated FVIII inhibitor titer, depending on the criteria in combination with an outlined deviation of the kinetic profile from the theoretical kinetic model.

Abbreviation: FVIII, factor VIII.

^aB–D refer to the generated kinetics as presented in Figure 3, showing either a kinetic profile with a higher slope (Figure 3B) or a lower slope (Figure 3C,D) at 50% residual activity.

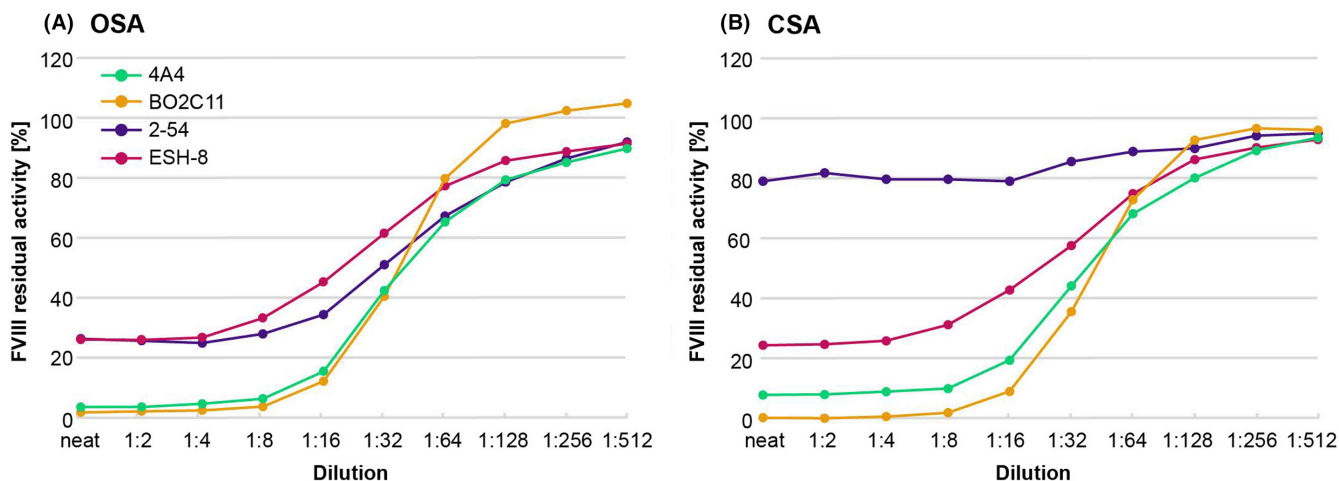


FIGURE 1 Inhibition kinetics of different FVIII monoclonal antibodies at different concentrations (concentration-related study). Different FVIII monoclonal antibodies (4A4, BO2C11, 2-54, ESH-8) were diluted and mixed with a consistent amount of FVIII (neat: 1 µg/ml); after incubation, the FVIII residual activity was measured using an OSA (A) and CSA (B). CSA, chromogenic substrate assay; FVIII, factor VIII; OSA, one-stage clotting assay.

by the theoretical kinetic model (Figure 3A), while the other FVIII inhibitor kinetic profiles showed deviations from the theoretical kinetic model (Figure 3B–D). The Type II antibodies 2-54 and ESH-8 did not completely inactivate FVIII, resulting in a plateau phase around 25% FVIII residual activity (Figure 3C,D). The deviations from the theoretical kinetic model were explored by comparing the slope values for each curve after sigmoidal regression at 50% FVIII residual activity (Table 3). The curves for the theoretical kinetic model had a consistent slope of 24% at 50% FVIII residual activity independent of the inhibitor titer. While 4A4 had a similar value to the theoretical kinetic model (mean slope, $25.2 \pm 1.37\%$), the other Type I inhibitor, BO2C11, had a

higher value (mean slope, $36.0 \pm 1.61\%$). In contrast, the Type II inhibitors, 2-54 and ESH-8, had lower values than the theoretical kinetic model (mean slope, $16.7 \pm 1.51\%$ and $17.2 \pm 1.00\%$, respectively; Table 3).

3.3 | Impact of deviating kinetics on different titer calculation criteria

The different criteria and methods used for titer calculation are summarized in Table 2. The criteria were analyzed according to the observed kinetic profiles of the FVIII antibodies (Figure 3).

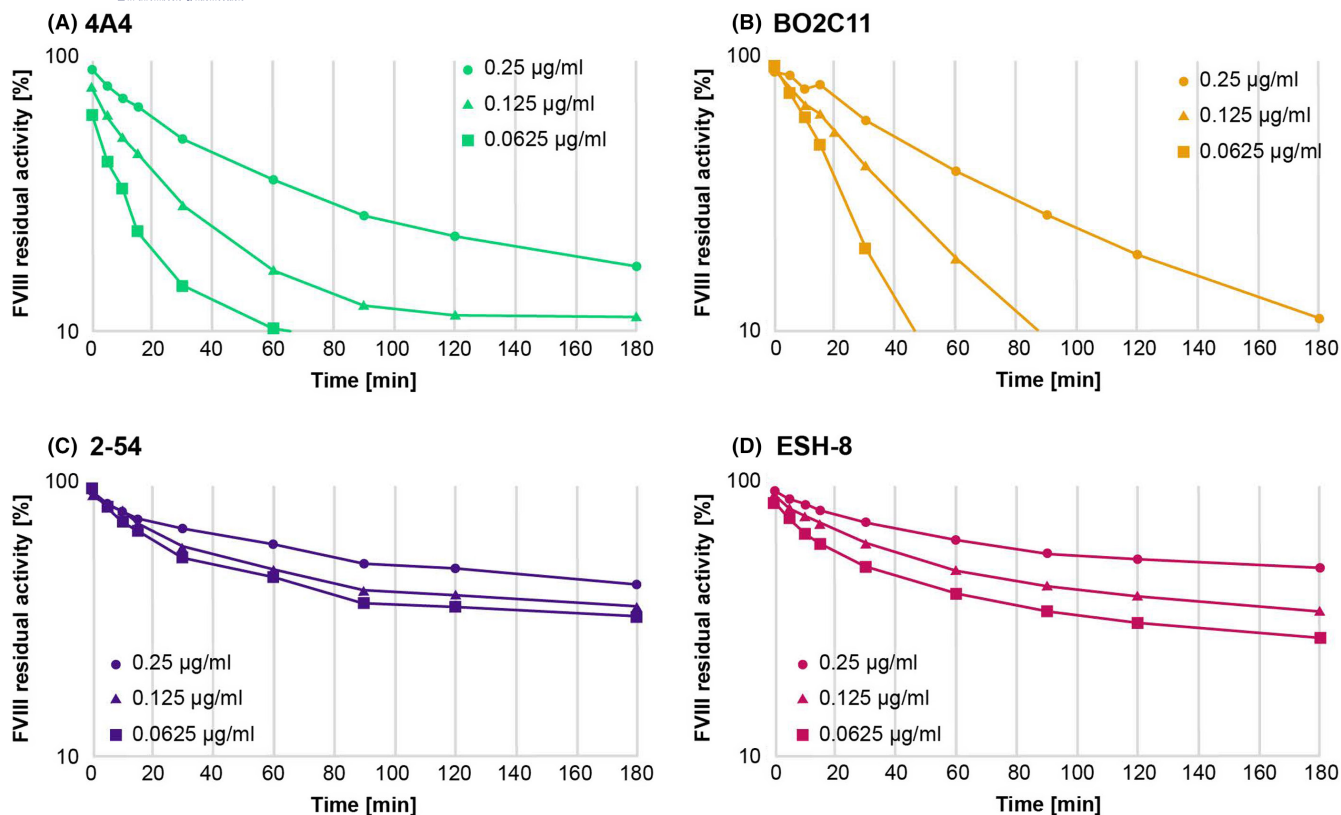


FIGURE 2 Inhibition kinetics of different FVIII monoclonal antibodies by time course (time-related study). Different monoclonal FVIII antibodies (4A4, BO2C11, 2-54, ESH-8) were diluted and mixed with a consistent amount of FVIII. The inhibition reaction was stopped at different time points and the FVIII residual activity was measured using an OSA (A–D) and CSA (A, B, and D). CSA, chromogenic substrate assay; FVIII, factor VIII; OSA, one-stage clotting assay.

Criteria 1 through 3 describe different strategies to select FVIII residual activities to calculate the corresponding inhibitor titer using the theoretical kinetic model. Whenever the measured FVIII residual activity data perfectly fit the theoretical kinetic model (e.g., 4A4), comparable FVIII inhibitor titers are generated independent of the criterion used. In contrast, when the measured FVIII residual activity deviated from the theoretical kinetic model (e.g., BO2C11, 2-54, ESH-8), the FVIII inhibitor titer can be overestimated or underestimated using Criteria 1 through 3. For antibodies with a lower slope value than the theoretical kinetic model values (Figure 3C,D), FVIII residual activities greater than 50% are normally lower than reflected by the theoretical kinetic model, potentially leading to an overestimated FVIII titer quantification. Conversely, residual activities less than 50% are greater than theoretical kinetic model values and can cause underestimation. For antibodies with a greater slope than the theoretical kinetic model (Figure 3B), residual activities greater than 50% lead to underestimated FVIII titer quantification, and residual activities less than 50% lead to overestimated FVIII titer quantification. The greatest deviation is observed when the titer is calculated according to Criteria 1 and 2 for Type II antibodies with a long plateau phase greater than 25% FVIII residual activity. In this case, values of the plateau are used for titer calculation according to the criteria.

Titer calculation using Criteria 4 and 5 is independent of the theoretical kinetic model, as the measured FVIII residual activities are not directly converted to inhibitor titers, but use the measured values to calculate a theoretical inhibitor dilution that correlates to 50% residual activity. Nevertheless, the calculation for antibodies with a greater slope might not be possible using Criterion 4 due to the value limitation (no calculation is possible when only one FVIII residual activity value is between 25% and 75%). In contrast, Criterion 5 uses all measured values to determine the dilution that results in 50% residual activity. In Criterion 5, the sigmoidal regression reflects the kinetic profiles of the different antibodies; therefore, deviating slopes and incomplete inhibition have no impact on the calculated titer result (Figure S2).

4 | DISCUSSION

This study analyzed the kinetic profiles of four different FVIII monoclonal antibodies at clinically relevant concentrations and their impact on the inhibitor titer calculation in the modified Nijmegen-Bethesda assay, using different available criteria and methodologies for titer quantification. The kinetic profile of the Type I FVIII antibody 4A4 was close to the theoretical kinetic model, while the kinetic profiles of the other Type I FVIII antibody, BO2C11, and the

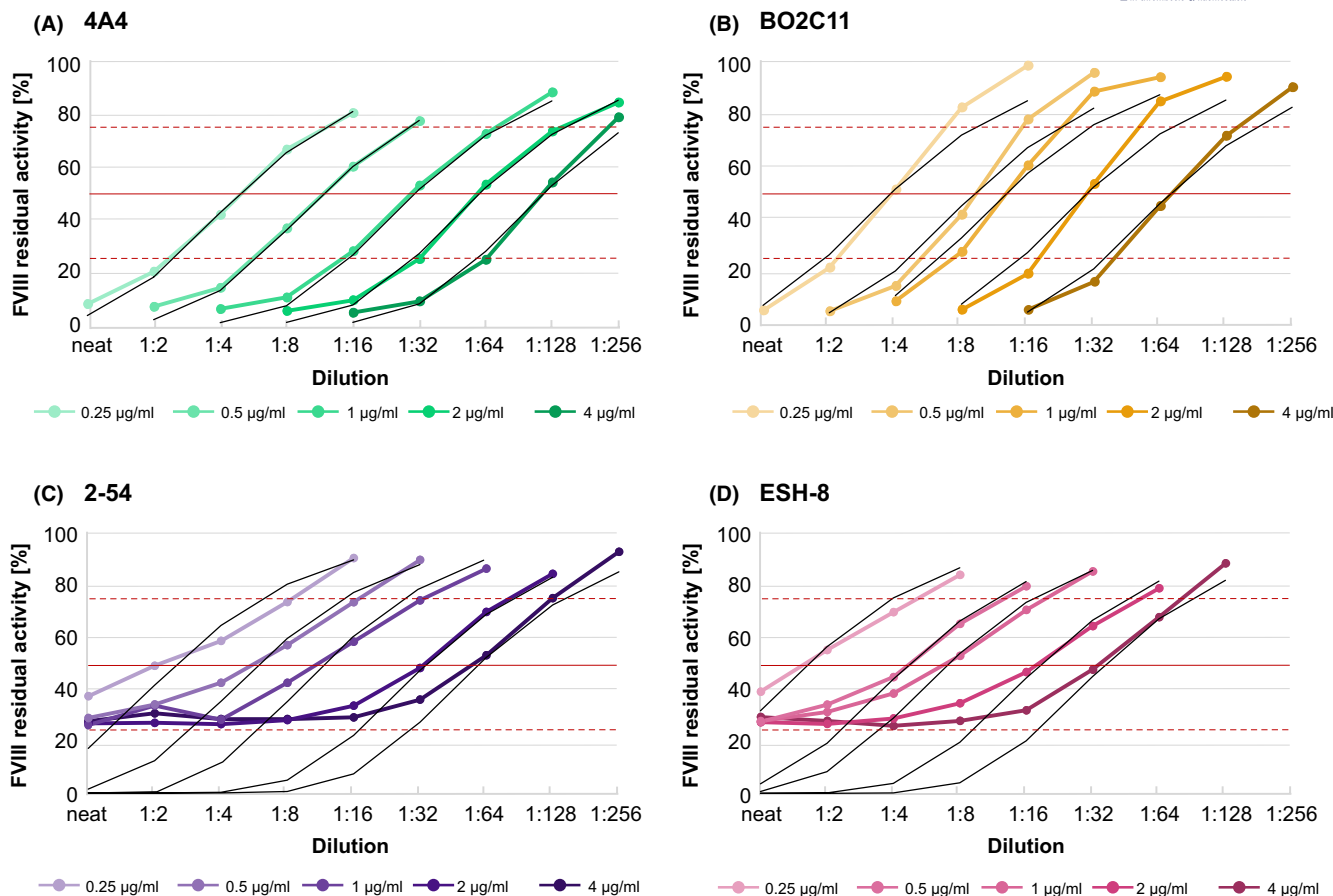


FIGURE 3 Inhibition kinetics of different FVIII monoclonal antibodies in relation to the theoretical kinetic model. Modified Nijmegen–Bethesda assay analysis of two Type I (4A4 [A], BO2C11 [B]) and two Type II FVIII antibodies (2-54 [C], ESH-8 [D]) using five different concentrations (0.25–4 µg/ml). The measured FVIII residual activities of each dilution (colored lines) are plotted against the theoretical kinetic model (black lines) where every residual activity would lead to the same calculated titer result. The solid red line represents a FVIII residual activity of 50% and the dashed red lines represent the commonly accepted FVIII residual activity limits of 25%–75% for which a titer calculation is recommended. FVIII, factor VIII.

TABLE 3 Slope calculations at 50% residual activity for different FVIII monoclonal antibodies

FVIII antibody	FVIII antibody concentration, µg/ml					Mean slope ± SEM
	0.25	0.5	1	2	4	
4A4	24.1%	22.9%	24.3%	24.3%	30.6%	25.2 ± 1.37%
BO2C11	37.0%	39.5%	35.4%	37.8%	30.1%	36.0 ± 1.61%
2-54	12.3%	15.7%	15.5%	18.8%	21.1%	16.7 ± 1.51%
ESH-8	15.9%	16.9%	16.1%	15.8%	21.1%	17.2 ± 1.00%
Theoretical kinetic model ^a	24%	24%	24%	24%	24%	24%

Abbreviations: FVIII, factor VIII; SEM, standard error of the mean.

^aThe theoretical kinetic model had a consistent slope of 24% at 50% FVIII residual activity independent of the inhibitor titer.

two Type II FVIII antibodies, 2-54 and ESH-8, showed significant deviations from the theoretical kinetic model. The introduced Criterion 5 may provide the most reliable titer for most types of FVIII antibodies, especially those with kinetic profiles that deviate from the theoretical kinetic model.

The mechanism of action of the Type II FVIII antibody 2-54 involves suppression of the thrombin-catalyzed activation of FVIII.²⁴ Artificially high values of thrombin, such as those used in

the CSA, are shown to overcome this inhibition²⁵; thus, the inhibitory capacity of 2-54 was detectable only using the OSA. In real-world clinical practice, other substances that may be present in the patient plasma sample, such as emicizumab, therapeutic anticoagulants, and lupus anticoagulants, can also interfere with the measurement of FVIII activity using either OSA or CSA. Thus, verifying the FVIII inhibitor titer using both the OSA and CSA could be beneficial.

The concentration-related profiles of the four different FVIII monoclonal antibodies showed a near-complete or incomplete inactivation of FVIII residual activity as expected, according to the respective inhibitor type. In contrast, the linear relationship between logarithm of FVIII residual activity and incubation time reported for Type I antibodies¹¹ in the time-related profile was seen only for BO2C11. In this study, concentration-related inhibition appeared to be more discriminatory than time-related inhibition for Type I and II FVIII antibodies, in line with previous work by Ling et al.²⁶

The Nijmegen–Bethesda assay was originally developed to quantify Type I FVIII inhibitors only.²⁷ Consequently, FVIII inhibitor titers for Type II FVIII inhibitors should be interpreted with caution.^{28,29} In this study, the lower slope value and incomplete FVIII inactivation for Type II FVIII antibodies were not reflected by the theoretical kinetic model used in the modified Nijmegen–Bethesda assay for titer quantification. In addition, the Type I FVIII antibody BO2C11 displayed significant deviations from the theoretical kinetic model. The observed deviations of the kinetic profiles relative to the theoretical kinetic model had an impact on the FVIII inhibitor titer calculation, dependent on the criteria and method used.

Following analysis of different FVIII titer calculation methodologies, Criteria 1 (titer of first dilution FVIII residual activity greater than 25%) and 2 (mean titer value of residual activities 25%–75%) were identified as the most error-prone criteria, especially for Type II FVIII antibodies. The commonly used Criterion 3 (titer of dilution closest to FVIII residual activity of 50%) can give the most accurate titer when the data include a dilution with a FVIII residual activity value close to 50%.^{2,14,20,21} However, it is not possible to know in advance what the outcome of the experiment will be when preparing samples for analysis (i.e., whether a FVIII residual activity value of close to 50% will be achieved). The key factor that influences the accuracy of the calculation in Criterion 3 is how far from a FVIII residual activity of 50% the values are; therefore, for FVIII antibodies with kinetic profiles that deviate from the theoretical kinetic model and produce values that are not close to FVIII residual activity of 50%, there is a risk of significant overestimation or underestimation of the inhibitor titer. In this case, repeat testing with additional dilutions would result in a more accurate result. In contrast, Criteria 4 and 5 determine the inhibitor titer by calculating the theoretical dilution, giving a FVIII residual activity of 50% by interpolation or regression, respectively. In this study, the sigmoidal regression in Criterion 5 reflects all different kinetic profiles of the analyzed FVIII antibodies. In addition, the regression including all measured values is more robust against outliers. For low-titer antibodies (0.6–1 BU/ml) that have greater than 50% FVIII residual activity in the undiluted sample, only the upper part of the sigmoidal curve is covered by measured values. To improve the analysis of those antibodies, fixing the bottom value in the sigmoidal regression depending on the FVIII inhibitor type (full vs. incomplete inhibition) would be helpful.

In this study, the performance of a panel of different criteria used for inhibitor titer calculation was analyzed. A

relative standardized titer calculation that uses sigmoidal regression, Criterion 5, was also included. A normal plasma pool spiked with FVIII monoclonal antibodies with well-defined biochemical features was used to model patient samples; however, it cannot be excluded that patient samples containing a mixture of antibodies with different epitope affinity and specificity might behave differently.^{30,31} Moreover, the monoclonal antibodies used in this study do not represent the full diversity of polyclonal FVIII inhibitors seen clinically. Nevertheless, the four antibodies used in this study showed typical kinetic profiles seen in samples from people with hemophilia A containing Type I and Type II inhibitors. Therefore, this study provides a good overview of the impact of different kinetic profiles on FVIII inhibitor titer quantification. Future studies are required to validate Criterion 5 in a clinical setting, including titer quantification of patient samples with a wide range of inhibitor types and concentrations.

5 | CONCLUSION

The choice of a suitable FVIII activity assay should be based on the mechanisms of action of the FVIII monoclonal antibodies of interest and with consideration of any interfering substances that may be present in the patient's plasma. The kinetic profile of Type I and II FVIII antibodies can deviate from the theoretical kinetic model used for titer calculation in the modified Nijmegen–Bethesda assay. These discrepancies could lead to differences in the calculated FVIII inhibitor titers, depending on the criteria and methodologies used. For FVIII antibodies with kinetic profiles that deviate from the theoretical kinetic model, it is particularly important for accurate quantification to achieve a value close to 50% FVIII residual activity, either by measurement and/or remeasurement, or by theoretical calculation as shown in this study.

AUTHOR CONTRIBUTIONS

C. Ketteler, I. Hoffmann, D. Chen, A. Tiede, and N. Richter contributed to the conception/design of the study. C. Ketteler and I. Hoffmann were involved in data acquisition. C. Ketteler, I. Hoffmann, S. Davidson, A. Tiede, and N. Richter were involved in analysis/interpretation of data. All authors were involved in manuscript writing and/or critically reviewing the manuscript and approving the final version for submission.

ACKNOWLEDGMENTS

The authors thank Precision BioLogic Inc. for providing the CRYOcheck Factor VIII Inhibitor Kits. Third-party medical writing support, under the direction of the authors, was provided by Heather Small, PhD, of Ashfield MedComms (Macclesfield, UK), an Inizio Company, and was funded by Roche Diagnostics International Ltd (Rotkreuz, Switzerland). COBAS and COBAS T are trademarks of Roche. All other product names and trademarks are the property of their respective owners.

FUNDING INFORMATION

The study was supported by Roche Diagnostics GmbH.

RELATIONSHIP DISCLOSURE

C.K., I.H., and N.R. are employees of Roche Diagnostics GmbH. N.R. and I.H. hold nonvoting equity securities in F. Hoffmann–La Roche. S.D. is an employee of Roche Diagnostics International Ltd. D.C. is an employee of Genentech, Inc., a member of the Roche Group. A.T. has received research grants or honoraria for lectures or consultancy from Bayer, Biotest, Chugai, CSL Behring, Novo Nordisk, Octapharma, Pfizer, Roche, SOBI, and Takeda.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the main figures or in the [Supplementary Material](#) of this article.

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How to cite this article: Ketteler C, Hoffmann I, Davidson S, Chen D, Tiede A, Richter N. Impact of different factor VIII inhibitor kinetic profiles on the inhibitor titer quantification using the modified Nijmegen–Bethesda assay. *Res Pract Thromb Haemost.* 2022;6:e12799. doi: [10.1002/rth2.12799](https://doi.org/10.1002/rth2.12799)

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