




METHODOLOGICAL ARTICLE

Selective human factor VIII activity measurement after analytical in-line purification

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Abstract

Background: It is essential to measure the activity of factor VIII (FVIII) throughout the life cycle of a coagulation FVIII concentrate. Such measurement in nonclinical pharmacokinetic studies is potentially biased by the presence of endogenous nonhuman FVIII, and certain manufacturing process-related additives can also impact the assay performance. Finally, the presence of FVIII activity-mimicking antibodies poses challenges when measuring FVIII in samples. Therefore, we developed an antibody-based chromogenic FVIII assay, which facilitates the selective and sensitive activity measurement of human FVIII in the presence of animal plasma and interfering agents.

Methods: Plate-bound monoclonal anti-FVIII antibody specifically captured human FVIII, which was then measured with a chromogenic activity assay. A human reference plasma preparation was used to construct the calibration curve. Spike recovery was carried out in a citrated cynomolgus monkey plasma-solvent/detergent mixture and in the presence of the bispecific antibody emicizumab.

Results: The calibration curve ranged from 3.03 to 97.0 mIU FVIII/ml and was obtained repeatedly with good accuracy. B domain-deleted and full-length FVIII did not differ in their responses. Recovery of spiked human FVIII in citrated cynomolgus monkey plasma was 102.7%, while neither native monkey plasma nor the other animal specimen tested showed any activity. Solvent/detergent solution and the bispecific antibody emicizumab had no influence on the assay.

Conclusion: Combining antibody-mediated specific capture of human FVIII and a chromogenic activity assay resulted in a selective and sensitive measurement of human FVIII with no interference by endogenous, nonhuman FVIII, manufacturing process additives, or an FVIII activity-mimicking antibody.

KEYWORDS

animals, drug evaluation, emicizumab, factor VIII, hemophilia A, humans, preclinical

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Essentials

- Factor VIII (FVIII) activity measurement is essential for hemophilia treatment and drug development.
- Assay interference by endogenous animal FVIII can confound data evaluation.
- Analytical human FVIII-specific in-line purification overcomes this limitation.
- The robust method described demonstrated acceptable performance characteristics.

1 | INTRODUCTION

Accurate measurement of factor FVIII (FVIII) activity is undoubtedly of utmost importance in FVIII product development and patients' treatment. From patients' perspective, FVIII measurement is first required for diagnosing hemophilia A and then for monitoring FVIII replacement therapy. To support the development and manufacturing of the replacement products, it is mandatory to measure the FVIII activity in the course of nonclinical and clinical studies. Such studies address postinfusion bioavailability and pharmacokinetic behavior. Finally, the potency assignment to release the products requires measurement of FVIII activity. The first assays for antihemophilic factor, described in 1948, were based on acceleration of the whole-blood coagulation time,¹ but this approach rapidly turned out to lack the sensitivity required. Graham et al.² first proposed using prothrombin, which markedly increased the assay's sensitivity, in 1951. Two years later, Pitney and Dacie³ applied recalcified hemophilic plasma for measuring antihemophilic factor levels in human fibrinogen fractions. This basic principle of recalcification was refined by the addition of a crude thromboplastin preparation,⁴ thus more or less creating the first one-stage assay (OSA) for FVIII. At that time, the so-called thromboplastin-generation test,⁵ which had been standardized by using a bovine reference preparation,⁶ was the most sensitive test available for diagnosing FVIII deficiency.

Currently, three assay types for FVIII activity measurement are established:^{7,8} the one-stage and two-stage clotting assays and the chromogenic substrate assay (CSA). The one-stage assay uses correction of the prolonged activated partial thromboplastin time (aPTT) obtained by the FVIII-containing test sample diluted into FVIII-deficient plasma, which is nowadays prepared via immunoadsorption of FVIII from normal plasma.^{9,10} Apart from this, the assay requires an aPTT reagent, containing the contact activator such as ellagic acid, kaolin, silica, celite, or polyphenols and phospholipids, either from natural sources or synthetic ones. A variety of different reagents are commercially available. After contact activation, the assay fully relies on the intrinsic pathway ending with the clotting of fibrinogen. Relative standard deviations (RSDs) range from 5% to 10% with a lower limit of quantification (LLOQ) of 0.005 IU/ml. Heparin,¹¹ direct oral anticoagulants,¹² and FVIII activity-mimicking antibodies have been shown to influence the assay.¹³ The OSA is still the most often used FVIII assay, the two-stage clotting assay having lost its importance after the chromogenic substrate assay was introduced.¹⁴ The two-stage clotting and the chromogenic assay both share an initial incubation step. Subsequently, in the two-stage clotting assay, the action of the prothrombinase complex is measured by clot formation, while the tenase complex generated is determined

by measuring the chromogenic factor Xa (FXa) activity. The expensive chromogenic assay, which is usually considered technically challenging and complex, is less easily automated. It shows an LLOQ of 0.01 IU/ml, while relative standard deviations are commonly between 5% and 10%. Direct thrombin inhibitors and heparin do not influence the chromogenic assay, but the presence of direct FXa inhibitors or FVIII activity-mimicking antibodies inhibits or enhances the activity measured with the chromogenic assay.^{15,16} Using bovine factor X (FX) has been shown to overcome the assay interference described for the bispecific antibody emicizumab.^{17,18} Discrepancies between results from the one-stage and the chromogenic assay are well described and have been extensively reviewed.^{19,20}

Recently, a new type of chromogenic assay, termed the *modification-dependent activity assay* (MDAA),²¹ has been developed. This assay enables selective measurement of PEGylated²²⁻²⁴ or polysialylated²⁵⁻²⁷ recombinant FVIII in the presence of nonmodified FVIII. The MDAA uses a modification-recognizing capturing agent, which currently is an antibody against polyethylene glycol or polysialic acid that selectively binds modified FVIII in the presence of endogenous, nonmodified FVIII. The activity of thus in-line purified modified FVIII can be measured by a conventional chromogenic assay. This approach to measuring modified FVIII was also shown to overcome the sometimes-challenging differentiation between endogenous nonhuman and human FVIII, which can obviously contribute to bias in pharmacokinetic studies.²⁸

Here, we extended this basic concept of chromogenic activity measurement after in-line purification of modified FVIII to nonmodified FVIII, as we had been able to identify a suitable antihuman FVIII monoclonal antibody for this purification step. This assay, termed *antibody-bound chromogenic substrate assay* for FVIII (ABC-SA), not only enables measurement of human FVIII without any interference from endogenous FVIII derived from common laboratory animals but also removes other interferences described for the conventional chromogenic assay. Among them, the reversal of the interference by FVIII activity-mimicking agents, irrespective of the chromogenic assay reagents used, seems to be the most noteworthy.

2 | MATERIALS AND METHODS

2.1 | Materials

The following monoclonal anti-FVIII antibodies were used: GMA-8024, GMA-8023, and GMA-012, all from Green Mountain Antibodies, ESH-4, ESH-8, and N55195 (American Diagnostica), and RFF-VIIIC/5²⁹ and RFF-VIIC/8²⁹ from Bio-Rad. MaxiSorp F96

flat-bottom plates were obtained from ThermoFisher Scientific. All analytical-grade buffer chemicals were purchased from VWR; Tween 20 (EIA grade) and nonfat dry milk (blotting-grade blocker) were from Bio-Rad, and benzamidine hydrochloride monohydrate from Sigma. The chromogenic assay reagents were from Takeda Manufacturing Austria (commercially available from Technoclone); a detailed method description is supplied in the Appendix S1. The human plasma standard ORKL17 was from Siemens, and the normal human reference plasma preparations used were from Precision BioLogic (CRYOCheck, Coachrom) and George King Bio-Medical Inc. Citrated plasma samples (sheep, goat, cynomolgus and rhesus monkey, rabbit, and rats) and serum samples (mouse, swine, hamster, and guinea pig) from laboratory animals were from Rockland. The bispecific anti-factor IXa (FIXa)/FX antibody emicizumab (Hemlibra) was from Roche. Finally, the solvent/detergent (S/D) solution investigated contained 1% Triton X-100, 0.3% Polysorbate 80 and 0.3% tri-n-butylphosphate (TnBP), all purchased from VWR. Plasma-derived von Willebrand factor (VWF)-FVIII concentrate IMMUNATE S/D (Takeda Manufacturing Austria), recombinant full-length FVIII ADVATE (Takeda Manufacturing Austria) and B domain-deleted (BDD) recombinant FVIII (rFVIII Refacto; Pfizer) were used as the FVIII source.

2.2 | Methods

2.2.1 | Antibody-based chromogenic substrate assay for FVIII

A detailed test protocol for the FVIII ABCSA is provided in Appendix S1. Briefly, 100 µl/well antibody GMA-8024 was coated at 4°C overnight. The washed plate (phosphate-buffered saline [PBS], 0.05% Tween 20 [PBST]) was blocked with dilution buffer [PBST with 0.1% non-fat dry milk, 2 mM benzamidine, 650 mM NaCl]. The washed plate was incubated with the serial dilution series of the standard and the samples at room temperature (RT) for 1 h. After a washing step, the chromogenic assay was carried out on the plate. Briefly, 40 µl/well reagent A (diluted 1 + 1 with FVIII dilution buffer) was loaded; reagent B (20 µl/well) was subsequently added, and the plate was incubated at RT for 15 min. Substrate (100 µl/well) was added, and after incubation at RT for 25 min, the stopping solution (20% acetic acid) was added (40 µl/well). The CSA procedure was implemented on a Precision 2000 pipetting system (Bio-Tek, Szabo) and carried out automatically. The plate was measured at 405 nm (reference wavelength, 620 nm). Quantitative evaluation was based on a double-logarithmic calibration curve established between the mean blank-corrected optical densities (ODs) of the duplicates and the FVIII concentrations of the six nonzero assay calibrators. Standard human plasma from Siemens ORKL17 was used to construct the calibration curve ranging from 3 to 97 mIU FVIII/ml.

2.2.2 | Selection of a capturing antibody

There are two indispensable requirements for the capturing antibody: selectivity for human FVIII and no interference with the chromogenic FVIII assay. The latter requirement was investigated for eight monoclonal antibodies, which were coated at 4°C overnight to the wells of a NUNC F96 microplate. After a washing step with PBST and blocking the wells, FVIII samples containing 188, 94 and 47 mIU/ml were incubated at RT for 1 h. Then, the plate was washed, and the chromogenic assay carried out as described above.

2.2.3 | Influence of FVIII integrity and nonhuman animal specimens on the assay

While our main interest was to measure full-length FVIII, we nevertheless investigated if BDD rFVIII could be measured with a similar performance or if the missing B domain unexpectedly impacted the capture efficiency. For this reason, we prepared calibration curves using BDD rFVIII. According to the manufacturer's specification, the antibody GMA-8024 finally selected only binds human FVIII at its A2 domain. We nevertheless checked 1/10-diluted citrated sheep, goat, cynomolgus monkey, rhesus monkey, rabbit, and rat plasma samples for signal generation. Furthermore, we also analyzed 1/10-diluted mouse, swine, hamster, and guinea pig serum samples to exclude any interaction with animal serum components resulting in false-positive signals.

2.2.4 | Assay performance studies

Animal specimens (sheep, goat, cynomolgus monkey, rhesus monkey, rabbit, and rat plasma; and mouse, swine, hamster, and guinea pig serum) were spiked with 0.1 U/ml of human FVIII. These samples were measured in the final dilution of 1/10 to determine the FVIII recovery. Moreover, we spiked a citrated cynomolgus monkey plasma pool with human full-length and BDD rFVIII (approximately 0.08 IU/ml) and determined the accuracy and precision for the measurement of the low human FVIII levels in the presence of normal endogenous (animal) FVIII levels. This also allowed us to calculate the assay's total error, as outlined by the European Medicines Agency (EMA) guideline on bioanalytical assay validation.³⁰ Furthermore, assay repeatability was determined by six measurements of the full-length and BDD rFVIII preparation. Finally, data on the intermediate precision were collected over an observation period of 6 months, during which three operators carried out a total of 108 measurements of the assay control preparation with a FVIII level of about 0.6 IU/ml. These data were also used to check for the assay robustness with regard to a possible influence of the operator on the assay.

2.2.5 | Influence of S/D reagents and emicizumab

The ABCSA provides the option to also measure FVIII activity in sample matrices so far inaccessible for direct measurement because of considerable interference with the CSA. This option arises from the fact that interfering substances can be removed after capturing FVIII to a solid support, as long as such substances do not impact the capturing step itself. The S/D solution (1% Triton X-100, 0.3% Polysorbate 80, 0.3% TnBP) is an example of a specific matrix that is incompatible with the chromogenic assay but with none of the components expected to interfere with the antibody-mediated capture of FVIII. To prove this reasonable assumption, samples containing 1 IU FVIII/ml were prepared with and without S/D solution and comparatively analyzed. Furthermore, the probable influence of the single components was checked at FVIII concentrations of 10, 1 and 0.1 IU/ml. FVIII activity-mimicking bispecific anti-FIXa/FX antibodies interfere with the FVIII chromogenic assay when reagents of human origin are used. Supposedly, antibody-based capturing of human FVIII followed by the chromogenic assay also provides the option to measure FVIII activity in samples, despite the presence of FVIII activity-mimicking antibodies. To prove this reasonable assumption, normal human plasma was spiked with therapeutically relevant concentrations of emicizumab (60, 200, and 600 nM).

3 | RESULTS

3.1 | Selection of appropriate capturing antibody

Figure 1 shows a schematic representation of the reaction sequence of the FVIII ABCSA. After FVIII has been captured by the monoclonal antibody, bound FVIII released from its tight complex

with VWF³¹ is activated by thrombin and exerts its FIXa cofactor activity. Both initial events, complex release, and thrombin activation, proceed without any obvious limitation in solution but could be impeded when FVIII has been adsorbed by a solid phase-bound antibody. As the probable impact depends mainly on the binding epitope of the antibody, we decided to check monoclonal antibodies, predominantly such with binding epitopes disclosed. Figure 2 shows the signals obtained for three FVIII concentrations: 188, 94, and 47 mIU/ml. Heterogenous but in all instances dose-dependent responses were obtained. Two of these antibodies, GMA-8024 and GMA-8023, demonstrated higher signals than the six others. Both antibodies bind to FVIII's heavy chain within the A2 domain and have been reported to show no interference in an aPPT assay. We were prompted to select GMA-8024 due to its slightly higher signal in all three FVIII concentrations tested. Interestingly, GMA-012, binding as well to the A2 domain, showed a clearly lower response, while the two antibodies RFF-VIIIc/5 and RFF-VIIIc/8, binding to the light and heavy chain, respectively, showed almost no response.

3.2 | Assay calibration curve

The EMA guideline for bioanalytical assay validation³⁰ recommends at least six nonzero standards for the calibration curves of ligand-binding assays, without giving any preferences for the mode of curve fitting. Based on our MDA and ELISA³² experiences, we relied on a log-log fitting calibration model rather than a 4/5-parameter fitting model. These curves were constructed by using a human standard plasma supplied by Siemens, showing a FVIII concentration of 0.98 IU/ml. The log-log six-point calibration curve ranged from 3 to 97 mIU FVIII/ml. Figure 3A shows the mean calibration curve obtained in 108 independent runs, carried out by three operators. The mean slope and y-intercept showed RSDs of 4.6% and 5.3%,

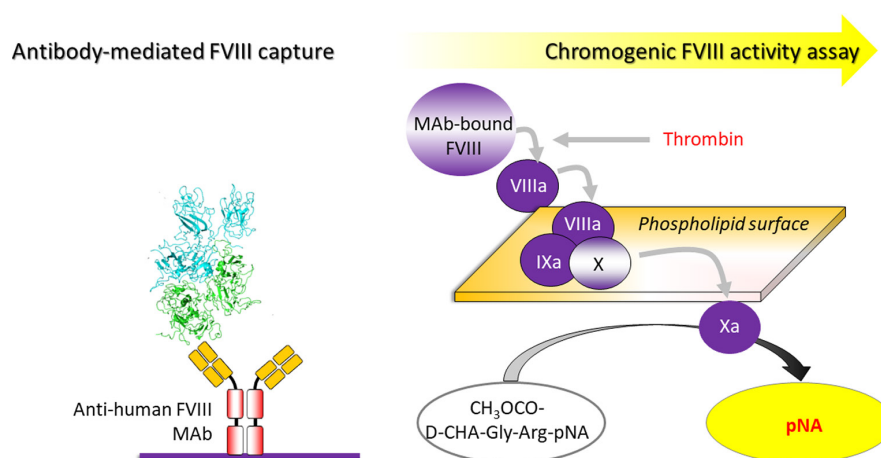


FIGURE 1 Assay principle. The assay principle of the antibody-based chromogenic substrate assay (ABCSA) for factor VIII (FVIII) is shown. After the selective capture of human FVIII via a physically adsorbed anti-A2 FVIII monoclonal antibody (left part), the chromogenic assay factor IX (FIX) cofactor assay (right part) is carried out with the solid-phase adsorbed FVIII. Nonhuman FVIII and other possibly interfering substances are removed by a washing step done before the CSA. The capture step is done in the presence of the reversible protease inhibitor benzamidine to protect FVIII against proteolytic degradation. MAB, monoclonal antibody; pNA, p-nitroanilide.

FIGURE 2 Capturing antibody selection. The eight monoclonal antibodies were physically adsorbed overnight at 4°C to the wells of a Nunc microplate at a concentration of 10 µg/ml in phosphate-buffered saline. After a blocking step, the wells were incubated with samples containing three factor VIII (FVIII) levels (188, 94, and 47 mIU/ml) and the chromogenic substrate assay was carried out. Bars show the mean blank-corrected optical densities (ODs) measured.

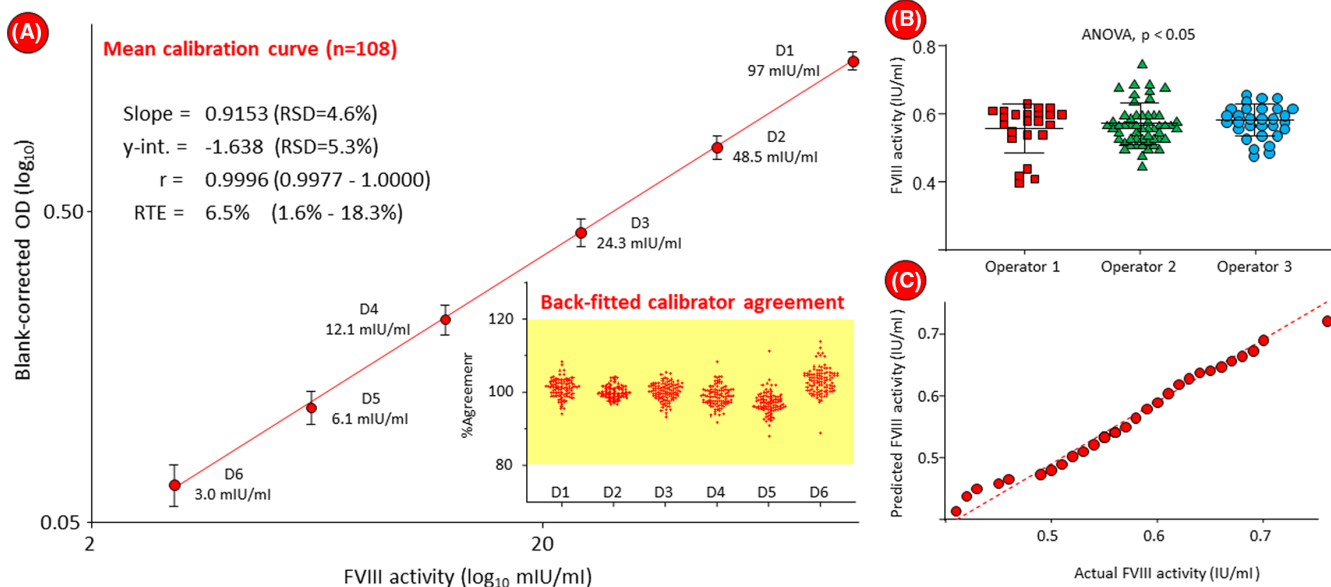
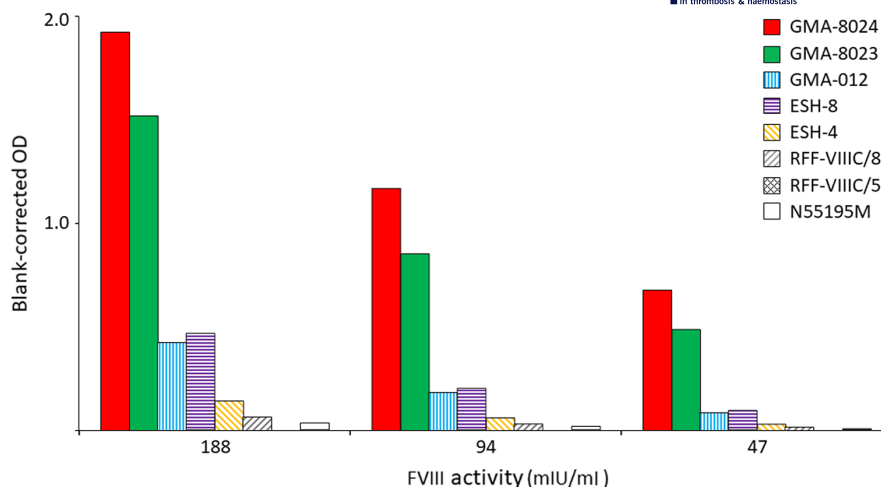


FIGURE 3 Assay calibration curves and assay control performance. Panel A shows the mean log-log calibration curve ranging from 3 to 97 mIU/ml factor VIII (FVIII). Error bars mark the single standard deviation of the blank-corrected optical densities (ODs). The upper insert shows the calibration curve characteristics slope, y-intercept, correlation coefficient *r* and relative total error RTE, while the lower insert gives the agreement of the back-fitted assay calibrators. Panels B and C present data obtained for the assay control over a period of 6 months: Panel B compares the results obtained by three operators with no significant difference between these results; Panel C shows a quantile-quantile plot for the data demonstrating their normal distribution. RSD, relative standard deviation; RTE, relative total error.

respectively, demonstrating the low variability of these curves, characterized also by a good quality of fitting as shown by their high correlation coefficients and low relative total errors (RTEs). A mean RTE of 6.5% with individual values ranging from 1.6% to 18.3% proved the selection of an adequate calibration curve-fitting model. Agreement of the back-fitted assay calibrators is shown in the insert of Figure 3A: All individual values were clearly within the 100%±20% defined by the EMA guideline for a valid calibration curve. Thus, the sensitive calibration curve allowing the unambiguous discrimination of 3 mIU/ml FVIII from blank samples could be accurately and reproducibly constructed. With regard to the assay sensitivity, the ABCSA for FVIII did not differ obviously from the standard chromogenic assay carried out in solution, despite the limited accessibility.

3.3 | Assay response for BDD FVIII

We compared the assay response for full-length and BDD FVIII on the basis of the calibration curve characteristics obtained (Table 1). These curves demonstrated similar ranges and shapes, and the latter were evaluated on the basis of their slopes and y-intercepts, which differed by 8.7% and 3.0%, respectively. For the slopes, this difference was lower than 9.2%, representing the two-fold standard deviation (SD) determined for the mean slope of the full-length FVIII calibration curve, while the y-intercept differed even less than the respective single SD. The mean correlation coefficient of 0.9989 and the mean relative total error of 12.0% confirmed the accuracy of the curve-fitting model applied and also when BDD FVIII was used. In essence, similar slopes obtained for

the full-length and BDD FVIII calibration curve evidenced that the capture step worked with similar efficiency for the full-length and the truncated FVIII molecules.

3.4 | Assay performance studies

The EMA guideline for bioanalytical assay validation³⁰ comprehensively summarizes the requirements for bioanalytical assays, discriminating between chromatographic and ligand binding assays. Although activity assays are not mentioned explicitly, it seems reasonable to classify such assay types as ligand-binding assays

TABLE 1 Comparison of assay calibrations curves obtained with full-length and BDD FVIII

Characteristic	BDD FVIII (n = 4)	Full-length FVIII (n = 108)	%Δ
Upper FVIII level (mIU/ml)	93.8	97.0	3.4
Lower FVIII level (mIU/ml)	2.9	3.0	3.4
Slope (mean)	0.9987	0.9153	8.7
y-intercept (mean)	-1.69	-1.64	3.0
r (mean)	0.9989	0.9996	0.1
RTE (mean)	12.0	6.5	N/A
Maximum RTE	17.0	18.3	N/A

Note: Slope, y-intercept, correlation coefficient *r*, and RTE of the log-log calibration curves obtained with full length and BDD FVIII were compared on the basis of their means. The RTE was calculated according to $RTE = (|x_N - x_M| + 2 SD) / x_N \times 100$. x_N and x_M represent the nominal and the measured mean, respectively, and SD the standard deviation of x_M . To obtain the x_M , the mean blank-corrected optical densities of the individual dilutions were back-fitted on the curve, normalized by multiplication with the respective dilution and finally averaged. The column “%Δ” shows the relative differences determined. Abbreviations: BDD, B domain-deleted; FVIII, factor VIII; N/A, not applicable; RTE, relative total error.

because the activity measurement requires an initial binding interaction of at least two partners. We followed the definitions provided by the guideline for the common assay characteristics in our assay performance study including specificity/selectivity, accuracy, precision, and linearity.

3.4.1 | Specificity for human FVIII

The specificity of the human FVIII ABCSA was confirmed by measuring different citrated laboratory plasma and serum samples from laboratory animals at the minimum dilution of 1/10 (Table 2). Even at the lowest dilution tested, none of the samples tested showed ODs that were significantly higher than the OD measured for the blank. Only sheep and rabbit plasma samples showed slightly higher ODs, but the relative differences were as low as 0.5% and 2.7%, respectively, and can thus be deemed to be within the assay variability. All animal sample ODs, however, were at least 20% lower than those of the corresponding assay standard D6 containing 2.9 mIU FVIII/ml. These data confirmed the selectivity of the ABCSA for human FVIII.

3.4.2 | Accuracy

The animal specimens (citrated plasma and serum) were spiked with 0.1 IU/ml of human FVIII (Table 3). The FVIII recoveries obtained were good, that is, within the commonly accepted 80%–120% range.³⁰ In particular, the recoveries varied from 83.3% (goat plasma) to 116.9% (rat plasma). There was no signal from endogenous (animal) FVIII, which would have increased the recovery. Furthermore, recovery of spiked human recombinant full-length FVIII (0.082 IU/ml) and BDD FVIII (0.088 IU/ml) was investigated in citrated cynomolgus plasma samples. Again, the citrated

Animal specimen	OD			Relative differences	
	D6	Blank	Sample	ΔBlank%	ΔD6%
Sheep (plasma)	0.248	0.195	0.196	0.5	-21.0
Goat (plasma)	0.248	0.195	0.181	-7.2	-27.0
Cynomolgus (plasma)	0.248	0.195	0.175	-10.3	-29.4
Rabbit (plasma)	0.245	0.188	0.193	2.7	-21.2
Rat (plasma)	0.245	0.188	0.184	-2.1	-24.9
Rhesus (plasma)	0.245	0.199	0.195	-2.0	-20.4
Mouse (serum)	0.245	0.188	0.188	0.0	-23.3
Swine (serum)	0.256	0.198	0.196	-1.0	-23.4
Hamster (serum)	0.256	0.198	0.193	-2.5	-24.6
Guinea pig (serum)	0.261	0.199	0.196	-1.5	-24.9

TABLE 2 Selectivity investigation for animal plasma and serum specimens

Note: ODs are shown for D6, the assay standard with the lowest FVIII concentration (2.9 mIU/ml), the blank and the 1/10-dilution of the animal specimen sample. The columns “ΔBlank%” and “ΔD6%” show the relative differences calculated for the sample and the blank and D6, respectively. Abbreviations: FVIII, factor VIII; OD, optical density.

TABLE 3 Recovery of human FVIII (100 mIU/ml) spiked to animal specimens (citrated plasma and serum)

Animal specimen	Recovery in % (100 mIU/ml)
Sheep (plasma)	97.2
Goat (plasma)	83.3
Cynomolgus (plasma)	89.0
Rabbit (plasma)	95.8
Rat (plasma)	116.9
Rhesus (plasma)	98.5
Mouse (serum)	86.7
Swine (serum)	88.9
Hamster (serum)	108.0
Guinea pig (serum)	114.8

Note: Recovery is shown as a percent of the spiked FVIII concentration of 0.1 IU/ml. FVIII concentrations for the samples were obtained as means of the results obtained for the individual dilutions of the dilution series when those provided ODs, which were within the range of the calibration curve.

Abbreviations: FVIII, factor VIII; OD, optical density.

cynomolgus plasma sample did not show any response. The FVIII concentrations found differed by less than 10% from the expected ones, with mean recoveries of $102.7\% \pm 2.8\%$ and $92.4\% \pm 2.4\%$ (mean \pm SD, $n = 3$) for full-length and BDD FVIII, respectively. The total errors calculated from these data were 5.5% and 10.0% for full-length and BDD FVIII, respectively. These errors are far below the EMA guideline acceptance criterion of $\leq 30\%$.³⁰ Further data showing successful use of the human FVIII ABCSA for the pharmacokinetic analysis of the PEGylated FVIII preparation Adynovate in cynomolgus monkeys and minipigs are provided as supplemental data (Figure S1).

3.4.3 | Precision analysis

The assay's repeatability was investigated by triplicate measurement of spiked citrated cynomolgus monkey plasma samples at FVIII concentrations of about 0.1 IU/ml. The RSDs of the triplicate testing did not exceed 3%. Furthermore, full length (approximately 30 IU/ml) and BDD FVIII (approximately 1700 IU/ml) were subjected to a sixfold measurement in one run. The means showed the low RSDs of 3.0% and 1.8% for the full-length and the BDD rFVIII, respectively. Intermediate precision, finally, was addressed by the repeated analysis of the assay control (Figure 3B,C) over a period of 6 months. A total number of 108 measurements was carried out by three operators resulting in a mean FVIII concentration of 0.57 ± 0.06 IU/ml (RSD, 10.4%). The data were normally distributed as checked with the D'Agostino-Pearson test ($p = 0.12$; GraphPad Prism 8.3). ANOVA (GraphPad Prism 8.3) showed that there was no statistically significant difference between the results obtained by the three operators ($p = 0.34$).

3.4.4 | Linearity analysis

Calibration curve standards can be prepared for measuring FVIII in the matrix of animal specimens by using the respective blank animal specimen or test buffer. To apply the latter approach, adequate parallelism of the samples' dilution-response curves to that of the assay standard is a prerequisite. The use of calibration curves based on buffer dilution not only increases the assay robustness but also lessens its complexity because it reduces the number of biological reagents required and renders unnecessary the preparation of animal specimen-specific calibration curves. To establish this simplification, we checked the parallelism of dilution-response curves obtained for animal specimens spiked with 0.1 IU/ml human FVIII and FVIII diluted in buffer (Figure 4). The dilution-response curves obtained for all animal specimens investigated at their minimum dilution of 1/10 were adequately parallel to those of the respective assay standard in the buffer matrix. Thus, despite the substantial, though proportionally decreasing, levels of animal protein present during the antibody capture, their slopes differed by less than 8% from that of the assay standard, where no animal protein was present. Furthermore, the linearity of these curves was good as shown by their squared correlation coefficients, which were at least 0.992. These data demonstrated that the animal protein matrix had no essential influence on the assay, thus supporting the important assay simplification of using a buffer-based calibration curve.

3.5 | Influence of S/D reagents

The S/D mixture (1% Triton X-100, 0.3% Polysorbate 80, 0.3% TnBP) is an example of a matrix that is incompatible with the chromogenic FVIII assay. Its presence, however, is not expected to interfere with the antibody-driven FVIII capture. To prove this reasonable assumption, human FVIII solutions were measured in the presence and absence of S/D solution and their individual compounds (Figure 5). Almost identical, overlapping dilution-response curves were obtained with slopes differing by only 0.5%. We furthermore spiked three FVIII solutions (10, 1, and 0.1 IU/ml) with the S/D mixture and the individual components at their levels present in the S/D mixture (Table 4). Adequate recoveries, that is, within an 80%–120% range, were found for all spiked samples, even at the low FVIII concentration of 0.1 IU/ml. None of the individual components of the S/D solution showed considerable impact on the assay.

3.6 | Measurement in the presence of the FVIII activity-mimicking antibody emicizumab

Bispecific anti-FIXa/FX antibodies interfere with chromogenic FVIII measurement if assays that use human coagulation factors are applied. Antibody-based capturing of human FVIII followed by the chromogenic assay provides the option to also measure FVIII activity in human plasma containing FVIII activity-mimicking antibodies.

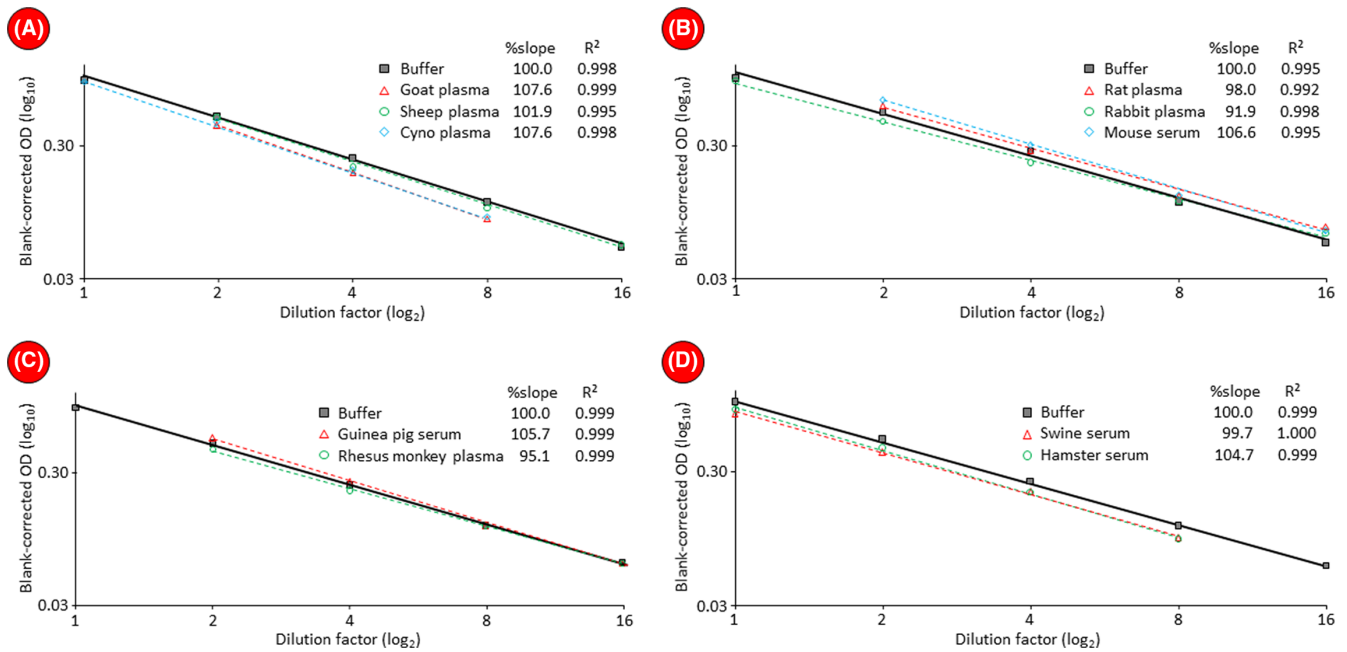


FIGURE 4 Assay parallelism in animal plasma and serum samples. Dilution-response curves (log/log) are shown for goat, sheep, and cynomolgus monkey plasma (A), rat and rabbit plasma and mouse serum (B), guinea pig serum and rhesus monkey plasma (C), and swine and hamster serum (D). The black curve (full line) represents the dilution-response curve obtained for the assay standard, diluted in buffer only.

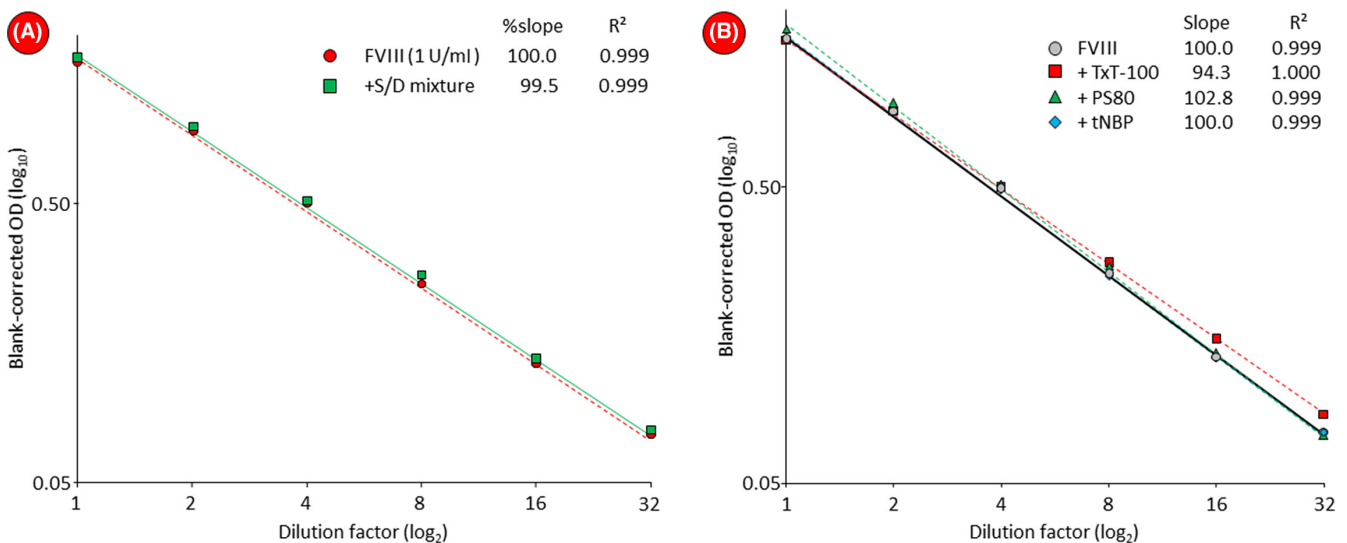


FIGURE 5 Influence of solvent-detergent solution and individual reagents. Dilution-response curves (log/log) are shown for factor VIII (FVIII) and FVIII in the solvent/detergent (S/D) mixture (A) and in the presence of the individual components (B). Inserts show the relative respective slopes of the dilution response curves, expressed as a percentage of that obtained for the FVIII solution in buffer, and their squared correlations coefficients. OD, optical density.

As expected, the addition of the therapeutically relevant concentrations 9, 30, and 90 µg/ml (60, 200, and 600 nM) of emicizumab to normal human plasma did not influence the FVIII activity measured in a human normal plasma: FVIII levels ranged from 0.81 to 0.86 IU/ml FVIII. Emicizumab itself, measured at a concentration of 90 µg/ml did not show any response. FVIII recoveries in the presence of emicizumab were 98.8% at a 60-nM emicizumab level, and 104.4% and 101.7% at 200 and 600 nM, respectively. The dose-response curves obtained (Figure 6) reasonably allowed any remarkable influence of emicizumab on the FVIII ABCSA to be excluded. These

results provide evidence that the assay allows selective measurement of human FVIII activity in patient plasma containing bispecific anti-FIXa/FX antibodies.

4 | DISCUSSION

The development of chromogenic assays for measuring FVIII activity without doubt opened new options for product development and somehow paved the way for the development of the FVIII

activity-mimicking bispecific antibody, which was selected on the generation of chromogenic FXa activity.³³ Like all other coagulation assays, the chromogenic assay proceeds in solution, which allows unrestricted and mutual access for all reaction partners. Consequently, the reaction rates depend in the first instance exclusively on the reaction partners' solution concentrations and the solution temperature. The FVIII ABCSA described here differs distinctly from such assays because FVIII is selectively captured by an antibody to a solid phase. It therefore seemed interesting to estimate the amount of FVIII available for the chromogenic assay. Consequently, a total average amount of 400 ng antibody/cm² well surface was determined³⁴ translating under the coating conditions applied to a total amount of 0.23 nM IgG/well.³⁵ Presupposing the formation of an equimolar complex, a maximum binding capacity of about 0.3 U FVIII/well can be estimated assuming a plasma FVIII concentration of 100 ng/ml³⁶ with a molecular weight of 150 kDa. This rough calculation is based on several expectations, including the full functional capacity of the plate-bound antibody, which might not be the case,³⁷ and the equimolar ratio for the antibody-FVIII complex formation, although there are theoretically two binding sites per IgG molecule available for binding. However, the fact that Fab parts of the physically adsorbed IgG have also been shown to directly contact the

solid phase³⁵ supports the notion of an equimolar IgG-FVIII immune complex.

Another important fact relates to the accessibility of the FVIII bound to the solid phase: Without doubt, there will be lower exchange rates for the reaction partners due to the diffusion-limiting effects of the solid phase compared with the solution. There are theoretical considerations describing antigen and antibody interactions at surfaces³⁸ that demonstrate there are restrictions, especially if macromolecules are involved. Thus, the rate of the initial antibody binding was shown to become diffusion rate limited and affected by steric hindrance. Overall, the reaction kinetic observed in heterologous systems clearly differ from that of the corresponding homogeneous, that is, liquid system. Stenberg and Nygren³⁹ and Stenberg et al.⁴⁰ presented models that addressed this diffusion rate-limited reaction on the surface, while Mushens and Scott⁴¹ clearly demonstrated the benefits of orbital shaking compared with static incubation conditions.

A heterologous system for measuring activated protein in plasma has been described.⁴² The so-called enzyme capture assay (ECA) applied an anti-protein C antibody in the presence of the reversible inhibitor benzamidine. After removal of the inhibitor, the amidolytic activity of activated protein C was determined with a suitable chromogenic substrate. The authors reported a sensitive standard curve and successfully used the ECA for measuring activated protein C in human plasma specimens. This enzyme capture assay can be considered as the simplest variant of a heterologous test because only the hydrolysis of the low-molecular-weight chromogenic substrate is carried out by the surface-bound activated protein C. Restrictions in accessibility will result in slower substrate hydrolysis rates than observed in solution, but as only one step is concerned, this reduction in reaction rate could be compensated by simply increasing the reaction time or the substrate concentration. By contrast, the FVIII ABCSA requires a well-defined, complex sequence of events for the final generation of the chromogenic FXa signal.

The data presented here for the FVIII ABCSA demonstrate that despite the restrictions discussed above, an adequate sensitivity was nevertheless reached that did not differ essentially from that of

TABLE 4 Influence of the S/D mixture and their individual components on FVIII ABCSA

Compound	Recovery (%)		
	10 IU/ml	1 IU/ml	0.1 IU/ml
S/D mixture	109.0	105.0	100.0
Triton X-100 (1%)	N/D	106.0	100.0
Polysorbate 80 (0.3%)	N/D	103.0	110.0
Tri-n-butylphosphate (0.3%)	N/D	98.0	90.0

Note: Recovery is shown as a percent of the nominal FVIII concentration.

Abbreviations: ABCSA, antibody-bound chromogenic substrate assay; FVIII, factor VIII; N/D, not determined; S/D, solvent/detergent.

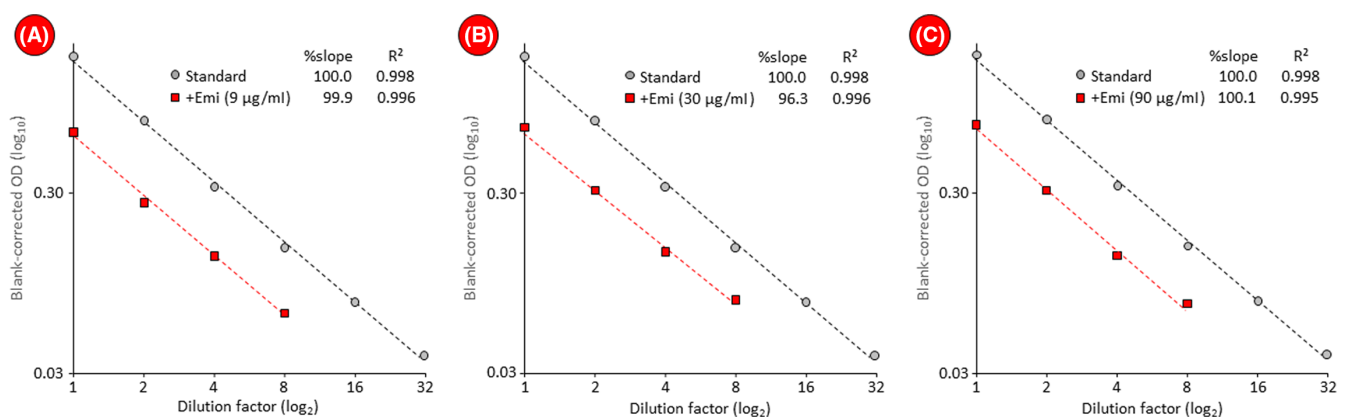


FIGURE 6 Influence of emicizumab. Panels A and B compare the log-log dilution-response curves of normal plasma and normal plasma spiked with 9 and 30 µg/ml emicizumab, while panel C shows the same data for an emicizumab level of 90 µg/ml. OD, optical density.

a conventional FVIII chromogenic assay. Our understanding is that there were several reasons for this finding, including the choice of the capturing antibody, the composition of the dilution buffer, and the modifications introduced to the chromogenic assay itself. Using continuous orbital shaking and increasing the reaction time for the CSA seemed to have successfully compensated for these limitations discussed above. Similarly, using a dilution buffer with an NaCl concentration of 650mM was beneficial, as these conditions favored the dissociation of the VWF-FVIII complex. The essential requirement for releasing FVIII from its tight complex with VWF during thrombin activation has been described.⁴³

A similar assay sensitivity had been reported for the MDAs. In these assays, however, modified FVIII is captured via the polyethylene or polysialic acid moiety, which probably acts as a kind of spacer and confers a certain degree of mobility to FVIII. Clearly, binding FVIII via a plate-adsorbed anti-FVIII A2 domain antibody will result not only in a closer distance to the solid support but also most probably reduce the flexibility of the bound FVIII. Therefore, the assay sensitivity obtained was unexpected and seemed to be caused by the optimal choice of the capturing antibody and composition of the dilution buffer. Based on our experience gained during the MDAA development, we also added the nonspecific, reversible protease inhibitor benzamidine to the dilution buffer. This benzamidine addition was supposed to protect FVIII against proteolytic degradation during the initial capturing step, when the plasma matrix is still present. Antibody-bound FVIII, released from its complex with VWF, might be even more susceptible to proteolytic attack. Subsequently, however, the complete removal of benzamidine is a must as already traces of this inhibitor would impact the CSA. Therefore, extensive washing is essential after incubation with the samples/standards.

Finally, the specificity of the selected antibody for human FVIII allowed us to fully eliminate the detrimental impact of endogenous FVIII levels in non-hemophilia A models and interference of FVIII activity-mimicking compounds. Conventional coagulation and chromogenic FVIII activity assays are unable to differentiate between endogenous animal and administered (human) FVIII. A common approach to handle this limitation is the baseline subtraction method, meaning that the FVIII levels determined after the administration of human FVIII are corrected by subtraction of the baseline FVIII level, that is, the FVIII concentration measured before administration. This procedure assumes that the baseline concentration does not change over time, but this might be not the case because FVIII has been described as an acute-phase reactant.⁴⁴ Increased FVIII levels, controlled by interleukin-6,⁴⁵ have been reported after surgery and after administration of lipopolysaccharide to volunteers.⁴⁶ Changes in the endogenous FVIII levels will significantly bias the pharmacokinetic data obtained. This bias becomes even more important at later time points in pharmacokinetic studies when a substantial fraction of the administered FVIII has already been cleared and the difference between endogenous and administered FVIII reaches zero. The ABCSA developed does not require baseline correction because endogenous, nonhuman FVIII activity is not measured. Two representative examples for concentration versus time profiles, obtained for

the PEGylated recombinant FVIII preparation in cynomolgus monkey and minipigs, are shown in Appendix S1. Also, data presented clearly demonstrated that human plasma samples containing therapeutically relevant emicizumab concentrations could be measured without any interference, even though the ABCSA used human reagents. This is not the case for conventional chromogenic assays.

Overall, FVIII cofactor activity can be selectively measured in an animal plasma matrix containing endogenous FVIII by a heterogeneous assay that combines selective antibody-driven capture of FVIII followed by a chromogenic assay carried out with the solid-phase captured FVIII. This method is applicable for ex vivo plasma testing in the course of nonclinical studies where investigational FVIII products are administered on top of the endogenous FVIII present in the animal. Furthermore, the assay can be used for manufacturing-related matrices, where so far FVIII measurement has not been feasible by assay interference. In a clinical setting, finally, the assay enables human FVIII to be measured in the presence of FVIII activity-mimicking agents, such as bispecific antibodies, without interference by these agents.

AUTHOR CONTRIBUTIONS

Conceptualization: G. Schrenk and A. Weber; methodology: A. Engelmaier, H. Gritsch, G. Schrenk, and A. Weber; investigation: A. Engelmaier, M. Billwein, and C. Zlabinger; writing – original draft: A. Weber; writing – review and editing: M. Billwein, A. Engelmaier, H. Gritsch, G. Schrenk, A. Weber, and C. Zlabinger; visualization: A. Weber. All authors reviewed and agreed to the final manuscript.

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All authors are full-time employees of Baxalta Innovations GmbH, part of Takeda. G. Schrenk, H. Gritsch, and C. Zlabinger hold stocks of Takeda.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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