ORIGINAL ARTICLE

Detecting factor XIa in immune globulin products: Commutability of international reference materials for traditional and global hemostasis assays

Yideng Liang PhD | Joseph W. Jackson PhD (iD) | Samuel A. Woodle MD |
Stepan S. Surov PhD | Leonid A. Parunov PhD (iD) | Dorothy E. Scott MD |
Mark Weinstein PhD | Timothy K. Lee PhD | Mikhail V. Ovanesov PhD (iD)

Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, MD, USA

Correspondence
Mikhail V. Ovanesov, U.S. Food and Drug Administration, 10903 New Hampshire Ave, Silver Spring, MD 20993-0002.
Email: mikhail.ovanesov@fda.hhs.gov

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Abstract

Background: Activated coagulation factor XIa (FXIa) is an impurity and primary source of procoagulant activity in thrombosis-implicated immune globulin (IG) products. Several assays, of varying quality and precision are used to assess FXIa-like procoagulant activity in units relevant to their respective principles.

Objectives: To advance unified reporting, we sought to employ the World Health Organization reference reagents (RRs) to present the results of differing methodologies in units of FXIa activity and rank the sensitivity and robustness of these methodologies.

Methods: RR 11/236 served as a calibrator in several FXIa-sensitive blood coagulation tests: two commercial chromogenic FXIa assays (CAs); a nonactivated partial thromboplastin time (NaPTT); an in-house fibrin generation (FG) assay; an in-house thrombin generation (TG) assay; and an assay for FXIa- and kallikrein-like proteolytic activities based on cleavage of substrate SN13a. Some assays were tested in either normal or FXI-deficient plasma.

Results: Each method demonstrated a sigmoidal dose-response to RRs. NaPTT was the least sensitive to FXIa and the least precise; our in-house TG was the most sensitive; and the two CAs were the most precise. All methods, except for SN13a, which is less specific for thrombotic impurities, gave comparable (within 20% difference) FXIa activity assignments for IG lots.

Conclusions: Purified FXIa reference standards support quantitation of FXIa levels in IG products in all tested assay methodologies. This should help to standardize the measurement of thrombotic potentials in IG products and prevent products exhibiting high procoagulant activity from distribution for patient use. Further research is needed to address the effect of IG product-specific matrixes on assay performance.

Essentials

- Activated coagulation protein XIa (FXIa) is a main source of procoagulant activity in immune globulin products.
- Multiple methods test for FXIa activity, but report results in units respective to that method.
- World Health Organization reference reagent (RR) 11/236 yielded comparable FXIa activity assignments in all studied methods.
- RR 11/236 is a commutable reagent for both traditional and global hemostasis assays.

1 | INTRODUCTION

Activated coagulation factor XI (FXIa) plays a major role in the intrinsic (contact) pathway of the coagulation cascade via the activation of factor IX (FIX) to FIXa, which subsequently leads to the generation of thrombin.[1] FXI is activated by activated factor XII (FXIIa) in the contact phase of blood coagulation; thrombin in the positive feedback loop of the coagulation cascade; or by FXIa in an autoactivation reaction. All three reactions can be accelerated by negatively charged substances, which can be either physiological polyanions, for example, polyphosphates, extracellular DNA, and heparin; or artificial substances, for example, glass, kaolin, celite, or dextran sulfate.[2] The physiological importance of FXI is evident from the rare bleeding disorder hemophilia C caused by the deficiency of FXI. FXI is also recognized for its role in thrombosis.[3] Indeed, individuals with either FXI deficiency or elevated FXI levels had, respectively, overall lower or greater incidences of cardiovascular diseases, such as ischemic stroke and deep vein thromboembolism.[4-6]

Independent evidence supporting the potential role of FXIa in thrombosis came from the association between the presence of FXIa in FXI concentrates or immune globulin (IG) products and thrombotic adverse events (TAEs). A wide range of thrombotic complications, including myocardial infarction, deep vein thrombosis, and stroke, have been observed in patients treated with intravascular IG concentrates or early generations of FXI concentrates.[6-9] Notably, newer FXI concentrates that were manufactured with reduced FXIa levels did not elevate the markers of coagulation activation in humans and animals and were associated with a decrease in TAE frequency.[10] Similarly, several product recalls in 2010 and 2011 were initiated to remove IG product lots with high levels of FXIa from the market because of elevated incidences of TAEs. Following the recalls, manufacturing changes were introduced to control and reduce the amount of FXIa impurity in IG products. Product-related risk factors (manufacturing conditions, dosage, and route of administration) as well as patient-related risk factors (age, past thrombotic events, and coagulability potential) were also investigated.[11,12]

With evidence suggesting that limiting FXIa levels may improve the safety of IG products, drug manufacturers, national quality control laboratories, and other stakeholders have been developing assays to evaluate the activity of FXIa in these products. Nonactivated partial thromboplastin time (NaPTT), a traditional assay employed predominantly by manufacturers for the assessment and control of FIXa levels in prothrombin complex concentrates, was found to be

useful but not reliable.[13] An in-house and later commercial version of a chromogenic substrate assay (CA) for FXIa activity was developed involving the production of FIXa, which in turn activates factor X (FX) to FXa. The FXa activity is then measured using a chromogenic substrate. A global hemostasis assay of thrombin generation (TG) is another method suitable for measuring FXIa activity in IG products. In our laboratory, we successfully used an in-house TG assay to determine FXIa activity in TAE-associated IG product lots.[12,14]

Currently, commercial and in-house FXIa and global hemostasis assays are available to research and quality control laboratories to measure procoagulant activity. However, these assays often return differing results in parameters unique to that specific assay, making it difficult to ascertain the safe levels of FXIa activity in the product and to decide if the product is suitable for distribution. To address this need, the United Kingdom's National Institutes for Biological Standards and Controls (NIBSC) developed two FXIa reference materials, NIBSC 11/236 and NIBSC 13/100, which were adopted by the World Health Organization (WHO) as the WHO First International Reference Reagent,[13] and the WHO First International Standard for FXIa,[15] respectively. However, only the FXIa chromogenic assays were used for the calibration of these reference materials, and the applicability of calibration with FXIa reference material to global hemostasis assays has not been thoroughly investigated.

In this study, we sought to compare the sensitivity and range of traditional blood coagulation tests and global hemostasis assays and evaluate the commutability of WHO reference materials 11/236 and 13/100 in these FXIa activity assays. We have demonstrated alignment for chromogenic, NaPTT, microplate clotting (fibrin generation [FG]) and TG assays, for FXIa testing in IG products in both normal pooled plasma (NPP) or FXI-deficient plasma (FXI-DP). The data generated herein suggest that these assays can produce comparable and accurate results when calibrated with a FXIa standard, for example, NIBSC 11/236 or NIBSC 13/100, that may serve as commutable reference materials for all FXIa activity assays.

2 | MATERIALS AND METHODS

2.1 | Materials

WHO reference materials 11/236 and 13/100 were obtained from NIBSC (Potters Bar, UK). Purified in-house FXIa standard (IH-FXIa,

aliquot ~ 1 nM FXIa in Tris–bovine serum albumin [BSA] buffer) and substrate SN13a D-LPR-ANSNH-C3H7 2HCl[16] were purchased from Haematologic Technologies Inc (Essex Junction, VT, USA). An intramuscular IG product, IGIM Lot G, which contained about 0.3 IU/mL of FXIa and also kallikrein-like, non-FXIa impurities, was used as a control sample, and was supplied by the National Institutes of Health Pharmacy (Bethesda, MD, USA). Procoagulant reference materials made from IG preparations, codes C, D, E, and F,[15] were provided by the NIBSC.

2.2 | Commercial chromogenic substrate FXIa activity assays

Chromogenic substrate FXIa activity assays CA1 (Biophen FXIa; Hyphen Biomed, Aniara, West Chester, OH) and CA2 (ROX FXIa; Rossix AB, Molndal, Sweden) were performed according to the manufacturer's instructions except that they were downscaled to fit a half-area 96-well microplate format. Briefly, excess FIX was added to FXIa samples in the presence of phospholipids, and calcium allows for the subsequent activation of FIX to FIXa. This allows for the activation of FX to FXa, which is measured via a chromogenic substrate and is proportional to the amount of FXIa in the samples. Samples were diluted in Tris-BSA buffer (pH 7.4, FXIa chromogenic assay diluent, Biophen). CA1 and CA2 assay kinetics of absorbance increase was recorded on a Synergy H4 microplate reader (BioTek, Winooski, VT, USA) at 37°C. The maximal rate of substrate conversion within the first 10 minutes was used as the assay readout. In both assays, FXIa activity was calculated by comparing serially diluted samples against a calibration curve made with the frozen-thawed NIBSC 11/236.

2.3 | In-house fluorogenic substrate assay (SN13a)

The FXIa activity assay based on SN13a substrate was performed as described by Butenas et al.[17] Samples were mixed with substrate at a ratio of 1:1, and fluorescence was measured kinetically on a SpectraMax M2e microplate reader (Molecular Devices, San Jose, CA, USA) at 37°C. For each sample, the rate of substrate cleavage was calculated by taking the average rate over the time frame between 1 and 5 minutes.

2.4 | Nonactivated partial thromboplastin time

The assay was performed according to the European Pharmacopeia method, adapted to a half-area 96-well microplate design, using the UPTT reagent (Bio/Data Corporation, Horsham, PA, USA) and lyophilized normal pooled plasma (NPP, Biophen Normalplasma 2, Hyphen Biomed) or FXI-deficient plasma (FXI-DP; Affinity Biologicals, Ancaster, ON, Canada). The samples were diluted in Tris-BSA buffer

(Biophen) and added to plasma with a 96-channel pipettor (VIAFLO 96; Integra Biosciences, Hudson, NH, USA). Turbidity (492 nm) was measured on a Synergy H4 microplate reader (Biotek) at 37°C. The clot time was determined by an in-house software at a 15% level of the peak turbidity, which was normalized on the lowest and highest turbidity in the well.

2.5 | TG assays

The conditions of the TG-based assay were described previously.[18] Serially diluted samples (25% final volume [f.v.]) or FXIa standard (25% f.v., 0.01-0.125 mIU FXIa/mL final concentration [f.c.]) were arranged in a half-area 96-well microplate (assay plate). Human NPP or FXI-DP (50% f.v, Affinity Biologicals) was mixed with a fluorogenic substrate for thrombin Z-GGR-AMC (800 µM f.c., Bachem), phospholipid vesicles (12 µM f.c., Rossix AB), and recombinant tissue factor (TF, 0.1 pM f.c., ~1:60,000 dilution of RecombiPlasTin; Instrumentation Laboratory, Bedford, MA, USA). After recalcification via calcium chloride, 10 mM f.c., the reaction mixture was transferred to an assay plate using a VIAFLO 96-channel liquid handling system. AMC (7-amino-3-methylcoumarin) fluorescence (excitation at ~370 nm and emission at ~430 nm) was measured in a Biotek Synergy H4 (NPP experiments) or a Tecan F500 microplate reader (Tecan Austria GmbH, Grodig, Austria) (FXI-DP experiments) at 37°C. FXIa activity was calculated using the following TG assay readouts: thrombin peak height (TPH), area under the curve (AUC), lag time, time to thrombin peak (TTP) and peak TG velocity with the help of our in-house software, as previously described.[19,20] An internal thrombin calibrator (Stago, Parsippany, NJ, USA) was included in each microplate assay run to permit thrombin calibration using the calibrated automated thrombinography (CAT) algorithm.

2.6 | FG assay

Observation of TF-triggered fibrin clot formation in microplates (FG assay) was conducted simultaneously with the TG assay in the microplate reader by measuring the turbidity as absorbance at 492 nm. FXIa activity was calculated using two FG assay readouts, clot time and FG rate. Clot time was determined by in-house software at 45% level of the normalized lowest-to-peak turbidity.

2.7 | Statistical analysis

All samples were tested on one microplate (assay run) by the respective methods performed in parallel in assays on separate days, 2 to 7 days for each assay. FXIa activity was calculated as an average of activities derived from at least three serial dilutions of samples as described in Daniel et al[11] and Menis et al.[12] The assay readout for each dilution was compared with a calibration curve made with a
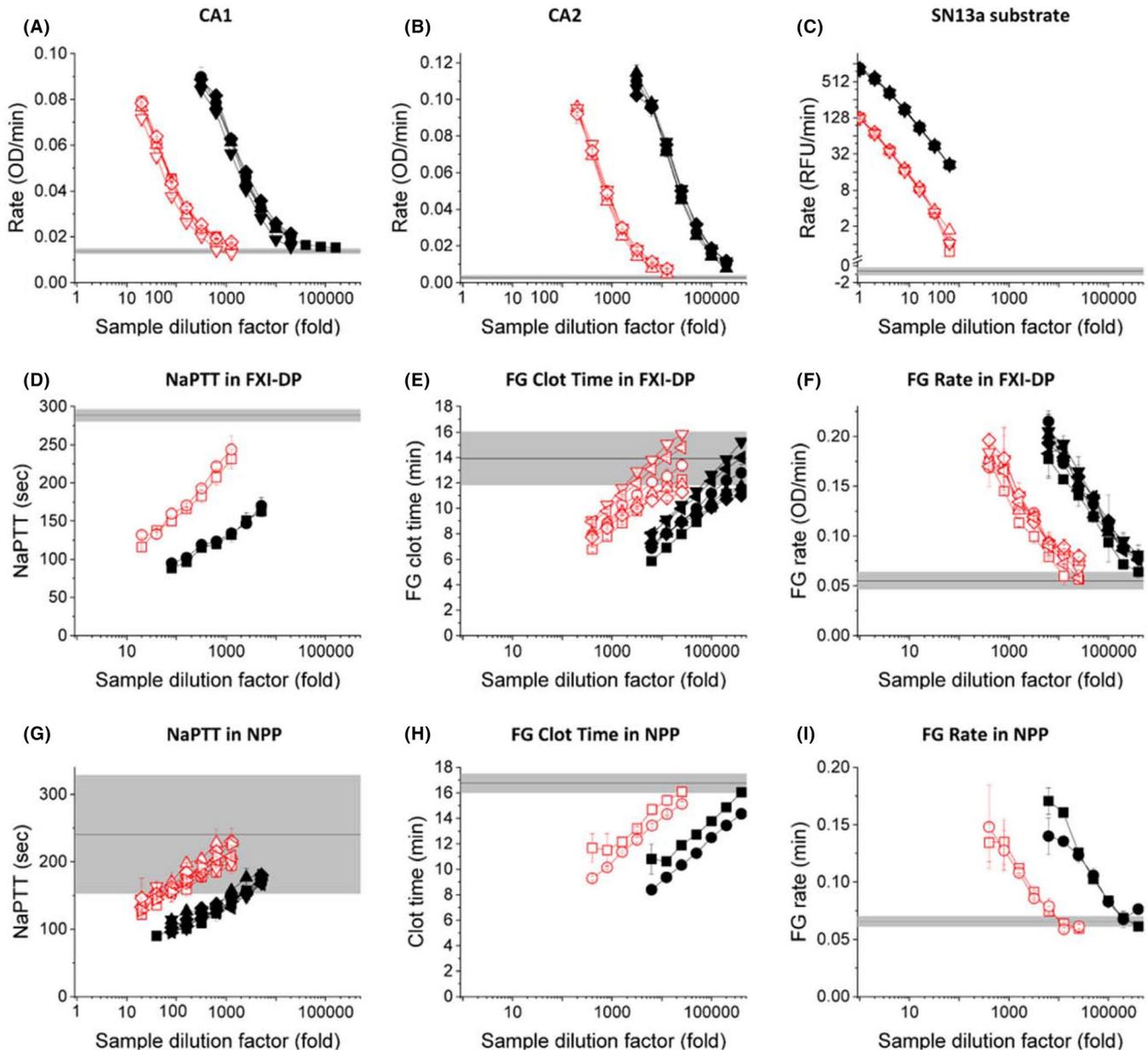
FIGURE 1 Parallelism of NISBC 11/236 and the procoagulant IGIM Lot G in multiple FXIa activity assays. The indicated dilutions of NIBSC 11/236 standard (black squares) and IGIM Lot G (red squares) were analyzed by multiple FXIa activity assays. A, CA1 (Biophen). B, CA2 (Rossix). C, SN13a substrate assay. D, NaPTT in FXI-DP. E and F, FG assay in FXI-DP. G, NaPTT in NPP. H and I, FG assay in NPP. Shown are means and SD for 2 replicate wells on one plate, n = 2-6 individual plates (runs) for each FXIa activity assay. Horizontal black line with gray shaded area is the blank value ± SD. CA1, Biophen chromogenic assay; CA2, Rossix chromogenic assay; FG, fibrin generation assay; FXIa, activated factor XI; FXI-DP, factor XI-deficient plasma; IGIM, intramuscular immunoglobulin; NaPTT, nonactivated partial prothrombin time; NIBSC, National Institutes for Biological Standards and Controls; NPP, normal pooled plasma.

frozen-thawed 11/236 FXIa standard (seven two-fold dilutions). Activity values for each sample were calculated using a cubic fit of the FXIa standard curve, and all samples were then normalized against the 11/236 sample (included on each plate) via the formula: [(calculated sample activity * 10)/ calculated 11/236 sample activity]. The unpaired t test was used to determine statistical significance of assay readouts, where a P value < .05 was significant. The limit of detection (LoD) and limit of quantitation (LoQ) were calculated to determine the sensitivities of each assay.

2.8 | LoD, LoQ, and linear range

LoD (expressed in mIU/mL of NIBSC 11/236), the smallest concentration of a FXIa that can be reliably detected by each analytical procedure, was estimated using the value obtained with the data from blank wells filled with assay diluent rather than sample and the lowest point on the calibration curve (lowcalcurve) as follows:

$$LoD = calibration\,curve\,(mean_{blank} + 1.645\,SD_{blank} + 1.645\,SD_{lowcalcurve}) \quad (1)$$

where SD$_{lowcalcurve}$ was calculated from repeated measurements of the lowest point on the calibration curve in assays on different days, and calibration curve is the linear regression line.

LoQ (expressed in mIU/mL) was estimated as the lowest point on the calibration curve with a coefficient of variation (CV) of < 35% and a recovery/accuracy value between 70% and 125%.

The linear range (expressed in mIU/mL) for the indicated assays was determined as the minimum and maximum values on the calibration curve that had a recovery/accuracy value (vs linear regression line) within 70% to 130%.

3 | RESULTS

3.1 | Substrate-based FXIa activity assays

To assess the commutability of 11/236 in these blood coagulation tests, serial dilutions of 11/236 were tested in comparison to those of procoagulant IGIM Lot G in three substrate-based assays for FXIa activity: two commercial multistage chromogenic substrate assays (CA1 and CA2) and a single-stage SN13a fluorogenic substrate assay. As observed in Figure 1A-C, all assays demonstrated parallelism between 11/236 and IGIM Lot G. Between the CAs, CA2 was more sensitive, generating similar rate results at lower concentrations than those from CA1. CA2 had a lower LoD and LoQ, suggesting that this assay was more sensitive (see Table S2).

To assess assay precision, in addition to IGIM Lot G, each microplate assay run included four in-range dilutions of freshly prepared vials of 11/236 and 13/100 and an aliquot of an in-house FXIa control (IH-FXIa). Table 1 shows the CVs for each sample, determined over four runs for each CA. The CVs generated from each assay over the four runs were below 7%, indicating that each assay was precise and had low run-to-run variability when testing IGIM Lot G or the FXIa reference reagent (RR).

It should be noted, however, that despite the low CVs, the SN13a assay produced remarkably overestimated activity measurements for the IGIM Lot G samples (Table 1). Unlike the commercial multistage CA methods that are based on cleavage of FXIa biological substrate, that is, coagulation FIX, specificity of the SN13a assay to FXIa is assured if FXIa is the only SN13a-cleaving enzyme in the tested sample. We found that SN13a is cleaved by kallikrein, FXIIa, and other coagulation enzymes (data not shown). Apart from FXIa, only kallikrein-like enzymes have been previously found in IG samples.[21] Consistently, the high signal in the SN13a assay could be corrected by preincubation of IG samples with a potent kallikrein inhibitor, Kallistop (American Diagnostica, Stamford, CT, USA) (see Figure S1). Therefore, the SN13a assay results are biased by the contribution of kallikrein-like impurities, which also react with the fluorogenic substrate.

3.2 | NaPTT FXIa activity assay

The NaPTT assay, a traditional global hemostasis method used for the assessment of activated coagulation factors and procoagulant impurities in plasma-derivedcoagulation factor concentrate products, was conducted on six occasions in lyophilized NPP, and on two occasions in FXI-DP. The NaPTT assay produced accurate activity assignments for both 11/236 and IGIM Lot G; however, the CVs and blank ranges (mean ± 1 SD for blank samples) produced from the six runs performed in NPP were high, indicating a well-known relatively high run-to-run variability in the NaPTT method (Figure 1D and G, blank values are shown as horizontal gray areas; Table 1). This increased CV may be due to endogenous FXI in NPP, which may support spontaneous contact activation, or reflect higher experimental replicates as compared to FXI-DP. Compared to the CA, the NaPTT assay was less sensitive, requiring more concentrated samples for more accurate assay readouts (Table S2).

TABLE 1 Results of testing by multiple assays for IGIM Lot G and 11/236 samples

Method	Assay readout	Assay Runs N	IGIM Lot G Mean, IU/mL	CV	11/236 Mean, IU/mL	CV
CA1	Rate	4	0.30	5.1%	10.0	6.1%
CA2	Rate	4	0.32	6.1%	10.4	6.9%
SN13a	Rate	4	1.08	5.6%	10.2	5.8%
NaPTT in FXI-DP	Clot time	2	0.30	13.9%	10.9	6.2%
NaPTT in NPP	Clot time	6	0.27	24.9%	10.0	11.9%
FG in FXI-DP	FGrate	6	0.41	11.2%	10.1	3.2%
FG in NPP	FGrate	2	0.41	7.3%	9.9	11.8%
TG FXI-DP (calibrated)	TPH	6	0.32	5.9%	10.7	5.2%

Abbreviations: CA1, Biophen chromogenic assay; CA2, Rossix chromogenic assay; FG, fibrin generation assay; FGrate, FG rate; FXI-DP, FXI-deficient plasma; IGIM, intramuscular immunoglobulin; NaPTT, nonactivated partial prothrombin time; NPP, normal pooled plasma; TG, thrombin generation assay; TPH, thrombin peak height.

3.3 | TF-triggered FG assay

We next sought to assess the suitability of our in-house FG assay for FXIa activity, which we performed for both NPP (two runs) and FIX-DP (six runs), and displayed the results as two FG parameters, clot time, and peak rate of clotting (FG rate). This global hemostasis assay combines the features of the NaPTT assay (detection of plasma clotting via turbidity measurement) and TG assay (TF-triggered coagulation in the presence of limited concentration of phosphatidyl serine:phosphatidyl choline reagent). FXIa produced a dose-dependent effect on the onset of clot time (Figure 1E,H) and the FG rate (Figure 1F, I). Normalized to the lowest and highest turbidity, FG curves were reproducible and orderly (Figure 2A, B), confirming that this simple assay is an accurate method for measuring FIXa activity. As demonstrated overall, the FG assay produced accurate activity values for IGIM Lot G, 11/236 and 13/100, and IH-FXIa control, although high CVs and blank ranges were observed similar to those in the NaPTT assay (Figure 1E, F, H, I, and Table 1).

3.4 | TG assay

TG, another global hemostasis assay, was measured using our in-house TG assay in both NPP and FXI-DP. Compared to traditional commercially available TG assays, we increased the concentrations of fluorogenic substrate (800 μM) and procoagulant lipids (12 μM) and decreased the concentration TF trigger (0.1 pM) to achieve a low but measurable baseline TG response in the absence of added FXIa, and a high response to low concentrations of FXIa. Further, we kept the volume of plasma at 50% of the final reaction mixture in every well of the microplate, and the concentration of calcium chloride was optimized to 10 mM as described previously.[22] Under these conditions, TG in blank wells peaked at 25 minutes and ~ 40 nM of thrombin, and samples with FXIa at and above 0.025 mIU/mL produced a reliable and dose-dependent effect on TG curves (Figure 2 C and D). Dose responses were similar between the 11/236 and IGIM Lot G samples in both FXI-DP (Figure 3) and NPP (Figure S2). The TG assay is traditionally assessed through several assay readouts thought to represent the different stages of TG: lag phase (lag time), propagation phase (TTP, velocity and TPH) and totality of the response (AUC, or endogenous thrombin potential) as demonstrated in Figure 3 and Figure S2. Good parallelism between 11/236 and IGIM Lot G was observed for all TG assay readouts. A small decrease in TPH for both the 11/236 and IGIM Lot G samples between FXI-DP and NPP was observed (Figure 3C and Figure S2, respectively). When compared to the other assays performed in this study, the TG assay was overall the most sensitive for FXIa activity (generating the lowest LoD values; see Table S2) and required higher dilutions of 11/236 to reach the linear range of the assay.

3.5 | Effect of internal thrombin calibration on TG assay precision

Traditional TG assays are calibrated in units of thrombin to assure consistency of reported assay results between different experiments and across laboratories. Since our assays are now calibrated with FXIa standard, thrombin calibration is no longer required. However, a commonly used CAT method employs internal thrombin calibration, which addresses artifacts of fluorogenic substrate consumption and the inner filter effect. These artifacts may result in the underestimation of the TG response to high procoagulant signals, for example, high concentrations of FXIa in the less diluted portions of the FXIa calibration curves. Application of CAT algorithm constitutes of a mathematical data manipulation step, which should be justified. Therefore, we compared the TG assay results before (uncalibrated data) and after CAT algorithm calibration (calibrated data).

Internal thrombin calibrator samples were included in each microplate to assess the corrective capacity of internal thrombin calibration on the run-to-run assessment of FXIa activity. The thrombin calibration of the TG curves produced no apparent effect on the parallelism between the 11/236 and IGIM Lot G samples in each assay run (Figure S3A). However, thrombin calibration resulted in an increased run-to-run variability in the dose-response curves (with more space in between the curves as compared to the uncalibrated curves; see Figure S3A) and higher run-to-run CV for both low and high dilutions of the 11/236 calibration curves. The effect of thrombin calibration was not limited to highly procoagulant samples and was universally applied to both low and high TPH values, suggesting that all wells on the microplate were corrected upwards for three of six assay runs (Figure S3C). This indicates that the internal thrombin calibration does not correct for substrate consumption in these experiments.

Interestingly, thrombin calibration produced slightly lower run-to-run CVs for activity values for 11/236 and IGIM Lot G (Table 1 and Figure S3C). The slightly lower CVs of FXIa values may be a chance occurrence indicative of an overall high variability of the TG assay rather than the effect of the FXIa calibration curve corrections.

3.6 | Comparison of dose responses between the CA and TG assays

Considering that the CA1, CA2, and TG assays were the most sensitive assays tested, we sought to compare the dose responses of 11/236 generated among the three assays. As shown in Figure 4A, different sensitivities are observed among the three assays. CA1 was the least sensitive, and the TG assay was the most sensitive regardless of assessment in NPP or FXI-DP (as demonstrated by the overlapping curves in both types of plasmas). Furthermore, comparison between the CA and TG assays (in both NPP and FIX-DP) demonstrate comparable dose-response curves (despite differences in sensitivity) suggesting that these assays are equally suitable
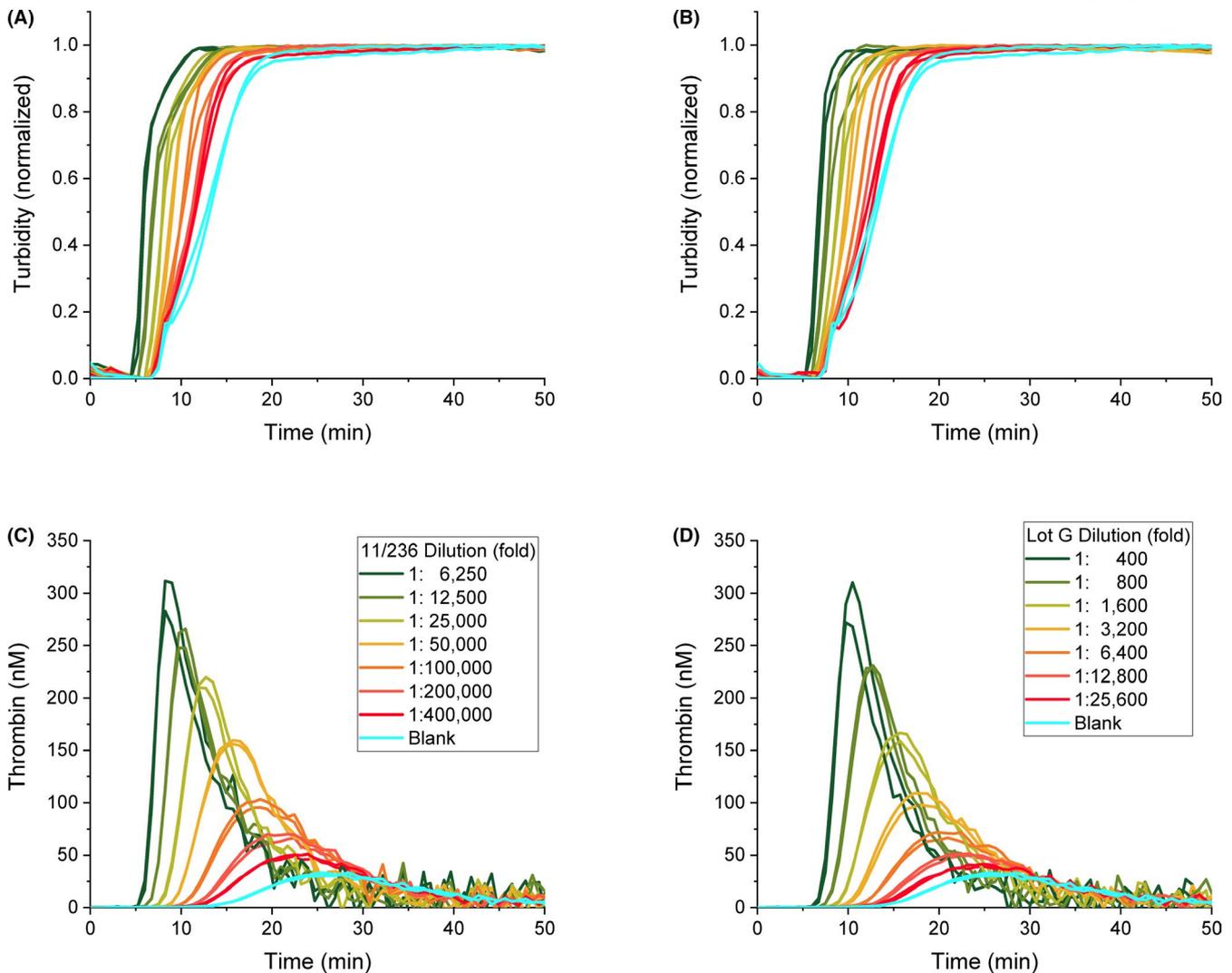
FIGURE 2 Assessment of 11/236 and IGIM Lot G via FG and TG assays. The indicated dilutions of 11/236 and IGIM Lot G samples were assessed via FG and TG assays. Normalized turbidity data from FG assay in A, 11/236; and B, IGIM Lot G samples. Calibrated TG data as thrombin (nM) from C, 11/236; and D, IGIM Lot G samples. Each dilution of both sample sets was run in duplicate. FG, fibrin generation assay; FXIa, activated factor XI; IGIM, intramuscular immunoglobulin; TG, thrombin generation

for assessing FXIa activity. When single runs of freshly prepared 11/236, FXIa samples, and IG samples (NIBSC Codes C, D, E, and F; see Materials and Methods) were performed simultaneously for the 2 CA and the TG assay (normalized to 11/236), they produced overlapping values, again supporting the use of both the traditional chromogenic substrate and global hemostasis TG assays for FXIa assessment (Figure 4B).

3.7 | Assay agreement on FXIa activity measurements

We next sought to compare the activity assignments generated by all these FXIa assays. Thus, we performed the TG, NaPTT, and the FG assays (either with NPP or FXI-DP), and the CA and SN13a substrate assays on either 9.8 IU/mL NIBSC 13/100 or ~0.3 IU/mL IGIM Lot G samples, after calibration with 11/236 as a reference

standard. We observed an accurate activity measurement of 13/100 at around 10 IU/mL for each of the indicated assays. In addition, all the assays, including the SN13a substrate assay, agreed on activity measurements of purified FXIa preparations (Figure 5A, Table 1). Similar agreements were observed with the IGIM Lot G, as all the assays returned values of around 0.3 IU/mL, except for the SN13a assay, which returned statistically significant higher values of > 1.0 IU/mL (Figure 5B). These higher SN13a values were most likely due to kallikrein-like impurities in the IGIM Lot G samples; SN13a is not a specific substrate for FXIa and has the capacity to react with kallikreins (Figure S1).

To compare the assays further, we tested several FXIa preparations and procoagulant IG products. All assays except for the SN13a correlated well in FXIa activity assignments (Figure 6A and B; see 45° black line). The SN13a assay gave higher results for IG products, in agreement with the previous observation that these products contain kallikrein impurities.

FXI-Deficient Plasma
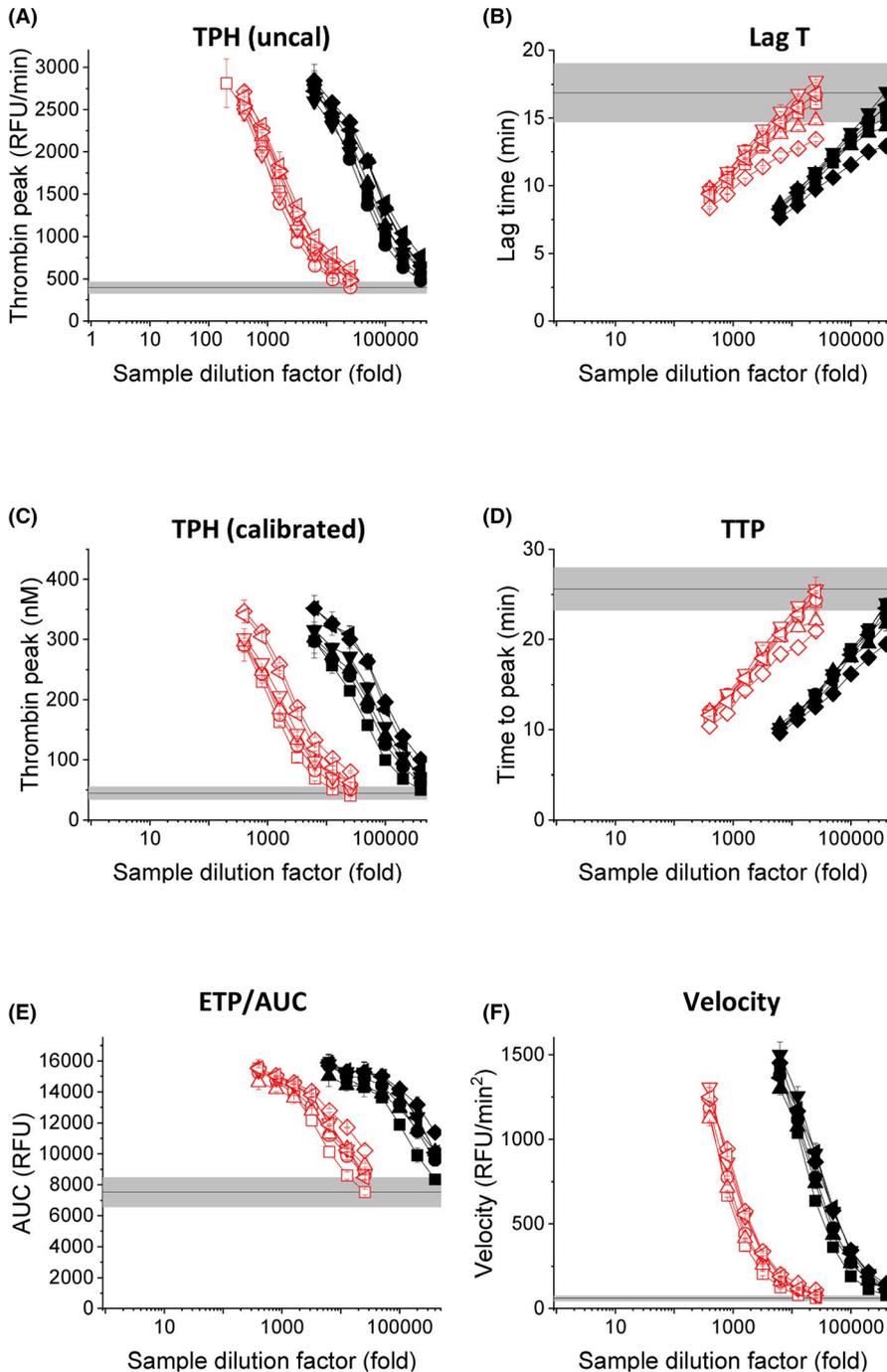
FIGURE 3 TG parameters in FXI-DP. The indicated dilutions of 11/236 (black) and IGIM Lot G (red) samples were assessed via the TG assay in FXI-DP (n = 6 replicates). The TG parameters A, thrombin peak height (TPH) uncalibrated FXI-DP; B, lag time (Lag T); C, TPH calibrated FXI-DP; D, time to peak (TTP); E, area under the curve (AUC); and F, velocity, were calculated for each condition. Horizontal black line with gray shaded area is the blank value ± SD. FXI-DP, factor XI-deficient plasma; IGIM, intramuscular immunoglobulin

4 | DISCUSSION

Plasma-derived IG products are essential therapeutics, yet several of these products have been found to be linked to TAEs, resulting in recalls of product around a decade ago.[11,12,21] It was determined that FXIa impurities are the major source of procoagulant activity and TAEs associated with IG products.[10] The safe acceptable levels of FXIa impurities are not known at this time, but evidence suggests that most thrombosis-implicated batches had >1 to 5 mIU/mL. Although the thrombotic potentials of these IG products are now tested by quality control laboratories, the assays performed are not uniform and may produce results in activity units relevant only to that defined assay, for example, clotting time in seconds. In this study, we have demonstrated that the WHO reference materials, NIBSC 11/236 and 13/100, are commutable as calibrators for multiple FXIa activity assays. Indeed, all the blood coagulation tests that we evaluated showed parallelism with 11/236, and all but the SN13a method produced comparable activity measurements.

We found the two commercial FXa substrate-based FXIa activity assays to be the most precise, whereas the global hemostasis
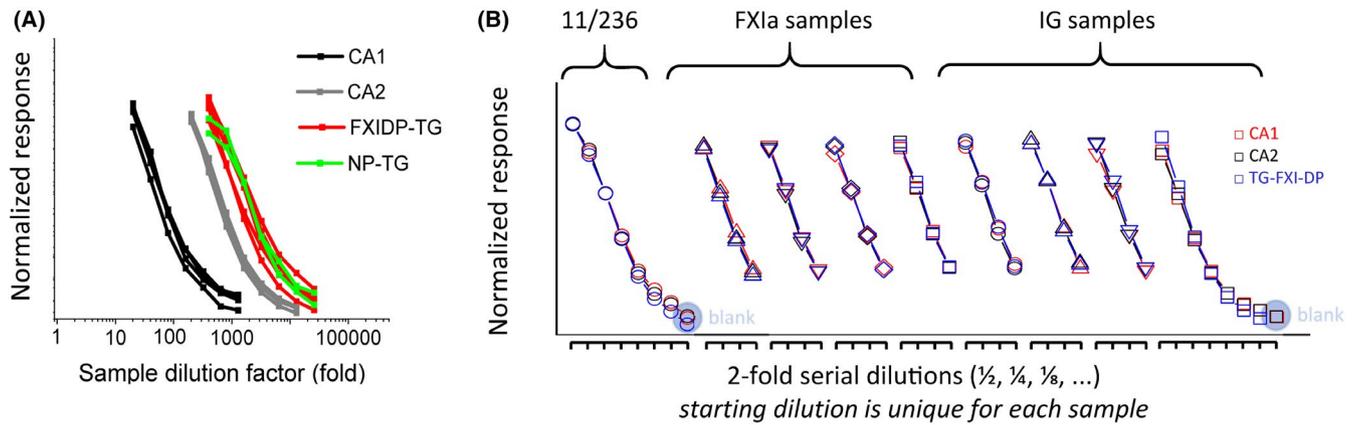
FIGURE 4 Comparison of CA and TG assays. A, Different sensitivities of the CAs and TG assays were observed using NIBSC 11/236. The CA2 (gray squares) had better sensitivity than CA1 (black squares). The TG assay was the most sensitive regardless of assessment in FXI-DP or in NPP. Regardless of sensitivity, all assays demonstrated comparable dose responses between assays. Data across all assay runs are shown. B, Single assay run on the three indicated methods demonstrates remarkable overlap of dose-responses despite different sensitivity in the reference reagent(11/236), 4 FXIa samples, and 4 IG samples (including the 3 samples provided by the NIBSC [13] and the IGIM Lot G). Data are normalized to the highest signal from 11/236 sample. CA1, Biophen chromogenic assay; CA2, Rossix chromogenic assay; FXIa, activated factor XI; FXI-DP, factor XI-deficient plasma; IGIM, intramuscular immunoglobulin; NIBSC, National Institutes for Biological Standards and Controls; NPP, normal pooled plasma; TG, thrombin generation.

TG assay was the most sensitive. In contrast, however, the SN13a substrate assay gave substantially higher values for the IG samples, despite accurately producing the target potency values for

11/236 and other FXIa samples. This finding is consistent with the assay's ability to detect various proteolytic enzymes.[16] Notably, the FXIa substrate for FXIa SN13a is also sensitive to kallikrein-like
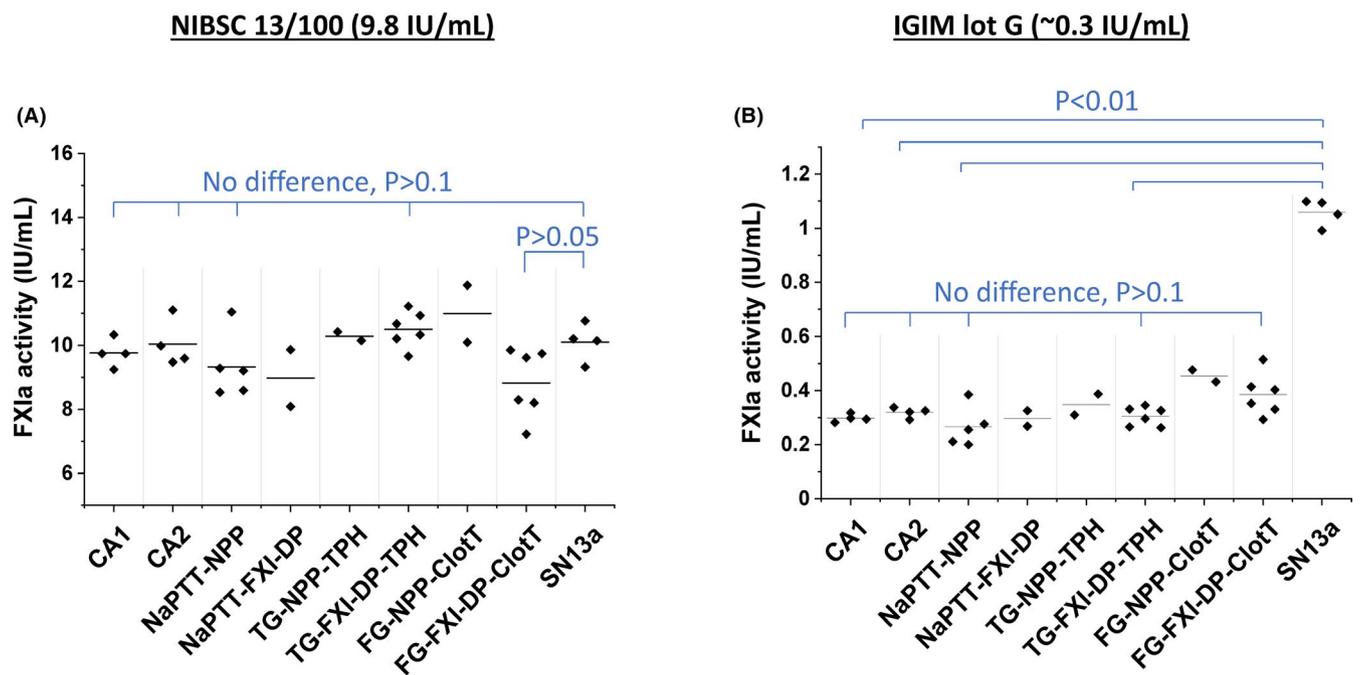
FIGURE 5 Comparison of activity from FXIa activity assays. A, 10 IU/mL frozen-thawed NIBSC 13/100; or B, ~0.3 IU/mL IGIM lot G samples were assessed for FXIa activity assignments via CA1, CA2, NaPTT in NPP or FXI-DP, both our in-house TG assay and FG assay in NPP or FXI-DP, and SN13a assay, using NIBSC 11/236 as a reference standard. All assays show agreement in 13/100 activity assignment of 10 IU/mL and IGIM Lot G at 0.3 U/mL, except for SN13a, which reported higher potency values for IGIM Lot G due to kallikrein-like impurities. Statistical significance was assessed for assays with at least 4 replicates via an unpaired *t* test, where *P* < .05 was deemed significant. CA1, Biophen chromogenic assay; CA2, Rossix chromogenic assay; FG, fibrin generation;FG-ClotT, fibrin generation clot time; FXIa, activated factor XI; FXI-DP, factor XI-deficient plasma; IGIM, intramuscular immunoglobulin; NaPTT, nonactivated partial prothrombin time; NIBSC, National Institutes for Biological Standards and Controls; NPP, normal pooled plasma; TG, thrombin generation; TPH, thrombin peak height

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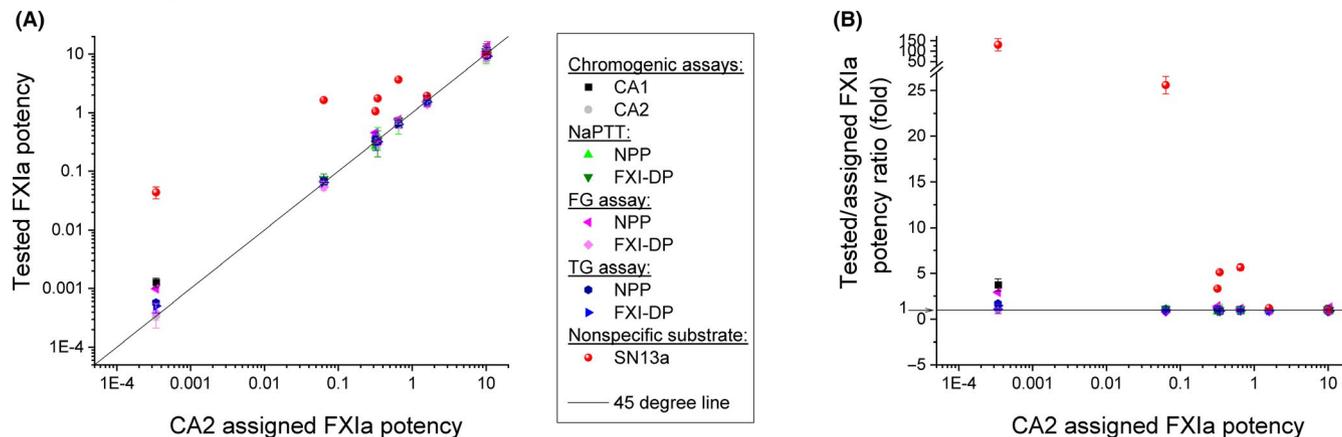
FIGURE 6 Activity correlation between FXIa activity assays. All the studied assays were subjected to correlation analysis for tested potency versus the CA2-assigned activity for FXIa. A, A correlation was observed between 8 of the 9 indicated FXIa activity assays for 11/236, as demonstrated by the diagonal black line, with the exception of SN13a, which reported higher FXIa activity values most likely due to the presence of kallikrein-like impurities. B, The ratio of the tested to assigned activity for all assays except SN13a correlated well with the assigned activity by CA2. CA1, Biophen chromogenic assay; CA2, Rossix chromogenic assay; FG, fibrin generation assay; FXIa, activated factor XI; FXI-DP, factor XI-deficient plasma; NaPTT, nonactivated partial prothrombin time; NPP, normal pooled plasma; TG, thrombin generation

impurities that are present in this IGIM product and many other IG lots.[21] Although the SN13a-based assay has previously been used to detect FXIa activity in blood of patients, this method was also the least sensitive of the modern CA and TG-based methods and even NaPTT-based assays, requiring undiluted concentrated samples. The data shown here support the usefulness of both the CA coagulation factor activity assays and global hemostasis assays such as TG and FG for measuring FXIa activity in IG samples. The results also suggest that the NIBSC reference materials, such as NIBSC 11/236, are commutable references for both traditional and global hemostasis assays.

The TG and clotting (FG) assays have been previously used to measure FXIa activity in plasma[23] and blood,[24] respectively. Calibration of these methods in international units of FXIa may be helpful in monitoring novel oral anticoagulants targeting FXIa. Several groups have demonstrated the potential for targeting FXIa to develop novel anticoagulant therapeutics. Indeed, FXIa deficiency protects mice from FeCl$_3$-induced thrombosis, and inhibition of FXIa by various small molecules prevents thrombosis in several animal models.[3,25,26] Furthermore, several FXIa inhibitors have reportedly been advanced for efficacy assessments in clinical trials.[27] Given that there are several FXIa inhibitors in clinical development, the availability of a common reagent, FXIa, calibrated in units of WHO standard for FXIa can help develop a common unit for FXIa inhibition, for example, 1 unit of inhibitor activity equals the amount of inhibitor sufficient to inhibit 1 IU of FXIa in 1 hour. Such a common inhibitor activity unit can then be used to calibrate the anti-FXIa activity assay, like the anti-FXa activity assay currently used by clinical laboratories for monitoring low-molecular-weight heparin and oral anticoagulants targeting FXa.

Although the mechanism of how FXIa is involved in thrombosis is still being investigated, there is strong evidence to support its role as a thrombogenic impurity in plasma-derived therapeutic proteins,

for example, IG products known to induce TAEs. Accurate determination of FXIa activity in product lots is therefore important in preventing TAEs in patients receiving IG products. Our work here demonstrates that all currently used assays for FXIa activity can be calibrated using a publicly available RR. The results of procoagulant activity tests can be expressed in international units for FXIa, which may allow for development of a consensus on the safe limits for procoagulant impurities or inform on the suitability of the assays for monitoring of product quality. The levels <1 mIU/mL are seen in marketed products showing acceptable safety.[11]

To ensure that the levels of FXIa activity remain low over the lifetime of the product or after manufacturing change, the manufacturers of IG products may choose to trend the changes in FXIa activity. For trending, the assay should be capable of quantification well below the current lot release limit. The CA and TG assays appear to be the most suitable for this purpose. Indeed, only two methods, the CA2 and TG assay in both NPP and FXI-DP, were both robust and sensitive in the low range of FXIa activities, below 1 mIU/mL. The NaPTT method and the clot-based FG assay appeared to be not as accurate or sensitive to detect levels of FXIa around 1 mIU/mL. While the accuracy and robustness of NaPTT can be addressed in the quality control laboratory through repeated measurements of the analyte, the insufficient sensitivity of NaPTT presents a problem for further application of this method in routine testing for FXIa activity.

The traditional global hemostasis assay, TG, has shown good sensitivity, accuracy, and range to FXIa, and exceeded that of the commercial CA1 assay, yielding comparable results to the CA2 method. Our study suggested that several TG parameters can be safely used as readout of the TG assay for detection of FXIa activity. However, contrary to the well-known impact of the substrate consumption and inner filter effect artifacts on the clinical laboratory version of the TG assay, correction of these effects by CAT

algorithm were associated with reduced robustness of the FXIa calibration curve, suggesting that thrombin calibration is not required for FXIa-calibrated TG assay. These results are likely limited to the FXIa testing applications, which are based on a response of a single lot of FXI-deficient or normal pool of plasma to multiple FXIa containing samples. In contrast, a clinical setting will compare multiple samples of patient plasma to a single TF-trigger, a setting where plasma-to-plasma variability may need to be corrected with CAT algorithm.

It should be noted that most of the assays employed in this study are not specific for FXIa and were chosen for evaluation of IG products based on prior evidence of FXIa as the primary prothrombotic substance in thrombogenic IG products. Good agreement between multiple orthogonal nonspecific assays have strengthened the notion of FXIa as the only significant procoagulant contaminant in tested IG products. In other applications, the procoagulant materials that contain unknown procoagulants should be investigated to determine the underlying root causes for responses in activity in observed assays. To identify the source of procoagulant activity, one would need to employ the use of specific coagulation enzyme inhibitors or specific factor-deficient plasma to ultimately pinpoint the factor(s) contributing to a procoagulant environment. For example, contamination of FIX concentrates with FIXa can be blocked by FVIII and FX deficiency and reproduced by FIXa spiking.

Our study has several limitations. First, the activity of sample IGIM Lot G, ~0.3 IU/mL, is at the high end of the range of FXIa activities observed in IG lots involved in TAEs. While some of the 2010 TAE-implicated thrombogenic lots had comparable activity, other lots had activities of as low as 0.001 to 0.010 IU/mL. Consequently, the sensitivity or specificity of some of the assays studied in our work, for example, SN13a and NaPTT, are insufficient to detect FXIa in these TAE-positive lots. Furthermore, we have not studied how matrix effects impact assay performance. The excipients used for the stabilization of IG concentrates may negatively affect the robustness and accuracy of the methods at the low range of FXIa activities in product samples. Finally, our in-house assays are optimized for robotic 96-channel pipettors. Automated pipettors may have the advantage of high throughput and intra-assay consistency, owing to consistency of timing and volumes dispensed across the wells of a 96-well plate. However, 96-channel pipettors are rarely used in hemostasis research; therefore, one might expect a greater method-to-method variability in traditional laboratories. To address these limitations, a collaborative study is being conducted to compare multiple in-house methods in well-qualified laboratories under the guidance of our laboratory and the NIBSC.

AUTHOR CONTRIBUTIONS

JWJ and MVO wrote the paper with help from MW, DES, and TKL. YL and SAW performed experiments and analyzed the data with help from MVO. SSS and LAP optimized 96-well assays and developed analysis software. MVO supervised the project.

ORCID

Joseph W. Jackson [iD] https://orcid.org/0000-0002-3826-2749
Leonid A. Parunov [iD] https://orcid.org/0000-0002-8892-8908
Mikhail V. Ovanesov [iD] https://orcid.org/0000-0003-2509-1781

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.