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Research Article

Neutralizing Antibody Assay Development with High Drug and Target Tolerance to Support Clinical Development of an Anti-TFPI Therapeutic Monoclonal Antibody

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Abstract. Immunogenicity is a major challenge for protein therapeutics which can potentially reduce drug efficacy and safety and is often being monitored by anti-drug antibody (ADA) and neutralizing antibody (NAb) assays. Circulating targets and residual drugs in matrices can have significant impacts on accuracy of results from ADA and NAb assays, and sufficient drug and target tolerance for these assays are necessary. Here, we report the development of a competitive ligand binding (CLB) NAb assay for an anti-TFPI (tissue factor pathway inhibitor) monoclonal antibody (PF-06741086) with high drug and target tolerance to support ongoing clinical studies. A double acid affinity capture elution approach was used to mitigate drug interference, and a robust target removal strategy was employed to enhance target tolerance. The validated NAb assay has sensitivity of 313 ng/mL, drug tolerance of 50 μg/mL, and target tolerance of 1200 ng/mL. A step-by-step tutorial of assay development is described in this manuscript along with the rationale for using a high drug/target tolerant NAb assay. The NAb assay cut point factor obtained was 0.78. Other assay performance characteristics, e.g., precision and selectivity, are also discussed. This validated method demonstrated a superior drug and target tolerance to warrant specific and precise characterization of the NAb responses in support of ongoing clinical studies.

KEY WORDS: drug tolerance; hemophilia; target tolerance and neutralizing antibody; TFPI.

INTRODUCTION

Monoclonal antibodies (mAb) with apparent advantages such as high specificity and low toxicity have become an important modality of medicines demonstrating clinical success in treating various diseases and conditions. A growing number of therapeutic monoclonal antibodies are being evaluated in preclinical or clinical development. Many biotherapeutics, including monoclonal antibodies, have been shown to trigger unwanted immunogenicity and, in some cases, have affected drug efficacy, exposure, and safety (1–5). Hence, immunogenicity assessment is required by regulatory agencies for approval of a therapeutic protein (6,7). Although immunogenicity of a therapeutic protein is comprised of both cellular (T cell) and humoral (antibody, B cell) responses, in practice, only the antibody response is usually assessed by the detection of anti-drug antibody (ADA) during drug development. Detection of ADA induction in patients can help to understand the implications of ADA responses in product safety and efficacy. Conventionally, a multi-tiered approach with sequential screening, confirmatory, titer, and neutralization assays is often conducted in clinical studies (4,5). In addition, the presence of neutralizing antibody (NAb) is often detected using a cell-based assay or a non-cell-based competitive ligand binding assay to monitor effects of ADA on biological activity of the drug.

The detection of NAb is highly dependent on key assay parameters such as sensitivity and specificity, as well as reagents, sampling time points, and patient condition; therefore, the NAb results can vary between assays. It is not possible to compare immunogenicity between different therapeutic proteins if not using assays with the same format and equivalent assay performance. Furthermore, various pharmacological factors including circulating drug and drug targets can interfere with detection of NAb and affect assay specificity (8,9). For example, high concentrations of circulating drug in the sample matrix can bind to target or NAb and result in false-positive or negative signals. In a CLB NAb assay, free soluble drug target can block the binding of detection drugs (assay reagents) to coated target on the assay plate and result in false-positive signals. Target interference may also cause false-negative results in other types of Nab assay, particularly cell-based.

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Therefore, it is important to have validated assays with appropriate sensitivity, specificity, and selectivity for accurate NAb assessment.

Tissue factor pathway inhibitor (TFPI) is a natural inhibitor for the human coagulation pathway and the antagonism of TFPI can increase coagulation activity. An anti-TFPI mAb, PF-06741086, is in clinical development for its potential use in prophylactic treatment of hemophilia A and B patients (10). To support clinical development of PF-06741086, we had developed a CLB assay for NAb analysis. The circulating plasma TFPI concentration is approximately 50 ng/mL and 80% of circulating TFPI is bound to lipoproteins which can link multiple TFPI molecules in vivo and create TFPI multimers with bivalent binding capacity (11). In addition, the maximum clinical dose of PF-06741086 was 440 mg intravenously, and a high concentration of circulating drug was anticipated. It has been demonstrated previously that anti-TFPI can prolong the half-life of endogenous TFPI and increase soluble TFPI in the plasma of monkeys (12). Subsequently, we observed 3X increase in total plasma TFPI levels in the clinic after administration of PF-06741086 at the highest dose treated (10). To eliminate potential target TFPI and free PF-06741086 interference in detection of anti-PF-06741086 neutralizing antibodies, we incorporated double acid treatment with affinity capture elution (ACE) along with a target blocking treatment in the CLB assay. We observed significant improvement in drug and target tolerance using this procedure. These strategies may be applicable to other monoclonal antibody therapeutics requiring an assay with high drug and target tolerance.

MATERIAL AND METHODS

Reagents, Matrix Samples, Software and Equipment

Recombinant human TFPI was obtained from Syngene (Bangalore, India). Pierce streptavidin-coated high binding capacity (SA-HBC) plates, Slide-a-lyzer™ cassettes with a 10-K molecular weight cut off (MWCO), Zeba™ spin desalting columns with a 40 K MWCO, and EZ-Link™Sulfo-*N*-hydroxysuccinimide (NHS)-LC biotin were obtained from Thermo Scientific (Hanover Park, IL). Human plasma (sodium citrate) was obtained from Bioreclamation IVT (Westbury, NY). High binding (HB) MSD® plates, Sulfo-TAG™ NHS-Ester ruthenium, and MSD® read buffer T (4X) were obtained from Meso Scale Discovery (Rockville, MD). Costar high binding plates were obtained from Corning (Corning, NY). NAb positive control (PC, a mouse monoclonal anti-drug antibody), drug (humanized anti-TFPI IgG), 7A4.D9 (TFPI blocker, mouse IgG1 specific to TFPI), biotin-drug, ruthenium-drug conjugate, wash buffer (50 mM Tris, 500 mM sodium chloride, 1 mM glycine, 0.05% polysorbate 20, pH 8.0), PBS (phosphate buffered saline, pH 7.2), block buffer (PBS containing 4% bovine serum albumin and 0.05% Tween-20), assay buffer (PBS containing 1% BSA and 0.05% Tween-20), deionized water, 100 mM glycine buffer, pH 2.0 and 1 M Tris buffer, pH 8.5 were supplied by Pfizer (Andover, MA). Developed plates were read on MSD® reader Sector Imager S 600, Model 1200, with Discovery Workbench Software (version 4.0, MSD®) by Meso Scale Discovery (Rockville, MD). Graphical figures were made in GraphPad Prism 7 (La Jolla, CA). JMP statistical software version 10 was used to identify outliers and distribution of individuals for normality by SAS Institute, Inc. (Cary, NC).

Biotin-Drug and Ruthenium-Drug Conjugation

Anti-TFPI therapeutic antibodies were coupled to EZ-Link™Sulfo-*N*-hydroxysuccinimide (NHS)-LC biotin at a molar coupling ratio (MCR) of 50:1. The reactions were performed at room temperature (RT) for 2 h and subsequently quenched with 1 M Tris, pH 9.0. Biotinylated antibodies were then dialyzed against PBS using 10 K MWCO Slide-a-lyzer™ cassettes for 18–24 h at 4°C.

Conjugation of anti-TFPI therapeutic antibodies to Sulfo-TAG™ NHS-Ester ruthenium was conducted at a MCR of 12:1 for 1 h at RT. Ruthenium-drug conjugate reagent was dialyzed against PBS using 40 K MWCO Zeba™ spin desalting columns per manufacturer's instructions. All the conjugated reagents were aliquoted and stored frozen at − 70°C.

CLB MSD®-Based NAb Assay Procedure

An ECL assay on the MSD® platform was used to assess NAb response against anti-TFPI mAb. Samples in sodium citrate plasma were diluted 1:2 with assay buffer, then further diluted 1:6 with ruthenium-drug conjugate (21.2 ng/mL in assay buffer). Samples were incubated overnight at room temperature. A high binding (HB) MSD® plate was coated with 50 μL/well of 0.4 μg/mL TFPI in PBS overnight at 4°C.

The next day, the MSD® plate was washed 4 times with wash buffer and blocked with 200 μL of block buffer for at least 1 h at RT without shaking. The MSD® plate was washed 4 times with wash buffer, and 70 μL/well of NAb and ruthenium-drug conjugate complexes were added to the MSD® plate. The MSD® plate was protected from light and incubated for 1 h at RT on the shaker. Ruthenium-drug conjugate bound to NAb was unable to bind to the TFPI-coated plate, resulting in a reduction of the final assay signal. The MSD® plate was washed 4 times with wash buffer, and 150 μL/well of 2X MSD® read buffer T (diluted from 4X with deionized water) was added to the MSD® plate and the plate was read on a MSD® reader Sector Imager S 600. Raw electrochemiluminescent signals (called Relative Light Units or RLU) were measured to represent assay responses.

CLB MSD®-Based NAb Assay Procedure with Sample Pretreatment

To increase drug and target tolerance, sample pretreatment (ACE along with plate-based immunodepletion of target) was performed prior to running the samples in the CLB NAb assay. Samples for screening assay were diluted 1:2 using assay buffer and incubated on a shaker for at least 5 min. For titer assay, samples were serially diluted threefold in plasma.

Forty (40) μL of diluted samples was treated with 150 μL of 100 mM glycine buffer, pH 2.0 in a 96-well polypropylene V-bottom plate at RT on a shaker (first acid step). After 5 min, 10 μL of biotin-drug conjugate (100 μg/mL in assay buffer) was added, and the samples were incubated for another 5 min.

Ninety (90) μL of the acidified samples was transferred in duplicate to a SA-HBC plate containing 9.5 μL/well of 1 M Tris buffer, pH 8.5, to neutralize the pH. The samples were incubated for 5 h at RT on a shaker to bind biotin-drug-ADA/Nab complexes to the SA-HBC plate and remove the free drug.

During the 5 h incubation of the samples on the SA-HBC plate, Costar high binding plates were coated with 100 μL/well of 15 μg/mL 7A4.D9 (mouse IgG1 specific to TFPI) in PBS buffer and incubated for 2 h at RT with agitation. Costar plates were washed 4 times with wash buffer and blocked with 200 μL of block buffer for 75 min at RT without shaking.

After biotin-drug-ADA/Nab complexes bound to the SA-HBC plates, the plates were washed 4 times with wash buffer and ADA/NAb were eluted by adding 85 μL/well of 100 mM glycine buffer and incubating for 5 min at RT on a shaker (second acid step). Blocked Costar plates coated with 7A4.D9 were washed 4 times with wash buffer, and 20 μL/well of a 1:1 solution of 1 M Tris Buffer, pH 8.5, and assay buffer were added to the Costar plates. Seventy (70) μL of the eluate from the SA-HBC plates was transferred to the Costar plate and incubated overnight at RT to capture free target.

Once target was sufficiently bound to the 7A4.D9 coated Costar plate, 70 μL of the supernatant was transferred to a 96-well polypropylene V-bottom plate containing 10 μL of ruthenium-drug conjugate (37.6 ng/mL in assay buffer). The plate was incubated overnight at RT in the dark. Meanwhile, a high binding MSD® plate was coated with 50 μL/well of Human TFPI (0.4 μg/mL in PBS buffer) and incubated overnight at 2–8°C without shaking. Next day, the CLB MSD® NAb assay was performed as described in the "CLB MSD®-Based NAb Assay Procedure" section.

Data Analysis for Assay Validation

Assay validation included assessment of the following parameters: (1) cut point determination; (2) sensitivity; (3) drug tolerance; (4) target tolerance; (5) selectivity and matrix interference; (6) dilution linearity; (7) precision and robustness (inter-analyst); and (8) stability. The method followed US Food and Drug Administration (FDA) guidance recommendations for the assay development for immunogenicity testing for therapeutic products and was validated using standard bioanalytical parameters and target acceptance criteria (4,7).

Cut Point Assessment

In order to determine whether or not a sample is positive or negative for NAb, a cut point must be determined. A NAb cut point is the signal that distinguishes NAb positive samples (signal equal to or below the cut point) and NAb negative samples (signal greater than the cut point). A sample is considered positive for NAb if the response generated by that samples is less than or equal to the cut point value. A plate cut point (floating cut point) is calculated by cut point factor x mean response of the NC of the individual plate. To generate an assay cut point, 50 individual treatment-naïve sodium citrate plasma samples from healthy donors were analyzed in three separate runs by two different analysts on two different days. Samples were prepared and analyzed following the procedures in the "CLB MSD®-Based NAb Assay Procedure with Sample Pre-treatment" section.

The resulting data set (150 points) was analyzed using the $\log_{10}$ transformed data set to determine outliers and the normality of the data distribution (4,13). The data set was then analyzed to allow for 1% of the subject samples to score positive in this CLB based NAb assay. If the mean responses of the subjects and the negative control have similar variability and the responses trend similarly, the use of a multiplication factor (n) linking the statistically-determined cut point value and the mean negative control value is justified to calculate cut point values for all plates during validation. Cut point was calculated as follows:

- Normally distributed data:

$$\text{Cut point} = \text{Anti}-\log\{(\text{Mean log transformed response of individual matrix samples} - 2.326^{*} \text{Standard Deviation of the Log individual samples})\}$$

- Not normally distributed data:

$$\text{Cut point} = 99^{\text{th}} \text{ percentile (with 1\% false positive)}$$

$$\text{Cut point factor} = \text{Cut point}/\text{mean response of the NC}$$

$$\text{Floating cut point} = \text{Cut point factor}^{*} \text{ mean response of the NC on individual plate}$$

Sensitivity

PC samples were prepared at final concentrations ranging from 3.70 to 8100 ng/mL, by diluting the mouse anti-drug monoclonal antibody in neat naïve human sodium citrate plasma. Unspiked plasma pool was used as negative control (NC). Estimation of the assay sensitivity was performed using PC and NC samples described in the "CLB MSD®-based NAb Assay Procedure with Sample Pre-treatment" section. The mass based sensitivity was defined as the concentration of the positive control at the dilution that generated a response equal to the assay cut point.

Drug Tolerance

Drug interference test samples were prepared by spiking drug at concentrations ranging from 20 to 50 μg/mL into pooled sodium citrate plasma with or without 1000 ng/mL of PC and incubated for 1 h at RT prior to being assessed.

Based on the cut point calculated for each plate, the concentration of the added drug which increases the signal above the cut point, thereby changing a positive result to a negative result, can be determined. The highest concentration of added drug, in the absence of PC, which generated an RLU above the cut point value (negative results), was reported as the assay drug tolerance.

Target Tolerance

To assess target tolerance of the assay, target interference samples were prepared by spiking TFPI concentrations ranging from 250 to 1200 ng/mL into sodium citrate plasma with or without 1 μg/mL of PC and incubated for 1 h at RT prior to being assessed in the assay.

At the highest target concentration tested (1200 ng/mL) in 100% plasma without added PC, there were no false-positive results seen. This indicates that the assay is tolerant to at least 1200 ng/mL of target in plasma samples, well above the levels expected in the clinic.

Selectivity and Recovery

Fifteen individual human sodium citrate plasma samples were spiked with PC to a concentration of 1000 ng/mL. The 15 individual plasma samples (spiked and unspiked), as well as the unspiked NC pool, were tested. The RLU values of the spiked plasma were compared to the normal pooled sodium citrate plasma control (NC). The % recovery of the NAb PC was calculated as 100*(RLU of spiked individual/spiked NC pool). The recovery (% differences between individuals and pool control) acceptable range was 75–125%. At least ten out of 15 individuals (2/3) must pass acceptance criteria. At least 12 out of 15 (80%) unspiked individuals should score negative.

Assay Precision and Robustness

Inter-day precision of the assay was analyzed from ten independent runs performed on multiple days. For each run, one set of replicates of the PC titration curve and 16 replicates of the NC were used during final data analysis. For titration, PC at 8100 ng/mL was diluted 1:2 in assay buffer, then serially diluted 1:3 in assay diluent (50% plasma in assay buffer) for a total of 8 dilution points followed by sample treatment as described in the "CLB MSD®-Based NAb Assay Procedure with Sample Pre-treatment" section. PC $\log_{10}$ titer was considered acceptable if it was within a log of the PC serial dilution factor (± 0.48, 0.48 is the $\log_{10}$ (3), where 3 is the dilution factor used in the assay).

To evaluate intra-plate and inter-plate variability of PC, five individual titrations of the positive control as described above were tested on the same plate. The test was performed using a total of two plates on 1 day by one analyst. Intra-plate precision was evaluated within each plate and inter-plate precision was evaluated between two tested plates.

The robustness of the assay was evaluated by two different analysts performing the assay with the PC titration and 16 replicates of the NC on two plates over 2 days. Between the two analysts, differences in titer values for PC sample should be a log of the PC serial dilution factor.

Dilution Linearity

To assess the dilution linearity, PC was spiked in sodium citrate plasma at concentration of 24.3 μg/mL. Dilution linearity samples were initially prepared by further diluting 1:2, 1:3, and 1:9 in 100% normal pooled human sodium citrate plasma and were further diluted as titration samples (followed by serial dilution 1:3 for a total of 8 dilution points). The difference of the $\log_{10}$ titer values among the PC and the dilution linearity samples were compared to determine the linearity of the assay.

Freeze-Thaw and Bench Top Stability in Sample Matrix

To evaluate freeze-thaw (F/T) stability of the positive control, three individual aliquots of the PC were thawed and kept at room temperature for 1 h. All aliquots were then refrozen at − 70°C overnight. The procedure was repeated for six freeze-thaw cycles. Aliquots were analyzed and compared to the positive control (1 F/T cycle).

To evaluate bench top or room temperature (RT) stability of the positive control, 3 individual positive control aliquots were thawed and kept at room temperature overnight (17.5 h). Aliquots were then refrozen at − 70°C for storage.

The freeze-thaw and bench top stability were considered acceptable if difference of PC log ($\log_{10}$) titer values of the treated and the untreated samples were within a log of the PC serial dilution factor ($\log_{10}$ (3) = 0.48).

RESULTS

A CLB NAb assay was developed to support clinical development of anti-TFPI therapeutic antibody. Based on projected therapeutic doses and the highest target concentration observed in healthy volunteers and the patient population, the drug tolerance level of up to 40 μg/ml and target tolerance level of up to 1 μg/mL is anticipated for the CLB NAb assay. To achieve such high drug and target tolerance, a NAb assay was developed by incorporating double acid affinity purification and target blocking steps (Fig. 1).

Impact of Sample Pre-treatment (Drug and Target Depletion)

The CLB NAb assay conditions on the MSD® platform were optimized using custom design of experiment (DoE, JMP). When PCs spiked in assay buffer and optimized TFPI and ruthenium-drug conjugate concentrations were applied, a reduction of assay signal was observed.

To evaluate NAb sensitivity, drug tolerance, and target tolerance, samples were prepared as described in the "Sensitivity," "Drug tolerance," and "Target tolerance" sections and tested in the assay as described in the "CLB MSD®-Based NAb Assay Procedure" section. Sodium citrate plasma interference was observed resulting in a lack of sufficient NAb PC dose response (Fig. 2a). Samples of PCs (either 500 or 1000 ng/mL) containing 50 μg/mL of drug were identified as false negative. In contrast, while samples spiked with 500 or 1000 ng/mL NAb PC containing 1200 ng/mL target were detected as positive, NC with 1200 ng/mL target was determined to be false positive.

After applying the sample pre-treatment ("CLB MSD®-Based NAb Assay Procedure with Sample Pre-treatment" section), PC at 500 and 1000 ng/mL containing 50 μg/mL drug were positive for NAb (negative without sample pre-treatment) and NC containing 1200 ng/mL target was negative for NAb (positive without sample pre-treatment) (Fig. 2b). The results indicate that sample pre-treatment using double acid elution approach and plate-based

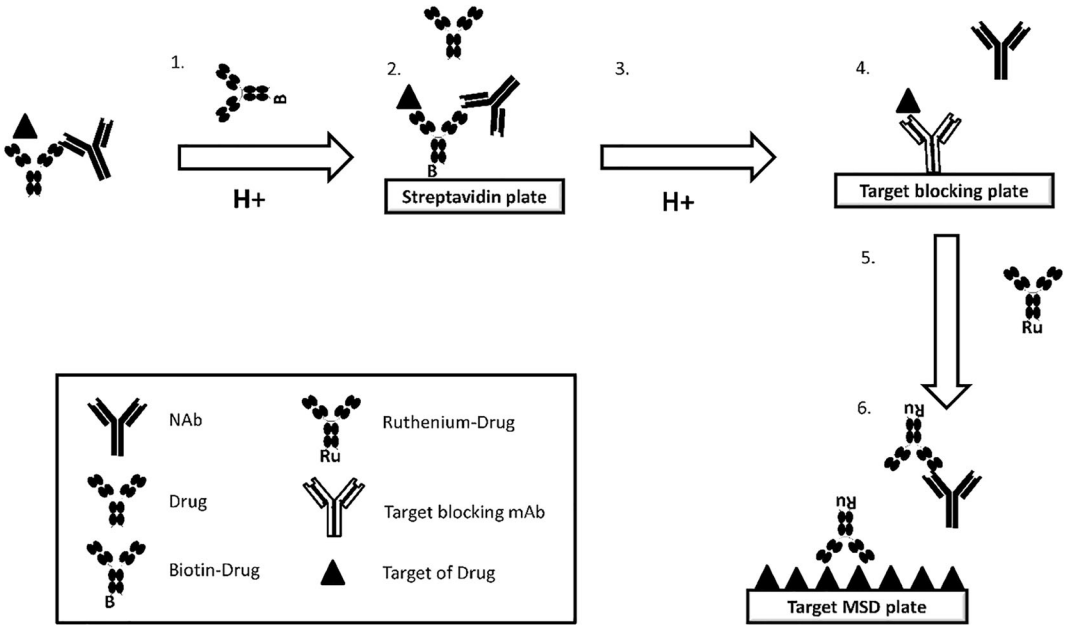


Fig. 1. Flow diagram of NAb assay format: competitive ligand binding assay with SA-HBC pre-treatment and target removal steps: 1. Acid dissociation and incubation of ADA/NAb with biotin-drug; 2. Neutralization of ADA/NAb-biotin-drug on SA-HBC plate; 3. Acid elution of ADA/NAb; 4. Neutralization of ADA/NAb on a plate coated with anti-target mAb (7A4.D9); 5. Supernatant containing ADA/NAb incubated with ruthenium-drug; 6. ADA/NAb complexed with ruthenium-drug incubated on target coated MSD plate

immunodepletion enabled accurate detection of NAb and improved the assay to mitigate drug and target interference when compared to the assay without sample pre-treatment.

Establishment of NAb Assay Cut Point

To determine the statistical cut point factor, 50 individual drug-naïve plasma samples were treated as described in the "CLB MSD®-Based NAb Assay Procedure with Sample Pre-treatment" section and analyzed on three separate days by two analysts. The individual sample response RLUs were log transformed for a total of 150 data points and analysis was performed by box plot in statistical software (JMP version 10) to identify and eliminate statistical outliers (Fig. 3a).

Two of the 150 individuals were determined to be statistical outliers and were not included in the assessment for normality using the Shapiro-Wilk test (Fig. 3b) or the cut point factor. The dataset was identified as normally distributed ($p = 0.1332$, which is > 0.05) for healthy individuals, and hence, the parametric approach was used in calculating the cut point factor. The validated screening cut point was determined to be 0.78.

Assay Sensitivity, Drug and Target Tolerance

The assay sensitivity was determined by interpolating the concentration of PC at the cut point (mean plasma concentration of the PC at the dilution that generates a response equal to the assay cut point). The assay sensitivity was estimated to be 313 ng/mL of PC in neat plasma, the concentration of NAb detectable in this assay (when using 0.78 as the cut-point factor, see "Establishment of NAb assay cut point" section) (Fig. 4a).

To assess drug tolerance, the concentration of drug that inhibits detection of the PC was assessed. PC diluted at 500, 1000 ng/mL, and NC were pre-incubated with 0, 10, 20, 30, 40, and 50 μg/mL of drug. The highest concentration of drug in which the positive control at 1 μg/mL still appeared positive for NAb (below the plate cut point) was 50 μg/mL (Fig. 4b). PC at 500 ng/mL appeared negative for NAb (above the plate cut point) upon the addition of 20 μg/mL of drug (false negative). Results indicated that PC at 1000 ng/mL can tolerate at least 50 μg/mL of drug.

To assess target tolerance, PC at 1 μg/mL and NC were pre-incubated with target (TFPI) diluted at 0, 250, 500, 1000, and 1200 ng/mL. At the highest concentration of target evaluated (1200 ng/mL), the NC still appeared negative (Fig. 4c). As expected, PC at 1 μg/mL was positive for NAb with all concentrations of target added.

Selectivity and Recovery

A robust NAb assay can detect and recover NAb within an acceptable range regardless of matrix interferences, known as matrix selectivity/recovery. To demonstrate matrix selectivity/recovery, 75–125% recovery of the PC is required.

A total of 15 individuals and NC pool were respectively spiked with 1000 ng/mL of PC to evaluate the recovery. All 15 individuals, when unspiked, were negative for NAb with RLUs above the plate cut point of 3100. Upon spiking with 1000 ng/mL of NAb PC, 87% of individuals (13 out of 15) were positive for NAb with RLUs below the plate cut point of 3100 (Fig. 5a), and 87% also met the 75–125% recovery criteria (Fig. 5b).

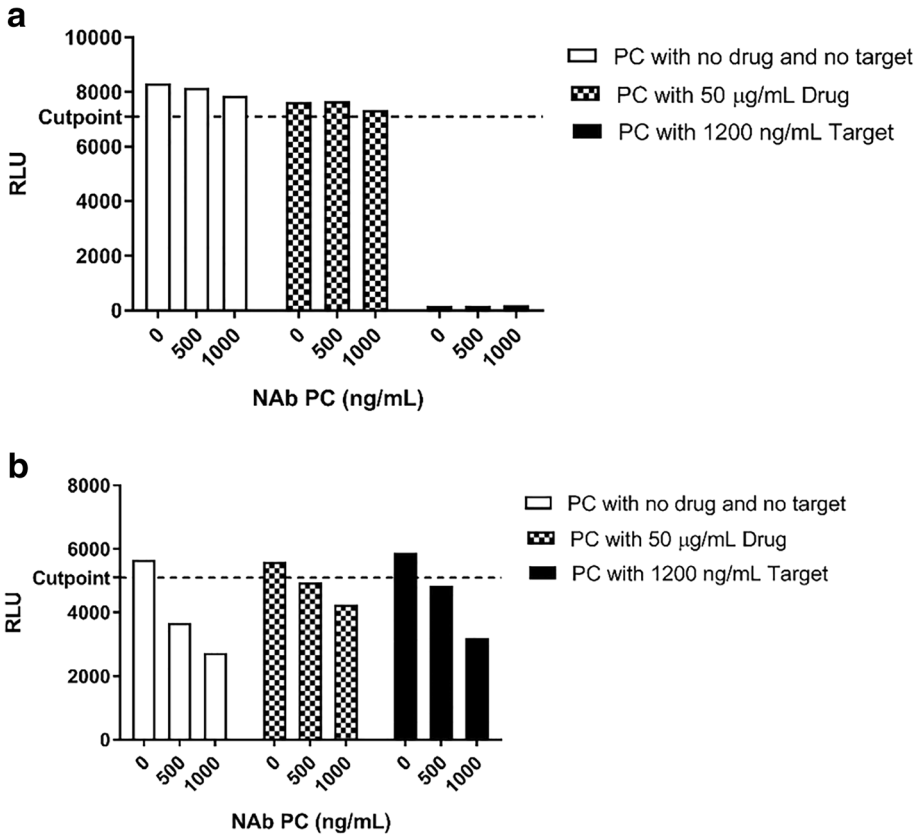


Fig. 2. NAb detection without and with sample pre-treatment was assessed by spiking NC and PC with 50 μg/mL drug or 1200 ng/mL target. The y-axis denotes the response readout in RLU and the x-axis denotes the NAb PC concentration (ng/mL) in 100% plasma. The dash line is the plate cut point. **a** CLB NAb assay without sample pre-treatment. Free drug resulted in false negatives and free target resulted in false positives. **b** CLB NAb assay with optimized sample pre-treatment procedure. All samples without NAb PC were negative. All samples spiked NAb PC 500 ng/mL or 1000 ng/mL were shown positive

Precision of Nab Assay and Robustness

Inter-day precision of the Nab assay was assessed and the data are shown in Table I. The mean $\log_{10}$ titer for the inter-day precision was 2.46 (Table I) which was within acceptable range (2.46 ± 0.48) for precision and robustness. To measure intra-plate and inter-plate variability, five individual titrations of the positive control were tested on the same plate. The intra-plate CVs were 3.8% and 2.9% for PC minimum RLU for plates 1 and 2 respectively and 5.2% and 3.9% for PC $\log_{10}$ titer. All PC $\log_{10}$ titers were within 2.46 ± 0.48. Inter-plate variability resulted in 3.9% CV for PC minimum RLU and 6.3% CV for inter-plate PC $\log_{10}$ titer (Table II).

The robustness of the assay was evaluated by having two different analysts perform the assay with the PC titration and 16 replicates of the NC on two plates over 2 days. The %CV for the ratio, NC RLU/PC (8.1 μg/mL) RLU, and the PC titer value between two analysts were 1.89 and 7.59, respectively. The data indicate that the assay is robust under the conditions tested (Table III).

Dilution Linearity

To demonstrate the ability of the NAb assay to provide reliable titer determination after factoring in dilutions, the positive control at 24.3 μg/mL was diluted to 1:2, 1:3, and 1:9 in assay matrix and titrated for a total of 8 dilution points. The %CV among Log_{10} titers of the PC (8.1 μg/mL) and dilution linearity samples was determined to be 3.9% (Table IV) and the $\log_{10}$ titers were within 2.46 ± 0.48, demonstrating dilution linearity of this NAb assay.

Freeze-Thaw and Room Temperature Stability in Matrix

To evaluate freeze-thaw (F/T) stability of the samples, aliquots of the positive control were analyzed with and compared to the untreated positive control (1 F/T cycle). No change in the $\log_{10}$ titer values of PC was detected after 6 freeze-thaw cycles. The % difference of the PC $\log_{10}$ titer values for freeze/thaw samples was within 3% compared to untreated sample. Min RLU % differences were 2.7, 6.2, and 4.3 for three independently tested samples when compared to the untreated sample (Table V).

When the bench top or RT stability was examined, Log_{10} titers of all positive controls were within 2.46 ± 0.48, and no significant change in the $\log_{10}$ titer values of positive control was detected after storage for up to 17.5 h at room temperature. Log_{10} titer % difference was under 3% and minimum RLU % difference values were 3.2, 16.1, and 1.3 when compared to the untreated sample.

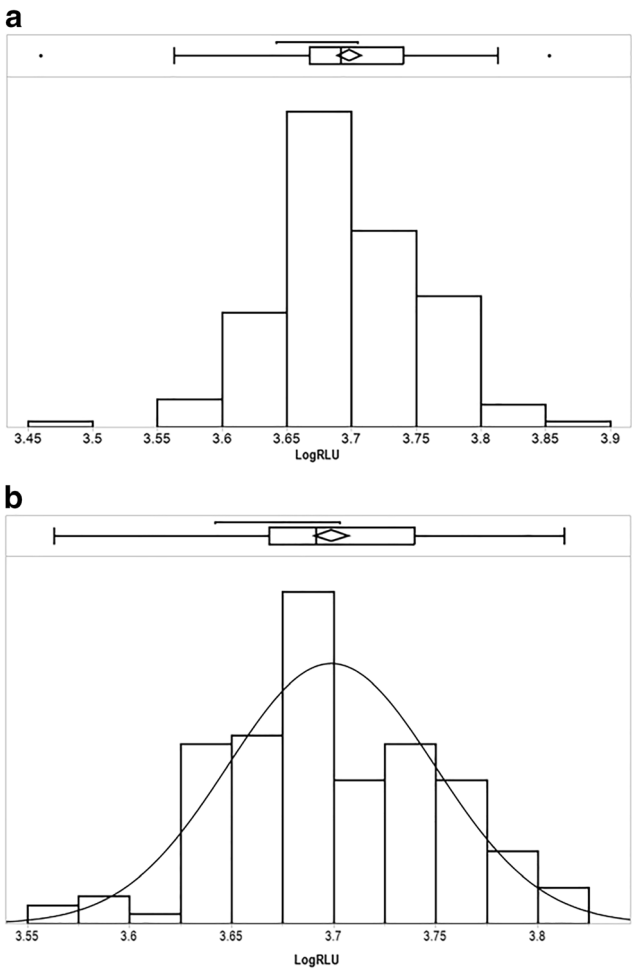
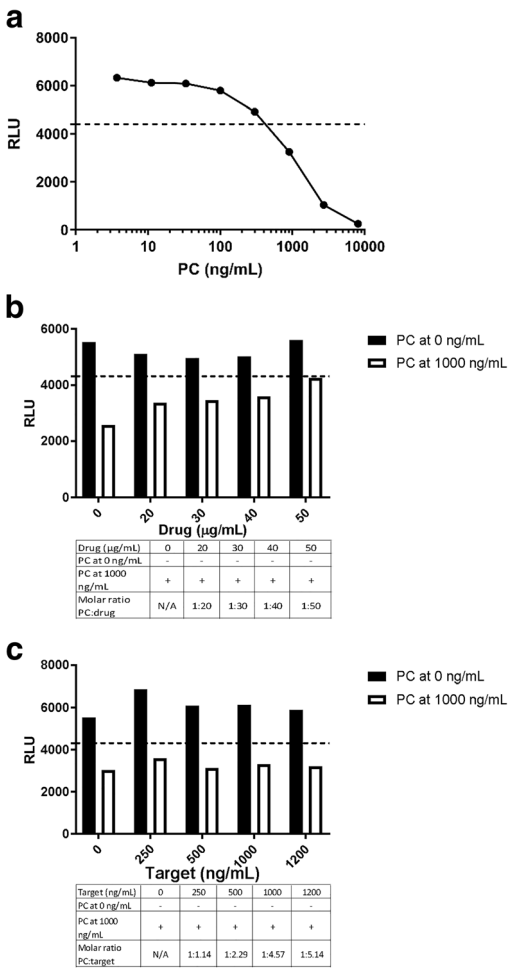


Fig. 3. Identifying outliers and determining normality. **a** Distribution of 150 data points identifying two outliers; **b** Distribution of data points after the removal of two outliers, normally distributed based on Shapiro-Wilk test. The density curve of normal distribution is overlaid on the histogram



Drug (µg/mL)	0	20	30	40	50
PC at 0 ng/mL	-	-	-	-	-
PC at 1000 ng/mL	+	+	+	+	+
Molar ratio PC:drug	N/A	1:20	1:30	1:40	1:50

Target (ng/mL)	0	250	500	1000	1200
PC at 0 ng/mL	-	-	-	-	-
PC at 1000 ng/mL	+	+	+	+	+
Molar ratio PC:target	N/A	1:1.14	1:2.29	1:4.57	1:5.14

Fig. 4. Assessment of the assay sensitivity and drug and target tolerances. Tests were conducted to evaluate assay sensitivity and drug and target tolerances. PC, drug, and target concentrations shown are all 100% plasma concentrations. Cut points were calculated as described in the "Establishment of NAb Assay Cut Point" section and are denoted by the dashed line. The y-axis denotes the assay signal (RLU). **a** The mass-based sensitivity of 313 ng/mL was determined by interpolating the concentration at the cut point. The x-axis denotes PC concentration (ng/mL). **b** Drug tolerance of the assay with 1000 ng/mL PC of at least 50 µg/mL of drug. No false-negative results of 1000 ng/mL PC obtained at up to 50 µg/mL of drug. The x-axis denotes drug concentration (µg/mL). **c** Target tolerance of the assay with 1000 ng/mL PC of at least 1200 ng/mL target. No false-positive results of 0 ng/mL PC obtained at up to 1200 ng/mL of target

NAb Assay Validation Characteristics

The acceptance criteria were determined during validation of the assay prior to the initiation of sample analysis. During validation, precision of the PC at 8100 ng/mL must have a CV ≤ 25% for both intra-plate and inter-plate values of the mean minimum RLU. CVs for the mean minimum RLU for intra-plate and inter-plate were 2.95–3.80% and 3.39%, respectively. The PC $\log_{10}$ titer must be within a log for intra-plate and inter-plate (± 0.48). In this assay, the intra-plate and inter-plate PC $\log_{10}$ titers were 2.55–2.67 and 2.67, respectively.

Mass-based sensitivity of this NAb assay was 313 ng/mL of PC. Drug tolerance of the assay was 20 µg/mL drug for 500 ng/mL of PC and 50 µg/mL of drug for 1000 ng/mL of PC. Target tolerance of the assay was 250 ng/mL (500 ng/mL of PC), 1200 ng/mL (1000 ng/mL of PC), and 1200 ng/mL in NC.

When individuals were spiked with 500 ng/mL of PC, 93% of the individuals recovered 75–125% of the PC. Of the individuals spiked with 1000 ng/mL of PC, 87% of the individuals recovered 75–125% of the PC. Stability of the PC was demonstrated at 6 freeze/thaw cycles and 24 h bench top stability with $\log_{10}$ titers of 2.41–2.48 and 2.43–2.50, respectively. Dilution linearity was demonstrated (up to a 1:9 dilution) with $\log_{10}$ titers of 2.38–2.60.

DISCUSSION

Endogenous TFPI is an inhibitor of the extrinsic coagulation pathway which can form complexes with FVIIa-TF, FXa to decrease clotting activity, and therefore blocking of TFPI by PF-06741086 has the potential to increase coagulation activity in hemophilia patients. PF-06741086 is a monoclonal antibody against the Kuniz-2 domain of human TFPI, and has no endogenous counterpart. The potential

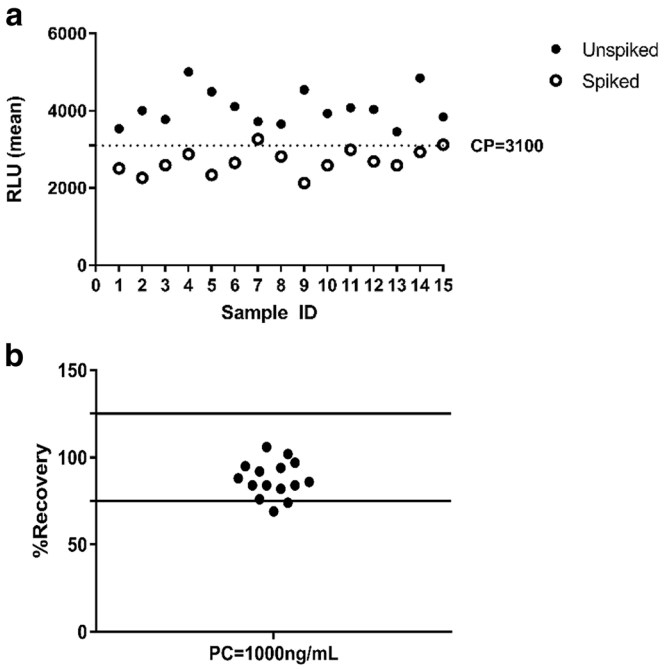


Fig. 5. Matrix selectivity/recovery was assessed by spiking 1000 ng/mL of NAb PC into 15 individuals. **a** Selectivity. One hundred percent of unspiked individuals were negative for NAb and 87% of individuals spiked with 1000 ng/mL of NAb were positive for NAb. Dashed line denotes assay cut point value. **b** Recovery. Eighty-seven percent of individuals spiked with 1000 ng/mL of NAb recovered between 75 and 125% of NAb. Solid line denotes acceptable range 100 ± 25%

adverse effects induced by anti-PF-06741086 antibodies are changes in drug exposure, pharmacological abrogation, hypersensitivity, and formation of immune complexes. In addition, endogenous TFPI forms oligomerization with lipoproteins and proteoglycans (11,14) and can exist as dimers or multimers in plasma which can potentially link the capture and detection reagents in a bridging ADA assay resulting in false ADA positive results. Unexpected ADA positive responses were detected in pre-dose samples when using standard ADA bridging assay format lacking sample pre-treatment step (data not shown) suggesting potential assay interference. Consequently, we developed a unique anti-TFPI specific mouse antibody, 7A4.D9 capable of binding epitope identical to that for PF-06741086. Because 7A4.D9 has murine framework regions and is different from PF-06741086 in complementary-determining regions (CDRs), it was possible to use 7A4.D9 as a target blocking reagent in both ADA and NAb assays. In addition to adding this target blocker, we incorporated double acid dissociation steps to increase drug tolerance.

The ADA and NAb measurement can vary depending on the reagents and procedures used in the detection methods. PF-06741086 was tested in phase I at doses from 30 to 440 mg and had an average drug concentration of 10 μg/mL for the 440 mg dose (10). Due to the typically long half-life of PF-06741086, high concentrations of drug and drug-bound TFPI were present in the biological matrix (100–

Table I. Inter-day Assay Precision

Run #	Ratio, NC RLU/PC (8.1 μg/mL) RLU	PC $\log_{10}$ titer
1	21.4	2.66
2	25.4	2.55
3	22.8	2.41
4	22.1	2.37
5	21.3	2.35
6	21.0	2.23
7	21.3	2.64
8	20.2	2.50
9	24.4	2.45
10	23.9	2.45
Mean	22.4	2.46
%CV	7.59	5.38

Table II. Intra- and Inter-plate Assay Precision

Parameters		PC (8.1 μg/mL) RLU		PC $\log_{10}$ titer	
Plates		Plate 1	Plate 2	Plate 1	Plate 2
Replicates	Rep 1	121	118	2.64	2.50
	Rep 2	119	120	2.83	2.55
	Rep 3	124	118	2.91	2.48
	Rep 4	112	125	2.90	2.72
	Rep 5	117	125	2.63	2.49
Intra-plate Precision	Mean	119	121	2.67	2.55
	%CV	3.80	2.94	5.17	3.92
Inter-plate Precision	Mean	120		2.67	
	%CV	3.39		6.29	

Table III. Inter-analyst Assay Precision

Parameters		Ratio, NC RLU/PC (8.1 μg/mL) RLU		PC log_{10} titer	
Analysts		Analyst 1	Analyst 2	Analyst 1	Analyst 2
Days	Day 1	21.5	22.8	2.66	2.41
	Day 2	21.4	21.3	2.64	2.35
Analyst precision	Mean	21.5	22.0	2.65	2.38
	%CV	0.29	5.02	0.53	1.78
Inter-analyst Precision	Mean	21.7		2.52	
	%CV	1.89		7.59	

300 ng/mL TFPI) after administration of the PF-06741086 (10). The drug tolerance target was set to exceed expected trough concentration of PF-06741086 based on the ADA/NAb sample collection time points in the clinical study. Therefore, the goal was to develop a NAb assay with drug tolerance ≥ 40 μg/mL and target tolerance ≥ 1000 ng/mL to reduce potential assay interference from both residual drug and soluble TFPI.

Methods aiming to address target interference in ADA or NAb assays, including various acid dissociation protocols, have been reported earlier (15–18). In this study, we combined sample pre-treatment step with double acid affinity purification of anti-drug antibodies and the target depletion step in order to minimize residual drug and circulating target interference in the assay. An initial acid dissociation of the sample is followed by incubation with biotin-drug in a streptavidin-coated microtiter plate containing neutralizing buffer. During this incubation period, the biotin-drug competes with any residual drug that may be present in the patient sample for binding to TFPI and ADA, if ADA are present. The biotin-drug binds to the streptavidin coated on the plate and is washed, removing drug and other interfering substances that may be present in the patient sample and retaining the TFPI and ADA on the plate. TFPI and ADA bound to the plate via the biotin-drug is subject to a second round of acid dissociation, thereby eluting the purified TFPI and ADA. The purified TFPI and ADA are then transferred into a microtiter plate pre-coated with target blocker. During this incubation time, TFPI binds target blocker and remains on the plate. The purified ADA undergoes a competitive inhibition ligand binding NAb assay.

Samples were tested in the assay with and without the pre-treatment steps. When pre-treatment steps were omitted, significant false-negative signals in the presence of high drug concentration, as well as false-positive signals, in the presence of soluble TFPI were observed (Fig. 2a). Application of the pre-treatment procedure not only improved drug and target tolerance of the assay, it also increased final sensitivity and specificity of the assay. Overall, the combination of double acid affinity purification and target mitigation pre-treatment reduces drug and target interference and increases assay sensitivity for anti-PF-06741086 NAb. The validated NAb assay displayed mass-based sensitivity of 313 ng/mL NAb PC in 100% sodium citrate plasma. In the validated NAb assay, 1000 ng/mL NAb PC demonstrated drug tolerance up to 50 μg/mL and target tolerance up to 1200 ng/mL.

In summary, a robust CLB NAb method was developed with steps that mitigated target interference by the addition of target blocking antibody. In addition, the drug tolerance was also improved by double acid affinity purification approach. This validated method demonstrated a superior drug and target tolerance for specific and precise characterization of clinical NAb responses. These approaches may be applicable to other NAb assays requiring high drug and target tolerance. Insufficient drug tolerance and target tolerance are two major concerns during immunogenicity assay development. If the NAb assay is unable to withstand the required drug and target tolerance, then many study samples may be reported as either false positive or false negative. By incorporating double acid affinity purification and target depletion pre-treatment to remove excess drug and target in samples, NAb assays may tolerate the high levels of drug and target tolerance required for the study while maintaining sufficient NAb sensitivity. The approaches described in this manuscript may provide operational advantages compared to bead-based methodologies with comparable drug and target tolerance while maintaining assay sensitivity.

Table IV. Dilution Linearity of the PC

Dilution linearity samples	PC (8.1 μg/mL)	PC (24.3 μg/mL)	PC (12.15 μg/mL, 1:2)	PC (8.1 μg/mL, 1:3)	PC (2.7 μg/mL, 1:9)
Log_{10} titer	2.60	2.38	2.39	2.49	2.39
Log_{10} titer Difference compared to PC	Not applicable	− 0.22	− 0.21	− 0.11	− 0.21
Coefficient of Variation	3.9%				

Table I dilution linearity was assessed by diluting PC at 24.3 μg/mL 1:2, 1:3, or 1:9 in human sodium citrate plasma. Log_{10} titers of the PC and dilution linearity samples were within 0.48.

Table V. Freeze/Thaw and Bench Top Stability of the Positive Control

Treatment	Treatment conditions	Log_{10} titer of Mu anti-TFPI Ab PC	Log_{10} titer % difference compared to the unexposed sample	Min RLU of Mu anti-TFPI Ab PC	Min RLU % difference compared to the unexposed sample
Freeze/thaw	Untreated	2.41	N/A	259	N/A
	6 cycles	2.48	2.90	266	2.70
	6 cycles	2.41	− 2.80	275	6.20
	6 cycles	2.44	1.20	270	4.30
Exposure to RT	Untreated	2.43	N/A	348	N/A
	Overnight RT	2.49	2.50	359	3.20
	Overnight RT	2.47	− 0.80	404	16.1
	Overnight RT	2.50	1.20	352	1.30

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