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Research Article

Approaches to Resolve False Reporting in Neutralizing Antibody Assays Caused by Reagent Leaching from Affinity Capture Elution Solid Phase

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Abstract. Insufficient drug tolerance presents a major challenge in the development of neutralizing antibody (NAb) assays for biotherapeutics. Sample pre-treatment using solid-phase extraction with acid dissociation (SPEAD) is widely reported to improve drug tolerance. In this paper, a case study is presented in which SPEAD was used in conjunction with a competitive ligand binding NAb assay format. A significant degree of biotin-drug conjugate leaching was observed resulting in the reporting of both false positive and false negative results in NAb assay. Mitigation steps have been evaluated to address drug/biotin-drug conjugate leaching. These steps included assessment of the streptavidin-coated plate in conjunction with biotin-drug conjugates at various biotin molar challenge ratios (MCR). In addition, an alternative method based on covalent capture of the drug on an aldehyde-activated plate was assessed. Both approaches were compared for the degree of drug/biotin-drug conjugate leaching during the second elution step of the SPEAD procedure. Moreover, the impact of various conditions on the assay performance was assessed, including elution pH, sample incubation time, and biotin MCR. For the covalent drug capture method, capture conditions were evaluated. Optimized conditions in both streptavidin capture and covalent capture methods enabled a significant reduction of drug/biotin-drug conjugate leaching. A streptavidin high binding capacity approach using biotin-drug conjugate with a MCR of 50:1 was chosen as the optimal method yielding a NAb assay with a fit for purpose sensitivity (153 ng/mL) and a drug tolerance of up to 50 μg/mL with 500 ng/mL PC.

KEY WORDS: ACE; anti-drug antibody; biotin-drug conjugate/drug leaching; drug tolerance; immunogenicity; neutralizing antibody; sample pre-treatment; SPEAD.

INTRODUCTION

Many therapeutic protein products have been approved for treating human diseases (1–3). However, all biological therapeutics have the potential to cause unwanted immunogenic responses and the development of anti-drug antibodies (ADA), with possible impact to the pharmacokinetic profile and efficacy of the drug (4–6). A specific type of ADA is neutralizing antibodies (NAb) which can interfere with drug-target interaction (7–9) or diminish the therapeutic protein pharmacological function (10,11). In some cases, NAbs may induce life-threatening adverse effects, *e.g.*, cross react with non-redundant endogenous counterparts or neutralize life-saving biotherapeutics (12–14).

Cell-based and competitive ligand binding (CLB) assays reflecting the *in vivo* therapeutic mechanisms of action are two common NAb assay formats for detection of drug-specific NAb in clinics. A high level of residual drug is often present in the samples collected for ADA and NAb assessment (15). To ensure assay accuracy, NAb assays with sufficient drug tolerance are required.

Sample pre-treatment methods such as ACE (drug coated on the plate) (16), SPEAD (biotin-drug conjugates captured on the streptavidin plate), (17) and BEAD (biotin-drug conjugates captured on the streptavidin bead) (18,19) have been successfully applied to reduce drug interference in the assays. In the SPEAD or BEAD protocols, pre-existing complexes of drug and ADA or NAb are first dissociated under low pH conditions. After the solution is neutralized, the biotin-drug conjugate competes with the free drug for binding to ADA or NAb. The biotin-drug complex with drug-specific ADA or NAb is then captured on streptavidin-coated

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solid phase (plates or beads). The captured ADA/NAb is eluted using a second low pH treatment. It is known that some degree of leaching of biotin-drug conjugate from streptavidin beads may occur at low pH (19,20). Generally biotin-drug conjugate leaching is not problematic for an ADA assay; however, it may interfere in a NAb assay confounding interpretation of assay results.

To develop a sensitive NAb assay for a monoclonal antibody therapeutic PF-06741086 (PF) with a high drug tolerance, two plate-based methods were explored and optimized. In this manuscript, various assay parameters and methodologies (a SPEAD-based method *vs.* a covalent drug capture method) to decrease biotin-PF conjugate/PF leaching were assessed. The data is presented and discussed. A SPEAD-based protocol based on use of biotin-PF conjugate with a high molar challenge ratio (MCR) and tolerating residual PF up to 50 μg/mL at the positive control concentration of 500 ng/mL is achieved.

MATERIALS

Equipment, Reagents, Assay Materials, and Matrix Samples

Humanized IgG therapeutic PF-06741086 (PF), NAb-positive control (PC, mouse anti-PF), biotin-PF, and ruthenium-PF were generated by Pfizer (Andover, MA). Human target to PF was produced by Syngene (Bangalore, India). Sodium borohydride ($NaBH_4$) was obtained from Fisher Chemical (Hanover Park, IL). BioFx TMB One Component HRP substrate was obtained from SurModics IVD, Inc. (Eden Prairie, MN). 3D aldehyde matrix–coated (3D AM) plates were obtained from Medical Surface (Natick, MA). Costar high binding (HB) polystyrene plates were purchased from Corning, Inc. (Corning, NY). Pierce streptavidin–coated high binding capacity (SA-HBC) plates, Slide-a-lyzer™ cassettes with a 10-K molecular weight cutoff (MWCO), and EZ-Link™Sulfo-*N*-hydroxysuccinimide (NHS)-LC biotin were obtained from Thermo Scientific (Hanover Park, IL). Goat anti-mouse IgG HRP was obtained from Southern Biotech (Birmingham, AL). Goat anti-human IgG HRP was obtained from Bethyl Labs (Montgomery, TX). Human plasma (sodium citrate) was obtained from BioreclamationIVT (Westbury, NY). High binding (HB) MSD plate, Sulfo-TAG NHS-Ester ruthenium and MSD read buffer (4×) were obtained from Meso Scale Discovery (Rockville, MD). Sulfuric acid (0.18 M), THST (Tris high salt buffer plus Tween-20), PBS-CMF (phosphate-buffered saline without magnesium or calcium), PBST (PBS containing 0.05% Tween-20), 4% BSA/PBST (PBST containing 4% bovine serum albumin), 1% BSA/PBST (PBST containing 1% BSA), Milli-Q water, 100 mM glycine pH 2.0, 300 mM acetic acid pH 3.0, 1 M Tris pH 8.5, 1 M Tris pH 9.0, 1 M Tris pH 10.0, and 2 N H_2SO_4 stop solution (sulfuric acid) were supplied by Pfizer (Andover, MA). DMSO was obtained from Sigma-Aldrich (Darmstadt, Germany). 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) or Spectramax 340 by Molecular Device (San Jose, CA).

Labeling of Antibodies

Biotin-PF Conjugate. The monoclonal therapeutic antibody (PF) was coupled to EZ-Link™Sulfo-*N*-hydroxysuccinimide (NHS)-LC biotin (Thermo Scientific) at MCR of 18, 50, or 100. The reaction was incubated for 2 h and subsequently quenched with 1 M Tris, pH 9.0. The resulting amide bond is stable at low pH. Biotinylated antibodies were purified by dialysis into PBS-CMF buffer, pH 7.2 through the utility of 10 K MWCO Slide-a-lyzer™ cassettes for 18–24 h at 4 °C. Reagent was aliquoted and stored frozen at − 70 °C. The incorporation ratios for those biotin-PF conjugate reagents were determined by LC-TOF, and they were 5.8, 11.4, and 21.2.

Ruthenium-PF Conjugate. Conjugation of PF to Sulfo-TAG NHS-Ester ruthenium (Meso-Scale Discovery) was conducted in the presence of excess amount of the label (12:1) for 1 h at ambient temperature. Ruthenium-PF conjugate reagent was dialyzed using PBS-CMF buffer, pH 7.2, applying 10 K MWCO Slide-a-Lyzer™ cassettes for 18–24 h at 4 °C. Reagent was aliquoted and stored frozen at − 70 °C.

Preparation of Streptavidin High Binding Capacity (SA-HBC) Plate with Non-covalently Bound Biotin-PF Conjugate

The SA-HBC plates were washed with THST (washing buffer) to remove weakly bound SA. A solution of 5 μg/mL biotin PF (MCR 18:1, 50:1, or 100:1) was prepared in 1% BSA/PBST and 100 μL/well added to the washed SA-HBC plate. The plate was incubated for 1 h at room temperature (RT).

Preparation of 3D AM Plate with Covalently Bound PF

3D AM plates were hydrated with 100 μL/well of water for 5 min. Water was removed and 100 μL/well of various concentrations of PF in PBS were added to the plate and incubated for 2 h. Plates were blocked with 4% BSA/PBST. PF-coated plates were treated with 100 μL/well of 1 mg/mL $NaBH_4$ for 1 h at RT.

METHODS

Detection of Biotin-PF Conjugate or PF Leaching

A volume of 110 μL/well of 100 mM glycine pH 2.0 or 300 mM acetic acid pH 3.0 was added to the PF-coated plates (biotin-PF conjugate for SA-HBC plate, PF for 3D AM plate) and incubated for 5 to 20 min. After incubation, 100 μL/well of eluate was neutralized on another SA-HBC plate or a HB plate with Tris buffer pH 8.5 or pH 10, respectively, and incubated for 2 h. The plates were then blocked with 150 μL/well of 4% BSA/ PBST for 1 to 2 h. One hundred microliters per well of 40 μg/mL goat anti-human IgG HRP in 1% BSA/PBST was added and incubated for 2 h. One hundred microliters per well of the enzyme substrate, 3,3′,5,5′-tetramethylbenzidine (TMB), was added to produce the colorimetric response. The reaction was stopped with 100 μL/well of 2 N sulfuric acid, and the optical density (OD) was measured at 450 nm on a Molecular Device Spectramax instrument.

Assessment of Binding Capacity of SA-HBC and 3D AM Plate

Biotin-PF conjugates with MCR of 18:1, 50:1, and 100:1 were diluted to final concentration of 0, 3, 4, 5, 10, or 20 μg/mL in 1% BSA/PBST. The biotin-PF conjugate solutions were incubated on SA-HBC plates for 1 h at RT as described in the "Preparation of Streptavidin High Binding Capacity (SA-HBC) Plate with Non-covalently Bound Biotin-PF Conjugate" section.

PF was covalently captured on 3D AM plates. PF solutions at final concentration of 0, 10, 20, or 40 μg/mL in PBS were incubated on the 3D AM plates for 2 or 4 h at RT, after which the plates were blocked and treated with $NaBH_4$ as described in the "Preparation of 3D AM Plate with Covalently Bound PF" section.

To assess plate-binding capacity, 100 μL/well of goat anti-human IgG HRP solution at 20 μg/mL or 40 μg/mL in 1% BSA/PBST was incubated on the SA-HBC and 3D AM plates, respectively, for 1 h at RT. Plates were washed with THST buffer, and 100 μL/well of TMB substrate was added to produce the colorimetric response. The reaction was stopped with 100 μL/well of 2 N sulfuric acid. The OD was measured at 450 nm on a Molecular Device Spectramax instrument.

Assessment of Binding Capacity of Biotin-PF-Coated SA-HBC Plate and PF-Coated 3D AM Plate to Capture NAb PC

The SA-HBC plates were coated with 5 μg/mL biotin-PF conjugate with MCR of 18:1, 50:1, and 100:1 for 1 h as described in the "Preparation of Streptavidin High Binding Capacity (SA-HBC) Plate with Non-covalently Bound Biotin-PF Conjugate" section. Alternatively, PF was covalently bound to the 3D AM plate with 20 μg/mL PF in PBS for 2 h as described in the "Preparation of 3D AM Plate with Covalently Bound PF" section.

To evaluate the capacity of the plates to bind NAb PC, 100 μL/well of NAb PC at 0, 62.5, 125, 250, or 500 ng/mL in 1% BSA/PBST was incubated on the prepared SA-HBC and 3D AM plates for 2 h. After washing plates with THST, 100 μL/well of 500 ng/mL goat anti-mouse IgG HRP in 1% BSA/PBST was incubated on the plates for 2 h. After washing the plates with THST, 100 μL/well of TMB substrate was added to produce the colorimetric response. The reaction was stopped with 100 μL/well of 2 N sulfuric acid. The OD was measured at 450 nm on a Molecular Device Spectramax instrument.

NAb PC Elution Efficiency

A 5 μ/mL biotin-PF conjugate solution was coated on two SA-HBC plates (identified as plates 1 and 2) as described in the "Preparation of Streptavidin High Binding Capacity (SA-HBC) Plate with Non-covalently Bound Biotin-PF Conjugate" section. Plate 1 was then incubated with 0 or 500 ng/mL of NAb PC solution as described in the "Assessment of Binding Capacity of Biotin-PF-Coated SA-HBC Plate and PF-Coated 3D AM Plate to Capture NAb PC" section.

A volume of 110 μL/well of 100 mM glycine, pH 2.0 or 300 mM acetic acid, pH 3.0, was added to plate 1 and incubated for 5 to 20 min at RT. After washing plate 2 with THST, 100 μL of eluate from plate 1 was transferred to plate 2 and neutralized with 10 μL/well of 1 M Tris at either pH 8.5 or pH 10 for glycine or acetic acid, respectively. Plate 2 was incubated for 2 h. After incubation, plate 2 was washed with THST; NAb PC material that was eluted and captured on the plate 2 was detected as described in the "Assessment of Binding Capacity of Biotin-PF-Coated SA-HBC Plate and PF-Coated 3D AM Plate to Capture NAb PC" section.

CLB MSD NAb Assay Procedure with Sample Pre-treatment

Various concentrations of NAb PC were spiked in human sodium citrate plasma to assess the assay sensitivity. Drug tolerance samples were prepared by spiking PF at various concentrations into human sodium citrate plasma containing 250 or 500 ng/mL of NAb PC. Fifty individual treatment-naïve plasma samples were used for cut point determination. Unspiked human plasma was used as the negative control (NC) sample.

Samples were diluted 1:2 in 1% BSA/PBST, then further diluted 1:4.75 in 100 mM glycine pH 2.0 and incubated for 5 min. After incubation, 190 μL acidified samples were incubated with 10 μL of 100 μg/mL biotin PF (MCR of 50:1) for 5 min. Then, 90 μL of samples was introduced to a SA-HBC plate containing 9.5 μL/well of 1 M Tris pH 8.5 and incubated for 5 h at RT. The plate was then washed with THST and incubated with 85 μL/well of 100 mM glycine pH 2.0 for 5 min to elute bound NAb. Seventy microliters of the acid eluate was neutralized with 20 μL of 21.2 ng/mL ruthenium-PF conjugate in neutralization buffer (1:1 of 1 M Tris pH 8.5:1% BSA/PBST) and incubated overnight at RT. Subsequently, 70 μL of the neutralized sample was transferred into a HB MSD assay plate (previously coated with 50 μL of 0.4 μg/mL target in PBS overnight at 4 °C, blocked with 4% BSA/PBST for at least 1 h at RT, and washed with THST) and incubated for 1 h. The HB MSD plate was washed with THST and read on a MSD reader Sector Imager S 600 after addition of 150 μL/well of TPA) read buffer 2× (diluted from 4× with Milli-Q water).

Data Analysis: Cut Point, Cut Point Factor, and Floating Cut Point Determination

Cut point is defined as the response level that distinguishes positive and negative samples. A sample is considered positive if the sample generated assay response is less than or equal to the cut point value. To define assay cut point, 50 individual treatment-naïve sodium citrate plasma samples were tested on each of three separate runs by two operators as described in the "CLB MSD NAb Assay Procedure with Sample Pre-treatment" section. Resulting data set was log transformed and normality of data distribution was assessed using the JMP statistical software (version 10, SAS Institute, Inc., Cary, NC) after outlier removal. Cut point was then calculated as follows (4,21,22):

- If the data distribution is not normal, the cut point was calculated using 99th percentile with 1% false positive

- For normal distribution, the cut point was calculated as follows:
- Anti-log{(mean log transformed response of individual matrix samples-2.326 × standard deviation of the log individual samples)}
- Cut point factor was calculated as follows:
- Cut point/mean response of the NC
- Floating cut point was calculated as follows:
- Cut point factor × mean response of the NC on individual plate

RESULTS

A CLB NAb assay was requested in support of a clinical development protocol for a therapeutic monoclonal antibody–based PF candidate that is designed to bind and inhibit a soluble circulating molecular target. Based on the PF dosing schedule, the CLB NAb assay drug tolerance of 40 μg/mL was required. To address the challenge, a SPEAD procedure was initially adapted. A biotin-PF conjugate was prepared with the MCR at 18:1.

In the method, the NAb is captured by biotin-PF conjugate after samples are pre-treated with low pH solution of 300 mM acetic acid (pH 3.0). The same low pH buffer was used to elute NAb from the capture streptavidin plate. After mixing and neutralization with the ruthenium-PF conjugate, eluted NAb was added to the MSD plate coated with the PF molecular target. Competition between PF and target *vs.* NAb for the binding to the ruthenium-PF conjugate resulted in a reduction of the assay signal as a function of NAb concentration in the sample. To monitor assay performance, a mouse monoclonal neutralizing antibody raised against the PF was used as a positive control (PC).

When PC sample was treated by low pH only (omitting the acid-capture-elution procedure) followed by the neutralization and analysis in the NAb assay, a reduction of assay signal was observed as a function of the NAb PC concentration (Fig. 1, closed circles). In contrast, assay signal dependency on the NAb PC did not follow the same trend when samples were subjected to the SPEAD pretreatment step described above (Fig. 1, open squares). Because treatment-naïve samples that did not contain drug were used in the test, it was suspected that observed lack of correlation is due to the leaching of the biotin-PF conjugate that took place during the second elution step of the SPEAD protocol. To investigate the suspected biotin-PF conjugate leaching, an ACE-like procedure based on the use of biotin-PF conjugate pre-captured on a streptavidin (SA-HBC) plate was applied.

Various low pH solutions (*e.g.*, 20 mM citric acid pH 3.0 with 150 mM NaCl and 200 mM glycine pH 2.7 with 100 mM NaCl) were applied during the wash step after the SA-HBC plate coating with the biotin-PF conjugate and prior to the NAb PC capture. To mitigate the effects of the biotin-PF conjugate leaching, various concentrations of acetic acid (*e.g.*, 10–100 mM) with varying concentrations of NaCl and BSA were evaluated as elution buffer. However, these methodologies not only reduced the impact from the biotin-PF conjugate leaching, but also led to a reduction in the NAb PC recovery (data not shown).

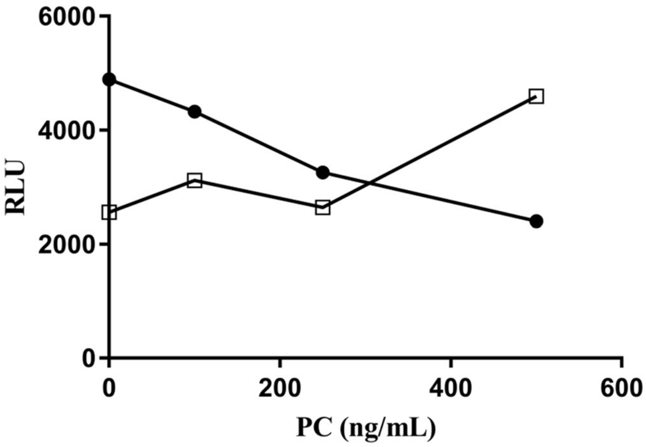

Fig. 1. Comparison of NAb PC response curve without and with sample pre-treatment (SPEAD, biotin-PF conjugate MCR 18:1) in CLB MSD format. The *x*-axis denotes the PC concentration in nanograms per milliliter. The *y*-axis denotes the signal of PC responses as relative light units (RLU). Closed circle: without sample pre-treatment; open square: with SPEAD sample pre-treatment

Due to the strong interaction between biotin and streptavidin, the dissociation of biotin-PF conjugate (MCR 18:1) from the SA-HBC plate at the tested conditions was not expected. As illustrated in Fig. 2, leaching of biotin-PF conjugate can generate false positive or false negative responses. Therefore, it is critical to identify conditions that can reduce biotin-PF conjugate leaching during the low pH elution step while allowing for an efficient recovery of the NAb PC material.

For a solid phase double acid elution assay to be successful, the chemistry securing PF to the solid phase needs to withstand harsh conditions of acid elution, *e.g.*, pH 2.0–3.0. The following two methods were evaluated:

- *SA-HBC*: streptavidin HBC plate–based capture of biotin-PF conjugate with higher MCR values, potentially allowing to increase avidity of biotin-PF binding to the plate;
- *3D AM*: 3D AM plate–based covalent capture of protein PF with or without Schiff base reduction reagent $NaBH_4$, allowing to stabilize PF binding to the plate.

Investigation of Biotin-PF Leaching and NAb PC Elution Efficiency Using a SA-HBC Approach

To first investigate the biotin-PF conjugate (MCR 18:1) leaching and NAb PC elution efficiency challenges with the SA-HBC approach, 100 mM glycine pH 2.0 and 300 mM acetic acid pH 3.0 were tested as elution buffers at varying incubation times as described in the "Detection of Biotin-PF Conjugate or PF Leaching" and "Nab PC Elution Efficiency" sections. The assessments of the leaching of biotin-PF conjugate and elution efficiency of NAb PC are shown in Fig. 3(a, b). SA-HBC plates were coated with 0 or 5 μg/mL of biotin-PF conjugate in 1% BSA/PBST. The 1% BSA/PBST buffer without biotin-PF conjugate was used as a blank. The sample to blank signal ratio was applied to monitor the degree of leaching. In order to determine the relative amount

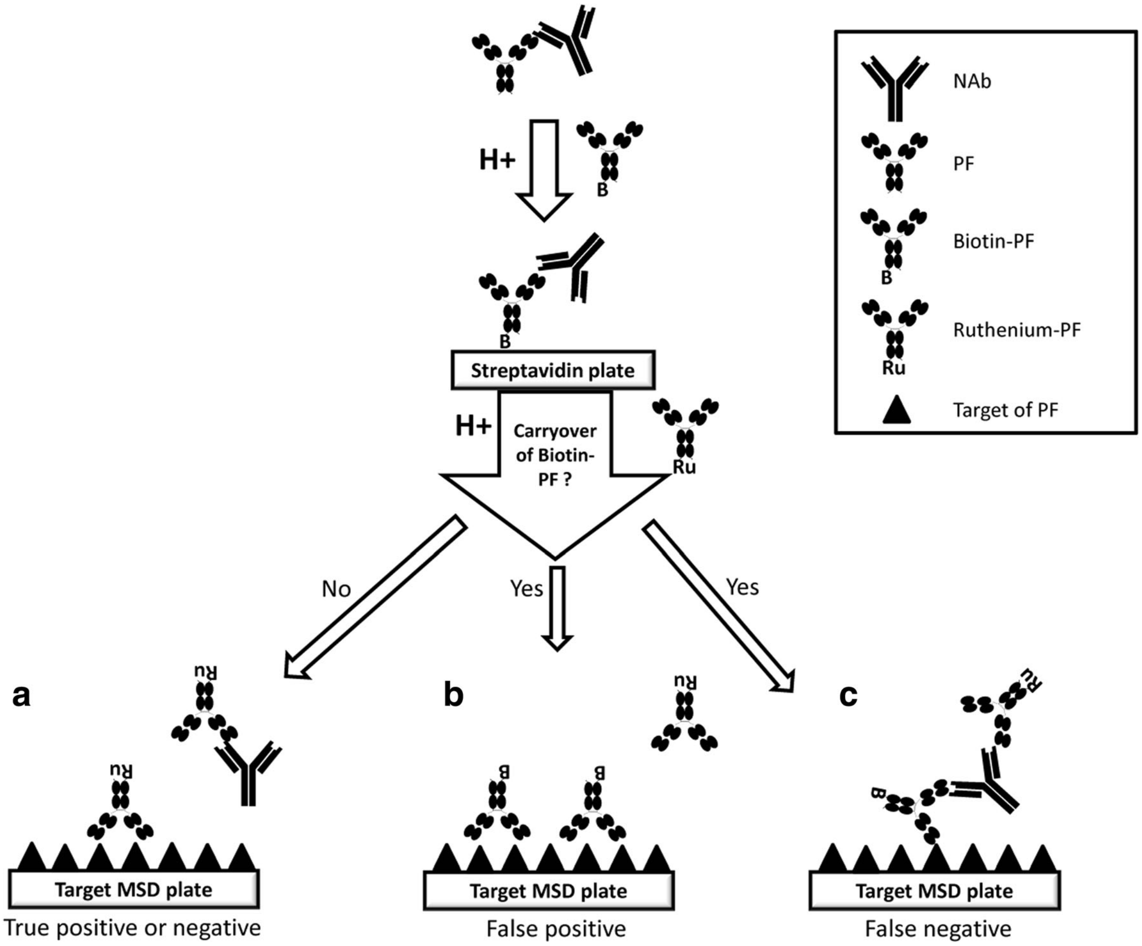

Fig. 2. Representative scenarios in a NAb assay with double acid affinity purification in sample pre-treatment to improve drug tolerance. **a** Presence or absence of NAb leading to a true positive signal or true negative signal. **b** Leaching of biotin-PF conjugates from 2nd acid elution step leading to false positive due to potential decrease in ruthenium-PF binding and a corresponding reduction in signal. **c** Leaching of biotin-PF conjugates leads to false negative due to the binding of biotin-PF/NAb/ruthenium-PF complexes to the target on the plate with a corresponding increase in signal

of biotin-PF conjugate leaching, the eluate was neutralized and the material was captured on a separate SA-HBC plate. The bound biotin-PF conjugate was detected using goat anti-human IgG HRP conjugate as described in the "Detection of Biotin-PF Conjugate or PF Leaching" section.

To assess NAb PC elution efficiency, a blank (0 ng/mL PC in 1% BSA/PBST) and 500 ng/mL of NAb PC in 1% BSA/PBST samples were prepared, captured on biotin-PF-coated SA-HBC plate, eluted, neutralized, and recaptured on a separate biotin-PF-pre-coated SA plate as depicted in the Fig. 3(b). The recaptured murine monoclonal NAb PC was detected using goat anti-mouse IgG HRP reagent as described in the "Nab PC Elution Efficiency" section and the ratio of signal to blank was used to monitor the PC elution efficiency.

The results of the biotin-PF conjugate leaching and PC elution efficiency using 100 mM glycine pH 2.0 and 300 mM acetic acid pH 3.0 are shown in Fig. 4(a, b). Figure 4(a) illustrates that the leaching of the biotin-PF conjugate at the MCR 18:1 may be dependent, in large part, on either the molar concentration or pH of the elution buffer. An increase in leaching was observed with 100 mM glycine pH 2.0 as compared to the 300 mM acetic acid pH 3.0. Leaching of the biotin-PF conjugate when using 100 mM glycine pH 2.0 increased as a function of the elution time, whereas limited impact of the elution time was observed for 300 mM acetic acid pH 3.0. Although 300 mM acetic acid pH 3.0 caused reduced level of leaching as compared to the 100 mM glycine pH 2.0 buffer, it is important to note that both conditions still resulted in a significant leaching.

As demonstrated in Fig. 4(b), the concentration and pH of the acidic solution have the greater impact on the recovery of the NAb PC *vs.* the elution time. There was an average of a 1.4-fold increase in the NAb PC sample signal to the blank ratio when the NAb PC was eluted with 100 mM glycine pH 2.0 as compared to the elution with 300 mM acetic acid pH 3.0. NAb PC was eluted more efficiently (generating a higher signal to blank ratio value) with the 100 mM glycine pH 2.0 as compared to the 300 mM acetic acid pH 3.0.

Impact of Different Biotin-PF Conjugate MCR on Leaching of Biotin-PF Conjugate in the SA-HBC Approach

To investigate if the biotin-PF conjugate MCR can impact leaching of the biotin-PF conjugate, reagents with MCR 18:1, 50:1, and 100:1 were prepared. SA-HBC plates

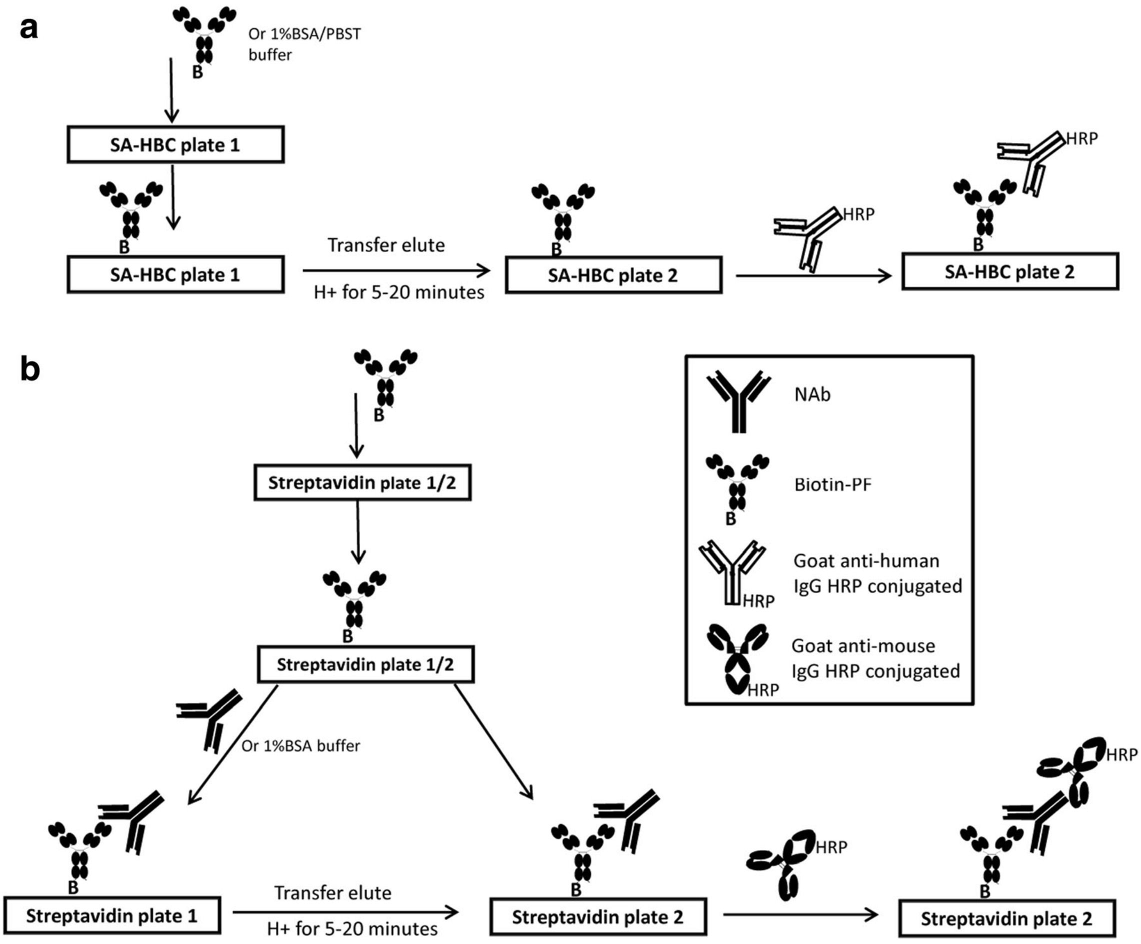

Fig. 3. Schematic of assay flow to evaluate leaching and elution efficiency. **a** Leaching of biotin-PF conjugate. **b** NAb PC elution

were coated with 5 μg/mL of biotin-PF conjugates diluted in 1% BSA/PBST. The 1% BSA/PBST buffer was used as blank solution. The ratio of signal generated by leached biotin-PF conjugate to the blank value served as a measure of biotin-PF leaching. Based on the data reported in the "Investigation of Biotin-PF Leaching and NAb PC Elution Efficiency Using a SA-HBC Approach" section, 100 mM glycine pH 2.0 was selected to produce optimal NAb PC elution efficiency. The effect of the incubation time was investigated in the range of 5 to 20 min as described in the "Detection of Biotin-PF Conjugate or PF Leaching" section. Figure 5 demonstrates that reagents with MCR 50:1 and 100:1 had minimal biotin-PF conjugate leaching detected when elution was conducted for 5 min as defined by a ratio of signal to blank approaching 1. As the elution time increased, there was a measurable increase in the leaching of the biotin-PF conjugate at MCR 50:1, while no detectable leaching was observed for the biotin-PF conjugate at MCR 100:1 up to 20 min of elution (Fig. 5). The biotin-PF conjugate at MCR 18:1 had a significant biotin-PF leaching as compared to the MCR 50:1 and MCR 100:1 reagents at all elution time points tested.

Based on the reduction of observed leaching for the biotin-conjugate reagents with MCR 50:1 and MCR 100:1, these preparations were investigated further to determine the impact of MCR on capacity of NAb PC binding.

Impact of Biotin-PF Conjugate MCR on the Capacity of SA-HBC Plate to Bind Biotin-PF Conjugate and Subsequent Capacity to Capture NAb PC

To determine if the MCR impacts the SA-HBC plate capacity to bind biotin-PF conjugate, reagent preparations at MCR 18:1, 50:1, or 100:1 were captured on SA-HBC plate as described in the "Assessment of Binding Capacity of SA-HBC and 3D AM Plate" section. The capacity of the SA-HBC plate to bind biotin-PF conjugate at all levels of MCR tested reached saturation at 5 μg/mL of biotin-PF reagent (shown in Fig. 6(a)). The MCR of the biotin-PF conjugate appears to have an inverse relationship to the signal as the biotin-PF conjugate at the MCR of 18:1 exhibited a slightly higher signal compared to the preparation at the MCR of 100:1. The increase in the number of biotin conjugated to the PF molecule at the MCR 50:1 and MCR 100:1 may have masked some epitopes available for the goat anti-human IgG HRP.

Next, the impact of biotin-PF conjugate MCR on the capacity of the biotin-PF-coated SA-HBC plate to bind NAb PC was investigated. Solutions of 5 μg/mL of biotin-PF

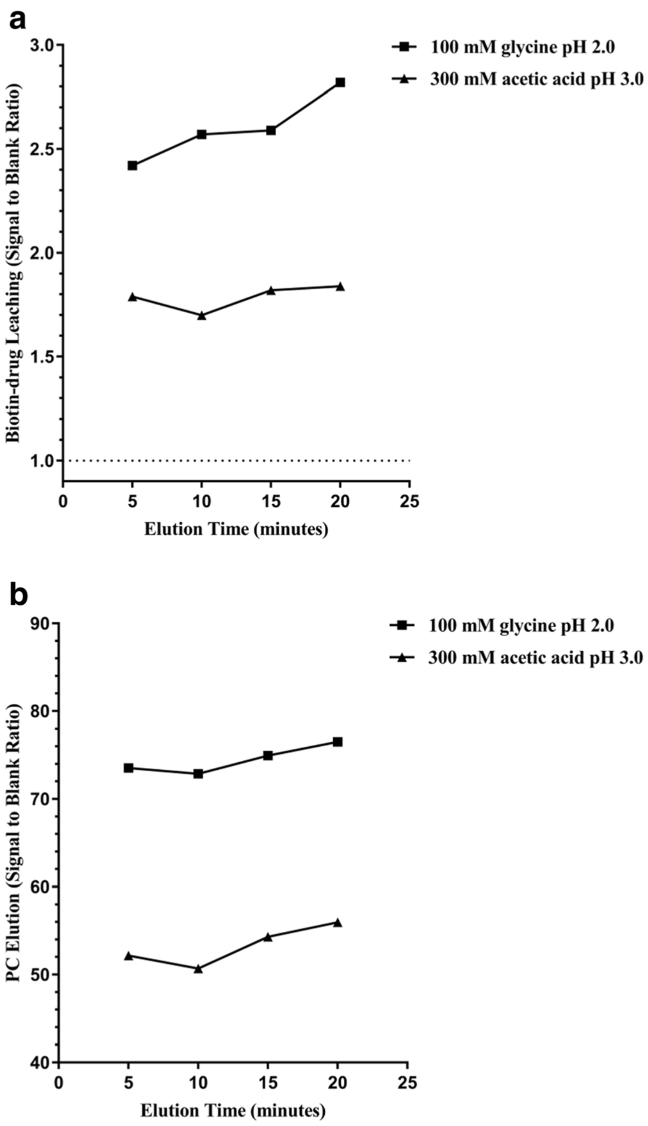

Fig. 4. Impact of molar concentration of the acid/pH and elution time on biotin-PF conjugate (MCR 18:1) leaching and NAb PC (500 ng/mL) elution efficiency in conventional SA-HBC approach. **a** biotin-PF conjugate leaching. **b** NAb PC (500 ng/mL) elution efficiency. Curves demonstrate the effects of 100 mM glycine pH 2.0 and 300 mM acetic acid pH 3.0 and the effects of elution time on biotin-PF conjugate leaching or NAb response in SA-HBC method. The *x*-axis denotes the acid elution time (minutes). The *y*-axis denotes biotin-PF conjugate leaching (reported as the ratio of the signal to blank; dotted line represents ratio of 1 where no leaching would be detected) **a** or NAb PC elution efficiency (reported as the ratio of signal to blank) **b**

conjugate at MCRs 18:1, 50:1, or 100:1 was coated on SA-HBC plate. Solutions of the NAb PC at 0, 62.5, 125, 250, or 500 ng/mL prepared in 1% BSA/PBST were added to the plate as described in the "Assessment of Binding Capacity of Biotin-PF-Coated SA-HBC Plate and PF-Coated 3D AM Plate to Capture NAb PC" section. NAb PC binding capacity of biotin-PF conjugate-coated SA-HBC plate reached up to 500 ng/mL of NAb PC reagent (Fig. 6(b)). The capacity of SA-HBC plates coated with biotin-PF conjugates at MCR 18:1, 50:1, and 100:1 to bind NAb PC were therefore comparable.

Impact of $NaBH_4$ Treatment in 3D AM Approach

The 3D AM approach utilizes an activated aldehyde surface microtiter plate as the solid phase. In the process of PF conjugation to the 3D AM plate, a Schiff base covalent bond is formed. The impact of a reducing agent, $NaBH_4$, on the stability of the PF conjugation was investigated. A PF solution at 0 or 20 μg/mL prepared in PBS was incubated on 3D AM aldehyde plate for 1 h. The plates were either left untreated or treated with the $NaBH_4$ solution. To determine stability of the formed covalent linker, the plates were incubated with a solution of 100 mM glycine pH 2.0 for 5 to 20 min. The eluate was transferred to a Costar HB plate and neutralized. Any leaching PF would be captured on the Costar HB plate followed by detection with goat anti-human IgG HRP. PBS buffer was used as a blank solution. The ratio of signal generated by the PF leached from the 3D AM plate to the signal generated by the blank served as a measure of PF leaching.

As illustrated in Fig. 7, leaching of the PF is dependent on treatment of the 3D AM plate with $NaBH_4$. $NaBH_4$ treatment significantly reduced the leaching of the PF during the elution step as compared to the 3D AM plate that did not undergo $NaBH_4$ treatment. Elution time of 5 to 20 min with 100 mM glycine pH 2.0 did not affect PF leaching from the $NaBH_4$-treated 3D AM plate. PF leaching from the $NaBH_4$ untreated 3D AM plate increased as a function with the elution time, most likely due to the hydrolysis of covalent Schiff bond, which is stabilized by the $NaBH_4$ reduction step. Thus, 3D AM plate with $NaBH_4$ treatment in the 3D AM approach was further selected to assess plate capacity to bind PF and subsequently NAb PC.

Capacity of 3D AM Plate to Bind PF and Subsequent Capacity to Capture NAb PC

PF was diluted to final concentration of 0, 10, 20, or 40 μg/mL in PBS and coated on hydrolyzed 3D AM plates for either 2 or 4 h. The bound PF was detected by goat anti-human IgG HRP as described in the "Assessment of Binding Capacity of SA-HBC and 3D AM Plate" section. The amount of PF the 3D AM plate captured continued to increase up to 40 μg/mL of PF without reaching a saturation point (Fig. 8(a)). A slight time-dependent increase in the binding capacity was observed at PF concentrations higher than 20 μg/mL.

A 20 μg/mL PF solution coated on 3D AM was utilized to determine plate capacity to specifically bind NAb PC reagent (see the "Preparation of 3D AM Plate with Covalently Bound PF" section for details). Plate was incubated with NAb PC at concentrations ranging between 0 and 500 ng/mL prepared in 1% BSA/PBST. Detection of bound NAb PC was performed using goat anti-mouse IgG-HRP (see the "Assessment of Binding Capacity of Biotin-PF-Coated SA-HBC Plate and PF-Coated 3D AM Plate to Capture NAb PC" section for details). An increase in the NAb PC binding was observed up to 500 ng/mL of the PC (Fig. 8(b)).

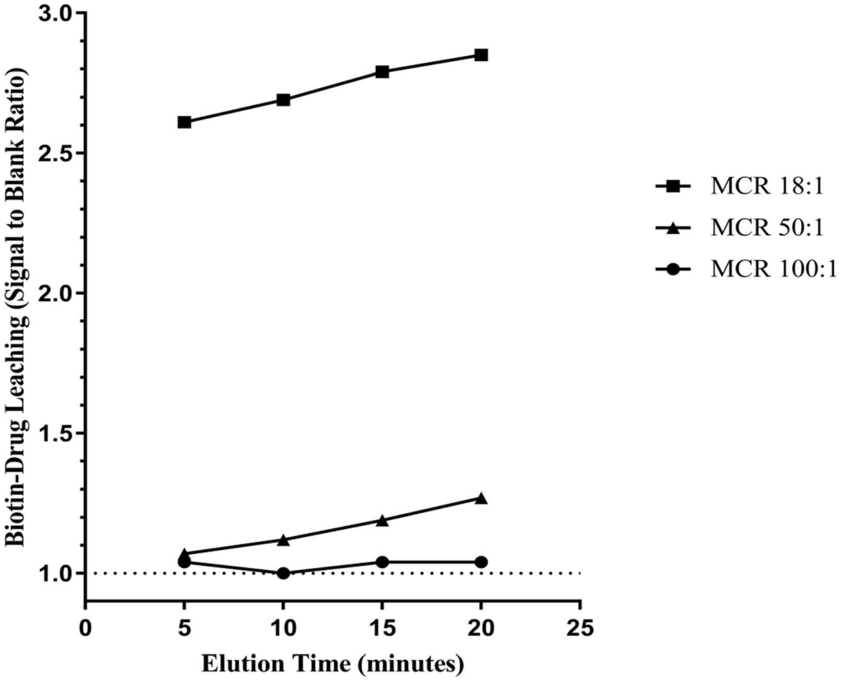

Fig. 5. Impact of the biotin-PF conjugate MCR (18:1, 50:1, and 100:1) and elution time on the leaching of biotin-PF conjugate in the SA-HBC method. The *x*-axis denotes the acid elution time (minutes). The *y*-axis denotes biotin-PF conjugate leaching (reported as the ratio of the signal to blank). Dotted line represents ratio of 1 where no leaching would be detected

Selection of Final Sample Pre-treatment Approach

SA-HBC (utilizing biotin-PF conjugate MCR 50:1) and 3D AM (including $NaHB_4$ reduction step, 3D AM method) protocols to capture PF and subsequently to bind NAb PC were compared (Figs. 6(b) and 8(b), respectively). Both approaches are equivalent in terms of minimizing biotin-PF conjugate or PF leaching.

The 3D AM protocol has a notably larger PF binding capacity compared to SA-HBC (40 *vs.* 5 μg/mL) and, therefore, may improve resulting drug tolerance characteristic of the final NAb detection method. The SA-HBC plates are ready to use and available from multiple vendors. The SA-HBC-based methodology offers a shorter assay time and can be adapted for a solution-based NAb assay protocol. The SA-HBC methodology requires a biotin-PF conjugate reagent vs. the 3D AM–based methodology where no reagent labeling is needed.

As described in the following sections, a NAb assay was developed based on the SA-HBC protocol utilizing biotin-PF conjugate with MCR 50:1.

Qualification of Assay Designed to Detect PF-Specific Neutralizing Antibody in Human Matrix

An assay was developed based on the SA-HBC protocol utilizing 50:1 MCR biotin-PF conjugate. Fifty (50) individual PF-naïve normal human sodium citrate plasma samples were analyzed in the method to determine assay-specific cut point as described in the "CLB MSD NAb Assay Procedure with Sample Pre-treatment" section. Samples were tested in three independent runs. The individual sample response signal values (RLUs) were log-transformed, analyzed to determine statistical outliers, and further analyzed to determine normality of the data set distribution (Fig. 9(a), the "Data Analysis: Cut Point, Cut Point Factor and Floating Cut Point Determination" section). Two out of 150 data points were determined to be statistical outliers by a box plot analysis using JMP software. After outlier removal, the data distribution was found to be normally distributed using Shapiro-Wilk test (p value = 0.0611). The parametric approach was used to calculate assay cut point factor allowing for a 1% false positive response rate (see the "Data Analysis: Cut Point, Cut Point Factor, and Floating Cut Point Determination" section for details). The floating cut point factor of 0.8 was established for the normal human sodium citrate sample population.

Sensitivity of the NAb assay was defined as the concentration of the NAb PC that generates an assay response equal to the floating cut point value. To assess the developed NAb assay sensitivity, NAb PC reagent was spiked into human plasma at concentrations ranging from 50 to 1000 ng/mL. Unspiked human sodium citrate plasma sample was used as a negative control (NC). Positive and negative control samples were assayed as described in the "CLB MSD NAb Assay Procedure with Sample Pre-treatment" section. The resulting mass-based sensitivity was estimated to be 153 ng/mL NAb PC in 100% human plasma (Fig. 9(b)).

To evaluate NAb assay drug tolerance, a sample of NC and samples spiked with NAb PC and PF were prepared and tested in the assay as described in the "CLB MSD NAb Assay Procedure with Sample Pre-treatment" section. A sample was

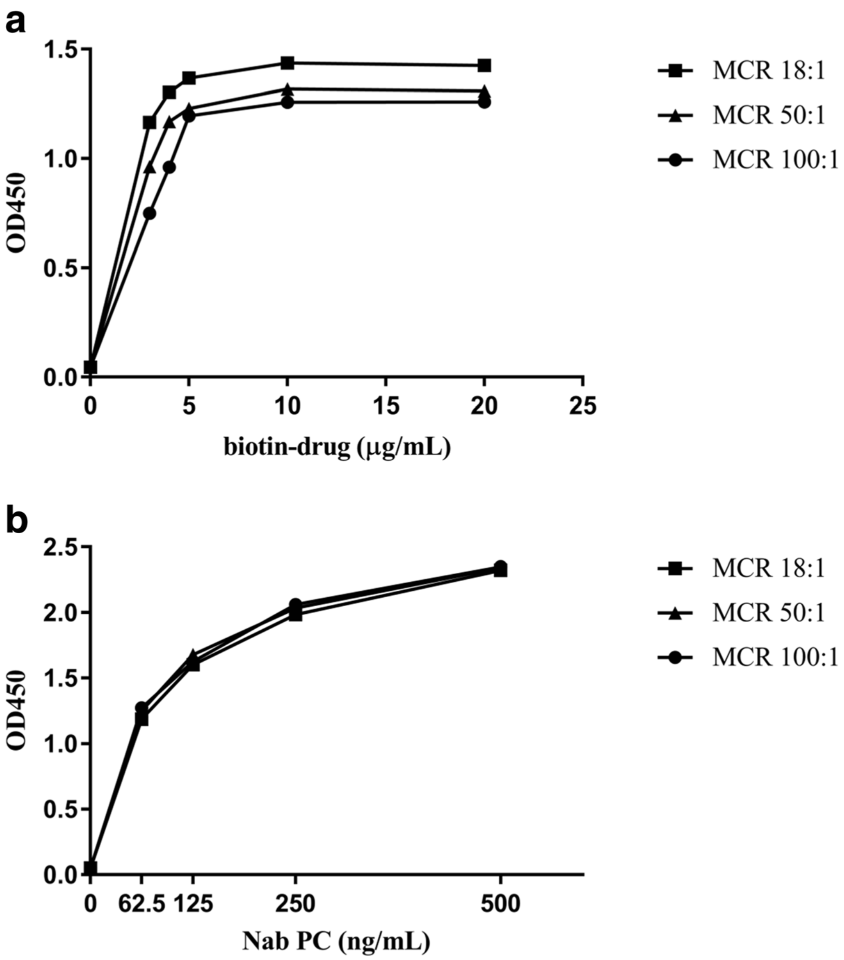

Fig. 6. Assessment of the binding capacity of SA-HBC plate. **a** Impact of the biotin-PF conjugate MCR on the SA-HBC place capacity to bind biotin-PF reagent. Conjugate preparations at MCR 18:1, 50:1, or 100:1 were captured on SA-HBC plate as described in the "Assessment of Binding Capacity of SA-HBC And 3D AM Plate" section. The *y*-axis denotes assay signal (OD450). The *x*-axis denotes the concentration of the biotin-PF conjugate tested (µg/mL). **b** Impact of biotin-PF conjugate MCR on the capacity of the biotin-PF-coated SA-HBC plate to bind NAb PC. The NAb PC reagent was titrated starting from 500 ng/mL as described in the "Assessment of Binding Capacity of Biotin-PF-Coated SA-HBC Plate and PF-Coated 3D AM Plate to Capture NAb PC" section. The *y*-axis denotes assay signal (OD450). The *x*-axis denotes the NAb PC concentration (ng/mL)

considered positive if it generated an assay signal at or below the assay cut point. NAb PC solutions prepared at 250 and 500 ng/mL in human sodium citrate plasma scored positive in the assay when spiked up to 40 µg/mL of PF (Fig. 9(c)).

DISCUSSION

An unwanted immunogenic response to a biotherapeutic can be detected in the form of anti-drug antibodies (ADA) and/or neutralizing anti-drug antibodies (NAb). The NAb response is a subset of the binding ADA response. Detection of the ADA and NAb responses is a common expectation during biotherapeutic drug development. One of the important characteristics of ADA and NAb assays is the ability to tolerate residual drug in the sample. A common approach to address such challenge is to apply a sample pre-treatment step, *e.g.*, an ACE, SPEAD, or BEAD procedure (16,17,19). However, a common challenge encountered during the development of NAb assays employing sample pre-treatment techniques is drug or biotin-drug conjugate leaching. The biotin-streptavidin interaction has a high binding affinity which is typically significantly higher as compared to common drug-ADA interactions ($K_d = 10^{-15}$ M) (17,23,24). Therefore, only the dissociation of the ADA from the biotinylated drug that is captured on a streptavidin solid phase is expected during the second elution step of a sample pre-treatment protocol. Some degree of biotin-drug conjugate leaching was observed which may in part be explained by a reduction of biotin-streptavidin binding affinity at low pH (25). The second elution step of a sample pre-treatment protocol is intended to isolate drug-specific antibody (ADA/NAb). Biotin-drug conjugate leached in the second elution step will therefore contaminate the ADA/NAb preparation and potentially impact performance of the final assay. Similarly, in methods based on immunocapture of ADA/NAb from a sample using drug that

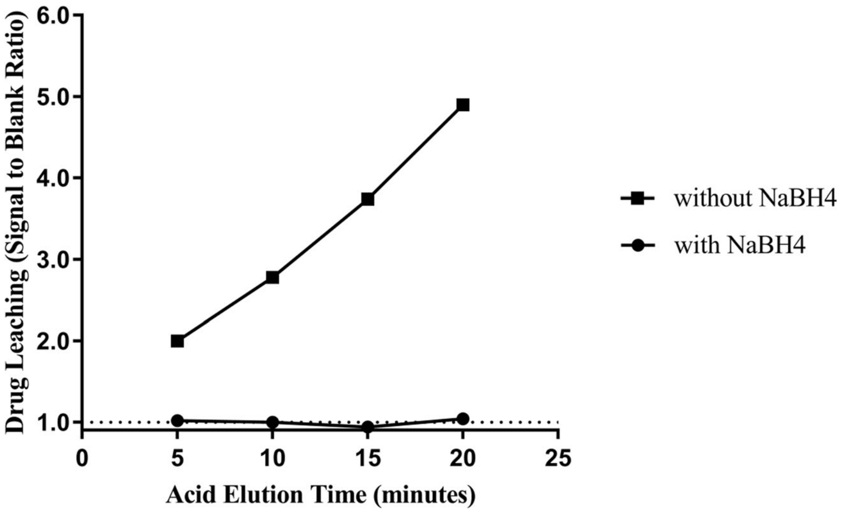

Fig. 7. PF leaching from the 3D AM plate with and without $NaBH_4$ treatment. The *x*-axis denotes the acid elution time (minutes). The *y*-axis denotes PF leaching (reported as the ratio of the signal to blank). Dotted line represents ratio of 1 where no leaching would be detected

is passively absorbed on a solid phase (*e.g.*, ACE), leaching of the drug molecule may lead to an impact on the performance of the resulting ADA/NAb assay.

In the present study, protocol modifications were assessed to address the issue of biotin-drug conjugate or drug leaching. Initially the leaching phenomenon was investigated under various conditions by varying wash buffer components, elution buffer pH, and incubation times. These did not lead to an impactful reduction of the observed degree of biotin-drug leaching.

Therefore, two methodologies were assessed: (a) SA-HBC with higher MCR biotin-drug conjugate protocol–based immunocapture of the ADA/NAb from a sample using streptavidin coated plate; (b) 3D AM with imine-forming reaction of aldehyde and amine on the biotherapeutic protein protocol–based immunocapture of the ADA/NAb from a sample using covalent capture of drug on 3D AM plate.

In the SA-HBC protocol, the low pH buffer composition (*e.g.*, glycine pH 2.0 *vs.* acetic acid pH 3.0), incubation time during the second elution step and the MCR (ranging from 18:1 to 100:1) were shown to have the most impact on the outcome of the assay. Improved efficiency of NAb PC capture/elution was obtained with glycine pH 2.0. The biotin-PF conjugate MCR characteristic was shown to have the strongest impact on the observed leaching. The conjugates at MCR 50:1 and 100:1 showed a significant improvement in the reduction of biotin-PF leaching as compared to the MCR 18:1 conjugate when tested using 100 mM glycine pH 2.0 and incubation times from 5 to 20 min during the second elution step. A limited increase in biotin-PF conjugate leaching was observed when using MCR 50:1 conjugate while the MCR 100:1 conjugate had no detectable leaching up to 20 min of treatment. The capacity of the SA-HBC plate to capture biotin-PF conjugate did not significantly change based on the MCR characteristic of the conjugate. Approximately 5 μg/mL of biotin-PF conjugate could be captured on a SA-HBC plate for the preparations at MCR 18:1, 50:1, and 100:1. The NAb PC capture/elution efficiency of the SA-HBC protocol was assessed by utilizing SA-HBC plates coated with biotin-PF conjugate with various MCRs at the saturation level of 5 μg/mL of the coating solution. The NAb PC capture/elution efficiency was not significantly modulated with a change in the MCR of the biotin-PF conjugate.

In the 3D AM protocol, drug covalent capture on the aldehyde-activated plate was shown to be significantly stabilized by a $NaBH_4$ reduction step leading to a reduction of drug leaching. No detectable PF leaching was observed up to 20 min of low pH elution step. In contrast, a notable amount of PF leaching was observed when the $NaBH_4$ reduction step was omitted and covalently captured drug on the 3D AM plate was treated using 100 mM glycine pH 2.0 for as little as 5 min. Sufficient capacity of the 3D AM plate to capture PF could be reached after 2 h of incubation with PF solution. In the 3D AM protocol that included the $NaBH_4$ reduction step, a plate coated with 20 μg/mL of PF was shown to bind up to 500 ng/mL of NAb PC.

Both approaches, SA-HBC and 3D AM, were shown to be comparable in reducing PF leaching and had adequate capacity to capture NAb PC reagent. Due to the relative operational complexity related to preparation of the 3D AM plate and the availability of the streptavidin-coated plates (*e.g.*, SA-HBC) from multiple vendors, the SA-HBC protocol was selected herein for further evaluation and implementation in the NAb assay.

These two approaches can be applied for either cell-based NAb or total ADA assays. The potential impact of residual buffer components on the cell viability should be evaluated for an individual assay protocol. Any bioanalytical method has its inherent advantages and disadvantages. One disadvantage of SPEAD method is a limited plate binding capacity as compared to the binding capacity of bead-based material utilized in the BEAD methods. However, SPEAD

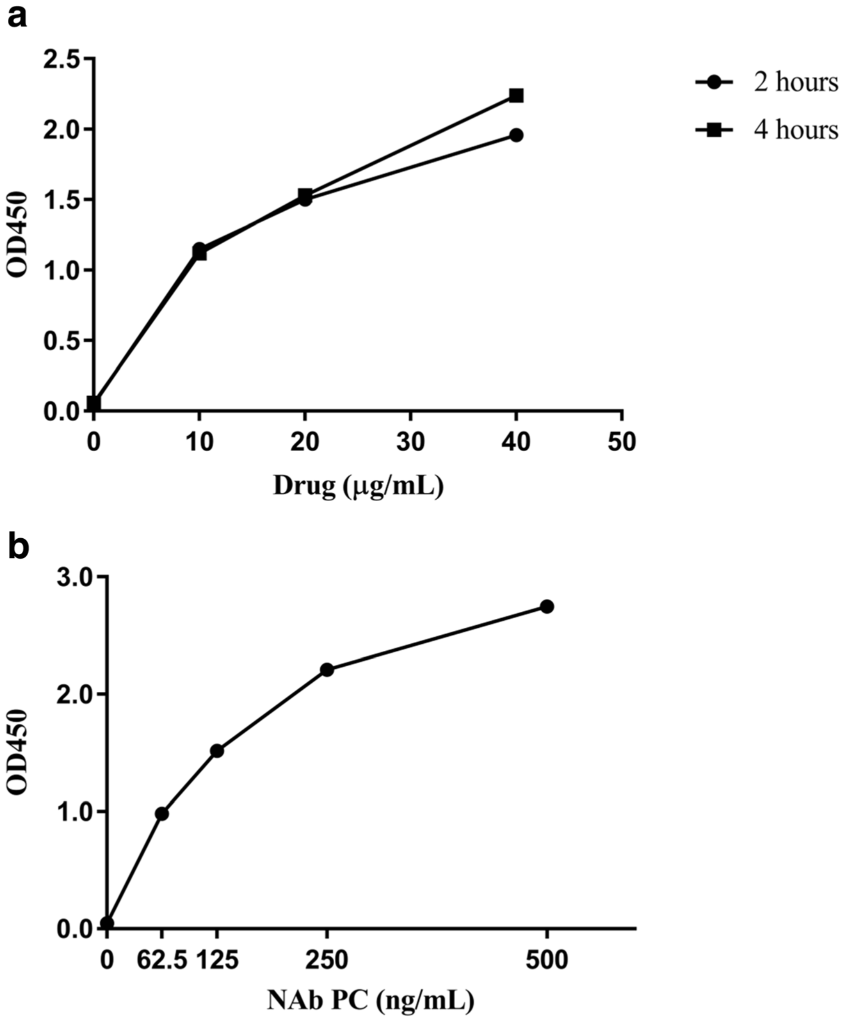

Fig. 8. 3D AM plate capacity to bind PF and subsequent capacity to capture PF-specific NAb PC. **a** Concentration dependence of the PF binding to a 3D AM plate. Coating was performed for 2 or 4 h. The *y*-axis denotes the assay signal (OD450). The *x*-axis denotes PF concentration (μg/mL). **b** Concentration dependence of the PF-specific NAb PC binding to a PF-coated 3D AM plate. The *y*-axis denotes the assay signal (OD450). The *x*-axis denotes NAb PC concentration (ng/mL)

protocol has general advantage of operational simplicity and material cost. Regardless whether the SPEAD or BEAD protocol is used, if free drug and biotin drug are complexed with NAb, the free drug may be leached to the final elute upon the 2nd acid dissociation treatment. The residual carried over drug may lead to false reporting in the NAb assay. Optimization of individual assay conditions may be needed to reduce the loss of NAb material, thus minimizing chances of false negative reporting in the NAb assay.

An assay was designed to detect anti-PF-specific neutralizing antibody using SA-HBC protocol described in this manuscript. In the assay, the 50:1 MCR biotin-drug conjugate was used to capture NAb from a sample. Based on the protocol, optimization data presented herein, the use of the 50:1 MCR biotin-drug conjugate significantly reduced the biotin-drug conjugate leaching in the secondary NAb elution step. The NAb was then detected in the non-cell-based competitive ligand binding assay. A SA-HBC protocol based on use of the 50:1 MCR biotin-drug conjugate produced a sufficiently sensitive assay capable of detecting 153 ng/mL of the NAb PC reagent and tolerating up to 50 μg/mL of residual drug in the presence of 500 ng/mL NAb PC reagent.

In summary, two protocols were investigated in order to reduce apparent leaching of the drug or biotin-drug conjugate from the ADA/NAb capture plate during a double acid elution assay method. The two protocols included a 3D AM plate where the drug is covalently captured based on the amine group interaction with aldehyde-activated plate surface. In the SA-HBC protocol, the biotin-drug conjugate is captured non-covalently by a strong streptavidin-biotin interaction. It was determined that with the use of a high molecular challenge ratio, biotin-drug conjugate preparation can significantly reduce elution of the biotin-drug conjugate from the SA-HBC plate. Overall, the two protocols provided a comparable degree of reduction of the drug (3D AM) or biotin-drug conjugate (SA-HBC) leaching. The SA-HBC

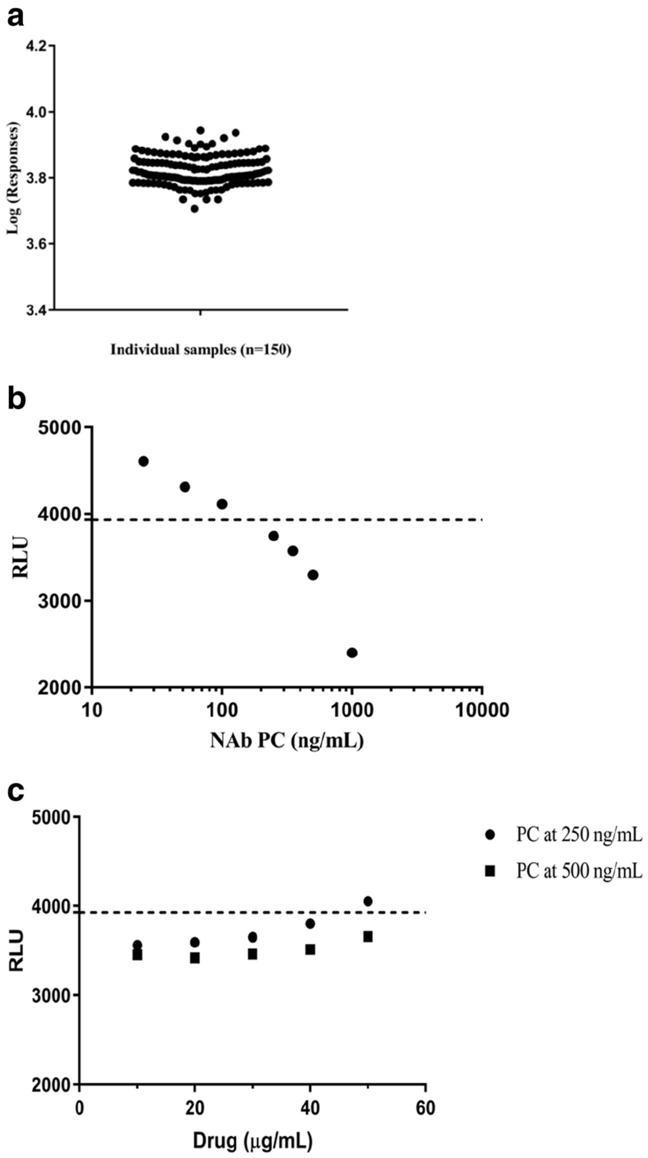

Fig. 9. Qualification of the assay designed to detect NAb specific to PF. Tests were conducted using SA-HBC protocol utilizing biotin-PF conjugate prepared at MCR 50:1. **a** Distribution of assay signal generated by analysis of 50 treatment-naïve human sodium citrate plasma samples. Samples were tested in three separate runs. **b** NAb PC titration profile. NAb PC was spiked into pooled human sodium citrate plasma at concentrations ranging from 50 to 1000 ng/mL. **c** Assessment of the assay ability to tolerate presence of residual PF (drug tolerance test). Drug tolerance was determined by analysis of samples spiked with the NAb PC at 250 and 500 ng/mL and supplemented with various amounts of PF, up to 50 μg/mL. The dashed line denotes assay cut point value

plate–based protocol was selected to develop a competitive ligand binding NAb assay with appropriateness and fit for purpose characteristics, including assay sensitivity and drug tolerance.

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