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Research Article

Anti-drug Antibody Assay Validation: Improved Reporting of the Assay Selectivity via Simpler Positive Control Recovery Data Analysis

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Abstract. Anti-drug antibody (ADA) assay selectivity is evaluated during assay validation to assess the potential for individual matrices to interfere with detection of ADA. While current EMA and FDA guideline documents suggest comparative analysis with and without matrix, they do not provide specific recommendations on the acceptance criteria such as an acceptable percent positive control (PC) recovery range or positive rate. Industry has adopted an approach where recovery of PC spiked sample is expected to fall within ± 20% (80 to 120%) *vs.* that for the PC material spiked in negative control matrix or assay buffer. Here, it is proposed that ADA assay selectivity evaluated using a qualitative assessment of PC recovery *vs.* a PK-like quantitative method may be more appropriate. The PC recovery test should focus on the reliability of the method to detect the low PC level in individual samples and avoid false-negative ADA reporting. Therefore, it is proposed that assessment of high PC level as well as the assessment of quantitative percent recovery (within ± 20%) should not be included in the test. The recovery test may be viewed as acceptable should a pre-selected number of individual samples (for example at least 8 or 9 out of 10) prepared at the low PC concentration of the assay score as ADA positive.

KEY WORDS: ADA; ADA assay selectivity; ADA assay validation; anti-drug antibody.

INTRODUCTION

Assessment of immune response potential is an integral element of biotherapeutic compound development. Most commonly, induction of humoral immune response to a biotherapeutic is evaluated by detecting immunoglobulin-based response with a drug-specific anti-drug antibody (ADA) test. Because an anti-drug immune response may lead to various safety and efficacy related clinical sequelae, it is expected that ADA assays should be developed with the reduced probability of reporting false ADA-negative results. A positive control (PC) reagent typically serves as an assay suitability control during ADA assay development, validation and production phases. The PC reagent is not an assay reference material as the typical ADA response is expected to be study subject dependent.

One of the elements of assay qualification and validation is an assessment of assay PC reagent recovery, also referred to as the ADA assay selectivity test, in which PC reagent is spiked into several treatment-naïve individual samples and reagent recovery is determined in the assay. The PC reagent recovery test demonstrates an appropriate degree of assay selectivity and lack of adverse impact of various matrix components on assay ability to detect ADA in samples. PC recovery is commonly conducted in study-relevant matrix as the prevalence of interfering components may vary and can be disease-state specific. Recovery test is typically conducted as part of the evaluation of the matrix interference and when determining assay minimal required dilution (MRD) value. Evaluation of both low and high concentrations of the PC (LPC and HPC levels) has been recommended.

Assessment criteria and acceptance thresholds vary between regulatory guidelines and industry publications, as presented in Table I. In the test, signal produced by the assay PC material spiked into an individual sample is compared with the signal produced by PC material when spiked into pooled negative control (NC) and/or assay buffer. It is critical to point out that current versions of EMA and FDA guideline documents do not contain direct and specific recommendations on the quantitative percent recovery range for PC spiked matrix samples but do state that an assessment is needed (1,2). A quantitative approach to PC recovery evaluation has been broadly applied by the industry based on previously published white paper with an acceptable range of recovery within ± 20% (*i.e.* 80 to 120%) (3). In the same publication, Shankar *et al.* suggested that an alternative approach may be possible. Yet, the semi-quantitative nature

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of ADA methods and polyclonal diverse type of a typical ADA response makes application of a strictly quantitative approach to assess recovery of PC reagent counterintuitive. Realizing the value of the ADA assay selectivity test when evaluating assay ability to detect low ADA concentrations and as a result reducing the risk of false ADA-negative reporting, we are proposing herein an alternative acceptance methodology for the test.

METHODS

ADA Assay Description, PF-76456267

An endogenous protein homologue fusion compound, designated here as PF-76456267, is developed for an anti-inflammation, immunomodulatory treatment. Anti-PF-76456267 antibodies were detected in human serum using bridging electrochemiluminescent (ECL) immunoassay method utilizing Meso Scale Discovery (MSD) platform. Samples, PCs and pooled NC were diluted 1:5 in the assay buffer and loaded into appropriate wells of a polypropylene plate. Samples were then acidified by the addition of an equal volume of 0.8% acetic acid (pH 3.5) and allowed to incubate for approximately 15 min. Samples were then neutralized by the addition of an equal volume of 2× master mix containing equal concentration of capture (PF-76456267—biotin) and detection (PF-76456267—ruthenium) conjugates along with neutralizing 1 M Tris buffer, pH 10. After approximately 1-h incubation at room temperature (RT), samples were transferred into a pre-blocked (using 4% bovine serum albumin (BSA) in phosphate-buffered saline (PBST), 0.05% (*v*/*v*) Tween-20) streptavidin MSD microplate and incubated for 1 h at RT. After a wash step, MSD tripropylamine-containing read buffer was added and chemiluminescence signals were acquired as relative light units (RLU). A polyclonal rabbit anti-PF-76456267 antibody was used as the PC reagent. The sensitivity limits of the screen and confirmatory assay were identified as 2.81 and 8.92 ng/mL, respectively, calculated as per Shankar *et al.* (3). A floating-type assay cut-point (cut-point factor 1.08) was defined by following recommendations described in the Shankar *et al.* (3). Anti-PF-76456267 ADA assay cut-point was determined based on analysis of 50 individual treatment-naïve matrix samples. Screening and confirmatory cut-point values were identified as described in the Shankar *et al.* (3). PC recovery in the anti-PF-76456267 ADA assay was calculated using Eq. 1 shown in the "Data Analysis Methodologies" section. Assay drug tolerance was assessed by supplementing pooled normal human serum samples spiked at the 100 ng/mL PC level with an increasing concentration of unlabeled PF-76456267 ranging from 0 to 100 μg/mL and was identified as 100 μg/mL of PF-76456267.

Table I. Regulatory Guidelines and Industry Recommendations for the PC Recovery Evaluation

Guideline/ manuscript	Proposed evaluation strategy	Specific acceptable range	Reference
EMA guideline, 2009	Matrix effect caused by reagents and substances present in sample assessed by "recovery" investigations based on observing effect on <PC> response. Investigation of impact for the full range of dilutions is suggested.	Not discussed	(1)
FDA guidance, 2019	Matrix components, including those introduced during sample collection, may have an effect on assay performance, affecting sensitivity and linearity. Spike and recovery studies are recommended as a part of minimal required dilution evaluation.	Not discussed	(2)
Shankar *et al.* 2008	Selectivity investigation is suggested based on comparison of PC recovery within normal and disease-state sera to determine potential interference by matrix components.	Up to 20% difference between responses observed in assay buffer and matrix samples is viewed as acceptable. Specific % recovery range may vary based on the PC used.	(3)

ADA Assay Description, PF-49761376

A monoclonal antibody-based compound, designated here as PF-49761376, is developed as a component of an immunomodulatory treatment. Anti-PF-49761376 antibodies were detected in human serum using ECL immunoassay method utilizing MSD platform. Samples, PCs and pooled NC were diluted 1:25 in the assay buffer and loaded into appropriate wells of a polypropylene plate. Samples were then acidified by the addition of an equal volume of 0.8% acetic acid (pH 3.5) and allowed to incubate for approximately 15 min. The samples were then neutralized by the addition of an equal volume of 2× master mix containing equal concentration of capture (PF-49761376—biotin) and detection (PF-76456267—ruthenium) conjugates along with the neutralizing 1 M Tris buffer, pH 10. After approximately 1-h incubation at RT, samples are transferred into a pre-blocked (4% BSA in PBST, 0.05% (*v*/*v*) Tween-20) streptavidin MSD microplate and incubated for 1 h at RT. After a wash step, MSD tripropylamine-containing read buffer was added and chemiluminescence signals were acquired (RLU). A monoclonal mouse anti-PF-49761376 antibody was used as the positive control reagent. The sensitivity limit of the screen assay was identified as 2.57 ng/mL calculated as per Shankar *et al.* (3). A floating-type assay cut-point (cut-point factor 1.12) was defined by following recommendations described in the Shankar *et al.* (3). Anti-PF-49761376 ADA assay cut-point was determined based on analysis of 50 individual treatment-naïve matrix samples.

Screening and confirmatory cut-point values were identified as described in the Shankar *et al.* (3). PC recovery in the PF-49761376 ADA assay was calculated using Eq. 2 shown in the "Data Analysis Methodologies" section. Assay drug tolerance was assessed by supplementing pooled normal human serum samples spiked at the 100 ng/mL PC level with an increasing concentration of unlabeled PF-49761376 ranging from 0 to 2000 μg/mL and was identified as approximately 500 μg/mL of PF-49761376.

Data Analysis Methodologies

PC recovery values were calculated based on analysis of individual sample-generated responses at a given PC concentration *vs.* signal generated by a PC spiked assay buffer sample (Eq. 1).

$$\%\text{Recovery} = \frac{(\text{IndSpiked} - \text{IndUnspiked})}{(\text{BufferSpiked} - \text{BufferUnspiked})} \cdot 100\% \quad (1)$$

Alternatively, PC recovery was calculated by comparing signals produced by individual PC spiked sample *vs.* signal generated by PC spiked normal serum pool (Eq. 2)

$$\%\text{Recovery} = \frac{(\text{IndSpiked} - \text{IndUnspiked})}{(\text{NCSpiked} - \text{NCUnspiked})} \cdot 100\% \quad (2)$$

where:

- Ind_Spiked represents signal obtained from an individual treatment-naïve sample when supplemented with a given PC concentration.
- Ind_Unspiked represents signal obtained from an individual treatment-naïve sample when not spiked with PC material.
- Buffer_Spiked represents signal obtained from a buffer sample when supplemented with a given PC concentration.
- Buffer_Unspiked represents signal obtained from a buffer sample when not spiked with PC material
- NC_Spiked represents signal obtained from negative control pool (NCP) matrix when spiked with a given PC concentration.
- NC_Unspiked represents signal obtained from NCP matrix when not spiked with PC material.

To calculate recovery % at the assay cut-point value, plate-specific cut-point RLU value was applied in place of the signal generated by an individual treatment-naïve sample supplemented with a given PC concentration as shown in the above Eqs. 1 and 2.

RESULTS

A drug-specific ADA assay was developed and validated in support of PF-76456267 which is an endogenous protein homologue fusion biotherapeutic. An assessment of PC recovery was conducted during anti-PF-76456267 ADA assay validation. In the assay, PC material was prepared using a polyclonal rabbit anti-PF-76456267 antibody. PC material was spiked into ten naïve human serum samples at two low PC concentrations: LPC1 (11.2 ng/mL) and LPC2 (14.4 ng/mL). These low PC concentrations were selected to approximate low ADA concentration in a study sample, approaching ADA assay sensitivity levels of 2.81 and 8.92 ng/mL for the screen and confirmatory tests, respectively. By assessing performance of low PC concentration sample, one may be able to evaluate ADA assay ability to avoid false-negative ADA reporting during production phase. Spiked samples were tested in the ADA method and generated assay signals were compared with that produced by the assay buffer spiked at the corresponding PC concentration (see Fig. 1). Obtained data are shown in two formats: as the raw assay signal RLU (Fig. 1a) and as % recovery (Fig. 1b). The 80 to 120% recovery range was applied in the test as the acceptance criteria. Many of the individual samples produced PC recovery values within the 80 to 120% range at both PC levels. Two samples at the LPC1 level produced % recovery above the 120% range (Fig. 1b). Three samples at the LPC1 and 6 samples at the LPC2 level produced % recovery below the 80% threshold. Although several of the samples recovered outside of the pre-defined 80–120% range, all samples spiked at the LPC1 and LPC2 concentrations generated signals above the assay cut-point and scored ADA positive. This clearly demonstrates the ability of the assay to reliably detect PC material at the 14.4 and 11.2 ng/mL concentrations. Further, the data indicate that in order to score ADA negative, PC recovery for a given sample on average would have to produce RLU value at or below the assay cut-point which corresponds to 59.4% and 31.7% recovery levels for the LPC1 and LPC2, respectively. Therefore, even when PC % recovery is well below 80%, the assay is capable of detecting low PC concentrations tested and avoiding false ADA-negative reporting. A separate example is shown in Fig. 2, where data related to the PC recovery in the ADA assay for a monoclonal antibody-based biotherapeutic PF-49761376 are presented. In the assay, PC material was prepared using a monoclonal mouse anti-PF-49761376 antibody. PC material was spiked into ten naïve human serum samples at the 10 ng/mL (LPC level) and 100 ng/mL PC (HPC level) concentrations. Similar to the PF-76456267 assay example, the low PC concentration was selected to approximate low ADA concentration in a study sample allowing to test for the assay ability to avoid false ADA-negative reporting during production phase. Spiked samples were tested in the ADA method and generated assay signals were compared with that produced by the corresponding concentration of the PC material prepared in the assay negative control matrix pool (Fig. 2). One sample spiked at the LPC level generated recovery values above the 120% threshold while none of the samples produced recovery values below the 80%. All the LPC spiked samples scored ADA positive (Fig. 2b). Four samples spiked at the 100 ng/mL of PC (HPC level) produced recovery values above the 120% and none were below 80%. In this assay, in order to score ADA negative, PC recovery for a given sample on average would have to produce RLU value at or below the assay cut-point which corresponds to 16.9% and 2.0% recovery levels for the 10 and 100 ng/mL PC, respectively. The drug tolerance assessment is another common test performed during ADA assay validation. Drug tolerance test allows better

understanding of the potential impact that residual drug in the sample may have on the ADA assay performance. During ADA drug tolerance test, increasing concentrations of exogenously introduced unlabeled drug are added to pooled assay matrix (*e.g.* human serum) spiked with a known amount of the ADA PC reagent. The ability of the assay to detect ADA PC reagent in the presence of the drug, as an interfering component, is evaluated over the tested drug concentration range. Common methodology involves analysis of samples prepared in assay matrix containing selected PC concentrations, *e.g.* LPC, 100 ng/mL or 250 ng/mL (1,2). There is typically no comparison done between signals generated by the assay buffer and matrix spiked PC samples during the ADA drug tolerance test. The highest drug concentration at which PC spiked matrix samples generate signal at or above the assay cut-point (*i.e.* score ADA positive) is declared as the ADA assay drug tolerance limit.

Although the ADA drug tolerance test aims to assess one specific type of interference (*i.e.* drug driven) and the PC recovery test is expected to evaluate potential interference from a range of often unknown matrix components the two tests have a sufficient degree of resemblance. The outcome of a typical ADA drug tolerance test is reported based on the ADA assay ability to score a given sample as ADA positive, *i.e.* avoid reporting false ADA-negative results. A similar approach could be proposed for the ADA PC recovery test. During drug tolerance assessment of the anti-PF-49761376 ADA assay, the NC pool samples spiked at 100 ng/mL of PC were supplemented with various concentrations of unlabeled PF-49761376 (Fig. 3). Results were compared with the signal generated by the PC spiked NC pool without the addition of the unlabeled PF-49761376 and expressed as % difference (Fig. 3b). Both the raw assay signal (Fig. 3a) and the % signal difference *vs.* the spiked PF-49761376 concentration profiles (Fig. 3b) show a gradual reduction in the reported values as a function of the PF-49761376 concentration. At approximately 500 μg/mL of PF-49761376 (estimated assay drug tolerance limit), the assay signal reached screening assay cut-point value. This corresponded to approximately 7.1% of the PC signal produced by the PC sample that was not spiked with the PF-49761376, a relative value that is significantly below the low threshold level considered for the PC recovery test (80%).It can be concluded that the use of the within ± 20% recovery criterion is not justified and not value added. It is proposed that PC recovery test should focus on the reliability of the method to detect the low PC level in individual samples and avoiding false-negative ADA reporting. Therefore, it is proposed that assessment of high PC level as well as the assessment of quantitative percent recovery (within ± 20%) should not be included in the test.

DISCUSSION

Strategies aiming to evaluate PC recovery have been proposed in the current versions of FDA and EMA guidelines (1,2) and industry white paper (3), and are summarized in Table I. A certain degree of matrix effect is expected based on the interactions between numerous matrix components and assay reagents, including PC material. PC spike and recovery tests conducted at various PC dilutions are proposed and analysis of target study population relevant samples is suggested. It is recognized that possible impact of matrix components will vary depending on the conditions of the assay, which may be optimized based on the observed outcome of the PC recovery test. Exogenous components of the buffer, *e.g.* anticoagulants, salts or detergents, as well as endogenous components present in the study matrix, *e.g.* rheumatoid factor, heterophilic antibodies or concomitant medication or target protein have been cited as potentially impacting the outcome of the test (4). It is recognized that concentration and type of PC used may impact observed recovery values. PC recovery testing is suggested in part to determine an appropriate assay minimal required dilution (MRD). Methods to calculate PC recovery values can be based on the analysis of difference of responses produced by matrix *vs.* buffer spiked PC samples (Eqs. 1 and 2) or by analysis of the ratio of the PC spiked individual *vs.* NC sample responses.

Current regulatory guidelines do not provide specific details around the acceptance criteria for the PC recovery test. The industry white paper publication by Shankar *et al.* (3) suggests that PC signal recovery in individual samples should fall within the 80–120% range for the majority of the samples tested (*e.g.* two-thirds). The origin of the proposed acceptable range is not clear. ADA assessment is broadly viewed as semi-quantitative and similarity with quantitative PK assay expectations may not be suitable. Calculation of % PC recovery relative to the buffer sample-generated signal assumes that a reasonable degree of parallelism between PC generated signal when tested in biological matrix and buffer is present. Lack of parallelism with the appropriate calibration curve would lead to an inability to back-calculate the concentration of analyte in an unknown sample (5,6). In the publication by Liang *et al.* a potential effect of PC control reagents affinity on the reported ADA assay sensitivity was evaluated to show a lack of linearity and parallelism of assay response generated by various control reagents (6). Polyclonality and study subject driven diversity of ADA response are commonly presented as the cause for the lack of parallelism and absence of a true ADA reference material. Admittedly, some degree of parallelism may be expected when the same material, specifically assay PC reagent, is spiked into various individual matrix samples. Yet, due to the high diversity of matrix composition and variable nature of interfering factors, the parallelism of response would need to be confirmed, a step that is not typically done. Additionally, the quantitative nature of the PC % recovery value assumes a linear proportionality in the assay response *vs.* PC concentration. Calculations are most commonly made using absolute assay signal without an interpolation of PC concentrations, an approach typically applied for a quantitative PK method (7). It can be concluded that PC recovery based on the absolute signal comparison in a semi-quantitative ADA assay is theoretically not appropriate.

A critical distinction between PC recovery and drug tolerance tests is that the nature of the interfering factor (unlabeled drug) is known and pre-defined in the case of the latter. The outcome of the ADA drug tolerance test is still expected to depend on the nature of the PC reagent, particularly its drug-binding affinity. For example, high-affinity monoclonal PC material is expected to be highly sensitive to the presence of unlabeled drug and as a result returning low apparent assay drug tolerance value. Various

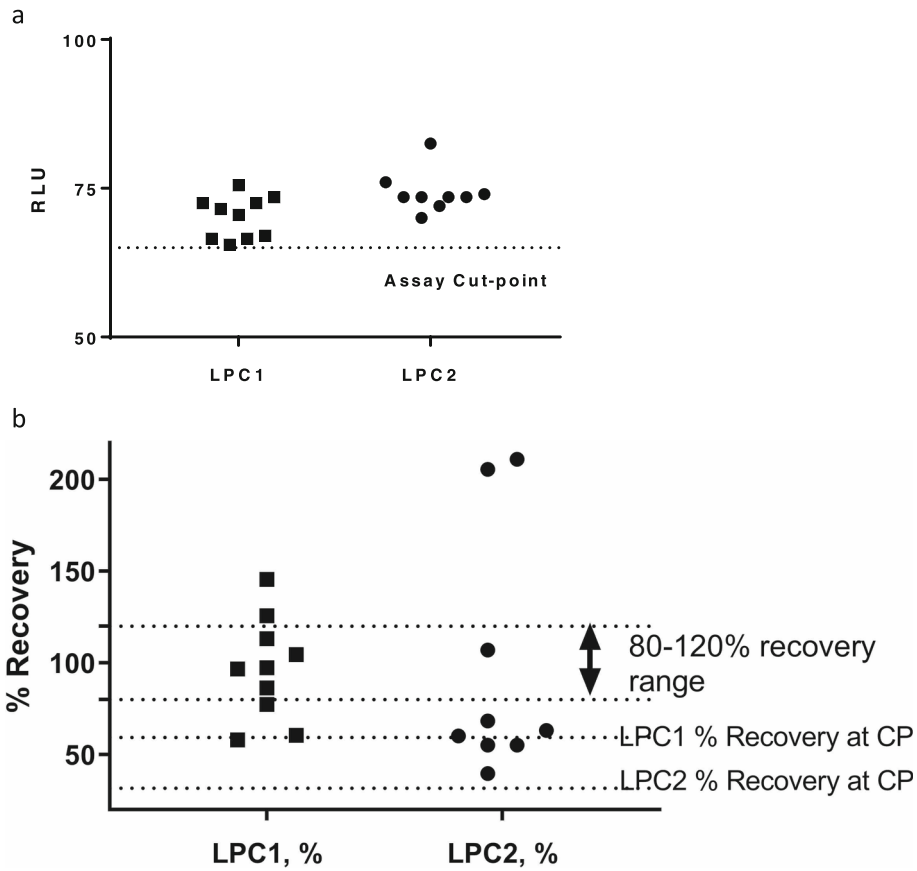

Fig. 1. Normal individual treatment-naïve samples (n = 10) were spiked at 11.2 ng/mL (LPC1) and 14.4 ng/mL (LPC2) of the assay PC. Panel **a** shows raw RLU values generated by the individual samples. Panel **b** shows corresponding % recovery values. The PC % recovery values were calculated based on the comparison of signal produced by the PC spiked individual samples vs. signal produced by the assay buffer sample spiked at the corresponding PC concentration. The dashed lines show assay cut-point (**a**), the 80 to 120% range of recovery, LPC1 and LPC2 recovery levels at the assay cut-point (**b**)

modifications to the assay conditions have been proposed to improve assay drug tolerance (8–10). The exact causes for abnormal PC recovery during ADA selectivity test vary and typically remain unknown. Various drug and assay reagent binding matrix components such as heterophilic antibodies, rheumatoid factors and specific pre-existing anti-drug antibodies have been associated with the loss of recovery (4). For example, because an ADA assay PC reagent is frequently a mouse monoclonal immunoglobulin, it is critical to understand how human anti-mouse antibody (HAMA), often found in human matrix, may impact assay performance. The presence of a substantial mouse sequence in the assay reagents (*e.g.* conjugated drug components) may lead to an analogous impact. The true anti-drug antibody found in an ADA-positive human sample will be lacking any non-human sequence and should not experience an impact due to the presence of HAMA. Additionally, an induction of a polyclonal ADA response is typically expected in study subjects. Blocking of a monoclonal antibody-based PC reagent interaction with the corresponding epitope on the drug molecule may result in a critical and substantial impact on the PC recovery value. Blocking of a single epitope on the drug protein may have limited impact on the ADA assay ability to detect a treatment-induced polyclonal ADA response due to a wide range of other remaining specificities. It can be concluded that the diverse nature of treatment-induced ADA response *vs.* typically a non-human monoclonal antibody PC material confounds a direct and quantitative translation of PC % recovery values to the assay ability to detect ADA in study incurred samples.

A well-designed ADA selectivity test should be able to evaluate presence of matrix interference for a given assay and whether the danger of false-negative ADA reporting can be avoided. If a high number of low PC spiked individual matrix samples score ADA negative, the method may need to be redeveloped. This may include an assessment of the specific nature of the interfering matrix component to determine its relevance. In contrast, false-positive reporting, particularly when using matrix samples spiked with assay PC material, is typically not a safety or regulatory concern. Over-recovery at the upper end of the recovery range (120%) may suggest a potential to produce a false ADA-positive result. The assay ability to detect and distinguish false-positive signal is typically evaluated in a separate assessment during analysis conducted to determine assay screening and confirmatory cut-points. High prevalence of ADA-positive responses during assessment of treatment-naïve individual matrix samples may indicate ADA assay potential to report high false ADA-positive score. Multiple reasons may lead to a high ADA-positive score

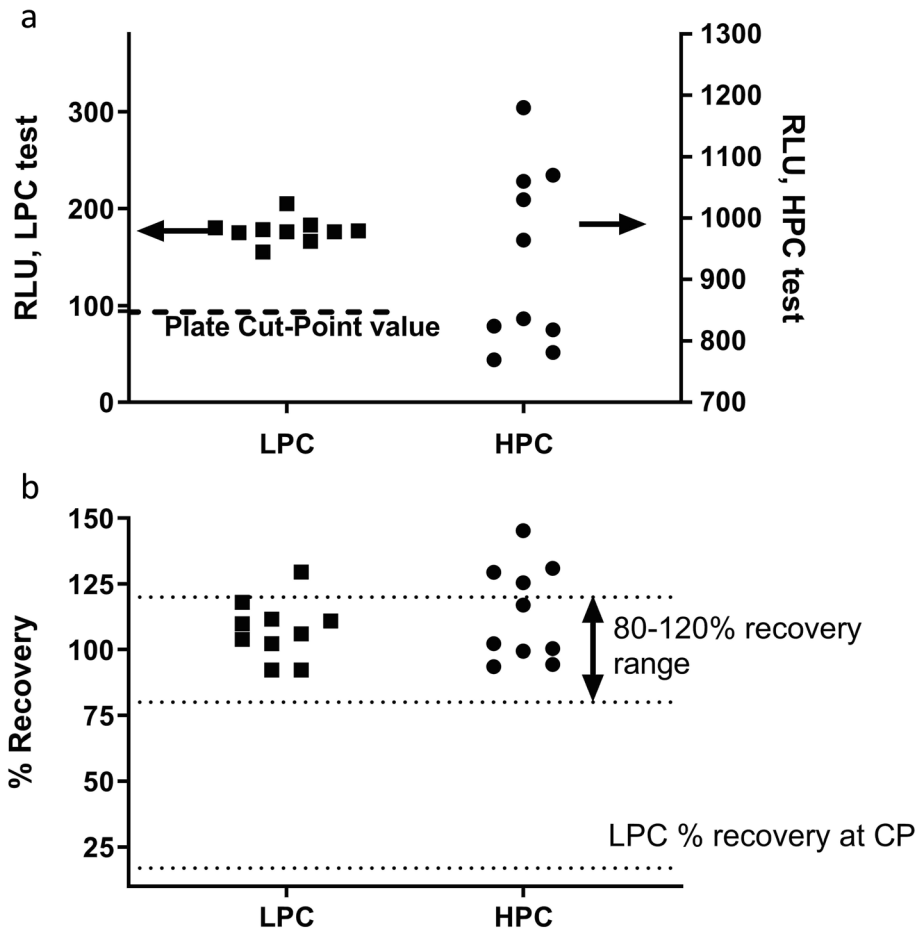

Fig. 2. Recovery test for the anti-PF-49761376 ADA assay. Data produced by normal individual treatment-naïve samples ($n = 10$) spiked at the 10 ng/mL (LPC) and 100 ng/mL (HPC) of the assay PC are shown. Panel **a** shows raw RLU values generated by the individual samples. Panel **b** shows corresponding % recovery values. The PC % recovery values were calculated based on the comparison of signal produced by PC spiked individual samples *vs.* signal produced by NC pool sample spiked at the corresponding PC concentration. The dashed lines show assay cut-point value (**a**) and the 80 to 120% range of recovery (**b**)

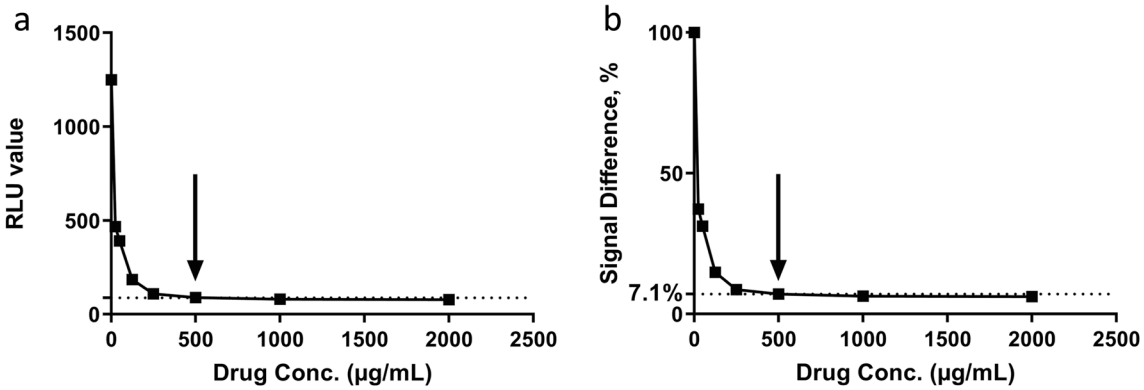

Fig. 3. Drug tolerance test for the anti-PF-49761376 ADA assay performed at the 100 ng/mL PC concentration. NC pool sample was spiked at 100 ng/mL PC and supplemented with increasing concentrations of unlabeled PF-49761376 material ranging from 0 to 2000 µg/mL. RLU values obtained and corresponding signal difference (relative to the PF-49761376 unspiked sample, %) are shown on **(a)** and **(b)**, respectively. Dashed lines indicate assay cut-point value (**a**) and signal difference (%) calculated at the assay cut-point value (**b**). Arrows indicate drug concentration that corresponds to the estimated anti-PF-49761376 ADA assay drug tolerance (approximately 500 µg/mL)

during the test. These have been discussed elsewhere and are commonly evaluated during assay development phase (11). The analysis of treatment-naïve individual matrix samples should allow to construct an assay with a limited potential for high false ADA-positive reporting. It can be concluded that the reason for and the value of the upper % recovery threshold in the PC recovery test is questionable.

Lack of linearity of PC response, semi-quantitative nature of ADA assays, diversity of ADA response in study subjects and broad and variable nature of assay interfering matrix factors contraindicate application of an exact and universal low recovery threshold level (80%). It appears that an approach based on the assay specific cut-point value will better represent ADA assay ability to tolerate matrix interference and produce an ADA-positive result when testing low PC concentration spiked samples. This approach will align with the ADA drug tolerance test.

An alternative to the quantitative acceptance criteria of the PC recovery (ADA assay selectivity) test is proposed herein. It is suggested to define the ADA assay selectivity test acceptance criterion as the ability of the method to score a pre-defined number (for example 8 or 9 out of 10) of LPC samples tested as positive. Evaluation of selectivity in this manner confirms the sensitivity of the method in individual treatment-naïve samples. It is proposed that a quantitative % recovery test should not be used to assess assay selectivity characteristic. The ADA selectivity test using HPC concentration samples may not be adding any significant value and can be completely removed. It is also proposed that the upper (120%) and lower (80%) recovery threshold requirements for the LPC samples should be removed as well.

The true selectivity test should be based on the assay ability to produce ADA-positive score at the appropriate PC concentration (LPC) when tested in a selected number of individual matrix samples. As an example, at least 8 or 9 out of 10 samples prepared at the LPC concentration in treatment-naïve individual matrix samples should score ADA positive to declare that the ADA assay PC recovery test is acceptable.

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