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Research Article

Anti-drug Antibody Assay Conditions Significantly Impact Assay Screen and Confirmatory Cut-Points

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Abstract. Assays for the detection and confirmation of anti-drug antibodies (ADA) are commonly used tools for assessing the immunogenicity of drug candidates in both clinical and nonclinical studies. During the development of such assays, it is typical to optimize the assay conditions based on factors such as sensitivity or signal/noise ratio (S/N) and is commonly done using an assay positive control (PC). However, even carefully optimized methods often suffer with problems due to low cut-point factors and failure to distinguish assay "noise" from a true biological response. In this paper, we describe an approach to assay development in which the impacts of assay conditions on the response and variability, both analytical and biological, of drug-naïve samples are tested by way of PC-independent assay condition optimization. Using two ADA methods as model systems, we examine the impact of minimum required dilution, assay reagent (labeled drug) concentrations, incubation time, assay, and wash buffer composition. We find that the choice of assay conditions, particularly the labeled drug concentration, can greatly affect the distribution of naïve sample responses and thus impact screening and confirmatory assay cut-points. In two case studies presented, screening assay cut-point (SCP) varied from 1.38 to 2.20 and 1.04 to 1.20 while the confirmatory assay cut-point (CCP) varied from 58.5 to 95.6% and 26.2 to 16.2% depending on the conditions tested. Some of the conditions produced unacceptably high CCP values. It is proposed that the degree of the observed impact of the assay conditions on SCP and CCP values depends on the compound nature and assay matrix composition and is likely connected with the diversity of interactions between drug protein and matrix components. Because it was also observed that higher assay SCP can associate with a loss of the PC-based assay sensitivity, additional assessment of the assay conditions would be required to determine an overall assay performance acceptability, including assay PC-based sensitivity, drug, and target tolerance characteristics. In conclusion, it is suggested that by assessing performance of treatment-naïve samples at various assay conditions, one can identify potential assay protocols that allow to avoid undesirably low screening (e.g., < 1.2) and confirmatory (e.g., $< 25\%$) cut-points.

KEY WORDS: ADA assay cut-point; anti-drug antibody; immunogenicity.

INTRODUCTION

Anti-drug antibody (ADA) assays are routinely applied to detect drug-specific antibodies as a part of evaluation of the immunogenicity risk for the biotherapeutic compound. In a tier-based approach, samples are commonly assessed in a screening protocol followed by a confirmatory test. Both tests utilize a threshold value (cut-point) to determine whether a given sample scores ADA positive *vs.* negative. Screen and confirmatory assay cut-points are determined based on statistical analysis of data sets obtained by testing individual treatment-naïve samples representing the relevant study population.

The statistical analysis of the data set is intended to determine signal variance and define boundaries that identify range of signal for a true negative sample. In a screening assay, a false-positive rate of 5% is typically expected to ensure that assays do not report false-negative results (1,2). The variability of the signals generated by individual samples is based on a combination of several factors. Overall, these can be separated into two categories: (1) those arising from analytical or system-specific variability of the signal and (2) biological diversity of samples. The analytical variability is a result of process deviations, even if minor, and includes the inherent robustness of the analytical platform used in the assay. Biological diversity of the sample-generated response is

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a combination of the high complexity and inter-subject variability of the matrix composition, variability of drug interactions with matrix components, and the disease state-based diversity of matrix composition.

While the analytical variability can be controlled by technological improvements and tight control over the execution of the method (systems, process), the biological component is highly unknown and drug dependent. For example, some post-translational modifications of the protein-based biologic may cause a greater degree of binding to existing matrix components. Examples include endogenous human IgG4 binding to altered IgG1 via Fc-Fc interaction (3), autoantibodies binding to constant domains of therapeutic monoclonal antibodies, including the anti-hinge domain (4), and endogenous antibodies to gal-1.3-gal and other glycans potentially found on drug proteins (5–10). Ability of soluble and multimeric drug target to cause a false-positive response in ADA assays has been broadly recognized and carefully addressed (11,12). Pre-existing matrix components capable of binding to the protein-based drug may exhibit low affinity of binding, as in the case of rheumatoid factors, but may be present in relatively high abundance (13,14).

Here we set out to evaluate how the ADA assay conditions may impact signal generated by an individual sample due to the ability to modulate either the analytical or the biological components of variability. As a consequence, we have assessed the impact of assay condition changes on the screening and confirmatory assay cut-point values and resulting acceptability of the method.

MATERIAL AND METHODS

Materials

Therapeutic drug candidate monoclonal antibodies referred here as PF-67786004 (IgG2 framework based) and PF-36137358 (IgG1 framework based), and PC reagent antibodies referred here as anti-PF-67786004 and anti-PF-36137358 and labeled PF-67786004 and PF-36137358 reagents were obtained from Pfizer, Inc. Biotinylated and ruthenylated reagents were prepared using conventional amine coupling chemistry. The coupling challenge ratio of 20:1 of biotin reagent over the molar concentration of compound was used for both PF-67786004 and PF-36137358. The coupling challenge ratio of 8:1 and 12:1 of ruthenylating reagent over the molar concentration of compound was used for PF-67786004 and PF-36137358, respectively. Other coupling challenge ratios were not evaluated. Negative control (NC) was a drug-naive human serum pool obtained from BioIVT (NY). Individual drug-naïve serum samples, including healthy and disease population samples, were obtained from BioIVT (NY). PC reagents were ligand-affinity purified mouse monoclonal antibodies.

MSD GOLD 96-well Streptavidin SECTOR Plates (catalog# L15SA), MSD GOLD SULFO-TAG NHS-Ester (catalog# R91AO), and Read Buffer T (catalog# R92TC) were purchased from Meso Scale Discovery (MD). Sulfo-NHS-LC-Biotinylation kit, cat#: 21327, and PBS-Casein, cat#: 37528, were obtained from Thermo-Fisher (MA). The KPL Milk Diluent Concentrate containing 2% nonfat dry milk in borate buffer, cat# 50-82-00, was obtained from SeraCare KPL (MA). Bovine serum albumin (BSA, cat#: 7030) was obtained from Sigma (MO). All other chemicals used for the assays were from various commercial sources.

THST wash buffer (50 mM Tris, 500 mM sodium chloride, 1 mM glycine, + 0.05% (*v*/*v*) Tween-20, pH 7.2 ± 0.1) and PBST wash buffer (1X PBS with 0.05% Tween-20) were used for plate washing, depending on the assay procedure used. Plate blocking buffer was 4% BSA (*w*/*v*) in PBST, 1% casein in PBST, or 0.2% KPL Milk in DI water as indicated. Assay buffer was 1% (*w*/*v*) BSA in PBST unless otherwise specified. Neutralizing buffer contained 200 mM Tris-base at pH 10.

Methods

ADA assays to detect PF-67786004 and PF-36137358 specific antibodies were electrochemilumiscence (ECL) bridging format based.

In the anti-PF-67786004 ADA method, NC, positive control solutions (HPC at 2000 ng/mL and LPC at 25 ng/mL) and individual serum samples were first treated with 300 mM acetic acid (pH 3.5) at 1:10 (MRD) for approximately 30 min followed by co-incubating with Master reagent Mix (MM) in the neutralizing buffer and equal amounts of biotinylated and ruthenylated PF-67786004 at concentrations as indicated in the procedure (concentrations shown are of each labeled drug prior to the 1:1.5 dilution in the well, which varied from 0.076 to 1 μg/mL) for approximately 1 h on a shaker. For confirmatory assay, following acid treatment samples were incubated with the MM in the absence and presence of 12 μg/mL of PF-67786004. Immunocomplexes between ADA, biotinylated and ruthenylated PF-67786004 were subsequently captured via the biotinylated PF-67786004 reagent binding to the streptavidin-coated pre-blocked MSD GOLD 96-well Streptavidin SECTOR plate during 1-h incubation at room temperature on a shaker. MSD plates were then washed three times with PBST or THST buffer and final detection of anti-PF-67786004 antibodies was conducted by addition of the 2X Read Buffer-T that contains tripropylamine (TPA) to produce an electrochemiluminescent signal detected on a MSD instrument. Each assay condition was tested once to include individual treatment-naïve samples in the presence of PC and NC reagents as indicated in "Assay Cut-Point Evaluation."

In the anti-PF-36137358 ADA method, NC, PCs (HPC at 4000 ng/mL and LPC at 180 ng/m), and individual serum samples were diluted at MRD 1:80 in the assay buffer and incubated overnight on a plate coated with an alternative antibody specific to the molecular target of the PF-36137358, therefore reducing soluble target interference from the samples. On the second day of the assay, the pre-treated controls and samples were transferred to a polypropylene microtiter plate and incubated with the MM containing equal amount of biotinylated and ruthenylated PF-36137358 at concentrations as indicated in the procedure (varying from 0.5 to 2.0 μg/mL each prior to the 1:1.5 dilution in the well) for up to 2 h on a shaker. For confirmatory assay, the controls and samples were mixed with the MM in the absence and presence of 150 μg/mL of PF-36137358 and incubated for up to 2 h on a shaker. Immunocomplexes between ADA, biotinylated and ruthenylated PF-36137358 were

subsequently captured via the biotinylated PF-36137358 reagent binding to the streptavidin-coated pre-blocked MSD GOLD 96-well Streptavidin SECTOR plate during 1-h incubation at room temperature on a shaker. MSD plates were then washed three times with PBST buffer and final detection of anti-PF-36137358 antibodies was conducted by the addition of 2X Read Buffer-T that contains TPA to produce an electrochemiluminescent signal detected on a MSD instrument. Each assay condition was tested two times to include individual treatment-naïve samples in the presence of PC and NC reagents as indicated in "Assay Cut-Point Evaluation."

Assay Sensitivity and LPC Determinations

To assess assay sensitivity, PC reagents were diluted in 100% human serum pool and then serially diluted using NC material with the final dilution that yielded assay signal below the screening assay cut-point. Assay sensitivity for the screening ADA assay was determined using a 95% upper confidence limit using Eq. (1) listed below. The LPC concentrations in each of the assays were determined using 99% upper confidence limit using Eq. (2) listed below.

$$\text{Sensitivity} = \text{mean PC concentrations at cut-point} + t_{0.05,df} * \text{SD} \quad (1)$$

$$\text{LPC concentration} = \text{mean PC concentration at cut-point} + t_{0.01,df} * \text{SD} \quad (2)$$

where

SD	standard deviation of the mean
df	degrees of freedom $(n-1)$
$t_{0.0x,df}$	t-distribution critical value with (df)

The anti-PF-67786004 ADA assay sensitivity was estimated as 20.4 ng/mL, and LPC concentration was identified as 25.0 ng/mL.

The anti-PF-36137358 ADA assay sensitivity was estimated as 149 ng/mL, and LPC concentration was identified as 180 ng/mL.

Investigational Procedures for Anti-PF-67786004 and PF-36137358 ADA Assays

To investigate factors that may impact anti-PF-67786004 ADA assay screening and confirmatory cut-point values, assay procedures were modified as presented in Table I. Investigation of anti-PF-36137358 effects included modification of the MM reagent concentrations only.

Assay Cut-Point Evaluation

For PF-67786004, the screening assay cut-point (SCP) and confirmatory assay cut-point (CCP) values for each of the assay procedures were evaluated by performing analysis of 59 drug-naïve individual normal human serum lots unless otherwise specified. Each sample was tested in duplicate, and individual sample data with duplicate CV > 20% were removed (less than 1% of samples across all plates and assays tested). Mean responses for each sample with intra-duplicate CV $\leq$ 20% were used for the data analysis. Initially, outliers were identified and removed using standard box plot approach (15). The ratio of mean response for a given sample and mean response of the assay NC (S/N) was calculated. The normality test was performed using JMP software (SAS, NC). It was determined that in the case of anti-PF-67786004 ADA, the assay data sets generated in all procedures tested were not normally distributed ($p < 0.05$ in Shapiro-Wilk's normality test). The screen cut-point factor (CPF) values were therefore established using the non-parametric method (95th percentile). To establish the CCP value, % inhibition of samples were calculated using the mean assay response generated by a given sample, tested with or without addition of the PF-67786004. The following formula was used:

$$\%\text{inhibition} = \left(1 - \frac{\text{sample response with PF-67786004 addition}}{\text{sample response without PF-67786004 addition}}\right) * 100\%$$

The CCP for each assay procedure investigated was established using 99% confident interval (upper limit) after removal of outliers.

For the PF-36137358, 28 samples were used for each assay condition investigated. The statistical procedures used for normality analysis and outlier removal are similar to what is described above for the anti-PF-67786004 ADA assay. The numbers of statistical outliers identified for individual conditions tested were as follows: 1, 2, 3, and 1 for 0.5, 1.0, 1.5, and 2.0 μg/mL of MM, respectively. In the case of the anti-PF-36137358 assay, the individual sample-generated signal distribution was determined to be normal for all assay procedures tested. Therefore, the SCP was established using 95% CI (one tail) approach. The CCP value was calculated as previously described for PF-67786004.

RESULTS

An ECL assay was developed to detect ADA specific to PF-67786004 in human serum in support of clinical studies. PF-67786004 is a human IgG2 framework-based monoclonal antibody drug candidate specific to a cell surface target found on tumor cells and is in development for an oncology indication. In the original version of the assay that followed protocol A described in Table I, up to 10% of pre-dose samples collected from patients with the disease tested positive in the ADA assay (data not shown). A different set of individual samples collected from treatment-naïve healthy volunteers was tested in the ensuing investigation to determine the reason for the observed pre-dose positive reactivity in the ADA assay. Approximately 17% of serum samples from treatment-naïve healthy individuals generated assay signal greater than the statistically defined SCP value when tested in the assay protocol A (Fig. 1). The assay-specific SCP was calculated based on analysis of individual treatment-naïve study relevant matrix samples by following industry-

Table I. Summary of Assay Conditions Used for Investigation of Anti-PF-67786004 ADA Assay. Procedures A Through J (Listed as A Through J) Applied to Investigate the Impact of Assay Conditions on the Anti-PF-67786004 ADA Assay. Master Mix Indicates the Combined Concentration of Assay Reagent Mixtures (Ruthenium and Biotin-Labeled PF-67786004). Buffer Compositions Are as per "MATERIAL AND METHODS" Section

	A	B	C	D	E	F	G	H	I	J
MRD	1:10	1:10	1:10	1:10	1:10	1:5	1:10	1:10	1:10	1:10
Master Mix (μg/mL)	0.076	0.076	0.076	0.076	0.2	0.076	0.1	0.2	0.5	1
Incubation time	1 h	1 h	1 h	o/n	o/n	1 h	1 h	1 h	1 h	1 h
Wash buffer	PBST	PBST	THST	THST	THST	PBST	THST	THST	THST	THST
Assay buffer and blocking buffer	4% BSA in PBST	1% casein in PBST	0.2% milk in DI water	0.2% milk in DI water	4% BSA in PBST	4% BSA in PBST	4% BSA in PBST	4% BSA in PBST	4% BSA in PBST	4% BSA in PBST
Samples tested/ outliers identified	59/7	59/8	40/5	40/4	59/6	59/5	59/4	59/6	59/10	59/5

accepted approaches as described in Shankar *et al.* (15) and based on analysis of 59 treatment-naïve healthy individual samples. Data were assessed for outliers and normality of distribution, followed by identification of the 95th percentile, allowing for up to 5% of false-positive response. During initial assay development, conditions were selected to produce an improved signal to noise ratio (S/N) that was assessed by comparing PC and NC sample results, an appropriate sensitivity of PC reagent detection and fit for purpose drug tolerance characteristics. The same set of samples was applied to determine SCP and CCP values which were established as 1.66 and 29.7%, respectively. The confirmatory analysis was conducted as per the "Methods" section in the presence of 12 μg/mL of PF-67786004.

To understand the cause for the observed pre-treatment ADA-like reactivity, a number of assay conditions were assessed for their ability to modulate the ADA assay signal. Parameters assessed that are most commonly assumed as potentially impactful for assay performance are shown in Table I. By varying assay capture/detector reagent concentrations, sample incubation times, and assay MRD, one may be able to modulate the ability of the assay to detect matrix components with various binding affinities, including anti-drug antibodies. In contrast, use of a harsher wash buffer or introduction of a different blocker component in the assay diluent may lead to a suppression of low-affinity binders. The experiment described herein was focused on assessment of the most critical and potentially highly impactful assay conditions and parameters. In the investigation of the anti-PF-67786004 ADA assay, the following conditions were assessed:

- Wash buffer type (PBST *vs.* THST)
- Sample incubation time (1 h at room temperature (RT) *vs.* 4C overnight (o/n))
- Capture/detector reagent concentration (0.076 to 1 μg/mL)

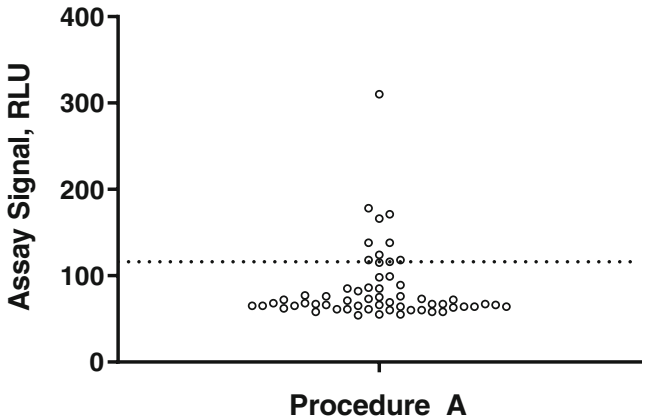


Fig. 1. Individual treatment-naïve sample generated signal distribution. Data from 59 treatment-naïve healthy individual samples tested in the anti-PF-67786004 ADA assay by following procedure A (original assay conditions). The screening assay cut-point (SCP) was assessed as described in the "Methods" section with the estimated value of 1.66 (plate cut-point 116 RLU). From 59 samples analyzed, 10 generated RLU values > 116 and therefore were identified as potentially ADA pre-dose positive. Assay SCP RLU value is identified by the dotted line

- Assay buffer and blocking buffer type (BSA, KPL Milk, casein)
- Minimum required dilution (MRD, 1:10, 1:5)

In these tests, an identical collection of 59 individual treatment-naïve samples (unless otherwise specified) as well as various preparations of the assay PC and NC pool were tested. Distributions of signal produced by individual samples when tested using a particular set of conditions are shown in Fig. 2. Notably, the impact varied greatly based on specific assay conditions, with some conditions resulting in significant modulation of the absolute signal. For example, assay procedures J, I, and H produced significantly broader ranges of signal distribution as compared to the assay procedures A, C, and D. In addition, the range of assay signal values varied from 50 to 140 RLU for procedure C, whereas the signal values ranged from 67 to 2492 RLU for procedure J. The most noticeable impact on the individual sample signal distribution was due to changes in the assay capture/detection reagent concentration.

The change in the assay signal generated by individual samples corresponded to modulation of the signal generated by the NC pool reagent (Fig. 3). The NC-generated mean signal varied from 58.2 to 530 RLU between procedures C and J (Fig. 3a). Critically, a change in the assay signal generated by the NC pool was not associated with a notable change in NC signal precision. Specifically, while the NC signal CV% varied, the value remained within the 5 to 15% range for majority of the assay procedures tested (Fig. 3b). The average NC CV% value was calculated to be 7.6%. All of the CV% values for the NC sample were well within the 20% range, which is generally viewed as acceptable. A narrow range of NC signal CV% observed in the test suggests the assay conditions had a limited impact on the analytical or system-specific variability of the assay.

Individual sample signal distribution data were used to calculate an assay procedure-specific SCP value. Calculations were conducted by following the industry-accepted approach as described in the "MATERIAL AND METHODS" section. Resulting multiplicative floating SCP factor values are presented in Fig. 4a. The SCP values varied from 1.39 (procedure C) to 2.31 (procedure H). No apparent correlation of the SCP values with NC CV% could be observed, as shown in Fig. 4b. In addition, as it is discussed below, limited modulation of the signal to noise value for the assay LPC sample *vs.* assay conditions was observed. It is generally accepted that sample to sample signal variability is a combination of the analytical variability of the method (analytical platform or system-specific variability) and inter-sample biological diversity. Lack of an apparent correlation between assay analytical variability as demonstrated by the NC CV% and assay procedure-specific CPF values suggests that the change in the assay conditions primarily impacted signal variance, as a direct result of inter-sample biological diversity (Fig. 4b). Similar to the experiment depicted in Fig. 2, the capture/detection reagent concentration change produced the greatest impact on the assay CPF (Fig. 4a).

Confirmatory tests are commonly applied to determine the specificity of the ADA signal detected in the initial screening ADA assay. Assay condition impact on the specificity of ADA-like reactivity was assessed by testing a separate set of 50 samples from healthy treatment-naïve individuals with and without addition of 12 μg/mL of unlabeled PF-67786004. Samples were tested using assay procedures listed in Fig. 5, focusing on procedures that previously showed a significant ability to modulate ADA screening assay signals. Similar to what is presented in Fig. 2, unspiked samples produced responses that varied significantly depending on the assay procedure used. As shown in Fig. 5a, addition of unlabeled PF-67786004 resulted in an effective inhibition of anti-PF-67786004 reactivity. Importantly, the RLUs generated by PF-67786004 spiked individual samples (inhibited assay signal, right half of Fig. 5a) fell within a relatively narrow range of values with a limited dependency on the assay procedure used in the test. The degree of signal inhibition for each sample was calculated as a % difference of signal produced by individual samples when tested neat (unspiked) *vs.* spiked with PF-67786004 as described in the

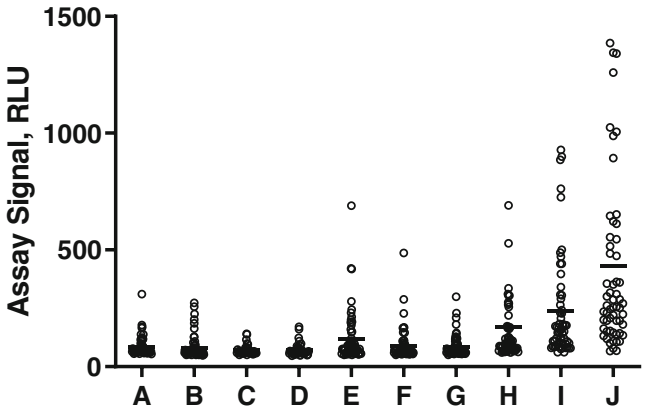


Fig. 2. Distribution of the screening ADA assay signal produced by individual treatment-naïve samples tested in the anti-PF-67786004 ADA assay by following procedures A through J (identified as A through J). Individual treatment-naïve samples were tested as described in the "Methods" section in the anti-PF-67786004 ADA assay by following assay procedures described in Table I. Horizontal bars represent mean values of the distributions

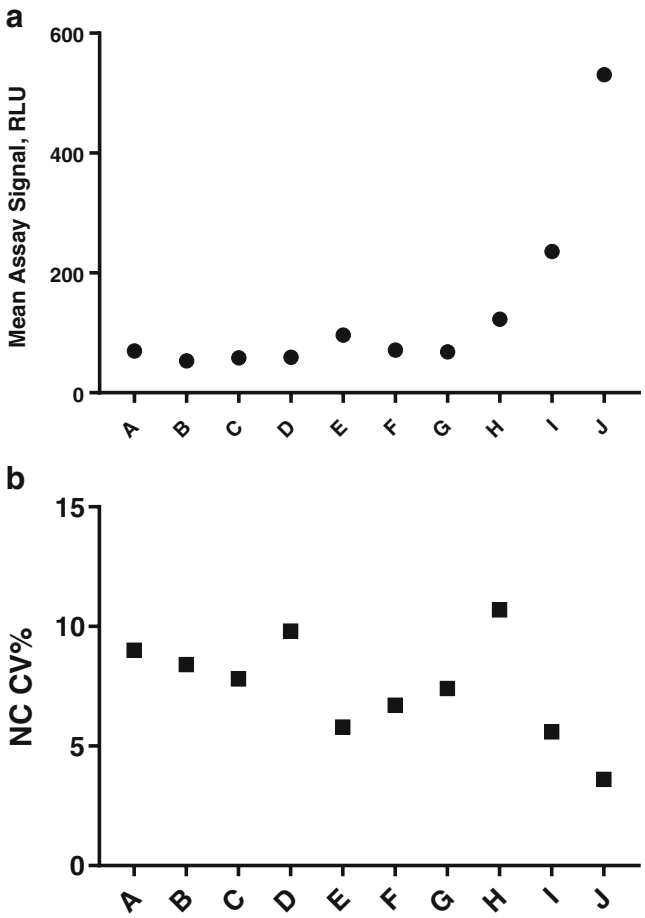


Fig. 3. Impact of assay procedures on the mean and variance (%CV) of NC signals in the anti-PF-67786004 ADA assay following assay procedures describe in Table I. A total of 4 NC replicates were included on each assay run (each plate). The mean and CV% of the mean NC responses from each run were calculated. **a** The mean assay signal generated by the NC samples over the different assay procedures tested. **b** NC CV% values for each of the assay procedures

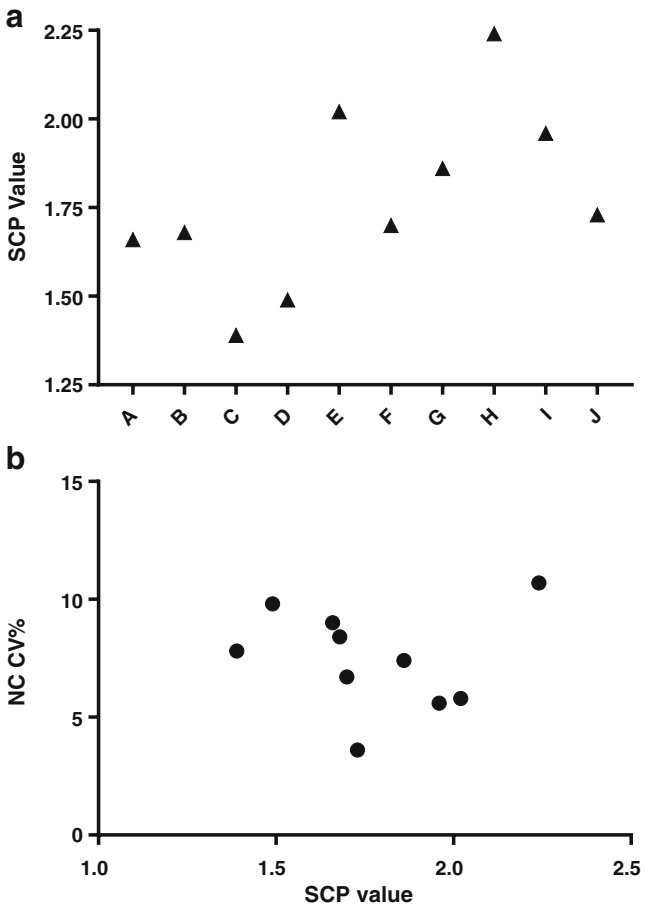


Fig. 4. Screening cut-point (SCP) values are modulated by the assay procedure conditions while there is no correlation between SCP and the assay-negative control precision (NC CV%). Individual treatment-naïve samples were tested as described in the "Methods" section in the anti-PF-67786004 ADA assay by following assay procedures described in Table I. SCP values were calculated as described in the "Methods" section. **a** Dependency of the SCP value on the ADA assay conditions. No apparent correlation of the SCP with the NC precision (CV%) could be observed as shown in **(b)**

"MATERIAL AND METHODS" section. The range of computed % inhibition values varied with the assay procedure (Fig. 5b). As an example, a significant spread of the RLU signals produced by unspiked samples tested under the procedure I combined with a narrow distribution of RLU signals obtained in the confirmatory test (samples were spiked with the PF-67786004) under the same assay procedure resulted to a significant spread of the % inhibition values, reaching as high as 90 to 100% signal inhibition for some of the samples (Fig. 5b). The mean % inhibition value varied from 17.0 to 54.1% between conditions A and I (Fig. 5b).

The ability of unlabeled PF-67786004 to effectively inhibit assay signal generated by an individual treatment-naïve sample resulted in an assay procedure-dependent range of % signal inhibition values. These results suggest that the assay conditions may mostly impact the biological, rather than the analytical variation of the signal generated in the screening and confirmatory tests.

The S/N at the low PC concentration was used to assess the impact of specific assay procedures on the assay ability to detect PC reagent at low (sub-100 ng/mL) concentrations. The LPC samples at 25 ng/mL of anti-PF-67786004 PC reagent were tested at various conditions listed in Fig. 6a. While the S/N values varied depending on the assay procedure used, the degree of the change was limited. In fact, S/N values were similar for the assay protocols H, E, and I, ranging from 2.25 to 2.41. Procedure A generated the lowest S/N value of 1.63. Similar tests conducted for the HPC sample (2000 ng/mL of PC) demonstrated a greater degree of S/N dependency on the assay conditions. The highest S/N was observed for procedure E (218) and the lowest for procedure A (80). All of the assay procedures resulted in an S/N ratio above 50 at the HPC concentration. Limited assessment of the anti-PF-67786004 ADA assay sensitivity was conducted by diluting assay PC reagent and following procedure described in the "Methods" section. Sensitivity parameters calculated for assay procedures A, E, and I were relatively similar ranging from 2.38 ng/mL (procedure I) to 8.69 ng/mL (procedure E). Assay sensitivity for the procedure H (20.3 ng/mL) approached the LPC concentration tested (25 ng/mL) although remained well under the required 100 ng/mL threshold. Procedure H also produced the highest value for

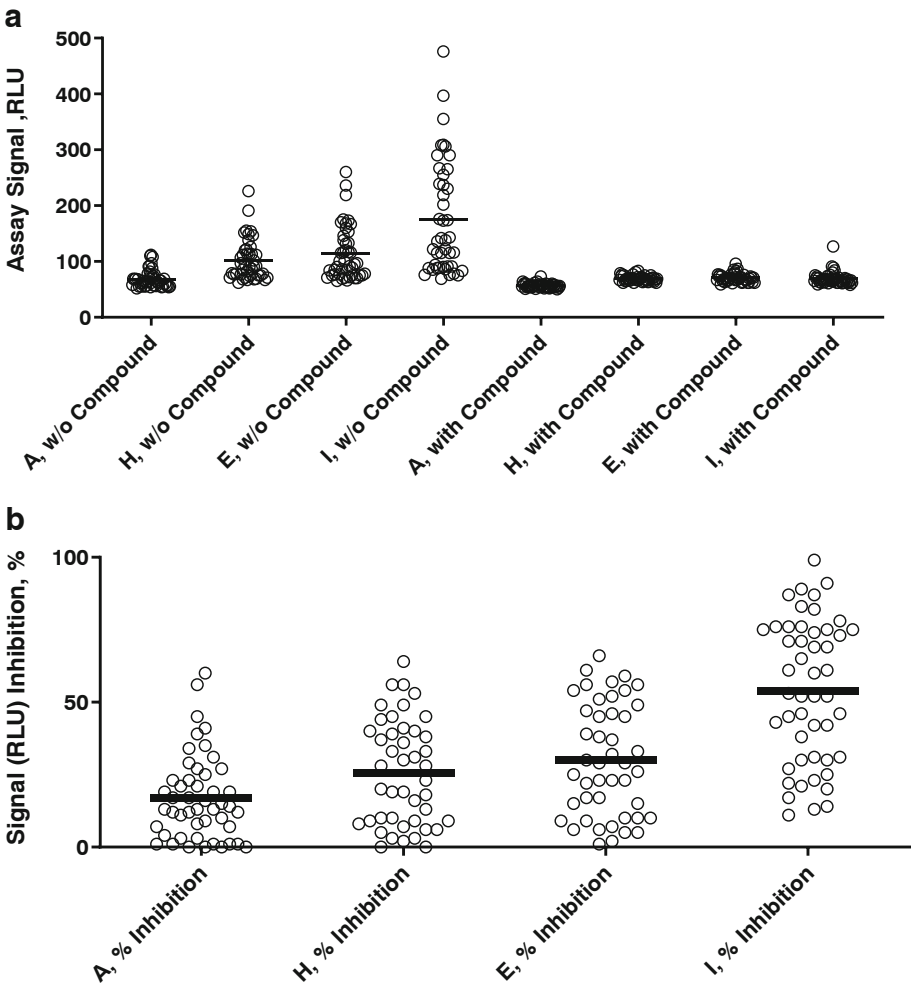


Fig. 5. Distribution of screening and confirmatory ADA assay signals produced by 50 samples from treatment-naïve healthy individuals. Samples were tested as described in the "Methods" section in the anti-PF-67786004 ADA protocols A, H, E, and I. **a** Assay signals (RLU) produced by individual samples when tested without (w/o) or with PF-67786004 addition. Horizontal bars represent mean values. **b** Distribution of the signal inhibition (%) values that were calculated by comparing assay signals generated by samples with and without addition of PF-67786004 (see "Methods" section for more details). Horizontal bars represent mean values of the distributions

the SCP suggesting a correlation between a rise of the SCP value and a relative loss of the PC-based assay sensitivity. This is additionally discussed later in the manuscript.

Data generated for the HPC, LPC, and NC pool samples in the confirmatory test were evaluated for evidence of impact of assay conditions on the reported % inhibition values produced by each control sample type. In stark contrast with the modest impact of assay conditions on the S/N generated by the LPC and HPC samples, % inhibition values were greatly dependent on the assay conditions, particularly for the LPC and NC samples. Most importantly, % inhibition varied greatly for NC samples and ranged from 15.3 to 74.2%, between procedures A and I, respectively (Fig. 6b). Significantly, the % inhibition for the NC sample exceeded 50% in protocol I, approaching the % inhibition reported for the LPC sample tested in the same procedure (79.8%). While the HPC produced at or close to 88% inhibition in all assay protocols, the LPC preparation generated % inhibition values ranged from 47.0 to 79.8%, between procedures A and I, respectively (Fig. 6b).

Individual treatment-naïve sample data sets generated for assay protocols A, H, E, and I in both screen and confirmatory tests were utilized to calculate corresponding SCP and CCP values (Fig. 7). Comparative analysis of SCP and CCP dependency on the assay procedures reveals an important observation. While some assay conditions produced similar SCP value, overall, both SCP and CCP values varied significantly across assay conditions tested. Low SCP and CCP values were reported for protocols A and I, 1.33 and 1.30, respectively, whereas assay protocols H and E generated measurably higher SCP values of 1.96 and 1.82, respectively. Therefore, a ~ 1.5-fold increase in the SCP values between assay procedures A and H can be produced based on changes in the assay conditions applied.

The CCP value was also modulated by assay conditions, with the lowest value reported for protocol A (58.5%).

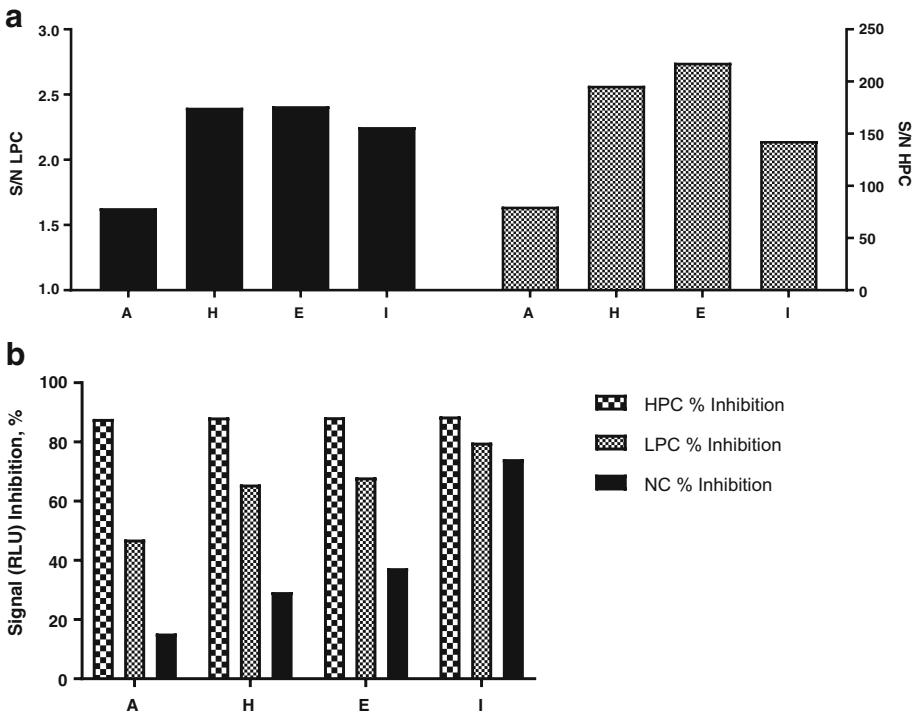
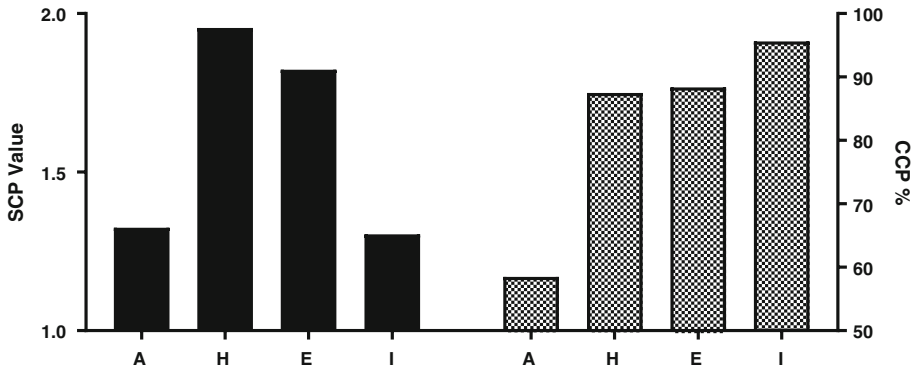


Fig. 6. Assay control performance as a function of assay conditions. Assay conditions used are described in Table I. The figure shows assay signals (RLU) produced by anti-PF-67786004 PC material spiked into pooled normal healthy human serum at 2000 ng/mL (HPC) and 25 ng/mL (LPC). The signal to noise ratio (S/N) of the mean assay signal produced by the LPC (solid bars) and HPC (dotted bars) samples *vs.* that produced by the NC sample and are shown in **(a)**. Assay signal inhibition (%) values were calculated by comparing assay signal generated by LPC, HPC, and NC samples tested with and without PF-67786004 addition and are shown in **(b)**

Protocols H, E, and I all generated high CCP values (at or above 85%), which is in significant excess of what is commonly viewed as an acceptable threshold of 50 to 60%. Remarkably, assay protocol I generated a CCP value of 95.6%, well in excess of what may be viewed as generally acceptable.



Tabulated data

Condition	Confirmatory CP%	Screen CP
A	58.5	1.33
H	87.5	1.96
E	88.4	1.82
I	95.6	1.30

Fig. 7. Anti-PF-67786004 ADA assay screening (SCP) and confirmatory (CCP) cut-point values depend on the conditions applied in the method. Assay procedure-specific SCP and CCP values were calculated as described in the "Methods" section

It is likely that the assay condition's ability to impact signals generated by a given individual treatment-naïve sample is dependent on the nature of the drug protein and its propensity to interact with various components of the assay matrix (e.g., endogenous serum factors). This hypothesis was evaluated using another monoclonal antibody-based drug candidate, PF-36137358, a human IgG1 framework monoclonal antibody specific to a soluble target and is in development for treatment of an oncological condition. Similar to the tests described earlier for the anti-PF-67786004 ADA assay, a separate set of 28 samples collected from healthy treatment-naïve individuals were tested under different assay conditions to determine the impact on the anti-PF-36137358 ADA method. While several assay condi-

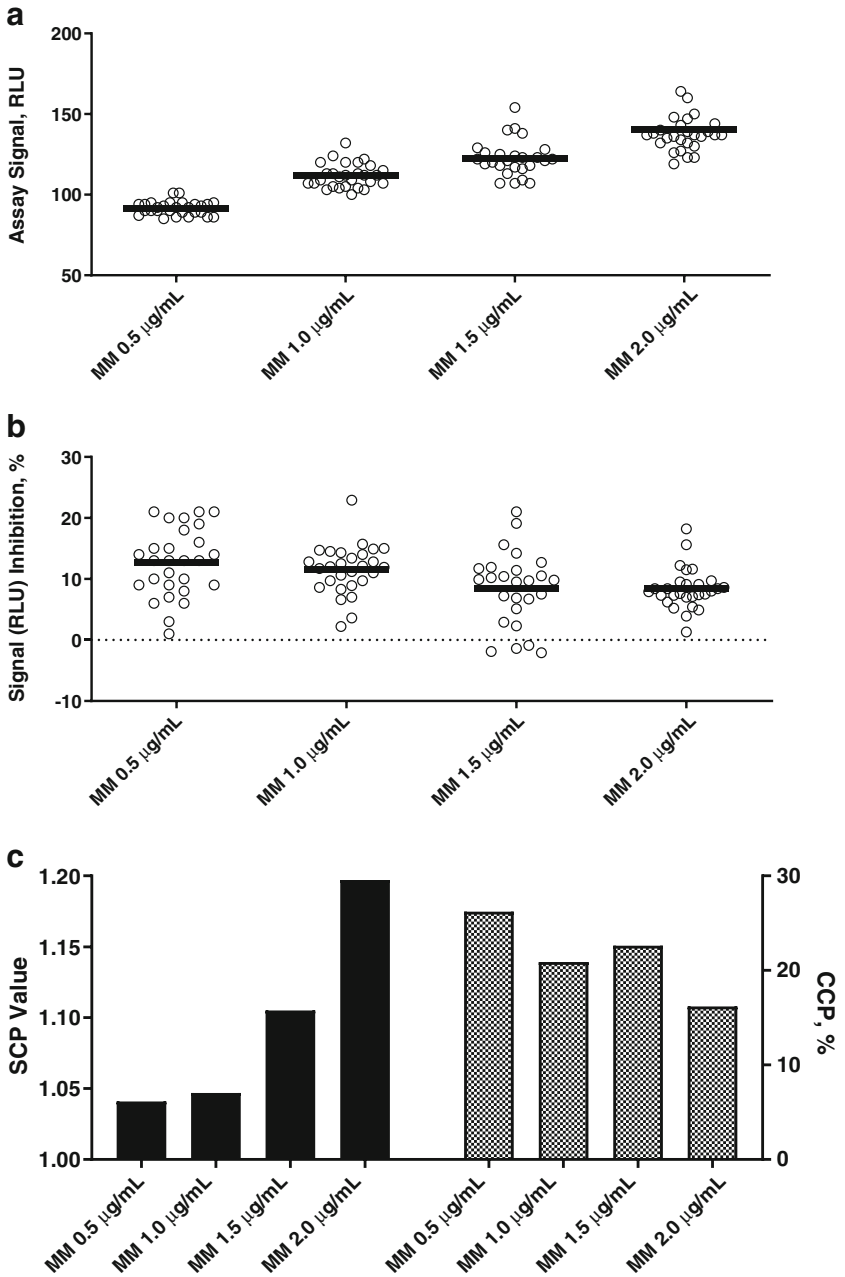


Fig. 8. Impacts of assay Master Mix reagent concentration on the performance of the anti-PF-36137358 ADA assay. Twenty-eight (28) individual samples collected from treatment-naïve healthy individuals were tested as described in the "Methods" section in the anti-PF-36137358 ADA assay. To determine the impact of the Master Mix (MM) reagent concentration on the assay performance, the MM concentrations were varied between 0.5 and 2.0 µg/mL as shown (**a**). To determine assay SCP and CCP parameters, individual samples were tested with and without PF-36137358 addition (**b**). The signal inhibition (%) values were calculated as described in the "Methods" section. Mean values for each of the distribution are shown (solid line). Procedure-specific SCP and CCP values are depicted in **(c)**

Table II. Assessment of the Anti-PF-36137358 ADA Assay Sensitivity as a Function of Assay Conditions. Impact of Varying Master Mix (MM) Concentrations on the PC-Based Screening Anti-PF-36137358 ADA Assay Sensitivity Was Evaluated. The MM Concentration Was Varied Between 0.5 and 2.0 μg/mL. Assay Sensitivity Was Calculated Using Procedure Described in the "Methods" Section Based on Data Collected in 2 Runs per Condition

Assay procedure type (MM concentration, μg/mL)	0.5	1.0	1.5	2.0
Screening assay cut-point	1.04	1.05	1.11	1.20
Screening test sensitivity (ng/mL)	11.7	50.9	118	158

tions were evaluated during anti-PF-67786004 ADA assay investigation, the concentration of the reagent mix (MM) appeared to produce the highest impact. The potential impact of the MM concentration was therefore the main focus during the anti-PF-36137358 ADA assay evaluation. As can be seen in Fig. 8a, a change in the MM reagent concentration resulted in modulation of the screening ADA assay signal generated by individual samples. Both mean and the distribution range of sample-generated signals increased as a function of MM concentration used in the assay. The mean RLU of sample signals varied from 91.5 RLU to 141 RLU with a similar change for the NC reagent signal from 96 to 143 RLU as the MM concentration increased from 0.5 to 2.0 μg/mL (concentrations prior to 1:1.5 dilution in the well).

Further, sample analysis in the presence of spiked unlabeled PF-36137358 (confirmatory test) revealed that the MM reagent concentration can modulate the degree of signal inhibition observed. Confirmatory test results shown in Fig. 8b demonstrated a slight reduction in the % inhibition generated by individual sample *vs.* MM reagent concentration. The mean inhibition value varied between approximately 12.7 and 8.4% for the assay protocols utilizing 0.5 to 2.0 μg/mL, respectively, of MM reagents. While variation of mean % inhibition as a function of MM concentration was limited, all samples generated % inhibition under 27% or less. Based on data generated using individual samples in the screening and confirmatory assays, the anti-PF-36137358 ADA assay SCP and CCP were calculated and are presented in Fig. 8c. The SCP values gradually increased as a function of the MM reagent concentration, ranging from 1.04 to 1.20 for 0.5 to 2 μg/mL of MM, respectively. The impact on the CCP was limited with calculated CCP values decreasing from 26.2 to 16.2% as the MM concentration rose while the mean % inhibition for the NC sample remained relatively flat ranging from 14.9 to 13.3% for the 0.5 to 2 μg/mL of MM-based conditions. The degree of the changes in the SCP and CCP values apparently correlated with the change of the inter-sample signal (RLU) and the % inhibition distribution variability. The MM concentration-associated change of the SCP also correlated with the change in the screening assay PC-based sensitivity which was evaluated following procedures described in the "Methods" section and is reported in Table II. The sensitivity values ranged from 11.7 to 158 ng/mL as the MM concentration increased from 0.5 to 2.0 μg/mL. It is also clear that the screening ADA assay PC-based sensitivity increased with the SCP value. Sensitivity value was above the 100 ng/mL threshold for the assay procedure where 2.0 μg/mL MM concentration was used. This clearly underscores the need for additional evaluation of the assay characteristics, including PC-based sensitivity, to determine final acceptability of the assay condition applied during validation phase. The anti-PF-36137358 assay was consequently validated using 1.5 μg/mL of MM with an acceptable PC-based sensitivity characteristic (data not shown).

Limited impact of assay conditions on the CCP value for the anti-PF-36137358 ADA assay contrasted with the significant dependency of the CCP in the case of the anti-PF-67786004 ADA protocol. This may be due to the specific nature of protein compounds and specifics of composition of the assay matrix. In addition, the two assay procedures are considerably different based on a number of factors, including the type of sample pre-treatment step and the nature of the reagents used. While some anti-PF-67786004 pre-existing reactivity was observed in the treatment-naïve individual samples, there was no evidence for anti-PF-36137358 pre-existing ADA-like reactivity in the study relevant individual matrix samples tested. Majority of the impact in the case of the anti-PF-36137358 ADA was generated based on the change of the MM reagent concentration.

One possible explanation for the observations from our study is that assay conditions can enhance low-affinity interactions and hence increase observed biological variability, and that this variability depends on the compound nature and matrix composition.

CONCLUSION

Signals generated by individual treatment-naïve samples in an ADA assay can depend on the nature of protein-based drugs and composition as well as the type and origin of the assay matrix. The difference between signals generated by individual samples in the assay is expected to incorporate both analytical variability of the assay and the biological diversity of individual sample matrix composition. The analytical variability of ADA methods is typically based on complexity of the assay procedure and the type of analytical platform applied. Analytical variability can be assessed by evaluating assay performance, including precision of the signal produced by assay controls. Inter-replicate assay control-sample signal precision is commonly expected to be within the 20% range. Most recently, improvements in analytical platforms and use of various automated or semi-automated systems have resulted in a significant improvement in the analytical precision of assays. Frequently, high analytical precision is further compounded by limited diversity of the biological matrix used in ADA assays. The result is exceedingly low screening cut-point factors, which present numerous challenges (16,17). For example, low ADA assay cut-point factors used during the study sample analysis phase may potentially result in reporting an

exceedingly high rate of false-positive ADA results, therefore diluting immunogenicity incidence data with spurious results and complicating data analysis (17). Use of a larger number of samples and/or analysis of study incurred samples has been viewed as potential solutions to this challenge, although both have inherent logistical and resource limitations. As an alternative, the biological diversity of the sample population tested during pre-study assay validation can be emphasized over the analytical variability of the assay, potentially reducing bias introduced by high analytical precision while appropriately representing the biology diversity of matrix. Taken together, this may reduce potential concerns related to cut-point values that fall below the inherent variability of the analytical platform and processes.

Here we present two examples demonstrating the impact of varying ADA assay conditions on the analytical and biological components of assay variability. In the first case study, PF-67786004 showed a low yet detectable degree of drug cross reacting pre-existing ADA-like reactivity which could be significantly enhanced by applying specific changes to the assay procedure. Condition changes in the anti-PF-67786004 ADA assay procedure described herein resulted in up to a 4.15-fold increase in the mean RLU signal and a considerable change in the inter-individual sample signal distribution. Consistent with the increase of ADA assay signal produced by treatment-naïve samples, a similar increase was observed for the assay NC material. Importantly, NC signal produced under various assay conditions did not lead to an increase in the NC signal precision. This was interpreted as a lack of direct correlation between assay conditions and procedures used and the observed analytical variability of the NC signal. The significantly broader range of assay signals produced under certain assay conditions was therefore attributed to modulation of the biological reactivity found in the individual samples. Biological diversity and the nature of the test compound together resulted in a notable change in the signal generated in the screen ADA assay, translating in a noticeable impact of the assay conditions on the screening cut-point value (SCP, tier 1). The SCP varied from 1.38 to 2.24 depending on the assay conditions tested which was an apparent consequence of the change in the distribution of signals generated by individual samples. The screen assay signal generated by individual samples was effectively inhibited in the presence of the unlabeled PF-67786004, consequentially leading to a greatly diverse range of assay signal inhibition (% signal change) which was highly depended on the assay conditions. As a result, a significant dependence of the mean % inhibition as well as the CCP (tier 2) on the assay conditions applied in the test was observed. The CCP value varied from 58.5 to 95.6% depending on the assay procedure applied. Overall, a noticeable dependency of the SCP and a significant dependency of the CCP on the assay conditions were observed for the anti-PF-67786004 ADA assay with some conditions producing unacceptably high CCP values (for example, 70% or higher).

In contrast, no pre-existing ADA-like reactivity was observed when treatment-naïve individual samples were tested in the anti-PF-36137358 ADA assay. Only a limited impact of the MM reagent concentration on the signals generated by individual samples in the anti-PF-36137358 ADA assay was observed. As a result, some modulation of the SCP and limited dependency of the CCP as a function of the MM concentration was detected. The screen and confirmatory assay cut-points varied from 1.04 to 1.20 and 26.2 to 16.2%, respectively, depending on the assay conditions tested. It was observed that assay condition-dependent change in the screening assay SCP correlated with the observed PC-based sensitivity in the anti-PF-36137358 ADA assay, where protocol that generated the highest SCP value also produced above the 100 ng/mL limit sensitivity of 158 ng/mL. Thus, after the initial condition selection, an additional evaluation is needed to determine an overall method acceptability, including assessment of assay PC-based sensitivity as well as drug and target tolerance characteristics. Typically, achieving desired PC-based sensitivity is not a significant concern with the reported ADA assay sensitivity known to be highly dependent on the PC reagent affinity for the drug (18). The anti-PF-36137358 ADA assay was later validated with the 1.5 μg/mL MM-based conditions, where an acceptable PC-based sensitivity characteristic was demonstrated (data not shown).

The sample to sample assay signal variability driven by biological diversity is likely to be driven by several factors, including the nature of the compound, presence of various matrix components, and the extent of low-affinity reactivity between PF-67786004 and these matrix components. Various domains present on the protein compound may lead to varying degrees of matrix component interactions. For example, rheumatoid factors present in some autoimmune disease serum may bind to the Fc domain of a monoclonal antibody-based biologic and/or autoantibodies may interact with glycan components of the compound (3,5–7). Although these interactions may exhibit low affinity of binding, high abundance of binding factors can easily result in a measurable ADA assay signal. By varying assay conditions, one may be able to modulate the ability of the assay to detect such low-affinity interactions, including low-affinity anti-drug antibody binding. Consequentially, assay conditions may effectively modulate distribution of the assay signal generated by individual samples in both screening and confirmatory ADA tests. Overall, an assessment of assay conditions during assay development enables a more thoughtful selection of an ADA protocol with fit for purpose screen and confirmatory cut-points.

Here we have shown that assay conditions can readily impact the range of signals produced by individual samples. We further show that assay cut-points, including SCP and CCP, can be affected by the assay conditions and procedures used. The degree of impact depends on the compound and matrix composition and is therefore largely driven by the biological diversity of the sample matrix. Viewed another way, this also suggests that assay conditions can significantly influence the ability of the assay to detect low-affinity interactions.

Therefore, we are proposing conduct of an initial assessment of assay conditions based on the performance of treatment-naïve individual matrix samples and not based on the performance of the assay PC. PC-based sensitivity will therefore not be the main driver for the assay conditions selection, which can lead to “supersensitive” assays. Further, we propose that when assessing assay condition impact on cut-point values, a combined analysis of estimated SCP and

CCP should be conducted with the goal to select appropriate cut-points, e.g., SCP ($1.2 < x < 2$) and CCP ($25\% < x < 60\%$). After this initial assessment, assay conditions will need to be verified to determine overall ADA assay performance acceptability, including assay PC-based sensitivity, drug and target tolerance characteristics.

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REFERENCES

1. FDA. Immunogenicity testing of therapeutic protein products—developing and validating assays for anti-drug antibody detection. Guidance for Industry. U.S. Department of Health and Human Services Food and Drug Administration. Center for Drug Evaluation and Research (CDER). Center for Biologics Evaluation and Research (CBER); 2019 [cited 2019]; 2019: Available from: https://www.fda.gov/ucm/groups/fdagov-public/@fdagov-drugs-gen/documents/document/ucm629728.pdf. Accessed 28 May 2019
2. Committee for Medicinal Products for Human Use C. Guideline on immunogenicity assessment of therapeutic proteins; 2017. Available from: https://www.ema.europa.eu/documents/scientific-guideline/guideline-immunogenicity-assessment-therapeutic-proteins-revision-1_en.pdf. Accessed 28 May 2019
3. Rispens T, Ooievaar-De Heer P, Vermeulen E, Schuurman J, van der Neut Kolfschoten M, Aalberse RC. Human IgG4 binds to IgG4 and conformationally altered IgG1 via Fc-Fc interactions. J Immunol. 2009;182(7):4275–81. https://doi.org/10.4049/jimmunol.0804338.
4. Rispens T, de Vrieze H, de Groot E, Wouters D, Stapel S, Wolbink GJ, *et al.*. Antibodies to constant domains of therapeutic monoclonal antibodies: anti-hinge antibodies in immunogenicity testing. J Immunol Methods. 2012;375(1–2):93–9. https://doi.org/10.1016/j.jim.2011.09.011.
5. Galili U. Anti-Gal: an abundant human natural antibody of multiple pathogeneses and clinical benefits. Immunology. 2013;140(1):1–11. https://doi.org/10.1111/imm.12110.
6. Galili U, Anaraki F, Thall A, Hill-Black C, Radic M. One percent of human circulating B lymphocytes are capable of producing the natural anti-Gal antibody. Blood. 1993;82(8):2485–93.
7. Arnold DF, Misbah SA. Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. N Engl J Med. 2008;358(25):2735; author reply -6. https://doi.org/10.1056/NEJMc080834.
8. Ghaderi D, Taylor RE, Padler-Karavani V, Diaz S, Varki A. Implications of the presence of N-glycolylneuraminic acid in recombinant therapeutic glycoproteins. Nat Biotechnol. 2010;28(8):863–7. https://doi.org/10.1038/nbt.1651.
9. van Ree R, Cabanes-Macheteau M, Akkerdaas J, Milazzo JP, Loutelier-Bourhis C, Rayon C, *et al.*. Beta(1,2)-xylose and alpha(1,3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens. J Biol Chem. 2000;275(15):11451–8.
10. Rieder F, Hahn P, Finsterhoelzl L, Schleder S, Wolf A, Dirmeier A, *et al.*. Clinical utility of anti-glycan antibodies in pediatric Crohn's disease in comparison with an adult cohort. Inflamm Bowel Dis. 2012;18(7):1221–31. https://doi.org/10.1002/ibd.21854.
11. Dai S, Schantz A, Clements-Egan A, Cannon M, Shankar G. Development of a method that eliminates false-positive results due to nerve growth factor interference in the assessment of fulranumab immunogenicity. AAPS J. 2014;16(3):464–77. https://doi.org/10.1208/s12248-014-9581-z.
12. Zhong ZD, Clements-Egan A, Gorovits B, Maia M, Sumner G, Theobald V, *et al.*. Drug target interference in immunogenicity assays: recommendations and mitigation strategies. AAPS J. 2017;19(6):1564–75. https://doi.org/10.1208/s12248-017-0148-7.
13. van Schie KA, Wolbink G-J, Rispens T. Cross-reactive and pre-existing antibodies to therapeutic antibodies—effects on treatment and immunogenicity. mAbs. 2015;7(4):662–71. https://doi.org/10.1080/19420862.2015.1048411.
14. Gorovits B, Clements-Egan A, Birchler M, Liang M, Myler H, Peng K, *et al.*. Pre-existing antibody: biotherapeutic modality-based review. AAPS J. 2016;18(2):311–20. https://doi.org/10.1208/s12248-016-9878-1.
15. Shankar G, Devanarayan V, Amaravadi L, Barrett YC, Bowsher R, Finco-Kent D, *et al.*. Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. J Pharm Biomed Anal. 2008;48(5):1267–81. https://doi.org/10.1016/j.jpba.2008.09.020.
16. Song S, Yang L, Trepicchio WL, Wyant T. Understanding the supersensitive anti-drug antibody assay: unexpected high anti-drug antibody incidence and its clinical relevance. J Immunol Res. 2016;3072586. https://doi.org/10.1155/2016/3072586.
17. Kubiak RJ, Zhang J, Ren P, Yang H, Roskos LK. Excessive outlier removal may result in cut points that are not suitable for immunogenicity assessments. J Immunol Methods. 2018;463:105–11. https://doi.org/10.1016/j.jim.2018.10.001.
18. Liang M, Klakamp SL, Funelas C, Lu H, Lam B, Herl C, *et al.*. Detection of high- and low-affinity antibodies against a human monoclonal antibody using various technology platforms. Assay and drug development technologies. 2007;5(5):655–62. https://doi.org/10.1089/adt.2007.089.