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Review

# Recommendations for the validation of cell-based assays used for the detection of neutralizing antibody immune responses elicited against biological therapeutics

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ABSTRACT

The administration of biological therapeutics may result in the development of anti-drug antibodies (ADAs) in treated subjects. In some cases, ADA responses may result in the loss of therapeutic efficacy due to the formation of neutralizing ADAs (NAbs). An important characteristic of anti-drug NAbs is their direct inhibitory effect on the pharmacological activity of the therapeutic. Neutralizing antibody responses are of particular concern for biologic products with an endogenous homolog whose activity can be potentially dampened or completely inhibited by the NAbs leading to an autoimmune-type deficiency syndrome. Therefore, it is important that ADAs are detected and characterized appropriately using sensitive and reliable methods. The design, development and optimization of cell-based assays used for detection of NAbs have been published previously by Gupta et al. 2007 [1]. This paper provides recommendations on best practices for the validation of cell-based NAb assay and suggested validation parameters based on the experience of the authors.



**Contents**



*Abbreviations:* ADA, anti-drug antibody; NAb, neutralizing antibody; FDA, Food and Drug Administration; EMEA, European Medicines Agency; ICH, International Conference on Harmonization; PC, positive control; LPC, low positive control; HPC, high positive control; SD, standard deviation; ANOVA, analysis of variance; DOE, Design of Experiment; LIMS, laboratory information management system.

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## 1. Introduction

Biological therapeutics being developed or marketed to combat human disease including recombinant proteins, peptides, nucleic acids and carbohydrates can induce an undesirable immune response resulting in the formation of ADAs. The development of ADAs does not necessarily signal an adverse event for the subject or patient, however these could impact the pharmacological and/or pharmacokinetic properties of the administered therapeutic. ADA-attributable adverse events include hypersensitivity reactions, immunologic cross-reactivity to endogenous homologs, reduced drug bioavailability and/or direct neutralization of the pharmacological activity of the administered biologic. The severity of the immune response, i.e. the incidence rate in the treated population as well as the magnitude of the clinical effect, influences the risk/benefit balance for the therapeutic [2]. For example, ADAs (neutralizing or non-neutralizing) can impact exposure by forming immune complexes with the drug and enhancing its clearance from circulation, thereby potentially impacting efficacy and/or raising the risk of type III hypersensitivity. Additionally, neutralizing antibodies (NAbs) can inhibit or block the biological/pharmacological activity of the drug *in vivo* and potentially also impact efficacy. Of greatest concern are antibodies that not only neutralize the administered drug but also neutralize an essential non-redundant endogenous homolog thereby impairing the body's normal function. Within the last decade, significant advancements have been made toward understanding the underlying mechanisms of immune responses to administered biologics. It is well recognized that numerous factors relating both to the administered product as well as the disease state of the study subjects could be contributing factors for unwanted immune responses towards biological therapeutics. Therefore, understanding the impact of ADA on drug safety, exposure and pharmacological activity represents good scientific practice, allows for a clearer interpretation of study data, and contributes to a better understanding of immunogenicity risk factors and risk mitigation [3].

Immunogenicity assays should be designed to detect polyclonal antibody responses generated against an administered product. For this, a two-step antibody testing and characterization strategy is warranted [2,4,5]. The first tier comprises screening and confirmatory immunoassays, capable of detecting ADAs that can bind to the drug product (binding ADAs). The approaches used to develop and validate such immunoassays have been described [4,6]. The second tier includes immunoassays that allow characterization of the detected ADAs, e.g. titer determination, isotyping, etc. Assays for NAb detection are also included in the second tier of assays and either cell-based or non-cell-based assays may be used to determine the impact of the detected ADAs on the biological activity of the therapeutic.

Cell-based NAb assays are *in vitro* methods designed to detect neutralization of drug function by NAbs present in sample matrix. Detection of drug neutralizing activity in such *in vitro* assays facilitates better understanding of the observed clinical effects of reduced pharmacological activity. In certain cases, it may be noted that NAbs detected *in vitro* do not appear to have an apparent effect *in vivo*. However, there is a regulatory expectation to provide correlative evidence of the presence of NAbs when the observed *in vivo* pharmacological behavior of the drug appears to be influenced by the immunogenicity of the drug.

At the present time, specific regulatory guidance governing the validation and performance acceptance criteria for immunogenicity assays is limited to the guidance issued by the EMEA in 2007 [7]. Draft guidance was also recently issued by the FDA [8]. In an effort to harmonize approaches that may be employed during development and validation of assays to support immunogenicity assessments, drug industry researchers have published several white papers outlining current industry practices [1,4–6]. A detailed overview of approaches that may be used for cell-based NAb assay development has been described previously [1]. The objective of this manuscript is to provide recommendations on approaches that may be used for validating cell-based NAb assays. In particular, this manuscript addresses the assay performance characteristics and best practices for the validation of qualitative (reporting positive/negative results) or quasi-quantitative (titer-based) NAb assays.

It is important to note that these recommendations may be applicable to a majority of cell-based NAb assays; however, certain situations may require modified approaches to assay validation. The validation of non-cell-based NAb assays is not addressed in this manuscript.

*Note*: These recommendations are based on the experience of the authors. They reflect scientific concepts that should assist assay developers to form a rational approach for the validation

of their specific assay. These recommendations are not intended for adoption in lieu of published regulatory guidance or direct discussions with the various regulatory agencies.

## 2. Neutralizing antibody assay formats

Direct and indirect NAb assay formats have been described in detail previously [1]. Briefly, NAb assays for biologics with agonist activity use the direct format while NAb assays for biologics with an antagonistic mechanism of action utilize the indirect assay format. A common approach to performing both types of assay formats includes using fixed concentrations of cells, drug in the selected assay matrix (i.e. a suitable dilution of test species serum), and ligand (if the drug is an antagonist). However, there may be cases where an approach using variable amounts of drug may be appropriate (Appendix A). The concentrations of each of the assay components are selected during assay optimization. One or more positive control antibodies capable of neutralizing drug activity may be needed to demonstrate that the assay can reliably detect neutralization of the drug used in the assay (Appendix B). The positive control is also used to monitor assay performance during routine use. Since the sensitivity of a NAb assay is highly dependent upon the selected drug concentration, particular attention should be given to the fixed drug concentration and the ability of positive control NAbs to inhibit it.

NAb assays may be conducted to obtain qualitative (positive/negative) or quasi-quantitative (e.g. titer) information. The term "quasi-quantitative" is applied to immunogenicity assays since these methods for assessing polyclonal ADAs lack a true reference control [4,9] and sample results are determined relative to an assay cut point. Positive control antibodies, usually obtained by immunizing animals are used to initiate assay development, to conduct assay validation and to monitor assay performance. In the qualitative assay format, the sample is tested at a single dilution, thereby allowing a higher throughput of sample analysis. A sample is considered positive or negative based on its signal relative to an assay cut point. Confirmatory NAb assay formats have been described in detail previously [1] and are referred to as matrix interference assays in the article. These assays may be used in conjunction with qualitative NAb assays to determine whether the detected neutralizing activity is due to NAb and not attributable to other factors that may be present in the serum.

Using the quasi-quantitative approach, a sample is tested at multiple serial dilutions to achieve a titer value. The highest dilution of test sample producing a positive result relative to an assay cut point represents the titer of neutralizing antibodies present in that sample. Many laboratories use a combination of the qualitative and quasi-quantitative modes by first screening samples in a qualitative NAb assay and then testing only the NAb positive samples in a titration assay. Another quasi-quantitative approach uses a NAb assay format that reports NAb activity on the basis of the amount of drug neutralized in the assay (Appendix A).

### 2.1. Assay controls

The relevant controls for use in direct and indirect NAb assays have been described in detail previously [1]. All assay controls should be prepared in assay matrix and should be included in all runs conducted during assay validation and study sample analysis. A 'run' is defined as an experiment that may consist of one or more assay plates tested using the same working preparations of cells and reagents.

At a minimum in an indirect NAb assay format, assay controls prepared in assay matrix should include: (a) cells, (b) cells + ligand, (c) cells + ligand + drug, and (d) cells + ligand + drug + positive control antibody. Similar controls (without ligand) should be employed for NAb assays utilizing the direct NAb assay format. In addition to functioning as system suitability controls (that ensure reliable performance of the assay), these controls also have functionality in making determinations of assay sensitivity (Section 3.2) and assay precision (Section 3.3). It is recommended that the number of replicates of each of the assay controls and samples prepared during validation or study sample analysis be similar.

As mentioned earlier, an appropriate positive control for a NAb assay is an ADA that inhibits the biological activity of the drug. Most laboratories utilize monoclonal or polyclonal antibodies produced in animals against the therapeutic of interest. The assay control that includes drug but lacks the positive control antibody functions as the negative control and the one that includes drug and positive control antibody represents the positive control in the NAb assay. These controls should be prepared in pooled matrix selected for assay validation. During assay validation and study sample analysis, each plate should include negative controls as well as 2–3 concentrations of the positive control antibody (prepared in undiluted pooled matrix) that should include the concentration representing the assay sensitivity and 1–2 higher concentrations expected to test positive in the assay. The controls containing the positive control antibodies are referred to as quality control (QC) samples in this paper. Inclusion of the low positive control (LPC: representing the assay sensitivity) is critical to confirm the relative sensitivity of the assay during routine assay performance and also provides assurance that the assay cut point set during validation is suitable for in-study sample testing. The establishment of the LPC antibody concentration is described in Section 3.2. In quasi-quantitative NAb assays, if the data will be reported as titers, the high positive control (HPC) concentration may be used as a surrogate to demonstrate dilutional linearity and titer precision. In a titration format, the HPC is typically evaluated as a full dilution curve while the negative control is evaluated as a single dilution in multiple replicates. Non-neutralizing antibody controls can be also be tested if available during assay development, included during assay validation to demonstrate NAb specificity and, if deemed necessary, utilized as a control during routine sample testing.

## 3. Assay validation

### 3.1. Cut point

The cut point of a NAb assay may be defined as an assay response value above (if the drug is an antagonist) or below (if the drug is an agonist) which a sample is defined to be positive for neutralizing activity. A detailed description and illustrations of the statistical methods used for evaluating cut points for immunogenicity screening assays have been provided previously [6]. Most of these details, such as the evaluation of distribution of donor sera for cut point determination, outlier testing, comparison of means and variances between assay runs, etc. are also applicable for deriving NAb assay cut points. Therefore, only a brief outline of the cut point evaluation process for NAb assays has been provided here.

#### 3.1.1. Qualitative NAb assay

The assay cut point should be derived using a statistically adequate number of individual sera (typically 30 or more) from the target disease population (if available) or from normal donors in the assay by testing them over a period of at least three days by a minimum of two operators. If a sufficient number of target disease sera are not available for deriving the assay cut point, normal sera or similar disease sera from a commercial source may be employed. The cut point established with these samples should later be compared with the performance of the pre-treatment (baseline) study

samples in the assay to ensure that is valid for the study population. This is accomplished by the statistical comparison of the distribution of the means and variances of these populations. The means are compared using mixed-effects ANOVA with population type (disease versus normal) as fixed effect and subjects nested within the population type as random effect. Other factors may also be included depending on the design used. The variances can be compared using Bartlett's or Levene's test. If upon evaluation, the variability of the disease sample distribution is significantly different from the naïve normal sample distribution, then a disease population specific cut point should be established. Additional considerations such as gender, age, etc. should be considered in the sample selection, depending on the nature of the intended target population.

The individual donor sera should be tested at the minimum required dilution established during method development and the obtained values should be inspected for outliers. The distribution of the assay response of the donor sera is an important consideration for establishing the cut point. If the distribution is approximately normal, based on the Shapiro-Wilk test, the mean and standard deviation (SD) can be calculated and the cut point can be calculated as the mean plus or minus 2.33 × SD (depending if a NAb positive response falls above or below the cut point) using the parametric method. The 2.33 value corresponds to a 1% false positive rate from one-side of the normal distribution. In cases where the NAb testing strategy includes a confirmatory step (Section 3.6), the cut point of the screening NAb assay could be calculated as the mean plus or minus 1.645 × SD (5% false positive rate), if desired. However if this approach is used, it is important that the confirmatory assay cut point calculation target no more than a 1% false positive rate.

Shapiro–Wilk tests for assessing the normality of distribution are readily available from most commercial software and provide a reasonable assurance about "adequate normality" when the sample size is not too small (>25). Even when this test is statistically significant (hence suggesting some "non-normality"), it is reasonable to consider the skewness-coefficient of the distribution. The skewness-coefficient provides a quantitative reflection of the nature of symmetry of the distribution. Typically, if this value is less than 1, the use of the normality assumption to define the cut-point as defined above is a reasonable approach. However, due to some degree of skewness, it may be safer to use the median value instead of the mean and "1.4826 times the Median Absolute Deviation" (1.4826 × MAD) instead of the SD. This is especially useful if the tails of the distribution (extreme ends) are long. If the skewness coefficient is greater than 1, the data can be transformed to achieve approximate normality and the cut point can be evaluated using the above formula on the transformed scale and later inverted back to the original scale. Additional rigorous statistical tests [10] for formally assessing the significance of skewness and tails (kurtosis) of the distribution may be used with the assistance of a trained statistician.

Data should be inspected for outliers in either the original scale or the transformed scale, depending on whichever scale is more symmetric. The criteria for identifying outliers can be based on the outlier box-plot or studentized residuals from the ANOVA [6]. The identified outliers should be excluded from the evaluation of cut point. The distribution of the data should be re-evaluated to confirm approximate symmetry/normality after excluding these outliers using methods described above.

To determine whether the cut point calculated during assay validation can be used during the bioanalysis (in-study) phase, the means and variances of the distribution should be compared between the assay runs using a mixed-effects model and Bartlett's test respectively [6]. If these are not significantly different, then the same cut point value may be used during the bioanalysis phase. The latter value is designated to be a "fixed cut point". If the assay run means are significantly different, then the use of the fixed cut point approach is not advisable. In this case, the cut point evaluated as above using these validation data should be divided (or subtracted) by (or from) the negative control pool. This is called a "multiplicative (or additive) correction factor". This factor may be multiplied (or added) to the negative control used during each run of the bioanalysis phase to define the run or plate-specific cut-point. Such a cut-point is called the "floating cut point". If the original data are found to be approximately symmetric/normal, then an additive correction factor may be used. If a log transformation is necessary to ensure approximate symmetry/normality of the distribution, then a multiplicative correction factor may be used. Further details on this and related approaches for other scenarios have been described by Shankar et al. [6].

#### 3.1.2. Quasi-quantitative NAb assay

Steps similar to those described above for qualitative assays are followed when establishing a cut point for quasi-quantitative (titer) results. Individual samples (typically 30 or more) containing HPC are analyzed at the minimal required dilution (often 1/10–1/20), followed by a reasonable dilution scheme (e.g. two-fold) maintained in the appropriate matrix in three assay runs by two or more analysts. Data for each dilution should be tabulated and analyzed for distribution and outliers, using methods described above for the qualitative assay. Data from each dilution are compared with respect to means and variances using mixed-effects ANOVA and Bartlett's test respectively, similar to the way the assay run means and variances are compared. If the means or variances are significantly different between the dilutions, the cut point should be calculated for each dilution, using the formulae/methods similar to those described above for the qualitative assay. Otherwise, an overall cut point can be determined by pooling the data across all dilutions. Other considerations listed for the qualitative assays above, such as comparison of assay run means and variances, normal versus disease population, etc. should also be evaluated.

### 3.2. Sensitivity

The purpose of defining the sensitivity of the NAb assay using a surrogate positive control antibody is to ensure that the assay performs as validated over time. The approach described below allows validation of (a) the selected positive control antibody and (b) the assay sensitivity. Sensitivity is defined as the lowest concentration of a positive control antibody that tests positive in the NAb assay. It is important to consider the desired reliability or consistency with which the particular concentration should test positive in the assay, e.g. at least 50% of the time, or 95% of the time, etc. [6]. The result obtained for this validation parameter is dependent on the neutralizing capacity of the positive control antibody used to conduct the experiments to derive this value. As stated earlier, the sensitivity of NAb assays is also largely dependent upon the concentration of drug used in the assay and these two characteristics bear an inverse relationship to each other.

Positive control antibody dilution curves should be prepared in pooled assay matrix and evaluated in at least three different runs by two operators for a total of six runs. It is recommended that more than one antibody curve be performed per run per operator. A dose-dependent inhibition of the drug-induced response by the antibody is sufficient for validating the neutralizing property of the positive control antibody.

For the validation of assay sensitivity, linear interpolation between values above and below the cut point, or the four-parameter logistic model should be used to analyze the resulting data. When a four-parameter model is used, at least six concentrations of the positive control antibody should be included in the dilution curve. One or more of these concentrations should fall

below the assay cut point to obtain an acceptable fit. The antibody concentration that corresponds to the cut point is then determined by interpolation. Typically, the average of the interpolated concentrations from replicate curves (derived from different plates over several days) is then reported as the sensitivity of the assay. In this case it should be recognized that a sample containing NAbs at an equivalent concentration would test positive only 50% of the time.

It is preferable to define sensitivity of the NAb assay at a concentration level that produces a positive result 95% of the time. This approach has been described in detail by Shankar et al. [6] and using the experimental approach described above, is also applicable for NAb assays. The concentration of the positive control antibody representing the assay sensitivity may be used to guide selection of a LPC that can be used to monitor assay performance in all assay runs during assay validation and sample analysis. It should also be noted that the reported assay sensitivity will vary enormously with different anti-drug antibodies and it is not possible to use one or more positive control antibodies to model the range of drug neutralization that may be observed with study samples. Therefore, the assay sensitivity value obtained during validation, while important for monitoring assay performance, should not be used to predict actual concentration values for antibodies that could be detected by the method in study samples (Appendix B). The use of multiple monoclonal neutralizing antibody positive controls with a range of neutralizing capacities, if available, is recommended for comparing the sensitivity of the neutralizing assay to the ADA screening assay.

### 3.3. Assay precision

Precision (intra-assay and inter-assay) is the quantitative expression of variability and provides a measure of the amount of random error that occurs during execution of an analytical procedure. Precision estimates are useful indicators of assay performance in the specified assay matrix.

#### 3.3.1. Qualitative NAb assay

*3.3.1.1. Intra-assay precision (repeatability).* Intra-assay precision is the closeness of agreement between results generated by consecutive analysis (replicate testing) of the same assay controls or samples under the same operating conditions by the same operator within an assay. Four to six independent preparations of negative control, low positive control and high positive QC samples in a single lot of pooled donor serum (normal or disease) are evaluated in duplicate or triplicate, in multiple positions on the same plate in a randomized manner, to determine the relevant sources contributing to response variability. The imprecision of the assay signal data (optical density, fluorescence unit, luminescence unit or level of inhibition of signal after normalization or interpolation), are calculated and reported as percent CV (= [(standard deviation/mean) $\times$ 100]). The percent CV values of the mean assay signal obtained with the various assay controls should suffice for assessment of intra-assay precision [12].

The %CV may vary depending on the technology used for readout, assay methodology, as well as procedural complexity. The expected target CV or pooled %CV for intra-assay precision therefore should be defined based on assay capability, as well as on intended use.

*3.3.1.2. Intermediate precision (overall precision).* Intermediate precision is the estimation of precision encompassing within-laboratory variations of assay runs, and therefore represents the overall precision of the assay. The experiment described above should be executed over multiple days with at least two operators especially if the sample testing will be executed in the study phase by more than one operator. The pooled intra-plate SD and the SD of the mean for each sample tested on multiple plates over multiple days can be used to calculate the intermediate precision assuming that most of the variability is attributable to plate variability and that the sample size is the same on every plate.

Intermediate precision is highly dependent on the assay methodology and procedural complexity. The target intermediate precision, therefore, should be defined based on assay capability, as well as on intended use (fit-for-purpose).

Using an alternate approach, inter-assay precision may also be assessed by deriving the mean, standard deviation and percent CV of the negative and positive controls from all the experiments (excluding those runs with an assignable operator or equipment error or with method variations purposely introduced for robustness testing) conducted during assay provided the plate location effects are negligible.

#### 3.3.2. Quasi-quantitative NAb assay

An assessment of the precision of the reported titers generally utilizes the LPC and 1–2 concentrations of the HPC. HPCs containing a higher concentration of the antibody will require more dilutions than those corresponding to the LPC. The HPCs (minimum of 3 independent preparations) should be diluted in a 2- or 3-fold titration series using undiluted pooled assay matrix as diluent and tested in the assay. For intra-assay precision, it is generally recommended that three titration curves of the low and high PC be analyzed by one operator on the same day. For inter-assay precision, analysis of 3 titration curves, each, of the low and high PC on a minimum of 2–3 different days by two operators is recommended. Titers are determined as a reciprocal value of the highest dilution of the PC that tests positive. Target titers can be determined and assigned to each low and high PC or can be calculated as mean values by averaging the titer values obtained for the low and high PCs in the precision assessment. Intra- and inter-assay precision of titers is then evaluated by comparing titers obtained for individually prepared curves to the target titer assigned for the low and high PC, respectively.

In general the acceptance criterion for the precision of titers is that the assigned titer value should be within one dilution of the target titer in independent titration series. This, however, will depend on the method capability, the dilution level (for instance, this criterion may be suitable for a 2 or 3-fold serial dilution assay format but not for a ten-fold serial dilution format) and the intended use of the reported titer data in the clinical setting. If using the calculated mean titer approach, occasionally, the mean titer may fall in between the dilutions since it is derived from observed values from multiple analyses. In the latter case, the $\pm$1 dilution rule needs to be modified. In such instances, titers observed for a defined positive control are rounded to the nearest dilution to yield the target titer.

### 3.4. Robustness

Robustness refers to the capacity of a method to remain unaffected by routine variations in assay procedures. Deliberate, small, and well-controlled changes are generally introduced to critical assay components or steps to determine their impact on NAb assay sensitivity and specificity. Robustness testing can be done following assay optimization during assay development or additional evaluation may be conducted during/after validation. Table 1, although not intended as a comprehensive list, lists some of the assay components that may be included for determining the robustness of a typical cell-based NAb assay. Laboratories should design their robustness testing based on their experience with that particular assay and its susceptibility to any unique conditions that could compromise its reliability.

Robustness experiments can be done using the checkerboard approach to test 1–2 changes in assay conditions at a time using assay controls or study samples. Statistical comparison of the results obtained can be conducted by using analysis of variance

**Table 1**
Assay variables for robustness testing.

| | |
|---|---|
| Tier 1 assay variables | Critical components (e.g., ligand concentration, drug lots)<br>Critical steps (e.g. incubation time, temperature)<br>Cell line (e.g. passage number, cell density) |
| Tier 2 assay variables | Positive control antibodies<br>Other assay conditions (e.g. assay plate stability prior to read, etc.)<br>Other reagents (fetal bovine serum, medium, assay plates, kits, etc.) |
| Tier 3 assay variables | Different instruments<br>Different operators |

The tier 1 assay variables include the most critical components and steps for an assay and any changes in these are most likely to impact the assay's sensitivity and specificity. Tier 2 and 3 assay variables also play important an important role in ensuring reliable assay performance.

(ANOVA). A multi-factorial Design of Experiment (DOE) approach [11] can also be used to study the impact on assay robustness if multiple assay parameters (e.g. concentrations of critical assay components, incubation conditions, etc.) are varied in a single experiment.

The level of robustness testing of an assay could vary depending on the intended use. For example, if a NAb assay will only be used to support a short toxicology study or if the samples from the study will be analyzed within a relatively short time frame and by the same operator(s) who developed and validated the assay, robustness testing may be limited in scope. On the other hand, robustness testing is of high importance if a NAb assay is to be used to support multiple large clinical studies lasting over several years, and if NAb sample testing is to be conducted by multiple operators. In the authors' experience, the data generated from robustness testing is most helpful when transferring assays between laboratories.

### 3.5. Ruggedness

Ruggedness refers to the reliability of an assay when performed by more than one laboratory and helps demonstrate the "transferability" of an assay. Ruggedness, termed 'reproducibility' by the ICH guideline [13] represents the precision of results obtained between different laboratories performing the same assay. Reproducibility of the method in a new laboratory should be demonstrated as part of the assay transfer qualification and/or validation of the method at the new facility.

Utilizing the knowledge gained during assay optimization, and use of the assay in the originator's laboratory, it is important to identify assay performance characteristics that are likely to be impacted by transfer of the method to a different lab and/or operator. Generally, an assay may need to be reproduced in a facility with operators that have different levels of experience and training. The performance of the system suitability controls should be assessed between laboratories to demonstrate comparable assay sensitivity between laboratories. If the sensitivity is not acceptable, the assay cut point may need to be established separately for the other laboratory. In addition, evaluation of a panel of blinded samples at the receiving laboratory can help demonstrate the concordance in results generated at different sites for the same samples.

The analysis of results from such an evaluation should be based on pre-defined acceptance criteria. Any significant change in the assay signal resulting in false negative or positive assessments at the receiving lab, performance differences of the QC samples (for e.g. increase in %CV of repeat measurements), or lack of concordance in blinded sample value assessments should be addressed to ensure that the assay performs as expected at the receiving site and per original validation of the method at the transferring site.

### 3.6. Assay specificity

Specificity is the ability of an assay to unequivocally detect the analyte of interest. For cell-based assays, specificity assessments should start with assessing the ability of the cell line to respond to structurally or functionally related agents that resemble the target or the receptor utilized by the assay and that may be expected to be present in the assay matrix. Key factors that govern the choice of the cell line to be used in the NAb assay have been detailed previously [1]. Assay optimization efforts should be made to determine that the selected assay endpoint (i.e., the assay signal) is specific for NAb detection. The impact of disease state sera on assay specificity is an important assessment since it can impact cell viability. The sterility of samples is a critical consideration for cell-based NAb assays since any microbial infection (e.g. mycoplasma or fungal) could impact assay performance and ultimately assay sensitivity.

Specificity testing during assay validation may include testing the behavior of irrelevant antibodies in the assay. Ideally these comprise drug specific non-neutralizing antibodies, in lieu of which, ADA against other related drugs may be utilized. A panel of antibodies that do not block drug activity may be screened in the assay along with the positive control antibody. The comparison may be made either by evaluating antibody curves or by spiking an excess concentration of these antibodies in pooled serum. The non-neutralizing antibodies should not yield a positive signal in the assay.

In certain instances, the specificity of the detected NAbs may have to be confirmed as a routine part of the assay since some structurally related molecules or cross-reacting components may be an integral part of the disease matrix. If a decision is made to include specificity or confirmatory testing on a routine basis during sample analysis, the publication by Gupta et al. [1] provide an in-depth overview of various assay formats that may be used for this purpose. These assays are referred to as matrix interference assays in the aforementioned paper and allow a confirmation of the fact if the observed inhibition of drug activity in the NAb assay is specific to the drug and may not be attributable to other factors that may mimic a NAb, e.g. soluble receptors, etc. If a decision is made to include a matrix interference NAb confirmatory assay during routine testing, the validation should follow the principles described in the assay cut point Section 3.1 described in this paper.

### 3.7. Selectivity/drug product interference

The presence of drug in test samples derived from drug-treated subjects interferes with the ability of immunogenicity assays to detect binding antibodies or neutralizing antibodies and thereby invariably results in falsely negative results. NAb assays generally tend to be more susceptible to drug presence than immunoassays used for detecting binding antibodies. The magnitude of interference is dependent on several factors, i.e. the concentration of drug, the characteristics of the positive control antibody, and the design of the assay, thus an establishment of a single "drug tolerance level" is not possible (Appendix C). Nevertheless, the extent of drug interference in the NAb should be addressed in the design of the assay and testing strategies. During assay development or optimization, drug interference may be assessed by using drug spiked into a positive control neutralizing antibody containing mock sample. The level of drug that will interfere with the detection of the antibody in the assay will be highly dependent on the concentration and other characteristics (affinity, avidity) of the antibodies in the positive control and those that are present in the test sample. Pertinent information regarding susceptibility of the assay to drug interference should be included in the validation report.

An example shown in Table 2 demonstrates that "drug tolerance levels" varied considerably even when a single positive control anti-

**Table 2**
Effect of NAb concentration on the drug tolerance level.

| Drug concentration added to sample (μg/mL) | Assay results of NAb positive control (log titer result) | | | |
|---|---|---|---|---|
| | Level I[a] | | Level II[b] | |
| | Log titer | Result | Log titer | Result |
| 0 | 2.13 | Positive | 2.97 | Positive |
| 0.01 | 1.65 | Positive | 3.01 | Positive |
| 0.10 | 1.53 | Positive | 2.91 | Positive |
| 1.0 | Below cut point | Negative | 2.99 | Positive |
| 10 | Below cut point | Negative | 2.66 | Positive |

An example of a drug tolerance study is shown in this table. Drug or vehicle was added in a small volume to a NAb LPC or to a HPC prepared at 9× concentration of the LPC. Prior to testing, the HPC was diluted 1/9 in assay matrix. After incubation, both samples were processed to measure NAb activity in the assay. NAb activity was still detectable even in the presence of 10 ug/mL of drug with the HPC, whereas addition of 1 or 10 μg/mL of drug rendered the NAbs undetectable in the LPC samples. As demonstrated here, the "drug tolerance level" may vary considerably even when the same positive control antibody is added at different levels to different concentrations of the drug and thus it is not possible to predict what level of drug will interfere with measurement of NAbs in study samples.

[a] NAb positive control at the LPC concentration.

[b] NAb positive control tested at 9× LPC concentration.

body was added at different levels to different concentrations of a drug prior to measurement in an assay. Since NAb assays tend to be highly susceptible to drug interference, attempting to detect or measure NAbs in samples from time points when drug is expected to be present is generally not recommended. Appendix C describes strategies for assessing NAb activity when analysis of samples containing drug is required.

To address matrix interference caused by concomitant medications, auto antibodies etc. the EMEA guidance [7] recommends validating methods to rule out matrix effects that can adversely affect the results obtained. Evaluation of the performance of the high and low PCs prepared in 10 individual disease sera along with the unspiked samples will provide information on this assay performance characteristic.

### 3.8. Stability

It is recognized that immunoglobulins are generally stable. However, individual clinical samples may display different stability properties thus the stability of QC samples does not necessarily reflect the stability of real preclinical or clinical samples. Shankar et al. [6] recommended that the stability of ADA can be approximated by the stability of serum or plasma immunoglobulin specific to any antigen. Thereby available clinical and non-clinical matrix samples, from each target species, should be characterized separately for stability and the ensuing results be extended to ADA assays of all drug programs in a research laboratory. Thus, it is not stipulated that sample stability for each drug-specific assay be separately validated.

However, it is important to understand the stability of QC samples which are used to monitor assay performance and setting assay acceptance criteria. Thus short and long term stability of QC samples and the impact of storage conditions (25 °C, 4 °C, −70 °C, etc.) should be tested as part of the NAb assay validation. The number of freeze/thaw cycles, storage lengths and temperatures should be tested and documented and should be based on the expected storage and use of samples. Acceptance criteria should be based on the time point when a positive sample at the assay's sensitivity no longer tests positive or a significant drop in the neutralizing activity is observed. Assay control trending data can also be used to demonstrate and monitor reagent stability.

Due to the difficulty of predicting real sample stability and the fact that there is no real control that can truly represent individual sample variation, it is highly recommended to closely monitor sample storage conditions and to limit the number of freeze/thaw cycles when possible. It is also recommended that several sample aliquots be available, and a fresh aliquot is used for NAb testing to maintain activity and relative sterility, when possible, although it is recognized that blood draws and matrix preparation do not lend to truly sterile sampling. If a sample requires processing prior to analysis in the NAb assay (e.g., sample pre-treatment through a protein A/G column etc.) the stability of the process controls should also be evaluated.

### 3.9. System suitability

An integral aspect of a method's utility is the verification of its overall effectiveness as an analytical system under actual conditions of use. The concept of system suitability testing is based on the premise that the equipment, reagents and samples, analytical operations and data collection constitute an integral system that can be evaluated as such. System suitability monitors and confirms that all critical reagents are performing adequately and the assay remains valid. System suitability test parameters will vary depending on the procedure but should capture key elements of the assay to ensure performance consistency.

The assay controls described in Section 2.1 should be included in every assay to set acceptance criteria and to ensure consistent method performance over time. Cell- based NAb assays typically require a broader spectrum of system suitability criteria than are needed for immunoassays. Depending on the biological mechanism, the neutralizing effect on the assay signal could be an overall increase or decrease. Assay controls (lacking positive control antibody) that correspond to assay background, minimum and maximum cellular responses should be monitored to identify any undesirable shifts in the dynamic range of the assay which could ultimately the assay's capability to reliably detect positive samples.

As mentioned earlier, two levels of positive control, LPC and HPC, can be used to determine assay acceptance criteria. A mid-level positive control may not be influenced to the same extent as the LPC and HPC, therefore using only a mid-level control can provide misleading information that an assay is performing adequately but yet may be drifting over time. Alternatively, the positive control at concentrations higher than the LPC may be run in a dilution series to cover multiple levels. Use of a non-neutralizing antibody as a negative control should also be considered as it could prevent reporting false-positive results from a failed assay.

Provisional assay control acceptance criteria for use during the validation may be determined from assay optimization or prevalidation studies, although not a requirement for validation. The detection range of the instrument should be considered when setting upper and lower boundaries of the assay signal obtained by the assay controls. Final assay acceptance criteria can be established using all assay control data generated during the validation.

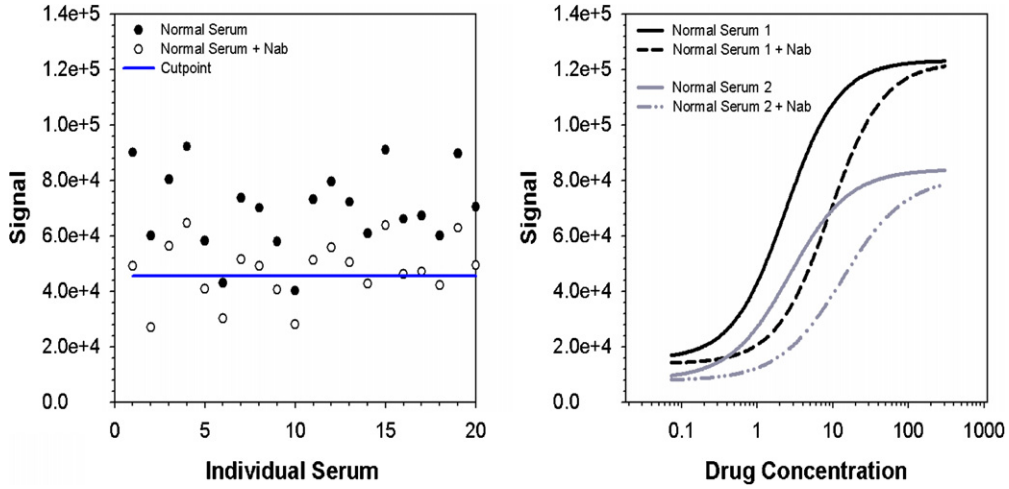


**Fig. 1.** Comparison of neutralizing bioassays using fixed (A) versus variable (B) drug concentrations. Panel A shows an example data set illustrating normal individual sera incubated with a fixed concentration of drug with and without neutralizing antibodies (NAb). The cut point is established based on a representative pooled sample and individual samples testing below the cut point are positive for NAb. The wide range of baseline responses within this population prevented the differentiation of neutralizing responses. In this scenario, 13 out of 20 (65%) samples tested as false negatives. B. Example data set illustrating individual sera 1 and 2 incubated with increasing concentrations of drug. Individuals were compared to their own baseline samples and a neutralization response was indicated by a change in the EC50 (shift in the curve to the right).

It is recommended that statistical analysis be used to compute control ranges, assay acceptance criteria and performance limits. Cell-based assays are known to inherently show more variability, therefore it is expected that assay development and optimization efforts will focus on establishing assay conditions that will identify the steps most likely to impact the performance of the assay over time and maintain the validated intra-assay and inter-assay precision. In all cases, controls should be monitored to ensure reliable results over time. Often these data are incorporated in the laboratory's LIMS system as part of quality monitoring. These controls can be used to identify short term and long term variations. Alternatively, software programs (e.g. MultiQC, WinSPC, www.dataworks.com) are available to track assay performance by monitoring control data relative to tolerance intervals. These assay QC profiles can provide insights into assay trends and identify data shifts that require further investigation.

## 4. Assay monitoring

A NAb assay method that supports a successful biotherapeutic program may be used for many years, as the biotherapeutic program advances through clinical development, possibly supporting trials in multiple populations and in post-marketing studies. New studies may involve testing the biotherapeutic in new study populations, or with new dosing regimens, or testing the biotherapeutic after modification of the manufacturing process or formulation. Since these types of changes may alter the immunogenicity risk profile, comparison of immunogenicity, including neutralizing antibody activity with historical data may be required. Thus it is important to maintain the assay in a validated state over time and to avoid drift in performance that could confound the data comparison between studies and over time. Variables likely to affect the performance and contribute to drift even for a relatively consistent assay include introduction of a new working cell bank, multiple changes in drug reference standard, critical reagent lots and instruments, incubation conditions, operators and laboratories. Therefore, good practices should be put into place early to support the potential use of the assay over an extended duration. Data acquired during the assay development, validation, and robustness testing, and the monitoring of assay controls over time during routine use and with multiple changes, will further enhance the understanding of the assay capabilities and limitations and its reliability for allowing historical comparisons. For this reason, the use of a software program to monitor control performance over time as mentioned earlier (Section 3.9) can be employed for assuring reliable performance of the assay during its lifecycle.

The laboratory should have defined processes for making changes in the assay. While major changes to the assay procedure (such as changing the cell line or assay endpoint) will require revalidation, most relatively less critical changes (such as the use of new reagent lots) will not. Qualification experiments should be performed before a change is introduced to assure that the change will result in assay performance that is consistent with historical data. The extent of the qualification may be determined based on historical performance of the assay and the expected potential impact of the change on the assay performance. When the change is qualified, the assay should pass acceptance criteria set during the validation process and should perform in a similar manner to historical data. The comparison to historical data is especially important to prevent assay drift If existing assay acceptance criteria cannot be met when the change is introduced, the acceptance criteria may require adjustment, however sufficient documentation will be needed to assure that this will not negatively affect the use of the assay for its intended purpose.

The appropriate design of a qualification experiment will depend upon a number of factors including the variability observed in the assay during validation and study support, degree of experience with the assay, variability that had been historically associated with the specific reagent and assay performance characteristics being measured, and whether the change will affect the assay acceptance criteria. A statistician should be consulted when feasible.

## 5. Conclusions

This paper provides an objective recommendation for best practices to be adopted in validating cell-based NAb assays based on the experience of the authors. The authors however recognize that alternate scientific methodologies and validation approaches may exist and be successfully employed. These recommendations are intended to promote standardization but are not intended for adoption in lieu of published regulatory guidance or direct discussions with the various regulatory agencies.

## Disclosure statement by authors

Shalini Gupta is employed by Amgen Inc. and owns Amgen stock.

Viswanath Devanarayan is employed by Abbott laboratories and owns Abbott stock.

Deborah Finco is employed by Pfizer Inc. and owns Pfizer stock.

George R. Gunn III is employed by Centocor Research and Development Inc. and owns Johnson & Johnson stock.

Susan Kirshner is employed by the Food and Drug Administration and has no actual or potential conflicts of interest.

Susan Richards is employed by Genzyme Corporation and owns Genzyme stock.

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An Song is employed by Genentech, a member of the Roche group and owns Roche stock.

Meena Subramanyam is employed by Biogen Idec and owns stock in the company.

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## Appendix A. Appendix A

### *A.1. NAb assay formats utilizing fixed and variable drug concentrations*

NAb assay formats commonly utilize a fixed concentration of drug and inhibition of the assay signal upon introduction of a test sample indicates the presence of NAbs in that sample. In general the authors recommend the use of this approach. However, in certain situations based on some authors' experience, the NAb assay could be designed to include varying drug concentrations. If the approach of using varying drug concentrations is employed, the presence of NAbs may be indicated by a shift in the EC50 of the drug curve or may be expressed as the amount of drug neutralized per unit volume of the test sample. These approaches are described in further detail below.

#### *A.1.1. Selection of a fixed drug concentration*

A fixed drug concentration is selected during the assay optimization phase and is generally the concentration that provides a reproducible signal above the assay background in the test matrix. Reproducibility may be evaluated by spiking the selected drug concentration into multiple target disease donor sera ($n = 5$) or pooled test species serum for demonstration of the reproducibility of the assay signal (signal/noise ratio). The response in the selected test conditions should meet the assay's pre-set signal to noise and precision estimate. An assessment of the signal-to-background (S/B) ratios over the course of assay optimization provides valuable information about the inter-day, inter-assay variability of the assay and may serve as a system suitability criterion for acceptable assay performance. In most cases, the fixed drug concentration selected should be one that consistently produces a reliable response, in most cases within the linear portion of the dose response curve, with acceptable precision and allows for an assay of acceptable sensitivity.

#### *A.1.2. Use of variable drug concentrations (EC50 shift as index of neutralization)*

In situations where adding a fixed amount of drug is not feasible (generally due to extensive patient to patient variability with a fixed amount of drug) one may consider an approach in which a single dilution of patient serum is combined with five to eight concentrations of drug (Fig. 1). The EC50 is calculated from the resulting concentration response curve. An increase in EC50 (post vs pre-exposure) indicates the presence of neutralizing antibodies. During validation, positive controls are evaluated for their ability to cause shifts in the EC50 and a statistically significant fold change in EC50, usually based on assay variability, can be determined. Due to possible patient-to-patient variability it is recommended that at least ten pre-immune patient serum samples spiked with the positive controls (multiple concentrations) be evaluated on at least three independent runs. Since the intent is to ascertain both biological and analytical variability, it is important to conduct the evaluation using multiple individual pre-immune serum samples and not just a single pool of human serum for this evaluation. Utilizing these data, a confidence interval based on the mean fold change and SD can be established, whereby a certain fold change in EC50 is defined as evidence of neutralizing activity. Due to sample volume requirements, low sample throughput, and the requirement for pre and post exposure sample to be run on the same plate, this method is best suited for qualitative assessment of neutralizing antibodies. When the sample volume is not limiting, a quasi-quantitative assessment could be performed by assessing the drug concentration response in multiple dilutions of the test sample.

#### *A.1.3. Use of fixed drug (change in EC50 as index of neutralization)*

Shifts in EC50 can be used in a variety of approaches for neutralizing bioassays. One method was described above using variable amounts of drug. Alternatively, in their evaluation of NAbs to GM-CSF, Wadhwa et al. [14] utilized EC50 shifts to determine the amount of serum needed to neutralize a fixed amount of drug (same as titer but instead of a cut point, the ED50 was used). The volume of serum required to neutralize the activity of 10 IU of cytokine was calculated using serum ED50 responses obtained by fitting common asymptotes and slope for all sera analyzed. This approach has been used to analyze responses to different GM-CSF preparations/products and may be applicable to other types of biologicals.

#### *A.1.4. Amount of drug neutralized in the assay as an index of neutralization*

The methodology for reporting data as an amount of drug neutralized per mL of serum generally requires a full drug concentration response curve as the standard curve. Samples designated for NAb testing are spiked with a known concentration of drug. Sample data (usually one or two tested dilutions) is interpolated against the drug dilution curve to determine the corresponding drug concentration detected in the sample. This value is then subtracted from the fixed concentration of drug used in the test system and adjusted for the minimal required dilution in order to determine the amount of drug neutralized per mL of serum. For example, if one added 50 ng/mL of drug to a test sample, however only detected 20 ng/mL in the assay (by back-calculation of a drug standard curve fitted to an appropriate mathematical model), they would determine that 30 ng/mL of drug were neutralized. If the sample was tested at a 1/100 dilution then the amount of drug neutralized per mL of patient serum would be 3 μg. This approach has been reported by several investigators [15–17]. When using this method, parallelism of samples being tested relative to the drug dilution curve must be considered. If samples are not parallel, the calculated results may be less accurate. These assays may require more intensive validation since in addition to cut point, stability, sensitivity, recovery and cell line stability, additional assay perfor-

mance characteristics including linearity, accuracy and precision of the drug standard curve, dilutional linearity, sample parallelism, etc. also require validation. The parallelism of the positive control antibody to the drug curve should be evaluated. Ideally, a variety of polyclonal and monoclonal positive controls should be evaluated. Validation approaches used for detection of drug in pharmacokinetic samples [12] may be used as a guide to design validation experiments. Assay precision should be based upon the amount of drug neutralized for a low, medium and high positive control. Typically multiple replicates (at least three) within a run and across at least three independent runs (preferably on different days) should be used to calculate precision. The drug standard curve acceptance criteria should utilize the EC50 and $R^2$ results from multiple runs of the curves over at least 3 days.

## Appendix B. Appendix B

### *B.1. NAb assay sensitivity*

Although a NAb assay is intended to detect all clinically relevant NAbs, it is not feasible to know the level of sensitivity required for achieving this until the assessment of the samples and eventual correlation to clinical sequelae [18]. Therefore, during validation the cell based NAb assay is demonstrated to be adequately sensitive with the use of one or more surrogates (positive controls). However, caution is required in the interpretation of these results because the conditions and environment of the *in vitro* assay systems, such as the use of cell lines over-expressing the target, may not be representative of the conditions in which the therapeutic protein, the NAb, and biological target interact *in vivo*. The ability of neutralizing anti-drug antibodies to influence the therapeutic effect *in vivo* is dependent on the properties of the antibody (affinity, avidity, cross-linking capability), pharmacokinetic and pharmacodynamic characteristics of the therapeutic protein (e.g. clearance, affinity for biological target, effective *in vivo* dose) and characteristics of the biological target (number and accessibility of receptors, sensitivity of signaling pathways, etc.) that may also not be completely understood. These properties are specific for the particular therapeutic protein, study population and disease indication, and therefore a target sensitivity level that would be suitable for all NAb assays cannot be ascertained. Moreover, there may be no direct correlation between the degrees of neutralization measured *in vitro* to that determined *in vivo*. It is important to consider the putative impact of NAbs for a given therapeutic as part of a risk-based approach to determine the level of sensitivity that should be demonstrated using the surrogate positive controls. In reality, the adequacy of the assay should be determined by its use in multiple studies and correlation of the assay results with clinical effects.

From a more practical perspective, evaluation of relative sensitivity during validation may demonstrate that the assay sensitivity will be consistent within a run (e.g. on different plates), over time, between laboratories (if appropriate), and with different sources of samples and cell passages. In addition, it may be desirable to understand the relative sensitivity of orthogonal methods used in immunogenicity testing (e.g. by comparing the sensitivity of the screening immunoassay and the NAb assay by using the same positive control antibody). This type of comparison should generally be conducted with a monoclonal antibody preparation because polyclonal antibody preparations may contain a mixture of neutralizing and non-neutralizing antibodies thereby complicating direct comparison of the sensitivity of the two types of assays.

## Appendix C. Appendix C

### *C.1. Strategies for assessment of NAbs in samples containing drug*

Attempting to detect or measure NAbs in samples from time points when drug is expected to be present is generally not recommended; however there may be some situations in which analysis of NAb activity in samples that are known or expected to contain drug may be necessary on an investigational basis. Therefore several approaches that may be used to conduct investigatory analysis are suggested below.

#### *C.1.1. Drug quantitation based NAb detection*

The drug quantitation-based NAb (DQ-NAb) assay is a recently described approach that is based on the detection of biologically active drug in test samples [19]. This approach is designed to address the acute susceptibility of NAb assays to the presence of circulating drug that hinders NAb detection and utilizes a 2-step approach. The first step includes a quantitative determination of the bioactivity of the circulating drug in immunoassay-positive samples. It is expected that if the sample contains clinically effective neutralizing antibodies, the bioactivity of any circulating drug will be impaired. The 2nd step of the DQ-NAb approach assesses the analytical recovery of a known concentration of drug into the same sample. Any loss or impairment of recovery of the added concentration of drug provides a confirmation that the antibodies detected by the immunoassay are neutralizing in nature. Both assay development and validation approaches have been detailed in the above referenced article [16].

#### *C.1.2. Acid pre-treatment*

If it is suspected that drug present in the sample may be interfering in the assay by binding with NAbs, it may be possible to disrupt and/or separate the drug-antibody complex using pre-treatment with acid to improve detection. Identification of conditions for acid disruption may be evaluated using positive and negative control samples. Treatment of samples with 100–500 mM acetic acid for 0.5–3 h may be evaluated. However conditions for optimal disruption of the complex are likely to be highly dependent on the characteristics (affinity, avidity, concentration) of the antibody positive control being measured; therefore, the selected conditions may not be appropriate for treatment of actual study samples. The caveat to this approach is that acid exposure treatment may decrease the binding of some antibodies and therefore if this method is used in routine analysis, it would be appropriate to analyze actual study samples without acid pre-treatment as well as with acid-pre-treatment. When employing acid-dissociation, the effect of incompletely neutralized (and therefore, acidic) samples on cellular response will have to be considered.

#### *C.1.3. Removal of drug*

In some cases, drug may be physically removed from the sample, especially following acid pretreatment. For low molecular weight drugs, physical separation methods may be possible (e.g. size exclusion columns or filters with the appropriate size separation cutoffs). For larger molecular weight drugs, removal of the drug may be attempted using affinity chromatography or depletion may be used to remove uncomplexed drug, especially after pre-treatment. For example, the sample may be pre-incubated with resin beads covalently coupled to either protein A or the drug plus control beads coupled with irrelevant protein of similar biochemical characteristics. After the pre-incubation period, the samples may be centrifuged to remove the beads and the supernatant tested in the assay. The caveat to this approach is that specific ADA could also be removed by the procedure.

#### *C.1.4. Evaluation of assay drug tolerance*

A NAb assay's ability to detect NAb in the presence of drug depends both on the concentration of interfering drug levels as well as the concentration of the NAbs present in the sample (Table 2). Since measurement of NAbs in the presence of drug is problematic, approaches to minimize drug interference should be considered

in design of study protocols and assay development. A study may be designed to include a wash-out period, or to follow patients for some time after drug treatment has been completed to collect samples for NAb analysis when drug levels are low or absent. However, intermittent dosing of drug may affect the immune response, therefore introduction of wash-out periods just for the purpose of collecting samples for ADA analysis is not recommended. Analysis of the baseline sample and an appropriate end-of-study sample after a drug washout period may provide sufficient evidence that NAb responses did or did not occur during the conduct of the study.

However when NAbs are detected after a drug-washout period, establishing when the onset of the NAb response occurred and the effect on exposure may be difficult. Since a strong immune response may be detected even during the dosing phase, it may be appropriate to collect dosing phase samples as well; these samples may be analyzed if detectable NAbs are expected or the samples may be saved for contingent analysis. For each sample collected for NAb assessment, samples should also be obtained to determine whether drug is present, with the understanding that the pharmacokinetic assay results may also be affected by the presence of ADA.

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