Review

Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics

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Abstract

The administration of biological therapeutics can evoke some level of immune response to the drug product in the receiving subjects. An immune response comprised of neutralizing antibodies can lead to loss of efficacy or potentially more serious clinical sequelae. Therefore, it is important to monitor the immunogenicity of biological therapeutics throughout the drug product development cycle. Immunoassays are typically used to screen for the presence and development of anti-drug product antibodies. However, in-vitro cell-based assays prove extremely useful for the characterization of immunoassay-positive samples to determine if the detected antibodies have neutralizing properties. This document provides scientific recommendations based on the experience of the authors for the development of cell-based assays for the detection of neutralizing antibodies in non-clinical and clinical studies.

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Keywords: Neutralizing antibody bioassay; Cell-based assay; Serum-based bioassay; Immunogenicity assay; NAb assay

Abbreviations: BrdU, bromodeoxyuridine; CAT, chloramphenicol acetyl transferase; CV, coefficient of variance; ECL, electrochemiluminescence; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; EPO, erythropoietin; FACS, flow activated cell sorting; GFP, Green Fluorescent Protein; HSA, human serum albumin; Ig, immunoglobulin; IL-1, interleukin-1; KIRA, kinase induced receptor activation; MAb, monoclonal antibody; MGDF, megakaryocyte growth and development factor; mRNA, messenger ribonucleic acid; MTT, [3-(4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide]; NAb, neutralizing antibody; RIA, radioimmunoassay; PRCA, pure red cell aplasia; rHuEPO, recombinant human EPO; SPA, scintillation proximity assay; TNF, tumor necrosis factor.

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1. Introduction

The development of antibodies to biological therapeutics upon their administration in study subjects is not an uncommon occurrence (Porter, 2000). On a case-by-case basis, the antibody response may be inconsequential or may have a dramatic effect on the efficacy and/or safety of the drug product if the antibodies alter drug pharmacokinetics or are of a neutralizing nature. Neutralizing antibodies (NAbs) block the biological activity of the therapeutic molecule by either binding directly to epitope(s) that lie within the active site of the therapeutic molecule or by blocking its active site by steric hindrance due to binding to epitope(s) that may lie in close proximity to the active site. While, in certain cases NAb presence may not result in a clinical effect, at sufficient NAb levels in other cases, a decrease in efficacy may be observed which may require administration of higher doses of the
drug product in order to achieve similar efficacy. In certain cases non-neutralizing antibodies may also reduce clinical response (Hjelm Skog et al., 2001) therefore it is important to correlate immunogenicity assay data with clinical effects even though the detected antibodies appear to be non-neutralizing in vitro.

Neutralizing antibodies to interferon products, where the endogenous protein exists in multiple forms, have been shown to reduce or abrogate efficacy of the drug product, but have no other apparent adverse effects (Steis et al., 1988). However, NAb formation has a serious outcome when the antibodies not only neutralize the administered drug product but cross-react with and neutralize the biological activity of a homologous, non-redundant, endogenous protein. Examples of this phenomenon include the development of severe thrombocytopenia in healthy volunteers administered with pegylated MGDF that induced a NAb response which cross-reacted with endogenous thrombopoietin (Li et al., 2001) and the recent occurrence of pure red cell aplasia resulting from NAbs against administered erythropoietin (Cassadevall et al., 2002).

Determination of NAb development in non-clinical studies is also useful since NAbs prevent the drug product from being active, making the presence or absence of any toxicological findings difficult to interpret. In situations where immunogenicity of the drug product poses a patient safety risk, evaluation of NAb incidence is useful both for the non-clinical and clinical evaluation of product safety. Therefore, it is important to develop reliable NAb assays of appropriate sensitivity, specificity, and robustness that can detect and distinguish NAbs from non-neutralizing antibodies in non-clinical or clinical samples collected for antibody testing. In certain cases, detected NAbs may require quantitation; however, due to the lack of appropriate reference standards for immunogenicity assays, any effort to quantitate anti-drug product antibodies yields a quasi-quantitative result (Mire-Sluis et al. 2004).

Typically, immunogenicity testing for biological therapeutics uses a tiered assay approach (Swanson, 2003; 2004; Wadhwa et al., 2003). Samples withdrawn prior to dosing (pre dose) and appropriate post-treatment samples (post dose) designated for antibody testing are initially analyzed using an immunoassay for the presence of anti-drug product antibodies. The recommendations for the design and optimization of these immunoassays have been published recently (Mire-Sluis et al., 2004). Samples yielding a positive result in the immunoassay are subsequently tested for anti-drug neutralizing activity using an in-vitro cell-based assay or a non-cell-based competitive ligand binding assay.

The objective of this manuscript is to provide recommendations on the development, optimization, and qualification of cell-based assays for assessing the neutralizing capacity of anti-drug product antibodies by using a fixed concentration of drug product in the NAb assay. A background on NAb assay formats, a risk-based approach to assay design, and the choice of potential assay endpoints are presented. In addition, specific considerations for parameters for assay optimization and qualification are discussed. Since cell-based assays are extremely sensitive to changes in the cell culture medium, introduction of test species serum into the assay may cause interference leading to erroneous results; therefore, a description of matrix interference assay formats is also included. 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 design, optimization, and qualification. Recommendations on the validation parameters of cell-based NAb assays will be described in a future manuscript.  

2. General principles for NAb assay design

2.1. Principle of a cell-based NAb assay

An in-vitro cell-based assay provides the most appropriate biological model for the assessment of anti-drug product neutralizing antibodies since it most closely mimics the mechanism by which neutralizing antibodies may exert their effect in vivo. A cell-based NAb assay can be defined as an in-vitro assay utilizing cultured cells that interact with or respond to the drug either directly or indirectly in a measurable manner in the presence of test species matrix for the detection of anti-drug product neutralizing antibodies.

The detection of NAbs is based on the principle that any sample containing NAbs would reduce or abolish the biological activity associated with a known concentration of drug product used in a cell-based NAb assay.

2.2. Impact of drug's mode of action on NAb assay design

The mechanisms by which most biological therapeutics exert their desired effect in vivo could be broadly classified by their ability to function as agonists or

Note: These recommendations are based on the experience of the authors. They reflect scientific concepts that should assist assay developers form a rationale for the development of their specific assay. They are not intended for use in lieu of published FDA regulations and guidance, or direct discussions with the various regulatory agencies.
antagonists. Biological therapeutics with agonistic properties include cytokines, growth factors, hormones, agonistic monoclonal antibodies, etc. that exert their effect by directly binding to receptors on the target cell surface and inducing a measurable response. Biological therapeutics with antagonistic properties include antagonistic MAbs and soluble receptor based therapeutics that act by blocking a ligand that binds to the target receptor expressed on the surface of responsive cells. In simple terms, agonists induce a response in a direct manner while antagonists influence a cell’s response in an indirect manner. This distinction plays an important role in cell-based NAb assay design and the choice of critical assay components that comprise the NAB assay system. In this paper, cell-based NAb assays for therapeutics with agonistic or antagonistic properties have been described as direct and indirect NAb assays, respectively (Sections 4.1 and 4.2). The critical components of a direct NAb assay include: (a) a drug-responsive cell line, (b) the drug product, (c) a positive control NAb, and (d) test species serum that represents the matrix of the biological samples that require testing. The critical components of an indirect NAb assay include (a) a cell line that responds to the ligand that is blocked by the drug product, (b) ligand, (c) drug product, (d) positive control NAb and (e) test species serum.

2.3. Risk-based approach to cell-based NAb testing

The overall cell-based NAb assay testing scheme requires a risk-based analysis of potential harm to patients should a NAB response occur. Listings of drug product characteristics that contribute towards its immunogenicity resulting in high or low risk to the patient are provided in Table 1. For instance, high-risk products may be defined as those that have a non-redundant endogenous counterpart. For such products, it is recommended that a sensitive NAB assay that closely mimics the biological activity of the endogenous protein be implemented. In addition, it would be necessary to determine whether antibodies to the drug product cross-react with the endogenous protein. If so, then NAB assays for both the drug product and the endogenous protein would require implementation. The ability to perform this assessment is dependent upon the availability of sufficient quantities of the purified native endogenous protein. Alternatively, a recombinant form of the endogenous protein may be used. For low-risk products such as those with no endogenous counterpart (e.g. MAb therapeutics, bacterially derived enzymes), the requirements for NAB assay sensitivity may be more flexible.

2.4. Utility of matrix interference assays in NAb testing

The introduction of test species serum or plasma can have a profound effect on the performance of a cell-based assay. Known or unknown factors present in test matrices could interfere with the performance of cell-based NAb assays by (a) affecting the cells in their basal state or in their ability to respond to the drug, or (b) affecting the potency of the drug in a stimulatory or inhibitory fashion. The impact of test species serum on both these critical NAb assay components could result in a loss of specificity of the NAB assay system. Test matrix originating from different subjects and disease populations could introduce additional interference which may require characterization to discern between a true NAb positive result and non-specific matrix interference. The use of a matrix interference assay in the NAB testing strategy is recommended to assist in differentiating between drug-specific NABs and other non-specific inhibitory factor(s). Details on matrix interference assay formats are provided in Section 4.5.

3. Assay design: considerations and critical components

3.1. Selection of the cell line

A good starting point for choosing the cell line for the NAB assay is often the one used for the biological potency assay. The major advantage of this approach is that optimization of the cell line maintenance, culture conditions, culture media, etc, has already been achieved. However, the adaptability of the potency assay to the presence of test species serum and response to the drug

<table>
<thead>
<tr>
<th>Characteristics of drug products that influence the risk of inducing an immune response</th>
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</thead>
<tbody>
<tr>
<td>Lower risk product characteristics</td>
</tr>
<tr>
<td>No endogenous counterpart</td>
</tr>
<tr>
<td>Redundant endogenous counterpart</td>
</tr>
<tr>
<td>Immunosuppressed patients</td>
</tr>
<tr>
<td>Single dosing</td>
</tr>
<tr>
<td>‘Humanized’ product</td>
</tr>
<tr>
<td>Intravenous administration</td>
</tr>
<tr>
<td>HSA containing</td>
</tr>
<tr>
<td>Highly pure</td>
</tr>
<tr>
<td>No aggregates</td>
</tr>
</tbody>
</table>

*a The risk described here refers to the formation of immunogenic product/HSA adducts. In some cases, the presence of HSA absorbed or prevented interactions of other components with protein products that could have led to an immunogenic potential.
product to yield a sufficiently sensitive NAb assay requires consideration. Ideally, the cell line should yield a functional endpoint upon treatment with drug product. The selected endpoint should represent some aspect of the drug product’s mechanism of action. Optimally, the assay should be simple to perform and the biological endpoint should be tolerant to test species serum and work over a wide range of drug product concentrations. Cell lines transfected with target receptors can also be used and may offer the advantage of higher receptor density and thus enhanced signaling. Both natural and engineered cell lines should be well characterized to ensure responsiveness to the drug product during continuous culture.

Since NAb assays may be needed for non-clinical, clinical, and post-marketing surveillance studies, the selected line (if several are available to choose from) should be able to tolerate serum from different species planned to be used in the drug development program. The specificity of the cell line’s response to the target ligand or drug product in the presence of animal and human serum should be evaluated during assay development to minimize major re-development efforts to support the different types of studies.

The conditions used for routine maintenance of the cell line should be given careful consideration and should be controlled to obtain consistent results. As an example, cell passage number can have a major impact on assay variability. As shown in Fig. 1, a decline in assay precision was observed in the behavior of an assay control as cells with higher passage numbers were used in the assay. It is recommended that a cell bank be established early on in the assay development process to ensure continuous availability of a consistent cell source at the culture age required for optimal assay performance. This requires the establishment of appropriate acceptance criteria for cells to allow their use in assays.

3.2. Selection of the assay endpoint

In general, assay endpoints could utilize (i) early or (ii) late biological responses that are triggered in the cells upon treatment with the drug product or by the drug-inhibitable ligand in NAb assays. Examples of NAb assay endpoints using early biological responses include (i) binding of the drug product to its target on the cell surface, (ii) internalization of the drug product, (iii) phosphorylation of intracellular substrates, (iv) protein trafficking, etc. Assay endpoints based on late biological events detect the ability of NAbs to interfere with drug product-induced downstream events such as cell proliferation, cell death, apoptosis, cell differentiation, or the cellular induction of measurable products such as cytokines or enzymes. Since these assays measure terminal cellular responses, the assay duration may be long and it can take several days to obtain final results. Assay endpoints based on nuclear events such as changes in mRNA expression or reporter gene expression may fall into the early or late category based upon the kinetics of drug product-induced expression of the mRNA or reporter gene. The most commonly employed assay endpoints that may be utilized for NAb assays are shown in Table 2.

3.3. Positive control antibody

The positive control antibody for a NAb assay must neutralize the biological activity of the drug product in vitro. The ideal positive control would be antisera from humans that mount a NAb response to the administered

![Fig. 1. Effect of cell passage number on precision of a positive control included routinely in an assay. The coefficient of variation (%CV) of the neutralizing antibody titers were determined from the weekly analysis of the replicates of an assay positive control (pooled Rhesus antisera) in a Direct NAb assay format. Cells were maintained in routine culture starting with a thaw from a working cell bank at passage 27 and assayed starting at passage 30 through passage 45.](image)
drug product; however, such antisera are unlikely to be available during the assay development phase. Consequently, positive controls are generally antisera obtained from hyper-immunized animals. Polyclonal antibodies can be appropriately purified and spiked back into a suitable matrix at a specific concentration for use as positive controls. If polyclonal NAbS are not available, monoclonal NAbS can be produced and used as a positive control, either individually or as a “cocktail.”

Evaluation of the utility of the positive control antibody used for the screening immunoassay for suitability to act as a NAb assay positive control is

<table>
<thead>
<tr>
<th>Assay endpoint</th>
<th>Assay platform</th>
<th>NAb action</th>
<th>Relevant assay considerations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell surface interactions</td>
<td>Fluorescence activated cell sorting (FACS)</td>
<td>• Interferes with binding of drug to target site on the cell, or</td>
<td>• Requires labeling of drug with a fluorescent tag. Important to determine the impact of labeling of drug on its biological activity</td>
<td>Griffith et al. (1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Affects internalization of drug product into the cell</td>
<td>• Need cell line that naturally expresses or is stably transfected with the target receptor or substrate</td>
<td>(Sadick et al., 1999; Steele-Perkins et al., 1988; Kaplan et al., 1991; Sadick et al., 1997)</td>
</tr>
<tr>
<td>Phosphorylation of intracellular substrates</td>
<td>KIRA, ELISA</td>
<td>• Affects drug-induced phosphorylation of target receptor/substrate, or</td>
<td>• Need phosphorylation site-specific antibodies</td>
<td>(Ashby et al., 2004; Almholt et al., 2004; Moon et al., 2001)</td>
</tr>
<tr>
<td>Intracellular trafficking</td>
<td>Fluorescence imaging</td>
<td>• Affects ligand-induced phosphorylation by blocking drug</td>
<td>• Need cell line engineered with GFP-tagged reporter molecule. GFP fluorescence may be very susceptible to test species serum</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Affects movement of signaling molecules (eg, transcription factors, etc.) from one cellular compartment to another</td>
<td>• Need fluorescently tagged antibodies to the signaling molecule that redistributes within the cell</td>
<td></td>
</tr>
<tr>
<td>Cell proliferation</td>
<td>Radioactive (tritiated thymidine uptake)</td>
<td>• Affects cell proliferation induced by drug, or</td>
<td>• Need cell line that is drug-dependent for growth</td>
<td>(Wei et al., 2004; Mossman, 1983; Byth et al., 2001; Shevach, 1997; Gearing and Bird, 1987; Kitamura et al., 1989)</td>
</tr>
<tr>
<td></td>
<td>Non-radioactive (BrdU/ELISA, MTT, WST, Alam dye, etc)</td>
<td>• Affects ligand-induced cell proliferation by blocking drug, or</td>
<td>• Response to other stimuli useful for developing an alternative stimulus matrix interference assay</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Affects anti-proliferative effect of drug</td>
<td>• Need cell line that is dependent upon ligand for growth</td>
<td></td>
</tr>
<tr>
<td>Protein expression</td>
<td>ELISA, EIA, RIA, ECL, SPA</td>
<td>• Influences protein expression by drug, or</td>
<td>• The protein can be measured in the cell supernatant or in cell lysate using an ELISA</td>
<td>(Musco et al., 1998; Zhu et al., 1999)</td>
</tr>
<tr>
<td>mRNA expression</td>
<td>Branched DNA technology</td>
<td>• Affects mRNA expression of target protein, or</td>
<td>• The mRNA is measured in cell lysate which tends to be viscous and may require optimization to reduce assay variability</td>
<td>Sakamoto et al. (2003)</td>
</tr>
<tr>
<td>Reporter gene expression</td>
<td>Luciferase, β-galactosidase, chloramphenicol transacetylase (CAT)</td>
<td>• Affects reporter gene expression by drug product, or</td>
<td>• Need to construct reporter gene cell line that can respond in presence of test species serum</td>
<td>(Wu et al., 2003; Littlejohn et al., 2003)</td>
</tr>
</tbody>
</table>
recommended provided sufficient quantities are available. All pertinent documentation such as source, purification protocols, concentration (if measurable) should be procured. Since pools of antibodies from the same immunized animal may be different, lot-to-lot qualification is essential. Attempts should be made to determine the stability of the positive control antibody upon long-term storage and consideration may be given to preparing smaller aliquots to avoid repeated freeze/thaw cycles.

4. Assay development: assay formats

As mentioned in Section 2.2, the choice of NAb assay format depends upon the mode of action of the drug. Similarly, the format of any matrix interference assay is also guided by the influence of the drug product on the cellular response. The consideration of whether the sample will be tested in a qualitative or quasi-quantitative mode also determines the choice of assay format that is most appropriate.

4.1. Direct NAb assay

A direct NAb assay format is used for drug products that directly exert a biological effect on the cell (e.g. drug product-induced proliferation). This assay format is composed of the cell line possessing the appropriate receptor, the drug product, and the sample matrix (test species serum) containing NAbs (Table 3). For example, if a drug product induces cell proliferation, this assay is based on the principle that if a sample contains detectable levels of NAb, it would inhibit the response resulting in a lowered assay signal (Kelley et al., 2005). In the direct NAb assay format, there is potential for interactions between the matrix/NAb and the drug product (i.e. drug product binding by antibody), the drug product and the cell line (i.e. drug product/receptor association) and the cell line with the matrix (matrix effects).

Assay controls for direct NAb assays are shown in Table 3. These basic assay controls would allow monitoring of the interactions between the various critical components of the assay. If the drug product causes an increase in signal, tracking the fold-increase in signal obtained with the control that contains cells and drug product proves useful in monitoring cell culture quality and/or drug product activity. The control that contains cells, drug product, and positive control antibody would allow monitoring of the reactivity of the positive control antibody and the assay overall. The control that only contains cells is a good indicator of basal cell activity in the assay matrix. Aberrant values may provide an indication of either changes in the assay matrix that could be having an adverse effect on the cells or that the cell population may have undergone a drift that may affect the assay background and/or the ability of the cells to respond to the drug product.

4.2. Indirect NAb assay

NAb assays for drug products including MAb therapeutics, soluble receptors, receptor antagonists, etc., often fall into the indirect NAb assay format category where the antigen for the therapeutic antibody or the ligand for the soluble receptor is required for the assay. An indirect NAb assay for an anti-cytokine MAb would have to be designed to contain the serum matrix, the cytokine-responsive cell line, the cytokine and the drug product, all of which have the potential to interact with each other (Table 3). If the cytokine induces the expression of a target mRNA in the cell line, the drug product would lower the mRNA expression; however, a sample containing detectable levels of NAb would inhibit the drug thereby allowing the cytokine to induce the target mRNA, resulting in an enhanced assay signal. In certain cases, it may be possible that a NAb to a

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Types of NAb assay formats and relevant assay controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay format</td>
<td>Drug product's mechanism of action</td>
</tr>
<tr>
<td>Direct</td>
<td>Acts directly on the cells to induce a biological response</td>
</tr>
<tr>
<td>Indirect</td>
<td>Acts by interfering or enhancing the binding of a ligand to a receptor on the cell surface</td>
</tr>
</tbody>
</table>

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soluble receptor type drug product may interact with the receptor expressed on the cell surface and produce an agonistic response. An agonistic NAb in this case would function like a ligand and may escape detection in the NAb assay. If agonistic NABs are suspected to be present in the sample, it may be important to consider incubating the cells only with the serum sample to assess its ability to induce the assay signal in the absence of added ligand. Indirect NAb assay formats become even more complex if additional biological components are required, for example the requirement of a secondary analyte to assist in receptor binding of the antigen and/or drug product, or to synergize the cellular response being measured.

Assay controls that could be utilized in indirect NAb assays are shown in Table 3. If the drug’s ability to block the action of a ligand on the cell line results in a lowered signal, tracking the reactivity of the control that contains cells, ligand, and drug product relative to the control that contains only the cells and ligand would provide an indication of drug product and/or ligand activity between assays. As mentioned in the previous section, the control that contains only cells constitutes the assay background and is important to monitor.

4.3. Qualitative NAb assay

In this approach, the sample is tested at a single dilution in the assay, generally at the lowest dilution where the assay matrix interference is minimal, and allows for a higher throughput of sample analysis. This approach is useful for screening samples for neutralizing activity and typically yields a Positive/Negative result and is often used for non-clinical studies. Clinical samples may also be tested using this approach. In a direct NAb assay, the sample is allowed to incubate with the drug product before addition to the cells. For an indirect NAb assay, the sample is allowed to incubate with the drug product followed by incubation with the ligand before addition to the cells. The final concentration of the serum, drug, and ligand (if required) in the assay is equivalent to those determined during assay optimization (Section 5). A sample is considered to have neutralizing activity in the final validated assay if it inhibits drug product activity above or below the assay’s established threshold (Fig. 2).

4.4. Quasi-quantitative NAb assay

Relative quantitation of NAb present in a serum sample can also be determined by performing a dilution series and establishing a titer value. This approach can be useful when one needs to assess changes in NAb over time. Also titer values allow for further characterization of potential impact of antibody responses, such as whether there is a titer threshold above which an association with changes in surrogate markers or clinical parameters could be made. This approach is also useful.
for conducting comparisons of NAb development and incidence between studies. It must be stressed that the antigen–antibody interaction is a dynamic process dependent on the concentration of antibody and drug product. In addition, a test serum sample most likely consists of a mixture of antibodies, including binding antibodies, which can vary in class and affinity. Consequently, similar to immunoassays for antibodies, the quasi-quantitative approach provides for a relative assessment of the amount of NAb present.

Usually, the first step involves an initial dilution of the test serum sample with medium plus appropriate tissue culture supplements, however not containing test species serum. Subsequently a 2-fold or other appropriately selected dilution series is prepared in medium containing pooled test species serum in order to maintain an equivalent serum matrix across the dilutions. Each dilution of the test sample is incubated with a fixed concentration of the drug product (and ligand, if applicable) before addition to the cells. Serum sample without drug spike is also included as a control to determine the background of the sample. If decreased activity is detected in the test sample, its neutralizing activity may ultimately be reported as a titer value, which corresponds to the logarithm or the reciprocal of the last dilution before it tests negative (Fig. 3).

A panel of 20–50 normal human donor (or test species) serum samples should be analyzed at the various dilutions to determine background interference due to serum matrix. The mean +/-1.645 SD can be set as a provisional cut point for each dilution. The assay readout from the patient dilution series is compared to these cut points and the highest dilution that has neutralizing activity before reaching normal serum assay reactivity is considered the titer. Alternatively, if the data is reported as percent neutralization, a general percent inhibition of assay response attributable to normal human serum matrix (based on the panel of donor sera) can be established as the assay threshold using appropriate statistical approaches and the patient dilution series compared to this assay threshold. The highest dilution of the patient’s sample that continues to yield inhibition greater than the assay threshold would be considered the titer.

4.5. Matrix interference assays

A matrix interference assay may prove useful in discriminating a true NAb response from other interfering factors that may be present in the serum sample that could mimic a NAb effect in the absence of NAbs. If it is planned to incorporate a matrix interference assay into the testing strategy, its development should occur in parallel with the primary NAb assay (Fig. 4). The following are four general strategies that could be considered when developing a matrix interference assay. The recommendations for some relevant assay controls for these 4 approaches are included in Table 4. Additional novel strategies and accompanying assay controls for matrix interference assays may also be possible.

4.5.1. Alternative stimulus assay

This type of matrix interference assay may be developed with a cell line that elicits the same response...
as the drug product in the presence of another stimulant (e.g., another cytokine, growth factor, hormone, etc.). This approach may be employed when using pleiotropic cell lines, which respond to multiple cytokines, e.g., TF-1 cells (Kitamura et al., 1989). Therefore, while selecting a cell line during assay development, efforts should be made to gather as much information as possible about the ability of the cell line to yield a

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**Table 4**

Types of matrix interference assays for detection of drug-specific neutralizing antibodies

<table>
<thead>
<tr>
<th>Matrix interference assay type</th>
<th>Matrix interference assay format</th>
<th>NAb assay format compatible with</th>
<th>Drug products applicable to</th>
<th>Assay controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternative stimulus</td>
<td>Effect of sample on response induced by another stimulus that the cell line responds to in the same manner as the drug product</td>
<td>Direct</td>
<td>Cytokines (e.g. EPO), growth factors, agonistic Mabs, soluble receptors, etc.</td>
<td>(i) Cells alone (ii) Cells + alternative stimulus</td>
</tr>
<tr>
<td>Sample-induced response</td>
<td>Effect of sample on cells in the absence of added drug product or ligand</td>
<td>Indirect</td>
<td>All types of biological therapeutics</td>
<td>(i) Cell alone (ii) Positive control antibody spiked into pooled serum and immunodepleted by immunoadsorbent resin (iii) Positive control antibody spiked into pooled serum and treated with unconjugated resin (iv) Cells alone (v) Cells + drug ± ligand (vi) Cells + drug ± ligand + positive control antibody</td>
</tr>
<tr>
<td>Immunodepletion</td>
<td>Treatment of the sample with an immunoabsorbent followed by retest of the unbound fraction in the NAb assay</td>
<td>Direct or indirect</td>
<td>All types of biological therapeutics</td>
<td>(i) Assay controls (ii) Positive control antibody spiked into pooled serum and treated with unconjugated resin</td>
</tr>
<tr>
<td>Immunocompetition</td>
<td>Treatment of the sample with excess drug product followed by retest in the NAb assay</td>
<td>Direct or indirect</td>
<td>All types of biological therapeutics</td>
<td>(i) Cells alone (ii) Cells + drug ± ligand (iii) Cells + drug ± ligand + positive control antibody</td>
</tr>
</tbody>
</table>

*Wei et al. 2004.*
similar response as the drug product when treated with another stimulant. The concentration of the other stimulant used in the matrix interference assay should be carefully selected and is recommended to be such that it yields a similar response to that observed with the selected concentration of the drug product in the NAb assay. A sample containing drug product-specific NAb would be expected only to inhibit the response induced by the drug product and not the response induced by the alternative stimulus. This type of matrix interference assay generally complements direct NAb assays and has been used for the detection of anti-EPO neutralizing antibodies (Wei et al., 2004). However, with NAb assays that use drug product-induced proliferation as an assay endpoint, samples from certain subjects may contain cytostatic agents at sufficiently high levels that may result in an inhibitory response in the alternative stimulus matrix interference assay, giving rise to the possibility of false negative results (Fig. 4). In such a situation, it is advisable to use the immunodepletion matrix interference assay described below (Section 4.5.3) which could help discern if the drug product inhibition observed in the NAb assay is attributable to an antibody moiety or not.

4.5.2. Sample-induced response assay

This approach is generally complementary to indirect NAb assays. In this approach, samples that test inhibitory in the NAb assay may be tested for their effect on the cells in the absence of added ligand and drug. Using the example of a MAb drug product that blocks the action of a cytokine which stimulates the expression of a target mRNA in the cell line, a sample containing detectable levels of NAb would induce an assay signal that corresponds with cytokine-induced mRNA expression. However, if the sample has elevated levels of cytokine due to the disease state of the serum, a false positive result may be obtained in the NAb assay. Testing the sample for its effect on the cell line in the absence of added drug or ligand allows the determination if the response observed in the NAb assay is specific to the drug or not. In cases where there is evidence that the NAb may be agonistic, this matrix interference assay approach may yield false negatives and it would be advisable to use the immunodepletion matrix interference assay approach to confirm the presence of NAbs in the test sample.

4.5.3. Immunodepletion assay

This assay type involves an immunodepletion step in which a sample that tests inhibitory in the NAb assay would require a pre-treatment with a solid phase capable of binding to the heavy and/or light chains of immunoglobulin molecules (e.g., Protein G or Protein L conjugated agarose or sepharose beads). Test samples are tested with or without treatment with conjugated resin in the primary NAb assay. The ability to demonstrate that such a pre-treatment removes neutralizing activity from an inhibitory sample provides verification that the neutralizing activity was due to antibodies. Immunodepletion assays may be useful if the other two types of matrix interference assays described above are not feasible, or yield an unacceptable rate of false positives or negatives due to serum interference in the primary NAb assay.

Careful consideration should be given to the choice of conjugated resin keeping in mind the source of the positive control antibody used for the assay. Protein L possesses better affinity for the human kappa light chain fraction of all human immunoglobulins (Igs) and their isotypes (De Chateau et al., 1993). Protein A and Protein G bind well to all human IgG isotypes but have poor affinity for other Ig subclasses and isotypes (Bjorck and Kronvall, 1984; Hakoda et al., 1994; Soundarajan et al., 2005) and therefore may not remove other classes of immunoglobulins which may be potentially contributing to a specific neutralizing response. Experiments conducted during assay development should demonstrate the effectiveness of the resin for removing the positive control antibody spiked into pooled or individual donor sera.

4.5.4. Immunocompetition assay

Certain NAb assays may be coupled to a matrix interference assay in which the sample is retested in the NAb assay after being spiked with an excess amount of the drug product. If the sample contains drug product-specific NAbs, they will be bound to the exogenously added drug product and unavailable to inhibit the assay endpoint in the NAb assay. For these assays, it is important to establish the amount of drug to be introduced into the sample to block the NAbs in a reliable and reproducible manner. The extent of NAb inhibition necessary to ascertain the true presence of NAbs in a sample would also need to be established.

The key point to consider for this approach is the amount of drug product that is utilized in the NAb assay. If the amount of drug product used in the NAb assay corresponds to the concentration that yields maximum (~ 100%) response in the dose response curve, this matrix interference assay approach may work. However, if the concentration of drug product lies within the lower region of the dose response curve (which is recommended for NAb assays), this approach may give
confounding results, since the addition of exogenous drug in addition to blocking the NAbs may exert its own effect on the cells and/or ligand, thereby yielding data that may be difficult to interpret.

5. Assay optimization

Following development of the prototype assay, it is important to follow a rigorous assay optimization phase to arrive at a final robust assay. To facilitate assay optimization, one might consider employing a factorial design approach. Such a mathematical approach allows one to evaluate assay performance and assay robustness and to optimize assay parameters under diverse conditions with minimal resources. Some considerations that are useful for cell-based assays in general are included in Table 5. Considerations specific for cell-based NAb assays are described below.

5.1. Selection of drug product concentration

5.1.1. Direct NAb assay

The sensitivity of a direct NAb assay depends upon the concentration of drug product that will be used in the assay for neutralization by test samples. As a general rule, the amount of drug product used in the assay should be the lowest possible concentration that elicits \( \geq 50\% \) response and lies on the linear portion of the dose response curve. This may be evaluated by testing the effect of the drug at several concentrations in the assay matrix, preferably, so that both upper and lower plateaus can be observed.

5.1.2. Indirect NAb assay

The sensitivity of an indirect NAb assay depends upon the concentration of ligand that will be used in the assay, which further facilitates the selection of the drug product concentration that will be used for evaluation of neutralization by test samples. Similar to the considerations employed for the selection of drug product concentration in a direct NAb assay, the amount of ligand used in the assay may be the lowest possible concentration that elicits \( \geq 50\% \) response. This may be evaluated by testing the effect of the ligand at several concentrations in the assay matrix. The lowest concentration of the ligand that yields \( \geq 50\% \) of the total response may be a suitable one to select. Next, one should test the ability of the drug product to inhibit the selected ligand concentration in the assay matrix using several, ideally using a concentration range, so that both

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Considerations</th>
<th>Suggestions for optimization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell preparation</td>
<td>• Cell bank</td>
<td>Create a cell bank with cells that have not been subjected to extensive passaging</td>
</tr>
<tr>
<td></td>
<td>• Cell number</td>
<td>Perform a 2-fold serial dilution titer series to determine the cell number that yields (i) low background and (ii) maximal response to drug product</td>
</tr>
<tr>
<td></td>
<td>• Viability</td>
<td>Monitor viability using a microscope and standard cell staining techniques; check performance of cells as they recover from freeze-thaw, select viability state that yields maximal response to drug product</td>
</tr>
<tr>
<td></td>
<td>• Morphology</td>
<td>Perform regular visual inspection of cells, if unexpected change in morphology is noticed it is advisable to initiate a new batch of cultured cells</td>
</tr>
<tr>
<td></td>
<td>• Density</td>
<td>Establish a cell culture density during routine cell maintenance that yields optimum results to drug product</td>
</tr>
<tr>
<td></td>
<td>• Confluence at time of assay</td>
<td>This feature is pertinent to adherent cells that prefer a certain level of confluence to maintain their ability to respond to stimuli. Under-confluence may yield a low response while over-confluence may give high background values in the assay. Establish the confluence level that yields cells which give an expected response to drug product</td>
</tr>
<tr>
<td></td>
<td>• Passage number</td>
<td>Monitor cell response to drug product periodically during the span of cell culture for a particular batch. Establish the range of passage numbers that give optimum results to the drug product</td>
</tr>
<tr>
<td>Washing conditions</td>
<td>• Cell pre-treatment</td>
<td>Evaluate usefulness of synchronizing cell culture (serum-starving) to obtain optimal results</td>
</tr>
<tr>
<td></td>
<td>• Adherent cells</td>
<td>Monitor tightness of cell adherence to the plate if washing is involved. Use gentle washing to avoid any cell loss and to control assay variability</td>
</tr>
<tr>
<td>96-well assay plates</td>
<td>• Non-adherent cells</td>
<td>Use gentle centrifugation to wash</td>
</tr>
<tr>
<td></td>
<td>• Clear</td>
<td>Useful for plating/growing cells in a colorimetric assay</td>
</tr>
<tr>
<td></td>
<td>• Black</td>
<td>Useful for fluorescence-based assays</td>
</tr>
<tr>
<td></td>
<td>• White</td>
<td>Useful for luminescence-based assays</td>
</tr>
<tr>
<td>Assay plate configuration</td>
<td>• Placement of samples and controls</td>
<td>Avoid edge wells, include multiple replicates (( \geq 3 )) of samples and controls to control assay precision</td>
</tr>
</tbody>
</table>
upper and lower plateaus are achieved. The concentration of the drug product that alters ligand function by at least 50% may be a suitable concentration to select.

5.2. Selection of sample matrix dilution

Determination of the appropriate dilution of assay matrix is an important part of NAb assay optimization because this will dictate the minimal test sample dilution and therefore the assay sensitivity. The considerations for defining the minimum dilution used for the antibody screening immunoassay described by Mire-Sluis et al. (2004) may also be applied to cell-based NAb assays. The effect of sample matrix on the ability of the cells to respond to drug product or ligand should be evaluated at multiple dilutions preferably by different pools of the test species serum. The dilution of sample matrix that has a minimal effect on the cellular response should be selected and further evaluated using the positive control antibody spiked into sample matrix. A NAb assay should be able to detect the antibody and to differentiate it in the presence of assay matrix components that may be expected to be present. These might include complement, coagulation factors, soluble receptors, lipids, concomitant medications, the endogenous homologous counterpart, as well as the administered drug product.

Factors such as soluble receptors, that either bind the drug product, or act directly on the cells, can interfere in the assay, leading to false positive results. Alternatively, certain factors may alter the response of the cells in such a manner that may mask the presence of a neutralizing antibody in the sample. Matrix interference assays can help to determine the presence of such interfering substances (Section 4.5). Assessment of matrix effects during assay development can be accomplished using pooled sera. Ideally, the assay matrix should be defined using multiple individual sera due to heterogeneity of the various possible interfering substances. Diseased state sera may contain additional factors that may be encountered during analysis of study samples and whenever possible should be evaluated.

If lipemic, hemolyzed, incompletely clotted and preferably disease state sera from naïve subjects are available they should be screened in the assay both with and without the addition of antibody. If the unspiked sera give a response in the assay and/or the sera interferes in the detection of the antibody, the analytical procedure should state that samples compromised in such a manner may not yield reliable results and may have to be excluded from testing.

5.3. Assay response range

The observed signal to background ratio of the cellular response to the drug product and ligand in direct and indirect NAb assays, respectively, and the variability associated with the ratio, can provide an early indication of the assay’s performance. Assays demonstrating \( \leq 5 \)-fold change in signal upon treatment with the selected drug product or ligand concentration in pooled serum might not give the range of assay response necessary to observe an inhibitory effect due to NAbs. The evaluation of the \( Z' \) factor for the drug product/ligand-induced cellular response may be useful for assessing assay performance quality. The \( Z' \) factor is a coefficient that takes into account the dynamic range of the assay signal and the data variation associated with the signal measurements (Zhang et al., 1999).

5.4. Assay robustness

The robustness of the NAb assay should be gauged during assay development. The variability of the drug product dose response curve, its range or shape from day to day and/or the behavior of assay controls under minor deliberate changes in certain assay conditions (e.g. incubation time) may provide some early information on developing strategies for the final assay format.

5.5. Assay incubation conditions

For NAb assays it is important that the positive control antibody or the sample be allowed to incubate with the drug product for an established period of time at an appropriate temperature to allow binding to occur, before addition of the mixture to the cells. A range of incubation times and temperature may be studied to determine the conditions that favor these binding events as well as those that are optimum for maximal cell response to the drug product or ligand. In general, an incubation of 30 min to an hour at room temperature or 37 °C is usually sufficient to allow drug–antibody binding to occur; however, this would need to be evaluated for each assay.

6. Assay qualification

Assay development and optimization may be followed or integrated with a qualification phase that allows a better understanding of assay performance and determines the ability of the assay to stand the test of validation. The experiments performed during this qualification phase allow the emergence of the
acceptance criteria to be included in the validation protocol. The information gathered during the assay qualification phase can be extremely useful for drafting the NAb analytical procedure that will ultimately undergo validation. The types of experiments that can be performed during this phase are described below.

6.1. Characteristics of the drug product standard curve

The primary NAb assay format relies on the ability of the cells to respond to the drug product. Hence the drug product dose response relationship should be well characterized in the selected assay matrix. The mathematical fit that best describes the dose response relationship may be explored. Usually a 4-parameter logistic fit aptly describes the dose response relationship for cell-based assays. A 5-parameter logistic fit generally provides a better fit for dose–response curves with asymmetrical asymptotes and/or inconsistent variances across the dose range. An estimate of the asymptotes, slope, linear region and ED50 functions may be obtained using the selected mathematical model. The estimates for accuracy and precision of the individual drug product concentrations that comprise the standard curve standards may be derived. The estimates obtained for the drug product concentration selected for routine use in the NAb assay (control consisting of cells + drug product ± ligand; Table 3) allows the setting of the desirable acceptance criteria for this control in routine use.

6.2. Characteristics of the positive control antibody curve

The positive control antibody should be titrated to characterize the drug product inhibitory profile of the antibody. A dose-dependent inhibition of the selected drug product concentration should be evident. The positive control antibody curves should be prepared in neat serum at selected concentrations and tested for their ability to inhibit the drug product at the selected sample matrix concentration. The repeatability of the positive control antibody curves could be assessed in pooled and/or individual donor sera by determining the (i) ED50, (ii) ED90, etc. Precision estimates should be made on the critical portions of the curves depending on the assay format. Separately prepared quality control samples consisting of antibody concentrations that are not included in the titration curve may be included. The ED50 provides an estimate of the concentration of antibody required to cause a 50% inhibition of the selected drug product in pooled serum and may provide an early indication of assay sensitivity in that particular assay matrix. The precision of the various antibody concentrations is usually a useful indicator of assay performance in the assay matrix.

6.3. Assay cut points for the NAb assay

A NAb assay cut point may be defined as the level of response of the assay above or below which a sample is defined to be negative or positive for neutralizing activity towards the drug product. Establishment of a statistically derived assay cut point for the NAb assay allows the emergence of a criterion to be used for terming samples “positive” or “negative” for the presence of neutralizing activity.

6.3.1. Blank donor sera

A limited number of drug naïve individual healthy donor or target untreated diseased state sera (n=10 to 20, if possible) should be screened in the assay to establish a preliminary threshold response value that serves as the assay cut point. The cut point value may be obtained using appropriate statistical approaches: parametric, non-parametric, or a robust alternative. It is important to point out that the assay cut point for cell-based NAb assays must take into account the effect of the expected NAb on the assay endpoint. If the drug product were a cytokine that induces proliferation, a NAb would result in reduced proliferation or reduced assay signal in the NAb assay. For such an assay, using parametric or non-parametric approaches, lower bound of a one-sided 95% prediction interval may serve as the assay cut point for the tested population. If the drug product were a MAb therapeutic that blocks ligand-induced response, a NAb would result in increased response or an enhanced assay signal. For such an assay, using parametric or non-parametric approaches, the upper bound of a one-sided 95% prediction interval may serve as the assay cut point for the tested population. An outlier test may be performed to remove aberrant values to ensure that the assay cut point does not yield a high number of false positives or false negatives.

The screening of multiple donor sera in the NAb assay in this manner also allows an early assessment of the robustness of the assay response in the presence of sera from different sources, thereby qualifying the appropriateness of the selected serum matrix. If high variability of response is observed within the tested donors, it is recommended to evaluate a more dilute assay matrix, within limits so that assay sensitivity is not compromised.
6.3.2. Ratio of antibody spiked and blank donor sera (post to pre ratio)

If the assay cut point developed with blank donor sera shows too much inter-donor variability that cannot be overcome with further dilution of the assay matrix, an alternative approach might be acceptable. The concentration of positive control antibody that shows a consistent 1.5 to 2 fold inhibition of the drug product in the experiments conducted to qualify the antibody (Section 6.2) may be spiked into 10 to 20 individual donor sera. These spiked sera (surrogate post doses) may be tested in the NAb assay along with their unspiked counterparts (surrogate pre doses) and the results obtained may be used to compute post/pre ratios for this donor population. The lower bound of a one-sided 95% prediction interval for these post/pre ratios may be used as the assay cut point. If false negatives are still obtained, the lowest post/pre ratio obtained from the tested population may be used as the assay cut point. The underlying goal should be to obtain an assay that has the lowest possible rate of false negatives. For high-risk products, false negatives should not be acceptable and if the assay continues to yield false negatives, efforts should be made to evaluate the critical components of the assay and if needed a new assay with a different cell line may need to be designed.

6.4. Assay cut point for the matrix interference assay

The matrix interference assay cut point may be defined as the level of response of the assay above or below which a sample may be deemed to have non-specific activity that could artificially mimic a NAb response.

6.4.1. Alternative stimulus assay

The ability of the cells to respond to another stimulus that yields a similar response to that obtained by treating the cells with the drug product should be well characterized in the selected assay matrix. Similar to the primary NAb assay where the drug product concentration is held constant, the concentration of the other stimulant should be held constant in the matrix interference assay format. For the assay cut point, 10 to 20 blank donor sera may be tested in the assay with the selected concentration of the other stimulant to derive the mean response level of the tested population. This approach to assessing if any matrix interference has occurred complements direct NAb assays used for cytokine and growth factor-based drug products that (i) generally require factor-dependent cell lines (Table 4), and (ii) the presence of a NAb is associated with a drop in the assay signal. Therefore, using parametric or non-parametric approaches described in Section 6.3.1, the lower bound of a one-sided 95% prediction interval, may serve as the matrix interference assay cut point for the tested population. If a sample yields an assay response that lies below the matrix interference assay cut point it would be considered inhibitory to the other stimulant, thereby indicating that the sample contains non-specific inhibitory activity (Fig. 4). A sample that contains drug-specific NAbs and is relatively free of non-specific serum factors that could affect the assay endpoint, would be expected to yield an assay response value that lies above the matrix interference assay cut point.

6.4.2. Sample-induced response

A limited number of blank naive individual healthy donor or target naive diseased state sera (n = 10 to 20, if possible) should be screened with the cells, in the assay matrix. The addition of drug or ligand is not required in this approach. Similar to the approach used for deriving the NAb assay cut point with blank donor sera (Section 6.3.1) the matrix interference assay cut point value for this type may be obtained using appropriate statistical approaches: parametric, non-parametric, or a robust alternative. Since this matrix interference assay is more suited for indirect NAb assays in which the presence of NAb correlates with an enhanced assay signal, using parametric or non-parametric approaches as described in Section 6.3.1, the upper bound of a one-sided 95% prediction interval may serve as the assay cut point for the tested population. If a sample contains non-specific activity that mimics a NAb response in the assay, it would yield a response value that lies above the matrix interference assay cut point. However, if a sample contains drug-specific NAb and lacks any confounding non-specific factors, it should yield a response value that lies below the matrix interference assay cut point (Fig. 4). However, as mentioned earlier, agonistic NAbs elicited to soluble receptor therapeutics may yield false negative results in these circumstances.

It has been observed with drug products that require indirect NAb assays (e.g., IL-1- or TNF-blocking therapies), occasionally the naive untreated disease state serum may have high levels of ligand (IL-1 or TNF, respectively) that may yield high response values in the corresponding matrix interference assay where the sample is incubated with the cell (sample-induced response matrix interference assay, Table 4). In such a situation, high inter-donor variability may be observed while deriving the assay cut point. An alternative approach that compares the behavior of the sample in
the NAb and the matrix interference assays could be adopted. This can be accomplished by deriving ratios of the response obtained in the NAb assay and the matrix interference assay (NAb/Matrix interference assay). However, this approach requires parallel testing of the samples in the NAb and matrix interference assays. Statistical approaches similar to those used to derive the NAb assay cut point described in Section 6.3.1 with blank donor serum may be utilized to obtain the NAb/Matrix interference assay cut point. Typically, if a sample contains clinically relevant levels of NAb, the response obtained in the NAb assay should exceed the response observed in the interference assay.

6.5. Assay sensitivity

Upon establishment of the assay cut point, the positive control antibody curves prepared in pooled or disease state (if available) neat serum and tested at the sample matrix concentration should be examined to identify the lowest antibody concentration that yields a positive result. This concentration serves as the initial assessment of the assay’s sensitivity. An assessment of the assay sensitivity should also be made in pooled and individual naïve donor serum.

The reliability of the assay to detect the concentration of the positive control antibody established above at the assay’s level of sensitivity should be assessed. This may be accomplished by spiking that particular concentration of the antibody into a limited number of individual donor sera. If, due to serum interference, all spiked samples do not test positive, attempts should be made to determine the concentration of antibody that consistently tests positive in the assay. This concentration can serve as a more definitive assessment of the assay’s sensitivity.

6.6. Assay standardization

Since reference material or standards are not readily available for most immunogenicity assays, it is very difficult to compare antibody results among laboratories. Assay formats can vary and may significantly differ in sensitivity, specificity and precision from one lab to the next. This is particularly relevant for cell-based assays where different cell lines, passage numbers, and serum matrixes can influence assay results. Proficiency testing or Alternative Assessment procedures, as proposed by the Clinical and Laboratory Standards Institute (www.nccls.org), can be considered as an important part of quality management. Split-sample procedures can be implemented to obtain comparative results. This approach calls for the same samples to be analyzed by participating laboratories to evaluate inter-laboratory agreement and testing errors.

7. Conclusions

Cell-based NAb assays provide a valuable tool for the detection of anti-drug product neutralizing antibodies elicited to biological therapeutics. These assays tend to be complex; therefore this manuscript focused on providing information that would aid the assay developer during assay development, optimization, and qualification. The key activities that may be undertaken during these 3 phases have been summarized in Table 6. Assay validation ensues upon the completion of assay qualification. Some validation parameters that have
been discussed among the authors while working on this manuscript are included in Table 6.

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References


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