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*Review Article*

# Recommendations for the Development of Cell-Based Anti-Viral Vector Neutralizing Antibody Assays

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***Abstract.*** Viral vector–based gene therapies (GTx) have received significant attention in the recent years and the number of ongoing GTx clinical trials is increasing. A platform of choice for many of these studies is adeno-associated virus (AAV). All humans may be exposed to natural AAV infections and could mount an immune response against the virus. Consequently, there can be a high prevalence of pre-existing anti-AAV immunity. This presents a potential limitation for AAV-based GTx due to the potential for AAV-specific antibodies to reduce the efficacy of the GTx. Therefore, appropriate assessment of potential subjects enrolled in these studies should include evaluation for the presence and degree of anti-AAV immunity, including anti-AAV neutralizing antibodies (NAb). Recommendations for the development and validation of cell-based anti-AAV NAb detection methods, including considerations related to selection of appropriate cell line, surrogate vector/reporter gene, assay matrix and controls, and methodologies for calculating assay cut-point are discussed herein. General recommendations for the key assay validation parameters are provided as well as considerations for the development of NAb diagnostic tests. This manuscript is produced by a group of scientists involved in GTx therapeutic development representing various companies. It is our intent to provide recommendations and guidance to industrial and academic laboratories working on viral vector based GTx modalities with the goal of achieving a more consistent approach to anti-AAV NAb assessment.

**KEY WORDS:** Neutralizing Antibody; Viral vector Gene Therapy; Anti-Capsid Immune Response; Cell-Based Assay.

## INTRODUCTION

As adeno-associated virus (AAV)–based gene therapies (GTx) continue to move into clinical development at an ever-increasing rate, understanding patient-specific responses impacting efficacy will become of even greater importance to ensure successful clinical outcomes in diverse patient populations. One potential limitation for AAV-GTx is the presence of pre-existing anti-AAV antibodies. Pre-existing anti-AAV antibodies commonly arise as a consequence of natural, non-pathogenic, AAV infection. The seroprevalence of anti-AAV antibodies varies between the different AAV serotypes, with geographical region, and with the age of the individual. Additionally, the titer of anti-AAV antibodies will vary over time within an individual. Further, it is expected that patients without detectable, pre-existing anti-AAV antibody will seroconvert to being antibody positive following administration of AAV GTx vectors. Treatment-induced antibody responses may preclude retreatment of patients using the same GTx and this may present a significant constraint for GTxs which experience a decrease of product activity over time. In non-human primate (NHP) studies, treatment-induced antibodies are demonstrated to be significantly elevated compared with pre-existing antibody levels that presumably result from environmental exposure [1]. Notably, mice that were passively immunized by administering human serum containing anti-AAV antibodies mounted lower levels of anti-AAV neutralizing antibodies post-AAV administration compared with control animals. The mechanism for this different response is unknown,

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although it is reasonable to speculate that this is due to a difference in the clearance of AAVs in the presence of pre-existing anti-AAV antibodies [2, 3]. It is also important to note that other factors in the blood may hinder vector transduction efficiency. These non-antibody-based inhibitors of transduction have not been fully characterized and may include shed AAV receptors, small molecules, or innate immune mechanisms [4–6].

Regardless of the nature of the inhibitory factors, they may operate in one or more ways to prevent vector uptake into target tissue, inhibition of endosomal escape, interference with nuclear trafficking, or otherwise inhibit capsid processing and transfer of genetic material to the target cell nucleus [7]. Figure 1 describes the steps involved in viral vector transduction and areas where vector-specific antibody or other factors may inhibit AAV gene transfer [4, 8–20]. Even though the mechanisms for the inhibition of vector uptake and transgene expression may be diverse, these factors are collectively described as neutralizing. This article focuses on cell-based assays designed to detect anti-AAV antibodies that may neutralize the efficacy of AAV-based GTx. In addition to antibody-based immunity, a possibility of pre-existing and treatment-induced cellular immune response against GTx viral vector has been reported [21–23] and is commonly assessed by Enzyme-Linked ImmunoSpot analytical platform [23]. The potential impact of viral vector cellular immune response on GTx efficacy and safety at this time is fully understood and will be outside of the scope for this review manuscript.

Multiple assay methodologies have been developed to measure AAV gene transfer inhibitory factors. Prior publications variously refer to the readout for these assays as total antibody titers (TAb), neutralizing antibodies (NAb), neutralizing titers, neutralizing factors, or inhibitors of transduction [24–30]. The NAbs are a sub-class of the total antibodies, along with non-neutralizing antibodies. The diversity of assays (including assay methodologies, sensitivities, and titer calculations) limits the utility of comparing results across studies.

It is generally understood that pre-existing anti-AAV antibodies have a negative impact on the efficacy of viral vector GTx; however, the influence of pre-existing antibodies on the outcome of AAV gene transfer has not been fully elucidated, and no clear or consistent relationship between antibody titers, neutralizing or total, and the impact on efficacy has been demonstrated. In fact, various methodologies for AAV GTx treatment enrollment are currently applied by sponsors in ongoing clinical trials where some are based on the presence of anti-AAV NAb, some based on the presence of total anti-AAV antibody (TAb), and some do not apply either NAB or TAb. For example, neutralizing titers as low as 1:1 have been described as having an inhibitory impact on AAV vector transduction [31]. In some cases, low titers have resulted in decreased therapeutic efficacy when compared with patients with no evidence of pre-existing NAbs [21], and titers as low as 1:5 have completely blocked liver transduction [32]. However, not all studies in published literature indicate that low antibody titers have a negative impact on efficacy. A recent study in NHP has reported a threshold of an anti-AAV9 Ab titer (detected in a TAb assay) of 1:400 above which a diminishing transduction of an AAV9 GTx was observed [33]. The concern that the presence of pre-existing antibodies may negatively impact the safety and efficacy of AAV GTx has led to the use of anti-AAV antibody titer as an exclusion criterion in clinical trials and for therapeutic use [34]. However, the suitability of using an anti-AAV Ab titer is unclear. In clinical trials of an anti-AAV5-hFIX GTx, it has been noted that there was no impact of titers of pre-existing anti-AAV5 antibodies (detected in a NAb assay) that were < 1:340 (the highest titer observed) on the treatment efficacy, which has led to the inclusion of patients in subsequent studies regardless of their pre-existing anti-AAV5 antibody status or titer [35]. Therefore, published evidence points to contradictory results for the impact of anti-AAV antibodies on the efficacy of AAV GTx. Whether this is due to the different viral vectors used, different patient populations, source of animals used, the route of administration, differences in the assays used, or how the titers are calculated and reported is unknown.

Cell-based assays have been developed to detect antibodies capable of neutralizing vector transduction [28]. These assays are commonly used to identify animals (usually NHP) or patients with no, or low, titers of pre-existing neutralizing antibodies in order to maximize the possibility of cellular transduction. This article describes the design and conduct of cell-based neutralizing antibody (NAb) assays used for the development of AAV-based GTx, although some of the principles will apply to other viral vectors such as Adenovirus (Ad), herpes simplex virus (HSV), and Lentivirus. In addition, the important factors that should be taken into account when developing and validating cell-based anti-AAV NAb assays will be summarized. The use of these assays applies equally to the assessment of pre-existing and treatment-emergent antibodies.

Assessing the different methodologies and understanding assay variability is an important first step in developing a validated antibody test method. Ideally, it would be beneficial to be able to standardize the format and conduct of NAb assays in order to compare the data generated across different AAV-based GTx of the same serotype. There are several factors that currently would preclude generating a standard assay that could be used for all AAV GTx of the same serotype. These factors include, but are not limited to:

- Lack of true reference standard materials
- Lack of a standardized cell line and/or other critical assay components
- Differences in sample matrix, sample collection, processing, and storage
- Lack of a harmonized analytical procedure

## CURRENT ANTI-AAV NEUTRALIZING ANTIBODY ASSAY METHODOLOGIES

A number of different methods have been developed for the detection of neutralizing anti-AAV antibodies and their impact on the efficacy of the AAV GTx. The majority of the published NAb methods describe *in vitro* cell-based methods where the level of transduction in animal [32] or human [24, 27–29, 36, 37] cells is measured by the expression of a reporter gene carried by an AAV vector. The most common reporter genes used in cell-based NAb assays are, from least to most sensitive, green fluorescent protein (GFP), LacZ, and luciferase [29, 38, 39]. Alternatively, the AAV GTx itself can be used in the neutralization assay, with the endpoint being expression of the transgene mRNA or protein (i.e., in a similar or identical manner to an activity assay used in AAV GTx release testing). The ability of the assay to detect low titer NAb may depend on the specific characteristics of the reporter gene used in the assay [40]. Although NAb assays may be fundamentally similar to drug product release tests designed to

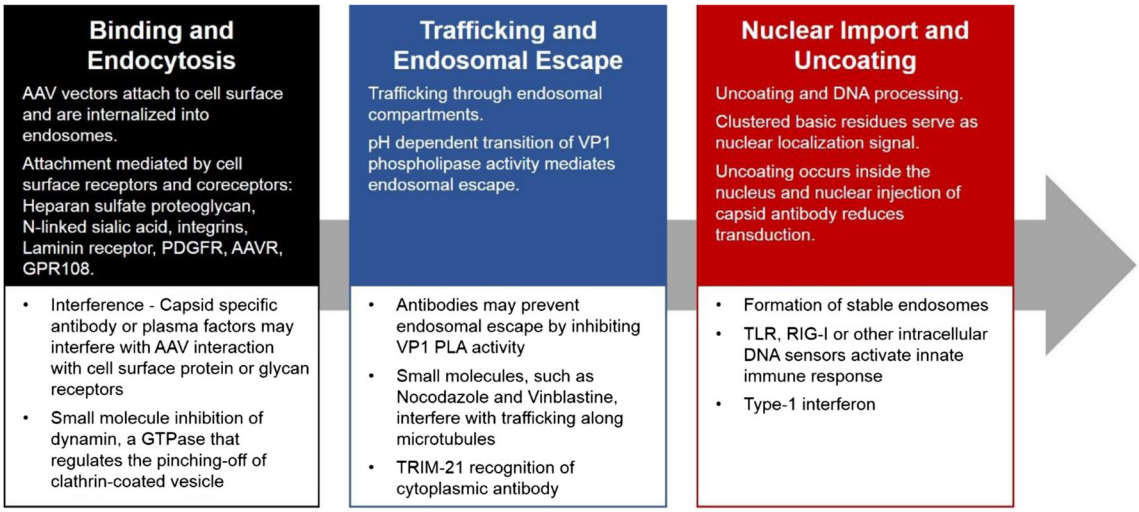


**Fig. 1.** Steps involved in viral vector transduction and areas where vector specific antibody or other factors may inhibit transgene expression [4, 8–20]

evaluate AAV GTx potency, the latter are typically not based on the use of a reporter gene. Recently, alternative NAb methods have been developed that do not rely on the measurement of transgene expression. These methods generally assess the binding of AAV vectors to the target cells and do not evaluate all the steps involved in the vector transduction that could potentially be affected by the presence of anti-AAV-specific NAbs [38] or their uptake by cells [2].

Cell-based NAb assays are low throughput and lengthy (i.e., taking 2 or more days). In the example described by Kruzik *et al.* [41], the NAb assay involves several steps, including seeding of cells and, after an appropriate incubation period, infection with a helper adenovirus 5 in diluted serum samples pre-incubated with AAV luciferase reporter construct. The ability of the NAb that may be present in samples to inhibit viral vector construct's ability to infect cells is then assessed by measuring the activity of the expressed luciferase reporter protein.

Of special interest are the approaches used to determine the levels of NAbs. Many labs determine the level of NAbs present in a sample by serially diluting the sample and plotting the fold dilution against the corresponding level of the reporter gene expression. Using the resulting curves, the "50% inhibition titers" are determined based on the highest sample dilution achieving the 50% inhibition of the viral vector transduction in comparison with the negative control (NC) sample and are considered to be a measure of the NAb activity [27, 29]. This approach is similar to that taken to assess the potency of an antibody for its target (i.e., the calculation of the $IC_{50}$). The calculation of 50% inhibition may not reflect a biologically relevant patient response, but is used to ensure an accurate determination of the values by measuring the response in the linear phase of the sigmoidal concentration effect relationship where small changes in concentration may have a large impact on the $IC_{50}$ readout. Although a biologically relevant patient response may occur at 90% inhibition, it is not possible to determine accurately the concentration that causes this level of inhibition due to the relatively small change in response that occurs over a relatively large range of concentration. Additionally, assay variability limits the ability of an assay to measure accurately the response at low and high levels of inhibition. The approach of determining NAb titer based on the 50% inhibition threshold is well established, relatively simple and convenient, and broadly applied. This approach also has several drawbacks and, ultimately, clinical (or animal) data correlating NAb titers and efficacy are required in order to be able to properly interpret the data generated from the *in vitro* NAb assay [21, 42]. Alternative approaches to assess NAb presence based on statistically defined cut-point are discussed later in the manuscript [28, 29].

Another point to note is that these approaches are described as measuring NAb present in a sample. However, without the use of a step that confirms the neutralization is specifically due to the presence of an antibody, the data from such assays should be reported as "neutralizing activity" rather than being attributed to NAbs. Although this alternative definition can be regarded as being pedantic, it is important to note this distinction in order to avoid confusion as to what the assay specifically measures. The immunoglobulin nature of observed inhibition activity may be confirmed by testing a sample after IgG/IgM components of the assay matrix are removed. This approach will remove both neutralizing and non-neutralizing antibodies. Since, non-neutralizing anti-AAV antibodies have shown to increase the transduction efficiency of some AAV-based GTx [32], the value of data generated after the removal of anti-AAV antibodies is questionable. From a patient perspective, what is important is the extent of neutralization that would prevent clinical benefit. From a drug development perspective, what is important is whether transduction inhibition is due to the presence of neutralization antibodies or other factors and how these can be mitigated to allow effective treatment.

## CONSIDERATIONS FOR ASSAY DESIGN

### Viral Reporter Vectors

The AAV vector used to assess the impact of neutralizing factors on transduction is a critical aspect of the assay design. As mentioned previously, the majority of the cell-based NAb assays use a surrogate viral vector to assess the impact of neutralizing

factors on the transduction of the viral delivery-based GTx. These surrogates use the same AAV serotype as that used for the GTx treatment. However, instead of carrying the therapeutic transgene, the surrogate vector contains a transgene cassette for the expression of an enzyme or other type of a reporter gene, including LacZ, luciferase, and GFP. The luciferase-based reporter gene constructs are recommended as these are easy to use and are highly sensitive [43]. The applicability of the surrogate vector should be established experimentally by showing that addition of the GTx of interest reduces the expression of the reporter gene by competing for the same uptake mechanism(s). The results of such experiments should be documented in the method performance report.

The amount of reporter GTx viral vector spiked into the sample relative to the number of cells used in the test (viral vector particle per cell) is one of the critical factors defining NAb assay sensitivity. To provide the desired assay sensitivity, the optimal amount of viral vector used should be determined based upon the presumed amount of detectable anti-AAV antibodies that will be present in the samples, a knowledge of the transduction efficiency of the viral reporter GTx vector, and a knowledge of the relationship of extent of transgene protein expression and signal response in the detection assay.

## Cell Line

A variety of cell types have been used for cell-based NAb assays used in support of GTx therapeutics. These include HEK293, HeLa, and HuH7 cell lines with HEK293 being the most widespread. Often, the cell type chosen for a NAb assay is the same as that used for the potency assay as part of the Drug Product batch release testing.

Here, it is proposed that when possible, HEK293 cells should be used assuming that the AAV serotype used for the GTx can adequately and reproducibly transduce this cell line. The ability of both the GTx viral vector and the reporter viral vector to transduce the cell line should be demonstrated experimentally and documented in the method development or validation report. Better understanding of the mechanism of the AAV-based GTx viral vector cellular uptake could lead to a selection of a cell line to be used in an assay that is more efficient at monitoring the efficiency of the viral vector transduction which, in turn, could result in a more sensitive and overall more appropriate NAb assay.

In addition to the cell type, other critical methodological details need to be considered. These include cell density per well, cell passage number, duration of incubation with the viral vector and tested sample, viability and confluence level of the cells used in the test, and a need for any cell line pre-treatment, e.g., with chemical such as etoposide. The impact of these variables on the performance of an assay is unknown. Consequently, best practices cannot be recommended at this point in time. However, the bioanalyst should, wherever possible, assess the impact of these factors on the performance of the assay and document whether they have an impact or not in the method performance report.

## Assay Controls

Similar to other immunogenicity assessment assays, methods designed to detect anti-GTx NAb activity require suitability controls, including positive (PC) and negative (NC) controls. Both polyclonal and monoclonal anti-viral vector-specific PC reagents have been used [28]. A selection of non-human commercial antibodies developed against a particular viral serotype is available, including AAV5, AAV8, or AAV9. These reagents tend to be polyclonal. The use of commercially available PC reagents is generally regarded as acceptable although the use of proprietary PC reagents may be needed, particularly when working with non-natural (i.e., engineered) vector serotypes. When using a PC reagent, regardless of whether it is commercially available or proprietary, it is critical to demonstrate the ability of the reagent to effectively neutralize the viral vector of interest. From a practical point of view, a monoclonal reagent that inhibits the viral vector from binding to a cell surface protein, being internalized, and subsequently transfecting the cell, may be preferred although utility of a monoclonal PC can be limited if multiple mechanisms of cellular uptake of the viral vector into the cell are possible. Polyclonal PCs also have limitations as these will contain a mixture of neutralizing and non-neutralizing anti-viral vector antibodies. Changes in the relative amounts of neutralizing and non-neutralizing antibodies may change the overall transduction efficiency and hence make it challenging to generate an acceptable positive control.

Due to the presence of pre-existing anti-AAV-antibodies, it is unlikely that a commercial pooled serum would be feasible for the preparation of a NC reagent. The NC reagent is commonly produced by pooling individual sera from several (e.g., 20) NAb-negative donors. Alternatively, individual samples may be screened for the presence of AAV-specific TAb. Consequently, the preparation of the assay NC pool will require the collection of a large amount of serum samples from many subjects. Alternatively, the NC reagent could be generated by depleting sera of the anti-AAV antibodies by passing the serum over a column or incubating the serum with magnetic beads to which the relevant AAV serotype is immobilized. However, it should be noted that this approach has the potential to generate a NC reagent that may not be representative of a true biological matrix as it is artificially depleted of any material that can bind to the AAV viral vector, including any binding antibody as well as other unknown factors resulting in an increased risk that all test samples would be reported as positive for the anti-AAV antibodies. The presence of AAVs in the pooled sera may also have an impact on the response in the NAb assay by either binding to the anti-AAV antibodies or by competing with the viral vector containing the reporter gene for uptake into the cell.

In order to monitor assay performance, each assay run is expected to include NC and PC samples where PC is commonly tested at low (LPC), and high (HPC) concentrations. The use of a mid-concentration positive control is optional. Alternatively, PC sample may be diluted as part of the titration test of screen-positive samples.

The PC simply functions to show that the assay is performing acceptably and is not used to quantitate the level of NAb that is present in an unknown sample; serial dilution of the PC may not be required.

## Assay Matrix

The most readily collected assay matrices are serum and plasma. Consequently, these matrices have been used for the

determination of the presence of anti-AAV antibodies. While relevant for the determination of the presence or absence of pre-existing anti-AAV antibodies for GTx that are administered systemically, they may be less relevant for GTx that are administered directly into tissues. Matrices such as cerebrospinal fluid (CSF, for intrathecal administration), synovial fluid (for intra-articular administration), or vitreous humor or aqueous humor (for intraocular administration) may potentially be more relevant for the determination of the presence of pre-existing anti-AAV antibodies in these cases, although collection of such samples may be challenging. Whereas it is feasible to collect plasma or serum samples and screen those samples to determine whether a potential patient has a low titer of anti-AAV antibodies and hence is eligible for the administration of the GTx, it is not feasible to do so for GTx administered directly into a tissue. It may not be ethical to obtain vitreous, CSF, or synovial fluid without the benefit of the potential efficacy derived from the administration of the GTx. Due to logistical challenges related to the need to deliver samples to a bioanalytical laboratory for analysis, the potential patient may be subject to two separate surgeries. Consequently, it is important to understand the relationship between the titer of anti-AAV antibodies in these special matrices and serum or plasma so that serum or plasma could be used as a surrogate for the anti-AAV antibody titers at the site of treatment. In an example of intracerebroventricularly administered cerliponase alfa enzyme replacement therapy, transient and low titer anti-drug antibodies (ADAs) were detected in the CSF [44]. ADAs in the CSF were lower in titer than those observed in the plasma and were not detected when plasma samples were determined to be ADA-negative. In this case, it may be concluded that antibody in tissue is likely to be derived from a peripheral source and a *de novo* antibody production in tissues other than lymph nodes is not likely. Based on this example and in general, it can be appropriate to view plasma or serum as a surrogate for tissue antibody presence.

The need for a surrogate matrix may also be based on the inability to generate a large pool of NC material for tissue-based matrices such as vitreous fluid.

For serum and plasma samples, it is important to understand the potential impact of anti-coagulants on the NAb assay performance. For example, heparin is known to interfere with the viral vector transduction of HeLa or HEK293 cells and therefore, heparin-containing plasma matrix should be avoided, or the use of heparin prior to the administration of the GTx may need to be contraindicated [45]. It has also been noted that glycans may be involved in the uptake of certain AAVs by cells [46].

It is important to collect sufficient volume of blood (or other matrices) that will enable analysis of the sample. Although the amount of serum or plasma that is required for the NAb or binding antibody assay is relatively small (e.g., 50 to 200 μL), the total volume of sample may be impactful for some population types, for example in pediatric studies where the overall blood volume that can be collected will be extremely limited.

### Sample Dilution Scheme

The most commonly used dilution scheme for a titration assay involves serial two- or threefold dilutions. The number of serial dilutions can vary from assay to assay; however, 7 dilution steps are commonly used. The minimal required dilution (MRD) of the assay is directly related to the resulting NAb assay sensitivity and is one of the critical assay quality attributes. MRD values in the range of 1:5 to 1:20 have been described [39, 47] and we recommend that an MRD of less than 1:20 is used. Limitations on the sample volume that can be collected may have an impact on the selection of the MRD to be used. Based on the FDA Immunogenicity Testing of Therapeutic Protein Products guidance recommendations, all sample dilutions, including initial MRD step and other sample pre-treatment steps, if applicable, should be included in the calculation of the final reported titer value [48].

Anti-AAV NAb titers can range from very low to very high values. Anti-AAV2 NAb titers ranging from < 1:2 to 1:28,000 have been observed in serum from healthy donors [49]. Consequently, a large number of dilution steps may be required to determine the NAb titer if the accurate determination of high titers is required. The inclusion of a large number of dilution steps in the assay will have an impact on the throughput of the assay and may be prohibitively expensive. An alternative approach may be to use twofold dilutions for the first 2 or 3 dilution steps (i.e., if an accurate assessment of low titer antibodies is required) with a fourfold or higher dilution scheme being used for subsequent dilution steps. Alternative approaches such as reporting titers as being greater than the highest dilution tested or the utilization of a dilution scheme based upon the results of a binding Ab titer assay could be considered. Such approaches will help to increase throughput and lower costs. For example, when conducting pre-treatment screening of patients, an exact NAb titer value may not be required, unless titer value is used for subject stratification purposes. If subjects with positive titer (as defined in study protocol) are excluded, it may be appropriate to state that a subject titer is greater than the inclusion titer value.

## KEY VALIDATION PARAMETERS

### Sensitivity

The sensitivity of the anti-AAV NAb assay should be calculated in a similar manner to that of other anti-drug antibody assays. The approach to the determination of the assay sensitivity has previously been described in detail [50].

Although current regulatory guidance [48] recommends that the ADA assay has a sensitivity of 100 ng/mL of the anti-therapeutic antibody, this may not be feasible or advisable for an anti-AAV NAb assay. The intrinsic variability of a cell-based assay may make it difficult to achieve this desired sensitivity [51]. Additionally, there is no clear understanding of the relevance of the desired level of sensitivity to the clinical signals, including treatment safety and efficacy. The required sensitivity of the therapeutic specific assay will depend upon the levels of the AAV GTx present in the sample, the levels of endogenous AAV present in the sample, and the number of anti-AAV antibodies that are required to

ablate the ability of the one single viral vector to transduce the cell. Currently, there is a dearth of information on many of these factors and hence, it is not possible to make an accurate assessment of the required assay sensitivity.

### Specificity of Neutralizing Activity

As discussed previously, the overall neutralizing activity of a sample results from a balance of the neutralization of viral vector transduction by antibodies and non-antibody factors and the potential enhancement of transduction by binding antibodies. Consequently, although it may be of interest to show that the neutralization activity is due specifically to anti-AAV antibodies, such a confirmatory step may not be necessary. If an irrelevant antibody is used to determine the specificity of the NAb assay, it is advisable to use a monoclonal antibody that does not recognize any AAVs. The use of pooled sera may be confounded by cross-reactivity of anti-AAV antibodies with multiple AAV serotypes (e.g., AAV2, AAV6, AAV8, and AAV9) [52].

If it is necessary to show that the neutralization activity is due to anti-AAV antibodies, several approaches can be taken. Firstly, a non-specific antibody-mediated neutralization can be evaluated by the use of an irrelevant antibody. Secondly, the immunoglobulin fraction can be depleted from the sample by passing the sample over a column or magnetic beads containing Protein A, Protein G, or Protein L. Thirdly, a conventional competitive inhibition approach can be used, where an empty vector or vector containing an irrelevant transgene is added to compete with the reporter gene-bearing viral vector [28].

It should be noted that determining the specificity of the response will be costly and time-consuming and may use large amounts of assay reagents. Consequently, the sponsor should determine whether such efforts markedly add to the interpretation of the data prior to performing such steps.

### Precision

Monitoring of the precision of the assay is commonly based on the assessment of the intra- and inter-assay %CV of the raw signal and signal/noise (S/N) ratios. Each sample can be tested as a duplicate or higher number of replicates. Inter-assay assessment needs to include several (e.g., 3 or more) plates tested by 2 or more analysts on different days. For cell-based assays, an intra- and inter-assay precision of less than 25% and 35%, respectively, is recommended [51]. The exact value of the %CV may depend on the control type, e.g., HPC and LPC. For example, tighter precision limits (e.g., 20% CV) can be set for the mid-level PC (if used) and HPC as these are expected to generate a relatively high degree of inhibition in the NAb assay. A higher %CV limit (e.g., not to exceed 30% for intra assay) may be set for the LPC and/or NC.

### Selectivity

Selectivity is an important quality attribute of the assay. Selectivity assessment commonly includes testing of the LPC reagent recovery in samples collected from healthy or study-relevant individuals. Ideally, 10 or more NAb-negative samples should be selected for the assessment.

In the selectivity assessment, at least 80% of selected NAb-negative individual samples should score positive when spiked with the LPC (e.g., 8 out of 10 tested samples score NAb-positive) and similarly 80% of unspiked individual samples should score NAb-negative.

### Matrix Interference

Related to the selectivity assessment, it is recommended to consider evaluation of assay performance in samples of varying quality (e.g., hemolytic, lipemic, and icteric sera). Although the FDA guidance on method validation of immunogenicity assays [48] states that the impact of factors such as hemoglobin (hemolysis), lipids (lipemia), and bilirubin (icterus) should be evaluated, it is unclear as to how such compounds will interfere with an ELISA or cell-based assay, especially given the marked sample dilution that is performed on the unknown sample. Generally, the evaluation of these interferences is not viewed as a critical test and can be omitted from the method validation. Of greater importance will be the impact of certain medications or anticoagulants (e.g., heparin) used for sample collection as has been discussed previously in this article. Criteria for the assay acceptance should be similar to that used in the selectivity test.

### Assay Cut-Point

The assay cut-point is a protocol-specific parameter that defines whether the sample is potentially reported as being negative or positive. In a tiered approach, samples that are considered positive in the initial screening test may be then tested at multiple dilutions to determine antibody titer value. Sample NAb positivity and the titer value are defined based on the method-specific cut-point parameter. The latter can be designed based on the sample's ability to produce 50% inhibition of the viral vector transduction. Alternatively, a statistically defined assay cut-point can be computed as presented below. When selecting the 50% inhibition threshold-based cut-point value, one needs to appreciate that such a high level of inhibition bears the risk of missing the low positive samples. For example, inhibition levels in the range between 25 and 50%, albeit lower, may still indicate the presence of neutralizing anti-AAV antibodies. To those sponsors who prefer to use the % inhibition threshold for the NAb positivity, it is recommended to explore, validate, and implement the lowest reproducible inhibition percentage that is greater than the inter-assay precision (e.g., 20% CV).

The statistically defined assay cut-point value is typically defined during assay validation. Methodologies applied for calculating statistically defined cut-points for anti-drug antibody-detecting methods have been broadly discussed for protein-based biotherapeutics with several industry white papers and regulatory agency guidelines available [48, 50, 53]. In alignment with the general recommendations for the determination of anti-protein biotherapeutic antibodies, we propose to apply a statistically determined cut-point derived based on data generated by testing, when feasible, approximately 50 individual matrix samples from subjects that are negative for anti-AAV antibodies that could be

defined as producing < 50% inhibition of viral vector transduction. A high percentage of therapeutic treatment-naïve individuals may be expected to be NAb-positive due to environmental exposure to the AAVs [24]. High number of NAb positive samples among randomly selected samples may significantly complicate the analysis of data generated during statistical cut-point determination. One approach can be to remove any samples generating greater than 50% signal inhibition in the assay as these may be assumed as true NAb-positive. The remaining selection of the samples will then be used to produce the data required for the cut-point calculations. One needs to realize that this approach may require initial analysis of a large number of individual samples in order to find 50 matrix samples with no anti-AAV positivity. Alternative methods to selecting individual samples and analyzing data generated in the initial cut-point evaluation are actively discussed within the industry [54–56].

Other matrix factors may exhibit NAb-like activity in the assay [28]. Whether the signal observed in the NAb assay is caused by antibodies or is a result of other factors can be confirmed by applying immunoglobulin depletion (e.g., with Protein A/G/L) from the sample.

## DATA REPORTING

Detection of anti-AAV antibodies is commonly conducted in a tiered-based approach in which samples are initially screened for possible NAb positivity and those that are deemed screen positive are assessed in multiple dilution test to determine final antibody titer value. The final reportable antibody score (positive/negative) and the semi-quantitative titer value are reported. As previously discussed, a 50% viral vector inhibition threshold or a statistically set method-specific cut-point may be used in both screen and titer tiers of the assay. Regardless of the type of cut-point that is used for determining NAb positivity, the results from the NAb assay will be commonly reported as a titer. Alternative approaches such as the % neutralization/μL matrix at a particular dilution can be employed. Both positive/negative status of the sample and antibody titer value can be used to determine possible correlation with the clinical outcome and therefore, NAb status for all samples tested is expected.

## COMPANION DIAGNOSTIC OR SIMILAR LABORATORY DEVELOPED TESTS

The presence of pre-existing antibodies (neutralizing or binding) has been used as an exclusion criterion in some clinical trials. If a test is used to select patients for inclusion in the clinical trial based upon their pre-study anti-AAV antibody titer, it is logical that such a test will be required once the drug has been approved since there will be no efficacy or safety data available for the use of the GTx in patients with anti-AAV titers higher than the level used as the exclusion criterion. Under such circumstances, a relevant test (companion diagnostic, CDx, or a laboratory-developed test, LDT) may be required at the time of submission of the marketing application for the GTx product [57]. The availability of a companion diagnostic assay will be of great importance when determining the timing of re-administration of the GTx, if re-administration is indeed required. The details of the development of a CDx are beyond the scope of this manuscript. However, there are several important points to be considered regarding what would make a successful companion diagnostic test.

As stated in the FDA guidance titled "In Vitro Companion Diagnostic Devices," IVD companion diagnostics are, by definition, essential for the safe and effective use of a corresponding therapeutic product and may be used to: (1) identify patients who are most likely to benefit from the therapeutic product; (2) identify patients likely to be at increased risk for serious adverse reactions as a result of treatment with the therapeutic product; (3) monitor response to treatment with the therapeutic product for the purpose of adjusting treatment (e.g., schedule, dose, discontinuation) to achieve improved safety or effectiveness; or (4) identify patients in the population for whom the therapeutic product has been adequately studied and found to be safe and effective [58].

The ideal companion diagnostic is one that is robust and can be used at the point of care. As currently configured, cell-based NAb assays may not be able to meet this ideal. Alternatively, TAb assays that may be applied for pre-treatment assessment of patients are quicker and less laborious in execution, factors that would facilitate development as a CDx. In clinical trials for Zolgensma®, subjects were required to have a pre-study anti-AAV9 antibody of ≤ 1:50 as measured using a binding ELISA assay. Few subjects were excluded from the clinical trial based on this exclusion criterion due the age of the patient population (< 2 years of age). At this age, the patients will have had limited environmental exposure to AAV and hence will likely have low to no antibody titers. This criterion also applies to therapeutic use of Zolgensma® [34].

## SUMMARY AND CONCLUSIONS

Neutralizing anti-AAV antibodies are a sub-class of the total anti-AAV antibodies which, along with non-neutralizing antibodies, occur due to natural exposure to wild type AAVs and/or to administration of the AAV vectors. The inhibitory effects of NAbs on the transgene expression (i.e., expression of mRNA or protein) have been clearly demonstrated in animals *in vivo* [28, 59–61]. However, *in vivo* methods have limited utility and were not employed in human clinical studies where inhibitory effects of anti-AAV NAbs were observed as well [21, 31, 32], thus limiting our ability to explore *in vitro* test correlates of inhibition. *In vitro* methods are more practical and investigators are encouraged to assess levels of NAbs prior to and after the administration of the AAV GTx. However, different assay methodologies, assay sensitivities, the manner in which the assay titers are calculated, and data are reported, limiting the utility of comparing results across studies. This paper provides recommendations for development and validation of the cell-based methods for the detection and measurement of anti-AAV NAbs including selection of cell lines and culture conditions, selection of surrogate vectors and reporter genes, matrix selection, characterization of positive and negative NAb controls, and determinations of assay cut-point. Provided are also general recommendations on the key assay validation parameters such sensitivity, specificity, precision, selectivity,

and interference. Industry-wide experiences with the cell-based assays for neutralizing antibodies against recombinant therapeutic proteins were used, wherever appropriate, as the basis for these recommendations. This paper is a result of a consensus between multiple scientists involved with various aspects of GTx. It is hoped that recommendations outlined here will help assay developers and clinicians alike to apply more harmonized strategies for immunogenicity assessment of GTx products.

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## GLOSSARY

AAV adeno-associated virus

AAV binding (total) antibodies (TAb) immunoglobulins able to specifically bind to AAV capsid protein epitopes. These antibodies may be neutralizing or non-neutralizing

AAV neutralizing antibodies (NAb) immunoglobulins able to specifically bind to AAV capsid protein epitopes and inhibit one or more critical steps involved in AAV infectivity and cell transduction

Companion diagnostic (CDx) an *in vitro* test method which provides information that is essential for the safe and effective use of a corresponding drug or biologic product

Gene therapy (GTx) a technique that uses genes, or genetic modification, to treat or prevent disease

Transgenea gene that is transferred due to GTx treatment

Transgene expression of mRNA or protein

Viral vector viral based tools used to deliver genetical material to cells

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