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Confirmatory detection of neutralizing antibodies to AAV gene therapy using a cell-based transduction inhibition assay

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Successful treatment with adeno-associated virus (AAV)-based gene therapies can be limited by pre-existing anti-AAV antibodies. Cell-based transduction inhibition (TI) assays are useful to characterize the neutralizing potential of anti-AAV antibodies in patient samples. While these assays are commonly used, they are not specific for neutralizing antibodies (NAbs) against AAV, also detecting non-antibody-based factors that inhibit AAV transduction *in vitro* but may not substantially decrease efficacy *in vivo*. This paper describes the development and bioanalytical validation of a confirmatory assay to improve the specificity of detecting anti-AAV5 NAbs in cell-based TI assays. Samples that screen positive for transduction inhibitors are subsequently depleted of all classes of immunoglobulins using agarose resins conjugated with protein A, G, and L (AGL), which restores AAV5 transduction for NAb-containing samples. Unconjugated agarose resin serves as a mock control for non-specific depletion effects and facilitates normalization of the transduction efficiencies between an AGL- and mock-treated sample; the normalized value is termed the AGL/mock ratio. During validation, a confirmatory cut point for the AGL/mock ratio was derived; sensitivity, precision, selectivity, and matrix interference were also assessed. This confirmatory TI assay facilitates a characterization of humoral immunity to AAV gene therapy by reliably distinguishing NAbs from non-antibody-based neutralizing factors.

INTRODUCTION

In recent years, treatment of human genetic diseases with adeno-associated virus (AAV)-based gene therapy has been pursued with great interest by an increasing number of academic groups and pharmaceutical companies. While the first gene therapies have now been approved in the United States,[1,2] multiple non-clinical studies and evaluations of clinical trial participants have shown that the success of AAV-based gene therapy can be limited by pre-existing humoral immunity.[3–5] Natural AAV infections and resulting anti-AAV antibody formation are common but vary depending on AAV serotype, with anti-AAV5 antibodies ranking among those with the lowest seroprevalance,[6] the age of individuals, and their geographic region.[7,8] It is therefore important to understand the immune status of patients prior to enrolling them into AAV-based gene therapy trials. In addition, assessing changes in anti-AAV immunity after gene therapy administration can help inform strategies for potential re-dosing.

Pre-existing humoral immunity can be easily assessed using immunoassays that detect the presence of total anti-AAV antibodies (TAbs).[6,9] Non-clinical and clinical studies strongly suggest that results from anti-AAV TAb assays suffice for patient stratification in AAV gene therapy trials.[5,10,11] Nonetheless, cell-based AAV transduction inhibition (TI) assays remain valuable to characterize the neutralizing potential of the detected antibodies.[5,9,10] While TI assays are capable of detecting the presence and levels of inhibitory factors that interfere with AAV transduction, they are typically not designed to distinguish between antibody-based and non-antibody-based inhibition. As with other assay methodologies, it is important to implement a confirmation assay to verify that the inhibition measured in a cell-based TI assay truly stems from anti-AAV NAbs. The impact of non-antibody factors in serum on AAV transduction, including commonly prescribed drugs, has been described previously.[12–16] It is less clear what impact, if any, these factors have in patients, when compared to anti-AAV NAbs that oftentimes lead to reduced efficacy.[5,11,17]

Several bioanalytical strategies can be devised to characterize anti-AAV neutralizing factors in cell-based TI assays. This includes the physical depletion of test sample matrix using resins conjugated with the AAV capsid of interest[18] or with immunoglobulin-binding molecules, such as protein A, protein G, and protein L. Protein G/L has been used previously to confirm the presence of NAbs against other therapeutic modalities.[19] Alternatively, empty or generic AAV capsids added to the test sample can competitively absorb and functionally deplete anti-AAV antibodies.[20] Determining a suitable confirmation strategy for NAb assays depends on the goal of the confirmation step. Using AAV-coupled resins or empty AAV capsids will physically or functionally deplete transduction inhibitors that specifically interact with a particular AAV capsid; however, both antibodies and non-antibody-based factors would be eliminated.

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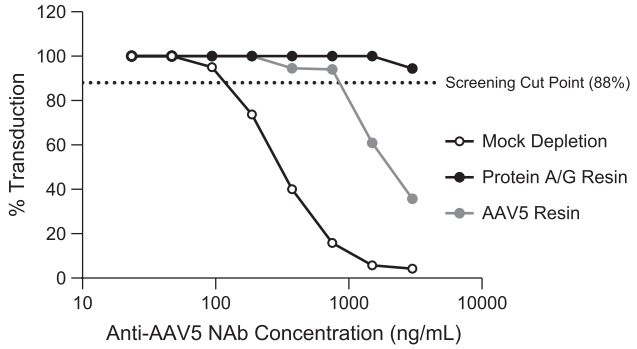

Figure 1. Comparison of anti-AAV5 NAb depletion using protein A/G versus AAV5-conjugated resin

A 1:2 titration of anti-AAV5 NAb from 3,000 ng/mL to 23.4 ng/mL, in normal human plasma, was converted to serum and filtered over either protein A/G-conjugated, AAV5-conjugated, or unconjugated (mock depletion) resins. Each color circle indicates a different depletion condition. The screening cut point is indicated at 88% transduction. Depletion with protein A/G-conjugated resin resulted in an increase in percent transduction above the screening cut point at all concentrations of anti-AAV5 NAb tested. The AAV5-conjugated resin increased percent transduction above the screening cut point for all anti-AAV5 NAb concentrations ≤750 ng/mL, while only NAb concentrations ≤93.8 ng/mL allowed for percent transduction above the screening cut point when mock depleted.

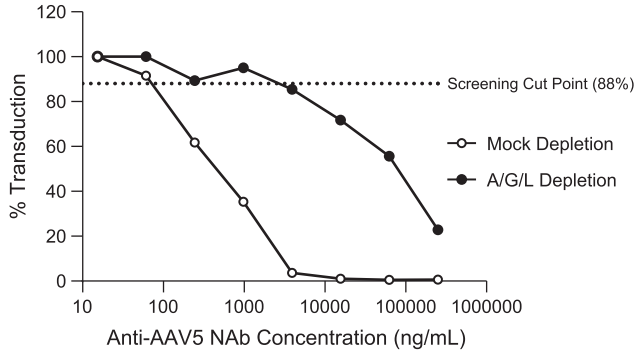

Figure 2. Depletion of high-titer NAb using protein A/G/L-conjugated resin

A 1:4 titration of anti-AAV5 NAb from 250,000 ng/mL to 15.3 ng/mL in normal human plasma was converted to serum, and A/G/L and mock depleted. The screening cut point is indicated at 88% transduction. The protein A/G/L-conjugated resin increased percent transduction above the screening cut point for all anti-AAV5 NAb concentrations ≤977 ng/mL, while only NAb concentrations ≤61.0 ng/mL allowed for percent transduction above the screening cut point in the mock depletion sample.

Therefore, this procedure does not provide insights into the molecular classification of the detected inhibitors. To confirm the presence of anti-AAV NAbs, we therefore selected protein A, G, and L conjugated agarose resins that specifically deplete immunoglobulins of various isotypes from human plasma samples, thereby restoring efficient transduction in a cell-based AAV5-Luciferase TI assay. In conjunction, unconjugated (mock) resin is used to control for non-specific depletion of other inhibitory plasma components. We considered both regulatory guidances[21] and industry white papers[22–25] to validate the performance of this new method. During bioanalytical method validation, we established a confirmatory cut point using samples from 50 hemophilia A (HA) donors. Selectivity in HA and healthy individuals, as well as matrix interference in hemolytic and lipemic samples, was assessed. Sensitivity was determined based on the validated cut point. Precision was determined for high and low positive control antibody concentrations. The method was deemed fit for purpose based on the results of this validation and can be used to characterize pre-existing as well as treatment-induced humoral anti-AAV5 immunity in gene therapy target populations.

RESULTS

Comparison of protein A/G- and AAV5-coupled resins in restoring AAV5 transduction

To compare the efficiency by which protein A/G- and AAV5-coupled resins remove capsid-specific antibodies and thus restore AAV5 transduction, we prepared a serial dilution of a monoclonal anti-AAV5 antibody (clone ADK5b) in healthy human male pooled plasma. Plasma samples were tested in cell-based TI assays, following depletion with protein A/G-coupled, AAV5-coupled, or unconjugated (mock) resin (Figure 1). The percent transduction increased when the sample was depleted using protein A/G and AAV5-coupled resins in comparison to the mock resin, but the protein A/G resin showed greater depletion capacity: The protein A/G resin was able to restore AAV5 transduction above the assay screening cut point of 88% transduction at all concentrations of antibody tested (up to 3,000 ng/mL), while the AAV5-coupled resin was capable of restoring AAV5 transduction for antibody concentrations up to 750 ng/mL.

Establishment of the AGL/mock ratio to evaluate restored AAV5 transduction

To evaluate the best detection algorithm to confirm a sample as positive for AAV5 NAbs, a serial dilution of a monoclonal anti-AAV5 antibody (clone ADK5b) was prepared in normal human male pooled plasma, followed by conversion to serum and protein A/G/L or mock depletion (Figure 2). Due to the propensity of plasma to clot when exposed to agarose resin, plasma samples were converted to serum by formation and removal of a fibrin clot before antibody depletion to ensure consistency and prevent loss of sample. AAV5 transduction in most protein A/G/L-depleted samples was highly efficient. However, at the highest concentrations of antibody tested (≥3,906 ng/mL), the transduction of the AAV5-Luciferase vector remained partially inhibited following protein A/G/L depletion, with a percent transduction below the screening cut point (88% transduction). This is likely due to incomplete antibody removal. Nonetheless, a clear increase in transduction for the protein A/G/L-depleted sample was observed, when compared to the mock depleted sample. These results suggested that evaluating samples based on the restoration of transduction efficiency to levels above the screening cut point may not be the most appropriate detection algorithm for all NAb-positive samples, in particular not for those with very high antibody titers. We therefore established an alternative detection algorithm, the AGL/mock ratio, which is calculated as the percent transduction of a sample following protein A/G/L depletion divided by the percent transduction of the

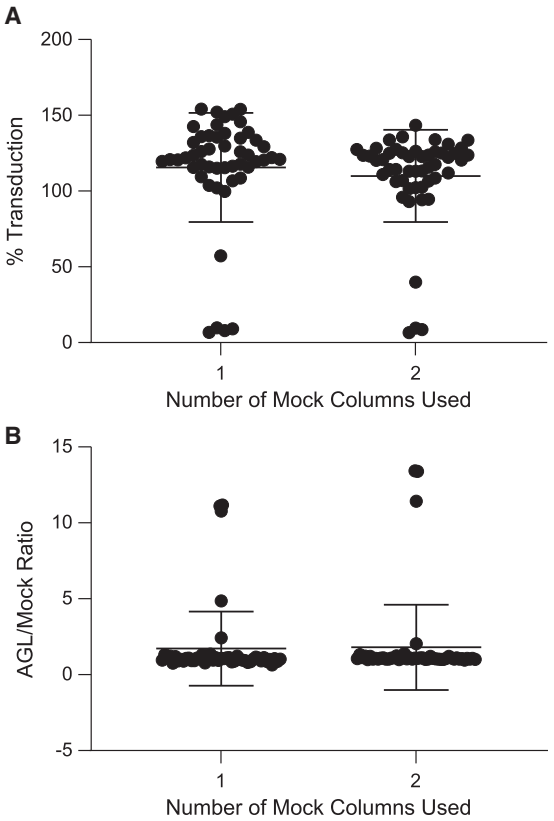

Figure 3. Comparison of using one versus two mock depletion columns

Citrated plasma from 50 normal male human donors (without anti-AAV5 antibody spike) was converted to serum and protein A/G/L depleted or mock depleted using either one or two unconjugated resin columns. The percent transduction and ratio of AGL/mock depletion was calculated for each individual and is plotted with the mean and standard deviation for all 50 individuals by the number of mock depletions. (A) The percent transduction following mock depletion did not change significantly between one and two rounds ($p = 0.0608$, paired t test). (B) The observed mean AGL/mock ratio did not change significantly ($p = 0.4266$, paired t test) depending on the number of mock depletions, indicating the suitability of using only one mock column for depletion.

same sample following mock depletion. This provides a relative rather than absolute measure for the increase in transduction observed after immunoglobulin removal from the test sample.

Two-column depletion process using protein A/G/L for immunoglobulin removal

Based on the comparison of depletion resins and considering the established specificity of protein A and protein G for various classes of immunoglobulins, we moved forward with the protein A/G depletion resin to confirm the presence of anti-AAV NAbs. In addition, a protein L-coupled depletion resin was added to increase the efficient removal of non-IgG antibodies, such as IgM. For ease of use, we chose NAb protein A/G and NAb protein L spin columns to remove antibodies. These resins are supplied as two different columns by the manufacturer, which would theoretically also require the parallel use of two unconjugated mock control columns. To establish an appropriate, but also user-friendly control process for the two-column protein A/G/L depletion, we compared a single round of mock depletion to two rounds of mock depletion for 50 human serum-converted plasma samples. There was no remarkable difference in the percent transduction (Figure 3A) or AGL/mock ratio (Figure 3B) determined following a single mock depletion versus two mock depletions. Therefore, only one mock depletion column was used for all validation experiments and subsequent sample testing.

Validation of the confirmatory TI assay cut point

Dual cut points for screening and confirmatory assay steps were established per FDA Guidance for Immunogenicity Assay Validation.[21] The use of dual cut points allows for the screening cut point to have a higher false-positive rate, thus increasing the likelihood of detecting low positive samples. False-positive and low positive samples are subsequently discriminated by using the confirmatory cut point. To establish the confirmatory assay cut point, i.e., the value of the AGL/mock ratio, at or above which samples are confirmed positive for anti-AAV5 NAbs, plasma samples from 50 drug-naïve, hemophilia A (HA) individuals were tested in four assay runs, performed by three analysts over three days. HA plasma was chosen since it represented the intended gene therapy target population. The AGL/mock ratio was determined for each individual, represented in Figure 4A. Biological outliers, defined as individuals whose AGL/mock ratio was greater than that of the low-concentration positive control (LPC; pooled normal plasma spiked with 200 ng/mL anti-AAV5 antibodies) in more than two runs, were removed from subsequent analyses, so as to not artificially skew the cut point.[22] Patients with pre-existing AAV5 Nab reactivity in a clinical setting would not be eligible for treatment with drug product.[6,7] In addition, statistical outliers identified by boxplot and samples with a coefficient of variation (%CV) between replicate wells greater than 25% were also excluded. The exclusion of statistical outliers is commonly used when setting a statistical cut point, so that the cut point is not artificially inflated or deflated.[23] Samples with high %CV are excluded since their reliability is uncertain and they may represent artificially high or low data points. The distribution of the remaining results was normal in three of four assay runs. The overall distribution of these results is shown in Figure 4B. Means and variances were not significantly different between runs before outlier removal ($p = 0.578$ and $p = 0.089$, respectively). However, upon outlier removal both the means and variances differed significantly ($p = 1.15 \times 10^{-5}$ and $p = 0.00056$, respectively). To account for these differences, the global mean and variance were not used. Instead, the cut point was calculated separately for each run using a parametric approach targeting the 99th percentile distribution limit and then averaged across the four runs. Based on this calculation, the validated confirmatory cut point was established as an AGL/mock ratio of 1.30.

Selectivity of the confirmatory TI assay

Although the cut point was established using hemophilia A donors, the suitability of this method was subsequently demonstrated for healthy individuals by confirming selectivity using samples

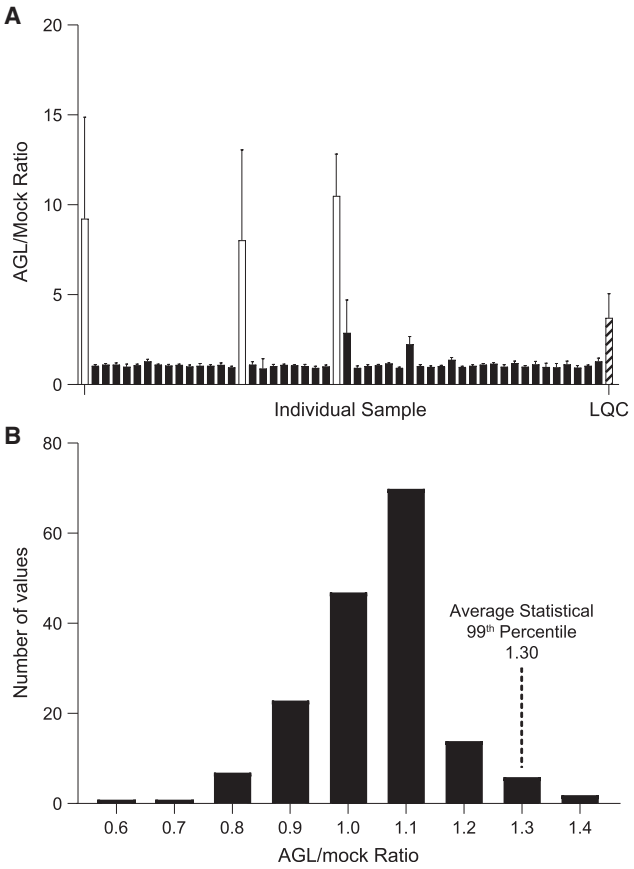

Figure 4. Distribution of responses for confirmatory cut point evaluation

Citrated plasma samples from 50 normal male human donors, as well as normal pooled plasma spiked with anti-AAV5 NAb at the LPC concentration (200 ng/mL), were converted to serum, protein A/G/L, or mock depleted and tested over four different occasions. The AGL/mock ratio was calculated for each sample. (A) The mean and standard deviation of the AGL/mock ratio was plotted for each individual, and any individual with a ratio greater than the LQC ratio was flagged as an outlier. (B) The pooled distribution of AGL/mock ratios (outliers excluded) was plotted, and the confirmatory cut point was established as the statistical 99th percentile.

from 14 randomly selected healthy donors, which were tested alongside 17 additional randomly selected HA donors (Figures 5A and 5B). These samples were tested either unspiked or spiked with 200 ng/mL of a monoclonal anti-AAV5 antibody (ADK5b). Six of 14 healthy and seven of 17 HA samples that were left unspiked had a percent transduction in the mock sample that fell below the screening cut point of 88%, indicating that they contained pre-existing AAV5 inhibitors. Most of these samples also confirmed positive, with six of six healthy and five of seven HA showing an AGL/mock ratio greater than 1.30. None of the remaining healthy (n = 8) or HA (n = 10) samples without pre-existing immunity confirmed positive when left unspiked. All individual donor samples spiked with 200 ng/mL of anti-AAV5 antibodies screened and confirmed positive, demonstrating the selectivity of the method for detecting anti-AAV5 antibodies in both HA and healthy donor matrices.

Assessment of hemolytic and lipemic matrix interference

To assess the potential impact of hemolysis on test results, hemoglobin was added to 10 of the 14 randomly selected healthy individual donor plasma samples to a final concentration of 1,100 mg/dL. These samples were then left unspiked or spiked with anti-AAV5 antibodies (ADK5b) at 200 ng/mL and tested. Of the unspiked samples, nine of 10 individual mock samples showed a percent transduction greater than 88% (i.e., nine of 10 screened negative), with eight of those nine samples having an AGL/mock ratio less than the confirmatory cut point (Figure 5C). Overall, nine (90%) of 10 donor samples screened and/or confirmed negative, as expected. 10 of 10 (100%) antibody-spiked donor samples screened and confirmed positive, achieving AGL/mock ratios above the confirmatory cut point of 1.30. These findings demonstrate that there was no interference due to hemolysis in the confirmation assay.

The potential effect of lipemia was also assessed using the same 10 individual donor samples that were used to assess hemolytic interference, spiked with a final concentration of 300 mg/dL human lipid (SyntheChol NS0 Supplement). These samples were also tested unspiked or spiked with 200 ng/mL anti-AAV5 antibodies. 10 of 10 (100%) unspiked donor samples screened negative, with a mock percent transduction greater than 88% and did not confirm, in accordance with the expected negative results (Figure 5D). While three of 10 donor samples spiked with anti-AAV5 antibodies screened positive, zero of 10 (0%) spiked donor samples screened and confirmed positive, demonstrating that lipemia greatly interferes with the results of the confirmatory assay. Therefore, lipemic plasma samples should not be tested using this method.

Sensitivity of the confirmatory TI assay

The sensitivity of the confirmatory assay was assessed by diluting anti-AAV5 antibodies (ADK5b) from 23.4 to 3,000 ng/mL in normal human male pooled plasma, followed by conversion to serum, protein A/G/L or mock depletion of each dilution sample, and testing in the cell-based TI assay. The AGL/mock ratio was determined for each sample. A total of six independently prepared curves were tested by two different analysts over two days. The mean ratio at each antibody concentration was calculated, and sensitivity was evaluated by determining the limit of detection (LOD), calculated as the concentration of anti-AAV5 antibodies interpolated at the confirmatory cut point of 1.30, which was equal to 26.4 ng/mL of anti-AAV5 antibodies in neat human plasma (Figure 6).

Precision of the confirmatory TI assay

Precision was determined using normal human pooled plasma spiked at two concentrations of the positive control anti-AAV5 antibody (ADK5b), which were chosen near the lower and upper limits of the linear portion of the titration curve. 200 ng/mL antibody was used as the LPC and 3,000 ng/mL was used as the high-concentration positive control (HPC). All controls were run in two replicates on each assay plate, with each replicate tested in duplicate wells. To evaluate intra-assay precision, LPC and HPC set from a given run were compared by a one-way analysis of variance (ANOVA). Inter-assay precision was also evaluated by ANOVA for all runs,

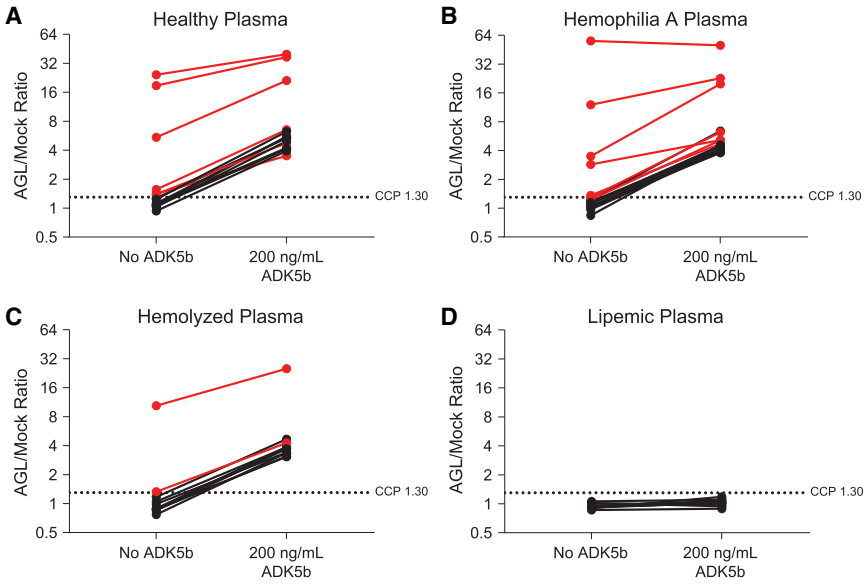

Figure 5. Selectivity and matrix interference evaluation

The effect of possible matrix interference on evaluating the presence of anti-AAV5 NAbs using the AGL/mock ratio was assessed in (A) 14 individual healthy male human plasma samples, (B) 17 plasma samples from hemophilia A patients, (C) 10 individual healthy plasma samples spiked with 1,100 mg/dL hemoglobin, and (D) 10 individual healthy plasma samples spiked with 300 mg/dL lipid. All samples were assessed neat and with 200 ng/mL anti-AAV5 NAb (ADK5b). Samples in red, including six of 14 (43%) healthy, seven of 17 (41%) hemophilia A, and two of 10 (20%) hemoglobin-spiked samples, had ratios above the confirmatory cut point when unspiked. All healthy, hemophilia A, and hemoglobin-spiked samples confirmed positive when spiked with ADK5b. Lipid-spiked samples did not confirm positive with or without the addition of ADK5b.

with at least six sets of positive controls at each level. Results are presented in Table 1. LPC intra-assay precision was calculated as 24.8%, whereas the inter-assay precision was 26.7%. For the HPC, intra-assay precision was determined to be 46.1% and inter-assay precision was 107.3%. While the HPC precision did not meet the <25.0% and <35.0% criteria recommended for intra-assay and inter-assay precision, respectively,[25] these results were acceptable because the confirmatory TI assay is mainly intended to provide a qualitative (positive/negative) rather than quantitative assessment of antibodies. The variability observed at the HPC concentration of anti-AAV antibodies could be affected by differences in the exact maximal immunoglobulin binding capacity that may vary slightly between two columns. More importantly, all HPC samples consistently confirmed positive, demonstrating the capacity to reproducibly deplete even higher antibody levels. In addition, the AGL/mock ratios of HPCs exceeded those of LPCs in all experiments, consistent with the higher quantities of antibody present in the HPC. Hence, the observed limited precision at the HPC level was considered acceptable since there was no impact on the reported qualitative confirmatory results.

DISCUSSION

Anti-AAV antibodies can occur in response to both wild-type AAV infection as well as treatment with AAV-based gene therapy. Since the presence of anti-AAV antibodies can have a negative impact on transduction *in vivo*,[3,5] prospective gene therapy patients are oftentimes screened to establish eligibility. Patient screening can be performed using TAb or TI assays; in clinical trials sponsored by BioMarin, TAb assays are preferred for screening under routine clinical operations, due to their less complex format, higher-throughput, broader detection of various types of antibodies (neutralizing and non-neutralizing), lower susceptibility to interfering matrix effects,[9] and observed association with gene therapy outcomes in non-human primates.[5]

Nonetheless, cell-based TI assays offer an opportunity to further explore the neutralizing potential of anti-AAV antibodies, not only in prospective but also in treated gene therapy patients. These assays may be particularly helpful in studies that evaluate novel methods for eliminating these antibodies prior to AAV administration or re-dosing, such as plasmapheresis or other forms of immunodepletion.[18,26–28] The ability to unambiguously distinguish neutralizing antibodies (NAbs) from other inhibitory matrix components is therefore a critical part of interpreting positive titer results obtained in cell-based TI screening assays. While NAbs are likely to impact successful transduction *in vivo*, other matrix components may inhibit transduction only in the *in vitro* cell culture system.[5,12]

To unambiguously identify anti-AAV5 NAbs, we chose to evaluate samples by comparing the restored transduction of samples following protein A/G/L depletion to that upon mock depletion. Mock depletion served as a control for the non-specific removal of non-antibody-based inhibitory factors from plasma, which could improve transduction and falsely resemble the removal of anti-AAV NAbs. To this end, we used a protein AGL/mock ratio, similar to the method described previously by Sanchez, et al.,[19] to determine whether TI is due to the presence of NAb. An alternative method could be to compare protein AGL and mock depleted samples by calculating percentage immunodepletion, as described Jolicoeur and Tacey.[29]

An advantage to using commercially available protein A/G/L-conjugated resin is the ability to expand the analyses of patient antibodies beyond just detection. NAb spin columns are designed for the capture and subsequent elution of antibodies from cell culture or other liquid matrices. Following the depletion of antibodies from patient plasma, a simple elution step as described in the column manufacturer's protocol would make the depleted NAbs readily available for further characterization, including isotyping or surface plasmon resonance to assess capsid binding affinity and avidity, although it is important

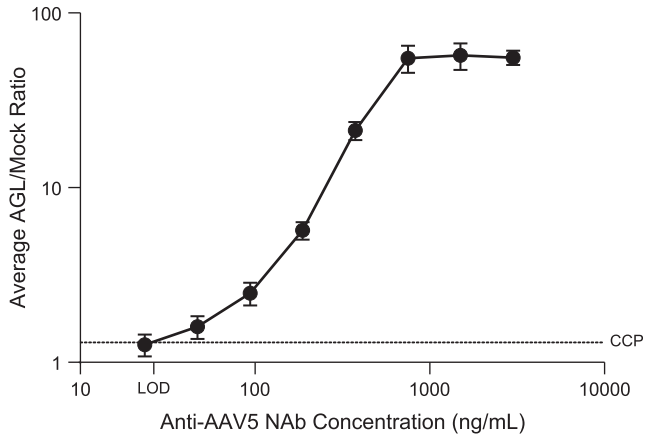

Figure 6. Sensitivity of the confirmatory assay

Six independent 1:2 titrations of anti-AAV5 NAb from 3,000 ng/mL to 23.4 ng/mL in normal human plasma were subjected to serum conversion, AGL or mock depleted. The AGL/mock depletion ratio was calculated for each dilution. The mean AGL/mock ratio for all six curves was calculated and plotted along with the standard deviation. The sensitivity (LOD) was determined as the concentration of anti-AAV5 NAb at the confirmatory cut point, 26.4 ng/mL.

to consider the effect that acid-elution may have on antibody specificity.[30]

AAV vectors elicit strong humoral immune responses, and thus, clinical test samples may contain very high levels of anti-AAV NAbs after dose administration.[31–33] This raises the concern that test samples with very high concentrations of anti-AAV5 NAb might exceed the binding capacity of the depletion columns, thus leading to false negative confirmatory results. During clinical sample analysis using the confirmatory TI assay, we found it most effective to first dilute screen-positive test samples to achieve a titer less than that of the HPC, given the validated detectability of HPC-spiked samples in the confirmatory assay, i.e., given that the HPC consistently confirmed with an AGL/mock ratio greater than the confirmatory cut point during method validation. By first diluting high titer samples, we successfully mitigated the risk that the AGL/mock ratio would remain below 1.30 due to incomplete immunoglobulin depletion.

A variety of strategies to identify and characterize neutralizing factors against AAV-based gene therapy exist. In an attempt to harmonize measurements of pre-existing immunity, industry experts started to provide guidance on the best strategies for developing and validating cell-based *in vitro* assays to detect anti-AAV NAbs.[25] In presenting this manuscript, we hope to offer an additional opportunity for researchers to evaluate and utilize the described testing and validation strategies, which we have found to be valuable for anti-AAV5 NAb characterization. Common criteria for how to validate the performance of analytical methods developed by different organizations are essential, as many in the field have expressed a desire to standardize immunological assays. These efforts should eventually allow for a better comparison of results across different AAV-based gene therapy trials.

MATERIALS AND METHODS

Materials

HEK293T/17 cell were purchased from ATCC (Manassas, VA). High-glucose Dulbecco's modified eagle medium (DMEM) was sourced from Lonza (Basel, Switzerland). Hyclone Fetal Bovine Serum (FBS), NAb protein A/G Spin columns (0.2 mL), NAb protein L Spin columns (0.2 mL), and Pierce Control Agarose Resin were supplied by Thermo Fisher Scientific (Waltham, MA). Multiscreen Durapore 0.22 μm Hydrophilic Low Protein Binding Membrane Filter plates, BSA, human hemoglobin, and SyntheChol NS0 Supplement were obtained from Millipore Sigma (St. Louis, MO). Etoposide was purchased from Enzo Life Sciences (Farmingdale, NY). NHS-activated Sepharose 4 Fast Flow was purchased from GE Healthcare (Chicago, IL). AAV5-CMV-Luciferase reporter virus was manufactured in sf9 cells by Virovek (Hayward, CA). Steady-Glo Luciferase reagent was purchased from Promega (Madison, WI). The positive control mouse anti-AAV5 monoclonal antibody (clone ADK5b) was obtained from LifeSpan Biosciences (Seattle, WA). Hemophilia A plasma was obtained under consent from donors enrolled in a study of natural seroprevalence of AAV antibodies. Healthy, drug-naïve human male plasma was obtained from BioIVT (Westbury, NY). Dade Actin FSL Activated PTT Reagent was purchased from Siemens (Erlangen, Germany). Recombinant Factor VIII (Xyntha) was obtained from Wyeth/Pfizer (New York, NY). Spin-X filter centrifuge tubes and phosphate-buffered saline (PBS, without calcium and magnesium, pH 7.4) were purchased from Corning (Corning, NY).

Conversion of plasma samples to serum

Fibrin clot formation in control-spiked plasma, as well as HA and healthy individual plasma samples, was induced by mixing 250 μL of plasma with 30 μL of Dade Actin FSL Activated PTT Reagent, 10 μL of 500 IU/mL recombinant human FVIII (Xyntha), and 10 μL of 0.3M $CaCl_2$. Samples were incubated for 1 min at 37°C until clot formation. Samples were then centrifuged for 1 min at 4,000 *x g* to pellet the clot, and the resulting serum supernatant was collected and used for column depletion. Xyntha was added to aid in clotting, particularly in HA samples, which lack functional FVIII. Although clot formation occurred in healthy plasma without the addition of Xyntha, it was still added to all samples to ensure identical treatment.

Generation of AAV5-coupled Sepharose resin

All centrifugation steps were performed at 1000 *x g* for 1 min unless otherwise indicated. Three milliliters of NHS-activated Sepharose 4 Fast Flow was washed with 40 mL of cold 1 mM HCl (pH 8.5) and centrifuged. The supernatant was discarded and 3 mL of 0.2 M $NaHCO_3$, 0.5 M NaCl (pH 8.3) with 6 mg of AAV5-CMV-Luciferase was added and incubated at room temperature for 2 h with rotation. The solution was then centrifuged and the supernatant was removed. To block, the AAV5-coupled resin was suspended in 3 mL Tris-HCl (pH 8.5) and incubated at room temperature for 2 h with rotation. Following this incubation, 9 mL of Tris-HCl (pH 8.5) was added and the solution was centrifuged. The supernatant was removed, and the resin was washed three times with 9 mL of 0.1 M acetate

Table 1. Intra-assay and inter-assay precision

	Mean AGL/mock ratio	
Validation run number	LPC	HPC
1	4.43	101.18
2	3.96	104.01
3	5.19	47.91
4	4.67	107.53
5	4.68	117.57
6	5.40	26.75
7	5.84	NA
Overall mean ratio	4.92	88.77
Intra-assay precision	24.8%	46.1%
Inter-assay precision	26.7%	107.3%

AGL, protein A/G/L; LPC, low-concentration quality control; HPC, high-concentration quality control; NA, not applicable.

buffer, 0.5M NaCl (pH 5). The resin was stored in 3 mL Tris-HCl (pH 8.5) at 4°C for subsequent use.

AAV5-coupled Sepharose resin depletion

All centrifugation steps were performed at 3,000 *x g* for 2 min unless otherwise indicated. AAV5-coupled Sepharose (1 mL) was washed three times with 1 mL of DMEM supplemented with 1% BSA and suspended in DMEM with 1% BSA to a final volume of 1 mL. 100 µL per sample of coupled Sepharose was add per well of a Durapore membrane filter plate and centrifuged for 2 min at 1000 *x g*. Flowthrough was collected in a 96-well round bottom plate and discarded. Plasma samples were diluted 1:2.5 with DMEM +1% BSA and transferred to the filter plate containing the coupled resin. The plate was incubated at room temperature for 10 min with shaking, before centrifugation. The flowthrough was collected in a new 96-well round bottom plate and assayed in the cell-based AAV5 TI assay.

Protein AGL column depletion

All centrifugation steps were performed at 5,000 *x g* for 1 min unless otherwise indicated. NAb protein A/G and protein L spin columns were equilibrated using PBS. 200 µL of Pierce Control Agarose Resin was added to a Spin-X filter centrifuge tube to create the mock depletion column. This resin was also equilibrated with PBS. Serum-converted plasma samples were divided in half by volume. One-half was added first to a protein A/G spin column and incubated for 10 min at room temperature. The columns were then centrifuged, and the flowthrough was retained and added next to a protein L spin column. This was again incubated on the column for 10 min at room temperature before centrifugation and collection. The collected volume was then assayed in the cell-based AAV5 TI assay. The second half of the sample volume was applied to mock depletion column and incubated for 10 min at room temperature before centrifugation, collection, and subsequent testing in the same AAV5 TI assay.

Cell-based AAV5 TI screening assay for human plasma/serum

The transduction inhibition assay for human plasma samples was performed as described previously.[9] Briefly, HEK293T/17 cells were thawed from frozen single-use vials and plated in white, clear bottom 96-well tissue culture plates, avoiding all edge wells. Cells were seeded at a density of 40,000 cell/well in DMEM supplemented with 10% FBS (not heat-inactivated). On the following day, samples and quality controls were mixed 1:1 with 25,000 vg/cell of AAV5-CMV-Luciferase vector in DMEM with 1% BSA and incubated for 30 min at room temperature. 25 µL of this transduction mix was added to cells in duplicate wells. Plates were then incubated for 1 h at 37°C before the addition of etoposide to a final concentration of 20 µM in DMEM with 10% FBS. Following 2 days of incubation, luminescence was measured using Steady-Glo Luciferase reagent. The percent transduction was calculated as the RLU of the A/G-, A/G/L-, AAV5-, or mock-depleted sample divided by the RLU of the control pooled plasma (mock depleted) assayed on the same plate. A screening cut point of 88% transduction delineates whether a sample is positive or negative for AAV5 inhibitors (higher percent transduction being negative, and lower percent transduction being positive). This screening cut point, targeting a false-positive rate of 5%, was determined following published recommendations for bioanalytical assay validation.[22,23]

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AUTHOR CONTRIBUTIONS

T.K., S.T., and C.V. designed and implemented the development and validation study experiments. T.K., S.T., and C.V. analyzed the data. T.K, and C.V wrote the manuscript. S.T., B.R.L., and S.J.Z. reviewed and edited the manuscript.

DECLARATION OF INTERESTS

T.K., B.R.L., S.J.Z., and C.V. are current employees and/or shareholders of BioMarin Pharmaceutical, Inc. S.T. is a current employee of GenoSafe SAS.

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