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A sensitive AAV transduction inhibition assay assists evaluation of critical factors for detection and concordance of pre-existing antibodies

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Pre-existing antibodies to viral capsids may have a negative impact on the efficacy and safety of adeno-associated virus (AAV)-based gene therapies. Total antibody (TAb) and/or cell-based transduction inhibition (TI) assays have been used to exclude seropositive individuals in clinical studies. Published AAV seroprevalence and patient enrollment criteria regarding antibody status lack comparability between assay formats, hindering a direct cross-study comparison. To identify critical factors impacting TI assay detection of AAV neutralizing antibodies (NAbs), we created a reporter construct expressing NanoLuc® luciferase (Nluc) that enabled a more sensitive and robust detection of AAV6 NAbs than using firefly luciferase. Assessment of additional factors including multiplicity of infection, cell lines, viral production, and capsid purity revealed the reporter is the major determinant of assay sensitivity impacting NAb detection. The Nluc reporter was further used to assess seroprevalence to AAV5, 8, and 9. Last, we compared AAV6 Nluc TI with two TAb assay formats. A higher correlation of Nluc TI was observed with direct binding (90%) than with the more sensitive bridging TAb assay (65%), suggesting both assay sensitivity and TAb formats contribute to AAV seropositivity concordance. Our results support a need to standardize assay formats to ensure proper assessment of pre-existing AAV immunity.

INTRODUCTION

Recombinant adeno-associated virus (AAV) vectors have emerged as one of the most safe and promising gene delivery platforms for treating patients with genetic and acquired diseases. The success of AAV vectors in mediating gene transfer and expression are showcased by the recent approval of voretigene neparvovec (Luxturna®) for use in pediatric and adult patients with inherited retinal disease, onasemnogene abeparvovec (Zolgensma®) for pediatric patients with spinal muscular atrophy, and etranacogene dezaparvovec (Hemgenix®) for adults with hemophilia B. A major challenge to a wider application of *in vivo* AAV gene therapy is the humoral immunity to AAV capsids as a consequence of natural exposure to wild-type AAVs prior to the administration of AAV gene therapy. These pre-existing anti-AAV antibodies have been shown to decrease the transduction efficiency of recombinant AAV vectors carrying therapeutic transgenes.[1–3] Rare findings of serious adverse events such as thrombotic microangiopathy have been reported, possibly caused by the formation of large immune complexes and complement activation.[4–7]

Methods to measure pre-existing anti-AAV capsid antibodies for seroprevalence studies generally include total antibody (TAb) and neutralizing antibody (NAb) assays.[8] TAb assays are typically plate-based immunoassays allowing detection of all antibodies capable of binding to AAV capsids, including NAbs and non-NAbs. TAbs may impact AAV vector clearance, activate complement pathways, and neutralize AAV transduction. TAb assays have the advantage of ease of use, less variability, and generally higher sensitivity than NAb assays. NAbs are commonly detected by cell-based transduction inhibition (TI) assays (therefore, NAb and TI are interchangeable terminology unless otherwise described in this study) using an AAV reporter vector. Compared with TAb assays, TI assays are functional measurements of neutralizing activities in the test samples, including both antibody and non-antibody neutralizing factors.[9] However, cell-based TI assays have inherently higher assay variability, are more time consuming to develop and run, and may be unable to detect low levels of neutralizing activities due to lower sensitivity than TAb assays. Pre-existing antibodies developed by a majority of individuals are NAbs, which can be detected by both NAb and TAb assays.[10,11] Currently, both TAb and cell-based TI assays have been implemented in AAV gene therapy clinical trials for patient enrollment.[8,12,13]

Many different assay cutoffs have been utilized to determine clinical trial patient eligibility, even for the same AAV serotype and route of administration.[12] The antibody enrollment threshold has ranged from 1:1 to 1:1,200 for NAb assays and from 1:5 to 1:1,600 for TAb assays in different clinical trials.[12] This makes cross-trial comparison of antibody assays and clinical study results very challenging. In addition, the anti-AAV antibody seroprevalence results of same serotypes

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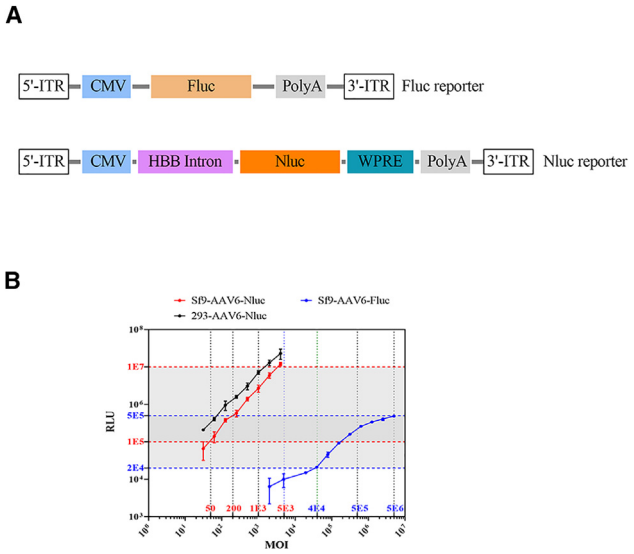

Figure 1. Development of a sensitive AAV6 TI assay

(A) Schematic illustration of Fluc and Nluc reporter expression constructs for AAV6 TI assays. The Fluc reporter construct contained a CMV promoter and a polyA sequence. The Nluc reporter construct replaced the transgene with the Nluc, which produces a stronger luminescence signal than the Fluc. Additionally, the Nluc construct included HBB intron and a 3′-enhancer element, a mutant WPRE, to increase luciferase expression. (B) Transduction of HT1080 cells by Nluc and Fluc AAV6 reporters with titration of viral MOI. Reporter transgene expression was measured by luminescence and expressed as RLUs. The AAV6-Nluc reporters were produced from both Sf9/rBV and HEK293 systems and the AAV6-Fluc reporter were from the Sf9/rBV system only. All data are presented as mean ± SD of duplicate wells in one representative experiment out of three independent runs. The red and blue numbers on the axes mark out the MOIs of the Nluc and Fluc reporters (on the x axis) that generate the luciferase expression at the corresponding RLU signal levels (as indicated on the y axis), respectively. ITR, inverted terminal repeat.

can vary significantly from laboratory to laboratory, which is partially ascribed to differences in the assay methods. A typical cell-based TI assay measures the *in vitro* transduction efficiency of an AAV reporter vector preincubated with human serum or plasma in a cell line culture system. Currently, there is no regulatory guidance or harmonization of assay conditions, selection of cutoff, or critical reagents and their characterization. For example, different cell lines, reporter genes and multiplicity of infection (MOI) for AAV transduction, reporter vector production systems (insect vs. mammalian cells) and empty capsid contents (vs. full capsid ratios) can vary among laboratories. In addition, there are no uniform definitions for seropositivity or standardized critical reagents, including positive control and negative control matrixes, for cross-laboratory comparison. Furthermore, it is not clear if a more sensitive assay would be more suitable for patient enrollment, since it is controversial whether enrollment criteria based on anti-AAV antibody levels have an impact on safety and/or efficacy in clinical trials of AAV gene therapy.[1–3,12,14]

Here we present a qualified AAV6 TI assay utilizing a reporter construct expressing NanoLuc® luciferase (Nluc), which enabled a robust and sensitive detection of anti-AAV6 neutralizing activity. We compared the sensitivity of the Nluc TI assay to a previously established TI assay using a reporter construct expressing firefly luciferase (Fluc) and then evaluated the impact of multiple assay factors on TI assay readouts. In addition, we adapted the Nluc reporter for AAV5, AAV8, and AAV9 and compared pre-existing NAb prevalence in healthy donors using this TI assay format. Furthermore, considering the current debate on whether TAb or TI assays are more suitable for clinical enrollment, we compared the seropositivity concordance of the AAV6 Nluc TI assay with the two TAb assays using capsid direct-binding colorimetric ELISA or electrochemiluminescence (ECL) bridging formats. Our results suggest that both assay sensitivity and format can impact evaluation of pre-existing anti-AAV6 antibodies, and high concordance can be achieved between AAV6 TAb and TI assays with comparable assay sensitivity and appropriate assay formats.

RESULTS

Development and qualification of a sensitive AAV6 TI assay using an Nluc reporter construct

We previously described a validated AAV6 TI assay for patient enrollment in support of AAV6-based clinical studies.[13] This assay utilized the U-87MG human glioblastoma cell line and an AAV6 vector construct encoding a Fluc reporter gene, which was well suited for patient eligibility screening in several liver-directed AAV6 gene therapy clinical trials.[13] We have since developed a new AAV6 TI assay using a reporter construct expressing high levels of Nluc, which also emits stronger luminescence signals than regular luciferase.[15] The new reporter expression cassette incorporated two elements, a human beta globin (HBB) intron and a mutated variant of woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) at 5′- and 3′ UTRs, respectively, to increase transgene expression (Figure 1A).[16] The Nluc reporter construct was packaged with an AAV6 capsid using a triple transfection method in HEK293 cells. The resultant AAV6-Nluc was titrated to different MOI (defined as viral copy number per cell for transduction) for cell line screening. Out of eight cell lines tested, the human fibrosarcoma cell line HT1080 showed the highest AAV6 transduction signals when compared with the other cell lines, such as Huh7 and HepG2 (Figure S1A for representative data), and thus was selected for further assay development. The AAV6-Nluc vector was later produced using a recombinant baculovirus infected Sf9 cell (Sf9/rBV) system, which showed a slightly less potent titration curve than using the HEK293 method (Figure 1B). The Nluc reporter from both preparations required a much lower MOI range (i.e., MOI of 50-5E3 copies/cell for Sf9-AAV6-Nluc versus MOI of 4E4-5E6 copies/cell for Sf9-AAV6-Fluc), and yet achieved a much higher signal of relative luminescence units (RLUs) with a much wider dynamic range (1E5–1E7 RLUs for Sf9-AAV6-Nluc versus 2E4–5E5 RLUs for Sf9-AAV6-Fluc) (Figure 1B).

To select the optimal MOI for TI assay development, we balanced the considerations for sensitivity and variability of the assay performance. A TI assay with lower MOI (fewer copies of reporter viruses per cell for transduction) is expected to achieve higher assay sensitivity.

Table 1. Comparison of Fluc and Nluc AAV6 TI assay sensitivities using three positive control samples

	Anti-AAV6 positive control samples (in neat NCS)		
AAV6 TI assay	ADK6 (ng/mL)	Positive human serum pool (titer)	Purified human IgG from positive sample (mg/mL)
Fluc	1180[a]	1:29	68.3
Nluc	508	1:131	14.3

ADK6, purified mouse monoclonal anti-AAV6 antibody.
[a]Data from previous publication.[13]

However, we found that the intra-replicate coefficient of variation (CV) was frequently higher than 30% at an MOI of 50, although the Nluc luciferase expression levels were greater than 1E5 RLU (Figure S1B), still approximately 100-fold higher than the background signal. The assay variability was improved with an MOI of 200, and further improved with an MOI of 1,000, with approximately 92% of samples showing an intra-replicate CV of less than 30% based on an evaluation of 104 commercial healthy human sera in 6 runs (data not shown). We speculated that a sporadic high intra-replicate CV found with an MOI of 50 could be caused by random viral non-specific adsorption to assay containers and/or small pipetting variability amplified by low viral concentrations. To ensure intra-replicate precision, we selected an MOI of 1,000 for the final assay condition.

Matrix components in serum samples may interfere with the detection of the anti-AAV6-neutralizing activities. We therefore followed the U.S. Food and Drug Administration immunogenicity assay development guidance and optimized the sample minimal required dilution (MRD) to decrease such interference while maintaining sufficient assay sensitivity suitable for differentiating positive from negative samples.[17] Based on titration profiles of individual naive human serum samples and performance of spiked commercial anti-AAV6 antibody in negative control serum (NCS), we selected MRD 20 (10-fold dilution of serum samples in dilution medium followed by a 2-fold dilution with reporter virus) as the final condition to achieve optimal assay performance (data not shown).

With the selected MOI for AAV6-Nluc reporter virus and sample MRD, we conducted assay qualification to evaluate assay cutoff, sensitivity, precision, specificity, and selectivity. The assay cutoff was determined using 112 healthy human sera consisting of mixed gender, ethnicity, and U.S. geographic origins. All donor samples were evaluated following a balanced design of assay runs per industry recommendation for development of immunogenicity assays.[18,19] As shown previously for the Fluc TI assay,[13] the Nluc TI assay normalized response (NR) of the 112 healthy donors also showed a bimodal distribution (Figure S2). The population with high NR values was considered as low or free of anti-AAV6 neutralizing activities and used for TI assay cutoff calculations by B2S Life Sciences (Franklin, IN, USA) using a statistical method described previously.[13] The parametric NR cutoff of 0.2 was derived after applying a 0.1% false-positive error rate and inclusion of minimum significant ratio metric. This cutoff was used during assay qualification for parameter evaluations.

The Nluc TI assay can detect the positive control monoclonal antibody ADK6 at a concentration of 17.9 ng/mL with good intra- and inter-assay precision of less than 25% CV. Assay specificity was examined using an irrelevant anti-GFP antibody that showed no inhibition of AAV6-Nluc transduction at 25 ng/mL. Last, assay selectivity was demonstrated in individual seronegative sera spiked with ADK6 at 18 ng/mL or unspiked (see the assay qualification results summarized in Table S1).

To further confirm the specificity of the neutralizing activity, we conjugated AAV6 empty capsids produced from HEK293 cells to magnetic beads and pre-incubated with a human serum pool containing high levels of anti-AAV6 neutralizing activity to deplete neutralizing factors. The AAV6 neutralizing activity in the depleted sample was significantly decreased compared with the untreated serum pool (Figure S3), suggesting that the Nluc TI assay detects anti-AAV6-neutralizing activities with minimal effect from other matrix components.

Evaluation of sensitivity for AAV6 TI assays using Fluc and Nluc reporters

During assay qualification, the sensitivity of AAV6-Nluc TI assay was determined using an anti-AAV6 mouse hybridoma supernatant from clone ADK6, based on the vendor-provided antibody concentration. Once the protein A/G-purified ADK6 monoclonal antibody was available from the vendor, we reassessed the sensitivity of the Nluc TI assay to be 508 ng/mL (Table 1), which is approximately 2-fold more sensitive than the reported clinical screening assay using the Fluc reporter and the same purified ADK6 clone.[13] To further understand the impact of the luciferase reporters on assay sensitivity, we compared the two methods by titrating two positive sample reagents containing polyclonal anti-AAV6 antibodies, including a pool of anti-AAV6 seropositive human sera and the purified immunoglobin G (IgG) from a human serum with a strong anti-AAV6 TI activity. The Nluc TI assay was 4- to 5-fold more sensitive than the Fluc TI assay at the assay cutoffs of 0.20 and 0.34, respectively. Therefore, our results from three positive controls suggested that the Nluc assay is more sensitive than the Fluc TI assay (Table 1).

The two TI assays differed in multiple conditions, including MOI, reporter construct, cell line, and the sample MRD, which prompted us to further investigate the impact of these and other assay conditions on AAV6 TI assay performance.

Impact of reporter MOIs, reporter constructs, and cell lines on detection of AAV6 neutralizing activity

To identify the key assay parameters affecting detection of AAV6-neutralizing activities, commercial serum samples from 60 healthy individuals and/or 56 factor VIII (FVIII)-deficient hemophilia A donors were tested under various conditions.

We first evaluated how reporter viral copy concentrations affect AAV6-Nluc assay sensitivity using human intravenous immunoglobulin (IVIG) and tested MOIs ranging from 200 to 10,000. MOIs of greater than 10,000 would produce a signal outside of the instrument

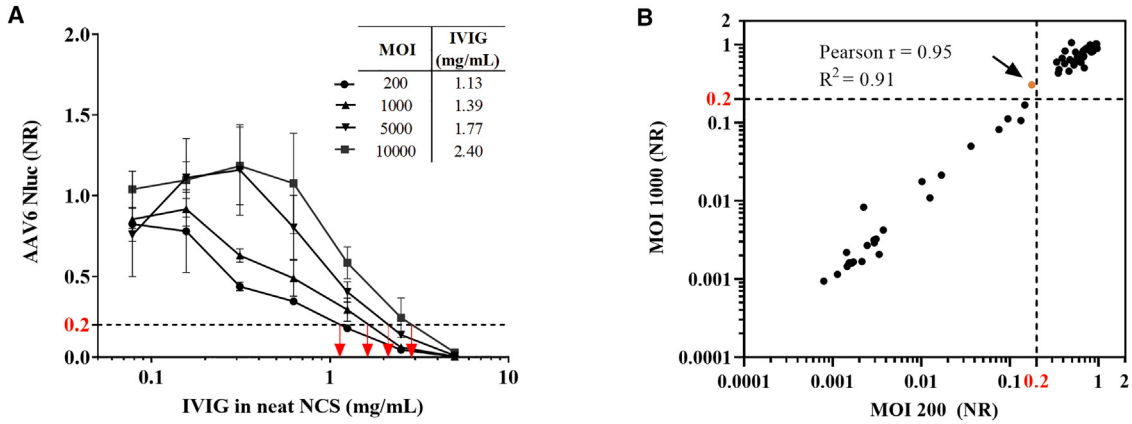

Figure 2. Impact of reporter virus concentrations on AAV6 Nluc TI assay sensitivity and seroprevalence evaluation

(A) Human IVIG was serially diluted in an NCS pool and tested in the AAV6 Nluc TI assay using different MOIs of the reporter vector. TI assay results are presented as the mean ± SD of the NR from duplicate wells in one representative experiment out of two independent runs. The IVIG concentrations yielding 80% of TI (NR cutoff of 0.2) for the respective MOI conditions are indicated by the red arrows. The mean IVIG concentrations at the NR cutoff from the two independent runs are presented in the inset. (B) Correlation of AAV6-Nluc TI assay results for 60 commercial human sera. Each sample was tested using AAV6 Nluc reporter at MOIs of 200 and 1,000. The black arrow indicates one sample (the orange dot) with discordant AAV6 NAb seropositivity between two assay conditions using an NR cutoff of 0.2.

detection range, therefore these levels were not tested. Although higher MOI seemed to yield a lower sensitivity (Figure 2A) when comparing MOIs of 200 versus 1,000 at the middle and linear ranges of the titration curve (Figure 1B, red curve), seropositivity of 60 human donors were highly correlated (Pearson r = 0.95, R^2 = 0.91). Only one sample showed a discrepancy of being positive (close to the NR cutoff of 0.2) for an MOI of 200 but negative for an MOI of 1,000, possibly because of a small difference in sensitivities (1.13 mg/mL for an MOI of 200 vs. 1.39 mg/mL for an MOI of 1,000) (Figure 2B). These results suggest that MOIs within the optimal range do not significantly impact AAV-neutralizing activity detection in individual serum samples.

The AAV6 TI assay was also conducted using two different reporter constructs (Nluc at an MOI of 1,000 or Fluc at an MOI of 5E5) to transduce the same cell line (HT1080). Thirteen of 56 human serum samples (23.2%) determined to be positive in the Nluc assay tested negative in the Fluc assay (Figure 3, orange dots). This discordance was likely related to the Nluc TI assay having a higher sensitivity than the Fluc assay, as indicated in Table 1. Consistent with this hypothesis, no sample tested negative in the Nluc assay but positive in the Fluc TI assay.

To evaluate the impact of cell line on NAb detection, the AAV6 Nluc assay was performed using different cell lines, including HT1080 (the selected cell line for the Nluc TI assay), U-87MG (used in the previously reported Fluc assay),[13] and 293T (the most used cell line in TI assay).[20] The NR values from all three cell lines transduced with the same amount of Nluc reporter virus (MOI of 1,000) exhibited good correlation (Figures 4A and 4B), even though 293T cells yielded more than 10-fold lower RLU signals than the HT1080 and U-87MG cells (Figure S1B). Only 5 of 116 samples (4.3%) were identified as different seropositivity status using different cell lines, two of which (D3 and D5) may be due to assay variability since the NR values were close to the cutoff of 0.2 (Figure 4C).

Impact of viral production systems and empty capsids on AAV6 TI assay performance

Triple plasmid transfection of HEK293 and baculovirus transduced Sf9 (Sf9/rBV) cells are currently the most common AAV production systems for gene therapy. Compared with the Sf9/rBV system, the HEK293 system is easier and faster for producing reporter viruses to facilitate early-stage AAV TI assay development. The Sf9/rBV system has been increasingly used to manufacture AAV vectors for use in clinical studies due to advantages of lower empty/full capsid ratios and better yields and scalability.[12] To determine if AAV reporters produced from the two systems are equivalent in neutralizing activity detection, we tested 60 commercial human serum samples using the two AAV6 reporter viruses. Although the HEK293-produced reporter vector seemed to be more potent than the Sf9/rBV-produced vector (generating an approximately 2.5-fold higher RLU signal) (Figure 1B), AAV6 NR values measured by both reporter viruses were highly correlated (Pearson r = 0.98) and showed concordant seropositivity for all samples (Figure 5A).

During recombinant AAV vector production, empty capsids (referred to in this paper as partially encapsulated or absence of transgene genome) can represent a significant portion of total capsids, although the Sf9/rBV system may produce lower levels of empty capsids than the HEK293 cell production system. Depending on the purification methods, the abundance of empty capsids can be as high as 10-fold of the full capsid product,[21,22] or nearly absent. We measured the full/empty capsid ratio of the two AAV6-Nluc vectors produced from the HEK293 and Sf9/rBV systems by anion-exchange HPLC and found comparable 10%–20% empty capsids in both reporter viruses (data not shown).[23] To test whether increased empty capsid

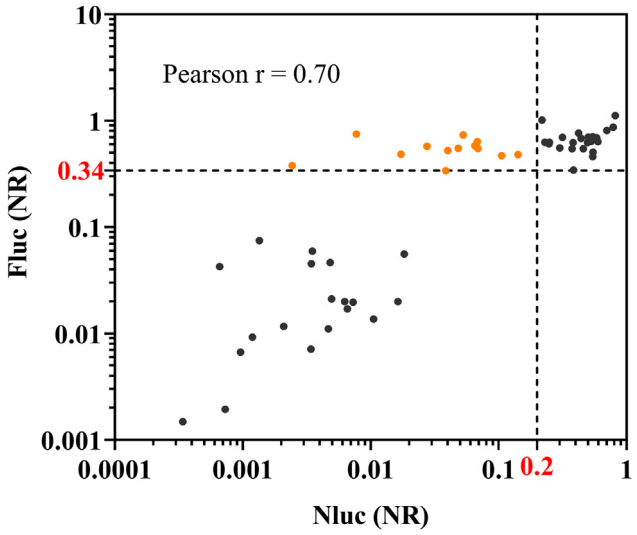

Figure 3. Impact of reporter constructs on detection of AAV6 neutralizing activity

Commercial human serum samples from 56 donors were tested using AAV6 Fluc and Nluc reporters. The NR values from both AAV6 TI assays were correlated using cutoffs of 0.34 and 0.20, respectively. The orange dots represent discordant serum samples, all of which are AAV6-Nluc positive and AAV6-Fluc negative.

impurity could change the AAV6 TI assay readout, a range of AAV6 empty capsids were spiked into a fixed concentration of AAV6-Nluc reporter produced from the HEK293 system and its impact on the reporter transduction at an MOI of 1,000 was assessed. There were no significant differences in transduction signals in the presence of empty capsids spiked at a range of 20%–100% of the reporter capsids compared with the reporter virus transduction alone (Figure 5B). We also evaluated 60 human donor sera using AAV6-Nluc with empty capsids spiked in at 80% of the Nluc reporter capsids (44% of the total capsids) and showed that 58 of 60 had concordance between the two reporter assay readouts (Pearson r = 0.94). The two subjects scored differently had NR values close to the cutoff of 0.2 (0.16 vs. 0.23 and 0.32 vs. 0.17), which most likely was due to assay variability (Figure 5C).

Detection of neutralizing activity against different AAV serotypes using Nluc reporter for seroprevalence comparison

The Nluc reporter construct used in the AAV6 TI assay can be easily adapted to other AAV serotypes for seroprevalence studies. The Nluc reporter construct was packaged into respective capsids for AAV5, 8, and 9 in HEK293 cells and an MOI titration was conducted for each reporter virus. MOIs of 7.6E3, 1E4, and 1.5E4 vector copies/cell were selected for AAV5-, 8-, and 9-Nluc reporters, respectively, to ensure Nluc expression levels within an optimal RLU range of 1E5 to 1E7 (Figure 6A). As a proof of concept, IVIG was titrated in the four TI assays with different serotypes. Based on the IVIG concentration corresponding with an NR cutoff of 0.2, the relative neutralizing activity against the four serotypes was estimated to be AAV6 > AAV9 ≈ AAV8 > AAV5 (Figure 6B). In addition, the prevalence of pre-existing neutralizing activity was evaluated in a second population of 112 healthy human serum samples different from the panel used for AAV6-Nluc TI assay qualification (Figure S2) and showed the neutralizing activity seroprevalence for AAV5, 8, and 9 as 40%, 42%, and 40%, respectively, which was relatively lower than that for AAV6 (57%) (Figure 6C). It is worth noting that 42 of the 112 donors (38%) were anti-AAV TI positive for all four serotypes, while 13 of 112 donors (12%) were TI positive for only one serotype (AAV6) (data not shown), suggesting significant co-prevalence and/or cross-reactivity of AAV antibodies against different serotypes even within individual donors, which is consistent with the previous report.[24]

Impact of assay sensitivity and TAb assay formats on seropositivity concordance

To understand the correlation of the AAV6 TI and TAb assay readouts, two TAb assay formats were evaluated using either direct-binding colorimetric ELISA or an ECL-based bridging assay method (Figure 7A). The direct binding ELISA has comparable sensitivity, while the ECL bridging TAb assay is much more sensitive than the Nluc TI assay (data not shown). AAV6 antibody seropositivity of 60 human donor sera detected by the Nluc TI assay were compared with each of the two TAb assay results. Of the test population, 54 donor samples (90%) were either double positive or double negative in both the Nluc TI and the direct-binding ELISA, while only 39 donors (65%) showed concordant seropositivity by the Nluc TI and the ECL bridging TAb assay (Figure 7B). Therefore, the Nluc TI assay had better concordance with the direct-binding ELISA than with the ECL bridging TAb assay.

The upper right quadrant for TAb (+)/TI (−) samples represents non-NAbs. The ECL bridging TAb assay detected more TAb (+)/TI (−) discordant donors than the direct-binding ELISA (16 vs. 5, or 26.7% vs. 8.3% in the upper right quadrant, Figure 7B), which is consistent with higher sensitivity of the ECL bridging TAb assay than the direct-binding ELISA. The lower left quadrant for TAb (−)/TI (+) may represent non-antibody neutralizing factors that can be detected by the AAV6 TI assay as previously reported.[9] Only one TAb (−)/TI (+) donor was not detected by the direct-binding ELISA (Figure 7C, D1), while five were not detected by the ECL bridging TAb assay, even though it was more sensitive (Figure 7C, D2–D6). Interestingly, one discordant sample with a Nluc TI assay titer of 1:865 and direct-binding ELISA signal of 11.95 (significantly higher than the TAb assay cutoff of 1.73) was not detected by the ECL bridging assay (Figure 7C, D4). These results suggested a differential antibody detectability of the two AAV6 TAb assay formats, which in turn impacted the seropositivity concordance with the Nluc TI assay readouts.

DISCUSSION

A qualified AAV6 TI assay was presented using a Nluc reporter expression construct, which produces a higher luminescence signal than a regular Fluc reporter construct. This signal increase allowed a lower MOI and wider dynamic range, resulting in an improved

A

Positive Sample ID	AAV6-Nluc NR (<0.2)		
	HT1080	U-87 MG	293T
POS 1	0.0003		
POS 2			
POS 3			
POS 4			
POS 5			
POS 6			
POS 7			
POS 8			
POS 9			
POS 10			
POS 11			
POS 12			
POS 13			
POS 14			
POS 15			
POS 16			
POS 17			
POS 18			
POS 19			
POS 20			
POS 21			
POS 22			
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POS 34			
POS 35			
POS 36			
POS 37			
POS 38			
POS 39			
POS 40			
POS 41			
POS 42			
POS 43			
POS 44			
POS 45			
POS 46			
POS 47			
POS 48			
POS 49			
POS 50			0.19
POS 51			
POS 52			
POS 53			
POS 54			
POS 55			
POS 56			
POS 57			

B

Negative Sample ID	AAV6-Nluc NR (≥0.2)		
	HT1080	U-87 MG	293T
NEG 1			
NEG 2			
NEG 3			0.21
NEG 4			
NEG 5			
NEG 6			
NEG 7			
NEG 8			
NEG 9			
NEG 10			
NEG 11			
NEG 12			
NEG 13			
NEG 14			
NEG 15			
NEG 16			
NEG 17			
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NEG 34			
NEG 35			
NEG 36			
NEG 37			
NEG 38			
NEG 39			1.35
NEG 40			
NEG 41			
NEG 42			
NEG 43			
NEG 44			
NEG 45			
NEG 46			
NEG 47			
NEG 48			
NEG 49			
NEG 50			
NEG 51			
NEG 52			
NEG 53			
NEG 54			

C

Discordant Samples	HT1080 (NR)	NAb Status	U-87 MG (NR)	NAb Status	293T (NR)	NAb Status
D1	0.04	POS	0.09	POS	0.28	NEG
D2	0.05	POS	0.06	POS	0.21	NEG
D3	0.23	NEG	0.19	POS	0.25	NEG
D4	0.42	NEG	0.05	POS	0.72	NEG
D5	0.14	POS	0.21	NEG	0.22	NEG

Figure 4. Impact of cell lines on detection of AAV6 neutralizing activity

Commercial human serum samples from 116 donors were tested for anti-AAV6 neutralizing activity. Three cell lines (HT1080, U-87 MG, and 293T) were compared using AAV6 Nluc TI assay. (A) Fifty-seven positive samples with all three cell lines. (B) Fifty-four negative samples with all three cell lines. (C) Five discordant samples with respective NR values and NAb status shown for all three cell lines. The bar sizes correlate with the NR values in respective sample tables, with red bars for positive samples, green bars for negative samples, and orange bars for discordant samples. Note that only the lowest and highest NR values in the positive (A) and negative (B) sample tables were presented. D, discordant; NEG, negative; POS, positive.

assay sensitivity compared with the AAV6 Fluc TI assay.[13] In addition, the Nluc reporter construct was packaged with capsids for other serotypes AAV5, 8, and 9 and MOIs were selected for each viral reporter transduction based on comparable RLU signal ranges across serotypes. This enabled the cross-serotype seroprevalence comparison in a more standardized manner. Variables such as of cell line, reporter viral transduction concentration, reporter viral production cell system, and empty capsid content were evaluated to better understand conditions and assay reagents that may affect pre-existing AAV neutralizing activity detection. Our results indicate that TI assay sensitivity and detectability of neutralizing activity largely depend on the reporter construct, and are less impacted by viral production system, empty capsid content in reporter vector, and cell line, provided that the cell line has reasonable transduction efficiency. Furthermore, to address TAb and TI assay correlation, a comparison of seropositivity of individual donors detected by the Nluc TI assay to those measured by two AAV6 TAb assays with different formats (direct binding vs. bridging) and detection technologies (ELISA vs. ECL) was conducted. With appropriate assay format and sensitivity, pre-existing AAV6 antibodies detected by TAb or TI assays can be highly concordant.

AAV TAb and TI assay sensitivity can be determined by using anti-AAV capsid monoclonal and/or polyclonal antibodies that are either commercially available or proprietarily produced. The three positive controls used in this study included a monoclonal antibody specific to AAV6, a pool of AAV6 NAb-positive human sera, and immunoglobins purified from an AAV6 NAb-positive serum using protein A/G. The sensitivities of the Nluc compared with the Fluc TI assay determined using these positive controls varied between 2.2- and 5-fold. This difference may be attributed to monoclonal antibodies not representing all epitopes and/or affinities of natural anti-AAV6 antibodies existing in individual human donors for making the positive controls. It is worth noting that positive control antibodies are only used for assay characterization and in-study assay performance evaluation. Compared with polyclonal antibody controls, monoclonal antibodies have the advantages of better lot-to-lot consistency and availability in large quantities when used as positive controls for long-term monitoring of TI assay performance.

As shown in this study, although the sensitivities of AAV6 Nluc TI assay determined by IVIG decreased slightly as MOIs increased from 200 to 10,000, the seropositivity status of individual human donors were highly concordant as tested at MOIs of 200 and 1,000, suggesting that MOI selections in the linear range can produce similar results. It is expected that high MOIs in the saturation phase will

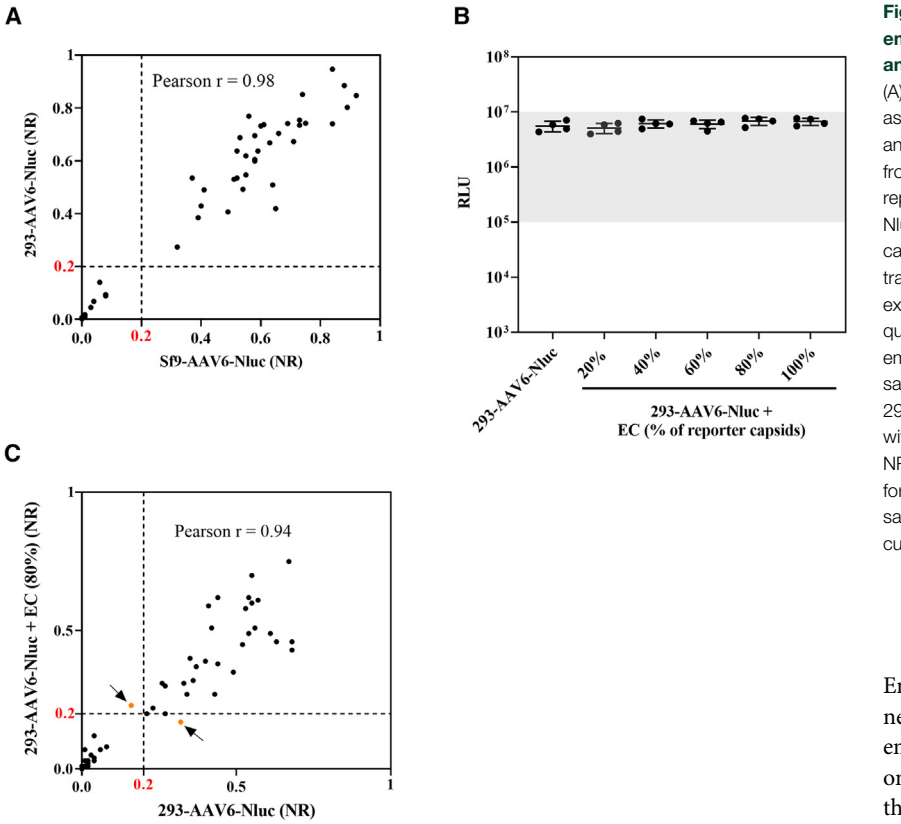


Figure 5. Impact of viral production cell systems and empty capsid contents on AAV6-Nluc transduction and seroprevalence evaluation

(A) Sixty human donor sera were tested in the AAV6 Nluc TI assay, using reporter vectors produced from the HEK293 and the Sf9/rBV systems. The NR values of each sample from both tests showed good correlation. (B) AAV6-Nluc reporter produced from the HEK293 system (293-AAV6-Nluc) were tested alone or in the presence of empty capsids with indicated ratios to the reporter capsids for transduction efficiency. The luminescence signals of expressed Nlucs are presented as means ± SD of quadruplicates and showed no effect on transduction by empty capsids compared with the reporter only. (C) The same 60 human donor sera from (A) were tested in the 293-AAV6-Nluc TI assay, using the reporter only or spiked with empty capsids at 80% of the reporter capsids. The NR values of each sample from both tests were plotted for correlation. Black arrows indicate that only two samples (the orange dots) with NR values close to the cutoff of 0.2 had discordant AAV6 NAb status.

render poor assay sensitivity. It is worth noting that the ultimate TI assay sensitivity required for patient enrollment in AAV gene therapy needs to take into account multiple factors, including serotypes, vector doses, and administration routes, as well as target tissues and therapeutic indications. The successful achievement of therapeutic efficacy in patients enrolled by the less sensitive TI assays validated the clinical relevance of their applications in patient enrollment.[13,14]

While both HEK293 and Sf9/rBV cell systems are commonly used for AAV therapeutic vector production, the HEK293 system is more convenient to be implemented in AAV TI assay reporter virus preparation at the early stage of AAV drug development. AAV reporter vectors produced by the two production systems may have difference in physiochemical attributes and post-translational modifications as recently reported,[25] which may result in different detectability of neutralizing activities. In this work, we evaluated whether production differences impact the AAV6 TI assay results. Consistent with the recent findings on comparable *in vitro* and *in vivo* biological functions of AAV products from both production systems,[25] the seropositivity of 60 human sera detected by the AAV6 Nluc reporters produced from HEK293 and Sf9/rBV systems showed no differences, although the transduction signals differed by approximately 2.5-fold. These results indicate that the reporter virus production system for the anti-AAV antibody screening assay may not need to match that of the clinical AAV product.

Empty capsids are a common impurity component of AAV vector products, and the level of empty capsids in AAV vector production depends on the purification method. Previous studies on the impact of empty capsids are controversial, ranging from no impact to either increased or decreased AAV transduction.[26–28] These discrepancies are likely caused by the different testing systems (*in vivo* vs. *in vitro*) and/or different levels and types of empty capsids present in the assays. The current study detected no significant impact of empty capsids on either AAV6-Nluc transduction efficiency or detection of anti-AAV6 neutralizing activity with empty capsid content up to 44% of total capsids, which suggests that empty capsid impurities at the level tested do not affect the AAV6 TI assay performance.

The impacts of pre-existing AAV immunity on transgene expression have been reported in both preclinical and clinical studies. For example, pre-existing AAV NAbs could inhibit AAV transduction at a titer of 1:1 in human subjects,[2] or completely blocked liver transduction at a titer of 1:5 in non-human primates.[3] Whereas in other studies, transgene expression could be achieved at a therapeutic level with a pre-existing AAV5 NAb titer up to 1:1,030 in non-human primates and 1:340 in human subjects.[14] Multiple parameters, such as serotype-specific titer threshold, AAV dose and route of administration, have been proposed to explain these discrepancies. In addition, method variation across clinical studies is also an important contributing factor. Assays to detect TAbs and/or neutralization activities have been used in clinical enrollment for AAV-based gene therapies.[8,12,13] Both assays have advantages and disadvantages for implementation in a bioanalytical setting supporting gene therapy drug development.[11] The ECL bridging TAb assay format has been reported to screen patients with pre-existing anti-AAV5 antibodies.[9] Here we

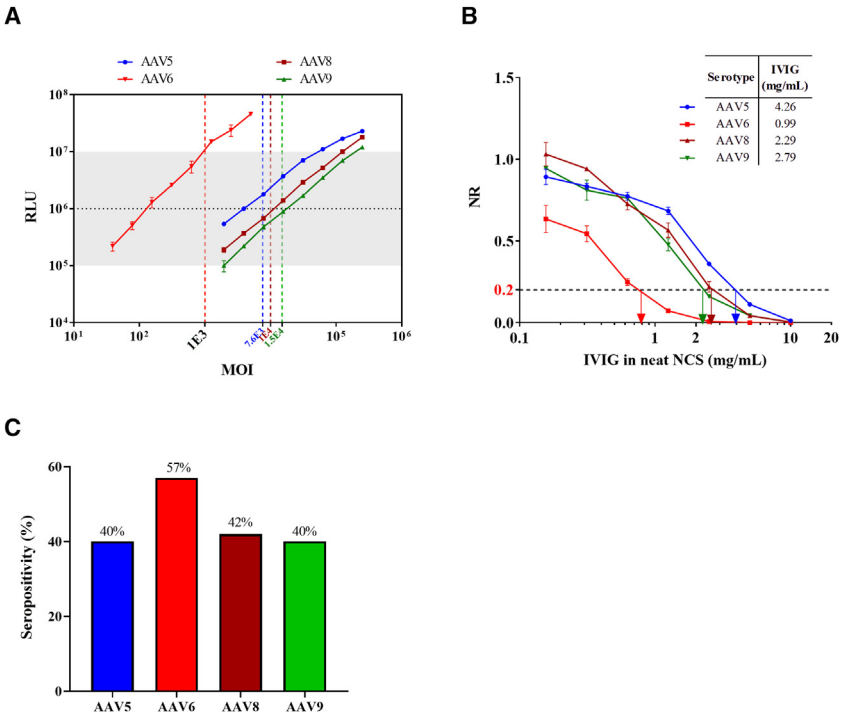


Figure 6. Detection of neutralizing activity against different AAV serotypes using Nluc reporter for seroprevalence comparison

(A) Transduction of HT1080 cell line by AAV5-, 6-, 8-, and 9-Nluc reporters produced from the HEK293 system with titration of reporter viral MOI. Reporter transgene expression was measured by luminescence and expressed as RLUs. The MOIs selected for respective AAV Nluc TI assays yielded luciferase expression within optimal RLU range of 1E5 to 1E7. (B) Human IVIG was serially diluted in NCS pool and tested in respective AAV Nluc TI assays. The IVIG concentrations yielding 80% of TI (correlated with an NR cutoff of 0.2) for respective TI assays are shown in the inset. The luminescence signals of expressed Nluc in (A) and NRs in (B) are presented as the mean ± SD of duplicates in one representative experiment out of two independent runs. The mean IVIG concentrations at the NR cutoff from two independent runs are presented in the inset. (C) 112 healthy donor serum samples were tested in AAV5-, 6-, 8-, and 9-Nluc TI assays for seroprevalence evaluation using an NR cutoff of 0.2.

also developed a direct binding ELISA TAb assay and compared the two TAb assay formats with the Nluc TI assay in detection of pre-existing anti-AAV6 antibodies. The data showed that the direct binding ELISA has better concordance with the Nluc TI assay than the ECL bridging TAb assay. The ECL bridging assay is more sensitive, whereas the direct binding ELISA has similar sensitivity as the Nluc TI assay when measured by the human IgG purified from an AAV6-seropositive human serum (data not shown). Therefore, the discrepancy of 26.7% ECL bridging TAb (+)/TI (−) versus 8.3% ELISA direct-binding TAb (+)/TI (−) is most likely attributed to different assay sensitivity. Even though the ECL bridging TAb is more sensitive than the Nluc TI assay, it detected more discordant TAb (−)/TI (+) samples than the direct binding ELISA (8.3% vs. 1.7%), suggesting that the bridging TAb assay format may be unable to detect some antibody isotypes in the donor serum samples, as discussed further below.

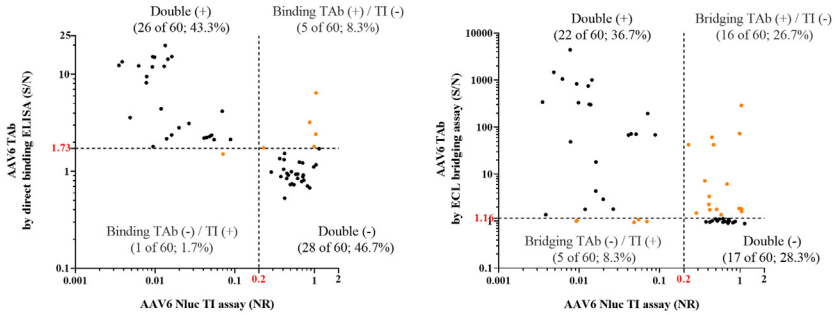
All four IgG subclasses can be produced against AAV serotypes in human populations as a result of natural exposure, with IgG1 as the predominant and IgG4 as the minor subclasses.[29,30] It is known that IgG4 undergoes dynamic Fab arm exchange,[31] and the resulting monovalent IgG4 can be detected by the binding assay, but not the bridging assay, because of its dependence on the bi-valency of the anti-drug antibody. It remains to be characterized if IgG4 represents the main IgG subclass in the anti-AAV6 humoral response of the discordant sample that tested negative in ECL bridging assay yet positive with high NAb TI titer and high ELISA direct-binding TAb signal. Another possibility for the discordance could be the epitope masking of the AAV6 capsids caused by the labeling step required in the bridging TAb, but not needed in the direct-binding TAb assay.

Higher rates of TI (+)/TAb (−) discordancy between ECL bridging TAb and TI assays were reported for anti-AAV5 than anti-AAV6 antibody detection reported here in healthy donors (24% vs. 8.3%), although the ECL bridging TAb and TI assays for AAV5 antibody detection had similar sensitivities,[9] suggesting that the presence of non-antibody neutralizing factors may be serotype dependent. In another report, an ECL-based direct-binding AAV8 TAb confirmatory assay achieved high concordance (approximately 98%) with the cell-based NAb assay that had comparable sensitivities.[32] These results suggested that the direct-binding and bridging assay formats using the same detection technology can still differ in detection of anti-AAV TAbs, although both ECL assays showed comparable sensitivities to their respective AAV TI assays. In addition, correlation between direct-binding TAb and TI assay can also vary. Compared with 90% concordance detected for AAV6 antibodies in this work, higher correlations were reported for AAV5 (99%) and AAV8 (94%), while lower correlations was seen for AAV2 (76%).[24] Taken together, correlations between AAV TI and TAb assay readouts may be serotype specific and can depend on assay sensitivity and assay formats. Additionally, the implementation of a confirmatory step to the screening TAb assay may improve the correlation.[32]

The AAV-binding TAb assay was reported to be universally applicable to all serotypes and used in pre-screening of non-human primates in animal studies and patients in clinic[33]; however, appropriate characterization of the antibody detection reagents is required to ensure that the assay reliably detects at a minimum isotypes IgG and IgM to evaluate recent and longer time exposures. The clinical enrollment assay cutoff often needs to be established based on preclinical animal studies. Since both TI and

A

B



C

TAb (-) Nluc TI (+)	ELISA Binding TAb (S/N)	ECL Bridging TAb (S/N)	Nluc TI Titer
D1	1.51	**195.94**	1:65
D2	**2.13**	1.09	1:124
D3	**2.29**	0.95	1:34
D4	**11.95**	1.00	1:865
D5	**1.80**	1.04	1:40
D6	**4.17**	0.99	1:42

Figure 7. Impact of assay sensitivity and TAb assay formats on TAb and TI correlation

(A) Schematic illustration of assay formats for detection of anti-AAV6 TAb (created with BioRender.com). Plate-coated AAV6 capsids are utilized for capturing the anti-AAV6 antibodies in human serum samples. In the left-side format, the assay signals are detected by addition of HRP-conjugated anti-human IgG and the colorimetric development following addition of HRP substrates. In the right-side format, anti-AAV6 antibodies in human serum samples can bridge the plate-coated and the SULFO-tagged AAV6 capsids, and ECL signals can be measured when the SULFO-TAG is electrochemically stimulated. (B) Sixty human donor sera were tested in direct-binding ELISA and ECL bridging assays. The resultant TAb assay signals were plotted against the AAV6 Nluc TI assay readouts for anti-AAV6 antibody status comparison using assay cutoffs as indicated on the respective axes. Orange dots in each graph indicate the discordant samples. (C) The six discordant donor sera shown in the lower left quadrants of both graphs in (B) are presented. The positive TAb signals (S/N greater than the cutoff for the respective TAb assays) are shown in bold with underlines. HRP, horseradish peroxidase; S/N, sample signal normalized to negative control signal.

bridging TAb assays do not require species-specific reagents (detection antibody and positive controls) and their critical reagents are applicable to both animal and human assays, better translatability of assay performance from animal to human studies can be achieved.

In summary, the sensitive cell-based AAV6 TI assay we reported here allowed the identification of critical TI assay factors for detection of pre-existing AAV6 NAbs in human sera and can be adapted as a unified TI assay format for cross-serotype prevalence comparison. Our analysis of AAV6 TI and TAb assays in two formats revealed that both assay sensitivity and TAb assay formats may impact seropositivity concordance detected by different methods, thus emphasizes the significance of anti-AAV antibody assay standardization. The results further supported a need to conduct comparative analysis of different AAV antibody detection methods and characterize the true factors contributed to seropositivity discrepancy, which will enable the selection of appropriate clinical enrollment methods including companion diagnostic assays, and ensure proper identification of eligible patients for receiving AAV-based gene therapies.

MATERIALS AND METHODS

Cell culture

HT1080, 293T, and U-87MG cells were purchased from ATCC (Manassas, VA, USA). HT1080 and U-87MG cells were cultured in minimum essential media (MEM) (Gibco, Grand Island, NY, USA) and 293T cells in Dulbecco's modified eagle's medium, both of which were supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin-streptomycin (Lonza, Morristown, NJ, USA). All cells were cultivated in a humidified 37°C incubator, 5% CO_2.

AAV luciferase vectors

The Nluc reporter construct contains Nluc (Promega, Sunnyvale, CA, USA) under the control of a cytomegalovirus (CMV) promoter with addition of a 5′-HBB intron and a 3′-mutant WPRE by Gibson cloning. The Fluc reporter contains a *Photinus pyralis* Fluc driven by a CMV promoter. Triple transfection of HEK cells was performed in 10-layer CellSTACK chambers (Corning, Corning, NY, USA) to produce AAV5, 6, 8, and 9 report vectors containing the Nluc construct. A nonclinical, small-scale Sf9/rBV expression system using methods proprietary to contracted research organization (CRO) Virovek (Hayward, CA, USA) was performed to produce AAV6-Nluc and Fluc reporter vectors. All AAV reporter viruses were formulated in buffer containing PBS and 0.001% Pluronic F-68. AAV copy concentration were determined by qPCR.

Human serum samples

Individual human serum samples from healthy donors were purchased from BioIVT (Westbury, NY, USA), Discovery Life Sciences (Los Osos, CA, USA), and Golden West Biologicals (Temecula, CA, USA). Donors varied in ethnicity (Black, White, Hispanic, and Asian), ages, and geographical locations in the United States. Two populations of 112 healthy donors were used for AAV6-Nluc TI assay qualification and AAV seroprevalence tests. Sixty healthy donors were randomly selected from the two populations for additional tests shown in the paper. A population of FVIII-deficient sera from hemophilia A patients were purchased through custom collection from HRF, Inc (Raleigh, NC, USA). All serum samples were tested without heat inactivation.

Positive and negative controls

The pooled positive human sera were selected from healthy donors using AAV6-Nluc TI assay. The purified positive human serum IgG was prepared by Nab protein A/G spin kit (Thermo Fisher Scientific, Waltham, MA, USA) using a positive human serum with high TAb titers. The mouse monoclonal anti-AAV6 antibody (ADK6) was purchased from Progen (Wayne, PA, USA). IVIG was purchased from Carimune NG CSL Behring. NCS for AAV Nluc TI assay was generated by pooling serum from healthy individuals with tested luciferase responses comparable to the measured luciferase responses in the cell culture media (normalized ratio ranged from 0.9 to 1.3). All analyses were performed using sample signals normalized to NCS signals unless otherwise specified in the text and presented as the NR. The positive controls were spiked into NCS stock and subjected to serial dilutions for test in the AAV TI or TAb assays. The assay sensitivity was determined by testing a minimum of three individually prepared titration curves and calculated as the mean concentrations of purified antibodies or the mean dilution folds (reciprocally defined as titers) of pooled human positive control sera at the intersection of respective assay cutoffs using two-point linear regression curve fitting.

AAV TI/NAb assay

The cell-based TI assay (also referred as NAb assay in this study) described in this work varied in cell line, reporter vector, and other assay conditions as mentioned in the text. In general, viable cells in log phase growth and with a passage number no more than 15 were plated at 1E4 or 2E4 cells per well on 96-well culture plates and allowed for 24 ± 2 h growth at 37°C, 5% CO_2 prior to the transfection step. On the transduction day, the serum samples were initially prediluted 5-fold (for Fluc TI assay) or 10-fold (for Nluc TI assay) in dilution media (cell basal growth media with 1% bovine serum albumin [BSA]), followed by another 2-fold dilution in dilution media containing AAV reporter virus at an MOI as indicated in the text (1:1 mix at 75 μL each to reach 150 μL in total volume). The mix of AAV reporter virus and sample were incubated at 37°C, 5% CO_2 for 50 ± 5 min. Following incubation, all media from the plated wells were removed via aspiration. We added 50 μL of complete culture media and 50 μL of the reporter virus/sample mix to the plate in duplicate, bringing final volume to 100 μL. The plates were incubated in a humidified 37°C incubator, 5% CO_2 for 24 ± 2 h. On the following day, for cells transduced with Fluc reporter, 100 μL of One-Glo reagent/buffer mixture (Promega) was added to each well to lyse cells at room temperature for 7–10 min. For cells transduced with the Nluc reporters, the culture media in each well were removed and 50 μL Dulbecco's PBS (DPBS) per well was added to the culture plates followed by 50 μL Nano-Glo luciferase assay reagent (Promega). The cells were lysed for 8–10 min at room temperature with the first 3 min on a microplate shaker. We mirror transferred 100 μL One-Glo reagent lysates or 50 μL Nano-Glo reagent lysates per well to the Corning 96-well white opaque reading plates (MilliporeSigma, Burlington, MA, USA) for reading luminescence on the microplate reader VarioSkan Lux (Thermo Fisher Scientific) or SpectraMax I3X (Molecular Devices, San Jose, CA, USA). The sample luminescence signals were divided by the mean luminescence signals of wells transduced with AAV reporter in the presence of NCS on the same plate for normalization and expressed as NR for reporting.

AAV6 TAb assays

Direct binding ELISA

High binding 96-well microplates (Corning) were coated with 1.5E9 capsids of AAV6 vector in DPBS and incubated at 2°C–8°C overnight. After washing with 300 μL/well PBS containing 0.05% Tween 20 (PBST), the wells were blocked with 200 μL/well PBST containing 0.5% BSA. After three washes with PBST, 100-μL controls and samples were added into each well and incubated for 2 h. After washing with PBST, 100 μL of 20 ng/mL horseradish peroxidase-conjugated goat anti-human IgG (H + L) (Jackson ImmunoResearch, West Grove, PA, USA) prepared in PBST containing 0.5% BSA was added to each well and incubated for 1 h. Antibody binding was detected using TMB 2-Component Microwell Peroxidase Substrate (KPL Protein Research Products, Washington, DC, USA). The plate was read at both 450 and 650 nm. The reading at 650 nm was subtracted from reading at 450 nm for each well and used for data analysis.

ECL bridging assay

The method was developed at a CRO using a method similar to previously reported,[9] except with unlabeled and SULFO-TAG labeled AAV6 capsids as coating and detection reagents, respectively.

Depletion of anti-AAV6 neutralizing activity

AAV6 empty capsid (AAV6-EC) produced from the HEK293 cell system was cross-linked to Pierce NHS-Activated Magnetic Beads (Thermo Fisher Scientific) in KingFisher Fle×96 deep-well magnetic particle processor (Thermo Fisher Scientific). We incubated 25 μL of AAV6-EC coupled magnetic beads with 1 mL of serial dilutions of a pooled serum sample from positive healthy human donors, using automated depletion protocol run on the KingFisher Flex 96 instrument.

DATA AND CODE AVAILABILITY

Data generated can be found within the published article and its supplemental files.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2023.101126.

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AUTHOR CONTRIBUTIONS

Y.P., J.Z., L.C., J.H., and Y.L. contributed to assay development; Y.P., M.R., J.Z., and S.N. performed the experiments; Y.P. and Y.L. designed the experiments and wrote the paper; supervision, Y.P., L.C., K.M., and Y.L. All authors have reviewed and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare the following competing financial interests: Y.P, M.R., J.Z., S.N., L.C., J.H., K.M., and Y.L. are full-time current or former employees and own stocks of Sangamo Therapeutics, Inc.

REFERENCES

1. Manno, C.S., Pierce, G.F., Arruda, V.R., Glader, B., Ragni, M., Rasko, J.J., Ozelo, M.C., Hoots, K., Blatt, P., Konkle, B., et al. (2006). Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat. Med. *12*, 342–347. https://doi.org/10.1038/nm1358.

2. George, L.A., Sullivan, S.K., Giermasz, A., Rasko, J.E.J., Samelson-Jones, B.J., Ducore, J., Cuker, A., Sullivan, L.M., Majumdar, S., Teitel, J., et al. (2017). Hemophilia B Gene Therapy with a High-Specific-Activity Factor IX Variant. N. Engl. J. Med. *377*, 2215–2227. https://doi.org/10.1056/nejmoa1708538.

3. Jiang, H., Couto, L.B., Patarroyo-White, S., Liu, T., Nagy, D., Vargas, J.A., Zhou, S., Scallan, C.D., Sommer, J., Vijay, S., et al. (2006). Effects of transient immunosuppression on adenoassociated, virus-mediated, liver-directed gene transfer in rhesus macaques and implications for human gene therapy. Blood *108*, 3321–3328. https://doi.org/10.1182/blood-2006-04-017913.

4. Zaiss, A.K., Cotter, M.J., White, L.R., Clark, S.A., Wong, N.C.W., Holers, V.M., Bartlett, J.S., and Muruve, D.A. (2008). Complement Is an Essential Component of the Immune Response to Adeno-Associated Virus Vectors. J. Virol. *82*, 2727–2740. https://doi.org/10.1128/jvi.01990-07.

5. Pipe, S., Leebeek, F.W.G., Ferreira, V., Sawyer, E.K., and Pasi, J. (2019). Clinical Considerations for Capsid Choice in the Development of Liver-Targeted AAV-Based Gene Transfer. Mol. Ther. Methods Clin. Dev. *15*, 170–178. https://doi.org/10.1016/j.omtm.2019.08.015.

6. Flotte, T.R. (2020). Revisiting the "new" inflammatory toxicities of adeno-associated virus vectors. Hum. Gene Ther. *31*, 398–399. https://doi.org/10.1089/hum.2020.29117.trf.

7. Chand, D.H., Zaidman, C., Arya, K., Millner, R., Farrar, M.A., Mackie, F.E., Goedeker, N.L., Dharnidharka, V.R., Dandamudi, R., and Reyna, S.P. (2021). Thrombotic Microangiopathy Following Onasemnogene Abeparvovec for Spinal Muscular Atrophy: A Case Series. J. Pediatr. *231*, 265–268. https://doi.org/10.1016/j.jpeds.2020.11.054.

8. Gross, D.A., Tedesco, N., Leborgne, C., and Ronzitti, G. (2022). Overcoming the Challenges Imposed by Humoral Immunity to AAV Vectors to Achieve Safe and Efficient Gene Transfer in Seropositive Patients. Front. Immunol. *13*, 857276–857312. https://doi.org/10.3389/fimmu.2022.857276.

9. Falese, L., Sandza, K., Yates, B., Triffault, S., Gangar, S., Long, B., Tsuruda, L., Carter, B., Vettermann, C., Zoog, S.J., and Fong, S. (2017). Strategy to detect pre-existing immunity to AAV gene therapy. Gene Ther. *24*, 768–778. https://doi.org/10.1038/gt.2017.95.

10. Gardner, M.R., Mendes, D.E., Muniz, C.P., Martinez-Navio, J.M., Fuchs, S.P., Gao, G., and Desrosiers, R.C. (2022). High concordance of ELISA and neutralization assays allows for the detection of antibodies to individual AAV serotypes. Mol. Ther. Methods Clin. Dev. *24*, 199–206. https://doi.org/10.1016/j.omtm.2022.01.003.

11. Schulz, M., Levy, D.I., Petropoulos, C.J., Bashirians, G., Winburn, I., Mahn, M., Somanathan, S., Cheng, S.H., and Byrne, B.J. (2023). Binding and neutralizing anti-AAV antibodies: Detection and implications for rAAV-mediated gene therapy. Mol. Ther. *31*, 616–630. https://doi.org/10.1016/j.ymthe.2023.01.010.

12. Shen, W., Liu, S., and Ou, L. (2022). rAAV immunogenicity, toxicity, and durability in 255 clinical trials: A meta-analysis. Front. Immunol. *13*, 1001263–1001313. https://doi.org/10.3389/fimmu.2022.1001263.

13. Cao, L., Ledeboer, A., Pan, Y., Lu, Y., and Meyer, K. (2023). Clinical enrollment assay to detect preexisting neutralizing antibodies to AAV6 with demonstrated transgene expression in gene therapy trials. Gene Ther. *30*, 150–159. https://doi.org/10.1038/s41434-022-00353-2.

14. Majowicz, A., Nijmeijer, B., Lampen, M.H., Spronck, L., de Haan, M., Petry, H., van Deventer, S.J., Meyer, C., Tangelder, M., and Ferreira, V. (2019). Therapeutic hFIX Activity Achieved after Single AAV5-hFIX Treatment in Hemophilia B Patients and NHPs with Pre-existing Anti-AAV5 NABs. Mol. Ther. Methods Clin. Dev. *14*, 27–36. https://doi.org/10.1016/j.omtm.2019.05.009.

15. England, C.G., Ehlerding, E.B., and Cai, W. (2016). NanoLuc: A Small Luciferase is Brightening up the Field of Bioluminescence. Bioconjug. Chem. *27*, 1175–1187. https://doi.org/10.1021/acs.bioconjchem.6b00112.

16. Yasuda, M., Huston, M.W., Pagant, S., Gan, L., St Martin, S., Sproul, S., Richards, D., Ballaron, S., Hettini, K., Ledeboer, A., et al. (2020). AAV2/6 Gene Therapy in a Murine Model of Fabry Disease Results in Supraphysiological Enzyme Activity and Effective Substrate Reduction. Mol. Ther. Methods Clin. Dev. *18*, 607–619. https://doi.org/10.1016/j.omtm.2020.07.002.

17. FDA (2019). Immunogenicity testing of therapeutic protein products —developing and validating assays for anti-drug antibody detection. Guidance for Industry, 1–33.

18. Shankar, G., Devanarayan, V., Amaravadi, L., Barrett, Y.C., Bowsher, R., Finco-Kent, D., Fiscella, M., Gorovits, B., Kirschner, S., Moxness, M., et al. (2008). Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. J. Pharm. Biomed. Anal. *48*, 1267–1281. https://doi.org/10.1016/j.jpba.2008.09.020.

19. Gupta, S., Devanarayan, V., Finco, D., Gunn, G.R., 3rd, Kirshner, S., Richards, S., Rup, B., Song, A., and Subramanyam, M. (2011). Recommendations for the validation of cell-based assays used for the detection of neutralizing antibody immune responses elicited against biological therapeutics. J. Pharm. Biomed. Anal. *55*, 878–888. https://doi.org/10.1016/j.jpba.2011.03.038.

20. Gorovits, B., Fiscella, M., Havert, M., Koren, E., Long, B., Milton, M., and Purushothama, S. (2020). Recommendations for the Development of Cell-Based Anti-Viral Vector Neutralizing Antibody Assays. AAPS J. *22*, 24. https://doi.org/10.1208/s12248-019-0403-1.

21. Nathwani, A.C., Tuddenham, E.G.D., Rangarajan, S., Rosales, C., McIntosh, J., Linch, D.C., Chowdary, P., Riddell, A., Pie, A.J., Harrington, C., et al. (2011). Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. N. Engl. J. Med. *365*, 2357–2365. https://doi.org/10.1056/NEJMoa1108046.

22. Wright, J.F. (2014). AAV empty capsids: For better or for worse? Mol. Ther. *22*, 1–2. https://doi.org/10.1038/mt.2013.268.

23. Khatwani, S.L., Pavlova, A., and Pirot, Z. (2021). Anion-exchange HPLC assay for separation and quantification of empty and full capsids in multiple adeno-associated virus serotypes. Mol. Ther. Methods Clin. Dev. *21*, 548–558. https://doi.org/10.1016/j.omtm.2021.04.003.

24. Kruzik, A., Fetahagic, D., Hartlieb, B., Dorn, S., Koppensteiner, H., Horling, F.M., Scheiflinger, F., Reipert, B.M., and de la Rosa, M. (2019). Prevalence of Anti-Adeno-Associated Virus Immune Responses in International Cohorts of Healthy Donors. Mol. Ther. Methods Clin. Dev. *14*, 126–133. https://doi.org/10.1016/j.omtm.2019.05.014.

25. Liu, S.S., Chen, H., Li, J., Peraramelli, S., Luo, N., Chen, A., Dai, M., Liu, F., Yu, Y., Leib, R.D., et al. (2023). Systematic Comparison of rAAV Vectors Manufactured by Large-Scale Suspension Cultures of Sf9 and HEK293 Cells. Mol. Ther. *31*, 60.

26. Baatartsogt, N., Kashiwakura, Y., Hayakawa, M., Kamoshita, N., Hiramoto, T., Mizukami, H., and Ohmori, T. (2021). A sensitive and reproducible cell-based assay via secNanoLuc to detect neutralizing antibody against adeno-associated virus vector capsid. Mol. Ther. Methods Clin. Dev. *22*, 162–171. https://doi.org/10.1016/j.omtm.2021.06.004.

27. Mingozzi, F., Anguela, X.M., Pavani, G., Chen, Y., Davidson, R.J., Hui, D.J., Yazicioglu, M., Elkouby, L., Hinderer, C.J., Faella, A., et al. (2013). Overcoming pre-existing humoral immunity to AAV using capsid decoys. Sci. Transl. Med. *5*, 194ra92. https://doi.org/10.1126/scitranslmed.3005795.

28. Gao, K., Li, M., Zhong, L., Su, Q., Li, J., Li, S., He, R., Zhang, Y., Hendricks, G., Wang, J., and Gao, G. (2014). Empty virions in AAV8 vector preparations reduce transduction efficiency and may cause total viral particle dose-limiting side effects. Mol. Ther. Methods Clin. Dev. *1*, 20139. https://doi.org/10.1038/mtm.2013.9.

29. Boutin, S., Monteilhet, V., Veron, P., Leborgne, C., Benveniste, O., Montus, M.F., and Masurier, C. (2010). Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: Implications for gene therapy using AAV vectors. Hum. Gene Ther. *21*, 704–712. https://doi.org/10.1089/hum.2009.182.

30. Murphy, S.L., Li, H., Mingozzi, F., Sabatino, D.E., Hui, D.J., Edmonson, S.A., and High, K.A. (2009). Diverse IgG subclass responses to adeno-associated virus infection and vector administration. J. Med. Virol. *81*, 65–74. https://doi.org/10.1002/jmv.21360.

31. van der Neut Kolfschoten, M., Schuurman, J., Losen, M., Bleeker, W.K., Martínez-Martínez, P., Vermeulen, E., den Bleker, T.H., Wiegman, L., Vink, T., Aarden, L.A., et al. (2007). Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. Science *317*, 1554–1557. https://doi.org/10.1126/science.1144603.

32. Dai, Y., Dong, H., Gleason, C., Mora, J., Kolaitis, G., Balasubramanian, N., Surapaneni, S., Kozhich, A., and Jawa, V. (2023). Comparison of Pre-existing Anti-AAV8 Total Antibody Screening and Confirmatory Assays with a Cell-Based Neutralizing Assay in Normal Human Serum. AAPS J. *25*, 35. https://doi.org/10.1208/s12248-023-00805-6.

33. Haar, J., Blazevic, D., Strobel, B., Kreuz, S., and Michelfelder, S. (2022). MSD-based assays facilitate a rapid and quantitative serostatus profiling for the presence of anti-AAV antibodies. Mol. Ther. Methods Clin. Dev. *25*, 360–369. https://doi.org/10.1016/j.omtm.2022.04.008.