Validation of Anti-Adeno Associated Virus Serotype rh10 (AAVrh.10) Total and Neutralizing Antibody Immunogenicity Assays

POSTER #873

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BACKGROUND AND PURPOSE

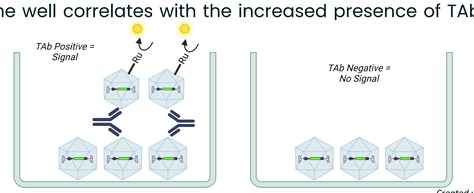
Immunogenicity assessments as inclusion/exclusion criteria to predict investigative drug safety and efficacy are critical in the development of Adeno-Associated Virus (AAV)-based gene therapies. Although multiple assay formats which measure distinct endpoints (i.e., binding versus neutralizing antibody activity) are applicable and being used in this context, it is not clear which assays should be selected and at what stage of drug development these methods should be implemented.

Here, we provide data for the validation of a total antibody (TAb) assay and a neutralizing antibody (NAb) assay for detection of anti-AAVrh.10 antibodies with the intent to use these assays for screening patients prior to enrollment in an AAVrh.10 gene therapy clinical study. The AAVrh.10 TAb and NAb validation data sets are compared in terms of minimum sample dilution, cut point, assay sensitivity, drug tolerance, and precision. Further, a concordance analysis was performed to determine if the assays produced similar results when used for screening a population of potential patients. This comparison is meant to provide insight into the utility of each assay as a potential tool for inclusion of select individuals into clinical studies.

METHODS – *TAB ASSAY*

The TAb assay was a sequential bridging ECLIA method. Briefly, MSD standard binding plates were coated with unlabeled AAVrh.10 for capture, followed by blocking then incubation with human serum and detection with a ruthenium labeled AAVrh.10-Luc (Ru-AAV) and MSD Read Buffer T. Increase in signal within the well correlates with the increased presence of TAb in the sample.

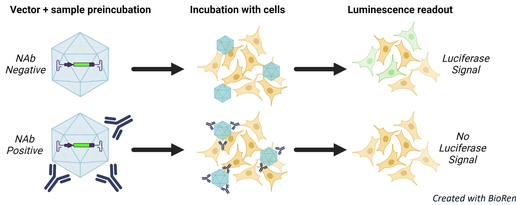
Figure 1. Method Schematic for TAb assay. TAb present in human serum samples binds to the AAV on the plate and is detected with Ru-AAV, resulting in a signal.



METHODS – *NAB ASSAY*

For the NAb assay, an AAVrh.10 vector containing a firefly luciferase reporter gene was applied to a transduction permissive cell line, 2V6.11. Cells were seeded in 96-well Poly-D-Lysine coated plates and incubated overnight at 37ºC/5% CO_2. The next day, AAVrh.10-Luc vector was mixed with human serum samples, added to the cell plate and placed back in the incubator for 24hrs. After incubation, Promega One-Glo was added to the plate before measuring luminescence on a BioTek Cytation3 plate reader.

Figure 2. Method Schematic for NAb assay. The presence of NAb in human serum samples inhibits the luminescence activity of the virus, thereby reducing signal in the assay



RESULTS – *ASSAY VALIDATION DATA SETS*

Both assay formats (TAb and NAb) were validated to evaluate cut point (CP) determinations, sensitivity, precision, drug tolerance, and selectivity. The validation performance parameters of the two assays are summarized in Table 1. Both the TAb and the NAb assays screening CPs were determined in accordance with the 2019 FDA immunogenicity guidance. A summary of the calculations and values are provided in Table 2 and additional data from the CP data sets are shown in Figure 3. Sensitivity data for each method is shown in Figure 4.

Validation Parameter	TAb Results	NAb Results
Minimum Required Dilution (MRD)	1:75	1:4
Cut-Point (CP)	1.21	0.587
Sensitivity	8.9ng/mL	77.7 ng/mL
Intra/Inter Precision	Pass; CVs >20%*	Pass; CVs <20%
Drug Tolerance at LPC	7.2E11vp/mL	10014 MOI
Specificity (anti-AAV2)	N/A	Pass; Screen Neg.

**higher CVs observed due to potentiating effect of the SPC in NHS; all controls rank ordered*

Table 1. Summary of validation results for each assay

CP Calculations	TAb Value	NAb Value
Total # of Assay Values (samples × runs)	392 (56x7)	276 (46x6)
Values excluded, %CV >20%	8	3
Analytical Outliers	17	12
Biological Outliers	40	23
Final # Values Used	327	238

Table 2. CP calculation parameters for each assay

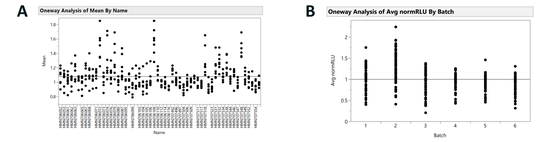
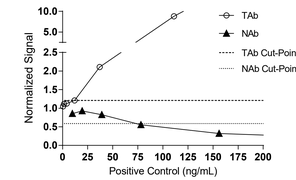


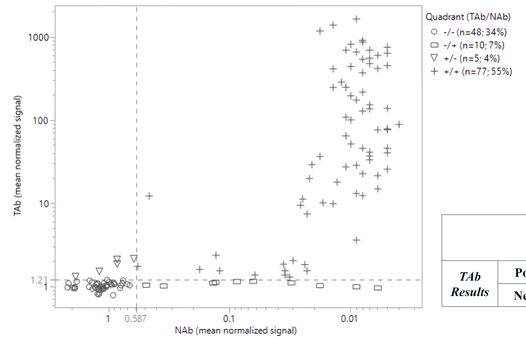
Figure 3. CP Sample Distribution Data for **A.** TAb and **B.** NAb Assays



Sensitivity was evaluated in the anti-AAVrh.10 TAb assay by assaying a series of dilutions (open circles) of the positive control at the 1:75 MRD. The data shown are the mean of six independent runs. The TAb cut-point is depicted by the dashed line (1.21). Sensitivity of the anti-AAVrh.10 NAb assay was determined by assaying the PC at a series of dilutions (closed triangles) at the 1:4 MRD. The data shown are the mean of six independent runs. The NAb cut-point is depicted by the dotted line (0.587).

Figure 4. Sensitivity Determinations for TAb and NAb Assays.

RESULTS – *CONCORDANCE ANALYSIS*



		NAb Results	
		Neg	Pos
TAb Results	Pos	5	77
	Neg	48	10

Figure 5. Correlation of TAb and NAb results. Serum samples at a dilution of 1:75 in the TAb and 1:4 in the NAb. CPs for each assay are shown by dashed lines. Positive or negative values are denoted by the symbol corresponding to the figure legend. A Cohen's Kappa correlation coefficient was determined to be 0.78 which denotes substantial agreement.

140 serum samples were screened in both assays. Of these, 34% (48 samples) screened negative in both assays (TAb-/NAb-), whereas 55% (77 samples) screened positive in both assays (TAb+/NAb+). Five samples (3.6%) that screened positive in the TAb assay did not demonstrate positivity for neutralizing antibodies to AAVrh.10 (TAb+/NAb-), while 10 samples (7.1%) that screened positive in the NAb assay were negative in the TAb assay (TAb-/NAb+, Figure 5). Further evaluations were performed to understand non-concordant results; the antibody titer response for samples that screened positive in the NAb assay and negative in the TAb assay are shown in Figure 6.

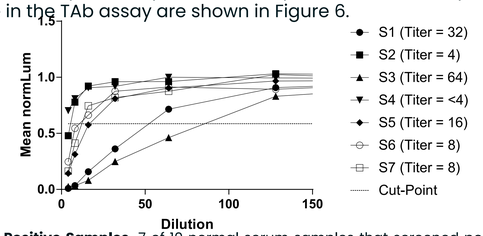


Figure 6: Titration of NAb Positive Samples. 7 of 10 normal serum samples that screened positive in the NAb assay (S1-S7) were titrated (titration analysis performed in the NAb assay format). Each sample was diluted in normal human serum pool in a two-fold dilution series, then diluted 1:2 in assay media before being mixed 1:2 with the AAVrh.10-Luc vector to achieve the assay MRD of 4. The titer for each sample that crossed the cut-point is displayed next to each sample in the graph legend.

CONCLUSIONS

The TAb and NAb assays for detection of anti-AAVrh.10 antibodies were successfully validated, meeting the regulatory expectations for all parameters evaluated, including sensitivity, drug tolerance, and precision. Both assays were shown to be appropriate for use as a regulated method to test clinical samples. Additionally, the concordance data we present herein suggests a strong positive correlation between TAb and NAb assay results, with nearly all detected antibodies demonstrating neutralizing activity (of the 5 TAb+/NAb- samples, 4 were negative in the TAb assay when titered, data not shown). The TAb-/NAb+ samples may reflect the presence of non-antibody or other uncharacterized serum components; the clinical relevance of these factors is unknown. These data demonstrate the suitability of either method as a screening assay for patient enrollment into AAVrh.10 gene therapy clinical studies, providing the industry with key information to determine utility of these assays for improved selection of patients during clinical study enrollment and as a potential companion diagnostic for AAVrh.10 gene therapies.