A Single Neutralizing Antibody Assay for Detection of Anti-AAV9 Antibodies in Minipig and Human Serum

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BACKGROUND AND PURPOSE

The field of gene therapy has grown significantly in the last decade with Adeno-Associated Virus (AAV)-based therapeutics becoming a common vector for this modality. A critical component of designing effective preclinical strategies for AAV-based therapeutics is evaluating pre-existing anti-AAV antibodies as these can have a major impact on translatability of preclinical data. Monitoring strategies for this include screening animals with neutralizing antibody (NAb) immunoassays for animal selection or stratification.

Minipigs are useful alternatives to rodents for preclinical studies due to their physiological similarity to humans, and their utility for AAV therapeutic testing is well established. Assays that easily translate between preclinical species such as minipigs to humans in clinical testing can save time and resources for programs. To this end, we describe an AAV9 NAb assay that can be utilized across species. This versatile assay provides a rapidly implemented screening solution for use with both minipig serum samples and human serum samples

METHOD

This anti-AAV9 NAb assay uses an AAV9 construct expressing firefly luciferase (AAV9-FLuc), a transduction-permissive epithelial cell line, and an AAV9 neutralizing surrogate positive control (SPC) antibody. Briefly, AAV9-FLuc is pre-incubated with serum samples, this mixture is then added to cells in a 96 well plate and incubated for 24 hrs. After incubation, luminescence is detected with a plate reader. NAb activity inhibits AAV9-FLuc transduction resulting in loss of signal. The normalized luminescence signal is compared to a statistically derived threshold (cut point) to determine if a sample is positive of negative.

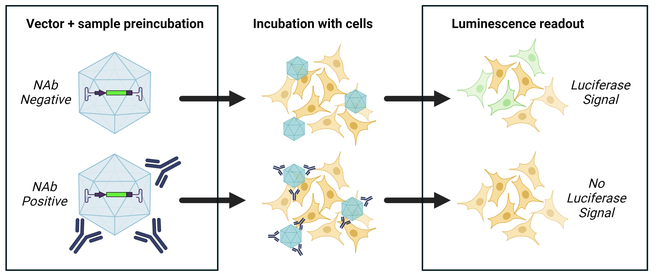

Figure 1. Diagram of NAb Immunoassay Format

Image created with BioRender.com

RESULTS – *VALIDATION OF HUMAN AAV9 NAB ASSAY*

During development of the human AAV9 NAb assay, the optimal cell line, source of AAV9 vector, transduction conditions (temperature/time/media), and minimum sample dilution (MRD) were selected. Individual human serum samples were screened to identify NAb negative samples for use as a negative control pool. During validation, the multiplicity of infection (MOI) of 6000 vg/cell was shown to provide a suitable signal window (Fig. 2). The assay cut points (CP) were established for screening and confirmatory tiers (Table 1 and Fig. 3) and the sensitivity of the assay was established using the surrogate positive control (SPC) (Fig. 4). The full performance parameters of the assay evaluated during the GxP validation included assay cut point, sensitivity, selectivity, specificity, inter/intra-assay precision, short-term stability, and robustness. All validation parameters met acceptance criteria according to current regulatory expectations (Table 2).

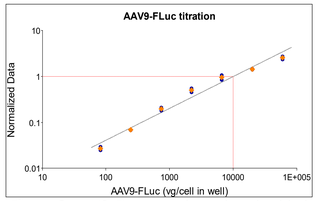

Figure 2. AAV9-FLuc was titrated and added to cells to confirm the MOI of 6000 vg/cell fell within the linear range of the vector titration curve as plotted on a log-log scale; vector potency was too low to resolve an upper asymptote for a 4PL fit

Cut point summary	Data points
Total	300
Biological outliers (9x6)	54
CV outliers	6
Analytical outliers	11
Total in assessment	229
Final screening CP (5% FPR)	0.695
Final confirmatory CP (1% FPR)	0.566

Table 1. 50 NHS samples were run 6 times in duplicates (two analysts, three runs each). A linear-mixed-effects analysis of variance (ANOVA) model was used to investigate sources of variation in log transformed normRLU values. After exclusion of outliers, the distribution of untransformed normalized results per batch were assessed for normality using Shapiro-Wilks test. The 1% and 5% quantiles (1% and 5% false positive rates for confirmatory testing and screening) were calculated using JMP's custom quartile function for each batch. The average of the 6 batches was reported as the assay cut point.

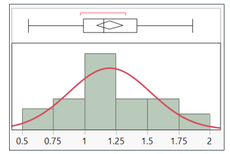

Figure 3. Example CP distribution

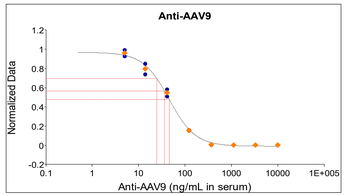

Figure 4. Sensitivity was assessed by evaluating an 8-point titration curve of SPC diluted 3-fold in normal human serum (NHS) starting at 10000 ng/mL; the interpolated concentration value corresponding to the EC50 and the cut points of 0.695 and 0.566 are shown.

Validation Parameter	Result
Minimum Required Dilution	2-fold
Screening Cut Point (SCP)	0.695
Confirmatory Cut Point (CCP)	0.566
Sensitivity at SCP	26.5 ng/mL
Sensitivity at CCP	38.3 ng/mL
Titration Range	Up to 4374-fold
Selectivity (high and low spikes)	Pass
Inter-Assay Precision at LPC	17.9%
Intra-Assay Precision at LPC	7.4-20.0%

Table 2. The final assay performance across all parameters tested during the GxP assay validation is listed.

RESULTS – *MINIPIG AAV9 ASSAY*

To ensure the MOI used for the human assay was appropriate for minipig samples, a titration of AAV9-FLuc was evaluated and the MOI of 1000 vg/cell was selected as this gave a high signal-to-noise window (Fig. 5). Minipig serum at MRD2 and MRD5 were tested and the MRD was adjusted to 1:5 for the assay (Fig. 6). Individual minipig samples at MRD 1:5 were evaluated and the seroprevalence of anti-AAV9 NAbs was ~10%. A titration curve of SPC was prepared in prescreened negative minipig serum pool for evaluating sensitivity (Fig. 7) and individual or pooled negative samples were spiked with SPC to evaluate selectivity (not shown). An estimated cut point of 0.7 normRLU was used to evaluate the data sets. Based on this estimate, the sensitivity of the assay was ~675ng/mL and all selectivity samples tested at 1000ng/mL SPC were positive for NAb. Assay parameters are summarized in Table 3.

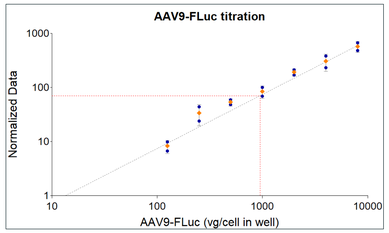

Figure 5. AAV9-FLuc was titrated and added to cells to evaluate the MOI; 1000 vg/cell fell in the linear range of the titration curve as plotted on a log-log scale

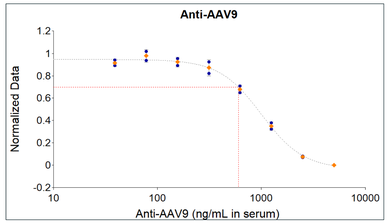

Figure 7. Sensitivity was assessed with an 8-point titration curve of SPC diluted 2-fold in NAb negative minipig serum starting at 5000 ng/mL; the interpolated concentration value at the CP of 0.70 is shown.

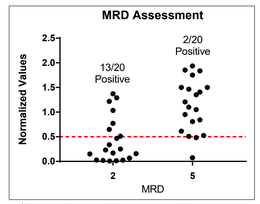

Figure 6. Individual minipig serum samples were analyzed at MRD2 vs MRD5. A cutoff of 0.5 was used to approximate the NAb positive rate.

Parameter	Result
MRD	5-fold
Estimated CP	0.700
Sensitivity (ng/mL)	~675
Selectivity	Pass

Table 3. The final assay performance across all parameters tested during the assay optimization in minipig serum.

CONCLUSIONS

Here, we provide an example of a human AAV NAb assay that was readily implemented in a pre-clinical animal species. The use of the assay in the new matrix required only three development runs to optimize the method and establish the appropriate per-formance of the assay in the new matrix. In the future, designing assays that can be readily applied to a variety of animal species as well as humans would greatly reduce cost and increase efficiency for the transition of bioanalytical assays from non-clinical studies to first-in-human trials.

ACKNOWLEDGEMENTS

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