VALIDATION OF AN *IN VITRO* CELL-BASED ASSAY FOR THE DETECTION OF NEUTRALIZING ANTIBODIES AGAINST ADENO-ASSOCIATED VIRUS 9 IN CYNOMOLGUS MONKEY

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PURPOSE

Adeno-associated virus has been widely recognized as a valuable vector for gene therapy. There has been increasing enthusiasm for developing AAV-based gene therapies around the world. Despite the safety profile of AAV, the high prevalence of naturally occurring AAV infections correlates with a high incidence of pre-existing neutralizing antibodies against AAV in individuals, which decreases cell transduction efficiency and is thought to be one of the factors in early clinical failures. Besides humans, non-human primates are also natural hosts of AAV. Therefore, it is critical to screen for the presence of AAV neutralizing antibodies in non-human primates and set an exclusion criterion for participation in preclinical trials.

Here, we reported the development and validation of a cell-based assay to determine the neutralizing antibodies against AAV9, which could be used to prescreen animal subjects with pre-existing Nabs in preclinical studies. In this assay, flow cytometry was utilized to measure the inhibition of the AAV transduction rate, which streamlined the analytical procedure and improved the efficiency of sample analysis. Moreover, a battery of parameters has been systemically optimized and standardized to ensure the reliability and reproducibility of the assay. Ultimately, a fully validated method for AAV9 neutralizing antibodies was established, which was then used to support a preclinical GLP toxicology study.

RESULTS

Method development and optimization

During the assay development, we have tested several cell lines (HeLa, HEK293, HEK293T, and Huh7 cells), a range of cell densities (1×105, 1.5×105, 2×105, 4×105cells/mL), a variety of multiplicity of infections (MOIs, 40000, 20000, 10000 and 5000), and different incubation time to optimize the transduction efficiency for the AAV9 in vitro. In addition, the minimum required dilution factors (MRD, 1:20, 1:10, 1:5) of cynomolgus monkey serum samples were tested in the feasibility runs to balance the sensitivity and specificity. After systemic optimization, we found that HEK293T with the density of 1.5×105 cells/mL, MOI of 20000, and MRD of 1:5 provided the best assay performance for AAV9 neutralizing antibody detection. The method development result was illustrated in Figure 1 A to D.

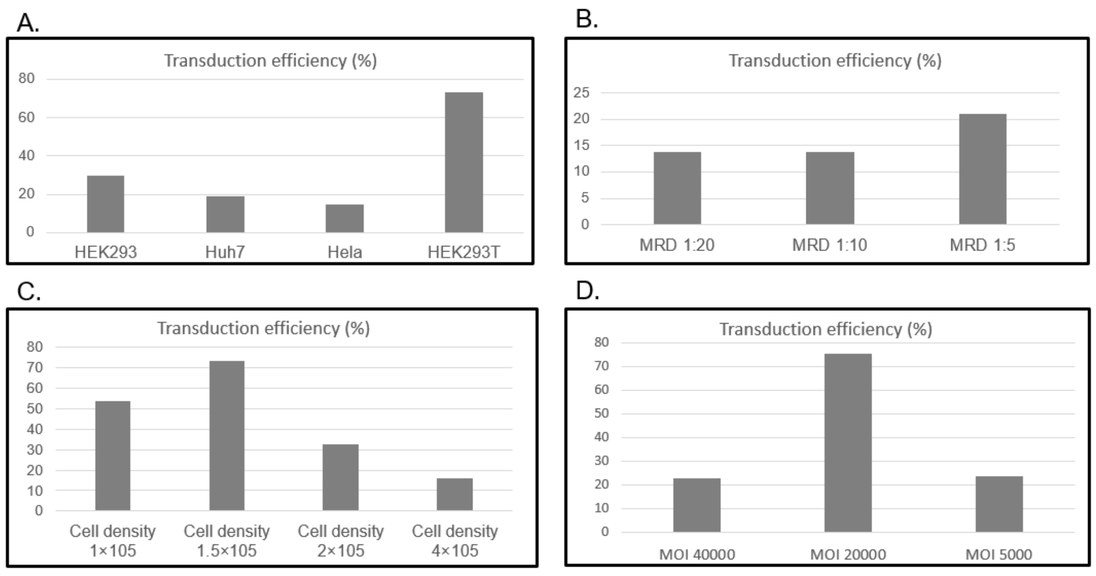
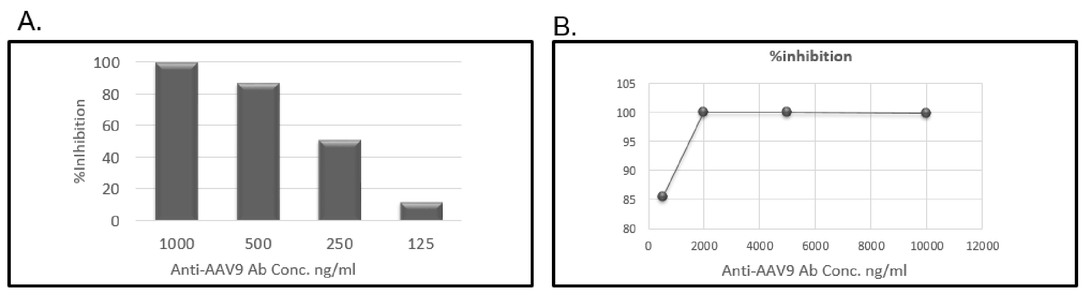


Figure 1. *The optimization of assay parameters during method development: A) cell line, B) MRD, C) cell density, and D) MOI.*

Method validation

A method validation was conducted in line with current guidelines and whitepaper recommendations. Our results showed that in monkey serum, the assay sensitivity reached 250 ng/mL with a cut point of 50% inhibition. No hook effect was observed when evaluating a set of different concentration of the positive control antibody (10000 ng/mL~ 50 ng/mL) in pooled cynomolgus monkey serum. Drug tolerance test revealed that 500 ng/mL Anti-AAV9 (intact particle) mouse recombinant IgG 1 could tolerate 2x10^10 GC/mL AAV9. The intra/inter-precision and robustness were assessed. All parameter acceptance criteria meet regulatory requirements. The method validation result was illustrated in Figure 2 A to D.



C.

AAV9 Titer (vg/mL)	Spiked at PC (2000 ng/mL)	AAV9 Titer (vg/mL)	Spiked at PC (500 ng/mL)
1E+12	10.4	5E+10	52.4
5E+11	68.0	2.5E+10	73.4
2.5E+11	92.5	1E+10	80.4
5E+10	99.9	1E+09	87.5
0	100.0	0	88.8
Cut Point		50.0%	

D.

Assay Control	Intra-assay (CV%)Max	Inter-assay (CV%)
High Positive control	0.3	0.2
Low Positive control	5.3	3.4
Medium+AAV9	2.1	2.2
Negative Control	7	5.7

Figure 2. *The assay performance and validation. A) Sensitivity determination. B) Hook effect evaluation. C) Drug tolerance assessment. D) Precision evaluation*

Fifty cynomolgus monkey serum samples have been collected and tested for the presence of anti-AAV9 neutralizing antibody. Twenty-three out of fifty cynomolgus monkey were tested positive and thus excluded for participation in pre-clinical study. The screening result was illustrated in Figure 3.

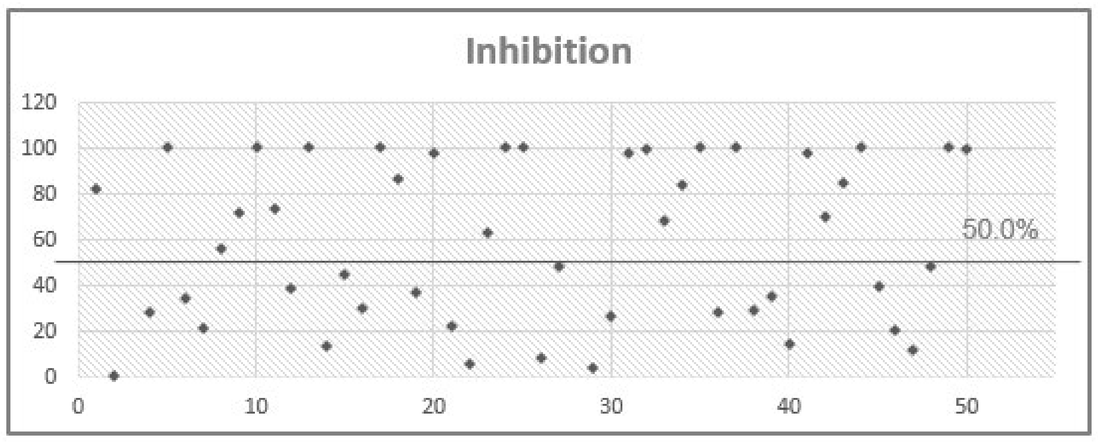


Figure 3. *Screening for anti-AAV9 neutralizing antibody in cynomolgus monkey serum samples.*

METHODS

AAV9 carrying a GFP gene was utilized to transduce target cells in vitro in the presence of anti-AAV9 positive control antibody (Anti-AAV9 (intact particle) mouse recombinant IgG 1 (clone ADK9-1R)) or cynomolgus monkey serum samples. The flow cytometry was used to quantify the percentage of GFP-expressing cells as an indication of AAV transduction. The transduction inhibition rate is calculated according to the following formula: Inhibition rate (inhibition%) = 100 - [(transduction efficiency of test sample – the mean transduction efficiency of current batch blank sample) / (the mean transduction efficiency of current batch negative controls – the mean transduction efficiency of current batch blank samples)] * 100.

CONCLUSION

A flow cytometry method for detecting anti-AAV9 neutralizing antibodies in cynomolgus monkey serum has been developed after a systemic optimization. A rigorous validation process following current guidelines and whitepaper recommendations has been completed. This method is considered valid for supporting the GLP preclinical toxicology studies.

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