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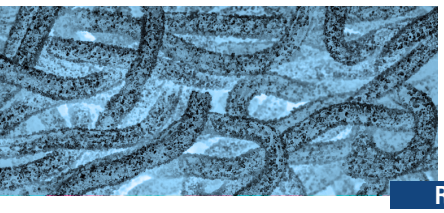

RESEARCH MODELS AND SERVICES

Summary Report of Adeno-Associated Virus Serotype-2 (AAV-2) Neutralizing Antibody (NAb) Assay Qualification Report

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Purpose and Background:

Adeno-associated viruses (AAVs) are harmless viruses which were first discovered in the 1960s. These viruses have become critical components in the development of gene therapies for patients. In the past five years, US regulators approved two landmark gene therapies – one for a rare, inherited form of blindness and the other for a genetic condition that progressively destroys motor neurons – that were delivered using AAVs.[1] In the US clinical trials database, there are currently more than 200 gene therapy trials employing a viral vector, the vast majority of which are AAV vectors.

We know the most about AAV2, which serves as a prototype for the AAV family and is the one most commonly used in gene therapies. There are currently 13 known serotypes of AAV which differ in their ability to infect certain tissues or cell types. This makes AAV a useful system for preferentially transferring a gene therapy to a specific cell type.[2,3]

While viral vectors have led the way in many clinical and preclinical successes, a number of challenges limit their true potential, such as pre-exposure of people to one or many of the 13 canonical AAV serotypes. This can result in the activation of the immune system to produce neutralizing antibodies (NAbs) against AAV, preventing AAV viral vectors from delivering their cargo to the appropriate target in the body.[4-7] As a result, the viruses being used to shuttle the gene therapies into cells can unwittingly interfere with their clinical success.

This is not just a problem for patients. Animals, including mice and some large animal species used to test gene therapies, can also have pre-existing immunity to the viruses used as vectors. This undermines the potential efficacy of the drug being tested and biases the outcome of the study. To prevent this, scientists have developed a workaround to this problem by screening animals for antibodies prior to including them in a study.

AAV can be detected via serological or PCR testing. Due to a high degree of both sequence (65-99%) and structural (95-99%) identity across the canonical AAVs, it can sometimes be difficult to distinguish between certain AAV serotypes by PCR. Because AAV is so prevalent, it can be challenging to identify enough seronegative participant animals for studies using total antibody detection methods, such as ELISA. As a result, cell-based AAV NAb assays are commonly used rather than traditional total antibody detection methods. Serum can be used for these assays, and heat inactivation of a sample is highly recommended to neutralize factors in the blood that can alter the transduction of AAV viral particles, which can confound results.

Cell-based NAb assays were developed by Charles River Laboratories' Research Animal Diagnostic Services (RADS) to assist in routine screening of animals for detection of AAV2-specific NAbs. The purposes of this qualification is to perform AAV NAb assays on positive and negative sera and demonstrate that AAV NAb screening and titer assays are sensitive and specific.

Materials and Methods

Cells were plated in a 96-well plate and infected with AAV2 viral vector, containing a plasmid with a luciferase expression cassette, in the presence of sample sera. After incubation to allow for the infection of cells with the virus and expression of luciferase, substrate was added to the cell wells. Luciferase cleaves the substrate, resulting in a luminescent signal that was measured using a plate reader. The signal of sera samples was divided by that of NAb-free controls, giving a neutralization percentage. Sera samples with a neutralization percentage equal to or below 50% are considered to have neutralizing antibody

Positive controls were made using commercially acquired anti-AAV2 neutralizing antibody diluted in PBS. Negative controls consisted of only the AAV2 viral vector in media.

Briefly, analytical sensitivity, also known as limit of detection (LOD), diagnostic sensitivity, and diagnostic specificity of the AAV2 NAb assay were evaluated. The LOD comparison between two technicians was done by the evaluation of four positive sera, each serially diluted twofold into standard assay media, with a total of eight dilutions being analyzed. In addition, analytical specificity of the AAV2 NAb assay was evaluated by testing antibodies against several other AAV serotypes, including AAV1, AAV5, AAV6, AAV8, and AAV9.

Diagnostic sensitivity and reproducibility were measured by analyzing eight known positive sera samples tested by two technicians (Tech 1 and Tech 2) performing three replicate AAV2 NAb assay runs on different days for a total of six runs. In addition, diagnostic specificity was measured by analyzing eight known negative test samples in the same six runs.

Prevalence of AAV2-neutralizing antibodies was also examined in a total of 91 macaque field sera samples from a North American colony.

Results Summary

Assay Cutoffs and Interpretation Guidelines

Samples were designated as AAV2 NAb positive if the RLU signal was less than or equal to 50% of the average of negative control cells infected with AAV2 viral vector in the absence of anti-AAV2 NAb.

Limit of Detection (LOD)

To assess the analytical sensitivity (LOD) and compare the titer reproducibility of the AAV2 NAb assay, four positive sera were individually titrated and tested. The neutralization percentage of each titer for two technicians over two runs were compared. The graphs for analytical performance (Figure 1) below show titration curves of serially-diluted AAV2 NAb positive samples. Data

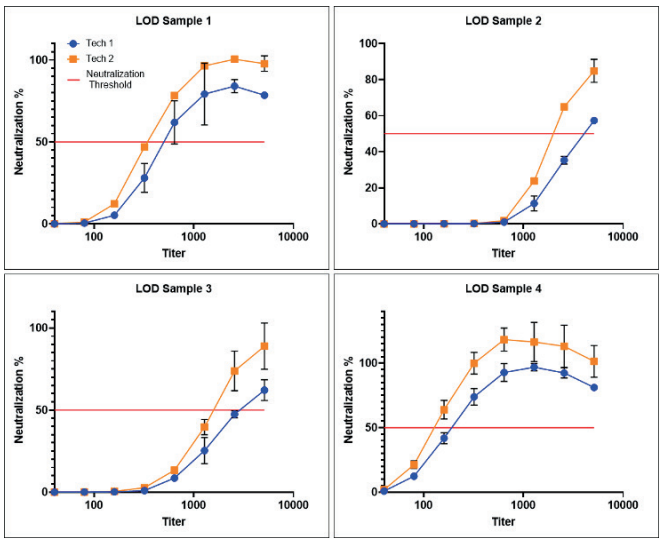


Figure 1. LOD Titration of AAV2 Nab-Positive Sera. Eight serial twofold dilutions of AAV2 Nab-positive sera samples were analyzed by AAV2 NAb assay. Titers for duplicate runs of four samples were compared between two techs. The neutralization threshold line indicates 50% neutralization, below which samples are considered to be AAV2 NAb positive.

Table 1. LOD Titration of AAV2 NAb Positive Sera. Four AAV2 Nab-positive sera samples were twofold serially diluted from 1:40 to 1:5120 for a total of eight dilutions. The last dilution at which AAV2 NAb was detected for each sample is shown for each Tech across two replicates (Trial 1 and Trial 2). A pass indicates the LOD for a sample is within one twofold dilution between Tech 1 and Tech 2.

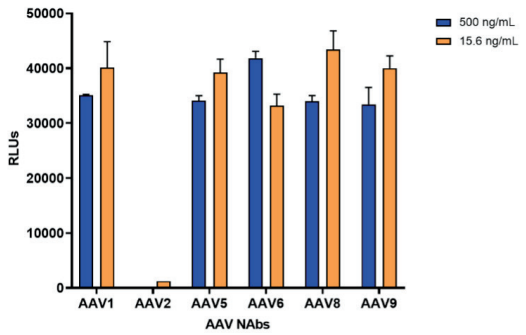
	Trial 1			Trial 2		
Sample ID	**Tech 1 LOD**	**Tech 2 LOD**	**Pass/Fail**	**Tech 1 LOD**	**Tech 2 LOD**	**Pass/Fail**
1	640	320	**Pass**	320	320	**Pass**
2	2560	1280	**Pass**	2560	1280	**Pass**
3	2560	1280	**Pass**	2560	1280	**Pass**
4	160	80	**Pass**	160	80	**Pass**

(% neutralization) between two techs was similar for all four samples and crossed the neutralization threshold within one twofold dilution of each other (Table 1).

Analytical Specificity (Selectivity)

Analytical specificity of the AAV2 NAb assay was assessed by testing neutralizing antibodies against different serotypes including AAV1, AAV5, AAV6, AAV8, and AAV9 (Figure 2). Antibodies specific to only the AAV2 serotype were able to block AAV2 viral infection (lower RLU for AAV2 antibodies) in the assays, confirming high analytical specificity of the AAV2 NAb assay.

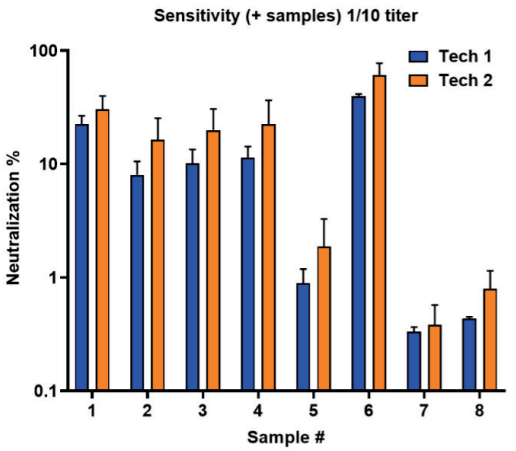
Figure 2. AAV2 Viral Vector Neutralization Specificity Test (Analytical Specificity). Cells were infected with AAV2 viral vector in the presence of AAV neutralizing antibodies for six serotypes (AAV1, AAV2, AAV5, AAV6, AAV8, and AAV9) at two concentrations (500 ng/mL and 15.6 ng/mL).



Diagnostic Sensitivity, Specificity, and Repeatability

To determine AAV2 NAb assay sensitivity, eight known positive antiserum samples were analyzed. Samples were run independently at 1/10, 1/20, and 1/40 dilutions by both Tech 1 and Tech 2 on three separate days. The calculated average neutralization percentages for each tech matched well (Figure 3).

Figure 3. Eight known AAV2 Nab-positive sera were tested in three separate runs by each of two technicians. The average neutralization percentages of these samples for the 1/10 titer are shown.



Assay sensitivity was calculated by comparing the actual number of measured positive samples to the total number of expected positive samples for both Tech 1 and Tech 2 for each of their three test runs (Table 2). Diagnostic specificity was calculated by comparing the total number of measured negative samples at three dilutions (1:10, 1:20, and 1:40) for the eight known negative samples by both Tech 1 and Tech 2 for each of their three test runs to the total number of expected negative samples. The known negative samples were selected from previously tested negative AAV2 NAb NHP sera samples (Table 2).

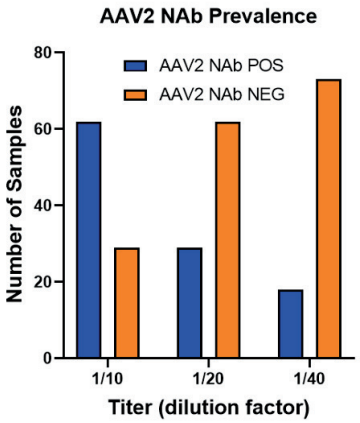
Table 2. Eight known AAV2 Nab-positive sera and eight known AAV2 Nab-negative sera were tested in three separate runs by each of two technicians. Dilutions with less than or equal to 50% signal inhibition were considered positive. Samples were considered positive if the 1/10 dilution was positive. Samples were considered negative only if all three dilutions (1/10, 1/20, and 1/40) were negative.

	Sensitivity	Specificity
	Positives	Negatives
#	46	48
Total Assays Tested	48	48
%	**95.8%**	**100.0%**

Prevalence of AAV2 NAb in Nonhuman Primates (NHPs)

Prevalence of AAV2 NAb was very high in 91 macaque field sera samples from a North American colony, as shown in Figure 4. Data is shown as the number of positive and negative animals at each sera dilution (1/10, 1/20, and 1/40). Weak positives containing only a small concentration of AAV2 NAb are evident as the number of positive samples decreases with each dilution (Figure 4).

Figure 4. Prevalence of AAV2 NAb in 91 Macaque Field Sera Samples from a North American Colony.



Conclusion

While conducting animal studies and human trials, it is important to know whether neutralizing antibodies for a specific AAV are present in their blood because it can impact whether they are a good study candidate for being dosed. This study was undertaken to develop an AAV2 NAb detection assay for routine prescreening of NHPs used for gene therapy studies.

Results from qualification of the AAV2 NAb assay show a high sensitivity, specificity, and reproducibility. The overall diagnostic sensitivity and specificity of the AAV2 NAb assay was found to be greater than 95% and greater than 99%, respectively (Table 2). LOD between two techs was similar for all four samples and crossed the neutralization threshold within one twofold dilution of each other (Table 1). Analytical specificity of the AAV2 NAb assay was very high with no cross reactivity observed by other AAV serotype antibodies, including those against AAV1, AAV5, AAV6, AAV8, and AAV9 serotypes.

In conclusion, we have validated a cell-based AAV2 NAb assay which is highly sensitive and specific for routine screening for the presence of AAV2-neutralizing antibodies in NHPs. The data demonstrates that the AAV2 NAb assay can be used for routine monitoring of NHP colonies and can be performed in a user-friendly and high-throughput format.

References

1. https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products
2. Arun Srivastava (2016). *In vivo* tissue-tropism of adeno-associated viral vectors. Curr Opin Virol. 21: 75–80.
3. Wang D, Tai PWL, Gao G. Adeno-associated virus vector as a platform for gene therapy delivery. *Nat Rev Drug Discov*. 2019;18(5):358–378.
4. Cotmore SF, Tattersall P. (2014) Parvoviridae: General Features. In Reference Module in Biomedical Sciences. 90-97.
5. Kleopatra Rapti, Vedell Louis-Jeune, Erik Kohlbrenner, Kiyotake Ishikawa, Dennis Ladage, Sergei Zolotukhin, Roger J Hajjar, and Thomas Weber (2012) Neutralizing Antibodies Against AAV Serotypes 1, 2, 6, and 9 in Sera of Commonly Used Animal Models. Mol. Ther. 20, 73-83.
6. Yagi H, Mizukami H, Tsukahara T, Urabe M, Hamada H, Kume A, Yoshikawa H, Ozawa K. Prevalence of Neutralizing Antibodies Against Adeno-Associated Virus 2, 8 and 9 in NonHuman Primate Colonies (2010). Molecular Therapy. 18: S214
7. Boutin S, Monteilhet V, Veron P, Leborgne C, Benveniste O, Montus MF, 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.

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