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METHOD DEVELOPMENT AND VALIDATION OF A CELL-BASED ASSAY FOR THE DETECTION OF ANTI-AAV NEUTRALIZING ANTIBODIES IN HUMAN SERUM

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PURPOSE

AAV is known for its low immunogenicity, high safety profile, wide host range, long-term transgenic expression, and the ability to target specific tissue sites for gene delivery. These characteristics make AAV-based vectors highly promising for gene therapy applications in humans.

In recent years, many AAV gene therapy products have been approved by regulatory authorities while more are in different stages of clinical trials. However, the production of neutralizing antibodies against AAV in human may block the transduction of AAV, thereby impacting the efficacy of therapeutic products.

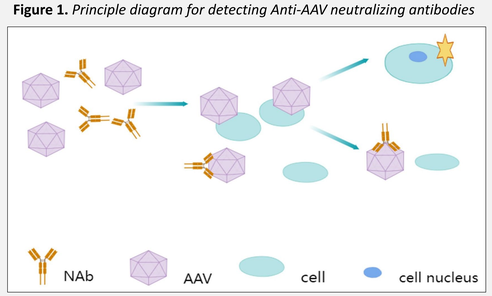
The current published AAV neutralizing antibody analysis methods either lack high sensitivity or miss comprehensive validation data following principles of immunogenicity validation guidance, which limits their applications in drug development or clinical testing.

In this study, a series of cell-based methods for detecting neutralizing antibodies against different serotypes of AAV (AAV2, AAV8 and AAV9) in human serum have been established.

METHOD

AAV carrying the luciferase reporter gene infected 2V6.11 cells and expressed luciferase within the cells to product luminescence signal. With the presence of neutralizing antibodies (NAb) against AAV in human serum, the luminescence signal will decrease. By measuring the luminescence signal in the presence and absence of neutralizing antibodies, the presence and quantity of neutralizing antibodies against AAV was determined in the tested sample. The assay procedure is as following: 2V6.11 cells were coated on cell culture plate overnight at 37℃,5% CO2 incubator. Test samples were mixed with luciferase-modified AAVs. Then they were transferred to the cell coated plate and incubated overnight at 37℃. After that, the luminescence signal was detected with luciferase detection reagent.

Figure 1. *Principle diagram for detecting Anti-AAV neutralizing antibodies*

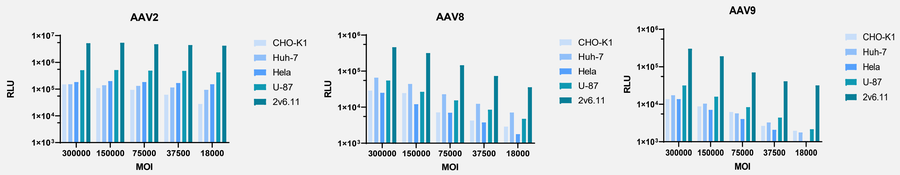


RESULTS

Screen cell lines

Five cell lines were tested for AAV infection efficacy, including CHO-K1, Huh-7, Hela, U-87, and 2v6.11. Among these, the 2v6.11 cell line exhibited the highest infection efficacy for AAV2, AAV8, and AAV9 transduction.

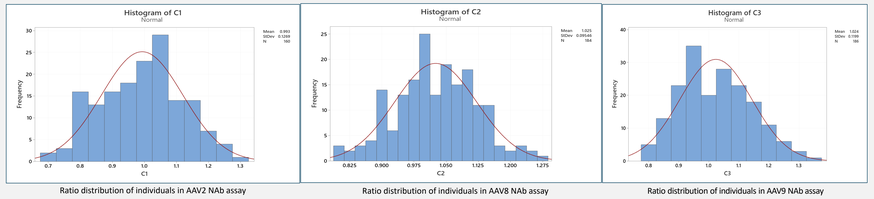
Figure 2. *Infection efficiency of different AAVs to different cell lines*



Individual serums screen and cut point determination

Individual sera were screened for multiple times to obtain a pool of sera free from pre-existing antibodies. Individuals with over 50% inhibition were excluded from the preparation of the serum pool. Based on our data, the prevalence of AAV2, AAV8 and AAV9 neutralizing antibody were around 70%, 28% and 34%, respectively. Finally, screened individuals were tested for CPF determination and signal/noise ratio of all three assays were normally distributed. The cut point factor of AAV2, AAV8, and AAV9 are determined as 0.70, 0.80, 0.74.

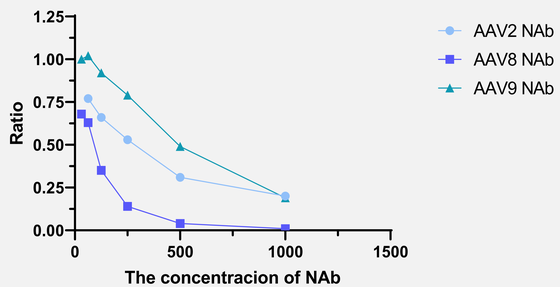
Figure 3. *Ratio distribution of individuals in different AAVs NAb assay*



Sensitivity

The positive control antibodies were spiked into pooled human serum at an initial concentration of 1000 ng/ml. Then, they were serially diluted with pooled human serum. These samples were analyzed by two analysts over a period of three days. The sensitivity for detecting anti-AAV2, AAV8, and AAV9 neutralizing antibodies reached to 100 ng/mL, 30 ng/mL, and 300 ng/mL, respectively.

Figure 4. *Examples of assay sensitivity*



Selectivity

Normal individual serums, lipemic and hemolysis serums were tested in these three NAb assays, the results were shown in below table. At least 80% normal individual serums met the acceptance criteria: unspiked samples were negative, spiked samples were positive; at least 2/3 hemolysis and lipemic serums met the acceptance criteria: unspiked samples were negative, spiked samples were positive. Normal and hemolysis serum met the acceptance criteria for AAV2, AAV8 and AAV9 assays. AAV2 assay passed the lipemic selectivity test, AAV8 and AAV9 assays did not.

Table 1. *Selectivity results of AAV NAb assays*

Serum	AAV2			AAV8			AAV9		
	Unspiked	HPC	LPC	Unspiked	HPC	LPC	Unspiked	HPC	LPC
Normal	10/10	8/10	9/10	10/10	10/10	10/10	10/10	10/10	9/10
Lipemic	3/3	3/3	3/3	3/3	0/3	0/3	3/3	0/3	0/3
Hemolysis	2/3	2/3	3/3	3/3	3/3	3/3	2/3	3/3	3/3

RESULTS (CONTINUED)

Precision

Precision runs were tested by 6 sets of PCs and NCS in each runs in six runs by 2 analysts in 3 days. Titer precision was tested by 3 sets dilution curves in one plate, and neutralizing antibody titer was reported at the first dilution at 50% or greater inhibition of the luciferase expression was measured. Intra and inter precision of three assays were all within 30%.

Table 2. *Precision results of AAV NAb assays*

Positive Control	AAV2		AAV8		AAV9	
	Inter CV%	Intra CV%	Inter CV%	Intra CV%	Inter CV%	Intra CV%
HPC	23.8	10.0-20.0	12.8	4.7-13.6	17.1	5.4-15.0
LPC	18.9	8.7-19.2	13.0	5.1-14.5	18.9	8.0-22.7
NC	27.6	5.0-14.6	18.3	13.0	21.2	8.3-15.8
TPC	Titer range:4-8	Titer range:2-8	Titer range:2	Titer range:1-2	Titer range:2	Titer range:1-2

Stability

Three sets of stability samples for each level (HPC, LPC, NC) were prepared independently and analyzed for each stability test. It showed that short stability (2-8℃,RT), 8 times freeze, and thaw testing all passed.

Table 3. *Stability results of AAV NAb assays*

Conditions	AAV2	AAV8	AAV9
2-8℃	72 hours	72 hours	72 hours
RT	72 hours	72 hours	72 hours
F/T(-20℃ and -70℃)	8 cycles	8 cycles	8 cycles

CONCLUSION

Based on above results, we have established a series of cell-based methods for detecting multiple serotypes of AAV neutralizing antibodies in human serum. These methods have high sensitivity and reliability for detecting neutralizing antibodies of AAV2, AAV8 and AAV9, and meet the requirements of health authority guidelines of immunogenicity, thus could be used for subject screening and clinical sample analysis of AAV therapeutic products.

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