Development of a Highly Sensitive Cell-based Infectivity Assay for the Assessment of AAV Vector Shedding in Clinical Serum Samples

Alisa Bowen[1], Theresa Seitel[1], Brian Long[1], Melissa Calton[1]
[1] 4D Molecular Therapeutics, Inc., Emeryville, CA, USA

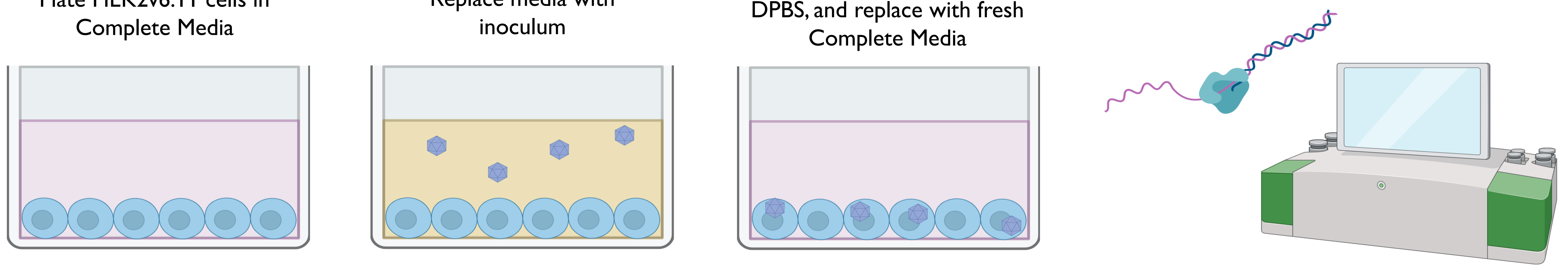
Introduction

Assessment of AAV vector shedding post-administration is required by regulatory health agencies and has traditionally been characterized by qPCR, a method that detects both transduced and encapsidated, potentially transduction-competent vector DNA, but does not discriminate between the two. As a follow-up to the qPCR method, clinical study sponsors have been compelled to investigate with an assay that specifically characterizes the infectious potential of shed material[1-3]. Characterization of shed material may have added relevance for AAV gene therapies administered intravenously, as vector genomes have been detected in matrices such as blood and semen out to 12 weeks[4]. Cell-based transduction methods are ideal to evaluate this infectivity question, as they simulate the entire AAV vector transduction process from entry into the cell, endosomal escape, nuclear transport, double-strand synthesis, and productive expression of vector mRNA and corresponding transgene proteins. However, previous attempts to develop infectivity assays in relevant shedding matrices have been limited by the sensitivity of protein-based readouts[5]. To achieve a clinically meaningful sensitivity that is specific for transduction-competent vector DNA, the assay described here employs cell-based transduction paired with RT-ddPCR as the endpoint detection. These molecular readouts are expected to be more sensitive than traditional readouts that detect the expressed transgene protein.

Methods

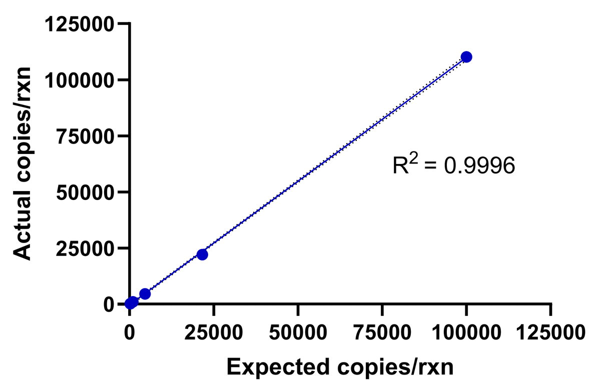
This assay was developed with the AAV-permissive cell line HEK293 2v6.11 (*Johns Hopkins*) to detect transduction-competent viral particles in human serum following administration of AAV gene therapy. Incubation of the cells with a therapeutic AAV spiked into serum results in cellular transduction of the vector and subsequent expression of transgene mRNA. To measure productive transduction, cells were lysed for RNA extraction, followed by cDNA preparation by reverse transcription. RT-ddPCR was performed with an exon-exon spanning amplicon to quantify vector transcripts. The primer set was designed to target a splice junction, creating a sequence that is not found either in vector DNA or human genomic DNA. This strategy is intended to facilitate unambiguous detection of productive transduction events that lead to mRNA expression, without interference from other nucleic acid sequences anticipated to be present in clinical test samples.

The forward primer sits in the chicken beta actin Exon1 from the CAG promoter, while the reverse primer sits in the transgene (Tg) Exon 2. The probe spans the exon-exon junction.



Results

Range of Quantification



Sample	%CV	%RE
STD1	1.0%	9.2%
STD2	0.1%	2.1%
STD3	0.7%	-1.4%
STD4	0.0%	-5.1%
STD5	7.5%	-15.0%
STD6	15.0%	-13.9%
STD7	28.9%	8.3%

Linear Range:
1.00E5 – 1.00E1 vg/rxn

Intra- & Inter-assay Precision/Accuracy

	HQC			LQC		
Sample	1	2	3	1	2	3
Replicate	n=3			n=3		
Expected vg/rxn	3750.0			187.5		
Average vg/rxn	3890.4	3852.3	4036.9	185.4	171.2	186.0
Intra-assay %CV	0.2%	1.6%	0.5%	3.5%	7.1%	2.1%
Intra-assay %RE	3.4%	1.1%	6.7%	-1.1%	-9.5%	-0.8%
Inter-assay %CV	2.0%			3.8%		
Intra-assay %RE	4.5%			-3.7%		

Specificity

Cross-reactive?	
HEK RNA	No
HEK DNA	No
Vector DNA	No

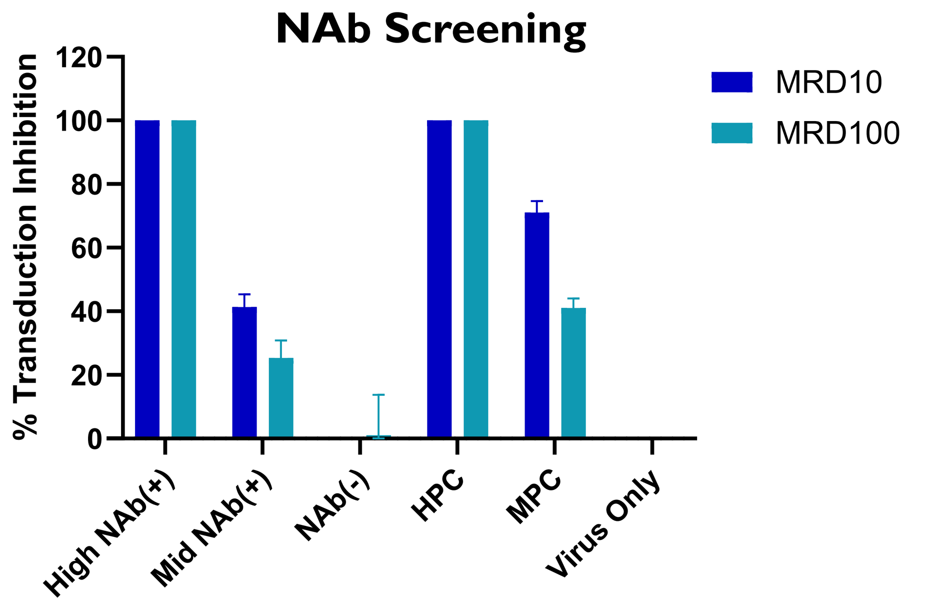
Detection Range (LOQ, LOD)

	DP1	DP2	DP3	DP4	DP5	DP6	DP7	NTC
Target copies	**160**	**80**	**40**	**20**	**10**	**5**	**2.5**	**0**
Replicate 1	102.7	92.5	32.3	13.1	10.0	0.0	2.5	0.0
Replicate 2	181.9	95.8	43.5	22.0	12.1	5.0	0.0	0.0
Replicate 3	195.0	101.7	27.8	19.5	15.3	8.4	1.2	0.0
Replicate 4	180.6	78.6	49.3	25.1	9.4	6.0	2.4	0.0
Replicate 5	165.8	67.6	52.2	29.5	7.2	6.0	1.2	1.1
Replicate 6	169.8	90.7	41.4	11.0	16.8	2.5	3.6	0.0
Replicate 7	173.3	113.4	52.0	15.9	11.9	4.8	4.7	0.0
Replicate 8	190.0	82.5	57.0	21.2	10.3	9.4	3.4	0.0
Replicate 9	163.6	96.5	40.8	23.3	7.4	3.7	2.4	0.0
Replicate 10	199.0	60.8	48.6	19.6	12.1	3.7	2.4	2.2
Average	**172.2**	**88.0**	**44.5**	**20.0**	**11.2**	**4.9**	**2.4**	**0.3**
%CV	**15.0%**	**17.1%**	**19.6%**	**26.3%**	**26.2%**	**52.8%**	**54.6%**	**N/A**

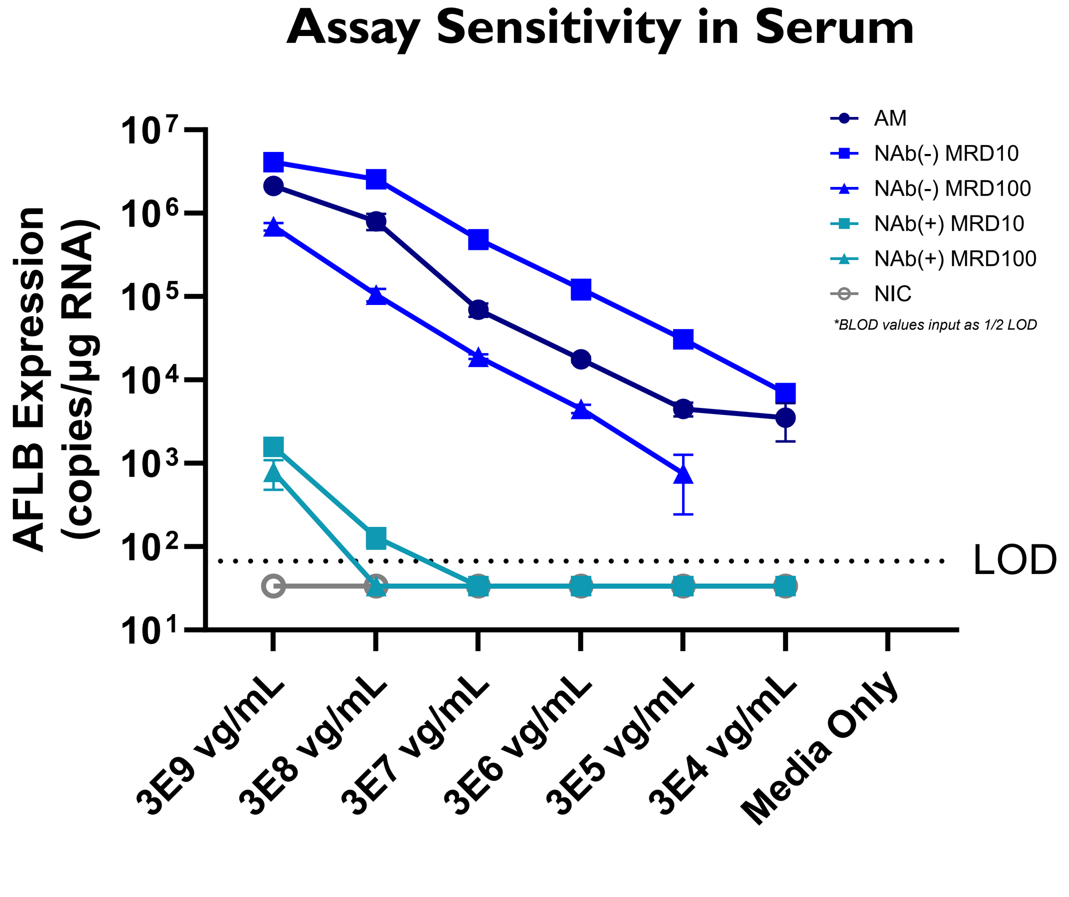
Limit of Quantitation:
10 vg/rxn
(lowest dilution point where CV < 30%)

Limit of Detection:
6.7 vg/rxn

NAb Screening



Assay Sensitivity in Serum



NAb(-) Serum Sensitivity: 3E4 vg/mL serum at MRD10
NAb(+) Serum Sensitivity: 3E8 vg/mL serum at MRD10

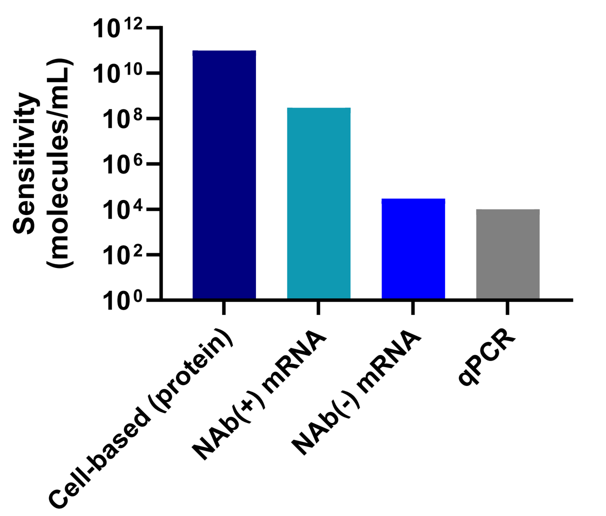
To characterize the performance of the exon-exon junction spanning primer set in a representative shedding matrix, key parameters such as range of quantification, accuracy, precision, limit of detection (LOD), and specificity were evaluated using a synthetic cDNA template spiked into human serum. This RT-ddPCR assay demonstrated excellent performance in all parameters, ensuring that this molecular readout is capable of reliably detecting vector mRNA transcripts in the presence of human serum.

The overall assay sensitivity of *in vitro* transduction at a serum dilution of 1:10 (MRD10) paired with the RT-ddPCR readout was established to be 3E4 vg/mL NAb(-) serum and 3E8 vg/mL NAb(+) serum, demonstrating significantly improved sensitivity over previously published methods employing protein-based endpoint detection.

Conclusions

An analytical assay with appropriate sensitivity and specificity is necessary to characterize the vector shedding profile of AAV gene therapies. The advantage of a cell-based infectivity assay is that it will only detect material that is potentially transduction competent. While infectivity assays utilizing protein detection are inherently less sensitive than conventional qPCR assays, a highly selective mRNA readout by RT-ddPCR proved key to achieving a clinically relevant assay sensitivity, even in NAb(+) serum. Previous reports in both NHP and clinical studies have established that immune responses to the AAV capsid begin to accumulate 1-2 weeks following systemic IV administration, which can be highly neutralizing and pose signification inhibition to the transduction competency of any shed virus. Though this method was developed in serum as a representative shedding matrix, future work can expand on characterization in other matrices such as tears, nasal and throat swabs, urine, feces, or semen. With this assay, a comprehensive characterization of shed material can be used to understand the infectious potential of circulating AAVs in serum and assess the risk of horizontal transmission.

Comparison of Readout Sensitivities



Therapeutic Name	Max shed vg concentration	Clearance Time
onasemnogene abeparvovec	1.0E12 vg/mL feces	8 wks
etranacogene dezaparvovec	n/a	159 wks
delandistrogene moxeparvovec	5.5E10 vg/mL serum	8 wks
aloctocogene roxaparvovec	2.0E11 vg/mL blood	12 wks
fidanacogene elaparvovec	n/a	42wks

References

1. Center for Biologics Evaluation and Research (CBER) Guidance for Industry, "Design and Analysis of Shedding Studies for Virus or Bacteria-based Gene Therapy and Oncolytic Products." August 2015.

2. Center for Biologics Evaluation and Research (CBER) Guidance for Industry, "Long Term Follow-Up After Administration of Human Gene Therapy Products." January 2020.

3. European Medicines Agency (EMEA) ICH Considerations, "General principles to address virus and vector shedding." July 2009.

4. Agarwal, S., Sandza, K., Koziol, E., Akeefe, H., Yang, F., Holcomb, J., Robinson, T. M., Jayaram, K., Russell, C. B., Zoog, S., Vettermann, C., & Henshaw, J. (2024). Blood biodistribution and vector shedding of Valoctocogene Roxaparvovec in people with severe hemophilia a. *Blood Advances*, *8*(17), 4606–4615. https://doi.org/10.1182/bloodadvances.2024013150.

5. Sandza, K., Clark, A., Koziol, E., Akeefe, H., Yang, F., Holcomb, J., Patton, K., Hammon, K., Mitchell, N., Wong, W. Y., Zoog, S. J., Kim, B., Henshaw, J., & Vettermann, C., (2021). Ultra-sensitive AAV capsid detection by immunocapture-based qPCR following factor VIII gene transfer. *Gene Therapy*, *29*(1–2), 94–105., 2021. https://doi.org/10.1038/s41434-021-00287-1.