Genome integrity analysis of adeno-associated viruses (AAVs)

Featuring the BioPhase 8800 system and the RNA 9000 Purity & Integrity kit

Marcia Santos, Fang Wang, Jane Luo and Sahana Mollah

BioPharma
SCIEX, USA

Introduction

In a world where drug development pipelines are growing exponentially, analytical scientists are looking for faster and innovative ways to remove bottlenecks without compromising the accuracy and precision of the analyses. This work demonstrates it is possible to evaluate the genome integrity and purity of adeno-associated viruses (AAVs) irrespective of their serotype in a high-throughput format by combining a chemistry kit with a multi-capillary electrophoresis system. Our results revealed that this assay is sensitive to lot-to-lot variations of full AAV8 samples (Figure 1), without interference from sample matrices (Figure 3). This work also demonstrates excellent inter-capillary reproducibility (Figure 4 and table 1) with %RSD values better than 0.2% for migration time (MT) and 1.6 % for corrected peak area (CPA) for the 8 markers in the ssRNA ladder standard.

An AAV is a non-enveloped virus with an icosahedral protein shell capsid of approximately 20 nm in diameter harboring a single-stranded DNA genome of about 4.7 kb in length.

Due to its low pathogenicity and immunogenicity, broad tropism and persistent transgene expression in proliferating and quiescent cells, AAV is an attractive choice for creating viral

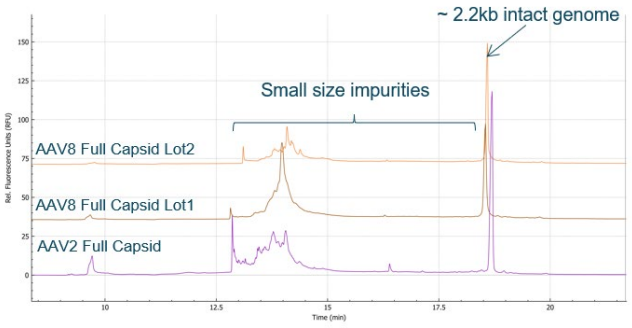

Figure 1. Overlay e-gram of AAV genome analysis from AAV of different serotypes. From top to bottom: AAV8 full capsid lot 2 (orange), AAV8 full capsid lot 1(brown) and AAV2 full capsid (purple).

vectors for gene therapy for various diseases.[1] Verification of genome size has been traditionally done by denaturing agarose-gel electrophoresis and southern blot.[2] Both are time consuming and have limited resolution on size determination.[2,3]

Key features

- Assessment of AAV intact genome size and purity without interference from sample preparation matrices
- The assay is sensitive to lot-to-lot variations and is independent of the serotype of AAV, which makes this assay attractive to QC
- Kitted and ready-to-use reagents and consumables help simplify operation and minimize user deviation
- Unique ssRNA ladder allows for accurate size determination over an extended size range
- Excellent inter-capillary reproducibility for both ssRNA ladder and intact genome with %RSD values for migration time and % corrected area <0.20% and <1.60%, for the ladder peaks, respectively

Methods

Materials: The RNA 9000 Purity & Integrity kit (PN C48231), which includes nucleic acid extended range gel, acid wash/regenerating solution, CE grade water, SYBR Green II RNA Gel Stain* and a ssRNA Ladder were procured from SCIEX (Framingham, MA). Sample Plate Packs (P/N 5080311), Reagent Plate Packs (PN 5080314), Sample Loading Solution (SLS) (P/N 608082), and a 30 cm BFS Capillary Cartridge – 8 x 30 cm (PN 5080121) all for BioPhase were also procured from SCIEX (Framingham, MA). QIAquick PCR purification kit (PN 28104) was from Qiagen (Germantown, MD). AAV Samples including, packaged AAV8 of pAV-CMV-GFP lot 1 (1X10^13 GC/mL) and lot 2 (2.23X10^13GC/mL), Packaged AAV2 of pAV-CMV-GFP (2X10^13 GC/mL) and AAV8 of pAV-CMV-GFP Empty Capsid were from Vigene Biosciences, (Rockville, MD) and (Hayward, CA 94541). AAV formulation buffer (1X PBS with 0.001% Pluronic F68) were from Vigene Biosciences (Rockville, MD).

Sample preparation for the ssRNA ladder: ssRNA ladder standard was prepared according to the RNA 9000 Purity & Integrity kit user guide. Briefly, 30 µL of the ssRNA Ladder was added to a 720 µL of sample loading solution and mixed well. The sample was denatured with heat using a thermal cycler at 70°C for 5 minutes, then placed on ice to cool down. 90 µL of the mixture was transferred into each well on the sample plate.

AAV sample and blank preparation for genome extraction: This work focused on high-throughput AAV genome analysis of purified samples. Therefore, the AAV samples were prepared following a simplified workflow for genome release and purification which does not include any extra step to remove potential residual nucleic acids impurities. In summary, 20 µL of the each AAV sample was mixed thoroughly with the binding buffer from the QIAquick PCR purification kit. Nucleic acids were purified following manufacturer's instructions with minor modifications as needed. 10 µL of the eluted AAV genome solution was mixed with 40 µL of nuclease-free water and 50 µL of sample loading solution and heated at 70°C for 5 minutes and immediately placed on ice for 5 minutes to cool down. 90 µL of the sample were transferred in to each well on the sample plates. The sample blank was processed similarly, except that sample buffer was used rather than AAV.

Important sample preparation note: filter tips were used for all steps to minimize unintended nucleic acid degradation.

BioPhase 8800 system methods: Figure 2 shows the BioPhase 8800 system methods for the conditioning of the cartridge (panel A), separation of the ssRNA ladder in the kit (Panel B), and shutdown and cartridge storage (panel C). It is worth noting that the injection time for the extracted genome from the AAV sample was increased from 3 seconds used for the ladder to 10 seconds due to the lower concentration of the extracted genome relative to the ladder.

Panel A. Conditioning method.

Method Duration: 42.0 min. Number of Actions: 7

Settings	Capillary Cartridge: 20.0 °C, Wait Capillary Length: 30.0 cm Capillary Type: Bare Fused Silica Current Limit: 100 µA , Enabled		Sample Storage: 10.0 °C Detector Type: LIF, 520 nm, Wait,… Peak Width: 1 sec. Data Rate: 8 Hz		
Rinse	Duration: 5.0 min. 50.0 psi			Inlet: Water Outlet: Waste	
Rinse	Duration: 5.0 min. 20.0 psi			Inlet: Acidic Conditioni… Outlet: Waste	
Rinse	Duration: 2.0 min. 20.0 psi			Inlet: Water Outlet: Waste	
Rinse	Duration: 10.0 min. 50.0 psi			Inlet: Nucleic Acid Exte… Outlet: Waste	
Wait	Duration: 0.0 min.			Inlet: Water Dip 1 Outlet: Water Dip 1	
Wait	Duration: 0.0 min.			Inlet: Water Dip 2 Outlet: Water Dip 2	
Separate	Duration: 20.0 min. -6.0 kV Ramp Time: 2.0 min. Autozero: 8.0 min. Disable Data Collection			Inlet: Nucleic Acid Exte… Outlet: Nucleic Acid Exte…	

Panel B. Separation method.

Method Duration: 31.0 min. Number of Actions: 8

Settings	Capillary Cartridge: 30.0 °C, Wait Capillary Length: 30.0 cm Capillary Type: Bare Fused Silica Current Limit: 100 µA , Enabled		Sample Storage: 10.0 °C, Wait Detector Type: LIF, 520 nm, Wait,… Peak Width: 1 sec. Data Rate: 8 Hz		
Rinse	Duration: 1.0 min. 70.0 psi			Inlet: Acidic Conditioni… Outlet: Waste	
Rinse	Duration: 1.0 min. 70.0 psi			Inlet: Water Outlet: Waste	
Rinse	Duration: 5.0 min. 50.0 psi			Inlet: Nucleic Acid Exte… Outlet: Waste	
Separate	Duration: 2.0 min. -30.0 kV Ramp Time: 0.2 min. Disable Data Collection			Inlet: Nucleic Acid Exte… Outlet: Nucleic Acid Exte…	
Wait	Duration: 0.0 min.			Inlet: Water Dip 1 Outlet: Water Dip 1	
Inject	Duration: 3 sec. -5.0 kV	Plate: Sample		Outlet: Nucleic Acid Exte…	
Wait	Duration: 0.0 min.			Inlet: Water Dip 2 Outlet: Water Dip 2	
Separate	Duration: 22.0 min. -6.0 kV Ramp Time: 2.0 min. Autozero: 8.0 min., Advance after: 6 acti…			Inlet: Nucleic Acid Exte… Outlet: Nucleic Acid Exte…	

Panel C. Shutdown method.

Method Duration: 20.0 min Number of Actions: 4

Settings	Capillary Cartridge: 20.0 °C, Wait Capillary Length: 30.0 cm Capillary Type: Bare Fused Silica Current Limit: 600 µA		Sample Storage: 10.0 °C Detector Type: LIF, 520 nm, Wait, PMT Gain: 100 Peak Width: 2 sec Data Rate: 4 Hz	
Rinse	Duration: 5.0 min 20.0 psi			Inlet: HCl Outlet: Waste
Rinse	Duration: 5.0 min 50.0 psi			Inlet: Water Rinse Outlet: Waste
Wait	Duration: 0.0 min			Inlet: Water Dip 1 Outlet: Water Dip 1
LIFLaser	OFF			

Figure 2. *The BioPhase 8800 system methods for the ssRNA standard analysis. When analyzing extracted genome from the AAV samples, the injection time was increased from 3 seconds to 10 seconds under -5 kV in Panel B.*

Instrumentation: The BioPhase 8800 system equipped with LIF detection has excitation wavelength at 488 nm and emission at 520 nm was from SCIEX (Framingham, MA).

Data acquisition and processing: Data was acquired and processed using the BioPhase software version 1.1.

Results and discussion

Study of sample preparation matrices interference

In this section, we demonstrate that AAV formulation buffer and sample buffer diluent (SLS) does not interfere with the analysis of the genome integrity. For this analysis, we prepared 2 sample blanks. One with the sample formulation buffer and the other with the extraction blank. The extraction blank consists of the AAV formulation buffer processed as if it was the AAV sample. These blanks were processed in parallel with the ssRNA ladder. Figure 3 shows the overlay of the electropherograms with the ssRNA ladder on top, followed by the sample buffer and the extraction blank. No peaks were detected in either the sample buffer or the extraction blank sample.

Inter-capillary reproducibility of migration time and corrected peak area% for the ssRNA ladder

The repeatability of the injection across capillaries was evaluated using the ssRNA ladder. Figure 4 illustrates the overlay of the ssRNA ladder obtained from capillaries A–H in one single injection and Table 1 summarizes the %RSD values for the MT and corrected peak area (CPA). It is worth noting the exceptional inter-capillary reproducibility with %RSD for migration time and CPA better than 0.20 and 1.60%, respectively.

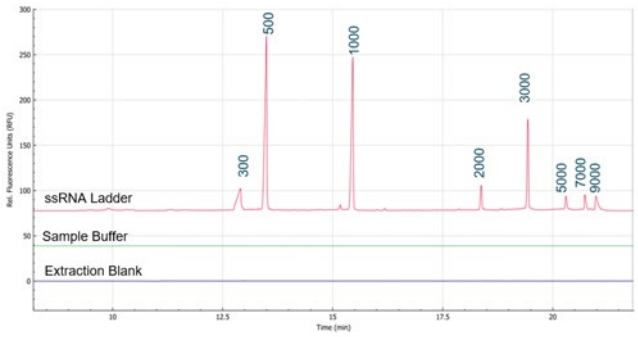

Figure 3. Comparison of electropherograms of ssRNA ladder (top, red), extraction blank (middle, green) and sample buffer (bottom, purple).

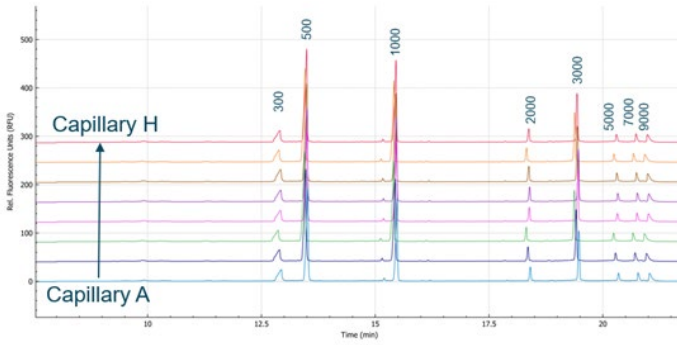

Figure 4. Reproducibility of migration time and corrected peak area% for the ssRNA ladder. Overlay e-grams of a single injection on the 8 capillaries.

Table 1. Inter-capillary reproducibility (RSD%) of migration time (MT) and corrected peak area% (CPA%) of each RNA marker in the ssRNA ladder. N=8.

Marker	Average MT	%RSD MT	Average corrected peak area%	%RSD corrected peak area %
300	*12.91*	*0.18*	*9.39*	*1.21*
500	*13.49*	*0.18*	*39.76*	*0.27*
1000	*15.45*	*0.17*	*28.16*	*0.39*
2000	*18.37*	*0.17*	*3.14*	*0.66*
3000	*19.43*	*0.18*	*10.88*	*0.91*
5000	*20.30*	*0.19*	*1.85*	*0.95*
7000	*20.72*	*0.19*	*1.90*	*1.52*
9000	*20.98*	*0.19*	*2.69*	*0.84*

Analysis of the released genome from different AAV serotypes

Figure 1 shows an overlay of the released genome from an AAV 2 and 2 lots of AAV 8 capsids. According to the manufacturer, the encapsulated genome is the same and the theoretical size is around 2.8 kilobases for all 3 AAVs. Our results revealed the size is around 2.2 kilobases based on a point-to-point calibration with external RNA markers. The results show a good correlation to the theoretical value (Figure 5). We also observed the presence of small size impurities that could be degraded nucleic acids from the host cell DNA and from the plasmid vectors that were not well separated from the full AAV capsids.

Most notably, Figure 1 also shows that this assay can elucidate significant differences in the small size impurities region from lot-to-lot for AAV 8 and for different serotypes, making this method and chemistry amenable to QC operations.

Figure 5 shows an overlay of 3 electropherograms obtained with the ssRNA ladder, the released genome from an AAV 8 capsid and the empty capsid fractions. Excellent resolution of fragments of different sizes was demonstrated in all 3 traces.

The electropherogram corresponds to the empty AAV 8 capsid showing the presence of minor peaks around 2.2 kilobases. We speculate that the presence of genomic material in an empty capsid may be due to the contamination of a small amount of full capsids as this sample was collected via iodixanol gradient centrifugation. In addition, a slightly more intense peak was observed with a size of around 1.7 kilobases, indicating the presence of potential partial capsid in this sample.

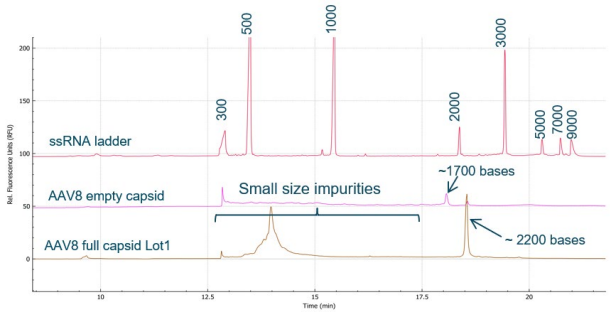

Figure 5. Comparison of electropherograms of ssRNA ladder (top, red), genome extraction from AAV 8 Empty (middle, pink) and corresponding genome extraction from Full (bottom, purple).

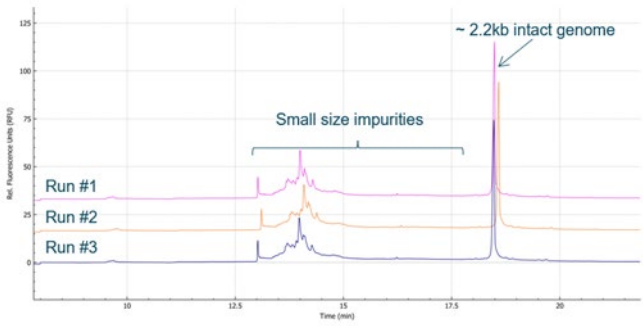

Figure 6. Triplicate injections of extracted genome from AAV8-lot 2 Full capsid.

Figure 6 shows the injection reproducibility of the assay with intact genome released from the AAV 8 – lot 2 sample. The peaks between 12.8 and 17.5 minutes are the small size impurities (below 2.2 kilobases). The %RSD for CPA for intact genome peak for all three samples was below 1%.

Conclusions

- RNA 9000 Purity & Integrity kit with ssRNA ladder allows for the assessment of genome size and purity of AAV regardless of the serotype
- The genome integrity assay can detect lot-to-lot variation, a quality attractive to QC operations
- Excellent inter-capillary reproducibility with %RSD of < 0.20% for MT and <1.60% for CPA
- Sample preparation matrices do not interfere with the reliability of the genome integrity assessment

References

1. C. F. de la Camara, M. E. McClements and R. E. MacLaren, Accurate Quantification of AAV Vector Genomes by Quantitative PCR, Genes (Basel). 2021 Apr; 12(4): 601. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8074223/
2. B. Dong, H. Nakai, W. Xiao, Characterization of Genome Integrity for Oversized Recombinant AAV Vector. Molecular Therapy, 2010, 18(1):87–92
3. G. G. Mironov, A. V. Chechik, R. Ozer, J. C. Bell, M. V. Berezovski, Viral Quantitative Capillary Electrophoresis for Counting Intact Viruses. Analytical Chemistry, 2011,83(13):5431-5

The SCIEX clinical diagnostic portfolio is For In Vitro Diagnostic Use. Rx Only. Product(s) not available in all countries. For information on availability, please contact your local sales representative or refer to https://sciex.com/diagnostics. All other products are For Research Use Only. Not for use in Diagnostic Procedures.

Trademarks and/or registered trademarks mentioned herein, including associated logos, are the property of AB Sciex Pte. Ltd. or their respective owners in the United States and/or certain other countries (see www.sciex.com/trademarks).

© 2022 DH Tech. Dev. Pte. Ltd. RUO-MKT-02-14621-A

*SYBR™ is a trademark of the Life Technologies Corporation. SYBR™ Green II RNA Gel Stain is not available for resale.

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices