Determination of genome titer and integrity of adeno-associated virus (AAV) reference standards using the QIAcuity® Digital PCR System

Jonathan Thorn[1], Anida Mesihovic Karamitsos[1], Dana Holzinger[2], Katharina Hammer[2], Lisa Mutz[2], Andreas Hecker[1], Francesca Di Pasquale[1], Domenica Martorana[1]
[1]QIAGEN GmbH, QIAGEN Strasse 1, 40724, Hilden, Germany; [2]PROGEN Biotechnik GmbH, Maaßstraße 30, 69123 Heidelberg, Germany

Standardization and reproducibility in gene therapy analytics using digital PCR (dPCR)

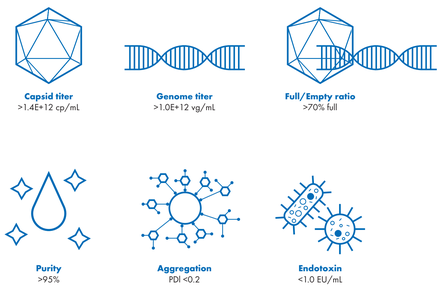
Recombinant adeno-associated virus (rAAV) vectors are widely used as vehicles for gene therapy in both clinical and research applications. To help ensure product safety, reference standard materials (RSMs) are key. The use of viral vector RSMs is particularly important to normalize laboratory internal controls, for the calibration of medical products and procedures, and as best practices for manufacturing and testing of advanced therapy medicinal products (ATMPs). Additionally, AAV RSMs are often used for assay validation and optimization.

In general, the use of well-characterized AAV RSMs and orthogonal methods is indispensable to meet current authority guidelines. Since the production of these is not achievable for many companies, it is important to have commercially available AAV RSMs for the most frequently used AAV serotypes. Underlying these standards is a comprehensive characterization process including determination of vector genome and capsid titer, filling grade, purity and aggregation. One of the critical quality attributes of AAV reference materials is the viral vector genome titer which is usually determined using qPCR.

Here, we demonstrate a streamlined digital PCR-based workflow for quantification of genome titers of commercially available, high-quality rAAV reference standards that enables complete characterization with increased precision, accuracy and robustness. Additionally, the integrity of the viral vector genome can be assessed, ensuring quality and safety of the final product.

Quality attributes of PROGEN's AAV standard material

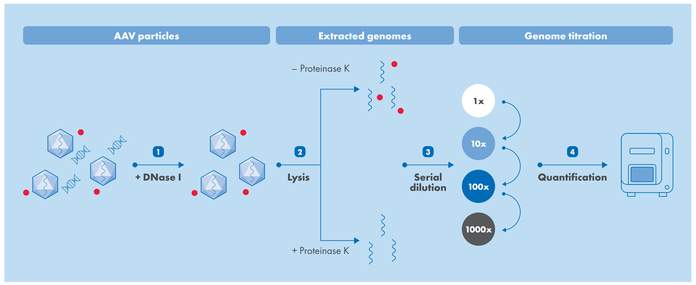
PROGEN has established a comprehensive characterization process for eGFP-filled AAV standards for the serotypes 1–3, 5–6, 8–9, rh10, and rh74. The quality control ensures well-characterized high-quality AAV standard material, which can be easily implemented as reference material in a variety of assays to prove the validity of the corresponding assay.



Comprehensive characterization of PROGEN's AAV reference standards. Characterization procedure includes determination of capsid and viral genome titer using PROGEN ELISA and qPCR, respectively. The filling grade is measured by charge detection mass spectrometry and purity by silver staining. Aggregation is analyzed by dynamic light scattering and endotoxin levels by spectrophotometric measurement (LAL kinetic chromogenic assay). cp/mL: Capsid particles per milliliter; EU/mL: Endotoxin units per milliliter; PDI: Polydispersity index; vg/mL: Vector genomes per milliliter.

AAV standards genome titer and integrity determination following the CGT Viral Vector workflow

AAV reference standards of different serotypes and suppliers are efficiently processed in four standardized steps for genome titration and genome integrity determination on a QIAcuity dPCR System.



1. Removal of DNA impurities such as host cell DNA or plasmids
2. Extraction of vector genome via lysis – with or without Proteinase K digestion
3. Serial dilution of vector genomes – linearity over a broad range
4. Absolute determination of vector titers using digital PCR with gene-of-interest or backbone assays

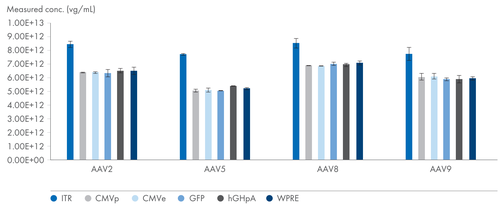
Vector genome · DNA impurity · Host protein

AAV standards processing, titer and integrity determination using the QIAGEN CGT Viral Vector Lysis Kit and CGT dPCR Assays: In a first step, AAV samples are treated with DNase I to remove DNA impurities. In step 2, DNase I-treated samples are lysed in the presence or absence of Proteinase K. In step 3, the lysates are serially diluted. Finally, in step 4, the viral titers and integrity status are determined using a QIAcuity dPCR instrument.

Accurate, precise determination of genome titers and integrity of PROGEN's AAV standards using the QIAcuity dPCR System

AAV reference standards are crucial tools for CGT analytics. Besides the determination of the viral vector genome titer, the assessment of genome integrity is playing an increasingly important role. Digital PCR with high multiplex capabilities enables the analysis of both critical attributes.

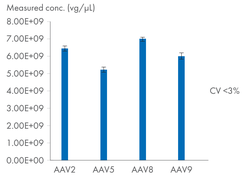
A



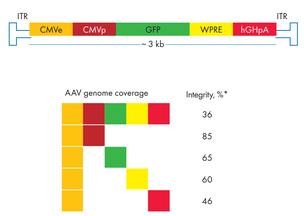
Genome targets	AAV2 (vg/mL)	AAV5 (vg/mL)	AAV8 (vg/mL)	AAV9 (vg/mL)
ITR	8.45×10^{12}	7.71×10^{12}	8.53×10^{12}	7.75×10^{12}
CMVp	6.39×10^{12}	5.07×10^{12}	6.88×10^{12}	6.09×10^{12}
CMVe	6.40×10^{12}	5.12×10^{12}	6.90×10^{12}	6.11×10^{12}
GFP	6.36×10^{12}	5.10×10^{12}	7.03×10^{12}	5.92×10^{12}
hGHpA	6.53×10^{12}	5.44×10^{12}	6.95×10^{12}	5.90×10^{12}
WPRE	6.52×10^{12}	5.24×10^{12}	7.10×10^{12}	5.97×10^{12}

The non-ITR genome targets of PROGEN's AAV standards show comparable quantification. AAV2, AAV5, AAV8 and AAV9 standards were processed using the CGT Viral Vector Lysis Kit and quantified on a QIAcuity dPCR System using 8.5k nanoplates and CGT dPCR Assays. The CGT dPCR Assays were run in 2 triplex reactions in the FAM™, HEX™ and Cy5® channels (ITR, CMVe, CMVp and GFP, WPRE, hGHpA). The samples were serially diluted and 2 dilutions with 3 technical replicates each were used for the determination of the genome titer.

B



C



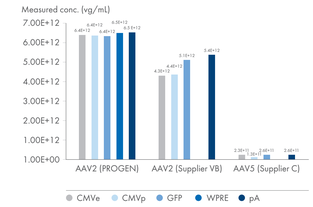
Genome titer determination with high precision. B Reference standards (PROGEN) were processed using the CGT Viral Vector Lysis Kit and quantified using the CGT dPCR Assays in 2 triplex reactions (ITR, CMVe, CMVp and GFP, WPRE, hGHpA) on a QIAcuity dPCR System. Coefficient of variation (CV) between the non-ITR targets is shown. Maximum deviation from mean between targets <5% for AAV5 and <2% for AAV2, AAV8 and AAV9. **C** Genome integrity was determined using the QIAcuity Software Suite version 2.5. Genome targets were run in a 5-plex reaction. The analysis of genome integrity was performed including all 5 channels or in groups of 2 spanning different parts of the genome.

* AAV2 standard was processed using CGT Viral Vector Lysis Kit. Processing includes enzymatic removal of ITR secondary structures and takes place outside of the QIAcuity nanoplate partitions, which may lead to reduced integrity scores.

Comparing genome titers and integrity of different AAV reference standards using dPCR

Quantification of multiple targets on the genome increases insights on sample titer and quality. AAV packaging is error prone and elimination of mispackaged or incomplete particles is difficult to achieve during manufacturing and downstream clean-up. Digital PCR allows in-depth analysis of AAV samples and standards.

A



B

AAV PROGEN

AAV suppliers VB and C

Standard	Integrity, %* 5' end of genome	Integrity, %* 3' end of genome
AAV2 (PROGEN)	85	84 (~380 bp)
AAV2 (Supplier VB)	88	70 (~200 bp)
AAV5 (Supplier C)	52	57 (~450 bp)

The viral vector genome titer does not necessarily reflect the genome integrity status. A AAV standards from different suppliers (PROGEN, VB, C) were processed using the CGT Viral Vector Lysis Kit and quantified using the CGT dPCR Assays in multiplex reactions on a QIAcuity dPCR System. **B** Genome integrity of the 5' and 3' end of the genomes was determined using the QIAcuity Software Suite version 2.5. Integrity was analyzed in a duplex setup (5' end towards GFP and GFP towards 3' end). Distance between targets is indicated in case of differences between standards.

* AAV standards were processed using the CGT Viral Vector Lysis Kit. Processing includes enzymatic removal of ITR secondary structures and takes place outside of the QIAcuity nanoplate partitions which may lead to reduced integrity scores.

Conclusions

- Viral vectors, such as AAVs, can be reliably processed using the CGT Viral Vector Lysis Kit. Genome titers and integrity can be accurately determined using dPCR and the CGT dPCR Assays.
- Thoroughly characterized AAV reference standards like PROGEN's standard material are crucial for accurate analytics of viral vectors.
- QIAcuity Software Suite version 2.5 enables access to insights into genome integrity.
- Viral vector genome titers do not necessarily reflect the genome integrity status.
- The more targets are analyzed in a multiplex reaction, the more comprehensive the integrity score.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit instructions for use or user operator manual. QIAGEN instructions for use and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services (or your local distributor).

Sample to Insight