Developing reliable AAV standards for ELISA

Besir H, Loos N, Hammer K, Razov A, Queitsch I, Kuhlendahl S
Progen Biotechnik GmbH, Heidelberg

PROGEN passion for research

Abstract

A growing number of academic and industrial labs are using AAV (adeno-associated viruses) vectors for the development of gene therapies leading to an increase in the demand for reliable, reproducible and robust analytical AAV tools for R&D and manufacturing. A dependable and reproducible quantification of rAAV titers is needed to enable safe and effective AAV gene therapies. Current quantification methods for rAAV vector preparations include qPCR, digital droplet PCR (ddPCR) for measuring DNA, Dot Blot and ELISA for measuring intact viral capsids. Here we show the development of an internal standard for calibrating an AAV Titration ELISA. A calibrated conventional sandwich ELISA is a robust and reliable tool for the quantification of rAAV preparations in terms of intra- and inter-assay variability and ease of use.

Introduction

Gene therapies often use viruses such as AAV as vectors to deliver desired genes into target cells. Quantification of purified AAV vector preparations is a critical step for clinical applications, to minimize an immunogenic response and to maximize gene transfer into target cells. So far, international reference material only exists for AAV2 and AAV8. However, the development of standardized AAV capsid material is an essential requirement for the calibration of all AAV titration ELISA assays to ensure the reliable quantification of rAAV vector preparations. Here, we show an example of the development of an internal AAV5 gold standard by using electron microscopy, qPCR and ddPCR. The AAV5 standard was then utilized to calibrate the AAV5 ELISA kit control for reliable and reproducible quantification of intact AAV5 capsids.

Methods & Results

To characterize and quantify purified AAV5 vector preparations, the purity was confirmed by SDS-PAGE, the ratio of filled and empty capsids was determined by electron microscopy (EM) followed by qPCR to measure viral gene amount. The preparation of AAV5 contains a GFP transgene. The results of these experiments were taken as basis for determination of the total capsid titer of the gold standard.

Development of an AAV5 Internal Gold Standard

1. Preparation of a pure AAV5 internal standard

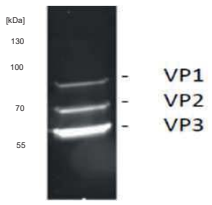
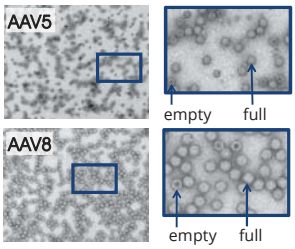


Figure 1. *SDS-PAGE with separated AAV5 viral protein preparations.*

The AAV5 preparation generated for standaridization was tested for high purity on SDS-PAGE to confirm size, ratio an purity of viral proteins.

2. Determination of filled/empty capsids ratio by EM



AAV5 Sample		
Image	Total capsids counted	% filled capsids
1	415	86
2	418	80
3	393	79
4	476	87
5	442	82
6	488	85
Average		83
CV		4%

Figure 2. *Comparison AAV5 sample and reference AAV8 (WL217S) capsids by EM. AAV5 micrograph; AAV8 (WL217S) micrograph, which was used to characterize the current AAV8 RSM material from ATCC [Ayuso et al., 2014]. Samples were stained with uranyl acetate.* ***Table:*** *Determination of filled and empty AAV5 capsids by EM. Multiple EM images AAV samples were taken and full and empty capsids were counted.*

On average, the AAV5 preparation contains 83% full capsids with a coefficient of validation (CV) of 4%.

3. Determination of the AAV5 gold standard capsid particle titer by qPCR

		Mean (qPCR-Titer)	CV (qPCR)
A	AAV5 (3 Labs, 16 runs)	7,1E+12 vg/mL	18%
B	rAAV2 RSM (ATCC)	3,8E+10 vg/mL	78%
C	rAAV8 RSM (ATCC)	9,6E+11 vg/mL	113%

Figure 3. *Comparison of AAV5 qPCR titers with ATCC standards for AAV2 and AAV8.* ***A*** *3 independent labs provided qPCR / ddPCR data for the AAV5 standard material.* ***B/C*** *Published qPCR data for ATCC standard material AAV2 [Lock et al., 2010] and AAV8 [Ayuso et al., 2014].*

The CV of the AAV5 qPCR data is well within range of published intra-lab variations of ATCC reference standard material for AAV2 and AAV8.

4. Calculation of the final AAV5 titer

The capsid particle titer of the AAV5 material was calculated from qPCR titer and % filled capsids (EM):

7.1E+12 vg/mL (qPCR titer) ÷ 0.83 (% filled capsids) = 8.6E+12 capsids/mL

This characterized AAV5 gold standard was then used to calibrate Kit Controls for the AAV5 Titration ELISA.

Calibration of the AAV5 Titration ELISA

5. Principle of the AAV5 Tiration ELISA

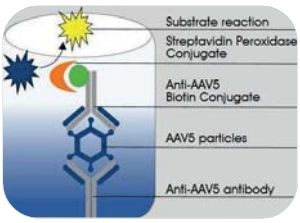


Figure 4. *Principle of the AAV5 Titration ELISA.*

The conventional sandwich ELISA uses a microtiter plate coated with monoclonal antibodies to capture only intact, assembled AAV5 capsids. The biotin/ streptavidin peroxidase color reaction of the detecting antibody allows the photometrical determination of the precise titer of infective vectors.

6. Calibration of the Kit Control

The ELISA Kit Control is a serial dilution of empty AAV5 particles. The resulting curve is used to determine the titer of unknown samples. The Kit Control is aligned with the established gold standard.

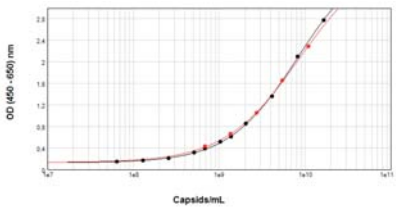


Figure 5. *Alignment of the Kit Control with the established gold standard in the AAV5 Titration ELISA. Aligned curves of gold standard material and Kit Control, measured with the AAV5 Titration ELISA (OD vs. concentration). Black: Curve of the gold standard material, Red: Curve of the Kit Control.*

7. Parameters of the calibrated AAV5 Titration ELISA

A: Lot-to-lot consistency

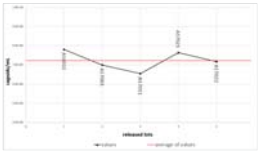


Figure 6. ***A*** *Lot-to-lot consistency. The same sample was measured in different lots of AAV5 Titration ELISA,* ***B*** *Inter-assay variability. An established internal control is measured in the same lots on different days.* ***C*** *Inter-assay variability. Replicates of one sample were measured in the same experiment and plate.*

B: Inter-assay variance

Sample No.	P1	P2	P3
No. of test days	n = 5	n = 5	n = 5
Average reading [Capsids/mL]	1.6E+10	1.0E+10	7.0E+09
CV	6%	4%	5%

C: Intra-assay variability

Sample No.	P1	P2	P3
Tested Replicates	n = 24	n = 24	n = 24
Average OD	2.0	1.4	0.8
Average Reading [Capsids/mL]	2.0E+10	9.4E+09	5.0E+09
CV	5%	5%	4%

With a lot-to-lot variability of < 15% and intra-/inter-assay variability of CV 5% each, we conclude that the AAV ELISA has a very high accuracy and reproducibility.

Conclusion

qPCR and electron microscopy are established and adequate methods for the development and characterization of purified AAV internal gold standards when reference materials are lacking. The AAV5 internal standard in this study was successfully used to calibrate ELISA kit controls. With a high lot-to-lot consistency and low range of intra- and inter-assay variability, the resulting ELISA is a robust and accurate tool for the determination of AAV5 titers. The assay thereby fulfills the requirements for a reliable quantification of rAAV preparations in AAV5 gene therapy research and development.

References

Lock, M. et al. Characterization of a recombinant adeno-associated virus type 2 Reference Standard Material. Hum. Gene Ther. 21, 1273–1285 (2010).
Ayuso, E. et al. Manufacturing and characterization of a recombinant adeno-associated virus type 8 reference standard material. Hum. Gene Ther. 25, 977–87 (2014).

Acknowledgements

We thank Sirion Biotechnik GmbH, München and Prof. Grimm, BioQuant, Heidelberg, for qPCR-titer data, as well as the EM Core Facility (Dr. Karsten Richter) of the DKFZ, Heidelberg, for the EM image analysis.