



The New AAV3 Titration ELISA

- continued tradition of reliable AAV titer determination -



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Abstract

AAV vectors are frequently used by academic and industrial labs for the development of gene therapies. A growing number of serotypes are being used for various diseases. To ensure safety and efficacy of these therapies, a robust and reliable quantification of AAV titers is indispensable. Meeting this need of the gene therapy community, PROGEN developed a new AAV3 Titration ELISA for reliable determination of total capsid titer. The AAV3 ELISA was developed following the well-established workflow for other PROGEN AAV serotype-specific ELISAs. A purified AAV3 gold standard material was generated and quantified by multiple qPCR, dPCR and EM experiments in several labs to determine the ratio filled/empty capsid and quantification of viral DNA. Each lot of AAV3 Titration ELISAs is calibrated using this internal gold standard and compared to previous lots in order to ensure minimal lot-to-lot variation.

Introduction

Quantification of purified AAV vector preparations is a critical step for clinical applications of gene therapies, to minimize an immunogenic response and to maximize gene transfer into target cells. So far, international reference material only exists for AAV2 and AAV8^{1,2}. 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 AAV preparations. Here, we show the development of an internal AAV3 gold standard by using electron microscopy, as well as qPCR techniques. The AAV3 standard was then utilized to calibrate the AAV3 ELISA kit control for reliable and reproducible quantification of intact AAV3 capsids.

Methods & Results

To characterize and quantify purified AAV3 vector preparations, the purity was confirmed by SDS-PAGE, the ratio of filled and empty capsids was determined by negative stain electron microscopy (EM) and cryo EM followed by qPCR / digital PCR (dPCR) to measure viral gene amount. The preparation of AAV3 contains a GFP transgene. The results of these experiments were taken as basis for the determination of the total capsid titer of the gold standard.

Development of an AAV3 Internal Gold Standard

1. Preparation of a pure AAV3 internal standard

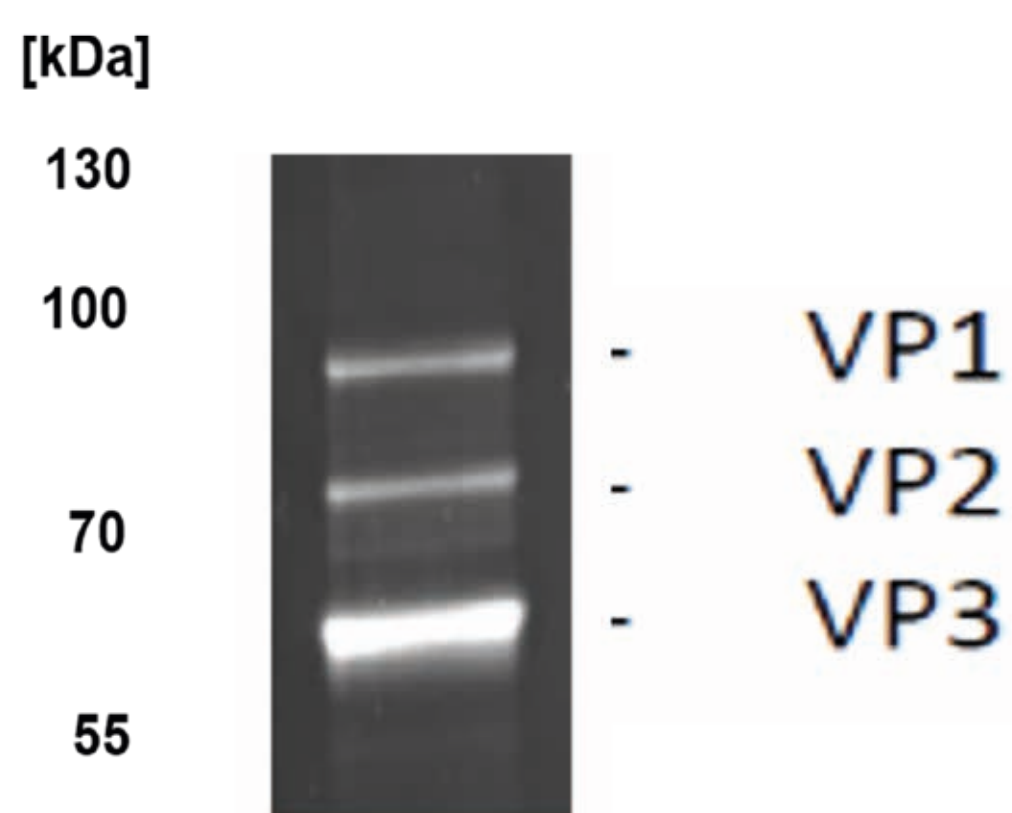


Figure 1. SDS-PAGE with separated AAV3 viral capsid proteins.

The AAV3 preparation generated for standardization was tested for high purity on SDS-PAGE to confirm size, ratio and purity of viral capsid proteins.

2. Determination of filled/empty capsid ratio by neg. stain EM and cryo EM

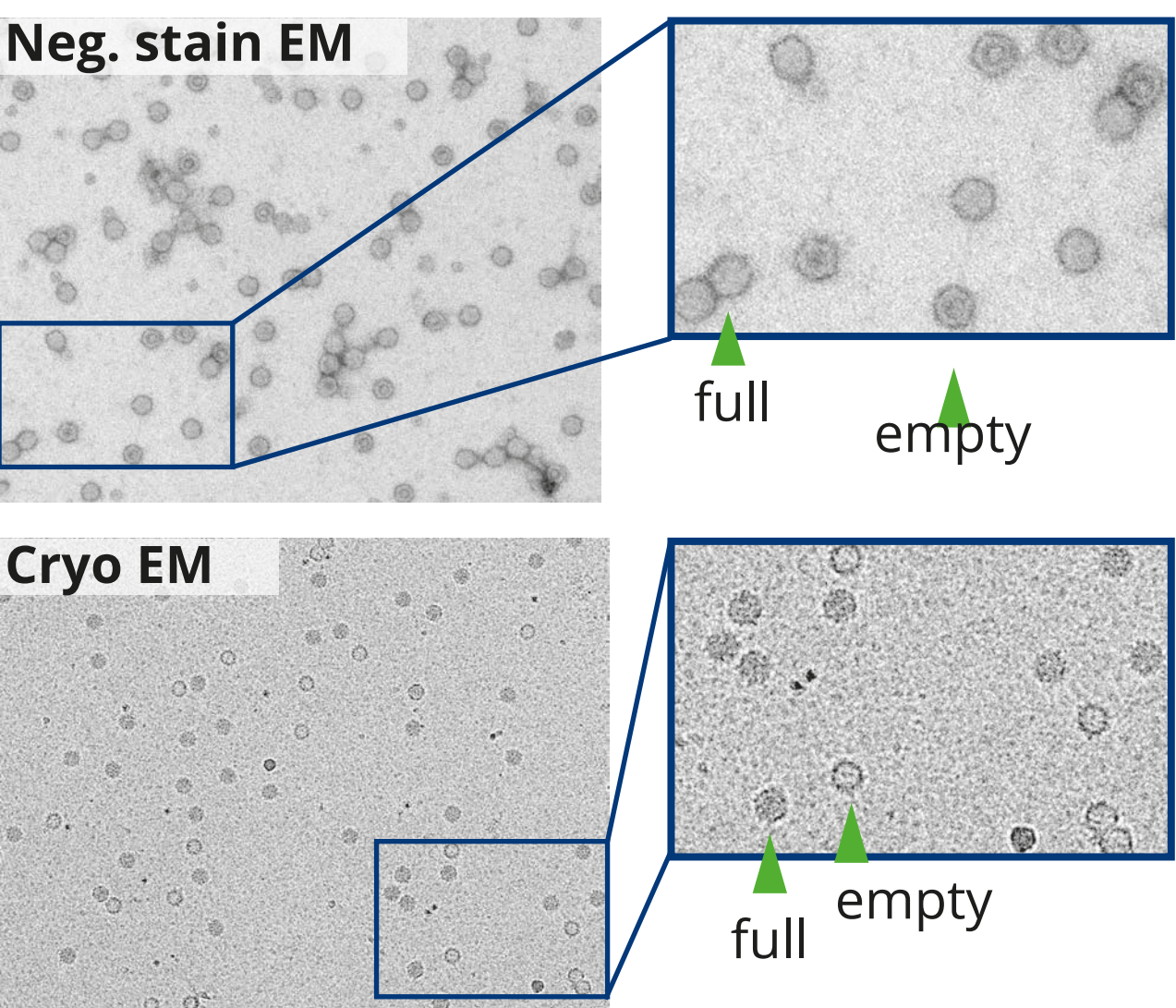


Figure 2. Comparison AAV3 samples imaged by negative stain EM and cryo EM, respectively.

On average, the AAV3 gold standard preparation contains ~70% full capsids. The ratio of filled and empty capsids is comparable for both, negative stain EM and cryo EM.

| AAV3 Sample | | |
|-------------------|-----------------------|------------------|
| image | total capsids counted | % filled capsids |
| negative stain EM | 5571 | 68 % |
| cryo EM | 1690 | 71 % |

Table1. Comparison of counted AAV3 particles in negative stain EM vs. cryo EM. Multiple EM images of the same AAV3 preparation were taken and full and empty capsids were counted.

3. Determination of the AAV3 gold standard viral genome titer by qPCR/dPCR

| | | Mean (PCR-Titer) | CV (PCR) |
|---|------------------------|------------------|----------|
| A | AAV3 (3 Labs, 18 runs) | 3.8E+12 vg/ml | 28% |
| B | rAAV2 RSM (ATCC) | 3,8E+10 vg/ml | 78% |
| C | rAAV8 RSM (ATCC) | 9,6E+11 vg/ml | 113% |

Table 2. Comparison of AAV3 qPCR titers with ATCC standards for AAV2 and AAV8. **A** 3 independent labs provided qPCR/dPCR data for the AAV3 standard material. **B&C** Published qPCR data for ATCC standard material AAV2¹ and AAV8².

The CV of the AAV3 qPCR/dPCR data is well within the range of published intra-lab variations of ATCC reference standard material for AAV2 and AAV8.

4. Calculation of the final AAV3 reference titer

The capsid particle titer of the AAV3 material was calculated from PCR titer and % filled capsids (EM):

$$3.8E+12 \text{ vg/ml (qPCR/dPCR titer)} \div 0.7 (\% \text{ filled capsids}) = 5.5E+12 \text{ capsids/ml}$$

This characterized AAV3 gold standard material was then used to calibrate Kit Controls for the AAV3 Titration ELISA.

AAV3 Titration ELISA

5. Principle of the AAV3 Titration ELISA

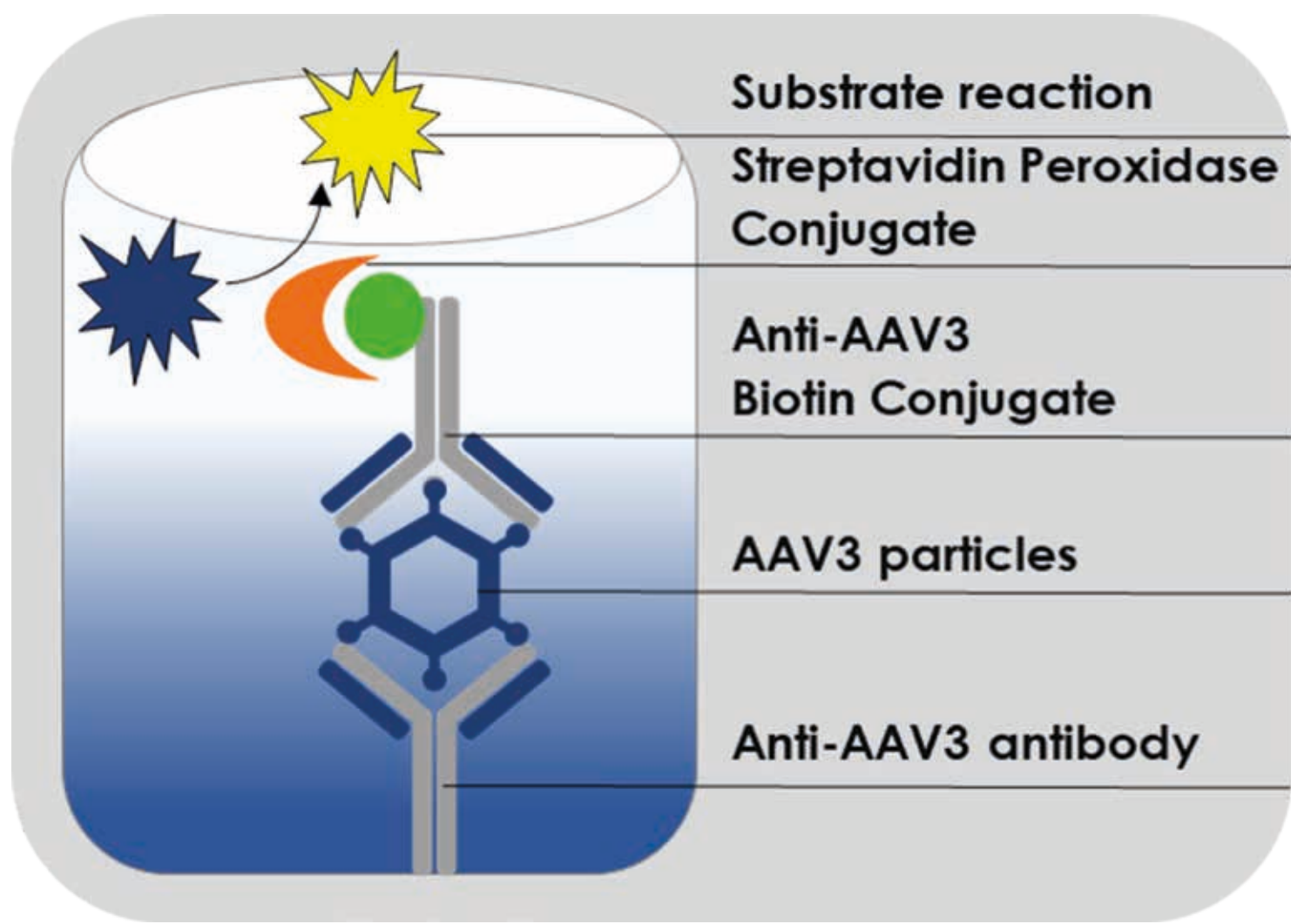


Figure 3. Principle of the AAV3 Titration ELISA.

The conventional sandwich ELISA uses a microtiter plate coated with a monoclonal antibody to capture only intact, assembled AAV3 capsids. The biotin/streptavidin peroxidase color reaction of the detecting antibody allows the photometric determination of the precise titer of intact vectors.

6. Calibration of the AAV3 Kit Control

The ELISA Kit Control is a serial dilution of empty AAV3 particles with known concentration. The resulting curve is used to determine the titer of unknown samples. The Kit Control is calibrated with the established gold standard.

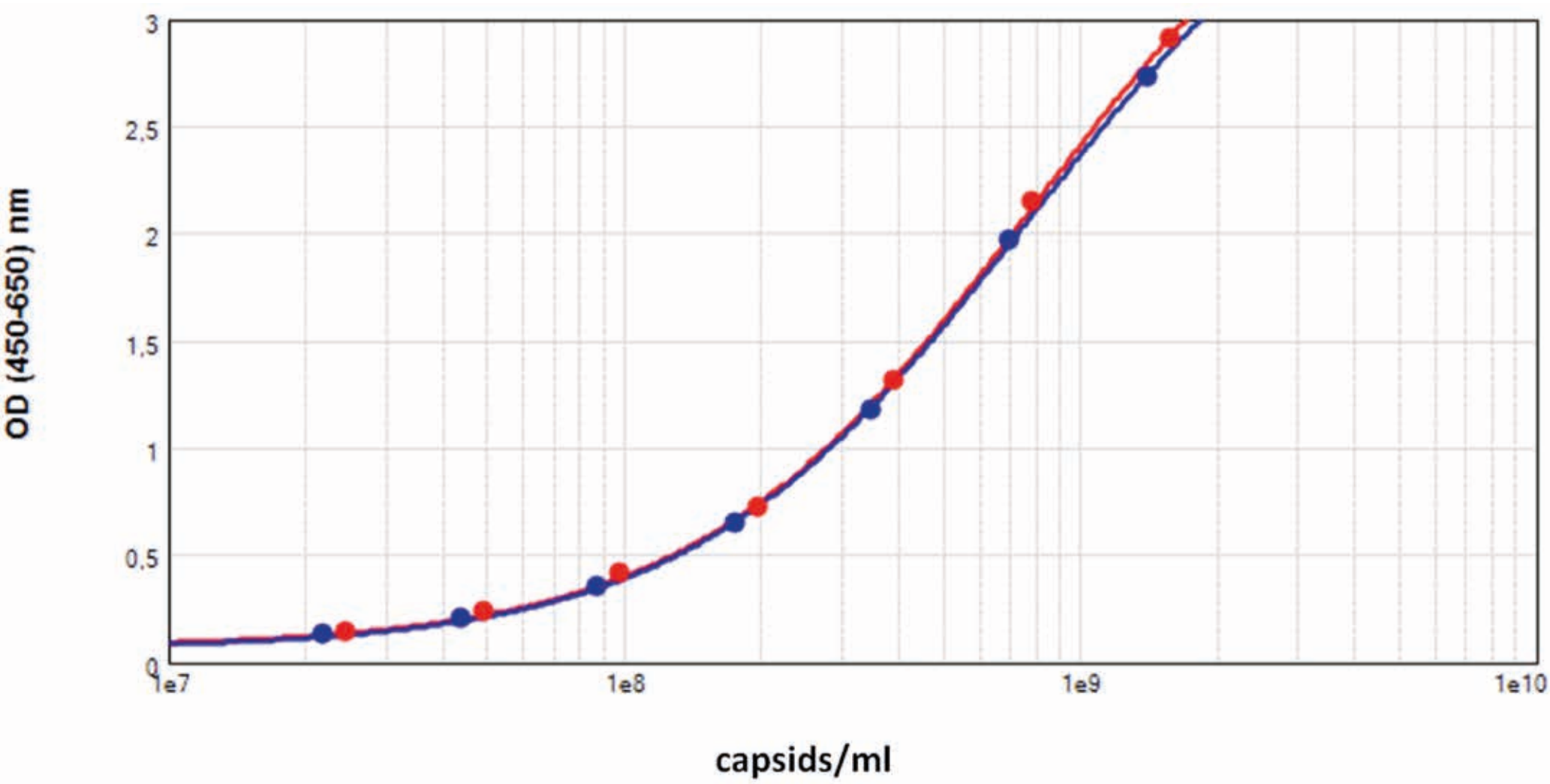


Figure 4. Aligned curves of the Kit Control (blue curve) with the established gold standard (red curve) measured with the AAV3 Titration ELISA (OD vs. concentration).

7. Parameters of the calibrated AAV3 Titration ELISA

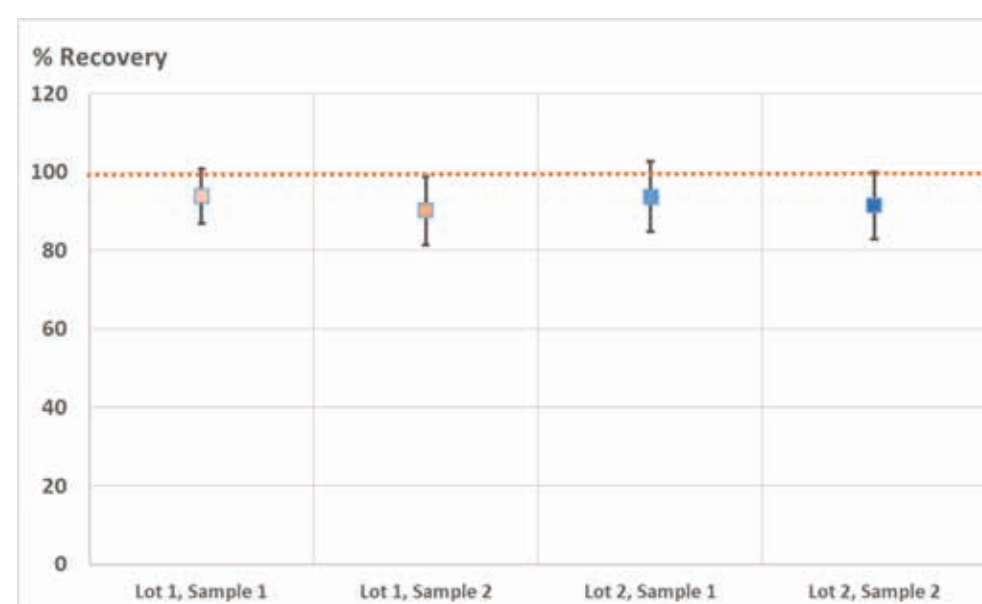
A: Inter-assay variance

| Sample No. | P1 | P2 | P3 | P4 |
|------------------------------|---------|---------|---------|---------|
| No. of test days | n = 5 | n = 5 | n = 5 | n = 5 |
| Average reading [Capsids/ml] | 9.0E+08 | 4.9E+08 | 3.5E+08 | 2.1E+08 |
| CV | 3% | 4% | 3% | 6% |

B: Intra-assay variance

| Sample No. | P1 | P2 | P3 |
|------------------------------|---------|---------|---------|
| Tested Replicates | n = 24 | n = 24 | n = 24 |
| Average OD | 1.9 | 1.2 | 0.8 |
| Average Reading [Capsids/ml] | 6.7E+08 | 3.4E+08 | 2.1E+08 |
| CV | 2.5% | 2.5% | 2.6% |

C: Lot-to-lot consistency



A Inter-assay variability. An established internal control is measured in the same lots on different days.
B Intra-assay variability. Replicates of one sample were measured in the same experiment and plate.
C Lot-to-lot consistency. The same samples were measured in different lots of AAV3 Titration ELISA.

With a lot-to-lot variability of < 15% and intra- and inter-assay variability of CV < 3% and < 6%, respectively, we confirm that the AAV3 Titration ELISA has a high accuracy and reproducibility.

Conclusion

PCR and electron microscopy are established and adequate methods for the development and characterization of purified AAV internal gold standards when reference materials are lacking. Both negative stain EM and cryo EM methods can be used to determine the ratio of DNA-filled and empty capsids. In our experiments, both EM methods resulted in comparable values. The AAV3 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 AAV3 titers. The assay thereby fulfills the requirements for a reliable quantification of rAAV preparations in AAV3 gene therapy research and development.

References

- ¹Lock, M. et al. Characterization of a recombinant adeno-associated virus type 2 Reference Standard Material. Hum. Gene Ther. 21, 1273–1285 (2010).
²Ayuso, 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 and Prof. Dirk Grimm for qPCR and dPCR data, respectively, as well as the EM Core Facility (Dr. Karsten Richter) of the DKFZ, Heidelberg, and cryo EM facility (Dr. Felix Weis) of the EMBL Heidelberg for the negative stain EM and cryo EM image analysis, respectively.