TrueCourse Biosciences

TECHNICAL NOTE — CRYO-TEM

Cryo-TEM Characterization of Adeno-Associated Virus (AAV) Capsid Ratios

Direct, single-particle determination of the empty-to-full capsid ratio — a critical quality attribute for rAAV gene-therapy formulations.

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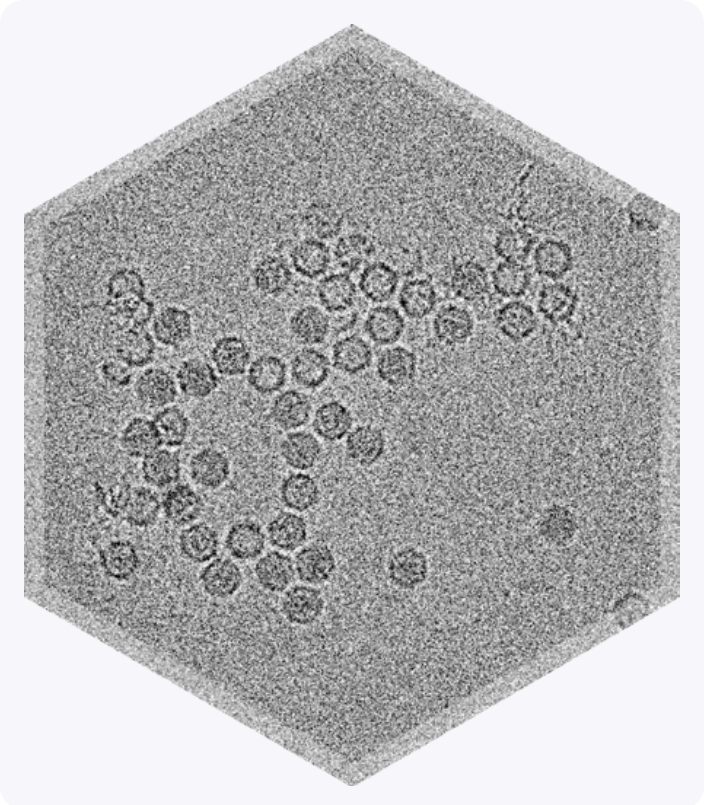
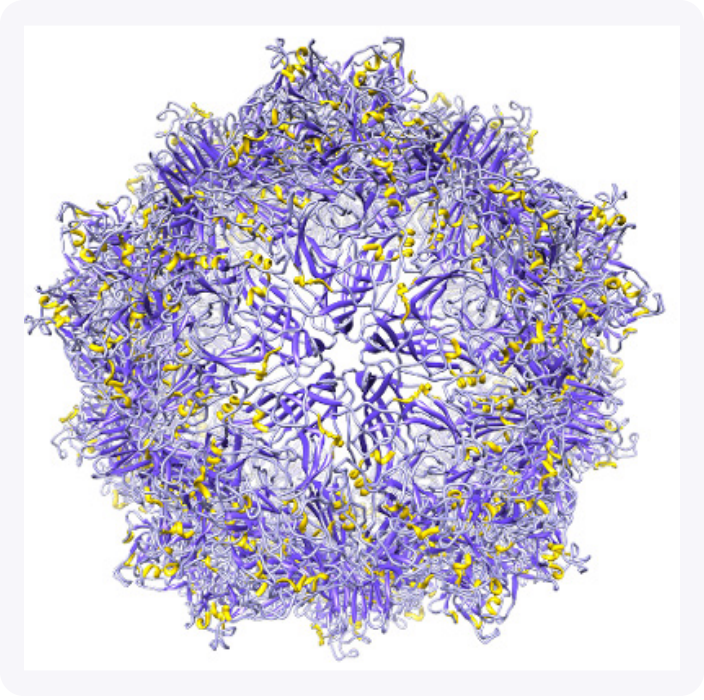
Recombinant adeno-associated viruses (rAAVs) have emerged as a leading viral vector platform for gene therapy applications due to their favorable safety profile, broad tissue tropism, and ability to achieve sustained transgene expression. The formulation process for rAAV therapies involves optimization of numerous factors and the assessment of several critical quality attributes. Some of the most important characterization parameters for an rAAV formulation are titer, aggregation, and the empty-to-full capsid ratio.

Introduction

The empty-to-full capsid ratio directly impacts both the safety and efficacy of rAAV-based therapeutics. Empty capsids, which lack the therapeutic genetic payload, compete with full capsids for cellular receptors and can trigger unwanted immune responses without providing therapeutic benefit. An elevated empty-to-full ratio reduces the effective dose of a formulation while potentially increasing immunogenicity and adverse reactions.

Consequently, regulatory agencies require the determination of this ratio as part of any characterization profile. Numerous advanced analytical techniques have been employed for determining the empty-to-full ratio. Some of these techniques, such as SEC-MALS, UV/Vis, and PCR-ELISA, cannot distinguish partially filled capsids, and therefore the interpretation of results from these methods is difficult. Other techniques, including analytical ultracentrifugation (AUC), charge detection mass spectroscopy, mass photometry, and cryo-transmission electron microscopy (cryo-TEM) are capable of detecting populations of partially filled capsids, making the determination of empty-to-full ratio more robust and reliable.





Example cryo-TEM image & 3D reconstruction to 1.8 Å.

Cryo-Transmission Electron Microscopy

Of the various techniques that are commonly employed to ensure that AAV formulations meet stringent pharmaceutical standards for clinical and commercial use, cryo-TEM is unique in that it allows direct observation of individual AAV particles, rather than assessing samples in bulk. One advantage of this approach is that several critical quality attributes can be assessed simultaneously for a sample, including the empty-to-full ratio, aggregation, the presence of contaminants, the presence of unencapsulated DNA, and capsid integrity (Figure 1).

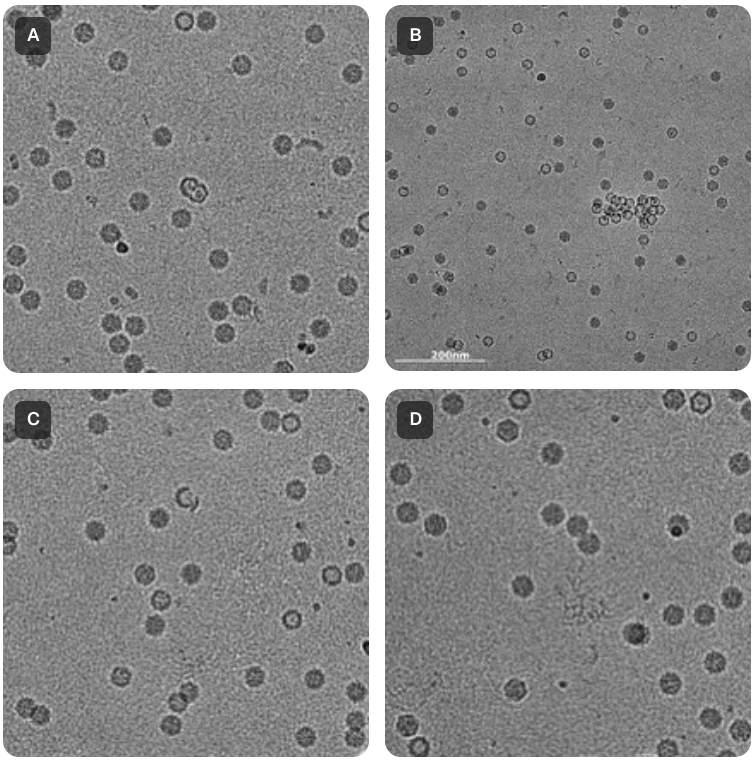


Figure 1. Cryo-TEM imaging can reveal several critical quality attributes during the characterization of rAAV formulations. A) malformed dimeric capsid, B) particle aggregate, C) broken capsid, D) unencapsulated DNA.

These sample characteristics can all be unambiguously directly observed in cryo-TEM images. Traditionally, negative staining TEM has been used to characterize rAAV samples; however, there are certain disadvantages associated with this approach as it requires drying samples in a vacuum and it depends on staining the samples with heavy metal to generate indirect particle contrast.

Conversely, cryo-TEM preserves particles in their native, hydrated state and the interior density of particles as seen in images is directly correlated to the size of the genetic payload (Figure 2). This makes interpretation of the cryo-TEM data more straightforward than negative stain TEM and increases accuracy in determining the empty-to-full ratio.

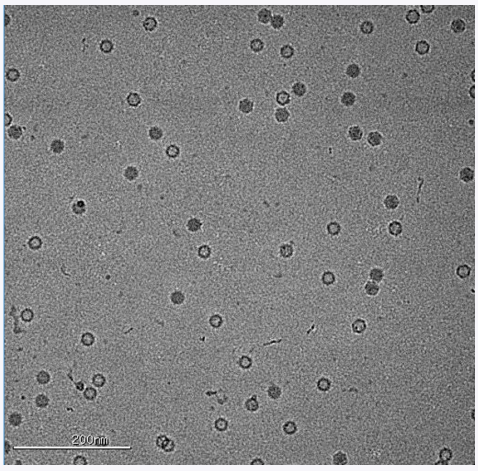


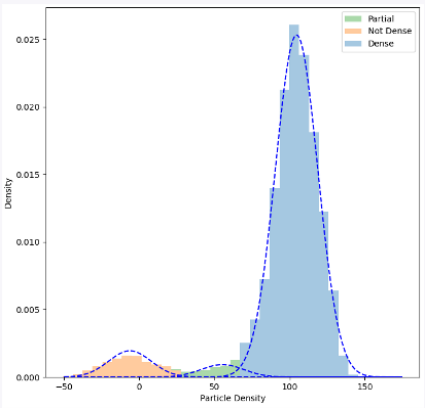
Figure 2. Loading status of AAV particles is clearly visible in cryo-TEM images. The contrast of particles is directly correlated with the mass of each particle. Particles loaded with DNA appear dense (dark), unloaded particles have a low-density interior (not dense), while partially filled particles have an intermediate density.

Sample Requirements for Cryo-TEM

Most rAAV samples are compatible with cryo-TEM imaging and analysis.

- The matrix typically used for rAAV formulations rarely presents challenges for cryo-TEM sample preparation or imaging.
- The technique is compatible with titers as low as 1E11 particles/mL.
- Only a small sample volume is required — a cryo-TEM grid is prepared for analysis with just 3 µL of sample.
- The assay clearly distinguishes empty particles from full particles with payloads as small as 2.4 kb in length (Figure 3).
- Empty-to-full analysis can be validated and assays can be performed under cGMP conditions.

~2.5 kb

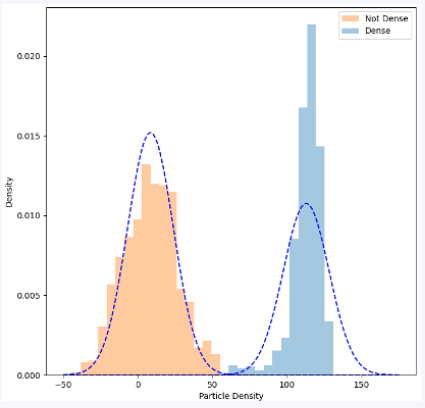


Dense 90.3%

Partial 2.8%

Not Dense 6.9%

~4 kb

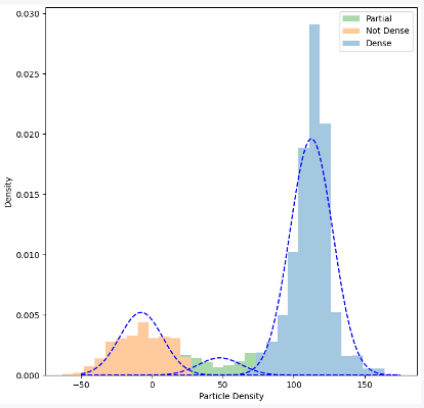


Dense 74.6%

Partial 5.1%

Not Dense 20.3%

~5 kb



Dense 41.7%

Partial 0%

Not Dense 58.3%

Figure 3. Empty-to-full ratio determination is robust across a wide range of payload sizes. Populations of empty and full particles are clearly resolvable in samples containing 5 kb, 4 kb, and 2 kb payloads. Small populations of partially filled particles are detectable, as seen in the 2.5 kb and 4 kb samples. The determination of the dense, partial, and non-dense populations is performed fully automatically based on statistical analysis of the underlying particle-density data.

Assessing Method Performance

The automated nature of sample preparation and data analysis for empty-to-full ratio determination ensures consistent and unbiased results. Extensive testing has been completed to characterize the performance of the assay. When repeated measurements are made of a single sample, the relative standard deviation is <1%. Even when measurements are repeated across multiple days and with multiple lab technicians, the intermediate precision is <5% (Figure 5, page 7).

When compared with another gold-standard orthogonal technique (AUC), the cryo-TEM method results are very similar to AUC across a wide range of empty-to-full ratios (Figure 4). Furthermore, the analysis has been demonstrated to work for formulations with payloads ranging from 2 kb to 5 kb. Populations of empty and full particles are clearly resolvable across all these payload sizes and, if present, populations of partially filled particles are also detectable.

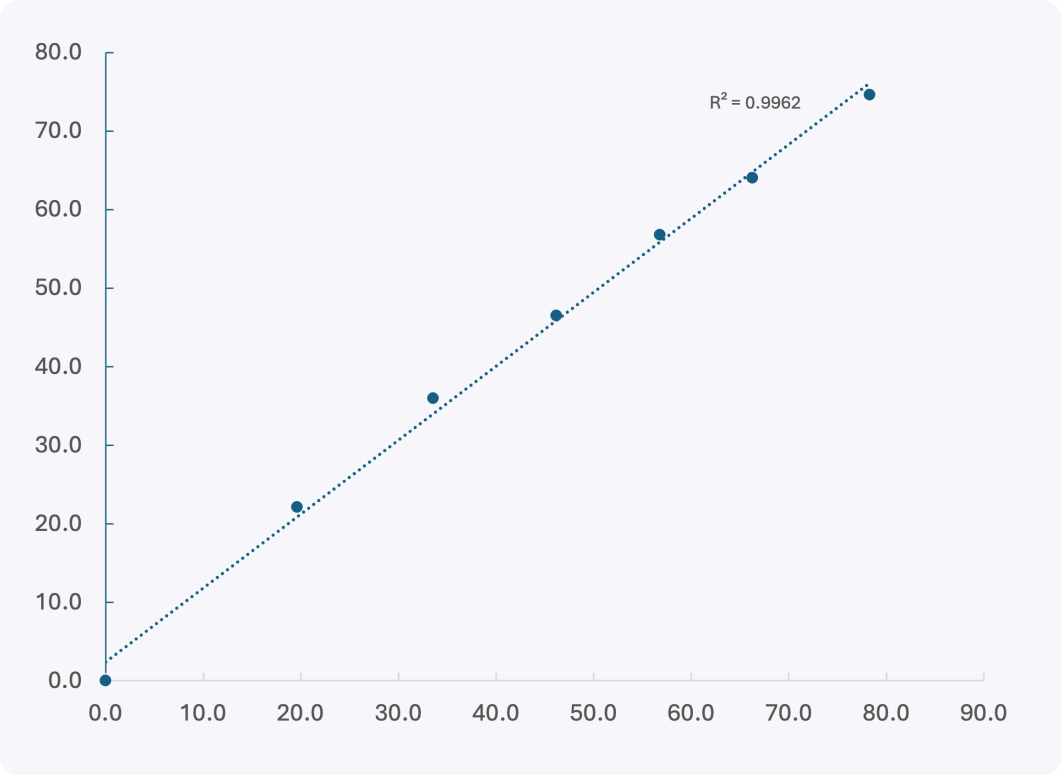


Figure 4. Linearity of the cryo-TEM analysis has been established relative to analytical ultracentrifugation. A series of AAV samples were analyzed by AUC and cryo-TEM, consisting of a full sample, an empty sample, and a range of intermediate samples produced by combining the two. There is a very high degree of correlation between the percentage of full capsids determined by the two techniques across the full range of samples.

Automated Workflow for Achieving Consistent Results

The determination of the empty-to-full ratio begins with sample preparation and data collection. The sample is applied to a TEM grid and a robotic system performs vitrification in liquid nitrogen-cooled ethane. This automation ensures highly repeatable sample preparation. The sample is then held at cryogenic temperatures while transferred to the microscope and throughout imaging. Once a set of images has been collected, they are automatically analyzed by software developed in-house: it scans all images to detect AAV particles and determine the interior density of each, with high accuracy and no detectable picking bias. After a sufficiently large sample of particles has been measured (a minimum of 1,500 particles), the software automatically detects sub-populations according to density — empty, partial, and full — resulting in an empty-to-full ratio determination for the sample.

Grid	Day	Lab Tech	Grid Lot	Percent Dense	Percent Partial	Percent Not Dense
1	1	1	1	77.3%	5.4%	17.3%
2	1	1	1	78.2%	5.4%	16.4%
3	1	1	1	77.0%	5.9%	17.2%
4	2	2	1	77.4%	5.6%	17.0%
5	2	2	2	73.5%	6.1%	20.4%
6	3	3	2	74.6%	5.1%	20.3%

REPEATABILITY (DAY 1)

Mean = 77.5% · SD = 0.6% · %RSD = 0.8%

INTERMEDIATE PRECISION

Mean = 76.3% · SD = 1.8% · %RSD = 2.4%

Figure 5. Cryo-TEM empty-to-full ratio determination is highly precise. Multiple tests of a single sample, either with identical testing conditions or while varying experimental conditions, reveal very high repeatability and intermediate precision for cryo-TEM determination of the percentage of full particles.

AAV Analysis at TrueCourse

TrueCourse has extensive experience characterizing complex nanoparticle drug formulations since 2007, and is a GMP-compliant cryo-TEM service provider in North America. Our team can provide imaging results and well-documented reports within as little as one week. In addition to empty-to-full ratio determination for AAVs, our characterization studies can also assess other critical quality attributes such as aggregation, broken or malformed capsids, unencapsulated DNA, or the presence of other contaminants. Beyond the quality attributes discussed in this technical note, we can rapidly perform high-resolution 3D structural analysis of rAAVs that can reveal residue-level effects of capsid protein engineering.

For more information, or to speak to one of our cryo-TEM experts, please email us at info@truecoursebio.com.

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