Simplifying Progress

Analyzing AAV – A Story of Problems and Solutions

Tim Steffens[1], Julian Droste[1], Niklas Krämer[1], Ben Tyrrell[2], Aimee Mincher[2], Alyssa Vetter[1], Catriona Thomson[2], Kathrin Teschner[1], Sandra Klausing[1], Stefan Northoff[1]

1.Sartorius Xell GmbH, Waldweg 21, 33758 Schloß Holte-Stukenbrock, Germany
2. Sartorius Stedim BioOutsource Ltd., 1 Technology Terrace, Todd Campus, West of Scotland Science Park, Glasgow, G20 0XA, Scotland
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Introduction

The application of adeno-associated virus (AAV) vectors for gene therapy delivery is a rapidly growing market with numerous novel drugs in the pipeline.[1] However, there is still a need for the development of new or optimized processes and methods. In particular, further characterization of rAAVs, as well as small-scale purification methods, downstream processing and quality control measurements need to be established, improved or streamlined. Here we present a comparison of different state-of-the-art as well as a newly developed analytical methods for the determination of different AAV quality attributes, such as the genomic and capsid titer, as well as full:empty ratio.

Methods

ddPCR: Bio-Rad QX200 ddPCR-system with suitable probes; ELISA: Specific Kits by Progen; Eppendorf epMotion 5075; read-out with a Thermo Varioskan Flash. SEC-MALS: DAWN8 MALS detector and Astra Software by Wyatt Technology with an Agilent HPLC. Affinity Chromatography: Thermo AAVX affinity resin, and an Agilent HPLC as chromatography system. AUC (analytical ultracentrifugation): Optima AUC by Beckman-Coulter with different AAV reference samples.

AAV samples: If not stated differently, we used samples generated in-house with a commercial cell line in HEK VIP NB medium. Further information can be found at posters Krämer et al. and Teschner et al.

Results

AAV capsid titers for everyone - ELISA

ELISA is a very common and accessible method for the determination of AAV capsid titers, as dedicated kits can be bought. Unfortunately, results generated by ELISA can suffer from high correlation of variations (CV)[1]. To tackle that problem, we developed a (semi-)automated pipetting protocol applying a liquid handling robot.

Table 1: *Difference of CV Values Between Manual and Automated Pipetting. Furthermore, the General Accuracy Improves When the Automated Method Is Used. AAV 2 reference was used.*

	Manual	Automated
CV (5 replicates)	18.5%	2.2%
Deviation from theoretical value	16.1%	-4.6%

Figure 1: *To Show the Precision of the Automated Method for Different Serotypes, Four Measurements for AAV2, AAV5 and AAV8 Were Conducted. Each With a Dedicated Elisa Kit.*

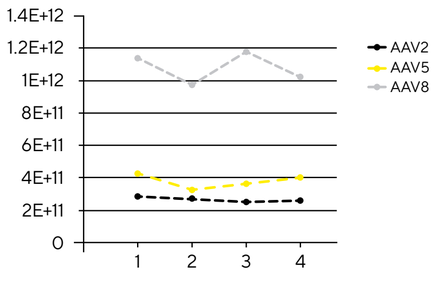
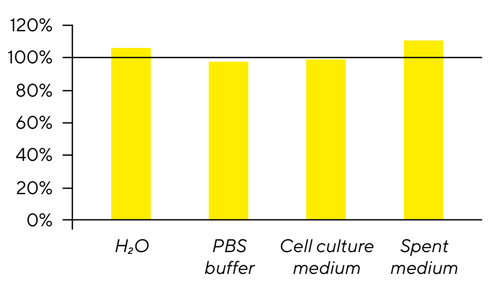


Figure 2: *AAV Samples May Be Measured in Different Matrices, Therefore We Tested the Robustness Against Different Samples. Each Result Is a Mean Value of Two Measurements.*



Vector genomes drop by drop - ddPCR

Next to the capsid titer, the vector genome (VG) titer is an important attribute for AAV analyses. Commonly used are qPCR and ddPCR, and the generated results are not necessarily alike.[1] This marks the importance to not change the methodology during any kind of development. We generally choose ddPCR, since we observed a higher robustness.

Table 2: *Displayed Are Different Method Parameters That Were Tested During the Methods Validation for Different Serotypes. Displayed Are Mean Values of 3 (Linearity) Or 5 Replicates.*

probe | serotype	Accuracy	Precision	Linearity	Range (copies/µL)
Probe 1 // AAV2	17.90%	1.90%	R2 > 0.998	2 – 6,600
Probe 1 // AAV5	2.10%	3.30%	R2 > 0.999	2 – 4,400
Probe 1 // AAV8	5.20%	3.00%	R2 > 0.999	8 – 7,800

The only thing you need? – SEC-MALS

SEC-MALS is a powerful method when working with AAV. It allows the collection of several critical quality attributes (CQA), like total capsids, full capsids, molecular weight and aggregation within one or maybe two runs, even without external calibration. However, a method for AAV analysis without disadvantages is yet to be found. SEC-MALS is not suitable for high-throughput analyses and the samples should be as pure as possible in order to get good results.

Figure 3: *Left: Chromatogram of a Crude AAV Sample With AAVMonomer and AAVMultimer Peaks. Right: Chromatogram of a Purified AAV Sample With a Clear AAV Monomer Peak.*

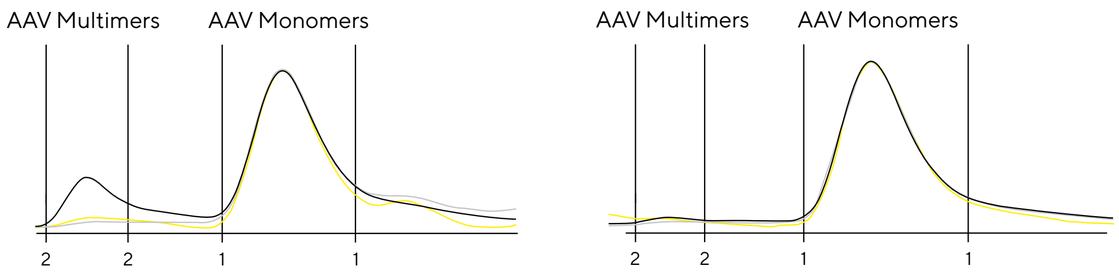


Table 3: *Displayed Are Typical Results That Can Be Gained With SEC-MALS. Here We Tested How Robust AAV8 Is Against Different Storage Temperatures (1 Week Storage). Results were confirmed by additional measurement.*

		Particles/mL				Radius (nm)	
Serotype	Storage	Total	Full	Empty	Full to Total Ratio (Vg/Cp)	Monomer	Aggregates
AAV8	-20°C	2.89E+12	1.19E+11	2.78E+12	4.1%	13.8	42.3
	4°C	2.40E+12	6.27E+10	2.33E+12	2.6%	13.9	42.2

There is another alternative – Affinity Chromatography

Next to the known methods we have developed an in-house affinity chromatography method. It shows to be suitable for the analysis of capsid as well as genome titers and, therefore, also for full:empty analyses. We tested the method for AAV2, AAV5 and AAV8. Furthermore, raw samples like culture supernatant can directly be used for analysis. The method also allows the purification of AAV for further analytics, as the affinity method is non-destructive. A drawback is that the sample volume needed for a reliable analysis increases when titers are low.

Figure 4: *Shown Are the Linear Correlations of Several Dilutions of an AAV8 Sample. We Measured Capsid as Well as Genome Titers on Two Different Days.*

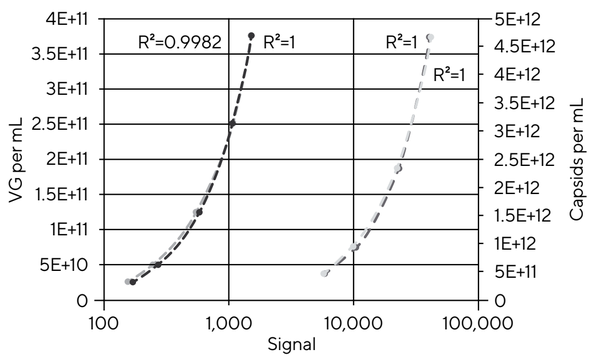


Table 4: *The Method Was Compared to Known Elisa Results. Different Aliquots of the Same Sample Were Measured on Different Days, to Show the Intermediate Precision. Overall for AAV8 the Correlation of Variation Is 2.2%.*

Culture sample	Capsid titer per mL (Affinity chromatography)	Deviation to reference ELISA [%]
AAV8 A, day 1	4.33E+12	7.2
AAV8 B, day 1	4.53E+12	3.0
AAV8 C, day 1	4.58E+12	1.9
AAV8 D, day 2	4.34E+12	7.0
AAV8 E, day 2	4.42E+12	5.4
AAV5 A, day 1	7.02E+11	3.9
AAV5 B, day 2	6.80E+11	0.6

So many methods, are they comparable?

In order to compare the methods at hand we used samples of AAV2, AAV5 and AAV8, cleared the culture broth with two different methods (Poster: Teschner et al.) and used the resulting supernatants for ddPCR, ELISA, SEC-MALS and the Affinity Chromatography. Additionally, we purified the AAV5 extract as proof of concept and measured SEC-MALS as compared to the Affinity Chromatography. The resulting data are displayed in the tables.

Table 5: *Comparisson of analytical methods using cell culture samples (first table) and purified samples (second table).*

Crude extract	Total capsids			Full capsids | VG			Empty | Full Ratio		
Sample	SEC-MALS*	Affinity (in-house)	ELISA	SEC-MALS*	Affinity (in-house)	ddPCR	SEC-MALS*	Affinity (in-house)	ddPCR | ELISA
AAV8 protocol 1	4.00E+12	5.66E+12	4.06E+12	1.92E+11	2.04E+11	2.76E+11	4.8%	3.6%	6.1%
AAV8 protocol 2	3.89E+12	4.99E+12	5.12E+12	2.05E+11	2.00E+11	2.66E+11	5.3%	4.0%	5.2%
AAV2 protocol 1	N/A	2.89E+12	3.47E+12	N/A	6.08E+10	1.51E+10	N/A	2.1%	0.6%
AAV2 protocol 2	N/A	2.46E+12	4.68E+12	N/A	5.67E+10	1.27E+11	N/A	2.3%	2.8%
AAV5 protocol 1	N/A	2.55E+12	5.78E+12	N/A	1.37E+11	2.36E+11	N/A	5.2%	3.9%
AAV5 protocol 2	N/A	2.34E+12	5.98E+12	N/A	1.10E+11	2.36E+11	N/A	4.7%	4.2%

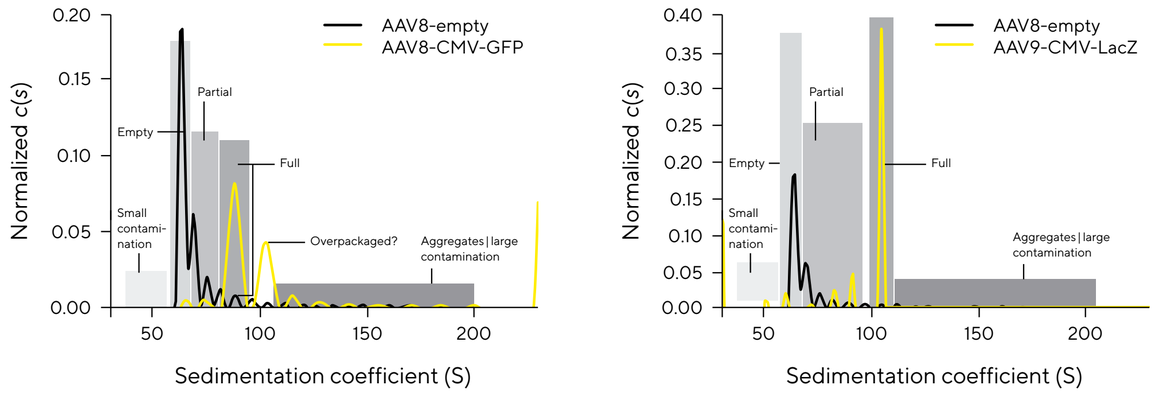
*SEC-MALS is better used for purified samples and was therefore only applied to AAV8 as proof of concept.

Purified extract	Total capsids		Full capsids | VG		Empty | Full Ratio	
Sample	SEC-MALS	Affinity (in-house)	SEC-MALS	Affinity (in-house)	SEC-MALS	Affinity (in-house)
AAV5 protocol 1	1.80E+12	1.10E+12	6.98E+10	4.47E+10	3.9%	4.0%
AAV5 protocol 2	1.90E+12	1.15E+12	6.12E+10	4.81E+10	3.2%	4.2%

More for the future - AUC

We are in the late stages of developing a platform method to generate full:empty ratios of AAV via the AUC. Separation of AAV capsids is obtained by spinning at high speeds due to the difference in the weight of AAV capsids - the presence or absence of DNA causes this difference. Along with defining full and empty capsids, the system has the sensitivity to resolve capsids with differing sizes of DNA. This resolution allows the AUC to dig deep into the characteriztion of AAV samples.

Figure 5: *Empty AAV8 Samples Have a Predominant Peak at ~60S But Also Contains a Range of Partially Filled Capsids. When an AAV8-CMV-GFP Sample Is Overlaid We Can See the Main Population at ~90S. Of Note Is That the AUC Can Also Resolve Overpackaged Capsids (S Value~ 105S), as Well as Partial and Aggregated Species. Furthermore, You Can See an AAV9-CMV-LacZ Sample That Contains More DNA Than an AAV-CMV-GFP Sample and, Therefore, the Main Peak Comes Out as Expected at 105S, Demonstrating the Ability of the AUC to Resolve Samples With Differing Sizes of DNA Inserts.*



Summary | Conclusion

If the message of this poster could be reduced to one sentence it is this: Know your methods! All of the presented methods show variations as compared to one another. Like so often, there are different abilities and limitations for each technique and while the precision of each method can be improved, it is hard to get good insights into their accuracy, due to the lack of certified references. It is yet to be determined if certain methods will become a gold standard or if the combination of different methods for the analysis of AAV attributes need to be used. Until then, we will build a broad analytical platform with a wide range of methods in order to get the best out of every AAV sample.

References

Andreas L. Gimpel et al., Analytical methods for process and product characterization of recombinant adeno-associated virus-based gene therapies. Molecular Therapy: Methods & Clinical Development Vol. 20 March 2021.