APPLICATION NOTE

Complementing Platforms that Enable Multiparametric Characterization of AAV Capsids

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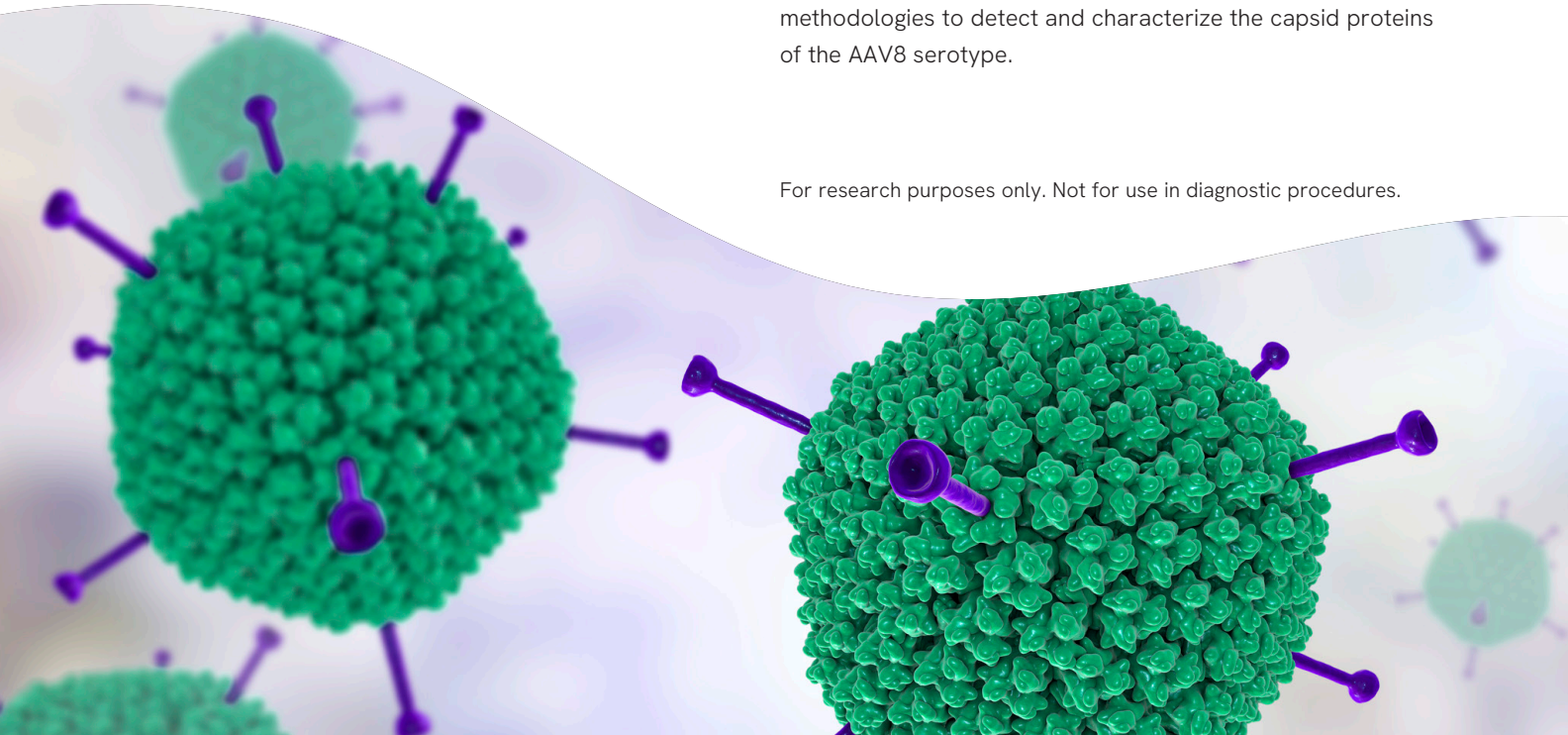
Introduction

AAV (Adeno-associated virus) is a small, replication-deficient, non-enveloped parvovirus.[1] The AAV capsid is formed from cap viral gene products VP1, VP2, and VP3, which are structural proteins that assemble into a 60-subunit and form a ~25 nm capsid. The three capsid proteins encapsulate a ~4.7 kb genome at a VP3:VP2:VP1 ratio of 50:5:5.[2] AAV was first discovered as a contaminant in adenovirus isolates.[3] It was later determined that helper viruses, like adenovirus, are required for AAV replication in host cells. AAVs are not known to cause disease in animals or humans.[4] Because of their unique properties, including low toxicity and high gene transfer efficiency, AAVs have been developed as a vector for human gene therapy.[5] Recombinant DNA technologies have been used to genetically engineer AAVs (rAAVs) to deliver therapeutic genes to the cells of target tissues to treat a variety of monogenic diseases in humans.[6] Revvity has designed, tested, and manufactured various analytical tools to quantify and characterize AAV in crude and purified samples during AAV research, process development, and manufacturing pipelines. Characterization of various AAV serotypes using conventional and next generation technologies has been the focus of researchers and bioanalytical scientists within the cell and gene therapy community to deliver critical quality attributes along the workflow. In this study we report the application of higher throughput assays that leverage bead-based AlphaLISA® and the LabChip® GXII Touch™ microfluidics-based CE technology. Both technologies have been evaluated as orthogonal methodologies to detect and characterize the capsid proteins of the AAV8 serotype.

For research purposes only. Not for use in diagnostic procedures.



Methods

AlphaLISA AAV Capsid Detection Kits

AlphaLISA AAV Capsid Detection Kits are a family of no-wash immunoassays that measure AAV in Viral Particle/ mL (VP/mL) units, which represents the intact AAV capsid. This is different from the VP structural proteins, which are the building blocks of the AAV capsid. The AlphaLISA immunoassay can measure AAV8 concentrations in buffers as well as in analyte matrices with a high protein background, such as cell culture media and cell lysates. AlphaLISA kits are available for AAV1, AAV2, AAV3B, AAV5, AAV6, AAV8, and AAV9 serotypes. Each kit contains AAV serotype-specific antibodies, Acceptor beads, and Donor beads. The Acceptor beads in the AAV1 and AAV6 kits are conjugated to an anti-HRP antibody which recognizes and binds to an HRP-conjugated anti- AAV antibody. The Acceptor beads in all other AlphaLISA AAV kits are directly conjugated to an anti-AAV antibody. All AlphaLISA AAV kits also contain biotinylated anti-AAV antibodies that bind streptavidin-coated Donor beads. When the AlphaLISA assay reagents are combined with the analyte, the Donor and Acceptor beads come into proximity. Excitation of the Donor beads at 680 nm releases singlet oxygen that diffuses into the analyte matrix and interacts with the Acceptor beads, facilitating a chemiluminescent reaction, resulting in an emission λ_{max} at 615 nm which can be read on an Alpha-capable multimode plate reader. An illustration of the reaction mechanism for all AlphaLISA AAV Capsid Detection Kits is provided in Figure 1.

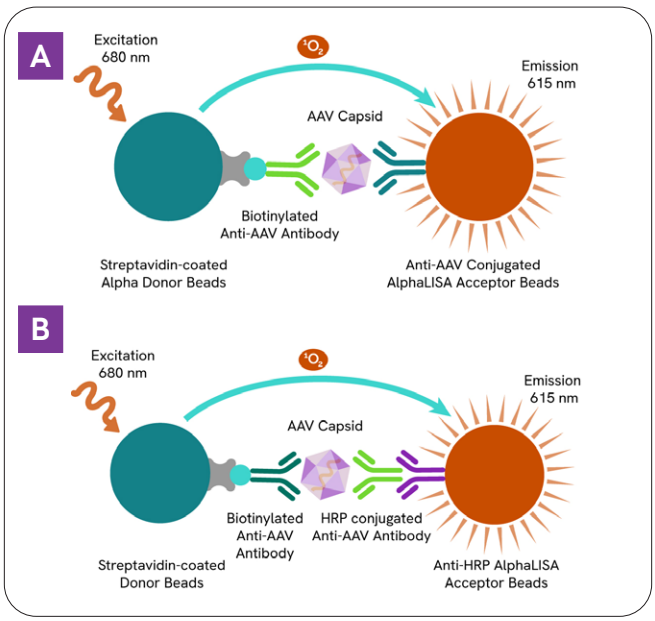


Figure 1: Schematic of AlphaLISA AAV Capsid Detection Kits. A.) General AlphaLISA AAV Capsid Detection Kit. B.) AAV1 and AAV6 Capsid Detection Kits.

LabChip GXII Touch Protein Characterization System

The LabChip GXII Touch instrument is an automated biomolecular characterization system that uses chip-based microfluidic electrophoresis separation technology to measure size, percent purity and concentration for both proteins and nucleic acids. The LabChip GXII Touch instrument can analyze samples in a high-throughput manner by sipping nanoliters of analyte mixture through a capillary onto the chip from 96-well or 384-well format microplates. AAV-specific workflows have been developed utilizing the LabChip technology (for DNA or protein assays) and related reagent kits to assess the concentration, purity, VP stoichiometry, and empty/full ratio of the AAV capsid in purified samples. A schematic measurement of AAV using the LabChip Pico Protein Assay is shown in Figure 2 below.

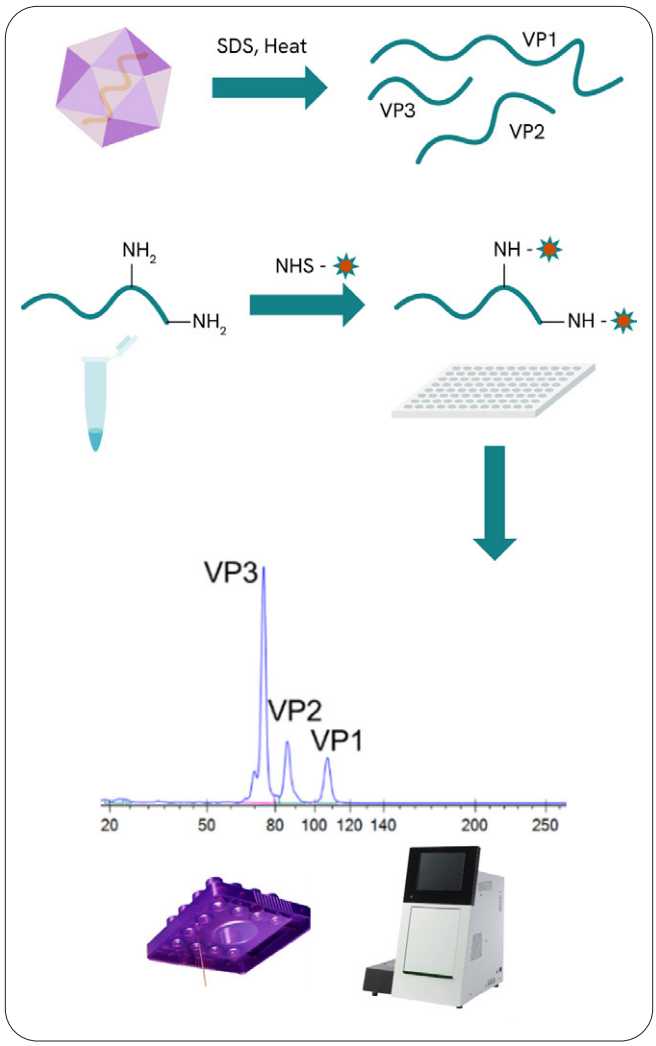


Figure 2: A) Schematic illustrating the dissociation of AAV8 particles into VP3, VP2 and VP1 monomers for protein analysis on LabChip GXII Touch instrument. B) Schematic of Pico Protein Assay for LabChip GXII Touch instrument. Proteins are covalently labeled (amino groups shown) with dye using NHS-ester coupling reaction. Samples are electrophoretically separated and analyzed in plate-format on the LabChip GXII Touch instrument.

AAV8 particles generated from recombinant viral expression cassettes containing CMV-eGFP DNA element (eGFP gene driven under a CMV promoter, SIRION Biotech, a Revvity company) were measured using both AlphaLISA AAV8 Capsid Detection Kit (catalog # AL3180) and Pico Protein Assay run on LabChip GXII Touch instrument (catalog # 760498).

Results & Discussion

AlphaLISA AAV8 Capsid Detection Kit can measure both empty and full AAV8 Viral Particles

The CMV-eGFP AAV8 standard (SIRION Biotech) was reconstituted in PBS and 0.001% Pluronic to 1.00E+13 Viral Genome/mL (VG/mL) and stored at -80 °C until required. The standard curve dilution matrix on page 7 of the AAV8 AlphaLISA kit Technical Data Sheet (TDS) was used to dilute both the empty (provided with the kit) and loaded AAV8 standards in DNase and RNase free water to equivalent concentrations in VP/mL and VG/mL (Table 1). AAV standards and all AlphaLISA reagents were diluted in Immunoassay buffer (IAB), provided with the kit. The default protocol with a 10 µL sample volume was used to achieve the highest possible assay sensitivity. Samples and reagents were pipetted into a 384-well AlphaPlate, light gray (catalog # 6005350). The CMV-eGFP AAV8 concentration in VP/mL was interpolated from the empty AAV8 capsid standard curve. The signal from 12 background wells loaded with IAB instead of sample was used to calculate the LDL and LLOQ provided in Table 1. The Alpha Signal and VP/mL concentrations for both empty and full capsid AAV8 standards were plotted in the graph below (Figure 3).

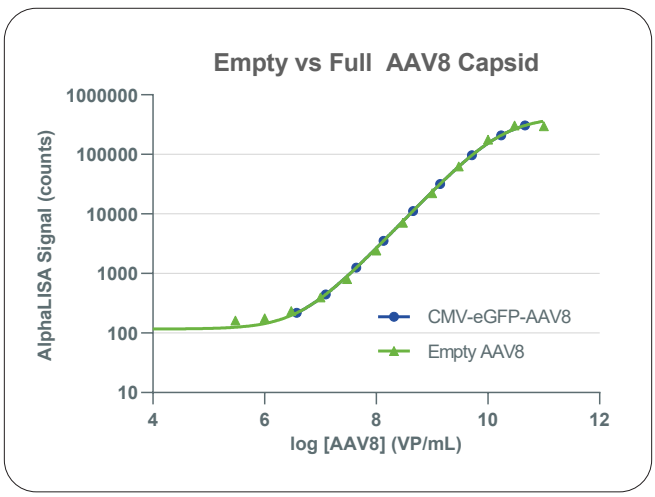


Figure 3: AlphaLISA AAV8 Capsid Detection Kit standard curve of empty and CMV-eGFP DNA full AAV8 (SIRION Biotech). XY scatter plot of empty and full AAV8. The curve was fitted using nonlinear regression with a 4-parameter logistic equation (sigmoidal dose-response with variable slope) with 1/Y2 data weighting.

The calculated LDL (7.75E+05 VP/mL) is lower than the reported LDL of 2.26E+06 VP/mL for the AlphaLISA AAV8 Capsid Detection Kit. The linear range of the empty AAV8 standard was determined to be between 1.00E+10 to 1.00E+07 VP/mL, with an R2 value of 0.99. The raw AlphaLISA Signal (counts) and interpolated AAV8 concentration in VP/mL for the CMV-eGFP AAV8 standards and signal from empty AAV8 standards (Table 1) were plotted on the same graph using GraphPad Prism 9.4.1, resulting in a perfectly superimposed standard curve (Figure 3). This demonstrates that both empty and full AAV8 capsids can be equally detected and quantified using the AlphaLISA AAV Capsid Detection Kit and cannot be differentiated.

LabChip GXII Touch instrument and Pico Protein Assay can measure the concentration and VP3:VP2:VP1 ratio of AAV capsids in purified samples

The LabChip Pico Protein Assay is an electrophoresis assay that uses covalent labeling of denatured proteins, resulting in a linear range of 10 pg/µL to 100 ng/µL (4 logs). The precision of the Pico Protein Assay is illustrated in the overlayed electropherograms shown in Figure 4A, generated from repeated sampling of 1.0E+13 VP/mL CMV-eGFP AAV8. AAV8 capsid proteins VP3, VP2 and VP1 peaks are clearly visible and fully resolved in the electropherogram with retention times resulting from the differences in their respective molecular weights. The small peak directly preceding the larger VP3 peak in Figures 4A & 4B represents an N-terminally truncated variant of the VP3 protein believed to be generated by differential scanning of the position of the initiation codon in the VP3 gene.[7] The difference of size between the VP3 and VP3 variant peak is ~600 Da. The ability of the LabChip GXII Touch instrument to resolve these peaks using a separation channel of ~15 mm, resulting in a short analysis time, represents an inherent strength of this microfluidic technology. A serial dilution of CMV-eGFP AAV8 was made from a stock solution of 1.0E+13 VP/mL down to 4.57E+09 VP/mL using PBS and 0.001% Pluronic. The overlayed electropherograms at each dilution are provided in Figure 4B. The VP3 peak (VP3 variant peak included) was detected at a concentration of 4.12E+10 VP/mL with an R^2 value of 0.99 based on the theoretical concentration in VP/mL (Figure 4B inset).

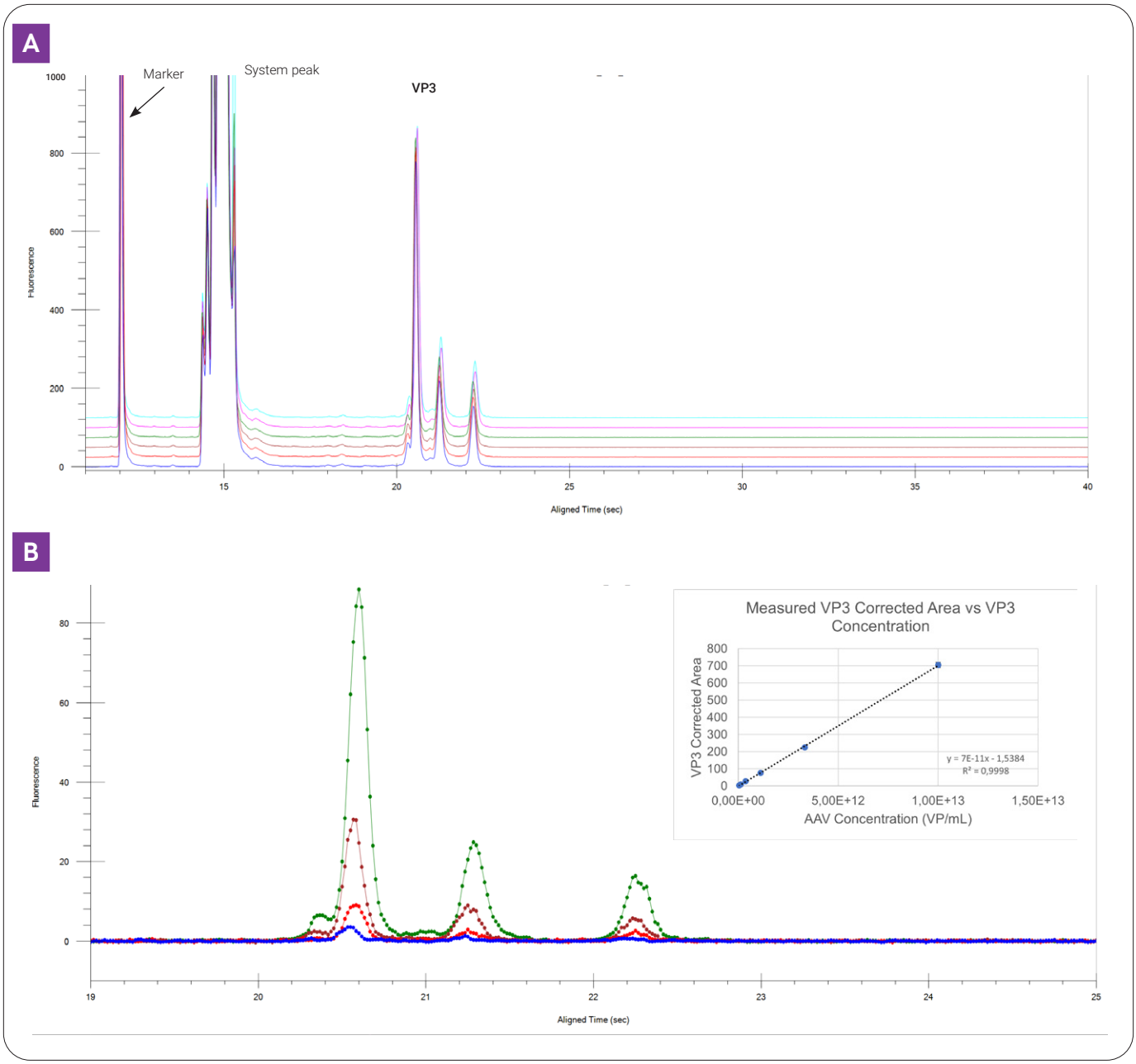


Figure 4: A) Overlay electropherogram of AAV8 sample at concentration 1E+13 VP/mL. B) Example traces of AAV8 proteins at lower concentration. At 4.1E+10 VP/mL, the VP3 protein is detected with signal greater than 3X noise. At 1.2E+11 VP/mL, the VP3, VP2 and VP1 proteins are detected with signal greater than 3x baseline. A linear response (R2 = 0.99) was obtained for the concentration range from 4.1E+10 to 1.1E+12 VP/mL (inset). Each data point is an average of 6 sips, with error bars showing the standard deviation.

AlphaLISA immunoassays and LabChip GXII Touch assays offer complementary methods for analyzing the AAV capsid in both crude and purified samples

AlphaLISA and LabChip GXII Touch assays are complementary technologies that can provide important and unique quantitative insights about AAV samples at various stages along the gene therapy development workflow. AlphaLISA and LabChip GXII Touch technologies offer robust and rapid methods of AAV capsid quantification and characterization over a broad concentration range in a high-throughput manner when used together to complement data. The infographic in Figure 5 shows when AlphaLISA or LabChip GXII Touch technology can be used in a conventional AAV process development and production pipeline while highlighting important assay parameters.

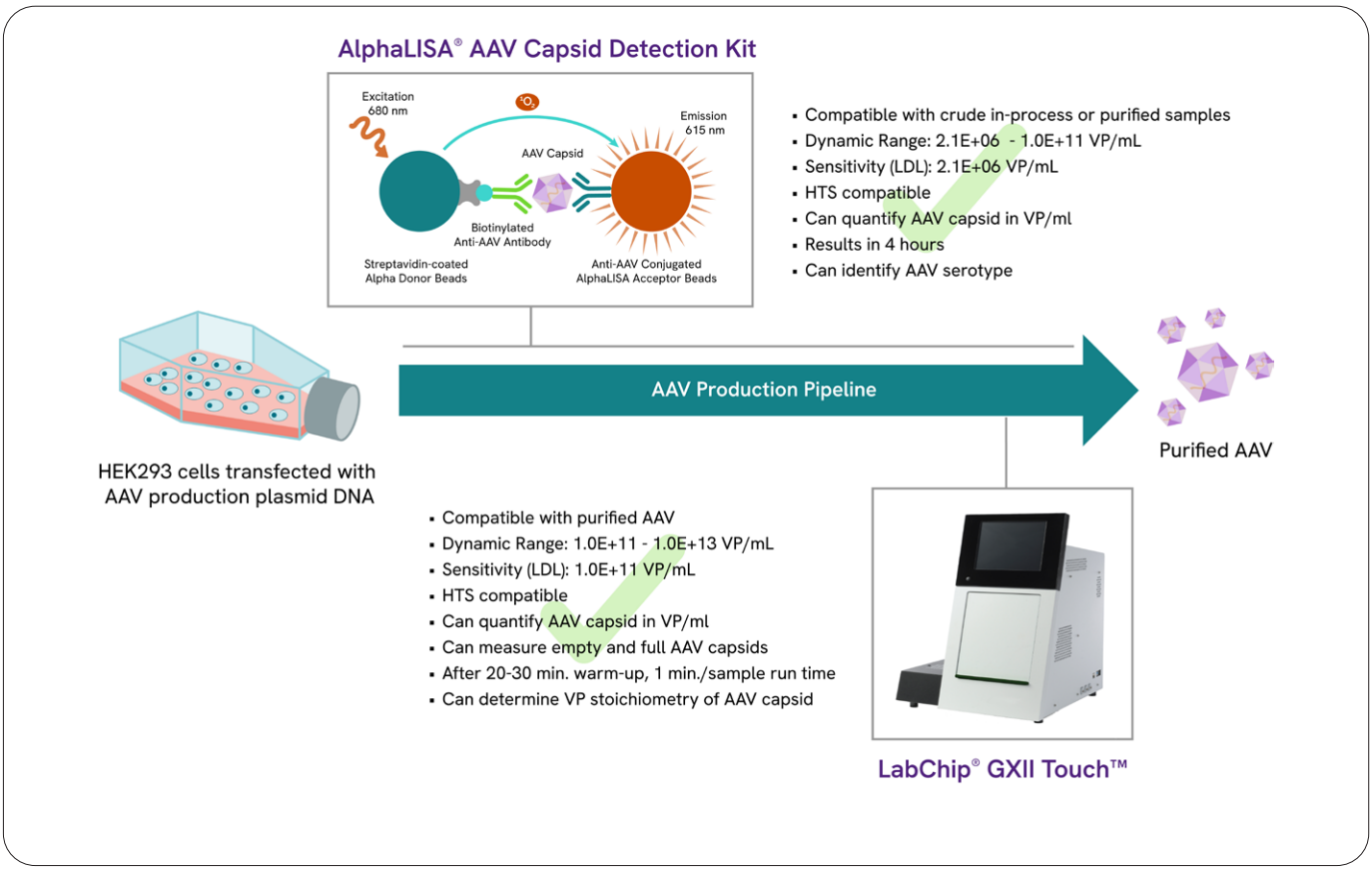


Figure 5: A summary outlining how AlphaLISA and LabChip technologies can be used to analyze samples containing AAV capsids produced in a typical AAV process development and production pipeline.

Both methods can rapidly quantify the AAV capsid concentration in an unknown sample as well as measure both empty and full capsids. The two technologies complement to extend analyte detection. For example, as shown above for the AAV8 system, the AlphaLISA Capsid Detection Kit detects at a lower LDL (2.1E+06 VP/mL) and offers a broader dynamic range compared to the LabChip Pico Protein Assay. However, the LabChip instrument facilitates detection at higher AAV capsid concentrations (≥ 4.0E+10 VP/mL) which further extends the dynamic range. Therefore, when AlphaLISA and LabChip technologies are used in parallel, the combined dynamic range for AAV8 quantification extends to ~7 orders of magnitude. In addition, it can also assess sample purity and provide the relative VP protein stoichiometry of the AAV capsid. However, the LabChip technology cannot distinguish between capsids of different serotypes. Fortunately, this can be accomplished using the high-throughput, capsid-specific, quantitative capability of the AlphaLISA AAV Capsid Detection Kits. Furthermore, AlphaLISA AAV kits can detect analytes in matrices with a high protein background, such as cell culture media, cell lysate, as well as common buffers. This offers an opportunity to detect and quantify the AAV capsid in both relatively crude or impure samples, as well as purified AAV encountered across the entirety of the process development and production pipline. In addition, a recent study has shown that the LabChip GXII Touch instrument can be used with the Pico Protein Assay and other LabChip-compatible assays to measure the concentration of encapsulated viral genome to determine the ratio of empty/full capsids in a AAV sample.[8]

Summary

As shown above, the use of complementary assay platforms (AlphaLISA and LabChip GXII Touch systems), enabled the identification and quantification of the AAV capsid over an extended concentration range in VP/mL representing ~7 orders of magnitude. In addition to measuring the AAV capsid concentration, the LabChip instrument can further characterize AAV preparations for purity, capsid protein (VP3:VP2:VP1) ratios as well as analytical insights into empty/full proportions. Compared to the conventional methods of AAV analysis, such as TEM, mass spectrometry or traditional CE, the next generation systems we reported above are powerful orthogonal tools to be used to obtain multiparametric data from AAV workflows.

Additional notes related performance:

1. Due to the cross-reactivity of some AlphaLISA AAV Capsid Detection Kits, the kit TDS should be consulted prior to using it for serotype identification purposes.
2. Certain analyte matrices may contain components that interfere with the AlphaLISA immunoassay, like free biotin, often found in RPMI medium. Fortunately, the AlphaLISA AAV Capsid Detection Kit TDS contains an alternate protocol, using a lower sample volume to reduce compound interference.
3. The LabChip GXII Touch and Pico Protein Assay can detect and measure impurities in AAV samples, however, it is recommended that the AAV sample be as pure as possible to ensure accurate and reproducible results.

References

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Appendix

Table 1: AlphaLISA results from empty AAV8 standard provided with the kit and CMV-eGFP AAV8 (SIRION Biotech). LDL and LLOQ were calculated from 10 wells and are consistent with values provided in the AlphaLISA AAV Capsid Detection Kit TDS. The concentration of the CMV-eGFP AAV8 stock was provided in VG/mL and was diluted to the concentrations shown. The concentration of CMV-eGFP AAV8 was interpolated using the empty AAV8 standard curve and given in VP/mL.

	Empty AAV8 Capsid (standard)				CMV-eGFP AAV8				
	[AAV8] (VP/mL)	Average AlphaLISA Signal (counts)	St. Dev.	CV	[AAV8] (VG/mL)	Average AlphaLISA Signal (counts)	St. Dev.	CV	Interpolated [AAV8] (VP/ mL)
	1.00E+11	297810	8673.6	2.91%	1.00E+11	306017	4005.9	1.31%	4.64E+10
	3.00E+10	306378	4427.9	1.45%	3.00E+10	208882	5139.5	2.46%	1.73E+10
Linear range	1.00E+10	176628	6651.0	3.77%	1.00E+10	96636	2223.5	2.30%	5.18E+09
	3.00E+09	62526	4765.1	7.62%	3.00E+09	31914	177.2	0.56%	1.40E+09
	1.00E+09	22202	853.1	3.84%	1.00E+09	11185	907.4	8.11%	4.55E+08
	3.00E+08	7085	291.0	4.11%	3.00E+08	3536	232.8	6.58%	1.35E+08
	1.00E+08	2438	71.6	2.94%	1.00E+08	1251	82.2	6.57%	4.39E+07
	3.00E+07	808	58.1	7.19%	3.00E+07	443	21.6	4.88%	1.24E+07
	1.00E+07	395	82.1	20.80%	1.00E+07	218	56.5	25.97%	3.76E+06
	3.00E+06	234	65.6	28.03%	3.00E+06	147	8.5	5.81%	1.11E+06
	1.00E+06	236	106.7	45.16%	1.00E+06	186	58.7	31.62%	
	3.00E+05	137	70.7	51.50%	3.00E+05	116	26.0	22.41%	
Background	1.00E+03	102	10.4	10.22%	Background: 10 wells, no analyte. --> LDL: 7.75E+05 VP/mL --> LLOQ: 4.07E+06 VP/mL				

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