APPLICATION NOTE

AN2004: Why and how to quantify AAV aggregates by FFF-MALS

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Summary

The percentage of aggregate is a critical quality attribute (CQA) of DNA-based therapeutics delivered by the engineered adeno-associated virus (AAV). This application note compares two platforms for analytical separations—size exclusion chromatography (SEC) and field-flow fractionation (FFF)—along with three online detection methods—UV, fluorescence, and multi-angle light scattering (MALS). The results demonstrate that FFF-MALS is the most appropriate method for quantifying all aggregates of AAV-mediated products, from dimer, trimer, and small oligomers to large aggregates.

Introduction

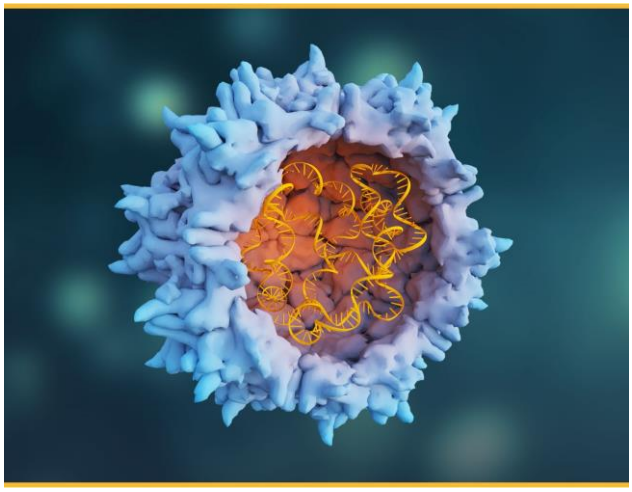
Owing to its recent medical successes, AAV has emerged as the most popular gene vector for delivering small gene therapeutics[1]. Ensuring the safety and efficacy of an AAV-encapsidated DNA product requires the identification and quantification of its CQAs, which must be monitored throughout the development and production cycle[2].

AAV aggregation depends on many factors including serotype, capsid titer, empty/full ratio, formulation buffer composition, storage, and stress conditions. Aggregates in an AAV product—just as in protein-based therapeutics—may decrease efficacy and increase immunogenicity, which may lead to immune-related adverse effects. Hence the degree of aggregation is one of the CQAs that must be monitored throughout the AAV product lifecycle.[3,4].

Size exclusion chromatography with ultra-violet absorption detection (SEC-UV) has been widely used to quantify the aggregation in therapeutic proteins and has been considered for AAV-based gene therapy products as well. For AAVs, the addition of a fluorescence detector (FLD) to the SEC-UV system will enhance detection sensitivity due to intrinsic fluorescence of the analyte. A MALS detector is often added as well, to help understand the aggregation profile and measure other AAV CQAs[5-8].

FFF is a size-based separation technique, orthogonal to SEC, that does not incorporate a stationary phase or an affinity-dependent mechanism of action. Combined FFF and SEC data constitutes comprehensive evidence to convince regulatory authorities that all aggregates are detected and quantified in the therapeutic product[4,8].

We discuss the strengths and limitations of these separation and detection tools for aggregate quantification and reveal some specific details for correctly quantifying AAV aggregates by MALS.



Materials and Methods

AAV samples were produced in-house at Novartis Gene Therapies and consist of four preparations with descending level of aggregation. The key sample properties are summarized in Table 1.

These preparations were pre-screened by DLS using a DynaPro® Plate Reader with DYNAMICS® software, to assess size and size distribution, and to confirm measurable differences in aggregation.

Table 1. Details of the AAV samples used in this note.

Sample ID	Type of Capsid	Aggregation level
Sample A	Empty	Level 4
Sample B	Empty	Level 3
Sample C	Empty	Level 2
Sample D	Full	Level 1

SEC-MALS-UV-FLD instrumentation

An Acquity UPLC system from Waters Corp., equipped with FLD and PDA UV detectors, was employed for SEC separation and quantification. For some measurements, a DAWN® MALS detector was also added for molecular weight (MW) and size analysis. An appropriate SEC column was used to resolve AAV monomer and its oligomers with phosphate buffer saline as the mobile phase. Data from UV absorbance at 280 nm and fluorescence with 280 nm excitation/350 nm emission were collected with Empower software. ASTRA® software was used to collect and analyze MALS data.

FFF-MALS-UV-FLD instrumentation

FFF separation was carried out with an Eclipse™ FFF instrument and separation channel supported by an Agilent 1260 Infinity II HPLC pump and autosampler. Online detectors included a DAWN MALS instrument, an Optilab® dRI detector, and Agilent 1260 Infinity II FLD and MWD UV detectors. The FFF system was controlled by OpenLab with the Eclipse plug-in, while data were collected and processed by ASTRA. Separation took place in an Eclipse short channel with a 350 µm spacer and regenerated cellulose (RC) membrane (10 kDa cutoff). Phosphate-buffered saline (PBS) was used as the running buffer at a detector flow of 0.6 mL/min.

An optimal FFF separation method was developed. For analysis, at least two injections were made for each sample to assess the reproducibility of the method. The injection amounts were approximately $6x10^{11}$ AAV particles for all SEC and FFF runs. AAV particle concentration and the total volume of particles contained in each peak were calculated from MALS data with ASTRA's *Number Density* method. The sphere model was used with correction factors applied to account for the non-spherical shape of the different aggregates[9].

Results and Discussion

Though an AAV, with an approximate radius of 13 nm, is much larger than most proteins, SEC with a large pore size column (e.g., 450 to 2000 Å) is still appropriate for separating AAV monomer, small aggregates, and fragments. However, as we have learned from working with protein therapeutics, large aggregates can be dissociated by column shear, dilution, or solvent exchange, or directly removed by the column acting as a filter[10,11]. Because of this limitation of SEC, FFF is required to assess aggregation of AAV products, even more so than for protein therapeutics.

SEC removes large AAV aggregates

All four AAV samples were analyzed by SEC and FFF with UV and FLD detectors. The UV and FLD traces from both SEC and FFF of Sample A (the sample containing the most aggregates) are shown in Figure 1. Note that the elution order in FFF is reversed relative to SEC: larger particles elute earlier in SEC but later in FFF. The peak corresponding to large aggregates was only observed in FFF (39 to 46 minutes) and not in SEC (before 10.5 minutes). These results imply that SEC—even using a column packed with large pore sized beads—cannot preserve and properly elute the large aggregates.

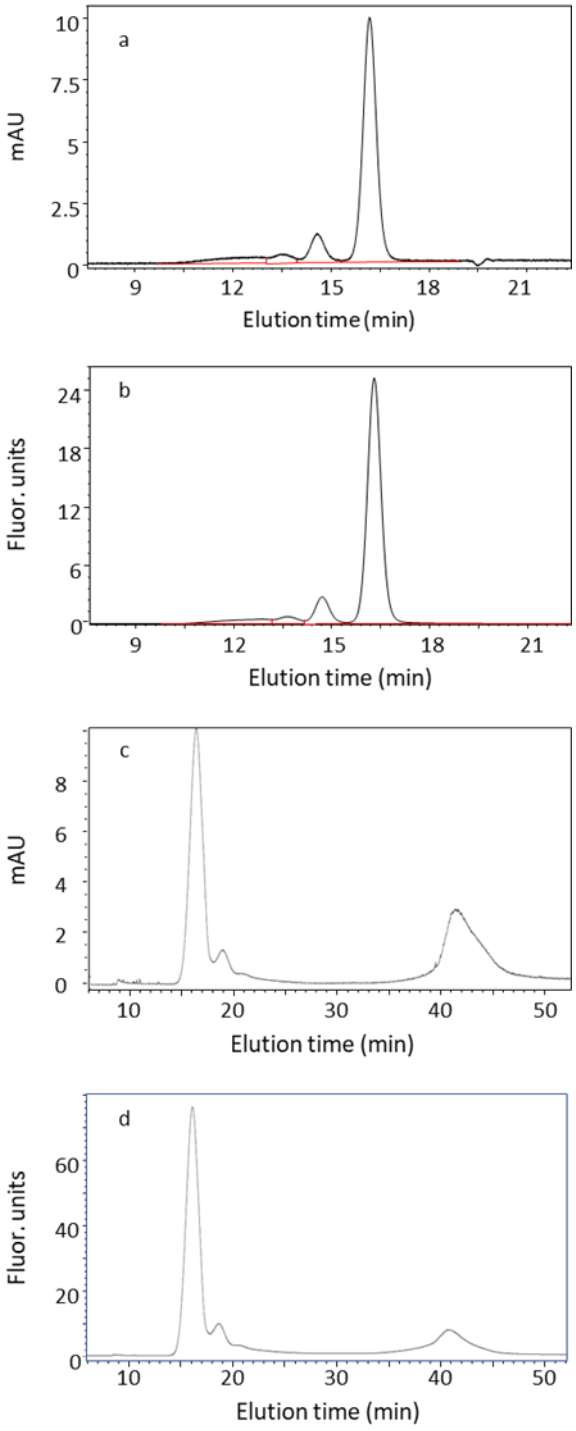


Figure 1. SEC-UV (a) and SEC-FLD (b) chromatograms and FFF-UV (c) and FFF-FLD (d) fractograms of AAV Sample A.

We compare the MALS data obtained from SEC and FFF for Samples A, B and C in Figure 2, where the radius determined by MALS is overlaid with the LS fractogram, plotted against elution time. The radius plotted here is the geometric radius, calculated by fitting angular data to ASTRA's sphere model. Under the conditions used for these measurements, SEC provided better resolution than FFF between monomer and dimer.

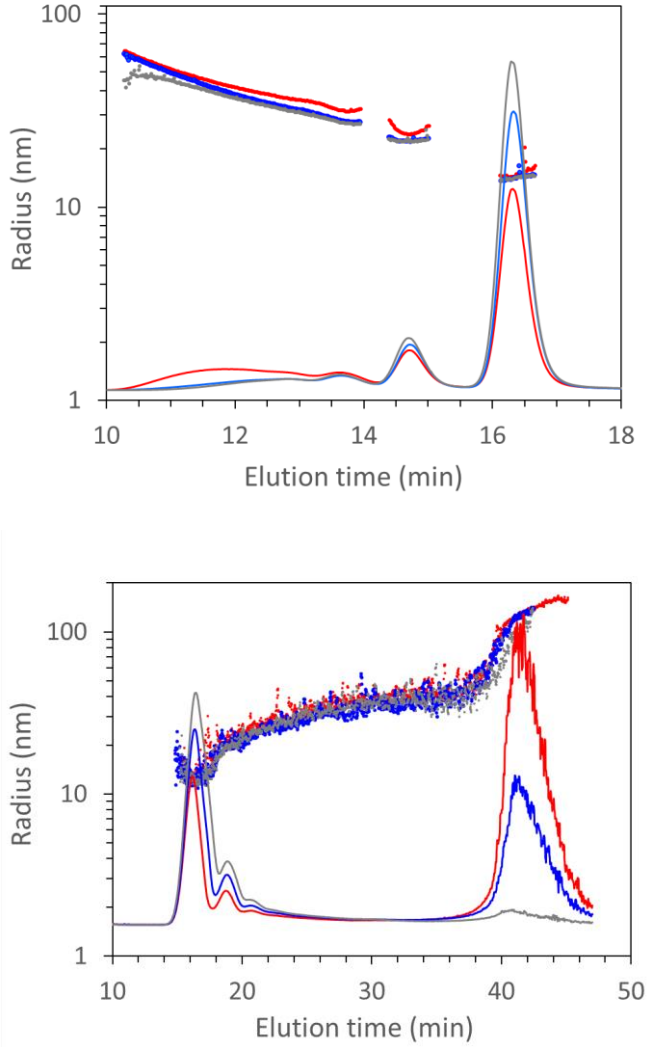


Figure 2. Radius versus elution time, overlaid with LS traces obtained from SEC-MALS (top) and FFF-MALS (bottom), for Samples A (red), B (blue) and C (gray), with descending degree of aggregation. SEC provides baseline separation of monomer and dimer, whereas FFF does not.

At the same elution time, radii from different samples were different in SEC but similar in FFF. This indicates that eluted aggregates were not as well separated in SEC as in FFF. The apparent large radii of dimer and trimer from Sample A in SEC are due to the co-eluted larger aggregates, which are prominent from 10 to 13 minutes but are believed to bleed into the dimer and trimer peaks. In addition, aggregates with radii greater than 60 nm (which will be referred to as large aggregates, L.A., in this note) were only detected by FFF-MALS, consistent with the other reports that the large aggregates are

removed by the column packing or column frits and possibly degraded by shearing[8].

Quantification of small oligomers is similar across methods

We then set out to compare the quantity of monomer, dimer, trimer, and oligomers, excluding the large aggregates, as determined by the three different detectors following the SEC and FFF separations. The results are shown in Figure 3, which plots the mass percentage of different AAV oligomeric states obtained under different combinations of separation platform and detection mode. Quantitation of mass percentage by UV and FLD is based on peak area, whereas quantitation by MALS makes use of the total particle volume calculation (provided by ASTRA software), corrected to account for oligomeric shape. Details of the correction will be discussed in the next section. Note that mass percentage and volume percentage are nearly identical quantities (after shape correction). Average values from duplicate injections were found to have a typical relative standard deviation of less than 5%.

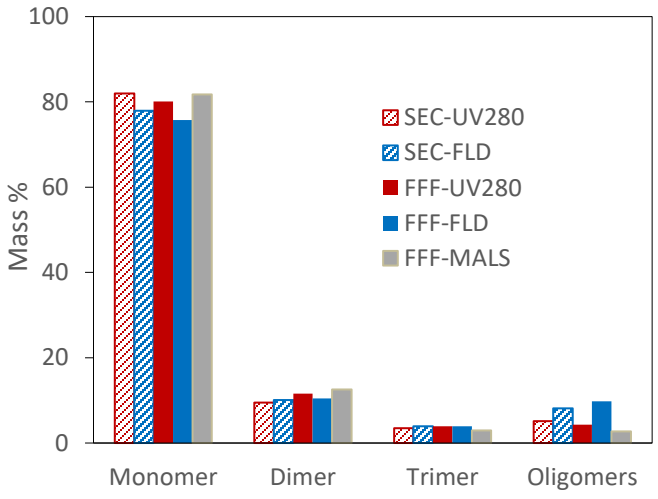


Figure 3. Quantitation of AAV oligomeric states in Sample A by SEC (stripped bars) and FFF (solid bars) using UV (red), FLD (blue), and MALS (gray) analyses.

Ignoring large aggregates, the mass percentages of monomer, dimer, and trimer from these five methods agree relatively well. For example, the measured monomer percentage varies across the methods from 74% to 82%, dimer percentage changes from 9% to 12% and trimer percentage from 3% to 4%. However, the mass percentage of oligomers differs noticeably: 3% from FFF-MALS, 4-5% by UV, and 8-10% by FLD. Note that the 'oligomer' peak includes tetramer, pentamer and larger aggregates, constrained to a radius of less than 60 nm. The average radius of the oligomers is about 36 nm, large enough to cause a scattering effect in UV and FLD signals which likely contributed the apparently higher mass percentage from these detectors, relative to the MALS result.[12]

The results in Figure 3 suggest that SEC-UV and SEC-FLD are adequate for quantifying the mass percentage of AAV monomer, dimer, and trimer. They are not appropriate, however, for quantifying larger AAV oligomers due to scattering artifacts. We will discuss similar observations with large aggregates in the next section.

UV and FLD overestimate large aggregates

The complete mass percentage results of all the AAV species–including the large aggregates–are tabulated in Table 2. As discussed above, this table reveals that the large aggregates were not detected when SEC served as the separation platform. Additionally, due to scattering of incident UV light in the respective detectors, quantitation by UV or FLD overestimates the mass percentage of large oligomers and especially of large aggregates.

Table 2. Mass percentage of AAV monomer (M), dimer (D), trimer (T), oligomer (O), and large aggregates (L.A.) of Sample A obtained from SEC and FFF with different detectors.

	M%	D%	T%	O%	L.A.%
SEC-UV	82.0	3.4	3.4	5.1	0.0
SEC-FLD	77.9	10.1	3.9	8.1	0.0
FFF-UV	48.6	7.0	2.1	1.9	40.4
FFF-FLD	61.4	9.1	3.0	6.4	20.1
FFF-MALS	86.8	6.4	1.4	2.2	3.2

To understand these results more fully, we plotted the mass fraction of each aggregate type normalized to monomer mass in Figure 4. It is evident that quantitation with UV and FLD overestimates the amount of aggregates larger than trimer, and greatly overestimates the L.A. fraction. These data and graphs enable us to conclude that FFF-MALS is the only method, among the five methods discussed in this note, that can properly separate and quantify **all** aggregates in an AAV sample.

FFF-MALS is the most appropriate method to quantify all AAV aggregates

As the most appropriate method for quantifying all AAV aggregates, FFF-MALS was then used to determine the mass percentage of each aggregate species in all four AAV samples listed in Table 1. The MALS fractograms from duplicate injections are shown in Figure 5.

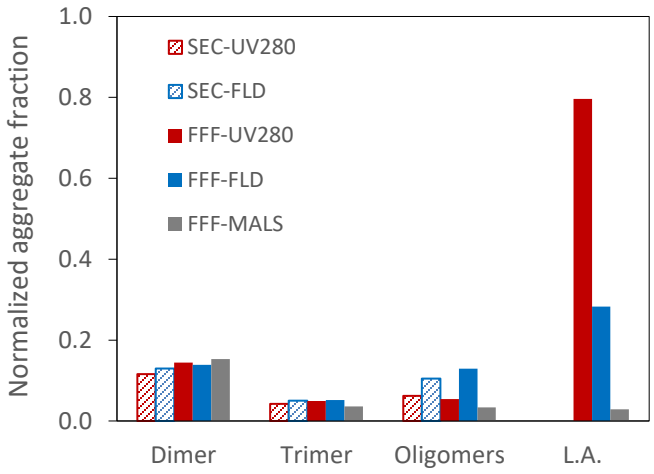


Figure 4. Aggregate fraction normalized to monomer mass in Sample A, separated by SEC' (stripped bars) or FFF (solid bars) and analyzed by UV (red), FLD (blue) or MALS (gray).

The perfectly overlapped traces from duplicate injections demonstrate the excellent reproducibility of the FFF-MALS method. The flat trace from a blank injection (injection of mobile phase), which were collected at the end of the data collection sequence, show negligible carryover under the FFF method employed.

Mass percentage is typically used to quantify aggregates. For UV and FLD, mass percentage is based on the areas of monomer and small oligomer peaks. UV and FLD detectors, however, cannot measure reliable mass percentage of large aggregates, as discussed in the previous section. MALS data and ASTRA software determine size, number of particles, and total volume of particles in each of the designated peak regions, enabling the peaks to be both unequivocally identified and quantified in terms of mass percentage.

For FFF-MALS, mass percentage is related to the total volume of particles in each peak, normally calculated under the assumption of uniform spheres with the same densities. However, AAV aggregates, especially small oligomers like dimer and trimer, deviate from a spherical model such that corrections must be applied. The correction factors for each type of aggregate are detailed in Table 3. We applied the shape correction factor to FFF-MALS data from all four AAV, with the results shown in Figure 6.

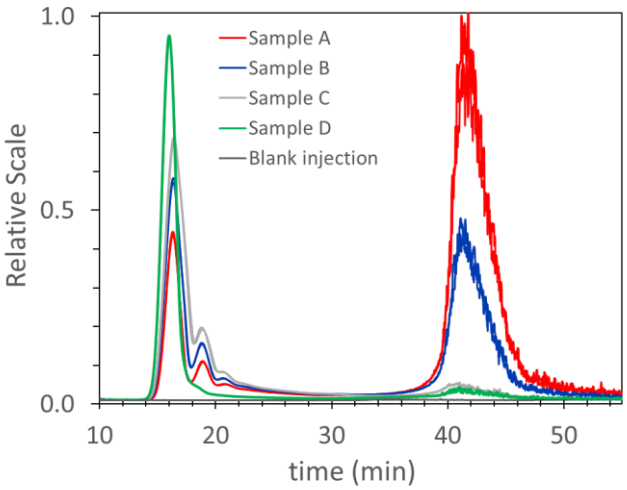


Figure 5. MALS fractograms of four AAV samples (red, blue, gray, and green traces), 2x each, and a 'blank' injection (black trace). Near-perfectly superimposed fractograms from duplicate injections demonstrate the reproducibility of the method.

Table 3. Correction factor of volume of particle for monomer, dimer, trimer, oligomers, and large aggregates (L.A.).

Aggregation state	Correction factor for volume
Monomer	1.00
Dimer	2.22
Trimer	1.96
Oligomer	1.39
L.A.	1.10

From Figure 6, we readily conclude that the concentration of large aggregates decreases in the order from Sample A to Sample D, consistent with the expected trend. For Sample D, the full capsid sample, there are no measurable large aggregates, and monomer accounts for 98.7% of the total injected mass. The aggregation trend found in the FFF-MALS results are consistent with the one observed from the DLS measurements (not shown).

Though FFF-MALS is the tool of choice for characterizing and quantifying all sizes of AAV aggregates, it may not always be required. When it has been demonstrated that large aggregate formation is not detected under typical

storage conditions, SEC-MALS is adequate for routine aggregate monitoring.

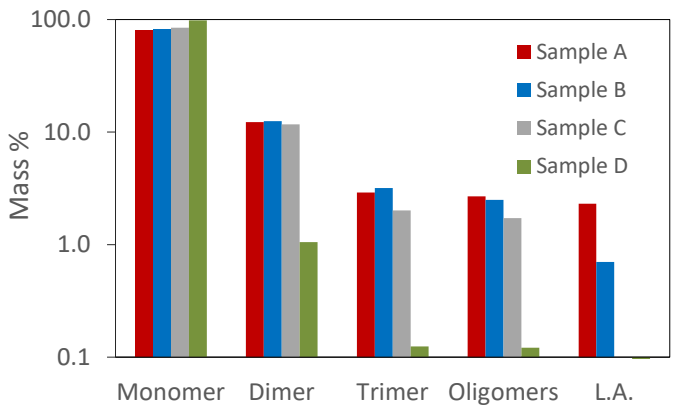


Figure 6. Mass percentage (logarithmic scale) of each aggregate type for the four AAV samples.

Conclusions

As more and more AAV-delivered gene therapy products come near to their final stages of clinical trials and begin commercialization, it is critical to accurately assess the amount of all aggregates in these products. Though SEC-UV and SEC-FLD are appropriate for measuring small aggregates such as dimer and trimer, they are inadequate for quantifying large oligomers and larger aggregates that may be present.

Large aggregates are shown to be susceptible to alteration and removal by the SEC column, making FFF the separation tool of choice for quantifying all the aggregates in an AAV sample. Furthermore, UV and FLD are not recommended for quantification by FFF. Due to scattering artifacts in these detectors, they overstate the quantity of larger aggregates. Applying the modified number density analysis to FFF-MALS data is the most appropriate method for measuring the mass percentage of aggregates, from dimer to large aggregates. With aggregates and other critical quality attributes accurately quantified and monitored, the safety and efficacy of AAV products can then be determined with greater certainty.

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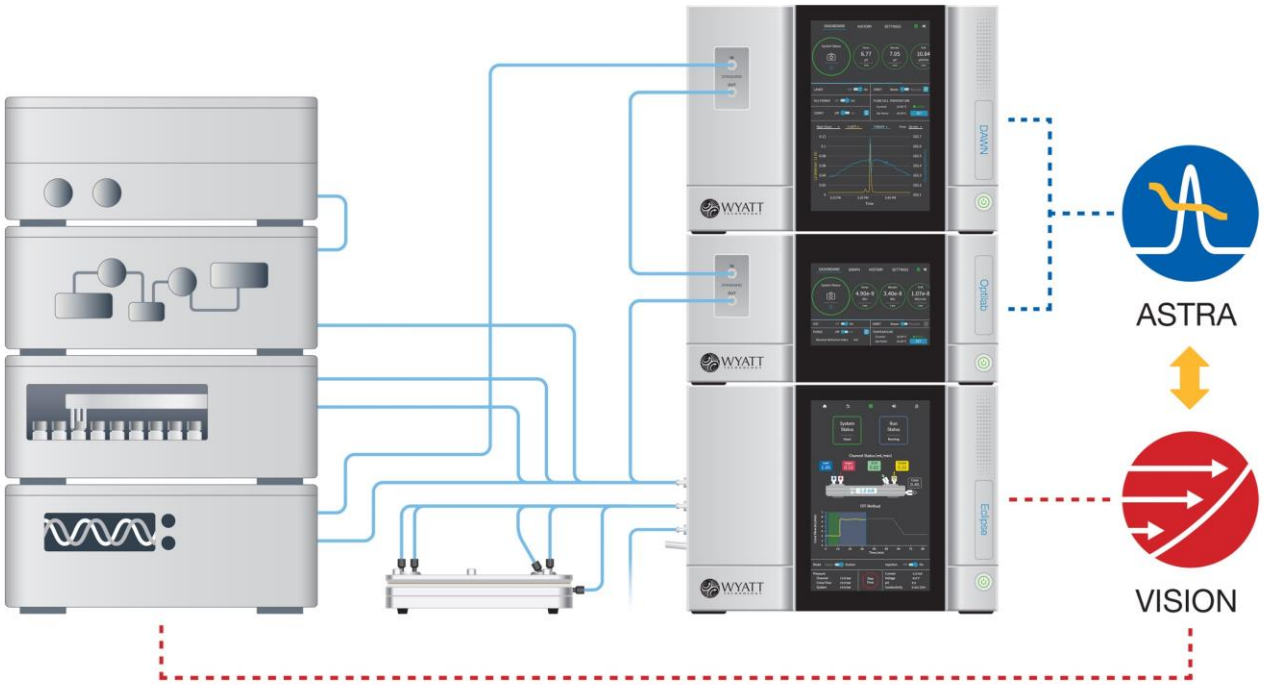

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