Application Note

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Determining Adeno-Associated Virus Critical Quality Attributes

Using size exclusion chromatography and multiple detectors

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Abstract

Adeno-associated virus (AAV) vectors enable efficient gene delivery with selective tissue tropism, making them valuable tools for treating genetic diseases. To ensure their safety and efficacy, various critical quality attributes (CQAs) have to be monitored. In this application note, size exclusion chromatography (SEC) using a widepore Agilent AdvanceBio column (500 Å) in combination with multiple detectors is introduced to study these CQAs. Upon interrogating the different detector signals, peak purity, empty-full ratio, molecular weight (MW), size, aggregates, and fragments can be assessed.

Introduction

Originally discovered in the mid-1960s as a contaminant in adenovirus preparations, AAV has emerged as a widely used gene therapy vector. AAVs are 20 to 30 nm, nonenveloped, and nonpathogenic viruses that can package a single-stranded deoxyribonucleic acid (ssDNA) transgene with a size up to 4.7 kb. Three different viral proteins (VP1–3) form the icosahedral macromolecular capsid structure, comprising a total of 60 VP copies in a ratio 1:1:10 (VP1:VP2:VP3). Several naturally occurring AAV serotypes exist, all exhibiting specific tropism features, which are leveraged in gene therapy to target different types of cells or tissues.[1]

The recombinant production of AAVs for therapeutic use is an inherently complex, multistage process that leads to batch-to-batch variability in terms of quality, yield, and purity. It is crucial to control heterogeneity in the preparation of the resulting vector. For example, the presence of empty capsids in AAV formulations can impair the potency and induce immune responses.[2] At the same time, it is plausible that a certain percentage of AAVs contain either a partial genome fragment or DNA impurities originating from the host cell. A certain fraction (up to 30%) may also contain overfilled capsids, which can typically occur when relatively small (± 2 kb) genomes are packaged. Furthermore, AAV aggregation can severely impact bioavailability and concurrent distribution, as well as immunogenicity. As such, the AAV empty-full ratio and aggregation are CQAs that must be carefully monitored.

Several methods have been developed to determine the fraction of empty capsids, including optical density measurements at 260 and 280 nm, capsid enzyme-linked immunosorbent assay in tandem with a quantitative PCR assay, and anion-exchange chromatography. Some techniques exist to study both aggregation and empty-full ratio, such as negative stain transmission electron microscopy or cryogenic electron microscopy, static/dynamic light scattering, analytical ultracentrifugation, charge detection mass spectrometry, mass photometry, and size exclusion chromatography-multiangle light scattering detection (SEC-MALS). SEC-MALS can simultaneously determine the MW of aggregates and fragment impurities (large MW dynamic range attributed to the pore size) and the empty-full ratio from minute sample amounts in sub-20-minute timeframes.

In this application note, an Agilent AdvanceBio SEC 500 Å column was used in combination with ultraviolet (UV), fluorescence detection (FLD), refractive index (RI), and MALS detection to probe various structural features of an AAV5.[3]

Experimental

Materials

Type I water was produced using an Arium Pro ultrapure lab water system from Sartorius (Bohemia, NY, USA). Sodium chloride (≥ 99.0%, ReagentPlus), sodium phosphate dibasic (≥ 99.0%, ACS reagent) and sodium phosphate monobasic monohydrate (≥ 99.0%, ACS reagent) were purchased from Sigma-Aldrich (St. Louis, MO, USA). AAVs were sourced from Virovek (Houston, TX, USA). See Table 1.

Table 1. AAVs used in this application note.

Sample Name	DNA	Titer (vg/mL)	Host
AAV5-full	CMV-GFP	2×10^{13}	Insect Sf9 cells
AAV5-empty	–	1×10^{13}	Insect Sf9 cells

Sample preparation

AAV samples were thawed and aliquoted to an HPLC vial, without dilution, under a laminar flow cabinet.

Instrumentation and method

Details of the instrument configuration can be found in Table 2. Method parameters are summarized in Table 3. Data were acquired and processed in Agilent OpenLab CDS version 2.7 and ASTRA 8.2.2 from Wyatt Technology.

Table 2. Details of the instrument configuration used.

Parameter	Value
Pump	Agilent 1260 Infinity II bio-inert pump (p/n G5654A)
Autosampler	Agilent 1260 Infinity II bio-inert multisampler (p/n G5668A)
Column Compartment	Agilent 1260 Infinity II multicolumn thermostat (p/n G7116A)
Diode Array Detector	Agilent 1260 Infinity II diode array detector (p/n G7115A)
UV Flow Cell	Agilent bio-inert flow cell, 13 µL, 10 mm path length (p/n G5615–60022)
Fluorescence Detector	Agilent 1260 Infinity II FLD (p/n G7121B)
FLD Flow Cell	Agilent FLD flow cell, standard 8 µL (p/n G1321-60005)
MALS Detector	miniDAWN MALS detector (Wyatt Technology)
RI Detector	Agilent 1260 Infinity RI detector (p/n G1362A)

Table 3. LC method parameters.

Parameter	Value
Column	Agilent AdvanceBio SEC 500 Å, 4.6 × 300 mm, 2.7 µm (p/n PL1580-5325)
Flow Rate	0.35 mL/min
Mobile Phase	50 mM phosphate buffer, 400 mM NaCl, pH 7.2
Column Temperature	25 °C
Autosampler Temperature	4 °C
Injection	1 µL (2 µL for MALS)
Diode Array Detection	260/4 nm, 5 Hz 280/4 nm, 5 Hz
FLD	Ex 280 nm, Em 348 nm, 4.63 Hz
MALS Detection	Laser 658 nm, detection angles: 49°, 90°, 131°
RI Detection	35 °C, 2.31 Hz
Run Time	24 minutes

Results and discussion

SEC, in combination with a range of detectors, is one of the most prominent approaches for the determination of CQAs in AAV samples. UV detection allows the assessment of peak purity by interrogating the peak heights or areas recorded at 280 nm (protein content) and 260 nm (nucleic acid content). It has been described that the A_{260}:A_{280} ratio is nonlinearly correlated with the percentage of empty capsids.[4] For filled AAVs, this ratio ranges between 1.2 and 1.4, whereas empty capsids typically have an A_{260}:A_{280} ratio of 0.6 to 0.7. An A_{260}:A_{280} ratio around 1.8 is expected for "pure" DNA. Figure 1 shows the UV 260 and 280 nm chromatograms of an AAV5-full and AAV5-empty sample, respectively. Upon comparing both wavelengths, one can qualitatively observe that the UV 280 signal is most intense for the empty capsid sample. This indicates a decreased (< 1) A_{260}:A_{280} ratio.

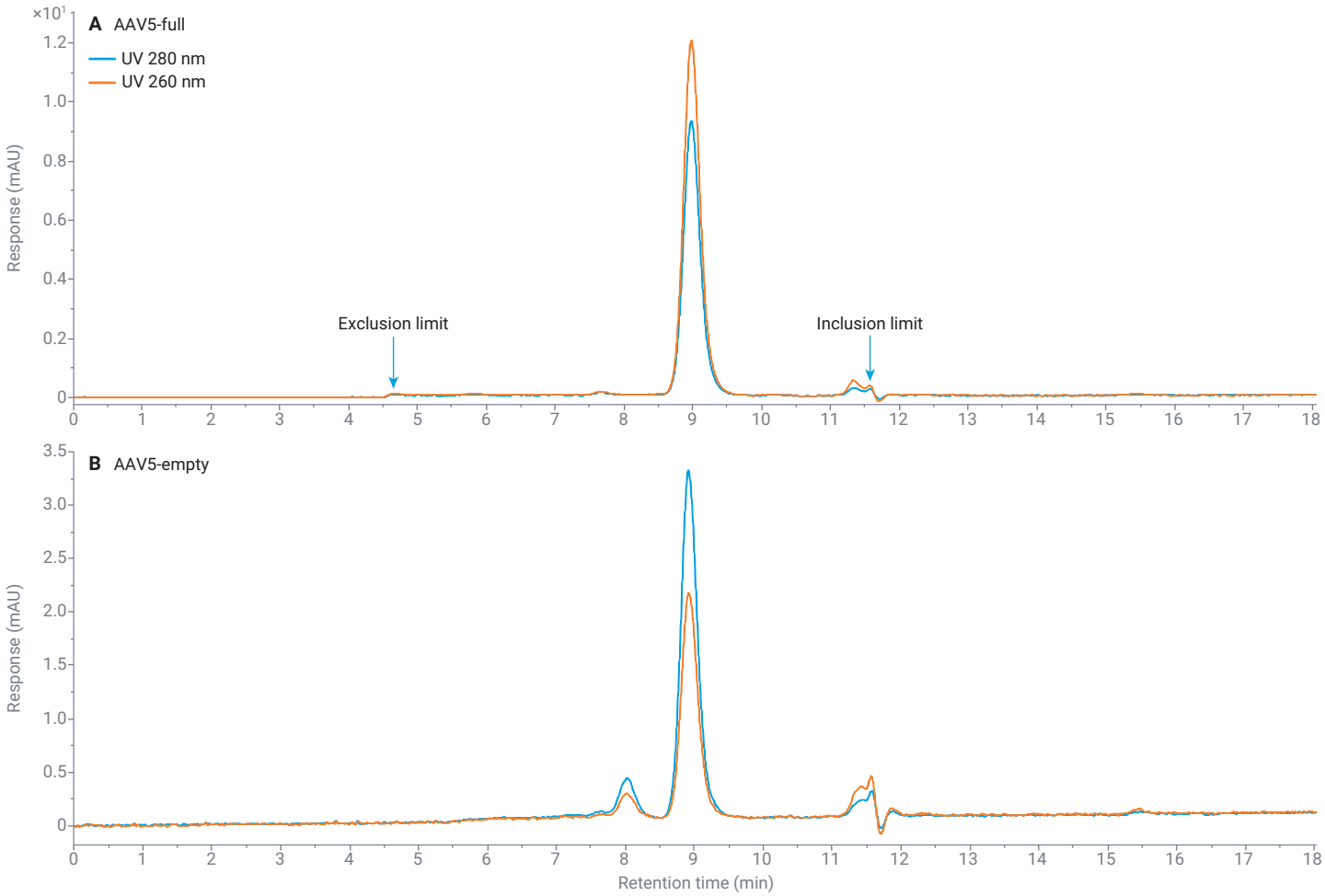

Figure 1. UV chromatograms recorded at 280 nm (blue) and 260 nm (orange) of AAV5-full (A) and AAV5-empty (B), enabling peak purity assessment based on an A_{260}:A_{280} ratio. Note that the decreased signal intensity for AAV5-empty is due to the lower particle concentration and the absence of UV-absorption of DNA.

Table 4 provides the calculated ratios based on the peak heights, which were seen to excellently follow observations from literature. The USP resolution (based on peak width at half height) between the aggregate and main peak of AAV5-full was calculated to be 2.9, showing that the CQA was well resolved. The major aggregates showed the same A_{260}:A_{280} ratio as the main peak for both AAV5-full and empty, which amounted to 1.0% and 8.6%, respectively, of the total peak area.

Table 4. Peak heights and A_{260}:A_{280} ratios calculated for AAV5-full and AAV5-empty samples.

Sample Name	Relative Peak Area (%)	Peak Height A_{260} (mAU)	Peak Height A_{280} (mAU)	A_{260}:A_{280} Ratio (/)
AAV5-full (Aggregate)	1.011	0.12378	0.10148	1.22
AAV5-full (Main Peak)	98.989	11.98925	9.28036	1.29
AAV5-empty (Aggregate)	8.552	0.21324	0.34371	0.62
AAV5-empty (Main Peak)	91.448	2.10302	3.2595	0.65

Incorporating FLD in the instrument setup with excitation at 280 nm and emission at 348 nm, which is selective for tryptophan residues, provided a highly specific and sensitive detection method for monitoring AAV capsids. When comparing the UV 280 nm with the FLD chromatogram (Figure 2), a significant gain in sensitivity for the aggregates and main peak could be observed. Interestingly, the major aggregate species in AAV5-empty were smaller than that of AAV5-full. A small difference in elution time of the main peak was also evident for both samples, which can be explained by the presence or absence of the DNA payload.

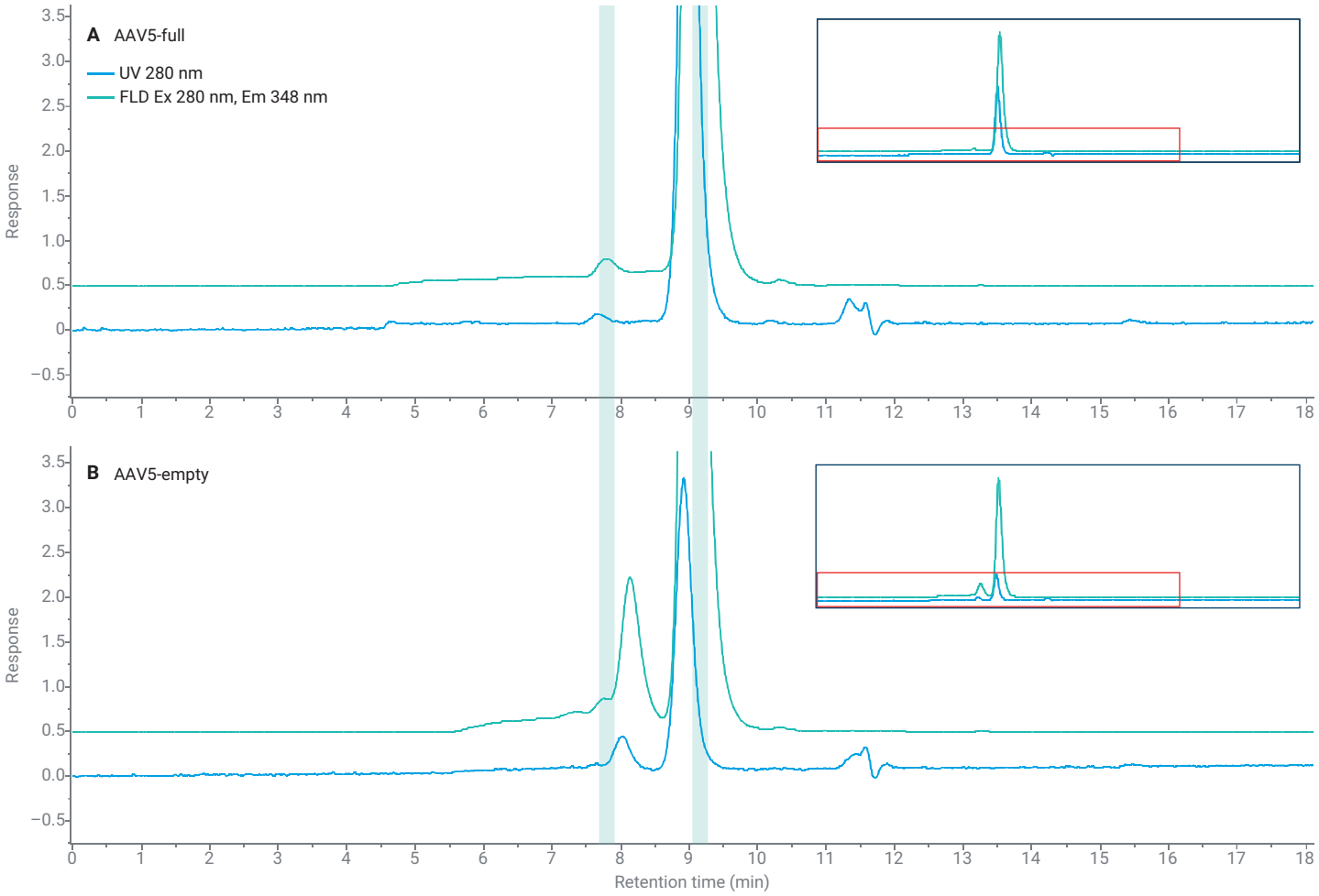

Figure 2. Zoom of UV and FLD chromatograms of AAV5-full (A) and AAV5-empty (B), showing the increased sensitivity. The highlighted areas based on the FLD chromatogram of AAV5-full make it possible to compare the elution times of the aggregate and main peak with those of the AAV5-empty sample.

Using SEC-UV-MALS-RI allowed for a more detailed analysis of several features of AAVs, including capsid/genome MW along with their respective concentrations, polydispersity, empty-full capsid ratio, radius of gyration, and aggregates or fragments. A 100% empty AAV standard of the same serotype as the AAV sample of interest was required to determine the capsid extinction coefficient (using RI or UV 260 nm and UV 280 nm). Next, a filled AAV sample was used to determine the extinction coefficient of the nucleic acid, allowing determination of the CQAs of the same serotype as the used AAV standards. The light scattering (LS) detector is ideally suited to detect aggregates in solution, since the MALS response scales directly with molecular weight.[5] This could immediately be observed when comparing the LS chromatograms in Figure 3, where the AAV5-empty sample is shown to contain multiple aggregates compared to AAV5-full, eluting from 5 to 7.5 minutes. A dissimilarity in size between the main aggregates in both chromatograms, as also observed using FLD, was again apparent.

Given the well-known icosahedral arrangement of 60 viral proteins in 1:1:10 molar ratio, the theoretical MW for the capsid is approximately 3.8 MDa. The theoretical MW of the encapsulated CMV-GFP promotor and genome with 2.5 kb was approximately 773 kDa. It was shown that the calculated MW for the empty AAV capsid sample (3,798.4 kDa) was in line with the theoretical MW (Table 5). The MW of the nucleic acids for AAV5-full was 884.5 kDa, which is slightly higher than the theoretically calculated MW. This might be the result of a coeluting fraction of overfilled AAVs with that of the main AAV5-CMV-GFP capsid peak, resulting in an overestimation of the nucleic acid MW. Assuming a "double" filling of the 2.5 kb genome, it could be estimated that the overfilled AAV5 fraction was on the order of 15%. Based on SEC-MALS, 89.8% of the capsids of the AAV5-full sample were filled. The observed radius of gyration (R_g) predicted a size of roughly 22 nm, which is close to the well-known value of 25 nm for AAV capsids.

Table 5. Calculated parameters from SEC-MALS for AAV samples.

Sample Name	R_g (nm)	MW Total (kDa)	MW Protein (kDa)	MW Nucleic Acid (kDa)	Empty-Full Ratio (%)
AAV5-Full (Main Peak)	11.0	4311.6	3427.1	884.5	89.8
AAV5-Empty (Main Peak)	11.7	3798.4	3791.1	6.7	0.5

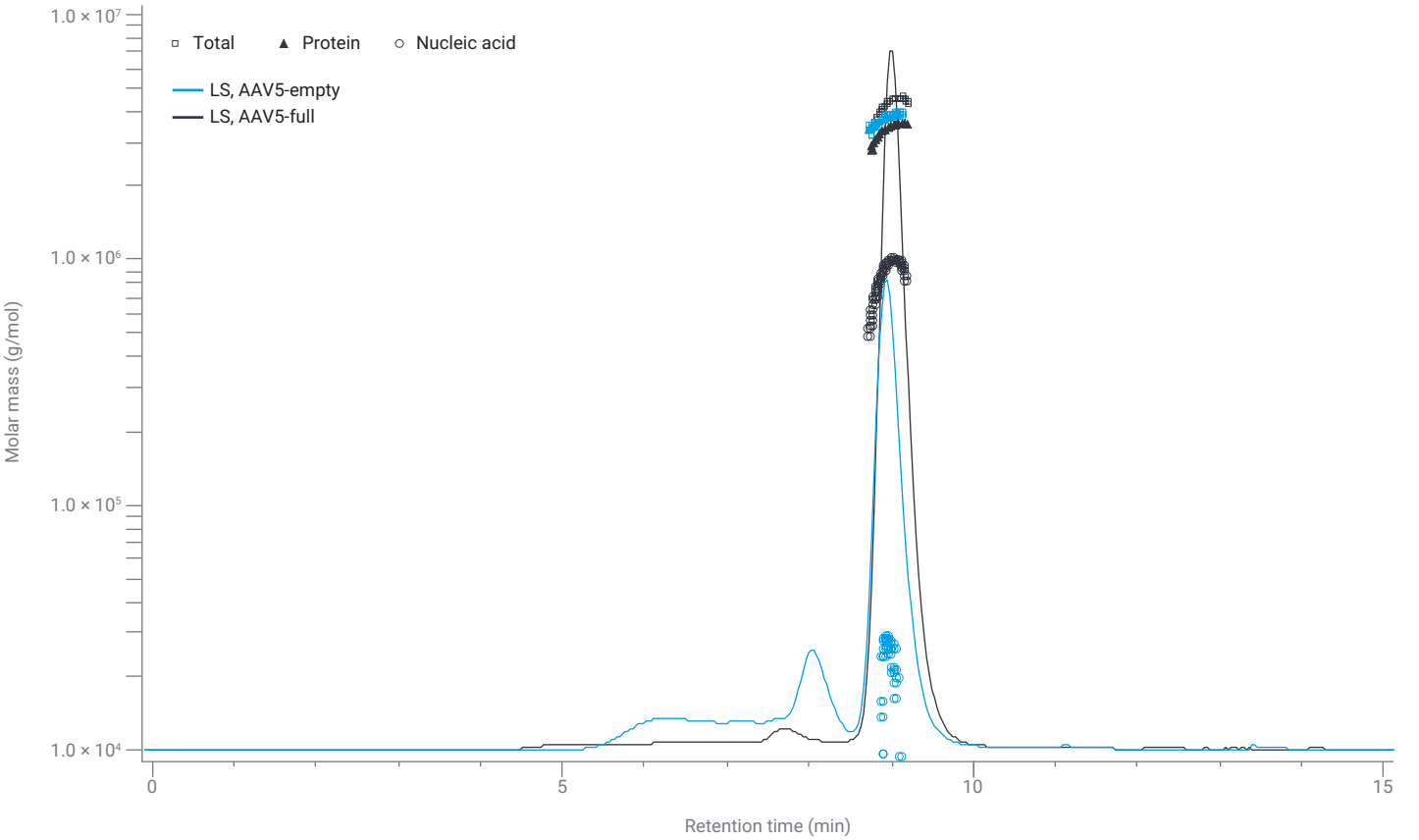

Figure 3. SEC-MALS experiment showing the LS signal of AAV5-full (black) and AAV5-empty sample (blue). MWs of capsid, nucleic acid, and AAV total, as displayed in Table 5, were calculated following calibration using the empty AAV5 standard.

Conclusion

This application note demonstrates how SEC, combined with multiple detectors, enables multi-attribute analysis of AAV samples. The Agilent AdvanceBio SEC 500 Å column provides excellent performance to study various structural features of AAVs, including a detailed study of aggregation. Based on FLD, a gain in sensitivity can be achieved allowing to monitor minor aggregates and impurities at reduced sample amounts. The assessment of peak purity and capsid filling can be performed when interrogating the A_{260}:A_{280} ratio. When hyphenating SEC to MALS detection, it is possible to determine the MW of the capsid and genome, AAV size, and empty-full ratio.

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DE-011847

Printed in the USA, January 9, 2026
5994-8897EN

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