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Routine Monitoring of DNA/mRNA Therapeutics With the Alliance™ iS Bio HPLC System and Ultra Wide-Pore GTxResolve™ Premier SEC Column

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Abstract

The development of gene therapy, especially in quality control (QC) environments, requires modern analytical technologies to improve efficiency and safety. Size exclusion chromatography (SEC) is a prevalent method used for the characterization and monitoring of size variants in biopharmaceuticals. While size exclusion chromatography is considered one of the most effective methods for the separation of mRNA from its potential aggregates, challenges such as peak broadening, caused by system dispersion, and inadequate pore size of the column particles can impede method development and reduce performance. In this study, the Alliance iS Bio HPLC System was evaluated on its ability to support manufacturing labs in the routine analysis of mRNA therapeutics. In summary, when using Waters™ ultra-wide pore particle GTxResolve Premier SEC Column, the lower system dispersion of the Alliance iS Bio HPLC System offers comparable performance to the ACQUITY™ Premier UPLC™ System in SEC separation and is an ideal HPLC platform for downstream analysis of DNA/mRNA therapeutics using size exclusion chromatography.

Benefits

- Lower system dispersion of the Alliance iS Bio HPLC System offers improved SEC performance for efficient method development and migration
- GTxResolve Premier SEC Columns offer high resolution SEC separations to support DNA/mRNA analysis in QC environments
- Alliance iS Bio HPLC System provides consistent and reliable performance for routine monitoring of impurities in DNA/mRNA therapeutics

Introduction

Gene therapy, which involves treating disease by transferring genetic material into cells, has a wide range of applications including cancer treatment, genetic disorders, and immunotherapy. A notable recent application is the messenger RNA (mRNA) vaccine developed to reduce the transmission of the COVID-19 virus.[1] While these advancements are promising, it is important to acknowledge that gene therapy continues to be a rapidly evolving field with ongoing research and development, and the introduction of these new modalities to market presents its own set of challenges. Similar to other biopharmaceuticals, mRNA has the potential to self-associate and form aggregates which can impact drug product safety.

SEC is a prevalent method used for the characterization and monitoring of size variants in biopharmaceuticals. However, the substantial size difference between gene therapy drugs and more traditional biotherapeutics prevents the use of conventional SEC columns optimized for smaller analytes. As a technique that relies on accessible pore volume to effectively separate analytes by size, SEC columns with ultra-wide pores are needed to resolve the relatively large mRNA molecules, which can range from hundreds to thousands of nucleotides in length, from their potential aggregates.[2] Further more, as an isocratic technique, SEC is sensitive to system volume artifacts that can contribute to extra-column peak dispersion resulting in loss of chromatographic resolution.[3] Therefore, selecting the appropriate column and system in the development and manufacturing of these new modalities is critical to ensure quality indicating attributes such as aggregates are characterized and monitored accurately throughout the lifecycle of the drug product.

The Alliance iS Bio HPLC System, a next-generation HPLC system featuring lower system dispersion and MaxPeak™ High Performance Surfaces (HPS) Technology, is a biocompatible and exceedingly inert HPLC platform designed for biopharmaceutical manufacturing environments. This study will evaluate the Alliance iS

Bio HPLC Systems suitability to support manufacturing labs in the routine analysis of mRNA therapeutics. To fully utilize system performance, SEC separations will be performed using the ultra-wide pore particle GTxResolve Premier SEC Column. Peak dispersion and resolution will be used as comparative metrics to determine system suitability for the routine monitoring of mRNA and its associated aggregates.

Experimental

DNA ladders were purchased from New England Biolabs (Cat. No: N3236L) with a size range of 50 to 1350 base pairs (bp). The EGFP mRNA (997 nucleotides) was purchased from TriLink BioTechnologies at 1 mg/mL in 1 mM sodium citrate buffer. The samples were used without further dilution.

LC System Conditions

LC system:	Alliance iS Bio HPLC System, TUV with an analytical 10 mm flow cell and a passive preheater	
	ACQUITY Premier System, QSM variant, TUV with a 5 mm flow cell and CH-30 Column heater.	
	Alliance e2695 HPLC System, 2489 TUV with an analytical 10 mm flow cell and a passive preheater	
Detection:	λ = 260 nm	
Column:	GTxResolve Premier SEC Column, 1000 Å, 3 µm, 4.6 × 300 mm (p/n: 186010736); GTxResolve Premier SEC Column, 1000 Å, 3 µm, 7.8 × 300 mm (p/n: 186010738)	
	DNA ladders	mRNA
Column temp:	50 °C	25 °C
Sample temp:	6 °C	6 °C
Injection volume:	10 µL	2.5 µL
Flow rate:	0.080 mL/min (4.6 mm column) 0.230 mL/min (7.8 mm column)	0.100 mL/min (4.6 mm column) 0.288 mL/min (7.8 mm column)
Mobile phase:	2X PBS in water	50 mM Tris and 200mM KCl in water
Software:	Empower 3.8.1	

Results and Discussion

As drug candidates migrate downstream, associated analysis methods are frequently transferred with them to ensure quality indicating attributes identified in characterization and development phases are monitored appropriately as part of manufacturing activity. As an isocratic method, scaling or migration of SEC methods represent a unique challenge in that they are more sensitive to pre-column dispersion effects since analytes do not adsorb to the stationary phase at injection as they would in a gradient-based method. In this respect, the impact of system dispersion on chromatographic performance should be considered as part of SEC method development on column selection in manufacturing environments.

In this study, system dispersion was experimentally determined for an ACQUITY Premier System, which represents an upstream UPLC platform, as well as the Alliance iS Bio HPLC System, and an Alliance e2695 HPLC System. These latter two systems are representative of modern and legacy platforms typically deployed downstream in QC or manufacturing labs. To support mRNA SEC separations at elevated temperatures using a longer 30 cm column, both the ACQUITY Premier System and Alliance e2695 HPLC System were configured with a 30CH-A column heater. System dispersion was measured by injecting 1 mg/mL caffeine under isocratic conditions using a zero-volume union in place of the column and measuring the peak width at 4.4% peak height and multiplied by flow rate to determine peak volume.[4] Using the 5σ value, peak dispersion was determined to be 12.6 µL for the ACQUITY Premier System, 26.2 µL for the Alliance iS Bio HPLC System and 92.0 µL for the Alliance e2695 System.

To determine the impact of system dispersion on SEC, commercially purchased DNA ladders were separated using a GTxResolve Premier SEC Column (1000 Å, 4.6 x 300 mm) using two times PBS mobile phase. As shown in Figure 1, in terms of accessible pore volume, the DNA ladder eluted in the expected order with the longest DNA bp having the lowest accessibility eluting first at ~25 minutes and the shortest sequence (50 bp) having the highest accessibility eluting last at 44 minutes. Examining the DNA ladders across systems, the effect of system volume is clearly evident. The chromatography shows that both the ACQUITY Premier System and Alliance iS Bio HPLC System yielded similar separations for all bp with sharp and symmetrical peaks, regardless of the co-elution of the densely packed mid-sized 550–916 bp species. However, the Alliance e2695 System shows lower performance in terms of peak broadening and overall loss of resolution between DNA species when using the smaller *i.d.* 4.6 x 300 mm column. Using the 50 bp peak as a fully resolved peak as an example, peak width was observed to increase with system dispersion although not necessarily in a proportional manner. However, the data trend illustrates the impact of system dispersion on SEC chromatographic performance where the lower system dispersion Alliance iS Bio System was able to better match the ACQUITY Premier Systems performance when coupled with the smaller *i.d*. GTxResolve Premier SEC Column (1000 Å, 4.6 x 300 mm) as compared to the Alliance e2695 HPLC System. In contrast, as shown in Figure 2, similar chromatographic performance could be achieved on the Alliance e2695 HPLC System when the method was scaled to use the larger *i.d.* GTxResolve Premier SEC Column (1000 Å, 7.8 x 300 mm) to help manage the large system dispersion of the legacy HPLC System albeit at a higher flow rate (0.080 mL/min vs 0.230 mL/min) and increased solvent use.

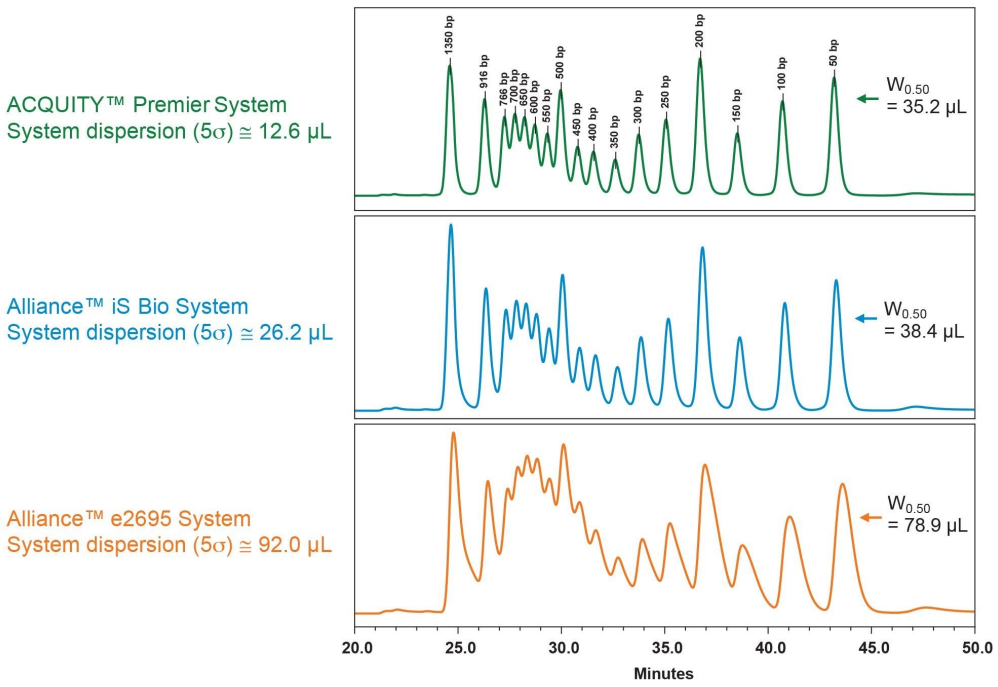

Figure 1. 5-sigma extra-column dispersion volumes (5σ) based on peak width at 4.4% peak height and the SEC-based separation of DNA ladders using the GTxResolve Premier SEC 1000 Å 4.6 x 300 mm, 3 µm Column with 2X PBS on ACQUITY Premier System, Alliance iS Bio HPLC System, and Alliance e2695 System.

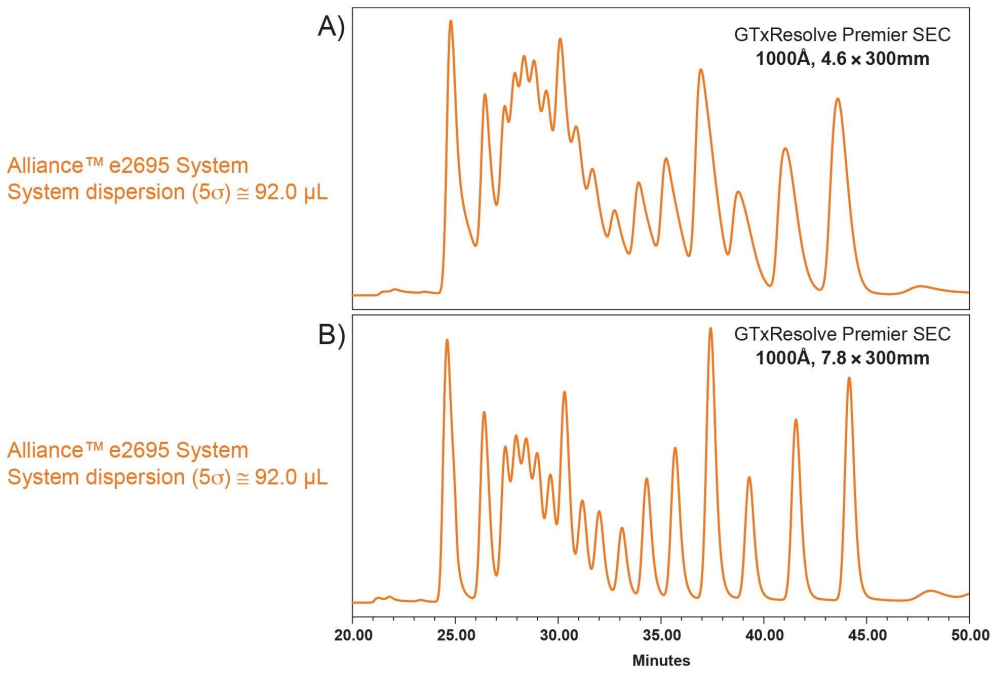

Figure 2. DNA ladder separated on a Alliance e2695 HPLC using the A) GTxResolve Premier SEC Column, 1000 Å 4.6 x 300 mm, 3 µm Column and the B) GTxResolve Premier SEC Column, 1000 Å 7.8 x 300 mm, 3 µm Column.

Using commonly accepted models based on hydrodynamic radius in solution, a plot of normalized retention volume (V_R/V_C) vs molecular weight was made to determine the effective working range of the GTxResolve Premier SEC Column.[5] As shown in Figure 3, using the data from the Alliance iS Bio HPLC System, the experimental DNA ladder data matched well with the modeled data (solid line), where K_{SEC} represents the distribution coefficient of analyte between the accessible pore volume and interstitial volume of the particles. The effective working range of the column is characterized by the linear portion of the plotted data between the total exclusion volume (K_{SEC}=0) and total inclusion volume (K_{SEC}=1). In this case, the GTxResolve Premier SEC Column was determined to be well suited for the separation of small to moderately sized mRNA samples within the tested range of (50–1500 bp).

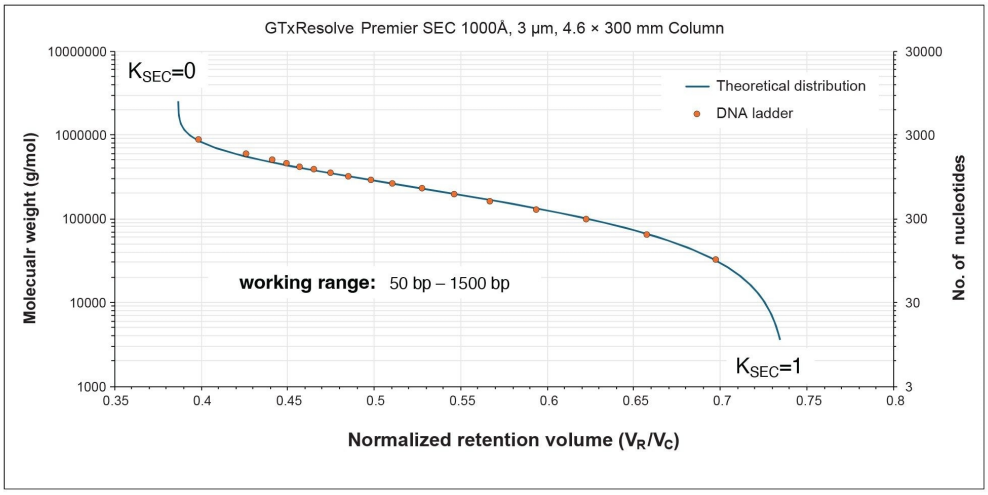

Figure 3. GTxResolve Premier SEC 1000 Å 3 µm Column calibration curve using 50 bp DNA ladders data collected on the Alliance iS Bio HPLC System.

To evaluate the suitability of the Alliance iS HPLC System configured with a GTxResolve Premier SEC Column in the routine analysis of mRNA, a 997 nucleotides EGFP mRNA sample from TriLink BioTechnologies was analyzed for the presence of aggregates. For comparison, the mRNA sample was run across the ACQUITY Premier System, Alliance iS Bio HPLC System and the Alliance e2695 System. As shown in Figure 4A, a main peak at approximately 28.75 minutes was observed as the mRNA monomer. Higher molecular weight species (HMWS) were found eluting between 19 and 27 minutes (20.61±0.19 % Area), most likely corresponding to mRNA aggregates.[2] Smaller nucleotide fragments or excipients, also known as low molecular weight species (LMWS), were observed to elute at approximately 34.25 min and accounted for less than 0.5 % of total peak area. Similar to the DNA ladder results, the Alliance iS Bio HPLC System (Figure 4B) was able to closely match the resolution between the HMWS and monomer in peak-to-valley resolution when compared to the representative upstream system (p/v=2.3 vs. p/v=2.2) when using the smaller *i.d.* GTxResolve Premier SEC column (1000 Å, 4.6 x 300 mm). While not as noticeable as the DNA ladders, the Alliance e2695 HPLC System (Figure 4C) exhibited almost a 25% loss in resolution (p/v=1.6) when running the same SEC method and column. Similar to before, separation performance for the mRNA surrogate was recovered when using the larger *i.d.* GTxResolve Premier SEC column (1000 Å, 7.8 x 300 mm) on the legacy HPLC system (Figure 5A, bottom panel). While both HPLC systems were observed to deliver acceptable performance in terms of resolution when paired with the appropriate column, the Alliance iS Bio HPLC System was able to deliver the same quality of separation with the added benefit of using

less solvent for reduced overall operating costs. Furthermore, the separation of mRNA and its associated aggregates was consistent and reproducible on the Alliance iS Bio System as evidenced by the low peak area % RSD for the HMWS (% RSD=0.93%), which is ideal for instruments deployed in manufacturing environments (Figure 5B). More noteworthy was the platform's ability to deliver consistent results (% RSD=4.8%) on the LMWS which accounted for less than 0.5% of total peak area. In summary, the Alliance iS Bio HPLC System outperformed the legacy Alliance e2695 System in terms of cost savings and exhibits comparable performance to the ACQUITY Premier UPLC System in SEC separations with its lower system dispersion and is an ideal HPLC platform for downstream analysis of DNA/mRNA therapeutics using size exclusion chromatography.

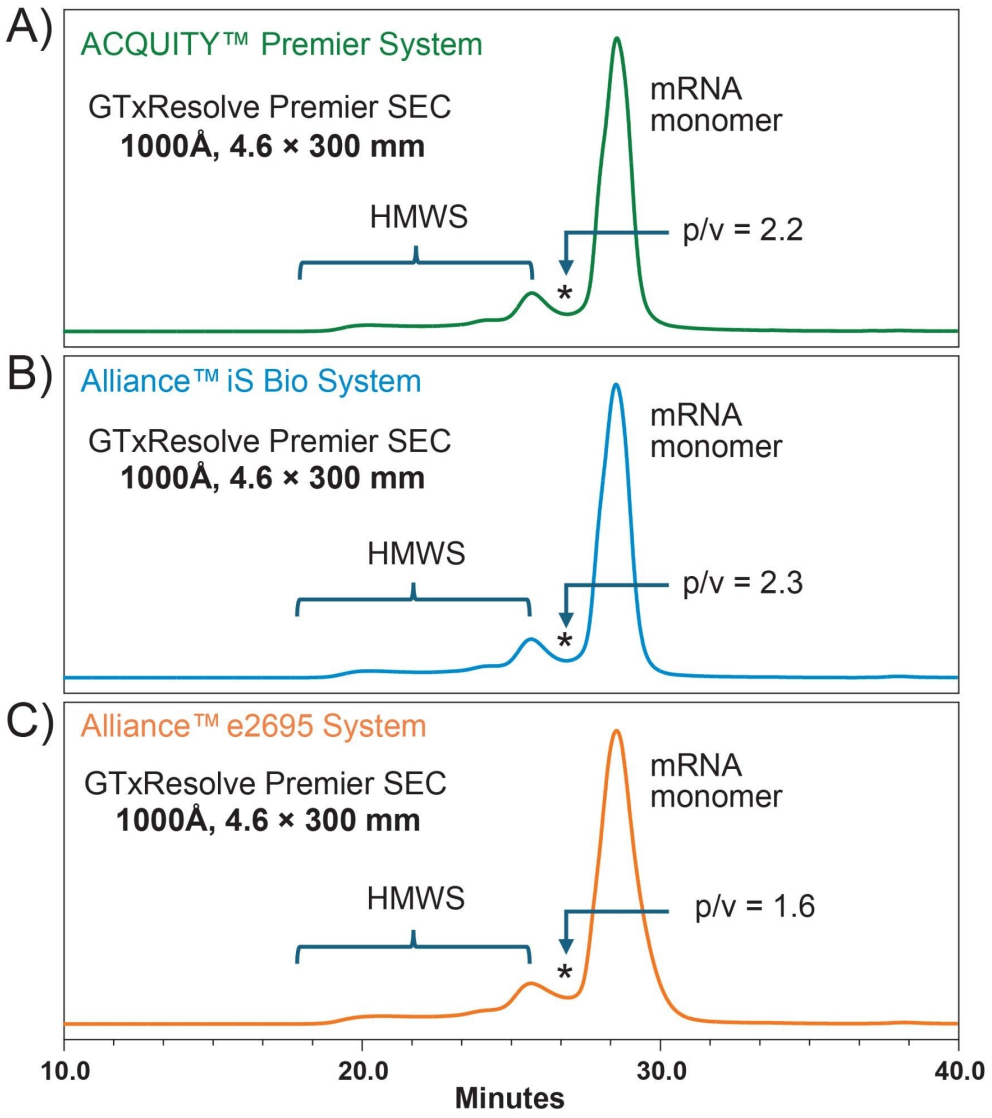

Figure 4. A) SEC separation of 1 mg/mL EGFP mRNA via different LC platforms with 50 mM Tris and 200 mM potassium chloride using the GTxResolve Premier SEC 1000 Å, 4.6 x 300 mm, 3 µm Column.

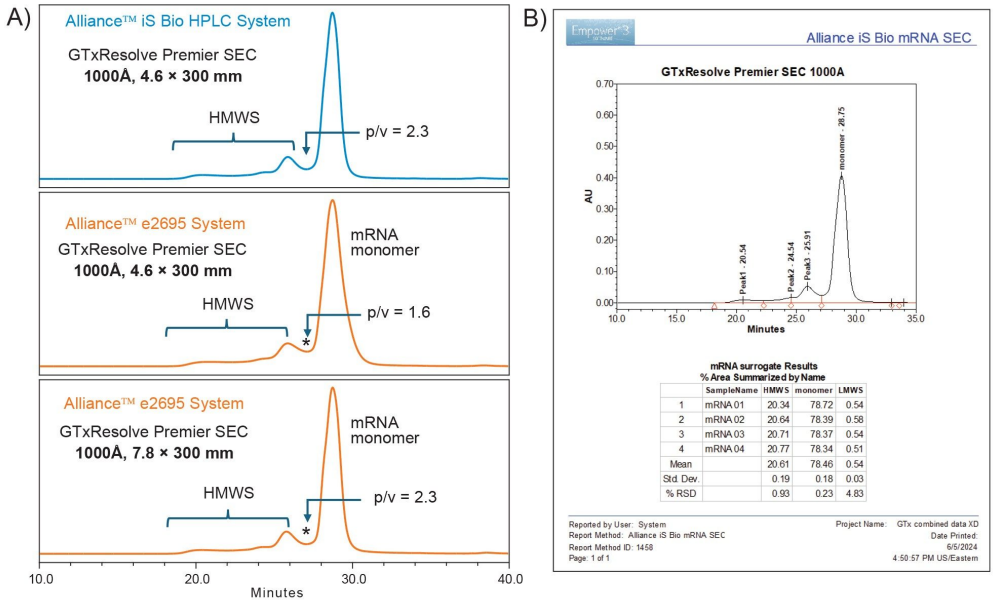

Figure 5. Comparison of SEC separations of EGFP mRNA performed on the Alliance iS Bio HPLC System and Alliance e2695 System using GTxResolve Premier SEC 1000 Å in 4.6 x 300 mm, 3 µm and 7.8 x 300 mm, 3 µm formats. B) Empower 3 report of mRNA data collected on the Alliance iS Bio HPLC System.

Conclusion

Reliable and consistent methods for monitoring quality indicators are crucial for ensuring the safety of drug products. This study demonstrated the effectiveness of the Alliance iS Bio HPLC System in supporting the routine analysis of gene therapies in quality control environments. As a next-generation HPLC system, the lower dispersion of the Alliance iS Bio HPLC System offers comparable results to the ACQUITY Premier UPLC System while reducing operating costs. The Alliance iS Bio HPLC System, configured with an ultra-wide pore particle GTxResolve Premier SEC Column, provided exceptional reproducibility for low-abundant species (total peak area %≤0.5%) with % R.S.D.≤5%. In conclusion, the Alliance iS Bio HPLC System, with its low dispersion and consistent performance, is well positioned for downstream analysis of new modality applications for QC environments.

References

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