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Application Note

Chromatography Solutions for AAV Full and Empty Capsid Separation

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Introduction

Adeno-associated viruses (AAV) have emerged as a promising viral vector for gene therapy templates. Yet despite the expanded adoption of this modality, several process challenges create inefficiencies during AAV manufacturing. A critical hurdle is the separation of full and empty AAV capsids during downstream processing. Empty capsid impurities, which lack the desired genomic content and therefore provide no therapeutic benefit to the patient, should be removed during purification. However, the similar characteristics of empty and full capsids make separation quite challenging; the full capsids feature only a slightly lower isoelectric point (pI) and a slightly higher density compared to the empty capsids **(Table 1).**

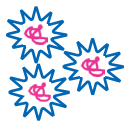
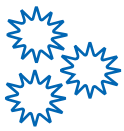
	Full Capsids	Empty Capsids
Approximate isoelectric point (pI)	5.9	6.3
Approximate density	1.40 g/cm^3	1.32 g/cm^3
		

Table 1. Properties of full and empty AAV capsids

Effective separation of full and empty AAV capsids requires implementation of purification strategies that differ from standard approaches used for traditional modalities, such as antibodies. Here, we present two case studies describing such approaches. In **Case Study 1**, anion exchange resins were evaluated for their ability to separate full and empty capsids using a non-traditional hybrid elution gradient. **Case Study 2** describes the online detection capabilities of a single-use chromatography system, demonstrating feasibility of real-time UV ratio detection for improved process control in viral vector manufacturing.

Case Study 1: Use of Fractogel® Ion Exchange Resin for AAV Full and Empty Capsid Separation

Fractogel® TMAE (S) and TMAE (M) resins are tentacular resins featuring strong anion exchange chemistries affixed to a methacrylate base bead. These resins were explored for their ability to separate empty and full capsids in an AAV8 serotype feed. Recommendations for a unique hybrid elution gradient, which incorporates elements of both gradient and step elution, are discussed.

Materials

Feed material:

- AAV8 serotype material was provided by an industry collaborator. The feed was purified by affinity chromatography and adjusted to pH 9 and approximately 5 mS/cm prior to anion exchange chromatography experiments. The feed contained approximately 1.8E+13 vp/mL and 15% full capsids.

Resins:

- Fractogel® TMAE (S) resin (particle size 24 – 40 µm), packed in a 5 mL CV column (10 cm bed height).
- Fractogel® TMAE (M) resin (particle size 48 – 60 µm), packed in a 5 mL CV column (10 cm bed height).

Buffers:

- Buffer A: 20 mM Tris + 50 mM Ammonium Acetate, pH 9
- Buffer B: 20 mM Tris + 500mM Ammonium Acetate, pH 9
- Full capsid step elution buffer: 20 mM Tris, 500mM Ammonium Acetate, 500 mM NaCl, pH 9.
- Strip: 20 mM Tris + 1.5M NaCl, pH 9
- CIP: 0.5 M NaOH

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Analytics:

- UV absorbance monitoring at 280 nm (A280) and 254 nm (A254). Full capsids are expected to absorb more strongly at 254 nm due to genomic DNA content. Therefore, a UV signal in which A254/A280 > 1 suggests enriched full capsids, while < 1 suggests enriched empty capsids.
- Enzyme linked immunosorbent assay (ELISA), for measurement of total viral particles (VP/mL)
- Digital droplet polymerase chain reaction (ddPCR), for measurement of total viral genomes (VG/mL)
- Size exclusion chromatography with multiangle light scattering detector (SEC-MALS), for combined measurement of total viral particles and total viral genomes.

Discussion of Methods and Results

The first step in developing a hybrid gradient elution strategy is to execute a simple linear conductivity gradient. Here, linear conductivity gradients (starting with 100% Buffer A and transitioning to 100% Buffer B over 20 column volumes (CV)) were applied to both Fractogel® TMAE (S) and Fractogel® TMAE (M) columns after loading each to 1E+13 VP/mL resin. The linear gradients were performed at a 4-minute residence time. Results are shown in **Figure 1**.

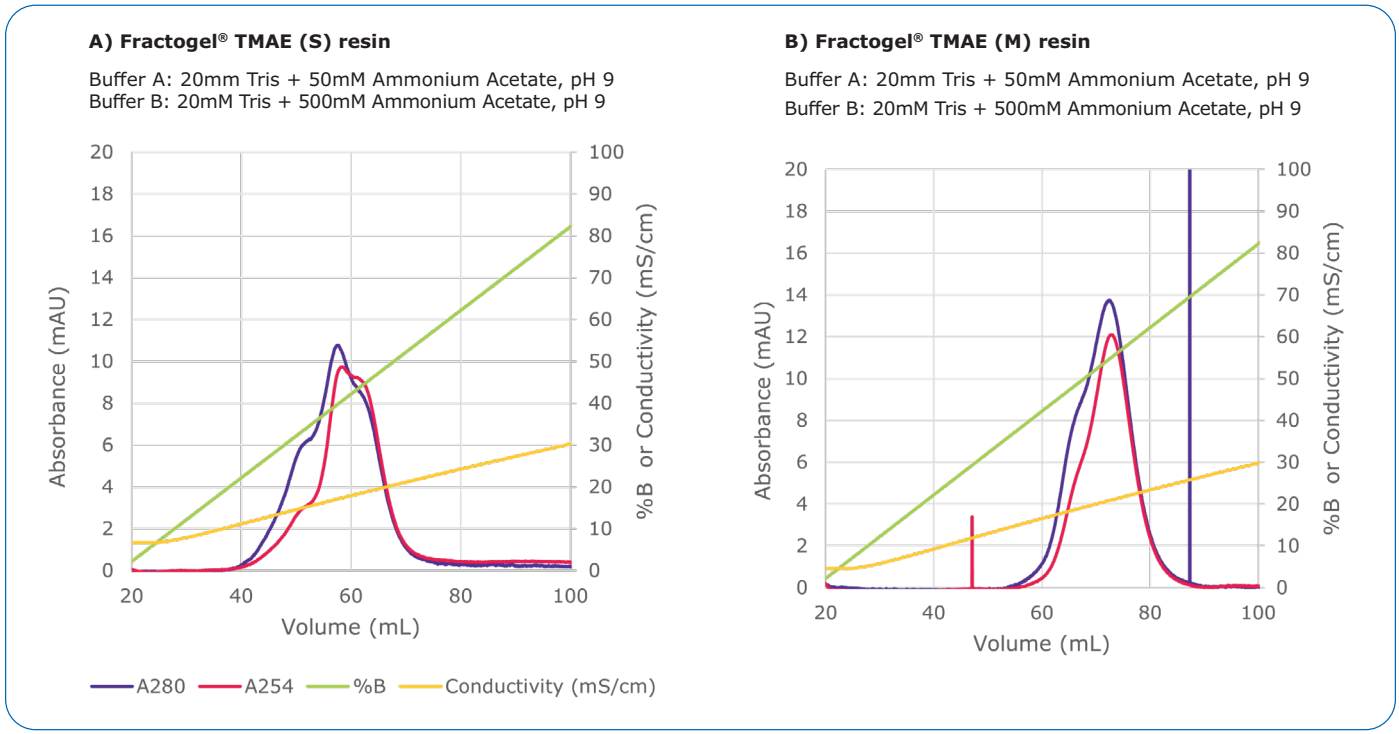


Figure 1. Linear conductivity gradient elution of AAV capsids at 1E+13 VP/mL loading.

As shown in **Figure 1**, the two Fractogel® TMAE resins resulted in different elution profiles. While both resins feature a strong anion exchange chemistry, with comparable ligand density, porosity, and base matrix features, a key difference is bead size. For the smaller diameter Fractogel® TMAE (S) resin (24-40 µm bead diameter), the right-hand side of the elution peak exhibited a brief period in which A254 signal exceeded the A280 signal, suggesting enriched full capsids **(Figure 1A)**. In contrast, the larger diameter Fractogel® TMAE (M) resin (48-60 µm bead diameter) did not show evidence of full capsid enrichment **(Figure 1B)**. Based on this linear gradient elution data, the smaller bead diameter Fractogel® TMAE (S) resin was selected as an optimal resin for further evaluation.

Next, a hybrid elution gradient strategy was explored to improve purity and yield for this challenging separation. Based on the **Figure 1A** absorbance chromatogram, empty capsids were observed to elute prior to full capsids. Therefore, the hybrid purification strategy was designed to selectively remove empty capsids in a gradient conductivity wash up to a critical conductivity level, referred to as the "critical %B buffer". If this gradient wash conductivity is too high, the user risks early elution of full AAV capsids; if it is too low, the user will not completely remove empty capsid impurities from the product stream. Thus, proper identification of the critical %B buffer is paramount to success. After the empty capsids are removed via gradient wash, the full capsid product can be recovered in a simple step elution.

Using this approach, five hybrid elution experiments were performed at critical %B values ranging from 28% B to 36.3% B. These values were selected as they represented regions along the left-hand side of the linear gradient elution peak where enriched empty capsids are expected **(Figure 2A)**. Following the equilibration, load (1E+13 VP/mL resin), and wash steps, a 5 CV empty capsid wash gradient was followed by a 5 CV hold at the critical % B buffer **(Figure 2B)**. Full capsids were then eluted by step elution, and the resin was cleaned and sanitized.

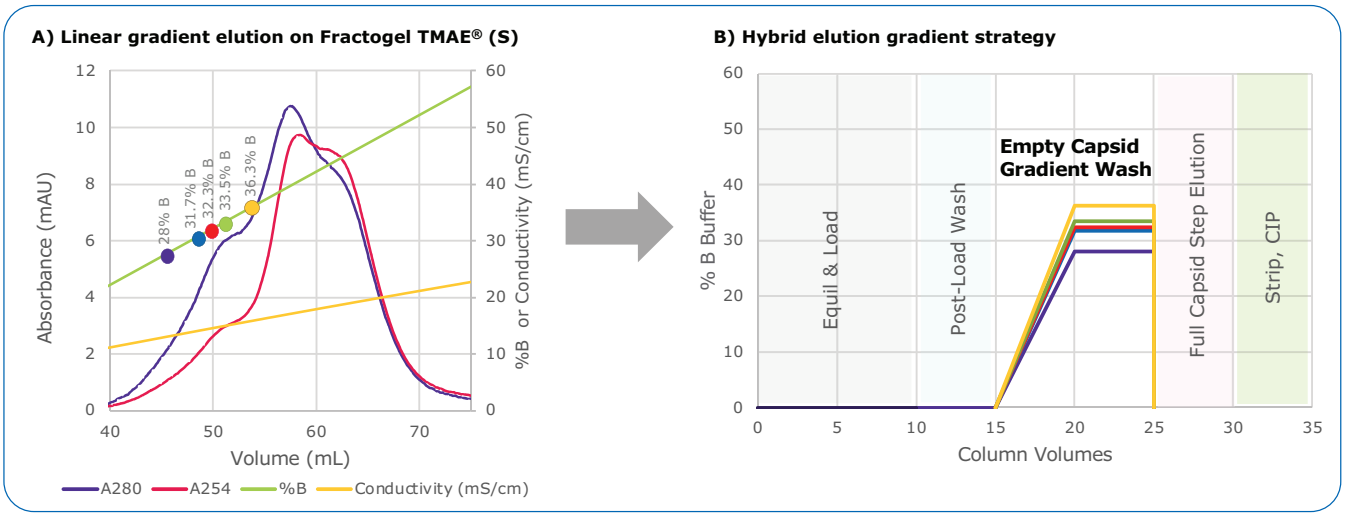


Figure 2. Development of a hybrid gradient elution strategy. A range of buffer concentrations which result in empty capsid elution (A) are explored in a hybrid gradient strategy (B).

Figure 3 shows elution peak profiles for the full capsid step elution after empty capsid wash at each of the five critical % B values. The highest UV signal (nearly 200 mAU) was observed after gradient wash to 28% Buffer B. Note that the A254 signal exceeded A280 for this condition, suggesting enriched full capsids in the elution peak. Empty capsid wash at %B values > 30% exhibited reduced UV signal magnitude, suggesting low recovery. Further experiments evaluating wash buffers < 28% B did not show proper enrichment of full capsids based on UV ratio (data not shown). Therefore, for this AAV8 feed, a hybrid gradient elution utilizing empty capsid wash to 28% B appears to be optimal. Surprisingly, the gradient wash was more effective than a simple step wash to 28% B, which removed too many full capsids and lowered elution yield (data not shown).

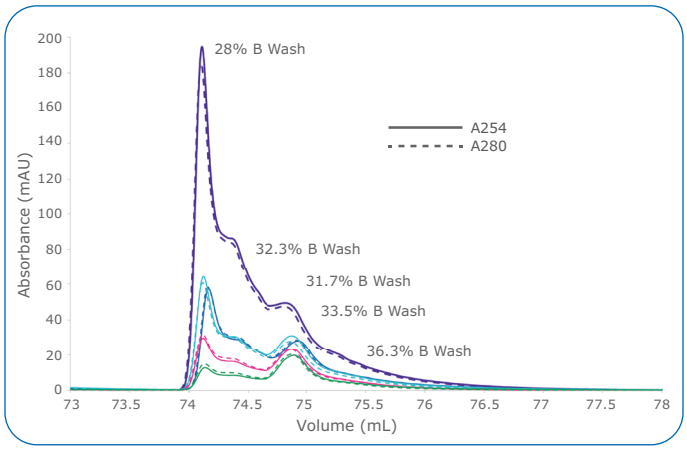


Figure 3. A comparison of full capsid step elution peaks following gradient wash at 5 different Buffer B concentrations

Figure 4 shows the full chromatogram for the 28% B wash condition. The A280 signal exceeded A254 signal during the gradient wash, indicating removal of empty capsid impurities. The full capsid product was then eluted by step change in conductivity.

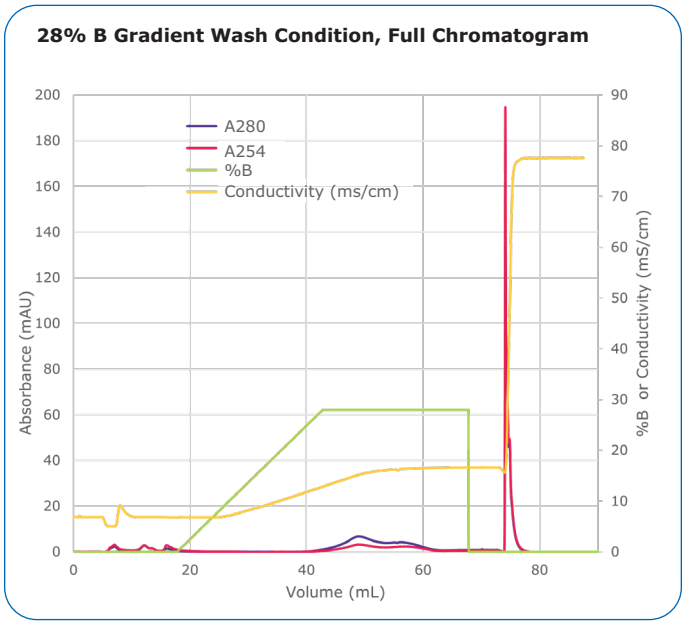


Figure 4. Full chromatogram showing hybrid gradient elution using 28% B empty capsid wash and Fractogel® TMAE (S) resin.

The step elution product peak from **Figure 4** was collected and analyzed for full capsid content by SEC-MALS, ddPCR, and ELISA offline assays **(Table 2)**. The ddPCR/ELISA assays reported 42% full capsids in the eluate, while the SEC-MALS approach resulted in enrichment to 54% full capsids, representing an enrichment factor of 2.8x and 2.7x, respectively.

	ddPCR/ELISA assay	SEC-MALS assay	
	Full Capsid % Purity	Full Capsid % Purity	Full Capsid % Yield
AAV8 Feed	15%	20%	N/A
Eluate after 28% B Gradient Wash	42%	54%	42%

Table 2. Offline analytical results for the elution pool following a hybrid gradient wash to 28% B.

The nearly 3-fold increase in full capsid content was achieved with 42% product yield. Optimization experiments to further improve yield may include exploration of elution salt type (for example, using sodium acetate instead of ammonium acetate), longer residence time, or taller column bed height.

Case Study 2: UV Ratio Detection using the Mobius® FlexReady XMO Chromatography System

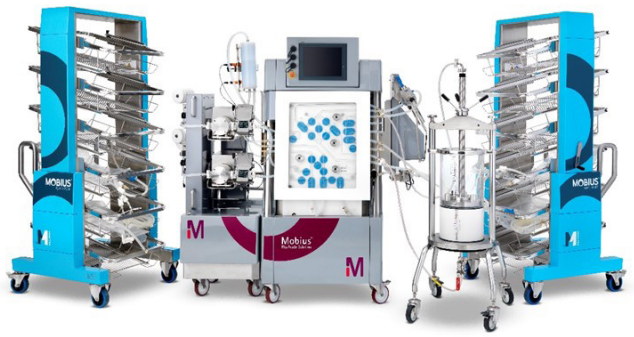
The data in Case Study 1 demonstrates the use of UV absorbance ratios for AAV polishing chromatography. Monitoring the ratio of two UV wavelengths in real-time allows for easy identification of product and impurity elution windows. However, while UV wavelength detection is universal in bioprocess chromatography systems, the practice of employing automated UV ratio calculation for improved process control is not widespread. The Mobius® FlexReady XMO Chromatography system is uniquely capable of performing online UV ratio detection in real-time, allowing end users to incorporate this parameter into their process control scheme. This feature is demonstrated in the following case study.

Materials

- Mobius® FlexReady XMO Single-Use Chromatography System, equipped with dual wavelength UV ratio capabilities at 254 nm and 280 nm.
- 0.16 g/L Caffeine (MilliporeSigma CAT# C0750) for monitoring at 280 nm wavelength
- 0.11 g/L Uracil (MilliporeSigma CAT# U0750) for monitoring at 254 nm wavelength

Discussion of Methods and Results

The feasibility of using real-time UV ratio detection for automatic product collection was evaluated on the Mobius® FlexReady XMO chromatography system. Model solutions of caffeine and uracil were formulated to simulate UV absorption at 254 nm (uracil, representing full AAV capsids) and 280 nm (caffeine, representing empty AAV capsids). Caffeine was connected to the system's primary pump, and uracil was connected to the system's secondary pump. A gradient was then applied to modulate UV absorbance within a mock process run. During this run, the system's Common Control Platform (CCP) software was used to monitor the UV ratio in real-time and to



automate product collection. Note that in Case Study 2, the UV ratio was defined as A280/A254, and therefore full capsid product is indicated by a UV ratio < 1.

Chromatogram trends for this feasibility trial are shown in **Figure 5**. The trial began with 90% caffeine flowing through the UV detector, resulting in a dominant A280 signal (represented by indicator AI014) ; the A280/A254 UV ratio (black trendline in **Figure 5**) was near 1.4 during this time. After a few minutes, a gradient was applied to gradually increase the flow of uracil to the UV detector. This corresponded with a linear increase in A254 signal (represented by indicator AI010), as well as a decrease in the A280/A254 UV ratio. At approximately the 15:11 timepoint on the X-axis, the UV ratio reached a value of 0.99, indicating A254 signal was greater than the A280 signal. This UV ratio value also prompted the system's CCP programming to engage collection of effluent material through a dedicated outlet valve, as noted by a vertical dashed line on the chromatogram. Fraction collection of the "product" pool continued if the A280/A254 ratio was < 1. After the gradient reached 90% uracil (UV ratio near 0.4), caffeine was gradually re-introduced back into the system. At approximately 15:30, the UV ratio reached 1.01, indicating A280 signal was greater than A254. At this time the system's CCP programming directed effluent material back to waste, ensuring separate collection of the A254 "product" pool.

While this experiment was conducted with model feed solutions, it demonstrates the feasibility of using the Mobius® FlexReady XMO system for automated product collection with real-time detection of 254 nm and 280 nm UV ratios. This strategy can be applied to viral vector manufacturing for improved process control, eliminating the need to collect many fractions during a purification run for subsequent pooling.

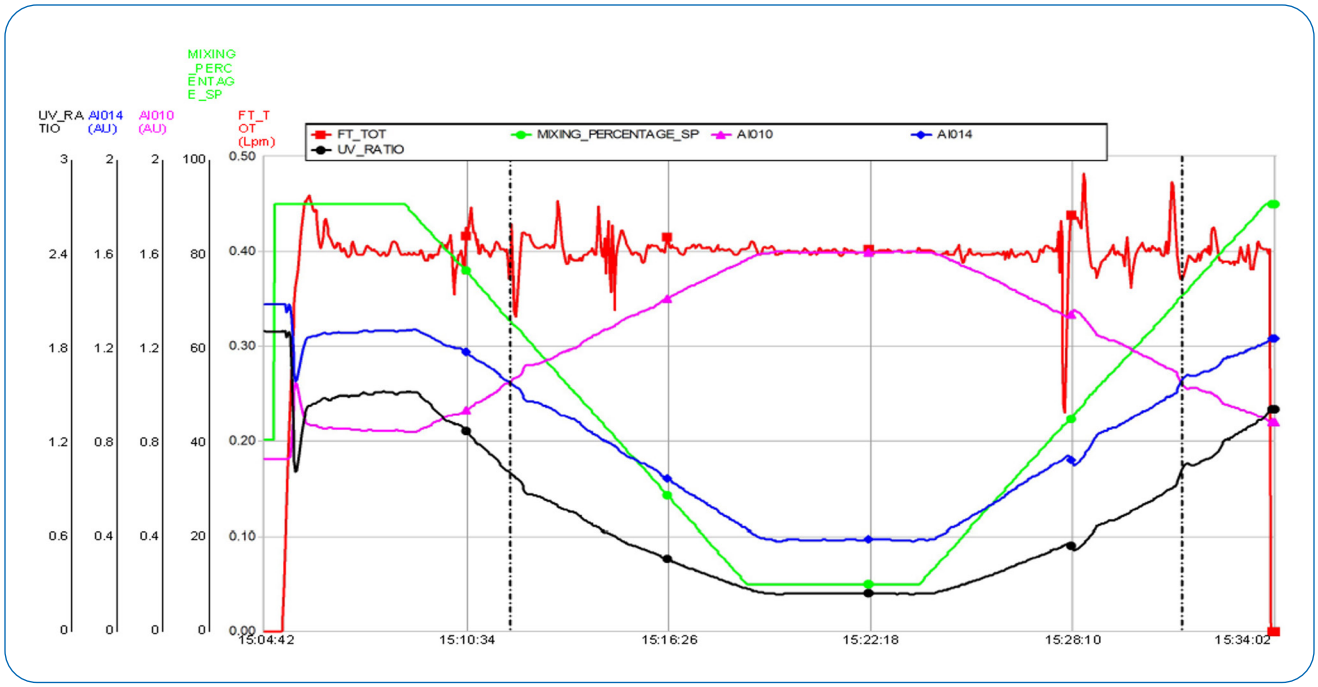


Figure 5. Chromatogram trends showing feasibility of real-time UV ratio detection for improved process control the Mobius® FlexReady XMO Chromatography system. Signal AI010 = 254nm absorbance; AI014 = 280nm absorbance; and UV_RATIO = 280nm / 254 nm"

Conclusions

Due to their similar surface properties, separation of empty and full AAV capsids is especially challenging. Effective purification requires exploration of alternative approaches which differ from traditional purification methods. Here we have demonstrated feasibility of AAV capsid purification using Fractogel® TMAE (S) resin, a tentacular anion exchange resin featuring a relatively small base bead diameter. Optimal results were achieved when applying a unique hybrid gradient elution strategy to this resin, resulting in nearly 3-fold increase in full AAV capsid content. We have also demonstrated a method of improved process control by utilizing real-time UV ratio detection capabilities on the Mobius® FlexReady XMO single-use chromatography system. As the AAV industry continues to grow, tools such as these can be implemented for improved purification of full and empty capsids.

Fractogel® resin may not be used in commercial manufacturing processes for the separation of empty and full AAV capsids, such AAV capsids are intended for human therapeutic use and which use has received regulatory authority approval for commercial sale, without prior consent by the owner of EP 2 277 996 (Genzyme Corporation, Cambridge, MA) and/or US 8,137,948 (Genzyme Corporation, Framingham, MA) and/or US 9,528,126 (Genzyme Corporation, Cambridge, MA). Purchase constitutes an agreement by the purchaser that the product may not be used in commercial manufacturing processes for the separation of empty and full AAV capsids, such AAV capsids are intended for human therapeutic use and which use has received regulatory authority approval for commercial sale.

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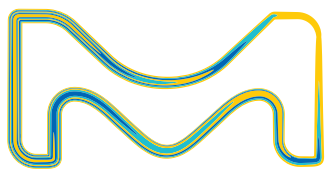

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