

ABSTRACT

Recombinant adeno-associated virus (rAAV) is a popular choice as a gene therapy delivery vector due to its sustained transient expression levels, safety and low immunogenicity. It is used in the treatment of numerous genetic disorders with more than 225 conducted clinical trials and 6 FDA approved gene therapy products.¹

Forge Biologics utilizes cesium chloride (CsCl) density gradient ultracentrifugation as a platform process step to enrich full AAV vector capsids. CsCl density gradient ultracentrifugation is a gold-standard for the separation of the full/empty capsids. Although the process is commonly used as an industry standard, scale-up, processing times, consistency and reproducibility are some critical challenges associated with the use of this technology. Key bottlenecks include manual filling, precise tube balancing, longer spin times, and inconsistent manual tube sealing that can impact process consistency and reproducibility.

To address these challenges, in collaboration with the Beckman Coulter Life Sciences we evaluated the use of a pre-formed continuous CsCl gradient using the OptiMATE Gradient Maker (Beckman Coulter Life Sciences) and compared it to our established CsCl isopycnic density gradient process with respect to processing time, recovery and product quality. The use of a pre-formed gradient significantly shortens the process time while maintaining resolution and improving process throughput. The separated full capsids were recovered using the OptiXTRACT sample recovery tool (Beckman Coulter Life Sciences) and tested for recovery efficiency and purity. We found that the recovery efficiency of full AAV capsids was similar >75% in both cases. The purity of the full capsids was approximately 88% in both cases as analyzed by analytical ultracentrifugation.

Given the high production costs for AAV, it is important to minimize process times and reduce operator-dependent variability. Overall, the results indicate that the automated gradient maker performs comparably to our established CsCl platform in both recovery and product quality, while offering significant time savings and reproducibility for large-scale operations.

AAV DOWNSTREAM PURIFICATION PROCESS FLOW

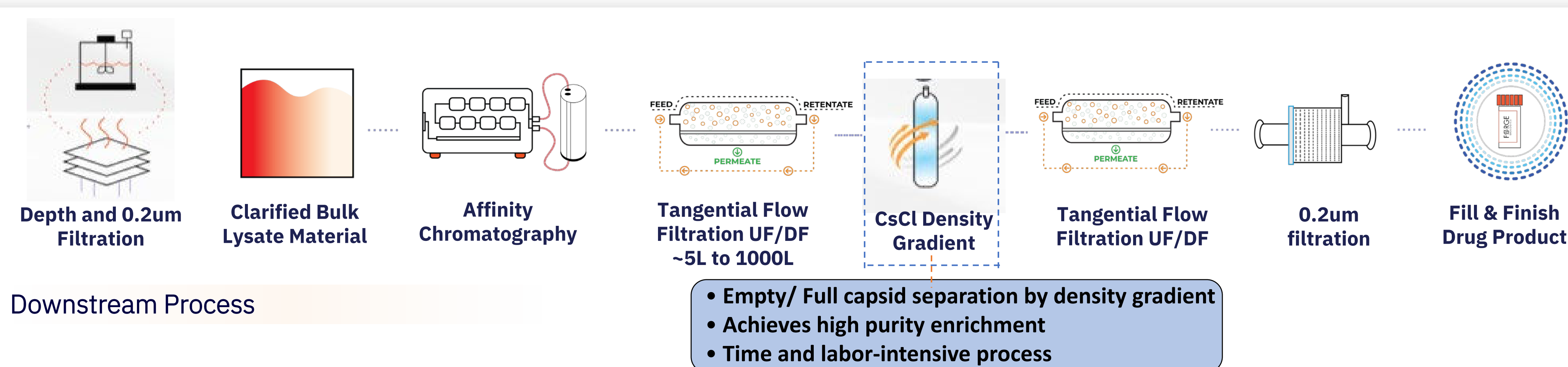


Figure 1. AAV Purification Workflow. The process includes clarification, affinity capture, concentration and buffer exchange using TFF, CsCl density gradient empty/full capsid separation, final formulation TFF, final filtration, and fill-finish to produce the Drug Product.

CURRENT CsCl PROCESS CHALLENGES

- Manual Filling:** Trapped air bubbles during manual filling can deform ultracentrifugation tubes and disturb CsCl gradients, reducing separation quality.
- Balancing Tubes:** Precise balancing of tubes is required to maintain integrity during high-speed centrifugation which is tedious, time-consuming, and prone to operator error process.
- Long Spin Times:** Self gradient ultracentrifugation runs often exceed 16 hours, extending the total process times to ~2-3 days which significantly limits manufacturing throughput.
- Variability during Sealing:** Manual tube sealing can lead to improper closures, causing leaks, material loss, compromising tube integrity, and increasing process failure risk.
- Operator-to-Operator Variability:** Multiple manual steps introduce operator-to-operator variability, making standardization difficult and reducing process reproducibility in manufacturing.

OptiMATE Gradient Maker FEATURES

- Automated Gradient Formation & Pre-Formed Linear Gradients:** Enables precise, reproducible stepwise and linear gradient generation while eliminating manual variability. Pre-formed gradients significantly shorten ultracentrifugation spin times, improving throughput.
- Automated Tube Sealing & Reduced Operator Burden:** Standardizes tube sealing to minimize leakage and ensure integrity, while reducing manual steps such as filling and balancing. This lowers operator workload and variability across runs.
- Reagent Flexibility:** Compatible with multiple separation media including Cesium Chloride (CsCl), Iodixanol (IDX), and sucrose.
- Ease of Tech Transfer:** Backed by published methods, enabling smooth transition of processes from development to GMP-scale manufacturing.
- Enhanced Process Robustness:** Combining automation, reduced spin times, and standardized handling strengthens scalability, safety, and reproducibility of AAV full/empty capsid separations.

OptiMATE SYSTEM AND TRADITIONAL PROCESS SETUP

- OptiMATE System Process:** User defined gradient parameters, such as upper and lower limit range for density, gradient type (linear or stepwise), and total tube volume. The OptiMATE system is programmed to automatically fill the tubes and seal them.
- Traditional CsCl Process (Forge Platform):** Includes Manual mixing of CsCl with AAV to target a specific refractive index and manual tube fill and sealing.

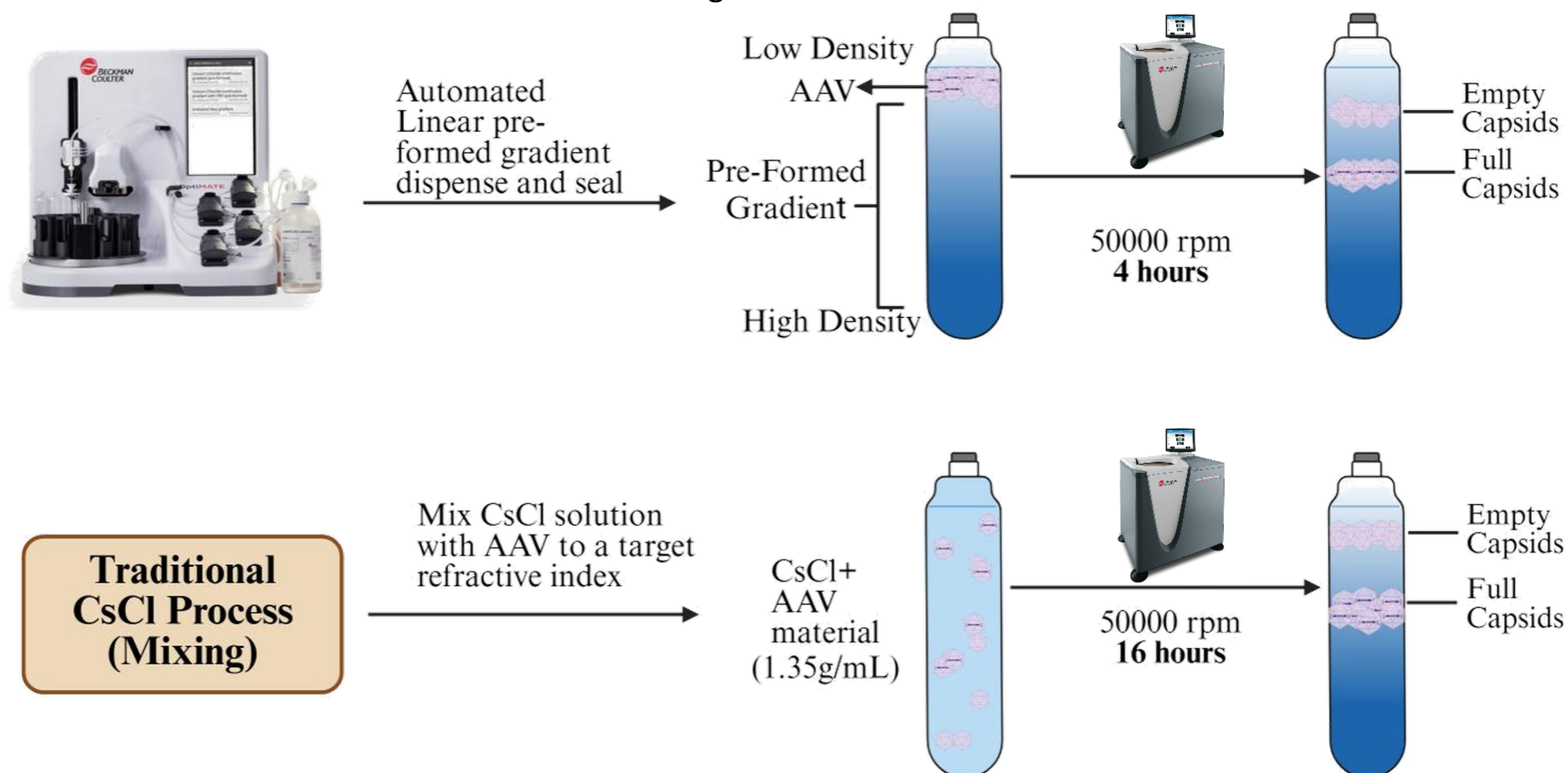


Figure 2. Comparison of CsCl Gradient Approaches. Pre-formed gradient (OptiMATE system) enables faster separation of full and empty capsids compared to self-formed gradient (Traditional), reducing spin time while maintaining resolution.

Parameters	Experiment 1 OptiMATE System Process	Experiment 2 Forge Platform Process (Traditional)
RPM	50,000	50,000
Temperature	17 °C	17 °C
Tubes	2x39.2 mL	2x39.2 mL
Refractive Index	1.3710-1.3730	1.3709
Neutralized Eluate (mL)	40.8 mL (20.4 mL each tube)	27 mL
Spin Time	4 h	≥16 h

Table 1. CsCl Run Parameters. Comparison between pre-formed and self-formed gradient process parameters



Figure 3. OptiMATE instrument setup. Ready to start CsCl tube filling and sealing



Figure 4. Tubes post-sealing using the OptiMATE system. Produced uniform and reliable tube seal

RESULTS

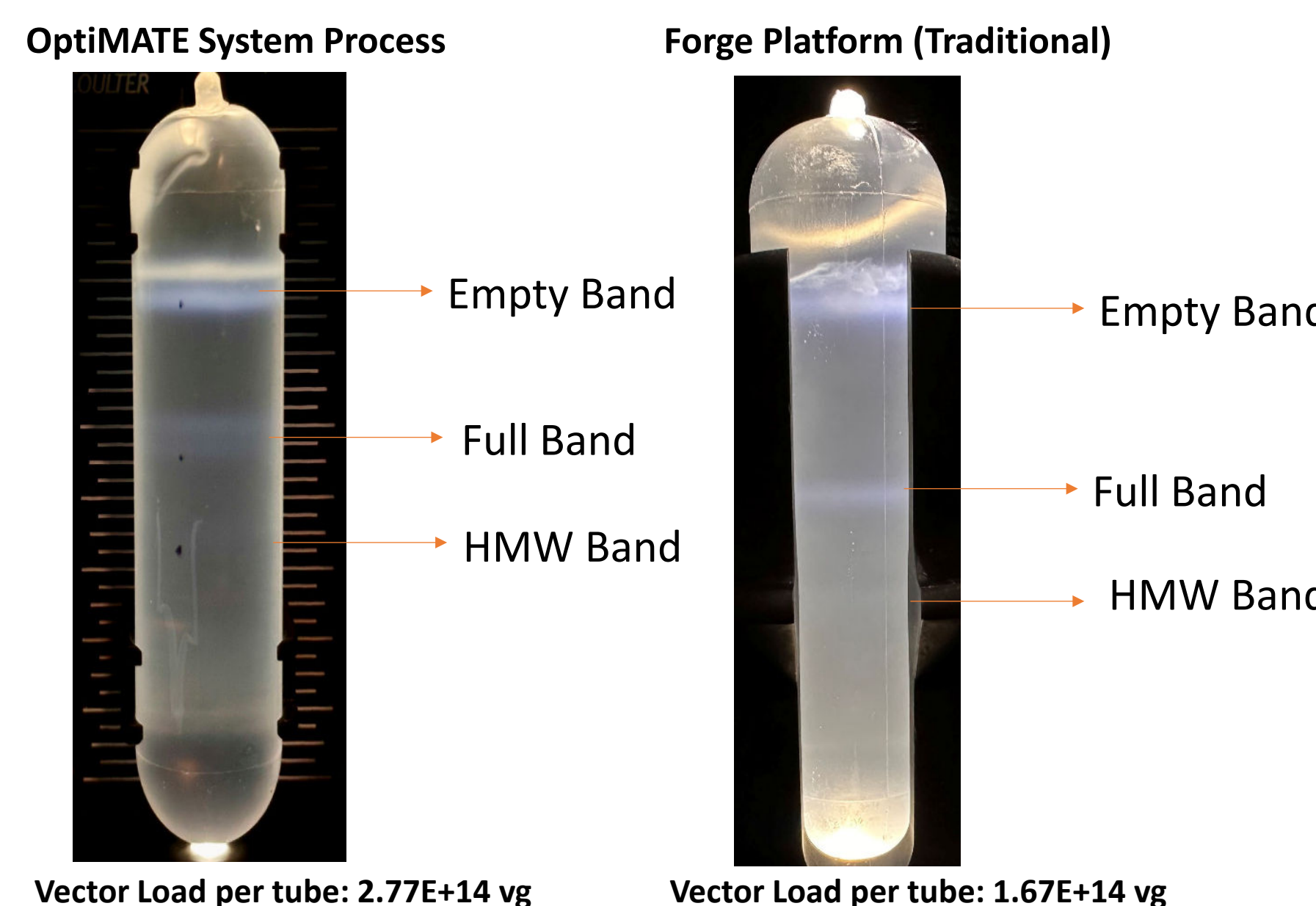


Figure 5. CsCl Tube post ultracentrifugation spin. Three separate bands (Empty, Full and High Molecular Weight) were observed post-spin.

Condition	Sample	ddPCR Titer (vg/mL)	Yield (vg)	Step Recovery (%)
OptiMATE System Process	CsCl Load	1.36E+13	5.54E+14	N/A
OptiMATE System Process	CsCl Full Band	3.23E+13	4.16E+14	75.09
Forge Platform (Traditional)	CsCl Load	4.44E+12	3.33E+14	N/A
Forge Platform (Traditional)	CsCl Full Band	1.93E+13	2.41E+14	72.37

Table 2. Table summarizes ddPCR titers, yield, and step recoveries between the OptiMATE System and Forge Platform processes (Traditional).

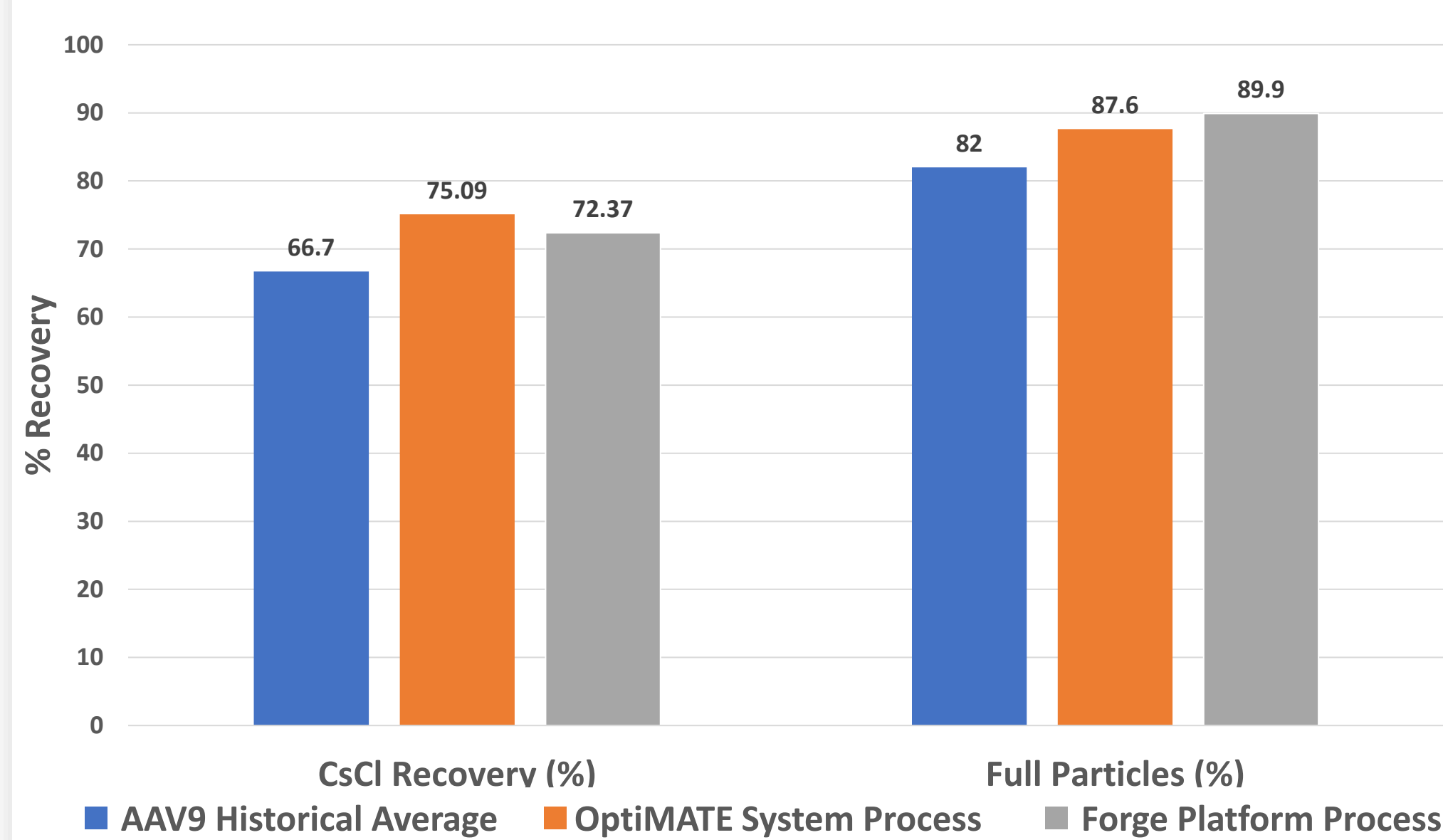


Figure 6. Comparison of %Full band recovery and % Full Particles by AUC. CsCl recovery was comparable between OptiMATE System (75.09%) and Forge Platform (72.37%), with both being above historical averages (66.7%). % Full particles were also comparable (OptiMATE System 87.6%, Forge Platform 89.9%) relative to historical averages (82%).

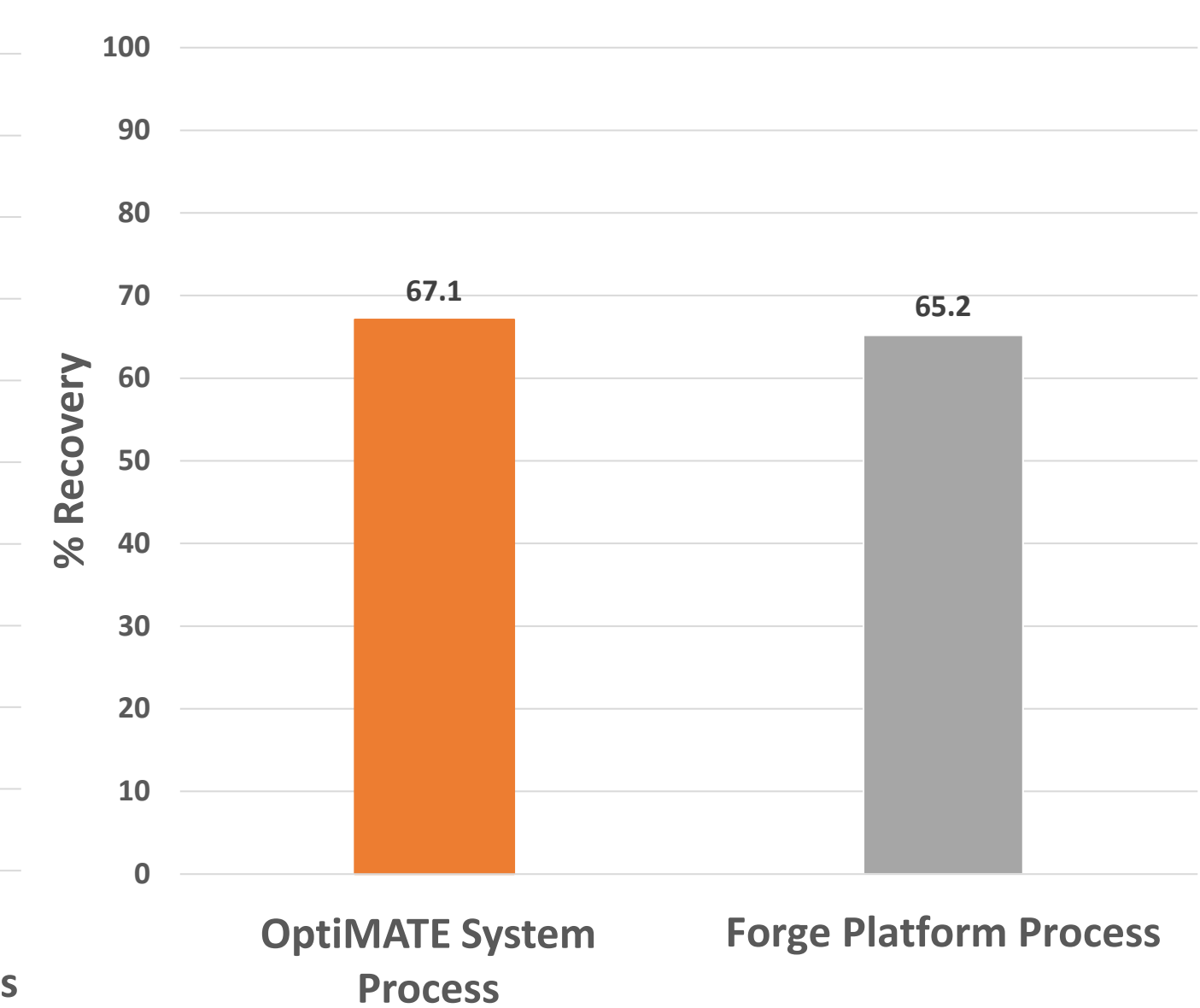


Figure 7. Recovery from CsCl Load to Final Formulation. Recovery was 67.1% with OptiMATE System and 65.2% with Forge Platform, demonstrating comparable performance between the two processes.

OptiXTRACT Sample Recovery Aid

Eliminate Safety Concerns

Simple slide rail ensures tubes can be punctured without the need to hold the tube

Reduce Error & Improve Consistency

Graduations and a dimmable light allow for accurate, reproducible piercing and band extraction

Enhanced Flexibility

Supports multiple Luer-Lok™ syringes with customizable positions and is compatible with various tube sizes

Suitable for sterile operation

Small equipment footprint that fits easily inside a biosafety cabinet; sterile syringe adapters, perfect for single use



Key Takeaways

- The results from the gradient maker testing show **similar recoveries and quality attributes** to the Traditional Forge platform CsCl process.
- Shorter processing times**, makes it possible to perform more spins per day.
- Preconfigured methods ensure **consistent and reproducible** results across different lots.
- Provides **uniform and reliable sealing** across tubes, removing operator-dependent variation.
- Eliminates bubble formation while filling the tubes. **Removes the need for balancing** the tubes.
- The OptiXTRACT** extraction tool offers excellent stability for band collection, consistent pull based on distance and removes operator variability.

Conclusion

Overall, the results indicate that the outcomes using the automated gradient maker are comparable to the Forge platform process, both in terms of recovery and product quality, while offering significant time savings and reproducibility for large-scale operations.