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Rapid, higher-scale purification of AAV with pre-formed gradients using the OptiMATE Gradient Maker

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High-resolution purification in 5 hours

Empty & full capsids

Adeno-associated viruses (AAVs) are powerful delivery vectors for gene therapies.[1] It is critical to separate fully loaded AAV capsids from empty capsids prior to gene delivery applications. Cesium chloride (CsCl) density gradient ultracentrifugation (DGUC) remains the gold standard[2,3] for this separation and can routinely provide > 90% full AAV particles. We have previously demonstrated that effective separation by CsCl DGUC, which has a long ultracentrifugation run time of ≥ 16 hours,[4] can be achieved in 5 hours using pre-formed density gradients generated by the OptiMATE Gradient Maker. This significantly reduces overall process time from 2-3 days to just 1 day.

While this was demonstrated for 39 mL Quick-Seal tubes in a commonly used Type 70 Ti rotor (capacity: 312 mL), a key factor to test is if this reduction in run time using pre-formed density gradients remains applicable when scaling up to higher-volume capacity rotors. In this application note, we tested the ability of OptiMATE Gradient Maker-generated pre-formed gradients to enable DGUC separation of AAV full capsids from empties in a similar 5-hour ultracentrifugation run time for Quick-Seal tubes in Type 50.2 Ti (capacity: 468 mL) and Type 45 Ti (capacity: 564 mL) rotors.

Methods

Gradient Profiling

We first compared density gradient profiles between tubes created by the OptiMATE Gradient Maker and tubes created manually (Figure 1). For this experiment, 39 and 94 mL Quick-Seal Round-Top Ultra-Clear tubes were used for the Type 50.2 Ti and Type 45 Ti rotors, respectively.

The parameters used for density gradient profile generation are shown in Table 1. Methods were created on the OptiMATE instrument to dispense linear density gradients between pre-defined upper- and lower-limit densities (with average tube density of 1.35 g/mL) using OptiMATE Cesium Chloride Solution and nanopure water as the diluent. These upper and lower limits were defined using the protocol described in a previous study.[4] In parallel, tubes were manually filled with a homogeneous mixture of OptiMATE CsCl Solution and water to a starting density of 1.35 g/mL and sealed using a Cordless Tube Topper. The tubes were centrifuged in an Optima XPN-90 ultracentrifuge. The OptiMATE Gradient Maker-dispensed pre-formed linear density gradient tubes (hereafter referred to as pre-formed gradient tubes) were centrifuged for 4 hours, while the homogeneous 1.35 g/mL density CsCl tubes (hereafter referred to as self-forming gradient tubes) were centrifuged for 20 hours to allow the density gradient to self-form. After centrifugation, tube contents were fractionated and recovered via bottom puncture, and fractions were analyzed using a refractometer to assess density gradient profiles.

Parameter	39 mL tubes	94 mL tubes
Total volume (mL)	39	94
Type tube	Quick-Seal® Round-Top Ultra-Clear	
Rotor	Type 50.2 Ti	Type 45 Ti
Ultracentrifugation speed (rpm)	50,000	43,000
Ultracentrifugation speed (x g)	301,311	214,382
Average density (g/mL)	1.35	
Upper limit density of pre-formed gradient (g /mL)	1.15	1.24
Lower limit density of pre-formed gradient (g /mL)	1.55	1.46
Approximate fraction sizes collected (mL)	1. 7	

Table 1: Parameters for CsCl density gradient ultracentrifugation for self-forming (manual) and pre-formed gradients (OptiMATE Gradient Maker).

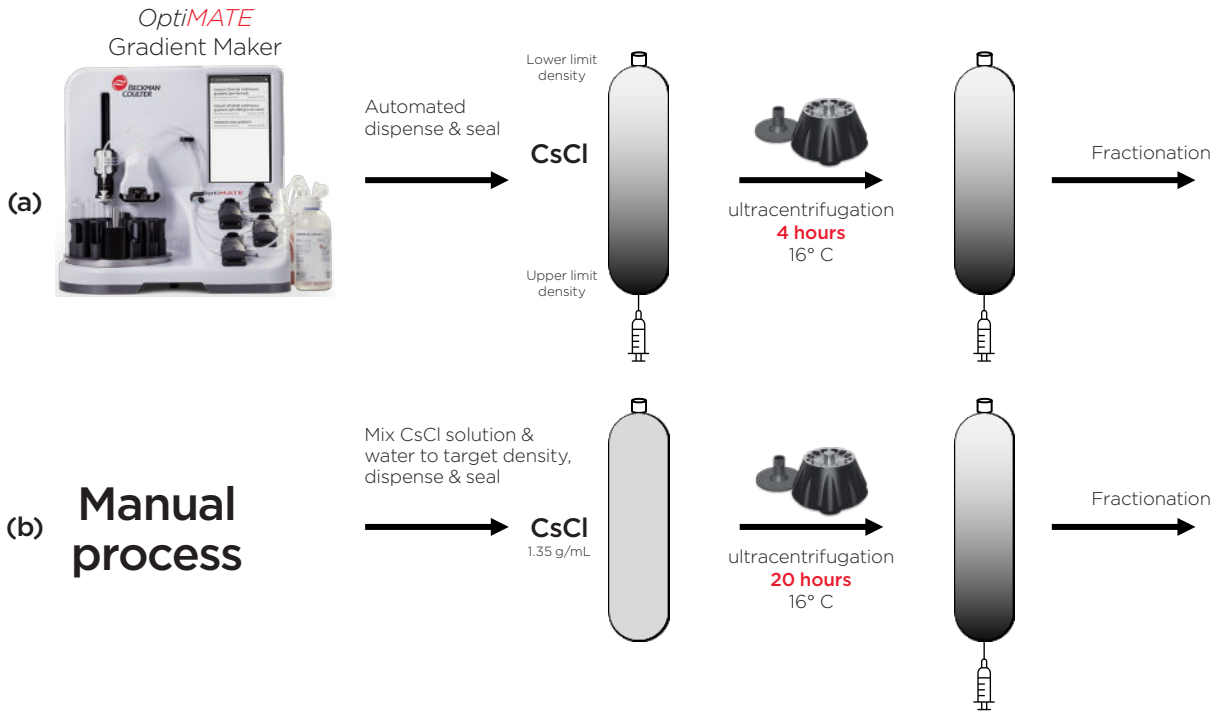


Figure 1: Scheme for density gradient control experiment comparing (a) automated dispense of a pre-formed CsCl gradient and (b) manual dispense of a self-forming CsCl gradient. Ultracentrifugation speeds and lower and upper density limits are as defined in Table 1. Syringes represent bottom fractionation.

AAV Purification

AAV serotype 9 (AAV9) was expressed using triple transfection in HEK-293 cells. Following expression, cells were lysed and centrifuged, and the crude AAV was subjected to affinity chromatography for initial enrichment of all capsid species. This semi-purified material was used as the AAV sample for the experiment.

For both self-forming and pre-formed CsCl density gradients, OptiMATE Cesium Chloride reagent was used, with the AAV sample as the diluent (Figure 2). 2×10^{13} viral genomes (vgs) of AAV were loaded per 39 mL tube and 4×10^{14} vgs of AAV were loaded per 94 mL tube. Care was taken to ensure that, despite differences in the preparation method for the pre-formed and self-forming density gradients, an equal amount of AAV was used per tube. The same process parameters used for the density gradient profile generation (Table 1) were used to prepare tubes for AAV purification. In this case, however, an ultracentrifugation run time of 5 hours was used for the pre-formed density gradient, as we expected that to yield sharper (less diffuse) bands. The entire process is detailed in Figure 2.

After centrifugation, the quality of the separation of AAV full-capsid bands vs empty capsid bands was imaged, as visual identification of separation drives effective extraction of the purified bands.

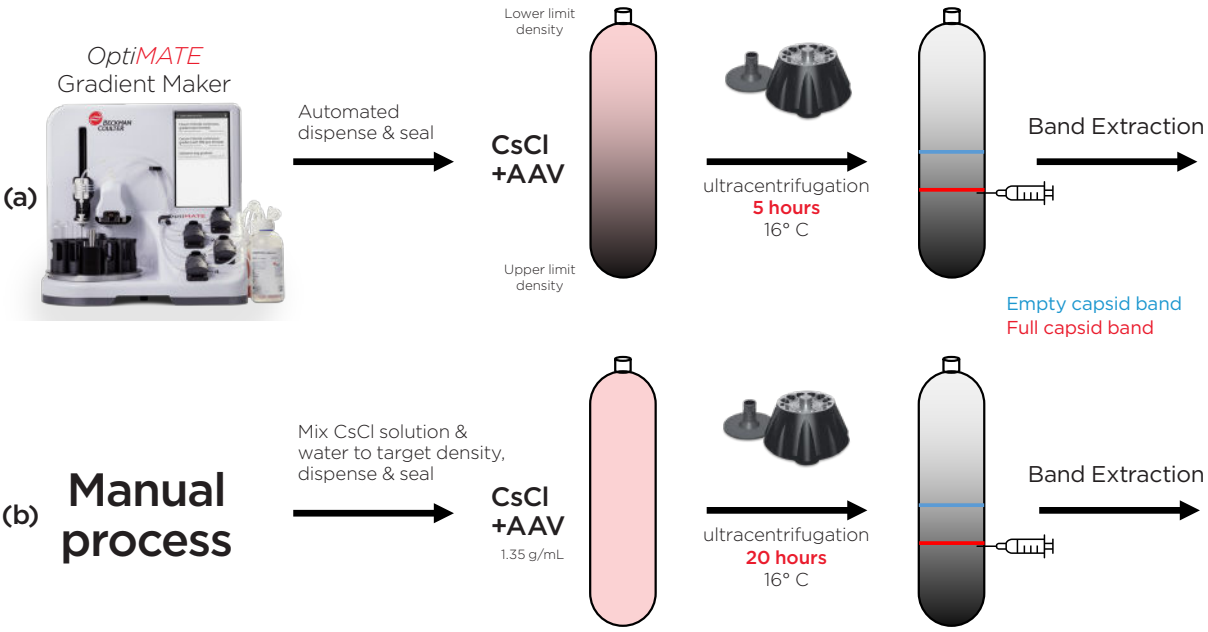
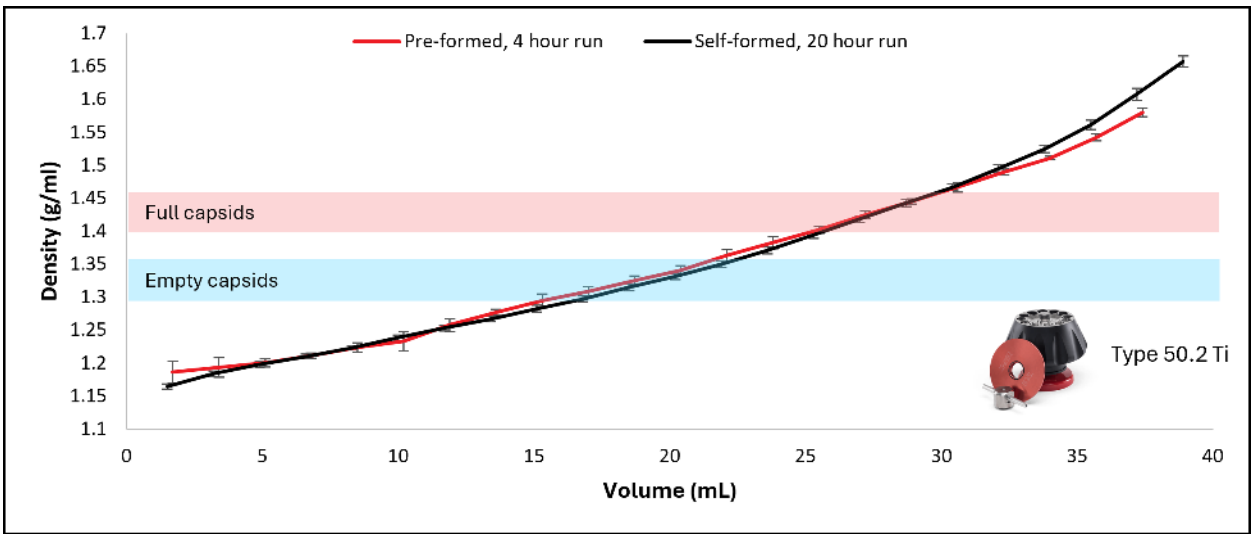


Figure 2: Scheme for AAV purification comparing (a) automated dispense of a pre-formed CsCl gradient and (b) manual dispense of a self-forming CsCl gradient. Ultracentrifugation speeds and lower and upper density limits are as defined in Table 1. The AAV sample is resolved into visible bands (empty capsids blue; full capsids red) at the end of the centrifugation run, which are recovered by puncturing the tube from the side with a syringe and extracting the band.

Results

Density profile

The density gradient profiles for the dispensed tubes after ultracentrifugation are shown in Figure 5. The profiles for the pre-formed gradients have a good overlap with the curves for the self-forming gradients after the ultracentrifugation run up to 1.4 g/mL density for both tube sizes (though there is more separation between curves overall for the 94 mL tubes). The pre-formed gradients would therefore be expected to adequately retain resolution in separating empty and full capsids despite having just 20% of the centrifugation time (4 hours vs 20 hours). There continues to be some difference in the gradient profile between the pre-formed and the self-forming gradient toward the bottom of the tube as observed with previous studies.[4] This may not be a concern for the purification of full and empty AAV capsids but can potentially be improved by centrifuging the tubes longer, if required. Additionally, in general, the less steep slope of the pre-formed gradient after the 4-hour run may allow pre-formed gradients to retain equivalent or better resolution than a self-forming gradient.



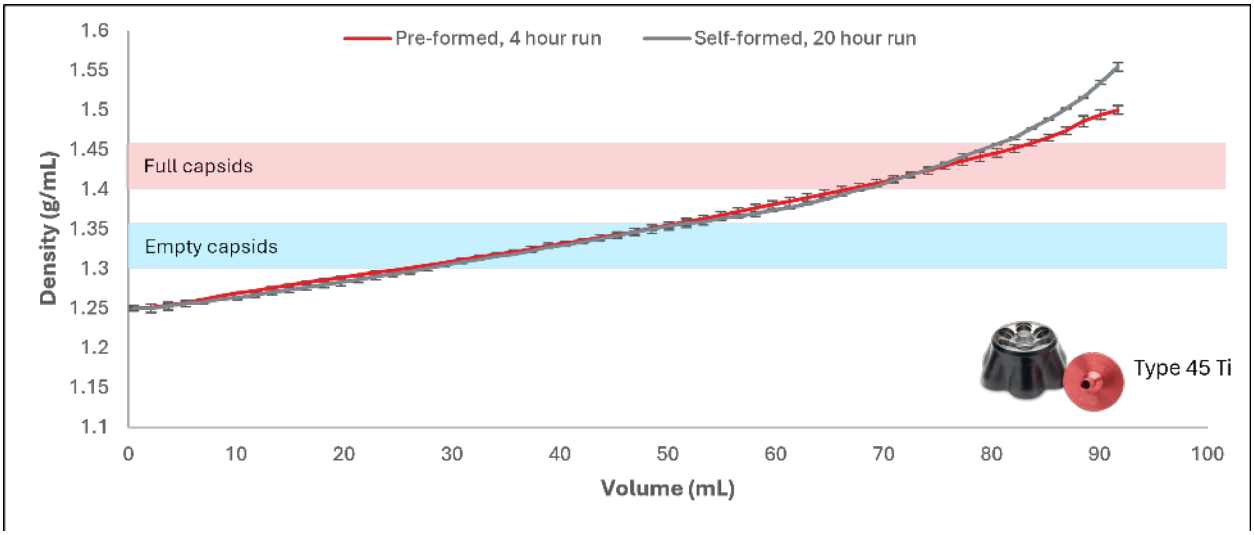


Figure 5: Comparison of gradient profiles from 39 mL Quick-Seal tubes from a Type 50.2 rotor ultracentrifugation (top) and 94 mL Quick-Seal tubes from a Type 45 Ti rotor ultracentrifugation (bottom). Both plots have curves for OptiMATE Gradient Maker-dispensed pre-formed CsCl density gradient centrifuged for 4 hours (red) versus a manually dispensed homogeneous CsCl density gradient that self-forms during a 20-hour centrifugation (black). Error bars are standard deviation. The blue and red bands represent the expected position range of the empty and full capsid bands.

AAV purification

The results of the purification of AAV samples by this established pre-formed and self-forming gradient profile are shown in figure 6.

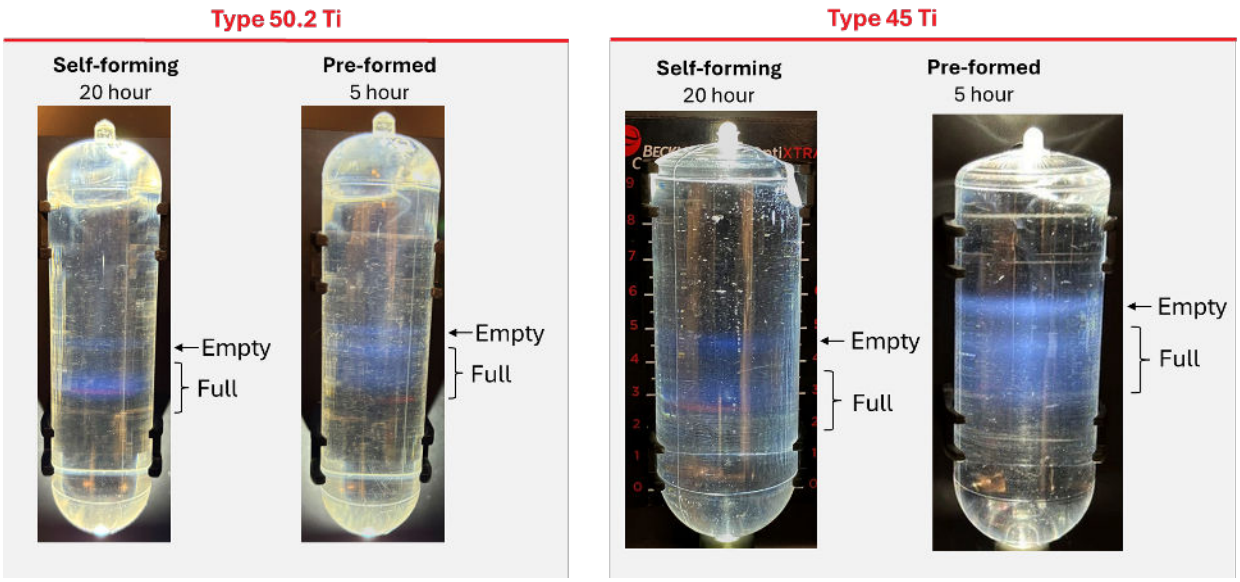


Figure 6: Representative images of AAV bands after ultracentrifugation from self-forming density gradients run for 20 hours and pre-formed density gradients run for 5 hours in 39 mL tubes (left) and 94 mL tubes (right).

There are differences in the band profiles between the self-forming density gradient tubes and the pre-formed density gradient tubes, particularly in the spread (i.e., sharpness) of the partially filled and full-capsid bands, as well as the separation between these bands and the empty capsid band. However, the degree of separation between the empty capsid band and the full- capsid bands is still adequate for visual observation and recovery by syringe puncture and extraction. The resolution of bands from these images is similar to our observations from previous studies,[4] where we used analytical ultracentrifugation to confirm that the purity of recovered full capsids is around 90% from the pre-formed gradients despite a significantly shorter ultracentrifugation run time of 5 hours. With more optimization of pre-formed density gradients, improved visual separation of empty, partially filled and full capsids can be achieved.

Conclusions

We have previously demonstrated effective ultracentrifugation run time reduction via use of pre-formed gradients dispensed by the OptiMATE Gradient Maker, which eliminates one of the key pain points in CsCl DGUC.[3] Extending this process to higher-capacity rotors is important to demonstrate the continued applicability of the process at higher scales of production. The Type 45 Ti rotor enables use of fewer tubes and therefore less time and effort in the extraction process, while the Type 50.2 Ti rotor enables more 39 mL tubes to be used in comparison to the Type 70 Ti rotor (12 versus 8), providing users with increased flexibility in planning their purification. Users are encouraged to conduct their own optimization studies (using our previously described process[4]), as we believe the time savings realized can be greater in some cases with different OptiMATE methods that are specific to their individual hardware and consumable configurations.

References

1. Wang, Jiang-Hui, et al. “Adeno-Associated Virus as a Delivery Vector for Gene Therapy of Human Diseases.” Signal Transduction and Targeted Therapy, vol. 9, no. 1, Apr. 2024, pp. 1–33

2. Nascimento, André, et al. “Purification of AAV8 through a Scalable Two-Step Monolithic Chromatography Approach.” Journal of Chromatography A, vol. 1740, Jan. 2025, p. 465586

3. Strobel, Benjamin, et al. “Comparative Analysis of Cesium Chloride- and Iodixanol-Based Purification of Recombinant Adeno-Associated Viral Vectors for Preclinical Applications.” Human Gene Therapy Methods, vol. 26, no. 4, Aug. 2015, pp. 147–57

4. Venkatakrishnan, Jasti and Sternisha. Application note: Rapid, Automated Purification of Adeno-Associated Virus using the OptiMATE Gradient Maker.

Item Description	Part number
Quick-Seal Round-Top Ultra-Clear Tube, 39 mL	344326
Quick-Seal Round-Top Ultra-Clear Tube, 100 mL	345778
OptiMATE Cesium Chloride Solution	D01357
Type 45 Ti rotor	339160
Type 50.2 Ti rotor	337901
Optima XPN-90 - IVD	A99842

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