Center for Breakthrough Medicines

Technical White Paper

Determining Ideal Plasmid Complexation Parameters to Optimize AAV Vector Manufacturing

Outline

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WHY DNA COMPLEXATION MATTERS

More than 50 gene therapies could be approved in the next several years, most of which rely on viral vectors for delivery of the active genetic material.[1] In addition, close to 60% of the 500-plus gene and gene-modified cell therapies in clinical trials in 2021 target more prevalent diseases. Furthermore, these treatments are generally administered systemically, require large doses, and have the potential to serve as first-line therapies. [2,3]

After several years of quiet commercial approvals, Q4 2022 saw a wave of viral mediated gene therapies approved including Bluebird (lentivirus), Uniqure/CSL (AAV), Biomarin (AAV), Ferring (Adenovirus). Viral-vector demand is expected to increase by 100- to 1000-fold. To ensure long-term success of gene therapies, therefore, manufacturers of viral vectors must achieve robust, readily scalable, cost-effective manufacturing processes.

Most direct gene therapies leverage adeno-associated viral (AAV) vectors as delivery vehicles, complex products whose functional performance is impacted by numerous biophysical-characteristics. While packaging and producer cell lines provide the most robust and scalable solutions for AAV vector manufacturing, their development remains challenging. Coinfection methods using the insect-based baculovirus expression vector system (BEVS) and human-derived herpes simplex virus (HSV) Type 1 systems are also readily scalable and result in high yields of fully functional vectors, but have extended development timelines, may present quality issues, and/or can be challenging to purify.

Transient transfection of three plasmids (rep, cap, and helper) in HEK293 cells facilitated by a transfection reagent is therefore the most widely used method for AAV vector production. It has, in fact, been shown effective for all AAV serotypes and is a regulatory-validated approach. The fact that transfection occurs over a fairly short time also allows for rapid development of optimum processes and thus shorter time to the clinic and the market.[4] Other platforms for AAV manufacturing are summarized in **Table 1**.

	Triple Transfection (adherent)	Triple Transfection (suspension)	Baculovirus-infected producer cell line	Herpes virus co-infection	Adenovirus-infected producer cell line
REP/CAP	Plasmid	Plasmid	Integrated in cell line	First rHSV	Integrated in cell line
ITR-transgene	Plasmid	Plasmid	BEV	Second rHSV	Integrated in cell line
Helper genes	Plasmid	Plasmid	BEV	rHSVs (above)	Wt adenovirus
Cell line	HEK293 (adherent)	HEK293 (suspension)	Sf9 insect cells	BHK (suspension)	HeLa S3 (suspension)
Production system	CellFactory, roller, CellCube	Wave reactor (tens of liters)	200 L stirred tank reactor	10L wave reactor	250L stirred tank reactor
Scalability	-	++	+++	+++	+++
Safety concerns	None	None	None	Contaminating Helper Virus	Contaminating Wild-type Helper Virus
Advantages	Quick to produce in small scale Helper virus-free AAV		Added safety of insect cells & virus	No stable cell line required Efficient large scale production	Same helper virus for all production runs Efficient large scale production
Challenges	Low scalability of triple transfection		Potentially low BEV stability	2 HSV helper viruses to produce Sensitive to production condictions	Stable producer cell line to produce for each project

Table 1. Current manufacturing platforms for AAV production

Transient transfection involves multiple steps **(Figure 2)**, including cell expansion and growth, formation of the plasmid DNA-transfection reagent complex and transfer of the complex into the bioreactor (at large scale). Viral particles are then generated once the DNA is transfected into the cells and delivered to the nuclei, where it is transcribed and translated to enable expression of the appropriate viral proteins.

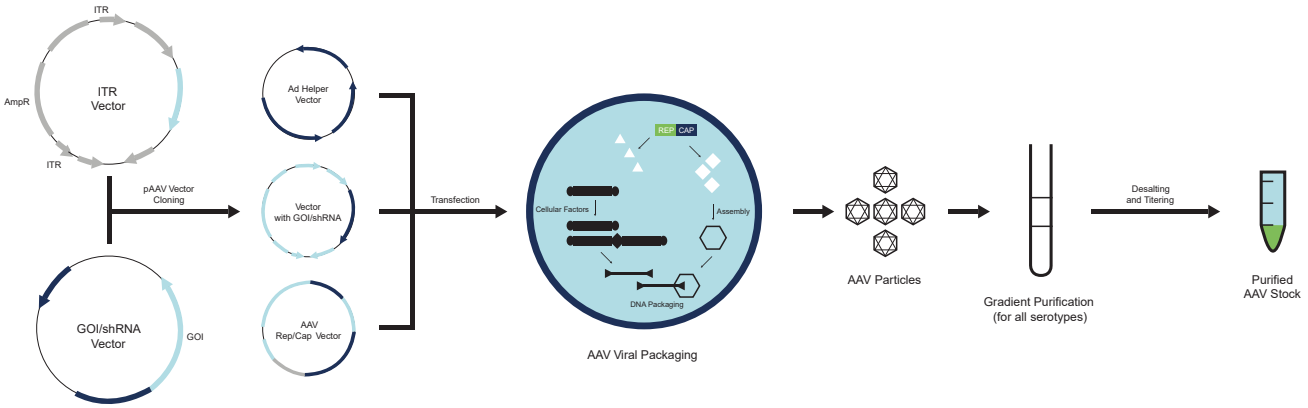


Figure 2. Schematics representation of the steps involved to generate recombinant AAV by the triple transfection method using plasmid DNA

Transfection reagents are necessary for transient transfection because naked DNA is not readily transported across cell membranes. Initially, calcium phosphate was used. Next-generation solutions included lipid-based lipofectamine reagents and versions of the linear polymer polyethyleneimine (PEI). More advanced transfection reagents designed specifically for AAV and lentiviral (LV) vector production via transient transfection are now available that combine the properties of lipid- and polyamine-based compounds for improved performance. These reagents not only aid in transport of DNA across the cell membrane, but also delivery to the nucleus, and do so with reduced cytotoxicity. A comprehensive overview describing both viral and non-viral methods for nucleic acid transfection was recently reviewed by Fus-Kujawa et al.[5]

However, even for advanced transfection reagents, complexes formed with plasmid DNA tend to be unstable and shear-sensitive, limiting the stirring speed for mixing, the rate at which they can be transferred from the mixing tank to the bioreactor, and the time they remain available for transfection into HEK293 cells.

Optimization of the complexation processes is therefore essential to optimizing the efficiency and productivity of the overall transient transfection process.

BUILDING CRITICAL PROCESS PARAMETER KNOW-HOW

Traditionally, optimization of transient transfection processes has involved investigation of process parameters individually. More recently, the application of quality-by-design (QbD) approaches that leverage design of experiment (DoE) studies have become increasingly common given the complexity of the process.

A QbD strategy makes it possible to identify the critical process parameters that directly impact viral vector critical quality attributes.[6,7,8] Even DoE studies that explore only a few of the most important parameters simultaneously can lead to more robust, efficient, and productive transient transfection processes.[9,10]

Three main parameters can impact the complexation step:

1. the complexation reagent
2. the ratio of transfection reagent to plasmid DNA
3. complexation time

These three factors influence the size of the complexes that are generated, which has been linked transfection efficiency. Past literature has found that the ideal particle size for optimal transport across cell membranes is 400 – 500 nm.[11] Thus it can be assumed that transporting DNA/reagent complexes with larger macromolecular structures would be inefficient.

The transfection reagent to plasmid ratio will influence the kinetics of complexation and thus complexation efficiency. Using smaller quantities of plasmids and/or transfection reagent to achieve optimum transfection efficiency would provide a mechanism for reducing the cost of goods for the transfection process.

Complexation time, while not an issue at R&D scale where only small volumes are involved, can become significant when scaling a transient transfection reaction from 50L scale to 200-500L and beyond. The stability of the plasmid DNA/transfection reagent complexes becomes a critical parameter, as transfer of large volumes of complex mixture generally takes 30 to 60 minutes. During that time, reduce degrative shear forces to avoid inefficiencies in transfection that results in product loss.

Finally, the choice of transfection reagent can have an impact on the efficiency of the complexation step and ultimately the productivity of the transfection process. Reagents based on lipofectamine, polyethyleimine (PEI) and lipid-polymer complexes are now available. PEI is generally considered the gold standard transfection reagent and is suitable for use with many virus types in both adherent and suspension cell culture. FectorVIR® AAV (Polyplus) is a reagent designed specifically for suspension-based transient transfection of AAV vectors and is experiencing increasing use as the industry transitions to larger bioreactor processes. Both reagents are available in research and GMP grades, facilitating tech transfer from the lab to commercial scale. Product 2 and Product 3 are additional multicomponent (cationic lipid polymer) transfection reagents for research-scale transient transfection in various mammalian cells (including both HEK293 and Chinese hamster ovary, or CHO).

To obtain a greater understanding of how these three parameters impact the complexation process, a series of experiments was conducted. Complexation processes were performed for different periods of time using a range of plasmid (for AAV generation)/reagent ratios and the four different transfection reagents.

STUDY METHODS

Complexation reactions were performed following the instructions/recommendations of the transfection reagent manufacturers. All mixing was performed in test tubes on the benchtop at room temperature. Only the ratio of plasmid DNA to transfection reagent and the complexation time were modified.

For each experiment, a sample of DNA alone was used as the control. To evaluate the kinetics of DNA-transfection reagent complexation, reactions using fixed ratios of complexation reagent to DNA (volume: weight) were allowed to proceed for 60 minutes, with samples analyzed at 0, 5, 15, 30, and 60 minutes. Dose-response studies were performed by allowing complexation to proceed for 30 minutes using volume: weight ratios of complexation reagent to DNA ranging from 0.1:1 to 3.0:1 (0.1:1, 1:0.5, 0.6:1, 0.7:1, 0.8:1, 0.9:1, 1.0:1, 2.0:1, and 3.0:1).

Gel electrophoresis leveraging SYBR Gold staining at a gel loading of 20 µl was used to determine the level of free DNA present in the samples and the complexation efficiency. The isothermal stability of DNA-transfection reagent complexes was measured using the Uncle (Unchained Labs) dynamic light scattering (DLS) system for 60 minutes. DNA-transfection reagent complex formation was then quantified using an Aura (halo labs) particle size analyzer.

Triple transient transfection reactions were performed to evaluate the performance of different complexation mixtures, media, mixing times, and DNA-complexation reagent ratios. For these studies, AAV2 or AAV8 vectors were produced using the pAAV-CMV-GFP packaging plasmid containing the CMV reporter gene, which causes expression of green fluorescent protein (GFP).

Fluorescence detection of the generated viral particles was achieved using the EVOS M7000 Imaging System (Life Technologies) at a magnification of 4x. Viral titers were determined using the Stunner system (Unchained Labs) with UV detection (254 and 280 nm).

EXPERIMENT RESULTS

Impact of Complexation Time and DNA:Reagent Ratio

To explore the kinetics of the complexation reaction, plasmid DNA was mixed in a test with FectorVIR® AAV, Product 1, Product 2 or Product 3 at various transfection reagent: DNA weight: volume ratios ranging from 0.1:1 to 1:1 for 60 minutes. Samples were collected at time 0 and after 5, 15, 30, and 60 minutes **(Figure 3)**. These samples, as well as control samples containing only the DNA alone (143.4 uL Master Mix), were analyzed using gel electrophoresis with SYBR Gold 1%. Continuous DLS analyses were also performed using the Uncle™ system.

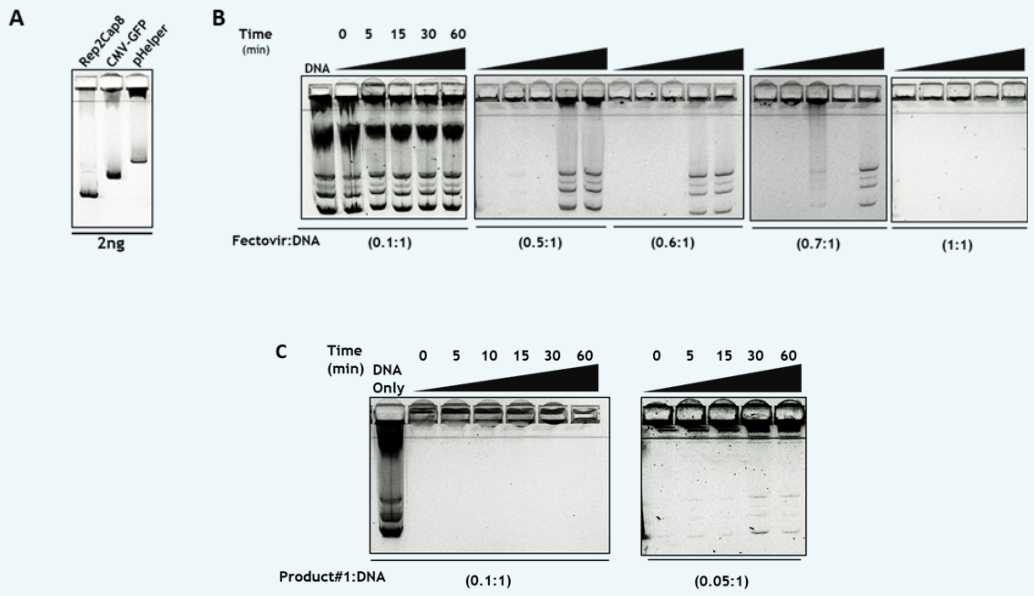


Figure 3. Gel electrophoresis results for samples of complexation mixtures containing various DNA: reagent ratios over time. Panel A: Loading controls for plasmid DNA used. Panel B: FectorVIR® AAV and Panel C: Product 1 ratio at indicated reagent: DNA ratio

For a FectorVIR® AAV:DNA ratio of 0.1:1, there is insufficient complexation agent, and free DNA is present even after 60 minutes. At ratios of 0.5:1, 0.6:1, and 0.7:1, complexation occurs immediately, but is not maintained, and eventually free DNA is again present in the solution. Only at a FectorVIR® AAV:DNA ratio of 1:1 is the complexation mixture sufficiently stable to maintain complex formation for the entire duration of the experiment.

With Product 1, however, even at a low reagent: DNA ratio of 0.1:1, all of the DNA is bound with the transfection reagent for the full 60 minutes. It is only at a ratio of 0.05:1 that the presence of low levels of DNA is detected.

The stability of the complexation mixtures was then evaluated using continuous dynamic light scattering, which revealed the average diameter of the generated complexes over time. The results are presented in Figure 2 for all four transfection reagents. As expected, the particle sizes for the mixtures with only DNA and only reagent were the smallest.

Impact of DNA:Complexation Reagent Ratio on Complex Formation

The dose-response behavior of complex formation between plasmid DNA and the different transfection reagents was then evaluated at a fixed reaction time of 30 minutes. transfection reagent:DNA ratios (volume:weight) again ranged from 0.1:1 to 3:1. Gel electrophoresis results using SYBR Gold staining are shown in **Figure 4**.

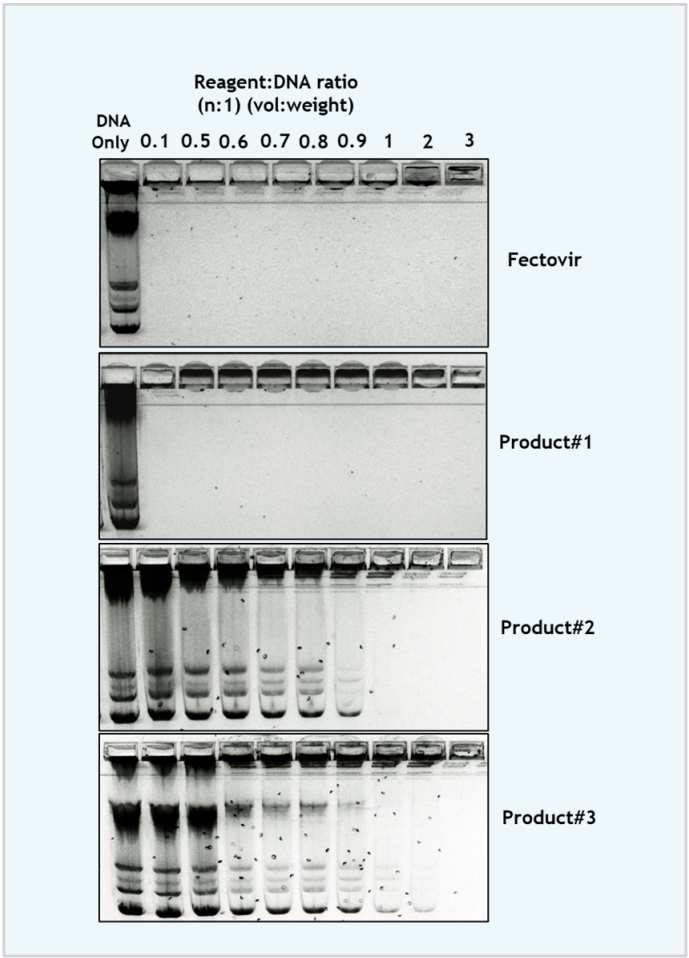


Figure 4. Dose-response study of DNA-transfection reagent complexation at 30min.

This study confirmed the results obtained during the analysis of the complexation kinetics for FectoVIR® AAV (data not shown) and Product 1. With FectoVIR® AAV, no free DNA was present after 30 minutes for reagent:DNA ratios of 0.5:1 and greater, while effective complexation occurred with Product 1 even at a low reagent:DNA ratio of 0.1:1 **(Figure 3)**. In fact, very little free DNA was present with a Product 1:DNA ratio of 0.05:1. Only when a ratio of 0.01:1 was employed was free DNA observed after 30 minutes of stirring (data not shown). A ratio of 0.1:1 Product 1 still represents a net positive charge: weight ratio, thus the efficiency of complexation at these very low ratios is not unexpected.

The results for the Product 2 and 3 reagents were similar. A minimum 1:1 ratio of reagent to DNA was required to avoid having any measurable free DNA in the mixture at 30 minutes. With Product 2, no free DNA was detected at a 1:1 ratio. With Product 3, complete absence of free DNA after 30 minutes of stirring was only observed for a 3:1 reagent:DNA ratio. The fully optimized Gibco™ AAV-MAX Helper-Free AAV Production System (Thermo Fisher Scientific) showed similar results to FectoVIR® AAV. As indicated, lower amounts of reagent (0.1:1) resulted in incomplete complexation, which was expected, with higher ratios (as recommended by the manufacturer) providing the best results.

Complexes with particle sizes reported in the literature to be ideal for transport across cell membranes were obtained at FectroVIR® AAV:DNA ratios of 0.5:1 and 0.6:1 with the largest complexes (800-1000 nm average diameter; maximum 1 micron diameter detection limit on Uncle) observed for reagent:DNA ratios between 0.7:1 to 1:1. Meanwhile, almost all of the Product 1:DNA ratios resulted in large complexes with diameters greater than 800 nm. Only at a 0.1:1 reagent:DNA ratio were particles of 300-400 nm in diameter generated.

The Products 2 and 3 reagents afforded different results. In this case, only the reagent:DNA ratio of 3:1 or greater (data not shown) resulted in large diameter particles (>1000 nm). On the other hand, small particles, below 200 nm in diameter, were obtained when the reagent:DNA ratio was 0.1:1. It is interesting to note that while Fectovir® and Product 1 all produced very large complexes at 1:1, only Product 2 and Product 3 reagents demonstrated ideally sized complexes in the 400nm range. Only when excess reagent was provided (3:1 ratio) did we start to observe larger particles. While these reagents all serve as excellent reagents for complexing DNA, the biophysical properties of how these reagents work is evident in the various distribution of particle sizes **(Figure 5)**.

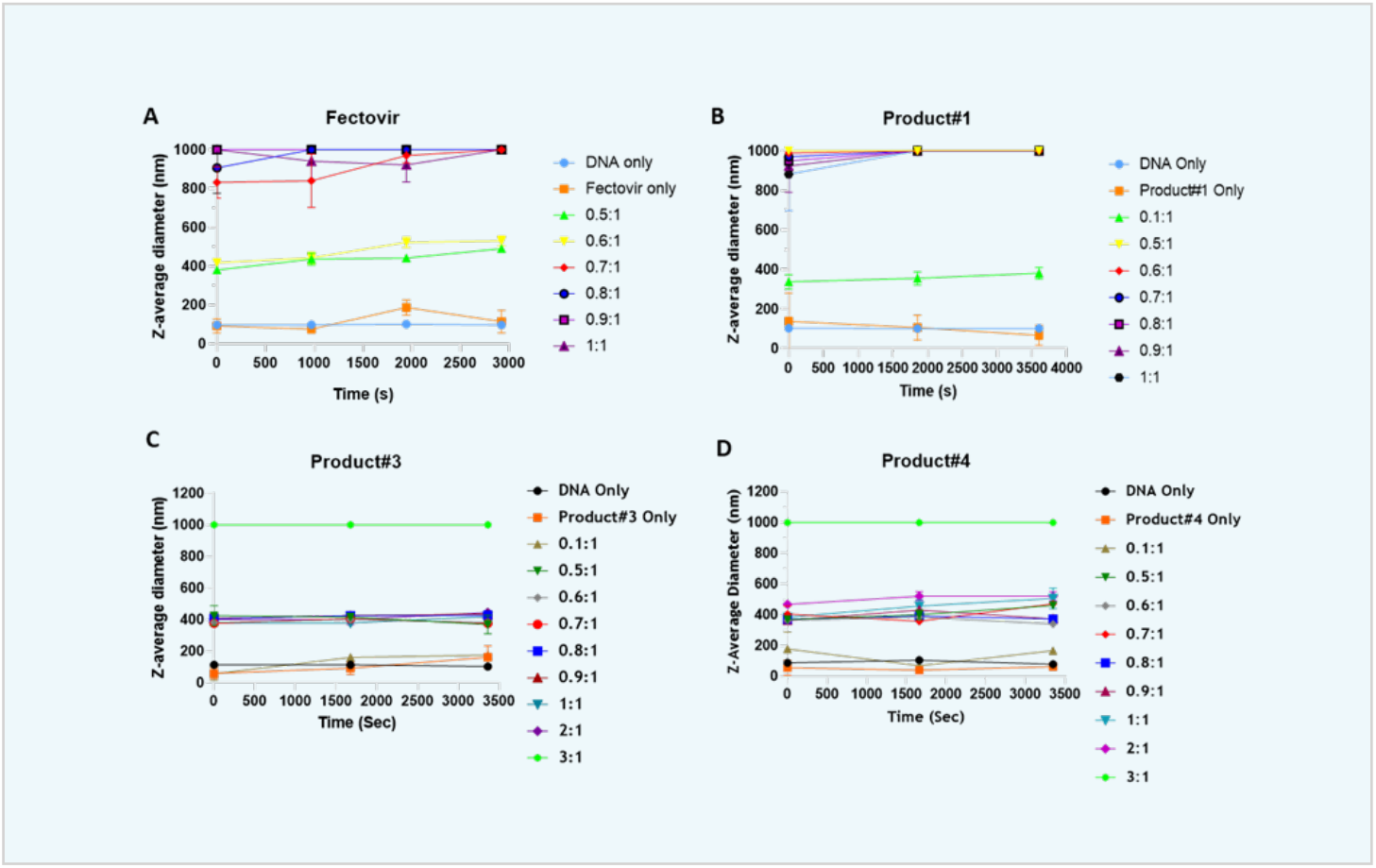


Figure 5: Isothermal stability (25°C) of DNA:reagent complexes over time determined using dynamic light scattering. A: FectorVIR® AAV; B: Product 1; C: Product 2; D: Product 3.

Given the limited detection range for DLS only enables detection of particles up to 1 micron in size, particle size distribution was subsequently reexamined using the Aura system, which allows detection of larger, subvisible particles ranging from 1 to 25 microns in diameter. The Halo Aura system uses sophisticated Background Membrane Imaging to meticulously quantify particles of various sizes. Moreover, the Aura can compartmentalize particle counts in 1-micron increments, conferring an advantage when seeking to examine much larger particle sizes and distributions.

Each dataset in **Figure 6** includes average counts of particles with diameters of 2-25 m for the medium, DNA, and reagent alone along with results for FectoVIR® AAV:DNA ratios ranging from 0.1:1 to 1:1. To simplify the analysis, particle counts ranging from 2-10 and 11-25 micron intervals were combined and plotted as a function of time. As shown in **Figure 4**, and as expected based on the UncleTM results, complexation using FectoVIR® AAV indicated a very rapid complexation event occurring immediately after mixing, keeping in mind that processing time from mixing to read-out is between 0-5 minutes.

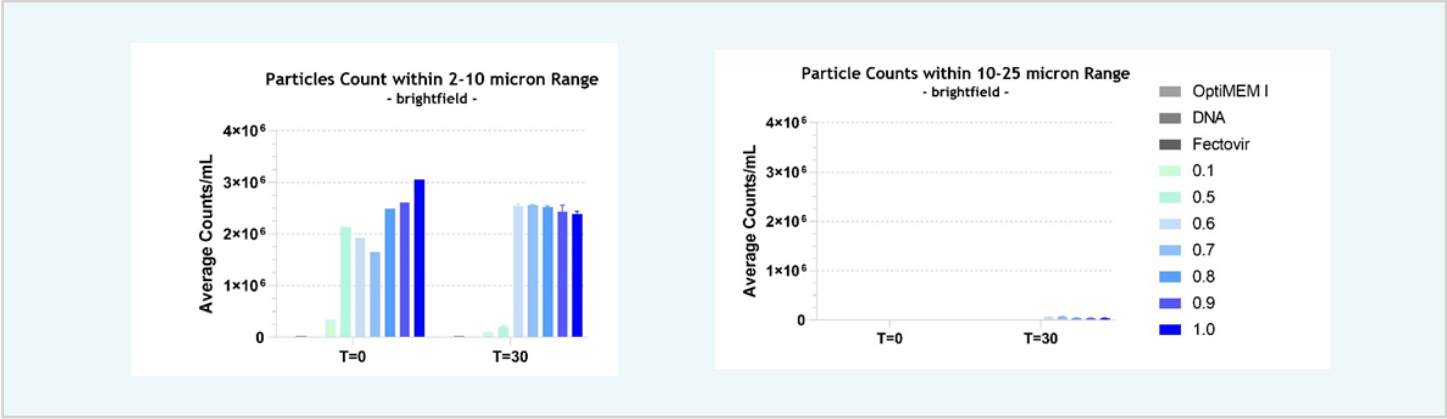


Figure 6: DNA-FectoVIR® AAV Complex Formation Quantification using the Halo Aura System. Detection of sub-visible particles at time 0 and 30min post-complexation sizes between 2-10 microns (left panel); and 10-25 microns (right panel).

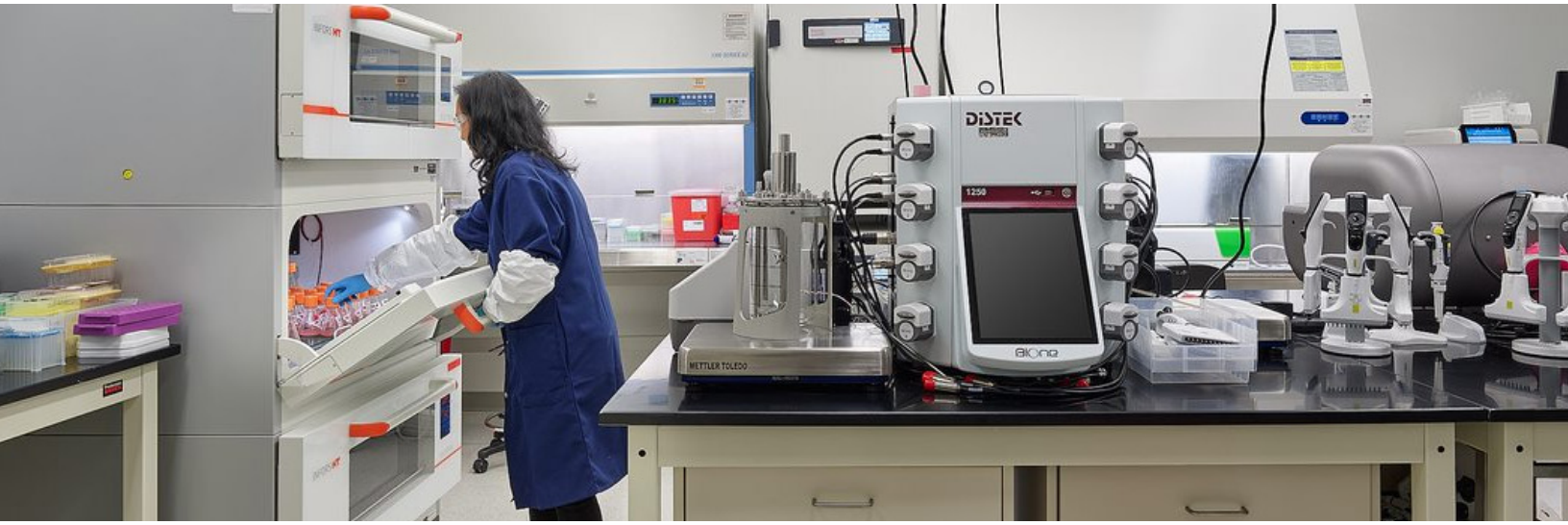
At time zero, brightfield analysis revealed high particle counts in the 2-10 micron range for all FectoVIR® AAV:DNA ratios with the exception of 0.1:1. As time progressed (T=30min), a uniform and stable distribution of particles was observed between 2-10 microns for FectoVIR® AAV:DNA ratios > 0.6:1, with few particles detected for ratios of 0.1:1 and 0.5:1, suggesting that these low complexation ratios result in highly unstable particles. This data is consistent with the results shown in **Figure 3 and 4**.

Although there were some very large particles (11-25 microns) detected, the totality of these particles represented an insignificant quantity relative to all of the particles between 2-25 microns. As expected, no subsvisible particles were observed for the negative controls (samples containing only DNA, medium, or FectoVIR® AAV)

Impact of Complexation Parameters on Transfection Efficiency

Having gained a better understanding of the complexation reaction kinetics for various transfection reagents, CBM next sought to address how specific kinetics of this reaction impact the efficiency of virus production during the transient triple transfection step.

For this purpose, the cis-packaging plasmid pAAV-ITR-CMV-GFP-IRES-fLuc was used to monitor transfection efficiency as a function of GFP expression driven by the constitutive CMV promoter within the transfected cells. The cis plasmid was engineered at CBM to include intact ITRs as determined by NGS sequencing and a 4.5kb packaged insert, which is close to the recommended 4.7kb packaging capacity of AAV. This design strategy for the cis plasmid was anticipated to facilitate an efficient increase in packaged particles. Cell viability and viable cell counts were determined 72 hours after transfection for the four transfection reagents prepared with stirring in Gibco™ Viral Production Medium for 5 or 15 minutes using a 1:1 reagent: DNA ratio. Examination of viability demonstrated very mild toxicity at 1:1 ratio for both FectorVIR® AAV and Product 1 and a concomitant attenuation of cell growth with the cells hovering close to the seeding density of this experiment (1.5e6 cells/mL). Interestingly, we did not observe any direct impact on viability for either of Product 2 and Product 3 reagents and during the course of the 72h transfection, the cells doubled at their normal rate of ~24h.



Next, we sought to address how the ratio of transfection reagent:DNA, across all four transfection reagents and three different complexation ratios (0.1:1, 0.6:1, 1:1) impacts virus titers. These three ranges were determined based on the results obtained from Figures 2 and 3, with the expectation that 0.1:1 would be very inefficient, 1:1 represents optimal and 0.6:1 could represent an alternative mimimal effective amount. We monitored all parameters during virus production, including genome tites, particle counts, viability, and viable cell density. As expected, at a 1:1 ratio, all 4 reagents yielded acceptable titers ($1e^{11}$ – $1e^{12}$ VG/mL) of DNAse-resistant particles (DRPs) measured by dPCR using the Qiacuty® (Qiagen) platform (see CBM whitepaper on this topic) **(Figure 7A)**. Capsid ELISA using the Progen AAV8 titer kits, showed a similar range of virus particle titers (mid $1e^{11}$ – $1e^{12}$) **(Figure 7B)**. At 0.1:1 complexation ratio for all the reagents tested, we observed a precipitous decline (~2-3log loss) in overall VG titers, as well as a consistent ~2-3 log loss in VG titers. To some extent, the loss in particles approached the LLOD (lower limit of detection) for the ELISA assay. This was particularly evident for Product 1, where we could not detect capsid particles at all at 0.1:1 ratio.

Full Particle percentage was calculated by dividing the VG titers by VP titers. Surprisingly, we found that Product 2 at a 1:1 ratio yielded ~63% full particles, nearly double that observed with Fectovir® AAV (30-36%). Product 1 and Product 2 yielded similar results (12-15%) **(Figure 7C)**. FectoVIR® AAV providing the highest amounts close to $1e^{12}$ VG/mL.

Further inspection of viability and viable cell density yielded consistent results that as complexation reagent amount decreases, overall cell health is improved **(Figure 7D & 7E)**. Given that all 4 reagents yielded similar viral titers at 1:1, the reduction of viability is mostly likely a result of toxicity to the reagent and not because of excessive virus production. Using slightly lower amounts of complexation reagent:DNA (0.6:1) which was sufficent to complex DNA using either FectoVIR® AAV and Product 1 but not Product 2 or 3, we observed a slight increase in full particles, compared to 1:1 (FectoVIR® AAV 36% vs 28.5%; Product 1 14% vs 12%) and an expected decrease for the Product 2 & 3 reagents (Product 2 14% to 2.5%; Product 3 63% to 9%). These results are somewhat expected based on the efficiency of these reagents to complex DNA as shown in **Figures 3 & 4**.Moreover, there was improvement in viability using reduced amounts of reagents (FectoVIR® AAV 84% (1:1) vs 93% (0.6:1); Product 1 87% vs 94%), again consistent with the toxicity noted for these compounds. It is worth noting that a ratio of 0.1:1 is not recommended by any of the manufacturers for these reagents, which is consistent with our experimental observations.

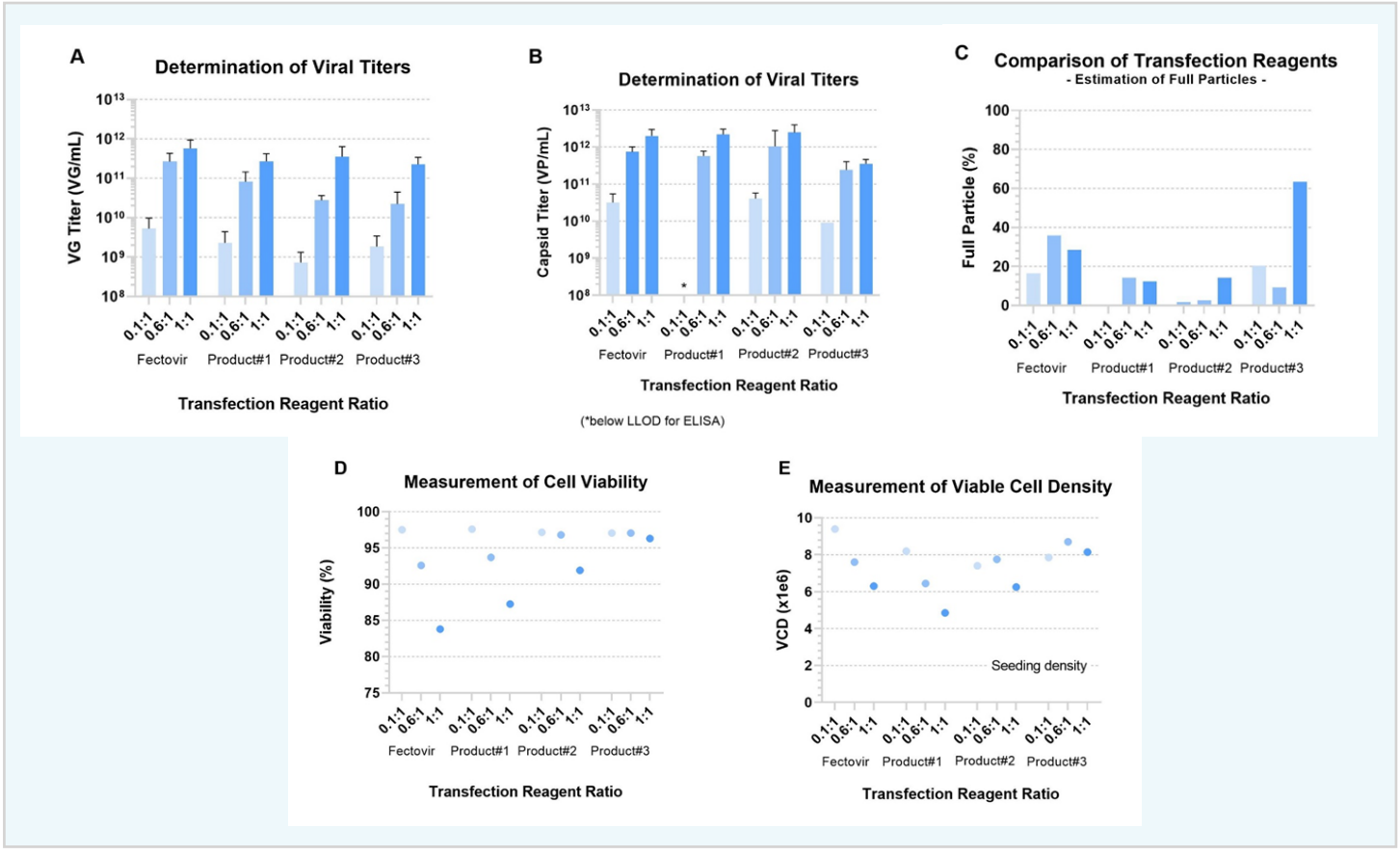


Figure 7. Evaluation of Complexation Reagent on Virus Production. FectorVIR® AAV, Product 1, Product 2, and Product 3 were tested at three ratios to assess impact on virus production and cell viability. 48h post-transfection cells were lysed, treated with DNAse and assessed for A: DNAse resistant particle titers by PCR (VG/mL), B: capsid ELISA (VP/mL), C: Calculation of Full Particles, D: viability and E: viable cell density (ViCell).

The efficiency of transfection was monitored by GFP expression of the cis plasmid by viewing the cells containing viral particles using the EVOS M7000 Imaging System (Life Technologies) with both Brightfield and EVOS™ Light Cube, GFP 2.0. An example set of images is presented in **Figure 8**. Robust GFP-positivity was observed for all 1:1 complex, while only the FectoVIR® AAV and Product 1 reagents showed increased positivity at a reagent: DNA ratio of 0.6:1. Product 2was comparable at both ratios, while the Product 3 reagent showed a significant drop-off to near undetectable levels of GFP expression at 0.6:1 which is also not recommended by the vendor. No GFP color was observed for 0.1:1 complexation ratio for all reagents tested. These results are consistent with the observed virus expression data reported in **Figure 8C**.

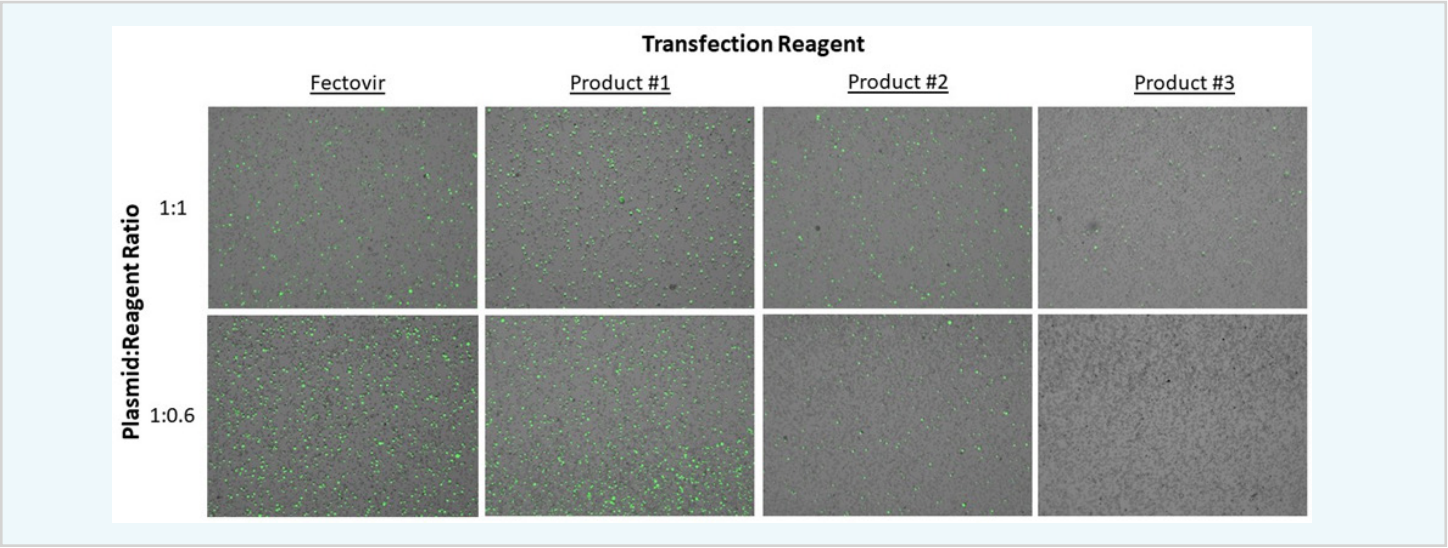


Figure 8. Imaging results for cells before and 72 hours post transfection of AAV8 vectors using a variety of reagent:DNA complexes

To extend these observations from small scale (30mL) cultures we opted to evaluate the performance of each of these reagents at a slightly larger scale (200mL) and incorporated subsequent down-stream purification and viral analytics. We decided to also include FectoVIR® AAV at a 0.6:1 ratio based on the interesting findings that we observed from the results in **Figure 7C**, in which we observed an increase in full particle percentage, relative to the 1:1 ratio. Triple-transfection of cells was performed on the same day, using the same source of DNA and transfection conditions. Likewise, all preps were harvested identically, and purified within 24h to avoid any potential issues with aggregation loss that would erroneously diminish viral titers. We also incorporate high salt in our lysis conditions which greatly diminishes potential aggregation.

The results shown in **Figure 9A** represent the elution fraction, mAU/260/280, for each of the transfection mixes. VP/mL titers as indicated were determined using the Unchained Labs Stunner. Based on these results, we observed >log10 difference in titers ranging from $6.1e^{14}$ VP/mL (FectoVIR® AAV 0.6:1) to $5.7e^{13}$ VP/mL (Product 3). Product 1 and Product 2 produced comparable titers $2.2e^{14}$ & $3e^{14}$ VP/mL, respectively, Fectovir AAV (1:1) was slightly higher ($4.5e^{14}$ VP/mL) while FectoVIR® AAV (0.6:1) produced the highest ($6.1e^{14}$ VP/mL).

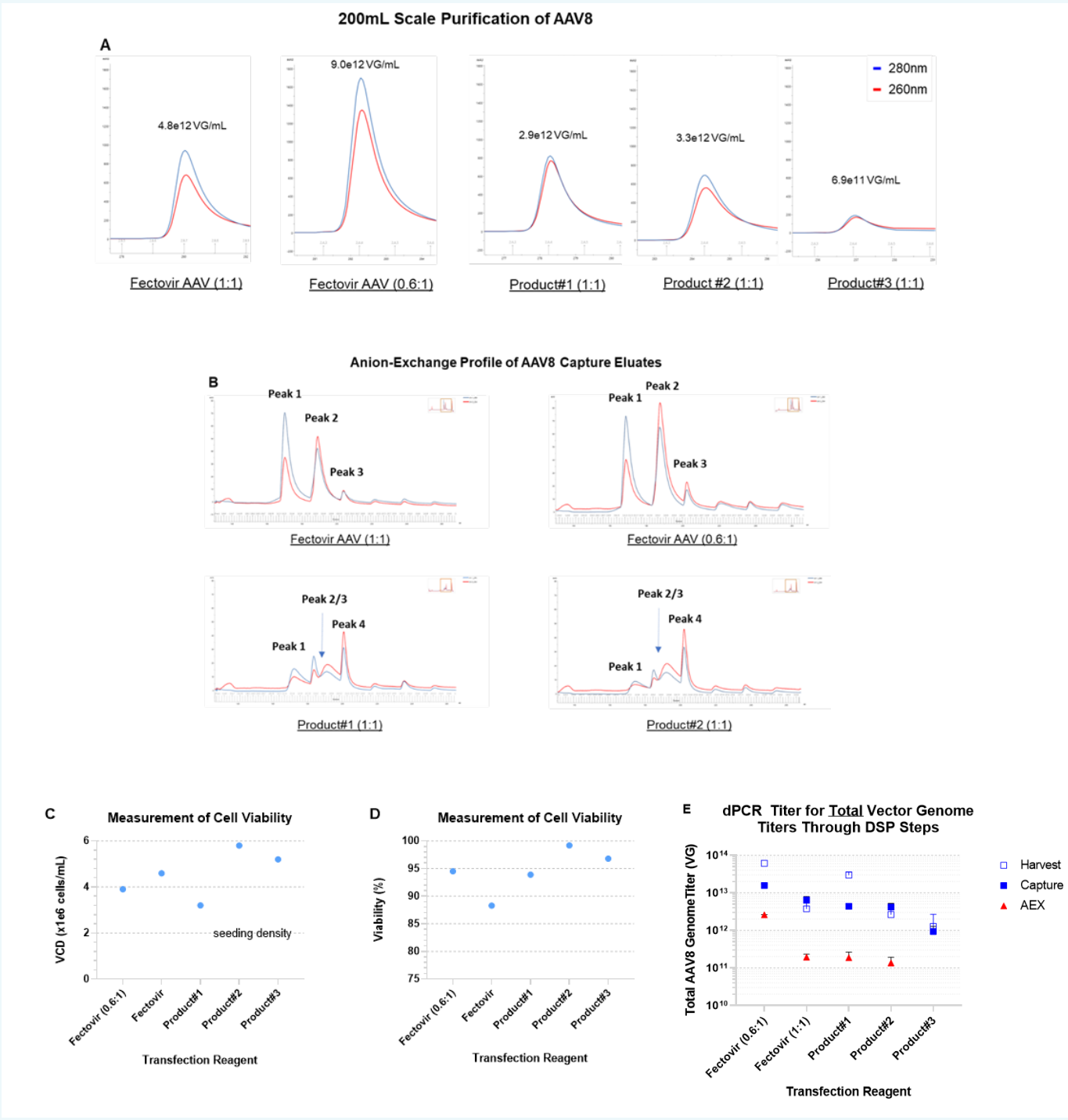


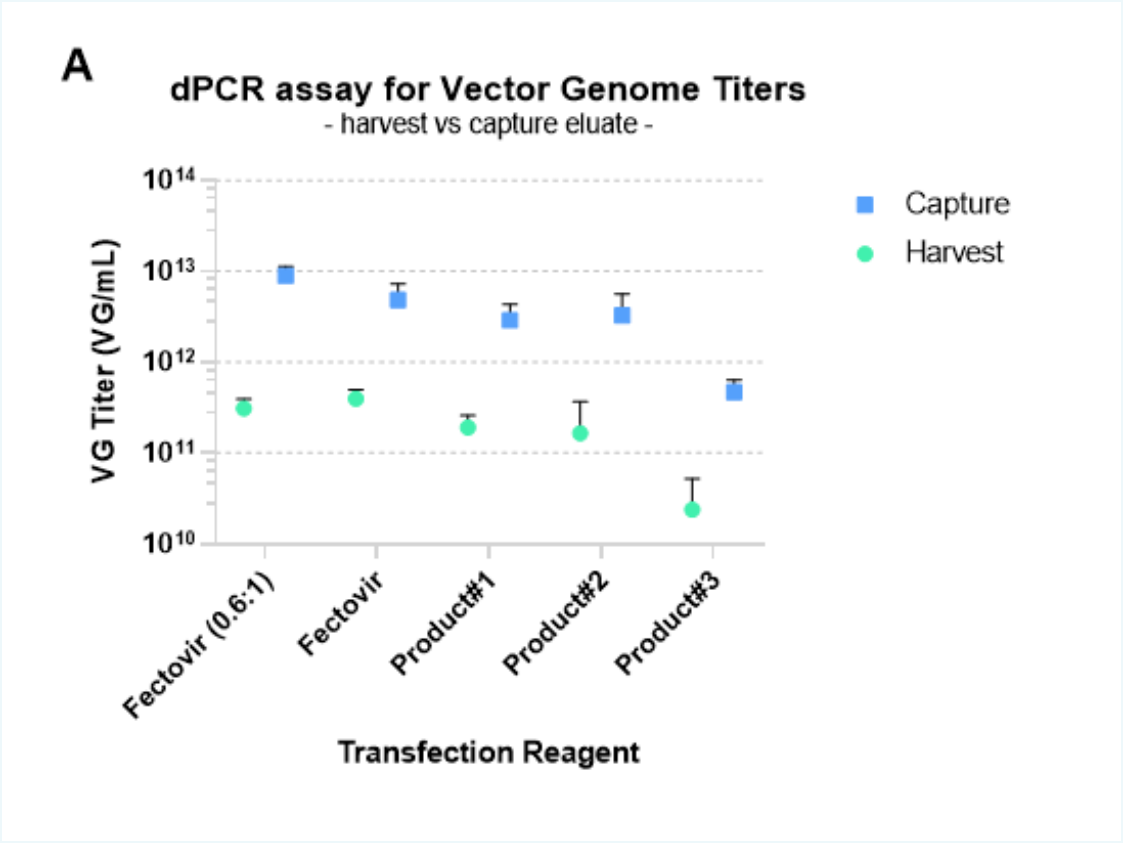
Figure 9. Evaluation of Transfection Reagents in 200mL shake flasks with downstream purification. AAV8 was produced using by triple-transfection using the indicated transfection reagent at a 1:1 ratio (or 0.6:1 FectoVIR® AAV:DNA as indicated). Cells were harvested 48h post-transfection, purified by A) Affinity-Chromatography using the Repligen's AviPURE AAV8 column and B) polished using a MustangQ anion-exchange resin. At time of harvest, C) viable cell density and D) viability were recorded.

To gain a better understanding as to whether the transfection reagent impacts the composition of AAV8 particles produced, we polished the AVIPure capture eluates using an increasing NaCl, 9-step gradient on a MustangQ Anion Exchange resin **Figure 9B**. Much to our surprise, we found that the method of transfection has a significant impact on particle distribution. FectoVIR® AAV at either ratio produced 3 predominant peaks of empty (peak 1, fraction C3), full (peak 2, fraction D3), and other (peak 3, fraction E1) as determined by 260/280 ratio (peak inversion of 260/280 observed between peaks 1 and 2/3). Moreover, at a FectoVIR® AAV 0.6:1, we saw that the full peak 2 was more abundant than what observed at FectoVIR® AAV 1:1. Surprisingly, we saw a much different pattern of particle distribution for Product 1 and Product 2. As mentioned, these reagents yielded similar VP/mL titers upon capture, but as shown in in the AEX trace, we did not observe 3 distinct peaks. Instead, we saw roughly 4 peaks (peak 1, fraction C6; peak 2, fraction D2; peak 3, fraction D7; and peak 4, fraction E1). More importantly, we saw compression of peaks 2-4, which was not present in any of the FectoVIR® AAV samples. While we did observe peak 260/280 peak inversion between peaks 2/3 and 4/5, we were not able to separate distinct peaks and troughs which would make larger scale separation of distinct empty/full peaks challenging. Moreover, Product 1 and Product 2 produced what appears to be two distinct populations of packaged viruses, peaks 3 and 4. It is interesting to note that while we saw a distinct empty peak (fraction C3, peak 1) for FectoVIR® AAV, this peak was split between two distinct fractions for Product 1 and Product 2 (peak 1 and 2, fractions C6 and D2, respectively) with an even more pronounced shift if full particle elution at peak 2, fraction C3 for Fectovir® AAV vs peaks D7 and E1 for Product 1 and Product 2. Extensive characterization by AUC/MS/NGS would need to be performed to understand the difference between these two populations of virus and what effect transfection reagents impart on packaging.

The slight discrepancy in virus titers as well as the AEX-profile of particle distribution is a characteristic of the reagent mechanism of how they function versus an impact on cells. As noted in **Figure 9C and D**, we observed excellent viable cell density (1-2 doublings) and viability (~95%) indicating that significant titer gain or loss was not due to the overall health of the cultures. This was clearly evident for Product 3, where we observed nearly 2 complete cell doublings (based on 24h for AAVMAX, VPC2.0 cells) and >95% viability, yet this reagent produced > log10 less titer than Fectovir® AAV (0.6:1) which showed only a 1x doubling of cells and a viability <95%. Similarly, Fectovir® AAV (1:1) which also showed the second highest titer also had the lowest viability <90%.

Quantification of vector genomes from bulk harvest as well as peak fraction from AKTA-affinity capture eluates was performed using dPCR **(Figure 10B, left panel)**. All reagents, with the exception of Product 3 yielded consistent VG titers from bulk harvest, also in line with the results we observed in small-scale cultures **(Figure 7A)**. We also observed consistent yields of recovered VG titers for Fectovir® AAV, Product 1, and Product 2 at 1:1 reagent:DNA ratios, while slightly higher, and lower, virus titers were observed for Fectovir® AAV (0.6:1) and Product 3, respectively.

Further inspection of capsid protein VP1, VP2, and VP3 ratios was examined by polyacrylamide gel electrophoresis coupled with Coomasie staining. We observed the expected stoichiometry of VP1≈VP2<VP3 for AAV with no other detectable protein contaminants **(Figure 10B, right panel)**. While we saw compression and alterations of peaks following AKTA-AEX separation, the explanation for why this happened cannot be explained by VP capsid stoichiometry alterations. As shown, we observed similar capsid staining patterns across all the fractions inspected by Coomassie staining. These results suggest that alterations in DNA-packaging are contributing to the peak distribution noted in the AEX profile. Further inspection using Next-Generation Sequencing technology is warranted.



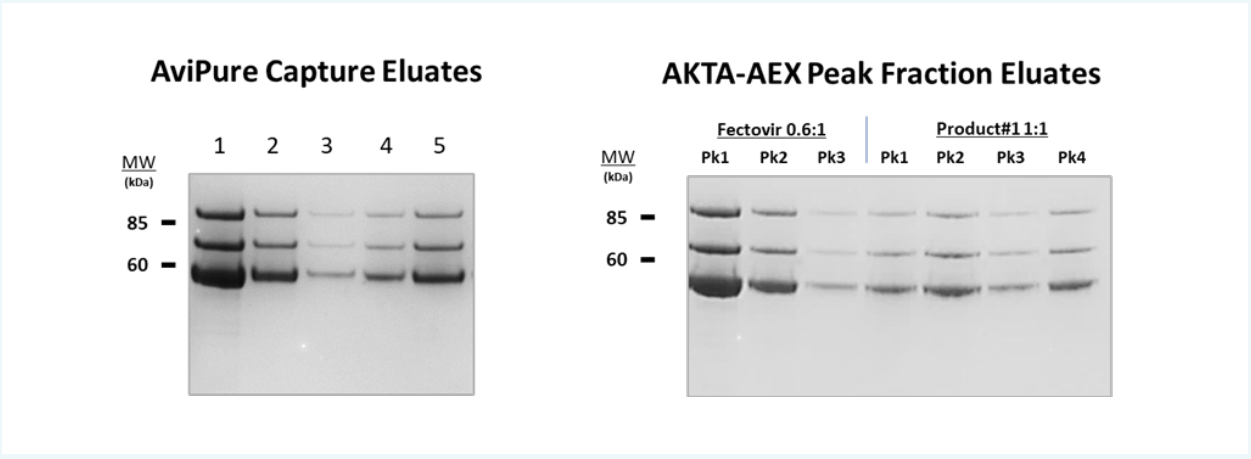


Figure 10. Characterization of purified AAV.
A. Quantification of vector genomes by dPCR was performed on harvest and capture AKTA-affinity capture eluates.
B. Visualization of capsid stoichiometry of peak fractions from AKTA-affinity and -AEX was examined by silver staining (right panel): 1, Fectovir® AAV (1:1); 2, Fectovir® AAV (0.6:1); 3, Product 1 (1:1); 4, Produict 3; 5, Product 2.

TAKEAWAYS FOR PLATFORM PROCESSES

Preparation of complexation reagents is a key first step in transient transfection for the production of viral vectors used in gene and gene-modified cell therapies and viral-vectored vaccines. The series of experiments described clearly demonstrate the importance of understanding the CPPs of the complexation process. The complexation (stirring) time, ratio of plasmid DNA to transfection reagent, and the choice of reagent all impact transfection efficiency and overall performance.

These factors are important because it is essential that the DNA/reagent complexes remain stable during not only initial mixing, but while the complexation mixture is added to the bioreactor. On the laboratory scale, time is not a factor because volumes are small and transfer times are short. On a commercial scale, however, much larger complexation volumes are involved. Mixing and transfer of these large volumes must be done carefully and at a sufficiently slow rate to avoid shear forces that can damage the sensitive DNA/reagent complexes. Generally, the complexes must be stable for up to 60 minutes or more.

In general, the series of studies performed with FectoVIR® AAV, Product 1, 2, and 3 reagents did contribute to a greater understanding of the initial plasmid DNA-transfection reagent complexation step performed prior to transient transfection. The main conclusions to be drawn are as follows:

- The choice of transfection reagent has a direct impact on the performance of the transfection step.
- The complexation step may be influenced by different reagents, some which provide better transfection efficiencies
- The DNA:transfection reagent ratio is a critical process parameter.
- Complexation time has an impact on the transfection efficiency for FectorVIR® AAV and the Product 2 and 3 reagents.
- The average particle sizes of DNA:reagent complexes formed under optimum conditions are much larger than the 300-500-nm range reported in the literature as being ideal for transport across cell membranes in an in vivo setting

Greater understanding of the process parameters critical to DNA:transfection reagent complexation enables the development of optimum, robust, efficient, and scalable transient transfection processes for the generation of viral vectors. This process knowledge is applicable across different AAV vectors, and potentially other vector types as well.

Center for Breakthrough Medicines is well-positioned to design complexation steps and transient transfection processes that exhibit improved performance. With the right level of understanding, we are able to provide advice on not only aspects of process development, but reagents and consumables that will be optimal for each customer process. Our experts are also aware of the major challenges to achieving large-scale viral vector manufacturing and work closely with customers to identify solutions to overcome these issues. Clients benefit from more rapid development of cost-efficient, viral-vector manufacturing solutions that afford higher product titers.

Center for Breakthrough Medicines

ABOUT THE CENTER FOR BREAKTHROUGH MEDICINES

CBM is a cell and gene therapy contract development and manufacturing organization (CDMO) based in the heart of Philadelphia's Cellicon Valley. CBM offers preclinical through commercial manufacturing capabilities including process development, plasmid DNA, viral vector manufacturing, cell banking, cell processing, and a full suite of complimentary testing and analytical capabilities. Through a single-source, end-to-end solution, CBM accelerates time to market without compromising quality.

Co-locating manufacturing, process development and analytical services prevents delays and handling errors. CBM's aim was to create one campus, one building, one manufacturing site. Our purpose built 700,000 sq. ft. manufacturing center is future-proofed in terms of infrastructure within and around the site. The current facility sits on over 1 million sq. ft. of space, allowing for future expansion to match the growing demand of the cell and gene therapy industry. Internally, the suites have been designed so that complementary services and labs are adjacent or nearby, to ensure we can accelerate time to market without compromising quality.

Scan or click the QR code to learn more about our process development

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