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Gene Therapy Manufacturing 2.0

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Abstract

Over the past century, therapeutics developed from plant extracts to chemically defined small molecules and then on to large molecule based recombinant proteins. After decades of relentless scientific efforts, gene and cell therapy, whose promise was envisioned for decades, now fuel the next step change of the therapeutic landscape, opening entirely new routes towards previously unaddressable disease states. Approvals across multiple therapeutic types now include Maci, Kymriah, Yescarta and Tecartus for cell therapies and Luxturna and Zolgensma® for *in vivo* gene therapies. Together, this set of therapies has catalyzed the initiation of hundreds of clinical trials that will surely herald the arrival of more successful treatments within the next decade. Luxturna, which addresses retinal dystrophy, and Zolgensma®, which addresses spinal muscular atrophy, represent breakthrough therapies with important, but small, patient numbers. As gene therapies mature, more common diseases, such as solid tumor cancers, will emerge with far greater patient numbers.

In order to leverage the clear and vast potential of gene and cell therapies for common disease states, manufacturing processes need to augment both productivity and efficiency. Early manufacturing processes were developed in research labs with technologies not designed for industrial scale-up. This whitepaper, which exclusively considers gene therapy, refers to this precursive work that enabled the production of early gene therapy clinical and commercial batches as Gene Therapy Manufacturing 1.0 (GTM 1.0). The early success of early gene therapy therapeutics, coupled with the limitations of GTM 1.0, have created a significant supply chain gap in which viral vector demand exceeds the current supply-and that gap is increasing. Significant technical developments that increase productivity and efficiency are immediately required to meet current demands and then fuel future endeavors. This review summarizes a select number of GTM 1.0 challenges and outlines potential Gene Therapy Manufacturing 2.0 (GTM 2.0) productivity, scalability and safety improvements.

Manufacturing demand gap

The large number of variables that determine gene therapy demand, including indication, prescribed dosage and number of patients impede calculation of a definitive future value. Several researchers have generated predictive models, however, in order to better understand different complex scenarios. Quinn *et al.* from MIT developed a baseline model towards predicting the number of approved therapies and patient populations across different diseases (1). Depending on the condition, the number of patients for each therapy was allowed to vary from 100 (e.g. ophthalmology treatment) to above 20,000 (e.g. neurological treatment). The output of the model estimated “350,000 patients are likely to be treated using 30 to 60 different gene therapies by 2030.” The number of annually treatable patients in 2030 was estimated to reach 50,000, which most likely represents an increase of at least ten-fold the number of current patients. In a second model, Rininger *et al.* (2) estimated manufacturing demand and capacity for AAV and lentiviral vectors by compiling dose per patient across a series of therapeutic indications, enabling deduction of an estimated annual AAV and lentiviral production demand. For example, they estimate the average dosage for a hematological cancer indication to be 5×10^8 TU of lentivirus per patient. Assuming we can consider at least 15,000 patients with such a condition to be treated per

year, an annual production of 7.5 x 10^{12} TU of lentivirus will be required. For muscular dystrophy, the authors estimate the average dosage at 3.9 x 10^{15} VG of AAV per person. With an incidence of approximately one in every 5,600 to 7,700 male between the ages of five to 24 (3) in the US, a worldwide population of 10,000 patients with Duchenne muscular dystrophy can be envisaged, which would lead to the need for ~4 x 10^{19} VG of AAV. While the models of Quinn et.al. and Rininger et al. are constructed with different goals, strategies, and assumptions, both come to the same conclusion; a viral vector supply demand gap either already exists and or will develop soon.

The viral vector supply demand gap stems from the fact that viral vector manufacturing technologies and processes are largely and non-optimally re-purposed from recombinant protein and monoclonal antibody (mAb) processes. Prioritization of time to market over optimization compound the gap between supply and demand. The time-consuming iterative process of optimization is largely forgone as low yielding processes are often considered “good enough” as soon as the desired quantity of viral vectors is produced. Later, in commercial manufacturing, this becomes a significant process bottleneck.

The impact of the shortage varies widely across the different indications. For example, the aggregate manufacturing demand of a hematological cancer, estimated to require 7.5 x 10^{12} TU of lentivirus per year, can likely be met by existing GTM 1.0 technologies. A 100 m^2 fixed bed adherent bioreactor with a 30% overall yield run twenty times in one year will most likely meet such a demand.

Consideration of 10,000 muscular dystrophy patients per year paints a different picture. Again, using approximate numbers, a single 3000 L STR delivering twenty batches per year at 1x10^{10} AAV vg/day/ml with a 30% overall process yield might deliver ~5x10^{17} VG of AAV. Scaling out that process to meet the 4 x10^{19} would then require an unreasonable 80 (3000 L) bioreactors, each delivering twenty batches per year.

Each order of magnitude of viral vector yield increase will create new opportunities for the field. A tenfold yield increase will lower costs and expand access of existing therapies to a more equitable set of patients as well as enable expansion of the global pipeline. A 100- fold improvement will bring indications such as muscular dystrophy and others within reach as manufacturable solutions. Finally, a 1000X improvement could pilot gene therapy into an era where it addresses common diseases with even larger patient populations and completely revolutionize modern healthcare.

Limited productivity and scalability with transient expression and adherent cell culture

Current upstream viral vector manufacturing processes primarily are based on transient expression, which, although convenient in a lab setting, typically suffer from low productivity and a complexity that can challenge manufacturing reproducibility. For AAV and lentivirus, cell specific productivities in the range of 1 - 5 x 10^4 VG/cell (5) and 1-10 TU/cell (6) respectively, have been reported using transient expression. Most systems require three to four DNA plasmids, each of which accrues complexity. Optimization across multiple parameters such as the ratio of DNA to transfection agent, the ratio of DNA to cells and the incubation time, for multiple plasmids, swells the number of resources required for DOE execution as well as the risk of deviation during unit operation execution. Moreover, transfection reagents can impact cell growth, with viable cell density generally dropping after transfection, meaning that the very agents that enable transfection also decrease the cells available for production. This complexity translates to significant cost when manufacturing high quality plasmids for clinical applications, causing multi-plasmid transfection as a significant contributor to overall process cost (4).

Through improved understanding of cellular metabolism and protein secretion, multi-fold cell specific productivity has been achieved for recombinant proteins and mAbs. Extending this approach to gene therapy would represent an effective solution to the demand supply gap but will unlikely

align with the rapid timelines of the current clinical pipeline. Two decades were required to realize current cell specific 10 - 100X productivity increased for mAbs. Given that viral vectors are biochemically more complex than most recombinant proteins and mAbs, a similar amount of time should be expected to achieve comparable gains in viral vector cell specific productivity. Moreover, a cell specific productivity solution for one product will unlikely be broadly applicable given the nuances of metabolism and viral vector molecular.

Adherent based cell culture processes represent an effective means of generating gene therapy product at lab scale with eventual challenges at manufacturing scale. While easy to grow and maintain with media exchange, adherent cultures suffer from low cell density (~1 x 10^6 cells/ml) and flatware dimensional limitations. The largest commercially available flatware device (~2.5 m^2, ~6 L media volume) provides less than 3 x 10^{14} VG and 6 x 10^{10} TU of AAV and lentivirus respectively. Increasing the device area 100 X to 250 m^2 or more, towards meeting the demand gap, does not represent a practical or executable solution. Scaling-out, as opposed to scaling-up, with a large number of small vessels carries its own obstacles that include: significant capital equipment costs for automation, increased complexity, manufacturing footprint and labor. As a step in the forward direction, a new generation of fixed-bed bioreactors has been shown to increase viral vector yields per bioreactor 100X to ~2 x 10^{16} VG for AAV (7) and ~1 x 10^{12} TU for lentivirus (8), but this is still not enough to meet the demand. With the largest fixed-bed bioreactor currently at 500 m^2, scale-up to the equivalent of 2000 - 3000 L STR remains out of reach.

Non-optimized downstream leads to low yields

Translation of filtration and chromatography methods developed from biological drug processes to viral vector processes does in fact lead to production of the desired product, but recoveries fall far below expected levels. While the cumulative downstream yield for a mAb typically reaches 80%, viral vectors often achieve only 30%, with some as low as 10%. With greater structural and biochemical complexity than proteins, viral vectors have fewer methods that can be applied and a more limited set of stable buffer and temperature conditions. For example, low pH and high conductivity buffer conditions frequently applied to proteins, lead to virus inactivation, such as the fragile enveloped lentivirus. Most product loss occurs during non-optimized chromatographic separation methods that require a compromise between purity and yield.

Affinity resins efficiently remove host cell proteins (HCPs) and DNA impurities from AAV but are challenged by the diversity of AAV serotypes. Not all capsids bind to currently commercially available AAV resins and those that do bind are recovered with variable yields, with published results ranging from 50% - 90% (9, 10).

The AAV polishing step functions primarily to separate capsid lacking DNA (empty) from plasmid containing DNA (full). While nucleic acid is packed within the capsid, its presence does alter the effective isoelectric point of the overall virus sufficiently for, in most cases, at least partial separation of the two species by ion exchange. Partial separation, however, forces an operational choice between yield and purity; a higher overall yield may be achieved with a lower purity of the full capsid; vice versa, a higher purity of full capsid may be achieved with a lower overall yield. As purity is typically prioritized, yield for this critical step suffers. Using ion exchange chromatography to separate empty from full capsids also increases development, validation, and scale-up times due to the sheer number of variables that include, but are not limited to format (resins, membranes, and monoliths), chemistry (strong or weak anionic and cationic groups at different ligand densities), and separation conditions (pH, conductivity, buffer composition, gradient, or step elution).

Affinity ligands have not yet been developed for lentivirus, leaving ion exchange chromatography as the primary method and size exclusion chromatography (SEC) a sub-optimal secondary method. Forgoing traditional concentration and diafiltration by UF/DF after harvest and processing clarified conditioned through membrane-based ion exchange adsorbers provides an alternative solution for

lentivirus purification. The smaller elution volumes that follow membrane chromatography then allow a second column chromatography step to be executed at a slower flow rate. Filtration steps in the downstream process, such as clarification using depth filtration (11, 12) and product concentration and formulation using tangential flow filtration (TFF) (13) also frequently lead to product loss. Adsorption to the filter media, shear stress and hold-up volume all represent mechanisms of product loss or inactivation during processing.

Process safety

Technologies for GTM 1.0 technologies lack adequate tools for contaminant removal and process safety. Because the product itself is a virus or viral vector, therapeutic protein-based contaminant (bacteria or adventitious viruses) removal techniques such low pH viral inactivation and sterile/virus filtrations are not always compatible. This drives a strong need for contamination prevention over removal.

Furthermore, analytical technologies for in-line monitoring of the product critical quality attributes (CQAs) are limited for gene therapy. In general, analytical methods become more complicated with increasing molecular complexity. Methods such as mass spectrometry required decades to adapt from small molecules to recombinant proteins. Analogous adaptions of existing methods and new analytical technologies will be required for gene and cell therapy. Those methods will then need to be brought in-line to monitor the critical quality attributes (CQA) because pH, conductivity and pressure conditions do not directly measure gene and cell therapy product quality. Most gene therapy analytical methods are conducted off-line and some of them, such as quantitation of AAV capsids by transmission electronic microscopy (TEM), can take up to two weeks.

Leaping to Gene Therapy Manufacturing 2.0

Benefits of intensified suspension cell culture with producer cell lines

Several gene therapy companies have already identified the need to upgrade GTM 1.0 to fulfill the supply-demand gap. Innovative technologies are being rapidly adopted and implemented to fast-track the transition to GTM 2.0, with some significant gains already realized.

Most efforts towards GTM 2.0 focus on increasing cell culture productivity using approaches like those developed for therapeutic proteins. Many of those historical improvements, such as media optimization and feed strategies led to incremental gains, rather than transformative, change. The most critical cell culture step for gene therapy to achieve, which also happened in recombinant protein production, will be to transition adherent cells to a suspension mode. While adherent cells provide rapid access to modest amounts of material with a facile harvest method that reduces impurities, scale-up challenges prevent broad applicability at either the clinical or commercial manufacturing stage. Repligen data indicates that 60% of gene therapy companies have already transitioned from adherent platforms to suspension mode, 30% are supporting both platforms and 10% remain committed to adherent. Investment in adaptation to suspension cells can provide significant benefits towards facilitating process scale-up, expanding cell culture volume and increasing virus titers. Suspension cells also add an element of safety as they are typically grown in serum-free and chemically-defined media. The lower virus titers often observed in suspension mode, however, necessitate the use of an additional solution for further process improvements.

Cell retention devices, such at the XCell ATF® technology, can increase bioreactor viable cell density (VCD) to greater than 300 x 10^6 cells/ml and this, in turn, can be applied to increase the overall capacity of a facility in several different ways. When connected to the production bioreactor, a cell retention device can be applied to augment productivity in either a discrete or continuous mode. In a discrete mode, the cell retention device enables multiple harvests at defined time points from a single bioreactor, often referred to as “fed-batch intensification.” In a continuous perfusion mode,

product is harvested from the bioreactor throughout the production run, commonly referred to as "perfusion." In either mode, the production bioreactor output per day increases while downtime decreases.

The cell retention device further intensifies upstream operations by executing both the harvest and clarification steps at the same time. Each cell retention device contains a 0.2 - 0.65 µm filter, through which conditioned media passes as permeate. Harvested material may therefore pass directly from harvest to chromatography operations, without necessitating centrifugation or depth filtration, creating additional processes efficiencies.

Cell retention devices can also be used to increase cell densities of seed train bioreactors. A recent study by Cytovance® Biologics demonstrated an order of magnitude productivity gain during viral vector manufacturing by increasing VCD. VCD for the standard cell culture volume reached ~1.3 x 10^6 cells/ml whereas VCD for the intensified processes increased 10-fold to ~1.2 x 10^7 cells/ml. The increase in VCD translated to an even greater increase in harvested capsids. The standard fed-batch process yielded 1 - 3 x 10^{10} capsids/ml, the XCell ATF® intensified process increased that yield approximately 100-fold to 4-10 x 10^{12} capsids/ml.

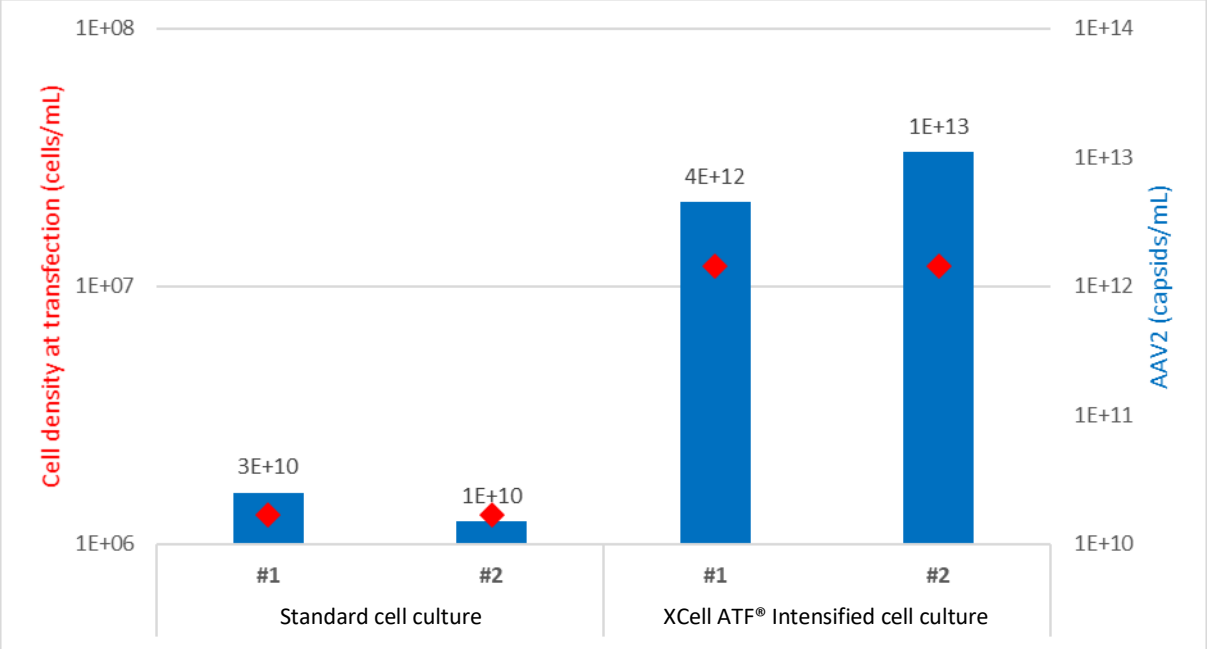

Figure 1. Intensification of AAV2 production process with XCell ATF® (data adapted from (14), courtesy of Cytovance® Biologics). The AAV2 productivity of a perfusion process using the XCell ATF® cell intensification device (immediately prior to` transfection) increased capsid production > 100-fold relative to the standard batch process.

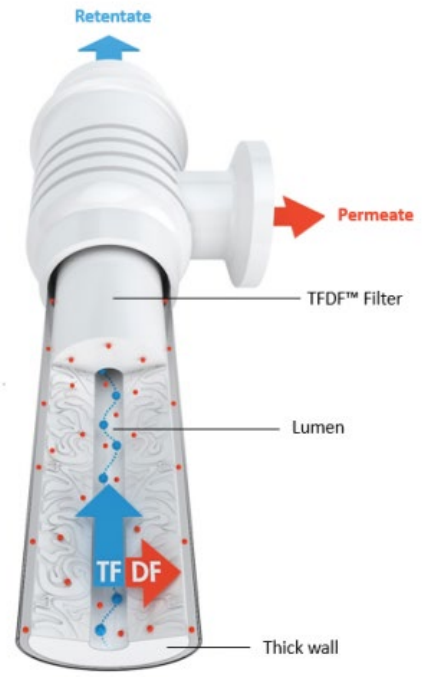

Figure 2. Tangential flow depth filtration (TFDF®) operates a depth filter in tangential mode. Cell culture medium passes through the lumen of the tubular depth filter. Permeate passes through the depth filter wall and exits a permeate port. Retentate exits the lumen and directed back to the bioreactor.

Cell retention can also be achieved using the novel TFDF® technology, which has already demonstrated exceptional performance increasing lentivirus productivity. TFDF® technology combines tangential flow and depth filtration in a single-use and closed filtration device. A thick-walled tubular depth filter combined with a 2 - 5 µm pore size captures cells and cell debris with negligible product retention. Tangential flow reduces fouling by directing the majority of cells and cell debris towards the retentate and away from the filter (Figure 2). The unprecedented combination of tangential flow and depth filtration synergize into a highly clarified solution that is robust towards fouling. A recent publication from Oxford Biomedica describes a more than two-fold lentiviral productivity increase compared to the standard batch process harvest using TFDF® (15). For a single harvest of a standard fed-batch bioreactor, depth filtration yielded ~70% yield while harvest with TFDF® was ~90%. More importantly, because TFDF® functions in tangential mode with low shear stress, rather than as a dead filter, intact cells are retained in the bioreactor. Replenishing the bioreactor with fresh media enabled the cells to continue virus production with a second harvest just a few days later. The combined benefits of a higher yield per harvest and the extension of a second harvest culminated in an ~250% TFDF® yield increase relative to the depth filtration based single harvest process. TFDF® technology can also reduce the risk of virus inactivation by enabling a series of rapid harvests that remove product from the bioreactor as opposed to accumulation of the lentivirus in the bioreactor with high cell density and elevated temperature.

To date, cell culture intensification has been primarily applied during cell expansion prior to transfection in transient systems. The development of stable cell lines that eliminate the need for plasmid transfection will help realize the full benefit of cell culture intensification. Early examples of stable cell lines for both lentivirus (6, 16) and AAV (9) have been reported. Packaging cell lines with viral capsid genes has also been presented as a viable means of reducing dependency on the transfection step. They will still require transfection of the plasmid carrying the gene of interest, but a single plasmid transfection would greatly simplify the process and reduce the overall manufacturing timeline as compared to the current multi-plasmid transfection process. Transition

from transfected transient expression with adherent cells to a stable expressing suspension platform could potentially supply the ten or even 100-fold upstream productivity required to define GTM 2.0.

High throughput tools for Rapid downstream process development

Order of magnitude increases in upstream viral vector yield will require comparable step change improvements in the downstream process. Clarification, an often overlooked opportunity for process intensification, can now be addressed by multiple technologies to increase yield, decrease resource requirements, or couple clarification directly with harvest. One of the newest filtration modes developed, TFDF®, can be applied to clarification (as well as bioreactor intensification). By directing the vast majority of cells back to the bioreactor in a circulating loop, rather into a dead-end depth filter, TFDF® profoundly increases filter capacity and flux (Figure 3). The increased filter capacity allows TFDF® to clarify high cell density cell cultures with a small filter surface, which is important as cell densities continue to increase. Simply scaling the surface area of a traditional depth filter to accommodate higher bioreactor cell density, dramatically escalates clarification resource requirements that include: operator time, cost, buffer, WFI, suite space and GMP storage space . Reduction of the filter size ten-fold reduces each of these considerations on a case-by-case basis and can play a key role in intensifying not only a process, but an entire facility, to deliver more product per day per square foot of facility space. Moreover, the TFDF® filter also ships dry and gamma irradiated within an integrated flow path that does not require flushing with WFI or buffer.

Figure 3. KrosFlo® TFDF® System circulates cell culture media and cells in a loop from the bioreactor through the lumen of the TFDF® filter and back to the bioreactor. The reduced number of cells entering the walls of the tubular depth filter significantly increases capacity.

Development of downstream techniques specific to gene therapy entities would be highly beneficial, but as they are unlikely to be available in the next two to three years, rapid optimization of existing methods for this new application is required. High throughput tools offer an available and easily implementable means of compensating for the lack of time allocated to downstream process optimization rendered by speed-to-market prioritization. Miniaturized pre-packed columns, OPUS® RoboColumn® Columns being the most widely used, enable automated chromatography process development using a 96-well plate format and robotic liquid handling workstations. A large number of different resins, chemistries and purification process parameters can be screened in just a few hours with microliter sample volumes for each experiment. Pre-packed columns play a continued role during process scale-up. High-quality column packing with batch reproducibility save operator preparation time but, more importantly, facilitate validation, create scheduling flexibility and

accelerate tech transfer, ultimately making a significant time and CAPEX savings for the overall program.

Optimization of TFF for product concentration and formulation starts with the selection of either hollow fiber or flat sheet cassette as a filter format. Product sensitivity to shear stress represents the key parameter that guides this decision. Hollow fibers generate lower shear forces due to their open structure. The hollow fiber format is generally preferred for enveloped viruses like lentivirus, which can be easily inactivated from excessive shear stress. For more stable viruses like AAV, the cassette format is equally effective and, towards shortening process time, can even increases the filtrate flux with a limited filter surface area. Defining the molecular weight cutoff (MWCO) of the membrane or fiber dictates sieving and, consequently, yield as well as the level of impurity removal. Large viral vectors like lentivirus (~120 nm) can be processed efficiently with 500 - 750 kDa MWCO membranes. Smaller viruses, such as AAV (~25 nm) pair best with a MWCO between 100 and 300 kDa. Optimization of process parameters (transmembrane pressure or TMP, cross flow, flux) then minimizes processing time and membrane surface area. In tandem with selection of automated system hardware at the appropriate scale, yield will be maximized, time will be reduced and process deviations due to human error mitigated.

Closed and single-use flow paths for improved process safety

Keeping the unit operations as closed as possible will reduce the risk of contamination. Gamma-irradiated, single-use, pre-assembled, process flow paths offer a low bio-burden, convenient and immediately implementable solution for contamination mitigation. The capabilities of the supply chain to generate diverse configurations are expanding. ProConnex® Flow Paths, for example, can be built from a library of over 250 components, including tubing, bags and/or containers, filters, sensors, pump heads and sterile connectors. The flexibility in flow path design enables the benefits of a closed system to be applied to a broad set of process variations throughout the workflow.

Better analytics for improved process monitoring

Process analytical technologies for in-line process monitoring provide process safety, operational convenience and also make the process more robust. By nature of the chemical composition, viral vectors require CQAs different than mAbs and proteins, therefore demand distinct analytical techniques for CQA monitoring. Process parameters commonly used for therapeutic protein processes, such as pressure, pH, conductivity, and UV are also used for viral vector processes, but they are not effective indicators of product stability or purity.

As an example, protein concentration during a UF/DF unit operation is typically determined by withdrawing a sample from a flow path followed by an error prone and time-consuming cuvette based UV-Vis Spectrometer measurement. Such methods create a challenge during the end of concentration unit operations when small changes in volume can impart large changes on product concentration. The tedious and iterative process of withdrawing a sample, diluting the sample, and measuring concentration forces the operator to balance process control against process time. If process control is prioritized, then concentration will be halted until analytical data is available, leading to an extension of overall time to completion. Alternatively, if process time is prioritized, then concentration will be allowed to continue during analysis with creation of a lag between the actual product concentration in the experimental flow path and that analyzed in the UV-Vis spectrometer, potentially leading to overconcentration.

UV-Vis spectroscopy measurements have been greatly simplified with the development of Slope Spectroscopy® technology, which removes the need for serial dilution during sample preparation. The Beer-Lambert equation (A=e*C*l). describes absorbance(a) as the product of the extinction coefficient (e), concentration (c) and path length (l). Slope Spectroscopy® technology varies the path length while keeping the sample concentration fixed, as opposed to traditional UV-Vis methods that

maintain a fixed path length while varying sample concentration (through serial dilution). Streamlining this one part of the analytical method creates multiple cascading benefits at both the method and program levels. The UV-Vis standard operating procedure (SOP) document length significantly decreases and time to result shortens from one to two hours to five to ten minutes. The faster time to result allows nearly immediate access to analytical data for critical decision making, enabling the concentration unit operation to complete quickly with robust monitoring. For processes that are transferred between multiple groups and/or sites, this single step towards simplification and robustness can save months of tech transfer time through removal of idiosyncratic operator pipetting and serial dilution steps.

Slope Spectroscopy® technology recently took another significant step forward towards GTM 2.0 with engineering of an in-line instrument that integrates within a UF/DF flow path (Figure 4). In-line monitoring allows unparalleled process control, which can be an important factor for stability compromised therapeutics such as viral vectors. Initial data acquired during an AAV concentration and diafiltration unit operation using a FlowVPE® Slope Spectrometer® demonstrated correlation between in-line acquired UV-Vis data and off-line acquired digital droplet QPCR (ddQPCR) (Figure 5).

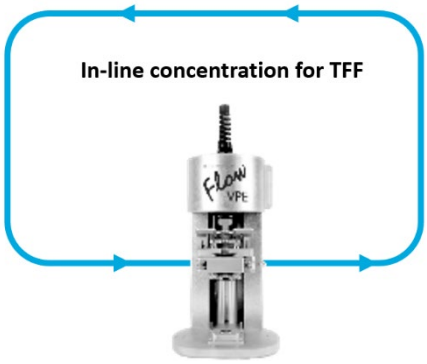

Figure 4. The Flow VPE® Device integrates into a TFF flow path for in-line measurement of product concentration.

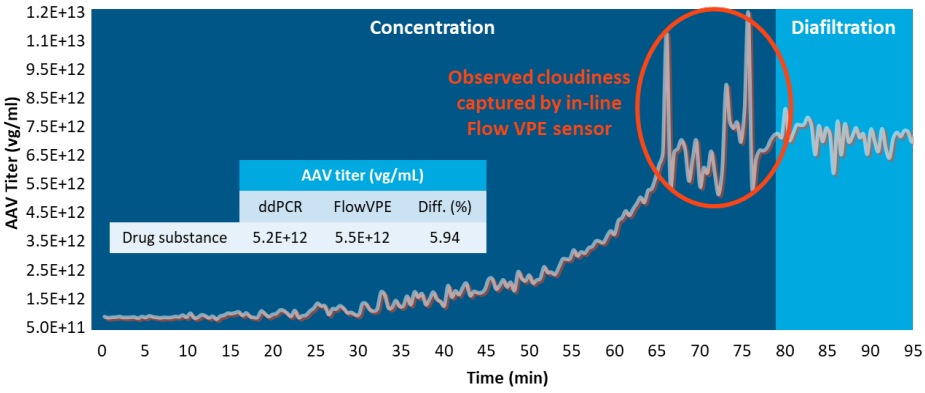

Figure 5. In-line measurement of AAV titer during final concentration and diafiltration process steps using the Slope Spectroscopy® FlowVPE® technique. In-line monitoring enables rapid adjustment as process deviation occurs (e.g. cloudiness observed, indicating potential virus aggregation, requires stopping concentration and/or changing buffer).

Monitoring of the concentration step from 0 to 65 min followed a steady increase in titer from 1.0 x 10^{12} vg/ml to approximately 6.0 x 10^{12} vg/ml. Deviation of the smooth increasing curve to a signal spike at 65 min highlights the value of in-line monitoring as it coincides with sample cloudiness that

could indicate virus aggregation. With this observation, diafiltration was initiated at 80 minutes. In early development, accurate determination of the critical aggregation or precipitation concentration for an SOP can save significant time and material. In a manufacturing setting, access to real-time data improves product quality, reduces process deviations, and accelerates the overall process time.

Conclusions

The manufacturing processes that enabled the first landmark gene therapies to successfully complete clinical trials and regulatory approvals lack the efficiency and productivity required to meet current, let alone future, demand. These processes, referred to here as Gene Therapy Manufacturing 1.0 (GTM 1.0), limit productivity and scalability with transient expression, adherent cell culture, low purification yields and off-line measurement of CQAs. Process optimization of existing methods and innovation of new technologies will lead us to Gene Therapy Manufacturing 2.0 with a ten to 100-fold increase in overall productivity. A that point, stable cell-line producer cells, perfusion intensified suspension cell culture, product specific affinity resins, fully closed downstream operations and novel analytical technologies will mostly likely be incorporated. Emergence Of Gene Therapy Manufacturing 2.0 within the next five years will help secure the supply chain of gene therapies and help address currently unmet life-threatening diseases.

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