PALL Biotech

White Paper

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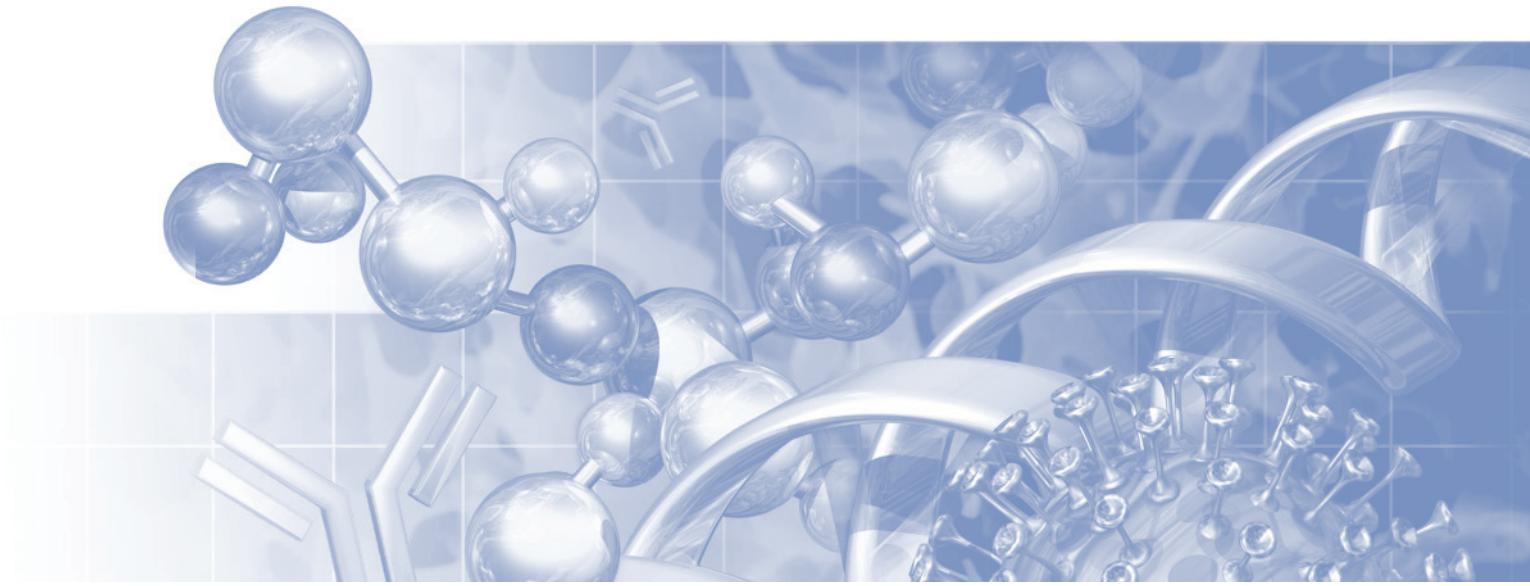
An end-to-end process for large-scale adenovirus manufacturing for gene therapy



*Filtration. Separation. Solution.*SM

Abstract

Recent advances in personalized regenerative medicine have been significant. However, further developments are needed to support market and regulatory demands, particularly the development of manufacturing processes to support large scale batches of high quality viral vectors. Here we describe the development of a robust integrated adenoviral manufacturing platform using adherent cells. A step-by-step scalable approach was implemented starting from laboratory scale to develop a fully integrated and reproducible process. Such a well-controlled operational manufacturing process allowed the production of large amounts of high quality viral vector in compliance with GMP requirements suitable for the clinical trials. Moreover, it was also possible to apply the same manufacturing process to more than one recombinant adenoviral vector without further optimization thereby accelerating therapy development time and reducing overall development costs.

Introduction

With over 650 clinical trials reported[1], and with the recent approval of autologous cell therapy products such as Kymriah♦ and Yescarta♦, and gene therapy product Luxturna♦, there is little doubt that cell and gene therapy is booming. As the technology matures scientifically, therapies with viral vectors are advancing through clinical trials towards commercialization, bringing an increasing demand for preclinical and clinical grade viral vectors[2]. Expansion of viral manufacturing capacity requires scalable production methods and manufacturing systems that are tailored to specific viruses. This, in turn, will facilitate the production of large quantities of virus needed to support commercial demand in full compliance with regulatory requirements[3].

So, what are the limitations? The main bottleneck for viral vector manufacturing is scalability – both upstream and downstream. Adherent cell culture vessels such as flasks, cell stacks and cell factories are difficult to scale up due to the manpower required for handling, as large numbers need to be manipulated, leading to an increased contamination risk, and decreased reproducibility and yield. Thus, scale up requires switching either to a suspension system or to higher density adherent cell culture bioreactors. Downstream purification methods must also be scaled to match upstream output. This usually requires large process modifications, such as the replacement of a centrifugation step with chromatography. However, even when equipment is available in relevant sizes, maintaining product quality and yield through optimized conditions at each step often remains a challenge[4]. For upstream development, accurate control of parameters such as temperature, availability of nutrients, and other environmental factors is crucial throughout the process to prevent cell stress and aggregation, in particular for adenovirus production[5,6].

This whitepaper describes the development of a robust, scalable and reproducible GMP-compliant virus expression and purification platform for a regenerative cell therapy company Orgenesis♦ to support both preclinical studies and a Phase I clinical trial with ten patients. Orgenesis is developing an adenovirus-based therapy for diabetic patients using patients' liver biopsies to generate Autologous Insulin-Producing (AIP) cells similar to beta cells found in the pancreas. Cell reprogramming is achieved by transducing liver-derived cells with three recombinant adenoviral vectors each coding for one of the following transcription factors: PDX-1, NEUROD1 and MAFA. The manufacturing process of these three recombinant adenoviral vectors (Ad5) was transferred from cell culture flasks, to the iCELLis® Nano bioreactor, a scalable, industrial plug-and-play fixed-bed bioreactor. Furthermore, a scalable downstream purification process was developed involving depth filtration, ion exchange chromatography, tangential flow filtration and sterile filtration.

The challenge

For Orgenesis' cell therapy, liver-derived cells are taken from the patients prior to being expanded and transdifferentiated following sequential transduction of three recombinant Ad5 vectors carrying the genes *Pdx-1, NeuroD1 and MafA*. The combined expression of those three genes induces differentiation of the cells into AIP cells, which are then transplanted to the patient via implantation, to express insulin upon glucose challenge in diabetic patients.

The clinical protocol requires 2 x 10^7 liver-derived cells from the biopsy in order to be able to transplant 2 x 10^8 AIP cells after expansion and cell reprogramming. The challenge here was to produce sufficient quantities of highly purified viral particles to transduce all the patient's liver-derived cells: up to 2 x 10^{12} IFU of each recombinant adenovirus are required in order to treat each patient. As the virus to cell ratio was different for each virus, the total amount of adenovirus needed for a Phase I trial enrolling ten patients in one clinical site, was 7.2 x 10^{13} IFU for Ad5-Pdx-1, 1.8 x 10^{13} IFU for Ad5-NeuroD1 and 3.6 x 10^{12} IFU for Ad5-MafA.

Overview of the large-scale virus manufacturing process

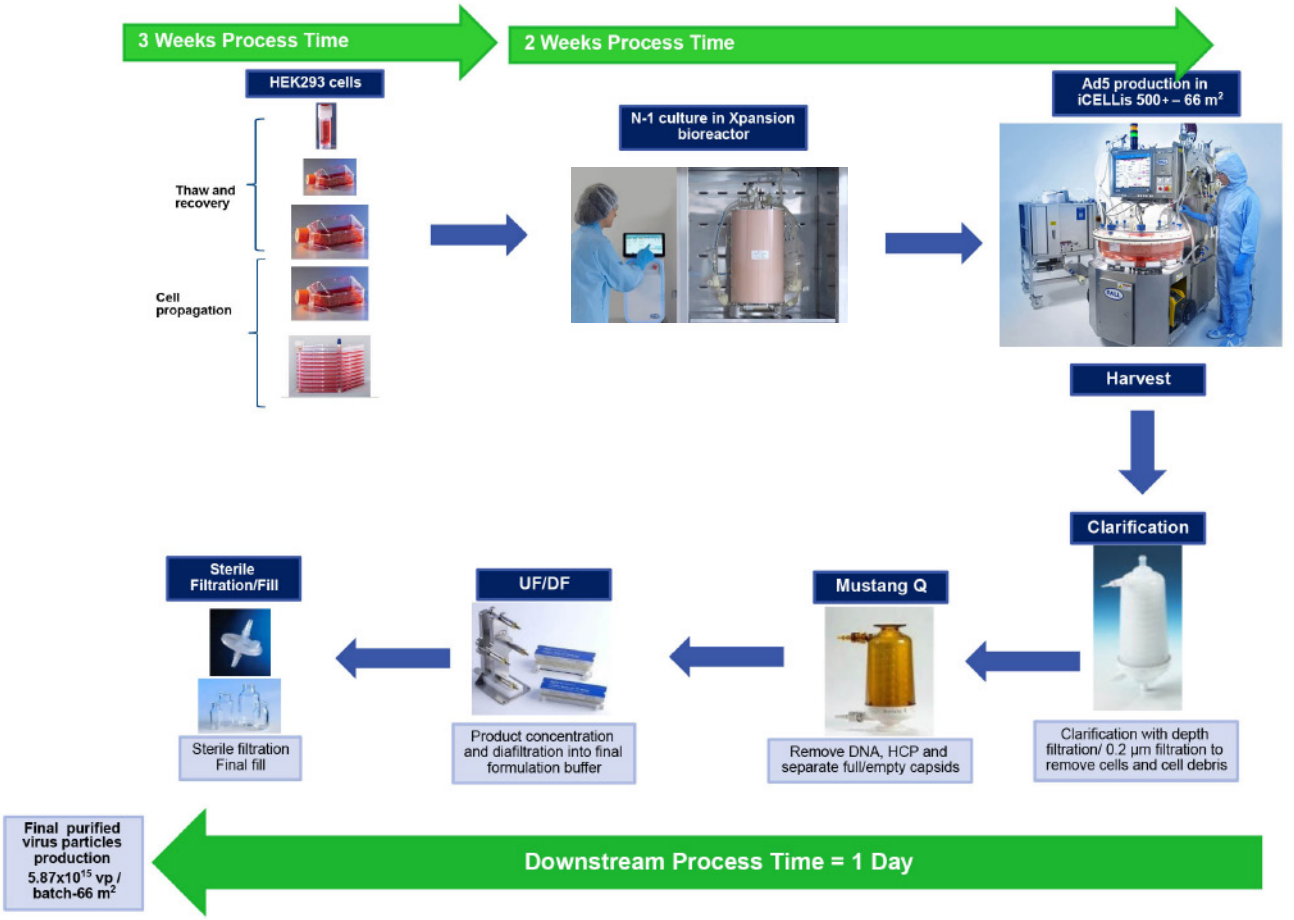
The development of the manufacturing process included:

1) The development and optimization of a robust large-scale adenovirus production process for adherent cells, applicable to all three adenoviruses and yielding high viral titers, including a scalable seed train.
2) The development and optimization of a purification process in order to obtain a high quality adenovirus preparation.

Figure 1 describes the efficient fully integrated industrial recombinant adenovirus manufacturing process, which takes approximately five weeks for one production run.

Figure 1

Detailed steps of a fully integrated adenovirus virus manufacturing process using an iCELLis 500+ bioreactor. The HEK293 cells are first propagated and expanded in T flasks then transferred to the Xpansion™ 200 bioreactor, prior to seeding in the iCELLis 500+ fixed-bed bioreactor. Those cells are infected with a 'hot' starter virus preparation grown separately in the iCELLis Nano bioreactor. The recombinant adenovirus, expressed in the cell culture medium is then harvested through a series of purification steps.

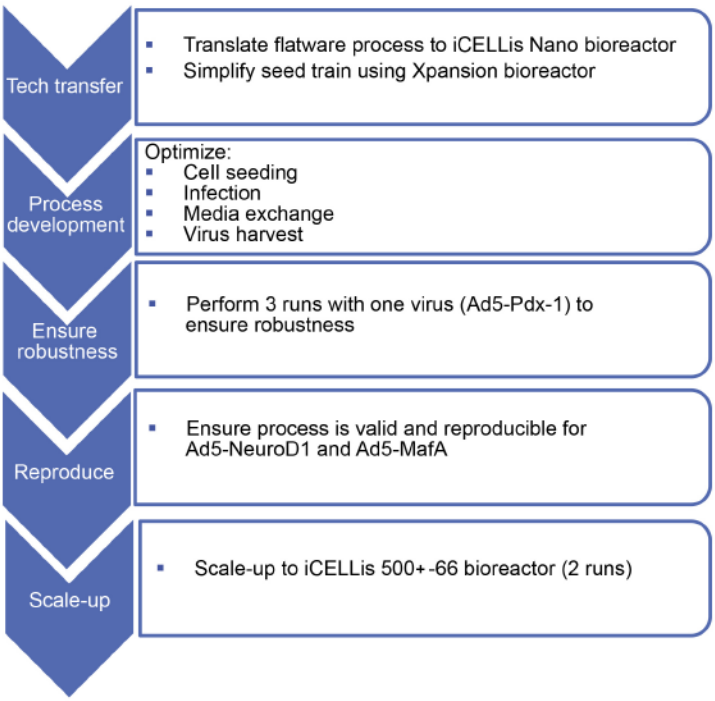


Upstream process development: Linear scalability and increased productivity

The process to transfer a laboratory technology to an industrial manufacturing scale is shown in Figure 2.

Figure 2

Upstream process development. From flatware to iCELLis 500+ bioreactor.



1) **Tech transfer:** First the flatware cell culture process needs to be reproduced at Pall facilities. In this work, the T175 culture system was replaced with an easy to manipulate, disposable large-scale adherent bioreactor, the iCELLis bioreactor, which is well suited for the production of a wide range of viruses, including oncolytic viruses, lentiviruses, adenoviruses and AAV[6,7,8]. It is an easy to manipulate, three-dimensional, controlled and perfusable system composed of polyester fiber carriers with low shear stress for adherent cells. As a result, cells can grow to high cell density while maintaining optimal gas exchange and nutrient supply. In addition, when used in recirculation mode the bioreactor prevents the accumulation of by-products such as ammonia and lactic acid, thereby improving cell culture conditions and cell growth. This in turns increases infection efficiency and subsequent recombinant virus expression. The bioreactor is available in different formats, facilitating the scale-up process: the iCELLis Nano bioreactor; a small benchtop model designed for initial process development; and the iCELLis 500+ bioreactor for large-scale production. Each one can be fitted with different sizes of macrocarrier beds for a range of cell growth surfaces. For the process developed here, the virus production protocol was first optimized on the iCELLis Nano bioreactor prior to transfer to the iCELLis 500+ bioreactor.

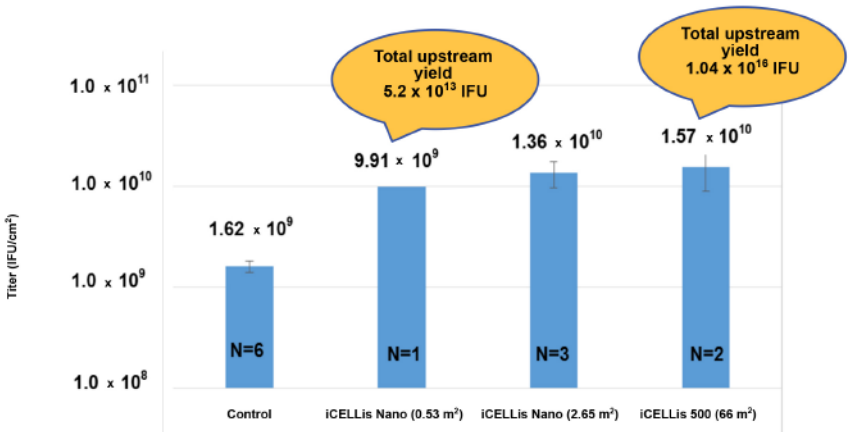
2) **Seed train simplification:** Due to the high cell density required for virus production in the iCELLis bioreactor, the initial propagation and expansion of the HEK293 cells also needs to be optimized. It is crucial to obtain the right cell density and to ensure the cells are in the exponential growth phase for adaptation and infection in the larger iCELLis 500+ bioreactor. For this scale up, the propagation was performed in the Xpansion bioreactor, a single-use bioreactor with stacked multi-plates, also available in different formats. It has an automated aeration system, ideal for large-scale production of adherent cells. With a surface up to 122,400 cm^2, the larger model Xpansion 200 bioreactor allows the propagation of cells to seed the iCELLis 500+ bioreactor.

3) **Process development:** After seeding and growth into the iCELLis bioreactor, cell culture conditions were optimized to achieve similar or higher productivity. Key process parameters were defined including media, temperature, pH, media linear speed (which affects nutrient usage by the cells and gas exchange), aeration etc., in preparation for infection. The HEK293 were grown for 4 days prior to infection, which was performed using a starter viral preparation. For the large production runs in the iCELLis 500+ bioreactor, the starter virus was grown in the iCELLis Nano bioreactor. Following infection, the cells are then grown under stringent temperature and media conditions (132 L recirculation media feeds into the iCELLis 500+ bioreactor - 66 m^2 scale) for three days before harvest and purification of the recombinant adenovirus (see below for downstream purification process).

4) **Reproducibility:** Once the bioreactor conditions were established, three production runs were performed for the Ad5-Pdx-1 adenovirus in the iCELLis Nano bioreactor using different bed depths/cell culture surface, to demonstrate the robustness of the process. The viral titer obtained was higher than that obtained in cells stacks 10 (CS10). The increase in viral titer from the flatware control to the iCELLis bioreactor is attributed to various key process parameters including the increase in cell density achieved through well-controlled and optimized cell culture conditions in the bioreactor (Figure 3).

Figure 3

Recombinant Ad5-Pdx-1 viral titers obtained following virus production in different size vessels and bioreactors: T 175 flask (control), iCELLis Nano bioreactor with 2 cm fixed bed (0.53 m^2), iCELLis Nano bioreactor with 10 cm fixed-bed (2.65 m^2), iCELLis 500+ bioreactor with 2 cm fixed-bed (66 m^2).



5) **Versatility:** The experimental parameters established for expression of Ad5-Pdx-1 in the iCELLis Nano bioreactor were used to express other recombinant adenovirus, namely Ad5-NeuroD1 and Ad5-MafA. Comparable viral titers were obtained from these runs compared to the optimization runs showing the versatility and the reproducibility of the production platform that can be applied to a wide number of viral vector therapeutic candidates (data not shown).

6) **Scale up:** Production was then up scaled in the iCELLis 500+ bioreactor with two runs expressing recombinant adenovirus using the optimized conditions previously developed in the iCELLis Nano bioreactor. The viral titer was comparable to that obtained at the small-scale bioreactor, demonstrating that the experimental parameters are easily transferable to a larger vessel. Furthermore, when scaling up 125 fold, from iCELLis Nano bioreactor 0.53 m^2 of cell culture surface to iCELLis 500+ bioreactor – 66 m^2, the specific productivity of viral vector remained of the same, demonstrating the linearity and robustness of the process.

Downstream process development: Effective adenovirus purification

Downstream processing is where most protocol modifications are required during scale up (see Figure 4 for a comparison between conventional laboratory and industrial purification). During the purification steps, the recombinant adenovirus secreted in the cell culture media is separated from any cells or debris, cell proteins, virus-related impurities and residual DNA. Inactive empty and incomplete viral capsids are also separated from full capsids as they reduce the infectious viral titer. The viral preparation is then concentrated and transferred to the final formulation buffer. Finally, the viral preparation is filter-sterilized and filled into vials as the drug substance.

The conventional downstream processing for virus purification is monotonous, labor-intensive and time-consuming, taking 3 to 4 days (Figure 4); virus recovery yields are also insufficient.

An outline of the industrialized process is as follows:

1) **Depth filtration:** Viruses are harvested directly onto a depth filter (Supracap™ 100 capsules or Stax™ capsules depending on the scale) for clarification.
2) **Ion exchange membrane chromatography:** The clarified virus preparation is then loaded onto an anion-exchange chromatography membrane (Mustang® Q) for effective removal of the double-stranded DNA and cellular proteins from the preparation (Figure 5). In addition, inactive empty, incomplete or broken viral particles that occur during virus assembly are also removed by exploiting differences in surface charge between full, and empty or broken particles. Figure 6 shows the enrichment of full viral capsids in the preparation. Overall, Ad5 virus recovery was > 95%.
3) **Tangential flow filtration (TFF):** The virus preparation needs to be concentrated and the buffer exchanged. This step was carried out using the Cadence™ TFF system, which concentrated the virus preparation five-fold.
4) **Sterile filtration:** Finally, the concentrated virus was passed through a 0.22 µm filter.

The overall virus recovery was higher than 65% at the end of the purification process. This process can be scaled to meet patient demand effectively, particularly as purification time is now reduced from three to four days, to just one day.

Figure 4

Steps of virus purification. Comparison of conventional (laboratory) and industrial downstream processes.

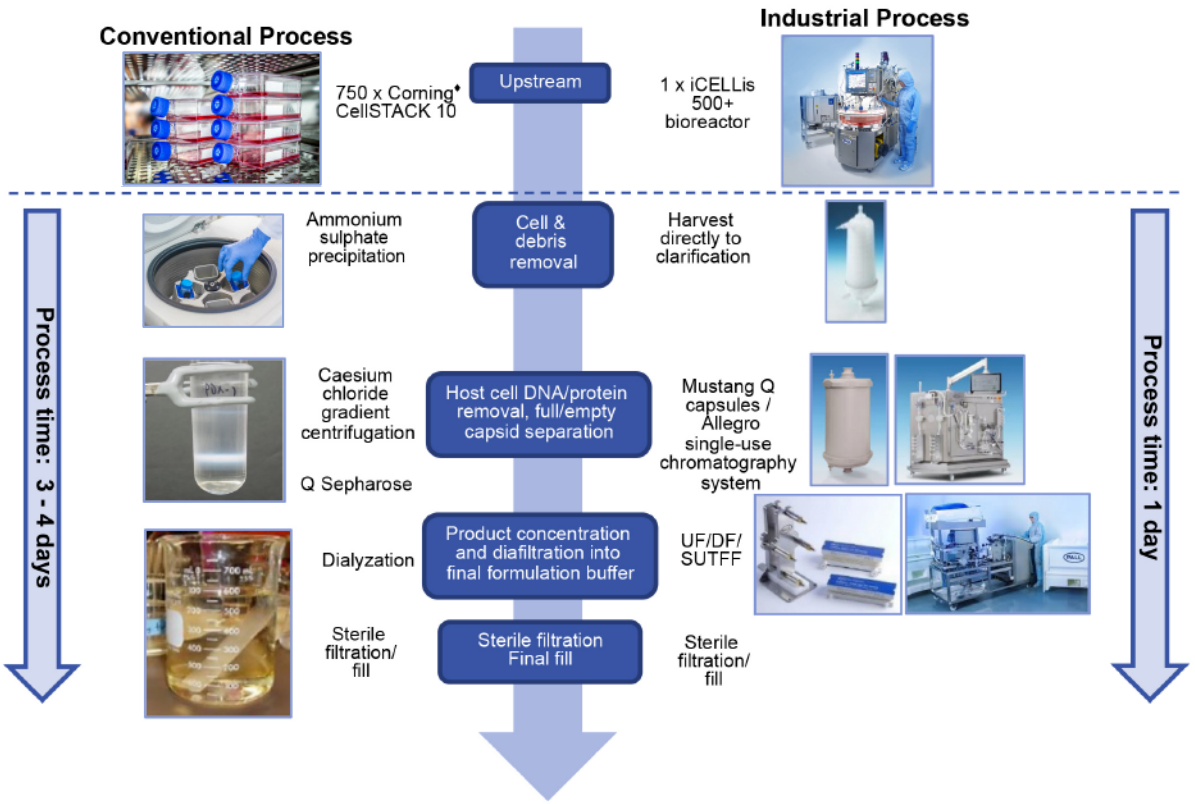


Figure 5

Host cell protein (HCP) and DNA clearance (dsDNA) of the Ad5-Pdx-1 viral preparation as measured before and after ion exchange membrane chromatography using Mustang Q.

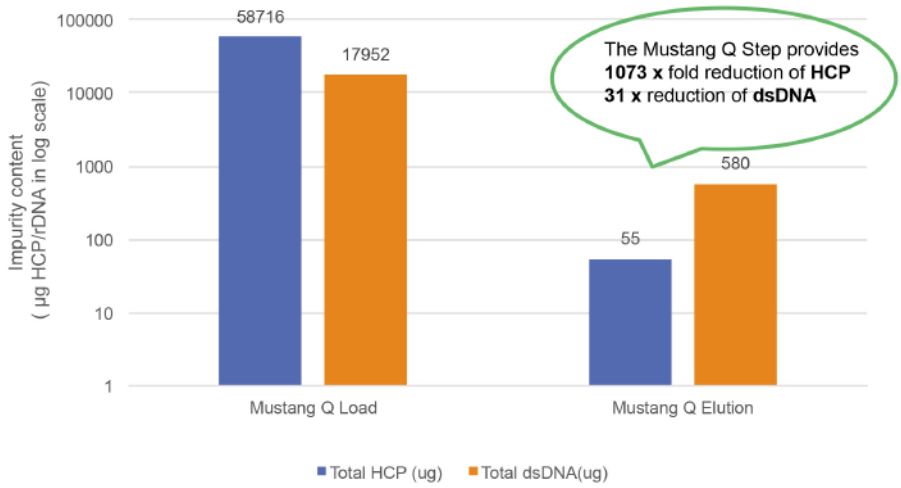


Figure 6

Transmission electron microscopy of adenovirus preparation to evaluate full, empty and broken capsids in the sample post Mustang Q capsules elution. A. Mustang Q load. B. Mustang Q elution.

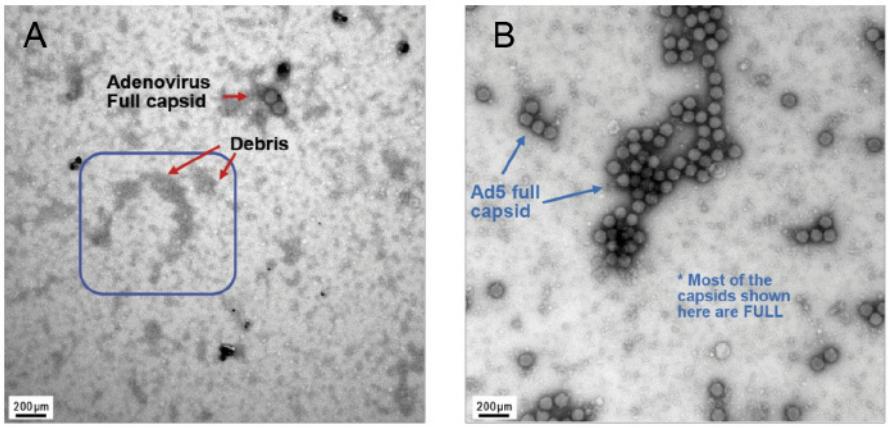
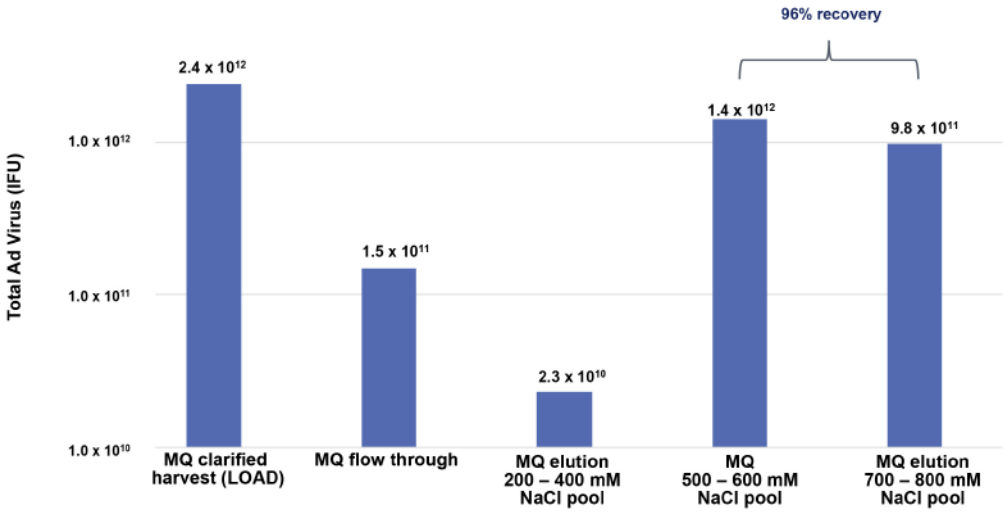


Figure 7

Ad5-PDX-1 Virus infectivity using the Adeno-X♦ rapid titer kit, before and after ion exchange chromatography using a Mustang Q chromatography capsule, in the clarified preparation, the flow through and three pooled eluted fractions.



Final virus preparation: Quality and functionality

Manufacturing for clinical purposes requires the production of highly pure and biologically active vectors that meet regulatory requirements.

The highly pure viral preparation was obtained through a rigorous downstream purification process based on ion-exchange chromatography. In addition to the volume reduction of about 15 x fold, this method selectively allowed clearance of serum and host proteins (1000-fold reduction), and removal of double stranded DNA (30-fold reduction as shown in Figure 5), leaving the preparation almost free of contaminants (Figure 8). This step also enabled efficient removal of empty and incomplete viral capsids, enriching the preparation for full viral capsids (90%), which are functional and able to transduce the liver-derived cells effectively without any cell toxicity (Figure 6 and Figure 9). For large runs, the overall purification yields were higher than 65%, showing the efficiency of the process and reaching the level of purity required for GMP-compliance in clinical trials.

A robust manufacturing process should also demonstrate batch-to-batch reproducibility with comparable activity of the final virus preparation. As shown in Figure 9, the recombinant virus batches produced in the iCELLis bioreactor were all functional as they transduce liver derived cells to deliver the genes of interest with comparable efficiency. The transduced genes were expressed (Figure 9A) and triggered expression of pancreatic genes that were not present in the liver cell initially (Figure 9B). This confirms that the adenoviruses are functional as they induced cell reprogramming converting liver cells into pancreatic cells. Furthermore, the consistency of the results at different Multiplicity of Infections (MOIs) shows the robustness of the process.

Taken together, these results shown that the activity and the purity of the large-scale virus preparations were comparable to those obtained using a conventional method. Once in place, the industrial manufacturing process, presents a significant gain in time for each run. Furthermore, given the ability to apply the manufacturing process to more than one vector, the process is efficient, reducing overall treatment development time and costs.

Figure 8

Purification of recombinant Ad5-Pdx-1 expressed in HEK293 grown in the iCELLis Nano bioreactor. The SDS-PAGE shows the purification steps from crude extract to final sterile virus preparation. The overall virus recovery yield in this experiment was 65%.

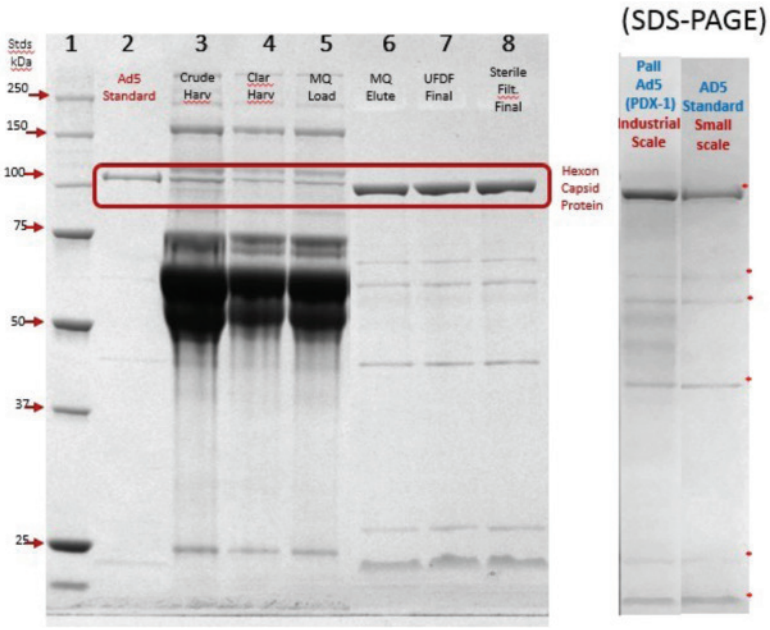
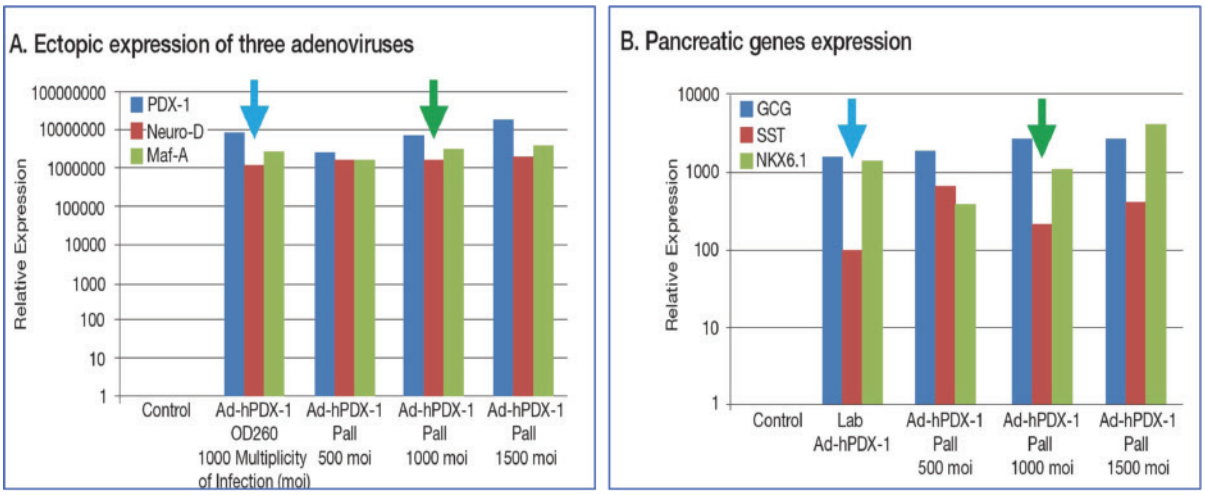


Figure 9

Gene expression as measure by real time PCR, following transduction of primary liver-derived biopsy cells using all three recombinant Ad5 vector subsequently. (A) Expression of PDX-1, NEUROD and MAFA. (B) Expression of pancreatic genes (gcg, sst and nkx 6.1) confirming transdifferentiation of the liver-derived cells.



Conclusion

With the rapid development of novel regenerative medicine treatments, the need for virus production capacity has increased significantly. Manufacturers are now required to produce a range of highly purified and well-characterized viral preparations in large quantities to address market demand and regulatory constraints. To do so, they need to develop end to end, scalable and GMP-compliant manufacturing processes such as the one described here enabling the production of three large batches of purified recombinant adenoviral vectors. This production process is robust, reproducible, scalable and much faster. This is particularly advantageous for therapeutic companies such as Orgenesis, for which the availability of GMP-compliant scalable and transposable processes significantly accelerates clinical trials mitigating the developmental risks and associated costs.

Materials and methods

The viral vectors used were replication incompetent adenovirus serotype 5 expressing three different transgenes (Pdx-1, NeuroD1 and MafA) (Orgenesis). The recombinant adenoviruses were expressed in HEK293 (ATCC) grown in DMEM with 8% serum and grown for 4 days prior to infection.

The HEK293 cells were expanded in the Xpansion multiplate bioreactor. Viral vectors were produced using the iCELLis bioreactor systems of varying sizes. Small-scale runs were performed in the iCELLis Nano bioreactor with different bed-sizes: 0.53 m^2, 1.07 m^2, 2.65 m^2 and 4 m^2. Large-scale runs were performed in the iCELLis 500+ (66 m^2) bioreactor. The iCELLis Nano bioreactor full starter kit and iCELLis 500+ bioreactor full starter kit, with CPC connectors were also used in conjunction with the relevant bioreactor systems for large scale runs.

Each cell extract was clarified using a Seitz V100P depth filter in Supracap 100 capsules or in Stax capsules, followed by 0.2 µm filtration using Supor® EKV filters in a Mini Kleenpak™ capsule. A Mustang Q 60 mL membrane volume capsule was used for anion exchange chromatography. Each purified bioprocess fluid was then subjected to ultrafiltration/diafiltration (UF/DF) using a Cadence inline diafiltration module with 100 kDa molecular weight cut-off (MWCO). Finally, the virus preparation was sterile filtered through Supor EKV filters.

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