The COVID-19 pandemic has caused millions of deaths and devastated communities across the globe. Vaccines will play a key role in bringing the pandemic under control, with successful clinical trials, authorizations and roll-out having now occurred in several countries. However, large-scale manufacturing of such vaccines remains a bottleneck to delivering doses to billions of people at risk of infection, as well as producing new versions of the vaccines that target variants. Here we discuss the current status of manufacturing, focusing on the adenovirus-vectored vaccine developed by the University of Oxford and how the process for manufacturing it was developed.

**COVID-19**

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused the novel coronavirus outbreak that emerged in the end of 2019 (COVID-19) and led to the pandemic that continues to this day. The WHO reports that confirmed COVID-19 cases are approaching 200 million and deaths exceed 4 million, although both numbers are likely underestimates.

Historically, vaccination has been a successful approach to limiting or eradicating various infectious diseases. While there has been early success with several vaccines, it is unclear which immune responses are key to protecting from infection, transmission, disease and death, and how long such responses last after vaccination or natural infection. It is also possible that emerging variants may render these immune responses less effective, making eradication of SARS-CoV-2 challenging.

Several vaccine candidates have gained conditional approvals for use in multiple jurisdictions, including BNT162B2 (Pfizer and BioNTech), mRNA-1273 (Moderna), JNJ-78436735 (Johnson & Johnson) and AZD1222 (AstraZeneca and University of Oxford). All of these vaccines deliver genetic instructions encoding the spike antigen of the SARS-CoV-2 virus, which plays a key role in helping the virus enter our cells and was identified early as the most likely target of effective immune responses. BNT162B2 and mRNA-1273 utilize nucleoside-modified messenger RNA (mRNA), whereas JNJ-78436735 and AZD1222 are using genetically modified adenoviral vectors based on adenoviruses isolated from humans or chimpanzees, respectively.

One and a half years after the first reported cases of COVID-19, over 2bn doses of vaccine have been administered, despite many predictions in early 2020 that such an achievement was impossible. But with 10bn more doses needed, plus uncertainty over whether further boosters will be required to maintain immunity and improve protection against emerging variants, vaccine manufacturing remains one of the most vital challenges facing humanity today.

**Adenovirus-vectored vaccines**

Adenoviruses are an excellent platform for modern vaccine candidates due to their attractive biological properties: deletion of the E1 and E3 regions make them replication defective outside of the helper cells and gives a high-level assurance against generation of replication-competent virus; excellent safety profile across numerous clinical trials involving a range of diseases; multiple transgenes encoding the antigen of interest or other genes that enhance immune responses can be incorporated into the adenoviral vector; most importantly they induce potent antigen-specific cellular and humoral immunity in all age groups. However, when using human adenovirus these immune responses might be impeded by the pre-existing immunity to human adenovirus in the population; thus using simian-origin adenovirus will circumvent any problems of pre-existing immunity.

Once an adenovirus vector-based vaccine is developed to fight an infectious disease such as COVID-19, the manufacturing process is required to produce doses for clinical trials and vaccinating the world. As seen with the pandemic, the demand for doses against a new pathogen can be astronomical and push beyond the limits of global manufacturing capacity. The key attributes desired for an optimal manufacturing process are as follows:

1. scalable: a large number of doses will be required to vaccinate the world, therefore the process should work at a large scale (scale up);
2. robust: the process can be applied at any scale and consistently results in product that is within the specifications for quality;
3. simplicity: the ease of executing the manufacturing process influences how quickly it can be adapted to various manufacturing sites all over the world (scale-
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out) so that the vaccines can be made locally without relying on overseas supply;
4. economical: a good process will make the vaccine affordable not only for the high-income but also for low- and middle-income countries.

To ensure quality and safety of any vaccine, we need good manufacturing practice (GMP)-compliant production processes. GMP regulations ensure human medicines are manufactured appropriately and influence all steps of the process, including the quality of input materials and equipment, staff training, quality management systems, in-process quality assurance, and quality control testing. Regardless of the scale of manufacturing, GMP regulations must be met at all times.

Manufacturing adenovirus-vectored vaccines

For adenovirus-vectored vaccines, generally the drug substance manufacturing process can be separated into two parts: cell thawing and amplification of virus in the cells are included in the upstream process (USP) and virus purification is included in the downstream process (DSP).

The upstream process

To begin the USP, a replication-deficient master viral seed is required. This initial viral stock will be used to infect helper cells such as human embryonic kidney (HEK) 293 cells or PerC.6 cells, which express the adenovirus E1 gene that encodes viral activating factors required for virus amplification. Traditionally, this USP can be accomplished using cell culture flasks at laboratory scale, such as shake flasks for suspension cells, or roller bottles and T-flasks for adherent cells. However, this process is not scalable because to produce more vaccine you need more and more flasks. These processes also require a lot of optimizing for each individual vaccine being developed. At an industrial scale USPs based largely on suspension HEK293 cells and PerC.6 cell lines, grown in single-use, stirred-tank bioreactors, overcome the scalability problems. Although some optimization of the process is still needed, using larger and larger bioreactors (scale-up) tends to have little impact on the manufacturing process while massively increasing

Figure 1. Upstream process development in our lab with a parallel system showing two 1-L bioreactors. This system allows for batch or fed-batch experiments to be run in up to four bioreactors simultaneously, allowing us to compare the impact of altering parameters like MOI or timing of infection on vaccine yield.
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In order to generate an adequate number of cells to inoculate a bioreactor, a seed train is required for cell expansion into larger and larger volumes. The design of the seed train involves several optimized parameters of cell maintenance from the thawing of the master or working cell bank (MCB/WCB), volume of cells or vessel sizes (flask for pre-inoculation and different size bioreactors post-inoculation), density of the cells, and passaging time.

Once the targeted cell parameters are reached, the cells are then ready for infection with the virus (vaccine) seed. The amount of virus added to the cells is predetermined by the multiplicity of infection (MOI) – the ratio of infectious virus added per cell. The virus seed will replicate inside the helper cells by hijacking the cell machinery and utilizing the nutrients from the medium.

There are several different modes of upstream based on the source of nutrients which cells primarily utilize during virus amplification: batch, fed batch (Figure 1), or perfusion (Figure 2). In batch mode, the cells use the existing medium they have grown in as the primary source of nutrients. In fed batch, cells also utilize a supplement made up of concentrated nutrients, typically a mixture of amino acids, glucose, or protein hydrolysates, added to the medium. In perfusion, there is continuous exchange of medium with a controlled addition of fresh medium and removal of spent medium, maintaining a more constant environment for the cells throughout the USP. The suspension-adapted HEK293 cells are commonly used in batch-mode, stirred-tank reactor USPs, although the highest volumetric yields have been reported in high-density perfusion-based processes in PerC.6 cells.

Once the projected virus titre is reached, the infected cells are then ready to be harvested. Most of the virus will be cell associated; therefore, cell lysis is required to release the virus from the cells and cell debris. This can be achieved by repeatedly freeze-thawing the cell-pellet or mixing the cell culture in detergent containing lysis buffer. When lysing cells from a bioreactor, the addition of lysis buffer is the most effective method. During this process, an endonuclease such as benzonase is typically added to reduce the contaminating host cell-free nucleic acid to oligonucleotides of between three and five bases in length. The addition of benzonase is also important in reducing the virus aggregation and viscosity of lysate due to the release of nucleic acid during cell lysis.

The simple method of density gradient ultracentrifugation using caesium chloride (CsCl) can be used as the primary method to separate the host cell's contaminants from the virus; however, this conventional method is labour intensive, time consuming, and not scalable, all of which contribute to limited production throughput in a manufacturing facility. Therefore, large-scale manufacturing facilities typically perform adenovirus purification using several steps comprising clarification, followed by varying combinations of chromatography, ultrafiltration, and diafiltration.

Clarification may be carried out by a filtration step, typically with the use of depth filters, removing the cell debris and other impurities from the harvested adenovirus. Purification of adenovirus typically involves at least one round of anion exchange chromatography which is the step that removes the majority of the host cell proteins. This separation method utilizes the negatively charged residue of the virus which will bind to positively charged membrane or resins inside the column whilst the remaining contaminants will pass through the column. The virus can then be eluted from the column by increasing the concentration of salt in the elution buffer, displacing the virus bound to the negative ions. Ultrafiltration and diafiltration can be performed with the tangential flow filtration (TFF; Figure 3) technique. During TFF, small products of cell lysis, salts and soluble impurities are removed while retaining the virus and transferring it to a new buffer. TFF is a membrane filtration mode also known as cross-flow filtration, where...
the flow of the solution (feedstream) passes in parallel to the membrane whereas a portion of the solution may pass through the membrane (permeate) and the remainder of the solution (retentate) will be recirculated back to the feed reservoir. The molecules that are retained or passed through the membranes are largely determined by the molecular weight cut-off (MWCO) of the membrane of choice. In different adenovirus manufacturing processes there may be TFF steps before and/or after chromatography. The final stage of the DSP is a sterile filtration step. The resulting vaccine solution, referred to as the drug substance, is then put into vials for distribution, a process known as fill and finish.

Challenges in developing the USP for this vaccine included taking cells that preferred to grow on surfaces (adherent cells) and adapting them to growing floating in culture media in a bioreactor (suspension cells), finding the best media for growing cells that could be supplied at massive scales and optimizing the conditions to ensure maximal cell densities combined with vaccine yield per cell. We continue to optimize these conditions, e.g., looking at how different MOIs or the use of perfusion can increase yields.

The core technical challenges we faced in the DSP have been around optimizing the filtration and purification processes. The lysing of the host cells to release the vaccine generates a significant amount of debris that has to be removed by clarification before going through the purification process. The problem is that the debris can clog the filter causing vaccine losses, therefore, optimizing the filter throughput is critical for a robust DSP. As vaccine is pumped across a filter, the shear stress may cause the vaccine particles to aggregate or lose functionality, thus an optimized flow rate is needed.

Scale-up is always a challenge, as the process carried out in the lab using a 3-L bioreactor can have issues when trying to replicate it using 1000-L+ bioreactors. For example, during the filtering and purification DSP, the vaccine spends time being pumped across membranes and held between different steps. At lab scale, these processes are simple, they can be done quickly at room
temperature and the vaccine returned to the ideal 4°C in just minutes. However, the manufacturing scale process is slower, meaning that a combination of efficient working patterns and temperature-controlled storage and processes are needed. So as scale-up happens, maintaining vaccine quality by balancing the need to do things quickly to keep temperatures low, while not damaging vaccine particles by applying excessive shear stress, is important.

Scale-out, where we want identical vaccine products produced at multiple locations, raises challenges also. There can be equipment differences, such as different sites using different size bioreactors, which means the whole process must be robust enough to perform optimally across such variables. The speed at which this happened also applied pressure to supply chains, as the demand for equipment and consumables such as culture media and buffers was enormous.

**Conclusion**

Due to the pandemic, there is a drive for large pharmaceutical companies to develop efficient, robust and economical manufacturing processes to facilitate the migration of adenovirus-based vaccines from the bench to the bedside. The traditional methods of shake-flask-based USPs and ultracentrifugation-based DSPs are simple and robust; however, they are limited to laboratory-scale pre-clinical preparation and small-scale manufacturing for early phase clinical trials. The bioreactor-based suspension cell culture process is one way of increasing scale-up from the upstream side, whilst the combination of TFF and chromatography for DSP allows rapid, simple and low-cost purification with significant advantages over the CsCl method in terms of suitability in scale-up, and clinical translation (Figure 4). With minimal further optimization and validation for each individual vaccine, these platform technologies can be readily transferred into a GMP-compliant protocol that can yield large quantities and be rolled out to a range of manufacturers.

**Further reading**

Further reading (Continued)


Carina is a postdoctoral research scientist in vaccine development focusing on viral vector vaccines at the University of Oxford. She has been working as the lead scientist in process development for large-scale GMP manufacturing of the Oxford/AstraZeneca COVID-19 vaccine, which the team managed to develop in record time. Since then, that manufacturing technology has been transferred to multiple GMP manufacturing facilities around the world, enabling clinical trials and global vaccine supply to dozens of countries. Email: Carina.joe@ndm.ox.ac.uk

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