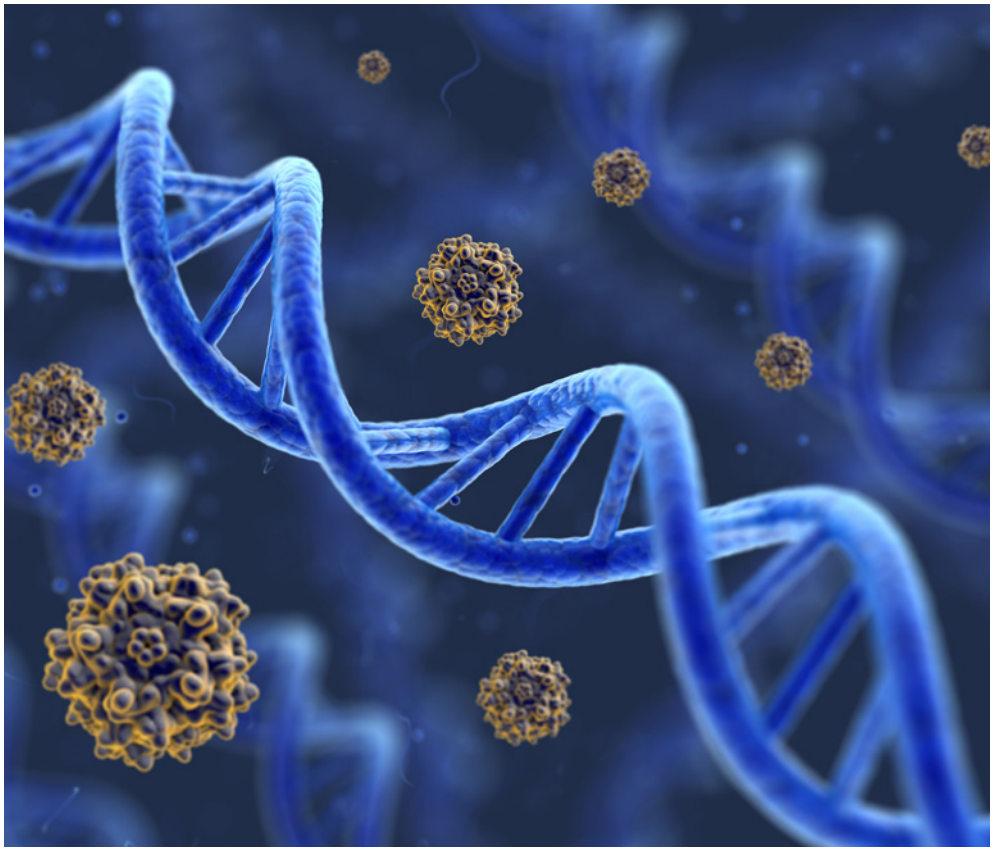
GROWING PAINS FOR GENE THERAPY MANUFACTURING

With the first gene therapies on the market and dozens more in trials, the race is on to improve the PRODUCTION PROCESS



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In December 2017, the US Food & Drug Administration (FDA) approved Luxturna from Spark Therapeutics — the first gene therapy to win market approval in the US. Several other gene therapy programs are following close behind, an indicator of a field poised for rapid growth. Yet success brings new obstacles, and after overcoming decades of setbacks, gene therapy's pioneers face the challenge of manufacturing cutting-edge treatments at scale.

The linchpin of every gene therapy is the vector, and many of today's therapies, including Luxturna, are based around recombinant adeno-associated viruses (rAAVs), which have generally proven safe in humans and capable of efficiently delivering DNA to a variety of tissues. At least 17 different companies are conducting clinical trials for rAAV-based gene therapies, with three programs now in phase-3 trials.

This surge in interest has brought a boom in demand for clinical-grade preparations of rAAV, leaving many companies scrambling to secure reliable vector production. As rAAV demand goes unmet, companies face lost opportunities, patient access to existing treatments is reduced, and development plans for new gene-therapeutic entities stall.

While most viruses freely procreate upon infecting host cells, rAAV needs a 'helper' to replicate. Historically, this has entailed coinfection of rAAV-producing cells with another virus, like adenovirus, but various workarounds are now available. Nevertheless, only a fraction of the resulting preparation represents fully-functional viruses containing the gene therapy construct. In many cases, routinely ~50% of these particles are empty protein shells containing no DNA. This requires careful purification, as empty viral particles can increase the risk of toxic immune responses and reduce the effectiveness of therapy.

This low efficiency also creates significantly more challenges for manufacturers, as many gene therapy treatments require huge numbers of fully packaged viral particles — particularly as one transitions from experimental proof-of-concept to clinical testing. For example, BioMarin's valoctocogene roxaparvovec (now in phase-3 trials) is being given to hemophilia A patients at a dose of 6×10^{13} viral particles per kilogram of body weight. Many thousands of liters of cultured cells will be needed to manufacture therapy for the 40 adults in this trial cohort, and if the drug is approved, the company will need to scale up manufacturing dramatically to treat the 20,000+ Americans afflicted with hemophilia A.

TABLE 1: AAV PRODUCTION OPTIONS

Pros and cons of various recombinant adeno-associated virus (rAAV) manufacturing strategies. rAAV cannot replicate without a helper virus, and early manufacturing efforts entailed coinfection of host cells with adenovirus or herpesvirus. Newer strategies replace these with plasmids containing key helper virus genes, or combine all necessary genetic elements into an insect cell-specific baculovirus vector. Production is most efficient in free-floating suspension cells, but substrate-attached adherent cell lines can also achieve reasonable viral output.

AAV MANUFACTURING TECHNOLOGY	KEY STRENGTHS	KEY DRAWBACKS	PRODUCTION CELL LINE CHOICES	
			ADHERENT	SUSPENSION
Helper virus	■ Highly scalable ■ Serum-free media ■ Efficient production in suspension culture	■ Helper virus contamination ■ Long lead time for cell line and virus seed generation ■ May require serum-containing media	HEK293/293T HeLa	HEK293/293T-s HeLa-s
Helper-free triple transfection	■ No helper virus contamination ■ Rapidly produce virus in small scale ■ Simple procedure	■ May require serum-containing media ■ Large proportion of empty capsids ■ Supply of plasmids for large-scale production can be costly	HEK293/293T	HEK293/293T-s
Baculovirus	■ Highly scalable Serum-free media ■ Efficient production in suspension culture	■ Baculovirus virus contamination ■ Baculovirus instability ■ Long lead time for cell line and virus seed generation	-	*sf9*

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BioMarin is one of very few companies opting to bring rAAV manufacturing in-house. Most others are turning to contract manufacturers, such as Vigene Biosciences, who have a reliable track record of clinical batch manufacturing and can provide dedicated resources for rAAV products produced using current good-manufacturing practices (cGMP).

This series will review and compare manufacturing technologies for relieving the bottleneck of rAAV cGMP production. Table 1 offers a summary of the three prevailing technologies. Although supply chain uncertainty is unlikely to ease soon, continual technological advances and steady accumulation of expertise in the rAAV space should ultimately ensure gene therapy a secure place in the clinical arsenal. ■

Vigene Biosciences
Excellence in Gene Delivery

A BUG IN THE SYSTEM

Insect cell-based viral vector production offers advantages for efficiency and safety, even if it requires some fine-tuning to achieve PEAK PERFORMANCE

The fall armyworm's cells make great vector-production biofactories.

KOCHIEVMV/GETTY IMAGES

The idea of producing a medicine within insect cells destined for human use may sound odd, and the fall armyworm (*Spodoptera frugiperda*) is nobody's idea of a standard laboratory model. However, cells derived from these larval moths are easy to culture and can be readily infected with baculovirus — an insect pathogen that can be reprogrammed to churn out enormous amounts of adeno-associated virus (AAV) for use in gene therapy. Indeed, the first gene therapy to reach the clinic — Glybera, from Amsterdam-based uniQure — was produced from baculovirus-infected insect cells for both clinical testing and commercial release.

Baculovirus was already in widespread use for producing protein-based drugs when, in 2002, Robert Kotin and colleagues at the US National Heart, Lung, and Blood Institute first demonstrated its suitability for AAV manufacturing. They infected Sf9 cell lines — derived from the fall armyworm — with three different baculoviruses: two containing essential genes for AAV particle production (*rep* and *cap*), and one containing the transgene sequence intended for delivery (Figure 1a). In this manufacturing process, the baculoviruses play a dual role, functioning as the 'helper' virus normally required for replication, as well as the vehicle for AAV genetic material. In their initial demonstration, Kotin's team achieved levels of productivity comparable with existing AAV manufacturing approaches — on the order of 50,000 functional viral particles per cell.

The baculovirus production strategy offers a number of advantages. First, Sf9 cells can be cultivated at high densities as free-floating suspensions in large bioreactors with volumes of up to 200 litres, enabling far more efficient AAV production than adherent cell cultures. These cells can also be grown under serum-free conditions, which improves biosafety by eliminating the presence of potentially immunogenic or toxic animal-derived proteins. Furthermore, baculovirus cannot replicate in human cells, though it is still necessary to purify out any unwanted viral particles as a prelude to clinical use.

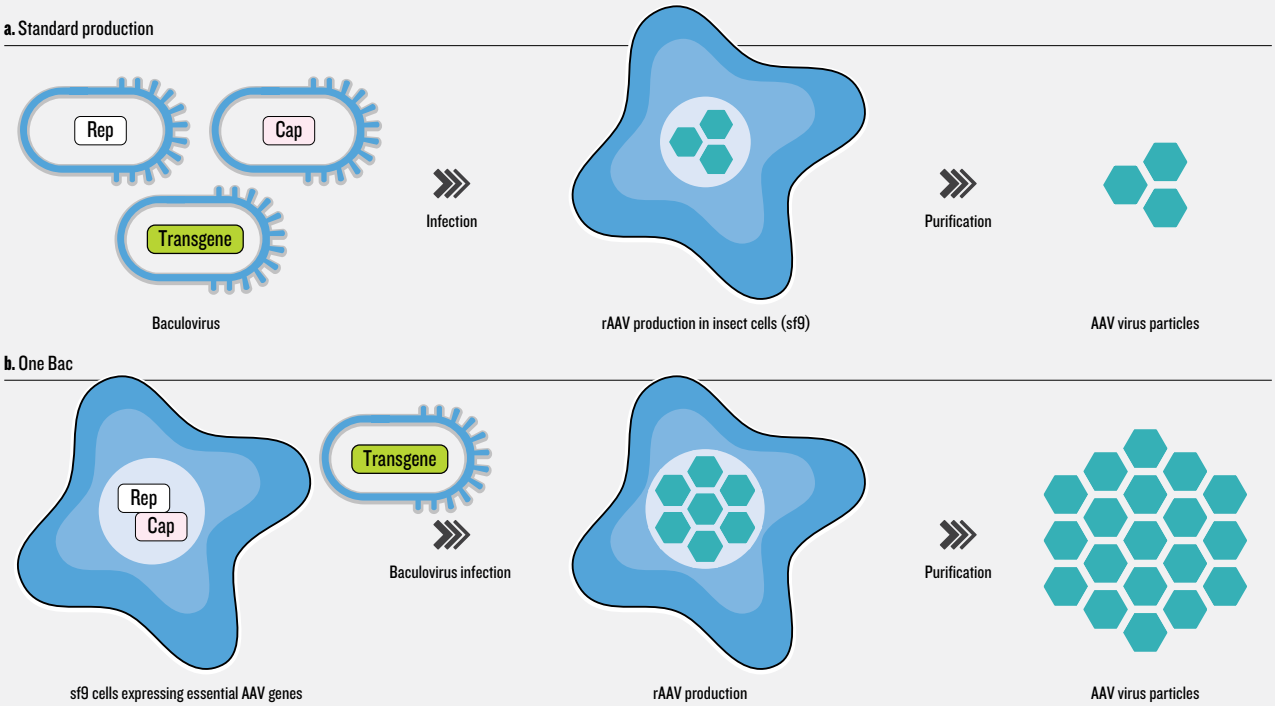
THE FIRST GENE THERAPY TO REACH THE CLINIC, GLYBERA, WAS PRODUCED FROM BACULOVIRUS-INFECTED INSECT CELLS

However, there are also significant limitations to baculovirus that require fine-tuning of the cultivation process. For example, the baculovirus genome can become unstable at high viral concentrations — and loss of the *rep* gene brings gene therapy vector production to a halt. This problem has been addressed to some extent by introducing genetic modifications to the baculovirus construct carrying this gene, which can improve genomic stability and prevent unwanted recombination.

The requirement for simultaneous infection of Sf9 cells with the three different baculovirus constructs also introduces complexity, and several groups have developed strategies intended to simplify the procedure. Perhaps the most notable is the BAC-to-AAV technology developed by Virovek, a company based

FIGURE 1: BACULOVIRUS-MEDIATED PRODUCTION OF AAV

(a) In conventional production systems, insect-derived cells (Sf9) are infected with a trio of viruses—two carrying the essential adeno-associated virus (AAV) genes *rep* and *cap*, and one containing the therapeutic transgene. This stimulates the Sf9 cells to produce infectious AAV particles. (b) The OneBac system simplifies this procedure by using Sf9 cells that have been genetically modified to carry *rep* and *cap*. These cells can produce large quantities of AAV after infection with a single baculovirus carrying the transgene.



in Hayward, California. This system employs specially-designed baculoviral constructs that are fine-tuned to ensure production of AAV component proteins at an optimal ratio for efficient viral assembly. This system also confers additional stability on the baculovirus genome, and gives rise to AAV vectors that achieve higher levels of infectivity — potentially enabling greater therapeutic efficacy when introduced into patients.

The OneBac platform, developed by Regine Heilbronn and colleagues at Charité Universitätsmedizin Berlin, employs Sf9 cells that have been genetically modified so that they already contain the *rep* and *cap* genes.

SF9 CELLS CAN BE CULTIVATED AT HIGH DENSITIES IN LARGE BIOREACTORS

Upon infection with a single baculovirus containing the full genome of AAV plus the therapeutic gene of interest, these cells produce AAV particles with an output that exceeds conventional baculovirus systems by an order of magnitude (Figure 1b). The first iteration of this system was unsuitable for clinical production because of a tendency to produce vector particles that contain unwanted baculovirus DNA sequences, introducing foreign proteins that could create safety issues in patients. Heilbronn's team has since devised a newer version, OneBac 2.0, which appears to eliminate this 'collateral packaging', although it remains, at present, unproven as a large-scale manufacturing technique.

Despite these technical challenges, the benefits of baculovirus-based manufacturing have made this approach a popular commercial alternative to the standard 'triple-transfection' AAV strategy. For example, BioMarin has used this manufacturing approach to support the clinical testing of valoctocogene roxaparvovec — one of the most advanced candidates in the gene therapy clinical pipeline. This AAV-based treatment for hemophilia A has demonstrated impressive performance in patients during phase I and II testing, and is now undergoing a pair of pivotal phase III trials. Accordingly, a growing number of companies are recognizing the possibility that this approach could help ameliorate some of the difficulties of AAV production. ■

GOOD HELP IS HARD TO FIND

Infecting mammalian cells with helper viruses greatly boosts AAV vector production for gene therapy, but creates EXTRA WORK IN PURIFICATION

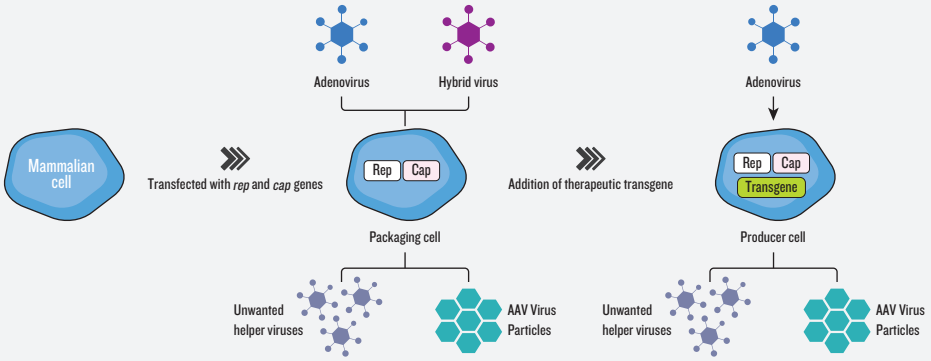
When Robert Atchison and his colleagues at the University of Pittsburgh first stumbled across adeno-associated viruses (AAV) in 1965, they initially supposed they were merely defective byproducts of the well-studied adenovirus. These AAV particles could not replicate within host cells on their own, but could when the cells were also infected with adenovirus. It has since become clear that AAV is actually a 'dependovirus' that can only be produced with assistance from other helper viruses.

AAV offers a number of advantages for gene therapy: it can efficiently deliver genetic material into patients' cells without causing cell damage or illness, and its inability to independently reproduce prevents uncontrolled generation of new viruses within a host. A 1984 paper by Paul Hermonat and Nicholas Muzyczka offered the first demonstration that AAV might provide a safe and effective vehicle for transgene delivery, with helper adenovirus infection enabling vector production in mammalian cells. This method offered good proof-of-concept, but was too inefficient for large-scale manufacturing. What's more, it needed extensive clean-up to eliminate adenovirus particles that might otherwise trigger a pathological or immune response in patients.

Newer iterations of the helper-virus-assisted approach improve performance. One strategy converts mammalian cells into 'packaging cells' by genetically modifying them to express the AAV genes *rep* and *cap*, which help copy and package viral genetic material. For gene therapy manufacturing, one can convert packaging cells to 'producer cells' by further modifying their genomes to include the therapeutic transgene along with sequences that signal for the transgene to be bundled into the AAV particle (see Figure 1).

FIGURE 1: PACKAGER AND PRODUCER CELLS

Mammalian cells (either HEK293, HeLa or A549) can be modified to express the adeno-associated virus (AAV) genes *rep* and *cap*, and become 'packaging cells' to help create recombinant AAV particles. By adding a therapeutic transgene, packaging cells can become 'producer cells', pumping out rAAV without the need for an additional hybrid helper virus.



Producer cell lines serve as durable rAAV factories: upon infection with adenovirus, they will churn out fully functional gene therapy particles. This technique is compatible with cells grown in free-floating suspension cultures under serum-free conditions, achieving an output of 5,000–50,000 viral genomes per cell. However, it is also very labor-intensive, as each new gene-therapy product requires a new producer-cell line.

Alternatively, some groups stay with the packaging cells, which they co-infect with two different adenoviruses: one wild-type, and one containing the AAV gene-therapy elements. In this scenario, a single packaging-cell line can be used to generate many different gene therapy constructs. However, it has not yet been used in large-scale manufacturing. Furthermore, both the packaging- and producer-cell approaches also create adenovirus particles, requiring an extra purification step to eliminate the unwanted helpers. This problem can be partially remedied by using adenoviruses that have been genetically modified to be temperature-sensitive or replication-defective.

AAV is not exclusively reliant on adenovirus; Friedrich Weindler and Regine Heilbronn demonstrated in 1991 that herpes simplex virus (HSV) can also play the role of helper. This method, in development by Applied Genetic Technologies Corporation (AGTC), requires no packaging-cell line; instead, suspension-cultured mammalian cells are infected with two modified HSV particles. One contains the transgene of interest, and the other holds the essential AAV genes. This approach is faster and simpler than the adenovirus-based methods, and can achieve AAV output levels that rival or exceed producer-cell systems. However, contamination is a recurrent problem of helper-mediated production systems and, despite using replication-defective HSV constructs, purification remains essential.

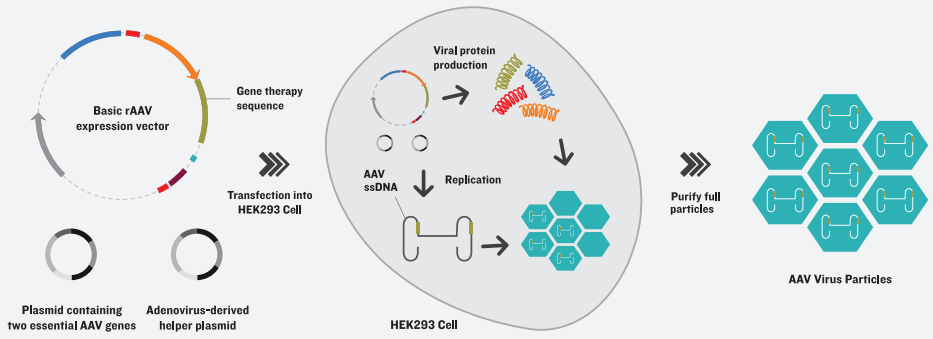
In spite of these potential risks, the high titer production by HSV is attractive to the developers of the therapies that require high-dose AAVs, such as those for Duchenne Muscular Dystrophy. In addition to AGTC, Solid Biosciences has adopted an HSV production system for its AAV production. Good help appears to be on the way. ■

TRIPLING DOWN ON EFFICIENT GENE THERAPY PRODUCTION

Three is the magic number when it comes to manufacturing vectors for NOVEL THERAPIES

FIGURE 1. OVERVIEW OF THE TRIPLE-TRANSFECTION STRATEGY FOR AAV PRODUCTION

This approach eliminates the need for a 'helper' adenovirus by introducing key genes in a DNA plasmid (top left), accompanied by two other plasmids (bottom left). The three plasmids are transfected into the production cell line (middle), which produces mature AAV capsids containing the transgene of interest. Once purified (right), these viral particles can infect patient cells to deliver their DNA payload, but cannot replicate further.



Given the considerable time and effort companies have invested in gene therapy, most prefer to play it safe when it comes to manufacturing. Accordingly, the large majority of recombinant adeno-associated virus (rAAV)-based therapies—which account for most gene therapies—are still produced with a method developed more than two decades ago, known as the 'triple transfection' technique.

One of the central challenges of rAAV production is the need for a 'helper virus' to facilitate replication within a host mammalian cell. In the early days of rAAV, cultured cells would first be infected with adenovirus, after which they would be transfected with two DNA plasmids: one containing a pair of essential rAAV genes, and the other a sequence to be employed for the gene therapy in question. This approach yields a reasonable output of fully-assembled, gene therapy-ready rAAV particles. However, these must then be separated from the also-abundant adenovirus particles that contaminate the resulting virus preparation, and can put patients at risk.

In 1998, two different research groups—one led by R. Jude Samulski at the University of North Carolina and the other by Peter Colosi at Avigen—determined that they could avoid these hassles by shifting key functions of the adenovirus on to a third, 'helper' plasmid. In this approach, researchers can simply transfect their preferred host cell—in most cases, human embryonic kidney (HEK) 293 cells—with all three plasmids in a stepwise procedure (see Figure 1). The combined activity of the adenovirus-derived genes on the helper plasmid with the rAAV genes on the second plasmid proved sufficient to generate functional rAAV particles containing the transgene from the third plasmid.

MODULAR CONSTRUCTION

Besides eliminating unwanted adenoviruses, this procedure has several advantages. There are numerous different serotypes of rAAV with distinct properties and capacity to infect different human organs, and researchers can readily swap different genetic elements into this system in a modular fashion to determine which virus is generated. Furthermore, the transfection procedures used to deliver the three plasmids to cells are robust, well-established, and can be performed at manufacturing scale.

Today, at least 11 companies are producing experimental or clinically-approved rAAV-based therapies using this approach, including Spark Therapeutics' Luxturna and a pair of other therapies now in phase 3 testing from Avexis and Gensight. Many contract manufacturers, such as Vigene Biosciences, also adopt this approach, which can be routinely scaled up to generate HEK293 cultures in volumes of up to 500L per bioreactor, generating rAAV at an output of 10^5 viral genomes per cell.

The biggest limitation to this approach is scalability. HEK293 cells are typically maintained in adherent cultures, but far greater culture volumes are possible with cells floating free in suspension. To address this, many teams have developed various strategies for producing suspension cultures of triple-transfected HEK293 cells. Published results initially demonstrated equivalent productivity to adherent culture in a 20L volume, but in principle could scale up to culture volumes of 200L or more to achieve far greater rAAV productivity.

Triple transfection technology has propelled commercial launch of rAAV-based gene therapy for eye-disease patients across the US. Despite newer and more efficient systems emerging, this approach will almost certainly remain instrumental for the manufacture of rare disease gene therapies as well as for proof-of-concept clinical trial materials. ■

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