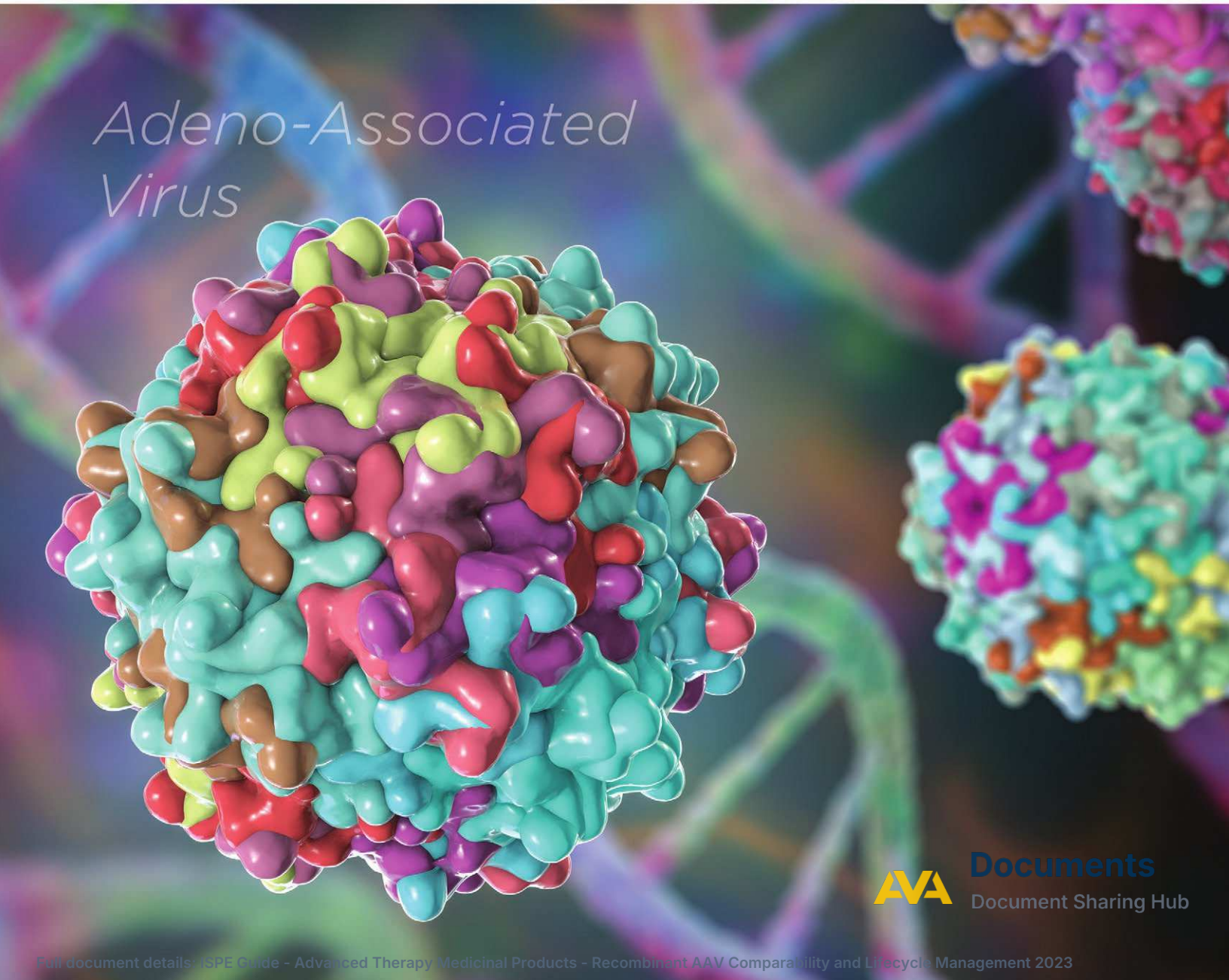
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GUIDE:

Advanced Therapy Medicinal Products (ATMPs)

Recombinant AAV Comparability and Lifecycle Management



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GUIDE:

Advanced Therapy Medicinal Products (ATMPs)

Recombinant AAV Comparability and Lifecycle Management

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Preface

In June of 2021, the ISPE Advanced Therapy Medicinal Product (ATMP) Community of Practice (CoP) was created with the vision and mission to expand the growing technical knowledge on these new modalities.

There has been a surge of recombinant Adeno Associated Viral (rAAV) vector products, and as these products progress through clinical phases, there have been comparability challenges noted by industry, and by health authorities. Comparability must be demonstrated by manufacturers when changes are introduced to the production process, to ensure product safety, identity, strength, quality, and purity.

EMA and FDA draft guidelines have provided considerations for evaluating the comparability of rAAV vector products. The collection of expertise across the ISPE ATMP CoP has enabled the design of a harmonized approach on comparability for rAAVs. The aim of this ISPE Guide is to provide the current understanding and best practices on rAAV comparability exercises, to offer manufacturers a standardized approach for developing process and product comparability strategies. This ISPE Guide has been created by a team of industry experts with global experience. It is intended as a reference and a practical guide for addressing common challenges that may arise when demonstrating comparability across the rAAV product lifecycle.

Discussion of proprietary technologies is included to convey practical information to the readers. In no way does this Guide endorse or promote the use of these items.

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Company affiliations are as of the final draft of the Guide.

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1 Introduction

One of the most significant challenges facing manufacturers is demonstrating comparability for Advanced Therapy Medicinal Products (ATMPs), such as for recombinant Adeno Associated Viral (rAAV) vector products. These are highly complex emerging therapeutics that provide patient benefits by expanding pharmacological possibilities, ranging from treating previously untreatable diseases to reducing the frequency of patient hospital visits and/or slowing disease progression. Industrializing the production and analytical systems requires addressing process challenges throughout the product's lifecycle, with the aim that the therapy proves effective for patients in a clinical setting and obtains health authority approval. Notably, landmark health agency approvals for AAV-based therapies in recent years have validated AAV's position in the pharmaceutical repertoire for treatment of human disease. With increasing commercialization, there is a growing need to consolidate current thinking for comparability to support manufacturers embarking on rAAV production.

The life sciences industry continues to experience significant growth in the advanced therapy sector as a result of intensifying scientific progress and achievement; however, manufacturing technologies, practices, and standards have struggled to keep pace. While the manufacturing know-how behind many biologics (such as monoclonal antibodies) stands on the shoulders of countless experts with decades of experience, it is important to recognize insights that translate well, and those that do not, into the new frontiers of AAV. Product comparability is one such challenge reported across the industry and this Guide serves to accelerate the translation, development, and adoption of standardized practices, as experts race to implement process improvements, expand scale, and work to eliminate sources of process variability.

rAAVs have become a leading candidate platform for the therapeutic delivery of *in vivo* gene therapy due in part to a strong safety profile and their ability to sustain gene expression. Although rAAVs may be utilized in many pharmaceutical applications, this Guide primarily focuses on rAAVs intended as a final product for *in vivo* gene therapy. The mechanism of action is largely intended to silence a gene (often in the case of mutated genes), replace an undesired gene with a target gene, increase expression of an existing gene, or express a novel or synthetic gene. Manufacturers utilizing rAAV outside of these contexts may consider these points; however, it is of note that varying considerations are appropriate based on the intended application of rAAV technology.

1.1 Regulatory Framework on Comparability

ICH Q5E [1] states:

> *"The goal of the comparability exercise is to ensure the quality, safety, and efficacy of drug product produced by a changed manufacturing process, through collection and evaluation of the relevant data to determine whether there might be any adverse impact on the drug product due to the manufacturing process changes."*

This is often referenced as manufacturers develop internal comparability strategies. In December 2019, EMA released a Q&A on comparability considerations for ATMPs [2] and recommended that the manufacturer consider process comparison in addition to product comparison if relevant, emphasizing that specific attributes should be examined at certain stages during the manufacturing process, rather than solely the DP stage. They also highlight the need for a robust comparability plan for new manufacturing sites, leveraging data from process parameters and in-process controls.

Comparability studies are recommended when significant changes are introduced in manufacturing and/or analytical methods, including major equipment changes, raw material changes, and/or introducing the process into a new facility, that is, changes that may affect product quality and/or stability. As development of a product progresses through the discovery/Research and Discovery (R&D) stage, clinical trial phases, and commercial launch, product knowledge and understanding increases. It is advised to perform product characterization studies early in development as these data can be used to ensure product and process consistency throughout manufacturing and is critical to support comparability assessments in later development.

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Developing a panel of suitable analytical assays for comparability is highly recommended from the first steps of development and should include additional characterization tests (including orthogonal methods). During the early phases of nonclinical and clinical studies, comparability testing should focus on assessing the impact to the product safety profile. However, when considering changes in later development, particularly when confirmatory clinical studies[1] have been performed, the principles per ICH Q5E [1] should be applied. Here, a higher degree of sameness is expected, and a more comprehensive analytical package should be in place. If analytical comparability cannot be adequately demonstrated, then data from nonclinical or clinical studies may be required.

1.2 Issues with rAAV Comparability

The life sciences industry has witnessed comparability issues manifest throughout the clinical trial phases. While these issues were not attributed to process changes per se, they highlight the novelty of these products and the learnings needed to wield them with the same mastery seen in many biologics. AAV vectors are not considered "well-characterized" products. This creates a challenging landscape for demonstrating comparability – and with rapidly evolving technology and manufacturing platforms, there is a need to drive consensus within industry and share industry manufacturing standards.

AAV is a non-pathogenic, replication-defective, and non-enveloped virus belonging to the *Parvoviridae* family. It is currently the most common gene therapeutic modality used to deliver viral-based gene therapy *in vivo* [3]. AAV consists of a single-stranded DNA genome that is packaged in a capsid, commonly containing three structural proteins, VP1, VP2, and VP3, that are assembled into a 60-subunit three-dimensional icosahedral structure to encapsulate the viral genome consisting of ~4.8 kilobases.

The complex structure may contain multiple sites susceptible to post-translational and chemical modifications to the capsid as well as the therapeutic sequence. Such modifications, as well as mis-assembly, fragmentation and/or aggregation can occur at various levels during the manufacturing process including cell culture, purification, formulation, and/or storage under selected conditions. The inherent complexity and typically heterogeneous nature of rAAV products make consistent product quality challenging since any change has the potential to impact product quality, safety, and efficacy. As a result, process-change risk assessments are more critical for pivotal clinical material, requiring increased product and process understanding of Critical Quality Attributes (CQAs) and associated Critical Process Parameters (CPPs). While traditionally any process change that could affect CQAs should be considered a major change, the novelty of AAV vectors requires broadening the traditional comparability assessment to holistically understand the impact of the change to the process and product. Compared to recombinant protein biologics, both product and process understanding for rAAV products is still very limited; consequently, there is a challenge in CQA and CPP establishment, including tolerance ranges and understanding the CQAs that have a direct effect on patient safety and product quality. To achieve comparable products, the underpinning scientific knowledge surrounding CQA and CPP needs to be generated and optimized, along with a suite of standard analytical methods.

1.3 Purpose and Scope

The purpose of this Guide is to provide industry with recommendations when designing a comparability strategy for rAAV vector products, taking into consideration that rAAV vectors are a major *in vivo* gene therapy subtype evaluated in clinical trials [3]. This Guide focuses on rAAV products used for *in vivo* gene therapy and their manufacturing processes considering process changes throughout the product lifecycle and analytical method changes.

This ISPE Guide aligns with FDA and EMA guidelines and requirements for rAAV vector comparability. It provides considerations for evaluating the comparability of rAAV vector products pre and post-change to the manufacturing process and proposes strategies to address the unique challenges posed by this new class of products when assessing their comparability.

[1] Confirmatory clinical studies are typically studies intended to provide primary evidence of effectiveness to support a marketing application.

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1.4 Structure of the Guide

This Guide is divided as follows:

- Chapter 2: rAAV Gene Therapy Manufacturing Processes

  This chapter describes various manufacturing platforms, considerations for process parameters, process trending and process capability.

- Chapter 3: Analytical Techniques

  This chapter focuses on analytical techniques that may be applicable to rAAV comparability studies.

- Chapter 4: Comparability

  This chapter reviews the comparability concept and strategy at various stages.

- Chapter 5: Appendix 1 – Case Studies

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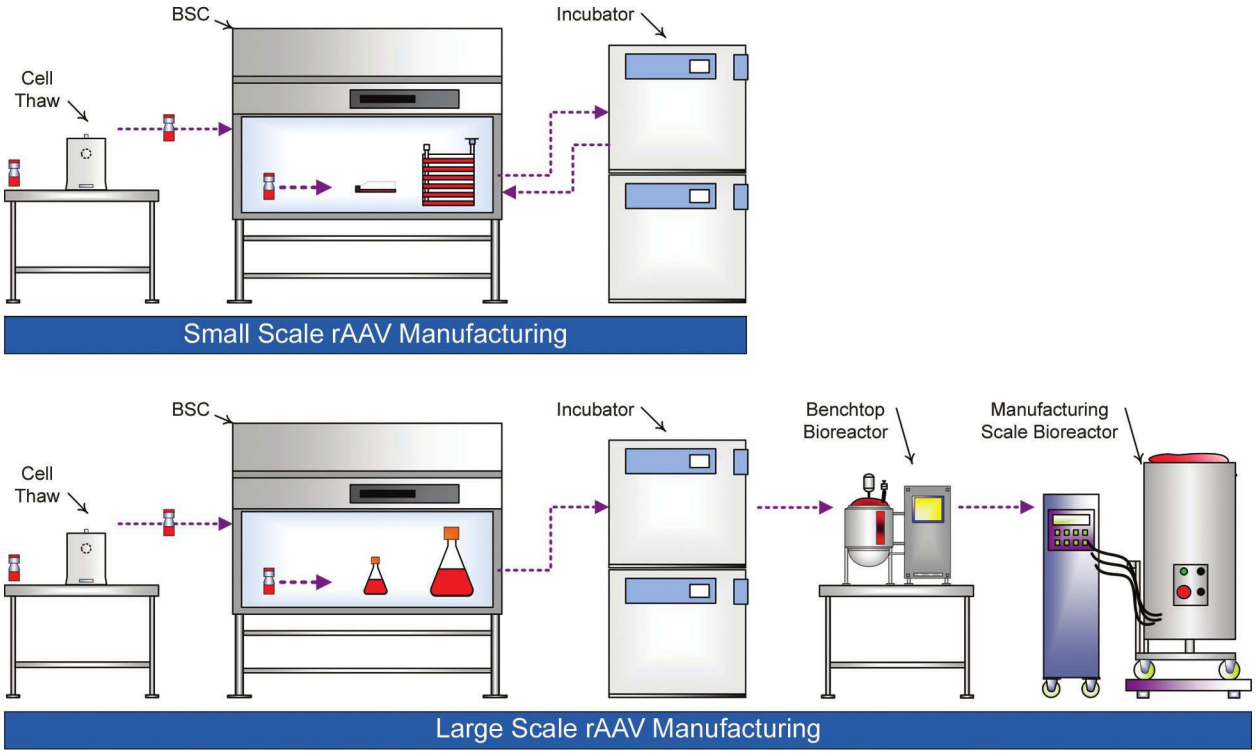
2 rAAV Gene Therapy Manufacturing Processes

2.1 Introduction

This chapter presents key aspects of the rAAV gene therapy manufacturing, including process understanding, unit operations, CPPs, process trending, sampling, and Quality Control (QC).

rAAV manufacturing has significantly progressed over the last few decades. While initial manufacturing processes were developed for the laboratory scale, the recent emergence and rise of gene therapy products has pushed for new processes to enable commercial viability [4]. Production methods originally developed for the laboratory scale have presented challenges with scaling. To answer the call, multiple platforms have been developed to produce and purify rAAV products. The upstream platform selection determines the host cell type and method of gene delivery. Historically, small-scale production methods utilized adherent HEK293 cells and triple-transfection methods due to the laboratory scale development and product quantity requirement. Process advancements such as suspension culture adaptation, stable producer cell lines, and insect cell production platforms have enabled large-scale manufacturing of rAAV products (see Figure 2.1).

Figure 2.1: Example of Small Scale versus Large Scale Upstream Manufacturing Formats



In general, the rAAV manufacturing process can be divided into key unit operations, including cell expansion, gene delivery, harvest, purification, drug substance bulk fill, and DP fill finish. While variations exist depending on the production platform, this chapter presents a holistic overview of the purpose and differences of the common unit operations encountered across various rAAV manufacturing platforms. Key process parameters and process trending are also discussed.

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2.2 Process Understanding

2.2.1 Background

Several of the unit operations within the rAAV manufacturing process share similarities, drawing upon the knowledge base of established biologics such as Monoclonal Antibody (mAb) products (i.e., cell expansion, clarification, chromatography). While the aforementioned unit operations adhere to an industry standard biologics manufacturing platform, rAAV manufacturing processes today are considerably more variable in the selection of host cell type (i.e., HEK293, SF9, HeLa) as well as in the rAAV gene delivery method (transient transfection of packaging cells, etc.). Each of these platforms has advantages, disadvantages, and an impact on the upstream and downstream operations, including differences in impurity profiles depending on the platform used.

Additionally, platform selection will impact the manufacturing scale that can be achieved. The vector demand for small clinical and/or orphan drug programs can be met with small-scale rAAV production formats as shown in Figure 2.1; however, large-scale rAAV manufacturing is required for programs with large patient populations. HEK293 and transient transfection are the most widely adopted host cell type and rAAV genes delivery method, yet scale-up challenges associated with this manufacturing platform, such as transfection efficiency and rAAV yield, have driven process evolution and development of alternative platforms [5].

As the demand of rAAV manufacturing increases, the scale-out versus scale-up of unit operations has been an increased topic of discussion. While traditional and proven methods of using adherent cell cultures exist, the cell-culture volume limitations lend this operation to a scale-out approach to increase manufacturing capacity. Recent innovations in the rAAV manufacturing space, however, are enabling further scale-up approaches with the increased use of large-scale suspension or adherent cell expansion bioreactor platforms (see Figure 2.2). Hybrid approaches for scale-up and scale-out can also be implemented to increase rAAV product output, for example by scaling-out multiple production bioreactors at the highest achievable volumetric scale.

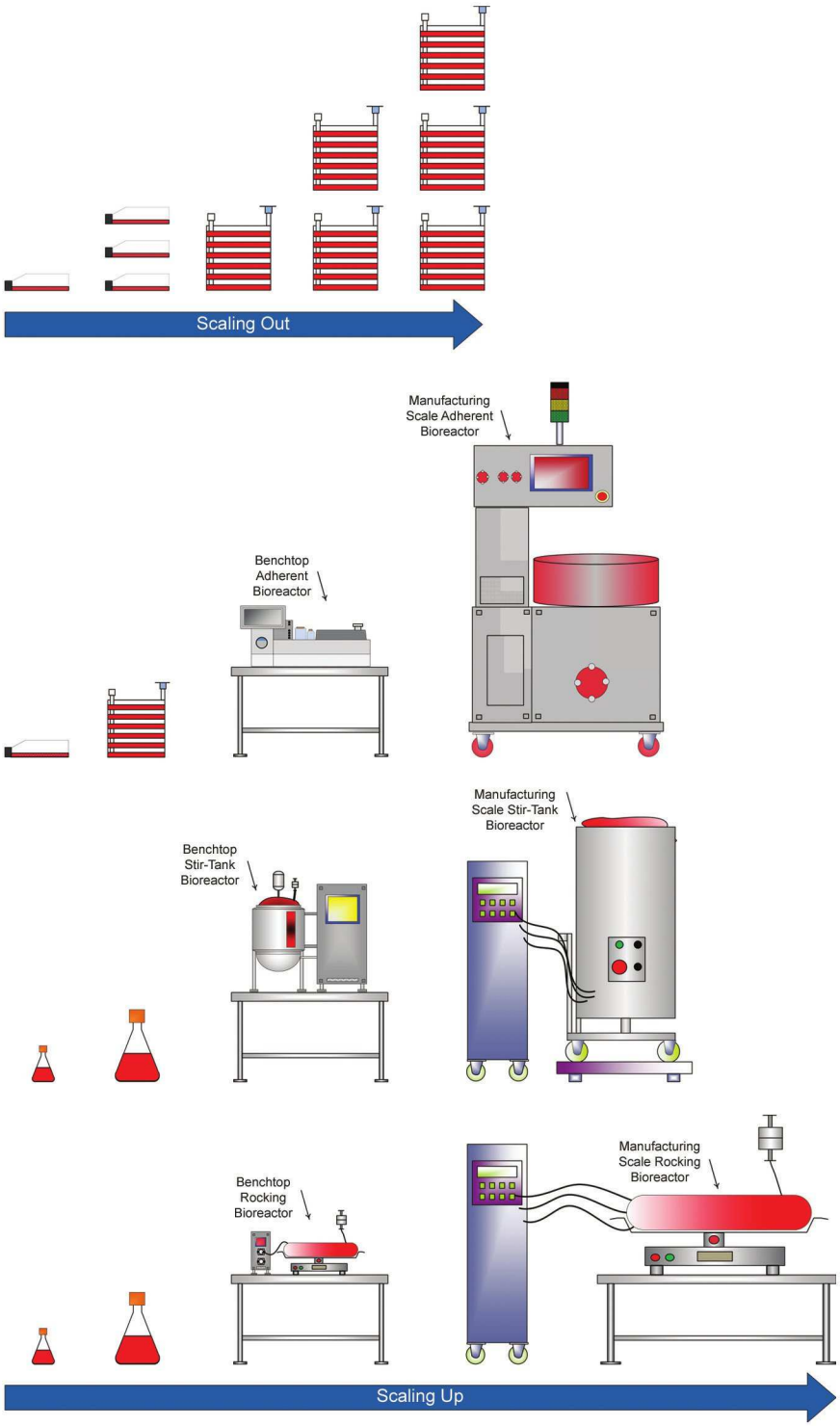
Scaling-up upstream and downstream operations to meet manufacturing demand is a proven approach in the biologics field. Challenges unique to the upstream scale-up of rAAV manufacturing include scaling of the gene delivery method (transient transfection, stable cells, etc.), lysis, and clarification [5] [R4]. Following harvest and clarification, downstream purification bears similarities to other biologics processes with challenges unique to rAAV products such as the capture of various AAV serotypes, the separation of empty/full capsids, and the removal of residual impurities [6].

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Figure 2.2: Scale-Out (Adherent) versus Scale-Up (Adherent and Suspension)



The scale and the need for short and numerous production campaigns of rAAV manufacturing unit operations lends them well to the adoption of single-use equipment [6]. Utilization of single-use technology and ensuring process closure with closed fluid transfer and sampling consumables can enable a more robust process design and a flexible facility design that takes advantage of simplified equipment and suite turnover between product batches [7].

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When designing single-use equipment and systems typical engineering design practices should be followed. Design considerations such as pressure and flow ratings, material compatibility, equipment layout, tubing supports and routing, component standardization, as well as the need for engineering documentation such as specifications and process drawings (piping and instrumentation diagrams, process flow diagrams, general arrangements) are a few examples of items that should be evaluated. When unable to ensure closure throughout the entire process, appropriate validation of the open process steps is required to protect the product from contamination [8]. To render the open process step functionally closed, validated cleaning procedures may include *in situ* chemical flushes/ sanitization, and/or performing aseptic connections under laminar flow of a Biological Safety Cabinet (BSC). Alternatively, the open process step can be performed in an isolator.

Single-use equipment components require an evaluation of the potential impact of leachables and extractables on rAAV product purity and will be included in the final product impurities tests (See Chapter 3 for further details on leachable and extractable testing). Stainless steel systems are not commonly used for rAAV manufacturing due to the risk of viral vector product contamination. Process closure and single-use equipment technology are essential to minimizing product contamination and cross-contamination between different products, ultimately ensuring rAAV product quality and safety.

The following sections provide an overview of the manufacturing processes developed across the rAAV gene therapy field to help contextualize the broad possibilities of process changes impacting product quality and comparability strategies discussed in Chapter 4.

2.2.2 Unit Operation Alignment

This section provides a high-level outline of a representative rAAV gene therapy manufacturing process and their nuances given the variety of cell types, upstream platforms, gene delivery methods, and purification techniques. Typical rAAV manufacturing processes utilize suspension HEK293 or SF9 cells to package rAAV, followed by affinity capture and ion exchange polishing chromatography downstream steps. However, a given manufacturing process can leverage a combination of orthogonal approaches, presenting a challenge to describe a single unified rAAV manufacturing process. Instead, the sections below present the general unit operation categories and provide an overview of the variations that can be encountered for each unit operation, starting with initial cell bank generation, selection of platform and gene delivery method, downstream purification, and DP fill and finish. A generalized block flow diagram is shown in Figure 2.3.

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Figure 2.3: High-Level Outline of a rAAV Gene Therapy Manufacturing Process

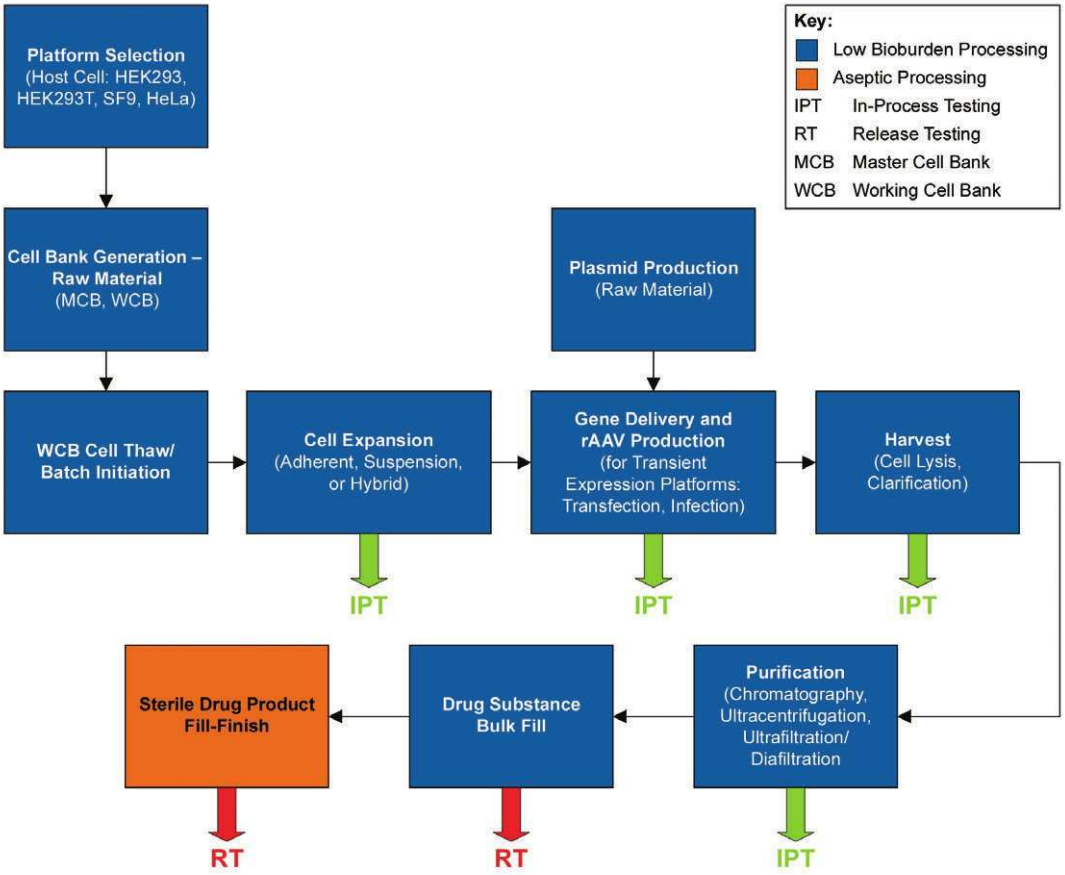


Figure 2.3 shows an overview of the rAAV process, and the potential associated In-Process Testing (IPT) and Release Testing (RT) points. Examples of potential IPT testing are cells count and viability, bioburden, mycoplasma, Mycobacteria, *in vitro* adventitious virus testing, genome titer, total DNA, full/empty capsid, and process-related impurities testing. Examples of potential Drug Substance (DS) release testing are identity, biological activity, rAAV genome titer, purity and impurities, bioburden, and endotoxin. Examples of potential Drug Product (DP) release testing are appearance, osmolarity, pH, biological activity, quantity (virus titer, infectious virus titer), purity and impurities, safety testing (endotoxin, sterility, CCI, particulates, etc.). See Chapter 3 for details on assay methods and further descriptions. Note that cell bank generation and plasmid production are included as raw materials for reference only and are outside the scope of this Guide.

2.2.2.1 Cell Lines

This section focuses on available rAAV packaging cell lines commonly used for clinical and commercial manufacturing and is not intended to be exhaustive. Associated platform technologies are described in Section 2.2.2.2. Table 2.1 summarizes attributes for the cell line options discussed, including common attributes, advantages, and disadvantages.

The most widely used manufacturing platform for rAAV utilizes mammalian cells isolated from Human Embryonic Kidney (HEK), commonly known as HEK293. These cells were transformed with a sheared Adenovirus 5 (Ad5) genome. A 4 kb fragment of the Ad5 genome was stably integrated, resulting in constitutive expression of the Ad5 E1a and E1b genes [9]. HEK293T cells are a well-known derivative of the HEK293 cell line, created by stably transfecting the SV40 large T antigen into HEK293 cells. The SV40 T antigen transformation enabled HEK293T cells to produce large quantities of recombinant protein from plasmid vectors containing the SV40 promoter, making them more efficient for viral vector production than the parent HEK293 cell line. HEK293T cells have been shown

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to have higher proliferation and improved transfection efficiency compared to HEK293 cells [10]. Both HEK293 and HEK293T cell lines are tumorigenic; however, HEK293T cells present an elevated safety concern due to the potential of additional SV40-mediated tumorigenicity [11, 12]. The HEK293 and HEK293T cell lines have been successfully adapted for suspension growth and subcloned to facilitate scale-up and higher production yields, respectively [13]. Adherent format systems are also readily available to scale platforms developed in a 2D adherent HEK293(T) culture.

Resembling the mode of HEK293 immortalization, human amniocyte cells have been isolated by routine amniocentesis procedures and subsequently transformed with a defined gene fragment encoding the essential Adenovirus E1a and E1b genes to create attractive cell lines that enable scalable high-titer production of viral vectors such as Adenovirus and rAAV [14, 15]. Amniocyte cells have been subcloned to isolate high-yielding producers and scaled using suspension bioreactors [15].

Baby Hamster Kidney (BHK) cells were derived from newborn hamsters using single-cell isolation and immortalized via polyoma virus transformation [17]. The commonly used BHK-21 (C-13) clonal cell line has widespread applications ranging from vaccine production to rabies testing and rAAV production. BHK cells adapted to suspension culture provide another scalable option for clinical and commercial manufacturing [18, 19].

HeLa cells, were the first cultured immortal human cells. They were isolated from cervical adenocarcinoma tissue collected at Johns Hopkins Hospital in 1951 [20]. Since their discovery and propagation, HeLa cells have been widely used in scientific research and development. Their high rate of proliferation has made HeLa cells an attractive candidate for manufacturing biologics and viral vectors, resulting in the engineering of multiple derivatives of the original cell line in both adherent and suspension formats [21].

In addition to mammalian cells, several insect cell lines have been developed as rAAV packaging cells. The most commonly used SF9 cells were isolated from the pupal ovarian tissue of Spodoptera Frugiperda, a fall armyworm and major agricultural pest throughout the American continents [22]. The SF9 cell line has been successfully cultured in suspension and was adopted in scalable Baculovirus manufacturing platforms to produce rAAV [23].

Table 2.1: Advantages and Disadvantages of Commonly Used rAAV Packaging Cell Lines

Cell Line	Attributes	Challenges and Considerations
HEK293(T)	• Largest existing body of clinical/commercial rAAV manufacturing data • Commercially available cell lines (transient or stable expression) • Transformed with 4 kb Ad5 genome fragment, expresses E1a and E1b genes • HEK293T derivative transformed with SV40 large T antigen	• Challenging transition from adherent to suspension • Tumorigenic potential • Prone to phenotypic and genotypic drift at high passage
Human Amniocyte	• Naturally adapted for suspension growth • Reported higher virus yields compared to HEK293 • Transformed with a defined gene fragment encoding only Ad E1a and E1b genes • Commercially available cell lines (transient or stable expression)	• Limited adoption for rAAV manufacturing • Tumorigenic potential
BHK	• Transformed with polyoma virus • Widely used (vaccine production *in vitro* diagnostics, manufacturing)	• Limited adoption for rAAV manufacturing • Tumorigenic potential • Challenging transition from adherent to suspension

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Table 2.1: Advantages and Disadvantages of Commonly Used rAAV Packaging Cell Lines (continued)

Cell Line	Attributes	Challenges and Considerations
HeLa	• Widely used in R&D and manufacturing • Commercially available cell lines (transient or stable expression)	• Tumorigenic potential • Challenging transition from adherent to suspension
SF9	• Amenable to 2D and 3D culture • Lower incubation temperature compared to mammalian cells (27°C–28°C) • No CO_2 gassing/sparging requirement • Non-mammalian, therefore, less of a viral safety concern compared to human-derived cell lines	• Insect glycosylation profile differs from human cell lines

2.2.2.2 Expression Platforms

This section reviews commonly used methods to express rAAV genes in the various cell lines described in Section 2.2.2.1. Transient transfection refers to introducing nucleic acids into cells without the foreign genes integrating into the host cell genome. The resulting gene expression is therefore transient, as the transfected nucleic acids are lost with cell division. A common way to transfect mammalian cells such as HEK293 and BHK involves complexing negatively charged plasmid DNA with a cationic polymer-based transfection reagent, such as Polyethylenimine (PEI) or other commercially available transfection reagents. The resulting positively charged complex binds to anionic cell surface moieties and is taken up by endocytosis. Plasmid DNA can also be complexed with cationic lipids to form liposome complexes that fuse with the cell's plasma membrane to deliver the desired genes, a method known as lipofection.

Both PEI and lipofection methods require preparation and the addition of the transfection mixture within a narrow, validated window of time to achieve effective consistent transfection efficiency. The scale-up of transfection methods presents logistical challenges such as mixing the transfection mixture, fluid transfer, and mixing dynamics within the bioreactor vessel, which must all be addressed to maintain sterility and transfection efficiency. See Section 4.3.2.1 for further discussion and details on transfection method comparability and efficiency.

Alternatively, plasmid DNA can be transiently introduced by electroporation methods, which use an electric field to temporarily permeabilize the cell membrane, allowing for uptake of DNA into the cell. This technology started as a cuvette-based benchtop format but has expanded into a scalable single-use format offered by several manufacturers, enabling its use in large-scale processes. Electroporation methods can simplify downstream purification and comparability studies as they do not introduce potential residual transfection reagents.

While the capacity to electroporate the cell count equivalent of a 500 L bioreactor has been developed, the volumetric restriction imposed by electroporation methodology presents a significant challenge when attempting to scale-up electroporation protocols. This technology has been scaled to process up to 200 billion cells in a 1 L volume, however, this requires introducing live cell Tangential Flow Filtration (TFF) buffer exchange and concentration steps ahead of electroporation [24]. Such operations can be difficult to optimize as they require specialized equipment, introduce shear stress, and can decrease cell viability. Adoption of this technology has been demonstrated for engineering small-scale products such as CAR-T cells and can be considered for manufacturing rAAV products that serve a small patient population. Bridging the gap to large-scale commercial rAAV manufacturing will require the development of instruments capable of handling 1000 L bioreactors in combination with scalable live cell TFF methods.

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The genes required to produce and package rAAV are typically supplied with three separate plasmids in a method referred to as “triple transfection.” Once the transfection complex is formed, it can be delivered to either adherent or suspension cells, which express genes encoded by the plasmid DNA. The replicase and capsid genes are provided with a trans-plasmid. The expression cassette containing the therapeutic transgene (rAAV genome flanked by Inverted Terminal Repeat (ITR) sequences) is delivered with a cis-plasmid to be packaged into rAAV capsids. The Adenovirus (Ad) helper genes required for replication, mRNA processing and translation (E2A, E4, VA RNA) are delivered by a helper plasmid. Using a three-plasmid expression system improves product safety by mitigating the risk of generating replication-competent AAV and reducing cytotoxicity to the packaging cells resulting from large plasmids. Eliminating the need for a helper virus by supplying an Ad helper plasmid also improves product safety and simplifies the downstream purification process.

Stable cell producer lines express AAV by having all AAV producing components within the starting cell host. This process typically does not use a transfection method (plasmids) or any helper virus to express the AAV gene of interest as the capsid and transgene are stably integrated into the cell genome [25, 26]. There are several methods of growth including both adherent and suspension-based platforms. This process typically has a chemical induction step that triggers the cell line to begin the expression of the rAAV and gene of interest. Common cell hosts for this method include HEK293 and Human amniocytes [26].

HeLa cells also provide several options for use as stable rAAV producer cells. HeLa producer cell platforms are engineered to express AAV rep and cap, and to stably integrate the therapeutic transgene (rAAV genome flanked by ITR sequences). Ad has been utilized as a helper virus to provide the genes required to accomplish AAV replication [28]. Ad-AAV hybrids or replication-deficient Herpes Simplex Virus (HSV) type 1 have also been introduced to circumvent the issues presented by Ad and can be successfully used as helper viruses in scalable HeLa cell platforms. In the examples above and below, Ad and HSV are process impurities that must be inactivated and removed by downstream purification steps [29, 30].

rAAV production can also be accomplished by means of viral infection. Mentioned above, replication-deficient HSV1 can not only serve as a helper virus, but also as the primary vector delivering the genes to produce and package rAAV. HSV1-based platforms have been established using two rHSV1 vectors, one carrying the ITR-gene of interest construct and the second carrying the AAV rep and cap construct. Coinfection of these rHSV1 vectors in HEK293 and BHK cells has been shown to generate robust yields of high purity rAAV [18].

The most commonly used viral infection rAAV manufacturing platform takes advantage of Baculoviruses, which naturally infect SF9 cells. Similar to the rHSV1 platform described above, Recombinant Baculovirus (rBV) vectors are used to infect SF9 cells to initiate vector production. SF9 cells lend themselves to expressing AAV rep and cap, while the rBV itself also supplies sufficient viral helper genes for AAV replication and packaging. Early generations of this platform utilized two rBV vectors, one supplying the ITR-gene of interest construct and the other carrying AAV rep and cap [31]. The OneBac system was later established to circumvent observed issues with Baculovirus instability. Briefly, SF9 cells are first engineered to stably express the AAV rep and cap genes under the control of the Baculovirus polyhedron promoter polh and cis-acting element hr2-0.9. The stable cells are then infected with a rBV carrying the ITR-gene of interest construct, which also induces expression of rep and cap [32]. During early harvest steps, residual rBV is inactivated by the lysis detergent and fully removed by downstream purification. See Section 2.2.2.3 for details concerning the generation of precursor Bacmid DNA required to produce rBV.

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Table 2.2: Advantages and Disadvantages of Commonly Used Expression Platforms

Expression Platform	Advantages	Disadvantages
Transient Transfection	• Avoids helper virus • Low investment during process development	• Scalability and consistency challenges • Costly GMP-grade transfection reagents • Transfection efficiency difficult to scale
Electroporation	• Avoids helper virus • Potential lower cost of manufacturing – no transfection reagent • High reproducibility	• Scalability limitations dictated by electroporation equipment capacity • Costly equipment
Helper Virus Infection	• Lower plasmid DNA requirement	• Helper virus process impurities can present downstream challenges • Genome instability (e.g., SF9 – rBV platforms)
Stable Cell Lines/ Inducible Cell Lines	• Avoids helper virus • Inducible expression • Eliminates plasmid DNA packaging impurities	• Costly and time-consuming cell line development

2.2.2.3 DNA Production (Plasmid, rBV Plasmid, neDNA™, Doggybone™ or Equivalent)

DNA production can be via biological methods (*E. coli* fermentation) or synthetic methods (enzymatic process).

For a plasmid DNA (pDNA) manufacturing process, initially the gene of interest is inserted into the pDNA, which is introduced into host bacteria (typically *E. coli*) in a process called transformation, usually by electroporation or chemically competent *E. coli.* The host *E. coli* cells replicate during fermentation, making copies of the plasmid and producing high yields of the desired plasmids. Once the *E. coli* cells have reached the desired density, they are harvested, and the pDNA is extracted, typically by alkaline lysis. The lysate is then clarified, and a TFF step is performed to exchange the lysate into the appropriate chromatography buffer. pDNA is purified using chromatography, followed by formulation and concentration using ultrafiltration/diafiltration. Finally, the plasmid is sterile filtered before QC testing to ensure the product is of sufficient purity.

For neDNA™, Doggybone™ or equivalent DNA manufacturing processes, the process is mainly based on enzymatic steps. *Corresponding* DNA can be defined as a minimal, linear, double-stranded, and covalently closed DNA construct, manufactured by enzymatic technology. The enzymatic process combines the use of two enzymes, Phi29 DNA polymerase and a protelomerase, to generate covalently closed, linear DNA constructs known as dbDNA, ne DNA. The process begins with a circular double-stranded DNA molecule (e.g., a precursor plasmid) containing the sequence of interest flanked on each side by a 56 bp palindromic protelomerase recognition sequence. The DNA starting material is then denatured and Phi29 DNA polymerase is primed. Then Phi29 initiates Rolling Circle Amplification (RCA) of this template, creating double-stranded concatemeric repeats of the original construct. Later protelomerase is added, binding to the recognition sites flanking the sequence of interest and performing a cleavage-joining reaction that results in monomeric double-stranded, linear, covalently closed DNA constructs. One of a panel of common restriction enzymes is added to cut undesired backbone DNA sequences, exposing open ended DNA that can be removed through digestion with exonuclease. Finally, the dbDNA is purified from small fragments and reaction components with size separation to leave only the dbDNA sequence of interest. The resulting dbDNA construct can be used as a starting material for a further amplification reaction.

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Bacmid DNA Manufacturing Process

SF9-Baculovirus expression systems require the production of rBV plasmid DNA known as “bacmid DNA” as a precursor material. The bacmid DNA encodes the AAV replicase and capsid genes as well as the transgene of interest, which can be supplied as a single bacmid or multiple distinct bacmids [33]. The generation of bacmid DNA is accomplished by transforming *E. coli* cells stably expressing the Baculovirus genome as a bacmid, with a donor plasmid encoding the desired AAV genes and/or gene of interest to create recombinant bacmid DNA using molecular tools such as site-specific transposition. Following antibiotic selection of *E. coli* carrying the recombinant bacmid DNA, the cells are expanded and the bacmid DNA is isolated via typical alkaline lysis and column chromatography purification methods.

Process impurities resulting from bacmid DNA manufacturing include residual antibiotics, bacterial Host Cell Protein (HCP) and DNA, bacterial endotoxin, culture media components, purification buffers, and chromatography media ligands. Care should be taken to remove associated process impurities during bacmid purification to minimize carryover into the rAAV manufacturing process. Purified bacmid(s) are transfected into SF9 cells to generate the rBV seed stock(s) used to infect naive SF9 cells for rAAV production [34].

2.2.2.4 MCB/WCB Generation

Master Cell Banks (MCB) and Working Cell Banks (WCB) allow for the generation of identical cell lines for use in GMP manufacturing spaces. MCBs are generated under GMP conditions to produce a single pool of cells from a single source [35]. WCBs are derived from one or more MCB vials under defined conditions to expand the subculture [36]. MCB cryogenic vials should be allocated for QC testing and confirmation of identity, count, viability, absence of contaminants as well as other testing requirements. WCB vials are then utilized in the manufacturing processes to provide a consistent starting point for the cellular expansion unit operations. The WCB and MCB vials are stored under tight low-temperature control (typically < -130°C with liquid nitrogen in vapor phase as the cooling medium or with an ultra-cool mechanical driven cryo-freezer). Backup and redundant sources of cooling are often considered. Characterization testing and stability should be performed on the MCB and WCB in accordance with testing strategies outlined in ICH Q5D [35] and the FDA Guidance for Industry: Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications [35, 37].

2.2.2.5 Cell Expansion

Cellular expansion is the first step in rAAV manufacturing and begins with the thaw of a WCB vial into an appropriate cell-culture vessel. The cells are then progressively expanded to production scale bioreactors until the desired cell count for rAAV production is reached. Regardless of whether adherent or suspension-based systems are used, cell expansion steps utilize a consistent seeding and passage density in order to maintain the cells actively proliferating within a consistent population doubling time across sequential passages, critical for achieving a reproducible expansion process.

Choosing an adherent or suspension cell line for rAAV production is another important factor in platform selection, as the reproducibility of the expansion process depends upon the culture format. Changes in cell culture raw materials, particularly of biological origin, should be risk assessed to determine whether a comparability study is required (see adventitious agents testing Section 3.3.1 and comparability study in Section 4.3.5).

In adherent-based processes, cells are grown on a substrate as a monolayer. Typical technologies include tissue culture flasks, cell factories, roller bottles, and commercially available fixed-bed or plate-based reactors [38]. The rationale for developing adherent cell-culture processes is based on the reduced process development required for the manufacturing scale-out approach. When small-scale processes are developed and need to increase in scale, limitations in incubated vessel sizes drive the need for scale-out rather than scale-up to increase the overall surface area [38]. Scalable single-use fixed-bed adherent bioreactor systems are readily available in the range of up to 500–600 m² per reactor, and 1000 m² systems are in development. Process variability, however, can be a concern for batch-to-batch consistency within individual adherent reactors.

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Adherent cells can also be grown with a hybrid format by adhering to microcarriers that are cultured in suspension. Microcarriers are small spherical particles made from materials such as polymers, glucan, or glass, and range in size, typically between 60–250 µm. By providing an attachment surface for adherent cell growth, microcarriers circumvent the scale-up limitation of flatware and allow for the scale-up of adherent cells grown on microcarriers in 3D suspension flasks and large-scale suspension bioreactors [39]. Microcarriers have been widely adopted for use in pharmaceutical manufacturing processes exceeding scales of 2000 L. Large-scale culture of HEK293 cells and rAAV production using microcarriers has also been reported, expanding the availability of formats for rAAV manufacturing.

Microcarrier culture has associated limitations and challenges that must also be considered and addressed during process development. Passaging during cell expansion requires trypsinization akin to cells grown on flatware. This unit operation grows in complexity with increasing bioreactor volumes because perfusion or closed single use centrifugation steps must be added to facilitate introducing and removing cell dissociation reagents. Further, the size of the microcarrier particles can render the attached cells more susceptible to shear stress compared to single-cell suspension, and agitation parameters must therefore be carefully examined during development [40, 41].

Suspension-based processes involve free-floating cells in a cell-culture medium to promote growth. This type of cellular expansion relies on multiple technologies ranging from simple flasks to large-scale single-use bioreactor systems. Initial shake flask expansions occur in single-use shake flasks and agitated in an incubator. Sequential expansions can occur in either rocking motion bioreactors of up to 200 L or in Stirred Tank Reactors (STR). While adherent-based processes have been the choice for rAAV commercial manufacturing initially due to the ease of continuing development starting with adherent R&D processes, there is a push towards suspension-based processes in the ranges of 500–6000 L. There is the potential for future rAAV products to require large batch sizes resembling the scale of biologics manufacturing, and 7500 L single-use STR systems are now available to begin addressing future needs. Ultimately, while suspension-based processes could take longer to develop and refine, there are multiple benefits including larger batch sizes, simplified handling, higher reproducibility of transfection efficiency, and ultimately more consistent product quality across batches.

The implementation and usage of process intensification steps, which allows medium exchange to prevent nutrient depletion while removing growth-inhibiting compounds, during cellular expansion steps and post induction/ transfection could allow for high production titers and vector quantities [42]. Perfusion processes use a filtration system to perfuse spent media (while retaining bulk viable cells) and replace it with fresh media to enable increased cell proliferation and higher cell concentrations. Typical technologies include Alternating Tangential Flow (ATF) and TFF. These concentration steps can occur at multiple steps in the cellular expansion process to reduce the quantity of expansion steps or increase cell concentration at the production step to increase product quantity [42]. Perfusion processes can also occur post-transfection to increase cell and rAAV concentration.

Another method of cell growth can be a hybrid of the two options discussed above: mixing suspension and adherent processes throughout a cell-growth phase. In this method, often the cells begin in a suspension-mode of cell growth in flasks (or equivalent) within shaker incubators. Once the cells grow to a volume exceeding a typical incubator capacity, they are transferred to a rocking motion bioreactor for further growth passages. Next, the cells may be transferred to an adherent-style growth method, often strategically for the gene delivery step, such as a fixed-bed bioreactor where the cells will adhere to a surface within the fixed bed. During the adherent-style bioreactor step with a fixed-bed, growth media and other materials may pass over the cells, allowing further growth, often in a recirculating pattern. At the next step, the cells are transfected (in the case of a triple-transfection process) where the triple-transfection cocktail passes over the cells in a favorable mass flow pattern (see Section 2.2.2.2). This method allows for suspension cell growth while final cell growth, gene transfer, and expression and delivery occur in an adherent-state. [43] A further benefit of this method is that the cells are retained on the fixed-bed bioreactor, resulting in less clarification/harvesting requirements to remove cellular debris.

2.2.2.6 Harvesting

Following the expansion and production of rAAV through the gene delivery method chosen, the cells may or may not need to be lysed to release the viral vector. Because the cell lysis step contributes to process impurities, clarification operations are crucial to help provide initial purification of the rAAV product from cellular impurities. Various steps within the clarification methods but are similar to large scale biological production.

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Cell lysis releases the AAV vector product from the host cells. This is typically accomplished using a chemical lysing agent such as a detergent and an endonuclease to degrade host cell DNA and non-encapsulated DNA, helping to reduce host cell DNA impurities [44]. These agents are usually added directly to the bioreactor of choice and adequately mixed to ensure complete lysis. While chemical lysis agents are typically used due to their scalability and use in suspension platforms, physical methods such as microfluidizer lysis can also be utilized [44]. To further promote flocculation for easier decantation, use of sodium-citrate, citric acid, and polycations ($MgCl_2$ and $CaCl_2$) may be employed. This lowers the amount of debris entering the depth filters and may reduce the size of the depth filter required by reducing the overall surface area requirements for depth filtration during clarification. Flocculation methods combined with centrifugation instead of depth filtration have been employed at small scale and could be developed further for large scale rAAV manufacturing [45].

Following lysis, clarification removes large cellular debris. This is typically accomplished through either filtration or centrifugation. Centrifugation allows for clarification by applying centrifugal force to separate particles based on density and/or particle size. While centrifugation is often used for small-scale production, it can present challenges when scaling for larger processes and using single-use technology [46].

Depth filtration filters remove cellular debris. Initial wetting of the filter is required to remove loose particles leftover from the manufacturing process prior to flushing product. Due to the nature of the filters, this is usually a fully single-use system that allows the system to remain a closed process with the assistance of presterilized depth filtration or rendered closed through a sanitization method. These steps often utilize similar principles as in normal flow filtration described in Section 2.2.2.7.

Following the clarification steps, a low bioburden filter (0.45 um or 0.22 um) is often used to further polish the cell lysate or excreted rAAV. Optionally, an intermediate ultrafiltration/diafiltration step could be completed directly afterward to allow for a 5–10 time concentration of product.

2.2.2.7 Purification

Much like with biologic products, purification is a requirement for any rAAV process before being administered to a patient to separate the product from impurities, potential adventitious agents, and added process components. Purification operations must not negatively alter the product.

A combination of unit operations may be chosen including normal flow filtration, ultrafiltration/diafiltration, TFF, ultracentrifugation, chromatography, and viral filtration/nanofiltration steps. Large-scale rAAV GMP manufacturing for use in humans predominantly utilizes chromatography purification techniques, however orthogonal purification methods such as ultracentrifugation or a combination of chromatography and ultracentrifugation can also be employed.

Normal Flow Filtration/Dead-End Filtration

Normal flow filtration/dead-end filtration examples include processes around harvesting, flocculation/clarification, prefilters to other unit operations, viral filtration, bulk fill, and DP formulation. Typically, normal flow filtration occurs around the harvesting step of a rAAV process to remove cell debris and other components from the upstream process. This step may be followed by an additional polishing filter typically using a 0.45 µm or 0.22 µm pore size. Prefilters in the process before specific unit operations may protect equipment from any large particles. Typically, this occurs before a chromatography step, or following any “hold steps” where the product has been refrigerated or stored at ambient temperature for an extended period to reduce/lower the turbidity of the product. Additional normal flow filtration operations occur for viral filtration.

Normal flow filtration/dead-end filtration is used at the end of the DS process, when the product will be stored for an extended period as “drug substance.” Typically, the finished product is filtered through a 0.2 µm pore size filter before storing in the bulk container that will hold the frozen product. At the DP formulation step before DP filling, the rAAV product is typically filtered once more through a 0.2 µm filter. Typically, this filter is tested to validate and ensure integrity throughout the installation and processing steps. This step is traditionally the boundary where the rAAV product classified as a “low bioburden process” becomes an “aseptic process.”

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Ultracentrifugation

Ultracentrifugation is a common technique for purifying viruses, including rAAV, via lysate migration through density gradients to facilitate virus sedimentation at the appropriate density. This step may be batch-based (more common in research-grade operations) or continuous and automated for large-scale manufacturing, and is used to purify rAAV from cell lysate and/or further separate empty and full capsids (polishing ultracentrifugation).

Iodixanol and Cesium Chloride (CsCl) are commonly used gradients for the purification of rAAV. Iodixanol has been reported to achieve higher vector purity, however, vector preparations contain higher percentages of empty capsids compared to purification using a CsCl gradient. While CsCl methods were historically used to purify vectors directly from cell lysate, they are complex and time-consuming as they involve Polyethylene Glycol (PEG) precipitation and are not easily scalable [47]. As a result, CsCl techniques are now more commonly used to separate empty and full capsids following iodixanol gradient purification or affinity capture chromatography.

In the small-scale, batch-based format, ultracentrifuge tubes are typically prepared and loaded manually within a BSC to keep these open process steps aseptic. The extraction of rAAV with a batch-based ultracentrifugation process includes the manual removal of the viral regions or bands observed in the ultracentrifuge tube, typically via a hypodermic needle within the BSC.

Scalable continuous-flow ultracentrifugation systems using density gradients have been on the market for decades and have been historically utilized as a workhorse for influenza purification in the vaccine industry. The purification principle is identical to the batch-based technique; however, this format is scalable and enables the continuous single or multi-pass purification of large lysate volumes in the range of 50–200 L per spin. rAAV extraction following centrifugation is accomplished by automated fraction collection and can be controlled by UV or refractive index measurement. These systems can be fully closed and integrated with automated fluid handlers using a sterile single-use fluid path [48, 49]. The rotor casing and rotor core on the other hand are reusable assemblies made of stainless steel and inert polymers, respectively. The ultracentrifuge components and flow path therefore require cleaning validation, performed with an inactivation agent, including but not limited to, NaOH and 70% IPA. While the centrifugation approaches described above are less popular for rAAV GMP manufacturing than chromatography, they are robust and can be successfully implemented with proper risk assessments and risk-based cleaning validation strategies developed around justifiable and realistic acceptance criteria [50].

Polishing ultracentrifugation may be used for empty/full capsid separation in place of chromatography polishing steps and commonly uses CsCl gradients. Purification processes using CsCl methods require careful consideration and evaluation of residual CsCl in the DS and DP as CsCl poses significant safety risks [51]. Often, before and after the ultracentrifuge step, there are Ultrafiltration/Diafiltration (UFDF) steps for buffer exchange into a centrifugation buffer medium as well as a buffer exchange out of the centrifugation buffer medium and into the formulation buffer.

Chromatography

Chromatography is a common purification step utilized by the biopharmaceutical industry. Within the rAAV purification process, it is utilized for AAV capture, impurities clearance (purification/polishing), as well as empty/full capsid separation to enrich for full capsids. In a rAAV purification process, there are typically 1–3 chromatography steps used and flow through or bind/elute methods may be deployed.

Chromatography has two major modes of function, “bind/elute” and “flowthrough” mode. In a bind and elute mode of operation, the viral vector is pumped through the chromatography skid and loaded onto the column. Once the product is loaded to the column, and the remaining impurities have passed through or been “washed” through the column, the product is “eluted” off the column matrix, and the viral vector transfers to the next major purification step. In a flow through mode of operation, the viral vector passes through the chromatography matrix while the impurities are bound to the column.

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Affinity Chromatography [52]

Affinity chromatography is based on an affinity ligand that binds AAV and from which AAV can be released/eluted. It often has the highest specificity of chromatography operations. For AAV, it is specific to the serotype used in the production of that specific product. AAV affinity chromatography typically utilizes a rigid cross-lined agarose-base matrix coupled to a dextran sulfate ligand. Other ligands may also be utilized for the purification of various other serotypes. Dextran sulfate ligands are commonly used for purification of AAV1, AAV2, AAV3, and AAV5 serotypes. Buffers used for this process are typically Tris-HCl based at pH 8.0 and are used for product loading and washing. The wash buffer may also contain NaCl. For elution of the product, a pH gradient from acidic to basic conditions is utilized. These buffers may contain sodium acetate (acidic) and arginine (basic).

While affinity is helpful to bind the rAAV product, it does not differentiate between empty/full capsids, thus further purification steps may be required. Affinity chromatography is always operated in a bind/elute mode.

Typical affinity chromatography steps include equilibration, load, wash, elution, and inactivation (before disposal).

Ion Exchange Chromatography

Ion exchange chromatography is a common method for biologics purification that is often found within rAAV purification processes. Ion exchange separates molecules depending on the molecule's net surface charge and will interact with a chromatography medium based on differences in overall charge, charge density, and surface charge distribution. Ion exchange chromatography may be used in either a bind/elute or flowthrough method of chromatography operations. rAAV purification processes leveraging ion exchange chromatography typically utilize a rigid, agarose medium with dextran surface extenders. This medium is typically functionalized with a variety of quaternized amines or PEIs.

The bind/elute mode of separation works by binding product of interest components to the chromatography medium based on electrostatic charges. If the medium has negatively charged functional groups (as in cation exchange) then components with positively charged ions will bind to it. If the medium has positively charged functional groups (as in anion exchange) then the components with negatively charged ions will bind to it. Once the product is bound, the medium is washed, and non-bound material is washed through. Following the wash steps, the bound material is eluted under conditions of increasing ionic strength or change in pH. With increasing salt concentration, salt ions in the buffer compete for binding with the charges on the medium, and the bound material is displaced and eluted. Alternatively, when the pH is changed, bound proteins are titrated and eventually become noncharged or have the same charge as the ligand, leading to repulsion and elution of the bound protein. Typical buffers in such a process are Tris based buffers (pH 9.0) utilized for equilibration, loading, and washing. Polycations, such as $MgCl_2$, are frequently utilized and a linear buffer gradient of increasing NaCl concentration is typically employed for elution and efficient separation by exploiting the charge differences of empty and full capsids.

In flowthrough mode, the pH of the product of interest and buffer is selected to modify the charge of the rAAV target or the chromatography medium such that the rAAV will not bind but will rather flow through the column, leaving most impurities bound. In this mode of operation, additional wash steps increase impurity removal and there is no product of interest elution steps necessary. [53]

For example, the product will be washed through from the first chromatography column while impurities will remain bound to this first column. Then, the product will bind to the second chromatography column, and residual impurities will be washed through the second column. The product is then eluted from the second chromatography column in series, then going on to further purification steps.

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Specific to rAAV purification processes, ion exchange chromatography is often used for empty/full capsid differentiation and empty capsid removal. The ion exchange process is typically determined by percentage of empty/full capsids, typically performed by a qPCR IPT (or similar technology). Within the AAV serotypes, full capsids typically have a lower isoelectric point than the empty capsids, thus allowing the ion exchange principles described above to be applied. Ion exchange chromatography skids typically have gradient elution capabilities and 260 nm and 280 nm UV sensors to perform inline spectrophotometry to detect measurements of DNA and protein, respectively.

A key advantage to ion exchange empty/full capsid differentiation and removal is that it is a directly "scale-up" approach compared to ultracentrifugation, which is often a scale-out approach (batch mode). Continuous ultracentrifugation methods are available and can be found described in the ultracentrifugation section. A key disadvantage to ion exchange chromatography is that it may be difficult to distinguish between "full capsids" and "functionally full capsids" and "partially full capsids" in regard to rAAV purification.

Hydrophobic Interaction Chromatography (HIC)

HIC targets the hydrophobicity of the binding element. This type of chromatography typically operates in a bind/elute method. It can be a serotype independent method of chromatography purification [54]. While not as typically seen in rAAV chromatography methods as ion exchange and affinity chromatography, it can be utilized in development processes for a scalable and simpler method of purification (as opposed to highly specific chromatography resins) [55].

Resin-Based Columns versus Monolithic Columns

rAAV production may use resin-based chromatography or monolithic chromatography methods. Resin-based matrices will be specified for that use and the column can be packed for various resin types and methods. Monolithic chromatography columns are a single solid, connected matrix and are specified with different channel sizes that may allow more laminar mass flow properties through the chromatography column.

Other Considerations

Additional considerations apply if chromatography columns are reused to process multiple upstream batches during a single-product manufacturing campaign. In this scenario, the chromatography columns would be regenerated following elution, placed in a storage buffer, sanitized, and equilibrated before use in accordance with column hold time studies and cleaning validation.

Nanofiltration

Nanofiltration is a step that may be utilized in a rAAV purification process to remove potential adventitious agents. It is typically a process step employing a normal/dead-end flow filtration or TFF step with specific viral-retaining filters. AAV has the ability to flow through 35 nm filters, allowing separation of larger impurities. The objective of this step is further impurity removal and adventitious agent reduction. Integrity testing of this step, if it exists in a process, is typically critical and may contain quantitative or qualitative integrity testing methods.

UFDF/Buffer Exchange

Ultrafiltration/Diafiltration (UFDF) is a purification step usually included in rAAV purification processes that use TFF methods. It is commonly utilized following harvesting clarification, single use centrifugation, or throughout the purification process where a buffer exchange step is required. UFDF steps concentrate product through volume reduction, and exchange buffers through diafiltration. UFDF as a polishing step typically removes low molecular weight impurities and liquids that may not be captured through a filtration process, while retaining higher molecular weight soluble components, typically the product. UFDF also provides means and methods for buffer exchange that may be required for multiple purposes including preparation for chromatography steps, ultracentrifugation steps, freeze/thaw strategies, formulation steps, etc. TFF is a step commonly found around harvest or DS Bulk Fill where product can be further concentrated, if necessary. Integrity testing of the UFDF system or UFDF/TFF cassettes may be required pending company risk assessments and quality risk management strategies.

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2.2.2.8 *Drug Substance Bulk Fill*

rAAV product bulk filling is traditionally a manual process with automated processes emerging. Due to the small volumes typically associated with rAAV DS products currently, limiting hold-up volumes within single-use assemblies and processes are an important aspect to consider. Minimizing any tubing length or processes that could lead to product loss are important. This leads to the high value as well of rAAV products. rAAV DS products have the possibility of being sterile filtered prior to filling. DS bulk fill products can be filled into bag, rigid polymeric, or glass containers. Following the filling into the bulk DS container, step down freezers may be utilized to maintain appropriate product storage conditions, typically between -20°C and -80°C. Then, long-term storage conditions will be maintained in a -80°C freezer.

Maintaining process closure for manual bulk fill processes can be a challenge. Performing these operations within a BSC, or in an isolator, to maintain environmental and product sterility is an important aspect to consider. These operations are typically performed in locally protected or Grade A/ISO 5 environments.

2.2.2.9 *Drug Product Fill Finish [56–59]*

Formulation

Before fill/finish can occur, rAAV DS may be stored in cold conditions (2°C – 8°C) or thawed if frozen, formulated, and sterile filtered. The rAAV DS is typically thawed via refrigerator, thawing device, or in a closed bag in ambient cleanroom conditions.

Final formulation buffers and excipients are added and mixed into the DS bag before final filtration, which typically occurs through a sterile 0.22 µm filter. These excipients are typically classified by the FDA's GRAS list (Generally Regarded as Safe) [60]. In regard to EU GMP Annex 1 [61], the integrity of the sterilized filter assembly should be verified by integrity testing before use (pre-use post sterilization integrity test or PUPSIT), to check for damage and loss of integrity caused by the filter preparation prior to use [55]. Redundant filtration or the flow path of filters in series may be considered as an operational strategy to mitigate the risk of one filter filling. Filter hold-up volume must also be considered as DP volumes to be filtered are often low and product loss can be substantial. Once the product passes through the final 0.22 µm filter, the process is converted from a "no or low bioburden" DS process to an "aseptic and sterile" DP process and aseptic processing conditions must be followed. The formulation step may or may not pool multiple DS lots together into one DP lot to be processed. Closed processing and open manipulations under aseptic conditions are required.

Aseptic Filling

Once formulation is complete, the rAAV DP is ready for fill/finish step. rAAV DP is typically filled in vials that may be performed by manual, semiautomated, or automated filling processes. The aseptic filling process must follow the same principles as other biologics aseptic filling processes including but not limited to component preparation, filling, stoppering, capping, crimping, coding, Container Closure Integrity Testing (CCIT), inspection, media fills/challenges, etc. Aseptic filling must be performed in Grade A/ISO-5 conditions and may occur under a BSC, open Restricted Access Barrier System (RABS), closed RABS, or isolator, and must consider biosafety principles and EU GMP Annex 1 [61] and PIC/S Annex 1 [62], throughout the DP process. For filling of small DP volumes, a filling line using 100% ready-to-use vials can be selected. Trends in the industry include the use of gloveless isolators and robotic filling technologies.

Labeling, Freezing and Packaging

After the filling process, rAAV DP can be immediately labeled at ambient conditions and frozen in -80°C freezers for storage. Alternatively, rAAV DP can be immediately frozen following filling and then cold-chain labeled. Cold-chain labeling has specific challenges including adherence of the label to the vial as well as temperature control of the vial into and out of the cold-chain labeling environmental conditions. [63-65]

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The packaging process may occur in a multitude of ways depending on the product, such as a product where each dosage is consistent from patient to patient, including retinal products. In this case, the final container type (vial) is packaged in primary and secondary packaging, and frozen until ready for final shipment.

Conversely, a rAAV product may be packaged in a patient-specific method in which the rAAV product is dosed to a patient-by-patient weight or other considered patient-based characteristics, sometimes found in pediatric products. If this is the case, the rAAV product that has been filled may be in patient-specific product packaging (multiple same-size vials, or different vial sizes, pending on the patient-specific requirements). rAAV is typically frozen once in primary and secondary packaging and typically has a dry-ice process for shipment from the manufacturing center to the final destination. Shipping verification and validation should be considered as in other biologics products.

2.2.2.10 Freeze/Thaw

Cell Bank

The generation of cell banks is considered the beginning of the GMP manufacturing process, and each bank must go through a rigorous process of characterization and testing to confirm identity, purity, suitability, and stability for use in manufacturing.

Storage of the primary cell lines as frozen stocks and their subsequent use for generating the drug ensures same provenance of the starting cell culture for each manufacturing run. Furthermore, these stocks are tested, and quality controlled ahead of time to ensure reliability, reproducibility, and safety of the product being manufactured.

For very long-term storage of cells over several years, the typical strategy is to prepare a MCB that can be used to generate a working stock or a WCB (comprised of several hundred vials) as needed periodically [66]. This two-tiered approach enables users to generate multiple banks of cells without compromising the passage history and lineage of the original cell line or stock.

It is recommended to transfer the cryovials to vapor phase liquid nitrogen storage once frozen to at least -130°C or below for storage in at least two different facilities under temperature alarmed containers for over several years and decades [67]. Although the intracellular solution is, theoretically, safely vitrified after controlled rate freezing, excursions of -80°C freezers may reach high enough temperatures to jeopardize the vitrification and samples are best held at vapor phase liquid nitrogen temperature if storage is to be prolonged. Many cells stored above -80°C are not stable, probably because traces of unfrozen solution may still exist [68].

Thawing cells at 37°C for 1–2 min followed by dilution of the cryopreserved cell culture into thaw medium works for most animal cells. After thawing, some cells may require dilution of the cryoprotectant to prevent osmotic shock. In some cases, thawed cells may also be centrifuged to completely exchange the freezing medium with the growth medium [69]. It is important to note that often the processes described above occur in an open manner. It is recommended that the procedures occur in a protected environment with appropriate active particle monitoring.

Process Intermediates [70]

Freezing process intermediate applications are typically post lysis and clarification and prior to the continuation of downstream processing. A TFF concentration operation can be performed on the clarified lysate to achieve higher product concentration, while optionally adding a buffer exchange to improve product stability during freezing [70]. Following freezing and thawing in a campaign mode, the sub-batches can be pooled to allow a larger batch size for downstream processing of multiple smaller upstream batches.

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The benefit to freezing process intermediates prior to downstream processing is to decouple the process. Decoupling the process allows for a more just-in-time processing for when unit operations are available, demand is present coupling the process in time. Additionally, being able to pool prior to downstream allows for a higher concentration of large volume processing and for multiple upstream batches be performed on one unit operation [70]. While these benefits can be seen to help decouple processes and improve processing time, studies need to be performed to show product stability at the temperatures proposed and inherent risks of pooling multiple sub-batches from a data and GMP perspective. In this case, it is important to risk assess loss of titer and/or variability in freeze/thaw recovery. One must consider the potential impurities for all sub-batches to be pooled in this case.

Bulk Drug Substance

Frozen DS storage is a conservative and generally preferred method when extended storage is needed, due to the benefits of circumventing potential product degradation at 4°C, extending product shelf life, and decreasing the potential for microbial growth. Several DS batches can be frozen, then thawed, and finished according to DP batch size required. However, freeze-thaw stresses may arise during these processes by cold and heat denaturation, by freeze concentration (cryo-concentration), and by interaction of solutes with the ice-liquid interface [71–74]. These stresses may cause precipitation of buffer resulting in pH shifts [75, 76], and concentration of salt to destabilize the product [77], all of which could lead to a loss of potency. Minimizing these stresses to achieve maximum product stability should be the primary objective when developing a freeze-thaw process. Additionally, freezing and thawing rates are considered CPPs because they impact product stability and activity. Consequently, DS stability studies will establish how long bulk DS can be kept frozen prior to DP formulation. In addition, studies of thawing time frame impact on DS bulk stability are required to control the thawing process step.

Developing a robust freeze-thaw process to maintain product stability is essential to product preservation and storage. Freeze-thaw, when coupled with disposable bags, provides some advantages over the traditional rigid container system including operational flexibility and a reduction in capital requirements. Frozen bags can be conveniently stored in qualified and monitored freezers of various formats. A single-use bag system can be designed to provide a shorter freezing distance for a given volume than commercially available rigid containers, thereby significantly reducing the resistance to heat transfer [78]. Handling and transport configurations are available to facilitate shipping. Single-use systems could virtually eliminate the risks of cross-contamination, particularly in multiple product facilities. The use of disposables combined with freeze-thaw production technology is therefore gaining attention.

Drug Product

The frozen storage temperature for biologic products generally falls in the range of −80°C to −20°C, but the preferred range for rAAV DP is −60°C to −80°C. The selected storage temperature is ultimately defined by product stability studies performed following ICH Q1A(R2) [79] and Q1E [80]. Future development of DS formulation allowing stable rAAV products at −20°C versus −80°C will help long-term energy and equipment cost savings. In addition, it will simplify operational logistics for rAAV product storage and shipment.

2.2.3 Other Considerations

Throughout the DS and DP manufacturing processes, including bulk storage or any hold steps, the production process may need to be protected from UV light to reduce potential oxidation and deamination effects. This parameter is often difficult to quantify. Post-gene transfer rAAV titers may have variable effects due to UV light. It may be necessary to limit UV light exposure where possible throughout the process.

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